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Viral vector-mediated gene transfer in fundamental and applied cardiovascular research

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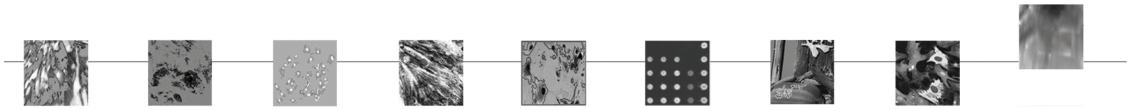


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Chapter 9

Summary, conclusions and future directions

Samenvatting, conclusies en vervolgonderzoek

9.1 Summary and conclusions

The structure and function of cells is determined -to a large extent- by the repertoire of genes that are expressed in those cells. To treat various cardiac diseases, modification of gene expression for the purpose of increased or decreased expression of a particular gene, is regarded as a potential therapy. As a vehicle to introduce the gene of choice into the heart cell, viral vectors have given the most promising results. This thesis describes studies that are undertaken to investigate how viral vectors may be used to efficiently target cardiac cells and what the effects of certain genetic interventions are on the (patho)physiology of heart cells.

Chapter 1 presents an overview of current viral vector technology and how these vectors are used in basic cardiac research and as novel treatment options for cardiac diseases. The three most commonly used vector systems for *in vitro* and *in vivo* gene transfer are introduced, followed by several methods that can be used to modify viral vectors in order to specifically target the heart. Furthermore, several modes of intervention, *i.e.* overexpression or downregulation of endogenous gene expression, are discussed. The final parts of this chapter focus on the application of viral vector technology in basic cardiac research and the potential application of viral vector-mediated gene transfer to the heart to treat several cardiac diseases.

Chapter 2 describes how forced expression of the skeletal muscle-specific transcription factor MyoD in human mesenchymal stem cells (hMSCs) can improve the potential of these cells to deliver the dystrophin gene to Duchenne muscular dystrophy (DMD) myoblasts through cell fusion. Transduction of hMSCs with a lentiviral vector encoding MyoD led to the expression of several early and late skeletal muscle markers as assessed by immunofluorescent staining and reverse transcription PCR. Furthermore, the percentage of MyoD expressing hMSCs that fused with myoblasts to form myotubes was higher than the percentage of fused hMSCs that did not express MyoD. Next, MyoD-expressing hMSCs were transduced with an adenoviral vector encoding full-length dystrophin to test whether these hMSCs could restore dystrophin expression in DMD myoblasts. Immunofluorescent staining for dystrophin showed that dystrophin was present only in myotubes that had fused with hMSCs. Moreover, dystrophin expression was properly localized along the plasma membrane. Expression of β -dystroglycan, part of the dystrophin-associated glycoprotein complex, was stronger in mosaic myotubes than myotubes made up exclusively of myoblasts, indicating that delivery of dystrophin to DMD myotubes could rescue the formation of this complex. These results show that the delivery of transgenes through cellular fusion is feasible and may provide a novel treatment for DMD.

Chapter 3 studies whether stimulation of integrins on neonatal rat cardiomyocytes (nr-CMCs) promotes the uptake of macromolecules by these cells. Therefore, the uptake of Texas Red-labeled ovalbumin (OTR) or Texas Red-labeled dextran (DTR) was quantified. Integrin stimulation by the pentapeptide GRGDS, containing the RGD motif, resulted in increased uptake of both OTR and DTR, which was dependent on their concentration in the medium. In addition, the uptake of these macromolecules correlated with the concentration of GRGDS and incubation time. The uptake of OTR as a result of GRGDS stimulation was mediated by macropinocytosis, as wortmannin, an inhibitor of this process,

was shown to decrease OTR uptake after GRGDS stimulation. Next, the role of dysferlin in macromolecule uptake was assessed. Immunofluorescent staining of dysferlin revealed that the size of dysferlin patches in nrCMCs was increased after GRGDS stimulation compared to untreated cells. However, this increase in patch size was not observed when beating of nrCMCs was inhibited, indicating that membrane disruptions are involved in macromolecule uptake. Knock-down of dysferlin expression using lentiviral vectors encoding short hairpin (sh)RNAs targeting the dysferlin mRNA resulted in a reduction of GRGDS-induced OTR uptake, suggesting that dysferlin is required for macropinocytosis. As cell stretch increases the incidence of membrane disruptions, nrCMCs that were stretched showed more uptake of OTR than non-stretched nrCMCs and this increase was dependent on the degree of stretch that was applied. In conclusion this research shows that integrin stimulation leads to uptake of macromolecules via macropinocytosis, which seems to be mediated by dysferlin involved in repair of membrane disruptions.

Chapter 4 describes a study in which the effect of changes in the bioavailability of nitric oxide (NO) on integrin stimulation-induced hypertrophy in nrCMCs was tested. Therefore, in GRGDS-stimulated nrCMCs NO-synthase (NOS)1 was inhibited by incubation with L-NAME or by transduction with a lentiviral vector encoding either protein inhibitor of NOS1 (PIN) or a shRNA targeting the NOS1 mRNA. All three interventions resulted in an inhibition of the hypertrophic effect and the degree of inhibition was dependent on NO availability. Furthermore, it was shown that exogenous NO administration could bypass NOS1 inhibition, leading to hypertrophy in GRGDS-stimulated nrCMCs. Induction of NOS2 by lipopolysaccharide (LPS) resulted in hypertrophy, which was dependent on the level of NOS2 expression. Hypertrophy was abrogated completely by ODQ, an inhibitor of soluble guanylate cyclase (sGC), but the hypertrophic response was restored upon coadministration of 6-Br-cGMP, an analogue of cGMP which is the product of the sGC-catalyzed reaction. Since sGC is NO responsive, this indicates the importance of NO-signaling in the hypertrophic response to integrin stimulation. In addition, it was investigated whether the signaling pathway leading to hypertrophic growth is mediated by Akt1. Activation of Akt1 occurs via phosphorylation of Akt1. The P-Akt1/Akt1 ratio was increased upon GRGDS stimulation and was significantly decreased by administration of L-NAME. Furthermore, PI3K inhibition upstream of Akt1 by wortmannin led to a complete abolishment of GRGDS-induced hypertrophy. Overexpression of constitutively active Akt1 using a lentiviral vector induced hypertrophy in nrCMCs, whereas lentiviral vector-mediated expression of a dominant-negative version of Akt1 inhibited GRGDS-induced hypertrophy. Together these results imply that PI3K/Akt1 signaling is involved in integrin stimulation-induced hypertrophy in nrCMCs. In addition, a model is proposed that links integrin stimulation, NO signaling and PI3K/Akt1 signaling and results in physiological hypertrophy.

In chapter 5, the hypothesis is tested whether inhibition of coupling between myofibroblasts (MFBs) and CMCs reduces the development of arrhythmias in fibrotic cultures. It was shown that the conduction velocity was significantly slower in fibrotic cultures compared to control cultures. Moreover, the incidence of ectopic activity and reentrant tachyarrhythmias was higher in fibrotic cultures compared to control cultures. Immunofluorescent staining showed that Cx43 was present between adjacent MFBs and CMCs. The resting membrane potential of the CMCs in fibrotic cultures was significantly less nega-

tive than that of CMCs in control cultures, which increases the risk of reentry. To test our hypothesis, MFBs were transduced with a lentiviral vector encoding a shRNA targeting Cx43. In MFBs that expressed the Cx43-shRNA, Cx43 expression was decreased by 53% and significantly less dye transfer through gap junctions was observed. The membrane potential of CMCs in fibrotic cultures containing MFBs with downregulated Cx43 was significantly more negative than the membrane potential of CMCs in fibrotic cultures containing MFBs that received a control shRNA. The conduction velocity in cultures containing MFBs in which Cx43 was knocked-down was significantly higher than in cultures containing MFBs that received a control shRNA, and ectopic activity and reentrant tachyarrhythmias were decreased by 32.8% and 44.1%, respectively. In conclusion, functional uncoupling of MFBs and CMCs through the knock-down of Cx43 can prevent the occurrence of arrhythmias in fibrotic cultures. These results indicate that the myofibroblasts in the myocardium can be considered a novel target for anti-arrhythmic therapy.

Chapter 6 describes the role of gap junctional coupling in transdifferentiation of hMSCs into CMCs. In contrast to adult hMSCs, fetal hMSCs are able to differentiate into CMCs when cultured in co-culture with CMCs. The finding that fetal hMSCs display a high Cx43 expression which allows these cells to couple to CMCs, whereas adult hMSCs have almost no Cx43 expression, may account for this difference. In order to elucidate this further, Cx43 was knocked-down in fetal hMSCs, while Cx43 was expressed in adult hMSCs using lentiviral vectors. Dye transfer measurements using calcein showed that a higher percentage of adult Cx43-expressing hMSCs was calcein-positive and that the intensity of the dye was stronger compared to control adult hMSCs, whereas in fetal hMSCs the opposite effect was observed when Cx43 was knocked-down. Immunofluorescent staining of α -actinin was used to assess the differentiation of hMSCs towards CMCs. Although ~2.2% of control fetal hMSCs were α -actinin positive, none of the Cx43 knock-down fetal hMSCs was α -actinin positive, indicating that Cx43-mediated coupling to surrounding CMCs is necessary for cardiac differentiation of hMSCs. In contrast, differentiation of adult hMSCs was not observed, even when Cx43 was expressed. To test whether expression of another gap junction protein Cx45 could rescue the differentiation potential of fetal hMSCs in which Cx43 was knocked-down, the latter cells were transduced with a lentiviral vector encoding Cx45. Both the percentage of calcein-positive cells and the dye intensity were markedly increased. Moreover, ~1.6% of these cells stained positive for α -actinin. Together these results show that gap junctional coupling between fetal hMSCs and CMCs is necessary for the differentiation of hMSCs to CMCs. It is proposed that small molecules are able to travel between CMCs and hMSCs via gap junctions and elicit differentiation. Adult hMSCs, however, did not differentiate towards CMCs, likely because these less primitive cells have lost part of their differentiation capacity due to changes in their epigenetic makeup.

Chapter 7 describes a study which aimed to develop adeno-associated virus (AAV) vectors for the transduction of myocardial scar fibroblasts (MSFs). A prerequisite for AAV vectors as a gene transfer vector to scar tissue is that the therapeutic transgene should exclusively be expressed in scar fibroblasts and not in the surviving CMCs. Therefore, we aimed to determine the optimal promoter and AAV serotype that result in high transgene expression in MSFs but not in CMCs. Nine different promoters were tested for their activity in human, mouse and rat MSFs, as well as in nrCMCs. In human MSFs the human

CMV promoter displayed the highest activity, whereas in mouse and rat MSFs, the human EF1 α promoter showed the highest activity. The RSV promoter gave the highest transgene expression in nrCMCs. Subsequently, we tested five distinct serotypes to determine which serotype would most efficiently transduce rat MSFs or nrCMCs. This comparison revealed that AAV serotype 2 transduced rat MSFs most efficiently and AAV serotype 9 transduced nrCMCs most specifically. These results demonstrate that the most specific transgene expression in rat MSFs can be achieved by transduction with an AAV vector of serotype 2 containing a transgene driven by the EF1 α promoter, whereas nrCMCs can best be transduced by an AAV serotype 9 vector containing a transgene driven by the RSV promoter. To confirm these results, optimized AAV vectors encoding GFP for rat MSFs or the red fluorescent protein FP650 for nrCMCs were generated. As expected, the intensity of the fluorescent signal of cells transduced with the AAV2.EF1 α .GFP vector was 4 times higher in rat MSF cultures than in nrCMC cultures. Transduction of rat MSFs with the AAV9.RSV.FP650 vector did not result in significant transgene expression, whereas high levels of expression were measured in nrCMCs. These results indeed confirm that our optimized AAV vectors are able to specifically target MSFs and CMCs in the rat. Whether the same results can be obtained *in vivo* requires further experiments.

The experiments described in this thesis emphasize the enormous potential of gene therapy to treat various illnesses using viral vectors. Illnesses that have been studied include Duchenne muscular dystrophy and cardiac arrhythmias as a result of cardiac fibrosis. Furthermore, genetic modification using viral vectors is a strong tool in elucidating (patho)physiological mechanisms, such as integrin physiology of CMCs, the role of NO in hypertrophy of CMCs, and the role of cell-cell contact in stem cell differentiation. For application of viral vectors *in vivo*, issues like the safety of viral vectors, efficient targeting of the heart and the duration of gene expression deserve further study.

9.2 Future directions

The limited regenerative capacity of the heart upon injury and the finding that cells administered to the heart as cell replacement therapy do not differentiate into CMCs have prompted research to find ways to differentiate primitive cells to CMCs *in vitro*. Human MSCs that were endowed with the skeletal muscle-specific transcription factor MyoD expressed some skeletal muscle markers and were more prone to fuse with myoblasts in co-culture to form myotubes than control hMSCs. These results show the potential of genetically modified stem cells to differentiate towards a muscle cell fate. Although none of the known cardiomyogenic transcription factors are able to elicit full differentiation of stem cells, combinations of transcription factors have shown to be sufficient to differentiate murine cells to CMCs. Further research on cultures of human MSCs should clarify what combination of factors is required to differentiate these cells to CMCs. The finding that more primitive stem cells, *i.e.* fetal MSCs, more readily differentiate into CMCs than adult MSCs would favor the use of the former cells in such experiments.

Besides the use of cardiomyogenic transcription factors, improved gap junctional coupling between engrafted MSCs and the surrounding CMCs could result in cardiomyogenic

differentiation of these MSCs. Fetal MSCs in co-culture with nrCMCs were able to differentiate to CMCs, but this ability was abolished when Cx43 expression was knocked-down. It is hypothesized that the gap junctions allow the transfer of cardiomyogenic signals from CMCs to MSCs, thereby inducing differentiation of MSCs. It would be of interest to investigate what the nature of the exact signals and mechanisms are that are responsible for this process. Forced expression of Cx43 in transplanted cells for cell replacement therapy could thus improve the differentiation of engrafted cells, although some pitfalls have to be taken into account. Firstly, the transplanted cells should have the capability to differentiate into CMCs, as it was also shown that endowing adult MSCs with Cx43 did not result in cardiomyogenic differentiation of these cells in co-culture with nrCMCs. Secondly, it was shown that gap junctional coupling of nrCMCs to undifferentiated cells diminishes the membrane potential in the CMCs, which causes ectopic activity and reentrant tachyarrhythmias. Injection of MSCs that express Cx43, but do not differentiate into CMCs, could therefore have a detrimental effect on cardiac function. These results again stress the need to identify ways to improve the cardiomyogenic potential of autologous MSCs before injection into the heart of the patient.

Apart from the use of gene therapy to improve cardiac cell therapy, genetic modification of myocardial scar tissue in patients that suffered a myocardial infarction may ameliorate cardiac function. The scar fibroblasts and the extracellular matrix excreted by these cells present an anatomical conduction block, leading to dyssynchrony and potential arrhythmias. Genetically modifying the scar fibroblasts by introducing a viral vector expressing ion channel proteins, such as Cx43, may improve conduction across the scar and thereby reduce dyssynchrony. In order to increase the efficiency and reduce the adverse effects of the delivery of the therapeutic gene to the scar tissue, a suitable viral vector is required. Over the last years, AAV vectors in particular have shown great potential as vector system to target the heart. Since it would be undesirable to transduce healthy CMCs with the therapeutic vector, as this could lead to supraphysiological levels of the transgene in these cells, this thesis describes the generation of an AAV vector that specifically targets fibroblasts. The usefulness of this vector *in vivo* has to be tested in a rat model of myocardial infarction. Future experiments should test the effect of the introduction of ion channel genes into the scar fibroblasts. In addition, AAV vectors have to be further optimized to better target scar fibroblasts.