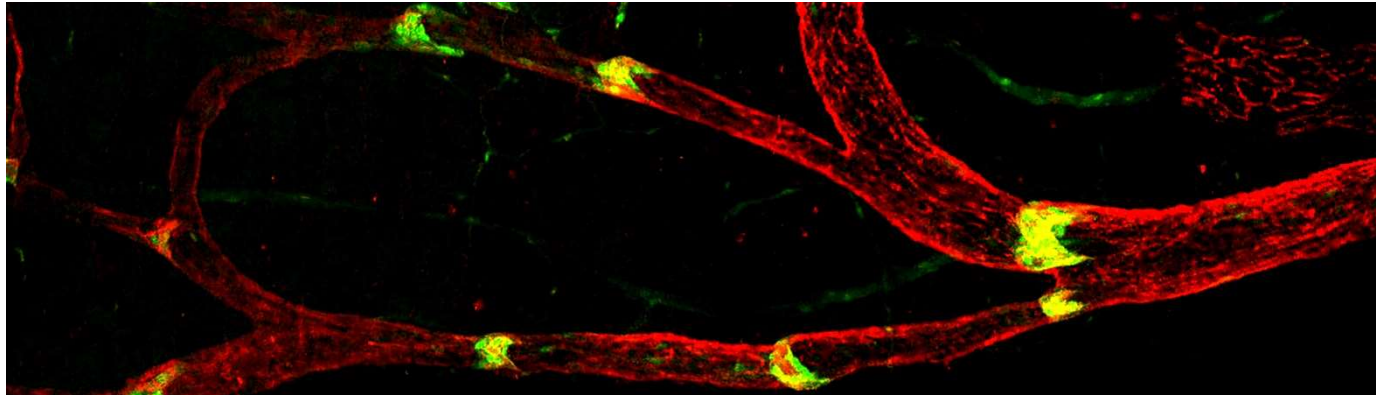
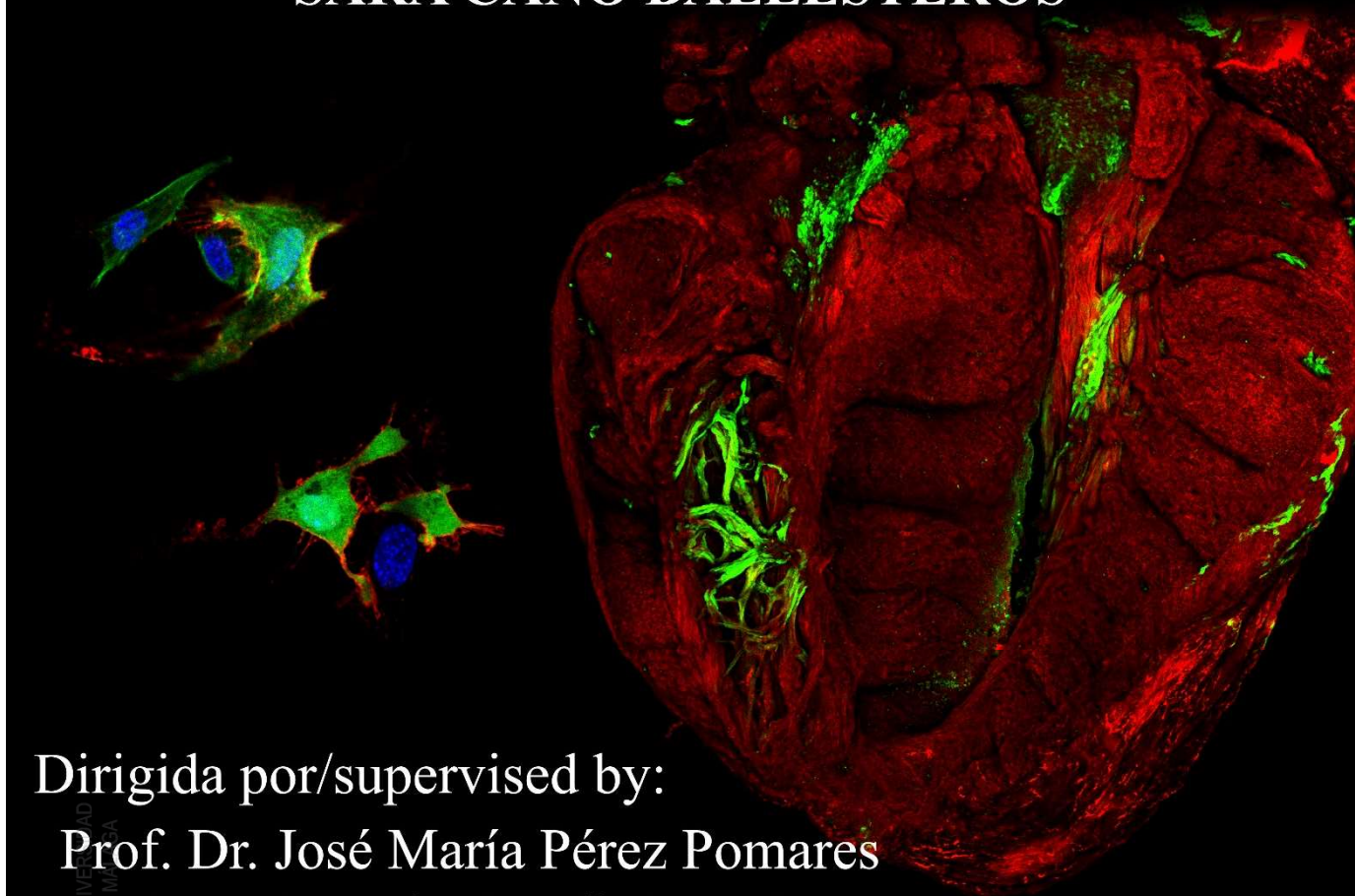


TESIS DOCTORAL / PHD THESIS

FSP1/S100A4 delineates distinct cardiac cell populations

SARA CANO BALLESTEROS



Dirigida por/supervised by:
Prof. Dr. José María Pérez Pomares
Dr. Juan Antonio Guadix Domínguez

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Tesis doctoral

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DEPARTAMENTO DE BIOLOGÍA ANIMAL

FACULTAD DE CIENCIAS

UNIVERSIDAD DE MÁLAGA

DECLARACIÓN DE AUTORÍA Y ORIGINALIDAD DE LA TESIS PRESENTADA PARA OBTENER EL TÍTULO DE DOCTOR

D./Dña SARA CANO BALLESTEROS
Estudiante del programa de doctorado BIOLOGÍA CELULAR Y MOLECULAR de la Universidad de Málaga, autor/a de la tesis, presentada para la obtención del título de doctor por la Universidad de Málaga, titulada: FSP1/S100A4 DELINEATES DISTINCT CARDIAC CELL POPULATIONS

Realizada bajo la tutorización de JOSÉ MARÍA PÉREZ POMARES y dirección de JOSÉ MARÍA PÉREZ POMARES Y JUAN ANTONIO GUADIX DOMÍNGUEZ (si tuviera varios directores deberá hacer constar el nombre de todos)

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Dña. Sara Cano Ballesteros, Licenciada en Biología, ha realizado en el Departamento de Biología Animal de la Facultad de Ciencias de la Universidad de Málaga las investigaciones contenidas en la siguiente memoria de Tesis Doctoral, titulada: “FSP1/S100A4 delineates distinct cardiac cell populations”.

Como directores de la tesis, consideramos que la presente memoria reúne todos los requisitos para ser sometida a juicio de la Comisión correspondiente, por lo que autorizamos su exposición y defensa pública para la obtención del Grado de Doctor en Biología con la mención de Doctor Internacional.

Y para que así conste, en cumplimiento de las disposiciones vigentes, firmamos la presente acreditación en Málaga, a 15 de noviembre de 2020.

Prof. Dr. José María Pérez Pomares

Dr. Juan Antonio Guadix Domínguez

Este trabajo ha sido realizado en el laboratorio “Desarrollo y Enfermedad Cardiovascular” (DeCA) perteneciente al Departamento de Biología Animal de la Facultad de Ciencias de la Universidad de Málaga, bajo la supervisión del Prof. Dr. José María Pérez Pomares y del Dr. Juan Antonio Guadix Domínguez. Parte de dicho trabajo ha sido realizado durante un periodo de corta estancia financiada por la Universidad de en el grupo “Applied Stem Cell Technologies” del centro University of Twente (Enschede, Países bajos) bajo la supervisión del Prof. Dr. Robert Passier.

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Además, parte de los resultados de esta investigación han sido publicados en revistas científicas y expuestos en congresos nacionales e internacionales en presentaciones de tipo póster:

Publicaciones en revistas científicas:

1. **Cano-Ballesteros, S.**, Palmquist-Gomes, P., Marín-Sedeño, E., Guadix, J. A. & Pérez-Pomares, J. M. 2020. Fsp1 cardiac embryonic

expression delineates atrioventricular endocardial cushion, coronary venous and lymphatic valve development. *Journal of Anatomy, in press.* doi: 10.1111/joa.13306

2. Ruiz-Villalba A., Mattiotti A., Gunst Q.D., **Cano-Ballesteros S.**, van den Hoff M.J., Ruijter J.M. 2017. Reference genes for gene expression studies in the mouse heart. *Scientific Reports. 2;7(1):24.* doi: 10.1038/s41598-017-00043-9.

Otras publicaciones:

1. **Sara Cano-Ballesteros**, Juan Antonio Guadix, José María Pérez-Pomares. 2018. La proteína Fsp1/S100A4 especifica distintas poblaciones cardíacas. Libro de actas del Workshop Internacional "Metabolic Reprogramming as a Target for Cancer and Other Diseases". *UMA editorial, Universidad de Málaga.* ISBN: 978-84-17449-56-8

Presentaciones en congresos científicos:

1. **Sara Cano-Ballesteros**, Juan Antonio Guadix, José María Pérez-Pomares. Characterization of FSP1+ cell populations in the developing and adult heart. ESC Working Group on Development, Anatomy and Pathology *2019. Málaga, Spain, 2019.* Póster.
2. **Sara Cano-Ballesteros**, Juan Antonio Guadix, José María Pérez-Pomares. FSP1/S100A4 identifies distinct non-myocardial populations. Metabolic Reprograming as a Target for Cancer and Other Diseases, Málaga, Spain, 2018. Póster.

Y una vez que la tormenta termine, no recordarás cómo lo lograste, cómo sobreviviste. Ni siquiera estarás seguro si la tormenta ha terminado realmente. Pero una cosa es segura. Cuando salgas de esa tormenta, no serás la misma persona que entró en ella. De eso se trata esta tormenta.

Haruki Murakami

The first part of this book, my doctoral thesis, is focused to thank all the people that made it possible. Let me do it in Spanish.

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A mi compañero de vida, Paul. Desde aquel 18 de noviembre en el que nos conocimos, siempre has sido mi brújula y mi mayor apoyo. A lo largo de estos 10 años hemos crecido juntos y hemos formado una familia que me hace sentir la mujer más feliz y afortunada del mundo. En este mundo de locos, nosotros somos dos locos felices que luchan por sus sueños y no se detienen ante las adversidades. Aquí, en París, en Costa Rica, o donde nos lleve el destino, porque estando contigo siempre estoy en casa. Gracias por ser mi hogar y mi refugio, por conocerme y entenderme, y por quererme tan bien como lo haces. Gracias por dar tanto de tí para ayudarme en este camino, por no haber soltado mi mano en ningún momento y por haber sido siempre mi inspiración, en la ciencia y en la vida. Esta tesis es también tuya.

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Contents

Chapter 1: Introduction

1.1. Heart development

1.1.1. Cardiogenesis and primary heart tube formation

The vertebrate heart, which is the earliest-functional organ in the Vertebrate embryo, has a major mesodermal origin (Brand, 2003). In the mouse embryo, approximately at embryonic developmental stage E5.75, the expression of the T-box transcription factor Eomesodermin (Eomes) in the prospective posterior part of the epiblast identifies the location of cardiovascular system precursors before gastrulation (Costello et al., 2011; Russ et al., 2000). After gastrulation, a subset of the primitive mesoderm is specified as precardiac mesoderm. This process involves the activation of *Eomes*, encoding for a transcription factor crucial to the activation of the transcription factor *Mesp1*, widely considered as the earliest cardiac progenitor marker (Saga et al., 1999). At E7.5, cardiac progenitors distribute forming a characteristic cardiac crescent formed by the convergence of Mesp1+ cells at the embryonic midline. Differentiation of these cells into cardiac muscle and endothelium begins with the activation of a complex regulatory program involving the transcription factors Nkx2.5, Gata4, SRF and Mef2c. A portion of these heart progenitors, known as the first heart field (FHF), transforms into the tubular heart primordium. These cells will further express the myocardial potassium channel HCN4 (Später et al., 2013) and the transcription factor Tbx5 (Bruneau et al., 1999) and progressively differentiate into the endocardium and myocardium of the left ventricle and parts of the atria (Zaffran et al., 2004).

1.1.2. Cardiac primordium formation

The progressive incorporation of other cardiac precursor populations located at the subpharyngeal mesoderm, known as the second heart field (SHF), will give rise to the myocardial, endocardial, smooth muscle and endothelial cells of the right ventricle, as well as to the cardiac inflow (sinus venosus and atria) and outflow (aortic and pulmonary arteries) domains (Cai et al., 2003; Christoffels et al., 2006; Galli et al., 2008; Kelly and Evans, 2010; Mjaatvedt et al., 2001; Waldo et al., 2001). This SHF cardiac progenitor lineage first expresses the transcription factor Islet1 (Cai et al., 2003; Zaffran et al., 2004) and then other ones like Mef2c (Lin et al., 1997), Tbx1 or Tbx5 (De Bono et al., 2018). As the developing ventricle elongates, it bends and twists to the right in a process named heart looping (Patten, 1922), which should be considered as the first morphological manifestation of vertebrate embryonic left-right asymmetry (Fig.1) (Desgrange et al., 2018; Hamada and Tam, 2014; Le Garrec et al., 2017; Taber, 2006).

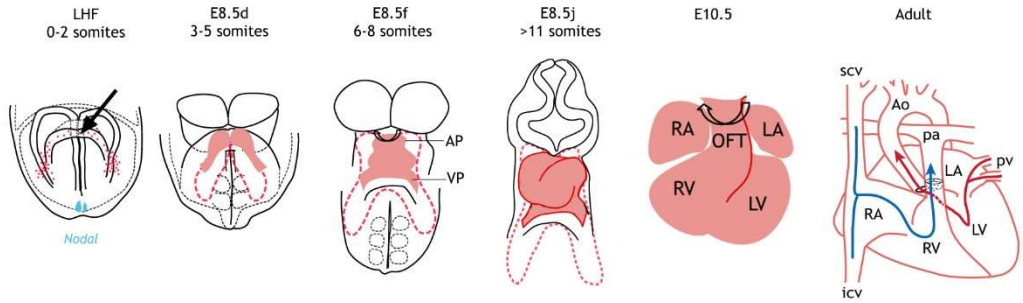

Figure 1: Sequential summary of heart development. The heart originates from the cardiac crescent at the embryonic midline. Heart looping breaks this early symmetry of the embryo and generates a four-chambered heart, with two atria and two ventricles. LA, left atrium; LV, left ventricle; OFT, outflow tract; RA, right atrium; RV, right ventricle. Modified from Desgrange et al., 2018.

The looped heart tube will remodel into a four chambered heart by the combination of complex series of phenomena involving the formation of cardiac valves and septa and the expansion of cardiac chamber domains

(ventricles and atria) occurring between stages E9 and E12 in the mouse developing heart (Christoffels et al., 2000). This latter process, known as heart ballooning, results in the enormous growth of the ventral side of the outer curvature of the heart and the maturation of the primitive myocardium of this region into a working myocardium displaying fast conduction; during this process the primitive myocardium at the inner curvature retains its primitive phenotype (Moorman and Christoffels, 2003) (Fig. 2).

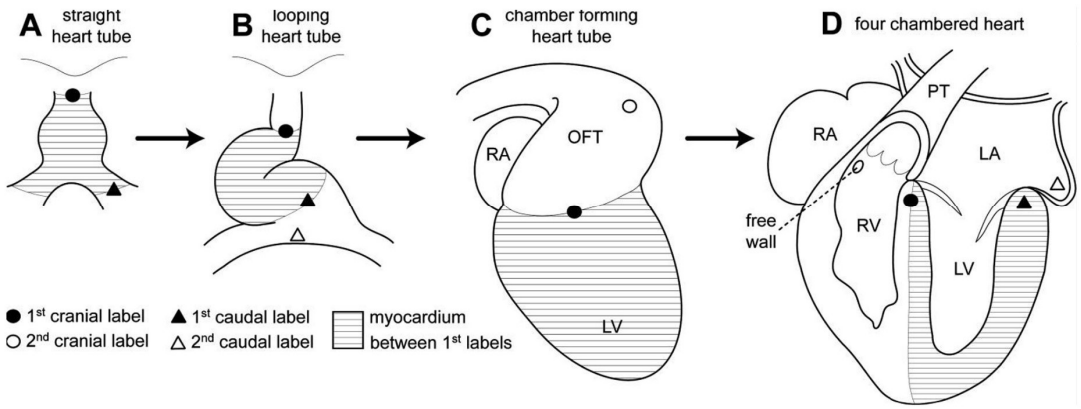

Figure 2: Ballooning model of cardiac chamber formation. Ventral views of a straight (a) and a looped (b) heart tube. Right (c) and ventral (d) view of a four-chambered heart. From van den Berg and Moorman, 2009.

All the events we have described generate a heart outline formed by myocardium and endocardial cells (the characteristic vascular endothelium lining the cardiac cavities) only. The heart, however, is still missing an external tissue layer, called epicardium, which has a delayed development with respect to the myocardium and the endocardium. The epicardium derives from a group of mesodermal progenitor cells, the proepicardium (PE), forming at E9.5 at the posterior limit of the heart tube (Virágh and Challice, 1981). The progressive attachment and migration of PE cells to the naked myocardium is an interesting process that varies between species. Different studies suggest that zebrafish and mouse embryonic epicardium is formed by the eventual attachment of intrapericardial free floating

proepicardial derived cell clusters to the myocardial surface (Andrés-Delgado et al., 2019; Komiyama et al., 1987). Other studies evidenced a direct attachment of the proepicardium to the atrioventricular groove in the chick embryo, allowing for the continuous migration of epicardial progenitors to the myocardial surface (Nahirney et al., 2003). PE cell transference to the myocardial surface will result in the formation of the epicardium, a monolayered squamous epithelium covering the surface of the whole heart.

In all studied vertebrate models, the epicardial coverage of the myocardium also allows for the spreading of some cells enclosed in the core of the PE. In parallel, the activation of an epithelial to mesenchymal transition (EMT) process (Hay, 1995) in the primitive epicardium takes place (Dettman et al., 1998; Missinato et al., 2015; Pérez-Pomares et al., 1998; von Gise and Pu, 2012). This EMT results in the migration and differentiation of epicardial derived mesenchymal cells (Zamora et al., 2007). These epicardial derived cells (EPDC) contribute to a wide variety of cardiac cell types, including cardiac fibroblasts (Acharya et al., 2012; Lie-Venema et al., 2007; Vega-Hernández et al., 2011), endothelial cells (Katz et al., 2012), smooth muscle cells (Smart et al., 2007) and adipocytes (Yamaguchi et al., 2015; Zangi et al., 2016). Although several studies suggest the embryonic epicardium could represent a potential source of functional cardiomyocytes (Cai et al., 2008; Smart et al., 2011; Zhou et al., 2008), this contribution remains controversial (Christoffels et al., 2009).

During heart development, the molecular communication between these three cardiac layers (endocardium, myocardium and epicardium) is essential for the proper formation and growth of the heart walls. Both epicardial (Chen et al., 2002; Pennisi et al., 2003; Stuckmann et al., 2003) and endocardial (Brutsaert, 2003; Narmoneva et al., 2004) paracrine or juxtacrine signals are key to myocardial growth and maturation (MacGrogan et al., 2016; Olivey and Svensson, 2010). This reveals the importance of

tissue interactions during heart development, which have been shown to be required for the formation of cardiac structures relevant to this thesis such us the atrio-ventricular (AV) valves, the cardiac conduction system and cardiac coronary vasculature.

1.1.3. Morphogenesis: shaping cardiac structure and function

Cardiac valves

Adult cardiac valves form from structures named cardiac cushions (Markwald et al., 1977; Person et al., 2005). During mouse development, these primitive cushions are observed at E10.5 at the atrioventricular (AV) and conoventricular (CV) regions. These structures are endocardial-lined extensive accumulations of extracellular matrix (ECM, a.k.a. cardiac jelly), mainly including chondroitin sulphate proteoglycans and hyaluronan. Endocardial EMT will seed this cardiac jelly with valvuloseptal mesenchymal cells that sustain ECM synthesis and will eventually give rise to adult valvular interstitial cells (VICs) (De Lange et al., 2004; Hinton and Yutzey, 2011; Lincoln et al., 2004). Valves cushion formation is a dynamic process regulated by different signaling pathways including BMP (Luna-Zurita et al., 2010), Notch (MacGrogan et al., 2016) and TGFß (Camenisch et al., 2002); and their inhibitors Nfatc1 (De La Pompa et al., 1998; Ranger et al., 1998), Nf1/Ras (Lakkis and Epstein, 1998) and Fog2 (Flagg et al., 2007).

For many years, an endocardial origin for all valvuloseptal cells was generally assumed (Eisenberg and Markwald, 1995). However, recent studies demonstrated that circulating bone marrow-derived cells (Hajdu et al., 2011), neural crest-derived cells (Jiang et al., 2000; Phillips et al., 2013), and/or epicardial-derived cells (Wessels et al., 2012) contribute to the valvuloseptal population. From E14.5 onwards, the gradual remodeling of

endocardial cushions results in the formation of the leaflets of cardiac valves (Fig. 3). Postnatal remodeling of these leaflets is key to comply with the increasing vascular pressures of the adult heart as well as for the efficient functioning of blood circulation through the heart (Kruithof et al., 2007; Leu et al., 2001).

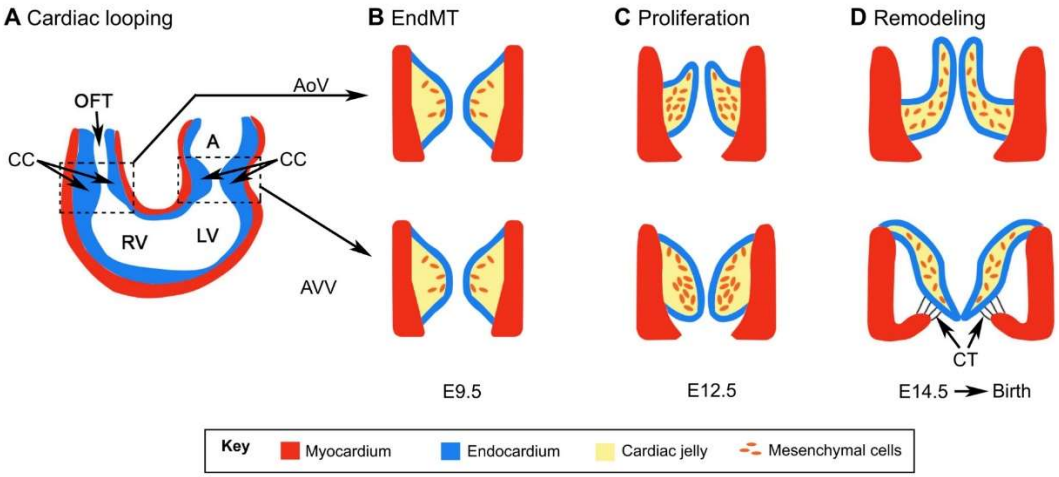

Figure 3. Valve cushions formation. The tubular heart consists of an inner endocardial (blue) and an outer myocardial (red) layer. The cardiac cushions form at the OFT (left box) and the atrioventricular (right box) canals (A). During the formation of the cushions, the endocardial cells acquire a mesenchymal phenotype (B), known as valvular interstitial cells (VICs, orange). VICs proliferate and secrete ECM components in the primitive valves (C). Finally, the valves undergo further remodeling during the maturation process (D). From Geng et al., 2017.

In addition to the cellular component of these cardiac valves, extracellular matrix in these structures is formed by a unique and dynamic mixture of proteins, glycosaminoglycans and proteoglycans (Grande-Allen et al., 2007; Wang et al., 2014). This cardiac jelly is secreted by both myocardial cells lining the cushion regions (Krug et al., 1987) and endocardial cells (Wirrig et al., 2007). The consequent remodeling of these cushions by an increase in both protease and hyaluronidase activity allows the reorganization of the constituents of the matrix during cushions growth and

differentiation to form the mature mitral, tricuspid, aortic and pulmonary valves (Snarr et al., 2008).

Cardiac conduction system

As cardiac valves regulate the unidirectional blood flow, the cardiac conduction system (CCS) of the heart generates to coordinate the electrical impulse that causes the rhythmic and coordinated contractions of the atria and ventricles (Mikawa and Hurtado, 2007). The functional components of the CCS include; 1) the sinoatrial node (SAN), also known as the pacemaker, which is responsible for the initialization of electrical current that stimulates heart muscle contraction; 2) the intermodal atrial tracts, including interatrial bundle or tract in charge of distributing the electrical current in the atrial walls; 3) the atrioventricular node (AVN) that delays conduction between atria and ventricles; 4) the His bundle and the right and left bundle branches transporting the electrical current through the interventricular septum to the ventricular wall; and 5) the peripheral ventricular conduction system (Purkinje fibers) that activates ventricular cardiomyocyte contraction.

In the adult heart, the SAN is located between the superior caval vein and the dorsal right atrial wall. Although all cardiac muscle cells have the potential to rhythmically beat, SAN cells are able to spontaneously create an action potential (electrical impulse) 70-80 times per minute (on average) in human hearts (Jaffe et al., 2018). This unique characteristic makes the SAN the primary pacemaker of the heart, generating an initial electrical impulse that rapidly spreads through the atria (Keith and Flack, 1907) to then reach the AVN, located next to the tricuspid valve in the triangle of Koch (Anderson and Ho, 2003). This AVN delays the impulse, separating atrial and ventricular chamber contraction in time. The impulse leaving the AVN is rapidly propagated through the bundle of His, the right and left bundle branches, and

the peripheral ventricular conduction system (Purkinje fiber network) that rapidly transmits the impulse throughout the ventricle, from its apical region to the basal one (Fig.4).

Three main models have been proposed to explain the formation of the conduction system, the “multiple ring theory”, the “recruitment model” and the “early specification model” of progenitor cells (Christoffels and Moorman, 2009). These models suggest that CCS may form by a differential proliferation rate in the cells of the tubular heart (Mirzoyev et al., 2010), the continuous recruitment and differentiation of cardiomyocytes (Cheng et al., 1999; Gourdie et al., 2003; Pennisi et al., 2002), or the progressive differentiation and proliferation of an embryonic myocyte subpopulation (Christoffels and Moorman, 2009), respectively.

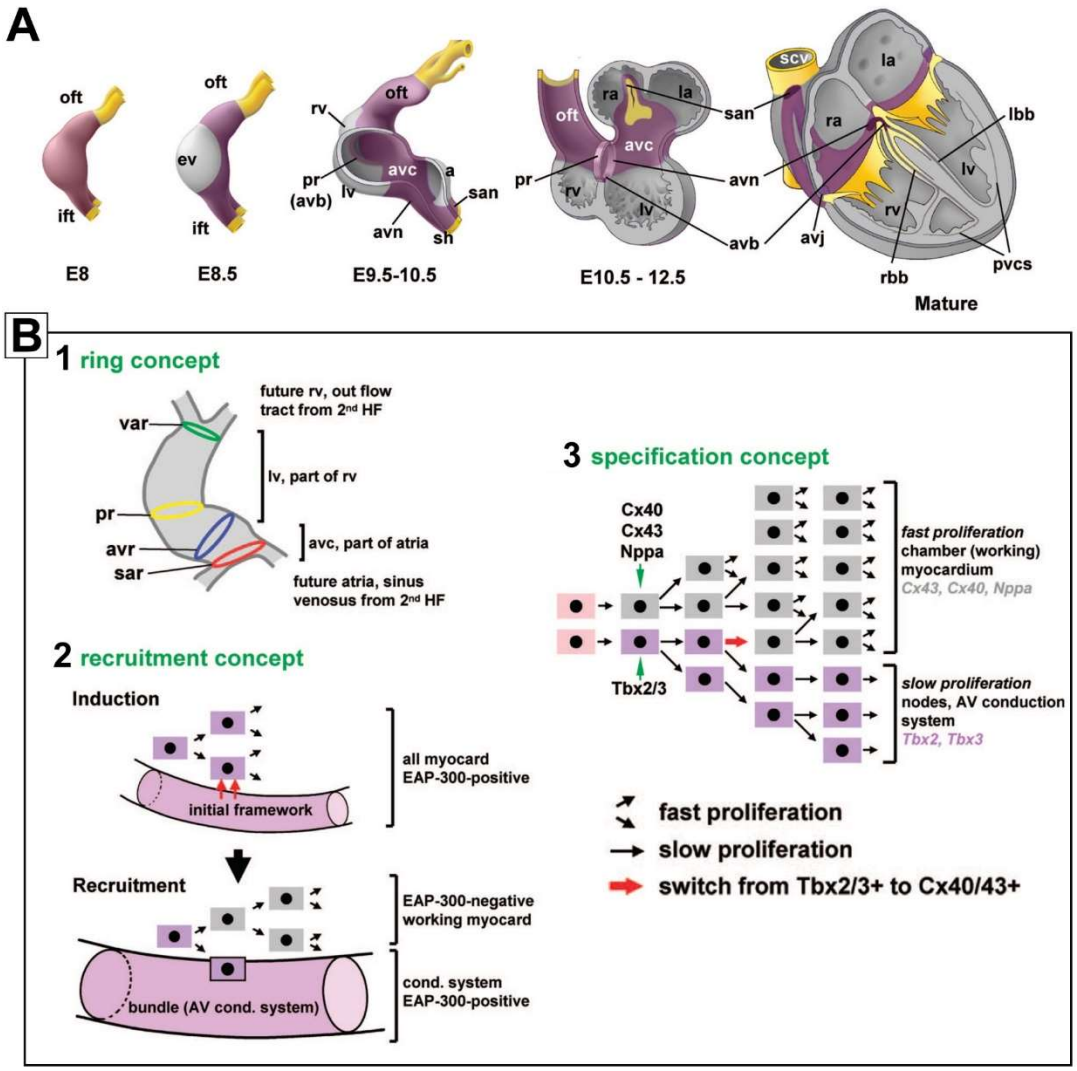

Figure 4. Cardiac conduction system. Sinus horn myocardium contributes to the sinoatrial node (SAN), atrioventricular canal myocardium to the atrioventricular node (AVN) and the atrioventricular junction (AVI). The ventricular septum crest part of the primary ring (PR) will form the atrioventricular bundle (AVB). The four ring model for the development of the conduction system is represented in the context of the tubular heart (B1). The recruitment model with two steps, induction and recruitment of the myocardial cells to the conduction system (B2). In the early specification model, the myocardium of the heart tube (pink) express either conduction system or chamber genes. The expression of conduction system factors confers an undifferentiated and low proliferative phenotype (purple). These cells differentiate to nodes or conduction tissues, whereas the loss of these expression factors allows for the differentiation into chamber myocardium (gray) (B3). Modified from Christoffels and Moorman, 2009.

Despite all the discrepancies on the cellular origin of CCS, it is clear that the CCS is formed from primitive embryonic heart tube cardiomyocytes (Cheng et al., 1999; Gourdie et al., 1995). Indeed, while conduction system precursor cells maintain a primitive gene expression profile and present a low proliferative activity, adjacent cells differentiate and form the mature-beating myocardium (as known as working myocardium). The early specification of conduction system precursor cells requires the expression of Tbx2 and Tbx3 as repressors of the working-conventional myocardial fate (Bakker et al., 2010; Mohan et al., 2018). Some of these CCS genes, such as the pacemaker channel Hcn4, are widely expressed in all conduction system cells, especially in the developing SAN (Garcia-Frigola et al., 2003).

Coronary vessels

The proper vascularization of every organ is important to maintain its homeostasis (Rehman and Rehman, 2019). The heart is vascularized by the coronary vascular system, a vascular network of arteries, veins and capillaries that irrigates the myocardium. Major adult coronary vessels are formed by three concentric tissue layers: tunica intima, tunica media and tunica adventitia. The innermost layer (tunica intima) is formed by endothelial cells in contact with the vascular lumen and the subendothelial extracellular matrix. The accumulation of smooth muscle cells (SMC), pericytes and elastic collagen fibers form the tunica media that is thicker in arteries as compared to veins. Finally, the outermost tunica adventitia, is formed by the assembly of ECM compounds, fibroblasts and other cell types (Fig. 5).

Coronary arteries have a thick and elastic muscular wall submitted to the high arterial pressure of the blood and lack valves. On the contrary, veins have a thin medial layer, and are a blood reservoir and display valvular structures, which are critical for the efficient return of blood to the heart,

particularly from hind limbs (Bazigou and Makinen, 2013). This returning path of deep veins is also driven by the combinatory role of skeletal muscle contraction (Alimi et al., 1994), arterial pulsation and the pump generated by the ankle joint motion (Kugler et al., 2001). Correct irrigation of the myocardium by coronary arteries and veins is essential to maintain the tissue homeostasis during the adulthood. A failure in this irrigation process, either by congenital or acquired cardiac diseases, may threaten the health of the individual or even result in death.

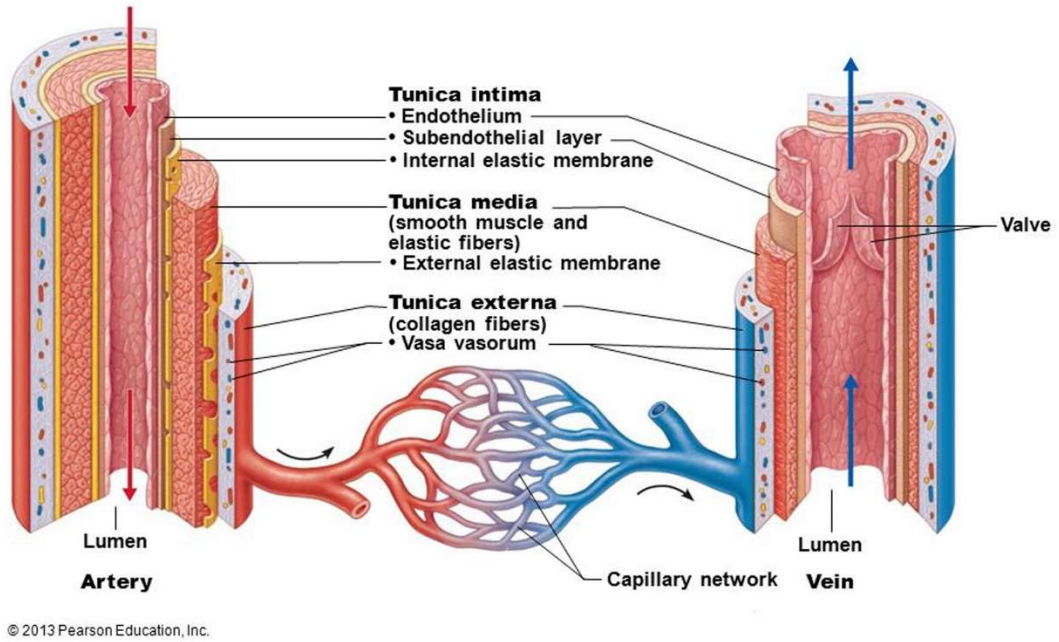

Figure 5. Cellular components of arteries and veins. The wall of the vessels consists of three concentric layers. The inner layer, named tunica intima, is mainly formed by endothelial cells and their basement membrane. In veins, this layer is characterized by the presence of valves. Tunica media is composed of smooth muscle cells surrounding by a collagenous extracellular matrix. The outermost layer, tunica adventitia, contains the fibrous connective tissue. Modified from: © 2013 Pearson Education (https://images.slideplayer.com/25/8101608/slides/slide_4.jpg).

To better understand congenital diseases affecting this coronary vasculature, it is important to analyse the cellular origin of these vessels during their ontogeny. It has been proposed that the coronary endothelium

forms by the assemblage of endothelial precursors from the sinus venosus endocardium (Red-Horse et al., 2010), the developing epicardium (Mikawa and Fischman, 1992; Pérez-Pomares et al., 1998), or the ventricular endocardium (Wu et al., 2012). In particular, recent studies have suggested the simultaneous contribution of endothelial progenitors from multiple embryonic sources as the origin of this coronary vascular network (Cano et al., 2016; Palmquist-Gomes et al., 2018). A high developmental plasticity has also been suggested during this early vascularization of the developing heart, as evidenced by the suggested reprograming ability of venous cells to arterial fates during ontogenesis (Red-Horse et al., 2010; Su et al., 2018).

Endothelial cells of both arteries and veins share the expression of CD31 endothelial marker, but other genes can be used to specifically identify endothelial cells of arteries (e.g. Notch1, EphrinB2 and Neuropilin 1) (del Monte et al., 2011; le Noble et al., 2003) or veins (e.g. EphrinB4, COUP-TFII, Vascular endothelial growth factor receptor 3, Neuropilin 2 and Apelin Receptor) (Red-Horse et al., 2010).

Lymphatic vessels

There are two vascular systems in mammals, the blood vascular and the lymphatic one. They are integrated in cycle in which the blood transports oxygen and nutrients to the tissues, and the lympha transports fluids and macromolecules from tissues back to the blood circulation. This balance is key to maintain fluid homeostasis (Oliver and Srinivasan, 2008).

The classic studies on the origin of the lymphatic vessels suggested a venous origin for the whole mammalian lymphatic system (Sabin, 1902). Recent studies, however, suggest that although the transdifferentiation of venous progenitors is the predominant origin of cardiac lymphatic endothelial cells, other extracardiac progenitor cell populations, including some located

in the embryonic yolk sac (Klotz et al., 2015) and the SHF (Lioux et al., 2020) may also contribute to the formation of the developing lymphatic vasculature.

In mouse embryos, the lymphatic vascular system develops shortly after blood circulation is established (Kume, 2015). Most lymphatic endothelial cells (LECs) originate from somatic venous endothelial cells (VECs) (Sabin, 1902; Srinivasan et al., 2007; Stone and Stainier, 2019; Yang et al., 2012) and their differentiation is characterized by the expression of *prospero-related homeobox protein 1* (Prox1) (Wigle and Oliver, 1999), *vascular endothelial growth factor receptor-3* (VEGFR-3) (Kaipainen et al., 1995) and *lymphatic vessel endothelial hyaluronan receptor 1* (Lyve-1) (Banerji et al., 1999).

Around E9.5, lymphatic progenitors migrate from the anterior cardinal vein forming the lymphatic sacs and primitive vessels, which will then proliferate and sprout until the formation of the lymphatic plexus. Between E15.5 and E16, this plexus is remodeled to give rise to the lymphatic vascular network, including capillaries and the collecting vessels that present smooth muscle cells (SMC) and intraluminal valves (Kume, 2015). These intraluminal valves prevent the backflow of the lymphatic fluid, as occurs in the case of venous valves, which allow the unidirectional blood flow in the veins. The morphogenetic process in both types of valves is similar and includes four distinct phases: initiation, condensation, elongation, and maturation (Kampmeier and La Fleur Birch, 1927; Sabine et al., 2012). Although venous valves originate in the embryo and lymphatic valves form postnatally (Bazigou and Makinen, 2013), valve-forming cells in vascular networks share a common expression profile (Table 1) (Bazigou et al., 2011). Such profile includes the expression of Prox1, Foxc2, NFATc1 and Connexin37 (Cx37) (Table 1) (Mellor et al., 2007; Norrmén et al., 2011, 2009; Petrova et al., 2004; Sabine et al., 2012). Foxc2 and Prox1 are independently expressed (Petrova et al., 2004) and both control valve formation by regulating Cx37

expression and calcineurin activation (Munger et al., 2016; Sabine et al., 2012). In the case of the lymphatic valves, mechanical forces of the lymph flow (Sweet et al., 2015) and some other genes like GATA2 (Kazenwadel et al., 2015; Riaj Mahamud et al., 2019) have also been described as important factors for the correct development and maturation of these vascular structures.

	VECs expression	Developmental stages	Developmental time-points		
			Initiation	Circumferential valve territory	Maturation and elongation
Lymphatic	Prox1 Foxc2 NFatc1 Gata2 α9 integrin Vegfr-2 Vegfr-3 Ephrin-B2 Laminin α5 Collagen IV Podocalyxin	1) Initiation 2) Circumferential valve territory 3) Valve leaflet elongation and maturation 4) Condensation 5) Leaflet elongation	E15 (stage 1)	E16-E17 (stage 2)	E18-P0 (stage 3)
Venous	Prox1 Integrin α9 Ephrin-B2 Laminin α5 Collagen IV	0) Rounding up of cells 1) Cell alignment 2) Formation of a circular shelf 3) Formation of first commissure 4) Formation of second commissure	E18-P0 (stages 0-1)	P1-P3 (stage 2)	P4 (stage 3-4)

Table 1. Venous and lymphatic valves development. Modified from Bazigou and Mäkinen, 2013.

Although lymphatic and venous valves seem to form in a similar way (Fig. 6), both vascular networks are quite different in their function and developmental formation. Since the function and origin of the lymphatic

vasculature has been detailed above, in the next section we will focus on the origin and morphogenesis of the coronary vascular system.

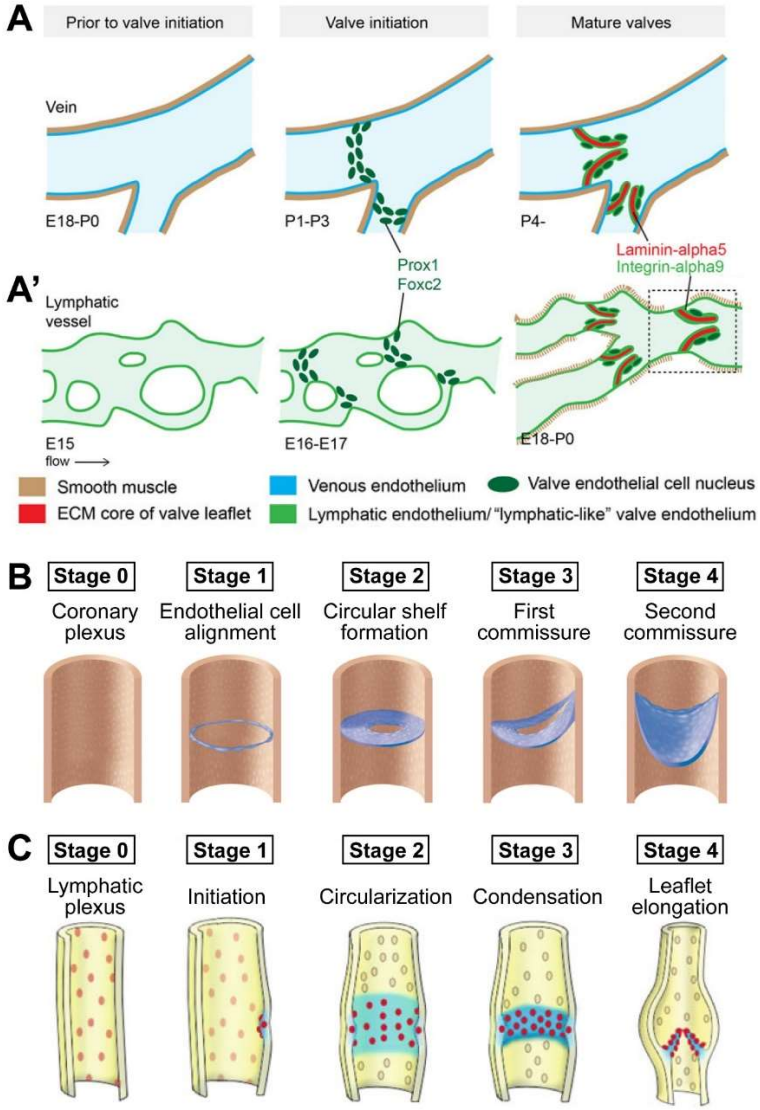

Figure 6. Developmental origin and morphogenesis of venous and lymphatic valves. Valve initiation in both veins (**A**, blue) and lymphatic vessels (**A'**, green) is characterized by the high expression level of Prox1 and Foxc2, followed by formation of leaflets with two layers of endothelial cells expressing Integrin-alpha9 attached to Laminin-alpha5 positive matrix core. Five developmental stages (from stage 0 to stage 4) were described for venous (**B**) and lymphatics valves (**C**). **A** was modified from Bazigou and Mäkinen, 2013; **B** was modified from Bazigou et al., 2011; **C** was modified from Sabine and Petrova, 2014.

1.2. Cardiovascular diseases

Any alteration of the cellular and molecular mechanisms that control heart morphogenesis may lead to congenital heart diseases (CHD). These CHD affect around 1% of newborns (Van Der Linde et al., 2011) and their embryological origin remains poorly understood in most cases (Pérez-Pomares et al., 2016). Congenital coronary anomalies (CA) are a type of CHD closely linked to other congenital cardiac conditions such as ventricular non-compaction (Branton et al., 2011; Nagumo et al., 2015) or even some cardiomyopathies (Guadix and Pérez-Pomares, 2016).

In addition to CHD, other cardiac diseases can be originated after birth. These are normally referred-to as acquired heart diseases (AHD). Although AHD in children (e.g. rheumatic heart and Kawasaki disease) have a low incidence in the human population (Lin and Wu, 2018; Watkins et al., 2017) the severity of these malformations requires a better understanding of their cellular and molecular origin. The pathological progression of many AHD involves a group of interstitial cells named cardiac fibroblasts. An excessive proliferation and secretory activity of these cells lead to fibrosis, a pathologic process relevant to a wide range of adult heart pathologies, including subaortic stenosis (Mohan et al., 2016), tropical endomyocardial fibrosis (Grimaldi et al., 2016), pericardial disease (Khandaker et al., 2010), myocardial calcification (Pillai et al., 2017), dilated cardiomyopathy (Ohtani et al., 1995), arrhythmogenic cardiomyopathy (Segura-Rodríguez et al., 2020), or myocardial infarction (Joshi et al., 2015). Among these human pathologies, the ischemic cardiomyopathy known as myocardial infarction (MI) represents a prevalent disease in the Western population. The response of cardiomyocyte death due to ischemia evokes a primary reparative response requiring cardiac fibroblast activation, proliferation and subsequent

massive synthesis of ECM molecules. The detailed characterization of the cardiac fibroblast population, known to be heterogeneous (Zeisberg and Kalluri, 2010), is a key issue in the understanding of MI pathological response.

1.3. Cardiac fibroblasts

As previously discussed, cardiac fibroblasts are key cells in the pathological progression of adult heart diseases. Fibroblasts are frequently defined as spindle/stellate-shaped cells that synthesize high amounts of collagen and are essential to counterbalance cardiac mechanical instability (Boor and Floege, 2012; Lafyatis, 2014). In the steady-state heart, cardiac fibroblasts (CFs) are located in the cardiac interstitium, a complex dynamic microenvironment formed by the extracellular space between cardiomyocytes and a great variety on non-myocytes cardiac cells (Pogontke et al., 2019). In the mouse heart, it has been reported that cardiac interstitial cells (CICs) represent around 45% of all the cells of the adult heart; less than 20% of these cells are considered to be fibroblasts (Banerjee et al., 2007; Pinto et al., 2016).

Recent studies have shown that EPDCs are the major source of CFs in the cardiac interstitium during development (Acharya et al., 2012; Lie-Venema et al., 2007; Wessels and Pérez-Pomares, 2004). These epicardial-derived fibroblasts mostly accumulate in the ventricular interstitium (Ruiz-Villalba et al., 2015), and normally participate in the formation of the annulus fibrosus and cardiac valve primordia (Wessels et al., 2012). While it is generally accepted that embryonic CFs derive from the epicardium via EMT, it is unclear whether any EMT-mediated contribution to the adult cardiac fibroblast population occurs in response to pathological conditions (Ruiz-

Villalba et al., 2015). In addition to the epicardium, there are other cell sources that may give rise to ventricular fibroblasts, including bone-marrow-derived circulating cells (Haudek et al., 2006), neural crest-derived cells (Bergwerff et al., 1998; Phillips et al., 2013) and the ventricular endocardium (Moore-Morris et al., 2014; Zeisberg et al., 2007).

It is relevant to consider that cardiac fibroblasts can be found at two different physiological states, either synthetizing high amounts of ECM molecules (activated fibroblasts or myofibroblasts) or in a quiescent state (inactivated fibroblasts). Under pathological conditions, myofibroblasts secrete high amounts of ECM proteins in a relatively short period of time (Kalluri and Zeisberg, 2006), whereas healthy fibroblasts secrete low levels of collagen along extended periods of time. The excessive deposition of ECM components, including collagen, leads to a pathological condition known as fibrosis.

The high relevance of fibrotic processes in cardiac pathological conditions emphasizes the necessity of progressing in the characterization of fibroblast diversity and function. Although cardiac fibroblasts have been studied in terms of the combined expression of different cellular markers (including αSMA, DDR-2 or fibroblast-specific protein, FSP1), there is not a true single specific molecular marker for fibroblasts (Ivey and Tallquist, 2016). This is also the case for myofibroblasts markers. The protein α-SMA is commonly used to characterize myofibroblasts (table 2), but it is also expressed by smooth muscle cells, pericytes and myoepithelial cells (Arnoldi et al., 2012). Fibroblasts and SMC also share the expression of other molecules like cadherins, transgelin (SM22α), vimentin and FSP1, especially in the context of tissue repair (Arnoldi et al., 2012; Brisset et al., 2007; Chimori et al., 2000; Hinz and Gabbiani, 2003; Ivanov et al., 2001; Matsuyoshi and Imamura, 1997; Solway et al., 1995).

Cardiac fibroblasts marker	Utility
aSMA	Marker for cardiac myofibroblasts in hypertrophic and fibrotic hearts
CD90	Mesenchymal cell surface marker
DDR2	Marker of mesenchymal cells, important for fibroblasts proliferation and migration
FSP1	Widely used as a fibroblast specific marker, but its specificity is discussed during this thesis
MEFSK4	Described in mouse cardiac fibroblasts also expressing PDGFRα and *Col1a1*.

Table 2. Markers used to characterize cardiac fibroblasts relevant in this thesis. These and more fibroblast markers were reviewed in Ivey and Tallquist, 2016.

1.4. Fibroblast-Specific Protein-1 (FSP1/S100A4)

1.4.1. S100 protein family

FSP1, also known as S100A4, belongs to the S100 protein family. First identified by Moore in 1965 (Moore, 1965), the S100 protein family (named after their solubility in saturated ammonium sulfate) includes multifunctional calcium binding proteins with a molecular mass of 10–12 KDa. S100 proteins participate in different cellular functions such as cell contraction, proliferation, differentiation, inflammation, and motility, among others (Donato et al., 2013), and it is generally accepted that proteins belonging to this family are not functionally interchangeable.

All S100 family members can interact with and regulate the activity of other proteins through an EF-hand domain, a helix-loop-helix motif that often

binds to metal ions (Lewit-Bentley and Réty, 2000). Several S100 proteins have been found in the extracellular space, where they participate in local intercellular communication (autocrine and paracrine) or coordinate biological events over long distances traveling through the systemic circulation (Schäfer and Heizmann, 1996). Since they lack the signal peptide for secretion via the conventional Golgi-mediated pathway, S100 proteins are supposed to be translocated or secreted via a different, Golgi-independent pathway (Donato et al., 2013; Hermann et al., 2012; Leclerc and Heizmann, 2011). Extracellular S100 proteins interact with a variety of cell-surface receptors including receptor for advanced glycosylation end products (RAGE; also known as AGER), G protein-coupled receptors, Toll-like receptor 4 (TLR4), scavenger receptors, fibroblast growth factor receptor 1 (FGFR1), CD166 antigen (a.k.a. ALCAM), interleukin-10 receptor (IL-10R), extracellular matrix metalloproteinase inducer (EMMPRIN, a.k.a. basigin) and the bioactive sphingolipid ceramide 1-phosphate (Dmytriyeva et al., 2012; Donato et al., 2013; Hankins et al., 2013; Hibino et al., 2013; von Bauer et al., 2013). Currently, 21 proteins belonging S100 family have been described in Vertebrates. S100 genes are highly conserved between human and mouse, including the number of genes, their chromosomal localization and their expression patterns (Fig. 7, A) (Marenholz et al., 2004).

1.4.2. FSP1 protein structure

FSP1 is a 101 amino acid peptide. Biologically active FSP1 normally forms symmetric homodimers stabilized by noncovalent interactions (Moore, 1965; Vallely et al., 2002). Each monomer is formed by two EF-hand calcium-binding domains: a N-terminus that binds calcium via backbone carbonyl oxygen atoms, and a C-terminus domain (Garrett et al., 2006; Heizmann and Cox, 1998) (Fig. 7, B). The N-terminus Ca^{2+} binding domain (pseudo EF-

hand) is composed of 14 amino acids, while the C-terminal canonical EF-hand is composed of 12 amino acid residues and binds Ca^{2+} with higher affinity (Rezvanpour et al., 2009). Binding of FSP1 to Ca^{2+} mediates a large conformational change in the three-dimensional structure of the dimeric protein, giving access to hydrophobic protein-protein interaction sites (Pathuri et al., 2008; Santamaria-Kisiel et al., 2006). This open conformation of the protein allows for the interaction between FSP1 and p53 (Orre et al., 2013), nonmuscle myosin IIA (Li and Bresnick, 2006; Ramagopal et al., 2013), tropomyosin (Takenaga et al., 1994), liprin ß1 (Kriajevska et al., 2002), methionine aminopeptidase 2 (Endo et al., 2002), rhotekin (Chen et al., 2013) and S100A1 (Wang et al., 2005). It has been reported that, in some cases, the addition of Ca^{2+} reduces the complex formation, as occurs with the binding between FSP1 and annexin A2 (Semov et al., 2005).

1.4.3. FSP1 functions

FSP1 is considered to be a cytoplasmic protein mainly, although it has been also described in cell nucleus (Flatmark et al., 2003; Hsieh et al., 2004) and in the extracellular space (Cabezón et al., 2007). The majority of FSP1 functional studies have focused on its role during cancer progression and metastasis. Indeed, FSP1-myosin IIA interactions are known to influence cell migration (Li et al., 2003) during metastasis (Zhang et al., 2005). This involvement in cell motility has also been described in other non-metastatic cell types, such as macrophages (Dulyaninova et al., 2018; Li et al., 2010) or endothelial cells (Ochiya et al., 2014). Moreover, it has been reported that FSP1-Rhotekin complex induces the formation of thicker F-actin fibers lamellipodia ruffles, conferring an invasive phenotype in cancer cells (Chen et al., 2013). Finally, as an extracellular protein, FSP1 stimulates the migration of endothelial cells (Schmidt-Hansen et al., 2004), smooth muscle

cells (Spiekerkoetter et al., 2009), T lymphocytes (Grum-Schwensen et al., 2010) and fibroblasts (Cunningham et al., 2010) probably through the binding to some promiscuous surface receptors such as the receptor for advanced glycation end products (RAGE) (Fig. 7, C) or toll-like receptor 4 as well as to annexin II or heparan sulfate proteoglycans (Björk et al., 2013; Kiryushko et al., 2006; Semov et al., 2005; Yammani et al., 2006). In addition to cell migration, the expression of *Fsp1* in endothelial cells has also been related to angiogenic phenomena (Ambartsumian et al., 2001; Semov et al., 2005), and lymphatics integrity (Norrmén et al., 2010), but further research is required to unveil new functions for this protein.

1.4.4. FSP1 expression in cardiac tissues

FSP1 has often been defined as specific fibroblast marker (Lawson et al., 2005; Zeisberg et al., 2007). This protein, however, has been recently described in other cell types, such as lymphocytes, macrophages, or endothelial cells, making controversial the use of FSP1 as a marker for a single specific cell type (Kong et al., 2013; Le Hir et al., 2005; Osterreicher et al., 2011). Moreover, many of these results have proven that not all fibroblasts express FSP1. This is in accordance with the well-known phenotypical and functional diversity of fibroblasts (Ruiz-Villalba et al., 2020; Tallquist and Molkentin, 2017).

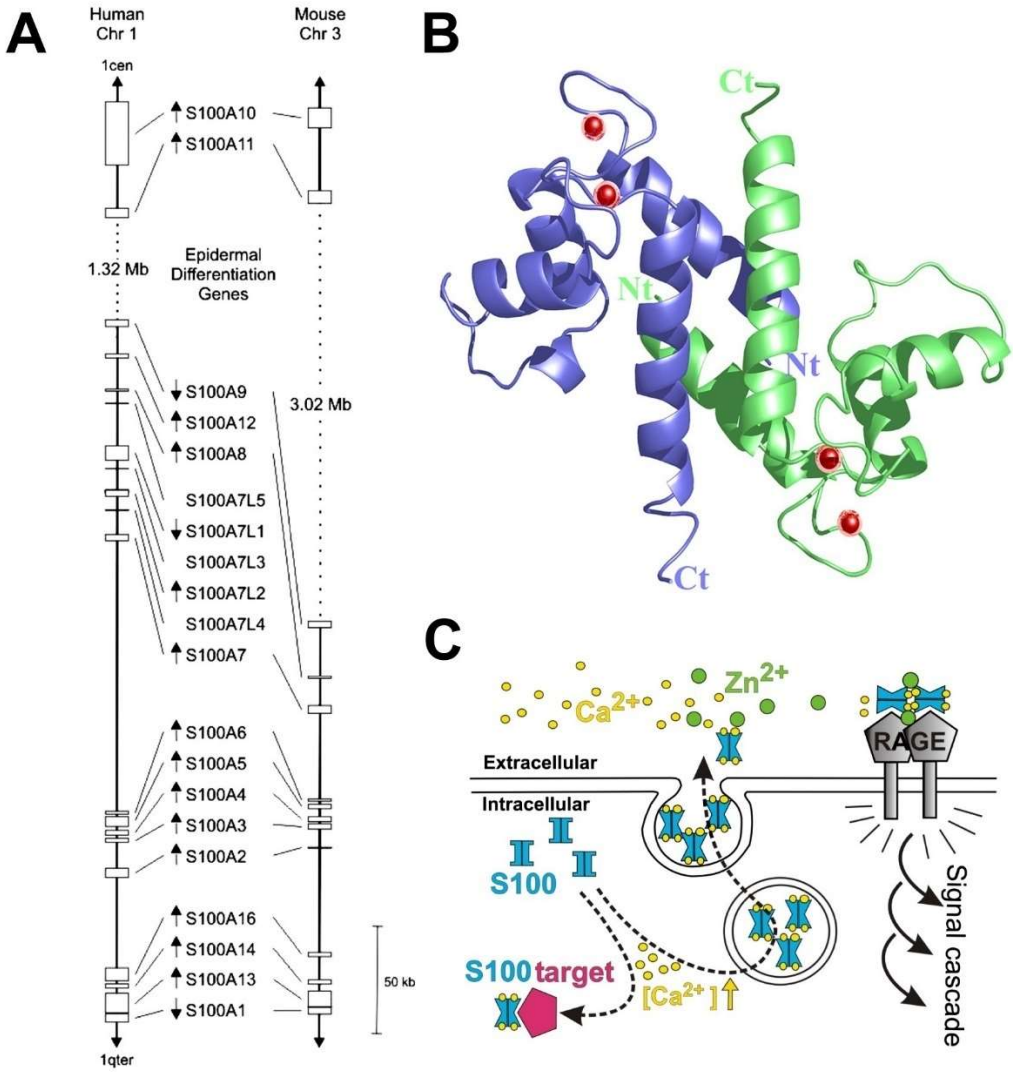

Figure 7. Location, structure and function of FSP1/S100A4 gene and protein. Human S100 genes are located on 1q21 chromosome meanwhile mouse ones are located on 3F1-F2 chromosome (A). In both genomes, a cluster of epidermal differentiation genes is located between S100A9 and S100A11 (A). S100A7-like genes were present in human and absent in mice (A). Crystal structure of FSP1 active homodimer (B) reveals the tridimensional shape of monomer A (blue) and monomer B (green), represented in the calcium-bound form with calcium ions as red spheres. In the intracellular space, S100 proteins regulate the biological activity of the target protein by a Ca2+-dependent binding (C). Ca2+ ions also induce the secretion of S100 proteins to the extracellular space, promoting their interaction with RAGE receptors. A and C were modified from Marenholz, Heizmann and Fritz, 2004; B was modified from Pathuri, Vogeley and Luecke, 2008.

1.4.5. FSP1 in pathological processes (cancer and myocardial infarction)

The FSP1 protein is involved in several pathologic processes including cancer and cardiovascular diseases, two of the main leading causes of death in Western countries (Palmquist-Gomes et al., 2019). As previously discussed, FSP1 is implicated in a broad range of cellular phenomena, including cell migration and inflammation, both extremely relevant to the pathophysiology of several diseases, (i.e. metastasis in cancer and cardiac ventricular scarring after myocardial infarction; Fig. 8).

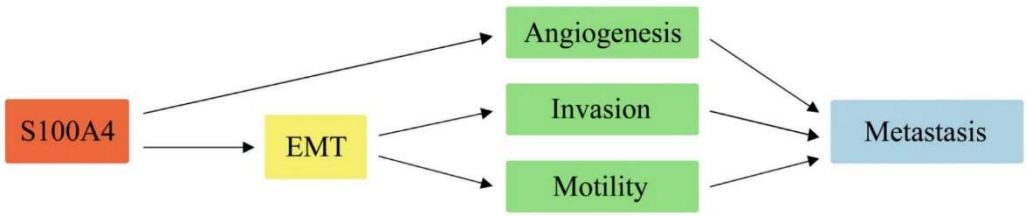

Figure 8. S100A4/FSP1 involvement in metastatic progression. FSP1 stimulates angiogenesis, and EMT, which probably promote cell motility and invasion. All of them contribute to the metastatic stimulation. Modified from Boye and Mælandsmo, 2010.

Metastasis is the leading cause of cancer progression, resistance to therapies and patient death. During this process, cancer cells spread from the primary tumor to surrounding tissues and then to distant organs (Fei et al., 2017; Seyfried and Huysentruyt, 2013). The relationship between FSP1 and cancer was first studied in human breast cancer (Rudland et al., 2000), and later on in other tissues such as the liver (Taylor et al., 2002) or the brain (Zakaria et al., 2016). Due to FSP1 role in promoting cell migration some authors consider this protein as a good marker for metastatic potential in primary tumors (Bailey et al., 2007; Chow et al., 2017; Micalizzi et al., 2010; Nieto, 2013; Ning et al., 2018; Xu et al., 2016). The extracellular activity of

FSP1 includes angiogenesis induction, immune cell recruitment in growing tumor-lesions or the stimulation of cytokine and growth factors secretion into the tumor microenvironment (Boye and Mælandsmo, 2010; Nasser et al., 2015) (Fig. 8).

FSP1 was also described in the adult ischemic heart (Fig. 9). Myocardial infarction (MI) is the massive death of myocardial muscle, usually associated with ischemia and subsequent oxygen and nutrient deprivation. After myocardial infarction, the massive loss of working cardiomyocytes is compensated by the formation of a non-contractile fibrous scar that expands chronically (Jessup and Brozena, 2003). This process, known as ventricular remodeling, starts with the dilation of the ventricular wall during the inflammatory phase (first 72 hours), which is followed by a proliferative phase where quiescent fibroblasts mature into secretory myofibroblasts. The massive synthesis and deposition of collagen and other ECM molecules in the maturation phase, leads the formation of a fibrotic scar (Anversa et al., 1985; Erlebacher et al., 1984, 1982; McKay et al., 1986). Although this fibrotic process is needed to prevent the rupture of the ventricular wall, the progression of the post-MI akinetic scar increases ventricular wall stiffness and decreases its mechano-electric coupling, eventually leading to the loss of heart wall contractile properties, arrhythmias and cardiac failure (Braunwald and Pfeffer, 1991). Cardiac fibroblasts are therefore pivotal to the modulation of inflammatory, proliferative, and maturation phases of ventricular remodeling due to their high resistance to ischemic death, wide distribution and secretory ability of both ECM proteins and pro-inflammatory mediators (Shinde and Frangogiannis, 2014). Relevant to this thesis, post-MI scar includes high numbers of FSP1-expressing fibroblast-like cells (Kong et al., 2013), but not all these FSP1-positive cells are described as cardiac fibroblasts (van Berlo et al., 2014). Finally, increased extracellular levels of FSP1 were described in plasma after cardiac ischemia, suggesting this

protein as a powerful clinical biomarker for the early detection of cardiac ischemic events (Gong et al., 2015).

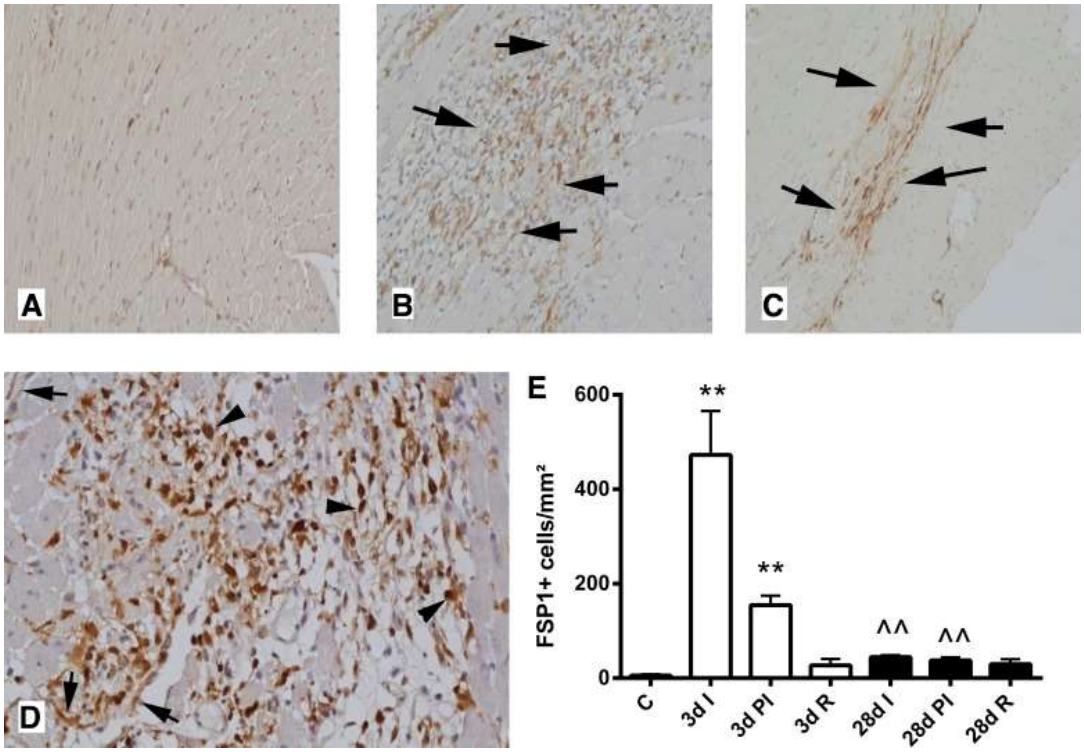

Figure 9. FSP1 in the infarcted heart. The presence of FSP1⁺ cells in the mouse infarcted heart was described by anti-GFP immunohistochemical methods on *Fsp1*^{GFP} mice ventricles. As compared to control undamaged hearts (A), high amounts of GFP⁺ cells were described in the ischemic ventricle 3 days (B, arrows) and 28 days (C, D, arrows) after myocardial infarction. Quantitative analyses revealed high amounts of these FSP1⁺ cells in the infarcted heart 3 days post injury (E). Infarcted area (I); Peri-infarcted area (PI); Remote remodeling myocardium (R). From Kong et al., 2013.

1.5. Developmental research approaches

One of the main assumptions of this thesis is that research on the embryonic origins of cardiovascular cells is instrumental to understand their adult biology. We would like to suggest that the response of adult cardiac cells to a variety of homeostatic and pathologic stimuli strongly depend on

their ontogenetic background, so that the careful study of cardiac cell differentiation from its embryonic progenitors would help us to better understand the pathophysiology of different cardiac human diseases. For this purpose, non-human animal models are required to study the *in vivo* cellular and molecular mechanisms involved in disease origin and progression.

Classical technologies in the developmental biology field include mouse transgenesis, allowing for permanent cell tracing as well as for loss- or gain-of function experiments. The combinatory use of these animal models and advanced techniques to study protein (e.g. immunohistochemistry, Western blot, FACS) and gene expression (qPCR, ISH) has proven to be powerful methods to obtain *in vivo* results. In this thesis many of these protocols are used to study lineage relationships between *Fsp1*-expressing cells and clarify its specific functions. In addition to these *in vivo* approaches, *in vitro* approximations were also performed to functionally test our working hypotheses. For this, human pluripotent stem cells (hiPSC) were cultured and differentiated to different cardiac cell types. After differentiating embryonic stem cells from mouse blastocyst inner cell mass (Evans and Kaufman, 1981; Martin, 1981), the Thomson laboratory succeed in deriving the first human embryonic stem cell (hESC) line. The use of hESCs in biomedical research is ethically and politically controversial since it involves the destruction of human embryos (Lo and Parham, 2009). The use of these cells has been superseded by the use of induced pluripotent stem (iPS), which can be obtained after reprogramming mouse (Takahashi and Yamanaka, 2006) and human (Takahashi et al., 2007) adult somatic cells through the forced expression of the transcription factors Oct3/4, Sox2, c-Myc, and Klf4. By using these same four factors, it is currently possible to obtain iPSC from peripheral blood mononuclear cells (Kim et al., 2016), keratinocytes (Aasen et al., 2008), liver and stomach cells (Aoi et al., 2008) and epicardium-derived cells (Paulitschek et al., 2017).

In this PhD thesis, the role of *Fsp1* gene has been studied during heart development by using immunochemical analyses and the *Fsp1*^{GFP} mouse transgenic strain. Additional in vitro analyses by using human cultured cells were performed to confirm the results obtained in mouse embryos.

Hypothesis and objectives

The main hypothesis of this thesis is that FSP1 would not represent a fibroblast specific marker in a cardiovascular context. In particular, we will study the role of the protein FSP1/S100A4 in embryonic and adult heart tissues. The main objectives of this thesis are:

Objective 1. To analyse *Fsp1* expression in cardiac fibroblasts.
Objective 2. To analyse *Fsp1* expression in other cardiac cell types.
Objective 3. To test the functional relevance of *Fsp1* in adult cardiac tissues.

Chapter 2: Material and Methods

Mice

All animals used in this study were handled in compliance with institutional and European Union guidelines for animal care and welfare under a specific experimental procedure approved by the Ethics Committee of the University of Malaga and BIONAND.

Fsp1-GFP

To study the origin of fibroblasts during tissue fibrosis in the heart, Iwano and colleagues created the transgenic line *Fsp1*^{GFP} (Iwano et al., 2002). This is the same transgenic mouse line in this thesis.

Generation of G2Gata4^{CRE} x Fsp1$^{LoxP/LoxP+/+}$

Heterozygote *G2Gata4*^{CRE} mice were crossed twice (F1, F2) with homozygotes *Fsp1*$^{LoxP/LoxP}$ (RBRC05699 from Riken) to obtain *G2Gata4*^{CRE} x *Fsp1*$^{LoxP/LoxP}$ animals. These mice display *Fsp1* deletion in *G2Gata4+* lineage. Both mouse lines were maintained and bred at the BIONAND animal facility.

Genotyping

DNA was extracted from embryos and adult mice. The following primers (Table 3) were used.

Gene	**Orientation**	**Sequence**
Fsp1-GFP	Forward	GTGATTTGGGTCATGCTCAG

Fsp1-GFP	Reverse	GAACAGCTCCTCGCCCTTGC
*Fsp1*LoxP/LoxP	Forward	ACATCAACCAACAGCAAGAG
*Fsp1*LoxP/LoxP	Reverse	CCCTTAAGCCTATCCCAAAG
*Fsp1*LoxP/LoxP WT	Forward	GACTTCCAGGAGTACTGTGTC
*Fsp1*LoxP/LoxP WT	Reverse	GCCGCTAGCAGTGCTATCAG
Wt1-Cre	Forward	AGATATCTCACGTACTGACGG
Wt1-Cre	Reverse	TTTCTAGACACCTTGAACCAC
G2-Gata4-Cre	Forward	TGCCACGACCAAGTGACAGC
G2-Gata4-Cre	Reverse	CCAGGTTACGGATATAGTTCATG

Table 3. sq-PCR primers used for mice genotyping.

Isolation of adult cardiac interstitial cells

Fsp1^{GFP} adult hearts were perfused and washed with PBS (1,504g Na_2HPO_4, 0,43g KH_2PO_4, 7,22g NaCl, pH 7,3-7,4). Ventricles were removed, placed in 1 mL of cytometry buffer (2% FBS, 1% HEPES, 0,2% EDTA in PBS, pH 8) and PBS (1:1) and mechanically minced. Samples were centrifuged (fast spin), the supernatant was removed, and cells were washed in 1 mL of PBS. Samples were centrifugated again (fast spin) and the pellet was resuspended and incubated in 1 mL of Liberase TH (05401135001, Roche) for three times, 5 minutes each at 38ºC, rocking (1000 rpm). The supernatant was diluted in FBS:PBS (1:10) and filtered using a 40µM diameter cell strainer. Filtered sample was centrifuged (5 minutes, 500g), and the pellet was resuspended in cytometry buffer for analysis.

Isolation of mouse embryonic fibroblasts (MEFs)

E13.5 embryos were isolated on sterile PBS. After removing head and internal organs of the embryos, the remaining tissue was minced and

digested in trypsin (1mL per embryo) for 20 minutes at 37ºC. After trypsin incubation, the supernatant was placed in a new tube and the same volume of MEF culture medium (DMEM supplemented with pyruvate, 10% FBS, 1% P/S, 1% L-Glut, 1% Non-essential Amino Acids) was added.

Isolation of femoral vein

Using small surgical scissors, make a curved incision in the skin over the femoral vein. The vein was separated together with a small piece of muscle from the rest of the muscle. The tissue was fixed in 4 % PFA diluted in PBS during 30 minutes at RT and cryopreserved in sucrose (15-30%), embedded in OCT resin (TissueTek) and sectioned in a cryostat (10μm).

GFP$^+$ bone marrow transplanted mice

Busulfan (1,4-Butanediol dimethanesulfonate, Sigma-Aldrich) is a myelosuppresive agent used to improve the efficiency of a hematomoietic graft in newborn mice. Pregnant females were injected intraperitoneally on days 17.5 and 18.5 of gestation with 15 mg busulfan/Kg. Newborns at stage P1 were injected through the facial vein with cells suspended in 50 µL of DPBS + 1% FCS + 1% P/S (Cañete et al., 2017; Sands and Barker, 2000). One month after transplantation, the efficiency of the hematopoietic engraftment was ensured by analyzing GFP fluorescence in the peripheral blood of treated mice by flow cytometry.

Myocardial infarction induced by left anterior descent (LAD) coronary artery ligation

The bone marrow transplant was performed as previously described in the literature (Ruiz-Villalba et al., 2015), using the Fsp1-GFP mice as donor (Fig. 17A). The surgical procedure for LAD ligation was performed by Gloria Abizanda, veterinary of the Hematology and Cell Therapy Area in the University of Navarra, Pamplona. Myocardial infarction (MI) was induced by ligation of the left anterior descending (LAD) coronary artery of 20-24 weeks old mice as described elsewhere (Aranguren et al., 2008). Briefly, mice were anesthetized through vaporized isoflurane (4%). The mice were orally intubated using a 20G intravenous catheter, mechanically ventilated and placed on a heating pad to maintain body temperature. A left thoracotomy was performed at the fourth-fifth intercostal space, where muscles were dissected. LAD was permanently ligated using a 7/0 unabsorbable ethilon suture. Sham operated mice (without LAD permanent occlusion) were used as negative control. After visual verification of anemia and akinesia of the apex and anterior-lateral wall to ensure coronary occlusion, the thorax was closed in layers. After de-intubation, mice were kept warm until fully recovered.

Inmunohistochemical characterization of infarcted hearts and quantification

After sacrificing the animals by cervical dislocation, the hearts were injected with phosphate-buffered saline (PBS), rapidly perfused with freshly dissolved 4% (w/v) paraformaldehyde (PFA) and then stored in the same

fixative for 12 hours in at 4ºC. The fixed hearts were washed in cold PBS, cryoprotected, frozen and sectioned as described before (cryostat).
Slides processed for immunohistochemistry were extensively washed in PBS, blocked 60 minutes in SBT and incubated at 4ºC overnight in the primary antibody (Table 4) diluted in SBT. Only slides with non-conjugated primary antibodies were incubated again in a secondary Cy5/Alexa Fluor 647 conjugated IgG (1:100, Jackson Inmuno Research), for one hour at room temperature. Finally, samples were counterstained with DAPI (SIGMA) and analysed under a SP5 laser confocal microscope (LEICA). Circulating FSP1 positive cells were estimated with the IMARIS® software. All the circulating cells were counted (CD45+) and co-located with FSP1 (GFP+). A t-test was used to assess statistical significance.

Flow cytometry

Cell suspensions were incubated with fluorochrome-conjugated antibodies (Table 4) for 30 min on ice. After washing, the labelled cells were analysed in a FACS verse flow cytometer. For fluorescence activated cell sorting, the labelled cells were resuspended in 1 mL of working solution and sorted in a MoFlo cell sorter. Hoechst 33342 staining of bone marrow cells to identify the side population was performed according to standard protocols.

Immunocytochemistry

Cells were fixed with 4% paraformaldehyde for 20 minutes, washed three times with PBS, and blocked with SBT for 1 hour at room temperature (RT). Samples were then incubated in primary antibodies (Table 4) overnight at 4ºC, washed in PBS three times (5 minutes each), and incubated with the

appropriate fluorochrome-coupled secondary antibodies (Table 5) at 1:100 dilution and the DAPI nuclear counterstain for 1 hour (RT). Cells were finally washed in PBS three times (5 minutes each) and they were visualized using Leica SP5 laser confocal microscope.

Epitope	**Host**	**Clonality**	**Dilution**	**Reference**
HCN4	Rabbit	Polyclonal	1/50	Alomone APC-052
Lyve-1	Rat	Monoclonal	1/100	Santa Cruz Biotechnology sc-65647
Troponin I	Rabbit	Polyclonal	1/100	Santa Cruz Biotechnology sc-15368
Endomucin	Rat	Polyclonal	1/500	Santa Cruz Biotechnology sc-65495
Cardiac troponin T	Mouse	Monoclonal	1/100	DSHB CT3
GFP	Chicken	Polyclonal	1/500	Abcam ab13970
FSP1	Rabbit	Polyclonal	1/100	DAKO A5114
CD90.2	Rat	Monoclonal	1/100	EBioscience 17-0902-82
CD31	Rat	Monoclonal	1/100	BD Pharmingen 550274
WT1	Mouse	Monoclonal	1/100	Millipore MAB4234

Contactin 2	Mouse	Monoclonal	1/100	Santa Cruz Biotechnology sc-376780
Pro-COL1A2	Goat	Polyclonal	1/100	Santa Cruz Biotechnology sc-8787
CD45	Rat		1/100	Miltenyi, 130-102-544
α-smooth muscle actin	Mouse	Monoclonal	1/200	SIGMA A2547
Cytokeratin	Rabbit	Polyclonal	1/200	DAKO Z0622
mEF-SK4	Rat	Monoclonal	1/50	Miltenyi 130-120-802

Table 4. Primary antibodies used in this thesis.

Epitope	**Host**	**Conjugated molecule**	**Dilution**	**Reference**
Mouse IgG	Goat	FITC	1/200	SIGMA F2012
Mouse IgG	Donkey	Alexa Fluor® 647	1/200	Jackson IR 715-605-151
Rat IgG	Donkey	Alexa Fluor® 488	1/200	Jackson IR 712-545-153
Rabbit IgG	Goat	Alexa Fluor® 647	1/200	Jackson IR 711-605-152
Rabbit IgG	Goat	Biotin	1/200	SIGMA B7389

Table 5. Secondary antibodies used in this thesis.

hiPSC culture

The wild-type hiPSC line SFLB6 (Zhang et al., 2014) were cultured on vitronectin-coated (A14700, Thermo Fisher) plates, and the differentiation

was induced on 6 well matrigel-coated (354230, Corning) plates for qPCR or in 96 matrigel-coated plates for immunocytochemistry.

Differentiation to endothelial-like cells

SFLB6 cells were harvested and resuspended on day 0 in BPEL medium containing 20ng/mL bone morphogenetic protein 4 (BMP4) (R&D Systems), 20 ng/ml activin-A (Miltenyi), 1.5 μM GSK3 inhibitor CHIR99021 (Axon Medchem). At day 3, refresh with 5 μM XAV939, 50 ng/mL VEGF and 10 μM SB 431542. Repeat this refreshment with 50 ng/mL VEGF and 10 μM SB 431542 at day 6 and day 9 (Fig. 18A) (Giacomelli et al., 2017).

Differentiation to epicardial-like and fibroblasts-like derived cells

SFLB6 cells were harvested and resuspended on day 0 in BPEL medium containing 20ng/mL bone morphogenetic protein 4 (BMP4) (R&D Systems), 20 ng/ml activin-A (Miltenyi), 1.5 μM GSK3 inhibitor CHIR99021 (Axon Medchem). At day 3, cells were refreshed with 5 μM XAV939, 1 μM retinoic acid (RA) and 30 ng/mL BMP4. At day 6, refreshed again with 1 μM RA and 30 ng/mL BMP4. At day 9, cells were passaged 250K into a 6 well fibronectin coated plate in BPEL supplemented with 10 μM TGFß inhibitor SB431542. Finally, the cells were refreshed the day after the passage with BPEL, then again at day 10, and induced to differentiate into fibroblast-epicardial derivedlike cells with 10 ng/mL fibroblasts growth factor 2 (FGF2) (Guadix et al., 2017).

hESCs culture and differentiation to cardiomyocytes-like cells

hESCs NKX2.5$^{EGFP/+}$-COUP-TFII$^{mCherry/+}$ dual reporter line, were differentiated toward atrial or ventricular cardiomyocytes like using a spin EB

protocol. Briefly, undifferenciated cells were harvested and resuspended on day 0 in BPEL medium containing 30 ng/mL Activin-A (R&D Systems), 30 ng/mL BMP4 (R&D Systems), 40 ng/ml stem cell factor (SCF) (Stem Cell Technologies), 30 ng/mL Activin-A and 1.5 µM CHIR 99021 (Axon Medchem). EBs were refreshed on day 3 with BPEL and then transferred to gelatin-coated dishes on day 7.

For efficient directed cardiac differentiation towards the atrial fate, 1 µM RA was added to the EBs from day 4 to day 7 without additional medium changes (Devalla et al., 2015).

Real time qPCR analyses

The cells of interest were recollected, and RNA was isolated (Macherey-Nagel, 740955), quantified with NanoDrop and retrotranscribed into cDNA (iScript™ cDNA Synthesis Kit, BioRad, 1708890). qPCR was carried out using SYBR Green (Applied Biosystems) and the CFX384-real time PCR detection system. The following primers were used to analyse the expression levels:

Gene	**Orientation**	**Sequence**
CD31	Forward	GCATCGTGGTCAACATAACAGAA
CD31	Reverse	GATGGAGCAGGACAGGTTCAG
WT1	Forward	ATAGGCCAGGGCATGTGTATGTGT
WT1	Reverse	AGTTGCCTGGCAGAACTACATCCT
FSP1	Forward	GAGCTGCCCAGCTTCTTG
FSP1	Reverse	TGCAGGACAGGAAGACACAG
hARP	Forward	CACCATTGAAATCCTGAGTGATGT
hARP	Reverse	TGACCAGCCCAAAGGAGAAG

NKX2.5	Forward	TTCCCGCCGCCCCCGCCTTCTAT
NKX2.5	Reverse	CGCTCCGCGTTGTCCGCCTCTGT
KCNJ3	Forward	AAAAACGATGACCCCAAAGA
KCNJ3	Reverse	TGTCGTCATCCTAGAAGGCA

Table 6. qPCR primers used to validate cell differentiation.

Flow cytometry (FACS) and sorting

G^+/M^+ were purified into four populations using a BD ARIA III flow cytometer after exclusion of dead cells and cell debris according to side and forward scatter. After sorting, cells were immediately lysed for RNA isolation or re-plated for functional analysis onto Matrigel-coated glass coverslips into TID medium (Birket et al., 2015).

Human Umbilical Vein Endothelial Cells (HUVEC) culture

HUVEC (CC-2935, Lonza) were maintained in EGM-2 medium (CC-3162, Lonza) at a maximum of nine passages.

Human Umbilical Vein Endothelial Cells (HUVEC) transfection

HUVECs cells were seeded in a confluence of 5 millions per well and they were simultaneously transfected, in two biological replicates, with 5 µg of *FSP1* overexpression vector (HG10185-ANR, Sino Biological) using Lipofectamine® 3000 (L3000015, ThermoFisher) according to manufacturer's instructions.

RNA isolation and PCR screening

After 24 hours, RNA was extracted from the transfected HUVEC using the Direct-Zol RNA Miniprep (R2051, Zymo Research) and was retrotranscribed into cDNA with PrimeScript™ RT Reagent Kit (RR047B, Takara Bio). Correctly modified cells were confirmed by the *FSP1* expression PCR-based analysis. The rt-qPCR was performed with TB Green Premix Ex Taq II (Tli RNase H Plus) (RR280B, Takara Bio) and the primers of interest (Table 7). The raw data were exported and analysed using the LinRegPCR program (Ruijter et al., 2009). The graphs represent the geometric mean of two different biological replicates, and the error bars are generated by the standard error of the mean (SEM).

Gene	**Orientation**	**Sequence**
FSP1	Forward	GCATCGTGGTCAACATAACAGAA
FSP1	Reverse	GATGGAGCAGGACAGGTTCAG
VEGFR3	Forward	CCCACGCAGACATCAAGACG
VEGFR3	Reverse	TGCAGAACTCCACGATCAGC
FOXC2	Forward	CCTCCTGGTATCTCAACCACA
FOXC2	Reverse	GAGGGTCGAGTTCTCAATCCC
PROX1	Forward	AAAGTCAAATGTACTCCGCAAGC
PROX1	Reverse	CTGGGAAATTATGGTTGCTCCT

Table 7. qPCR primers.

Histological analyses

Fsp1^{GFP}, *G2Gata4*^{CRE} x *Fsp1* $^{LoxP/LoxP}$ embryonic mice were staged from the time point of vaginal plug observation. Pregnant females were

sacrificed using cervical dislocation; embryos were extracted from the uterus and washed in cold (4ºC) PBS.

Adult hearts were obtained by sacrificing the donors as described; after thoracotomy, the hearts were perfused using a 21G hypodermic needle and the heart were excised. All samples were extensively washed in PBS before fixation. Adult mouse hearts were fixed in 4 % PFA diluted in PBS during 12 hours at 4 ºC and embryonic hearts were fixed depending on their size (Table 8). The tissues were cryoprotected in 15 % and 30 % sucrose/PBS solutions; snap frozen in liquid nitrogen-cooled isopentane and embedded in OCT for cryostate sections. Or they were dehydrated in an ethanolic series, butanol and then embedded in paraffin for microtome sections. Between 10- and 12- µm thick sections were obtained with both methods and they were collected on a-lysine-coated or poly-l-lysine-coated slides, respectively.

Stage	**9.5-10**	**11-12**	**13-14**	**15-16**	**16-18**	**Adult**
PFA 4% fixation	2h/RT	4h/RT	6h/RT	8h/RT	ON/4ºC	ON/4ºC

Table 8. Fixation times. Abbreviations: RT, room temperature; ON, overnight.

Immunohistochemistry

Immunofluorescence analyses of histological samples were performed by blocking non-specific binding sites with 16% sheep serum, 1% bovine serum albumin and 0.5% Triton X-100 in PBS (SBT) and incubating tissue slides or whole mount hearts with primary antibodies (Table 4) overnight at 4ºC. Then, samples were washed in PBS (3 x 5 min for tissue sections; 3 x 1h for whole hearts), incubated with the secondary antibody (Table 5) for 1h (tissue sections) or overnight (whole hearts). Then samples

were washed in PBS, mounted in a PBS: glycerol (1:1) solution and analysed under a SP5 laser confocal microscope (LEICA). FSP1 epitopes were unasked with TEG buffer (0.12% trizma base and 0.02% EGTA diluted in distiller water; pH 8.95-9.1) in a pressure cooker for 10 minutes. For single immunoperoxidase staining, endogenous peroxidase activity was quenched incubating the sections for 30min in 3% hydrogen peroxide. After washing, endogenous biotin was blocked with a specific avidin–biotin blocking kit (Vector SP2001). Nonspecific binding sites were saturated for 1h with 16% sheep serum, 1% bovine serum albumin, and 0.5% Triton X-100 in Tris-PBS (SBT) at room temperature (RT). Slides were then incubated overnight at 4°C in the primary antibody, HCN4 (Table 4), washed in PBS (3 x 5min), incubated for 1hr at RT in biotin conjugated goat anti-rabbit Immunoglobulin G (IgG; B7389; Sigma-Aldrich) and washed again in PBS. After a final incubation (1h, RT) in streptavidin–peroxidase complex (S5512; Sigma-Aldrich), sections were washed, and peroxidase activity was detected using SIGMAFAST 3,3′-diaminobenzidine tablets (D4293; Sigma-Aldrich). Tissues were counterstained with Harris haematoxylin.

Trichrome staining

Tissue sections were dewaxed in xylene (3 x 10′), hydrated in ethanol series, and rinsed in distilled water (5 min each step). Samples were then submitted to Mallory's trichrome staining, including the sequential incubation of the samples in corrosive sublimate (distilled water saturated with mercury chloride) for 30 min, 1% acid fucsin for 30 s, 1% phosphomolybdic acid for 75 s and Mallory's liquid (2.5% orange G, 2% oxalic acid, and 0.5% aniline blue) for 45 s. All samples were washed with distilled water between staining steps.

Quantifications

Distances between adult venous valves were measured using the ImageJ software. These values were measured from the central point of each GFP+ structure to the next one, by analysing whole mount images of seven different adult hearts (n = 7). Each replicate would correspond to the mean value of all measured distances over one adult heart surface.

Ventricular thickness was measured on Mallory's trichrome stained images by using ImageJ software. At least 3 tissue sections of 5 mutant and 5 wild type embryos were analysed. A mean value of all measurements per embryo was represented as a replicate.

Chapter 3: Results

3.1. *Fsp1* promoter-driven GFP accumulation recapitulates FSP1 protein expression in embryonic and adult cardiac tissues

GFP expression in *Fsp1*^{GFP} transgenic mice faithfully recapitulates FSP1 protein fibroblast expression, as shown by the colocalization of the GFP reporter with the FSP1 protein in cardiac ventricular interstitial cells (Fig.10). The colocalization of the GFP reporter with the FSP1 protein was evident in mouse embryonic fibroblasts (MEFs), isolated from E13.5 *Fsp1*^{GFP} mice (Fig10A-C). The expression of CD90, a well-known mesenchymal marker, was shown in a subpopulation, about 14,41±2,6%, of these GFP^{+} MEFs at passage 2 (Fig.10B-C). Adult cardiac ventricular GFP^{+} interstitial cells display a spindle-shaped phenotype (Fig.10D-E). This cell compartment includes both bona fide cardiac fibroblasts (GFP^{+}/ProCOL1^{+}) (Fig.10F) and infiltrated blood-borne cells (GFP+/CD45^{+}) (Fig.10G). In addition to these *Fsp1*$^{+}$/CD45^{+} circulating cells, *Fsp1*$^{+}$/CD41^{+} structures lacking nuclei were observed (Fig.10H). FACS analyses of digested adult hearts (n=3) showed a 2±0.69% of GFP+ cells (Fig.10I). Mesenchymal (CD90, mEF-SK4), endothelial (CD31), and circulating (CD45) cellular markers were observed in this GFP+ cardiac cell population (Fig.10I).

3.2. Embryonic and postnatal AV cushions express *Fsp1*

To investigate *Fsp1* expression pattern in the endocardium and other cardiac endothelia we have used a well-established *Fsp1*^{GFP} murine transgenic line (Iwano et al., 2002). These transgenic mice express GFP under the control of the murine *Fsp1/S100A4* gene promoter. Earliest *Fsp1* cardiac expression was identified in the atrioventricular (AV; Fig.2A) and

proximal cardiac outflow tract (OFT) valve primordia (a.k.a. endocardial cushions; Fig.11B) of E11.5 mouse embryos. At this stage, only a few OFT endocardial cells were found to be GFP+ (Fig. 11C). GFP expression, however, was frequent in the AV endocardium ($CD31^+$, Fig. 11D). The majority of E11.5 AV or OFT GFP^+ cells were found within the mesenchymal core of the cushions (Fig. 11D).

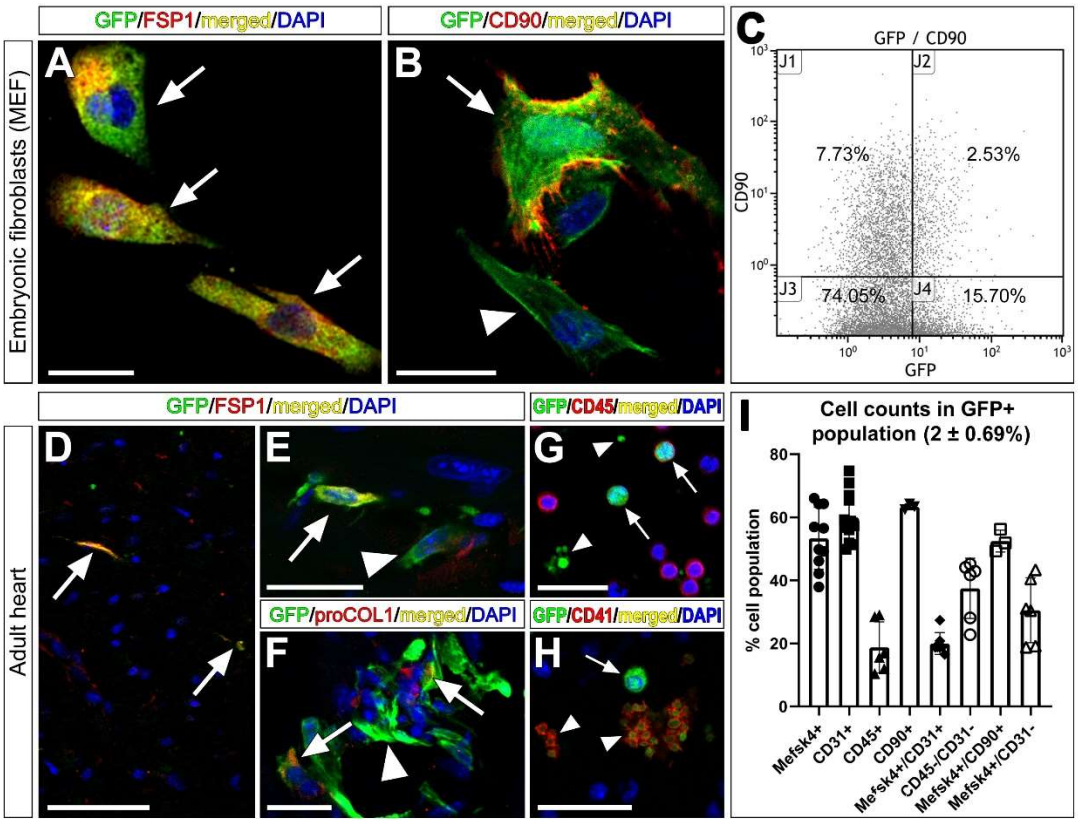

Figure 10. Fsp1-GFP reporter expression in embryonic and adult fibroblasts. GFP expression was observed in embryonic fibroblasts (MEF) extracted from E13.5 mouse embryos and expanded in vitro (A-C). FSP1 (A) and CD90 (B,C) proteins were observed in the GFP+ population of these cells (arrows), whereas not all GFP+ cells were CD90+ (B, arrowhead). Co-localization of GFP transgenic reporter (green) and FSP1 protein (red) was observed in elongated fibroblast-like cells in the adult heart (D,E, arrows). ProCOL1 was observed in some of these GFP positive cells (F). $GFP^+/CD45^+$ infiltrated cells (G) and $GFP^+/CD41^+$ non-nucleated cell elements (H) were observed in the adult heart (G). Different percentages of mesenchymal (CD90, mEF-SK4), endothelial (CD31), and circulating cells (CD45) were observed in the cardiac GFP+ cell population of the adult heart (I). **Scale bars:** A,B,E,F,G,H =25μm; D=50μm.

Colocalization of GFP and the FSP1 protein confirms that the *Fsp1*^{GFP} transgene faithfully recapitulates native *Fsp1* spatio-temporal expression pattern in the cardiac OFT (Fig. 11E). At E13.5, GFP expression was conspicuous in AV and OFT cushion mesenchymal cells (Fig. 11F,G and Fig. 11H, respectively). Expression of GFP in AV (Fig. 11G,G') and OFT (Fig.11 I,I') CD31+ endocardial cells is also evident at these stages. GFP expression was maintained at perinatal and postnatal stages (ED18.5, Fig. 11J,K). Endomucin (END) counterstaining of GFP+ AV valve mesenchyme revealed FSP1 protein accumulation in both endocardial (END+) and (END−) mesenchymal cells (Fig. 11J). GFP/FSP1 colocalization was evident in both tissues (Fig. 11K). This same expression pattern was observed in 4-day-old postnatal hearts (P4, Fig. 11L-O).

3.3. GFP is expressed in a subset of cardiac endothelial cells

Cardiac venous endothelial cell Fsp1 *expression delineates venous valves*

Detailed analysis of GFP expression in the heart revealed the presence of significant numbers of GFP+ cells associated to the prenatal and postnatal coronary vasculature at E15.5 (Fig. 12A-F'). No GFP+ coronary cells could be recorded in the coronary endothelium before this time point. These GFP+ coronary cells were evenly distributed in the coronary tree. At these stages, the majority of GFP+ cells in the developing cardiac walls were nonvascular ones (CD31^{-}; Fig. 12A).

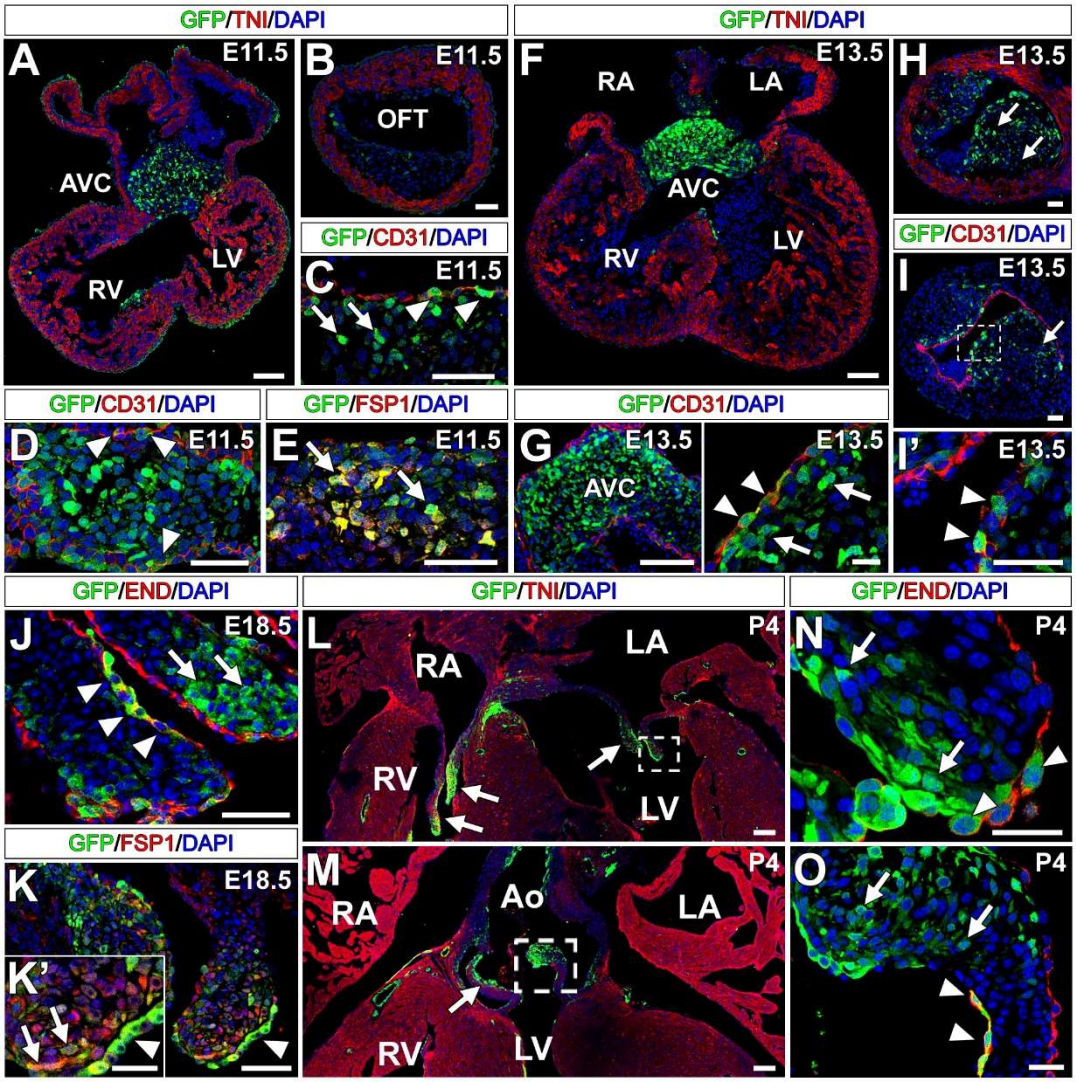

Figure 11. *Fsp1* in developing and adult atrioventricular valves. GFP expression was first observed the developing atrio-ventricular (A, arrows) and cardiac outflow tract (B, arrows) valve primordia/cushions of E11.5 *Fsp1*^{GFP} embryos. Both endocardial and mesenchymal cells in the OFT (C, arrowheads and arrows, respectively) and AV (D, arrowheads and arrows, respectively) were GFP+. GFP and FSP1 protein extensively colocalize in these tissues (E, arrows). At E13.5, GFP expression was robustly expressed in cardiac AV cells (F,G, arrows). Both endocardial and cushion mesenchymal cells were GFP+ (G', arrows and arrowheads, respectively). E13.5 OFT cushions also are GFP+ (H,I, arrows); GFP expression is evident in endocardial and cushion mesenchymal cells (I, arrows; I', arrowheads). At E18.5, FSP1 protein strongly colocalized with the GFP reporter in endocardium and mesenchyme of the forming AV leaflets (J, arrowheads and arrows, respectively). FSP1 protein and GFP expressions overlapped in both the endocardium (K, arrowhead) and the cushion mesenchyme (K', arrows). Postnatal (P4) AV valves (L, arrows) and arterial valves (M, arrows) strongly expressed GFP. Boxed areas in L and M are

magnified in N and O, respectively, to show GFP expression in maintained in both endocardial (End+, arrowheads) and mesenchymal (End−, arrows) cell subsets. **Scale bars:** A,F,G,L,M = 100 μm; B,C,D,E,H,I,I',J,K = 50 μm; K', N, O = 25 μm; G'=10 μm. **Abbreviations:** AV, atrioventricular; AVC, atrioventricular canal; LA, left atrium; LV, left ventricle; OFT, cardiac outflow tract; RA, right atrium; RV, right ventricle**.**

Endothelial GFP expression was first found in discrete populations of vascular endothelial cells (Fig. 12B-F'), both on the subepicardium (heart surface, Fig. 12B,D) and intramyocardially (Fig. 12C,E); the GFP reporter and the FSP1 protein extensively colocalized in these blood vessels (Fig. 12F,F'). By E18.5, GFP expression was observed at specific locations of the CD31+ coronary vasculature (Fig. 12G), while adult GFP vascular endothelial expression was restricted to cardiac venous valves and some discrete endothelial domains close to these structures (Figure 12H-J). GFP+ valves in the adult heart vascular network appeared regularly along the vessel lumen, so that the average distance between these valves was 200 μm (Fig. 12K).

Cardiac lymphatic endothelial cells express Fsp1

The first consistent GFP expression in cardiac lymphatic vessels (LYVE1^{+}) was found at E18.5 and shows a clear morphological trend to concentrate on specific regions of the developing lymphatic vessels (Fig. 12L,M). Although we were not able to co-localize GFP and a specific marker for developing valves, GFP expression was progressively confined to structures morphologically similar to lymphatic valves from postnatal stages to adulthood (Fig. 12M,N). Double counterstain with IB4 lectin (for vascular endothelium) and Lyve1 (for lymphatic endothelium) in adult hearts showed that both lymphatic and blood vessels simultaneously expressed the GFP reporter (Fig. 12O-O').

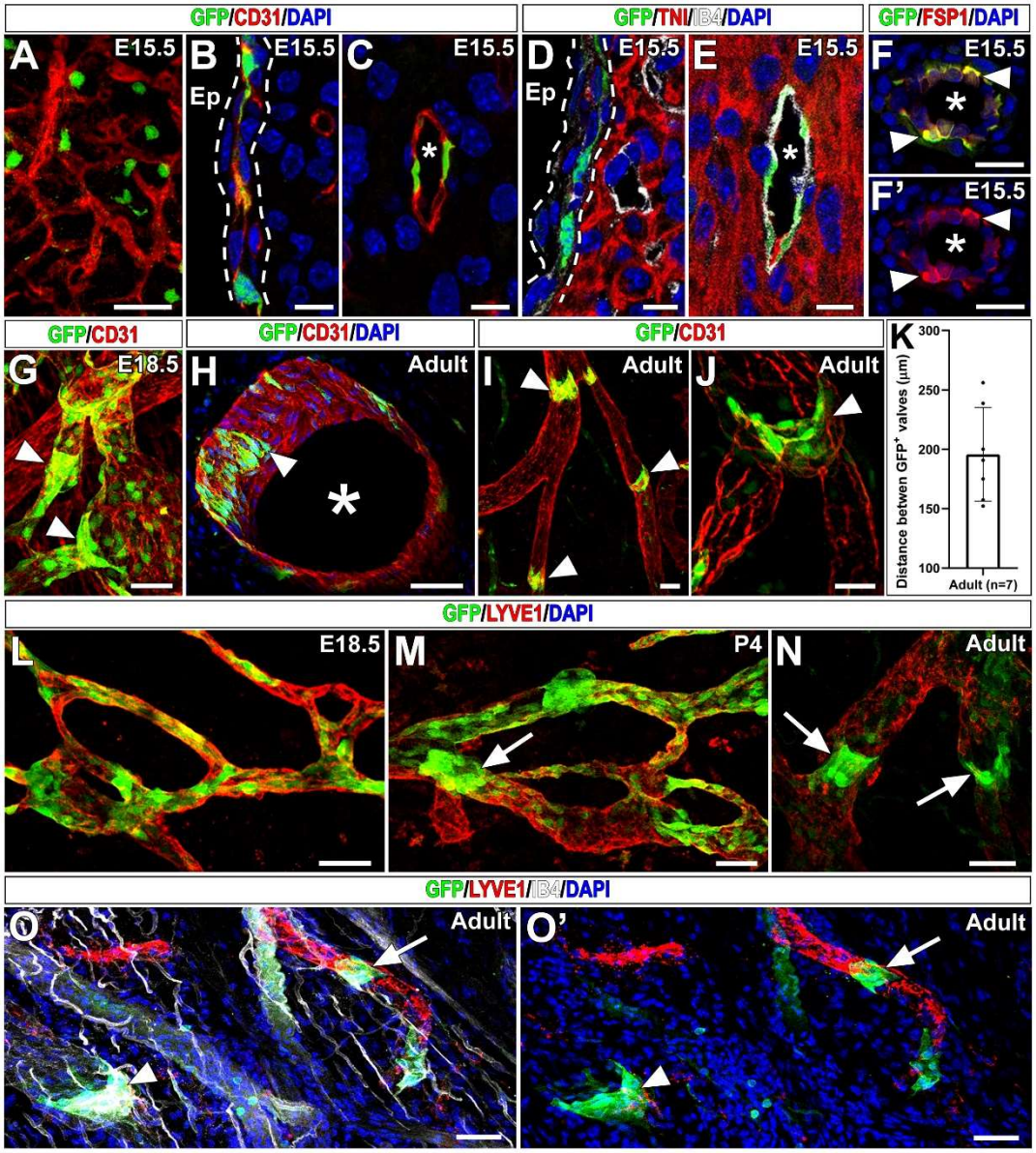

Figure 12. *Fsp1* expression in developing and adult coronary and lymphatic vascular systems. At E15.5, most cardiac GFP+ cells were found associated to but not integrated into the CD31+ developing coronary endothelium (A, arrowheads). First GFP+ vascular endothelial cells (either CD31+ or IB4+) were found in the subepicardial (B, D; the subepicardial space is marked by dashed lines) and intramyocardial (C, E) coronary vessels of the AV and interventricular groove (asterisks mark the vascular lumen); colocation of GFP and FSP1 was obvious in these cells (F,F', arrowheads). At E18.5, GFP expression was widely observed along the CD31+ coronary endothelium (G). Vibratome sectioning of adult hearts revealed a patterned distribution of these GFP+ cells in discrete CD31+ venous endothelial domains (H, arrowhead; the asterisk marks the vessel lumen) and structures that had the unambiguous 3D morphology of venous valves (I,J, arrowheads). These GFP+ endothelial domains were spatially patterned (K, arrowheads). At E18.5, GFP signal was

evident in the developing Lyve1+ lymphatic endothelium (L). Postnatal (M) and adult (N) cardiac Lyve1+ lymphatics, showed GFP signal restricted to lymphatic valves (M-O', arrows) and IB4+/Lyve1− venous valves (O,O', arrowheads). **Scale bars:** A,G,H,I,L,M,N,O,O' = 50 μm; F,F',J = 25 μm; B,C,D,E = 10 μm. **Abbreviations:** Ep, epicardium.

3.4. Mouse femoral vein also express *Fsp1*

GFP+ cells were observed along the femoral vein of *Fsp1*^{GFP} mice (Fig.13). GFP+ valve-resembling structures were found in this tissue (Fig.13A). GFP+ endothelial cells (CD31+) were also observed in the endothelial lining of the vein wall (Fig.13B, C).

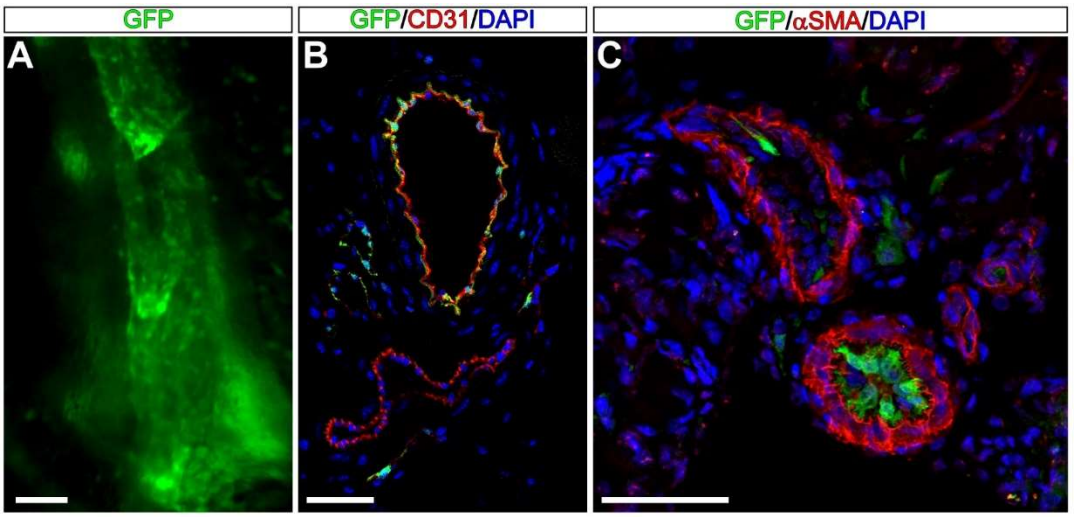

Figure 13. GFP expression in the femoral vein. GFP expression was observed in the femoral vein of *Fsp1*^{GFP} adult mice (A-C). GFP+ cells were located resembling venous valves (A). The endothelial nature of these cells was evidenced by CD31 marker (B) and by their location (C), forming the inner lining of the vessel. **Scale bars:** 100μm.

3.5. Fsp1 overexpression in endothelial cell culture

Transfection of HUVEC cells with a plasmid vector directing overexpression of Fsp1 gene generates differences in the trend of the expression profile. Although no statistical analyses were performed due to the low number of replicates (n=2), after 24 hours, the analysis by qPCR suggests an increase in the expression trend of the lymphatic valve marker

Foxc2 (Fig.14B). However, the transfection does not induce variation in the expression trend of the valve marker Prox1 (Fig.14C) and seems to indicate a decrease in the expression marker of the lymphangiogenesis factor Vegfr3 (Fig.14D).

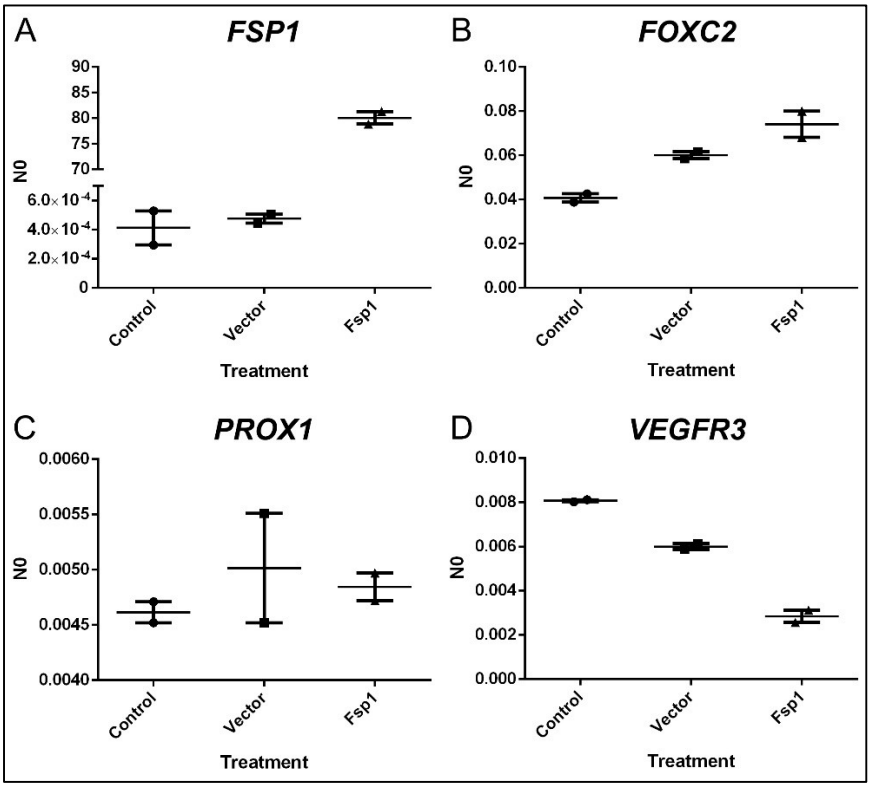

Figure 14. HUVEC transfection with Fsp1 overexpression plasmid. qPCR analyses of HUVEC cells showed the expression trend of different lymphatics markers. No statistical analyses were performed due to the low number of replicates (n=2). The graphs represent the geometric mean of two different biological replicates, and the error bars are generated by the standard error of the mean (SEM) for the FSP1 (A), FOXC2 (B), PROX1 (C) and VEGFR3 (D) expression.

3.6. Conditional deletion of Fsp1 in proepicardial cells does not disturb epicardial and myocardial development

Fsp1 gene was conditionally deleted in epicardial progenitors located in the embryonic ST/PE by using *G2-Gata4*^{CRE} mouse transgenic strain, as previously reported in the literature (Cano et al., 2016). No evident morphological anomalies were observed in transgenic embryonic hearts (Fig. 15A,B). At E12.5, significant differences were observed in the compact ventricular myocardium thickness of the left ventricle as compared to the right one in wild type embryos, while this difference was not significant in transgenic embryos (n=5) (Fig.15C).

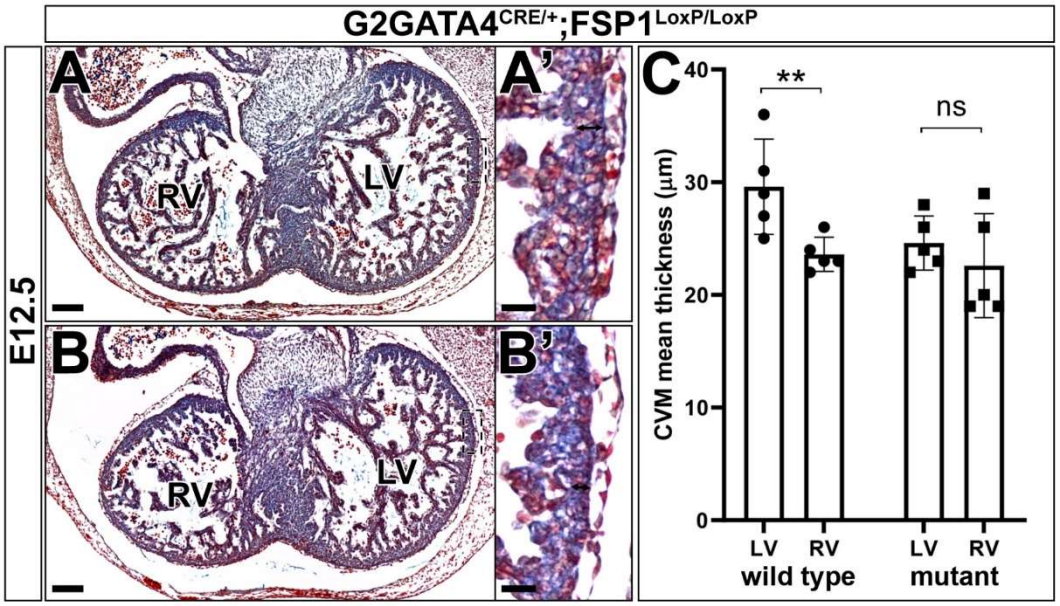

Figure 15. *Fsp1* conditional deletion in ST/PE cells does not disrupt heart development. *G2Gata4*^{CRE};*Fsp1*$^{LoxP/LoxP}$ mutant embryos were isolated at E12.5 and compared to stage-matched wild type ones. At E12.5, no evident differences were observed in neither heart morphology nor in the ventricular wall of wildtype (A-A') and mutant (B-B') embryos. Quantifications of the compact ventricular myocardium (CVM) thickness evidenced significant differences in the left ventricle (LV) as compared to the right one (RV) in wild type embryos ($p<0.05$), while no significant differences were observed in mutant hearts ($p=0.42$) (n=5 in both cases). **Scale bars:** 100µm. **Abbreviations:** LV, left ventricle; RV, right ventricle.

3.7. GFP+ (*Fsp1*+) bone marrow derived cells contribute to the AV valves, but do not form coronary venous valves

Previous studies in our group showed the presence of FSP1 protein in circulating cells (Ruiz-Villalba et al., 2015). In order to characterize this population, we have performed bone marrow transplantation using *Fsp1*^{GFP} mice as donors. Since GFP$^+$/CD45$^+$ cells were observed in the bone marrow of *Fsp1*^{GFP} mice (Fig.16A, A'), we decided to analyse the contribution of GFP+ bone marrow derived cells (BMC) to the adult heart. Blood lineage reconstitutions in busulfan-myeloablated neonatal mice were performed to efficiently trace bone marrow GFP$^+$ cells in the host heart (Fig.16B).

Circulating cells of one-month old mice were analysed by flow cytometry and considered as efficiently transplanted with more than 40% of GFP+ circulating cells (see Table 9). In these treated adult hearts, bone marrow-derived circulating cells were not observed within the forming venous valves (Fig.16C). Instead, accumulations of GFP$^+$/CD45$^+$ cells were observed in both the myocardial interstitium (Fig.16D, D') and atrioventricular valves (Fig.16E, E').

Sample	Female 0	Female 1	Male 0	Male 1	Male 2	Male 3	Male 4
% GFP	79.51	45.31	73.73	14.39	84.18	57.29	86.72

Table 9. Percentage of GFP in each sample after transplantation experiment.

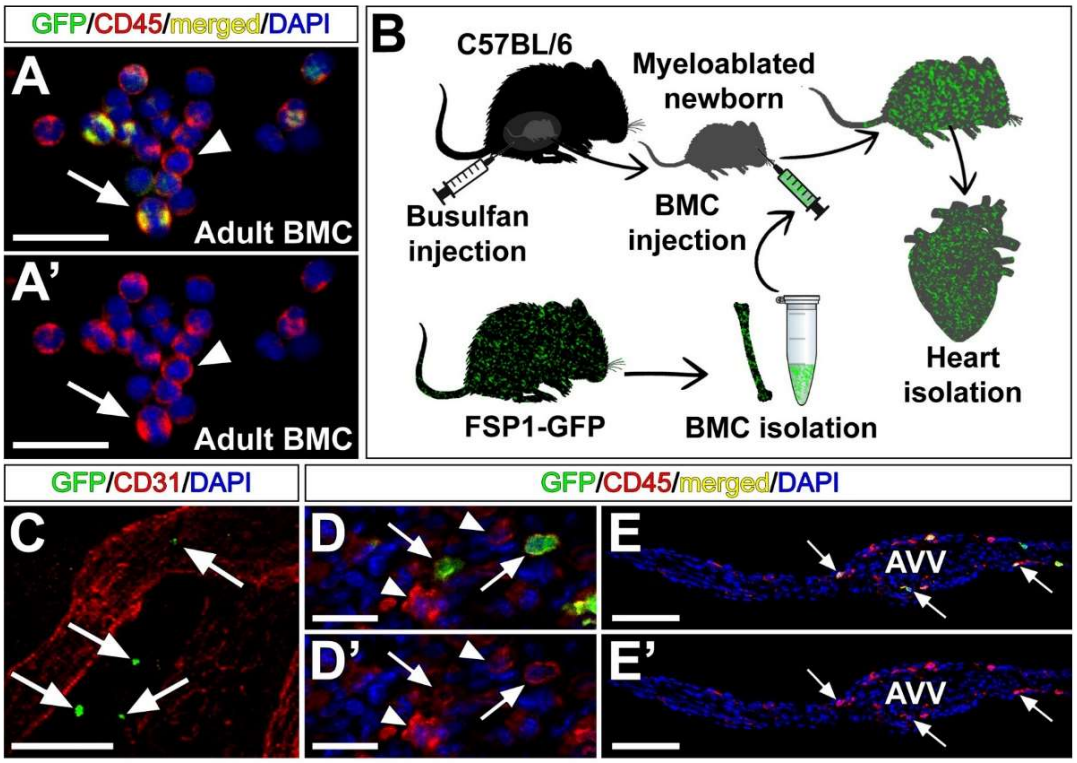

Figure 16. Bone marrow derived cells contribution to the adult heart. GFP⁺/CD45⁺ cells were observed in bone marrow cells (BMC) suspensions extracted from adult *Fsp1*^{GFP} mice (A,A', arrows). These cells represent a subpopulation of all CD45⁺ cells (A,A', arrowheads). Bone marrow cells (BMC) were isolated from *Fsp1*^{GFP} mice and injected in busulfan-myeloablated C57BL/6 neonatal mice (B). Heart organs were isolated one month after bone marrow injection (B). CD31 whole mount immunostaining procedures of these tissues showed an extravascular location for these GFP+ cells (C). Both whole mount and sections immunostainings were used to find GFP⁺/CD45⁺ cells in the heart surface (D,D') and in atrioventricular valves (E,E'), respectively. **Scale bars:** A,A',D,D'=25μm; C,E,E'=100μm. **Abbreviations:** AVV, atrio-ventricular valves.

3.8. Fsp1 in myocardial infarction

Wild type mice were irradiated and transplanted with *Fsp1*^{GFP} bone marrow derived cells (Fig.17A). Myocardial infarction was induced by the permanent ligation of the left descendent coronary artery (LDA) one month after the irradiation and bone marrow transplantation procedures (Fig.17A). *Fsp1*^{GFP} bone marrow derived cells were observed in the ventricular wall 7 days after the myocardial infarction (Fig.17B, B'). CD45 marker was

observed in some of these GFP+ cells (Fig.17D-G). The percentage of GFP$^+$ and GFP$^+$/CD45$^+$ cells was measured in both infarcted and sham operated hearts at 7 and 30 days after the procedure (Fig.17H). A significant increase of both GFP$^+$ and GFP$^+$/CD45$^+$ cells was observed at 7- and 30-days post infarction as compared to sham operated hearts (Fig.17H). Although GFP+ cells were observed in the remote zone of infarcted hearts, this region was not analysed and compared to the infarcted one.

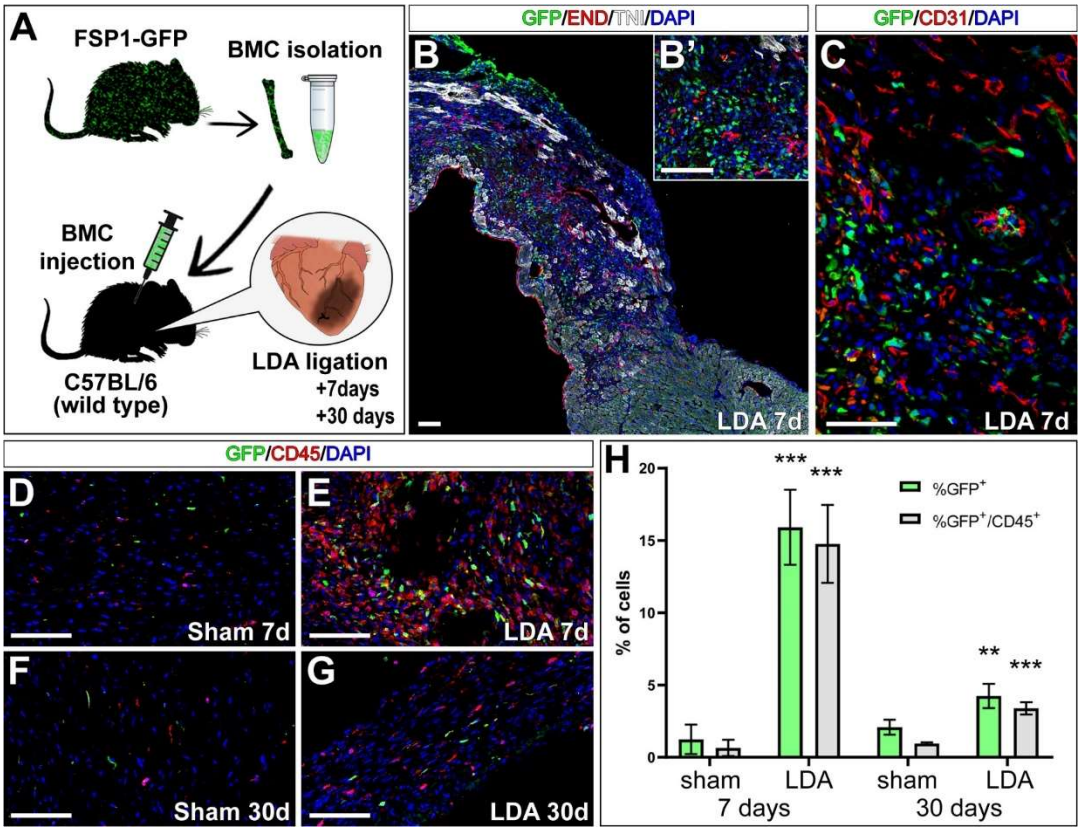

Figure 17. Fsp1-GFP+ bone marrow-derived cells are recruited to the damaged heart. Bone marrow cells were isolated from *Fsp1*^{GFP} mice and transplanted to irradiated wild type adult mice (n=3 for each stage). Coronary ligation was performed to these mice one month later (A). GFP$^+$ cells were observed in the injured region 7 days after coronary ligation (B, B'). GFP$^+$/CD31$^+$ cells were observed in the infarcted area (C). CD45 marker was used to identify circulating cells in the heart of sham operated animals and ligated adult mice 7 and 30 days after coronary ligation (D, E and F, G, respectively). Percentages of GFP$^+$ and GFP$^+$/CD45$^+$ cells were significantly higher 7 days after ligation in damaged hearts as compared to sham operated ones (H; $p<0.01$). The percentages of both GFP$^+$ and GFP$^{+/}$CD45$^+$ cells were also significantly higher ($p<0.05$ and $p<0.01$, respectively) in the

infarcted zone of the damaged hearts at 30 days after ablation as compared to sham operated controls (H) **Scale bars:** B,B',D,E,F,G=100µm; C=50µm. **Abbreviations:** LDA, left descendant artery coronary ligation.

3.9. Fsp1 is expressed in a subset of myocardial cells

In *Fsp1*^{GFP} mice, at E16.5, the expression of GFP was evident in discrete cell populations of the atrioventricular myocardium, coinciding with the anatomical location of the atrioventricular node (AVN) (Fig.18A) and the distal tip of some ventricular trabeculae (Fig.18A-C). These prenatal (E16.5) cells co-express the GFP reporter together with both the cardiac conduction marker HCN4 (Fig.18B) and the FSP1 protein (Fig.18C).

Although we were not able to co-localize FSP1 and HCN4 expression at E16.5, we demonstrate that the Fsp1 expressing cells show typical properties of the cardiac conduction system, including their sub-endocardial location (Fig.18D) and the expression of myocardial markers such as troponin I (TNI) (Fig.18E) at postnatal stages. All these GFP+ cells at the tip of the trabecula present the myocyte-defining sarcomeres (Fig.18F) and express Contactin-2 (Fig.18G), evidencing their contribution to the developing Purkinje fibers.

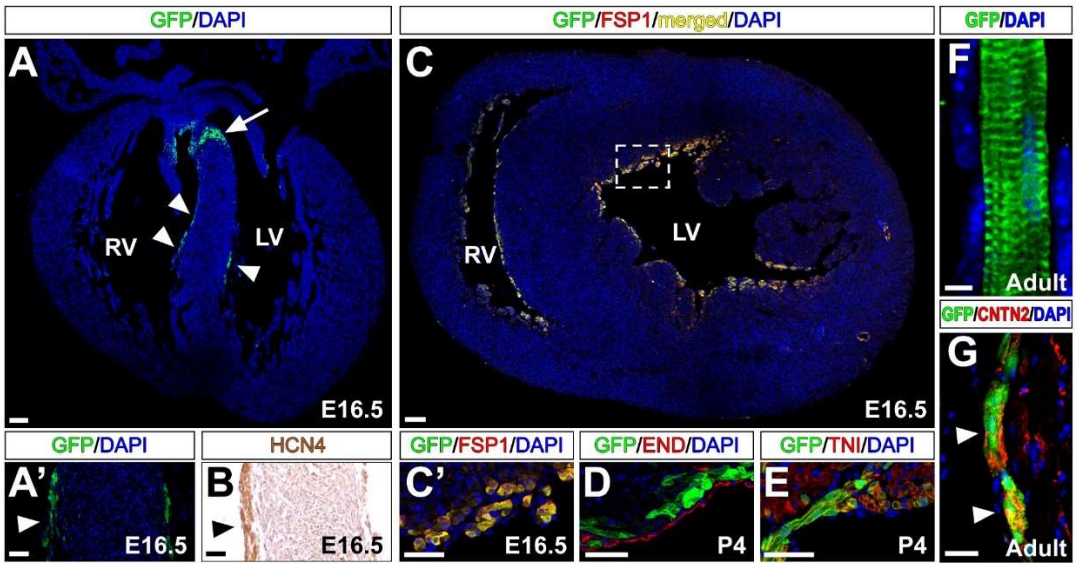

Figure 18. GFP expression in the cardiac conduction system. At 16.5, GFP was observed in diverse regions of the interventricular septum of *Fsp1*^{GFP} mice, including the AV node region (A, arrow) and fibers in a sub-endocardial location (A,A', arrowheads). HCN4 expression was found in these sub-endocardial cells (B, arrowhead). Co-expression of GFP and FSP1 was evident in these sub-endocardial cells (C,C'). The sub-endocardial location and the myocyte nature of these cells were evidenced by the endomucin (END) endocardial protein (D) and troponin (TNI) myocardial marker (E), respectively. A higher magnification allowed us to confirm the presence of sarcomeres in these cells (F). Finally, contactin-2 (CNTN2) was observed in these GFP+ cells (G). **Scale bars:** A,C=100μm; A',B,C',D,E=50μm; F=5μm; G= 25μm. **Abbreviations:** CNTN2, contactin-2; END, endomucin; GFP, green fluorescent protein; LV, left ventricle; RV, right ventricle; TnI, troponin I.

3.10. Different types of hiPSC-derived cardiac cells express *Fsp1*

To assess whether Fsp1 expression is affected during a differentiation process, human induced pluripotent stem cells (hiPSC) were cultured and differentiated into different cell types following previously described protocols (Fig.19A). These experiments would give us new insights on how Fsp1 expression can be induced by multiple differentiation processes. The differentiation of these hiPSCs to endothelial, epicardial, fibroblast, and cardiomyocyte-like cells was evidenced by using specific antibodies for each

cell type (Fig.19B-F). This differentiation to cardiomyocytes was additionally evidenced in the DRAGGN cell strain by the expression of GFP, since these cells were Nkx2-5^{GFP} (Fig.19F) and mRuby, Since these cells were ACTN^{mRuby} (Fig.19F-G''). An additional validation of these differentiated cells by analysing the expression of specific gene sets was performed (Fig.S1).

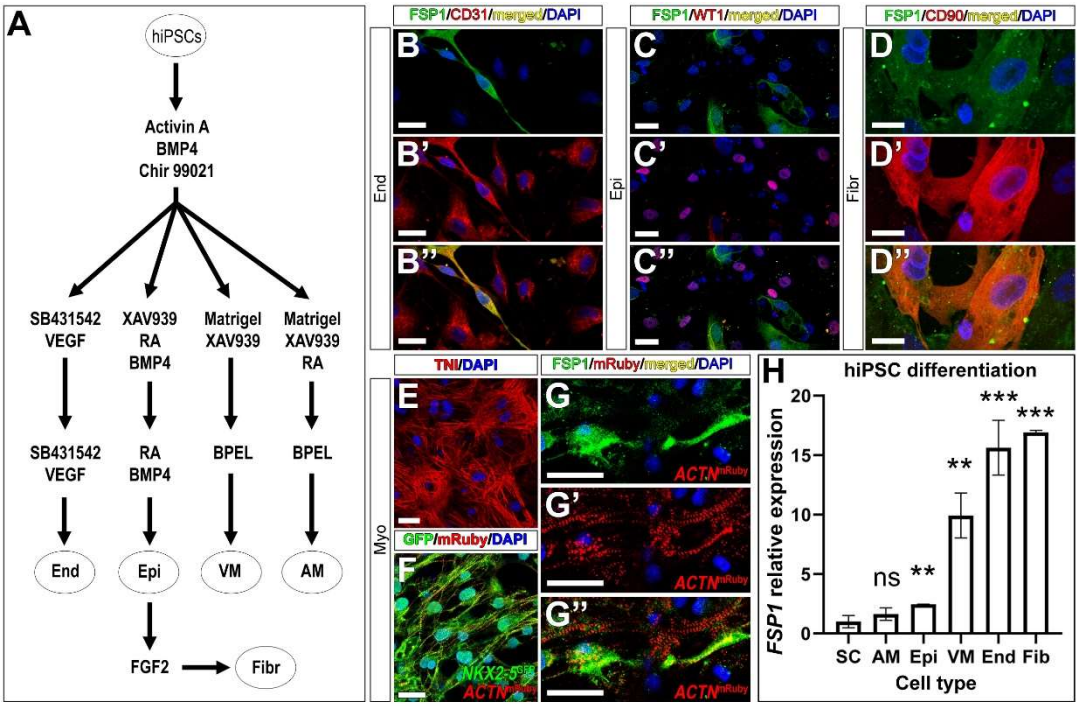

Figure 19. *FSP1* is expressed in multiple human differentiated cell populations. hiPSCs were differentiated to multiple cell types following a previously described differentiation protocol (A). FSP1 protein was observed in all hiPSCs differentiated populations (B-G). These cells were characterized by the expression of CD31 (endothelial-like cells; B), WT1 (epicardial-like cells; C), CD90 (fibroblasts-like cells; D) and troponin TNI (cardiomyocytes; E). Differentiation of DRAGGN cells to cardiomyocytes was analysed by GFP and mRuby expression, associated to *NKX2-5* (F) and alpha-actinin (*ACTN*; G) reporter genes, respectively. FSP1 was observed in some of these DRAGGN derived cardiomyocytes (G-G''). qPCR analyses of all hiPSCs differentiated populations revealed a significant increase of *FSP1* expression in epicardial and ventricular myocardial cells ($p<0.05$) as well as in endocardial and fibroblast -like cells ($p<0.01$) (H). No significant differences of *FSP1* expression levels were observed in atrial myocardial -like cells ($p=0.21$). **Scale bars:** 25µm. **Abbreviations:** AM, atrial myocardium; End, endocardium; Epi, epicardium; Fib, fibroblasts; Myo, myocardium; VM, ventricular myocardium.

Different expression levels of the FSP1 protein were observed in differentiated cells (Fig.19B-D,F). Accordingly, *FSP1* expression was significantly higher in differentiated cell populations as compared to undifferentiated ones (Fig.19H). The highest *FSP1* expression was observed in fibroblasts and endothelial-like cells, followed by ventricular cardiomyocytes and epicardial -like cells (Fig.19H). No significant differences were observed in *FSP1* expression in atrial cardiomyocyte-like cells as compared to undifferentiated stem cells (Fig.19H). All quantifications were performed based on three biological replicates and three technical replicates.

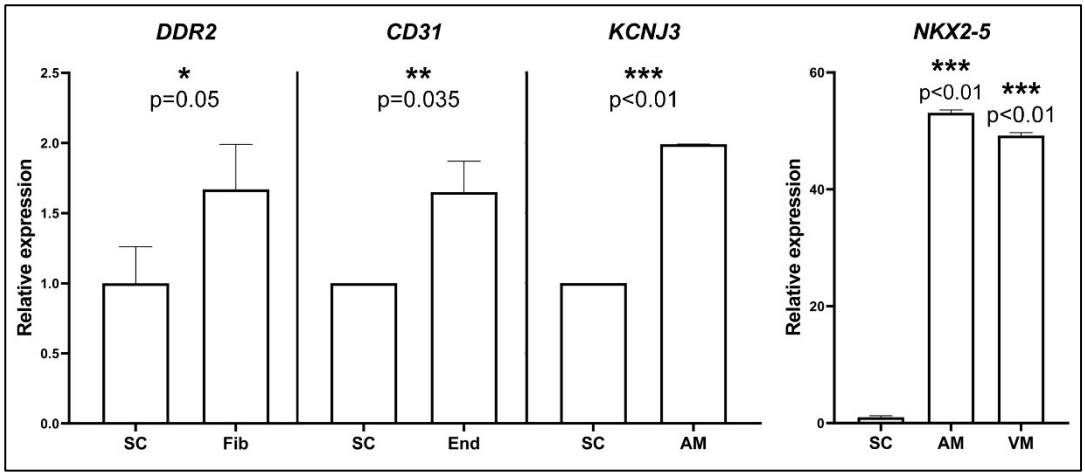

Figure S1. qPCR validation for hiPSC differentiation. qPCR analyses of hiPSCs differentiated populations revealed a significant increase of *DDR2*, *CD31* and *KCNJ3* expression in fibroblasts (Fib), endothelial cells (End) and atrial myocytes (AM), respectively. In addition, a significant increase of NKX2-5 was observed in atrial (AM) and ventricular myocytes (VM). All values were compared to the expression of each gene in undifferentiated stem cells (SC). **Abbreviations:** AM, atrial myocytes; End, endothelial cells; Fib, fibroblasts; VM, ventricular myocytes.

Chapter 4: Discussion and conclusions

Embryonic morphogenesis is a complex process that requires the spatio-temporal coordination of large number of regulatory genetic networks, associated transcriptional programs, and the orchestration of multiple cell signals. All these molecular events result in the establishment of the embryonic body plan, the differentiation and diversification of specialized tissues from undifferentiated progenitors, and the completion of the intricate morphogenetic phenomena that result in mature tissues and organs. The vast majority of proteins studied in the context of embryonic development are transcription factors, i.e. DNA-binding proteins able to transactivate or repress gene transcription or growth factors and the elements of their transduction machinery. However, an increasing number of evidences suggest that other protein types could be key effectors to the differentiation and functional maturation of tissues and organs. Therefore, besides the evident relevance of identifying upstream activators of developmental processes, it is also necessary to characterize and understand the role of downstream molecular effectors of the triggering. This could be of extreme relevance for our knowledge on how sophisticated anatomical structures are formed. In this thesis, we will study the involvement of the protein FSP1/S100A4 in cardiac development.

4.1. FSP1 is involved in multiple biological events

FSP1 is a low molecular weight protein (10-12 kDa) that normally forms symmetric homodimers stabilized by non-covalent interactions (Moore, 1965). This protein has been reported to be involved in a wide range of biological processes such as cell proliferation, migration (Okada et al., 1997) and tissue inflammation (Li et al., 2010), but the detailed molecular basis for

its role in these phenomena is far from being understood. The pleiotropic functions of FSP1 could be partially explained by the existence of different protein isoforms (Kiss et al., 2016), their ability to acquire different conformations depending on its binding to calcium (Pathuri et al., 2008), or by the wide range of binding proteins (Garrett et al., 2006).

The formation of FSP1-methionine aminopeptidase-2 heterodimers (Ma et al., 2011; Wang et al., 2003) and the association between FSP1 with myosin IIA (Li and Bresnick, 2006) or p53 (He et al., 2019; Orre et al., 2013) give us two different examples of how FSP1 regulate cell proliferation and migration phenomena, respectively. In addition, FSP1 was specifically associated with the growth and integrity of endothelial lymphatic valves by the modulation of liprin ß1 protein phosphorylation (Kriajevska et al., 2002; Norrmén et al., 2010). Altogether, a high ubiquity in the location and function of FSP1 protein suggests us FSP1 as a protein-specific modulator, instead of being the final effector of its proposed functions.

4.2. Searching for tools to study Fsp1 biological functions

Cellular protein expression levels are highly variable. In the embryo, these variations can be related to both differentiation stage and physiological status (e.g. quiescent versus activated) of the cells. Protein expression levels can occasionally be low, making difficult their analysis by using immunohistochemical techniques. The poor quality of available antibodies usually represents an additional problem on the analysis of certain proteins. During the first phase of this thesis, various FSP1 antibodies were tested, but only one of them provided a consistent, reproducible staining (see materials and methods). However, since we wanted to make sure we were not missing cells with low FSP1 expression levels in our study, we decided to combine

immunohistochemistry with an additional tool to evaluate *Fsp1* expression. We thus used a previously described transgenic mouse line in which the GFP reporter expression is under the control of the *Fsp1* promoter (Iwano et al., 2002). This mouse allows for the real time assessment of *Fsp1* expression in all tissue types (Jang et al., 2013; Sinha et al., 2018).

In our screening of *Fsp1* expression in cardiac tissues, we have found that a majority of GFP^+ cells also express significant levels of the FSP1 protein, meaning that GFP reporter expression faithfully recapitulates natural *Fsp1* expression pattern. We have however discovered that some GFP^+ cells did not show FSP1 protein accumulation. We believe this finding can result from the presence of low FSP1 protein amounts under the detection threshold of immunohistochemical techniques. It is also possible that the antibody we have used does not identify all FSP1 protein isoforms; this alternative explanation would be especially relevant if we consider that non-conventional secretion of the FSP1 protein has been reported (Prudovsky et al., 2008), so that the intracellular GFP and the extracellular FSP1 protein forms would be unlikely to overlap in space. Another possible explanation could be the activation of the transcription, but the mRNA is never translated into protein. We also found some positive FSP1 cells that were GFP negative. This fact could be explained by both the half-life life of each protein itself, estimated in 26h for GFP (Corish and Tyler-Smith, 1999) and 85.5h (Rivard et al., 2007) for FSP1 and by the high homology between different S100 proteins, which increases the cross-reactivity of antibodies (Boye and Mælandsmo, 2010).

4.3. Fsp1 in the developing heart

As previously indicated, FSP1 has been considered as a fibroblast marker for years (Strutz, 1995). The early motivation of this thesis indeed was to study the biological functions of this protein in cardiac fibroblasts, under the assumption of its expression must be restricted to this same cell population. However, it soon became evident that *Fsp1* was also expressed by discrete groups of different cardiac cells. Our findings corroborate previous disperse evidences describing *Fsp1* expression in some endothelial cells (Gibbs et al., 1995), neurons (Kiryushko et al., 2006), macrophages (Osterreicher et al., 2011), and bone marrow-derived cells (Cheng et al., 2012; Ruiz-Villalba et al., 2015). In this work, we describe *Fsp1* expression in multiple cell types in the developing and adult mouse heart. These cells include cardiac fibroblasts, circulating cells ($CD45^+$), platelets ($CD41^+$) and other cell endothelial and myocardial populations as discussed below.

4.3.1. On the fibroblast cell type and FSP1

FSP1 cardiac expression has widely been associated to fibroblast cells. Indeed, FSP1 has been used as a fibroblast specific marker (FSP1 stands for 'Fibroblast Specific Protein'), in addition to other molecules like DDR2, POSTN or CD90. However, recent studies demonstrate that none of these markers are universally expressed by all fibroblasts (Guerrero-Juarez et al., 2019; Ruiz-Villalba et al., 2020). These issues related to the use of a single molecule to assign a cell identity have been discussed elsewhere (Cano et al., 2016), and are relevant to this thesis. The results shown in this thesis confirm that *Fsp1* is expressed in cardiac fibroblasts, but also

demonstrate that other non-fibroblastic cells would display strong *Fsp1* expression.

All the findings quoted above strongly suggest the necessity of re-evaluating the specificity of these so-called fibroblast markers, allowing for the re-interpretation of these molecules as dynamic indicators of the developmental or physiological status of fibroblasts. Accordingly, not all fibroblasts express CD90 (Koumas et al., 2003), which is often considered as a marker for relatively undifferentiated mesenchymal cell types (Dominici et al., 2006; Lin et al., 2013). This could, for example, explain the low prevalence of CD90 expression found in FSP1-expressing fibroblasts in this thesis. Another important conclusion regarding this discussion is that fibroblast populations could be more heterogeneous than previously thought. This cellular heterogeneity and diversity has been previously suggested and linked to a differential ontogenetic origin of fibroblasts (Ruiz-Villalba et al., 2015; Zeisberg and Kalluri, 2010).

4.3.2. Fsp1 in endothelial cells

In this thesis we have described the highly patterned expression of *Fsp1* in three different cardiac endothelial populations: the endocardium, the coronary vascular endothelium, and the lymphatic endothelium.

The endocardium of the atrioventricular (AV) and conoventricular (CV) regions is necessary for the formation of cardiac valves. Activation of AV and CV endocardial epithelial-to-mesenchymal transition (EMT) is instrumental in the appearance of the first valvuloseptal mesenchymal cells. These valvuloseptal cell populations will progressively differentiate in a characteristic population of fibroblasts that actively synthesize ECM molecules, especially collagens, and are responsible for the morphological transformation of immature cardiac valve primordia (a.k.a. endocardial

cushions) in the functional fibrous leaflets of adult cardiac valves. With time, the adult cardiac valves become decellularized and only a few fibroblastic cells remain in the valvular leaflets. Most of these cells, known as Valvular Interstitial Cells (VIC), are quiescent and have a fibroblast phenotype, while minor populations of myofibroblasts and smooth muscle cells-like can also be found in cardiac valves (Bairati and DeBiasi, 1981; Rabkin-Aikawa et al., 2004; Taylor et al., 2003). Most interestingly, these cells can be activated to a secretory myofibroblast phenotype under pathological stimulations (Chester and Taylor, 2007). Our report of *Fsp1* expression in the E11.5 AV and OFT endocardial cushions is compatible with the described role of FSP1 protein in EMT (Schäfer and Heizmann, 1996), as well as with fibroblast activation (Ruiz-Villalba et al., 2015).

Taken together, the local expression of Fsp1 in these (but no other) endocardial cells, and the reported involvement of FSP1 in EMT phenomena (Okada et al., 1997), strongly suggest this protein could play a significant role in endocardial EMT, but further research is necessary to confirm this hypothesis (e.g. loss-of-function experiments). We cannot, however, exclude the participation of this molecule in other parallel phenomena like the mobilization of the cushion mesenchyme in the developing valves.

4.3.3. FSP1 in cardiac vascular and lymphatic endothelium

In our screening of FSP1 expression in developing embryonic tissues we found FSP1 protein expression in some cardiac vascular and lymphatic cells. This expression, which primarily affects to some subsets of these cells only, becomes progressively restricted to the valvular structures of both coronary and lymphatic vascular networks.

The formation of coronary and lymphatic vascular systems is intensely researched. The coronary vascular tree is formed by the assembly and

coordinated differentiation of endothelial cells of multiple origins. The primitive coronary vasculature will be remodeled into a mature functional cardiac vascular circuitry under the control of instructive signals provided by secreted factors from the epicardium and myocardium (Olivey and Svensson, 2010). On contrary, an extracardiac origin has been proposed for some heart lymphatic vessels (Lioux et al., 2020). In addition, transdifferentiation of venous endothelial cells represents the major source of lymphendothelial cells during lymphatic system growth and remodeling. Cardiac lymphatic vessels are known to form from a heterogeneous pool of progenitors including venous endothelium (Srinivasan et al., 2007) and extracardiac cell sources (Lioux et al., 2020). Cardiac lymphatic vessel formation follows coronary morphogenesis in time (Klotz et al., 2015).

Previous results from other research groups have suggested a role for *Fsp1* in vascular development (Ochiya et al., 2014). Since the activation of cell migration is a common feature in vessels and valves development, it is tempting to speculate that FSP1 expression could be involved in the mobilization of cells during venous and lymphatic morphogenesis. It is also possible that FSP1 could be involved in the local modulation of cell proliferation, an idea that is in accordance with the reported interaction of FSP1 with P53 (He et al., 2019; Orre et al., 2013). In summary, our results show that these two vascular elements of the adult heart (i.e. coronary and lymphatic vascular systems) share the expression of *Fsp1* gene. Nevertheless, additional research is needed to clarify whether *Fsp1* is actively involved in the spatial patterning and/or the morphogenesis of cardiac venous valves.

Our results also show the expression of *Fsp1* in different subsets of endothelial cells, including cardiac veins and lymphatic valves. We suggest that *Fsp1* role in the development of these structures could be related with the initiation of venous and lymphatic endothelial cell protrusion towards the

vessel lumen, an event marking the first steps of venous and lymphatic valve leaflet morphogenesis (Bazigou et al., 2011). The molecular bases of this process are unknown, but is reasonable to speculate that FSP1 could contribute to maintain the integrity of these delicate structures through the modulation of Liprin ß1 (Kriajevska et al., 2002). Interestingly, Liprin forms protein complexes with CASK, also known to interact with connexins (Márquez-Rosado et al., 2012). This idea would fit the characteristic expression of connexins in lymphatic vessels, and disrupted valve development in lymphatic-specific Connexin43 deletion (Munger et al., 2017).

4.3.4. Testing a possible origin for Fsp1+ *endocardial and endothelial cells in the developing heart.*

As indicated, Fsp1 expression was also conspicuous in some of the endocardial-derived mesenchymal cells that populate the forming cardiac valvular primordia. This expression starts as early as E11.5, continues postnatally, and could be related to the mobilization and active migration of cells taking place at the mesenchymal core of developing valves. However, since some studies had reported the recruitment of circulating cells to the forming cardiac valves perinatally (Hajdu et al., 2011), and some blood-borne cells (including monocytes/macrophages) are known to express FSP1, we aimed at demonstrating that FSP1^{+} valvuloseptal cells were not derived from the transdifferentiation of circulating cells. In order to do so, we transplanted donor GFP bone marrow cells into postnatal hosts submitted to busulfan myeloablation (Cañete et al., 2017; Sands and Barker, 2000), but we did not find any of those cells in the endocardium or the valvuloseptal mesenchyme of the host atrioventricular (AV) valves.

Similar additional analyses were performed to study the contribution of GFP$^+$ bone marrow-derived cells to these tissues. Our results confirm that FSP1$^+$ bone marrow derived cells would not contribute to the endothelium of cardiac vein or lymphatic valves. Notwithstanding this conclusion, we should consider that both myeloablation and blood reconstitution were performed just before birth (E18.5) and postnatally, respectively, after the developmental formation of most venous (Bazigou and Makinen, 2013) and lymphatic valves (Sabine and Petrova, 2014) take place. Therefore, it is also possible that we did not find blood-borne cells recruited to cardiac chamber valvular structures or in venous/lymphatic valves because these structures were already formed when the GFP$^+$ cells were injected.

4.3.5. FSP1 is expressed in cardiomyocytes of the cardiac conduction system

In this work, we observed robust *Fsp1* expression in the cardiac conduction system (CCS), including atrio-ventricular nodes, the His bundles and the distal (Purkinje) fibers. This unique cardiac pacemaker system controls the coordinated contraction of cardiac chambers and is a key element to adult cardiac physiology. CCS cells form from the transdifferentiation of poorly proliferating, primitive cardiomyocytes (Mohan et al., 2018; Moorman et al., 1998). Three different developmental models have been proposed to explain the differentiation and morphogenesis of the CCS: 1) the differential proliferation rate of tubular heart cells (Mirzoyev et al., 2010), 2) the continuous recruitment and differentiation of cardiomyocytes (Gang Cheng et al., 1999; Gourdie et al., 2003; Pennisi et al., 2002), and 3) the progressive differentiation and proliferation of an embryonic myocyte population (Christoffels and Moorman, 2009). Although

the different populations of the CCS are genetically and morphologically characterized, there are some important developmental details that remain unknown (van Weerd and Christoffels, 2016), like the genes that are regulated by the Tbx3 and Tbx18 (McNally and Svensson, 2009), the discovery of some key factors involved in the formation and function (Fishman, 2020) or the etiology of some related diseases like the peripartum cardiomyopathy (Chinweuba and Rutkofsky, 2020).

To our knowledge, this is the first report of *Fsp1* expression in the developing CCS. Although specific functions of FSP1 protein during CCS differentiation are unknown, FSP1 interactions with muscle structural proteins were previously described, including myosin II heavy chain isoform A and actin (Chen et al., 2001; Ford and Zain, 1995; Grigorian et al., 2001; Kriajevska et al., 2002; Takenaga et al., 1994; Watanabe et al., 1992). Since the ventricular conduction system and nodal cells have a poor contractile apparatus (i.e. they have few sarcomeres) we would like to suggest that FSP1 could be involved in the transition from primitive cardiomyocytes to CCS cells.

4.3.6. Biological implications

Taken together, the results discussed in sections 4.3 indicate that Fsp1 is not a marker for a single cell type (Fig.20), but rather a potential developmental “maker” and/or homeostatic modulator that may have specific embryonic and postnatal functions. This is crucial to correctly interpret results from studies dissecting the cellular dynamics in post-infarction ventricular remodeling (Lawson et al., 2005; Ruiz-Villalba et al., 2015; Schneider et al., 2007). This is so because assuming Fsp1 is a specific fibroblast marker may lead to the wrong interpretation of circulating cells transdifferentiating to fibroblasts in response to pathologic stimuli, as found in some recent reports

(Haider et al., 2019). This is therefore important to consider the high amounts of CD45+ and CD41+ *bona fide* circulating cells that are recruited in the damaged heart after a myocardial infarction that express or could potentially express FSP1. Regarding these circulating cells, in this thesis we describe high amounts of bone marrow derived FSP1+ (GFP+) cells in the diseased heart after MI in chimeric mice. We therefore suggest these cells should be considered in future analyses of the cellular diversity of the post-infarction scar.

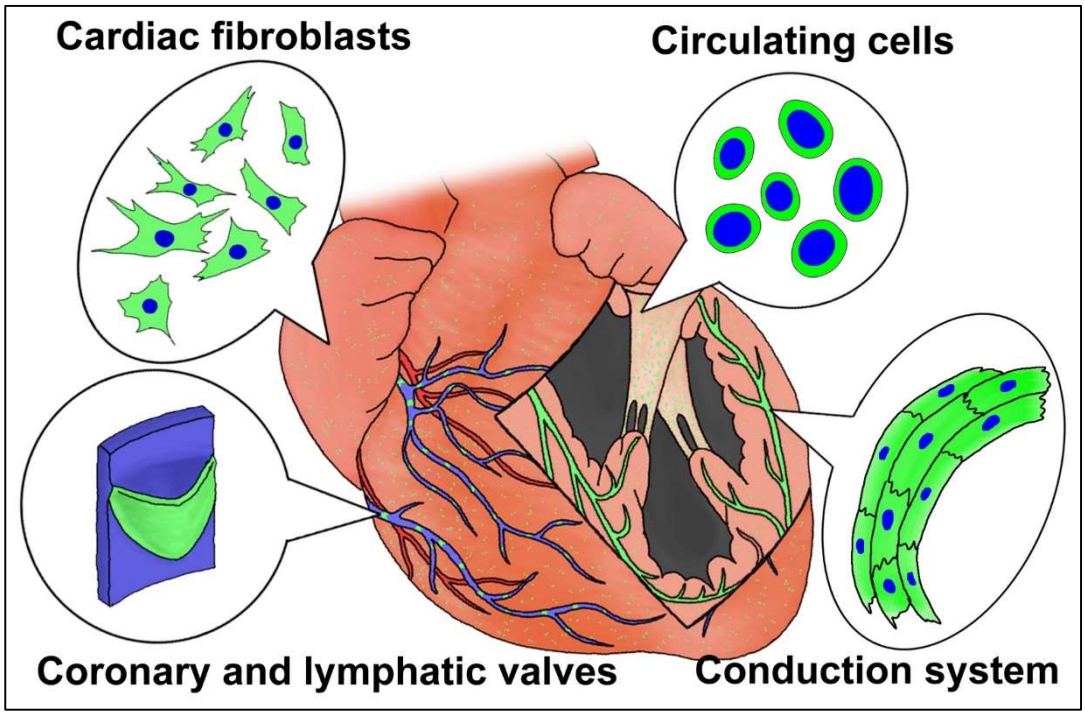

Figure 20. FSP1 is expressed in different cardiac cell types. In the mouse heart, FSP1 expression was observed in cardiac fibroblasts as well as in other cell populations, including circulating cells, cardiac conduction system cells and coronary and lymphatic endothelial valves.

4.4. *In vitro* experimental models allow to study *FSP1* functions

Cell culture is a powerful experimental approach to study cell biology. Nevertheless, these experimental approaches present some limitations that could bias the interpretation of obtained results. These limitations are mostly based on observed differences in behavior and survival of cultured cells as compared to those cells in their biological niche (Ram Singh, 2012). These variations may strongly influence the ability of cultured cells to compensate stress situations (Hoehme et al., 2010).

In our work, *FSP1* overexpression in human umbilical venous endothelial cells (HUVEC) induced cell death in approximately 48 hours, restricting our analyses to the first 24 hours after transfection. As FSP1 is not a transcription factor, its overexpression does not cause changes in the cellular transcription at short term. Rather, it induces variations in cytoplasmic signaling pathways, which may eventually have an effect on transcription. Therefore, it is logical to expect small variations in the expression profile of the cells in a short period of time. Although several studies have suggested some specific functions for FSP1 during vascular growth, no hypotheses are available on its role during vascular valve formation. Therefore, we wanted to test the effect of *FSP1* overexpression on these human venous endothelial cells. This *FSP1* overexpression resulted in the increase of the *FOXC2* trend expression and the decrease of the *VEGFR3* trend expression. Both markers, *FOXC2* and *VEGFR3*, are involved in lymphangiogenesis but their expression does not overlap in all types of embryonic or adult lymphatic cells (Dagenais et al., 2004). *FOXC2* controls the formation of lymphatic collecting vessels (Norrmén et al., 2009), and their valves (Sabine et al., 2012). While *VEGFR3* is key for the sprouting and motility of lymphatic cells during

lymphangiogenesis both in embryonic and adult tissues (Matsumoto et al., 2013; Zhang et al., 2018). These data suggest that *FSP1* could be implicated in the final fate of the lymphatic cells, promoting a valve cell phenotype via the overexpression of *FOXC2*.

During this thesis, additional *in vitro* analyses were performed to model *FSP1* expression in cultured human iPSCs. These cells were differentiated to specific cardiac cell types and *FSP1* expression patterns were studied in all undifferentiated and differentiated cell types. We found a higher *FSP1* expression in differentiated cell populations than in undifferentiated ones. Concretely, high *FSP1* expression levels were observed in fibroblasts, endothelial cells, and ventricular cardiomyocytes.

All these *in vitro* results suggested again *FSP1* as a modulator gene, implied in the differentiation of multiple cell types including fibroblasts, cardiomyocytes, and endothelial cells.

4.5. Loss-of-function (LOF) approaches allows for a better understanding of FSP1 in concrete cell populations

CRE-Lox technology represents a powerful tool for spatial and temporal control of gene expression, allowing for DNA modification in a specific cell type. In this modification, CRE recombinase enzyme mediates the deletion of DNA specific regions by recognizing LoxP sequences (Sauer and Henderson, 1988). This method could help to avoid the early lethality associated to some classical knockout mice through the conditional gene ablation in specific cell populations.

Fsp1 knockout mice (*Fsp1*$^{-/-}$) are born with an abnormal sex ratio, suggesting a certain level of embryonic lethality (Ambartsumian et al., 2005; Luo et al., 2015). Although no evident differences are observed in postnatal

mutant mice by routine histological analyses, around 10% of adult mice develop tumors (Naaman et al., 2004), but show a reduced metastatic potential (Grum-Schwensen et al., 2005). Different *Fsp1* transgenic strains have been used to identify *Fsp1* function during tissue fibrosis in different organs (Ackerman et al., 2019; Osterreicher et al., 2011; W. Zhang et al., 2018), including the adult heart (Tamaki et al., 2013). During this thesis, we have crossed *G2Gata4*^{CRE} with *Fsp1LoxP/LoxP* mice to abrogate *Fsp1* expression in the proepicardium. Although anomalies in heart development were not evident and epicardium develops properly in *G2Gata4*^{CRE};*FSP1*$^{Loxp/Loxp}$ mutant embryos, a thinner compact myocardium was observed in the left ventricle of these mutant hearts at E12.5. These results suggest that a small fraction of epicardial and/or epicardial derived cells have been abrogated, slightly affecting the compaction of the developing myocardium. Regarding the previously discussed heterogeneity of cardiac fibroblast populations, it is logical to think that FSP1 may not play a key role in cardiac development (i.e chamber formation, epicardial growth and EPDCs segregation), but further experiments are needed to better understand the percentage of epicardial-derived fibroblasts that express FSP1 and how this ablation could influence heart development.

Future perspectives:

1) **To analyse in detail *Fsp1* deletion in (pro)epicardial cells.** Additional analyses are needed to better understand how *Fsp1* is abrogated in proepicardial and epicardial cells in *G2Gata4*^{CRE}; *Fsp1*$^{LoxP/LoxP}$ embryos. Since *G2Gata4* expression was described in proepicardial cells (Cano et al., 2016), and our data suggest epicardium formation is not impaired in these mutants, *Fsp1* ablation

would not interfere in the migration and attachment of proepicardial cells over the embryonic myocardium surface. In this work, mild differences in compact myocardium thickness were observed at E12.5, but these mutant embryos develop and birth normally, with no evident myocardial malformations (data not shown). As the main objective of these experiments was to analyse the experimental deletion of epicardial derived FSP1-positive fibroblasts, we should confirm that these FSP1+ fibroblast populations are affected in mutant mice. For this purpose, we suggest the quantification and comparison of fibroblast-like cells in wild type and mutant embryos as future experiments, by using different FSP1, CD90 and procollagen (COL1A1) antibodies. These comparisons would elucidate how epicardial derived fibroblast populations are affected by the ablation of *Fsp1* gene early in development. Other markers like TCF21, described in epicardial derived fibroblasts (Acharya et al., 2012), could be additionally used for this purpose. These results could be validated by using other transgenic lines previously reported in the literature, like $Wt1^{Cre}$ (Zhou and Pu, 2012) which could be combined with our $Fsp1^{LoxP/LoxP}$ mouse strain to abrogate *Fsp1* expression in epicardial cells.

2) **To analyse epicardial FSP1 deletion in pathological conditions.** Since we did not observe major morphological defects in $G2Gata4^{CRE}$; $Fsp1^{LoxP/LoxP}$ mice and adult mutants were found to be viable, we believe that submitting them to cardiac ischemia using a classical left coronary anterior descendant artery (LDA) ligation method could trigger a differential response in pathological conditions. The dynamic response of FSP1 expression during ventricular remodeling has been

already characterized (Kong et al., 2013), but we aim at establishing a cellular definition to these findings.

3) **To analyse the effects of *Fsp1* deletion in adult cardiomyocytes by stress tests.** To analyse whether *Fsp1* LOF affects CCS performance, we deleted *Fsp1* using the same *Fsp1*$^{loxP/loxP}$ line and a well-known myocardial driver (*cTnI*^{CRE}). Since these mice develop and born with an anatomically normal heart (data not shown), we believe that *Fsp1* ablation in mature cardiomyocytes (expressing cardiac troponin) does not affect the normal patterning and formation of the CCS. However, further research is needed to check on the possible impact of Fsp1 loss of function in the adult heart (e.g. by physiologically challenging the CCS. Some CCS derived diseases, as arrhythmias, could not affect to heart contraction ability if mice are in steady-state conditions. A deeper characterization of the conduction system function (i.e. effort-based test and electrocardiogram) will be necessary to describe the role of *Fsp1* in the adult heart conduction system of *cTnI*^{CRE};*Fsp1*$^{LoxP/LoxP}$ mice (and also in *Nkx2-5*^{CRE};*Fsp1*$^{LoxP/LoxP}$ mice).

Conclussions:

1) FSP1 is not a marker for a single cell type (e.g. cardiac fibroblasts), but rather a developmental “maker” with specific embryonic and postnatal functions showing a defined spatio-temporal expression pattern.

2) There is a wide range of *Fsp1*-expressing cells in the developing heart, including cardiac fibroblasts, endothelial cells (coronary and lymphatics), cardiac conduction system cardiomyocytes (purkinje fibres and AV node cells) and circulating cells (nucleated cells and platelets).

3) *Fsp1* expression increases as a consequence of cell differentiation to epicardium, myocardium and endothelium in human cultured cells.

4) *Fsp1* overexpression may implicate the differentiation and/or commitment of endothelial progenitor cells to lymphatic fates.

5) *Fsp1* has no evident functional relevance in epicardium formation and myocardium maturation.

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Resumen en español / Spanish summary

1. Introducción

1.1. Desarrollo cardíaco

1.1.1. Cardiogénesis

El corazón de los Vertebrados es el primer órgano funcional durante el desarrollo embrionario. En el ratón, aproximadamente en el día de gestación embrionaria (E) 7.5, una serie de progenitores cardíacos de naturaleza mesodérmica converge en la línea media ventral del embrión, generando el creciente cardíaco. Este creciente de aspecto semilunar adquiere una forma tubular, con un polo venoso posterior y un polo arterial anterior. Este corazón tubular primitivo se forma gracias a la contribución organizada de dos poblaciones celulares conocidas como campo cardiaco primario (del inglés *First Heart Field*, FHF) y campo cardiaco secundario (del inglés *Second Heart Field*, SHF) que se diferenciarán en regiones concretas del corazón adulto. El FHF contribuye a la formación del ventrículo izquierdo, mientras que los progenitores del SHF dan lugar al tracto de entrada (del inglés *inflow tract,* IFT) y al tracto de salida (del inglés *outflow tract,* OFT). El resto del corazón primitivo, incluyendo el ventrículo derecho y ambos atrios, derecho e izquierdo, derivan de una contribución celular mixta, procedente de ambos campos cardíacos.

1.1.2. Crecimiento y morfogénesis

El corazón tubular se curva en un proceso conocido como torsión (proceso que en inglés se conoce como *looping*), que permite el alineamiento de los territorios que formarán el corazón tetracameral adulto.

A partir de este momento, el crecimiento del corazón embrionario se produce por la proliferación de los cardiomiocitos atriales y ventriculares en la curvatura externa del primordio, un proceso conocido como *ballooning.* En este momento, se llevará a cabo el desarrollo de los cojines endocárdicos (tejido primordial que dará lugar a las válvulas cardíacas) y la formación de los septos que separarán la cavidad cardíaca primitiva en cuatro cámaras. En el embrión de ratón, estos procesos comienzan aproximadamente en E10.5, con la formación del septo primario atrial y con la activación de los mecanismos de transición epitelio-mesénquima (del inglés *Epithelial to Mesenchymal Transition,* EMT) del endocardio atrioventricular y conal. Esta transformación localizada del endocardio genera una población de células mesenquimales valvuloseptales. Estas células mesenquimales son las progenitoras de las válvulas mitral y tricúspide, y de la porción membranosa del septo ventricular.

Además de estos eventos que permiten una morfogénesis correcta del tejido cardíaco a nivel anatómico, no debemos dejar de lado la importancia del desarrollo del corazón desde un punto de vista fisiológico. El corazón es un órgano que, a pesar de ser funcional a estadios tempranos del desarrollo, debe desarrollar las distintas estructuras que permiten 1) la correcta irrigación del músculo y 2) la difusión del impulso eléctrico del corazón adulto, que permite coordinar su latido. Estos eventos se llevan a cabo gracias a la vasculatura coronaria y al sistema de conducción cardíaco, respectivamente.

1.2. Vascularización del corazón embrionario

A lo largo del desarrollo del corazón embrionario, el aumento progresivo del grosor de las paredes del ventrículo supone un impedimento

físico para la difusión del oxígeno desde el lumen hasta las células que forman este miocardio. Desde una perspectiva evolutiva, el aumento en grosor del ventrículo favoreció la aparición de un sistema vascular coronario encargado de irrigar cada una de las células que forman el corazón adulto. A través de mecanismos de angiogénesis y vasculogénesis, progenitores endoteliales de diferentes orígenes embrionarios se organizan para formar los vasos coronarios de distinto calibre, que son remodelados antes del nacimiento en un sistema vascular funcional. Se han descrito dos tipos diferentes de sistemas vasculares en mamíferos, la vasculatura sanguínea y la vasculatura linfática. Estos vasos se encuentran estrechamente interconectados entre sí en un ciclo continuo en el cual la sangre transporta oxígeno y nutrientes a los tejidos, mientras que la linfa transporta fluidos y macromoléculas desde los tejidos hacia a la circulación sanguínea. Durante el desarrollo temprano, la vasculatura linfática se desarrolla a partir de las células endoteliales procedentes de las venas, una diferenciación que se caracteriza por la expresión de factores como el receptor vascular VEGFR-3 (del inglés *vascular endothelial growth factor receptor-3*), el receptor linfático Lyve-1 (del inglés *lymphatic vessel endothelial hyaluronan receptor 1*) y la proteína Prox1 (del inglés *prospero-related protein 1*).

Las principales diferencias entre el endotelio linfático y el endotelio de la vasculatura sanguínea han sido ampliamente estudiadas en los capilares, vasos de paredes finas compuestas exclusivamente por una capa de células de naturaleza endotelial. Las células endoteliales linfáticas (del inglés *lymphatic endothelial cells*, LECs) difieren en muchos aspectos de las células endoteliales de la vasculatura sanguínea (del inglés *blood vascular endothelial cells,* BECs). La diferencia principal es que los capilares linfáticos no presentan pericitos, las células que envuelven a los capilares sanguíneos. Las uniones entre células también son diferentes en ambos tipos de capilares; las BECs están comunicadas por uniones tipo

“cremallera” mientras que las LECs presentan discontinuidades que permiten el traspaso de fluidos y de ciertos leucocitos. Otra diferencia fundamental entre ambos tipos de vasculatura es la circulación del fluido por su interior; por un lado, la circulación de la sangre en los organismos adultos está impulsada por el latido del corazón, mientras que el retorno de la linfa está facilitado por la contracción de los músculos esqueléticos o la pulsación arterial. Las válvulas linfáticas son fundamentales para facilitar el retorno eficiente desde las extremidades inferiores, donde las fuerzas gravitacionales son más fuertes.

En la vasculatura linfática, existen diferentes formas de permitir el flujo; los vasos linfáticos pueden bombear la linfa con la ayuda de compresiones externas, como por ejemplo la contracción del músculo esquelético, o pueden hacerlo por contracciones del propio vaso linfático. La capacidad intrínseca de contracción no está presente en todos los vasos linfáticos, y estos vasos no contráctiles son los que pueden presentar válvulas para permitir el flujo unidireccional del fluido. El desarrollo morfogenético de las válvulas venosas y linfáticas ocurre de manera similar, y está regulado por mecanismos moleculares que implican a las proteínas integrina α9, efrina-B2 y efrina-B4.

1.3. Coordinación de la contracción cardíaca: el sistema de conducción

El impulso eléctrico que coordina las contracciones de atrios y ventrículos en el corazón es generado y controlado por el sistema de conducción cardíaco (del inglés Cardiac Conduction System, CCS). Este CCS está formado por células de naturaleza miocárdica (cardiomiocitos), que forman estructuras como el nodo sinoatrial, el nodo atrioventricular, el

haz de His, y las fibras de Purkinje. El nodo sinoatrial (del inglés *Sino-Atrial Node,* SAN) genera el impulso eléctrico de conducción, el nodo atrioventricular (del inglés *Atrio-Ventricular Node*, AVN) retrasa el impulso y permite la contracción acompasada de atrios y ventrículos y, por último, la acción sincrónica del Haz de His y de las fibras de Purkinje transmite rápidamente el impulso hacia los ventrículos. El sistema de conducción se desarrolla a partir de cardiomiocitos que derivan de un progenitor miocárdico común, ya presente en el corazón tubular embrionario. De hecho, existen dos modelos de formación del sistema de conducción cardíaco: 1) el modelo de reclutamiento y 2) el modelo de especificación temprana. El primero de ellos sugiere que el sistema de conducción cardíaco se desarrolla gracias a un reclutamiento progresivo de células progenitoras multipotentes, mientras que el segundo modelo propone que una subpoblación de miocitos embrionarios está destinada a diferenciarse y a formar los componentes del sistema de conducción.

El desarrollo cardiovascular, y en concreto la especificación de los cardiomiocitos, es un proceso complejo que implica la interacción de numerosas proteínas como TGFß2, BMP2, FGF, YAP, NKX2-5 y ISLET 1. Dada la complejidad del corazón, alteraciones que afecten la formación del órgano o la fisiología del corazón adulto pueden desencadenar en enfermedades cardiovasculares.

1.4. Enfermedades cardiovasculares

Durante la morfogénesis cardiaca, pueden producirse defectos que conllevan el desarrollo de una enfermedad congénita. Este tipo de enfermedades congénitas del corazón se han descrito en aproximadamente el 1% de los recién nacidos. Otro tipo de enfermedades que afectan al

órgano pueden aparecer durante la vida adulta. Este tipo de anomalías se denominan enfermedades cardíacas adquiridas, siendo el infarto de miocardio la más común. El infarto de miocardio es la muerte masiva de músculo cardiaco, generalmente asociado con fenómenos de isquemia. Como respuesta fisiológica del órgano, el músculo muerto es reemplazado por una cicatriz fibrótica no contráctil que se expande progresivamente, en un proceso conocido como remodelación ventricular. Esta cicatriz está empobrecida en cardiomiocitos, y enriquecida en un tipo de células denominadas fibroblastos.

1.5. Fibroblastos cardíacos

Los fibroblastos cardíacos son las células más abundantes del corazón. Son células de forma estrellada que sintetizan y secretan una amplia variedad de los componentes de la matriz extracelular, incluyendo el colágeno. Estas células están localizadas en el espacio que hay entre los cardiomiocitos, denominado intersticio cardiaco. Se ha estimado que este tipo celular representa alrededor del 20% de las células intersticiales del corazón adulto del ratón. Estudios recientes han demostrado que las células derivadas de epicardio son la fuente principal de fibroblastos cardíacos durante el desarrollo. Además del epicardio, otros tipos celulares como las células derivadas de la médula ósea, las células derivadas de la cresta neural y las células derivadas de endocardio, se han propuesto como origen celular de los fibroblastos cardíacos. Los fibroblastos pueden encontrarse en dos estados fisiológicos diferentes, un estado inactivado o quiescente y un estado activado conocido como miofibroblasto. Cuando los fibroblastos se encuentran en estado activado, la deposición excesiva de colágeno por parte de este tipo celular desencadena una condición patológica conocida

como fibrosis. A pesar de que el análisis de este tipo celular mediante el uso combinado de una gran variedad de marcadores (por ejemplo, CD90, DDR2, CADH11, VIM, FN, POSTN, FSP1, αSMA o TCF21) ha permitido el estudio del comportamiento de los miofibroblastos en el corazón, no existe un marcador específico para la identificación de fibroblastos o miofibroblastos. Esto se debe a que cada marcador identifica solo un subconjunto de células dentro de la población total de fibroblastos. Entre todos, el factor FSP1/S100A4 es el más usado para la identificación de este tipo celular.

1.6. La proteína FSP1/S100A4

1.6.1. Familia de proteínas S100

La proteína FSP1 (del inglés *Fibroblasts specific protein 1*), pertenece a la familia de proteínas S100 de unión a calcio, una familia de proteínas pequeñas (10-12 kDa) sin actividad enzimática, que pueden ser secretadas al espacio extracelular. Estas proteínas S100 participan en diferentes funciones celulares tales como contracción, proliferación, diferenciación, inflamación, y movimiento celular.

1.6.2. Estructura de la proteina FSP1

Esta proteína fue descubierta por el grupo del Dr. Ebradize en 1989 y fue nombrada como metastasina-153. Actualmente también es conocida como S100A4, p9KA, pEL-98, 18ª2, CAPL y calvasculina. FSP1 es un péptido de 101 aminoácidos que forma homodímeros simétricos estabilizados por interacciones no covalentes. Cada monómero está formado por dos dominios de unión a calcio. La unión del ión Ca2+ genera

un cambio conformacional en la estructura tridimensional de la proteína, permitiendo la interacción hidrofóbica entre Fsp1 y otras proteínas de unión, incluyendo p53, miosina IIA, tropomiosina, liprina ß1, metionina aminopeptidasa 2, rhotekina, anexina A2 y S100A1.

1.6.3. Funciones de Fsp1

La mayor parte de los estudios sobre las funciones de FSP1 se han centrado en su implicación durante la progresión del cáncer y la metástasis. De hecho, la interacción FSP1-miosina IIA está implicada en el movimiento celular de las células cancerígenas y su capacidad de metastatizar. En el espacio extracelular, FSP1 puede estimular la migración de células endoteliales, células musculares lisas, linfocitos y fibroblastos. En células endoteliales, la expresión de *Fsp1* también se ha relacionado con la angiogénesis y con la integridad linfática.

1.6.4. Fsp1 en procesos patológicos

FSP1 es una proteína implicada en distintos procesos patológicos, incluyendo el cáncer y las enfermedades cardiovasculares. La metástasis es la causa principal de la progresión del cáncer. Debido a la implicación de FSP1 en la migración celular, algunos autores la han propuesto como un buen marcador pronóstico de la capacidad metastásica en tumores primarios.

La expresión de *Fsp1* también ha sido descrita durante el infarto de miocardio. La cicatriz producida tras el infarto de miocardio incluye altas cantidades de células que expresan FSP1, mayormente fibroblastos y células endoteliales. Sin embargo, a pesar del uso de la proteína FSP1 en el estudio de la fibrosis, cada vez más evidencias cuestionan la especificidad

de FSP1 como un marcador específico de fibroblastos, sugiriendo que otros tipos celulares presentan dicho marcador.

En esta tesis doctoral, se ha estudiado el papel del gen *Fsp1* durante el desarrollo cardiaco gracias al uso de la cepa transgénica de ratón *Fsp1*^{GFP}. Además, se han realizado análisis *in vitro* adicionales de células cultivadas humanas para confirmar los resultados obtenidos en embriones de ratón.

2. Resultados

2.1. La proteína verde fluorescente GFP recapitula la expresión de FSP1 en tejidos cardíacos embrionarios y adultos de ratones transgénicos *Fsp1*^{GFP}

El gen de la proteína verde fluorescente GFP se encuentra bajo la acción del promotor del gen *Fsp1* en los ratones transgénicos *Fsp1*^{GFP} usados en esta tesis doctoral. Esto significa que la presencia de GFP implica la expresión de *Fsp1* en la célula. Este hecho se ha evidenciado gracias a la co-localización de GFP y la proteína FSP1 en células intersticiales, tanto fibroblastos como células circulantes, del ventrículo cardiaco en desarrollo y en estadios postnatales. El análisis por citometría (FACS) de los corazones adultos (n=3) muestra la presencia de GFP en un 2±0.69% de las células. La colocalización entre ambas proteínas también es evidente en fibroblastos embrionarios en cultivo.

2.2. Las válvulas cardíacas de los ratones *Fsp1*^{GFP} presentan GFP en su estadio embrionario y postnatal

En esta tesis, la expresión más temprana de *Fsp1* se ha en el canal atrioventricular y en el tracto de salida del corazón en desarrollo en embriones E11.5. La expresión de GFP en estos estadios y a estadios posteriores (E13.5) es evidente en las células endocárdicas de los cojines del canal atrioventricular y del tracto de salida. Esta expresión de GFP en estos cojines (territorio valvular presuntivo) colocaliza con la proteína FSP1 y se mantiene hasta estadios postnatales.

2.3. Diferentes poblaciones de células endoteliales cardíacas presentan GFP

En nuestro estudio hemos mostrado que a E15.5, la proteína GFP se encuentra en poblaciones discretas de células endoteliales en vasos coronarios. A estadios posteriores (E18.5) y en corazones adultos, estas células endoteliales GFP+ se encuentran formando estructuras siguiendo un patrón a lo largo de la vasculatura coronaria, formando las válvulas endoteliales de dichos vasos.

Además, el marcador LYVE1 nos ha permitido identificar células endoteliales GFP+ en vasos linfáticos. La expresión temprana de GFP en estos vasos linfáticos cardiacos, a un estadio de E18.5, se encuentra en células endoteliales a lo largo de dicha vasculatura. Al igual que ocurre con la vasculatura coronaria, se aprecia un patrón espacial de estas células GFP+ en la linfa, formando válvulas en el interior de los vasos linfáticos a estadios postnatales y adultos.

2.4. *Fsp1* se expresa en las válvulas de la vena femoral

Dado que las válvulas endoteliales se encuentran en otros grandes vasos sanguíneos, como es la vena femoral, en esta tesis se ha querido estudiar la expresión de Fsp1 en estas estructuras extra cardiacas. Así, se han encontrado células GFP+ en la vena femoral de ratones transgénicos *Fsp1*^{GFP}, formando estructuras similares a las válvulas endoteliales. Algunas de estas células son de naturaleza endotelial (GFP+/CD31+) y se sitúan bajo la túnica media de la pared venosa.

2.5. La sobreexpresión de *Fsp1* en células endoteliales *in vitro* no implica su diferenciación

En esta tesis doctoral, se ha inducido la expresión de *Fsp1* en células endoteliales *in vitro* con el fin de determinar si la expresión de dicho gen induce la diferenciación del endotelio hacia un linaje venoso y/o linfático. En este experimento se han utilizado células humanas cultivadas e inmortalizadas a partir de células endoteliales obtenidas de la vena umbilical (del inglés Human Umbilical Vein Endothelial Cells, HUVEC). Estas células HUVEC se transfectaron con un plásmido que induce la sobreexpresión del gen *FSP1*. Tras 24 horas, el análisis por qPCR revela que la sobreexpresión de *FSP1* produce un incremento en la tendencia de la expresión de *FOXC2*, y una disminución en la tendencia de la expresión de *VEGFR3*. No se han realizado estudios estadísticos puesto que sólo se han obtenido dos réplicas biológicas de este experimento.

2.6. La deleción condicional de Fsp1 en proepicardio y células epicárdicas no afecta al desarrollo del epicardio ni del miocardio

La deleción condicional del gen *Fsp1* en progenitores epicárdicos se llevó a cabo gracias al uso combinado de ratones transgénicos *G2-Gata4*^{CRE} y *Fsp1*$^{LoxP/LoxP}$. No se han encontrado alteraciones morfológicas graves en los corazones embrionarios de los ratones mutantes a estadios tempranos del desarrollo (E12.5). Sin embargo, se ha encontrado una compactación menor de lo esperado en el ventrículo izquierdo de los embriones mutantes de ratón.

2.7. Las células GFP+ derivadas de la médula ósea de ratones *Fsp1*^{GFP} contribuyen a la formación de las válvulas atrioventriculares, pero no se incorporan al endotelio vascular coronario

Dado que en esta tesis doctoral se describen células circulantes (CD45+) que presentan la proteína GFP en el corazón adulto y en la médula ósea de ratones *Fsp1*^{GFP}, se han llevado a cabo experimentos quiméricos con el fin de determinar si esas células derivadas de la médula ósea (del inglés *Bone Marrow Derived Cells*, BMDC) contribuyen a la formación del endotelio vascular coronario. Para ello, se ha aplicado un protocolo de mieloablación química a ratones adultos de fenotipo silvestre, con el fin de eliminar células del linaje hematopoiético. Acto seguido, se ha reconstituído dicho linaje sanguíneo mediante el trasplante de células derivadas de la médula ósea de ratones *Fsp1*^{GFP}. Gracias a esta metodología se han

obtenido ratones quiméricos en los que se pueden trazar las células GFP+ derivadas de la médula ósea del mutante *Fsp1*^{GFP} en el ratón de fenotipo silvestre. La eficiencia de la reconstitución se ha estimado mediante el análisis del porcentaje de células circulantes GFP+ un mes después del trasplante. Solo se han considerado animales reconstituidos exitosamente los que presentan más de un 40% de células GFP+ en la sangre. Este experimento demostró que no hay una contribución activa de las células circulantes derivadas de la médula ósea al endotelio coronario a estadios postnatales. Sin embargo, se observaron células GFP+/CD45+ tanto en el intersticio cardiaco como en las válvulas atrioventriculares.

2.8. Células Fsp1-GFP+ derivadas de la médula ósea se alojan en la zona dañada tras un infarto de miocardio

Siguiendo el mismo protocolo descrito en el apartado anterior, ratones de fenotipo silvestre fueron irradiados y trasplantados con las células de la médula ósea de ratones *Fsp1*^{GFP}. Posteriormente fueron sometidos a una ligadura coronaria permanente (método usado para reproducir un infarto de miocardio en el modelo murino) y analizados en distintos estadios tras el infarto. En estos ratones quiméricos, se observaron aumentos significativos de células GFP+ (derivadas de la médula ósea y expresando *Fsp1*) en la pared ventricular 7 y 30 días después del infarto de miocardio, teniendo como punto de comparación los corazones de los ratones quiméricos no dañados. Los valores máximos de células GFP+ se encontraron en los corazones infartados a 7 días tras el infarto. Algunas de estas células GFP+ se caracterizan por la presencia del marcador CD45.

2.9. Expresión de Fsp1 en células miocárdicas

En esta tesis se ha descrito la presencia de la proteína GFP en células de naturaleza miocárdica (cardiomiocitos) en el ventrículo de ratones *Fsp1*^{GFP}. Durant el desarrollo embrionario (E16.5), poblaciones de células discretas del miocardio atrioventricular de ratones *Fsp1*^{GFP} presentaban la proteína GFP. Estas células coinciden espacialmente con la ubicación anatómica del nodo atrioventricular (AVN) y de las fibras de Purkinje del sistema de conducción cardíaco. Estas células GFP+ se caracterizan por la presencia de los marcadores HCN4 y CTNT2 (marcadores del sistema de conducción), la proteína FSP1, el marcador TNI (marcador de musculatura cardiaca), y la presencia de sarcómeros.

2.10. La diferenciación de células humanas pluripotentes (hiPSC) a células cardíacas implica la sobreexpresión del gen *Fsp1*

Como se explica en los párrafos anteriores, en este trabajo se ha descrito la expresión del gen *Fsp1* en diferentes tipos celulares del corazón del ratón, incluyendo fibroblastos, células endoteliales, células circulantes y células del sistema de conducción. Un experimento adicional en la tesis revela que las células en cultivo expresan Fsp1 como consecuencia de su diferenciación hacia alguno de estos linajes. Este experimento consistió en el cultivo y diferenciación de células madre humanas pluripotentes inducidas (del inglés *human induced Pluripotent StemCells*, hiPSC). Estas células se diferenciaron en varios tipos de células presentes en el corazón, incluyendo células endoteliales, fibroblastos, cardiomiocitos y células epicárdicas, siguiendo una serie de protocolos descritos en la literatura. El uso de

anticuerpos específicos para cada tipo celular confirmó la diferenciación exitosa de estas células madre humanas. Además, se observaron diferentes niveles de expresión de la proteína FSP1 en estas células mediante ensayos de inmunofluorescencia, y se confirmó mediante técnicas de expresión génica (qPCR) un aumento significativo de la expresión del gen Fsp1 en fibroblastos, células endoteliales, cardiomiocitos ventriculares y células epicárdicas, tras su diferenciación. Sin embargo, no se observó un aumento significativo en la expresión del gen *Fsp1* en cardiomiocitos atriales tras su diferenciación.

3. Discusión

3.1. Fsp1 está implicada en un amplio rango de eventos biológicos

FSP1 es una proteína de bajo peso molecular implicada en un amplio rango de procesos biológicos, pero se desconocen los detalles de las bases moleculares que la implican en estas funciones. Las funciones pleiotrópicas de FSP1 pueden deberse a la existencia de diferentes isoformas de la proteína, a su habilidad de adquirir diferentes conformaciones en función de su unión a calcio, o al amplio rango de proteínas que pueden interaccionar con ésta. La formación de los heterodímeros FSP1-metionina aminopeptidasa 2, FSP1-miosina IIA o FSP1-p53, son ejemplos de cómo FSP1 puede regular la proliferación celular o la migración, respectivamente. Además, FSP1 se ha relacionado con el crecimiento y la integridad de las válvulas linfáticas a través de la modulación de la fosforilación de la proteinna liprina ß1. En conjunto, la alta ubicuidad en la localización y la función de FSP1 sugiere a esta proteína como un modulador específico de

la actividad proteica, en lugar de ser el efector final de las funciones celulares en las que está implicada.

3.2. Diferentes herramientas en el estudio de las funciones biológicas de FSP1

Una misma célula puede sintetizar una cantidad variable de proteínas. En el embrión, esta variabilidad se puede relacionar con el estado de diferenciación y/o estado fisiológico de la célula. La síntesis proteica puede ser ocasionalmente baja, haciendo difícil su reconocimiento por técnicas inmunohistoquímicas. Esta dificultad es incluso mayor si los anticuerpos disponibles no reconocen de manera idónea el epítopo. Por este motivo, en esta tesis doctoral se ha combinado el análisis de la proteína FSP1 mediante técnicas inmunohistoquímicas con una herramienta adicional, el uso de una línea transgénica de ratón en la cual el gen de la proteína fluorescente verde (GFP) se expresa bajo el control del promotor del gen *Fsp1/S100A4*. Estos ratones permiten la evaluación en tiempo real de la expresión de *Fsp1* en todos los tejidos mediante el análisis de la proteína GFP. En este estudio, se ha descrito la proteína FSP1 mediante técnicas inmunohistoquímicas en una gran cantidad de células GFP+, lo cual significa que la expresión del gen *Fsp1* en el transgénico recapitula satisfactoriamente el patrón de expresión natural de la proteína FSP1. Sin embargo, se han encontrado algunas células GFP+ que no presentan acumulación de la proteína FSP1. Pensamos que este resultado puede deberse a 1) la presencia de cantidades de Fsp1 por debajo del umbral de detección de la técnica inmunohistoquímica empleada o 2) que el anticuerpo utilizado no identifique todas las isoformas de la proteína FSP1. Por otro lado, hemos encontrado células Fsp1+ que no presentan GFP. Este hecho puede explicarse debido

a 1) los diferentes tiempos de vida media de ambas proteínas, estimada en 26 horas para la proteína GFP y 85.5 horas para FSP1, o 2) la alta homología entre FSP1 y otras proteínas de la familia S100, lo que puede producir la reactividad cruzada de los anticuerpos.

3.3. Fsp1 durante el desarrollo cardiaco

Al comienzo de esta tesis, se hizo evidente que diferentes tipos de células cardíacas podían expresar *Fsp1*. Nuestros resultados corroboran evidencias previas que describen la expresión de *Fsp1* en células endoteliales, neuronas, macrófagos, y células derivadas de medula ósea en otros tejidos. En este trabajo, se ha descrito la expresión de *Fsp1* en múltiples tipos celulares incluyendo fibroblastos cardíacos, células circulantes, plaquetas, células endoteliales y células de naturaleza miocárdica (sistema de conducción).

3.3.1. FSP1 en fibroblastos cardíacos

La expresión de *Fsp1* en el corazón se ha relacionado de forma rutinaria con un tipo celular concreto: los fibroblastos cardíacos. De hecho, la proteína FSP1 se ha utilizado de manera rutinaria como un marcador específico de fibroblastos (de ahí su nombre, del inglés *Fibroblast Specific Protein 1*, FSP1). Sin embargo, estudios recientes demuestran que no existe un marcador exclusivo de este tipo celular. Los resultados mostrados en esta tesis confirman que la expresión de *Fsp1* no es exclusiva de fibroblastos cardíacos, ya que también se demuestra que otras células de naturaleza no fibroblástica expresan altas cantidades de *Fsp1*. Por ello, los datos obtenidos en esta tesis doctoral sugieren la necesidad de reevaluar la

especificidad de los comúnmente utilizados como marcadores de fibroblastos. Un ejemplo es CD90, ausente en algunas poblaciones de fibroblastos y considerado un marcador de células mesenquimales indiferenciadas. Por consiguiente, una conclusión importante a destacar en esta discusión es que la población de fibroblastos cardíacos puede ser más heterogénea de lo que se consideraba hasta la fecha.

3.3.2. FSP1 en el endotelio

En esta tesis, la expresión del gen *Fsp1* se ha descrito en tres poblaciones endoteliales diferentes: el endocardio, el endotelio vascular coronario y el endotelio linfático. La proteína FSP1 se ha observado en el endocardio de las regiones atrioventricular y conoventricular del corazón en desarrollo. El endocardio de estas regiones es necesario para la formación de las válvulas cardíacas, siendo la activación de la transición epitelio mesénquima fundamental en el origen de estas primeras células valvuloseptales. Los resultados obtenidos sugieren que la proteína FSP1 puede jugar un papel importante en la transición de las células endocárdicas hacia células mesenquimales.

Además, en este estudio se ha descrito la expresión de *Fsp1* en células endoteliales de la vasculatura coronaria y linfática. Esta expresión se restringe progresivamente a las estructuras valvulares tanto de vasos coronarios como linfáticos. Resultados previos de otros grupos de investigación que investigan la formación de ambos sistemas vasculares sugieren la implicación de FSP1 en el desarrollo del endotelio vascular. Puesto que la activación de la migración celular es una característica común en el desarrollo de los vasos y las válvulas, resulta lógico pensar que la expresión de FSP1 pueda estar involucrada en la movilización celular durante la morfogénesis venosa y linfática. También es posible que la

proteína FSP1 pueda estar involucrada en la modulación local de la proliferación celular, una idea que concuerda con la relación descrita entre FSP1 y P53. Además, en el caso de las válvulas linfáticas, pensamos que la proteína FSP1 puede estar implicada en el crecimiento y la integridad de los vasos linfáticos gracias a su interacción con la proteína liprina ß1.

3.3.3. Expresión de Fsp1 en cardiomiocitos del sistema de conducción

En este trabajo, hemos observado una expresión robusta de *Fsp1* en el sistema de conducción cardíaco, incluyendo células del nodo atrioventricular, del haz de His y de las fibras de Purkinje. Las células del sistema de conducción se forman a partir de la transdiferenciación de cardiomiocitos primitivos. En nuestro conocimiento, este es el primer estudio que demuestra la expresión de *Fsp1* en las células del sistema de conducción cardiaco. Aunque las funciones específicas de *Fsp1* en este sistema son desconocidas, la interacción de FSP1 con proteínas musculares estructurales ha sido descrita previamente, incluyendo la miosina IIA y la actina. Puesto que las células del sistema de conducción ventricular y las células de los nodos tienen un aparato contráctil poco desarrollado, sugerimos que *Fsp1* pueda estar involucrado en la transición de cardiomiocitos primitivos hacia células del sistema de conducción, pero estudios adicionales son necesarios para confirmar este punto.

3.4. Análisis del origen de las células endocárdicas y endoteliales que expresan Fsp1

Como se ha indicado previamente, la expresión de *Fsp1* se puede

observar en algunas células endocárdicas que forman el primordio de las válvulas cardíacas. Esta expresión comienza a estadios tempranos del desarrollo de las válvulas (E11.5), y continúa a estadios postnatales. Sin embargo, puesto que algunos estudios han demostrado un reclutamiento de células circulantes durante el desarrollo de las válvulas cardíacas, y algunas células del linaje sanguíneo expresan *Fsp1*, en esta tesis doctoral hemos intentado analizar si las células GFP+ valvuloseptales en ratones *Fsp1*^{GFP} derivan de la migración y transdiferenciación de células circulantes, mediante el análisis de ratones quiméricos, como se explica anteriormente. A pesar de que nuestros resultados confirman que las células derivadas de médula ósea no contribuyen a la formación del endotelio de las válvulas venosas o linfáticas, debemos considerar el hecho de que tanto la mieloablación como la reconstitución sanguínea se han realizado a estadios avanzados del desarrollo (E18.5) y postnatales (P1), respectivamente. Por tanto, es posible que una gran parte de las válvulas linfáticas y venosas ya estuviesen formadas cuando las células *Fsp1*^{GFP} fueron trasplantadas.

3.5. Modelos experimentales in vitro permiten el estudio de las funciones de *Fsp1*

Los cultivos celulares son una poderosa aproximación para estudiar la biología celular. Sin embargo, estas aproximaciones presentan algunas limitaciones que pueden sesgar la interpretación de los resultados. Estas limitaciones consisten principalmente en las diferencias observadas entre el comportamiento y la supervivencia de células cultivadas en comparación con las células que se encuentran en su nicho biológico. En nuestro trabajo, la sobreexpresión de *FSP1* en células endoteliales venosas derivadas de cordón umbilical humano (HUVEC) provocó la muerte de las células en

cultivo en tan solo 48 horas, restringiendo nuestra posibilidad de análisis a 24 horas tras la sobreexpresión del gen. Puesto que *FSP1* no es un factor de transcripción, es lógico esperar pequeñas variaciones en el perfil de expresión celular en un corto periodo de tiempo. Aunque muchos estudios han sugerido algunas funciones específicas de FSP1 durante el crecimiento vascular, no se han realizado hipótesis sobre el papel de FSP1 durante la formación de las válvulas endoteliales. Por ello, quisimos testar el efecto de la sobreexpresión de *FSP1* en estas células endoteliales humanas. Este experimento resultó en el aumento en la tendencia de la expresión de *FOXC2*, que controla la formación de los vasos linfáticos colectores y sus válvulas; mientras que disminuyó la tendencia de la expresión de *VEGFR3*, un factor clave para la motilidad de las células linfáticas.

Estos datos sugieren que *FSP1* podría estar implicado en el destino de las células linfáticas, promoviendo el fenotipo válvulas de las células linfáticas a través de la sobreexpresión de *FOXC2*.

A lo largo de esta tesis, se realizaron análisis adicionales in vitro sobre la expresión de *FSP1* en células humanas pluripotentes inducidas (hiPSC). Estas células fueron diferenciadas hacia distintos tipos celulares cardíacos y se analizó la expresión de *FSP1* tanto en estas células diferenciadas como en las células pluripotentes. Concretamente, altos niveles de expresión de Fsp1 fueron observados en fibroblastos, células endoteliales, y cardiomiocitos ventriculares. Todos estos resultados *in vitro* sugieren a *FSP1* como un gen modulador, implicado en la diferenciación de múltiples tipos celulares incluyendo fibroblastos, cardiomiocitos y células endoteliales.

3.6. Los análisis de pérdida de función permiten un mayor conocimiento de la función de *Fsp1* en poblaciones celulares concretas

La tecnología Cre-Lox representa una herramienta poderosa que permite la modificación del perfil de expresión génico en un tipo celular específico. En esta modificación, la enzima Cre recombinasa media la deleción de regiones específicas del DNA flanqueada por secuencias loxP. Este método permite solventar la letalidad temprana asociada a los clásicos ratones KO (*knockouts;* relativo a la pérdida sistémica de la expresión de un gen determinado).

El ratón transgénico *Fsp1*-KO presenta un cierto nivel de letalidad embrionaria. A pesar de que no se han encontrado anomalías morfológicas postnatales, alrededor de un 10% de los ratones adultos desarrollaron tumores sin potencial metastásico. En esta tesis, hemos cruzado las líneas transgénicas *G2Gata4*^{CRE} y *cTnT*^{CRE} con *Fsp1*^{FLOX} para eliminar la expresión de *Fsp1* en el proepicardio y el miocardio, respectivamente. A pesar de que no se encontraron anomalías graves en el desarrollo del corazón y el epicardio se desarrolla correctamente en los ratones mutantes *G2Gata4*^{CRE};*Fsp1*$^{Loxp/Loxp}$, se observó un descenso significativo en el grosor de la capa compacta del ventrículo izquierdo a estadios E12.5. Teniendo en cuenta la discutida heterogeneidad de la población de fibroblastos cardíacos, es lógico pensar que *Fsp1* puede no jugar un papel relevante en el desarrollo cardíaco, así como en la diferenciación de fibroblastos a partir del epicardio. De todos modos, experimentos adicionales son necesarios para conocer el porcentaje de fibroblastos derivados de epicardio que expresan *Fsp1*.

Para analizar si la pérdida de FSP1 afecta al desarrollo del sistema de

conducción cardíaco, deleccionamos FSP1 utilizando la línea transgénica cTnI^{CRE}. Puesto que estos ratones se desarrollan y nacen con un corazón anatómicamente normal (datos no mostrados), pensamos que la ablación de *Fsp1* en cardiomiocitos maduros no afectaría al desarrollo del sistema de conducción cardíaco. Por tanto, nos gustaría proponer la línea transgénica *Nkx2-5*^{CRE} para los próximos experimentos, debido a la expresión temprana de *Nkx2-5* en los progenitores del sistema de conducción del corazón embrionario.

Perspectivas futuras

1) **Estudiar en detalle la deleción de *Fsp1* en células del proepicardio y epicardio**. Puesto que la expresión del gen *G2Gata4* ha sido descrita en el septo transverso y en células proepicárdicas, y los ratones *G2Gata4*^{CRE};*Fsp1*$^{LoxP/LoxP}$ no presentan anomalías en el epicardio, podemos intuir que la ablación de *Fsp1* no interfiere en la migración de las células derivadas del proepicardio y su posterior adhesión a la superficie del miocardio embrionario. Puesto que el objetivo principal de este experimento era analizar el efecto de la ablación de los fibroblastos derivados de epicardio que expresan *Fsp1*, debemos confirmar que esta población de fibroblastos está afectada en los ratones mutantes. Con este propósito, sugerimos la cuantificación y comparación de células fibroblásticas entre embriones controles y mutantes como futuros experimentos, con el uso de anticuerpos que reconozcan las proteínas FSP1, CD90 y procolágeno (COL1A1). Estos resultados podrían revelar cómo los fibroblastos derivados del epicardio son afectados por la ablación del gen *Fsp1* en el desarrollo temprano del corazón. Otros genes como

TCF21, descritos en fibroblastos derivados de epicardio, pueden ser utilizado adicionalmente para este propósito.

2) **Estudiar la deleción de *Fsp1* en condiciones patológicas.** Dado que no hemos encontrado defectos morfológicos letales en ratones *G2Gata4*^{CRE}; *Fsp1*$^{LoxP/LoxP}$, pensamos que someter a los ratones mutantes adultos a un infarto de miocardio usando un método clásico de ligadura coronaria permanente, podría desencadenar una respuesta diferente de la habitual.
3) **Testar la deleción de *Fsp1* en cardiomiocitos por ensayos de comportamiento.** Algunas patologías del sistema de conducción cardíaco, como las arritmias, podría no afectar a la capacidad de contracción en condiciones de reposo de ratones estabulados. Una caracterización más avanzada de la funcionalidad del sistema de conducción, por ejemplo, mediante el uso de pruebas de esfuerzo y electrocardiogramas en ratones mutantes *cTnT*^{CRE};*Fsp1*$^{LoxP/LoxP}$, podrían permitir describir la implicación de *Fsp1* en la capacidad de contracción.