The building blocks for cardiac repair : isolation and differentiation of progenitor cells from the human heart

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SCOPE

CARDIAC DISEASE

Cardiovascular disease is the leading cause of death globally and its major contributor is coronary heart disease (World Health Organization 2016). The latter is caused by diminished flow of oxygen-rich blood to the heart muscle due to obstruction of a coronary artery. Eventually this can lead to a myocardial infarction or a heart attack and heart failure. Upon cardiac damage a sequence of events occurs from inflammation, loss of cardiac tissue to scar formation resulting in severely reduced cardiac output or death. The only therapy that will cure the fundamental loss of functional heart muscle is replacing scar tissue with de novo myocardium via cardiac regeneration.

CARDIAC REGENERATION

"If there were no regeneration there could be no life and if everything regenerated there would be no death" (Goss 1969). Regeneration is defined as the regrowth of lost or destroyed body parts or organs. Although animals from all walks of life are able to regenerate, from a clinical point of view we are only at the beginning of understanding the cellular and molecular mechanisms underlying this process [1]. Furthermore, while some mammalian organs can regenerate to some extent, the heart is lacking an intrinsic ability to cope with the loss of cardiomyocyte and tissue architecture. Regeneration of the heart is not possible without cells that can differentiate and organize into a working myocardium. Therefore, stem or progenitor cells have emerged as a valuable source for cardiac-repair strategies where they can be induced to proliferate and differentiate into the different cell types needed for cardiac repair. The ultimate stem or progenitor cell source for cardiac regeneration comes from the cardiac lineage itself [2].

PROGENITORS FOR CARDIAC REGENERATION

The heart is a four chambered and never resting muscular pump dedicated to meet the body's need for oxygen and nutrients. The cardiac wall consists of three layers: the endocardium or inner layer, the myocardium and the epicardium which covers the surface of the heart. The longstanding dogma is that the heart harbors no intrinsic regenerative capacity. However, this dogma was challenged by the identification of a pool of cardiac progenitor cells (CPCs) located in the interstitial space of the myocardium [3] and to date a number of different, and potentially distinct, CPC populations have been described. Several of these populations are able to differentiate into the three cell types needed for generation of a de novo myocardium, cardiomyocytes, endothelial and smooth muscle cells [4]. In addition to the CPCs, the epicardium has been shown to contain a progenitor cell population [5]. During development, epicardial derived cells (EPDCs) actively contribute to heart formation by undergoing epithelial to mesenchymal transition (EMT) and differentiate into fibroblasts, smooth muscle cells and potentially endothelial cells. The adult epicardium is quiescent. However, upon injury this layer re-activates a developmental gene program and, to some extent, recapitulates its embryonic function [6]. In conclusion, EPDCs and CPCs can contribute to cardiac regeneration via EMT and differentiation in a multipotent manner. Thereby they can be considered the seeds of cardiac tissue regeneration. However, before fully understanding and appreciating the potential of CPCs and EPDCs in cardiac repair, a pure population of cells needs to be isolated.

ISOLATION OF HUMAN CPCS AND EPDCS

Previously our group has isolated CPCs from the human heart using an antibody against stem cell antigen-1 (Sca-1). These Sca-1+ CPCs can be induced to differentiate into the three cell types needed for the generation of de novo myocardium, including beating cardiomyocytes [7,8]. However, the absence of a human Sca-1 homologue [9] has hampered the clinical application of these CPCs. Therefore, using whole-cell immunization, a panel of monoclonal antibodies (mAbs) was generated against human Sca-1+ CPCs (Figure 1). Together, these mAbs provide the possibility of using multiple markers to obtain homogenous sub-fractions of CPCs. As a consequence, these CPCs are better characterized, based on their surface markers, which is an important step in using these cells for cell-based cardiac repair. To explore the suitability of these mAbs (mAb C19, mAb C1096 and mAb C573) in the identification and isolation of a human CPC population, immunofluorescent stainings, cell isolations and differentiation assays were conducted to identify the cell type they recognize in the human heart. Our data suggested that, using mAb C102 or mAb C1465, CPC-like cells could not be efficiently derived (data not shown).

One of the important questions in the EPDC field is if we can extrapolate the

ability of the fetal epicardium to support myocardial growth and differentiation to the adult setting? However, the major obstacles to answer this question has been the inability to consistently isolate a pure population of human EPDCs from both fetal and adult heart tissue, and maintain these cells in a stable epithelial state *in vitro*. Therefore, we established a culture protocol allowing the direct comparison of fetal to adult human EPDCs to understand their intrinsic behavior and ability to undergo EMT.

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Clone	Isotype	Human CPC binding?	Human fetal fibroblast binding?	Human ES cells binding?
mAb C19	IgM	+++	-	-
mAb C1096	IgG1	+++	-	-
mAb C573	IgM	+++	-	+
mAb C102	IgG3	+++	-	-
mAb C1465	IgG3	+++	-	-

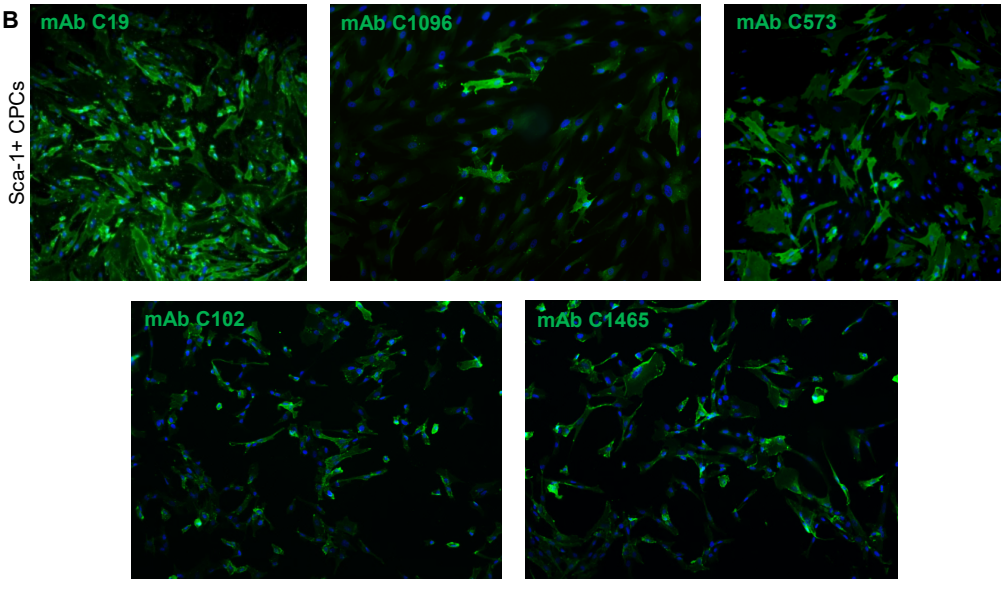

Figure 1.1: **Overview of mAbs with high affinity for human Sca-1+ CPCs.** (A) Panel of mAbs that was raised against human Sca-1+ CPCs. (B) Immunofluorescent stainings revealed that human Sca-1+ CPCs are positive for these mAbs. From this panel, mAb C19 is described in chapter 3, mAb C1096 in chapter 4 and mAb C573 in chapter 5.

THE TGFβ SIGNALING PATHWAY

Upon their isolation, CPCs and EPDCs can embark the differentiation and EMT path and the transforming growth factor beta (TGFβ) superfamily plays a pivotal role during both processes. The TGFβ superfamily includes the TGFβ and Bone Morphogenetic Proteins (BMP) signaling pathways which function through phosphorylation and nuclear localization of the small mothers against decapentaplegic (SMAD) proteins (Figure 2). Starting upstream in the TGFβ/BMP pathway a TGFβ or BMP ligand binds to a heterotetramer of two type I and two type II serine-threonine kinase receptors. Upon ligand binding, signaling is initiated by transphosphorylation of the type I receptor by the type II receptor. To date, there are seven type I receptors (the activin receptor-like kinases 1-7; ALK1-7) and five type II receptors known in mammals which in different combinations can bind different ligands. On most cells, TGFβ (TGFβ1, β2 and β3) binding leads to activation of the ALK5 (TGFβRI) receptor kinase. For activation of the BMP pathway, on the other hand, a BMP ligand activates the type I receptor ALK1, ALK2, ALK3 or ALK6. An active receptor kinase results in phosphorylation and activation of SMAD proteins. Originally, these proteins can be divided into the TGFβ (SMAD2 and SMAD3) and BMP (SMAD1, 5, 8) mediated SMADs. A complex of three SMAD proteins, including the common SMAD4 protein, transduces the signal to the nucleus by interacting with a variety of transcription factors leading to transcription of target genes.

TGFβ signaling is tightly controled in a spatial and temporal manner. How a cell will respond and which genes will be activated is depending on, amongst others, the ligand concentration and duration of the signal, e.g. TGFβ can either be pro-angiogenic or anti-angiogenic. Germline deletion of members of the TGFβ signaling pathway often results in embryonic lethality due to cardiovascular defects demonstrating the important role of this pathway in cardiovascular development and homeostasis [10,11]. Therefore, elucidating the function of the TGFβ pathway in human CPC and EPDC behavior is a step forward in directing their fate in favor of cardiac regeneration.

THE TGFβ CO-RECEPTOR ENDOGLIN

The cellular response to TGFβ stimulation is not only defined by a type I and a type II receptor but also by a type III receptor including Endoglin (CD105). Endoglin mainly functions as a co-receptor facilitating binding of TGFβ1/3 and BMP9 to the ALK1 receptor which together with Endoglin is abundantly expressed on endothelial cells. On these cells, TGFβ binds to a receptor complex consisting two TGFβ type II receptors, in which ALK1 counteracts ALK5 mediated signaling [12] and Endoglin is required for TGFβ/ALK1 signaling leading to pSMAD1/5 activation [13]. This promotes the migration and proliferation of endothelial cells, while signaling via TGFβ/ALK5/SMAD2/3 inhibits these processes [14]. As such, Endoglin seems

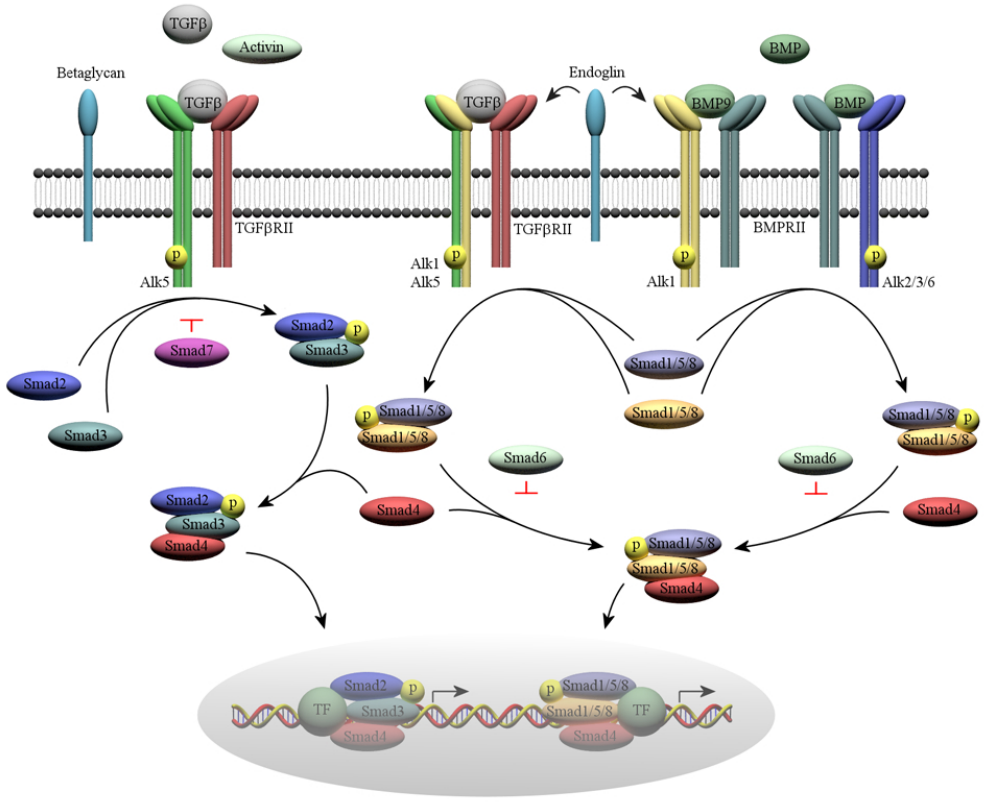

Figure 1.2: **Schematic representation of the TGFβ/BMP signaling pathway (adapted from [10]).**

to play an important role in fine-tuning the state of endothelial cells [15]. However, in addition to activated endothelial cells, Endoglin is expressed by, amongst others, smooth muscle cells, mononuclear cells and various stem cell populations [16], including embryonic stem cells. Mice heterozygous for Endoglin display defective vascular repair following myocardial infarction and Endoglin knockout embryos die due to vascular abnormalities [17–20]. In addition, mononuclear cells from Endoglin heterozygous patients are impaired in their homing ability to the infarcted heart [17,21]. This indicates that Endoglin plays a key role during cardiac regeneration and it has become clear that the importance of Endoglin goes beyond its role during angiogenesis.

WHAT CAN WE LEARN FROM THE EMBRYO?

"Study the past if you would define the future" (Confucius). To understand how adult progenitors will react to certain signals and what they are able to differentiate

into, we could look back to the path they once travelled during embryonic development. For example, the extraembryonic visceral endoderm (VE) interacts with the nascent mesoderm to induce the cardiac fate. To recapitulate this developmental process, VE-like cells (e.g. XEN cells) were used to induce cardiogenesis of human and mouse embryonic stem cells and they identified an array of factors that may support myocardial differentiation [22,23]. Furthermore, during development the epicardium provides several cardiac cell types and instructive signals to the developing myocardium [6]. Given that EPDCs once actively contributed to developing the myocardium, they may be able to do this again in adult life. Eventually, we do not have to accept the progenitors own decision but actively direct their behavior by 'telling' them what to do.

OUTLINE OF THIS THESIS: FROM PROGENITOR CELL ISOLATION TO REGENERATION MECHANISMS

This thesis is directed to the various aspects contributing to cell-based cardiac repair. **Part 1 (chapter 3-6)** describes the isolation of progenitor populations (CPCs and EPDCs) from the human heart. Having isolated human CPCs and EPDCs, **part 2 (chapter 7-10)** provides an insight in the molecular mechanisms underlying (cardiomyocyte) differentiation and EMT, the two processes via which these progenitor cells can contribute to cardiac regeneration (overview is given in Figure 3).

An overview of the current options to regenerate the heart and restore its contractile force after injury is given in **chapter 2**. One of these options is using CPCs, isolated by their ability to bind to the anti-mouse Sca-1 antibody (Sca-1+ CPCs which are also called cardiomyocyte progenitor cells or CMPCs), and the ability of these cells to differentiate into the three major cell types of the heart [7,8,24]. However, there is no human homologue of Sca-1 which makes it impossible to pursue this isolation method in a clinically relevant setting. Therefore, we raised a panel of monoclonal antibodies (mAbs) against Sca-1+ CPCs. We tested their ability to isolate a multipotent CPC population from the human heart. MAb C19 is described in **chapter 3**, mAb C1096 in **chapter 4** and mAb C573 in **chapter 5**. In addition, mAb C19 and mAb C1096 recognize glycosylated residues and glycan motifs are proposed as the next generation of CPC markers in **chapter 4**. Furthermore, a novel method to isolate and expand the other progenitor population of the heart, EPDCs, is presented in **chapter 6** and **appendix 2**.

An important part in cardiac repair, is the ability of CPCs and EPDCs to contribute to cardiac regeneration via cardiomyocyte differentiation and epicardial EMT, respectively **(Part 2)**. We studied human CPC to cardiomyocyte differentiation, based on deep sequencing data, in **chapter 7** and suggest that the TGFβ signaling pathway and microRNA 424 play an important role during this process. Next, we questioned whether extraembryonic endoderm-derived (XEN) cells can support

CPC to cardiomyocyte differentiation. Therefore, we provide a review about XEN cells in **chapter 8** and introduce these cells as an *in vitro* tool for human cardiomyocyte differentiation in **appendix 3**. The EPDC-isolation method described in chapter 6 allows us to directly compare fetal and adult EPDCs which we implemented by investigating their corresponding ability to undergo EMT. Since the TGFβ signaling pathway plays a pivotal part in regulating EMT, this pathway was studied and compared between fetal and adult EPDCs in **chapter 9**. We observed Endoglin expression in human EPDCs. Given that this TGFβ co-receptor has been linked to pluripotency and cellular activation, we studied epicardial behavior after myocardial infarction in mice heterozygous for Endoglin in **chapter 10**. Furthermore, we took a closer look at the potential role of Endoglin in pluripotent stem cells in **appendix 4**. Finally, in **chapter 11** the results and topics described in this thesis are taken together and discussed.

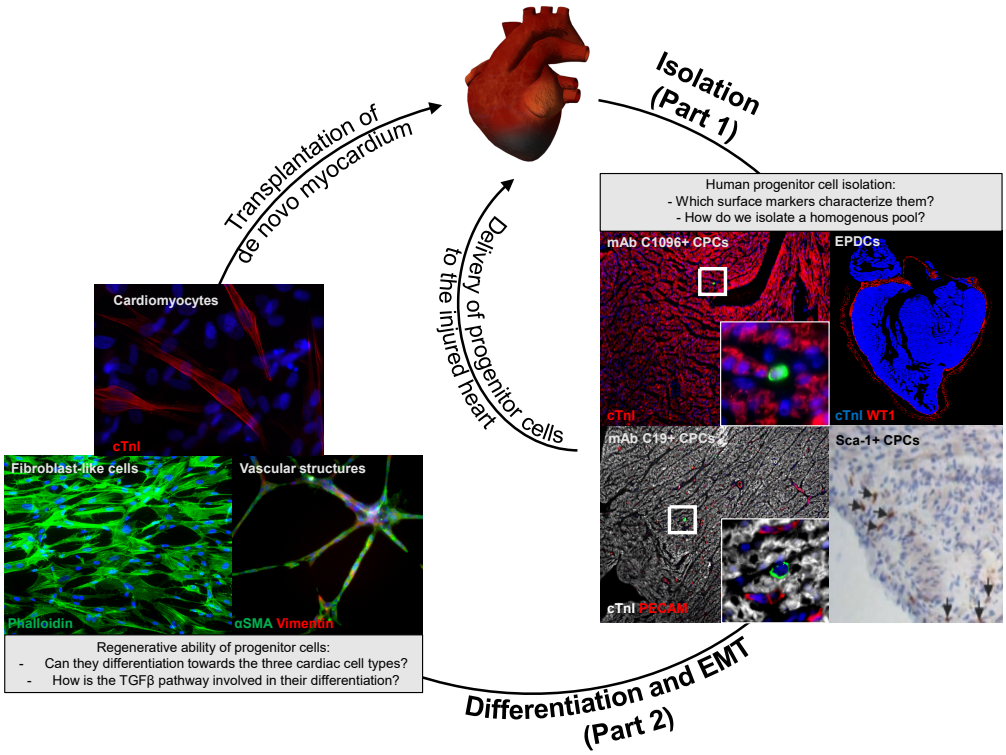

Figure 1.3: **The building blocks for cardiac regeneration.** Depicted are the various progenitor populations which can be isolated from the human heart, as described in this thesis. Sca-1+, mAb C1096+ or mAb C19+ cardiac progenitor cells or CPCs can be obtained from the myocardial layer. In addition, the epicardial layer, which is marked by expression of the transcription factor Wilms tumor 1 (WT1), is the source for epicardial-derived cells or EPDCs. These progenitor populations can differentiate or undergo EMT and give rise to cardiomyocytes, fibroblast-like cells or vascular structures. The TGFβ signaling pathway plays a pivotal role during these processes.

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