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**Title:** Cellular communication in cardiac regeneration
**Issue Date:** 2019-06-12
General Discussion
Myocardial infarction leads to a long and complex interplay of processes within the cardiac tissue. In short, ischemia in the cardiac wall results in cell death, which triggers an inflammatory reaction, necessary for the removal of cell debris, but also affecting the cardiac ECM structure. In order to compensate for the loss in cardiomyocytes and to strengthen the cardiac wall, (myo) fibroblasts migrate to the infarcted area and form collagen rich scar tissue. New blood vessels are formed in an attempt to restore blood flow. Lastly, the unaffected myocardium compensates for the loss in contractile force by, for example, hypertrophic remodelling. Even though this does not result in repair of the myocardial wall, it encompasses the best efforts of a tissue under strain to maintain its function. Regenerative therapy for the heart involves a treatment that should alter these processes in such a way that the injured area regains its functionality, without loss of cardiac output. The aim of my thesis was to explore different processes involved in cardiac repair and develop a possible therapy for cardiac regeneration.

**Regenerative processes for cardiac repair**

The different cellular responses after MI each serve an important role to regenerate the heart. However, the balance between these cellular responses should be precisely tuned, otherwise complete repair is not possible. Cell death is an immediate consequence of ischemia and reperfusion, and is the major factor for the loss in cardiomyocyte numbers. Protecting cells from apoptosis and necrosis by inducing survival pathways would therefore reduce the initial infarct size. However, in order to completely heal the infarct, new cardiomyocytes are needed within the myocardium via stimulation of proliferation of endogenous cells and/or transplantation of exogenous cells. The heart was long thought to be a post-mitotic organ, but the research by Bergmann et.al. showed that the mature heart was capable of self-renewal, albeit only a very low rate of 0.3-1% per year. Following this finding, multiple means were explored to induce cardiac proliferation, among which also affecting YAP (Yes-associated protein) in the Hippo pathway. Besides proliferation of cardiomyocytes, cardiac progenitor cells are also able to replace the lost cardiomyocytes by their ability to migrate to the infarcted region, expand and differentiate. However, their number is not sufficient to produce the necessary number of new cardiomyocytes.

Since an MI also leads to the deterioration of blood vessels, neo-angiogenesis is an important process for restoring the blood flow to the infarcted area. Without new blood vessels, any newly formed cardiomyocytes is unable to survive. Even though a hypoxic environment already induces angiogenesis, via production of e.g. VEGF, further stimulation of this process might lead to complete neovascularization of the affected area.

The extracellular matrix also undergoes extensive remodelling throughout the healing process of the infarcted ventricle. Firstly, inflammatory cells that homed to myocardium in response to the hypoxia and cell death, not only clear cell debris, but also release proteases such as e.g. MMPs. These proteases break down parts of the ECM, which on the one hand makes it easier for migrating cells to enter the tissue, but on the other hand leads to weakening of the cardiac wall. Myofibroblasts that migrated to the site of injury produce new ECM, which helps to stabilize the cardiac wall and prevent rupture. However, without new cardiomyocytes being incorporated, the production of new matrix molecules will only result in a collagen rich scar, not recapitulating the normal cardiac ECM.
organization. Therefore, the remodelled matrix does not provide a suitable environment for cardiomyocytes to form new contractile tissue.

**Paracrine signalling after MI**

Cell-based therapy after MI has shown in the past that injection of cells can stimulate the regenerative process of the injured myocardium at multiple levels. Besides directly contributing cells to the newly formed tissue – e.g. endothelial cells and cardiomyocytes – they also influence the resident cells during the different phases of wound healing after MI. Although large numbers of cells are injected, only a small percentage of the initial cells injected can be found in the heart 3 months post-MI. The lack of cell retention contradicts the beneficial effects on cardiac function, angiogenesis and fibrosis. This supports the theory that the paracrine factors secreted by the transplanted cells are an important contributor to the beneficial effect seen after MI. Paracrine factors, such as cytokines and extracellular vesicles (EVs), are able to influence cellular processes that will enhance tissue survival after MI, such as apoptosis and proliferation. Using conditioned medium from stem and progenitor cells, especially the vesicle fraction, demonstrated that this was sufficient to reduce infarction size, stimulate angiogenesis, and improve cardiac function to the same extent as injecting the cells. These observation make EVs, such as exosomes, an interesting factor for future therapies. Cell therapy has several disadvantages in a clinical setting, such as immune response and retention. Utilizing their secreted factors can overcome these problems and therefore be an interesting approach for future therapy. We do however need to understand which component in this conditioned medium, such as cytokines or EVs, are necessary for this paracrine effect.

**The impact of Endoglin**

**Angiogenesis**

One interesting factor in the aftermath of an MI is endoglin. Endoglin is a co-receptor in the TGF-β pathway and thus involved in TGF-β related processes, such as angiogenesis. Angiogenesis is an important process for regeneration and understanding the underlying mechanisms to stimulate this is essential in order to improve therapies. Although VEGF is the key regulator of angiogenesis, many other signalling pathways, like TGF-β, modulate the growth and stabilization of new vessels. TGF-β has a biphasic effect on angiogenesis, depending on the receptor that transduces the signal: binding of TGF-β to ALK5/TGF-β receptor 2 (TGF-βRII) inhibits angiogenesis, while signalling via ALK1/TGF-βRII is pro-angiogenic. Endoglin is a coreceptor for ALK1 and signalling via this pathway leads to Smad 1/5/8 phosphorylation, while it inhibits the anti-angiogenic signal via Alk5/TGF-βRII and Smad2/3. Inhibition of endoglin by neutralizing antibodies reduces neo-vascularization, which underlines the importance of endoglin in angiogenesis, and makes it an attractive strategy for anti-angiogenic therapies. Research has shown that endoglin can also interact with the VEGF receptor, which resulted in retention of the VEGF-receptor on the surface of the cell membrane and thus reduced degradation of the VEGF-receptor. A neutralising antibody against endoglin inhibited VEGF signalling and tip cell formation. These results corroborate our findings, showing that endoglin is a crucial factor in angiogenesis, as shown in chapter 4. Even though vasculogenesis did occur
when differentiating endoglin deficient embryonic stem cells using embryoid bodies, the reduced endoglin levels prevented the formation of a complex vascular network.\textsuperscript{22}

Because of the strong pro-angiogenic effect of endoglin, it is no surprise that the expression of endoglin is upregulated after MI. One week post-MI, endoglin is predominantly expressed by the endothelial cells in the border zone of the infarction, and not present in the scar myofibroblasts, confirming involvement in neovascularization.\textsuperscript{23} Besides endoglin upregulation after MI in vivo, expression of endoglin was also increased in endothelial cells in hypoxia \textit{in vitro}. The increase in endoglin resulted in enhanced endoglin/ALK1 signalling.\textsuperscript{24} Comparison of the angiogenic response of wild type mice with mice that are heterozygous for endoglin (Eng\^{+/-}) showed that endoglin expression and the number of newly formed vessels was higher in wild type mice after MI, and long term follow-up revealed reduced cardiac function in Eng\^{+/-} mice compared to wild type animals.\textsuperscript{23}

\textbf{Fibrosis and remodelling}
While endoglin is supportive of cardiac repair when taking into account its stimulating role in angiogenesis, it can have adverse effects during cardiac remodelling and fibrosis. Endoglin is expressed not only on endothelial cells, but also on fibroblasts. As TGF-β is a well-known factor in fibrosis, it is not surprising that an increase in endoglin correlates with an increase in cardiac fibrosis.\textsuperscript{25} Interestingly, reduced endoglin levels was also reported to limited fibrosis in a cardiac overload model.\textsuperscript{26} Transplantation of cardiospheres, generated from rat heart explants outgrowths, into the myocardium one month after MI showed a decrease in hypertrophy and an increase in cardiac function, relating to a decrease in TGF-β signalling via Smad 2/3. This change in TGF-β signalling was caused by an increase in soluble endoglin, cleaved from the cardiospheres and fibroblasts by MMP14. \textit{In vitro} coculture of cardiospheres with fibroblasts indeed showed that the presence of soluble endoglin was able to reduce TGF-β\textsubscript{1} induced Smad2/3 signalling.\textsuperscript{27} Since soluble endoglin can interfere with TGF-β\textsubscript{1} signalling, this might abrogate the pro-fibrotic effects of TGF-β in the remodelling phase of wound repair post-MI. Therefore, increasing endoglin is desirable in a time-dependent manner, in order to stimulate only the pro-angiogenic effect.

\textbf{Influence of EVs on regeneration}

\textbf{Angiogenesis}
Angiogenesis is not only stimulated by cytokines released by the cells in the infarcted area, but also by the proteins and miRNAs present in EVs secreted by the injured cells. Studies in tumour research have shown that EVs can play an important role in angiogenesis. Vessel growth is essential for tumour progression and it is therefore not surprising that tumour cells release EVs that contain factors to stimulate angiogenesis. These pro-angiogenic factors include VEGF and microRNAs miR-21 and miR-126-3p.\textsuperscript{28–31} EVs from tumour cells containing pro-proliferative mRNA have been shown to increase the mitosis of HUVECS.\textsuperscript{32} Furthermore, uptake of tumour EVs by endothelial cells \textit{in vivo} increases the permeability of the blood vessels, which in turn enhanced the formation of metastasis.\textsuperscript{33}
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The composition and content of EVs, i.e. the miRNAs, proteins and receptors present in and on the vesicles, is (partly) reflective of the cytoplasmic and membrane composition of the donor cell at a certain moment in time. Any change in the physiology and environment of the cell, e.g. a drop in oxygen levels, will result in a change in the composition in the EVs they secrete. Hypoxia induces stabilization of Hif1-α followed by induction of VEGF and Angiopoietin 2 transcription within the hypoxic cell. Therefore it is no surprise that this increase in angiogenic factors is mirrored within the EVs they secrete. EVs from glioblastoma cells, cultured in hypoxic conditions, were shown to stabilize tube structures of ECs under hypoxia. Moreover, EVs from hypoxic glioma cells induce the secretion of growth factors and cytokines by endothelial cells and stimulate pericyte activation and migration. Not only the content but also the number of EVs secreted by cells is influenced by hypoxia, for example a two fold increase in exosome secretion by MDA-MB231 cells after 24 hours of 0.1% O₂.

In our studies, we have found that EVs from CPCs are capable of inducing angiogenesis both in vitro and in vivo. This pro-angiogenic effect was highly dependent on the presence of the extracellular MMP inducer EMMPRIN on the EVs. We demonstrated that EMMPRIN was a key player in stimulating angiogenesis, as EVs with reduced EMMPRIN levels failed to induce migration and tube formation of endothelial cells. Moreover, the general infiltration of cells and the formation of blood vessels in a matrigel plug was decreased when EMMPRIN depleted from the EVs (see chapter 5). Other reports have shown that EVs from embryonic stem cells, cardiosphere derived cells (CDCs) and MSCs are also able to stimulate the proliferation of endothelial cells, corroborating our in vitro results. EVs from embryonic stem cells, human bone marrow cells and human CPCs increased vessel density when injected into the myocardial wall after myocardial infarction, demonstrating that EVs also induce angiogenesis in a hypoxic and damaged environment in vivo.

Analysis of the paracrine effects of CDCs showed that endoglin is one of the factors responsible for its proangiogenic effect. Knockout of endoglin in CDCs led to a decrease in the pro-angiogenic paracrine effect of CDCs in in vitro assays. Moreover, a decrease in vessel density was observed in the border zone of the infarction after injection of endoglin KO CDCs. We have shown that EVs from CPCs have high levels of endoglin in chapter 6. Injection of CPC EVs after MI resulted in an increase in the expression of endoglin in the myocardial wall, partly overlapping with the uptake of EVs. This suggests that delivery and/or upregulation of endoglin is beneficial for the stimulation of angiogenesis.

Cell protection

Protection of cardiac cells from ischemic damage shortly after myocardial infarction helps to preserve cardiac tissue and therefore will maintain cardiac function in the long term. In the last decade, transplantation of cells, e.g. CPCs, MSCs and ES cells, have shown a beneficial effect on cardiac function, but the studies have been ambivalent about the influence on infarct size shortly after MI. In order to understand these long-term effects, we sought to understand how CPCs and the EVs they secrete affect the myocardium shortly after injection. In our experiments (see chapter 6), we have observed a decrease in infarct size 48 hours after MI in the animals injected with CPCs. This effect is lost when Rab27A is knocked-down in the injected CPCs, Rab27a knockdown severely reduces exosome secretion, indicating that the effect on infarct size of transplanted CPCs
is due to the vesicles secreted. Although studies have shown that injection of EVs mimics the effect of cell injection on infarct size in the long term, we already observed a reduction in infarct size 48 hours after injection of CPC-derived EVs. Thus, not only are the EVs an important part of the cardioprotective effect of CPCs, the vesicles alone are already capable of reducing the infarct size. Differences in EV secretion or effectiveness by the different cell types transplanted might explain why not all studies have detected a decrease in infarct size after cell transplantation compared to injecting a high concentration of EVs directly into the myocardium.

The studies of Ibrahim et al. and Wang et al. show that pre-treatment of CDCs or MSCs with the exosome secretion inhibitor GW4689 successfully decreased exosome secretion and abrogated their \textit{in vitro} cardioprotective effects. After cell transplantation, the GW4689 cells were unable to reduce infarct size and improve cardiac function. Parameters such as viable cardiac tissue and wall thickness were decreased while scar mass was increased. Systemic blocking of exosome secretion in HSP-20 overexpressing cardiomyocytes also failed to increase cardiac function in a mouse model of diabetes, compared to injection of EVs from the HSP-20 overexpressing cardiomyocytes. These results corroborate that the effects seen two days after cell transplantation are due to the secretion of EVs by these cells.

Evaluation of the miRNA content of EVs has revealed that multiple cell survival miRNAs are present in EVs, of which miR210 and miR21 are mentioned regularly. Multiple of these miRNAs have targets in the pAkt pathway. This pathway has been documented to be involved in cell survival after MI. Moreover, one of the earliest studies with MSC exosome injection after MI showed an increase in pAkt after 24 hours. In vitro analysis of the downstream effects of EV uptake resulted predominantly an increase in pTEN-pAkt signalling. It was shown that the miRNAs present in the respective EVs were responsible when similar results were obtained with transfection of mimics or miRNA. Altogether this is indicative of cardioprotective effect of EVs through the pAkt pathway.

**Proliferation and migration**
Proliferation is essential for complete regeneration after myocardial infarction in order to replace the lost cells. New cardiomyocytes can restore contractile units, while proliferation and migration of endothelial cells provide new blood vessels and fibroblasts help to strengthen the cardiac wall and aid in cell alignment. All together, these cells are able to restore cardiac function. However, since their endogenous proliferation and regeneration is not sufficient to restore the normal cardiac tissue, (myo) fibroblasts populate the infarct area and produce a collagen rich scar to strengthen the cardiac wall and replace the lost cells.

Proliferation is known to be influenced by EVs, which is mostly studied using tumour cells. EVs from gastric cancer cells are able to induce proliferation of these cells in an Akt depending manner. Mast cells secrete EVs able to induce proliferation and stimulate the migration of lung cancer cells, via activation of the PI3/AKT pathway due to the presence of KIT, a growth factor receptor, on the EVs. Furthermore, EVs from T cells are able to promote the invasion of tumour cells by inducing MMP9 expression and activating the ERK and NF-kB pathways, both \textit{in vivo} and \textit{in vitro}. The sentinel lymph nodes can be
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stimulated by tumour EVs to induce the expression of pro-angiogenic genes, as seen in melanomas. Together with an increase in matrix proteins, this enhances tumour recruitment and growth. Vesicles can also influence bone marrow cells to enhance tumour growth and increase the vascular density in the tumour mass.

Even though these effects on proliferation and migration are detrimental in tumour progression, it does confirm that exosome signalling is able to influence the proliferation and migration of cardiovascular cells needed for cardiac regeneration. EVs for cardiac repair can in theory be isolated from any cell type, but all possible (side) effects of these EVs on the recipient cells need to be investigated. Although EVs isolated from tumour cells are very potent and stimulate many beneficial effects necessary for cardiac regeneration, the chance of oncogenic activity makes them less suitable for a potential therapy. Therefore, isolating EVs from cardiac specific donor cells such as CPCs reduces the possibility of transferring unwanted stimuli. Adverse effects of EVs are often related to their systemic administration and can therefore be decreased when EVs are locally delivered. Modification of EVs through hypoxia and overexpression or knockdown of specific proteins can further increase the effectiveness of the EVs, but any undesirable effects of the modification should be thoroughly investigated.

EVs from various cell sources have been studied for their effect on cardiac regeneration and an increase in cell proliferation was observed. Endothelial cell proliferation is increased after stimulation with MSC or cardiomyocyte derived EVs. Both MSC and cardiospheres derived EVs were able to increase proliferation of cardiomyocytes, while EVs from ES cells and rat MSCs stimulated the proliferation of CPCs. Studies aiming to identify the mediators of cardiovascular cell proliferation showed miRNAs, such as miR-291, miR146a and miR147 are responsible for this effect. In vivo proliferation has only been analysed in two studies. Khan et.al. show that treatment after MI with ES-derived EVs increases the proliferation of cardiac progenitor cells in the heart. Zhu et. al. also saw a slight increase in CPC numbers as well as proliferating cardiomyocytes, which failed to reach significance. We have shown that EVs from CPCs are able to increase cell proliferation in the infarct border zone of the heart, 48 hours after MI, explaining why the infarct size is reduced. In depth analysis of which cells respond to exosome treatment reveals that primarily the number of proliferating cardiomyocytes and endothelial cells is increased (see chapter 6). These cell types are vital for restoring cardiac tissue contractility and viability.

Boundaries and challenges of regenerative therapy

Cardiac regeneration requires an interplay of several processes. We have shown the importance of endoglin and EVs in angiogenesis and the potential of EVs on infarct reduction and proliferation, as shown in chapters 4, 5 and 6. Endoglin can be an interesting target for a therapy, as its role in angiogenesis and vessel stability is well known. However, angiogenesis is not the only process endoglin and the TGF-β pathway play a role in. For example, homing of inflammatory cells and tissue fibrosis are also affected by a change in endoglin/TGF-β signalling. Therefore, any treatment changing this signalling pathway should be applied locally and in a time-specific manner.
EVs have the advantage that they are rapidly taken up by cells, not having the problem of retention seen with cell transplantation. However, this rapid uptake does imply a short term and short range effect of the EVs, which could be circumvented by multiple treatments or a slow release delivery system. Though quantification of total EVs uptake is very difficult in order to compare to percentage of cells retained in the heart, the spread of the exosome uptake clearly shows that a large area of the heart can be targeted. For translation into a clinical setting, challenges such as the number of EVs and injection strategies must be addressed. Furthermore, patient details such as sex, age and comorbidities may also affect EVs dose and effectiveness. One study has compared intracoronary injection vs intramyocardial injection of cardiosphere-derived EVs in a large animal model.

Figure 1: Hypothesized potential of EV treatment after MI. A therapy with EVs has the potential to increase the number of cardiomyocytes, promote angiogenesis and reduce scar formation, which is accompanied by a smaller infarct size and a sustained cardiac function.
They concluded that only direct injection into the myocardium resulted in preservation of cardiac function. They also observed a decrease in scar size one month after MI.64 This large animal study shows that intramyocardial injections of EVs are safe and can lead to long-term effect on scar size.

**Future perspectives**
The ultimate goal is to develop a ready, off-the-shelf, universal therapy able to regenerate the heart after MI. The studies done so far have shown that endoglin is a promising pro-angiogenic target and that EVs from several cell sources are able to beneficially influence the heart after MI in preclinical models. Yet several questions remain before the leap to the clinic can be made. Ideally, EVs should be used directly after the occurrence of an MI, at the time of opening the vessel, to serve as a direct cardiac protection agent and deliver the first regenerative stimuli. An overview of the potential implications of EV treatment after MI is shown in Figure 1. How EVs can best be isolated and stored in order to fulfill this requirement needs to be investigated. Since EVs are sensitive to changes in the cells environment, cell culture and exosome isolation needs to be optimised and standardised. Nevertheless, this also provides possibilities to modify and improve the isolated EVs, for example by harvesting more angiogenic EVs from hypoxic cells. Furthermore, artificial methods can be used to increase the cardioprotective effect of EVs. Genetic manipulation of donor cells can increase the levels of beneficial RNAs and proteins in the secreted EVs. This type of modification requires a more detailed knowledge about the exosomal content and transmembrane proteins in the exosomal membrane, in order to find the most suitable targets for modification. An interesting direction for EV modification would be to introduce specific cardiac homing proteins to the vesicles, which would make intra-venous administration possible for cardiac treatment. Lastly, more knowledge is needed about autosomal and autologous application of EVs. EVs from MSCs and CPCs are immunosuppressive according to the first reports, but in-depth analysis of their effect on the immune system is needed before moving to the clinic.65,66

**Conclusion**
Complete restoration of cardiac function is the aim of regenerative therapies. An all-encompassing therapy might not be attainable yet, but our increasing knowledge of the factors involved in regeneration paves the way for smaller, targeted treatments. In this thesis, we explored these regenerative processes and different means to enhance them. We found that endoglin is a crucial factor for angiogenesis. Reduction in endoglin levels is sufficient to prevent correct vessel and network formation, making endoglin an interesting target for pro-angiogenic therapies. Angiogenesis was also stimulated by the EVs secreted by our CPCs. Furthermore, these CPC-derived vesicles are able to convey cardioprotective effects leading to a decrease in infarct size and an increase in proliferation of cardiac cells. More research into their content and the cellular and systemic consequences of intra-cardiac injection will ultimately lead to the most efficient therapeutic agents.
References


52. Giricz, Z. *et al.* Cardioprotection by remote ischemic preconditioning of the rat heart


