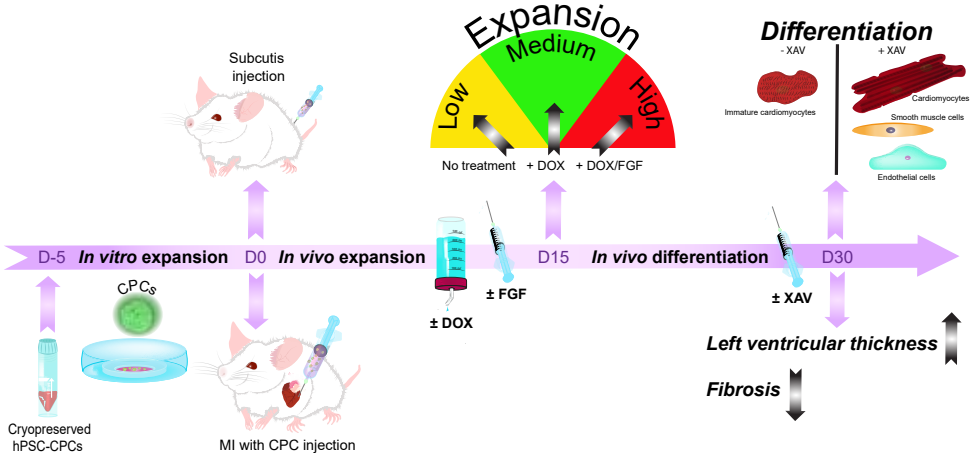
Guide to the heart: Differentiation of human pluripotent stem cells towards multiple cardiac subtypes

Schwach, V.


Expansion
Medium
Low
High
No treatment
+ DOX
+ DOX/FGF
Differentiation
- XAV
+ XAV
Immature cardiomyocytes
Cardiomyocytes
Smooth muscle cells
Endothelial cells
Subcutis injection
D-5 In vitro expansion
D0 In vivo expansion
D15 In vivo differentiation
D30
CPCs
± DOX
± FGF
± XAV
Cryopreserved hPSC-CPCs
MI with CPC injection
Left ventricular thickness
Fibrosis

Chapter 6:

Expandable human cardiovascular progenitors from stem cells in regenerating mouse heart after myocardial infarction

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Abstract

Cardiovascular diseases, including myocardial infarction (MI), are a major cause of mortality and morbidity worldwide due in large part to the low regenerative capacity of the adult human heart. Human pluripotent stem cell (hPSC)-derived cardiovascular progenitor cells (CPCs) are a potential cell source for cardiac repair. This study aimed to determine whether doxycycline (DOX)-inducible (Tet-On-MYC) hPSC-derived CPCs could be directed to proliferate then differentiate *in vivo*, in a drug-regulated manner, and thus achieve large-scale remuscularization and coincident revascularization of the heart. We further aimed to determine the impact of creating large grafts on cardiac remodeling and function in a mouse model of MI.

First, CPCs were injected at a non-cardiac site under the skin of immunocompromised mice to assess their commitment to the cardiovascular lineage and ability to self-renew or differentiate when instructed by systemically delivered factors including DOX and basic fibroblast growth factor (bFGF). We then transitioned to intramyocardial injection in mice subjected to MI and assessed whether expandable CPCs could have a reparative effect. Transplanted CPCs expanded robustly in the subcutis and myocardium using the antibiotic-inducible transgene system in conjunction with bFGF. Upon withdrawal of these self-renewal factors, CPCs differentiated with high efficiency at both sites into the major cardiac lineages including cardiomyocytes, endothelial cells and smooth muscle cells. After MI, although cardiac function was not improved, engraftment of CPCs in the heart significantly reduced fibrosis in the infarcted area and prevented left ventricular remodeling.

Replacement of large areas of muscle may be required to regenerate the heart of patients following MI. Our model has demonstrated the potential of proliferating hPSC-derived CPCs to achieve such repair. This creates an important opportunity to understand the biology of these cells, the determinants of graft size and downstream functional outcomes; all necessary for maximizing clinical success.

Introduction

Cardiovascular diseases, including myocardial infarction (MI), are a major and enduring cause of death worldwide, despite great advances in modern medicine. MI results in the loss of up to 1 billion contractile cardiomyocytes (CMs) and since these divide only slowly in adult heart, their early replacement has been proposed as an approach to prevent later heart failure (Bergmann et al., 2009). Human pluripotent stem cell-derived cells (hPSC) are already being actively explored as a source of replacement cardiac cells (Bellamy et al., 2014; Chong et al., 2014; Fernandes et al., 2015; van Laake et al., 2007, 2009; Laflamme et al., 2007; Liu et al., 2018; Shiba et al., 2016; Vandergriff et al., 2014); since they are now widely available and can be derived efficiently in large numbers in bioreactors (Kempf et al., 2016). However, washout from the transplantation site and low survival can limit graft size whilst coupling with native (resident) cardiomyocytes can cause arrhythmias (Chong and Murry, 2014). Human adult stem cells (like bone-marrow stromal cells) have also been examined as alternatives both in animal models and patients and although demonstrated as safe, they are not retained in the heart for more than a few days and meta-analysis of multiple large scale clinical trials, has indicated contradictory results on clinically significant long-term improvements in cardiac function (Gyöngyösi et al., 2015). In contrast to non-cardiomyocyte stem cells, hPSCs can differentiate into all of the different CM subtypes of the human heart (Burridge et al., 2014; Devalla et al., 2015; Mummery et al., 2012) as well as epicardial cells and their derivatives (Guadix et al., 2017; Iyer et al., 2015; Witty et al., 2014) and (cardiac) vascular endothelial cells (ECs) (Giacomelli et al., 2017; Orlova et al., 2014). Transplantation of hPSC-derived CMs (hPSC-CMs) after MI is thus among the more promising approaches to restoring contractile function of the heart but the issue of graft size is one hurdle that needs to be addressed. To promote the formation of larger grafts of new myocardium with coincident revascularization, we hypothesized that multipotent cardiovascular progenitor cells (CPCs) may represent an appropriate source of cardiac cells for heart repair. CPCs can be effectively isolated from differentiating hPSCs and the transplantation of PDGFRα$^+$ KDR$^+$ hPSC-CPCs in a rat model of MI, was functionally beneficial, but failed to increase neovascularization or graft size beyond the transplantation of differentiated CMs (Fernandes et al., 2015; Zhang et al., 2016). Thus, it remains uncertain whether CPCs can be induced to expand and undergo multi-lineage differentiation *in situ* after transplantation and whether

this could be beneficial in regenerating the injured heart. We previously reported the derivation of CPCs from a human embryonic stem cell line (hESC) (Birket et al., 2015) containing an NKX2.5$^{eGFP/+}$ reporter (Elliott et al., 2011) in which we had inserted a doxycycline (DOX)-inducible (Tet-On-MYC) construct. CPCs selected on day 6 of differentiation from this Tet-On-MYC-NKX-2.5$^{eGFP/+}$ hESC line could be expanded *in vitro* by mitogen stimulation in the presence of DOX. Upon DOX removal, the CPCs responded predictably to developmental signaling pathways and could undergo highly efficient myocardial and vascular differentiation in appropriate conditions. This created an opportunity to investigate whether this "switchable" system could operate similarly *in vivo*, so that DOX in the presence of mitogens would induce CPC proliferation to produce large grafts *in situ* and after DOX removal, cardiac cell differentiation could be induced. Here, we showed that this approach was effective in CPCs injected under the skin and in the heart of mice given DOX in the drinking water and that systemic delivery of growth factors differentiation towards the cardiac lineage, improving ventricular thickness and decreased fibrosis.

Results

Human stem cell-derived cardiovascular progenitors expand *in vivo* after subcutaneous injection

Since DOX-inducible NKX2.5$^{eGFP/+}$ CPCs could robustly expand *in vitro*, we examined whether this could also occur *in vivo*. As an accessible transplantation site in which the graft size could be monitored over time through palpation without sacrificing the mouse, we chose injection just under the mouse skin (subcutis). Following *in vitro* expansion for 5 days, approximately 500.000 cryopreserved CPCs (containing ±90% NKX2.5^{eGFP}-positive CPCs; Figure S1) in spheres were mixed with 150 µl CPC medium plus 10% Corning™ Matrigel™ GFR (growth factor reduced) Membrane Matrix (354277, BD Bioscience) and injected subcutaneously into the left and right flank of immune-deficient NSG mice that were primed with DOX one week prior to CPC injection (Figure 6.1A). To recapitulate the conditions of CPC expansion *in vitro* as closely as possible, mice were randomized into 3 treatment groups: 1) No treatment, 2) 2 mg/ml DOX in drinking water (+ DOX condition) and 3) 2 mg/ml DOX in drinking water in combination with subcutaneous delivery of 20 µg FGF every 2 days (48 h) (+ DOX/FGF condition). As demonstrated by prominent expression of the proliferation marker Ki-67 in subcutaneous plugs 15 days post treatment, only few CPCs were positive in the groups without DOX or DOX only (No treatment or + DOX), whilst with DOX and FGF (+ DOX/FGF) many CPCs were Ki-67 positive and formed large grafts indicating proliferative expansion and that CPCs were DOX and FGF sensitive *in vivo* (Figure 6.1B). The human origin of the cells was evidenced by staining for human β1-integrin (Figure 6.1B).

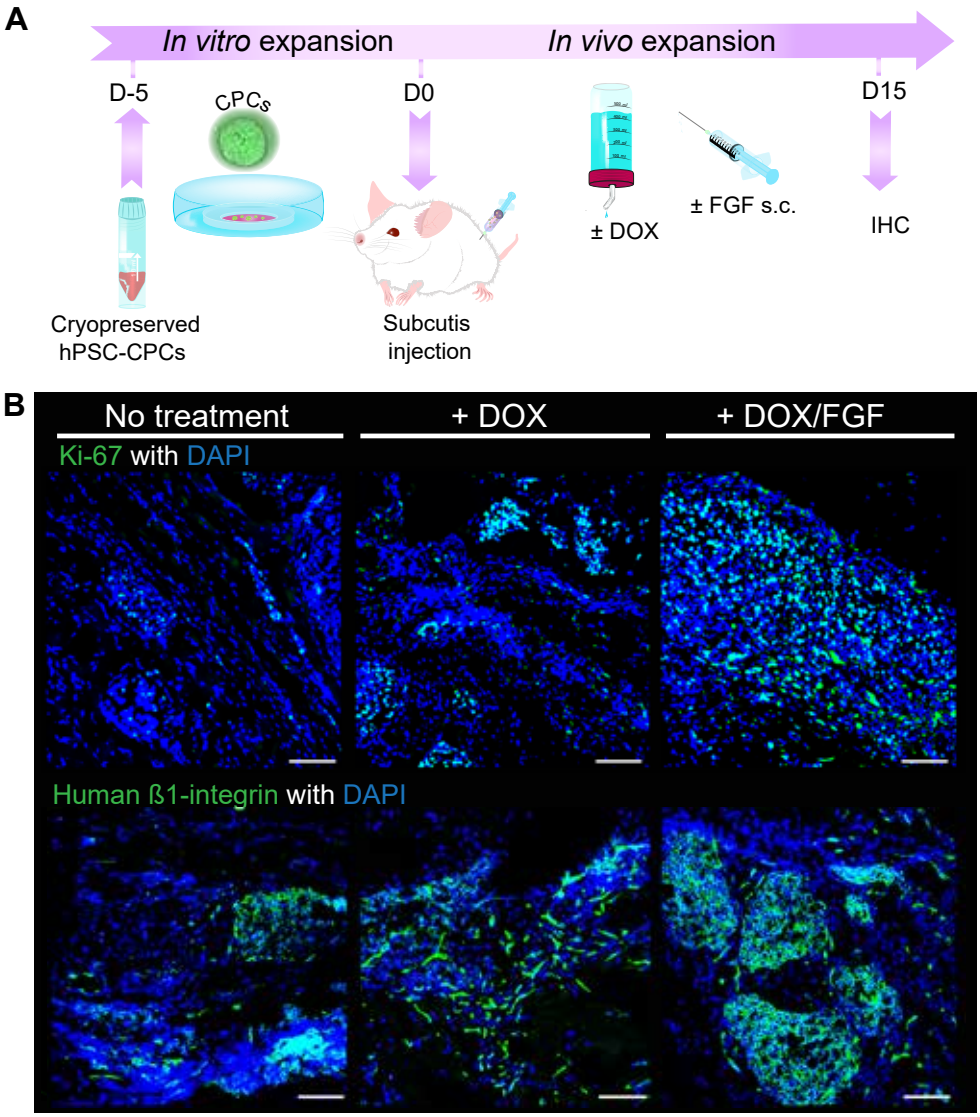
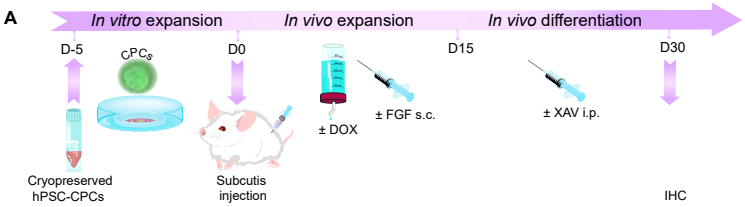


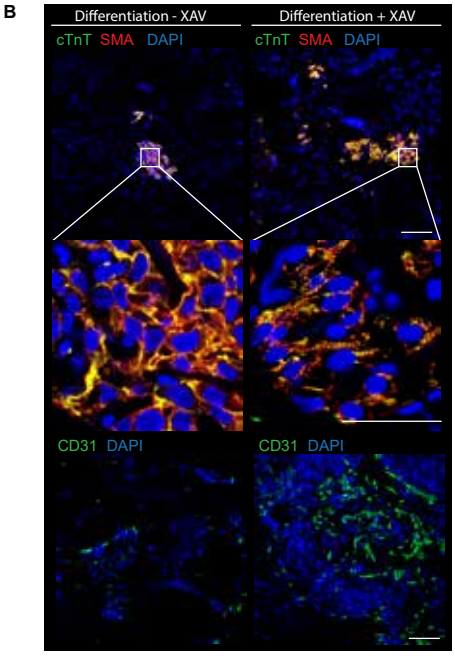
Figure 6.1: Human cardiovascular progenitors (CPCs) expand after subcutis injection *in vivo*. A) Schematic of the subcutaneous (s.c.) CPC injection and doxycycline (DOX)/ fibroblast-growth-factor (FGF) induced expansion in NSG mice. Cryopreserved CPCs undergo expansion *in vitro* for 5 days in presence of Dox and FGF before subcutis injection followed by 15 days of *in vivo* expansion period. After that plugs were evaluated by immunohistochemistry (ICH). B) DOX and FGF treatment induced CPC proliferation *in vivo* as visualized by immunostaining for proliferation marker Ki-67 together with Human β1-integrin after no treatment, DOX only (+DOX) or DOX/FGF (+DOX/FGF) for 15 days; scale bar = 100 µm.

Human stem cell-derived cardiovascular progenitors expand and differentiate *in vivo* after subcutaneous injection at a non-cardiac site

Since DOX-inducible NKX2.5$^{eGFP/+}$ CPCs efficiently differentiate to the cardiac lineage *in vitro* after withdrawal of DOX, an *in vivo* protocol was developed to direct CPCs towards cardiovascular cells by intraperitoneal injection of 50 μg of the WNT-signaling inhibitor XAV939 every three days in combination with decreasing doses of FGF (2 μg FGF) for an additional 15 days (Figure 6.2A). Injection of the WNT-pathway inhibitor XAV939 promoted efficient differentiation of CPCs into functional cardiomyocytes (CMs), co-expressing endogenous GFP from the NKX2.5$^{eGFP/+}$ reporter, cardiac troponin T (cTnT) with the typical striated pattern and α-actinin. In addition, this protocol yielded multipotent differentiation with cells expressing PECAM (CD31) or smooth muscle actin (SMA), indicating human ECs or smooth muscle cells (SMCs or activated fibroblasts) (Figure 6.2B and C).

Together this confirmed that human stem cell-derived CPCs are capable of expanding and differentiating towards the cardiovascular lineage *in vivo* after injection at an extramyocardial site under the skin.


A
In vitro expansion
In vivo expansion
In vivo differentiation
D-5
CPCs
D0
D15
D30
± DOX
± FGF s.c.
± XAV i.p.
Cryopreserved hPSC-CPCs
Subcutis injection
IHC


B
Differentiation - XAV
Differentiation + XAV
cTnT SMA DAPI
cTnT SMA DAPI
CD31 DAPI
CD31 DAPI

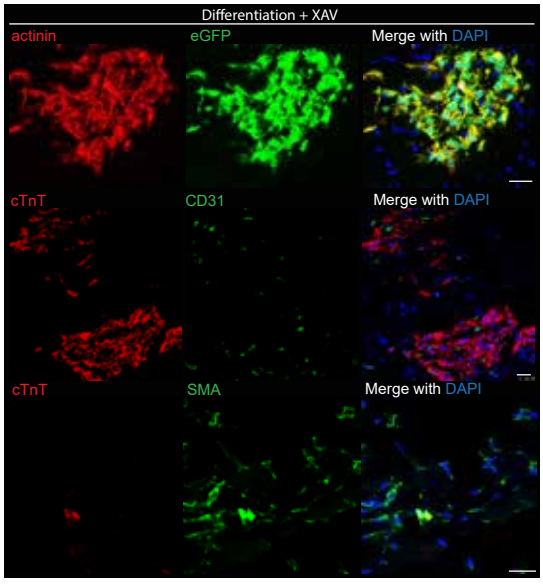

C
Differentiation + XAV
actinin
eGFP
Merge with DAPI
cTnT
CD31
Merge with DAPI
cTnT
SMA
Merge with DAPI

Figure 6.2: CPCs differentiate towards the cardiovascular lineage *in vivo* in response to WNT-inhibition after subcutis injection. A) Schematic of the subcutaneous (s.c.) CPC injection and DOX/FGF induced expansion, followed by directed differentiation to the cardiac lineage by intraperitioneal (i.p.) injection of XAV939. Cryopreserved CPCs undergo expansion *in vitro* for 5 days in presence of Dox and FGF before subcutis injection. Mice were treated for 15 days with DOX/FGF during *in vivo* expansion period followed by 15 days of XAV for differentiation. After that plugs were evaluated for differentiation capacity by ICH. B) WNT-inhibition via systemic administration of XAV939 induces differentiation to human cardiac troponin T (cTnT)-positive CMs (upper and middle panel) and CD31-positive ECs (lower panel) as visualized in samples without XAV (Differentiation – XAV) or with XAV (Differentiation + XAV); scale bar = 100 μm in upper panel and lower panel; scale bar = 25 μm in middle panel. C) CPCs differentiate into CMs, ECs and SMCs. Representative images of human cTnT (red), eGFP (green) together with nuclear stain DAPI (blue), cTnT (red) with human CD31 (green) and cTnT (red) with smooth muscle actin (SMA) together with nuclear stain DAPI; scale bar = 25 μm.

Human stem cell-derived cardiovascular progenitors expand and form large grafts composed of CMs, ECs and SMCs *in vivo* after MI in mice

To evaluate if CPCs could be similarly expanded after transplantation into the heart post MI, we then assessed the expansion and differentiation potential of human CPCs in the heart after intramyocardial injection in immune-deficient mice that had undergone acute MI by permanent ligation of the left ascending coronary artery (LAD) (Figure 6.3A). As with the subcutaneous injections, NKX2.5eGFP-expressing cryopreserved CPCs were injected after *in vitro* expansion for 5 days. Approximately 500.000 CPCs in spheres were mixed with 50 μl of CPC medium plus 10% Corning™ Matrigel™ GFR (growth factor reduced) Membrane Matrix (354277, BD Bioscience) before intramyocardial injection in three myocardial sites around the infarcted area, immediately after performing the ligation. One week prior to injection, mice received DOX, then *in vivo* CPC expansion continued for 15 or 30 days with DOX/FGF, followed by 15 days of XAV939 injections to inhibit WNT-signaling and promote differentiation towards the cardiac lineage *in vivo* (see supplementary table 1 for experimental conditions).

GFP+ CPCs were monitored by bright field and fluorescent microscopy (Figure 6.3B). After serial sectioning of the hearts (8 μm), sections were stained for human ß1-integrin and the graft volume was quantified by manual tracing of graft borders (Figure S2A). We found significantly larger grafts after treatment with DOX and FGF for 15 days compared to those in animals receiving no treatment with a graft volume increasing from 0.02 ±0.01 mm^3 without treatment to 0.32 ±0.005 mm^3 with DOX/FGF stimulation (Figure 6.3C and D). As with the subcutaneous cell transplants, prominent expression of Ki-67 was evident indicating active proliferation that led to expansion of the grafts (Figure S2B). Unexpectedly, we found that grafts in mice treated for 15 days with DOX/FGF were larger than grafts in mice treated for 30 days (0.32 mm^3 and 0.22 mm^3) (Figure 6.3C and D) even though there was no significant difference and despite no increase in apoptosis as shown by immunostaining for apoptosis markers Caspase and TUNEL which revealed some apoptotic cells in the graft area after 15, as well as 30 days of *in vivo* expansion (Figure S2C).

After *in vivo* expansion and XAV driven differentiation, the CPCs had clearly differentiated to CMs; as evidenced by double staining with human ß1-integrin and cTnT showed that more than 90% of the cells in the β1-integrin$^+$-grafts were cardiac troponin positive (Figure 6.3E and F). In addition however, we found that the cTnT-negative graft area stained positively for SMA which accounted for 7% (Expansion of 15 days) and 11% (Expansion of 30 days) of the graft area, suggesting that these cells are SMCs or activated myofibroblasts (Figure 6.3G and H). Also, as in the subcutaneous injections, staining for human specific CD31 revealed that CPCs have the potential to differentiate into blood vessel ECs (Figure 6.3I). Quantification of the CD31$^+$ pixels per graft showed that about 5.4 ±2% of the graft was vascularized by human ECs (Figure 6.3J). We observed no difference in the number of CD31$^+$ ECs between 15 and 30 day expansion (Figure 6.3I and J). Together, CPCs appeared to have efficiently differentiated into cTnT$^+$ CMs, CD31$^+$ ECs, as well as SMA$^+$ SMCs.

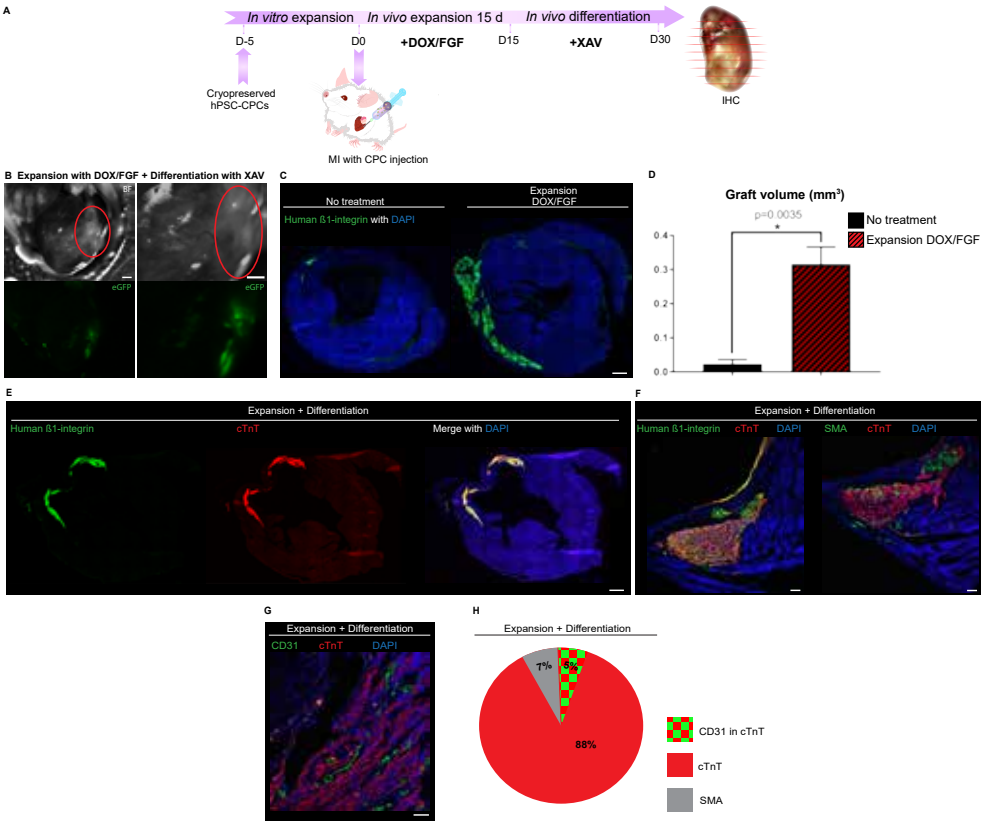


Figure 6.3: CPCs form large grafts composed of CMs, ECs and SMCs after intramyocardial injection post myocardial infarction (MI) *in vivo*. A) Schematic representation of intramyocardial CPC injection post MI and subsequent analysis with 15 (upper panel) or 30 days (lower panel) of expansion. Cryopreserved CPCs follows *in vitro* expansion before injection during induction of MI via permanent ligation of the left coronary artery as a model for MI in mice. Mice were treated with DOX/FGF for either 15 or 30 days as *in vivo* expansion period followed by differentiation phase for another 15 days by injection of XAV939. After 12 weeks cardiac sections were evaluated for graft size and differentiation capacity by immunohistochemistry (ICH). B) Visualization of infarct area by bright field microscopy (BF) and intramyocardial grafts by endogenous GFP (eGFP); infarct area marked in red; scale bar = 500 µm. C) Representative images of grafts visualized by human ß1-integrin staining (green) together with nuclear stain DAPI (blue) after no treatment and DOX/FGF treatment for 15 or 30 days; scale bar = 500 µm. D) Quantification of graft volume; data is expressed as means ±SEM.; one-way ANOVA with Tukey's multiple comparisons

test was applied for differences in means between groups. Statistical significance was defined as P<0.05; n= 3 for no treatment, n=10 for DOX/FGF treatment. E) Representative scans of grafts stained for cTnT (red) together with human ß1-integrin (green) and nuclear stain DAPI (blue) after 15 (upper panel) or 30 (lower panel) days *in vivo* expansion in presence of DOX and FGF followed by 15 days of *in vivo* differentiation with XAV939; scale bar = 500 µm. F) Quantification of cTnT+ area per graft volume; data is expressed as means ±SEM.; one-way ANOVA with Tukey's multiple comparisons test was applied for differences in means between groups. Statistical significance was defined as P<0.05. G) Representative confocal immunofluorescent pictures of grafts stained for human ß1-integrin (green), cTnT (red) (left panel) and cTnT (red) with smooth-muscle-actin (SMA) (green) (right panel) and DAPI (blue); scale bar = 75 µm. H) Pie chart to illustrate the contribution to the graft composition of $cTnT^+$ or SMA^+ cells after 15 or 30 day expansion. I) Representative confocal pictures of cardiac grafts stained for the human-specific EC marker CD31 (green) and cTnT (red) with nuclear stain DAPI (blue) after 15 (left panel) or 30 (right panel) days of *in vivo* expansion followed by 15 days of *in vivo* differentiation; scale bar = 25 µm. J) Quantification of $CD31^+$ per cTnT area; data is expressed as means ±SEM.; one-way ANOVA with Tukey's multiple comparisons test was applied for differences in means between groups. Statistical significance was defined as P<0.05.

CPC grafts remarkably diminished fibrosis and left ventricular remodeling after MI, but did not improve cardiac function

As a final step, we investigated the effect on cardiac function, fibrosis and left ventricular remodeling. Quantification of the ejection fraction by magnetic-resonance-imaging (MRI) was performed 3 days after CPC injection (D3), after the expansion period (D30 or D45) and 90 days after injection (D90) (Figure 6.4A). Ejection fraction was compared between sham operated mice (sham/control), mice subjected to MI without injection of CPCs (MI), mice with MI and injected CPCs (MI+CPCs), as well as mice with MI and Matrigel injection (MI+Matrigel). Already after 3 days, we found a significant reduction of the ejection fraction in mice subjected to MI when compared to sham mice, but no significant difference between mice with MI without or with injected CPCs or Matrigel only, suggesting that the injection of CPCs did not improve cardiac function following acute MI (Figure 6.4B).

Fibrosis resulting from MI was determined by Sirius red staining after sectioning the hearts, animals with no histologically detectable MI being excluded from analysis. The MI resulted in formation of large fibrotic scars detectable by Sirius Red staining. Sirius red in serial sections of the left ventricle in mice subjected to MI with and without CPC expansion showed that the injection of CPCs remarkably diminished the amount of fibrosis about 50% (Percent of fibrosis relative to left ventricular volume: MI = 41%; MI+CPCs = 19% (Figure 6.4C and D and Suppl. Figure 4A).

To evaluate impact on left ventricular remodeling, we assessed the left ventricular wall thickness by cardiac MRI in a 12-segment model of the left ventricle (Figure 6.4E). Average wall thickness was reduced from ~0.88 ±0.11 mm in sham mice to 0.70 ±0.1 mm in mice with acute MI and was restored to 0.94 ±0.5 mm mice with MI plus injection of CPCs in segments of the grafted area (Figure 6.4F). Similarly, average wall thickness per segment was preserved as a result of the transplantation (Figure 6.4G).

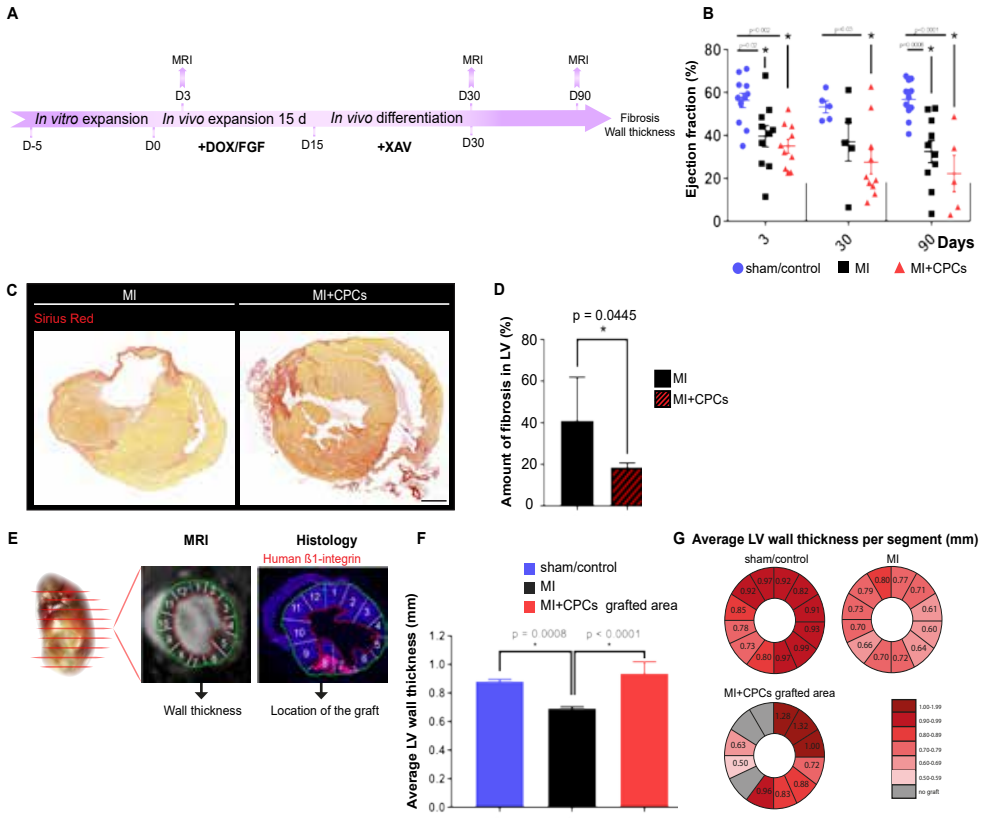


Figure 6.4: Injection of CPCs did not improve cardiac function, but significantly reduced amount of fibrosis and left ventricular remodeling after MI. A) To evaluate cardiac function, MRI was performed at 3 days after injection, after the expansion period (30 or 45 days) and 12 weeks after injection before evaluation of cardiac fibrosis and wall thickness. B) Quantification of cardiac function by ejection fraction; data is expressed as means ± SEM. and individual data points of each animal; one-way ANOVA with Tukey's multiple comparisons test was applied for differences in means between groups. Statistical significance was defined as $P<0.05$. C) Representative scans of grafts stained for Sirius red to visualize cardiac fibrosis in mice with MI only (MI) or MI with injected CPCs (MI+CPCs); scale bar = 1000 μm. D) Quantification of fibrosis volume in percent of left ventricular volume after MI or MI + CPCs; data is expressed as means ±SEM.; one-way ANOVA with Tukey's multiple comparisons test was applied for differences in means between groups. Statistical significance was defined as $P<0.05$. E) Schematic of the evaluation of ventricular wall thickness by cardiac MRI in a 12-segment model of the left ventricle. F) Average wall thickness in mice with acute MI and mice with MI plus injection of CPCs in segments of the grafted area; data is expressed as means ±SEM.; one-way ANOVA with Tukey's multiple comparisons test was applied for differences in means between groups. Statistical significance was defined as $P<0.05$. G) Average wall thickness in each segment of the left ventricle.

Discussion

In this study we investigated whether defined hPSC-derived CPCs expressing a DOX-inducible c-MYC construct could expand and differentiate to cardiovascular cells *in vivo* in mice with view to investigating whether increased graft size improves cardiac function or remodeling after MI but circumventing challenges that arise from transplanting large cells numbers to the heart. We found that: (i) CPCs could be expanded in response to DOX-induced c-MYC together with FGF signaling, (ii) CPCs differentiated *in vivo* outside the cardiac microenvironment after systemic administration of WNT-inhibitor XAV939, (iii) large grafts could form *in vivo* in the heart after transplanting relatively few CPCs and this was the result of CPC proliferation *in situ*, (iv) CPCs could undergo trilineage differentiation in both extra- and intramyocardial grafts, (v) ultimately graft size was probably limited by available vasculature, (vi) whilst cardiac function did not improve, there was significant decrease in fibrosis and significant increase of left ventricular thickness as a result of the transplantation.

Since CPCs were able to expand and differentiate to cardiovascular cells in a non-cardiac microenvironment suboptimal for the cardiac lineage, this subcutis model for *in vivo* differentiation may be extremely useful for elucidating different cardiac factors for *in vivo* expansion, cardiac differentiation, as well as maturation in less invasive setting compared to heart injections. In addition, new biomaterials could be more easily tested in the subcutis site.

Upon intramyocardial injection, we identified that grafts of mice treated for 15 days were larger in contrast to grafts of mice of 30 day expansion. Importantly, CPCs were able to vascularize the cardiac graft while in comparable studies utilizing non-expandable CPCs only sparse vessels were found (Fernandes et al., 2015). However, in contrast to human heart tissue, where most CMs are in direct contact with ECs, the formation of vessels was limited to some areas. Therefore, it is possible that the inadequate amount of blood vessel supply limit graft expansion of grafts after 30 day expansion. *In vitro* CPCs did not reach a senescent state for more than 80 doublings (approximately 20 days) (Birket et al., 2015). Therefore, we cannot exclude the possibility that CPCs undergo senescence beyond 20 days.

Remarkably, expression of cTnT in cardiac grafts showed that the large majority of the graft was composed of CMs while the remainder of the cells in the graft stained positive for SMA suggesting that the identity of these cells was SMCs or activated fibroblasts. In general, we found a higher percentage of SMA-positive cells after expansion of 30 days when compared to 15 day expansion. Similar to these *in vivo* results, *in vitro* CPCs yielded highest percentage of CMs within first passage, while at higher doubling levels cardiac differentiation potential dropped (beyond 78 doublings, only SMCs were generated) (Birket et al., 2015). CPCs significantly reduced fibrosis in the infarcted area to approximately 50%. These results were comparable in grafts expanded for 15 or 30 day expansion, suggesting that the reduction in fibrosis is independent of the graft size. Additionally, we found attenuated left ventricular remodeling around the graft area. Since amount of newly formed human vessels was comparable in both groups, a possible explanation is that the revascularization by human vessels had a major beneficial effect through the delivery of paracrine factors to the infarcted area. Despite these beneficial effects, CPC grafts failed to improve cardiac function as quantified by the ejection fraction of the left ventricle by MRI at different time points of expansion and differentiation. It is well known that species differences between mice and human, such as differences in cardiac physiology including heart rate and distribution of contractile proteins or ion channels (Kaese and Verheule, 2012; De Sousa Lopes et al., 2006), prevent the coupling of human CMs to the murine host tissue, which is a pre-requisite for proper rhythm control. Thus, the uncoupling between large human grafts and murine cardiac tissue might induce a conduction block and hinder cardiac function. In the future, it would be interesting to assess if hPSC-derived CPCs would have a beneficial effect on cardiac function in larger animal models, such as pig or sheep. Moreover, CPCs have been injected onto hearts within a matrigel plug, a gelatinous mixture of laminin and collagen, which could hinder self-organization of cells, as well as coupling to host myocardium. In contrast, engineered cardiac patches from cells within carefully designed scaffold materials have proven superior because they support self-organization of transplanted cells and contractile function of CMs, vascularization, as well as matrix remodeling within the graft (Chen et al., 2010; Dvir et al., 2012; Tiburcy et al., 2017). Thus, it would be valuable to investigate the *in vivo* expansion and differentiation of CPCs engrafted in different biodegradable materials, or via targeted delivery and their contribution for improved cardiac function post MI.

In summary, we have shown that Tet-On-MYC NKX2.5$^{eGFP/+}$ hESC-derived CPCs can be expanded and differentiated to cardiovascular cells in a controlled manner in both cardiac and non-cardiac niches in response to DOX-induced cMYC together with FGF. This allowed the limits to graft size to be explored and the effects of large grafts to be examined on mouse hearts after MI without the damage normally induced by injecting large numbers of cells and the substantial cell loss from washout. Moreover, in a mouse model of experimental heart failure, we have observed beneficial effects on cardiac remodeling as evidenced by increased vascularization, decreased cardiac fibrosis and left ventricular remodeling. However, these beneficial effects on cardiac remodeling did not lead to improved heart function, which could be explained by suboptimal electrical communication between mouse and human CMs. Experiments in larger animals may provide more insight whether expanded and differentiated CPCs can lead to heart repair and improved function and whether this is a feasible approach for cell-based therapies in patients with myocardial injury post MI.

Materials and Methods

CPC culture

To generate CPCs, Tet-On-MYC NKX2.$^{5eGFP/+}$ hESCs were differentiated as embryoid bodies (EBs) as previously described12. At day 6, EBs were dissociated using TrypLE Select (Invitrogen) and cryopreserved in BPEL medium containing 1 µg/ml DOX (Sigma-Aldrich), 5 µM SB431542 (Sigma-Aldrich), 100 ng/ml LONG R3 IGF-1 (Sigma-Aldrich), 1 µM SAG (Millipore), 1 ng/ml bFGF (Miltenyi Biotech), 20 ng/ml BMP4 (R&D) (hereafter called CPC medium) plus 10% DMSO and 1 µM fasudil. Thawed CPCs were expanded in CPC medium on Matrigel for 5 days before transplantation. GFP expression was verified by FACS.

In vivo expansion and differentiation of CPCs in immuno-deficient mice

8-9 weeks old male NSG mice (NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ, Charles River) were used for cell injections for both the subcutaneous and the intramyocardial protocol. See supplementary methods for dosage and regimen of DOX and growth factors.

Subcutaneous injection of CPCs in NSG mice

Approximately 1000 spheres (500.000 CPCs) in CPC medium (150 µl) were mixed with 10% Corning™ Matrigel™ GFR (growth factor reduced) Membrane Matrix (354277, BD Bioscience) (150 µl), and subcutaneously injected into the right and left flank area of each mouse.

Induction of MI in NSG mice and intra-myocardial injection of CPCs

MI was performed as previously reported (see supplementary methods). Approximately 1000 cardiospheres (500.000 CPCs) were suspended in a

total of 50 µl of CPC medium containing 10% Matrigel and injected directly into 3 points of the surrounding border zone.

Cardiac Magnetic resonance imaging (MRI) and left ventricular wall thickness evaluation

Cardiac magnetic resonance imaging (MRI) was performed under general anesthesia and using the methodology further explained in the supplementary material. Left ventricular wall thickness were calculated using Mass4mice. Briefly, the RV insertion point was marked in all the image slices, from basal to apex, and the left ventricle (LV) was divided into 12 segments (clockwise) per slice, where segments 1 to 8 correspond to the lateral wall and 9 to 12 to the septal wall. Segments containing the graft were identified histologically using a staining for β1-integrin and matched with the segments provided in Mass4mice images along all the slices.

Isolation and histology of CPC plugs and hearts

Animals were euthanized by CO2; CPC plugs or removed hearts were isolated and fixated for cryosectioning for 4 h in 4% neutral buffered formaldehyde at room temperature (RT), then 2 h in 15% sucrose-PBS (RT) followed by overnight 30% sucrose-PBS solution (4°C) before embedding in Tissue-Tek® OCT compound (Sakura® Finetek) at -80°C. 8 µm serial sections (1:10) were prepared for all the samples.

IF and Sirius Red staining

See supplementary methods with supplementary table 2 for information on stainings and antibodies.

Imaging

Brightfield and fluorescent slides were scanned on a 'Panoramic' MIDI digital scanner (3DHISTECH, Budapest, Hungary) using the provided software 'Panoramic Viewer' (3DHISTECH). Additional imaging was performed on a Leica TCS SP8 upright confocal microscope (Leica Microsystems, Wetzlar, Germany).

Quantification of graft size, fibrosis, cTnT, SMA and CD31 vessel area

The total graft volume, the proportion of cardiac and smooth muscle areas, as well as the vessel area per cardiac graft were determined as described in supplementary methods.

Statistical Analysis

Statistical significance ($p<0.05$) was detected by one- or two way ANOVA or Mann-Whitney test. Statistical analysis was performed using Graph Pad Prism 7.0 software. Data is presented as means ± SEM.

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