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Differential chamber-specific expression and regulation of long non-coding RNAs during cardiac development

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A B S T R A C T

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 also reported to 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. 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 differentially regulated by *Nkx2.5*, *Srf* and *Mef2c*, Pitx2 > Wnt > miRNA signaling pathway and angiotensin II and thyroid hormone administration. Importantly isoform-specific expression and distinct nuclear *vs* cytoplasmic localization of *Braveheart*, *Carmen* and *Fendrr* 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 novel roles of these lncRNAs in different temporal and tissue-restricted fashion during cardiogenesis.

1. Introduction

Cardiovascular development is a complex developmental process leading to the formation of a four-chambered heart [1,2]. 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)[3]. At E10.5, five distinct regions can be delineated in the embryonic heart, the inflow tract, the embryonic atrial chambers, the atrioventricular canal, the ventricular chambers and the outflow tract [4]. 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 [3]. Failure or impaired development of these developmental processes leads to congenital heart diseases that, in many cases, are incompatible with life [5,6].

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 [7]. Soon thereafter, cardiac-specific transcription factors are activated, such as *Nkx2.5*, *Gata4*, *Mef2c* and *Srf*, orchestrating early steps of cardiogenesis [8,9]. Genetic deletion of *Nkx2.5* and *Mef2c*, respectively, results in embryonic lethality within the early steps of cardiac looping [10–12], *Gata4* null mutants display impaired development of the precardiac mesoderm [13] and *Srf* is essential for mesoderm formation, and thus subsequently for cardiac morphogenesis [14,15]. As development proceeds, additional transcription factors play essential roles in cardiogenesis, such as several T-box family members [16–25], Pitx2 [26–28] and Hand1/Hand2 [29–32]. Impaired transcriptional activation of any of these transcription factors results in developmental defects causing congenital heart diseases ranging from life-threating malformations such as Tetralogy of Fallot to milder defects such as atrial or ventricular septal defects [33]. 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 [34–38]. Non-coding RNAs are broadly classified into two distinct categories, small non-coding RNAs (< 200 nt) and large non-coding RNAs (> 200 nt). Among the first category, microRNAs are the most representative group and the most well-studied [39,40]. Post-transcriptional regulatory mechanisms

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driven by microRNAs involve annealing by homologous base-paring with mRNAs transcript, promoting thus protein translation blockage and/or mRNA degradation [41–43]. 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 [44–46]. However, lncRNAs display a variety of transcriptional and post-transcriptional functions, ranging from scaffold platform, recruitment of activating or repressing epigenetic factors or as transcriptional enhancers, thus influencing transcriptional regulation or modulating ribosome adherence to protein coding RNAs and thus modulating protein translation [39,47]. In addition, lncRNA-microRNA interactions also occurs, being the most well-studied mechanism the sponge of microRNAs [39] that in turn indirectly influences mRNA stability.

At present, functional significance of lncRNAs 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 [48–52], *Charme* and *Chast* in cardiac remodeling [53,54] and *Myheart*, *H19* and *Chaer* in cardiac hypertrophy [55–58]. In addition several lncRNAs have also been reported to enhance cardiomyocyte proliferation and repair, such as *Cpr* [59], *NR_045363* [50,51], *Crrl* [60] and *uc.167* [61]. Furthermore several lncRNAs have been implicated in key developmental processes related to cardiac development, such as *Braveheart* [62,63], *Fendrr* [64], *Carmen* [65] *Upperhand* [66], *Terminator*, *Alien* and *Punisher* [67]. However, their tissue distribution during embryonic cardiac development remains poorly elucidated.

In particular, *Braveheart* is required for early cardiogenesis, playing a fundamental role in conversion of nascent mesoderm into the cardiomyogenic lineage, by modulating *Mesp1* function [62,63,68]. Similarly, *Carmen* is essential for cardiac precursor specification and additionally it is re-activated in heart failure [65,69]. *Fendrr*, a lncRNA located in the vicinity of *Foxh1*, a transcription factor with restricted expression to the lateral plate mesoderm during early embryogenesis, is fundamental for correct cardiac development. *Fendrr* deficient mice display cardiac hypoplasia [64]. In addition to its role in cardiac development, multiple studies demonstrate that impaired *Fendrr* expression is associated to different oncogenic processes [70–74]. *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 [67]. *Miat* and *H19* have been implicated in distinct cardiac pathological conditions such as myocardial infarction and ischemia [75–85], 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 [41,43,86] as well as by pro-arrhythmogenic and pro-hypertrophic substrates such as angiotensin II/norepinephrine and thyroid hormone administration [87,121]. 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 *Braveheart*, *Carmen* and *Fendrr* during chamber morphogenesis. Endocardial and transient epicardial and myocardial expression of *H19* during cardiogenesis is also documented. In summary our data provide novel insights into the regulation and tissue-specific expression of lncRNAs during heart development.

2. Materials & methods

2.1. 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. Right and left ventricular free walls of E14.5 and adult hearts, respectively, were also dissected and processed as reported above.

$Pitx2^{floxed}$ and NppaCre transgenic mouse lines, and generation of conditional atrial (NppaCre) mutant mice have been previously described [42,86,122,123]. Three different conditions were used for the NppaCrePitx2 mice: wild-type Cre controls ($NppaCre^{-}Pitx2^{fl/fl}$) and atrial-specific heterozygous ($NppaCre^{+}Pitx2^{fl/-}$) and homozygous ($NppaCre^{+}Pitx2^{-/-}$) mice. 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.

2.2. 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 [86]. 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 heterozygous ($NppaCre^{+}Pitx2^{fl/-}$) and homozygous ($NppaCre^{+}Pitx2^{-/-}$) mice were analyzed by qPCR, displaying in all cases 60–70% reduction in Pitx2c expression in $NppaCre^{+}Pitx2^{-/-}$ samples.

2.3. 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 1 h 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 1 h 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.

2.4. 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 [41–43]. 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 [88,89] and normalized in all cases taking as 100% the wild-type (control) value, as previously described [86].

2.5. 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 [88] and control measurements were normalized to represent 100% as previously described [86].

2.6. 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 [42,86,87]. 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 [90], cultured accordingly and treated with angiotensin II (Sigma), norepinephrine (Sigma), T3 and T4 (Sigma) hormone, respectively, as previously reported [42].

2.7. microRNA pull-down assays

Biotinylated *miR-29* probe was synthesized by IDT technologies. Whole-cell lysates (500 µg) from HL1 cells were incubated with 1 µg of biotinylated RNA for 2 h at room temperature. Complexes were isolated with Streptavidin-coupled Dynabeads (Invitrogen) following the protocol published by Panda et al. [91]. RNA associated to biotinylated miR-29 was isolated using *Direct-zol™ RNA Miniprep Plus* (Zymo research) following the manufacturer's instructions and qRT-PCR was subsequently performed as detailed above. Biotinylated *miR-23* was used as a negative control.

2.8. 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.

2.9. 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 pGEM-T 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. [92].

2.10. 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. $P < .05$ was considered statistically significant.

3. Results

3.1. Expression profile of lncRNAs 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, using primers that were common to all distinct lncRNAs isoforms, respectively. 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 (Fig. 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 (Fig. 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 (Fig. 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 (Fig. 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 (Fig. 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 (Fig. 1F). Overall, these data demonstrate a distinct developmental behaviour of these lncRNA during cardiac development. In addition, we also explore if differential left/right expression was applicable in the developing and adult ventricles. As reported in Supplementary Fig. 1, *Braveheart*, *Carmen* and *H19* displayed differential left/right expression in the embryonic stage while *H19* and *Alien* displayed similar differences in the adult ventricle. 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.

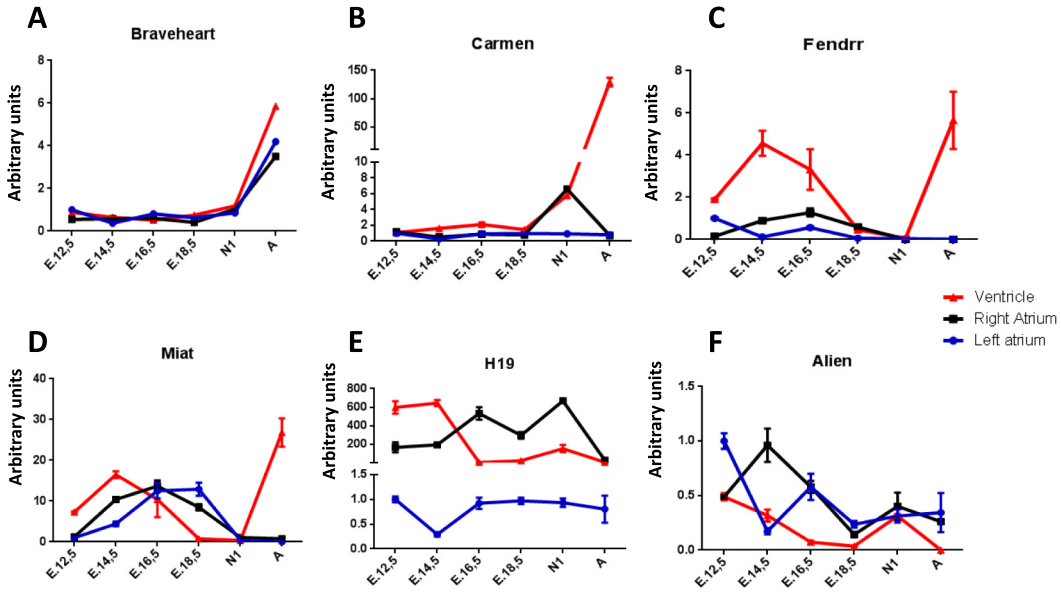


Fig. 1. Developmental expression profiles of *Braveheart* (panel A), *Carmen* (panel B), *Fendrr* (panel C), *Miat* (panel D), *H19* (panel E) and *Alien* (panel F) from E12,5 to adulthood in right atrium (RA), left atrium (LA) and ventricles (V), respectively as revealed by qPCR. Note that *Braveheart*, *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.

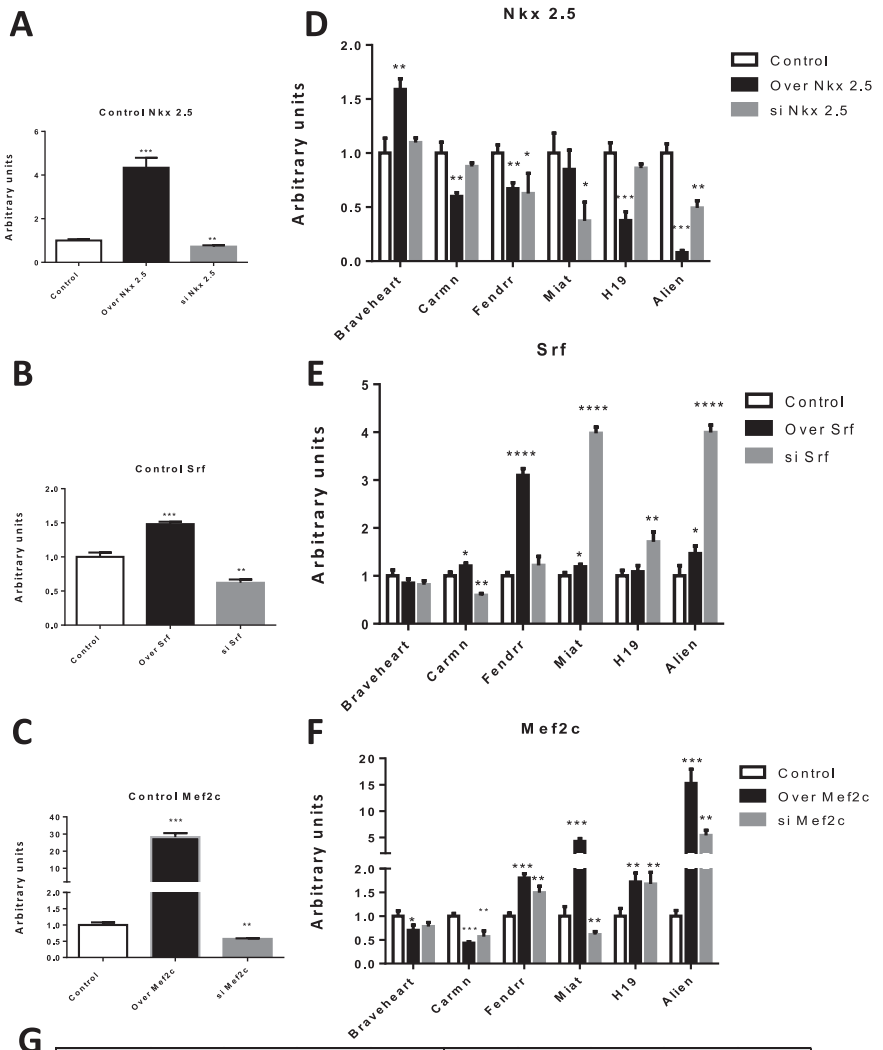
3.2. Transcriptional regulation of lncRNAs expression by Nkx2.5, Mef2c and Srf

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 Fig. 2A–C. Bioinformatic analyses demonstrate putative binding sites for *Nkx2.5*, *Srf* and *Mef2c* in upstream sequences of these lncRNAs (Supplementary Table 3). *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* (Fig. 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 (Fig. 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* (Fig. 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, our data 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 (Fig. 2G). Thus, importantly, impaired expression of these transcription factors in pathological conditions such as cardiac hypertrophy, will therefore have an impact on lncRNA expression.

3.3. 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. In particular, Pitx2 regulates expression of Wnt8 and Wnt11 that in turn regulate expression of distinct microRNAs with potential to modulate cardiac ion channel expression. Importantly, a complementary cross-talk between Wnt8 and Wnt11 is established on the regulation of these microRNAs [41,43,86,121]. 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 (Fig. 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* (Fig. 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 deficient mice (Fig. 3G and Supplementary Fig. 2). Expression of *Braveheart* and *Carmen* display are significantly downregulated in NppaCrePitx2 deficient 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 deficient mice (Fig. 3G). In its important to highlight that trends of dose-dependent expression driven by Pitx2 is only observed in a small subset of lncRNAs in both gain and loss-of-function assays (Supplementary Figs. 2 and 3) supporting the notion that precise *Pitx2* levels are required for normal expression and/or that additional compensatory mechanisms are operative. Overall, these data therefore reinforce the evidences that *Pitx2* regulates the expression of these lncRNAs (Fig. 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* (Fig. 3E). On the other hand, *Wnt11* gain-of function resulted in up-regulation of *Carmen*, *Fendrr*,



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	Mef2c	Nkx2.5	Srf			Mef2c	Nkx2.5	Srf	
over-expression	+++	+++		*Braveheart*	over-express	+++		+++	*Miat*
inhibition					inhibition	+++	+++	+++	
over-expression	+++	+++	+++	*Carmen*	over-express	+++	+++		*H19*
inhibition	+++		+++		inhibition	+++		+++	
over-expression	+++	+++	+++	*Fendrr*	over-express	+++	+++	+++	*Alien*
inhibition	+++	+++			inhibition	+++	+++	+++	

Fig. 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 and loss-of-function, respectively, in lncRNA expression. Student's *t*-test *p < .05, **p < .01, ***p < .005, ****p < .001.

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 (Fig. 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 (Fig. 3H), opening up the possibility that impaired expression of these lncRNAs might play a role in arrhythmogenesis.

3.4. microRNA regulation of lncRNA expression

It is widely accepted that lncRNA can modulate microRNA function by acting as sponges [82,83,93,94]. 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 miR-1, miR-133 and miR-29 in atrial cardiomyocytes and analyzed their expression levels by qPCR (Fig. 4A).

miR-1 mimic administration led to up-regulation of *Carmen*, *Fendrr* and *H19*, while *Braveheart*, *Alien* and *Miat* displayed no significant differences (Fig. 4B–G). miR-29 gain-of-function led to up-regulation of *Braveheart* and *Fendrr*, down-regulation of *Miat*, while *Carmen*, *Alien* and *H19* were not significantly different (Fig. 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 (Fig. 4B–G). In order to dissect the molecular interactions between microRNAs and lncRNAs we performed miR-29 pull-down assays. miR-29 pull-down assays demonstrate a direct interaction between miR-29 and *Braveheart*, *Fendrr* and *H19*, while no interaction was observed for *Carmen* (Supplementary Fig. 4). No detectable signals were recorded for *Miat* and *Alien*, respectively (data not shown). These data revealed that microRNAs could provide novel regulatory mechanisms controlling lncRNA expression, increasing and/or decreasing their expression levels in cardiomyocytes both by direct and indirect pathways. Furthermore, these data reinforces the notion that these lncRNAs might be deregulated in atrial arrhythmogenesis.

Angiotensin and thyroid hormone administration distinctly alters lncRNA expression.

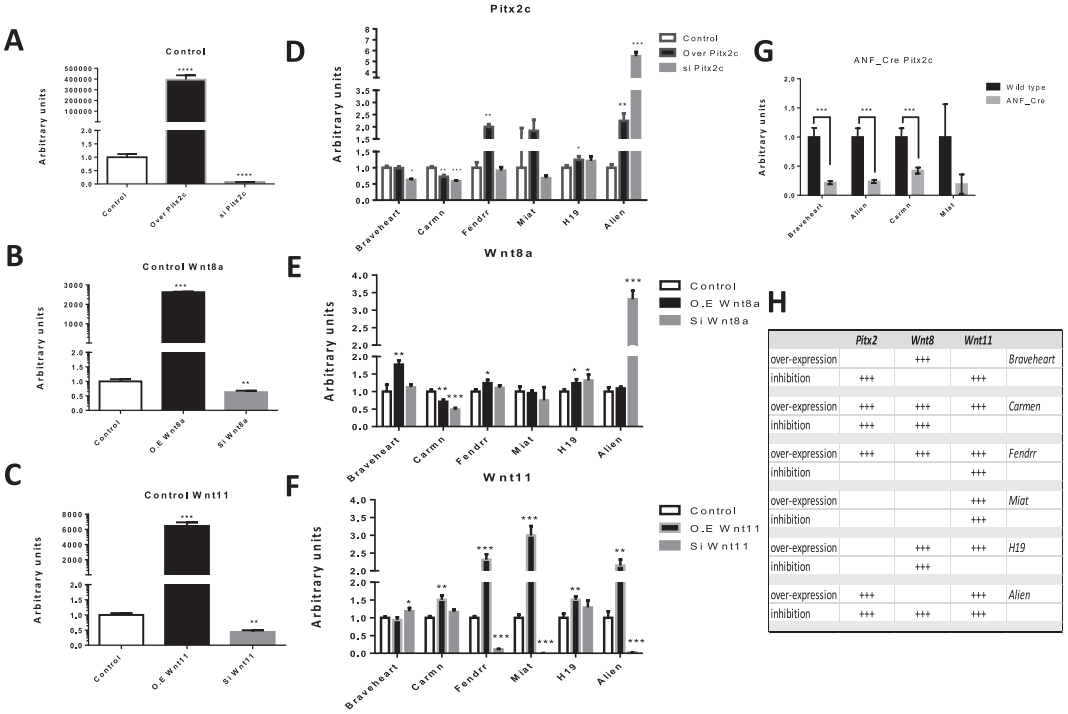

Fig. 3. qPCR analyses of lncRNA expression in *Pitx2*, *Wnt8* and *Wnt11* gain and loss-of-function assays in HL1 atrial cardiomyocytes. Panel A, B and C display *Pitx2*, *Wnt8* and *Wnt11* expression in gain-of-function (over) and loss-of-function (si) assays, demonstrating significant up- and down-regulation 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 and loss-of-function, respectively, in lncRNA expression. Student's *t*-test *p < .05, **p < .01, ***p < .005, ****p < .001.

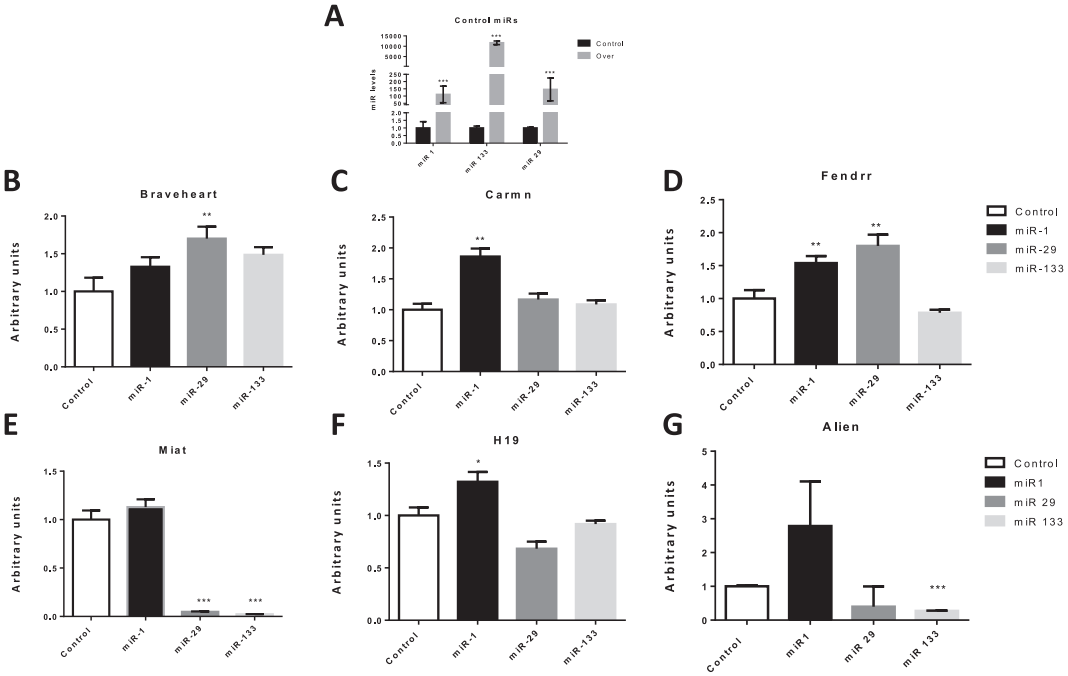

Fig. 4. qPCR analyses of lncRNA expression in *miR-1*, *miR-29* and *miR-133* gain-of-function assays in HL1 atrial cardiomyocytes. Panel A display *miR-1*, *miR-29* and *miR-133* expression in gain-of-function (over) assays, demonstrating significant up-regulation of these microRNAs, respectively. Panels 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. Student's *t*-test *p < .05, **p < .01, ***p < .005.

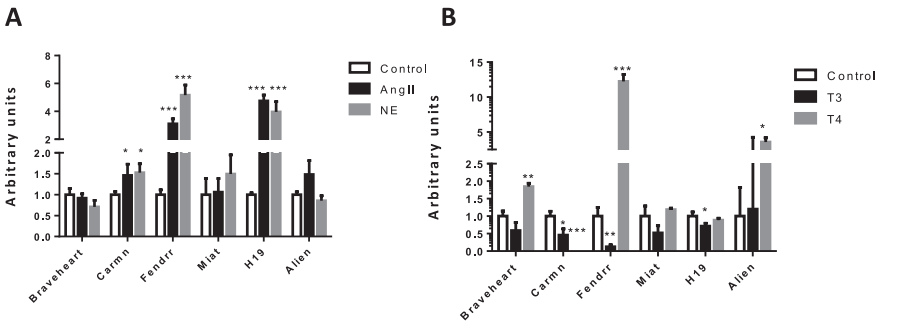

Fig. 5. qPCR analyses of *Braveheart*, *Carmen*, *Fendrr*, *Miat*, *H19* and *Alien*, after angiotensin II (AngII) or norepinephrine (NE) (panel A), and T3 or T4 thyroid hormone (panel B) administration in HL1 atrial cardiomyocytes, respectively. Observe that AngII and NE treatment enhanced *Carmen*, *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. Student's *t*-test *p < .05, **p < .01, ***p < .005.

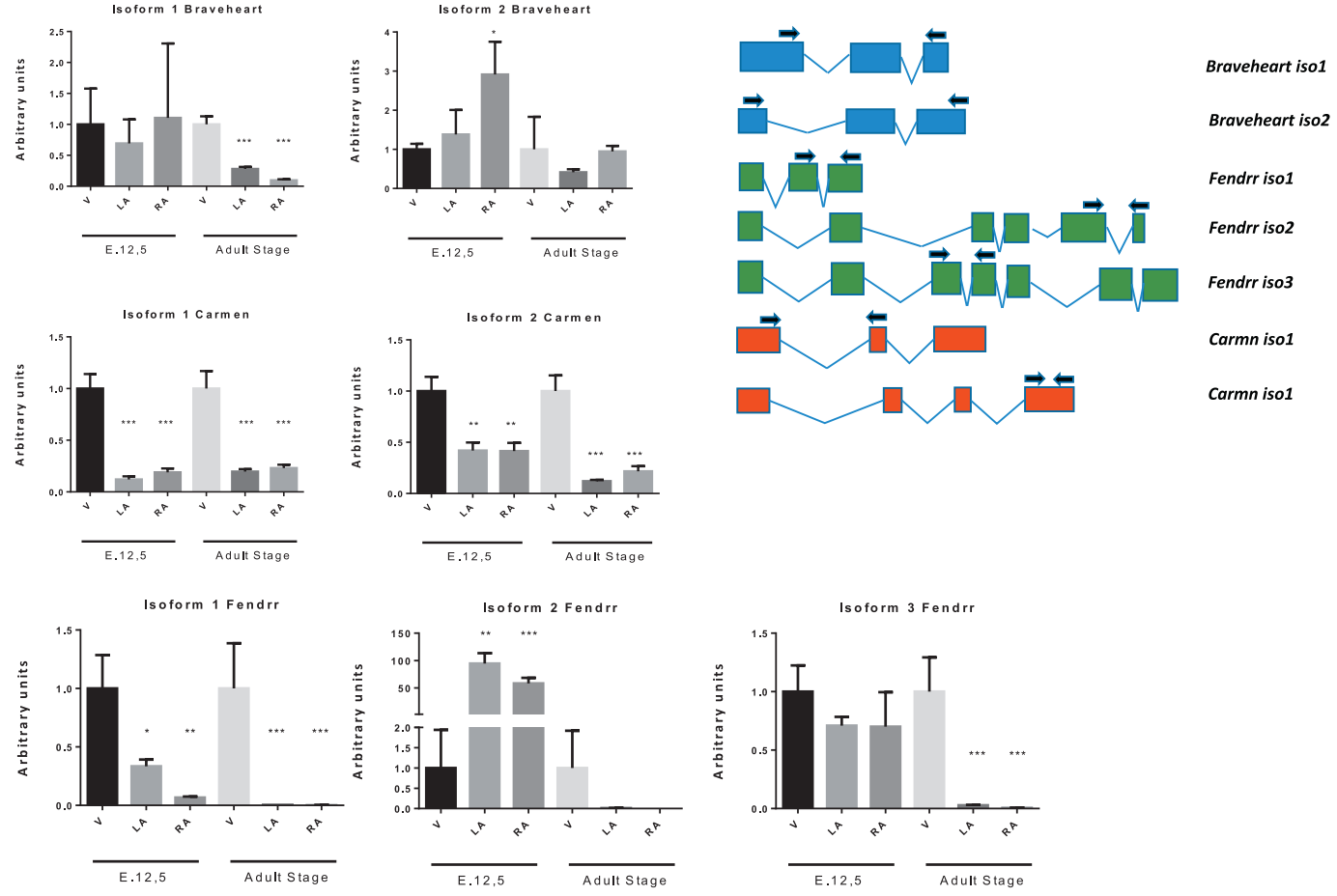

Fig. 6. PCR analyses of *Braveheart*, *Carmen and Fendrr* isoform expression in embryonic and adult RA, LA and V. Observe that *Braveheart* isoform 1 and 2 and *Carmen* 2 and *Fendrr* isoforms 1, 2 and 3 display differentially expression profiles in embryonic and adult tissues as well as in left and right atrial chambers. Student's *t*-test *p < .05, **p < .01, ***p < .005, ****p < .001.

To further investigate the plausible involvement of these lncRNAs in cardiac pathophysiology we treated HL1 atrial cardiomyocytes 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 (Fig. 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 (Fig. 5B). Overall, these data demonstrate that pro-arrhythmogenic and pro-hypertrophic substrates can modulate the expression of these lncRNAs.

3.5. 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, Braveheart isoform 1 is similarly expressed in the embryonic (E12.5) right and left atria and ventricles, while is significantly decreased in adult right and left atria as compared to the ventricles. Braveheart isoform 2 is enhanced in the embryonic right atrium but display no significant differences in right

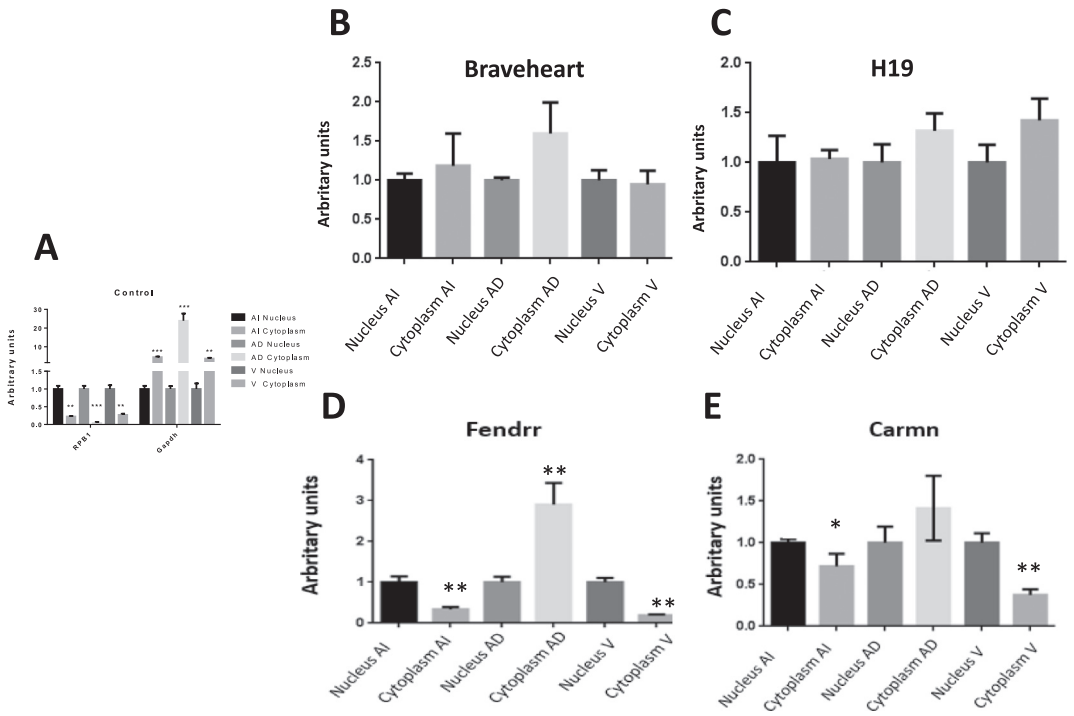

Fig. 7. qPCR analyses of *Braveheart*, *H19*, *Carmen*, *Fendrr* in nuclear and cytoplasmic subcellular extracts of E12.5 embryos corresponding to RA, LA and V. Panel A illustrate control qPCR expression of *Rpb1* in the nucleus and *Gapdh* in the cytoplasm in all tissues and samples analyzed. Observe 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). Student's *t*-test *p < .05, **p < .01, ***p < .005.

and left atria and ventricles in the adulthood (Fig. 6). *Carmen* displays two distinct isoforms. Both isoforms, *i.e. Carmen* isoform 1 and Carmen isoform 2 are expressed preferentially in the ventricles, both in embryonic and adult stages, while expression in right and left atrium is decreased. On the other hand, *Carmen* isoform 2 is similarly expressed in all tissues and stages analyzed (Fig. 6). Finally, *Fendrr* display three distinct isoforms. *Fendrr* isoform 1 is preferentially expressed in the embryonic and adult ventricles, with almost negligible expression in embryonic and adult right and left atria. *Fendrr* isoform 2 is highly expressed in embryonic right and left atria, but not in the adult right and left atria while *Fendrr* isoform 3 is displays similar expression levels in embryonic left and right atria and ventricles while in the adult is only detectable in the ventricles, with negligible expression in the right and left atria (Fig. 6). These data demonstrate that *Braveheart*, *Carmen* and *Fendrr* display tissue and time-dependent differential expression of their isoforms.

3.6. 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 Fig. 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 (Fig. 7B). *H19*, on the other hand, displays a similar distribution as *Braveheart*, *i.e.* similarly expressed in all tissues analyzed (Fig. 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 (Fig. 7D-E). Similarly, differential nuclear and cytoplasmic expression is identified for *Braveheart*, *Carmen* and *Fendrr* isoforms as illustrated in Supplementary Fig. 5. 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.

3.7. 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 (Fig. 8). H19 expression at E12.5 is observed in all cardiac tissue layers including the endocardium, myocardium and epicardium (Fig. 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 (Fig. 9A, F–G) as compared to control Mlc2v hybridization (Fig. 9H). Epicardial expression is observed similarly in the atrial and ventricular lining (Fig. 9E,G). At E14.5, H19 is mainly restricted to the endocardium and epicardium, and similarly observed in the ventricular endocardium and valve leaflets (Fig. 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 (Fig. 10) and the atrioventricular valve mesenchymal component (Fig. 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 (Fig. 11). Right atrial endocardium displays a prominent H19 expression in contract to left atrium, where H19 is scarce.

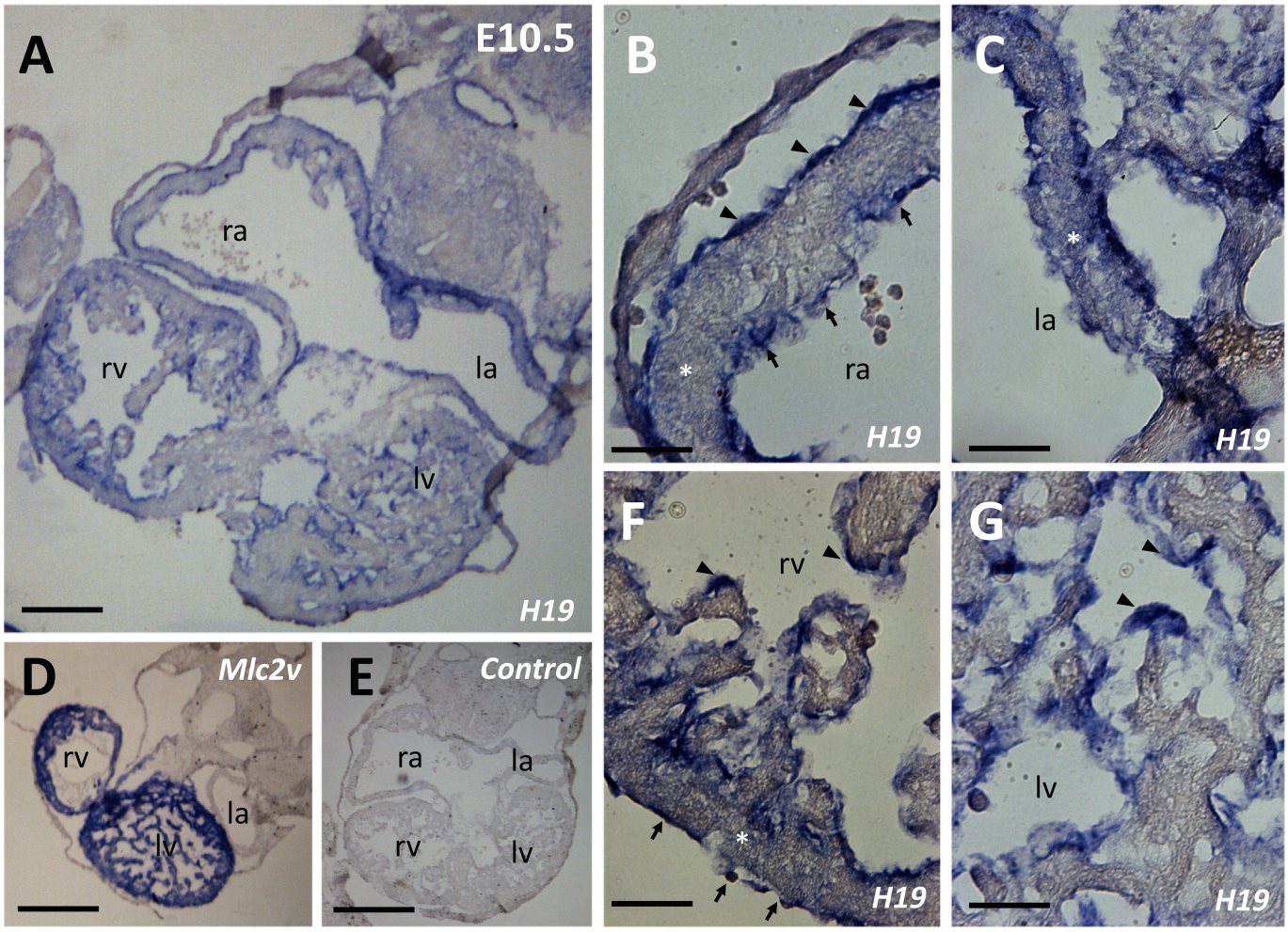

Fig. 8. *In situ* hybridization analyses of H19 expression in mouse E10.5 embryonic heart. Observe endocardial, myocardial and epicardial expression (panels A–C,F–G). Panel D is a positive control *in situ* hybridization against Mlc2v delineating the ventricular myocardium. Panel E is a negative control. Bars in panels A, D, E represents 200 μm. Bars in panels, B,C F and G represents 20 μm.

Atrioventricular valve only display H19 expression confined to the endocardial lining. No expression in the epicardium is observed anymore. Myocardial and cardiac fibroblasts remain 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.

4. 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 [1,2]. During these morphogenetic changes, atrial and ventricular chambered are formed, each of them displaying distinct left and right components [3,95]. Concomitant with these developmental transformations, 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 [5,124]. 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 [39,47]. Such distinct regulatory mechanism can simultaneously occur because many lncRNAs display dual nuclear and cytoplasmic subcellular localization [96,97]. 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

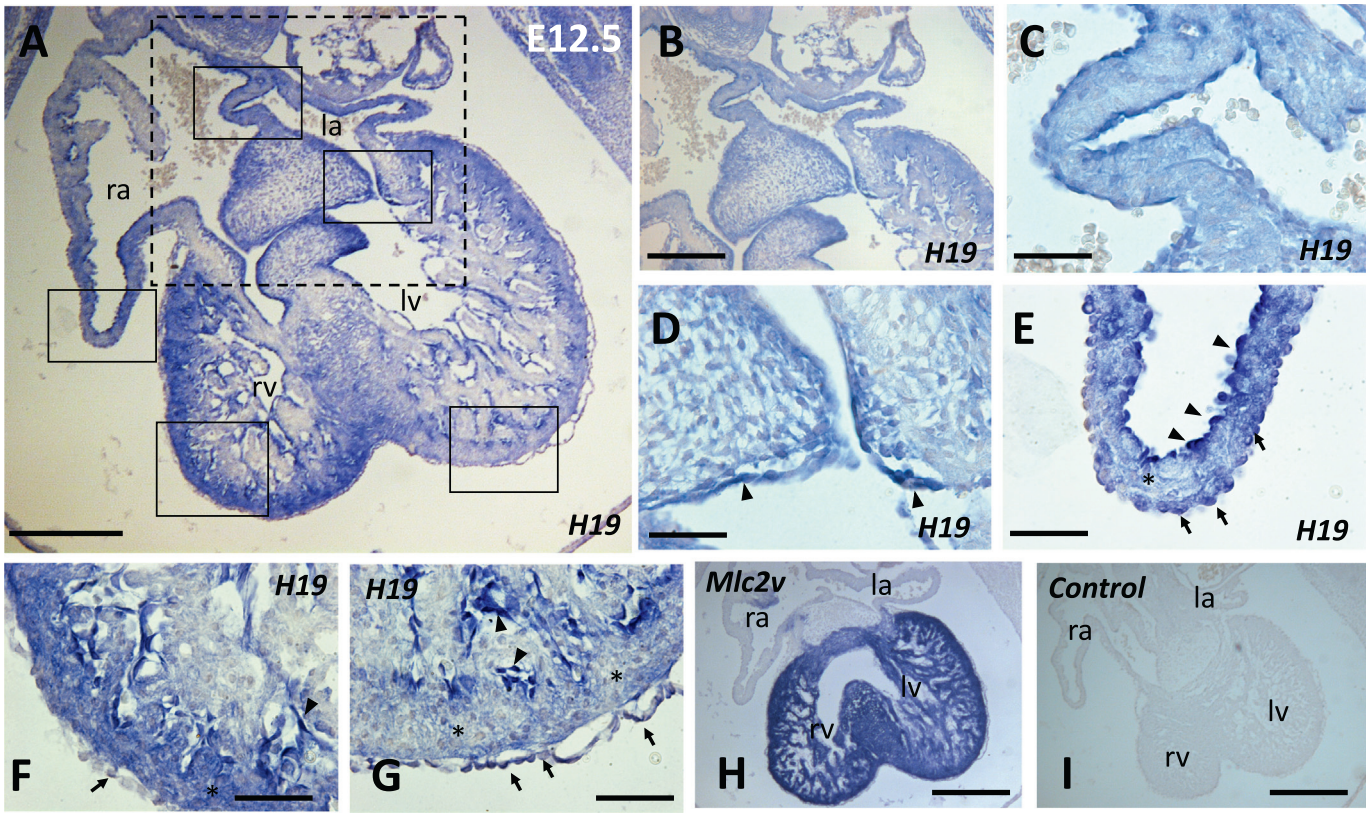

Fig. 9. *In situ* hybridization analyses of H19 expression in mouse E12.5 embryonic heart. Observe endocardial, myocardial and epicardial expression (panels A–G). Panel H is a positive control *in situ* hybridization against Mlc2v delineating the ventricular myocardium. Panel I is a negative control. Bars in panels A, H and I represents 200 μm. Bars in panels, B, G and F represents 20 μm. Bars in panels, C, D and E represents 10 μm.

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* [5]. We tested if cardiac-enriched lncRNAs were similarly regulated by these transcription factors using gain and loss-of-function assays. While usage of HL1 cardiomyocytes has some limitations, 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 [98–101] 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 [57,78,79,85,102–104]. We tested if *Braveheart*, *Fendrr*, *Carmen* and *Alien* were distinctly regulated by key signaling pathways leading to cardiac arrhythmias, *i.e.* Pitx2 > Wnt > miRNA [41,43,86,105,106,121], or cardiovascular risk factors such as hypertension, *i.e.* angiotensin/norepinephrine administration [107,108] or hyperthyroidism, *i.e.* T3/T4 hormone treatment [109], that also increased the possibility of developing cardiac arrhythmias [110]. 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 [86]. In addition, *Wnt8* and *Wnt11* can modulate expression of all lncRNA analyzed [41,43,121], 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 [125] 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 [82,83,93] while *Miat* sponges miR-22 in diabetic cardiomyopathy [94]. 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 [41,43,86,121]. 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. As a proof-of-principle, miR-29 pull-down assays demonstrate that both direct and indirect microRNA modulation of lncRNA is operative. Our data suggest that direct microRNA-lncRNA interactions favour lncRNA stabilization while indirect regulation seems to be responsible for lncRNA downregulation, although additional experiments are required to fully dissect these regulatory pathways. Secondly, they further support the plausible role of these lncRNAs in cardiac arrhythmias, in line with previous report linking miR-29 and *H19* cross-talk in cardiac hypoxia and myocardial infarction [82–84,111,112].

LncRNAs display a genomic structure similar to protein-coding RNAs, with intron and exons as well as alternative splicing [45,46]. To date, our current understanding of the distinct isoform specific

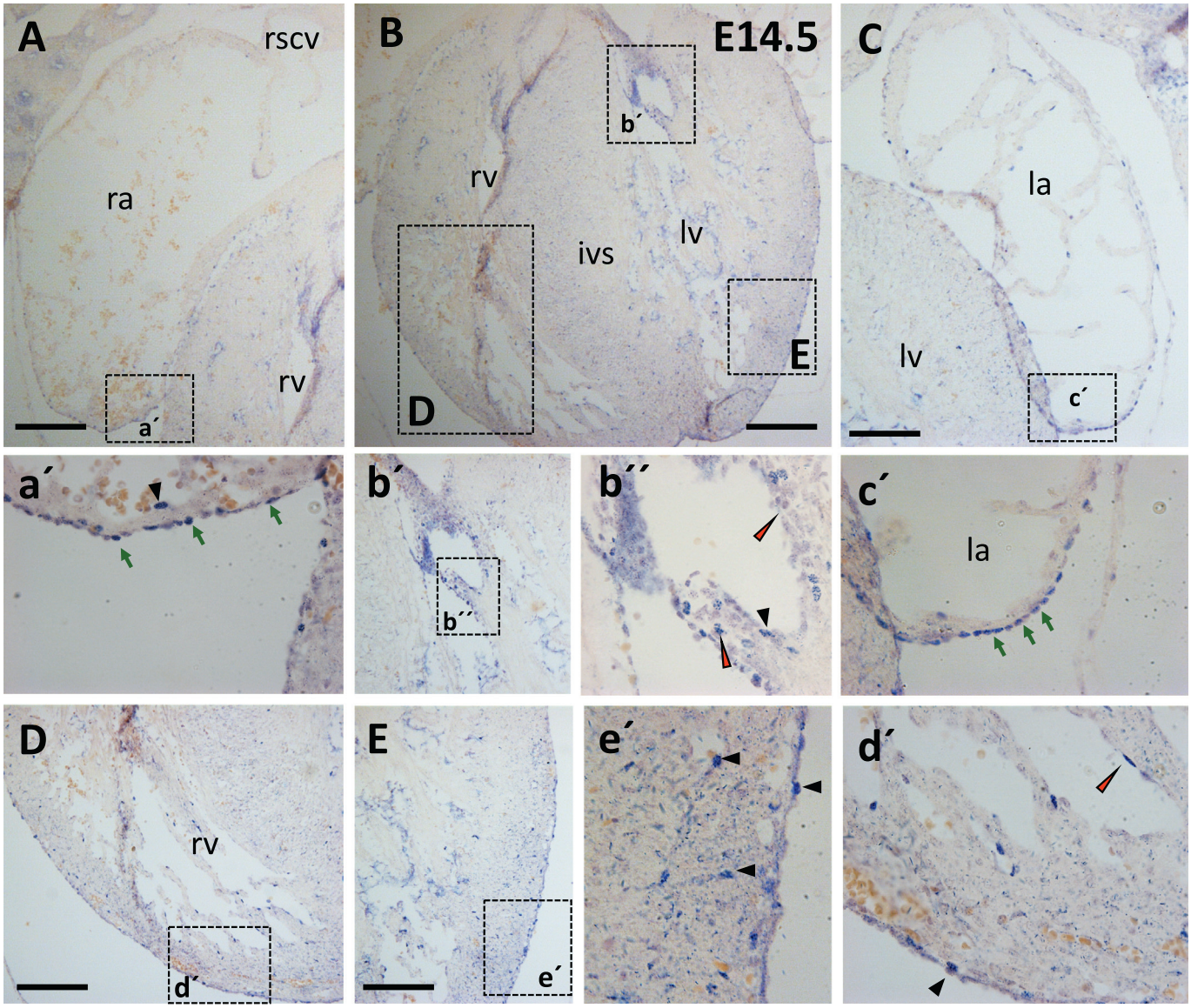

Fig. 10. *In situ* hybridization analyses of H19 expression in mouse E14.5 embryonic heart. Observe the epicardial (arrows) and endocardial (arrowheads) expression in the right atrium (panels A and a´) and left atrium (panel C and c´). Expression in the ventricular chambers (panel B) is restricted to the endocardium (panels D, d´, E and e´), the valve leaflets (panels b'and b´´) and the epicardium (panels e'and d´). Bars represents 200 μm.

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*, *Fendrr* and *Carmen* isoforms display differential tissue and time-dependent expression. Furthermore, each distinct isoform displays differential nuclear and cytoplasmic expression supporting the notion that distinct isoforms might be directing different transcriptional and post-transcriptional regulatory functions within distinct cardiac compartment at the same developmental stage.

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 [113–115], while in the cardiovascular system *H19* expression have only been reported in ischemic heart failure [55].

LncRNAs expression levels are on average 10-fold lower than those of protein coding RNAs (mRNAs) limiting their detection level. We have indeed tried to do *in situ* hybridization for several lncRNAs obtaining unsatisfactory results for all but H19, since H19 levels are 2–3 fold higher that the other lncRNAs analyzed (Supplementary Fig. 6). We report herein tissue distribution of *H19* 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)([116–118]: [119]). Our data

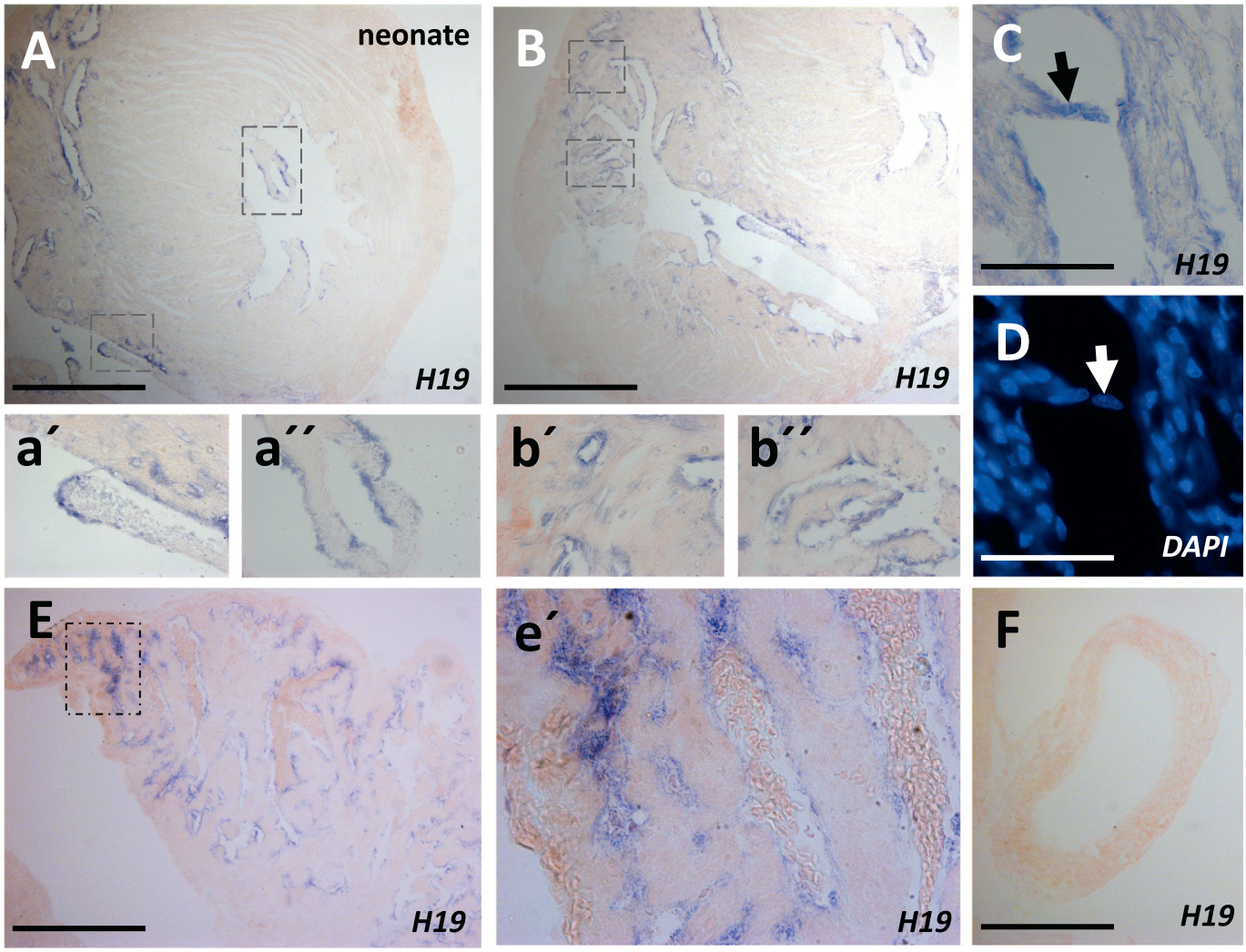

Fig. 11. *In situ* hybridization analyses of H19 expression in mouse neonatal heart. Observe the endocardial expression in the right and left ventricles (panels A-B, b´, b´´) and tricuspid (panel a´) and mitral (panel a´´) valves. Observe that expression is mainly cytoplasmic (panel C). Panel D represents 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). Bars in panels A, B, E and F represents 200 μm. Bars in panels, C and D represents 10 μm.

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 [120].

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 evidences 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.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbagrm.2019.194435.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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