Tesis doctoral

PhD thesis

PERICORONARY CELL RESPONSE TO CARDIAC DAMAGE: BLOOD-BORNE CELLS AND NEUROVASCULAR INTERACTIONS

Dña. Cristina Pogontke Díaz

Dirigida por/ Supervised by:

Prof. José María Pérez Pomares

Dr. Juan Antonio Guadix Domínguez

UNIVERSIDAD DE MÁLAGA

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FACULTAD DE CIENCIAS

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

D./Dña CRISTINA POGONTKE DÍAZ
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: PERICORONARY CELL RESPONSE TO CARDIAC DAMAGE: BLOOD-BORNE CELLS AND NEUROVASCULAR INTERACTIONS

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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Facultad de Ciencias
Departamento de Biología Animal

El Prof. José María Pérez Pomares, Catedrático del Departamento de Biología Animal de la Universidad de Málaga y el Dr. Juan Antonio Guadix Domínguez, Profesor Contratado Doctor en el mismo departamento, acreditan que:

Dña. Cristina Pogontke Díaz, 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: “PERICORONARY CELL RESPONSE TO CARDIAC DAMAGE: BLOOD-BORNE CELLS AND NEUROVASCULAR INTERACTIONS”.

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 enero de 2021.

Prof. José María Pérez Pomares Dr. Juan Antonio Guadix Domínguez

Este trabajo ha sido realizado en el laboratorio “Desarrollo y Enfermedad Cardiovascular” (DeCA), adscrito al Departamento de Biología Animal de la Facultad de Ciencias de la Universidad de Málaga, bajo la supervisión del Prof. José María Pérez Pomares y del Dr. Juan Antonio Guadix Domínguez. La investigación predoctoral de Cristina Pogontke Díaz se ha realizado al amparo de una beca de Formación de Personal Universitario (FPU) del Ministerio de Educación (FPU2015-03846).

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LIST OF FREQUENT ABBREVIATIONS

αSMA: Smooth muscle actin
BDNF: Brain-derived growth factor
BM: Basement membrane
BMDC: Bone marrow derived cell
CD31: Platelet and endothelial cell adhesion molecule 1 (a.k.a. PECAM1)
CF: Cardiac fibroblast
CI: Cardiac interstitium
CK: Cytokeratin
CSC: Cardiac stem cell
ECM: Extracellular Matrix
EMT: Epithelial-mesenchymal transition
EPDC: Epicardial derived cell
HSC: Hematopoietic stem cell
MHC: Myosin Heavy Chain
NGF: Neurotrophic growth factor
NO: Nitric oxide
NSC: Neural stem cell
NT3: Neurotrophin-3
OTF: Outflow tract
$P75^{NTR}$: p75 neurotrophin factor receptor
PDGFB: platelet derived growth factor B
PE: Proepicardium
PHF: Primary heart field
SGT: stellate ganglia
SMC: smooth muscle cell
SHF: Secondary Heart Field
TRK: Tyrosine kinase receptor
WT1: Wilms tumor 1
ZO1: Tight junction protein 1

INDEX

INTRODUCTION

"Fear tends to come from ignorance. Once I knew what the problem was, it was just a problem, nothing to fear."

— Patrick Rothfuss, The Name of the Wind

I.1. BACKGROUND

The heart is the first organ to develop and perform its functions during embryonic development, since early cardiac function is mandatory to guarantee the proper distribution of nutrients and oxygen to the continuously growing tissues of the embryo. Cardiac performance will remain crucial to the homeostasis of tissues and organs throughout the whole life of the organism, and deficiencies in cardiac performance often result in severe diseases. Cardiac activity critically depends on the integration of the functions of a great variety of specialized cells; these cells interact in space and time, first to build the embryonic cardiac primordium, and then to complete their differentiation into mature, fully functional cellular units. Understanding these first stages of heart morphogenesis is necessary for the further analysis of cardiac tissue complexity. Therefore, the second part of this introduction (I.2) will summarize the events controlling the formation of the cardiac system and detail the specific origins of major cardiac cell types. Then, the third section of the introduction (I.3) is dedicated to the cardiac interstitium, the extracellular space that forms between the muscular fibres of the heart, as this is one of the main subjects of this Ph.D. thesis. The cardiac interstitium hosts a great variety of cells relevant to understand cardiac homeostasis and responses to pathologic conditions. The fourth and final section of this chapter (I.4) will elaborate on cardiac reparative and regenerative responses of to injury.

I.2. THE CELLULAR AND MOLECULAR PRINCIPLES OF HEART DEVELOPMENT

I.2.1. Cardiogenesis

The specification of cardiac progenitor cells from the primary mesoderm shortly follows gastrulation, i.e. the ingression of epiblastic cells through the primitive streak. In the vertebrate amniote embryo, cardiac progenitor cells are originally located in the posterior regions of the lateral epiblast and ingress early, at the mid-streak stage (Figure 1A). As cardiac precursors leave the primitive streak, they migrate laterally and anteriorly from the midline to locate under the head folds. From this movement, two bilateral subsets of cardiac progenitors (left and right) are primarily formed (Antin et al., 1994; Kinder et al., 2001; Tam et al., 1997), although they soon fuse their most anterior ends medially forming a characteristic cardiac crescent (Jiang et al., 1998; Saga et al., 1999). This cardiac crescent will lie in the splanchnic layer of the lateral plate mesoderm (Figure 1B), once this mesodermal subpopulation has split into two distinct domains, the splanchnopleura and the somatopleura.

I.2.2. Formation of the cardiac tube: myocardium and endocardium

At these stages (E7.5-E8.5 in mice), two important cell types start to differentiate simultaneously from the cardiac crescent progenitors. Some of these precardiac progenitors, in response to endodermal signals including BMP and FGF molecules (Lough & Sugi, 2000; Sugi & Markwald, 1996), detach from their neighbouring cells, acquire a transitory mesenchymal phenotype, invade the underlying extracellular matrix, attach to the fibronectin-rich basal surface of the endoderm (Wiens, 1996), and finally regain a characteristic squamous, epithelial phenotype. These are the primitive endocardial cells, a special type of vascular endothelial cells that soon will form a continuous endocardial cell layer (Figures 1B´and B´´). This process of segregation of the cardiac crescent mesoderm into two

different cell types is considered to be, together with epiblast ingression at gastrulation and neural crest cell formation, one of the first examples of epithelial-to-mesenchymal transitions (EMT, see Hay, 2005) that can be recorded in the early embryo.

Quiescent precardiac epithelial cells, i.e. those that do not undergo EMT, progressively initiate their differentiation into cardiomyocytes, the striated muscular cells of the heart (Figures 1B´and B´´).

In amniotes, folding and invagination of the endoderm occurs on both the rostrocaudal and mediolateral axes, and consequently, the dorso-ventral organization of the cardiac tissues is inverted. Initially, the myocardial progenitors lie dorsally to the endocardial progenitors, but once the foregut has closed, endocardial cells are found dorsally to myocardial cells.

Cardiac progenitor cells at the cardiac crescent are not homogeneous. Multiple studies developed during the last two decades have revealed that, at least, two different subsets of cardiac progenitors form the cardiac mesoderm. These two populations have been dubbed Primary Heart Field (PHF) and Secondary Heart Field (SHF) with the SHF initially laying medially with respect to the PHF. These two developmental lineages of cardiac progenitors are regulated by complex transcriptional networks that differ between these two cardiac fields, so that early PHF are characterized by the expression of genes such us Nkx2.5, SRF, Mef2c or Gata4, whereas SHF cells specifically express Isl1 and Tbx1 (Meilhac & Buckingham, 2018).

PHF cells are the first ones to differentiate and form the primitive cardiac primordium, which has a tubular shape. This process starts at the bilaterally located splanchnopleural epithelial cells that contain the cardiac progenitors. These cells soon merge at the midline (around E7.5 in mouse), with the fusion process extending both rostrally and caudally (Moreno-Rodriguez et al., 2006) in close association to the forming foregut (Lough & Sugi, 2000; Matsui et al., 2005) (Figure 1C). As this primary heart tube appears, the SHF comes to lie anteriorly and posteriorly to the

cardiac anlage, distributed all through the subpharyngeal space. Multiple cell tracing experiments have revealed that SHF form the outflow tract myocardium, the right ventricle and an important part of the cardiac inflow (atria and sinus venosus), while PHF derivatives basically contribute to the left ventricle and atria (Galli et al., 2008; Kelly et al., 2001; Meilhac et al., 2004; Zaffran et al., 2004) (Figure 1D).

When this first stage of heart morphogenesis is completed, the heart consists of two concentric layers of cells, the outer myocardial one, characterized by the expression of myosins (mostly MHC), and the inner endocardial one, expressing the characteristic set of endothelial markers (VEGFR2, CD31, among others) (Harris & Black, 2010). Between the myocardium and the endocardium, the cardiac jelly, a highly hydrated extracellular matrix is found (Wessels & Markwald, 2000).

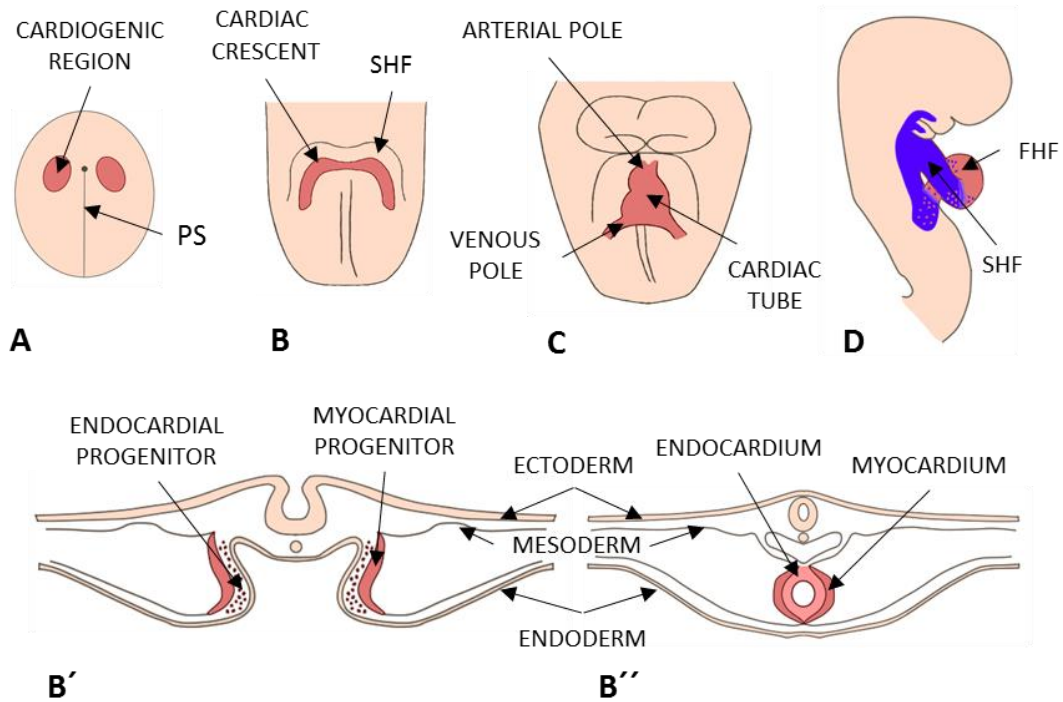

Figure 1. An overview of cardiac development. (A) Cardiac progenitor cells migrate to the midline to form the cardiac crescent. **(B)** Cardiac progenitors include endocardial and myocardial precursors **(B´ and B´´)**. **(C)** The developing heart then forms a linear tube with the contribution of the PHF. **(D)**. Subsequently, SHF cells progressively incorporate to the looped heart tube at both the venous and arterial poles Abbreviations: PHF = primary heart field; PS = primitive streak; SHF = secondary heart field. Modified from Buckingham et al., 2005; Lough & Sugi, 2000.

I.2.3. Epicardial development

In vertebrates, soon after the embryonic tubular heart appears, a third epithelial tissue layer, the epicardium, forms over the heart surface in contact with the fluid of the pericardial cavity (E9.5-11.5 in mice). This monolayered mesothelium arises from an extracardiac splanchnic mesodermal cell population called the proepicardium (PE). In mammals, proepicardial cells appear bilaterally, on the posterior limit of the left and right sinus horns (Figure 2A), and then become restricted to a single proepicardial cluster over the septum transversum since both PE primordia fuse in the midline (Schulte et al., 2007) (Figure 2B).

Little is known about the lineage relationship that the PE holds with the PHF and SHF. Although cells in the PE are known to express the cardiogenic transcription factor Nkx2.5 during their differentiation (Ma et al., 2008) epicardial cells strongly express molecules not related to myocardial or endocardial cell lineages, as the Wilms tumor suppressor 1 (Wt1), Podoplanin (Pdpn) or Tbx18 (Cai et al., 2008; Christoffels et al., 2009; Guadix et al., 2011; Mahtab et al., 2008). Surprisingly, these same genes are also common in the kidney mesodermal progenitors. This and other findings in the most primitive vertebrates known (the agnathan lamprey) (Pombal et al., 2008) has led to the hypothesis of the PE being an evolutionary derivative of the vertebrate pronephric glomeruli (Pérez-Pomares et al., 2009).

Histologically, the PE is composed of at least two distinct cell types, an external mesothelial epithelium and an inner mesenchymal core, which is also rich in extracellular matrix, both highly proliferative (Pérez-Pomares et al., 1998; Wessels & Pérez-Pomares, 2004). As soon as the PE forms, cells from its mesothelial surface start to be transferred to the beating myocardial surface (Figure 2C). In avian embryos, an important part of this cell transfer is accomplished through the formation of conspicuous adhesive proepicardial villi to the cardiac surface (Ishii et al., 2010; Männer, 1992). Studies in fish (Muñoz-Chápuli et al., 1997) and mouse embryos (Komiyama et al., 1987), however, suggest that in these species the PE does not develop well-extended villi, and instead generates short protrusions that ultimately transform into proepicardium cysts that detach from the mesothelium. These cysts

are thus released as free-floating, multicellular aggregates or vesicles into the coelomic space, where they contact with the myocardial surface, attaching to the cardiomyocytes (Männer et al., 2001). Whether this process is developmentally patterned remains unknown, although studies in the zebrafish suggest that heartbeat-driven pericardiac fluid forces could be key in controlling epicardial formation from the PE (Peralta et al., 2013).

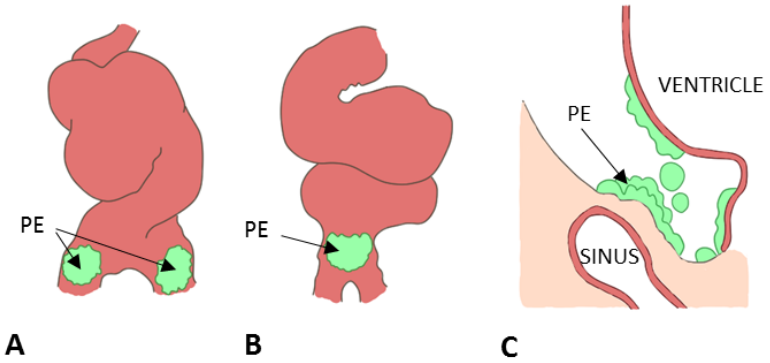

Figure 2. **Proepicardium in the developmental heart. (A)** Original bilateral location of the epicardial progenitor cells and **(B)** formation of the single proepicardium. **(C)** Different cellular mechanisms allow for the transference of proepicardial cells to the bare myocardial surface. Abbreviations: PE = proepicardium. Modified from Rosenthal & Harvey, 2010.

Once these proepicardial cells attach to the cardiomyocytes, the epicardial epithelium spreads over the heart surface in a process that is partially mediated by α4 integrin, present in the epicardium (Yang et al., 1995), and its ligand the vascular cell adhesion molecule-1 (VCAM1), present in the myocardium (Kwee et al., 1995). Then, a subpopulation of epicardial cells initiate a process of EMT. This implies extensive changes in epicardial cells, including the substitution of the epithelial intermediate cytoskeletal filament cytokeratin for the mesenchymal vimentin (Pérez-Pomares et al., 1997) as well as the E-cadherin adhesion molecule for N-cadherin (Martínez-Estrada et al., 2010). Once detached from the epicardial epithelium, these cells invade the extracellular space between the myocardium and the epicardium. This new space, called the subepicardium, is more prominent at the atrioventricular (AV), conoventricular (CV) and interventricular (IV) junctions, and is key for early cardiac coronary vascularization (see below). The mesenchymal cells that form after

epicardial EMT are known as epicardial-derived cells (EPDCs) (Gittenberger-de Groot et al., 1998; Pérez-Pomares et al., 2016).

Lineage analysis of avian (Palmquist-Gomes et al., 2018) and mouse (Pérez-Pomares et al., 2016) (pro)epicardial derivatives has revealed that cells originated within the PE colonize the compact and trabecular myocardium and give rise to diverse cell types, including fibroblasts, vascular smooth muscle and endothelial cells of the coronary blood vessels (Figure 3). The contribution of EPDC to the coronary endothelium is, however, significantly lower in the mouse than in the avian embryo (Cano et al., 2016).

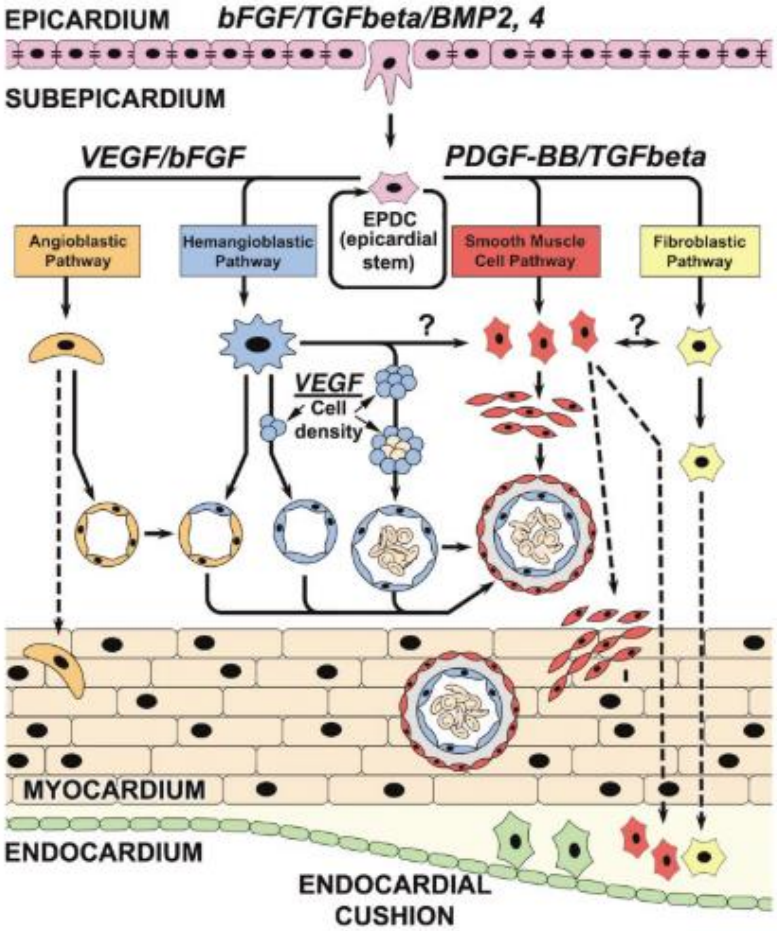

Figure 3. Differentiation pathways of EPDCs. Epicardial cells invade the subepicardium and differentiate into diverse cell types under the regulation of various growth factors. From Wessels & Pérez-Pomares, 2004.

As mentioned before, although some of the molecules implicated in the formation the EPDC have been studied, little is known about the molecular signals and transcriptional regulators that trigger and modulate epicardial EMT. One molecule that seems to play a key role in the regulation of this process is Wt1, a zinc-finger protein implicated in development, tissue homeostasis and disease of multiple organs, including the heart.

As previously indicated, Wt1 expression is characteristic of the PE, the epicardium, and the EPDCs, in this latter case only until they reach their final destination and start differentiation. The relevant role played by Wt1 in epicardial biology was evidenced by the multiple defects of Wt1-null mice. In these mutants, the epicardium does not form correctly, coronary blood vessels are lost, their ventricular muscular walls are abnormally thin so that the animals die around E12.5 from massive haemorrhage (Guadix et al., 2011). Wt1 pattern expression, among that of other genes, has inspired the development of genetic technologies for the tracing and the study of the fate of PE, epicardial cells and their derivatives during heart morphogenesis and homeostasis like those based in the Cre/LoxP technology (Cano et al., 2016; Del Monte et al., 2011; Zhou et al., 2008).

I.2.4. Cardiac looping and chamber growth

Once formed, the straight heart tube hangs along the dorsal midline freely suspended from the foregut by a double mesothelial membrane referred-to as the primary dorsal mesocardium. Shortly after the heart tube forms, the mid-portion of the dorsal mesocardium disintegrates, so that the heart remains attached to the pericardial roof through the arterial pole of the heart (cardiac outflow) and the remnants of the dorsal mesocardium at the cardiac venous pole (Wessels et al., 2000). The persisting dorsal mesocardium is the area in which the pulmonary vein develops and forms a gateway for mesenchymal cell populations (neural crest cells) to migrate into the venous pole of the heart (Drake et al., 2006; George et al., 2020).

This partial disintegration of the dorsal mesocardium is a crucial event, as it allows the straight heart tube to bend in a process known as cardiac looping. This process implies the rightward bending of the cardiac tube (R-loop) and the anterior displacement of the originally posterior venous pole (Männer, 2009) resulting in the re-alignment of different cardiac domains, which is necessary for the completion of cardiac septation and the establishment of two parallel, pulmonary and aortic blood circuitries in the higher vertebrate heart (Ocaña et al., 2017).

As cardiac development proceeds, the expansion of local domains of the myocardium become distinguishable in the outer curvature of the cardiac tube. These regions will form the cardiac chambers (atria and ventricles). According to the ballooning model of cardiac chamber formation, atria and ventricles form by the massive proliferation of cardiomyocytes. In contrast, the cardiac outflow (OFT) and AV myocardium do not proliferate and retain a primitive contractile activity, and important parts of these tissues will differentiate into the cardiac conduction (pacemaker) tissues during late development (Christoffels et al., 2010). This process is tightly regulated by Tbx transcription factors: the primary cardiac chamber cardiomyocyte proliferation program is transcriptionally promoted by the interaction of Nkx2.5 and Tbx5, whereas OFT and AV myocardial proliferation is blocked by the transcriptional repression exerted by Nkx2.5 and Tbx2/3 interaction. As a result of this process, the chamber myocardium expands and grows, forming an inner mesh of myocardial strands lined by endocardium known as trabeculae, that give the developing ventricular myocardium a characteristic “spongy” appearance. Trabeculation is the earliest phenotypical expression of cardiac chamber formation, especially in the case of cardiac ventricles (so far, only the molecular mechanisms that regulate ventricular trabeculation have been studied in depth). Reports from the last decade confirm that endocardial Notch signalling, mediates the secretion of Neuregulin (Nrg1), thus inducing the formation of the ventricular trabecular layer (del Monte-Nieto et al., 2018; Grego-Bessa et al., 2007).

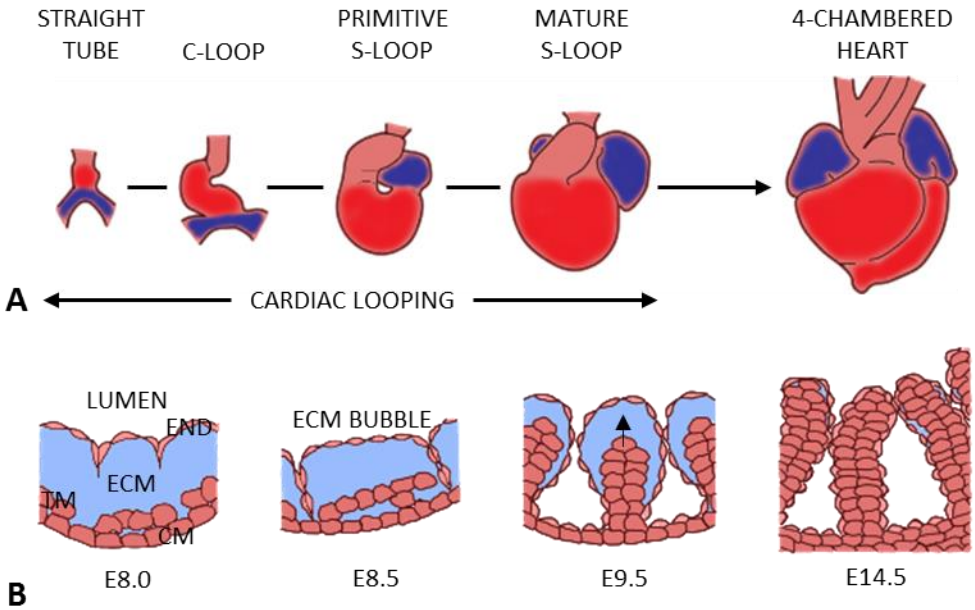

Figure 4. **Cardiac looping and trabeculation. (A)** Phases of the cardiac looping, with transformation of the straight tube-like heart into a helical structure with cardiac septation and double circuit. **(B)** Trabeculation stages, including formation of cardiomyocyte perpendicular structures, contact of the endocardium to the compact myocardium and degradation of extracellular matrix. Abbreviations: CM = compact myocardium; ECM = extracellular matrix; END = endocardium; TM = trabecular myocardium. Modified from Männer, 2004 (A) and del Monte-Nieto et al., 2018 (B).

I.2.5. Epicardium and coronary blood vessel development

During trabeculation and wall thickening, coronary vascular system formation occurs. The process starts with the formation of a primitive endothelial plexus formed by endothelial cells recruited from the sinus venosus and ventricular endocardium (Red-Horse et al., 2010; Wu et al., 2012), the septum transversum/PE, (Cano et al., 2016), and the aortic sac (Palmquist-Gomes et al., 2018).

Early stages of coronary development take place through the combined action of different blood vessel growth mechanisms. Both vasculogenesis (assembly of isolated vascular progenitors known as angioblasts to form blood vessels *de novo*, see Risau & Flamme, 1995) and angiogenesis (formation of new blood vessels from pre-existing ones, see Folkman, 1971; Folkman & Haudenschild, 1980) cooperate to form the highly patterned coronary vascular bed (Pérez-Pomares et al., 2016).

Several growth factors regulate vasculogenesis and angiogenic sprouting, including vascular endothelial growth factor (VEGF), fibroblast growth factors (bFGF and FGF2) and angiopoietins (Tomanek et al., 2001; Tomanek et al., 2002).

Coronary blood vessel stabilization, as it happens in the non-cardiac vasculature, is tightly dependent from the formation of the vascular wall. This event is concomitant with a dramatic reduction in endothelial cell proliferation, sharp morphological changes in endothelial cell shape and organization, and the recruitment of vascular wall cell components (Cleaver & Krieg, 2010), particularly smooth muscle cells and pericytes, two cell types that will be studied in detail in section I.3 of this introduction. Soluble platelet derived growth factor B (PDGF-B), TGFβ and membrane-bound ephrins have been shown to finely regulate the recruitment of mural cells and the spatial patterning of the vascular bed (Van Dijk et al., 2015).

Once the formation of the blood vessel wall is completed, a sophisticated tree of blood vessels composed of arteries, arterioles, capillaries, venules and veins is assembled. These blood vessels are in charge of supplying and draining blood from the cardiac muscle (Figure 5A). Large arteries and veins display a multi-layered conformation, with different tissues arranged concentrically. The tunica intima is the innermost layer and is composed of the endothelium, which lines the lumen of the blood vessel, the endothelial basement membrane and the internal elastic tissue. Surrounding the tunica intima, the tunica media is found, formed by smooth muscle and elastic extracellular fibres. Finally, the outermost tunica adventitia, formed by connective tissue and large amounts of fibroblasts, covers the surface of the blood vessels (Figure 5B). Smaller vessels tend to show a less complex vascular wall arrangement (the adventitia is often lost) and capillaries, which are distributed all through the interstitial space, only have a discontinuous layer of pericytes in contact with endothelial cells (Martin-Robles & Pérez-Pomares, 2016).

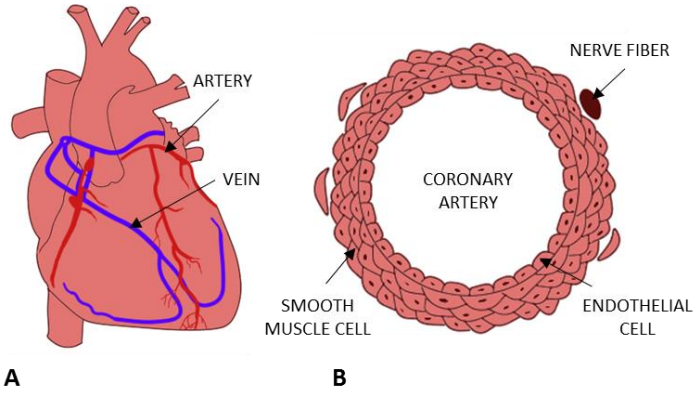

Figure 5. **Coronary blood system**. **(A)** Distribution of the main coronary vessel in the adult heart (coronary arteries and veins). **(B)** Cellular composition of a mature coronary artery.

I.2.6. Myocardial maturation

Although the basic cardiac structure is almost complete around birth, at these perinatal stages, embryonic and foetal cardiomyocytes are considered immature cells, and it is only a few weeks after birth that full cardiomyocyte maturation occurs. In amniote vertebrates (but not in anamniote ones), cardiomyocyte maturation is paralleled by the marked decrease of cardiomyocyte proliferation (Soonpaa et al., 2015). This decrease is indeed so abrupt that cardiomyocytes are not able to complete their last mitotic round, remaining binucleated in the case of the mouse (each nucleus being diploid) or with a single tetra- or poliploid nucleus as in humans (Kikuchi & Poss, 2012; Matrone et al., 2017). During the first 3-4 days after birth, the percentage of binucleated myocytes in the mouse heart roughly increases from 4% to 17%, so that while cardiac myocyte volume remains stable, cardiomyocyte cell number rapidly increases. After day 3, the number of myocytes in the heart remains relatively constant, suggesting that cell division stops between day 3 and day 4. In accordance with this finding, cardiomyocyte binucleation proceeds at a high rate from day 4 onwards, approaching the numbers of the adult heart by day 12 after birth.

Full myocardial maturation requires myofibril stabilization, the effective electrical coupling of cardiomyocytes and a metabolic shift from glycolytic to oxidative phosphorylation (Guo & Pu, 2020; Murry et al., 2002; Slaats et al., 2020).

Remarkably, optimal cardiomyocyte contractile performance is reached progressively after birth through a ‘beating training’ that can be partially mimicked *in vitro* using culture systems that modify the biophysical conditions of the cell (Schwach & Passier, 2019; Veerman et al., 2015).

I.3. THE CARDIAC INTERSTITIUM

For years, cardiomyocytes have been considered the most abundant cell type in the heart (Moore et al., 1980; Vliegen et al., 1991). However, recent studies have quantified the relative proportion of different cardiac cell types in humans and have determined that cardiac muscle cells represent around 30% of the complete cardiac cell population only (Bergmann et al., 2015; Pinto et al., 2016). In the myocardium, the space not occupied by cardiomyocytes is called the cardiac interstitium (CI) and it comprises not only the non-myocyte cells living between the cardiac muscle fibres (cardiac interstitial cells, CICs), but also an abundant extracellular matrix.

I.3.1. The structure of the cardiac interstitium

This CI, whose definitive organization is established during neonatal development, plays an essential role in the regulation of development and homeostasis of the myocardium (Krenning et al., 2010; Pérez-Pomares & de la Pompa, 2011; Pogontke et al., 2019) and in the adaptive response of the heart to pathological conditions (Ruiz-Villalba et al., 2015; Takeda et al., 2011).

The CI includes large numbers of cells whose size is significantly smaller than that of cardiomyocytes, immersed in a rich, fibrillar extracellular matrix including collagens (mainly types I and III), proteoglycans, non-collagenous glycoproteins, growth factors, multiple cytokines and extracellular proteases (Bergmann et al., 2015; Borg et al., 1996; Frangogiannis, 2017; Meschiari et al., 2017) (Figure 6).

Endothelial cells. As mentioned above, endothelial cells (approximately 58% of CICs) line the lumen of the coronary vessels, and are the main cell type in the cardiac capillary network.

As blood vessels mature, endothelial cells synthetise multiple extracellular matrix proteins developing the endothelial basement membrane (BM). This structure supports the basal surface of endothelial cells, and critically contributes to maintain the cell polarity and regulate the endothelial cell proliferation, adhesion and

differentiation. In the adult, endothelial cell interacts simultaneously with circulating cells and cells present in the vascular wall as smooth muscle cells and fibroblasts exercising important functions in vascular homeostasis. Moreover, being the interface between blood and tissue, they are mostly susceptible to changes in blood composition and in blood flow, playing a central role in the mechanisms underlying the onset and progression of vascular disorders (Michiels, 2003).

Vascular Smooth Muscle Cell (vSMC). Following the morphological changes associated with endothelial vascular remodelling, mesenchymal progenitors for mural cells are recruited to provide mechanical and physiological support to the endothelium. There are several important signalling pathways involved in smooth muscle cells recruitment, differentiation and maturation, some of the most relevant ones being platelet-derived growth factor (PDGF)-BB and transforming growth factor-β (TGFβ) (Hao et al., 2003; Hirschi et al., 1998).

In the adult heart, SMC typically have elongated and flattened nuclei and locate themselves covering the whole endothelial surface. These tunica media cells express a set of up-regulated smooth muscle markers, including cytoskeletal and contractile proteins, such as smooth muscle α-actin (αSMA), smooth muscle myosin heavy chain (SM-MHC), calponin and SM22α (Salmon et al., 2012). SMCs not only play a key role in maintaining vascular structure but also perform various functions as regulate vascular tone and hence, blood flow.

Pericytes. By definition, pericytes are cells embedded within the vascular BM of small blood vessels and do not form a continuous cell layer, extending primary cytoplasmatic processes along the abluminal surface of the endothelial tube. These membrane projections usually span several endothelial cells and occasionally bridge neighbouring capillary branches. Similar to vSMCs, pericytes contain contractile filaments composed of vimentin and alpha-smooth muscle actin (αSMA) and are capable of vasomotion regulation, being primarily distinguished from vSMCs by their specific perivascular morphology. They typically maintain a rounded nuclear structure and embrace the endothelial surface with multiple extensions. Membrane markers

for pericytes include platelet-derived growth factor receptor B (PDGFRB), CD146, aminopeptidases A and N (CD13), endoglin (CD105), and neuron-glial 2 (NG2). Common cytosolic markers for pericyte identification include αSMA, non-muscle myosin, desmin, vimentin and nestin. NG2 has been successfully used in combination with alpha-smooth muscle actin (αSMA) to identify three main subsets of human pericytes: the capillary ($NG2^+/\alpha SMA^-$), the venule ($NG2^-/\alpha SMA^+$) and the arteriole ($NG2^+/\alpha SMA^+$) ones.

It is generally accepted that pericytes belong to the same ontogenetic lineage of vSMC (Nees et al., 2012). These same studies also suggest a close ontogenic relationship between mural cells (pericytes and vSMC) and fibroblasts in many organs. In the heart, some cells like coronary pericytes, vSMC, adventitial and ventricular interstitial fibroblasts share a common epicardial origin (Cano et al., 2016; Ruiz-Villalba et al., 2015; Volz et al., 2015). Moreover, some studies have suggested that some pericytes might indeed be multipotent stem and/or progenitor cells. Indeed, pericytes also exhibit surface markers of mesenchymal stem/progenitors cells (CD44, CD73, CD90 and CD105) (Armulik et al., 2011).

Fibroblasts. Cardiac fibroblasts (CF) represent only 10% of the total number of cells in the adult homeostatic cardiac walls, although their numbers massively increase in pathological contexts (Palmquist-Gomes et al., 2019; Ruiz-Villalba et al., 2015, 2020). Under physiological equilibrium, CF are the stromal cells responsible for the synthesis and regulation of a major part of cardiac ECM, playing a critical role in the maintenance of the heart by supporting cardiomyocytes and other cardiac tissues (e.g. coronary blood vessels and capillaries). Moreover, these cells are involved in the modulation of cardiomyocyte electrical activity (Rohr, 2011) and CF-secreted ECM molecules are believed to be necessary to the setting of the cardiac immune equilibrium. However, CF have been reported to secrete TGFβ, VEGF, TNF-α, IL-1β, IL-6, and angiotensin-II, all of which are important molecules related to different pro-inflammatory, pro-fibrotic and pro-angiogenetic processes that affect the myocardium and interfere with active cardiac cell signalling (LaFramboise et al., 2007; Rossini et al., 2008; Souders et al., 2009; Yokoyama et al., 1999).

Blood-borne cells. The vertebrate circulatory system includes diverse cell types with different functions, mainly cells specialized in gas transport (erythrocytes), blood clotting (thrombocytes), and immune response or tissue repair (leukocytes). Leukocytes are classified into granulocytes or polymorphonuclear leukocytes depending on the morphology of their cell nuclei and relative cytoplasmic inclusions. Among these cells, monocytes are essential mediators of tissue response to damage, as monocytes travel to the site of infection or injury and, once there, they differentiate into macrophages capable of phagocytizing pathogenic or damaged cells and further activating complex inflammatory responses that involve lymphocyte populations responsible for specific immunity (Hartenstein & Mandal, 2006).

The adult mouse heart harbors all major leukocyte cell types, from mononuclear phagocytes to neutrophils and B and T cells, but specific functions of some of these cells in health conditions is poorly understood (Sampaio-Pinto et al., 2020). Macrophages, in particular, have been shown to be the most numerous and relevant, displaying active phagocytosis, producing trophic and immune-related factors and modulating cardiac conduction system activity.

In the adult vertebrate, all these cell types originate through a process called haematopoiesis from hematopoietic stem cells (HSC) that are found in a very low proportion in the bone marrow (Orkin & Zon, 2008). Rounded in morphology, small in size and with a relatively large nucleus, they are uncommon in the bloodstream and generally only present in elevated numbers under pathological conditions (Hartenstein & Mandal, 2006). These cells are characterized by being capable of self-renewal by asymmetric division and also of giving rise to different parents hematopoietic (no longer self-renewing capacity) that eventually differentiate into matured cells (Challen et al., 2009).

In the embryo, however, at least two major sources of blood cells have been identified, an extraembryonic one including the yolk sac/placenta and an intraembryonic one (the aorta-gonad-mesonephros region or AGM). Although the AGM is considered to be the origin of adult definitive blood cells (Golub & Cumano,

2013), recent studies indicate that extracardiac blood progenitors significantly contribute to the adult macrophage population (Bajpai et al., 2018).

Nerves. The innervation of the heart refers to the network of nerves that are responsible for the functioning of the heart, composed of sympathetic and parasympathetic fibres from the autonomic branch of the peripheral nervous system. The embryonic cardiac neural crest is known to be the origin of the major part of cardiac nervous tracts (Hutson & Kirby, 2003). In relation to the sympathetic nervous system, these nerve fibres originate primarily in the stellate ganglia (SGT), the second most anterior pair of sympathetic ganglia located at C7/T1 level. Sympathetic nerves synapse close to the sinoatrial (SA) node (the pacemaker of the heart) to increase heart rate, and directly on the myocardium to increase contractility, both via release of norepinephrine. Previous studies have shown that arterial vSMCs mediate proximal sympathetic axon extension by secretion of artemin, neurotrophins and endothelins (Coote & Chauhan, 2016; Manousiouthakis et al., 2014; Nam et al., 2013).

Cardiac stem cells and progenitors. During the last twenty years, many laboratories have reported on the identification of cardiac multipotent cells with residence in the cardiac interstitium. These cells were described to be able to differentiate into different cardiovascular cell types (cardiomyocytes, fibroblasts, endothelial and smooth muscle cells). Many of these multipotent cells were first identified by their expression of markers like c-Kit (Beltrami et al., 2003; Quaini et al., 2002), Sca-1 (Matsuura et al., 2004; Oh et al., 2003), SSEA-1 (Ott et al., 2007), Bmi-1 (Valiente-Alandi et al., 2016) or by their characteristic extrusion of the Hoechst 33342 dye via the ATP-binding cassette transporter Abcg2 (side population cells) (Martin et al., 2004; Pfister et al., 2005; Tomita et al., 2005).

Since all these markers are known to be expressed by well-characterized stem cells on non-cardiac origins (Berrill et al., 2004; Bradfute et al., 2005; Capela & Temple, 2002; Leong et al., 2008; Park et al., 2003; Sangiorgi & Capecchi, 2008; Thoren et al., 2008) the term cardiac stem cells (CSC) was rapidly coined to refer to these heart cells. However, it soon became evident that self-renewal of these cells

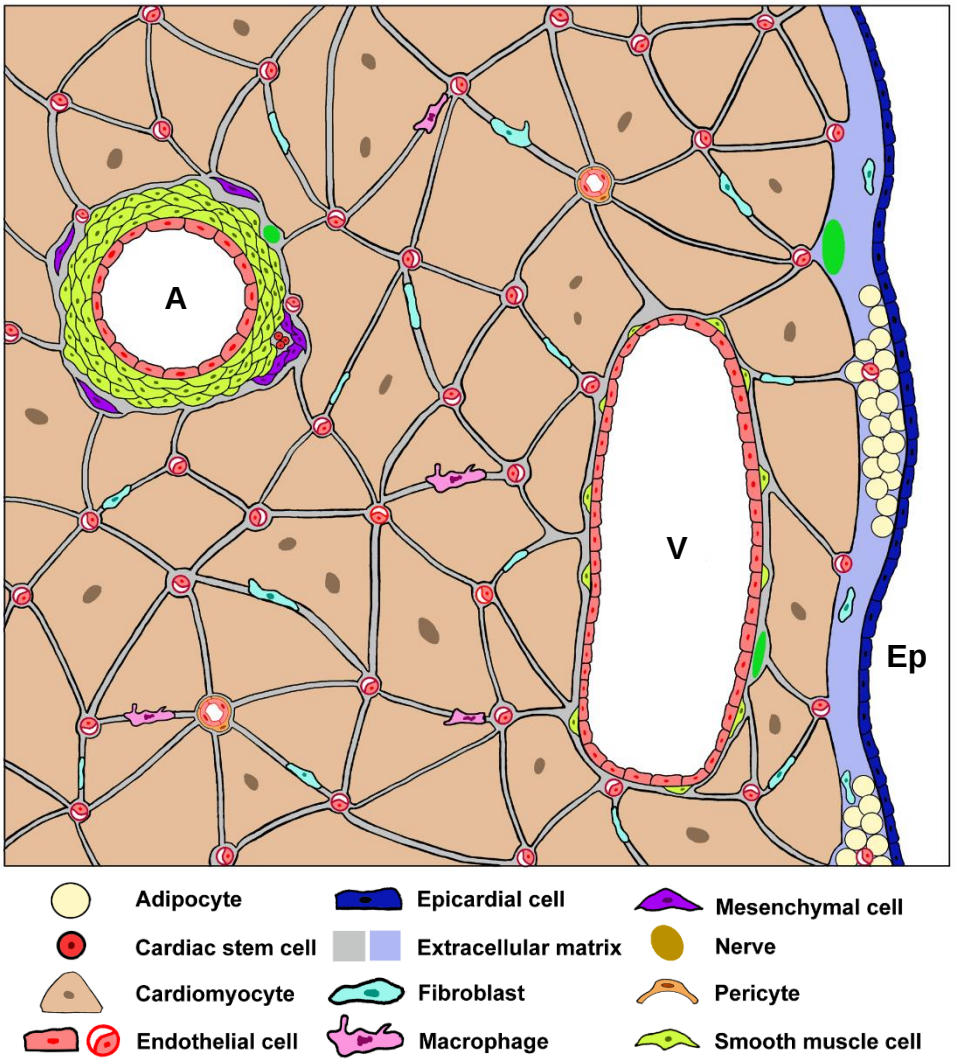

Figure 6. A cellular map model for the cardiac interstitium. The cartoon depicts the cell diversity and configuration of the cardiac interstitium. Abbreviations: A = artery; Ep = epicardium; V = vein. Modified from Pogontke et al., 2019.

(a sine qua non feature of true stem cells) was not easy to prove, and therefore some other authors preferred to call them cardiac progenitor cells (Bianconi et al., 2018). Their actual capacity to perform as real CSC is extremely controversial and will be profusely analysed in the next chapter.

I.3.2. The neuro-vascular motif

The trophic interaction between different tissues is essential to the coordination of animal functions. This aspect has been especially studied in the neurobiology field, which has developed a remarkable interest in the impact of non-neural cells and tissues in the activity of the nervous system. In this context, the concept of neurovascular unit was coined, highlighting the physiological and pathophysiological relevance of vascular and neural interactions in diverse organs, including the heart (Egawa et al., 2016).

It has for long been known that neural activity locally regulates blood flow changes through neurotransmitters; the sympathetic nerves secrete norepinephrine (NE), adenosine triphosphate (ATP) and neuropeptide Y (NPY) that perform as vasoconstrictors, whereas the parasympathetic nerves produce acetylcholine (Ach) and calcitonin gene-related peptide (CGRP) that can mediate vasodilation (Ebert & Stowe, 1996; Guyenet, 2006). Nevertheless, the idea that the perivascular microenvironment (often referred-to as niche) could play a central role in complex phenomena like neurogenesis is a relatively recent concept (Ohab et al., 2006).

The neurovascular unit is composed of neurons, glial cells (astrocytes, oligodendrocytes and microglia), endothelial cells and pericytes (Hawkins & Davis, 2005). Classical secreted mediators of neuro-vascular function include Nitric Oxide (NO), SDF1, VEGF, and PDGF-B (Egawa et al., 2016). Interestingly, other factors like neurotrophins (e.g. NGF or BDNF, see next section), that were considered specific proteins of the nervous system, have also been involved in this process; and is now accepted these molecules could play important roles in the interaction of nervous cells with other tissues, including blood vessels.

I.3.3. Neurotrophins

In mammals, four members of the neurotrophin family have been identified: nerve growth factor (NGF), brain derived neurotrophic factor (BDNF), neurotrophin 3 (NT3) and neurotrophin 4/5 (NT4/5). They are members of the Cysteine Knot

Superfamily of proteins (CKS) and are all synthesized as precursor proteins. These precursor forms are processed intracellularly by convertases (furin, PACE4 and PC5 for constitutive secretion; PC1 and PC2 for regulated secretion), or extracellularly by plasmin or matrix metalloproteinases (MMP3 and MMP7) to yield functional, mature neurotrophins. NGF, NT3 and NT4/5 are secreted constitutively by nervous cells, while BDNF is processed and secreted in a regulated way (Al-Qudah & Al-Dwairi, 2016).

Neurotrophins signal through two types of specific transmembrane receptors, the high affinity tyrosine receptor kinases (Trks) and the low affinity neurotrophin receptor p75NTR. Three Trk proteins has been identified in mammals so far: TrkA, TrkB and TrkC, displaying 87% of homology at the aminoacid level. The four neurotrophin ligands exhibit specificity in their interaction with the three Trk receptors: NGF activates TrkA, BDNF and NT4/5 activates TrkB, and NT3 efficiently TrkC, as well as other Trk receptors, albeit with less efficiency (Clary & Reichardt, 1994; Davies et al., 1995; Klein et al., 1991; Strohmaier et al., 1996). The p75NTR receptor binds proneurotrophins with high efficiency and all mature neurotrophins with low efficiency (Reichardt, 2006).

Proneurotrophins and neurotrophin binding to their receptors activates complex signalling cascades in the nervous system. Neurotrophin interaction with the Trk receptors induces transphosphorylation of the tyrosines of the receptors, triggering signals through different signalling pathways like Ras/MAPK, PI3-kinase/Akt or PLC-gamma1. Neurotrophin ligand interaction with the p75NTR receptor, that requires the presence of sortilin, induces activation of several signalling pathways related to apoptosis and cell survival, mediated through association of diverse adaptor proteins, including Traf6, neurotrophin receptor-interacting factor (NRIF), melanoma associated antigen (MAGE), neurotrophin receptor p75 interacting MAGE homologue (NRAGE), Schwann cell factor 2 (SC1), and RhoDI. Most interestingly, P75NTR activation in the absence of activated Trk receptors leads Jun kinase signalling cascade promotion, which results in p52 activation, Fas ligand production and apoptosis (Kaplan & Miller, 2000; Reichardt, 2006) (Figure 7).

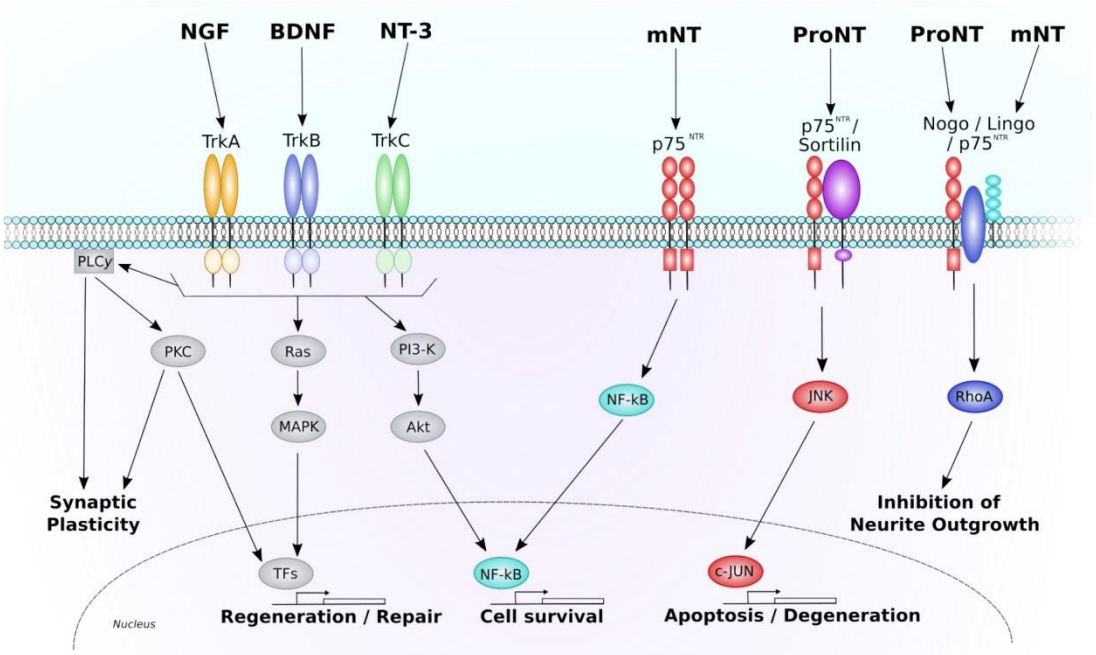

Figure 7. Main members of the neurotrophin family. This diagram depicts the major intracellular signalling pathways associated through each neurotrophin receptor. From Houlton et al., 2019.

Although this complex system of neurotrophin pathway signalling has been primarily analysed in the nervous system, neurotrophin interaction has been also observed in other tissues as the hematopoietic, the urinary or the circulatory system. Particularly, in the developing and adult heart, the diverse neurotrophins and receptors have been observed in diverse cell types although their relevance in the cardiac function has not been yet deeply analysed in some cases:

NGF-TrkA. In the heart, NGF expression increases during development, concurring with the arrival of the sympathetic nerve fibers from the SGT, that express TrkA. This signaling is indispensable for cardiac sympathetic nerve formation, since deletion of cardiac NGF or nerve TrkA inhibits nerve migration inducing embryonic lethality (Cao et al., 2000; Fagan et al., 1996; Ieda et al., 2006). During adulthood, NGF is expressed by endothelial cells, smooth muscle cells and cardiomyocytes (Donovan et al., 1995; Kaye et al., 2000), where they seem to be implicated in

process of angiogenesis, migration or cell homeostasis (Cantarella et al., 2002; Caporali et al., 2008; Kraemer et al., 1999).

BDNF-TrkB. BDNF-TrkB interaction is required for cardiac microvessel stabilization in perinatal and adult stages, with BDNF-expressing endothelial cells regulating migration of TrkB-expressing pericytes/SMCs. As a matter of fact, BDNF/TrkB deficiency results in a reduction in endothelial cell-cell contacts and in endothelial cell apoptosis, leading to ventricular wall hemorrhage, depressed cardiac contractility and early postnatal death (Anastasia et al., 2014; Donovan et al., 2000). Moreover, in adulthood, BDNF and TrkB are also expressed by cardiomyocytes with functions related to cell survival and performance (Feng et al., 2015; Okada et al., 2012).

NT3-TrkC. In early development, NT3-TrkC signalling is implicated in cardiac neural crest migration, with TrkC expression in neural crest cells (Youn et al., 2003). Moreover, TrkC is also expressed by immature cardiomyocytes, where is important in cardiomyocyte proliferation and conduction system formation (Donovan et al., 1996; Hiltunen et al., 1996; Kawaguchi-Manabe et al., 2007; Nakahashi et al., 2000). As consequence, $NT3^{-/-}$ mutants die shortly after birth with cardiac dilation, ventricular septal defects and valvular and sinus venosus defects (Donovan et al., 1996). During adulthood, NT3 is expressed by coronary vessels and cardiomyocytes, while TrkC is only present in cardiomyocytes (Kawaguchi-Manabe et al., 2007).

All neurotrophin forms are expressed by the cardiac vascular cells during development or adulthood, with described significance in the cardiac neurovascular band in some circumstances. Neurovascular interactions have been intensively researched in regard to their relevance in sustaining the central nervous system niches identified for neural stem cells (NSC). The NSC niche is characteristic neurovascular, so that the close association of NSC to the vascular wall has been noted to be pivotal to the maintenance of the niche system (Licht & Keshet, 2015). Relevant to this thesis, since NT3 was described to be produced by endothelial cells and be involved in neovascularization phenomena (Cristofaro et al., 2010), reports followed on the role of this particular neurotrophin in the milieu where neural stem

cells reside. In particular, it was found that endothelial-secreted NT3 induces the rapid phosphorylation of endothelial nitric oxide (NO), leading to the production of NO in B1 adult NSC. This, in turn, has a cytostatic effect on this cell population (Chou & Modo, 2020; Delgado et al., 2014).

I.4. CARDIAC REGENERATION AND REPAIR

For more than twenty years, overstatements on the ability of the mammalian heart to regenerate after significant damage have sustained controversy in the field. Recent joint efforts by specialists have, however, clarified key aspects on mammalian heart responses to injury, in many cases considering the comparison between different vertebrates as a rationale for the study of these phenomena (Beffagna, 2019; de Wit et al., 2020; Eschenhagen, 2007). In this section of the introduction, we will briefly summarize our current knowledge on vertebrate heart reparative and regenerative responses.

I.4.1. Tissue repair versus regeneration

A brief note on the differences between tissue regeneration and repair is necessary to critically consider some of the information that follows. Regeneration implies the full substitution of dead or impaired tissue by a new functional tissue that is fully equivalent to the original one. On the contrary, repair involves the substitution of one tissue type by another one supporting the integrity of living structures but unable to develop the same functions (Galliot et al., 2017). Unfortunately, both terms are often used interchangeably (for a discussion on this see Palmquist-Gomes et al., 2018). In this introduction we will stick to these two definitions when referring to tissue repair and regeneration, but as we will immediately discuss, these two phenomena are not mutually excluding.

I.4.2. Cardiac regeneration in anamniote vertebrates

Most of the work carried out on anamniote vertebrate regeneration focused on the zebrafish (*Danio rerio*). It was not until a reliable method to cause a local, reproducible cardiac injury was devised that research on teleostean regeneration really started (Belling et al., 2020; Poss et al., 2002). In any case, both tissue resection or cryoinjury affecting a significant part of the zebrafish ventricle results in

massive cardiomyocyte death followed by a reparative fibrosis/scarring response that is soon resolved in favour of re-differentiated cardiomyocytes that restore cardiac function and therefore completes regeneration (Belling et al., 2020).

Results from a great variety of experiments using the zebrafish as animal model were primarily accepted to be a general corpus of evidence for heart regeneration in all teleostean species. However, quite soon other studies using different fish models like medaka (*Oryzias latipes*) indicated that heart regeneration was not universal among bony fishes (Ito et al., 2014), and that even populations of the same species could behave in a completely different manner in response to damage (Stockdale et al., 2018). To explain the variable cardiac regenerative responses between teleosteans, it has been argued that unbalanced cardiomyocyte proliferation and scar formation, as well as a significantly different management of immune responses, underlie these different responses (Lai et al., 2019).

It is now accepted that the sustained postnatal proliferation of cardiomyocytes and the ability of some quiescent cardiac muscle cells to re-entry the cell cycle are key factors for proper zebrafish heart regeneration (Zhu et al., 2021; Zuppo & Tsang, 2020). Notwithstanding, many other works have also identified extracellular matrix remodelling as an equally critical event during the regenerative process (Sanz-Morejón & Mercader, 2020). Indeed, the balance between cardiomyocyte proliferation and reparative fibrosis seems to be the key driving force behind heart regeneration, and systemic aspects like metabolism regulation are not alien to this event (Honkoop et al., 2019).

Other anamniote vertebrates (mostly amphibians) have been shown to be able to, at least, partially regenerate their damaged hearts (Bettencourt-Dias et al., 2003), but discussions on the specific molecular and cellular mechanisms that guide such regeneration remain open (Brockes & Kumar, 2002; de Wit et al., 2020; Liao et al., 2018). It is also unclear how the cardiac regenerative properties of these animals relate to mammalian (and most specifically human) responses to injury. This is a relevant question for different reasons. First because the anamniote cardiac histoarchitecture is very different from the amniote vertebrate one (e.g. the heart is

mostly trabeculated). Second because the experimental protocols used to injure anamniote animal models do not accurately reproduce the pathophysiology (e.g. ischemia) of human cardiac diseases involving the massive loss of myocardium. Finally, it is difficult to explain how the regenerative properties of inner organs like the heart could have been conserved through evolution in the absence of the selective pressure provided by common pathologic stimuli cardiac disease. In this regard, recent studies suggest that environmental conditions could be relevant to understand the conservation of regenerative responses in the vertebrate heart (Sakaguchi et al., 2020).

I.4.3. Does cardiac regeneration in amniote vertebrates exist?

It has become a common place in the cardiovascular biomedical field to talk about 'cardiac regeneration' when discussing possible solutions to severe forms of human cardiac disease like myocardial infarction. Unfortunately, multiple studies have unambiguously shown that the adult mammalian heart, including that of humans, does not spontaneously regenerate after losing a significant mass of cardiomyocytes (Deshmukh et al., 2019). Therefore, after massive cardiomyocyte death, the remaining living cardiomyocytes become overworked and experience chronically elevated biomechanical stress that induces further cardiomyocyte death, which ultimately leads to pathological heart remodelling (primary a reparative fibrosis that transforms into a disabling scar), heart failure and death. So far, heart transplantation is the only effective treatment currently available for end-stage cardiac failure. According to the OMS, more than 7.4 million of people died in 2019 due to myocardial infarction, so the search for regenerative therapies to repair or regenerate the damaged heart is an urgent necessity.

I.4.4. Evidence supporting mammalian cardiac endogenous regenerative potential

Although achieving mammalian (and thus human) 'heart regeneration' is a dreamt goal rather than a reality, there is evidence enough to suggest that the

mammalian heart encloses certain regenerative potential that, for unknown reasons, is not harnessed into a robust regenerative response after damage (Palmquist-Gomes et al., 2019). These evidences are as follows:

1) *The mammalian postnatal heart is transiently able to regenerate*. The loss of massive proliferation capacity in cardiomyocytes occur soon after birth in rodents (Alvarez et al., 2019; Li et al., 1996; Walsh et al., 2010), or throughout the last weeks of gestation in humans (Huttenbach et al., 2001; Mayhew et al., 1997), concurring with the transition from a hyperplasic growth (growth by cardiomyocyte proliferation) to a hypertrophic growth in the cardiac muscle, exactly in that moment when cardiomyocytes stop increasing their number, but start to grow in size. This conversion reflects in the regenerative ability of the murine heart during foetal (Drenckhahn et al., 2008) and perinatal stages, compared to more postnatal and adult stages (Porrello et al., 2011, 2013). The real extent of this regenerative capacity remains controversial, and differences reported between different laboratories have been claimed to depend on technical discrepancies (Andersen et al., 2014; Mahmoud et al., 2015; Sen & Sadek, 2015). The maturation of early postnatal cardiomyocytes seems to occur rapidly. One of the most striking changes during cardiomyocyte maturation due to its implications in the adult organism, as mentioned above, is cardiomyocyte proliferation inhibition for polyploidization and maturational hypertrophy. In murine cardiomyocytes, 90% cardiomyocytes present 2 diploid nuclei due to karyokinesis without cytokinesis (Soonpaa et al., 1996; Walsh et al., 2010). whereas in adult humans 75% cardiomyocytes are mononuclear, but the majority of these nuclei are polyploid because of DNA endoreplication without karyokinesis (Mollova et al., 2013; Olivetti et al., 1996). In cardiomyocytes, DNA content and polyploidy seem substantially related to the regenerative capacity of the heart after an injury (González-Rosa et al., 2018; Patterson et al., 2017).

2) *The adult heart displays significant cardiomyocyte turnover*. It has been discovered that adult human cardiomyocytes emerge slowly but constantly during adulthood (Bergmann et al., 2009, 2015; Mollova et al., 2013), although there are discrepancies about the extent and function of this phenomenon. Mollova and

colleagues (Mollova et al., 2013), using stereological methods, observed that during the first year of life human cardiac muscle cells proliferate at a rate of 0.016% and that this rate decreases until the age of 20 eventually becoming undetectable. This proliferation contributes to heart growth increasing cardiomyocyte number in 3.4 fold. However, according to Bergmann and colleagues (Bergmann et al., 2015), who used a complex mathematical model based on ^{14}C incorporation to DNA, postnatal cardiomyocyte turnover is the highest (1-5%) during the first decade of life, declining to 0.8% per year during the second decade and to 0.3% per year at the age of 75. These authors also observed that the number of cardiomyocytes does not change during life, so these new cardiac muscle cells ought to substitute dead cardiomyocytes renewing the myocardium. It has been suggested that differences in cardiomyocyte labelling methods and strategies to select tissue samples might account for the discrepancies found between these two studies (Lázár et al., 2017).

Disagreement between experts persists regarding the origin of new cardiomyocytes in the adult heart. It is important to consider that cardiomyocyte cell cycle activity does not necessarily associate with proliferation, as it can also reflect pathological hypertrophy, polyploidization or polynucleation, and this is an aspect that can distort the interpretation of experimental results. Thus, classical markers of proliferation as nucleotide analogue incorporation (e.g. BrdU or EdU) or sarcomere disassembly/dedifferentiation cannot be used as markers for proliferation without combining this approach with other techniques like clonal analysis (Leone et al., 2015). The difficulty of this task is reflected in the controversies observed between different studies (Porrello et al., 2013; Senyo et al., 2013). For example, Senyo and colleagues combined two different pulse-chase approaches and genetic fate-mapping with stable isotope labelling (^{15}N-thymidine) and multi-isotope imaging mass spectrometry analyse cardiomyocyte proliferation. Ten weeks after ^{15}N-thymidine administration to 2 month-old mice, it was observed that 17% of the 4.4% of cardiomyocytes that had incorporated ^{15}N were diploid and mononucleated, and this finding was interpreted to be consistent with the generation of new cardiomyocytes. As these cells were, as pre-existing cardiomyocytes, also predominantly GFP^+ (83%),

it was strongly suggested that new cardiomyocytes derived from differentiated cardiomyocytes at a slow rate of 0.76% per year (Senyo et al., 2013). Despite the specific differences identified between scientific reports, all these results show that, in homeostatic conditions, formation of new cardiomyocytes from pre-existing cardiomyocytes occurs in a very low rate. This is in accordance with the reported technical difficulties in the identification of adult proliferative cardiomyocytes using powerful technologies like single-cell RNAseq transcriptomics (Kretzschmar et al., 2018; Li et al., 2019).

I.4.5. Identity of cardiac cells expressing stemness markers

As mentioned above, during the last twenty years several authors have reported on the existence of pluripotent CSC with myogenic potential in the adult heart (Beltrami et al., 2003; Marino et al., 2019). Many of these multipotent cells were first identified by their expression of diverse markers like c-Kit, Sca-1, SSEA-, Bmi-1 or by their characteristic extrusion of the Hoechst 33342 dye via the ATP-binding cassette transporter Abcg2 (side population cells).

1) ***c-Kit***. CD117 or c-Kit is a type III receptor tyrosine kinase normally activated by binding of its ligand, the stem cell factor (SCF), leading to a phosphorylation cascade that regulates apoptosis, cell differentiation, proliferation, chemotaxis and cell adhesion. c-Kit is expressed in mast cells, some hematopoietic stem cells, germ cells, melanocytes, Cajal cells of the gastrointestinal tract, epithelial cells, some cerebellar neurons and in diverse carcinomas (Miettinen & Lasota, 2005). During murine cardiac development, the number of c-Kit$^+$ in the heart has been reported to progressively increase from E14.5 until postnatal stages (Fransioli et al., 2008; Tallini et al., 2009). These cells were described to form part of the cardiac interstitium, where they organized into little clusters in association with diverse progenitor cells, endothelial cells, smooth muscle cells and less frequently, fibroblasts and cardiomyocytes as shown by immunostaining with connexin 43 and diverse cadherins (Bearzi et al., 2009; Beltrami et al., 2003). It was a major statement of these studies that cardiac c-Kit$^+$ cells did not express the CD45 pan-leukocyte

marker, ruling out the possibility of these cells being blood-borne cells of bone marrow origin (Beltrami et al., 2003; Ellison et al., 2011; Limana et al., 2005; Vicinanza et al., 2017).

2) ***Sca1***. Stem cell antigen-1 or Sca1 is a glycosyl phosphatidylinositol-anchored cell surface protein (GPI-APC) of the LY6 gene family, expressed by stem/progenitor cell from a variety of tissues such as hematopoietic tissue, mammary gland, skin muscle and testis (Holmes et al., 2007). $Sca1^+/CD45^-/CD31^-$ cells (10-33% of the non-myocyte compartment) are usually localized in the cardiac interstitium associated to the basal lamina of cardiomyocytes or in the adventitia of coronary vessels (Dey et al., 2013; Fioret et al., 2014; Rosenblatt-Velin et al., 2005; Uchida et al., 2013). Recent studies consider the cells identified with this phenotype as a heterogenous population, since diverse subpopulations with different protein profiles can be identified. Some of these cells express other markers associated with stemness, as c-Kit, CD34, ABCG2, SOX2 or PDGFRα (Noseda et al., 2015; Oh et al., 2003; Uchida et al., 2013), while others seem to be more related to committed lineages as cardiomyocytes ($GATA4^+$, MEF^+ or $TIF1^+$) (Oh et al., 2003; Wu et al., 2012) or endothelial cells ($Cdh5^+$, $VEGFR2^+$) (Noseda et al., 2015). A variable proportion $Sca1^+$ cells localized in the adult heart belong to the hematopoietic lineage, as they express the pan-leukocyte marker CD45, or to the endothelial lineage as they express CD31 (Matsuura et al., 2004; Oh et al., 2003; Rosenblatt-Velin et al., 2005; Valente et al., 2014). $Sca1^+$ cells robustly differentiate into endothelial cells *in vitro* after treatment with VEGF or PDGF BB ($CD31^+$, vWF^+, $Caveolin^+$), and formation of endothelial tubules can be observed if cells are culture in 3D matrices like Matrigel (Takamiya et al., 2011; Wang et al., 2006).

3) ***Abcg2*** **and** ***Mdr1*** (Hoeschst 33342 efflux). Side population (SP) cells, characterized by their ability to efflux Hoechst dye (Hoeschst 33342), have been isolated though flow cytometry analysis from multiple adult tissues including skeletal muscle, bone marrow, liver, lung, kidney, and brain. In these tissues they are considered to be progenitor cells due to their capability to adopt alternative phenotypes in permissive environments (Martin et al., 2004). The SP phenotype is

linked to the presence of two ATP-binding cassette (ABC) transporters, ABCG2 and MDR1, which seem to be regulated in an age-dependent manner in cardiac SP cells. During murine embryonic development, Abcg2 RNA is profusely expressed in cardiac tissues at E8.5, but this expression decreases at midgestation (E11.5-E13.5), being progressively restricted to a little cell population (2-3.5% of total cardiac cells) in postnatal stages (Martin et al., 2004; Oyama et al., 2007; Tomita et al., 2005). This number decreases with time until reaching 0.02-1.2% of nonmyocyte population cardiac cells only (Hierlihy et al., 2002; Liang et al., 2010; Oyama et al., 2007; Tomita et al., 2005; Yamahara et al., 2008; Yoon et al., 2007). Most cardiac $Sca1^+$ cells have been reported to locate close to coronary vessels (Yamahara et al., 2008). SP cells have frequently been classified in two groups. $SP/CD45^+$ cells are accepted to derive from blood lineages, whereas $SP/CD45^-$ cells are considered by many authors a subpopulation of cardiac $Sca1^+$ cells because they express Sca1 in a high proportion (43-92% of SP cells are $Sca1^+$) (Liang et al., 2011; Martin et al., 2004; Mouquet et al., 2005; Pfister et al., 2005; Tomita et al., 2005; Yamahara et al., 2008; Yoon et al., 2007). SP cells do not show expression of other stemness markers such us c-Kit or the hematopoietic CD34 (Liang et al., 2011; Martin et al., 2004; Mouquet et al., 2005; Pfister et al., 2005; Tomita et al., 2005; Yoon et al., 2007).

In addition to these three markers, other molecules like the transcription factors Isl1 or Bmi1 (see section I.3.1) have also been used to characterize populations of so-called cardiac stem cells. Interestingly enough, researchers have intensively searched to characterize the *in vitro* properties of these cells. In this regard, some of them were found to form cardiospheres, which were described as clonal-derived, non-adherent clusters of cells including cardiac stem cells and different cardiovascular cell types. Messina and colleagues were the first researchers to describe cardiospheres from mice and human samples, rounded cell clusters with clonogenic, self-renewal and differentiation capacity. According to their protocol, when cardiac tissue fragments are cultured as explants after a mild enzymatic digestion, a layer of fibroblast-like cell from well-adherent explants starts to emerge, after which some small, round, phase bright cells begin to migrate over it (Messina

et al., 2004). These forming cardiosphere forming cells do not seem to derive from dedifferentiated cardiomyocytes according to lineage tracing experiments using the MerCreMer technology or from hematopoietic cells, as they seem to be $CD45^-$ (Davis et al., 2009, 2010). Cardiospheres are enriched in the mesenchymal marker CD90 (Davis et al., 2009, 2010), contain cells expressing the stemness markers c-Kit or Abcg2, the endothelial markers CD31, CD34, CD133 and VEGFR2, and some markers for immature cardiomyocytes, although this latter result has been disputed (Eschenhagen et al., 2017; Menasché, 2018).

I.4.6 The myth of cardiac stem cells (CSC)

CSC were deemed by many scientists to be the main source of new cardiomyocytes in the adult heart and, as such, they were characterized, isolated, expanded and used in cell-based experimental therapies to treat the diseased heart (Gude & Sussman, 2018; Keith & Bolli, 2015). Nevertheless, controversy always existed on the properties of these CSC, most especially in the case of the cell pool characterized by the expression of c-Kit. These $c\text{-}Kit^+$ CSC were thought to be self-renewing, clonogenic, and multipotent, being able to give rise to a minimum of three different cardiogenic cell lineages (myocytes, smooth muscle cells, and endothelial cells) (Beltrami et al., 2003). Furthermore, $c\text{-}Kit^+$ CSC, when grown in suspension, were expected to form cardiospheres. The biological relevance of these cells in the homeostatic turnover of adult cardiac myocytes was first challenged and then refuted (Sultana et al., 2015; Van Berlo et al., 2014). The most striking news on these CSC followed, as prestigious journals identified high levels of fraud in the research of Dr. P. Anversa, who discovered and published most of the work related to $c\text{-}Kit^+$ CSC (Servick, 2014). This led to the withdrawal of more than 30 papers from this laboratory, and the scandal was publicized by prestigious non-scientific media (see, https://www.nytimes.com/2018/10/15/health/piero-anversa-fraud-retractions.html).

As it can be inferred from the previous discussion, the central question then is: Do CSC really exist? From the analysis of recently published data, it can be inferred that CSC properties, as described above for $c\text{-}Kit^+$ cells, are a myth.

However, the presence of cells expressing markers like Abcg2 (characteristic of the 'side population' stem cluster), c-Kit, Sca1, Isl1 or Bmi1 (Herrero et al., 2018; Laugwitz et al., 2005; Matsuura et al., 2004; Pfister et al., 2005; Wang et al., 2006), all of them associated to the CSC concept and known to be expressed by true stem cells in other tissues, remains undisputed. Then, a second crucial question must be issued: what are those cardiac cells expressing stem cell markers? Cardiac-isolated c-Kit^{+} cells are rare in the healthy heart, but far more frequent in the context of cardiac diseases involving an inflammatory response (Ruiz-Villalba et al., 2015). Since c-Kit is a well-established marker for hematopoietic stem cells and progenitors (Rojas-Sutterlin et al., 2014), c-Kit^{+} cardiac cells are also likely to be bone marrow derivatives. Cells expressing the transcription factor Isl1, are mostly found in the adult outflow tract, the atria and the right ventricle (Laugwitz et al., 2005), and seem to be a remnant of SHF embryonic progenitors (Cai et al., 2003) and not real stem cells. Sca1^{+} cells are also frequent in the heart, but their co-expression of molecules like Tie-2, Ang-1 and CD31 denotes a close relationship with endothelial and blood lineages rather than a multipotent profile. Cells with a 'side population' (SP) phenotype, characterized by the ability to efflux the Hoechst 33342 dye in an ATP-binding cassette (ABC) transporter dependent manner, have been described within the cardiac Sca1^{+} fraction (c-Kit^{low}, CD34^{low} and CD45^{low}). Finally, cardiac Bmi1^{+} cells, which were originally described as holding myogenic potential (Valiente-Alandi et al., 2015) have been recently shown to represent a population of endothelial progenitors (Herrero et al., 2018). All these data, taken together, suggest that the heart does not home true pluripotent cardiac stem cells, but could host some progenitor-like cells with a more restricted differentiation potential. This hypothesis, however, needs further evaluation.

HYPOTHESIS & OBJECTIVES

HYPOTHESIS

The general working hypothesis of this Ph.D. thesis is that the organization of the cardiac ventricular interstitium (including tissue histoarchitecture and tissue molecular crosstalk) is key to the homing of putative cardiovascular progenitor cells with restricted differentiation potential. These cells are responsive to pathologic stimuli and are involved in tissue repair after cardiac damage.

OBJECTIVES

1. To map the cardiac distribution and abundance of putative cardiovascular progenitor cells (c-Kit^{+}; Sca1^{+}) in the embryonic, postnatal and adult cardiac interstitium considering their possible ontogenetic relationship with the epicardial cell lineage.

2. To study neurovascular interactions in the cardiac interstitium with a special focus in the pericoronary environment.

3. To evaluate *in vitro* the impact of neurotrophic factor signalling in relevant cardiac interstitium cell types.

4. To test the *in vivo* response of cardiac interstitial cells in: a) an experimental model of cardiac damage; b) a genetic model of deficient neurotrophic signalling.

MATHERIAL & METHODS

"The most expensive part of building is the mistakes."

— **Ken Follett, The Pillars of the Earth**

II.1. 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 Málaga and BIONAND. All mouse lines were maintained and bred at the University of Málaga and BIONAND animal facilities. All embryos were staged considering the moment of vaginal plug observation, which was designated as E0.5.

II.2. Wilm´s Tumor 1 (Wt1)-Rosa26R (Wt1Cre-YFP) mice

Wt1/IRES/GFP-Cre (Wt1Cre, del Monte et al., 2011) and B6.129X1-Gt(ROSA)26Sor$^{tm1(EYFP)Cas}$/J (Rosa26R-eYFP, Srinivas et al., 2001) have been used in this thesis.

Succinctly, to develop Wt1/IRES/GFP-Cre, an IRES/GFP-Cre cassette was inserted 17 bp downstream of the translation stop site of the Wilms Tumour Gene 1 (Wt1) gene in the BAC clone RP23-266M16 of the mouse RPCI-23 (C57Bl/6J) BAC library. The resulting BAC clone was used to generate independent transgenic mouse lines expressing Cre in the epicardial lineage from the proepicardial stage. For B6.129X1-Gt(ROSA)26Sor$^{tm1(EYFO)Cas}$/J, a targeting vector was designed to contain the Enhanced Yellow Fluorescent Protein gene (from the pEYFP-N1 plasmid, Clonetech) downstream of a loxP-flanked stop sequence. This entire construct was inserted into the Gt(ROSA)26Sor locus via electroporation of 129X1/SvJ-derived JM-1 embryonic stem cells. Homozygote Wt1/IRES/GFP-Cre mice were crossed with homozygote B6.129X1-Gt(ROSA)26Sor$^{tm1(EYFO)Cos}$/J, and recombination activated permanent reporter enhanced Yellow Fluorescent Protein (eYFP^{+}) expression in the Wt1^{+} cell linage (Wt1Cre-YFP^{+}) in both embryos and adult mice (Figure 8).

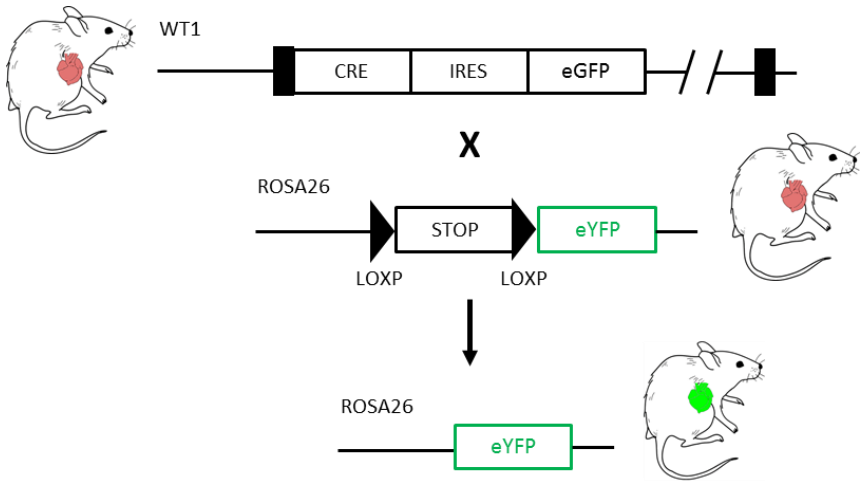

Figure 8. Generation of Wilm´s Tumor 1 (Wt1)-Rosa26R (Wt1Cre-YFP) mice.

II.3. NT3$^{LacZNeo/+}$

To obtain insights into the expression of neurotrophin-3 (NT3) in the mouse heart, the NT3$^{LacZNeo/+}$ mouse line was used. In these mice, the *Escherichia coli* lacZ gene was inserted into the neurotrophin-3 locus (NT3^{LacZNeo}, Fariñas et al., 1994), so that β-galactosidase production is under control of the NT3 promoter in the context of its normal chromosomal regulatory environment. In these animals, histochemical measurement of β-galactosidase provides a simple, sensitive method to determine which cells express NT3.

II.4. mRFP-Bone marrow transplantation

Hematopoietic stem cell transplantation in adult mice was used to study the role of blood-borne cells in the organization of the cardiac interstitium.

For mRFP^{+} bone marrow transplantation into adult recipients, 12- to 16-week-old C57Bl/6J mice (The Jackson Laboratory) were used. Receptor mice were X-irradiated at Dr. Jose Carlos Segovia´s laboratory (CIEMAT, Madrid) under a myeloablative regime. In detail, a Philips MG324 X-ray equipment (Philips, Hamburg, Germany) set at 300kV, 10mA, and delivering a total dose 10Gy, split into two doses,

4 hours apart, at a dose of 1.03 Gy/min, was used. Bone marrow cells were collected from pCX-mRFP1 mice (Zhu et al., 2005) by flushing tibias and femurs with phosphate-buffered saline (PBS), adjusted at 10^7 cells/mL and infused into anesthetized mice via retro-orbital injection with a 25-gauge needle containing PBS (Figure 9). One and three months after transplantation, the mRFP expression of the hematopoietic engraftment was analysed by flow cytometry from peripheral blood samples.

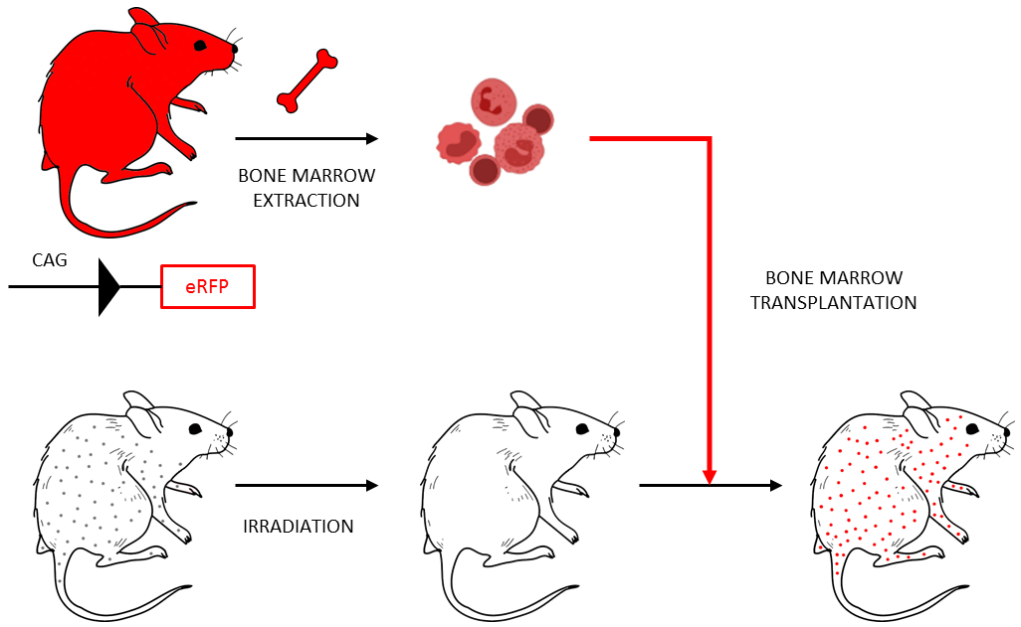

Figure 9. mRFP-Bone marrow transplantation scheme.

II.5. Model of chronic cardiac fibrosis induced by angiotensin-II

Cardiac fibrosis was induced in mice by infusing angiotensin-II (1.5 mg/kg/day during 14 days in 0.9%ClNa); and only 0.9%ClNa in controls using Alzet mini osmotic pumps (Model 2002, Durect Co.) according to the manufacturer´s protocol. In brief, after mice anaesthesia with sevoflurane a small area of the dorsal surface of the animal (back) was shaved, cleaned with 70% ethanol, and the osmotic pumps subcutaneously inserted using scalpel and forceps. Then, a thin suture was used to maintain the osmotic pump in position and close the incision.

In 2-month old Wt1Cre-YFP^{+} and NT3$^{LacZNeo/+}$ male and female mice (n=2), the hearts were perfused (PBS) and excised after 2 weeks of treatment, and then fixed for immunohistochemical analysis as described below.

In 4-month old NT3$^{LacZNeo/+}$ male mice (n=4), cardiac damage was confirmed by high-resolution magnetic resonance spectroscopy and imaging, using the Bruker BioSpec 9.4T animal MRI system. All image analysis was done using the freely available software Segment version 3.0 (http://segment.heiberg.se). 4 weeks after treatment onset, cardiac blood samples were obtained through cardiac puncture and hearts were then perfused (PBS), excised, and fixed for posterior immunohistochemical analysis.

II.6. Cardiac troponin analysis

To quantitatively detect cardiac troponin levels in blood samples, mouse cardiac troponin I (cTn-I) ELISA Kit (Cusabio, CSB-E08421) was employed, according to manufacturer indications. Data analysis was performed with the Curve Expert v1.4 software.

II.7. Histology and immunohistochemistry

To study aspects related to mouse cardiac embryogenesis, pregnant females were sacrificed using cervical dislocation, and embryos were extracted from the uterus and washed in PBS. For adult heart isolation, mice were sacrificed as described, submitted to thoracotomy, the hearts perfused with PBS using a 25G hypodermic needle, and rapidly perfused with freshly prepared 4% paraformaldehyde (PFA) in PBS. After excision, embryos and adult hearts completed a fixation cycle immersed in 4% PFA overnight (4ºC). After 3 washes in PBS (5 minutes each), the tissues were cryoprotected in 15% and 30% sucrose/PBS solutions, transferred to OCT, and frozen in liquid N_2-cooled 2-methylbutane. Ten-micron thick sections were obtained in a cryostat and collected on a-lysine-treated slides.

To initiate the immunohistochemical protocol, tissue cryosections were rehydrated in PBS and non-specific IgG binding sites were blocked with 10% horse serum, 1.5% BSA and 0.5% Triton X-100 in TPBS and incubated overnight (ON) in the proper primary antibodies (see Table 1) diluted in TPBS, at 4°C. After incubation in the primary antibodies, samples were washed in PBS (3x5 minutes), and incubated again at room temperature for 1 hour in the selected secondary antibody (see Table 2). Negative controls were performed incubating the samples with fluorochrome-conjugated secondary antibodies only (see Table 2). Cell nuclei were counterstained with 4´, 6-diamidino-2-phenylindole (DAPI) (Sigma).

Epitope	Host	Dilution	Reference	
αSMA	Rabbit	1:100	Abcam	Ab5694
αSMA	Mouse	1:100	Sigma	A2518
βIIITubulin	Mouse	1:100	Biolegend	801213
CD31	Rat (MEC13.3)	1:100	BDPharmingen	550274
CD45APC	Rat (30F11)	1:100	Miltenyi	130-097-962
Cytokeratin	Rabbit	1:100	Dako	Z0622
c-Kit	Goat	1:100	RyDSystem	Af1356
Fibronectin	Rabbit	1:100	Sigma	F3648
LectinTRITC	--------	1:100	Sigma	L5294
NT3	Rabbit	1:100	Santa Cruz	SC547
Procollagen	Goat (Y-18)	1:100	Santa Cruz	SC8787
Sca1Af647	Rat (E-13)	1:100	Biolegend	122518
Sca1Af647	Rat (D7)	1:100	Biolegend	108117
TrkB	Rabbit	1:100	Millipore	07-225
ZO1	Mouse	1:100	DSHB	R26.4C

Table 1. Primary antibodies used for tissue immunohistochemistry.

Epitope	Host	Dilution	Reference	
GoatFITC	Rabbit	1:400	Sigma	B7367
GoatTRITC	Donkey	1:400	Jackson	705-025-147
GoatCy5	Donkey	1:400	Jackson	705-175-147
MouseFITC	Goat	1:400	Sigma	F2012
MouseTRITC	Goat	1:400	Sigma	T5393
MouseAf647	Donkey	1:400	Jackson	715-605-151
RabbitFITC	Goat	1:400	Sigma	F9887
RabbitTRITC	Goat	1:400	Sigma	T6778
RabbitCy5	Donkey	1:400	Jackson	711-605-152
RatAf488	Donkey	1:400	Jackson	712-545-153
RatTRITC	Donkey	1:400	Jackson	712-025-153
RatCy5	Donkey	1:400	Jackson	712-605-150

Table 2. Secondary antibodies used for tissue immunohistochemistry.

All images were captured in a Leica SP5 laser confocal microscopy. In the case of Neurotrophin (NT3) immunostainings, antigen retrieval in citrate buffer (10mM citric acid, 0.05% Tween 20, pH 6) was performed before incubation with the primary antibody.

II.8. Whole Mount immunochemistry

For adult hearts, ventricular walls were isolated using surgical scissors and mounted after being slightly compressed between the bottom of a 4-well plate and a coverslip during fixation (4% PFA in PBS at 4ºC ON). This manipulation helps to flatten the ventricular surface, thus optimizing confocal microscope analysis of structures located at the heart surface. After several washes in PBS, non-specific IgG binding sites were blocked with SBT and samples were incubated ON for 3 days with CD31 and βIIITubulin primary antibodies (Table 1) in TPBS. Then, the samples were washed in TPBS and incubated again (2 hours-ON at 4˚C) in secondary antibodies (see Table 2). Negative controls were performed incubating with fluorochrome-conjugated secondary antibodies (see Table 2) without the primary antibody. Cell

nuclei were counterstained with DAPI (Sigma). All images were captured in a Leica SP5 laser confocal microscopy.

II.9. Cell proliferation assay

For cell proliferation analysis, EdU (5´-ethynyl-2´-deoxyuridine, Abcam Ab146186), a nucleoside analogue of thymidine that is incorporated into the cell nuclei during active DNA synthesis, was used. In short, adult mice were injected intraperitoneally with 50mg EdU/kg body weight (diluted in 0.9% ClNa) 1 hour before euthanasia. Rehydrated slides were submitted to click-iT reaction and washed in PBS (3x5 minutes) before DAPI co-staining.

EdU incorporation was recorded using a Leica SP5 laser confocal microscopy, and cardiac ventricular proliferating cells were counted using the IMARIS software. All nuclei ($DAPI^+$) and proliferating nuclei ($DAPI^+/EdU^+$) were considered to calculate the cell proliferation rate. Statistical significance was analysed by the Student´s t-test and shown as the mean plus standard deviation ($p<0.05$).

II.10. Flow cytometry (FACS)

Adult heart ventricles (n=3) were cut into small pieces using iridectomy scissors (<1mm). These tissue fragments were dissociated through sequential steps in 37°C pre-warmed Liberase TH (0.5mg/ml, Merck) with intense agitation; the supernatant was collected after every disaggregation step. The enzymatic reaction was blocked in DMEM+10%FBS and the supernatant was filtered through a 40µm filter (BD biosciences) to remove cardiomyocytes. Red blood cell lysis was performed using 8.3g/L ammonium chloride in 0.01M Tris-HCl buffer (pH7.2) followed by two washing steps with PBS for 5 minutes.

Cells were incubated in the proper primary antibodies (see Table 3) diluted in 2%FBS, 1%HEPES in PBS for 15 minutes, using isotype antibodies as negative controls. For the exclusion of non-viable cells in flow cytometry analysis, 7AAD (EBioscience) was employed.

Epitope	Host	Dilution	Reference	
CD11bAf700	Rat (M1/70)	1:250	Ebioscience	56-0112-80
CD31APC	Rat (MEC13.3)	1:250	Ebioscience	17-0311-82
CD34Af700	Rat (RAM34)	1:150	BDBioscience	560518
CD45PE	Rat (30F11)	1:250	EBioscience	12-0451-82
CD146PE	Rat (SA023G11)	1:150	Biolegend	150605
CCR2BV421	Rat (ME-9F1)	1:200	Biolegend	134703
c-KitAPC-780	Rat (2B8)	1:200	Ebioscience	47-1171-80
CXCR3PE-Cy7	Hamster (173)	1:500	Biolegend	126515
F4/80Af660	Rat (BM8)	1:200	Ebioscience	50-4801-80
Sca1SB436	Rat (D7)	1:150	Ebioscience	62-5981-80

Table 3. Primary antibodies used in FACS analysis.

Results were recorded in a *Gallios* flow cytometer (Beckman Coulter) and analysed using the *Kaluza* software (Figure 10A-E´). First, the population was selected according to Forward Scatter/Side Scatter (FS/SS) parameters, reducing the number of non-cell events and cell clusters (Figure 10A), then doublets were removed (Figure 10B), and alive cells were gated using 7AAD threshold (Figure 10C).

Finally, the remanent events were distributed in relation to their fluorescence and molecule expression (Figure 10D-E´). Statistical significance was analysed using the Student´s t-test and the results shown as mean plus standard deviation ($p<0.05$).

II.11. Cell culture. HUVECs

Human umbilical vein endothelial cells (HUVEC) were isolated from umbilical cords as previously described (Kubota et al., 1988), and grown on gelatin-coated dishes in EGM™-2 Endothelial Cell Growth Medium-2 BulletKit™ (Lonza) with 0.1% pen/strep in humidified, 5% CO_2 atmosphere. Passages three to seven were used

for experiments (cells were sub-cultured using trypsin-EDTA and seeded onto gelatin-coated flasks).

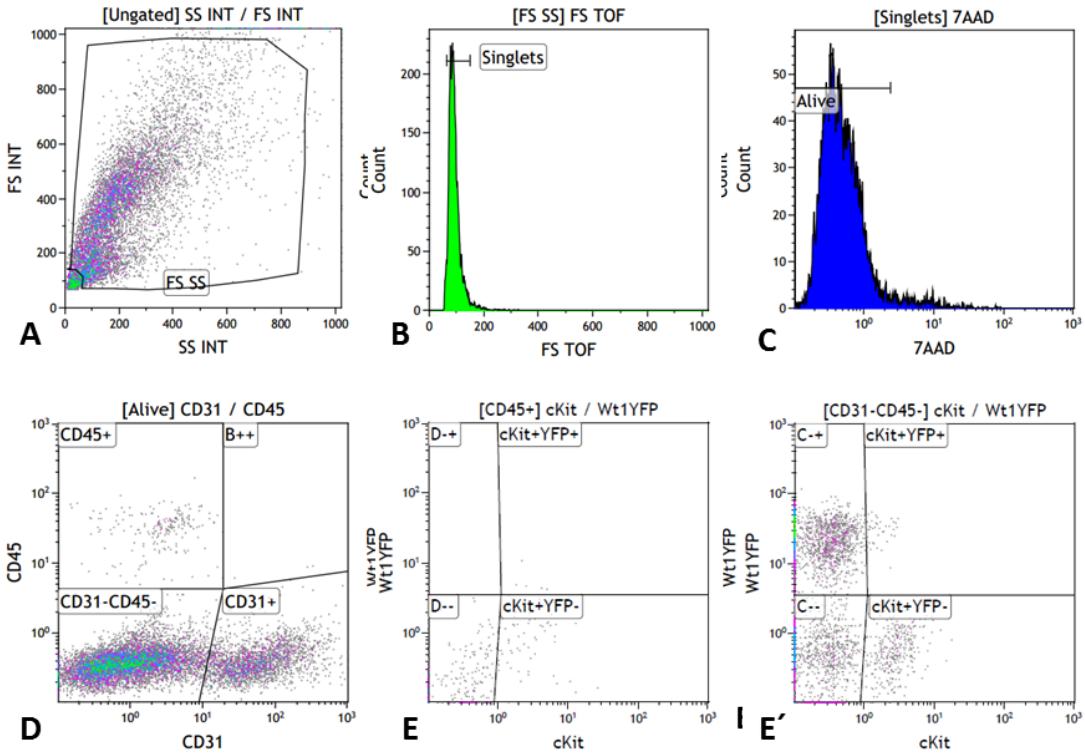

Figure 10. **FACS analysis and parameters studied**. **(A)** FS/SS distribution and gating of the events of interest. **(B)** In the previous selection, FS TOF was used to gate the singlets, that were afterwards analyzed **(C)** according to their 7AAD fluorescence. **(D-E´)** The selected cells were analyzed depending on their CD31/CD45 expression.

For cell treatment with neurotrophins and proliferation assay, HUVECs were seed in 96 well plates at 2500 cells/well in 50µl of EGM^{TM}-2, cultured for 24-36 hours and then put them in starving conditions with ECM ^{TM}-2 with 1% FBS. After 6h, neurotrophic factors Recombinant Murine β-NGF (Peprotech), Recombinant Human/Murine/Rat BDNF (Peprotech) and Recombinant Human NT-3 (Peprotech) were added at 1ng/ml, 10ng/ml and 100ng/ml final concentrations for 18h in culture conditions. EGM^{TM}-2 medium was used as positive control.

For cell proliferation analysis, EdU (5´-ethynyl-2´-deoxyuridine, Abcam Ab146186) was added at a 10µM final concentration and incubated for 10min. 2 washes in PBS were performed and cell were fixed in PFA4% for 15min. Cell plates

were submitted to the click-iT reaction and co-stained with DAPI. EdU incorporation was recorded using high content screening (Operetta, PerkinElmer). All nuclei ($DAPI^+$) and proliferating nuclei ($DAPI^+/EdU^+$) were considered to calculate the cell proliferation rate. Statistical significance was analysed by the Student´s t-test and shown as the mean plus standard deviation ($p<0.05$).

II.12. Cell culture. Epicardial Derived Interstitial Cells (EPDC)

E11.5 mouse (C57BL/6) embryonic hearts were dissected in EBSS (GIBCO) using forceps, iridectomy scissors and sharpened tungsten needles and let to attach to 0.1% gelatin coated-coverslips in high glucose DMEM supplemented with 10% FBS, 1% L-glutamine,100U/mL of penicillin and 100mg/mL streptomycin. Hearts were removed after an attachment period of 24 hours, leaving characteristic halo of epicardial cells attached to the substrate. These epicardial cells were cultured for an extra period of 48 hours before induce starving statement with reduced FBS (1%) ON.

Neurotrophic factors Recombinant Murine β-NGF (Peprotech), Recombinant Human/Murine/Rat BDNF (Peprotech) and Recombinant Human NT-3 (Peprotech) were added at 100ng/ml for 48h in culture conditions before Dent's fixation (methanol:DMSO, 4:1) and immunohistochemical characterization (Table 1-2).

II.13. Cell culture of cardiac interstitial progenitors

Isolated ventricular myocardial tissue from C57Bl/6J mice was minced into 0.5-1 mm^3 pieces, washed with Ca^{2+}-Mg^{2+}- free phosphate-buffered solution (PBS) (Invitrogen), and incubated three times (5 minutes each, 37°C) in 0.2% trypsin (Invitrogen) and 0.1% collagenase IV (Sigma). After digestion, cells from the supernatant were discarded, and the remaining tissue fragments were isolated, washed 3 times with complete explant medium (CEM) (Iscove´s Modified Dulbecco´s Medium/IMDM supplemented with 10% fetal calf serum, 100U/ml penicillin G, 100µg/ml streptomycin, 2mM L-glutamine and 0.1mM 2-mercaptoethanol). Finally,

these tissue fragments were cultured as explants over bovine fibronectin-coated multiwell plates (25µg/ml, Sigma) in CEM (37°C and 5% CO_2) for 3-4 weeks (CEM medium was changed every 2 days).

Once the incubation period was completed, a layer of fibroblast-like cells outgrows from the adhered explants. Over these cells, small cells identified easily identified under bright-field phase had grown. Sample collection was performed by pooling the cells from two washes with Ca^{2+}-Mg^{2+}- free PBS, one wash with 0.53mM EDTA and one additional wash with 0.5g/L trypsin and 0.53mM EDTA (2-3´) at room temperature.

The cells obtained were seeded at 0.5 to $2x10^5$ cell/ml in poly-D-lysine-coated multiwell plates in 35% complete IMDM/65% DMEM-Ham F-12 mix containing 2% B27, 0.1mM mercaptoethanol, 10ng/ml EGF, 20ng/ml bFGF, 40nM cardiotrophin-1, 40nM thrombin, antibiotics and L-Glu. Isolation of free-floating cell clusters was performed at least 4 times at 6- to 10-day intervals form the same explants. Floating cell clusters were observed after 24 hours (Messina et al., 2004), collected by mild rubbing and fixed in 4% PFA in PBS at 4ºC ON. Then, PFA excess was removed by 3 washes in PBS and immunohistochemical procedures were assessed as mentioned above.

RESULTS & DISCUSSION

"Philip had always believed that hard work should be rewarded by good food."

— Ken Follett, The Pillars of the Earth

III.1. MAPPING PUTATIVE CARDIAC PROGENITOR CELLS IN THE CARDIAC INTERSTITIUM

III.1.1. c-Kit positive cells in the developing heart

During the early phases of cardiac chamber development (E11.5), small, rounded c-Kit$^+$ cells were frequently observed in the forming ventricles and atria (Figure 11A-D). To delimitate the ventricular and atrial walls, the cardiac epithelia (the outer epicardium and the inner endocardium) were counterstained (Figure 11A-B). For the epicardium the expression of the YFP reporter in Wt1Cre-YFP mice was used (please, see Material & Methods for further details), whereas the endocardium was highlighted by immunofluorescence techniques, using antibodies against the endothelial marker CD31, also known as Platelet Endothelial Cell Adhesion Molecule (PECAM-1). Cardiac c-Kit$^+$ cells were always observed in the lumen of the cardiac chambers. No expression of c-Kit was observed in the forming cardiac ventricular or atrial walls. The absence of interstitial c-Kit$^+$ cells in the cardiac chamber walls at this stage may relate with the poor development of the cardiac interstitium in the E11.5 heart (Pogontke et al., 2019; Ruiz-Villalba et al., 2015). This interpretation is supported by other studies, that found c-Kit$^+$ in the heart from E14.5 onwards (Bearzi et al., 2009; Beltrami et al., 2003).

It is well-known that c-Kit is expressed in different cell types present in the blood, including very primitive blood progenitors, and is even considered a stem cell marker for the hematopoietic lineage (Escribano et al., 1998). The rounded cellular phenotype and luminal location of the E11.5 c-Kit$^+$ cardiac cells described above strongly suggest they are blood-borne cells. To examine this possibility, we used anti-CD45 (also known as protein tyrosin phosphatase receptor type C) antibodies to identify leukocytic cells, as CD45 is accepted to be a pan-leukocytic marker. We found that both c-Kit$^-$/CD45$^+$ (data not shown), c-Kit$^+$/CD45$^-$ and c-Kit$^+$/CD45$^+$ (Figure 11C-D) were present in the lumen of cardiac chambers. The presence of c-Kit$^+$/CD45$^+$ cells in the heart, confirm, at least partially, the blood lineage origin of these c-Kit expressing cells.

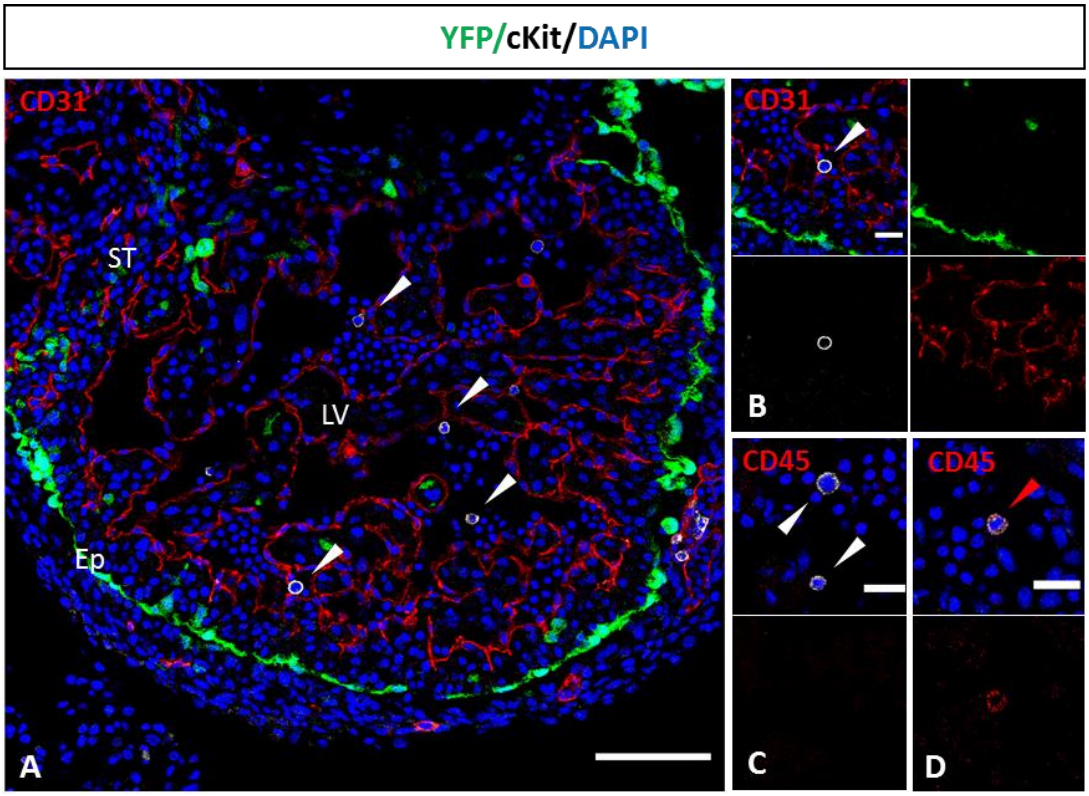

Figure 11. **Localization and characterization of c-Kit⁺ cells in E11.5 embryos.** Wt1YFP mouse hearts display permanent expression of the YFP reporter in Wt1⁺ lineage cells. **(A-B)** Small and rounded c-Kit⁺ cells **(arrowheads)** are located in the lumen of the forming cardiac chambers, lined by the CD31⁺ endocardium. **(C-D)** Some circulating c-Kit⁺ cells express the hematopoietic marker CD45 **(red arrowheads)**. Scale bars: **A**, 100 μm; **B-D**, 25 μm. Abbreviations; CD31/PECAM1 = platelet and endothelial cell adhesion molecule 1; CD45 = protein tyrosine phosphatase receptor type C; c-Kit = KIT proto-oncogene receptor tyrosine kinase; DAPI = 4',6-diamidino-2-fenilindol; Ep = epicardium; LV = left ventricle; ST= septum transversum; YFP = yellow fluorescent protein.

At these early stages CD45$^+$ circulating cells cannot derive from bone marrow, since this hematopoietic environment has not started its development at these anatomical locations in these stages. Therefore, these cells probably represent some immature blood cell linage from the yolk sac, the placenta, the aorta-gonad-mesonephros region (AGM region) or the foetal liver, as these are the main haematopoietic sources in the E11.5 mouse embryo (Dieterlen-Lièvre et al., 2010; Sampaio-Pinto et al., 2020).

Starting at E13.5 (Figure 12A-F), besides luminal c-Kit$^+$ cells (Figure 12B), low numbers of interstitial c-Kit$^+$ cells started to be recorded in the epicardium/subepicardium, which can be easily identified by their YFP expression (Figure 12C-F). The quick development of the EPDC population is known to be critical for the conditioning of the subepicardial and cardiac ventricular interstitium (Pogontke et al., 2019); and at this developmental stage, the rapid growth of the subepicardium and its colonization by EPDCs becomes evident (see Cano et al., 2016). This rapid expansion of the subepicardial space, affecting both to EPDCs and the extracellular space, may explain the increasing recruitment of c-Kit$^+$ cells to this microenvironment (Pogontke et al., 2019). Most E13.5 cardiac interstitial c-Kit$^+$ cells were located close to endothelial cells, suggesting that the coronary vasculature is one of the preferred pathways used for the migration of c-Kit$^+$ cells to the subepicardial space and the nascent cardiac interstitium. In this regard it is important to consider that the full perfusion of the embryonic coronary vascular system is known to occur between E13.5 and E14.5 (Théveniau-Rissy et al., 2016).

Occasionally, E13.5 cardiac c-Kit$^+$ cells also expressed the endothelial marker CD31, and these c-Kit$^+$/CD31$^+$ cells were often located next to other c-Kit$^-$/CD31$^+$ cells, forming small subepicardial clusters including 2-4 cells (Figure 12D). Co-expression of c-Kit and CD31 has been previously reported for some endothelial cell progenitors, both *in vitro* (Broudy et al., 1994; Fang et al., 2012; Matsui et al., 2004) and *in vivo* (Liu et al., 2015; Ren et al., 2019), and we therefore would like to suggest that our cardiac c-Kit$^+$/CD31$^+$ cells may belong to this cell population. Alternatively, taking into account that c-Kit and low expression levels of CD31 are characteristic of some

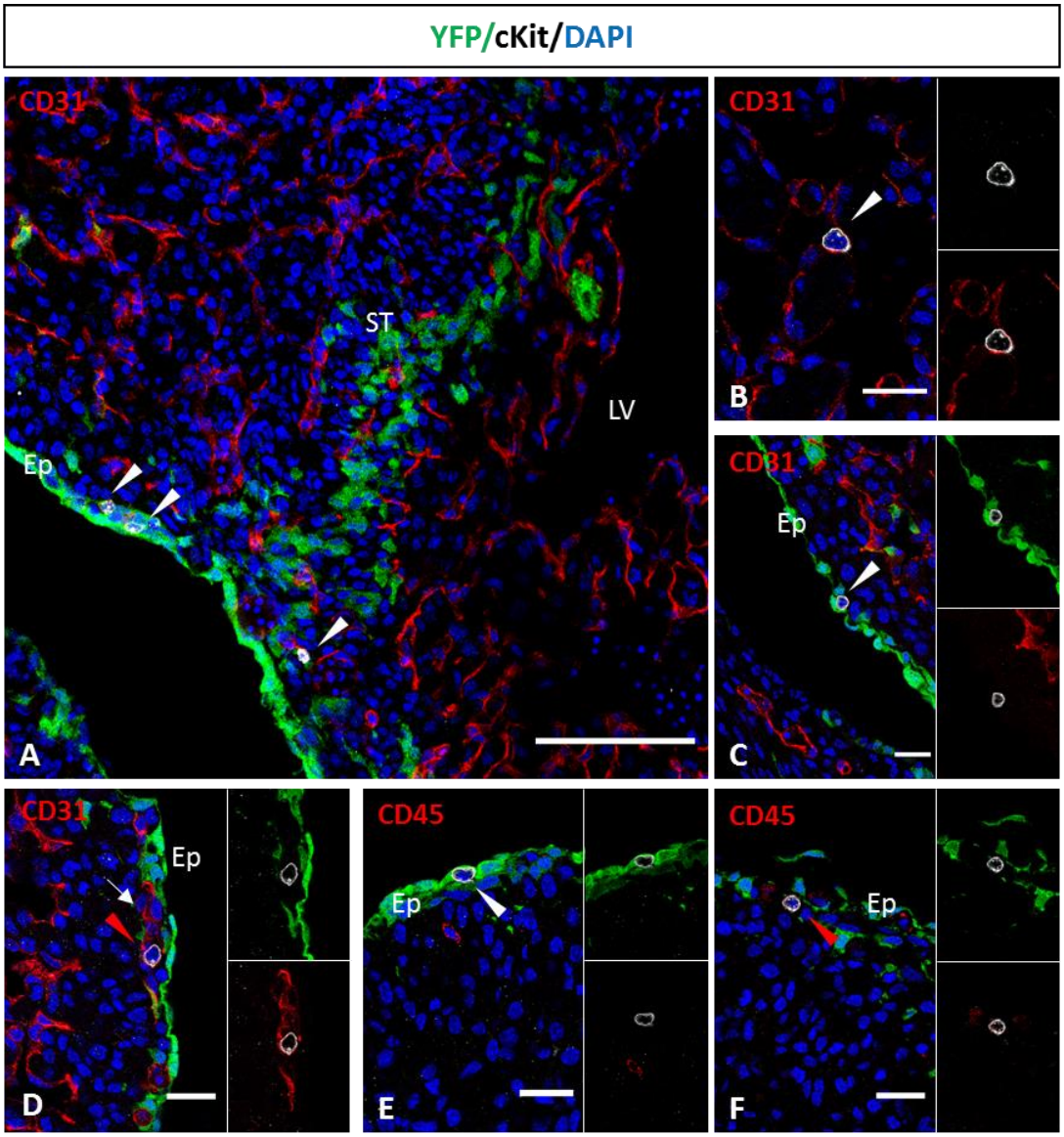

Figure 12. **Localization and characterization of c-Kit⁺ cells in E13.5 mouse embryos.** Wt1Cre-YFP mice hearts display permanent expression of the YFP reporter in Wt1⁺ lineage. **(A, B)** Small and rounded c-Kit⁺ cells **(arrowheads)** are located in the lumen of the forming cardiac chambers **(A, C-F)**, but also in the epicardium/subepicardium close to YFP⁺ cells and to c-Kit-/CD31⁺ cells **(D, arrows)**. Some of these latter cells are $CD31^{low}$ **(D, red arrowheads)** and/or CD45⁺ **(E-F, arrowheads)**. Scale bars: **A**, 100 μm; **B-F**, 25 μm. Abbreviations: CD31/PECAM1 = platelet and endothelial cell adhesion molecule 1; CD45 = protein tyrosine phosphatase receptor type C; c-Kit = KIT proto-oncogene receptor tyrosine kinase; DAPI = 4',6-diamidino-2-fenilindol; Ep = epicardium; LV = left ventricle; ST = septum transversum; YFP = yellow fluorescent protein.

hematopoietic progenitor cells (Baumann et al., 2004; Boisset et al., 2010), it is also possible that the cells we have described represent a definite fraction of immature hematopoietic progenitors.

As reported for circulating c-Kit$^+$ cells in the lumen of cardiac chambers, interstitial c-Kit$^+$ cells can co-express the hematopoietic marker CD45 (Figure 12E-F), a finding that relates these cells with blood-related cell lineages. The identification of c-Kit$^+$/CD45$^-$ cells is, however, more complex. In the heart, c-Kit$^+$/CD45$^-$ cells have been for long regarded to be multipotent cardiac stem cells (CSC) (Beltrami et al., 2003). As emphasized in the introduction to this thesis, the existence of cardiac resident multipotent stem cells has been discredited for different reasons (Servick, 2014). Therefore, uncertainty on the real nature of these cells remains, since the presence in heart tissues of cells expressing the so-called "stem marker" c-Kit has never been disputed. Some authors had previously identified c-Kit$^+$/CD45$^-$ cells as endothelial progenitors (Sandstedt et al., 2010); the presence of these cells in the subepicardium, which is a vascularized cardiac domain at these embryonic stages (E13.5) (Cano et al., 2016), may partially explain the presence of these cells in the embryonic heart. This is a plausible option, but it is also necessary to consider that CD45 expression has been found to be highly dynamic (Pogontke et al., 2019), and therefore the possibility of CD45$^+$ blood cells progressively loosing CD45 expression when recruited to certain environments cannot be ruled out.

Once the primitive coronary plexus has connected to the aortic root, and the muscularization of the main coronary arterial trunks is evident (Pérez-Pomares et al., 2016b), c-Kit$^+$ cells started to be observed inside small vessels and capillaries of the forming coronary plexus. At E15.5 (Figure 13A-E), CD31 endothelial counterstain allows for the identification of circulating versus extravasated c-Kit$^+$ cells (Figure 13B). At these stages, extravascular subepicardial c-Kit$^+$ cells located in the subepicardium progressively enlarge and flatten, changing their phenotype from a rounded, non-adherent one to a characteristic fusiform shape. This transformation suggests the activation of the mechanisms that allow some blood-circulating cells to invade solid tissues under specific circumstances. No c-Kit$^+$/CD31$^+$ cells were

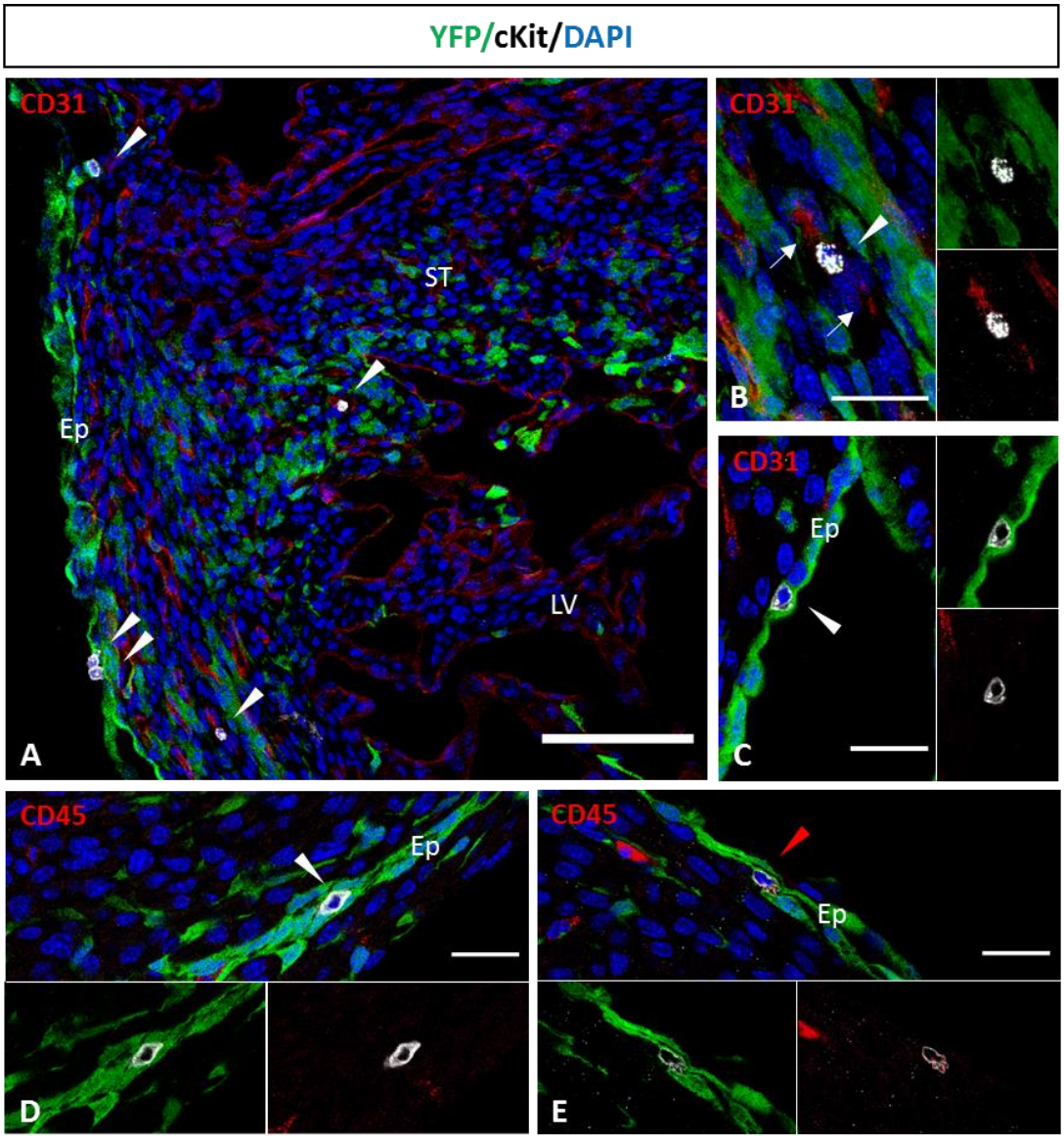

Figure 13. **Localization and characterization of cardiac c-Kit⁺ cells in E15.5 mouse embryos.** Wt1Cre-YFP mice hearts display permanent expression of the YFP reporter in Wt1⁺ lineage cells. **(A, B)** c-Kit⁺ cells **(arrowheads)** are located inside small coronary vessels, lined by CD31⁺ coronary endothelium **(B, arrows)**, as well as in the epicardium/subepicardium **(C)**, where they associate with YFP⁺ epicardial cells **(D and E).** Some c-Kit⁺ cells co-localize the pan-leukocytic marker CD45 **(red arrowheads)**. Scale bars: **A**, 200 μm; **B-E**, 25 μm. Abbreviations: CD31/PECAM1 = platelet and endothelial cell adhesion molecule 1; CD45 = protein tyrosine phosphatase receptor type C; c-Kit = KIT proto-oncogene receptor tyrosine kinase; DAPI = 4',6-diamidino-2-fenilindol; Ep = epicardium; LV = left ventricle; ST = septum transversum; YFP = yellow fluorescent protein.

observed at this stage (Figure 13C), but it is unclear whether this relates to the complex dynamics of coronary embryonic vascular development with proliferation and posterior differentiation of multipotent endothelial cells into mature endothelial cells (Cano et al., 2016; Palmquist-Gomes et al., 2018).

As found at earlier developmental stages, CD45 expression was either absent (Figure 13D) or present (Figure 13E) in the c-Kit$^+$ cell fraction.

To refine the quantification and characterization of cardiac c-Kit$^+$ cells, FACS analysis was performed on E15.5 cells from the ventricular walls, including embryonic cardiomyocytes (Figure 14A-E). This analytical approach revealed that, c-Kit$^+$/YFP$^-$ cells represent 11.57±5.74% of the total CD31$^+$ endothelial population (Figure 14B). This finding is in accordance with previous observations indicating that c-Kit$^+$ cells also expressing CD31 are frequent in the hearts of E13.5 embryos, often clustered with c-Kit$^-$/CD31$^+$ cells. In contrast, a portion of the CD31$^+$ cell population (0.64±0.52%) was identified to be c-Kit$^+$/YFP$^+$, thus suggesting the epicardial (Wt1$^+$) origin of some cardiac c-Kit$^+$ cells. We believe that this result should be taken with care, as it might represent a residual, non-specific binding of anti-c-Kit antibodies to YFP$^+$ cells due to the low percentage observed. As far as the CD45$^+$ population is concerned, a high percentage of them (65.22±12.87%) express c-Kit, something expected in a context of high hematopoietic proliferation and colonization of diverse tissues by the hematopoietic cells, especially macrophages, as show the expression of the well-known macrophage molecule F4/80 by some of these cells (Figure 14D). In any case, it is important to indicate here that some authors have regarded this cell fraction (c-Kit$^+$/CD45$^+$) as not relevant in the context of the analysis of cardiac progenitors (Ellison et al., 2011; Limana et al., 2005), a criterion that might result from the biased search for non-blood-related c-Kit$^+$ cells in the heart.

FACS screening of the CD31$^-$/CD45$^-$ fraction also revealed the presence of c-Kit$^+$/YFP$^-$ cells (8.30±5.12%) and c-Kit$^+$/YFP$^+$ cells (8.32±2.05%) (Figure 14C). Both populations contain cells expressing the stem/mesenchymal markers CD34 and Sca1, supporting the possibility that this fraction contains multipotent cells (Sydney et al., 2014). The c-Kit$^+$/YFP$^-$ fraction seems to be enriched in F4/80 and CXCR3

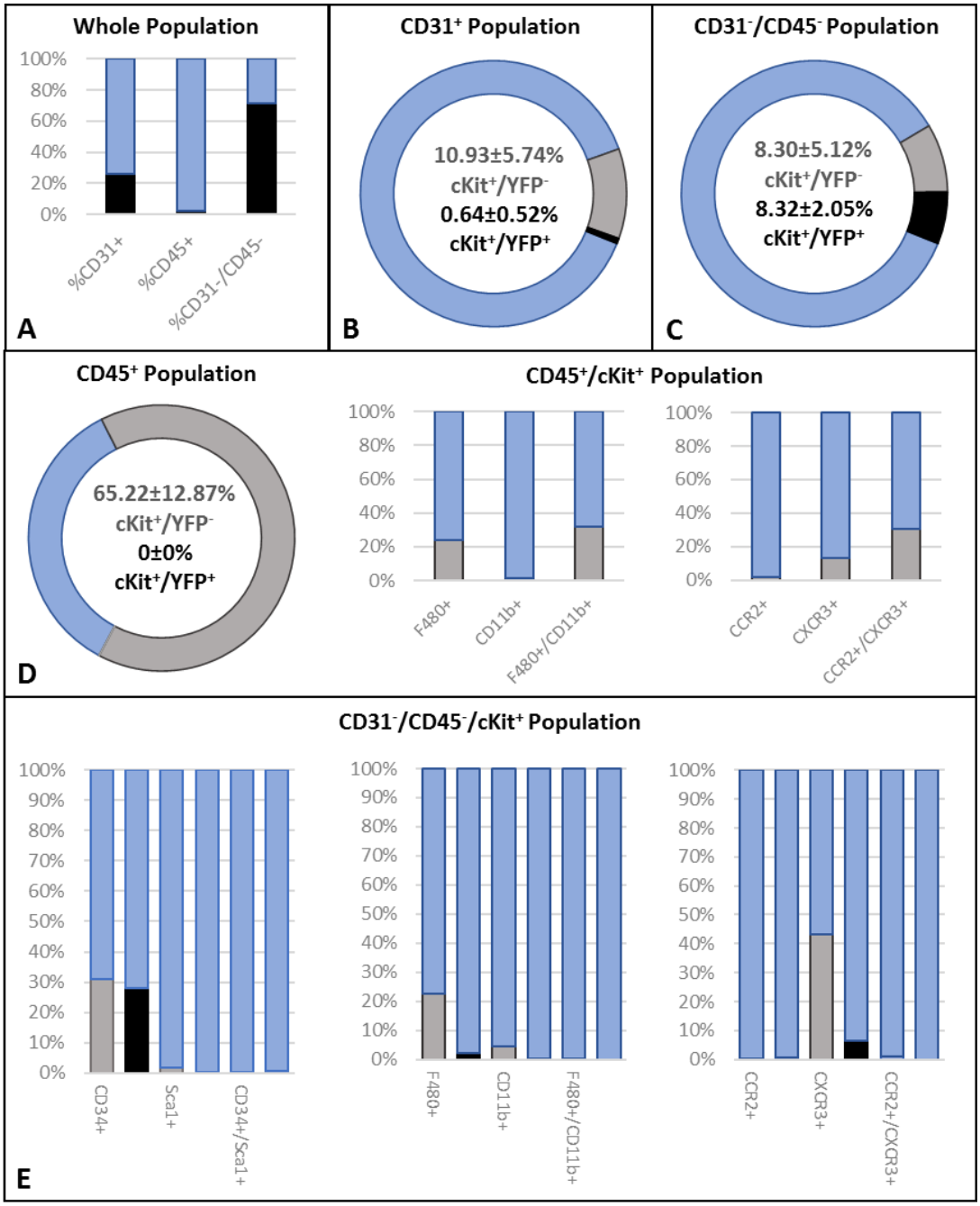

Figure 14. **FACS analysis of c-Kit$^+$ cells at E15.5 in Wt1YFP mice with permanent YFP expression in the Wt1$^+$ cell lineage**. **(A)** CD31$^+$, CD45$^+$ and CD31$^-$/CD45$^-$ cardiac cell pools are represented in relation to the whole cardiac cell population. c-Kit$^+$/YFP$^-$ (grey) and c-Kit$^+$/YFP$^+$ (black) cell percentages are shown in CD31$^+$ **(B)**, CD45$^+$ **(D)** and CD31$^-$/CD45$^-$ **(C)** cardiac cell fractions. Characterization of CD45$^+$/c-Kit$^+$ **(D)** and CD31$^-$/CD45$^-$/c-Kit$^+$ **(E)** cells is also represented (*p<0.05). Abbreviations: CCR2 = C-C motif chemokine receptor 2; CD11b = Integrin alpha M; CD31/PECAM1 = platelet and endothelial cell adhesion molecule 1; CD34 = cluster of differentiation 34; CD45 = protein tyrosine phosphatase receptor type C; c-Kit = KIT proto-oncogene receptor tyrosine kinase; CXCR3 = C-X-C motif chemokine receptor 3; F4/80 = EGF-like module-containing mucin-like hormone receptor-like 1; Sca1 = stem cell antigen-1; YFP = yellow fluorescent protein.

expression (Figure 14E), although we found no significant differences between these two populations, most probably due to the high individual (intraspecific) variability of our samples ($P<0.05$).

We consider CXCR3 expression in these cells of especial interest. CXCR3 is known to be tightly associated to lymphocytic phenotypes (Altara et al., 2016) and we therefore assume that many of these c-Kit$^+$/YFP$^-$ cells belong to the lymphocytic (CXCR3$^+$) or monocytic/macrophagic (F4/80$^+$) blood lineages. Nevertheless, the specific origin of these c-Kit$^+$/YFP$^-$ cells remain unknown. They could belong to the early hematopoietic lineage, since CD34 expression, frequent in these cells, is also characteristic of short term hematopoietic stem cells present in the adult heart (Ruiz-Villalba 2015) and has been demonstrated that Wt1 is also expressed in restricted blood cell populations (Cunningham et al., 2013), but further characterization would be necessary to prove this point in cardiac cells.

The histological distribution of c-Kit$^+$ cells in the cardiac walls remains unchanged at prenatal stages (E18.5). FACS analysis show that in the CD31$^-$/CD45$^-$ fraction the proportion of c-Kit$^+$/YFP$^-$ remains stable (10.97±2.05%), while the percentage of c-Kit$^+$/YFP$^+$ is significantly decreased (1.70±0.55%). In both populations, the expression of CD34 shows an increasing trend, although non significantly ($p<0.05$) (Supplementary Figure 1). The biological reason for this increment remains unknown.

III.1.2. c-Kit-positive cells in the postnatal and adult heart

After birth (postnatal day 1, P1; Figure 15A-D), the number of cardiac c-Kit$^+$ cell was found to have increased after a detailed immunohistochemical inspection of cardiac tissues, especially in the subepicardium (Figure 15B-C, white arrows). For the first time, c-Kit$^+$ cells were found infiltrated in the ventricular interstitium, close to coronary vessels (Figure 15A) or in the subepicardial space (Figure 15B). The extravascular location of these cells was confirmed by CD31 counterstaining.

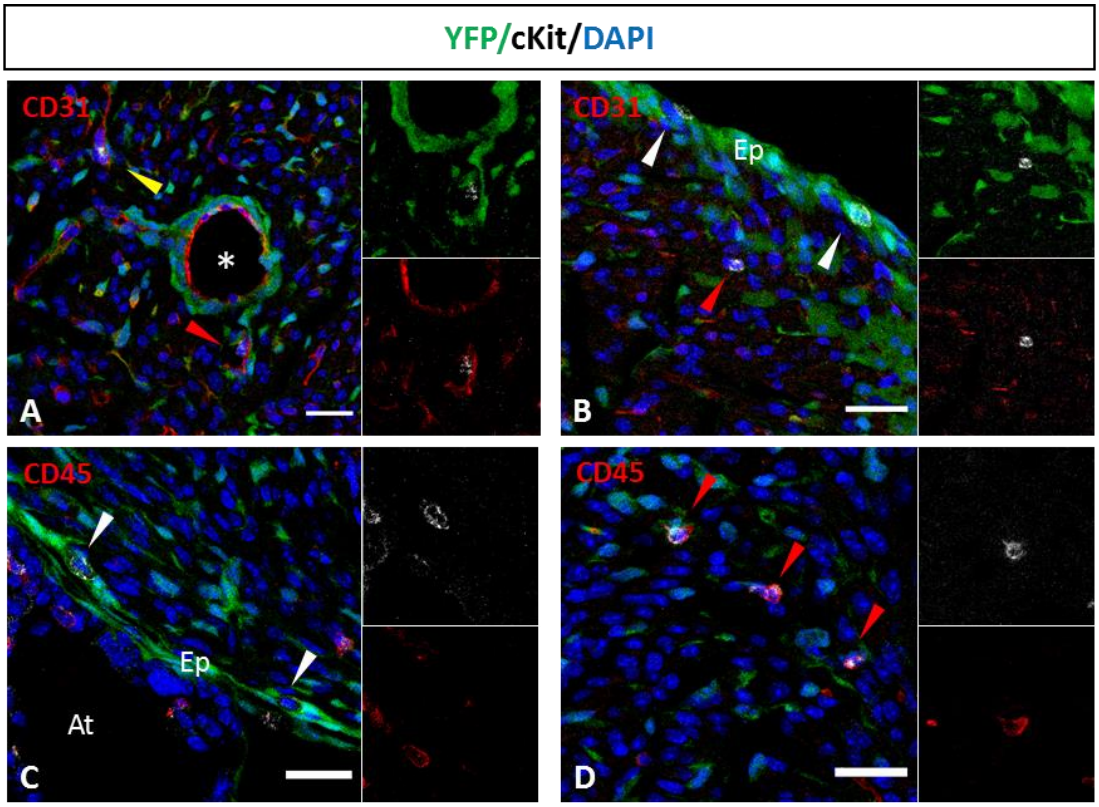

Figure 15. **Localization and characterization of cardiac c-Kit⁺ cells at P1.** Wt1Cre-YFP mouse hearts show permanent expression of YFP in Wt1⁺ lineage cells. c-Kit⁺ cells **(arrowheads)** are located inside small coronary vessels **(A, yellow arrowhead)**, lined by CD31⁺ coronary endothelium, in the epicardium/subepicardium in close association to Wt1-YFP⁺ epicardial cells **(B, white arrowheads)** and in the cardiac interstitium **(A, B, red arrowheads)**. Some c-Kit⁺ cells co-localize the hematopoietic marker CD45 **(C, D, red arrowheads)**. Scale bars: **A-D,** 25 µm. Abbreviations: Asterisk (*) = coronary vessel; At = Atrium; CD31/PECAM1 = platelet and endothelial cell adhesion molecule 1; CD45 = protein tyrosine phosphatase receptor type C; c-Kit = KIT proto-oncogene receptor tyrosine kinase; DAPI = 4',6-diamidino-2-fenilindol; Ep = epicardium; YFP = yellow fluorescent protein.

In accordance with the results previously described for the developing heart, low numbers of c-Kit$^+$/CD45$^+$ cells were also identified in the subepicardial space or the postnatal ventricular interstitium (Figure 15C-D). This result strongly argues in favour of a hematopoietic origin for these cells and infiltration of them through the endothelial layer into the cardiac interstitium.

FACS analysis of cardiac c-Kit$^+$ cells at P1 (Figure 16A-E) revealed a significant decrease of c-Kit$^+$ cells in the endothelial CD31$^+$ fraction as compared to prenatal stages (2.05±0.75%) (Figure 16B). Besides the increase of the CD31$^+$ fraction in the whole heart, the analysis reveals a reduction in the endothelial progenitor pool due to their differentiation into mature endothelial cells. The leukocytic CD45$^+$ fraction also shows a reduced amount of c-Kit expression (Figure 16D); cells in this fraction were characterized by their expression of monocyte/lymphocyte/macrophage markers as CD11b, F4/80 or CCR2. This increase in c-Kit$^+$/CCR2$^+$ cells in the CD45$^+$ fraction is likely to be related to the progressive postnatal increase in cardiac tissues of macrophages of circulating origin (CCR2$^+$) as compared to primitive embryonic ones, known to be CCR2$^-$ (Bajpai et al., 2018).

Opposite to what we observed in our immunohistological analysis of cardiac tissues, the proportion of c-Kit$^+$/YFP$^-$ (15.88±5.78%) or that of c-Kit$^+$/YFP$^+$ (3.52±1.05%) cells does not significantly increase in the postnatal heart (Figure 16B); as observed in E18.5 tissues, most of these cells express CD34 and a high proportion are CXCR3$^+$ also. At P7, the characteristics of the c-Kit$^+$ cardiac cell population regarding CCXR3, CCR2 and CD34 expression were similar to the ones described for P1 c-Kit$^+$ cells. The total number of c-Kit$^+$ cells in the ventricular cell extracts, however, was clearly reduced as compared to data from P1 (Supplementary Figure 2), perhaps reflecting the dilution of c-Kit$^+$ cell abundance with respect to the massively expanding endothelial and mural interstitial cell populations.

In the adult heart (2-3 months-old tissue samples), c-Kit$^+$ cells were analysed according to their localization in the heart (ventricular apex, mid ventricle, and atrioventricular region; Figure 17A). No significant differences between the cardiac

domains analysed was observed. Regarding the vascular wall, important numbers of c-Kit$^+$ cells were found close to epicardial/subepicardial cells (Figure 17B, B') or the ventricular interstitium (Figure 17C, C'), either isolated or in contact with the walls of coronary vessels. CD31, αSMA and CD45 antibodies were used to counterstain the vascular endothelium, the vascular smooth muscle, and leukocytes, respectively (Figure 17D-G).

In order to confirm the hematopoietic origin of cardiac c-Kit$^+$ cells in a CD45-expression independent manner, adult bone marrow transplants were performed using pCX-mRFP1 mice as donors. This manipulation renders all bone marrow derived cells permanently mRFP$^+$. Immunohistochemical inspection of cardiac tissues after this bone marrow transplantation procedure revealed that no c-Kit$^+$/mRFP$^+$ cells were identified by immunohistochemistry present in the adult myocardium (data not shown). This is a surprising result, since an important part of the c-Kit$^+$ cells present in the cardiac interstitium are CD45$^+$ and are therefore must very likely to derive from the bone marrow. The most plausible explanation for this phenomenon is that the new transplanted cells are not able to incorporate into the ventricle walls for unknown reasons; it is possible that the native cells were not depleted during the irradiation process (their proliferation rate is low), so that these cells would continue occupying the interstitial space, preventing the immigration of mRFP$^+$ cells to this environment. It is also possible that the transplanted cells lack the necessary mechanisms to incorporate into the cardiac interstitium.

The adult cardiac c-Kit$^+$ cell pool (Figure 18A-E) was also profiled using FACS. The number of c-Kit$^+$ cells in the endothelial fraction remained low (1.53±2.17%), although they are still present in the adult heart (Figure 18B). In contrast, blood-borne cells were significantly reduced (1.60±1.31%), and many of such cells express markers such as F4/80, CD11b, CCR2 and CXCR3, a finding that suggests they are progressing in their differentiation towards a mature phenotype (Figure 18D).

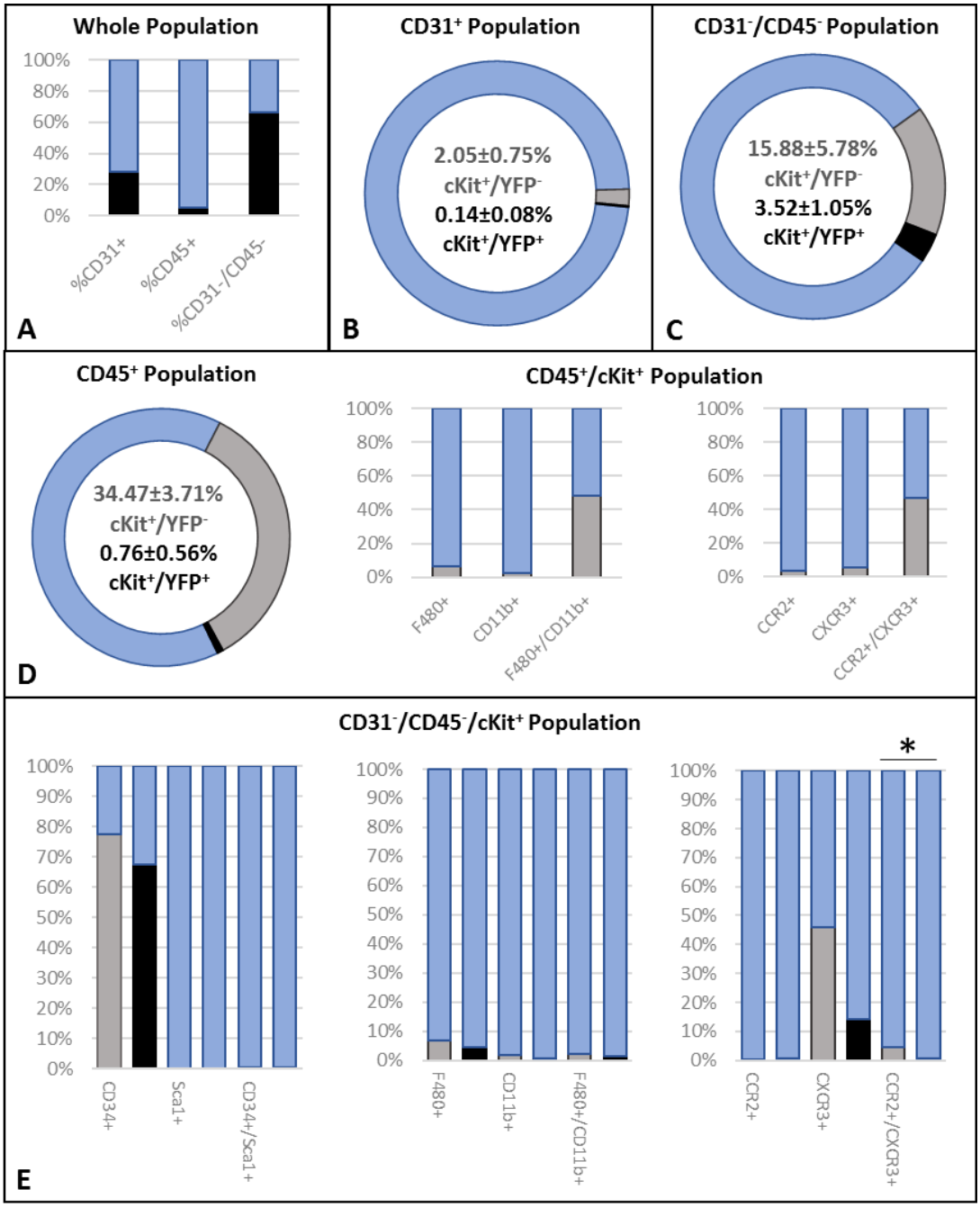

Figure 16. **FACS analysis of cardiac c-Kit$^+$ cells at P1 in Wt1YFP mice**. **(A)** CD31$^+$, CD45$^+$ and CD31$^-$/CD45$^-$ representation with respect to the whole cell population, with c-Kit$^+$/YFP$^-$ (grey) and c-Kit$^+$/YFP$^+$ (black) percentage shown for CD31$^+$ **(B)**, CD31$^-$/CD45$^-$ **(C),** and CD45$^+$ cell fractions **(D)**. Characterization of CD45$^+$/c-Kit$^+$ **(D)** and CD31$^-$/CD45$^-$/c-Kit$^+$ **(E)** cells (*p<0.05). Abbreviations: CCR2 = C-C motif chemokine receptor 2; CD11b = Integrin alpha M; CD31/PECAM1 = platelet and endothelial cell adhesion molecule 1; CD34 = cluster of differentiation 34; CD45 = protein tyrosine phosphatase receptor type C; c-Kit = KIT proto-oncogene receptor tyrosine kinase; CXCR3 = C-X-C motif chemokine receptor 3; F4/80 = EGF-like module-containing mucin-like hormone receptor-like 1; Sca1 = stem cell antigen-1; YFP = yellow fluorescent protein.

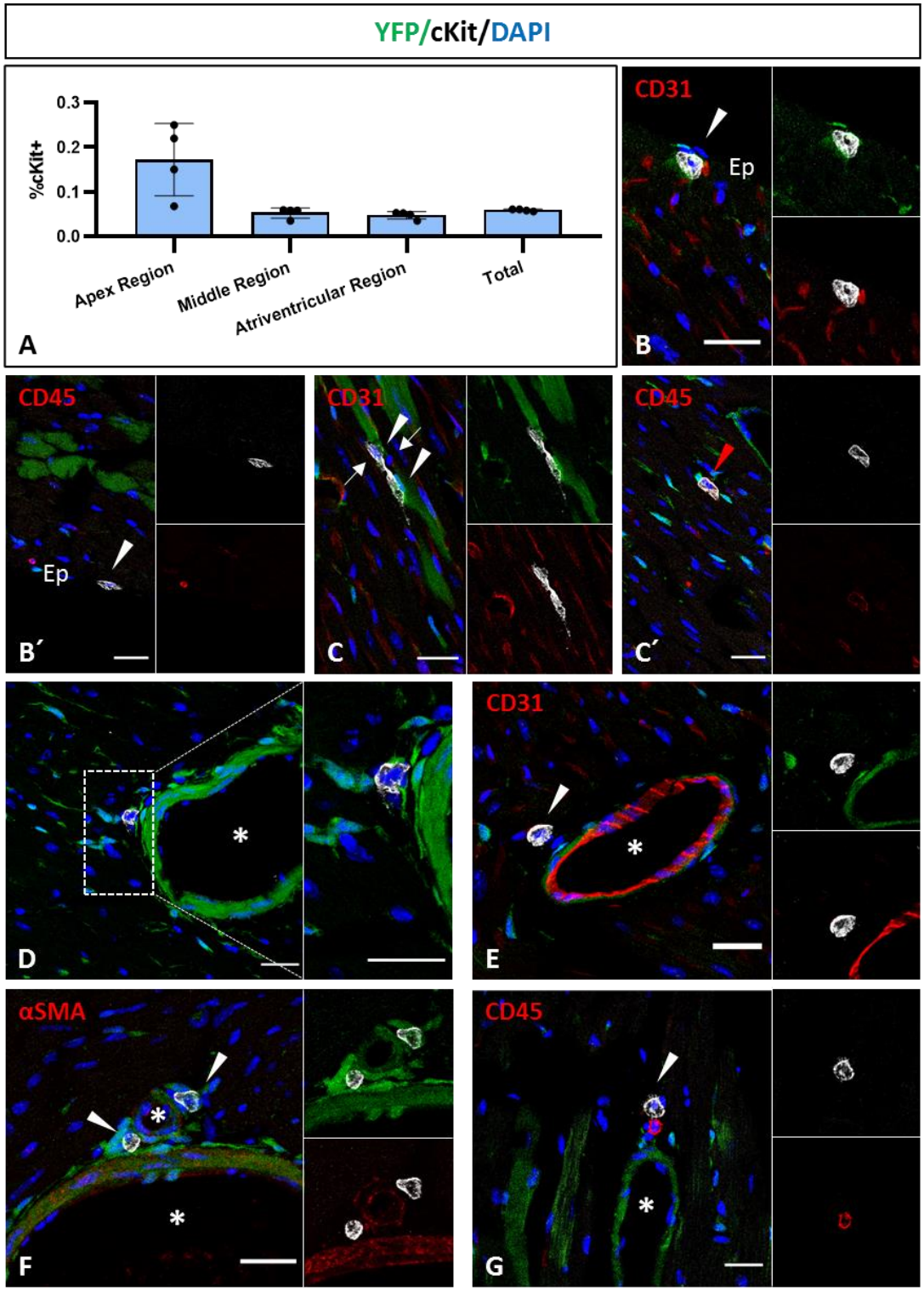

Figure 17. **Localization and characterization of cardiac c-Kit$^+$ cells in the adult heart.** Wt1YFP adult mouse hearts displayed permanent expression of the YFP reporter in Wt1$^+$ lineage cells. A significant number of c-Kit$^+$ cells are identified in different regions of the heart (**A**). **(B-G)** c-Kit$^+$ cells **(arrowheads)** are located in the epicardium/subepicardium, either associated with Wt1YFP$^+$ epicardial cells **(B and B´)** and in the cardiac interstitium **(C-G)**. In the cardiac interstitium are close to small capillaries CD31$^+$ **(C-C´, arrows)**, or next to **(D-G) (E)** CD31$^-$ **(F)** αSMA$^-$ adventitia of coronary vessels **(asterisk)**. **(B´, C´ and G)** Regardless their localization, some c-Kit$^+$ cells colocalize the hematopoietic marker CD45 **(red arrowheads).** Scale bars: **B-G,** 25 μm. Abbreviations: Asterisk (*) = coronary vessel; αSMA = smooth muscle actin; CD31/PECAM1 = platelet and endothelial cell adhesion molecule 1; CD45 = protein tyrosine phosphatase receptor type C; c-Kit = KIT proto-oncogene receptor tyrosine kinase; DAPI = 4',6-diamidino-2-fenilindol; Ep = epicardium; YFP = yellow fluorescent protein.

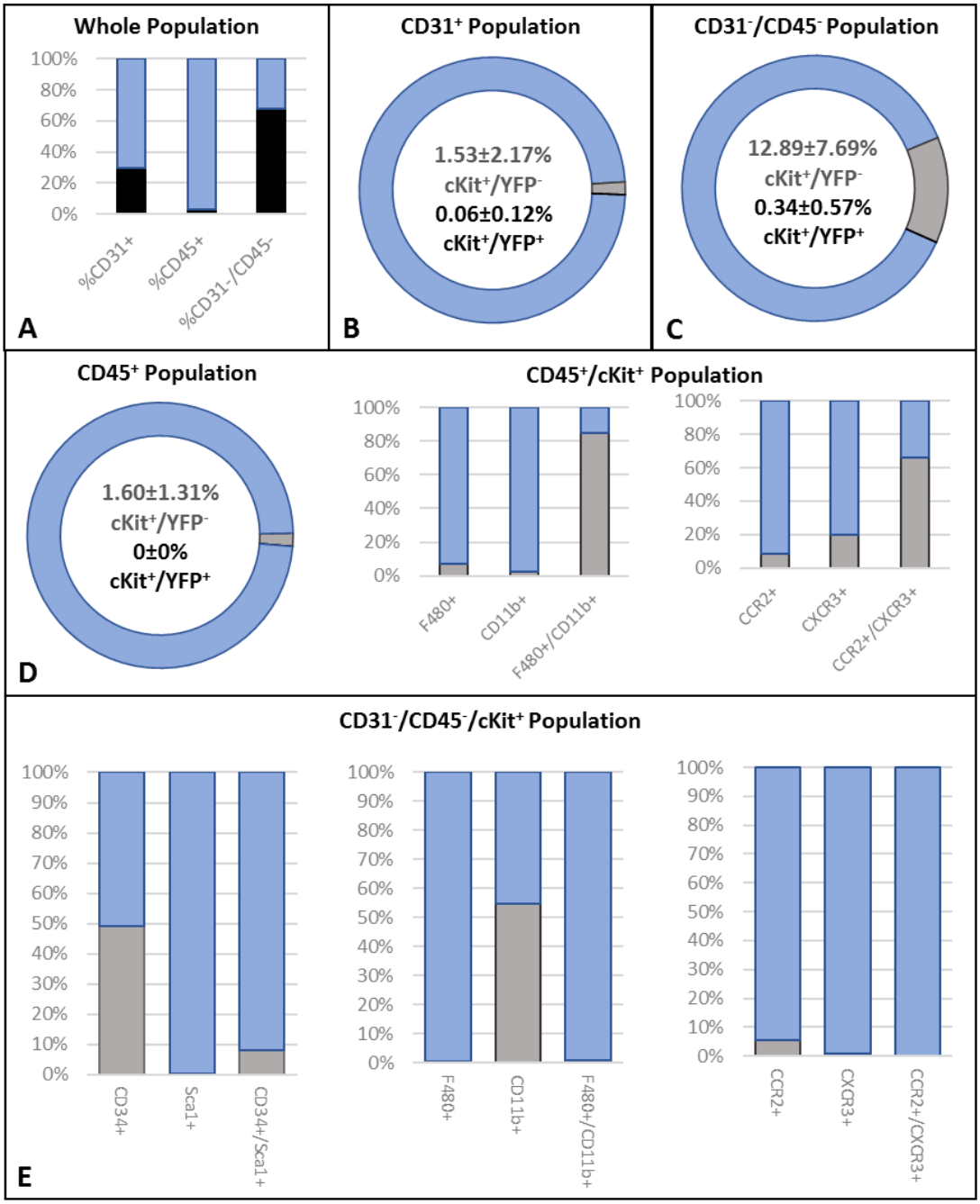

Figure 18. **FACS analysis of adult cardiac c-Kit$^+$ cells in Wt1YFP mice**. **(A)** CD31$^+$, CD45$^+$ and CD31$^-$/CD45$^-$ percentage representation with respect to the whole cell population. c-Kit$^+$/YFP$^-$ cells (grey) and c-Kit$^+$/YFP$^+$ cells (black) are shown in relation to the CD31$^+$ **(B)**, the CD45$^+$ **(D)** and **the** CD31$^-$/CD45$^-$ **(C)** fractions. **(E)** Characterization of CD45$^+$/c-Kit$^+$ **(D)** and CD31$^-$/CD45$^-$/c-Kit$^+$ cells. No significant differences have been observed between male and female samples (*p<0.05). Abbreviations: CCR2 = C-C motif chemokine receptor 2; CD11b = Integrin alpha M; CD31/PECAM1 = platelet and endothelial cell adhesion molecule 1; CD34 = cluster of differentiation 34; CD45 = protein tyrosine phosphatase receptor type C; c-Kit = KIT proto-oncogene receptor tyrosine kinase; CXCR3 = C-X-C motif chemokine receptor 3; F4/80 = EGF-like module-containing mucin-like hormone receptor-like 1; Sca1 = stem cell antigen-1; YFP = yellow fluorescent protein.

Regarding the adult $CD31^-/CD45^-$ cell fraction (Figure 18C), the number of c-Kit^+/YFP^- cells remained stable. However, it should be noted that this analysis did not include cardiomyocytes; therefore, it is likely that the real number of this cells is actually lower, as supported by the immunohistological quantification previously shown (Figure 17A). Significant changes were observed in this population as compared with late embryonic (E18.5) and early postnatal (P1) ones (Figure 18D). In particular, the expression of the CD11b marker, which is preferentially expressed by monocytes, is remarkably increased. This finding suggests a significant recruitment of monocytes to the cardiac ventricular walls after the first week of postnatal life. On a final note, it is noteworthy the absence of c-Kit^+/YFP^+ cells in the adult heart, especially since it is not possible to know their fate with the performed analysis. They could have experimented apoptosis, incorporated into the circulatory system or most probably, lost their c-Kit expression due to dynamic cellular mechanisms. What is clear is that these cells cannot be implicated in the maintenance of the adult cardiac interstitium.

All these results are summarized in Figure 19, with the proportion of c-Kit^+/YFP^- and c-Kit^+/YFP^+ in the $CD31^-/CD45^-$ fraction represented through development and adulthood (Figure 19A) and the characterization of these cells in relation with diverse molecule expression at different stages (Figure 19B) (statistical significance is indicated, $p<0.05$).

III.1.3. Sca1-positive cells in the developing heart

The first cardiac $Sca1^+$ cells were identified in the embryonic heart tissues at E15.5. These cells were found to be isolated in the cardiac ventricular interstitium, displaying a spindle-shaped phenotype (Figure 20A). The number of $Sca1^+$ cardiac interstitial cells increased between E17.5 and E18.5; most of these cells were found to be closely associated to epicardial lineage YFP^+ interstitial and coronary cells. The co-localization of the YFP reporter with the Sca1 marker was not evident in this cell population (Figure 20B-C). Taken together, these results indicate that at these stages cardiac $Sca1^+$ cells are not related to the $Wt1^+$ lineage.

FACS analysis of E15.5 heart cellular extracts showed that, at this stage, the percentage of $Sca1^+$ cells were very low, confirming what was observed in our immunohistochemical analysis. In detail, $Sca1^+$ cells suppose only 0.84±0.57%, 2.60±1.68% and 0.06±0.05% of the $CD31^+$, $CD45^+$ and $CD31^-/CD45^-$ populations, respectively (Supplementary Figure 3).

Remarkably, the presence of $Sca1^+$ cells in the heart increases significantly at E18.5 (Figure 21A-D) ($p<0.05$), especially in the $CD31^+$ fraction, with 30.48±2.33% of $Sca1^+/YFP^-$ and 3.54±0.60% $Sca1^+/YFP^+$ cells (Figure 21B). The presence of $Sca1^+/YFP^+$ cells oppose to what was observed by immunohistology, where no co-staining of Sca1 and YFP was noticed, possibly due to the lower specifity of this technique. Since the expression of Sca1 has been related to certain endothelial populations in the heart (Vagnozzi et al., 2018), we interpret that these cells belong to the endothelial lineage. Nevertheless, it should not be ignored that Sca1 and CD31 are also expressed in determined subpopulations of the blood lineage (Baumann et al., 2004; Boisset et al., 2010). Accordingly, some of these cells could be identified as blood progenitors, although further research is needed to confirm the role of these cells in the cardiac interstitium. Finally, Sca1 expression also increased in the blood population, identified by the pan-leukocyte marker CD45 (13.25±1.34%) (Figure 21C), although this specific cell fraction was not considered for further analysis.

When the $CD31^-/CD45^-$ fraction was measured (Figure 21D), only 1.74±0.15% of the cells were found to be $Sca1^+$. This is a low number of cells, especially if we consider the predominance of this marker in the other cardiac populations considered. The $Sca1^+/YFP^+$ cells, although reflected in our analysis, were very scarce, and therefore their actual existence as an independent cardiac cell population should be carefully considered, as these cells it might represent a residual, non-specific binding of anti-c-Kit antibodies to YFP^+ cells. Regarding $Sca1^+/YFP^-$ cells, many of them expressed CD34, c-Kit and/or CXCR3. We believe these cells represent hematopoietic progenitors (Holmes et al., 2007). Their blood-related nature must be evaluated independently from their CD45 pan-leukocyte marker expression

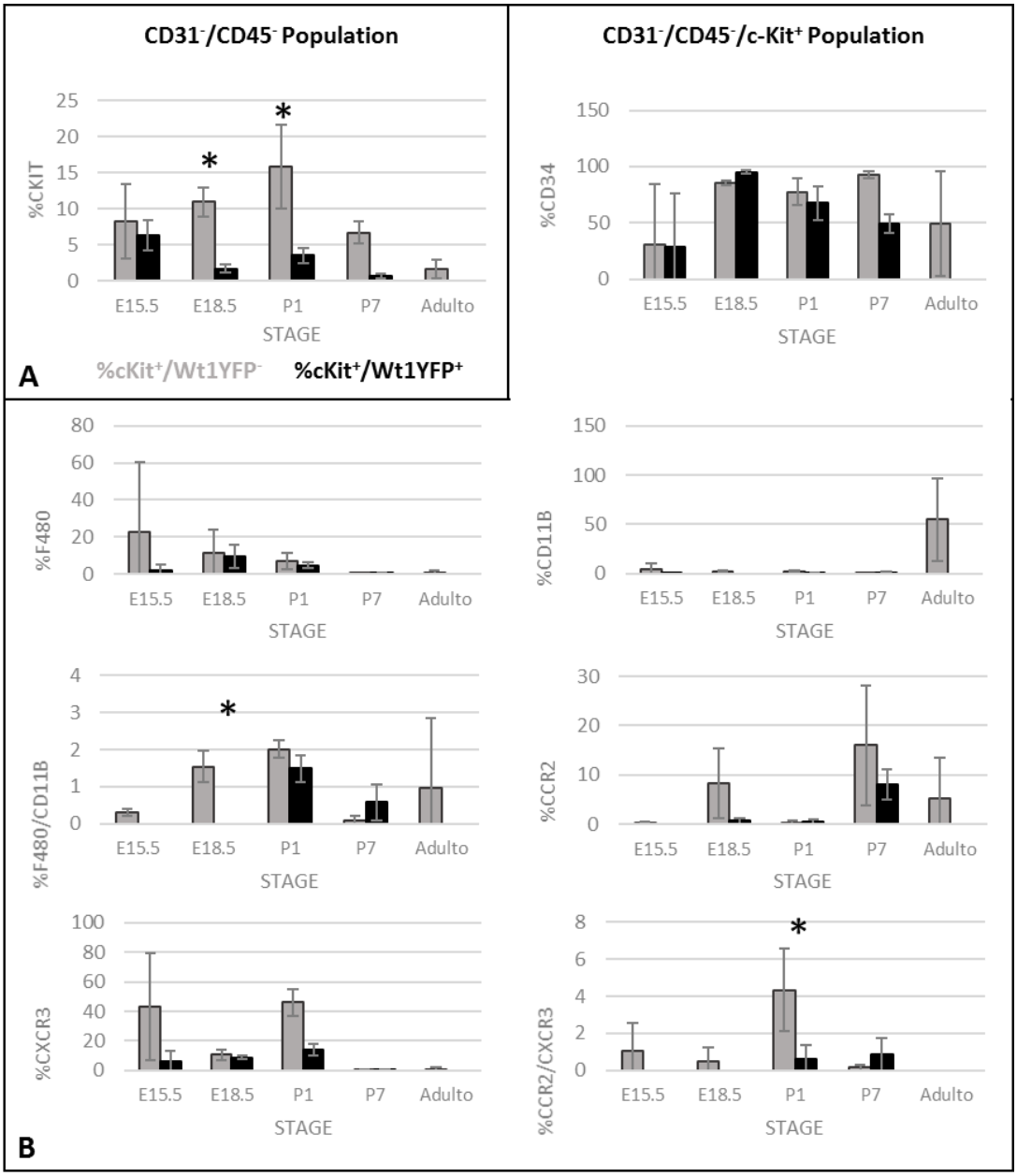

Figure 19. FACS characterization of cardiac c-Kit$^+$ cells (E15.5, E18.5, P1, P7 and adult) in Wt1YFP mice. (A) Proportion of c-Kit$^+$/YFP$^-$ (grey) and c-Kit$^+$/YFP$^+$ (black) cells in the CD31$^-$/CD45$^-$ cardiac population. **(B)** Characterization of c-Kit$^+$/YFP$^-$ and c-Kit$^+$/YFP$^+$ cells according to their expression of selected molecules (*$p < 0.05$). CCR2 = C-C motif chemokine receptor 2; CD31/PECAM1 = platelet and endothelial cell adhesion molecule 1; CD34 = cluster of differentiation 34; CD45 = protein tyrosine phosphatase receptor type C; c-Kit = KIT proto-oncogene receptor tyrosine kinase; CXCR3 = C-X-C motif chemokine receptor 3; F4/80 = EGF-like module-containing mucin-like hormone receptor-like 1; YFP = yellow fluorescent protein.

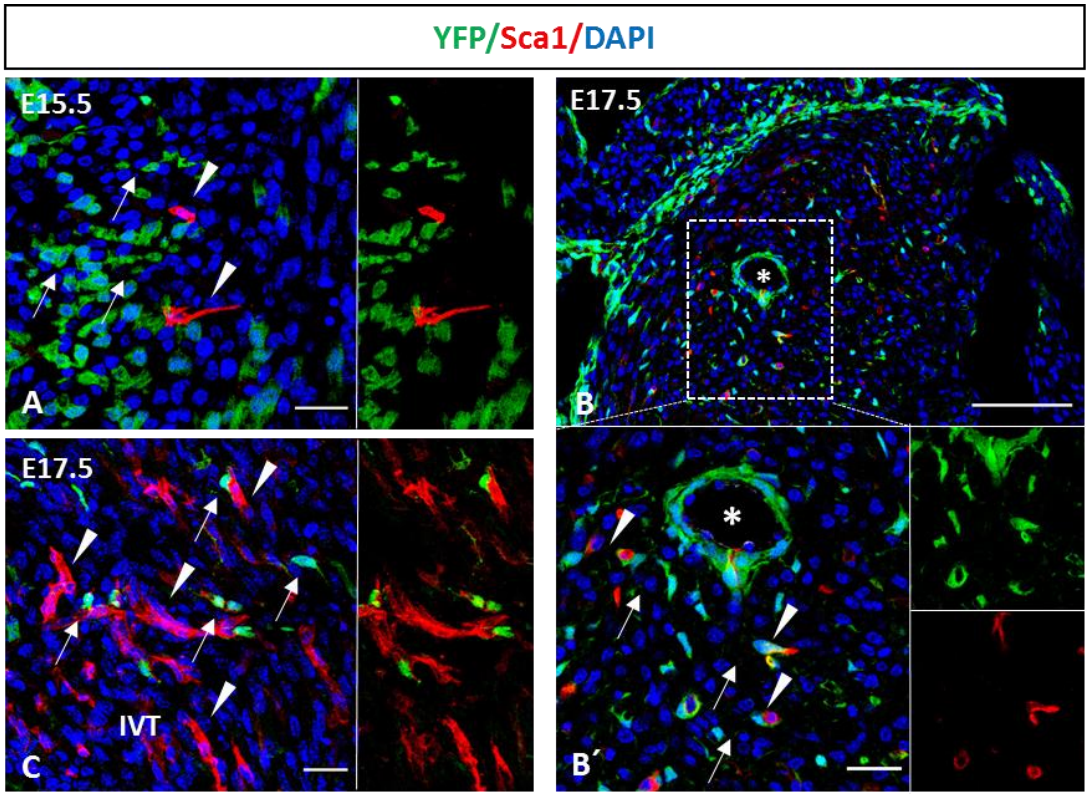

Figure 20. **Localization and characterization of E15.5 and 17.5 cardiac Sca1⁺ cells.** Wt1YFP mouse heart tissues show permanent expression of the YFP reporter in Wt1⁺ lineage cells. **(A)** Sca1⁺ cells are observed at E15.5 in the developing myocardium **(arrowheads)**; these cells do not show co-expression of YFP **(arrows)**. **(B-C)** At E17.5, Sca1⁺ cells locate in the cardiac interstitium **(B-B´)** of the cardiac chamber walls including the interventricular septum **(C)**. These cells often adopt a tubular-like structure in close association with YFP⁺ cells. Scale bars: **A, B´, C**, 25 µm; **B**, 100 µm. Abbreviations: Asterisk (*) = coronary vessel; = DAPI = 4',6-diamidino-2-fenilindol; IVS= interventricular septum; Sca1 = stem cell antigen-1; YFP = yellow fluorescent protein.

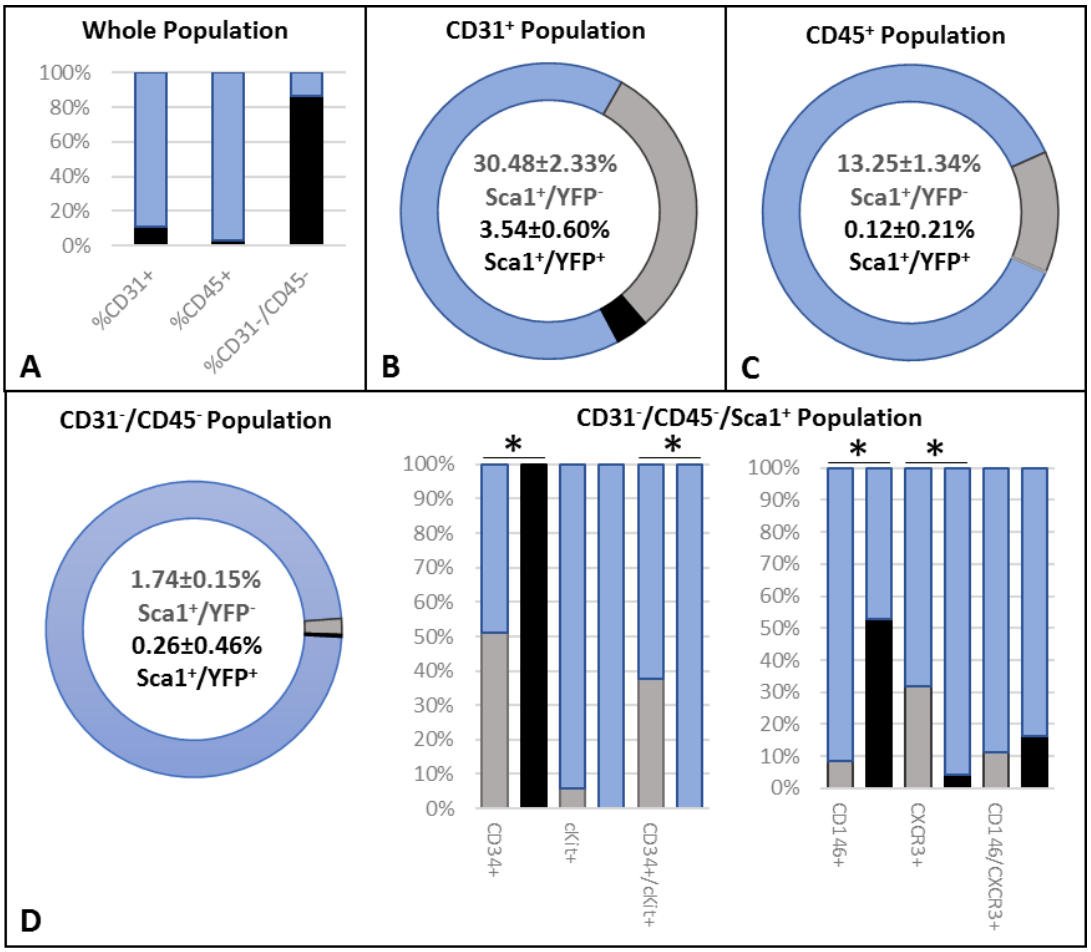

Figure 21. **FACS analysis of E18.5 cardiac Sca1⁺ cells.** Wt1YFP mouse hearts display permanent expression of the YFP reporter in Wt1⁺ lineage cells. **(A)** Representation of the CD31⁺, CD45⁺ and CD31⁻/CD45⁻ cell fractions in the whole population. Sca1+/YFP- (grey) and Sca1⁺/YFP⁺ (black) percentages are represented with respect to CD31⁺ **(B)**, CD45⁺ **(C)** and CD31⁻/CD45⁻ **(D, left) cell** fractions. **(D, right)** Characterization of CD31⁻/CD45⁻/Sca1⁺ cells. (*p<0.05). Abbreviations: CCR2 = C-C motif chemokine receptor 2; CD146 = cluster of differentiation 146; CD31/PECAM1 = platelet and endothelial cell adhesion molecule 1; CD34 = cluster of differentiation 34; CD45 = protein tyrosine phosphatase receptor type C; c-Kit = KIT proto-oncogene receptor tyrosine kinase; Sca1 = stem cell antigen-1; YFP = yellow fluorescent protein.

since, as already discussed, CD45 expression levels have been found to be highly dynamic depending on the environment. Conversely, the expression of CD146, the melanoma cell adhesion molecule (MCAM) present in diverse vascular cells as pericytes (Ruiz-Villalba et al., 2020), was reduced, indicating that a these stages this multipotent population are not contributing to the mural population.

III.1.4. Sca1-positive cells in the postnatal and adult heart

As the histological quantification of endothelial cells (a predominant cell type within the $Sca1^+$ cardiac interstitial cell fraction) has been shown to be poorly accurate when compared to flow cytometry quantification methods (Cano et al., 2016), FACS was used as the main method to analyse the cardiac $Sca1^+$ cell population in the postnatal and adult heart.

At P1 (Supplementary Figure 4), the proportion of Sca1 expressing cells in the endothelial $CD31^+$ subpopulation is high, especially when compared to their embryonic abundance ($p<0.05$). In detail, 60.84±2.57% of cells are $Sca1^+/YFP^-$ and 12.24±1.19% are $Sca1^+/YFP^+$. This confirms that Sca1 expression is closely associated to the endothelial phenotype (Xu et al., 2018). Regarding the $CD31^-/CD45^-$ fraction, the number of $Sca1^+$ cells remained low (1.37±0.22%), with no significant differences in comparison to embryonic stages. In the $Sca1^-/YFP^-$ subpopulation the expression of c-Kit and CXCR3 was significantly reduced, perhaps due to the acquisition of a more mature phenotype, although further research is necessary to confirm this statement.

A week later (P7) (Figure 22A-D), most of the cells in the $CD31^+$ population were also $Sca1^+$ (Figure 22B). Most importantly, a significant increase in the proportion of $Sca1^+$ cells was observed in the $CD31^-/CD45^-$ fraction (Figure 22D).

Specifically, the proportion of $Sca1^+/YFP^+$ cells changed from 0.037±0.07% at P1 to 7.65±0.35% at P7 in the non-endothelial/non-blood-related population. This is a remarkable finding, since the presence of epicardial derived (YFP^+) $Sca1^+$ cells

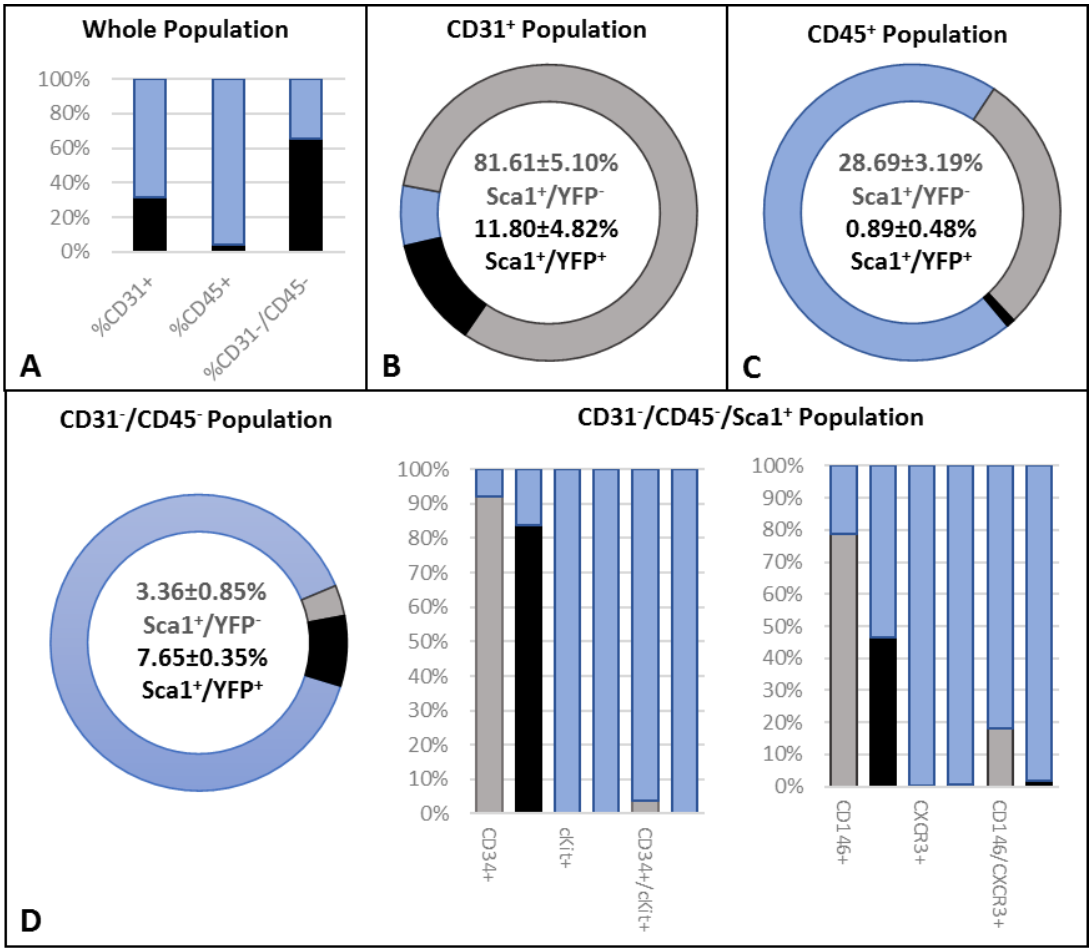

Figure 22. FACS analysis of P7cardiac Sca1⁺ cells. Wt1YFP mouse hearts showed permanent expression of the YFP reporter in Wt1⁺ lineage cells. **(A)** CD31⁺, CD45⁺ and CD31⁻/CD45⁻ representation in the whole cardiac cell population. Sca1⁺/YFP⁻ (grey) and Sca1⁺/YFP⁺ (black) percentages are represented with respect to total CD31⁺ **(B)**, CD45⁺**(C)**, and CD31⁻/CD45⁻ cell fractions **(D, left)**. **(D, right)** Characterization of CD31⁻/CD45⁻/Sca1⁺ cells. (*p<0.05). Abbreviations: CCR2 = C-C motif chemokine receptor 2; CD146 = cluster of differentiation 146; CD31/PECAM1 = platelet and endothelial cell adhesion molecule 1; CD34 = cluster of differentiation 34; CD45 = protein tyrosine phosphatase receptor type C; c-Kit = KIT proto-oncogene receptor tyrosine kinase; Sca1 = stem cell antigen-1; YFP = yellow fluorescent protein.

in the cardiac interstitium (in particular those that do not belong to the endothelial lineage, $CD31^-$) has not been previously described.

This finding indeed rises numerous questions about the origin and nature of these cells in the neonatal heart. As mentioned previously in the introduction, Wt1 is highly expressed by epicardial and EPDCs during cardiac development until EPDCs differentiation and mature phenotype acquisition, therefore most probably these postnatal $Sca1^+/YFP^+$ cells surge by high proliferation of the preexisting $Sca1^+/YFP^+$ cells. Nevertheless, it is not possible to forget the possibility that their increasing number can be related to a *de novo* expression of Wt1 in the Sca1 population. In fact, low levels of Wt1 have been described in homeostatic adult endothelial cells in scarce capillaries and coronary veins, where this transcription factor can be related to endothelial proliferation (Duim et al., 2015).

Regarding their phenotype, $Sca1^+/YFP^+$ show no significant differences in comparison with the $Sca1^+/YFP^-$ fraction; both populations show high levels of CD34 and CD146 expression. This finding is interesting, since the concurrent expression of both CD34 and CD146 has been reported to form part of the molecular signature of adipocytic progenitors (Zimmerlin et al., 2013), a cell type that is abundant in the adult heart and is known to be ontogenetically related with the epicardium (Yamaguchi et al., 2015).

In the adult heart (2-3 months-old tissue samples) (Figure 23A-C), virtually all $Sca1^+$ cells were identified by their binding to the vascular specific agglutinin IB4 from *Gliffonia simplifolia*, a finding that confirms their endothelial nature (Figure 23A). No colocalization between Sca1 and αSMA was found at any time (Figure 23B). The analysis of Sca1 expression in adult animal transplanted with RFP^+ bone marrow cells was unable to identify $Sca1^+/mRFP^+$ cells, therefore ruling out the contribution of blood-borne cells to the cardiac $Sca1^+$ fraction during adulthood (Figure 23C).

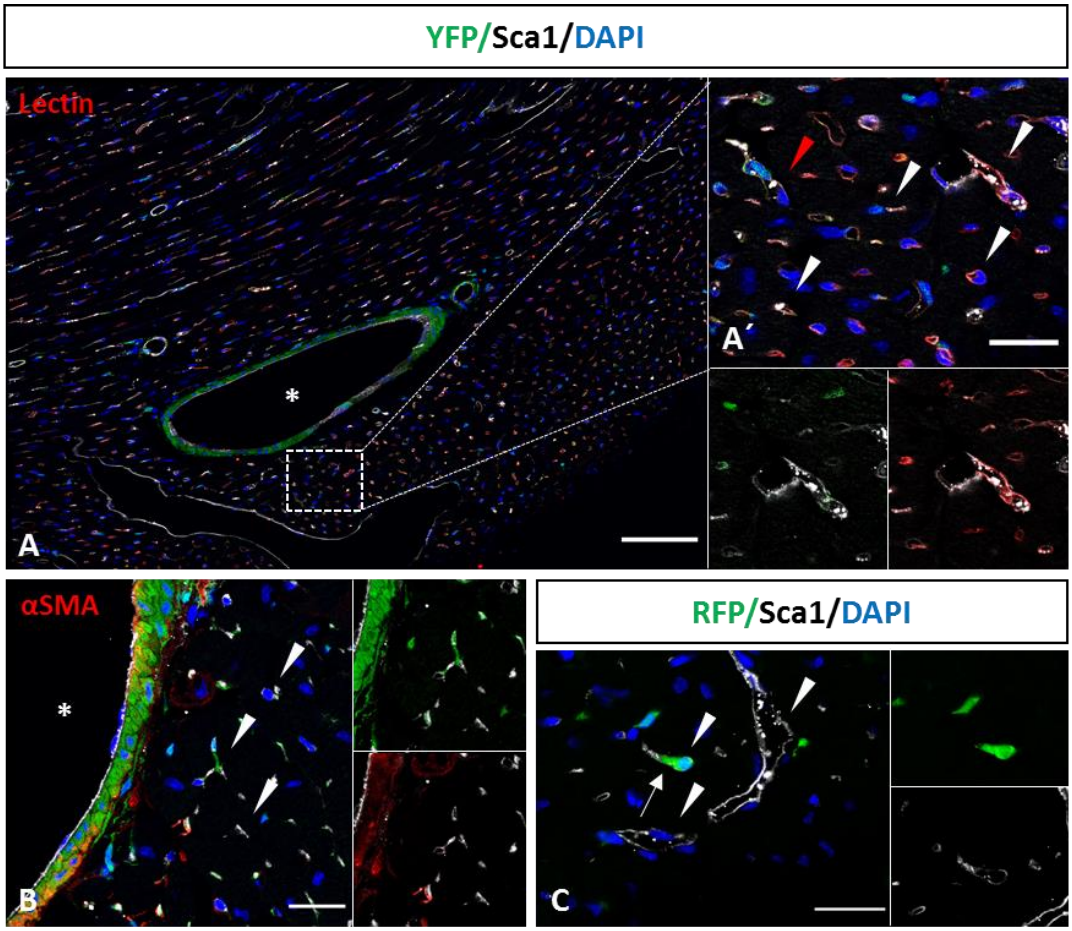

Figure 23. Localization and characterization of cardiac Sca1⁺ cells in adult mice. (A-C) Sca1⁺ cells **(A´, arrowheads)** are integrated in the endothelium of coronary vessels and in the cardiac interstitium **(A´),** where they form part of small vessels (IB4/*G. simplicifolia* lectin⁺) and are αSMA⁻ **(B)**. **(A-B)** Some of these Sca1⁺ cells are from epicardial **(A´, red arrowhead). (C)** In BMRFP mice with adult bone marrow transplant, no co-expression of Sca1 **(arrowheads)** and RFP **(arrow)** has been observed. Scale bars: **A**, 100 μm; **A´-C**, 25 μm. Abbreviations: asterisk (*) = coronary vessel; αSMA = smooth muscle actin; DAPI = 4',6-diamidino-2-fenilindol; Lectin = IB4/G *simplicifolia*; RFP = red fluorescent protein; Sca1 = stem cell antigen-1; YFP = yellow fluorescent protein

FACS analysis of these same adult samples (Figure 24A-D) showed that not all Sca1$^+$ cells belong to the endothelial lineage (Figure 24B), as CD45$^+$ (Figure 24C) and CD31$^-$/CD45$^-$ (Figure 24D) cell fractions also contained Sca1 expressing cells, perhaps in an insufficient number to be detected by immunohistochemical routines.

Inside the CD31$^-$/CD45$^-$ fraction, the group of cardiac interstitial cells deemed to contain the cells with wide progenitor properties (Valente et al., 2014), the balance between Sca1$^+$YFP$^-$ cells and Sca1$^+$/YFP$^+$ ones seemed to have inverted in comparison with the perinatal stages. Nevertheless, this apparent increase of the Sca1$^+$/YFP$^-$ population and the decrease of the Sca1$^+$/YFP$^+$ population are not statistically significant, probably due to the variability existent among samples.

The presence of both populations (Sca1$^+$/YFP$^-$; Sca1$^+$/YFP$^+$) in the adult ventricular interstitium supports the idea that Wt1$^+$ lineage (epicardial-derived) cells could contribute to the cardiac progenitor pool, as previously suggested (Muñoz-Chápuli et al., 2002; Smits et al., 2018; Wessels & Pérez-Pomares, 2004). In accordance to this hypothesis, these two populations show a sustained expression of CD34, particularly in the Wt1YFP$^+$ cell subset. As previously mentioned, CD34 is regarded as a stem cell marker in many contexts and has been used by some authors, in combination with other markers like CD45 and CD31 to identify stem/progenitor cells in the cardiac interstitium (Sydney et al. 2014). However, CD34 is also expressed in other endothelial, adipocytic or mesenchymal cell lineages. Therefore, the expression of this molecule alone is not enough to determine if both Sca1$^+$ populations fulfil the criteria to be considered as cardiac progenitor cells or if their functions in the cardiac interstitium are different and may vary depending on their ontogenetic origin.

Figure 25 summarizes the changes recorded in the Sca1$^+$ cardiac population from embryonic stages to adulthood, with the proportion of c-Kit$^+$/YFP$^-$ and c-Kit$^+$/YFP$^+$ in the CD31$^-$/CD45$^-$ fraction (Figure 25A) and the characterization of these cells in relation with diverse molecule expression at different stages (Figure 25B) (statistical significance is indicated, $p<0.05$).

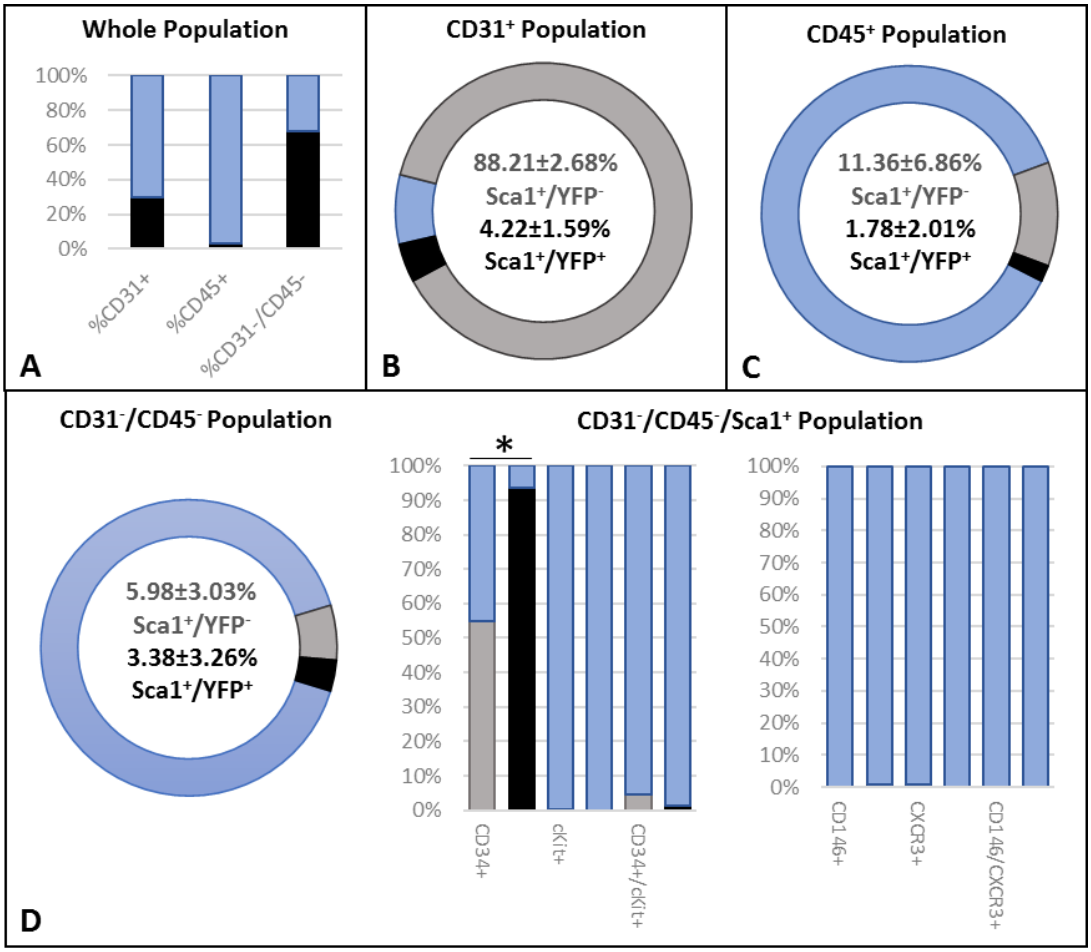

Figure 24. FACS analysis of adult cardiac Sca1⁺ cells. Wt1YFP mouse hearts showed permanent expression of the YFP reporter in Wt1⁺ lineage cells. **(A)** CD31⁺, CD45⁺ and CD31⁻/CD45⁻ representation with respect to the whole cardiac interstitial population. Sca1⁺/YFP⁻ (grey) and Sca1⁺/YFP⁺ (black) percentages are shown as part of the CD31⁺ **(B)**, CD45⁺ **(C)** and CD31⁻/CD45⁻ fractions **(D, left)**. **(D, right)** Characterization of CD31⁻/CD45⁻/Sca1⁺ cells. (*p<0.05). Abbreviations: CCR2 = C-C motif chemokine receptor 2; CD146 = cluster of differentiation 146; CD31/PECAM1 = platelet and endothelial cell adhesion molecule 1; CD34 = cluster of differentiation 34; CD45 = protein tyrosine phosphatase receptor type C; c-Kit = KIT proto-oncogene receptor tyrosine kinase; Sca1 = stem cell antigen-1; YFP = yellow fluorescent protein.

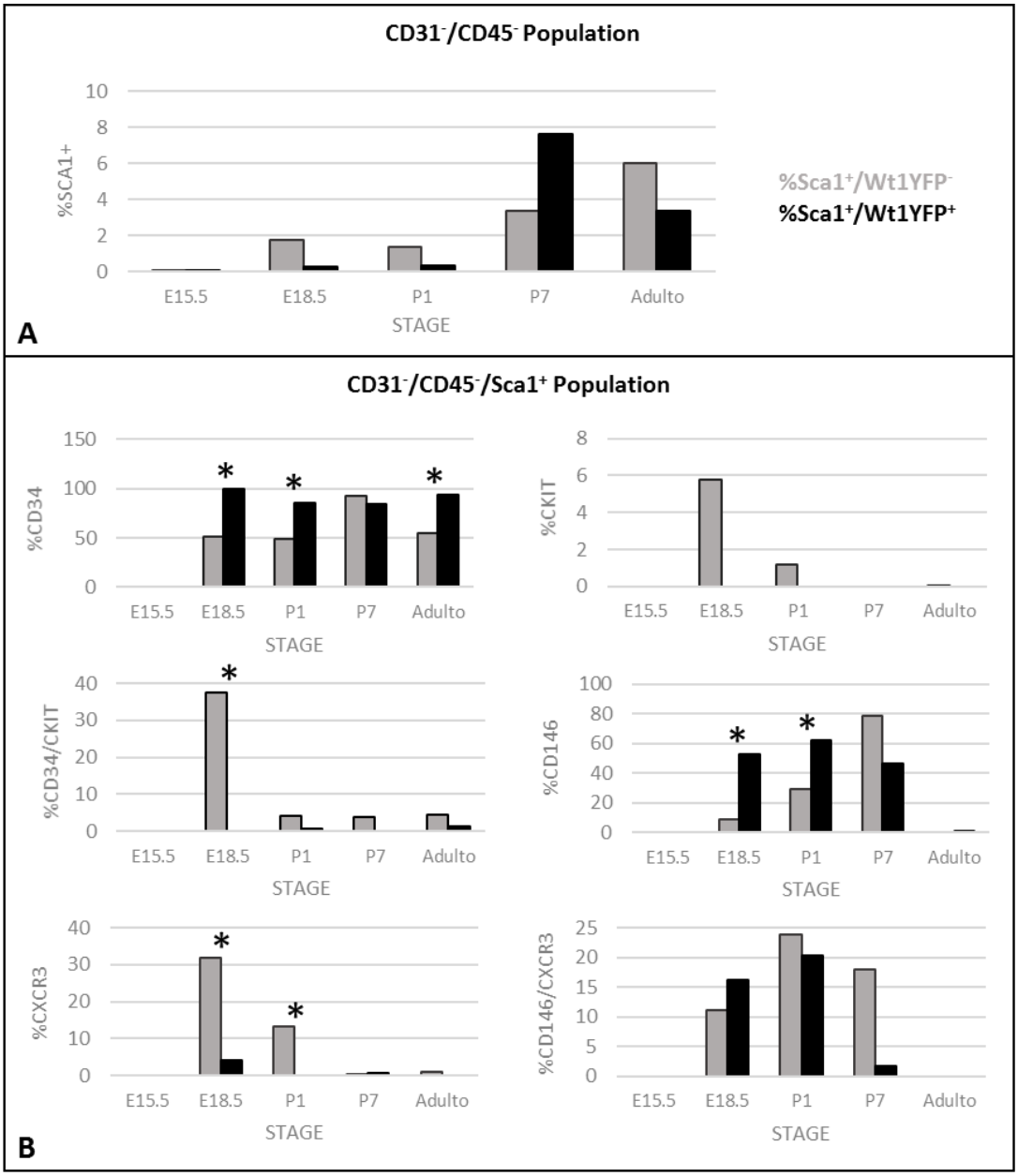

Figure 25. FACS characterization of E15.5, E18.5, P1, P7 and adult cardiac Sca1$^+$ cells at in Wt1YFP mice. (A) Proportion of Sca1$^+$/YFP$^-$ (grey) and Sca1$^+$/YFP$^+$ (black) cells in the CD31$^-$/CD45$^-$ cardiac population. **(B)** Characterization of Sca1$^+$/YFP$^-$ and Sca1$^+$/YFP$^+$ cells according to their molecular expression profile (*$p < 0.05$). Abbreviations: CCR2 = C-C motif chemokine receptor 2; CD146 = cluster of differentiation 146; CD31/PECAM1 = platelet and endothelial cell adhesion molecule 1; CD34 = cluster of differentiation 34; CD45 = protein tyrosine phosphatase receptor type C; c-Kit = KIT proto-oncogene receptor tyrosine kinase; Sca1 = stem cell antigen-1; YFP = yellow fluorescent protein.

III.2. NEUROVASCULAR SCAFFOLDING OF THE CARDIAC INTERSTITIUM

In the previous section of this chapter (III.1) we studied the diversity of cardiac interstitial cells as based on the expression of two molecular markers classically associated to cell progenitor phenotypes (c-Kit and Sca1). In the second section, we will study the organization of cardiac neurovascular associations (i.e. closely related cardiac nerves and coronary blood vessels), classically referred-to as *neurovascular bundles* (Walker et al., 2015) for two different reasons. First because our immunohistochemical analysis showed that c-Kit$^+$ and Sca1$^+$ cells frequently distribute around or even integrate in the coronary vasculature. Second, because neurovascular bundles have been considered to be essential in the definition of stem cell/progenitor niche-like microenvironments (Obernier & Alvarez-Buylla, 2019), and proposed to be key in the organization of the cardiac interstitium (Nam et al., 2013).

III.2.1. Immunohistological analysis of cardiac neurovascular bands in embryonic and adult hearts

Whole mount βIII-Tubulin/CD31 immunostainings revealed that the first cardiac nerves (βIII-Tubulin$^+$) appear on the dorsal surface of the developing heart at E13.5, in close proximity to the cardiac inflow (Figure 26A). At E15.5, these nerves extend towards the cardiac apex following the major coronary vascular branches on the dorsal aspect of the heart (Figure 26B), whereas the ventral side of the organ remains devoid of cardiac nerves (data not shown). Two days after (E17.5), the nerve fibers have reached the cardiac apex on the dorsal side of the heart (Figure 26C), and the nerve growth and extension start to be evident on the ventral aspect of the heart (Figure 26C'). The rapid ventricular expansion of these nerves towards the most distal part of the heart and their ultimate distribution in the ventricular wall strongly suggest that they belong to the sympathetic component of cardiac nerves (Nam et al., 2013).

The close interaction observed between the developing cardiac nerves and coronary blood vessels is likely to result from the fact that both structures require the activity of similar molecules (e.g. ephrins, semaphorins or neurotrophins) during their embryonic development and normal physiological interaction (Hasan, 2013; Nam et al., 2013; Ward & LaManna, 2004). Moreover, during embryonic development, nerves are prone to grow over cell paths rich in laminin (Nakazawa et al., 2013), an essential molecule of the embryonic developing blood vessels (Risau & Flamme, 1995), something that partially explains the concurrent parallel paths followed by both blood vessels and nerves.

In the adult heart (2-3 months-old), the close interaction between cardiac sympathetic nerves and coronary blood vessels remained evident in whole mount immunostained (βIII-Tubulin/CD31) hearts (Figure 27A-C), both in the subepicardial space and compact myocardium, the locations where the major coronary vascular tracts are located (Lavine et al., 2008; Red-Horse et al., 2010) (Figure 27A). When cardiac progenitor cell markers (c-Kit and Sca1) were analysed in the context of the neurovascular association, it was observed that the c-Kit$^+$ cells located close to coronary vessels, were frequently also associated to small cardiac nerves (βIII-Tubulin$^+$) (Figure 27B, B'), which are the ones that regulate the vascular tone in the coronary vessels. Otherwise, cardiac Sca1$^+$ cells were also found to be close to cardiac nerves, but in this case, all cells found to express CD31 and formed part of the coronary endothelium, as mentioned in the previous section (Figure 27C).

III.2.2. Neurotrophin signaling elements in the cardiac interstitium: NT3 expression

As previously discussed, (Introduction I.3.3.), all neurotrophins are expressed in the developing and adult heart, although their relevance is not completely understood. For instance, NT3 function has been described in neural crest migration and cardiomyocyte proliferation (Donovan et al., 1996; Youn et al., 2003), but little is known about its possible significance in the formation and maintenance of neurovascular bands, as described in the central nervous system. Moreover, in this

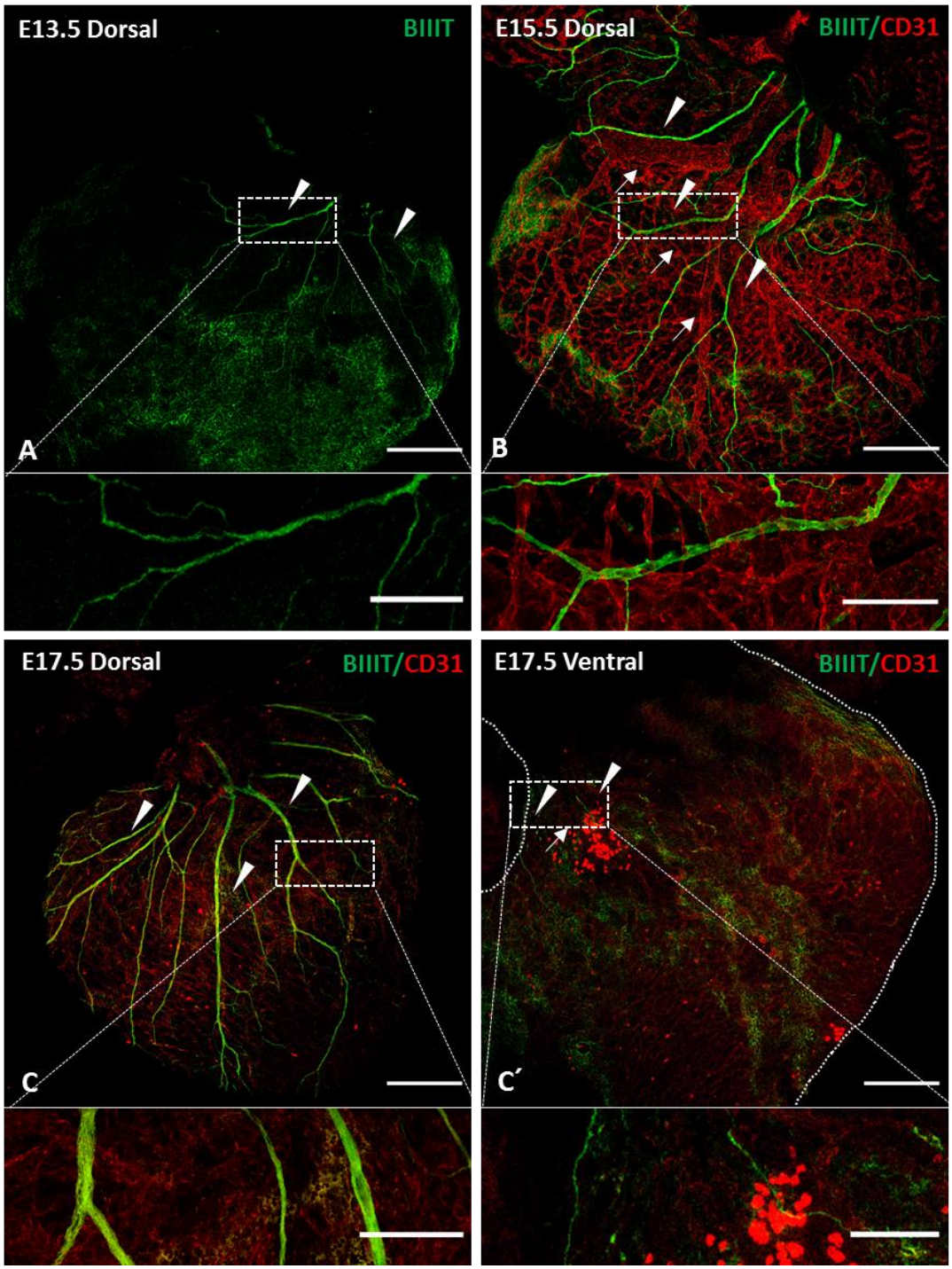

Figure 26. **Vessel and sympathetic nerve growth during cardiac development. (A)** Sympathetic innervation **(arrowheads)** starts at E13.5 in the dorsal region of the developing heart, close to sinus venosus. **(B)** At E15.5, sympathetic nerves spread through the dorsal wall close to coronary vessels **(arrows)**. **(C)** At E17.5, sympathetic nerves have reached **(C)** distal areas of the dorsal region and begin to develop in the basal region of the cardiac ventral wall **(C')**. Scale bars: **A-C´,** 250 µm; **magnifications**, 100 µm. Abbreviations: BIIIT = tubulin beta class III; CD31/PECAM1 = platelet and endothelial cell adhesion molecule 1.

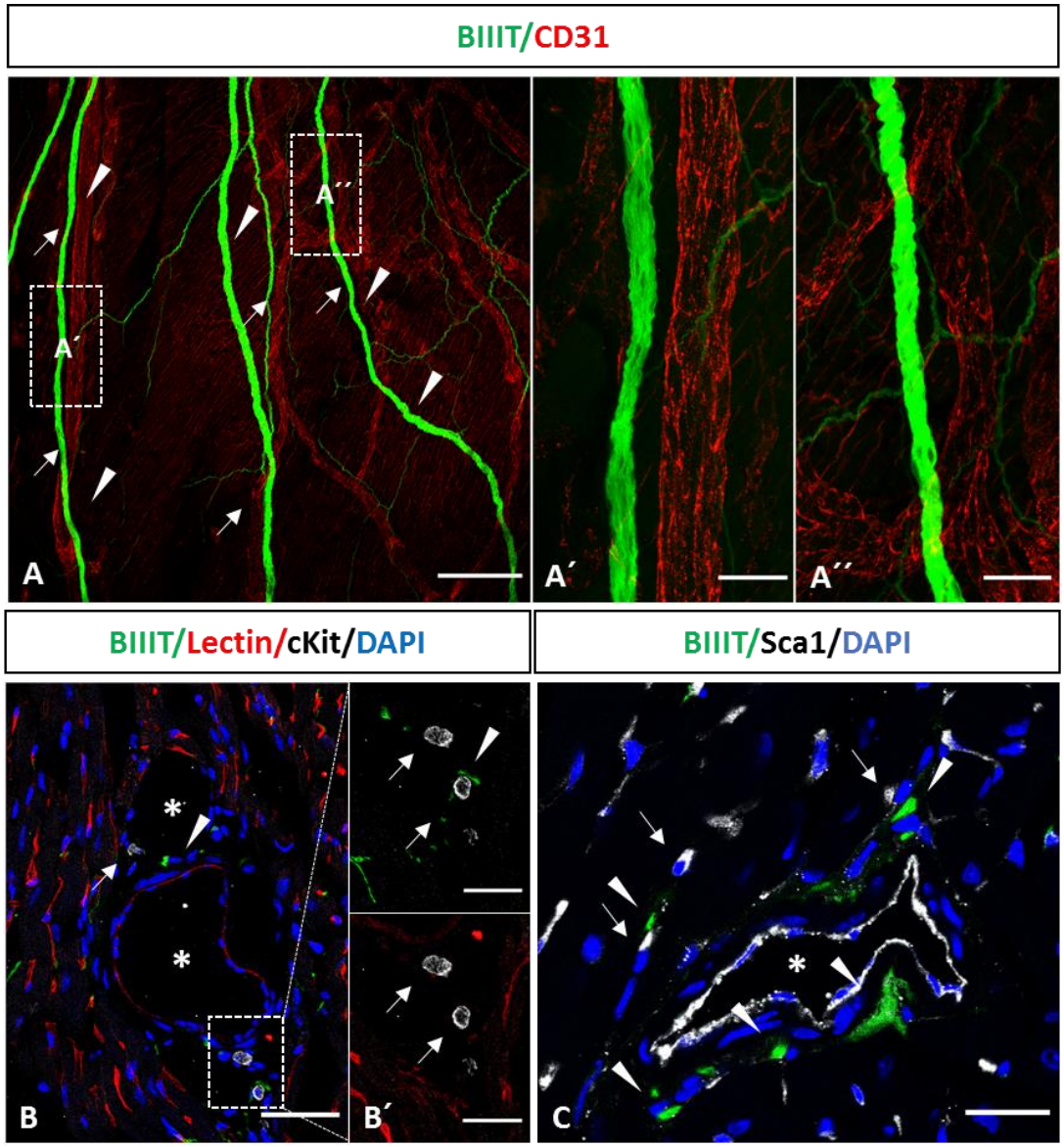

Figure 27. Neurovascular band and relation with c-Kit⁺ and Sca1⁺ cells in adult hearts. Sympathetic cardiac nerves **(A-A´´, arrowheads)** and large coronary vessels (arrows) form neurovascular bundles in the adult heart. Cardiac c-Kit⁺ cells **(B-B´, arrows)** are located adjacent to large coronary vessels, sometimes close to nerve fibers. On some occasions, cardiac Sca1⁺ cells **(C, arrowheads)** are also located in the vicinity of nerve fibers. Scale bars: **A**, 100 µm; **A´-B**, 50 µm; **B´-C**, 25 µm. Abbreviations: βIIITubulin = tubulin beta class III; CD31/PECAM1 = platelet and endothelial cell adhesion molecule 1; c-Kit = KIT proto-oncogene receptor tyrosine kinase; DAPI = 4',6-diamidino-2-fenilindol; Lectin = IB4/G *simplicifolia*; Sca1 = stem cell antigen-1.

case, NT3 is implicated in the preservation of the niche system (Chou & Modo, 2020; Delgado et al., 2014).

During early cardiac embryonic development, NT3 was found to be intensely expressed by compact and trabecular cardiomyocytes and some circulating cells (E11.5) (Figure 28A, compare to negative control). This latter circulating cell NT3 immunoreactivity is more likely to relate to the blood plasma contents of this neurotrophic factor (Nikolaou et al., 2006) than to the active expression of the molecule by blood cells. NT3 expression decreased in the compact myocardium at E13.5, while NT3 trabecular expression remained evident in the ventricles (Figure 28B) until E15.5 (Figure 28C). The adult myocardium (2-3 months-old hearts) but not blood cells, continued to express low NT3 levels (Figure 28D-E').

These results agree with previous reports, where embryonic NT3 cardiac expression show to be essential to the proper formation of the organ, since NT3 mutants have dilated hearts and poorly trabeculated hearts (Donovan et al., 1996). Surprisingly, an excessive NT3 signalling results in cardiomyocyte hypertrophy (Kawaguchi-Manabe et al., 2007). On the other hand, the suggested role of NT3 in cardiac neural crest cell migration (Youn et al., 2003) could also explain the poor innervation of NT3 mutant hearts (Donovan et al., 1996), since cardiac nerves are a primary neural crest cell derivative (Hutson & Kirby, 2003).

III.2.3. Neurotrophin signaling elements in the cardiac interstitium: the TrkB receptor

BDNF and its receptor, TrkB, are well known to be expressed in endothelial and smooth muscle cells during cardiac development and adulthood (Anastasia et al., 2014; Donovan et al., 2000). Nevertheless, their relation with the neurovascular fibers, in the context of the neurovascular band and a potential niche microenvironment, are not fully understood in the cardiac tissue.

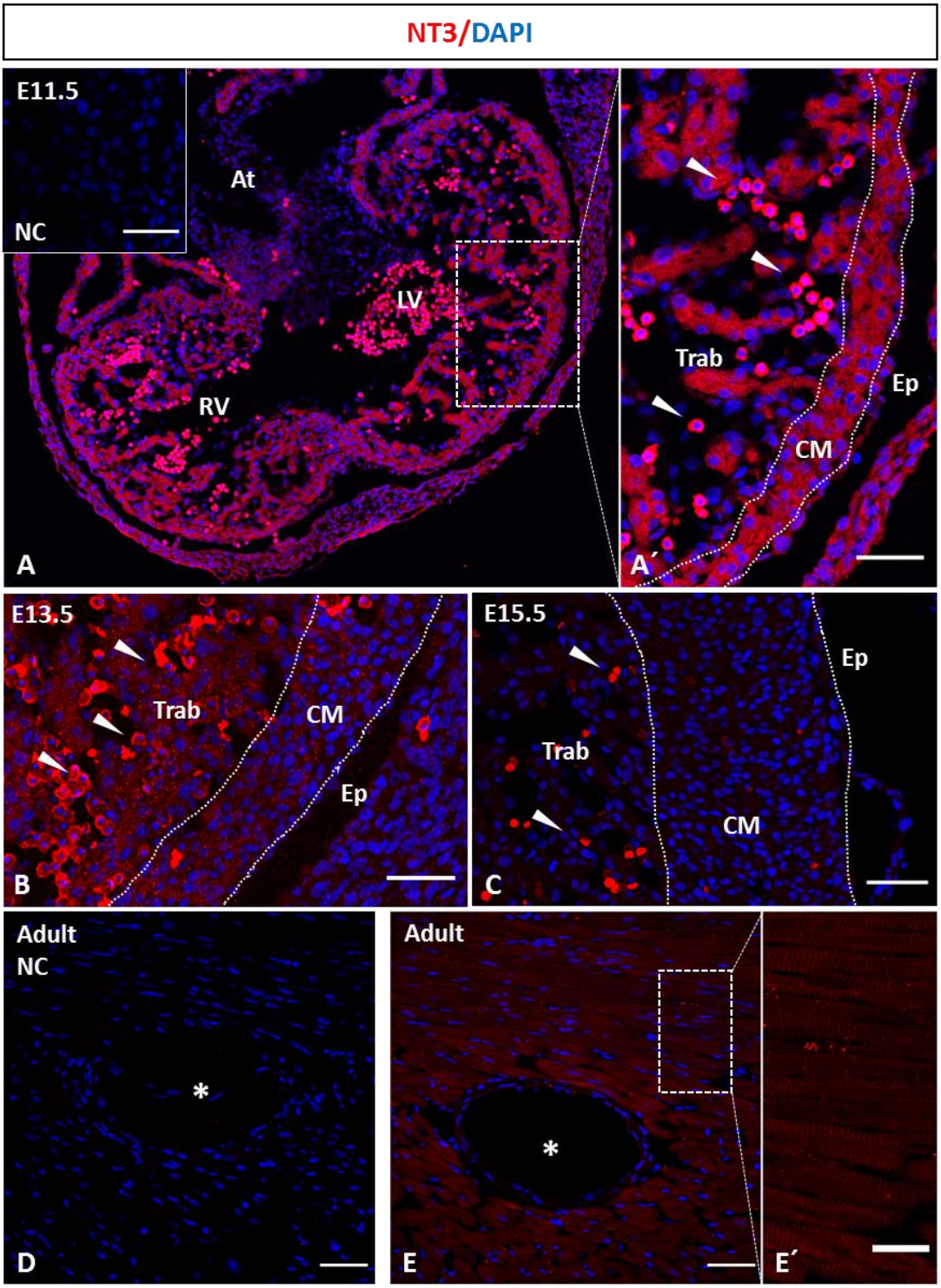

Figure 28. **Localization and characterization of cardiac NT3^{+} cells.** Embryonic and adult C57Bl6/J mouse hearts were studied. During early cardiac development (E11.5), NT3 expression is observed in all cardiac ventricular cardiomyocytes, both in compact and trabecular myocardial layers **(A-A´)**. Some blood cells also showed NT3 expression **(A', white arrowheads)**. NT3 levels decrease first in the compact myocardium around E13.5 **(B)** and then in the trabecular myocardium at E15.5 **(C)**. **(D-E')** In the adult heart, as compared to the negative control **(D)**, NT3 is low expressed in adult cardiomyocytes and coronary vessels **(E, asterisk)** was identified. Scale bars: **A,** 250 µm; **A´-E**, 50 µm; **E´**, 25 µm. Abbreviations: At = Atrium; CM = compact myocardium; DAPI = 4',6-diamidino-2-fenilindol; Ep = epicardium; LV = Left ventricle; NC = negative control; NT3 = neurotrophin 3; RV = right ventricle; Trab= trabecular myocardium.

TrkB was found to be conspicuously expressed in the developing heart. Detectable TrkB levels were first identified in the embryonic myocardium starting at E11.5 (Figure 29A). TrkB was strongly expressed in the growing cardiac nerves in E13.5 hearts, whose cardiomyocytes remained expressing this receptor (Figure 29B). Between E15.5 (Figure 29C) and E18.5 (Figure 29D), TrkB cardiomyocyte expression was still evident, but the strongest TrkB immunoreactivity was identified in the vascular endothelial and smooth muscle cells of the developing coronary blood vessels, including large numbers of YFP^+ cells. This expression pattern did not change significantly in postnatal stages (P1, P14) (Figure 29E, F).

As indicated above (Introduction I.3.3), TrkB expression has been described previously in maturing vessels, specifically in the smooth muscle cells where TrkB is implicated in cell migration (Anastasia et al., 2014; Donovan et al., 2000). Nevertheless, in this case endothelial TrkB expression was also observed and it could be related, as occurs with other neurotrophins (e.g. NGF), with autocrine signalling and endothelial homeostasis.

Relevant to this thesis, other laboratories have shown that the transcriptional forms of Wt1, a master molecule in the regulation of epicardial biology, modulates TrkB gene expression (Wagner et al., 2005). In accordance with this finding, Wt1 mutants have less EPDC and disrupted coronary development (Guadix et al., 2011) and TrkB-null mice have severe vascular defects related to the formation of the intimal and medial layers (Anastasia et al., 2014).

In the adult heart (2-3 months-old), TrkB was expressed by different cell types, including cardiomyocytes (Figure 30A-B), $CD31^+$ coronary vascular endothelial cells (Figure 30C), and αSMA^+ medial smooth muscle cells (Figure 30D), but also in some isolated cells in the ventricular interstitium (Figure 30A-B). The consistent YFP expression found in the majority of coronary smooth muscle, fibroblastic adventitial and ventricular interstitial cells argued in favour of their epicardial (Wt1-lineage) origin (Figure 30A-B). The counterstain with βIII-Tubulin showed the frequent association of $TrkB^+$ cells with cardiac nerves (Figure 30E). Pericoronary $c\text{-}Kit^+$ interstitial cells often appeared in close association with adventitial $TrkB^+$ cells (Figure 30F). The

preferential distribution of these cells close to neurovascular bundles supports our idea of neurovascular domains being involved in the maintenance of an interstitial/perivascular microenvironment suitable for the homing of cells with potential progenitor properties (Pogontke et al., 2019).

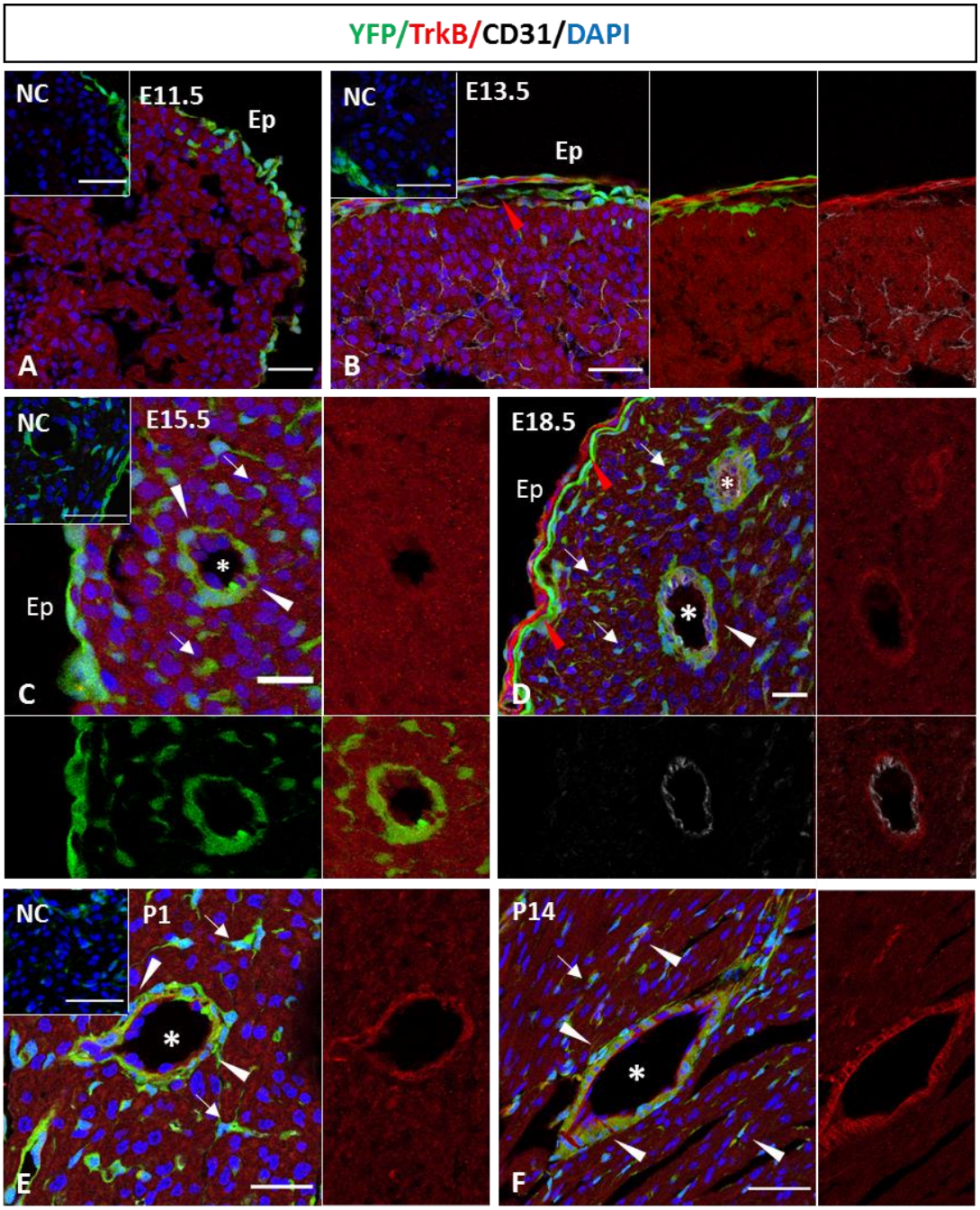

Figure 29. **Localization and characterization of cardiac TrkB^{+} cells during embryonic development.** Wt1Cre-YFP mice displayed permanent YFP expresion in Wt1^{+} lineage cells. **(A)** During early cardiac development (E11.5), TrkB expression is observed in the cardiac interstitium. This expression pattern is maintained at E13.5 **(B)**. NT3 expression is increased in E13.5 developing sympathetic nerves **(red arrowheads)**. Once large diameter vessels are formed (around E15.5), TrkB is also expressed in coronary vessels **(C, asterisk)**, including YFP^{+} EPDC **(C, white arrowheads**) that have incorporated into the TrkB^{-}/YFP^{+} vessel wall cells (**C, arrows**). **(D-E)** Around birth, coronary endothelial and smooth muscle cells express significant levels of TrkB. **(F)** During postnatal stages, TrkB is also highly expressed in some adventitial and interstitial EPDCs. Scale bars: **A, B, and F**, 50 µm; **C-E**, 25 µm. Abbreviations: Asterisk (*) = coronary vessel; CD31/PECAM1 = platelet and endothelial cell adhesion molecule 1; DAPI = 4',6-diamidino-2-fenilindol; Ep = epicardium; NC = Negative Control; TrkB = tyrosine receptor kinase 2; YFP = yellow fluorescent protein.

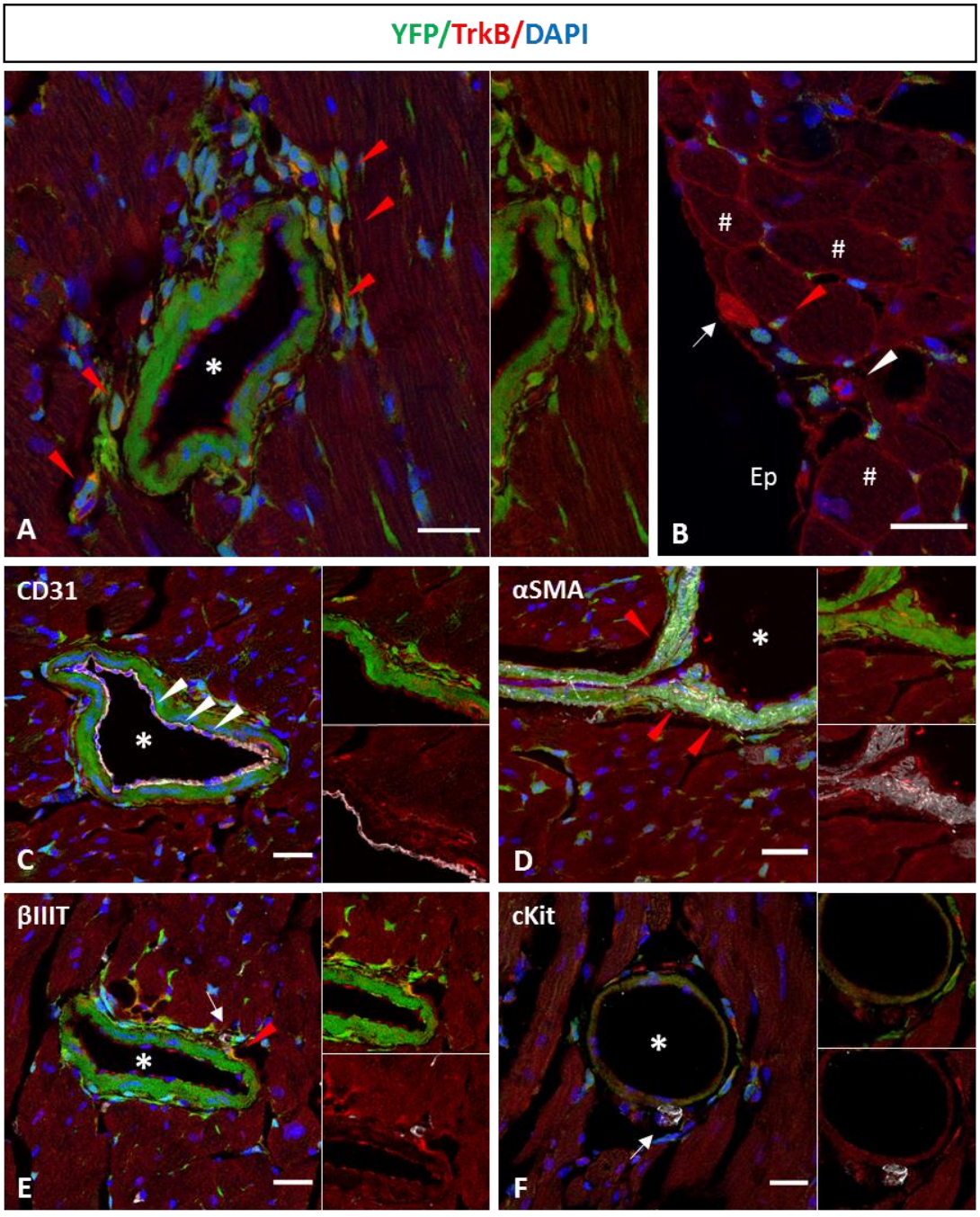

Figure 30. **Localization and characterization of cardiac TrkB$^+$ cells in adult hearts.** Wt1Cre-YFP mouse hearts show permanent expression of the YFP reporter in Wt1$^+$ lineage cells. Coronary vessels **(A, asterisk)**, cardiomyocytes **(B, #)**, nerve fibers **(B, arrows)** and some cardiac interstitial cells **(B, arroweads),** express TrkB. Some TrkB$^+$ coronary vessels and ventricular interstitial cells cells are YFP$^+$ **(B, red arrowheads)**. **(C-F)** In large coronary vessels, TrkB is expressed by CD31$^+$ endothelial cells **(C)**, α-SMA$^+$ smooth muscle cells **(D)** and CD31$^-$αSMA$^-$ adventitial cells, but not in small nerve fibres **(E, arrow)** or c-Kit$^+$ cells **(F, arrow)**. Scale bars: **A-F**, 25 μm. Abbreviations: αSMA = smooth muscle actin; βIIITubulin = tubulin beta class III; Asterisk (*) = coronary vessel; CD31/PECAM1 = platelet and endothelial cell adhesion molecule 1; cKit = KIT proto-oncogene receptor tyrosine kinase; DAPI = 4',6-diamidino-2-fenilindol; Ep = epicardium; TrkB = tyrosine receptor kinase 2; YFP = yellow fluorescent protein.

III.3. *IN VITRO* EVALUATION OF NEUROTROFIN MODULATION OF CARDIAC INTERSTITIAL CELLS

In section III.2 of this memory we have studied the organization of neurovascular units in the developing, postnatal and adult heart stages, as well as their relationship with putative cardiovascular progenitor cells (c-Kit$^+$ and Sca1$^+$). To evaluate the possible impact of NT3 in the cardiac interstitium, we devised an *in vitro* strategy aiming at testing the effect of different neurotrophic factors on the proliferation of endothelial cells and epicardial-derived cells, some of the most relevant cell types found in this cardiac microenvironment.

III.3.1. HUVECs

Human umbilical vein endothelial cells (HUVECs) were used to analyse the impact of the diverse neurotrophic factors in an endothelial cell model, specifically in relation to their capacity to induce cell proliferation. As explained previously in Material & Methods section, different concentrations of NGF, BDNF and NT3 were tested; and cell proliferation in the HUVECs was assessed using the click-iT EdU assay (Figure 31). First, EdU incorporation to HUVEC cells was assessed by routine microscopic evaluation of the growing cells, taking advantage of the fluorescent reaction that follows the development of the EdU-click-iT reaction (Figure 31A). Then, a high content screening approach (Operetta, PerkinElmer) was used to quantify EdU incorporation to HUVEC. We observed that only 100ng/ml NT3 was able to trigger a statistically significant change in HUVEC proliferation compared to control medium without growth factors (Figure 31B). This result thus suggested that in this *in vitro* cell model, endothelial growth may be sensible to neurotrophic factor doses, as only high doses (100ng/ml) of NT3 were able to induce cell proliferation in a significant way ($p<0.001$).

This possible implication of NT3 in endothelial homeostasis and proliferation can be related to their expression in the adult cardiac endothelium (Kawaguchi-Manabe et al., 2007), since in this cell type, as occurs with other endothelial

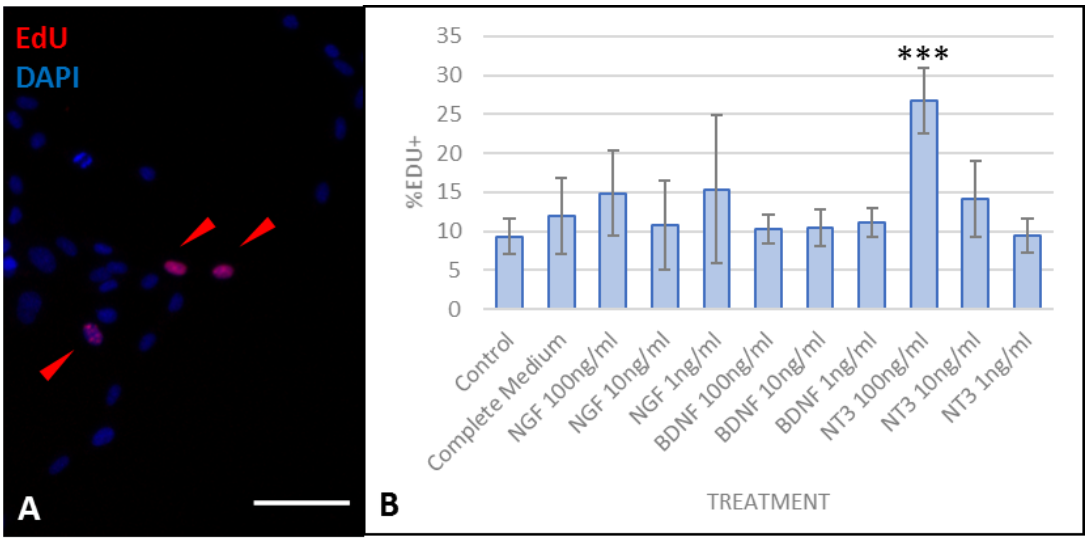

Figure 31. **Proliferation in Human Umbilical Vein Endothelial Cells (HUVECs) after neurotrophic factor treatment**. **(A)** Proliferating cells with incorporation of EdU and posterior EdU-click-iT reaction **(red arrowheads)**. **(B)** Proliferation rate in diverse treatment conditions **(n=1, 7 duplicates)** (***p<0.001). Scale bar: 25 µm**.** Abbreviations: DAPI = 4',6-diamidino-2-fenilindol; EdU = (5-ethynyl-2´-deoxyuridine).

molecules, NT3 could be exerting an autocrine function. However, it is not possible to ignore the possibility that addition to autocrine signalling the surrounding cells, that also expresses NT3, could be exerting a paracrine signalling, as occurs with other neurotrophins.

It also calls attention that previous studies show that NGF is also implicated in process of endothelial angiogenesis, migration or cell homeostasis, and that is able to induce significant cell proliferation at doses of 50ng/ml in HUVECs (Cantarella et al., 2002). Nevertheless, we have no observed significant differences in this experiment.

III.3.2. Epicardial explants

To test the effect of the neurotrophic factors in epicardial cells, an *in vitro* epicardial explant system was set up and primary culture of E11.5 embryonic epicardium were performed (Figure 32A). After 24 hours, epicardial cells were found to outgrow from the explant forming a squamous epithelial monolayer (Figure 32B) that remained once the heart was removed (Figure 32C). These cells, which retained the characteristic cytokeratin expression of native *in vivo* epicardium (Pérez-Pomares et al., 1997), did massively proliferate in culture (Figure 32D-D´´) as previously described by Ruiz-Villalba and colleagues (Ruiz-Villalba et al., 2013).

The epithelial integrity of the explants after treatment with neurotrophic factors (NGF, BDNF and NT3, all of them at 100ng/ml) was assessed using anti-cytokeratin (CK) and anti-ZO-1 antibodies (Figure 33A-D). Two days after the treatment with these neurotrophic factors, epicardial cells, both in the centre and the edge of the explant were found to remain epithelial, with a marked expression of both the epithelial intermediate filament CK and the apical cell junction marker ZO-1 (Figure 33B, D); only NGF-treated cells did lose their epithelial phenotype, as evidenced by

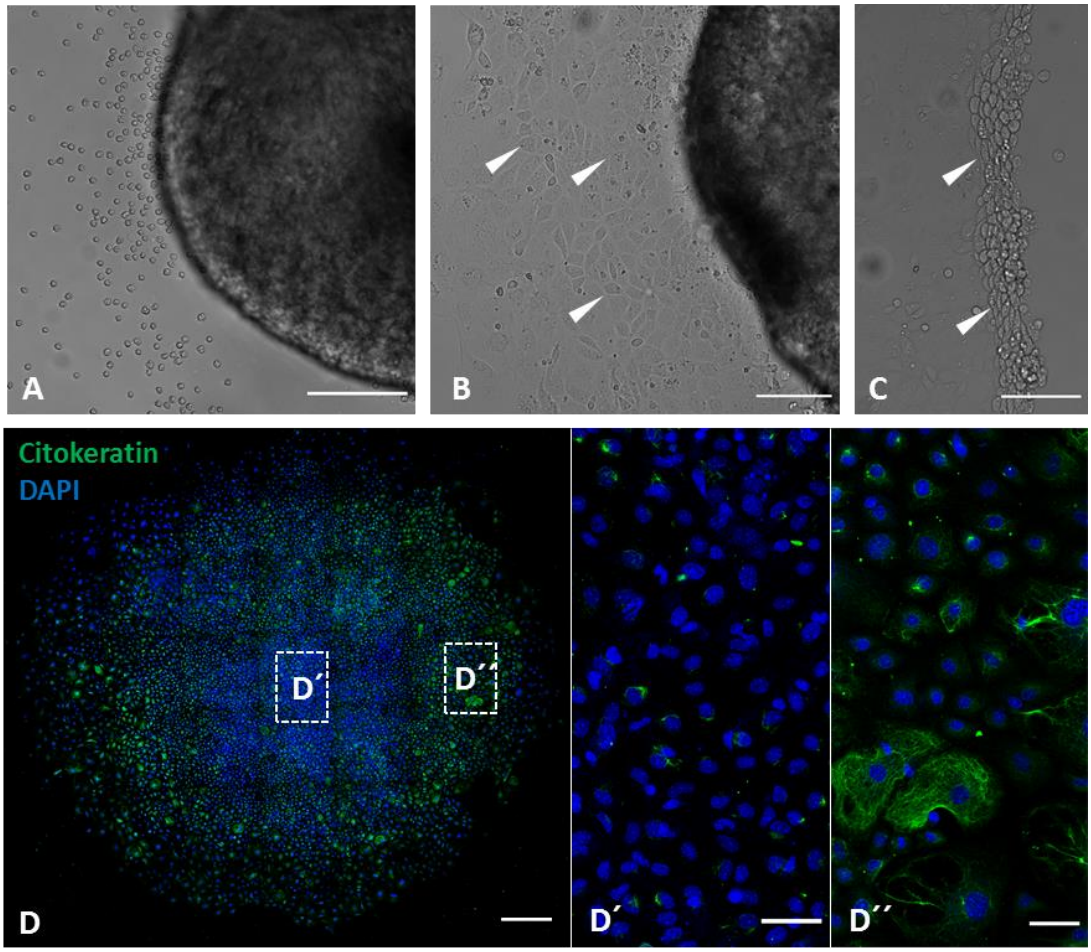

Figure 32. **Characterization of embryonic epicardium primary cultures. (A)** Whole heart explants at day 0. Some red blood cells surround the explanted tissue, but the substrate does not show adhesive cells on it. **(B)** Whole heart explant 24 hours after the initiation of the experiment. A clear epicardial cell halo starts to grow on gelatine-coated coverslips **(arrowheads). (C)** Epicardial cells remain over the substrate after removing the explant. **(D-D´´)** Epicardial cells after 48 hours of culture have expanded but maintain their characteristic cytokeratin expression. Scale bars: **A, B, C, D´ and D´´,** 50 µm; **D**, 500 µm. Abbreviations: DAPI = 4',6-diamidino-2-fenilindol.

a marked decrease of CK and ZO-1 expression (Figure 33B´). To evaluate whether neurotrophic factor stimulation has an impact on the differentiation of epicardial cellular derivatives into cardiovascular cell types, cultured epicardial explants were immunostained with anti-aSMA (smooth muscle marker), pro-collagen I (fibroblast marker) and CD31 (endothelial marker) antibodies. The expression of $aSMA^+$ cells is clearly increased in epicardial explants treated with the three neurotrophic factors used in this assay (Figure 33E-H'), most notably in the case of NGF (Figure 33F, F'). Neither pro-collagen (Figure 34A-H') nor CD31 expression (Supplementary Figure 5) was found to be substantially different in neurotrophic factor-treated epicardial explanted cells as compared to control cultures.

These results suggest that NGF might be involved in promoting and sustaining the mesenchymal phenotype of epicardial cellular derivatives. In this regard, it is important to consider that activation of epithelial-to-mesenchymal transition (EMT) is required for the generation of EPDC during cardiac embryonic development (Dettman et al., 1998; Pérez-Pomares et al., 1998). Also, this neurotrophin seems to stimulate the differentiation/proliferation of these mesenchymal cells into more differentiated αSMA expressing cells, in contrast to what was observed in other studies in which NGF was only able to induce migration (Kraemer et al., 1999).

III.3.3. Culture of cardiac interstitial cells: formation of spherical bodies

To test whether some interstitial cells displayed a functional progenitor-like profile we adapted and developed a protocol for the *in vitro* culture of cardiac cells as spherical cell bodies. This approach was inspired by multiple studies suggesting that non-myocyte cardiac cells present in the cardiac interstitium could be grown *in vitro* to give rise to the so-called *cardiospheres* (Ashur & Frishman, 2018). The original idea of these cardiospheres originating from a multipotent $c\text{-}Kit^+$ or $Sca1^+$ cardiac stem cell with cardiomyocytic potential is not accepted anymore (Neidig et al., 2018), so we aimed at testing whether these structures actually form *in vitro* and then to characterize their nature.

The procedure was started by explanting small pieces of adult ventricular heart tissue (please, refer to the Material & Methods section for detailed information on the procedure, see Figure 35A) over fibronectin-coated plates or coverslips. Around four or five days to the end of the first week after the initiation of the cultures, a clear halo of fibroblastic like cells was seen growing from the explant onto the culture substrate (Figure 35B). This process continued until the second week in culture, when round, poorly-adherent and strongly light refracting cells were identified growing over the fusiform cells described above (Figure 35C-F). Immunohistochemical screening of these cells showed substantial Wilms' tumour suppressor 1 (Wt1) protein accumulation in some of the cellular nuclei (Figure 35D), indicating reactivation of this transcription factor. In this regard, as mentioned in the introduction, Wt1 is expressed in the heart during cardiac development in the proepicardium/epicardium and EPDCs, but then this expression is downregulated once the EDPCs differentiate. Nevertheless, Wt1 reactivation has been observed in EPDCs, as endothelial cells, in hypoxia conditions such as occurs after myocardial infarction. In these conditions, Wt1 seem to be implicated in cell proliferation (Duim et al., 2015; Wagner et al., 2008).

$CD45^+$ cells were also identified in this *in vitro* cell population, indicating contamination of this coronary culture with blood-borne cells (Figure 35E, E'). $CD31^+$ cells could only be recorded in the original ventricular tissue explant and not in the halo of growing cells (Figure 35F, F'). Thus, in this situation, just non-endothelial mesenchymal cells contribute to the Wt1 reactivated pool, in contraposition to what occurs in hypoxia conditions, indicating that other mechanisms must be implicated in Wt1 reactivation.

After 3 weeks in culture, the round and poorly adherent cells previously described were isolated from the medium, seeded onto fresh poly-l-lysine-coated plates, and grown for 24 additional hours. Inspection of the cultures allowed for the identification of multicellular spherical cell bodies (cell clusters) displaying a restricted adhesion to the substrate (Figure 35G-I). Some of the cells enclosed in these interstitial cell bodies were $CD31^+$, αSMA^+ or $CD90^+$ (Figure 35H, I), suggesting a

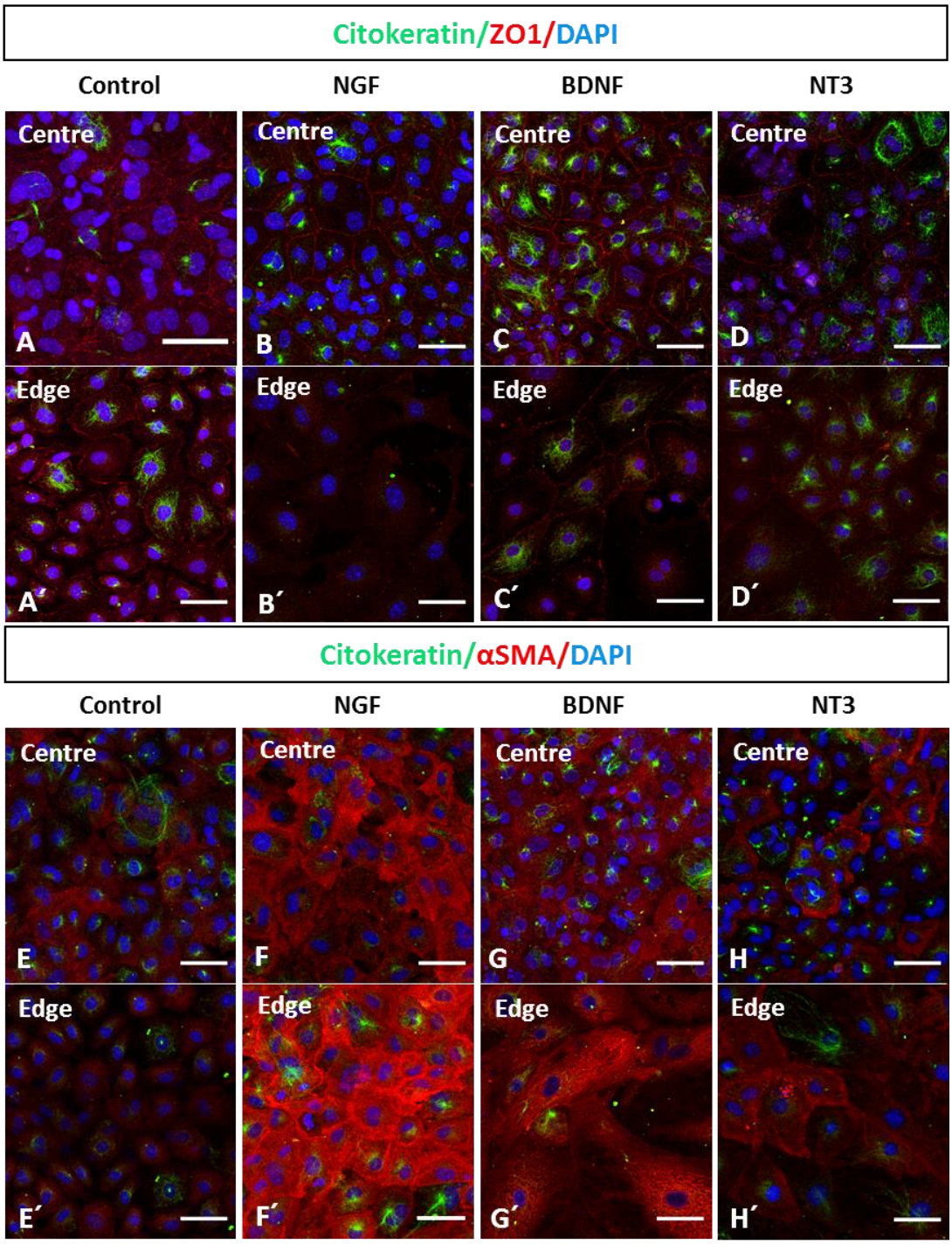

Figure 33. **Characterization of primary culture of embryonic epicardium after treatment with neurotrophic factors. (A-H´)** Differences related to morphology and molecule expression are observed between the centre of the epicardial cell monolayers and their edges. Expression of ZO1 is evident **(A-A´)** in control conditions as well as in **(B-D´)** neurotrophin treated samples, showing a trend to reduce epithelial markers at the border in treated cultures, particularly after NGF treatment **(B´)**. Expression of αSMA has been observed **(E-E´)** in control conditions as well as in neurotrophic factor-treated samples **(F-H´)**. Increased expression of this smooth muscle cell marker was evident **(F-F´)** after NGF treatment **(G-G´)**, in particular in the periphery of the cultures. Scale bars: **A-H´**, 50 μm. Abbreviations: αSMA = smooth muscle actin; DAPI = 4',6-diamidino-2-fenilindol.

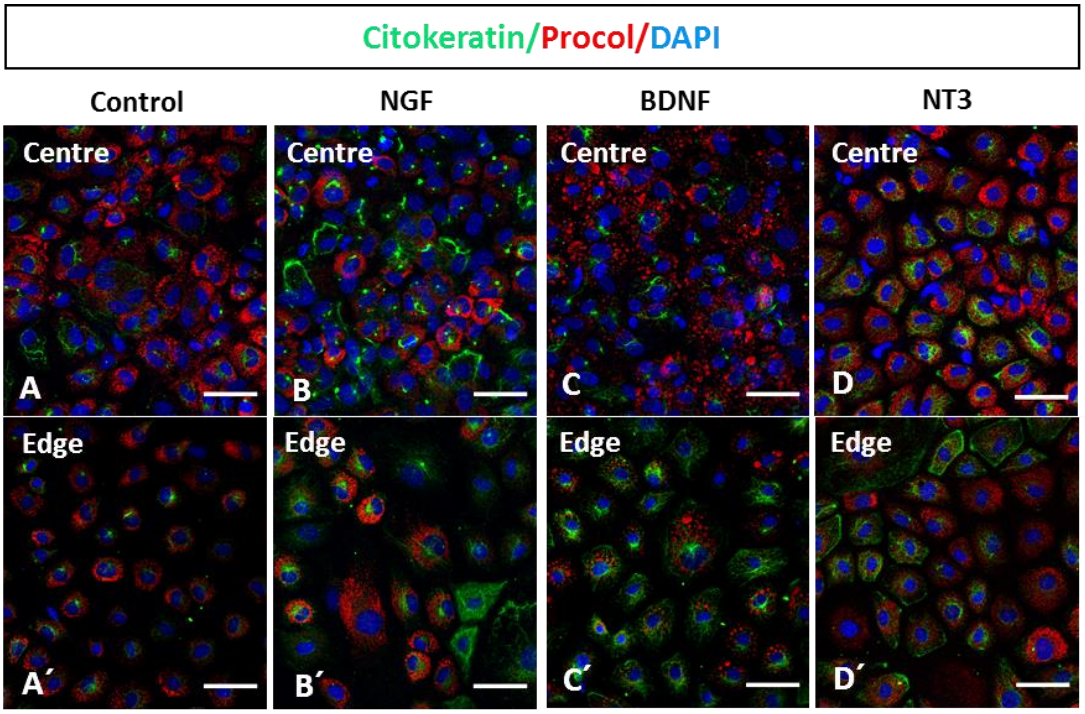

Figure 34. Characterization of primary culture of embryonic epicardium with neurotrophin treatment. (A-D') Procollagen synthesis is evident in control conditions **(A-A')** as well as in neurotrophin treated explants **(B-D')**, but its intracellular distribution expression pattern differs between the two culture conditions **(C-C')**. Scale bars: **A-D´**, 50 µm. Abbreviations: DAPI = 4',6-diamidino-2-fenilindol.

certain cell diversity for the cells that formed the spherical cell bodies *in vitro*, most likely in the vascular and connective tissue range.

Since single cell techniques have not been used in this experiment, the spheric cell body formation could have been occurred through proliferation and posterior differentiation of a single cell, or through reorganization of several cells with different properties. Previous studies support this first possibility, as single cell suspensions have been able to forma spheric cell bodies in a low proportion (Tomita et al., 2005). Moreover, as mentioned above, no $CD31^+$ cells were transferred into the spheric cell body induction medium, but CD31 expressing cells have been observed in the forming bodies. Altogether, these results suggest that the heart contains some cell progenitors with a restricted differentiation potential, most likely lacking the ability to differentiate into cardiac muscle cells.

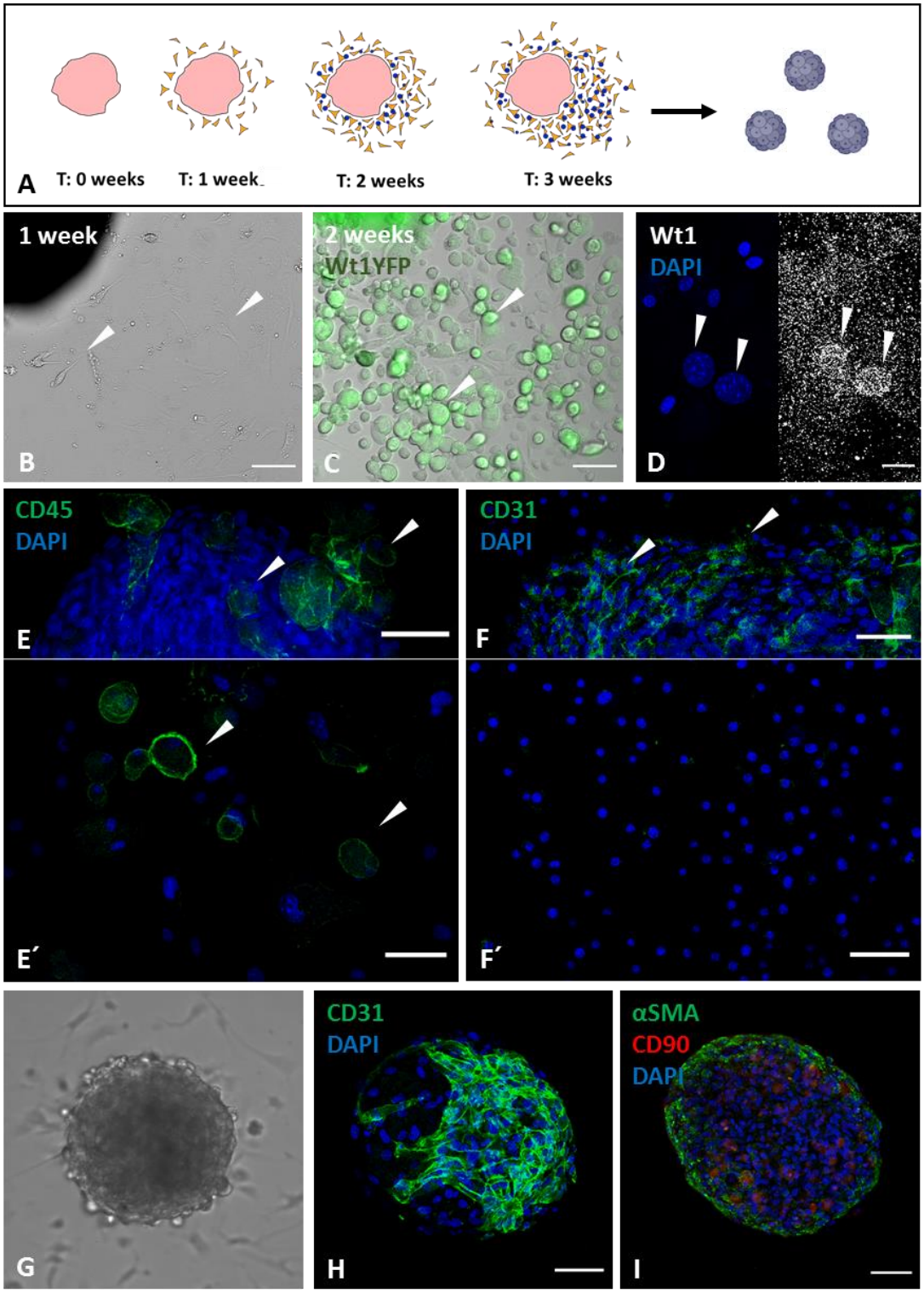

Figure 35. **Characterization cardiac interstitial-derived cells *in vitro*: formation of spheric bodies. (A-F')** Digested cardiac tissue explants are cultured and fibroblast-like cells appears few days after **(B, arrowheads).** The rise of small and bright round cells followed; explants derived from Wt1Cre-YFP^{+} hearts yield some round cells that express the YFP reporter **(C, arrowheads).** Wt1 protein accumulates in the nuclei of some of these cells (**D**, **arrowheads**). **(E-E')** Some CD45^{+} cells **(arrowheads)** were identified in the original ventricular explant **(E)** and in the cells outgrowing **(E')** the explant. At these stages of the culture, endothelial CD31^{+} cells **(arrowheads)** were observed in the original explanted tissue only **(F)**. **(G)** Multicellular spherical body formation is observed after additional culture *in vitro*. CD31- **(H)** and αSMA-expressing cells **(I)** are detected in the periphery of these spherical bodies, while CD90 is present in the internal core of the structure. Scale bars: **B-C, G,** 50 μm; **D-F´, H-I,** 25 μm. Abbreviations: DAPI = 4',6-diamidino-2-fenilindol. αSMA = smooth muscle actin; CD31/PECAM1 = platelet and endothelial cell adhesion molecule 1; CD45 = protein tyrosine phosphatase receptor type C; CD90 = Thy-1 cell surface antigen; DAPI = 4',6-diamidino-2-fenilindol; Wt1 = Wilms tumor 1.

III.4. *IN VIVO* EVALUATION OF INTERSTITIAL REMODELLING IN AN EXPERIMENTAL MODEL OF CARDIAC DAMAGE

In this final section of the Results and Discussion chapter of my thesis, specific aspects of cardiac interstitium interference after experimental injury and genetic disruption of normal NT3 signalling have been studied.

III.4.1. Evaluation of the interstitial structure in an experimental model of cardiac damage

Intradermic Angiotensin II (AngII) infusion in adult mice is a classical method to reproduce cardiac damage (Bader, 2002). In normal cardiac conditions, AngII is synthesized in the heart through the renin-angiotensin system (RAS), where perform fundamental functions inducing vasoconstriction or increased cardiac contraction after sympathetic nervous system stimulation. Nevertheless, in pathological conditions like pressure overload or myocardium infarction, an excess of AngII production occurs, intensifying smooth muscle cell proliferation and migration, fibroblast proliferation and collagen synthesis, which derives in pathological cardiac fibrosis (De Mello & Jan Danser, 2000).

To test the effect of AngII in the cardiac interstitium we used osmotic pumps to infuse AngII in adult mice. This experiment induced severe perivascular fibrosis, which can be easily identified as an intense red colour after classical picrosirius red tissue staining (Figure 36A, A'). Immunohistochemical evaluation of the cardiac tissue using anti-fibronectin antibodies confirmed the fibrotic nature of this pericoronary tissue (Figure 36B, B'). The tracking of the YFP reporter confirms that an important proportion of fibrotic perivascular cells are epicardial derived cells that do not belong to the endothelial or smooth muscle lineage, since they do not express neither the endothelial marker CD31 nor the αSMA smooth muscle marker (Figure 36C, C'). These findings are in accordance with the previously described massive

contribution of epicardial derivatives (from a $Wt1^+$ lineage) to the cardiac fibroblast cell compartment (Ruiz-Villalba et al., 2015).

Anti-CD45 antibodies highlighted the presence of significant numbers of leukocytes in the perivascular fibrous tissue, suggesting the activation of inflammatory-like phenomena in the expanding fibrotic tissue. However, neither c-Kit^+ (Figure 36D, D') nor $Sca1^+$ cells could be recorded in these same tissues (Figure 36E, E'), so that it is reasonable to conclude that this specific type of cardiac injury does not activate the recruitment or expansion of putative c-Kit^+ or $Sca1^+$ cell progenitors, as has been described for other damage models with massive death of cardiomyocytes (Bearzi et al., 2009; Ellison et al., 2013; Uchida et al., 2013; Wang et al., 2006).

III.4.2. Evaluation of the interstitial structure in an experimental model for reduced NT3 signalling

To study the effect of diminished NT3 signalling in the cardiac interstitium, since as demonstrated above NT3 expression is maintained through adulthood with unknown functions, $NT3^{LacZNeo}$ mice, which are known to show a 50% reduction of NT3 synthesis (Delgado et al., 2014), were used as experimental models. Remarkably, changes in NT3 expression are not evident in $NT3^{LacZNeo}$ mutants after NT3 immunohistochemical inspection (Figure 37A). The evaluation of coronary blood vessels using anti-CD31, αSMA, βIII-Tubulin, TrkB of c-Kit antibodies did not reveal any significant change in the composition of the vessels or their neighbouring perivascular cells (Figure 37B-E'). Moreover, the evaluation of EdU incorporation in cardiac tissue sections did not show significant differences between wild type and $NT3^{LacZNeo}$ samples either (Figure 37F).

To circumvent the known limitation of cell quantification in tissue sections, small pieces of the ventricular wall of adult wild type and $NT3^{LacZNeo}$ mice were dissected and dissociated as described in the Material and Methods section. FACS analysis of ventricular cell extracts did not reveal significant differences between wild

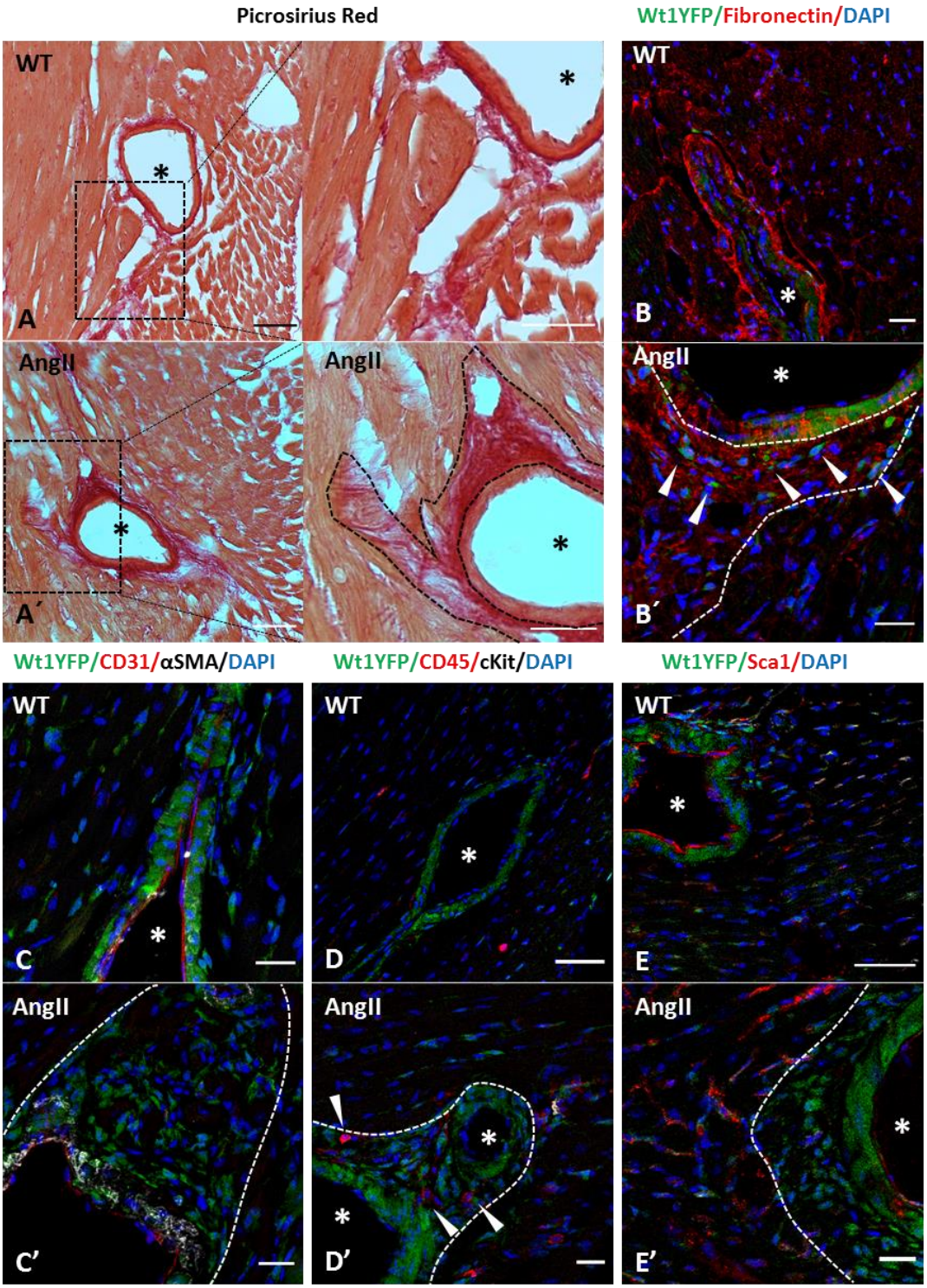

Figure 36. **Characterization of AngII-induced fibrosis in adult hearts**. Wt1-Cre-YFP mouse hearts display permanent expression of the YFP reporter in $Wt1^+$ lineage cells. **(A-B)** AngII-induced fibrosis is confirmed by **(A-A')** extensive picrosirius red perivascular staining, as well as by changes in the expression of molecules related to the extracellular matrix such as fibronectin **(B-B')**. In AngII-treated treated mice, numerous EPDCs $Wt1YFP^+$ **(B-E', arrowheads)** and $CD45^+$ cells **(D-D', arrowheads)** are embedded in the coronary perivascular fibrotic tissue**.** No expression of endothelial $CD31^+$ **(C-C')**, smooth muscle αSMA^+, or stem c-Kit^+/$Sca1^+$ cells **(D-D')** is observed in the affected tissue. Scale bars: **A-E'** 50 μm. Abbreviations: αSMA = smooth muscle actin; Asterisk (*) = coronary vessel; CD31/PECAM1 = platelet and endothelial cell adhesion molecule 1; CD45 = protein tyrosine phosphatase receptor type C; c-Kit = KIT proto-oncogene receptor tyrosine kinase; DAPI = 4',6-diamidino-2-fenilindol; Sca1 = stem cell antigen 1; YFP = yellow fluorescent protein.

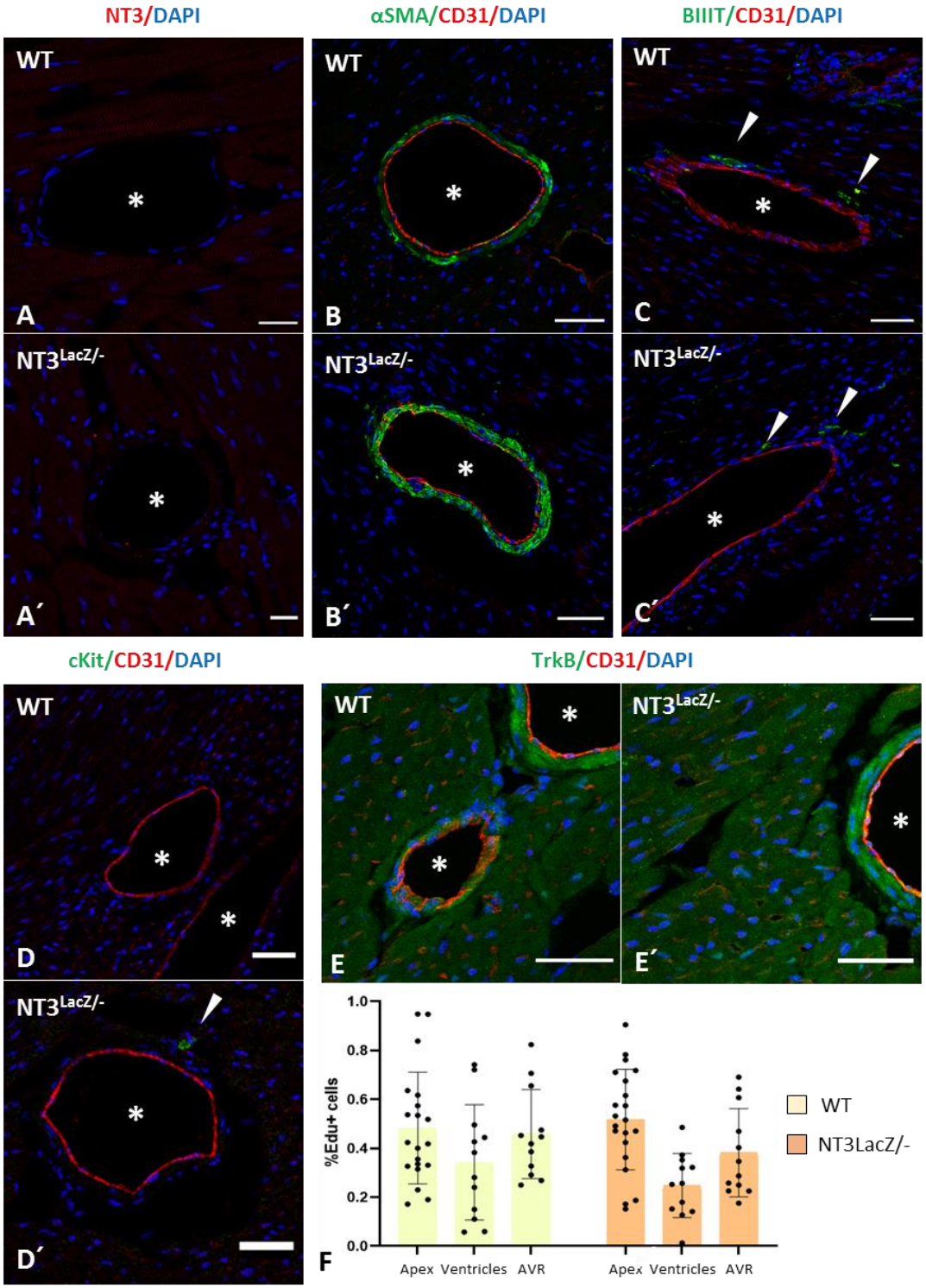

Figure 37. **Characterization of the adult NT3$^{LacZ/-}$ cardiac interstitium**. **(A)** Reduction of NT3 synthesis in these mutants cannot be confirmed using immunohistochemical approaches. No large-scale morphological or immunohistochemical differences have been detected in NT3$^{LacZ/-}$ large coronary vessels **(B-F, asterisk)** as compared to wild type samples in relation to endothelial CD31^{+} cells **(B-B')**, smooth muscle αSMA^{+} cells, bIIITubulin^{+} nerves **(C-C', arrowheads),** c-Kit expression **(D-D', arrowhead)** or TrkB expression **(E-E')**. **(F)** EdU quantification does not show significant changes in cell proliferation in diverse regions of the heart ($p < 0.05$). Scale bars: **A-E'**, 25 μm. Abbreviations: αSMA = smooth muscle actin; Asterisk (*) = coronary vessel; CD31/PECAM1 = platelet and endothelial cell adhesion molecule 1; c-Kit = KIT proto-oncogene receptor tyrosine kinase; DAPI = 4',6-diamidino-2-fenilindol; EdU = 5-Ethynyl-2´-deoxyuridine; NT3 = neurotrophin 3; TrkB = tyrosine receptor kinase 2.

type and $NT3^{LacZNeo}$ mutants in relation to the distribution of the main cell lineages analysed (endothelial and hematopoietic lineages) or the number of putative progenitor c-Kit^+/$Sca1^+$ cells in these lineages (Figure 38A-B), except when the percentage of $Sca1^+$ cells in the $CD45^+$ fraction was considered (Figure 38B) ($p<0.05$). This significant increase in the number of blood-borne Sca1 expressing cells could be caused by a higher migration and incorporation of these cells into the cardiac interstitium, mediated by altered cell signalling. Nevertheless, it is crucial remind that in the $NT3^{LacZNeo}$ model, the NT3 reduction occurs at a systemic level, thus it could also affect to the haematopoiesis system stimulating the production of $CD45^+$/$Sca1^+$ cells or inhibiting their differentiation into $CD45^+$/$Sca1^-$ cells. As a matter of fact, in previous studies the implication of neurotrophin factor in haematopoiesis has been demonstrated (Vega et al., 2003).

III.4.3. Evaluation of the interstitial structure in an experimental model for reduced NT3 signalling and cardial damage

Since no significant differences have been observed in the $NT3^{LacZNeo}$ mutant in homeostatic conditions when immunohistological and FACS techniques has been used (except $CD45^+$/$Sca1^+$ cells), the remained question is whether when the homeostatic conditions are broken and a cardiac damage occurs, this NT3 reduction could be crucial in the cardiac reparation/regeneration.

In order to answer this question, intradermic AngII infusion were performed in WT and $NT3^{LacZNeo}$ mice for 15 days, with a total duration of the experiment of 30 days. During this period, cardiac condition was assessed through magnetic resonance imaging (MRI) and diverse cardiac parameters as ventricular mass (VM), blood volume (V) and stroke volume (SV). Of these, ventricle stroke volume is the one that best reflects the cardiac function, identified as the ejection capacity of the heart in every cardiac cycle. No significant differences were detected between

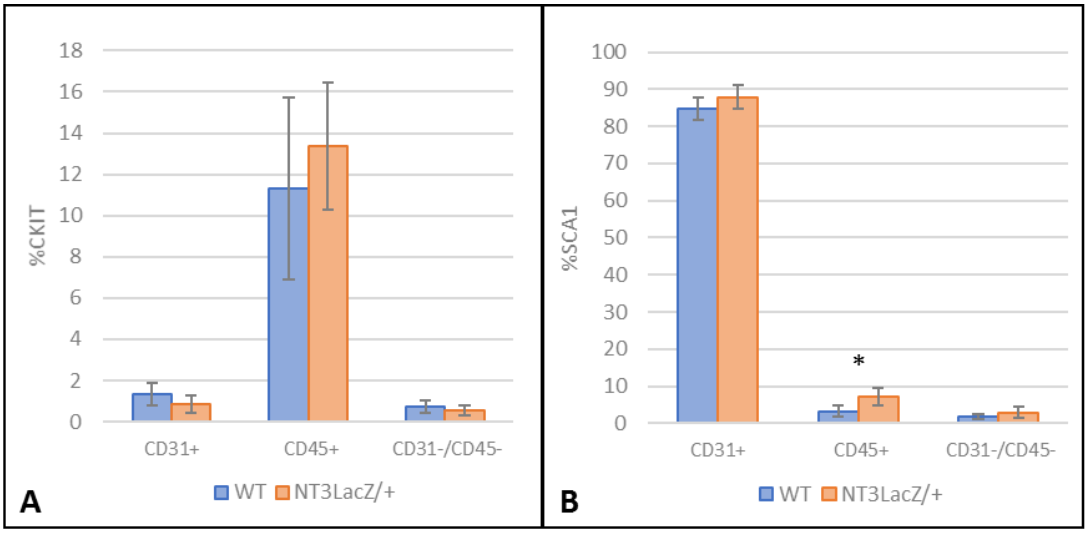

Figure 38. FACS analysis of c-Kit^{+}/Sca1^{+} cells at adult stage in NT3LacZ/+ model. **(A)** c-Kit quantification in CD31^{+}, CD45^{+} and CD31^{-}/CD45^{-} fractions and **(B)** Sca1 quantification in CD31^{+}, CD45^{+} and CD31^{-}/CD45^{-} fractions (*p<0.05). Abbreviations: CD31/PECAM1 = platelet and endothelial cell adhesion molecule 1; CD45 = protein tyrosine phosphatase receptor type C; c-Kit = KIT proto-oncogene receptor tyrosine kinase; Sca1 = stem cell antigen-1.

models (WT vs. NT3 deletion) or among experimental days, probably due to the sample variability derived from the difficulty of the technique, but it is possible to observe a trend with a decrease in the cardiac function at the end of the angiotensin treatment and a posterior minor recovering (Figure 39A).

After 30 days, the intracardiac blood was collected though cardiac punction and cardiac troponin levels were measured through ELISA (Figure 39B). Since troponin I is released in the blood after cardiomyocyte death, blood troponin concentration is a great unit measure for cardiac damage, but no differences were observed between WT and $NT3^{LacZNeo}$. Because of the low levels of cardiac troponin I, AngII does not seem to induce massive cardiomyocyte death as compared with others models, both in WT and $NT3^{LacZNeo}$ (Engle et al., 2009). Altogether, these results suggest that partial deletion of NT3 does not affect cardiovascular response to AngII damage.

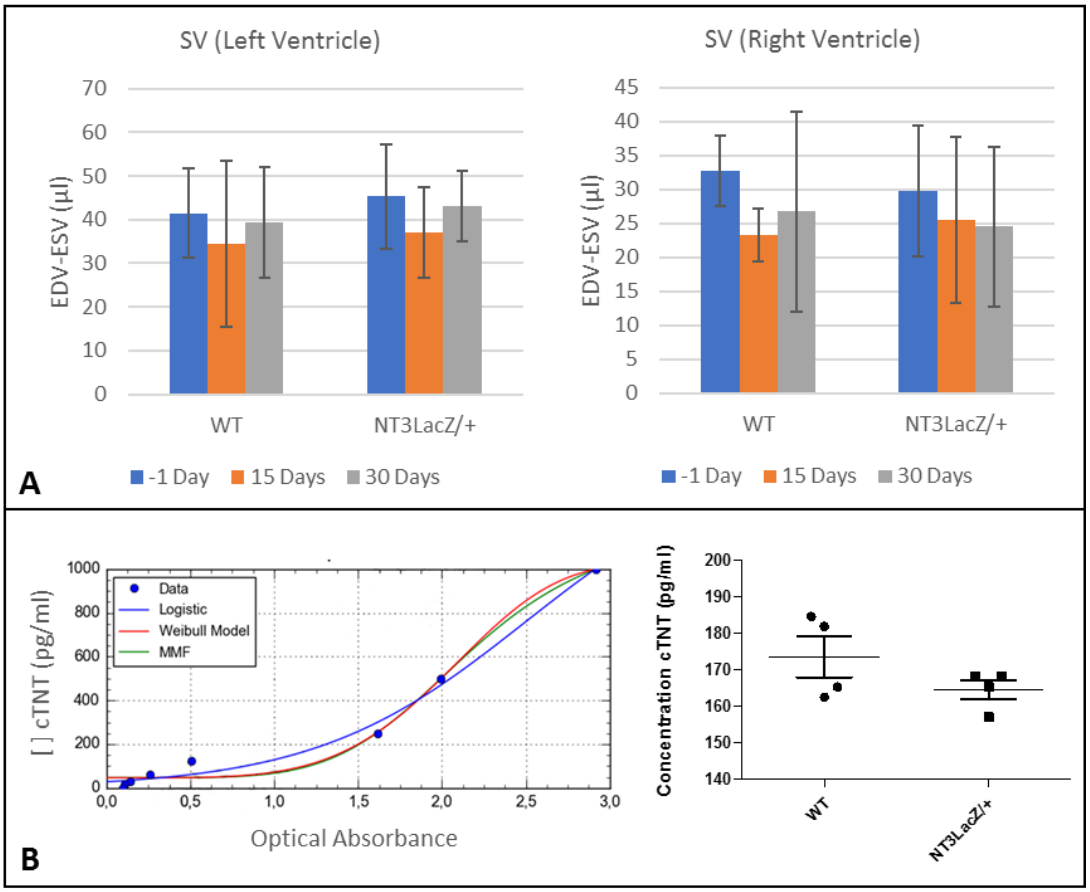

Figure 39. Characterization of Wt/NT3^{LacZNeo} mice after angiontensin II treatment. (A) Stroke Volume measurement -1, 15 and 30 days after experiment onset does not show significant differences in cardiac function (*p<0.05). **(B)** Troponin I concentration in blood at 30 days (*p<0.05).

SUPPLEMENTARY FIGURES

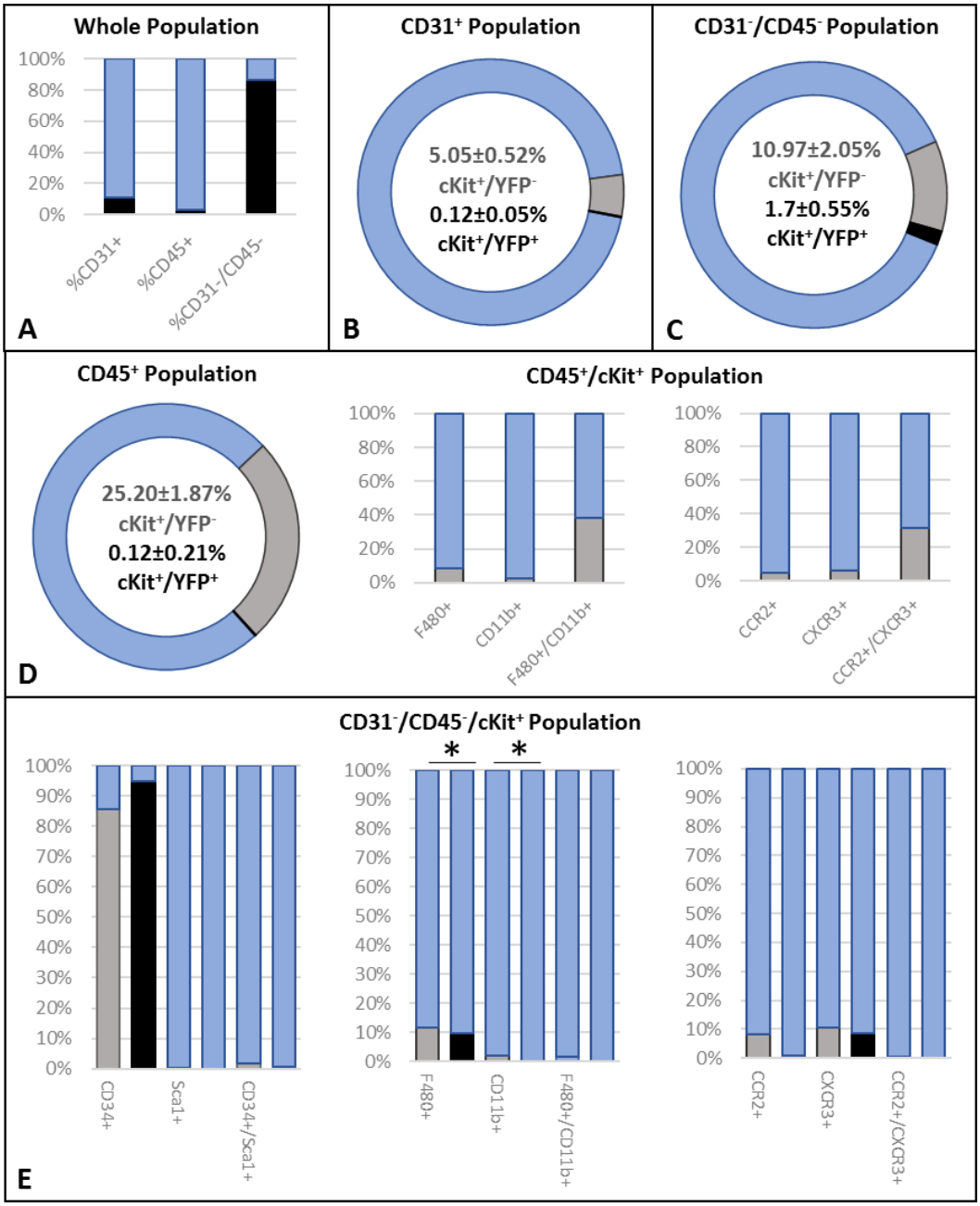

Figure 1S. **FACS analysis of c-Kit$^+$ cells at E18.5 in Wt1YFP mice with permanent YFP expression in the Wt1$^+$ cell lineage**. **(A)** CD31$^+$, CD45$^+$ and CD31$^-$/CD45$^-$ cardiac cell pools are represented in relation to the whole cardiac cell population. c-Kit$^+$/YFP$^-$ (grey) and c-Kit$^+$/YFP$^+$ (black) cell percentages are shown in CD31$^+$ **(B)**, CD45$^+$ **(D)** and CD31$^-$/CD45$^-$ **(C)** cardiac cell fractions. Characterization of CD45$^+$/c-Kit$^+$ **(D)** and CD31$^-$/CD45$^-$/c-Kit$^+$ **(E)** cells is also represented (*p<0.05). Abbreviations: CCR2 = C-C motif chemokine receptor 2; CD11b = Integrin alpha M; CD31/PECAM1 = platelet and endothelial cell adhesion molecule 1; CD34 = cluster of differentiation 34; CD45 = protein tyrosine phosphatase receptor type C; c-Kit = KIT proto-oncogene receptor tyrosine kinase; CXCR3 = C-X-C motif chemokine receptor 3; F4/80 = EGF-like module-containing mucin-like hormone receptor-like 1; Sca1 = stem cell antigen-1; YFP = yellow fluorescent protein.

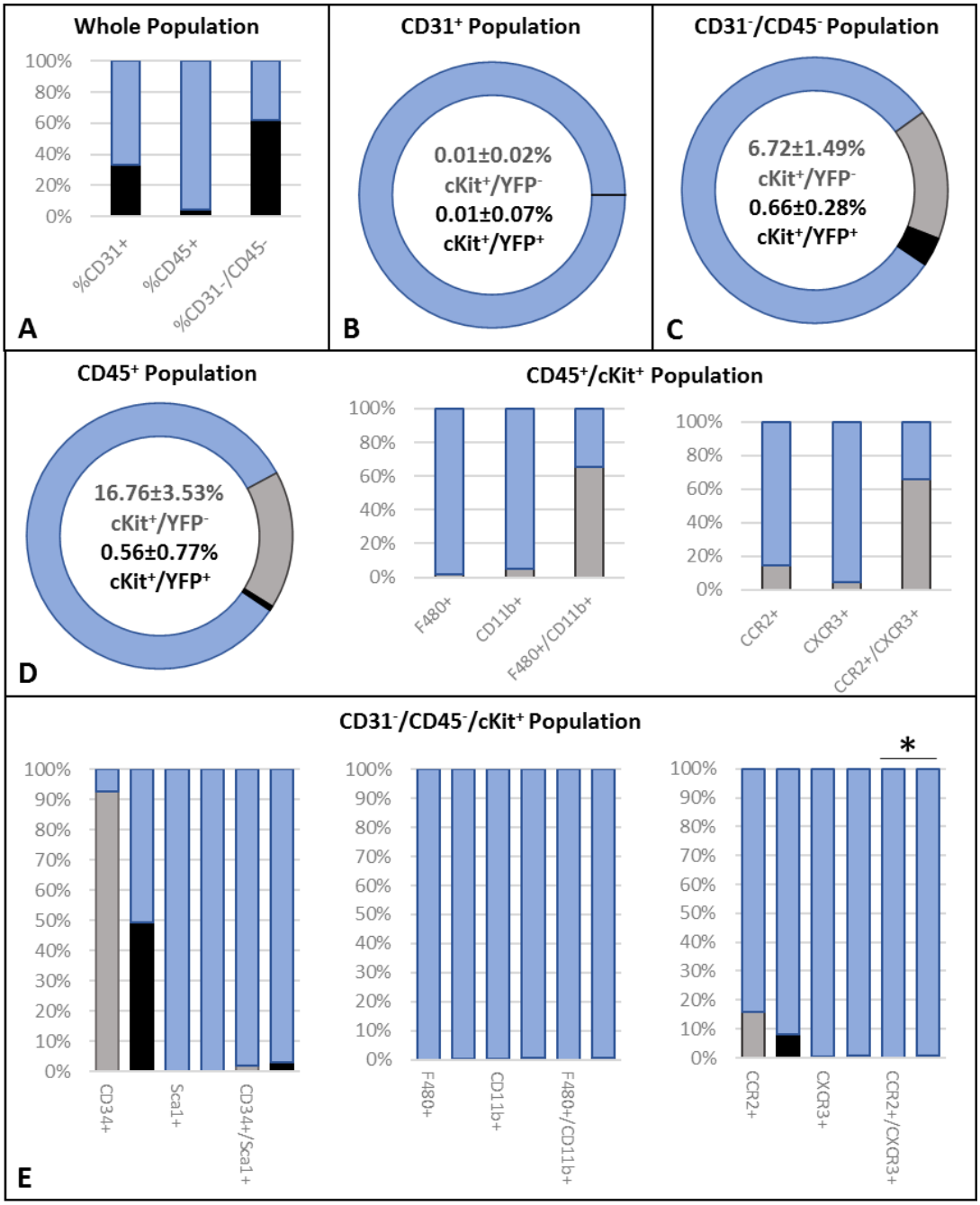

Figure 2S. **FACS analysis of c-Kit$^+$ cells at P7 in Wt1YFP mice with permanent YFP expression in the Wt1$^+$ cell lineage**. **(A)** CD31$^+$, CD45$^+$ and CD31$^-$/CD45$^-$ cardiac cell pools are represented in relation to the whole cardiac cell population. c-Kit$^+$/YFP$^-$ (grey) and c-Kit$^+$/YFP$^+$ (black) cell percentages are shown in CD31$^+$ **(B)**, CD45$^+$ **(D)** and CD31$^-$/CD45$^-$ **(C)** cardiac cell fractions. Characterization of CD45$^+$/c-Kit$^+$ **(D)** and CD31$^-$/CD45$^-$/c-Kit$^+$ **(E)** cells is also represented (*p<0.05). Abbreviations: CCR2 = C-C motif chemokine receptor 2; CD11b = Integrin alpha M; CD31/PECAM1 = platelet and endothelial cell adhesion molecule 1; CD34 = cluster of differentiation 34; CD45 = protein tyrosine phosphatase receptor type C; c-Kit = KIT proto-oncogene receptor tyrosine kinase; CXCR3 = C-X-C motif chemokine receptor 3; F4/80 = EGF-like module-containing mucin-like hormone receptor-like 1; Sca1 = stem cell antigen-1; YFP = yellow fluorescent protein.

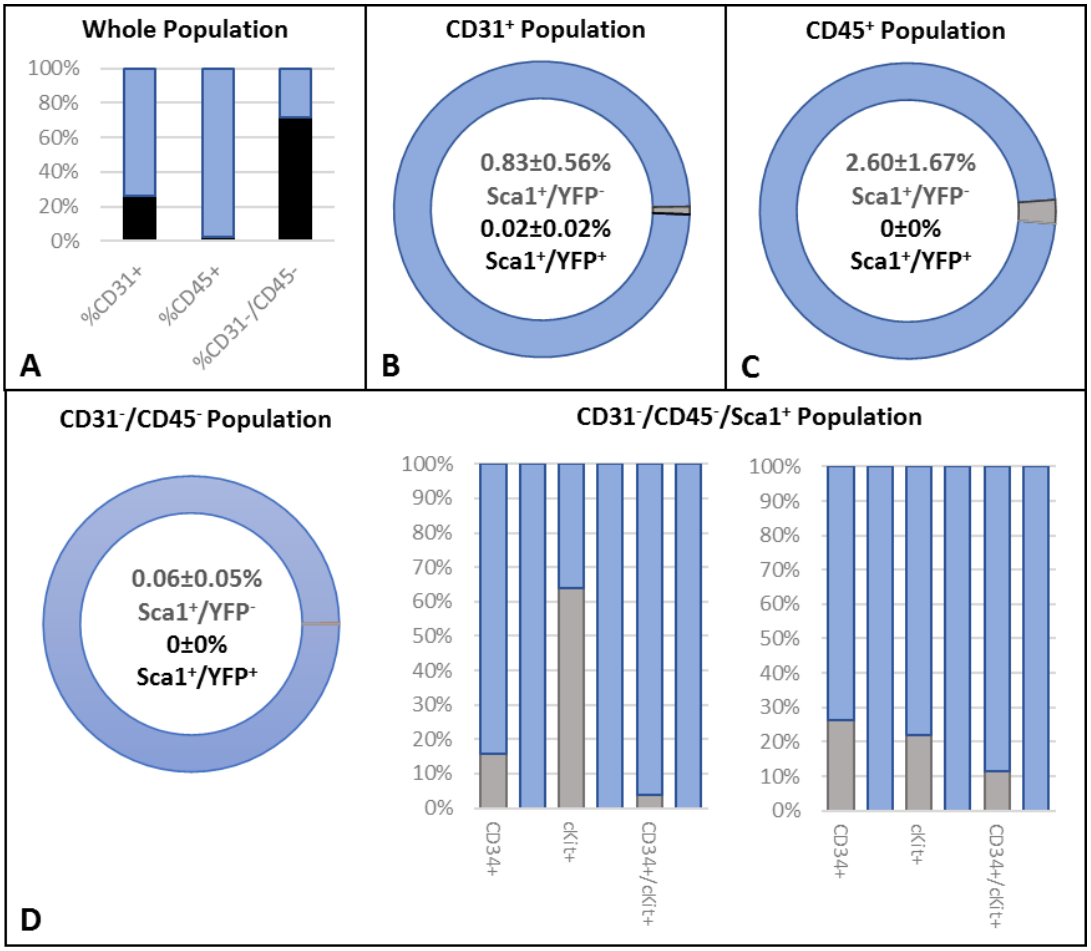

Figure 3S. **FACS analysis of E15.5 cardiac Sca1$^+$ cells.** Wt1YFP mouse hearts display permanent expression of the YFP reporter in Wt1$^+$ lineage cells. **(A)** Representation of the CD31$^+$, CD45$^+$ and CD31$^-$/CD45$^-$ cell fractions in the whole population. Sca1+/YFP- (grey) and Sca1$^+$/YFP$^+$ (black) percentages are represented with respect to CD31$^+$ **(B)**, CD45$^+$ **(C)** and CD31$^-$/CD45$^-$ **(D, left) cell** fractions. **(D, right)** Characterization of CD31$^-$/CD45$^-$/Sca1$^+$ cells. (*p<0.05). Abbreviations: CCR2 = C-C motif chemokine receptor 2; CD146 = cluster of differentiation 146; CD31/PECAM1 = platelet and endothelial cell adhesion molecule 1; CD34 = cluster of differentiation 34; CD45 = protein tyrosine phosphatase receptor type C; c-Kit = KIT proto-oncogene receptor tyrosine kinase; Sca1 = stem cell antigen-1; YFP = yellow fluorescent protein.

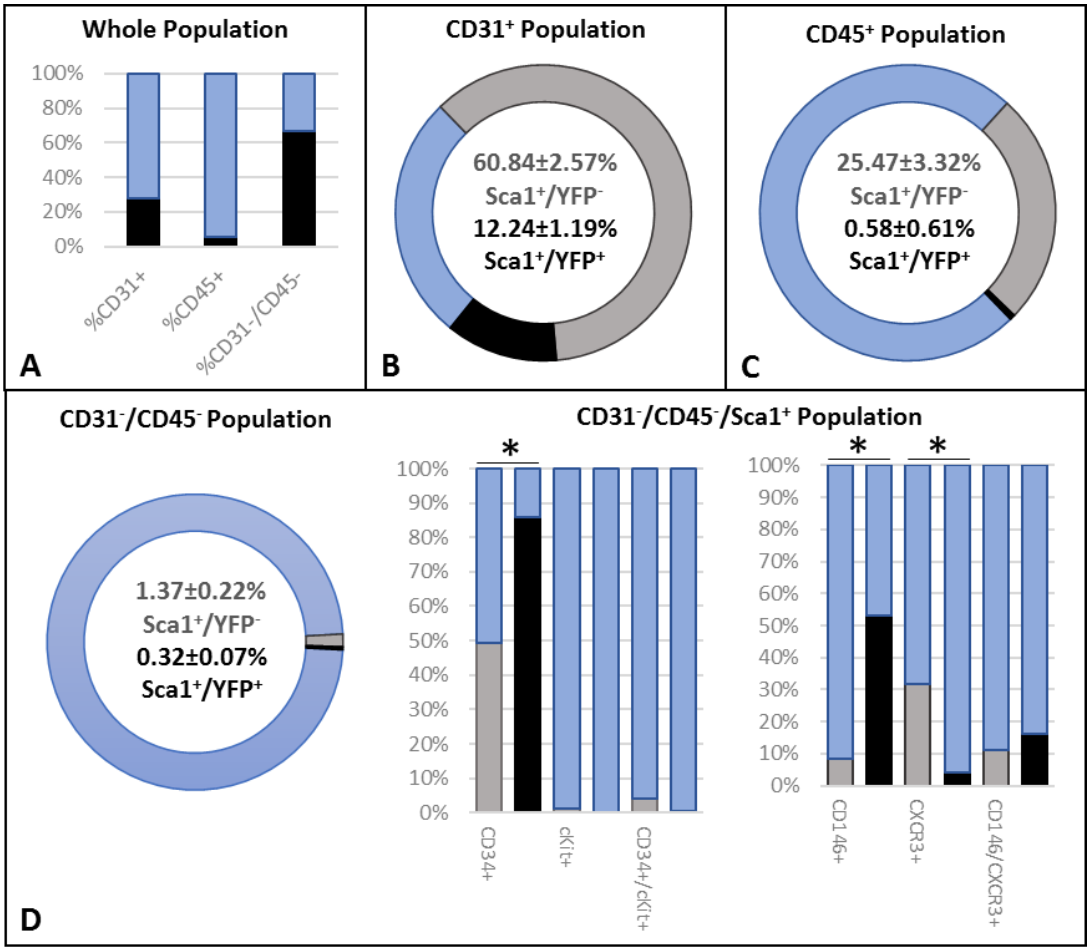

Figure 4S. **FACS analysis of P7 cardiac Sca1⁺ cells.** Wt1YFP mouse hearts display permanent expression of the YFP reporter in Wt1⁺ lineage cells. **(A)** Representation of the CD31⁺, CD45⁺ and CD31⁻/CD45⁻ cell fractions in the whole population. Sca1+/YFP- (grey) and Sca1⁺/YFP⁺ (black) percentages are represented with respect to CD31⁺ **(B)**, CD45⁺ **(C)** and CD31⁻/CD45⁻ **(D, left) cell** fractions. **(D, right)** Characterization of CD31⁻/CD45⁻/Sca1⁺ cells. (*p<0.05). Abbreviations: CCR2 = C-C motif chemokine receptor 2; CD146 = cluster of differentiation 146; CD31/PECAM1 = platelet and endothelial cell adhesion molecule 1; CD34 = cluster of differentiation 34; CD45 = protein tyrosine phosphatase receptor type C; c-Kit = KIT proto-oncogene receptor tyrosine kinase; Sca1 = stem cell antigen-1; YFP = yellow fluorescent protein.

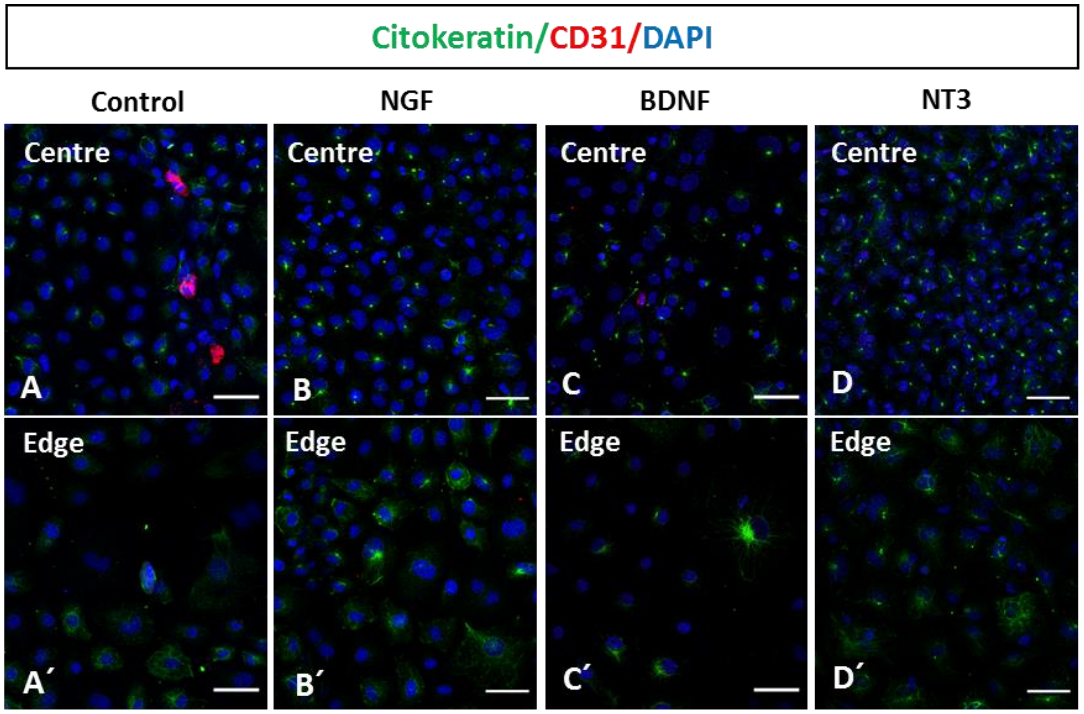

Figure 5S. **Characterization of primary culture of embryonic epicardium with neurotrophin treatment. (A-D´)** CD31 expression is restricted to scarce cells **(A-A´)** in control conditions as well as **(B-D´)** neurotrophin treated explants with similar proportion. Scale bars: **A-D´,** 50 µm. CD31/PECAM = platelet and endothelial cell adhesion molecule 1; DAPI = 4',6-diamidino-2-fenilindol.

CONCLUSIONS

CONCLUSION 1. The embryonic, postnatal and adult cardiac interstitium contains low but significant numbers of c-Kit$^+$ and Sca1$^+$ cells. These c-Kit$^+$ cells often associate to the wall of coronary blood vessels.

CONCLUSION 2. Some c-Kit$^+$ cells express molecular markers (e.g., CD45 or CD34) that indicate they belong to blood cell lineages.

CONCLUSION 3. A small fraction of cardiac Sca1$^+$ cells express the YFP reporter in transgenic Wt1Cre-RosaYFP mice. This strongly suggests an epicardial origin for these cells.

CONCLUSION 4. Sca1$^+$ cells express molecular markers (e.g., CD45 or CD31) that relate them with blood or endothelial cell lineages.

CONCLUSION 5. Cardiac NT3 expression in embryonic and adult cardiac tissues is restricted to cardiomyocytes and some circulating blood cells.

CONCLUSION 6. In the heart, the neurotrophic factor receptor TrkB is mildly expressed in the myocardium and strongly in cardiac nerves and coronary blood vessels (endothelial and smooth muscle cells).

CONCLUSION 7. NT3 (100ng/ml) promotes the proliferation of endothelial cells *in vitro* (HUVEC). Other neurotrophic factors tested (BDNF, NGF) did not have the same effect than NT3.

CONCLUSION 8. NGF (100ng/ml) induces the downregulation of the epithelial phenotype of embryonic epicardial cells explanted *in vitro,* a finding that suggests a role for this molecule in epicardial epithelial-to-mesenchymal transition. Other neurotrophic factors tested (BDNF, NT3) did not have the same effect than NGF.

CONCLUSION 9. Some cardiac interstitial cells can be cultured as multicellular spherical bodies *in vitro*. These structures are poorly adherent and may precede from cells that express the Wt1 gene, a master regulator of epicardial cells.

So far, no significant numbers of c-Kit$^+$ and/or Sca1$^+$ cells have been identified in these *in vitro* spherical cell clusters.

CONCLUSION 10. Endothelial and smooth muscle cells spontaneously differentiate within these pluricellular bodies, that closely resemble the so-called *cardiospheres* described in the literature.

CONCLUSION 11. Intradermic Angiotensin II infusion in Wt1Cre-RosaYFP mice induces a pericoronary vascular fibrosis extensively formed by YFP$^+$ fibroblasts, e.g., fibroblasts of epicardial origin.

CONCLUSION 12. Significant numbers of CD45$^+$ but not of c-Kit$^+$ or Sca1$^+$ cells are found in the fibrotic perivascular domains of Angiotensin II-treated mice.

CONCLUSION 13. Genetic deletion of one NT3 allele (reducing normal NT3 signalling by a 50%) does not modify the cardiac interstitium in homeostatic conditions but apparently reduces cardiac fibrosis when treated with angiotensin II.

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SUMMARY (IN SPANISH)
RESUMEN (EN CASTELLANO)

RESPUESTA DE LA CÉLULA PERICORONARIA AL DAÑO CARDÍACO: CELULAS SANGUÍNEAS E INTERACCIONES NEUROVASCULARES. RESUMEN

Durante los últimos 15 años, diferentes laboratorios han descrito la existencia de células madre cardíacas (CSC, del inglés *Cardiac Stem Cells*) pluripotentes residentes en el intersticio cardíaco, que es el espacio extracelular situado entre las fibras musculares del corazón. Las CSC, cuyo origen embrionario nunca fue descrito de manera sistemática, fueron caracterizadas por la expresión de un número finito de marcadores moleculares (fundamentalmente c-Kit, Sca1, Bmi1, Isl1) y su potencial de diferenciación valorado in vitro e in vivo. Diversos estudios concluyeron que las CSC eran capaces de originar distintos tipos celulares cardiovasculares como células endoteliales, células musculares lisas y cardiomiocitos. Esta conclusión permitió considerar a las CSC, al mismo tiempo, como el origen de la renovación de cardiomiocitos durante la vida adulta y como una esperanza para la regeneración terapéutica del corazón enfermo. Sin embargo, diversos estudios han demostrado de manera convincente que, si bien existen células cardíacas residentes que expresan marcadores asociados con la multipotencia (como c-Kit o Sca1), esas células no son pluripotentes y, sobre todo, no tienen capacidad para diferenciarse en cardiomiocitos. Los principales objetivos de esta tesis son estudiar estos tipos celulares en relación a su origen embrionario así como su papel en el espacio intersticial pericoronario en el que se encuentran y estudiar si el microambiente intersticial podría actuar como un nicho de soporte para progenitores celulares con potencial de diferenciación restringido. En particular, estudiaremos los vasos sanguíneos del sistema vascular coronario y los nervios periféricos cardíacos como componentes fundamentales del intersticio cardíaco.

1. Introducción

1.1. Desarrollo embrionario del corazón

El desarrollo del corazón es un evento dinámico y progresivo. A lo largo de la embriogénesis diversas poblaciones mesenquimáticas originalmente indiferenciadas y localizadas fuera del primordio cardiaco se incorporan a éste para dar lugar a nuevo músculo o contribuir al desarrollo del sistema coronario y a las poblaciones intersticiales (no miocárdicas) del corazón. Al menos una parte de estas células mesenquimáticas parecen proceder o estar estrechamente relacionadas con el epicardio, el tejido celómico que recubre la superficie cardíaca.

El epicardio es un tejido imprescindible para el correcto desarrollo cardiaco. Además de recubrir y proteger la superficie externa del corazón, el epicardio genera -a través de una transición epitelio-mesénquima- un conjunto de células mesenquimáticas, pluripotentes y con propiedades invasivas que contribuyen al desarrollo del endotelio, la musculatura lisa y la capa adventicia de los vasos coronarios, así como al tejido fibroso (intersticial) del corazón. Por otro lado, estudios recientes indican que a partir de un momento determinado en el desarrollo, las propiedades proliferativas del miocardio ventricular no son completamente autónomas, sino que dependen de una regulación paracrina debida al epicardio, de forma tal que la alteración de las propiedades señalizadoras del tejido epicárdico provocan una grave hipoplasia ventricular.

1.2. Capacidad regenerativa en el corazón de mamíferos

Durante muchos años el corazón adulto de mamíferos ha sido considerado un órgano postmitótico, ya que poco después del nacimiento se produce la transición desde una fase de crecimiento hiperplásico (crecimiento por proliferación de cardiomiocitos) a una fase de crecimiento hipertrófico en el que no aumenta el número de cardiomiocitos sino su tamaño. Esta transición está correlacionada con la maduración de los cardiomiocitos, consistente en la duplicación del material

genético sin citocinesis, el incremento en la densidad de microfibrillas y la aparición de discos intercalares maduros. En muchos casos se ha considerado que este cambio marcaba la pérdida de las propiedades regenerativas en favor de los mecanismos reparativos del órgano (fibrosis y cicatrización). De hecho, en ratones neonatos de 1 día, la resección del ápex supone la formación primaria de un coágulo de sangre que es sustituido progresivamente por matriz extracelular y células musculares gracias a la proliferación de los cardiomiocitos existentes, mientras que en neonatos de 7 días se produce una fibrosis similar a la de un organismo adulto con pérdida de masa miocárdica y ausencia de regeneración real.

Sin embargo, durante los últimos quince años se ha descubierto que en circunstancias normales el miocardio adulto de los mamíferos se renueva lenta pero constantemente. Esta renovación se ha atribuido tanto a pequeñas poblaciones de cardiomiocitos inmaduros y proliferativos como a diferentes tipos de células cardíacas residentes con perfil de células madre o progenitores, denominadas por algunos investigadores células madre cardíacas (CSC, del inglés *Cardiac Stem Cell*). Estas células se caracterizan por su capacidad de autorrenovación, formación de cardiosferas y diferenciación clonal en diferentes tipos celulares cardiovasculares (células endoteliales, células musculares lisas, cardiomiocitos y fibroblastos). Algunas de estas células también expresan diversos marcadores como c-Kit, Bmi-1 o Sca-1, asociados a la condición troncal.

1.3. Células madre cardíacas: origen y caracterización

El origen de estas CSC es muy discutido. De hecho, aún se desconoce si los marcadores moleculares mencionados anteriormente identifican a linajes ontogenéticos diferentes o a distintos estados fisiológicos o de diferenciación de un mismo linaje[11]. Esta cuestión es de gran importancia, ya que el origen embrionario de estas células podría determinar, en gran medida, su respuesta a señales moleculares y estímulos diversos (patológicos o no).

Numerosos investigadores consideran a los progenitores epicárdicos embrionarios, contenidos en el proepicardio, como un posible origen de CSC. El proepicardio, un grupo de células de origen celómico no incluido en el creciente cardíaco y situado en la región caudal del corazón en desarrollo, da lugar a un epicardio primitivo cuando las células proepicárdicas se adhieren a la superficie miocárdica mediante vellosidades y cubren el miocardio formando un epitelio monoestratificado. Inmediatamente este epicardio primitivo experimenta una transición epitelio-mesénquima, con lo que algunas células epiteliales sufren un cambio fenotípico profundo y se transforman en células mesenquimáticas que reciben el nombre de EPDC (del inglés, *Epicardial-Derived Cell*), que migrarán hacia el subepicardio primero (matriz extracelular situada entre el epicardio y el miocardio) y posteriormente hacia el miocardio, donde contribuyen al sistema de vasos que irriga el corazón y que conocemos como sistema vascular coronario. Por lo tanto, el epicardio se genera de forma independiente con respecto al miocardio y al otro epitelio cardíaco, el endocardio (ambos derivados del campo cardíaco primario y secundario). La médula ósea también ha sido considerada posible fuente de CSC. Durante las últimas fases del desarrollo embrionario de ratón y la vida adulta se produce la incorporación de células derivadas de médula ósea a las paredes cardíacas, especialmente alrededor de las arterias coronarias. En algunas ocasiones se ha observado que estas células, caracterizadas por la expresión de CD45 o CD34, también expresan algunos de los marcadores mencionados anteriormente como c-Kit y Sca-1.

Dentro de las células con fenotipo de CSC, las células c-Kit^{+} son las mejor caracterizadas desde su descubrimiento a principios de siglo. Con excepción de Sultana et al. 2015[1], la mayoría de los estudios han establecido un número muy bajo de estas células en el corazón adulto, de las cuáles una proporción variable expresan CD45 y por tanto son derivadas de médula ósea. Las células c-Kit^{+}/CD45^{-} se caracterizan por una expresión elevada de marcadores mesenquimales (CD90 y CD166), marcadores de diferenciación a cardiomiocitos (Nkx2.5 y GATA4) y de otros marcadores de células madre o progenitoras como Bmi-1, Flk-1 o MDR1. Sin

embargo, son negativas para Isl-1 o Sca-1. *In vitro* se comportan como células clonogénicas con capacidad de autorenovación y multipotencia, ya que pueden formar cardiosferas y dar lugar a células endoteliales, de musculatura lisa o cardiomiocitos con un fenotipo inmaduro, pero se ha demostrado que *in vivo* su capacidad para originar cardiomiocitos es muy limitada, tanto en condiciones de homeostasis como tras un daño isquémico. Estos datos se consideran en la actualidad como una refutación consistente de la existencia de CSC tal y como fueron originalmente descrita (células pluripotentes con capacidad de diferenciarse en cardiomiocitos).

Sca-1, otra de las moléculas asociadas a las CSC, se expresa en el 14-17% de la fracción no miocítica adulta, en células localizadas junto a la lámina basal de los vasos coronarios o asociadas a cardiomiocitos. Un elevado porcentaje no expresa otros marcadores de células madre como c-Kit, Flt-1 o Flk-1, pero sí marcadores relacionados con la diferenciación endotelial o cardiomiocítica. La proporción de células Sca-1$^+$/CD45$^+$ del total de células Sca-1$^+$ varía según el estudio, caracterizándose la población Sca1$^+$/CD45$^-$ por ser capaz en cultivo de diferenciarse en los tres linajes principales con un fenotipo en la mayoría de los casos inmaduro. *In vivo* tienen una escasa capacidad para formar cardiomiocitos, tanto en homeostasis como después de un daño.

Algunas células Sca-1$^+$ destacan por su capacidad intrínseca para expulsar el colorante Hoechst gracias a que poseen transportadores de tipo ATP, como el transportador Abcg2. Esta población, denominada *Side Population* (SP), disminuye con la edad del animal[16,26] y se caracteriza por no expresar marcadores de células derivadas de médula ósea (CD45, CD34), pero sí expresar en un porcentaje variable marcadores de linaje endotelial (CD31 o Tie2) o de linaje cardiomiocítico (Nkx2.5, GATA4, αSMA). En cocultivo con cardiomiocitos maduros son capaces de originar cardiomiocitos MEF2C$^+$, GATA4$^+$, α-actinina$^+$ y Troponina T$^+$. Se ha observado que tras un infarto de miocardio su número disminuye rápidamente por diferenciación a células endoteliales CD31$^+$ y vWF$^+$, pero poco después la población original se recupera por división celular de la población remanente

Las células cardíacas Bmi1^{+} también parecen constituir otra subpoblación de células Sca-1^{+}/CD31^{+} localizadas perivascularmente o en el intersticio entre cardiomiocitos. Tanto *in vitro* como *in vivo* (homeostasis e infarto de miocardio) pueden diferenciarse en endotelio, musculatura lisa y cardiomiocitos.

1.4. El concepto de nicho. Nervios y vasos

Las verdaderas células madre y también muchos progenitores celulares se localizan en nichos. Un nicho se define como el microambiente específico en el que estas células mantienen sus propiedades singulares, incluyendo su potencial de desarrollo y habilidad para la auto-renovación. Los nichos de células madre están normalmente formados por: 1) células madre, 2) células derivadas de células madre/progenitores en diferenciación, 3) células de soporte que regulan el comportamiento de las células madre mediante señalización endocrina y paracrina, 4) matriz extracelular característica, y 5) moléculas señalizadoras que modulan las propiedades de las células madre. En el corazón, algunas de las funciones del nicho pueden ser realizadas por cardiomiocitos y células con apariencia fibroblástica, una idea que es respaldada por la existencia de complejos de unión entre las células intersticiales de fenotipo fibroblástico y las CSC.

Si el intersticio cardíaco actúa como un nicho real, es muy posible que las estructuras vasculares participen en su organización, como ocurre en otros órganos que albergan células madre como el hígado, el cerebro, el bazo o el hueso. De acuerdo con esto, otro dominio cardíaco extensivamente vascularizado como el epicardio ha sido también considerado como nicho cardíaco. Un posible elemento adicional en el establecimiento del nicho cardíaco podría ser el sistema nervioso simpático, dada su asociación con los vasos sanguíneos en numerosos órganos. En el corazón de ratón, la inervación simpática comienza a 13,5dpc junto a las venas de gran diámetro (EPHB4^{+}) situadas entre el epicardio y la superficie del miocardio (el subepicardio). Posteriormente, estas fibras invaden el miocardio donde se desarrollan las arterias coronarias (endotelio EfrinaB2^{+}). Esta relación entre los dos

tejidos depende de una señalización cruzada dependiente de neurotrofinas y endotelinas.

1.5. Neurotrofinas

Descrita por primera vez en el sistema nervioso, la familia de neurotrofinas está formada por cuatro polipéptidos muy relacionados en mamíferos: factor de crecimiento nervioso (en inglés *nerve growth factor*, NGF), factor neurotrófico derivado del cerebro (en inglés *brain derived neurotrophic factor*, BDNF), NT-3 y NT4/5. Todas estas formas son sintetizadas como proteínas precursoras que son procesadas intracelularmente por furinas o extracelularmente por metaloproteinasas dando lugar a neurotrofinas maduras que se asocian como homodímeros[44,45]. Las formas maduras de neurotrofinas son reconocidas por receptores del tipo tirosina quinasa, concretamente por los miembros de la familia Trk: TrkA, TrkB y TrkC y sus respectivas isoformas truncadas. Estos receptores, que actúan como homodímeros, se caracterizan por su elevada homología en el dominio intracelular difiriendo en la fracción extracelular responsable del reconocimiento de la neurotrofina. Así, TrkA actúa como receptor de NGF, ambos BDNF y NT4/5 activan a TrkB y NT-3 es el principal ligando de TrkC, aunque también pueden asociarse a TrkA y TrkB con menor eficiencia. En ausencia de receptores TrkB las neurotrofinas también pueden interactuar con el receptor $p75^{NTR}$, implicado en la apoptosis y muerte celular.

Aunque el estudio de las neurotrofinas y sus receptores en el corazón de mamíferos aún se encuentra en un estado poco desarrollado, la información existente refleja la gran importancia que estas moléculas poseen tanto en el desarrollo como en mantenimiento de la homeostasis adulta.

Una de las neurotrofinas mejor caracterizadas en la actualidad es NGF, dada su importancia en la formación del sistema nervioso simpático cardíaco. En ratones, NGF comienza a expresarse a partir de 15,5dpc en la musculatura lisa de los vasos coronarios, ejerciendo una señalización paracrina sobre los nervios simpáticos procedentes del ganglio estrellado que acaban de contactar con la superficie del

corazón. Esto tiene como resultado que los nervios simpáticos se extiendan sobre los vasos coronarios según el patrón mencionado anteriormente. Posteriormente, la señalización ejercida por las células musculares lisas disminuye, pero su expresión aumenta en los cardiomiocitos adultos donde parece ejercer una señalización autocrina importante para la supervivencia y homeostasis de éstos.

La expresión de BDNF y TrkB también comienza en el endotelio y musculatura lisa de los vasos coronarios, durante la embriogénesis tardía. Esta localización, así como los experimentos de inhibición realizados, sugieren que BDNF actúa de forma autocrina sobre las células endoteliales promoviendo su supervivencia y de forma paracrina sobre pericitos y células musculares lisas promoviendo su reclutamiento hacia los vasos y la producción de matriz extracelular. TrkB también se expresa en los cardiomiocitos adultos, aunque su importancia en la actividad miocárdica no está demostrada.

NT-3, por el contrario, es una neurotrofina con una función significativa en la proliferación temprana de los cardiomiocitos inmaduros durante las primeras fases del desarrollo del corazón, limitándose posteriormente su expresión a los vasos coronarios durante el estadio adulto. Dada la ausencia de TrkC en arterias y venas coronarias, es posible que NT-3 ejerza su función a través de TrkB.

2. Hipótesis y Objetivos

La hipótesis básica de este trabajo es que el espacio intersticial, especialmente el espacio intersticial pericoronario, determina un auténtico nicho para diversos tipos de CSC, de forma tal que la modulación de las propiedades del nicho podría permitir activar una respuesta regenerativa endógena en el corazón dañado. Los objetivos científicos concretos se detallan a continuación:

Objetivo 1: Analizar la ontogenia de diversas poblaciones de células madre cardíacas o progenitores cardíacos (c-Kit, Sca-1, y Bmi-1).

Objetivo 2: Estudiar la localización de las diversas células madre cardíacas en el corazón fetal, postnatal y adulto, especialmente en relación a los vasos sanguíneos del sistema coronario y a los nervios cardíacos.

Objetivo 3: Valorar el papel las señales moleculares en el mantenimiento de un posible nicho de progenitores cardíacos en el corazón adulto con un énfasis especial en el papel de las neurotrofinas y de las señales derivadas del endotelio y de la pared muscular de los vasos.

Objetivo 4: Identificar la dinámica y la respuesta específica de las células madre y progenitores residentes en el corazón enfermo.

3. Resultados y discusión

Los experimentos desarrollados y sus resultados se presentan a continuación de manera resumida en relación con los objetivos científicos descritos anteriormente.

Objetivo 1:

Tarea 1: Analizar la relación ontogenética de los progenitores cardiovasculares c-Kit, Sca-1, Bmi1 e Islet-1 con los linajes celulares epicárdicos mediante el uso de trazado genético Cre/LoxP. Se han usado líneas de ratones trangénicos ($Wt1^{Cre}$ y $G2GATA4^{Cre}$) que tras su cruzamiento con la línea *reporter* $^{ROSA26}YFP$ permiten el trazado permanente de las células del linaje epicárdico (células $Wt1^{CreYFP+}$ y $G2GATA4^{CreYFP+}$) tanto en el corazón embrionario como en el corazón adulto. Se ha estudiado la relación que los progenitores cardiovasculares identificados en el corazón mantienen con el linaje epicárdico. Usando anticuerpos específicos para identificar a las células c-Kit^+, Sca-1^+, o Bmi-1^+ mediante inmunohistoquímica y citometría de flujo procedentes de corazones embrionarios (E9,5-18,5), neonatos y adultos y se ha determinado que son pocas las células derivadas de epicardio (YFP^+) que expresan los marcadores c-Kit o Bmi-1, aunque estas células siempre se encuentran en asociación cercana al epicardio, lo que sugiere un papel trófico para este tejido en relación a las células c-Kit^+ o $Bmi1^+$. Al contrario de lo que pasa con estos marcadores, se ha identificado un número

significativo de células de linaje epicárdico (YFP^+) que también expresan Sca1, especialmente en torno a las paredes de los vasos coronarios. Estos resultados vinculan al menos una parte de las células $Sca1^+$ a un linaje embrionario multipotente como el epicárdico.

Tarea 2: Estudiar la relación ontogenética de los progenitores cardiovasculares c-Kit, Sca-1, y Bmi-1 con los linajes celulares derivados de la médula ósea mediante el uso de trasplante de médula de donantes transgénicos. Se han usado como receptores ratones de linaje C57BL/6 así como ratones procedentes del cruce de las líneas $Wt1^{Cre}$ y $^{ROSA26}YFP$, ambos irradiados con rayos X. Los receptores fueron trasplantados con médula ósea procedente de ratones donantes que expresan de forma constitutiva la proteína fluorescente roja (RFP). En el caso de los trasplantes en receptores postnatales, los individuos fueron sometidos a mieloablación mediante un tratamiento con busulfán. Se emplearon anticuerpos específicos y técnicas de inmunohistoquímica y citometría de flujo para identificar a las células que co-expresan RFP y cualquiera de los marcadores de CSC/progenitores descritos en la tarea 1 (c-Kit^+, Sca-1^+ o Bmi-1^+). Los resultados de estos experimentos indican que al menos una parte de las células que expresan estos marcadores son derivados de la médula ósea. Esto debe ser interpretado como que al menos una parte de las células consideradas como progenitores cardiovasculares residentes en el corazón son de estirpe sanguínea.

Objetivo 2:

Tarea 3. Generar un mapa de la localización de los diversos tipos de CSC/progenitores cardíacos en el corazón fetal, postnatal y adulto, especialmente en relación con los vasos coronarios y los nervios cardíacos, con el fin de caracterizar los componentes celulares de un hipotético nicho pericoronario. Se han usado los modelos experimentales anteriormente descritos para analizar mediante inmunohistoquímica corazones embrionarios (E9,5-18,5), neonatos y adultos y caracterizar la distribución de las células c-Kit^+, Sca-1^+, Bmi-1^+ respecto a los vasos coronarios de origen epicárdico (YFP^+). Se ha prestado especial atención a la localización de estas células en la íntima endotelial ($CD31^+$, lectina), la media

muscular (αSMA^{+}) o la adventicia fibrosa. También se estudiará la localización de estas células respecto a los nervios cardíacos ($\beta III\text{-tubulina}^{+}$). Los resultados se representan y valoran de manera detallada en las figuras de la tesis.

Objetivo 3:

Tarea 4. Definir la localización espacial de señales moleculares (neurotrofinas) implicadas en el mantenimiento de un posible nicho de células madre cardíacas en el ratón adulto. Utilizando de forma combinada técnicas de inmunohistoquímica, citometría de flujo, hibridación *in situ* y qRT-PCR se han analizado corazones adultos de una línea de ratones control (C57BL/6) y en ratones $Wt1^{CreROSA26}YFP$ para determinar la expresión de diversas neurotrofinas (NGF, BDNF y NT-3) y receptores de neurotrofinas (TrkA, TrkB y TrkC) en los vasos coronarios y las CSC/progenitores cardíacos. Nuestros resultados muestran la expresión estable de NT3 por parte de los cardiomiocitos durante el desarrollo embrionario. La expresión del receptor de neurotrofinas TrkB se ha identificado en el endotelio y las paredes de los vasos coronarios. Por otro lado, también se ha utilizado el ratón transgénico $NT3^{LacZNeo}$, que produce la mitad de la neurotrofina habitual, para estudiar la proliferación celular y organización de las paredes de los vasos coronarios subepicárdicos e intersticiales.

Tarea 5. Determinar in vitro *las capacidades clonogénicas, de renovación y de diferenciación de las diferentes poblaciones de CSC, así como la influencia de las neurotrofinas sobre estas características.* Se han usado técnicas de formación de cardiosferas para obtener células formadoras de estas colonias a partir de corazones adultos de ratón (tanto normales como transgénicas, en este último caso con la intención de marcar genéticamente los linajes celulares epicárdicos y derivados de la médula ósea). Las cardiosferas han sido caracterizadas según su expresión c-Kit, Sca-1 y Bmi-1 y posteriormente cultivadas con el fin de estudiar su potencial de diferenciación. Además, en este medio se añadieron neurotrofinas o inhibidores de neurotrofinas con el fin de determinar su efecto sobre estas células.

Objetivo 4:

Tarea 6. Identificar la dinámica y la respuesta específica de las CSC/progenitores cardíacos de diferentes orígenes al daño por infusión de angiotensina. Se han utilizado bombas osmóticas para infundir Angiotensina II en ratones y causar daño cardíaco. Una vez analizada mediante inmunohistoquímica la distribución de distintos tipos de CSC/progenitores cardiovasculares en diferentes momentos de la remodelación ventricular, estudiaremos fenómenos de activación (p.ej. proliferación y expresión de factores de transcripción cardiogénicos como Nkx2.5 o Mef2c).