Enhancing epicardial EMT to repair the heart

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Discussion

REGENERATION IS NOT AN EASY TASK

Each year 34.000 patients are hospitalized in the Netherlands because of a myocardial infarction (1). The inability of cardiac tissue to regenerate causes these patients to suffer from a dysfunctional heart which can ultimately progress into fatal heart failure. Regenerating the mammalian heart has therefore been a dynamic research area over the past decades, characterized by trial and error. The first decade of this century was dominated by research into adult stem cells, that reside in the bone marrow or in the heart itself. The ultimate goal was to show that these cells are capable of differentiating into cardiomyocytes after transplanting the cells into the heart. After 20 years of research, we now know that although it may be possible to generate cardiac cells out of adult stem cells, the numbers are most likely not clinically relevant (2). The initial success of stem cell injections has been attributed to stem cell derived paracrine factors but may be predominantly due to activation of the innate immune response, as transplantation of dead cells generates comparable beneficial effects to living cells (3). The extensive research into adult stem cells revealed the main hick ups of cardiac regeneration: 1) there is no adult cardiac stem cell that can replenish cardiomyocytes in the injured heart (4), 2) also failing angiogenesis (5) and dysregulated inflammatory responses (6) play an essential role in the limited regenerative response of the heart, and 3) it is particularly difficult to administer any solution containing cells into a heart without it being flushed out in one heartbeat (7). Although we now realize that simply delivering stem cells into the heart does not regenerate the tissue, the research did provide a lot of knowledge where to start. The focus of the research field steered towards stimulation of endogenous mechanisms to initiate cardiac tissue formation, and biomaterials to deliver cells and factors to the heart without washout. The epicardium is such an endogenous source of progenitor cells that can participate in myocardial growth and the formation of proper vasculature.

IMPORTANCE OF EPIMT

In all regenerative processes there is a similar sequence of events. It starts with an inflammatory phase, aiming at clearing all dead cells by macrophages and neutrophils. This is followed by a proliferative phase, in which fibroblasts provide support by producing matrix. Finally, remodelling of the scar region takes place. In the mammalian heart, this remodelling results in cardiac fibrosis. However, in some animal species, such as the zebrafish, remodelling can lead to regeneration. This final regenerative process often mimics the development of the organ (8). In this regard, the epicardium

is of interest as it plays a major role in cardiac development (reviewed in Chapter 2). As such, enhancing recapitulation of embryonic epicardial behaviour could be a target for therapeutic intervention for the injured heart. One of the main features of epicardial cells is their plastic phenotype, allowing them to switch from being an epithelial cell on the outside of the heart to becoming a motile mesenchymal cell – a process called epithelial to mesenchymal transition (epiMT). We hypothesized that epiMT is an essential step for induction of the beneficial effects that the epicardium exerts on the developing myocardium. This is based on the fact that the foetal epicardial cells, which are actively involved in tissue formation, are much more prone to undergo epiMT in vitro than the less active adult cells (9). In addition, recent papers exploited single cell sequencing to describe the trajectory of epiMT in the developing heart (10–12), showing that epiMT must be completed before cells can start to differentiate and fate specification can take place. This suggests that epiMT is an essential first step in the epicardial contribution to the heart. Moreover, one of the rare studies comparing pre- and post-EMT epicardial cells performed by Quijada et al. found that epiMT is vital for maturation of the vasculature and fate specification of the endothelial cells during development (13). These studies all point to a vital role for epiMT in tissue development which may serve as a blueprint for enhancing tissue formation in the injured adult heart. Besides a potential role in repair, dysregulated epiMT has been described as a driving factor in fibrosis and fibro-fatty remodelling of the myocardium leading to atrial fibrillation (14–16) and arrhythmogenic cardiomyopathy (17). The fact that epiMT is a fundamental process in epicardial behaviour in disease and repair demonstrates that understanding its regulation is essential in the identification of cardiac therapies.

REGULATION OF EPIMT

The regulation of epiMT has been studied extensively, revealing that the main regulatory pathways are TGFβ, PDGF, FGF and Wnt signalling. Over the years, more detailed studies have shown that Retinoic acid, WT1, and TCF21 are important downstream players of epiMT regulation (reviewed in (18)). Additionally, an array of factors has been identified that intervene with these pathways, for example, the extracellular matrix (ECM) component Agrin was identified as an epiMT regulator that signals via β-catenin and WT1 (19), and hypoxia was demonstrated to stimulate epiMT by increasing TGFβ expression levels (20). All epiMT inducers have in common that they activate at least one of the so called EMT-transcription factors (EMT-TFs), which are SNAIL, SLUG, ZEB1, ZEB2, or TWIST. These factors are essential in the orchestration of

epiMT by repressing epithelial characteristics, such as the downregulation of E-cadherin, and inducing mesenchymal features.

In our cell culture model, we have shown multiple times that TGFβ robustly induces epiMT =. However, the pleiotropic nature of this signalling pathway, including its role in the development of severe fibrosis, makes it a less desirable target for therapeutic purposes. Therefore, we aimed at finding other epiMT inducers. In this thesis we describe the identification of three novel inducers of epiMT: Activin/ALK4 signalling, TBBz, and Oltipraz Metabolite M2 (M2). Activin/ALK4 was identified based on analysis of TGFβ related pathways in our bi-directional cell culture system allowing us to identify both stimulators and inhibitors of epiMT. TBBz and M2 were identified in a phenotypic small molecule screen using the human adult epicardial cells. Of all the epiMT inducers that passed by during the course of these experiments, we could not identify one common pathway that would explain the induction of epiMT for all of them. Only Activin/ALK4 is related to a well-known epiMT inducer, which is TGFβ. Because of this, we expected Activin to induce epiMT via TGFβ or TGFβ-associated signalling. However, although we found that ALK4-induced epiMT results in activation of SNAIL, similar to the downstream effect of TGFβ, the effects of Activin/ALK4 are TGFβ independent. The question that remains is how the downstream signalling is conveyed after ALK4 activation. Because of the lack of αSMA upregulation, it is to be expected that Activin/ALK4 induced epiMT follows a path different from TGFβ. Therefore, we assume that a SMAD-independent pathway is activated, resulting in the activation of SNAIL, leading to epiMT. For the other epiMT inducers, TBBz and M2, it was less clear via which pathway they induce epiMT. TBBz is labelled as a CK2 inhibitor and M2 as LXRα inhibitor. Based on our sequencing data, we anticipate that M2 induces epiMT via expression of FOXQ1 which is a TGFβ-related transcription factor (21) and may therefore be indirectly involved in TGFβ signalling. This was difficult to assess since a combination of M2 and SB was toxic to the cells. Regarding TBBz, although it is labelled as specific inhibitor of CK2, we could not find any effect on CK2 activity in our cell culture model. We revealed that TBBz induces epiMT most likely indirectly via epigenetic changes, which lead to histone methylation modifications that ultimately result in induction of SLUG. This is in line with a recent publication that the epicardial cells undergo major changes in the chromatin accessibility upon activation in zebrafish hearts (22). Interestingly, Activin and TGFβ both induce SNAIL, while TBBz and M2 act via SLUG which demonstrates that a distinct set of transcription factors is involved. This shows that TBBz and M2 do not elicit epiMT by indirect activation of TGFβ signalling but suggest that different pathways are involved. Future research into the exact epigenetic mechanisms should reveal the detailed mechanisms and

may also provide options to use other histone modificators that may be less toxic to cells than TBBz.

A general remark that can be made regarding research using stimulants and inhibitors is that it is very tricky to claim that a molecule has a specific target. Often, it is known what the intended target of a molecule is, but it is difficult to exclude any off-target effects. To deal with these inconsistencies, we used multiple approaches to identify the actual targets of our compounds. In case of TBBz, labelled as a specific CK2 inhibitor that is elaborately studied, we found no clear effect on the predicted target. We measured the effect of TBBz on CK2 activity and compared its effect with other CK2 inhibitors. Additionally, we performed RNA sequencing as an unbiased attempt to identify its direct target, pointing to a role for TBBz in histone modification. Although a specific targets of TBBz have been described (23), to our knowledge, the role of TBBz in histone methylation has not been described before warranting further investigations into the mechanism. Regarding SB431542 (SB), it is known that it blocks the kinase activity of ALK4, 5, and 7 but it is most often referred to as a TGFβ blocker or an ALK5 inhibitor. This 'mislabelling' of the inhibitor in scientific literature probably caused the role of ALK4 in epiMT to be neglected. For SB, we have tried to selectively demonstrate the role of ALK5 and ALK4 in epicardial cells using siRNAs, but these experiments were hampered by technical difficulties. Therefore, we chose to use specific ligand-receptor inhibitors, FST for ALK4 and TGFβ capture antibody for ALK5, to study the specific role of ALK4 in epiMT. To conclude, the described mode of action of a compound can only serve as a starting point for studying its mechanism. Accurate and thorough reporting of compound effects could help to smoothen this search.

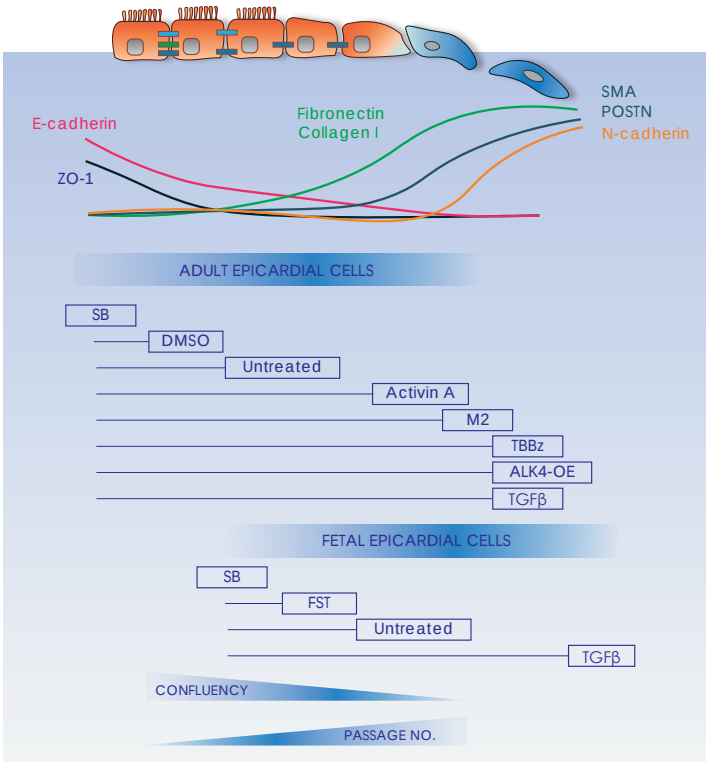

Fig. 1 | Factors that influence the plasticity of epicardial cells. A schematic overview of the epiMT axis and how factors influence the epiMT status of adult and foetal epicardial cells. The epiMT markers are an interpretation of differences in expression that we have observed in our studies. We find that there is a gradient of marker expression along the epiMT axis that allows us to discriminate between intermediate epiMT stages. Importantly, it also shows that some markers are already differentially expressed in untreated cells. The markers represent a schematic interpretation of multiple experiments and are not intended to be fully accurate for every individual cell stimulation.

EPIMT CELL CULTURE MODEL

Studying the regulation of epiMT is often performed in vitro. Using the right cell culture model, understanding it, and recognising its strong points and limitations is essential to obtain useful and translational data. In chapter 3 we describe a detailed protocol for the isolation and culture of human adult and foetal primary epicardial cells that have been used extensively to study epiMT. Although epiMT is often presented as a binary process where cells are either epithelial or mesenchymal (also in this thesis), in reality epiMT resembles a much more plastic process with several

intermediate states between the epithelial and mesenchymal phenotype (24)(Fig. 1). When performing epiMT experiments, we observed that a range of factors push the epicardial cells leftward or rightward on this epiMT axis (Fig. 1). An example of this is the effect of SB, which is routinely added to our epicardial cell culture. Removal of SB from the cell culture medium of adult cells already morphologically changes the cells, as the cells lose some adhesion molecules and downregulate Zonula occludens-1 (ZO-1) at the cell border (data not shown), indicating a shift to the right on the epiMT axis (Fig. 1). Remarkably, removing SB from foetal cells pushes the cells much further to the right, towards a mesenchymal phenotype. The fact that foetal cells are more prone to epiMT suggests that they are intrinsically already further on the epiMT gradient compared to adult cells (Fig. 1). This is supported by the fact that foetal epicardial cells in the presence of SB already express lower levels of E-cadherin, and higher levels of mesenchymal markers Vimentin and TCF21 compared to adult cells (9). We have not been able to identify the molecular explanation of this disparity between foetal and adult epicardial cells. However, our recent finding that TBBz induces histone modifications implies that epiMT induction also depends on the epigenetic landscape of the cell, which is supported by literature (24). A divergent epigenetic status in foetal cells may well explain its position on the epiMT axis. A first step towards proving this concept is shown in Figure 2, where TBBz stimulation pushes adult cells towards an epigenetic status that is comparable to foetal cells.

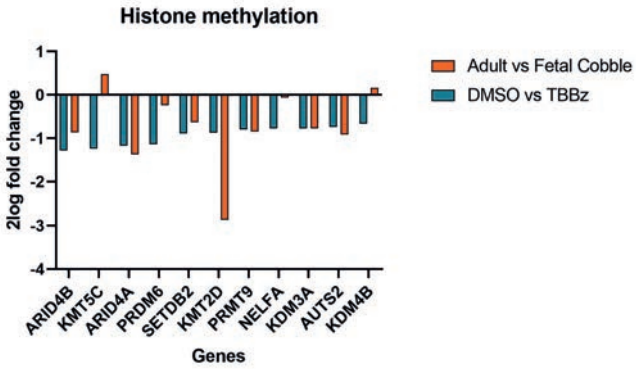

Fig. 2 | TBBz pushes adult epicardial cells towards a foetal histone methylation gene profile. Genes involved in histone methylation that were differentially expressed in TBBz vs DMSO treated adult epicardial cells (3 hours stimulation, see chapter 4 for details) were listed. The expression of these genes was established in a dataset of adult and foetal cobblestone epicardial cells cultured in SB431542. The fact that 9 out of 11 genes follow the same pattern (gene expression is decreased in both TBBz treated adult cells and in foetal cells) points to a mesenchymal histone methylation status.

We exploited these difference in epiMT status between foetal and adult epicardial cells in our in vitro experiments. For example, the different status of adult and foetal cells allowed us to simultaneously study the effects of stimulants and inhibitors of pathways potentially involved in epiMT. The addition of SB, which helps to maintain an epithelial state, as a control condition provided a reliable negative control. In addition, the concept of intermediate stages of epiMT also helps to explain some of the results, e.g. the fact that we find only partial inhibition of FST on foetal epiMT. Interestingly, we also found that DMSO, a solvent for almost all our stimulants (including SB), seems to push the cells leftwards on the epiMT scale. This has been found by others before (25) and suggests that a low concentration of DMSO could be preferable in all epiMT experiments. But despite the presence of it, TBBz and M2 (both dissolved in DMSO) were able to induce epiMT. In practice, there are also other factors that influence the position on this epiMT-gradient. An example of this is that the occurrence of epiMT is highly dependent on the confluency of the cell culture plate, and the cell passage (Fig. 1). We postulate that also the status of the heart where we isolate the epicardium from (diseased or healthy, age, gender) is relevant in this setting. It is therefore essential to resemble this complexity in the in vitro study design, by using primary cells derived from multiple cell isolations to make sure that we observe a robust and translational result.

Differentiation of EPDCs

Besides the regulation of epiMT itself, it is also relevant to consider the differentiation capacity of the generated mesenchymal cells and their potential contribution to tissue formation. Differentiation capacity is in general influenced by a combination of the cell itself and by the biochemical and mechanistic input from the local environment (26). As described in chapter 2, in the developing heart epicardial derived cells (EPDCs) mainly differentiate into smooth muscle cells, (myo-) fibroblasts and pericytes. While epiMT in the mammalian adult hearts has been described to be beneficial for repair (see Chapter 2), the actual contribution of epiMT derived cells remains uncertain. Noteworthy in this context is that in the mammalian heart epiMT has been linked to the induction of fibrosis in the atria (15). Interestingly, the process of atrial fibrosis was related to increased levels of Activin A secreted by epicardial adipose tissue (EAT) (27). It suggests that Activin pushes induces epicardial cells into epiMT whereafter the EPDCs differentiate towards a fibroblast phenotype involved in atrial fibrosis. Moreover, in an environment of stress, such as an infarct, EPDCs can also become adipogenic (28–30). These findings raise the question whether stimulating epiMT in the adult mammalian heart will ultimately be beneficial for the patient. There are a few things to consider in this context. Firstly, epiMT has

been related to cardiac generation in mammals and regeneration in zebrafish. An example of this is NRG-1 secretion by post-epiMT cells that is essential for zebrafish regeneration, as described by us (chapter 7) and others (31,32). We have shown that NRG-1 secretion is PRRX1 dependent in zebrafish and in human cells. PRRX1 was almost absent in patient cardiac tissue samples, which could indicate that the lack of PRRX1-dependent NRG-1 expression hampers mammalian cardiac repair resulting in a fibrotic response. It would be interesting to investigate whether overexpression of PRRX1 in diseased mammalian hearts could affect fibrosis. Secondly, although differentiation towards fibroblasts is mainly considered a bad thing because it is often related to the development of fibrosis and arrhythmogenic substrate, in many organs fibroblasts and their secreted ECM lie at the base of repair and regeneration (33). Thirdly, it is likely that EPDCs will sense their environment, whereby the cues derived from the ECM as well as the stiffness of underlying tissue will influence their differentiation. Pathological remodelling is by itself not a disease but a reaction to a diseased environment. Therefore, targeting the microenvironment in order to steer the EPDC towards the desired cell type will be of relevance for epiMT to be beneficial to cardiac repair.

APPLICATION OF IDENTIFIED FACTORS IN A MOUSE MODEL – TOWARDS A THERAPEUTIC APPROACH

The next step towards an epicardial based regenerative therapy is the application of epiMT stimulating factors to the infarcted heart. The simplest method for this is systemic application, via injection or orally. However, given that induction of EMT is undesirable in most organs due to development of fibroses and metastatic cancer, local application is preferred. Local therapy in the heart is often injected into the heart muscle. But the difficulty with injecting something into the heart is that it is expelled within a few contractions (7). Therefore, a biomaterial is essential to keep the factor in the heart for a certain time-period. The additional benefit of a biomaterial is the possibility of timed release, thereby prolonging the therapy with a single intervention. Interestingly, because of the convenient location of the epicardium at the outside of the heart, it is relatively easy to apply a drug-releasing patch directly on top of the epicardium instead of a potential harmful injection into the heart muscle. This would mainly be beneficial when the patch is self-adhesive and does not require stiches or glue, and moreover when the release is uni-directional to secure specific release to the epicardium. Although quite extensive research has been performed to develop epicardial patches (reviewed in (34)), most of them aim to target the underlying myocardium or have the goal to deliver additional contractile units by generating patches

containing cardiac cells. In our research, we wanted to hit the epicardium itself. We aimed for a robust protocol to prepare a patch that can easily be reproduced and a highly adhesive patch that does not require stitches or glue. Therefore, we have optimized two drug releasing patches that could be applied onto the epicardium. The TISSEEL method relies on the gellification of fibrinogen upon mixing with thrombin, which provides a hydrogel that can be picked up and placed on the heart. The TISSEEL constituents can be mixed with many different types of factors prior to solidification and provides a robust protocol that is easily applicable during in vivo experiments. The supramolecular UPy-catechol patch consists of a UPy-PEG molecule that has been functionalized with a catechol group that provides outstanding binding to tissue. The hydrogel is mixed with the compound of interest and subsequently solidified on top of a UPy-PEG electrospun layer. We use this layer to easily pick up the patch and to protect the lungs. However, this layer could easily be adjusted to an impermeable layer, allowing for unidirectional release from the patch.

In the research described in this thesis, we aimed for identifying factors that interfere with epiMT in vitro whereafter we intended to test this in vivo in the injured mouse heart using a patch. One of the identified pathways was Activin/ALK4 signalling. Given the fact that Activin is broadly expressed in the infarcted heart (35), viral overexpression of ALK4 specifically in the epicardium could be a potential solution to induce epiMT. We executed a study in a mouse model for myocardial infarction to compare a TISSEEL patch containing control virus to a TISSEEL patch containing a constitutively active ALK4 adenovirus. Unfortunately, we encountered difficulties with inefficient viral transduction and therefore we were not able to draw any conclusions. In a second mouse study, we aimed to determine the effect of epiMT-inducing small molecules that were identified in chapter 4. We applied TISSEEL patches containing TBBz and M2 onto the injured mouse heart and we used DMSO and SB eluting patches as controls. Unfortunately, no differences were found in heart function or infarct size between DMSO and TBBz/M2 stimulated hearts after 4 weeks. To determine if the release of compounds from the patch was sufficient, we established the release of SB after 7 days which should be detectable by measuring SMA and pSMAD2 in the underlying infarcted area. However, we did not find a difference between SB and DMSO. There are three possible explanations for the fact that we were not able to observe an effect of epiMT-inducing factors in vivo. The first is that the compounds simply do not exert any effect on the heart. Although this is a realistic option for our newly identified compounds, it is less plausible for SB, given the fact that blocking of TGFβ signalling in the heart has been demonstrated to have major effects on SMA expression, ECM production and fibrosis (reviewed in (36)). The second possibility is

that there is a flaw in the design of the mouse studies, e.g. the timing of the experiment, the release properties of the patch in vivo, the concentration of the factors that was used, or the methods applied for functional analysis. These reasons warrant further investigation before any conclusions can be drawn about the usefulness of these patches to target the epicardium. The third explanation is that the epicardium does not easily take up certain compounds from the outside. Given its barrier function, it would not be surprising that the epicardium displays a reduced permeability, resulting in a suboptimal targeting efficiency. Targeting the epicardium from the inside of the heart by injecting a hydrogel containing the factor of interest would be an alternative approach to cope with this. Interesting to mention in this case is that a peptide has been identified that actively targets the epicardium and could serve as a cargo to deliver small molecules (37). Although injection into the heart muscle is less optimal compared to an epicardial applied patch, delivery of this peptide in the heart should specifically hit the epicardium and therefore will be local and circumvent the current issues.

THE FUTURE OF EPICARDIAL RESEARCH

In animals that display regenerative capacity, such as newts who can fully regrow amputated limbs, an essential element of regeneration is the blastema, a mass of progenitor cells able to grow and differentiate into new tissue. A blastema coordinates the transition from the initial wound healing response, consisting of the inflammatory and proliferative phase, to regeneration. The blastema consists of a group of fibroblast derived progenitor cells, among others PRRX1+ cells (38), which is surrounded by ECM and covered by an epithelium. Regenerative processes within the blastema are coordinated by paracrine signalling, among others CXCR4-CXCL12 signalling (39) and ECM ques such as Tenascin-C and hyaluronic acid (40) . Although a one-to-one comparison with a cardiac blastema may not be applicable due to the lack of pluripotent stem cells in the heart, blastema formation may shed light on the actual role of the epicardium in the developing and diseased heart, which is to secure a microenvironment where regeneration can take place. There are phenotypic similarities between epicardial reactivation and blastema formation, such as the epithelial layer (the epicardium) with subepicardial ECM production, Tenascin-C and hyaluronic acid expression (14,41), CXCR4-CXCL12 signalling (18), and the presence of PRRX1+ mesenchymal cells (this thesis).

The concept that the epicardium functions as a blastema is in line with the dynamics in the epicardial research field. For several years the general idea was that epicardial cells would contribute to cardiac repair by increasing the number of epicardial derived cardiomyocytes and endothelial cells, either by endogenous stimulation towards these cell lineages or via cell transplantation of isolated epicardial cells (42–46). However, although these studies show beneficial effects of epicardial cells on cardiac repair, the absolute numbers of cells that differentiate towards cardiomyocytes or endothelial cells turned out to be non-existent or minimal at best and could not explain the observed improvement in cardiac function. The cellular epicardial contribution to fibroblast and SMCs has been demonstrated multiple times (see chapter 3), but most cardiac fibroblasts in the infarcted myocardium seem to derive from pre-existing fibroblasts instead of the epicardial layer (47). The past few years, the focus of epicardium-driven repair research has switched from cellular contribution towards a regulatory role for the epicardium during disease and repair. This regulatory role was demonstrated when the beneficial effects of epicardial cell injection (46) were attributed to epicardial cell derived secretome instead of a cellular contribution (48). Interestingly, in this study mesenchymal EPDC-derived secretome was injected, indicating that the beneficial effects derive from post-epiMT cells. There is a wide array of observations pointing towards the regulatory role of these mesenchymal EPDCs as opposed to pre-epiMT epithelial epicardial cells. Firstly, multiple studies have shown that epicardial cells secrete factors that promote cardiomyocyte proliferation and maturation (49,50), e.g. Follistatin-like 1 (51). In this manuscript we describe a similar finding, namely the secretion of NRG-1 by post-epiMT epicardial cells. Secondly, EPDCs are essential for vessel maturation, both by a cellular contribution (pericytes) and a regulatory contribution (13). And thirdly, epicardial derived spindles secrete ECM components, which are essential for a proper repair response, such as periostin (52) and fibronectin (53). Together, this shows that the epicardium, and particularly the mesenchymal epicardial cells, are central players in the tissue generating response of the heart, not solely by providing cells but mainly by regulating the processes necessary to generate a fully functioning tissue. Therefore, in my opinion, epicardial research should focus on increasing epiMT, as we describe in this thesis, and on studying how EPDCs orchestrate the post-injury response. Finetuning this response by investigating both the developing heart and the regenerating zebrafish heart may be the key to optimize the epicardial response to repair.

A specific therapeutic approach to benefit from the regulatory role of the epicardium is the use of engineered heart tissue (EHT). EHTs consist of beating cardiomyocytes which are produced in vitro and subsequently applied to the outside of the injured

heart for cardiac support (54). Pre-clinical experiments have been exceptionally convincing, and the engineered tissues are now being tested in patients (55). Interestingly, numerous studies have come to the conclusion that epicardial cells highly improve the maturation of such engineered tissues (50,56–58), demonstrating once more the significance of the epicardium as regulator of cardiac tissue generation. There is also an indirect value in the optimization of EHTs, since development of highly matured cardiac microtissues can be used for research purposes, such as high throughput screening, toxicity tests, and, in combination with patient derived induced pluripotent stem cells (IPSCs), for disease modelling (56).

The bottleneck of epicardial research is the translation of preclinical findings towards a therapeutical approach in patients, mainly due to the size of the human heart and the ratio between the number of epicardial cells and the amount of myocardial tissue. Size issues become more pronounced when taking into the account that patients often have massive epicardial fat deposition and thickening of the sub-epicardium. It is difficult to envision how epicardial cells or epicardial derived factors should reach the infarcted heart. Mouse models are often used for this type of research, but mice barely develop EAT (59), and are young and healthy while patients often suffer from additional comorbidities. Currently there is very little information available about the endogenous role of the epicardium in the human repair response to injury, let alone if the epicardial layer is deployable for cardiac repair. The main challenge for the coming years is to translate findings from cell and animal research to the human heart. As a first step we used primary human cells for our studies instead of animal cells or cell lines. Using epicardial cells derived from multiple patients includes the biological variability present in patients. A rapidly upcoming approach to study patient characteristics is the use of IPSC derived epicardial cells. The benefit of this is the option to specifically study a patient derived genotype combined with their isogenic control. In contrast, the value of patient derived primary epicardial cells is that these represent cells of the patient in its current situation, e.g. exposed to adipose tissue, medication, etc. This will presumably be more representative for the end-user of developed therapy compared to a more development-like IPSC.

Using this cell culture model we were able to easily study signalling pathways because almost all parameters could be controlled and read-outs were relatively strict and clear. This allowed us to investigate the regulation of epiMT in detail. Another significant benefit of in vitro models is the low costs per sample compared to organoids or animal models, and as a result, the possibility of high throughput screening, as we did in chapter 4. The next step in translational research is to study the behaviour

of the cell in its natural context, as it has been shown numerous times that cell-cell interactions and cell-ECM interactions are essential for cell behaviour (60). Therefore, validation in tissue makes the in vitro finding much stronger. However, findings in tissue often reflect a single timepoint, making it difficult to determine the processes that occur, and the cell types that are involved. Therefore, the combination of in/ex vivo and in vitro results will provide the most robust data. In our studies, we aimed for validation of our findings in mouse and human tissue. We used embryonic mouse hearts to validate Activin induced epicardial invasion and we determined PRRX1 expression in human hearts to translate findings from zebrafish studies to the human heart.

Potential opportunities to study the epicardium in the human heart lie within the culture of human tissue. One example of this is tissue culture of specimens from diseased human hearts (61) or human heart auricles (15). These approaches are high throughput and could include semi-healthy versus diseased tissue. Because it is difficult to obtain fresh healthy adult ventricular tissue, the use of healthy and diseased pig hearts may be a good alternative since one heart can provide a large number of slices to study ex vivo, and porcine hearts resemble human hearts in size. Interestingly, a recent paper describes a method to specifically study the epicardium in heart slices (62), which is a highly promising approach to bridge the gap between cell culture models, small animal models, and the patient. In addition, the ongoing progress in omics such as single cell sequencing, spatially-resolved proteomics and Tomo-seq will provide a wide potential to study processes over time in single cells derived from tissue.

CONCLUSION

In this thesis, we describe the isolation and culture of human primary epicardial cells and we demonstrate an extensive list of experiments to better understand the process of epiMT. While studying the signalling cascade of epiMT, we found that other factors than the pleiotropic TGFβ ligand can be used for the induction of epiMT. Furthermore, we showed that it is feasible to set up a screen using primary epicardial cells to reveal novel regulatory mechanisms of epiMT. In addition, we used the epiMT cell culture model to translate zebrafish findings to the human setting. Finally, we have explored the use of epicardial, compound eluting patches which may serve as a first step for further investigation into how factors identified based on their potential to stimulate the epicardium can be exploited to repair the injured heart.

Overall, we have described that the epicardium is an attractive therapeutic target for the injured heart, we have shown how to study it and we have provided novel approaches for stimulating this cell population to enhance cardiac repair.

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