Enhancing epicardial EMT to repair the heart

Dronkers, E.

1

Introduction

THE LIMITED REGENERATIVE CAPACITY OF THE HEART

The human body is in a continuous process of losing and renewing cells. Some organs are successful tissue regenerators: the gut epithelium replenishes every 4-6 days, the liver can fully regrow itself when partially resected, and – most visible in day to day life – the skin can completely restore (small) wounds without leaving a scar. These regenerative processes can be divided into two types: physiological regeneration (maintenance, e.g. gut epithelium, blood cells) and reparative regeneration (e.g. liver and skin regeneration upon injury). Unfortunately, some tissues have very limited reparative abilities. One of the organs in the human body with the least effective reparative capacity is the heart. Although cardiac tissue can maintain itself during the first decades of life by an annual cell renewal of 1% of the cardiac muscle cells (cardiomyocytes)(1), it cannot cope with massive loss of tissue upon wounding. Such a loss of tissue can happen when people experience a myocardial infarction (MI), a phenomenon that occurred 70.000 times in the Netherlands in 2020 (2). During the course of an MI, one of the coronary arteries becomes obstructed, which prevents oxygen and nutrients to reach downstream tissue. As a consequence, the tissue dies and the heart, which cannot stop beating and therefore has no time for proper wound healing, replaces the dead cells within the injured area by a non-contractile fibrotic scar. Although the scar is initially supportive and prevents tissue rupture, the heart's decreased contractility results in insufficient output to provide the required amount of oxygen and nutrient rich blood to the body. Having to work harder, to maintain the body's demand, the cardiac muscle becomes exhausted, leading to stiffening of the cardiac wall and ultimately to a progressive loss of cardiac pump function. This progressive and incurable disease, characterized by fatigue and shortness of breath, is known as heart failure which can be fatal if left untreated. In the Netherlands, 1 out of 3 patients who experienced an MI develops heart failure within a few years (3). Due to adequate first aid policies, nowadays less people acutely die of MI (2).

However, this brings about an increasing number of patients with a scarred heart who are at risk of developing heart failure (4). Therapy includes life-style changes and medication to lower the demand on the heart and thereby slow the progression towards heart failure. Currently, the only resolving treatment for patients with heart failure is partial or full replacement of cardiac function, either by a Left-ventricular assist device (LVAD), heart transplantation or, recently added to this list of curative treatments in the Netherlands, the total artificial heart (TAH). Although highly promising, these solutions are not without risk or burden for the patient, necessitate life-long immunosuppressive drugs and require either scarce donor hearts or a cardiac device which is still considered less optimal than a biological heart (5). A much more elegant and effective solution would be to target the actual problem and restore the damaged heart tissue.

The development of the heart

To repair an injured heart, the lost tissue needs to be replaced, meaning that cardiomyocytes must be substituted, and vasculature renewed. To achieve this, mechanisms need to be identified that support tissue repair. One way to study cardiac tissue renewal is to compare the mammalian adult heart with its counterpart that is perfectly able to form cardiac tissue: the embryonic heart.

The human embryonic heart is the first functional organ with a blood circulation already present after 4 weeks. The primitive heart initiates as a primitive tube, that starts to form a loop and to twist in such a way that it results in the structure of what will become a four chambered heart consisting of two ventricles and two atria. Progenitor cells migrate towards the heart to populate the heart, aiming to form a strong cardiac wall and to create a network of vessels and nerves. Cells that partake in the formation of the heart originate from three sources (reviewed in Tan 2020). The first one is the so-called cardiac mesoderm that gives rise to cardiac progenitors which provide cardiomyocytes and the endocardial cells that form the inner lining of the heart. The second source is the neural crest, which cells mainly contribute to cardiac innervation and valve formation. In this thesis, we focus on the third source of cells that populate the heart, which is the pro-epicardium, a transient structure at the base of the heart. Between week 4 and 6 of human development, pro-epicardial cells migrate towards the heart and start to populate the outside of the heart forming the epicardium (6,7). At that moment, the heart is still a thin-walled and poorly vascularized structure. However, when the epicardium has covered the heart, it starts to participate in cardiac development in several manners. First, epicardial cells undergo epithelial to mesenchymal transition (epiMT, see below), a process that transforms the

epicardial cell into a mesenchymal cell with the ability to migrate into the myocardium (8). Although there is debate about which cell types derive from the epicardium, it is generally accepted the epicardial-derived cells (EPDCs) differentiate into smooth muscle cells that cover developing vessels and thereby contribute to the maturation of the coronary system (9). Furthermore, EPDCs differentiate towards cardiac fibroblast securing the stability and organization of the heart. Second, the biochemical cross talk between epicardial cells and myocardial cells direct the cardiomyocytes in their growth (10,11). Altogether, the epicardial layer plays a crucial role in the formation of the heart (see Fig. 1). Most importantly, it comprises the endogenous capacity to contribute to cardiac tissue formation. Stimulating this endogenous capacity in the adult heart may be a way to repair injured tissue.

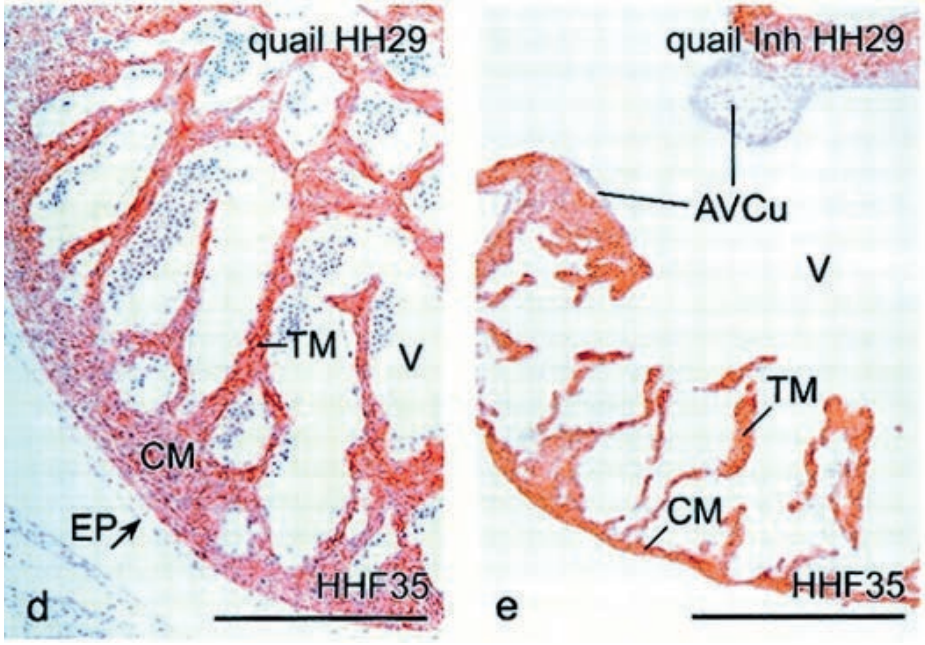

Fig. 1 | Crucial role of the epicardium during cardiac development. The left picture represents a normal quail heart, covered by the epicardium (EP) and properly developed compact myocardium (CM). The right picture shows a quail heart in which epicardial formation was blocked by preventing pro-epicardial outgrowth. The absence of the epicardium, as can be appreciated by the lack of an outer layer, prevents proper development of the myocardium, shown by the thin-walled compact myocardium (CM). Derived from (9).

Epicardial epithelial to mesenchymal transition (epiMT)

As stated before, an essential step of epicardial behavior is epiMT, allowing the cells to migrate and to conduct a variety of tasks. We refer to epicardial EMT as epiMT to make a clear distinction with the general EMT process, a common overarching process that does not solely occur in the epicardium, but is involved in tissue development and wound healing, and when dysregulated, can lead to severe pathological diseases such as fibrosis and metastatic cancer. EpiMT generally involves the epicardium, an epithelial tissue lining which is characterized by firm cell-cell adhesions, such as E-cadherin (12), and strong attachment to a basal membrane. If epiMT is initiated, structural changes start to occur. These include losing cell-cell adhesions and changes in the actin skeleton that prepares the cell to migrate (13). Because the cell needs to pass the basal membrane before migrating into underlying tissue, the production of matrix metalloproteases (MMPs) is increased to free the way. Furthermore, mesenchymal markers such as αSMA and Vimentin are upregulated (14,15). Phenotypically, epicardial cells undergo a major change from a squamous epithelial cell, defined by a clear apical and basal side and the nucleus centered in a web of the cytoskeleton, towards an elongated spindle-shaped cell that lacks polarization.

EpiMT and TGFβ signaling

If we zoom in on the regulation of epiMT, it starts with an initiator. One of the best known initiators is Transforming Growth Factor β (TGFβ), a pleiotropic factor involved in the regulation of critical cellular processes such as proliferation, apoptosis, and differentiation. TGFβ signaling starts by binding of one of the ligands (TGFβ1,2 or 3) to the TGFβ type 2 receptor which initiates pairing with the TGFβ type I receptor ALK5. Upon activation of the ALK5 kinase, the intracellular SMAD2/3 proteins become phosphorylated and consequently bind SMAD4, translocate to the nucleus where the SMAD complex acts as a co-factor that regulates gene transcription. Inhibitory SMADs (SMAD6 and SMAD7) can prevent transmission of the signal by competing with SMAD4 (reviewed in (16)). Besides the SMAD-dependent signaling, TGFβ is also known to interact with other pathways, such as MAPK/JNK and PI3K/Akt (reviewed in (17)), independent of SMAD, which is referred to as 'SMAD-independent' or 'non-canonical' signaling. The intricacy of this pathway allows for regulation at multiple levels, e.g. availability of receptors, balance between inhibitory and phosphorylated SMADs, transcriptional environment in the nucleus, etc. Hence, this explains how it is possible that TGFβ regulates so many processes, dependent on cellular context. In the context of EMT, TGFβ is known to reduce cell adhesion protein E-cadherin and increase mesenchymal markers such as Smooth muscle actin. It is doing so via the induction of EMT-transcription factors (EMT-TFs), such as Snail (Reviewed in (18)).

TGFβ is essential for epicardial behavior during cardiac development. This was demonstrated by the observation that absence of ALK5 results in loose epicardium and an underdeveloped heart (19). Furthermore, TGFβ induces epiMT in epicardial cells in vitro (20). TGFβ signaling and its related pathways are therefore an interesting starting point to study the regulation of epiMT.

Epicardial reactivation in the injured heart

A decade after the functional characterization of the embryonic epicardium at the end of the 20th century, it was discovered in 2006 that the epicardium does not solely play a role during development but also becomes active directly after ischemic injury in the adult heart. The initial study of Lepilina et al. demonstrated the contribution of the epicardium to cardiac regeneration in the partially resected zebrafish heart (21). Further research into the re-activation of the epicardium after injury showed that epicardial cells start to recapitulate developmental behavior, including upregulation of EpiMT genes (22). Studies in mice showed the relevance of embryonic recapitulation; mice with an epicardium-specific knockdown of β-catenin displayed diminished epicardial thickening and worsened cardiac function compared to wildtype mice (23). Although this response resembles fetal epicardial activities, there are also differences. For example, in the adult injured heart, the (sub-) epicardial layer thickens upon epicardial activation which barely occurs in the fetal heart. Furthermore, there appears to be less invasion of EPDCs into adult myocardial tissue compared to the fetal heart (24). Given that the epicardium has a proven record of contributing to tissue formation during development, optimizing the epicardial recapitulation may serve as an interesting target to increase cardiac repair. Therefore, studies were performed to exploit its potential to contribute to repair. Smart et al. showed that priming the epicardium with Thymosin β4 increased the number of epicardial cells and improved cardiac repair in the injured mouse heart (25). Moreover, local epicardial treatment with mesenchymal stem cell-derived exosomes increased epiMT which was related to improved cardiac repair (26). To summarize, inhibition of epiMT hampers cardiac repair, while stimulation of epiMT corresponds to an improved reparative response, demonstrating a window of opportunity to enhance epicardium-driven repair via epiMT. Therefore, the aim of my thesis is to find ways to boost epiMT in the injured heart. In this thesis, we describe a cell culture model which allows us to study epiMT. Using this model, we identify novel epiMT regulators. Because EMT is also involved in pathological remodeling, application of an epiMT stimulator should be transient and local. Therefore, we describe a method to locally administer these factors to the injured mouse heart (see Fig.2).

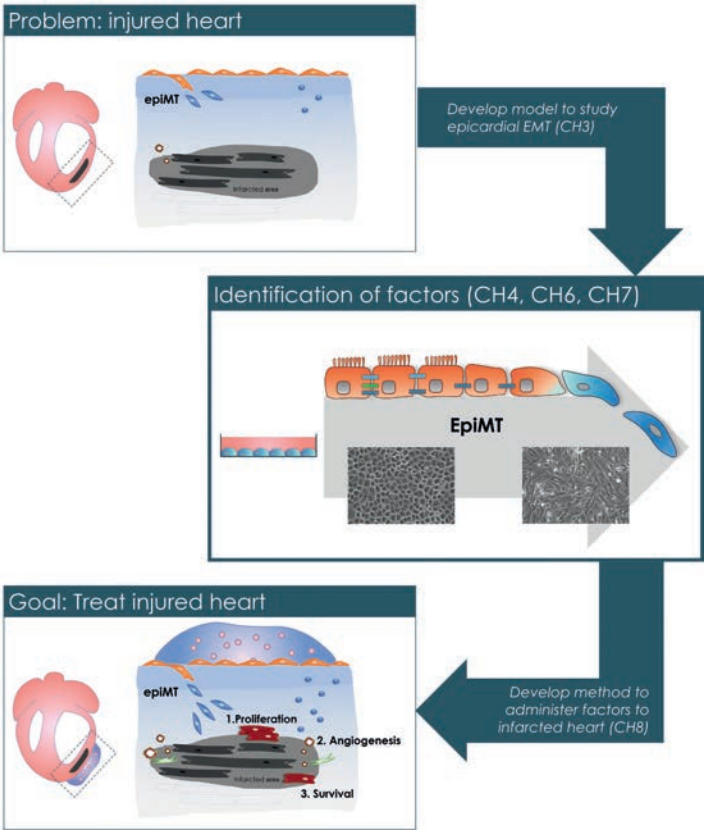

Fig. 2 | Overview of thesis

Scope

The ultimate goal is to enhance epicardium-driven repair after myocardial infarction. In this thesis, we aim to identify approaches to enhance epicardial EMT (epiMT).

Chapter 2 comprises a literature overview of epicardial behavior during development and disease. We describe that epiMT is an essential part of epicardial behavior. To study epiMT, we have developed an *in vitro* cell culture model of human primary epicardial cells, which is described in **chapter 3**. In **chapter 4**, we exploit these epicardial cells in a phenotypic compound screen resulting in the identification of novel inducers of epiMT.

One of the main regulators of EMT are members of the TGFβ family. In **chapter 5** we summarize what is already known about the role of TGFβ family signaling in epiMT.

Although the role of TGFβ in epicardial behaviour has been elaborately described, we conclude that other factors such as BMP and Activin may also be involved. Therefore, we used our cell culture model to study these pathways in epiMT in **chapter 6**, revealing that Activin and ALK4 signaling are regulators of epiMT.

Once cells have undergone EpiMT, they have the ability to invade underlying tissue and contribute to the generation of cardiac tissue. In **chapter 7** we describe how epicardial mesenchymal cells may contribute to cardiac repair by investigating the role of epicardial PRRX1 in fetal mesenchymal EPDCs and human tissue. We show that PRRX1 regulates NRG1 secretion, which is beneficial for cardiac regeneration. Furthermore, we demonstrate that PRRX1 is scarcely present in injured human cardiac tissue.

To be able to demonstrate the effectiveness of the identified factors on epiMT in the injured heart, a biomaterial needed to be developed that releases the factor locally to the epicardium in a timely manner. Therefore, in **chapter 8** we describe two types of self-adhering biomaterials that can be mixed with any compound and applied to the mouse heart.

Finally, in **chapter 9** the results and conclusions of this thesis are discussed.

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