UNIVERSIDAD DE JAÉN

FACULTAD DE CIENCIAS
EXPERIMENTALES
DEPARTAMENTO DE BIOLOGÍA

DOCTORAL THESIS

Functional Roles of microRNA Interactive Networks in Epicardium Development

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Jaén, 5th November, 2020

UNIVERSIDAD DE JAÉN

Área de Biología Celular

Departamento de Biología Experimental

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Dr. D. Diego Franco Jaime, Profesor Titular del Área de Biología Celular del Departamento de biología Experimental de la Universidad de Jaén.

CERTIFICA:

Que el trabajo presentado para optar al Grado de Doctor en Biología Molecular y Celular titulado ***"Functional Role of microRNA Interactive Networks in Epicardium Development"*** por Don Ángel Dueñas Lechuga y llevado a cabo en los laboratorios de Biología Celular del Departamento de Biología Experimental de la Universidad de Jaén, se ha realizado bajo mi dirección y que reúne los requisitos para proceder a la lectura y defensa del mismo.

Para que así conste, firma la presente:

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En Jaén, a 5 de Noviembre de 2020

“Aconseja a un amigo como cortesano viejo

Don Juan, no se le dar a un hombre nada
de cuanto van y viene, es cuerdo efeto;
que toda la quietud del que es discreto
en solo este aforismo está fundada.

¿Qué Gobierno, qué ejército, qué armada
corre por vuestra cuenta? Lo perfeto
es el descuido y el tener secreto
cuando da pesadumbre y cuando enfada.

Nunca os halléis en juntas ni en corrillos,
que es cuerdo de las bestias el rodeo,
ni en estas ruedas de amolar cuchillos.

Haced de la virtud secreto empleo;
que yo en mi pobre hogar, con dos librillos,
ni murmuro, ni temo, ni deseo.”

Soneto CLIII
(1632)

Lope de Vega Carpio

Tras este largo viaje que concluye aquí, con la redacción de este manuscrito, hay mucho camino andado, mucha batalla luchada y mucha gente a la que dar las gracias.

He de agradecer a Amelia Aránega el brindarme la oportunidad de formar parte del Grupo de Cardiovascular y Miogénesis para poder desarrollar este trabajo.

A Diego Franco, mi director de tesis, con lo que eso implica, palabras mayores, el cual, también se ha comportado como algo más a lo largo del trayecto. Gracias por enseñarme, por guiarme y por la infinita indulgencia y paciencia que has mostrado.

A Esther Ocaña, mi directora de máster, mi jefa en el hospital, la persona que me animó a doctorarme y que me contagió esa chispa para trabajar en ciencia.

A Fernando Bonet. Mi maestro de laboratorio, la persona que me enseñó los intríngulis de lo que posteriormente se iba a convertir en este proyecto de tesis. Gracias por tu amabilidad, dedicación y paciencia con el nuevo cuando tú ya te estabas yendo.

Agradecer a Dani, Jose y Estefanía vuestro compañerismo, esfuerzo, buen hacer y amistad durante los años que hemos estado trabajando en el laboratorio. Vuestra dedicación y apoyo ha sido ejemplar y estoy orgulloso de haber tenido semejantes compañeros de trabajo.

A Robert Kelly por darme la bienvenida en su equipo en el IBDM de Marsella. Gracias por los conocimientos y experiencia adquirida y por tu calidad humana. Del mismo modo agradecer a Jun Takeuchi y a su equipo en la TMDU en Tokio, durante mi estancia allí y en especial a Yutaro Hori, por hacerlo venir 3 veces en semana a enseñarme bioinformática y sus entresijos cuando terminaba su jornada laboral en la Universidad de Tokio, quedándonos hasta las tantas, cosido de pies a cabeza por mis infinitas dudas y preguntas.

Un agradecimiento a todos los que están y han estado en el Grupo, Paco, Jorge, Feli, Almudena, Mohamed, Fabio, y en especial a Pilar y Victoria, que habéis hecho para nosotros fácil lo difícil desde la oficina de administración.

A mi buena amiga Rocío Herrero de Genética, a la que siempre andaba pidiéndole Eva Green de estraperlo cuando necesitaba poner una PCR y en el grupo andábamos caninos, por eso, por estar ahí siempre y por mucho más, gracias.

Finalmente, no me puedo dejar atrás a la persona que más ha hecho para que no tirara la toalla en tiempos duros, a la que quiero con locura y que sin ella es muy probable que este proyecto no se hubiera hecho realidad jamás, mi mujer, Yukako.

A todos, gracias.

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1.ACKNOWLEDGMENTS AND FUNDING

This work was supported by grants from the Ministry of Science, Innovation and Universities for the Spanish Government to AA and DF (BFU2015-67131P) and grants-in-aid from the Junta de Andalucía Regional Council (CTS-446) to AA.

The International Research Stays at the Institut of Developmental Biology in Marseille (France) and the Tokyo Medical and Dental University in Bunkyo City, Tokyo (Japan) have been supported and financed by grants-in-aid from Universidad de Jaén Doctoral School through the EDUJA programs in 2015 and 2019, respectively.

2. ABBREVIATIONS

AVC: Atrio-ventricular Canal
ceRNAs: competing endogenous RNAs
circRNAs: circular RNAs
CM: Cardiomyocytes
CoSMCs: Coronary Smooth Muscle Cells
DAPI: 4-6 Diamidino-2-phenylindole
DAVID: Database for Annotation, Visualization and Integrated Discovery
DEG(s): Differential Expressed Genes
DMEM: Dubelco's Modified Eagle's Medium
EBSS: Earle's Balanced Salt Solution
EE: Embrionary Epicardium
EMC: Extracellular Matrix Components
EMT-TFs: Epithelial to Mesenchymal Transition Transcription Factors
EMT: Epithelial to Mesenchymal transition
EPDCs: Epicardial Derived Cells
FACS: Fluorescence-activated cell sorting
GFP: Green Fluorescent Protein
GO BP: Gene Ontology Biological Process
GO CC: Gene Ontology Cellular Component
GO: Gene Ontology
HH: Hamilton & Hamburger
IHC Immunohistochemical
KEGG: Kyoto Encyclopedia of Genes and Genomes
lncRNAs: long non-coding RNAs
MET: Mensenchymal to Epithelial Transition
MREs: microRNA response elements
ncRNA: non-coding RNAs
ncRNAs: non-coding RNAs
NF-kB: Nuclear Factor Kappa-light-chain-enhancer of activated B cells
PAR and SCRIB: Polarity Protein Complexes involved in Cell Migration and Invasion
PBS: Phosphate Buffered Saline
PE: Proepicardium
PIWI: *Drosophila* P-element Induced Wimpy
q-RT-PCR: quantitative Real Time Polimerase Chain Reaction
RBPs: RNA Binding Proteins
siRNAs: small nuclear RNAs
SMCs: Smooth Muscle Cells
ST: Septum Transversum
TMM: Trimmed Mean of M-values
TPM: Transcripts per Million
UTR: Untranslated Region

3. ABSTRACT

The heart is the first organ that acquires functionality in vertebrate embryos. The tubular heart starts beating at a very early developmental stage and later bends and expands to conform the tetracameral chambers of the adult heart. Population of cells that are external to the primordial cardiac fields migrate into the developing heart, contributing to its sculpture, such as the derivatives of the epicardium.The proepicardium (PE) is a transitory cauliflower-like structure located between the sinus census and the posterior lateral mesenchyme that with development will form the embryonic epicardium. Cells derived from the proepicardium migrate to the neighboring embryonic heart and progressively cover the most external surface, leading to the formation of the embryonic epicardium. Evidence in chicken have demonstrated that epicardial derived cells can contribute to fibroblasts, endothelial and smooth muscle cells. Isolation of the developing PE and *ex vivo* culture spontaneously leads to cardiomyocyte differentiation, a process enhanced by *Bmp* but hampered by *Fgf* administration. In this study, we carried out a characterization of the coding and noncoding gene expression at two critical stages during mouse proepicardium and embryonic epicardium development, identifying a large array of mRNA, microRNAs and lncRNAs differentially expressed in both stages and involved in the regulation of preferential biological pathways. In addition, we also provide a comprehensive characterization of the expression profile of multiple microRNAs during epicardial development in chicken. Subsequently, we identified that *miR-125*, *miR-146, miR-195* and *miR-223* selectively enhance cardiomyogenesis in the PE/ST and in the embryonic epicardium, process driven by *Smurf1* and *Foxp1*. In addition, we identified three novel long non-coding RNAs with enhanced expression in the PE/ST, complementary regulated by *Bm*p and *Fgf* as well as microRNAs that selectively promote cardiomyogenesis supporting a role of these lncRNAs in microRNA-mediated cardiomyogenesis of the PE/ST cells.

4. INTRODUCTION

4.1. An introduction to Early Cardiac Morphogenesis

The heart is the first organ that becomes functional in the vertebrate embryo. Approximately 24h after its induction the precardiac mesoderm forms a primitive tubular heart, which starts beating at stage Hamburger & Hamilton (HH) 10 in the chick (Hamburger & Hamilton, 1951). The heart is an asymmetric structure, which receives its polarity from the three body axes, antero-posterior (A-P), dorsal-ventral (D-V), and left-right (L-R) (Brand, T., 2003). The heart undergoes multiple morphogenetic processes, which are particularly governed by the L-R axis. Several cell populations external to the initial cardiogenic fields migrate into the developing heart, providing additional cell types and playing a major role in sculpturing the embryonic heart (Yelon, 2001).

While primary cardiogenic induction is complete by HH8, classical studies suggested that recruitment of splanchnic mesodermal cells to the cardiac cell lineage continues at the arterial pole until HH22 in chickens and E11.5 in mice (Kelly & Buckingham, 2002), suggesting a progressive movement of cells from the pharyngeal mesoderm into the growing arterial pole of the mouse heart between E7.5 and E10.5 (Kelly et al., 2001), while the distal outflow tract originates anterior to the heart tube in the chick embryo (Mjaatvedt et al., 2001). After cardiac mesoderm is specified, the two heart fields fuse and form a single heart tube at the embryonic midline. Tubular heart formation is dependent on the closure of the anterior intestinal portal (AIP). Importantly, the heart maintains its physiologic pumping function while it is continuously remodelled until the four chambered organ is formed.

4.1.1. Formation of the Early Heart Tube

Cells that give rise to the early heart tube are specified and differentiated in the lateral anterior splanchnic mesoderm as a result of combinatorial signals from surrounding tissues. Cranial mesoderm is derived from progenitor cells that activate the bHLH transcription factor *Mesp1* in the primitive streak, under control of the *T-box* factor *Eomesodermin* (Saga et al., 2000, Costello et al., 2011). The pattern of inductive signals from adjacent endoderm and overlying ectoderm, together with inhibitory signals from the embryonic midline and posterior region of the embryo, refine the sites in which the cardiomyogenic transcriptional program is first activated (Marvin et al., 2001, Harvey, 2002, Lopez-Sanchez & Garcia-Martinez, 2011). These signals include the bone morphogenetic protein (*Bmp*), fibroblasts growth factor (*Fgf*) and *Wnt* signals. In addition, short range signals like fibronectin mediated cascades, lead to the activation of key upstream transcriptional regulators of the cardiac phenotype. Among these it is important to highlight transcriptions factors such as *Nkx2-5*, *Gata4* and *Tbx5*, and chromatin remodelling proteins *Smarcd3, Gata 4 and Tbx5*, all of which has been shown to be sufficient to drive cardiomyogenesis in noncardiogenic regions of the embryo (Takeuchi & Bruneau, 2009). Convergence of left and right precardiac regions in the embryonic

anterior midline results in the formation of the cardiac crescent and linear heart tube. These early events in heart morphogenesis must be considered in the context of broad embryonic morphogenetic events including embryonic coelom formation and ventral folding of the embryo associated with foregut closure.

In the early myocardium, in which cardiac contractions commence, myocardial specification and differentiation are ongoing processes that continue in splanchnic mesoderm throughout the time of heart tube elongation and cardiac looping. Thus, initial growth of the heart tube is driven by cells that are added along its entire length (Kelly & Buchingham, 2002). These cells maintain continuity with the early heart tube at the arterial and venous poles of the heart, positioned anteriorly and posteriorly respectively. Fluorescent dye labelling and proliferation studies have revealed that these cells divide rapidly and are displaced towards the poles of the heart tube where they progressively differentiate as the linear dimensions of the heart rapidly increasing during looping morphogenesis (Abulssa & Kirby, 2008, van den Berg et al., 2009). Addition of these progenitor cells to the heart may in fact be a major driver of rightward looping. The rate of displacement of these cells toward the poles of the heart tube has been estimate at 70 μm/h in avian embryos (van den Berg et al., 2009). These subpharyngeal cells are termed the second heart field (SHF) and have been shown by genetic tracing and retrospective lineage analysis in the mouse to contribute to a large part of the definitive heart including atrial and inflow myocardium at the venous pole and right ventricular and outflow tract myocardium at the arterial pole (Buckingham et al., 2005, Kelly, 2012). In contrast, early differentiating cardiac cells forming the cardiac crescent are termed the first heart field (FHF) and give rise to part of the left ventricle. The embryonic heart is thus comprised of cardiomyocytes derived from the cardiac crescent, i.e. FHF comprising the linear heart, as well as those derived from the pharyngeal mesoderm, i.e. SHF. Furthermore, a population of late differentiating SHF cardiomyocytes has been found to add to poles in frog and fish hearts suggesting that this mechanism for heart tube elongation is evolutionarily conserved across vertebrate species (Zhou et al., 2011).

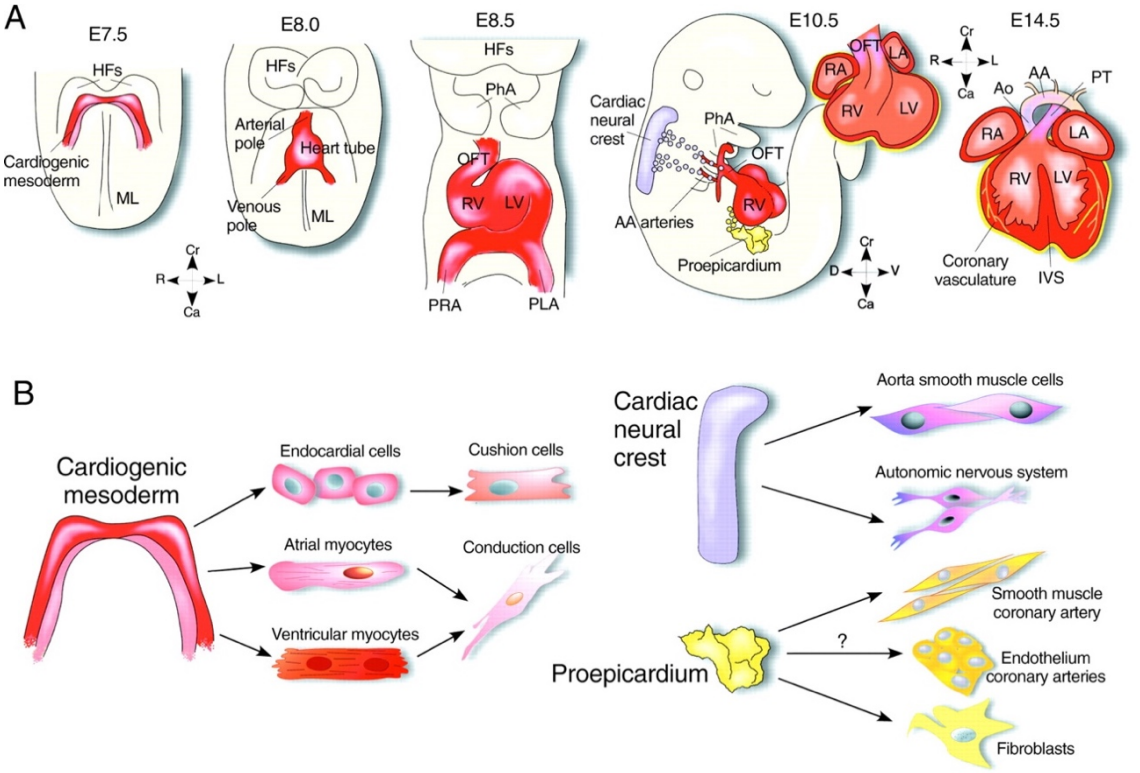

Figure 1. (A) Contribution of the three populations of embryonic heart cardiogenic heart progenitors. (B) Cardiac cell types that arise through the lineage diversification of the three embryonic precursor pools in the heart. (Image from Laugwitz et al., 2008).

4.1.2. Contribution of the A-P and D-V axes to the developing heart

The linear heart tube is essentially polarized along the A-P axis (van Mierop, 1967; Satin et al., 1988; Kamino et al., 1981; Yutzey et al., 1994; 1995; Garcia-Martinez et al., 1993; Lyons, 1994; Moorman et al., 1995; Franco et al., 1998). The number of genes and signals showing a heterogeneous distribution along the A-P axis is steadily growing and includes, among others *Tbx5* (Bruneau et al., 1999), *Gata4* (Malketin et al., 1997), *Hrt1* and *Hrt2* (Nagagawa et al., 1999). An additional candidate for A-P polarity defining pathway is the retinoic acid signalling system, which has been shown to be dynamically involved in the specification of posterior sinoatrial structures and the regulation of gene expression along the A-P axis (Xavier-Neto et al., 1999). *Irx4* expression pattern may demarcate the limits of the A-P region in the linear heart in which the ventricular chamber myocardium is to be formed

This implies that the combinatorial actions of A-P signals such as retinoic acid and/or Irx4 and a second signal along the D-V axis defining sites of the chamber myocardium within the linear and looping heart (Christoffels et al., 2000).

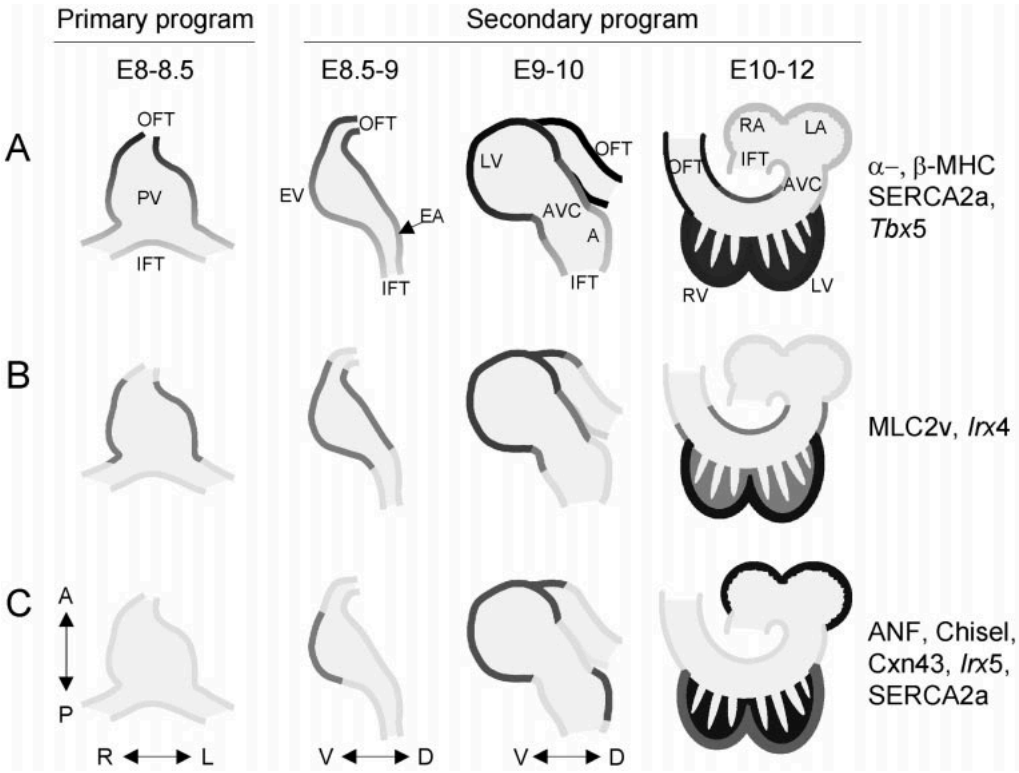

Figure 2. Schematic of the expression patterns of genes during chamber formation. The primary program of expression is found in the linear heart. At E8.5-9 the secondary program is initiated. Upregulation of several gene expression is observed at the ventral side of the linear heart. At E9-9.5 at the dorsolateral side at the level of the atria. During these stages, the genes already expressed in the linear heart essentially do not alter their original pattern along the A-P axis. From E10 onward a clear expansion of chamber myocardium is seen that is accompanied by upregulation of genes like *SERCA2* and I*rx4*). (Image from Christoffels et al., 2000).

In *Drosophila*, maternal genes first set up the A-P axis, which subsequently, though the use of several gene cascades gives an identity to the embryonic segments The *Drosophila* heart is composed of a simpler linear heart tube subdivided into an anterior positioned aorta and a posterior heart, having a much larger cavity than the aorta. It has been demonstrated that Hox genes play an important role on the identity to the embryonic segments of *Drosophila* (Lovato et al., 2002) and thus also to its heart (Lo et al., 2002). In avian embryos, several anterior Hox genes are expressed within the early heart forming field (Searcy and Yutzey, 1998) and retinoic acid, which is known to affect A-P axis specific gene expression within the heart also alters Hox genes expression.

Hoxd-3 expression is detected in the heart forming region of embryos prior to heart tube formation. Expression of *Hoxa-4, Hoxd-3* and *Hoxb-5* is increased in cardiogenic tissue treated with RA in culture conditions that also produced changes in positionally restricted cardiomyogenic phenotypes (Searcy & Yutzey, 1998). *Hox* genes expressed in cardiac explants exhibited distinct sensitivities to RA when compared to genes, such as *Nkx-2.5*, that are involved in cardiac commitment and differentiation. These studies support a role for *Hox* genes in early heart patterning and suggest that positional

information in the cardiogenic region is regulated by mechanisms distinct from early heart lineage specification. (Searcy & Yutzey, 1998).

Furthermore, alteration of *Hox* gene expression and expansion of *AMHC-1* expression in anterior cardiac progenitor with RA treatment after stage 7 suggests that distinct regulatory pathways are involved in cardiac myocyte determination vs A-P patterning of the cardiogenic region. The presence of restricted patterns of *Hox* gene expression in the lateral plate, including the heart-forming region of the gastrulating embryo, and the sensitivity of these expression patterns to *RA* treatment are suggestive of a role for *Hox* genes in establishing the early A-P polarity of the chick primitive heart (Searcy & Yutzey, 1998).

Contribution of the L-R axis to the developing heart

Animals that show external bilateral symmetry, display a hinderance in the observation of multiple internal left-right asymmetries that are fundamental for proper organ packaging and function (Raya and Izpisua-Belmonte, 2006; Lopez-Gracia and Ros, 2007). The heart is the first organ that displays left-right (L-R) asymmetry. At HH9, bilateral symmetric heart tubes are established. Transformation of straight heart tube into a C-shape heart loop starts at HH10 (Manner, 2000). Early looping morphogenesis involves two different processes that are intertwined: the primitive ventricular region bends towards its ventral side and simultaneously rotates to the right. Thereby, the original left and right sides of the straight heart tube become the ventral and dorsal sides of the post-looping ventricle. After ventricular looping has started the primitive conus is also displaced to the right. Later looping steps involve the shift of the ventricular position from its originally cranial to its definitive caudal position and the positioning of the conus from its right lateral position with respect to the atria toward its definitive ventral position. This whole process is highly conserved from *Xenopus* to humans.

Morphological asymmetries of the vertebrate heart are actually at the end of complicated process of L-R axis determination, which has its origin much earlier than cardiac looping (Hamada et al., 2002). In *Xenopus*, a proposed upstream mechanism involves transport of L-R determinants through gap junction channels (Levin and Mercola, 1999). An alternative model in *Xenopus* implicates the early activation of *Vg1* signalling pathway on the left side and an antagonistic *Bmp* pathway on the right side (Ramdsdell and Yost, 1999). *Vg1* signalling probably occurs during early gastrulation and requires ligand presentation by ectodermal cells via *syndecan-2* to the migrating mesoderm (Kramer and Yost, 2002).

In amniotes, the node is central for the establishment of the L-R asymmetry. By HH4, the node is morphologically an asymmetric structure in the chick embryo (Dathe et al., 2002). Several asymmetric expression domains have been described in Hensen's node. A right sided asymmetric *activinβ* signal in the epiblast induces Bmp4 and antagonizes Shh

expression, thereby *Shh* is restricted to the left (Levin et al., 1995). *Bmp4 i*nduces the expression of *Fgf8* and ultimately *c5nR*, which functions as a repressor of the left identity on the right side (Isaac et al., 1997). Several additional determinants are active within Hensen's node, including *N-Cadherin* on the right side, which limits *Wnt9C*-induced *β-catenin* signalling to the left side (Garcia Castro et al., 2000). Ultimately, the *TGF-β* molecule *Nodal* is induced exclusively on the left side in a domain adjacent to Hensen's node (Levin et al., 1995). Subsequently, *Nodal* is expressed in a second and much larger expression domain in the lateral plate mesoderm which actually depends on the presence of *Bmp2* (Fischer et al., 2002). *Bmp2* is required for the expression of the competence factor CFC in the lateral plate mesoderm, and *Nodal* requires the presence of CFC on the surface of responsive cells in the chick (Slange et al., 2001). *Nodal* establishes the expression of *Lefty* in the midline and for this induction the presence of the cofactor CFC is also required. *Lefty* is involved in limiting *Nodal* expression and preventing spreading of left-sided signals to the right side. The bicoid-type homeobox gene *Pitx2* acts downstream of *Nodal* in the left LPM. Both overexpression and ablation of *Pitx2* cause laterality defects (Campione et al., 1999). However, *Pitx2* expression does not control cardiac looping.

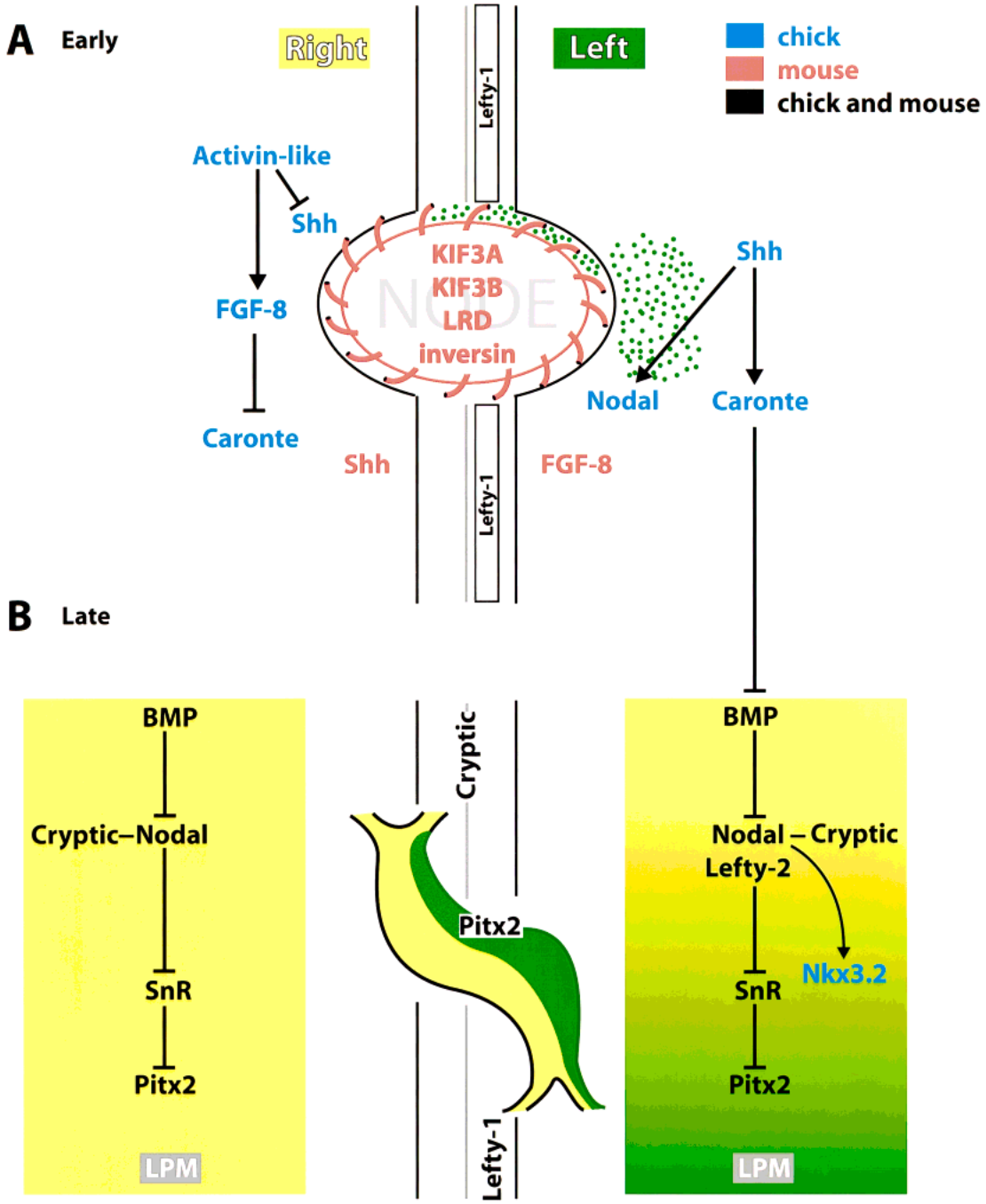

Figure 3. Regulation of left-right (LR) asymmetry. Early asymmetric gene expression around the node **(A)** results in activation or repression of *Shh* or *Fgf8*-dependent pathways on the right or left (ventral view). Early roles of *Shh* and *Fgf8* are reversed in mouse and chick (see colours). Leftward flow of morphogens (green dots) by nodal cilia establishes the asymmetric gradient around the node in mice. Expression of *Lefty-1* near the midline may serve as a barrier to maintain left-sided asymmetry of morphogens. At later stages, LR asymmetric information at the node is transferred to the lateral plate mesoderm by Caronte. Caronte relieves *Bmp* inhibition on the left, initiating a cascade of events that culminates in *Pitx2* expression in the left lateral plate mesoderm and on the left side of the heart tube **(B)**. Consequently, a "left" signal (in green) appears to be propagated to overcome a default "rightness" program (in yellow).

Introduction

Several aspects of L-R axis determination in the mouse appear to be different from the chick. Shh for example is symmetrically expressed in the node (Meyer & Martin, 1999). Nonetheless, genetic evidence suggests that Nodal expression in the left LPM requires a functional *hedgehog* signalling pathway (Zhang et al., 2001). *Fgf8* in the mouse appears to be a left determinant and Nodal is expressed on both sides of the node being only slightly asymmetric. Another transcription factor downstream of Nodal is the left LPM is the homeobox gene *Nkx3.2*, which is asymmetrically expressed on the left in the chick but on the right side in the mouse (Schneider et al., 1999).

The asymmetric expression of *Pitx2* within the LPM depends on Nodal and is mediated by a binding site for the homeobox transcription factor *Nkx2* (Shiratori et al., 2001). The *Nkx-2* binding site is dispensable for the initiation but necessary for the maintenance of *Pitx2*. During early cardiac development, *Pitx2* is expressed in the left portion of the cardiac crescent and in the left side of the heart tube (Campione et al., 2001). Through cardiac looping, *Pitx2* is present in the left atrium, in the ventral portion of the ventricles, and in the left-ventral part of the outflow tract. The *Pitx2* null mutant displays numerous cardiac malformations, including right atrial isomerism, atrial septal defects (ASD), abnormal AV septation, and abnormal arterio-ventricular connections (Kitamura et al., 1995). Based on the analysis of the allelic series of *Pitx2*, it is apparent that the heart requires relatively low concentrations of *Pitx2* function for normal cardiac morphogenesis to occur (Liu et al., 2001). This could be the reason for the observed low frequency of heart defects in patients with Rieger syndrome, an autosomal dominant disorder with mutations in the *Pitx2* gene (Semina et al., 1996). One obvious way how *Pitx2* regulate cardiac morphogenesis is through the control of cell proliferation. Indeed, there is enough data that indicate that, in the outflow tract, Pitx2 induces cell proliferation through activation of *Cyclin-D2* expression (Kioussi et al., 2002).

As mentioned above, in vertebrates, left identity is mediated by left-specific *Nodal-Pitx2* axis that is repressed on the right-hand side by the epithelial mesenchymal transition (EMT) inducer *Snail* (Patel et al., 1997: Murray & Gridley, 2006). Despite some existing evidence (Patel et al., 1997; Schlueter & Brand, 2009), it remains unclear whether an equivalent instructive pathway provides right-hand specific information to the embryo. Recently, Ocaña et al. (2017) showed that, in zebrafish, *Bmp* mediates the L-R asymmetric activation of another EMT inducer, *Prrx1a*, in the lateral plate mesoderm with higher levels on the right. *Prrx1a* drives L-R differential cell movements towards the midline, leading to a leftward displacement of the cardiac posterior pole though an actomyosin-dependent mechanism. Downregulation of *Prrx1a* prevents heart looping and leads to mesocardia. Two parallel and mutually repressed pathways, respectively driven by *Nodal* and *Bmp* on the left and right lateral plate mesoderm, converge on the asymmetric activation to govern heart morphogenesis. This mechanism is conserved in the chicken embryo. In the mouse embryo, *Snail1* acts in a similar fashion to as *Prrx1* does in zebrafish. Thus, a differential L-R EMT produces asymmetric cell movements and forces, more prominent from the right, that drive heart laterality in vertebrates.

4.1.3. Cardiac Looping

In a complex morphogenetic progression, the linear tube gradually transforms into a contracting chambered heart in which atrial and ventricular chamber myocardium, a conduction system and physically separated systemic and pulmonary blood streams are delineated.

During looping of the linear heart an array of distinct compartments becomes visible, which are dubbed outflow tract (OFT), embryonic right ventricle (RV), embryonic left ventricle (LV), atria and sinus venosus (Anderson et al., 1978). These compartments typically indicated as segments of the heart tube, represent key structures in the developing heart, since they roughly correspond to their functional counterparts in the fully formed mature heart. Fate mapping studies showed that the anteroposterior (A-P) order of compartments within the tubular heart corresponds to their A-P position within the heart-forming region (Garcia-Martinez et al., 1993). Several cardiac genes have been reported to display an apparent segmental expression pattern since distinct atrial and ventricular structures are formed (Franco et al., 1998). The relevance of segments therefore seems clear, since they are associated with functionally distinct compartments and distinct transcriptional domains. These observations have led to the classical and commonly held concept of the developing heart as a linear array of segments (Anderson et al., 1978; De la Cruz et al., 1998).

However, several observations need to be incorporated into this classical segmental concept of cardiac development. First, the entire linear heart is characterized by a low density of gap-junction proteins and by slow and uniformly spreading conduction velocities of action potentials. Only after looping does the forming chamber myocardium of the atria and ventricles become characterized by much higher conduction velocities and density of gap junctions (de Jong et al., 1992; Moorman et al., 1998). Second, during the transformation of the linear heart into a four chambered structure, the right atrium becomes directly connected to the right ventricle and the left ventricle to the OFT. This remodelling would require trans-differentiation of the left ventricular segment. Furthermore, these segments are seen only at the outer curvature of the looped heart tube and not at the inner curvature (Netter, 1969; Steding et al., 1990). They are not found in the trabeculated atrial and ventricular compartments at the outer curvature.

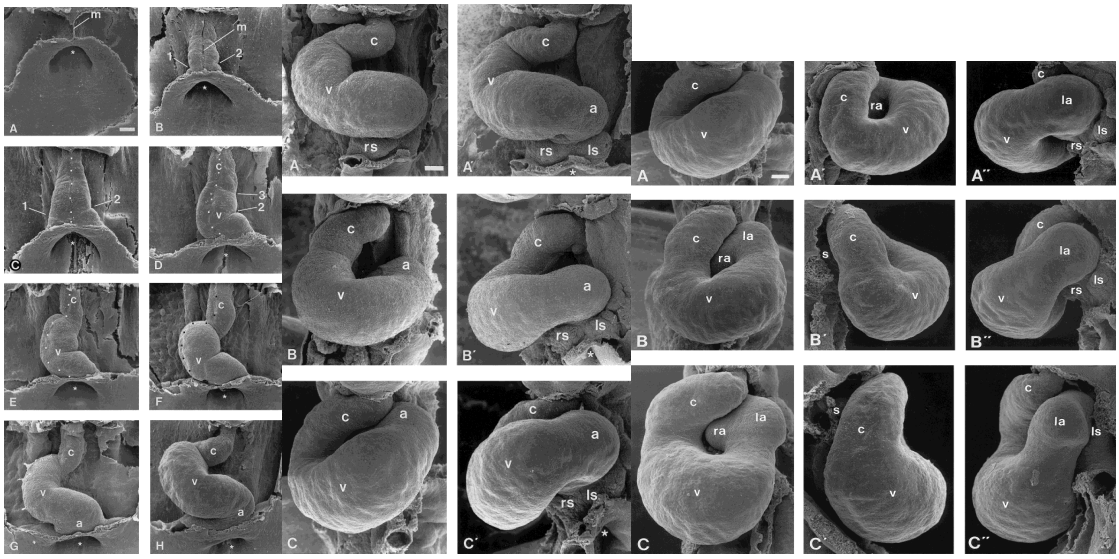

Figure 5. Positional and morphological changes of the embryonic heart tube during the transformation from the c-shaped into the s-shaped loop. HH-stages 14 (A-A'), 15 (B-B') and 16 (C-C').
(Middle picture) (1) Shortening of the distance between the caudal wall of the primitive conus and the cranial wall of the primitive atria (A-C and A'-C'); (2) the shift of the primitive ventricular bend from its original position cranial from the primitive atria toward its definitive position caudal to the atria (A-C, A'-C'). These positional changes are accompanied by the morphological appearance of the sinus venosus, by the appearance of the lateral expansions of the atrial region and by general growth of the heart tube.
(Right picture). HH-stages 16 (A, A', A"), 17 (B, B, B") and 18 (C, C', C"). Ventral (A, B, C), right lateral (A', B', C') and left lateral (A", B", C") views. The shift of the primitive ventricular bend its original position cranial from the primitive atria toward its definitive position caudal to the atria is complete at HH-stage 18 (C, C', C"). **s**, sinus venosus; **rs**, right horn of the sinus venosus; **ls**, left horn of the sinus venosus; **c**, primitive conus; **v**, primitive ventricular bend; **ra**, atrium; **la**, left atrium.

An important outcome of these patterning events is the emergence of chamber specific transcriptional programs and proliferative centers, on the outer curvature of the embryonic heart, resulting in the development of the right and left atrial and ventricular chambers through a process termed ballooning morphogenesis (Christoffels et al., 2002).

All the above-mentioned considerations, in conjunction with observed patterns of expression of chamber specific genes encoding atrial natriuretic factor (*Anf*) sarcoplasmic reticulum Ca2+ ATPase (*Serca2a*), Chisel, myosin light chain *Mlc-2v*, β-myosin heavy chain (*Mhc*) and transcription factors *Irx4, Irx5, Hand1* and *Tbx5*, provide a direct relation to the formation of chambers in the linear heart through early looping stages. Although the developmental patterns of expression of most of these genes have been described, emphasis in previous studies was placed on expression in the fully formed embryonic atrial and ventricular myocardium, which constitutes the bulk of the mass of the foetal and adult heart. Expression in the inflow tract (IFT), AVC, OFT, and especially the inner curvature, has been severely underemphasized, even though these structures form major components of the early embryonic heart and are of profound importance to the proper alignment of the cardiac chambers. Further studies revealed (Christoffels et al., 2000) that the onset of the transcriptional program that is associated with the formation of ventricular and atrial chamber myocardium is restricted to specific sites of the linear heart that are

the outer curvature of the looped heart. The inner curvature does not participate in the initiation of the ventricular and atrial transcriptional programs.

4.1.4. Ballooning model for chamber formation

Taking into account the concept of cardiac growth by addition of cardiac precursor cells, the developing heart could be represented as a transversally segregated structure, to which additional parts are added in a manner as occurs during somitogenesis (Davis, 1927; De la Cruz et al., 1989; Markwald et al., 1998). Another option is to consider the developing heart as a tube with an inflow and an outflow, in which the caudally added cells have been under the control of retinoic acid to provide them with caudal atrial identity (Harvey, 2002a; Simoes-Costa et al., 2005). The latter model is essentially a bipartite model, comprising only atrial and ventricular components arranged along the cranio-caudal axis. In this respect, it is well-established that the cardiac tube is patterned along a caudal-to-cranial or inflow to-outflow, axis (Osmond et al., 1991; Yutzey et al., 1994; Xavier-Neto et al., 1999). Such patterning permits formation of the atrial chamber at the caudal inflow end of the tube, and the ventricular chamber at the cranial or outflow end. Patterns of gene expression, and the localized formation of atrial and ventricular trabeculated myocardium at the outer curvatures of the heart, do not support a model of cardiac growth that depends exclusively on the specification of cranio-caudally arranged transverse components (Christoffels et al., 2000; Moorman et al., 2000; Moorman and Christoffels, 2003a). The definitive chambers are also patterned along the dorso-ventral axis (Moorman and Christoffels, 2003a).

Subsequent to the formation of the primary tube, exclusively at the ventral side of the forming heart tube, a focus of cells increases in size. These cells then reinitiate proliferation (Soufan et al., 2006). This observation is in line with earlier studies (Rychter et al., 1979) in which growth from such a proliferative center had been proposed. With further differentiation of the ventricle, a transmural pattern of proliferation emerges. The cardiomyocytes in the compact layer display a very short cell-cycle time of 8,5 hours, whereas the trabeculated myocardium displays a much slower rate of proliferation (van den Berg et al., 2009). A recent study used clonal analysis to address the mode of growth of the chambers in the mouse (Meilhac et al., 2004b). The presence of small and large elongated clones in the ventricular region indicated directional clonal growth of the chambers. The basis of this conclusion is that growth rates in the developing heart are regionally and temporally homogeneous (Meilhac et al., 2004b). Nonetheless, in embryonic chick hearts was observed remarkably patterned ventricular growth, with highest proliferation in the region where the endocardium approaches the myocardium, and with proliferation gradually tapering off towards the periphery of this focus.

Whereas the differentiation of cardiac precursors into cardiomyocytes shows an inverse relationship between proliferation and differentiation which is remarkably reminiscent of differentiation of skeletal muscle (Stokdale and Holtzer, 1961), the

formation of the ventricle shows simultaneous proliferation and differentiation. The rapidly-proliferating region of ventricular expansion is also distinguished by the expression of ventricular chamber-specific genes, as is already well-documented in the mouse (O'Dell et al., 1994; van Kempen et al., 1996; Delorne et al., 1997; Dunwoodie et al., 1998; Christoffels et al., 2000; Palmer et al., 2001; Houweling et al., 2002). Together, these proliferations, lineage and gene expression data demonstrate the presence of a program for morphogenetic patterning of the definitive cardiac chambers along both the caudal-cranial and dorso-ventral axes (Christoffels et al., 2000; Moorman et al., 2000; Moorman and Christoffels, 2003a). This regional

In summary, this regional growth and differentiation of chamber myocardium is now well-recognized as the ballooning model, as opposed to the earlier model of segregated differentiation of chamber myocardium within transversely arranged compartments of the heart tube, each transverse part considered to represent a compartment as present in the formed heart.

4.1.5. Atrial and Ventricular Chamber Morphogenesis

From HH26-28 in chicken embryos, addition of pharyngeal mesoderm to the poles of the heart is complete and there is a shift from proliferation in extracardiac progenitor cell populations to intracardiac proliferation as the main driver of cardiac growth. The emergence of patterned gene expression domains in the embryonic heart drives chamber morphogenesis and initiates septation as well as conduction system differentiation. Atrial and ventricular working chamber myocardium develops on the outer curvature of the embryonic heart while cardiac cushions develop from the endocardium underlying atrioventricular canal and outflow tract myocardium (Moorman & Christoffels, 2003). *Bmp* and *Notch* signalling play upstream roles restricting the expression of transcription factors of the *T-box* gene family to different regions of the developing heart. Transcriptional repressors such as *Tbx2* and *Tbx3* play a role in cardiac cushions formation and working myocardial program repression in atrioventricular myocardium (Habets et al., 2002). In fact, overlapping expression patterns of the *T-box* transcription factors encoding genes plays a central role in the emergence of cardiac form and early establishment of the cardiac conduction system (Hoogaars et al., 2007; Greulich et al., 2001). The *T-box* transcriptional activators *Tbx20* and *Tbx5* restrict *Tbx2* and *Tbx3* expression to the atrioventricular canal region and compete with these factors for interaction with core myocardial transcription factors such as *Nkx2-5* (Singh et al., 2009).

Ventricular wall growth is accompanied by the development of distinct trabeculated and compact myocardial layers. Analysis of patterns of proliferation during formation of the four chambered mouse heart has revealed that proliferative centers at the base of the trabecules contribute to growth of the compact myocardial layer (de Boer et al., 2012). Growth of the ventricular myocardium occurs in response to *Notch* and *Neurogulin*

signalling from endocardium at the base of the trabecular pits and *Fgf* signals from the epicardium and endocardium (Grego-Bessa et al., 2007).

In the chick heart, as development proceeds, (Yamagishi et al., 1999) some endothelial cells in both the OT and AV regions transform their phenotype and become mesenchymal. This embryonic phenomenon leads to the formation of the endocardial cushion tissue of the valves and septa of the adult heart (Markwald et al., 1984, 1990). In vitro assays using a three-dimensional collagen gel culture system have indicated that mesenchyme formation in the AV canal is prompted by a stimulus produced by the sub-adjacent AV myocardium (Runyan and Markwald, 1983; Krug et al., 1985, 1987; Mjaavedt et al., 1987, 1991; Mjaavedt and Markwald, 1989). The regulation of ventricular growth through signals from adjacent endocardium is response to hemodynamic and flow related forces highlights the interplay between form and function in cardiac morphogenesis.

4.1.6. Septation of the Outflow Tract

The outflow tract of the heart connects the developing right ventricle with the arterial segment, and hence the arteries in the pharyngeal arches. (Thompson and Fizharris, 1984). (Thompson and Fitzharris, 1979; Thompson et al., 1983). The outflow tract itself undergoes a complex process of septation and valvar formation to produce the ventricular outlets, whereas the arterial segment gives rise to the systemic and pulmonary arterial channel of the adult. The fate of the endocardial cushions (Davies, 1927; Kramer, 1942; Patten et al., 1948; Markwald, 1984) found within the outflow tract is crucial to these processes.

In the chick, there are three endocardial cushions within the distal segment of the outflow tract (Qayyum et al., 2001). These endocardial cushions give rise to the leaflets of the arterial valves and to the walls of their supporting *sinuses of Valsava*. The proximal cushions fuse initially to form an intracardiac septum, but, as the cushions become muscularised within the outflow tract, they lose their septal location. Thus, it is not possible to identify a discrete muscular outlet septum in the normal heart of the chick, a fact that is also true of mammals.

The leaflets and sinuses of the arterial valves develop from the distal cushions of the outflow tract just proximal to the initial level of transition between the myocardial cuff and the arterial wall. The subsequent change in the level of the ventriculoarterial boundary in the walls of the maturing valvar sinuses suggests either that the original myocardium has continued physically to retract, or else that it has transdifferentiated into fibroelastic tissue. Further studies are required to elucidate these events and to determine the mechanisms of formation of the valvar leaflets as opposed to the arterial sinuses and the steps involved in the separation of the aortic and pulmonary components.

4.1.7. Ventricular and Atrial Septation

Within the ventricular chamber, an interventricular muscular septum emerges and grows superiorly to fuse with AV cushions, dividing the ventricular chamber into left and right ventricles (Anderson et al., 2003a; Moorman et al., 2003). This muscular septum also connects with OFT cushions to separate the ventricular outlets.

4.1.8. Morphogenetic Remodelling of the Ventricular Chambers, the Formation of the Trabecular and Compact Compartments

Trabecular morphogenesis is a key morphologic event during cardiogenesis and contributes to the formation of a competent ventricular wall. Lack of trabeculation results in embryonic lethality (Wu et al., 2018). The trabecular morphogenesis is a multistep process that includes, but not limited to, trabecular initiation, proliferation/growth, specification and compaction.

The pattern of primitive trabecular ridges runs dorsoventrally, circumferential to the heart tube and appears similar in different species. This occurs in the chick at stage HH16-17, in mice at E10.5 and in humans at the end of the fourth week of gestation (Thompson et al., 2000). The spacing of such trabecular sheets, might be in essence a process of asymmetry break (Stewart & Golubitsky, 1993). Whatever the forces underlying their formation, early trabeculations effectively increase surface area, enabling the myocardial mass to increase in the absence of a coronary circulation and may serve to route blood flow as separate ventricular streams evolve or become functional (van Mierop & Kutsche, 1984).

After bucking, patterns of trabeculation specific for the morphologically left, as opposed to the right, ventricles become apparent at the beginning of ventricular septation, but the differences are more pronounced in birds than in mammals. In the avian left ventricle, the trabeculations are thicker and more sheet-like than in the right ventricle (Rychter & Rycherova, 1981; Sedmera et al., 1997). Trabecular remodelling nonetheless, starts before the completion of septation in birds, but it is postponed after its completion in mammals. Apart from enhancing contractility (Chalice & Viragh, 1973), the trabeculations are also important in coordinating intraventricular conduction (de Jong et al., 1992) and in compartmentalization of the blood in the heart prior to its septation (Hoogers et al., 1995).

With the completion of ventricular septation, the increase in ventricular volumes, and concomitant luminal increase, the trabeculations effectively become compressed within the ventricular wall. This results in a significant increase of proportion and thickness of the compact myocardium (Sedmera, 1997).

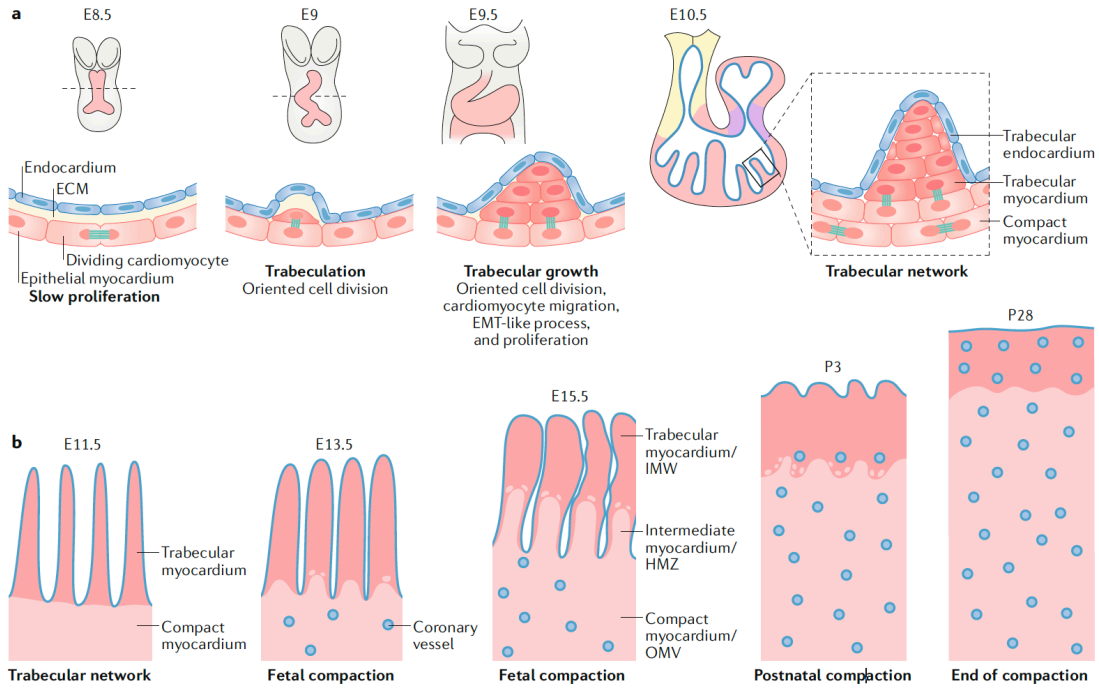

Figure 5. (A). Depiction of the process of trabeculation, beginning at embryonic day 8.5 (E8.5). At E9, the trabeculae, appear bulging towards the ventricular lumen. Various cellular mechanisms, including oriented cell division are involved in the formation of these protrusions. At E9.5-E10.5 a growing trabecular network is formed by oriented cell division, cardiomyocyte migration EMT-like process and proliferation. A rapidly dividing outer layer of compact myocardium and a slowly dividing inner trabecular myocardium are defined at this stage. (B) At E11.5, trabeculae form a complex network. At E13.5, compactation begins and trabeculae integrate into the compact myocardium. Compactation continues postnatally and concludes at postnatal day 28 in mice. (Image from MacGrogan et al., 2018).

Marked developmental modifications occur in the compact myocardium itself which are significant in terms of both structure and function. Much of the increase in myocardial mass, and hence in pumping function, is due to the continuing formation of trabeculations (Wenink et al., 1996). The initial outer compact layer also serves as a source of new cells. Its proliferative activity is higher, and its level of differentiation lower, than in the newly formed trabeculations themselves (Thompon et al., 1995; Mikawa et al., 1992). The growth of the heart is mainly by apposition at the periphery. But as the thickness of the compact layer is limited by diffusion of the oxygen and nutrients from the inside the innermost cells move into the trabeculations, where they continue to proliferate though at a slower rate.

Subsequent thickening occurs due to compactation of trabeculations, this process coinciding with invasion by the developing coronary vasculature from the epicardium (Vranken Peeters et al., 1997). The stimulus for the angioblast invasion comes from mediators such as fibroblasts and vascular endothelial growth factors. Interestingly, the process of vascularization itself does not seem to be dependent of ventricular load (Rongish et al., 1996). The contribution of the compact layer to the total myocardial mass

then becomes much more significant than that of the remaining luminal trabeculations (Jouk et al., 1996).

Although initial formation of the chambers involves little more than a local widening of the original primary heart tube, it is almost immediately followed by the formation of trabeculations which are most conspicuous at the apices of the forming ventricles; they are more limited in the forming atrial chambers, where they form the pectinate muscles. In the mouse, the ventricle, including the thin-walled outer layer, is almost entirely composed of phenotypic trabecular myocardium. At this stage, markers like *Cx40* and Natriuretic pro-peptide (*Nppa*), are expressed in the entire ventricular myocardial component, but become confined to the trabeculations as the compact myocardium thickens. *Cx43*, on the other hand, remains expressed in both the trabecular and compact layers of the ventricular wall (Christoffels et al., 2004a). Many genes that are confined to the atrial myocardium later in development remain expressed in the trabecular component, whereas calcium-handling genes are upregulated in the compact myocardium and remain low in the trabecular component (Franco et al., 1997; Moorman et al., 1998).

The developing compact layer in contrast, is highly proliferative, acquiring its adult phenotype at birth when the proliferative activity ceases. Lineage studies in chicks demonstrated that single labelled cells of the cardiogenic mesoderm form cone-shaped clones spanning the entire thickness of the ventricular wall (Mikawa et al., 1991). The shared lineage of the trabecular and the compact layers of the developing ventricular walls imply that local cues are responsible for further differentiation of the compact working myocardium among others. Indeed, endocardial-myocardial interactions regulate trabeculae formation and withdrawal from the cell-cycle whereas epicardial-myocardial interactions regulate proliferation, growth and compact wall formation (Grego-Besa et al., 2007; Smith and Bader, 2007). *Notch* signaling is crucially involved in the interaction between endocardium and myocardium (Grego-Bessa et al., 2007). The process involves *Bmp10*-dependent proliferative activity in the trabeculations and *EphrinB2*-dependent activation of the *neuregulin/ErbB* pathway, which leads to the differentiation of the trabeculations.

4.1.9. Development of the Cardiac Conduction System

The right side of the heart receives deoxygenated blood and sends it to the lungs while the left side of the heart receives blood from the lungs and pumps it into the rest of the body. The right and left atria and right and left ventricular chambers contract and relax synchronously leading to a rythmic heartbeat. To pump blood throughout the body, the muscles of the heart must be perfectly coordinated to squeeze the blood in the right direction, at the right time, at the right pressure. Thus, the heart activity is coordinated by electrical impulses governed by the cardiac system.

Introduction

The electrical activity of the heart is generated by pacemaker cells localized in the sino-atrial node (SAN) in the dorsal region of the right atrium. From SAN, electrical activity spreads rapidly to both atria, provoking simultaneous atrial contractions. The atria and ventricles are electrically isolated by the annulus fibrosus, composed of non-myocardial cells (Anderson et al., 2003). In order to reach the ventricles, the electrical impulse follows a specialized route, the atrioventricular node (AVN), where its conduction velocity is reduced to delay the spread of electrical activity and allow atrial relaxation before ventricular activation. Electrical activity is then rapidly propagated to the ventricular apex through a specialized fast conducting system or ventricular conduction system (VCS), including the atrioventricular bundle (AVB), also known as the bundle of His, the right and left bundle branches, on either side of the interventricular septum, and a complex network of Purkinje fibers, ramifying over the subendocardial ventricular surface. The VCS plays a critical role in coordinating the heartbeat by rapidly conducting the electrical impulse to the ventricular apex in order to activate contraction from the apex and maximize efficiency of expulsion of the blood through the great arteries at the base of the heart. Conductive cells can be distinguished from working myocardium by the presence of a poor contractile apparatus with few sarcomeres, enriched glycogen content and reduced numbers of T-tubules (Viragh & Chalice, 1997). The function of conductive cells demonstrates specific electrical properties, including rapid conduction (Anumonwo et al., 2001), however, it is the global anatomy of the VCS that orchestrates co-ordination of the heartbeats.

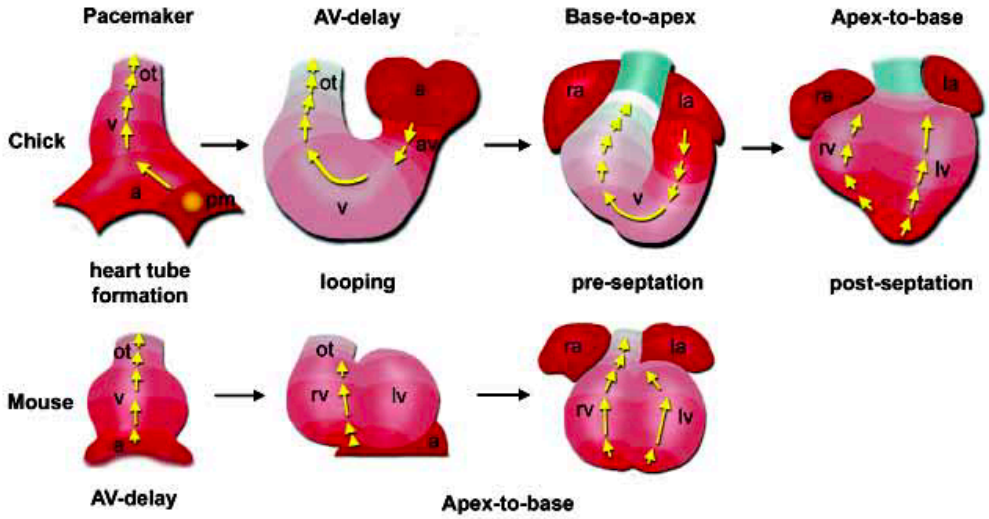

Figure 6. Cardiomyocytes in the presumptive sinoatrial node are the first to become electrically active and effectively become the primitive "pacemaker" for the rest of the heart tube. Note that as septation completes, there is a dramatic shift in the wave of impulse dissemination (yellow arrows). (Image from Penissi et al., 2002).

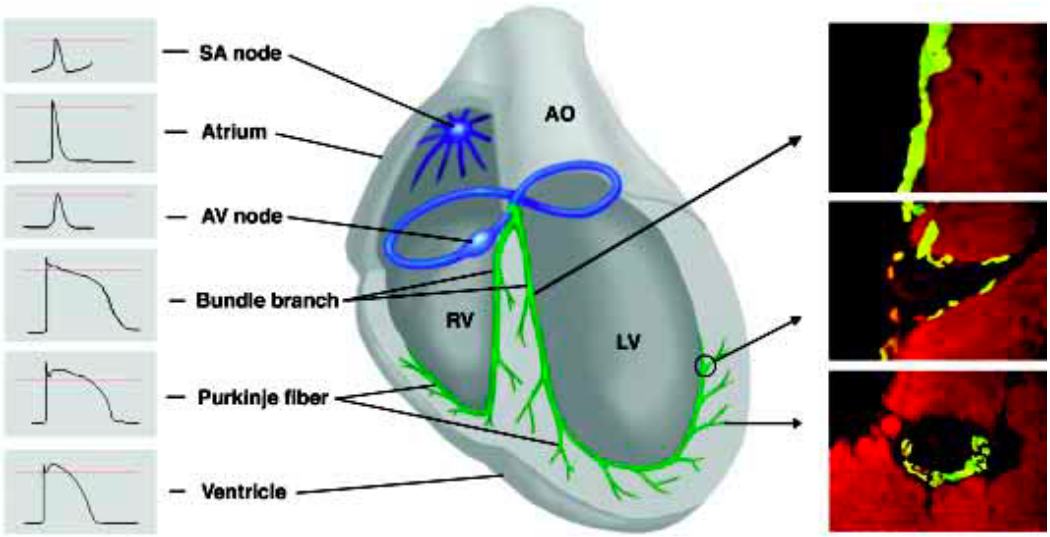

Figure 7. (A). Action potentials for components of the chick heart. (B). IF pictures that detect slow tonic myosin heavy chain in Purkinje fibers (green) and myosin-binding protein C in ventricular myocytes (red). (C) Subendocardial Purkinje fibers. (D). Branch point from subendocardial Purkinje fibers € Intramural Purkinje fibers in different parts of the heart (Image from Pennisi et al., 2002)

4.2. Development of the Proepicardium and the formation of the Epicardium

The outermost layer of the heart is the epicardium. The early embryonic heart lacks an epicardium and consists thus of only two cell layers, the endocardium and the myocardium. The epicardium derives from the Proepicardium (PE), a transitory cell cluster that develops at the junction between the forming sinus venosus and the posterior undifferentiated lateral plate mesenchyme (Rossy et al., 2001; Ishii et al., 2007; Schulte et al., 2007) at HH stage 15.

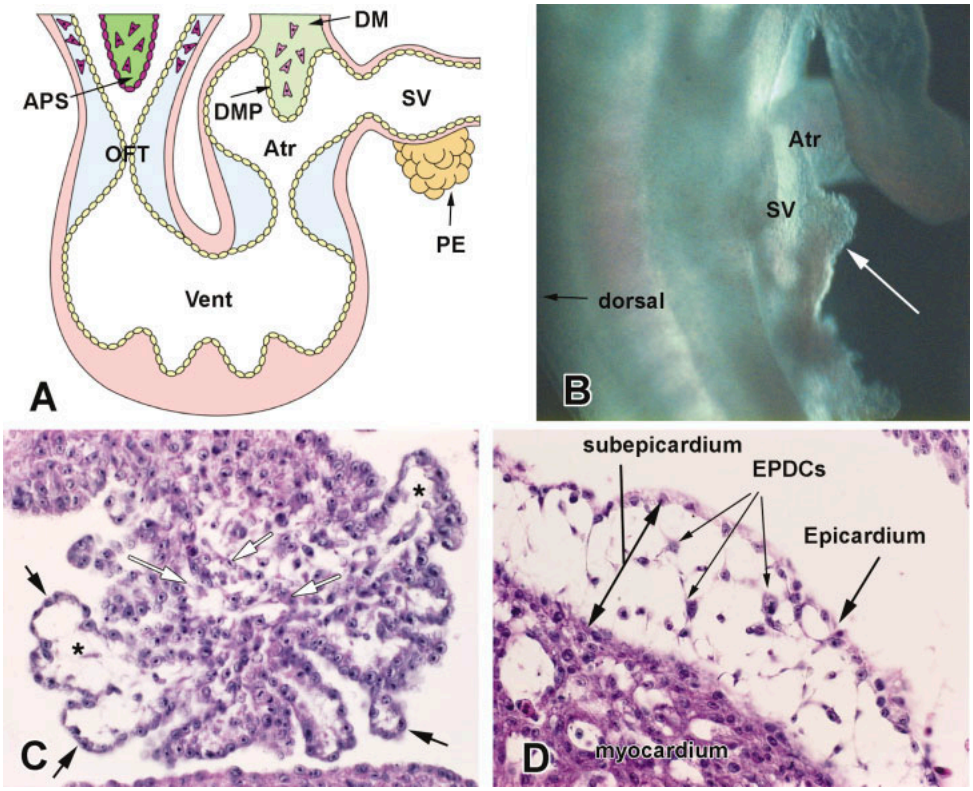

Figure 8. Origin and fate of the proepicardium. (A) Non precardiac cell populations that contribute to the development of the heart. In the OFT, neural crest cells play an important role in the formation of the aortico pulmonary septum (APS), and in the atrial segment (Atr), the dorsal mesenchymal protrusion (DMP), a cell population that is continuous with the mesenchyme of the dorsal mesocardium (DM), plays a part in the separation of the left vs. the right atrium. On the inferior aspect of the sinus venosus (SV) the epicardial precursor tissue, or proepicardium (PE). (B) HH17 chick embryo demonstrating the proepicardium at the margin of sinus venosus and liver and caudal to the developing atrium. (C) Shows the anatomy of the PE. The black arrows point to the mesothelial epithelium. The white arrows indicate the mesenchymal cells within the proepicardium. The asterisks illustrate the spaces within the proepicardium filled with ECM. (D) Advanced stage of epicardial development in HH24 chick heart. The ventricular epicardium (arrows) is generating EPDCs by epicardial EMT that migrate into the subepicardium. (Image from Wessels and Perez-Pomares, 2004).

In its mature form the PE has been described in the chick to have a cauliflower or grape-like appearance due to the formation of multiple villi. It consists of an outgrowth of cuboidal mesothelial cells overlying an internal cluster of mesenchymal cells, which is embedded into an extensive amount of extracellular matrix (Nhimey et al, 2003). Underneath is a stratified cuboidal cell layer, which is adjacent to the endocardial layer of the sinus horns (Nahimey et al., 2006). Between HH15 and HH17, PE grows in size and villous projections extend from the PE towards the dorsal aspect of the inner curvature of the heart tube, ultimately contacting at the AV junction and forming a tissue bridge. Subsequently, PE cells will migrate onto the heart and spread over the surface forming a single squamous epithelium which is termed the epicardium. Underneath the epicardium an extracellular-rich sub-epicardial mesenchyme forms. Some of this mesenchyme is believed to be a direct descendent of the proepicardial mesenchyme (Nahimey et al., 2006). Myocardial signals induce epicardial cells to undergo an

epithelial-to-mesenchymal transformation (EMT) and either populate the sub-epicardial mesenchyme or migrate into the myocardial wall.

In the chick embryo, the PE in an asymmetric structure, which predominantly develops on the right sinus horn (Schlueter, Manner & Brand, 2006). On the left side this proepicardial outgrowth is severely retarded (Schulte et al., 2007; Schlueter et al., 2006, Torlopp et al., 2010). A right sided pathway was identified that is responsible for asymmetric PE development (Schlueter et al., 2009). This pathway involves Fgf8, which is expressed on the right side of Hensen's node (Isaac et al., 1997). Fgf8 is able to induce the transcriptional repressor Snai1 in the right lateral plate mesoderm, which has the ability to suppress the expression of the left-side determinant Pitx2 (Patel et al., 1999).

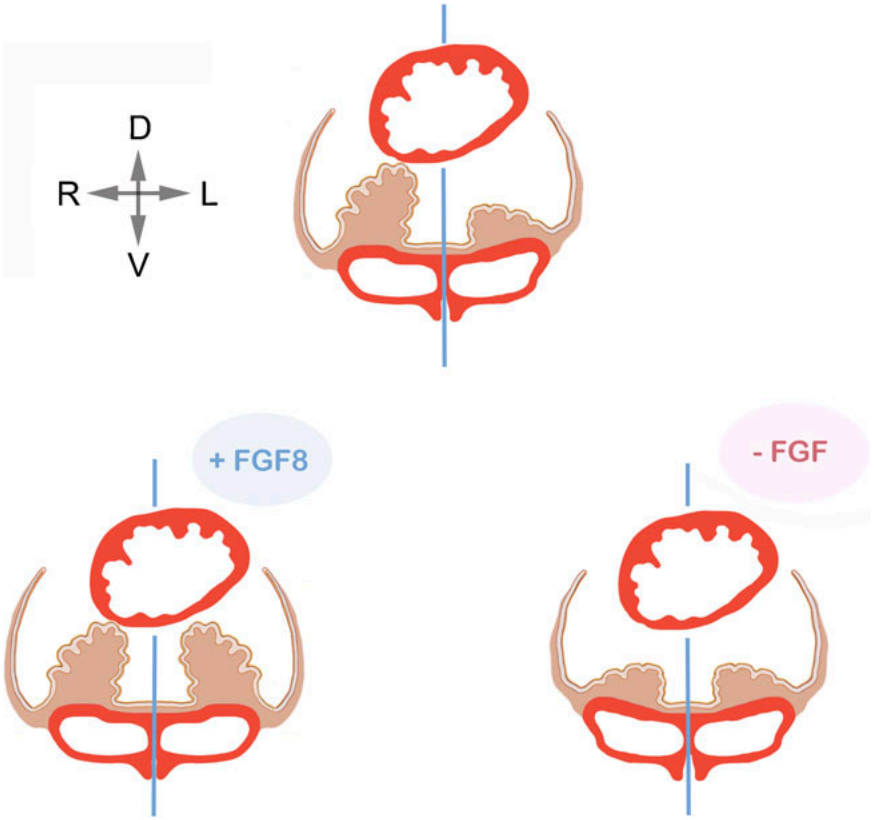

Figure 9. Depiction of a chick embryo at HH17. The PE on the right side makes contact to the dorsal surface of the heart. The left PE is smaller and does not establish contact to the heart. Implantation of *Fgf8* on the left side induces the formation of two equally sized proepicardia on both body sides. Inhibition of F*gf8* on the right inhibits PE formation (Image from Schlueter & Brand, 2012).

In mice, PE starts to form at E.8,5 (Schulte et al., 2007; Rodgers et al., 2008). It was proposed that in mice, proepicardial villi are not connected to the ventricle via an extracellular matrix bridge but are released as free-floating vesicles, which pass the pericardial cavity establishing epicardial islands on the surface of the heart (Schulte et al., 2007; Komiyama et al., 1987; Hirose et al., 2006). This principle was however recently challenged by a descriptive analysis performed by Rodgers et al. (2008) in which they were able to show that murine proepicardial vesicles are indeed in direct contact with the myocardium. However, free vesicles were also observed, in particular in the region of the

atrioventricular sulcus. The findings resulted in a model in which proepicardial villous projections attach to the heart, passively elongate by retraction of the beating heart tube, ultimately collapse and leave a vesicular patchwork on the myocardial surface (Rodgers et al., 2008). Furthermore, data from rat embryos also confirm a matrix-rich connection between the PE and myocardium which might be similar to that found in the chick (Nestbitt et al., 2006).

A more significant difference between mouse and chick is the bilateral formation of the murine PE anlagen on the sinus venosus, which subsequently fuse ventrally at the midline before the cells colonize the ventricle (Schulte et al., 2007). The right-sidded *Fgf8/Snai1* pathway that was proposed in the chick for induction of the PE however is not deployed in a similar fashion in the mouse. This might also be reflected by the different strategies of the left-right asymmetry generation in the mouse and the chick (Schlueter et al., 2007). The mechanisms of the PE induction in mice remain unknown to a large extent.

Curiously, PE in lower vertebrate embryos shares similar morphological features as compared to the chick embryo. Xenopus PE can be first found at stage 41, equivalent to approximately 3 days of embryonic development (Jahr et al., 2008). It is strictly formed on the right side as a cluster of mesothelial cells that colonizes the ventricle via a distinct and persisting tissue-bridge (Jahr et al., 2008). Asymmetric right-sided tissue bridge formation is also described in a related amphibian species, the *axototl* (Fransen et al., 1990).

In zebrafish, the epicardium covering the ventricle derives mainly from the atrioventricular canal proepicardial cluster (avcPE) and to lesser extent, from the venous pole proepicardial cluster (vpePE) (Peralta, Gonzalez-Rosa, Marques and Mercader., 2014). Transfer of cells from these two PE clusters occurs through their release into the pericardial cavity and subsequent adhesion to the ventricle. Additionally, some cells from the cranial pericardial wall adhere directly to the ventricular surface. In contrast the bulbus arteriosus (BA) aka the outflow tract, is covered by cephalic pericardial mesothelial cells that do not undergo a change in cells shape and are not derived from the PE> (Peralta, Gonzalez-Rosa, Marques and Mercader., 2014).

The sturgeon is another, yet more primitive fish species, in which proepicardial development has been investigated in detail. Around four days post hatching the PE can be observed as a bilateral cluster of cells between the sinus venosus and the ducts of Cuvier (Icardo et al., 2009). These two clusters fuse at the midline and subsequently attach to the heart. Interestingly, different strategies are used to colonize the different regions of the heart. The conus myocardium is covered by a cohesive proepicardial epithelium. In contrast, free-floating vesicles apparently colonize the ventricle. However, a mechanism similar to the murine epicardium formation should not be excluded. In accordance to higher vertebrates, the bulbus epicardium appears to be derived from mesothelium of the pericardial wall. The tissue bridges that are formed during

development persist and connect the coronary vascular plexus and the sinus venosus and some are even found to carry cardiac innervation.

One of the most primitive organisms studied to date is the lamprey. The lamprey develops two bilateral PE anlagen around 17 days post fertilization but only the right cell cluster forms a transient tissue bridge to transfer cells onto the heart (Pombal et al., 2008). In later stages, after the epicardium has formed, the two cell clusters give rise to the pronephric external glomeruli, which become highly vascularized. The relation to the pronephros might be a primitive feature represented in agnathans and potentially might reveal the evolutionary origin of proepicardial cells.

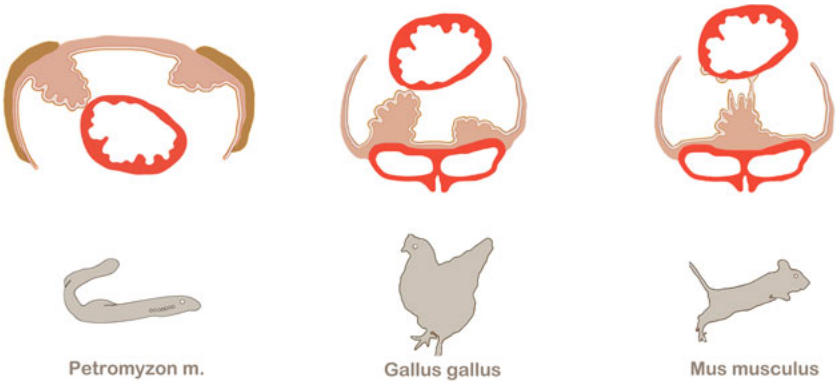

Figure 10. The PE is formed as an asymmetrical or symmetrical outgrowth of mesothelial cells. Left in the lamprey mesothelial cells of the pronephric external glomerulus form a tissue bridge on the right body side, which arises from the coelomic wall, Middle in the chick embryo a right sided tissue bridge is established from the PE that develops on the right sinus horn. Right PE development in the mouse is symmetrical with two bilateral anlagen on either sinus horn fusing at the middle (Image from Schlueter & Brand., 2012).

In summary, the occurrence of a distinctive cluster of PE cells secondarily colonize the heart is an intriguing feature of cardiac development and is found in all vertebrates Interestingly, PE formation across the different vertebrate classes, displays also some significant differences, most likely due to species-specific morphological peculiarities, including asymmetrical development and the process of cell transfer to the heart (Schlueter & Brand, 2011).

4.2.1. Signaling Pathways Involved in PE Specification

Various signals determine the outgrowth and precise location of the PE. In the chick embryo, two PE primordia appear bilaterally, but only the right one develops. This asymmetry is proposed to be under the control of the right determining molecular system including *Fgf-8Snai1* (Schlueter et al., 2009). *Fgfs* are also suggested to support PE proliferation, thereby sustaining PE identity and preventing PE cells from differentiating into cardiomyocytes (Kruithof et al., 2006; Torlopp et al., 2010). Lower *Bmp2*

concentrations also maintain PE identity by promoting expression of *Tbx18* and *Wt1*, whereas higher *Bmp2* concentrations induce the differentiation of PE cells into cardiomyocytes (Kruithof et al., 2006; Schlueter et al., 2006). Bmp has also been identified as an important signal in PE specification in zebrafish (Liu et al., 2010), due to inhibition of *Bmp* signaling through the expression of *Tbx18*, indicating that *Bmp2* alone is not enough to drive mesodermal commitment to PE fate (Liu et al. 2009). In addition, *Bmp2* expression is crucial for attachment of PE cells to the heart (Ishii et al., 2010). All these events require highly patterned expression of *Bmp2* in the PE/septum transversum region (Kruithof et al., 2011) and thus it can be postulated to play a role in establishing the posterior myocardial limit of the developing heart.

Formation of the epicardial sheet requires that PE cells attach to the myocardial surface. In avian embryos, this is accomplished by formation of a PE tissue bridge over the myocardium. Ectopic *Bmp2* expression in chicken myocardium suggests that *Bmps* in the atrioventricular (AV) canal direct PE protrusion toward the heart tube (Ishii et al., 2010). In mammals the predominant mode of PE ell transfer to the heart seems to be the release of PE cell cysts to the pericardial cavity, with the PE connecting to the heart relatively late in epicardial development (Viragh et al., 1981; Manner, 1992).

One PE cells adhere to the myocardial surface they flatten and spread to form the epithelial monolayer. PE cell polarity is an important aspect of this process, because inactivation of the cell polarity protein PAR3 severely impairs epicardial development (Hirose et al., 2006). In PAR3 mutants, baso-apical cell polarity is disrupted and the cells that bud from the PE do not form the cysts that would normally form the primitive epicardial epithelium.

As development proceeds, the epicardium grows over the heart in a fixed spatiotemporal pattern, starting at dorsal AV groove (Vrancken et al., 1995; Wengerhoff et al., 2010). Examination of the distribution of *α4-integrin* ligand vascular cell adhesion molecule-1 (VCAM-1) indicates that CAM-1 is expressed in the developing myocardium and *α4-integrin* in the epicardium. This complementary pattern suggests that *α4*/VCAM-1 interaction stabilizes adhesion of epicardium to myocardium and enables superficial migration. Targeted inactivation of VCAM-1 (Kwee et al., 1995) or *α4-integrin* (Yang et al., 1995) in mice confirmed this hypothesis, with mutants dying at approximately E14,5 and displaying impaired formation of epicardium and coronary vessels. Interfering with *α4-integrin* in the chick increases the invasive behaviour of epicardial mesenchymal derivatives, indicating a role for *α4-integrin* in modulating epicardial-derived cell migration (Dettman et al., 2003). Further studies in the chick show that communication between integrin receptors facilitates epicardial cell adhesion and subepicardial matrix organizations (Pae et al., 2008). Additional evidences on the role of key molecules involved in epicardial-myocardial interaction comes from genetically engineered mice, including herein *Rxra, Gata4* and *Fog2*. Targeted inactivation of *Rxra* in mice is embryonic lethal between 13.5 and E15.5 and affected embryos have a thin compact myocardium layer (Krastner et al., 1994; Kubalak et al., 2002), while deletion of *Fog2* a

cofactor for *Gata* transcription factor, display normal epicardium development at E12.5 but it does not undergo epithelial-to mesenchymal transition (EMT) and thus coronary vessel formation is impaired, resulting in embryonic death at approximately E14.5 (Tevosian et al., 2000).

4.2.2.-Derivatives of the Embryonic Epicardium

The epicardium is embryologically formed by the outgrowth of proepicardial cells over the naked heart tube (Lie-Venema et al., 2007). Following epithelial-to-mesenchymal transition, subsequently migrate into the myocardium and differentiate. During this process they are thus dubbed EPDCs.

EPDCs once they go through the sub-epicardial space continue their journey into the developing heart. Seminal approaches using quail-chick chimeras demonstrate that quail EPDCs contribute to distinct cardiac cell lineages, such as endothelial and smooth muscle cells in the coronary vasculature, endocardial mesenchymal cells in the atrioventricular cushions and also cardiac fibroblasts (Poelmann et al., 1993; Dettman et al., 1998). Since the experimental model used was a heterologous chimera, multiple criticisms arose as which was indeed the real contribution of these cells. Supporting evidences were generated using retroviral-defective cell lineage tracing experiments in chicken hearts, providing similar results (Mikawa & Gourdie. 1996); i.e. Contribution to vascular endothelial cells, smooth muscle cells and cardiac fibroblasts. Contribution to endocardial cushions is scarce, although it has been proposed that these cells are important for the correct development of the atrioventricular junctions and the annulus fibrosus (Lie-Venema et al., 2008; Zhou et al., 2010; Lockhart et al., 2014). More recently, a contribution to cardiac resident stem cells (mesenchymal-like) has also been reported (Chong et al., 2001). In all cases, contribution to the developing myocardium was never observed (Poelmann et al., 1993; Mikawa & Gourdie, 1996; Perez-Pomares et al., 2002). Surprisingly, in vitro PE culture experiments demonstrated that cardiomyocytes could be derived from these precursor cell pools (Kruithof et al., 2006).

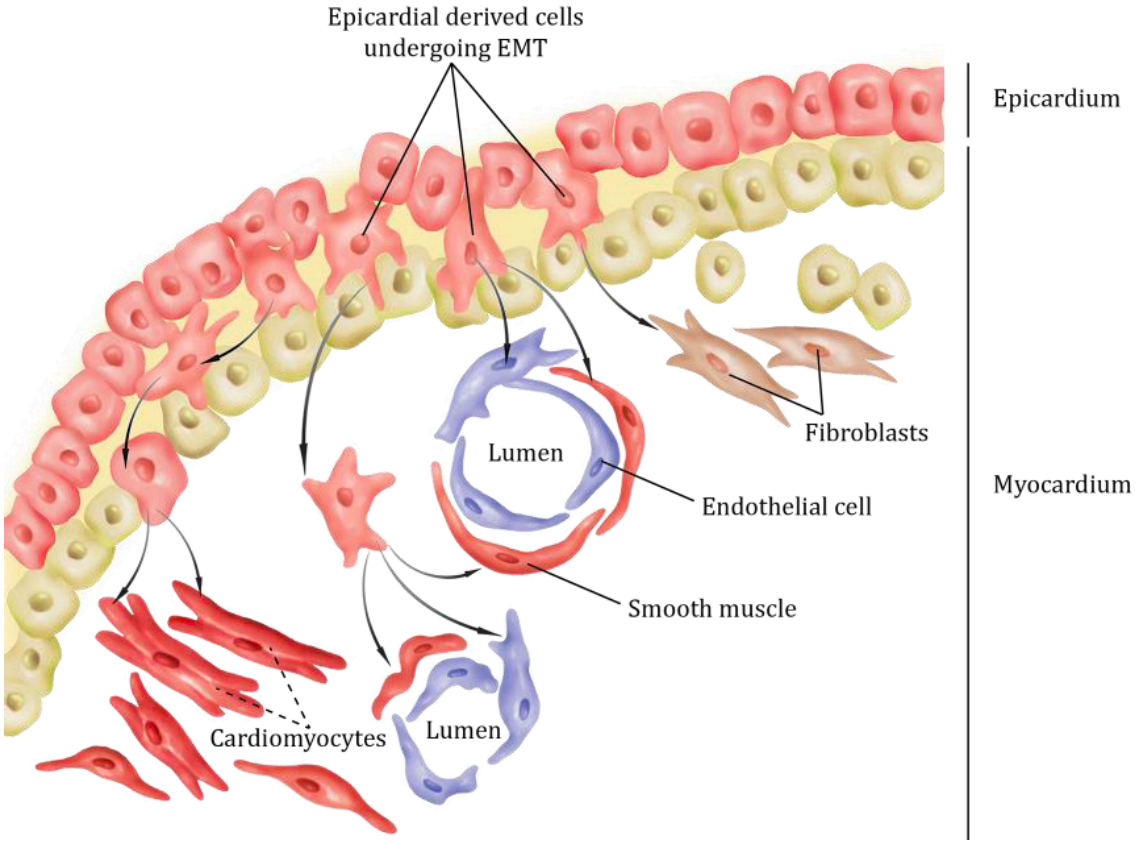

Figure 11. Epicardial derivatives contribute to cardiac lineages Epicardial cells undergo epithelial-to-mesenchymal transition (EMT), delaminate from the epicardium, invade the underlying myocardium and differentiate into various cardiac lineages, including fibroblasts, smooth muscle cells, endothelial cells and cardiomyocytes. (Image from Singh and Epstein, 2013).

Genetic lineage tracing in mice provided evidences on the contribution of the PE/embryonic epicardium to the mature murine heart. Several lineage tracing approaches were documented, in most cases, using *Cre/LoxP* conditional activation of the reporter genes. In this settings, *Tbx-18* lineage tracing demonstrated a contribution to all the previously reported EPDC-derived lineages but surprisingly, also to the cardiomyocyte lineage Whereas, these studies claimed that epicardial *Tbx-18* positive cells contributed in vivo to ventricular cardiomyocytes (Cai et al., 2008) it was previously reported that fetal cardiomyocytes also expressed *Tbx-18* (Franco et al., 2006; Christoffels et al., 2009; Zeng et al., 2011) and thus those *Tbx-18* positive epicardial lineage tracing experiments were dubious.

Epicardial *Wt1* positive derived cells have also been reported to contribute to endothelial and myocardial cells (Zhou et al., 2008; Zhou and Pu., 2001). Evidence for *Wt1* positive cells in the embryonic heart has also been reported but excluding cardiomyocytes (Zeng et al., 2001) yet more recent evidence demonstrated that *Wt1*-derived cardiomyocytes can be traced in the developing heart before PE/epicardial formation (Rudat and Kospert, 2012, 2012; Cano et al., 2016), thus questioning the epicardial contribution to the developing cardiac muscle. On the other hand, prove that

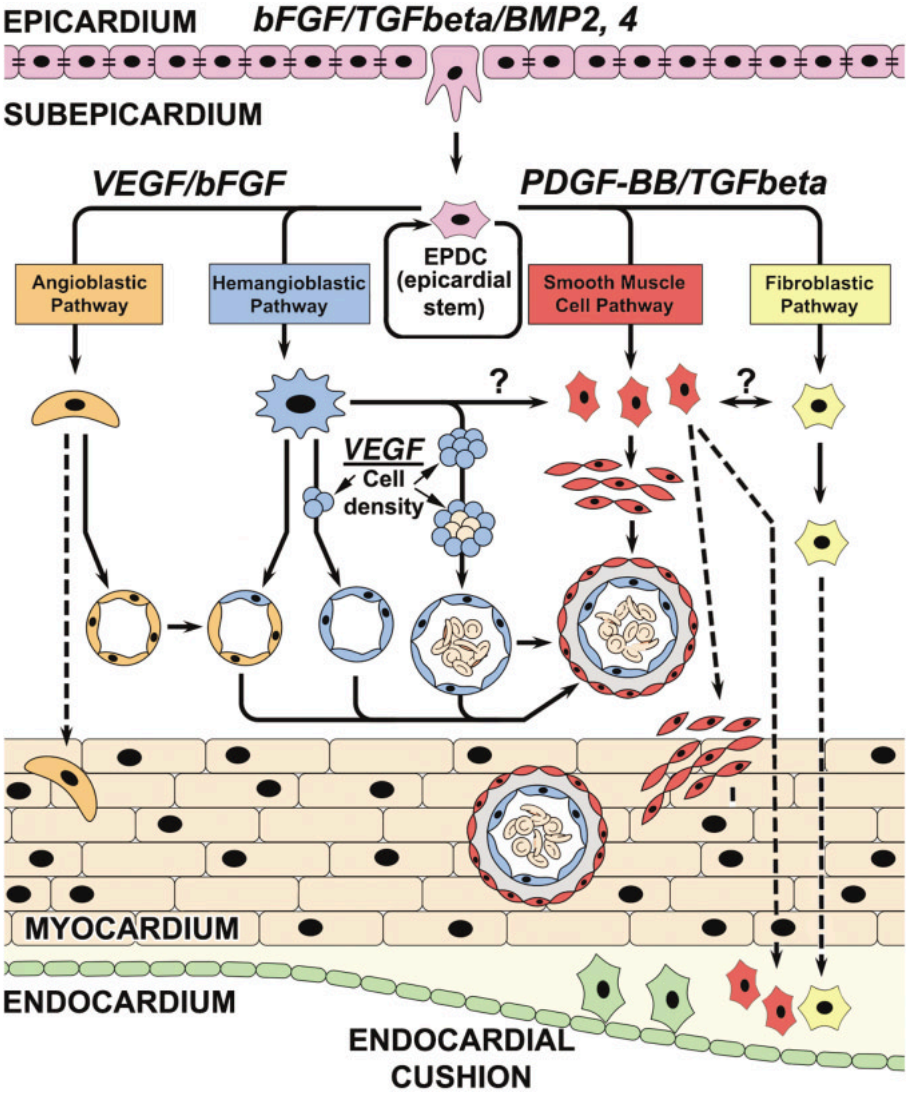

Figure 12. Differentiation pathways of EPDCs. Bmp and TGF-β are involved in the induction/regulation of epicardial (pink) EMT. Furthermore, VEGF, *Fgf*, PDGF and TGF-β play a role in the regulation of EPDC differentiation (isolated pink cell). Specifically, VEGF and *Fgf* are responsible for the differentiation of EPDCs into angioblasts (orange) and hemangioblasts (blue). A subset of clustered hemangioblasts can, dependent on their exposure to VEGF, further differentiate into endothelial cells, whereas other will transdifferentiate into hemopoietic cells (yellow). These processes are crucial for the initial phases of coronary vasculogenesis/angiogenesis. The growth factors PDGF and TGFβ1 are thought to be responsible for the differentiation of EPDCs into fibroblasts (yellow)/smooth muscle (red) pathways. It is also shown where in the embryonic heart EPDCs can be found. (Image from Wessels and Perez-Pomares, 2004).

epicardial cells do not contribute to myocardium in zebrafish comes from *Tcf21*-tracing (Kikuchi et al., 2011) and transplant experiment (Gonzalez-Rosa et al., 2012), in which a contribution to the perivascular beds is reported. While these data might support the notion that epicardial cells can contribute to the formation of cardiomyocytes in vivo, yet these evidences remain controversial, mainly because the limitation on the use of *Cre*-based techniques as a bone fide fate mapping approach (Christoffels et al., 2009).

Additional controversies have also arisen regarding the contribution of EPDCs to other vascular components. To date, it seems clear that EPDCs mostly contribute to vascular endothelial cells have also been challenged by additional Cre-based fate mapping to provide substantial contribution to the developing vascular endothelium in mice (Merki et al., 2005; Cai et al., 2008; Zhou et al., 2008). More recent experiments described that coronary endothelial lining was mostly entirely derived from the sinus venosus endothelium as revealed by an *Apelin-Cre* mice (Red -Horse et al., 2010), a VEGF-dependent process (Chen et al., 2014). Furthermore, additional evidences reported that ventricular endocardial cells also can contribute to the coronary vasculature (Wu et al., 2012) as revealed by *Nfat1-Cre* lineage tracing. Importantly, the usage of novel proepicardial lineage tracing markers such as *Scleraxis-Cre*, *Semaphorin-3D-Cre* and *Fabp4-CreER* drivers (Katz et al., 2012; He et al., 2014) demonstrate contribution to the coronary vasculature. In fact, reconciling evidences reported by Chen et al (2014) determined that sinus-venosus (SV) derived coronary vasculature (70%) whereas the ventral aspects were mostly endocardial derived (70-80%) with just a small (20%) but uniform contributions from the epicardium. These data are in line with a recent report that similarly estimated a 20% contribution from the proepicardium (Cano et al., 2016). Interestingly, a significant proportion of SV-derived and endocardial-derived cells displayed overlapping patterns with PE-derived cells, suggesting a common lineage origin. These data support the notion that multiple precursors cell population, contribute to the formation of the cardiac vasculature in mice, in contrast to avian hearts, in which the epicardial-derived contributions is large and undisputed. Lineage relationships between these three distinct coronary vasculature components remain nonetheless to be fully elucidated in mice.

Epicardial cells display distinct divergent and overlapping expression patterns of *Wt1, Nfact1, Tbx-18* in chicken and murine hearts (Braitsch et al., 2012), providing a heterogeneous panel of potentially distinct cardiac stem cells. Whereas to date it remains elusive when and how epicardial cells becomes specific to their prospective lineage, it is increasing clear that multiple factors paly pivotal roles in this process. In particular PDGFR-β is important for epicardial migration and for the development of coronary vascular smooth muscle cells (Mellgren et al., 2008; Bax et al., 2009; Smith et al., 2001), retinoic acid and VEGF primers endothelial vs. smooth muscle differentiation (Guadix et al., 2006) and *Fgf10* and *Fgfr2b* are essential for cardiac fibroblast formation (Vega-Hernandez et al., 2011). In addition, *Po1/Tfc21* is regulated by retinoic acid and inhibits differentiation of EPDCs into smooth muscle cells in chicken and mice (Braitsch et al., 2012) while *Wnt* signalling is also important for epicardial specification as *Dkk1* and *Dkk2* mouse mutants display impaired epicardial development (Phillips et al., 2008) as well as MAPK kinase genetic inactivation (Liberatore & Yutzey., 2004; Craig et al., 2010). Other signalling pathways, such as CXCL12/CXC4 are also crucial for cardiac vascular development (Cavallero et al., 2015). Furthermore, *Hippo* signalling mediated by *Yap/Taz* modulates *Tbx18* expression in the epicardium controlling their contribution to the coronary vasculature (Singh et al., 2016). Several other molecules have also been reported to be critical for coronary artery formation such as *tenascin-C* (Ando et al., 2011)

and *Nephrin* (Wagner et al., 2011), particularly for smooth muscle recruitment to those cardiac vessels. Overall these findings highlight the complexity of distinct signalling pathways and molecules governing the coronary vasculature development.

4.2.3. Contributions of Epicardial Derived Cells in Cardiac Looping

Little is known about the contributions of EPDCs in the beginning process of dextral looping during embryonic development (Ramsdell, 2005; Taber, 2006; Linask and Vanauker, 2007) but the inner curvature, which is obviously involved in the cellular movements during cardiac looping, is the first site of EPDCs and endothelial cell entry into the myocardium (Lie-Venema et al., 2005) at a developmental stage just preceding the last phase of looping as HH20-24. Disorders related to the last looping phase may arise with defects in epicardial development (Gittenberger-de Groot et al., 2000). Further research on the matter is required nonetheless.

4.2.4. Functional Roles of Epicardial Derived Cells in Coronary Development

In avian species, coronary vessels start to form from developmental stage HH23-HH25 onward. Liver-derived endothelial precursor cells travelling along with the PE and the epicardium enter the sub-epicardial mesenchyme and give rise to the early endothelial capillaries of the coronary plexus by vasculogenesis (Viragh et al., 1993; Pearlmann et al., 1993; Kattman, Dettman and Bristow, 2004; Lie-Venema et al., 2005; Waldo et al., 1990). The initial endothelial vessel network is in open contact with the sinus venosus and starts growing from the dorsal side of the sub-epicardial mesenchyme of the atrioventricular sulcus. From here, it encircles the atrioventricular groove towards the ventral side and is connected to the sinus venosus at this time point. Although smooth muscle components have been observed at stage HH27 (Lattan, Dettman and Bristow, 2004), stabilization of the vascular network by SMCs and fibroblasts takes place after arterial connection of the peritruncal coronary network by ingrowth of the coronary stems into the aortic wall at stage HH32 (Waldo et al., 1990; Rogers et al., 2989). Retroviral labelling experiments showed that the SMCs and adventitial fibroblasts of the coronary system originate from the PE (Mikawa and Gourdie, 1996; Mikawa and Fischman, 1992) and confirmed by several studies in quail-chicken chimeras (Manner, 1999; Vrancken Peeters et al., 1999; Gittenberger-de Groot et al., 1998; Dettman et al., 1998). Recruitment of SMCs and pericytes to the developing vessel network is primarily regulated by PDGF-β and its receptor PDFG-β (von Tell et al., 1993), expressed by coronary endothelium and thus attracting EPDCs respectively (van den Akker et al., 2005). TGF-β and serum response factor (SFR) are involved in SMC differentiation of the recruited EPDCs (Compton et al., 2006; Landerholm et al., 1999; Lu et al., 2001). The role of EPDCs in

coronary development was further studied by manipulation of the epicardial contribution to the developing heart, which resulted in impaired development of the coronary arteries.

Despite reports on a common epicardial origin of the coronary endothelium and the coronary media and adventitia (Wessels and Perez-Pomares., 2004; Perez-Pomares et al., 2002; Guadix et al., 2006), only SMCs and fibroblasts of the coronary system are truly derived from EPDCs. The endothelium of the coronary vasculature is actually liver derived, since PE transplants without a tiny piece of liver will never give rise to endothelial cells formation in the recipient heart (Poelman et al., 1993). The separate origins of the coronary endothelial and SMCs were also recognized in retroviral tracing experiments, which never showed labelling of both endothelium and SMCs in the same coronary segment (Mikawa & Gourdie, 1996). Reporter gene expression under control of the epicardium-specific *Gata-5* reporter in the coronary smooth muscle layer, but not in the endothelium, provided additional evidence for a non-epicardial origin of the endothelial coronary component. The fact that endothelial cells reach the heart via PE (Viragh et al., 1993; Poelmann et al., 1993) and can be found there already at stage HH16-HH17 (Kattan et al., 2004; Lie Venema et al., 2005) may well explain findings of a seemingly common epicardial origin of endothelium and mesenchymal coronary contributors in studies with proepicardial explants of similar developmental stages.

The differentiation of proepicardial/epicardial cells into Coronary Smooth Muscle Cells was first reported in a series of studies based on a retroviral in vivo labelling (Mikawa and Fischman, 1992; Mikawa and Gourdie, 1996). Subsequently, a number of studies using quail/chick proepicardial transplantations confirmed and expanded these observations (Dettman et al., 1998; Gittenberger-de Groot et al., 1998; Manner et al., 1999; Vrancken Peeters et al., 1999; Perez-Pomares et al., 2002a, 2002b). In vitro studies indicate that in presence of serum, EPDCs can differentiate into CoSMCs (Landerholm et al., 1999). Furthermore, CoSMCs are regulated by a *Rho-A*-mediated reorganization of α-actin filaments associated with *p160 rho-kinase* activity (Lu et al., 2001). Yamada et al., (2009) provided evidence for SMC differentiation after PDGF- βB induction from an early embryonic bipotential ancestor characterized as a *flk-1*/VEGFR-2 positive cell type. In the absence of PDGF- βB these cells would differentiate into endothelium after VEFG induction.

4.2.5. Contribution of Epicardial Derived Cells to Cardiac Fibroblasts

During cardiac development, diverse non-myocardial cells colonize the extracellular space surrounding cardiomyocytes These cells produce extracellular matrix (EMC) components, including collagen and fibronectin, and the cells and their milieu constitute the cardiac interstitium (Kanekar et al., 1998). Interstitial ECM strongly influences cell shape and function and is crucial for transmitting information from the extracellular environment during development and in the onset of disease (Kanekar et al., 1998, Souders et al., 2009).

Although most cardiac interstitial cells are thought to be cardiac fibroblasts, multiple studies have shown that these cells represent a heterogeneous population. The function of cardiac fibroblasts is to maintain ECM homeostasis and cardiac vessels, and even contribute to correct cardiac electrophysiologic activity (Krenning, Zeisberg and Kalluri, 2010). Morphology is no a sufficiently rigorous criterion for identifying fribroblastic cells, because the shape of a fibroblast changes depending on its position and physiological status. The EPDC produced by epicardial EMT are considered a major source of cardiac fibroblasts (Norris et al., 2008) and several studies in avian (Gittenberger-de Groot et al., 1998; Mikawa and Gourdie, 1996; Mikawa and Fischmann, 1992; Manner, 1999) indicate that the embryonic epicardium is the first and main contributor of embryonic cardiac fibroblasts.

The interaction of cardiac fibroblasts with cardiomyocytes in the embryonic and adult heart is an important issue. A report demonstrates that the emergence of ventricular myocardium stimulates the proliferation of cardiomyocytes progenitors (Ieda et al., 2009). Culturing embryonic cardiomyocytes and cardiac fibroblasts together or cardiomyocytes alone, these authors observed strong stimulatory effects from fibroblasts on cardiomyocytes proliferation Gene expression profiling revealed that cardiac fibroblasts express high levels of growth factors, cytokines and EMC components. Examination of the effect of different EMC molecules on cardiomyocytes proliferation revealed that fibronectin and collagen potently stimulate embryonic cardiomyocyte proliferation. These molecules signal through integrins, and embryonic β1-integrin expressed on cardiomyocytes has been reported to mediate this proliferative effect via activation of the *Erk1/2* and *Pi3K/Akt* pathways *Nkx2-5-Cre* mediated myocardial deletion of *β-integrin* corroborates this finding in vivo, resulting in weak myocardial proliferation, a thin compact layer at E.14,5 and perinatal death (Ieda et al., 2009).

4.2.6. Contributions of Epicardial Derived Cells to Myocardial Architecture

EPDCs are essential for normal myocardial architecture, which is evident from the numerous studies in which mechanical (Gittenberger-de Groot, Vrancken Peeters, Bergwerff, Mentink and Poelmann, 2000; Eralp et al., 2005; Perez-Pomares, Munoz-Chapuli and Wessels (2001) or genetic manipulation (Kwee et al., 1995; Yang, Rayburn and Hynes, 1995; Lie Venema et al., 2003, Merki et al., 2005; Tevosian et al., 2000; Kastner et al.,. 1994; Watt et al., 2004) of epicardium resulted in a thin ventricular myocardium. There is a close spatiotemporal relationship between the deployment of EPDCs into the myocardium and the onset of the formation of the ventricular compact zone (Gittenberger-de Groot et al., 1998).

The signaling pathway of the vitamin A metabolite retinoic acid is the best described paracrine effector route by which EPDCs influence myocardial development. Initiated by

the finding that mice deficient for the retinoic acid receptor RXRα showed a thin ventricular myocardium failed to form, subsequent in vitro studies showed that retinoic acid treatment releases a trophic factor from EPDCs that promotes cardiomyocyte proliferation via activation of intracellular P13K and *Erk* signal transduction pathway (Chen et al., 2002; Kang and Sucov, 2005). The finding that the key enzyme in retinoic acid synthesis*, Raldh2* is expressed in the epicardium and EPDCs (Perez-Pomares et al., 2002; Xavier-Neto, Shapiro, Houghton and Rosenthal, 2000), and that epicardium-specific (Merki et al., 2005) but not myocardium-specific (Chen, Kubalak and Chien, 1998), RXRα knockout mice have a thin ventricular myocardium indicate that this instructive feature of EPDCs is regulated by an autocrine loop. *Fgfs* are among the trophic factors that are upregulated in the epicardium by retinoic acid (Chen et al., 2002; Chen, Kubalak and Chien, 1998; Stuckmann, Evans and Lassar, 2003). In turn, *Fgf2, Fgf-9* and *Fgf-16*, signalling to the myocardium through the *Fgf* receptors 1 and 2, were shown to enhance cardiomyocyte proliferation (Corda et al., 1997; Laine et al., 2005; Pennisi, Ballard and Mikawa, 2003). Additional candidates that have been identified as factors involved in the cross-talk between myocardium and epicardium that are influencing myocardial architecture are ET-1 (Eid, de Bold, Chen and de Bold AJ, 1994; Kelly et al., 1990) *Fog-2* (Tevosian et al., 2000) and erythropoietin (Stuckmann, Evans and Lassar, 2003).

Although extracellular matrix biology is essential for myocardial architecture during development and remodelling (Corda et al., 2000), only few studies have focused on the extracellular matrix as a signalling vehicle. In the RXRα knockout mouse, defective epicardial development was associated with an increase in fibronectin deposition (Jenkins et al., 2005). The finding that the extracellular matrix protein periostin is expressed by fibroblasts and by mesenchymal cells that differentiate into fibroblasts (Kruzynska-Freitag et al., 2001; Norris et al., 2004) and colocalize with the presence of EPDCs in the epicardium, the sub-epicardium, the atrioventricular valves and interstitial cells within the ventricle (Gittenberger-de Groot et al., 1998) opens up a new avenue for the research on the role of EPDCs in myocardial development.

Paracrine signals originating from the developing epicardium play a critical role for myocardial growth during heart development (Pennisi et al., 2003; Lavine et al., 2005). The early myocardium consists of an outer, highly mitotic, compact zone and an inner trabecular zone with lower mitotic activity. Both microsurgical and genetic inhibition of epicardium formation gives rise to a decrease in myocyte proliferation, accounting for a thin-wall compact myocardium, (Manner et al., 1993; Moore et al., 1999; Yang et al., 2005). Genetic data in the mouse identified a non-cell autonomous role for epicardium-derived retinoic acid and erythropoietin (EPO) signalling, which are both involved in compact layer expansion (Sucov et al., 1994; Kastner et al., 1994; Wu et al., 1999). Similar conclusions have been obtained for the chick heart (Stuckmann et al., 2003). Recent data in mice have further modified this model of growth control of compact layer expansion. Retinoic acid signalling is not actually active in the epicardium but in the liver stimulating in this tissue the secretion of EPO, which binds to the epicardial EPO receptor,

resulting in the secretion of *Ifg2*, which acts as a ligand involved in compact layer expansion (Li et al., 2011; Brade et al., 2011). In addition, fibroblasts growth factor (*Fgf*) receptor mediated signalling is important or myocyte proliferation in the developing heart (Lavine et al., 2005, Lavine et al., 2006; Mima et al., 1995; Mikawa et al., 1995), whereby retinoic acid signalling induces epicardial Fgf secretion (Merki et al., 2005; Chen et al., 2002).

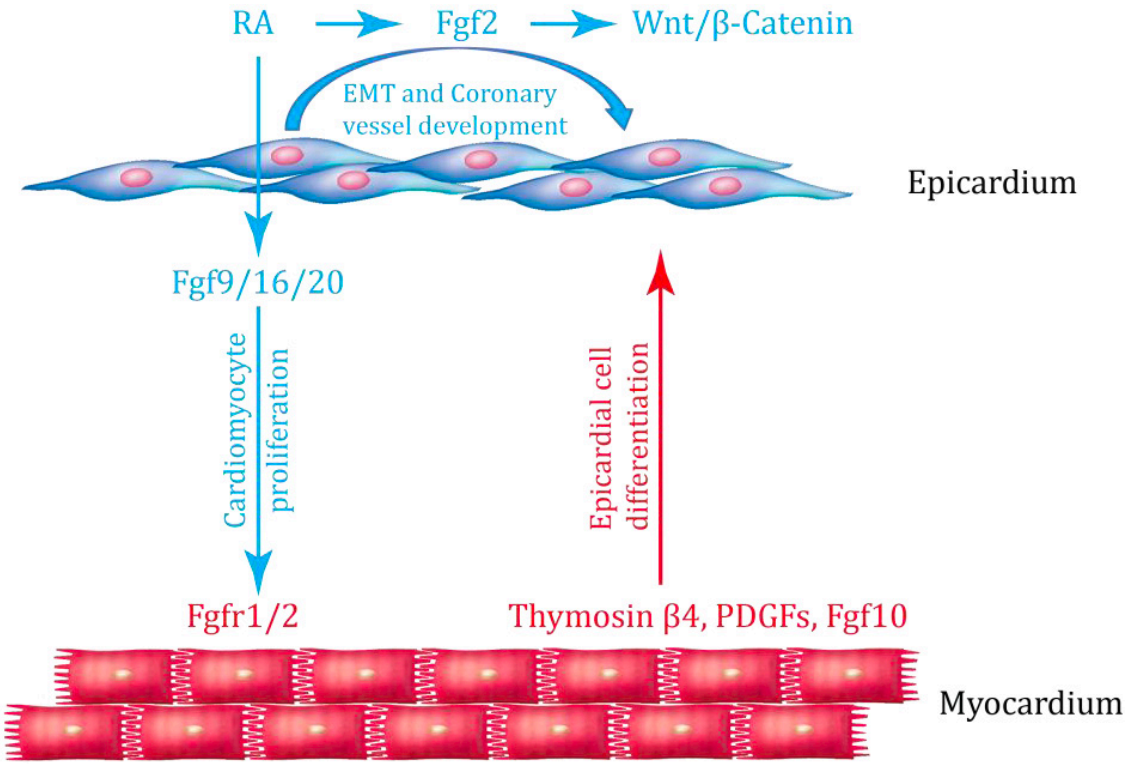

Figure 13. Reciprocal interactions between epicardium and myocardium during cardiac development. Retinoic acid (RA) modulates epicardial EMT and coronary vascular development by regulating *Fgf2* and *Wnt/β-catenin* signalling. RA also activates *Fgf9* expression in the epicardium which signals via receptors *Fgfr1* to regulate myocardial proliferation and differentiation. Signals from the myocardium, such as *Thymosin β4*, platelet-derived growth factors (PDGFs) and fibroblast growth factors (*Fgfs*), regulate epicardial cell differentiation. (Image from Singh and Epstein, 2013)

4.2.7. Role of Epicardium in the Injured and Regenerating Heart

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Within the adult heart, the epicardium represents, the outermost layer, i.e. a simple epithelial monolayer. For many years, the functional role of this monolayer has been neglected as it was considered as an external cover devoid of any functional meaning. The discovery that epicardial precursors can differentiate into beating cardiomyocytes has branded the epicardium as a source of cardiac stem cells with therapeutic potential (Wessel & Perez-Pomares., 2004; Perez-Pomares et al., 2006; Winter et al., 2009). In addition, it has been reported that the adult epicardium plays a pivotal role in cardiac

regeneration (Bollini et al., 2011; 2015; Schlueter & Brand, 2012; Masters and Riley, 2014; Kennedy-Lydon and Rosenthal, 2015).

Pioneer work by Kruithoff et al. (2006) described that the embryonic chicken PE if isolated and placed in appropriate cell culture conditions, was capable of giving rise to beating cardiomyocytes. Such in vitro conditions could be further enhanced by *Bmp* and blocked by *Fgf* administration. Thus, these data opened up the possibility that the epicardium could serve as an in vivo source of potential cardiomyocytes if the appropriate signals would be instructed. Importantly, Smart et al., (2011) demonstrated that adult epicardial derived cells, if previously primed with *thymosin β4*, eventually generated functionally beating cardiomyocytes in an ischemic heart, yet the proportion of de novo integrated cells was rather spurious and the instructive mechanisms remains rather obscure (Gaizer et al., 2013). Nevertheless, as a proof of principle approach it highly valuable. This work introduced a novel concept of an activated epicardium, a condition by which embryonic epicardial markers such as *Wt1* and *Tbx18* are re-expressed in the adult epicardium (Huang et al., 2012; van Wiik et al., 2012; Braitsch et al., 2013; Bollini et al., 2014; Aguiar and Brunt, 2015) in response to distinct biological stimuli such as thymosin β4 (Smart et al., 2012; Smart and Riley, 2012) stem cell factor (SCF) (Xiang et al., 2014) and prokineticins (Urayama et al., 2008) among others. In addition, this activated epicardium secretes paracrine factors that modulate myocardial injury response (Zhou et al., 2011; Foglio et al., 2015).

While it is documented that the human heart has a limited capacity to regenerate (Bergmann et al., 2009) it is also highly acknowledged that the new heart can also widely regenerate by other means (Becker et al., 1974). Furthermore, the adult zebrafish can also regenerate (Poss et al., 2002) and the epicardium provides a pivotal role during this regeneration process (Gemberling et al., 2015; Wang et al., 2015). Molecular analyses have demonstrated that the epicardium becomes activated as soon as the heart is injured and such activation provides instructive signals that promote cardiomyocyte proliferation, revascularization and tissue repair (Lien et al., 2006, 2012; Marin-Huez et al., 2016). During this process a transitory scar stage occurs and is subsequently replaced by fully functional and integrated cardiomyocytes (Gonzalez-Rosa et al., 2011; Mercer et al., 2013; Itou et al., 2014; Marro et al., 2016).

Further analyses in adult zebrafish identified that *Wnt/β-catenin* is crucial promoting formation of cardiac fibroblasts and hence cardiac repair (Duan et al., 2012). Several studies have identified key molecules modulating this regeneration capacity. For example, *Nrg1*, acts as a mitogenic agent in cardiomyocytes following injury during cardiac zebrafish regeneration (Gemnberling et al., 2015). *Notch* (Zhao et al., 2014), *Raldh2* (Itou et al., 2014) and myocardial *NF-kB* (Karra et al., 2015) are also essential for heart regeneration in zebrafish. Hydrogen peroxide (Han et al., 2014) has been reported to prime heart regeneration and telomerase has been identified as instrumental for zebrafish regeneration (Bernarek et al., 2015) but still it remains to be stablished if these factors are modulated by the epicardium. More recently, it has been demonstrated that epicardial

regeneration is guided by the cardiac outflow tract and hedgehog signalling (Wang et al., 2015) and single cell transcriptome of the epicardium has identified *caveolin-1* as essential factor in regenerating zebrafish heart (Cao and Poss, 2016). Moreover, re-expression of epicardial developmental genes and enhanced EMT in response to injury has been widely demonstrated (Lepilina et al., 2006; Kim et al., 2010; Gonzalez-Rosa et al., 2011; Schnabel et al., 2011). These data suggest that complex regulatory networks control zebrafish regeneration (Rodius et al., 2016) positioning the epicardium as a key tissue layer for regeneration Thus, these data will be highly instrumental to search or novel ways to heal the injured heart.

In adult mice, the regenerative capacity has been lost and the injured heart responds by generating a fibrous scar which is derived from pre-existing epicardial cells (Zhou et al., 2008; Duan et al., 2012) as well as de novo recruited bone marrow-borne circulating cells (Ruiz-Villalba et al., 2015). Interestingly, full regeneration is achieved at early developmental stages, i.e., on the first week of life, in which the epicardium is also a highly instructive player (Porrelo et al., 2011) and *thymosin-β4* priming increases the time window for mammalian heart regeneration (Rui et al., 2014). In addition, a role for *Wnt* signalling has also been identified in the regenerating heart in mice (Mizutani et al., 2016).

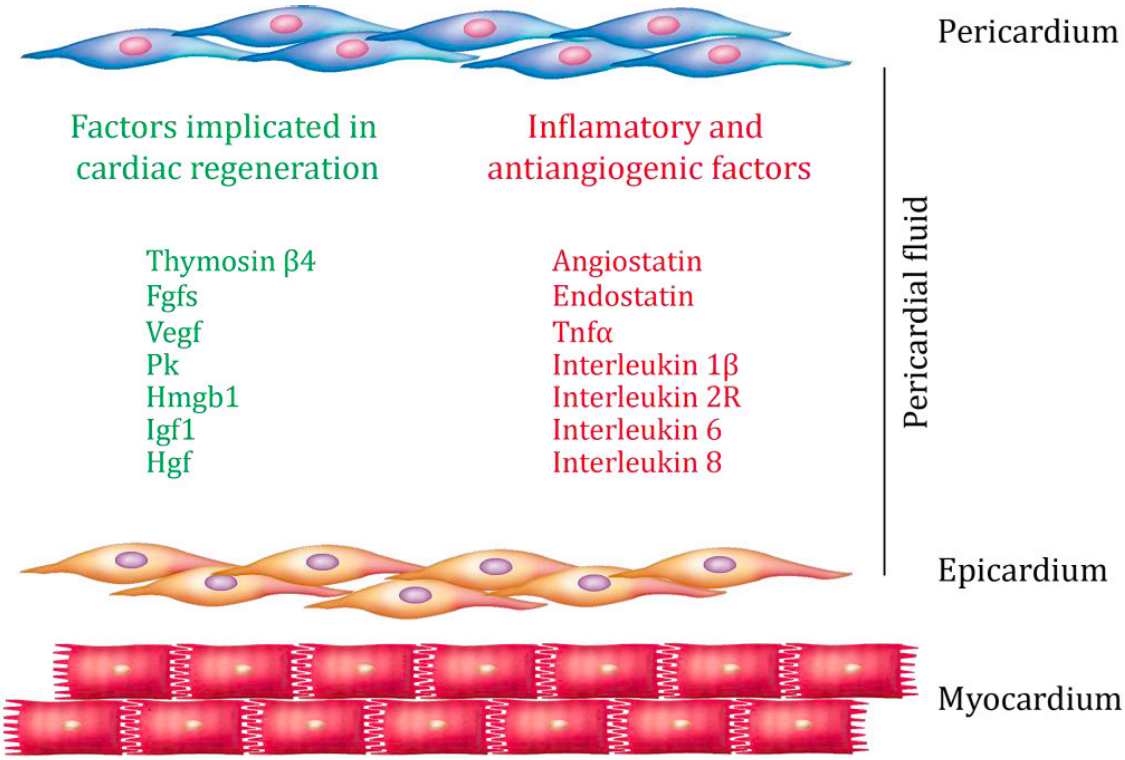

Figure 14. Regenerative and inflammatory response after myocardial injury. Factors accumulate in the pericardial fluid after myocardial infarction that may affect both epicardial and myocardial function and which are implicated in cardiac regeneration and inflammation. Image from Singh and Epstein, 2013).

4.2.8. Molecular Mechanisms Controlling Epicardial EMT

EMT is a fundamental process during which polarized epithelial cells convert into motile mesenchymal cells. This reversible process involves profound changes in cell morphology and behaviour, enabling cells to move into the interior of the embryo, travel long distances and participate in the formation of internal organs (Huang & Nieto, 2009; Nieto, 2013). The activation of this trans-differentiation program depends on contextual microenvironmental signals and is orchestrated by a network of EMT-inducing transcription factors that interact with epigenetic regulators to control the expression of proteins involved in cell polarity, cell-cell contact, cytoskeleton structures and extracellular matrix degradation, including the repression of key epithelial genes (Tam & Weinberg, 2013). The loss of *E-cadherin* expression is considered a crucial event in EMT, and EMT-TFs can be classified based on their ability to repress *E-cadherin* directly or indirectly. Direct repressors include zinc finger proteins of the *Snail* superfamily, such as *Snai1, Snai2 Snai3*; zinc finger and *E-box* binding proteins of the *Zeb* family, such as *Zeb1* (*Tcf8*) and *Zeb2* (*Sip1*); the bHLH factor E47; and the Kruppel-like factor *Klf8*. Factors such as the *Twist* bHLH proteins (*Twist1* and *Twist2*), the homeobox proteins goosecoid (*Gsc*) and *Six1*, the *bHLH* factor *E2.2* and the forkhead box protein *FoxC2* repress *E-cadherin* transcription indirectly (Thiery, Acloquem Huang & Nieto, 2009).

The first steps of epithelial-mesenchymal transition (EMT) are the disassembly of epithelial cell-cell contacts, that is tight junctions, adherens junctions, desmosomes and gap junctions, and the loss of cell polarity through the disruption of the *Crumbs*, partitioning defective (PAR) and *Scribble* (SCRIB) polarity complexes. The expression of epithelial genes is repressed, concomitantly with the activation of mesenchymal gene expression (Thiery & Sleeman, 2006; Yilmaz & Christofori, 2009, Yilmaz & Christofori, 2010). Next, the epithelial actin architecture reorganizes, and cells acquire motility and invasive capacities by forming lamellipodia and filopodia, and by expressing matrix metalloproteinases (MMPs) that can degrade extracellular matrix (ECM) proteins. The process of mesenchymal-epithelial transition (MET) enables the cells that have undergone EMT to revert its phenotype to the epithelial state (Kalluri & Weinberg, 2009; Lim & Thiery, 2012; Zeisberg & Kalluri, 2013).

Three types of EMT can be discerned depending on the physiological tissue context (Kalluri & Weinberg, 2009). Type 1 EMT occurs in embryogenesis and organ development (Lim & Thiery, 2012), type 2 EMT is important for tissue regeneration and organ fibrosis (Kalluri & Weinberg, 2009; Lim & Thiery, 2012) and type 3 EMT is associated with cancer progression and cancer stem cell properties (Scheel & Weinberg, 2012). MET also contributes to development and generation of metastatic carcinomas (Brabletz, Jung, Spaderna, Hlubek & Kirchner, 2005; Hanahan & Weinberg, 2011). Epicardial EMT belongs to the type 1 EMT.

Many cells undergoing EMT also express α-smooth muscle actin, a molecule associated with the onset of smooth muscle differentiation. However, embryonic α-smooth muscle actin expression is also strongly related to activation of a migratory program in mesenchymal cells, a feature of cells undergoing EMT (Luna-Zurita et al., 2010; Syagner, 2001) and is considered a hallmark of the myofibroblastic cell type in adults (Masszi and Kapus, 2010).

Various myocardial signals promote epicardial EMT. Transforming growth factor-β was originally described as negative regulator (Morabito et al., 2001) but more recent studies indicate this growth factor promotes EMT (Compton et al., 2007). The role of *Fgfs* in epicardial EMT (Morabito et al., 2001) is difficult to distinguish from the other functions of these potent mitogens (Yun et al., 2010) and *Fgfs* are also suggested to be required for EPDC migration into the myocardium (Pennisi and Mikawa, 2009). Other growth factors involved in epicardial biology, such as *Vegf* and platelet-derived growth factor (*Pdgf)*- βB have not been demonstrated to directly affect epicardial EMT but instead can control cell fate decisions taken by EPDC once EMT is completed (Smith et al., 2011).

A key regulator of gene expression changes during epicardial EMT is *Wt1*. This transcription factor is expressed in many cells of the early PE and epicardium and EPDC (Moore et al., 1999). Epicardial-specific *Wt1* binds and transactivates the *Snai1* promoter, thus reducing E-cadherin levels (Martinez-Estrada et al., 2010). In addition, *β-catenin* is also required for epicardial EMT (Zamora et al., 2007).

4.2.9. Molecular Mechanisms Controlling EPDC Differentiation

Initial formation of the epicardium, epicardial EMT and EPDC lineage determination are regulated by a complex network of transcription factors, including the zinc finger transcription factors *Wt1, Snai1* and *Snai2* as well as the bHLH transcription factors *Tcf21, Scleraxis, Twist1* and *Hand2* **(Figure 15)** (Braistch & Yutzey, 2013). Additional factors including *Tbx18, Nfact1, Sox9* and C/EBP, regulate aspects of EPDC lineage development. Signalling pathways such as Retinoic Acid signalling pathway and transcription factors together regulate EPDC behaviour and differentiation into cardiac fibroblasts and vascular SMCs.

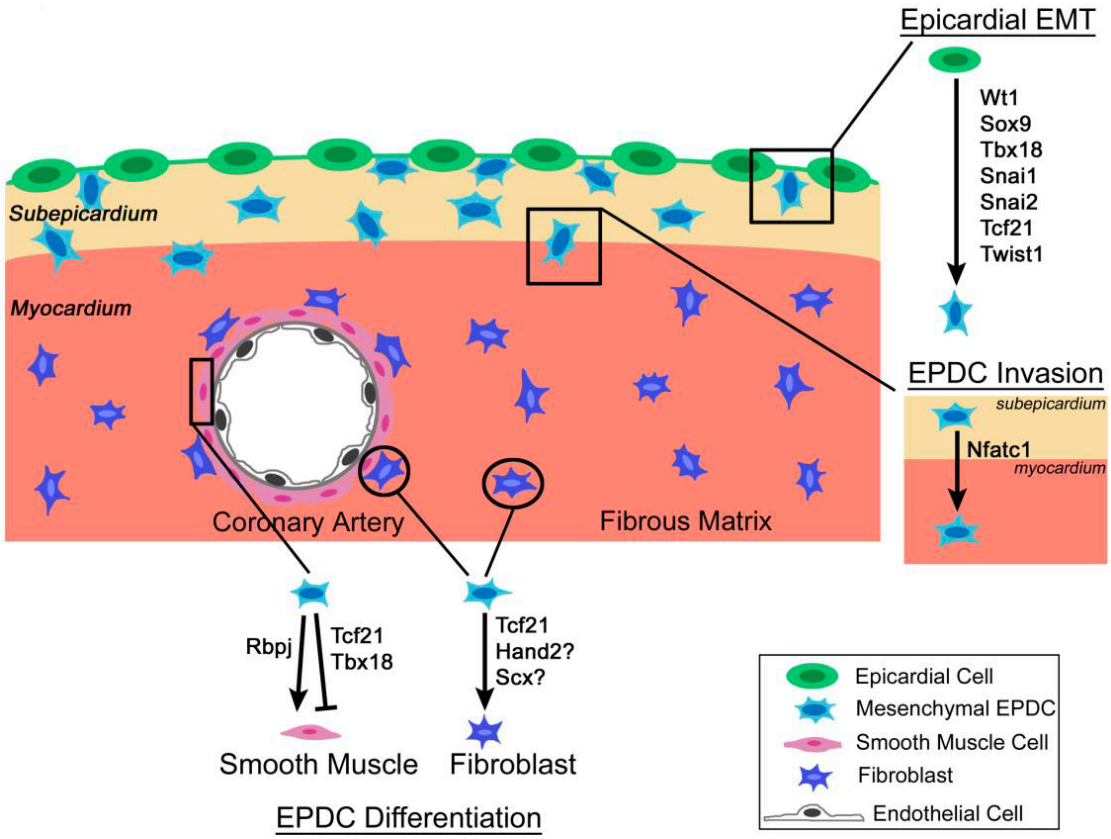

Figure 15. Depiction of the transcription factor regulation of epicardial cells during embryonic heart development. Transcription factors are expressed during epicardial EMT, EPDC lineage specification and EPDC differentiation into vascular smooth muscle cells and cardiac fibroblasts (Image from Braitsch and Yutzey, 2013).

4.2.10. The Role of Retinoic Acid Signalling in EPDCs differentiation

The epicardial epithelium in the chick embryo expresses the RA-synthesizing enzyme Raldh2 as well as the sub-epicardial mesenchymal EPDCs (Perez-Pomares et al., 2002). However, as soon as they migrate into the myocardium, a process that starts around HH26, RALDH2 expression is reduced significantly. Thus, the EPDCs found in the AV endocardial cushions, many of which express *caldesmon*, do not express *Raldh2*. In addition, the terminally differentiated coronary endothelium of EPDC origin (sub-epicardial as well as intramyocardial) which is characterized by the expression of the endothelial specific marker QH1 (in quail), generally does not express *Raldh2* (Perez-Pomares et al., 2002). Therefore, the loss of expression of *Raldh2* is related to the organization of EPDCs in recognizable structures and the upregulation of molecular markers of cell lineage-specific differentiation. When epicardial development is delayed, a decrease in the overall number of EPDCs and hence also the number of *Raldh2* positive cells is observed (Perez-Pomares et al., 2002). This reduction in the number of cells correlates with malformations of the structures in which they are normally found. These malformations include the "thin myocardial syndrome", abnormal coronary vessels, and dysmorphic AV cushions. This spectrum of malformations closely resembles that seen in

mice deficient for genes involved in epicardial development (Dyson et al., 1995; Kwee et al., 1995; Yang et al., 1995; Moore et al., 1999; Wu et al., 1999; Tevosian et al., 2000).

Targeted inactivation of *Raldh2* in mice causes embryonic death, embryos showing interrupted heart development (Niderreither et al., 1999). RA is identified as an epicardial factor required for CM proliferation (Stuckman et al., 2003). Inhibition of RA signalling in cultured heart slices or removal of the epicardium from these slices reduces CM proliferation. RA does not activate proliferation directly, but rather induces the production of a proliferative signal in epicardial cells (Stuckmann et al., 2003). Deletion of the nuclear *Rxra* receptor gene in mice results in myocardial hypoplasia and death (Sucov et al., 1994) and conditional *Rxra* inactivation in epicardial-derived cells using Gata-5Cre driver causes a partially penetrant lethal phenotype characterized by a detached epicardium and a thin sub-epicardial mesenchyme layer secondary to reduce *Fgf2* production in the epicardium. Formation of the coronary arteries is impaired in this model, implying defective angiogenesis and ventricular cells maintain expression of early cardiac differentiation markers. This suggests that the epicardium not only supports CM proliferation but also instructs myocardial cell differentiation (Merki et al., 2005). RA signalling via *Rxra* thus sustains heart development by stimulating the epicardium to produce signals that promote CM proliferation.

The Role of Fibroblast Growth Factor Signalling in EPDC differentiation

Various *Fgf* family members are expressed in the epicardium including *Fgf1*, *Fgf2, Fgf4, Fgf16* and *Fgf20* (Morabito et al., 2001; Lavine et al., 2005; Pennisi et al., 2003; Sugi et al., 2003). In mice *Fgf9*, *Fgf1*6 and *Fgf2*0 are expressed in epicardium in partially overlapping patterns starting at E10 (Laine et al., 2005). *Fgf9* mutant mice die at birth and show a hypoplastic left ventricle associated with weak CM proliferation (Lavine et al., 2006). Interestingly, *Fgf9* is induced by RA in primary epicardial cell cultures, linking epicardial RA signalling and myocardial proliferation. Embryonic stem cells (EC) express *Fgfr1* and *Fgfr2*, suggesting that they are able to process *Fgf* signals (Lavine et al., 2005). These data suggest that *Fgfs* are part of the epicardium-to-myocardial signalling that regulates myocardial proliferation and, indirectly, coronary vasculogenesis (Lavine et al., 2005). A report shows that *Fgf/Fgfr2b* signalling from myocardium to epicardium is crucial for EPDC migration into compact myocardium and also for myocardial growth (Vega-Hernandez et al., 2011) indicating that the myocardium also influences the epicardium. In this regard coordination of myocardial and epicardial activities by *Fgf* signalling has been proposed to vascularize new CM during heart regeneration in zebrafish (Lepilina et al., 2006).

4.2.11. The Role of *Hedgehog* Signalling in EPDC differentiation

Sonic hedgehog (*Shh*) is a *Fgf* target during coronary vasculogenesis (Lavine et al., 2006). At E12.5, Shh is expressed in epicardial cells of the AV groove and the base of the heart, later spreading to the rest of the epicardium (Lavine et al., 2006). Its receptor, Patch (Ptc), is expressed in the AV groove and CM at the base of the heart, suggesting that the myocardium responds to epicardial-derived Shh signals. Epicardial Shh expression is delayed in conditional knockouts of *Fgf9* and *Fgfr1/2* and the associated delay in coronary plexus formation is rescued *ex vivo* by exogenous *Shh* (Lavine et al., 2006). Furthermore, inhibition of *Hh* signalling blocks coronary plexus formation and conditional inactivation of the *Hh* downstream effector Smoothened also leads to defective coronary plexus formation at E13.5 (Lavine et al., 2008). Together, these data suggest that epicardial *Fgf* activate myocardial *Fgfr*, which by a still unknown mechanism activate Shh expression in the epicardium.

4.2.12. The Role of *Notch* Signalling in EPDC differentiation

Notch pathway is key to the development of mouse epicardium and coronary vessels (Grieskamp et al., 2011; del Monte et al., 2011). Various conditional mutants combine with epicardial-specific drivers have been used to ascertain the function of *Notch* in the epicardium and its derivatives. Grieskamp et al. (2011) demonstrated that the differentiation potential of mutant EPDC is compromised and that Notch signalling is required for differentiation of perivascular cells into smooth muscle cells.

4.3. Transcriptional and Post-transcriptional regulation of Cardiac Development

The combination of complex morphogenetic events necessary for cardiogenesis and its superimposed hemodynamic influences may contribute to the sensitivity of the hearts to perturbations (Srivastava, 2003). These processes are controlled by transcriptional factors and act as regulators having an impact in the morphogenesis of the developing heart (Bruneau, 2002). Transcriptional factors and growth factors such as *Notch, Wt1, Hedgehog, Fgf, Bmp,* among others have been already described on previous chapters of this Thesis project.

During the past several decades it has become increasingly clear that transcriptional and post-transcriptional processes play a major role in specifying the proteome of eukaryotic cells and tissues. Regulation can occur at different phases in the life history of an mRNA: splicing, polyadenylation, capping nuclear export subcellular transport/localization, translation and stability. All of these processes are directed by RNA-binding proteins (RBPs) and non-coding RNAs (ncRNAs) (Lipshitz, Claycomb & Smibert, 2017). We provide herein a state-of-the art overview of the importance of post-transcriptional regulation by ncRNAs of cardiac development.

4.3.1. Post-transcriptional Regulation of Cardiac Development

Expression of a gene can be controlled at many levels, including transcription, mRNA splicing, mRNA stability, translation and post-transcriptional events such as protein stability and modification (Day and Tuite, 1998). The underlying theme is that post-transcriptional gene regulation relies on specific RNA-protein and /or RNA-RNA interactions that either result in the targeted degradation of mRNA or prevent access of the ribosome to the translation start codon (Day and Tuite, 1998).

Non-coding RNAs are important players of post-transcriptional regulation. The earliest ncRNAs identified were ribosomal RNAs. More recently discovered ncRNAs include small nuclear RNAs, (siRNAs); microRNAs; PIWI-associated RNAs (Aravin et al., 2006) and long non-coding RNAs (lncRNAs) which include competing endogenous RNAs (ceRNAs) (Tray et al., 2011), circular RNAs (circRNAs) (Zarphiropoulos, 1997), and transcribed pseudogenes (Poliseno et al., 2010). From all these ncRNAs mentioned above, microRNAs have been extensively studied during the last two decades and have a very important role on cardiac development and disease. On the other hand, lncRNAs are a relative new subject of study, with promising possibilities on the regulation of key processes on heart development and disease.

4.3.2. The Role of microRNAs in cardiac development

MicroRNAs are small ≅22 nucleotide (nt) single-stranded, endogenously encoded, ncRNAs that serve a critical role in post-transcriptional regulation of protein expression, and thus contribute to a wide range of biological processes and in the development of disease. This regulation is so important to normal physiology that more than 60% of human protein-coding genes are under selective evolutionary pressure to maintain microRNA binding sites, also called microRNA response elements (MREs), in their 3' untranslated regions (3'UTR) (Friedman et al., 2009). microiRNAs are encoded, transcribed and loaded into ribonucleoprotein-silencing machinery. Serving as the target recognition component of the ribonucleoprotein-silencing machinery, microRNAs identify specific transcripts, in a sequence-specific manner, for translational repression and transcript destabilization. Thousands of putative miRNAs have been identified in the human genome, with hundreds having been experimentally validated to effect known targets (Yan and Jiao, 2016).

MicroRNAs are encoded in the metazoan genome in a variety of ways (Berezikow et al., 2007; Cai et al., 2004; Rodriguez et al., 2004; Weber et al., 2005). microRNAs that are processed from retained portions of their host transcript are processed as pri-microRNAs (Lee et al., 2002). Pri-miRNAs are processed to precursor miRNAs (pre-miRNAs), which are 70nt hairpin loop structures. The step from pri-miRNA to the 70nt pre-microRNA defines one end of the mature microRNA (Lee et al., 2003). Pre-

microRNAs, processed from pri-miRNAs are exported from the nucleus by the Exportin-5-Ran-GTP shuttle (Bohnsack et al., 2004; Lund et al., 2004; Yi et al., 2003). Exportation is also an important checkpoint of pre-microRNA processing, where those pre-microRNAs with stem loops of sufficient length and with 3'-overhangs are recognized by the enzyme and exported from the nucleus to cytoplasmic processing (Lung and Dhalberg., 2006; Zeng and Cullen., 2004).

Pre-miRNAs are further processed in the cytoplasm, where they are cleaved to their final 22nt form of mature miRNA by *Dicer1,* a class 3 *Ribonuclease III* (Grishok et al., 2001; Hutvagner et al., 2001; Ketting et al., 2001; Lee et al., 2003). Mature microRNAs are loaded into the RISC complex which can thereafter search for mRNA transcript base complementarity (Hammond, 2015; Shen and Hung, 2015; Dueñas, Aranega and Franco, 2017).

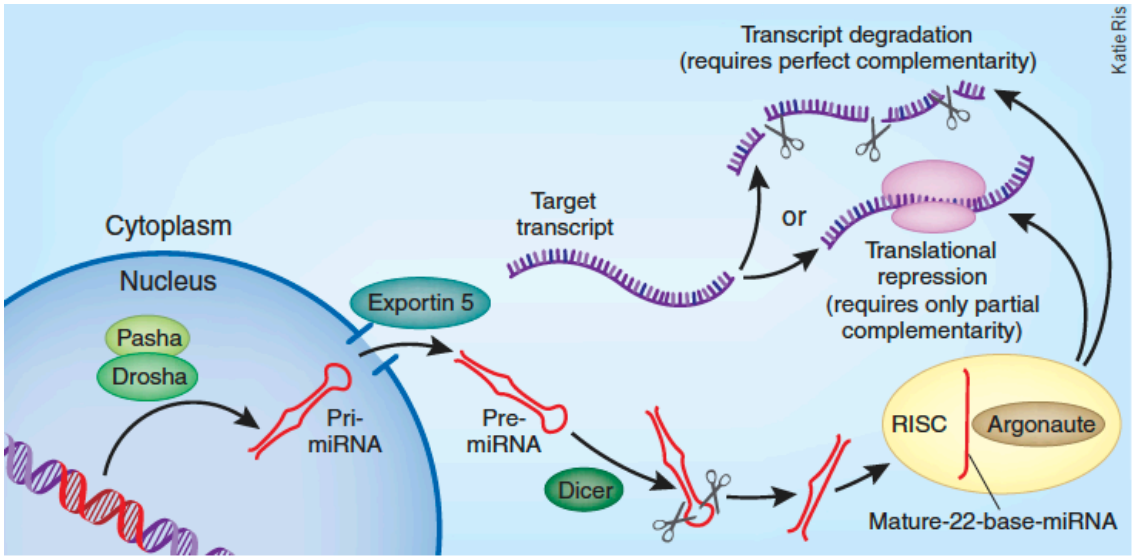

Figure 16. microRNA biogenesis. A primary microRNA (pri-miRNA) transcript is encoded in the cell's DNA and transcribed in the nucleus, processed by and exported into the cytoplasm where it is further processed by Dicer. After strand separation, the mature microRNA represses protein production either by blocking translation or causing transcript degradation. (Image from Mack, 2007)

The importance of microRNAs in heart biology was revealed by blocking the expression of all the microRNAs in the cardiovascular system (Hata et al., 2010). This effect was obtained through tissue-specific deletion of genes essential to microRNA biogenesis, such as *Drosha Dgcr8, Ago2* or *Dicer*. Mutant mice resulted in death during early gestation due to severe developmental defects of the heart and blood vessels (Hata et al., 2010). To date, the functional role of microRNA in cardiac development is limited to a small fraction of all microRNAs expressed during cardiac morphogenesis. Many microRNAs have been shown to be critical for cardiomyocyte development. *miR-1* negatively regulates cardiac growth and differentiation by inhibiting the translation of the transcription factor HAND-2 (Zhao et al., 2005), critical for ventricular cardiomyocyte expansion (Zhao et al., 2005; McFadden et al., 2005; Srivastava et al., 2001; Yamagishi

et al., 2005). Similarly, *miR-133* negatively regulates cardiomyocyte proliferation by inhibiting its targets *Cyclin-D2* and Serum Response Factor (SRF) (Liu et al., 2008), but when overexpressed in embryonic cardiomyocytes causes embryonic lethality due to reduced cardiomyocyte proliferation (Liu et al., 2008). In addition to the two most abundantly expressed microRNAs in the heart, multiple other microRNAs have been reported to regulate cardiomyocyte proliferation and apoptosis. These include the *miR-17-92* cluster, the *miR-15* family and the *miR-590, miR-199a, miR-320* and *miR-98/-128/-142* (Liu et al., 2010; Malizia et al., 2011; Peng et al., 2014).

4.3.3. The Role of microRNAs in Epicardium Development

The roles of microRNAs during epicardial development have been scarcely studied. Mice with an epicardial-specific deletion of *Dicer1* die immediately after birth, exhibiting coronary vasculature defects due to an impaired EMT, and displaying reduced epicardial cell proliferation and differentiation (Signh et al., 2011; Bronnum et al., 2013). Specifically, *miR-21* positively modulates fibrogenic EMT in epicardial mesothelial cells (EMCs) by targeting *Programmed Cell Death 4* (*Pdcd4*) and *Sprouty Homolog 1* (*Spry1*) (Bronnum et al., 2013). The overexpression of *miR-21* in non-stimulated EMCs leads to a reduction in the expression of the mesenchymal biomarker *E-cadherin*, while the knockdown of endogenous *miR-21* during *Tgf-β* induced EMT promotes the expression of *E-cadherin* (Bronnum et al., 2013). In contrast to *miR-21, miR-31* negatively regulates fibrogenic EMT in EMCs by directly repressing the cardiac progenitor gene *Isl1*. In non-stimulated EMCs, *Isl1* promotes mesenchymal features. In *Tgf-β* induced EMCs, *miR-31* reduces *Isl1* and thereby inhibits the EMT (Bropnnum et al., 2013). In summary, the regulation of epicardial gene expression by microRNAs plays indispensable roles in cardiogenesis (Ucar et al., 2012; Thum et al., 2011).

4.3.4. The Role of lncRNAs in Cardiac Development

Advances in genomics technology over recent years have led to the surprising discovery that the genome is far more pervasively transcribed than was previously appreciated. Much of the newly discovered transcriptome appears to represent long non-coding RNA (lncRNA), a heterogeneous group of largely uncharacterized transcripts (Kapranov et al., 2001; Hoon et al., 2015). LncRNAs share many features with coding transcripts, such as the presence of epigenetic marks indicating differential expression (Guttman et al., 2009), the presence of introns and the existence of splice variants. Many, but not all, lncRNAs are polyadenylated, and there is evidence indicating that many lncRNAs exist in both polyadenylated and non-polyadenylated forms (Hanguaer et al., 2013). LncRNAs can overlap coding genes, and indeed, it is estimated that 20% of human transcripts display sense-antisense pairs (Chen et al., 2004). These transcripts may overlap the entire gene

or only a part of it, and non-coding transcripts may originate from either the sense or antisense strands (Pandey et al., 2008; Pastori et al., 2014).

While the vast majority of lncRNA do not yet have any known function, there is an increasing understanding of the functions of a small number of characterized lncRNAs. LncRNAs can act at many different levels of gene expression and their functions are highly diverse. This diversity reflects the versatility of RNA itself by folding into a variety of secondary structures that can bind to a large number of substrates in a highly specific manner (Geisler et al., 2013). In addition, without the need for translation, ncRNA expressions is highly dynamic and can be rapidly up-or-down-regulated to modulate gene expression (Geisler et al., 2013).

While many imprinted lncRNAs appear to act in cis, regulating nearby loci, there is also evidence that lncRNAs can act in trans. Details on the precise mechanisms underlying these activities are unclear. It seems that the lncRNA must serve two functions: it must bind to a protein or protein complex or at least facilitate the formation of a complex, perhaps acting as a scaffold (Tsai et al., 2010); and the other is, the lncRNA must be able to target this complex to a specific DNA sequence. Thus, the function of the lncRNA is to provide specificity to the chromatin modifying enzymes, acting as a tethered scaffold.

The involvement of lncRNAs regulation in development has been recently investigated. Hundreds of lncRNAs have been found to be cardiac-specific during development or in pathological conditions (Ounzain et al., 2014; Ounzain et al., 2015), although their function role have only been studied by a handful of them. The first lncRNA identified as a key regulator in cardiac development in the mouse embryo was *Braveheart* (*Bvht*) (Klattenhoff et al., 2013). Its expression has been detected in cardiac mesoderm and cardiomyocytes, playing an essential role on the transition from nascent to cardiac mesoderm and subsequently in cardiomyocyte differentiation, Indeed, during embryonic stem cell differentiation the depletion of *Bvht*, results in loss of beating cardiomyocytes and deactivation of genes specifying key cardiac transcription factors.

Another identified lncRNA that acts as a critical regulator in cardiac development is *Fendrr* (*Fox adjacent non-coding developmental regulatory RNA*) (Grote et al., 2013). It is exclusively expressed in the lateral plate mesoderm and regulates its differentiation into the heart and ventral body wall. Disruption of the *Fendrr* transcript results in embryonic lethality, partially due to heart failure (Grote et al., 2013; Sauvageau et al., 2013). Similar to *Bvht, Fendrr* functions as a chromatin modulator interacting with the repressing complex PCR2 with the difference that they likely counteract each other in order to regulate the PCR2 binding on these cardiac lineage-determining gene promoters.

Kurvan et al., (2015) identified two nobel human lncRNAs that are also necessary in cardiac development and are partially. Conserved across vertebrates. TERMINATOR was found crucial for pluripotency and early mesendodermal differentiation and survival,

while *Alien* was found crucial for the correct mesodermal specification and cardiac chamber formation.

To date, the regulation of epicardium development by lncRNAs is largely unknown as well as expression and characterization data. A better understanding of how lncRNAs act during epicardium development would advance greatly the knowledge regarding the molecular mechanisms of cardiogenesis and disease (Ucar et al., 2012; Thum et al., 2001).

5. GENERAL AND SPECIFIC OBJECTIVES

5. General and Specific Objectives

General Objective

The present doctoral thesis ***general objective*** is to analyse the role of microRNAs in the molecular processes involved during the transition from proepicardium to embryonic epicardium and their contribution to the different cell lineages derived from them.

Specific Objectives

The present doctoral thesis ***specific objectives*** are:

Analyse the microRNAs different expression profiles in the chicken proepicardium as well as the chicken embryonic epicardium development stages.

Analyse the functional contribution of different microRNAs to the modulation of cell differentiation in the proepicardium and embryonic epicardium different cell lineages of chicken embryos, particularly in cardiomyogenic and fibrogenic cell lineages as well as epithelial-mesenchymal transition.

Identification of microRNAs that favour cardiomyogenic differentiation and the study of the underlying molecular pathways.

Analysis through massive sequencing techniques the expression profiles of mRNAs, microRNAs and lncRNAs in mouse proepicardial to epicardial transition.

Find a genetic interaction network of the proepicardial to embryonic epicardial transition in the mouse embryo.

6. RESULTS

6.1. CHAPTER 1

MiR-195 enhances cardiomyogenic differentiation of the proepicardium/septum transversum by *Smurf1* and *Foxp1* modulation

6.1. Chapter 1

MiR-195 enhances cardiomyogenic differentiation of the proepicardium/septum transversum by Smurf1 and Foxp1 modulation.

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Abstract

Cardiovascular development is a complex developmental process in which multiple cell lineages are involved, namely the deployment of first and second heart fields. Beside the contribution of these cardiogenic fields, extracardiac inputs to the developing heart are provided by the migrating cardiac neural crest cells and the proepicardial derived cells. The proepicardium (PE) is a transitory cauliflower-like structure located between the cardiac and hepatic primordia. The PE is constituted by an internal mesenchymal component surrounded by an external epithelial lining. With development, cells derived from the proepicardium migrate to the neighboring embryonic heart and progressive cover the most external surface, leading to the formation of the embryonic epicardium. Experimental evidence in chicken have nicely demonstrated that epicardial derived cells can distinctly contribute to fibroblasts, endothelial and smooth muscle cells. Surprisingly, isolation of the developing PE anlage and *ex vivo* culturing spontaneously lead to differentiation into beating cardiomyocytes, a process that is enhanced by Bmp but halted by *Fgf* administration. In this study we provide a comprehensive characterization of the developmental expression profile of multiple microRNAs during epicardial development in chicken. Subsequently, we identified that *miR-125, miR-146, miR-195* and *miR-223* selectively enhance cardiomyogenesis both in the PE/ST explants as well as in the embryonic epicardium, a *Smurf1* and *Foxp1*-driven process. In addition, we identified three novel long non-coding RNAs with enhanced expression in the PE/ST, that are complementary regulated by *Bmp* and *Fgf* administration and well as by microRNAs that selectively promote cardiomyogenesis, supporting a pivotal role of these long non coding RNAs in microRNA-mediated cardiomyogenesis of the PE/ST cells.

Introduction

Cardiovascular development is a complex developmental process in which multiple cell lineages are involved (Christoffels & Moorman, 2003). Soon after gastrulation, bilateral sets of procardiogenic cells align into the embryonic midline configuring a linear cardiac straight tube (Lopez-Sanchez & Garcia-Martinez, 2011). These cellular populations constitute the first heart field and will essentially contribute to the future left ventricle (Vincent & Buckingham et al., 2010; Kelly et al., 2014). Additional cardiogenic progenitor cells emanate from the medial structures in the gastrulating embryo configuring the second heart field and contributing through both cardiac poles to the addition of the right ventricle and outflow at the arterial pole, and the atrioventricular canal and right and left atrial appendages at the venous poles (Kelly et al., 2001; Waldo et al. 2001; Kelly et al., 2014). Beside these cardiogenic fields, extracardiac contribution to the developing heart are provided by the migrating cardiac neural crest cells and the proepicardial derived cells (Hutson & Kirby, 2003; Waldo et al., 2005; Kirby & Hutson, 2010; Perez-Pomares et al., 1998; Carmona et al., 2010/; Winter & Gittenberger-de Groot, 2007; Gittenberger-de Groot et al., 2010). Neural crest cells transiently contribute to the septation of the outflow tract at the arterial pole while at the venous pole they constitutively contribute to the cardiac ganglia (Kirby & Stewart, 1983; Waldo et al., 2005; Kirby & Hutson, 2010).

The proepicardium (PE) is a transitory cauliflower-like structure located between the cardiac and hepatic primordia. The PE is constituted by an internal mesenchymal component surrounded by an external epithelial lining. With development, cells derived from the proepicardium migrate to the developing neighbor embryonic heart and progressive cover the most external surface in a highly organized manner in chicken (Winter & Gittenberger-de Groot, 2007; Gittenberger-de Groot et al., 2010). In other species, such as zebrafish and mice, small PE-derived vesicle are detached and subsequently set down into distinct regions of the developing heart resulting in a more serendipitous external covering of the ventricular and atrial chambers, finally forming the embryonic epicardium (Peralta et al., 2013, 2014; Plavicki et al., 2014). Subsequently, the embryonic epicardium is trigger by the underlying myocardium to proceed with an epithelial-to-mesenchymal transition (EMT), migrate into the subepicardial space, leading to the so-called epicardial derived cells (EPDCs) and thereafter migrate into the myocardial layer and start a process of cell differentiation into mainly three distinct cell types, endothelial and smooth muscle cells of the coronary vasculature and fibroblasts and fibrocytes constituting the cardiac fibroskeleton and the outmost layer of the coronary vasculature, the adventitia layer (Perez-Pomares et al., 1998; Carmona et al., 2010; Winter & Gittenberger-de Groot, 2007; Gittenberger-de Groot et al., 2010). Experimental evidence in chicken have nicely demonstrated that EPCDs can distinctly contribute to all these cell types, while controversial findings have been reported in mice, supporting the notion that a limited, but yet significant contribution to the endothelial vasculature is epicardially derived (Merki et al., 2005; Red-Horse et al., 2010; Tian et al., 2013; Chen et al., 2014; Cano et al., 2016). Similarly, a confounding factor remains as whether PE

cells and/or EPDCs are already specified to become either cell type (Poelmann et al., 1993; Valder & Olson, 1004; Mikawa & Gourdie, 1996; Cossette & Misra, 2011; Niderla-Bielinska et al., 2015; Jankowska-Steifer et al., 2018).

Surprisingly, isolation of the developing PE anlage and culturing ex vivo spontaneously lead to differentiation of beating cardiomyocytes, a process that is enhanced by Bmp but halted by *Fgf* administration (Kruithof et al., 2006). These observations lead to postulate that the PE cells have the capacity to become cardiomyocytes but are triggered to adopt a distinct cell fate, opening the possibility of searching for strategies that unlock this halted fate. More recently, in vivo contribution to the myocardial lineage has been reported (Cai et al., 2008; Zhou et al., 2008), yet these findings are controversial. Epicardial *Tbx18*+ cells were reported to contribute to ventricular cardiomyocytes in vivo (Cai et al., 2008) using *Cre/LoxP* lineage tracing strategies. However, it was previously reported that fetal cardiomyocytes also expressed Tbx18 (Franco et al., 2006; Christoffels et al., 2009) and thus those *Tbx18*+ epicardial lineage tracing experiments were dubious. Similarly, epicardial *Wt1*+ derived cells have also been reported to contribute to endothelial cells and to the myocardium (Zhou et al., 2008), but *Wt1*-derived cardiomyocytes can be traced in the developing heart before PE/epicardial formation (Cano et al., 2016), thus questioning the epicardial contribution to the developing cardiac muscle. Overall, these data demonstrate the lack of contribution of the embryonic epicardium to the developing myocardium. Importantly, adult epicardium can be triggered to be converted into adult cardiomyocytes by thymosin-β4 priming (Smart et al., 2011), opening novel therapeutic opportunities.

MicroRNAs are a subclass of non-coding RNAs widely and extensively expressed in different tissues during embryonic development, homeostasis and diseases (Exposito-Villen et al., 2018). microRNAs are small RNA molecules of 22-24nt in length, transcribed by RNA polymerase II, 5′-capped and 3′-poliadenylated. MicroRNAs control post-transcriptional regulation by base-paired complementary binding to the 3′UTRs of coding RNAs leading to mRNA degradation and/or protein translational blockage (Callis et al., 2007; Callis & Wang, 2008; Bonet et al., 2013; Yan & Jiao, 2016). Multiple evidences demonstrated the pivotal role of microRNAs during cardiovascular development as evidenced by seminal studies demonstrating that deletion of a single microRNA, i.e. *miR-1* and *miR-126*, respectively, led to embryonic lethality with severe cardiovascular defects (Zhao et al., 2007; Fish et al. 2008). Furthermore, manipulation of a discrete number of microRNAs can influence cell fate determination (Jayawardena et al., 2012; Porrello et al. 2013).

Evidence of the functional importance of microRNAs in the development of the epicardium was provided by selective inhibition of the key microRNA processing ribonuclease *Dicer* in the epicardial tissue, resulting in thin myocardium and impaired vascular development (Singh et al., 2011). These data suggest that microRNAs are involved in the cell fate determination process of the embryonic epicardium. However, the functional role of microRNAs in PE remains elusive. In this study we provide a comprehensive characterization of the developmental expression profile of multiple

microRNAs during PE and epicardial development in chicken. Subsequently, we identified that *miR-125*, *miR-146*, *miR-195* and *miR-223* selectively enhance cardiomyogenesis both in PE/ST explants as well as in embryonic epicardium cultures, a *Smurf1*- and *Foxp1*-driven process. In addition we identified novel three novel long non-coding RNAs with enhanced expression in the PE/ST, that are complementary regulated by *Bmp* and *Fgf* administration and well as by microRNAs that selectively promote cardiomyogenesis, supporting a pivotal role of these long non coding RNAs in microRNA-mediated cardiomyogenesis of the PE/ST cells.

Materials & Methods

Tissue Isolation and Culture

Experimental protocols with chicken embryos were performed in agreement with the Spanish law in application of EU Guidelines for animal research. These protocols conformed to the Guide for Care and Use of Laboratory Animals, published by the Us National Institutes of Health (NIH publication no. 85-23). Approved consent of the Ethic Committee of the University of Jaen was obtained prior to the initiation of the study. Fertilized eggs from white Leghorn chickens (Granja Santa Isabel, Cordoba, Spain) were incubated at 37.5°C and 50% humidity for 2-7 days. Embryos were harvested and classified at different developmental stages (HH17, HH24 and HH32) according to Hamburguer and Hamilton. Embryos were removed from the egg by cutting the blastocyst margin with iridectomy scissors and placing them into Earle's balanced salt solution (EBSS) (Gibco). For qPCR analyses, HH17 embryonic hearts and PE/STs, respectively, were isolated, pooled and directly stored at -80°C until used. HH24 and HH32 hearts were isolated, cultured as described by Ramesh et al., collected, pooled and stored at -80°C until used. Epicardial identity was validated by *Wt1* and *Tbx18* immunohistochemistry, resulting in >80% cells positive for these markers. For *in vitro* explants cultures, chicken HH17 were dissected in Earle's balanced salt solution (EBSS) (Gibco) and culture into collagen gels as previously described or, alternatively, cultured in hanging drops until collected, pooled and stored at -80°C until used.

MicroRNA and siRNA Transfection

HH17 PE explants were culture don collagen gels or hanging drops for 24 hrs at 37°C in a cell culture incubator before pre-miRNAs (micro RNA precursors) or siRNA transfections respectively, as previously described (Bonet et al., 2015)). HH24 and HH32 epicardial cells were cultured for 72 hours after the myocardial layer was removed and then transfected. Pre-miRNAs transfections were carried out with Lipofectamine 2000 (Invitrogen), following the manufacturer's guidelines. Briefly, 85 nM of pre-miRNA were applied to the explants (3-5 explants per well) for 24 hours. siRNA transfections

were either processed for qRT-PCR or immunohistochemical (IHC) analyses. Negative controls, i.e HH17 explants treated only with Lipofectamine were run in parallel. To perform IHC analyses, the explants were fixed with 1% PFA for 2 hours at 4°C, rinsed for three times in PBS during 10 min, and stored in PBs at 4°C. Each experimental condition was carried out in isolated tissues from at least 30 embryos. In all cases, 3-5 independent biological replicates were analyzed.

Growth factors and thymosin-β4 administration

PE HH17 explants and HH24 embryonic epicardial primary cultures were treated for 24h with Bmp2, Fgf2, Fgf8 and thymosin-β4 (Prospec, East Brunswick, NJ, USA), respectively, as reported by Hinkel et al. (2015). Tissue explants were collected and processed according for qPCR and/or immunohistochemistry. Each experimental condition was carried out in isolated tissues from at least 30 embryos. In all cases, 3-5 independent biological replicates were analyzed.

Cell migration assays

Embryonic epicardial (HH24) primary cell cultures were established similarly as reported by Ramesh et al. (2016) but using chicken hearts. Briefly, HH24 embryonic heart were isolate from the developing embryo and the inferior ventricular half was dissected, plated upside down into tissue culture dishes and incubated into DMEM supplemented with glutamine culture media for 48 hours. At this stage, emerging epicardial epithelial sheet starting to develop. Transfections with corresponding pre-miRNAs, scrambled and negative controls, respectively, were carried out and immediately placed into the culturing chamber of the time-lapse laser confocal microscope and provided adequate cell tissue culture conditions. Time lapse analyses was carried for 48h, with images taken every 30 minutes. Each experimental condition was carried out in isolated tissues from at least 39 embryos. In all cases, 3-5 independent biological replicates were analyzed.

RNA isolation and qPCR

All q-RT-PCR experiments followed MIQE guidelines (Bustin et al.,2009) and similarly as previously reported (28, 31). Briefly, RNA was extracted and purified by using Trizol reactive (Invitrogen) according to the manufacturer's instructions. For mRNA expression measurements, 1μg of total RNA was used for retro-transcription with Maxima First Strand cDNA Synthesis Kit for RT-qPCR (Thermo Scientific). Real time PCR experiments were performed with 1μL of cDNA, SsoFast EvaGreen mix and corresponding primer sets as described on Suplementary Table 1. For microRNA expression analyses, 20 ng of total RNA was used for tetro-transcription with Universal cDNA Synthesis Kit II (Exiqon) and the resulting cDNA was diluted 1/80. Real time PCR

experiments were performed with 1 μL of diluted cDNA, ExiLENT SYBR Green master mix (Exiqon) and corresponding primer sets described on Supplementary Table 1. All qPCRs were performed using CFX384TM thermocycler (Bio-Rad) following the manufacture's recommentadions. The relative level of expression of each gene was calculated as described by Livak & Schmittgen (Livak & Schimittgen 2001) using Gadph and Gusb as internal control for mRNA expression analyses and 5S and 6U for microRNA expression analyses, respectively. Each PCR reaction was carried out in triplicate and repeated in at least three distinct biological samples to obtain representative means. Heatmaps were obtained using the Multi Experimenter Viewer (version 4.9.0 – Windows 10), of the TM4 software suite (Saed et al., 2006). Previously, the normalize function was applied to microRNA expression data, which transform the values using the mean and the standard deviation of the row of the matrix to which the value belongs using the following formula: Value= [(Value) – Mean(Row)]/[Standard deviation(Row)].

Immunofluorescence analysis by Confocal scanning laser microscopy

Immunofluorescence analyses were performed as previously reported (Bonet et al., 2015). Briefly, control and experimental HH17 PE explants and HH24, HH32 epicardial cell cultures were collected after the corresponding treatment, rinsed in PBS for 10 min at room temperature and fixed with 1% PFA for 2 hours at 4°C. After fixation, the samples were rinsed three times (10 min each) in PBS at room temperature ant them permeabilized with 1% Triton X-100 in PBS for 30 min at room temperature. To block nonspecific binding sites, PBS containing 5% goat serum and 1% bovine serum albumin (Sigma) was applied to the explants overnight at 4°C. As primary antibody, a polyclonal goat anti-cardiac troponin I (Hytest) was used, diluted (1:200) in PBS to remove excess primary antibody and incubated overnight at 4°C with Alexa-Fluor 546 anti-goat (1:100; Invitrogen) as secondary antibody. After incubation with the secondary antibody, the explants were rinsed as described above. Finally, the explants and/or epicardial cell cultures, respectively, were incubated with DAPI (1:1000; Sigma) for 7 min at room temperature and rinsed three times in PBS for 5 min each. Explants were stored in PBS in darkness at 4 °C until analyzed using a Leica TCS SP5 II confocal scanning laser microscope.

Statistical analyses

For statistical analyses of datasets, unpaired Student's t-tests were used, as previously reported (Bonet et al., 2015). Significance levels or P values are stated in each corresponding figure legend. P,0,05 was considered statistically significant.

Results

MicroRNA Profiling During PE/ST and Embryonic Epicardium Development

As previously reported, microRNA function is essential to PE/ST development and contribution to the developing heart (Singh et al., 2011). In order to identify differentially expressed microRNAs during epicardial development we have analyzed the expression of 37 distinct microRNAs in three epicardial differentiation stages; HH17 PE/ST explants and HH24 and HH32 cultured embryonic epicardial cells, respectively. Among these 37 microRNAs, 16 display differential expression in all distinct epicardial stages (*miR-21, miR-29a, miR-100, miR-106b, miR-125b, miR-130a, miR-146, miR-148b, miR-195, miR-200a, miR-200c, miR-202, miR-208b, miR-214, miR-429, miR-503) while the remaining 21 (miR-1, miR-15b, miR-16, miR-22, miR-23a, miR-23b, miR-25, miR-26, miR-27a, miR-31, miR-34a, miR-34c, miR-39, miR-128, miR-130b, miR-185, miR-199a, miR-203a, miR-223, miR-328, miR-448*) display no expression in the PE/embryonic epicardium. Among those displaying differential expression in the developing epicardium, three distinct developmental profiles were observed; a) ten microRNAs were increased over time (*miR-21, miR-29, miR-106b, miR-130a, miR-148, miR-195, miR-200a, miR-200c, miR-208b and miR-429*) being barely detectable in the PE/ST explants while significantly increasing as development proceeds (**Figure 1A**) b) three microRNAs were decreased over time (*miR-100, miR-125 and miR-503*), *i.e.* highly expressed in ST/PE explants while progressively decreasing in HH24 and HH32 stages (**Figure 1B**), and c) three microRNAs (miR-146, miR-202 and miR-214) displayed moderate expression levels in HH17 PE/ST, peaking at HH24 and decreasing thereafter at HH32 (**Figure 1C**). Overall these data demonstrate a wide dynamic expression profile of distinct microRNAs, suggesting distinct functional roles during epicardial development.

MicroRNA Mimics Modulate Cell Lineage Marker Expression in the PE/ST

MicroRNAs play modulatory roles in multiple aspects of embryonic development and therefore also during cardiac formation. We have previously reported a pivotal role for *miR-23b* and *miR-199* during epithelial-to-mesenchymal transition in cardiac valvulogenesis (Bonet et al., 2015). We herein tested whether microRNA administration can influence cell lineage determination of the PE/ST. Chicken HH17 PE/ST explants were isolated **(Figure 2A-E)** and cultured in collagen gels as previously described (Bonet et al., 2015). Treatment of the PE/ST explants was carried with nine distinct microRNAs (*miR-21, miR-23b, miR-27b, miR-100, miR-125, miR-126, miR-146, miR-195 and miR-223*), displaying distinct expression profiles during embryonic epicardial development, for 48h and tissues were either processed for qPCR or immunohistochemistry and confocal image analyses, respectively.

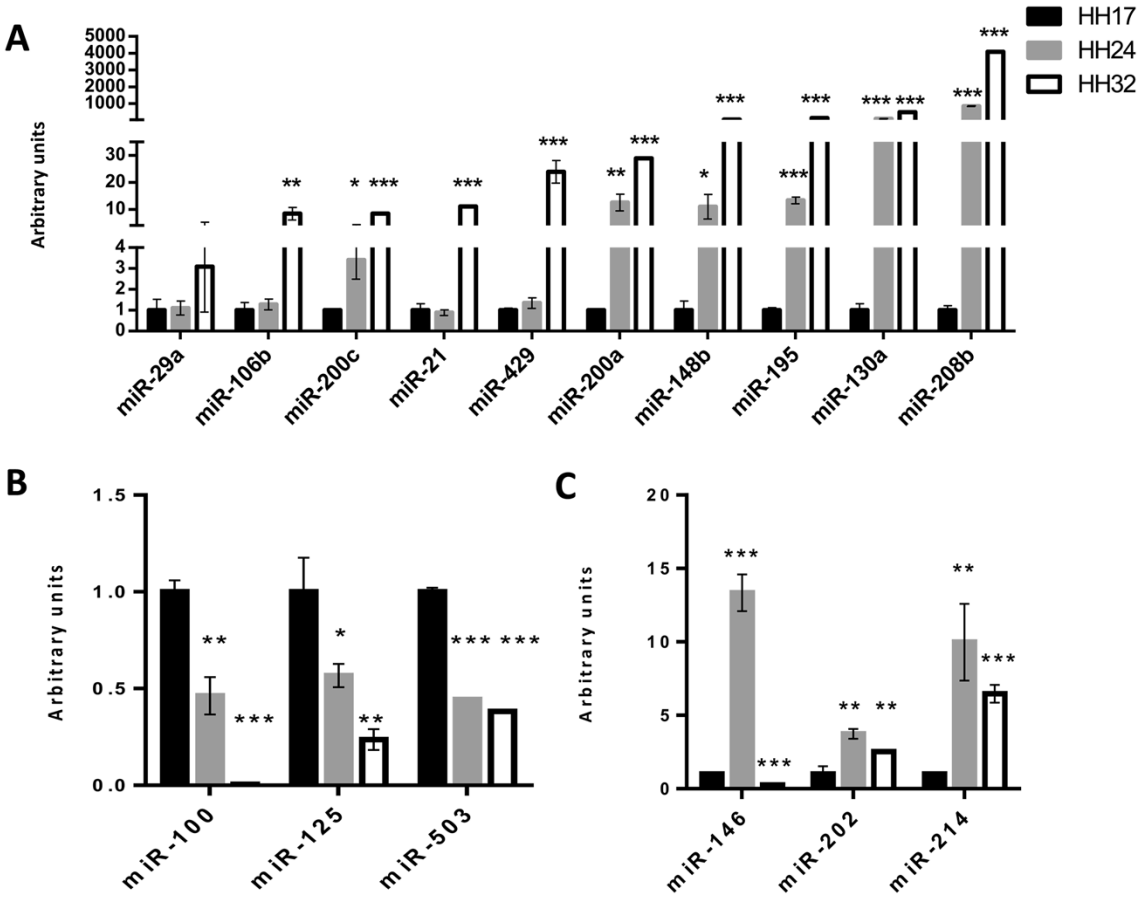

Figure 1. microRNA expression profile during PE and epicardium development. qPCR analyses of the differential expression of microRNAs during PE and epicardium development. Panel **A** illustrate microRNAs with increasing expression ranging from PE HH17 to embryonic epicardium at HH32. Panel **B** illustrate microRNAs with decreasing expression ranging from PE HH17 to embryonic epicardium at HH32. Panel **C** illustrate microRNAs with increased expression ranging from PE HH17 to embryonic epicardium at HH24 but decreasing at H32. HH17 PE were collected from >30 embryos and pooled to performed RNA isolation. Similarly, HH24 and HH32 epicardial cells were collected from >30 ventricular explants. In all cases, three distinct biological replicates of pooled PE/ST, HH24 and HH32 samples were subsequently tested by qPCR.

In all cases, at least 30 PS/ST explants were treated for each experimental condition that were subsequently pooled. qPCR analyses were performed for markers of early (*Mef2c, Nkx2.5 and Gata4*) and terminal cardiogenic differentiation (*Mhy15* and *Tnnt2*), epithelial to mesenchymal transition (*Snail, Slug, Cdh1, Cdh2, Cdh5*), and fibrogenesis (*Col1a1*). Analyses were always performed in 3-5 distinct biological replicates for each microRNA treatment. Overexpression of microRNA mimics were validated by qPCR as illustrated in **Supplementary Figure 1**. Expression of early cardiomyogenic differentiation markers such as *Mef2c, Nkx2.5* and/or *Gata4* were selectively down-regulated (or not altered) by *miR-21, miR-23b, miR-27b, miR-100, miR-195, miR-125 while miR-126 and miR-146*, respectively, enhanced expression of *Nkx2.5* and *miR-223* administration increased expression of all three early cardiomyogenic markers. Cardiogenic terminal differentiation markers were significantly up-regulated in PE/ST treated with *miR-223, miR-195, miR-125* and *miR-146*, respectively while they were selectively inhibited by *miR-23b, miR-27b* and *miR-126* but not significantly altered by

miR-100 and *miR-21* administration (**Figure 2F**). These data were further corroborated by immunohistochemical detection of cardiac troponin T as illustrated in (**Figure 2G-R).** Curiously, in most cases that terminal differentiation is enhanced, a selective down-regulation of most of the early cardiogenic markers is observed (*miR-195, miR-125* and *miR-146*), except for *miR-223* that were equally enhanced.

PE/ST cells migrate into the nude myocardium and activate an epithelial-to-mesenchymal transition that is pivotal for it subsequent migration into the embryonic myocardium. However, it is unclear if EMT is required for PE/ST differentiation. Thus, we tested if EMT also display significant differences after microRNA administration particularly those promoting cardiomyogenesis. *miR-195* and *miR-23* treatment decreased EMT markers such as *Snail* and/or *Slug*, whereas *miR-27b, miR-21, miR-100, miR-146, miR-125, miR-126* and *miR-223* increased those EMT transcriptional activators (**Supplementary Figure 2**). Our data support the notion that EMT and cardiomyogenic differentiation are not mutually exclusive biological process, i.e both can concur simultaneously. Curiously, expression of cell-cell junctional proteins, i.e. *Cdh1, Chd2* and *Chd5*, was not always concomitantly up-regulated or down-regulated as the EMT transcriptional activators (**Supplementary Figure 2**), suggesting that different molecular mechanisms drive activation and cell-cell uncoupling, in line with previous reports (Bonet et al., 2015) and/or that transcriptional overriding after microRNA administration is operative as suggested by Hill et al. (2014).

Fibrogenic differentiation, as assessed by *Col1a1* expression, was significantly increased after *miR-23b, miR-27b, miR-195* and *miR-223*, administration whereas *miR-100* and *miR-125* treatment decreased *Col1a1* expression (**Suplementary Figure 2**). No significant changes were observed for *miR-21, miR-146* and *miR-126* administration, respectively (**Supplementary Figure 2**). Overall these data demonstrate that microRNA treatment can distinctly modulate cell differentiation behavior including cardiomyocyte, epithelial to mesenchymal transition and fibroblast differentiation.

In sum, these data demonstrate that single microRNA administration can exert different cell differentiation modulatory roles; e.g. *miR-23* inhibits cardiogenesis and epithelial-mesenchymal transition while enhances fibrogenesis while *miR-195* enhances terminal cardiomyocyte differentiation and fibrosis while inhibits epithelial-to-mesenchymal transition.

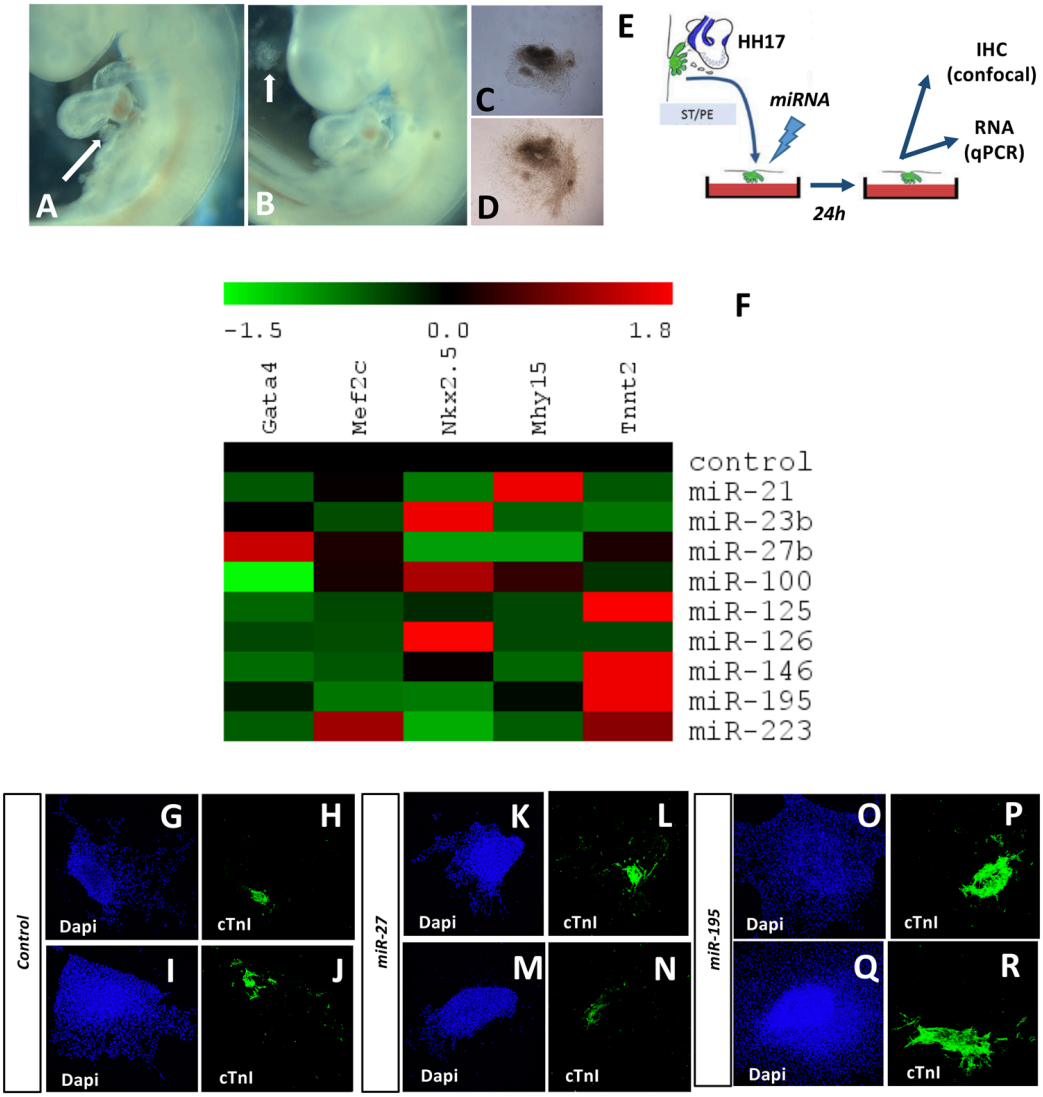

Figure 2. Modulation of cardiomyogenic potential of PE/ST explants by microRNA mimics administration Panels A–D. Representative image of chicken HH17 embryo before (panel A) and after (panel B) PE excision. Arrows demarcates the PE. Panel **C** illustrates PE culturing just right after dissection (panel C) and 24 h after culturing (panel D). Panel E represents a schematic overview of the experimental design. Panel F shows
qPCR results of cardiomyogenic (*Nkx2.5, Mef2c, Gata4, Mhy15, Tnnt2*) markers expression after microRNA mimic administration in HH17 PE/ST explants. Observe that *miR-23* and *miR-27* over-expression leads to down-regulation of all cardiomyogenic markers, miR-100 does not modify most of them and *miR-223, miR-195, miR-125* and *miR-146* increased terminally differentiation markers such as *cardiac troponin T* (*Tnnt2*).
Confocal image analyses of *cTnI* expression in controls (panels G-J), *miR-27* (panels K-N) and *miR-195* (panels O-R) treated HH17 PE/ST explants. Observe that *miR-195* administration selectively increases the overall cTnI immunohistochemical signal (panel P and R). HH17 PE were dissected from >30 embryos, treated with the corresponding microRNA mimics and subsequently pooled to perform RNA isolation. On each case, three-to five distinct biological replicates were subsequently tested by qPCR.

Modulatory Effects of *miR-195* and *miR-233* is Partially Promoted in the Embryonic Epicardium

In order to dissect if the modulatory roles exerted by distinct microRNAs in the HH17 PE/ST explants is also applicable to the embryonic epicardium, HH24 epicardial explants were analyzed after microRNA over-expression of a selected number of microRNAs, i.e. those reporting enhanced cardiomyogenesis *miR-125, miR-126, miR-146, miR-195* and *miR-223*, and *miR-100* as a negative control. Regulation of early myogenic markers is partially discordant as compared to PE/ST explants. For example, *miR-100, miR-125* and *miR-126* administration displayed decreased *Nkx2.5* expression in EE HH24 cells while no changes or increased is observed in HH17 PE/ST explants **(Figure 3A)**. On the other hand, concordant modulation is observed for *Gata4* after *miR-100, miR-125* and *miR-146*, as well as for *Mef2c* after *miR-125, miR-146* and *miR-223* **(Figure 3A)**. Importantly, up-regulation of terminally differentiation markers such as *Tnnt2* and *Mhy5* as also concordantly observed after *miR-195* and *miR-223*, demonstrating that cardiomyogenesis is similarly enhanced in EE HH24 and PE/ST explants.

Epithelial-to mesenchymal transition also display concordant expression for Slug expression following microRNA administration in HH24 embryonic epicardial cells, in particular for *miR-100, miR-125, miR-126* and *miR-146* while opposite regulatory patterns are observed after *miR-195* and *miR-223* **(Figure 4B)**. Similar discordant patterns are observed for *Cdh5* expression except for *miR-223* **(Figure 4B)**.

Expression of the fibrogenic marker *Col1a1*, display similar concordant patterns in EE HH24 and PE/ST explants after administration of *miR-100* and *miR-125*, whereas discordant patterns were observed for all the other microRNAs tested **(Supplementary Figure 2)**. Overall these data demonstrate that *miR-195* and *miR-223* maintain their potential to enhance cardiomyogenesis in the embryonic epicardium and while the loose their ability to promote epithelial-to mesenchymal transition and enhance fibrogenesis in EE HH24 as compared to PE/ST explants. Thus, these data suggest a plausible therapeutic usage of *miR-195* and *miR-223* to enhance cardiomyogenesis without compromising putative adverse events such as EMT and fibrosis promotion.

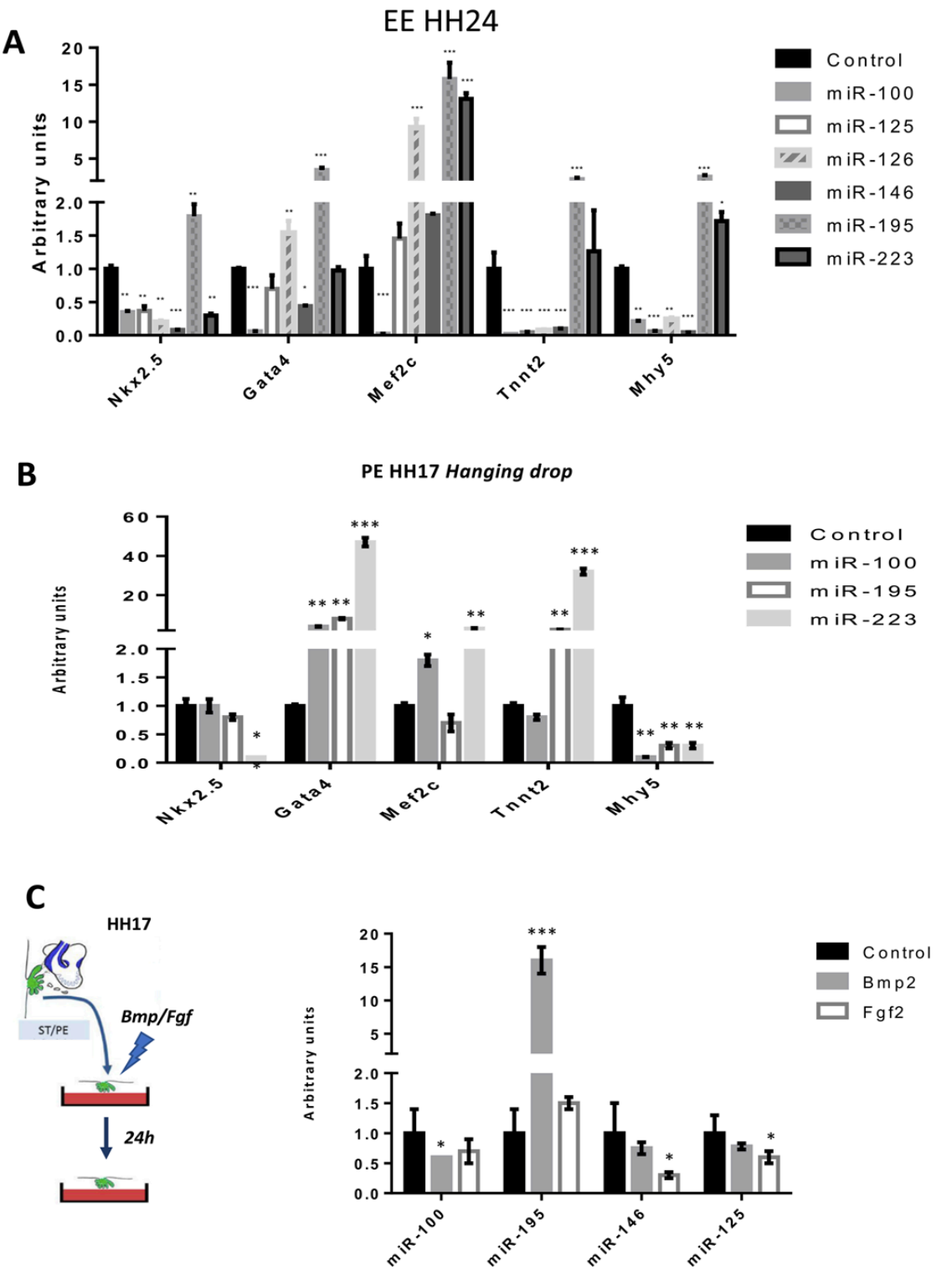

Figure 3. Modulation of cardiomyogenic potential of HH24 embryonic epicardium and HH17 PE/ST in hanging drops by microRNA mimics administration. Modulation of microRNAs by *Bmp* and *Fgf* signaling. Panel **A** represents qPCR analyses of HH24 embryonic epicardium treated with microRNA mimics. Observe that *miR-195* and *miR-223* selectively enhance expression of terminally differentiation markers such as *Tnnt2* and *Mhy15*. Panel **B** represents qPCR analyses of HH17 PE/ST explants cultured in handing drops and treated with microRNA mimics. Observe that *miR-195* and *miR-223* selectively enhance expression of terminally differentiation marker *Tnnt2*, while administration of *miR-100* does not changes it expression. Panel **C** qPCR analyses of HH17 PE/ST explants cultured in handing drops and treated with *Bmp2* and *Fgf2,* respectively. Observe that *Bmp2* selectively enhances expression of *miR-195* while no significant differences are observed after *Fgf2* administration. HH17 PE were dissected from >30 embryos,

treated with the corresponding microRNA mimics and/or *Bmp/Fgf* treatment, respectively, and subsequently pooled to perform RNA isolation. On each case, three-to-five distinct biological replicates were subsequently tested by qPCR.

In addition, we have also tested if these modulatory effects were similarly occurring in the PE HH17 cultured in hanging drop. For this purpose, we assayed only those microRNAs displaying enhanced cardiomyogenesis in PE/ST explants and HH24 EE cultures, i.e. *miR-195* and *miR-223*, and *miR-100* as a negative control. Briefly, PE HH17 were dissected, set into hanging drops and concomitantly transfected with distinct microRNA mimics. After 24h, RNA was isolated and cell lineage markers were assessed by qPCR. Our data demonstrate that administration of *miR-100* does not enhance the expression of terminally differentiation cardiomyogenic marker cardiac troponin while *miR-195* and *miR-223* significantly increased it **(Figure 3B),** in line with previous data in PE HH17 cultured in collagen matrices (**Figure 2F**) and HH24 EE cell cultures (**Figure 3A**). Surprisingly, no enhancement was observed for *Mhy5*, probably due do time-specific differences in the onset of expression of these cardiomyogenic markers. Thus, our data revealed that cell-matrix interactions are not required for microRNA-mediated cardiomyogenesis.

Bmp and *Fgf* can Distinctly Modulate microRNA Expression in the Developing Proepicardium

Several growth factors members of the *Bmp* and *Fgf* families have been reported to distinctly modulate cell lineage specification in cardiogenic mesoderm into proepicardial and myocardial cells, respectively (Kruithof et al., 2006). In particular, *Bmp2* promotes differentiation of the septum transversum mesoderm into myocardial cells whereas *Fgf2* enhances proepicardial lineage commitment, a process that is intricately regulated by a complex feed-back loop involving several other Bmp and *Fgf* family members (Kruithof et al., 2006). We experimentally tested whether *Bmp* and *Fgf* signaling in the developing PE/ST influence the expression of distinct microRNAs with potential to modulate PE/ST cell differentiation as reported above. HH17 PE/ST tissues were dissected and cultured in hanging drops. Administration of Bmp2 significantly increased expression of *miR-195* while blocked expression of *miR-100* and no significant differences were observed for *miR-146* and *miR-125*. On the other hand, *Fgf2* administration selectively blocked *miR-125*, miR-100, miR-125, miR-146 but enhanced *miR-195* expression (**Figure 3C**). These data illustrate that distinct administration of Bmp and *Fgf* signaling influence miRNA expression in the developing PE/ST tissues.

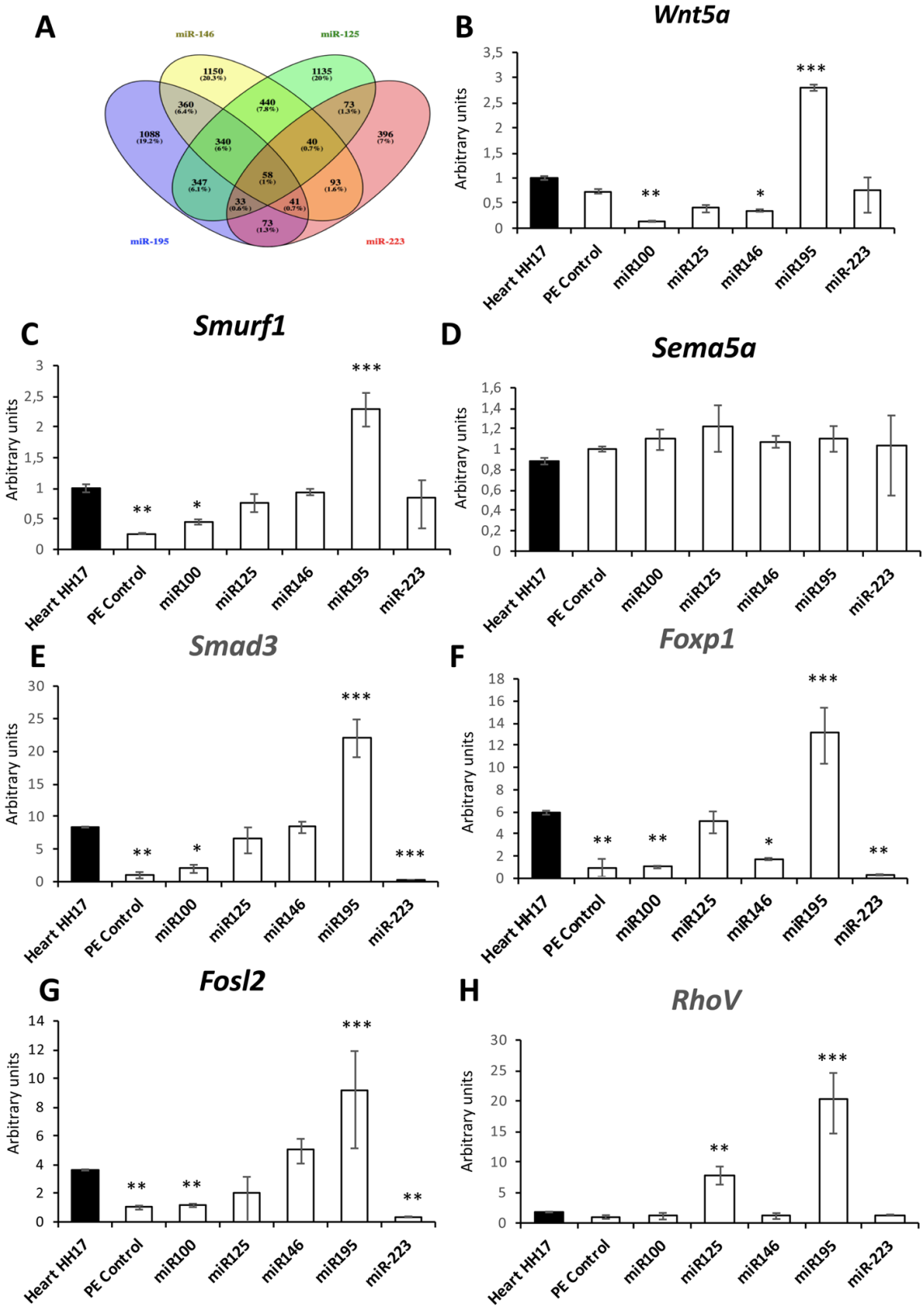

Figure 4. Potential targets driving PE/ST explants cardiomyogenesis. Panel **A.** Venny diagram of shared potential targets *of miR-146, miR126, miR-195* and *miR-223*. Observe that only 58 potential targets (representing 1%) were shared between these four microRNAs. Panels **B-H.** qPCR analyses of the expression of *Wnt5a* (panel B), *Smurf1* (panel C), *Sema5a* (panel D), *Smad3* (panel E), *Foxp1* (panel F), *Fols2* (panel G) and *RhoV* (panel H) in HH17 hearts, HH17 control PE/ST explants and PE/ST explants treated with *miR-100, miR-125, miR-146, miR-195* and *miR-223*. Observe that over-expression of *miR-195*

increases expression of *Wnt5a, Smurf1, Smad3, Foxp1, Fols2* and *RhoV,* while no significant differences were observed for Sema5a. On the contrary*, miR-100* over-expression lead to significant downregulation of *Wnt5a, Smurf1*, up-regulation of *Smad3* and no significant differences of *Sema5a, Foxp1, Fosl2* and *RhoV*. HH17 PE were dissected from >30 embryos, treated with the corresponding microRNA mimics and subsequently pooled to performed RNA isolation. On each case, three distinct biological replicates were subsequently tested by qPCR.

Search for Common microRNA-mRNA Pathways Modulating Cardiogenic Lineage Commitment

MicroRNAs can modulate multiple mRNA transcripts, ranging from hundreds to thousands of targets (Bartel, 2009). Distinct in silico algorisms can predict micro-mRNA interaction based on sequence complementary, biophysical interaction models and evolutionary conservation (i.e. TargetScan; http://www.targetscan.org/vert_72/ and MirWalk; http://mirwalk.umm.uni-heidelberg.de). We have demonstrated that over-expression of *miR-195, miR-125*, *miR-146* and *miR-223* respectively, in HH17 PE explants can enhance cardiomyocyte terminal differentiation. We therefore thought that they might share common targets governing these phenotypic changes. We selected all putative mRNA targets of *miR-195, miR-125, miR-146* and *miR-223* using MirWalk software and we identified all shared targets between these microRNAs. A total of 58 (1% all putative targets) mRNAs were identified for all four microRNAs, while 454 (8% all putative targets) were shared in three out of four microRNAs (**Figure 4A**). We subsequently scrutinized all genes (512 target genes; 9% all putative targets) with previous cited reports playing a role in myogenesis, and selected short list of seven transcripts (Wnt5a, Smurf1, Sema5a, Smad3, Foxp1, Fosl2, RhoV) for further testing their implication in PE/ST-derived cardiomyogenesis. We then tested if these genes were modulated by *miR-195, miR-125, miR-146* or *miR-223* over-expression, respectively, in HH17 PE explants. In addition, *miR-100* over-expression was also assayed as a negative control of cardiomyogenic inhibition and HH17 embryonic heart expression was also included to compare the relative expression of these genes in proepicardial and myocardial cells.

Comparative analyses of shared targets in the HH17 PE and embryonic heart demonstrate that *Smurf1, Smad3, Foxp1, Fosl2, RhoV* are enriched in the PE as compared to the embryonic heart, whereas *Wnt5a* and *Sema5a* display no significant differences **(Figure 4B-H**). Over-expression of *miR-100* selectively increased *Smad3*, decreased *Wnt5a* and *Smurf1* while no significant differences were observed for *Sema5a, Foxp1, Fosl2* and *RhoV* **(Figure 4B-H**). Administration of *miR-125* resulted in down-regulation of *Wnt5a*, up-regulation of *Smad3, Foxp1* and *RhoV*, while no significant differences were observed for *Smurf1*, *Sema5a* and *Fols2*. Similarly, *miR-146* over-expression resulted in down-regulation of *Wnt5a*, up-regulation of *Fosl2*, whereas all the other putative targets display no changes **(Figure 4B-H)**. Administration of *miR-195* to HH17 PE explants resulted in up-regulation of *Wnt5a*, *Smurf1, Smad3, Foxp1,* Fosl2 and *RhoV,* but no significant changes were detected for *Sema5a* **(Figure 4B-H).** Overall these data

demonstrated that most of the shared targets are enriched in the HH17 PE. In addition, *miR-125, miR-146* and *miR-195* selectively modulate expression of these genes as predicted, except for *Sema5a*. Furthermore, our data illustrate that *miR-195* exerts up-regulation of multiple genes involved in early cardiomyogenesis, in line with our results demonstrating that *miR-195* over-expression in HH17 PE explants enhances cardiomyogenesis, whereas *miR-100* blocks several of them, in line with HH17 PE explants over-expression assays. Among those shared targets, up-regulation of *Fosl2* and *Smad3* is exerted by both *miR-146* and *miR-195*, whereas up-regulation of *RhoV* and *Foxp1* is exerted by *miR-125* and *miR-195*, and *Wnt5a* and *Smurf1* by *miR-195* posing those genes as good candidates to explain the phenotypic consequence of driving cardiomyogenic differentiation upon microRNA over-expression.

Smad3 and *Smurf1*, but not *Fols2*, are Essential for *miR-195* Driven Cardiomyogenesis in PE/ST Explants

In order to test the functional role of these genes in *miR-195* promoted cardiomyogenesis, loss-of-function experiments were performed in presence or absence of *miR-195* administration. siRNAs were successfully designed and validated against *Fols2, Smad3* and *Smurf1* while either failed for *Wnt5a* and *Foxp1* either on the experiment design itself or the validation. As illustrated in (**Figure 5A)**, successful inhibition was obtained for *Smad3, Smurf1* and *Fols2,* respectively. Interestingly, *miR-195* administration rescued siRNA inhibition of *Smad3* and *Smurf1* but not *Fols2*. Subsequently we tested if siRNA silencing leads to impair cardiomyogenesis in PE/ST explants and if silencing was rescued by *miR-195* administration by measuring early (*Mef2c, Gata4* and *Nkx2.5*) and terminally (*Tnnt2*) differentiation cardiomyocyte markers by qPCR. Our data demonstrate that Smad3 silencing significantly blocked the expression of *Mef2c, Gata4, Nkx2.5* and *Tnnt2,* while *miR-195* administration in this setting only partially rescued expression of *Nkx2.5* and *Gata4* but without reaching control levels (**Figure 5B**). Similarly, *Smurf1* siRNA significantly down-regulates *Nkx2.5*, *Gata4* and *Tnnt2*, but surprisingly up-regulates *Mef2c. MiR-195* administration on *Smurf1* siRNA treated explants partially rescued *Mef2c* to basal control levels but it was unable to recover *Nkx2.5, Gata4* and *Tnnt2* (**Figure 5C**). Immunohistochemical analyses against cardiac troponin T corroborated these findings **(Figure 5E-G)**. Finally, *Fols2* siRNAs display no significant differences on *Mef2c* and *Gata4* expression while *Nkx2.5* was decreased and *Tnnt2* was increased. Moreover, *miR-195* administration in *Fols2* siRNA treated PE/ST explants significantly up-regulated all cardiomyogenic markers (**Figure 5D**). These data demonstrate that *Smad3* and *Smurf1* expression is critical for enhanced cardiomyogenesis in PE/ST explants while Fols2 seems to be dispensable.

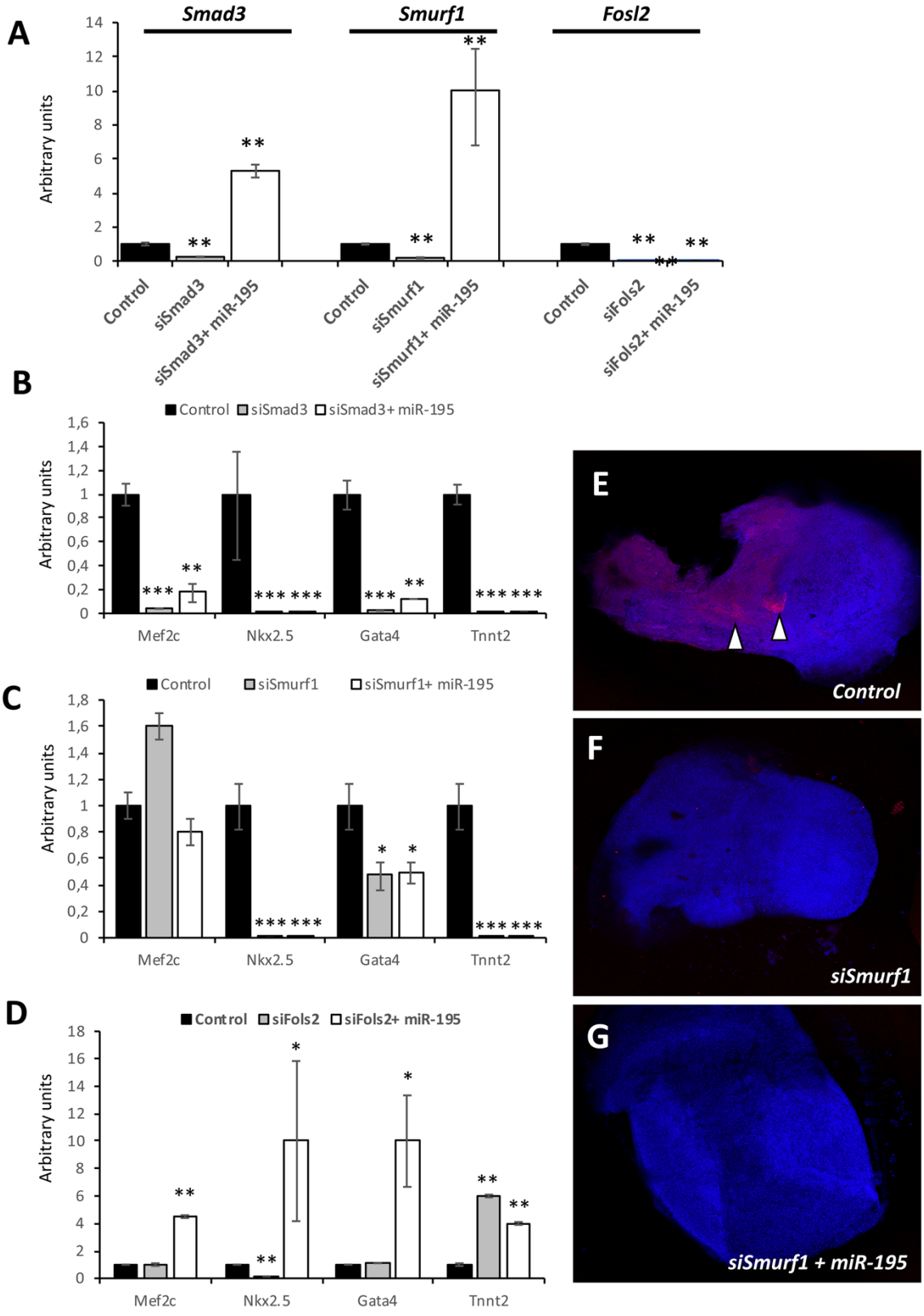

Figure 5. *Smad3* and *Smurf1*, but not *Fols2*, are essential for *miR-195* driven cardiomyogenesis in PE/ST explants. Panel **A.** qPCR analyses of *Smad3, Smurf1* and *Fols2* in control, siRNA treated and siRNA plus *miR-195* overexpression. Observe that siRNAs against *Smad3, Smurf1* and *Fols2* significantly decrease *Smad3, Smurf1* and *Fols2,* respectively. Observe also that *miR-195* administration can rescue *Smad3* and *Smurf1* upregulation but not *Fols2*. Panel **B.** qPCR analyses of cardiomyogenic markers in control, *siSmad3* treated and *siSmad3* plus *miR-195* overexpression. Observe that expression of *Mef2c, Nkx2.5, Gata4* and *Tnnt2* is severely impaired in both *siSmad3* treated and siSmad3 plus *miR-195* overexpression conditions.

Panel **C.** qPCR analyses of cardiomyogenic markers in control, *siSmurf1* treated and *siSmurf1* plus *miR-195* overexpression. Observe that expression of *Nkx2.5, Gata4* and *Tnnt2* is severely impaired in both *siSmurf1* treated and *siSmurf1 plus miR-195* overexpression conditions. Panel **D**. qPCR analyses of cardiomyogenic markers in control*, siFols2* treated and *siFols2* plus *miR-195* overexpression. Observe that expression of *Mef2c, Gata4* is not altered, *Nkx2.5* is diminished but *Tnnt2* is significantly increased in *siSmurf1* conditions. Furthermore, *Mef2c, Nkx2.5, Gata4* and *Tnnt2* expression is significantly increased in *siSmurf1* plus *miR-195* overexpression conditions. HH17 PE were dissected from >30 embryos, treated with the corresponding microRNA mimics, siRNA or the combination of both (microRNA mimic and siRNA) and subsequently pooled to performed RNA isolation. On each case, three distinct biological replicates were subsequently tested by qPCR**.**

Bmp4_53170 and *Fgf8_57126* are Modulated by *miR-195* in PE/ST Explants

Long non-coding RNAs are emerging as novel molecules playing essential roles in gene expression regulation in multiple biological contexts (Dahariya et al., 2019; Cruz-Miranda et al., 2019; Samudyata et al., 2018). Several studies have provided evidence that lncRNAs can play regulatory roles affecting neighboring genes (Hori et al., 2018; He et al., 2016; Schultz et al., 2015). We have identified nine distinct annotated lncRNAs neighboring key regulatory factors (*Bmp2, Bmp4, Wt1, Fgf2, Fgf8, Tcf21*) involved in PE development in the chicken genome **(Figure 6A)** and we have assessed their expression in PE as compared to age-matched developing heart. Three distinct patterns were observed, those displaying no significant differences (*Bmp2_33140, Bmp2_53839, Wt1_74077*), those with decreased expression in PE (*Fgf2_56708, Tcf21_48334*) and those with increased expression in PE (*Wt1_76127, Bmp4_53170 and Fgf8_57126*) as compared to HH17 embryonic heart **(Figure 6B)**. We subsequently assessed if those lncRNAs with enhanced expression in PE were modulated by cardiomyogenic enhancing signals provided by Bmp administration or PE signals provided by *Fgf* administration (Kruithof et al., 2006). We observed that *Wt1_76127* was significantly down-regulated by Fgf8 while up-regulated by *Bmp4* administration. Similarly, *Bmp4_53170* was down-regulated by *Fgf8* while up-regulated by both *Bmp2* and *Bmp4.* On the other hand, *Fgf8_57126* was up-regulated by *Fgf2* and *Fgf8* while significantly down-regulated by *Bmp2* and *Bmp4* **(Figure 6D)**. Overall these data demonstrate that these lncRNAs are complementary modulate by *Fgf* and *Bmp* signaling suggesting a plausible role in PE/ST derived cardiomyogenesis. In addition, we also tested if *thymosin-β4*, an epicardial to myocardial priming agent (Smart et al., 2011) could modulate the expression these lncRNAs. Interestingly, *thymosin-β4* enhanced *Bmp4_53170* while inhibited *Wt1_76127* and *Fgf8_57126* expression in PE/ST explants **(Figure 6C)**, further supporting a plausible role in PE/ST derived cardiomyogenesis, yet additional experiments are required to fully understand their functional role in this context.

Given the plausible role of these lncRNAs in PE/ST derived cardiomyogenesis, we tested if those microRNAs enhancing (*miR-195* and *miR-223*) or blocking (*miR-23* and *miR-27*) cardiomyogenesis are capable of modulating their expression. PE/ST explants treated with *miR-23* and *miR-27* significantly inhibited expression *of Wt1_76127* but did not modify expression of *Bmp4_53170* and *Fgf_57126*. On the other hand, *miR-*

195 significantly enhanced *Wt1_76127* while inhibited *Bmp4_53170*, but no differences were observed for *Fgf8_57126*. *MiR-223* administration significantly blocked *Wt1_76127* while *Bmp4_53170* and *Fgf8_57126* display no significant differences. These data demonstrate that microRNAs can regulate these lncRNAs and furthermore, microRNAs promoting vs inhibiting cardiomyogenesis display complementary regulatory roles, particularly on *Wt1_76127* and *Bmp4_53170*, further reinforcing their plausible role in PE/ST derived cardiomyogenesis.

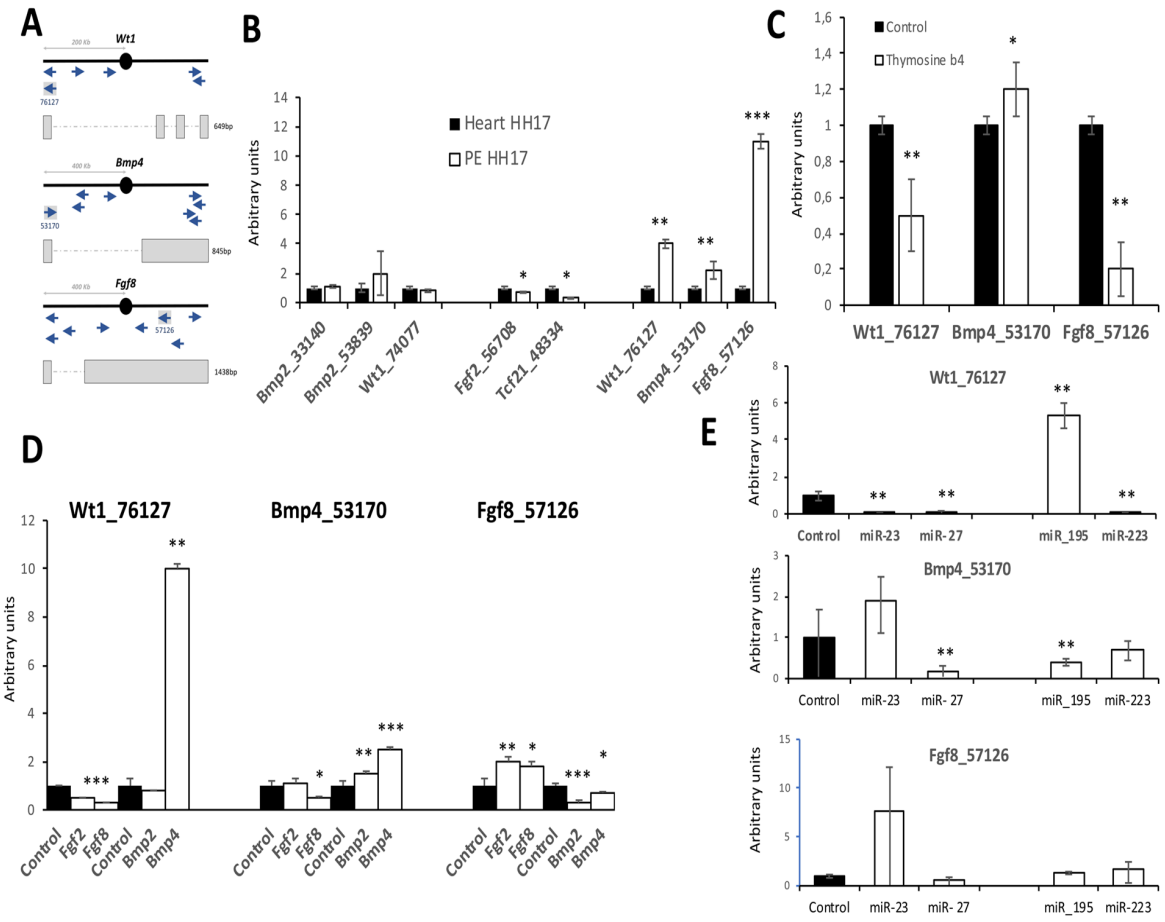

Figure 6. LcnRNA expression and modulation in PE/ST explants Panel **A.** Schematic representation of the genomic location of annotated lncRNAs within *Wt1, Bmp4* and *Fgf8* loci in chicken. Panel **B.** qPCR analyses of the expression of lncRNAs in HH17 embryonic heart and PE/ST, respectively. Observe that *Wt1_76127, Bmp4_53170* and *Fgf8_57126* display significant increased expression in the PE/ST as compared to the embryonic heart at the same developmental stage (HH17). Panel **C**. qPCR analyses of *Wt1_76127, Bmp4_53170* and *Fgf8_57126* in *thymosin-β4* treated PE/ST explants. Observe that *thymosin-β4* treatment increases *Bmp4_53170* while decreased *Wt1_76127* and *Fgf8_57126* expression. Panel **D**. qPCR analyses of *Wt1_76127, Bmp4_53170* and *Fgf8_57126* in *Bmp2, Bmp4, Fgf2* and *Fgf8* treated PE/ST explants, respectively. Observe that *Bmp* signaling decreased while *Fgf* signaling increased the expression of *Wt1_76127, Bmp4_53170,* while *Fgf8_57126* displays the opposite regulatory modulation by *Bmp and Fgf* signaling. Observe that *miR-195* increases *Wt1_76127* while decreases *Bmp4_53170* expression. On the other hand, *miR-23* and *miR-27* decreases *Wt1_76127* while does not modify the expression of *Bmp4_53170*. Panel **E.** qPCR analyses of *Wt1_76127, Bmp4_53170* and *Fgf8_57126* in *miR-23, miR-27*, *miR-195* and *miR-223* treated PE/ST explants, respectively. HH17 PE and HH17 embryonic hearts were collected from >30 embryos and pooled to performed RNA isolation. In all cases, three distinct biological replicates of pooled HH17 PE/ST and HH17 heart samples were subsequently tested by qPCR (panel A). HH17 PE/ST were dissected from >30 embryos, treated with *thymosin-beta4* (panel C), the corresponding

Bmp/Fgf growth factor (panel D), and/or microRNA mimics treatment (panel E), respectively and subsequently pooled to performed RNA isolation. On each case, three
distinct biological replicates were subsequently tested by qPCR.

Discussion

Differential expression of microRNAs have been widely reported in distinct biological settings including homeostatic and pathological contexts (Lim et al., 2005: Ji et al., 2007; Zeiger et al. 2010). Within the cardiovascular system, several studies have provided evidences of the differential expression of microRNAs during cardiogenesis (Wilson et al., 2010; Chinchilla et al., 2011; Cao et al., 2013). However, to date, microRNA profiling of the proepicardium and/or epicardium is still missing. We provide herein evidence that multiple microRNAs display differential expression during the process of PE and epicardium formation. A large subset of microRNAs display increasing expression, supporting a plausible role blocking or inhibiting the expression of mRNA target genes during PE to embryonic epicardial transition. On the other hand, a small subset display decreased expression supporting a role in releasing repression of inductive signals while a similar subset display transition peak expression in HH24 as compared to HH32 embryonic epicardium, suggesting a plausible modulatory role in this transition, probably affecting thus epicardial to mesenchymal transition onset (Perez-Pomares et al., 2002; van Tuyn et al., 2007; Smith et al., 2011). Thus, these data provide an entry site to start dissecting the functional roles of microRNAs during epicardial development.

Seminal evidences on the functional role of microRNAs in epicardial development was provided by Singh et al. (2011) by selective deletion of Dicer, a ribonuclease involved in microRNA maturation, in the embryonic epicardium. However, understanding of the functional role of discrete microRNAs in the epicardium have only been provided for *miR-31* and *miR-21*, both of them directing fibrogenic EMT by distinctly modulating *Islet1* (Bronnum et al., 2012) and *Pcd4/Spry1* (Bronnum et al., 2013) expression, respectively. Importantly, to the best of our knowledge this is first evidence reporting cardiomyocyte cell fate modulation of the PE/ST. A significant enhancement of cardiomyocyte terminal differentiation was provided by administration of *miR-223* and *miR-195* mimics, a weaker activation was provided by *miR-125* and *miR-146* while only activation of early cardiogenic markers but not terminal differentiation was obtained for *miR-126*. On the other hand, *miR-23* and *miR-27* selectively inhibited cardiomyogenesis while *miR-100* and *miR-21* essentially displayed not significant enhancement. These data therefore evidence the differential microRNA modulation of PE/ST cardiomyogenesis.

Previous studies reported the involvement of *miR-23* and *miR-27* in both cardiac development and pathology (Chinchilla et al., 2011; Langedijk et al., 2013; Kou et al., 2016; Oikawa et al., 2018; Liu et al., 2018; Wang et al., 2018), while *miR-100* has only been reported as a protective agent of cardiomyocyte apoptosis (Chen et al., 2015). On the other hand, *miR-223* and *miR-195* have been reported in distinct cardiac pathologies

(Lu et al., 2010; Zhu et al., 2011; Dai et al., 2014; Wang et al., 2015; Chen et al., 2016; Liu et al., 2016; Zhang et al., 2018; Wang et al., 2018) but no evidences on their functional role during cardiac development have been described so far. Furthermore, scarce evidences on the role of *miR-125* (Wang et al., 2014) and/or *miR-146* (Zhang et al. 2018) in cardiac development and pathology have been reported. On the other hand, *miR-126* represents a vascular specific microRNA and *miR-126* deficient zebrafish are embryonic lethal (Fish et al., 2008). Furthermore, additional functional roles for *miR-126* in the vasculature have been extensively reported (Sun et al., 2018; Chistiakov et al. 2016; Potus et al., 2015; Schober et al., 2014). Importantly a functional role in cardiomyocytes, particularly in apoptosis, is recently emerging (Shi et al., 2013; Jiang et al. 2014; Suresh Babu et al., 2016). Our data demonstrate that a more enhanced cardiomyogenic differentiation is exerted by *miR-195* and *miR-223* as compared to miR-*125, miR-146* and *miR-126, while miR-23* and *miR-27* blocked such cardiomyogenic differentiation. Furthermore, our findings open up the possibility of exploring these microRNAs as therapeutic tools to enhance cardiomyogenesis.

Regulation of cardiac transcription factors such as *Mef2c, Gata4* and *Nkx2.5* by microRNAs have been reported in different biological contexts (Yao et al., 2013; Liang et al.,2016; Wu et al., 2016). In striated muscle, *miR-27* and *miR-125* distinctly regulate *Mef2c* in cardiac and skeletal muscle cells (Chinchilla et al., 2011; Lozano-Velasco et al. 2015). Curiously, *miR-223* downregulation leads to *Mef2c* upregulation in leukemia (Agatheeswaran et al., 2012) while no evidences have been reported for *miR-195* and/or *miR-146* modulating the expression of these early cardiomyogenic differentiation markers in striated muscle. In this study we demonstrate for the first time the regulatory role of these microRNAs modulating expression of early and terminally differentiation cardiomyogenic markers in both PE/ST and epicardial cell cultures, enhancing thus their potential therapeutic usage.

An integral developmental process linked to PE and epicardium morphogenesis is driven by an epithelial to mesenchymal transition that provides mechanistic clues to these cells favoring their integration into the embryonic myocardium and subsequently differentiation into distinct cell types, such as fibroblasts smooth muscle cells and endothelial cells (Smith et al., 2011). In this study we further investigated how administration of these microRNAs influence EMT and fibrogenic differentiation. Our data demonstrate that miR-195 and miR-23 can selectively down-regulate expression of EMT inducers such as *Snail* and *Slug* and up-regulate *Cdh1* expression without modulating *Cdh2* and *Chd5*, in line with previous reports in other biological contexts (Singh et al., 2015; Liu et al., 2015; Yu et al., 2017; Yang et al., 2017). On the other hand, all the other microRNAs tested (*i.e. miR-21, miR-27, miR-100, miR-125, miR-126, miR-223 and miR-146*) resulted in *Snail* and/or *Slug* up-regulation while effects on *Cdh* expression is not always concomitant. Several of these microRNAs have been reported to promote EMT (*miR-27, miR-100*; Zhang et al.2011; Chen et al., 2014) while other can either promote or inhibit it in different biological contexts (*miR-100, miR-126, miR-223; miR-21* Wang et al., 2014; Jiang et al., 2017; Jia et al., 2018; Liu et al., 2019; Ding et al.,

2018; Tang et al., 2015; Ma et al. 2015; Li et al., 2016; Dai et al., 2019; Yang et al. 2018), in line with our findings. Importantly, we provide evidence for the first time on the involvement of *miR-125* and *miR-146* in EMT regulation. Our data suggest that up-regulation of EMT inducers and subsequent cytoskeletal remodeling represent uncoupled events in this setting, in line with previous reports during AV EMT modulation by microRNAs (Bonet et al., 2015), alternatively that additional time is required to see such transcriptional changes in *Cdh* expression or that transcriptional overriding effects by microRNAs over-expression is occurring (Hill et al., 2014). Thus, additional experiments are required to fully elucidate this apparently discordant findings. Furthermore, our data demonstrate that EMT is not required to PE/ST cardiomyogenic differentiation, since administration of *miR-223* can simultaneously induce both processes. *i.e*. EMT and cardiomyogenesis.

Importantly, we demonstrate herein that a single microRNA can exert different regulatory aspects in both PE/ST explants and HH24 EE cell cultures. *miR-23* can block cardiomyogenic differentiation and EMT while promotes fibrogenic differentiation, *miR-195* enhances cardiomyogenesis while blocking EMT but promoting fibrogenic differentiation while, *miR-223* can promote all three developmental processes, providing thus a therapeutic potential for cardiomyogenic regeneration.

To understand the molecular mechanisms that drive promotion of cardiomyogenic terminal differentiation by *miR-223, miR-195, miR-125* and *miR-146* administration, we search for common shared putative targets. A short list of seven genes (*Wnt5a, Smurf1, Sema5a, Smad3, Foxp1, Fosl2* and *RhoV)* previously involved in myogenesis (Wang et al., 2004; Suwanabol et al., 2012; Mazzotta et al., 2016; Jahangiri et al., 2016; Koefoed et al. 2018) were assessed, demonstrating that *miR-195* overexpression lead to up-regulation of all these genes, except Sema5a, further supporting their plausible involvement in *miR-195* driven cardiomyogenesis in PE/ST explants. Furthermore, silencing of *Smad3* and *Smurf1* lead to significant downregulation of early and terminally differentiation markers. Importantly, application of *miR-195* in s*iSmad3* and *siSmurf1* treated PE/ST explants was unable to rescue the expression of cardiomyogenic lineage markers. Thus, these data demonstrate for the first time that *miR-195* application modulates expression of *Smad3* and *Smurf1*, factors that are essential to promote cardiomyogenesis. It remains unclear whether Smad3 and *Smurf1* up-regulation by miR-195 is a direct or an indirect effect. Future experiments will be designed to unravel the molecular mechanisms, although it is important to highlight that microRNAs can directly increase mRNA stability (Daimi et al., 2015). Overall, these data provide novel insights into the molecular mechanisms whereby *miR-195* administration exerts increased cell differentiation into the cardiomyogenic lineage, *i.e.* by regulating the expression of *Smad3* and *Smurf1*.

Long non-coding RNAs represents a novel emerging class of non-coding RNAs with highly diverse cellular functions (Caley et al., 2010). Tissue-specific expression of lncRNAs has been widely reported in distinct biological settings, including the

cardiovascular system (Greco & Condorelli, 2015). Seminal studies by Klatenhoff et al. (2013) reported the functional role of Braveheart, a mesoderm-restricted lncRNA essential for normal lateral plate mesoderm formation and thus cardiac development. Similarly, the function role of handful set of lncRNAs have been reported such as *Fendrr, Carmen, Upperhand* and *Tbx5ua* (Grote et al., 2013; Ounzain et al., 2015; Anderson et al., 2016; Hori et al., 2018). However, to date, no lncRNAs has been reported during PE and epicardium formation. We provide herein a systematic analysis of lncRNAs neighboring key growth factors and transcription factors involved in PE and epicardium development and we identify three lncRNAs with enhanced expression in the PE. Secondly, we demonstrate that all three of them and distinctly regulated by cardiomyogenic inductive signals such as *thymosin-β4* (Smart et al., 2011) and Bmp (Kruithoff et al., 2003) administration as well as by repressive signals, *i.e. Fgf* signaling. These data support a plausible role for these lncRNAs in PE/ST cardiomyogenic differentiation. Regulatory effects of lncRNAs upon microRNAs has been widely reported in the cardiovascular system (Zhang et al., 2018) as well as in other biological settings (Li et al., 2018ab). However, evidence of microRNA regulation of lncRNAs is still scarce. We provide herein evidences for the first time that microRNAs can modulate the expression of lncRNAs. *MiR-195* administration exerts opposite regulatory effects as compared to *miR-23* and *miR-27* supporting a role for these lncRNAs in PE/ST *miR-195* driven cardiomyogenesis. Surprisingly, *miR-223* did not affect the expression of these lncRNAs, suggesting a microRNA-specific modulation. In sum, our data opened up new pathways to identify and dissect the functional role of microRNAs and lncRNAs in PE/ST development as well as for their putative application as therapeutic tools to enhance myocardial formation from the epicardium.

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6.2 CHAPTER 2

Gene regulatory networks involved in proepicardium to embryonic epicardium transition

6.2. Chapter 2

Gene regulatory networks involved in proepicardium to embryonic epicardium transition

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Abstract

The heart is the first organ that becomes functional in the vertebrate embryo. The precardiac mesoderm forms a primitive tubular heart, which starts beating at stage approximately embryonic day (E) 8.0-E.8,5 in mice and subsequently invariably bends to the right configuring prospective atrial and ventricular chambers. Thereafter, each embryonic cardiac region will be separated into distinct left and right components. In addition, cell populations external to the initial cardiogenic fields also migrate into the developing heart, providing roles in sculpturing the embryonic heart, such as the epicardium and its derivatives.

The proepicardium (PE) is a transitory cell cluster that develops at the junction between the sinus venosus (SV) and the posterior undifferentiated lateral plate mesenchyme, that subsequently migrate onto the heart and spread over the surface forming a single squamous epithelium, i.e. the epicardium. Our understanding of the cell lineage contribution of the epicardium and the epicardial derived cells has greatly increased over the last decade, while our understanding of the molecular determinants remains poorly understood. In this study, we carried out a comprehensive characterization of the coding and non-coding gene expression hallmarks at two critical timepoints during proepicardium (PE) and embryonic epicardium (EE) development. Our data identified a large array of mRNA, microRNAs and lncRNAs differentially expressed in PE vs EE. GO analyses demonstrate that such DEGs preferentially regulate distinct biological pathways. Furthermore, gene regulatory networks based on microRNA-mRNA complementary expression identified important novel pathways plausible involved in PE to EE transition.

Introduction

The heart is the first organ that becomes functional in the vertebrate embryo. Approximately 24 h after its induction, the precardiac mesoderm forms a primitive tubular heart, which starts beating at stage approximately embryonic day (E)8.0-E.8,5 in mice. 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).In addition, cell populations external to the initial cardiogenic fields also migrate into the developing heart, providing roles in sculpturing the embryonic heart (Yelon, 2001), i.e the cardiac neural crest and the epicardium and its derivatives.

The proepicardium (PE) is a transitory cell cluster that develops at the junction between the sinus venosus (SV) and the posterior undifferentiated lateral plate mesenchyme (Rossy et al., 2001; Ishii et al., 2007; Schulte et al., 2007) at E8,5-E9.0 in the mouse embryo (Schulte et al., 2007; Rodgers et al., 2008). Myocardial signals induce epicardial cells to undergo an epithelial-to-mesenchymal transformation (EMT) and either populate the sub-epicardial mesenchyme or migrate into the myocardial wall. In chicken embryos, PE grows in size and villous projections extend towards the dorsal aspect of the cardiac inner curvature, ultimately contacting at the AV junction and forming a tissue bridge. Subsequently, PE cells migrate onto the heart and spread over the surface forming a single squamous epithelium which is termed the epicardium (Nahimey et al., 2006). Importantly, two PE primordia are initially formed bilaterally, but only the right one develops. This asymmetry is proposed to be under the control of the right determining molecular system including Fgf-8Snai1 (Schlueter et al., 2009). Fgfs are also suggested to support PE proliferation, thereby sustaining PE identity and preventing PE cells from differentiating into cardiomyocytes (Kruithof et al., 2006; Torlopp et al., 2010). Low Bmp2 concentrations also maintain PE identity by promoting expression of Tbx18 and Wt1, whereas high Bmp2 concentrations induce the differentiation of PE cells into cardiomyocytes (Kruithof et al., 2006; Schlueter et al., 2006). All these events require highly patterned expression of Bmp2 in the PE/septum transversum region (Kruithof et al., 2011) and thus it can be postulated to play a role in establishing the posterior myocardial limit of the developing heart.

In mouse embryos, proepicardial villous projections attach to the heart, passively elongate by retraction of the beating heart tube, ultimately collapse and leave a vesicular patchwork on the myocardial surface (Rodgers et al., 2008). In lower vertebrate embryos the PE share similar morphological features as in chicken embryo. *Xenopus* PE can be first found approximately 3 days of embryonic development (Jahr et al., 2008), exclusively strictly located on the right side as a cluster of mesothelial cells that colonizes

the ventricle via a distinct and persisting tissue-bridge (Jahr et al., 2008). In zebrafish, the epicardium covering the ventricle derives mainly from the atrioventricular canal proepicardial cluster (avcPE) and to lesser extent, from the venous pole proepicardial cluster (vcePE) (Peralta, Gonzalez-Rosa, Marques and Mercader., 2014). Transfer of cells from these two PE clusters occurs through their release into the pericardial cavity and subsequent adhesion to the ventricle. In the sturgeon proepicardial development is observed as a bilateral cluster of cells between the sinus venosus and the ducts of Cuvier (Icardo et al., 2009). Curiously, the most primitive chordate studied to date, i.e. the lamprey, develops bilateral PE anlagen around 17 days post fertilization but only the right cell cluster forms a transient tissue bridge to the heart (Pombal et al., 2008). In summary, the occurrence of a distinctive cluster of PE cells secondarily colonize the heart is feature found in all vertebrates. PE formation across the different vertebrate classes, displays also some significant differences, due to species-specific morphological peculiarities, including asymmetrical development and distinct cell transfer processes to the heart (Schlueter & Brand, 2011).

The epicardium is the outermost layer of the heart. For many years, the functional role of this monolayer was considered as an external cover devoid of any functional meaning, but in later studies the discovery that epicardial precursors are essential contributors to the developing heart, can differentiate into beating cardiomyocytes and it has branded the epicardium as a source of cardiac stem cells with therapeutic potential and cardiac regeneration. The epicardium is embryologically formed by the outgrowth of proepicardial cells over the naked heart tube (Lie-Venema et al., 2007) that subsequently migrate into the myocardium and differentiate into different cell types, configuring thus the epicardial-derived cells (EPDCs).

Seminal approaches demonstrate that EPDCs contribute to distinct cardiac cell lineages, such as endothelial and smooth muscle cells in the coronary vasculature, endocardial mesenchymal cells in the atrioventricular cushions and also cardiac fibroblasts (Poelmann et al., 1993; Mikawa & Gourdie, 1996; Dettman et al., 1998). Contribution to endocardial cushions is scarce, although it has been proposed that these cells are important for the correct development of the atrioventricular junctions and the annulus fibrosus (Lie-Venema et al., 2008; Zhou et al., 2010; Lockhart et al., 2014). More recently, a contribution to cardiac resident stem cells (mesenchymal-like) has also been reported (Chong et al., 2001) as well as to the cardiomyocyte lineage (Zhou and Pu, 2001; Cai et al., 2008, Zhou et al., 2008), yet this latter point remains highly controversial (Christoffels et al. 2009; Dueñas et al., 2017).

While our understanding of the cell lineage contribution of the epicardial derived cells has greatly increased over the last decade, our understanding of the molecular determinants that contribute to such cell fate decision remains poorly understood. In this study, we carried out a comprehensive characterization of the coding and non-coding gene expression hallmarks at two critical timepoints during proepicardium (PE) and embryonic epicardium (EE) development by RNAseq analyses in mouse embryos. Our

data identified a large array of mRNA, microRNAs and lncRNAs differentially expressed in PE vs EE. GO analyses demonstrate that such DEGs preferentially regulate distinct biological pathways in PE vs EE. Furthermore, gene regulatory networks based on microRNA-mRNA complementary expression identified important novel pathways plausible involved in PE to EE transition.

Materials & Methods

Mouse Lines and Tissue Collection

Previously described $Wt1^{GFP/+}$ mice used in this study (refs). Pregnant $Wt1^{GFP/+}$ female mice were harvested to E9.5 and to E10.5, respectively. E9.5 PE were manually dissected, pooled and stored in liquid nitrogen until used. E10.5 EE was FACS-sorted as previously described, pooled and stored in liquid nitrogen until used. At least 3-5 litters were used on each developmental stage until sufficient tissues were collected that would guarantee optimal RNA isolation.

MiRNAseq Library Preparation, Sequencing and Proccesing of FastQ Files

500 pg of total RNA were used to generate barcoded miRNA-seq libraries using the *Bioo NEXTflex Small RNA* (BiooScientific). Briefly, 3´ and 5´ SR adapters were first ligated to the RNA sample. Next, reverse transcription followed by PCR amplification was used to enrich cDNA fragments with adapters at both ends. Adapter-ligated cDNA fragments from different samples were pooled and run in a 6% polyacrilamide gel. The 147 nt band, corresponding to the pooled miRNA libraries, was purified from the gel. Finally, the quantity and quality of the pooled miRNA libraries were determined using the Agilent 2100 Bioanalyzer High Sensitivity DNA chip. Libraries were sequenced on a HiSeq 2500 (Illumina) and processed with RTA v1.18.66.3. FastQ files for each sample were obtained using bcl2fastq v2.20.0.422 software (Illumina). Sequencing reads were aligned to the mouse reference genome (mm10) with HISAT2 v2.10.0 (Kim *et al.* 2015) and then extracted the miRNA counts with featureCounts (Liao *et al*. 2014) and miRBase (Kozomara *et al*. 2014) GFF3 for mouse. Raw counts were normalized with TPM (Transcripts per Million) and TMM (Trimmed Mean of M-values) methods, transformed into log2 expression (log2(rawCount+1)) and compared to calculate fold-change and corrected pValue. The limits for the differential expression were Log2FC > 0.584 (1.5x) and corrected pValue < 0.05. Only miRNAs detected in the three replicates of any condition were use in the analysis.

mRNAseq Library Preparation, Sequencing and Proccesing of FastQ Files

First 2.5 ng of total RNA were used to amplify the cDNA using the *SMART-Seq v4 Ultra Low Input RNA Kit* (Clontech-Takara). 1 ng of amplified cDNA was used to generate barcoded libraries using the *Nextera XT DNA library preparation kit* (Illumina). Basically, cDNA is fragmented, and adapters are added in a single reaction followed by an amplification and clean up. The size of the libraries was checked using the Agilent 2100 Bioanalyzer High Sensitivity DNA chip and their concentration was determined using the Qubit® fluorometer (ThermoFisher Scientific). Libraries were sequenced on a HiSeq 2500 (Illumina) and processed with RTA v1.18.66.3. FastQ files for each sample were obtained using bcl2fastq v2.20.0.422 software (Illumina). Sequencing reads were aligned to the mouse reference transcriptome (mm10 v92) and quantified with RSem v1.3.1 (Li and Dewey. 2011). Raw counts were normalized with TPM (Transcripts per Million) and TMM (Trimmed Mean of M-values) methods, transformed into log2 expression (log2(rawCount+1)) and compared to calculate fold-change and corrected pValue. The limits for the differential expression were Log2FC > 0.584 (1.5x) and corrected pValue < 0.05. Only mRNAs detected in three transcriptomes were use in the analysis.

RNA Isolation and qPCR

RNA samples from the same pools used for RNAseq libraries construction were used; namely, those corresponding to E9.5 PE cells and E10.5 FACS sorted EE cells. All qRT-PCR experiments followed MIQE guidelines (Bustin et al., 2009) and similarly as previously reported (Bonet et al., 2015; Lozano-Velasco et al., 2015). Briefly, RNA was extracted and purified by using Trizol reactive (Invitrogen) according to the manufacturer's instructions. For mRNA expression measurements, 1 μg of total RNA was used for retro-transcription with Maxima First Strand cDNA Synthesis Kit for RT-qPCR (Thermo Scientific). Real time PCR experiments were performed with 1 μL of cDNA, SsoFast EvaGreen mix and corresponding primer sets as described on Supplementary Table 1. All qPCRs were performed using a CFX384TM thermocycler (Bio-Rad) following the manufacturer's recommendations. The relative level of expression of each gene was calculated as described by Livak & Schmittgen (2001) using *Gapdh* and *Gusb* as internal control for mRNA expression analyses and *5S* and *6U* for microRNA expression analyses, respectively. Each PCR reaction was carried out in triplicate and repeated in at least three distinct biological samples to obtain representative means.

Results

RNAseq Libraries and Output Data

Mouse heterozygous Wt1-GFP E9.5 PE were manually dissected (n=20) and pooled to obtain three independent biological samples. In addition, mouse E10.5 Wt1-GFP embryonic hearts were minced and GFP+ cells were FACS-sorted and pooled, obtaining

three independent biological samples (n=3-5 hearts per each biological sample). RNAseq libraries for detection of microRNAs and mRNA/lncRNAs in these two distinct stages of epicardial development were constructed and sequenced. microRNA libraries yielded an average of $5*10^6$ reads ($5{,}25*10^6 \pm 1*10^6$) and mRNA/lncRNA libraries an average of $35*10^6$ reads ($36{,}5*10^6 \pm 2{,}5*10^6$) mRNA reads alignment was successfully obtained in approximately 85-90% of the total input resulting on the identification of ~12500 genes. microRNA reads alignment yielded lower inputs, of 40-50% of the total and identified ~200 microRNA expressed in both conditions.

Analyses of mRNA and microRNA Differentially Expressed Genes

In order to identify those genes that might be involved in governing the transition between the proepicardium and its detachment and subsequent attachment to the myocardial surface, we have identified those differentially expressed genes (DEGs), including therein microRNAs, mRNAs and lncRNAs, using as a selection criteria those genes displaying a log2 FC >1 and FDR $p<0.05$. Under these conditions, we have identified 536 mRNA up-regulated in the proepicardium (PE) as compared to the embryonic epicardium (EE) (**Table 1**), whereas 750 display the opposite pattern, down-regulated in the PE as compared to the EE (**Table 2**) (**Figure 1A**). Validation analyses by qPCR confirmed the differential expression of these DEGs (**Figures 1C and 1D**). In this context, it is important to highlight that transcription factors such as *Hoxb1* and *Prox1* are enriched in the PE9.5, whereas *Spry1* and *Hey2* are enriched in the EE at 10.5.

Gene ontology analyses using DAVID functional annotation chart of those DEGs that are up-regulated in the PE display significant association with genes involved in GO terms such as angiogenesis, positive regulation of cell migration, regulation of cell shape and cell adhesion (GO BP), extracellular space and extracellular exosome (GO CC) as well as to KEGG pathways such as focal adhesion, regulation of actin cytoskeleton and cytokine-cytokine receptor interaction (**Table 3**). On the other hand, gene ontology analyses of those DEGs that are up- regulated in the EE10.5 display significant association with genes involved in GO terms such cholesterol and triglyceride homeostasis, retinoid and steroid metabolic processes (GO BP), extracellular space, membrane and cell surface (GO CC) as well as KEGG terms such as complement and coagulation cascades and cell adhesion molecules (**Table 4**). These data illustrate that differential expression of genes in PE and EE are involved in distinct biological pathways, which are consistent with cell-cell interaction and cell migration at the PE stage and cholesterol/triglyceride homeostasis and retinoid and steroid metabolism at EE stage.

Analyses of differentially expressed microRNAs identified 10 microRNAs that are highly expressed in the PE9.5 as compared to the EE10.5, whereas 8 microRNAs display the opposite pattern, i.e. low expression in PE9.5 and high expression in EE10.5 (**Table 5**)(**Figure 1B**). Gene ontology analyses of differentially expressed microRNAs using DAVID functional annotation revealed significant association to KEGG pathways such

as microRNAs in cancer for both PE9.5 and EE10.5 as detailed in **Table 6**. Curiously, only one GO BP term is shared, i.e. gene silencing by microRNAs, whereas the others are exclusively specific for each developmental condition (**Table 6**). These data therefore suggest distinct functional role for up-regulated microRNAs in PE9.5 as compared to EE10.5, in line with previous findings in mRNA GO analyses.

LncRNAs display an averaged lower expression levels as compared to protein coding RNAs. Even so, our RNAseq analyses identified 119 lncRNAs that are highly expressed in the PE9.5 as compared to the EE10.5 (**Table 7)**, whereas 329 lcnRNAs display the opposite pattern, i.e. low expression in PE9.5 and high expression in EE10.5 (**Table 8**). These data demonstrate therefore an important contribution of lncRNAs to DEGs in the PE to EE transition, yet their functional implications remain to be elucidated.

Most Abundantly Expressed microRNA and mRNA Expression in PE to EE Transition

Differentially expressed genes provide hints about those genes that might be relevant for the progression from PE to EE. Additionally, RNAseq data provide an important repository of data that allow to identify those highly abundant genes in both conditions, providing therefore clues about their importance within the global developmental process. We have therefore identified those highly expressed mRNA, microRNAs and lncRNAs in PE and EE, respectively, taking as such those genes that display higher expression levels from the sum of the mean and two standard deviations. **Tables 9 to 14** display the mRNA, microRNAs and lncRNAs with highest expression data and their corresponding GO analyses, respectively. Among those 16417 genes detected by RNAseq in the PE and EE, 531 genes were highly expressed in the PE and 476 genes in the EE. Most of these genes, i.e. 386 (62%) were shared in both conditions, whereas 145 (33%) were exclusively highly expressed in the PE and 90 (15%) in the EE (**Table 9**) (**Figure 1E).** GO BP analyses display of most expressed mRNAs shared between PE and EE stages were protein folding, toxin transport, positive regulation of protein localization to Cajal body (**Table 10**) whereas for those PE restricted ones were mRNA processing, cell cycle and mitotic nuclear division as shown in **Table 11**, and EE restricted genes display distinct GO BP pathways, highlighting apoptotic process, skeletal muscle development and response to iron ion (**Table 12**). Importantly, none of the GO BP terms are similar in shared vs PE/EE specific ones, whereas only one GO CC term is shared for all three, extracellular exosome (**Tables 10-12**).

We also conducted the identification of those most up-regulated microRNAs in PE and EE stages, respectively. Application of the mean plus two standard deviations threshold identify 4 highly expressed microRNAs in PE and 4 microRNAs in EE (**Table 13**). Comparison between them unrevealed that only 2 (33%) were shared in both conditions, whereas 2 (33%) where uniquely highly expressed in PE and only 2 (33%) were in EE (**Figure 1F**). These data demonstrate a highly dynamic profile of microRNAs

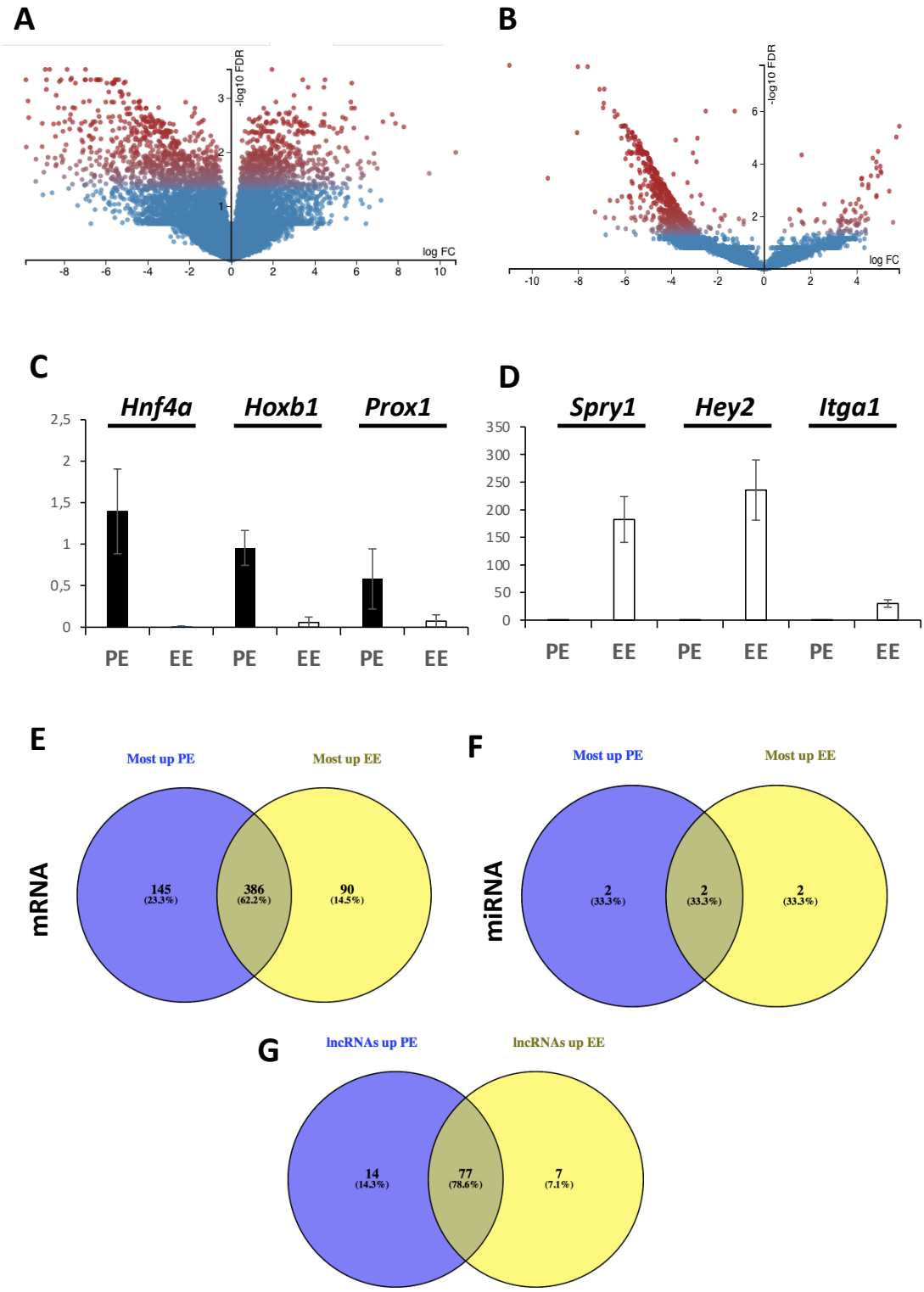

Figure1. Representation of differentially expressed genes (DEGs) involved in the epicardium transition, detachment and attachment to the myocardial surface processes, including mRNAs and lncRNAs (panel A) and microRNAs (panel B). Genes displaying a log2 FC >1 and FDR p<0.05 are only taken into account for a relevant significance. Validation analysis by qPCR of DEGs (mRNAs) (panels C and D). Panels **E-G** are

Venny representation of most abundantly expressed genes in PE and EE, representing mRNA (panel E), microRNAs (panel F) and lncRNAs (panel G).

during PE EE development, in contrast to a more conservative profile in protein coding RNAs.

Similarly, we also conducted the identification of those most up-regulated lncRNAs in PE and EE stages, respectively. Application of the mean plus two standard deviations threshold identify 91 highly expressed lncRNAs in PE and 84 lncRNAs in EE (**Table 14**). Comparison between them unrevealed that most of them were shared in both conditions, 77 (79%), whereas 14 (14%) where uniquely highly expressed in PE and only 7 (7%) were in EE (**Figure 1G**).

Unravelling Novel Growth Factor Signaling Pathways in PE to EE Transition

Previous studies have demonstrated a fundamental role of distinct signaling pathways in PE and EE development, such as Bmp2/Bmp4 and Fgf2/Fgf8 signaling among others, mainly based on candidate approaches (Kruithof et al., 2006; Schlueter et al., 2006). Using RNAseq technology provide us with a unique tool to systematically screen the relative contribution of all distinct family members of the signaling pathways. We have therefore search for the expression profiles of all embryonic signaling pathways, including therein *Bmp, Fgf, Tfg, Wnt, Notch and Hh* signaling. Previous studies identified *Bmp2* and *Bmp4* as key elements governing ST/PE development (Kruithof et al., 2006). We provide herein evidences that beside *Bmp2* and *Bmp4*, also *Bmp5*, *Bmp7* and *Bmp10* are highly expressed in PE/EE, suggesting a plausible role for these growth factors in PE/EE development. Furthermore, *Bmpr1a* is the most abundant *Bmp* receptor, followed by *Bmpr2* and *Bmpr1b*, respectively, illustrating the preferentially pathway used in these cells **(Figure 2A)**.

Previous studies highlighted the importance of *Fgf2* and *Fgf8* in ST/PE development (Kruithof et al., 2006;). We provide herein evidences that several additional *Fgf* family members are highly expressed in the PE/EE such as *Fgf5, Fgf7, Fgf9, Fgf10, Fgf18* and *Fgf22*. Importantly, Fgf8 is not detected while *Fgf2* is preferentially detected in EE10.5, suggesting some temporal and/or species-specific differences in the functional role of these *Fgf* members during PE/EE development. In relation to the *Fgf* receptors, all of them are expressed, with significant differences both in levels and stage specific, e.g. *Fgf1r* and *Fgfr4* are exclusively detected in PE but not in EE, while *Fgf2r* and *Fgf3r* are similarly expressed at both stages **(Figure 2A)**.

Similarly, as for Bmp and *Fgf, Tgf* signaling has previously reported to modulate PE development (Olivey et al., 2006). We demonstrate herein that *Tfg-α* is highly expressed in PE while *Tfg-β2 and Tfg-β3* are the most abundant ligands expressed in the EE. Among the Tgf-beta receptors, *Tgfbr1* and *Tgfbr3* are prominently expressed in the PE, while

Tfg2r is similarly expressed in both PE and EE stages **(Figure 2A)**. Overall, these data demonstrate novel insights into the role *Tgf* signaling in the transition from PE to EE.

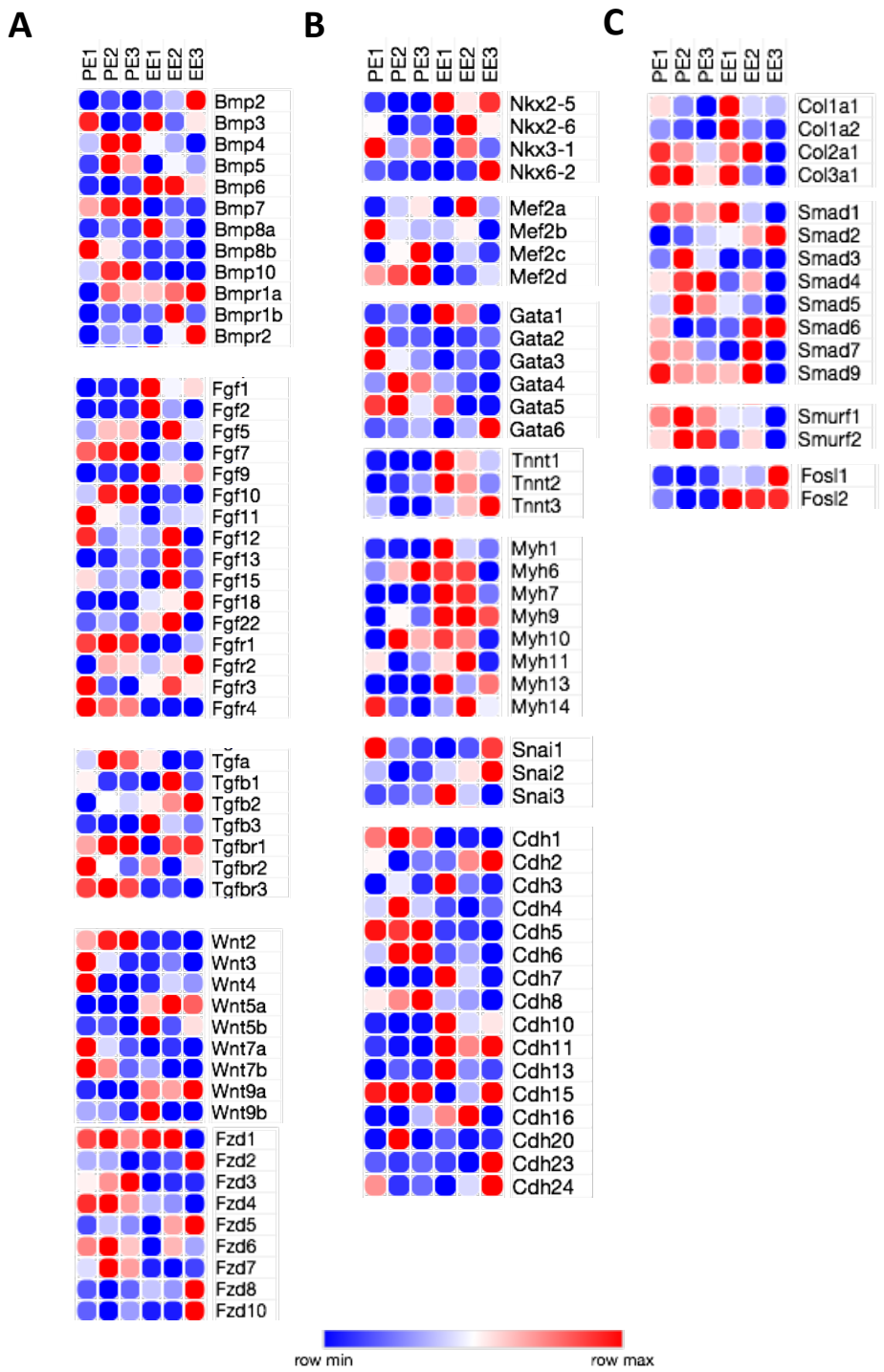

Figure 2. Panel **A.** Heatmap representation of *Bmp, Fgf, Tgf* and *Wnt* signalling during PE to EE transition. *Bmp2, Bmp4, Bmp5, Bmp7* and *Bmp10* are highly expressed in PE/EE, being also *Bmpr1a* the most highly

expressed *Bmp* receptor, followed by *Bmpr2* and *Bmpr1b. Fgf5, Fgf7, Fgf9, Fgf10, Fgf18* and *Fgf22* are highly expressed in the PE/EE. *Fgf8* is not detected at all, while *Fgf2 i*s preferentially detected in EE10.5. All of the *Fgf* receptors are expressed. Significant differences are observed in *Fgfr1* and *Fgfr4,* which are observed exclusively in PE but not in EE. Others such as *Fgfr2* and *Fgfr3* are expressed at both stages. *Tfga* is expressed in PE, *Tgfb2* and *Tgfb3* are most abundant ligands expressed in EE. *Tgfbr1* and *Tgfbr3* are predominantly expressed in PE; *Tgfbr2* is similarly expressed in PE and EE. *Wnt2, Wnt5a, Wnt5b* and *Wnt9a* are highly expressed in PE/EE with different expression patterns in PE and EE stages**.** Panel **B.** Heatmap representation of transcriptional regulators of the *Nkx, Mef2* and *Gata* families as well as EMT related markers of the *Snai* and cadherin families during PE to EE transition. *Nkx2.5* expression is high in EE compared to PE, while in *Nkx2.6, Nkx3.1* and *Nkx6.2* such expression does not elucidate any specific trend between stages. *Mef2a* and *Mef2c* expression profiles are the most representatives with similar patterns in both stages. *Gata1* expression is higher in EE, while *Gata4* and *Gata6* display a similar expression pattern in both stages. In the other hand the receptors *Gatad1, Gatad2b* and *Gatad2a* show a similar display in PE/EE, while *Gata5* expression comparatively is low. *Snai2* is highly expressed in both stages but *Snai1* and *Snai3* display the opposite expression pattern. *Twist1* and *Twist2* are similarly expressed at low levels while *Zeb1, Zeb2* are highly expressed. *Prrx1* is also highly expressed but with a lower fold chain. *Cdh1, Cdh2, Cdh3, Cdh6* and *Cdh11* are the only genes that show expression profiles. From them, *Cdh1* shows a higher expression in PE, while *Cdh2* and *Cdh3* display a similar expression pattern in PE/EE. *Cdh11* is overexpressed in EE, while *Cdh6* is more expressed in the PE**.** Panel **C.** Heatmap representation of *collagen, Smad*, *Smurf* and *Fosl* family members during PE to EE transition.

Wnt signaling on the other hand has not been directly related to date with PE/EE development. Our data reveal that three distinct *Wnt* signaling member, *Wnt2, Wnt5a, Wnt5b* and *Wnt9* are highly expressed in the PE/EE, displaying all of them differential expression between PE and EE stages **(Figure 2A)**. These data therefore identify for the first time the plausible role of *Wnt* signaling in PE/EE development and moreover it suggests a stage-specific role. A wide variety of *Frizzled* receptors are expressed during the PE to EE transition. Importantly, it is important to highlight that *Fzd3, Fzd4, Fzd6* and *Fzd7* display high expression levels in the PE decreasing in the EE, while only *Fzd5* displays the opposite expression pattern.

Transcriptional Control of Proepicardium (PE) to Embryonic Epicardium (EE) Transition

Beside growth factors, several transcription factors have been involved in PE/EE. In fact, several reports have identified that lineage-restricted transcription factors such as *Gata* and *Tbx5* family members are expressed already in the PE suggesting that distinct cell lineages are already determine at this stage (Hatcher et al. 2004; Basson et al., 2004; Watt et al., 2004; Zhou et al. 2008). We have scrutinized the expression profiles of cardiomyogenic transcription factors (*Nkx, Mef2* and *Gata*) and differentiation markers (troponin and myosins) (**Figure 2B**).

For *Nkx* members, expression is detected for *Nkx2.5, Nkx2.6, Nkx3*.1 and *Nkx6.2*, displaying *Nkx2.5* a trend to high expression in PE as compared to EE **(Figure 2B)**. Among the *Mef2* family members, *Mef2a* and *Mef2c* are the most represented ones **(Figure 2B)**, while for the *Gata* family members, all six members are identified, being

Gata1, Gata4 and *Gata6* the most highly expressed **(Figure 2B)**. *Troponin* constitute genuine molecular markers of the striated muscle, while myosin displays a wider molecular class of contractile molecules, being distributed in basically all cell types. Our RNAseq analyses demonstrate that all three troponin types are expressed at low levels in the PE only peaking up in the EE. In the case of the myosin, both non-muscle and striated muscle myosin display relatively high expression levels in both PE and EE; e.g. *Mhy7*, *Mhy9* and *Mhy13* are more prominently expressed in the EE while *Mhy6* and *Mhy10* are similarly expressed at both stages (**Figure 2B**). Overall these data suggest that both the PE and EE are heterogeneous cell population displaying striated muscle-specific gene expression characteristics.

Profiling of EMT Regulatory Pathways

PE cells are meant to actively engage into an epithelial-to-mesenchymal transition (EMT) during their journey to the embryonic epicardium as well as during the subsequent phase of migration into the embryonic myocardium leading to the formation of the epicardial derived cells. Several transcription factors have been previously involved in PE/EE EMT, such as *Snail* family members. We provide herein evidences that *Snai2* is highly expressed but not *Snai1* and *Snai3*, in line with previous reports **(Figure 2B)**.

Cadherins are also involved in the EMT process, often displaying an inverse expression profile to the EMT regulators such as *Snail, Zeb* and *Twist*. Our data demonstrate that multiple cadherin types are expressed in the PE and EE tissues, displaying distinct expression pattern*; Cdh1, Chd5, Chd6* and *Chd8* are mostly expressed only in the PE whereas *Chd2, Chd10* and *Cdh11* display complementary pattern (**Figure 2B**). Cell-matrix interaction are also crucial to EMT development. A key component of the extracellular matrix is formed by collagens. We provide evidence herein that PE and EE cells produce distinct collagen types, particularly *Col2a1* and *Col3a1* are abundantly expressed in the PE while their expression diminishes in the EE, while *Col1a1* and *Col1a2* display similar profile at both stages.

In Chapter I, we have demonstrated that miRNAs such as *miR-195* and *miR-223* are capable of modulating cell fate differentiation of PE cells, increasing their commitment to the cardiomyogenic lineage. Furthermore, we demonstrate that *miR-195* directs such actions by modulating *Smad3* and *Smurf1* expression but not *Fosl2*. We scrutinized herein also the expression levels of *Smad, Smurf* and *Fosl* family members in the PE to EE transition. *Smad1-9* are all expressed in PE/EE providing novel clues about the plausible functional role of several *Smads* that have not been previously reported in PE to EE transition (**Figure 2C**). Curiously all but *Smad3* are similarly expressed at both stages. *Smurf1* and *Smurf2* are highly expressed in the PE declining their expression in the EE whereas *Fosl1* and *Fosl2* display the opposite pattern (**Figure 2C**).

Identification of microRNA-mRNA *cross-talk* in PE EE Development

Previous studies have identified microRNA-mRNA *cross-talk* correlations by searching for opposite patterns between mRNA and microRNAs in distinct experimental conditions. Using miRComb software, we have searched for all putative candidate microRNAs that target each of the differentially expressed mRNA within high expression in the PE as compared to the EE. Among those, we have subsequently identified those microRNAs that displaying the opposite pattern, i.e. low in PE and high in EE. **Figure 3** illustrates such microRNA-mRNA gene regulatory networks. Five distinct microRNAs with enhanced expression in the PE (*miR-205, miR-200, miR-122, miR-10, miR-375 and miR-409*) display complementary pattern to distinct mRNAs with the opposite pattern (PE<EE). *miR-205* and *miR-200* display a wide range of interactions, supporting a more relevant functional role, as compared to *miR-122, miR-10, miR-375* and *miR-409*. On the other hand, five distinct microRNA with enhanced expression in the EE (*miR-877, let-7b, let-7c, miR-351, miR-760*) display complementary pattern to distinct mRNAs with the opposite pattern (PE>EE). *Let-7b, let-7c* and *miR-351* display a wide range of interactions, respectively, as compared to *miR-877* and *miR-760*. Overall, these data identify novel putative microRNA-mRNA interactions that might be functionally important during PE/EE development.

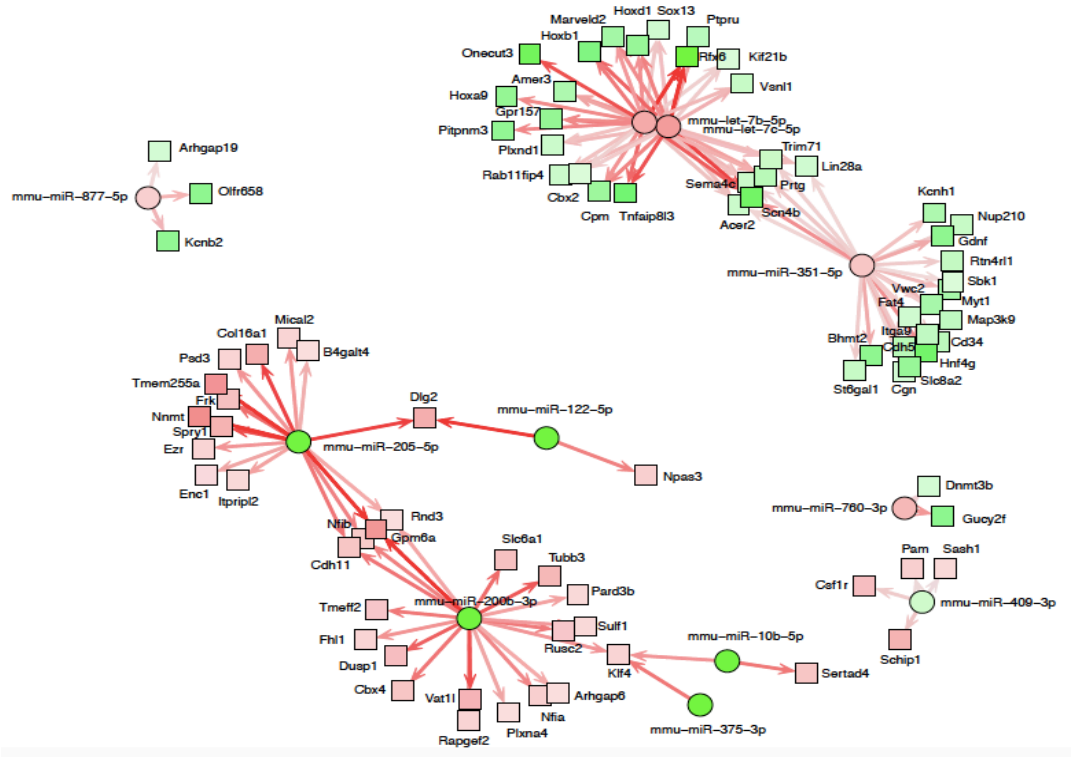

Figure 3. Schematic representation of microRNA-mRNA interactions in PE and EE developmental stages as revealed by miRComb software. Observe that five distinct microRNAs with enhanced expression (green) in the PE (*miR-205, miR-200, miR-122, miR-10, miR-375 and miR-409*) display complementary pattern to distinct mRNAs with the opposite pattern (red; PE<EE). *miR-205* and *miR-200* display a wider range of

interactions as compared to *miR-122, miR-10, miR-375* and *miR-409.* Similarly, five distinct microRNAs with enhanced expression (red) in the EE (*miR-877, let-7b, let-7c, miR-351, miR-760*) display complementary pattern to distinct mRNAs with the opposite pattern (green; PE>EE). *Let-7b, let-7c* and *miR-351* display wider range of interactions as compared to miR-877 and miR-760.

Discussion

In this study we provided a comprehensive analysis of gene expression during PE to EE transition, identifying a large number of differentially expressed mRNAs, microRNAs and lncRNAs during this developmental process. Such a comprehensive catalogue will allow us to unravel novel gene regulatory networks involved in PE to EE development. Our findings identified *Raldh2, Tcf21* and *Tbx18* differential expressed in PE/EE stage, in line with previous reports that demonstrate their key functional role of these genes during PE development (Carmona et al. 2001; Perez-Pomares et al., 2002; Ishii et al., 2007; Martinez-Estrada et al., 2010; Guadix et al., 2011; Wu et al., 2013; Mommersteeg et al., 2010), validating thus our RNAseq approach. More importantly, our data identified novel differentially expressed transcription factors such as *Hoxb1* that was not previously reported in the PE/EE development and Prox1 that was scarcely previously reported to be expressed in the PE/EE (Wilting et al., 2007; Niderla-Bielińska et al., 2015). Thus, our data support a plausible functional role in PE>EE transition. GO analyses of DEG during PE>EE transition demonstrate that PE up-regulated genes are mostly associated to cell-cell interaction and cell migration while EE up-regulated genes are associated to cholesterol/triglyceride homeostasis and retinoid and steroid metabolism. These data illustrate therefore that differential expression of genes in PE and EE are involved in distinct biological pathways. Our data thus identified new players involved in these cellular and molecular regulatory pathways involved, ensuring new experimental strategies to be pursued.

MicroRNAs play fundamental roles in multiple biological context (Bartel, 2009, 2018), including the epicardium, as recently reported by Singh et al., (2011), yet our understanding of the functional role of discrete microRNAs in this context remains scarce (Brønnum et al. 2013; Dueñas et al., 2017). We have identified 8 microRNAs up-regulated in the PE (*mmu-miR-7071-5p, mmu-miR-6236, mmu-let-7c-5p, mmu-miR-483-5p, mmu-let-7b-5p, mmu-miR-760-3p, mmu-miR-351-5p, mmu-miR-877-5p*) and 10 microRNAs up-regulated in the EE (*mmu-miR-375-3p, mmu-miR-122-5p, mmu-miR-122b-3p, mmu-miR-292a-3p, mmu-miR-292b-5p, mmu-miR-200b-3p, mmu-miR-10b-5p, mmu-miR-205-5p, mmu-miR-412-3p, mmu-miR-409-3p*), and none of them have been previously reported to be expressed and/or to play any functional role during PE/EE development. *Let-7b* have been repetitively detected in human pericardial fluid exosomes promoting angiogenesis (Beltrami et al., 2017) and also in human heart failure pericardial fluid it was significantly up-regulated (Kuosmanen et al., 2015). Thus, our data provide for the first time the assessment of microRNAs-enriched expression during PE>EE development. While none of these microRNAs have been previously reported in PE/EE

development, many of them have been reported to play fundamental roles in biological processes that are essential in PE/EE and its subsequent cell diversification, i.e. epithelial to-mesenchymal transition (*let-7c;* Hou et al., 2018; Li et al., 2009; *miR-483;* Zhang et al., 2019; Song et al.,2014; *let-7b;* Sohn et al., 2016; *miR-375;* Wang et al., 2016; Fu et al.,2017; *miR-122;* Wang et al., 2020; Duan et al. 2018; Jin et al., 2016; *miR-200;* Liu et al., 2018: Choi et al., 2017; Sulaiman et al., 2016, Bracken et al., 2015; Hilmarsdottir et al., 2014; *miR-10;* Zhang et al., 2015; *miR-205;* Xiao et al., 2019; Zhang et al., 2019; Dai et al., 2019; *miR-409;* Josson et al., 2014), displaying both negative and positive regulation of EMT mainly in oncogenic processes. GO analyses of DEG microRNAs display similarly distinct regulatory pathways in PE vs EE, in line with mRNA DEG data. Thus, these data support the notion and open up the possibility to explore the fundamental roles that these microRNAs might play in PE/EE development.

LncRNAs are relatively new players in gene regulatory mechanisms. To date, no evidence of differential expression and/or functional role for lncRNAs have been reported in the proepicardium and/or epicardium. Yet our RNAseq analyses identified 119 lncRNAs that are highly expressed in the PE9.5 as compared to the EE10.5 and whereas 329 lncRNAs with low expression in PE9.5 and high expression in EE10.5. Thus, our data represent an open field to dissect the functional role of lncRNAs during epicardial development. Importantly, lncRNA-microRNA interactions have been wide reported for DEG microRNAs identified in this study, including pivotal roles in EMT progression (Li et al., 2018; Shu et al., 2018; Hajarnis et al., 2015; Exposito-Villen et al., 2019).

In addition to the identification of DEGs, our RNAseq data allowed us to identify those most abundantly expressed genes in each developmental condition, i.e. PE and EE, respectively. Highly abundant genes might provide clues about the most relevant pathways operating within these cells at these developmental stages. Interestingly, a bulk of the most abundantly expressed genes (mRNAs and lncRNAs) are shared in both conditions, supporting conserved signaling pathways, such as protein folding, toxin transport, positive regulation of protein localization to Cajal body and extracellular exosome. In this context, it is importantly to highly the key signaling role of exosomes in distinct normal and pathological conditions (Mun et al., 2019; Li et al., 2020; Chen et al., 2020; Wang et al., 2020; Pan et al., 2020; Dougherty et al., 2020), including the heart (Kuosmanen et al., 2015;Chistiakov et al., 2016; Beltrami et al., 2017; Sahoo et al., 2018; Pellegrini et al., 2020). Thus, it is plausible that PE an EE development might signal during cardiac development by generating exosome that might mediate epicardial-myocardial communication, a hypothesis that will be pursued in future experiments. Importantly, a subset of highly abundant genes are specific to each developmental conditions, supporting the notion that they would play specific stage-specific developmental roles. In fact, PE restricted ones are mainly involved in mRNA processing, cell cycle and mitotic nuclear division whereas EE restricted genes display distinct GO BP pathways, highlighting apoptotic process, skeletal muscle development and response to iron ion. Future experiments will be designed to unravel the role of these stage-specific pathways during PE/EE development.

The morphogenetic processes leading to the development of the proepicardium and its subsequent conversion of the embryonic epicardium have been thoroughly addressed in different experimental models such as the sturgeon (Icardo et al., 2009), bony fishes (Peralta et al. 2013, 2014; Liu et al., 2010), chicken (Gittenberger-de Groot et al., 1998; Perez-Pomares et al., 1998; Vrancken-Peeters et al. 1995, 1999; Männer et al., 2001, 2005; Nahirney et al. 2003), rat (Nesbitt et al.,2006) and mice (Schulte et al., 2007; Cossette & Misra, 2011; Plavicki et al., 2014; Niderla-Bielińska et al., 2015, 2016; Jankowska-Steifer et al., 2018). *Bmp* and *Fgf* signaling have been identified play essential roles on the formation of the proepicardium (Kruithof et al., 2006; Schlueter et al., 2006, 2009; Liu & Stainier, 2010; Ishii et al., 2010; Svensson, 2010; Andres-Delgado et al., 2019), particularly for *Bmp2/Bmp4*, *Fgf2/Fgf8*. Our RNAseq data demonstrate that beside the high expression levels of several of these previously reported *Bmp/Fgf* family members, we also identified that *Bmp5, Bmp7* and *Bmp10* are highly expressed in the PE. To date no information is available about the functional role of Bmp5 and Bmp10 whereas no effect on PE EMT has been reported for *Bmp7* (Olivey et al., 2006). *Fgf7* and *Fgf10* also display high PE expression yet no functional role in PE development have been reported in mice while in chicken it seems to have a modest effect (Torlopp et al., 2010). Tgf and Wnt signalling contribution to PE development have been scarcely investigated (Olivey et al., 2006; Zamora et al., 2007; Buermans et al., 2010). Our data demonstrate that *Tgf-α* and *Wnt2/Wnt7* are highlight expressed in the PE but declining in the EE, supporting a plausible role for these signaling factors in PE to EE development.

Besides growth factors, transcriptional activation of key cardiovascular-enriched transcription factors have also been reported during PE formation, such as *Tbx5* and Gata4 among others (Hatcher et al. 2004; Basson et al., 2004; Watt et al., 2004; Zhou et al. 2008). Here we provide evidences that *Nkx3.1, Mef2d* and *Gata5* are also highly expressed in the PE. Additional experiments will provide evidences on their plausible role in PE development.

Several studies have reported that cell specification of distinct epicardial-derived cell already occurs at the proepicardium stage (Hatcher et al. 2004; Basson et al., 2004; Watt et al., 2004; Zhou et al. 2008; Wilting et al., 2007; Cossette & Misra, 2011; Niderla-Bielińska et al., 2015, 2016; Jankowska-Steifer et al., 2018). Particularly controversial is the evidence of whether the PE can give rise to cardiomyocytes (Cai et al., 2008; Christoffels et al., 2009). Our data revealed that several types of myosin, including non-muscle (*Myh10*) and muscle (*Mhy6*) myosins are expressed in both developmental stages, while troponin expression is only observed in the EE stage. These data support the notion that the cardiac-specific expression program might be already initiated at the proepicardial stage.

Epithelial to mesenchymal transition (EMT) is a key developmental process involved in multiple biological contexts such as heart development, including PE/EE development and its subsequent developmental stages (Morabito et al., 2001; Perez-Pomares et al., 2002; Compton et al., 2006; Olivey et al., 2006; Pennisi & Mikawa, 2009; Artamonov et

al., 2015; Singh et al., 2016). Whether EMT is already initiated in PE remains controversial. We provide herein evidences that key components of EMT initiation such as *Snai1* and *Snai2* are distinctly expressed during PE to EE transition. In addition, differential expression of key cadherin components such as *Cdh1* (*E-cadherin*) and *Cdh5* (*VE-cadherin*), previously reported to be involved in EMT remodeling (Kokkinos et al. 2010; Wong et al., 2018; Loh et al. 2019; Song et al., 2019; Bure et al., 2019), are identified during PE to EE transition. Thus, these data suggest that EMT program is already activated at the PE stage. Importantly, additional cadherins are also distinctly expressed during PE to EE transition, such as *Cdh6* (*K-cadherin*), *Cdh8* and *Chd15* (*M-cadherin*). None of these cadherins (*Cdh6, Chd8, Cdh15*) have been previously reported to be expressed during cardiac development, thus our data support new evidences to investigate their functional role during PE/EE development.

It is increasingly acknowledged that complex gene regulatory networks regulate multiple biological events, including cardiovascular development. Until recently such regulatory networks were mainly based on growth factor signaling cascades, transcription factor activators and repressor and target genes. Over the last decades our increasing understanding of the genome complexity has unraveled novel players in such gene regulatory networks, i.e. non-coding RNAs such as microRNAs and lncRNAs. Our RNAseq data have provided us with a highly valuable snapshot of the differential expression of mRNAs, microRNAs and lncRNAs during mouse PE to EE transition. An exploratory analysis using miRComb software provided us with plausible miRNA-mRNAs interactions based on their complementary pattern on PE vs EE expression. We identified five distinct microRNAs with enhanced expression in the PE (*miR-205, miR-200, miR-122, miR-10, miR-375 and miR-409*) display complementary pattern to distinct mRNAs with the opposite pattern (PE<EE). It is important to highlight in this context the *miR-205/Spry1* interaction, since it might be an importantly regulator of growth factor signaling (Huebert et al., 2004; Lagha et al., 2008; Lee et al., 2010) and EMT progression (Brønnum et al. 2013). Furthermore, *miR-200* and *miR-205* might co-regulate *Cdh11/Nfib* and *Rnd3/Gpm6a* supporting a functional role on EMT (Schneider et al., 2012; Yao et al., 2016; Chen et al., 2019) and cell migration (Jie et al., 2015; Honda et al., 2017; Gu et al., 2018; Formoso et al., 2015, 2017; Alvarez Julia et al., 2016), respectively. Curiously, Klf4 is potentially regulated by *miR-200*, *miR-10* and *miR-375*, yet its plausible functional role in PE/EE development remains unclear. Similarly, we identified five distinct microRNA with enhanced expression in the EE (*miR-877, let-7b, let-7c, miR-351, miR-760*). *Let-7b, let-7c* and *miR-351* display wider range of interactions, as compared to *miR-877* and *miR-760*. Importantly, *let-7b* and *let7c* might co-regulate *Hoxb1* and *Hoxd1* expression, yet their functional implication on PE/EE transition remains to be elucidated. To date, *Hoxb1,* but not *Hoxd1*, have been reported to be expressed during cardiac development (Bertrand et al., 2011 Roux et al., 2015; Zaffran et al., 2018). Furthermore, *let7b, let7c* and *miR-351* might co-regulate *Trim71*, *Lin28a, Sema4c, Acer2, Scn4b* and *Prtg* expression. *Trim71, Lin28a* and *Sema4* have been widely reported to play a role in cell migration in different biological contexts (Chang et al., 2012;Meda et al., 2012; Yin et al., 2016; Nguyen et al., 2017; Ren et al., 2017;Sun et al., 2017; Li et al., 2019), and

evidence of regulation for *Trim71* and *Lin28a* by *let-7* have also been reported (Lin et al., 2007). *Acer2* has been recently reported to induce autophagy and apoptosis (Wang et al., 2017). Scn4b, an auxiliary subunit of sodium channels is widely expressed (Bouza & Isom, 2018; Miyazaki et al., 2014; Aman et al., 2009) yet evidence supporting a functional role beyond its electrophysiological coupling have been reported such as controlling proliferation and metastasis (Dai et al., 2020) and cell migration (Bon et al. 2016). *Protogenin* (*Prtg*) have been implicated in preventing apoptosis (Wang et al., 2013) and differentiation (Wong et al., 2010) in the neural cells and promoting cell adhesion in paraxial mesoderm (Ito et al., 2011). Thus, these data support the notion that *let7b, let7c* and *miR-351* might actively regulate key developmental processes such as proliferation, cell migration and differentiation during PE to EE transition.

Overall, these data identify novel putative microRNA-mRNA interactions that might be functionally important during PE/EE development. Future experiments will be designed to investigate the functional role of such gene regulatory networks. In addition, efforts will be also performed to construct lncRNA-mRNA and lncRNA-microRNA gene regulatory networks during PE to EE transition.

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7. DISCUSSION

Discussion

Cardiovascular development is a complex developmental process in which multiple cell lineages are involved. Extracardiac inputs to the developing heart such as the epicardial derived cells (EPDCs) represents a capital contributor. With development, cells derived from the proepicardium migrate towards the heart, leading to the formation of the embryonic epicardium (Peralta et al., 2013; Plavicki et al., 2014). Fibroblasts, endothelial and smooth muscle cells (Perez-Pomares et al., 1998, Carmona et al., 2010; Winter & Wittenberger-de Groot, 2007; Gittenberger-de Groot et al., 2010) are subsequently derived from the embryonic epicardium and curiously also differentiated beating cardiomyocytes but only in isolated *ex vivo* cultures (Kruithof et al., 2006), where growth factors, such as *Fgfs* and *Bmps* play a fundamental role. Proepicardium to embryonic epicardium transition and subsequently epicardial derived cells diversification are regulated by both transcriptional and post-transcriptional regulators. In this context, non-coding RNAs are emerging as novel players in cardiovascular development (Callis et al., 2007; Callis & Wang, 2008; Bonet et al., 2013; Yan & Jiao, 2016; Caley et al., 2010).

7.1. Differential mRNA gene expression during PE and EE development

In this study (Chapter II), we provide a comprehensive analysis of gene expression during the proepicardium to embryonic epicardium (PE to EE) transition in mice, identifying a large number of differentially expressed genes (DEGs), including mRNAs, microRNAs and lncRNAs that might be involved in the process. Analyses of DEG mRNAs allowed us to identify *Raldh2, Tfc21* and *Tbx18* as DEGs in PE/EE stages, in line with previous reports that demonstrate their key functional role during PE development (Carmona et al., 2001; Perez-Pomares et al., 2002; Ishii et al., 2007; Martinez-Estrada et al., 2010; Guadix et al., 2011; Wu et al., 2013; Mommersteeg et al., 2010). Our data also identified novel differentially expressed transcription factors such as *Hoxb1* that was not previously reported in the PE to EE development and *Prox1* that was sparsely reported to be expressed at these stages (Wilting et al., 2007; Nideria-Bielinska et al., 2015). Gene Ontology analyses of mRNA DEGs during PE to EE transition demonstrate that PE up-regulated genes are mostly associated with cell-cell interaction and cell migration, supporting thus a pivotal role on biological events such as EMT, of capital importance during PE to EE transition as previously demonstrated (Singh et al., 2013), while EE up-regulated genes are mostly associated to cholesterol/triglyceride homeostasis and retinoid and steroid metabolism. The functional relevance of such association remains to be further investigated. In sum, our data illustrate differential expression of genes in PE and EE that are involved in distinct biological pathways.

In addition to the identification of mRNA DEGs, our RNAseq approach allowed us to identify those most abundantly expressed genes in PE and EE stages, respectively, providing information about the most relevant pathways operating within these developmental stages. A bulk of the most abundantly expressed genes (mRNAs) are

shared between both stages, supporting conserved signalling pathways, such as protein folding, toxin transport, positive regulation to Cajal body and extracellular exosomes (Mun et al., 2019; Li et al., 2020; Chen et al., 2020; Wang et al., 2020; Pan et al., 2020; Dougherty et al., 2020). These findings are especially interesting due to the fact of the paramount control that exosomes exert via their surface-receptor mediated cell signalling. It is also important to remark, that a subset of these highly abundant genes are specific to each developmental condition, supporting the notion that they would play stage-specific developmental roles. In fact, PE restricted genes are mainly involved in mRNA processing, cell cycle and mitotic nuclear division, whereas EE restricted genes display distinct GO BP pathways such as skeletal muscle development and response to Fe2+ and the apoptotic process. These findings are quite interesting due to the importance of apoptotic events and the pathological consequences of its dysregulation as promising assets for novel therapies in different biological contexts, and thus it deserves to be further explored in the PE to EE transition.

Bmp and *Fgf* signaling have been identified to play essential roles in the formation of the PE (Kruithoff et al., 2006; Schlueter et al., 2006, 2009; Liu & Stalnier, 2010; Ishii et al., 2010; Svensson, 2010; Andres-Delgado et al., 2019), particularly on the existing balance between *Bmp2/Bmp4* and *Fgf2/Fgf8*. Beside the high expression levels of these previously reported *Bmp/Fgf* family members, our data in Chapter II further identified *Bmp5, Bmp 7* and *Bmp 10* as highly expressed genes. *Fgf7* and *Fgf10* also display high PE expression while no functional description in PE development in mice have been reported, in chicken it seems to have just a modest effect (Torlopp et al., 2010). *Tfg* and *Wnt* signaling contribution to PE development is barely known (Olivey et al., 2006; Zamora et al., 2007; Buermans et al., 2010), but our data demonstrate that *Fgf-α* and *Wnt2/Wnt7* are highly expressed in the PE but not in the EE, supporting a plausible role for these factors in the PE to EE transition.

7.2. Differential microRNA gene expression during PE and EE development

Differential expression of microRNAs have been widely reported in distinct biological settings including homeostatic and pathological contexts (Lim et al., 2005; Ji et al., 2007; Cao et al., 2013). One of them is the cardiac development (Wilson et al., 2010; Chinchilla et al., 2011; Cao et al., 2013), even though microRNAs profiling of the PE and EE had not been done yet to date. In Chapter I, using a candidate approach in chicken embryos, we provided evidence that multiple microRNAs display differential expression during PE and EE development. A large subset of microRNAs are up-regulated, increasing their expression, supporting a plausible role blocking or inhibiting the expression of mRNA target genes during PE to EE transition. On the other hand, another subset of microRNAs, display decreased expression, support roles in releasing repression of inductive signals while other show transition peaks of expression in HH24 as compared to HH32 EE stage, suggesting a plausible modulatory role in this event, probably affecting EMT onset (Perez-Pomares et al., 2002; van Tuyn et al., 2007; Smith et al., 2011).

To get further insights into the plausible functional role of microRNAs in PE and EE development, we scrutinized their expression profiles by RNAseq analyses in mouse embryos (Chapter II). We have identified 8 microRNAs up-regulated in the PE and 10 microRNAs up-regulated in the EE. Importantly, none of them have been previously reported to be expressed and/or play any functional role during the PE to EE transition, highlighting the novelty of our findings. *Let-7b* is one of them and has been detected in human pericardial fluid where it was significantly up-regulated (Kuosmanen et al., 2015). While none of all these microRNAs have been reported in the PE to EE, many of them play fundamental roles in biological processes such as cadherin control, cell polarity, cytoskeleton reorganization, mechanisms that are very important in EMT, and essential process in EE development. Importantly, GO analyses of microRNA DEGs show a similar pattern over pathway regulation PE *vs* EE, in line with mRNA DEGs data.

To sum up, it is interesting to point the existence of DEGs in the PE and EE with distinct functions, some of them, directly related to the heart development and the PE to EE transition control, while for others the exact function remains to be further elucidated. Similarly, we also identified DEG microRNAs at both stages of development. Their differential expression pattern might imply a potential regulatory function in the transition from PE to EE, even though the target genes and exact function for these microRNAs remain largely unknown, pending new pathways to be researched.

7.3. Dissecting the functional role of microRNAs in PE to EE development

Understanding the functional role of discrete microRNAs in the epicardium have only been provided for *miR-31* and *miR-21*, both of them controlling fibrogenic EMT by distinctly modulating *Islet1* (Bronnum et al., 2012) and *Pcd4/Spry1* (Bronumm et al., 2013) expression, respectively. Importantly, to the best of our knowledge, in Chapter I evidence reporting cardiomyocyte cell fate modulation by microRNAs during PE differentiation is presented for the first time. As stated in our results, a significant enhancement of cardiomyocyte terminal differentiation was provided by administration of *miR-223* and *miR-195* mimics, while a weaker activation was provided by *miR-125* and *miR-146* while only activation of early cardiogenic markers but not terminal differentiation was obtained for *miR-126*. On the other hand, *miR-23* and *miR-27* selectively inhibited cardiomyogenesis while *miR-100* and *miR-21* essentially display not significant enhancement. These data evidence the differential modulation by overexpression of these microRNAs in the PE-derived cardiomyogenic process.

Previous studies have reported the involvement of *miR-23* and *miR-27* in both cardiac development and pathology (Chinchilla et al., 2011), while miR-100 has only been reported as a protective agent of cardiomyocyte apoptosis (Chen et al., 2015). On the other hand, *miR-223* and *miR-195* have been reported in distinct cardiac pathologies (Lu et al., 2010; Zhu et al., 2011; Dai et al., 2014; Wang et al., 2015; Chen et al., 2016; Liu

et al., 2016; Zhang et al., 2018; Wang et al., 2018) but there is no evidence on their functional role during cardiac development so far. Furthermore, scarce evidences on the role of *miR-125* (Wang et al., 2014) and/or *miR-146* (Zhang et al., 2018) in cardiac development and pathology have been reported. On the other hand, *miR-126* represents a vascular specific microRNA and this fact was demonstrated by the embryonic lethality of *miR-126* deficient zebrafish (Fish et al., 2008). Furthermore, additional functional roles for *miR-126* in the vasculature have been extensively reported (Sun et al., 2018; Chistianov et al., 2016; Potus et al., 2015; Shcoeber et al., 2014). Our data demonstrate that an enhanced cardiomyogenic differentiation is exerted by *miR-195* and *miR-223* as compared to *miR-125*, *miR-146* and *miR-126*, while *miR-23* and *miR-27* blocked such cardiomyogenic differentiation. Thus, our findings open up the possibility of exploring these microRNAs as therapeutic tools to enhance cardiomyogenesis.

To understand the molecular mechanisms that drive promotion of cardiomyogenic terminal differentiation by *miR-223*, *miR-195*, *miR-125* and *miR-146* administration, we searched for common shared putative targets. A short list of seen genes (*Wnt5a, Smurf1, Sema5a, Smad2, Foxp1, Fosl2 and RhoV*) previously reported to be involved in cardiomyogenesis (Wang et al., 2004; Suwanabol et al., 2012; Mazzota et al., 2016; Jahangiri et al., 2016; Koefoed et al., 2018) were assessed, demonstrating that *miR-195* overexpression leads to up-regulation of all these genes, except *Sema5a*, further supporting their plausible involvement in *miR-195* driven cardiomyogenesis in PE to EE transition explants. Furthermore, silencing of *Smurf1* and *Foxp1* lead to significant downregulation of early and terminally differentiation markers. Thus, these data demonstrate for the first time that *miR-195* administration modulates expression of *Smurf1* and *Foxp1*, factors that are essential to promote cardiomyogenesis. Future experiments will allow to clarify whether *Smurf1* and *Foxp1* up-regulation by *miR-195* is directly or indirectly modulated.

Regulation of cardiac transcriptional factors such as *Mef2c, Gata4 and Nkx2.5* by microRNAs have been reported in different biological contexts (Yao et al., 2013; Liana et al., 2016; Wu et al., 2016). In striated muscle, *miR-27* and *miR-125* distinctly regulates *Mef2c* in cardiac and skeletal muscle cells (Chinchilla et al., 2011; Lozano-Velasco et al., 2015). Curiously, *miR-223* downregulation leads to *Mef2c* up-regulation in leukemia (Agatheeswaran et al., 2012) while no evidences have been reported for *miR-195* and/*or miR-146* modulating the expression of these early cardiomyogenic differentiation markers in striated muscle. Other factors such as *Tbx5* and *Gata4* among others (Hatcher et al., 2004; Basson et al., 2004; Watt et al., 2004; Zhou et al., 2008) have been reported during PE formation. In Chapter II, we provide evidence that *Nkx3.1*, *Mef2d* and *Gata5* are also highly expressed in mouse PE. Several studies have concluded that cell specification of distinct EPDCs already occurs at the PE stage (Hatcher et al., 2004; Basson et al., 2004; Watt et al., 2004; Zhou et al., 2008; Wilting et al., 2007; Cossete & Misra, 2011; Nideria-Bielinska et al., 2015, 2016; Jankowska et al., 2018), but it is controversial at some point the evidence of whether the PE can differentiate into cardiomyocytes *in vivo* (Cai et al., 2008; Christoffels et al., 2009). We revealed in Chapter

II that several types of myosin, including non-muscle type such as *Myh10* and muscle myosin such as *Myh6* are expressed in both developmental stages, while troponin expression is only observed in the EE stage. These data support the notion that the cardiac-specific expression programme might be already initiated at the PE stage. All in all, our data demonstrate for the first time the regulatory role of these microRNAs modulating expression of early and terminally differentiation cardiomyogenic markers in both PE and epicardial cell cultures as reported in Chapter I. Furthermore, high expression levels of cardiac-enriched transcription factors and sarcomeric proteins in PE/EE transition in mice (Chapter II) further underscore the plausible myocardial lineage contribution of PP/EE origin.

EMT is a key developmental process involved in multiple biological processes such as heart development, including the PE to EE development and its subsequent stages, providing mechanistic clues to the EPDCs, favouring their integration into the embryonic myocardium and subsequently differentiation into cell types such as endothelial cells, smooth muscle cells and fibroblasts (Smith et al., 2011; Morabito et al., 2001; Perez-Pomares et al., 2002; Compton et al., 2006; Olivey et al., 2006; Pennisi & Mikawa, 2009; Artamonov et al., 2015; Singh et al., 2016). In Chapter I proof that key components of EMT initiators such as *Snai1* and *Snai2* are expressed during PE to EE transition are provided, respectively. In this regard, we further investigated how the administration of these microRNAs influence EMT and fibrogenic differentiation. As a result, *miR-195* and *miR-23* can selectively down-regulate expression of EMT inducers such as *Snai1* and *Snai2* and up-regulate *Cdh1* expression without modulating *Cdh2* and *Cdh5*, in line with previous descriptions in other biological contexts (Singh et al., 2015; Liu et al., 2015; Yu et al., 2017; Yang et al., 2017). On the other hand, all the other microRNAs tested such as *miR-21, miR-27, miR-100, miR-125, miR-126, miR-223* and *miR-146* resulted in *Snail* and/or *Slug* up-regulation, while effects on cadherin expression is not always concomitant. Several of these microRNAs have been reported to promote EMT such as *miR-27, miR-100* (Zhang et al., 2011; Chen et al., 2014) while others can either promote or inhibit in different biological contexts (*miR-100, miR-126, miR-223, miR-21*) (Wang et al., 2014; Jiang et al., 2017; Jia et al., 2018; Liu et al., 2019; Ding et al., 2018; yang et al., 2018), in line with our findings. Importantly, we provide evidence for the first time on the involvement of *miR-125* and *miR-146* in EMT regulation. We suggest that up-regulation of EMT promoters and subsequent cytoskeletal remodelling represent uncoupled events in this setting, in line with previous reports during atrioventricular (AV) EMT modulation by microRNAs (Bonet et al., 2015). An alternative explanation would be that additional time is required to see such transcriptional changes in cadherin expression or the transcriptional overriding effects by microRNAs over-expression is occurring (Hill et al., 2014). Moreover, our research demonstrates that EMT is not required for PE to EE cardiomyogenic differentiation, since administration of *miR-223* can simultaneously induce both processes, i.e. EMT and cardiomyogenesis.

In summary, there is a number of microRNAs involved in cardiac development that have been described, though the microRNAs functional role in PE and its cell type

differentiation into the EE have been quite scarce to date. Our results in Chapter I revealed that the administration of microRNA mimics enhance cardiomyogenesis modulating genes such as *Smurf1* and *Foxp1*, while other microRNA mimics control the expression of cadherins and the EMT activators *Snail* and *Slug*. This fact, reinforce the idea that cardiomyogenesis in PE to EE transition can be EMT independent.

7.4. Identifying the emerging role of lncRNAs in PE to EE development

Long non-coding RNAs represent a novel emerging class of non-coding RNAs with highly diverse cellular functions (Caley et al., 2010). To date, no lncRNAs have been reported during PE and EE formation. In Chapter I and Chapter II of this study, we make a first approach to dissect the functional role of lncRNAs during epicardial development. We provide herein a systematic analysis of lncRNAs neighbouring key growth factors and transcription factors involved in PE and EE development and we identified three lncRNAs with enhanced expression in the PE as described in Chapter I. Secondly, we demonstrate that all three of them are distinctly regulated by cardiomyogenic promoting signals such as thymosin-β4 (Smart et al., 2011) and Bmp administration (Kruithoff et al., 2003) as well as by cardiomyogenic repressive signals such as Fgf signaling.

Importantly, evidence of microRNA regulation of lncRNAs is still sparse. In Chapter I we provide evidence for the first time that microRNAs can modulate expression of PE-enriched lncRNAs. *MiR-195* administration exerts opposite regulatory effects as compare to *miR-23* and *miR-27* supporting the role for these lncRNAs in PE to EE *miR-195* cardiomyogenesis. Surprisingly, *miR-223* did not affect the expression of these lncRNAs, suggesting a microRNA-specific modulation. In addition, identification of lncRNAs in Chapter II only provide a very limited number of lncRNAs with statistically significant differential expression. This fact might indeed be due to the 10-fold lower expression levels as average of lncRNAs expression as compared to mRNA levels or to the limitation of the bioinformatic approach used in this study. Future experiments will be focused on identifying the lncRNA DEGs during PE to EE transition. To sum up, our data opened up new pathways to identify and dissect the functional role of microRNAs and lncRNAs in the PE to EE development.

7.5. Gene regulatory networks in PE to EE transition

Non-coding RNAs play a capital role in the regulation of biological events such as heart development. Our data provide a glimpse of the differential expression of mRNAs, microRNAs and lncRNAs during the mouse PE to EE transition. Using *miRComb* software, we obtained a microRNA-mRNA interaction network based on their complementary expression pattern at PE vs. EE stage. We identified 5 microRNAs with enhanced expression in PE, displaying a complementary pattern to distinct mRNAs. It is important to remark in this context the *miR-205/Spry1* interaction, since it might be an

important regulator of growth factor signalling based on previous studies (Huebert et al., 2004; Lagha et al., 2008; Lee et al., 2010) and EMT progression (Bronnum et al., 2013). Furthermore, other microRNAs such as *miR-200* and *miR-205*, co-regulate EMT (Schneider et al., 2012; Yao et al., 2016; Chen et al., 2019) and cell migration (Jie et al., 2015; Honda et al., 2017; Gu et al., 2018; Formoso et al., 2015, 2017; Alvarez Julia et al., 2016). Similarly, in our RNAseq approach, we identified five distinct microRNAs with enhanced expression in the EE, co-regulating genes reported during cardiac development such as *Hoxb1*, genes implicated in cell migration in different biological contexts and sodium channel auxiliary subunit (*Scn4b*), that provides a quite relevant function to the cardiomyocyte contractile process, yet their functional implications on the PE to EE transition remain as yet unknown.

8. CONCLUSIONS

Conclusions

First Conclusion

We have identified a microRNA differential expression pattern at three epicardial developmental stages in chicken, ranging from PE/ST (HH17) to EE cultured embryo explants (HH24 and HH32), observing three expression pattern profiles; i) those increasing, ii) those decreasing and iii) those peaking at intermediate developmental stages. These findings support the notion that distinct microRNAs are involved in different developmental processes during epicardium formation.

Second Conclusion

We have determined that overexpression of *miR-125, miR-146, miR-195* and *miR-223* mimics enhance cardiomyogenesis in PE/ST and EE chicken culture explants, a process that *Smurf1* and *Foxp-1*-dependent for *miR-195* mimics treated PE/ST explants. On the other hand, no substantial changes and/or cardiomyogenic blocking effect was observed by *miR-21, miR-23, miR-27* and *miR-100 mimic* overexpression, respectively. Overall these data further underscore the notion that distinct microRNAs are involved in different developmental processes during epicardium formation.

Third Conclusion

We demonstrate that *miR-195* driven cardiomyogenesis occurs independently of the EMT process. Therefore, these data support the notion that PE cells can be directly specified into the cardiomyogenic lineage without the requirement of migrating and covering the myocardial surface and subsequently undergoing EMT, leading thus to EPDCs.

Fourth Conclusion

We have identified a total of 531 genes that are differentially expressed in the mouse PE and 476 in the EE. Identification of DEGs such as *Raldh2, Tfc-21* and *Wnt*, previously reported to be highly enriched in PE to EE transition, validate our approach. Importantly, we have identified novel DEG transcription factors such *Hoxb1* and *Prox1* in the PE, and *Spry1* and *Hey2* in the EE, supporting a plausible role of these genes in the PE to EE transition.

Fifth Conclusion

Our RNAseq approach in mouse PE and EE developmental stages, have allowed us to identify additional members of *Bmp* (*Bmp5, Bmp7, Bmp10*), *Fgf (Fgf5, Fgf7, Fgf9, Fgf10, Fgf18, Fgf22)*, *Tgf* (*Tgf-α, Tgf-β2, Tgf-β3*) and *Wnt (Wnt2, Wnt5a, Wnt5b, Wnt9)* signaling families described for the first time as DEG in the PE and EE, supporting the notion that they might play novel functional roles on the PE to EE transition.

Sixth Conclusion

We have determined the existence of a microRNA-mRNA interaction network between those mRNA and microRNA displaying complementary expression patterns, such as *miR-205/Spry1* network interaction that might play a role in EMT during PE/EE development. Thus, our microRNA-mRNA interaction network opens up new pathways for future research discovering novel regulatory pathways between microRNAs and their potential target genes during PE to EE transition.

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10. CURRICULUM VITAE

Curriculum Vitae

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- **Perfil**

Científico Biomédico con 9 años de experiencia en el campo Médico y de la Investigación Biomédica, principalmente en la Regulación de la Expresión Génica y Biología del Desarrollo del Corazón**.**

- **Educación**

PhD, Universidad de Jaén, Jaén, España
Noviembre 2013 – Noviembre 2020

Máster en Biotecnología y Biomedicina, Universidad de Jaén, Jaén España
Septiembre 2010 - Diciembre 2011

Licenciado en Biología, Universidad de Jaén, Jaén España
Septiembre 1997 – Diciembre 2001

Habilidades
Cultivos celulares/ explantes
Epigenética
Regulación de la Expresión Génica
Técnicas de Biología Molecular
Técnicas de Análisis de Imagen
Bioinformática

- **Formación Adicional**

Curso en técnicas de análisis de imagen, Universidad de Jaén
Octubre 2014 – Noviembre 2014

Curso en Cultivos Celulares, Universidad de Jaén
Mayo 2016 – Junio 2016

Curso de Electroforesis en geles de agarosa y poliacrilamida, Universidad de Jaén
Abril 2016 – Mayo 2016

Curso en Citometría de Flujo, Universidad de Jaén

Abril 2016 – Mayo 2016

Curso en técnicas de detección de anticuerpos ELISA, Universidad de Jaén
Mayo 2016 – Junio 2016

- **Competencias Adicionales**

Alta experiencia en el uso y manejo de programas informáticos MS Office, así como otras herramientas utilizadas en el análisis de datos científicos tales como GraphPad Prism, Thermo-Fisher Software, Leica Inc. Software para análisis de imágenes, Cytoscape e IGV entre otros, así como una dilatada experiencia en el manejo y uso de bases de datos entre los que destacan Gene Ontology, miR-Base, JASPAR y PHAST.

- **Experiencia Profesional**

Investigador/ Estudiante de Doctorado en la Universidad de Jaén
Octubre 2013 – Presente

Estudio del papel modulador de los microRNAs y lncRNAs en el desarrollo del epicardio en los modelos de pollo y ratón

Investigador en la Tokyo Medical and Dental University, Tokio, JP
Junio 2019 – Diciembre 2019

Determinación del potencial modulador del lncRNA Tbx5-AS1 así como identificación de nuevos lncRNAs asociados al gen Nkx2-5 y su potencial efecto regulador sobre el mismo.

Investigador en el Institut de Biologie du Développement de Marseille, France
Octubre 2015 – Febrero 2016

Determinación de la contribución a las diferentes líneas celulares en modelos de embriones ratón a partir del estudio del proceso de EMT que tiene lugar en las células primordiales de linaje en el Campo Secundario del Corazón

Investigador en el Laboratorio de Inmunidad y Alergia del Centro Hospitalario de Jaén
Abril 2011 – Diciembre 2012

Estudio de la relación existente entre pacientes crónicos de hepatitis C y la presencia de marcadores de enfermedades autoinmunes en la sangre periférica.

Técnico de Laboratorio en North Cumbria University Hospital-NHS, Carlisle, UK
Abril 2005 – Abril 2006

Realización de técnicas rutinarias en la manipulación y procesamiento de muestras de sangre y tejidos humanos para su posterior análisis, así como el manejo de la maquinaria de laboratorio necesaria para técnicas de laboratorio tales como PCR y técnicas de determinación de anticuerpos.

- **Contribuciones**

Participación en Weinstein Cardiovascular Development and Regeneration Conference 2018, Nara, JP
Mayo 2018

MiR-195b Enhances Cardiomyogenic Differentiation via Modulation of Wnt5A/Fosl2 on Chicken PE/PS explants

Participación en el Congreso de Análisis Clínico de Barcelona 2012, España
Abril 2012

Marcadores de Autoinmunidad en Pacientes Crónicos de Hepatitis-C

- **Idiomas**
  Español nivel nativo
  Inglés C1
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- **Links**
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- **Méritos**

Obtención de Ayudas de la EDUJA para la realización de estancias de investigación en Universidades Extranjeras para mención internacional de la tesis doctoral
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