Universidad de Jaén

Escuela de Doctorado

TESIS DOCTORAL

Análisis de la expresión y de la modulación de lncRNAs en el desarrollo cardiaco

PRESENTADA POR:

Carlos Garcia Padilla

DIRIGIDA POR:

Diego Franco Jaime

JAÉN, 13 de Mayo de 2019

Universidad de Jaén

Área de Biología celular

Departamento de Biología Experimental

Facultad de Ciencias Experimentales

El Dr. Diego Franco Jaime, Profesor Titular del Área de Biología celular de la Universidad de Jaén certifica que la Tesis Doctoral titulada: **"Análisis de la expresión y de la modulación de lncRNAs en el desarrollo cardiaco"**, que presenta Carlos García Padilla para optar al Grado de Doctor con Mención Internacional, ha sido realizada bajo su dirección, reuniendo a su juicio, los requisitos exigidos para esta presentación.

Jaén, 13 de Mayo de 2019

Dr Diego Franco Jaime

Memoria presentada por el graduado

Carlos García Padilla

Mayo de 2019

Índice

Resumen /Summary **3-8**
Resumen 3-5
Summary 6-8
Introducción **9-45**
Desarrollo cardiaco 9-11
Regulación molecular del desarrollo cardiaco 12-25
Factores de crecimiento 12-16
Factores de transcripción 16-25
Regulación post-transcripcional del desarrollo cardiaco 25-45
MicroRNAs 26-27
Funciones de los microRNAs en el desarrollo cardiaco 27-30
LncRNAs 30-36
Papel de los lncRNAs en el desarrollo cardiaco 36-43
Papel de los lncRNAs en patologías cardiacas 43-45
Objetivos /Aims 47-48
Objetivos 47
Aims 48
Capitulo I: Differential chamber-specific expression and regulation of long non coding RNAs during cardiac development and disease 49-94
Capitulo II: Identification of Novel Long non coding RNAs regulated by Pitx2>Wnt>microRNA Signaling Pathway involved in Atrial Fibrillation 95-135
Discusión 136-151
La expresión de lncRNAs muestra patrones dinámicos durante el desarrollo cardiaco136-138
La expresión de lncRNAs asociados a Pitx2>Wnt muestra patrones cámara-específicos durante el desarrollo cardiaco 138-139
La localización subcelular de Carmen y Fendrr pero no de Braveheart y H19, muestra diferencias significativas en las distintas cámaras cardiacas durante el desarrollo 139-140
H19 se expresa dinámicamente en distintos tipos celulares durante el desarrollo cardiaco 140-143
Expresión diferencial de las distintas isoformas de Fendrr y Carmen, pero no de Braveheart, durante el desarrollo cardiaco 143
Modulación de la expresion de lncRNAs por factores de transcripción cardiacos 143-144
Modulación de lcnRNAs por Pitx2c>Wnt>microRNAs 144-146
Modulación de lcnRNAs asociados a Wnt8/Wnt11 por Pitx2c>Wnt>microRNAs 146-148

Regulacion de lcnRNAs por factores prohipertroficos y proarritmogenicos 148-149

Wnt11_Gm_44934 interacciona con proteínas citoesqueleticas y miosinas que se encuentran desreguladas en procesos hipertróficos. 149-151

Conclusiones / Conclusions **152-157**

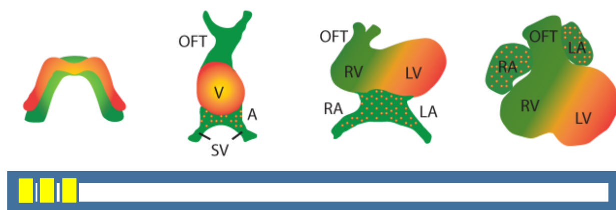
Conclusiones.. 152-154

Conclusions.. 155-157

Bibliografia **158-186**

Anexos.. **187-260**

RESUMEN/ABSTRACT



1. Resumen

El desarrollo del corazón es un proceso complejo que se encuentra regulado espacio-temporalmente tanto a nivel transcripcional como a nivel post-transcripcional. El papel de los factores de transcripción cardiacos como *Nkx2.5, Srf, Mef2c* o *Pitx2c* ha sido descrito ampliamente durante la cardiogénesis. En los últimos años se ha señalado la importancia del RNA no codificante en la modulación de las rutas de señalización que median el desarrollo cardiaco. En este contexto se han descrito varios microRNAs, miR-1, miR-133, miR-503/322, cuyo papel es esencial para una correcta cardiogénesis. Sin embargo, el papel de los RNA no codificantes de cadena larga (*lncRNAs*) aún no ha sido estudiado de manera exhaustiva durante la cardiogénesis. Múltiples estudios han posicionado a los *lncRNAs* como importantes reguladores tanto a nivel transcripcional como post-transcripcional en muchos procesos celulares. Asimismo se han descrito varios *lncRNAs* que juegan un papel en la cardiogénesis y/o en patologías cardiacas aunque aún se requiere de un mayor conocimiento para comprender su papel en estos procesos.

En el primer capítulo que conforma esta Tesis se ha estudiado la expresión de distintos *lncRNAs* candidatos en las distintas cámaras cardiacas durante el desarrollo cardiaco, así como su modulación por factores de transcripción que median la cardiogénesis. Del mismo modo se ha explorado el posible papel de estos *lncRNAs* en la ruta de señalización pro-arritmogénica y pro-hipertrófica mediada por *Pix2>Wnt>miRNAs*, angiotensina II/norepinefrina y la hormona tiroidea, respectivamente.

Asimismo, atendiendo a su localización genómica, los *lncRNAs* pueden modular la expresión génica de los genes que se encuentran adyacentes a estos. En el segundo capítulo de esta Tesis, analizamos el papel de *lncRNAs* localizados en el entorno genómico de *Pitx2, Wnt8a* y *Wnt11*, genes que habían sido relacionados con fibrilación atrial. Exploramos la posible expresión de estos *lncRNAs* en las distintas cámaras cardiacas durante el desarrollo cardiaco y la etapa adulta. Del mismo modo que para los *lncRNAs* candidatos, exploramos el posible papel de estos lncRNAs en la ruta de señalización pro-arritmogénica y pro-hipertrófica mediada por *Pix2>Wnt>miRNAs*, angiotensina II/norepinefrina y la hormona tiroidea, respectivamente.

Capítulo I: Expresión camara especifica y modulación de lncRNAs en el desarrollo y patologías cardiacas

En este primer capitulo se ha puesto de manifiesto los perfiles de expresión en las distintas cámaras cardiacas desde los ED12,5 hasta la etapa adulta de distintos *lncRNAs* descritos en cardiogénesis temprana, tales como *Braveheart, Carmen, Fendrr* y *Alien*, o en patologías cardiacas, tales como *Miat* y *H19*. Mientras que *Braveheart, Carmen* y *Fendrr* muestran un perfil de expresión adulto, *Alien, Miat* y *H19* muestran perfiles de expresión embrionarios. Asimismo el estudio de la expresión de las distintas isoformas de *Braveheart, Carmen* y *Fendrr* muestra que tanto las isoformas de *Carmen* como las de *Fendrr* se expresan de manera diferencial en las distintas cámaras cardiacas durante la etapa embrionaria y adulta, lo que sugiere distintos papeles funcionales de estas isoformas. Además, los ensayos de hibridación *in situ* demostraron que *H19* se expresa dinámicamente en el miocardio, en el epicardio y en el endocardio sugiriendo un papel de este *lncRNAs* en la maduración de los distintos tipos celulares cardiacos.

El estudio del posible papel modulador de los factores de transcripción sobre los *lncRNAs* estudiados sugiere que estos pueden formar parte de las rutas de señalización que median la cardiogénesis. Del mismo modo estos *lncRNAs* son modulados por la ruta de señalización Pitx2>Wnt>microRNAs y por factores pro-hipertróficos y pro-arritmogénicos, lo que sugiere que pueden tener un papel en distintos procesos patológicos cardiacos.

Capitulo II: Identificación de lncRNAs en el desarrollo cardiaco regulados por la ruta de señalización Pitx2>Wnt>microRNAs involucrada en fibrilación atrial

El estudio de los *lncRNAs* que se encontraban cercanos a *Pitx2, Wnt8a* y *Wnt11* nos ha permitido identificar cinco *lncRNAs* con una expresión diferencial durante el desarrollo cardiaco no descritos hasta la fecha. Wnt11_Gm_45188, Wnt11_Gm_44934 y Wnt8_2010110K18Rik muestran una expresión auricular diferencial respecto a los ventrículos, sugiriendo un papel en la maduración de las cámaras auriculares. Asimismo

estos *lncRNAs* son modulados por la ruta de señalización *Pitx2>Wnt>miRNAs* y por factores pro-hipertróficos y pro-arritmogónicos, lo que sugiere que estos *lncRNAs* pueden jugar algún tipo de papel en estas patologías cardiacas.

Ensayos de *pull-down* de Wnt11_Gm_44934 y el posterior análisis por espectometría de masas indican que este *lncRNA* interacciona con proteínas citoplasmáticas asociadas con hipertrofia cardiaca y arritmias entre las que destaca MHY6 así como con proteínas del citoesqueleto como la TAL1, ACTN1 y ACTN4. Además, Wnt11_Gm_44934 también interacciona con proteínas mitocondriales, nucleares y endosomales lo que sugiere que este lncRNA puede jugar múltiples papeles en el músculo cardiaco.

Summary

The development of the heart is a complex process tighly regulated space-temporal both transcriptional level and post-transcriptional level. The role of cardiac transcription factors such as *Nkx2.5, Srf, Mef2c* or *Pitx2c* has been widely described in cardiogenesis. In recent years, the importance of non-coding RNA in the modulation of signaling pathways that mediates cardiac development has been highlighted. In this context, several microRNAs have been described, miR-1, miR-133, miR-503/322, whose role is essential for a correct cardiogenesis. However, the role of long-chain non-coding RNAs has not yet been exhaustively studied during cardiogenesis. Multiple studies have positioned out *lncRNAs* as important regulators both at the transcriptional and post-transcriptional levels in many cellular processes. Several *lncRNAs* have also been described that play a role in cardiogenesis and / or cardiac pathologies, although greater knowledge is still required to understand their role in these processes.

In the first chapter of this thesis, the expression of candidate *lncRNAs* in the different cardiac chambers during cardiac development was studied, as well as their modulation by transcription factors that mediate cardiogenesis. The possible role of these *lncRNAs* in the pro-arrhythmogenic and pro-hypertrophic signaling pathway mediated by Pix2> Wnt> miRNAs, angiotensin II / norepinephrine and thyroid hormone, respectively, has been explored.

Also, by using them, gene expression of the genes that are adjacent to *these* can be modulated. In the second chapter of this thesis, we analyzed the role of lncRNAs located in the genomic environment of *Pitx2*, *Wnt8a* and *Wnt11*, genes that had been related to atrial fibrillation. To explore the possible expression of these *lncRNAs* in the different cardiac chambers during cardiac development and the adult stage. In the same way as for the candidate *lncRNAs*, we explored the possible role of these *lncRNAs* in the pro-arrhythmogenic and pro-hypertrophic signaling pathway mediated by Pixt2> Wnt> miRNAs, angiotensin II / norepinephrine and thyroid hormone.

Chapter I: Differential chamber-specific expression and regulation of long non coding RNAs during cardiac development and disease

In this first chapter we have published the profiles of expression in the different cardiac chambers from the ED12,5 to the adult stage of the *lncRNAs* in early cardiogenesis, such as *Braveheart*, *Carmen*, *Fendrr* and *Alien*, or in cardiac pathologies, such as *Miat* and *H19*. While *Braveheart*, *Carmen* and *Fendrr* show a profile of adult expression, *Alien*, *Miat* and *H19* present embryonic expression profiles. Likewise, the study of the expression of the different isoforms of *Braveheart*, *Carmen* and *Fendrr* shows that both the isoforms of *Carmen* and *Fendrr* are expressed differentially in the different cardiac cards in the embryonic and adult stage. In addition, in situ hybridization assays will demonstrate that *H19* is expressed dynamically in the myocardium, in the epicardium and in the endocardium suggesting a role for this *lncRNAs* in the maturation of the different cardiac cell types.

The study of the possible modulating role of transcription factors on the *lncRNAs* studied so that they can be part of the signaling pathways that mediate cardiogenesis. In the same way, these *lncRNAs* are modulated by the Pitx2 > Wnt> microRNAs signaling pathway and by the pro-hypertrophic and pro-arrhythmogenic factors, which you have to have a role in the different cardiac pathological processes.

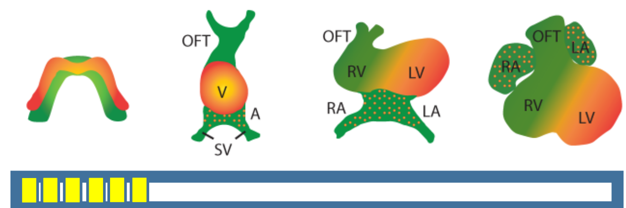
Chapter II: Identification of Novel Long non coding RNAs regulated by Pitx2>Wnt>microRNA Signaling Pathway involved in Atrial Fibrillation

The study of the lncRNAs that have been found in *Pitx2*, *Wnt8a* and *Wnt11* has allowed us to identify five lncRNAs with a differential expression in cardiac development not to date. *Wnt11_Gm_45188, Wnt11_Gm_44934* and *Wnt8_2010110K18Rik* for their differential atrial expression in relation to the ventricles, suggesting a role in the maturation of the atrial chambers. These lncRNAs are also modulated by the Pitx2> Wnt> miRNAs signaling pathway and by the pro-hypertrophic and pro-arrhythmogonic factors, which implies that these lncRNAs may be the type of role in these cardiac pathologies.

Wnt11_Gm_44934 RNA pulldown assys and subsequent analysis by media spectrometry indicate that this lncRNA interacts with cytoplasmic proteins and with cardiac hypertrophy and arrhythmias among which among which MHY6 stands out as well as

with cytoskeletal proteins such as TAL1, ACTN1 and ACTN4. In addition, *Wnt11_Gm_44934* also interacts with mitochondrial, nuclear and endosomal proteins suggesting that this lncRNA can play multiple roles in the cardiac muscle.

INTRODUCCIÓN



2. Introducción

1. Desarrollo cardiaco

El corazón es el primer órgano en formarse durante el desarrollo embrionario y en adquirir una funcionalidad esencial, tanto para la vida fetal como adulta, siendo vital en la distribución de los nutrientes y oxigeno así como en la eliminación de los productos de desecho del organismo.

El desarrollo cardiaco es un proceso complejo que se encuentra altamente regulado, tanto temporal como espacialmente, en el que participan tres fuentes celulares distintas de progenitores cardiacos: 1) mesodermo cardiogénico, 2) proepicardio y 3) células procedentes de la cresta neural cardiaca (Figura 1), que darán lugar a las distintas poblaciones celulares que conforman el corazón (Brade et al., 2013).

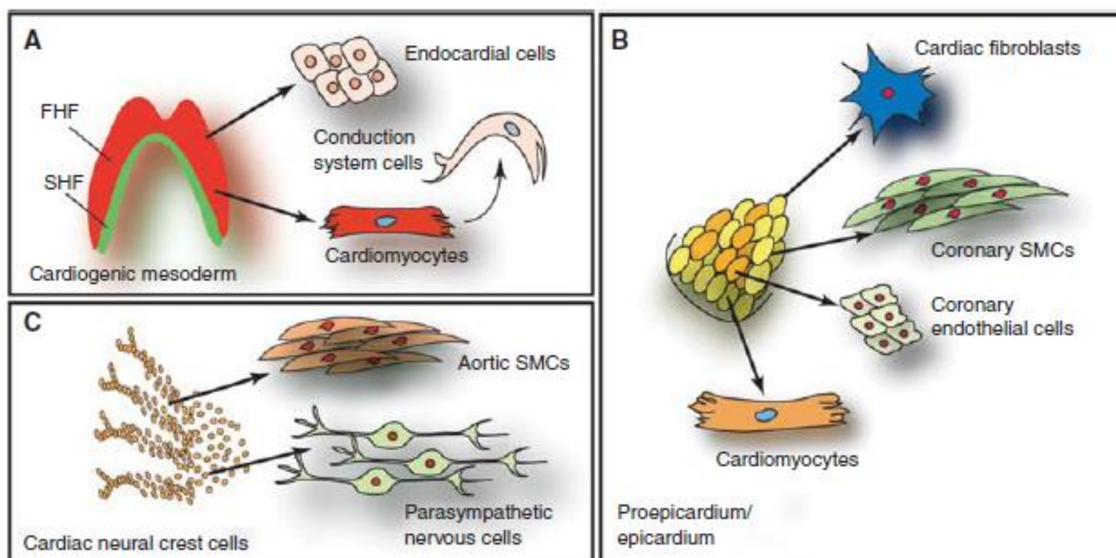


Figura 1. Progenitores cardiacos. (A) Mesodermo cardiogénico: Formado por el campo cardiaco primario y secundario, constituye la principal fuente de cardiomiocitos que pueblan el miocardio. Adicionalmente también es fuente de células endocárdicas y de células que forman parte del sistema de conducción cardiaco. (B) Proepicardio/epicardio: Los progenitores que se originan de esta fuente celular formaran el epicardio y se diferenciaran a fibroblastos cardiacos que se entremezclan con el miocardio, células musculares lisas y células endoteliales de los vasos coronarios así como algunos miocitos localizados en el septo atrioventricular. (C) Células de la cresta neural cardiaca: Originan las células musculares lisas distales del tracto de salida y el puente aórtico pulmonar así como el sistema nervioso autónomo del corazón. Procedencia de la imagen: Brade et al. (2013).

Durante la gastrulación, los precursores del mesodermo cardiogénico, migran fuera de línea primitiva, hacia la región antero-lateral del embrión, denominada mesodermo esplácnico. En este mesodermo, una subpoblación celular del mesodermo cardiogénico conocida como campo cardiaco primario constituye las crestas cardiacas (Watanabe y Buckingham, 2010). Hacia el día embrionario (ED) 8 del desarrollo del ratón (aproximadamente tres semanas en humano), se produce la fusión de las crestas cardiacas en la línea media ventral del embrión, formando el tubo lineal cardiaco primitivo. Este tubo cardiaco, que comienza a latir, está constituido por una capa miocárdica exterior y una capa endocárdica interior separadas por una matriz extracelular (Buckingham et al., 2005). En este mismo estadio, una segunda subpoblación celular, conocida como campo secundario cardiaco (SHF), que procede del mesodermo faríngeo y del mesodermo cardiaco dorsal, comienza a unirse a ambos polos del tubo cardiaco. La adicción de esta subpoblación al tubo cardiaco participa de forma activa en su elongación. Se distingue a su vez un SHF anterior que contribuye al polo arterial y un SHF posterior que contribuye al polo venoso (Kelly et al., 2014). En ED8,5 el tubo cardiaco comienza a curvarse hacia la derecha y a experimentar una proliferación regional del miocardio localizado en la capa más externa del corazón embrionario que contribuye a la formación de las futuras cámaras auriculares y ventriculares. Las células cardiacas procedentes del campo secundario cardiaco siguen uniéndose al tubo cardiaco en crecimiento hasta aproximadamente el estadio ED10,5-ED11,5. En este punto, el corazón tiene un aspecto tetracameral constituido por dos aurículas y dos ventrículos, donde las células derivadas del campo cardiaco primario (FHF) constituyen el miocardio del ventrículo izquierdo y de una pequeña parte de las aurículas mientras que las células derivadas del SHF constituyen la mayor parte del miocardio auricular, del ventrículo derecho y del tracto de salida (Rana et al., 2013).

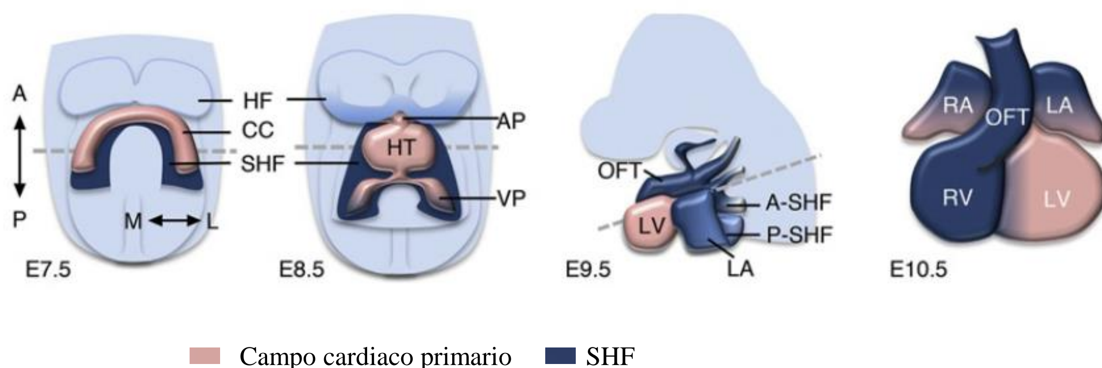


Figura 2. Contribución de FHF y SHF al miocardio de las distintas cámaras cardiacas. (CC): CresTa cardiaca; (HF): Pliegues cefálicos; (SHF): SHF; (HT): Tubo cardiaco; (AP): Polo arterial del tubo cardiaco; (VP): Polo venoso del tubo cardiaco; Procedencia de la imagen: Rana et al. (2013).

Entre ED9,5 y ED11,5, un subconjunto de células endocárdicas localizado en el canal atrioventricular y en el tracto de salida experimentan un proceso transición epitelio-mesénquima (EMT), en respuesta a señales procedentes del miocardio subyacente, para formar el mesénquima de los cojines endocárdicos. Estos cojines endocárdicos actúan como primordios tanto de las válvulas como de los septos cardiacos, facilitando el flujo sanguíneo unidireccional en el corazón embrionario. Aproximadamente, hacia el estadio ED11 los cojines endocárdicos sufren un fuerte proceso de remodelación que conduce a la maduración tanto de las válvulas como de los septos situados entre las aurículas y los ventrículos, así como entre los ventrículos y el tracto de salida (Lin et al., 2012).

Dos conjuntos de progenitores celulares adicionales al mesodermo cardiogénico son necesarios para el correcto desarrollo cardiaco. Por un lado, las células procedentes de la cresta neural cardiaca (CNCCs) son esenciales en la formación de los grandes vasos (arteria aorta y pulmonar, respectivamente) al diferenciarse en las células musculares lisas que formaran dichas arterias, así como en el desarrollo y posterior septación del tracto de salida al constituir el septo aorticopulmonar que separará la aorta del tronco pulmonar (Keyte y Hutson, 2012). Al mismo tiempo, las CNCCs son vitales para la formación del sistema parasimpático cardiaco y parece ser que contribuyen a una pequeña población de cardiomiocitos ventriculares (Hutson y Kirby, 2007).

Por otro lado, el proepicardio, una estructura transitoria formada cerca del seno venoso, constituye la fuente progenitora que dará lugar al epicardio que rodea al miocardio. Del epicardio derivan los fibroblastos miocárdicos y las células de la musculatura lisa de la vasculatura coronaria. Además existen evidencias de un pequeño número de cardiomiocitos ventriculares que pueden derivar del epicardio (Cano et al., 2016; Dueñas et al., 2018).

1. Regulación molecular del desarrollo cardiaco

El desarrollo del corazón está sujeto a una intensa y precisa regulación molecular tanto a nivel transcripcional como post-transcripcional. La regulación transcripcional está mediada principalmente por factores de crecimiento, factores de transcripción y por complejos remodeladores de la cromatina mientras que la regulación post-transcripcional está mediada por RNAs no codificantes, entre los que destacan los microRNAs (*miRs*) y una clase de RNA no codificantes de cadena larga denominados *long non coding* RNAs (*lncRNAs*). Ambas regulaciones orquestan la expresión y traducción de los genes necesarios para un correcto desarrollo cardiaco. Distintas mutaciones genéticas en estos genes cardiacos o una alteración en las cascadas génicas que conforman, suelen ser el origen de la mayoría de las enfermedades cardiacas congénitas y pueden aumentar la predisposición a sufrir enfermedades cardiovasculares o incluso ser causa de letalidad embrionaria (Calcagni et al., 2017).

1.1.Factores de crecimiento

El ectodermo y endodermo emiten señales mediante la secreción de diferentes factores de crecimiento para activar o inhibir las distintas rutas de señalización que modulan la diferenciación, especificación y desarrollo de los progenitores cardiovasculares. Del mismo modo, las diferentes regiones del corazón se comunican entre sí a través de señales paracrinas para una correcta coordinación de la cardiogénesis. Entre los factores de crecimiento más importantes que median este proceso se encuentran: 1) Proteínas morfogenéticas del hueso (BMPs), 2) Factores de crecimiento de fibroblastos (FGFs) y 3) Vías de señalización por Wnt.

1.1.1. Proteínas morfogenéticas del hueso (BMPs)

Distintos modelos mutantes para diferentes BMPs han revelado la importancia de estos factores de crecimiento en el desarrollo cardiaco. Así, ratones mutantes para *Bmp2* muestran una letalidad embrionaria entre los días de gestación ED7,5 y ED10,5 y una localización exocelómica cardiaca (Zhang y Bradley, 1996). Bmp2 ha sido descrito como un factor de especificación cardiaca al inducir la expresión de forma ectópica de *Nkx2.5*

y *Gata4*, además de jugar un papel esencial en la diferenciación del SHF al modular la expresión del *cluster* miR-17-92, que es a su vez, clave en la represión de genes progenitores cardiacos dado que promueven la diferenciación miocárdica (Waldo et al., 2001; Wang et al., 2010). *Bmp2* participa en el modelado miocárdico al dirigir la expresión de *Tbx2* en el miocardio atrioventricular, siendo requerido para la inhibición de genes cámara-específicos (Christoffels et al., 2004; Shirai et al., 2009). A su vez *Bmp2* ha sido identificado como un regulador clave en la inducción de la transición epitelio-mesénquima (EMT), siendo necesario para la expresión de *Has2*, componente crucial de la gelatina cardiaca y necesario para el EMT en el endocardio. *Bmp2* también juega un papel en las últimas fases de la morfogénesis de las válvulas cardiacas (Sugi et al., 2004; Luna-Zurita et al., 2010).

Del mismo modo, ratones mutantes para *Bmp4* muestran una letalidad embrionaria entre los días de gestación ED6,5 y ED9,5 mostrando defectos en la diferenciación del mesodermo (Winnier et al., 1995). *Bmp4* induce la formación del mesodermo cardiogénico incrementando la expresión de la proteína *Brachyury*, *Cdx-2* y *Nanog* mientras al mismo tiempo inhibe la diferenciación del endodermo (Bernardo et al., 2011). La delección selectiva de *Bmp4* en los cardiomiocitos genera mutantes con defectos en el septo interventricular y en el canal atrioventricular que producen un doble de salida de ventrículo derecho (DORV) (Jiao et al., 2003). Además, *Bmp4* es necesario para la correcta septación del tracto de salida y para la correcta proliferación del mesénquima de los cojines endocárdicos (Liu et al., 2004).

Los miembros *Bmp5, Bmp6* y *Bmp7* pertenecen a la misma subfamilia, por lo que la perdida de función de uno de ellos es compensada por los demás. Sin embargo, diferentes combinaciones en la eliminación de estos miembros han demostrado su papel en la cardiogénesis. Así, ratones mutantes para *Bmp5* y *Bmp7* presentan una letalidad embrionaria en ED10,5 mostrando defectos en la formación de los cojines endocárdicos así como defectos en la septación de las cámaras cardiacas (Solloway y Robertson, 1999). Ratones mutantes para *Bmp6* y *Bmp7* presentan una letalidad embrionaria entre ED10,5 y ED15,5, mostrando defectos en la septación ventricular y hipoplasia en los ventrículos con una trabeculación reducida (Kim et al., 2001).

Por último, los ratones deficientes para *Bmp10* presentan una letalidad embrionaria hacia ED9,5 como consecuencia de un desarrollo incompleto de los ventrículos, en los

que se observa profunda hipoplasia de las paredes, ausencia en la trabeculación miocárdica y proliferación anormal de los cojines endocárdicos en el tracto de salida y en el canal atrioventricular (Chen et al., 2004).

1.1.2. Factores de crecimiento fibroblasticos (FGFs)

Los FGFs han sido descritos como importantes mediadores de señales paracrinas necesarios para la correcta comunicación entre los distintos progenitores cardiacos. Entre todos los miembros de esta familia de factores de crecimiento se han señalado a *Fgf8*, *Fgf9, Fgf10, Fgf15, Fgf16 y Fgf19* como importantes reguladores del desarrollo cardiaco (Itot et al., 2016)

La delección sistémica de *Fgf8* se traduce en la pérdida de todas las estructuras derivadas del mesodermo y del endodermo embrionario y por tanto en una letalidad embrionaria muy temprana, revelando un papel en la especificación temprana de estos tejidos (Sun et al., 1999). *Fgf8* se expresa en la parte anterior del SHF, en los progenitores cardiacos que forman parte del polo arterial que constituirán el tracto de salida y el ventrículo derecho. En concordancia con esta expresión, mutantes condicionales para este factor de crecimiento en el SHF presentan defectos en la torsión del tubo cardiaco, en el tracto de salida y en la diferenciación del SHF lo que demuestra la necesidad del mismo para un correcto desarrollo de los cardiomiocitos derivados del SHF (Abu-Issa et al., 2002; Ilagan et al., 2006). Interesantemente, la expresión de *Fgf8* en la parte anterior del SHF se solapa con la expresión de otro factor de crecimiento fibroblástico, *Fgf10*. Ratones deficientes para *Fgf10* presentan corazones con una morfología ventricular alterada en los que los cardiomiocitos del ventrículo derecho muestran una proliferación incorrecta, lo que sugiere un papel de este Fgf en la regulación de la proliferación de los cardiomiocitos derivados del SHF. Además, *Fgf10* es esencial para el movimiento de los fibroblastos, derivados del epicardio, hacia el miocardio (Watanabe et al., 2010).

El fenotipo de los mutantes para *Fgf9* y *Fgf16* es similar entre si y se caracteriza por defectos en la proliferación de los cardiomiocitos, lo que apoya un papel de estos Fgfs en la regulación del crecimiento de los cardiomiocitos (Lavine et al., 2005; Hotta et al., 2008). Sin embargo cuando el *Fgf16* es deleccionado en la cepa *Swiss Black*, los embriones no son viables y presentan una letalidad en ED11,5. Los corazones mutantes

de estos embriones se caracterizan por defectos cardiacos entre los que se encuentran una dilatación en las cámaras cardiacas, paredes más delgadas y una reducida trabeculación (Lu et al., 2008). Esta diferencia en el fenotipo es explicado por el diferente fondo genético de ambas cepas que, mientras en una de ellas solventa la perdida de *fgf16*, en la otra cepa esta pérdida es letal (Lu et al., 2010).

Tanto *Fgf15* como *Fgf19* se expresan en los arcos faríngeos durante el desarrollo embrionario. La eliminación de estos Fgfs se traduce en una letalidad embrionaria entre ED13,5 y 7 días postnatales. Los corazones mutantes presentan un alineamiento erróneo de la arteria aorta y el tronco pulmonar. Este fenotipo cardiaco es correlacionado con defectos tempranos en el desarrollo del tracto de salida lo que sugiere un papel de estos Fgfs en el mismo (Vincent et al., 2005).

1.1.3. Vía de señalización Wnt

La señalización por Wnt puede ser mediada por dos vías distintas, la vía canónica mediada por la β-catenina y la vía no canónica mediada principalmente por la ruta de señalización JNK. Ambas vías son de vital importancia para la organogénesis del corazón (Hoppler y Moon, 2014)

La vía canónica Wnt/β-catenina tiene dos efectos contrapuestos durante el desarrollo temprano del corazón. En primer lugar promueve la inducción de los progenitores cardiacos modulando positivamente la expresión de *Brachyury* por acción de los ligandos *Wnt3a* y *Wnt8a*, mientras que más tarde inhibe el destino cardiaco adquirido por los progenitores cardiacos (Mazzota et al., 2016; Cohen et al., 2007). La supresión de esta vía en el SHF mediante la delección de la β-catenina reduce el número de progenitores cardiacos derivados del SHF y la expresión de *Islet-1*, lo que sugiere una modulación positiva del desarrollo de este campo cardiaco. Sin embargo, la activación forzada de la vía canónica en el SHF se traduce en un desarrollo erróneo del mismo, lo que confirma que esta vía juega tanto una modulación positiva como negativa del desarrollo del SHF en su desarrollo. Acorde con este papel bivalente, el ligando *Wnt2* media la activación de la vía Wnt/β-catenina en la parte posterior del SHF siendo necesaria para la proliferación de los cardiomiocitos que derivan de esta región cardiaca (Tian et al., 2010),

Wnt2 también es necesario para la diferenciación de los cardiomiocitos, aunque en este contexto, *Wnt2* activa la vía no canónica Jnk (Onizuka et al., 2012).

Al igual que la vía canónica, la vía Wnt/Jnk, denominada como no canónica, media la inducción del mesodermo cardiogénico. Los ligandos *Wnt5a* y *Wnt5b* regulan la expresión de *Mesp1* en el mesodermo precardiogénico mientras que los ligandos *Wnt2, Wnt5a* y *Wnt11* han mostrado ser de vital importancia para la diferenciación de los cardiomiocitos derivados del SHF. Así, delección de *Wnt5a* y *Wnt11* se traduce en una pérdida de los progenitores cardiacos derivados del SHF (Wang et al., 2006; Cohen et al., 2012; Mazzota et al., 2016).

1.2. Factores de transcripción cardiacos

En vertebrados, el programa transcripcional que media la especificación del mesodermo esplácnico hacia mesodermo cardiogénico se inicia con la expresión del factor de transcripción *Eomesodermina* (Arnold et al., 2008). La expresión de *Eomesodermina* en la línea primitiva es necesaria para la activación de *Mesp1*, el cual ha sido identificado como un regulador clave en la especificación y migración de las células mesodérmicas que darán dar lugar a los campos cardiacos, siendo considerado el primer marcador de diferenciación cardiaca (Costello et al., 2011). *Mesp1* se expresa por primera vez en el mesodermo hacia ED6,25 y su expresión desaparece antes de que las crestas cardiacas se hayan formado por completo. *Mesp1* induce la especificación y migración del mesodermo cardiogénico reduciendo la expresión de genes pluripotenciales y aumentando la expresión de factores de transcripción cardiacos tales como *Gata4*, *Nkx2.5*, *Mef2c y Smarcd3* (Bondue y Blanpain, 2010). *Mesp2*, otro miembro de la familia Mesp, también se expresa en la capa mesodérmica que dará lugar al mesodermo cardiaco (Saga et al., 2000), aunque su potencial cardiogénico es menor que el de *Mesp1*. En mutantes nulos para *Mesp1*, el fenotipo es compensado por *Mesp2*, que se incrementa en ausencia de *Mesp1*. Sin embargo en mutantes nulos para ambos, el mesodermo cardiogénico está ausente y estos ratones presentan una letalidad embrionaria en ED9,5 (Kitajima et al., 2000; Saga et al., 2000). Recientemente, Lescroat et al. (2014) demostraron que las células que conforman ambos campos cardiacos, expresan *Mesp1* en diferentes espacios

de tiempo durante su especificación, sugiriendo la existencia de un progenitor común existente en el epiblasto antes de que comience la gastrulación.

La inducción por parte de *Mesp1* de la expresión de los factores de transcripción cardiacos *Gata4*, *Nkx2.5* y *Mef2c* promueve la diferenciación y desarrollo de los progenitores cardiacos hacia un corazón funcional. Ensayos de ganancia y pérdida de función tanto *in vivo* como *in vitro* ha señalado la importancia de estos factores de transcripción en la adquisición del destino celular cardiaco.

Gata4 se expresa por primera vez, al comienzo de la gastrulación, en el endodermo extraembrionario. Más tarde su expresión queda restringida al mesodermo que dará lugar al miocardio y al endodermo subyacente. La expresión de *Gata4* en las células miocárdicas en desarrollo es observada por primera vez en las crestas cardiacas, y continua hasta el miocardio adulto, mientras que la expresión temprana endocárdica queda restringida a las células mesenquimáticas de los cojines endocárdicos que darán lugar a las válvulas cardiacas. *Gata4* también se expresa en el epicardio y en los vasos coronarios que derivan de este (Nemer et al., 2003; Rojas et al., 2005).

La pérdida de función de *Gata4* ha puesto de manifiesto la importancia de este factor de transcripción tanto en el desarrollo del miocardio como del endocardio. Los mutantes nulos para *Gata4* presentan una letalidad embrionaria en ED9,5-10 provocando una alteración severa del desarrollo cardiaco. Los corazones mutantes se caracterizan por un fenotipo hipoplásico con un miocardio delgado, ausencia del proepicardio y de los cojines endocárdicos (Watt et al., 2004). Este fenotipo hipoplásico también se observa en mutantes murinos con ganancia de función para *Gata4*. En este caso, los embriones presentan una letalidad embrionario en ED12,5 y además de mostrar una menor proliferación miocárdica, los corazones mutantes se caracterizan por defectos en la septación y en la formación de las válvulas así como por presentar un canal atrioventricular común y doble tracto de salida (Crispino et al., 2001). La hipoplasia miocárdica en ambos modelos sugiere que *Gata4* modula de forma muy precisa la proliferación de los cardiomiocitos durante el desarrollo (Pu et al., 2004; Zeisberg et al., 2005).

Gata4 ha sido descrito como un importante regulador e inductor de las rutas de señalización cardiacas, siendo necesario para la activación de factores de transcripción

claves para el desarrollo del corazón. Así durante el desarrollo temprano del corazón, *Gata4* en concierto con *Smad* activa a *Nkx2.5*. *Nkx2.5* interacciona a su vez con el propio *Gata4* para modular conjuntamente la activación de otros factores de transcripción, interactuando con las regiones promotoras de estos últimos (Kuo et al., 1997; Kuo et al., 1999).

El papel de *Nkx2.5* ha sido ampliamente estudiado durante el desarrollo cardiaco. Este factor de transcripción se expresa de forma continua en el miocardio en formación, tanto en el FHF como en el SHF (Lints et al., 1993; Stanley et al., 2004). Los ratones mutantes para *Nkx2.5* muestran una letalidad embrionaria en ED10,5, lo que indica que no es esencial para la especificación o formación del tubo cardiaco. Sin embargo, la cardiogénesis es bloqueada durante la torsión cardiaca y los corazones mutantes se caracterizan por la presencia de un único ventrículo primitivo y un tracto de salida anómalo (Lyons et al ,1995; Tanaka et al., 1999).

Los defectos en el tracto de salida son atribuidos a la existencia de un *feedback* negativo establecido entre *Nkx2.5* y la vía de señalización *Bmp2/Smad1* que regula la proliferación y diferenciación del SHF. La importancia de este *feedback* ha sido puesta de manifiesto gracias al estudio de las delecciones de *Nkx2.5* y *Smad1* en modelos murinos, respectivamente. Ambas perdidas de función tienen efectos contrarios entre sí, ya que mientras los mutantes para *Nkx2.5* presentan una reducida proliferación celular en el SHF posterior, los mutantes para *Smad1* presentan una mayor proliferación celular en este campo con una consecuente mayor elongación del tubo cardiaco. Además en los mutantes para *Nkx2.5* la expresión tanto de *Bmp2* como *Smad1* se encuentran incrementadas y la perdida de función de *Smad1* en estos mutantes rescata el fenotipo observado en el tracto de salida. Estos datos en su conjunto, permiten elaborar un modelo de *feedback* negativo en el SHF, por el cual *Bmp2* vía *Smad1* regula por un lado la inducción de la expresión de *Nkx2.5* como parte del programa cardiogénico y por otro lado la supresión de la proliferación en los progenitores de este campo. Posteriormente, *Nkx2.5* suprime la expresión de *Bmp2* y la activación de *Smad1* regulando así el balance temporal entre especificación y proliferación del SHF (Prall et al., 2007).

El ventrículo primitivo que caracteriza a los corazones mutantes para *Nkx2.5* está asociado a una disminución del factor de transcripción *Hand1*, lo que sugiere que *Nkx2.5* es necesario para una correcta expresión tanto en el espacio como en el tiempo de este

factor de transcripción. *Hand1*, que juega un papel clave en la regulación del balance entre proliferación y diferenciación de los cardiomiocitos (Biben and Harvey, 1997; Risebro et al., 2006) y de hecho los mutantes para *Hand1* presentan un fenotipo muy parecido a los mutantes para *Nkx2.5* (Firulli et al., 1998; Riley et al., 1998).

El correcto desarrollo del sistema de conducción ventricular requiere también de la expresión de *Nkx2.5*. Los mutantes homocigotos para este factor de transcripción carecen del nódulo atrioventricular mientras que los mutantes heterocigotos presentan un número muy reducido de fibras de Purkinje. La delección condicional de *Nkx2.5* en el ventrículo genera un fenotipo muy similar al que se observa en enfermedades del sistema de conducción en humanos, como una degeneración del sistema de conducción cardiaca y una hipertrabeculación (Pashmforoush et al., 2004).

Además, *Nkx2.5* juega un importante papel en la formación y en el mantenimiento de los límites entre las regiones del corazón embrionario junto con los factores T-box. *Nkx 2.5* mantiene la demarcación funcional entre el nodo sinoatrial, encargado de generar los impulsos nerviosos del corazón, y el miocardio atrial adyacente, mediante la inhibición de la expresión de *Tbx3*. *Tbx3* bloquea la expresión de los marcadores genéticos miocárdicos auriculares. Por lo tanto, la inhibición de *Tbx3* dependiente de *Nkx2.5* en el miocardio atrial evita que las células musculares atriales adquieran un fenotipo electrogénico (Mommersteeg et al ,2007a; Mommersteeg et al ,2007b).

Mef2c tiene un papel importante en la miogénesis del corazón. La expresión de este factor de transcripción se detecta en estadios muy tempranos del desarrollo, en los progenitores del musculo cardiaco y esquelético. Esta expresión es dependiente de *Gata4*. Wang et al. (2001) describieron la presencia de un promotor específico y responsable de la activación de *Mef2c* en las células miocárdicas del SHF, siendo activado desde una fase muy temprana del desarrollo cardiaco por *Gata4* y de *Islet1*, un factor de transcripción que delimita esta región del miocardio. La deficiencia de *Mef2c* se traduce en una letalidad embrionaria en ED10, 5 y en defectos cardiacos de las estructuras derivadas del campo secundario como la pérdida de un ventrículo derecho bien definido, lo que sugiere que es necesario para el desarrollo de los cardiomiocitos que derivan del SHF (Lin et al,. 1997; Lin et al., 1998; Verzi et al,. 2005). *Mef2c* activa la transcripción de genes estructurales del musculo cardiaco vitales para la función de este, como la troponina cardiaca, α-actina, α-MHC o Mlc2v entre otros. La pérdida de *Mef2c* genera la

disminución de algunos de estos genes pero no de todos. Esta disminución parcial es explicada por la presencia de otras isoformas de la familia Mef2 que rescatan parcialmente la perdida de función de *Mef2c*. La delección condicional en estadios más tardíos de la cardiogénesis, usando modelos murinos *cTnT-Cre*, genera embriones viables, lo que indica que el papel de *Mef2c* es necesario para el desarrollo temprano del miocardio pero no para el desarrollo tardío del mismo (Vong et al, 2005).

La regionalización y compartimentación que exhibe el corazón adulto es el resultado de la activación e inhibición de varias cascadas de señalización durante el desarrollo embrionario de este. Estas cascadas de señalización son moduladas en gran medida por la familia T-box (*Tbx*). Los miembros *Tbx1*, *Tbx2*, *Tbx3*, *Tbx5*, *Tbx18* y *Tbx20* tienen una expresión abundante y compartimentada en el corazón embrionario y juegan un papel vital en la regionalización del corazón, en el desarrollo de las cámaras cardiacas y en el sistema de conducción cardiaco (Papaioannou et al., 2014).

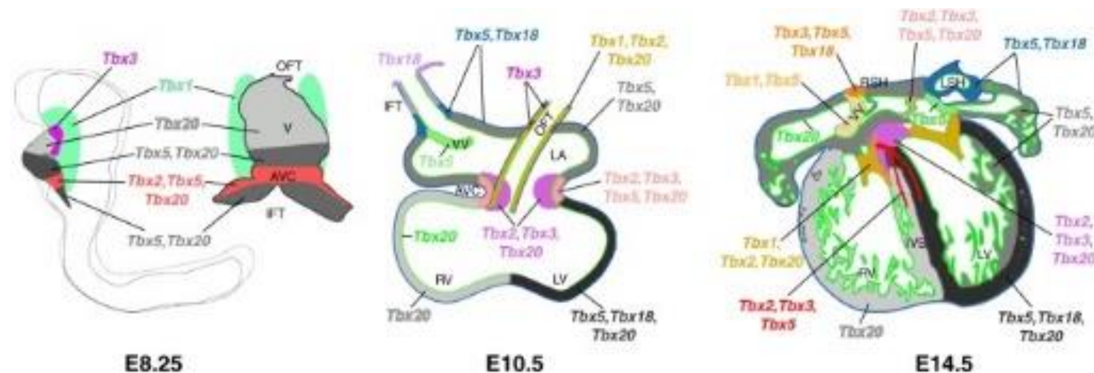


Figura 3. Expresión de los distintos miembros de la familia T-box durante el desarrollo cardiaco. Notese la regionalización tan especificada de cada uno de los miembros T-box en las distintas etapas de la cardiogénesis. Imagen tomada de Papaioannou et al., (2014).

Ensayos de pérdida de función en modelos murinos de *Tbx5* y *Tbx20* han puesto de manifiesto la importancia de ambos miembros en la morfogénesis de las cámaras cardiacas. La pérdida de uno de estos dos miembros se traduce en una letalidad embrionaria hacia la mitad de la gestación con un pobre desarrollo de las cámaras cardiacas (Bruneau et al., 1999; Stennard et al., 2005). *Tbx5* se expresa en los derivados de la región posterior del campo cardiaco primario e interacciona con *Nkx2.5* y *Gata4* para promover la activación de genes miocárdicos cámara-específicos como *Nppa* o *Gja5* (Bruneau et al., 2001). De forma similar, *Tbx20* promueve la expresión de genes cámara específicos en el miocardio derivado del FHF. *Tbx20* también regula la formación del

canal atrioventricular en su etapa más temprana y la transición epitelio-mesénquima (EMT) del endocardio modulando la activación de *Bmp2* (Cai et al., 2011). Además en el endocardio, *Tbx20* es necesario para que la vía de señalización Wnt sea activada durante la formación de los cojines endocárdicos y posteriormente en la remodelación de las válvulas cardiacas (Cai et al., 2013)

A diferencia de *Tbx5* y *Tbx20*, *Tbx2* y *Tbx3* se co-expresan en el miocardio del canal atrioventricular. Tanto la expresión de *Tbx2* como de *Tbx3* suprime la proliferación celular y reprime la expresión de los genes miocárdicos cámara-específicos estableciendo diferencias entre el miocardio de las cámaras cardiacas y del resto del corazón. La expresión de *Tbx2* y *Tbx3* no se solapan en el tiempo pero si en el espacio durante el desarrollo cardiaco. De hecho cuando *Tbx2* comienza a dejar de expresarse, *Tbx3* comienza a hacerlo, lo que sugiere que mientras *Tbx2* reprime la expresión de los genes miocárdicos cámara-específicos durante las primeras fases de la cardiogénesis, *Tbx3* lo hace en fases más tardías manteniendo los límites entre las cámaras cardiacas y el sistema de conducción cardiaco (Habets et al ,2002; Christoffels et al., 2004; Hoogaars et al., 2007). Además, *Tbx2* y *Tbx3*, junto con *Tbx1* modulan el desarrollo del polo arterial del corazón y sus estructuras derivadas (Mesbah et al., 2012).

Por último, *Tbx18* se expresa en el proepicardio, en los precursores del miocardio del seno venoso y el nódulo sinoatrial. Ratones deficientes para el *Tbx18* mueren debido a defectos en el retorno venoso hacia el corazón, a un retraso en la diferenciación miocárdica y a la ausencia del nódulo sinoatrial. El epicardio y los vasos coronarios que derivan de este también presentan defectos incompatibles con la vida (Greulich et al., 2011; Wu et al., 2013).

Además de los factores de transcripción ya mencionados, la cardiogénesis requiere algunos más factores de transcripción que son necesarios para que esta se desarrolle de manera correcta, entre los cuales hemos de destacar Srf y Pitx2.

Srf juega un papel clave en el desarrollo del miocardio, modulando la expresión de proteínas que hacen posible que esta capa del corazón adquiera el fenotipo contráctil que la caracteriza. Durante la cardiogénesis, *Srf* se expresa en las crestas cardiacas, en el tubo cardiaco y posteriormente en el miocardio de las cámaras cardiacas (Barron et al., 2005). La deficiencia de *Srf* se traduce en una letalidad embrionaria debida a una

insuficiencia cardiaca durante la maduración de las cámaras. Además, la ausencia de *Srf* provoca la disminución de la alfa actina cardiaca y de proteínas accesorias que forman parte de los sarcómeros, generando cardiomiocitos sin capacidad contráctil. De hecho, *Srf* orquesta la biogénesis de los sarcómeros desde múltiples niveles, modulando la expresión de remodeladores de la cromatina, proteínas miofibrilares y proteínas que conducen el impulso entre las células, siendo la expresión de *Srf* necesaria para un correcto desarrollo de los sarcómeros y para la generación del primer latido embrionario (Niu et al., 2008).

Srf por si solo tiene una capacidad muy limitada de activar los promotores de los genes musculares cardiacos y requiere de la participación de *Gata4* y *Nkx2.5* entre otros factores para poder llevar a cabo su función dentro del programa miogénico. Durante el desarrollo, la expresión de estos tres factores de transcripción coinciden y es necesaria la interacción entre ellos para la activación del promotor de la alfa actina cardiaca (Sepulveda et al., 1998; Sepulveda et al., 2003; Chen and Schwartz, 1996; Schwartz, 2010).

Srf también ha sido señalado como un importante inductor de determinados microRNAs con un papel crucial en el desarrollo miogénico temprano del corazón como miR-1 o miR-503, como se verá más adelante. Estos microRNAs tienen un elemento CArG en su región promotora, al que se une *Srf* activándolos y promoviendo la expresión de estos miRNAs (Zhao et al 2007; Niu et al., 2008).

En el ratón, el gen *Pitx2* presenta 3 isoformas, *Pitx2a, Pitx2b y Pitx2c.* Mientras que las dos primeras isoformas comparten el mismo promotor, *Pitx2c* tiene un promotor alternativo situado aguas arriba del exón 4 (Gage et al., 1999; Schweickert et al., 2000). Además en humanos hay una cuarta isoforma, *PITX2D*, que actua como dominante negativo de las demás isoformas (Cox et al., 2002). Las isoformas de *Pitx2* se expresan de forma simétrica en el mesénquima craneal a partir del ED 7. Sin embargo, la isoforma *Pitx2c* se expresa de forma asimétrica en el lado izquierdo de la placa lateral mesodérmica manteniéndose durante la cardiogénesis y la etapa adulta (Chinchilla et al., 2011; Kirchhof et al., 2011; Wang et al., 2010).

Es importante destacar que el corazón es el primer órgano en mostrar una asimetría izquierda-derecha durante el desarrollo. Diversos análisis genéticos han puesto de manifiesto que la asimetría morfológica durante la organogénesis viene precedida de

una asimetría molecular (Spéder et al., 2007; Bakkers et al., 2009; Namigai et al., 2014). Esta asimetría molecular comienza a detectarse en la región nodal del embrión y posteriormente en la lámina lateral del mesodermo (LMP), y es modulada por la vía de señalización Nodal>Pitx2 (Vandenberg y Levin, 2013).

La expresión de *Pitx2c* durante la cardiogénesis es detectada por primera vez en la cresta cardiaca izquierda pero no en la derecha. En el tubo cardiaco, esta expresión queda restringida en el lado izquierdo del mismo, manteniéndose durante la torsión cardiaca en las estructuras cardiacas derivadas lado izquierdo del tubo cardiaco. En el ED10,5, *Pitx2c* se expresa en el tracto de salida izquierdo, en el miocardio de la aurícula izquierda y en el miocardio localizado en la porción lateral izquierda del canal atrioventricular, en la porción ventral de ambos ventrículos y en la región izquierda del tracto de salida. Posteriormente, durante la etapa posnatal y adulta, la expresión de Pitx2c se queda restringida esencialmente a la aurícula izquierda (Franco y Campione, 2003).

Ratones deficientes para el gen *Pitx2* presentan una letalidad embrionaria hacia la mitad de la gestación. Los embriones deficientes para Pitx2 exhiben un cierre incompleto de la pared corporal y graves defectos cardiacos. Los corazones mutantes se caracterizan por un isomerismo auricular derecho (RAI), en el que la aurícula izquierda es similar a la aurícula derecha, tanto en su localización como en su forma, y en el que ambas cámaras auriculares se encuentran unidas debido a la ausencia del septo interatrial. Asimismo, los corazones mutantes para Pitx2 muestran un remodelado incorrecto de la región interna del canal atrioventricular así como una errónea rotación y elongación del tracto de salida lo que conduce a defectos en el septo interventricular y en el alineamiento de los ventrículos con los vasos arteriales. Además, estos corazones presentan un desarrollo incorrecto de las venas pulmonares y del nodo sinoatrial (Mommersteeg et al., 2007a; Mommersteeg et al., 2007b).

La delección específica de cada una de las isoformas de Pitx2 en modelos murinos han puesto de manifiesto que todos los defectos observados en el sistema cardiovascular son consecuencia de la pérdida de función de *Pitx2c*, y no a *Pitx2a* o *Pitx2b* (Liu et al 2002).

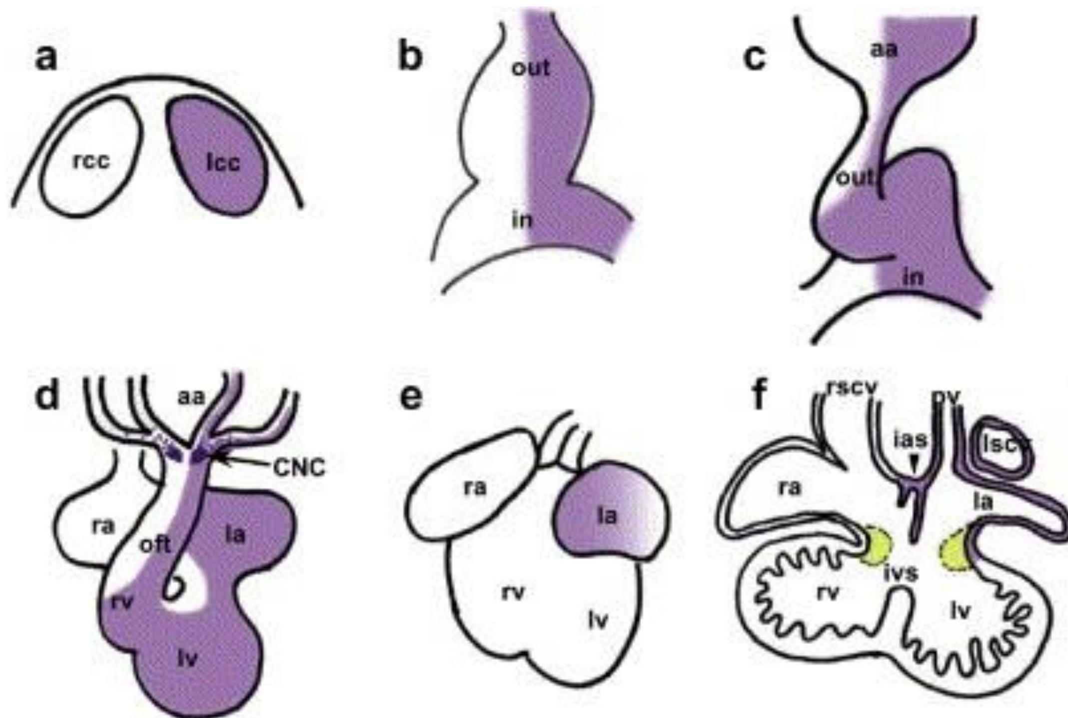


Figura 4. Imagen representativa de la expresión de Pitx2c durante la cardiogénesis. Imagen tomada de Franco y Campione, 2003.

Múltiples estudios asociativos del genoma (GWAS) han identificado y relacionado varios polimorfimos de un solo nucleótido (SNPs) localizados en el cromosoma 4p25 con una mayor predisposición a padecer fibrilación atrial (AF), la enfermedad arritmogénica más frecuente en seres humanos (Gudbjartsson et al, 2007; Ellinor et al., 2012; Hakin y Shen, 2014). Estos SNPs se encuentran en la vecindad de *Pitx2*, lo que junto con el papel de *Pitx2* en la cardiogénesis, sugiere un posible rol de Pitx2 en AF. En línea con este posible papel, la haploinsuficiencia de *Pitx2c* en modelos murinos predispone a padecer AF (Wang et al., 2010). Por otro lado, Chinchilla et al., (2011) pusieron de manifiesto que la expresión de *Pitx2c* se encontraba reducida en pacientes humanos que padecían AF. Además, la delección selectiva de *Pitx2c* en las aurículas genera defectos electrofisiológicos que aumentan el riesgo a padecer AF. Estos datos apoyan que una reducción en la dosis génica de Pitx2c aumenta la predisposición a padecer AF.

Más recientemente, Lozano et al., (2016) ha demostrado que la insuficiencia de *Pitx2c* regula la expresión de *Wnt8* que a su vez modula una compleja cascada de señalización que tiene un gran impacto en la homeostasis de los canales de calcio y en eventos pro-arritmogénicos .

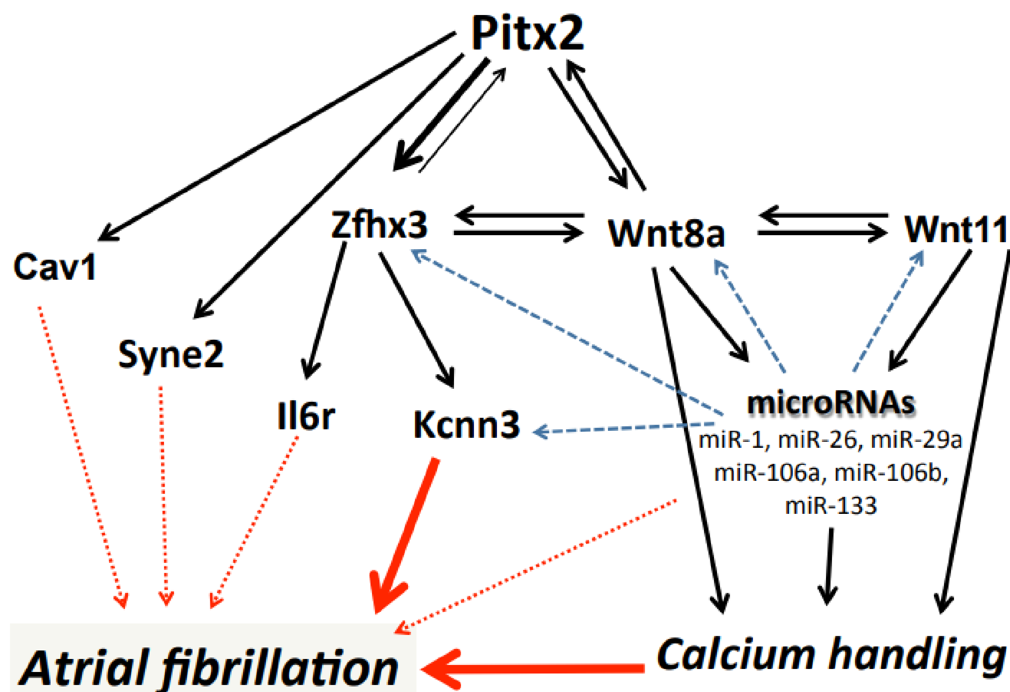


Figura 5. Cascada de señalización que conduce a AF. Notese como Pitx2 adquiere una posición central en esta cascada modulando la expresión de muchos de sus integrantes. Imagen tomada de Lozano et al., 2016.

2. Regulación post-transcripcional del desarrollo cardiaco

En los últimos años se ha demostrado que todos los procesos celulares están regulados post-transcripcionalmente y que esta modulación es necesaria para una correcta señalización de las distintas cascadas génicas que median dichos procesos. Múltiples estudios han señalado que las cascadas génicas cardiacas están sujetas a una intensa regulación post-transcripcional ejercida en su mayor parte por RNAs no codificantes (ncRNAs).

Los ncRNAs pueden ser divididos en dos clases en función de su tamaño: 1) RNA no codificantes con una longitud menor de 200 nucleótidos, entre los que destacan los microRNAs y 2) RNA no codificantes con una longitud mayor de 200 nucleótidos, *long non coding RNAs* (*lncRNAs*). El papel de los microRNAs en el desarrollo cardiaco ha sido ampliamente estudiado, posicionándose como reguladores cruciales en las diferentes

fases de la cardiogénesis (Yan y Jiao, 2016), mientras que el papel de los *lncRNAs* ha comenzado a ser estudiado recientemente (Klattenhoff et al., 2013; Grote et al., 2013; Ounzain et al., 2015; Anderson et al., 2016).

2.1. MicroRNAs

Los microRNAs (miRNAs) son pequeñas moléculas de 22-24 ribonucleótidos que no codifican proteína y que tienen la capacidad de regular la expresión génica post-transcripcionalmente al interactuar con el mRNA modulando su estabilidad y/o traducción a proteínas.

La biogénesis de los microRNAs constituye un proceso dinámico que incluye la participación de diferentes complejos con acción endonucleasa. Inicialmente, los microRNAs son transcritos en su mayoría por la RNA polimerasa II en una larga cadena de RNA precursora que presenta una caperuza en el extremo 5’ y una cola de poly (A) en su extremo 3’, pudiendo alcanzar hasta una kilobase de longitud y contener diferentes microRNAs en su estructura (Cai et al., 2004). Esta cadena de RNA precursora recibe el nombre de pri-miRNA. La porción codificadora del microRNA en el pri-miRNA forma una horquilla que es reconocida y escindida por el complejo *Drosha* y su cofactor *Dgcr8* en el núcleo (Landthaler et al., 2004). La horquilla escindida recibe el nombre de pre-miRNA y es exportada al citoplasma por un transportador dependiente de RAN-GTP, donde es reconocida por el complejo *Dicer* que corta la molécula de pre-miRNA formando un microRNA maduro de doble cadena. La cadena menos estable termodinámicamente es introducida en el complejo RISC, mientras que la otra cadena es degradada, aunque en algunos casos ambas cadenas son incorporadas. El complejo RISC media la interacción entre el miRNA y el mRNA produciendo como consecuencia la represión o desestabilización de este último (Katz et al, 2016).

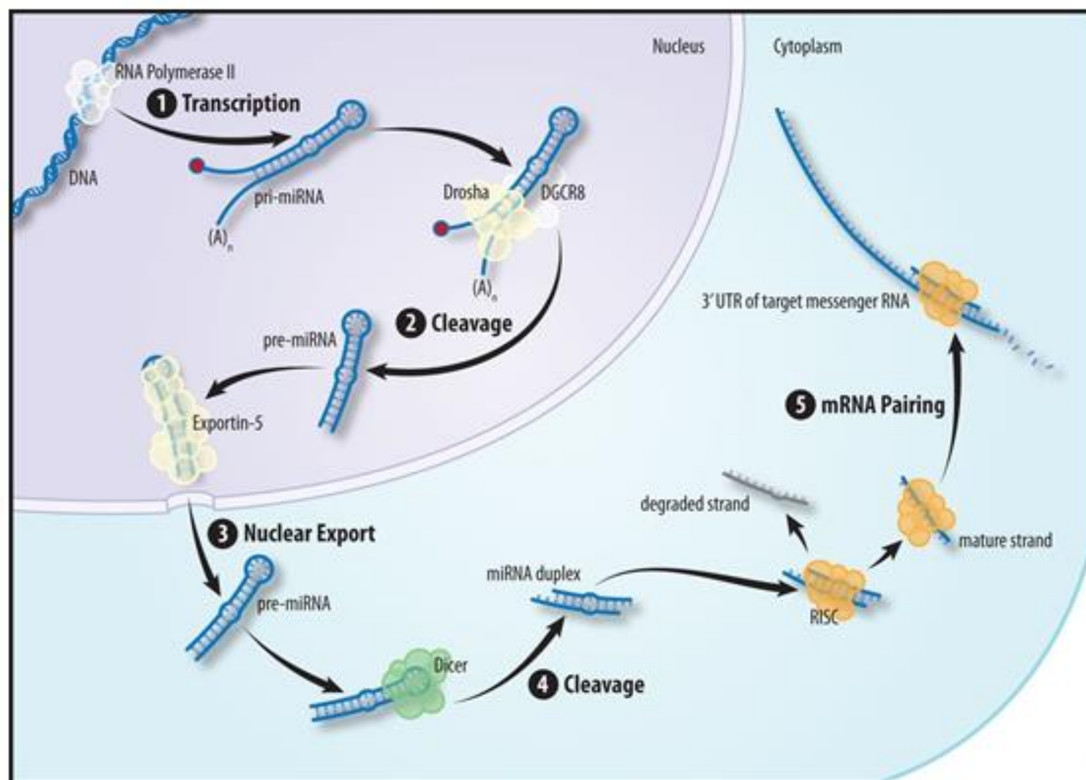


Figura 6. Representación esquemática de la biogénesis y función de los microRNAs. Imagen tomada de Katz et al (2016).

Los microRNAs, en su mayoría, ejercen su función uniéndose a la región 3'UTR de su mRNA diana causando la degradación de este. Así mismo, el complejo RISC unido al microRNA puede interaccionar con la maquinaria transcripcional o promover la deadenilación del mRNA lo que en ambos casos reprime la traducción del mismo, por lo que son importantes reguladores de los niveles de expresión de las proteínas (Rajewsky, 2006; Ezzeddine et al., 2007).

2.2. Funciones de los microRNAs durante el desarrollo cardiaco

La necesidad e importancia de la biogénesis de los microRNAs para un correcto desarrollo cardiaco ha sido demostrada usando distintos modelos de pérdida de función de *Dicer1* en distintas fases de la cardiogénesis. La delección de esta endonucleasa en los distintos modelos estudiados se traduce en una letalidad embrionaria y en diferentes anomalías cardiacas incompatibles con la vida. Así la delección específica de *Dicer1* en los progenitores cardiacos en un modelo *Nkx2.5-Cre* provoca un retraso en el desarrollo

ventricular y una letalidad embrionaria en ED12,5. Del mismo modo, la delección miocárdica de *Dicer1* en un modelo *cTnT-Cre* provoca defectos en el desarrollo de las paredes ventriculares, en las que se observa una baja compactación, acompañada de una reducida proliferación celular, un incremento en la muerte celular y una letalidad embrionaria en ED15,5.

Ensayos similares en los que se ha eliminado *Dicer1* en las crestas neurales cardiacas o en el epicardio tienen como consecuencia la adquisición de fenotipos cardiacos severos. La delección de *Dicer1* en las crestas neurales cardiacas genera defectos en la aorta y en la septación ventricular así como doble tracto de salida (Huang et al, 2010a). Mientras que el fenotipo observado en la delección epicárdica se caracteriza por defectos en el desarrollo vascular así como una reducida diferenciación y proliferación de células epicárdicas (Huang et al, 2010b). El conjunto de estos fenotipos evidencia un papel crucial de los microRNAs durante el desarrollo cardiaco (Zhao et al., 2007; Chen et al., 2008; Saxena y Tabin, 2010; Peng et al., 2014)

En línea con el papel de los microRNAs en la cardiogénesis, se han descrito varios microRNAs implicados en la regulación de las rutas de señalización cardiacas. Las familias de miR-1, miR-133, miR 17-92 son claros ejemplos de microRNAs necesarios para un correcto desarrollo cardiaco (Zhao et al., 2005; Chen et al., 2006; Meder et al., 2008; Mendell, 2008; Wang et al., 2010).

Las familias miR-1 y miR-133 se expresan de manera específica en el músculo cardiaco y esquelético durante el desarrollo embrionario y en la etapa adulta. Ambos derivan del mismo *locus* génico y muestran una alta conservación entre diferentes especies (Chen et al., 2006). En el corazón, la transcripción de ambos microRNAs está regulada por *Srf* y *Mef2* mientras que en el musculo esquelético su expresión esta mediada por *MyoD* (Liu et al., 2007).

El microRNA miR-1 juega un papel clave en la cardiogénesis al modular negativamente la traducción de *Hand2*, un factor de transcripción crítico para la correcta expansión de los cardiomiocitos ventriculares (Zhao et al., 2007; McFadden et al., 2005). Ensayos de ganancia de función de este miRNA en modelos murinos, han mostrado una disminución de la proliferación de cardiomiocitos y defectos en la trabeculación similares a los observados en ratones deficientes para *Hand2* (Meder et al., 2008). Del mismo

modo, ratones deficientes para el miR-1 muestran defectos en la morfogénesis cardiaca, en el sistema de conducción y una hipoplasia ventricular (Zhao et al., 2005). Estos datos sugieren un papel clave de miR-1 en la regulación de la proliferación de los cardiomiocitos durante el desarrollo.

De forma similar, el microRNA miR-133 participa en la regulación de la proliferación de los cardiomiocitos modulando negativamente la traducción de la ciclina D2 y de *Srf*. La familia miR-133 está formada por dos isoformas, miR-133a-1 y miR-133a-2. Ratones deficientes para una u otra no presentan fenotípicos cardiacos anómalos, lo que sugiere una compensación por parte de una isoforma cuando la otra está ausente. Sin embargo ratones deficientes para ambas isoformas presentan una letalidad embrionaria temprana y defectos en el septo ventricular. A diferencia del fenotipo observado en los ratones doble mutantes, la sobreexpresión del miR-133 provoca una letalidad embrionaria debida a una reducción en la proliferación de los cardiomiocitos (Lui et al., 2008).

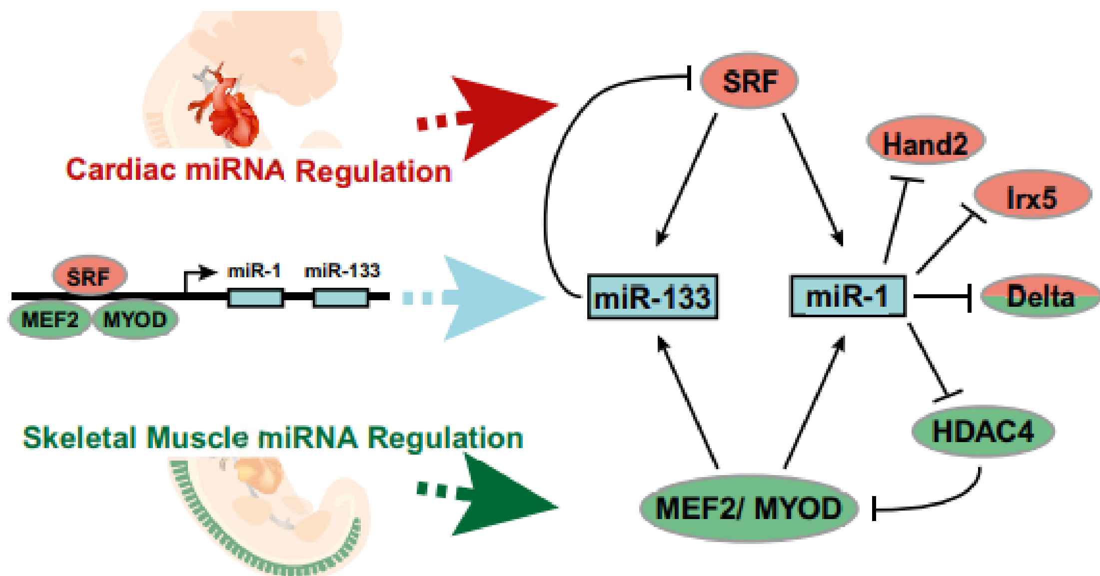


Figura 7. Regulación de la expresión de miR1/133 durante el desarrollo embrionario. Imagen tomada de Cordes and Srivastava, 2010.

Por otro lado el *cluster* miR-17-92 que codifica 6 microRNAs, miR-17, miR-18a, miR-19a, miR-20a, miR-19b-1 y miR-92a-1, promueve la diferenciación miocárdica del SHF reprimiendo *Islet1* y *Tbx1* al unirse a su región 3'UTR respectivamente. Ratones deficientes para este *cluster* mueren a los pocos días de nacer y muestran doble salida de

ventrículo derecho (DORV) así como defectos en la septación ventricular. Del mismo modo los embriones nulos presentan una inhibición de la diferenciación de los cardiomiocitos y un aumento de la expresión de *Islet1* y *Tbx1*. Acorde con estas observaciones, la sobreexpresión transgénica del *cluster* miR-17-92 reduce drásticamente la expresión de *Islet1* y *Tbx1*. La transcripción de este *cluster* en el SHF está mediada por *Bmp2* y *Bmp4* (Mendell, 2008; Wang et al., 2010).

Recientemente se han señalado a dos microRNAs, miR-322 y miR-503 como importantes reguladores de la diferenciación de los cardiomiocitos. Estos microRNAs promueven la diferenciación de los cardiomiocitos reforzando el destino celular adquirido por el mesodermo cardiogénico. Los microRNAs miR-322 y miR-503 se expresan en los progenitores cardiacos y su expresión está restringida a aquellas células que previamente han expresado *Mesp1*, ya que su transcripción esta modulada por este factor de transcripción. Estos miRNAs actúan inhibiendo la traducción de *Celf1*, que es expresado en los tejidos derivados del neuroectodermo y que juega un papel en la diferenciación de estos. La acción de estos microRNAs proporcionan un ejemplo de como *Mesp1* reprime decisiones celulares en las mismas fases del desarrollo (Shen et al., 2017).

2.3. Long non coding RNAs

El desarrollo de nuevas técnicas de secuenciación masiva ha conducido a la anotación y descubrimiento de un gran número de *long non coding* RNAs. Las estimaciones iniciales indicaban que el genoma humano poseía 9640 miembros de esta clase de moléculas de RNA. Sin embargo, las últimas estimaciones indican un total de 96308 genes que transcriben *lncRNAs* en todo el genoma humano (Derrien et al., 2012; Fang et al., 2017). Esta cifra advierte que el número de genes que transcriben *lncRNAs*, es tres veces superior al número de genes que transcriben RNA mensajeros con capacidad de codificar proteínas, lo que sugiere la importancia de esta clase de RNA no codificantes en los procesos celulares. Importancia que está comenzado a ser puesta de manifiesto en múltiples contextos y procesos (Schhmitz et al., 2016; Rosa y Ballarino, 2011; Wapinsky y Chang, 2011).

Los *lncRNAs* son moléculas de RNA con una longitud superior a 200 nucleótidos, que no tienen la capacidad de codificar proteínas. Al igual que los RNA mensajeros, estas

moléculas de RNA son trascritas por la RNA polimerasa II y están sujetas a modificaciones post-transcripcionales como la adicción de una cola de poly (A) en el extremo 3' o una caperuza en el extremo 5'. Existe un grupo minoritario de *lncRNAs* que son trascritos por la RNA polimerasa III y que no se encuentran poliadenilados (Diece et al., 2007). Estructuralmente están compuestos por exones e intrones y se encuentran sujetos a procesos de *splicing* alternativo. En comparación con los RNA mensajeros, el número y la longitud de los exones de los *lncRNAs* es menor, y de igual modo se encuentran mucho menos conservados entre las especies. Sin embargo, los intrones de los *lncRNAs* están altamente conservados y presentan las mismas señales en sus extremos que los intrones de los RNA mensajeros, necesarias para ser reconocidos por la maquinaria de *splicing* (Derrien et al., 2012). Si bien la mayoría de los *lncRNAs* se encuentran en el genoma nuclear, se han descrito varios ejemplos de *lncRNAs* que se localizan en el genoma mitocondrial. Estos *lncRNAs* mitocondriales son transcritos y procesados por la maquinaria transcripcional mitocondrial pero se encuentran bajo la regulación de proteínas nucleares (Rackham et al., 2011).

Aunque los *lncRNAs* son definidos como moléculas de RNA sin capacidad para codificar proteínas, existen varios ejemplos de *lncRNAs* que contienen pequeños marcos de lectura abiertos (ORFs), a los que pueden unirse ribosomas y sintetizar pequeños péptidos (Derrien et al 2012; Nelson et al 2016).

Los *lncRNAs* tienen una expresión muy restringida tanto en el tejido como en el tiempo en que se expresan, en comparación con los RNA mensajeros, aunque tienen niveles de expresión mucho menores que estos últimos. Esta expresión tan específica sugiere la participación de estas moléculas de RNA en múltiples procesos celulares que se encuentran regulados de manera muy precisa, lo que ha sido evidenciado por múltiples estudios (Engreitz et al., 2016; Gloss y Dinger, 2016). A nivel celular, *lncRNAs* pueden localizarse tanto en el núcleo como en el citoplasma, incluso pueden localizarse en ambos compartimentos celulares. Su localización celular es un reflejo del papel funcional que ejercen. Así, los *lncRNAs* citoplasmáticos, suelen actuar como reguladores post-trancripcionales mientras los *lncRNAs* nucleares suelen actuar como reguladores transcripcionales (Chen, 2016).

Una de las características que mejor define a los *lncRNAs* es su escaso grado de conservación entre especies. La conservación de los *lncRNAs* es tan pobre que, muchos

de ellos no se encuentran conservados entre especies de la misma familia (Johnsson et al., 2014). Este escaso grado de conservación queda ejemplificada por *Braveheart*, un *lncRNA* especifico de ratón (*mus musculus*) (Klattenhoff et al., 2013). Sin embargo, a pesar del escaso grado de conservación entre especies, la comparación de los sitios de *splicing* sugieren que los *lncRNAs* se encuentran evolutivamente conservados siendo la mayoría de estos, tan antiguos como los mamíferos (Derrien et al., 2012).

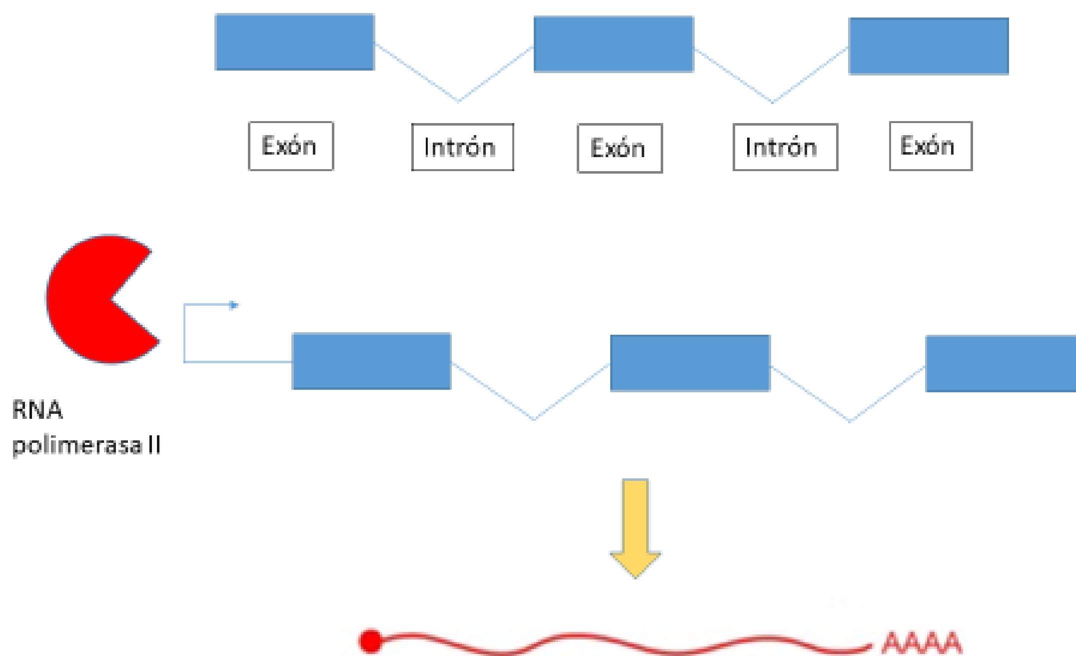


Figura 8. Estructura y biogénesis de los lncRNAs

2.3.1. Clasificación de lncRNAs

Los *lncRNAs* pueden ser clasificados en cinco grupos bien establecidos, en función de su localización genómica y los elementos génicos que tienen a su alrededor: 1) *LncRNAs* que son transcritos desde el mismo promotor que un gen que se encuentra adyacente a estos. Este tipo de *lncRNA* puede ser transcrito en dirección 3' o 5' y puede ser transcrito desde la misma cadena de ADN o desde la cadena complementaria. Tanto la expresión del *lncRNA* como del RNA mensajero del gen que se encuentra adyacente suelen estar correlacionada siendo la expresión del RNA mensajero modulada por la acción del

lncRNA. 2) *LncRNAs* que se encuentran localizados entre dos genes codificantes para proteína a una distancia entre ambos aproximadamente de 10 kb, en denominados desiertos genómicos. Estos *lncRNAs* reciben el nombre de *long non coding* RNAs intergénicos (*lincRNAs*) (Ulitsky y Bartel, 2013). 3) LncRNAs que son transcritos desde intrones de genes que codifican para proteínas o desde regiones promotores. Existe una subclase de *lncRNAs* intrónicos denominados, snoRNAs. Esta subclase no tiene la estructura típica del resto de *lncRNAs* y tiene una localización exclusivamente nuclear (Yin et al., 2012). La expresión de los *lncRNAs* que derivan de regiones promotoras se correlaciona con la expresión de los promotores activos procedentes de estas regiones, así como con la expresión de los genes dianas de estos promotores activos (Ounzain et al., 2015; Ounzain y Pedrazzini, 2015). 4) *LncRNAs* circulares (circRNAs) que pueden generarse durante el *splicing* alternativo de genes que codifican proteínas (Memczak et al., 2013). 5) Finalmente, hay otra clase de *lncRNAs* que contiene microRNAs dentro de su estructura genética, como ejemplifica *H19*, cuyo primer exón codifica el microRNA miR-675 (Keniry et al., 2012).

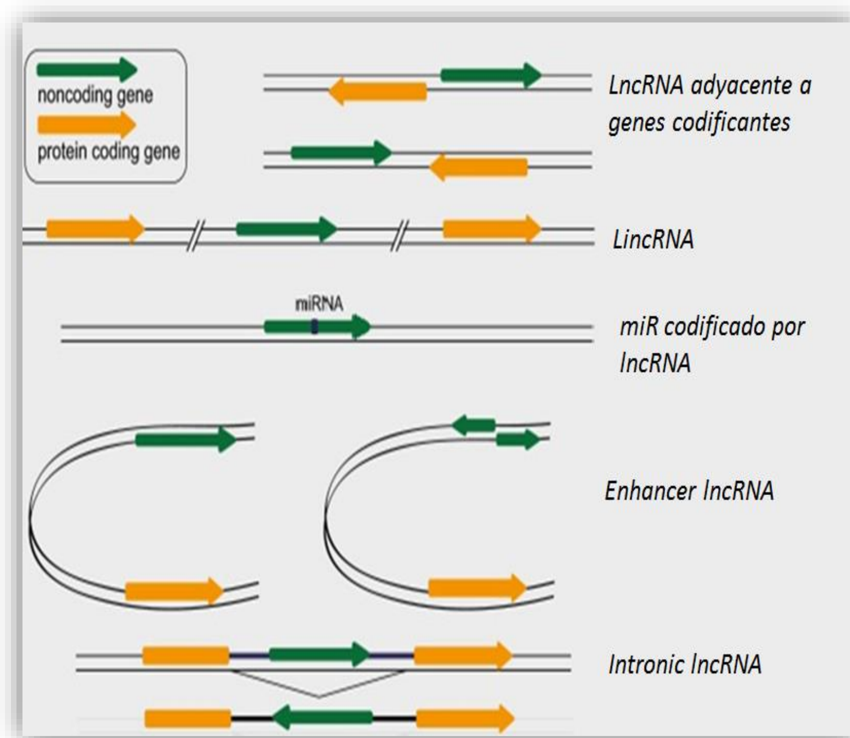


Figura 9. Clasificación de los lncRNAs en función de su estructura genómica y de los genes que se encuentran en sus alrededores. Imagen modificada de Schmitz et al., 2016.

2.3.2. Funciones de los lncRNAs

La complejidad que exhiben los *lncRNAs* tanto en su estructura como en su localización celular se refleja también en sus distintos modos de acción, pudiendo actuar tanto como reguladores a nivel transcripcional como reguladores a nivel post-transcripcional.

A nivel transcripcional, los *lncRNAs* pueden modular el escenario epigenético de la célula a través de diferentes vías. Se han descrito hasta cuatro tipos de acciones distintas que ejercen los *lncRNAs* en este contexto. Algunos *lncRNAs* pueden actuar como guías moleculares, uniéndose, bien a factores de transcripción o a subunidades proteicas de complejos remodeladores de la cromatina, y dirigirlos hacia sus dianas génicas, promoviendo o reprimiendo la actividad génica en función de si los complejos son activadores (como el complejo MLL) o represores (como el complejo PRC2). Este tipo de acción puede ser llevada a cabo en *cis* (i.e *Xist*) o en *trans* (i.e *HOTAIR*) (Hasegawa et al., 2010; Gupta et al., 2010).

Se han descrito varios *lncRNAs* que pueden actuar como *scaffold* de diferentes complejos facilitando el ensamblaje y siendo partes funcionales de los mismos. *ANRIL*, un *lncRNA* que ha sido descrito como un factor de riesgo de en enfermedades coronarias promueve el silenciamiento del locus INK4b-ARF-INK4a al actuar como plataforma para el ensamblaje y siendo parte funcional del complejo represor PRC (Tsai et al., 2010; Aguilo et al., 2011).

Muchos de los *lncRNAs* son denominados como *lncRNAs* potenciadores dado que pueden actuar como potenciadores de la transcripción, promoviendo y manteniendo la conformación génica necesaria para que la maquinaria transcripcional pueda acceder a las regiones promotoras (Mousavi et al., 2013). De forma similar, otros *lncRNAs* pueden actuar como represores de la expresión genética al impedir el acceso de la RNA polimerasa a los promotores. En este contexto, *Playrr*, un *lncRNA* situado aguas arriba de *Pitx2*, reprime la expresión de *Pitx2c* al interaccionar con su promotor e impidiendo que sea accesible para la RNA polimerasa II (Welsh et al., 2015).

Por último, otros *lncRNAs* pueden actuar como moléculas inhibidoras al competir con factores de transcripción o complejos remodeladores de la cromatina por sus dianas. *Myheart*, es un *lncRNA* cardiaco que ejerce actúa como una molécula inhibidora al evitar

que el complejo BRG1-BAF, que está involucrado en el desarrollo de la hipertrofia cardiaca, se una a sus dianas. (Han et al., 2014).

A nivel post-transcripcional, los *lncRNAs* pueden regular la expresión génica interactuando con otros RNA no codificantes como microRNAs, con la maquinaria transcripcional o con los RNA mensajeros (Yoon et al., 2013).

Algunos *lncRNAs* nucleares pueden participar en la maduración de los pre-RNA mensajeros al interaccionar con importantes factores que median el *splicing* alternativo. Por ejemplo, *Malat1* interactúa con el factor de *splicing* SR (serina/arginina) modulando la concentración y distribución del mismo en el núcleo permitiendo un correcto *splicing* alternativo (Tripathi et al., 2010).

Existen ejemplos de *lncRNAs* que interaccionan con la secuencia de los RNA mensajeros, generando dúplex con ellos modulando así, la estabilidad y la traducción de los mismos. Que esta interacción promueva o no la estabilidad del RNA mensajero, depende de la eficiencia de la misma. De este modo, una interacción RNA-RNA completa, promueve la estabilidad del RNA mensajero mientras que si la interacción es incompleta, el RNA mensajero es degradado y por tanto la traducción del mismo es inhibida (Kim et al., 2005; Kim et al., 2007).

Un pequeño número de *lncRNAs* modula la traducción de los RNA mensajeros al asociarse con los ribosomas. Por ejemplo, *LincRNA-21*, un *lncRNA* que se encuentra co-distribuido con ribosomas, reprime la traducción de varios RNA mensajeros, al unirse a la secuencia de estos e impidiendo que puedan ser reconocidos y traducidos por los ribosomas (Yoon et al., 2012). Además, se han descrito varios *lncRNAs* que pueden interaccionar con la maquinaria transcripcional y modular su actividad. Así, el *lncRNA BC1* interactúa a través de la región 3'UTR de sus dianas con distintos represores de la traducción, inhibiendo así el ensamblaje del complejo de iniciación de la traducción (Wang et al., 2005). Por el contrario, el *lncRNA Uchl1*, promueve la generación de complejos poli-ribosomales que potencian la traducción del RNA mensajero del gen adyacente a *Uchl1* (Carrieri et al., 2012).

Por último, se han descrito multitud de *lncRNAs* que modulan la función de los microRNAs, bien interaccionado con ellos y evitando que se unan a sus dianas o modulando su expresión al servir como reservorios genéticos de algunos de ellos. Por

ejemplo, *linc-MD1* puede secuestrar a los microRNAs miR-133 y mR-135, promoviendo la expresión de las dianas de estos últimos en el musculo, como *Maml1* o *Mef2c*, entre otros (Cesana et al., 2011). En el contexto cardiaco, *H19*, un *lncRNA* ampliamente estudiado, modula la expresión del microRNA miR-675, que se encuentra codificado en su primer exón (Liu et al., 2016)

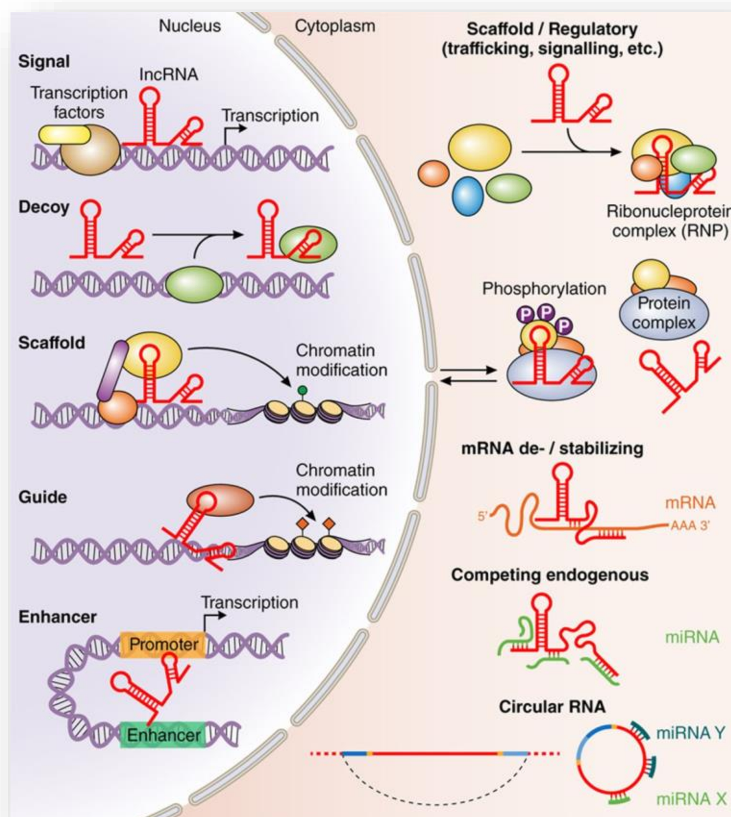


Figura 10. Principales funciones de los lcnRNAs en la célula a nivel trancripcional como post-transcripcional. Imagen tomada de Bär, C et al, 2016

2.4.Papel de los lncRNAs durante el desarrollo cardiaco

Distintos estudios han demostrado el papel de diferentes *lncRNAs* en el desarrollo cardiaco, regulando tanto transcripcional como post-transcricionalmente las cascadas de señalización que median la cardiogénesis. Hasta la fecha se han identificado ocho

lncRNAs cuya funcion es necesaria para un correcto desarrollo cardiaco; *Braveheart*, *Carmen*, *Fendrr*, *Alien*, *Tbx5as*, *Upperhand*, y *LncRNA u-167*.

Braveheart es un *long non coding* RNA especifico de ratón cuya función, es clave para la adquisición del compromiso celular cardiaco y para la correcta expresión del programa génico de diferenciación cardiaco. *Braveheart* actua aguas arriba de *Mesp1* y es requerido para la activación y expresión de este factor de transcripción. La delección de *Braveheart* tiene como consecuencia un fallo en la activación de factores claves para el desarrollo del corazón y para la diferenciación de los cardiomiocitos como *Mesp1*, *Hand1*, *Hand2*, *Nkx2*.5 y *Tbx20*. Estos datos sugieren que *Braveheart* modula positivamente la activación de la cascada de señalización gobernada por *Mesp1*, promoviendo la especificación del mesodermo cardiogénico (Klattenhoff et al., 2013). Además, la sobreexpresión de *Braveheart* en celulas mesenquimales pluripotentes provoca una diferenciación de estas hacia un fenotipo cardiogenico al aumentar la expresión de factores de transcripción cardiacos claves durante la cardiogenesis como *Gata4*, *Nkx2*.5 o *Islet*-1 asi como genes claves involucrados en procesos de EMT como *Snail* o *Twist* y proteinas cardiacas estructurales como troponina cardiaca *cTnT* (Hou et al., 2017). *Braveheart* tambien es necesario para el correcto mantenienimento de la homeostasis en los cardiomiocitos asi como para el mantenimiento de su destino celular (Klattenhoff et al., 2013).

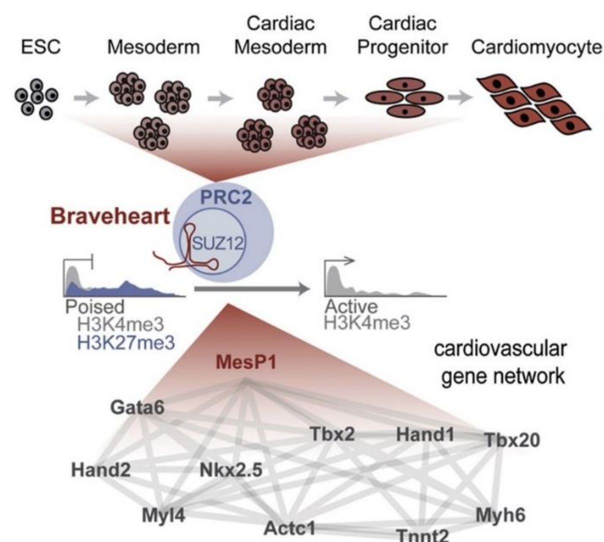


Figura 11. Braveheart es necesario para la adquisión del destino celular cardiaco y para para la correcta expresión y activación de Mesp1 y de la cascada genica que este factor de transcripción modula. Imagen tomada de Klattenhoff et al., 2013.

Curiosamente, la expresión de *Braveheart* es modulada por otro *lncRNA* especifico del tejido cardiaco, *Carmen* (Ounzain et al., 2015). Ounzain et al. (2015) identificaron en el transcriptoma de celulas progenitoras cardiacas humanas, la expresión diferencial de 570 *lncRNAs*, muchos de los cuales se encontraban asociados a regiones potenciadoras que se encuentran activas durante la cardiogenesis. Entre todos los *lncRNAs* identificados, destaca *Carmen*, un *lncRNA* cardio-especifico que se encuentra asociado a una región super potenciadora del genoma, activa durante el desarrollo cardiaco. *Carmen* se expresa tanto en corazones fetales como en corazones adultos y se encuentra conservado entre los mamiferos. *Carmen* juega un papel clave en la cardiogenesis temprana al regular la cascada de señalización que media la especificación y diferenciación del mesodermo precardiogenico modulando la expresión de los genes que la conforman . Ademas *Carmen* regula la expresion de factores clave pluripotenciales como *Nanog* y *Sox2*, lo que sugiere un papel bivalente de este *lncRNA* durante el desarrollo cardiaco, tanto en la especificación y diferenciación de mesodermo cardiogenico como en la pluripotencia celular. *Carmen* actua en *trans* interaccionando con las subunidades SUZ12 y EZH2 del complejo represor PRC2, quien metila la lisina 27 de la histona H3, generando una marca represora en el genoma necesaria para una correcta diferenciación cardiaca (Ounzain et al., 2015). Ademas, al igual que *Braveheart*, *Carmen* tambien es necesario tanto para el mantenimiento de la homeostasis de los cardiomiocitos como para el mantenimiento del destino celular de estos (Ounzain et al., 2015).

Grote el al. (2013) identificaron un *lncRNA* cuya expresión se encuentra restringida a la placa lateral mesodermica, al que denominaron *Fendrr*, y cuya función es esencial para el correcto desarrollo de las estructuras que derivan de esta, en especial el corazón y las paredes corporales. *Fendrr* se encuentra localizado 1250 pares de bases aguas arriba del gen *Foxf1* y es co-expresado junto con este factor de transcripción, que es de vital importancia para la correcta diferenciación de la placa lateral mesodermica hacia el mesodermo esplacnico y el mesodermo somático. Ratones deficientes para *Fendrr* muestran una letalidad embrionaria en ED13,5. Los embriones mutantes presentan una función cardiaca erronea, una acumulacion de sangre en las cámaras cardíacas derechas y un descenso del grosor de las paredes corporales, como consecuencia

de una hipoplasia en los cardiomiocitos. Esta hipoplasia conduce al desarrollo de paredes corporales y del septo interventriucular demasiado delgadas como para resistir las variaciones en la presión sanguinea.

Fendrr actua en *trans* como un elementor regulador epigenético interactuando con complejos remodelodores de la cromatina tanto represores, el complejo PRC2, como activadores, el complejo TrxG/MLL. La interacción de *Fendrr* con estos complejos remodeladores conlleva al estableciendo de un *ratio* tanto de marcas represivas como activadoras en los promotores de *Pitx2*, *Foxf1* y *Irx3*, los cuales juegan un papel vital en el desarrollo del mesodermo. El establecimiento de este *ratio* determina los patrones de expresión de los genes diana de *Fendrr* en la placa lateral mesodermica y en los tejidos derivados de esta. Estudios *in silico* han puesto de manifiesto la existencia de una secuencia de 40 nucleotidos en la estructura de *Fendrr* con la capacidad de unirse directamente a los promotores de *Pitx2* y *Foxf1* y generar un heteroduplex ARN-ADN, aunque esta unión ha de ser comprobada experimentalmente y aun no se tienen datos de la naturaleza de la misma (Grote et al., 2013).

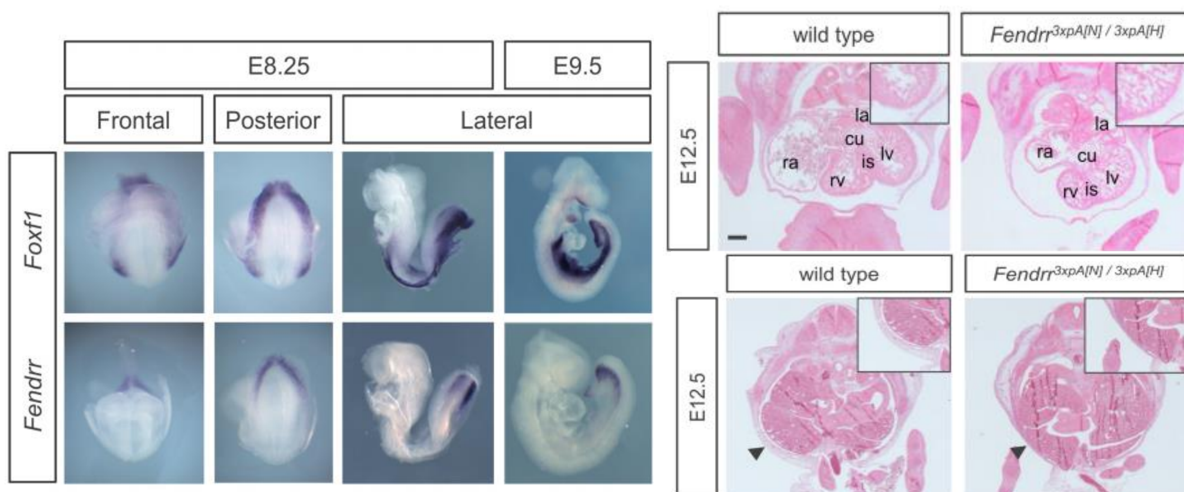


Figura 12. Expresión de Fendrr durante el desarrollo y defectos cardiacos asociados al mutante knock-out. (A) La expresión de Fendrr durante el desarrollo cardiaco queda restringida al extremo caudal de la placa mesodermica lateral; (B) Hipoplasia de los cardiomiocitos de las camaras cardiacas; (C) Descenso del grosor de las paredes corporales. Imagen tomada de Grote el a, 2013.

Al igual que *Fendrr*, *Alien* es necesario para el correcto desarrollo de las estructuras derivadas del mesodermo. Este *lncRNA* especifico de ratón es co-expresado en los progenitores vasculares derivados del alantoides y de la placa lateral mesodermica junto con genes involucrados en el desarrollo de la musculatura esquelética y en la morfogénesis cardiaca como *Brachyury*, *Eomes*, *MixL1* y *Gata4*. Ratones deficientes para este lncRNA presentan deficiencias en el desarrollo de los tejidos derivados del mesodermo entre los que destacan defectos en el desarrollo del sistema vascular y en la formación de las camaras cardíacas. Sin embargo el mecanismo de acción de este *lncRNA* durante el desarrollo cardiaco no ha sido elucidado y es necesario un mayor estudio del mismo (Kurian et al., 2015).

Estudios de RNA-seq llevados a cabo durante distintas fases de la cardiogenesis han identificado la expresión diferencial de 279 *lncRNAs* (Hori et al., 2018). Algunos de estos *lncRNAs* se encuentran en la vecindad de factores de transcripción claves cardiacos, como *Gata4*, *Nkx2.5*, *Tbx5*, *Tbox20*, *Irx3*, *Hand2* o *Irx5* y comparten un promotor bidireccional con estos (Hori et al., 2018). Hasta la fecha, los *lncRNAs* asociados a *Tbx5*, *Tbx5as*, y a *Hand2*, *Upperhand*, han sido señalados como factores clave para un correcto desarrollo cardiaco.

Tbx5as se encuentra en el mismo locus que *Tbx5* y es transcrito en dirección 3'-5' en relación con este factor de transcripción constituyendo un ejemplo de un *lncRNA* antisentido. Este *lncRNA* esta altamente conservado en los mamiferos. *Tbx5as* se expresa en todas las camaras cardiacas sin embargo presenta una expresión mayor en la aurícula derecha que en la izquierda. Ratones deficientes para *Tbx5as* muentran una letalidad embrionaria en ED13,5. Los corazones mutantes presentan una hipoplasia de los cardiomiocitos en el ventriculo derecho y defectos en la trabecuación ventricular y en los primodios del septo interventricular. La funcionalidad de este lncRNA aun no se he descrito y es necesario un mayor estudio molecular del mismo para comprender el papel de este en la cardiogenesis (Hori et al., 2018).

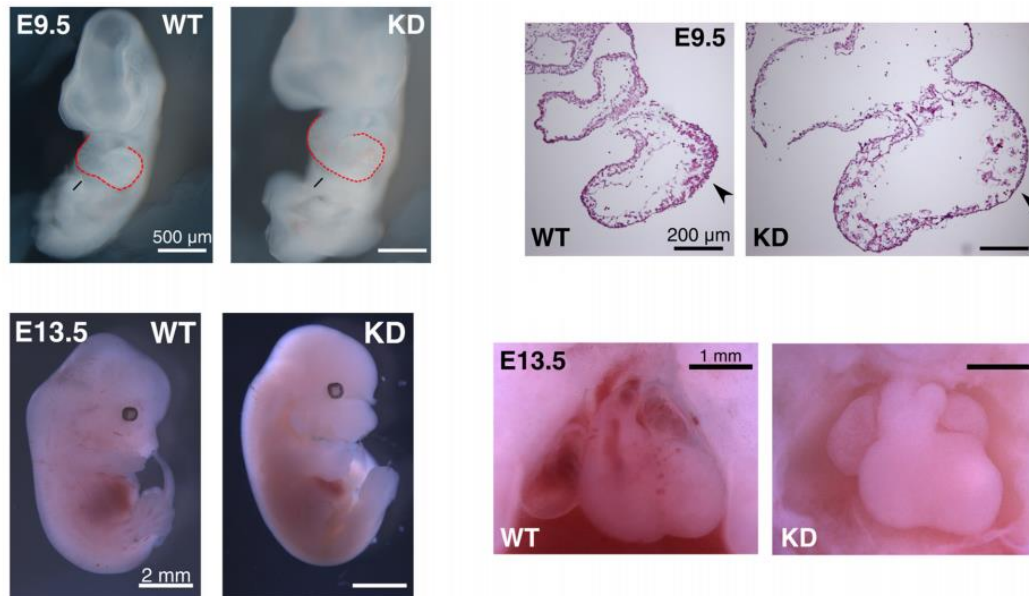


Figura 13. Defectos observados en ratones deficientes para Tbx5as. (A y B) Los corazones mutantes presentan una hipoplasia de los cardiomiocitos y una trabeculación reducida. (C) Los ratones mutantes presentan el mismo tamaño corporal (D) Las paredes ventriculares de los ratones mutantes parecen tener un menor grosor. Imagen tomada de Hori et al., 2018.

A diferencia de *Tbxas5*, que no es requerido para la correcta expresión de *Tbx5*, *Upperhand*, si es necesario para la expresión de *Hand2*. Este lncRNA se encuentra a 150 pares de bases aguas arriba de *Hand2* y ambos comparten el mismo promotor bidireccional. *Upperhand* es co-expresado espacio-temporalmente junto con *Hand2*. Ratones deficientes para *Upperhand* presentan una letalidad embrionaria y un fenotipo cardiaco similar al observado en los ratones deficientes para *Hand2,* caracterizandose por un hipoplasia ventricular derecha que se traduce en un desarrollo incorrecto de esta camara cardiaca (Yamagashi et al., 2001). Los embriones mutantes fallan a la hora de establecer la marca epigenetica H3K27 en el *locus Hand2-Uph*, que es necesaria para su correcta expresión. Del mismo modo, el factor de transcripción *Gata4*, que es requerido para la expresión de *Hand2*, es incapaz de unirse a la región promotora de este último (Mcfadden et al., 2000). Análisis de inmunoprecipitación de ADN en los embriones deficientes para *Upperhand* demostraron que tanto el establecimiento de la marca epigenetica H3K27 como la unión de *Gata4* al promotor de *Hand2* regulan negativamente la transcripción de este último al impedir la función de la RNA polimerasa II. Curiosamente, ensayos *in vitro* en cardiomiocitos atriales HL1 han señalado que los

transcritos maduros de *Upperhand* no son necesarios para la expresión de *Hand2*, lo que sugiere que es la transcripción de *Upperhand* y no su expresión, lo que modula la expresión de *Hand2*. En conjunto estos datos sugieren que la transcripción de *Upperhand* es necesaria para la expresión de *Hand2* modulando el establecimiento de la marca epigenetica H3K27 en su *locus* y la unión de *Gata4* al promotor de *Hand2* (Anderson et al., 2016).

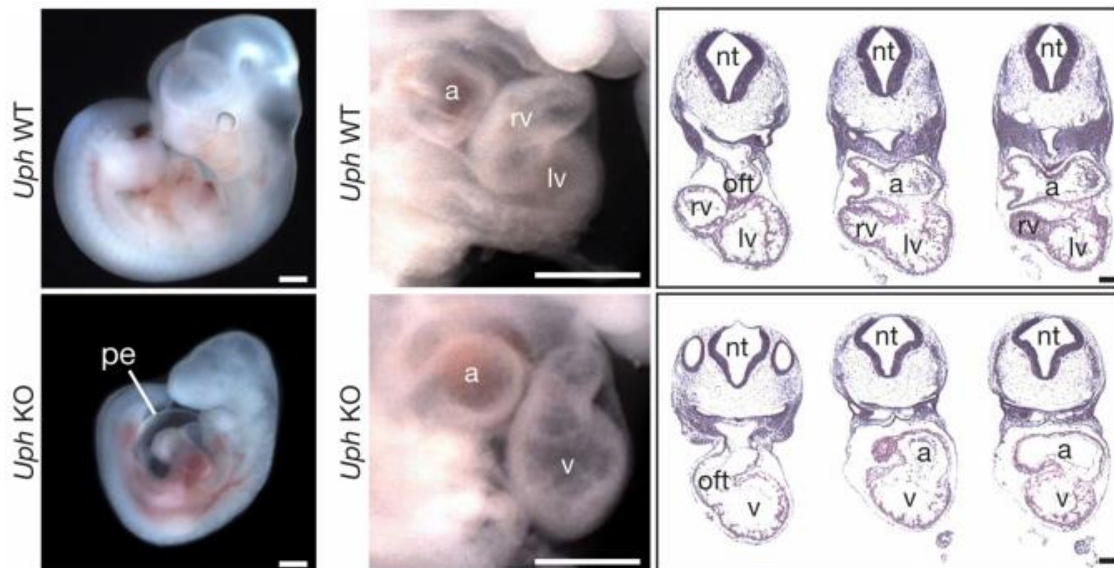


Figura 14. Defectos observados en ratones deficientes para Upperhand. (A) En ED 10,5, los embriones exhiben defectos en el pericardio y un tamaño menor en comparación con los wild type. (B) Los corazones mutantes exhiben un unico ventriculo a diferencia de los wild type. Este fenotipo es muy similar al exhibido por ratones deficientes para Hand2. (C) Secciones histologicas de la región rostro-caudal destacan la ausencia de un ventriculo derecho bien definido. Imagen tomada de Anderson et al., 2016.

Dado que muchas de las enfermedades congenitas del corazón son debidas a defectos en las cascadas genicas que regulan la cardiogenesis, el estudio del transcriptoma de pacientes con CHD puede dar arrojar información de que genes pueden estar involucrados en dichas enfermedades y por ende pueden tener un papel en el desarrollo cardiaco (Bruneau y Srivastava, 2014). En este contexto, Song et al. (2013) analizaron el transcriptoma de pacientes con defectos en el septo ventricular (VSD) e identificaron 270 *lncRNAs* que estaban espresados diferencialmente en relación con personas que no parecian esta enfermedad. Entre ellos, Song et al. (2013) centraron tu atención en un

lncRNA que se encontraba en la cadena de ADN opuesta a *Mef2c*, y al que denominaron como *LncRNA-uc. 167*. Este lncRNA está altamente conservado en Mamiferos y su expresión sigue un patrón inverso a la expresión *Mef2c*, tanto durante el desarrollo cardiaco como durante la diferenciaión *in vitro* de celulas P19 hacia cardiomiocitos. Ensayos *in vitro* han puesto de manifiesto que la sobreexpresión de este lncRNA inhibe a *Mef2c* además de inhibir la diferenciación de los cardiomiocitos a partir de celulas P19, incrementa la apoptosis y disminuye la proliferación de las mismas. El efecto de la sobreexpresión de *LncRNA uc-167* es parcialmente reducido por la sobreexpresión de *Mef2c*. Estos datos sugieren que este lncRNA puede participar en las cascadas de señalización cardiacas, aunque es necesario un mayor conocimiento del papel de este durante la cardiogenesis y de una posible interacción con *Mef2c*.

2.5.Papel de lncRNAs en enfermedades cardiovasculares

Multiples estudios han señalado a los lncRNAs como importantes reguladores de procesos patológicos del sistema cardiovascular, entre los que se encuentran cardiomiopatias hipertroficas, la fibrosis cardiaca, enfermedades valvulares y la fibrilación atrial. Estos estudios han demostrado la importancia de los *lncRNAs* en el mantenimiento de la homeostasis y la función cardiaca (Guo et al., 2016; Devaux et al., 2017; Hermans-Beijnsberger et al., 2018; Zhang et al., 2018).

Se han descrito varios *lncRNAs* que regulan la respuesta hipertrófica miocardica, ejerciendo un papel protector o de riesgo respecto a esta, respectivamente. *H19*, *Myheart* y *Hrcr* han demostrado tener un papel protector frente a la hipertrodia cardiaca. Por ejemplo, *H19* actua como un regulador negativo de la respuesta hipertrófica al modular la expresión cardiaca de CaMKIIδ (Liu et al, 2016). Asimismo, estudios genéticos de pacientes que padecen cardiomiopaticas pro-hipertróficas han identificado 2 SNPs en la estructura genica de *H19* que aumentan el riesgo a padecer este tipo de cardiomiopatias (Gomez et al 2018).

Por el contratio *Miat*, *Chast*, *Chaer* y *Chrf* han demostrado tener un papel pro hipertrofico (Viereck et al, 2016; Wang et al, 2016; Wang, 2014). *Miat* es considerado un lncRNA pro-hipertrófico siendo necesario para que la respuesta hipertrófica se desarrolle en los cardiomiocitos (Li et al, 208). *Miat* actua como esponja de dos microRNAs que

actúan como represores de la hipertrofia cardíaca. Por un lado, *Miat* secuestra al microRNA miR-150, un importante represor hipertrófico, impidiendiendo que este se una a sus dianas, modulando asi positivamente la respuesta hipertrófica (Zhu et al, 2016). Del mismo modo, *Miat* se une al microRNA miR-93 e impide que este reprima la traducción del receptor tipo toll 4 (*Tlr4*), un importante factor pro-hipertrófico (Li et al, 2018).

Se han descrito varios lncRNAs relacionados con con la fibrosis cardiaca entre los que se encuentran *Wisper, GAS5, Meg, H19 y Miat*. Por ejemplo, *Wisper*, un lncRNA que se expresa en los fibroblastos cardiacos y que se encuentra conservado entre los mamiferos, es necesario para la supervivencia de este tipo celular y para el mantenimiento de la cascada de señalización profibrótica y por ende es necesario para el desarrollo de la fibrosis cardiaca. De hecho la atenuación in *vivo* de este lncRNAs mediante *GapmeRs* reduce la fibrosis cardiaca que se produce tras un infarto de miocardio, sugiriendo que *Wisper* puede ser una importante diana farmacologica (Micheletti et al., 2017).

H19 y *Miat* tambien han sido considerados como factores pro-fibróticos. *H19* modula positivamente la fibrosis al impedir que el microRNA miR-445 se una a la región 3' UTR del factor *Ctgf* que regula positivamente la expresión de proteinas fibróticas como la proteina alfa-SMA y las fibras colagenas I y III (Huang et al., 2017). *Miat* ha sido señalado como un importante regulador de la fibrosis cardiaca que aparece tras el infarto de miocardio. La expresión de este *lncRNA* se encuentra incrementada en modelos murinos tras el infarto de miocardio. Esta sobreexpresión viene acompañada de la desregulación de tres factores fibroticos, una disminución de la expresión del microRNA miR-24, que tiene un carácter antifibrotico y un incremento en la expresión de la proteina furina y de Tfg-β, que tienen un carácter pro-fibrótico (Yue et al., 2017). Esta desregulación promueve la proliferación de los fibroblastos, el deposito de colágeno y por ende la fibrosis cardíaca. En linea con estos datos, el silenciamiento endogeno de *Miat* tiene como consecuencia una reducción de la fibrosis cardiaca mejorando la función cardiaco y restableciendo la expresión de los factores fibroticos desregulados. Ademas, los ensayos *in vitro* recapitulan lo observado *in vivo*. En conjunto estos datos, apoyan un papel clave de *Miat* como factor pro-fibrotico (Qu et al., 2017).

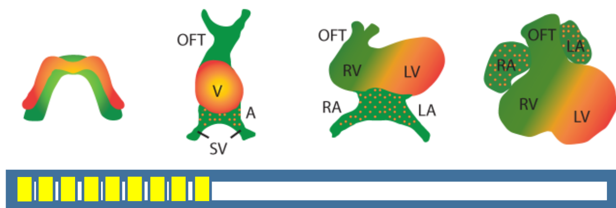
Se ha demostrado que *HOTAIR*, *IFNG-AS1* y *H19* juegan un papel vital en la homeostais valvular (Carrion et al., 2014; Hadji et al., 2016; Xu et al,. 2018). Hadji et al. (2016) demostraron que el promotor de este *lncRNA* se encuentra desregulado en

pacientes que padecen una calcificación en la valvula aórtica. Esta desregulación en el promotor de *H19* provoca un aumento en la expresión del mismo, que promueve la diferenciación osteogenica valvular inhibiendo la expresión de *Notch1*, sugiriendo un papel de *H19* en la homeostasis valvular.

Por otro lado, el estudio del posible papel de lncRNAs en enfermedades arritmogenéticas esta comenzado a ser explorado. Varios estudios, han identificado un subconjunto de lncRNAs que se encuentran desregulados en AF, lo que sugiere un posible papel de estos en la patogenesis de dicha enfermedad (Ruan et al, 2015; Li et al, 2017; Mei et al, 2018; Wu et al., 2019). En linea con estas observaciones, Gore-Ponter et al (2016), estudiando la asociación entre el gen *Pitx2* y los SNPs en el contexto patologico de la fibrilación atrial, identificaron un *lncRNA* intergénico especófico de humano adyacente a *Pitx2*, al que denominaron *Pancr*. Este lncRNA se co-expresa junto con *Pitx2c* en la auricula izquierda de adultos y en el ojo en menor medida. *Pancr* regula positivamente los niveles de mRNA de *Pitx2c* durante el desarrollo de los cardiomiocitos, por un mecanismo aun desconocido y por tnato es necesario un mayor conocimiento de *Pancr* y de la regulación que este ejerce sobre *Pitx2c*.

En su conjunto podemos describir el desarrollo cardiaco como un proceso complejo que se encuentra mediado por multiples factores que regulan espacio-temporalmente la diferenciación, especificación y maduración de los progenitores cardiacos. El papel de los distintos factores de transcripción que median la cardiogenesis ha sido amplimente descrito , al igual que el papel de los factores de crecimiento. En los ultimos años, nuestro conocimiento sobre el genoma no codificante ha aumentado de manera exponencial, señalando a este como un importante regulador de múltiples procesos celulares. En el contexto de la cardiogénesis, se han descrito varios microRNAs esenciales, miR-1 o miR 133. Sin embargo, el papel que en tienen los *lncRNAs* en la cardiogenesis esta comenzado a ser explorado. Dada la versatilidad de los procesos reguladores que pueden controlar esta clase de RNA, su abundacia en el genoma y el numero creciente de estudios en los que se demuestra su papel en la patogenesis cardiaca es de capital importancia estudiar el papel de estos en el desarrollo cardiaco y/o patogenesis.

OBJETIVOS/AIMS



3. Objetivos

El desarrollo del corazón es un proceso complejo que se encuentra altamente regulado en el tiempo y en el espacio, tanto transcripcional como post-transcripcionalmente. En los últimos años se ha puesto de manifiesto el papel regulador en la cardiogénesis de los RNA no codificantes, en especial de los microRNAs. Sin embargo, el papel de los lncRNAs esta comenzado a ser explorado en este contexto. A pesar del elevado número de lncRNAs presentes en el genoma y del papel de estos en múltiples procesos celulares, hasta la fecha solo se ha descrito un pequeño número de *lncRNAs* que participan en el desarrollo cardiaco, aunque el conocimiento de la naturaleza de esta regulación aún necesita de un mayor estudio. Es fundamental seguir explorando el papel de aquellos lncRNAs que se han relacionado con el desarrollo cardiaco así como buscar nuevos lncRNAs hasta ahora no descritos que puedan jugar algún tipo de papel modulador durante la cardiogénesis. Por ello, nos hemos propuesto los siguientes objetivos específicos:

Primer objetivo

Estudiar los perfiles de expresión de lncRNAs candidatos en las distintas cámaras cardiacas durante el desarrollo embrionario y en la etapa adulta.

Segundo objetivo

Analizar la modulación de lncRNAs candidatos por parte de factores de transcripción claves en la cardiogénesis.

Tercero objetivo

Estudiar el papel de lncRNAs candidatos en la ruta de señalización pro-arritmogénica mediada por Pitx2>Wnt>microRNAs

Cuarto objetivo

Estudiar la expresión de lncRNAs candidatos en respuesta al tratamiento de agentes pro-arritmogénicos y pro-hipertróficos tales como la angiotensina II/norepinefrina y la hormona tiroidea.

Quinto objetivo

Analizar la expresión y distribución tisular de lncRNAs candidatos

Aims

The cardiac development is a process highly regulated in time and space, both transcriptionally and post-transcriptionally. Over last years, the regulatory role of non-coding RNAs in heart development, especially microRNAs, has become elucited. However, the role of *lncRNAs* has been initiated and explored in this context. Despite the high number of *lncRNAs* present in the genome and the role of these in multiple cellular processes, to date has only been highlighted in a small number of *lncRNAs* involved in cardiac development, although in the knowledge of the nature of this condition is still in need of further study. It is essential to continue exploring the role of the *lncRNAs* that have been related to cardiac development. Therefore, we have proposed the following specific aims:

First aim

To study the expression profiles of candidate lncRNAs in the different cardiac chambers during embryonic development and in the adult stage.

Second aim

To analyze the modulation of candidate lncRNAs by key transcription factors in cardiogenesis.

Third aim

To study the role of candidate lncRNAs in the pro-arrhythmogenic signaling pathway mediated by Pitx2> Wnt> microRNAs

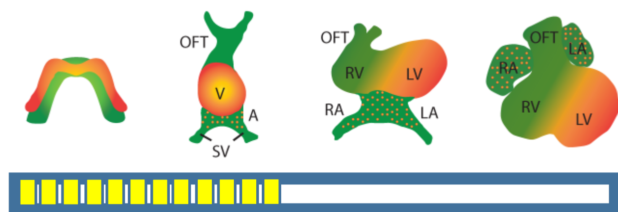
Fourth aim

To study the expression of candidate lncRNAs in response to the treatment of pro-arrhythmogenic and pro-hypertrophic agents such as angiotensin II / norepinephrine and thyroid hormone.

Fifth aim

To analyze the expression and tissue distribution of candidate lncRNAs

CAPITULO I



5. Capítulo 1: Differential chamber-specific expression and regulation of long non coding RNAs during cardiac development and disease

Abstract

Cardiovascular development is governed by a complex interplay between inducting signals such as Bmps and Fgfs leading to activation of cardiac specific transcription factors such as *Nkx2.5*, *Mef2c* and *Srf* that orchestrate the initial steps of cardiogenesis. Over the last decade we have witnessed the discovery of novel layers of gene regulation, i.e. post-transcriptional regulation exerted by non-coding RNAs. The function role of small non coding RNAs has been widely demonstrated, e.g. miR-1 knockout display several cardiovascular abnormalities during embryogenesis. More recently long non coding RNAs have been reported to also modulate gene expression and function in the developing heart as exemplified by the embryonic lethal phenotypes of *Fendrr* and *Braveheart* knock out mice, respectively. In this study, we investigated the differential expression profile during cardiogenesis of previously reported lncRNAs in heart development and disease. Our data revealed that *Braveheart, Fendrr, Carmen* display a preferential adult expression while *Miat, Alien, H19* preferentially display chamber-specific expression at embryonic stages. We also demonstrated that these lncRNAs are distinctly regulated by *Nkx2.5*, *Srf* and *Mef2c*, the pro-arrhythmogenic Pitx2>Wnt>miRNA signaling pathway as well as by pro-arrhythmogenic and pro-hypertrophic stimuli such as angiotensin II and thyroid hormone administration. Importantly isoform-specific expression and distinct nuclear *vs* cytoplasmic localization of *Fendrr* and *Carmen* during chamber morphogenesis is observed, suggesting distinct functional roles of these lncRNAs in atrial and ventricular chambers. Furthermore, we demonstrate by *in situ* hybridization a dynamic epicardial, myocardial and endocardial expression of *H19* during cardiac development. Overall our data support pivotal roles of these lncRNAs in different temporal and tissue-restricted fashion during cardiogenesis.

Introduction

Cardiovascular development is a complex developmental process leading to the formation of a four-chambered heart (Moorman & Christoffels, 2001; Moorman et al., 2004). In mice, early cardiogenesis is initiated as bilateral precardiac mesoderm precursors are specified (E7.5) and migrate towards the embryonic midline to fuse and configure an early straight cardiac tube (E8). Subsequently, the embryonic cardiac tube invariably displays a rightward looping configuring prospective atrial and ventricular chambers (E9.5)(Franco et al., 2014). At E10.5, five distinct regions can be delineated in the embryonic heart, the inflow tract, the embryonic atrial chamber, the atrioventricular canal, the ventricular chambers and the outflow tract (Christoffels et al., 2000). From this stage onwards, each embryonic cardiac region will be separated into distinct left and right components, providing thus a double circuitry with distinct inlet and outlet connections (Franco et al., 2014). Failure or impaired development of these developmental processes leads to congenital heart diseases that in many cases are incompatible with life (Srivastava, 2006; Bruneau, 2008).

Over the last decade, we have witnessed great advance on the discovery of the cellular and molecular mechanisms driving cardiac development. Growth factors such as Bmp, Fgf and Wnt play essential roles regulating and delimiting the early developmental stages of precardiac mesoderm precursors (Lopez-Sanchez & Garcia-Martinez, 2011). Soon thereafter, cardiac-specific transcription factors are activated, such as *Nkx2.5, Gata4, Mef2c* and *Srf*, orchestrating early steps of cardiogenesis (Harvey, 1999; Xin et al., 2013). Genetic deletion of *Nkx2.5* and *Mef2c*, respectively, results in embryonic lethality within the early steps of cardiac looping (Lyons et al., 1995; Biben & Harvey, 1997; Lin et al., 1997), *Gata4* null mutants display impaired development of the precardiac mesoderm (Kuo et al., 1997) and *Srf* is essential for mesoderm formation, and thus subsequently for cardiac morphogenesis (Arsenian et al., 1998; Miano et al., 2004). As development process, additional transcription factors play essential roles in cardiogenesis, such as several Tbox family members (Vitelli et al. 2002, Jerome & Papaioannou et al., 2001; Ryan & Chin, 2003; Xu et al., 2004; Liao et al., 2004; Habets et al., 2002; Harrelson et al., 2004; Ribeiro et al., 2007; Bruneau et al., 2001; Takeuchi et al., 2003), Pitx2 (Kitamura et al., 1999; Campione et al., 2001; Campione & Franco, 2016) and Hand1/Hand2 regional identity (Srivastava et al., 1995; 1997; Thomas et al., 1998, Firulli et al. 1998). Impaired transcriptional activation of any of these transcription factors results in developmental defects causing congenital heart diseases ranging from

life-threating such as Tetralogy of Fallot to milder defects such as atrial or ventricular septal defects (Sperling, 2011). Thus, overall, these data demonstrate a key role of transcriptional regulation governing cardiac development and disease.

Over the last years we have evidenced a novel revolution in the concept of the regulation of gene regulatory networks. ENCODE and NONCODE platforms have unraveled that beside protein-coding genes, a much large proportion of the genome is also transcribed but not translated, configuring thus the non-coding RNAs (Davis et al., 2018; Liu et al., 2005; Zhao et al., 2016; Diehl & Boyle, 2016; Fang et al., 2018). Non-coding RNAs are broadly classified into two distinct categories, small non-coding RNAs (<200 nt) and large non-coding RNAs (>200nt). Among the first category, microRNAs are the most representative group and the most well-studied (Huang, 2018; Islas & Moreno-Cuevas, 2018). Post-transcriptional regulatory mechanisms driven by microRNAs involve annealing by homologous base-paring with mRNAs transcript promoting protein translation and/or mRNA degradation (Lozano-Velasco et al., 2015ab, 2016). Long non-coding RNAs (lncRNAs) constitute a more diverse group of non-coding RNAs with structural similarities to protein-coding RNAs as they contain intron and exons and are transcriptionally spliced (Haemmig et al., 2007; Sallam et al., 2018; Garcia-Padilla et al., 2018). However, functionally lncRNAs display a variety of transcriptional and post-transcriptional functions, ranging from scaffold function, recruiting activating or repressive epigenetic factors or as transcriptional enhancers, thus influencing transcriptional regulation or modulating ribosome adherence to protein coding RNAs and thus modulating protein translation (Huang, 2018; Zampetaki et al., 2018). In addition, lncRNA-microRNA interactions also occurs, by sponging microRNA actions (Huang, 2018), and thus indirectly influencing mRNA stability.

At present, the functional significance of lncRNA in cardiac development and diseases is emerging. Several lncRNAs have been associated with distinct cardiac pathological conditions, such as *Crnde, Homeobox A11, Wisper* and *Meg3* in cardiac fibrosis (Zheng et al., 2019; Wang et al., 2019; Micheletti et al., 2017; Piccoli et al., 2017), *Charme* and *Chast* in cardiac remodeling (Ballerino et al., 2018 Viereck et al., 2016) and *Myheart, H19* and *Chaer* in cardiac hypertrophy (Han et al., 2014, Liu et al., 2016; Greco et al., 2016; Wang et al., 2016). In addition several lncRNAs have also been reported to enhance cardiomyocyte proliferation and repair, such as *Cpr* (Ponnusamy et al., 2019), *NR_045363* (Wang et al., 2019), *Crrl* (Chen et al., 2018) and *uc.167* (Song et al., 2016). Furthermore several lncRNAs have been implicated in key developmental processes

related to cardiac development, such as *Braveheart* (Klattenhof et al., 2013; Hou et al., 2017), *Fendrr* (Grote et al., 2013ab), *Carmen* (Ounzain et al., 2015) *Upperhand* (Anderson et al., 2016), *Terminator, Alien* and *Punisher* (Kurian et al., 2015). However, their tissue distribution during embryonic cardiac development remains poorly elucidated.

In particular, *Braveheart* is required for early cardiogenesis, play a fundamental role in the conversion of the nascent mesoderm into the cardiomyogenic lineage, by modulating *Mesp1* function (Klantenhoff et al., 2013; Xue et al., 2016; Hou et al., 2017). Similarly, *Carmen* is essential for cardiac precursor specification and additionally it is re-activated in heart failure (Ounzain et al., 2015; Plaissance et al., 2016). *Fendrr*, a lncRNA located in the vicinity of *Foxh1* with restricted expression to the lateral plate mesoderm during early embryogenesis is fundamental for correct cardiac development. *Fendrr* deficient mice display cardiac hypoplasia (Grote et al., 2013). In addition to its role in cardiac development, multiple studies demonstrate that impaired *Fendrr* expression is associated to different oncogenic processes (Shi et al., 2019; He et al., 2019; Gyuyte et al., 2018; Zhao et al., 2018; Dong et al., 2018). *Alien* is specifically expressed in vascular progenitors during cardiovascular differentiation. Morpholino-mediated downregulation of Alien in zebrafish resulted in impairment of multiple mesoderm derivatives, including vascular patterning and cardiac chamber morphogenesis (Kurian et al., 2015). *Miat* and *H19* have been implicated in distinct cardiac pathological conditions such as myocardial infarction and ischemia (Li et al., 2018ab; Qu et al., 2017; Zhu et al., 2016; Frade et al., 2016; Xiong et al., 2019; Zhang et al., 2018; Bitarafan et al., 2019; Huang et al., 2017), but their plausible relevance in other cardiac pathophysiological conditions such as cardiac arrhythmias remains to be elucidated.

In this study, we sought to investigate the differential expression profile of these previously characterized lncRNAs in cardiac development, analyze their regulation by cardiac-enriched transcription factors such as *Nkx2.5*, *Srf* and *Mef2c* and by pro-arrhythmogenic Pitx2>Wnt>miRNA signaling pathway (Chinchilla et al., 2011; Lozano-Velasco et al., 2015) as well as by pro-arrhythmogenic and pro-hypertrophic substrates such as angiotensin II/norepinephrine and thyroid hormone administration (Hernandez-Torres et al., 2015; Lozano-Velasco et al., 2017). Our data revealed a subset of lncRNAs with preferential expression embryonic stages (*Miat, Alien, H19*) while others (*Braveheart, Fendrr, Carmen*) in adult stages. These lncRNAs are distinctly regulated by transcription factors involved in early cardiac specification, microRNAs and distinct

signaling pathway supporting the putative role in cardiac pathophysiology, particularly in arrhythmias. In addition we demonstrate a dynamic isoform-specific expression and relative nuclear/cytoplasmic localization of *Fendrr* and *Carmen* during chamber morphogenesis and a endocardial and transient epicardial and myocardial expression of H19 during cardiogenesis. In summary our data provide novel insights into the regulation and tissue-specific expression of lncRNAs during heart development.

Materials & Methods

Mouse breeding and tissue sampling

CD1 mice were bred and embryos were collected at different embryonic developmental stages, ranging from embryonic day (E) E12.5 to E18.5. Neonatal day 1 and adult hearts were also collected. Briefly, embryonic and postnatal hearts were dissected into right atrium, left atrium and ventricular chambers, pooled and stored in liquid nitrogen until used.

Pitx2^{floxed} and NppaCre transgenic mouse lines, and generation of conditional atrial (NppaCre) mutant mice was previously described (Gage et al., 1999; de Lange et al., 2003; Chinchilla et al., 2011; Lozano-Velasco et al., 2016). Two different conditions were used for the NppaCrePitx2 mice: wild-type Cre controls (NppaCre2Pitx2$^{fl/fl}$) and atrial-specific homozygous (NppaCre+Pitx2$^{-/-}$). This investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health. The study was approved by the University of Jaen Bioethics Committee.

Mouse genotyping and phenotyping

DNA for PCR screening was extracted from adult ear and/or tail samples and from embryonic yolk sacs. Screening of Cre and Pitx2 floxed alleles was routinely done using used specific primers as previously described (Chinchilla et al. 2011). Cycling conditions for Cre were as follows; 5 min at 95°C, 35 cycles of 30s at 95°C, 30s at 60°C and 90s at 72°C, and for Pitx2 as follows; 5 min at 95°C. 40 cycles of 30s at 95°C, 30s at 60°C and 90s at 72°C, followed by a final extension step of 10 min at 72°C, respectively. In addition, expression of Pitx2 in left atrial samples of wild-type Cre controls (NppaCre2Pitx2$^{fl/fl}$) and atrial-specific homozygous (NppaCre+Pitx2$^{-/-}$) were analyzed by qPCR, displaying in all cases 60-70% reduction in Pitx2c expression in NppaCre+Pitx2$^{-/-}$ samples.

RNA isolation and cDNA synthesis

Genetically modified Pitx2 mice, and their corresponding controls, were sacrificed by cervical dislocation. Adult hearts were carefully dissected and briefly rinsed in Ringer's solution. Left atrium tissue samples were collected for each experimental condition, immediately snap-frozen in liquid nitrogen, and stored at -80°C until used. Pooled

samples of at least three independent mice were processed for each condition, respectively. Three independent pooled samples were further processed for RNA isolation and qPCR analyses. Mouse CD1 pooled right atria, left atria and ventricular chambers were processed similarly on each developmental stages, as previously detailed. Total RNA was isolated using Trizol (Roche) according to manufacture's guidelines and DNase treated using RNase-Free DNase (Roche) for 1h at 30°C. In all cases, at least three distinct pooled samples were used to perform the corresponding qRT-PCR experiments.

First strand cDNA was synthesized at 50°C for 1h using 1 µg of RNA, oligo-dT primers and Superscript III Reverse Transcriptase (Invitrogen) according to manufacture's guidelines. Negative controls to assess genomic contamination were performed for each sample, without reverse transcriptase, which resulted in all cases in no detectable amplification product.

qPCR analyses (mRNA and lncRNA)

RT-PCR was performed in Mx3005Tm QPCR System with an MxPro QPCR Software 3.00 (Stratagene) and SyBR Green detection system. Reactions were performed in 96-well plates with optical sealing tape (Cultek) in 20 µL total volume containing SYBR Green Mix (Finnzymes) and the corresponding cDNA. Two internal controls, mouse Gusb and Gapdh, were used in parallel for each run and represented as previously described (Lozano-Velasco et al., 2015ab, 2016). Amplification conditions were as follows: denaturisation step of 95°C for 10 min, followed by 40 cycles of 95°C for 30s, 60°C for 30s, 72°C for 30s; with final elongation step of 72°C for 10 min. All primers were designed to span exon-exon boundaries using online Primer3 software Primer3input (primer3 www.Cgi v 0.2). Primer sequences are provided in **Supplementary Table 1** No amplifications were observed in PCR control reactions containing only water as the template. Each PCR reaction was performed at least three times to obtain representative averages. The Livak method was used to analyze the relative quantification RT-PCR data (Livak & Schmittgen, 2001; Schmittgen & Livak, 2008) and normalized in all cases taking as 100% the wild-type (control) value, as previously described (Chinchilla et al., 2011).

qPCR analyses (microRNA)

microRNA qRT-PCR was performed using Exiqon LNA microRNA qRT-PCR primers and detection kit according to manufacturer's guidelines. All reactions were always run

in triplicates using 5S as normalizing control, as recommended by the manufacturer. SyBR Green was used as quantification system on a Stratagene Q-Max 2005P qRT-PCR thermocycler. Relative measurements were calculated as described by Livak & Schmittgen (2001) and control measurements were normalized to represent 100% as previously described (Chinchilla et al., 2011).

Plasmid, siRNA, microRNA mimics cell transfections

HL-1 cells (6×10^5 cells per well) were transfected with plasmids containing expression constructs for Pitx2c, Wnt8a (Addgene), Wnt11a (Addgene, Cambridge, MA, USA), Srf, Nkx2.5 and Mef2c, respectively, as well as for pre-miR-1, pre-miR-133, pre-miR-29 (Exiqon) or siRNA-Pitx2c, siRNA-Wnt8a, siRNA-Wnt11a, siRNA-Srf, siRNA-Nkx2.5, siRNA-Mef2c (Sigma, Aldrich, Munich, Germany) as previously described (Chinchilla et al., 2011; Hernandez-Torres et al., 2015; Lozano-Velasco et al., 2016). siRNA sequences are provided in **Supplementary Table 2.**

Cell culture and angiotensin II, norepinephrine and thyroid hormone treatment

HL-1 cells and primary cultures of mouse fetal (E17.5) cardiomyocytes were isolated using standard procedures (Daimi et al., 2015), cultures accordingly and treated with angiotensin II (Sigma), norepinephrine (Sigma), T3 and T4 (Sigma) hormone, respectively, as previously reported (Lozano-Velasco et al., 2016).

Nuclear/cytoplasmatic distribution

Cytoplasmic and nuclear RNA fractions from atrial and ventricles samples were isolated with Cytoplasmic & Nuclear RNA Purification Kit (Norgen, Belmont, CA, USA) following the manufacturer's instructions. After RNA isolation, qPCR for nuclear enriched *Rpb1* marker and cytoplasmic *Gapdh* marker were performed to validate enrichment on each subcellular fractions. qPCR for distinct lncRNAs was subsequently performed as detailed above.

In situ hybridization

Embryonic and fetal tissues were fixed overnight in a cold solution of 4% formaldehyde in PBS. After fixation, tissues were dehydrated in increasing alcohol series ending in a 1-butanol step, before embedment into paraplast. Samples tissues were sectioned (10 µM thick) and mounted into 3-aminopropyltriethoxysilane-coated glasses. Probes RNA were

generated using T7 and SP6 polymerase in presence of digoxigenin-11-UTP labelling nucleotides (DIG RNA Labeling Mix Roche #11277073910) from pGEMT plasmids containing last exon of H19 gene (**Supplementary Table 1**). Control RNA probes were Mcl2v and Mcl2a mRNAs and hybridization was performed as previously described for mRNAs transcripts by Franco et al. (2000).

Statistical analyses

For statistical analyses of datasets, unpaired Student's t-tests were used. Significance levels or P values are stated in each corresponding figure legend. $P < 0.05$ was considered statistically significant.

Results

Expression profile of lcnRNAs during cardiogenesis

We have analyzed the developmental expression profile of six distinct lncRNAs previously reported to play a fundamental role in cardiac development and disease in three distinct cardiac regions, right atrium, left atrium and ventricular chambers ranging from E12.5 to adulthood by qPCR. Our data demonstrate that *Braveheart* display basal expression levels during all embryonic stages within all distinct cardiac structures, peaking its expression only in adult stages (**Figure 1A**). *Carmen* displays basal expression levels in all cardiac chambers during embryonic development, similar to *Braveheart*, peaking at neonatal stages in the right atrium and ventricles. Curiously, in the adult stage, increased expression is detected in the ventricular chambers, while in the right atrium is set back to basal levels. Thus, only a transient up-regulation is observed in the right, but not in the left atrium, at neonatal stages (**Figure 1B**). *Fendrr* displays a prominent ventricular expression at early embryonic stages, declining in fetal and neonatal stages but peaking again in the adulthood. Importantly, only basal expression is observed in both right and left atrial during embryonic, fetal and adult stages (**Figure 1C**). *Miat* display a highly dynamic expression during embryonic development, in all three distinct cardiac chambers analyzed, while in the adulthood, *Miat* prominent expression is only observed in the ventricular chambers (**Figure 1D**). Similarly, *H19* displays also a highly dynamic expression in embryonic stages, declining in neonatal and adult stages but maintaining overt right *vs* left enhanced expression in the atrial chambers. Importantly a highly prominent expression is observed in right atrium and ventricular chambers during all stages analyzed, while expression in left atrium is significantly lower (**Figure 1E**). Finally, *Alien* display a predominant embryonic expression, particularly higher in early embryonic stages (E12.5-E14.5) but declining in neonatal and adult stages (**Figure 1F**). Overall, these data demonstrate a distinct developmental behaviour of these lncRNA during cardiac development. Importantly, *Braveheart*, *Carmen*, *Fendrr, H19* and *Miat* display a prominent and chamber-specific expression in the adult ventricular chambers. *Braveheart*, in all three adult cardiac chambers, *Carmen*, *Fendrr* and *Miat* exclusively in the ventricular chambers while *H19* is mainly restricted to right atrium and ventricular chambers in the adult heart.

Transcriptional regulation of lncRNAs expression by Nkx2.5, Mef2c and Srf

In order to dissect the functional role of early cardiac specification transcription factors, such as *Nkx2.5, Srf*, and *Mef2c,* in the regulatory mechanisms driving lncRNAs, we performed gain and loss-of-function assays for these transcription factors in HL1 cardiomyocytes and analyzed their expression by qPCR as reported in **Figure 2A-C**. *Nkx2.5* overexpression significantly upregulated *Braveheart* expression, while *Fendrr, Carmen*, *H19* and *Alien* were down-regulated. *Nkx2.5* silencing provide significant down-regulation of *Fendrr*, *Miat* and *Alien*. No significant changes were observed for *Braveheart, Carmen* and *H19* (**Figure 2D**). *Srf* overexpression significantly upregulated Carmen, *Fendrr, Miat* and *Alien*, while *Braveheart* and *H19* display no significant differences. *Srf* inhibition by siRNAs lead to up-regulation of *Miat, H19* and *Alien* while *Carmen* was down-regulated and *Braveheart* and *Fendrr* were not significantly altered (**Figure 2E**). Mef2c gain-of-function significantly up-regulated *Fendrr, Miat, H19* and *Alien* and while down-regulated *Braveheart* and *Carmen*. On the other hand, *Mef2c* silencing up-regulates *Fendrr*, *H19* and *Alien,* down-regulates *Carmen* and *Miat* while no significant changes were observed for *Braveheart* (**Figure 2F**). Surprisingly, in several cases, *Nkx2.5, Mef2c* and *Srf* gain and loss-of-function assays, respectively, resulted in similar up-regulation and down-regulation of the lncRNAs analyzed. While the precise molecular mechanisms remains to be established, it provide evidence that subtle changes in the expression of these transcription factors results in lncRNAs deregulation. In summary, these data illustrate that key transcription factors such as *Nkx2.5, Mef2c* and *Srf* distinctly regulate lncRNA expression in cardiomyocytes (**Figure 2G**). Thus, importantly, impaired expression of these transcription factors in pathological conditions such as cardiac hypertrophy, will therefore have an impact on lncRNA expression.

Regulation of lncRNAs by Pitx2>Wnt>microRNA pathway

We have previously reported that Pitx2>Wnt>microRNA signaling pathway plays a fundamental role in the onset of cardiac arrhythmias (Chinchilla et al., 2011; Lozano-Velasco et al., 2015, 2017). We sought to evaluate if modulation of this signaling pathway will impact on the expression of these lncRNAs and thus might therefore implicated in this cardiac pathophysiology. For this purpose, we performed gain and loss-of-function assays of *Pitx2*, *Wnt8* and *Wnt11* in HL1 atrial cardiomyocytes, respectively (**Figure 3A-C**). Pitx2 gain-of-function revealed up-regulation of *Fendrr, H19* and *Alien*, down-regulation of *Carmen*, but no significant differences were observed for other lncRNAs analyzed. Importantly, *Pitx2* silencing only resulted in up-regulation of *Alien,* down-

regulation of *Braveheart* and *Carmen* and no significant differences for *Fendrr, Miat* and *H19* (**Figure 3D**). To further support the Pitx2 regulatory role, expression of deregulated lncRNAs after siRNA-Pitx2 administration was also evaluated in the adult left atrial chambers of NppaCrePitx2 haploinsufficient mice. Expression of *Braveheart* and *Carmen* display are significantly downregulated in NppaCrePitx2 haploinsufficient mice while *Miat* display no significant differences, in line with Pitx2 loss-of-function data in HL1 atrial cardiomyocytes. Surprisingly, *Alien* instead of being up-regulated also displays decreased expression in NppaCrePitx2 happloinsufficient mice (**Figure 3G**). Overall, these data therefore reinforce the evidences that Pitx2 regulates the expression of these lncRNAs (**Figure 3H**), and support the notion that they might be involved in signaling pathways associated to cardiac arrhythmias.

Wnt8 gain-of-function resulted in up-regulation of *Braveheart, Fendrr* and *H19*, while *Carmen* was down-regulated and *Miat* and *Alien* displayed no significant differences. Silencing of *Wnt8* lead to up-regulation of *H19* and *Alien*, down-regulation of *Carmen* but no significant differences of *Braveheart*, *Fendrr* and *Miat* (**Figure 3E**). On the other hand, *Wnt11* gain-of function resulted in up-regulation of *Carmen, Fendrr, Miat*, *H19* and no significant differences for *Braveheart* expression. Wnt11 inhibition by siRNA led to up-regulation of *Braveheart,* while *Fendrr, Miat,* and *Alien* were down-regulated. *Carmen* and *H19* display no significant differences (**Figure 3F**). Importantly, *Carmen* and *Alien* expression is complementary regulated by Wnt8 and Wnt11, respectively. Overall, these data demonstrate that Wnt signaling significantly impacts on these lncRNA expression (**Figure 3H**), opening up the possibility that impaired expression of these lncRNAs might play a role in arrhythmogenesis.

microRNA regulation of lncRNA expression

It is widely accepted that lncRNA can modulate microRNA function by acting as sponges (Zhou et al., 2017; Zhang et al., 2018ab; He et al., 2018). However, it is less evident that lncRNAs might be, similar to mRNAs, deregulated by microRNAs. We sought to investigate if previously reported microRNAs involved in atrial arrhythmias, downstream of the Pitx2>Wnt signaling pathway, might indeed modulate expression of the lncRNAs previously analyzed. We therefore over-expressed miR1, miR-133 and miR-29 in atrial cardiomyocytes and analyzed their expression levels by qPCR (**Figure 4A**).

miR-1 mimic administration led to up-regulation of *Carmen, Fendrr* and *H19*, while *Braveheart, Alien* and *Miat* displayed no significant differences (**Figure 4B-G**).

miR-29 gain-of-function led to up-regulation of *Braveheart* and *Fendrr* but down-regulation of *Miat*, while *Carmen, Alien* and *H19* were not significantly different (**Figure 4B-G**). Finally, miR-133 over-expression resulted in significant down-regulation of *Miat* and *Alien* while all the other lncRNAs analyzed displayed no significant differences (**Figure 4B-G**). These data revealed that microRNAs could provide novel regulatory mechanisms controlling lncRNA expression, increasing and/or decreasing their expression levels in cardiomyocytes. Furthermore, these data reinforces the notion that these lncRNAs might be deregulated in atrial arrhythmogenesis.

Angiotensin and thyroid hormone administration distinctly alters lncRNA expression

To further investigate the plausible involvement of these lncRNAs in cardiac pathophysiology we treated atrial cardiomyocyte with pro-arrhythmogenic and pro-hypertrophic substrates such as angiotensin II and norepinephrine, respectively, and evaluate the expression levels of these lncRNAs by qPCR. Both, angiotensin II and norepinephrine treatment resulted in selective up-regulation of *Carmen, Fendrr* and *H19*, while *Braveheart, Miat* and *Alien* display no significant differences (**Figure 5A**).

On the other hand, T4 thyroid hormone administration in HL1 atrial cardiomyocytes significantly up-regulates expression of *Braveheart, Fendrr* and *Alien,* down-regulates *Carmen* expression while no significant differences are observed for *Miat* and *H19* expression, while T3 thyroid hormone administration resulted in no significant differences for any of the lncRNAs analyzed, except for *Carmen*, *Fendrr* and *H19* that were significantly downregulated (**Figure 5B**). Overall, these data demonstrate that pro-arrhythmogenic and pro-hypertrophic substrates can modulate the expression of these lncRNAs.

Differential isoform lncRNA expression embryonic and adult heart

lncRNAs display a genomic structure similar to protein-coding RNAs and they are also subjected to alternative splicing. We selected those lncRNAs with differential expression between embryonic and adult stages (*Braveheart, Fendrr* and *Carmen*) to discern if differential expression of their isoforms was occurring between those stages. *Braveheart* displays two distinct isoforms and both are similarly expressed in embryonic and adult right atrium, left atrium and ventricular tissues (**Figure 6**). *Fendrr* display three distinct isoforms. *Fendrr* isoform 1 is embryonic and adult ventricles, but only expressed in

embryonic, but not adult, right and left atria. *Fendrr* isoform 2 is only expressed in embryonic, but not adult, tissues while *Fendrr* isoform 3 is only observed in embryonic left atria and adult ventricles (**Figure 6**). Finally, *Carmen* displays two distinct isoforms. *Carmen* isoform 1 is expressed in all embryonic tissues analyzed, i.e. right atrium, left atrium and ventricles while expression in adult right and left atrium is decreased as compared to adult ventricles. On the other hand, *Carmen* isoform 2 is similarly expressed in all tissues and stages analyzed (**Figure 6**). These data demonstrate that *Fendrr* and *Carmen* display tissue and time-dependent differential expression of their isoforms.

Nuclear/cytoplasmatic distribution of lncRNAs

lncRNAs display an dual subcellular localization within the nucleus and the cytoplasm, providing distinct functional properties, i.e. transcriptional vs post-transcriptional roles, respectively. We investigated the relative expression of four distinct lncRNAs, i.e. those with differential expression in embryonic and adult heart, *Braveheart*, *Fendrr* and *Carmen*, as well as one sustained expression in embryonic and adult stages, *H19*. Nuclear and cytoplasmic enrichment was validated by qPCR analyses of *Rpb1* and *Gapdh* markers, respectively, as illustrated in **Figure 7A**. Nuclear and cytoplasmic distribution was analyzed in three distinct cardiac compartment of E14.5 mouse embryos, right atrium, left atrium and ventricles. *Braveheart* displays similar expression within the cytoplasm and nucleus in all tissues analyzed (**Figure 7B**). *H19*, on the other hand, displays a similar distribution as *Braveheart*, i.e. similarly expressed in all tissues analyzed (**Figure 7C**). *Fendrr*, as well as *Carmen*, display enriched nuclear localization in the left atrium and ventricles, whereas in the right atrium, expression is preferentially localized in the cytoplasm (**Figure 7D-E**). These data demonstrate differential nuclear vs cytoplasmic distribution within distinct cardiac compartment, suggesting that notion that the same lncRNA can be displaying distinct transcriptional *vs* post-transcriptional roles within different cardiac compartment at the same developmental stage.

Tissue distribution of H19 is restricted to the developing endocardium and transiently to the embryonic epicardium

We analyzed H19 tissue distribution during cardiogenesis ranging from E10.5 to adulthood. At E10.5, expression of H19 is distinctly observed in the external epicardial and internal endocardial lining of both atrial and ventricular chambers. Myocardial expression is most prominent in the right ventricular chamber (**Figure 8**). H19 expression

at E12.5 is observed in all cardiac tissue layers including the endocardium, myocardium and epicardium (**Figure 9A-G**). Within the atrioventricular valve cushions, H19 expression is observed in the endocardial lining as well as in the mesenchymal component. Within the ventricular myocardium, prominent expression is observed in the right as compared to the left ventricular chamber (**Figure 9A, F-G**) as compared to control Mlc2v hybridization (**Figure 9H**). Epicardial expression is observed similarly in the atrial and ventricular lining (**Figure 9E,G**). At E14.5, H19 is mainly restricted to the endocardium and epicardium, and similarly observed in the ventricular endocardium and valve leaflets (**Figure 10A-E**) while myocardial expression is no longer observed. Importantly, H19 expression is mainly confined to the nuclei. Additionally, cytoplasmic expression is also observed in the ventricular epicardial lining (**Figure 10**) and the atrioventricular valve mesenchymal component (**Figure 10**). A similar expression profile is observed at E16.5 (data not shown). Neonatal hearts display a much restricted expression pattern for H19, confined to the ventricular endocardium but mainly localized on the trabecular crypts (**Figure 11**). Right atrial endocardium display a prominent H19 expression in contract to left atrium, where H19 is scarce. Atrioventricular valve only display H19 expression confined to the endocardial lining. No expression in the epicardium is observed anymore. Myocardial and cardiac fibroblasts remains mainly negative for H19 expression. Importantly, H19 expression in the neonatal heart is no longer restricted to the nuclei, displaying a most robust cytoplasmic localization. These data demonstrate that H19 is mainly expressed in the developing and adult endocardium, with prominent contribution during atrioventricular valve development as well as a transient expression in the developing embryonic epicardium.

Discussion

Cardiac development is a complex developmental process initiated with the formation of cardiac straight tube that subsequently becomes remodeled into a four-chambered organ (Moorman & Christoffels, 2001; Moorman et al., 2004). During these morphogenetic changes, atrial and ventricular chambered are formed, each of them displaying distinct left and right components (Campione & Franco, 2003; Franco et al., 2014). Concomitant with these developmental transformation, regionalized expression of key cardiac enriched transcription factors and structural proteins occurs, providing developmental cues to model distinct functional cardiac chambers during embryonic, fetal and adult heart (Franco et al., 1997; Bruneau, 2008). We provide herein evidence that lncRNAs also display regionalized expression patterns during development. For example, *Fendrr* display enhanced ventricular expression in early embryonic stages as well as in adult stages as compared to atrial expression while *H19* display enhanced right atrial and ventricular expression at all stages analyzed as compared to relatively low expression in the left atrium. Furthermore, it is also important to highlight these lncRNAs display a dynamic expression profile during cardiogenesis, e.g. *Braveheart*, *Fendrr* and *Carmen* display basal expression levels during embryonic stages peaking up only in adulthood while *Miat, Alien* and *H19* display a more robust embryonic expression. Therefore these data demonstrate chamber- and temporal-specific expression of these lncRNAs, suggesting that they can play chamber-specific roles in a stage-specific manner.

LncRNAs can modulate transcriptional and post-transcriptional regulation (Huang, 2018; Zampetaki et al., 2018). Such distinct regulatory mechanism can simultaneously occur because many lncRNAs display dual nuclear and cytoplasmic subcellular localization (Coassin et al., 2014; Das et al., 2018). Our data demonstrate that *Braveheart* and *H19* display similar nuclear and cytoplasmic distribution in all cardiac chamber analyzed, supporting the notion that can exert transcriptional and post-transcriptional regulatory mechanisms at this stage. Our *in situ* hybridization further demonstrates dual nuclear and cytoplasmic localization of H19, displaying temporal and tissues-specific heterogeneity. Importantly, *Fendrr* and *Carmen* display enhanced nuclear expression in the left atrium and ventricles, while in the right atrium the opposite subcellular distribution is demonstrated. While further experiments are required to dissect the molecular mechanisms driven by these lncRNAs, our data suggest that distinct transcriptional *vs* post-transcriptional regulatory mechanisms can be exerted by the same lncRNA in distinct cardiac chambers at similar developmental stages.

Tissue specific expression of multiple cardiomyocyte specific genes such as sarcomeric and cytoskeletal genes are transcriptionally regulated by a core set of cardiac enriched transcription factors, including therein *Nkx2.5, Mef2c* and *Srf* (Bruneau, 2008). We tested if cardiac-enriched lncRNAs were similarly regulated by these transcription factors using gain and loss-of-function assays. Our loss-of-function data demonstrate that *Nkx2.5*, *Mef2c* and *Srf* are indispensable for the expression of *Carmen, H19* and *Alien,* while *Srf* and *Mef2c* are indispensable for *Braveheart* and *Fendrr* expression. *Miat* is primarily regulated by *Nkx2.5* and *Srf*. Importantly, over-expression of any of these cardiac enriched transcription factor is capable of modulating expression of these lncRNAs, suggesting that subtle changes on the expression of these transcription factors can modulate lncRNA expression in cardiomyocytes. In this context, it is important to highlight that cardiac pathologies such as cardiac hypertrophy provoke *Mef2c* and *Srf* deregulation (Kucia et al., 2004; Kuwahara et al., 2007; Small et al., 2010; Tritsch et al., 2013) and such impaired expression will therefore impact lncRNA expression.

Impaired *Miat* and *H19* expression have been previously associated with cardiac pathologies such as cardiac hypertrophy and dilated cardiomyopathy (Zhu et al., 2016; Liu et al., 2016; Zhang et al., 2017; Li et al., 2018). We tested if *Braveheart, Fendrr, Carmen* and *Alien* were distinctly regulated by key signaling pathways leading to cardiac arrhythmias, i.e. Pitx2>Wnt>miRNA (Yang et al., 2007; Terentyev et al., 2009; Chinchilla et al., 2011; Lozano-Velasco et al., 2015, 2017), or cardiovascular risk factors such as hypertension, i.e. angiotensin/norepinephrine administration (Danilczyk & Penniger, 2006; Oudit et al., 2003) or hyperthyroidism, i.e. T3/T4 hormone treatment (Kahaly & Dillmann, 2005), that also increased the possibility of developing cardiac arrhythmias (Baumgartner et al., 2017). Our data demonstrate a key regulatory role for *Pitx2* directing *Braveheart, Carmen* and *Alien*, while *Fendrr* was only impaired by *Pitx2* over-expression. *Pitx2* is dispensable for *Miat* and *H19* expression in cardiomyocytes. These data were further reinforced in Pitx2 deficient mice that display cardiac arrhythmias (Chinchilla et al., 2011). In addition, *Wnt8* and *Wnt11* can modulate expression of all lncRNA analyzed (Lozano-Velasco et al., 2015, 2017), further supporting their plausible role in atrial arrhythmias. Moreover, AngII and NE administration invariably increased expression of *Fendrr, Carmen*, and *H19* while T4 treatment increased *Braveheart, Alien* and *Fendrr*, supporting a plausible link between these cardiovascular risk factors, impaired lncRNAs expression and the onset of cardiac arrhythmias. In this context, our data are in line with previous report demonstrating up-

regulation of *Fendrr* and *Carmen* upon AngII administration (Kontaraki et al., 2018) and we further provide evidences that H19 is also upregulated.

To date, it has been widely demonstrated that lncRNAs can modulate microRNA expression and function by acting as sponges of these microRNAs. *Fendrr* can sponge miR-761, miR-214 and miR-18, respectively, in distinct cancers (Zhang et al., 2018ab; He et al., 2018) while *Miat* sponges miR-22 in diabetic cardiomyopathy (Zhou et al., 2017). However, whether microRNAs can directly modulate lncRNA expression remains unclear. We therefore explored the modulatory role of distinct microRNAs with pro-arrhythmogenic potential such as miR-1, miR-29 and miR-133 (Chinchilla et al., 2011; Lozano-Velasco et al., 2015; 2017). Our data demonstrate that all lncRNAs can be distinctly modulate by these microRNAs, displaying in most cases up-regulation such as *Fendrr* and *H19* for miR-1 administration, while in other cases down-regulation was recorded, such as for *Miat* after miR-29 and miR-133 administration, respectively. While it remains to be established if such modulation is directly or indirectly, these data demonstrate that lcnRNA expression is also regulated by microRNAs and secondly, it further support the plausible role of these lncRNAs in cardiac arrhythmias, in line with previous report linking miR-29 and H19 cross-talks in cardiac hypoxia and myocardial infarction (Yu & Dong, 2018; Grabmaier et al., 2018; Zhang et al., 2018).

LncRNAs display a genomic structure similar to protein-coding RNAs, with intron and exons as well as suffering alternative splicing (Haemming et al., 2007; Sallam et al., 2018). To date, our current understanding of the distinct isoform specific expression and function is rather limited. We have explored in this study the isoform-specific expression of three distinct lncRNAs with differential expression in embryonic and adult stages, i.e. *Braveheart, Carmen* and *Fendrr*. Our data demonstrate that both *Braveheart* isoforms are expressed in all chambers at embryonic and adult stages. On the other hand, *Fendrr* and *Carmn* isoforms display differential tissue and time-dependent expression. In this context, it is important to highlight that differential nuclear and cytoplasmic expression was observed for *Fendrr* and *Carmen* but not for *Braveheart*. Thus, it is plausible to speculate that distinct isoforms might be directing different transcriptional and post-transcriptional regulatory functions within distinct cardiac compartment at the same developmental stage. Further experiments are required to clarify this hypothesis.

One of the major caveats analyzing lncRNA function is to discern their tissue-specific distribution. LncRNAs are expressed on average 10-100 fold lower that protein-coding RNAs, and thus their tissue distribution by in situ hybridization is in many cases

challenging. Previous studies have reported expression of H19 in distinct pathological settings, particularly in cancer (Matouk et al., 2007; Zhang et al., 2016; Guo et al., 2018), while in the cardiovascular system H19 expression have only been reported in ischemic heart failure (Greco et al., 2016).

We report herein tissue distribution of *H19* and *Braveheart* at different developmental stages during cardiogenesis. We provide evidence that H19 is transiently expressed in the epicardium and myocardium while expression in the developing endocardium is observed at all stages analyzed. Curiously, H19 displays a preferential nuclear localization in the early stages of heart development but shifts to a cytoplasmic localization in neonates. Importantly, nuclear localization is also observed in the endocardial lining of the atrioventricular valves while a more prominent cytoplasmic expression is observed in the underlying mesenchymal component. A transient epicardial expression is also observed, with prominent nuclear localization the atrial epicardial lining while in the ventricular epicardium, scattered cells display nuclear localization while the vast majority are mostly cytoplasmic. Endocardial valve and epicardial lining display similar morphogenetic events leading to epithelial to mesenchymal transition (EMT)(Perez-Pomarez et al., 2002; Camenish et al., 2002; Stevens et al., 2006: Exposito-Villen et al., 2018). Our data demonstrate a dual and heterogeneous nuclear and cytoplasmic expression in these tissues suggesting a plausible role of H19 in cardiovascular EMT whereby nuclear to cytoplasmic translocation coincide with epithelial-to-mesenchymal transition and thus with transcriptional to post-transcriptional control of the EMT regulatory mechanisms. Myocardial expression is only observed at early developmental stages, displaying subtle but significant left/right asymmetric ventricular expression, as previously reported for several sarcomeric proteins (Zammit et al., 2000).

In summary, we provide herein evidence of distinct temporal and tissue-specific expression, nuclear/cytoplasmic and isoform distribution of distinct lncRNAs during cardiogenesis. In addition, we provide evidence of distinct transcriptional regulation by cardiac enriched transcription factors as well as key signaling pathways linked to cardiac pathophysiology, particularly to cardiac arrhythmias. Our data provide therefore novel insights into the plausible role of these lncRNAs in cardiac development and diseases.

References

Anderson, K.M.; Anderson, D.M.; McAnally, J.R.; Shelton, J.M.; Bassel-Duby, R.; Olson, E.N. Transcription of the non-coding RNA upperhand controls Hand2 expression and heart development. Nature 2016, 539, 433–436.

Arsenian S, Weinhold B, Oelgeschläger M, Rüther U, Nordheim A. Serum response factor is essential for mesoderm formation during mouse embryogenesis. EMBO J. 1998 Nov 2;17(21):6289-99

Ballarino, M.; Cipriano, A.; Tita, R.; Santini, T.; Desideri, F.; Morlando, M.; Colantoni, A.; Carrieri, C.; Nicoletti, C.; Musarò, A.; et al. Deficiency in the nuclear long noncoding RNA Charme causes myogenic defects and heart remodeling in mice. EMBO J. 2018, 37, e99697.

Baumgartner C, da Costa BR, Collet TH, Feller M, Floriani C, Bauer DC, Cappola AR, Heckbert SR, Ceresini G, Gussekloo J, den Elzen WPJ, Peeters RP, Luben R, Völzke H, Dörr M, Walsh JP, Bremner A, Iacoviello M, Macfarlane P, Heeringa J, Stott DJ, Westendorp RGJ, Khaw KT, Magnani JW, Aujesky D, Rodondi N; Thyroid Studies Collaboration. Thyroid Function Within the Normal Range, Subclinical Hypothyroidism, and the Risk of Atrial Fibrillation. Circulation. 2017 Nov 28;136(22):2100-2116

Biben C, Harvey RP. Homeodomain factor Nkx2-5 controls left/right asymmetric expression of bHLH gene eHand during murine heart development. Genes Dev. 1997 Jun 1;11(11):1357-69.

Bitarafan S, Yari M, Broumand MA, Ghaderian SMH, Rahimi M, Mirfakhraie R, Azizi F, Omrani MD. Association of Increased Levels of lncRNA H19 in PBMCs with Risk of Coronary Artery Disease. Cell J. 2019 Jan;20(4):564-568.

Bruneau BG, Nemer G, Schmitt JP, Charron F, Robitaille L, Caron S, Conner DA, Gessler M, Nemer M, Seidman CE, Seidman JG. A murine model of Holt-Oram syndrome defines roles of the T-box transcription factor Tbx5 in cardiogenesis and disease. Cell. 2001 Sep 21;106(6):709-21.

Bruneau BG. The developmental genetics of congenital heart disease. Nature. 2008 Feb 21;451(7181):943-8. doi: 10.1038/nature06801.

Camenisch TD, Molin DG, Person A, Runyan RB, Gittenberger-de Groot AC, McDonald JA, Klewer SE. Temporal and distinct TGFbeta ligand requirements during mouse and avian endocardial cushion morphogenesis. Dev Biol. 2002 Aug 1;248(1):170-81.

Campione M, Franco D. Current Perspectives in Cardiac Laterality. J Cardiovasc Dev Dis. 2016 Dec 9;3(4). pii: E34.

Campione M, Ros MA, Icardo JM, Piedra E, Christoffels VM, Schweickert A, Blum M, Franco D, Moorman AF. Pitx2 expression defines a left cardiac lineage of cells: evidence for atrial and ventricular molecular isomerism in the iv/iv mice. Dev Biol. 2001 Mar 1;231(1):252-64.

Chen, G.; Li, H.; Li, X.; Li, B.; Zhong, L.; Huang, S.; Zheng, H.; Li, M.; Jin, G.; Liao, W.; et al. Loss of long non-coding RNA CRRL promotes cardiomyocyte regeneration

and improves cardiac repair by functioning as a competing endogenous RNA. J. Mol. Cell. Cardiol. 2018, 122, 152–164.

Chinchilla A, Daimi H, Lozano-Velasco E, Dominguez JN, Caballero R, Delpón E, Tamargo J, Cinca J, Hove-Madsen L, Aranega AE, Franco D. PITX2 insufficiency leads to atrial electrical and structural remodeling linked to arrhythmogenesis. Circ Cardiovasc Genet. 2011 Jun;4(3):269-79.

Christoffels VM, Habets PE, Franco D, Campione M, de Jong F, Lamers WH, Bao ZZ, Palmer S, Biben C, Harvey RP, Moorman AF. Chamber formation and morphogenesis in the developing mammalian heart. Dev Biol. 2000 Jul 15;223(2):266-78.

Coassin SR, Orjalo AV Jr, Semaan SJ, Johansson HE. Simultaneous detection of nuclear and cytoplasmic RNA variants utilizing Stellaris® RNA fluorescence in situ hybridization in adherent cells. Methods Mol Biol. 2014;1211:189-99.

Daimi H, Lozano-Velasco E, Haj Khelil A, Chibani JB, Barana A, Amorós I, González de la Fuente M, Caballero R, Aranega A, Franco D. Regulation of SCN5A by microRNAs: miR-219 modulates SCN5A transcript expression and the effects of flecainide intoxication in mice. Heart Rhythm. 2015 Jun;12(6):1333-42.

Danilczyk U, Penninger JM. Angiotensin-converting enzyme II in the heart and the kidney. Circ Res. 2006 Mar 3;98(4):463-71.

Das S, Zhang E, Senapati P, Amaram V, Reddy MA, Stapleton K, Leung A, Lanting L, Wang M, Chen Z, Kato M, Oh HJ, Guo Q, Zhang X, Zhang B, Zhang H, Zhao Q, Wang W, Wu Y, Natarajan R. A Novel Angiotensin II-Induced Long Noncoding RNA Giver Regulates Oxidative Stress, Inflammation, and Proliferation in Vascular Smooth Muscle Cells. Circ Res. 2018 Dec 7;123(12):1298-1312.

Davis CA, Hitz BC, Sloan CA, Chan ET, Davidson JM, Gabdank I, Hilton JA, Jain K, Baymuradov UK, Narayanan AK, Onate KC, Graham K, Miyasato SR, Dreszer TR, Strattan JS, Jolanki O, Tanaka FY, Cherry JM. The Encyclopedia of DNA elements (ENCODE): data portal update. Nucleic Acids Res. 2018 Jan 4;46(D1):D794-D801.

Diehl AG, Boyle AP. Deciphering ENCODE. Trends Genet. 2016 Apr;32(4):238-249.

Dong B, Zhou B, Sun Z, Huang S, Han L, Nie H, Chen G, Liu S, Zhang Y, Bao N, Yang X, Feng H. LncRNA-FENDRR mediates VEGFA to promote the apoptosis of brain microvascular endothelial cells via regulating miR-126 in mice with hypertensive intracerebral hemorrhage. Microcirculation. 2018 Nov;25(8):e12499.

Expósito-Villén A, E Aránega A, Franco D. Functional Role of Non-Coding RNAs during Epithelial-To-Mesenchymal Transition. Noncoding RNA. 2018 May 28;4(2). pii: E14.

Fang S, Zhang L, Guo J, Niu Y, Wu Y, Li H, Zhao L, Li X, Teng X, Sun X, Sun L, Zhang MQ, Chen R, Zhao Y. NONCODEV5: a comprehensive annotation database for long non-coding RNAs. Nucleic Acids Res. 2018 Jan 4;46(D1):D308-D314.

Firulli AB, McFadden DG, Lin Q, Srivastava D, Olson EN. Heart and extra-embryonic mesodermal defects in mouse embryos lacking the bHLH transcription factor Hand1. Nat Genet. 1998 Mar;18(3):266-70.

Frade AF, Laugier L, Ferreira LR, Baron MA, Benvenuti LA, Teixeira PC, Navarro IC, Cabantous S, Ferreira FM, da Silva Cândido D, Gaiotto FA, Bacal F, Pomerantzeff P, Santos RH, Kalil J, Cunha-Neto E, Chevillard C. Myocardial Infarction-Associated

Transcript, a Long Noncoding RNA, Is Overexpressed During Dilated Cardiomyopathy Due to Chronic Chagas Disease. J Infect Dis. 2016 Jul 1;214(1):161-5.

Franco D, Campione M, Kelly R, Zammit PS, Buckingham M, Lamers WH, Moorman AF. Multiple transcriptional domains, with distinct left and right components, in the atrial chambers of the developing heart. Circ Res. 2000 Nov 24;87(11):984-91.

Franco D, Campione M. The role of Pitx2 during cardiac development. Linking left-right signaling and congenital heart diseases. Trends Cardiovasc Med. 2003 May;13(4):157-63

Franco D, Christoffels VM, Campione M. Homeobox transcription factor Pitx2: The rise of an asymmetry gene in cardiogenesis and arrhythmogenesis. Trends Cardiovasc Med. 2014 Jan;24(1):23-31.

Garcia-Padilla C, Aranega AE, Franco D. The role of long non-coding RNAs in cardiac development and disease. Genetics, 2018, 5(2): 124-140. doi: 10.3934/genet.2018.2.124.

Grabmaier U, Clauss S, Gross L, Klier I, Franz WM, Steinbeck G, Wakili R, Theiss HD, Brenner C. Diagnostic and prognostic value of miR-1 and miR-29b on adverse ventricular remodeling after acute myocardial infarction – The SITAGRAMI-miR analysis. Int J Cardiol. 2017 Oct 1;244:30-36.

Greco S, Zaccagnini G, Perfetti A, Fuschi P, Valaperta R, Voellenkle C, Castelvecchio S, Gaetano C, Finato N, Beltrami AP, Menicanti L, Martelli F. Long noncoding RNA dysregulation in ischemic heart failure. J Transl Med. 2016 Jun 18;14(1):183.

Greco S, Zaccagnini G, Perfetti A, Fuschi P, Valaperta R, Voellenkle C, Castelvecchio S, Gaetano C, Finato N, Beltrami AP, Menicanti L, Martelli F. Long noncoding RNA dysregulation in ischemic heart failure. J Transl Med. 2016 Jun 18;14(1):183.

Grote, P.; Wittler, L.; Hendrix, D.; Koch, F.; Währisch, S.; Beisaw, A.; Macura, K.; Bläss, G.; Kellis, M.; Werber, M.; et al. The tissue-specific lncRNA Fendrr is an essential regulator of heart and body wall development in the mouse. Dev. Cell 2013, 24, 206–214.

Guo JR, Yin L, Chen YQ, Jin XJ, Zhou X, Zhu NN, Liu XQ, Wei HW, Duan LS. Autologous blood transfusion augments impaired wound healing in diabetic mice by enhancing lncRNA H19 expression via the HIF-1α signaling pathway. Cell Commun Signal. 2018 Nov 20;16(1):84.

Gyvyte U, Kupcinskas J, Juzenas S, Inciuraite R, Poskiene L, Salteniene V, Link A, Fassan M, Franke A, Kupcinskas L, Skieceviciene J. Identification of long intergenic non-coding RNAs (lincRNAs) deregulated in gastrointestinal stromal tumors (GISTs). PLoS One. 2018 Dec 17;13(12):e0209342.

Habets PE, Moorman AF, Clout DE, van Roon MA, Lingbeek M, van Lohuizen M, Campione M, Christoffels VM. Cooperative action of Tbx2 and Nkx2.5 inhibits ANF expression in the atrioventricular canal: implications for cardiac chamber formation. Genes Dev. 2002 May 15;16(10):1234-46.

Haemmig S, Simion V, Yang D, Deng Y, Feinberg MW. Long noncoding RNAs in cardiovascular disease, diagnosis, and therapy. Curr Opin Cardiol. 2017 Nov;32(6):776-783.

Han P, Li W, Lin CH, Yang J, Shang C, Nuernberg ST, Jin KK, Xu W, Lin CY, Lin CJ, Xiong Y, Chien H, Zhou B, Ashley E, Bernstein D, Chen PS, Chen HV, Quertermous T, Chang CP. A long noncoding RNA protects the heart from pathological hypertrophy. Nature. 2014 Oct 2;514(7520):102-106.

Harrelson Z, Kelly RG, Goldin SN, Gibson-Brown JJ, Bollag RJ, Silver LM, Papaioannou VE. Tbx2 is essential for patterning the atrioventricular canal and for morphogenesis of the outflow tract during heart development. Development. 2004 Oct;131(20):5041-52.

Harvey RP. Seeking a regulatory roadmap for heart morphogenesis. Semin Cell Dev Biol. 1999 Feb;10(1):99-107

He W, Zhong G, Wang P, Jiang C, Jiang N, Huang J. Downregulation of long noncoding RNA FENDRR predicts poor prognosis in renal cell carcinoma. Oncol Lett. 2019 Jan;17(1):103-112.

He Z, Wang X, Huang C, Gao Y, Yang C, Zeng P, Chen Z. The FENDRR/miR-214-3P/TET2 axis affects cell malignant activity via RASSF1A methylation in gastric cancer. Am J Transl Res. 2018 Oct 15;10(10):3211-3223.

Hernandez-Torres F, Aranega AE, Franco D. Identification of regulatory elements directing miR-23a-miR-27a-miR-24-2 transcriptional regulation in response to muscle hypertrophic stimuli. Biochim Biophys Acta. 2014 Sep;1839(9):885-97.

Hou J, Long H, Zhou C, Zheng S, Wu H, Guo T, Wu Q, Zhong T, Wang T. Long noncoding RNA Braveheart promotes cardiogenic differentiation of mesenchymal stem cells in vitro. Stem Cell Res Ther. 2017 Jan 17;8(1):4.

Huang Y. The novel regulatory role of lncRNA-miRNA-mRNA axis in cardiovascular diseases. J Cell Mol Med. 2018 Dec;22(12):5768-5775. doi: 10.1111/jcmm.13866.

Huang ZW, Tian LH, Yang B, Guo RM. Long Noncoding RNA H19 Acts as a Competing Endogenous RNA to Mediate CTGF Expression by Sponging miR-455 in Cardiac Fibrosis. DNA Cell Biol. 2017 Sep;36(9):759-766.

Islas JF, Moreno-Cuevas JE. A MicroRNA Perspective on Cardiovascular Development and Diseases: An Update. Int J Mol Sci. 2018 Jul 17;19(7). pii:E2075.

Jerome LA, Papaioannou VE. DiGeorge syndrome phenotype in mice mutant for the T-box gene, Tbx1. Nat Genet. 2001 Mar;27(3):286-91.

Kahaly GJ, Dillmann WH. Thyroid hormone action in the heart. Endocr Rev. 2005 Aug;26(5):704-28.

Kitamura K, Miura H, Miyagawa-Tomita S, Yanazawa M, Katoh-Fukui Y, Suzuki R, Ohuchi H, Suehiro A, Motegi Y, Nakahara Y, Kondo S, Yokoyama M. Mouse Pitx2 deficiency leads to anomalies of the ventral body wall, heart, extra- and periocular mesoderm and right pulmonary isomerism. Development. 1999 Dec;126(24):5749-58.

Klattenhoff, C.A.; Scheuermann, J.C.; Surface, L.E.; Bradley, R.K.; Fields, P.A.; Steinhauser, M.L.; Ding, H.; Butty, V.L.; Torrey, L.; Haas, S.; et al. Braveheart, a long noncoding RNA required for cardiovascular lineage commitment. Cell 2013, 152, 570–583.

Kucia M, Dawn B, Hunt G, Guo Y, Wysoczynski M, Majka M, Ratajczak J, Rezzoug F, Ildstad ST, Bolli R, Ratajczak MZ. Cells expressing early cardiac markers reside in

the bone marrow and are mobilized into the peripheral blood after myocardial infarction. Circ Res. 2004 Dec 10;95(12):1191-9.

Kuo CT, Morrisey EE, Anandappa R, Sigrist K, Lu MM, Parmacek MS, Soudais C, Leiden JM. GATA4 transcription factor is required for ventral morphogenesis and heart tube formation. Genes Dev. 1997 Apr 15;11(8):1048-60

Kurian, L.; Aguirre, A.; Sancho-Martinez, I.; Benner, C.; Hishida, T.; Nguyen, T.B.; Reddy, P.; Nivet, E.; Krause, M.N.; Nelles, D.A.; et al. Identification of novel long noncoding RNAs underlying vertebrate cardiovascular development. Circulation 2015, 131, 1278–1290.

Kuwahara K, Teg Pipes GC, McAnally J, Richardson JA, Hill JA, Bassel-Duby R, Olson EN. Modulation of adverse cardiac remodeling by STARS, a mediator of MEF2 signaling and SRF activity. J Clin Invest. 2007 May;117(5):1324-34

Li L, Wang Q, Yuan Z, Chen A, Liu Z, Li H, Wang Z. Long non-coding RNA H19 contributes to hypoxia-induced CPC injury by suppressing Sirt1 through miR-200a-3p. Acta Biochim Biophys Sin (Shanghai). 2018 Oct 1;50(10):950-959.

Li Y, Wang J, Sun L, Zhu S. LncRNA myocardial infarction-associated transcript (MIAT) contributed to cardiac hypertrophy by regulating TLR4 via miR-93. Eur J. Pharmacol. 2018 Jan 5;818:508-517.

Li Z, Liu Y, Guo X, Sun G, Ma Q, Dai Y, Zhu G, Sun Y. Long noncoding RNA myocardial infarction-associated transcript is associated with the microRNA-150-5p/P300 pathway in cardiac hypertrophy. Int J Mol Med. 2018 Sep;42(3):1265-1272.

Liao J, Kochilas L, Nowotschin S, Arnold JS, Aggarwal VS, Epstein JA, Brown MC, Adams J, Morrow BE. Full spectrum of malformations in velo-cardio-facial syndrome/DiGeorge syndrome mouse models by altering Tbx1 dosage. Hum Mol Genet. 2004 Aug 1;13(15):1577-85.

Lin Q, Schwarz J, Bucana C, Olson EN. Control of mouse cardiac morphogenesis and myogenesis by transcription factor MEF2C. Science. 1997 May 30;276(5317):1404-7

Liu C, Bai B, Skogerbø G, Cai L, Deng W, Zhang Y, Bu D, Zhao Y, Chen R. NONCODE: an integrated knowledge database of non-coding RNAs. Nucleic Acids Res. 2005 Jan 1;33(Database issue):D112-5.

Liu L, An X, Li Z, Song Y, Li L, Zuo S, Liu N, Yang G, Wang H, Cheng X, Zhang Y, Yang X, Wang J. The H19 long noncoding RNA is a novel negative regulator of cardiomyocyte hypertrophy. Cardiovasc Res. 2016 Jul 1;111(1):56-65.

Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods. 2001 Dec;25(4):402-8.

López-Sánchez C, García-Martínez V. Molecular determinants of cardiac specification. Cardiovasc Res. 2011 Jul 15;91(2):185-95.

Lozano-Velasco E, Galiano-Torres J, Jodar-Garcia A, Aranega AE, Franco D. miR-27 and miR-125 Distinctly Regulate Muscle-Enriched Transcription Factors in Cardiac and Skeletal Myocytes. Biomed Res Int. 2015;2015:391306.

Lozano-Velasco E, Hernández-Torres F, Daimi H, Serra SA, Herraiz A, Hove-Madsen L, Aránega A, Franco D. Pitx2 impairs calcium handling in a dose-dependent manner by modulating Wnt signalling. Cardiovasc Res. 2016 Jan 1;109(1):55-66.

Lozano-Velasco E, Vallejo D, Esteban FJ, Doherty C, Hernández-Torres F, Franco D, Aránega AE. A Pitx2-MicroRNA Pathway Modulates Cell Proliferation in Myoblasts and Skeletal-Muscle Satellite Cells and Promotes Their Commitment to a Myogenic Cell Fate. Mol Cell Biol. 2015 Sep 1;35(17):2892-909.

Lyons I, Parsons LM, Hartley L, Li R, Andrews JE, Robb L, Harvey RP. Myogenic and morphogenetic defects in the heart tubes of murine embryos lacking the homeo box gene Nkx2-5. Genes Dev. 1995 Jul 1;9(13):1654-66.

Matouk IJ, DeGroot N, Mezan S, Ayesh S, Abu-lail R, Hochberg A, Galun E. The H19 non-coding RNA is essential for human tumor growth. PLoS One. 2007 Sep 5;2(9):e845.

Miano JM, Ramanan N, Georger MA, de Mesy Bentley KL, Emerson RL, Balza RO Jr, Xiao Q, Weiler H, Ginty DD, Misra RP. Restricted inactivation of serum response factor to the cardiovascular system. Proc Natl Acad Sci U S A. 2004 Dec 7;101(49):17132-7.

Micheletti, R.; Plaisance, I.; Abraham, B.J.; Sarre, A.; Ting, C.C.; Alexanian, M.; Maric, D.; Maison, D.; Nemir, M.; Young, R.A.; et al. The long noncoding RNA Wisper controls cardiac fibrosis and remodeling. Sci. Transl. Med. 2017, 9, eaai9118.

Moorman AF, Christoffels VM. Cardiac chamber formation: development, genes, and evolution. Physiol Rev. 2003 Oct;83(4):1223-67.

Moorman AF, Soufan AT, Hagoort J, de Boer PA, Christoffels VM. Development of the building plan of the heart. Ann N Y Acad Sci. 2004 May;1015:171-81.

Oudit GY, Crackower MA, Backx PH, Penninger JM. The role of ACE2 in cardiovascular physiology. Trends Cardiovasc Med. 2003 Apr;13(3):93-101.

Ounzain, S.; Micheletti, R.; Arnan, C.; Plaisance, I.; Cecchi, D.; Schroen, B.; Reverter, F.; Alexanian, M.; Gonzales, C.; Ng, S.Y.; et al. CARMEN, a human super enhancer-associated long noncoding RNA controlling cardiac specification, differentiation and homeostasis. J. Mol. Cell. Cardiol. 2015, 89 Pt A, 98–112.

Pérez-Pomares JM, Phelps A, Sedmerova M, Carmona R, González-Iriarte M, Muñoz-Chápuli R, Wessels A. Experimental studies on the spatiotemporal expression of WT1 and RALDH2 in the embryonic avian heart: a model for the regulation of myocardial and valvuloseptal development by epicardially derived cells (EPDCs). Dev Biol. 2002 Jul 15;247(2):307-26.

Piccoli, M.T.; Gupta, S.K.; Viereck, J.; Foinquinos, A.; Samolovac, S.; Kramer, F.L.; Garg, A.; Remke, J.; Zimmer, K.; Batkai, S.; et al. Inhibition of the Cardiac Fibroblast-Enriched lncRNA Meg3 Prevents Cardiac Fibrosis and Diastolic Dysfunction. Circ. Res. 2017, 121, 575–583.

Plaisance I, Perruchoud S, Fernandez-Tenorio M, Gonzales C, Ounzain S, Ruchat P, Nemir M, Niggli E, Pedrazzini T. Cardiomyocyte Lineage Specification in Adult Human Cardiac Precursor Cells Via Modulation of Enhancer-Associated Long Noncoding RNA Expression. JACC Basic Transl Sci. 2016 Oct;1(6):472-493.

Ponnusamy, M.; Liu, F.; Zhang, Y.H.; Li, R.B.; Zhai, M.; Liu, F.; Zhou, L.Y.; Liu, C.Y.; Yan, K.W.; Dong, Y.H.; et al. The Long Non-Coding RNA CPR Regulates Cardiomyocyte Proliferation and Cardiac Repair. Circulation. 2019, doi:10.1161/CIRCULATIONAHA.118.035832.

Qu X, Du Y, Shu Y, Gao M, Sun F, Luo S, Yang T, Zhan L, Yuan Y, Chu W, Pan Z, Wang Z, Yang B, Lu Y. MIAT Is a Pro-fibrotic Long Non-coding RNA Governing Cardiac Fibrosis in Post-infarct Myocardium. Sci Rep. 2017 Feb 15;7:42657.

Ribeiro I, Kawakami Y, Büscher D, Raya A, Rodríguez-León J, Morita M, Rodríguez Esteban C, Izpisúa Belmonte JC. Tbx2 and Tbx3 regulate the dynamics of cell proliferation during heart remodeling. PLoS One. 2007 Apr 25;2(4):e398.

Ryan K, Chin AJ. T-box genes and cardiac development. Birth Defects Res C Embryo Today. 2003 Feb;69(1):25-37.

Sallam T, Sandhu J, Tontonoz P. Long Noncoding RNA Discovery in Cardiovascular Disease: Decoding Form to Function. Circ Res. 2018 Jan 5;122(1):155-166.

Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative C(T) method. Nat Protoc. 2008;3(6):1101-8.

Shi Y, Chen C, Xu Y, Liu Y, Zhang H, Liu Y. LncRNA FENDRR promotes high-glucose-induced proliferation and angiogenesis of human retinal endothelial cells. Biosci Biotechnol Biochem. 2019 Jan 30:1-7.

Small EM, Thatcher JE, Sutherland LB, Kinoshita H, Gerard RD, Richardson JA, Dimaio JM, Sadek H, Kuwahara K, Olson EN. Myocardin-related transcription factor-a controls myofibroblast activation and fibrosis in response to myocardial infarction. Circ Res. 2010 Jul 23;107(2):294-304.

Song, G.; Shen, Y.; Ruan, Z.; Li, X.; Chen, Y.; Yuan, W.; Ding, X.; Zhu, L.; Qian, L. LncRNA-uc.167 influences cell proliferation, apoptosis and differentiation of P19 cells by regulating Mef2c. Gene 2016, 590, 97–108.

Sperling SR. Systems biology approaches to heart development and congenital heart disease. Cardiovasc Res. 2011 Jul 15;91(2):269-78.

Srivastava D, Cserjesi P, Olson EN. A subclass of bHLH proteins required for cardiac morphogenesis. Science. 1995 Dec 22;270(5244):1995-9.

Srivastava D, Thomas T, Lin Q, Kirby ML, Brown D, Olson EN. Regulation of cardiac mesodermal and neural crest development by the bHLH transcription factor, dHAND. Nat Genet. 1997 Jun;16(2):154-60.

Srivastava D. Genetic regulation of cardiogenesis and congenital heart disease. Annu Rev Pathol. 2006;1:199-213.

Stevens MV, Parker P, Vaillancourt RR, Camenisch TD. MEKK4 regulates developmental EMT in the embryonic heart. Dev Dyn. 2006 Oct;235(10):2761-70.

Takeuchi JK, Ohgi M, Koshiba-Takeuchi K, Shiratori H, Sakaki I, Ogura K, Saijoh Y, Ogura T. Tbx5 specifies the left/right ventricles and ventricular septum position during cardiogenesis. Development. 2003 Dec;130(24):5953-64.

Terentyev D, Belevych AE, Terentyeva R, Martin MM, Malana GE, Kuhn DE, Abdellatif M, Feldman DS, Elton TS, Györke S. miR-1 overexpression enhances Ca(2+) release and promotes cardiac arrhythmogenesis by targeting PP2A regulatory subunit

B56alpha and causing CaMKII-dependent hyperphosphorylation of RyR2. Circ Res. 2009 Feb 27;104(4):514-21.

Thomas T, Yamagishi H, Overbeek PA, Olson EN, Srivastava D. The bHLH factors, dHAND and eHAND, specify pulmonary and systemic cardiac ventricles independent of left-right sidedness. Dev Biol. 1998 Apr 15;196(2):228-36.

Tritsch E, Mallat Y, Lefebvre F, Diguet N, Escoubet B, Blanc J, De Windt LJ, Catalucci D, Vandecasteele G, Li Z, Mericskay M. An SRF/miR-1 axis regulates NCX1 and annexin A5 protein levels in the normal and failing heart. Cardiovasc Res. 2013 Jun 1;98(3):372-80

Viereck, J.; Kumarswamy, R.; Foinquinos, A.; Xiao, K.; Avramopoulos, P.; Kunz, M.; Dittrich, M.; Maetzig, T.; Zimmer, K.; Remke, J.; et al. Long noncoding RNA Chast promotes cardiac remodeling. Sci. Transl. Med. 2016, 8, 326ra22.

Vitelli F, Morishima M, Taddei I, Lindsay EA, Baldini A. Tbx1 mutation causes multiple cardiovascular defects and disrupts neural crest and cranial nerve migratory pathways. Hum Mol Genet. 2002 Apr 15;11(8):915-22.

Wang, J.; Chen, X.; Shen, D.; Ge, D.; Chen, J.; Pei, J.; Li, Y.; Yue, Z.; Feng, J.; Chu, M.; et al. A long noncoding RNA NR_045363 controls cardiomyocyte proliferation and cardiac repair. J. Mol. Cell. Cardiol. 2019, 127, 105–114.

Wang, J.; Liu, X.; Zhuang, Q.; Pan, R.; Zou, L.; Cen, Z.; Tang, L. Long noncoding RNA homeobox A11 antisense promotes transforming growth factor β1-induced fibrogenesis in cardiac fibroblasts. Mol. Med. Rep. 2019, doi:10.3892/mmr.2019.9891.

Wang, Z.; Zhang, X.J.; Ji, Y.X.; Zhang, P.; Deng, K.Q.; Gong, J.; Ren, S.; Wang, X.; Chen, I.; Wang, H.; et al. The long noncoding RNA Chaer defines an epigenetic checkpoint in cardiac hypertrophy. Nat. Med. 2016, 22, 1131–1139.

Xin M, Olson EN, Bassel-Duby R. Mending broken hearts: cardiac development as a basis for adult heart regeneration and repair. Nat Rev Mol Cell Biol. 2013 Aug;14(8):529-41.

Xiong G, Jiang X, Song T. The overexpression of lncRNA H19 as a diagnostic marker for coronary artery disease. Rev Assoc Med Bras (1992). 2019 Feb;65(2):110-117.

Xu H, Morishima M, Wylie JN, Schwartz RJ, Bruneau BG, Lindsay EA, Baldini A. Tbx1 has a dual role in the morphogenesis of the cardiac outflow tract.Development. 2004 Jul;131(13):3217-27. Epub 2004 Jun 2. PubMed PMID: 15175244.

Xue Z, Hennelly S, Doyle B, Gulati AA, Novikova IV, Sanbonmatsu KY, Boyer LA. A G-Rich Motif in the lncRNA Braveheart Interacts with a Zinc-Finger Transcription Factor to Specify the Cardiovascular Lineage. Mol Cell. 2016 Oct 6;64(1):37-50

Yang B, Lin H, Xiao J, Lu Y, Luo X, Li B, Zhang Y, Xu C, Bai Y, Wang H, Chen G, Wang Z. The muscle-specific microRNA miR-1 regulates cardiac arrhythmogenic potential by targeting GJA1 and KCNJ2. Nat Med. 2007 Apr;13(4):486-91.

Yu BY, Dong B. LncRNA H19 regulates cardiomyocyte apoptosis and acute myocardial infarction by targeting miR-29b. Int J Cardiol. 2018 Nov 15;271:25.

Zammit PS, Kelly RG, Franco D, Brown N, Moorman AF, Buckingham ME. Suppression of atrial myosin gene expression occurs independently in the left and right ventricles of the developing mouse heart. Dev Dyn. 2000 Jan;217(1):75-85.

Zampetaki A, Albrecht A, Steinhofel K. Long Non-coding RNA Structure and Function: Is There a Link? Front Physiol. 2018 Aug 24;9:1201.

Zhang G, Han G, Zhang X, Yu Q, Li Z, Li Z, Li J. Long non-coding RNA FENDRR reduces prostate cancer malignancy by competitively binding miR-18a-5p with RUNX1. Biomarkers. 2018 Jul;23(5):435-445.

Zhang MY, Zhang ZL, Cui HX, Wang RK, Fu L. Long non-coding RNA FENDRR inhibits NSCLC cell growth and aggressiveness by sponging miR-761. Eur Rev Med Pharmacol Sci. 2018 Dec;22(23):8324-8332.

Zhang X, Cheng L, Xu L, Zhang Y, Yang Y, Fu Q, Mi W, Li H. The Lncrna, H19 Mediates the Protective Effect of Hypoxia Postconditioning Against Hypoxia-Reoxygenation Injury to Senescent Cardiomyocytes by Targeting MicroRNA-29b-3p. Shock. 2018 Jun 27.

Zhang Y, Zhang M, Xu W, Chen J, Zhou X. The long non-coding RNA H19 promotes cardiomyocyte apoptosis in dilated cardiomyopathy. Oncotarget. 2017 Apr 25;8(17):28588-28594.

Zhang Z, Gao W, Long QQ, Zhang J, Li YF, Liu DC, Yan JJ, Yang ZJ, Wang LS. Increased plasma levels of lncRNA H19 and LIPCAR are associated with increased risk of coronary artery disease in a Chinese population. Sci Rep. 2017 Aug 8;7(1):7491.

Zhang Z, Weaver DL, Olsen D, deKay J, Peng Z, Ashikaga T, Evans MF. Long non-coding RNA chromogenic in situ hybridisation signal pattern correlation with breast tumour pathology. J Clin Pathol. 2016 Jan;69(1):76-81.

Zhao D, Ge H, Ma B, Xue D, Zhang W, Li Z, Sun H. The interaction between ANXA2 and lncRNA Fendrr promotes cell apoptosis in caerulein-induced acute pancreatitis. J Cell Biochem. 2018 Nov 26.

Zhao Y, Li H, Fang S, Kang Y, Wu W, Hao Y, Li Z, Bu D, Sun N, Zhang MQ, Chen R. NONCODE 2016: an informative and valuable data source of long non-coding RNAs. Nucleic Acids Res. 2016 Jan 4;44(D1):D203-8.

Zheng, D.; Zhang, Y.; Hu, Y.; Guan, J.; Xu, L.; Xiao, W.; Zhong, Q.; Ren, C.; Lu, J.; Liang, J.; et al. Long noncoding RNA Crnde attenuates cardiac fibrosis via Smad3-Crnde negative feedback in diabetic cardiomyopathy. FEBS J. 2019, doi:10.1111/febs.14780.

Zhou X, Zhang W, Jin M, Chen J, Xu W, Kong X. lncRNA MIAT functions as a competing endogenous RNA to upregulate DAPK2 by sponging miR-22-3p in diabetic cardiomyopathy. Cell Death Dis. 2017 Jul 13;8(7):e2929.

Zhu XH, Yuan YX, Rao SL, Wang P. LncRNA MIAT enhances cardiac hypertrophy partly through sponging miR-150. Eur Rev Med Pharmacol Sci. 2016 Sep;20(17):3653-60.

Figure legends

Figure 1. Developmental expression profiles of *Braveheart* (panel A), *Carmen* (panel B), *Fendrr* (panel C), *Miat* (panel D), *H19* (panel E) and *Alien* (panel F) from E12,5 to adulthood in right atrium (RA), left atrium (LA) and ventricles (V), respectively as revealed by qPCR. Note that *Braveheart, Carmen* and *Fendrr* display preferentially higher expression levels in the adult heart, while *Miat*, *H19* and *Alien* display a more dynamic expression profile during embryonic stages while decreasing in adulthood. It is also worth highlighting that several lncRNAs display chamber-specific expression such as e.g. *Carmen* and *Fendrr* that are preferentially expressed in the ventricles or *H19* in the right atrium and ventricles.

Figure 2. qPCR analyses of lncRNA expression in *Nkx2.5, Srf* and *Mef2c* gain and loss-of-function assays in HL1 atrial cardiomyocytes. Panel A, B and C display *Nkx2,5, Srf* and *Mef2c* expression in gain-of-function (over) and loss-of-function (si) assays, demonstrating significant up- and down-regulation of these transcription factors, respectively. Panel D, E and F display qPCR analyses of *Braveheart, Carmen, Fendrr, Miat, H19* and *Alien* in *Nkx2,5, Srf* and *Mef2c* gain (over) and loss-of-function (si) assays. Observe that both, gain and loss-of-function assays distinct regulate expression of these lncRNAs. Panel G schematically represents the influence of transcription factor gain-of-function (upper circles) and loss-of-function (lower circles), respectively, in lncRNA expression.

Figure 3. qPCR analyses of lncRNA expression in *Pitx2, Wnt8* and *Wnt11* gain and loss-of-function assays in HL1 atrial cardiomyocytes. Panel A, B and C display *Pitx2, Wnt8* and *Wnt11* expression in gain-of-function (over) and loss-of-function (si) assays, demonstrating significant up- and down-regulation of these factors, respectively. Panel D, E and F display qPCR analyses of *Braveheart, Carmen, Fendrr, Miat, H19* and *Alien* in *Pitx2, Wnt8* and *Wnt11* gain (over) and loss-of-function (si) assays. Observe that both, gain and loss-of-function assays distinct regulate expression of these lncRNAs. Panel G schematically represents the influence of transcription factor/ligand gain-of-function (upper circles) and loss-of-function (lower circles), respectively, in lncRNA expression.

Figure 4. qPCR analyses of lncRNA expression in *miR-1, miR-29* and *miR-133* gain-of-function assays in HL1 atrial cardiomyocytes. Panel A display *miR-1, miR-29* and *miR-133* expression in gain-of-function (over) assays, demonstrating significant up-regulation of these microRNAs, respectively. Panels B to F display qPCR analyses of *Braveheart, Carmen, Fendrr, Miat, H19* and *Alien,* in *miR-1, miR-29* and *miR-133* gain-of-function assays. Observe that *miR-1* significantly up-regulates *Carmen*, F*endrr* and H19 while *miR-29* up-regulates *Fendrr*. On the other hand, *miR-29* and *miR-133* significantly down-regulates *Miat*. Panel G display *miR-1, miR-29* and *miR-133* expression in gain-of-function assays, demonstrating significant up-regulation of these microRNAs, respectively.

Figure 5. qPCR analyses of *Braveheart, Carmen, Fendrr, Miat, H19* and *Alien,* after angiotensin II (AngII) or norepinephrine (NE) (panel A), and T3 or T4 thyroid hormone (panel B) administration in HL1 atrial cardiomyocytes, respectively. Observe that AngII and NE treatment enhanced *Carmen, Fendrr* and *H19* expression. T4 administration up-regulates *Braveheart, Fendrr* and *Alien* and downregulates *Carmen*, while T3 does not significantly alter lncRNA expression except for *Carmen*, *Fendrr* and *H19*, when a significant downregulation is observed.

Figure 6. PCR analyses of *Braveheart, Carmen and Fendrr* isoform expression in embryonic and adult RA, LA and V. Observe that *Braveheart* isoform 1 and 2 and *Carmen* 2 are similarly expressed in all tissues analyzed while *Fendrr* isoforms 1, 2 and 3 and *Carmen* isoform 1 display differentially expression profiles in embryonic and adult tissues as well as in left and right atrial chambers.

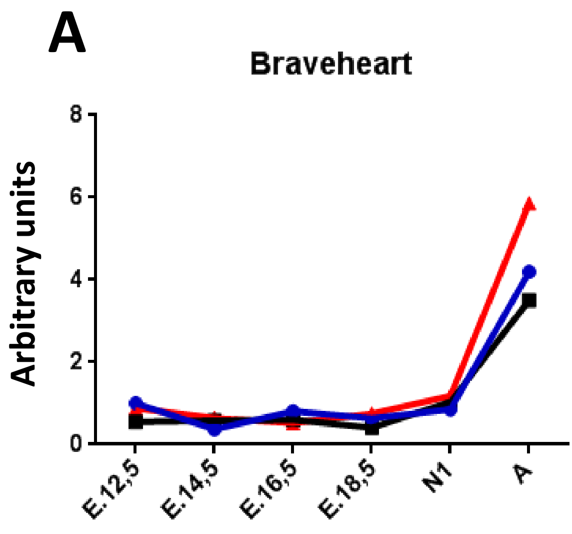
Figure 7. qPCR analyses of *Braveheart, H19, Carmen, Fendrr* in nuclear and cytoplasmic subcellular extracts of E12.5 embryos corresponding to RA, LA and V. Panel A illustrate control qPCR expression of *Rpb1* in the nucleus and *Gapdh* in the cytoplasm in all tissues and samples analyzed. Observe that *Braveheart* and *H19* display similar distribution in the nuclear and cytoplasmic fractions, while *Fendrr* and *Carmen* display enhanced cytoplasmic expression in the right atrium while enhanced nuclear localization in the left atrium and ventricles (Panels B-E).

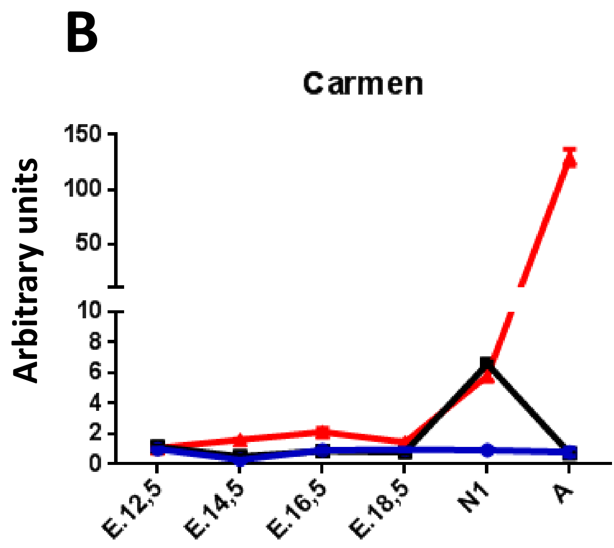
Figure 8. *In situ* hybridization analyses of H19 expression in mouse E10.5 embryonic heart. Observe endocardial, myocardial and epicardial expression (panels A-C,F-G). Panel D is a positive control *in situ* hybridization against Mlc2v delineating the ventricular myocardium. Panel E is a negative control.

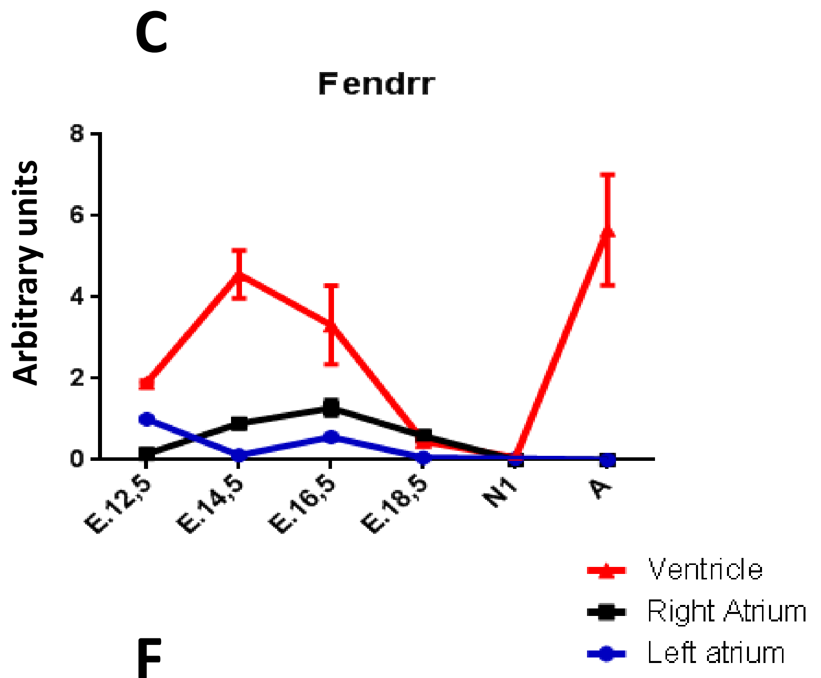
Figure 9. *In situ* hybridization analyses of H19 expression in mouse E12.5 embryonic heart. Observe endocardial, myocardial and epicardial expression (panels A-G). Panel H is a positive control *in situ* hybridization against Mlc2v delineating the ventricular myocardium. Panel I is a negative control.

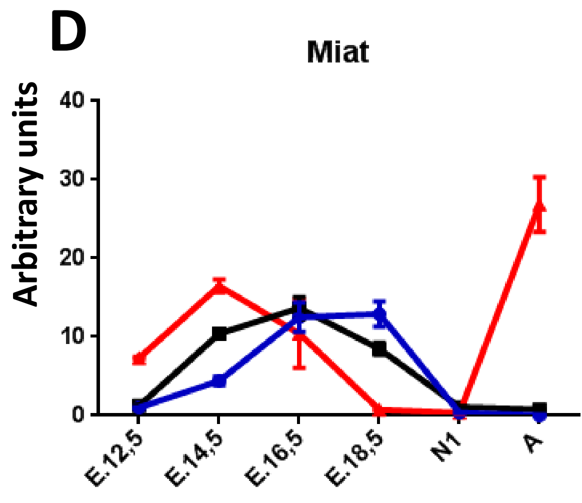
Figure 10. *In situ* hybridization analyses of H19 expression in mouse E14.5 embryonic heart. Observe the epicardial (arrows) and endocardial (arrowheads) expression in the right atrium (panels A and a´) and left atrium (panel C and c´). Expression in the ventricular chambers (panel B) is restricted to the endocardium (panels D, d´, E and e´), the valve leaflets (panels b´and b´´) and the epicardium (panels e´and d´).

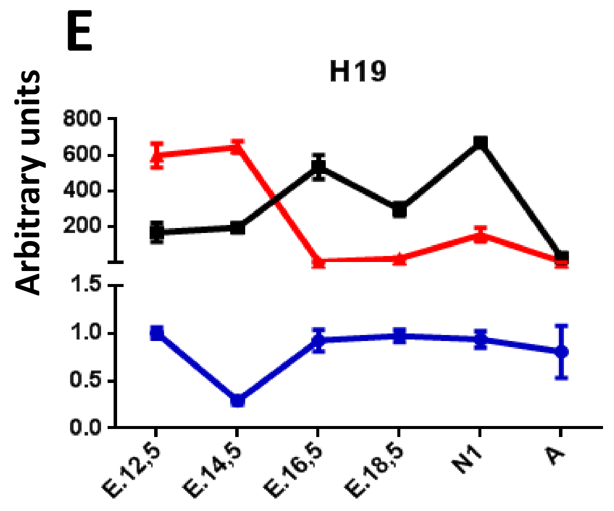
Figure 11. *In situ* hybridization analyses of H19 expression in mouse neonatal heart. Observe the endocardial expression in the right and left ventricles (panels A-B, b´, b´´) and tricuspid (panel a´) and mitral (panel a´´) valves. Observe that expression is mainly cytoplasmic (panel C). Panel D represents a DAPI nuclear staining of panel C. H19 expression is also observed in the endocardium of the right atrium (panel E and e´), but not in the left atrium (panel F). It is noteworthy that the epicardium display no H19 expression at this stage (panel A, B and E).

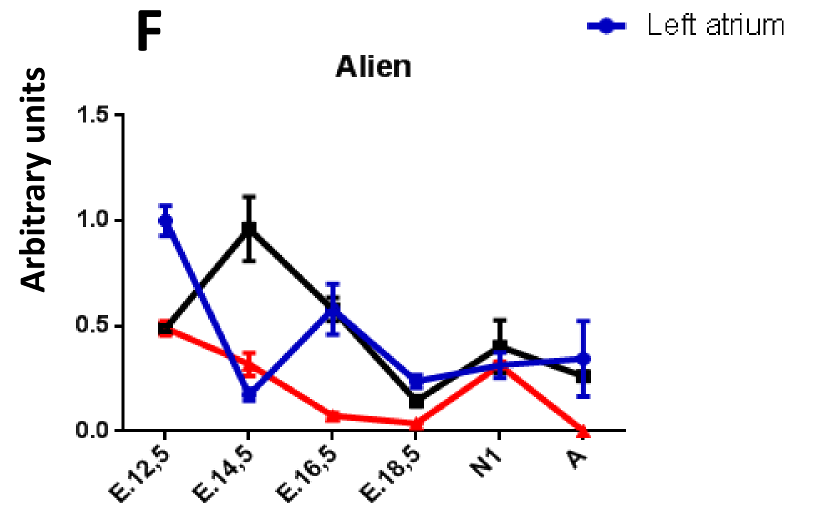






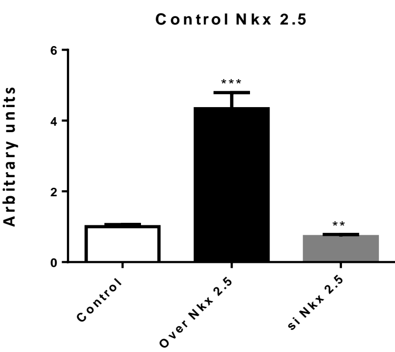




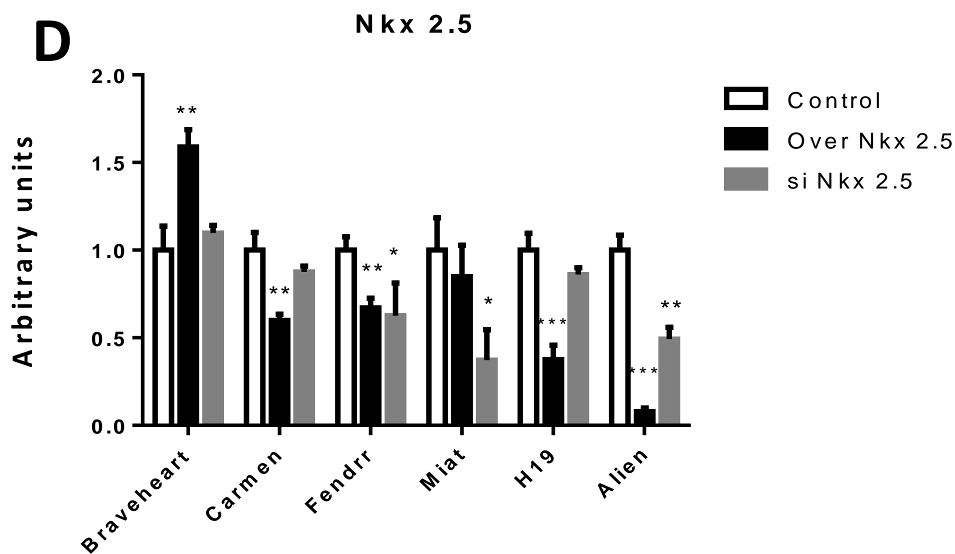


Garcia-Padilla et al., Figure 1

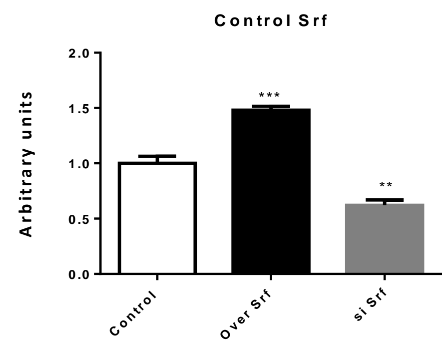
A



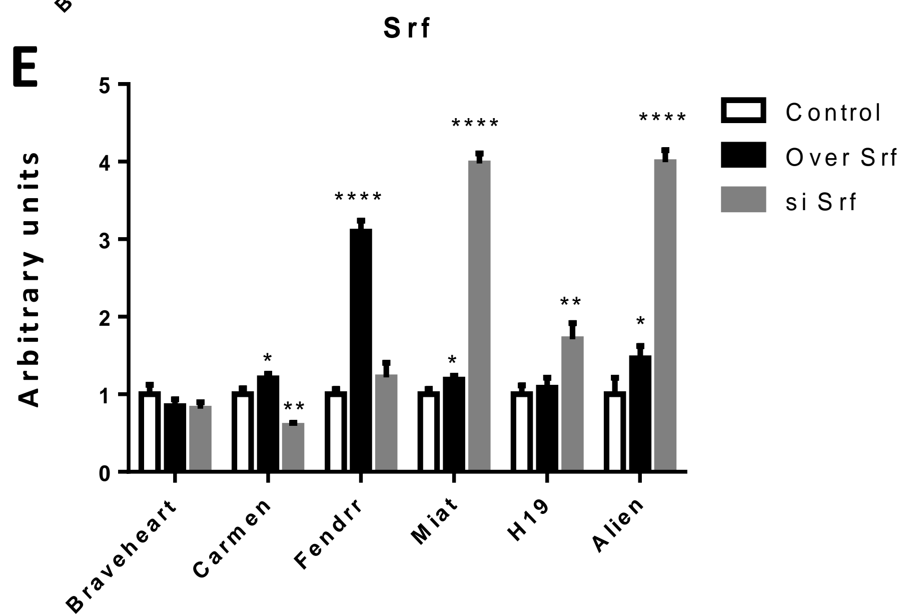
D



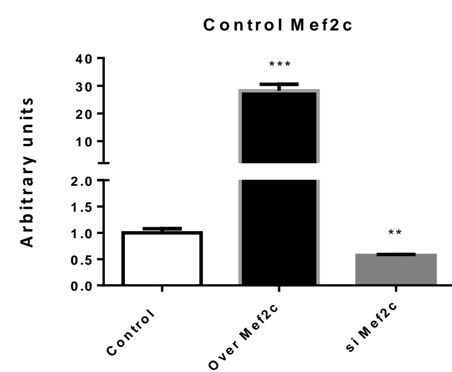
B



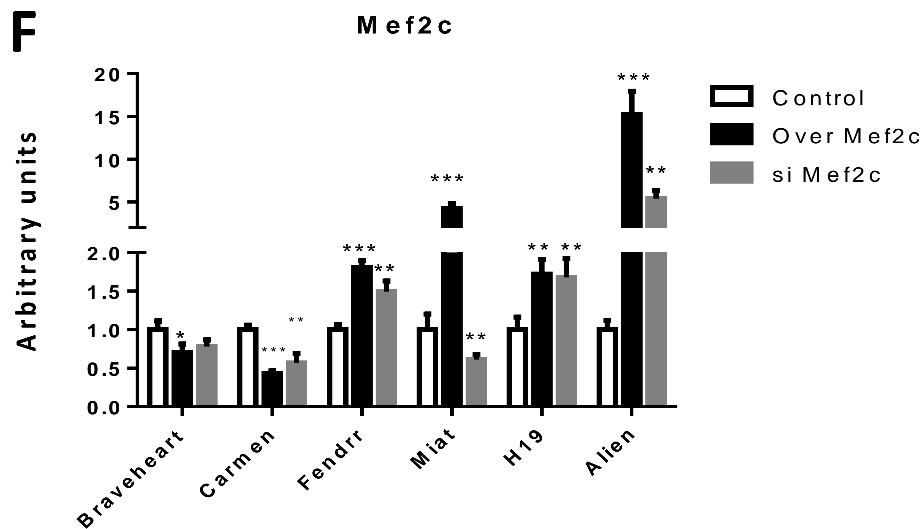
E



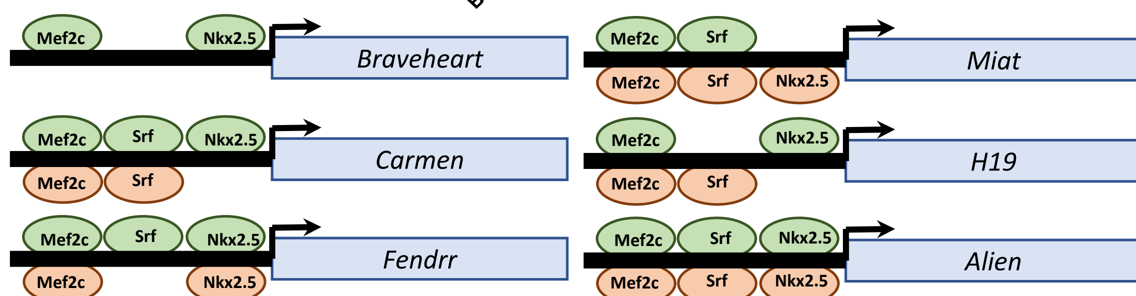
C



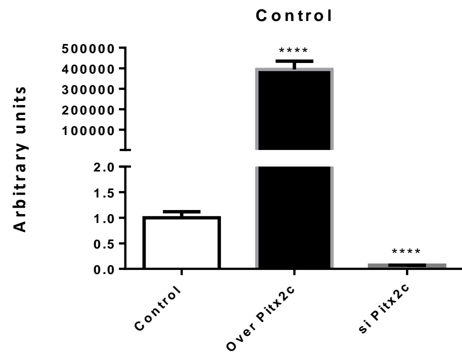
F

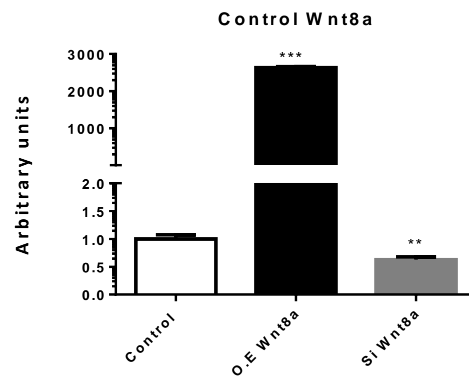


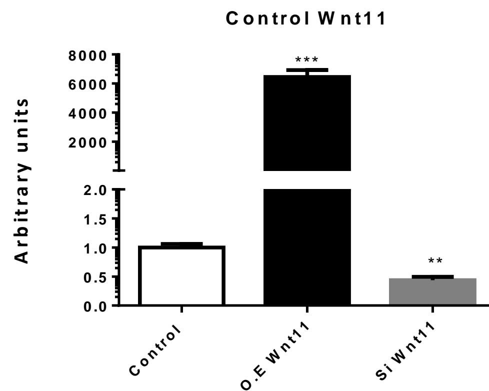
G

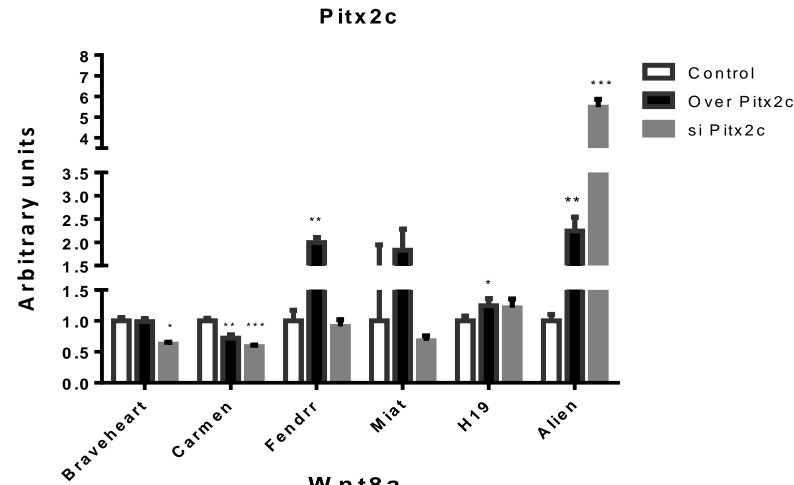


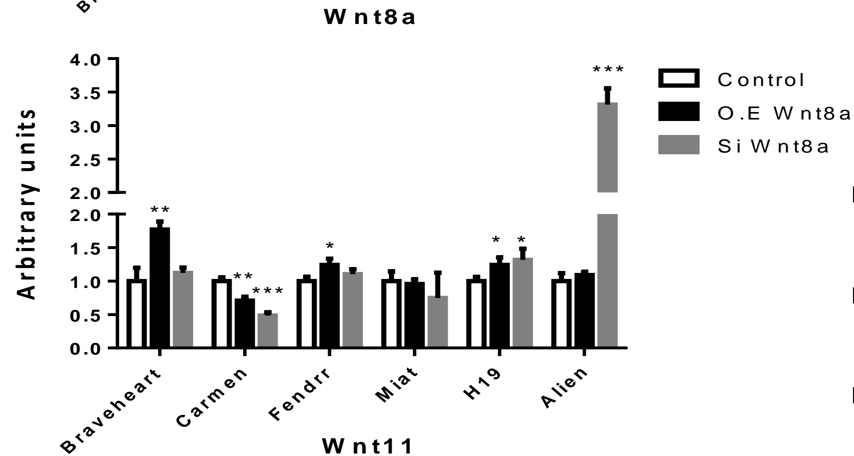
Garcia-Padilla et al., Figure 2

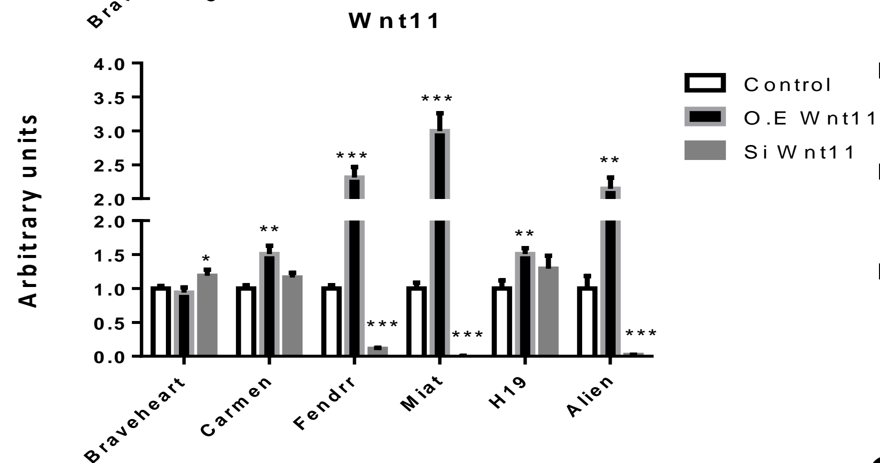


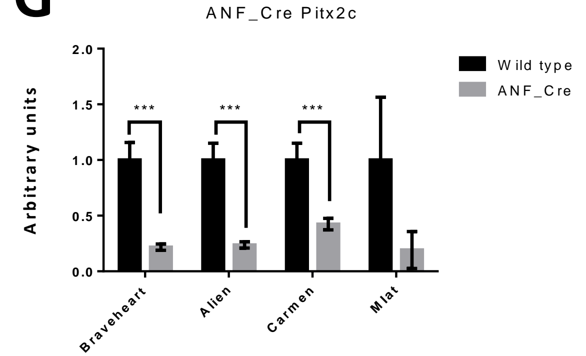


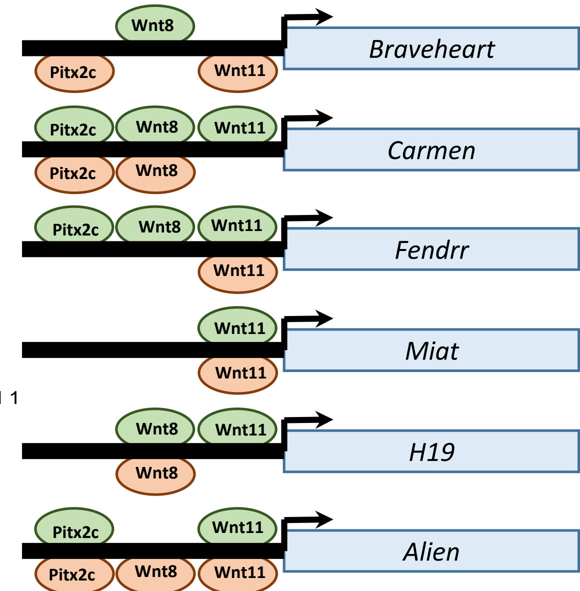




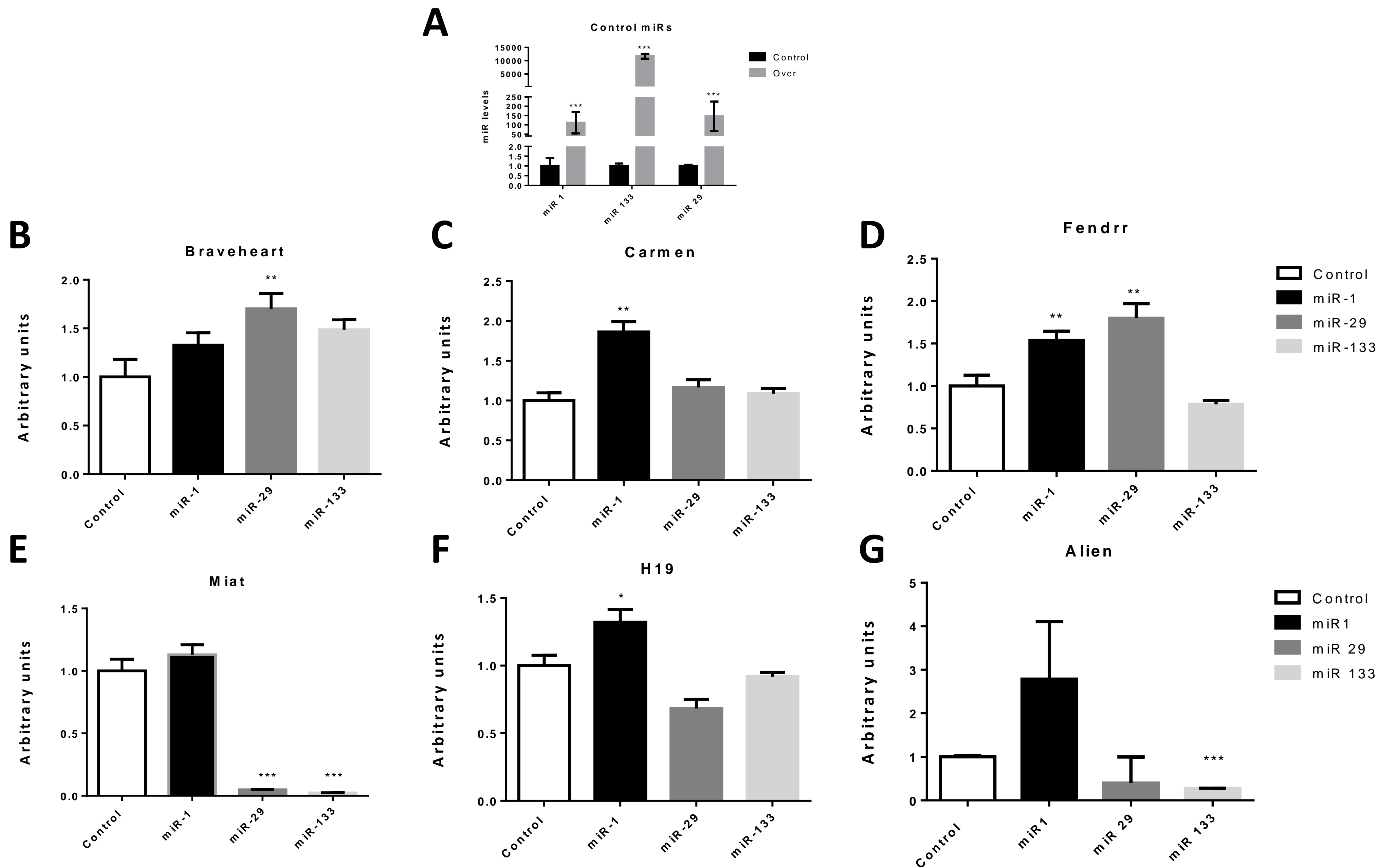






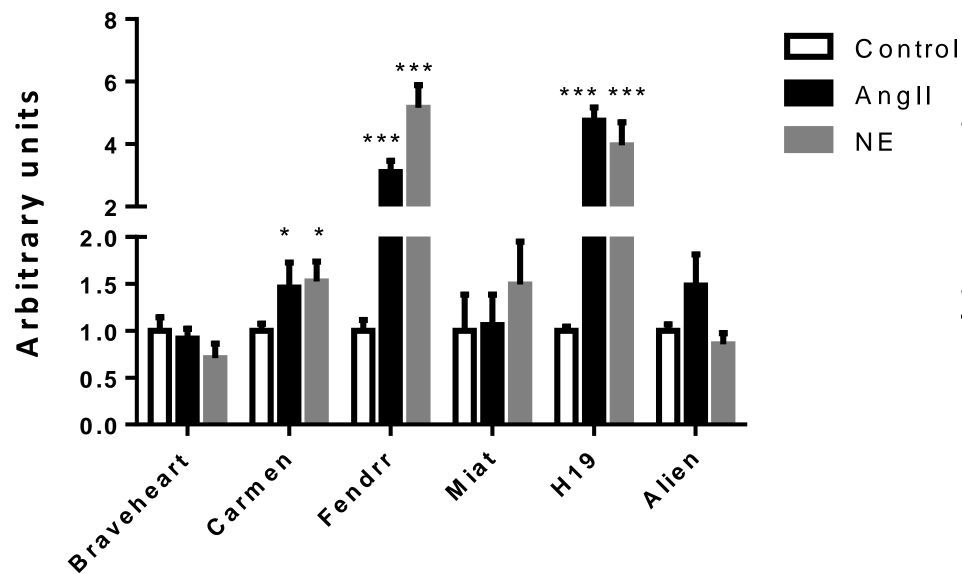


Garcia-Padilla et al., Figure 3

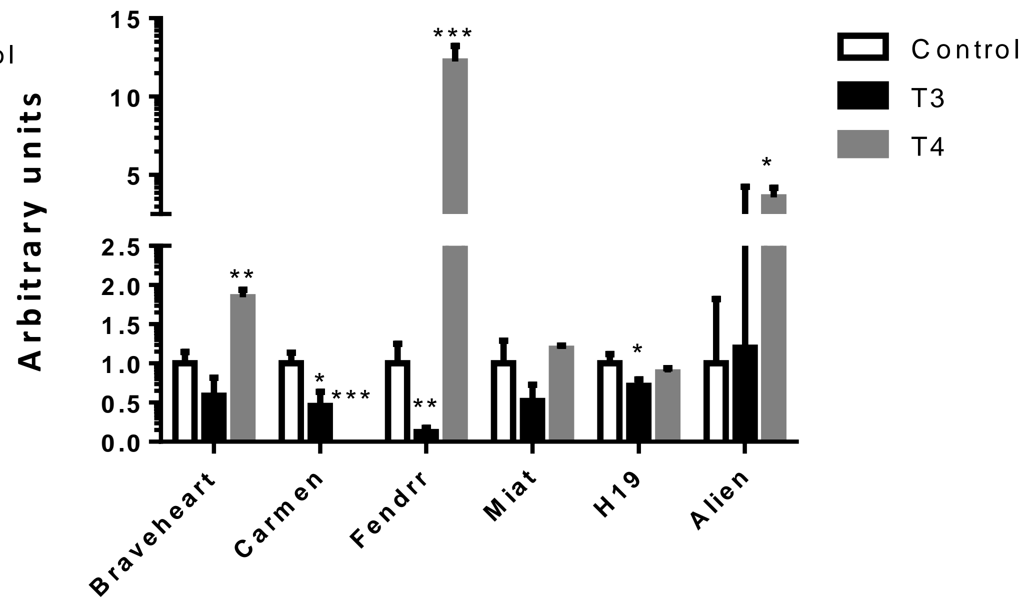


Garcia-Padilla et al., Figure 4

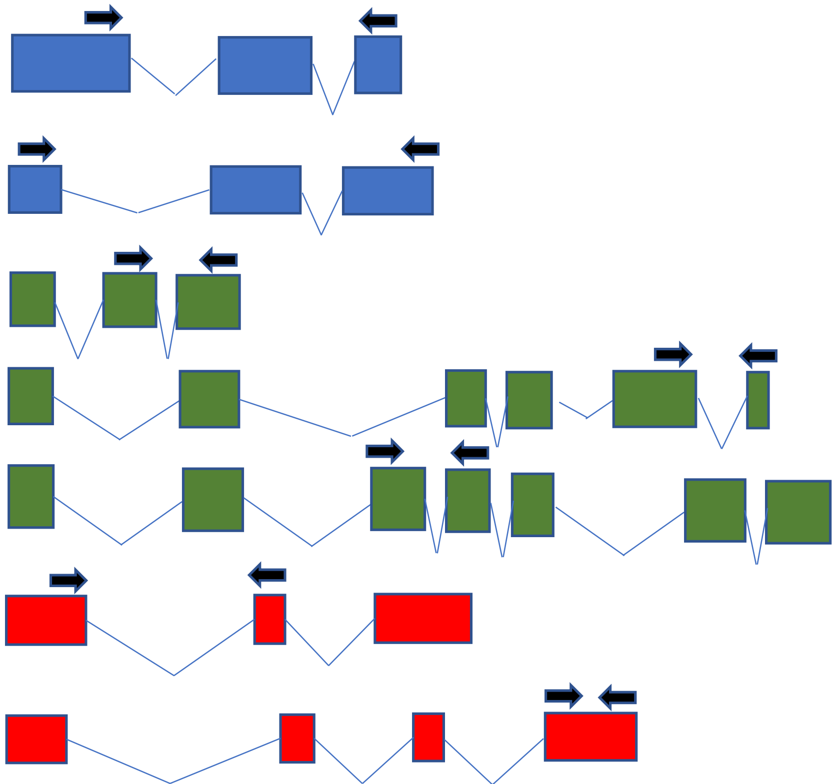
A

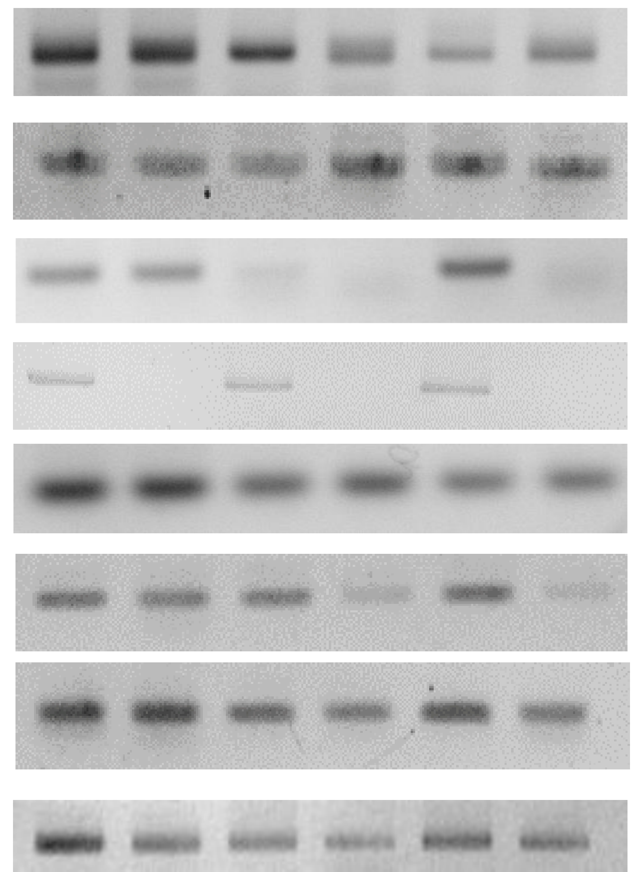


B

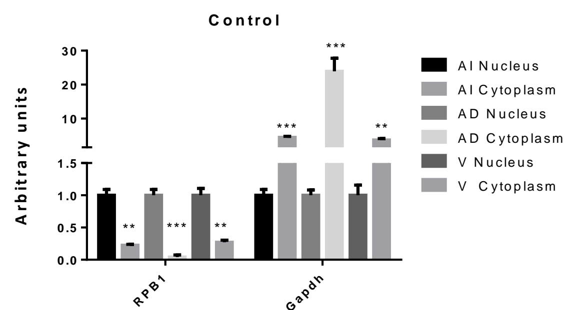


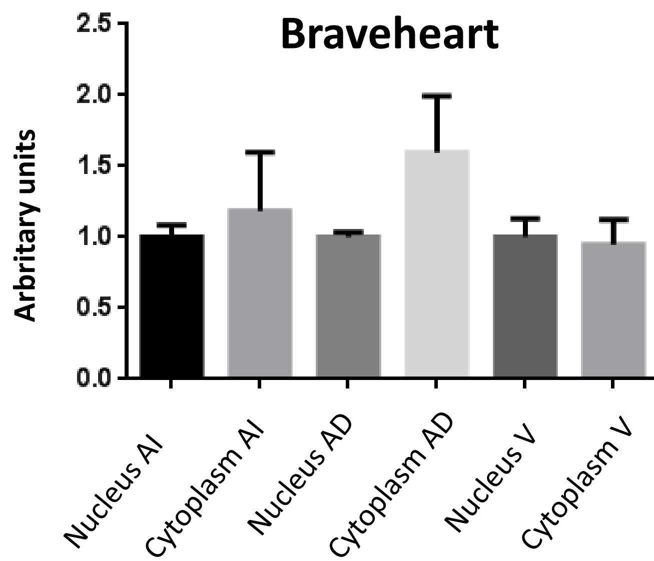
Garcia-Padilla et al., Figure 5

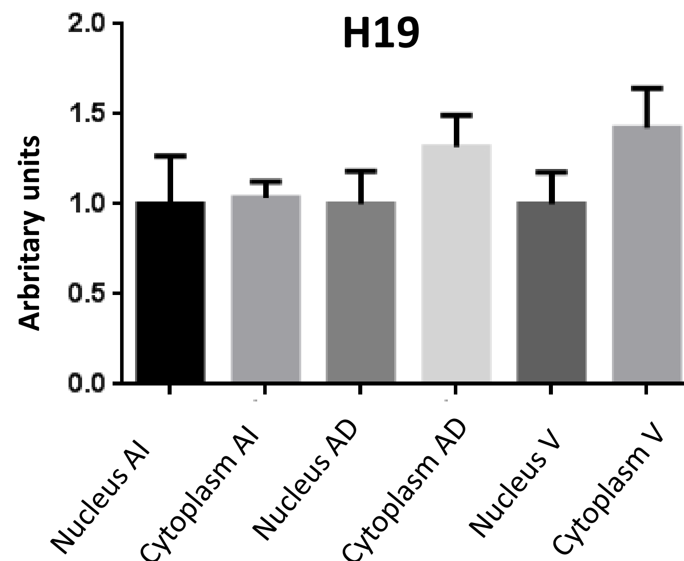


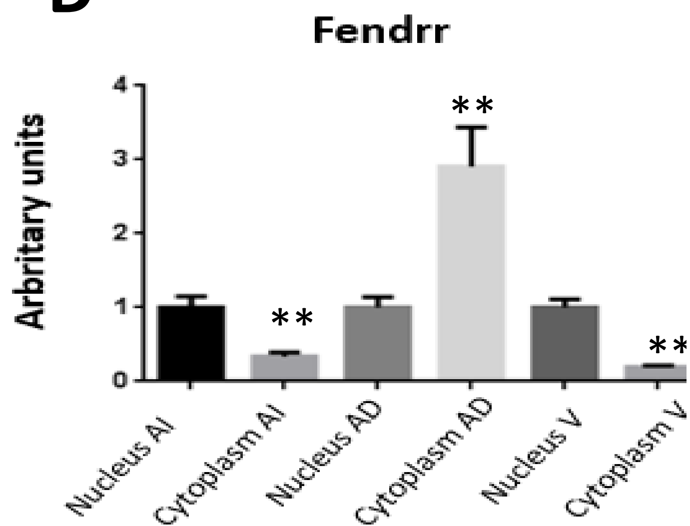


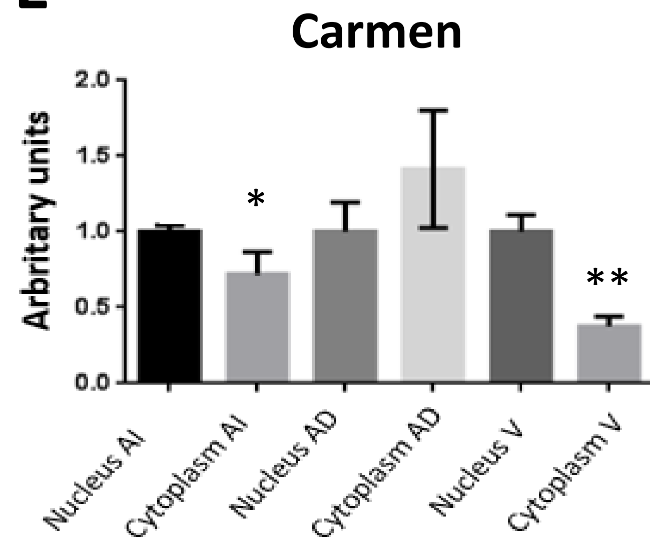
Garcia-Padilla et al., Figure 6



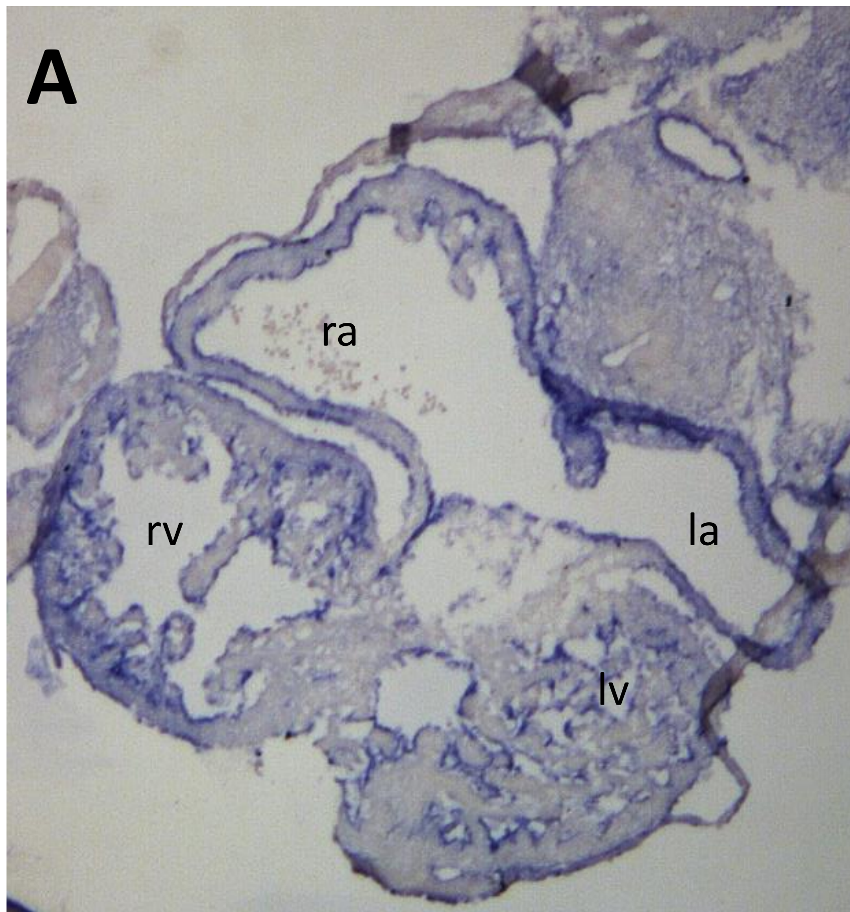


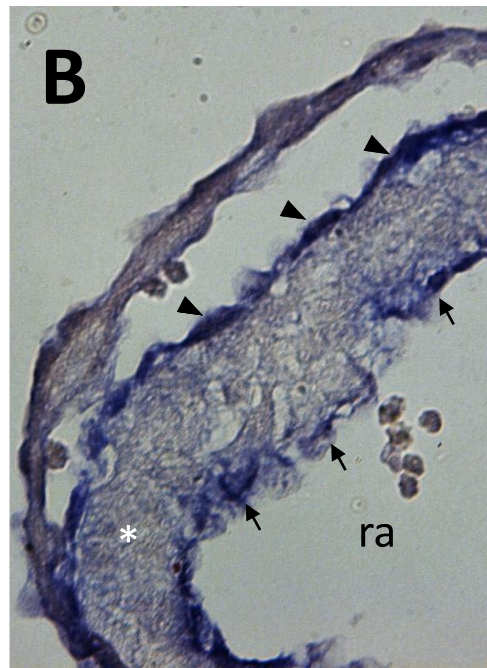


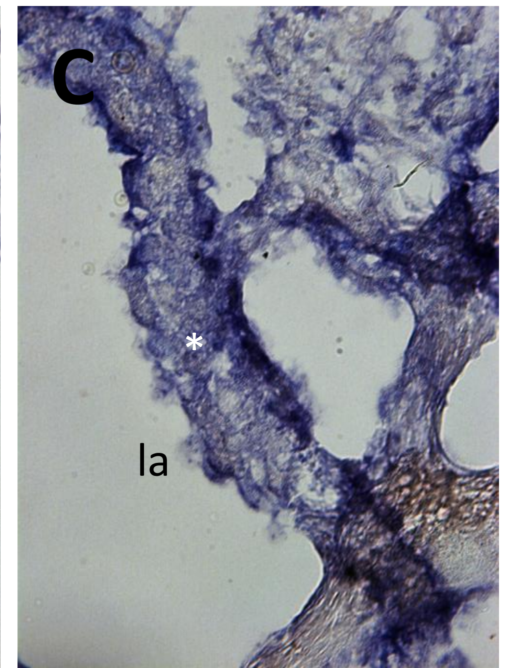


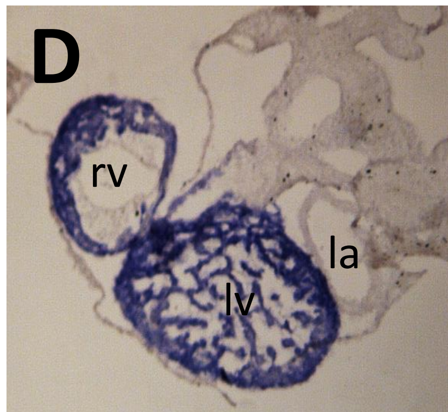


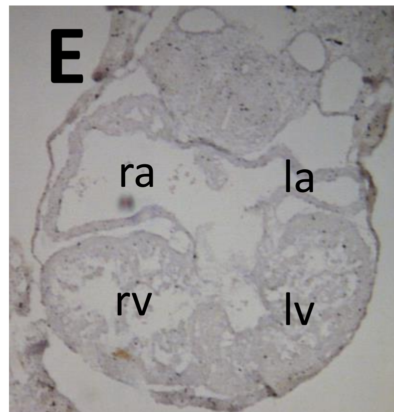
Garcia-Padilla et al., Figure 7

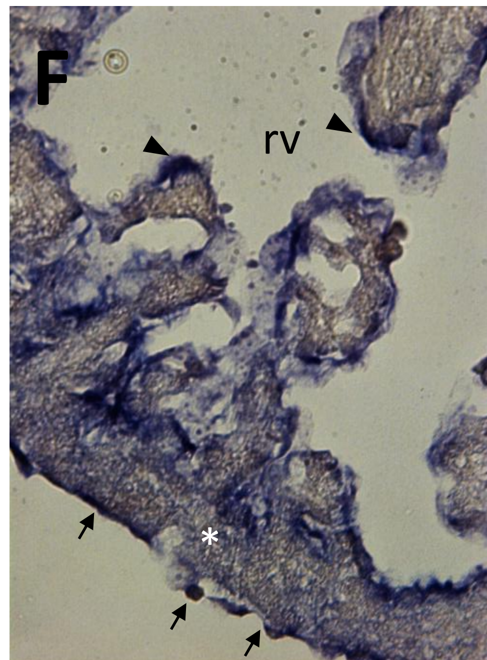


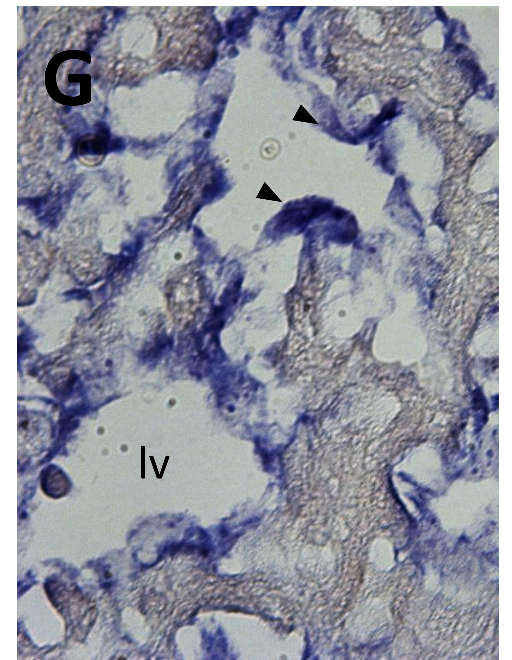




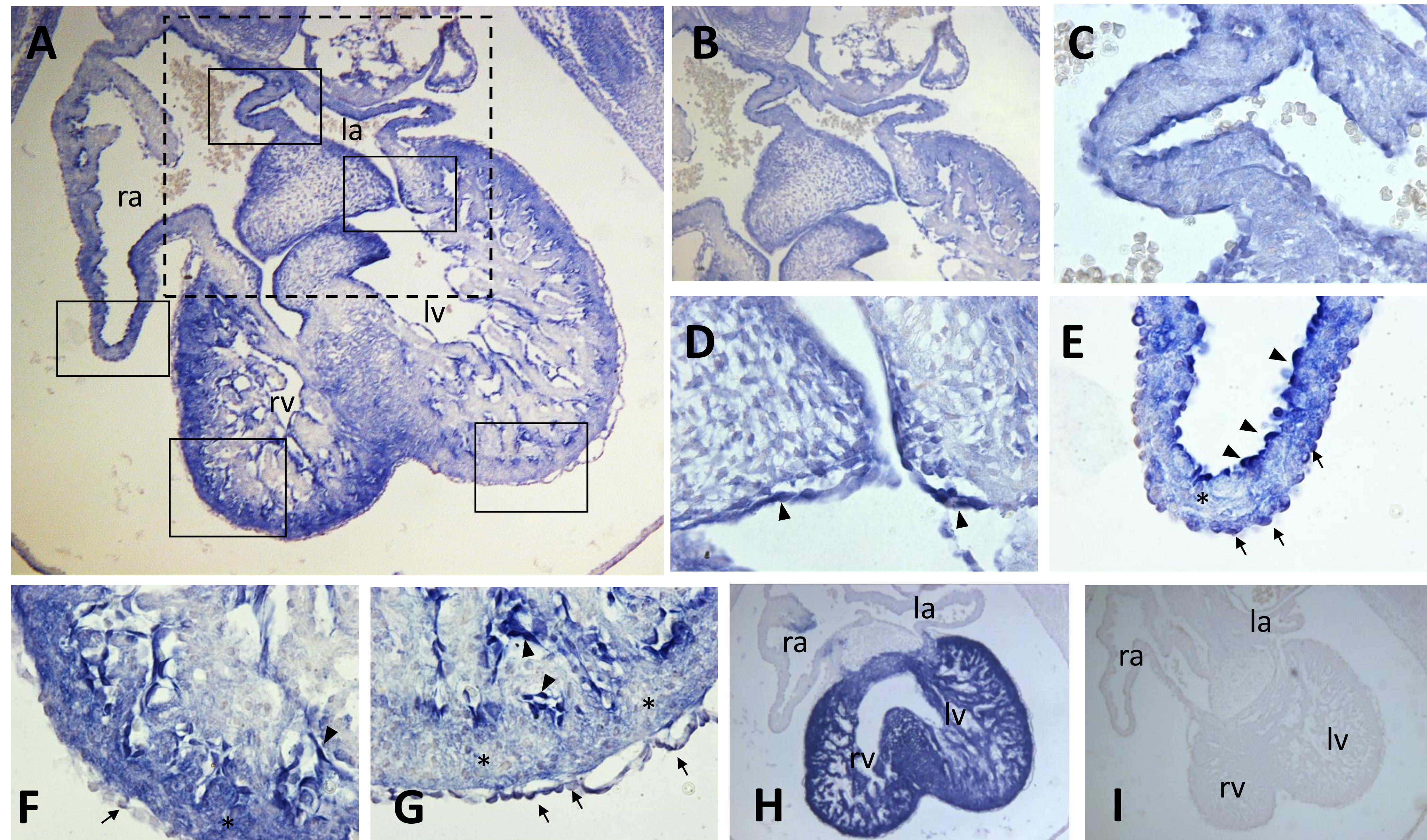




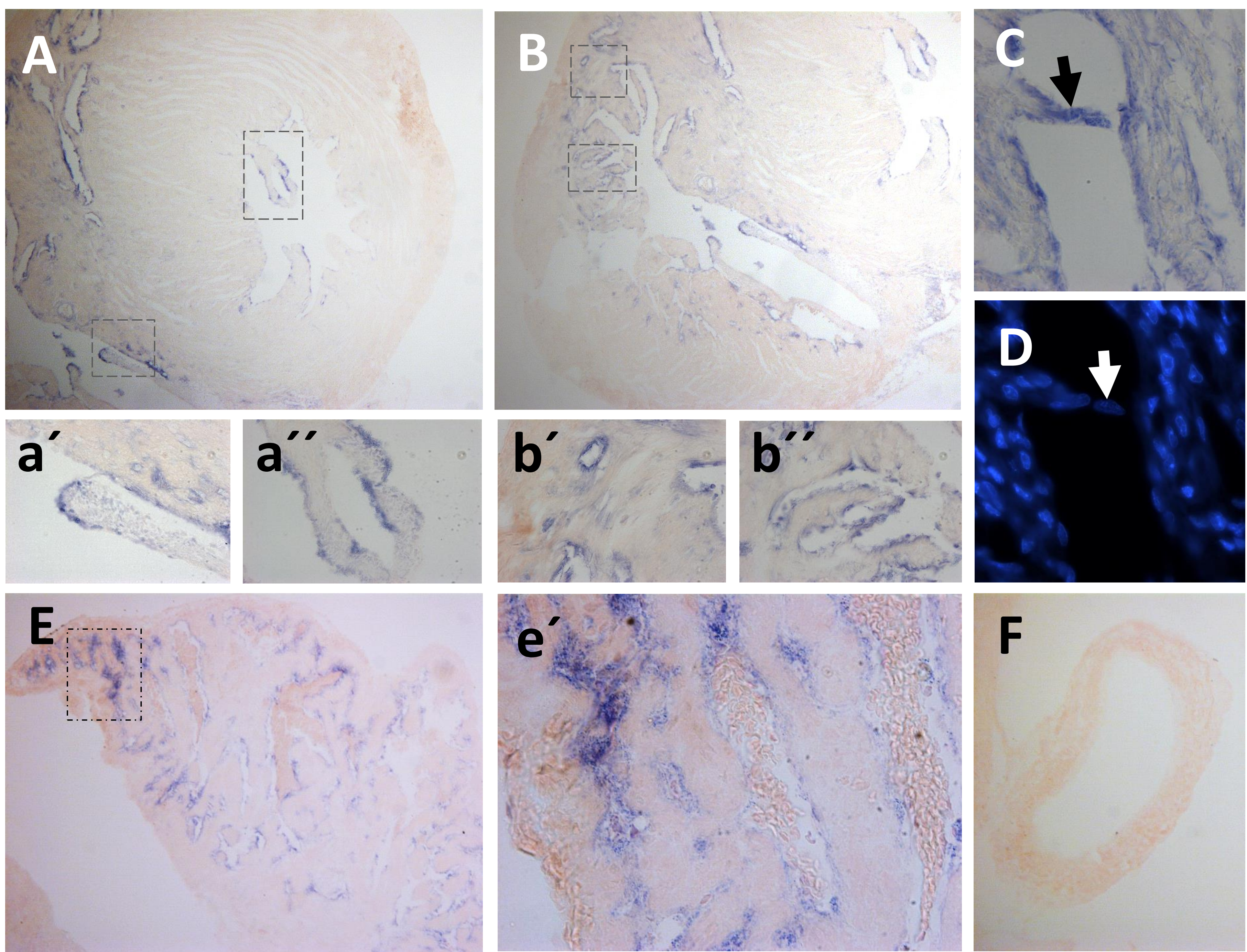




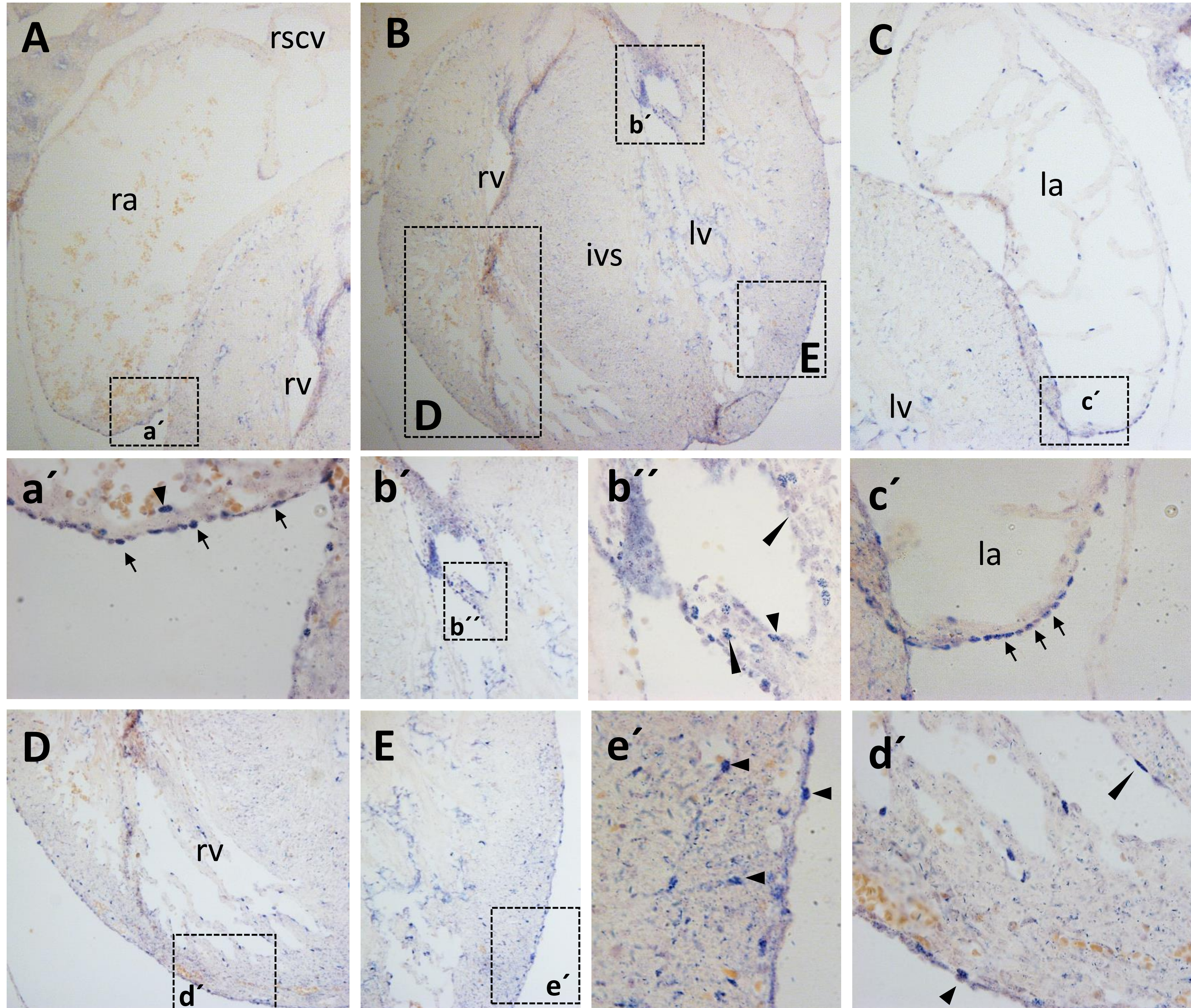
Garcia-Padilla et al., Figure 8



Garcia-Padilla et al., Figure 9

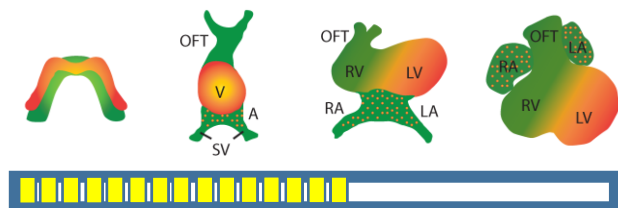


Garcia-Padilla et al., Figure 11



Garcia-Padilla et al., Figure 10

CAPITULO II



6. Capítulo II: Identification of Novel Long non coding RNAs regulated by Pitx2>Wnt>microRNA Signaling Pathway involved in Atrial Fibrillation

Abstract

Atrial fibrillation is the most prevalent cardiac arrhythmia in humans. Genetic and genomic analyses have recently demonstrated that the homeobox transcription factor Pitx2 plays a fundamental role regulating expression of distinct growth factors, microRNAs and ion channels leading to morphological and molecular alterations that promote the onset of atrial fibrillation. We now address the plausible contribution of a novel class of non-coding RNA in this Pitx2>Wnt>miRNA signaling pathway, i.e. long non coding RNAs (lncRNAs). *In silico* analyses of annotated lncRNAs in the vicinity of Pitx2, Wnt8 and Wnt11 chromosomal loci identified five novel lncRNAs with differential expression during cardiac development. Among them, Wnt11_45188, Wnt11_44934 and Wnt8_2010110K18Rik displayed preferential atrial-specific expression during embryogenesis. In addition, Wnt11_44653 displayed moderate expression during embryogenesis but peaked preferentially in the right atrium vs left atrium and ventricle in adulthood. Wnt11_45188, Wnt11_44934 and Wnt8_2010110K18Rik were distinctly regulated by Pitx2, Wnt8 and Wnt11 and Wnt8_2010110K18Rik is severely up-regulated in conditional atrial-specific Pitx2 deficient mice. We also demonstrate that arrhythmogenic microRNAs such as miR-1, miR-133 and miR-29 distinctly regulate these lncRNAs. Furthermore, pro-arrhythmogenic and pro-hypertrophic substrate administration to atrial cardiomyocyte cell cultures such as angiotensin II/norepinephrine invariably leads to significant up-regulation of these three newly identified lncRNAs, while thyroid hormone administration significantly enhances Wnt11_44934 and diminishes Wnt11_45188 and Wnt8_2010110K18Rik expression. These data support distinct modulatory roles of AF cardiovascular risk factors, i.e. hypertension and hyperthyroidism to the regulation of these lncRNAs. Overall, we have identified three novel lncRNAs that are distinctly regulated by Pitx2>Wnt>miRNA signaling pathway and pro-arrhythmogenic and pro-hypertrophic substrates indicating that they might be therefore implicated in gene regulatory networks leading to atrial arrhythmogenesis.

Introduction

Atrial fibrillation (AF) is the most common cardiac arrhythmia, with an incidence of 2-3% in the general population that rises up to 8-10% in the elderly. Genetics studies of linkage analyses have identified point mutations in several ion channels with key roles in the configuration of the cardiac action potential, as the culprit genes leading to AF onset. However, these genetic defects only explain a minority (<10%) of all AF cases. Seminal work by Gudbjartsson et al. (2007) using genome wide association studies (GWAS) revealed that risk variants in 4q25 locus were highly associated to lone AF, postulating that regulatory elements within this locus might influence the neighboring gene, i.e. the homeobox transcription factor Pitx2, leading therefore to impaired cardiac function and thus atrial fibrillation. Subsequent GWAS studies and meta-GWAS analyses has exponentially increased our understanding of the plausible genetic substrates of AF, with the identification of more than 90 risk variants associated to AF (refs). Importantly, in all cases the most significant risk variants remain those at 4q25 locus. Experimental studies have provided evidences that 4q25 physically interacts with Pitx2 promoter (Aguirre et al., 2015), supporting key regulatory roles as suggested by Gudbjartsson et al. (2007). In addition, systemic Pitx2 loss of function experiments demonstrate increased susceptibility to atrial arrhythmias (Wang et al., 2010; Kirchoff et al., 2011) while conditional atrial-specific deletion of Pitx2, resulting in Pitx2 insufficiency, lead to spontaneous atrial arrhythmias (Chinchilla et al., 2011). Subsequent studies demonstrate that Pitx2 insufficiency lead to remodeling of several meta-GWAS associated genes, such as Wnt8, which in turned regulated Wnt11, leading to microRNA deregulating and thereafter ion channel impaired expression and function (Chinchilla et al., 2011; Lozano-Velasco et al., 2016, 2019). These data demonstrate a pivotal role of the Pitx2>Wnt>microRNA pathway as regulatory mechanism leading to AF. In addition, experimental model of induced AF resulted in Pitx2 downregulation, supporting the notion of self-perpetuating Pitx2 downregulation in the context of AF progression (Torrado et al., 2015). Moreover, risk cardiovascular factors contributing to increase frequency of AF onset in the human population, such as hypertension, hyperthyroidism and antioxidant redox imbalance, consistently alters Pitx2>Wnt>microRNA pathway, demonstrating a molecular link between these AF risk factors and the causative signaling pathways involved (Lozano-Velasco et al., 2017).

Over the last decade we have witnessed a novel paradigm in the control of gene regulatory networks by the identification of novel types of non-coding RNAs with

regulatory potential. A large array of evidences have demonstrated the essential regulatory role of microRNAs in cardiovascular development and diseases, including therein seminal studies on the role of microRNAs regulating cardiac ion channels and AF itself. The complexity of the regulatory roles of non-coding have greatly enlarged with the discovery of long non coding RNAs (lncRNAs). Long non-coding RNAs display essentially no potential to code for proteins, although they are structurally similar to mRNAs. LncRNAs are transcribed using the same pathways; i.e. RNA polymerase II, have typical histone modifications, 5' terminal cap and 3' terminal poly(A) tails (Wapinski & Chang, 2011). LncRNAs are constituted by exons and introns and are often spliced. LncRNAs display low expression levels yet with increased tissue and time specificity as compared to the protein-coding genes (Engreitz et al., 2016). Such specificity suggests an important role for these transcripts tightly defining distinct cellular events (Gloss & Dinger, 2016; Bär et al., 2016). At the cellular level, lncRNAs can be located both in the cytoplasm and nucleus. Cytoplasmic enriched lncRNAs have mainly a role in post-transcriptional regulation whereas nuclearly located lncRNAs predominantly play a role in transcriptional gene regulation. Importantly, lncRNAs are dynamic molecules that can be located in the nucleus but translocate and act in the cytoplasm (Chen, 2016). Several studies have been performed exploring the functional role of particular cardiac enriched-lncRNAs during cardiogenesis such as those reporting C*armen, Braveheart and Fendrr*. *Carmen* is located upstream of miR-143 and miR-145, two microRNA involved in cardiovascular development (Ouzain et al., 2016;Boucher et al., 2011). Although *Carmen*, miR-143 and miR-145 are located within the same genomic locus, they are expressed as independent transcripts. *Carmen* is expressed both in fetal and adult hearts and it is well conserved between mammalian species. *Carmen* directly acts during the earliest steps of cardiac lineage commitment regulating cardiac differentiation from nascent mesoderm by modulating the expression downstream of *Mesp1* cardiac gene network (Ouzain et al., 2015). *Braveheart* acts as a key regulator in cardiac lineage commitment, upstream of *Mesp1,* and it is required for proper cardiac gene expression in mice. Depletion of *Braveheart* results in failure of activation of key cardiac factors necessary for correct heart development and cardiomyocyte differentiation (Klatenhoff et al., 2013). *Fendrr* is differentially and transiently expressed at the caudal end of the nascent lateral plate mesoderm, being necessary for the correct development of tissues derived from it, particularly the heart and body walls (Mahlapuu et al., 2001; Grote et al., 2011).

In the context of atrial fibrillation, several reports have provided evidences of differential lncRNAs expression (Ruan et al., 2015; Su et al., 2018; Mei et al. 2018, Qian et al. 2018) but scarce informatio is available about their tissue distribution and regulatory mechanisms. Gore-Panter et al. (2016) identified a long intergenetic non-coding RNA adjacent to *PITX2*, dubbed as *PANCR*. *PANCR* is expressed in the adult left atrium and in lower levels in the adult eye, and shows a coordinate expression with *PITX2C*, during the differentiation of cardiomyocytes regulating positively the expression of *PITX2C* mRNA by a yet unknown mechanism. Interestingly, *PANCR* have been reported in human tissues but no orthologues are found in other mammalian species such as mice. Therefore, *PANCR* seems to be a human specific lincRNA (Gore-Panter et al. 2016).

In this study, we address the plausible regulatory role of the Pitx2>Wnt>miRNA signaling pathway in long non coding RNAs expression. *In silico* analyses of annotated lncRNAs in the vicinity of Pitx2, Wnt8 and Wnt11 chromosomal loci identified five novel lncRNAs with differential expression during cardiac development in mice. Three of them displayed preferential atrial-specific expression during embryogenesis and are distinctly regulated by Pitx2, Wnt8 and Wnt11. We also demonstrate that miR-1, miR-133 and miR-29 over-expression distinctly regulate these lncRNAs. Furthermore, pro-arrhythmogenic and pro-hypertrophic substrates such as angiotensin II, norepinephrine and thyroid hormone administration distinctly regulate their expression. In summary, we have identified three novel lncRNAs with enhanced atrial expression that are distinctly regulated signaling pathways leading to atrial arrhythmogenesis.

Materials & Methods

Mouse breeding and tissue sampling

CD1 mice were bred and embryos were collected at different embryonic developmental stages, ranging from embryonic day (E) E12.5 to E18.5. Neonatal and adult hearts were also collected. Briefly, embryonic and postnatal hearts were dissected into right atrium, left atrium and ventricular chambers, pooled and stored in liquid nitrogen until used.

Pitx2^{floxed} and NppaCre transgenic mouse lines, and generation of conditional atrial (NppaCre) mutant mice was previously described (Gage et al., 1999; de Lange et al., 2003; Chinchilla et al., 2011; Lozano-Velasco et al., 2016). Two different conditions were used for the NppaCrePitx2 mice: wild-type Cre controls (NppaCre2Pitx2$^{fl/fl}$) and atrial-specific homozygous (NppaCre+Pitx2$^{-/-}$). This investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health. The study was approved by the University of Jaén Bioethics Committee.

Mouse genotyping and phenotyping

DNA for PCR screening was extracted from adult ear and/or tail samples. Screening of Cre and Pitx2 floxed alleles was routinely done using used specific primers as previously described (Chinchilla et al. 2011). Cycling conditions for Cre were as follows; 5 min at 95°C, 35 cycles of 30s at 95°C, 30s at 60°C and 90s at 72°C, and for Pitx2 as follows; 5 min at 95°C. 40 cycles of 30s at 95°C, 30s at 60°C and 90s at 72°C, followed by a final extension step of 10 min at 72°C, respectively. In addition, expression of Pitx2 in left atrial samples of wild-type Cre controls (NppaCre2Pitx2$^{fl/fl}$) and atrial-specific homozygous (NppaCre+Pitx2$^{-/-}$) were analyzed by qPCR, displaying in all cases 60-70% reduction in Pitx2c expression in NppaCre+Pitx2$^{-/-}$ samples.

RNA isolation and cDNA synthesis

Genetically modified Pitx2 mice, and their corresponding controls, were sacrificed by cervical dislocation. Adult hearts were carefully dissected and briefly rinsed in Ringer's solution. Left atrium tissue samples were collected for each experimental condition, immediately snap-frozen in liquid nitrogen, and stored at -80°C until used. Pooled samples of at least three independent mice were processed for each condition, respectively. Three independent pooled samples were further processed for RNA

isolation and qPCR analyses. Total RNA was isolated using Trizol (Roche) according to manufacture's guidelines and DNase treated using RNase-Free DNase (Roche) for 1h at 30°C. In all cases, at least three distinct pooled samples were used to perform the corresponding qRT-PCR experiments.

First strand cDNA was synthesized at 50°C for 1h using 1 µg of RNA, oligo-dT primers and Superscript III Reverse Transcriptase (Invitrogen) according to manufacture's guidelines. Negative controls to assess genomic contamination were performed for each sample, without reverse transcriptase, which resulted in all cases in no detectable amplification product.

qPCR analyses (mRNA and lncRNA)

RT-PCR was performed in Mx3005Tm QPCR System with an MxPro QPCR Software 3.00 (Stratagene) and SyBR Green detection system. Reactions were performed in 96-well plates with optical sealing tape (Cultek) in 20 µL total volume containing SYBR Green Mix (Finnzymes) and the corresponding cDNA. Two internal controls, mouse Gusb and Gapdh, were used in parallel for each run and represented as previously described [refs]. Amplification conditions were as follows: denaturisation step of 95°C for 10 min, followed by 40 cycles of 95°C for 30s, 60°C for 30s, 72°C for 30s; with final elongation step of 72°C for 10 min. All primers were designed to span exon-exon boundaries using online Primer3 software Primer3input (http://bioinfo.ut.ee/primer3-0.4.0/). Primer sequences are provided in **Supplementary Table 1**. No amplifications were observed in PCR control reactions containing only water as the template. Each PCR reaction was performed at least three times to obtain representative averages. The Livak method was used to analyze the relative quantification RT-PCR data (Livak & Schmittgen, 2001) and normalized in all cases taking as 100% the wild-type (control) value, as previously described (Chinchilla et al., 2011).

qPCR analyses (microRNA)

microRNA qRT-PCR was performed using Exiqon LNA microRNA qRT-PCR primers and detection kit according to manufacturer's guidelines. All reactions were always run in triplicates using 5S as normalizing control, as recommended by the manufacturer. SyBR Green was used as quantification system on a Stratagene Q-Max 2005P qRT-PCR thermocycler. Relative measurements were calculated as described by Livak &

Schmittgen (2001) and control measurements were normalized to represent 100% as previously described (Chinchilla et al., 2011).

Plasmid, siRNA, microRNA mimics cell transfections

HL-1 cells (6×10^5 cells per well) were transfected with plasmids containing expression constructs for Pitx2c, Wnt8a (Addgene), Wnt11a (Addgene, Cambridge, MA, USA) and with pre-miR-1, pre-miR-133, pre-miR-29 (Exiqon) or siRNA-Pitx2c, siRNA-Wnt8a, siRNA-Wnt11a (Sigma, Aldrich, Munich, Germany), respectively, as previously described (Chinchilla et al., 2011; Lozano-Velasco et al., 2011). siRNA sequences are provided in **Supplementary Table 2.**

Cell culture and angiotensin II, norepinephrine and thyroid hormone treatment

HL-1 cells and primary cultures of mouse fetal (E17.5) cardiomyocytes were isolated using standard procedures (Daimi et al., 2015), cultures accordingly and treated with angiotensin II (AngII), norepinephrine (NE) and thyroid T3/T4 hormone, respectively, as previously reported (Wang et al., 2003; Lozano-Velasco et al., 2017).

lcnRNA pull down assays

Biotinylated RNA of exón 1 and exon2 of Gm_44934 of Exon 1 and Exon 2 were synthesize from PCR fragment using specific forward primers that contained the T7 RNA polymerase promoter sequence [(T7), CCAAGCTTCTAATACGACTCACTATAGGGAGA]. After purifying the DNA template, biotinylated transcripts were synthesized using MaxiScript T7 kit (Ambion); whole-cell lysates (500 µg) from HL1 cells were incubated with 1 µg of biotinylated RNA for 2 hours at room temperature. Complexes were isolated with Streptavidin-coupled Dynabeads (Invitrogen) and were analyzed by MS.

For MS analysis, peptide mixtures from each sample were loaded onto a peptide trap cartridge and eluted onto a reversed-phase PicoFrit column (New Objective, Woburn, MA, USA). Eluted peptides were ionized and sprayed into the mass spectrometer, using a Nanospray Flex Ion Source ES071 (Thermo Scientific). The LC/MS/MS analysis of samples were carried out using a Thermo Scientific Q-Exactive hybrid Quadrupole-Orbitrap Mass Spectrometer and a Thermo Dionex UltiMate 3000 RSLCnano System. Proteins were identified using the Thermo Proteome Discoverer 1.4.1 platform. Database

search against public mouse protein database from NCBI was performed through the Proteome Discoverer 1.4.1 platform.

Statistical analyses

For statistical analyses of datasets, unpaired Student's t-tests were used. Significance levels or P values are stated in each corresponding figure legend. $P < 0.05$ was considered statistically significant.

Results

Identification of novel long non coding RNAs putatively involved in Pitx2>Wnt>microRNA signaling pathway

Previous studies have demonstrated that long non coding RNAs can act locally regulating neighboring genes (Villegas & Zaphiropoulos, 2015; Dempsey et al., 2018; Hori et al., 2018; Hitachi et al., 2019). We therefore scrutinized mouse genome to identify lncRNAs in close vicinity to genes previously identified to play a pivotal role in the onset of atrial fibrillation, particularly Pitx2>Wnt>microRNA pathway (Chinchilla et al., 2011; Lozano-Velasco et al., 2015, 2017). In particular, we search lncRNAs close to Pitx2, Wnt8 and Wnt11. We identified no lncRNAs in the Pitx2 locus, besides the previously reported *Playrr* lncRNA (Welsch et al., 2015), while we identified two lncRNAs adjacent to Wnt8 and four to Wnt11 (**Figure 1A**). Subsequently, we profiled the expression of these lncRNAs in right atrial, left atrial and ventricular tissues of mouse embryos ranging from E12.5 to E18.5, neonate and adult hearts. Among seven lncRNAs interrogated, including herein *Playrr*, only five of them displayed detectable expression in the cardiac tissues (**Figure 1B-F**). Importantly, three of them displayed a preferential embryonic expression, confined to the atrial chambers, while only basal ventricular expression was detected. In this context, it is important to highlight that Wnt11_Gm45188 displays atrial specific expression, similarly in both right and left atrial chambers and peaking at E16.5 (**Figure 1B**), Wnt11_Gm44934 display an left atrial restricted expression (**Figure 1D**), with peak levels at E16.5, while Wnt8a_2010110K18Rik display also atrial restricted expression with a preferential left *vs* right expression also at E16.5 (**Figure 1F**). In all cases expression in neonatal and adult heart is severely downregulated as compared to the embryonic stages. Importantly, Wnt11_Gm44653 display preferential right atrial expression in all stages analyzed (**Figure 1C**), including adulthood, but also presents a transient peak expression at neonatal stages in the ventricular chambers. On the contrary, Wnt11_06 display preferential embryonic expression but it is not restricted to the atrial chambers (**Figure 1E**), showing a dynamic expression pattern in right atrial, left atrial and ventricular tissues at different developmental time points. *Playrr* display a prominent expression in the early developmental stages declining as development proceeds (**Figure 1G**). In summary, these data demonstrate the identification of three novel lncRNAs, i.e. Wnt11_Gm45188, Wnt11_Gm44934 and Wnt8a_2010110K18Rik, with preferential expression in the embryonic atrial chambers.

LncRNA regulatory roles exerted by Pitx2, Wnt8 and Wnt11

To dissect the functional role of Pitx2, Wnt8 and Wnt11 on the regulation of these newly identified lncRNAs, gain- and loss-of-function experiments were carried out in both atrial HL1 cardiomyocytes (**Figure 2A-C**).

Pitx2c over-expression and silencing significantly up-regulates Wnt11_Gm45188, Wnt11_Gm44653 and Wnt11_06, down-regulates Wnt8a_2010110K18Rik while Wnt11_Gm44934 display no significant differences (**Figure 2E**). Wnt8a over-expression significantly decreases all lncRNAs analyzed except Wnt8a_2010110K18Rik. Wnt8a siRNA silencing significantly decreases Wnt11_Gm45188 and Wnt11_Gm44934, up-regulates Wnt11_06 while Wnt11_Gm44653 and Wnt8a_2010110K18Rik display no significant differences. On the other hand, Wnt11 over-expression significantly up-regulates all lncRNA analyzed except Wnt11_Gm45188 that is down-regulated. Importantly, Wnt11 siRNA silencing also up-regulated all lncRNAs analyzed except Wnt11_Gm44934. These data demonstrate that Pitx2, Wnt8a and Wnt11 distinctly regulate lncRNA expression. It is importantly to highlight that Pitx2 silencing up-regulates Wnt11_Gm44653 and Wnt11_06, while Wnt8 and Wnt11 over-expression display complementary patterns on the regulation of Wnt11_Gm44653 and Wnt11_06 expression, in line with our previous findings in NppaCrePitx2$^{-/-}$ mice (Lozano-Velasco et al., 2015). In addition, although the precise molecular mechanism remains to be elucidated, it is importantly to highlight that both Pitx2 gain- and loss-function similarly affect lncRNAs, demonstrating that subtle changes in Pitx2 expression greatly influence lncRNAs. Such observations are also applicable for Wnt8 and Wnt11 in most cases.

To further support the functional regulatory role of Pitx2 in the expression of these lncRNAs, we analyzed their expression levels in atrial-specific conditional Pitx2 mouse model NppaCrePitx2$^{-/-}$. Adult left atrial samples were used for the analyzed and verification of Pitx2 loss-of-function was performed by genotype analyses and Pitx2c qPCR . As previously NppaCrePitx2$^{-/-}$ mice display 70-80% reduction of Pitx2c expression in the left atrial chamber (**Figure 2D**), displaying therefore Pitx2 insufficiency. We focused our attention to those lncRNAs displaying an atrial-specific expression, i.e. Wnt11_Gm44934, Wnt11_Gm45188 and Wnt8a_2010110K18Rik. In this context, Wnt11_Gm45188 is severely up-regulated while Wnt11_Gm44939 and

Wnt8a_2010Rik display no significant differences (**Figure 2**). These data support previous observations in HL1 cardiomyocytes that Pitx2 regulates Wnt11_Gm45188 while does not alter Wnt11_Gm44939. Surprisingly, Wnt8a_2010Rik does not display significant changes in NppaCrePitx2$^{-/-}$ mice, suggesting additional source of Wnt8a_2010Rik expression rather than the atrial cardiomyocytes. Additional studies are required to clarify this point.

AF related microRNAs modulate lncRNA expression

We have previously shown that Pitx2>Wnt signaling regulate the expression of a large subset of microRNAs with pivotal roles regulating ion channel expression/function and thus having an important impact on onset of atrial arrhythmogenesis (Chinchilla et al., 2011; Lozano-Velasco et al., 2015, 2017). We therefore tested is AF-related microRNAs such as miR-1, miR-133 and miR-29 are capable of modulating the expression of these lncRNAs. We over-expressed with microRNAs in HL1 atrial cardiomyocytes and analyzed lncRNA expression by qPCR (**Figure 3D**). Our data demonstrate that Wnt11_Gm45188 is up-regulated by miR-1 and miR-29 administration, respectively, while miR-133 treatment leads to significant down-regulation (**Figure 3A**). miR-133 and miR-29 administration leads to severe down-regulation of Wnt11_Gm44934 in HL1 cardiomyocytes, while miR-1 does not alter it expression (**Figure 3B**). Finally, miR-1 up-regulates Wnt8a_2010110K18Rik expression while miR-133 and miR-29 administration leads to no significant changes (**Figure 3C**). In addition, we also demonstrate that *Playrr* is significantly down-regulated by miR-1, miR-133 and miR-29 over-expression (**Figure 3E**). These data demonstrate that distinct microRNAs exert different regulatory properties in these lncRNAs and moreover, these data support a plausible functional role of these lncRNAs in the Pitx2>Wnt>microRNA pathways, previously reported to play a fundamental role in the onset of atrial fibrillation.

Angiotensin II and norepinephrine treatment significantly up-regulates AF-associated lncRNAs

We have previously demonstrate that AF associated cardiovascular risk factors such as hypertension and hyperthyroidism distinct modulate Pitx2>Wnt>microRNA signaling pathway. We herein tested if these newly identified lncRNAs are regulated by AngII and/or NE administration. HL1 atrial cardiomyocytes were treated with AngII and NE, respectively, as previously reported (Lozano-Velasco et al., 2017), and lncRNA

expression was assessed by qPCR. Our data invariably demonstrate that both AngII and NE administration, respectively, significantly enhance Wnt11_Gm45188, Wnt8a_2010110K18Rik and Wnt11_Gm44934 (**Figure 4A-C**). These data therefore also support a plausible role of these lncRNAs in AF pathophysiology.

Thyroid hormone administration distinctly regulated AF-associated lncRNAs

Impaired thyroid levels have been consistently reported as risk cardiovascular factor associated with increased prevalence of atrial arrhythmias. We herein tested if T3 and T4 administration can modulate AF-associated lncRNAs. Our data demonstrate that Wnt11_Gm45188 is down-regulated by T4 but not by T3 administration (**Figure 4D**). On the other hand, both T3 and T4 administration significantly up-regulates expression of Wnt11_Gm44934 while decreases Wnt8a_2010110K18Rik expression (**Figure 4E-F**). In line with the observations after AngII and NE treatment, thyroid administration also modulate AF-associated lncRNAs, supporting the notion of a plausible role for these lncRNAs in AF pathophysiology.

Wnt11_Gm44934 lcnRNA pull down assays

In order to dissect the lncRNA-protein interactions of the atrial-specific Wnt11_Gm44934 lncRNA, pull-down assays were performed and associated proteins were identified by LC-MS/MS. A total of 40 proteins were identified (Table 1) significantly interacting with Wnt11_Gm44934. Among these, approximately 40% are cytoplasmic proteins, 12% are nuclear and 20% are mitochondrial. These data suggest that Wnt11_Gm44934 acts prominently as a cytoplasmic lncRNAs. Among these cytoplasmic protein, it is important to highlight the physical interaction between Wnt11_Gm44934 and three distinct myosin proteins, MHY6, MHY9 and MHY10. These data support the notion that Wnt11_Gm44934 plays a fundamental post-transcriptional role. MHY6 is an structural protein forming the part of the sarcomere and it is frequently altered in cardiac pathological conditions such as cardiac hypertrophy. Additionally, several cytoskeletal proteins RAB1A, RAB2A, RAB7A, ACTN1, ACTN4, TLN1 and ITB1 are also associated to Wnt11_Gm44934. Importantly several of these proteins are linked to Wnt non-canonical signaling pathway. At the nuclear levels, Wnt11_Gm44934 interacts with ROA2, ROA1, DXC39B and MBBIA. These data suggest that Wnt11_Gm44934 can also exert transcriptional regulatory mechanisms, although at present the functional roles of these interacting proteins is poorly understood.

Surprisingly, Wnt11_Gm44934 also interacts with a large array of mitochondrial proteins, such as KAD2, ETFD, DHE3, VDAC2, IVD, CYC and SDHB, as well as with proteins located in other subcellular organelles, particularly at the ER (RTN-4, CALU, TXDN5). The nature of such interactions remains to be explored.

Discussion

Atrial fibrillation is the most common cardiac arrhythmia in humans, with an estimate incidence of 2-4% in the general population, but rising up to 8-10% in the elderly. Mechanistically, atrial fibrillation is initiated by impaired electrical activity that is most frequently originated in the left atrium, particularly in the vicinity of the pulmonary veins entrance. Atrial fibrillation onset leads to structural and electrical remodeling of both left and right atrial chambers leading thereafter to self-perpetuation. We identified herein four distinct lncRNAs with preferential expression in the atrial chambers, three of them confined to the embryonic stages, with a fourth lncRNA that is also preferentially expressed in the adult right atrium. These data therefore suggest that these lncRNAs might potentially be involved in atrial arrhythmogenesis.

Over the last decades, our understanding of the genetic substrates underlying atrial fibrillation have greatly increased. A large number of studies provided evidences on the causal relationship between distinct point mutations in multiple genes coding for ion channels and the occurrence of atrial fibrillation. More recently, a seminal paper by Gudbjartsson et al. (2007) identified risk variants at 4q25 highly associated to lone AF and subsequently corroborated in distinct AF cohort studies (Henningsen et al., 2011; Kiliszek et al., 2011; Olensen et al., 2012; Parvez et al., 2013; Mohanty et al., 2013). Curiously, 4q25 risk variants are located 150 kb downstream of the homeobox transcription factor PITX2. Experimental studies in mice demonstrated that impaired Pitx2 function triggered atrial arrhythmias susceptibility (Wang et al., 2010; Kirchhof et al., 2011; Chinchilla et al., 2011). Wang et al. (2010) firstly demonstrated that Pitx2 loss-of-function increased AF susceptibility due to aberrant *Shox2* and *Tbx3* expression in the developing heart. Kirchhof et al. (2011) reported similar findings and demonstrated that multiple ion channels expression were equally impaired. Importantly, in both cases, no AF episodes occurred under basal conditions. Using an atrial-specific Pitx2 conditional transgenic mouse line, Chinchilla et al. (2011) demonstrated abnormal ECGs at rest as well as impaied SCN5A (I_{Na}), KCNJ2 (I_{K1}) and KCNJ12 (I_{K1}) expression and function. These observations were subsequently corroborated by Tao et al. (2014) using an adult Pitx2 conditional approach. These data suggest that an embryonic impairment of Pitx2 might predispose to atrial arrhythmias while an adult deficiency will be already causative of atrial fibrillation. In this study we therefore tested whether these lncRNAs that display prominent atrial-specific expression were modulated of Pitx2. Our data demonstrate that

Pitx2 enhances the expression of Wnt11_Gm_45188, Wnt11_Gm_44653, Wnt11_06 while significantly decreases expression of Wnt8a_2010110K18Rik supporting the plausible role of these lncRNAs in a Pitx2 regulated cascade. Analyses in atrial-specific Pitx2 conditional transgenic mice further support the regulatory role of Pitx2 directly Wnt11_Gm_45188 up-regulation whereas no significantly changes are observed for Wnt11_Gm_44934. Surprisingly, divergent results are observed for Wnt8a_2010110K18Rik in atrial-specific Pitx2 conditional transgenic mice. It might be therefore possible that additional regulatory mechanisms driven by Pitx2 is exerted in non-cardiomyocyte cells. Additional experiments will be required to reconcile these findings.

Besides *PITX2*, additional GWAS have enlightened other genes putatively involved in AF pathophysiology, including *KCNN3, ZFHX3, IL6R, CAV1, HCN4, SYNE2, SYNPOL2, PRRX1* and *WTN8A* among others (Gudbjartsson et al. 2009; Kääb et al., 2009; Schnabel et al., 2011; Ellinor et al., 2013). To date more that 90 genes have been associated to AF by GWAS analyses (Roselli et al., 2018). Using strategies of gain and loss-of-function, Lozano-Velasco et al. (2015) demonstrate that *Pitx2* can direct expression of *Wnt8a*, but not *Zfhx3*. Importantly, abnormal *Wnt8a* expressed is documented only in *Pitx2* loss-of-function models with ECG alterations at rest, suggesting that Wnt signaling is fundamental for the AF susceptibility *vs* triggering capacity. In this study we therefore analyzed if *Wnt8* and *Wnt11* could modulate lncRNA expression as they exert synergistic and complementary regulatory mechanisms in the Pitx2>Wnt>microRNA signaling pathway as previously reported by Lozano-Velasco et al. (2015; 2017). Our data demonstrate that Wnt8 *vs* Wnt11 over-expression, respectively can complementary modulate Wnt11_Gm_44653, Wnt11_Gm_44934 and Wnt11_06 expression. Therefore, expression of Wnt11_Gm_44653 and Wnt11_06 is regulated by both Pitx2 and Wnt whereas Wnt11_Gm_44934 is exclusively regulated by Wnt signaling. Thus, these data reinforce the notion of a plausible role for these lncRNAs in Pitx2>Wnt signaling and thus in atrial arrhythmias.

Several studies have unraveled that Pitx2-regulated microRNAs can also contribute to AF pathophysiology. Chinchilla et al. (2011) demonstrated impaired modulation of Kcnj2 and Kcnj12 by miR-1, underlie abnormal resting membrane potential configuration in Pitx2 deficient mice. Lozano-Velasco et al. (2015) identified a large number of microRNAs modulated by Pitx2, most of which were previously reported to be associated to AF in humans . miR-1, miR-133, miR-21, miR-26, miR-29 and miR-

106b were previously reported to regulate calcium (CACNA1C, RYR2), sodium (SCN5A), potassium (KCNJ2, KCNE1, KCNB2), cation (HCN4) channel subunits, respectively (revised in Lozano-Velasco et al., 2019). Importantly, microRNA regulation by Pitx2 is exerted by a balance interplay between Wnt8 and Wnt11 expression (Lozano-Velasco et al., 2015). We therefore tested in this study whether over-expression of Pitx2-regulated microRNAs can influence lncRNAs expression. Our data demonstrate that that microRNA over-expression can modulate lncRNAs. In particular, miR-1 over-expression up-regulated Wnt11_Gm_45188 and Wnt8a_2010110K18Rik while Wnt11_Gm_44934 is not altered. In addition, miR-29 over-expression also significantly increases Wnt11_Gm_45188, while miR-133 significantly decreases Wnt11_Gm_45188 and Wnt11_Gm_44934. These data demonstrate that Wnt11_Gm_45188 and Wnt8a_2010110K18Rik, besides being modulated by Pitx2 and Wnt, they are also regulated by these key pro-arrhythmogenic microRNAs, particularly miR-1 and to a lesser extend miR-29. Thus, these data reinforce the notion of a plausible role for these lncRNAs in atrial arrhythmias. Moreover, we demonstrate for the first time that microRNAs can modulate lncRNAs, mimicking their action as in mRNA transcripts, although it remains to be established if such modulatory roles are directly or indirectly exerted.

Cardiovascular risk factors such hypertension (HTN), hyperthyroidism (HTD), diabetes and obesity have been repetitively demonstrated to promote onset of atrial fibrillation, respectively (Zhang et al., 2014; Anumonwo & Kalifa, 2014; Goudis et al., 2015). Furthermore, the occurrence of AF can be also triggered by preceding cardiovascular diseases such as hypertrophic cardiomyopathy and valvular heart diseases (Anumonwo & Kalifa, 2014; Yadaya et al., 2014; Kumar et al., 2015). Importantly, Pitx2 is up-regulated in cardiac hypertophy (Su et al., 2014) as ewll as in heart failure (Torrado et al., 2014). We have recently provide evidence on the modulatory role of upstream pathways influencing PITX2 in the context of AF. HTD but not HTN elicits a complex impairment of PITX2>Wnt>microRNA signaling which leads to abnormal ion channel expression. We herein tested whether AngII and NE treatment modulate lncRNA expression. Our data demonstrate that all lncRNAs analyzed are up-regulated upon AngII and NE administration, therefore these data support the plausible role of these lncRNAs in atrial arrhythmias as well as in cardiac hypertrophy.

In addition, we also analyzed if thyroid hormone could influence their expression. Surprisingly, thyroid hormone distinctly modulate lncRNA expression as compared to

AngII/NE administration. Wnt11_Gm_45188 was significantly down-regulated after T3 administration as well as Wnt8a_2010110K18Rik after both T3 and T4 administration, respectively. On the other hand, Wnt11_Gm_44934 was significantly up-regulated after both T3 and T4 administration, respectively. Thus, Wnt11_Gm_45188 and Wnt8a_2010110K18Rik is distinctly regulated by the pro-arrhythmogenic and pro-hypertrophic substrates AngII/NE vs T3/T4. Thus these data therefore further reinforce the plausible role of these lncRNAs in atrial arrhythmias as they display differential modulation by AngII/NE vs T3/T4 administration.

In order to start understanding the functional roles of these lncRNAs in cardiac development, pull down assays for the lncRNA Wnt11_Gm_44934 were performed in HL1 atrial cardiomyocytes. A total of 40 proteins were identified that significantly interact with Wnt11_Gm44934. Most of these proteins are cytoplasmic (>40%), suggesting a prominent post-transcriptional role for Wnt11_Gm44934. Two major set of cytoplasmic proteins were found, myosin proteins (MHY6, MHY9 and MHY10) and cytoskeletal proteins (RAB1A, RAB2A, RAB7A, ACTN1, ACTN4 and TLN1). Point mutations in MHY6 have been associated to cardiac hypertrophy, dilated cardiomyopathy, atrial fibrillation and congenital heart diseases (Carniel et al., 2005; Hershberger et al., 2010; Holm et al., 2011; Granados-Riveron et al., 2011; Nielsen et al., 2018). In addition, SNV in the proximity of MHY6 have been associated with atrial fibrillation and impaired heart rate (Holm et al., 2010,). Experimental studies in mice further support the functional role of Mhy6 in cardiac pathophysiology (Jiang et al., 2013). RAB7A has been implicated in controlling beta-adrenergic receptor endosome recycling (Noon et al., 2017) and impaired beta-adrenergic stimulation is frequently associated to AF. ACTN1 and TLN1 abnormal expression leads to dilated cardiomyopathy (Jia et al., 2016; Manso et al., 2013; 2017). In particular, TLN1 knock-out display decreased beta1-integrin expression (another Wnt11_Gm44934 interacting protein), costameric instability and cardiac hypertrophy (Manso et al., 2017). Importantly, two additional cytoplasmic Wnt11_Gm44934 interacting protein are also lined to cardiac hypertrophy (UBA1 and PPIA)(Satoh et al., 2011; Wang et al., 2013; Shu et al., 2018). Overall, these evidences support that impairment of Wnt11_Gm44934 might also lead to cardiac arrhythmias and/or hypertrophy by deregulating one or several interacting proteins.

At the nuclear levels, Wnt11_Gm44934 interacts with ROA2, ROA1, DXC39B and MBBIA, supporting the notion that Wnt11_Gm44934 can also exert transcriptional

regulatory mechanisms. At present the functional roles of these interacting proteins is poorly understood. Surprisingly, Wnt11_Gm44934 also interacts with a large array of mitochondrial proteins (KAD2, PGAM1, DHE3, CPT2, IVD, CYC and SDHB) and endoplasmatic reticulum proteins (HYEP, NB5R3, RTN-4, CALU, ETFD and TXDN5). While the nature and functional consequences of such interactions remains to be explored, it is importantly to highlight that impaired expression of SDHD, CPT2, RTN4, ETFD and CATD have been also associated to hypertrophic and/or dilated cardiomyopathy (Zhou et al., 2009; Tang et al., 2014; Roselló-Lletí et al., 2014; Sasagawa et al., 2016; Pereyra et al., 2017), reinforcing the notion that Wnt11_Gm44934 might play a role in cardiac hypertrophy.

In summary, we have identified five novel lncRNA that are differentially expressed in the developing and adult heart. Three of them display atrial-specific expression during embryogenesis and a fourth one remains to be differentially expressed in the right atrium in adulthood. Pitx2>Wnt>miRNAs signaling pathway can distinctly modulate the expression of these lncRNAs. In addition, pro-hypertrophic and pro-arrhythmogenic pathways such as those exerted by AngII/NE and thyroid hormone administration distinctly regulated also the expression of these lncRNAs. Pull-down assays demonstrate that Wnt11_Gm44934 interacts primarily with cytoplasmic proteins that, if impaired are associated to cardiac arrhythmias and hypertrophy. Overall, these data suggest a plausible role of these lncRNAs in cardiac arrhythmogenesis and hypertrophy.

Figure legends

Figure 1. Panel A. Schematic representation of the mouse chromosomal localization of lncRNAs situated in the vicinity of Pitx2, Wnt11 and Wnt8 genes. Panels B-G. qPCR analyses of Wnt11_Gm45188, Wnt11_Gm44934, Wnt8a_2010110K18Rik, Wnt11_Gm44653, Wnt11_06 and Playrr, respectively. Observe that Wnt11_Gm45188, Wnt11_Gm44934 and Wnt8a_2010110K18Rik display a atrial-specific expression during embryogenesis, while Wnt11_Gm44653 is mainly expressed in the left atrium in the adulthood.

Figure 2. qPCR analyses of lncRNA expression in *Pitx2, Wnt8* and *Wnt11* gain and loss-of-function assays in HL1 atrial cardiomyocytes. Panel A, B and C display *Pitx2, Wnt8* and *Wnt11* expression in gain-of-function (over) and loss-of-function (si) assays, demonstrating significant up- and down-regulation of these factors, respectively. Panel D, E and F display qPCR analyses of Wnt11_Gm45188, Wnt11_Gm44653, Wnt11_Gm44934, Wnt11_06 and Wnt8a_2010110K18Rik in *Pitx2, Wnt8* and *Wnt11* gain (over) and loss-of-function (si) assays. Observe that both, gain and loss-of-function assays distinct regulate expression of these lncRNAs. Panel D display Pitx2c expression in NppaCrePitx2$^{-/-}$ adult left atrial chambers. Panel H displays Wnt11_Gm45188, Wnt11_Gm44934 and Wnt8a_2010110K18Rik in NppaCrePitx2$^{-/-}$ adult left atrial chambers.

Figure 3. qPCR analyses of lncRNA expression in miR-1, miR-29 and miR-133 gain-of-function assays in HL1 atrial cardiomyocytes. Panels A to C display qPCR analyses of Wnt11_Gm45188, Wnt11_Gm44934 and Wnt8a_2010110K18Rik in miR-1, miR-29 and miR-133 gain-of-function assays. Observe that miR-1 significantly up-regulates Wnt11_Gm45188 and Wnt8a_2010110K18Rik while no significant differences are observed for Wnt11_Gm44934. miR-29 significantly up-regulates Wnt11_Gm45188, down-regulates Wnt11_Gm44934 while no changes are observed for Wnt8a_2010110K18Rik. miR-133 significantly down-regulates Wnt11_Gm45188, Wnt11_Gm44934 but no significant changes are observed for Wnt8a_2010110K18Rik. Panel D display miR-1, miR-29 and miR-133 expression in gain-of-function assays, demonstrating significant up-regulation of these microRNAs, respectively. Panel E display qPCR analyses of *Playrr* in miR-1, miR-29 and miR-133 gain-of-function assays. In all these conditions, *Playrr* expression in significantly decreased.

Figure 4. qPCR analyses of Wnt11_Gm45188, Wnt11_Gm44934 and Wnt8a_2010110K18Rik after angiotensin II (AngII) or norepinephrine (NE) (panels A-C), and T3 or T4 thyroid hormone (panels D-F) administration in HL1 atrial cardiomyocytes, respectively. Observe that AngII and NE treatment enhances Wnt11_Gm45188, Wnt11_Gm44934 and Wnt8a_2010110K18Rik expression. T4 administration up-regulates Wnt11_Gm44934 and downregulates Wnt8a_2010110K18Rik while no changes are observed for Wnt11_Gm45188. T3 administration significantly down-regulates Wnt11_Gm45188 and Wnt8a_2010110K18Rik and up-regulates Wnt11_Gm44934.

Table 1. Identification of Wnt11_Gm_44934 interacting proteins as revealed by pull-down assays followed by LC-MS/MS analyses. Observed that 40 interacting proteins were identified, most of them are located in the cytoplasm, with a small but significant fraction is also observed in other cellular organelles such as the nucleus, mitochondria and endoplasmatic reticulum.

References

Aguirre LA, Alonso ME, Badía-Careaga C, Rollán I, Arias C, Fernández-Miñán A, López-Jiménez E, Aránega A, Gómez-Skarmeta JL, Franco D, Manzanares M. Long-range regulatory interactions at the 4q25 atrial fibrillation risk locus involve PITX2c and ENPEP. BMC Biol. 2015 Apr 17;13:26.

Anumonwo JM, Kalifa J. Risk factors and genetics of atrial fibrillation. Cardiol Clin. 2014; 32:485-494.

Bär C, Chatterjee S, Thum, T (2016). Long Noncoding RNAs in Cardiovascular Pathology, Diagnosis, and Therapy. Circulation 134(19): 1484-1499.

Boucher J M, Peterson S M, Urs S, et al (2011). The miR-143/145 cluster is a novel transcriptional target of Jagged-1/Notch signaling in vascular smooth muscle cells. Journal of Biological Chemistry 286(32): 28312-28321.

Carniel E, Taylor MR, Sinagra G, Di Lenarda A, Ku L, Fain PR, Boucek MM, Cavanaugh J, Miocic S, Slavov D, Graw SL, Feiger J, Zhu XZ, Dao D, Ferguson DA, Bristow MR, Mestroni L. Alpha-myosin heavy chain: a sarcomeric gene associated with dilated and hypertrophic phenotypes of cardiomyopathy. Circulation. 2005 Jul 5;112(1):54-9.

Chen L. (2016). Linking Long Noncoding RNA Localization and Function. Trends in Biochemical Sciences 41(9): 761-772.

Chinchilla A, Daimi H, Lozano-Velasco E, Dominguez JN, Caballero R, Delpón E, Tamargo J, Cinca J, Hove-Madsen L, Aranega AE, Franco D. PITX2 insufficiency leads to atrial electrical and structural remodeling linked to arrhythmogenesis. Circ Cardiovasc Genet. 2011 Jun;4(3):269-79.

Chinchilla A, Daimi H, Lozano-Velasco E, Dominguez JN, Caballero R, Delpón E, Tamargo J, Cinca J, Hove-Madsen L, Aranega AE, Franco D. PITX2 insufficiency leads to atrial electrical and structural remodeling linked to arrhythmogenesis. Circ Cardiovasc Genet. 2011;4:269-279.

Daimi H, Lozano-Velasco E, Haj Khelil A, Chibani JB, Barana A, Amorós I, et al. Regulation of SCN5A by microRNAs: miR-219 modulates SCN5A transcript expression and the effects of flecainide intoxication in mice. Heart Rhythm. 2015;12:1333–42.

de Lange FJ, Moorman AF, Christoffels VM. Atrial cardiomyocyte-specific expression of Cre recombinase driven by an Nppa gene fragment. *Genesis*. 2003;37:1–4.

Dempsey J, Zhang A, Cui JY. Coordinate regulation of long non-coding RNAs and protein-coding genes in germ-free mice. BMC Genomics. 2018 Nov 21;19(1):834.

Dieci G, Fiorino G, Castelnuovo M, et al (2007). The expanding RNA polymerase III transcriptome. Trends in Genetics 23(12): 614-622.

Ellinor PT, Lunetta KL, Glazer NL, Pfeufer A, Alonso A, Chung MK, Sinner MF, de Bakker PI, Mueller M, Lubitz SA, Fox E, Darbar D, Smith NL, Smith JD, Schnabel RB, Soliman EZ, Rice KM, Van Wagoner DR, Beckmann BM, van Noord C, Wang K, Ehret GB, Rotter JI, Hazen SL, Steinbeck G, Smith AV, Launer LJ, Harris TB, Makino S, Nelis M, Milan DJ, Perz S, Esko T, Köttgen A, Moebus S, Newton-Cheh C, Li M, Möhlenkamp S, Wang TJ, Kao WH, Vasan RS, Nöthen MM, MacRae CA, Stricker BH, Hofman A, Uitterlinden AG, Levy D, Boerwinkle E, Metspalu A, Topol EJ, Chakravarti A, Gudnason V, Psaty BM, Roden DM, Meitinger T, Wichmann HE, Witteman JC, Barnard J, Arking DE, Benjamin EJ, Heckbert SR, Kääb S. Common variants in KCNN3 are associated with lone atrial fibrillation. Nat Genet. 2010;42:240-244.

Engreitz J M, Ollikainen N, Guttman M. (2016) Long non-coding RNAs: spatial amplifiers that control nuclear structure and gene expression. Nature Reviews Molecular Cell Biology.

Franco D, Chinchilla A, Aránega AE. Transgenic insights linking pitx2 and atrial arrhythmias. Front Physiol. 2012 Jun 12;3:206.

Franco D, Chinchilla A, Daimi H, Dominguez JN, Aránega A. Modulation of conductive elements by Pitx2 and their impact on atrial arrhythmogenesis. Cardiovasc Res. 2011 Jul 15;91(2):223-31.

Franco D, Christoffels VM, Campione M. Homeobox transcription factor Pitx2: The rise of an asymmetry gene in cardiogenesis and arrhythmogenesis. Trends Cardiovasc Med. 2014 Jan;24(1):23-31.

Franco D, Sedmera D, Lozano-Velasco E. Multiple Roles of Pitx2 in Cardiac Development and Disease. J Cardiovasc Dev Dis. 2017 Oct 11;4(4). pii: E16.

Gage PJ, Suh H, Camper SA. Dosage requirement of Pitx2 for development of multiple organs. *Development*. 1999;126:4643–4651

Gloss B S, Dinger M E. (2016).The specificity of long noncoding RNA expression. Biochimicaet Biophysica Acta (BBA)-Gene Regulatory Mechanisms 1859(1): 16-22.

Gore-Panter S R, Hsu J, Barnard J, et al (2016). PANCR, the PITX2 Adjacent noncoding RNA, is expressed in human left atria and regulates PITX2c expression. Circulation: Arrhythmia and Electrophysiology, 9(1): e003197.

Gore-Panter SR, Hsu J, Barnard J, Moravec CS, Van Wagoner DR, Chung MK, Smith JD. PANCR, the PITX2 Adjacent Noncoding RNA, Is Expressed in Human Left Atria and Regulates PITX2c Expression. Circ Arrhythm Electrophysiol. 2016 Jan;9(1):e003197.

Goudis CA, Korantzopoulos P, Ntalas IV, Kallergis EM, Ketikoglou DG. Obesity and atrial fibrillation: A comprehensive review of the pathophysiological mechanisms and links. J Cardiol. 2015;S0914-5087

Granados-Riveron JT, Ghosh TK, Pope M, Bu'Lock F, Thornborough C, Eason J, Kirk EP, Fatkin D, Feneley MP, Harvey RP, Armour JA, David Brook J. Alpha-cardiac myosin heavy chain (MYH6) mutations affecting myofibril formation are associated with congenital heart defects. Hum Mol Genet. 2010 Oct 15;19(20):4007-16.

Grote P, Herrmann B G. (2013). The long non-coding RNA Fendrr links epigenetic control mechanisms to gene regulatory networks in mammalian embryogenesis. RNA biology 10(10):1579-1585.

Grote P, Wittler L, Hendrix D, Koch F, Währisch S, Beisaw A, Macura K, Bläss G, Kellis M, Werber M, Herrmann BG. The tissue-specific lncRNA Fendrr is an essential regulator of heart and body wall development in the mouse. Dev Cell. 2013 Jan 28;24(2):206-14.

Gudbjartsson DF, Arnar DO, Helgadottir A, Gretarsdottir S, Holm H, Sigurdsson A, Jonasdottir A, Baker A, Thorleifsson G, Kristjansson K, Palsson A, Blondal T, Sulem P, Backman VM, Hardarson GA, Palsdottir E, Helgason A, Sigurjonsdottir R, Sverrisson JT, Kostulas K, Ng MC, Baum L, So WY, Wong KS, Chan JC, Furie KL, Greenberg SM, Sale M, Kelly P, MacRae CA, Smith EE, Rosand J, Hillert J, Ma RC, Ellinor PT, Thorgeirsson G, Gulcher JR, Kong A, Thorsteinsdottir U, Stefansson K. Variants conferring risk of atrial fibrillation on chromosome 4q25. Nature. 2007; 448:353-357.

Gudbjartsson DF, Arnar DO, Helgadottir A, Gretarsdottir S, Holm H, Sigurdsson A, Jonasdottir A, Baker A, Thorleifsson G, Kristjansson K, Palsson A, Blondal T, Sulem P, Backman VM, Hardarson GA, Palsdottir E, Helgason A, Sigurjonsdottir R, Sverrisson JT, Kostulas K, Ng MC, Baum L, So WY, Wong KS, Chan JC, Furie KL, Greenberg SM, Sale M, Kelly P, MacRae CA, Smith EE, Rosand J, Hillert J, Ma RC,

Ellinor PT, Thorgeirsson G, Gulcher JR, Kong A, Thorsteinsdottir U, Stefansson K. Variants conferring risk of atrial fibrillation on chromosome 4q25. Nature. 2007; 448:353-357.

Gudbjartsson DF, Holm H, Gretarsdottir S, Thorleifsson G, Walters GB, Thorgeirsson G, Gulcher J, Mathiesen EB, Njølstad I, Nyrnes A, Wilsgaard T, Hald EM, Hveem K, Stoltenberg C, Kucera G, Stubblefield T, Carter S, Roden D, Ng MC, Baum L, So WY, Wong KS, Chan JC, Gieger C, Wichmann HE, Gschwendtner A, Dichgans M, Kuhlenbäumer G, Berger K, Ringelstein EB, Bevan S, Markus HS, Kostulas K, Hillert J, Sveinbjörnsdóttir S, Valdimarsson EM, Løchen ML, Ma RC, Darbar D, Kong A, Arnar DO, Thorsteinsdottir U, Stefansson K. A sequence variant in ZFHX3 on 16q22 associates with atrial fibrillation and ischemic stroke. Nat Genet. 2009;41:876-878.

Henningsen KM, Olesen MS, Haunsoe S, Svendsen JH. Association of rs2200733 at 4q25 with early onset of lone atrial fibrillation in young patients. Scand Cardiovasc J. 2011;45:324-326.

Herraiz-Martínez A, Llach A, Tarifa C, Gandía J, Jiménez-Sabado V, Lozano-Velasco E, Serra SA, Vallmitjana A, Vázquez Ruiz de Castroviejo E, Benítez R, Aranega A, Muñoz-Guijosa C, Franco D, Cinca J, Hove-Madsen L. The 4q25 variant rs13143308T links risk of atrial fibrillation to defective calcium homoeostasis. Cardiovasc Res. 2019 Mar 1;115(3):578-589.

Hershberger RE, Norton N, Morales A, Li D, Siegfried JD, Gonzalez-Quintana J. Coding sequence rare variants identified in MYBPC3, MYH6, TPM1, TNNC1, and TNNI3 from 312 patients with familial or idiopathic dilated cardiomyopathy. Circ Cardiovasc Genet. 2010 Apr;3(2):155-61.

Hitachi K, Nakatani M, Takasaki A, Ouchi Y, Uezumi A, Ageta H, Inagaki H, Kurahashi H, Tsuchida K. Myogenin promoter-associated lncRNA Myoparr is essential for myogenic differentiation. EMBO Rep. 2019 Mar;20(3). pii: e47468.

Holm H, Gudbjartsson DF, Arnar DO, Thorleifsson G, Thorgeirsson G, Stefansdottir H, Gudjonsson SA, Jonasdottir A, Mathiesen EB, Njølstad I, Nyrnes A, Wilsgaard T, Hald EM, Hveem K, Stoltenberg C, Løchen ML, Kong A, Thorsteinsdottir U, Stefansson K. Several common variants modulate heart rate, PR interval and QRS duration. Nat Genet. 2010 Feb;42(2):117-22.

Holm H, Gudbjartsson DF, Sulem P, Masson G, Helgadottir HT, Zanon C, Magnusson OT, Helgason A, Saemundsdottir J, Gylfason A, Stefansdottir H, Gretarsdottir S, Matthiasson SE, Thorgeirsson GM, Jonasdottir A, Sigurdsson A, Stefansson H, Werge

T, Rafnar T, Kiemeney LA, Parvez B, Muhammad R, Roden DM, Darbar D, Thorleifsson G, Walters GB, Kong A, Thorsteinsdottir U, Arnar DO, Stefansson K. A rare variant in MYH6 is associated with high risk of sick sinus syndrome. Nat Genet. 2011 Mar 6;43(4):316-20.

Hori Y, Tanimoto Y, Takahashi S, Furukawa T, Koshiba-Takeuchi K, Takeuchi JK. Important cardiac transcription factor genes are accompanied by bidirectional long non-coding RNAs. BMC Genomics. 2018 Dec 27;19(1):967.

Jia Y, Chang HC, Schipma MJ, Liu J, Shete V, Liu N, Sato T, Thorp EB, Barger PM, Zhu YJ, Viswakarma N, Kanwar YS, Ardehali H, Thimmapaya B, Reddy JK. Cardiomyocyte-Specific Ablation of Med1 Subunit of the Mediator Complex Causes Lethal Dilated Cardiomyopathy in Mice. PLoS One. 2016 Aug 22;11(8):e0160755.

Jiang J, Wakimoto H, Seidman JG, Seidman CE. Allele-specific silencing of mutant Myh6 transcripts in mice suppresses hypertrophic cardiomyopathy. Science. 2013 Oct 4;342(6154):111-4.

Kääb S, Darbar D, van Noord C, Dupuis J, Pfeufer A, Newton-Cheh C, Schnabel R, Makino S, Sinner MF, Kannankeril PJ, Beckmann BM, Choudry S, Donahue BS, Heeringa J, Perz S, Lunetta KL, Larson MG, Levy D, MacRae CA, Ruskin JN, Wacker A, Schömig A, Wichmann HE, Steinbeck G, Meitinger T, Uitterlinden AG, Witteman JC, Roden DM, Benjamin EJ, Ellinor PT. Large scale replication and meta-analysis of variants on chromosome 4q25 associated with atrial fibrillation. Eur Heart J. 2009;30:813-819.

Kiliszek M, Franaszczyk M, Kozluk E, Lodzinski P, Piatkowska A, Broda G, Ploski R, Opolski G. Association between variants on chromosome 4q25, 16q22 and 1q21 and atrial fibrillation in the Polish population. PLoS One. 2011;6(7):e21790.

Kirchhof P, Kahr PC, Kaese S, Piccini I, Vokshi I, Scheld HH, Rotering H, Fortmueller L, Laakmann S, Verheule S, Schotten U, Fabritz L, Brown NA. PITX2c is expressed in the adult left atrium, and reducing Pitx2c expression promotes atrial fibrillation inducibility and complex changes in gene expression. Circ Cardiovasc Genet. 2011;4:123-133.

Kirchhof P, Kahr PC, Kaese S, Piccini I, Vokshi I, Scheld HH, Rotering H, Fortmueller L, Laakmann S, Verheule S, Schotten U, Fabritz L, Brown NA. PITX2c is expressed in the adult left atrium, and reducing Pitx2c expression promotes atrial fibrillation inducibility and complex changes in gene expression. Circ Cardiovasc Genet. 2011;4:123-133.

Klattenhoff, C A, Scheuermann J C, Surface L E, et al (2013) Braveheart, a long noncoding RNA required for cardiovascular lineage commitment. Cell 152(3): 570-583.

Kumar KR, Mandleywala SN, Link MS. Atrial and ventricular arrhythmias in hypertrophic cardiomyopathy. Card Electrophysiol Clin. 2015; 7:173-186.

Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 2001;25:402–408.

Lozano-Velasco E, Chinchilla A, Martínez-Fernández S, Hernández-Torres F, Navarro F, Lyons GE, et al. Pitx2c modulates cardiac-specific transcription factors networks in differentiating cardiomyocytes from murine embryonic stem cells. Cells Tissues Organs. 2011;194:349–62.

Lozano-Velasco E, Garcia-Padilla C, Aránega AE, Franco D. Genetics Of Atrial Fibrilation: In Search Of Novel Therapeutic Targets. Cardiovasc Hematol Disord Drug Targets. 2019 Feb 6.

Lozano-Velasco E, Hernández-Torres F, Daimi H, Serra SA, Herraiz A, Hove-Madsen L, Aránega A, Franco D. Pitx2 impairs calcium handling in a dose-dependent manner by modulating Wnt signalling. Cardiovasc Res. 2016 Jan 1;109(1):55-66.

Lozano-Velasco E, Hernández-Torres F, Daimi H, Serra SA, Herraiz A, Hove-Madsen L, Aránega A, Franco D. Pitx2 impairs calcium handling in a dose-dependent manner by modulating Wnt signalling. Cardiovasc Res. 2015;pii: cvv207.

Lozano-Velasco E, Wangensteen R, Quesada A, Garcia-Padilla C, Osorio JA, Ruiz-Torres MD, Aranega A, Franco D. Hyperthyroidism, but not hypertension, impairs PITX2 expression leading to Wnt-microRNA-ion channel remodeling. PLoS One. 2017 Dec 1;12(12):e0188473.

Mahlapuu M, Ormestad M, Enerback S, et al (2001). The forkhead transcription factor Foxf1 is required for differentiation of extra-embryonic and lateral plate mesoderm. Development 128(2): 155-166.

Manso AM, Li R, Monkley SJ, Cruz NM, Ong S, Lao DH, Koshman YE, Gu Y, Peterson KL, Chen J, Abel ED, Samarel AM, Critchley DR, Ross RS. Talin1 has unique expression versus talin 2 in the heart and modifies the hypertrophic response to pressure overload. J Biol Chem. 2013 Feb 8;288(6):4252-64.

Manso AM, Okada H, Sakamoto FM, Moreno E, Monkley SJ, Li R, Critchley DR, Ross RS. Loss of mouse cardiomyocyte talin-1 and talin-2 leads to β-1 integrin reduction,

costameric instability, and dilated cardiomyopathy. Proc Natl Acad Sci U S A. 2017 Jul 25;114(30):E6250-E6259.

Mei B, Liu H, Yang S, Liang MY, Yue Y, Huang SQ, Hou J, Chen GX, Wu ZK. Long non-coding RNA expression profile in permanent atrial fibrillation patients with rheumatic heart disease. Eur Rev Med Pharmacol Sci. 2018 Oct;22(20):6940-6947.

Mohanty S, Santangeli P, Bai R, Di Biase L, Mohanty P, Pump A, Natale A. Variant rs2200733 on chromosome 4q25 confers increased risk of atrial fibrillation: evidence from a meta-analysis. J Cardiovasc Electrophysiol. 2013;24:155-161.

Nielsen JB, Thorolfsdottir RB, Fritsche LG, Zhou W, Skov MW, Graham SE, Herron TJ, McCarthy S, Schmidt EM, Sveinbjornsson G, Surakka I, Mathis MR, Yamazaki M, Crawford RD, Gabrielsen ME, Skogholt AH, Holmen OL, Lin M, Wolford BN, Dey R, Dalen H, Sulem P, Chung JH, Backman JD, Arnar DO, Thorsteinsdottir U, Baras A, O'Dushlaine C, Holst AG, Wen X, Hornsby W, Dewey FE, Boehnke M, Kheterpal S, Mukherjee B, Lee S, Kang HM, Holm H, Kitzman J, Shavit JA, Jalife J, Brummett CM, Teslovich TM, Carey DJ, Gudbjartsson DF, Stefansson K, Abecasis GR, Hveem K, Willer CJ. Biobank-driven genomic discovery yields new insight into atrial fibrillation biology. Nat Genet. 2018 Sep;50(9):1234-1239.

Nooh MM, Bahouth SW. Two barcodes encoded by the type-1 PDZ and by phospho-Ser(312) regulate retromer/WASH-mediated sorting of the ß(1)-adrenergic receptor from endosomes to the plasma membrane. Cell Signal. 2017 Jan;29:192-208.

Olesen MS, Holst AG, Jabbari J, Nielsen JB, Christophersen IE, Sajadieh A, Haunsø S, Svendsen JH. Genetic loci on chromosomes 4q25, 7p31, and 12p12 are associated with onset of lone atrial fibrillation before the age of 40 years. Can J Cardiol. 2012;28:191-195.

Ounzain S, Micheletti R, Arnan C, et al (2015). CARMEN, a human super enhancer-associated long noncoding RNA controlling cardiac specification, differentiation and homeostasis. Journal of molecular and cellular cardiology 89: 98-112.

Ounzain S, Pedrazzini T, (2016). Long non-coding RNAs in heart failure: a promising future with much to learn. Annals of translational medicine 4(15):298.

Parvez B, Shoemaker MB, Muhammad R, Richardson R, Jiang L, Blair MA, Roden DM, Darbar D. Common genetic polymorphism at 4q25 locus predicts atrial fibrillation recurrence after successful cardioversion. Heart Rhythm. 2013 Jun;10(6):849-55.

Pereyra AS, Hasek LY, Harris KL, Berman AG, Damen FW, Goergen CJ, Ellis JM. Loss of cardiac carnitine palmitoyltransferase 2 results in rapamycin-resistant, acetylation-independent hypertrophy. J Biol Chem. 2017 Nov 10;292(45):18443-18456.

Qian C, Li H, Chang D, Wei B, Wang Y. Identification of functional lncRNAs in atrial fibrillation by integrative analysis of the lncRNA-mRNA network based on competing endogenous RNAs hypothesis. J Cell Physiol. 2018 Nov 27.

Roselli C, Chaffin MD, Weng LC, Aeschbacher S, Ahlberg G, Albert CM, Almgren P, Alonso A, Anderson CD, Aragam KG, Arking DE, Barnard J, Bartz TM, Benjamin EJ, Bihlmeyer NA, Bis JC, Bloom HL, Boerwinkle E, Bottinger EB, Brody JA, Calkins H, Campbell A, Cappola TP, Carlquist J, Chasman DI, Chen LY, Chen YI, Choi EK, Choi SH, Christophersen IE, Chung MK, Cole JW, Conen D, Cook J, Crijns HJ, Cutler MJ, Damrauer SM, Daniels BR, Darbar D, Delgado G, Denny JC, Dichgans M, Dörr M, Dudink EA, Dudley SC, Esa N, Esko T, Eskola M, Fatkin D, Felix SB, Ford I, Franco OH, Geelhoed B, Grewal RP, Gudnason V, Guo X, Gupta N, Gustafsson S, Gutmann R, Hamsten A, Harris TB, Hayward C, Heckbert SR, Hernesniemi J, Hocking LJ, Hofman A, Horimoto ARVR, Huang J, Huang PL, Huffman J, Ingelsson E, Ipek EG, Ito K, Jimenez-Conde J, Johnson R, Jukema JW, Kääb S, Kähönen M, Kamatani Y, Kane JP, Kastrati A, Kathiresan S, Katschnig-Winter P, Kavousi M, Kessler T, Kietselaer BL, Kirchhof P, Kleber ME, Knight S, Krieger JE, Kubo M, Launer LJ, Laurikka J, Lehtimäki T, Leineweber K, Lemaitre RN, Li M, Lim HE, Lin HJ, Lin H, Lind L, Lindgren CM, Lokki ML, London B, Loos RJF, Low SK, Lu Y, Lyytikäinen LP, Macfarlane PW, Magnusson PK, Mahajan A, Malik R, Mansur AJ, Marcus GM, Margolin L, Margulies KB, März W, McManus DD, Melander O, Mohanty S, Montgomery JA, Morley MP, Morris AP, Müller-Nurasyid M, Natale A, Nazarian S, Neumann B, Newton-Cheh C, Niemeijer MN, Nikus K, Nilsson P, Noordam R, Oellers H, Olesen MS, Orho-Melander M, Padmanabhan S, Pak HN, Paré G, Pedersen NL, Pera J, Pereira A, Porteous D, Psaty BM, Pulit SL, Pullinger CR, Rader DJ, Refsgaard L, Ribasés M, Ridker PM, Rienstra M, Risch L, Roden DM, Rosand J, Rosenberg MA, Rost N, Rotter JI, Saba S, Sandhu RK, Schnabel RB, Schramm K, Schunkert H, Schurman C, Scott SA, Seppälä I, Shaffer C, Shah S, Shalaby AA, Shim J, Shoemaker MB, Siland JE, Sinisalo J, Sinner MF, Slowik A, Smith AV, Smith BH, Smith JG, Smith JD, Smith NL, Soliman EZ, Sotoodehnia N, Stricker BH, Sun A, Sun H, Svendsen JH, Tanaka T, Tanriverdi K, Taylor KD, Teder-Laving M, Teumer A, Thériault S, Trompet S, Tucker NR, Tveit A,

Uitterlinden AG, Van Der Harst P, Van Gelder IC, Van Wagoner DR, Verweij N, Vlachopoulou E, Völker U, Wang B, Weeke PE, Weijs B, Weiss R, Weiss S, Wells QS, Wiggins KL, Wong JA, Woo D, Worrall BB, Yang PS, Yao J, Yoneda ZT, Zeller T, Zeng L, Lubitz SA, Lunetta KL, Ellinor PT. Multi-ethnic genome-wide association study for atrial fibrillation. Nat Genet. 2018 Jun 11;50(9):1225-1233.

Ruan Z, Sun X, Sheng H, Zhu L. Long non-coding RNA expression profile in atrial fibrillation. Int J Clin Exp Pathol. 2015 Jul 1;8(7):8402-10.

Sasagawa S, Nishimura Y, Okabe S, Murakami S, Ashikawa Y, Yuge M, Kawaguchi K, Kawase R, Okamoto R, Ito M, Tanaka T. Downregulation of GSTK1 Is a Common Mechanism Underlying Hypertrophic Cardiomyopathy. Front Pharmacol. 2016 Jun 14;7:162.

Satoh K, Nigro P, Zeidan A, Soe NN, Jaffré F, Oikawa M, O'Dell MR, Cui Z, Menon P, Lu Y, Mohan A, Yan C, Blaxall BC, Berk BC. Cyclophilin A promotes cardiac hypertrophy in apolipoprotein E-deficient mice. Arterioscler Thromb Vasc Biol. 2011 May;31(5):1116-23.

Schnabel RB, Kerr KF, Lubitz SA, Alkylbekova EL, Marcus GM, Sinner MF, Magnani JW, Wolf PA, Deo R, Lloyd-Jones DM, Lunetta KL, Mehra R, Levy D, Fox ER, Arking DE, Mosley TH, Müller-Nurasyid M, Young TR, Wichmann HE, Seshadri S, Farlow DN, Rotter JI, Soliman EZ, Glazer NL, Wilson JG, Breteler MM, Sotoodehnia N, Newton-Cheh C, Kääb S, Ellinor PT, Alonso A, Benjamin EJ, Heckbert SR;Candidate Gene Association Resource (CARe) Atrial Fibrillation/Electrocardiography Working Group. Large-scale candidate gene analysis in whites and African Americans identifies IL6R polymorphism in relation to atrial fibrillation: the National Heart, Lung, and Blood Institute's Candidate Gene Association Resource (CARe) project. Circ Cardiovasc Genet. 2011;4:557-564.

Shu Q, Lai S, Wang XM, Zhang YL, Yang XL, Bi HL, Li HH. Administration of ubiquitin-activating enzyme UBA1 inhibitor PYR-41 attenuates angiotensin II-induced cardiac remodeling in mice. Biochem Biophys Res Commun. 2018 Oct 20;505(1):317-324.

Su D, Jing S, Guan L, Li Q, Zhang H, Gao X, Ma X. Role of Nodal-PITX2C signaling pathway in glucose-induced cardiomyocyte hypertrophy. Biochem Cell Biol. 2014 Jun;92(3):183-90.

Su Y, Li L, Zhao S, Yue Y, Yang S. The long noncoding RNA expression profiles of paroxysmal atrial fibrillation identified by microarray analysis. Gene. 2018 Feb 5;642:125-134.

Tang Y, Mi C, Liu J, Gao F, Long J. Compromised mitochondrial remodeling in compensatory hypertrophied myocardium of spontaneously hypertensive rat. Cardiovasc Pathol. 2014 Mar-Apr;23(2):101-6.

Tao Y, Zhang M, Li L, Bai Y, Zhou Y, Moon AM, Kaminski HJ, Martin JF. Pitx2, an atrial fibrillation predisposition gene, directly regulates ion transport and intercalated disc genes. Circ Cardiovasc Genet. 2014;7:23-32.

Torrado M, Franco D, Hernández-Torres F, Crespo-Leiro MG, Iglesias-Gil C, Castro-Beiras A, Mikhailov AT. Pitx2c is reactivated in the failing myocardium and stimulates myf5 expression in cultured cardiomyocytes. PLoS One. 2014 Mar 4;9(3):e90561.

Torrado M, Franco D, Lozano-Velasco E, Hernández-Torres F, Calviño R, Aldama G, Centeno A, Castro-Beiras A, Mikhailov A. A MicroRNA-Transcription Factor Blueprint for Early Atrial Arrhythmogenic Remodeling. Biomed Res Int. 2015;2015:263151.

Villegas VE, Zaphiropoulos PG. Neighboring gene regulation by antisense long non-coding RNAs. Int J Mol Sci. 2015 Feb 3;16(2):3251-66.

Wang J, Klysik E, Sood S, Johnson RL, Wehrens XH, Martin JF. Pitx2 prevents susceptibility to atrial arrhythmias by inhibiting left-sided pacemaker specification. Proc Natl Acad Sci U S A. 2010 May 25;107(21):9753-8.

Wang J, Klysik E, Sood S, Johnson RL, Wehrens XH, Martin JF. Pitx2 prevents susceptibility to atrial arrhythmias by inhibiting left-sided pacemaker specification. Proc Natl Acad Sci U S A. 2010 May 25;107(21):9753-8.

Wang KC, Lim CH, McMillen IC, Duffield JA, Brooks DA, Morrison JL. Alteration of cardiac glucose metabolism in association to low birth weight: experimental evidence in lambs with left ventricular hypertrophy. Metabolism. 2013 Nov;62(11):1662-72.

Wang YG, Dedkova EN, Fiening JP, Ojamaa K, Blatter LA, Lipsius SL. Acute exposure to thyroid hormone increases Na+ current and intracellular Ca2+ in cat atrial myocytes. J Physiol. 2003 Jan 15;546(Pt 2):491–9

Wapinski O, Chang H. Y. (2011).Long noncoding RNAs and human disease. Trends in cell biology 21(6): 354-361.

Welsh IC, Kwak H, Chen FL, Werner M, Shopland LS, Danko CG, Lis JT, Zhang M, Martin JF, Kurpios NA. Chromatin Architecture of the Pitx2 Locus Requires CTCF- and Pitx2-Dependent Asymmetry that Mirrors Embryonic Gut Laterality. Cell Rep. 2015 Oct 13;13(2):337-49.

Yadava M, Hughey AB, Crawford TC. Postoperative atrial fibrillation: incidence, mechanisms, and clinical correlates. Cardiol Clin. 2014; 32:627-636.

Zhang Q, Liu T, Ng CY, Li G. Diabetes mellitus and atrial remodeling: mechanisms and potential upstream therapies. Cardiovasc Ther. 2014; 32:233-41.

Zhou B, Rao L, Li Y, Gao L, Li C, Chen Y, Xue H, Liang W, Lv M, Song Y, Peng Y, Zhang L. The association between dilated cardiomyopathy and RTN4 3'UTR insertion/deletion polymorphisms. Clin Chim Acta. 2009 Feb;400(1-2):21-4.

6. Discusión

El desarrollo del corazón es un proceso complejo que se inicia con la formación del tubo cardiaco a partir de dos subpoblaciones celulares procedentes del mesodermo espláncnico, el FHF y el SHF. Este tubo primitivo sufre una torsión y una remodelación conforme avanza el desarrollo que conduce a la formación de un corazón tetracameral, constituido por dos atrios y dos ventrículos funcional y morfológicamente diferentes (Moorman et al., 2004). Junto con estos cambios morfogenéticos, las distintas cámaras cardiacas presentan una expresión regionalizada de factores de transcripción cardiacos y de proteínas estructurales que median las diferencias funcionales y morfológicas observadas entre ellas, tanto en el corazón en desarrollo, como en el corazón fetal y en el adulto (Franco et al., 1997; Bruneau, 2008).

La expresión de lncRNAs muestra patrones dinámicos durante el desarrollo cardiaco

El análisis de los perfiles de expresión por qPCR de los distintos *lncRNAs* estudiados demuestra la expresión regionalizada de estos *lncRNAs* en las distintas cámaras cardiacas lo que sugiere que pueden tener algún papel en la especificación de funcional y/o morfológica de estas. Además de la regionalización observada, estos *lncRNAs* muestran perfiles de expresión dinámicos tanto en el desarrollo cardiaco como en la etapa adulta. *Braveheart*, *Carmen* y *Fendrr* muestran un patrón de expresión adulto mientras que *Miat*, *H19* y *Alien* muestran un patrón de expresión embrionario.

Braveheart y *Carmen* se expresan de manera basal durante el desarrollo embrionario a diferencia de la etapa adulta, donde su expresión es mucho más alta. Estos *lncRNAs* presentan el mismo perfil de expresión entre las cámaras cardiacas tanto en el desarrollo cardiaco como en la etapa adulta con la excepción de la aurícula derecha, donde la expresión de *Carmen* presenta niveles basales. Curiosamente, Ounzain et al. (2016) pusieron de manifiesto que la expresión de *Braveheart* era dependiente de la expresión de *Carmen* en la adquisición del destino celular cardiaco aunque esta modulación no fue estudiada en estadios de desarrollo posteriores ni en la etapa adulta. La pérdida de expresión de *Carmen* en la aurícula derecha adulta pero no de *Braveheart*, sugiere que la modulación positiva que se observa en las primeras etapas de la cardiogénesis, no es necesaria para la expresión de *Braveheart* en la etapa adulta en esta cámara cardiaca.

Fendrr, muestra una mayor expresión ventricular durante el desarrollo, entre los estadios ED12,5 y ED14,5 mientras que su expresión en las aurículas es significativamente menor. Curiosamente, la delección de *Fendrr* provoca una letalidad embrionaria en ED13,5, exhibiendo defectos en las paredes ventriculares y en el septo interventricular (Grote et al., 2013). La expresión ventricular máxima de *Fendrr* coincide con la maduración del septo interventricular que, junto con los defectos observados en los mutantes, sugieren que este *lncRNA* podría tener un papel en la septación.

La expresión ventricular de *Miat* decae en la etapa postnatal pero aumenta de manera drástica durante la etapa adulta, sugiriendo un papel de este *lncRNA* en la homeostasis ventricular adulta. De hecho, *Miat* ha sido relacionado con varias enfermedades cardiacas como el infarto de miocardio, la hipertrófica cardiaca o la fibrosis, procesos fisiopatológicos en los que la homeostasis ventricular está severamente alterada (Ishie et al., 2006; Devaux et al., 2014; Qu et al., 2017). El perfil de expresión preferentemente embrionario de *Miat* sugiere que este puede jugar un papel en el desarrollo embrionario, sin embargo los modelos *in vivo* deficientes para *Miat* no muestran un fenotipo cardiaco anómalo. Estos datos en su conjunto sugieren que si bien *Miat* puede jugar un papel en el desarrollo cardiaco este no es esencial, si bien también podría ser que su función fuese redundante o que el fenotipo generado sea sutil y haya pasado inadvertido (Ip et al., 2016).

Se ha descrito el papel de *Alien* en la especificación del mesodermo pero no se ha estudiado el papel de este *lncRNA* durante la cardiogénesis. Kurian et al (2015), demostró que la delección de este *lncRNA in vivo* conduce a una letalidad embrionaria en ED13,5 acompañada de graves defectos cardiacos. Los análisis por qPCR de *Alien* muestran una expresión muy dinámica en todas las cámaras cardiacas. Del mismo modo estos análisis muestran una expresión máxima en el ED12,5 que va decayendo conforme avanza el desarrollo lo que sugiere que la función de *Alien* se restringe al desarrollo. Esta expresión máxima coincide en el tiempo con el estadio donde acontece la letalidad embrionaria de los ratones mutantes lo que puede sugerir que en esta ventana del desarrollo, *Alien* podría jugar un papel esencial en el desarrollo cardiaco. Sin embargo seria necesario investigar los mecanismos moleculares subyacentes.

A diferencia de *Miat* y *Alien*, la expresión de *H19* se encuentra muy regionalizada durante el desarrollo. Así, la expresión de *H19* en los ventrículos y en la aurícula derecha es mucho mayor que la expresión observada en la aurícula izquierda. Es interesante

destacar que los mutantes deficientes para *H19* no muestran un fenotipo cardiaco visible lo que indica un papel no esencial de este lncRNA en el desarrollo cardiaco. Al igual que para *Miat*, también podría ser que su función fuese redundante o que el fenotipo generado sea sutil y haya pasado inadvertido. Futuros experimentos son necesarios para clarificar estas cuestiones.

La expresión de lncRNAs asociados a Pitx2>Wnt muestra patrones cámara-específicos durante el desarrollo cardiaco

Se han descrito multitud de ejemplos de *lncRNAs* que regulan genes que se encuentran adyacentes o cercanos a ellos (Sigova, et al., 2013). En base a este papel regulador de los *lncRNAs*, hemos explorado el entorno genómico del factor de transcripción *Pitx2* y de los factores de señalización *Wnt8* y *Wnt11*, factores que son claves en la ruta de señalización pro-arritmogénica *Pitx2>Wnt>microRNAs* (Lozano et al., 2016; Lozano et al., 2017). En la vecindad de estos *loci* génicos encontramos 5 lncRNAs no identificados hasta la fecha. En la vecindad de *Wnt11* identificamos a *Wnt11_06*, *Wnt11_44934*, *Wnt11_Gm_45188* y *Wnt11_Gm_44653*, en la vecindad de *Wnt8* identificamos a *Wnt8_2010110K18Rik*, y en la vecindad de *Pitx2* estudiamos *Playrr,* un *lncRNA* ya descrito pero cuya expresión en el corazón no había sido estudiada (Welsh et al, 2015). El análisis de la expresión espacio-temporal de estos *lncRNAs* muestran una expresión dinámica en las distintas cámaras cardiacas durante el desarrollo embrionario.

Entre ellos, *Wnt8_2010110K18Rik*, *Wnt11_45188*, *Wnt11_44934* muestran una expresión preferencialmente atrial durante el desarrollo embrionario. Mientras que *Wnt8a_2010110K18Rik* y *Wnt11_45188* muestran una expresión similar en ambas aurículas, *Wnt11_Gm 44934* muestra una expresión preferencialmente en la aurícula izquierda. Esta expresión cámara específica de estos tres *lncRNAs* durante el desarrollo cardiaco sugiere que podrían tener un papel en el desarrollo de la función específica de estas cámaras. Al contrario que *Wnt11_Gm_44934*, *Playrr* muestra una expresión preferencialmente en la aurícula derecha. *Playrr* ha sido descrito como un regulador negativo de la expresión de *Pitx2c* en el lado derecho del tracto digestivo, al impedir que el promotor de este sea accesible a la maquinaria transcripcional mientras que *Pitx2* reprime la expresión de este *lncRNA* en el margen izquierdo del estómago e intestino, (Welsh et al, 2015). Curiosamente, al igual que en el estómago y en el intestino, entre

otros muchos tejidos, la expresión de *Pitx2c* en el corazón se restringe al lado izquierdo. En concreto, la expresión de *Pitx2c* queda restringida a la aurícula izquierda tanto durante el desarrollo embrionario como en la etapa adulta (Campione et al., 2001; Bamforth et al., 2004; Franco et al, 2014; Davis et al, 2017). La modulación negativa de *Pitx2c* por parte de *Playrr* y la expresión contrapuesta en las aurículas de ambos sugieren que *Playrr* podría modular negativamente la expresión de *Pitx2c* en la aurícula derecha, reforzando así la expresión de *Pitx2c* en la aurícula izquierda.

Wnt11_06 y *Wnt11_Gm 44653* muestran un patrón más dinámico entre las distintas cámaras cardiacas durante el desarrollo cardiaco salvo con la diferencia de que mientras la expresión de *Wnt11_06* es claramente embrionaria, *Wnt11_Gm 44653* se expresa en la aurícula derecha de manera preferencial en el corazón adulto. La expresión en la aurícula derecha de *Wnt11_Gm 44653* sugiere un papel de este *lncRNA* en la homeostasis de la función de esa cámara cardiaca mientras que el perfil de expresión embrionario de *Wnt11_06* sugiere un posible papel de este durante el desarrollo. Atendiendo a la expresión cámara-especifica de *Wnt11_Gm_45188, Wnt11_Gm_44934, Wnt8a_2010110K18Rik* y por ello a su posible papel en el desarrollo atrial seria interesante estudiar el papel *in vivo* de estos lncRNAs en modelos murinos de perdida de función (*knock-out).*

La localización subcelular de Carmen y Fendrr pero no de Braveheart y H19, muestra diferencias significativas en las distintas cámaras cardiacas durante el desarrollo

La localización subcelular de los *lncRNAs* suele ser un reflejo de la función de estos. Así, los *lncRNAs* que se localizan preferentemente en el citoplasma ejercen una regulación post-transcripcional mientras que los *lncRNAs* que se localizan en el núcleo, ejercen una regulación transcripcional (Huang et al., 2018; Zampetaki et al, 2018). Se han descrito varios ejemplos de *lncRNAs* distribuidos de manera similar en el citoplasma y en el núcleo que regulan simultáneamente distintos procesos celulares tanto a nivel transcripcional como post-transcripcional (Coassin et al, 2014; Das et al, 2018).

El estudio de la expresión subcelular de *Braveheart* y *H19* en las distintas cámaras cardiacas durante el desarrollo demuestra que ambos se expresan de manera similar, tanto en el núcleo como en el citoplasma, lo que sugiere que ejercen su función, tanto a nivel transcripcional como a nivel post-transcripcional. Por el contrario, *Fendrr* y *Carmn*,

presentan una expresión preferencialmente nuclear en los ventrículos y la aurícula izquierda, mientras en la aurícula derecha muestran una expresión preferencialmente citoplasmática, sugiriendo un papel modulador transcripcional en ventrículos y aurícula izquierda y un papel modulador post-transcripcional en la aurícula derecha. Estos datos en su conjunto sugieren que *Fendrr* y *Carmn* pueden ejercer diferentes funciones cámara-específicas durante el desarrollo cardiaco.

***H19* se expresa dinámicamente en distintos tipos celulares durante el desarrollo cardiaco**

La localización subcelular de *H19* observada mediante qPCR así como el análisis del perfil de expresión se correlacionan con los resultados obtenidos mediante hibridación *in situ* (ISH) para *H19* durante el desarrollo cardiaco. La expresión de *H19* en ED10,5 se localiza tanto en el epicardio como en el endocardio de todas las cámaras cardiacas. En este mismo día embrionario una expresión muy leve es detectada en el miocardio ventricular derecho. En ED 12,5, la expresión de *H19* se detecta también en el miocardio, epicardio ventricular y en los cojines endocárdicos. Conforme el desarrollo avanza, la expresión de *H19* se va restringiendo al endocardio trabecular y al endocardio valvular. Así, en el ED 14,5, *H19* se expresa en el epicardio y en el endocardio valvular y trabecular. En la etapa postnatal, la expresión de *H19* se detecta en el endocardio de la aurícula derecha y de ambos ventrículos pero no en el endocardio de la aurícula izquierda.

La expresión de *H19* tanto en el epicardio, miocardio y endocardio durante el desarrollo sugiere un papel relevante y/o esencial de este *lncRNA* en la cardiogenesis. Actualmente se han generado dos modelos deficientes para *H19*, en los cuales se deleccionado el locus génico de *H19* junto con sus secuencias reguladoras y el gen *H19*, respectivamente (Leighton et al., 1995; Ripoche et al., 1997). Sorprendentemente, estos mutantes son viables y la única diferencia que presentan respecto a los ratones control es un aumento general de su tamaño corporal. Ensayos de la expresión génica en los mutantes han identificado la desregulación de un conjunto de genes involucrados en el crecimiento corporal entre los que destaca *Igf2* e *Igfr1*, cuya expresión se encuentra aumentada con respecto a los controles. Ensayos posteriores han puesto de manifiesto que *H19* es necesario para el mantenimiento de la impronta génica de *Igf2* ya que la eliminación de *H19* conlleva la expresión bi-alélica de *Igf2*. Además la regulación de

Igf2 por parte de *H19* ha sido demostrada durante la miogenesis esquelética y en diferentes estudios *in vitro* (Gabory et al, 2010; Kurukuti et al., 2006; Nordin et al., 2014).

Shen et al. (2014) demostraron que *Igf2* se expresa tanto en el endocardio como en el epicardio pero no en el miocardio a partir de ED10,5. La expresión de *Igf2* en el epicardio pero no en el endocardio es necesaria para una correcta proliferación y maduración de los cardiomiocitos al activar la ruta de señalización del ácido retinoico (RA) siendo considerado como el principal factor mitógeno que media via de señalización RA (Chen et al, 2002; Kang et al., 2005; Shen et al., 2014; Li et al, 2011). Recientemente, se ha puesto de manifiesto que *Igf1r* es el principal receptor de *Igf2* en el miocardio siendo expresado en este desde ED10,5 hasta ED14,5. Este receptor es necesario para la correcta proliferación y maduración de los cardiomiocitos mediada por *Igf2*. De hecho, los ratones mutantes para *Igf2* e *Igf1r* presentan un fenotipo similar caracterizado por un hipoplasia ventricular en las paredes ventriculares y en el septo interventricular (Shen et al., 2014; Wang et al., 2019). Curiosamente, Keniry et al. (2012) han puesto de manifiesto también que *H19* regula negativamente los niveles de RNA mensajero de *Igf1r*, a través del microRNA miR-675 en el tejido placentario.

Dado que *H19* también se expresa en el epicardio y en el endocardio al mismo tiempo que *Igf2* sería interesante estudiar si ambos factores muestran una co-expresión celular para poder determinar si la regulación documentada de *H19* sobre *Igf2* también está presente en el corazón en desarrollo. Del mismo modo, sería interesante co-localizar la expresión y traducción de *Igfr1* y *H19* en el miocardio, dado que los niveles de proteína de *Igfr1* se correlacionan negativamente con los niveles de expresión de *H19* durante el desarrollo ventricular. Cabe recordar la regulación negativa de este receptor por parte del microRNA miR-675 codificado en el primer exón de *H19* en la placenta. Además, la ISH muestra una mayor expresión citoplasmática de *H19* en los cardiomiocitos, lo que refuerza la posible modulación del RNA mensajero de *Igfr1* por parte de *H19* en el citoplasma.

En conjunto, los datos de ISH de *H19* junto con el papel de *Igf2/Igfr1* en la maduración y proliferación de los cardiomiocitos podrían sugerir que este *lncRNA* puede jugar un papel en la modulación de la cascada de señalización mediada por *Igf2* durante este proceso.

Por otro lado, es importante destacar la expresión de *H19* tanto en los primordios valvulares como en las válvulas cardiacas durante el desarrollo embrionario y en la etapa postnatal. En ED12,5 la expresión de *H19* se detectada en las células mesenquimáticas de los cojines endocárdicos, que posteriormente formaran las válvulas cardiacas. En ED14,5, *H19* se expresa en las válvulas cardiacas ya formadas y en las células mesenquimáticas que aún se están incorporando a estas. Esta expresión valvular también se detecta en el ED 16,5 y en la etapa postnatal. Hadji et al. (2014) demostraron que una metilación anómala en el promotor de *H19* conlleva a una calcificación valvular de la arteria aorta. Esta metilación anómala conduce a una sobreexpresión de *H19* que modula negativamente la expresión de *Notch1*. El papel de *Notch1* en el desarrollo y homeostasis valvular ha sido ampliamente descrito (MacGrogan et al., 2014; Garg, 2016; Koening et al., 2016; Wang et al,. 2017). La expresión tan marcada de *H19* en las válvulas cardiacas junto con la modulación negativa de *Notch1* por parte de este lncRNA permite hipotetizar que *H19* podría regular la expresión de *Notch1* durante el desarrollo valvular. Curiosamente el mutante de *H19* no presenta un fenotipo valvular, lo que implica que el posible incremento de *Notch1* debido a la ausencia de H19, no tendría un efecto en la cascada de señalización orquestada por *Notch1*. De hecho, hasta la fecha no se ha descrito un modelo *in vivo* de sobre-expresión de *Notch1* por lo que se desconoce si un posible incremento de este factor tiene un efecto en el desarrollo valvular. Sería interesante estudiar la co-expresión celular de ambos así como realizar ensayos de ganancia y pérdida de función de *H19* en explantes de cojines endocárdicos para explorar esta hipótesis. Asimismo un modelo *in vivo* en el que H19 se encuentre sobreexpresado podría arrojar más luz al posible papel modulador valvular de H19.

En línea con lo expuesto anteriormente en lo referente a un posible mecanismo regulador de *H19>Igf2* en la maduración de los cardiomiocitos, se ha descrito que ambos se encuentran sobre-expresados en pacientes que presentan calcificación de la válvula aortica (Zhu et al., 2014; Hadji et al, 2016). Asimismo se ha señalado que el proceso de calcificación recapitula en gran medida el programa embrionario que media el desarrollo valvular (Lerman et al., 2015). Cabe destacar que la expresión de *H19* en las válvulas es preferencialmente nuclear lo que indica que esta jugando un papel regulador de la transcripción. Estos datos en su conjunto sugieren que *H19* puede regular la expresión de *Igf2* durante el desarrollo valvular. Sin embargo se ha decir que los mutantes de *Igf2* no muestran defectos en el desarrollo de las válvulas por lo que, si bien *H19* puede modular

su expresión, esta no es esencial para un correcto desarrollo valvular, debido en gran medida por la existencia de la vía de señalización mediada por *Notch1* que es quien juega un papel esencial en este proceso.

Expresión diferencial de las distintas isoformas de Fendrr y Carmen, pero no de Braveheart, durante el desarrollo cardiaco

LncRNAs tienen una estructura genética muy similar a los RNA mensajeros y muchos de ellos presentan distintas isoformas como consecuencia de procesos de *splicing* alternativo (Haemming et al., 2007; Sallam et al., 2018). Hasta la fecha se desconoce si las distintas isoformas de los *lncRNAs* pueden ejercer funciones distintas, al igual que ocurre con las distintas isoformas de la mayoría de los RNA mensajeros (Ganassi et al., 2014). En este estudio hemos explorado la expresión de las distintas isoformas de *Fendrr*, *Braveheart* y *Carmen* en las distintas cámaras cardiacas durante el desarrollo embrionario y en el corazón adulto ya que estos se expresan de manera diferencial entre estas dos etapas. Nuestros datos demuestran que las dos isoformas de *Braveheart* se expresan de manera similar en todas las cámaras cardiacas tanto en el desarrollo como en la etapa adulta. Sin embargo, tanto las isoformas de *Fendrr* como las de *Carmen* muestran una expresión espacio-temporal entre ellas. Cabe destacar que *Fendrr* y *Carmen* presentan diferencias en cuanto a su localización subcelular en las distintas cámaras cardiacas, por lo que es posible hipotetizar que esas diferencias observadas en la localización celular pueden ser un reflejo del papel diferencial de las distintas isoformas de *Fendrr* y *Carmn* que podrían estar ejerciendo distintos papeles en la regulación transcripcional y post-transcripcional.

Modulación de la expresion de lncRNAs por factores de transcripción cardiacos

La expresión tejido especifica de múltiples genes claves para el desarrollo de los cardiomiocitos como aquellos que codifican proteínas citoesqueléticas o proteínas que constituyen los sarcómeros se encuentran regulados por un conjunto de factores de transcripción cardiacos entre los que destacan *Nkx2.5*, *Mef2c* y *Srf* (Meganathan et al., 2015). Usando ensayos de ganancia y pérdida de función en la línea celular HL1 de cardiomiocitos atriales de ratón, estudiamos una posible regulación de la expresión de los *lncRNAs* estudiados por parte de estos factores de transcripción. Los ensayos de perdida

de función de *Nkx2*.5, *Mef2c* y *Srf* demuestran que estos factores son indispensables para la expresión de *Carmen*, *H19* y *Alien* mientras que *Srf* y *Mef2c* son indispensables para la expresión de *Braveheart* y *Fendrr*. Asimismo, la perdida de función de *Nkx2.5* y *Mef2c* modula negativamente la expresión de *Miat,* lo que sugiere que ambos factores de transcripción son necesarios para la expresión de *Miat*. Sin embargo la pérdida de función de *Srf* sugiere que este reprime la expresión de *Miat*. La sobreexpresión de estos factores de transcripción es capaz de modular la expresión de estos *lncRNAs*, lo que sugiere que cambios en la expresión de estos factores de transcripción en respuesta a procesos patológicos cardiacos puede modular la expresión de aquellos *lncRNAs* que han sido relacionados con patologías cardiacas.

En este contexto es importante destacar la modulación de *Miat* por parte de *Mef2c* Los ensayos de ganancia de función demuestran que *Mef2c* modula positivamente la expresión de *Miat*. Es interesante destacar el papel de *Mef2c* y de *Miat* en el desarrollo de patologías hipertróficas. La expresión de *Mef2c* se encuentra incrementada en cardiomiopatías hipertróficas al igual que *Miat* (Alonso-montes et al., 2012; Xu et al., 2006; Wei al., 2017), por lo que es plausible sugerir que la expresión de *Miat* puede ser modulada por *Mef2c* en la respuesta hipertrófica cardiaca.

Al igual que *Miat*, la expresión de *H19* también se encuentra desregulada en procesos patológicos como la hipertrofia cardiaca o distintas cardiomiopatías. Asimismo, en diferentes patologías cardiacas, tales como la cardiomiopatía hipertrófica y/o dilatada (Zhu et al., 2016; Liu et al., 2016; Zhang et al., 2017; Li et al., 2018), el programa génico embrionario de las distintas cámaras cardiacas vuelve a reactivarse. Por ejemplo, *ANF*, el gen que codifica el péptido natriurético atrial y que presenta una expresión cámara-especifica no se expresa en el ventriculo del corazón adulto en condiciones normales, sin embargo su expresión aumenta en cardiomiopatías hipertróficas así como en enfermedades arritmogénicas (Horsthuis et al., 2008).

Modulación de lcnRNAs por Pitx2c>Wnt>microRNAs

Dado el perfil embrionario de muchos de *lncRNAs* descritos en este trabajo y la expresión cámara-especifica de alguno de ellos, junto con la necesidad de algunos de estos en el mantenimiento de la homeostasis nos planteamos el posible papel de la ruta de señalización pro-arritmogénica *Pitx2>Wnt>miRNAs* en la regulación de la expresión de

los *lncRNAs* estudiados (Lozano et al., 2016; 2017). Esta ruta de señalización está orquestada por *Pitx2*, que regula negativamente a *Wnt8a* y este a su vez establece un *feedback* positivo con *Wnt11*. La señalización *Pitx2>Wnt8/Wnt11* regula la expresión de distintos microRNAs que modulan a su vez la traducción de distintos canales iónicos involucrados en la configuración del potencial de acción cardiaco (Lozano et al., 2016; 2017).

Para conocer el papel de los lncRNAs que ocupan nuestro estudio, diseñamos ensayos de ganancia y de perdida de función de *Pitx2, Wnt8* y *Wnt11* en la línea celular HL1. La perdida de función de *Pitx2* demuestra que la expresión de *Braveheart, Carmen* y *Alien* es dependiente de este factor de transcripción. Curiosamente el estudio de la expresión *Braveheart* y *Carmen* en la aurícula izquierda de modelos *in vivo* en los que se ha eliminado específicamente la expresión atrial de *Pitx2* refuerzan estos datos. A diferencia de estos, la perdida de función de *Pitx2 in vitro* modula positivamente la expresión de *Alien*, lo que sugiere que se encuentra regulado negativamente por *Pitx2*. Sin embargo, los datos procedentes del estudio de la expresión de *Alien* en la aurícula izquierda de los mutantes contradice los datos obtenidos en los ensayos *in vitro*, mostrando una expresión muy disminuida con respecto al control.

Aunque los ensayos *in vitro* nos permiten explorar la modulación existente entre los genes, no son capaces de recapitular las condiciones fisiológicas en las que esta modulación se produce, debido en gran medida a que los distintos tipos celulares están continuamente interaccionando con el ambiente celular que le rodea, algo que no se puede imitar en ensayos *in vitro* en los que usan líneas celulares. Las diferencias entre los ensayos *in vitro* y los ensayos *in vivo* también pueden ser debidas a que en estos últimos la pérdida de función de *Pitx2* no solo tenga lugar en los cardiomiocitos auriculares sino también el resto de tipos celulares que expresan *Pitx2* (Hernández-Torres et al., 2015), a diferencia de los ensayos *in vitro*. En su conjunto, los análisis de expresión realizados en tejido *in vivo* recapitulan mejor la modulación de *Pitx2c*, por lo que podemos sugerir que la expresión de *Pitx2* es esencial para la correcta expresión de *Braveheart, Carmen* y *Alien* y no así para la expresión de *Miat, Fendrr* y *H19*.

La falta de función de *Wnt8* tiene el mismo efecto sobre la expresión de *Carmen* y *Alien* que la falta de función de *Pitx2*, mientras que la falta de función de *Wnt11* tiene efectos contrarios sobre la expresión de *Carmen* y *Alien* sugiriendo que *Wnt8* es necesario para la expresión de *Carmen* y *Alien* mientras que *Wnt11* reprime la expresión de estos.

La expresión de *Braveheart*, *H19* y *Fendrr* es modulada positivamente por *Wnt8* mientras que *Wnt11* es necesario para la expresión de *Fendrr* y *Miat*. Estos datos sugieren que los *lncRNAs* estudiados pueden jugar un papel en la ruta de señalización Pitx2>Wnt>miRNAs.

Se han descrito multitud de ejemplos de *lncRNAs* que inhiben la función de los microRNAs actuando como esponjas, uniéndose a ellos e impidiendo que estos puedan unirse a sus dianas en el citoplasma. Sin embargo no es claro si los microRNAs pueden modular los niveles de expresión de determinados lncRNAs de una manera similar a como lo hacen con los niveles de expresión de los RNA mensajeros. Para intentar arrojar luz sobre esta posible regulación, estudiamos si los microRNAs que se encuentran involucrados en arritmias auriculares y que se localizan aguas abajo de la ruta de señalización *Pitx2>Wnt>miRNAs* pueden modular los niveles de expresión de los lncRNAs analizados. Para ello, llevamos a cabo ensayos de sobreexpresión *in vitro* para miR-1, miR-29 y miR-133, respectivamente.

Estos ensayos demuestran que estos microRNAs modulan diferencialmente la expresión de los *lncRNAs* estudiados. La sobreexpresión del miR-1 modula positivamente la expresión de *Carmen*, *Fendrr* y *H19*. Esta sobreexpresión puede ser debida a que el miR-1 module negativamente a algún represor de la expresión de estos *lncRNAs*. Tanto la sobreexpresión del *miR-29* como del *miR-133* reduce casi en su totalidad los niveles de expresión de *Miat* mientras que la expresión de *Alien* se ve afectada negativamente solo en la sobreexpresión del miR-133. Estudios *in silico* de las secuencias de estos microRNAs y de los *lncRNAs* muestran que no existen regiones complementarias entre ambos, lo que sugiere que la modulación negativa observada puede ser indirecta.

Modulación de lncRNAs asociados a Wnt8/Wnt11 por Pitx2c>Wnt>microRNAs

El perfil de expresión auricular embrionario junto con la proximidad genómica de Wnt8a_2010110K18RIk, Wnt11_Gm44934 y Wnt11_Gm45188 a Wnt8a y Wnt11 nos hizo plantearnos una posible modulación de estos *lncRNAs* por la ruta de señalización *Pitx2c>Wnt>miRNAs*. Además, el hecho de que tanto Wnt11_Gm44934 y Wnt8a_2010110K18Rik tenga una expresión preferencial en la aurícula izquierda reforzó el posible papel de estos *lncRNAs* en esta ruta de señalización.

Los ensayos de perdida de función de *Pitx2* modulan negativamente la expresión de Wnt8a_2010110K18Rik mientras que tiene el efecto contrario en la expresión de Wnt11_Gm45188. A diferencias de estos dos, la expresión de Wnt11_Gm44934 no se encuentra alterada en la perdida de función de *Pitx2*. El estudio de la expresión Wnt11_Gm45188 y Wnt11_Gm44934 en la aurícula izquierda de modelos *in vivo* en los que se ha eliminado específicamente la expresión atrial de *Pitx2* refuerzan estos datos. Estos datos en su conjunto sugieren que *Pitx2* modula negativamente la expresión de Wnt11_Gm45188 mientras que la expresión de Wnt11_Gm44934 es independiente de *Pitx2*.

A diferencias de estos, como se ha descrito antes en *Alien*, la expresión de Wnt8a_2010110K18Rik en las aurículas mutantes contradice lo observado en los ensayos *in vitro*, demostrando que la perdida de función de *Pitx2 in vivo* conlleva una sobreexpresión de este *lncRNA*, lo que sugiere que este juega un papel represor en su expresión. Del mismo modo, la perdida de función de *Pitx2* en la aurícula izquierda genera un ambiente pro-hipertrófico y pro-arritmogénico. Dado el papel en la homeostasis de multitud de *lncRNAs*, no sería descartable pensar que la disminución de la expresión de Wnt8a_2010110K18RIk no está asociada directamente a la ruta de señalización mediada por *Pitx2* sino al ambiente pro-arritmogénico y pro-hipertrófico subyacente.

En línea con la modulación de Wnt11_Gm_45188 por parte de *Pitx2*, la sobreexpresión de *Wnt8* también modula negativamente la expresión de este *lncRNA*, en consonancia con los datos *in vivo*. En cuanto a Wnt8a_2010110K18Rik, su expresión no esta modulada por *Wnt8* pero si por Wnt11. La sobreexpresión de este aumenta la expresión de Wnt8a_2010110K18Rik, lo que sugiere que al igual que *Wnt11* modula positivamente la expresión de *Wnt8*, también puede ejercer esta modulación positiva en los elementos genéticos que se encuentra alrededor de este. Sería interesante testar si esta modulación positiva esta mediada por un *enhancer*. La presencia de un *enhancer* común que se encuentre bajo la regulación de la vía de señalización mediada por *Wnt11* y que sea capaz de promover la expresión de la región en la que Wnt8a y Wnt8a_2010110K18Rik se localizan, podría explicar esta regulación positiva.

Es interesante destacar que la expresión de *Wnt11_06* es modulada negativamente por *Wnt8*. Lozano et al. (2016) demostró que la expresión de *Wnt11* es modulada negativamente por *Wnt8*. Dado que este *lncRNA* se encuentra en la estructura génica de

Wnt11 y es derivado de un intrón durante el *splicing* alternativo, pudiese ser que tanto *Wnt11* y *Wnt11_06* sean modulados negativamente por *Wnt8a*.

En los ensayos de sobreexpresión de miR-1, miR-29 y miR-133 también se testó una posible modulación de la expresión de los *lncRNAs* identificados en la proximidad genómica de *Pitx2*, *Wnt8a* y *Wnt11* que presentaban una expresión aurícula específica, Wnt11_Gm45188, Wnt11_Gm44934, Wnt8a_2010110K18Rik y *Playrr*. La sobreexpresión del miR-1 modula positivamente la expresión de Wnt8a_2010110K18Rik mientras que modula negativamente la expresión de *Playrr*. La sobreexpresión de miR-29 modula positivamente la expresión de Wnt11_Gm_45188 mientras que modula negativamente la expresión de Wnt8a_2010110K18Rik, *Playrr* y Wnt11_Gm44934. Es interesante destacar que en esta ocasión los ensayos *in silico* si demostraron la existencia de una región complementaria entre el miR-29 y *Wnt8a_2010110K18Rik*, lo que sugiere que en esta ocasión la modulación negativa podría deberse a una mecanismo directo. Sin embargo es necesario un mayor estudio de esta interacción y un mayor conocimiento de la naturaleza de esta. Por último la sobreexpresión del miR-133 modula negativamente la expresión de todos los *lncRNAs* con una expresión aurícula-especifica, lo que sugiere que este microRNA puede regular a un regulador común que media la maduración de esta cámara.

En conjunto estos datos sugieren una regulación microRNA>lncRNA hasta la fecha desconocida. Además dado el carácter arritmogénico de estos microRNAs, es plausible considerar un posible papel de los lncRNAs estudiados en patologías arritmogénicas.

Regulacion de lcnRNAs por factores pro-hipertróficos y pro-arritmogénicos

Tanto la hipertensión como el hipertiroidismo son considerados como factores de riesgo en AF. Lozano et al. (2017) exploraron el impacto de estos dos factores de riesgo en la ruta de señalización orquestada por *Pitx2*, demostrando que si bien el hipertiroidismo afecta negativamente a la correcta expresión de esta ruta de señalización, la hipertensión tiene efecto solamente parcial sobre ella. Es importante resaltar en este contexto que una alteración de la ruta de señalización de Pitx2>Wnt>microRNAs aumenta la predisposición de padecer AF, señalando a esta ruta como un factor de riesgo genético en esta patología (Lozano-Velasco et al., 2015; 2017). Para estudiar si la expresión de los

lncRNAs estudiados, y en particular *Carmen* y *Alien* dada su regulación por *Pitx2c* y *Wnt8a/Wnt11*, se encuentran desregulados en estas dos patologías aislamos y cultivamos cardiomiocitos neonatales. Estos cardiomiocitos fueron tratados con triyodotirosina (T3) y tetrayodotirosina (T4) para simular el hipertiroidismo y con angiotensina II y NE para simular la hipertensión y la hipertrofia.

El tratamiento con T3 y T4 afecta negativamente la expresión de *Carmen* y *H19* mientras que la expresión de estos se ve incrementada en respuesta al tratamiento con angiotensina II y norepinefrina. Esto implica una regulación contrapuesta de *Carmen* y de *H19* en estas dos patologías y se encuentra en concordancia con el papel regulador que la ruta de señalización Pitx2>Wnt>microRNAs ejerce sobre *Carmen*, no así sobre *H19*. En contraposición la expresión de *Alien* y de *Fendrr* se ve aumentada tanto en respuesta a los factores hipertróficos como a la hormona tiroidea (T3 y T4). A diferencia de estos, la expresión de *Braveheart* no se ve afectada en condiciones prohipertróficas pero su expresión si se ve aumentada en el tratamiento con la hormona tiroidea lo que sugiere que si bien este *lncRNA* no juega un papel en patologías hipertróficas pero si en el hipertiroidismo.

En conjunto estos datos sugieren que tanto *Carmen*, *H19*, *Fendrr*, *Alien*, y *Braveheart* son desregulados en procesos patológicos hipertróficos y/o arritmogénicos, señalando a estos como importantes reguladores de la homeostasis.

El tratamiento con T3 y T4 modula negativamente tanto la expresión de *Wnt11_Gm_45188* como la de *Wnt8a_2010110K18Rik*. Contrariamente, el tratamiento con factores pro-hipertróficos aumenta la expresión de estos dos *lncRNAs* además de la expresión de *Wnt11_Gm_44934*. Estos datos en su conjunto sugieren que estos *lcnRNAs* están involucrados en la homeostasis fisiológica pudiendo tener un papel procesos patológicos hipertróficos y arritmogénicos.

Wnt11_Gm_44934 interacciona con proteínas citoesqueleticas y miosinas que se encuentran desreguladas en procesos hipertróficos.

Los ensayos de *pulldown* de Wnt11_Gm44934 en la línea celular HL-1 han demostrado que este *lncRNA* interacciona en su mayoría con proteínas citoplasmáticas, aunque también lo hace con proteínas nucleares y mitocondriales sugiriendo que

Wnt11_Gm44934 tiene un papel mayoritario en la regulación post-transcripcional de la célula aunque también pueda ejerce un papel en la regulación transcripcional de esta.

En las proteínas citoplasmáticas destacan las miosinas MHY6, MHY9 y MHY10, así como proteínas que constituyen el citoesqueleto tales como TAL1, ACTN1 y ACTN4. Ratones mutantes para las miosinas *Mhy6* y *Mhy10* exhiben diferentes enfermedades cardiacas congénitas y una hipertrofia cardiaca (England and Loughna, 2013). Además, *MHY6* ha sido relacionada con la fibrilación atrial (Bjornsson et al., 2018). La desregulación de estas miosinas junto con la desregulación de *Wnt11_Gm_44934* en condiciones hipertróficas, sugieren un papel regulador post-transcripcional de este *lncRNA* sobre *MHY6* y *MHY10*, en un contexto hipertrófico.

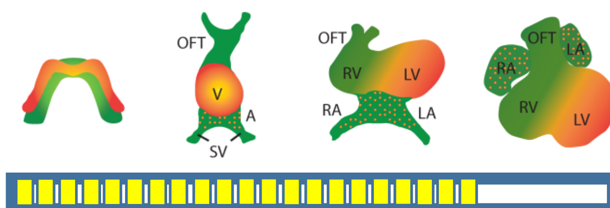
Entre las proteínas citoesqueléticas destacan la talina 1 (TAL1) y las actinas ACTN 1 y ACTN4. TAL1 fosforila a la β-integrina (ITB1), haciendo posible que esta se una al esqueleto citoplasmático celular, formado entre otras proteínas por las ACTN 1 y ACTN4 Curiosamente TAL1 muestra un patrón de expresión preferencialmente embrionario y una expresión basal en los cardiomiocitos adultos, al igual que Wnt11_Gm44934. Además TAL1 se encuentra sobre-expresada en la hipertrofia cardiaca y los mutantes para esta proteína presentan cardiomiopatías hipertróficas y una reducción en el nivel de proteína de β-integrina (Manso et al., 2013; 2017).

Asimismo es importante destacar la interacción entre Wnt11_Gm_44934 y UBA1, una ubiquitina cuya expresión en la hipertrofia cardiaca se encuentra aumentada (Shu et al., 2018). Es importante destacar que las ubiquitinas juegan un papel clave en la renovación proteica y que los cardiomiocitos están sujetos a una intensa remodelación citoesqueletica en hipertrofia cardiaca, que requiere de la participación de estas ubiquitinas (Li et al, 2018). Dada la interacción de Wnt11_Gm_44934 con UBA1 y con proteínas citoesqueléticas cuya expresión en la hipertrofia cardiaca se encuentran desreguladas es plausible hipotetizar que este *lncRNA* juega un papel en la remodelación y recambio de estas proteínas citoesqueléticas vía *UBA1* mediando posiblemente la interacción entre esta ubiquitina y las diferentes proteínas citoesqueléticas. Sin embargo, la naturaleza de este posible papel regulador necesita ser explorado.

A nivel nuclear, Wnt11_Gm44934 interacciona con ROA2, ROA1, DXC39B y MBBIA mientras que a nivel mitocondrial, Wnt11_Gm44934 interacciona con distintas proteínas específicas de este orgánulo como KAD2, ETFD, DHE3, VDAC2, CPT2, IVD,

CYC y SDHB. Además Wnt11_Gm_44934 interacciona con proteínas endosomales como RTN-4, CALU y TXDN5. La interacción de Wnt11_Gm44934 con proteínas de distintas sub-localizaciones celulares sugiere que la función de *lncRNA* no solo se restringe al núcleo o al citoplasma, jugando un papel en la regulación transcripcional y post-transcripcional, respectivamente, sino que también juega un papel en la modulación de proteínas específicas mitocondriales y del retículo endoplasmático. Curiosamente proteínas como CPT2 y RTN-4 se encuentran sobre-expresadas en patologías hipertróficas lo que refuerza más el posible papel modulador de Wnt11_Gm44934 en la hipertrófica cardiaca.

CONCLUSIONES/CONCLUSIONS



7. Conclusiones

Primera conclusión

Braveheart, *Carmen* y *Fendrr* se expresan tanto el desarrollo cardiaco como en la etapa adulta mostrando un perfil de expresión preferentemente adulto. La expresión de *Braveheart* y *Carmen* en las distintas cámaras cardiacas es bastante similar, divergiendo solo en el estadio neonatal y adulto. En contraposición, *Miat, Alien* y *H19* muestran una expresión dinámica en las distintas cámaras cardiacas exhibiendo un perfil de expresión preferentemente embrionario. Estos datos sugieren un papel de los *lncRNAs* estudiados en la cardiogénesis y en el mantenimiento de la homeostasis cardiaca.

Segunda conclusión

Los factores de transcripción cardiacos *Mef2c*, *Nkx2.5* y *Srf* modulan diferencialmente la expresión de los lncRNAs analizados. La falta de función de *Mef2c* demuestra que este factor de transcripción es esencial para la expresión de *Carmen* y *Miat* mientras que la falta de función de *Srf* demuestra su papel esencial para la expresión de *Carmen*. Por último la falta de función de *Nkx2.5* demuestra que es esencial para la expresión de *Fendrr*, *Miat* y *Alien*.

Tercera conclusión

Se ha identificado por primera vez la expresión en tejido cardiaco de los lncRNAs anotados Wnt11_Gm_45188, Wnt11_Gm_44934, Wnt11_2010110K18Rik, Wnt11_06 y Wnt11_Gm_44653, exhibiendo preferentemente un perfil de expresión embrionario. Es importante resaltar que mientras Wnt11_06 y Wnt11_Gm_44653 muestran una expresión dinámica entre las cámaras cardiacas, Wnt11_Gm_45188, Wnt11_Gm_44934, Wnt8a_2010110k18Rik se expresan de manera preferencialmente en las aurículas con respecto a los ventrículos durante el desarrollo embrionario. Estos datos sugieren que estos *lncRNAs* pueden jugar un papel importante en la cardiogénesis de las cámaras cardiacas atriales.

Cuarta conclusión

H19 se expresa en distintos tipos celulares durante el desarrollo cardiaco. En ED12,5, *H19* se expresa en el epicardio, miocardio y endocardio mientras que en ED14,5 se expresa solamente en el epicardio y endocardio ventricular y valvular. En la etapa posnatal, la expresión de *H19* queda restringida al endocardio ventricular y valvular. Estos datos sugieren que *H19* puede jugar un papel en el desarrollo y/o maduración de los distintos tipos celulares cardiacos.

Quinta conclusión

Las dos isoformas de *Braveheart* y *Carmen,* respectivamente, se expresan de manera similar en todas las cámaras cardiacas, tanto en el desarrollo embrionario como en la etapa adulta. Por el contrario, las tres isoformas de *Fendrr* muestran diferencias de expresión en las distintas cámaras cardiacas tanto en la etapa embrionaria como en la adulta. La expresión diferencial de las distintas isoformas de *Fendrr* sugiere que estas pueden jugar distintos papeles tanto en el desarrollo embrionario como en la etapa adulta.

Sexta conclusión

El factor de transcripción *Pitx2c* y los ligandos *Wnt8* y *Wnt11* modulan de forma diferencial la expresión de los *lncRNAs* analizados. Estos datos están en consonancia con los perfiles de expresión obtenidos en mutantes condicionales para *Pitx2*, con la excepción de *Alien*. La sobre-expresión de los microRNAs miR-1, miR-29 y miR-133, regulados a su vez por el factor de transcripción *Pitx2c*, modulan igualmente la expresión de los *lncRNAs* analizados. La modulación de los lncRNAs analizados por la ruta de señalización Pitx2>Wnt>microRNAs sugiere que estos pueden tener un papel en arritmogénesis.

Séptima conclusión

El tratamiento de cardiomiocitos con angiotensina II y norepinefrina aumenta los niveles de expresión de *Carmen, H19* y *Fendrr*. A su vez, el tratamiento con hormona tiroidea aumenta la expresión de *Braveheart, Fendrr* y *Alien* mientras que disminuye la expresión

de *Carmen* y *H19*. Asimismo el tratamiento con angiotensina II y norepinefrina aumenta los niveles de expresión de Wnt11_Gm_44934, Wnt_11_Gm_45188 y Wnt8a_2010110K18Rik. A su vez el tratamiento con hormona tiroidea disminuye la expresión de Wnt11_Gm_45188 y Wnt8a_2010110K18Rik. Estos datos, en su conjunto, sugieren que los *lncRNAs* analizados pueden ser modulados por un ambiente pro-hipertrófico y/o pro-arritmogénico.

Octava conclusión

Wnt11_Gm_44934 interacciona en su mayoría con proteínas citoplasmáticas aunque también lo hace en menor medida con proteínas nucleares, mitocondriales y endosomales sugiriendo un papel modulador bivalente de la regulación transcripcional y post-transcripcional. Del mismo modo, muchas de estas proteínas están relacionadas con hipertrofia cardiaca o distintas cardiomiopatías, lo que refuerza el *Wnt11_Gm_44934* en la hipertrofia cardiaca.

7. Conclusions

First conclusion

Braveheart, Carmen and Fendrr are expressed both in cardiac development and in the adult stage. These lncRNAs show an adult expression profile. The expression of Braveheart and Carmen in the different cardiac chambers is quite similar, diverging only in the neonatal and adult stage. In contrast, Miat, Alien and H19 show a dynamic expression in the different cardiac chambers exhibiting a preferentially embryonic expression profile. These data suggest a role the lncRNAs studied in cardiogenesis and in the maintenance of cardiac homeostasis.

Second conclusion

The cardiac transcription factors Mef2c, Nkx2.5 and Srf differentially modulate the expression of the analyzed lncRNAs. The lack of function of Mef2c demonstrates that this transcription factor is essential for the expression of Carmen and Miat whereas the lack of function of Srf demonstrates its essential role for the expression of Carmen. Finally, the lack of function of Nkx2.5 demonstrates that it is essential for the expression of Fendrr, Miat and Alien.

Third conclusion

The expression in cardiac tissue of the annotated lncRNAs Wnt11_Gm_45188, Wnt11_Gm_44934, Wnt8a_2010110K18Rik, Wnt11_06 and Wnt11_Gm_44653 has been published for the first time, exhibiting an embryonic expression profile. It is important to note that while Wnt11_06 and Gm_44653 show a dynamic expression between the cardiac chambers, Gm_45188, Gm_44934, 2010110k18Rik are expressed preferentially in the atria with respect to the ventricles during development. These are the data that are used in the cardiogenesis of atrial cardiac chambers.

Fourth conclusion

H19 is expressed in different cell types during cardiac development. In ED 12.5, H19 is expressed in the epicardium, myocardium and endocardium while in ED 14.5 it is expressed only in the epicardium and ventricular and valvular endocardium. In the postnatal stage, the expression of H19 is restricted to the ventricular and valvular endocardium. These data can not be played in the development and / or maturation of the different types of cardiac cells.

Fifth conclusion

The two isoforms of Braveheart and Carmen, respectively, are expressed in a similar manner in all cardiac chambers, both in development and in the adult stage. On the contrary, the three isoforms of the expression differences in the different cardiac chambers both in the stage and in the adult. The differential expression of Fendrr suggest that these can play different roles in both development and adulthood.

Sixth conclusion

The transcription factor Pitx2c and the ligands Wnt8 and Wnt11 modulate in a differential manner the expression of the analyzed lncRNAs. These data are consistent with the profiles of their expressions in the conditional mutants for Pitx2, with the exception of Alien. The overexpression of the microRNAs miR-1, miR-29 and miR-133, regulated in turn by the transcription factor Pitx2c, also modulate the expression of the analyzed lncRNAs. This modulation by the Pitx2 signaling pathway> Wnt> microRNAs of the lncRNAs analyzed suggest that these may have a role in arrhythmogenesis.

Seventh conclusion

The treatment of cardiomyocytes with angiotensin II and norepinephrine increases the expression levels of Carmn, H19 and Fendrr. In turn, the hormone treatment increases the expression of Braveheart, Fendrr and Alien while the expression of Carmn and H19.

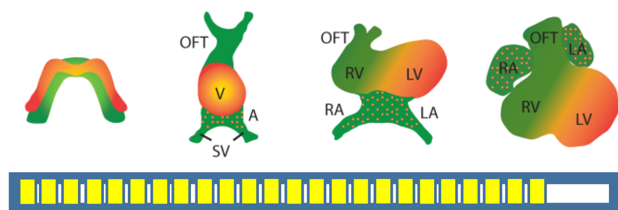
Also, treatment with angiotensin II and norepinephrine improves the expression levels of Wnt11_Gm_44934, Wnt_11_Gm_45188 and Wnt8a_2010110K18Rik. In turn, the hormone treatment reduces the expression of Wnt11_Gm_45188 and Wnt8a_2010110K18Rik.

These data suggest that lncRNAs analyzed can be modulated by a hypertrophic and pro arrhythmogenic environment.

Eight conclusion

Wnt11_Gm_44934 mostly interacts with cytoplasmic proteins, although it also interacts with mitocondrial, nuclear and endosomal proteins to a lesser extent, suggesting a bivalent modulator role of transcriptional and post-transcriptional regulation. Likewise, many of these proteins are related to cardiac hypertrophy or different cardiomyopathies, which reinforces the role of Wnt11_Gm_44934 in cardiac hypertrophy.

BIBLIOGRAFÍA



8. Bibliografía

Abu-Issa, R., Smyth, G., Smoak, I., Yamamura, K. I., and Meyers, E. N. (2002). Fgf8 is required for pharyngeal arch and cardiovascular development in the mouse. Development, 129(19), 4613-4625.

Aguilo, F., Zhou, M. M., and Walsh, M. J. (2011). Long noncoding RNA, polycomb, and the ghosts haunting INK4b-ARF-INK4a expression. Cancer research, 71(16), 5365-5369.

Alonso-Montes, C., Naves-Diaz, M., Fernandez-Martin, J. L., Rodriguez-Reguero, J., Moris, C., Coto, E., ... & Rodriguez, I. (2012). New polymorphisms in human MEF2C gene as potential modifier of hypertrophic cardiomyopathy. Molecular biology reports, 39(9), 8777-8785.

Anderson, K. M., Anderson, D. M., McAnally, J. R., Shelton, J. M., Bassel-Duby, R., and Olson, E. N. (2016). Transcription of the non-coding RNA upperhand controls Hand2 expression and heart development. Nature, 539(7629), 433.

Arnold, S. J., Hofmann, U. K., Bikoff, E. K., and Robertson, E. J. (2008). Pivotal roles for eomesodermin during axis formation, epithelium-to-mesenchyme transition and endoderm specification in the mouse. Development, 135(3), 501-511.

Bakkers, J., Verhoeven, M. C., and Abdelilah-Seyfried, S. (2009). Shaping the zebrafish heart: From left–right axis specification to epithelial tissue morphogenesis. Developmental biology, 330(2), 213-220.

Bakkers, J., Verhoeven, M. C., and Abdelilah-Seyfried, S. (2009). Shaping the zebrafish heart: From left–right axis specification to epithelial tissue morphogenesis. Developmental biology, 330(2), 213-220.

Bamforth, S. D., Bragança, J., Farthing, C. R., Schneider, J. E., Broadbent, C., Michell, A. C., ... & Anderson, R. H. (2004). Cited2 controls left-right patterning and heart development through a Nodal-Pitx2c pathway. Nature genetics, 36(11), 1189.

Bär, C., Chatterjee, S., & Thum, T. (2016). Long noncoding RNAs in cardiovascular pathology, diagnosis, and therapy. Circulation, 134(19), 1484-1499.

Barron, M. R., Belaguli, N. S., Zhang, S. X., Trinh, M., Iyer, D., Lough, J. W., Parmacek, M. S., Bruneau, B. G., and Schwartz, R. J. (2005). Serum response factor, an

enriched cardiac mesoderm obligatory factor, is a downstream gene target for Tbx genes. Journal of Biological Chemistry, 280(12), 11816-11828.

Bernardo, A. S., Faial, T., Gardner, L., Niakan, K. K., Ortmann, D., Senner, C. E., Callety, E. M., Trotter, M. W., Hemberger, M., Smith, J. C., Moffett, A., Bardwell, L. (2011). BRACHYURY and CDX2 mediate BMP-induced differentiation of human and mouse pluripotent stem cells into embryonic and extraembryonic lineages. Cell stem cell, 9(2), 144-155.

Biben, C., and Harvey, R. P. (1997). Homeodomain factor Nkx2-5 controls left/right asymmetric expression of bHLH gene eHand during murine heart development. Genes and development, 11(11), 1357-1369.

Bjornsson, T., Thorolfsdottir, R. B., Sveinbjornsson, G., Sulem, P., Norddahl, G. L., Helgadottir, A., ... & Adalsteinsdottir, B. (2018). A rare missense mutation in MYH6 associates with non-syndromic coarctation of the aorta. European heart journal, 39(34), 3243-3249.

Bondue, A., and Blanpain, C. (2010). Mesp1: a key regulator of cardiovascular lineage commitment. Circulation research, 107(12), 1414-1427.

Brade, T., Pane, L. S., Moretti, A., Chien, K. R., and Laugwitz, K. L. (2013). Embryonic heart progenitors and cardiogenesis. Cold Spring Harbor perspectives in medicine, 3(10), a013847.

Bruneau, B. G., and Srivastava, D. (2014). Congenital heart disease: entering a new era of human genetics. Circulation research, 114(4), 598-599.

Bruneau, B. G., Logan, M., Davis, N., Levi, T., Tabin, C. J., Seidman, J. G., and Seidman, C. E. (1999). Chamber-specific cardiac expression of Tbx5 and heart defects in Holt–Oram syndrome. Developmental biology, 211(1), 100-108.

Bruneau, B. G., Nemer, G., Schmitt, J. P., Charron, F., Robitaille, L., Caron, S., Conner, D. A., Gessler, M., Nemer, M., Seidman C. E., and Seidman J. G. (2001). A murine model of Holt-Oram syndrome defines roles of the T-box transcription factor Tbx5 in cardiogenesis and disease. Cell, 106(6), 709-721.

Buckingham, M., Meilhac, S., and Zaffran, S. (2005). Building the mammalian heart from two sources of myocardial cells. Nature Reviews Genetics, 6(11), 826.

Cai, X., Hagedorn, C. H., and Cullen, B. R. (2004). Human microRNAs are processed from capped, polyadenylated transcripts that can also function as mRNAs. Rna, 10(12), 1957-1966.

Cai, X., Nomura-Kitabayashi, A., Cai, W., Yan, J., Christoffels, V. M., and Cai, C. L. (2011). Myocardial Tbx20 regulates early atrioventricular canal formation and endocardial epithelial–mesenchymal transition via Bmp2. Developmental biology, 360(2), 381-390.

Cai, X., Zhang, W., Hu, J., Zhang, L., Sultana, N., Wu, B., Cai, W., Zhou, B., and Cai C. L. (2013). Tbx20 acts upstream of Wnt signaling to regulate endocardial cushion formation and valve remodeling during mouse cardiogenesis. Development, 140(15), 3176-3187.

Calcagni, G., Unolt, M., Digilio, M. C., Baban, A., Versacci, P., Tartaglia, M., and Marino, B. (2017). Congenital heart disease and genetic syndromes: new insights into molecular mechanisms. Expert review of molecular diagnostics, 17(9), 861-870.

Cano, E., Carmona, R., Ruiz-Villalba, A., Rojas, A., Chau, Y. Y., Wagner, K. D., ... and Pérez-Pomares, J. M. (2016). Extracardiac septum transversum/proepicardial endothelial cells pattern embryonic coronary arterio–venous connections. Proceedings of the National Academy of Sciences, 113(3), 656-661.

Carrieri, C., Cimatti, L., Biagioli, M., Beugnet, A., Zucchelli, S., Fedele, S. Pesce, E., Ferrer, I., Collavin, L., Santoro, C., Forrest, A. R., Carninci, P., Biffo, S., Stupka, E., and Gustincich S. (2012). Long non-coding antisense RNA controls Uchl1 translation through an embedded SINEB2 repeat. Nature, 491(7424), 454.

Carrion, K., Dyo, J., Patel, V., Sasik, R., Mohamed, S. A., Hardiman, G., & Nigam, V. (2014). The long non-coding HOTAIR is modulated by cyclic stretch and WNT/β-CATENIN in human aortic valve cells and is a novel repressor of calcification genes. PloS one, 9(5), e96577.

Cesana, M., Cacchiarelli, D., Legnini, I., Santini, T., Sthandier, O., Chinappi, M., Tramontano, A., and Bozzoni, I. (2011). A long noncoding RNA controls muscle differentiation by functioning as a competing endogenous RNA. Cell, 147(2), 358-369.

Chen, C. Y., and Schwartz, R. J. (1996). Recruitment of the tinman homolog Nkx-2.5 by serum response factor activates cardiac alpha-actin gene transcription. Molecular and cellular biology, 16(11), 6372-6384.

Chen, H., Shi, S., Acosta, L., Li, W., Lu, J., Bao, S., Chen, Z., Yang, Z.,Scheneider, M.D., CHien, K.R., Conway, S.J., Yoder, M.C., Haneline, L.S., Franco, D., and Shou, W. (2004). BMP10 is essential for maintaining cardiac growth during murine cardiogenesis. Development, 131(9), 2219-2231.

Chen, J. F., Mandel, E. M., Thomson, J. M., Wu, Q., Callis, T. E., Hammond, S. M., Conlon, F. L., and Wang, D. Z (2006). The role of microRNA-1 and microRNA-133 in skeletal muscle proliferation and differentiation. Nature genetics, 38(2), 228.

Chen, J. F., Murchison, E. P., Tang, R., Callis, T. E., Tatsuguchi, M., Deng, Z., Rojas, M., Hammond, S. M., Schneider, M. D., Selzman, C. H., Meissner, G., Patterson, C., Hannon, G. J., and Wang, D. Z. (2008). Targeted deletion of Dicer in the heart leads to dilated cardiomyopathy and heart failure. Proceedings of the National Academy of Sciences, 105(6), 2111-2116.

Chen, L. L. (2016). Linking long noncoding RNA localization and function. Trends in biochemical sciences, 41(9), 761-772.

Chen, T. H. P., Chang, T. C., Kang, J. O., Choudhary, B., Makita, T., Tran, C. M., Burch, J. B., Eid, H., and Sucov, H. M. (2002). Epicardial induction of fetal cardiomyocyte proliferation via a retinoic acid-inducible trophic factor. Developmental biology, 250(1), 198-207.

Chinchilla, A., Daimi, H., Lozano-Velasco, E., Dominguez, J. N., Caballero, R., Delpón, E., Tamargo, T., Cinca, J., Hove-Madsen, L., Aranega, A. E., and Franco, D. (2011). PITX2 insufficiency leads to atrial electrical and structural remodeling linked to arrhythmogenesis. Circulation: Cardiovascular Genetics, 4(3), 269-279.

Christoffels, V. M., Hoogaars, W. M., Tessari, A., Clout, D. E., Moorman, A. F., and Campione, M. (2004). T-box transcription factor Tbx2 represses differentiation and formation of the cardiac chambers. Developmental dynamics: an official publication of the American Association of Anatomists, 229(4), 763-770.

Cohen, E. D., Miller, M. F., Wang, Z., Moon, R. T., and Morrisey, E. E. (2012). Wnt5a and Wnt11 are essential for second heart field progenitor development. Development, 139(11), 1931-1940.

Cohen, E. D., Wang, Z., Lepore, J. J., Lu, M. M., Taketo, M. M., Epstein, D. J., and Morrisey, E. E. (2007). Wnt/β-catenin signaling promotes expansion of Isl-1–positive cardiac progenitor cells through regulation of FGF signaling. The Journal of clinical investigation, 117(7), 1794-1804.

Cordes, K. R., and Srivastava, D. (2009). MicroRNA regulation of cardiovascular development. Circulation research, 104(6), 724-732.

Costello, I., Pimeisl, I. M., Dräger, S., Bikoff, E. K., Robertson, E. J., and Arnold, S. J. (2011). The T-box transcription factor Eomesodermin acts upstream of Mesp1 to specify cardiac mesoderm during mouse gastrulation. Nature cell biology, 13(9), 1084.

Cox, C. J., Espinoza, H. M., McWilliams, B., Chappell, K., Morton, L., Hjalt, T. A., ... and Amendt, B. A. (2002). Differential regulation of gene expression by PITX2 isoforms. Journal of Biological Chemistry, 277(28), 25001-25010.

Cox, C. J., Espinoza, H. M., McWilliams, B., Chappell, K., Morton, L., Hjalt, T. A., Semina, E. V., and Amendt. B. A. (2002). Differential regulation of gene expression by PITX2 isoforms. Journal of Biological Chemistry, 277(28), 25001-25010.

Crispino, J. D., Lodish, M. B., Thurberg, B. L., Litovsky, S. H., Collins, T., Molkentin, J. D., and Orkin, S. H. (2001). Proper coronary vascular development and heart morphogenesis depend on interaction of GATA-4 with FOG cofactors. Genes and development, 15(7), 839-844.

Davis, A., Amin, N. M., Johnson, C., Bagley, K., Ghashghaei, H. T., & Nascone-Yoder, N. (2017). Stomach curvature is generated by left-right asymmetric gut morphogenesis. Development, 144(8), 1477-1483.

Derrien, T., Johnson, R., Bussotti, G., Tanzer, A., Djebali, S., Tilgner, H., Guernec, G., Martin, D., Merkel, A., Knowles, D. G., Lagarde, J., Veeravalli, L., Ruan, X., Ruan, Y., Lassmann, T., Carninci, P., Brown, J. B., Lipovich, L., Gonzalez, J. M., Thomas, M., Davis, C. A., Shiekhattar, R., Gingeras, T. R., Hubbard, T. J.,

Notredame, C., Harrow. J., and Guigó, R. (2012). The GENCODE v7 catalog of human long noncoding RNAs: analysis of their gene structure, evolution, and expression. Genome research, 22(9), 1775-1789.

Devaux, Y., Creemers, E. E., Boon, R. A., Werfel, S., Thum, T., Engelhardt, S., ... and Cardiolinc Network. (2017). Circular RNAs in heart failure. European journal of heart failure, 19(6), 701-709.

Dieci, G., Fiorino, G., Castelnuovo, M., Teichmann, M., and Pagano, A. (2007). The expanding RNA polymerase III transcriptome. TRENDS in Genetics, 23(12), 614-622.

Ellinor, P. T., Lunetta, K. L., Albert, C. M., Glazer, N. L., Ritchie, M. D., Smith, A. V., Arking, D. E., Müller-Nurasyid, M., Krijthe, B. P., Lubitz, S. A., Bis, J. C., Chung, M. K., Dörr, M., Ozaki, K., Roberts, J. D., Smith, J. G., Pfeufer, A., Sinner, M. F., Lohman, K., Ding, J., Smith, N. L., Smith, J. D., Rienstra, M., Rice, K. M., Van Wagoner, D. R., Magnani, J. W., Wakili, R., Clauss, S., Rotter, J. I., Steinbeck, G., Launer, L. J., Davies, R. W., Borkovich, M., Harris, T. B., Lin, H., Völker, U., Völzke, H., Milan, D. J., Hofman, A., Boerwinkle, E., Chen, L. Y., Soliman, E. Z., Voight, B. F., Li, G., Chakravarti, A., Kubo, M., Tedrow, U. B., Rose, L. M., Ridker, P. M., Conen, D., Tsunoda, T., Furukawa, T., Sotoodehnia, N., Xu, S., Kamatani, N., Levy, D., Nakamura, Y., Parvez, B., Mahida, S., Furie, K. L., Rosand, J., Muhammad, R., Psaty, B. M., Meitinger, T., Perz, S., Wichmann, H. E., Witteman, J. C., Kao, W. H., Kathiresan, S., Roden, D. M., Uitterlinden, A. G., Rivadeneira, F., McKnight, B., Sjögren, M., Newman, A. B., Liu, Y., Gollob, M. H., Melander, O., Tanaka, T., Stricker, B. H., Felix, S. B., Alonso, A., Darbar, D., Barnard, J., Chasman, D. I., Heckbert, S. R., Benjamin, E. J., Gudnason, V., and Kääb, S. (2012). Meta-analysis identifies six new susceptibility loci for atrial fibrillation. Nature genetics, 44(6), 670.

Engreitz, J. M., Ollikainen, N., & Guttman, M. (2016). Long non-coding RNAs: spatial amplifiers that control nuclear structure and gene expression. Nature Reviews Molecular Cell Biology, 17(12), 756.

Ezzeddine, N., Chang, T. C., Zhu, W., Yamashita, A., Chen, C. Y., Zhong, Z., Yamashita, Y., Zheng, D., and Shyu, A.B. (2007). Human TOB, an antiproliferative transcription factor, is a poly (A)-binding protein-dependent positive regulator of

cytoplasmic mRNA deadenylation. Molecular and cellular biology, 27(22), 7791-7801.

Fang, S., Zhang, L., Guo, J., Niu, Y., Wu, Y., Li, H., Zhao, H., Li, X., Teng, X., Sun, X., Sun, L., Zhang, M. Q., Chen, R., and Zhao, Y. (2017). NONCODEV5: a comprehensive annotation database for long non-coding RNAs. Nucleic acids research, 46(D1), D308-D314.

Firulli, A. B., McFadden, D. G., Lin, Q., Srivastava, D., and Olson, E. N. (1998). Heart and extra-embryonic mesodermal defects in mouse embryos lacking the bHLH transcription factor Hand1. Nature genetics, 18(3), 266.

Franco, D., and Campione, M. (2003). The role of pitx2 during cardiac development: Linking left–right signaling and congenital heart diseases. Trends in cardiovascular medicine, 13(4), 157-163.

Franco, D., Christoffels, V. M., and Campione, M. (2014). Homeobox transcription factor Pitx2: The rise of an asymmetry gene in cardiogenesis and arrhythmogenesis. Trends in cardiovascular medicine, 24(1), 23-31.

Gabory, A., Jammes, H., & Dandolo, L. (2010). The H19 locus: Role of an imprinted non-coding RNA in growth and development. Bioessays, 32(6), 473-480.

Gage, P. J., Suh, H., and Camper, S. A. (1999). Dosage requirement of Pitx2 for development of multiple organs. Development, 126(20), 4643-4651.

Gage, P. J., Suh, H., and Camper, S. A. (1999). The bicoid-related Pitx gene family in development. Mammalian Genome, 10(2), 197-200.

Ganassi, M., Badodi, S., Polacchini, A., Baruffaldi, F., Battini, R., Hughes, S. M., Hinits, Y., Molinari, S. (2014). Distinct functions of alternatively spliced isoforms encoded by zebrafish mef2ca and mef2cb. Biochimica et Biophysica Acta (BBA)-Gene Regulatory Mechanisms, 1839(7), 559-570.

Garg, V. (2016). Notch signaling in aortic valve development and disease. In Etiology and Morphogenesis of Congenital Heart Disease (pp. 371-376). Springer, Tokyo.

Gloss, B. S., and Dinger, M. E. (2016). The specificity of long noncoding RNA expression. Biochimica et Biophysica Acta (BBA)-Gene Regulatory Mechanisms, 1859(1), 16-22.

Gómez, J., Lorca, R., Reguero, J. R., Martín, M., Morís, C., Alonso, B., ... and Coto, E. (2018). Genetic variation at the long noncoding RNA H19 gene is associated with the risk of hypertrophic cardiomyopathy. Epigenomics, 10(7), 865-873.

Gore-Panter, S. R., Hsu, J., Barnard, J., Moravec, C. S., Van Wagoner, D. R., Chung, M. K., and Smith, J. D. (2016). PANCR, the PITX2 adjacent noncoding RNA, is expressed in human left atria and regulates PITX2c expression. Circulation: Arrhythmia and Electrophysiology, 9(1), e003197.

Greulich, F., Rudat, C., and Kispert, A. (2011). Mechanisms of T-box gene function in the developing heart. Cardiovascular research, 91(2), 212-222.

Grote, P., Wittler, L., Hendrix, D., Koch, F., Währisch, S., Beisaw, A., Macura, K., Bläss, G., Kellis, M., Werber, M., and Herrmann B. G (2013). The tissue-specific lncRNA Fendrr is an essential regulator of heart and body wall development in the mouse. Developmental cell, 24(2), 206-214.

Gudbjartsson, D. F., Arnar, D. O., Helgadottir, A., Gretarsdottir, S., Holm, H., Sigurdsson, A., Jonasdottir, A., Baker, A., Thorleifsson, G., Kristjansson, K., Palsson, A., Blondal, T., Sulem, P., Backman, V. M., Hardarson, G. A., Palsdottir, E., Helgason, A., Sigurjonsdottir, R., Sverrisson, J. T., Kostulas, K., Ng. M. C., Baum, L., So, W. Y., Wong, K. S., Chan, J. C., Furie, K. L., Greenberg, S. M., Sale ,M., Kelly, P., MacRae, C. A., Smith, E. E., Rosand, J., Hillert, J., Ma, R. C., Ellinor, P. T., Thorgeirsson, G., Gulcher, J. R., Kong, A., Thorsteinsdottir, U., and Stefansson, K. (2007). Variants conferring risk of atrial fibrillation on chromosome 4q25. Nature, 448(7151), 353.

Guo, Y., Luo, F., Liu, Q., and Xu, D. (2017). Regulatory non-coding RNAs in acute myocardial infarction. Journal of cellular and molecular medicine, 21(5), 1013-1023.

Gupta, R. A., Shah, N., Wang, K. C., Kim, J., Horlings, H. M., Wong, D. J., Tsai, M., Hung, T., Argani, P., Rinn, J. L., Wang, Y., Brzoska, P., Kong, B., Li, R., West, R. B., van de Vijver, M. J., Sukumar, S., and Chang, H. Y. (2010). Long non-coding RNA HOTAIR reprograms chromatin state to promote cancer metastasis. Nature, 464(7291), 1071.

Habets, P. E., Moorman, A. F., Clout, D. E., van Roon, M. A., Lingbeek, M., van Lohuizen, M., Campione, M., and Christoffels, V. M. (2002). Cooperative action of Tbx2 and Nkx2. 5 inhibits ANF expression in the atrioventricular canal: implications for cardiac chamber formation. Genes and development, 16(10), 1234-1246.

Hadji, F., Boulanger, M. C., Guay, S. P., Gaudreault, N., Amellah, S., Mkannez, G., ... and Pibarot, P. (2016). Altered DNA methylation of long noncoding RNA H19 in calcific aortic valve disease promotes mineralization by silencing NOTCH1. Circulation, 134(23), 1848-1862.

Haemmig, S., Simion, V., Yang, D., Deng, Y., & Feinberg, M. W. (2017). Long noncoding RNAs in cardiovascular disease, diagnosis, and therapy. Current opinion in cardiology, 32(6), 776-783.

Han, P., Li, W., Lin, C. H., Yang, J., Shang, C., Nurnberg, S. T., Jin, K. K., Xu, W., Lin, C. Y., Lin, C. J., Xiong, Y., Chien, H., Zhou, B., Ashley, E., Bernstein, D., Chen, P. S., Chen, H. V., Quertermous, T., and Chang, C. P. (2014). A long noncoding RNA protects the heart from pathological hypertrophy. Nature, 514(7520), 102.

Hasegawa, Y., Brockdorff, N., Kawano, S., Tsutui, K., Tsutui, K., and Nakagawa, S. (2010). The matrix protein hnRNP U is required for chromosomal localization of Xist RNA. Developmental cell, 19(3), 469-476.

Hermans-Beijnsberger, S., van Bilsen, M., and Schroen, B. (2018). Long non-coding RNAs in the failing heart and vasculature. Non-coding RNA research, 3(3), 118-130.

Hoogaars, W. M., Engel, A., Brons, J. F., Verkerk, A. O., de Lange, F. J., Wong, L. E., Bakker, M. L., Clout, D. E., Wakker, V., Barnett, P., Ravesloot, J. H., Moorman, A. F., Verheijck E. E., Christoffels, V. M.(2007). Tbx3 controls the sinoatrial node gene program and imposes pacemaker function on the atria. Genes and development, 21(9), 1098-1112.

Hoppler, S. P., and Moon, R. T. (2014). Wnt signaling in development and disease: molecular mechanisms and biological functions. John Wiley and Sons.

Hori, Y., Tanimoto, Y., Takahashi, S., Furukawa, T., Koshiba-Takeuchi, K., and Takeuchi, J. K. (2018). Important cardiac transcription factor genes are

accompanied by bidirectional long non-coding RNAs. BMC genomics, 19(1), 967.

Horsthuis, T., Houweling, A. C., Habets, P. E., de Lange, F. J., el Azzouzi, H., Clout, D. E., ... & Christoffels, V. M. (2008). Distinct Regulation of Developmental and Heart Disease–Induced Atrial Natriuretic Factor Expression by Two Separate Distal Sequences. Circulation research, 102(7), 849-859.

Hotta, Y., Sasaki, S., Konishi, M., Kinoshita, H., Kuwahara, K., Nakao, K., and Itoh, N. (2008). Fgf16 is required for cardiomyocyte proliferation in the mouse embryonic heart. Developmental Dynamics, 237(10), 2947-2954.

Hou, J., Long, H., Zhou, C., Zheng, S., Wu, H., Guo, T., ... and Wang, T. (2017). Long noncoding RNA Braveheart promotes cardiogenic differentiation of mesenchymal stem cells in vitro. Stem cell research and therapy, 8(1), 4.

Hou, J., Long, H., Zhou, C., Zheng, S., Wu, H., Guo, T., Wu, Q., Zhong, T., and Wang, T. (2017). Long noncoding RNA Braveheart promotes cardiogenic differentiation of mesenchymal stem cells in vitro. Stem cell research & therapy, 8(1), 4.

Huang, T., Liu, Y., Huang, M., Zhao, X., and Cheng, L. (2010). Wnt1-cre-mediated conditional loss of Dicer results in malformation of the midbrain and cerebellum and failure of neural crest and dopaminergic differentiation in mice. Journal of molecular cell biology, 2(3), 152-163.

Huang, Z. P., Chen, J. F., Regan, J. N., Maguire, C. T., Tang, R. H., Dong, X. R., Majesky, M. W., and Wang, D. Z (2010). Loss of microRNAs in neural crest leads to cardiovascular syndromes resembling human congenital heart defects. Arteriosclerosis, thrombosis, and vascular biology, 30(12), 2575-2586.

Huang, Z. W., Tian, L. H., Yang, B., and Guo, R. M. (2017). Long noncoding RNA H19 acts as a competing endogenous RNA to mediate CTGF expression by sponging miR-455 in cardiac fibrosis. DNA and cell biology, 36(9), 759-766.

Hutson, M. R., and Kirby, M. L. (2007). Model systems for the study of heart development and disease: cardiac neural crest and conotruncal malformations. In Seminars in cell and developmental biology (Vol. 18, No. 1, pp. 101-110). Academic Press.

Ilagan, R., Abu-Issa, R., Brown, D., Yang, Y. P., Jiao, K., Schwartz, R. J., ... and Meyers, E. N. (2006). Fgf8 is required for anterior heart field development. Development, 133(12), 2435-2445.

Ip, J. Y., Sone, M., Nashiki, C., Pan, Q., Kitaichi, K., Yanaka, K., ... & Nakagawa, S. (2016). Gomafu lncRNA knockout mice exhibit mild hyperactivity with enhanced responsiveness to the psychostimulant methamphetamine. Scientific reports, 6, 27204.

Itoh, N., Ohta, H., Nakayama, Y., and Konishi, M. (2016). Roles of FGF signals in heart development, health, and disease. Frontiers in cell and developmental biology, 4, 110.

Jiao, K., Kulessa, H., Tompkins, K., Zhou, Y., Batts, L., Baldwin, H. S., and Hogan, B. L. (2003). An essential role of Bmp4 in the atrioventricular septation of the mouse heart. Genes and development, 17(19), 2362-2367.

Johnsson, P., Lipovich, L., Grandér, D., and Morris, K. V. (2014). Evolutionary conservation of long non-coding RNAs; sequence, structure, function. Biochimica et Biophysica Acta (BBA)-General Subjects, 1840(3), 1063-1071.

Kang, J. O., and Sucov, H. M. (2005). Convergent proliferative response and divergent morphogenic pathways induced by epicardial and endocardial signaling in fetal heart development. Mechanisms of development, 122(1), 57-65.

Katz, M. G., Fargnoli, A. S., Kendle, A. P., Hajjar, R. J., and Bridges, C. R. (2015). The role of microRNAs in cardiac development and regenerative capacity. American Journal of Physiology-Heart and Circulatory Physiology, 310(5), H528-H541.

Kelly, R. G., Buckingham, M. E., and Moorman, A. F. (2014). Heart fields and cardiac morphogenesis. Cold Spring Harbor perspectives in medicine, 4(10), a015750.

Keniry, A., Oxley, D., Monnier, P., Kyba, M., Dandolo, L., Smits, G., and Reik, W. (2012). The H19 lincRNA is a developmental reservoir of miR-675 that suppresses growth and Igf1r. Nature cell biology, 14(7), 659.

Keyte, A., and Hutson, M. R. (2012). The neural crest in cardiac congenital anomalies. Differentiation, 84(1), 25-40.

Kim, R. Y., Robertson, E. J., and Solloway, M. J. (2001). Bmp6 and Bmp7 are required for cushion formation and septation in the developing mouse heart. Developmental biology, 235(2), 449-466.

Kim, Y. K., Furic, L., DesGroseillers, L., and Maquat, L. E. (2005). Mammalian Staufen1 recruits Upf1 to specific mRNA 3′ UTRs so as to elicit mRNA decay. Cell, 120(2), 195-208.

Kim, Y. K., Furic, L., Parisien, M., Major, F., DesGroseillers, L., and Maquat, L. E. (2007). Staufen1 regulates diverse classes of mammalian transcripts. The EMBO journal, 26(11), 2670-2681.

Kirchhof, P., Kahr, P. C., Kaese, S., Piccini, I., Vokshi, I., Scheld, H. H., Rotering, H., Fortmueller, L., Laakmann, S., Verheule, S., Schotten, U., Fabritz, L. and Brown N. A. (2011). PITX2c is expressed in the adult left atrium, and reducing Pitx2c expression promotes atrial fibrillation inducibility and complex changes in gene expression. Circulation: Cardiovascular Genetics, 4(2), 123-133.

Kitajima, S., Takagi, A., Inoue, T., and Saga, Y. (2000). MesP1 and MesP2 are essential for the development of cardiac mesoderm. Development, 127(15), 3215-3226.

Klattenhoff, C. A., Scheuermann, J. C., Surface, L. E., Bradley, R. K., Fields, P. A., Steinhauser, M. L., ... and Abo, R. (2013). Braveheart, a long noncoding RNA required for cardiovascular lineage commitment. Cell, 152(3), 570-583.

Koenig, S. N., Bosse, K., Majumdar, U., Bonachea, E. M., Radtke, F., & Garg, V. (2016). Endothelial Notch1 is required for proper development of the semilunar valves and cardiac outflow tract. Journal of the American Heart Association, 5(4), e003075.

Kuo, C. T., Morrisey, E. E., Anandappa, R., Sigrist, K., Lu, M. M., Parmacek, M.S., Soundais, C., and Leiden, J. M. (1997). GATA4 transcription factor is required for ventral morphogenesis and heart tube formation. Genes and development, 11(8), 1048-1060.

Kuo, H., Chen, J., Ruiz-Lozano, P., Zou, Y., Nemer, M., and Chien, K. R. (1999). Control of segmental expression of the cardiac-restricted ankyrin repeat protein gene by distinct regulatory pathways in murine cardiogenesis. Development, 126(19), 4223-4234.

Kurian, L., Aguirre, A., Sancho-Martinez, I., Benner, C., Hishida, T., Nguyen, T. B., Reddy, P., Nivet, E., Krause, M. N., Nelles, D. A., Esteban, C. R., Campistol, J. M., Yeo G. W., and Belmonte, J. C. (2015). Identification of novel long noncoding RNAs underlying vertebrate cardiovascular development. Circulation, 131(14), 1278-1290.

Kurukuti, S., Tiwari, V. K., Tavoosidana, G., Pugacheva, E., Murrell, A., Zhao, Z., Lobanenkov, V., Reik, W., and Ohlsson, R. (2006). CTCF binding at the H19 imprinting control region mediates maternally inherited higher-order chromatin conformation to restrict enhancer access to Igf2. Proceedings of the national academy of sciences, 103(28), 10684-10689.

Kurukuti, S., Tiwari, V. K., Tavoosidana, G., Pugacheva, E., Murrell, A., Zhao, Z., ... & Ohlsson, R. (2006). CTCF binding at the H19 imprinting control region mediates maternally inherited higher-order chromatin conformation to restrict enhancer access to Igf2. Proceedings of the national academy of sciences, 103(28), 10684-10689.

Landthaler, M., Yalcin, A., and Tuschl, T. (2004). The human DiGeorge syndrome critical region gene 8 and its D. melanogaster homolog are required for miRNA biogenesis. Current biology, 14(23), 2162-2167.

Lavine, K. J., Yu, K., White, A. C., Zhang, X., Smith, C., Partanen, J., and Ornitz, D. M. (2005). Endocardial and epicardial derived FGF signals regulate myocardial proliferation and differentiation in vivo. Developmental cell, 8(1), 85-95.

Leighton, P. A., Ingram, R. S., Eggenschwiler, J., Efstratiadis, A., and Tilghman, S. M. (1995). Disruption of imprinting caused by deletion of the H19 gene region in mice. Nature, 375(6526), 34.

Lerman, D. A., Prasad, S., and Alotti, N. (2015). Calcific aortic valve disease: molecular mechanisms and therapeutic approaches. European cardiology, 10(2), 108.

Lescroart, F., Chabab, S., Lin, X., Rulands, S., Paulissen, C., Rodolosse, A., ... and Simons, B. D. (2014). Early lineage restriction in temporally distinct populations of Mesp1 progenitors during mammalian heart development. Nature cell biology, 16(9), 829.

Li, J., Johnson, J. A., & Su, H. (2018). Ubiquitin and ubiquitin-like proteins in cardiac disease and protection. Current drug targets, 19(9), 989-1002

Li, P., Cavallero, S., Gu, Y., Chen, T. H., Hughes, J., Hassan, A. B., Brüning, J. C., Pashmforoush, M., and Sucov H. M. (2011). IGF signaling directs ventricular cardiomyocyte proliferation during embryonic heart development. Development, 138(9), 1795-1805.

Li, Y., Wang, J., Sun, L., and Zhu, S. (2018). LncRNA myocardial infarction-associated transcript (MIAT) contributed to cardiac hypertrophy by regulating TLR4 via miR-93. *European journal of pharmacology*, *818*, 508-517.

Li, Z., Wang, X., Wang, W., Du, J., Wei, J., Zhang, Y., ... and Hou, Y. (2017). Altered long non-coding RNA expression profile in rabbit atria with atrial fibrillation: TCONS_00075467 modulates atrial electrical remodeling by sponging miR-328 to regulate CACNA1C. Journal of molecular and cellular cardiology, 108, 73-85.

Lin, C. J., Lin, C. Y., Chen, C. H., Zhou, B., and Chang, C. P. (2012). Partitioning the heart: mechanisms of cardiac septation and valve development. Development, 139(18), 3277-3299.

Lin, Q., Lu, J., Yanagisawa, H., Webb, R., Lyons, G. E., Richardson, J. A., and Olson, E. N. (1998). Requirement of the MADS-box transcription factor MEF2C for vascular development. Development, 125(22), 4565-4574.

Lin, Q., Schwarz, J., Bucana, C., and Olson, E. N. (1997). Control of mouse cardiac morphogenesis and myogenesis by transcription factor MEF2C. Science, 276(5317), 1404-1407.

Lints, T. J., Parsons, L. M., Hartley, L., Lyons, I., and Harvey, R. P. (1993). Nkx-2.5: a novel murine homeobox gene expressed in early heart progenitor cells and their myogenic descendants. Development, 119(2), 419-431.

Liu, C., Liu, W., Palie, J., Lu, M. F., Brown, N. A., and Martin, J. F. (2002). Pitx2c patterns anterior myocardium and aortic arch vessels and is required for local cell movement into atrioventricular cushions. Development, 129(21), 5081-5091.

Liu, L., An, X., Li, Z., Song, Y., Li, L., Zuo, S., ... and Zhang, Y. (2016). The H19 long noncoding RNA is a novel negative regulator of cardiomyocyte hypertrophy. Cardiovascular research, 111(1), 56-65.

Liu, N., Bezprozvannaya, S., Williams, A. H., Qi, X., Richardson, J. A., Bassel-Duby, R., & Olson, E. N. (2008). microRNA-133a regulates cardiomyocyte proliferation and suppresses smooth muscle gene expression in the heart. Genes & development, 22(23), 3242-3254.

Liu, W., Selever, J., Wang, D., Lu, M. F., Moses, K. A., Schwartz, R. J., and Martin, J. F. (2004). Bmp4 signaling is required for outflow-tract septation and branchial-arch artery remodeling. Proceedings of the National Academy of Sciences, 101(13), 4489-4494.

Lozano-Velasco, E., Hernandez-Torres, F., Daimi, H., Serra, S., Herraiz, A., Hove-Madsen, L., Aránega, A. E., and Franco, D. (2015). Pitx2 impairs calcium handling in a dose-dependent manner by modulating Wnt signalling. Cardiovascular research, 109(1), 55-66.

Lozano-Velasco, E., Wangensteen, R., Quesada, A., Garcia-Padilla, C., Osorio, J. A., Ruiz-Torres, M. D., ... & Franco, D. (2017). Hyperthyroidism, but not hypertension, impairs PITX2 expression leading to Wnt-microRNA-ion channel remodeling. PloS one, 12(12), e0188473.

Lu, S. Y., Jin, Y., Li, X., Sheppard, P., Bock, M. E., Sheikh, F., ... and Cattini, P. A. (2010). Embryonic survival and severity of cardiac and craniofacial defects are affected by genetic background in fibroblast growth factor-16 null mice. DNA and cell biology, 29(8), 407-415.

Lu, S. Y., Sheikh, F., Sheppard, P. C., Fresnoza, A., Duckworth, M. L., Detillieux, K. A., and Cattini, P. A. (2008). FGF-16 is required for embryonic heart development. Biochemical and biophysical research communications, 373(2), 270-274.

Luna-Zurita, L., Prados, B., Grego-Bessa, J., Luxán, G., del Monte, G., Benguría, A., ... and de la Pompa, J. L. (2010). Integration of a Notch-dependent mesenchymal gene program and Bmp2-driven cell invasiveness regulates murine cardiac valve formation. The Journal of clinical investigation, 120(10), 3493-3507.

Lyons, I., Parsons, L. M., Hartley, L., Li, R., Andrews, J. E., Robb, L., and Harvey, R. P. (1995). Myogenic and morphogenetic defects in the heart tubes of murine embryos lacking the homeo box gene Nkx2-5. Genes and development, 9(13), 1654-1666.

MacGrogan, D., Luxán, G., Driessen-Mol, A., Bouten, C., Baaijens, F., and de la Pompa, J. L. (2014). How to make a heart valve: from embryonic development to bioengineering of living valve substitutes. Cold Spring Harbor perspectives in medicine, 4(11), a013912.

Manso, A. M., Li, R., Monkley, S. J., Cruz, N. M., Ong, S., Lao, D. H., ... & Abel, E. D. (2013). Talin1 has unique expression versus talin 2 in the heart and modifies the hypertrophic response to pressure overload. Journal of Biological Chemistry, 288(6), 4252-4264.

Mazzotta, S., Neves, C., Bonner, R. J., Bernardo, A. S., Docherty, K., and Hoppler, S. (2016). Distinctive roles of canonical and noncanonical Wnt signaling in human embryonic cardiomyocyte development. Stem cell reports, 7(4), 764-776.

Mazzotta, S., Neves, C., Bonner, R. J., Bernardo, A. S., Docherty, K., & Hoppler, S. (2016). Distinctive roles of canonical and noncanonical Wnt signaling in human embryonic cardiomyocyte development. Stem cell reports, 7(4), 764-776.

McFadden, D. G., Barbosa, A. C., Richardson, J. A., Schneider, M. D., Srivastava, D., and Olson, E. N. (2005). The Hand1 and Hand2 transcription factors regulate expansion of the embryonic cardiac ventricles in a gene dosage-dependent manner. Development, 132(1), 189-201.

McFadden, D. G., Charité, J., Richardson, J. A., Srivastava, D., Firulli, A. B., and Olson, E. N. (2000). A GATA-dependent right ventricular enhancer controls dHAND transcription in the developing heart. Development, 127(24), 5331-5341.

Meder, B., Katus, H. A., & Rottbauer, W. (2008). Right into the heart of microRNA-133a. Genes & development, 22(23), 3227-3231.

Meganathan, K., Sotiriadou, I., Natarajan, K., Hescheler, J., and Sachinidis, A. (2015). Signaling molecules, transcription growth factors and other regulators revealed from in-vivo and in-vitro models for the regulation of cardiac development. International journal of cardiology, 183, 117-128.

Mei, B., Liu, H., Yang, S., Liang, M. Y., Yue, Y., Huang, S. Q., ... and Wu, Z. K. (2018). Long non-coding RNA expression profile in permanent atrial fibrillation patients with rheumatic heart disease. European review for medical and pharmacological sciences, 22(20), 6940-6947.

Mendell, J. T. (2008). miRiad roles for the miR-17-92 cluster in development and disease. Cell, 133(2), 217-222.

Mesbah, K., Rana, M. S., Francou, A., Van Duijvenboden, K., Papaioannou, V. E., Moorman, A. F., Kelly, R., and Christoffels, V. M. (2011). Identification of a Tbx1/Tbx2/Tbx3 genetic pathway governing pharyngeal and arterial pole morphogenesis. Human molecular genetics, 21(6), 1217-1229.

Micheletti, R., Plaisance, I., Abraham, B. J., Sarre, A., Ting, C. C., Alexanian, M., ... and Schroen, B. (2017). The long noncoding RNA Wisper controls cardiac fibrosis and remodeling. Science translational medicine, 9(395), eaai9118.

Mommersteeg, M. T., Brown, N. A., Prall, O. W., de Gier-de Vries, C., Harvey, R. P., Moorman, A. F., and Christoffels, V. M. (2007). Pitx2c and Nkx2-5 are required for the formation and identity of the pulmonary myocardium. Circulation research, 101(9), 902-909.

Mommersteeg, M. T., Hoogaars, W. M., Prall, O. W., de Gier-de Vries, C., Wiese, C., Clout, D. E., Papaionnou, V.E., Brown, N.A., Harvey, R.P., Moorman, A.F., and Christoffels, V. M. (2007). Molecular pathway for the localized formation of the sinoatrial node. Circulation research, 100(3), 354-362.

Mousavi, K., Zare, H., Dell'Orso, S., Grontved, L., Gutierrez-Cruz, G., Derfoul, A., Hager, G. L., Sartorelli, V. (2013). eRNAs promote transcription by establishing chromatin accessibility at defined genomic loci. Molecular cell, 51(5), 606-617.

Namigai, E. K., Kenny, N. J., and Shimeld, S. M. (2014). Right across the tree of life: the evolution of left–right asymmetry in the Bilateria. Genesis, 52(6), 458-470.

Namigai, E. K., Kenny, N. J., and Shimeld, S. M. (2014). Right across the tree of life: the evolution of left–right asymmetry in the Bilateria. Genesis, 52(6), 458-470.

Nelson, B. R., Makarewich, C. A., Anderson, D. M., Winders, B. R., Troupes, C. D., Wu, F., Reese, A. L., McAnally, J. R., Chen, X., Kavalali, E. T., Cannon, S. C., Houser,

S. R., Bassel-Duby, R., and Olson, E. N. (2016). A peptide encoded by a transcript annotated as long noncoding RNA enhances SERCA activity in muscle. Science, 351(6270), 271-275.

Nemer, G., and Nemer, M. (2003). Transcriptional activation of BMP-4 and regulation of mammalian organogenesis by GATA-4 and-6. Developmental biology, 254(1), 131-148.

Niu, Z., Iyer, D., Conway, S. J., Martin, J. F., Ivey, K., Srivastava, D., Nordheim, A., and Schwartz, R. J. (2008). Serum response factor orchestrates nascent sarcomerogenesis and silences the biomineralization gene program in the heart. Proceedings of the National Academy of Sciences, 105(46), 17824-17829.

Nordin, M., Bergman, D., Halje, M., Engström, W., & Ward, A. (2014). Epigenetic regulation of the Igf2/H19 gene cluster. Cell proliferation, 47(3), 189-199.

Nordin, M., Bergman, D., Halje, M., Engström, W., and Ward, A. (2014). Epigenetic regulation of the Igf2/H19 gene cluster. Cell proliferation, 47(3), 189-199.

Onizuka, T., Yuasa, S., Kusumoto, D., Shimoji, K., Egashira, T., Ohno, Y., Kageyama, T., Tanaka, T., Hattori, F., Fujita, J., Ieda, M., Kimura, K., Makino, S., Sano, M., Kudo, A., and Fukuda K. (2012). Wnt2 accelerates cardiac myocyte differentiation from ES-cell derived mesodermal cells via non-canonical pathway. Journal of molecular and cellular cardiology, 52(3), 650-659.

Ounzain, S., and Pedrazzini, T. (2016). Long non-coding RNAs in heart failure: a promising future with much to learn. Annals of translational medicine, 4(15).

Ounzain, S., Micheletti, R., Arnan, C., Plaisance, I., Cecchi, D., Schroen, B., Reverter, F., Alexanian, M., Gonzales, C., Ng, S.Y., Bussotti, G., Pezzuto, I., Notredame, C., Heymans, S., Guigó, R., Johnson, R., and Pedrazzini, T. (2015). CARMEN, a human super enhancer-associated long noncoding RNA controlling cardiac specification, differentiation and homeostasis. Journal of molecular and cellular cardiology, 89, 98-112.

Ounzain, S., Micheletti, R., Beckmann, T., Schroen, B., Alexanian, M., Pezzuto, I., Crippa, S., Nemir, M., Sarre, A., Johnson, R., Dauvillier, J., Burdet, F., Ibberson, M., Guigó, R., Xenarios, I., Heymans, S., and Pedrazzini, T (2014). Genome-wide

profiling of the cardiac transcriptome after myocardial infarction identifies novel heart-specific long non-coding RNAs. European heart journal, 36(6), 353-368.

Papaioannou, V. E. (2014). The T-box gene family: emerging roles in development, stem cells and cancer. Development, 141(20), 3819-3833.

Pashmforoush, M., Lu, J. T., Chen, H., St Amand, T., Kondo, R., Pradervand, S., Evans, S.M., Clark, B., Feramisco, J.R., Giles. W., Ho, S.Y., Benson, D.W., Silberbach, M., Shou, W., and Chien, K.R (2004). Nkx2-5 pathways and congenital heart disease: loss of ventricular myocyte lineage specification leads to progressive cardiomyopathy and complete heart block. Cell, 117(3), 373-386.

Peng, Y., Song, L., Zhao, M., Harmelink, C., Debenedittis, P., Cui, X., Wang, Q., and Jiao, K. (2014). Critical roles of miRNA-mediated regulation of TGFβ signalling during mouse cardiogenesis. Cardiovascular research, 103(2), 258-267.

Prall, O. W., Menon, M. K., Solloway, M. J., Watanabe, Y., Zaffran, S., Bajolle, F., Biben, C., MacBride, J.J., Robertson, B.R., Chaulet, H., Stennard, F.A., Wise, N., Schaft, D., Wolstein, O., Furtado, M.B., Shiratori, H., Chien, K.R., Hamada, H., Black, B.L., Saga, Y., Robertson, E.J., Buckingham, M.E., and Harvey, R.P. (2007). An Nkx2-5/Bmp2/Smad1 negative feedback loop controls heart progenitor specification and proliferation. Cell, 128(5), 947-959.

Pu, W. T., Ishiwata, T., Juraszek, A. L., Ma, Q., and Izumo, S. (2004). GATA4 is a dosage-sensitive regulator of cardiac morphogenesis. Developmental biology, 275(1), 235-244.

Qu, X., Du, Y., Shu, Y., Gao, M., Sun, F., Luo, S., ... and Pan, Z. (2017). MIAT is a pro-fibrotic long non-coding RNA governing cardiac fibrosis in post-infarct myocardium. Scientific reports, 7, 42657.

Rackham, O., Shearwood, A. M. J., Mercer, T. R., Davies, S. M., Mattick, J. S., and Filipovska, A. (2011). Long noncoding RNAs are generated from the mitochondrial genome and regulated by nuclear-encoded proteins. Rna, 17(12), 2085-2093.

Rajewsky, N. (2006). microRNA target predictions in animals. Nature genetics, 38(6s), S8.

Rana, M. S., Christoffels, V. M., and Moorman, A. F. M. (2013). A molecular and genetic outline of cardiac morphogenesis. Acta physiologica, 207(4), 588-615.

Riley, P., Anaon-Cartwight, L., and Cross, J. C. (1998). The Hand1 bHLH transcription factor is essential for placentation and cardiac morphogenesis. Nature genetics, 18(3), 271.

Ripoche, M. A., Kress, C., Poirier, F., and Dandolo, L. (1997). Deletion of the H19 transcription unit reveals the existence of a putative imprinting control element. Genes & Development, 11(12), 1596-1604.

Risebro, C. A., Smart, N., Dupays, L., Breckenridge, R., Mohun, T. J., and Riley, P. R. (2006). Hand1 regulates cardiomyocyte proliferation versus differentiation in the developing heart. Development, 133(22), 4595-4606.

Rojas, A., De Val, S., Heidt, A. B., Xu, S. M., Bristow, J., and Black, B. L. (2005). Gata4 expression in lateral mesoderm is downstream of BMP4 and is activated directly by Forkhead and GATA transcription factors through a distal enhancer element. Development, 132(15), 3405-3417.

Rosa, A., and Ballarino, M. (2016). Long noncoding RNA regulation of pluripotency. Stem cells international,

Ruan, Z., Sun, X., Sheng, H., and Zhu, L. (2015). Long non-coding RNA expression profile in atrial fibrillation. International journal of clinical and experimental pathology, 8(7), 8402.

Saga, Y., Kitajima, S., and Miyagawa-Tomita, S. (2000). Mesp1 expression is the earliest sign of cardiovascular development. Trends in cardiovascular medicine, 10(8), 345-352.

Sallam, T., Sandhu, J., & Tontonoz, P. (2018). Long noncoding RNA discovery in cardiovascular disease: decoding form to function. Circulation research, 122(1), 155-166.

Saxena, A., and Tabin, C. J. (2010). miRNA-processing enzyme Dicer is necessary for cardiac outflow tract alignment and chamber septation. Proceedings of the National Academy of Sciences, 107(1), 87-91.

Schmitz, S. U., Grote, P., and Herrmann, B. G. (2016). Mechanisms of long noncoding RNA function in development and disease. Cellular and molecular life sciences, 73(13), 2491-2509.

Schwartz, R. J. (2010). Serum response factor and co-factors, roles in cardiac development. In Heart Development and Regeneration (pp. 617-649). Academic Press.

Schweickert, A., Campione, M., Steinbeisser, H., and Blum, M. (2000). Pitx2 isoforms: involvement of Pitx2c but not Pitx2a or Pitx2b in vertebrate left–right asymmetry. Mechanisms of development, 90(1), 41-51.

Schweickert, A., Campione, M., Steinbeisser, H., and Blum, M. (2000). Pitx2 isoforms: involvement of Pitx2c but not Pitx2a or Pitx2b in vertebrate left–right asymmetry. Mechanisms of development, 90(1), 41-51.

Sepulveda, J. L., Belaguli, N., Nigam, V., Chen, C. Y., Nemer, M., and Schwartz, R. J. (1998). GATA-4 and Nkx-2.5 coactivate Nkx-2 DNA binding targets: role for regulating early cardiac gene expression. Molecular and cellular biology, 18(6), 3405-3415.

Sepulveda, J. L., Vlahopoulos, S., Iyer, D., Belaguli, N., and Schwartz, R. J. (2002). Combinatorial expression of GATA4, Nkx2-5, and serum response factor directs early cardiac gene activity. Journal of Biological Chemistry, 277(28), 25775-25782.

Shen, H., Cavallero, S., Estrada, K. D., Sandovici, I., Kumar, S. R., Makita, T., Lien, C. L., Constancia, M., and Sucov, H. M. (2015). Extracardiac control of embryonic cardiomyocyte proliferation and ventricular wall expansion. Cardiovascular research, 105(3), 271-278.

Shen, X., Soibam, B., Benham, A., Xu, X., Chopra, M., Peng, X., Yu, W., Bao, W., Liang, R., Azares, A., Liu, P., Gunaratne, P. H., Mercola, M., Cooney, A. J., Schwartz, R. J., and Liu, Y. (2016). miR-322/-503 cluster is expressed in the earliest cardiac progenitor cells and drives cardiomyocyte specification. Proceedings of the National Academy of Sciences, 113(34), 9551-9556.

Shirai, M., Imanaka-Yoshida, K., Schneider, M. D., Schwartz, R. J., and Morisaki, T. (2009). T-box 2, a mediator of Bmp-Smad signaling, induced hyaluronan synthase

2 and Tgfβ2 expression and endocardial cushion formation. Proceedings of the National Academy of Sciences, 106(44), 18604-18609.

Shu, Q., Lai, S., Wang, X. M., Zhang, Y. L., Yang, X. L., Bi, H. L., & Li, H. H. (2018). Administration of ubiquitin-activating enzyme UBA1 inhibitor PYR-41 attenuates angiotensin II-induced cardiac remodeling in mice. Biochemical and biophysical research communications, 505(1), 317-324.

Solloway, M. J., and Robertson, E. J. (1999). Early embryonic lethality in Bmp5; Bmp7 double mutant mice suggests functional redundancy within the 60A subgroup. Development, 126(8), 1753-1768.

Song, G., Shen, Y., Ruan, Z., Li, X., Chen, Y., Yuan, W., Ding, X., Zhu, L., Qian, L. (2016). LncRNA-uc. 167 influences cell proliferation, apoptosis and differentiation of P19 cells by regulating Mef2c. Gene, 590(1), 97-108.

Song, G., Shen, Y., Zhu, J., Liu, H., Liu, M., Shen, Y. Q,. Zhu, S., Kong, X., Yu, Z., and Qian, L. (2013). Integrated analysis of dysregulated lncRNA expression in fetal cardiac tissues with ventricular septal defect. PloS one, 8(10), e77492.

Spéder, P., Petzoldt, A., Suzanne, M., and Noselli, S. (2007). Strategies to establish left/right asymmetry in vertebrates and invertebrates. Current opinion in genetics and development, 17(4), 351-358.

Spéder, P., Petzoldt, A., Suzanne, M., and Noselli, S. (2007). Strategies to establish left/right asymmetry in vertebrates and invertebrates. Current opinion in genetics and development, 17(4), 351-358.

Stanley, E. G., Biben, C., Elefanty, A., Barnett, L., Koentgen, F., Robb, L., and Harvey, R. P. (2004). Efficient Cre-mediated deletion in cardiac progenitor cells conferred by a 3'UTR-ires-Cre allele of the homeobox gene Nkx2-5. International Journal of Developmental Biology, 46(4), 431-439.

Stennard, F. A., Costa, M. W., Lai, D., Biben, C., Furtado, M. B., Solloway, M. J., Elliott, D. E., Prall, O. W., Black, B. L., Fatkin, D., and Harvey, R. P (2005). Murine T-box transcription factor Tbx20 acts as a repressor during heart development, and is essential for adult heart integrity, function and adaptation. Development, 132(10), 2451-2462.

Sugi, Y., Yamamura, H., Okagawa, H., and Markwald, R. R. (2004). Bone morphogenetic protein-2 can mediate myocardial regulation of atrioventricular cushion mesenchymal cell formation in mice. Developmental biology, 269(2), 505-518.

Sun, X., Meyers, E. N., Lewandoski, M., and Martin, G. R. (1999). Targeted disruption of Fgf8 causes failure of cell migration in the gastrulating mouse embryo. Genes and development, 13(14), 1834-1846.

Tanaka, M., Wechsler, S. B., Lee, I. W., Yamasaki, N., Lawitts, J. A., and Izumo, S. (1999). Complex modular cis-acting elements regulate expression of the cardiac specifying homeobox gene Csx/Nkx2. 5. Development, 126(7), 1439-1450.

Tian, Y., Cohen, E. D., and Morrisey, E. E. (2010). The importance of Wnt signaling in cardiovascular development. Pediatric cardiology, 31(3), 342-348.

Tripathi, V., Ellis, J. D., Shen, Z., Song, D. Y., Pan, Q., Watt, A. T., Freier, S. M., Bennett, C. F., Sharma, A., Bubulya, P. A., Blencowe, B. J., Prasanth, S. G., and Prasanth, K. V. (2010). The nuclear-retained noncoding RNA MALAT1 regulates alternative splicing by modulating SR splicing factor phosphorylation. Molecular cell, 39(6), 925-938.

Tsai, M. C., Manor, O., Wan, Y., Mosammaparast, N., Wang, J. K., Lan, F., Shi, Y., Segal, E., Chang, H. Y. (2010). Long noncoding RNA as modular scaffold of histone modification complexes. Science, 329(5992), 689-693.

Ulitsky, I., and Bartel, D. P. (2013). lincRNAs: genomics, evolution, and mechanisms. Cell, 154(1), 26-46.

Vandenberg, L. N., and Levin, M. (2010). Far from solved: a perspective on what we know about early mechanisms of left–right asymmetry. Developmental Dynamics, 239(12), 3131-3146.

Vandenberg, L. N., and Levin, M. (2013). A unified model for left–right asymmetry? Comparison and synthesis of molecular models of embryonic laterality. Developmental biology, 379(1), 1-15.

Verzi, M. P., McCulley, D. J., De Val, S., Dodou, E., and Black, B. L. (2005). The right ventricle, outflow tract, and ventricular septum comprise a restricted expression

domain within the secondary/anterior heart field. Developmental biology, 287(1), 134-145.

Viereck, J., Kumarswamy, R., Foinquinos, A., Xiao, K., Avramopoulos, P., Kunz, M., ... and Just, A. (2016). Long noncoding RNA Chast promotes cardiac remodeling. Science translational medicine, 8(326), 326ra22-326ra22.

Vincentz, J. W., McWhirter, J. R., Murre, C., Baldini, A., and Furuta, Y. (2005). Fgf15 is required for proper morphogenesis of the mouse cardiac outflow tract. Genesis, 41(4), 192-201.

Vong, L. H., Ragusa, M. J., and Schwarz, J. J. (2005). Generation of conditional Mef2cloxP/loxP mice for temporal-and tissue-specific analyses. genesis, 43(1), 43-48.

Waldo, K. L., Kumiski, D. H., Wallis, K. T., Stadt, H. A., Hutson, M. R., Platt, D. H., and Kirby, M. L. (2001). Conotruncal myocardium arises from a secondary heart field. Development, 128(16), 3179-3188.

Wang, D. Z., Valdez, M. R., McAnally, J., Richardson, J., and Olson, E. N. (2001). The Mef2c gene is a direct transcriptional target of myogenic bHLH and MEF2 proteins during skeletal muscle development. Development, 128(22), 4623-4633.

Wang, H., Gilner, J. B., Bautch, V. L., Wang, D. Z., Wainwright, B. J., Kirby, S. L., & Patterson, C. (2007). Wnt2 coordinates the commitment of mesoderm to hematopoietic, endothelial, and cardiac lineages in embryoid bodies. Journal of Biological Chemistry, 282(1), 782-791.

Wang, H., Iacoangeli, A., Lin, D., Williams, K., Denman, R. B., Hellen, C. U., and Tiedge, H. (2005). Dendritic BC1 RNA in translational control mechanisms. The Journal of cell biology, 171(5), 811-821.

Wang, J., Greene, S. B., Bonilla-Claudio, M., Tao, Y., Zhang, J., Bai, Y., ... and Martin, J. F. (2010). Bmp signaling regulates myocardial differentiation from cardiac progenitors through a MicroRNA-mediated mechanism. Developmental cell, 19(6), 903-912.

Wang, J., Klysik, E., Sood, S., Johnson, R. L., Wehrens, X. H., and Martin, J. F. (2010). Pitx2 prevents susceptibility to atrial arrhythmias by inhibiting left-sided

pacemaker specification. Proceedings of the National Academy of Sciences, 107(21), 9753-9758.

Wang, K., Liu, F., Zhou, L. Y., Long, B., Yuan, S. M., Wang, Y., ... and Li, P. F. (2014). The long noncoding RNA CHRF regulates cardiac hypertrophy by targeting miR-489. Circulation research, 114(9), 1377-1388.

Wang, K., Shen, H., Gan, P., Cavallero, S., Kumar, S. R., Lien, C. L., and Sucov, H. M. (2019). Differential roles of insulin like growth factor 1 receptor and insulin receptor during embryonic heart development. BMC developmental biology, 19(1), 5.

Wang, Y., Wu, B., Farrar, E., Lui, W., Lu, P., Zhang, D., Alfieri, C. M., Mao, K., Chu, M., Yang, D., Xu, D., Rauchman, M., Taylor, V., Conway, S. J., Yutzey, K. E., Butcher, J. T., and Zhou, B. (2017). Notch-Tnf signalling is required for development and homeostasis of arterial valves. European heart journal, 38(9), 675-686.

Wang, Z., Zhang, X. J., Ji, Y. X., Zhang, P., Deng, K. Q., Gong, J., ... and Gao, C. (2016). The long noncoding RNA Chaer defines an epigenetic checkpoint in cardiac hypertrophy. Nature medicine, 22(10), 1131.

Wapinski, O., and Chang, H. Y. (2011). Long noncoding RNAs and human disease. Trends in cell biology, 21(6), 354-361.

Watanabe, Y., and Buckingham, M. (2010). Heart fields and myocardial lineages. Annals Of The New York Academy Of Sciences, 1188, 15-24.

Watanabe, Y., Miyagawa-Tomita, S., Vincent, S. D., Kelly, R. G., Moon, A. M., and Buckingham, M. E. (2010). Role of mesodermal FGF8 and FGF10 overlaps in the development of the arterial pole of the heart and pharyngeal arch arteries. Circulation research, 106(3), 495.

Watt, A. J., Battle, M. A., Li, J., and Duncan, S. A. (2004). GATA4 is essential for formation of the proepicardium and regulates cardiogenesis. Proceedings of the National Academy of Sciences, 101(34), 12573-12578.

Wei, J., Joshi, S., Speransky, S., Crowley, C., Jayathilaka, N., Lei, X., ... & Gao, Y. (2017). Reversal of pathological cardiac hypertrophy via the MEF2-coregulator interface. JCI insight, 2(17).

Welsh, I. C., Kwak, H., Chen, F. L., Werner, M., Shopland, L. S., Danko, C., Lis, J. T., Zhang, M., Martin, J. F., Kurpios, N. A. (2015). Chromatin architecture of the Pitx2 locus requires CTCF-and Pitx2-dependent asymmetry that mirrors embryonic gut laterality. Cell reports, 13(2), 337-349.

Winnier, G., Blessing, M., Labosky, P. A., and Hogan, B. L. (1995). Bone morphogenetic protein-4 is required for mesoderm formation and patterning in the mouse. Genes and development, 9(17), 2105-2116.

Wu, J., Han, D., Shi, R., Chen, M., Sun, J., Tian, H., & Yan, Y. (2019). Identification of atrial fibrillation-associated lncRNAs in atria from patients with rheumatic mitral valve disease. Microscopy research and technique.

Wu, S. P., Dong, X. R., Regan, J. N., Su, C., and Majesky, M. W. (2013). Tbx18 regulates development of the epicardium and coronary vessels. Developmental biology, 383(2), 307-320.

Xu, J., Gong, N. L., Bodi, I., Aronow, B. J., Backx, P. H., and Molkentin, J. D. (2006). Myocyte enhancer factors 2A and 2C induce dilated cardiomyopathy in transgenic mice. Journal of Biological Chemistry, 281(14), 9152-9162.

Xu, Y., and Shao, B. (2018). Circulating lncRNA IFNG-AS1 expression correlates with increased disease risk, higher disease severity and elevated inflammation in patients with coronary artery disease. Journal of clinical laboratory analysis, 32(7), e22452.

Yan, S., and Jiao, K. (2016). Functions of miRNAs during mammalian heart. Development. International journal of molecular sciences, 17(5), 789.

Yin, Q. F., Yang, L., Zhang, Y., Xiang, J. F., Wu, Y. W., Carmichael, G. G., and Chen, L. L. (2012). Long noncoding RNAs with snoRNA ends. Molecular cell, 48(2), 219-230.

Yoon, J. H., Abdelmohsen, K., Srikantan, S., Yang, X., Martindale, J. L., De, S., Huarte, M., Zhan, M., Becker, K. G., and Gorospe, M. (2012). LincRNA-p21 suppresses target mRNA translation. Molecular cell, 47(4), 648-655.

Yue, Y., Meng, K., Pu, Y., and Zhang, X. (2017). Transforming growth factor beta (TGF-β) mediates cardiac fibrosis and induces diabetic cardiomyopathy. Diabetes research and clinical practice, 133, 124-130.

Zeisberg, E. M., Ma, Q., Juraszek, A. L., Moses, K., Schwartz, R. J., Izumo, S., and Pu, W. T. (2005). Morphogenesis of the right ventricle requires myocardial expression of Gata4. The Journal of clinical investigation, 115(6), 1522-1531.

Zhang, H., and Bradley, A. (1996). Mice deficient for BMP2 are nonviable and have defects in amnion/chorion and cardiac development. Development, 122(10), 2977-2986.

Zhang, Y., Luo, G., Zhang, Y., Zhang, M., Zhou, J., Gao, W., ... and Ni, B. (2018). Critical effects of long non-coding RNA on fibrosis diseases. Experimental and molecular medicine, 50(1), e428.

Zhao, Y., Ransom, J. F., Li, A., Vedantham, V., von Drehle, M., Muth, A. N., Tsuchihashi, T., McManus, M. T., Schwartz, R. J., and Srivastava, D. (2007). Dysregulation of cardiogenesis, cardiac conduction, and cell cycle in mice lacking miRNA-1-2. Cell, 129(2), 303-317.

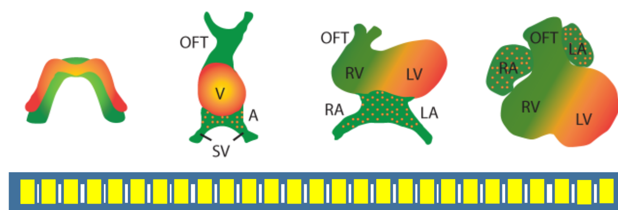
Zhao, Y., Samal, E., and Srivastava, D. (2005). Serum response factor regulates a muscle-specific microRNA that targets Hand2 during cardiogenesis. Nature, 436(7048), 214.

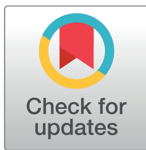
Zhu, D., Mackenzie, N. C., Millan, J. L., Farquharson, C., & MacRae, V. E. (2014). Upregulation of IGF2 expression during vascular calcification. Journal of molecular endocrinology, 52(2), 77.

Zhu, D., Mackenzie, N. C., Millan, J. L., Farquharson, C., and MacRae, V. E. (2014). Upregulation of IGF2 expression during vascular calcification. Journal of molecular endocrinology, 52(2), 77.

Zhu, X. H., Yuan, Y. X., Rao, S. L., and Wang, P. (2016). LncRNA MIAT enhances cardiac hypertrophy partly through sponging miR-150. Eur Rev Med Pharmacol Sci, 20(17), 3653.

ANEXOS





RESEARCH ARTICLE

Hyperthyroidism, but not hypertension, impairs PITX2 expression leading to Wnt-microRNA-ion channel remodeling

Estefanía Lozano-Velasco[1], Rosemary Wangensteen[2], Andrés Quesada[2], Carlos Garcia-Padilla[1], Julia A. Osorio[1], María Dolores Ruiz-Torres[1], Amelia Aranega[1], Diego Franco[1]*

1 Cardiac and Skeletal Muscle Development Group, Department of Experimental Biology, University of Jaen, Jaen, Spain, **2** Department of Health Sciences, University of Jaen, Jaen, Spain

* dfranco@ujaen.es

OPEN ACCESS

Citation: Lozano-Velasco E, Wangensteen R, Quesada A, Garcia-Padilla C, Osorio JA, Ruiz-Torres MD, et al. (2017) Hyperthyroidism, but not hypertension, impairs PITX2 expression leading to Wnt-microRNA-ion channel remodeling. PLoS ONE 12(12): e0188473. https://doi.org/10.1371/journal.pone.0188473

Editor: Johnson Rajasingh, University of Kansas Medical Center, UNITED STATES

Received: April 5, 2017

Accepted: November 7, 2017

Published: December 1, 2017

Copyright: © 2017 Lozano-Velasco et al. This is an open access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

Funding: This study was partially supported by grants BFU2012-38111, BFU2015-67131 (Ministerio de Economía y Competitividad, Gobierno de España), and CTS-1614 (Junta de Andalucia Regional Council) to DF and AA. This study was supported by the grant PI3/02384 from Carlos III Health Institute of Spain "Feder una

Abstract

PITX2 is a homeobox transcription factor involved in embryonic left/right signaling and more recently has been associated to cardiac arrhythmias. Genome wide association studies have pinpointed PITX2 as a major player underlying atrial fibrillation (AF). We have previously described that PITX2 expression is impaired in AF patients. Furthermore, distinct studies demonstrate that Pitx2 insufficiency leads to complex gene regulatory network remodeling, i.e. Wnt>microRNAs, leading to ion channel impairment and thus to arrhythmogenic events in mice. Whereas large body of evidences has been provided in recent years on PITX2 downstream signaling pathways, scarce information is available on upstream pathways influencing PITX2 in the context of AF. Multiple risk factors are associated to the onset of AF, such as e.g. hypertension (HTN), hyperthyroidism (HTD) and redox homeostasis impairment. In this study we have analyzed whether HTN, HTD and/or redox homeostasis impact on PITX2 and its downstream signaling pathways. Using rat models for spontaneous HTN (SHR) and experimentally-induced HTD we have observed that both cardiovascular risk factors lead to severe Pitx2 downregulation. Interesting HTD, but not SHR, leads to upregulation of Wnt signaling as well as deregulation of multiple microRNAs and ion channels as previously described in Pitx2 insufficiency models. In addition, redox signaling is impaired in HTD but not SHR, in line with similar findings in atrial-specific Pitx2 deficient mice. *In vitro* cell culture analyses using gain- and loss-of-function strategies demonstrate that Pitx2, Zfhx3 and Wnt signaling influence redox homeostasis in cardiomyocytes. Thus, redox homeostasis seems to play a pivotal role in this setting, providing a regulatory feedback loop. Overall these data demonstrate that HTD, but not HTN, can impair Pitx2>>Wnt pathway providing thus a molecular link to AF.

Introduction

Atrial fibrillation (AF) is the most frequent arrhythmogenic defect in the human population, with an estimate incidence of 2–4% in the general population but rising up to 10% in the elderly [1]. Genetic mutations in a large array of ion channel encoding genes have been

manera de hacer Europa" to RW. No additional external funding was received for this study. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing interests: The authors have declared that no competing interests exist.

described, although only representing <10% of AF cases [2–5]. Recently, genome wide association studies (GWAS) have identified a discrete number of risk variants linked to AF. In particular, SNPs located in chromosome region 4q25, thus in the vicinity of PITX2/ENPEP, display highest association significance [6], while other SNPs linked to ZFHX3 (16q22)[7–8], KCNN3 (1q21) [9] and IL6R (16q13) [10] display more modest significance. Functional evidences demonstrated that 4q25 genomic region containing these risk variants can interact with PITX2 and ENPEP promoter sequences [11]. However it remains elusive how variation within other SNPs (ZFHX3 (16q22), KCNN3 (1q21) and IL6R (16q13)) is mechanistically linked to AF.

Experimental analyses demonstrated that Pitx2 insufficiency leads to atrial arrhythmias [12–14] by modulating distinct ion channels that contribute to the configuration of the cardiac action potential [13–15], as well as cell-cell gap junctional and calcium handling proteins [13,16]. In addition, Pitx2 modulates expression of several GWAS associated genes, such as IL6R, KCNN3 and ZFHX3. Importantly it also regulates WNT8 expression which, in turn, modulates a complex gene regulatory network, including multiple microRNAs, with a large impact on calcium homeostasis control and pro-arrhythmogenic events [16].

It is well-established that the onset of an AF episode triggers subsequent and more severe AF episodes, leading to electrical and structural remodeling of the diseased heart, a condition quoted as "AF begets AF" [17]. Electrical remodeling involves progressive changes in the cardiac electrical properties, leading to early afterdepolarization, delayed afterdepolizations and/or changes in the action potential duration configuration [17], culminating thus in rotor formation [18]. In this context, a pivotal role of reactive oxidative stress has been recently reported [19–20]. Structural remodeling involves atrial dilation, fibrosis and/or inflammation [21] indirectly promoting rotor formation and thus electrical re-entry circuitries [18].

Cardiovascular risk factors such hypertension (HTN), hyperthyroidism (HTD), diabetes and obesity have been repetitively demonstrated to promote onset of atrial fibrillation, respectively [22–24]. Furthermore, the occurrence of AF can be also triggered by preceding cardiovascular diseases such as hypertrophic cardiomyopathy and valvular heart diseases [23,25–26]. Whereas large body of evidences has been provided in recent years on PITX2 downstream signaling pathways, scarce information is available on upstream pathways influencing PITX2 in the context of AF. A seminal study demonstrated that prolonged hypertension is capable of decreasing PITX2 expression [27] but the functional consequences of such changes remain unexplored. Furthermore, PITX2 has been recently reported to control redox homeostasis in skeletal muscle [28] as well as in the regenerating heart [29] but not linked to redox homeostasis and cardiac arrhythmogenic defects has been reported.

In this study we demonstrate that Pitx2 expression is impaired in HTN and HTD experimental models. Interestingly, HTD but not HTN elicits a complex impairment of PITX2>Wnt>microRNA signaling which leads to abnormal ion channel expression. Importantly, ROS signaling is also altered in Pitx2 deficient mice and impaired ROS signaling regulates Pitx2>Wnt>microRNA cascade. Overall, our data demonstrate a complex regulatory network of AF risk factors and PITX2 downstream signaling providing additional molecular insights linking pro-arrhythmogenic substrates and AF.

Materials & methods

Experimental models

The Pitx2^{floxed} and NppaCre transgenic mouse lines have been previously described [30–31]. Generation of conditional atrial (NppaCre) mutant mice has been previously described [14–15]. Three different conditions were used for the NppaCrePitx2 mice: wild-type Cre controls

(NppaCre2Pitx2$^{fl/fl}$), atrial-specific heterozygous (NppaCre+Pitx2$^{fl/-}$), and atrial-specific homozygous (NppaCre+Pitx2$^{-/-}$). This investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health. The study was approved by the University of Jaén Bioethics Committee.

Male Wistar rats weighing 250–280 g were maintained on standard chow and tap water *ad libitum*. Two groups of animals were analyzed; a) control and b) hyperthyroid rats as previously described [32]. Briefly, hyperthyroidism was induced by injecting s.c. thyroxine (75 Mg/rat/day) for four weeks. Spontaneously hypertensive rats (SHR) and their corresponding Wistar Kyoto controls (WKY) were purchased to Harlan Laboratories with 8 weeks old, and maintained on standard chow and tap water *ad libitum* during 24 weeks. Tail systolic BP (SBP) and heart rate (HR) were recorded once a month by using tail-cuff plethysmography in unanaesthetized rats (LE 5001-Pressure Meter, Letica SA, Barcelona, Spain) as illustrated in **S1 Fig**.

Mouse genotyping

DNA for PCR screening was extracted from adult ear and/or tail samples and from embryonic yolk sacs. Screening of Cre and Pitx2 floxed alleles was routinely done using used specific primers as previously described [16]. Cycling conditions for Cre were as follows; 5 min at 95˚C, 35 cycles of 30s at 95˚C, 30s at 60˚C and 90s at 72˚C, and for Pitx2 as follows; 5 min at 95˚C. 40 cycles of 30s at 95˚C, 30s at 60˚C and 90s at 72˚C, followed by a final extension step of 10 min at 72˚C, respectively.

Tissue and RNA isolation

When the experimental period was completed, rats were anaesthetized with thiobutabarbital (100 mg/kg IP, Inactin, Research Biochemicals International) and maintained at 37˚C on a servo-controlled heated rodent operating table. Heart tissue samples corresponding to left (LA) and right (RA) atrial appendages and ventricular chambers were collected, processed accordingly to RNA isolation and stored at -80˚C until used.

Genetically modified Pitx2 mice, and their corresponding controls, were sacrificed by cervical dislocation. Adult hearts were carefully dissected and briefly rinsed in Ringer's solution. Tissue samples corresponding to the RA and LA were collected for each experimental condition, immediately snap-frozen in liquid nitrogen, and stored at -80˚C until used. Pooled samples of at least three independent mice were processed for each condition, respectively. Three independent pooled samples were further processed for RNA isolation and qPCR analyses. Total RNA was isolated using Trizol (Roche) according to manufacture's guidelines and DNase treated using RNase-Free DNase (Roche) for 1h at 30˚C. In all cases, at least three distinct pooled samples were used to perform the corresponding qRT-PCR experiments.

First strand cDNA was synthesized at 50˚C for 1h using 1 μg of RNA, oligo-dT primers and Superscript III Reverse Transcriptase (Invitrogen) according to manufacture's guidelines. Negative controls to assess genomic contamination were performed for each sample, without reverse transcriptase, which resulted in all cases in no detectable amplification product.

qRT-PCR (mRNA)

RT-PCR was performed in Mx3005Tm QPCR System with an MxPro QPCR Software 3.00 (Stratagene) and SyBR Green detection system. Reactions were performed in 96-well plates with optical sealing tape (Cultek) in 20 μL total volume containing SYBR Green Mix (Finnzymes) and the corresponding cDNA. Three internal controls, mouse βactin, Gusb and GAPDH, were used in parallel for each run and represented as previously described [14,33].

Amplification conditions were as follows: denaturisation step of 95˚C for 10 min, followed by 40 cycles of 95˚C for 30s, 60˚C for 30s, 72˚C for 30s; with final elongation step of 72˚C for 10 min. All primers were designed to span exon-exon boundaries using online Primer3 software Primer3input (primer3 www.Cgi v 0.2). Primer sequences are provided in **S1 Table**. No amplifications were observed in PCR control reactions containing only water as the template. Each PCR reaction was performed at least three times to obtain representative averages. The Livak method was used to analyze the relative quantification RT-PCR data [33] and normalized in all cases taking as 100% the wild-type (control) value, as previously described [14].

qRT-PCR (microRNA)

microRNA qRT-PCR was performed using Exiqon LNA microRNA qRT-PCR primers and detection kit according to manufacturer's guidelines. All reactions were always run in triplicates using 5S as normalizing control, as recommended by the manufacturer. SyBR Green was used as quantification system on a Stratagene Q-Max 2005P qRT-PCR thermocycler. Relative measurements were calculated as described by Livak & Schmittgen [33] and control measurements were normalized to represent 100% as previously described [14].

Plasmid, microRNA and siRNA cell transfections

HL-1 cells (6×10^5 cells per well) were transfected with plasmids containing expression constructs for Pitx2, Wnt8a (Addgene), Wnt11a (Addgene, Cambridge, MA, USA), premiR-29a, pre-miR-200 (Exiqon) or siRNA-Pitx2c, siRNA-Zfhx3, siRNA-Enpep, siRNA-Sod2 (Sigma, Aldrich, Munich, Germany) as previously described [14,34]. Primary cultures of mouse fetal (E17.5) cardiomyocytes were isolated using standard procedures [35], cultures accordingly and treated with T4 hormone as previously reported [36]. siRNA sequences are provided in **S1 Table**.

Statistical analyses

For statistical analyses of datasets, unpaired Student's t-tests were used. Significance levels or P values are stated in each corresponding figure legend. $P < 0.05$ was considered statistically significant.

Results

Pitx2c>Wnt>microRNA signaling is severely impaired in experimental hyperthyroidism (HTD) rat model

We have analyzed the expression levels of the homeobox transcription factor *Pitx2c* in an experimental rat model of induced hyperthyroidism (HTD) [32]. As a consequence of HTD, this experimental model also displays moderate hypertension as documented in **S1 Fig**. qPCR analyzed demonstrated that *Pitx2c* is severely down-regulated in right and left atrial chambers (**Fig 1**). Similar findings are also observed in an *in vitro* model of HTD (T4 administration) using fetal primary cultures of cardiomyocytes (**S2 Fig**). Curiously, *Enpep* is also severely impaired in both atrial chambers (**Fig 1**). Since multiple evidences have demonstrated that Pitx2 differentially modulates expression of AF GWAS associated genes as well as multiple components of the cardiac action potential in the left atrium [14,16], we analyzed their expression in the HTD model. Our data demonstrate that *Wnt8* and *Wnt11* were significantly increased whereas *Zfhx3* and *Kcnn3* were decreased in HTD rats as compared to age-matched controls (**Fig 1**). Importantly, I_{Na} encoding genes, i.e. *Scn5a* and *Scn1b*, are severely decreased whereas resting membrane potential I_{K1} encoding genes (*Kcnj2* and *Kcnj12*) are up-regulated, as well as calcium handling

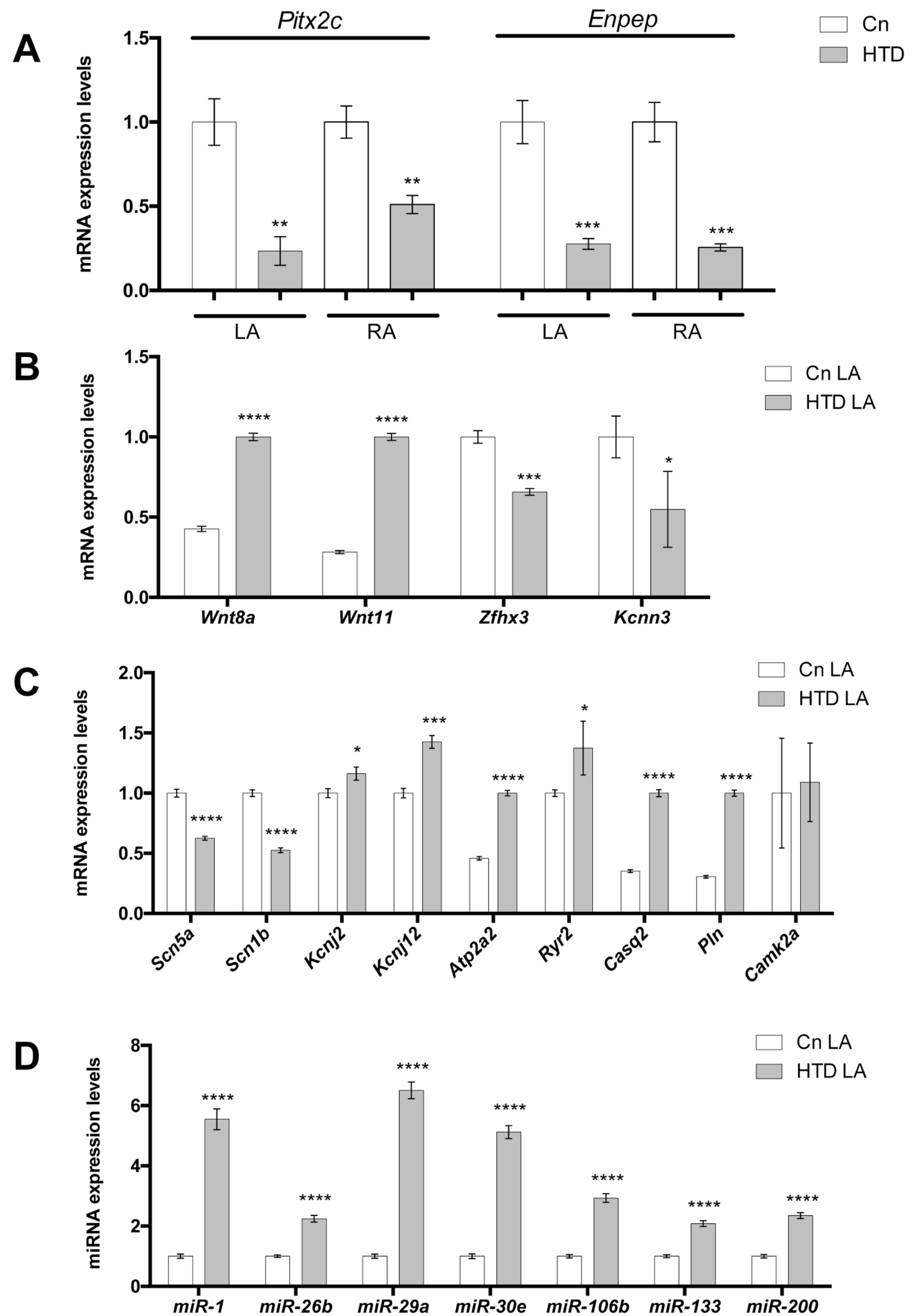


Fig 1. (A) *Pitx2* and *Enpep* gene expression in the left (LA) and right (RA) atrial chambers of experimental hyperthyroid rats (HTD) as compared to controls. Observe that *Pitx2* and *Enpep* are significantly decreased in both atrial chambers in HTD hearts. (B) AF GWAS-associated gene expression in the LA chamber of HTD hearts. Wnt8 and Wnt11 are significantly increased whereas Zfhx3 and Kcnn3 are decreased in HTD hearts as compared to controls. (C) Ion channel encoding genes display significant differential expression in HTD as compared to controls, while *Camk2a* display no significant differences. (D) Pitx2-regulated microRNAs display significant increased expression in HTD as compared to controls. In all cases, n = 6.*p<0.01, **p<0.05, ***p<0.001,****p<0.0001.

https://doi.org/10.1371/journal.pone.0188473.g001

proteins such as *Atp2a2* (Serca), *Ryr2*, *Casq2* and *Pln* while *Camk2a* display no significant differences (**Fig 1**). Overall these data suggest that HTD elicits down-regulation of Pitx2 and its downstream signaling pathway, mimicking the gene expression profile observed in atrial-specific Pitx2 deficient mice [14,16].

A pivotal role for post-transcriptional regulation by non-coding RNAs in cardiac development and disease is emerging [37] and we have provided evidence that Pitx2 controls a large set of microRNAs with key functional roles in cardiac electrophysiology [15]. We therefore tested whether distinct Pitx2-regulated microRNAs were deregulated in our HTD rat model. qPCR of left atrial chambers demonstrated that miR-1, miR-26b, miR-29a, miR-30e, miR-106b, miR-133 and miR-200 are up-regulated in HTD rats as compared to controls (**Fig 1**), demonstrating a similar microRNA expression profile as in atrial-specific Pitx2 deficient mice [14,16].

Pitx2 alone is impaired in experimental spontaneous hypertension (HTN)

We further explored if *Pitx2c* is also altered in another AF risk factor experimental model, i.e. spontaneous hypertensive SHR rats [38]. We therefore analyzed *Pitx2c* expression levels at two distinct developmental stages, 2 and 8 month old rats, respectively. Interestingly, *Pitx2c* expression was significantly decreased in right atrium but not in the left atrium of 2 month old SHR rats, whereas down-regulation was equally observed at 8 months (**Fig 2**). Curiously, *Enpep* expression was not significantly different in left and right atrial chambers at any of these stages (**Fig 2**). Since most significant differences on *Pitx2* expression were only observed at 8 months, subsequent analyses were only performed at this stage.

Analyses of AF GWAS associated genes demonstrate that *Wnt8*, *Zfhx3* and *Kcnn3* are significantly decreased in hypertensive rats whereas *Wnt11* display no significant difference in the left atrial chambers of HTN rats as compared to controls (**Fig 2**). In addition, analyses of ion channel encoding genes demonstrate that *Scn5a*, but not *Scn1b* was significantly increased (**Fig 2**). *Kcnj2*, *Kcnj12* and *Atp2a2* (Serca2) are down-regulated whereas *Ryr2*, *Casq2* and *Camk2a* display no significant differences (**Fig 2**) and only *Pln* is up-regulated in HTN left atrial chambers as compared to control normotensive rats (**Fig 2**). Thus, although Pitx2 is severely down-regulated in HTN rats, Pitx2-downstream signals, such as Wnt signaling or cardiac action potential determinants display either no changes or discordant changes as compared to atrial-specific Pitx2 deficient mice [14,16].

Following the same reasoning as for HTD experimental model, we assessed if Pitx2-downstream microRNA expression is impaired in HTN rats. Surprisingly none of the tested microRNAs, except for *miR-26b* and *miR-106b*, which in fact were decreased, display significant differences (**Fig 2**). Overall, these data demonstrate that while Pitx2 is impaired in HTN, its downstream pathways are mostly unaltered, demonstrating a discordant microRNA expression profile as compared to those revealed in atrial-specific Pitx2 deficient mice [14,16].

Enpep downregulation modulates PITX2 but not its downstream pathway

Given the fact that Enpep is deregulated in HTD experimental rats, it is widely expressed in cardiac regions that can contribute to AF and that genomic interaction between 4q25 AF risk variants containing sequences and the Enpep promoter have been reported in mice [11], it is plausible that ENPEP might have a role in AF predisposing factors. Furthermore, Pitx2 silencing in HL1 atrial cardiomyocytes also elicits *Enpep* downregulation (**S2 Fig**). We therefore silenced *Enpep* expression in HL1 atrial cardiomyocyte and evaluated expression of *Pitx2c* and Pitx2-Wnt downstream signaling. Our analyses demonstrated that *Enpep* silencing decreased

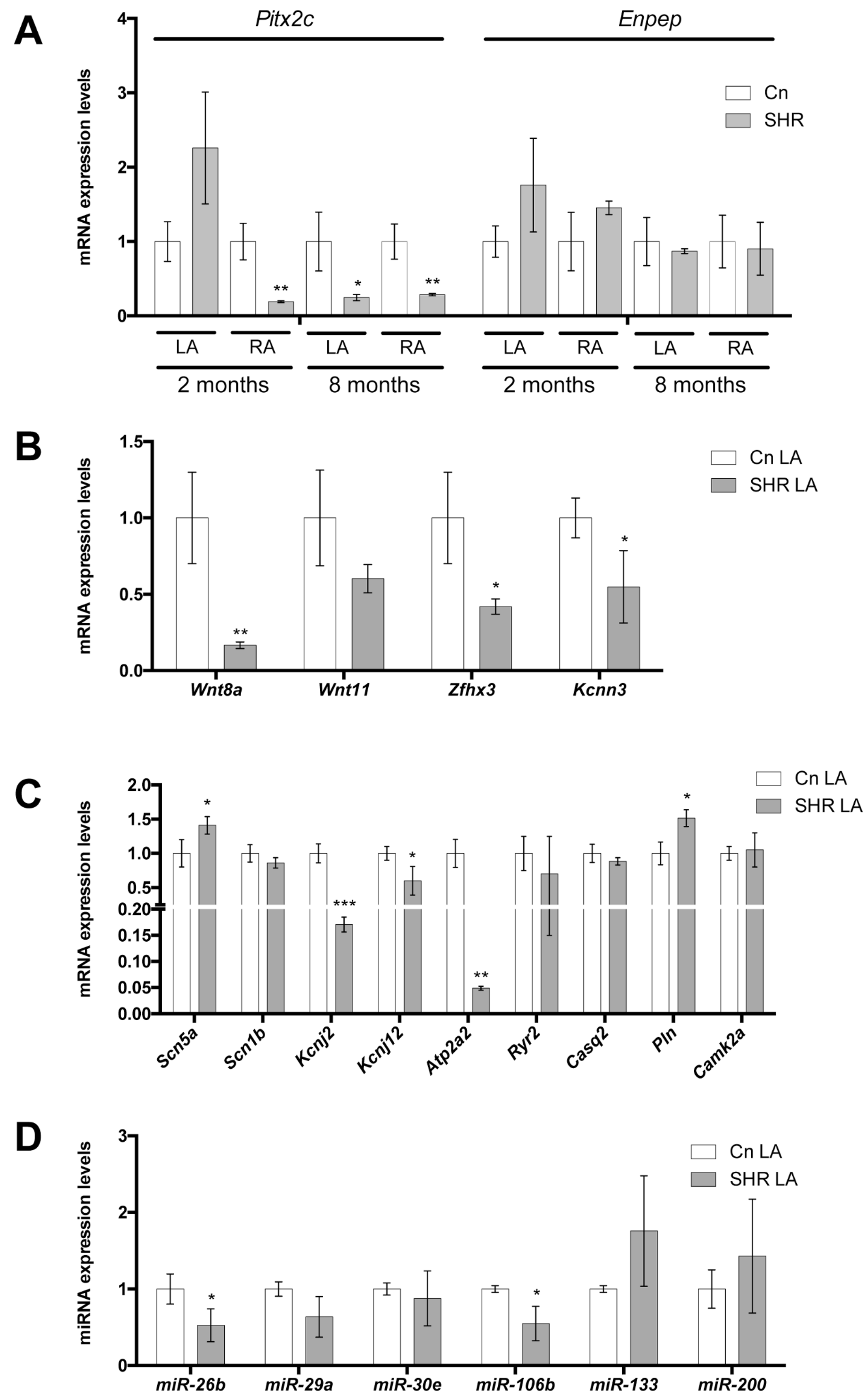


Fig 2. (A) *Pitx2* and *Enpep* gene expression in the adult left (LA) and right (RA) atrial chambers of spontaneous hypertensive rats (SHR) at 2 and 8 months, respectively, as compared to WHK controls. Observe that *Pitx2* is significantly decreased in both atrial chambers in SHR hearts at 8 months, whereas *Enpep* display no significant differences at any of the analyzed stages. (B) AF GWAS-associated gene expression in the LA chamber of HTD hearts. *Wnt8*, *Wnt11*, *Zfhx3* and *Kcnn3* are significantly decreased in HTD hearts as compared to controls. (C) Ion channel encoding genes such as *Kcnj2*, *Kcnj12*, *Atp2a2* (decreased), *Scn5a* and *Pln* (increased) display significant differential expression in HTD as compared to controls, while *Scn1b*, *Ryr2*, *Casq2* and Camk2a display no significant differences. (D) Pitx2-regulated

microRNAs display no significant differences, except for miR-26b and miR-106b which are significantly decreased, in HTD as compared to controls. In all cases, n = 6. *p<0.01, ** p<0.05, *** p<0.001.

https://doi.org/10.1371/journal.pone.0188473.g002

Pitx2 expression whereas *Zfhx3*, *Wnt8*, *Wnt11*, *Cacna1c* and *Scn5a* were unaltered (**Fig 3**). Thus these data demonstrate that Enpep contribution on the Pitx2-Wnt signaling pathways is limited to Pitx2 regulation.

Impaired ROS signaling contributes to Pitx2 pathophysiology

Reactive oxidative signaling (ROS) has been recently linked to AF [20,39] and experimental evidences demonstrated that impairing ROS can act as pro-arrhythmogenic factor [40]. We have therefore investigated if ROS is impaired in our atrial-specific Pitx2 mouse mutants as well as in AF risk factor HTD and SHR experimental models. qPCR demonstrate that several components of ROS signaling are impaired. In particular, we noticed that catalase (*Cat*), glutathione peroxidase (*Gpx)* and mitochondrial superoxide dysmutase (*Sod2*) were significantly down-regulated in the left atrial chamber of atrial-specific Pitx2 mouse mutants as compared to controls, whereas cytoplasmic superoxide dysmutase (*Sod1*) and glutathione reductase (*Gsr*) were unaltered (**Fig 4**). Subsequently we tested if ROS is impaired in SHR and/or HTD experimental models. Interestingly, HTD leads to up-regulation of *Cat* and *Sod2* but not any of the other ROS analyzed components (**Fig 4**) whereas HTN (SHR) elicited no significant changes in any of the studied ROS components (**Fig 4**). These data illustrate that Pitx2 insufficiency leads to altered ROS signaling, a condition that is minimally (HTN) or partially (HTD) impaired, respectively.

To test if *Pitx2c* can directly modulate ROS components, Pitx2 over-expression and silencing (siRNA) experiments were carried out in HL-1 atrial cardiomyocytes (**Fig 5**). Unexpectedly, Pitx2 over-expression did not influence *Cat* and *Sod2* expression whereas Pitx2 silencing slightly up-regulated *Cat* but not *Sod2*, as depicted in **Fig 5**. These data are in contrast with our previous findings in atrial-specific Pitx2 mutant mice (**Fig 4**). Thus it could be possible that Pitx2 does not directly target *Cat*/*Sod2* expression but else is modulated downstream of Pitx2. Mimicking Pitx2 insufficient model, we thus over-expressed Wnt8 and Wnt11, respectively and inhibited Zfhx3 by using siRNA in HL-1 atrial cardiomyocytes. Interestingly, both over-expression of Wnt signaling (Wnt8 and Wnt11) and inhibition of Zfhx3 (**Fig 5**), respectively, lead to significant downregulation of *Cat* and *Sod2* expression (**Fig 5**). These data therefore demonstrate that ROS signaling is modulated by Wnt and Zfhx3 signaling downstream Pitx2.

To test if the opposite pathways is operative, i.e. if ROS signaling alters Pitx2>Wnt pathway, we treated HL-1 atrial cardiomyocytes with hydrogen peroxidase (H_2O_2) at distinct incubation times ranging from 1h to 24h and we assessed if Pitx2, Wnt signaling and Zfhx3 is impaired. *Cat*, *Sod2* and also peroxiredoxines (*Prxd2*, *Prdx3*, *Prdx5* and *Prdx6*) were assayed in parallel as indicators of ROS signaling activity. Incubation times ranging from 1h to 6h display basically no significant changes in the expression level (data not shown), except for a transient up-regulation of *Wnt11* (3h/6h; **S3 Fig**). Curiously, sustained down-regulation of *Zfhx3* expression was observed at all time analyzed (**S3 Fig**). At 12h/24h of H_20_2 administration *Cat*, *Sod2*, *Pdrx2* (only 12h) and *Prdx5* were significantly decreased in treated cells as compared to controls, whereas *Prdx3* and *Prdx6* display no significant differences (**Fig 6**). These data suggest therefore that impaired ROS signaling was successfully achieved at 12h/24h of H_20_2 administration. Importantly, *Pitx2*, *Wnt8*, *Wnt11* and *Zfhx3* were severely down-regulated after H_20_2 administration at both 12h/24h (**Fig 6**). Thus these data demonstrate that ROS impairment can influence Pitx2>Wnt signaling. To further support these findings we selectively inhibited Sod2 expression by siRNA in HL1 atrial cardiomyocyte and assayed Pitx2>Wnt signaling components by qPCR. Sod2 inhibition leads to down-regulation of

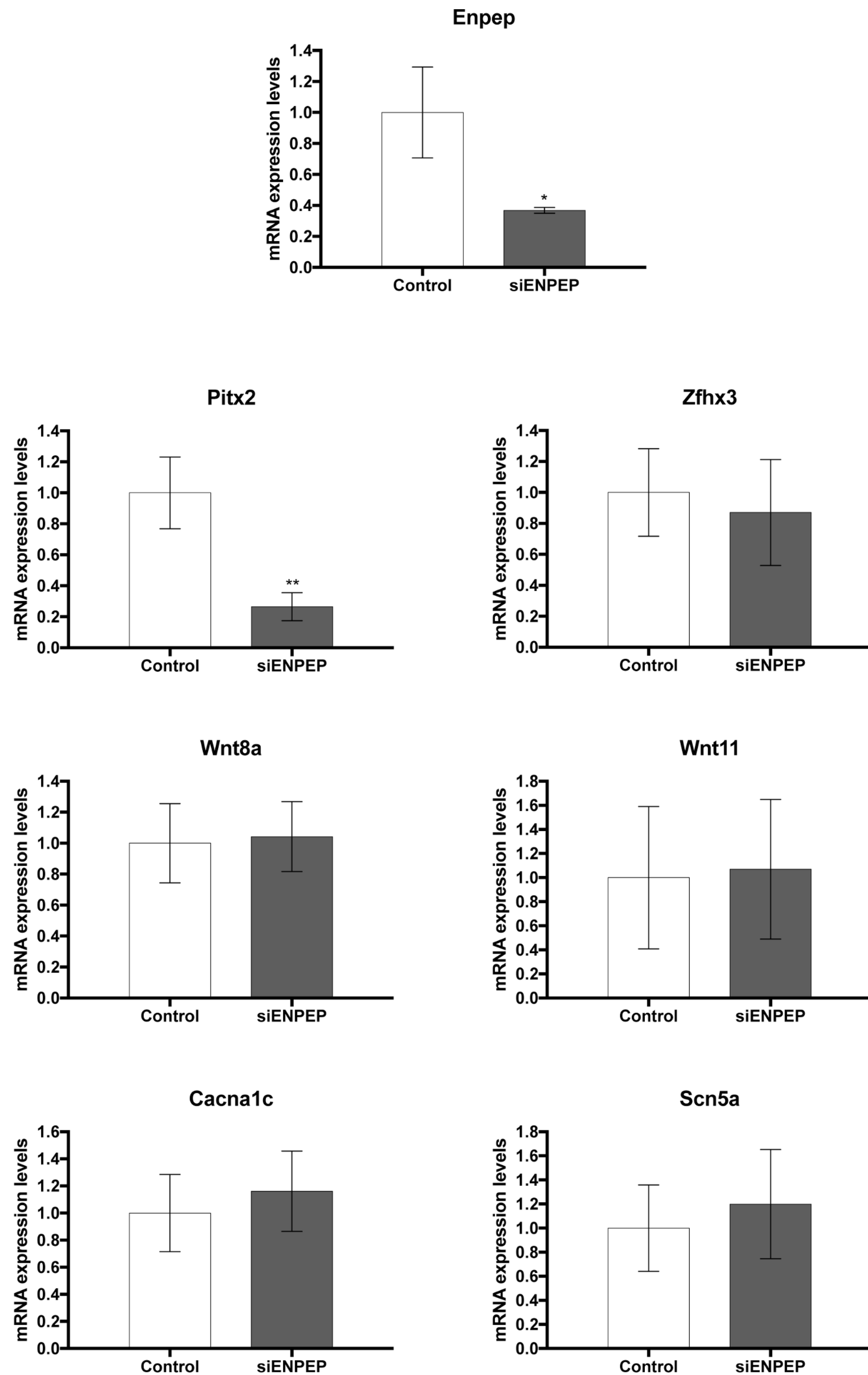


Fig 3. Gene expression analyses in Enpep silenced HL-1 atrial cardiomyocytes. Note that Enpep is decreased (siEnpep), leading to decreased expression of *Pitx2c* but not of *Wnt8a*, *Wnt11*, *Zfhx3*, *Cacna1c* or *Scn5a*. Representative values of three pooled replicates on three independent biological transfections. *p<0.01, **p<0.05.

https://doi.org/10.1371/journal.pone.0188473.g003

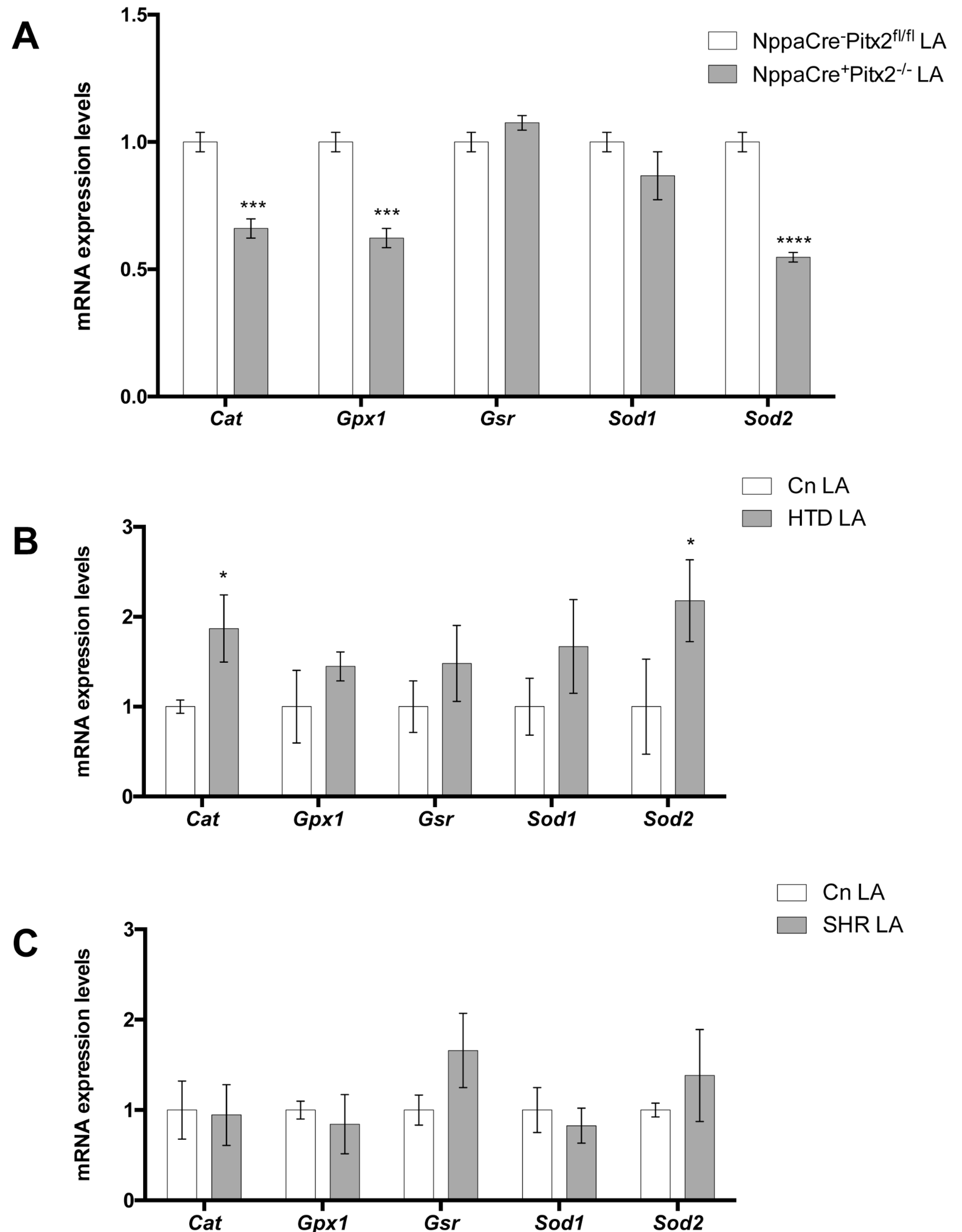


Fig 4. Redox signaling gene expression analyses of adult left atria (LA) corresponding to NppaCre^{+}Pitx2$^{-/-}$ (A), HTD (B) and SHR (C), respectively, as compared to their corresponding controls. Note that *Cat*, *Gpx1* and *Sod2* are significantly decreased in NppaCre^{+}Pitx2$^{-/-}$ as compared to NppaCre^{-}Pitx2$^{fl/fl}$ controls, *Cat* and *Sod2* are significantly increased in HTD mice whereas no changes are observed in SHR mice. In all cases, n = 6. *p<0.01, ***p<0.001, ****p<0.0001.

https://doi.org/10.1371/journal.pone.0188473.g004

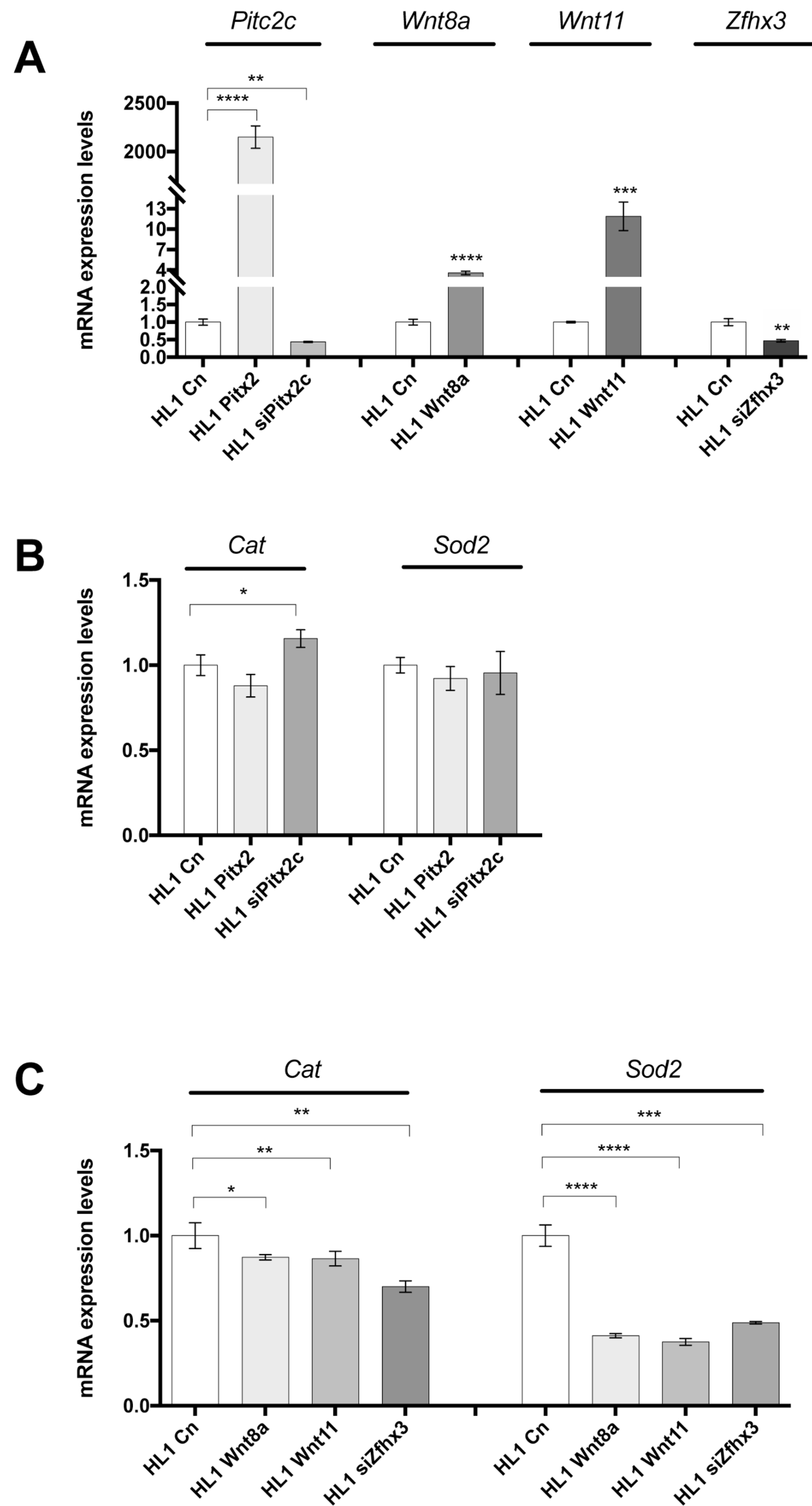


Fig 5. (A) Expression analyses of Pitx2c, Wnt8a, Wntt11 and Zfhx3 in gain- and loss-of-function experimental transfection of HL-1 atrial cardiomyocytes for Pitx2, Wnt8a, Wnt11 and Zfhx3, respectively. (B) Expression analyses of *Cat* and *Sod2* in *Pitx2* gain and loss-of-function experimental transfection of HL-1 atrial cardiomyocytes. Observe that only Pitx2c silencing (siPitx2c) significantly increases Cat expression. (C)

Expression analyses of *Cat* and *Sod2* in Wnt8a and Wnt11 gain of function transfections and Zfhx3 siRNA silencing (siZfhx3) of HL-1 atrial cardiomyocytes. Observe that *Cat* and *Sod2* are significantly decreased in all cases, as compared to controls. Representative values of three pooled replicates on three independent biological transfections. *p<0.01, **p<0.05, ***p<0.001, ****p<0.0001.

https://doi.org/10.1371/journal.pone.0188473.g005

Wnt8a, *Wnt11* and *Zfhx3* and surprisingly to up-regulation of *Pitx2* as depicted in **Fig 6**. While up-regulation of *Pitx2c* after Sod2 siRNA remains to be fully understood, our data reveal a complex interplay between Pitx2>Wnt and ROS signaling in atrial cardiomyocytes.

To further decipher the complex interplay between ROS and Pitx2 signaling, we experimentally test if Pitx2c gain- and loss-of-function was impaired by H_20_2 administration in HL-1 atrial cardiomyocytes, aiming to decipher which is the overruling signaling pathway. Pitx2c transfection lead to over-expression of *Pitx2c*, which was significantly diminished by H_20_2 administration at both experimental time points (12h/24h) (**Fig 7**). Similarly, *Pitx2c* expression was further inhibited by H_20_2 administration after Pitx2c siRNA silencing experiments (**Fig 7**), further demonstrating that H_20_2 administration significantly decreases *Pitx2c* expression.

In this experimental context, H_20_2 administration does not alter *Sod2* expression in Pitx2c gain and loss-of-function settings at 12 hours but it significantly decreases it at 24 hours (**Fig 7**). On the contrary, *Cat* expression is enhanced by H_20_2 administration in Pitx2 gain and loss-of-function settings at 12h but selectively decreased at 24hours only in Pitx2 loss-of-function conditions. These data suggest that H_20_2 administration cannot overrule Pitx2 modulation of *Cat*. In line with these findings only *Prdx5* is significantly decreased after H_20_2 administration in Pitx2c gain and loss-of-function settings at both 12 hours and 24 hours (**Fig 7**; **S4 Fig**), further supporting that H_20_2 administration overrules Pitx2c modulation of ROS signaling components.

In order to investigate if H_20_2 administration can also overrule Pitx2 modulation of its downstream targets, *Wnt8a*, *Wnt11* and *Zfhx3* expression was analyzed in this experimental setting. As it can be observed in **Fig 7**, H_20_2 administration prevented *Wnt8* and *Wnt11* up-regulation in Pitx2c siRNA silenced conditions at 24h but not at 12hours, demonstrating that H_20_2 administration can also overruled Wnt signaling up-regulation in absence of Pitx2c. On the other hand, *Zfhx3* was up-regulated after H_20_2 administration in Pitx2c silenced conditions at 24hours, demonstrating that H_20_2 administration can directly affect *Zfhx3* expression independently of Pitx2c expression levels. Overall these data further demonstrate that ROS signaling has a major impact on Pitx2>>Wnt signaling pathway. We further analyzed if ion channel expression would be affected in this experimental setting. qPCR analyses of *Cacna1c*, *Scn5a* and *Kcnj2* expression demonstrated significant down-regulation at both 12 and 24 hours after H_20_2 administration in Pitx2c silenced conditions (**Fig 8**). Importantly, such H_20_2 mediated down-regulation was only observed for *Kcnj2* in Pitx2c over-expressing cells, demonstrating a fine output balanced between ROS and Pitx2 function on the expression of distinct ion channels. Finally, we tested if microRNA expression would be also impaired in this context. A subset of Pitx2-modulated microRNA was assessed[16]. As depicted in **Fig 8**, *miR-1*, *miR-29a*, *miR-106b* and *miR-200a* was selectively inhibited by H_20_2 treated Pitx2-overexpressing cells but up-regulated in H_20_2 treated Pitx2 silenced cells at both time points (12h and 24h). These data illustrate that H_20_2 administration does not overrule Pitx2 function as regulator of microRNA expression.

Modulation of miR-29 and miR-200 alters cardiac action potential determinants

We have previously demonstrated that Pitx2 modulates expression of *miR-29* and *miR-200*, among other microRNAs [16] and furthermore we have demonstrated in this study that modulation of distinct ion channel is greatly influenced by H_20_2 administration while microRNA

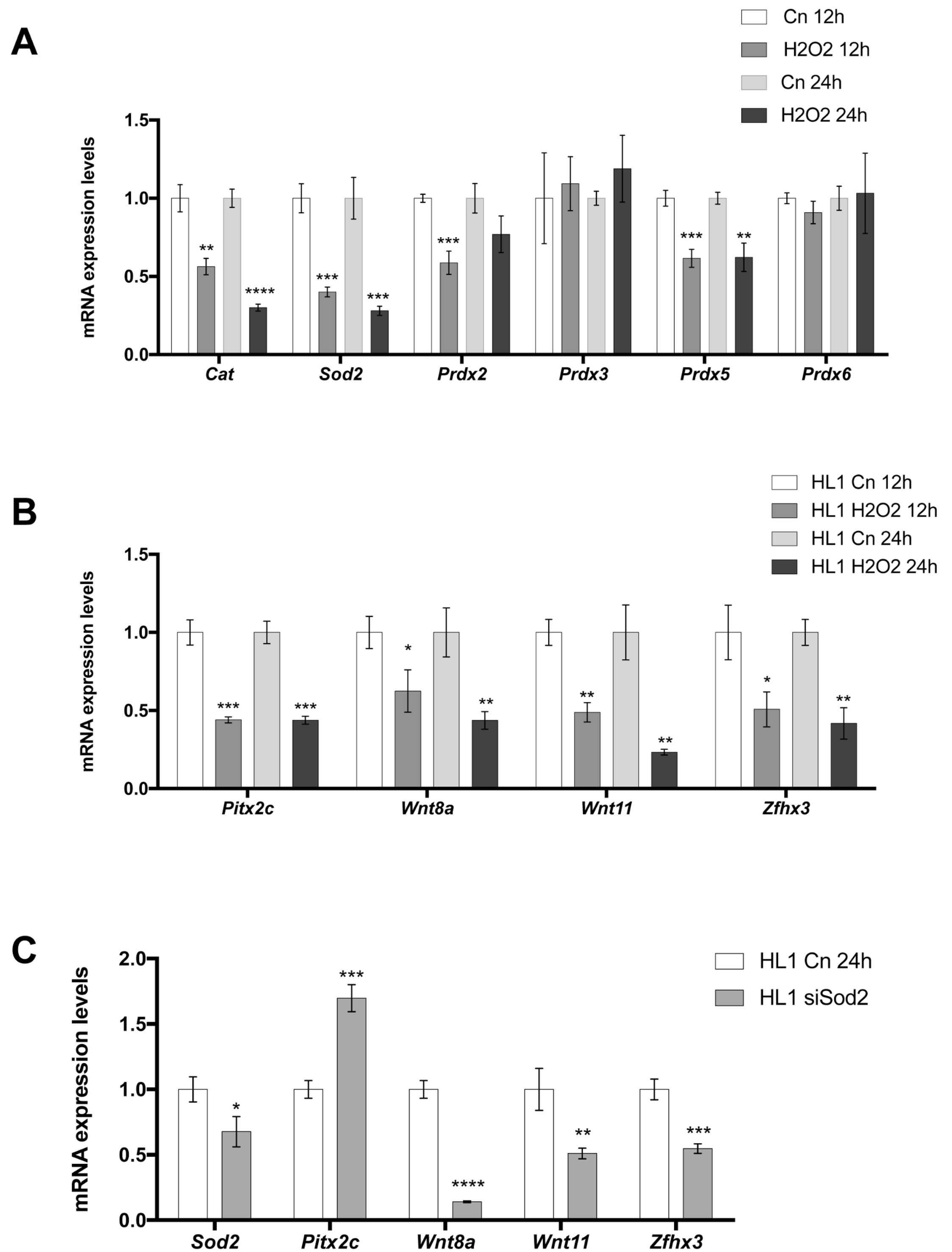
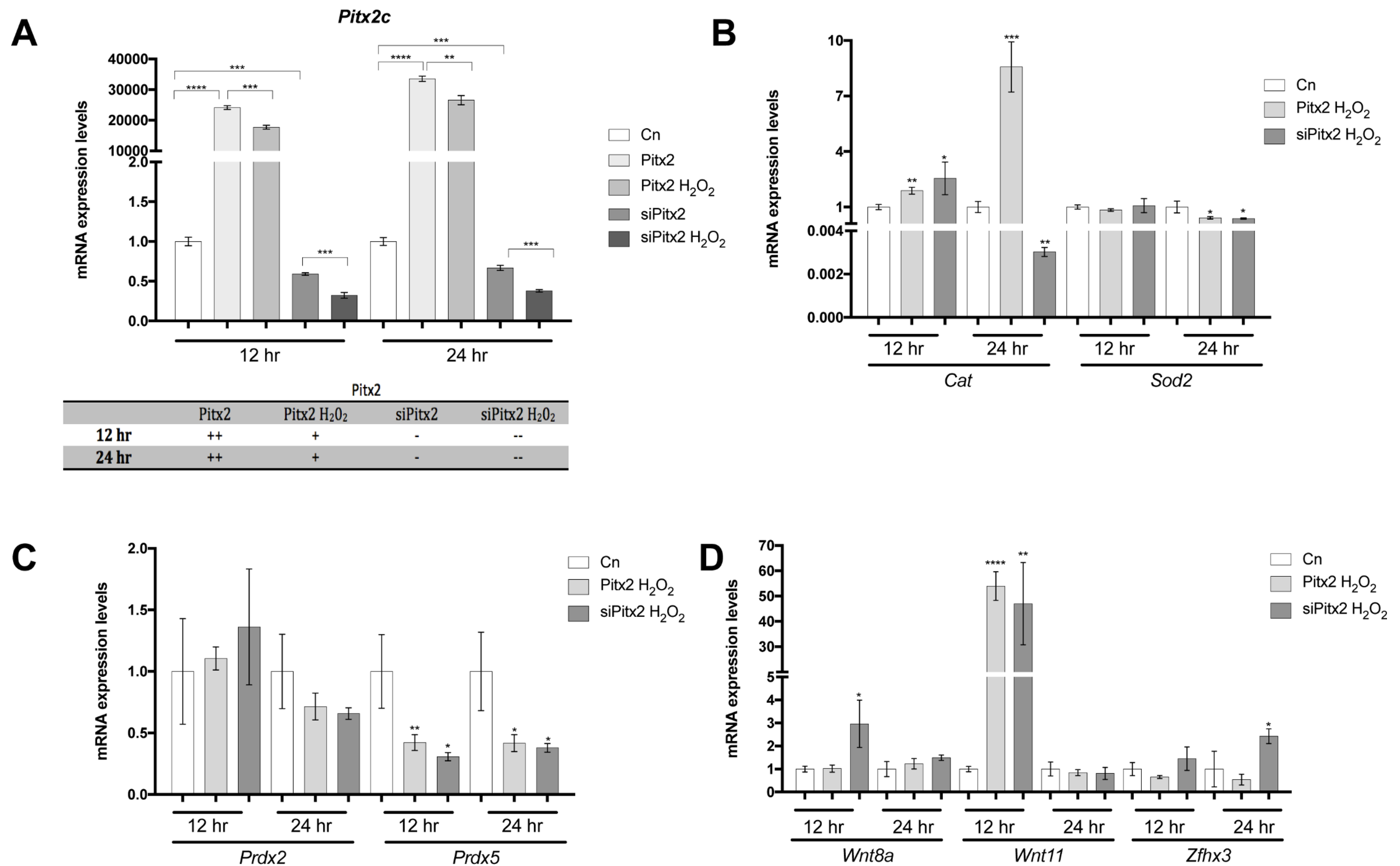


Fig 6. (A) Redox signaling gene expression analyses of H_20_2 treated HL-1 atrial cardiomyocytes at 12 h and 24h. Observe that *Cat*, *Sod2* and *Prdx5* are significantly decreased at 12h and 24h after H_20_2 administration, whereas *Prdx2* is only decreased at 12h but not at 24h. (B) Gene expression analyses of *Pitx2c*, *Wnt8a*, *Wnt11* and *Zfhx3* after H_20_2 administration. Observe that all of them are significantly decreased at 12h and 24h. (C) Gene expression analyses in Sod2 silenced HL-1 atrial cardiomyocytes. Note that Sod2 is decreased (siSod2), *Pitx2c* increases whereas *Wnt8a*, *Wnt11* and *Zfhx3* are significantly decreased. Representative values of three pooled replicates on three independent biological transfections. *p<0.01, **p<0.05, ***p<0.001, ****p<0.0001.

https://doi.org/10.1371/journal.pone.0188473.g006



	Pitx2	Pitx2 H2O2	siPitx2	siPitx2 H2O2
12 hr	++	+	-	--
24 hr	++	+	-	--

Fig 7. (A) Analyses of *Pitx2c* expression in Pitx2 gain and loss-of-function experiments with or without H_20_2 administration for 12h and 24h, respectively. Observe that H_20_2 administration significantly decreased Pitx2c expression in both Pitx2 overexpression and silencing conditions, at 12h and 24h. (B) Analyses of *Cat* and *Sod2* expression (B), *Prdx2* and *Prdx5* (C) and *Wnt8a*, *Wnt11* and *Zfhx3* (D) in Pitx2 gain and loss-of-function setting with H_20_2 administration for 12h and 24h, respectively. Observe that *Cat* is significantly increased by Pitx2 overexpression at 12h and 24h whereas is mildly increased and highly significantly decreased by Pitx2 siRNA treatment at 24h. On the other hand Sod2 is mildly decreased only at 24h in both conditions (B). *Prdx5*, but not *Prdx2*, is significantly decreased at 12h and 24h in both conditions (C). *Wnt8a* is only increased in Pitx2c siRNA conditions at 12h, *Wnt11* is increased in both conditions only at 12h while *Zfhx3* is significantly increased only by Pitx2c silencing at 24h. Representative values of three pooled replicates on three independent biological transfections. *p<0.01, **p<0.05, ***p<0.001, ****p<0.0001.

https://doi.org/10.1371/journal.pone.0188473.g007

signature is mostly dependent on Pitx2c but not H_20_2 administration. We provide herein evidences that *miR-29* and *miR-200* over-expression also contributes to ion channel expression remodeling. HL-1 atrial cardiomyocytes transfected with *miR-29* and *miR-200* (Fig 8) significantly down-regulate *Cacna1c*, *Hnc4* and *Ryr2* expression, while *Camk2a* was significantly decreased with *miR-200* but not *miR-29* (Fig 8). Thus these data demonstrate that *miR-29* and *miR-200* impaired expression also contributes to develop pro-arrhythmogenic substrates.

Discussion

Multiple risk factors are associated with the onset of AF [23], however understanding of the molecular causative links remains poorly elucidated. Among these risk factors, hyperthyroidism is highly linked to increased prevalence of AF [41–43]. It is widely documented that electrical properties of the atrial cardiomyocytes are functional impaired in hyperthyroid patients [41] as well as in distinct experimental models of hyperthyroidism [44–47]. In particular, there are ample evidences that TH deregulation can influence calcium and potassium channels [44–

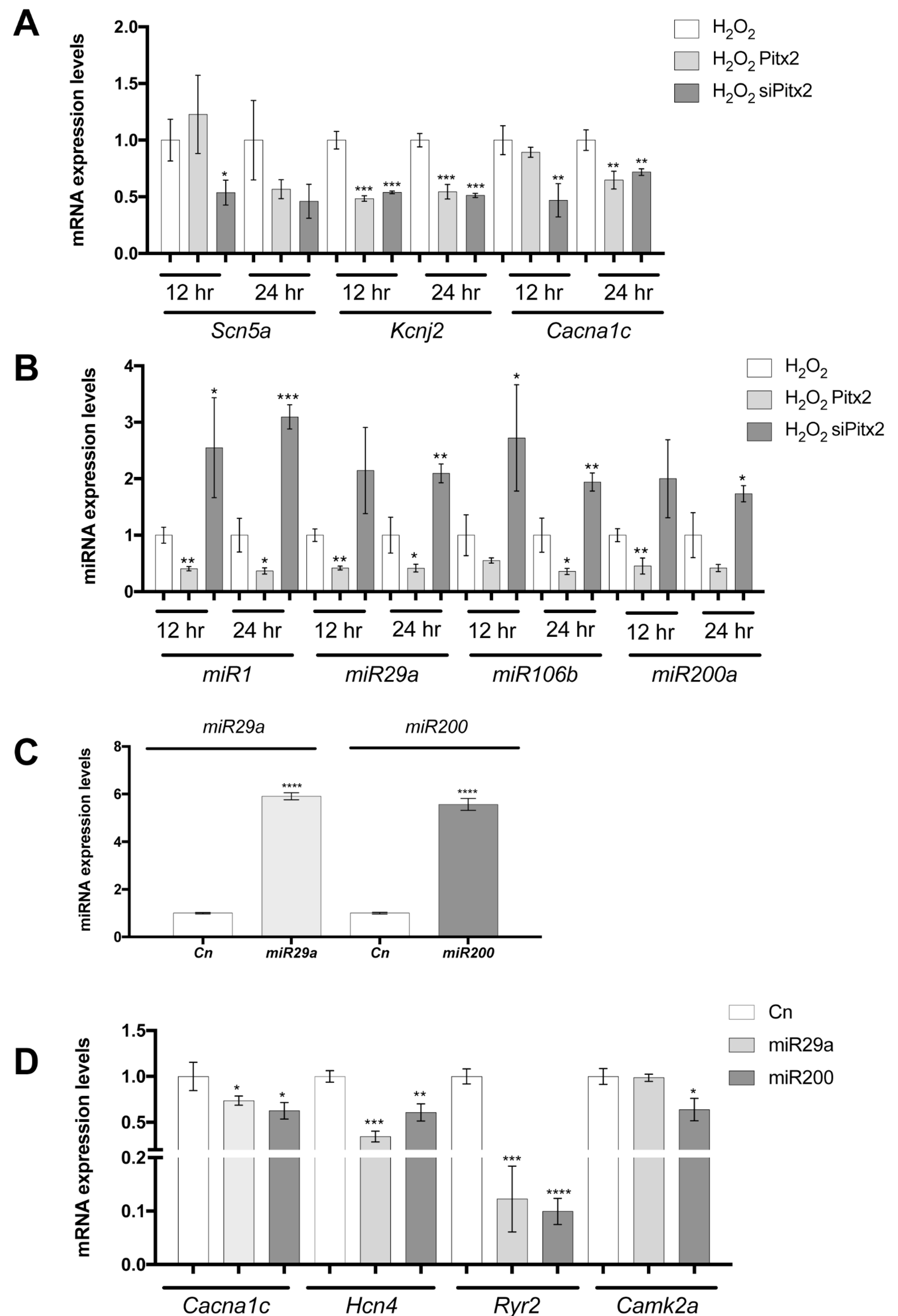


Fig 8. Analyses of *Scn5a*, *Kcnj2* and *Cacna1c* (A), *miR-1*, *miR-29a*, *miR-106b* and *miR-200b* (B) expression in Pitx2 gain and loss-of-function experiments after $H_2 0_2$ administration for 12h and 24h, respectively. Observe that most ion channel are significantly decreased in both experimental conditions at both time points (A) whereas microRNAs are all significantly decreased after Pitx2 over-expression and increased following Pitx2c silencing at 12h and 24h after $H_2 0_2$ administration. (C). *miR-29a* and *miR-200* expression in HL-1 atrial cardiomyocytes transfected cells. Observe that each microRNAs is significantly increased after corresponding pre-miRNA transfection. (D) Ion channel gene expression in *miR-29a* and *miR-200* transfected HL-1 atrial cardiomyocytes,

respectively. Observe that miR-29 and miR-200 over-expression leads to significant decreased of *Cacna1c*, *Hcn4*, *Ryr2* and *Camk2a* (except for miR-29a) expression. Representative values of three pooled replicates on three independent biological transfections. *p<0.01, **p<0.05, ***p<0.001, ****p<0.0001.

https://doi.org/10.1371/journal.pone.0188473.g008

47], whereas modulation of sodium channels is less documented [48]. Similarly, AF onset is also increased in HTN patients [23,49]. While extensive literature is available on the modulation of distinct ion channels in the vasculature of hypertensive patients [50–51], scarce evidence is available on cardiac ion channel deregulation and thus electrical impairment in the heart. Recently, generation of reactive oxygen species (ROS) has been associated to increase onset of AF [52–53] and dietary ROS reduction has been provided to be beneficial [54] on AF onset. Substantial evidence support ROS regulation of distinct cardiac ion channels [55–57], procuring thus a functional link between ROS signaling and impaired electrical activity leading to AF onset.

Thus, whereas there are evidence of functional impairment by these cardiovascular risk factors leading to AF, it largely remains unclear which are the molecular mechanisms driving AF in these contexts. Multiple point mutations in distinct ion channels have been reported to contribute to AF pathophysiology [58] yet covering less than 10% of AF cases. Genome-wide association studies have brought up novel candidate genes on AF pathophysiology among which most significantly associated risk variants are in the vicinity of the homeobox transcription factor PITX2 [6]. Genomic loci spanning within risk variants are capable of molecularly interacting with PITX2 as well as ENPEP regulatory elements [11], yet the specific role of these risk variants in AF remains elusive. Importantly, distinct Pitx2 loss-of-function experimental models demonstrated that this transcription factor controls a complex molecular signaling pathway that substantially modulates expression of multiple genes encoding ion channel and cell-cell proteins with pivotal role in cardiac electrophysiology [12–26]. We provide here first evidence that hyperthyroidism leads to decreased expression of PITX2 and ENPEP in the atrial chambers. Furthermore, we also demonstrated for the first time that impaired ROS signaling modulates PITX2 expression, while we corroborate previous findings that PITX2 expression, but not ENPEP, is significantly down-regulated in hypertensive rats [28]. Therefore these data demonstrate that PITX2 expression is impaired in HTD, a cardiovascular risk factor leading to AF, suggesting that PITX2 impairment could be pivotal provoking atrial arrhythmogenesis.

Pitx2 insufficiency in mice has been reported to distinctly modulate Sox2-Hcn4 expression [12] in the developing embryo leading to impaired conductive configuration and thus predisposition to AF. In addition, Pitx2 insufficiency in the adult heart has been proven to regulate I_{Na} and I_{K1} currents, leading to impaired ECG [14] and additionally to regulate calcium homeostasis by modulating Wnt signaling in a dose-dependent manner [16]. We provide herein evidences that HTD increases Wnt8/Wnt11 expression in the left atrial chamber while down-regulates Zfhx3 expression, mimicking thus similar results obtained in Pitx2 insufficient mice. Furthermore, genes encoding I_{Na} (*Scn5a*, *Scn1b*) were down-regulated, those encoding for I_{K1} (*Kcnj2*, *Kcnj12*) were up-regulated as well as those controlling calcium handling (*Ryr2*, *Atp2a2*, *Casq2*, *Plb*) were also up-regulated, in line with previous findings in atrial-specific conditional Pitx2 insufficient mice. Interestingly, such up-regulated Wnt signaling was not observed in the left atrial chamber of SHR rats (HTN model), neither such ion channel impaired expression. While Enpep is differentially expressed in HTN vs SHF left atrial chambers, Enpep provides no contribution to Wnt signaling or downstream ion channel expression. A plausible explanation might be that hypertension directly influences Wnt expression [59], counteracting Pitx2-mediated Wnt up-regulation. Importantly, HTD rats also course with

mild hypertension, supporting the notion that the specific molecular changes observed in HTD but not in SHR (HTN) are modulated by thyroid hormones. Additional experiments will be needed to further dissect these pathways.

We and other have previously reported the pivotal role of microRNAs as post-transcriptional regulatory mechanism driven by Pitx2 in the context of atrial arrhythmogenesis [57–58], and we therefore analyzed if expression of distinct cardiac enriched microRNAs were impaired in HTD and SHR (HTN model) rats. Importantly, we have observed that microRNA profiling in the left atrium of HTD, but not in SHR, rats, mimicking previous findings in atrial-specific conditional Pitx2 insufficient mice [16]. Several lines of evidence have already reported the key regulatory role of miR-1 [60–62], miR-26 [63], miR-106b [64], miR-133 [65–66] and miR-200 [64] in arrhythmogenesis. We provide herein additional evidence that these Pitx2-modulated HTD-regulated microRNAs modulate distinct ion channel expression with relevance in atrial electrophysiology. miR-29 over-expression in HL1 atrial cardiomyocyte deregulate *Cacna1c*, *Hnc4* and *Ryr2*, influencing therefore both the calcium handling and pacemaker activity, whereas miR-200 regulated *Cacna1c*, *Ryr2* and *Camk2a*, in addition to *Scn5a* as previously reported [64], impacting therefore also in calcium handling. Importantly, miR-29 and miR-200 are not significantly impaired in SHR atrial chambers, suggesting that Wnt-microRNA might be a pivotal candidate establishing fundamental differences between HTD and HTN in atrial arrhythmogenesis susceptibility.

It has been demonstrated that Pitx2 play a pivotal role regulating redox homeostasis in the adult skeletal muscle as well as during skeletal muscle regeneration [28]. More recently, Pitx2 has been demonstrated to play a pivotal role promoting resistance to ischemia by activating an antioxidant response in the adult heart [29]. We provide herein evidences of a complex interplay between Pitx2 and redox signaling. On the one hand, Pitx2 insufficiency leads to significant expression impairment of key redox components, such as *Cat* (catalase) and *Gpx* (Glutathione reductase) and *Sod2* (mitochondrial superoxide dismutase), in line with previous findings [28,29]. On the other hand, impaired redox homeostasis also significantly alters Pitx2 and its downstream AF signaling pathway, i.e. Wnt and Zfhx3 expression. Importantly, Wnt8/Wnt11 and Zfhx3 expression display significant decreased expression levels in both H_2O_2 and siRNA Sod2 atrial cardiomyocytes. Interestingly, redox signaling impairment is also observed in HTD but not in SHR (HTN model) rats, suggesting that redox impairment in the context of HTD can also contribute to atrial arrhythmogenesis in this setting.

Given the complex interplay between redox homeostasis and Pitx2 signaling, we have analyzed the molecular consequences of deregulating both pathways in atrial cardiomyocytes. We noticed that redox impairment significantly elicits down-regulation of Pitx2 independently of its expression levels, i.e. controls, Pitx2 over-expressing and Pitx2 silenced atrial cardiomyocytes. Furthermore, sustained H_2O_2 administration (24h) significantly blocked Pitx2-repressed Wnt expression and promotes Zfhx3 up-regulation. Whereas it is widely documented that redox signaling can compromise ion channel functioning and calcium homeostasis in cardiomyocytes [67], in our system we observed no influence of H_2O_2 administration on the regulatory impact of Pitx2 in distinct ion channels such as *Scn5a*, *Kcnj2* and *Cacna1c* as well as multiple Pitx2-regulated microRNAs such as miR-1, miR-26, miR-29 and miR-200, in which redox impairment impact is less documented [68].

In summary, we provide herein evidences that Pitx2-Wnt signaling pathway is impaired in HTD rats (**Fig 9**). Comparative analyses with Pitx2 insufficient models highlights the molecular hallmark similarities between HTD and atrial-specific Pitx2 deficient hearts, in particular Wnt upregulation, Zfhx3 down-regulation and redox signaling impairment. These data therefore support the notion that AF onset in HTN patients is mediated by Pitx2 impairment. Furthermore, we provide evidences of a complex interplay between Pitx2 and redox signaling

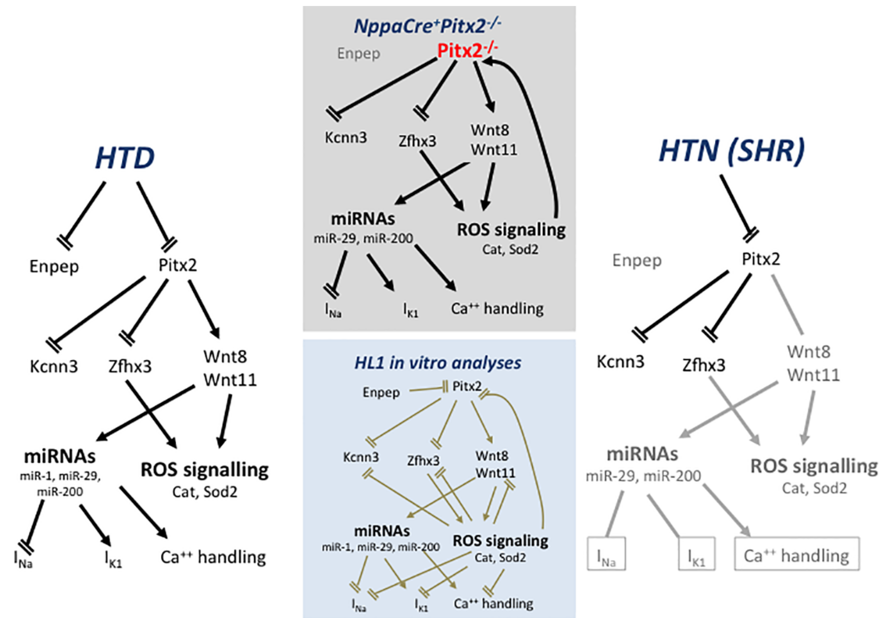


Fig 9. Schematic representation of the Pitx2>Wnt>ROS signaling pathway in NppaCrePitx2-/- insufficiency mice, as compared to experimental HTD and HTN (SHR) rat models. Observe that Pitx2 insufficiency leads deregulation of Zfhx3 and Wnt signaling, which subsequently leads to microRNA and ROS signaling deregulation and thus ion channel impairment. In HTD but not in HTN, Pitx2>Wnt>ROS signaling is also impaired. Furthermore, *in vitro* gain- and loss-of-function analyses (brown arrows) further support a complex retroactive gene regulatory network.

https://doi.org/10.1371/journal.pone.0188473.g009

highlighting therefore the complex molecular interactions priming molecular substrates that contribute to atrial arrhythmogenesis.

Supporting information

S1 Table. List of oligonucleotides for mRNA and microRNA detection by qPCR as well as oligonucleotide sequences used for siRNA silencing of *Enpep*, *Sod2* and *Pitx2c*, respectively.
(PDF)

S1 Fig. A) Systolic blood pressure (SBP) in mmHg and heart rate (HR) in beats per minute (BPM) measured in control and hyperthyroid (HTD) rats at the end of the experiment (n = 10 each group). Mean ± SEM is displayed. $^{*}p<0.001$ *vs* control group. B) Systolic blood pressure (SBP) in mmHg and heart rate (HR) in beats per minute (BPM) measured in Wistar-Kyoto (WKY) and spontaneously hypertensive rats (SHR) at the end of the experiment (n = 10 each group). Mean ± SEM is displayed. $^{*}p<0.01$, $^{**}p<0.001$ *vs* control group.
(TIFF)

S2 Fig. Analyses of Pitx2 expression in primary culture of fetal cardiomyocytes treated with T4 as compared to controls (panel A). Observe that Pitx2 is significantly decreased after T4 administration. Analyses of Pitx2 (panel B) and Enpep (panel C) in HL1 atrial cardiomyocytes after Pitx2 siRNA silencing. Observe that Pitx2 siRNA administration significantly decrease Pitx2 expression (panel B) and also Enpep expression (panel C). $^{*}p<0.01$, $^{***}p<0.001$, $^{****}p<0.0001$.
(TIF)

S3 Fig. Analyses of *Wnt11* and *Zfhx3* expression in H_20_2 treated HL-1 atrial cardiomyocytes at 1h, 3h and 6h, respectively. Observe that H_20_2 administration significantly increased *Wnt11* at 3h and 6h while significantly decreased *Zfhx3* expression at all experimental conditions analyzed. $^*p<0.01$, $^{**}p<0.05$, $^{****}p<0.0001$.
(TIF)

S4 Fig. Analyses of *Prdx3* and *Prdx6* expression in Pitx2 gain and loss-of-function experiments with or without H_20_2 administration for 12h and 24h, respectively. Observe that no significant differences are observed in *Prdx3* and *Prdx6* expression, except for *Prdx6* at 12h after treatment in Pitx2 silencing conditions. $^*p<0.01$.
(TIF)

Author Contributions

Conceptualization: Rosemary Wangensteen, Diego Franco.

Investigation: Estefanía Lozano-Velasco, Andrés Quesada, Carlos Garcia-Padilla, Julia A. Osorio, María Dolores Ruiz-Torres.

Methodology: Estefanía Lozano-Velasco, Andrés Quesada, Carlos Garcia-Padilla, Julia A. Osorio, María Dolores Ruiz-Torres.

Supervision: Rosemary Wangensteen, Diego Franco.

Writing – original draft: Diego Franco.

Writing – review & editing: Amelia Aranega, Diego Franco.

References

1. Hakim FA, Shen WK. Atrial fibrillation in the elderly: a review. *Future Cardiol*. 2014; 10: 745–758. https://doi.org/10.2217/fca.14.32 PMID: 25495816
2. Brugada R, Tapscott T, Czernuszewicz GZ, Marian AJ, Iglesias A, Mont L, et al. Identification of a genetic locus for familial atrial fibrillation. *N Engl J Med*. 1997; 336: 905–911. https://doi.org/10.1056/NEJM199703273361302 PMID: 9070470
3. Chen WJ, Yeh YH, Lin KH, Chang GJ, Kuo CT. Molecular characterization of thyroid hormone-inhibited atrial L-type calcium channel expression: implication for atrial fibrillation in hyperthyroidism. *Basic Res Cardiol*. 2011; 106:163–74. https://doi.org/10.1007/s00395-010-0149-5 PMID: 21234757
4. Yang Y, Xia M, Jin Q, Bendahhou S, Shi J, Chen Y, et al. Identification of a KCNE2 gain-of-function mutation in patients with familial atrial fibrillation. *Am J Hum Genet*. 2004; 75:899–905. https://doi.org/10.1086/425342 PMID: 15368194
5. Xia M, Jin Q, Bendahhou S, He Y, Larroque MM, Chen Y, et al. A Kir2.1 gain-of-function mutation underlies familial atrial fibrillation. *Biochem Biophys Res Commun*. 2005; 332:1012–1019. https://doi.org/10.1016/j.bbrc.2005.05.054 PMID: 15922306
6. Gudbjartsson DF, Arnar DO, Helgadottir A, Gretarsdottir S, Holm H, Sigurdsson A, et al. Variants conferring risk of atrial fibrillation on chromosome 4q25. *Nature*. 2007; 448:353–357. https://doi.org/10.1038/nature06007 PMID: 17603472
7. Benjamin EJ, Rice KM, Arking DE, Pfeufer A, van Noord C, Smith AV, et al. Variants in ZFHX3 are associated with atrial fibrillation in individuals of European ancestry. *Nat Genet*. 2009; 41:879–881. https://doi.org/10.1038/ng.416 PMID: 19597492
8. Gudbjartsson DF, Holm H, Gretarsdottir S, Thorleifsson G, Walters GB, Thorgeirsson G, et al. A sequence variant in ZFHX3 on 16q22 associates with atrial fibrillation and ischemic stroke. *Nat Genet*. 2009; 41:876–878. https://doi.org/10.1038/ng.417 PMID: 19597491
9. Ellinor PT, Lunetta KL, Glazer NL, Pfeufer A, Alonso A, Chung MK, et al. Common variants in KCNN3 are associated with lone atrial fibrillation. *Nat Genet*. 2010; 42:240–244. https://doi.org/10.1038/ng.537 PMID: 20173747
10. Schnabel RB, Kerr KF, Lubitz SA, Alkylbekova EL, Marcus GM, Sinner MF, et al. Atrial Fibrillation/Electrocardiography Working Group. Large-scale candidate gene analysis in whites and African

Americans identifies IL6R polymorphism in relation to atrial fibrillation: the National Heart, Lung, and Blood Institute's Candidate Gene Association Resource (CARe) project. *Circ Cardiovasc Genet*. 2011; 4:557–564. https://doi.org/10.1161/CIRCGENETICS.110.959197 PMID: 21846873

11. Aguirre LA, Alonso ME, Badía-Careaga C, Rollán I, Arias C, Fernández-Miñán A, et al. Long-range regulatory interactions at the 4q25 atrial fibrillation risk locus involve PITX2c and ENPEP. *BMC Biol*. 2015; 13:26–. https://doi.org/10.1186/s12915-015-0138-0 PMID: 25888893
12. Wang J, Bai Y, Li N, Ye W, Zhang M, Greene SB,et al. Pitx2-microRNA pathway that delimits sinoatrial node development and inhibits predisposition to atrial fibrillation. *PNAS* 2014; 111:9181–9186. https://doi.org/10.1073/pnas.1405411111 PMID: 24927531
13. Kirchhof P, Kahr PC, Kaese S, Piccini I, Vokshi I, Scheld HH, et al. PITX2c is expressed in the adult left atrium, and reducing Pitx2c expression promotes atrial fibrillation inducibility and complex changes in gene expression. *Circ Cardiovasc Genet*. 2011; 4:123–133. https://doi.org/10.1161/CIRCGENETICS.110.958058 PMID: 21282332
14. Chinchilla A, Daimi H, Lozano-Velasco E, Dominguez JN, Caballero R, Delpón E, et al. PITX2 insufficiency leads to atrial electrical and structural remodeling linked to arrhythmogenesis. *Circ Cardiovasc Genet*. 2011; 4:269–279. https://doi.org/10.1161/CIRCGENETICS.110.958116 PMID: 21511879
15. Tao Y, Zhang M, Li L, Bai Y, Zhou Y, Moon AM, et al. Pitx2, an atrial fibrillation predisposition gene, directly regulates ion transport and intercalated disc genes. Circ Cardiovasc Genet. 2014; 7:23–32. https://doi.org/10.1161/CIRCGENETICS.113.000259 PMID: 24395921
16. Lozano-Velasco E, Hernández-Torres F, Daimi H, Serra SA, Herraiz A, Hove-Madsen L, et al. Pitx2 impairs calcium handling in a dose-dependent manner by modulating Wnt signalling. *Cardiovasc Res*. 2016; 109:55–66. https://doi.org/10.1093/cvr/cvv207 PMID: 26243430
17. Berenfeld O, Jalife J. Mechanisms of atrial fibrillation: rotors, ionic determinants, and excitation frequency. *Cardiol Clin*. 2014; 32:495–506. https://doi.org/10.1016/j.ccl.2014.07.001 PMID: 25443232
18. Jalife J, Kaur K. Atrial remodeling, fibrosis, and atrial fibrillation. *Trends Cardiovasc Med*. 2015; 25:475–484. https://doi.org/10.1016/j.tcm.2014.12.015 PMID: 25661032
19. Wolke C, Bukowska A, Goette A, Lendeckel U. Redox control of cardiac remodeling in atrial fibrillation. *Biochim Biophys Acta*. 2015; 1850:1555–1565 https://doi.org/10.1016/j.bbagen.2014.12.012 PMID: 25513966
20. Reilly SN, Liu X, Carnicer R, Recalde A, Muszkiewicz A, Jayaram R, et al. Up-regulation of miR-31 in human atrial fibrillation begets the arrhythmia by depleting dystrophin and neuronal nitric oxide synthase. *Sci Transl Med*. 2016; 8:340ra74. https://doi.org/10.1126/scitranslmed.aac4296 PMID: 27225184
21. Dzeshka MS, Lip GY, Snezhitskiy V, Shantsila E. Cardiac Fibrosis in Patients With Atrial Fibrillation: Mechanisms and Clinical Implications. *J Am Coll Cardiol*. 2015; 66:943–959. https://doi.org/10.1016/j.jacc.2015.06.1313 PMID: 26293766
22. Zhang Q, Liu T, Ng CY, Li G. Diabetes mellitus and atrial remodeling: mechanisms and potential upstream therapies. *Cardiovasc Ther*. 2014; 32:233–41. https://doi.org/10.1111/1755-5922.12089 PMID: 25065462
23. Anumonwo JM, Kalifa J. Risk factors and genetics of atrial fibrillation. *Cardiol Clin*. 2014; 32:485–494. https://doi.org/10.1016/j.ccl.2014.07.007 PMID: 25443231
24. Goudis CA, Korantzopoulos P, Ntalas IV, Kallergis EM, Ketikoglou DG. Obesity and atrial fibrillation: A comprehensive review of the pathophysiological mechanisms and links. *J Cardiol*. 2015;S0914–5087
25. Yadava M, Hughey AB, Crawford TC. Postoperative atrial fibrillation: incidence, mechanisms, and clinical correlates. *Cardiol Clin*. 2014; 32:627–636. https://doi.org/10.1016/j.ccl.2014.07.002 PMID: 25443241
26. Kumar KR, Mandleywala SN, Link MS. Atrial and ventricular arrhythmias in hypertrophic cardiomyopathy. *Card Electrophysiol Clin*. 2015; 7:173–186. https://doi.org/10.1016/j.ccep.2015.03.002 PMID: 26002384
27. Scridon A, Fouilloux-Meugnier E, Loizon E, Rome S, Julien C, Barrès C, et al. Long-standing arterial hypertension is associated with Pitx2 down-regulation in a rat model of spontaneous atrial tachyarrhythmias. *Europace* 2015; 17:160–165. https://doi.org/10.1093/europace/euu139 PMID: 24908044
28. L'honoré A, Commère PH, Ouimette JF, Montarras D, Drouin J, Buckingham M. Redox regulation by Pitx2 and Pitx3 is critical for fetal myogenesis. *Dev Cell*. 2014; 29:392–405. https://doi.org/10.1016/j.devcel.2014.04.006 PMID: 24871946
29. Tao G, Kahr PC, Morikawa Y, Zhang M, Rahmani M, Heallen TR, et al. Pitx2 promotes heart repair by activating the antioxidant response after cardiac injury. *Nature*. 2016; 534:119–23. https://doi.org/10.1038/nature17959 PMID: 27251288

30. Gage PJ, Suh H, Camper SA. Dosage requirement of Pitx2 for development of multiple organs. *Development*. 1999; 126:4643–4651. PMID: 10498698

31. de Lange FJ, Moorman AF, Christoffels VM. Atrial cardiomyocyte-specific expression of Cre recombinase driven by an Nppa gene fragment. *Genesis*. 2003; 37:1–4. https://doi.org/10.1002/gene.10220 PMID: 14502570

32. Rodríguez-Gómez I, Banegas I, Wangensteen R, Quesada A, Jiménez R, Gómez-Morales M, et al. Influence of thyroid state on cardiac and renal capillary density and glomerular morphology in rats. *J Endocrinol*. 2013; 216:43–51. https://doi.org/10.1530/JOE-12-0208 PMID: 23048210

33. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 2001; 25:402–408. https://doi.org/10.1006/meth.2001.1262 PMID: 11846609

34. Lozano-Velasco E, Chinchilla A, Martínez-Fernández S, Hernández-Torres F, Navarro F, Lyons GE, et al. Pitx2c modulates cardiac-specific transcription factors networks in differentiating cardiomyocytes from murine embryonic stem cells. *Cells Tissues Organs*. 2011; 194:349–62. https://doi.org/10.1159/000323533 PMID: 21389672

35. Daimi H, Lozano-Velasco E, Haj Khelil A, Chibani JB, Barana A, Amorós I, et al. Regulation of SCN5A by microRNAs: miR-219 modulates SCN5A transcript expression and the effects of flecainide intoxication in mice. *Heart Rhythm*. 2015; 12:1333–42. https://doi.org/10.1016/j.hrthm.2015.02.018 PMID: 25701775

36. Wang YG, Dedkova EN, Fiening JP, Ojamaa K, Blatter LA, Lipsius SL. Acute exposure to thyroid hormone increases Na+ current and intracellular Ca2+ in cat atrial myocytes. J Physiol. 2003 Jan 15; 546 (Pt 2):491–9. https://doi.org/10.1113/jphysiol.2002.032847 PMID: 12527735

37. Philippen LE, Dirkx E, da Costa-Martins PA, De Windt LJ. Non-coding RNA in control of gene regulatory programs in cardiac development and disease. *J Mol Cell Cardiol*. 2015; 89:51–58. https://doi.org/10.1016/j.yjmcc.2015.03.014 PMID: 25820097

38. Okamoto AK.Development of a strain of spontaneously hypertensive rat. *Jap Circ J* 1963; 27:282–293. PMID: 13939773

39. Youn JY, Zhang J, Zhang Y, Chen H, Liu D, Ping P, et al. Oxidative stress in atrial fibrillation: an emerging role of NADPH oxidase. *J Mol Cell Cardiol*. 2013; 62:72–9. https://doi.org/10.1016/j.yjmcc.2013.04.019 PMID: 23643589

40. Violi F, Pastori D, Pignatelli P, Loffredo L. Antioxidants for prevention of atrial fibrillation: a potentially useful future therapeutic approach? A review of the literature and meta-analysis. *Europace*. 2014; 16:1107–1116. https://doi.org/10.1093/europace/euu040 PMID: 24706090

41. Biondi B, Kahaly GJ. Cardiovascular involvement in patients with different causes of hyperthyroidism. *Nat Rev Endocrinol*. 2010; 6:431–443. https://doi.org/10.1038/nrendo.2010.105 PMID: 20585347

42. Grais IM, Sowers JR. Thyroid and the heart. *Am J Med*. 2014; 127:691–8. https://doi.org/10.1016/j.amjmed.2014.03.009 PMID: 24662620

43. Kreuzberg U, Theissen P, Schicha H, Schröder F, Mehlhorn U, de Vivie ER, et al. Single-channel activity and expression of atrial L-type Ca(2+) channels in patients with latent hyperthyroidism. *Am J Physiol Heart Circ Physiol*. 2000; 278:H723–30. PMID: 10710339

44. Arai M, Otsu K, MacLennan DH, Alpert NR, Periasamy M. Effect of thyroid hormone on the expression of mRNA encoding sarcoplasmic reticulum proteins. *Circ Res*. 1991; 69:266–276. PMID: 1830516

45. Shimoni Y, Fiset C, Clark RB, Dixon JE, McKinnon D, Giles WR. Thyroid hormone regulates postnatal expression of transient K+ channel isoforms in rat ventricle. *J Physiol*. 1997; 500:65–73. PMID: 9097933

46. Mager S, Palti Y, Binah O. Mechanism of hyperthyroidism-induced modulation of the L-type Ca2+ current in guinea pig ventricular myocytes. *Pflugers Arch*. 1992; 421:425–430. PMID: 1334256

47. Jiang M, Xu A, Tokmakejian S, Narayanan N. Thyroid hormone-induced overexpression of functional ryanodine receptors in the rabbit heart. *Am J Physiol Heart Circ Physiol*. 2000; 278:H1429–1438. PMID: 10775119

48. Dudley SC Jr, Baumgarten CM. Bursting of cardiac sodium channels after acute exposure to 3,5,3'-triiodo-L-thyronine. *Circ Res*. 1993; 73:301–313. PMID: 8392449

49. Emdin CA, Callender T, Cao J, Rahimi K. Effect of antihypertensive agents on risk of atrial fibrillation: a meta-analysis of large-scale randomized trials. *Europace*. 2015; 17:701–710. https://doi.org/10.1093/europace/euv021 PMID: 25855678

50. Joseph BK, Thakali KM, Moore CL, Rhee SW. Ion channel remodeling in vascular smooth muscle during hypertension: Implications for novel therapeutic approaches. *Pharmacol Res*. 2013; 70:126–138. https://doi.org/10.1016/j.phrs.2013.01.008 PMID: 23376354

51. Pabbidi MR, Mazur O, Fan F, Farley JM, Gebremedhin D, Harder DR, et al. Enhanced large conductance K+ channel activity contributes to the impaired myogenic response in the cerebral vasculature of Fawn Hooded Hypertensive rats. *Am J Physiol Heart Circ Physiol*. 2014; 306:H989–H1000. https://doi.org/10.1152/ajpheart.00636.2013 PMID: 24464756

52. Montaigne D, Marechal X, Lefebvre P, Modine T, Fayad G, Dehondt H, et al. Mitochondrial dysfunction as an arrhythmogenic substrate: a translational proof-of-concept study in patients with metabolic syndrome in whom post-operative atrial fibrillation develops. *JACC*. 2013; 62:1466–1473. https://doi.org/10.1016/j.jacc.2013.03.061 PMID: 23644086

53. Anderson EJ, Efird JT, Davies SW, O'Neal WT, Darden TM, Thayne KA, et al. Monoamine oxidase is a major determinant of redox balancein human atrial myocardium and is associated with postoperative atrial fibrillation. *JAHA*. 2014; 3:e000713. https://doi.org/10.1161/JAHA.113.000713 PMID: 24572256

54. Martínez-González MÁ, Toledo E, Arós F, Fiol M, Corella D, Salas-Salvadó J, et al. Extravirgin olive oil consumption reduces risk of atrial fibrillation: the PREDIMED (Prevención con Dieta Mediterránea) trial. *Circulation*. 2014; 130:18–26. https://doi.org/10.1161/CIRCULATIONAHA.113.006921 PMID: 24787471

55. Zima AV, Blatter LA. Redox regulation of cardiac calcium channels and transporters. *Cardiovasc Res*. 2006; 71:310–21. https://doi.org/10.1016/j.cardiores.2006.02.019 PMID: 16581043

56. Wagner S, Rokita AG, Anderson ME, Maier LS. Redox regulation of sodium and calcium handling. *Antioxid Redox Signal*. 2013; 18:1063–77. https://doi.org/10.1089/ars.2012.4818 PMID: 22900788

57. Aggarwal NT, Makielski JC. Redox control of cardiac excitability. *Antioxid Redox Signal*. 2013; 18:432–68. https://doi.org/10.1089/ars.2011.4234 PMID: 22897788

58. Franco D, Chinchilla A, Daimi H, Dominguez JN, Aránega A. Modulation of conductive elements by Pitx2 and their impact on atrial arrhythmogenesis. *Cardiovasc Res*. 2011; 91:223–31. https://doi.org/10.1093/cvr/cvr078 PMID: 21427120

59. Laumanns IP, Fink L, Wilhelm J, Wolff JC, Mitnacht-Kraus R, Graef-Hoechst S, et al. The noncanonical WNT pathway is operative in idiopathic pulmonary arterial hypertension. *Am J Respir Cell Mol Biol*. 2009 40(6):683–91. https://doi.org/10.1165/rcmb.2008-0153OC PMID: 19029018

60. Terentyev D, Belevych AE, Terentyeva R, Martin MM, Malana GE, Kuhn DE, et al. miR-1 overexpression enhances Ca(2+) release and promotes cardiac arrhythmogenesis by targeting PP2A regulatory subunit B56alpha and causing CaMKII-dependent hyperphosphorylation of RyR2. *Circ Res*. 2009; 104:514–21. https://doi.org/10.1161/CIRCRESAHA.108.181651 PMID: 19131648

61. Jia X, Zheng S, Xie X, Zhang Y, Wang W, Wang Z, et al. MicroRNA-1 accelerates the shortening of atrial effective refractory period by regulating KCNE1 and KCNB2 expression: an atrial tachypacing rabbit model. *PLoS One*. 2013; 8:e85639. https://doi.org/10.1371/journal.pone.0085639 PMID: 24386485

62. Liao C, Gui Y, Guo Y, Xu D. The regulatory function of microRNA-1 in arrhythmias. *Mol Biosyst*. 2016; 12:328–33. https://doi.org/10.1039/c5mb00806a PMID: 26671473

63. Luo X, Pan Z, Shan H, Xiao J, Sun X, Wang N, et al. MicroRNA-26 governs profibrillatory inward-rectifier potassium current changes in atrial fibrillation. *J Clin Invest*. 2013; 123:1939–1951. https://doi.org/10.1172/JCI62185 PMID: 23543060

64. Daimi H, Lozano-Velasco E, Haj Khelil A, Chibani JB, Barana A, Amorós I, et al. Regulation of SCN5A by microRNAs: miR-219 modulates SCN5A transcript expression and the effects of flecainide intoxication in mice. *Heart Rhythm*. 2015; 12:1333–1342. https://doi.org/10.1016/j.hrthm.2015.02.018 PMID: 25701775

65. Shan H, Zhang Y, Cai B, Chen X, Fan Y, Yang L, et al. Upregulation of microRNA-1 and microRNA-133 contributes to arsenic-induced cardiac electrical remodeling. *Int J Cardiol*. 2013; 167:2798–2805. https://doi.org/10.1016/j.ijcard.2012.07.009 PMID: 22889704

66. Li YD, Hong YF, Yusufuaji Y, Tang BP, Zhou XH, Xu GJ, et al. Altered expression of hyperpolarization-activated cyclic nucleotide-gated channels and microRNA-1 and -133 in patients with age-associated atrial fibrillation. *Mol Med Rep*. 2015; 12:3243–8. https://doi.org/10.3892/mmr.2015.3831 PMID: 26005035

67. Köhler AC, Sag CM, Maier LS. Reactive oxygen species and excitation-contraction coupling in the context of cardiac pathology. *J Mol Cell Cardiol*. 2014; 73:92–102. https://doi.org/10.1016/j.yjmcc.2014.03.001 PMID: 24631768

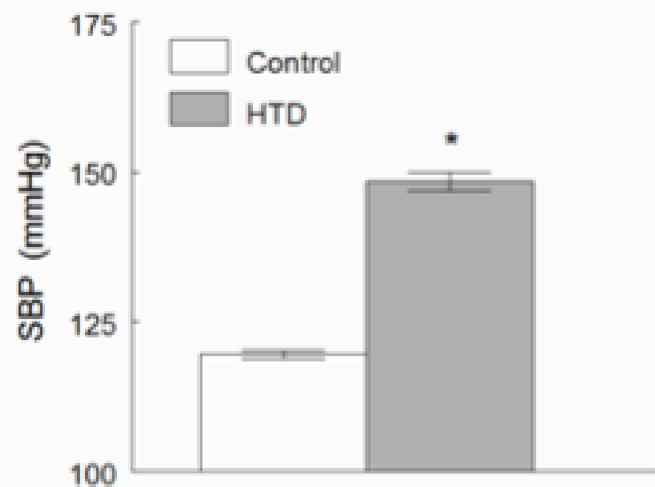
68. Cheng Y, Liu X, Zhang S, Lin Y, Yang J, Zhang C. MicroRNA-21 protects against the H(2)O(2)-induced injury on cardiac myocytes via its target gene PDCD4. *J Mol Cell Cardiol*. 2009; 47:5–14. https://doi.org/10.1016/j.yjmcc.2009.01.008 PMID: 19336275

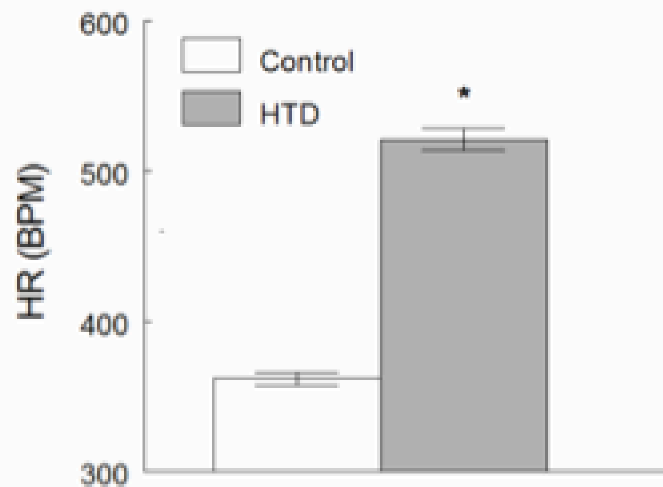
Table 1. Lozano-Velasco et al.

Gene	Specie	Oligo	Sequences
Atp2a2 (NM_001110139.2)	*Rattus novergicus*	Rn_Atp2a2_F	CTGTCCATGTCCCTCCACTT
		Rn_Atp2a2_R	CCTCCAGATAGTTCCGAGCA
Camk2a (NM_012519.2)	*Rattus novergicus*	Rn_Camk2a_F	TGGAAGGGATGGACTTTCAC
		Rn_Camk2a_R	ATTCTGCCACTTCCCATCAC
Cat (NM_012520.2)	*Rattus novergicus*	Rn_Cat_F	GAGAACATTGCCAACCACCT
		Rn_Cat_R	CTTTTCCCTTGGCAGCTATG
Casq2 (NM_017131.2)	*Rattus novergicus*	Rn_Casq2_F	TGGCTATGAGTTCCTGGAGA
		Rn_Casq2_R	CATTCACCACCCCAATCTGT
Enpep (NM_022251.2)	*Rattus novergicus*	Rn_Enpep_F	AAATGCTCAAAGACCCCAAT
		Rn_Enpep_R	CTGCAGCTCAGTGTTGAAGG
Gapdh (NM_017008.4)	*Rattus novergicus*	Rn_Gapdh_F	TCCCATTCTTCCACCTTTGA
		Rn_Gapdh_R	CCAGGGTTTCTTACTCCTTGG
Gpx1 (NM_030826.4)	*Rattus novergicus*	Rn_Gpx1_F	GTCCACCGTGTATGCCTTCT
		Rn_Gpx1_R	GAACTGATTGCACGGGAAAC
Gsr (NM_053906.2)	*Rattus novergicus*	Rn_Gsr_F	GGCCATATCCTAGTGGACGA
		Rn_Gsr_R	AGACCACGGTAGGGATGTTG
GusB (NM_017015.2)	*Rattus novergicus*	Rn_GusB_F	AGATGTACCAGAAGCCAATTATCC
		Rn_GusB_R	ATTCTTTTCGTTTCTCATCCAG
Kcnj2 (NM_017296.1)	*Rattus novergicus*	Rn_Kcnj2_F	TGAAAATGAAGTTGCCCTAACA
		Rn_Kcnj2_R	TCTCCGATTCTCGCCTTAAA
Kcnj12 (NM_053981.2)	*Rattus novergicus*	Rn_Kcnj12_F	GCCACTGACCGAGAAGTGC
		Rn_Kcnj12_R	GGCGCCTGACATGGTAAC
Kcnn3 (NM_019315.2)	*Rattus novergicus*	Rn_Kcnn3_F	ACCAACTGAGGGGTGTCAAG
		Rn_Kcnn3_R	ATTGAAGCTGGCTGTGAGGT
Pitx2 (NM_019334.2)	*Rattus novergicus*	Rn_Pitx2_F	CTGGAAGCCACTTTCCAGAG
		Rn_Pitx2_R	CAGCCCAATTGTTGTACGAG
Pln (NM_022707.2)	*Rattus novergicus*	Rn_Pln_F	GCTGAGCTCCCAGACTTCAC
		Rn_Pln_R	CATGATGCCAGGAAGACAAA
Ryr2 (NM_001191043.1)	*Rattus novergicus*	Rn_Ryr2_F	CTGTGGGATAGGCAACGACT
		Rn_Ryr2_R	GGGAAAAATTCCCAACACCT
Scn1b (NM_001271045.1)	*Rattus novergicus*	Rn_Scn1b_F	TATGGCATCCATCGTGTCAG
		Rn_Scn1b_R	CGCCTGTACAGTTCTCTTTGC
Scn5a (NM_001160162.1)	*Rattus novergicus*	Rn_Scn5a_F	ATGACGGAGGAGCAGAAGAA
		Rn_Scn5a_R	GGAACATGATGGTGACATCG
Sod1 (NM_017050.1)	*Rattus novergicus*	Rn_Sod1_F	CCACTGCAGGACCTCATTTT
		Rn_Sod1_R	CACCTTTGCCCAAGTCATCT
Sod2 (NM_017051.2)	*Rattus novergicus*	Rn_Sod2_F	CCGAGGAGAAGTACCACGAG
		Rn_Sod2_R	GCTTGATAGCCTCCAGCAAC
Wnt8a (NM_001106155.1)	*Rattus novergicus*	Rn_Wnt8a_F	CTGTGGCTGTGATGAGTCAAA
		Rn_Wnt8a_R	CAGCCCTGTTGTTGTGAAGA
Wnt11 (NM_080401.1)	*Rattus novergicus*	Rn_Wnt11_F	CAGGATCCCAAGCCAATAAA
		Rn_Wnt11_R	TAGGCCGGTGTACCACTTTC
Zfhx3 (XM_008772567.2)	*Rattus novergicus*	Rn_Zfhx3_F	CATGCTCAACAACAAGATCCA
		Rn_Zfhx3_R	GGACTCTAGACCTAGACACCGAAA
Cacna1c (NM_001159535.2)	*Mus musculus*	Mm_Cacna1c_F	TCCTGGTCTGAGGAGACGAC
		Mm_Cacna1c_R	GGTGGTGACCTCGATGAACT
Camk2a (NM_177407.4)	*Mus musculus*	Mn_Camk2a_F	AATGGCAGATCGTCCACTTC
		Mm_Camk2a_R	TGGCGACTTCTGTGAACAAG
Cat (NM_009804.2)	*Mus musculus*	Mm_Cat_F	ATCCAGGCTCTTCTGGACAA
		Mm_Cat_R	TCCATCCAGCGTTGATTACA
Gapdh (NM_008084.2)	*Mus musculus*	Mm_Gapdh_F	GGCATTGCTCTCAATGACAA
		Mm_Gapdh_R	TGTGAGGGAGATGCTCAGTG
Gusb (NM_010368.1)	*Mus musculus*	Mm_GusB_F	ACGCATCAGAAGCCGATTAT
		Mm_GusB_R	ACTCTCAGCGGTGACTGGTT

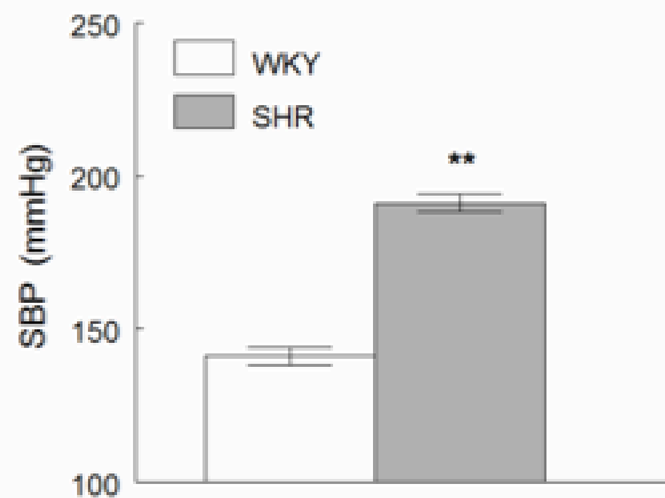
Hcn4 (NM_001081192.1)	*Mus musculus*	Mm_Hcn4_F	CAGCGTCAGAGCGGATACTT
		Mm_Hcn4_R	TGTGGAGGAGGATGGAGTTC
Kcnj2 (NM_008425.4)	*Mus musculus*	Mm_Kcnj2_F	TTGCTTCGGCTCATTCTCTT
		Mm_Kcnj2_F	AGAGATGGATGCTTCCGAGA
Pitx2c (NM_001042502.1)	*Mus musculus*	Mm_Pitx2c_F	CCTCACCCTTCTGTCACCAT
		Mm_Pitx2c_R	GCCCACATCCTCATTCTTTC
Prdx2 (NM_001317385.1)	*Mus musculus*	Mm_Prdx2_F	CTTCGCCAGATCACAGTCAA
		Mm_Prdx2_R	AAATCCAAGCTTCAGGCTCA
Prdx3 (NM_007452.2)	*Mus musculus*	Mm_Prdx3_F	TGGACACCAGAGTCCCCTAC
		Mm_Prdx3_R	TCAAGGCATTGGAAGGATTC
Prdx5 (NM_012021.2)	*Mus musculus*	Mm_Prdx5_F	TGGGAAGGCGACAGACTTAT
		Mm_Prdx5_R	CAGGGCCTCAGAGTTGAGAG
Prdx6 (NM_007453.4)	*Mus musculus*	Mm_Prdx6_F	TTTTGGCCCTGACAAGAAAC
		Mm_Prdx6_R	GAGGGTGGGAACTACCATCA
Ryr2 (NM_023868.2)	*Mus musculus*	Mm_Ryr2_F	TGGGATTGGCAACGATTATT
		Mm_Ryr2_R	GGGAAAAATTCCCAACACCT
Scn5a (NM_021544.4)	*Mus musculus*	Mm_Scn5a_F	CTTCACCAACAGCTGGAACA
		Mm_Scn5a_R	CATGACGAGGAAGAGGAGGA
Sod2 (NM_013671.3)	*Mus musculus*	Mm_Sod2_F	GCAAGGAACAACAGGCCTTA
		Mm_Sod2_R	AGCACCCCAGTCATAGTGCT
Wnt8a (NM_009290.2)	*Mus musculus*	Mm_Wnt8a_F	TTCGTGGACAGTTTGGAGAA
		Mm_Wnt8a_R	GCGGTCATACTTGGCCTTTA
Wnt11 (NM_001285792.1)	*Mus musculus*	Mm_Wnt11_F	GGCCTGTGAAGGACTCAGAA
		Mm_Wnt11_R	ACCACTCTGTCCGTGTAGGG
Zfhx3 (XM_006530585.3)	*Mus musculus*	Mm_Zfhx3_F	GACTGGCAGCTCAACAACAA
		Mm_Zfhx3_R	CCATCAGGTTCGGTTTAGGA
miR-1	*Mus musculus*	hsa-miR-1-3p	UGGAAUGUAAAGAAGUAUGUAU
miR-29a	*Mus musculus*	hsa-miR-29a-3p	UAGCACCAUCUGAAAUCGGUUA
miR-106b	*Mus musculus*	hsa-miR-106b-5p	UAAAGUGCUGACAGUGCAGAU
miR-200a	*Mus musculus*	hsa-miR-200a-3p	UAACACUGUCUGGUAACGAUGU
siEnpep	*Mus musculus*	siEnpep_sense	S 5’: GAUAGUUUAAGACCGAUCA
		siEnpep_asense	AS 3’: CUAUCAAAUUCUGGCUAGU
siSod2	*Mus musculus*	siSod2_sense	S 5’: CUUUCUCAGUAGCGGCAAA
		siSod2_asense	AS 3’: GAAAGAGUCAUCGCCGUUU
siPitx2c	*Mus musculus*	siPitx2c_sense	S 5': GUGCAUACAAUCUCCGAUA
		siPitx2c_asense	AS 3': CACGUAUGUUAGAGGCUAU

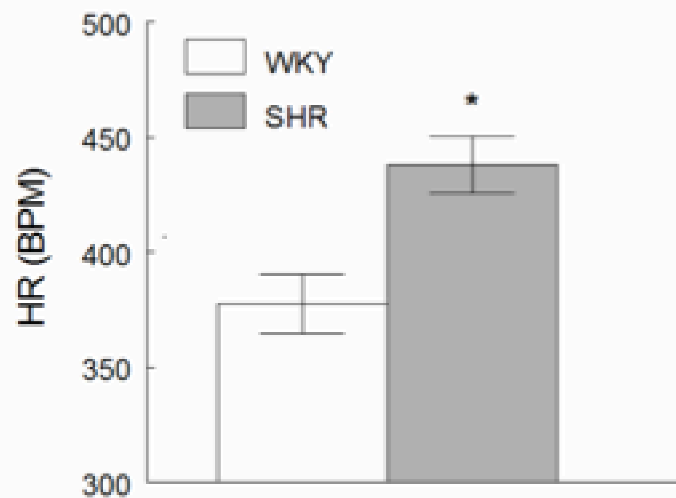
A



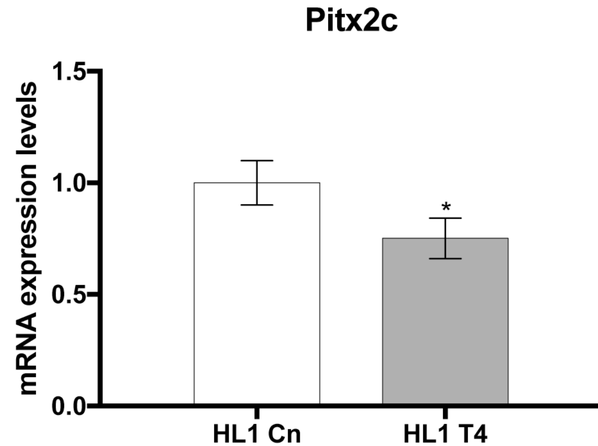


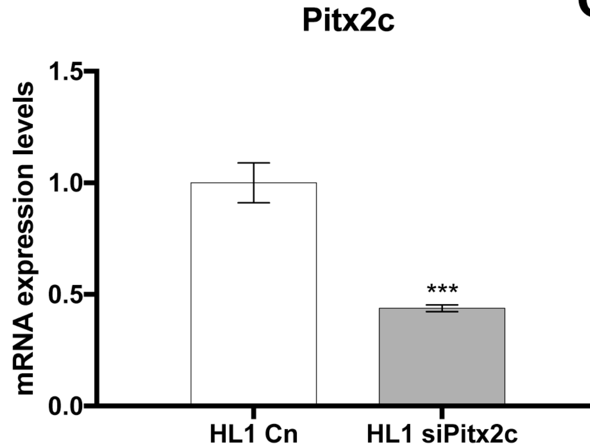
B

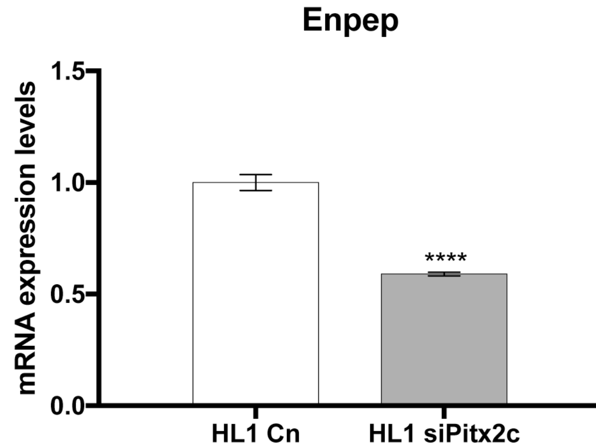


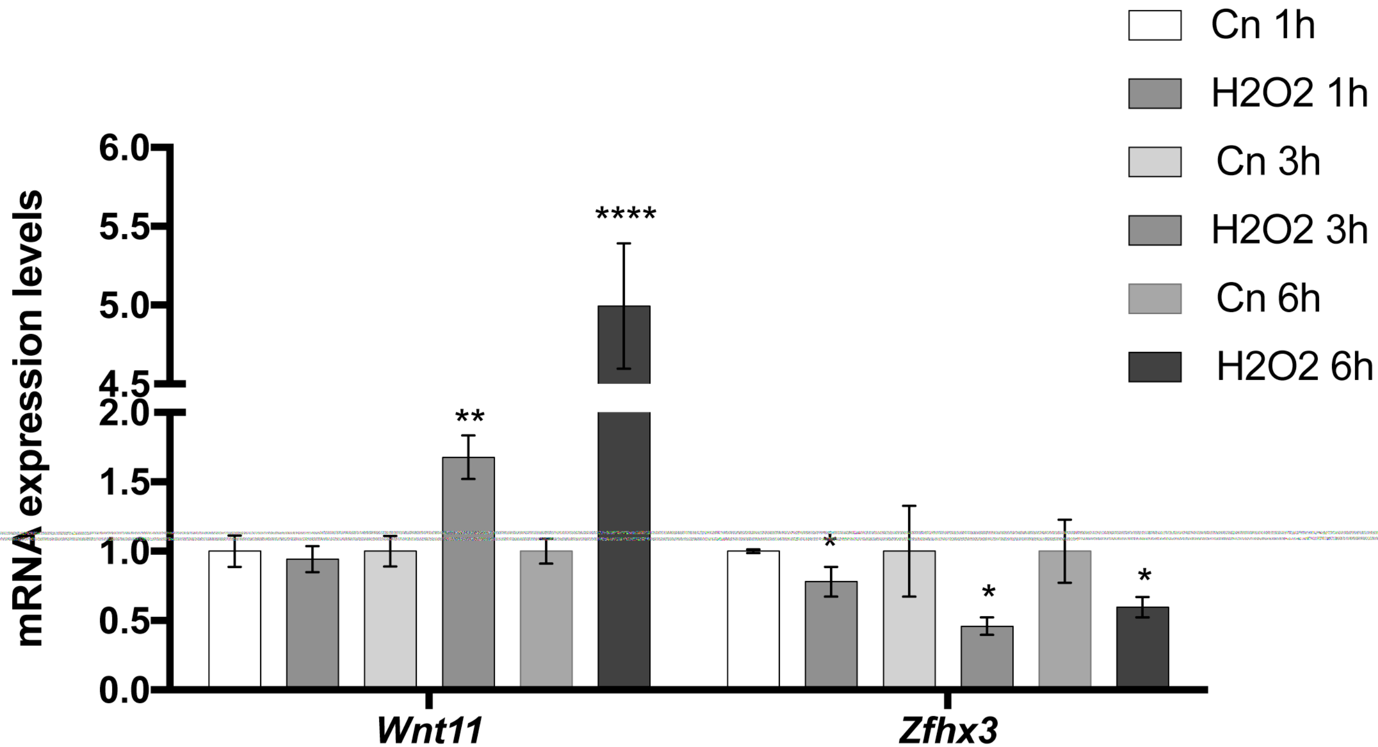


S1 Fig Lozano-Velasco et al.

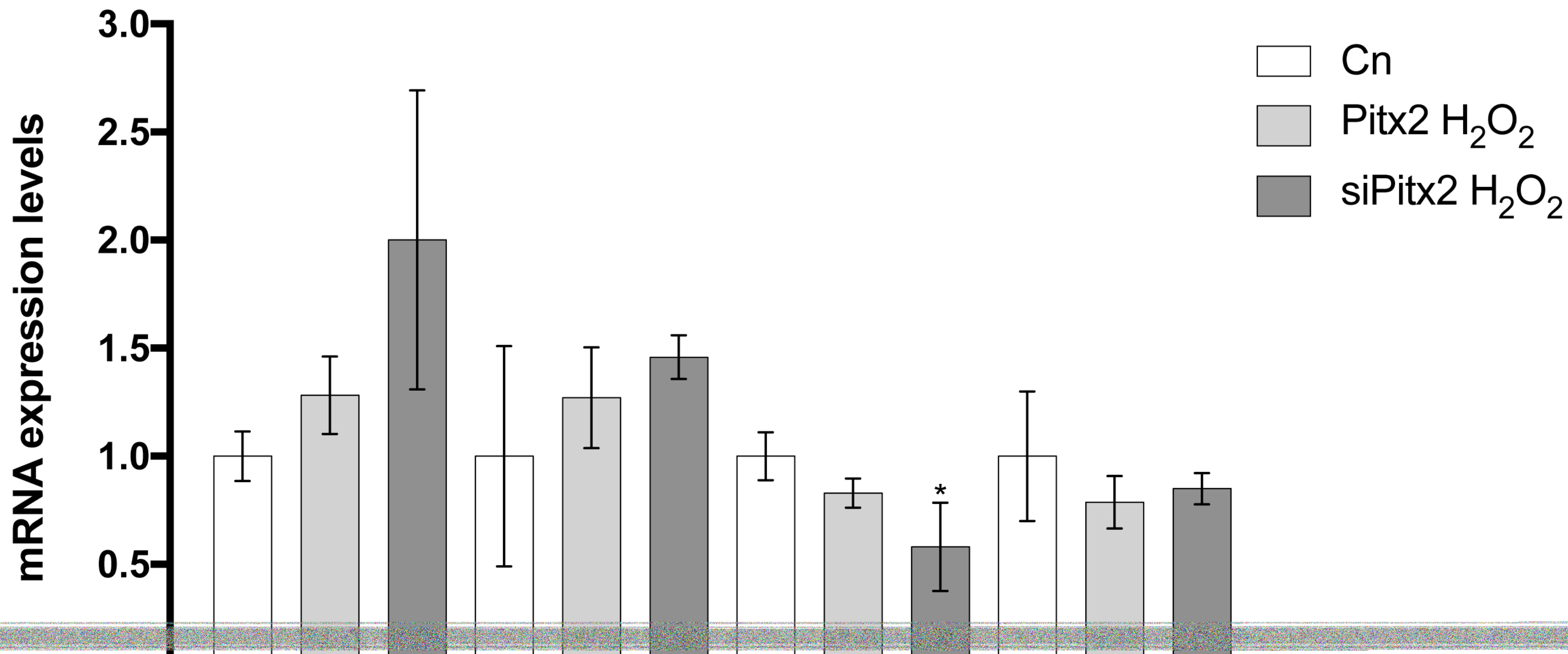

A
Pitx2c
mRNA expression levels
1.5
1.0
0.5
0.0
*
HL1 Cn
HL1 T4


B
Pitx2c
mRNA expression levels
1.5
1.0
0.5
0.0
***
HL1 Cn
HL1 siPitx2c


C
Enpep
mRNA expression levels
1.5
1.0
0.5
0.0
****
HL1 Cn
HL1 siPitx2c



mRNA expression levels
6.0
5.5
5.0
4.5
2.0
1.5
1.0
0.5
0.0
****
**
*
*
*
Wnt11
Zfhx3
Cn 1h
H2O2 1h
Cn 3h
H2O2 3h
Cn 6h
H2O2 6h



mRNA expression levels
3.0
2.5
2.0
1.5
1.0
0.5
*
Cn
Pitx2 H2O2
siPitx2 H2O2

Genetics

http://www.aimspress.com/journal/Genetics

AIMS Genetics, 5(2): 124–140.
DOI: 10.3934/genet.2018.2.124
Received: 30 October 2017
Accepted: 15 March 2018
Published: 26 March 2018

Review

The role of long non-coding RNAs in cardiac development and disease

Carlos García-Padilla, Amelia Aránega and Diego Franco*

Cardiovascular Development Group, Department of Experimental Biology, University of Jaén, Jaén, Spain

* **Correspondence:** Email: dfranco@ujaen.es; Tel: +953212763.

Abstract: Cells display a set of RNA molecules at one time point, reflecting thus the cellular transcriptional steady state, configuring therefore its transcriptome. It is basically composed of two different classes of RNA molecules; protein-coding RNAs (cRNAs) and protein non-coding RNAs (ncRNAs). Sequencing of the human genome and subsequently the ENCODE project identified that more than 80% of the genome is transcribed in some type of RNA. Importantly, only 3% of these transcripts correspond to protein-coding RNAs, pointing that ncRNAs are as important or even more as cRNAs. ncRNAs have pivotal roles in development, differentiation and disease. Non-coding RNAs can be classified into two distinct classes according to their length; i.e., small (<200 nt) and long (>200 nt) noncoding RNAs. The structure, biogenesis and functional roles of small non-coding RNA have been widely studied, particularly for microRNAs (miRNAs). In contrast to microRNAs, our current understanding of long non-coding RNAs (lncRNAs) is limited. In this manuscript, we provide state-of-the art review of the functional roles of long non-coding RNAs during cardiac development as well as an overview of the emerging role of these ncRNAs in distinct cardiac diseases.

Keywords: non coding RNAs; cardiac development; microRNAs; lcnRNAs

1. Introduction

The cell transcriptome can be defined as the set of RNA molecules present on it at one time point, reflecting thus the cellular transcriptional steady state. It is basically composed of two different classes of RNA molecules; protein-coding RNAs (cRNAs) and non-coding RNAs (ncRNAs) (Figure 1). For decades, scientists focused their attention on coding RNAs, while the non-coding RNAs were defined as the “dark matter” of the genome and were not considered important until recently. An exception to the rule was represented by tRNAs and rRNAs, which were widely studied given their

prominent role in protein translation. Sequencing of the human genome and subsequently the ENCODE project identified that more than 80% of the genome is transcribed in some type of RNA. Importantly, only 3% of these transcripts correspond to coding RNAs, pointing that ncRNAs are as important or even more as cRNAs [1,2]. Currently, it has been demonstrated that non-coding RNAs can perform multiple and important cellular functions acting thus as pivotal regulatory elements in development, differentiation and disease [3,4].

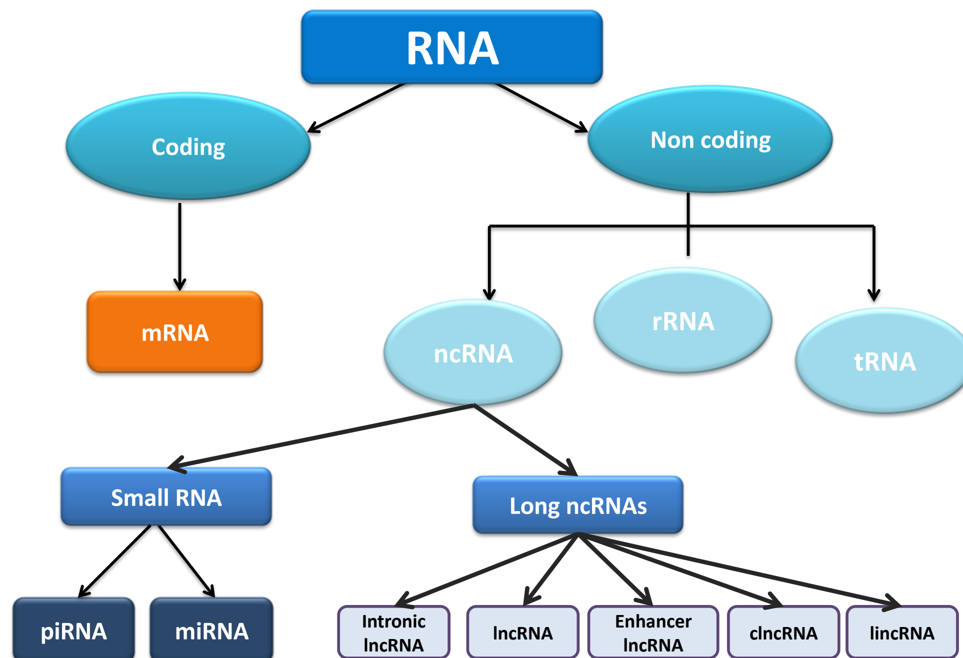


Figure 1. Schematic representation of the distinct classes of RNA molecules.

NcRNAs can be classified into two distinct classes according to their length: (1) Small noncoding RNAs, i.e., smaller than 200 nucleotides; including therein microRNAs, small nucleolar RNAs, piRNAs as well as transfer RNAs, and (2) Long non-coding RNAs, i.e., longer than 200 nucleotides [3], including a extensively variety of types as detailed below (Figure 1). The structure, biogenesis and functional roles of small non-coding RNA have been widely studied, particularly for microRNAs (miRNAs). MiRNAs have an average size of 20–22 nucleotides and act by binding to a target mRNA inducing thereafter degradation and/or inhibition of its translation, and thus negatively regulating gene expression. The functional role of miRNAs have been studied and demonstrated in multiple organisms and within multiple cellular contexts (see for a review; [5,6]).

In contrast to microRNAs, our current understanding of long non-coding RNAs (lncRNAs) is limited. The development of new massive sequencing techniques has led to the discovery and annotation of a large number of long non-coding RNAs. GENCODE annotation initially estimated the existence of 9640 lncRNA genes in the human genome [7] while recently the NONCODE database has increased this number up to 96,308 lncRNA genes [8]. Such estimates indicate that the number of lncRNAs is twice that coding genes, supporting an important role of these lcnRNA

transcripts in multiple biological contexts. Recently, lncRNAs have emerged as major players in regulating gene expression, both at transcriptional and post-transcriptional level and they have been implicated in development, stem cell differentiation, cellular homeostasis and disease [9–11].

2. Structure and cellular localization of lncrnas

Long non-coding RNAs display essentially no potential to code for proteins, although structurally are similar to mRNAs. They are transcribed using the same pathways; i.e., RNA polymerase II, have typical histone modifications, 5' terminal cap and 3' terminal poly(A) tails. LncRNAs are constituted by exons and intronsand are often spliced. Curiously, a minority of non-polyadenylated lncRNAs is transcribed from RNA polymerase III [12]. Unlike mRNAs, lncRNAs have lower number of exons (42% of lncRNA transcripts is composed by two exons compared with 6% protein-coding transcritps) are less conserved between species and on average slightly shorter. Interestingly, lncRNA promoters are more conserved than their exons and in fact almost as conserved as protein-coding gene promoters. Conversely, introns from lncRNAs are longer that those from protein-coding transcripts and are normally flanked by canonical splice sites (GT/AG), showing no differences in splicing signals as compared to protein-coding transcripts [7]. Although the vast majority of lncRNAs are located in the nuclear genome, lcnRNAs are also reported within the mitochondrial DNA. Mitochondrial encoded lncRNAs are transcribed and processed by mitochondrial transcriptional machinery but regulated by nuclear-encoded proteins [13].

Importantly, although lncRNAs they are referred as "non-coding", several lncRNAs contains short ORFs, can be engaged by ribosomes and thus can generate oligopeptides. Until recently, examples of lncRNAs that could generate small peptides were limited to sporadic cases. However recently, it is becoming increasingly acknowledged that a significant fraction of currently annotated lncRNAs is predicted to be capable of generating short peptides [14,15]. Among these long non-coding RNAs coding for small peptides, there are several examples, such as *Toddler* and *Dworf*, which are involved in mesoderm development [14–16].

LncRNAs display low expression levels yet with increased tissue and time specificity as compared to the protein-coding genes [17]. Such specificity suggests an important role of these transcripts in tightly defined cellular events as supported by several reports [18,19]. At the cellular level, lncRNAs can be located both in the cytoplasm and nucleus. Cytoplasmic enriched lncRNAs have mainly a role in post-transcriptional regulation whereas nuclearly located lncRNAs predominantly play a role in transcriptional gene regulation. Importantly, lncRNAs are dynamic molecules that can be located in the nucleus but translocate and act in the cytoplasm [20]. An example of such a dynamic behavior is represented by antisense *Uchl1* lncRNA, that partially overlapping *Uchl1* protein-coding gene, moving from the nucleus to the cytoplasm where it bind the 5' end of *Uchl1* mRNA promoting its translation under stress [1].

Other feature defining lncRNAs is their poor RNA sequence conservation across species, as exemplified by *Braveheart,* a mouse-specific lncRNA involved in early cardiogenesis, as detailed below [21]. However, despite poor conservation between species, the comparison of splice sites suggests that lncRNAs are evolutionarily conserved, showing that the majority of lncRNAs are, as least, as old as mammalian lineage [7].

3. Classification of lncRNAs

Classification of long non-coding RNA species differs between authors but at least five distinct groups can be distinguished. Broadly lcnRNAs are classified according to both, their position within the genome and relative location to neighboring genes. (a) lncRNAs transcribed from the same promotor as the adjacent protein coding gene. They are transcribed in both sense and antisense orientation and can be located in the same strand or opposite strand of protein coding gene. The expression of both is correlated and usually these lncRNAs modulates the expression of adjacent protein coding gene. (b) Long intergenic long non-coding RNAs (lincRNAs) are located between two protein-coding genes usually at distance of approximately 10 kb or in genomic desert as stand-alone genes. Since lincRNAs can be transcribed by their own promoter, they are classified as promoter-associated lncRNAs [22]. (c) LncRNAs can arise from intronic regions of protein coding genes, i.e., intronic lncRNA or enhancer regions, i.e., enhancer-associated lncRNA. There is a subclass of intronic lncRNAs, derived from these, known as sno-lncRNAs. Sno-lncRNAs do not have the typical structure observed in the majority of lncRNAs. They are not capped and nor polyadenilated and are flanked by small nucleolar RNAs at both extremes. The enhancer-associated lncRNAs are transcribed from enhancer region and their expression correlates with the expression of active enhancers. Also the expression enhancer lncRNAs correlate with expression of target genes showing a dynamic and specific patterning throughout differentiation and development [23,24]. (d) Alternative splicing of protein coding genes can generate a circular lncRNA, named circRNAs. These ncRNAs have a great regulatory potential but additional studies are required to fully understand their regulatory mechanisms [25]. For example *Hrcr*, a cardiac enriched circular lncRNA is a protective RNA against distinct molecular mechanisms leading to cardiac hypertrophy [26]. (e) Finally, there is a subclass of lncRNAs harboring microRNAs within their genetic structure, such as H19, which encode miR-675 in its first exon [27].

4. Function role of long non-coding RNAs

Long non-coding RNAs are defined as complex non-coding RNA molecules given their particularities affecting their structure as well as their dynamic expression pattern. Such a complexity is reflected in a wide variety of functions. LcnRNAs can act at both transcriptional and post-transcriptional regulation in multiple cellular processes as detailed below.

At the transcriptional level, lncRNAs can modulate the epigenetic landscape of the cell acting as different class of molecules. Currently, four different types of actions have been demonstrated as detail below. Some lncRNAs acting as guide, binding to transcription factors or protein subunits of chromatin remodeling complexes and direct them, as ribonucleoprotein complex, towards their genomic targets, promoting or suppressing gene activity depending on whether the guided complexes are activate (as MLL complex) or repressive complexes (as PRC2 complex). This class of lncRNAs can act in *cis* (i.e., *Xist*) or *trans* (i.e., *HOTAIR*) [28,29]. For example, *Fendrr,* a cardiac regulatory long non-coding RNA, acts as a guide for PCR2 and Trx/MLL directing them to *Foxf1* and *Pitx2* promoters and setting active and repressive marks [30].

Scaffold lncRNAs can acts as scaffold molecules for different complexes facilitating their assembly and being a functional component of it [31]. For example, *ANRIL*, a long non- coding RNA described as a risk factor for coronary disease acts as platform recruiting and interacting with polycomb complex (PRC1 and PRC2) to the INK4b-ARF-INK4a locus promoting its silencing [32].

Many of lncRNAs are dubbed enhancer lncRNAs since they can act as enhancers of transcription, promoting and maintaining the genomic3D conformation necessary for the transcriptional machinery to get access to promoter regions [33]. Similarly, other long non-coding RNAs can repress the formation of this genomic structures and therefore gene expression. An example of this functional role is *Playrr*, a long non-coding RNA encoded upstream of Pitx2 gene, that represses the expression of this homeodomain transcription factor in asymmetry pathway by interfering with the Pitx2 promoter [34]. The last class of transcriptional regulatory lncRNAs exert their function as decoy molecules competing with transcription factors or chromatin remodeling complexes for their genetic targets. Such interactions avoid that the latter can exert its function and thus indirectly inhibit transcription. *Terra*, telomeric repeat-containing RNA, physically binds to telomerase inverse transcriptase blocking the action of this enzyme and inhibiting telomere elongation [35]. In the cardiovascular context, *Myheart*, a cardiac lncRNA located in the murine myosin heavy chain 7 locus, sequestering BRG1-BAF complex avoiding that this complex can bind to targets [36].

On the other hand, long non-coding RNAs can also regulate gene expression at post-transcriptional level interacting with mRNAs, the translational machinery or other non-coding RNAs such as microRNAs [37]. Some nuclear lncRNAs can participate on pre-mRNA maturation by interacting with pivotal alternative splicing factors. For example, *Malat1* interact with SR (serine/argenine) splicing factors in the nuclear speckle domains and modulates the concentration and distribution of those factors on the nucleus providing proper alternative splicing [38]. As a subclass, these lncRNAs, are dubbed as specific regulators-alternative splicing (sno-lncRNAs). Sno-lncRNAs are located within the nucleolus and in Cajal bodies. This subclass of lncRNAs are associated a several FOX proteins, such as *Fox2* and regulate mRNA alternative splicing in stem cells [39].

Also, long non-coding RNAs can affect mRNA stability by base-pairing with them and altering their half-lives. Depending on efficacy of base pairing, the interaction can promote decoy or mRNA stability. Incomplete base-pairing normally promotes mRNA decoy whereas a full base pairing between both usually promotes mRNA stability and thus protein translation [40–43].

Interestingly many lncRNAs are associated with ribosomes and can therefore affect mRNA translation. For example, *LincRNA-21*, an lncRNA co-distributed with ribosomes, represses the translation of different mRNA targets by base pairing at distinct transcript regions, including coding and non-coding regions of mRNAs. This incorrect base pairing generates a complex between *LincRNA-21*target mRNA that interacts with different translation repressors [43]. Also, distinct lncRNAs can interact with the translation machinery by modulating its function. LcnRNA*BC1* interacts, through 3'UTR region, with several translation repressors, inhibiting the assembly of translation initiation complex [44]. On the contrary, *Uchl1*, an antisense lncRNA of *Uchl1* gene, promotes the active polysome generation at the *Uchl1* mRNA enhancing its translation [45]. In the cardiac context, some cytoplasmic lncRNAs act as decoy of mRNA such as *Hcrc* or *Chrr*. Also, lncRNAs can interact with the translation machinery by modulating this. So, BC1 interacts, through 3' untranslated region, with several represses translation inhibiting the assembly of translation initiation complex [44].

Finally, several lncRNAs can act upon microRNAs interacting with them and modulating post-transcriptional gene expression. *Linc-MD1* can sequester miR-133 and miR-135, enhancing the expression of their target mRNAs in skeletal muscle, such as *Maml1* and *Mef2c*, respectively, among others [46]. In addition, several lncRNAs harboring microRNAs in their genome structure have been described. For example, *H19* contains miR-675 in his first exon. Interestingly, the expression of this microRNA is regulated by *H19* [47].

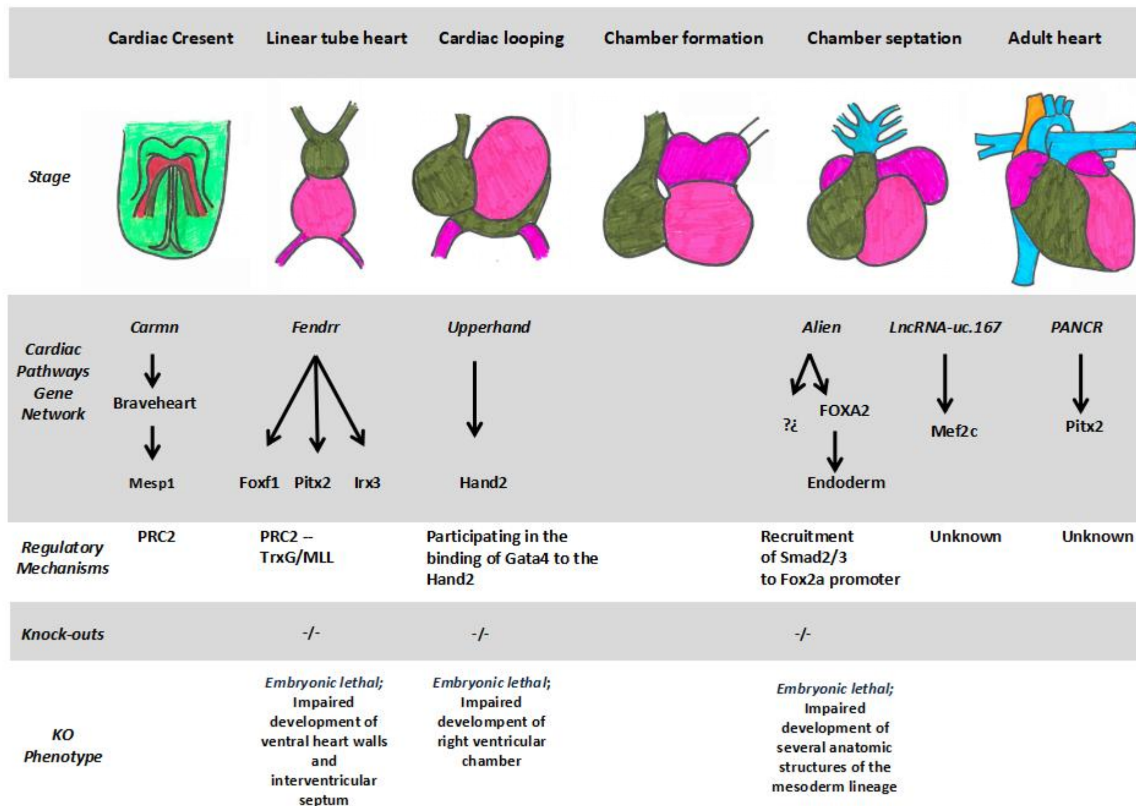


Figure 2. Schematic representation of the distinct stages of heart development from the bilateral sets of precardiac mesoderm (cardiac crescents) to the adult stage, illustrating the distinct long non-coding RNAs described to date and their corresponding molecular signaling pathways. Additional, if knock-out mice are available, the corresponding phenotype is briefly summarized.

5. The role of long non-coding RNA in cardiac development

The development of the heart is a complex morphogenetic process and thus highly regulated. The developing heart arises from sets of precursor cells during gastrulation (Figure 2) [48]. These cells progressively converge in the midline of the embryo, forming the early tubular heart [49]. The tubular heart grows and suffers a rightward displacement, a phenomenon dubbed cardiac looping [50], leading to the formation of the different cardiac regions and later a fully functional heart [51]. The molecular mechanisms underlying cardiac morphogenesis include the activation and expression of a several cardiac gene network pathways (CGNPs) evolutionarily conserved. The correct expression of CGNPs is closely regulated in time, specific and spatial patterning by different transcriptional (cardiac-enriched transcription factors, such as Mef2c, Gata4, Nkx2.5 and several members of the T-box family) and post-transcriptional factors (non-coding RNAs such as microRNAs) [52–55]. Cardiac transcription factors regulate the transcription of different elements that make up the cardiac transcription pathways forming part of them and regulating transcriptionally the cardiac development [53]. Post-transcriptionally, the role that non-coding RNAs can exert in the regulation of these pathways as well as in other aspects of cardiac development have been described [53–55]. Several microRNAs have been post-transcriptionally regulated in the

development of the heart interacting with various elements of cardiac signaling pathways [56]. Emerging evidences have pointed out lncRNAs as pivotal players in the heart development regulating transcriptionally and post-transcriptionally distinct cardiac signaling pathways. Transcriptomic analyses have identified a large number of differentially expressed lncRNAs during cardiomyogenic differentiation and proliferation [57,58]. For example, *HBL1*, a human cardiac-enriched lncRNA, negatively modulates cardiomyocyte differentiation from pluripotent stem cells by interacting with SOX2 and miR-1 [59]. Morever several studies have been performed exploring the functional role of particular cardiac enriched-lncRNAs during cardiogenesis. In particular, to date, seven cardiac lncRNAs have been analyzed in detailed; C*armn, Braveheart, Fendrr, Alien, Upperhand, LncRNA-uc.167* and *Pancr*. We provided here state-of-the art review of the functional role of these lncRNAs in heart development.

(a). Carmn

Ounzain et al. [60] profiled the lncRNA transcriptome of human cardiac precursor cells and identified a set of 570 lncRNAs differentially expressed during cardiac differentiation. Many of these lcnRNAs were associated with active cardiac enhancers and super enhancers [60]. Super enhancers are associated with increased production of enhancer-associated ncRNAs and with the enrichment of chromatin remodeling complexes and specific histone modifications [61]. Among these lcnRNAs, Ounzain et al. [62] focused their attention on *Carmn*, a super enhancer-associated lncRNA. *Carmn* is located upstream of miR-143 and miR-145, two microRNA involved in cardiovascular development [63,64]. Although *Carmn*, miR-143 and miR-145 are located within the same genomic locus, they are expressed as independent transcripts. *Carmn* is expressed both in fetal and adult hearts and it is well conserved between mammalian species. *Carmn* directly acts during the earliest steps of cardiac lineage commitment regulating cardiac differentiation from nascent mesoderm by modulating the expression downstream of mesp1-cardiac gene network [62]. Moreover *Carmn* modulates the expression of key factor of pluripotency suggesting a bivalent role both during early differentiation of nascent mesoderm as well as during pluripotency. Interestingly, the expression of *Carmn* in mice modulates, but not in human, the expression of other cardiac-associated lncRNAs, such as *Braveheart*, located downstream of *Carmn*, within the same genomic locus. Mechanistically, *Carmn* acts in *trans* by directly interacting with the polycomb repressive complex 2 (PRC2) through SUZ12, a core protein of this complex, and a EZH2, with mediates methylation of lysine 27 on histone 3, a repressive mark epigenetic that promote the correct cardiac differentiation. The role of this repressive complex in the development of heart has been well reported [65]. Thus *Carmn* exerts an epigenetic function altering the transcriptional landscape by interacting with repressive complex chromatin remodeling. Moreover, *Carmn* is necessary for cardiomyocyte homeostasis, maintaining a differentiated cardiac fate in mature cardiomyocytes [62].

(b). Braveheart

Braveheart is a mouse specific heart-associated lncRNA that plays a pivotal role during cardiac development. *Braveheart* acts as a key regulator in cardiac lineage commitment and it is required for proper cardiac gene expression in mice. *Braveheart* acts upstream of *Mesp1*, and it is nonetheless required for its activation within the same genetic pathway. Depletion of *Braveheart* results in failure

of activation of key cardiac factors necessary for correct heart development and cardiomyocyte differentiation. These evidences suggest that *Braveheart* is required to active Mesp1-driven gene expression program and to promote cardiac cell fate from nascent mesoderm. Moreover, *Braveheart* is necessary for fetal and neonatal cardiomyocyte homeostasis and the maintenance of cardiac fate of its [21]. Similarly evidences have also been demonstrated that *Braveheart is* necessary for ESC to acquire cardiac lineage commitment and differentiation into cardiomyocytes [21].

Recently, Xue et al. [66] have determined the secondary structure of *Braveheart* and showing that *Braveheart* adopts a high modular structure with a 5' AGIL motif that is required for correct mode of action of *Braveheart*. Importantly, this motif is necessary to for cardiovascular lineage commitment and proper ESC differentiation. These authors also demonstrated specific interactions between *Braveheart* and zinc-finger TF CNBP a negative regulator of the cardiac development program repressing the CM differentiation. Thus, it seems that *Braveheart* act as antagonist of CNBP to promote cardiovascular lineage commitment [66].

(c). Fendrr

Fendrr is differentially and transiently expressed at the caudal end of the nascent lateral plate mesoderm, being necessary for the correct development of tissues derived from it, especially the heart and the body walls. *Fendrr* is located 1250 base pairs upstream of *Foxf1* and is co-expressed with this transcription factor. *Foxf1* is of vital importance for the proper differentiation of lateral mesoderm in splanchnic mesoderm and somatic mesoderm [67]. Gene targeting approach has showed that the lack of *Fendrr* carries embryonic lethality at E13.75, characterized by an incorrect heart function, blood accumulated in the right heart chambers and a critical decrease in the thickness of the ventral body walls. The incorrect function of the heart is explained by a hypoplasia of the myocardium that leads to the development impair of ventral heart walls and interventricular septum that are too thin to be able to withstand the blood pressure. Mechanistically, *Fendrr* acts as epigenetic regulatory element by establishing a ratio of repressive and activate marks in the promotors of pivotal transcription factors involved in the mesoderm development, such as *Foxf1*, *Pitx2* and *Irx3.* Such epigenetic regulation is provided through interaction with chromatin remodeling complexes, the histone-modifying Polycomb repressive complex (PRC2) and Trithorax group/MLL protein complex (TrxG/MLL), respectively. The establishment of this ratio determinates the patterns of expression of *Fendrr* target genes in the nascent lateral plate mesoderm and in their descendants of by setting long-term epigenetic marks. *In silico* approaches have showed the existence of 40-nucleotide stretch in the *Fendrr* structure, which is able to interact with *Foxf1* and *Pitx2* promoters, thereby *Fendrr* seem to can be binds directly to those promoters via the formation of a dsDNA:RNA triplex structure. However this interaction needs to be experimentally validated [30,68].

(d). Alien

Alien is co-expressed in vascular progenitor cells derived from allantoides and lateral plate mesoderm along with genes involved in skeletal muscle development and heart morphogenesis. Gene targeting approaches have showed that the loss of *Alien* results in impaired development of several mesodermal derivatives. Among them defective vascular patterning and cardiac chamber formation is reported [69]. These observations suggest that *Alien* specially acts as pivotal regulator in the

cardiovascular development by exerting a function in an early stage of cardiovascular differentiation common to both vascular and cardiac progenitors. The molecular aspects of *Alien* function are unknown to date and thus it will be interesting to study the specific role of this lncRNA in the cardiovascular commitment [69].

On the other hand, *Alien* participates in the endoderm differentiation regulating positively the transcription of *FOXA2,* an important regulator of endoderm development, by facilitating *SMAD2/3* recruitment to the *FOXA2* promoter [70]. Thus Alien acts a versatile RNA molecule during the cardiovascular development.

(e). Upperhand

A recent study has showed that transcription of a promoter-associated lncRNA located near to *Hand2* is necessary for the expression of this transcription factor and proper heart development. This lncRNA is known as *Upperhand*, is located 150 bases pairs upstream of *Hand2* and shares a bidirectional promoter with this transcription factor. Interestingly, *Upperhand* locus contains a Hand2 associated cardiac enhancer within an intron. *Upperhand* is co-expressed in a temporal and tissue-specific pattern along with *Hand2* during embryonic development. *Upperhand* expression is enriched in the cytoplasm. The function of this lncRNA has been studied by gene targeting approach. *Upperhand* knockout (KO) mice display similar phenotype as*Hand2*KO mice, characterized by a development impairment of right ventricular chamber and embryonic lethality [71]. *Upperhand* KO embryo failed to establish H3K27ac marks in the Hand2-Uph locus and binding of *GATA4* to the *Hand2* cardiac enhancer is also impaired. This molecular interaction is required for the activation of *Hand2* cardiac enhancer and thus for Hand2 transcription [72,73]. Chromatin immunoprecipitation analyses (ChIP) in *Upperhand* KO embryo have showed that both the loss of the H3K27ac marks and the lack of Gata4 interaction with the *Hand2* cardiac enhancer negatively affects*Hand2* transcription, preventing the RNA polymerase II elongation within the *Hand2* locus. Interestingly, *in vitro* approaches in HL-1 have showed that the mature *Upperhand* transcripts are not required for *Hand2* expression suggesting that is the *Upperhand* transcription, the responsible of *Hand2* expression. Thus, these findings suggest that *Upperhand* transcription is required to the *Hand2* expression by participating in the establishing of the H3K27 marks and in the binding of *Gata4* to the *Hand2* associated cardiac enhancer both necessary to the proper hand2 transcription by the RNA polymerase II [74].

(f). LncRNA-uc.167

A screening of transcriptome of patients with ventricular septal defect (VSD), has showed the differential expression of a considerable number of lncRNAs [73]. Among them Song et al., [75] focused on *LncRNA-uc.167*, given its prominent expression in VSD patients. *LncRNA-uc.167* is located in the opposite strand of *Mef2c* and is well conversed between species. The expression of both follows an inverse pattern throughout cardiac development and also during the process of P19 cell differentiation into cardiomyocytes. Moreover, the overexpression of *lncRNA-uc.167* results in inhibition of *Mef2c* and absence of differentiation of P19 characterized by a higher level of apoptosis and a slower proliferation rate. The effects of *lncRNA-uc.167* overexpression are partially reduced by *Mef2c* overexpression, suggesting a functional relationship between them. Thus, those observations suggest that *LncRNA-uc.167* can participate in *Mef2c* signaling during heart development [76], yet

further experiments are required to fully understand the molecular mechanisms behind *lncRNA-uc.167* and *Mef2c* interaction.

(g). Pancr

Genome-wide association studies (GWAS) have associated several SNPs (risk variants) in distinct genetic loci with atrial fibrillation, including 4q25 genomic locus [77,78] where the homeobox transcription factor *Pitx2* is located. *Pitx2* is a pivotal player in embryonic left-right asymmetry pathway and cardiac development [79]. In this context, Gore-Panter et al. [80] exploring the possible relationship between *Pitx2* and these AF risk variants, identified a long intergenetic non-coding RNA adjacent to *Pitx2*, dubbed as *Pancr*. *Pancr* is expressed in the adult left atrium and in lower levels in the adult eye, and shows a coordinate expression with *Pitx2c*, during the differentiation of cardiomyocytes regulating positively the expression of *Pitx2c* mRNA by a yet unknown mechanism. Interestingly, *Pancr* have been reported in human tissues but no orthologues are found in other mammalian species such as mice. Thereby, *Pancr* seems to be a human specific lincRNA [80]. Since, the regulation of *Pitx2c* by *Pancr* it will be interesting to explore the role of this lincRNA in the cardiac development to provide more information of left-right asymmetry pathway.

6. Long non-coding RNAs in cardiac diseases

The role of long non-coding RNAs in disease is recently emerging. Over the last few years, the number of reports that have associated lncRNAs differential expression with some cardiac pathology has considerably increased [4]. Several transcriptomic studies in different species, using deep sequencing, have identified that multiple lncRNAs are deregulated in distinct cardiac pathologies (Figure 3), particularly in acute myocardium infarction [81,82], heart failure [83-85], cardiac fibrosis [86] and atrial fibrillation [87,88]. These studies have shown that lncRNAs are important players in the maintenance of cellular homeostasis and in disease processes, being their function necessary to correct physiological status.

Several studies have indicated the importance of lncRNAs in cardiac hypertrophy. For example, *Myhear*t, a lncRNA located in the murine myosin heavy chain 7 genomic locus, prevents cardiomyocyte hypertrophy by sequestering BRG1-BAF complex and therefore avoiding that this complex can bind to target genes and induce an hypertrophy response [36]. *Hrcr*, a cardiac circular lncRNA plays a protective role in the cardiac hypertrophy too, acting a decoy lncRNA by sequestering miR-223, considered as pro-hypertrophy factor [26]. Also, H19 and his encoded miR-675, play a protective role in hypertrophy cardiac by modulating cardiac CaMKIIδ expression in the hypertrophy response [27]. Other lncRNAs promote a pro-hypertrophy response in cardiomyocytes, such as *Chast, Chaer, Chrf* or *Miat*. *Chast*, cardiac hypertrophy–associated transcript, is overexpressed by hypertrophy stimuli and its overexpression, independently of pro-hypertrophic factor, is sufficient to activate the hypertrophy response in the cardiomyocytes both *in vivo* and *in vitro* [89]. *Chaer*, cardiac-hypertrophy-associated epigenetic regulator, promotes the hypertrophy by interacting directly with PRC2 and inhibiting the formation of transcriptional silent chromatin complex of pro-hypertrophic factors [90] *Chrf*, cardiac-hypertrophy-response factor, acts a sponge of miR-489, which target is mRNA of pro-hypertrophy factor *Myd88*, thereby promote the hypertrophy response by avoiding that miRNA can degrade *Myd88* transcripts [91]. Finally, *Miat*,

myocardial infarction–associated transcript, promote partly cardiac hypertrophy by sponging miR-150, an important miRNA with suppressor effect in the cardiac hypertrophy [92].

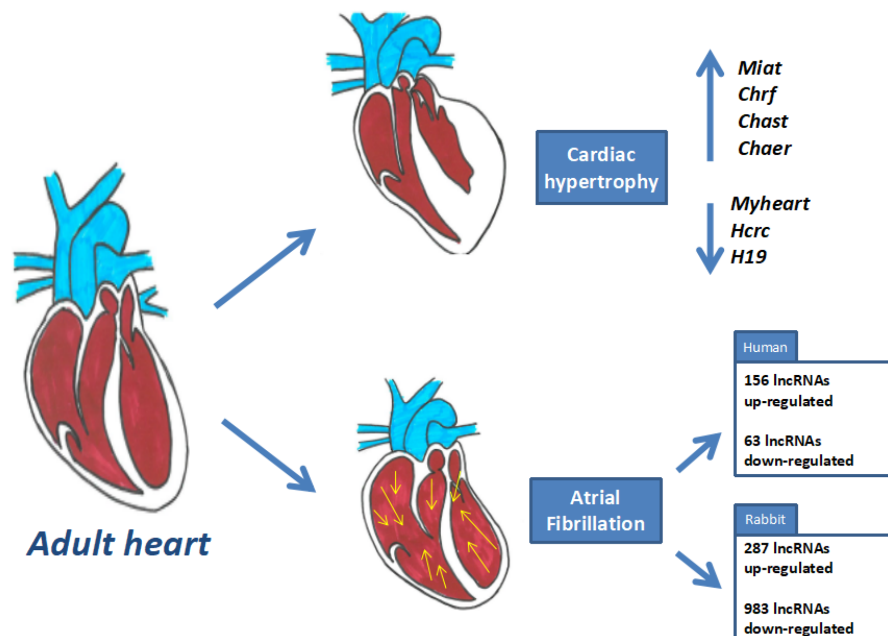


Figure 3. Schematic representation of the distinct cardiac pathophysiological conditions and the distinct long non-coding RNAs described to date.

Acute myocardial infarction, ultimately leading to heart failure, is frequently associated with a progressive accumulation of fibrotic depositions, i.e., cardiac fibrosis [86]. Differentially expressed lncRNAs have been identified in this fibrotic process. *Wisper*, a cardiac-fibroblast enriched lncRNA conserved between mouse and human, is necessary for the development of cardiac fibrosis. The expression of *Wisper* is required for survival and transdifferentiation of cardiac fibroblasts and maintaining the correct pro-fibrotic gene regulatory network. GapmeRs-mediated attenuation *in vivo* reduces the fibrotic process after myocardium infarct in mice, pointing to *Whisper* as potential therapeutic target [86]. Furthermore, two cardiac-fibroblast enriched lncRNAs modulate the fibrotic process through MMP2 (matrix metalloproteinase-2) modulation, a pro-fibrotic factor expressed in cardiac fibroblasts [93]. *Meg*, a lncRNA conserved in human and mice, promote cardiac fibrosis, upregulating MMP2 expression, while *Gas5* act as negative effector by sponging miR-21, which, in turn, positively regulates the fibrotic process by PTEN/MMP2 pathway [94]. Other examples of cardiac pro-fibrotic enriched sponges lncRNAs is exemplified by *Miat*, acting upon miR-24 and *H19*, acting upon miR-455 [95,96], respectively.

Interestingly, although the role of lncRNAs in cardiac arrhythmogenesis scarce, two different studies in humans and rabbits, respectively, have identified a subset of differentially expressed lncRNAs in atrial fibrillation, yet their functional roles remains to be established [87,88].

In addition to those functional roles in adult cardiac pathophysiology, several reports are also emerging in congenital heart diseases. Song et al. [75] have shown the different expression of up to 1500 lncRNAs between normal hearts and hearts from fetuses with ventricular septal defect, a

common congenital heart disease. The deregulation of these long non-coding RNAs supports the possible involvement of long non-coding in the development of CHD, however is necessary a greater knowledge about the biology of lnRNAs to understand the role of these in this kind of disease [75].

7. Conclusions

Emerging evidences suggest an important role of long non-coding RNA in cardiac development by regulating different cardiac gene network pathways. In this line of thinking, knockdown of distinct cardiac-enriched lncRNAs results in embryonic lethality, reflecting the pivotal role of lncRNAs in this process. However, understanding of the functional roles of long non-coding RNAs is still in its infancy. In the next coming years we will witness further insights into the diversity of regulatory roles of long non-coding RNAs and their interactions with epigenetic, transcriptional and post-transcriptional regulatory layers, not only during cardiovascular development, but also in cardiovascular pathology. In this context, several lncRNAs have been point out as important regulators of cardiac pathological processes such as cardiac hypertrophy. However, scarce information is available in electrophysiological disorders such as Brugada syndrome, LQT and SQT syndromes or arrhythmogenic right ventricular dysplasia (ARVD). Importantly, lncRNAs are identified in peripheral blood samples, opening the possibility of serving as diagnostic biomarkers of different cardiac disease [19]. Therefore, the identification of lncRNAs and the study of their functional roles, both in development and disease, highlight the important of non-coding RNAs as key regulatory elements.

Conflict of interest

The authors declare there is no conflict of interest.

References

1. Carninci P, Kasukawa T, Katayama S, et al. (2005) The transcriptional landscape of the mammalian genome. *Science* 309: 1559–1563.
2. Harrow J, Frankish A, Gonzalez JM, et al. (2012). GENCODE: the reference human genome annotation for The ENCODE Project. *Genome Res* 22: 1760–1774.
3. Esteller M (2011) Non-coding RNAs in human disease. *Nature Rev Gene* 12: 861–874.
4. Beermann J, Piccoli MT, Viereck J, et al. (2016) Non-coding RNAs in development and disease: background, mechanisms, and therapeutic approaches. *Physiol Rev* 96: 1297–1325.
5. Barwari T, Joshi A, Mayr M (2016) MicroRNAs in Cardiovascular Disease. *J Am College Cardiol* 68: 2577–2584.
6. Liu N, Olson EN (2010) MicroRNA regulatory networks in cardiovascular development. *Dev Cell* 18: 510–525.
7. Derrien T, Johnson R, Bussotti G, et al. (2012) The GENCODE v7 catalog of human long noncoding RNAs: analysis of their gene structure, evolution, and expression. *Genome Res* 22: 1775–1789.
8. Fang S, Zhang L, Guo J, et al. (2017) NONCODEv5: a comprehensive annotation database for long non coding RNAs. *Nucleic Acids Res* 46: D308–D314.

9. Schmitz SU, Grote P, Herrmann BG (2016) Mechanisms of long noncoding RNA function in development and disease. *Cell Mol Life Sci* 73: 2491–2509.
10. Rosa A, Ballarino M (2015) Long noncoding RNA regulation of pluripotency. *Stem Cells Int* 2016: 1–9.
11. Wapinski O, Chang HY (2011) Long noncoding RNAs and human disease. *Trends in Cell Biol* 21: 354–361.
12. Dieci G, Fiorino G, Castelnuovo M, et al. (2007) The expanding RNA polymerase III transcriptome. *Trends in Genet* 23: 614–622.
13. Rackham O, Shearwood AMJ, Mercer TR, et al. (2011) Long noncoding RNAs are generated from the mitochondrial genome and regulated by nuclear-encoded proteins. *RNA* 17: 2085–2093.
14. Pauli A, Norris ML, Valen E, et al. (2014) Toddler: an embryonic signal that promotes cell movement via Apelin receptors. *Science* 343: 1248636.
15. Pauli A, Valen E, Schier AF (2015) Identifying (non-) coding RNAs and small peptides: Challenges and opportunities. *BioEssays* 37: 103–112.
16. Nelson BR, Makarewich CA, Anderson DM, et al. (2016) A peptide encoded by a transcript annotated as long noncoding RNA enhances SERCA activity in muscle. *Science* 351: 271–275.
17. Engreitz JM, Ollikainen N, Guttman M (2016) Long non-coding RNAs: spatial amplifiers that control nuclear structure and gene expression. *Nat Rev Mol Cell Biol* 17: 756–770.
18. Gloss BS, Dinger ME (2016) The specificity of long noncoding RNA expression. *Biochim Biophysi Acta* 1859: 16–22.
19. Bär C, Chatterjee S, Thum T (2016) Long Noncoding RNAs in Cardiovascular Pathology, Diagnosis, and Therapy. *Circulation* 134: 1484–1499.
20. Chen LL (2016) Linking Long Noncoding RNA Localization and Function. *Trends in Biochem Sci* 41: 761–772.
21. Klattenhoff CA, Scheuermann JC, Surface LE, et al. (2013) Braveheart, a long noncoding RNA required for cardiovascular lineage commitment. *Cell* 152: 570–583.
22. Ulitsky I, Bartel DP (2013) lincRNAs: genomics, evolution, and mechanisms. *Cell* 154: 26–46.
23. Ounzain S, Pedrazzini T (2015) The promise of enhancer-associated long noncoding RNAs in cardiac regeneration. *Trends Cardiovasc Med* 25: 592–602.
24. Ounzain S, Burdet F, Ibberson M, et al. (2015) Discovery and functional characterization of cardiovascular long noncoding RNAs. *J Mol Cell Cardiol* 89: 17–26.
25. Memczak S, Jens M, Elefsinioti A, et al. (2013) Circular RNAs are a large class of animal RNAs with regulatory potency. *Nature* 495: 333–338.
26. Wang K, Long B, Liu F, et al. (2016) A circular RNA protects the heart from pathological hypertrophy and heart failure by targeting miR-223. *Euro Heart J* 37: 2602–2611.
27. Liu L, An X, Li Z, et al. (2016) The H19 long noncoding RNA is a novel negative regulator of cardiomyocyte hypertrophy. *Cardiovasc Res* 111: 56–65.
28. Hasegawa Y, Brockdorff N, Kawano S, et al. (2010) The matrix protein hnRNP U is required for chromosomal localization of Xist RNA. *Dev Cell* 19: 469–476.
29. Gupta RA, Shah N, Wang KC, et al. (2010) Long non-coding RNA HOTAIR reprograms chromatin state to promote cancer metastasis. *Nature* 464: 1071–1076.
30. Grote P, Wittler L, Hendrix D, et al. (2013) The tissue-specific lncRNA Fendrr is an essential regulator of heart and body wall development in the mouse. *Dev Cell* 24: 206–214.

31. Tsai M C, Manor O, Wan Y, et al. (2010) Long noncoding RNA as modular scaffold of histone modification complexes. *Science* 329: 689–693.
32. Aguilo F, Zhou MM, Walsh MJ (2011) Long noncoding RNA, polycomb, and the ghosts haunting INK4b-ARF-INK4a expression. *Cancer Res* 71: 5365–5369.
33. Mousavi K, Zare H, Dell'Orso S, et al. (2013) eRNAs promote transcription by establishing chromatin accessibility at defined genomic loci. *Mol Cell* 51: 606–617.
34. Welsh IC, Kwak H, Chen FL, et al. (2015) Chromatin architecture of the Pitx2 locus requires CTCF-and Pitx2-dependent asymmetry that mirrors embryonic gut laterality. *Cell Rep* 13: 337–349.
35. Redon S, Reichenbach P, Lingner J (2010) The non-coding RNA TERRA is a natural ligand and direct inhibitor of human telomerase. *Nucleic Acids Res* 38: 5797–5806.
36. Han P, Li W, Lin CH, et al. (2014) A long noncoding RNA protects the heart from pathological hypertrophy. *Nature* 514: 102–106.
37. Yoon JH, Abdelmohsen K, Gorospe M (2013) Posttranscriptional gene regulation by long noncoding RNA. *J Mol Biol* 425: 3723–3730.
38. Tripathi V, Ellis JD, Shen Z, et al. (2010) The nuclear-retained noncoding RNA MALAT1 regulates alternative splicing by modulating SR splicing factor phosphorylation. *Mol Cell* 39: 925–938.
39. Yin QF, Yang L, Zhang Y, et al. (2012) Long Noncoding RNAs with snoRNA Ends. *Mol Cell* 48: 219–230.
40. Kim YK, Furic L, Desgroseillers L, et al. (2005) Mammalian Staufen1 recruits Upf1 to specific mRNA 3'UTRs so as to elicit mRNA decay. *Cell* 120: 195–208
41. Kim YK, Furic L, Parisien M, et al. (2007) Staufen1 regulates diverse classes of mammalian transcripts. *EMBO* 26: 2670–2681
42. Faghihi MA, Modarresi F, Khalil AM, et al. (2008) Expression of a noncoding RNA is elevated in Alzheimer's disease and drives rapid feed-forward regulation of β-secretase expression. *Nat Med* 14: 723.
43. Faghihi MA, Zhang M, Huang J, et al. (2010) Evidence for natural antisense transcript-mediated inhibition of microRNA function. *Genome Biol* 11: R56.
44. Yoon JH, Abdelmohsen K, Srikantan S, et al. (2012) LincRNA-p21 suppresses target mRNA translation. *Mol Cell* 47: 648–655.
45. Wang H, Iacoangeli A, Lin D, et al. (2005) Dendritic BC1 RNA in translational control mechanisms. *J Cell Biol* 171: 811–821.
46. Carrieri C, Cimatti L, Biagioli M, et al. (2012) Long non-coding antisense RNA controls Uchl1 translation through an embedded SINEB2 repeat. *Nature* 491: 454–457.
47. Cesana M, Cacchiarelli D, Legnini I, et al. (2011) A long noncoding RNA controls muscle differentiation by functioning as a competing endogenous RNA. *Cell* 147: 358–369.
48. Keniry A, Oxley D, Monnier P, et al. (2012) The H19 lincRNA is a developmental reservoir of miR-675 which suppresses growth and Igf1r. *Nat Cell Biol* 14: 659.
49. Garry DJ, Olson EN (2006) A common progenitor at the heart of development. *Cell* 127: 1101–1104.
50. Wagner M, Siddiqui MAQ (2007) Signal transduction in early heart development (I): cardiogenic induction and heart tube formation. *Exp Biol Med* 232: 852–865.
51. Kelly RG, Buckingham ME, Moorman AF (2014) Heart fields and cardiac morphogenesis. *Cold Spring Harb Perspect Med* 4: a015750.

52. Christoffels VM, Habets PE, Franco D, et al. (2000) Chamber formation and morphogenesis in the developing mammalian heart. *Dev Biol* 223: 266–278.
53. Schonrock N, Harvey RP, Mattick JS (2012) Long noncoding RNAs in cardiac development and pathophysiology. *Circ Res* 111: 1349–1362.
54. Meganathan K, Sotiriadou I, Natarajan K, et al. (2015) Signaling molecules, transcription growth factors and other regulators revealed from in-vivo and in-vitro models for the regulation of cardiac development. *Int J Cardiol* 183: 117–128.
55. Stefani G, Slack FJ (2008) Small non-coding RNAs in animal development. *Nat Rev Mol Cell Biol* 9: 219–230.
56. Katz MG, Fargnoli AS, Kendle AP, et al. (2016) The role of microRNAs in cardiac development and regenerative capacity. *Am J Physiol Heart Circ Physiol* 310: H528–H541.
57. Li H, Jiang L, Yu Z, et al. (2017) The Role of a Novel Long Noncoding RNA TUC40-in Cardiomyocyte Induction and Maturation in P19 Cells. *Am J Med Sci* 354: 608–616.
58. Arnone B, Chen JY, Qin G (2017) Characterization and analysis of long non-coding rna (lncRNA) in In Vitro-and Ex Vivo-derived cardiac progenitor cells. *PloS One* 12: e0180096.
59. Liu J, Li Y, Lin B, et al. (2017) HBL1 Is a Human Long Noncoding RNA that Modulates Cardiomyocyte Development from Pluripotent Stem Cells by Counteracting MIR1. *Dev Cell* 42: 333–348.
60. Ounzain S, Micheletti R, Beckmann T, et al. (2014) Genome-wide profiling of the cardiac transcriptome after myocardial infarction identifies novel heart-specific long non-coding RNAs. *Eur Heart J* 36: 353–368.
61. Ounzain S, Pedrazzini T (2016) Long non-coding RNAs in heart failure: a promising future with much to learn. *Annals Trans Med* 4: 298.
62. Ounzain S, Micheletti R, Arnan C, et al. (2015) CARMEN, a human super enhancer-associated long noncoding RNA controlling cardiac specification, differentiation and homeostasis. *J Mol Cell Cardiol* 89: 98–112.
63. Boucher JM, Peterson SM, Urs S, et al. (2011) The miR-143/145 cluster is a novel transcriptional target of Jagged-1/Notch signaling in vascular smooth muscle cells. *J Biol Chem* 286: 28312–28321.
64. Cordes KR, Sheehy NT, White MP, et al. (2009) miR-145 and miR-143 regulate smooth muscle cell fate and plasticity. *Nature* 460: 705–710.
65. Mathiyalagan P, Keating ST, Du XJ, et al. (2014) Chromatin modifications remodel cardiac gene expression. *Cardiovasc Res* 103: 7–16.
66. Xue Z, Hennelly S, Doyle B, et al. (2016) A G-rich motif in the lncRNA braveheart interacts with a zinc-finger transcription factor to specify the cardiovascular lineage. *Mol Cell* 64: 37–50.
67. Mahlapuu M, Ormestad M, Enerback S, et al. (2001) The forkhead transcription factor Foxf1 is required for differentiation of extra-embryonic and lateral plate mesoderm. *Development* 128: 155–166.
68. Grote P, Herrmann BG (2013) The long non-coding RNA Fendrr links epigenetic control mechanisms to gene regulatory networks in mammalian embryogenesis. *RNA Biol* 10: 1579–1585.
79. Kurian L, Aguirre A, Sancho-Martinez I, et al. (2015) Identification of novel long non-coding RNAs underlying vertebrate cardiovascular development. *Circulation* 131: 1278–1290.
70. Jiang W, Liu Y, Liu R, et al. (2015) The lncRNA DEANR1 facilitates human endoderm differentiation by activating FOXA2 expression. *Cell Rep* 11: 137–148.

71. Yamagashi H, Olson EN, Srivastava D (2000) The basic helix-loop-helix transcription factor, dHAND, is required for vascular development. *J Clin Invest* 105: 261–270.
72. McFadden DG, Charité J, Richardson JA, et al. (2000) A GATA-dependent right ventricular enhancer controls dHAND transcription in the developing heart. *Development* 127: 5331–5341.
73. He A, Gu F, Hu Y, et al. (2014) Dynamic GATA4 enhancers shape the chromatin landscape central to heart development and disease. *Nat Commun* 5: 4907.
74. Anderson KM, Anderson DM, McAnally JR, et al. (2016) Transcription of the non-coding RNA upperhand controls Hand2 expression and heart development. *Nature* 539: 433–436.
75. Song G, Shen Y, Zhu J, et al. (2013) Integrated analysis of dysregulated lncRNA expression in fetal cardiac tissues with ventricular septal defect. *PloS One* 8: e77492.
76. Song G, Shen Y, Ruan Z, et al. (2016) LncRNA-uc. 167 influences cell proliferation, apoptosis and differentiation of P19 cells by regulating Mef2c. *Gene* 590: 97–108.
77. Gudbjartsson DF, Arnar DO, Helgadottir A, et al. (2007) Variants conferring risk of atrial fibrillation on chromosome 4q25. *Nature* 448: 353–357.
78. Ellinor PT, Lunetta KL, Albert CM, et al. (2012) Meta-analysis identifies six new susceptibility loci for atrial fibrillation. *Nat Genet* 44: 670–675.
79. Franco D, Christoffels VM, Campione M (2014) Homeobox transcription factor Pitx2: The rise of an asymmetry gene in cardiogenesis and arrhythmogenesis. *Trends Cardiovasc Med* 24: 23–31.
80. Gore-Panter SR, Hsu J, Barnard J, et al. (2016) PANCR, the PITX2 Adjacent noncoding RNA, is expressed in human left atria and regulates PITX2c expression. *Circ Arrhythm Electrophysiol* 9: e003197.
81. Guo Y, Luo F, Liu Q, et al. (2016) Regulatory non-coding RNAs in acute myocardial infarction. *J Cell Mol Med* 21: 1013–1023.
82. Vausort M, Wagner DR, Devaux Y (2014) Long Noncoding RNAs in Patients With Acute Myocardial InfarctionNovelty and Significance. *Circ Res* 115: 668–677.
83. Devaux Y, Creemers EE, Boon RA, et al. (2017) Circular RNAs in heart failure. *Eur J Heart Fail* 19: 701–709.
84. Greco S, Zaccagnini G, Perfetti A, et al. (2016) Long noncoding RNA dysregulation in ischemic heart failure. *J Transl Med* 14: 183.
85. Schiano C, Costa V, Aprile M, et al. (2017) Heart failure: Pilot transcriptomic analysis of cardiac tissue by RNA-sequencing. *Cardiol J* 24: 539–553.
86. Micheletti R, Plaisance I, Abraham BJ, et al. (2017) The long noncoding RNA Wisper controls cardiac fibrosis and remodeling. *Sci Transl Med* 9: eaai9118.
87. Li Z, Wang X, Wang W, et al. (2017) Altered long non-coding RNA expression profile in rabbit atria with atrial fibrillation: TCONS_00075467 modulates atrial electrical remodeling by sponging miR-328 to regulate CACNA1C. *J Mol Cell Cardiol* 108: 73–85.
88. Ruan Z, Sun X, Sheng H, et al. (2015) Long non-coding RNA expression profile in atrial fibrillation. *Int J Clin Exp Pathol* 8: 8402.
89. Viereck J, Kumarswamy R, Foinquinos A, et al. (2016) Long noncoding RNA Chast promotes cardiac remodeling. *Sci Transl Med* 8: 326ra22–326ra22.
90. Wang Z, Zhang XJ, Ji YX, et al. (2016) The long noncoding RNA Chaer defines an epigenetic checkpoint in cardiac hypertrophy. *Nat Med* 22: 1131–1139.

91. Wang K, Liu F, Zhou LY, et al. (2014) The Long Noncoding RNA CHRF Regulates Cardiac Hypertrophy by Targeting miR-489 Novelty and Significance. *Circ Res* 114: 1377–1388.
92. Zhu XH, Yuan YX, Rao SL, et al. (2016) Lncrna miat enhances cardiac hypertrophy partly through sponging mir-150. *Eur Rev Med Pharmacol Sci* 20: 3653.
93. Piccoli MT, Gupta SK, Viereck J, et al. (2017) Inhibition of the Cardiac Fibroblast–Enriched lncRNA Meg3 Prevents Cardiac Fibrosis and Diastolic Dysfunction Novelty and Significance. *Circ Res* 121: 575–583.
94. Tao H, Zhang JG, Qin RH, et al. (2017) LncRNA GAS5 controls cardiac fibroblast activation and fibrosis by targeting miR-21 via PTEN/MMP-2 signaling pathway. *Toxicology* 386: 11–18.
95. Qu X, Du Y, Shu Y, et al. (2017) MIAT is a pro-fibrotic long non-coding RNA governing cardiac fibrosis in post-infarct myocardium. *Sci Rep* 7: 42657.
96. Huang ZW, Tian LH, YangB, et al. (2017) Long noncoding RNA H19 acts as a competing endogenous RNA to mediate CTGF expression by sponging miR-455 in cardiac fibrosis. *DNA Cell Biol* 36: 759–766.

AIMS Press

© 2018 the Author(s), licensee AIMS Press. This is an open access article distributed under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/4.0)

CARDIOVASCULAR & HEMATOLOGICAL DISORDERS: DRUG TARGETS

GENETICS OF ATRIAL FIBRILATION: IN SEARCH OF NOVEL THERAPEUTIC TARGETS

Estefanía Lozano-Velasco, Carlos Garcia-Padilla, Amelia E. Aránega, Diego Franco

Cardiovascular Development Group, Department of Experimental Biology, University of Jaen, Spain

dfranco@ujaen.es

Abstract

Atrial fibrillation (AF) is the most frequent arrhythmogenic disease in humans, ranging from 2% in the general population and raising up to 10-12% in 80+ years. Genetic analyses of AF familiar cases have identified a series of point mutations in distinct ion channels, supporting a causative link. However, these genetic defects only explain a minority of AF patients. Genome-wide association studies identified single nucleotide polymorphisms (SNPs), close to PITX2 on 4q25 chromosome, that are highly associated to AF. Subsequent GWAS studies have identified several new loci, involving additional transcription and growth factors. Furthermore, these risk 4q25 SNPs serve as surrogate biomarkers to identify AF recurrence in distinct surgical and pharmacological interventions. Experimental studies have demonstrated an intricate signalling pathway supporting a key role of the homeobox transcription factor PITX2 as a transcriptional regulator. Furthermore, cardiovascular risk factors such hyperthyroidism, hypertension and redox homeostasis have been identified to modulate PITX2 driven gene regulatory networks. We provide herein a state-of-the-art review of the genetic bases of atrial fibrillation, our current understanding of the genetic regulatory networks involved in AF and its plausible usage for searching novel therapeutic targets.

Introduction

Atrial fibrillation (AF) is the most frequent arrhythmogenic syndrome in humans. Currently, the incidence of AF in the general population is 1-2%, raising up to almost 10-12% in the elderly [1]. The onset of AF might be triggered by forerunning cardiovascular risk factors such as diabetes, obesity, hypertension and hyperthyroidism [2-4] as well as by cardiovascular diseases such as valvular heart diseases and/or hypertrophic cardiomyopathy [5, 6]. In addition, obstructive sleep apnea, cardiac surgery and inflammatory processes can boosted it too [10]. Besides the global risk factors for AF, it is well known that AF initiation triggers additional and more severe AF episodes, generating electrical and structural remodeling of the diseased heart, a condition quoted as "AF begets AF" [11, 12]. Electrical remodeling leads to progressive changes in the cardiac electrical properties, triggering changes in the action potential duration (APD) configuration, early after depolarizations (EADs), and/or delayed after depolarizations (DADs) [13, 14], thus culminating in rotor formation [15]. Structural remodeling leads to inflammation, dilatation and/or atrial fibrosis [16], indirectly promoting therefore rotor formation and electrical re-entry circuitries [15]. Furthermore, AF might lead to further health problems, ranging from stroke and bleeding [17-19], dementia and /or cognitive decline [20, 21], ventricular dysfunction [22, 23] and even sudden death [24]. Therefore, these data highlight AF as an epidemic disease with substantial socio-economic burden worldwide.

Although epidemiological data invariably demonstrate the involvement of distinct risk factors for AF, unquestionable evidences in literature are also reported that no concurrent previous risk factors are diagnosed in a subset of AF patients, therefore AF can course in an isolated manner, a condition named lone AF [25, 26]. In this context, ground-breaking observations supporting that AF is also familial [27], strengthened the notion of a genetic component. Two complementary genetic approaches have been developed over time. Analyses of large AF familiar cases with genetic linkage analyses and subsequent screening of candidate genes pinpointed discrete point mutations in genes encoding for proteins enrolled in cardiac electrophysiology [28-30] **(Figure 1)**. Concomitantly, electrophysiological mapping and data modeling provided substantial understanding of AF pathophysiology [31-33]. Importantly, genetic identification of culprit AF genes only explained 10-15% of all AF patients. Therefore, new approaches to understand the genetic bases of AF were searched. Genome-wide association analyses (GWAS) opened up the identification of new AF-associated genes. Gubdjartsson et al. [34] firstly identified common risk variants associated with lone AF in Chinese and European cohorts. Successive GWAS and meta-GWAS studies have further

unraveled new candidate genes for AF [35-39] **(Figure 1)**. These studies have provided evidenced that the analyses of these risk variants can serve as biomarkers to predict the recurrence of AF upon several clinical interventions [40-46]. In this review we will provide a detailed analyses of the genetic bases of AF, ranging from early studies searching from point mutations in candidate genes to current whole genome strategies. Special emphasis will be provided to the transcription factor Pitx2 and its downstream signaling cascade in the context of AF and finally we will illustrate new roads to be driven on the upcoming important role of post-transcriptional regulation by non-coding RNAs and their plausible therapeutic applicability.

The contribution of the cardiac action potential to AF

The cardiac action potential is driven by inward and outward currents within the cardiomyocyte membrane. The action potential configuration is started by an upstroke depolarization of the sodium current, followed by a repolarization phase which is mediated by a series of different potassium currents, leading finally to the restoration of resting membrane potential in both atrial and ventricular cardiomyocytes. Within each action potential, conduction-contraction coupling is modulated by a complex calcium handling regulatory network. Curiously, nodal conductive cells exert different upstroke configuration that is governed by cation selective currents, while repolarization phase is similar to that observed in both working atrial and ventricular cardiomyocytes. Multiple mutations in the SCN5A gene, which codes for the I_{Na} current pore-forming subunits have been linked to AF, alone [47-51] or in combination to other additional cardiac pathologies [52-57]. Mutations in SCN1B, SCN2B, SCN3B and SCN4B beta subunits, respectively, have also been associated to AF [58-62]. These findings suggest that impaired I_{Na} current might promote AF, yet electrophysiological evidences are only available for a small subset of these point mutations. In addition, HNC4 mutations have also been reported in AF [63].

Mutations in genes encoding potassium channels involved in the cardiac action potential repolarization phase have also been linked to AF, such as those identified in KCNQ1 [28, 64-68], KCNA5 [69] and KCNE2 [30, 70] genes, respectively. These point mutations have been reported to shorten cardiac action potential repolarization, providing thus an electrophysiological substrate for the trigger of AF. Equally, impairment of the resting membrane potential have been demonstrated to promote AF initiation, in line with the large number of point mutations identified in KCNJ2 [29, 30, 71, 72] **(Figure 1)**.

Impaired calcium homeostasis has demonstrated to play an essential role as a triggering factor of AF by multiple lines of evidences [2, 73-75]. Mutations in RYR2 are linked to AF [76] concurrently with catecholaminergic polymorphic ventricular tachycardia [77, 78] **(Figure 1)**. Importantly, genetically engineered mouse model carrying the Ryr2 mutation leads to AF, further supporting the genetic evidences of impaired calcium homeostasis as a AF trigger [79].

Mutation in cell-cell interacting proteins such as GJA1 (connexin43) [80, 81] and GJA5 (connexin40) [82-85] gap junction proteins, which are critical for the coordinated transmission of the electrical impulse among cardiomyocytes, have also been identified in AF **(Figure 1)**. Pioneering studies described somatic cell (i.e. cardiomyocytes) mutations in connexin proteins linked to AF [82], and more recently germline mutations have also been reported [83-85]. Importantly, electrophysiological analyses of connexin mutations showed that impaired cardiomyocyte electrical transmission is impaired in the AF context [86-90], a condition that can lead to cardiac re-entry circuitries and thus to AF.

Transcriptional regulatory circuitries associated to AF

Beside mutations in those genes directly involved in cardiac electrophysiology, mutations in different transcription factors such as TBX5 [91], NKX2.5 [92-96], NKX2.6 [97] and PITX2 [98-100] have also been reported. TBX5 and NKX2.5 are transcription factors that exert pivotal roles during heart morphogenesis. Mutations in these genes have also been identified in patients with congenital heart diseases and, in addition, they are also linked by GWAS to other distinct electrophysiological defects of the heart [101-111]. Importantly among these transcription factors, PITX2 has emerged as a cornerstone in atrial fibrillation, as detailed below.

Genome-wide association studies (GWAS) have led to revolutionary genetic approaches on the understanding of AF acquisition, and to those of cardiac arrhythmias at large [112, 113]. Pioneered work by Gudbjartsson et al. [34] identified risk variants at 4q25 highly linked to lone AF and subsequently corroborated in distinct AF cohort studies [44, 114-117]. Curiously, 4q25 risk variants are located 150 kb downstream of the homeobox transcription factor PITX2. Subsequent experimental studies in mice demonstrated that impaired Pitx2 function triggered atrial arrhythmias susceptibility [118-120]. In the following paragraphs we will provide a detailed review of our current understanding of the functional role of the homeobox transcription factor PITX2 in AF.

The contribution of the homeobox transcription factor PITX2 to AF

PITX2 is a bicoid class of homeodomain transcription factor that play key roles in embryonic development and disease. Semina et al. [121] firstly identified 4q25 translocations association with Rieger syndrome, a human pathological condition coursing with mild craniofacial abnormalities and distinct eye defects, especially glaucoma and underdevelopment of the teeth. Gage & Camper [122] subsequently identified the PITX2 (RIEG) gene and reported two alternatively spliced mRNA products, i.e. PITX2A and PITX2B. PITX2 displayed ocular, cardiac, and craniofacial expression [123, 124], in line with its putative involvement in Rieger syndrome. Multiple evidences have now demonstrated an association of PITX2 mutations in Rieger syndrome patients [125]. Cox et al. [126] reported that PITX2A and PITX2B transcripts are generated by alternative splicing in humans, and PITX2C uses an alternative promoter. An fourth splice variant have been exclusively reported in humans, PITX2D, using the PITX2C promoter but lacking the N-terminus an therefore acting as a dominant negative isoform.

The earliest expression of the homeobox transcription factor PITX2 is observed soon after gastrulation. PITX2 is asymmetrically expressed in the left lateral plate mesoderm in multiple species, ranging from fish to mice and plays a key role on the establishment of embryonic left-right asymmetry [124, 127-129]. Gain-of-function experiments in chicken (and *Xenopus*) embryos supported a fundamental role for PITX2 in asymmetric development of the heart and gut [124, 127-128]. Importantly, left-right asymmetric determination is organ-specific and dose-dependent [130] distinctly affecting brain, heart [124], gut [130] and gonadal [131] morphogenesis. PITX2 loss-of function analyses in mice display normal rightward cardiac looping, suggesting that PITX2 is dispensable for early cardiac asymmetry break [132, 133]. PITX2$^{-/-}$ mice exhibited axial mesoderm hypoplasia, disruption of ventral body-wall splanchnic mesoderm formation as well as hypoproliferation of mandible, spleen, liver, periorbital musculature, and lens. These data demonstrated a multifaceted role for PITX2, including several aspects associated to Axenfeld-Rieger syndrome.

Importantly, Mommersteeg et al. [134] demonstrated that in PITX2c-null mice pulmonary myocardial sleeves were missing and furthermore reported that NKX2.5 and PITX2C are essential for the formation of the pulmonary myocardium. Furthermore, these authors also demonstrate a crucial role for PITX2 in the formation of the sinoatrial node [15]. Given the fact that pulmonary veins constitute a frequent foci of AF triggering, these evidences support that PITX2 is a cornerstone providing a molecular link between the GWAS risk variants identified by Gubarjtsson et al. [34] and a plausible morphogenetic substrate leading to AF.

The growing up role of Pitx2 in adult cardiac electrophysiology

As previously stated, Gubarjtsson et al. [34] identified risk variants adjacent to PITX2 gene that were highly associated to atrial fibrillation. Recently, evidences have emerged demonstrating a functional role of distinct genomic fragments containing risk variants [135] as well as indirectly regulating PITX2 expression via TFAP2 modulation [136]. Nowadays, multiple evidence demonstrate that distinct PITX2 insufficiency experimental models display impaired control of atrial electrophysiology [118-120, 137, 138].

Pitx2 can modulate the expression of several ion channels during heart development and adulthood. Wang et al. [119] firstly demonstrated that Pitx2 loss-of-function increased AF susceptibility. They further showed that Pitx2 haploinsufficiency led to aberrant expression of transcription factors such as *Shox2* and *Tbx3*, which are involved in sinoatrial morphogenesis. Therefore cardiac pacemaker structures were impaired. Surprisingly, no AF episodes occurred under basal conditions and no impairment on the ECG were observed. Subsequently, Kirchhof et al. [120] reported similar findings and demonstrated that multiple ion channels expression were equally impaired. However, the contribution of these ion channels to the cardiac action potential was limited and therefore the electrophysiological and molecular links were still scarce. Using distinct experimental approaches, i.e. an atrial-specific Pitx2 conditional transgenic mouse line, Chinchilla et al. [118] demonstrated abnormal ECGs at rest. Furthermore, increased resting membrane potential and prolonged action potential duration were recorded and the expression of multiple ion channels were also impaired, particularly SCN5A (I_{Na}), KCNJ2 (I_{K1}) and KCNJ12 (I_{K1}). Therefore, for the first time, these data demonstrate that abnormal function of PITX2 leads to molecular defects linked to electrophysiological defects and therefore to increased rate of atrial arrhythmias. More recently, Tao et al. [137] using a Pitx2 deletion conditional approach in the adult heart reported basal ECG alterations, in line with those reported by Chinchilla et al. [118]. More recently, it has been demonstrated that Pitx2 can functionally regulate I_{Ks} and I_{Ca} currents [139] and influence atrial membrane potential by interacting with distinct sodium channel blockers [140] and a cross-talk with Tbx5, another cardiac-enriched transcription factor with pivotal role in cardiac electrophysiology has been established [141]. In sum, these reports evidenced that Pitx2 insufficiency can promote and trigger electrophysiological and molecular defects leading to impaired depolarization, repolarization and resting membrane potential of the cardiac action potential **(Figure 2)**. Furthermore, they show that impairment of a single transcription factor can modulate a large array of ion channels associated

to AF. Thus Pitx2 is upstream on the hierarchical signaling pathway leading to AF and its aberrant function would explain a large fraction of AF onset [142, 143].

Beyond PITX2; the role of novel GWAS associated genes in AF

Besides PITX2, additional GWAS have enlightened other genes putatively involved in AF pathophysiology [37-39, 144-149] including KCNN3 [37], ZFHX3 [38], IL6R [39], CAV1, HCN4, SYNE2, SYNPOL2, PRRX1 and WTN8A among others. To date more that 30 genes have been associated to AF by GWAS analyses [148] **(Figure 1)**. Replication analyses have been confirmed for SYNE2 [150] and PRRX1 [151], while data on CAV1 are more controversial [43, 152]. No additional reports have been obtained for HCN4 and SYNPOL2 to date. Importantly, a 12-SNPs risk score have been developed that can individually identify the risk of AF and stroke [153].

While arguments for the possible functional link to AF have been addressed in all cases, experimental evidences are scarce to date. Genetic deletion of KCNN3 in mice show no overt cardiac electrophysiological defects, although KCNN3 overexpression leads to sudden death [154]. ZFHX3 functional role is equally scarce in the heart, although recent evidences demonstrate that atrial arrhythmias [155] and impaired pacing [151] leads to significant Zfhx3 expression defects. PRRX1 decreased expression has also been reported to trigger atrial arrhythmias in experimental models [156]. Similarly, impaired expression of CREB/CREM also leads to AF [157]. Importantly, risk variants associated to KCNN3, ZFHX3 and IL6R are located in intronic regions, with no evident functional consequences. Therefore, although functional roles for this risk variants on particular regulatory elements is hypothesized, functional demonstration remains to be tested. Importantly, point mutations in several of these genes (PITX2, ZFHX3, SYNE2, KCNN3) have been identified by *de novo* RNAseq in AF patients [149], strengthening their functional implication.

We recently investigated if those genes are regulated by PITX2 [158]. Using strategies of gain and loss-of-function, Lozano-Velasco et al. [138] demonstrate that PITX2 can direct ZFHX3, KCNN3 and IL6R expression. The functional role of ZFHX3 in atrial arrhythmias is basically unexplored, yet due to its transcriptional capacity it is possible that it might control some AF related genes. On the other hand, KCNN3 contribution to the cardiac action potential configuration, if any, seems to be minimal while the involvement of IL6R might be linked to inflammation, a biological process that if impaired might enhance AF onset, yet to date its links to AF remain to be further investigated.

We also analyzed if PITX2 can influence additional genes associated to AF by meta-GWAS. Previous studies demonstrated that PITX2 can influence HCN4 expression [119]. Recently, Lozano-

Velasco et al. [138] showed that CAV1, SYNE2 and WNT8A are regulated by PITX2 expression. Importantly, risk variants associated to CAV1 and SYNE2 have been identified in other cardiac electrophysiological disorders, yet their functional contribution remains enigmatic. Curiously, WNT signaling is extensively documented to play key roles during cardiac development and homeostasis [159-161], but not for WNT8A [161].

Given the pivotal signaling capacities of WNT8A and those at transcriptional level by ZFHX3 we investigated in any of those pathways can regulate the expression of cardiac ion channels. Gain- and loss-of-function studies unraveled that WNT8A, but not ZFHX3, can direct expression of calcium handling proteins [138]. Importantly, abnormal WNT8A expressed is documented only in Pitx2 loss-of-function models with ECG alterations at rest [138], suggesting that WNT signaling is fundamental for the susceptibility *vs* triggering capacity of the atrial chambers during the onset of AF.

Contribution of post-transcriptional regulatory mechanisms to AF

Gene regulatory networks have been classically associated as hierarchical interactions between master genes, such as transcription factors, and structural genes; in the heart context it would correspond to genes encoding ion channels configuring therefore the cardiac action potential or actin and myosin genes forming the sarcomere. Recently, we have witnessed the discovery of a novel layer of regulatory mechanisms, i.e. non-coding RNAs. Non-coding RNAs can be classified into small non-coding RNAs and long non-coding (lncRNAs) [151]. MicroRNAs have emerged as the larger group of small non coding RNAs, exerting a key role on of mRNA transcript post-transcriptional regulation [162, 163]. microRNAs are non-coding RNAs with an approximate length of 22-24 nucleotides that can trigger transcript degradation or block protein translation by binding to complementary seed sequence in the 3´UTR of mRNA transcripts [163]. Currently, the transcriptomic profile of microRNAs has been reported in both the developing and adult heart, in normal as well as also in pathological conditions [164, 165]. In AF, the microRNA fingerprint has been revealed in several settings [166-168], providing hints about their plausible use as biomarkers [169-173].

Several studies have unraveled that Pitx2-regulated microRNAs can also contribute to AF pathophysiology. Chinchilla et al. [118] demonstrated that impaired miR-1 expression, by modulating Kcnj2 and Kcnj12 post-transcriptional regulation, underlie abnormal resting membrane potential configuration in Pitx2 deficient mice. Huang et al. [174] confirmed the inhibition of miR-1 by Pitx2, which also negatively regulated Zfhx3. Wang et al. [175] and Chiang et al. [176], respectively, reported that *miR-17-92, miR-106b-25*, miR-335 and miR-423 are also regulated by Pitx2. Furthermore, *miR-*

17-92 and *miR-106b-25* genetic deletions respectively were more susceptible to pacing-induced atrial fibrillation as compared to wild-type controls. More recently, Lozano-Velasco et al. [138] identified a large number of microRNAs modulated by Pitx2, most of which were previously reported to be associated to AF in humans **(Figure 2)**. Importantly, miR-1, miR-133, miR-21, miR-106b and miR-26 were previously reported to regulate calcium (CACNA1C [177]; RYR2 [176]), sodium (SCN5A [178]), potassium (KCNJ2 [179], KCNE1 [180], KCNB2 [181]), cation (HCN4 [181]) channel subunits, respectively.

In addition to post-transcriptional regulatory mechanisms mediated by microRNA expression, a novel layer of complex post-transcriptional regulation is emerging with the identification of long non coding RNAs. Long non-coding RNAs are transcripts of 200 nucleotides or more in length that display essentially no potential to code for proteins. They are structurally similar to mRNAs, are transcribed by RNA polymerase II, have 5’ terminal cap and 3’ terminal poly(A) tails, typical histone modifications, structured by exons and introns and often spliced [182, 183]. Although the vast majority of lncRNAs are located in the nuclear genome, lncRNAs are also reported within the mitochondrial DNA. Mitochondrial encoded lncRNAs are transcribed and processed by mitochondrial transcriptional machinery but regulated by nuclear-encoded proteins [184, 185]. Increasing evidences are demonstrating that lncRNAs can serve as biomarkers in AF [186], both in paroxysmal [187] and permanent AF [188], using distinct methodological approaches such as microarrays [187-190] and next generation RNAseq [188,190]. In addition, seminal work by Gore-Panter et al. [192, 193] have identified a cardiac-enriched lncRNA in humans, PANCR, specifically expressed in the left atrium and regulated by PITX2 have been reported, although its functional implication in AF remains elusive [192, 193]. Mechanistically, two novel lncRNAs (TCONS_0032546 and TCONS_00026102) have been identified in the canine cardiac fat pads having influence on AF onset by modulating atrial effective refractory period [191] while TCONS_00075467 is differentially expressed in the atrial of experimentally induced AF in rabbits, sponging miR-328 and influencing thereafter the expression and function of CACNA1C [194]. Overall these data illustrate key regulatory roles of these novel class of RNAs that will enlighten our understanding of the molecular mechanisms driving AF in next coming years.

Conclusion and perspectives

In this review we have highlighted the complexity of risk factors influencing the onset of AF. Multiple evidences have shown that Pitx2 plays a critical role on the molecular mechanisms leading to AF. Importantly, cardiovascular risk factors such as hypertension [195], hyperthyroidism [196] and unbalanced redox homeostasis [197] impairs Pitx2 expression, providing thus a molecular link to increased AF frequency in these cardiac physiopathological conditions, yet it remains to be established if diabetes, obesity, valvular heart disease and/or obstructive sleep apnea also impairs Pitx2 and thus predisposes to AF, given their relevance role as AF risk factors.

Experimental mouse models robustly demonstrated that Pitx2 loss-of-function predisposes to atrial arrhythmogenesis in mice, yet controversial findings are found as whether PITX2 is impaired in AF in humans. Furthermore discrepancies also exist regarding the fact of whether Pitx2 predisposes or triggers AF. Our findings suggest that embryonic Pitx2 insufficiency predisposes whereas adult Pitx2 insufficiency triggers AF. In this context, activation/repression of key microRNAs and Wnt signaling seems to be pivotal. In the last decade, our understanding of the downstream pathways controlled by Pitx2 has greatly advanced providing therefore new targets for therapeutic intervention. In this context, the modulation of distinct microRNAs involved in the Pitx2-Wnt-microRNA signaling cascade are promising targets. In addition the discovery of a new layer of gene regulation by long non coding RNAs open up new possibilities to identify novel AF biomarkers as well as new AF therapeutic targets.

Importantly, Pitx2 is severely impaired in an experimental heart failure pig model [187] as well as in patients with dilated cardiomyopathy, highlighting the putative connection between ventricular dysfunction and AF. In this context it is importantly to highlight that 4q25 risk variants are also associated to ventricular fibrillation [198] supporting thus a role of Pitx2 in ventricular function [197, 199]. Furthermore, 4q25 risk variants are also associated to aged AF [200] and AF recurrence [201]. Curiously, a link between AF 4q25 risk variants and the occurrence of appendicitis has recently emerged [202], yet it remains unexplored if there are shared molecular and signaling pathways.

The advent of novel strategies for dissect the molecular pathways involved in AF fibrillation is emerging with the generation of patient-specific induced pluripotent stem cells as recently reported by Mora et al. [203] and Marczenke et al. [204]. These novel tools will greatly enhance our understanding of AF pathophysiology. In addition, integrative exploration of gene-gene regulatory networks will provide novel candidates involved in AF pathophysiology [174, 205].

In summary, current data emphasized the key role of PITX2 orchestrating different aspects that, if impaired, are pro-arrhythmogenic. Furthermore, they also open new therapeutic approaches that could eventually minimize the burden of AF.

Figure legends

Figure 1. Schematic representation of the distinct genetic mutations involves in AF by classical genetic approaches (i.e. before GWAS) and by recent genome wide association studies (after GWAS).

Figure 2. Schematic representation of the hierarchical relationship between the homeobox transcription factor PITX2 and the distinct transcriptional and post-transcriptional genes associated by GWAS analyses with AF. This representation summarizes the experimental data obtained by different seminal studies; i.e. Wang et al. [119], Kirchhof et al. [120], Chinchilla et al. [118] and Lozano-Velasco et al. [138].

References

[1] Hakim, F.A.; Shen, W.K. Atrial fibrillation in the elderly: A review. *Fut. Cardiol*., **2014**, *10*, 745-758.

[2] Abed, H.S.; Wittert, G.A. Obesity and atrial fibrillation. *Obes. Rev.,* **2013**, *14*, 929-938.

[3] Vargas-Uricoechea, H.; Sierra-Torres, C.H. Thyroid hormones and the heart. *Horm. Mol. Biol. Clin. Investig*., **2014**, *18,* 15-26.

[4] Goudis, C.A.; Korantzopoulos, P.; Ntalas, I.V.; Kallergis, E.M.; Liu, T.; Ketikoglou, D.G. Diabetes mellitus and atrial fibrillation: Pathophysiological mechanisms and potential upstream therapies. *Int. J. Cardiol.,* **2015**, *184*, 617-622.

[5] De Caterina, R.; Camm, A.J. What is 'valvular' atrial fibrillation? A reappraisal. *Eur. Heart J.,* **2014**, *35*, 3328-3335.

[6] Kumar, K.R.; Mandleywala, S.N.; Link, M.S. Atrial and ventricular arrhythmias in hypertrophic cardiomyopathy. *Card Electrophysiol Clin.,* **2015**, *7*, 173-186.

[7] Riber, L.P.; Larsen, T.B.; Christensen, T.D. Postoperative atrial fibrillation prophylaxis after lung surgery: systematic review and meta-analysis. *Ann Thorac Surg.,* **2014**, *98*, 1989-1997.

[8] Qaddoura, A.; Kabali, C.; Drew, D.; *et al.* Obstructive sleep apnea as a predictor of atrial fibrillation after coronary artery bypass grafting: a systematic review and meta-analysis. *Can J Cardiol*., **2014**, *30*, 1516-2522.

[9] Anumonwo, J.M.; Kalifa, J. Risk factors and genetics of atrial fibrillation. *Cardiol Clin.,* **2014**, *32*, 485-494.

[10] Yadava, M.; Hughey, A.B.; Crawford, T.C. Postoperative atrial fibrillation: incidence, mechanisms, and clinical correlates. *Cardiol Clin.,* **2014**, *32*, 627-636.

[11] Akoum, N.; Marrouche, N. Assessment and impact of cardiac fibrosis on atrial fibrillation. *Curr Cardiol Rep.,* **2014**, *16*, 518.

[12] Berenfeld, O.; Jalife, J. Mechanisms of atrial fibrillation: rotors, ionic determinants, and excitation frequency. *Cardiol Clin.,* **2014**, *32*, 495-506.

[13] Heijman, J.; Voigt, N.; Wehrens, X.H.; Dobrev, D. Calcium dysregulation in atrial fibrillation: the role of CaMKII. *Front Pharmacol.,* **2014**, *5,* 30.

[14] Wolke, C.; Bukowska, A.; Goette, A.; Lendeckel, U. Redox control of cardiac remodeling in atrial fibrillation. *Biochim Biophys Acta.,* **2015**, *1850,* 1555-1565.

[15] Jalife, J.; Kaur, K. Atrial remodeling, fibrosis, and atrial fibrillation. *Trends Cardiovasc Med.,* **2015**, *25*, 475-484.

[16] Corradi, D. Atrial fibrillation from the pathologist's perspective. *Cardiovasc Pathol.,* **2014**, 23, 71-84.

[17] Senoo, K.; Lane, D.; Lip, G.Y. Stroke and bleeding risk in atrial fibrillation. *Korean Circ J.,* **2014**, *44*, 281-290.

[18] Zimetbaum, P.; Waks, J.W.; Ellis, E.R.; Glotzer, T.V.; Passman, R.S. Role of atrial fibrillation burden in assessing thromboembolic risk. *Circ Arrhythm Electrophysiol.,* **2014**, *7*, 1223-1229.

[19] Hirsh, B.J.; Copeland-Halperin, R.S.; Halperin, J.L. Fibrotic atrial cardiomyopathy, atrial fibrillation, and thromboembolism: mechanistic links and clinical inferences. *J Am Coll Cardiol.,* **2015**, *65,* 2239-2251.

[20] Hui, D.S.; Morley, J.E.; Mikolajczak, P.C.; Lee, R. Atrial fibrillation: A major risk factor for cognitive decline. *Am Heart J.* **2015**, *169,* 448-456.

[21] Jacobs, V.; Cutler, M.J.; Day, J.D.; Bunch, T.J. Atrial fibrillation and dementia. *Trends Cardiovasc Med.,* **2015,** *25,* 44-51.

[22] Luong, C.; Barnes, M.E.; Tsang, T.S. Atrial fibrillation and heart failure: cause or effect? *Curr Heart Fail Rep.,* **2014,** *11,* 463-470.

[23] Wijesurendra, R.S.; Casadei, B. Atrial fibrillation: effects beyond the atrium? *Cardiovasc Res.,* **2015,** *105,* 238-247.

[24] Chen, L.Y.; Benditt, D.G.; Alonso, A. Atrial fibrillation and its association with sudden cardiac death. *Circ J.,* **2014**, *78,* 2588-2593.

[25] Potpara, T.S.; Lip, G.Y. A brief history of 'lone' atrial fibrillation: from 'a peculiar pulse irregularity' to a modern public health concern. *Curr Pharm Des.,* **2015**, *21,* 679-96.

[26] Tello-Montoliu, A.; Hernández-Romero, D.; Sanchez-Martínez, M.; Valdes, M.; Marín, F. Lone atrial fibrillation - a diagnosis of exclusion. *Curr Pharm Des.,* **2015**, *21,* 544-550.

[27] Brugada, R.; Tapscott, T.; Czernuszewicz, G.Z.; *et al.* Identification of a genetic locus for familial atrial fibrillation. *N Engl J Med.,* **1997**, *336,* 905-911.

[28] Chen, Y.H.; Xu, S.J.; Bendahhou, S.; *et al.* KCNQ1 gain-of-function mutation in familial atrial fibrillation. *Science.,* **2003**, *299,* 251-254.

[29] Xia, M.; Jin, Q.; Bendahhou, S.; He, Y.; *et al*. A Kir2.1 gain-of-function mutation underlies familial atrial fibrillation. *Biochem Biophys Res Commun.,* **2005,** *332,* 1012-1019.

[30] Yang, Y.; Xia, M.; Jin, Q.; *et al.* Identification of a KCNE2 gain-of-function mutation in patients with familial atrial fibrillation. *Am J Hum Genet.,* **2004,** *75,* 899-905.

[31] Berenfeld, O.; Zaitsev, A.V.; Mironov, S.F.; Pertsov, A.M.; Jalife, J. Frequency-dependent breakdown of wave propagation into fibrillatory conduction across the pectinate muscle network in the isolated sheep right atrium. *Circ Res.,* **2002**, *90,* 1173-1180.

[32] Chen, J.; Mandapati, R.; Berenfeld, O.; Skanes, A.C.; Gray, R.A.; Jalife, J. Dynamics of wavelets and their role in atrial fibrillation in the isolated sheep heart. *Cardiovasc Res.,* **2000,** *48,* 220-32.

[33] Mandapati, R.; Skanes, A.; Chen, J.; Berenfeld, O.; Jalife, J. Stable microreentrant sources as a mechanism of atrial fibrillation in the isolated sheep heart. *Circulation.,* **2000,** *101,* 194-199.

[34] Gudbjartsson, D.F.; Arnar, D.O.; Helgadottir, A.; *et al*. Variants conferring risk of atrial fibrillation on chromosome 4q25. *Nature.,* **2007,** *448,* 353-357.

[35] Benjamin, E.J.; Rice, K.M.; Arking, D.E.; *et al.* Variants in ZFHX3 are associated with atrial fibrillation in individuals of European ancestry. *Nat Genet.,* **2009,** *41,* 879-881.

[36] Ellinor, P.T.; Lunetta, K.L.; Albert, C.M.; *et al.* Meta-analysis identifies six new susceptibility loci for atrial fibrillation. *Nat Genet.,* **2012,** *44,* 670-675.

[37] Ellinor, P.T.; Lunetta, K.L.; Glazer, N.L.; *et al.* Common variants in KCNN3 are associated with lone atrial fibrillation. *Nat Genet.,* **2010,** *42,* 240-244.

[38] Gudbjartsson, D.F.; Holm, H.; Gretarsdottir, S.; *et al.* A sequence variant in ZFHX3 on 16q22 associates with atrial fibrillation and ischemic stroke. *Nat Genet.,* **2009,** *41,* 876-878.

[39] Schnabel, R.B.; Kerr, K.F.; Lubitz, S.A.; *et al.* Large-scale candidate gene analysis in whites and African Americans identifies IL6R polymorphism in relation to atrial fibrillation: the National Heart, Lung, and Blood Institute's Candidate Gene Association Resource (CARe) project. *Circ Cardiovasc Genet.,* **2011,** *4,* 557-564.

[40] H, J.; Zhu, W.; Yu, Y.; Hu, J.; Hong, K. Variant rs2200733 and rs10033464 on chromosome 4q25 are associated with increased risk of atrial fibrillation after catheter ablation: Evidence from a meta-analysis. *Cardiol J.,* **2018,** *25,* 628-638.

[41] Miyazaki, S.; Ebana, Y., Liu, L.; *et al.* Chromosome 4q25 variants and recurrence after second-generation cryoballoon ablation in patients with paroxysmal atrial fibrillation. *Int J Cardiol*., **2017**, *244,* 151-157.

[42] Zhao, L.Q.; Zhang, G.B.; Wen, Z.J.; *et al.* Common variants predict recurrence after nonfamilial atrial fibrillation ablation in Chinese Han population. *Int J Cardiol.,* **2017,** *227,* 360-366.

[43] Chen, F.; Yang, Y.; Zhang, R.; *et al.* Polymorphism rs2200733 at chromosome 4q25 is associated with atrial fibrillation recurrence after radiofrequency catheter ablation in the Chinese Han population. *Am J Transl Res.,* **2016,** *8,* 688-697.

[44] Parvez, B.; Shoemaker, M.B.; Muhammad, R.; *et al.* Common genetic polymorphism at 4q25 locus predicts atrial fibrillation recurrence after successful cardioversion. *Heart Rhythm.,* **2013,** *10*, 849-855.

[45] Benjamin Shoemaker, M.; Muhammad, R.; Parvez, B.; *et al.* Common atrial fibrillation risk alleles at 4q25 predict recurrence after catheter-based atrial fibrillation ablation. *Heart Rhythm.,* **2013,** *10,* 394-400.

[46] Husser, D.; Adams, V.; Piorkowski, C.; Hindricks, G.; Bollmann, A. Chromosome 4q25 variants and atrial fibrillation recurrence after catheter ablation. *J Am Coll Cardiol.,* **2010,** *55,* 747-753.

[47] Amin, A.S.; Bhuiyan, Z.A. SCN5A mutations in atrial fibrillation. *Heart Rhythm.,* **2010,** *7,* 1870-1871.

[48] Blana, A.; Kaese, S.; Fortmüller, L.; *et al.* Knock-in gain-of-function sodium channel mutation prolongs atrial action potentials and alters atrial vulnerability. *Heart Rhythm*., **2010,** *7,* 1862-1869.

[49] Laitinen-Forsblom, P.J.; Mäkynen, P.; Mäkynen, H.; *et al.* SCN5A mutation associated with cardiac conduction defect and atrial arrhythmias. *J Cardiovasc Electrophysiol.,* **2006,** *17,* 480-485.

[50] Li, Q.; Huang, H.; Liu, G.; *et al*. Gain-of-function mutation of Nav1.5 in atrial fibrillation enhances cellular excitability and lowers the threshold for action potential firing. *Biochem Biophys Res Commun.,* **2009**, *380,* 132-137.

[51] Makiyama, T.; Akao, M.; Shizuta, S.; *et al.* A novel SCN5A gain-of-function mutation M1875T associated with familial atrial fibrillation. *J Am Coll Cardiol.,* **2008**, *52,*1326-1334.

[52] Benito, B.; Brugada, R.; Perich, R.M.; *et al*. A mutation in the sodium channel is responsible for the association of long QT syndrome and familial atrial fibrillation. *Heart Rhythm*., **2008**, *5,* 1434-1440.

[53] Calloe, K.; Schmitt, N.; Grubb, S.; *et al.* Multiple arrhythmic syndromes in a newborn, owing to a novel mutation in SCN5A. *Can J Physiol Pharmacol.,* **2011,** *89,* 723-736.

[54] Dolz-Gaitón, P.; Núñez, M.; Núñez, L.; *et al.* Functional characterization of a novel frameshift mutation in the C-terminus of the Nav1.5 channel underlying a Brugada syndrome with variable expression in a Spanish family. *PLoS One.,* **2013,** *8,* e81493.

[55] Olson, T.M.; Michels, V.V.; Ballew, J.D.; *et al*. Sodium channel mutations and susceptibility to heart failure and atrial fibrillation. *JAMA.,* **2005,** *293,* 447-454.

[56] Ziyadeh-Isleem, A.; Clatot, J.; Duchatelet, S.; *et al*. A truncating SCN5A mutation combined with genetic variability causes sick sinus syndrome and early atrial fibrillation. *Heart Rhythm,* **2014,** *11,* 1015-1023.

[57] Rossenbacker, T.; Carroll, S.J.; Liu, H.; *et al*. Novel pore mutation in SCN5A manifests as a spectrum of phenotypes ranging from atrial flutter, conduction disease, and Brugada syndrome to sudden cardiac death. *Heart Rhythm.,* **2004,** *1,* 610-615.

[58] Watanabe, H.; Darbar, D.; Kaiser, D.W.; *et al.* Mutations in sodium channel β1- and β2-subunits associated with atrial fibrillation. *Circ Arrhythm Electrophysiol.,* **2009,** *2,* 268-275.

[59] Wang, P.; Yang, Q.; Wu, X.; *et al*. Functional dominant-negative mutation of sodium channel subunit gene SCN3B associated with atrial fibrillation in a Chinese GeneID population. *Biochem Biophys Res Commun.,* **2010,** *398,* 98-104.

[60] Olesen, M.S.; Jespersen, T.; Nielsen, J.B.; *et al*. Mutations in sodium channel β-subunit SCN3B are associated with early-onset lone atrial fibrillation. *Cardiovasc Res.,* **2011,** *89,* 786-793.

[61] Li, R.G.; Wang, Q.; Xu, Y.J.; *et al*. Mutations of the SCN4B-encoded sodium channel β4 subunit in familial atrial fibrillation. *Int J Mol Med.,* **2013,** *32,* 144-150.

[62] Olesen, M.S.; Holst, A.G.; Svendsen, J.H.; Haunsø, S.; Tfelt-Hansen, J. SCN1Bb R214Q found in 3 patients: 1 with Brugada syndrome and 2 with lone atrial fibrillation. *Heart Rhythm.* **2012,** *9,* 770-773.

[63] Macri, V.; Mahida, S.N.; Zhang, M.L.; *et al*. A novel trafficking-defective HCN4 mutation is associated with early-onset atrial fibrillation. *Heart Rhythm.,* **2014,** *11,* 1055-1062.

[64] Hong, K.; Piper, D.R.; Diaz-Valdecantos A.; *et al*. De novo KCNQ1 mutation responsible for atrial fibrillation and short QT syndrome in utero. *Cardiovasc Res.,* **2005,** *68,* 433-440.

[65] Lundby, A.; Ravn, L.S.; Svendsen, J.H.; Olesen, S.P.; Schmitt, N. KCNQ1 mutation Q147R is associated with atrial fibrillation and prolonged QT interval. *Heart Rhythm*., **2007,** *4,* 1532-1541.

[66] Kharche, S.; Adeniran, I.; Stott, J.; *et al*. Pro-arrhythmogenic effects of the S140G KCNQ1 mutation in human atrial fibrillation - insights from modelling. *J Physiol.,* **2012,** *590,* 4501-4514.

[67] Das, S.; Makino, S.; Melman, Y.F.; *et al*. Mutation in the S3 segment of KCNQ1 results in familial lone atrial fibrillation. *Heart Rhythm.,* **2009,** *6,* 1146-1153.

[68] El Harchi, A.; Zhang, H.; Hancox, J.C. The S140G KCNQ1 atrial fibrillation mutation affects 'I(KS)' profile during both atrial and ventricular action potentials. *J Physiol Pharmacol.,* **2010,** *61,* 759-764.

[69] Ravn, L.S.; Aizawa, Y.; Pollevick, G.D.; *et al*. Gain of function in IKs secondary to a mutation in KCNE5 associated with atrial fibrillation. *Heart Rhythm.,* **2008**, *5,* 427-435.

[70] Nielsen, J.B.; Bentzen, B.H.; Olesen, M.S.; *et al*. Gain-of-function mutations in potassium channel subunit KCNE2 associated with early-onset lone atrial fibrillation. *Biomark Med,* **2014,** *8,* 557-570.

[71] Deo, M.; Ruan, Y.; Pandit, S.V.; *et al*. KCNJ2 mutation in short QT syndrome 3 results in atrial fibrillation and ventricular proarrhythmia. *Proc Natl Acad Sci U S A.,* **2013,** *110,* 4291-4296.

[72] Kharche, S.; Garratt, C.J.; Boyett, M.R.; *et al*. Atrial proarrhythmia due to increased inward rectifier current (I(K1)) arising from KCNJ2 mutation--a simulation study. *Prog Biophys Mol Biol.,* **2008,** *98,* 186-197.

[73] Chelu, M.G.; Sarma, S.; Sood, S.; *et al*. Calmodulin kinase II-mediated sarcoplasmic reticulum Ca2+ leak promotes atrial fibrillation in mice. *J Clin Invest.,* **2009,** *119,* 1940-1951.

[74] Shan, J.; Xie, W.; Betzenhauser, M.; *et al*. Calcium leak through ryanodine receptors leads to atrial fibrillation in 3 mouse models of catecholaminergic polymorphic ventricular tachycardia. *Circ Res.,* **2012**, *111,* 708-717.

[75] Li, N.; Wang, T.; Wang, W.; *et al*. Inhibition of CaMKII phosphorylation of RyR2 prevents induction of atrial fibrillation in FKBP12.6 knockout mice. *Circ Res.,* **2012,** *110,* 465-470.

[76] Di Pino, A.; Caruso, E.; Costanzo, L.; Guccione, P. A novel RyR2 mutation in a 2-year-old baby presenting with atrial fibrillation, atrial flutter, and atrial ectopic tachycardia. *Heart Rhythm.,* **2014,** *11,* 1480-1483.

[77] Zhabyeyev, P.; Hiess, F.; Wang, R.; Liu, Y.; Wayne Chen, S.R.; Oudit, G.Y. S4153R is a gain-of-function mutation in the cardiac Ca(2+) release channel ryanodine receptor associated with catecholaminergic polymorphic ventricular tachycardia and paroxysmal atrial fibrillation. *Can J Cardiol.,* **2013,** *29,* 993-996.

[78] Kazemian, P.; Gollob, M.H.; Pantano, A.; Oudit, G.Y. A novel mutation in the RYR2 gene leading to catecholaminergic polymorphic ventricular tachycardia and paroxysmal atrial fibrillation: dose-dependent arrhythmia-event suppression by β-blocker therapy. *Can J Cardiol.,* **2011,** *27,* 870, e7-10.

[79] Zhang, Y.; Fraser, J.A.; Jeevaratnam, K.; *et al*. Acute atrial arrhythmogenicity and altered Ca(2+) homeostasis in murine RyR2-P2328S hearts. *Cardiovasc Res.,* **2011,** *89,* 794-804.

[80] Thibodeau, I.L.; Xu, J.; Li, Q.; *et al*. Paradigm of genetic mosaicism and lone atrial fibrillation: physiological characterization of a connexin 43-deletion mutant identified from atrial tissue. *Circulation.,* **2010,** *122,* 236-244.

[81] Tuomi, J.M.; Tyml, K.; Jones, D.L. Atrial tachycardia/fibrillation in the connexin 43 G60S mutant (Oculodentodigital dysplasia) mouse. *Am J Physiol Heart Circ Physiol.,* **2011,** *300,* H1402-1411.

[82] Delmar, M.; Makita, N. Cardiac connexins, mutations and arrhythmias. *Curr Opin Cardiol.,* **2012**, *27,* 236-241.

[83] Gollob, M.H.; Jones, D.L.; Krahn, A.D.; *et al*. Somatic mutations in the connexin 40 gene (GJA5) in atrial fibrillation. *N Engl J Med.,* **2006,** *354,* 2677-2688.

[84] Yang, Y.Q.; Liu, X.; Zhang, X.L.; *et al*. Novel connexin40 missense mutations in patients with familial atrial fibrillation. *Europace.,* **2010,** *12,* 1421-1427.

[85] Yang, Y.Q.; Zhang, X.L.; Wang, X.H.; *et al*. Connexin40 nonsense mutation in familial atrial fibrillation. *Int J Mol Med.,* **2010,** *26,* 605-610.

[86] Gemel, J.; Simon, A.R.; Patel, D.; *et al*. Degradation of a connexin40 mutant linked to atrial fibrillation is accelerated. *J Mol Cell Cardiol.,* **2014,** *74,* 330-339.

[87] Sun, Y.; Hills, M.D.; Ye, W.G.; Tong, X.; Bai, D. Atrial fibrillation-linked germline GJA5/connexin40 mutants showed an increased hemichannel function. *PLoS One.,* **2014,** *9,* e95125.

[88] Sun, Y.; Tong, X.; Chen, H.; *et al*. An atrial-fibrillation-linked connexin40 mutant is retained in the endoplasmic reticulum and impairs the function of atrial gap-junction channels. *Dis Model Mech.,* **2014**, *7,* 561-569.

[89] Patel, D.; Gemel, J.; Xu, Q.; *et al*. Atrial fibrillation-associated connexin40 mutants make hemichannels and synergistically form gap junction channels with novel properties. *FEBS Lett.,* **2014,** *588*, 1458-1464.

[90] Bai, D. Atrial fibrillation-linked GJA5/connexin40 mutants impaired gap junctions via different mechanisms. *FEBS Lett.,* **2014,** *588,* 1238-1243.

[91] Postma, A.V.; van de Meerakker, J.B.; Mathijssen, I.B.; *et al*. A gain-of-function TBX5 mutation is associated with atypical Holt-Oram syndrome and paroxysmal atrial fibrillation. *Circ Res.,* **2008**, *102,* 1433-1442.

[92] Gutierrez-Roelens, I.; De Roy, L.; Ovaert, C.; *et al*. A novel CSX/NKX2-5 mutation causes autosomal-dominant AV block: are atrial fibrillation and syncopes part of the phenotype? *Eur J Hum Genet.,* **2006,** *14,*1313-1316.

[93] Huang, R.T.; Xue, S.; Xu, Y.J.; Zhou, M.; Yang, Y.Q. A novel NKX2.5 loss-of-function mutation responsible for familial atrial fibrillation. *Int J Mol Med.,* **2013,** *31,* 1119-1126.

[94] Xie, W.H.; Chang, C.; Xu, Y.J. *et al*. Prevalence and spectrum of Nkx2.5 mutations associated with idiopathic atrial fibrillation. *Clinics.,* **2013,** *68,* 777-784.

[95] Yu, H.; Xu, J.H.; Song, H.M.; *et al.* Mutational spectrum of the NKX2-5 gene in patients with lone atrial fibrillation. *Int J Med Sci.,* **2014,** *11,* 554-563.

[96] Yuan, F.; Qiu, X.B.; Li, R.G.; *et al*. A novel NKX2-5 loss-of-function mutation predisposes to familial dilated cardiomyopathy and arrhythmias. *Int J Mol Med.,* **2015,** *35,* 478-486.

[97] Wang, J.; Zhang, D.F.; Sun, Y.M.; *et al.* NKX2-6 mutation predisposes to familial atrial fibrillation. *Int J Mol Med.,* **2014,** *34,* 1581-1590.

[98] Wang, J.; Zhang, D.F.; Sun, Y.M.; Yang, Y.Q. A novel PITX2c loss-of-function mutation associated with familial atrial fibrillation. *Eur J Med Genet.,* **2014,** *57*, 25-31.

[99] Zhou, Y.M.; Zheng, P.X.; Yang, Y.Q.; Ge, Z.M.; Kang, W.Q. A novel PITX2c loss-of-function mutation underlies lone atrial fibrillation. *Int J Mol Med.,* **2013**, *32,* 827-834.

[100] Tsai, C.T.; Hsieh, C.S.; Chang, S.N.; *et al*. Next-generation sequencing of nine atrial fibrillation candidate genes identified novel de novo mutations in patients with extreme trait of atrial fibrillation. *J Med Genet.,* **2015,** *52,* 28-36.

[101] Li, Q.Y.; Newbury-Ecob, R.A.; Terrett, J.A.; *et al*. Holt-Oram syndrome is caused by mutations in TBX5, a member of the Brachyury (T) gene family. *Nat Genet.,* **1997,** *15,* 21-29.

[102] Costa, M.W.; Guo, G.; Wolstein, O.; *et al*. Functional characterization of a novel mutation in NKX2-5 associated with congenital heart disease and adult-onset cardiomyopathy. *Circ Cardiovasc Genet.,* **2013,** *6,* 238-247.

[103] Qu, X.K.; Qiu, X.B.; Yuan, F.; *et al*. A novel NKX2.5 loss-of-function mutation associated with congenital bicuspid aortic valve. *Am J Cardiol.,* **2014,** *114,* 1891-1895.

[104] Wang, J.; Mao, J.H.; Ding, K.K.; *et al*. A novel NKX2.6 mutation associated with congenital ventricular septal defect. *Pediatr Cardiol.,* **2015,** *36,* 646-656.

[105] Lin, Y.; Guo, X.; Zhao, B.; *et al*. Association analysis identifies new risk loci for congenital heart disease in Chinese populations. *Nat Commun.,* **2015**, *6,* 8082.

[106] Chowdhury, R.; Ashraf, H.; Melanson, M.; *et al.* A Mouse Model of Human Congenital Heart Disease: Progressive Atrioventricular Block Induced by a Heterozygous Nkx2-5 Homeodomain Missense Mutation. *Circ Arrhythm Electrophysiol.,* **2015,** *8,* 1255-1264.

[107] Zhou, W.; Zhao, L.; Jiang, J.Q.; Jiang, W.F.; Yang, Y.Q.; Qiu, X.B. A novel TBX5 loss-of-function mutation associated with sporadic dilated cardiomyopathy. *Int J Mol Med.,* **2015,** *36,* 282-288.

[108] Zhao, L.; Ni, S.H.; Liu, X.Y.; *et al*. Prevalence and spectrum of Nkx2.6 mutations in patients with congenital heart disease. *Eur J Med Genet.,* **2014,** *57,* 579-586.

[109] Wang, J.; Zhang, D.F.; Sun, Y.M.; *et al*. NKX2-6 mutation predisposes to familial atrial fibrillation. *Int J Mol Med.,* **2014,** *34,* 1581-1590.

[110] Ta-Shma, A.; El-lahham, N.; Edvardson, S.; *et al*. Conotruncal malformations and absent thymus due to a deleterious NKX2-6 mutation. *J Med Genet.,* **2014,** *51,* 268-270.

[111] Heathcote, K.; Braybrook, C.; Abushaban, L.; *et al*. Common arterial trunk associated with a homeodomain mutation of NKX2.6. *Hum Mol Genet.,* **2005,** *14,* 585-593.

[112] Smith, J.G.; Magnani, J.W.; Palmer, C.; *et al*. Genome-wide association studies of the PR interval in African Americans. *PLoS Genet.,* **2011,** *7,* e1001304.

[113] den Hoed, M.; Eijgelsheim, M.; Esko, T.; *et al.* Identification of heart rate-associated loci and their effects on cardiac conduction and rhythm disorders. *Nat Genet.,* **2013,** *45,* 621-631.

[114] Mohanty, S.; Santangeli, P.; Bai, R.; *et al*. Variant rs2200733 on chromosome 4q25 confers increased risk of atrial fibrillation: evidence from a meta-analysis. *J Cardiovasc Electrophysiol.,* **2013,** *24,* 155-161.

[115] Olesen, M.S.; Holst, A.G.; Jabbari, J.; *et al*. Genetic loci on chromosomes 4q25, 7p31, and 12p12 are associated with onset of lone atrial fibrillation before the age of 40 years. *Can J Cardiol.,* **2012,** *28,* 191-195.

[116] Henningsen, K.M.; Olesen, M.S.; Haunsoe, S.; Svendsen, J.H. Association of rs2200733 at 4q25 with early onset of lone atrial fibrillation in young patients. *Scand Cardiovasc J.,* **2011,** *45,* 324-326.

[117] Kiliszek, M.; Franaszczyk, M.; Kozluk, E.; *et al*. Association between variants on chromosome 4q25, 16q22 and 1q21 and atrial fibrillation in the Polish population. *PLoS One.,* **2011,** *6,* e21790.

[118] Chinchilla, A.; Daimi, H.; Lozano-Velasco, E.; *et al.* PITX2 insufficiency leads to atrial electrical and structural remodeling linked to arrhythmogenesis. *Circ Cardiovasc Genet.,* **2011,** *4,* 269-279.

[119] Wang, J.; Klysik, E.; Sood, S.; Johnson, R.L.; Wehrens, X.H.; Martin, J.F. Pitx2 prevents susceptibility to atrial arrhythmias by inhibiting left-sided pacemaker specification. *Proc Natl Acad Sci U S A.,* ***2010*****,** *107,* 9753-9758.

[120] Kirchhof, P.; Kahr, P.C.; Kaese, S.; *et al*. PITX2c is expressed in the adult left atrium, and reducing Pitx2c expression promotes atrial fibrillation inducibility and complex changes in gene expression. *Circ Cardiovasc Genet.,* **2011,** *4,* 123-133.

[121] Semina, E.V.; Reiter, R.; Leysens, N.J.; *et al.* Cloning and characterization of a novel bicoid-related homeobox transcription factor gene, RIEG, involved in Rieger syndrome. *Nat Genet.,* **1996,** *14,* 392-399.

[122] Gage, P.J.; Camper, S.A. Pituitary homeobox 2, a novel member of the bicoid-related family of homeobox genes, is a potential regulator of anterior structure formation. *Hum Mol Genet.,* **1997,** *6,* 457-464.

[123] St Amand, T.R.; Ra, J.; Zhang, Y.; *et al.* Cloning and expression pattern of chicken PITX2: a new component in the SHH signaling pathway controlling embryonic heart looping. *Biochem Biophys Res Commun.,* **1998,** *247,* 100-105.

[124] Campione, M.; Steinbeisser, H.; Schweickert, A.; *et al.* The homeobox gene PITX2: mediator of asymmetric left-right signaling in vertebrate heart and gut looping. *Development.,* **1999,** *126,* 1225-1234.

[125] Tümer, Z.; Bach-Holm, D. Axenfeld-Rieger syndrome and spectrum of PITX2 and FOXC1 mutations. *Eur J Hum Genet.,* **2009,** *17,* 1527-1539.

[126] Cox, C.J.; Espinoza, H.M.; McWilliams, B.; *et al*. Differential regulation of gene expression by PITX2 isoforms. *J. Biol. Chem.,* **2002,** *77,* 25001-25010.

[127] Logan, M.; Pagan-Westphal, S.M.; Smith, D.M.; Paganessi, L.; Tabin, C.J. The transcription factor PITX2 mediates situs-specific morphogenesis in response to left-right asymmetric signals. *Cell.,* **1998,** *94,* 307-317.

[128] Piedra, M.E.; Icardo, J.M.; Albajar, M.; Rodriguez-Rey, J.C.; Ros, M.A. PITX2 participates in the late phase of the pathway controlling left-right asymmetry. *Cell.,* **1998,** *94,* 319-324.

[129] Long, S.; Ahmad, N.; Rebagliati, M. The zebrafish nodal-related gene southpaw is required for visceral and diencephalic left-right asymmetry. *Development.,* **2003,** *130,* 2303-2316.

[130] Bisgrove, B.W.; Essner, J.J.; Yost, H.J. Multiple pathways in the midline regulate concordant brain, heart and gut left-right asymmetry. *Development.,* **2000,** *127,* 3567-3579.

[131] Guioli, S.; Lovell-Badge, R. PITX2 controls asymmetric gonadal development in both sexes of the chick and can rescue the degeneration of the right ovary. *Development.,* **2007,** *134,* 4199-4208.

[132] Lin, C.R.; Kioussi, C.; O'Connell, S.; *et al.* PITX2 regulates lung asymmetry, cardiac positioning and pituitary and tooth morphogenesis. *Nature.,* **1999,** *401,* 279-282.

[133] Lu, M.F.; Pressman, C.; Dyer, R.; Johnson, R.L.; Martin, J.F. Function of Rieger syndrome gene in left-right asymmetry and craniofacial development. *Nature.,* **1999,** *401,* 276-278.

[134] Mommersteeg, M.T.; Brown, N.A.; Prall, O.W.J.; *et al.* PITX2c and Nkx2-5 are required for the formation and identity of the pulmonary myocardium. *Circ. Res.,* **2007,** *101,* 902-909.

[135] Aguirre, L.A.; Alonso, M.E.; Badía-Careaga, C.; *et al*. Long-range regulatory interactions at the 4q25 atrial fibrillation risk locus involve PITX2c and ENPEP. *BMC Biol.,* **2015,** *13,* 26.

[136] Ye, J.; Tucker, N.R.; Weng, L.C.; Clauss, S.; Lubitz, S.A.; Ellinor, P.T. A Functional Variant Associated with Atrial Fibrillation Regulates PITX2c Expression through TFAP2a. *Am J Hum Genet.,* **2016,** *99,* 1281-1291.

[137] Tao, Y.; Zhang, M.; Li, L.; *et al*. Pitx2, an atrial fibrillation predisposition gene, directly regulates ion transport and intercalated disc genes. *Circ Cardiovasc Genet.,* **2014,** *7,* 23-32.

[138] Lozano-Velasco, E.; Hernández-Torres, F.; Daimi, H.; *et al*. Pitx2 impairs calcium handling in a dose-dependent manner by modulating Wnt signalling. *Cardiovasc Res.,* **2016,** *109,* 55-66.

[139] Pérez-Hernández, M.; Matamoros, M.; Barana, A.; *et al*. Pitx2c increases in atrial myocytes from chronic atrial fibrillation patients enhancing IKs and decreasing ICa,L. *Cardiovasc Res.,* **2016,** *109,* 431-441.

[140] Syeda, F.; Holmes, A.P.; Yu T.Y.; *et al*. PITX2 Modulates Atrial Membrane Potential and the Antiarrhythmic Effects of Sodium-Channel Blockers. *J Am Coll Cardiol.,* **2016,** *68,* 1881-1894.

[141] Nadadur, R.D.; Broman, M.T.; Boukens, B.; *et al*. Pitx2 modulates a Tbx5-dependent gene regulatory network to maintain atrial rhythm. *Sci Transl Med.,* **2016,** *8,* 354ra115.

[142] Franco, D.; Aranega, A.E. PITX2 (Pituitary Homeobox Gene 2). *Encyclopedia of Signalling Molecules*, Springer Nature, pp. 4024-4032.

[143] Franco, D.; Chinchilla, A.; Aránega, A.E. Transgenic insights linking Pitx2 and atrial arrhythmias. *Front Physiol.,* **2012,** *3,* 206.

[144] Lee, J.Y.; Kim, T.H.; Yang, P.S.; *et al*. Korean atrial fibrillation network genome-wide association study for early-onset atrial fibrillation identifies novel susceptibility loci. *Eur Heart J.,* **2017,** *38,* 2586-2594.

[145] Thorolfsdottir, R.B.; Sveinbjornsson, G.; Sulem, P.; *et al*. A Missense Variant in PLEC Increases Risk of Atrial Fibrillation. *J Am Coll Cardiol.,* **2017,** *70,* 2157-2168.

[146] Nielsen, J.B.; Fritsche, L.G.; Zhou, W.; *et al*. Genome-wide Study of Atrial Fibrillation Identifies Seven Risk Loci and Highlights Biological Pathways and Regulatory Elements Involved in Cardiac Development. *Am J Hum Genet.,* **2018,** *102,* 103-115.

[147] Milan, D. The Com-PLEC-sity of Atrial Fibrillation Genetics. *J Am Coll Cardiol.,* **2017,** *70,* 2169-2170.

[148] Bapat, A.; Anderson, C.D.; Ellinor, P.T.; Lubitz, S.A. Genomic basis of atrial fibrillation. *Heart.,* **2018,** *104,* 201-206.

[149]. Tsai, C.T.; Hsieh, C.S.; Chang, S.N.; *et al*. Next-generation sequencing of nine atrial fibrillation candidate genes identified novel de novo mutations in patients with extreme trait of atrial fibrillation. *J Med Genet.*, **2015,** *52,* 28-36.

[150] Huang, Y.; Wang, C.; Yao, Y.; *et al*. Molecular Basis of Gene-Gene Interaction: Cyclic Cross-Regulation of Gene Expression and Post-GWAS Gene-Gene Interaction Involved in Atrial Fibrillation. *PLoS Genet.*, **2015,** *11,* e1005393.

[151] Kääb, S.; Darbar, D.; van Noord C.; *et al*. Large scale replication and meta-analysis of variants on chromosome 4q25 associated with atrial fibrillation. *Eur Heart J.,* **2009,** *30,* 813-819.

[152] Li, G.; Zhang, R.; Gao, L.; *et al*. Lack of association between rs3807989 in cav1 and atrial fibrillation. *Int J Clin Exp Pathol.,* **2014,** *7,* 4339-4344.

[153] Tada, H.; Shiffman, D.; Smith, J.G.; *et al.* Twelve-single nucleotide polymorphism genetic risk score identifies individuals at increased risk for future atrial fibrillation and stroke. *Stroke.,* **2014,** *45*, 2856-2862.

[154] Mahida, S.; Mills, R.W.; Tucker, N.R.; *et al*. Overexpression of KCNN3 results in sudden cardiac death. *Cardiovasc Res.,* **2014,** *101,* 326-334.

[155] Jiang, Q.; Ni, B.; Shi, J.; *et al*. Down-regulation of ATBF1 activates STAT3 signaling via PIAS3 in pacing-induced HL-1 atrial myocytes. *Biochem Biophys Res Commun.,* **2014,** *449,* 278-283.

[156] Tucker, N.R.; Dolmatova, E.V.; Lin, H.; *et al*. Diminished PRRX1 Expression Is Associated With Increased Risk of Atrial Fibrillation and Shortening of the Cardiac Action Potential. *Circ Cardiovasc Genet.,* **2017**, *10,* pii: e001902.

[157] Seidl, M.D.; Stein, J.; Hamer, S.; *et al.* Characterization of the Genetic Program Linked to the Development of Atrial Fibrillation in CREM-IbΔC-X Mice. *Circ Arrhythm Electrophysiol.,* **2017,** *10,* pii: e005075.

[158] Franco, D.; Lozano-Velasco, E.; Aranega, A. Gene regulatory networks in atrial fibrillation. *World J Med Genet.,* **2016,** *6,* 1-16.

[159] Nagy, I.I.; Railo, A.; Rapila, R.; *et al*. Wnt-11 signalling controls ventricular myocardium development by patterning N-cadherin and beta-catenin expression. *Cardiovasc Res.,* **2010,** *85,* 100-109.

[160] Pandur, P.; Läsche, M.; Eisenberg, L.M.; Kühl, M. Wnt-11 activation of a non-canonical Wnt signalling pathway is required for cardiogenesis. *Nature.,* **2002,** *418,* 636-641.

[161] Martin, A.; Maher, S.; Summerhurst, K.; Davidson, D.; Murphy, P. Differential deployment of paralogous Wnt genes in the mouse and chick embryo during development. *Evol Dev.,* **2012,** *14,* 178-195.

[162] Espinoza-Lewis, R.A.; Wang, D.Z. MicroRNAs in heart development. *Curr Top Dev Biol.,* **2012,** *100,* 279-317.

[163] Franco, D.; Aranega, A. Post-transcriptional regulatory mechanisms. In: *Clinic, Genetics and Molecular Pathways of Congenital Heart Diseases* Eds. Sperling S, Driscoll D, Kelly R. Springer., **2015.**

[164] Zhou, J.; Dong, X.; Zhou, Q. *et al*. microRNA expression profiling of heart tissue during fetal development. *Int J Mol Med.,* **2014,** *33,* 1250-1260.

[165] Hu, D.L.; Liu, Y.Q. Chen, F.K.; *et al*. Differential expression of microRNAs in cardiac myocytes compared to undifferentiated P19 cells. *Int J Mol Med.,* **2011,** *28,* 59-64.

[166] Synnergren, J.; Améen, C.; Lindahl, A.; Olsson, B.; Sartipy, P. Expression of microRNAs and their target mRNAs in human stem cell-derived cardiomyocyte clusters and in heart tissue. *Physiol Genomics.* **2011,** *43,* 581-594.

[167] Nishi, H.; Sakaguchi, T.; Miyagawa, S.; *et al*. Impact of microRNA expression in human atrial tissue in patients with atrial fibrillation undergoing cardiac surgery. *PLoS One.,* **2013,** *8,* e73397.

[168] Wang, J.; Wang, Y.; Han, J.; *et al*. Integrated analysis of microRNA and mRNA expression profiles in the left atrium of patients with nonvalvular paroxysmal atrial fibrillation: Role of miR-146b-5p in atrial fibrosis. *Heart Rhythm.,* **2015,** *12,* 1018-1026.

[169] Li, M.; Zhang, J. Circulating MicroRNAs: Potential and Emerging Biomarkers for Diagnosis of Cardiovascular and Cerebrovascular Diseases. *Biomed Res Int.,* **2015,** *2015,* 730535.

[170] Orenes-Piñero, E.; Montoro-García, S.; Patel, J.V.; Valdés, M.; Marín, F.; Lip, G.Y. Role of microRNAs in cardiac remodelling: new insights and future perspectives. *Int J Cardiol.,* **2013,** *167,* 1651-1659.

[171] Poudel, P.; Xu, Y.; Cui, Z.; Sharma, D.; Tian, B.; Paudel, S. Atrial fibrillation: recent advances in understanding the role of microRNAs in atrial remodeling with an electrophysiological overview. *Cardiology*., **2015,** *131,* 58-67.

[172] Gomes da Silva, A.M.; Silbiger, V.N. miRNAs as biomarkers of atrial fibrillation. *Biomarkers.,* **2014,** *19,* 631-636.

[173] Liu, Z.; Zhou, C.; Liu, Y.; Wang, S.; Ye, P.; Miao, X.; Xia, J. The expression levels of plasma microRNAs in atrial fibrillation patients. *PLoS One.,* **2012,** *7,* e44906.

[174] Huang, Y.; Wang, C.; Yao, Y.; *et al*. Molecular Basis of Gene-Gene Interaction: Cyclic Cross-Regulation of Gene Expression and Post-GWAS Gene-Gene Interaction Involved in Atrial Fibrillation. *PLoS Genet.,* **2015,** *11,* e1005393.

[175] Wang, J.; Bai, Y.; Li, N.; *et al*. Pitx2-microRNA pathway that delimits sinoatrial node development and inhibits predisposition to atrial fibrillation. *Proc Natl Acad Sci U S A.,* **2014,** *111,* 9181-9186.

[176] Chiang, D.Y.; Kongchan, N.; Beavers, D.L. *et al*. Loss of microRNA-106b-25 cluster promotes atrial fibrillation by enhancing ryanodine receptor type-2 expression and calcium release. *Circ Arrhythm Electrophysiol.,* **2014,** *7,* 1214-1222.

[177] Barana, A.; Matamoros, M.; Dolz-Gaitón, P.; *et al*. Chronic atrial fibrillation increases microRNA-21 in human atrial myocytes decreasing L-type calcium current. *Circ Arrhythm Electrophysiol.,* **2014,** *7,* 861-868.

[178] Daimi, H.; Lozano-Velasco, E.; Haj Khelil, A.; *et al*. Regulation of SCN5A by microRNAs: miR-219 modulates SCN5A transcript expression and the effects of flecainide intoxication in mice. *Heart Rhythm.,* **2015,** *12,* 1333-1342.

[179] Luo, X.; Pan, Z.; Shan, H; *et al*. MicroRNA-26 governs profibrillatory inward-rectifier potassium current changes in atrial fibrillation. *J Clin Invest.,* **2013,** *123,* 1939-1951.

[180] Jia, X; Zheng, S.; Xie, X.; *et al.* MicroRNA-1 accelerates the shortening of atrial effective refractory period by regulating KCNE1 and KCNB2 expression: an atrial tachypacing rabbit model. *PLoS One.,* **2013,** *8,* e85639.

[181] Li, Y.D.; Hong, Y.F.; Yusufuaji, Y.; *et al.* Altered expression of hyperpolarization-activated cyclic nucleotide-gated channels and microRNA-1 and -133 in patients with age-associated atrial fibrillation. *Mol Med Rep.,* **2015,** *12,* 3243-3248.

[182] Schmitz, S.U.; Grote, P.; Herrmann, B.G. Mechanisms of long noncoding RNA function in development and disease. *Cell. Mol. Life Sci.,* **2016,** *73,* 2491-2509.

[183] Rosa, A.; Ballarino, M. Long noncoding RNA regulation of pluripotency. *Stem cells Intern.,* **2015**

[184] Wapinski, O.; Chang, H. Y. Long noncoding RNAs and human disease. *Trends Cell Biol.,* **2011,** *21,* 354-361.

[185] Rackham, O.; Shearwood, A.M.J.; Mercer, T.R.; et al. Long noncoding RNAs are generated from the mitochondrial genome and regulated by nuclear-encoded proteins. *RNA.,* **2011,** *17,* 2085-2093.

[186] St Ecedil Pien, E.; Costa, M.C.; Kurc, S.; *et al.* The circulating non-coding RNA landscape for biomarker research: lessons and prospects from cardiovascular diseases. *Acta Pharmacol Sin.,* **2018,** *39,* 1085-1099.

[187] Su, Y.; Li, L.; Zhao, S.; *et al.* The long noncoding RNA expression profiles of paroxysmal atrial fibrillation identified by microarray analysis. *Gene.,* **2018,** *642,* 125-134.

[188] Yu, X.J.; Zou, L.H.; Jin, J.H.; *et al.* Long noncoding RNAs and novel inflammatory genes determined by RNA sequencing in human lymphocytes are up-regulated in permanent atrial fibrillation. *Am J Transl Res.,* **2017**, *9,* 2314-2326.

[189] Ruan, Z.; Sun, X.; Sheng, H.; *et al.* Long non-coding RNA expression profile in atrial fibrillation. *Int J Clin Exp Pathol.,* **2015,** *8,* 8402-8410.

[190] Xu, Y.; Huang, R.; Gu, J.; *et al.* Identification of long non-coding RNAs as novel biomarker and potential therapeutic target for atrial fibrillation in old adults. *Oncotarget.,* **2016,** *7,* 10803-10811.

[191] Wang, W.; Wang, X.; Zhang, Y.; *et al.* Transcriptome analysis of canine cardiac fat pads: involvement of two novel long non-coding RNAs in atrial fibrillation neural remodeling. *J Cell Biochem.,* **2015,** *116,* 809-821

[192] Gore-Panter, S.R.; Hsu, J.; Barnard, J.; *et al*. PANCR, the PITX2 Adjacent Noncoding RNA, Is Expressed in Human Left Atria and Regulates PITX2c Expression. *Circ Arrhythm Electrophysiol.,* **2016,** *9,* e003197.

[193] Holmes, A.P.; Kirchhof, P. Pitx2 Adjacent Noncoding RNA: A New, Long, Noncoding Kid on the 4q25 Block. *Circ Arrhythm Electrophysiol.,* **2016,** *9,* e003808.

[194] Li, Z.; Wang, X.; Wang, W.; *et al.* Altered long non-coding RNA expression profile in rabbit atria with atrial fibrillation: TCONS_00075467 modulates atrial electrical remodeling by sponging miR-328 to regulate CACNA1C. *J Mol Cell Cardiol.,* **2017,** *108,* 73-85.

[195] Scridon, A.; Fouilloux-Meugnier, E.; Loizon, E.; *et al.* Long-standing arterial hypertension is associated with Pitx2 down-regulation in a rat model of spontaneous atrial tachyarrhythmias. *Europace.,* **2015,** *17,* 160-165.

[196] Lozano-Velasco, E.; Wangensteen, R.; Quesada, A.; *et al.* Hyperthyroidism, but not hypertension, impairs PITX2 expression leading to Wnt-microRNA-ion channel remodeling. *PLoS One.,* **2017,** *12,* e0188473.

[197] Torrado, M.; Franco, D.; Hernández-Torres, F.; *et al.* Pitx2c is reactivated in the failing myocardium and stimulates myf5 expression in cultured cardiomyocytes. *PLoS One.,* **2014,** *9,* e90561.

[198] Jabbari, R.; Jabbari, J.; Glinge, C.; *et al.* Association of common genetic variants related to atrial fibrillation and the risk of ventricular fibrillation in the setting of first ST-elevation myocardial infarction. *BMC Med Genet.,* **2017,** *18,* 138.

[199] Chinchilla, A.; Esteban, F.J.; Lozano-Velasco, E.; *et al.* Ventricular chamber-specific Pitx2 insufficiency leads to cardiac hypertrophy and arrhythmias. *bioRxiv.,* **2018,** 253062.

[200] Weng, L.C.; Lunetta, K.L.; Müller-Nurasyid, M.; *et al.* Genetic Interactions with Age, Sex, Body Mass Index, and Hypertension in Relation to Atrial Fibrillation: The AFGen Consortium. *Sci Rep.,* **2017,** *7,* 11303.

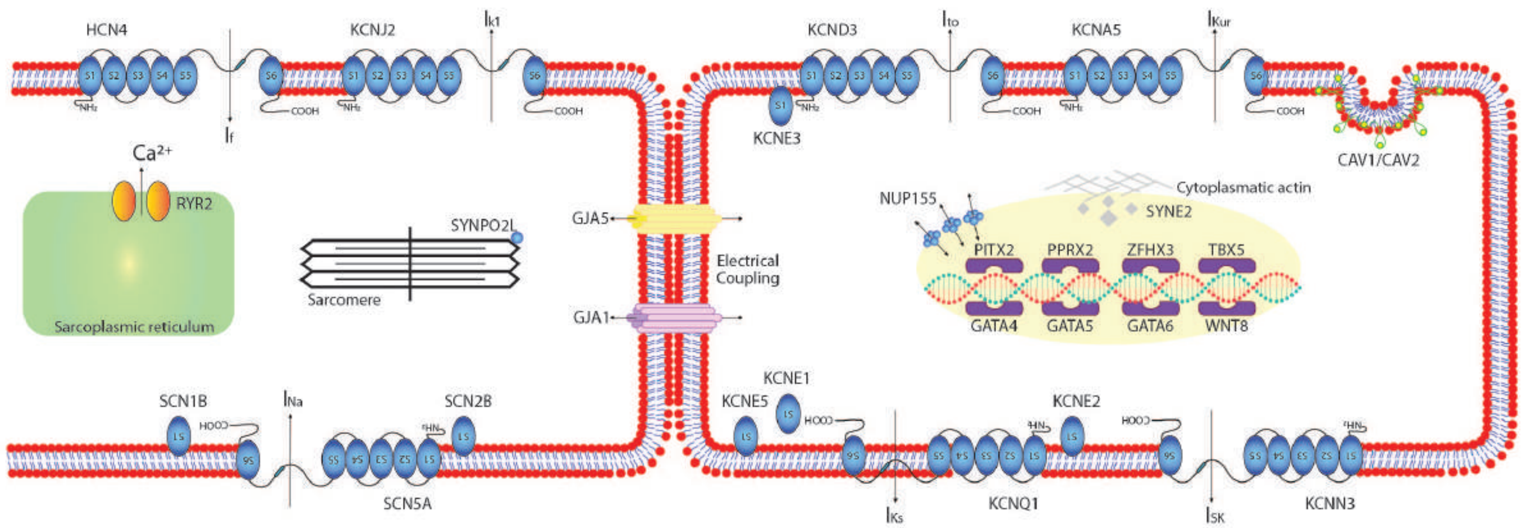
[201] Shoemaker, M.B.; Bollmann, A.; Lubitz, S.A.; *et al*. Common genetic variants and response to atrial fibrillation ablation. *Circ Arrhythm Electrophysiol.,* **2015,** *8,* 296-302.

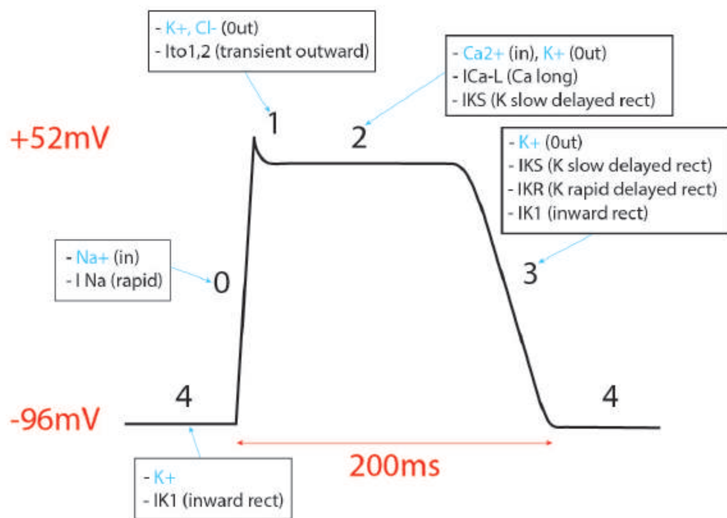
[202] Kristjansson, R.P.; Benonisdottir, S.; Oddsson, A.; *et al.* Sequence variant at 4q25 near PITX2 associates with appendicitis. *Sci Rep.,* **2017,** *7,* 3119.

[203] Mora, C.; Serzanti, M.; Giacomelli, A.; *et al.* Generation of induced pluripotent stem cells (iPSC) from an atrial fibrillation patient carrying a PITX2 p.M200V mutation. *Stem Cell Res.* **2017,** *24,* 8-11.

[204] Marczenke, M.; Fell, J.; Piccini, I.; *et al.* Generation and cardiac subtype-specific differentiation of PITX2-deficient human iPS cell lines for exploring familial atrial fibrillation. *Stem Cell Res.* **2017,** *21,* 26-28.

[205] Boutilier, J.K.; Taylor, R.L.; Mann, T.; *et al.* Gene Expression Networks in the Murine Pulmonary Myocardium Provide Insight into the Pathobiology of Atrial Fibrillation. *G3 (Bethesda).,* **2017,** *7,* 2999-3017.





Gene	Mutation	Phenotype
KCNQ1	S140G; Q147R	Shorten cardiac action potential repolarization
KCNE1	G25V; G60D	Shorten cardiac action potential repolarization
KCNE2	R27C; c.67A>T; c.157T>C	Shorten cardiac action potential repolarization
KCNE3	c.17G>A	Shorten cardiac action potential repolarization
KCNE5	L65F	Shorten cardiac action potential repolarization
KCNJ2	V93I; E299V	Impairment of the resting membrane potential
SCN5A	D127N; M1875; k1493R; Y1795C; R1860G	Depletion sodium current Early AF
SCN1B	c.254G>A; c.457G>A	Depletion sodium current
SCN2B	c.82C>T; c.83G>A	Depletion sodium current
SCN3C	A130V; R6K; L10P; M161T	Depletion sodium current
SCN4B	c.485G>T; c.496A>C	Dysfunctional sodoum current
HCN4	P257S	Faliure to traffick into cell membrane
RyR2	R176Q; S4153R	Leak of sarcoplasmic reticulum Ca2+
GJA5	p.V85I; p.L221L; p.L229M; p.Q49X	Imapiring gap-junction assembly and electrical coupling
GJA1	c.932delC; G60S	Impairing gap-junction formation both Cx40 and Cx43 and depletion in gap junctional coupling and in gap junction plaques