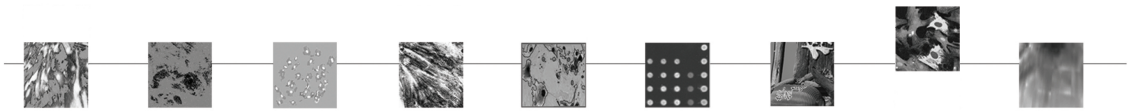

Chapter 8

Critical evaluation

Genetic modification has been an important tool in scientific research for several decades and in recent years has gained interest for its potential therapeutic applications. To deliver the gene(s) of interest to target cells, a variety of viral vector systems has been developed, the three most common of which are reviewed in chapter 1.

Communication between the extracellular matrix (ECM) and cardiomyocytes (CMCs) is essential for proper cardiac development and homeostasis.[1] Integrins on the plasma membrane of CMCs link these cells to the ECM and act as mechanotransducers.[2] The RGD motif present in various ECM proteins including fibronectin, tenascin and vitronectin is a ligand for integrins[3,4] and stimulation of contracting neonatal rat (nr) CMCs in culture with a pentapeptide containing this RGD motif (GRGDS) provides a model that allows the effects of CMC stretch to be studied *in vitro* (chapter 3). Besides the release of cardiac troponin I as a result of integrin stimulation[5], the uptake of macromolecules is induced as well. This uptake was shown to occur by macropinocytosis and required dysferlin. The role of dysferlin in membrane repair upon damage as a result of mechanical stress has been well established[6], but its role in macropinocytosis is new. Since macropinocytosis is independent of receptor molecules[7], formation of macropinosomes might be the result of membrane ruffling linked to dysferlin-mediated remodeling of the actin cytoskeleton during membrane repair.[8] However, additional research is needed to test this hypothesis and to gain insight into the precise mechanism by which dysferlin contributes to macropinocytosis.

Besides inducing the uptake and release of macromolecules, intensified stretch of CMCs may lead to cardiac hypertrophy. Stimulation of nrCMCs with GRGDS was indeed shown to lead to cardiac hypertrophy, which involved nitric oxide (NO).[9] In chapter 4, it is shown that the extent of hypertrophy is dependent on the availability of NO. Furthermore, the pathway that leads from NO to the hypertrophic response is partly elucidated and the involvement of AKT in this pathway suggests that integrin stimulation leads to physiological hypertrophy.[10] Nevertheless, the factor(s) linking integrin stimulation to activation of nitric oxide synthase (NOS)-1 remain(s) to be discovered. Focal adhesion kinase (FAK) is associated with integrin signaling as integrin stimulation with GRGDS resulted in FAK activation in nrCMCs.[9] However, phenylephrine (PE), an α1 adrenergic receptor agonist linked with pathological hypertrophy, was also shown to activate FAK. In a recent study, FAK overexpression in mice caused cardiac hypertrophy via the PI3K/AKT/mTOR pathway.[11] In accordance with our results, the authors concluded that Src was not involved in the FAK-dependent activation of PI3K and subsequent hypertrophic response. In contrast, FAK was found to interact directly with p85, the regulatory subunit of PI3K. Our results, however, show that NO signaling via sGC/cGMP is involved in integrin stimulation-dependent PI3K activation, as blockade of this pathway abolished integrin ligation-induced CMC hypertrophy. This suggests that other factors than FAK are involved in the signaling pathway that leads from integrin stimulation to NOS activation. A possible candidate link between integrin stimulation and NOS activation is integrin-linked kinase (ILK). ILK interacts with β-integrins and transduces mechanical stress to chemical signals.[12] Interestingly, ILK expression is increased in hypertrophic hearts compared to healthy hearts.13 Furthermore, ILK induced cardiac hypertrophy *in vitro* in nrCMC cultures and in a transgenic mouse model.[13,14] Moreover, ILK is involved in the activation of

AKT[15], possibly via NOS1

Activation of the PI3K/AKT signaling pathway has been considered a potential therapeutic strategy for heart failure.[16] As integrin stimulation appears to induce physiological hypertrophy via AKT, it could provide a novel way to treat patients suffering from hypertension, aortic constriction, aortic valve stenosis or heart failure due to myocardial infarction, all being -or leading to- cardiac pathologies associated with pathological hypertrophy. However, prolonged AKT activity has been associated with a worsening of cardiac function.[17] A dosed stimulation of the AKT pathway that would better mimic the effects of exercise would therefore be a more promising approach than uncontrolled activation of AKT. Whether AKT is able to reverse the effects of pathological hypertrophy should first be tested in CMCs that have been stimulated with PE.

Duchenne muscular dystrophy (DMD) is a severe muscle wasting disease caused by a defective dystrophin gene. As DMD is the most prevalent hereditary striated muscle disease and it is lethal at a relatively early age, a number of potential therapies are being developed.[18] Among these, cell therapy to complement the patient's diseased myofibers with a functional dystrophin gene has been proposed as a treatment for DMD.[19] We attempted to deliver the dystrophin gene to muscle fibers *in vitro* via the fusion of DMD myoblasts with dystrophin-expressing mesenchymal stem cells (MSCs) (chapter 2). Previous research had shown that MSCs are capable of fusing with myoblasts, but only at low frequency.[20] We therefore transduced the MSCs with a self-inactivating lentivirus vector (SIN-LV) encoding the myogenic transcription factor MyoD to increase their propensity to fuse with skeletal muscle cells. Although we showed *in vitro* that these genetically modified MSCs can deliver the dystrophin gene to myotubes, leading to dystrophin expression along the sarcolemma, we did not investigate whether they would survive *in vivo*. Lattanzi *et al.* showed that murine fibroblasts could be converted to the myogenic lineage by transducing these cells with an adenoviral vector carrying the MyoD gene.[21] Furthermore, Kimura *et al.* used MyoD to convert *mdx* mouse fibroblasts to the myogenic lineage.[22] In addition, both studies showed that the transduced fibroblasts contributed to myofibers after intramuscular injection *in vivo*. This indicates that cells that have been genetically modified in culture and partially differentiated towards the myogenic lineage are able to survive *in vivo* after being injected intramuscularly. Furthermore, several studies have shown that human cells expressing MyoD were also able to contribute to myofibers in mouse models.[23,24] Goudenege *et al.* demonstrated that stem cells derived from adipose tissue that expressed MyoD contributed to myofiber formation upon injury. Chaouch *et al.* combined myogenic conversion by forced MyoD expression with an exon skipping approach in skin fibroblasts from DMD patients. Human dystrophin expression was observed *in vivo* in mouse muscle after injection of converted DMD fibroblasts that received exon skipping treatment.

Several issues still have to be resolved before myogenically reprogrammed MSCs can be used in the clinic. Firstly, the expression of MyoD in the MSCs should be downregulated after their incorporation into myofibers, since MyoD is an early myogenic transcription factor that is not naturally expressed in myotubes.[25] This can, for instance, be achieved by applying the tamoxifen-induced MyoD-ER^{T2} fusion protein used in our study. This approach may be preferred over the use of an inducible promoter system, because

the fusion protein is of human origin and will therefore not cause an immune response, whereas an inducible promoter relies on the expression of the tetracycline repressor protein from *Escherichia coli*, which might be immunogenic. Furthermore, the response to the stimulus is more rapid compared to an inducible promoter and therefore a stricter control of transgene activity can be achieved. Activation of the protein as a result of the patient's pharmacotherapy should, however, be prevented. Placing the expression of the myogenic transcription factor gene under the control of a tissue-specific promoter that is active in MSCs but not in differentiated skeletal muscle cells may also be an elegant approach to prevent the accumulation of MyoD in myofibers at supraphysiological levels. Alternatively, myofiber-specific microRNAs may be utilized to stop transgene expression after fusion. This approach, however, depends either on the presence in the myofiber of sufficient amounts of microRNAs that can affect transgene expression from the fused MSC or on the ability of the converted MSC to produce these microRNAs. The use of a SIN-LV to transduce the MSCs has been associated with the risk of insertional oncogenesis, as these vectors integrate into the host cell genome.[26] Although this might not be a big issue as the myogenically converted MSCs become part of a terminally differentiated structure, SIN-LVs should only be used following extensive safety assessments. The use of adeno-asociated virus (AAV) vectors may provide an alternative to the use of LVs as the genomes of AAV vectors typically remain in the cell as episomes.

Cell therapy has gained further interest as a possible treatment option for cardiac diseases. MSCs from human adults have been used in clinical trials to enhance cardiac function after myocardial infarction, but treatment with these cells has so far yielded only minor improvements.[27] The slight functional benefit could in part be due to the inability of the injected cells to become CMCs with most of the beneficial effects being due to the production by the donor cells of paracrine factors that promote the survival of ischemic myocardium and neovascularization.[28] We indeed showed in a previous study that the cardiomyogenic differentiation potential of human MSCs (hMSCs) in co-culture with nrCMCs depends on the developmental stage of the donor tissue, *i.e.* differentiation into CMCs could be demonstrated for embryonic and fetal hMSCs but not for neonatal or adult hMSCs.[29] Moreover, a positive correlation was found between the connexin 43 (Cx43) expression levels of the different hMSC populations and their cardiomyogenic differentiation potential. Cx43 is the most abundant connexin in the heart and forms gap junctions between cardiac cells to facilitate myocardial impulse propagation.[30]

In chapter 6, definitive proof is provided that gap junctional coupling is required for the cardiomyogenic differentiation of fetal hMSCs in co-culture with nrCMCs. It seems that electrical and/or chemical signals from the surrounding nrCMCs pass through gap junctions and induce differentiation of hMSCs. The exact nature of the signals involved remains uncertain, although several studies have provided links. Hyperpolarization was shown to be sufficient for the differentiation of human CMC progenitor cells.[31] These cells may, however, be more prone to differentiate into CMCs than hMSCs and therefore require less stimulatory signals. Another possibility is that small molecules such as micro RNAs are transported through the gap junctions and alter gene expression in the recipient cells, resulting in their cardiomyogenic differentiation.[32] Furthermore, apart from gap junctional coupling, signal molecules might also be transferred via exosomes. Although

we demonstrated that gap junctional coupling is required for the differentiation of fetal hMSCs into CMCs, we did not investigate the possible contribution of exosome transport from the nrCMCs to the hMSCs to this process.

In addition, our results showed that overexpression of Cx43 in adult hMSCs did not induce cardiomyogenic differentiation of these cells in co-culture with nrCMCs even though dye transfer experiments revealed a large increase in gap junctional coupling between both cell types. It therefore appears that adult hMSCs are intrinsically less able to undergo cardiomyogenesis, possibly due to epigenetically controlled decreases in developmental plasticity.[33] Indeed fetal stem cells that had entered the maternal bloodstream homed to the heart and contributed to cardiac repair after myocardial infarction, indicating the potential of fetal cells to differentiate to cardiac cells.[34] This has implications for the use of hMSCs in the clinic, as "younger" cells may give better results. It might thus be worthwhile to use other sources than adult bone marrow or adipose tissue such as the umbilical cord or amniotic fluid to obtain autologous MSCs for clinical applications.

Cardiac fibrosis as a result of injury is caused by excessive fibroblast proliferation and ECM deposition and can lead to cardiac arrhythmias.[35] Myofibroblasts that are co-cultured with CMCs have been shown to induce ectopic activity[36], which might lead to ventricular fibrillation in the heart. Previously, we have shown that a higher percentage of fibroblasts in co-culture with nrCMCs corresponded with a higher incidence of reentry in these cultures.[37] Furthermore, the resting membrane potential of nrCMCs was significantly less negative in cultures with a high percentage of fibroblasts compared to cultures with few of these non-muscle cells. This finding contributed to the idea that electric coupling between fibroblasts and CMCs is responsible for the occurrence of reentry. In chapter 5 this hypothesis was tested by inhibiting the expression of the major ventricular gap junction gene Cx43 using short hairpin (sh) RNA-mediated RNA interference. Co-cultures of nrCMCs with fibroblasts that had been transduced with a SIN-LV encoding a shRNA targeting Cx43 showed less reentry, less ectopic activity and a higher conduction velocity through the culture compared to co-cultures with fibroblasts that had received a control SIN-LV. Ideally, these findings should be confirmed using another Cx43-specific shRNA or a dominant-negative version of Cx43 or by performing a phenotypic rescue experiment in which the effect of the shRNA is abolished. These extra experiments were, however, not feasible within the time available. Furthermore, the shRNA employed in the study described in chapter 5 has been successfully used before.[38]

Our findings suggest that disruption of gap junctional coupling between the fibroblasts and CMCs in the fibrotic heart may protect against the occurrence of arrhythmias. However, it remains to be investigated whether this strategy is beneficial *in vivo*. The presence of functional heterocellular gap junctions between CMCs and fibroblasts *in vivo* has been questioned[39], although direct data is lacking. Some *in vitro* studies have shown that the percentage of fibroblasts that is coupled to CMCs is low and that fibroblasts may affect conduction through other mechanisms, such as the activation of mechanosensitive channels or paracrine signaling.[39,40] Notwithstanding, $\sim$40$\%$ of fibroblasts in our co-cultures showed dye transfer, which is indicative for the presence of functional gap junctions. To study the effect of the inhibition of Cx43 expression in fibroblasts *in vivo* would be a logical next step.

In recent years, AAV vectors have emerged as one of the most promising vector system for the genetic modification of the heart.[41] Although several studies have focused on the transduction of the (rodent) heart as a whole[42-45], for the treatment of many cardiac disorders it would be beneficial to specifically target (or not target) one of the cell types present in the heart. The two major cardiac cell types, CMCs and fibroblasts, can be differentially targeted using an AAV serotype 2 (AAV2) vector pseudotyped with the capsid of AAV serotype 9 or 2, respectively (chapter 7). Furthermore, the use of either the RSV promoter or the human EF1α gene promoter provides extra specificity for CMCs and fibroblasts, respectively. However, even when these methods of transductional and transcriptional targeting were combined, some level of transgene expression was still present in the detargeted cell type. To be able to test the effect of the inhibition of Cx43 in fibroblasts, it is essential that Cx43 expression in the surrounding CMCs is unaffected. The specificity of AAV vectors for fibroblasts could therefore be further improved by incorporating micro RNA target sequences in the vector genome.[46] Two recent studies in which AAV vectors carrying target sequences to a liver-specific micro RNA were administered intravenously have demonstrated the usefulness of this method to selectively transduce the heart.[47,48] In a similar fashion, this method could be used to detarget CMCs by exploiting (C)MC-specific microRNAs, such as microRNA-1 or -133. Furthermore, it has been shown that the transduction of rodent fibroblasts by AAV2 vectors may be improved by mutating the capsid protein at several places to prevent the ubiquitination and proteasomal degradation of the vector particles.[49] Whether the improvement in transduction is exclusive for fibroblasts or can be observed in CMCs as well remains to be investigated. Furthermore, the transduction profiles of CMCs and fibroblasts *in vivo* may differ from the transduction profiles observed *in vitro*. The *in vitro* results should therefore first be verified *in vivo* before these AAV vectors are applied in studies that investigate the effect of potential therapeutic genes. Although it would have been desirable to also test the transduction of human CMCs with different AAV vector pseudotypes as compared with the human scar fibroblasts, this was not feasible due to the unavailability of these cells.

The production of AAV vectors in sufficient quantities for application in clinical trials is difficult and current protocols that are based on the transfection of 293T cells are cumbersome. Recently, it has been found that AAV vector particles do not only accumulate inside the producer cells but are also released into the culture medium, which could approximately double the yield compared to a protocol in which only the cells were harvested.[50] Nonetheless, this would be a minor improvement as we desire ~100 times higher yields to be able to initiate clinical trials in which up to 10^{13} particles are used.[51] As an alternative to an increase in particle yield, the particle to transduction ratio may be improved. One way to achieve this may be the use of self-complementary AAV vectors. These vectors do not require single strand to double strand conversion of the DNA and therefore exhibit an increased speed of transgene expression.[52] However, the capacity of these self-complementary vectors is halved, which makes this method unsuitable when larger transgenes are used. Recently it was shown that the single to double strand conversion of AAV vector DNA can be accelerated by co-transduction with a self-complementary AAV vector expressing protein phosphatase-5, which enhances second strand DNA synthesis by preventing the binding of FKBP52 to the viral DNA.[53] The harvesting of the viral

particles from cells remains complicated. By using a baculovirus vector-based production system with Sf9 insect cells much higher yields can be achieved.[54] At present, the latter production method combined with tangential flow filtration to concentrate AAV vector stocks seems best suited to produce the large, high-titered AAV vector stocks required for (pre)clinical studies.

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