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Cardiac progenitors repopulate left ventricular myocardium in an *ex vivo* 3D tissue model

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1 ZUSAMMENFASSUNG

Ischämische Herzerkrankungen gelten weltweit als die häufigste Todesursache. Bis heute existieren keine kurativen Therapiestrategien, da sich menschliches Myokard nach einer massiven Schädigung nicht mehr suffizient regenerieren kann. Deshalb hat die Erforschung der zugrunde liegenden Mechanismen und die Suche nach neuen therapeutischen Ansätzen in den letzten Jahren große wissenschaftliche Aufmerksamkeit erregt. In der vorliegenden Studie wurde das Potenzial humaner ventrikulärer Vorläuferzellen (HVPs) zum funktionellen Ersatz von absterbendem, nativem Herzmuskelgewebe in einem chimärem *ex vivo*-Langzeitmodell untersucht.

Hierfür wurde die *ex vivo*-Langzeitkultur von nativem und chimärem linksventrikulärem (LV) Myokard von Primaten (NHP) bis zu 50 Tage etabliert. Zur Herstellung von chimären Myokardschnitten wurden embryonale Stammzellen (ESCs) kultiviert, zu Tag 6 HVPs differenziert und auf natives LV NHP Myokard ausgesät. Hierbei konnte gezeigt werden, dass sich die Kontraktionskraft von nativen und chimären HVP-NHP Myokardschnitten anfangs ähnlich verhielt. Während der fortschreitenden Kultivierung starb jedoch das native Myokard ohne Hinzugabe von Zellen ab dem 15.-21. Tag sukzessive ab, während das chimäre Myokard einen Wiederanstieg der Kontraktionskraft mit konstantem Verlauf bis zum 50. Tag zeigte. In den Analysen der chimären Myokardschnitte zeigte sich, dass HVPs hochgradig proliferativ sind, expandieren können und sich vorwiegend in ventrikuläre Kardiomyozyten (CMs) differenzieren. Diese Ergebnisse verdeutlichen das Potenzial von HVPs, *ex-vivo* kultivierte native LV NHP Myokardschnitte neu zu besiedeln und deren strukturellen und funktionellen Eigenschaften nach einer chronischen Schädigung zu verbessern.

Insgesamt stellen HVPs eine vielversprechende Zellpopulation für die Regeneration von Myokardschäden dar. Dennoch sind weitere präklinische und klinische Studien erforderlich, um die verbleibenden Fragen zur Sicherheit und Anwendbarkeit von kardialen Vorläuferzellen zur Regeneration von Herzmuskelgewebe zu klären.

2 ABSTRACT

Ischemic heart disease is the leading cause of death worldwide. Currently, only symptomatic and non-curative therapeutic strategies exist for end-stage heart failure after myocardial infarction due to the inability of the adult mammalian myocardium to regenerate sufficiently after the massive death of cardiomyocytes (CMs). For this reason, new therapeutic approaches and the mechanisms that lead to cardiac repair have received considerable scholarly attention in recent years. The present study assesses the potential of human ventricular progenitors (HVPs) to repopulate and replace dying, native left ventricular (LV) non-human primate (NHP) myocardial tissue functionally in an *ex vivo* long-term chimeric tissue model.

An *ex vivo* long-term culture of native and HVP-co-cultured LV NHP myocardial slices was established in biomimetic culture chambers (BMCCs) for up to 50 days. Embryonic stem cells were differentiated into Day 6 HVPs and seeded onto native LV NHP myocardial slices in order to create co-cultured myocardial slices. It was shown that initially contractile force traces of native and co-cultured HVP-NHP heart slices act similarly. However, during the progress of the culture, the functional and structural properties of native LV NHP myocardial slices without HVPs deteriorated from Day 15 onwards, while co-cultured HVP-NHP myocardial slices regained contractile force between Day 15 and Day 21. The contractile force of these co-cultured myocardial slices remained stable until Day 50. In this setting, HVPs were found to be highly proliferative and capable of expanding and differentiating into ventricular CMs predominantly after being seeded onto native LV NHP myocardial slices. These findings outline the potential of HVPs to repopulate native LV NHP myocardial slices and to take over structural and functional properties in the setting of chronic damage to an *ex vivo* cultured myocardium.

HVPs are a promising source of cells for myocardial regeneration. Nevertheless, further preclinical and clinical studies are necessary to address the remaining questions about the safety and feasibility of using cardiac progenitors for cardiac repair in humans.

BMCC	Biomimetic culture chamber
ClCasp3	Cleaved Caspase-3
CM	Cardiomyocyte
CPC	Cardiac progenitor cell
cTNT	Cardiac Troponin T
ECM	Extracellular matrix
EdU	5-Ethynyl-2'-deoxyuridine
eGFP	Enhanced green fluorescent protein
ESC	Embryonic stem cell
FHF	First heart field
GFP	Green fluorescent protein
hESC	Human embryonic stem cell
hPSC	Human pluripotent stem cell
HVP	Human ventricular progenitor
iPSC	Induced pluripotent stem cell
LV	Left ventricular
MCM	Matrix culture medium
MLC2a	Myosin light chain 2 atrial
MLC2v	Myosin light chain 2 ventricular
mRNA	messenger RNA
NHP	Non-human primate
NSG	NOD scid gamma
NSM	Native slide medium
NYHA	New York Heart Association
PSC	Pluripotent stem cell
RNA	Ribonucleic acid
scRNA-seq	Single-cell RNA sequencing
SHF	Second heart field

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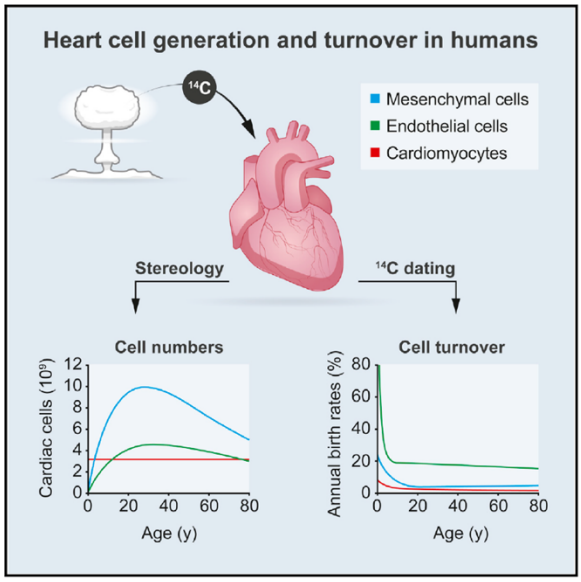
Ischemic heart disease is the leading cause of death worldwide. According to the results of the Global Burden of Disease Study, in the last two decades, the number of such deaths has increased and is expected to rise further in the future due to the aging of the population (Roth et al., 2018). The treatment and prevention of acute coronary syndromes, which are mostly followed by heart failure, has improved noticeably over the last few years. Nonetheless, in contrast to the results from zebrafish studies (Poss, Wilson, & Keating, 2002), adult human hearts lack the capacity to regenerate after the massive death of cardiomyocytes (CMs) caused by myocardial infarction. Instead of rebuilding vital myocardium, a collagen-based scar is predominantly formed in the area of the myocardial infarction (Prabhu & Frangogiannis, 2016). Currently, there are only symptomatic and non-curative therapeutic strategies for end-stage heart failure after myocardial infarction. To date, heart transplantation is the only curative approach. Its usefulness is limited considerably by the discrepancy between supply and demand: in 2021, 329 hearts were donated in Germany, while 727 patients were awaiting surgery (Deutsche Stiftung Organtransplantation, 2021; Hashimoto, Olson, & Bassel-Duby, 2018). For this reason, numerous research groups are studying cardiac regeneration.

6.1 CARDIAC REGENERATION: LIMITATIONS AND CURRENT THERAPEUTIC APPROACHES

Cardiac regenerative capabilities vary between organisms and type of injuries. Life-long regeneration has been observed in lower vertebrate-model species like zebrafish and urodele amphibians, such as newts (Laube, Heister, Scholz, Borchardt, & Braun, 2006; Poss et al., 2002), whereas adult mammalian hearts lose the capacity to regenerate shortly after birth (Bergmann et al., 2015). Although a higher renewal rate after injury has been hypothesised and discussed for many years, the human heart seems to react to injury with an increase in cardiomyocyte size, resulting in hypertrophy, rather than with an increase in the number of cells (Eschenhagen et al., 2017). Bergmann et al. used ^{14}C measurement and stereology to prove that the number of CMs does not change significantly during the human lifespan (Figure 1). In contrast, endothelial and, to an even larger extent, mesenchymal cells in the human heart exhibit an increasing turnover during the lifespan. Most notably, endothelial cells

renew themselves to a high degree even in adulthood. Conversely, cardiomyocyte generation and turnover decrease dramatically during the first days after birth and continue to decline thereafter, with the turnover rate falling to below <1 % annually (Bergmann et al., 2015).



Bergmann et al., Cell 2015. Copyright 2022 by Cell.

Figure 1. Heart cell generation and turnover in the human heart. Stereology has enabled the three-dimensional measurement of cell numbers in two-dimensional microscopic tissue sections of the left ventricular (LV) myocardium (left). Radiocarbon tracer technology has been used to quantify genomic ^{14}C concentrations in order to analyse cell turnover and to date the birth of human CMs (right). It has been shown that CMs lack the potential to generate and turnover, while the number of endothelial and, especially, mesenchymal cells exhibits a dynamic progression during the lifespan, with a peak between the ages of 20 and 30 (Bergmann et al., 2015).

The hallmarks of cardiac regeneration that need to be addressed to restore cardiac function sufficiently are as follows:

- the successful remuscularisation of the damaged area,
- electromechanical stability that avoids arrhythmias,
- angiogenesis from both pre-existing and induced structures,
- an extracellular matrix (ECM) and fibroblasts that prevent scarring,
- and the innate and adaptive immune systems (Bertero & Murry, 2018).

In this context, several therapeutic approaches have received much research attention, all with the aim of finding new strategies for replacing damaged myocardium (Figure 2). The promising therapeutical strategies for heart regeneration entail **1)** the induction of endogenous CM renewal (Bassat et al., 2017; Gabisonia et al., 2019; Wei et al., 2015), **2)** the direct reprogramming of non-CMs such as fibroblasts to the cardiac cell lineage *in vivo* (Srivastava & DeWitt, 2016), and **3)** the remuscularisation of the adult myocardium through directly transplanted human pluripotent stem cell (hPSC)-derived CMs or cardiac progenitor cells (CPCs) (Poch et al., 2022; Shiba et al., 2016).

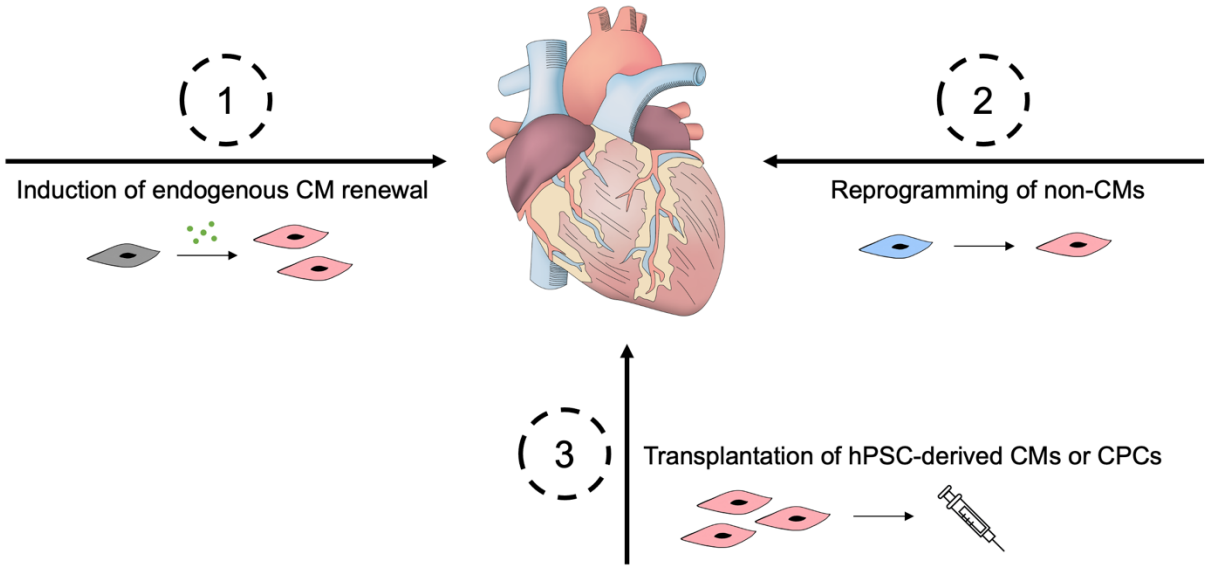


Figure 2. Therapeutic strategies for regenerating heart tissue. The three main strategies for regenerating damaged myocardium are 1) the induction of endogenous CM renewal, 2) the reprogramming of non-CMs such as fibroblasts, and 3) the direct transplantation of hPSC-CMs or CPCs. CMs = cardiomyocytes, hPSC-derived CMs = human pluripotent stem cell-derived cardiomyocytes, CPCs = cardiac progenitor cells.

In the past decades, the reprogramming of native non-CM tissue sources has been the subject of many clinical trials. However, the recognition of the drawbacks of those sources, which include the lack to form new CMs, the poor scalability, and the modest beneficial effects, has increasingly led scholars to consider other cell sources, particularly hPSC-derived CMs or CPCs, as attractive alternatives (Chien et al., 2019; Eschenhagen et al., 2017; Foo et al., 2018; Sadek & Olson, 2020).

Recent preclinical animal studies indicate that hPSC-derived CMs integrate into the hearts of small and large animals, such as pigs and non-human primates (NHPs). This engrafting is associated with electromechanical coupling and improvement in systolic LV function. Nevertheless, concerns about the immaturity of engrafted CMs, the resistance capacity of these cells, and potential ventricular arrhythmias remain (Chong et al., 2014; Shiba et al., 2016).

As stem cell therapies emerge as potential means of regenerating organs, three highly promising clinical trials have been initiated in different medical fields. First, hPSC-derived retinal pigment epithelial has been transplanted to prevent macular degeneration (Mehat et al., 2018). Second, multicellular hPSC-derived pancreatic beta cell progenitors have been implanted in patients with type-I or type-II diabetes to restore pancreatic function (Memon & Abdelalim, 2020). Third, hESC-derived CPCs, embedded in a fibrin hydrogel, have been transplanted onto the hearts of patients with severe heart failure during a coronary artery bypass procedure. Interestingly, the first results of this trial proved the feasibility and safety of generating a sufficiently large number of hESC-derived CPCs. Furthermore, there were no incidents of tumours or arrhythmias and they also revealed evidence of clinical improvement in myocardial function in a small cohort of six patients over a median follow-up period of 18 months (Menasché et al., 2018).

In conclusion, the new therapeutic approaches to regenerating damaged myocardium that have been tested in preclinical and clinical trials have shown promising results. Nevertheless, important issues remain that must be addressed. These include the tumorigenicity, the identification of an optimal timeframe for CPCs or CMs, the indirect impact of interventions on the tissue, and the costs of generating cells. In the future, novel perspectives on gene therapy, genome editing, and surgical automation will create new fields of research.

6.2 Human Embryonic Stem Cells (hESCs)

As noted in the preceding section, experimental approaches with hPSCs have shown promising results for heart regeneration in recent studies. HPSCs can be classified

according to their differentiation potential or by reference to their derivation from either reprogrammed somatic cells, which are also known as “induced pluripotent stem cells” (iPSCs) (Takahashi et al., 2007), or from developing blastocysts, which are called “embryonic stem cells” (ESCs) (Figure 3). Concentrating on ESCs, they can either be characterised as totipotent during the earliest eight-cell stage of the embryo or as pluripotent during the blastocyst phase of fetal development of a four-to-five-day-old embryo. The pluripotent phase is of significant interest for stem stell research (Abou-Saleh et al., 2018). Therefore, such ESCs are obtained from the inner cell mass of blastocysts and cultured for further indefinite division in order to generate a pool of stem cells that can be used in research and therapy (Thomson et al., 1998).

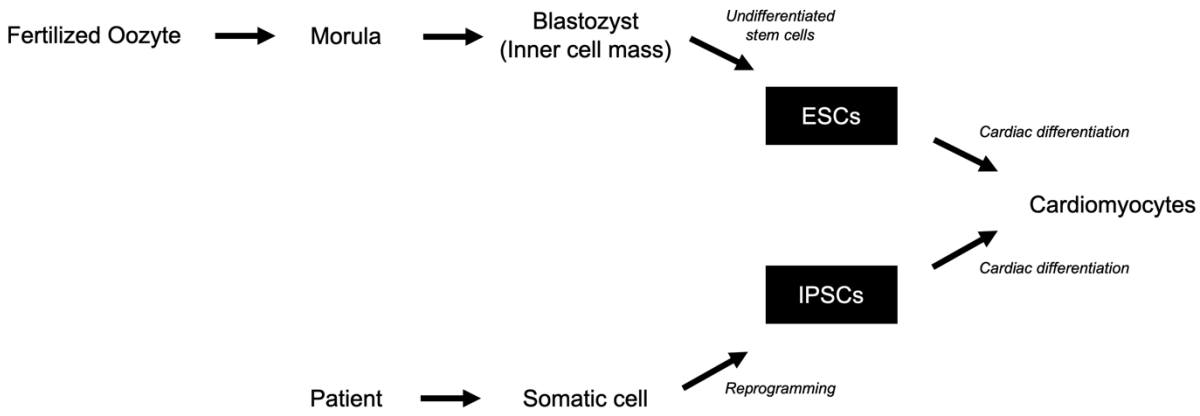


Figure 3. Derivation of hPSCs. CMs can be derived from the inner cell mass of blastocysts as ESCs or from iPSCs after the somatic cells of patients have been reprogrammed. In subsequent courses, they can be used for direct injection in patients or for tissue engineering. ESC = embryonic stem cell, iPSC = induced pluripotent stem cell.

Human ESCs (hESCs) in the pluripotent state have the potential to differentiate into almost every type of cell of ectodermal, mesodermal, or endodermal origin. The study of embryological development has become indispensable for understanding the differentiation strategies of various cell lineages, and for directing differentiation to defined cell types. This research requires the identification of relevant factors and their specific concentrations, combinations, and temporal windows of presence (Williams, Davis-Dusenbery, & Eggan, 2012). The use of defined differentiation programmes enables researchers from all fields to use ESCs, to culture them in a pluripotent state,

and to differentiate them into desired cell types, such as CMs, neurons, or liver cells (Figure 4) (Odorico, Kaufman, & Thomson, 2001).

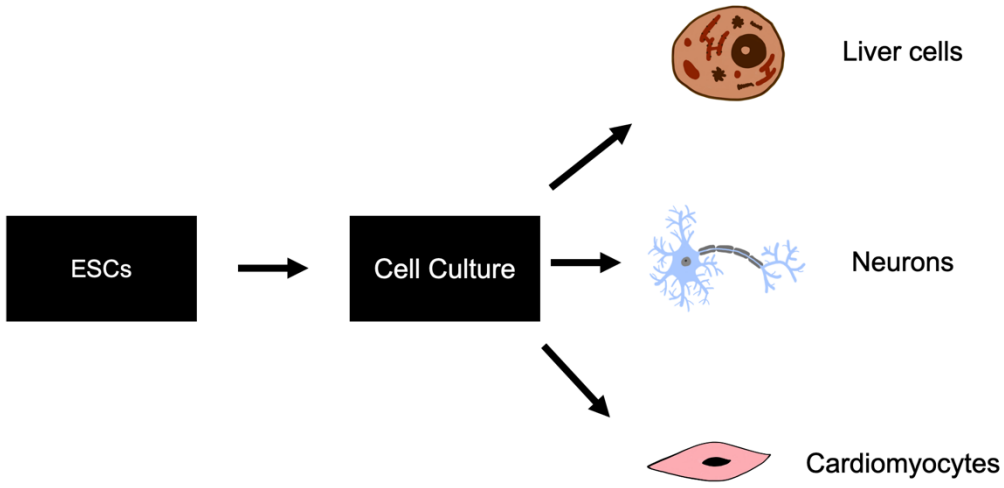


Figure 4. Culture and differentiation of ESCs. HESCs are obtained from the inner cell mass of the blastocyst, which corresponds to the phase of a four-to-five-year-old embryo. Thereafter, the stem cells are cultured. These cells still have the nearly unlimited potential to renew themselves and to differentiate into cells from all three germ layers, namely the endoderm (e.g. liver cells), the ectoderm (e.g. neurons or retinal cells), and the mesoderm (e.g. CMs). ESCs = embryonic stem cells.

The use of hESCs has excited discussion and controversy since the derivation of the first ESC line in 1998 because of the need to destroy the five-day-old embryo to obtain hESCs (King & Perrin, 2014). Nevertheless, despite the development of iPSC research, it is clear that further research on hESCs is urgently needed to study human cardiac development and achieve cardiac regeneration (Hyun, Hochedlinger, Jaenisch, & Yamanaka, 2007). Given the growing interest in hESCs research, several regulations have been passed, and numerous ethical committees have been founded in order to develop ethical guidelines for stem cell research in general and for the study of ESCs in particular. The model of the committee of the National Academy of Science in the US has been followed widely for this purpose (King & Perrin, 2014). Interestingly, the increasing amount of ESCs research has not resulted in an increase in the number of new ESC lines. It has been shown that, between 2008 and 2016, three ESC lines

were used in three quarters of all countries that did research with ESCs and that these cell lines were derived in the early years of ESC research (Guhr et al., 2018).

Due to their almost unlimited ability to renew and to differentiate into all somatic cell types, ESCs provide great potential for *in vitro* disease modeling, drug development, tissue engineering, and studies in regenerative medicine (Doğan, 2018). The strong proliferative drive of undifferentiated hESCs is a unique and cell defining feature. Nonetheless, it also leads to major safety concerns, as these constantly dividing cells have significantly higher risks of teratoma formation (Murry & Keller, 2008).

6.3 CARDIAC DIFFERENTIATION AND DERIVATION OF CARDIAC PROGENITORS

Several protocols for directing hPSC differentiation towards a cardiac lineage have been established based on the knowledge about signaling pathways that control the differentiation of heart cells during embryogenesis (Protze, Lee, & Keller, 2019). Gene-targeting studies and lineage tracing, which is a method for analysing the progeny of a marked single cell, its location, its differentiation status, and its signaling pathways (Kretzschmar & Watt, 2012), have been conducted with a view to developing a more profound understanding of the cardiac differentiation process during embryogenesis.

During human embryonic development, most of the mammalian heart is moulded by mesodermal progenitors in the first and second heart field (FHF and SHF). FHF cells, which are marked by HCN4 and NKX2.5, contribute to the contractile part of the primitive heart tube and to the generation of the cardiac muscle (Spater et al., 2013). $ISL1^{+}$ cardiac precursors originate from the SHF, can produce cells from the different cardiac lineages that are found in the right ventricle and the outflow tract, namely CMs, smooth muscle cells, and pacemaker cells. They exhibit considerable proliferation potential and migration both *in vivo* and *in vitro* (Karl-Ludwig Laugwitz, 2005; Moretti et al., 2006). Progenitors from both the FHF and the SHF seem to contribute, in varying degrees, to the atria (Cai et al., 2003). The concept of FHF and SHF thus explains the distinction between CMs from different compartments of the heart.

Some other transcription factors also define certain cardiac progenitor populations. For instance, Spalt-like gene 1 (Sall1), a zing-finger transcription factor, marks undifferentiated CPCs during development and regulates cardiac differentiation by suppressing the differentiation when being expressed continuously (Morita et al., 2016). Foxa2 identifies CPCs that predominantly differentiate into ventricular cells, with only few cells ending up in the atrial lineage (Bardot et al., 2017). Several reports have also revealed the potential of induced expandable CPCs from fibroblasts to self-renew, proliferate extensively, and differentiate into CMs, endothelial cells, and smooth muscle cells *in vivo* after reprogramming (Witman & Sahara, 2016; Y. Zhang et al., 2016).

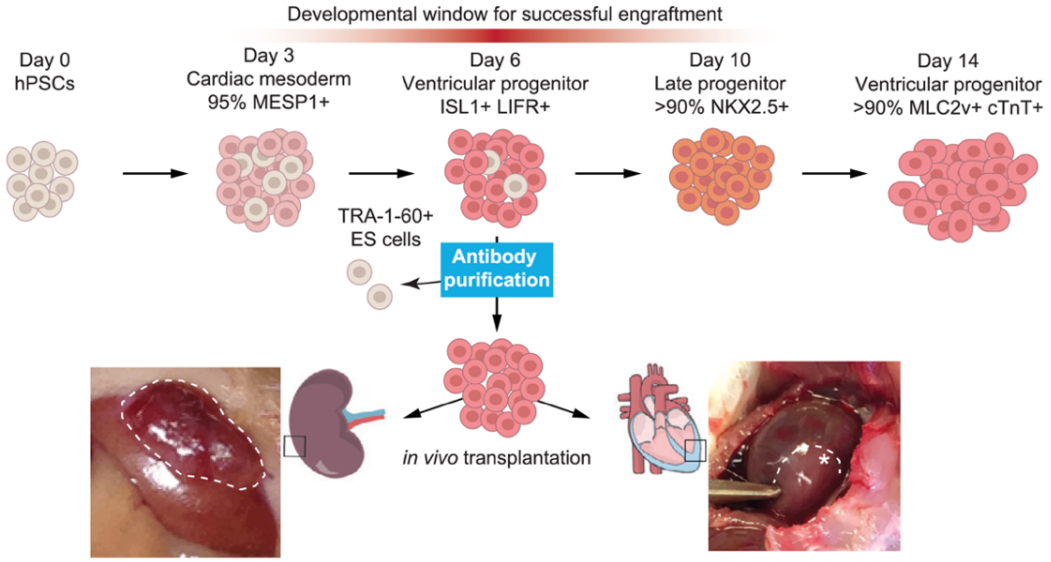
Driven by signalling pathways that are specific to particular cardiac lineages, hPSCs lose their pluripotent potential and differentiate into the defined cardiomyocyte cell type through the stage of the progenitor population. This knowledge is fundamental for developing cardiac differentiation protocols and so today, several cardiac differentiation protocols for the derivation of human ESC- or iPSC-derived CPCs and CMs exist, each with particular features and specifications (Burridge et al., 2014). Initially, the generation of merely heterogeneous cardiomyocyte populations that consist of atrial, ventricular, and pacemaker cells limited applicability (Mummery et al., 2012). Constant improvements in differentiation protocols over the last two decades have enabled the enrichment of more homogenous cell type populations of, for example, defined ventricular cell populations (Foo et al., 2018).

6.4 HUMAN VENTRICULAR PROGENITORS (HVPS)

Human ventricular progenitors (HVPs) form an already lineage-committed and plastic progenitor population during cardiovascular development. In their recent study, Foo et al. (2018) reported having made a breakthrough by establishing a protocol for the generation of a homogenous population of ventricular CMs by generating cardiac mesodermal progenitors that hold the potential to differentiate towards mature ventricular CMs. These ESC-derived $ISL1^{+}$ CPCs can expand, differentiate, aggregate, and develop into functional ventricular slices after sub-capsular transplantation onto kidneys without the use of supporting structures like gels or

matrices in *in vivo* mouse studies. The developmental timeframe was discovered as a key aspect of the self-assembly of HVPs into ventricular phenotypes. Day 6 of differentiation, which is also characterised by peak ISL1 expression, was highlighted as the ideal timeframe (Figure 5). Furthermore, the regenerative potential of HVPs was proven by a second experimental design. Eight months after transplantation of HVPs intramyocardially in (uninjured and injured) hearts of NOD scid gamma (NSG) mice, a stable, self-assembled, vascularized, and matured ventricular muscle graft was documented. Improvements in ejection fraction, wall thickness, contractility, and the end-systolic volume of the injured hearts were also observed (Foo et al., 2018).

Summarising the results from Foo et al., after being transplanted onto the kidneys or the (uninjured or injured) hearts of NSG mice, HVPs lost their progenitor properties and matured into ventricular CMs, forming a functional heart slice, and showing their strong potential for cell-based regenerative therapy. These results point out the unique properties of progenitors, which can sense and react to environmental cues.



Foo et al., Molecular Therapy 2018. Copyright 2022 by Molecular Therapy.

Figure 5. Experimental setting for *in vivo* differentiation, maturation, and transplantation of HVPs. HPSCs were first differentiated into Day 6 HVPs and in the subsequent course, after antibody purification, transplanted either sub-capsular onto kidneys or intra-myocardial into (uninjured or injured) mice hearts. Day 6 was determined as the optimal timeframe. Further differentiation of Day 6 HVPs led to

Day 14 CMs that were positive for MLC1v+ and cTNT+. hPSCs = human pluripotent stem cells, ES cells = embryonic stem cells.

6.5 HVP-DERIVED CARDIOMYOCYTES (CMS)

In their study, Foo et al. also reported on their investigations regarding the further differentiation and maturation of HVPs. Between Day 9 and Day 12, most cells showed uniform beating and were NKX2.5 positive, which is one of the earliest markers in the cardiac lineage and persists even in the adult myocardium. After 14 days, most of the cells were positive for the ventricular markers MLC2v (Myosin light chain 2 ventricular) and cardiac Troponin T (cTnT) and negative for the atrial marker MLC2a (Myosin light chain 2 ventricular), suggesting a ventricular differentiation of the progenitors (Figure 5). Electrophysiological measurements demonstrated that HVP-derived CMs (HVP-CMs) are a population of mature ventricular CMs that becomes quiescent by Day 21. Furthermore, on Day 21, the HVP-CMs responded to pharmacological substances similarly to native adult CMs, as exemplified by the shortening of the action potential after the application of acetylcholine. Taken together, from Day 19 on, HVP-CMs appeared to be relatively mature and to exhibit a ventricular phenotype (Foo et al., 2018).

Whereas in the previously explained study by Foo et al. HVPs that were injected in mice hearts produced the best results, Charles E. Murry's group demonstrated that hESC-derived CMs (hESC-CMs) can also remuscularise infarction areas in macaque hearts. They observed a significant recovery of the treated heart after four months, with improvements in ejection fraction and contractile function. Nevertheless, the ventricular arrhythmias, which were induced by the engrafted cells, were seen as the result of an autonomic pacemaker activity of the injected CMs. This side effect remains a serious safety concern for the clinical application of cell therapy (Chong et al., 2014; Liu et al., 2018). Instead of using hESC-CMs, Zhu et al. (2018) transplanted cardiovascular progenitors and observed an enhancement of NHP heart function in myocardial infarction areas. However, they could not achieve long-term remuscularisation due to cell rejection (Zhu et al., 2018).

Considerable scholarly attention has been devoted to the potential of ESC-derived CPCs and CMs for cardiac regeneration. Nevertheless, the promising results on the regenerative potential of HVPs, which were described in the previous chapters, need to be further investigated *in vitro* and *in vivo*. *In vivo* experiments on small and large animals demand considerable investments of time and resources. Accordingly, the possibility of mimicking *in vivo* conditions in *in vitro* models has aroused ever greater interest over the last decade. To that end, 3D culture models have been developed, and important progress in cardiac tissue engineering has been recorded.

6.6 Role of 3D culture models in cardiac regeneration studies

It is indispensable to test new therapeutic strategies *in vitro* to find solutions for effective cell therapy and to overcome the limitations of the regenerative potential of the human heart. Considering that, platforms for *in vitro* studies of cardiovascular development and regeneration have been improved steadily from 2D monolayer cultures to 3D engineered heart tissues (Tzatzalos, Abilez, Shukla, & Wu, 2016). The application of physiologic preload, electrical stimulation, and 3D multilayer cultivation has led to remarkable improvements in this field. The implementation of these biological requirements into standard culture conditions is referred to as biomimetic culture (Figure 6) (Radisic et al., 2007). The transposition of these principles onto cardiac tissue engineering has raised the prospect of producing an *ex vivo* model that can manage the complexity and the functional properties of heart tissue while enabling resources to be conserved and reducing the need for animal testing. Various systems have been developed and investigated over the last decade.

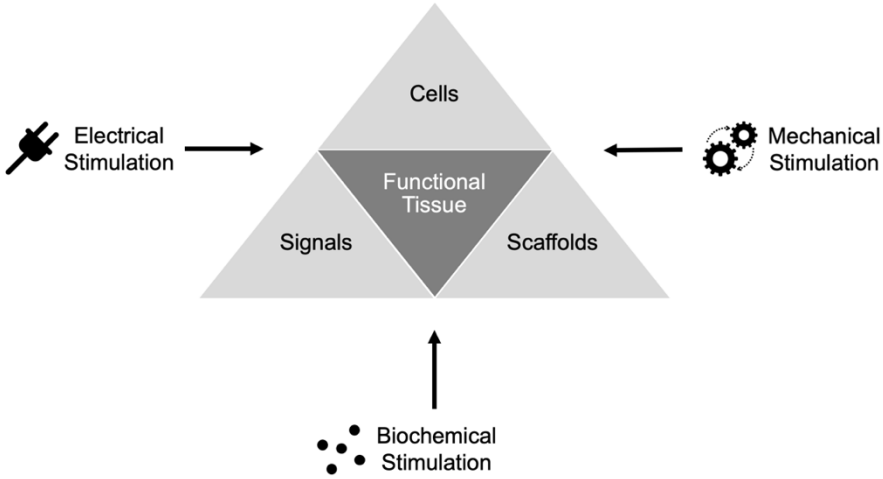


Figure 6. Principles of biomimetic culture. The construction of the ideal biomimetic culture system requires awareness of the biological requirements of an organism and their transfer to an *ex vivo* system. An ideal *in vitro* system, as exemplified by the case of cardiac tissue, is sensitive to the biochemical, electrical, and mechanical environmental signals of the native heart and follows the native cell responses.

In general, the *in vitro* generation of functional heart tissue from PSCs and the long-term cultivation of either engineered or native heart tissue is still limited (Sirabella, Cimetta, & Vunjak-Novakovic, 2015). Several biomimetic approaches have been developed in order to improve cardiac tissue cultivation, differentiation, cell type specification, and maturation (Vunjak-Novakovic & Scadden, 2011). By now, the field of cardiovascular research is increasingly focusing on 3D cultures of engineered heart tissues and the use of various types of scaffolds. Over the past decades, the use of polymeric materials, especially hydrogels, has been researched extensively because of their biocompatibility, the similarity between their structure and that of native ECM, their good usability, and their biochemical and biophysical features (Huang et al., 2017).

Another interesting idea in the use of 3D culture to model and analyse engineered heart tissues *in vitro* has to do with the employment of microfluidic models. Thus, organs on a chip were developed in recent studies. These microfluidic and microfabrication models enable modelling and analysing at a cellular-stage, functional-unit, and tissue level by observation at a microscopic size (Visone et al., 2016). For instance, an endothelialised myocardium-on-a-chip platform allowed the evaluation of the cardiovascular toxicity of drugs by using 3D bioprinting technology to produce endothelialised myocardium (Y. S. Zhang et al., 2016).

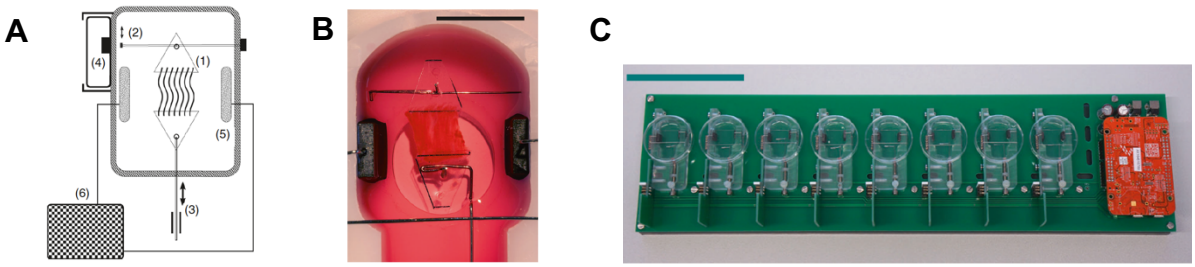
Other recent studies have explored the function of bioreactors in *in vitro* tissue engineering. For instance, Rogers et al. (2016) designed a biomimetic cardiac tissue model to improve the adaptation of human iPSC-derived CMs to the *in vivo* environmental factor of hemodynamic loading by installing a flow loop. Adjustments in the parameters “heart rate”, “pressure”, and “volume” enabled observation of a modified cellular response (Rogers, Fast, & Sethu, 2016). Tandon et al. (2013) reported on the development of a portable bioreactor with perfusion and electrical

control. Due to this bioreactor, the transportation of viable and sterile engineered tissue to hosts in medical operations or to collaborating laboratories could be improved (Tandon, Taubman, Cimetta, Saccenti, & Vunjak-Novakovic, 2013).

Just recently, 3D printed scaffolds and 3D bioprinting techniques have emerged as new technologies that can improve cardiac tissue engineering (Krane et al., 2021; Qasim, Haq, Kang, & Kim, 2019). In 2019, Noor et al. reported the successful printing of a 3D heart. This success was considered a breakthrough in 3D bioprinting because the heart was vascularised, perfusable, and matched to the patient immunologically. Therefore, cells from the omental tissue of a patient were isolated, reprogrammed, and differentiated into CMs and endothelial cells, while the extracellular matrix was used for a personalised hydrogel. In the following step, bioinks for the parenchymal cardiac tissue and blood vessels were constructed from these components. Nevertheless, the heart had the desired tissue architecture, but it was of no functional use. The main reasons were the simplified and limited blood vessel network as well as the tissue weakness (Noor et al., 2019). Consequently, it remains challenging to improve printing techniques and print a heart that can ultimately be transplanted in a patient.

6.7 Long-term culture of native or bioengineered cardiac tissue in biomimetic culture

Not only the 3D structure that surrounds CMs in native hearts is essential for adequate cellular differentiation and maturation, but also the culture conditions play a crucial role for the long-term culture of native or engineered heart tissue. Moving beyond the biomimetic fabrication methods that were described previously, Andreas Dendorfer and his group from the Walter-Brendel-Centre in Munich have invented a promising culture system. In their study, they reported on the construction of biomimetic culture chambers (BMCCs) that allow to culture human myocardium with a stable structure and function for up to four months (Figure 7). This culturing was achieved by mimicking *in vivo* environmental conditions, especially mechanical preload, tissue movement, and electrophysiological stimulation. In addition, the device enables contractile force to be measured dynamically (Fischer et al., 2019).



Fischer et al., Nature Communication 2019.

Figure 7. Design of BMCCs. A Outline of chamber: (1) plastic triangle, (2) magnetic tip on flexible wire, (3) linear attachment wire, (4) magnetic tip movement detector, (5) graphite electrodes, and (6) generator of a continuous stimulation pulse. **B** Chamber in use with clamped in myocardial slice. **C** Setup and organisation of BMCCs.

In detail, native LV myocardium is anchored to plastic triangles and cultured in BMCCs. The biomimetic system enables an elastic contraction because of the use of flexible wires, adequate oxygenation, and an electrical field stimulation. This culture system provides opportunities to study the long-term culture of myocardium, test interventions, genetic manipulation, and drug responses *ex vivo* (Fischer et al., 2019). The developed system provides an ideal environment not only for human native tissue, but also for the native myocardial tissues of other species, such as NHPs or pigs, and for engineered heart tissue that consists of decellularised extracellular matrices that are reseeded by hPSCs, as used in our laboratory in previous studies (Krane et al., 2021).

6.8 Biomimetic culture as maturational approaches for hPSC-derived CMs

The difficulty of achieving advanced maturation in PSC-derived CMs and whole engineered heart tissues has not been solved yet and has been addressed by senior scientists as one of the central challenges in the field of regenerative medicine, tissue engineering, *in vitro* disease modelling, and drug testing (Yang, Pabon, & Murry, 2014). Due to the immature and more fetal phenotype of PSC-CMs, routine clinical application is still limited (Robertson, Tran, & George, 2013). A number of studies in this domain have tried to develop new solutions for tissue engineering. Those studies consider long-term cultivation, 3D tissue engineering, and mechanical and electrical stimulation, as well as genetic, hormonal, and metabolic factors. But only the combination of these

factors has been shown to improve cell and tissue maturation (Sun & Nunes, 2017; Yang et al., 2014).

In a review article, Scuderi and Butcher outlined the differences in developmental maturation between PSC-CMs and native CMs (Table 1). First, they compared PSC-CMs to early or, at most, late fetal-like CMs. As far as morphology is concerned, PSC-CMs, like early fetal CMs, are round and single nucleated, whereas adult CMs are rod shaped and often binucleated. Moreover, the surface area of PSC-CMs ranges from 1,000 μm^2 to 1,300 μm^2, whereas native CMs can reach areas of between 10,000 μm^2 and 14,000 μm^2. The sarcomeres of PSC-CMs are less organised and aligned than those in adult CMs and even of late-stage fetal CMs, and sarcomere striations are indiscernible, which impacts the contractility of the cells. The contraction force of native CMs, at 10-50 nM/mm^2, is significantly larger than that of PSC-CMs, which is 0.1-0.5 nM/mm^2. PSC-CMs mostly contract asynchronously due to their immature electrical coupling and their more unstable resting membrane potential of between -50 to -60 mV, compared to the synchronous beating of adult CMs. The immature calcium handling properties of PSC-CMs are caused by the lack of a transverse tubules system. Furthermore, the mitochondria in PSC-CMs are fewer, smaller, and more disorganised than in adult CMs. Consequently, in PSC-CMs, energy is produced mainly by glycolysis, which is less efficient than the oxidative metabolism in adult CMs. In summary, the authors emphasise that the immature phenotype of differentiated PSC-CMs has limited the further development of tissue engineering and its clinical application so far (Scuderi & Butcher, 2017).

Since the fetal-like phenotype of PSC-CMs creates difficulties, considerable scholarly attention has been dedicated to find solutions for improving maturity. An important aspect is the derivation of stem cells and their maturation stage at time of transfer to *in vitro* systems or transplantation. Using early PSC-derived CMs (Ronaldson-Bouchard et al., 2018) or even progenitors (Foo et al., 2018; Ruan et al., 2015) can lead to an increased structural and functional maturation due to their higher proliferation and integration potentials. In fact, the hormonal environment plays a crucial role in the *in vivo* maturation of adult-like myocardium. Therefore, thyroid hormone supplementation (Jackman, Li, & Bursac, 2018) and combinations of thyroid and glucocorticoid hormones (Parikh et al., 2017) promote electrophysiological

maturation concerning T-tubule development, calcium handling, and excitation-contraction coupling.

		PSC-CMs	Native CMs
Morphology	Cell shape	Round, single-nucleated	Rod-shaped, often binucleated
	Surface area	1,000-1,300 μm^2	10,000-14,000 μm^2
Contractility	Sarcomere structure	Disarrayed	Highly aligned
	Sarcomere striatons	Indiscernible	I-bands, A-bands, M-line, H-zone, Z-disks
	Contraction Force	0.1-0.5 mN/mm^2	10-50 mN/mm^2
Electrophysiology	Contraction	Spontaneously, asynchronously	After stimulus, synchronously
	Resting membrane potential	-50 to -60 mV	-85 mV
Calcium Handling	Transverse tubules	No	Yes
	Sarcoplasmatic reticulum	Underdeveloped	Fully developed
Metabolism	Mitochondria	Immature, small, disorganized	Fully developed, bigger, organized
	Energy production	Glycolysis	Oxidative metabolism

According to Scuderi & Butcher, Frontiers in Cell and Developmental Biology 2017.

Table 1. Comparison between PSC-CMs and native CMs. Table 1 depicts general differences between PSC-derived CMs and native CMs in terms of morphology, contractility, electrophysiology, calcium handling and metabolism.

However, it is now generally accepted that mechanical and, particularly, electrical stimulation has the highest impact on PSC-derived cardiac tissue maturation. For example, it has been shown that biomimetic electrical stimulation regulates the beating properties of PSC-CMs and alters gene expression (Eng et al., 2016). In addition, it has been reported that electrical stimulation leads to increases in contractility and tissue stiffness, to improvements in the orientation of structural compartments, and to larger cells. When electrical and mechanical stimulation are combined, generalised gains in strength and increased expression of RYR2 and SERCA2 are observed, which suggests a matured excitation-contraction coupling (Ruan et al., 2016).

In conclusion, new biomimetic methods have been designed to account for the complexity of environmental factors in *in vitro* systems, as exemplified by the BMCCs that were described above (Fischer et al., 2019). These maturational approaches are

essential to enabling long-term myocardial cultures, modelling of diseases and testing of drugs in realistic conditions, and to finding solutions for the clinical application of stem cells and stem cell-derived tissues.

6.9 Hypothesis: HVPs successfully repopulate and mature inside native left ventricular (LV) non-human primate (NHP) myocardial slices

This thesis investigates the potential of HVPs to repopulate LV myocardial slices of NHPs. Whether HVPs can take over the structural and functional properties of the myocardium in an *ex vivo* long-term culture is highly relevant to the regeneration of cardiac tissue. Furthermore, the thesis assesses the structural and functional maturation of HVPs inside these native LV myocardial slices of NHPs. Conclusively, the aim is to show that HVPs are a promising cell source for stem cell therapy.

7.1 CELL CUTURE OF ESCS

Media and factors used

Accutase	Ref: Gibco, US (A1110501)
B27/-Insulin Supplement	Ref: Gibco, US (A1895601)
CHIR 98014	Ref: SelleckChem, US (CT98014)
CryoStor	Ref: Sigma-Aldrich, US (C2874)
Dimethylsulfoxid (DMSO)	Ref: Thermo Fisher, US (85190)
Essential 8^{TM} Basal Medium	Ref: Gibco, US (A1517001)
Fetal Bovine Serum (FBS)	Ref: Invitrogen (10270)
Matrigel	Ref: BD Biosciences, DE (354234)
PBS (without Ca^{2+} and Mg^{2+})	Ref: Gibco, US (14190144)
ROCK Inhibitor	Ref: Stemcell Technologies, CA (Y-27632)
RPMI 1640 Medium	Ref: Gibco, US (11835030)
Wnt C-59	Ref: SelleckChem, US (S7037)

E8 Medium

- Essential 8^{TM} Basal Medium
  (+) 15mM HEPES
  (+) L-Glutamine
  (+) Sodium Bicarbonate 1.743 g/L
- Essential 8^{TM} Supplement 50x
- Penicillin/Streptomycin (P/S) 0.5 %

The entire content of the Essential 8^{TM} Supplement, which was thawed (at room temperature for 1 h or at 2-8 °C overnight), was transferred to the bottle of Essential 8^{TM} Basal Medium, and 0.5 % P/S was added. Complete Essential 8^{TM} Medium can be stored at 2-8 °C for up to 2 weeks.

Cardiac differentiation basic medium

- RPMI 1640 Medium
- B27/-Insulin Supplement
- Penicillin/Streptomycin (P/S) 0.5 %

The entire content of a thawed B27/-Insulin Supplement was transferred to the bottle of RPMI 1640 Medium, and 0.5 % P/S was added.

7.1.1 Culture and passaging of ESCs

Maintaining ESCs

Guidelines and medium preparations:

- ESCs (ES03 Nkx2.5$^{eGFP/wt}$ and H9 Nkx2.5$^{eGFP/wt}$; ref: 3.04.02/0131, generously donated by Dr. David Elliott, MCRI Australia (Elliott et al., 2011)) were routinely maintained on ∅ 3.5 Matrigel-coated plates in E8 medium, with daily medium change adapted to single-cell culture in order to achieve cardiac differentiation in line with the protocol from Foo et al. (2018).
- Matrigel-coated plates and E8 medium supplemented by ROCK inhibitor at a concentration of 1 µl/ml were equilibrated at room temperature before usage to enhance cell survival.

A vial of frozen hPSCs was removed from liquid nitrogen storage (-80°C) and quickly thawed in a 37 °C water bath for 1 to 2 minutes until the last sliver of ice had melted. The content of the vial was placed in a 15 ml conical tube and filled with E8 medium before centrifugation for 5 min (296 RCF). The supernatant was aspirated. The remaining cell pellet was resuspended carefully in 1 ml of fresh E8 medium and plated on ∅ 3.5 Matrigel-coated plates, which had already been prepared with an additional 1 ml E8 medium that was supplemented by 2 µl/ml ROCK inhibitor. The ∅ 3.5 plates were placed into the incubator (37 °C, 5.0 % O_2, 5.0 % CO_2). The E8 medium was changed every day until the day of passaging.

Passaging of ESCs

Guidelines and medium preparations:

- The total amount of E8 medium and cell suspension were defined by reference to the required splitting ratio (e.g. 1:6, 1:14, or 1:20).
- As far as passaging is concerned, E8 basal medium, supplemented by 1:2,000 ROCK inhibitor, and 0.5 mM EDTA solution were prepared in advance.
- The preparation of the Matrigel-coated plates included equilibration at room temperature for few minutes and aspiration of the supernatant

After the cells had reached a confluency of 85-90 %, the differentiated colonies were removed from the plate by scratching under the microscope. The cells were passaged by dissociation into single cells using Accutase (a ratio of 1:6 or 1:9). Therefore, the E8 medium was aspirated, and the cells were washed twice with 1 ml PBS (-/-). The washing process was followed by the incubation of the plates with 0.5 ml of Accutase at 37 °C for 5 min. After the incubation was complete, the Accutase, including the cells, was pipetted up and down and transferred to a 15 ml conical tube. A total of 5-6 ml of E8 medium were added, and the tube was centrifuged for 4 min (RCF 296). The supernatant was aspirated, and the cell pellet was resuspended with E8 medium supplemented by 5 μM of ROCK inhibitor. The cells were passaged on a new Matrigel-coated six-well plate, in line with the splitting ratio, for 24 h before the E8 medium was changed. The plates were shaken vigorously in a horizontal direction and incubated at 37 °C (5.0 % O_2, 5.0 % CO_2). After between three to five days, the cells reached 85-100 % confluence and were ready for passaging again or differentiation.

7.1.2 Differentiation of ESCs into D6 HVPs

Guidelines and medium preparations:

- Differentiation was performed in line with the protocol of Foo et al. (2018) (Figure 8).
- Cardiac differentiation basic medium D0-D6: RPMI / +B27 -INS +P/S.
- Supplement for differentiation at Day 0: 1 μM CHIR-98014.
- Supplement for differentiation at Day 3: 2 μM Wnt-C59.

After dissociation with Accutase, the single ESCs were plated on Matrigel-coated 12-well plates in E8, supplemented by 1:2,000 ROCK inhibitor at a density of $1x10^6$ cells per well for 24 h. On the following day, the media were changed, and the cells were cultured further in E8.

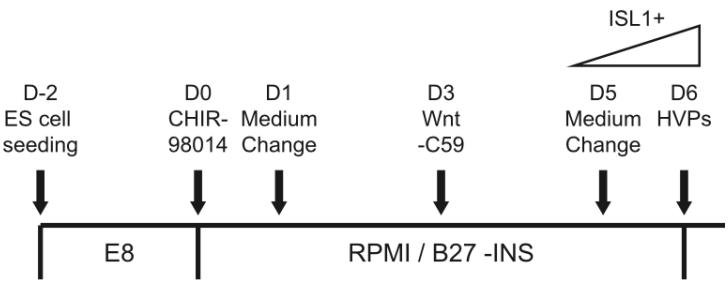
Differentiation Day 0:
After between three and five days of cell seeding in a 12-well plate with E8 medium, the cells reached full confluency, and differentiation was initiated by removing the E8 medium and by adding cardiac differentiation basic medium D0-D6 (RPMI / +B27 -INS +P/S) that was supplemented by 1 μM of GSK3 inhibitor CHIR-98014.

Differentiation Day 1:
After 24 h, the medium was changed to cardiac differentiation basic medium D0-D6 only.

Differentiation Day 3:
On the third day of differentiation, the cells were supplied with a combined medium that consisted of 1 ml of collected conditioned media and 1 ml of fresh cardiac differentiation basic medium D0-D6, supplemented by 2 μM of Wnt-C59.

Differentiation Day 5:
The medium of differentiation for Day 3 was replaced completely with cardiac differentiation basic medium D0-D6 only.

Differentiation Day 6:
The HVPs were collected for magnetic-activated cell sorting. Therefore, an average of $6x10^6$ HVPs per well were collected and dissociated into single cells by using Accutase. In the end, the single cells were cryopreserved in 1 ml of freezing medium (Cryostor, Sigma Aldrich, USA) at -80 °C for up to one year.



Foo et al., Molecular Therapy 2018. Copyright 2022 by Molecular Therapy.

Figure 8. Schematic protocol for the differentiation of ESCs into D6 HVPs. ESCs were plated on culture dishes and medium was changed every day. After confluency was reached, differentiation was initiated by adding CHIR-98014 to the cardiac differentiation basic medium on D0 and by adding Wnt-C59 on D3. Only the cardiac differentiation basic medium D0-D6 was changed additionally on D1 and D5. On D6, this differentiation protocol enabled the sufficient production of highly enriched ISL1+ HVPs and a purity of ~95 %. ES cell = Embryonic stem cell, HVPs = Human ventricular progenitors.

7.2 3D CO-CULTURE OF LV NHP HEART SLICES AND HVPS

For a co-culture to emerge, two or more different types of cells have to be cultured together. Here, this entails culturing native heart slices that still contain native CMs, non-CMs such as fibroblasts, endothelial cells, smooth muscle cells, neurons and immune cells, and an extracellular matrix together with differentiated ESCs-derived D6 HVPs in one system.

Media and factors used

Agarose	Ref: Thermo Fisher, US (17850)
B27/-Insulin Supplement	Ref: Gibco, US (A1895601)
2,3-Butanedione Monoxime (BDM)	Ref: Sigma-Aldrich, US (B0753)
DNase I from bovine pancreas	Ref: Sigma-Aldrich, US (DN25)
EDTA	Ref: Invitrogen, US (15575020)
HEPES	Ref: Gibco, US (15630106)
Histoacryl tissue glue	Ref: B. Braun, DE (69390)

Insulin/Transferrin/Selenium (100x)	Ref: Gibco, US (41400-045)
L-cysteine hydrochloride monohydrate	Ref: Sigma-Aldrich, US (C6852)
M199 Medium (1x)	Ref: Thermo Fisher, US (11043023)
β-Mercaptoethanol	Ref: Sigma-Aldrich, US (M6250)
Multicell Culture Inserts, 0.4 μm / 30 mm	Ref: Merk, DE (PICM03050)
PBS (without Ca^{2+} and Mg^{2+})	Ref: Gibco, US (14190144)
RPMI 1640 Medium	Ref: Thermo Fisher, US (11835030)
Trypsin Inhibitor	Ref: Sigma-Aldrich, US (T9253)

4 % Agarose

- Agarose 4 g
- MilliQ Water 100 ml

Cutting buffer

- NaCl 136 mM
- KCl 5.4 mM
- $MgCl_2 * 6\ H_2O$ 1 mM
- $NaH_2PO_4 * H_2O$ 0.33 mM
- Glucose * H_2O 10 mM
- $CaCl_2 * 2\ H_2O$ 0.9 mM
- BDM 30 mM
- HEPES 5 mM

The components were diluted in milliQ water. The pH of 7.4 was adjusted with NaOH 1 M. The solution was filtered and stored at 4 °C.

Matrix culture medium (MCM)

- RPMI 1640 Medium
- B27/-Insulin Supplement
- Penicillin/Streptomycin 0.5 %

The entire content of the thawed B27/-Insulin Supplement was transferred to a bottle of RPMI 1640 Medium, and 0.5 % P/S was added.

Native slide medium (NSM)

- M199 (1x)
  (+) Earle's Salts
  (+) L-Glutamine
  (+) 2.2 g/l Sodium Bicarbonate
- β-Mercaptoethanol 50 μM 0.5 %
- Insulin-Transferrin-Selenium (100x) 1 %
- Penicillin/Streptomycin 0.5 %

β-Mercaptoethanol, Insulin-Transferrin-Selenium and P/S were added to the M199 Medium and stored at 2-8 °C for up to 2 weeks.

7.2.1 EX-VIVO CULTURE OF NATIVE MYOCARDIAL LV NHP HEART SLICES

NHP cardiac tissue samples were shipped from the German primate centre in Göttingen (reference: 33.19-42502-04-16/2264), the Karolinska Institutet in Sweden (reference: N277/14), and the Walter Brendel Institute in Germany (reference: ROB-55.2-2532.Vet_02-14-184). The myocardial tissue was obtained from the left mid-ventricular transmural sections and immediately placed in a 30 mM 2,3-Butanedione-2-monoxime solution at 4 °C.

7.2.1.1 LV MYOCARDIUM PREPARATION

The LV NHP myocardial tissue specimen was transferred from the cardio care solution, in which the tissue had been transported, to a rubber cutting mat, and the endocardial, trabecular, and epicardial layers were trimmed with a scalpel or a razor blade (Wilkinson Classic). A tissue block with approximate dimension of 1 cm x 2 cm x 0.8 cm was cut, in line with the direction of the fibre and the best available morphology.

The 4 % agarose was cooked in a microwave until it liquefied and formed a homogenous mass. The sample had been placed in the centre of a ∅ 3.5 cm plate, and the block of heart tissue was embedded in cooled down 4 % agarose, with the epicardial surface facing down. After the gelification was complete, a block containing the embedded LV NHP heart tissue was cut straight by removing the excessive agarose.

7.2.1.2 VIBRATOME CUTTING

Before the Vibratome (VT1200S, Leica Biosystems, Germany) could be used for cutting, the settings had to be adjusted. A Vibro Check was completed, the speed was set to 0.7 mm/s, the amplitude to 1.5 mm, and the thickness to 300 μm.

The 4 % agarose block that contained the embedded LV NHP heart tissue block was glued with histoacryl tissue glue (B. Braun 69390) to the holder of the Vibratome and transferred into the cooling bath, which was filled with cutting buffer. The vibrating razor blade was aligned precisely, and slicing was performed at 4 °C. The 300 μm thick LV NHP heart slices were cut automatically and processed immediately (Figure 9).

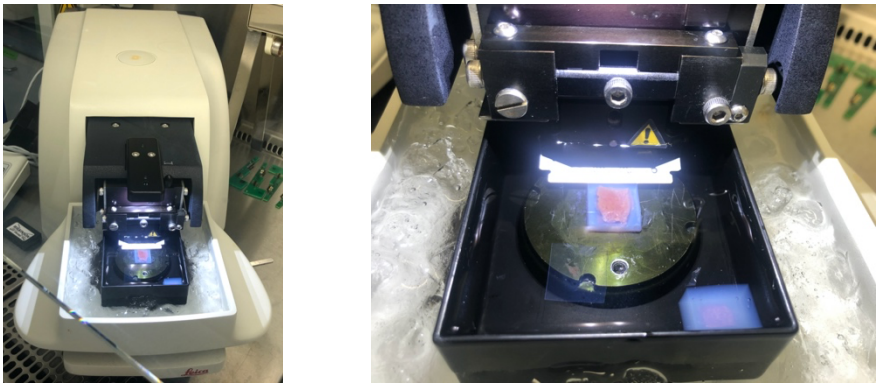


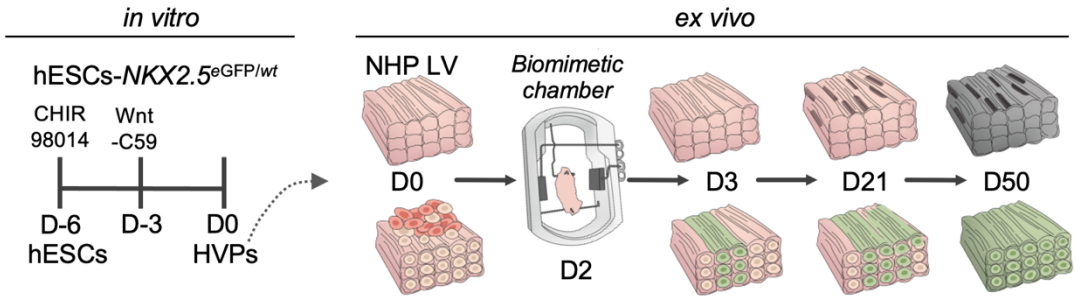
Figure 9. Vibratome in use. The prepared 4 % agarose block that contained the embedded LV NHP heart tissue was positioned in the cooling bath at 4 °C, where it was surrounded by ice. The slicing process was performed by the Vibratome, and the LV NHP heart slices were processed further immediately after cutting.

7.2.1.3 BIOMIMETIC CULTURE OF LV NHP HEART SLICES

The LV NHP heart slices were transferred from the vibratome cooling bath to the lid of a Petri dish. The surrounding agarose was removed carefully from the LV NHP heart slice. Thin plastic triangles were glued with histoacryl tissue glue onto the edges of the heart slice. The fixation was executed with consideration of the parallel orientation of the muscle fibres, and the protruding parts at the edges of the slice were trimmed with a scalpel to the size that was specified by the triangles. The slice with the attached plastic triangles could be removed from the lid of the Petri dish with help of a drop of cutting buffer. The constructed LV NHP heart slices were transferred from the lid of the Petri dish onto the chamber filters (multicell culture inserts 0.4 μm 30 mm) in a six-well plate to generate HVP-NHP heart slices. Alternatively, they were anchored directly to BMCCs, which were filled with NSM to serve as controls.

7.2.2 GENERATION OF HVP-NHP HEART SLICES

In order to generate co-cultured HVP-NHP heart slices, D6 HVPs had to be reseeded on the LV NHP heart slices, which were placed onto multicell culture inserts on a six-well plate, before being transferred to BMCCs (Figure 10).

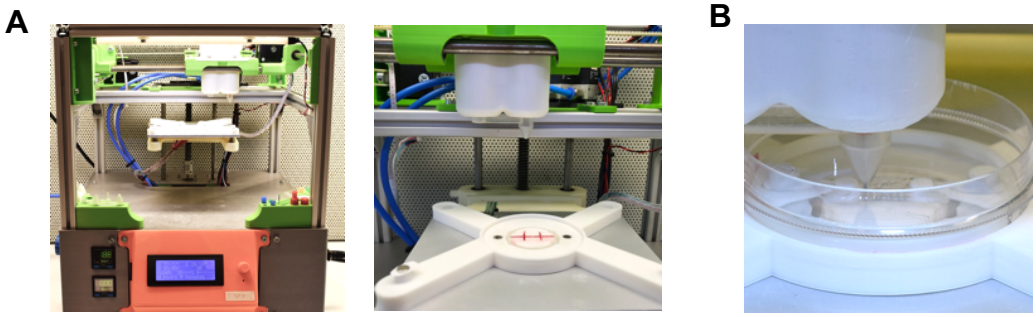


Poch et al., Nature Cell Biology 2022.

Figure 10. Schematic representation of the experimental design. *In vitro* differentiation of CPCs into D6 HVPs and their seeding onto native NHP heart slices for the purpose of synthesising co-cultured HVP-NHP heart slices *ex vivo*. hESCs = human embryonic stem cells, HVPs = human ventricular progenitors, NHP = non-human primate, LV = left ventricular.

7.2.2.1 SEEDING OF HVPS ON NATIVE NHP HEART SLICES

The previously enriched HVPs were thawed and centrifuged for 4 min (296 RCF) in 5 ml MCM, supplemented by 1:2,000 ROCK inhibitor at room temperature. The supernatant was aspirated, and the cell pellet was resuspended in MCM and diluted to achieve the required number of cells per vial. After that, the cells were centrifuged again for 4 min (296 RCF). In general, an estimated mortality rate of 30 % after thawing had to be considered for further seeding. The supernatant was removed, and the cells were seeded onto the native LV NHP heart slice by pipetting or by bioprinting (CANTER Bioprinter V4) with a 0.58 mm Luer-Lock nozzle (Vieweg Dosiertechnik) within a pluronic F-127 (Sigma Aldrich) frame (Figure 11). After pipetting, the samples were centrifuged for 2 min (RCF 296) in order to promote the integration of the cells. This step was repeated three or four times. In both procedures, a total of 500 µl of MCM was added under the filter, and an additional 500 µl of MCM was added two and four hours later. The six-well plate with the co-cultured heart slices was incubated at 37 °C (5.0 % O_2, 5.0 % CO_2 and 80 % humidity). After between 12 h and 24 h, rocking (60 rpm, 15° tilt angle) resumed.



Adapted from Poch et al., Nature Cell Biology 2022.

Figure 11. Bioprinting device. A Custom-made bioprinting device with pneumatic printhead. **B** Representative image of cell seeding on native NHP heart slices.

7.2.2.2 TRANSFERAL OF 3D HVP-NHP HEART SLICES TO BMCCS

After two days, the slices were transferred from the chamber filters into the BMCCs. Therefore, the components of the culture dish were sterilised, assembled manually,

and filled with 2.4 ml MCM. The HVP-NHP heart slice was anchored to the elastic spring wire by inserting one of the plastic triangles with a hole into the fixed wire pin and by inserting the other plastic triangle into the movable wire pin. The heart slice was submerged into the medium until it was covered with medium completely, and the culture dish was closed with a lid. The BMCC was transferred to the culture platform, which was installed in the incubator. The slices were subjected to the physiological preload of 1 mN once more, and, from Day 2 on, pacing was performed at 1 Hz (50 mA pulse current, 1 ms pulse duration). The medium was replaced every other day.

7.2.3 Long-term 3D culture of HVP-NHP heart slices

The long-term 3D culture was generated by using the system that was designed and constructed by Professor Dendorfer's research group at the Walter Brendel Centre at Klinikum Großhadern in Munich (Figure 12A) (Fischer et al., 2019). This culture platform enables the application of variable mechanical strain, electrical field stimulation, and the compilation of readouts of contractile forces that are specific to each BMCC that is installed at 37 °C in an organised setting in the incubator (5.0 % O_2, 5.0 % CO_2, and 80 % humidity).

The metal wires to which the co-cultured HVP-NHP heart slices and the control slices were attached were constructed differently. One metal wire was stable in order to adjust mechanical preload outside of the chambers, and the other was flexible (spring constant of 75 mN/mm). This decision was made in order to generate a linear relationship between the shortening of the slice and contraction forces. These signals were registered by the displacement of magnetic sensors and forwarded to the computer via USB. The computer recorded traces, which could then be tracked visually in the programme MyoDish (Figure 12B). Mechanical stimulation resulted from the continuous shaking on a rocker plate (60 rpm, 15° tilt angle). Two graphite electrodes provided electrical field stimulation. The pacing was performed at 0.5 Hz, with bipolar pulse currents of 50 mA that lasted 3 ms alternating with zero currents for 1 ms. The parameters could be adjusted through the custom programme MyoDish, as described by Fischer et al. (2019). Furthermore, 1.6 ml of 2.4 ml MCM was changed at 48 h intervals, and the slices were stimulated successively by individual impulses in order

to determine force-frequency relationships at 0.5, 1.0, 1.5, 2.0, and 2.5 Hz. Continuous readouts of the traces were accomplished by importing and analysing the recorded data through the software LabChart Reader (AD Instruments, Australia).

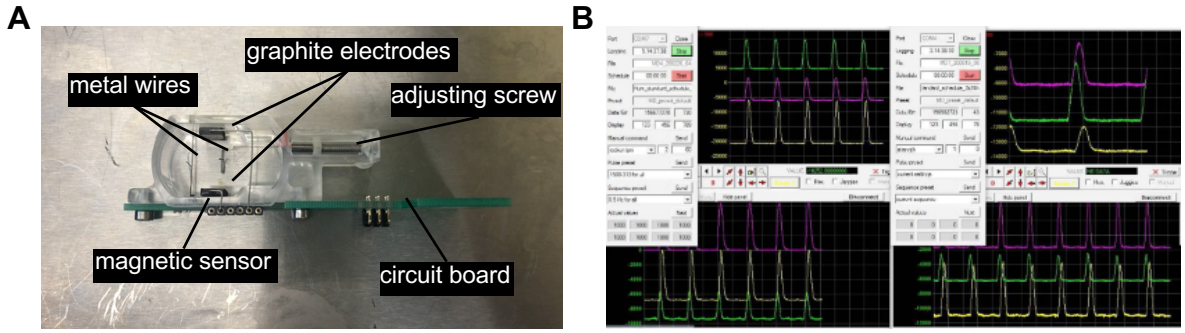


Figure 12. Hardware and software of the culture platform system. A Top view of BMCC. **B** Desktop view of programme MyoDish in use. Individual contractile traces can be observed.

7.3 Immunohistochemistry and flow cytometry of HVP-NHP heart slices

In order to demonstrate the migration and repopulation of D6 HVPs on LV NHP heart slices, the co-cultured slices were first fixed with paraformaldehyde (PFA) 4 %, frozen in O.C.T. Compound, cryosectioned, and stained for immunofluorescence analysis.

Media and factors used

2,3-Butanedione Monoxime (BDM)	Ref: Sigma-Aldrich, US (B0753)
Click-it EdU594 kit	Ref: Thermo Fisher, US (C10646)
Collagenase type II	Ref: Worthington, US (CLS2)
DAKO Mounting Solution	Ref: Agilent, US (S3023)
FACS kit	Ref: Invitrogen, US (C10420)
Fetal bovine serum (FBS)	Ref: Gibco, US (10270)
O.C.T. Compound Tissue-Tek	Ref: Sakure Finetek, NL (4583)
Paraformaldehyde (PFA) 4 %	Ref: Thermo Fisher, US (047392.9M)
PBS (with Ca^{2+} and Mg^{2+})	Ref: Gibco, US (14190144)
Triton X100	Ref: Thermo Fisher, US (85111)

7.3.1 CRYOSECTION OF CO-CULTURED LV HVP-NHP HEART SLICES

Preparation of samples:
BDM was added to the BMCCs for 10 min in order to decrease muscle hypercontraction before fixation and freezing. The co-cultured LV HVP-NHP heart slices were transferred from the BMCCs, the plastic triangles were removed from the edges, and the medium was aspirated. The slices were incubated in PFA 4 % for 20 min at room temperature. PFA was removed, and the slices were washed with PBS five times for 3 min. The samples were placed into a standard freezing mould and filled with O.C.T. Compound. The mould was transferred to a glass vessel that was filled with methyl butane, and freezing was accomplished by using liquid nitrogen.

Cryosection:
The frozen O.C.T. Compound block that contained the LV NHP heart slice was attached to the holder on the Cryotom. Sections that were 12 μm thick were cut manually throughout the whole slice and placed on glass coverslips for immunostaining.

7.3.2 IMMUNOSTAINING OF CRYOSECTIONED SLICES

Thin tissue slices that were 12 μm thick and were fixed on glass coverslips were dried for 20 min at room temperature. For fixation, the tissues were covered with PFA 4 % and incubated for another 20 min at room temperature before being washed and equilibrated with PBS (+/+) three times for 5 min. Blocking and cell permeabilization were conducted by incubating tissue slices with 0.1 % Triton X100 and 10 % FBS that was suspended in PBS (+/+) for 2 hours at room temperature.

Primary antibodies (Table 2) were diluted in 0.1 % Triton X100 and 1 % FBS that was suspended in PBS (+/+) and incubated, covered with Parafilm and a lid, at 4 °C overnight. After incubation, the coverslips were washed with 0.1 % Triton X100 in PBS (+/+) five times for 5 min.

Secondary antibodies (Table 2) of the appropriate species were diluted in 0.1 % Triton X100 and 1 % FBS that was suspended in PBS (+/+). They were then incubated again, protected from light and at room temperature, for 1 h. After three 5-min washes with PBS (+/+), Hoechst 33258 was added at a final concentration of 5 µg/mL in PBS at room temperature for 15 minutes in order to mark nuclear DNA. After having been washed once with PBS, the cells were mounted with fluorescence mounting medium. Images were generated by using a DMI6000-AF6000 Leica epifluorescence microscope or a SP8 confocal laser-scanning Leica microscope. The images were assigned with pseudocolours and processed with Image J.

Reagent	Source	Identifier	Concentration
First Antibodies			
Anti-Cardiac Troponin T, mouse monoclonal	Thermo Fisher Scientific	MA5-12960, Cl. 13-11	1:500 (IF)
Anti-CD31 (PECAM-1), sheep polyclonal	R&D systems	AF806	1:100 (IF)
Anti-Cleaved Caspase 3, rabbit monoclonal	Thermo Fisher Scientific	MA5-32015, Cl. SR01-02	1:100 (IF)
Anti-GFP, chicken polyclonal	Abcam	ab13970	1:500 (IF)
Anti-ISL1, mouse monoclonal	DHSB	Cl. 39.4D5	1:100 (IF, flow cytometry)
Anti-MLC2a AF647, mouse monoclonal	Synaptic Systems	311011 AT1, Cl. 56F5	1:100 (IF)
Anti-MLC2v, rabbit polyclonal	Proteintech	10906-1-AP	1:300 (IHC)
Second Antibodies			
Alexa Fluor 488, goat anti rabbit	Abcam	ab150077	1:250 (IF)
Alexa Fluor 488, donkey anti chicken	Jackson Immuno Research	703-545-155	1:100 (IF)
Alexa Fluor 647, goat anti mouse	Abcam	ab150115	1:250 (IF)
Alexa Fluor 647, donkey anti sheep	Abcam	ab150179	1:100 (IF)
Hoechst 33258 Staining Dye Solution	Abcam	ab228550	1:100 (IF)
Assays			
LIVE/DEAD viability/cytotoxicity kit	Thermo Fisher Scientific	L3224	2 µM Calcein AM 4 µM Ethidium homodimer-1

Table 2. Overview of fluorescent probes and antibodies. The probes and antibodies were used for the specific immunostaining of co-cultured HVP-NHP and native LV heart slices.

7.3.3 Fluorescence analysis using flow cytometry

Antibody-based detection of the nucleoside 5-ethynyl-2'-deoxyuridine (EdU), which is based on a click reaction, was employed to measure DNA synthesis directly in order to analyse the ability of HVPs to proliferate. Accordingly, the HVPs within the NHP heart slices were labelled by incubation with 10 µM EdU for 12 hours before being dissociated with 480 U/ml type-II Collagenase. Thereafter, fixation was conducted by incubating with 4 % PFA for 15 min at room temperature, followed by three PBS washes. The cells were processed with a FACS kit (Click-iT EdU594 Flow Cytometry Assay Kit, Thermo Fisher Scientific, C10646, 1 µM), according to its manual. Flow cytometry acquisition was performed with Gallios (Beckman Coulter), and the data were analysed with Kaluza Software (Beckman Coulter).

7.4 Statistical analysis

Statistical testing was performed with GraphPad Prism 9. The values of the statistical data are given as means ± SEM unless indicated otherwise. Two groups were compared by using an unpaired *t*-test. Three groups, or repeated measures, were analysed by using a one-way or multiple-way ANOVA. The significance thresholds were set as follows unless indicated otherwise: *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$.

8.1 STRUCTURAL AND FUNCTIONAL PROPERTIES OF NATIVE LV NHP HEART SLICES DETERIORATE IN THE LONG-TERM *EX VIVO* 3D CULTURE

After performing long-term culture of native LV NHP heart slices under the physiological preload of 1 mN and continuous pacing of 1 Hz, the heart slices were processed via cryosection, and immunohistochemistry was conducted in order to visualise various functional and structural properties. The immunofluorescent staining revealed the decreasing expression of cTnT during the culture of the heart slices from Day 7 to Day 21. CTnT is the tropomyosin-binding subunit of the troponin complex and is therefore important for the regulation of muscle contraction. The staining for activated cleaved Caspase-3 (ClCasp3) on Day 7, Day 14, and Day 21 provided evidence of the increased activation of this protease over time, which peaked on Day 14 (Figure 13A). The role of caspases in the apoptotic pathway of cells is well documented. The quantification of apoptosis was analysed by using a one-way ANOVA. The resultant chart quantitatively visualized the average increasing percentage of ClCasp3$^+$ CMs from ~5 % on Day 0 to ~25 % on Day 21 with a peak of ~29 % on Day 14 (Figure 13B).

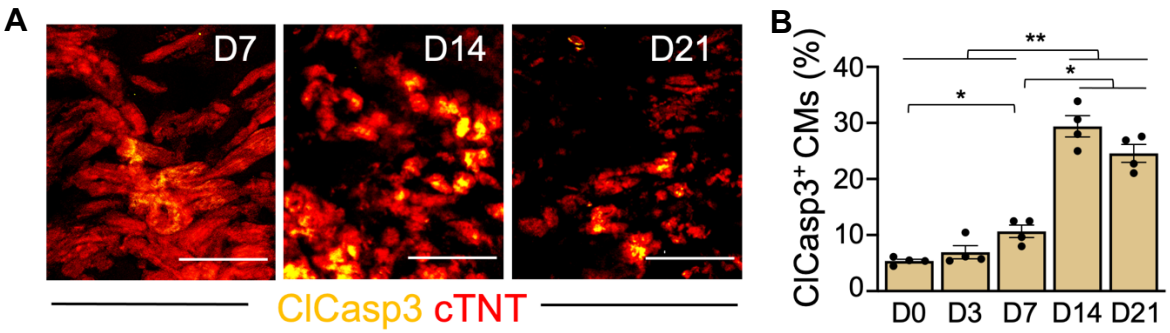


Figure 13. Staining for cTnT and ClCasp3. A Representative immunofluorescence images of cTnT and ClCasp3 in native LV NHP heart slices, cultured *ex vivo,* on D7, D14, and D21. Scale bar: 50 μm. **B** Percentages of ClCasp3$^+$ CMs on D0, D3, D7, D14, and D21 of culture, quantified by reference to immunofluorescence. The data are given as means ± SEM; n = 4 samples/time point; *p < 0.05, **p < 0.01 (one-way ANOVA).

Additional immunofluorescence analyses of Calcein and Ethidium Dimer staining were conducted on Day 7, Day 14, and Day 21 in order to evaluate the vitality of native LV NHP heart slices during further culture. Calcein is used as a marker to trace vital cells and to detect cell migration, whereas Ethidium Dimer marks dead or dying cells. The results showed that the number of Calcein-positive cells decreased from Day 7 to Day 14 and from Day 14 to Day 21. Conversely, Ethidium Dimer-positive cells exhibited the opposite tendency during the same period (Figure 14), which was indicative of the slow death of native LV NHP heart slices.

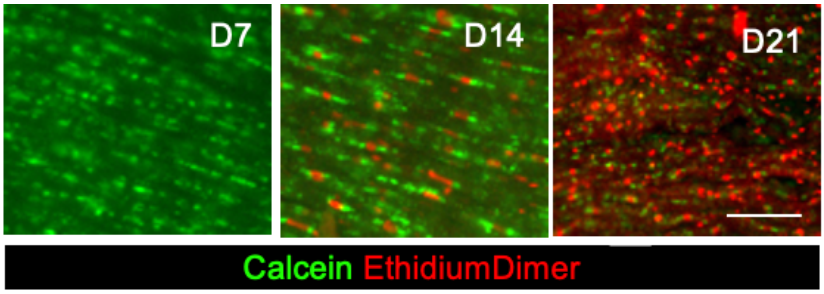


Figure 14. Representative life-analysis of Calcein and Ethidium Dimer staining. Fluorescent dyes used to label living (Calcein) and dead (Ethidium Dimer) cells from D7 to D21. Scale bar: 50 μm.

Furthermore, the contractile forces of native LV NHP heart slices were visualised by analysing continuous contractility traces in Lab Chart Reader over 21 days of culture. These traces were produced by contracting native LV NHP heart slices and detected by magnetic sensors. After the stressful transfer of myocardial slices at the beginning of biomimetic culture, the contractile forces increased from Day 0 to Day 7. From Day 7 to Day 14, the contractile forces initially remained stable. However, they deteriorated by more than 50 % around Day 14. Subsequently, further decreases could be observed until Day 21 or until no contractile forces could be detected anymore (Figure 15).

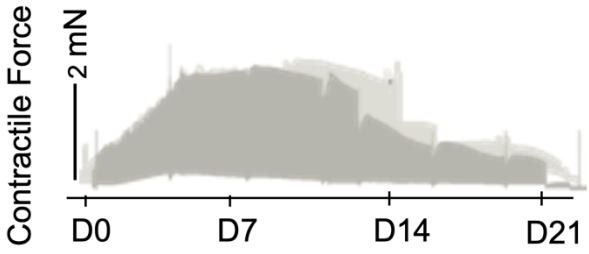


Figure 15. Representative contractility traces of two native LV NHP heart slices cultured *ex vivo* over 21 days. Contractility traces from two LV NHP heart slices were analysed in Lab Chart Reader. After an initial regain of contractile force during the first five to seven days, a stable phase followed before contractile force began to decrease progressively after D14.

8.2 HVPs PROLIFERATE AFTER SEEDING ON NATIVE LV NHP HEART SLICES

After having seeded Day 6 HVPs on native LV NHP heart slices, flow cytometry analyses of EdU fluorescence were performed on Day 7, Day 14, and Day 21 (Figure 16A and 16B) after gating for green fluorescent protein (GFP) positive cells. The measurement of EdU expression levels was examined to assay DNA synthesis in cell culture and to track proliferating cells. The population of Nkx2.5-GFP$^+$ cells only included marked and seeded Day 6 HVP cells. Between Day 0 and Day 3, the number of EdU$^+$ cells increased further and peaked at ~97 % (nearly 100 %) of all GFP$^+$ gated cells on Day 3. In the following period, the indicated proliferation rate decreased to ~59 % on Day 7 before recovering to ~66 % on Day 14. The further development of the proliferation rate was marked by a decline to ~29 % on Day 21.

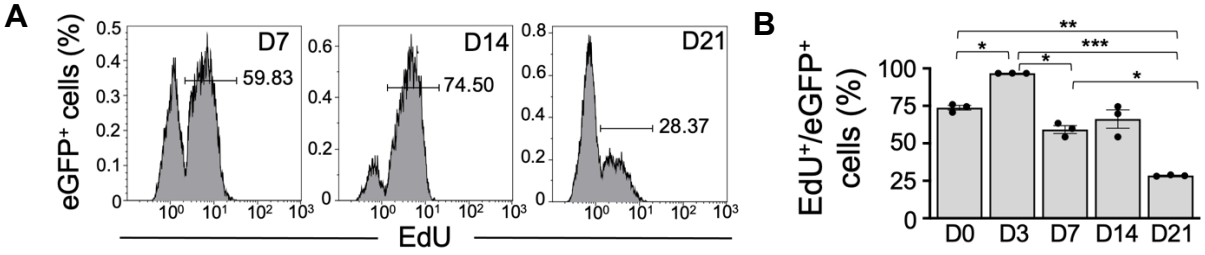


Figure 16. Flow cytometry analysis of proliferation rates. A Representative flow cytometry analysis for EdU in enhanced GFP$^+$ cells that were isolated at the indicated days of co-culture. **B** Average percentage of EdU$^+$/GFP$^+$ cells on D0, D3, D7, D14, and D21. Data are given as means ± SEM; $n = 3$ samples/time point; *$p < 0.05$, **$p < 0.005$, ***$p < 0.001$ (one-way ANOVA).

Conducting further experiments, dynamic changes of the EdU$^+$/GFP$^+$ ratio, which indicates the actively proliferating HVP population, compared to the ClCasp3$^+$/GFP$^-$

ratio, representing the dying native NHP myocardium over time, could be identified (Figure 17). In general, as mentioned previously, the first increase in the number of EdU^+/GFP^+ cells was followed by a decline of proliferating cells, with a renewed recovery on Day 14. Conversely, the number of $ClCasp3^+/GFP^-$ cells seemed to increase from ~5 % to ~11 % during the first seven days and reached its highest point on Day 14 (~29 %). After Day 14, the percentage of $ClCasp3^+/GFP^-$ cells stabilised.

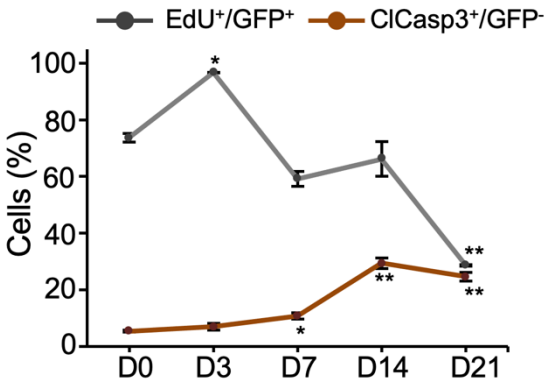


Figure 17. Percentage of EdU^+/GFP^+ and $ClCasp3^+/GFP^-$ cells. The figure displays the percentages of $EdU^+/eGFP^+$ and $ClCasp3^+/GFP^-$ cells during co-culture on indicated days. The data are given as means ± SEM; *n* = 3 samples/time point for EdU analysis, and *n* = 4 samples/time point for ClCasp3 analysis; **p* < 0.05, ***p* < 0.005 vs. D0 (one-way ANOVA).

8.3 HVPs EXPAND AND REPOPULATE NATIVE LV NHP HEART SLICES IN ORDER TO TAKE OVER FUNCTIONAL AND STRUCTURAL PROPERTIES

Beyond the ability of HVPs to proliferate after seeding on native LV NHP heart slices, they also showed expansion within the NHP heart slices. Enhanced GFP (eGFP) expression that was regulated by the NKX2.5 locus facilitated the live tracing of HVPs and the live eGFP imaging of HVPs and of their derivative CMs within the NHP heart slices. The stepwise migration and repopularisation of the native LV NHP heart slices can be displayed visually (Figure 18).

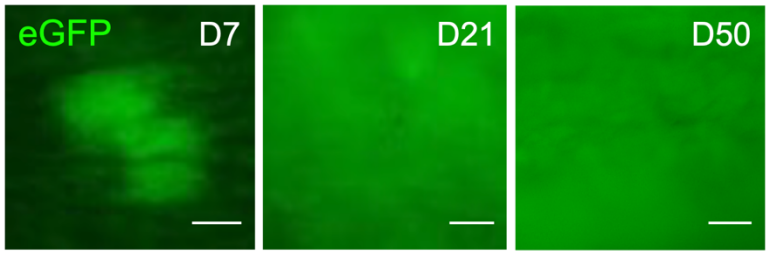


Figure 18. Live cell imaging of HVP expansion. The representative eGFP expression of HVPs showed progressive expansion and repopularisation of the native LV NHP heart slice. Scale bar: 200 μm.

The contractile forces readout from the long-term co-cultured HVP-NHP heart slices revealed the following results: a) from Day 0 to Day 14, the contractile force traces for native NHP heart slices and co-cultured HVP-NHP heart slices were similar, b) in contrast to the native NHP slices, the co-cultured HVP-NHP heart slices had regained their contractile force by Day 30 and retained it until Day 50 after the initial loss that occurred around Day 14 (Figure 19A and B).

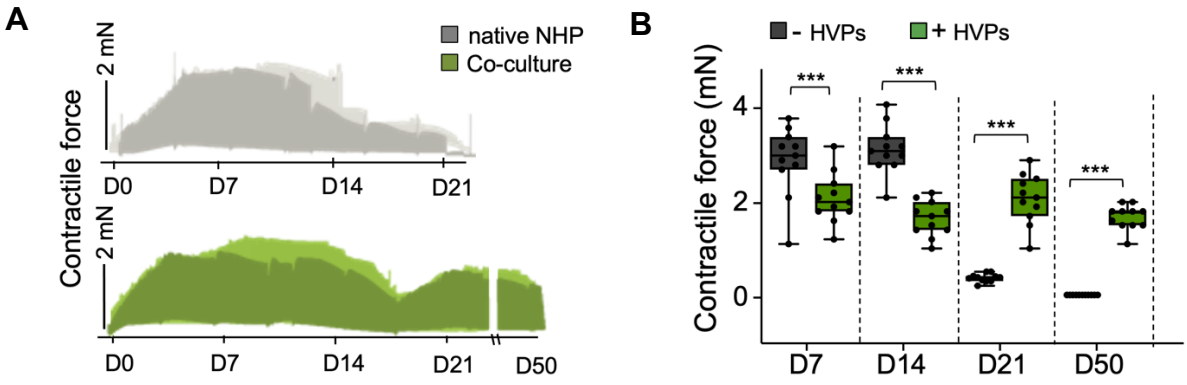


Figure 19. Continuous contractility recording of *ex vivo* cultured native NHP heart slices compared to HVP-NHP co-culture over 50 days. A Representative contractile force traces of *ex vivo* cultured native NHP heart slices and HVP-NHP co-cultured heart slices on indicated days of culture. **B** The box plot shows all data points as well as minima, maxima, medians, and quartiles; n = 11 slices/group, ***$p < 0.001$ (two-way ANOVA).

8.4 HVPs predominantly differentiate into ventricular CMs in HVP-NHP heart slices

The immunohistochemistry for atrial (MLC2a) and ventricular (MLC2v) cardiac muscle markers revealed that most of HVPs (GFP^+ cells) obtained a ventricular muscle identity over time in the HVP-NHP heart slices (Figure 20A). By Day 21, ~65 % were exclusively MLC2v positive, which is indicative of maturing ventricular CMs, whereas only ~15 % expressed both markers, a sign for immaturity. Alternatively, only ~20 % exclusively expressed $MLC2a^+$, a sign of atrial CMs. By Day 50, ~81 % of the cells had adapted a ventricular phenotype (Figure 20B). At that stage, the predominantly rod-shaped $GFP^+/MLC2v^+$ CMs, which had well aligned myofibrils, could be characterised as mature muscle cells (Figure 20A).

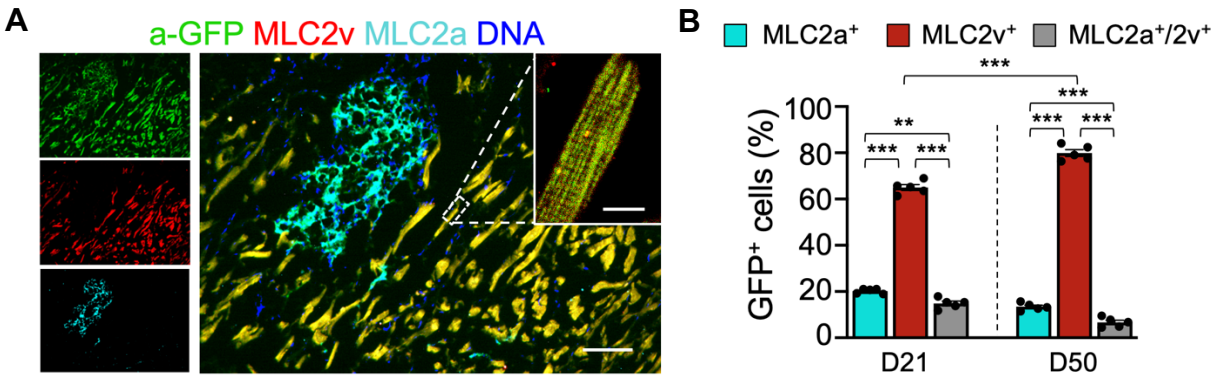


Figure 20. HVPs mature and adapt a ventricular phenotype within HVP-NHP heart slices. A Representative images of immunofluorescence analysis, reflecting the use of an anti-GFP antibody (a-GFP) for the identification of seeded HVPs, together with antibodies against MLC2a and MLC2v on D50 of biomimetic culture. Scale bar: 100 μm. **B** Percentage of GFP+ cells positive for MLC2v, MLC2a, and MLC2a+MLC2v, demonstrating the phenotypic maturation of D6 HVPs along the pathway to ventricular CMs between D21 and D50. The data are given as means ± SEM and as individual data points; n = 5 samples/time point; $^{**}p < 0{,}005$, $^{***}p < 0.001$ (two-way ANOVA).

The comparison between the GFP^+ cells on co-cultured HVP-NHP heart slices was sensitive to the expression of the cardiac muscle marker cTnT and Isl-1 on Day 7, Day 21, and Day 50. It also captured the maturation of seeded HVPs on native cardiac tissue. The high percentage of ~89 % cTnT positive cells on day 21 with an increase

to ~94 % by Day 50 suggested the further maturation of GFP$^+$-marked HVPs into ventricular CMs, which was supported by the downregulation of Isl-1 protein expression from ~6 % on Day 21 to ~2 % on Day 50 (Figure 21).

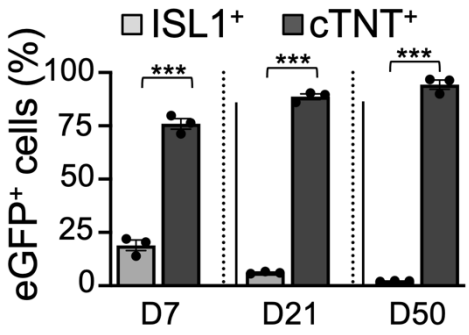


Figure 21. Percentage of GFP$^+$ cells positive for cTnT and Isl-1 on D7, D21, and D50 of culture. The percentage of cTnT$^+$ cells increased slightly from D7 to D21 and from D21 to D50, whereas Isl-1 expression decreased during the same time interval. The data are given as means ± SEM; n = 3 samples/time point; ***p < 0.001 (two-way ANOVA).

Several basic-research, preclinical and clinical studies have investigated the underlying complex mechanisms of cardiac regeneration, the benefits of various cell-based and cell-free therapies, and the feasibility and safety of therapeutic approaches. The long-term culture of native tissue is an important tool for *ex vivo* 3D cardiac disease modelling and regeneration approaches, and it provides an excellent opportunity to investigate cellular behaviour in a complex environment and, consequently, to increase the predictive accuracy of further tests in *in vivo* studies. In the present thesis, dying native LV NHP heart slices in a long-term *ex vivo* 3D culture were repopulated with lineage committed HVPs. The newly introduced cell source repopulated the native LV NHP heart slice, proliferated, and restored its functional and structural properties for up to 50 days in the *ex vivo* culture.

9.1 IMPORTANCE OF *EX VIVO* LONG-TERM CULTURE OF NATIVE AND CO-CULTURED HEART TISSUE FOR 3D CARDIAC DISEASE MODELLING AND REGENERATION APPROACHES

The present thesis showed that HVPs repopulate native LV myocardial slices successfully *ex vivo* and that they mature functionally during the time of long-term culture. The first task that had to be completed was the successful establishment of a 3D culture system that would enable the long-term culture of native myocardial slices *ex vivo* for up to 50 days. As the results show, the long-term culture of NHP cardiac tissue under the physiological preload of 1 mN and with continuous pacing at 1 Hz was feasible in our laboratory (Figure 15 and 19) by using the customised BMCCs designed by Professor Dendorfer's research group (Figure 7) (Fischer et al., 2019).

In their study, Fischer et al. (2019) reported that it is possible to culture and preserve human myocardial heart slices functionally for up to four months. During culture, the heart slices were subjected to a physiological preload of 1 mN, as well as to mechanical and electrical field stimulation. The histological analysis of the long-term cultured myocardial slices revealed well-aligned myofibrils, no fibrosis, and intact sarcomeres and transverse tubular systems. There were only few culture-related irregularities, relative to those observed on freshly prepared myocardial slices. Similarly, when

mRNA sequencing was performed, no significant changes in gene expression levels appeared in the long-term *ex vivo* cultured myocardial slices (Fischer et al., 2019). Watson et al. (2019) adopted an alternative approach. In this approach, besides electrophysiological stimulation, the importance of the mechanical preload of 2.2 μm was underlined. They demonstrated that the *ex vivo* maintenance of adult myocardial slices from rats, rabbits, and humans with heart failure can be prolonged by up to five days (Watson et al., 2019). It is evident from a comparison between the systems that were developed by Fischer et al. and by Watson et al. that mechanical preload as well as electrical and mechanical stimulation play crucial roles in maintaining native tissue *ex vivo*. Nevertheless, the informative value of the system invented by Watson et al. for long-term culture is limited because of the short period of *ex vivo* culture, which was only five days. Since the present thesis focused primarily on the long-term effects of HVPs in the setting of chronic damage to the myocardium, the system of Fischer et al. was the ideal system to work with because it enables to maintain myocardial slices for up to 50 days with an intact myocardial structure and without significant changes in gene expression levels.

In contrast to *ex vivo* systems, it is also possible to study cardiac regeneration and therapeutic approaches *in vitro*. Microfluidic device technology, such as organs on a chip, represents a potential platform to study cellular functions and interactions *in vitro* (Kimura, Sakai, & Fujii, 2018). Zhao et al. (2019) engineered the plastic platform Biowire II, which features chamber-specific drug response and gene expression. This approach combines organ-on-a-chip models and organoid self-assembly. Thus, the authors stimulated grown cardiac tissue from differentiated iPSCs biophysically. During the culture, they could refer to continous and non-invasive readouts of Ca^{2+} transients, active force, conduction velocity, and action potential (Zhao et al., 2019). However, *in vitro* systems appear to be inferior to *ex vivo* systems because of their lack of multicellularity and of complex architecture. Molecular mechanisms are analysed more accurately in this natural environment. Using the BMCC in our laboratory enabled adjustments to mechanical preload and electrical stimulation. While an *ex vivo* long-term culture of native cardiac tissue slices is the basis of this project, the identification of the molecular steps of HVP-mediated repair of chronic damage to myocardium was also an objective. Thus, an *ex vivo* 3D chimeric model was established in which HVPs were co-cultured with native NHP heart tissue that allowed

to study the regenerative potential of HVPs. Progressive cardiac tissue deterioration appeared, as indicated by the gradual loss of vitality and contractile force, which correlated with an increase in the incidence of apoptotic CM death over time, as visualised by the extent of staining for ClCasp3 and Calcein relative to that for Ethidium Dimer (Figure 13, Figure 14, and Figure 15). This created an ideal setting to evaluate cellular mechanisms of cardiac repair. The use of native tissue, compared to the Biowire II model (Zhao et al., 2019) or the organ-on-a-chip model (Kimura et al., 2018), has the advantage to study the cardiac regeneration in an even more natural setting. Until now, *in vivo* experiments were necessary to study the regenerative potential of CPCs in detail at an early stage of the research process which entail all disadvantages of small and large animal models, such as high costs, the need for special facilities and experts, and ethical concerns. Furthermore, detailed time-course tracing and single-cell resolutions are better feasible in *ex vivo* systems. This illustrates the need for and advantages of *ex vivo* long-term culture systems to study cardiac repair mechanism. Nevertheless, a final assessment of these therapeutic approaches for cardiac repair must be carried out through *in vivo* experiments on small or large animal models before those approaches can be applied to patients.

The possibility of long-term culture is essential for disease modelling, drug discovery, and the testing of cardiac regeneration strategies. Taken together, experimental models have to meet stable long-term culture conditions, continuous functional readouts, and comparable conditions as in the natural environment. An authentic representation of environmental factors must capture interactions with other cells, three-dimensionality, molecular signalling, and morphogens (Vunjak-Novakovic & Scadden, 2011). Mimicking the *in vivo* environment with continuous electromechanical stimulation and preload seems to be the most important factor for preventing rapid tissue dedifferentiation in *ex vivo* cultures (Fischer et al., 2019; Watson et al., 2019).

9.2 FUNCTIONAL AND STRUCTURAL MATURATION OF LINEAGE COMMITTED HVPS IN AN *EX VIVO* CO-CULTURED MYOCARDIAL TISSUE MODEL

The CPCs that were used in the study were characterised as $ISL1^{+}$ HVPs derived from $NKX2.5^{eGFP/wt}$ hESCs. After the HVPs were seeded on native primate tissue on Day 6

of differentiation, these cells showed their potential to be highly proliferative (Figure 16), and their ability to expand and repopulate the native tissue by tracing due to eGFP expression, which is regulated by the NKX2.5 locus (Figure 18). Remarkably, the gradual loss of contractile force until Day 14 was followed by a period of subsequent regain – but only in co-cultured NHP heart slices, whereas native slices deteriorated further (Figure 19).

In human embryonic development, the heart is formed from mesodermal progenitors in the FHF and SHF. Recent advances in biotechnology, such as lineage tracing, single-cell RNA sequencing, and genome editing have facilitated the identification of unknown cardiac progenitor populations (Bardot et al., 2017; Morita et al., 2016). The NKX2.5-gene encodes the homeobox protein Nkx2.5 and is expressed early in both FHF and SHF progenitors, whereas ISL1 only marks SHF progenitors which show migration during the embryonic development of the heart, can self-renew, and expand before differentiating into cardiovascular cells of diverse types, such as CMs, smooth muscle cells and endothelial cells (Bu et al., 2009; Moretti et al., 2006). These properties can explain the regenerative potential of the embryonic cardiac progenitor population of HVPs. In another study, ISL1+ cells were identified as endogenous adult CPCs, which are also called cardioblasts, in postnatal rat, mouse, and human myocardium with the ability to be expanded and differentiated into mature CMs (Laugwitz et al., 2005). Taken together, these findings help to understand the developmental pathway that runs from the progenitor cell to the differentiated cell type spatiotemporally, as well as the potential of these cells for heart regeneration.

The long-term culture of co-cultured HVP-NHP myocardial slices was enabled for up to 50 days in order to analyse the functional and structural maturation of HVPs. Furthermore, the lineage commitment of HVPs was studied by performing immunohistochemistry for MLC2v and MLC2a. The results showed that in the seeded HVP pool only few multipotent precursors deviated and developed into cell types other than ventricular CMs (Figure 20).

The previously established two-step protocol by Foo et al. (2019) enabled the generation of a highly enriched pool of $ISL1^{+}$ precursors that had lost their multipotent potential, could expand, and differentiated into ventricular CMs almost exclusively.

Day 6 of differentiation was identified as the best timeframe for reseeding HVPs (Foo et al., 2018). The use of this two-step protocol in the present study made it possible to generate a sufficiently large number of Day 6 HVPs for reseeding onto native NHP myocardial slices. Due to the establishment of a long-term culture of co-cultured myocardial slices using BMCCs (Fischer et al., 2019), it was feasible to follow the predominant patterns of differentiation and maturation of HVPs into ventricular CMs *ex vivo*.

Previous *in vivo* studies appear to support the notion that CPCs can be seeded onto native myocardium and, thus, proliferate, expand, and mature in a natural environment. Schwach V. et al. (2020) claimed in their study in mice that CPCs, after intra-myocardial transplantation into mice that had undergone myocardial infarction, could mediate cardiac repair and expand *in vivo* similarly to their behavior *in vitro* (Schwach et al., 2020). Menasché et al. (2018) went one step further and transplanted hESC-derived CPCs embedded into a fibrin hydrogel onto the hearts of human patients with severe heart failure during coronary artery bypass procedures. Hereby, the feasibility of enriching hESC-derived CPCs, their safety with no incidents of tumors or arrhythmias, their presumed maturation, and the improvements in myocardial function could be observed in a small cohort of six patients, with a median follow-up period of 18 months (Menasché et al., 2018). While these studies showed a wide consensus that CPCs mature functionally and structurally after being seeded onto native myocardium *in vivo,* both in animal models and in humans, the underlying mechanisms are not yet understood fully.

In order to achieve further progress and understand the underlying mechanisms, the *ex vivo* long-term culture that was described previously is ideal for performing analyses at a single cell level due to the accessibility of cells at different developmental stages. A molecular roadmap for HVP specifications and a cluster of subpopulations can be identified by profiling HVPs via single-cell RNA sequencing (scRNA-seq) at different points in time. ScRNA-seq, also known as single-cell transcriptomics, is a genomic approach to measuring mRNA concentration in specific cell populations of millions of cells simultaneously (Haque, Engel, Teichmann, & Lonnberg, 2017). Developmental pathways and transcriptional dynamics can thus be modelled through bioinformatic integration with published data. Using this approach in further experiments in our

research groups unraveled seven subpopulations of HVPs and their pseudotime trajectories from Day 0 to Day 21. Gene ontology enrichment analysis showed similarly to the results of immunohistochemistry and flow cytometry, but even more precisely, the activation of genes pointing to enhanced proliferation, cardiac growth, cell migration, and graft-host interaction (Poch et al., 2022). This method is becoming standard and had led to the identification of genes that are decisive for early cardiac development in, among others, *in vitro* and *ex vivo* studies on hESCs and mouse models (Lescroart et al., 2018; Sahara et al., 2019).

Combining the promising properties of HVPs, their lineage-committed differentiation potential, their high proliferation and expansion rates, their migratory tendencies, and their paracrine effects, leads to the question how HVPs can ultimately regenerate myocardial damage in adult mammalian hearts. In this way, HVPs may provide a therapeutic agent for cell-based therapy that is superior to cells of other types, such as CMs.

9.3 REGENERATIVE POTENTIAL OF HVPS IN *EX VIVO* CULTURED DYING MYOCARDIUM

The properties of a specific cardiac progenitor cell population and the interaction between native damaged tissue and regenerating cells define the potential to regenerate myocardial damages (Witman, Zhou, Grote Beverborg, Sahara, & Chien, 2020). In the present thesis, the embryonic cardiac progenitor population of Nkx2.5+/ISL1+ HVPs on Day 6 of differentiation was co-cultured *ex vivo* on native LV myocardial slices in BMCCs by using the previously developed two-step protocol that was devised by Foo et al. (2018), who also demonstrated that progenitors differentiate into ventricular CMs predominantly (Foo et al., 2018).

In the literature, there is a debate on the question of whether the regenerative potential of CPCs is superior to that of cells of other types, such as fully differentiated CMs (Ja et al., 2016). Recent studies have explored the differences of CPCs and fully differentiated CMs regarding their therapeutic potential for heart regeneration. Chong et al. (2014) reported a significant remuscularisation of ischemia-reperfusion-injured NHP hearts, electromechanical coupling and vascular structures between graft and

host, and progressive but incomplete maturation after an intra-myocardial injection of 1 billion hESC-CMs (Chong et al., 2014). In addition, Shiba et al. (2016) demonstrated the successful major histocompatibility complex-matched, allogeneic, intra-myocardial transplantation of iPCS-derived CMs in NHP directly after the induction of myocardial infarction. After four and twelve weeks, an improved cardiac contractile function was observed (Shiba et al., 2016). Romagnuolo et al. (2019) investigated the stable engraftment of hESC-derived CMs that were injected in immunosuppressed pigs, another large animal model, three weeks after myocardial infarction. They showed the further maturation of the implanted immature hESC-derived CMs over time and the development of connected vascular networks. However, there were only non-significant regional improvements in contractile function (Romagnuolo et al., 2019). All the previously mentioned three promising approaches to stem cell-derived CM injections after cardiac injury presented the same side effects of graft-related tachyarrhythmias several days after transplantation. These tachyarrhythmias were suspected to have been caused by focal mechanisms rather than re-entry incidents and limited the outcome of the studies (Chong et al., 2014; Romagnuolo et al., 2019; Shiba et al., 2016).

But what is the situation when comparing the transplantation of CPCs and CMs directly? Fernandes et al. (2015) performed a direct comparison of the regenerative potential of CPCs and CMs, showing that hESC-derived CMs and CPCs are similarly efficacious in regenerating cardiac tissue in a nude rat myocardial infarction model. Both were more efficient than human bone marrow mononuclear cells (hBM-MNCs). Although the authors hypothesised that CPCs have beneficial effects because of their ability to differentiate into cell types other than CMs *in situ*, such as vascular cells, and that these cells can contribute to more parts of the myocardium than CMs, no larger engraftment or increased number of vessels was observed in the infarcted heart (Fernandes et al., 2015). In summary, CMs showed improved cardiac functions in several studies but are hampered by their immature phenotype, the challenge of large-scale production, their lack to proliferate extensively and expand, and the risk of graft-related tachyarrhythmias. In particular, tachyarrhythmias appear to occur less frequently when CPCs are used for cardiac regeneration in different experimental approaches (Foo et al., 2018; Menasché et al., 2018), and were also absent in the

present *ex vivo* studies. However, further research has to be conducted in order to investigate the beneficial effects of either CPCs or CMs for regenerative therapy.

In the present study, the cardiac progenitor population of HVPs on Day 6 was used for co-culture in the setting of chronic damage to NHP myocardial slices *ex vivo*. Ongoing studies concentrate on the first clinical series that test regenerative CPC-based therapies in humans. In 2015, Menasché et al. published the first clinical case report in which they described the successful treatment of a 68-year-old female patient with severe heart failure after myocardial infarction. The treatment entailed transplanting ISL1+ hESC-derived CPCs that were embedded into a fibrin scaffold during a coronary bypass procedure. In the three-month follow-up, the patient exhibited improved cardiac function (the LV ejection fraction increased from 26 %, which is characterised as New York Heart Association (NYHA) Class III, to 36 %, which is considered as NYHA I) (Menasché et al., 2015). In a subsequent clinical trial with six patients, the authors demonstrated the technical feasibility of generating and transplanting highly purified CPCs and investigated the short- and medium-term safety of the procedure, showing neither teratomas nor arrhythmias. The study laid the foundation for the ongoing efficacy trial ESCORT (Transplantation of Human Embryonic Stem Cell-derived Progenitors in Severe Heart Failure; NCT02057900) (Menasché et al., 2018). Other clinical trials have focused on different cell types and administration techniques. For example, in the CADUCEUS trial (Cardiosphere-Derived Autologous Cells to Reverse Ventricular Dysfunction; NCT00893360), autologous cardiosphere-derived cells are infused intracoronary in patients with LV dysfunction between 1.5 and 3 months after myocardial infarction (Malliaras et al., 2014; Witman & Sahara, 2018).

Compared to the findings of other research groups, it appears that the collected data complement the existing knowledge and provide further evidence of the regenerative potential of HVPs in an *ex vivo* 3D chimeric model of chronic injury. HVPs, once equipped with the capacity to proliferate extensively and to migrate, repopulated and took over functional and structural properties of dying NHP cardiac tissue. Their properties can presumably be explained when their role in embryonic development is taken into consideration. This can also be one reason for their superior regenerative potential compared to CMs. It also remains important to consider the direct and indirect

effects of transplanted CPCs in the setting of damaged myocardium, with special focus on paracrine signalling.

9.4 Limitations of the study and outlook: HVPs as promising source for cell therapy after myocardial damage

Regenerative medicine for cardiovascular diseases has received lots of research attention during the last two decades. Numerous studies have investigated the basic mechanisms of cardiac regeneration, including cell products and paracrine factors, as well as their application in preclinical trials with animal models and in clinical studies with humans. The Transnational Alliance for Regenerative Therapies in Cardiovascular Syndromes (TACTICS) outlined the remaining challenges towards a successful regenerative therapy for cardiac repair in their global position paper. There, they state that the complex molecular, cellular, and tissue mechanisms that are involved in cardiac repair still need to be further investigated. In addition, TACTICS call for the international standardisation of preclinical studies with small and large animal models in order to improve the predictive usefulness of successful clinical trials. Furthermore, they demanded international collaborations and multicentre clinical trials to test the safety and efficacy of experimental therapies (Fernandez-Aviles et al., 2018; Fernandez-Aviles et al., 2017). The attention of all who work in the field should also be drawn to best practices, to the convenient use of scientific discourse, and to the need to avoid wasting years of research labour by chasing ideas from falsified studies, as Chien et al. (2019) argue regarding the questionable assumption of cardiac repair through the transdifferentiation of bone marrow cells or adult resident CPs (Chien et al., 2019).

Currently, several phase-I and phase-II clinical trials are ongoing or are still recruiting participants. Overall, the clinical endpoints from stem cell treatments of myocardial infarction have been evaluated as feasible and safe (Hao, Wang, & Wang, 2017). Several promising contributions to basic research and preclinical trials have examined the potential of stem cells, and especially CPCs, for regenerative therapy (Witman & Sahara, 2018). The ESCORT trial, the first in-human clinical trial, tested the potential of hESC-derived CPC for regenerative therapy. The results showed the technical

feasibility and short- and medium-term safety paving the way to broader long-term studies (Menasché et al., 2018). Even though CPCs showed beneficial effects in multiple studies due to their potential to proliferate, migrate, and take over functional and structural properties of dying myocardium, this research field is still in the preclinical stage because of the complex underlying mechanisms (such as integrating direct and indirect effects like paracrine factors) and safety concerns (such as the risk of uncontrolled proliferation, immunogenicity, or ventricular arrhythmias) (Fernandez-Aviles et al., 2017). Therefore, studies on large animal models, such as pigs, are not dispensable for narrowing the gap between results from preclinical studies and those from human clinical trials and, ultimately, for the development of a successful stem cell therapy that relies on CPCs in the near future. In one such study, our research group completed an intra-myocardial transplantation of hESC-derived HVPs into pigs that had suffered RFA injury (Poch et al., 2022). Nevertheless, long-term regeneration, as well as safety and ethical concerns, still have to be analysed in future research. Consequently, further research will be indispensable for identifying the optimal therapeutic approach to the regeneration of the human heart among various cell and cell-free based therapies, direct and indirect concepts, combinatorial treatments, and delivery methods.

The aim of the study was to identify the potential of HVPs to repopulate native LV myocardial slices. Therefore, the feasibility of an *ex vivo* long-term culture of NHP myocardial slices was demonstrated over a period of up to 50 days (*Section 8.1*). Thereby, the gradual loss of contractile force in the native LV NHP heart slices was recorded over time compared to preserved tissue characteristics in co-cultured HVP-NHP heart slices. This provided the basis for studying the regenerative potential of HVPs. After seeding HVPs on native LV NHP myocardial slices, their potential to proliferate was assessed *(Section 8.2*). Furthermore, the analysis of the co-cultured myocardial slices revealed the potential of HVPs to expand, to repopulate native heart slices, and even to take over functional and structural properties in the setting of a dying myocardium (*Section 8.3*). The concluding experiments provided evidence for the proposition that, after seeding on Day 6, the HVPs are lineage-committed and differentiate into ventricular CMs predominantly (Section 8.4). These results were obtained by culturing native and co-cultured NHP myocardial slices in BMCCs, by continuous readouts of contractile forces, and by immunohistochemistry and flow cytometry of the representative marker.

The results that are described in the present work support the findings of other research groups. In the literature, there is a broad consensus that stem cells, and especially CPCs, are a promising source of cells for cardiac repair. Without doubt, the long-term culture of native tissue is indispensable for *ex vivo* 3D cardiac disease modelling and regeneration approaches, and it provides an excellent opportunity to test the underlying mechanisms in a complex environment, thus increasing the predictive accuracy of other tests on animal models or even on humans. This also illustrates the unique advantages of *ex vivo* studies over *in vitro* and *in vivo* experiments (*Section 9.1*). Here, HVPs were shown to be a plastic cell population, and their potential to differentiate into ventricular CMs predominantly and to mature functionally and structurally was highlighted in an *ex vivo* 3D tissue model. These properties can be explained by a profound examination of the role of CPCs in early cardiogenesis (*Section 9.2*). Moreover, the regenerative potential of HVPs to repair damaged myocardium in the setting of a chronic-injury model was observed. It remains controversial, whether CPCs or fully differentiated CMs represent the superior

population for regeneration, and which important role paracrine effectors play in cardiac repair (*Section 9.3*). In conclusion, HVPs are considered as a promising source for cell therapy after myocardial damage, and it remains exciting how further preclinical and clinical studies contribute to the development of a successful stem cell therapy (*Section 9.4*). Hopefully, in the near future, this will lead to a curative treatment of ischemic heart disease, the leading cause of death worldwide.

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ES03 NKX2.5 GFP and H9 NKX2.5 GFP cell lines (reference: 3.04.02/0131) were given to the research group by Dr. David Elliott (MCRI, Australia). These ESCs lines were validated in the original studies (Elliott et al., 2011; Foo et al., 2018).

NHP cardiac tissue samples were part of a vaccination study in the control population. After termination of the study, hearts were shipped from the German primate centre, Göttingen (reference: 33.19-42502-04-16/2264) or Karolinska Institutet, Sweden (reference: N277/14). NHP hearts from Walter Brendel Institute, Germany were part of xenotransplantation experiments (reference: ROB-55.2-2532.Vet_02-14-184).