The myocardial potential of proepicardial cells: From development to cardiac regeneration
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The capacity of the heart to recover from damage is referred to as its regenerative capacity. Hearts of lower vertebrates, like fish and salamanders, have a high regenerative capacity compared to the hearts of mammals in which the regenerative capacity is very poor. In these hearts, ischemic myocardial cells are replaced by fibrotic scar tissue, which results in a reduced pumping capacity of the heart and eventually in cardiac failure. To fully recover from damage, new cardiomyocytes are needed in the heart. Therefore, insights into the developmental processes that regulate cardiomyocyte formation are of major clinical significance. In this thesis we have focused on the question which signaling molecules and signal transduction pathways are important regulators of the differentiation of progenitor cells into cardiomyocytes or non-myocardial cells.

During the development of the heart, signaling processes are operational that determine the differentiation of progenitor cells into myocardial or non-myocardial cells. With current techniques, it is possible to study these processes. The early embryonic heart is a simple peristaltic contracting tube. The wall of this tube is built up of cardiomyocytes only. In the adult heart, in contrast, cardiomyocytes comprise only 30% of the total number of cells, whereas non-myocardial cells, mainly fibroblasts, comprise the rest. The embryonic heart tube grows by the addition of cells at both its poles. During development, both myocardial and non-myocardial cells are added in this way to the heart, though by far the largest majority of non-myocardial cells are added via the proepicardium, a "cauliflower-like" structure, which develops at the inflow pole of the heart. The proepicardium forms villi that make contact with the heart tube and form a layer of cells that cover the entire heart, which lies "naked" within the pericardial cavity. This newly added outer-layer is called the epicardium, which is in itself a source of cells for the heart. During development the majority of the non-myocardial cells, like fibroblasts and coronary vessels cells, are derived from the epicardium. During the formation of the proepicardium an important process takes place in which progenitor cells are added to the heart as myocardial- or as non-myocardial cell. This is called a "lineage switch". It has been proposed that insights into the regulation of this switch could provide new ways that can be used to manipulate the process of scar formation after infarction, driving cells involved in this damage repair process to the myocardial lineage rather than the non-functional cells of the scar tissue.

In this thesis we provide insights into the diversification of proepicardial cells from cardiac precursor cells, the capacity of proepicardial cells to differentiate into myocardial cells and the signaling molecules that regulate the differentiation of proepicardial and myocardial cells. Finally, the role of the epicardium in response to a myocardial infarction in the adult heart is analyzed.

In chapter 1 an overview is given of the development of the heart. This chapter summarizes the role of the heart forming fields, proliferation in the embryonic heart and the contribution of cells from the endocardium, myocardium and epicardium to the forming
heart. Besides these developmental insights, this chapter describes the potential use of these insights in the development of new regenerative therapies.

Chapter 2 provides an overview of the role of Bone Morphogenetic Proteins (BMP), a family of signaling molecules, during heart development. BMPs play an important regulating role during the formation of the proepicardium, atrioventricular canal and the septa of the heart. Furthermore, BMPs play a major role in the regulation of the differentiation of stem cells into heart muscle cells.

The next four chapters concentrate on the regulation of the differentiation of the proepicardium. In chapter 3 we show that proepicardial cells, notwithstanding the major contribution to the non-myocardial component during the development of the heart, retain the capacity to differentiate into myocardial cells. The signaling molecules BMP and Fibroblast Growth factor (FGF) are important regulators in this differentiation. In chapter 4 it is shown by cell-labeling analysis, that the proepicardium and myocardium of the inflow of the heart, are derived form a common myocardial precursor population. The separation of these two lineages form a common precursor population is regulated by an intracellular interaction of BMP- and FGF-signaling transduction pathways. In the proepicardium, the differentiation into myocardial cells is inhibited by FGF-signaling via Erk which blocks the BMP-pathway via Smad. These insights enable us to stimulate the differentiation into myocardial cells, which we show in vitro and in vivo. In chapter 5 an overview is given on the common origin of the proepicardium and myocardium of the inflow pole of the heart. Based on traditional labeling and genetic labeling studies, described in literature, we argue that the proepicardium and myocardium share a common cardiac origin. In chapter 6 a genome-wide expression analysis of different proepicardial and epicardial developmental stages is described in which we searched for genes that play a role in the regulation of myocardial differentiation. In this search the Wnt signaling pathway was identified as an important pathway in the differentiation of cardiac progenitors into myocardial cells. The signaling molecule Wnt-inhibiting factor-1 (Wif1), a natural extracellular inhibitor of wnt-signaling, was found to stimulate myocardial differentiation both in vitro and in vivo.

The finding that proepicardial cells retain the capacity to differentiate into cardiomyocytes, and the insights into the signaling pathways that regulate this differentiation process, makes the epicardium an interesting population for regeneration of the adult heart. In chapter 7 this hypothesis is investigated in mice in which the epicardium is genetically labeled and a myocardial infarction is induced. The results of these experiments show that the epicardium, like the myocardium, dies within the infarcted area after a myocardial infarction. The epicardium, however, displays re-growth at the border zone of the infarcted area over the infarcted area within one day, and expresses genes that were also present during development of the epicardium from the proepicardium. Form this epicardium, mesenchymal cells are formed that differentiate not only into non-myocytes in the scar but also into myocardial cells, though the number of cardiomyocytes formed is not sufficient to fully regenerate the lost myocardium. The reactivation of the epicardium in
response to injury seems to be an evolutionary conserved mechanism. The same genes and signals that are activated during development, regulating the differentiation of precursor cells into myocardial or non-myocardial cells, are activated in the activated and newly formed epicardium. These experiments show that, in contrast to the common belief that the mammalian heart has absolutely no regenerative capacity, the mammalian heart can respond to damage, a response that although limited, does result in a small number of new cardiomyocytes. During this regenerative response embryonic processes are reactivated. It is our firm belief that greater insights into this process may lead to new therapies in which the endogenous regenerative response can be stimulated to fully recover the damaged heart. This is a new direction in the approach to find means to regenerate the impaired or damaged heart.

**Translational perspective**

The central question in the translation of the results presented in this thesis is how one can take these results into clinical practice, i.e. how the regenerative capacity of the heart can be stimulated. A second, though perhaps more basic question is why the regenerative response in lower vertebrates results in new functional cardiac muscle whilst in the mammalian heart this process derives only scar tissue.

There are several plausible explanations for this phenomenon. (1) Among vertebrates, fish and mammals show intriguing differences in their growth control properties with age. Under proper conditions, a variety of fish species, for example zebrafish, even retain the potential for unlimited or indeterminate growth. Mammals, on the other hand, possess a physical size restriction. The regenerative capacity is higher in growing, maturing animals compared to animals that have reached their maturity and have reached their maximal size. (2) Cold-blooded animals, like fish and salamanders, have a lower metabolism compared to warm-blooded animals. As a consequence of their higher metabolism, mammals have a higher body temperature and also a higher blood pressure. These characteristics make mammals more prone to infection of wounds and of bleeding caused by damage. Therefore, in mammals it is crucial that a wound is closed quickly, such that they are able to prevent bleeding and infection, taking the disabilities or limitations for granted. To realize this fast wound healing an inflammation reaction is activated after damage to close the wound. Although life saving this inflammatory response opposes proper regeneration. (3) Furthermore, as mentioned, mammals have to deal with totally different hemodynamic characteristics compared to the heart of a fish. Fish have a relative large blood-volume which is pumped around in low pressure. In mammals, on the other hand, their large energy requirements have, during evolution, resulted in a circulatory system which is characterized by a relative small blood volume, pumped around at high pressure. According to these hemodynamic characteristics, the hearts of mammals have a thick compact myocardial layer, while fish have a trabeculated sponge-like heart. Because of the high pressures the heart of a mammal has to bare the risk of rupture up on injury is great. Therefore evolution has chosen for a rapid repair response to
injury. Like the injured limbs, the ventricular wall of the heart has to be strengthened quickly to prevent rupture, even though this will ultimately be at the expense of heart contractility.

Our finding, that the mammalian heart does possess a level of regenerative capacity, is of interest both from an evolutionary aspect and from a clinical point of view. However, the reason underlying the apparent lack of capacity to complete this regenerative response might be related to the high pressure the heart still has to deal with. It is known that in the first week after a myocardial infarction the ventricular wall is most prone to rupture. To prevent this, fibrous formation can be considered a real lifesaver. Taking this into account one possible approach to allow sufficient regeneration in the wounded mammalian heart during any kind of regenerative therapy might be to adapt the hemodynamics in such a way that it is more comparable to a fish heart. For this purpose a ventricular assist device can be implanted, which is used in clinic to bridge the waiting period for a donor heart. In the unloaded heart, growth factors, described in this thesis, could be locally stimulated or applied to enhance the regenerative response to form myocardial cells at the expense of fibroblasts in the forming scar tissue. There are indications that the unloaded heart has a higher regenerative capacity. In an eleven month old girl that has had a heterotopic heart transplantation at the age of twelve, the donor heart could be removed because her own heart had recovered. Also in the adult heart, unloading is described to stimulate the regenerative response.

With this approach it might be possible, in the future, to permit mammalian heart recovery by combining a surgical intervention with local modulation of the relevant signal transduction pathways that drive myocardial formation. In this approach the insights into the regenerative mechanisms of lower vertebrates in combination with cardiac developmental insights (evo-devo) could finally result in a new rational therapy to recover the heart after ischemic damage.
References