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Author: Swildens, Jim

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

Issue Date: 2012-10-11

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

Jim Swildens

Colophon

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Cover design: R.F. Swildens

Layout: J. Swildens

Printed by: Proefschriftmaken.nl || Uitgeverij BOXPress

Published by: Uitgeverij BOXPress, 's-Hertogenbosch

ISBN: 978-90-8891-466-9

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

Proefschrift

ter verkrijging van
de graad van Doctor aan de Universiteit Leiden,
op gezag van Rector Magnificus prof.mr. P.F. van der Heijden,
volgens besluit van het College voor Promoties,
te verdedigen op donderdag 11 oktober 2012
klokke 13:45 uur

door

Jim Swildens

geboren te Rotterdam
in 1982

Promotiecommissie

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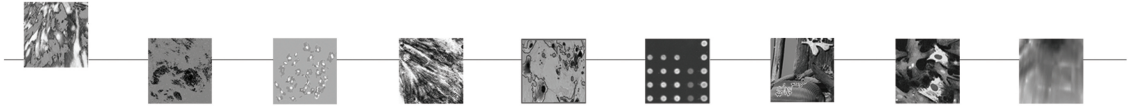
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Chapter 1

Introduction

1.1 Background

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Cardiovascular diseases (CVDs) are the leading cause of death worldwide. It is projected that the number of people dying from CVDs will increase with 30-40% over the next two decades.¹ In addition, the number of people suffering from well-known risk factors for CVDs, such as diabetes, obesity and high blood pressure is increasing, despite efforts to reduce these figures. Due to the lack of definitive cures for most CVDs current therapies focus primarily on alleviating the disease symptoms.

The heart consists of muscular, vascular and stromal (*i.e.* connective tissue) compartments. The smallest of these compartments in terms of cell numbers is the vascular compartment, which contains endothelial cells, pericytes and smooth muscle cells and is involved in supply of nutrients and oxygen to the heart and in removal of waste and carbon dioxide from the heart. The cells of the cardiac vasculature are greatly outnumbered by the cardiomyocytes (CMCs) in the muscular compartment of the heart and by the cardiac fibroblasts (CFBs), which represent the main interstitial cell type in the healthy heart. The muscular compartment of the heart is directly involved in the generation of contractile force. CMCs contract upon electrical stimulation, allowing the heart to pump blood through the body's circulation. These cells need a constant supply of oxygen and nutrients to remain active and die quickly after disruption of the blood supply as occurs during myocardial infarction (MI). Mature CMCs are terminally differentiated cells with very low regenerative capacity. Cardiac insults thus usually result in the permanent loss of CMCs and the replacement of dead CMCs by fibrotic tissue, leading to impaired cardiac function.² The cardiac interstitium not only plays a crucial role in maintaining the structural integrity of the heart but is also involved in mechanosensing/transduction and the regulation of CMC behavior. The CFBs in the cardiac connective tissue produce most of the heart's extracellular matrix (ECM)³ as well as many growth factors and cell surface molecules involved in homo- and heterocellular communication. In contrast to CMCs, CFBs display large phenotypic plasticity. In response to specific environmental stimuli CFBs become activated, start to proliferate and differentiate into (proto)myofibroblasts, which due to their contractile nature and biosynthetic activity are important for cardiac wound healing. Excessive proliferation of and ECM deposition by CFBs result in cardiac fibrosis that in turn can precipitate cardiac arrhythmias. The relative quantities of cells and ECM components in the stromal compartment of the heart are not constant but change in response to developmental, physiological and pathological stimuli. Likewise, the ratio between CMCs and CFBs differs between (i) mammalian species, (ii) atria and ventricles and (iii) developmental stages, and is decreased in many cardiac diseases.

Research into novel therapies that aim to cure cardiac diseases should focus on ways to prevent or reverse the loss of CMCs and/or the development of fibrosis and non-physiological hypertrophy. The growing knowledge of the molecular and cellular mechanisms involved in CVDs provides the opportunity to treat the underlying causes of disease. Genetic modification of cardiac tissue is a promising strategy to achieve this goal.

1.2 Current therapies

Chronic cardiac diseases, such as arrhythmias and heart failure, are in most cases incurable. This is mainly due to the poor understanding of the molecular and cellular mechanisms underlying these diseases and to the fact that cardiac remodeling, which is a major contributor to the clinical symptoms, cannot be reversed. Current therapies are, therefore, aimed primarily at preventing disease progression and managing symptoms.

Cardiac arrhythmias occur by several mechanisms, and are often difficult to treat. Current therapies include the use of anti-arrhythmic drugs to suppress the onset of arrhythmias. One cause of arrhythmias that is difficult to treat is cardiac fibrosis.⁴ Cardiac fibrosis may be secondary to MI; dead CMCs are replaced by scar tissue, whereas the surviving CMCs develop hypertrophic growth in response to overload.⁵ Cardiac fibrosis not only reduces myocardial compliance but also increases the risk of arrhythmias since fibrotic tissue is unexcitable and thereby creates a conduction block that may predispose for reentry. In addition, it has been shown that an excess of CFBs can induce cardiac ectopy through the partial depolarization of adjacent CMCs.⁶ Anti-fibrotic therapies may therefore show promise to treat arrhythmias. Current treatments of fibrosis-related arrhythmias include pharmacotherapy, using beta-blockers to lower heart rate, class III anti-arrhythmic drugs and angiotensin II-receptor blockers to reduce fibroblast proliferation.⁷ Alternatively, a pacemaker or implantable cardioverter-defibrillator (ICD) can be used to electrically stimulate the heart or treat arrhythmias by properly timed stimuli or shocks.⁸ These therapies effectively treat arrhythmias, but do not cure the underlying problem. In patients in whom the anatomical site(s) of arrhythmia can be located by electrophysiological study, ablation therapy can be used to destroy part of this tissue, thereby eliminating the source of arrhythmogenesis.⁹

There are many diseases and conditions that may lead to heart failure, and several therapies can alleviate the symptoms or even restore cardiac function. Medication may be prescribed to lower blood pressure, which decreases the workload of the heart. When heart failure is the result of valvular insufficiency, surgical therapy by valve replacement is often successful.¹⁰ In case of myocardial ischemia as a result of a stenosed or blocked coronary artery, either coronary bypass surgery or coronary angioplasty with or without stenting improves the delivery of oxygen to the heart.¹² If asynchronous contraction of left and right ventricles or left ventricular segments underlies heart failure, cardiac resynchronization therapy (CRT) using a dedicated pacemaker has been shown successful.^{12,13} In case of severe heart failure, patients may need an intra-aortic balloon pump (IABP), to improve myocardial perfusion, a left ventricular assist device (LVAD) to support or improve cardiac output¹⁴, or patients need a heart transplant. The latter therapy, however, suffers from a shortage of donor hearts.

1.3 Genetic modification

Modifying the gene expression profile of cells by introducing foreign DNA has generated much attention over recent years. Genetic modification has shown to be a valuable research tool and it has growing therapeutic potential. Using genetic modification, the effect of overexpression or knock-down of a single gene can be investigated, which pro-

Viral vector	Lentivirus	Adenovirus	Gutless adenovirus	Adeno-associated virus
Diameter	80-100 nm	70-90 nm	70-90 nm	»20 nm
Packaging capacity	< 10kb	5-11kb	»35kb	< 5kb
Large-scale production	difficult	very easy	easy	rather difficult
<i>In vivo</i> transgene expression	permanent	short-lived	long-lived	long-lived
Tissue penetration	poor	poor	poor	good
Immune response	absent	very strong	strong	weak

Table 1.1: Summary of commonly used viral vector systems for cardiac gene transfer.

1

vides insight into cardiac functioning at the molecular level. In addition, transgenic animal studies have been invaluable for understanding cardiac development, especially in elucidating the involvement of specific genes in congenital malformations.¹⁵ As a therapeutic tool, genetic modification may be employed for several purposes. It could be used to complement a missing function as a result of a genetic disorder, such as the dystrophin gene in Duchenne muscular dystrophy or the genes of several sarcomeric proteins that cause hypertrophic cardiomyopathy.^{16,17} Furthermore, a gene could be introduced that is normally not expressed in the target cell. This approach may be used to for non-muscle cells in the heart to acquire cardiomyogenic properties using cardiac transcription factors. Another application is to overexpress a protein that has a beneficial effect on the disease. This strategy is, for example, used to treat heart failure by overexpression of the sarcoplasmic reticulum Ca^{2+} -ATPase pump (SERCA2a) which improves cardiac function by increased uptake of Ca^{2+} ions from the cytoplasm to the sarcoplasmic reticulum after contraction.¹⁸ Alternatively, genetic modification could be used to inhibit expression of a protein that has a detrimental effect on the heart. The usefulness of this approach was demonstrated by Suckau *et al.*, who showed that symptoms of heart failure were reduced by lowering the level of the SERCA2a inhibitor phospholamban.¹⁹ Transfer of genes encoding so-called reprogramming factors into murine skin fibroblasts formed the basis for the generation of induced pluripotent stem cells (iPSs).²⁰ The rapid progress in iPS technologies as well as in embryonic and somatic stem cell research is boosting the search for innovative cell replacement therapies, that may improve cardiac function after MI.²¹⁻²³ Furthermore, iPS technologies provide the opportunity to study the differentiation of easy to obtain non-muscle cells to functional CMCs and to use these cells as models for cardiovascular disorders.^{24,25}

1.4 Vectors

In the past twenty years the techniques to genetically modify organisms have improved enormously. Refinement of existing methods as well as the development of new gene delivery vehicles has resulted in a large array of gene transfer vectors, characterized as viral or non-viral vectors. Each gene delivery system has specific advantages and drawbacks. Consequently, particular vector systems may be preferred depending on the research aim or therapeutic target.

This review will focus on the three most commonly used viral gene transfer systems (Table 1). Non-viral vectors used in the genetic modification of the heart are reviewed elsewhere.²⁶

1.4.1 Lentiviral vectors

Lentiviruses, such as human immunodeficiency virus type 1 (HIV-1) (figure 1a and b), are members of the retrovirus family.²⁷ Retrovirus particles are about 80-100 nm in diameter and contain a genome that consist of two single-stranded RNA (ssRNA) molecules that are associated with nucleocapsid proteins. This complex, together with the enzymes that are required for replication, is surrounded by a protein capsid, which itself is enclosed by the viral envelop, consisting of viral glycoproteins and lipids from the host cell. Upon infection of the host cell, the ssRNA is reverse transcribed into double-stranded DNA.²⁸ This so-called proviral DNA is subsequently inserted in a non-site-specific fashion into the host cell genome. This particular feature of retroviruses is the main advantage over other common viral vector systems as it allows permanent transgene expression in target tissues. Most retroviruses, however, can only cause a productive infection in dividing cells. This is an important drawback when one wants to apply these vectors for cardiac gene therapy as the large majority of CMCs in the adult heart have lost the capacity to divide. Lentiviruses, however, are able to replicate in non-dividing cells²⁹ and may therefore be more suitable than other retroviruses as vector to transduce (*i.e.* genetically modify) terminally differentiated CMCs.

Retroviral vectors have been widely used as gene transfer system in both experimental and therapeutic settings. Lentiviral vectors are mostly derived from HIV-1, the main causative agent of acquired immunodeficiency syndrome (AIDS). To convert HIV-1 from a potentially harmful virus into an innocuous viral vector, the proviral DNA is inserted into a plasmid followed by the removal of all viral genes and their replacement by one or sometimes two mono- or multicistronic heterologous gene expression units.³⁰ In order to be able to produce these vectors in the laboratory, lentivirus genes are provided in trans, by either co-transfecting helper/packaging plasmids or a complementing cell line.³¹ Production of lentiviral vectors by the co-transfection method is generally performed in human embryonic kidney (HEK) 293 cells carrying a stably integrated copy of the simian virus 40 genome.³² These so-called 293T cells are transfected with a lentiviral vector shuttle plasmid together with two (for second-generation packaging systems³³) or three (for third-generation packaging systems³⁴) helper plasmids (figure 1c). Alternatively, fourth-generation packaging systems like the Lenti-X system or the *trans* Lentiviral system are commercially available to date. A drawback of lentiviral vectors that are produced using these methods is that they cannot be easily produced in large quantities, like adenovirus vectors. A recently described protocol uses recombinant baculoviruses, carrying all the lentiviral elements required for particle production, to transduce 293T cells in suspension.³⁵ This method may provide an alternative to conventional lentiviral vector production methods when large yields of lentiviral vector particles are needed, as both the cell culture and the purification steps can be easily scaled up.

The viral sequences minimally needed in cis, *i.e.* present on the plasmid that is packaged, are the two long terminal repeats (LTRs), which are essential for viral replication and integration, the packaging signal (ψ), the rev-responsive element (RRE) and the complementary polypurine tract-central termination site (cPPT-CTS) necessary for nuclear export and import, respectively. A risk related to the high level of recombination in lentiviruses is that replication-competent virus particles may be formed. An improvement

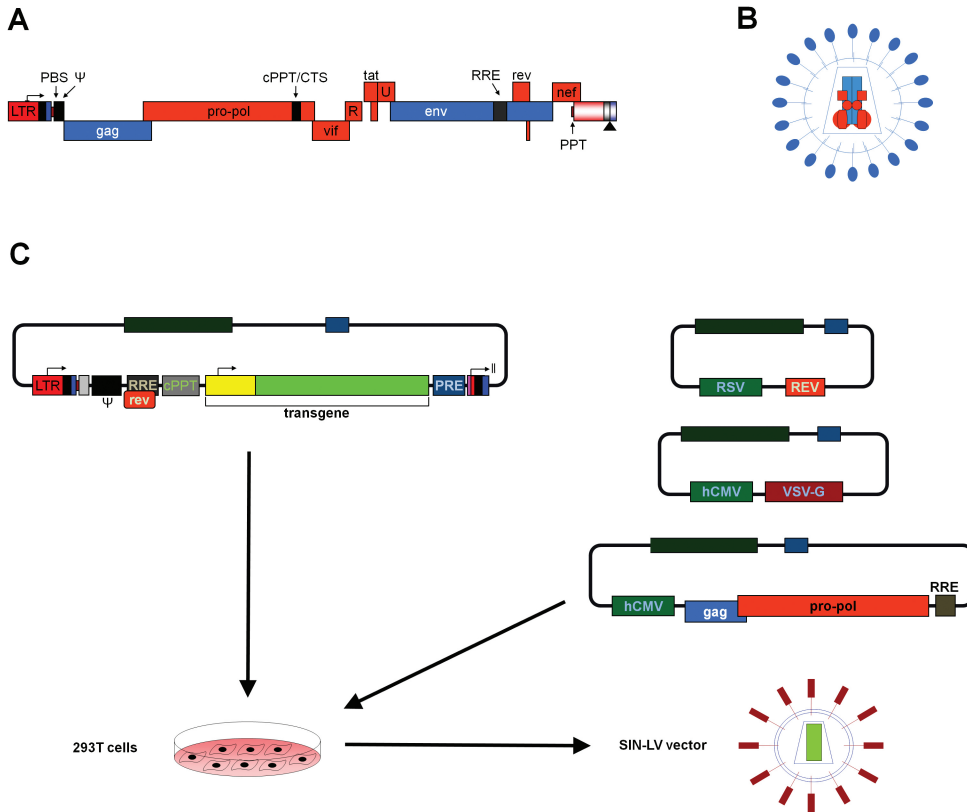


Figure 1.1: **A.** Schematic drawing of the HIV-1 genome. Blue boxes indicate genes encoding structural proteins, red boxes indicate non-structural proteins. ψ , packaging signal; LTR, long terminal repeat; cPPT-CTS, complementary polypurine tract-central termination site. **B.** Schematic drawing of a HIV-1 particle. **C.** Third generation SIN lentiviral vector production system.

of the lentiviral production system to overcome this risk was the development of self-inactivating (SIN) lentiviral vectors.^{36,37} In these SIN lentiviral vectors, the promoter activity from the 3'LTR is deleted. During reverse transcription of the lentiviral RNA genome, the 3'LTR is used as a template for both LTRs and the deletion is copied to the 5'LTR. As a result, the LTRs of these viral vectors lack transcriptional activity and thereby the risk of replication-competent recombinant formation is minimized. Lentivirus genes that need to be provided in *trans* are *gag*, encoding structural proteins, *pol*, which codes for important enzymes, *rev*, encoding a protein necessary for efficient *gag* and *pol* expression and *tat*, whose gene product enhances transcription of the viral genome. Lentiviral vectors produced with third-generation packaging systems have part of their 5'LTR replaced by the Rous sarcoma virus (RSV) or human cytomegalovirus immediate-early (hCMV-IE) promoter, resulting in high-level, Tat-independent viral genome synthesis. As a consequence *tat* is not needed in these production systems.

Wild-type HIV-1 particles infect target cells via its envelope protein gp120 that binds specifically to CD4 on the cell surface.³⁸ This limits HIV-1 infection to cells expressing CD4. Thus, in order for lentiviral vectors to infect cells that do not express CD4, gp120 has to be replaced by another envelope protein like the G-protein of vesicular stomatitis virus (VSV-G).³⁹ This protein endows lentiviral vectors with a broad tropism allowing transduction of many different cells types. In addition, VSV-G is more stable than gp120 facilitating the concentration of lentiviral vector particles. A drawback of lentiviral vectors is that they cannot be easily produced in large quantities, in contrast to adenoviral or AAV vectors (see below).

1.4.2 Adenoviral vectors

Adenoviruses are double-stranded DNA viruses that were first isolated from human adenoid tissue.⁴⁰ Currently, 56 distinct human adenovirus serotypes are known, divided over 7 groups (A-G).⁴¹ Adenoviruses are known to cause respiratory, gastrointestinal and ocular infections, but rarely lead to serious illness. The prototypic adenovirus, human adenovirus type 5 (HAdV-5) has a 35,938-bp genome containing 103-bp inverted terminal repeats (ITRs), which serve as origins of replication (figure 2a). The complex genome comprises 2 RNA polymerase III genes (*i.e.* VAI and VAII) and six early (E1a, E1b, E2a, E2b, E3 and E4), two intermediate (IX and IVa2) and five late (L1, L2, L3, L4 and L5) RNA polymerase II transcriptional units. The adenovirus particle is 80-90 nm in diameter and contains a genome that is packaged in a non-enveloped icosahedral capsid that contains 12 vertices from which trimers of the fiber protein extend and bind to target cell receptors. The main receptor by which the majority of human adenoviruses including HAdV-5 bind to cells is the Coxsackie virus B and adenovirus receptor (CAR).⁴² Other attachment receptors used by human adenoviruses include CD46, CD80, CD86, sialic acid and desmoglein-2.^{43,44} The most commonly used adenoviral vectors are based on HAdV-5. To apply adenoviruses as safe gene transfer vectors, they first need to be made replication-deficient. First-generation adenoviral vectors are therefore lacking early region 1 (E1), allowing insertion of up to 5 kb of foreign DNA. E1a products transactivate the promoters of other early regions and are necessary for viral replication, while E1b products are, amongst others, involved in the inhibition of apoptosis. E1-deleted vectors are produced in E1-complementing producer cell lines such as HEK 293⁴⁵, human embryonic retinoblasts (HER) 911⁴⁶ or PER.C6 cells.⁴⁷ First-generation adenoviral vectors often also have most of early region 3 (E3) deleted since this region is not needed for efficient viral replication in cultured cells and its deletion increases the capacity to harbor foreign DNA by 3 kb (figure 2b).⁴⁸ The main drawback of first-generation adenoviral vectors is that even in the absence of E1 proteins low levels of various adenovirus gene products are being produced, resulting in an immune response *in vivo*. To overcome this problem, vectors have been made in which multiple early genes have been deleted. Besides being devoid of E1 (and E3), these second-generation adenoviral vectors also lack (parts of) early region 2 (E2) and/or early region 4 (E4). The three E2 genes code for the adenovirus precursor terminal protein, polymerase and DNA-binding protein, which are essential for adenoviral DNA replication. Deletion of one or more E2 genes thus yields adenoviral vectors that are fully replication-deficient⁴⁹ and can only be produced in cell

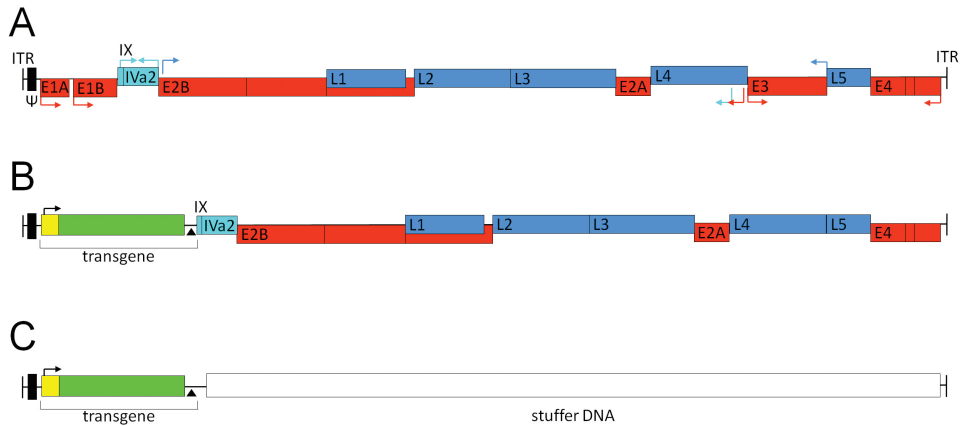


Figure 1.2: **A.** Schematic drawing of the HAdV-5 genome. The early (in red), intermediate (in light blue) and late (in dark blue) transcriptional units are indicated. ψ , packaging signal; ITR, inverted terminal repeat. **B.** Schematic drawing of a first-generation HAdV-5 vector genome lacking both the E1 and E3 region. **C.** Schematic drawing of a high-capacity or gutless HAdV-5 vector genome.

lines that complement the missing E1 and E2 functions. Of the E4 genes only E4orf3 or E4orf6 are required for adenovirus multiplication *in vitro*. Accordingly, either one of these genes should be retained in the genome of second-generation adenoviral vectors carrying deletions in E4 or should be provided in *trans*. Since some of the proteins encoded by these early genes are cytotoxic, the complementing cell lines that were developed in order to produce these vectors contain these genes under control of an inducible promoter.

It was thought that removal of these additional early region sequences would lead to a less severe immune response and a longer period of transgene expression in immunocompetent hosts. Studies comparing first- and second-generation adenoviral vectors have, however, yielded conflicting results.⁵⁰⁻⁵² In any case, even with second-generation adenoviral vectors low level expression of late adenovirus genes in target cells remains an issue. This led to the development of so-called gutless or helper-dependent adenoviral vectors, which are completely devoid of adenoviral genes and thus do not encode potentially cytotoxic and/or immunogenic adenovirus gene products (figure 2c). Gutless adenoviral vectors carry only the two ITRs and packaging signal from their parental virus and can harbor up to 37 kb of foreign DNA.⁵³ Their production typically depends on the (co-)infection of E1-complementing producer cells with a first-generation helper adenoviral vector to provide in *trans* all other adenovirus functions required for gutless vector DNA replication and encapsidation. As a consequence, crude gutless adenoviral vector preparations are heavily contaminated with helper adenoviral vector particles. Gutless adenoviral vector particles can subsequently be separated from adenoviral helper particles by ultracentrifugation on the basis of a difference in genome length/buoyant density. A major improvement in the production of gutless adenoviral vector was achieved by using the Cre/loxP recombination system. In this improved production system, the packaging signal of the helper adenoviral vector is flanked by loxP sites and the producer cells are

constitutively expressing the bacteriophage P1 cre gene.⁵⁴ Recognition of the loxP sites by the Cre recombinase leads to the selective removal of the packaging signal from most helper adenoviral vector genomes, which prevents their packaging in adenovirus capsid and hence greatly reduces the percentage of helper adenoviral vector particles in gutless adenoviral vector preparations. Gutless adenoviral vectors may hold promise for future gene therapy targeting the heart. The dissemination of HAdV-5 vectors through dense tissues is however poor and needs to be improved to efficiently target the heart. The use of fibers from other adenovirus serotypes or capsid modifications may enhance cardiac transduction. One possibility is the use of desmoglein-2-binding fibers present on some of the adenovirus serotypes from group B, as these fibers are significantly shorter than those of HAdV-5. Moreover, binding to desmoglein-2 led to the opening of intercellular junctions, which could further improve the spread of adenovirus vector particles through the heart.⁴⁴

1.4.3 Adeno-associated virus vectors

Adeno-associated virus (AAV) was first observed in an adenovirus stock.⁵⁵ Without a helper virus like adenovirus or herpesvirus, AAV replication is very inefficient in most cell types except for keratinocytes. Because of this, AAV is placed in a separate genus designated *Dependovirus* within the family *Parvoviridae*.⁵⁶ AAV has a non-enveloped icosahedral capsid of about 20 nm in diameter containing a 4.7-kb single-stranded DNA genome. The genome consists of two genes (*rep* and *cap*) flanked by ITRs, which, in the case of AAV serotype 2 (AAV-2), are 145 bp long (figure 3a). The *rep* gene encodes four enzymatic proteins involved in replication, encapsidation and host chromosomal DNA integration of the viral genome; *cap* encodes three structural proteins that make up the AAV capsid. The ITRs serve as origins of replication and contain the packaging signal.

Thus far, 13 serotypes and numerous variants of AAV have been described in primates.^{57,58} These serotypes vary mainly in the structure of their capsid proteins and are characterized as such because they do not cross-react with antibodies to other serotypes. The AAV serotypes show marked differences in cell tropism, which, amongst others, relate to differences in the utilization of attachment receptors on the surface of the target cells.⁵⁹ Although most human individuals contain AAV-specific antibodies⁶⁰, AAV has never been associated with human disease. In primate cells and in absence of a helper virus, the AAV genome is able to integrate into the host cell DNA at a specific locus on chromosome 19⁶¹ and maintained in a latent state. After infection with a helper virus, the AAV genome is excised and a lytic infection commences resulting in the production of new AAV particles.

The fact that AAV is non-pathogenic and replication-defective by nature has greatly stimulated its development into a viral vector. In AAV vectors, the ITRs are the only viral elements that are needed in cis. The *rep* and *cap* genes have to be provided in *trans* during vector production. The *rep* and *cap* genes are replaced by a transgene cassette of up to 4.5 kb in length. AAV vectors are most often produced by co-transfecting host cells with the AAV vector shuttle plasmid and two packaging plasmids, one containing the AAV *rep* and *cap* genes and one providing adenovirus helper functions.^{62,63} Alternatively, AAV vectors can be produced by using a single packaging plasmid expressing both AAV and

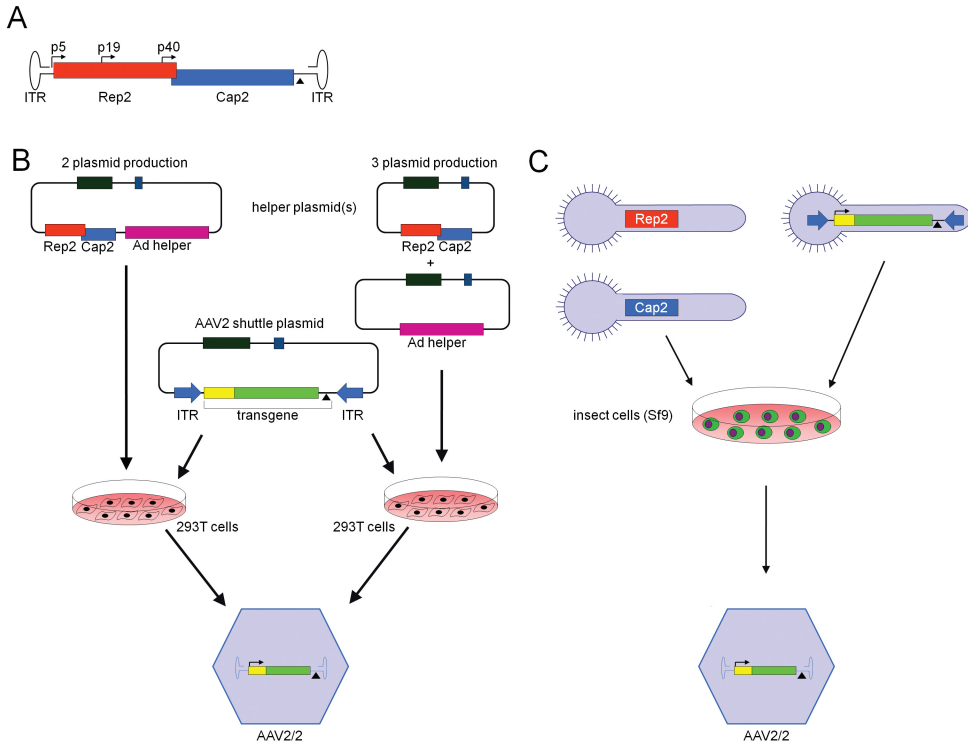


Figure 1.3: **A.** drawing of the AAV-2 genome. ITR, inverted terminal repeat. **B.** Overview of the two- plasmid or three-plasmid production of AAV vector in 293T cells. **C.** Production of AAV vector in insect cells using baculoviral vectors.

adenovirus helper genes (figure 3b).⁶⁴ AAV-2 vector genomes can be (cross-)packaged in capsids of other AAV serotypes. This process, which is also called pseudotyping, can be achieved by replacing the AAV-2 *cap* gene in the helper plasmid by that of another serotype. Pseudotyping allows the same AAV vector shuttle plasmid to be used to produce AAV vectors with different capsids and, as a consequence, different cell specificities.

A drawback of the use of AAV vectors for gene therapy is the slow transduction kinetics, and several rate-limiting steps in AAV vector transduction have been described.⁶⁵ After binding to the cellular receptor, the AAV vector particle is internalized via an endocytic process. Inside the endosome, the viral capsid has to be properly processed to allow for subsequent intracellular trafficking and endosomal escape. During this process, proteosomal degradation of viral particles may significantly reduce transduction. The AAV vector particle is then transported into the nucleus, where it is uncoated. Differences between the capsids of AAV serotypes cause some serotypes to be more affected by these processes than other serotypes.⁶⁶ A final rate-limiting step is that the single-stranded DNA genome first needs to be converted to double-stranded DNA to become transcriptionally active. This process can be bypassed by the use of self-complementary (sc)

AAV vectors that can fold on to themselves.⁶⁷ These vectors display increased speed of transduction *in vitro* and *in vivo*.⁶⁸ However, due to their specific design the packaging capacity of scAAV vectors is half that of conventional AAV vectors. scAAV vectors can thus only be used for the delivery of small transgenes. Single-strand to double-strand conversion is inhibited by the protein FKBP52 that binds to a specific sequence in the ITR. Dephosphorylation of FKBP52 by T-cell protein tyrosine phosphatase (TC-PTP) or protein phosphatase 5 (PP5) prevents the binding of FKBP52 to the ITR and improves second-strand DNA synthesis. A study by Jayandharan *et al.* demonstrated that transduction of a single-stranded AAV vector with a self-complementary AAV vector carrying either TC-PTP or PP5 increased transgene expression, indicating that this method can overcome this important rate-limiting step without the loss of transgene packaging capacity.⁶⁹

Although the production of high-titer ($\geq 10^{12}$ particles/mL) AAV vector preparations can be achieved using the aforementioned transient transfection methods, it is very hard to generate AAV vector particles in quantities sufficient for experiments in large animal models or for clinical applications. A major step towards large scale AAV vector production was the development of an insect cell-based production system.⁷⁰ This system uses recombinant baculoviruses encoding the AAV genes and the AAV vector genome to infect the insect-derived Sf9 cells (figure 3c). These cells are grown in suspension, thereby limiting the space required. Productions can be easily scaled up using bioreactors enabling the production of over 10^{14} vector particles per run.^{71,72}

1.5 Vector design

Viral vectors have shown their usefulness both *in vitro* and *in vivo*. The large variety of viral vectors that are available nowadays provide the opportunity to select, for each application, the vector system that is best suited. Nonetheless, it has become apparent that in many cases the way vectors are designed requires more refinement.⁷³ In most cases, the transgenes in viral vectors are cDNA-based and driven by strong constitutively active promoters, which are often derived from viruses. After transduction of target cells with such vectors it is impossible to regulate transgene expression levels, leading to non-physiological concentrations of transgene products and potentially adverse effects. For this reason, there is currently a focus on the development of vectors carrying transgenes with better control of their expression. This can be achieved by using tissue-specific promoters and other transcriptional and post-transcriptional control elements that allow endogenous regulation of transgene expression, or the implementation of inducible gene expression systems that can be controlled via exogenous stimuli.

Alternatively, the tropism of a vector can be modulated at the level of cell entry. In the case of AAV vectors this can easily be done by cross-packaging the vector genome in a capsid that allows efficient transduction of the target tissue. Other methods to better/more selectively transduce a particular tissue include the development of vectors with chimeric, chemically modified or mutated capsids.

1.5.1 (Tissue-specific) promoters

The most widely used promoter in viral vectors is the hCMV-IE promoter, since it drives very high levels of transgene expression in many mammalian cell types. The hCMV-IE promoter is, however, ubiquitously active, and is therefore less suited if transgene expression should be confined to either CMCs or CFBs. In addition, it was shown that in the lung transgene expression driven by the hCMV-IE promoter attenuates after one week, probable due to promoter silencing.⁷⁴ Besides the hCMV-IE promoter, several other viral promoters have been used. Of those promoters, the Rous sarcoma virus LTR (RSV-LTR) promoter has shown to be particularly active in CMCs.⁷⁵ This promoter could be useful when CMC-specific transgene expression without expression in the surrounding CFBs is desired.

In contrast to most viral promoters, cellular promoters are usually not silenced in cells expressing the corresponding gene and may provide transgene expression at a more physiological level. The promoters of the human eukaryotic translation elongation factor 1 alpha 1 (EEF1A1), ubiquitin C (UBC) and phosphoglycerate kinase 1 (PGK1) genes have been the promoters of choice in many studies, because they provide moderate to strong transgene expression in most human cell types over long periods of time.^{74,76,77} A potential drawback of these promoters is their lack of tissue specificity. Transgene expression in tissues other than the heart could lead to unwanted side-effects. In addition, body-wide transgene expression may increase the chance of an immune response against the transduced cells.⁷⁸ Promoters that limit transgene expression to the heart are to be preferred to lower this risk. Several CMC-specific genes have been identified. The promoters driving the genes encoding α -myosin heavy chain (MYH6), myosin light chain 2v (MYL2), cardiac troponin T (TNNT2) and cardiac Na⁺,Ca²⁺-exchanger (NCX1) have shown to display substantial activity only in CMCs.⁷⁹⁻⁸³ Moreover, it has been shown that MYL2 promoter activity and NCX1 promoter activity are confined to ventricular CMCs, conferring even more stringent specificity.⁷⁹⁻⁸⁰ A disadvantage of these tissue-specific promoters is that they are only weakly active. As a result, the transgene levels that are obtained using these promoters may not be sufficient to produce therapeutic effects. Incorporation of viral or cellular enhancer elements into the vector genome may help to generate adequate transgene levels using CMC-specific promoters.

1.5.2 Control elements

In the chromosome, gene expression is tightly regulated by control elements present upstream, within and/or downstream of the transcribed region of a gene.⁸⁴ To obtain physiological levels of transgene expression upon transduction with a viral vector, this vector would thus ideally contain the transgene in its own chromosomal context. Due to size limitations, this is often not feasible in the most commonly used viral vector systems. It would be possible, however, to incorporate individual cellular transcriptional control elements into the vector genome provided that such elements do not render the vector genome unstable and/or inhibit vector production as is often the case for retroviral vectors with their single-stranded RNA genomes. Of all vector systems mentioned, gutless adenoviral vectors are most suitable for the transfer of complete mammalian genes as they can

accommodate long stretches of foreign DNA in their double-stranded DNA genomes.

Control elements can either regulate the level of transcription or act post-transcriptionally. Transcription is dependent on the accessibility of the promoter to transcription factors. While large parts of the chromosomal DNA are packed in dense heterochromatin, transcriptionally active genes are located in regions of "open" euchromatin. Enhancer elements that have been implicated in retaining areas of accessible chromatin are known as locus control regions (LCRs) or matrix/scaffold attachment regions (MARs). Although the exact mechanisms by which these enhancers function is not completely understood, it has been shown that these regions cause hyperacetylation of the histones associated with the DNA and recruit parts of the transcription machinery, such as RNA polymerase II.^{85,86} Incorporation of these elements in the DNA of viral vectors has yielded promising results. Transduction of murine bone marrow cells with either lentiviral or AAV vectors containing LCR elements resulted in sustained transgene expression after integration of the vector DNA.^{87,88} In addition, MARs have been shown to increase transgene expression, although this effect is vector-dependent.⁸⁹ Apart from their effects in a chromosomal context, LCRs and MARs have also been shown to improve persistence and expression of transgenes incorporated in episomal plasmid vectors.⁹⁰⁻⁹² These elements could, therefore, also be useful in non-integrating vectors, like AAV or adenoviral vectors.

Besides these enhancer elements that are able to boost transgene expression at the transcriptional level, control elements that are involved in post-transcriptional regulation can be incorporated in the transcribed DNA. It has been established that inclusion of the 5' or 3' untranslated regions (UTRs) of a gene improves expression levels⁹³⁻⁹⁴, presumably by (1) stabilizing the structure of the transcript to prevent degradation, and (2) enhancing mRNA processing. One commonly used RNA element that can increase expression in many different contexts is the post-transcriptional regulatory element (PRE) from either human hepatitis B virus (HBV) or woodchuck hepatitis virus (WHV).^{95,96} Although the exact working mechanism of this element is not known, it appears to reduce transcriptional read-through and to modulate pre-mRNA processing as well as the nuclear export and translation of mRNA.⁹⁷⁻⁹⁹ Several studies using various vector systems have shown an upregulation of protein expression from PRE-containing transcripts compared to transcripts without such an element.⁹⁹⁻¹⁰¹ Together, these data indicate that the incorporation of a PRE in viral vectors targeting cardiac tissue could help improve the often low expression levels of transgenes driven by CMC- or fibroblast-specific promoters.

Another innovative strategy to obtain tissue-specific transgene expression uses endogenous microRNAs (miRNAs) to specifically silence transgene expression in non-target cells.¹⁰² miRNAs are short (mostly 21- or 22-nucleotide-long) non-coding RNAs that inhibit mRNA translation and/or induce mRNA decay by binding to complementary sequences in their target RNAs.¹⁰³ Over 1400 miRNA genes have thus far been identified in humans, but it is estimated that many more miRNAs are present in the genome.¹⁰⁴ These miRNAs have been implicated in many physiological and pathological processes, such as cardiac development, myocardial hypertrophy and heart failure.^{105,106} The knowledge that the expression of many miRNAs is to some extent tissue-specific has been used to design gene therapy vectors containing target sequences of miRNAs that are present in tissues which should **not** be targeted. This principle was shown to be effective *in vivo* after systemic delivery of a lentiviral vector containing a target sequence of

a miRNA expressed in cells of the hematopoietic lineage.¹⁰⁷ In this experiment, transgene expression in hematopoietic cells was suppressed, whereas expression in non-hematopoietic cells was unaffected. Likewise, this principle could be applied to gene therapy approaches, in which a certain heart cell type is targeted. Several miRNAs have been identified that are (cardiac) muscle cell- or fibroblast-specific. For example, miRNA-1, -133 and -206 are expressed in myocytes from all striated muscle, while miRNA-208 has been shown to be CMC-specific.^{108,109} In contrast, some miRNAs, such as miRNA-21 and -29 are preferentially expressed in CFBs, although CMCs also express low levels of these miRNAs.^{110,111} With respect to gene therapy of damaged hearts, miRNAs that are downregulated upon injury or disease deserve attention. Incorporation of target sequences of such miRNAs into the transgene would limit its expression to diseased cells lacking those miRNAs, while transgene expression in healthy tissue would be inhibited. Data from recent studies indicate that miRNA-133 and miRNA-590 target sequences could achieve this goal.^{112,113}

1.5.3 Vector capsid

Another method by which the viral vector tropism can be controlled includes the modulation of the capsid proteins that are involved in target cell binding.¹¹⁴ During the production of viral vectors, the genes coding for the capsid proteins are typically provided in *trans* by a helper plasmid. The tropism of a vector can therefore be modified by changing the capsid-encoding genes in the helper plasmid. Pseudotyping viral vectors in this way is an easy and straightforward method to obtain some level of transductional specificity. Lentiviral vectors are generally pseudotyped with the VSV-G glycoprotein, since the receptor of this protein is expressed on the surface of many different mammalian cell types, including the four main cell types in the heart (see above).³⁹ Due to their broad tropism, however, VSV-G pseudotyped lentiviral vectors do not appear to convey any level of specificity when targeting the heart. Research aimed to pseudotype lentiviral vectors with glycoproteins derived from other viruses has resulted in the discovery of several glycoproteins with specificity to a variety of tissues.¹¹⁵ Nevertheless, a pseudotyped lentiviral vector selectively targeting CMCs has not yet been discovered.

Like lentiviral vectors, commonly used adenoviral vectors display a broad tropism. Although more than 50 different serotypes of human adenovirus are known, most of them enter the cell by binding to the ubiquitously present CAR. Adenoviral vectors derived from HAdV-5 have been used *in vitro* and *in vivo* in animal models to transduce CMCs with great efficiency.^{116,117} Healthy human CMCs, however, are only poorly transduced by adenoviral vectors and show no detectable CAR expression on their surface.¹¹⁸ Of the fiber-modified HAdV-5 vectors, those carrying fiber shaft and knob domains of human adenovirus serotype 19a transduce skeletal muscle relatively well.¹¹⁹ Whether this also holds for cardiac muscle needs further investigation. Given the poor adenovirus targeting of human CMCs, several strategies have been developed to genetically alter the tropism of adenoviral vectors by mutation instead of exchange of the receptor-binding fiber domains. One of the strategies that could improve cardiac targeting involves the introduction of peptide ligands into the fiber or replacing (part of) the fiber by other protein moieties. Additionally, mutating or removing the CAR-binding motif in the fiber reduces background

transduction of other tissues that would normally be targeted. The feasibility of these approaches has been shown in general¹²⁰, but such vectors have not been tested on cardiac cell types.

The different AAV serotypes display diverse cell tropisms, which facilitates the development of AAV vectors targeting specific tissues. AAV-2 vectors pseudotyped with capsids of AAV serotypes 6, 8 and 9 (*i.e.* so-called AAV-2/6, AAV-2/8 and AAV-2/9 vectors) better transduce rat or mouse hearts than those endowed with capsids of other serotypes.^{121,122} Additionally, AAV-2/8 and AAV-2/9 vectors transduce tissues other than cardiac tissue considerably less efficiently. Research with neonatal rat CMC and CFB cultures showed that these cell types are preferentially transduced by different AAV vector pseudotypes.¹²³ This creates the opportunity to specifically target one of the two cardiac cell types using AAV vectors. The research published by Qi *et al.* also demonstrated that there is a difference between results obtained *in vivo* and *in vitro*, as AAV-2/2 vectors efficiently transduced both CMCs and CFBs *in vitro*, but intracardiac injection of AAV-2/2 vectors only resulted in very poor cardiac transduction *in vivo*. Thus, *in vitro* data should always be verified *in vivo*. Another confounding factor may be the existence of species-specific differences in the transduction profiles of a particular viral vector. This has been shown to be the case for AAV-2/2 vectors that were used to transduce NIH 3T3 (mouse fibroblast cell line) cells and HeLa cells (of human origin).¹²⁴ The AAV-2/2 vectors were less efficient in entering the nucleus of mouse cells compared to the human cells, resulting in lower protein expression levels in mouse cells. To improve the tissue-specific targeting of AAV vectors, attempts have been made to alter the capsid structure using methods similar to those used for adenoviral vectors. A commonly used approach exploits the high regional amino acid sequence similarity between the structural proteins of different AAV serotypes to create chimeric capsids combining features of multiple AAV serotypes. By applying this method, Yang *et al.* produced a chimeric AAV vector containing capsid sequences of serotypes 1, 6, 7 and 8 that displayed increased cardiac tropism compared to AAV vectors with AAV-6 and AAV-9 capsids.¹²⁵ However, this increase was observed after systemic injection but not after direct intramyocardial injection, indicating that the effect is due to enhanced transcytosis through the endothelial lining of the vasculature. Alternatively, unwanted transduction of the liver by AAV-2/2 can be reduced by mutating two amino acids of the capsid.¹²⁶ Each of these capsid modifications contributes to more selective targeting of cardiac tissue by AAV vectors. By combining these approaches with knowledge obtained from future studies it may be possible to generate AAV vectors for the highly selective transduction of myocardium *in vivo*.

1.5.4 Controllable systems

By applying the appropriate tissue-specific promoter, control elements, miRNA target sequences and/or capsid or envelope proteins, a specific cardiac cell type may be targeted. Apart from tissue-specific transgene expression, it might be needed to temporally regulate transgene expression in the target cell if the transgene product is required only for a short period of time and sustained transgene expression may be harmful. In addition, some genes can be toxic for viral vector producer cells and need to be silenced during vector production.

The Tet-on/off system is the most widely used inducible gene expression system. It depends on the commonly used antibiotic tetracycline or its analogue doxycycline as regulator. The required tetracycline levels are non-toxic and tetracycline is rapidly metabolized, allowing tight regulation of transgene expression.¹²⁷ This system utilizes two main components derived from *Escherichia coli*: the tetracycline repressor protein (TetR) and the tet operator (tetO) sequence. In the absence of tetracycline, TetR binds to tetO sequences upstream of the promoters in the Tet operon, thereby inhibiting its transcription. After binding of tetracycline, TetR undergoes a conformational change and is released from the tetO sequences resulting in the derepression of the promoters in the Tet operon. Several versions of this system have been developed based on different TetR fusion proteins.¹²⁸ Of these systems, the most promising one, as it can be used with both pol II and pol III promoters and allows simultaneous controlled expression of both transgenes and short hairpin (sh) RNAs, uses a fusion protein of TetR and the Krüppel-associated box (KRAB) domain.¹²⁹ It allows for the control of genes within a 2-3 kb region as binding of KRAB to the DNA leads to histone deacetylation and DNA methylation¹³⁰ and has been shown to possess a short response time both *in vitro* and *in vivo*. A potential problem associated with TetR-based inducible gene expression systems is the immunogenicity of the TetR fusion proteins. Furthermore, incorporation of tetO sequences and a TetR fusion protein expression unit decreases the transgene capacity of viral vectors.

Steroid hormone receptors (SHRs) provide a means to control transgene activity at the post-transcriptional level. Many transcription factors contain steroid hormone binding domains. These transcription factors are present in the cytoplasm and only travel to the nucleus after binding of a hormone to their ligand-binding domain. By fusing the ligand-binding domain of an SHR to a nuclear protein (domain) of interest, the activity of the latter becomes hormone-dependent.¹³¹ A drawback of this regulatory system is that endogenously present hormones may also activate the transgene product and that exogenously administered ligands can have many side-effects. The use of mifepristone and a mutated progesterone receptor may overcome many of these issues.¹³² Alternatively, the responsiveness of the transgene product to endogenously present hormones may be decreased by using a mutated version of the estrogen receptor which retains its ability to bind 4-hydroxytamoxifen.¹³³ For a more comprehensive overview of inducible gene expression systems, see *e.g.* Weber *et al.*¹³⁴

1.6 Modes of intervention

Genetic modification is used to either enhance or inhibit cellular processes. Overexpression of a particular protein or expression of a shRNA to inhibit the expression of a specific gene through RNA interference (RNAi) are most often used interventions.

1.6.1 Protein overexpression

Viral vector-mediated transgene expression is used for numerous purposes. It can be used as a treatment option in diseases that are caused by a loss-of-function mutation in a single gene. After the introduction of a recombinant gene coding for the functional protein, it can complement the genetic defect and alleviate the disease symptoms. In a

similar way, this method can be applied for various lines of cardiac research. Expression of a protein of interest enables the study of the involvement of this particular protein in signaling pathways or disease phenotypes. In case of proteins whose activity is regulated by post-translational modifications or interactions with co-factors, constitutively active mutants have been employed to avoid their functional shut-down by cellular control mechanisms. In addition, forced expression of cardiac transcription factor genes, such as myocardin, has been used to direct the differentiation of non-muscle cells towards cardiomyocytes.¹³⁵

1.6.2 Dominant-negative proteins

Functional knock-out of a gene can be achieved by expressing a variant of this gene that is mutated in one or more base pairs encoding a part of the protein that is essential for its function, *e.g.* a DNA- or protein-binding domain or a domain that exhibits catalytic activity. When such a protein variant is expressed at a high level, it may interfere with the function of the wild-type protein by competitive inhibition. This method of intervention is therefore called dominant-negative inhibition and it is used to appreciate the function of a protein by investigating the effects of its suppression. An advantage of this method over shRNA-mediated knock-down of gene expression (see below) is that generally a relatively high level of inhibition is achieved. A disadvantage of the use of dominant-negative proteins is the possibility that other factors, which bind the wild-type protein but are also important for other cellular processes, are sequestered by the high level of dominant-negative protein.

An alternative for using full-length dominant-negative proteins is the use of so-called microproteins to inhibit the function of proteins that normally function as dimers.¹³⁶ These microproteins only contain the dimerization motif but lack other domains, thus forming non-functional dimers.

1.6.3 Short hairpin RNAs

In a way similar to that described above for miRNAs, viral vector-delivered shRNAs are able to inhibit gene expression in a sequence-specific manner.¹³⁷ Viral vectors for RNAi typically contain a RNA polymerase III-based expression unit encoding a short RNA that is self-complementary and thus can fold onto itself to form a hairpin. This shRNA is processed into short interfering (si) RNAs, which are incorporated into an RNA-induced silencing complex (RISC). In this complex, the antisense siRNA binds specifically to the mRNA of its target gene, leading to mRNA degradation.

shRNAs are used for similar purposes as dominant-negative proteins, but the shRNA technology has the advantage that it can be used for all genes, irrespective of their function, and that for many genes validated shRNAs are commercially available. Nonetheless, even when a specific shRNA is a perfect match, the shRNA may result in modest knock-down of target gene expression. Furthermore, the shRNA may affect the expression of other genes which it should not target. To control for these so called off-target effects, the use of two different shRNAs targeting the same gene is preferred.

1.7 Research

The etiology of many cardiovascular diseases is often poorly understood. In order to develop therapies that cure these diseases, the underlying mechanisms need to be elucidated. Research into several important cellular processes that are involved in the development of serious health problems has profited from viral vector technology. Applications of genetic modification in different areas of cardiac research are discussed below.

1

1.7.1 Hypertrophy

Cardiac hypertrophy is defined as an abnormal increase in the size of CMCs, leading to an increased heart weight. In response to a higher workload, individual CMCs grow to enable the heart to generate more force. Cardiac hypertrophy may be a normal response to an increased workload, as is the case in professional cyclists and athletes, or during pregnancy. This type of hypertrophy is termed physiological, since it is generally harmless and reversible. In contrast, pathological hypertrophy can occur in response to pathological overloading of the heart and is a detrimental adaptation that may lead to cardiac dilatation, heart failure, arrhythmias and increased mortality.¹³⁸ Pathological hypertrophy can have many causes, some of which are associated with increased wall stress of one of the ventricles. Hypertension, aortic coarctation and stenosis, valvular regurgitation, myocarditis and the adaptation of non-infarcted myocardium in infarcted hearts are all common causes of pathological hypertrophy.

Cardiac hypertrophy research has focused on the distinction between physiological and pathological remodeling of the heart. Most information about the different pathways leading to the two types of hypertrophy has come from transgenic animal models. Results from those studies have led to the theoretical concept that physiological hypertrophy is mediated by signaling via the PI3K/AKT pathway, whereas protein kinase C and calcineurin are implicated in pathological hypertrophy.¹³⁹⁻¹⁴¹ Besides promoting physiological hypertrophy, the PI3K/AKT pathway has also been shown to reduce the hypertrophic response to pressure overload.¹⁴² Therefore, overexpression of one of the factors involved in the PI3K/AKT pathway is considered as a gene therapy approach to diminish pathological hypertrophy and its detrimental consequences.

Several gene therapy studies have been performed in animal models of cardiac hypertrophy that were aimed to diminish the hypertrophic stimulus rather than to reverse hypertrophy itself. Two studies targeted the renin-angiotensin system that is linked to hypertension.^{143,144} Kimura *et al.* used an AAV vector to deliver antisense DNA to suppress angiotensinogen in spontaneously hypertensive rats, while Pachori *et al.* used a retroviral vector to deliver angiotensin II type I receptor-antisense DNA to the TGR-(mREN2)27 hypertensive rat model. In both studies administration of the vector carrying the antisense DNA resulted in a delayed onset of cardiac hypertrophy. These studies illustrate the feasibility of gene delivery to treat cardiac hypertrophy by targeting the stimulus of cardiac overload.

1.7.2 Fibrosis

Cardiac fibrosis is associated with many cardiac diseases. It is caused by extensive fibroblast proliferation in combination with increased ECM deposition.³ In the healthy heart, fibroblasts and the ECM provide myocardium with proper passive mechanical properties. In addition, fibroblasts secrete growth factors that influence CMC function. Fibrosis in response to a pathological stimulus, however, is detrimental to cardiac homeostasis and may lead to arrhythmias, heart failure and sudden death.

The main goals of research into cardiac fibrosis are to (1) elucidate the mechanisms that cause fibroblasts to start proliferating excessively and become (proto)myofibroblasts, (2) determine the role of cardiac fibrosis in arrhythmias, and (3) explore ways to prevent or reverse cardiac fibrosis. Although there is still much to learn about the molecular basis of cardiac fibrosis, several studies have indicated that fibroblasts differentiate towards myofibroblasts under influence of certain hormones and cytokines.^{145,146} Myofibroblasts express several smooth muscle genes and are implicated in wound healing.¹⁴⁷ After wound repair, myofibroblasts undergo apoptosis, leaving behind patches of ECM. Under particular conditions, however, myofibroblasts persist and continue to deposit ECM, thereby contributing to fibrosis. Fate mapping experiments using genetically labeled cells have shown that these myofibroblasts originate from different cell lineages¹⁴⁸, but it remains to be seen whether these different origins have implications for the role of myofibroblasts in fibrosis and whether all sources of myofibroblasts are employed during cardiac fibrosis.

Cardiac fibrosis has many etiologies that determine the type of fibrosis, *i.e.* diffuse or focal. Specific treatment modalities may be required to treat these different types of fibrosis and the success of these therapies will depend on the cause and type of fibrosis. It is demonstrated that arrhythmias are reduced by inhibiting CFB proliferation *in vitro*⁶, but this still needs to be confirmed *in vivo*. Alternative strategies might be to either functionally uncouple fibroblasts from the surrounding CMCs or improve the conduction through the fibroblasts to prevent reentry. Although functional coupling between fibroblasts and CMCs has not been unequivocally established *in vivo*, fibroblasts are coupled to CMCs by gap junctions consisting of connexin 43 (Cx43) *in vitro*⁶. Uncoupling fibroblasts from surrounding CMCs by fibroblast-specific knock-down of Cx43 gene expression using a viral vector targeting fibroblasts and carrying a shRNA targeting Cx43 could therefore improve cardiac conduction and prevent arrhythmias. Alternatively, conduction through the fibroblasts could be enhanced by hyperpolarizing these cells and improving the coupling with surrounding CMCs. To this end, fibroblasts may be transduced with viral vectors carrying recombinant KCNJ2 and Cx43 genes.

1.7.3 Cardiac differentiation

The heart has a limited regenerative capacity. CMCs do not proliferate and are replaced by fibrotic tissue after myocardial damage. Intramyocardial injection of bone marrow cells has been shown to slightly improve the function of damaged hearts.¹⁴⁹⁻¹⁵¹ The injected cells, however, do not differentiate to CMCs and the observed improvement in cardiac function should thus be ascribed to other mechanisms, such as the secretion by the donor cells of paracrine factors that promote the survival of ischemic myocardium and

neovascularization. A major issue regarding cell therapy in the heart is the poor retention and survival of the transplanted cells. One way to improve donor cell engraftment is to transduce these cells before injection with a viral vector expressing a pro-survival factor or a factor that promotes migration and homing to the heart.¹⁵² An alternative strategy is to differentiate mesenchymal stem cells towards CMCs before injection. Results obtained *in vitro* have shown that some degree of differentiation can be achieved with extensive differentiation protocols, but full differentiation of these cells remains a problem.¹⁵³ Human embryonic stem cells (ESCs) and iPSCs can be differentiated into beating CMCs^{154,155}, and may therefore provide alternative cell sources for cardiac regeneration. Issues of potential immune rejection of non-autologous donor cells by the patient and the risk of teratoma formation by undifferentiated cells need to be addressed before these cells can be applied in the clinic. Human ESCs and iPSCs may also be used for *in vitro* drug screening and as cardiovascular disease models.^{24,156}

Viral vectors can be used to introduce genes encoding tissue-specific differentiation factors into cells to force them to differentiate into another cell type. In skeletal muscle research this principle has been successfully exploited by using the myogenic transcription factor MyoD to convert various cell types into skeletal muscle cells.¹⁵⁷ A single transcription factor that can induce cardiac differentiation has not been found and research has focused on differentiating primitive cells with a cocktail of factors. The molecular pathways and transcription factors that govern cardiac differentiation have been well described.^{158,159} Experiments in murine ESCs have shown that overexpression of the early transcription factor Mesp1 favored differentiation towards the cardiac lineage through direct regulation of cardiac-specific transcription factors.¹⁶⁰ Knock-down of the gene encoding Baf60c resulted in impaired heart formation in mouse embryos indicating a role of this chromatin remodeling factor in regulating cardiac differentiation.¹⁶¹ In addition, it was demonstrated that forced expression of Baf60c in combination with the cardiac transcription factors Gata4 and Tbx5 was sufficient to induce ectopic cardiac muscle formation in mouse mesoderm.¹⁶² Ieda *et al.* employed a MYH6 promoter-based reporter gene to determine the minimal set of transcription factors needed for differentiation of CFBs into CMCs.¹⁶³ Out of 14 investigated factors, they found Gata4, Mef2c and Tbx5 to be sufficient to induce cardiomyogenic differentiation. More primitive cells, such as fetal or embryonic stem cells, have proven to be more susceptible to cardiomyogenic differentiation than adult stem cells.¹⁶⁴ Furthermore, extra stimuli besides forced expression of cardiac transcription factor genes can be used. It has, for example, been shown that induction of BMP signaling promotes cardiac differentiation.¹⁶⁵

1.8 Clinical applications

The potential for gene-based therapies increases as a result of our growing knowledge of the molecular and cellular mechanisms underlying cardiovascular diseases. In animal models, numerous genes were targeted with good therapeutic results. Developments in viral vector technology have improved their safety and efficacy to a level that application in humans becomes feasible. This has prompted the initiation of several clinical gene therapy trials to treat a variety of cardiac illnesses.

1.8.1 Heart failure

In heart failure patients, the pump function of the heart is too weak to supply sufficient blood flow to meet the needs of the body. Gene therapeutic approaches have therefore aimed to improve this function by enhancing excitation-contraction coupling. The first gene therapy trial for patients with heart failure applied an AAV-1 vector to upregulate the expression of SERCA2a in CMCs.¹⁶⁶ SERCA2a is responsible for the uptake of Ca^{2+} ions from the cytoplasm to the sarcoplasmic reticulum after contraction, resulting in relaxation. Heart failure is typically associated with changes in the abundance and/or activity of SERCA2a¹⁶⁷ and in rodents with heart failure gene therapy with a first-generation SERCA2a-encoding adenoviral vector applied via the coronary arteries has been shown to improve the heart's pump function considerably.¹⁶⁸ SERCA2a gene delivery using AAV-1 proved to be safe and several patients with severe heart failure showed improvement upon treatment.^{166,169} Two new clinical trials using AAV-6 vectors to deliver the SERCA2a gene are planned.¹⁸ In addition, a clinical trial has started that aims to upregulate β -adrenergic signaling by injection of HAdV-5 vectors expressing adenylyl cyclase type 6.¹⁸ Adenylyl cyclase is the enzyme that, upon β -adrenergic receptor stimulation, increases intracellular cAMP concentration resulting in the activation of protein kinase A, which phosphorylates, among others, phospholamban. Phospholamban inhibits SERCA2a activity, but phosphorylation of phospholamban resolves this inhibition, leading to increased SERCA2a activity.

Apart from the genes that are used in current clinical trials, many other target genes involved in excitation-contraction coupling have been identified.¹⁸ An alternative strategy encompasses the prevention of CMC apoptosis using growth hormones or anti-apoptotic factors. The usefulness of human growth hormone and AKT1 gene transfer has been established in animal models^{170,171}, but the effects and safety of growth hormone gene transfer in humans are debated.¹⁷²

1.8.2 Angiogenesis

A major cause of heart failure is myocardial ischemia, an insufficient supply of blood to the myocardium caused by (partially) blocked coronary arteries. The formation of new blood vessels can increase the circulation in the affected region thereby improving myocardial performance. It has been shown in a canine model of ischemic heart disease and porcine models of hibernating myocardium and MI that the administration of angiogenic growth factors is beneficial for the heart.¹⁷³⁻¹⁷⁵ Angiogenic factors generally act in a paracrine fashion, which overcomes the need to transduce a large percentage of cells as long as their extracellular levels are sufficient. Most research has focused on two angiogenic factors: vascular endothelial growth factor (VEGF) and fibroblast growth factor-4 (FGF-4) and a number of clinical trials have been initiated using adenoviral vectors for transgene delivery. Several clinical studies have shown a positive effect of VEGF gene transfer on myocardial perfusion and angina symptoms in patients with myocardial ischemia.^{176,177} Adenoviral transfer of FGF-4 in patients with myocardial ischemia has produced mixed results.¹⁷⁸ Although a reduction in ischemic area and some improvement in an exercise stress test were observed in the AGENT1/2 trials, this could not be reproduced in the

larger AGENT3/4 trials.¹⁷⁹⁻¹⁸¹

The clinical benefit of gene transfer of angiogenic factors in patients with myocardial ischemia is still to be confirmed in larger studies, and several new clinical trials assessing the effect of VEGF and FGF-4 gene transfer have been initiated.¹⁸¹ In addition, other angiogenic factor genes, such as those encoding epidermal growth factor (EGF) and hypoxia inducible factor- α (HIF-1 α), are being considered as potential therapeutic transgenes. In order for angiogenic gene therapy to be effective, the transduction efficiency of the viral vectors that are used should be improved. Cardiotropic AAV vectors may prove to be a superior vector system compared to the currently used adenoviral vectors, as the AAV vector particles are smaller in size than adenovirus vector particles and therefore penetrate cardiac tissue better.

1.8.3 Arrhythmias

Contraction of CMCs is initiated by depolarization of their plasma membrane. CMC depolarization is the result of a complex interplay of several ion channels, particularly Na⁺, Ca²⁺ and K⁺ channels. Scar formation after MI may cause a disturbance of electrical conduction across the heart leading to phenomena such as slow conduction, conduction block and reentry, which serve as substrates for cardiac arrhythmias. Gene therapy may provide a means to prevent these arrhythmias by enhancing the capacity of the infarcted area to conduct an electrical signal. It was shown that forced expression of the transcription factor myocardin in ventricular scar fibroblasts enhances electrical conduction through these cells, via the upregulation of gap junction gene expression and increased gap junctional coupling.^{135,182} Furthermore, Lau *et al.* demonstrated that the introduction of HAdV-5 vectors encoding the skeletal muscle Na⁺ channel (SCN4A) into the border zone in a canine MI model led to an increase in conduction velocity and less ventricular arrhythmias.¹⁸³ Alternatively, the action potential duration of arrhythmic CMCs may be shortened by overexpressing certain inward rectifier K⁺ channels, thereby reducing the risk of early afterdepolarizations.¹⁸⁴

Another class of cardiac arrhythmias that could be treated using gene therapy is that caused by mutations in cardiac ion channel genes. Many genes are associated with arrhythmogenesis of which long-QT syndrome is the most prevalent.¹⁸⁵ Thirteen types of long-QT syndrome have so far been identified^{186,187}, each caused by the mutation of a gene encoding a protein involved in the generation of an action potential. In most cases, long-QT syndrome is caused by a mutation in one of the repolarizing K⁺ channel genes, especially KCNQ1 or KCNH2, which together comprise of >80% of mutations in long-QT patients. These mutations result in a loss-of-function of the ion channel and prolong action potential duration. Besides mutations in K⁺ channel genes, mutations in genes encoding depolarizing Na⁺ or Ca⁺ channels have been shown to cause long-QT syndrome. In most cases, these mutations result in a gain-of-function of these ion channels, thereby increasing the depolarizing current. Most of these patients suffer from long-QT type 3, which is caused by mutations in the SCN5A gene. In the case of a loss-of-function mutation, complementing CMCs with the wild-type version of the mutated gene may alleviate the symptoms caused by the disease. This principle was tested in a mouse model of long-QT syndrome.¹⁸⁸ These mice overexpress a truncated dominant-negative

Kv1.1 protein that interferes with Kv1.5-mediated K⁺ currents, leading to slower depolarization of the CMCs.¹⁸⁹ Forced overexpression of Kv1.5 by direct injection of adenoviral vectors into the myocardium shortened the action potential duration and reduced early afterdepolarizations.¹⁸⁹ Conversely, patients suffering from long-QT syndrome as a result of a gain-of-function mutation, *e.g.* in the SCN5A gene, might benefit from treatment with a gene expressing a dominant-negative version of Nav1.5 or with a shRNA that targets the mutant SCN5A gene in combination with an shRNA insensitive SCN5A gene encoding wild-type Nav1.5. However, the effect of such therapies has not been studied. The recent generation of an animal model with a gain-of-function mutation in SCN5A can aid research that aims to investigate the feasibility of gene therapy for long-QT type 3.¹⁹⁰

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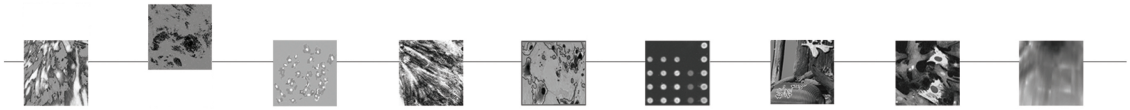
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Chapter 2

Genetic Complementation of Human Muscle Cells via Directed Stem Cell Fusion

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Molecular Therapy. 2008; 16:741-8.

Abstract

2

Duchenne muscular dystrophy (DMD) is caused by mutations in the X chromosome-linked *DMD* gene, which encodes the sarcolemma-stabilizing protein-dystrophin. Initial attempts at DMD therapy deployed muscle progenitor cells from healthy donors. The utilization of these cells is, however, hampered by their immunogenicity, while those from DMD patients are scarce and display limited *ex vivo* replication. Nonmuscle cells with myogenic capacity may offer valuable alternatives especially if, to allow autologous transplantation, they are amenable to genetic intervention. As a paradigm for therapeutic gene transfer by heterotypic cell fusion we are investigating whether human mesenchymal stem cells (hMSCs) can serve as donors of recombinant *DMD* genes for recipient human muscle cells. Here, we show that forced *MyoD* expression in hMSCs greatly increases their tendency to participate in human myotube formation turning them into improved DNA delivery vehicles. Efficient loading of hMSCs with recombinant *DMD* was achieved through a new tropism-modified high-capacity adenoviral (hcAd) vector directing striated muscle-specific synthesis of full-length dystrophin. This study introduces the principle of genetic complementation of gene-defective cells via directed cell fusion and provides an initial framework to test whether transient *MyoD* synthesis in autologous, gene-corrected hMSCs increases their potential for treating DMD and, possibly, other muscular dystrophies.

2.1 Introduction

Cell therapies are gaining much attention due to the perceived potential of these new treatment modalities to cure a wide variety of inherited illnesses. However, before non-hematopoietic cell therapies can enter the mainstream of medical practice, much research has still to be performed at both the fundamental and technological level. It is generally recognized that one of the main limitations in the development of cell-based therapies concerns the lack of methods that can reproducibly and quantitatively derive therapeutic cell types from cells that are either uncommitted to a particular fate or possess a phenotype different from the desired one. Thus, a great deal of effort is being put into devising procedures that can direct robust differentiation of target cells into cell types useful for the repair of specific tissues/organs.

Duchenne muscular dystrophy (DMD) afflicts ~1 in every 3,500 boys, making this lethal muscle-wasting disease the most prevalent hereditary muscle disorder. Strategies that aim at DMD therapy include (i) transplanting cells with myoregenerative capacity from healthy donors, (ii) restoring dystrophin synthesis from defective *DMD* transcripts by premature stop codon suppression or exon skipping, (iii) modulating the expression of nondefective endogenous genes such as upregulation of *utrophin* or downregulation of *myostatin*, and (iv) adding recombinant *DMD* genes via viral or nonviral gene transfer.^{1,2} The integration of gene- and cell-based approaches in DMD therapy increases the possibility of treating patients with their own cells. However, making autologous cell therapy for DMD viable requires much more research so as to establish (i) easily accessible cells with myoregenerative capacity, (ii) efficient gene carriers that can accommodate full-length, and thus fully functional, recombinant *DMD* genes, preferably under the control of striated muscle gene-specific promoters, and (iii) methods to disseminate the therapeutic cells throughout the musculature.

After birth, satellite cells and their descendants, referred to as myoblasts, constitute the natural reservoirs of muscle stem and progenitor cells, respectively. Accordingly, transplantation of allogeneic myoblasts has been at the forefront of DMD therapy research.³ Avoiding rejection of these cells requires strong and continuous immunosuppression,⁴ which increases the patient's risk of infections and is associated with nephrotoxicity. The deployment of gene-corrected autologous DMD myoblasts should obviate the need for immunosuppression but is hampered by the scarcity of functional satellite cells in DMD patients and their limited *ex vivo* expansion capacity.⁵ It is therefore important to investigate the myogenic potential of alternative cell types. Human mesenchymal stem cells (hMSCs) in particular are gaining much attention as therapeutic agents because they can easily be obtained from bone marrow or adipose tissue and can be greatly expanded *ex vivo*. Moreover, hMSCs can readily differentiate into specialized mesodermal cell types such as osteocytes, adipocytes, and chondrocytes. In addition, *in vitro* and *in vivo* experiments have demonstrated that at least a small fraction of these cells has myoregenerative capacity.⁶⁻⁹ We recently elaborated on these findings by showing that genetically modified hMSCs expressing recombinant full-length *DMD* genes can fuse with dystrophin-negative human muscle cells.¹⁰ Unfortunately, mosaic DMD myotubes derived from these heterotypic cell fusion events were rare indicating that few hMSCs display intrinsic myogenic fusion capacity.

In this study, we investigated whether (i) forced MyoD expression in hMSCs increases their proficiency to merge with DMD muscle cells and (ii) MyoD-stimulated hMSCs can be used as cellular vehicles for the delivery of recombinant genes encoding full-length dystrophin (Dys^{FL}).

2.2 Materials and methods

2.2.1 Cells

The hMSCs and human myoblasts used in this study were isolated from healthy individuals and DMD patients, respectively. The isolation, culture, and characterization of these cells have been detailed before.¹⁰⁻¹² The hMSCs were isolated from orthopedic surgery remnants from a healthy 38-year-old female. For all experiments, hMSCs of low passage number (<8) were employed. The human myoblasts used in this study were immortalized with *Bmi-1* and *TERT*.¹² Cultures of these diploid cells do not contain fibroblasts and show no obvious differences in myogenic differentiation capacity in comparison to primary myoblast cultures.

2.2.2 Viral vectors

The vesicular stomatitis virus G protein-pseudotyped self-inactivating human immunodeficiency virus type 1-based vectors CMVPRES (hereafter referred to as LV.eGFP),¹³ LV.hMyoD.eGFP, LV.mMyoD::ER^{T2}.eGFP, and LV.DsRed were produced in 293T cells with the aid of the packaging plasmids psPAX2 (Addgene, Cambridge, MA) and pLP/VSVG (Invitrogen, Breda, The Netherlands) as previously described.¹⁴ Lentivirus vector particles were purified by filtration through 0.45- μ m pore-sized cellulose acetate filters (Pall, Mijdrecht, The Netherlands) and centrifugation through 20% (wt/vol) sucrose (Merck, Nottingham, UK) cushions.¹⁴ The generation and purification of the Dys^{FL}-encoding and capsid-modified hcAd vector hcAd.F50.hrGFP.Dys^{FL} was carried out essentially as described for other hcAd-based vector systems using Per.tTA.Cre76 as producer cells¹⁵ and Ad.floxed^{1/2}.F50 as helper vector.¹⁶ The use of the latter reagent together with shuttle plasmid phcAd.Dys^{FL} yields Dys^{FL}-encoding hcAd particles whose fibers consist of knob and apical shaft domains from human Ad serotype 50 (species B) instead of the prototypic serotype 5 (species C). The resulting change in cell tropism overcomes the absence of the Coxsackie B virus and adenovirus receptor moiety on the surface of hMSCs allowing efficient gene transfer into these cells.^{10,11} Recombinant DNA techniques were performed using established methods. The complete nucleotide sequences of the shuttle plasmids used to generate LV.hMyoD.eGFP, LV.mMyoD::ER^{T2}.eGFP, LV.DsRed, and hcAd.F50.hrGFP.Dys^{FL} have been deposited in GenBank under accession numbers EU048697, EU048696, EU048695, and EU048698, respectively. The gene transfer activity of all vector stocks was determined by end-point titration on HeLa indicator cells using flow cytometric analysis of reporter gene expression as read-out. Consequently, vector titers are expressed in HeLa cell-transducing units per ml.

2.2.3 Establishment of cocultures of eGFP-labeled hMSCs and DsRed-marked DMD myoblasts

LV.DsRed was applied to DMD myoblasts in wells of 24-well culture plates at a multiplicity of infection of 30 HeLa cell-transducing units per cell. The 4-hour transduction procedure was carried out in 500 μ l of GM supplemented with 20 μ g/ml diethylaminoethyl-dextran sulfate (GE Healthcare, Leiden, The Netherlands). In parallel, hMSCs were transduced with LV.eGFP or with LV.hMyoD.eGFP as described in the Supplementary Materials and Methods, Supplementary Table S1. Three days later the cells were trypsinized and expanded in 25-cm² cell culture flasks (Greiner, Alphen aan den Rijn, The Netherlands). Two weeks after infection, 1.6×10^5 DsRed-marked DMD myoblasts were seeded in wells of 6-well culture plates alone or together with 4×10^4 hMSCs transduced either with LV.eGFP or with LV.hMyoD.eGFP. Four days later, the GM was replaced by DM. After incubation for 5 days in DM, the cocultures were analyzed by direct fluorescence microscopy.

2.2.4 Immunophenotyping

The surface antigen profile of human myoblasts, unmodified hMSCs and LV.mMyoD::ERT².eGFP-transduced hMSCs was determined by flow cytometry essentially as described before.^{11,17} All antibodies used were directly coupled to phycoerythrin and were purchased from Bioconnect, Huissen, The Netherlands (CD105), BD Biosciences (Breda, The Netherlands) (CD13, CD44, CD49e, CD73, and CD184) or Sanquin (Amsterdam, The Netherlands) (CD10, CD56, CD62P, and CD71). Isotype-matched, phycoerythrin-conjugated antibody controls from BD Biosciences were used to determine background fluorescence.

2.2.5 Analysis of skeletal muscle gene expression by IFM

hMSCs were transduced in triplicate with LV.eGFP or LV.hMyoD.eGFP as mentioned earlier and cultured for 16 days in GM, after which time, they were either exposed to DM or kept in GM for another 25 days. IFM was carried out with antibodies directed against desmin, skMHC-II, dystrophin, and MyoG as specified below.

2.2.6 Light microscopy and immunostainings

Phase-contrast microscopy, direct fluorescence microscopy, and IFM were performed with an Olympus IX51 inverse fluorescence microscope (Olympus, Zoeterwoude, The Netherlands). Images were captured by a ColorView II Peltier-cooled charge-coupled device camera (Olympus, Zoeterwoude, The Netherlands) and were archived using AnaLYSIS software (Soft Imaging Systems, Zoeterwoude, The Netherlands). In addition, direct fluorescence microscopy and IFM images of cells grown on glass cover slips were acquired by using a conventional Leica DM 5500 B fluorescence microscope (Leica, Rijswijk, The Netherlands) together with a CoolSnap K4 cooled charge-coupled device camera (Roper Scientific, Evry Cedex, France). The same specimens were also analyzed with a confocal Leica TCS SL laser scanning fluorescence microscope. Cells

were fixed with phosphate-buffered 4.0% (wt/vol) formaldehyde solution (pH 6.8-7.2; J.T. Baker; Deventer, The Netherlands) at room temperature for 30 minutes. After fixation, the cells were washed three times with phosphate-buffered saline (PBS) containing 10 mmol/l glycine (PBSG) and permeabilized with 0.1% (vol/vol) Triton X-100 (Sigma-Aldrich, Zwijndrecht, The Netherlands) in PBS for 5 minutes at room temperature. Following three washes with PBSG, aspecific antibody-binding sites were blocked by incubating the cells for at least 2 hours at room temperature with PBSG containing 5% 0.22- μ m filter-sterilized fetal bovine serum. Primary antibodies specific for desmin (clone D33; Dako, Heverlee, Belgium), skMHC-II (clone MY-32; Sigma-Aldrich, Zwijndrecht, The Netherlands), dystrophin (clone Dy8/6C5; Novocastra, Norwell, MA), MyoD (clone 5.8A; Dako), MyoG (clone F5D; Dako), and β -dystroglycan (clone 43DAG1/8D5; Novocastra, Norwell, MA) were diluted 1:50, 1:200, 1:20, 1:50, 1:50, and 1:40, respectively, in PBSG containing 5% 0.22- μ m filter-sterilized fetal bovine serum. The cells were incubated overnight with the primary antibodies at 4°C. Excess primary antibody was removed by three 5-minute washes with PBSG and the cells were labeled for 3-4 hours at 4°C with highly cross-absorbed, affinity-purified, AlexaFluor568-conjugated goat anti-mouse immunoglobulin G (Invitrogen, Breda, The Netherlands), diluted 1:500 in PBSG containing 5% 0.22- μ m filter-sterilized fetal bovine serum. The cells were again washed three times at room temperature with PBSG and their nuclei were stained by incubating for 10 minutes with 10 μ g/ml Hoechst 33342 (Invitrogen Breda, The Netherlands) in PBS, followed by four additional washes with PBS.

2.2.7 Analysis of mMyoD::ER^{T2} activity by IFM, heterotypic cell fusion assays and flow cytometry

After transduction with LV.mMyoD::ER^{T2}.eGFP at a multiplicity of infection of 5 HeLa cell-transducing units per cell, hMSCs were expanded for 2 weeks in GM. Parallel cultures of uninfected hMSCs served as controls. Next, the cells were kept for another 5 days in GM with or without 250 nmol/l of the synthetic drug 4-OHT (Sigma-Aldrich, Zwijndrecht, The Netherlands). Finally, uninfected and LV.mMyoD::ER^{T2}.eGFP-infected hMSCs were exposed for 72 hours to DM $\pm$ 250 nmol/l 4-OHT. Thus for a total of 8 days, cultures were either treated or not treated with 4-OHT. The effect of 4-OHT on the hMSCs was evaluated through desmin and skMHC-II immunostainings.

Twenty-nine days after infection, LV.mMyoD::ER^{T2}.eGFP-transduced hMSCs kept in GM were either exposed or not exposed to 4-OHT for an additional period of 8 days. Subsequently, 1×10^4 of untreated or 4-OHT-treated hMSCs derived from three independent cultures each were mixed with 5×10^4 DMD myoblasts. After 5 days in GM with or without 250 nmol/l 4-OHT, the cells were kept in 4-OHT-containing and drug-free DM, respectively. The frequency of mosaic myotubes in the presence and in the absence of 4-OHT was determined through fluorescence microscopy after the regular myoblast differentiation period. Finally, aliquots of hMSCs exposed to 4-OHT or kept in its absence, were subjected to flow cytometric analysis to determine cell-surface levels of CD10 and CD56.

2.2.8 Statistical analysis

Mean, SD, and SEM were computed using GraphPad Prism software (version 4.03). Student's *t*-test was used to compare data sets. $P < 0.05$ were considered significant.

2.3 Results

2.3.1 MyoD induces expression of skeletal muscle genes in hMSCs

Forced expression of a recombinant gene encoding the basic helix-loop-helix myogenic transcription factor MyoD, also known as Myf3, has been shown to activate skeletal muscle genes in various differentiated nonmuscle cell types.^{18,19} To investigate whether MyoD also upregulates expression of skeletal muscle genes in undifferentiated hMSCs, they were transduced with the bicistronic human immunodeficiency virus type 1-based vector LV.hMyoD.eGFP, which encodes human MyoD (hMyoD) and enhanced green fluorescent protein (eGFP), or with LV.eGFP, which only directs the synthesis of eGFP (Figure 1).

hMyoD was readily detected by immunofluorescence microscopy (IFM) to be exclusively found in the nuclei of LV.hMyoD.eGFP-treated hMSCs (Figure 2a). Next, through reverse transcription-polymerase chain reaction analysis, we searched for potentially hMyoD-responsive genes by targeting the RNA of the *desmin*, *Mef2C*, *MyoG*, *Myf5*, and *Myf6* genes. Figure 2b shows that *desmin* transcripts are already present in hMSCs transduced with LV.eGFP. *Desmin* gene expression is, nonetheless, upregulated after LV.hMyoD.eGFP infection. A much more striking induction was observed for *MyoG* and *Myf6* (Figure 2b). *Mef2C* transcripts, on the contrary, were not noticeably upregulated by *hMyoD* gene transfer since they were present in both hMSC populations at similar levels. Finally, *Myf5* messenger RNA was detected neither in MyoD-negative nor in MyoD-positive hMSCs (Figure 2b). We also tested the effect of ectopic *hMyoD* expression in hMSCs at the protein level. To this end, we performed desmin, fast-twitch skeletal myosin heavy chain-II (skMHC-II), dystrophin, and MyoG IFM on hMSCs transduced with LV.hMyoD.eGFP or LV.eGFP and cultured in growth medium (GM) or differentiation medium (DM) (Figure 3).

With the exception of dystrophin, which could not be detected regardless of the experimental setup used, the frequency of desmin-, skMHC-II-, or MyoG-positive cells was much higher in LV.hMyoD.eGFP-transduced hMSCs than in LV.eGFP-infected cells (Figure 3a). In fact, skMHC-II and MyoG were only detected in hMSCs transduced with LV.hMyoD.eGFP and not in cells infected with the control vector (Figure 3a). Quantification of desmin-, skMHC-II-, or MyoG-positive hMSCs revealed that myogenic induction was highest in cells exposed simultaneously to LV.hMyoD.eGFP and DM (Figure 3b). Moreover, LV.hMyoD.eGFP-treated cultures contained cells with a more slender morphology than those exposed to LV.eGFP and sporadically displayed syncytia with up to three nuclei (Figure 3a; white arrowheads). Thus, forced *hMyoD* expression can, at least to some extent, orchestrate the myogenic conversion of hMSCs.

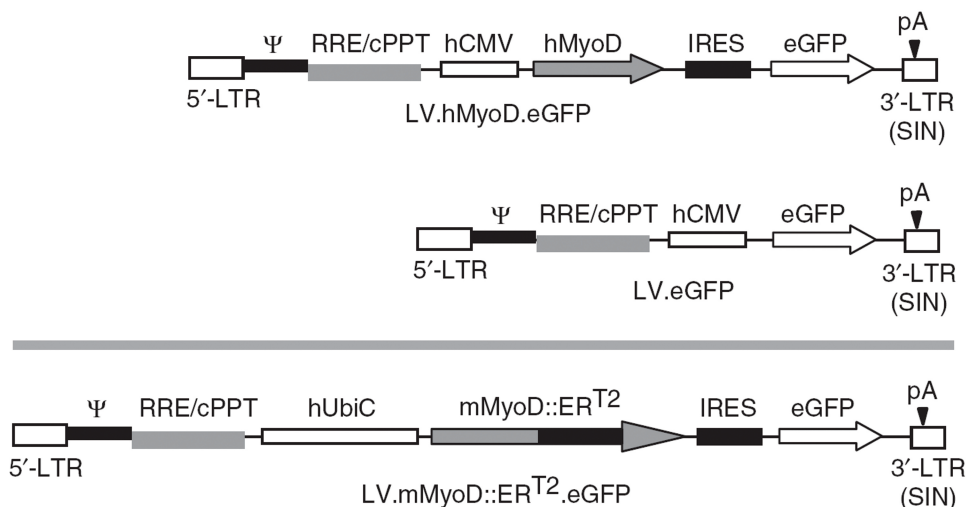


Figure 2.1: Genome structure of LV.hMyoD.eGFP, LV.eGFP and LV.mMyoD::ER^{T2}.eGFP. LV.hMyoD.eGFP encodes the human basic helix-loop-helix myogenic transcription factor MyoD (hMyoD) and, through the IRES, also directs the synthesis of eGFP. Expression of *hMyoD* and *eGFP* is driven by the human cytomegalovirus immediate-early gene promoter (hCMV). LV.mMyoD::ER^{T2}.eGFP encodes eGFP and a conditionally active version of murine MyoD (mMyoD) designated mMyoD::ER^{T2}. The *mMyoD::ER^{T2}* gene codes for a fusion product of mMyoD and a modified version of the ligand-binding domain of human estrogen receptor 1 named ER^{T2}. The superscript T2 denotes the presence of two point mutations in comparison to the original *mMyoD::ER* gene³⁶ that permits interaction of the fusion product with the synthetic hormone 4-hydroxytamoxifen (4-OHT) and not with natural estrogens. The ER^{T2} sequence in *mMyoD::ER^{T2}* is located between the coding regions for the bHLH and activation domains of mMyoD (solid box within gray arrow). *mMyoD::ER^{T2}* and *eGFP* expression are under the control of the human *ubiquitin C* gene promoter (hUbiC). In the absence of 4-OHT mMyoD::ER^{T2} is localized predominantly in the cytoplasm, while in its presence the protein is transported to the nucleus to exert its biological effects. ψ , packaging signal; eGFP, enhanced green fluorescent protein; IRES, encephalomyocarditis virus internal ribosomal entry site; LTR, long-terminal repeat; pA, polyadenylation signal; RRE/cPPT, Rev-responsive element and central polypurine tract; SIN, self-inactivating LTR (LTR with a deletion in the U3 region).

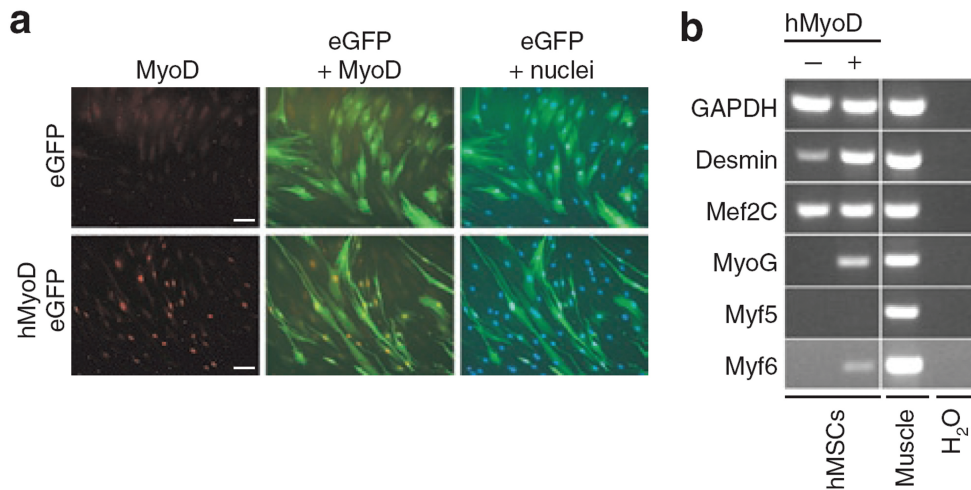


Figure 2.2: Analysis of human mesenchymal stem cells (hMSCs) transduced with LV.eGFP or LV.hMyoD.eGFP by fluorescence microscopy and reverse transcription-polymerase chain reaction (RT-PCR). (a) MyoD immunostaining (left and middle panels) and eGFP direct fluorescence microscopy (middle and right panels) of hMSCs transduced with LV.eGFP (upper panels) or with LV.hMyoD.eGFP (lower panels). The red human MyoD (hMyoD)-specific signal was combined with the green eGFP-specific fluorescence in the middle panels whereas in the right panels the eGFP-specific signal was overlaid with the blue fluorescence derived from hMSC nuclei labeled with the fluorochrome Hoechst 33342. Bars = 200 μ m. (b) RT-PCR analysis of hMyoD-reprogrammed hMSCs. Total cellular RNA from LV.eGFP- or LV.hMyoD.eGFP-transduced hMSCs maintained in growth medium (GM) was extracted 10 days after infection, reverse-transcribed and PCR amplified. Targeted transcripts are specified on the left. Skeletal muscle RNA from a human adult and water served as positive and negative controls, respectively. eGFP, enhanced green fluorescent protein; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

2.3.2 MyoD-transduced hMSCs have superior myogenic fusion capacity

We hypothesized that after MyoD-dependent reprogramming, hMSCs would become better fusion partners for neighboring DMD myoblasts undergoing differentiation. To test this hypothesis, we deployed an *ex vivo* system based on cocultures of hMSCs and DMD myoblasts.¹⁰ Before being mixed with DMD myoblasts, hMSCs were eGFP-labeled via infection with LV.hMyoD.eGFP or LV.eGFP. After the cocultures had been established, the cells were exposed to DM. Differentiated myotubes were detected by skMHC-II immunostaining (Figure 4a).

Merging of the eGFP- and skMHC-specific signals derived from cocultures initiated with MyoD-negative hMSCs revealed a few areas containing colabeled mature myotubes (data not shown), whereas in those containing hMyoD-positive hMSCs, multinucleated structures coexpressing eGFP and skMHC-II were ubiquitous (Figure 4a). Quantification of colabeled syncytia showed that ectopic hMyoD expression in hMSCs leads to a large increase in the frequency of mosaic myotubes (Figure 4b).

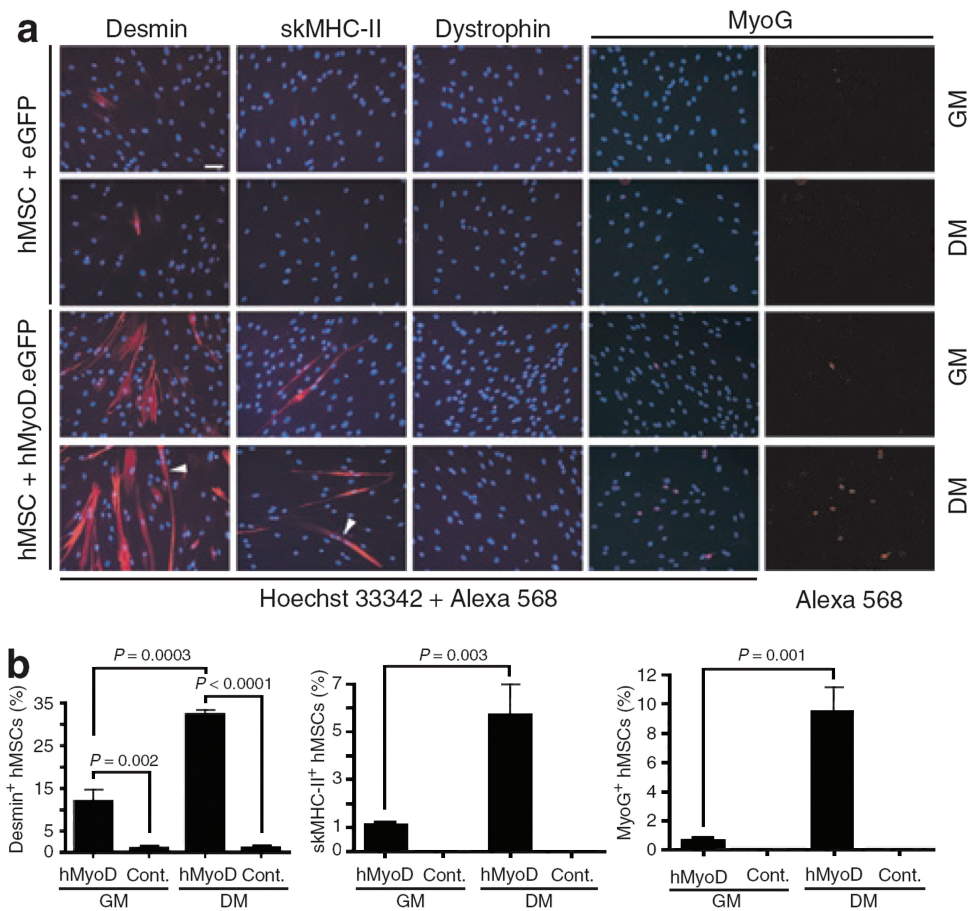


Figure 2.3: Immunodetection and quantification of human mesenchymal stem cells (hMSCs) synthesizing skeletal muscle proteins following human MyoD (hMyoD) gene delivery. (a) Analysis of hMyoD-directed differentiation of hMSCs by desmin, skeletal myosin heavy chain-II (skMHC-II), dystrophin and MyoG immunofluorescence microscopy. hMSCs were transduced in triplicate with LV.eGFP (upper two rows) or LV.hMyoD.eGFP (lower two rows). After a 16-day culture period the cells were either kept in growth medium (GM) or were exposed to differentiation medium (DM) for another 25 days. Nuclei were visualized with the aid of Hoechst 33342 counterstaining. (b) Frequency of hMSCs expressing desmin, skMHC-II, or MyoG in the cell cultures described in a was determined by counting cells positive for each marker protein present in randomly selected fields. Cumulative data are expressed as means $\pm$ SD. Relevant P values are also indicated. hMyoD, LV.hMyoD.eGFP-transduced hMSCs; Cont., LV.eGFP-transduced hMSCs. eGFP, enhanced green fluorescent protein.

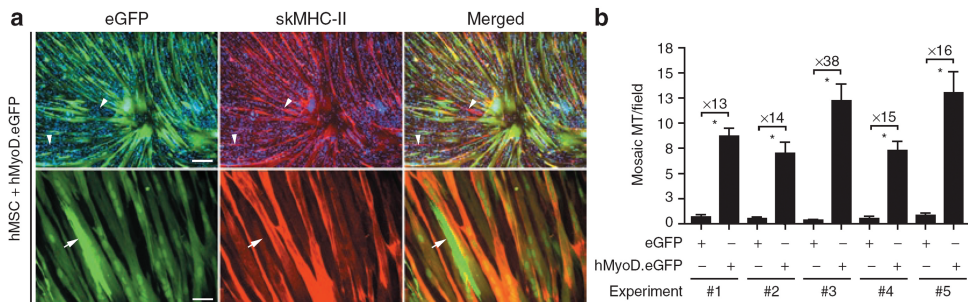


Figure 2.4: Light microscopy analysis of cocultures of Duchenne muscular dystrophy (DMD) myoblasts and enhanced GFP (eGFP)-tagged human mesenchymal stem cells (hMSCs). (a) eGFP direct fluorescence microscopy (left panels) and skeletal myosin heavy chain-II (skMHC-II) immunofluorescence microscopy (middle panels), 4 days after induction of differentiation. Arrowheads (upper panels) point at two examples of mature myotubes without a detectable contribution by hMSCs. Arrows (lower panels) point to an eGFP-positive hMSC among eGFP- and skMHC-II-labeled myotubes. The presence of skMHC-II in the eGFP-labeled syncytia as observed by overlaying both signals (right panels) indicates that they represent mature mosaic myotubes (MTs). The blue fluorescence in the upper panels corresponds to nuclei labeled with Hoechst 33342. The scale bars in the upper and lower panels correspond to 500 and 100 μm , respectively. (b) Frequency of eGFP- and skMHC-II-labeled MTs present in randomly selected fields of cocultures of DMD myoblasts and LV.hMyoD.eGFP- or LV.eGFP-transduced hMSCs from five independent experiments. Graph shows mean $\pm$ SEM from groups of a minimum of 25 and a maximum of 42 randomly selected fields. * $P < 0.0001$ versus LV.eGFP-transduced hMSC controls. The fold increase in mosaic MTs resulting from constitutive hMyoD expression in the hMSCs is indicated above each pair of bars. hMyoD, human MyoD.

The potential long-term deleterious effects of constitutive and high-level *MyoD* expression on myotubes or myofibers have not been thoroughly investigated. We therefore generated the bicistronic human immunodeficiency virus type 1-based vector LV.mMyoD::ER^{T2}.eGFP encoding eGFP and a version of murine *MyoD* (designated mMyoD::ER^{T2}) whose transactivating activity is controlled by the clinically applicable small-molecule drug 4-hydroxytamoxifen (4-OHT) (Figure 1). The functionality of the mMyoD::ER^{T2} construction was tested by transducing hMSCs with LV.mMyoD::ER^{T2}.eGFP and culturing the modified cells in the presence or absence of 250 nmol/l 4-OHT. IFM results depicted in Figure 5a reveal a clear 4-OHT-dependent upregulation of desmin and skMHC-II levels in cells exposed to LV.mMyoD::ER^{T2}.eGFP.

To further establish the functionality of the 4-OHT-controlled mMyoD::ER^{T2} fusion protein, we performed flow cytometric analysis of LV.mMyoD::ER^{T2}.eGFP-transduced hMSCs that had been cultured in medium with or without 250 nmol/l 4-OHT. For the purpose of comparison, we first screened human myoblasts and hMSCs by flow cytometry with a panel of antibodies to identify molecules differentially displayed on the surface of these two cell types. The resulting histograms revealed that CD10 (also known as CALLA) and CD56 (also known as NCAM) were abundantly decorating the surface of myoblasts (Figure 5b) while being very scarce on the plasma membrane of hMSCs (data not shown).

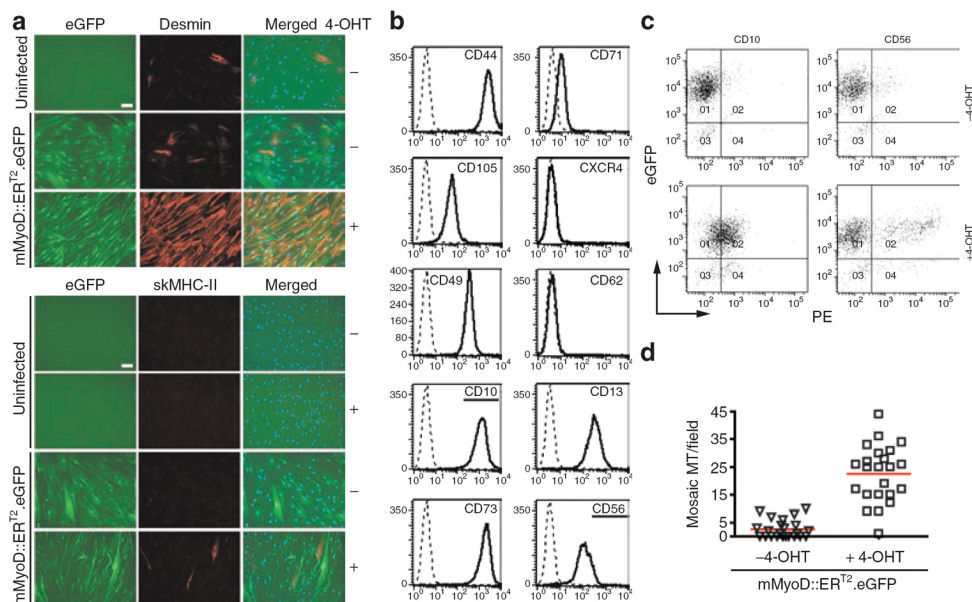


Figure 2.5: Effects of LV.mMyoD::ERT2.eGFP on human mesenchymal stem cells (hMSCs) cultured in the presence or absence of 4-hydroxytamoxifen (4-OHT). (a) Desmin and skeletal myosin heavy chain-II (skMHC-II) immunofluorescence microscopy of uninfected or LV.mMyoD::ERT^{T2}.eGFP-transduced hMSCs exposed (+) or not exposed (-) to 250 nmol/l 4-OHT. Bars = 200 μ m. (b) Flow cytometric analysis of human myoblasts identifying CD10 and CD56 (underlined) as antigens differentially expressed on the surface of hMSCs and human myoblasts. Targeted antigens are specified in the corresponding histograms (representing 10^4 events each). γ -Axis, cell counts; x -axis, phycoerythrin (PE) fluorescence. Broken and solid lines, control and specific antibodies, respectively. (c) Dot plot representation of CD10 and CD56 expression on the surface of LV.mMyoD::ERT^{T2}.eGFP-modified hMSCs cultured in the absence (-) or presence (+) of 250 nmol/l 4-OHT. γ -Axis, eGFP fluorescence; x -axis, PE fluorescence. (d) Frequency of mosaic myotubes (MTs) present in randomly selected fields ($n = 23$) of mature cocultures of Duchenne muscular dystrophy (DMD) myoblasts and LV.mMyoD::ERT^{T2}.eGFP-modified hMSCs maintained in medium without (-) or with (+) 250 nmol/l 4-OHT. eGFP, enhanced green fluorescent protein; ER^{T2}, 4-OHT-responsive variant of the ligand-binding domain of human estrogen receptor; mMyoD, murine MyoD.

consistent with a previous report.¹⁰ At this point, it is worth noting that, CD56 and CD10 have both been implicated in homotypic myoblast adhesion and fusion.^{20,21} Significantly, flow cytometric analysis showed a clear 4-OHT-dependent increase of CD10 and CD56 on the surface of LV.mMyoD::ER^{T2}.eGFP-transduced hMSCs (Figure 5c). Interestingly, these two factors were upregulated to different extents. While most MyoD-stimulated hMSCs expressed CD10, the corresponding cell-surface levels were relatively low. A much smaller fraction of MyoD-reprogrammed hMSCs expressed CD56 on their surface but the amount of this protein on a per cell basis was higher (Figure 5c). Regardless, one can conclude that mMyoD::ER^{T2} induces the expression on the surface of hMSCs of molecules present on the plasma membrane of bona fide muscle cells. Collectively, these data indicate that mMyoD::ER^{T2} exhibits myogenic reprogramming activity and is 4-OHT-responsive. Next, cocultures of DMD myoblasts and LV.mMyoD::ER^{T2}.eGFP-modified hMSCs were established and incubated with or without 4-OHT. After the regular differentiation period, the frequency of mosaic myotubes was on average much higher in cocultures exposed to 4-OHT than in those that did not receive the drug (Figure 5d). These data are consistent with the results obtained with the constitutively active form of hMyoD.

To definitely demonstrate that the eGFP-labeled myotubes arose through heterotypic cell fusion between hMyoD-stimulated hMSCs and DMD myoblasts and were not the result of homotypic cell fusion of MyoD-reprogrammed hMSCs under coculture conditions, LV.hMyoD.eGFP-transduced hMSCs were mixed with DMD myoblasts that had been labeled with a variant of the *Discosoma* sp. red fluorescent protein (DsRed).²² After the regular differentiation period, direct fluorescence microscopy was performed. Most areas in control cultures initiated with hMyoD-negative and eGFP-positive hMSCs together with DsRed-marked DMD myoblasts did not contain eGFP- or double-labeled myotubes (Figure 6, left column). In a few discrete regions, however, doubly marked myotubes could clearly be discerned in addition to the abundant DsRed-labeled syncytia (data not shown). On the contrary, the cocultures initiated with hMyoD- and eGFP-positive hMSCs together with DsRed-labeled DMD myoblasts contained many multinucleated structures expressing both *eGFP* and *DsRed* but again no large myotubes containing only the eGFP label were observed (Figure 6, right column). Co-localization of both markers in hybrid myotubes was confirmed by confocal microscopy. We hence conclude that, as previously shown for naïve hMSCs,¹⁰ MyoD-stimulated hMSCs participate in myogenesis predominantly via heterotypic cell fusion originating as end-products mosaic myotubes.

2.3.3 Recombinant DMD gene delivery into DMD myotubes by MyoD-transduced hMSCs

Taken together, the results described earlier demonstrate that forced *MyoD* expression in hMSCs (i) induces expression of skeletal muscle genes in these cells and (ii) increases significantly their recruitment by differentiating human myoblasts. These findings provide a rationale for exploiting MyoD-reprogrammed hMSCs as delivery vehicles of genes/proteins into diseased skeletal muscle. Toward this end, we investigated the use of *MyoD*-transduced hMSCs as carriers of full-length recombinant *DMD* (Dys^{FL}) genes into dystrophin-defective human muscle cells. Additionally, we studied whether Dys^{FL}-transduced hMSCs produce sufficient dystrophin to restore the proper localization

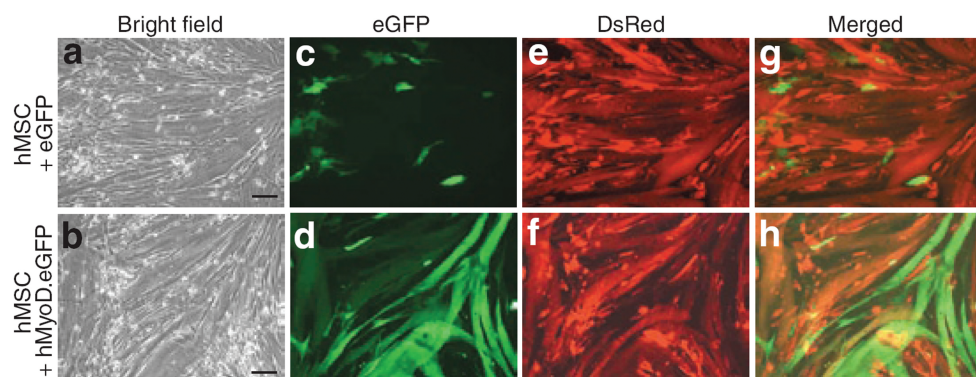


Figure 2.6: Live cell imaging of mature cultures of *Discosoma* sp. red fluorescent protein (DsRed)-tagged Duchenne muscular dystrophy (DMD) myoblasts and enhanced green fluorescent protein (eGFP)-labeled human mesenchymal stem cells (hMSCs). (a) and (b) Phase-contrast microscopy, (c) and (d) direct eGFP, and (e) and (f) DsRed fluorescence microscopy of DsRed-tagged DMD myoblasts mixed with human MyoD (hMyoD)-negative (left panels) or hMyoD-positive (right panels) eGFP-marked hMSCs 5 days after the addition of differentiation medium. The eGFP- and DsRed-specific signals were combined in (g) and (h). Bars = 200 μm .

of β -dystroglycan along the plasma membrane of mosaic myotubes. For this purpose, hMSCs were transduced with LV.hMyoD.eGFP or LV.eGFP alone or in combination with hcAd.F50.hrGFP.Dys^{FL}. hcAd.F50.hrGFP.Dys^{FL} is a tropism-modified high-capacity adenoviral (hcAd) vector in which Dys^{FL} expression is driven by a 5.2-kilobase fragment of the human *muscle creatine kinase* gene promoter. The use of the human *muscle creatine kinase* gene promoter leads to the synthesis of Dys^{FL} in differentiated myotubes but not in hMSCs, which may be important given the possible adverse effects of Dys^{FL} misexpression on mononuclear cells.²³ In addition, hcAd.F50.hrGFP.Dys^{FL} particles have their fiber domains tailored to by-pass the absence of the human Ad species C receptor (Coxsackie B virus and adenovirus receptor) on the surface of hMSCs.^{10,11} Analysis of the four different cultures by IFM revealed that dystrophin-positive myotubes were detected exclusively in mixed cultures initiated with LV.hMyoD.eGFP- and hcAd.F50.hrGFP.Dys^{FL}-transduced hMSCs (Figure 7a and data not shown). These results indicate that the endogenous *DMD loci* present in the nuclei of control and of MyoD-stimulated hMSCs do not give rise to detectable amounts of Dys^{FL} after their incorporation into DMD myotubes. While eGFP was evenly distributed over entire myotubes, dystrophin was often lining the plasma membrane of the syncytia suggesting its proper localization (see arrows in Figure 7a). Consistently, preferential membrane association of β -dystroglycan, a type I trans-membrane component of the dystrophin-associated glycoprotein complex, was observed in mosaic (see arrows in Figure 7b) but much less so in nonmosaic myotubes (see arrowheads in Figure 7b). In addition, the β -dystroglycan-specific staining was stronger in mosaic myotubes than in their nonmosaic counterparts (Figure 7b). These results indicate that introduction of Dys^{FL} into DMD myotubes by heterotypic cell fusion can rescue the dystrophin-associated glycoprotein complex at its normal location as observed after

more conventional genetic interventions such as those involving direct delivery and repair of recombinant and endogenous *DMD* messages, respectively.^{1,2}

2.4 Discussion

Forced *MyoD* expression induces skeletal myogenesis in various differentiated cell types of nonmuscle origin.^{18,19} In this study we report that undifferentiated hMSCs also acquire properties of muscle cells in response to ectopic *MyoD* expression. However, the differentiation of these cells along the myogenic lineage was found to be incomplete as demonstrated by IFM, reverse transcription-polymerase chain reaction analysis, and flow cytometry. Previous research has indicated that different cell types respond differently to forced *MyoD* expression.^{19,24-26} Myogenic conversion varies from undetectable *e.g.*, hepatocytes²⁴ to robust (*e.g.*, fibroblasts).²⁴⁻²⁶ Interestingly, ectopic *MyoD* expression causes some nonmuscle cell types to acquire an elongated shape without concomitant formation of extensive syncytia.^{19,25} These results are reminiscent of our data on monotypic hMSC cultures exposed to MyoD. In other cell types, however, forced MyoD synthesis triggers the formation of large myotubes.^{19,26} For instance, Ad vector-mediated delivery of *MyoD* into fetal dermal fibroblasts led to extensive homotypic cell fusion.²⁶ In our study, only a fraction of the *MyoD*-transduced hMSCs obtained properties of skeletal muscle cells suggesting the presence of negative regulators and/or the absence of positive regulators of myogenesis in the refractory cells. It is known, for example, that *Mef2C* and *Twist* can interact with MyoD-E proteins heterodimers to enhance and repress myogenesis, respectively.^{27,28} In this regard it is of note that *Mef2C* transcripts were detected by reverse transcription-polymerase chain reaction in both naïve and hMyoD-transduced hMSC cultures suggesting that (i) they are only present in the MyoD-responsive hMSC fraction, (ii) they are nonfunctional in the MyoD-insensitive cells, (iii) *Mef2C* activity is suppressed in the refractory cells, or (iv) *Mef2C* is not limiting.^{27,28}

As determined by IFM, forced *MyoD* expression in hMSCs did not suffice to induce discernable endogenous dystrophin synthesis. This situation applied to both single hMSCs as well as to hMSCs incorporated in myotubes. The lack of detectable amounts of dystrophin in MyoD-treated hMSCs is intriguing considering that after fusion with myotubes these cells are embedded in an environment supporting the skeletal muscle phenotype. Muscle differentiation is a temporally regulated process with certain gene products such as desmin accumulating early on, with others, including the dystrophin-associated glycoprotein complex components dystrophin and sarcoglycans, emerging later. A hypothesis consistent with our data is that the timeframe of the experiments might have been insufficient to induce significant expression of the very large *DMD* gene (cultured myotubes typically detach from their support 6-7 days after the initiation of myogenic differentiation). Alternatively, due to the partial genetic reprogramming of hMSCs, nonmuscle nuclei within myotubes may continue to express dominant-negative regulators of myogenesis such as those that block endogenous *DMD* gene expression by epigenetic mechanisms. This hypothesis might also explain the absence even long after transplantation of significant δ -sarcoglycan and dystrophin derived from wild-type bone marrow stem cell nuclei integrated into the myofibers of δ -sarcoglycan- and dystrophin-negative mice, respectively,

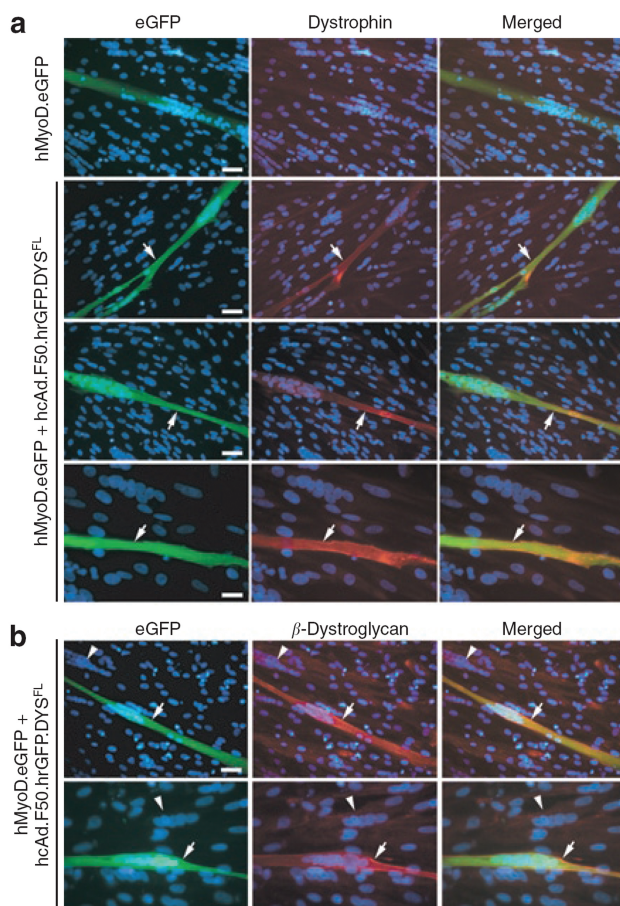


Figure 2.7: Genetic complementation of Duchenne muscular dystrophy (DMD) myotubes by *hMyoD*- and *Dys*^{FL}-expressing human mesenchymal stem cells (hMSCs). (a) hMSCs were transduced with LV.hMyoD.eGFP alone (upper row) or in combination with hcAd.F50.hrGFP.Dys^{FL} (lower three rows) and mixed with DMD myoblasts. After a 5-day differentiation period the cells were subjected to eGFP direct fluorescence microscopy (left column) and dystrophin immunofluorescence microscopy (IFM) (middle column). Signals derived from both analyses are combined in the right panels. Arrows point to myotubes displaying plasma membrane-associated dystrophin-specific staining. Bars = 100 μ m (upper three rows) and 50 μ m (lowest row). (b) hMSCs were transduced with LV.hMyoD.eGFP and hcAd.F50.hrGFP.Dys^{FL} and mixed with DMD myoblasts. After a 5-day differentiation period the cells were subjected to eGFP direct fluorescence microscopy (left column) and β -dystroglycan IFM (middle column). Signals derived from both analyses are combined in the right panels. Arrows point at myotubes displaying plasma membrane-associated β -dystroglycan-specific staining. Arrowheads indicate nonmosaic DMD myotubes showing diffuse β -dystroglycan labeling. Bars = 100 μ m (upper row) and 50 μ m (lower row). eGFP, enhanced green fluorescent protein; hMyoD, human MyoD.

even after long time post-transplantation.^{29,30}

Our results show that, cell-autonomous full-fledged differentiation of nonmuscle cells into bona fide muscle progenitor cells is not necessary in order for heterotypic cell fusion to occur. Instead, what one needs is the activation of the myogenic program to the extent that it endows the nonmuscle cells with factors required for their fusion with neighboring muscle cells. The set of molecules involved in cell adhesion/fusion in hMyoD-reprogrammed hMSCs, although incomplete for significant homotypic cell fusion to occur, can apparently be complemented in the presence of myoblasts undergoing canonical myogenic differentiation. Interestingly, we found that MyoD induced the expression on the surface of hMSCs of two membrane proteins (*i.e.*, CD10 and CD56) that have previously been reported to be engaged in myoblast differentiation, adhesion, and fusion.^{21,22}

By combining the use of MyoD-reprogrammed hMSCs with tropism-modified and Dys^{FL}-encoding hcAd vectors we accomplished the genetic rescue of DMD muscle cells without having to rely on epigenetic remodeling and subsequent activation of endogenous *DMD* loci in donor cell nuclei. Sufficient amounts of recombinant dystrophin can be introduced into DMD myotubes through heterotypic fusion with Dys^{FL}-expressing hMSCs to rescue β -dystroglycan at its normal location along the plasma membrane. Moreover, although dependent on the further development of safe and efficient hc vectors and despite the fact that recombinant dystrophin neoantigens may pose concerns, introducing Dys^{FL} into hMSCs has the distinct advantage of allowing transplantation of readily obtainable autologous cells. To minimize as much as possible the immunogenicity of the graft, noninternalized vector particles should be thoroughly removed and the transduced hMSCs might be cultured in the patient's own serum. Additional measures may entail the use of vectors directing Dys^{FL} expression from a late striated muscle gene-specific promoter. Regarding the neoantigenicity of recombinant dystrophin, it is relevant to mention that the occurrence in many DMD patients of so-called "revertant fibers"³¹ might aid in creating tolerance to the exogenous protein. In this context, it is also worth mentioning that hMSCs have been shown to display immunosuppressive properties.³² To the best of our knowledge this study demonstrates for the first time the directed differentiation of gene-corrected cells as a means of delivering therapeutic foreign DNA into another cell type through cell-cell fusion. These results provide a rationale for incorporating *MyoD* and Dys^{FL} expression units into a single vector particle. Such a vector might also hold value for direct DysFL transfer to dystrophic muscle since a substantial fraction of this tissue is fibrotic and thus composed of potentially MyoD-reprogrammable nonmuscle cells. However, for either application (*i.e.*, *ex vivo* or *in vivo* gene transfer) proper spatial and temporal control over the expression of *MyoD* is fundamental for harmonious skeletal muscle tissue development and repair. For instance, *MyoD* expression can trigger unscheduled cell cycle withdrawal.³³ Interestingly, within the time frame of our experiments we did not detect overt differences in the proliferation rate between LV.eGFP- and LV.hMyoD.eGFP-transduced hMSC cultures as determined by cell counts (data not shown). Until now, we do not know whether this is a consequence of (i) cell-specific factors, (ii) the relatively short time the cells were kept in GM or, (iii) MyoD levels. Clearly, further experiments directly probing cell cycle status endpoints are warranted to establish the impact of MyoD expression on the progression of hMSCs through mitosis. Moreover, although previous results clearly established that MyoD-null myoblasts have impaired dif-

ferentiation capacity,³⁴ recent data indicate that their MyoD-positive counterparts suffer from decreased survival after intramuscular administration.³⁵ Therefore, the synthesis or activity of MyoD should preferably be regulated. In this regard the 4-OHT-ER^{T2} approach tested in this study might constitute an interesting strategy especially if one considers that 4-OHT is already being deployed in the clinic. Furthermore, investigation of other myogenic regulatory factors (*e.g.*, Myf5) or even muscle stem cell specification factors (*e.g.*, Pax7) may provide valuable alternatives to MyoD. In any case, therapeutic approaches based on directed cell fusion of Dys^{FL}-positive autologous nonmuscle cells, should next be evaluated in combination with strategies aiming at tackling the pressing issue of body-wide dissemination of the transplanted cells. Finally, investigation of this gene transfer principle in treating diseases that depend on or benefit from skeletal muscle produced secretory proteins, such as the blood clotting factor deficiencies hemophilia A and B, may also be considered.

2.5 Acknowledgements

The authors are indebted to Richard J Bartlett (University of Miami), Sonia Pearson-White (University of Virginia), and Stephen J Tapscott (University of Washington) for providing the human *muscle creatine kinase* gene promoter, the *hMyoD* complementary DNA clone and the *mMyoD::ER* gene, respectively. We also thank Rob Nelissen (Leiden University Medical Center) for the steady supply of bone marrow as a source of hMSCs. The work presented was supported by research grants from the Dutch Prinses Beatrix Fonds (PBF; MAR04-0222) and the Association Française contre les Myopathies (AFM; 12259-SR-GROUPE E).

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2.7 Supplementary materials and methods

2.7.1 DNA transfections

HeLa cells were seeded in wells of 24-well plates (Greiner Bio-One) at a density of $0.8\text{--}1.0 \times 10^5$ cells per well. After an overnight incubation period, each sample received 350 ng of DNA mixtures in a total volume of 50 μl of 150 mM NaCl. The DNA mixtures contained 280 ng of pGL3.MCK.Luc, 4.6 nmol of LV shuttle plasmid and 4.6 nmol of either pcDNA3.1/Hygro(+) (Invitrogen) or a human Mef2C-encoding derivative thereof. A total amount of 350 ng of DNA was obtained by adding a plasmid lacking eukaryotic promoter elements. After the addition of 2.4 μl of ExGen 500 (Fermentas), the DNA mixtures were immediately shaken vigorously for 10 s and incubated for 15 min at room temperature. The DNA-polycation complexes were then diluted 10-fold in culture medium and added onto the HeLa cell monolayers. After 20 h at 37°C in a humidified air-10% CO₂ atmosphere, the transfection medium was replaced by regular culture medium. Three days post-transfection, cell lysates were prepared for measuring luciferase activity (see main text).

2.7.2 Intracellular HA tag-specific staining and flow cytometry

To ascertain that the transduction levels of cells expressing the various TF-encoding genes were similar, we employed the LVs LV.MyoCD::HA.eGFP, LV.MyoD::HA.eGFP and LV.MyoDCD::HA.eGFP encoding HA epitope-tagged versions of MyoCD, MyoD and MyoDCD, respectively. Expression of each of these proteins in target cells was measured by flow cytometry as detailed below. Suspensions of hMSCs transduced with each of the vectors were fixed for 10 min at 37°C in 400 μl of phosphate-buffered 4% formaldehyde solution (pH 7.0-7.4; KLINIPATH) and, subsequently, chilled on ice for 1 min. After being spun down for 5 min at 400 g and 4°C, the cells were resuspended in 400 μl of ice-cold 90% methanol and incubated on ice for 30 min while being gently swirled every 5-10 min. Next, aliquots containing $5\text{--}10 \times 10^4$ cells were diluted in 4 ml of phosphate-buffered saline (PBS)-0.1% bovine serum albumin (BSA), collected by centrifugation for 5 min at 400 g and 4°C and then incubated for 1 h at room temperature with a 1:20 dilution of AlexaFluor 647-conjugated anti-HA mouse monoclonal antibody HA-Tag (clone 6E2; Cell Signaling Technology) in PBS-0.1% BSA. Parallel samples to which no antibody was added served as negative controls. The cell suspensions were gently swirled every 10-15 min. After two consecutive washing steps consisting of adding 4 ml of PBS-0.1% BSA followed by 5-min centrifugations at 400 g and 4°C, the cells were resuspended in 250 μl of PBS-0.1% BSA and kept on ice until flow cytometric analysis for eGFP- and HA-specific fluorescence.

2.7.3 Quantification of MHCK7 promoter activity in haMSCs

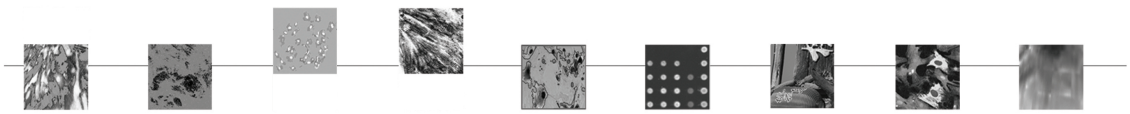
Thirty thousand haMSCs were incubated overnight in wells of 24-well plates with LV.PRE.pA+.Luc or LV.PRE.pA+.MHCK7.Luc at a multiplicity of infection (MOI) of 30 TU/cell. Mocktransduced haMSCs served as controls. The resulting haMSCLuc and haMSCMHCK7.Luc populations were amplified by sub-culturing. Subsequently, the cells were incubated with LV.LacZ.eGFP, LV.MyoD::HA.eGFP or LV.MyoDCD::HA.eGFP at an MOI of 25 HTU/cell and cultured in growth medium for 6 days. After another 6-day culture period in differentiation medium, cell lysates were prepared for measuring PpLuc activity. Mocktransduced haMSCs subjected to the same culture regimen served as negative control.

2.7.4 Quantification of CK8 promoter activity in human myocytes before and after differentiation

DMD myoblasts were seeded in wells of 24-well plates at a density of 2×10^5 cells per well. The following day these cells were transduced in duplicate with LVs carrying a promoterless, a human CMV promoter-driven or a CK8 promoter-based PpLuc expression unit at MOIs of 0.5, 2.0 and 8.0 TU/cell. At 48 h post-infection, the cells were rinsed once with 2 ml of PBS after which the cells in one sample of each pair of cell cultures were trypsinized and stored at -20°C whilst the cells in the other sample were exposed to differentiation medium for 4 days prior to trypsinization and storage at -20°C . After thawing and lysis of the cells, chemiluminescence measurements were performed.

2.7.5 Establishment and deployment of hfMSCCK8. μ Dys cultures

Thirty thousand hfMSCs in wells of 24-well plates were incubated overnight with LV.CK8.coh/ μ Dys at an MOI of 25 TU/cell. After expansion, the resulting hfMSCsCK8. μ Dys were seeded in wells of 24-well plates at a density of 3×10^4 cells/well and were incubated overnight with either LV.MyoD::HA.eGFP or with LV.MyoDCD::HA.eGFP at 25 HTU/cell. Unmodified hfMSCs seeded in parallel were transduced with the same dose of either of the two TF-encoding LVs. Mock-infected hfMSCs and hfMSCsCK8. μ Dys served as negative controls. Four days later, 35×10^4 cells from each of the various cell populations were seeded on glass cover slips in wells of 6-well plates (Greiner BioOne) either alone or together with 2×10^5 DMD myoblasts. After 4 days in their respective growth media, both monocultures and co-cultures were rinsed once with PBS and incubated in differentiation medium. After 4 additional days of culture, the cells were fixed and processed for dystrophin immunofluorescence microscopy as specified in the main text.



Prelude to chapters 3 and 4

2

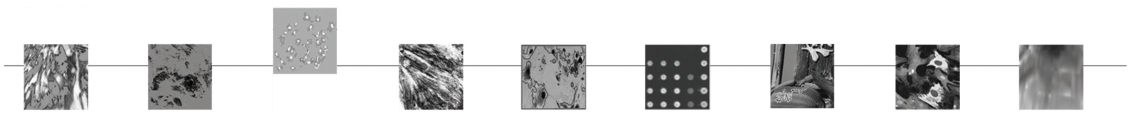
Integrins and integrin signaling

2

In the heart, cardiomyocytes (CMCs) are surrounded by a mesh of extracellular matrix (ECM). Besides providing structural support to the CMCs, the ECM is also involved in maintaining homeostasis in the healthy heart and in cardiac remodeling following myocardial injury.¹ CMC-ECM interactions are mainly mediated via integrins, which are transmembrane heterodimers consisting of an α - and a β -chain. There are 18 α - and 8 β -subunits that can form 24 different combinations with distinct binding properties.^{2,3} While the extracellular part of integrins bind various components of the ECM, such as fibronectin, laminin and collagen, the intracellular part is connected to the cytoskeleton via talin and α -actinin.⁴ In the heart, integrins are important as mechanotransducers that sense the mechanical forces of the beating heart and consequently induce signaling cascades in the CMC. Since integrins themselves do not possess enzymatic activity, they are linked to other signaling molecules, such as focal adhesion kinase (FAK), Src, integrin-linked kinase (ILK) and p130CAS.^{5,6} The effect of integrin stimulation can be studied *in vitro* by exposing CMCs to soluble integrin receptor ligands containing the Arg-Gly-Asp (RGD) sequence present in fibronectin and other ECM proteins.⁷ One of the effects of integrin stimulation that has been studied using these RGD containing peptides is the release of Ca^{2+} from the sarcoplasmic reticulum (SR). It was shown that integrin signaling led to Ca^{2+} release from the SR via ryanodine receptors.⁸ The release of Ca^{2+} leads to an increase in intracellular Ca^{2+} concentration, which triggers dysferlin-mediated sealing of plasma membrane ruptures caused by the contraction of the heart.⁹ Integrin stimulation has also been shown to induce cardiac hypertrophy.⁶ FAK and Src were shown to be activated upon integrin stimulation, which led to ERK1/2 activation.¹⁰ Furthermore, integrin-linked kinase (ILK) expression is increased in hypertrophic hearts and overexpression of ILK activates prohypertrophic signaling kinases.¹¹ In addition, it was shown that integrin stimulation-induced hypertrophy is nitric oxide-dependent.¹² Although the existing data indicate an important role for integrin signaling in cardiac hypertrophy, the exact pathways through which integrin signaling leads to hypertrophy remain to be clarified.

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Chapter 3

3

Integrin stimulation favors uptake of macromolecules by cardiomyocytes *in vitro*

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Cellular Physiology and Biochemistry. 2010; 26:999-1010.

Abstract

Previously, our research group showed that integrin stimulation induces release of cardiac troponin I from viable neonatal rat ventricular cardiomyocytes (NRCMs), but would it also stimulate uptake of exogenous macromolecules? For this purpose, beating NRCMs were incubated without or with an RGD motif-containing peptide (GRGDS) to stimulate integrins in the presence of Texas Red-conjugated ovalbumin (OTR; 45 kDa) or dextran (DTR; 70 kDa). After incubation periods of 8, 16 and 24 hours endocytosis of red label was quantified by fluorescence microscopy. Uptake of OTR and DTR by NRCMs was intensified by GRGDS treatment (p for trend >0.001 and 0.019 , respectively) and increased with duration of incubation ($p>0.001$ for both). The GRGDS-induced uptake of OTR by NRCMs correlated positively with OTR concentration ($p>0.001$). Experiments with pharmacological inhibitors of endocytosis indicated that in the absence of GRGDS, NRCMs take up OTR by the clathrin-mediated pathway of endocytosis while the GRGDS-dependent OTR uptake occurs by macropinocytosis. Cultures of NRCMs that were stretched cyclically showed ~4-fold increased uptake of OTR compared to stationary NRCMs. Immunofluorescence microscopy revealed that the dysferlin-positive PM areas in beating GRGDS-treated NRCMs were ~3-fold larger than in contracting NRCMs incubated with vehicle ($p>0.001$). However, in non-beating NRCMs exposure to GRGDS did not induce larger dysferlin-positive PM areas, nor did it stimulate uptake of OTR. After inhibition of *dysferlin* expression by short hairpin RNA-mediated RNA interference, OTR uptake by contracting NRCMs could no longer be stimulated via GRGDS treatment. We conclude that in NRCMs, stimulation of integrins by RGD motif-containing peptides or stretch cause uptake of labeled macromolecules. The latter process appears to depend on contractile behavior of the NRCMs and on the PM repair protein dysferlin, probably because of its role in macropinocytosis.

3.1 Introduction

The plasma membrane (PM) is a biological barrier between the extracellular and intracellular environment and is essential to the maintenance of cell integrity. Damage to this barrier leads to cell dysfunction or death. PM disruptions, however, commonly occur under physiological conditions in a wide variety of mechanically active mammalian tissues. Especially contractile cells such as vascular and intestinal smooth muscle cells, skeletal muscle cells¹ and cardiomyocytes endure frequent PM damage.² These cells hence possess an active repair mechanism to reseal PM lesions. Membrane repair is mediated by rapid Ca^{2+} -triggered exocytosis of various intracellular vesicles, such as lysosomes and enlargosomes, which create a membrane patch that reseals the PM lesion.^{3,4} Recent findings suggest a crucial role for dysferlin in this repair process, possibly as a Ca^{2+} sensor that triggers vesicle fusion.^{5,6}

Mechanical stretch causes integrin stimulation of cardiomyocytes and plays an important role in the development of myocardial hypertrophy.^{7,8} The mechanical forces exerted on cells are sensed by integrins, mechanotransducer proteins linking the extracellular matrix (ECM) to the intracellular cytoskeleton.⁹ The RGD motif present in fibronectin and in several other ECM proteins is a ligand of integrins.¹⁰ Oligopeptides containing this motif can be used to simulate myocardial mechanical stretch *in vitro*. Previously we have demonstrated that integrin stimulation of neonatal rat ventricular cardiomyocytes (NRCMs) using an RGD motif-containing pentapeptide (*i.e.* $\text{NH}_2\text{-Gly-Arg-Gly-Asp-Ser-COOH}$, abbreviated by GRGDS) resulted in (i) a rise in the intracellular concentrations of NO and Ca^{2+} ,¹¹ (ii) cardiomyocyte hypertrophy¹² and (iii) release of cTnI from viable NRCMs.¹³ Moreover, Kaye *et al.*¹⁴ have shown that release of basic fibroblast growth factor (bFGF) from electrically stimulated ventricular cardiomyocytes of adult rats was accompanied by uptake of 10-kilodalton (kDa) fluorescein-labeled dextran molecules from the culture medium.

In the present study, NRCM cultures were used to show that also integrin stimulation promotes uptake of extracellular macromolecules (*i.e.* Texas Red-conjugated ovalbumin [OTR] and Texas Red-labeled dextran [DTR]) by cardiomyocytes. In addition, we investigated whether this uptake is dependent on (i) the extracellular concentrations of the RGD motif-containing pentapeptide, (ii) the extracellular concentrations of the macromolecule used, and (iii) the duration of the incubation with the macromolecules. In a separate series of experiments, NRCMs were subjected to cyclic stretch to test its impact on the uptake of OTR. The effect of cardiomyocyte contraction on the endocytosis of macromolecules was studied using NRCM cultures treated with blebbistatin, a cell-permeable inhibitor of several class II myosins. To investigate the possible involvement of dysferlin in integrin stimulation-induced uptake of macromolecules by NRCMs, the size of PM-associated dysferlin-enriched patches was determined under various conditions. A lentivirus vector directing the synthesis of *dysferlin*-specific short hairpin (sh) RNA molecules was used to more directly study the role of dysferlin in the endocytosis of macromolecules. Finally, several different small-molecule inhibitors of endocytosis were applied to obtain information about the specific pathway(s) involved in the endocytosis of OTR by GRGDS-treated NRCMs and by their unstimulated counterparts.¹⁵

3.2 Methods

3.2.1 Cell culture

Primary cultures of NRCMs were prepared from the ventricles of 2-day-old Wistar rats as described previously.¹⁶ Briefly, ventricles were minced and cells were isolated enzymatically using collagenase type I (CLS, Worthington, Lakewood, NJ, USA) at 37°C. The cell suspension was centrifuged and the cell pellet was resuspended in Ham's F10 medium containing 10% fetal bovine serum (FBS), 10% horse serum (HS), 100 U/mL penicillin and 100 µg/mL streptomycin (all from Invitrogen, Breda, the Netherlands). The cells were seeded in 6-cm-diameter Primaria culture dishes (Becton Dickinson, Etten-Leur, the Netherlands), and after 45 min non-adherent cells representing the cardiomyocytes were collected and plated onto a rat-tail collagen type I (Sigma-Aldrich, Zwijndrecht, the Netherlands)-coated glass coverslips (Ø25 mm) in wells (Ø35 mm) of six-well plates (Costar, Corning Life Sciences, Amsterdam, the Netherlands) at a density of 6×10^5 cells per well. The NRCMs were incubated in a humidified incubator at 37°C and 5% CO₂ with so-called growth medium consisting of a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM; Invitrogen) and Ham's F10 medium containing 5% HS, penicillin (100 U/mL) and streptomycin (100 µg/mL) for 24 h. The next 24 h the cultures were incubated in the same medium supplemented with 5-bromo-2'-deoxyuridine (BrdU, 100 µmol/L; Sigma-Aldrich) to inhibit proliferation of cardiac fibroblasts. Subsequently, the cells were maintained in growth medium without BrdU for 24 h. This typically gave rise to cultures of spontaneously beating cardiomyocytes with a confluency of 60-70%, which were used for the experiments described below. The investigations had the approval of the Animal Experiments Committee of the LUMC, according to Dutch law.

3.2.2 Macromolecule uptake experiments

Three days after cell isolation, growth medium was changed to a 1:1 mixture of DMEM and Ham's F10 medium containing 2.5% HS, penicillin (100 U/mL) and streptomycin (100 µg/mL). The incubation experiments were started by adding OTR (45 kDa; Invitrogen) or DTR (70 kDa; Invitrogen) in the concentration range 0.01-0.5 µmol/L. These incubations were done in the absence or presence of various concentrations (100, 200 or 300 µg/mL) of the RGD motif-containing pentapeptide (GRGDS) (Sigma-Aldrich) to stimulate integrins.¹⁰ When indicated, the cell-permeable myosin class II inhibitor blebbistatin (Sigma-Aldrich) was added to the NRCM cultures at a final concentration of 10 µmol/L to stop cellular beating.¹⁷ Incubations lasted 8, 16 or 24 h. The reasons to select the combination of OTR and DTR for the macromolecule uptake experiments were twofold. Firstly, by selection of two macromolecules with different molecular weights the influence of cargo size on uptake efficiency could be investigated. Secondly, by studying the uptake of a Texas Red-labeled polysaccharide (*i.e.* the glucan dextran) as well as a Texas Red-conjugated polypeptide (*i.e.* the protein ovalbumin) possible differences in the uptake of different types of macromolecules could be uncovered, while the use of a similar label, *i.e.* Texas Red, will allow equal sensitivity of quantitative detection of uptake.

3.2.3 Cell stretch

For experiments with stretched NRCMs, these cells were cultured in stretch plates (Bio-Flex culture plates BF-3001C, FlexCell International Corporation, Dunn Labortechnik, Asbach, Germany) with flexible collagen type I-coated surfaces and placed in a stretch device (FX-4000T Flexcell Tension Plus System, FlexCell International Corporation) leading to biaxial stretch of all cells attached to the flexible bottom. Stretch was applied cyclically at 0.5 Hz for 24 h in a humidified incubator with 5% CO₂ at 37°C.¹⁸

3.2.4 Quantification of TexasRed-labeled macromolecules in NRCMs

At the end of the incubation with labeled macromolecules, the medium in the NRCM cultures was removed and the cells were washed twice with phosphate-buffered saline (PBS) containing 1% FBS. The NRCMs were then fixed in 4% buffered formaldehyde (Klinipath, Duiven, the Netherlands) for 30 min, and washed twice with PBS+1% FBS. Next, the coverslips with cells were mounted on microscope slides using Vectashield (Vector Laboratories, Burlingame, CA, USA). Cells cultured in stretch plates were processed in the same manner except that after the final wash step, they were covered with a drop of Vectashield and with a glass cover slide. Following uptake of TexasRed-labeled macromolecules, photographs of the NRCMs were taken using a fluorescence microscope (Nikon Eclipse, Nikon Europe, Badhoevedorp, the Netherlands) equipped with 100× oil-immersion objective and a digital camera (Nikon DXM 1200). Per cell culture, 10-50 fields were photographed. Images were analyzed quantitatively using the color intensity measurement routine of the Image-Pro Plus software (Media Cybernetics, Silver Spring, MD, USA) on all photographed cells available.

3.2.5 Immunocytochemistry

NRCM cultures were rinsed three times with ice-cold PBS + 1% FBS, fixed in 4% buffered formaldehyde for 30 min, washed twice with ice-cold PBS + 1% FBS, incubated with ice-cold PBS + 0.1% Triton X-100 (Sigma-Aldrich) for 15 min, washed twice with ice-cold PBS + 1% FBS, and incubated overnight with primary antibody (100× diluted in PBS) at 4°C. After washing twice with PBS + 1% FBS, the cells were incubated with secondary antibody (200× diluted) for 60 min. Following two wash steps with PBS + 1% FBS, the cultures were incubated with 10 µg/mL Hoechst 33342 (Invitrogen) in PBS +1% FBS for 8 min. After washing twice with PBS + 1% FBS, the coverslips with cells were mounted on microscope slides using Vectashield. To detect sarcomeric α -actinin, we used a mouse monoclonal antibody (clone: EA53; A7811; Sigma-Aldrich). Goat polyclonal antibodies (PABs) raised against a peptide mapping within an internal region of human dysferlin were purchased from Santa Cruz (sc-16635; Santa Cruz, CA, USA). Fluorescein isothiocyanate-conjugated donkey anti-goat IgG was from Santa Cruz (sc-2024) and Alexa568-coupled rabbit anti-mouse IgG was from Invitrogen (A11061).

Since dysferlin is a type II transmembrane protein with a very short cytoplasmic tail,¹⁹ the epitope recognized by the dysferlin-specific goat polyclonal antibodies should only be available for antibody binding in cells with a disrupted PM.

The NRCMs in the stretch plates were fixed and stained using the same methods. However, as the cells in the stretch plates were not on coverslips instead of mounting them on a microscope slide, they were covered with a drop of Vectashield and with a glass coverslip.

Images were produced using the same fluorescence microscope, camera, and off-line image analysis software as mentioned above, but now using the area measurement routine. The photographs of the dysferlin-stained NRCMs were acquired with a short exposure time to selectively record the signals associated with dysferlin-positive PM areas, the so-called patches.

3.2.6 Endocytosis

3

Chlorpromazine (Sigma-Aldrich) was used to inhibit clathrin-mediated endocytosis, as has been demonstrated in a rat alveolar type II epithelial cell line.²⁰ Filipin (Sigma-Aldrich) was used to inhibit caveolin-dependent endocytosis, a mechanism shown to be involved in the uptake of 155-kDa and 500-kDa dextrans by cultured primary bovine aortic endothelial cells.²¹ Wortmannin (Sigma-Aldrich) was used to inhibit macropinocytosis, a Ca^{2+} -dependent uptake mechanism of 70-kDa dextran and small ($\varnothing 20$ nm) latex beads.²²

3.2.7 Downregulation of dysferlin expression

Lentiviral vector shuttle constructs encoding shRNAs targeting the human (TRCN000000 0965) and mouse (TRCN0000111756) *dysferlin* genes were obtained from the Mission Library (Sigma-Aldrich). Both these shRNAs (which will be subsequently designated 965 and 756) contained a sense strand that perfectly matched with the coding sequence of the rat *dysferlin* gene and efficiently inhibited its expression as assessed by western blotting. As negative control, a lentiviral vector shuttle plasmid directing the synthesis of an *enhanced green fluorescent protein* (eGFP) gene-specific shRNA (SCH005, Sigma-Aldrich) was used. To allow for the easy assessment of transduction efficiencies, the puromycin N-acetyl transferase-coding sequence in these constructs was replaced by that of the green fluorescent protein of *Renilla reniformis* (hrGFPI). To this end, plasmid pLV.hPGK.hrGFPI (nucleotide sequence available upon request) was digested with the restriction endonucleases *SpeI* and *KpnI* and the resulting 1.1-kilobase (kb) DNA fragment containing the hrGFPI-coding sequence was combined with the 6.2-kb *SpeI*×*KpnI* fragment of each of the lentiviral vector shuttle constructs from the Mission library using T4 DNA ligase. Ligation mixtures were introduced in chemocompetent GeneHogs cells (Invitrogen) and large stocks of the correct plasmids were prepared with the aid of the JETSTAR 2.0. Plasmid Maxiprep Kit (Genomed, Löhne, Germany).

Vesicular stomatitis virus G protein-pseudotyped self-inactivating human immunodeficiency virus type I vectors (SIN-LVs) were produced in 293T cells as described previously.²³ The gene transfer activity of the vector stocks was determined by end-point titration on HeLa indicator cells using flow cytometric analysis of *hrGFPI* expression as read-out. The titers of the SIN-LV preparations are thus expressed in HeLa cell-transducing units (HTUs) per ml.

NRCMs were transduced with SIN-LV particles at a multiplicity of infection (MOI) of 10 HTUs per cell. The next day, the cultures were washed three times with PBS and provided with fresh growth medium. At 7 days post-transduction, the cells were used for OTR uptake experiments.

3.2.8 Statistics

Results are expressed as mean $\pm$ standard deviation (SD). Statistical analysis was performed by Student's *t*-test and by one-way analysis of variance (ANOVA) followed by Bonferroni's *post hoc* test. Non-parametric tests used were the Kruskal-Wallis test to quantify effects of trend, and Spearman's ρ to quantify effects of correlation. Differences were regarded as statistically significant if $p > 0.05$. SPSS16 for Windows (SPSS Inc, Chicago, IL, USA) was used for statistical analysis.

3.3 Results

3.3.1 Uptake of macromolecules: effect of macromolecule concentration

The uptake of both OTR and DTR by beating NRCMs was favored by integrin stimulation using the RGD motif-containing pentapeptide (figure 1).

The quantity of labeled macromolecules taken up in 24 h was dependent on their extracellular concentrations with, for OTR, a $p_{\text{trend}} > 0.001$ and $= 0.006$ in the presence and absence of GRGDS, respectively, and for DTR a $p_{\text{trend}} = 0.007$ and $= 0.052$ in the presence and absence of GRGDS, respectively. The positive effect of GRGDS treatment on OTR uptake was significant at all three protein doses tested (figure 2A). The effect of integrin stimulation on uptake of DTR only reached significance when the glucan was present at concentrations of 0.1 and 0.5 $\mu\text{mol/L}$ (figure 2B).

3.3.2 Uptake of macromolecules: effect of GRGDS concentration

The effect of GRGDS on uptake of TexasRed-labeled macromolecules by beating NRCMs was strongly dependent on the concentration of GRGDS applied, being evident for OTR as well as for DTR (figures 3A and B).

Uptake of OTR (0.3 $\mu\text{mol/L}$) after 24 h was 1.43-, 1.75- and 2.18-fold higher in the presence of 100, 200 and 300 $\mu\text{g/mL}$ of the RGD motif-containing pentapeptide than in its absence ($p_{\text{trend}} > 0.001$). For DTR (0.3 $\mu\text{mol/L}$) the uptake by NRCMs was 1.15-, 1.47- and 1.63-fold higher in the presence of 100, 200 and 300 $\mu\text{g/mL}$ of GRGDS than in its absence ($p_{\text{trend}} > 0.02$).

3.3.3 Uptake of macromolecules: effect of incubation period

The quantity of TexasRed-labeled macromolecules taken up by beating NRCMs in the absence and presence of GRGDS was strongly dependent on the duration of incubation, being evident for OTR as well as for DTR (figures 4A and B). P_{trend} was > 0.001 for OTR + GRGDS, 0.001 for OTR without GRGDS, > 0.001 for DTR + GRGDS, and 0.149 for DTR without integrin stimulation.

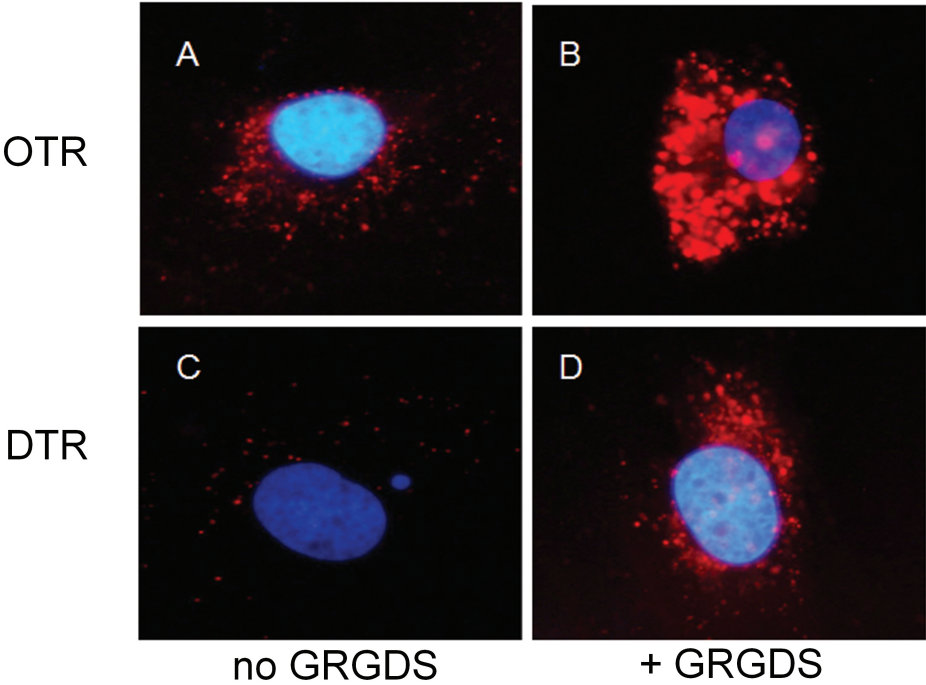


Figure 3.1: Uptake of OTR (0.1 $\mu\text{mol/L}$) (**A, B**) and DTR (0.3 $\mu\text{mol/L}$) (**C, D**) by NRCMs incubated with these TexasRed-labeled macromolecules for 24 h in the absence (**A, C**) or presence (**B, D**) of 300 $\mu\text{g/mL}$ of GRGDS.

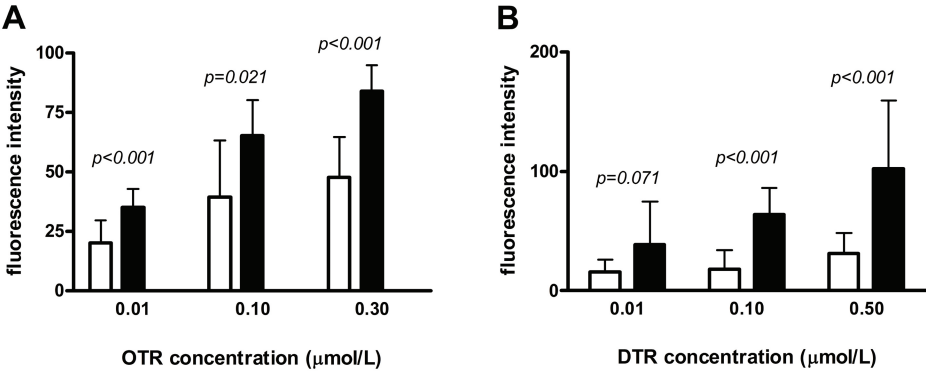


Figure 3.2: Uptake of OTR (**A**) and DTR (**B**) by NRCMs incubated with different concentrations of these TexasRed-labeled macromolecules for 24 h in the absence (white) or presence (black) of 300 $\mu\text{g/mL}$ of GRGDS. Number of data per bar: 8-10 for panel A en 10 for panel B. Indicated are mean values and SD.

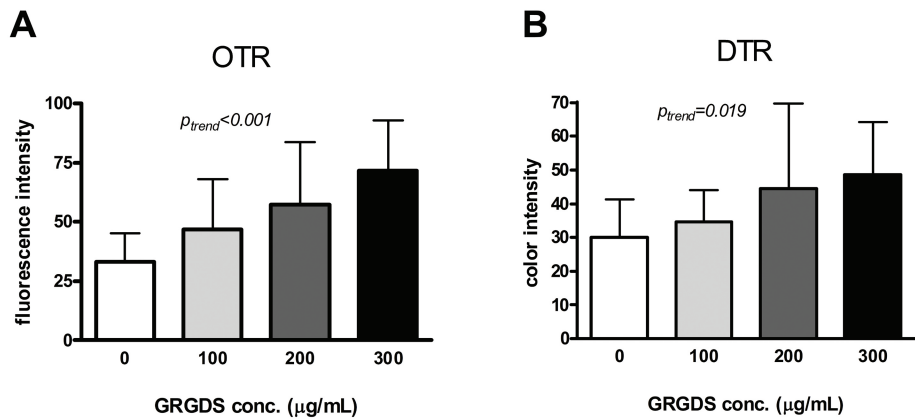


Figure 3.3: Uptake of OTR (0.3 $\mu\text{mol/L}$) (**A**) and DTR (0.3 $\mu\text{mol/L}$) (**B**) by NRCMs incubated with these TexasRed-labeled macromolecules for 24 h in the absence of GRGDS (left bars in both panels) and in the presence of 100, 200 or 300 $\mu\text{g/mL}$ of the RGD motif-containing pentapeptide. Number of data per bar: 24, 8, 8 and 8 moving from left to right in both panels. Indicated are mean values and SD.

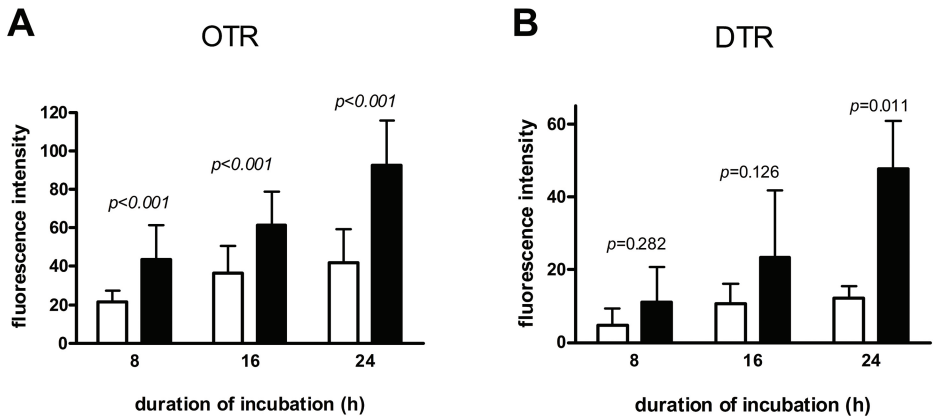


Figure 3.4: of OTR (0.3 $\mu\text{mol/L}$) (textbfA) and DTR (0.3 $\mu\text{mol/L}$) (textbfB) by NRCMs incubated with these TexasRed-labeled macromolecules for 8, 16 or 24 h in the absence (white) or presence (black) of 300 $\mu\text{g/mL}$ of GRGDS. Number of data per bar: 20 for panel A and 10 for panel B. Indicated are mean values and SD.

3.3.4 Mechanism of OTR uptake

To elucidate the mechanism(s) involved in the uptake of macromolecules by NRCMs, the cells were treated with pharmacological inhibitors of three different endocytic pathways. Chlorpromazine was used to inhibit clathrin-mediated endocytosis, whereas macropinocytosis and caveolin-dependent endocytosis were blocked with the phosphoinositide 3-kinase inhibitor wortmannin and the cholesterol-binding antibiotic filipin, respectively. The uptake of OTR by NRCMs in the absence of GRGDS was suppressed by chlorpromazine (15 $\mu\text{mol/L}$) by 31.9% ($p>0.05$), but was not affected by filipin (4 $\mu\text{g/mL}$) or by wortmannin (0.1 $\mu\text{mol/L}$). The dose-dependent effects of chlorpromazine and wortmannin on OTR uptake in the absence of integrin stimulation are presented in figures 5B and D. These data suggest that unstimulated NRCMs take up OTR by clathrin-mediated endocytosis.

The uptake of OTR in the presence of GRGDS was not sensitive to treatment of the NRCMs with filipin (4 $\mu\text{g/mL}$) but it was suppressed by 69.1% ($p>0.01$) and 44.1% ($p>0.05$) by chlorpromazine (15 $\mu\text{mol/L}$) and wortmannin (0.1 $\mu\text{mol/L}$), respectively. The integrin stimulation-dependent OTR uptake, being the difference between OTR uptake in the presence and in the absence of GRGDS, was inhibited by wortmannin ($\text{EC}_{50} = 276 \text{ nmol/L}$) but not by chlorpromazine (figures 5A and C). The GRGDS-independent uptake of OTR was inhibited by chlorpromazine ($\text{EC}_{50} = 23 \text{ } \mu\text{mol/L}$) but hardly by wortmannin (figures 5B and D). These results imply the involvement of macropinocytosis in the GRGDS-dependent OTR uptake by beating NRCMs. Thus, during integrin stimulation of NRCMs with GRGDS, basal OTR uptake by clathrin-mediated endocytosis is augmented by macropinocytosis.

3.3.5 Role of dysferlin in uptake of macromolecules

To study the role of dysferlin in the uptake of macromolecules by NRCMs, cells that had been incubated with Texas Red-labeled macromolecules for 24 h in the absence and presence of GRGDS were stained with antibodies directed against an epitope located in the large endodomain of dysferlin. NRCMs that were stimulated with the RGD motif-containing pentapeptide showed approximately 3-fold larger dysferlin-positive PM patches than their untreated counterparts (figures 6A-C). This finding suggests that the PM of NRCMs is sufficiently disrupted following fixation of the cells with formalin to allow excess of the dysferlin-specific antibodies to their intracellularly located epitope. Our results are also in line with previous reports indicating an important role for dysferlin-enriched PM patches in the repair of PM injury.⁴⁻⁶

Until now all our experiments were performed with cultures of contracting cardiomyocytes. As it has been argued that mechanical activity favors the occurrence of transient membrane disruptions,¹⁴ macromolecule uptake experiments in the presence and absence of GRGDS were performed in beating and in blebbistatin-treated (*i.e.* contraction-arrested) NRCMs. In non-beating NRCMs, the average size of dysferlin-positive PM patches did not differ between GRGDS-treated and unstimulated cells (figure 6C). Accordingly, in the absence of contractile activity the uptake of OTR was not enhanced by the presence of 300 $\mu\text{g/mL}$ GRGDS ($107.2 \pm 48.6\%$ and $94.5 \pm 59.1\%$ in the presence and absence of GRGDS, respectively; $p=\text{n.s.}$) and as high as uptake of OTR by beating

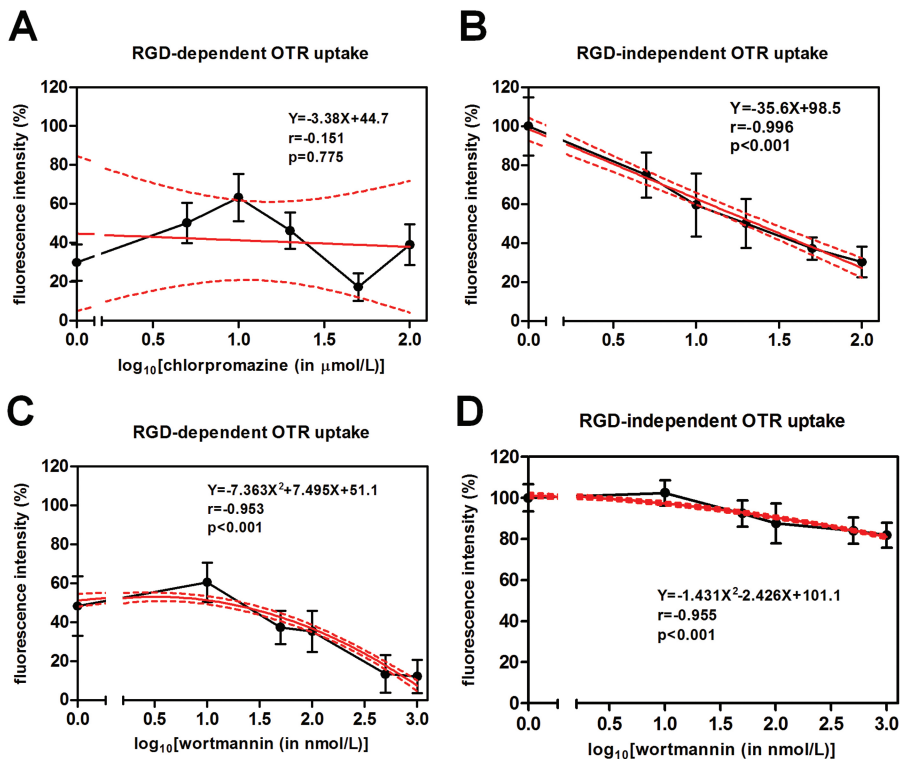


Figure 3.5: RGD-dependent (**A**, **C**) and RGD-independent (**B**, **D**) uptake of OTR ($0.3 \mu\text{mol/L}$) by NRCMs incubated for 24 h with different concentrations of chlorpromazine (**A**, **B**) or wortmannin (**C**, **D**). Chlorpromazine, an inhibitor of clathrin-mediated endocytosis, inhibited RGD-independent OTR uptake, whereas wortmannin, an inhibitor of macropinocytosis, suppressed RGD-dependent OTR internalization. Indicated are regression lines or curves plus 95% confidence limits; data points represent mean values $\pm$ SEM. Number of data per point: 7-12 for panels A and B, and 8-14 for panels C and D. OTR-associated fluorescence intensities are normalized to the value determined in NRCMs incubated for 24 h without GRGDS and without inhibitors.

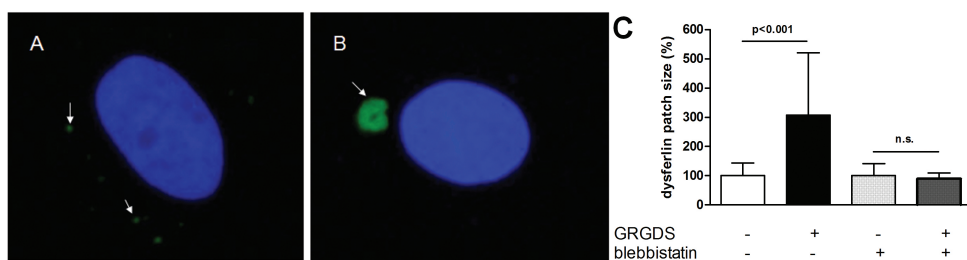


Figure 3.6: Size of dysferlin-positive PM patches in NRCMs after incubation for 24 h without (A) or with (B) 300 $\mu\text{g/mL}$ of GRGDS. (C) Size of dysferlin-positive PM patches after 24 h of incubation without or with 300 $\mu\text{g/mL}$ of the RGD motif-containing pentapeptide, in the absence or presence of 10 $\mu\text{mol/L}$ of the myosin class II inhibitor blebbistatin. Dysferlin patch sizes are normalized to the value determined in NRCMs incubated for 24 h without GRGDS and without blebbistatin. Number of data per bar: 100, 105, 7 and 7, moving from left to right. Indicated are mean values and SD.

NRCMs in the absence of GRGDS ($100 \pm 53.6\%$).

3.3.6 Effect of dysferlin level on OTR uptake

To study the effect of dysferlin level on OTR uptake, NRCMs were transduced with SIN-LVs directing the synthesis of *dysferlin*-specific shRNA 965 or 756 (SIN-LV965 and SIN-LV756) at an MOI of 10 HTUs per cell. Western blot analysis showed that this led to 86% reduction of dysferlin protein for shRNA 965 and to 79% reduction of dysferlin protein for shRNA 756. Contrary to NRCMs transduced with the SIN-LV encoding an *eGFP*-specific shRNA (negative control), NRCMs expressing the *dysferlin*-specific shRNAs displayed no extra OTR uptake upon GRGDS addition ($160.5 \pm 60.7\%$ in control vs. $109.8 \pm 36.5\%$ and $99.3 \pm 50.1\%$ in shRNA 965 and 756 transfected cells respectively; both $p > 0.001$ compared to control), while basal OTR uptake was unaffected ($105.2 \pm 29.0\%$ and $94.0 \pm 42.1\%$ in shRNA 965 and 756 transfected cells, respectively, both being not significantly different from 100%)(figure 7A). The transduction level of NRCMs with SIN-LV756 (reflected by the intensity of the hrGFPI-associated green fluorescence) was inversely correlated with cellular OTR uptake ($r = -0.70$, $p > 0.005$), demonstrating the inhibitory effect of dysferlin downregulation on internalization of OTR. SIN-LV756-transduced NRCMs revealed reduced levels of dysferlin-positive patches on the PM compared to the control cells both in the absence of GRGDS (44.3 vs. 704 a.u.) as well as in its presence (99.6 vs. 1468 a.u.) (figure 7B). Apparently, the lack of dysferlin had no effect on the GRGDS-independent uptake of OTR by clathrin-mediated endocytosis, but completely prevented integrin stimulation-dependent OTR uptake by macropinocytosis.

3.3.7 Uptake of macromolecules during stretch

NRCMs incubated in the presence of OTR (0.3 $\mu\text{mol/L}$) were either stretched for 24 h at amplitudes of 10, 15 and 20%, or left stationary in identical 6-well plates with collagen type I-coated silicone surfaces. The higher the stretch amplitude, the more OTR was

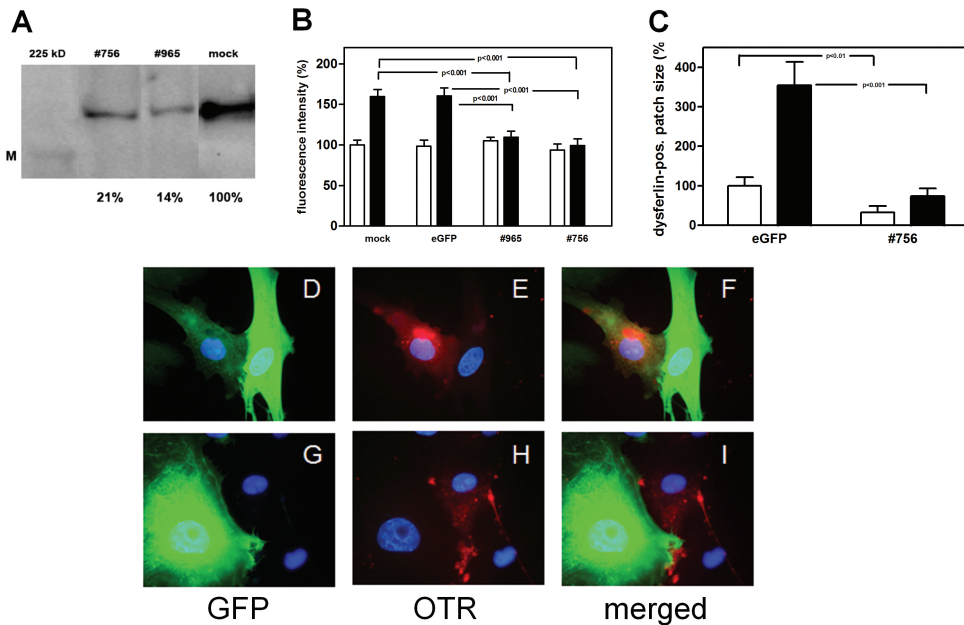


Figure 3.7: Before the start of the uptake experiment, NRCMs were either mock-transduced (mock) or infected with SIN-LVs encoding shRNAs specific for eGFP (eGFP) or dysferlin (#965 and #756) at an MOI of 10 HTUs per cell. **(A)** Western blot analysis indicated that dysferlin levels in the NRCMs transduced with SIN-LV965 or SIN-LV756 were 4- to 5-fold lower than those in mock-transduced NRCMs. **(B)** Uptake of OTR (0.3 $\mu\text{mol/L}$) by NRCMs incubated for 24 h in the absence (white) or presence (black) of 300 $\mu\text{g/mL}$ of GRGDS. OTR-associated fluorescence intensities are normalized to the value determined in mock-transduced NRCMs incubated for 24 h without GRGDS. Number of data per bar from left to right: 35, 43, 41, 39, 40, 27, 33 and 37, originating from 3 independent experiments. Indicated are mean values and SEM. **(C)** Dysferlin-positive patch areas in NRCMs transduced with the SIN-LV encoding an eGFP-specific shRNA (eGFP) and in NRCMs infected with SIN-LVs encoding shRNA molecules specific for dysferlin (#756) at an MOI of 10 HTUs per cell. NRCMs had been incubated for 24 h in the absence (white) or presence (black) of 300 $\mu\text{g/mL}$ of GRGDS. Dysferlin-positive patch areas are normalized to the value determined in NRCMs transduced with the SIN-LV encoding an eGFP-specific shRNA (eGFP) incubated for 24 h without GRGDS. Indicated are mean values and SEM. **(D-I)** NRCMs transduced with SIN-LV756, a bicistronic LV encoding hrGFPI and a dysferlin-specific shRNA were incubated for 24 h with OTR (0.3 $\mu\text{mol/L}$) in the presence of 300 $\mu\text{g/mL}$ of GRGDS. Comparison of the hrGFPI fluorescence (GFP, green; D and G) and the OTR-associated red fluorescence (E and H) revealed a inverse relationship between hrGFPI expression level (which is coupled to the shRNA756 expression level) and the intracellular OTR concentration. In other words, the lower the dysferlin expression, the lower the uptake of OTR during integrin stimulation. Panels F and I are merged images.

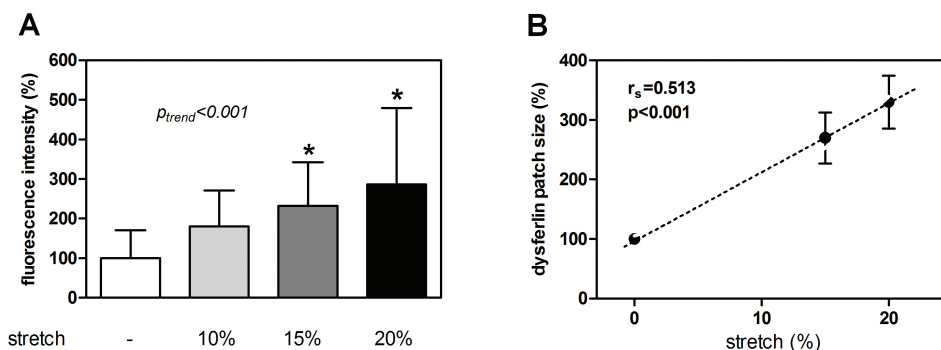


Figure 3.8: (A) Uptake of OTR (0.3 $\mu\text{mol/L}$) by NRCMs incubated for 24 h in the absence (-) or presence of 10%, 15% or 20% stretch. OTR-associated fluorescence intensity is normalized to the value determined in NRCMs incubated for 24 h without stretch. Number of data per bar from left to right: 36, 10, 14 and 10. Indicated are mean values and SD. * $p < 0.05$ vs. no stretch by Student t-test and Bonferroni correction. (B) Size of the dysferlin-positive PM patches in NRCMs after 24 h incubation in the absence or presence of 15% or 20% stretch. $r_s = 0.512$ indicates a high level of significance ($p < 0.001$). Dysferlin patch sizes are normalized to the value determined in NRCMs incubated for 24 h without stretch. Indicated are mean values $\pm$ SEM. The SEM of the values measured at 0% stretch is 7.2, and disappears within the size of the symbol. Number of data per point: 49 (no stretch), 27 (15% stretch) and 27 (20% stretch).

taken up ($p_{trend} > 0.001$) (figure 8A), and the larger was the dysferlin patch size ($r_s = 0.513$, $p > 0.001$) (figure 8B).

3.4 Discussion

The major findings of this study are that **1.** stimulation of integrins in NRCMs by an RGD-motif containing pentapeptide leads to extra uptake of macromolecules, **2.** the uptake of macromolecules by NRCMs, either stimulated by GRGDS or not, is dependent on the extracellular concentrations of these macromolecules, and **3.** on the duration of incubation with macromolecular cargo, **4.** the GRGDS-stimulated uptake of macromolecules by NRCMs is dependent on the extracellular concentrations of the pentapeptide, **5.** cell stretch stimulates the uptake of by NRCMs of macromolecules, **6.** uptake of OTR by NRCMs in the absence of GRGDS occurs by clathrin-mediated endocytosis, whereas **7.** macropinocytosis is involved in the uptake of OTR by NRCMs during integrin stimulation with RGD motif-containing pentapeptide, **8.** dysferlin-positive PM patches in NRCMs incubated with GRGDS are ~ 3 -fold larger than those observed in unstimulated NRCMs incubated without GRGDS, whereas **9.** contraction-arrested NRCMs do not show a GRGDS-dependent increase in dysferlin patch size, nor a GRGDS-dependent increase in OTR uptake, **10.** downregulation of *dysferlin* expression by shRNA-mediated RNA interference shRNA has no effect on basal OTR uptake but **11.** completely blocks the GRGDS-dependent OTR internalization.

We previously demonstrated that integrin stimulation promotes the release of cTnl from NRCMs.¹³ The present study indicates that integrin stimulation also facilitates the uptake of proteins (*i.e.* OTR) and other macromolecules (*i.e.* DTR) by cardiomyocytes. These findings may be explained by the theory of PM "wounding", which proposes that mechanical forces cause transient increases in PM permeability allowing macromolecules to both leave and enter the injured cells.^{2,14,24} The uptake of macromolecules by cardiomyocytes is apparently not carried out by cargo-specific uptake systems, as we found more or less similar results for the uptake of the protein ovalbumin and the branched glucose polymer dextran.

Although the uptake of OTR and DTR by NRCMs increased with their extracellular concentration, the uptake of these compounds cannot be explained by simple diffusion given the stimulatory effect of GRGDS on the internalization process. In fact, basal OTR uptake was sensitive to chlorpromazine, suggesting that it occurs via clathrin-mediated endocytosis.²⁰ Conversely, the RGD-dependent OTR uptake was sensitive to wortmannin, indicating the involvement of macropinocytosis.²² The observation that integrin stimulation triggers macropinocytosis is consistent with a previous report showing that human adenovirus serotype 2-induced macropinocytosis is integrin-dependent.²⁵ Apparently, macropinocytosis is also dysferlin-dependent, as lowering dysferlin levels by RNA interference inhibited RGD-dependent OTR uptake (figure 7A). While pharmacological inhibitors of endocytosis usually provide a good first indication of the specific pathway(s) involved in the uptake of a particular compound by cells, the results obtained with these drugs should be interpreted with caution due to possible pleiotropic effects and lack of specificity.²⁶ Accordingly, definitive proof for the role of clathrin-mediated endocytosis and macropinocytosis in the uptake of extracellular macromolecules by NRCMs awaits future studies involving the use of electron microscopy, dominant-negative versions of proteins with well-defined roles in endocytosis and/or RNA interference of genes engaged in specific endocytic pathways.

Protein release from vital cardiomyocytes is a well-known phenomenon. There is abundant literature about release of cardiac troponins in patients without acute coronary syndrome.²⁷⁻²⁹ Transient disruptions of PM integrity are now known to occur in a variety of mechanically active tissues under physiological conditions. These tissues include skin, gut, aorta, skeletal muscle and heart.³⁰ Approximately 25% of the cardiomyocytes examined in the normal rat heart had suffered constitutive non-lethal PM disruptions, as demonstrated by the intracellular presence of serum albumin.² Myocardial overloading, such as occurs during cardiac pressure or volume overload, increases this frequency of cardiomyocyte membrane wounding,^{1,2,14} probably mediated by integrins that transmit the mechanical force across the PM.³¹

The present study shows that uptake of OTR and DTR by NRCMs was intensified by integrin stimulation. Several mechanisms modulating endocytosis play a role in the uptake of extracellular macromolecules by cardiomyocytes, including the activation of stress-induced ion channels, mechanotransduction via integrin receptor binding to extracellular matrix components, and internal cytoskeletal tension.^{7,32-34} A mechanism for this macromolecule uptake behavior has been described in terms of contraction-induced resealable sarcolemmal membrane disruptions ("wounding") that have been observed in skeletal muscle as well as in isolated perfused rat hearts.^{1,2} Altered transient sarcolem-

mal permeability sufficient to release bFGF was inferred from the increased uptake of fluorescein-labeled dextran by ventricular cardiomyocytes of adult rats after pacing.¹⁴ Uptake of fluorescein-labeled dextran by paced cardiomyocytes was 3- to 50-fold higher than by unstimulated control cells depending on the specific preparation of cardiomyocytes and batch of fluorescently labeled dextran used.¹⁴ Along the same line, treatment with the β -adrenergic agonist isoproterenol stimulated the release of bFGF from beating adult rat hearts and was accompanied by an increased cardiac uptake of serum albumin from the circulation.² To determine whether membrane depolarization, mechanical activity, or both, were required to facilitate fluorescein-labeled dextran uptake, Kaye and co-workers¹⁴ used the L-type Ca^{2+} channel antagonist verapamil and the non-competitive inhibitor of class II myosin 2,3-butanedione monoxime (BDM) to block both Ca^{2+} currents and myofibrillar contractions or to exclusively interfere with myofibrillar contractions, respectively. In the presence of 10 $\mu\text{mol/L}$ of verapamil uptake of fluorescein-labeled dextran by cardiomyocytes exposed for 48 h to continuous uniform electric field stimulation was below that observed in quiescent control cells. Moreover, addition of BDM to a final concentration of 10 mmol/L caused uptake of fluorescein-labeled dextran by paced cardiomyocytes and by quiescent control cells to be equally low.¹⁴ Thus, it appears that mechanical activity favors the occurrence of transient PM disruptions and that these lesions facilitate the uptake of extracellular macromolecules by cardiomyocytes. Our finding that upon incubation with GRGDS non-beating NRCMs showed no significant increase in OTR uptake or dysferlin patch size is consistent with this idea (figure 6C). Our observation that the average size of the dysferlin-positive PM patches is ~ 3 -fold larger in GRGDS-treated beating NRCMs than in untreated beating NRCMs (figure 6A-C), suggests that integrin stimulation results in extra membrane wounding and its subsequent repair by a dysferlin-dependent resealing mechanism. Alternatively, dysferlin may play an active role in GRGDS-stimulated macropinocytosis.

The RGD-sequence is a ligand for integrins,¹⁰ and can be used to simulate myocardial mechanical stretch *in vitro*. The RGD-dependent uptake of OTR (figure 3A) and the RGD-dependent increase in the average size of the dysferlin-positive PM patches (figure 6A-C) have also been observed with stretch of the NRCMs. Stretch of NRCMs showed a stretch-dependent increase of OTR uptake and corresponding increase in dysferlin patch size (figure 8A and B). These observations indicate that spontaneously contractile NRCMs undergoing integrin stimulation by incubation with RGD-motif containing pentapeptide are a valuable model to study integrin-mediated processes such as occur during stretch of these cells *in vitro* and probably also in overload-induced hypertrophic and postinfarct remodeling *in vivo*.³⁶ However, the model depends on contractile activity as in non-beating NRCMs stimulation with GRGDS had no effect on OTR uptake nor on dysferlin patch size.

One of the responses of the PM of NRCMs to integrin stimulation is an increase in cellular permeability to macromolecules associated with an increase in dysferlin-positive PM areas. Dysferlin was the first identified member of a putative muscle-specific repair complex that permitted rapid resealing of PM disruptions induced by mechanical stress. In normal myofibers, dysferlin is predominantly localized in the PM and in subsarcolemmal vesicles.⁴ According to recent findings, membrane disruption causes influx of extracellular Ca^{2+} ions into muscle fibers. The resulting high intracellular Ca^{2+} concen-

tration triggers the aggregation of dysferlin-carrying repair vesicles and the migration of these vesicles toward the site of injury,³⁵ where they fuse with each other and with the PM. Dysferlin is thought to facilitate vesicle docking and fusion with the PM in this repair process, resulting in a membrane patch across the site of membrane disruption and a re-seal of the disrupted PM. The understanding of dysferlin and its role in membrane repair, signaling, muscle physiology, and pathophysiology has greatly improved in studies in patients and experimental animals with muscular dystrophy.^{35,37} However, further studies should elucidate the active role of dysferlin in the GRGDS-stimulated macropinocytosis of NRCMs.

Conclusion: In NRCMs, stimulation of integrins by a RGD motif-containing pentapeptide causes uptake of macromolecules through macropinocytosis. Dysferlin may have a role in this uptake process as reducing the intracellular levels of this membrane repair protein 4- to 5-fold through RNA interference prohibited GRGDS-induced uptake of OTR. Contractile activity is a crucial factor in the RGD-dependent uptake of OTR which may argue for the existence of a functional link between the repair of PM damage and macropinocytosis.

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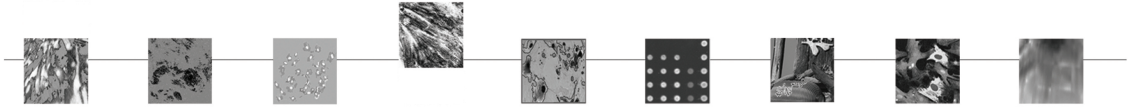
3.5 Acknowledgement

Minka Bax, Cindy Schutte and Lizet van der Valk are gratefully acknowledged for expert technical assistance.

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Chapter 4

Nitric oxide regulates integrin stimulation-induced hypertrophy in neonatal rat cardiomyocytes

4

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Abstract

Introduction Our research group previously showed that integrin stimulation with a RGD motif-containing pentapeptide (GRGDS) causes hypertrophy of neonatal rat cardiomyocytes (NRCMs).¹ **Purpose of the study** To investigate the effects of varying the NO-delivering capacity of NRCMs on GRGDS-induced hypertrophy. **Methods** NO production in NRCMs was either inhibited by treatment with the NO synthase (NOS) inhibitor L-NAME or by transduction with lentivirus vectors (LVs) encoding PIN (protein inhibitor of neuronal NOS [NOS1]) or a *NOS1*-specific short hairpin RNA (shNOS1) or was stimulated by incubation with the NO donor sodium nitroprusside (SNP) or with lipopolysaccharide (LPS), which triggers synthesis of inducible NOS (NOS2). The NRCMs were stained for nuclei and α -actinin to quantify cell surface area (CSA). Intracellular levels of PIN, shNOS1, NOS2, AKT1/2 (AKT), phospho-AKT1/2/3 (P-AKT) and phospho-S6 kinase were determined directly or indirectly by (immuno)fluorescence microscopy and/or western blotting. **Results** GRGDS-induced hypertrophy of NRCMs was dose-dependently blocked by L-NAME, which blockade could be overcome by SNP treatment. Suppression of NOS1 activity or production of PIN or shNOS1 inhibited GRGDS-induced hypertrophy, dependent on the expression level of the transgene. When either SNP or 8-Br-cGMP were administered together with GRGDS to *PIN*-transduced NRCMs, hypertrophy was rescued. Integrin stimulation-induced hypertrophy was associated with a 3- to 4-fold increase in P-AKT/AKT ratio, which increase was antagonized by L-NAME. Pharmacological inhibitors of soluble guanylate cyclase, phosphatidylinositol 3-kinase and AKT1/2/3 suppressed the GRGDS-induced increase in mean CSA as did the forced expression of a dominant-negative version of AKT1. AKT overexpression led to NRCM hypertrophy that was partly antagonized by rapamycin. LPS treatment without integrin stimulation also caused an increase in the average CSA of NRCMs, dependent on intracellular NOS2 level. LPS-induced NRCM hypertrophy was prevented by L-NAME. **Conclusion** NO is involved in the regulation of integrin stimulation-dependent hypertrophy of NRCMs and exerts at least part of its hypertrophic effects through activation of the PI3K/AKT signaling pathway.

4.1 Introduction

The contractile activity of cardiomyocytes leads to their periodic shortening and lengthening, but at the same time these cells experience the movements of neighboring cardiomyocytes. These movements are sensed by various mechanosensors in the sarcolemma including stretch-activated ion channels and integrins.² Integrins are heterodimeric transmembrane protein complexes directly linking the extracellular matrix (ECM) to the cytoskeleton.^{3,4} Binding of integrins by ECM components or ECM-like substances leads to cellular signaling involving, amongst others, p130 Crk-associated substrate (p130 CAS) family members, focal adhesion kinase (FAK), Src family kinases, Crk, the integrin-linked kinase (ILK)-PINCH-parvin complex, Nck⁵⁻⁹ and possibly neuronal NO synthase (nNOS/NOS1).^{1,10} Using neonatal rat cardiomyocyte (NRCM) cultures, our research group previously showed that integrin ligation by a pentapeptide (GRGDS) containing the Arg-Gly-Asp (RGD) sequence resulted in (i) elevated intracellular Ca²⁺ and NO concentrations¹⁰ and (ii) intracellular Ca²⁺ and NO concentration-dependent cellular hypertrophy.¹

In the present study, we examined whether integrin stimulation-induced hypertrophy of NRCMs could be modulated by changing the bioavailability of NO via (i) overexpression of a recombinant gene encoding the protein inhibitor of NOS1 of rats (PIN), (ii) short hairpin RNA (shRNA)-mediated inhibition of rat *NOS1* expression, (iii) lipopolysaccharide (LPS)-induced inducible NOS (iNOS/NOS2) production,¹¹ (iv) incubation with N^G-nitro-L-arginine methyl ester (L-NAME) which is a general inhibitor of all NOS isoforms including endothelial NOS (eNOS/NOS3) and (v) exogenous NO supplementation by the NO donor sodium nitroprusside (SNP). PIN, also called dynein light chain LC-8-type I, is a 89-amino-acid protein that inhibits NOS1 activity by destabilizing its dimeric structure.¹²

The recombinant *PIN* gene and the expression unit specifying the rat *NOS1*-specific shRNA (shNOS1) were introduced in the NRCMs using self-inactivating human immunodeficiency type 1 vectors (SIN-LVs) that also contained a *green fluorescent protein* (GFP) gene for quantifying transduction levels.

NO exerts its biological effects primarily via the intracellular NO receptor soluble guanylate cyclase (sGC), whose catalytic activity is enhanced by binding of NO leading to an increase in cGMP production and subsequent signaling via protein kinase G.¹³ We hence investigated the role of active sGC in GRGDS-induced NRCM hypertrophy by using a specific inhibitor of sGC, 1*H*-[1,2,4]oxadiazolo-[4,3-*a*]quinoxalin-1-one (ODQ)¹⁴ and a cell-permeable cGMP analogue, 8-Br-cGMP, to bypass the ODQ-imposed block of sGC activity.

Finally, to address the issue of whether integrin stimulation of cardiomyocytes leads to physiological or pathological hypertrophy,¹⁵⁻¹⁹ we studied the role of phosphoinositide-3-kinase (PI3K)/AKT signaling in the GRGDS-induced changes of NRCM surface area. To this end, we employed the PI3K inhibitor wortmannin, AKT-specific and phospho (P)-AKT-specific antibodies, the isoform-nonspecific AKT inhibitor SH-6, the mammalian target of rapamycin (mTOR) inhibitor rapamycin and LVs encoding either a dominant-negative version of murine AKT1 (DN-AKT1) or a constitutively active AKT1. Our results support a model in which integrin stimulation of NRCMs leads to NO-dependent phosphorylation of AKT and consequently a P-AKT-dependent physiological hypertrophic response.

4.2 Materials and methods

4.2.1 Cardiomyocyte cultures

Cultures of ventricular cardiomyocytes of two-day-old rats were prepared as described before.²⁰ Briefly, ventricular myocardium was dissociated with collagenase (CLS-2; Worthington, Lakewood, NJ, USA) and the resulting cell suspension was transferred to plastic dishes (Falcon Primaria; BD Biosciences, Breda, the Netherlands) to allow preferential attachment of non-cardiomyocytes. One h later, non-adherent cells were transferred to Petri dishes (Falcon Primaria; Ø35 mm), each containing a glass coverslip (Ø25 mm) coated with rat tail collagen type I solution (20 µg/mL; Sigma-Aldrich, Zwijndrecht, the Netherlands). The culture medium consisted of Ham's F10 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 10% heat-inactivated horse serum (HS), 100 U/mL penicillin and 100 µg/mL streptomycin (all from Invitrogen, Breda, the Netherlands). The culture medium was replaced 24 h after seeding of the cells by a 1:1 (v/v) mixture of Ham's F10 medium and Dulbecco's modification of Eagle's medium (DMEM; Invitrogen) containing 5% HS, antibiotics and 100 µmol/L 5-bromo-2-deoxyuridine (BrdU; Sigma-Aldrich) to inhibit proliferation of cardiac fibroblasts. The NRCM cultures were kept in a humidified incubator at 37 °C and 5% CO₂. The medium was replaced 24 h after the start of the BrdU treatment by a 1:1 (v/v) mixture of Ham's F10 medium and DMEM containing 2.5% HS and antibiotics. The experiments had the approval of the Animal Experiments Committee of the Leiden University Medical Center (LUMC).

4.2.2 Construction of SIN-LV shuttle plasmids

The coding sequence of the rat *PIN* gene was amplified from IMAGE clone 6887897 (imaGenes, Berlin, Germany) by polymerase chain reaction (PCR) in a total volume of 50 µL using 50 ng template, 0.5 µmol/L of forward (5'-CAGACGCGTATGTGCGACCGGAAGG-3') and 0.5 µmol/L of reverse (5'-CCACTCGAGTTAACCAGATTGAACAGGA-3') primer, 200 µmol/L of each dNTP, 1× Phusion HF buffer (Finnzymes, Espoo, Finland) and 1 U Phusion High-Fidelity DNA polymerase (Finnzymes). The PCR was performed according to the protocol provided with the DNA polymerase using an annealing temperature of 60°C and 35 amplification cycles. The 288-bp PCR product was purified from a 1% agarose gel using the JETSORB Gel Extraction Kit (GENOMED, Löhne, Germany), digested with MluI and XhoI and combined with the 8.4-kb MluI×XhoI fragment of pLV.CMV.IRES.eGFP²¹ using T4 DNA ligase (Fermentas, St. Leon-Rot, Germany). The resulting SIN-LV shuttle plasmid pLV.CMV.rPIN.IRES.eGFP and the starting construct pLV.CMV.IRES.eGFP (negative control) were used for generating SIN-LV particles as described below.

To repress rat *NOS1* expression, a SIN-LV shuttle construct encoding a shRNA targeting the mouse *NOS1* gene and matching perfectly with the coding sequence of the rat *NOS1* gene was obtained from the MISSION shRNA library (Sigma-Aldrich; clone TRCN0000072123). As negative control, another SIN-LV shuttle plasmid from the MISSION shRNA library (clone TRCN0000045425) encoding a shRNA targeting the human *NOS1* gene (CONsh) was used. The 5' end of this shRNA has 3 mismatches with the

rat *NOS1* gene and should therefore have no effect on *NOS1* expression in NRCMs. In both constructs, the puromycin N-acetyl transferase-coding sequence was replaced by that of the GFP of *Renilla reniformis* (hrGFPI) to allow easy assessment of transduction efficiencies. To this end, plasmid pLV.hPGK.hrGFPI (nucleotide sequence available upon request) was digested with *SpeI* and *KpnI* and the resulting 1.1-kb DNA fragment containing the hrGFPI-coding sequence was combined with the 6.2-kb *SpeI*×*KpnI* fragment of the two SIN-LV shuttle constructs from the Mission shRNA library using T4 DNA ligase.

To investigate the role of Akt1 signaling in the hypertrophic response to GRGDS stimulation, we constructed the SIN-LV shuttle plasmid pLV.CMV.iCA-AKT1.IRES.eGFP (nucleotide sequence available upon request) encoding the *Aequorea victoria* enhanced green fluorescent protein (eGFP) and a constitutively active version of human AKT1 (iCA-AKT1) whose activity is induced by 4-hydroxytamoxifen (4-OHT). The iCA-AKT1 protein was rendered constitutively active by adding the first 14 amino acids of the chicken Src protein, which encompass a myristoylation consensus sequence, to the N-terminus of human AKT1.²² In addition, the ligand-binding domain of mouse estrogen receptor 1 was fused to the C-terminus of human AKT1, causing it to bind to heat shock protein 90 (Hsp90) in the absence of 4-OHT. In the presence of 4-OHT, iCA-AKT1 is released from Hsp90 enabling it to mediate downstream signaling.

To block AKT signaling, a plasmid encoding a dominant-negative version of mouse AKT1 designated DN-AKT1 was obtained from Addgene (plasmid 16243).²³ This plasmid was digested with *SmaI* and *HincII* and the 1.4-kb fragment containing the DN-AKT1-coding sequence was combined with the 8.2-kb *SmaI* fragment of pLV.CMV.IRES.eGFP using T4 DNA ligase to generate pLV.CMV.DN-AKT1.IRES.eGFP.

All restriction enzymes were from Fermentas. Ligation mixtures were introduced in chemocompetent GeneHogs cells (Invitrogen) and large stocks of the correct plasmids were prepared with the aid of the JETSTAR 2.0 Plasmid Maxiprep Kit (GENOMED).

4.2.3 SIN-LV production

Vesicular stomatitis virus G protein-pseudotyped SIN-LVs were produced in 293T cells as described previously.²¹ The gene transfer activity of the vector stocks was determined by end-point titration on HeLa indicator cells using flow cytometric analysis of *eGFP* (LV.CMV.IRES.eGFP, LV.CMV.rPIN.IRES.eGFP, LV.CMV.iCA-AKT1.IRES.eGFP and LV.CMV.DN-AKT1.IRES.eGFP) or *hrGFPI* (shRNA-encoding SIN-LVs) expression as read-out. The titers of the SIN-LV preparations are thus expressed in HeLa cell-transducing units (HTUs) per mL.

Forty-eight to 72 h after seeding, the NRCMs were transduced with SIN-LV particles at a vector dose of 5 HTUs/cell in case of the eGFP-encoding SIN-LVs and of 10 HTUs/cell for the shRNA-specifying SIN-LVs (*i.e.* LV.shNOS1.PGK.hrGFPI and LV.CONsh.PGK.hrGFPI). The next day, the NRCMs were washed three times with phosphate-buffered saline (PBS) and subsequently provided with fresh culture medium. At 7 days post-transduction, the cultures were used for further experiments.

4.2.4 Experimental protocol

Integrin stimulation of NRCM cultures was accomplished by adding the pentapeptide GRGDS (Sigma-Aldrich; 10 mg/mL in PBS) to the culture medium to reach a final concentration of 300 μ g/mL. Cells that received PBS (30 μ L/mL of culture medium) only served as negative control. To pharmacologically inhibit NOS activity, we used L-NAME (Sigma-Aldrich; 10 mmol/L stock solution in PBS) at final concentrations of 5, 10, 20 or 100 μ mol/L. Exogenous NO was generated by adding SNP (pharmacy of LUMC; 20 mg/mL stock solution in PBS) to the culture medium at final concentrations of 5, 10, 20 or 40 μ g/mL. L-NAME was added 30 min prior to GRGDS addition and SNP was added just prior to GRGDS addition. To induce NOS2 activity, NRCMs were incubated with 10 μ g/mL LPS (Sigma-Aldrich; 10 mg/mL stock solution in PBS) for 48 h. At 24 h after the addition of LPS to the culture medium, the NRCMs were treated with 100 μ mol/L L-NAME or an equivalent volume of vehicle for another 24 h. Earlier, it has been demonstrated that NOS2 is detectable in cultured NRCMs already 6 h after exposure to 10 μ g/mL LPS.¹¹

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4.2.5 (Immuno)fluorescence microscopy

Twenty-four h after GRGDS addition, the NRCMs were rinsed 3 times with PBS + 1% FBS (PBSF), fixed by a 30-min incubation in 4% buffered formaldehyde (Klinipath, Duiven, the Netherlands), washed twice with PBSF and permeabilized by incubation for 30 min in PBS + 0.1% Triton X-100 (Sigma-Aldrich). After washing twice with PBSF, the cells were incubated overnight at 4°C with mouse monoclonal anti-sarcomeric α -actinin antibody (A7811; Sigma-Aldrich) diluted 1:400 in PBSF and, in a separate series of experiments, with rabbit anti-NOS2 antibodies (ab3523; Abcam, Cambridge Science Park, Cambridge, UK) at 1:400 dilution. After washing twice with PBSF, the cells were incubated with 500 $\times$ diluted secondary antibodies for 60 min. Rabbit anti-mouse IgG conjugated to AlexaFluor 568 (A11061; Invitrogen) was used for labeling the anti- α -actinin antibody and the anti-NOS2 antibodies were visualized using donkey anti-rabbit IgG conjugated to AlexaFluor 488 (A21206; Invitrogen). Following two wash steps with PBSF, the cultures were stained with Hoechst 33342 (Invitrogen; 10 μ g/mL in PBSF) for 8 min. After washing twice with PBSF, the coverslips were mounted on microscope slides using Vectashield (Vector Laboratories, Burlingame, CA, USA). Immunofluorescence images were produced using a fluorescence microscope (Nikon Eclipse; Nikon Europe, Badhoevedorp, the Netherlands) equipped with a digital camera (Nikon DXM 1200). As a measure of cell hypertrophy, NRCM surface area (CSA) was determined since average CSA has been proven to correlate well with other measures of NRCM hypertrophy, like [3 H]-leucine incorporation.²⁴ CSA was quantified by morphometric analysis of α -actinin-stained NRCMs using Image Pro Plus software (Media Cybernetics, Silver Spring, MD, USA). Typically, per experimental group the CSA of ≥ 5 randomly picked NRCMs per field were determined in multiple fields.

4.2.6 Assessment of the relationship between PIN level and NRCM hypertrophy

Of randomly selected LV.CMV.rPIN.IRES.eGFP-transduced NRCMs from 5 independent experiments we determined, with the aid of Image Pro Plus, the eGFP signal intensity and CSA as indirect and direct measures of PIN concentration and GRGDS-induced hypertrophy, respectively. Signal intensity of eGFP ranged from 0 to 70 arbitrary units (AUs) per cell. For several eGFP/PIN signal thresholds, the average CSA of all cells with an eGFP signal $\geq N$ AUs and the mean CSA of all cells with an eGFP signal intensity <20 AUs were calculated. The percentage inhibition of hypertrophy in the different subpopulations of cells displaying relatively higher eGFP signals and thus containing relatively more PIN was calculated for several signal thresholds N between 20 and 70 AUs using the formula: $100 - \{[\text{mean \% GRGDS-induced hypertrophy (eGFP threshold } \geq N)] / \text{mean \% GRGDS-induced hypertrophy (eGFP threshold } <20)] \times 100\}$.

4.2.7 Investigation of the relationship between shNOS1 level and NRCM hypertrophy

Of 40-50 NRCMs per coverslip we determined, with the aid of Image Pro Plus software, the hrGFPI signal intensity as an indirect measure of shNOS1 concentration and the CSA as a measure of hypertrophy. Signal intensity of hrGFPI ranged from 0 to 250 AUs per cell.

4.2.8 Assessment of the relationship between NOS2 level and NRCM hypertrophy

Of at least 50 NRCMs per coverslip CSA and NOS2 content were determined by analysis of immunofluoromicrographs using Image Pro Plus software. Signal intensity of NOS2 ranged from 25 to 55 AUs per cell.

4.2.9 Investigation of the role of sGC in GRGDS-induced NRCM hypertrophy

The major intracellular NO receptor is the enzyme sGC, which after binding of NO to its heme moiety increases its catalytic activity up to 400-fold.^{25,26} We used 1*H*-[1,2,4]oxadiazolo-[4,3-*a*]quinoxalin-1-one (ODQ; Sigma-Aldrich; 10 $\mu\text{mol/L}$) to selectively inhibit sGC¹⁴ and 8-Br-cGMP (Sigma-Aldrich; 0.5 mmol/L) to circumvent the blockade of sGC by ODQ.

4.2.10 Investigation of the role of Src family kinases (SFKs) in the transduction of integrin stimulation-dependent prohypertrophic signals

Since Src family kinases (SFKs) are important mediators of integrin stimulation-dependent cell signaling,^{9,27} we used the SFK inhibitor 4-amino-1-*tert*-butyl-3-(1'-naphthyl)pyra-

zolo [3,4-d]pyrimidine (PP1 analogue; 30 $\mu\text{mol/L}$; Merck Chemicals, Darmstadt, Germany) to test the importance of this non-receptor tyrosine kinase for GRGDS-induced NRCM hypertrophy.

4.2.11 Study of the involvement of PI3K in the transduction of integrin stimulation-dependent prohypertrophic signals

To investigate whether PI3K is implicated in GRGDS-induced NRCM hypertrophy, we analyzed the effect of various concentrations of the PI3K inhibitor wortmannin (10, 50, 100, 500 and 1000 nmol/L; Sigma-Aldrich; 1 mmol/L stock solution in PBS) on the average CSA of GRGDS-treated NRCMs. NRCMs not exposed to GRGDS were treated in parallel with the same range of wortmannin doses to distinguish between the effects of the PI3K inhibitor on integrin ligation-independent and on integrin stimulation-induced CSA. After 24 h of incubation, the average CSA of the NRCMs in the two cultures was x_n and $(x_n + y_n)$, respectively, with n representing one of the 5 wortmannin doses. Two parallel cultures of NRCMs were incubated without wortmannin, one without GRGDS and one with GRGDS. After 24 h of incubation the mean CSA of the cardiomyocytes in the two cultures was X and $(X + Y)$, respectively. The average CSA in the control culture without GRGDS and without wortmannin was set to 100% with the inhibitory effect of a particular dose of wortmannin on integrin ligation-independent CSA being equal to $(X - x_n)$. The inhibiting effect of a specific dose of wortmannin on GRGDS-induced CSA is equal to $(Y - y_n)$.

4.2.12 Study of the involvement of AKT in the transduction of integrin stimulation-dependent prohypertrophic signals

In NRCMs that had been incubated for 24 h without or with GRGDS (300 $\mu\text{g/mL}$) in the absence or presence of 100 $\mu\text{mol/L}$ of L-NAME, AKT and P-AKT levels were quantified by immunocytochemistry using 1:500 dilutions of goat anti-human-AKT1/2 (sc-1619; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and of rabbit anti-human-P-AKT1/2/3 (sc-7985-R; Santa Cruz Biotechnology) antibodies, respectively. The latter antibodies recognize an epitope including the phospho-serine residue in the AKT regulatory domain (Ser⁴³⁷ for human AKT1). The primary antibodies were detected by AlexaFluor 568-conjugated donkey anti-goat IgG (A11057; Invitrogen) and AlexaFluor 488-conjugated donkey anti-rabbit IgG, respectively, both at 1:500 dilution.

In a separate series of experiments, we prepared cell homogenates by scraping $\approx 6 \times 10^5$ cells in 0.5 mL ice-cold lysis buffer, after 24 h of incubation without or with GRGDS (300 $\mu\text{g/mL}$). Lysis buffer consisted of 50 mmol/L Tris-HCl (pH 8.0), 150 mmol/L NaCl, 1% Nonidet P-40 (Merck Chemicals), 1 mmol/L sodium orthovanadate (Sigma-Aldrich) + freshly added protease inhibitors (Protease Inhibitor Cocktail Tablets; Roche, Almere, the Netherlands). Cell homogenates were stored at -80°C until use in western blot analysis. To this end, proteins were separated in a NuPage 10% Bis-Tris gel (Invitrogen) with MOPS as running buffer, and electro-transferred onto a polyvinylidene fluoride membrane (Hybond-P, GE Healthcare Europe, Diegem, Belgium), which was processed essentially as described before.¹ As a molecular weight marker Full Range

Rainbow marker (RPN800E; GE-Healthcare Europe) was used. AKT1/2 and P-AKT1/2/3 were labeled with the same antibodies as used for their immunocytochemical detection. The anti-AKT1/2 and anti-P-AKT1/2/3 antibodies were used at dilutions of 1:10,000 and 1:100,000, respectively. As secondary antibodies we employed donkey anti-goat IgG conjugated to horseradish peroxidase (HRP; sc-2020, Santa Cruz Biotechnology] at a dilution of 1:20,000 and goat anti-rabbit IgG-HRP (sc-3837; Santa Cruz Biotechnology) at a dilution of 1:50,000. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used for normalization purposes. This housekeeping protein was detected using a mouse monoclonal anti-GAPDH antibody (MAB374; Millipore, Billerica, MA, USA) at a dilution of 1:100,000 and 100,000× diluted goat anti-mouse IgG-HRP (sc-2005; Santa Cruz Biotechnology). Chemiluminescence was induced with the aid of an ECL Advance Western Blotting Detection Kit (GE Healthcare Europe) and signal intensities were determined with the aid of the ChemiDoc XRS+ Imager (Bio-Rad Laboratories, Veenendaal, the Netherlands).

To test whether ribosomal protein S6 kinase (S6K) is part of the signaling pathway involved in integrin stimulation-induced cardiomyocyte hypertrophy and to corroborate that S6K acts downstream of AKT, NRCMs were incubated with and without GRGDS in the absence and presence of the AKT inhibitor SH-6 (20 μ mol/L; ENZO Life Sciences, Zandhoven, Belgium). Next, P-S6K levels were determined by immunocytology using rabbit anti-P-S6K antibodies (S6311; Sigma-Aldrich) at 1:250 dilution in combination with 500× diluted AlexaFluor 488-conjugated donkey anti-rabbit IgG.

Besides by using the phosphatidylinositol analogue SH-6, AKT inhibition was also accomplished by transducing NRCMs with a SIN-LV encoding DN-AKT1 and eGFP. In DN-AKT1 the lysine residue at position 179 is substituted by a methionine, resulting in a version of AKT1 that lacks kinase activity.²³ NRCMs transduced with LV.CMV.IRES.eGFP instead of LV.CMV.DN-AKT1.IRES.eGFP served as negative control in this experiment.

Mice overexpressing constitutively active AKT1 proteins demonstrated an increase of myocardial mass.^{24,28-30} To test the effects of constitutive high-level AKT signaling on cardiomyocyte hypertrophy, NRCMs were transduced with a bicistronic SIN-LV encoding iCA-AKT1 and eGFP. In the absence of 4-OHT, iCA-AKT1 is maintained in an inactive state through association with Hsp90. Incubation of the cells with 250 nmol/L 4-OHT (Sigma-Aldrich) causes disruption of the iCA-AKT1-Hsp90 complex leading to the activation of iCA-AKT1.

4.2.13 Statistics

Results are expressed as means $\pm$ standard error of the mean (SEM), unless stated otherwise. The significance of differences between means was calculated by Student's *t*-test and one-way analysis of variance followed by the Bonferroni's *post hoc* test whenever appropriate using SPSS16 for Windows (SPSS, Chicago, IL, USA). Linear and non-linear fits were calculated in Graph Pad Prism 4 (Graph Pad Software, La Jolla, CA, USA). Experimental results were always compared with data from control experiments performed at the same day. Differences were considered significant if $p < 0.05$.

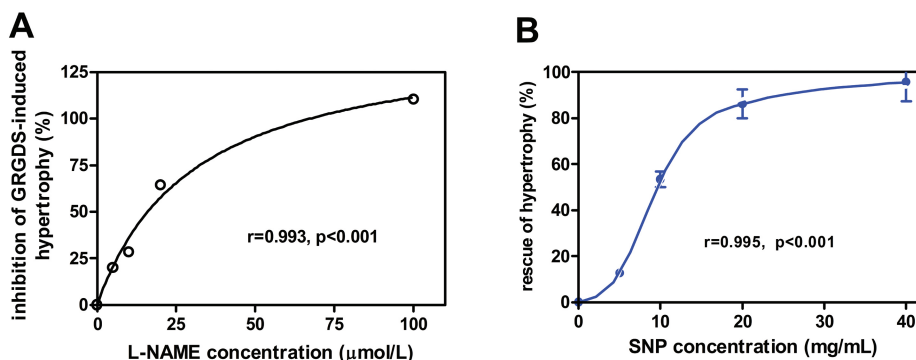


Figure 4.1: **A.** Percentage inhibition of GRGDS-induced NRCM hypertrophy is plotted against the L-NAME concentration. Per data point 51-67 cells were analyzed. **B.** Percentage stimulation of hypertrophy of NRCMs treated with GRGDS (300 $\mu\text{g/mL}$) plus L-NAME (100 $\mu\text{mol/L}$) by various doses of SNP. Per data point 42-88 cells were analyzed.

4

4.3 Results

4.3.1 The NOS inhibitor L-NAME represses GRGDS-induced NRCM hypertrophy

Previous work of our research group pointed to the involvement of NO signaling in the integrin stimulation-dependent hypertrophic response of cardiomyocytes.^{1,10} Using several concentrations of the NOS inhibitor L-NAME, ranging from 5 to 100 $\mu\text{mol/L}$, we found an inhibition of GRGDS-induced NRCM hypertrophy which was dependent on the L-NAME concentration (fig. 1A). By non-linear correlation analysis the correlation coefficient was 0.993 ($p<0.001$).

4.3.2 Exogenous NO donor administration can overcome L-NAME-dependent inhibition of GRGDS-induced NRCM hypertrophy

In NRCMs that were stimulated by GRGDS, hypertrophy was inhibited completely by 100 $\mu\text{mol/L}$ of L-NAME, but if combined with 5 to 40 $\mu\text{g/mL}$ of the NO donor SNP, inhibition was attenuated. Following treatment with 40 $\mu\text{g/mL}$ SNP, the average CSA in L-NAME-treated NRCMs went up to $95.6\pm 8.1\%$ of that of GRGDS-treated NRCMs not exposed to L-NAME or SNP ($r=0.995$; $p<0.001$) (fig. 1B).

4.3.3 Inhibition of NOS1 activity by forced PIN expression blocks GRGDS-induced NRCM hypertrophy

The observation that treatment of NRCMs with GRGDS peptides led to an increase in the amount of NOS1 molecules activated by phosphorylation at serine residue 1446¹ sug-

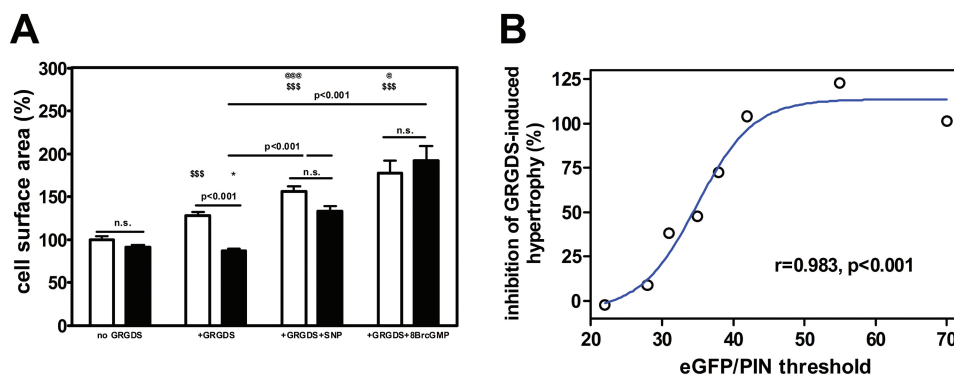


Figure 4.2: **A.** Mean CSAs ($\pm$ SEM) of NRCMs transduced with LV.CMV.IRES.eGFP (white bars) or with LV.CMV.rPIN.IRES.eGFP (black bars), treated for 24 h without GRGDS, with GRGDS (300 μ g/mL), with the combination of GRGDS (300 μ g/mL) and SNP (10 μ g/mL) and with the combination of GRGDS (300 μ g/mL) and 8-Br-cGMP. All bars are normalized to the average CSA of untransduced NRCMs incubated without GRGDS, SNP and 8-Br-cGMP. Numbers of cells analyzed are, from left to right, 128, 146, 178, 211, 109, 114, 10 and 10, obtained from 5 independent experiments. \$\$\$ $p < 0.001$ vs. "no PIN, no GRGDS"; @ $p < 0.05$ and @@@ $p < 0.001$ vs. "no PIN, +GRGDS". * n.s. vs. "+PIN, no GRGDS". **B.** Percentage inhibition of GRGDS-induced hypertrophy of NRCMs is plotted against the eGFP/PIN threshold value. Per data point, 31-46 cells were analyzed for eGFP intensity.

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gested an important role for this NOS isoform in integrin stimulation-dependent cardiomyocyte hypertrophy. To further investigate the contribution of NOS1 to GRGDS-induced cardiomyocyte hypertrophy, NRCMs were transduced with LV.CMV.rPIN.IRES.eGFP, a bicistronic SIN-LV coding for eGFP and the selective NOS1 inhibitor PIN or with the control vector LV.CMV.IRES.eGFP, which only directs the synthesis of eGFP. Averaged over 5 independent experiments, the pro-hypertrophic effect of the RGD motif-containing pentapeptide GRGDS was $28.0 \pm 3.8\%$ as determined from the CSAs of 178 NRCMs (fig. 2A). In NRCMs that had been transduced with LV.CMV.rPIN.IRES.eGFP, a nonsignificant decrease in average CSA was observed as compared to control cells both in the absence ($-9.1 \pm 2.9\%$) and presence ($-13.3 \pm 2.2\%$) of GRGDS. As expected from its site of action, the PIN-imposed block on GRGDS-induced hypertrophy was bypassed by SNP (10 μ g/mL) and by the cell-permeable cGMP analogue 8-Br-cGMP (0.5 mmol/L) resulting in increases in mean CSA of $33.4 \pm 6.2\%$, and of $92.2 \pm 17.0\%$, respectively, in comparison to LV.CMV.IRES.eGFP-transduced NRCMs not exposed to GRGDS (Fig. 2A).

In LV.CMV.rPIN.IRES.eGFP-transduced and GRGDS-treated NRCMs, eGFP signal intensity, an indirect measure of PIN expression level, correlated negatively with CSA by non-linear correlation analysis ($r = -0.49$; $n = 50$; $p < 0.001$). For several eGFP/PIN thresholds (between 22 and 70 AUs as determined by fluorescence microscopy), we determined the mean percentage of GRGDS-induced hypertrophy of all NRCMs above a certain eGFP/PIN threshold and divided that by the mean percentage of GRGDS-induced hypertrophy of all NRCMs below an eGFP/PIN threshold of 20. Non-linear correlation

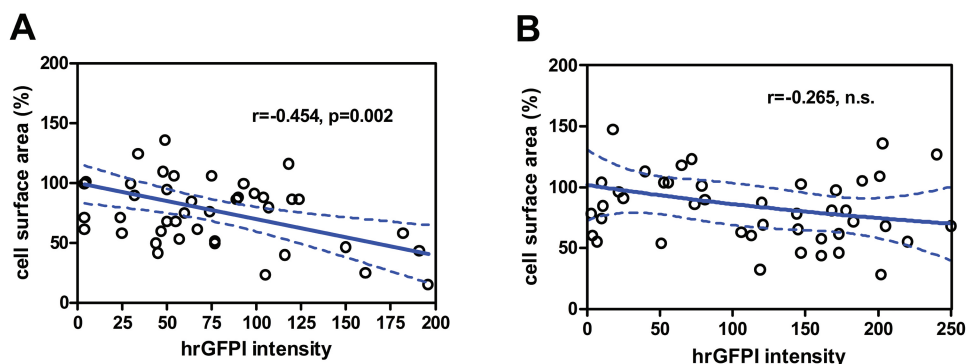


Figure 4.3: **A.** The CSA (AUs) of NRCMs transduced by LV.shNOS1.PGK.hrGFPI is negatively correlated with shNOS1 levels determined by measurement of hrGFPI signal intensity. Number of data points is 43. **B.** The CSA of NRCMs transduced by LV.CONsh.PGK.hrGFPI, which codes for a shRNA targeting a sequence in the human NOS1 transcripts that contains 3 mismatches with the rat NOS1 gene, does not correlate with hrGFPI signal intensity as an indirect measure of CONsh concentration. Number of data points is 45.

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analysis (fig. 2B) revealed that the extent of inhibition of GRGDS-induced NRCM hypertrophy by PIN was dependent on its intracellular concentration ($r = 0.983$; $p < 0.001$).

4.3.4 Knockdown of NOS1 expression by RNA interference inhibits GRGDS-induced NRCM hypertrophy

To confirm, by an independent method, the involvement of NOS1 in integrin stimulation-induced cardiomyocyte hypertrophy, NRCMs were transduced with a SIN-LV coding for a NOS1-specific shRNA or for a non-targeting control shRNA. In NRCMs that had been infected with LV.shNOS1.PGK.hrGFPI, hrGFPI signal intensity, an indirect measure of shNOS1 level, ranged from 0 to 200 AUs (as determined by fluorescence microscopy). In these cells, CSA after integrin stimulation was inversely correlated with hrGFPI signal intensity ($r = -0.454$; $n = 44$; $p = 0.002$) (fig. 3A). Conversely, in LV.CONsh.PGK.hrGFPI-transduced NRCMs that were treated with 300 $\mu\text{g/ml}$ GRGDS, no correlation between CSA and hrGFPI signal intensity was observed ($r = -0.265$; $n = 46$; $p = n.s.$) (fig. 3B).

4.3.5 Exogenous 8-Br-cGMP administration can overcome the inhibitory effect of suppressing sGC activity on GRGDS-induced NRCM hypertrophy

In NRCMs that were stimulated by GRGDS, hypertrophy was inhibited completely by 10 $\mu\text{mol/L}$ ODQ, an inhibitor of sGC (fig. 4). However, if 8-Br-cGMP (0.5 mmol/L) was coadministered with ODQ, hypertrophy occurred to an extent normally observed with GRGDS, and was independent of the presence of GRGDS (fig. 4), indicating that the

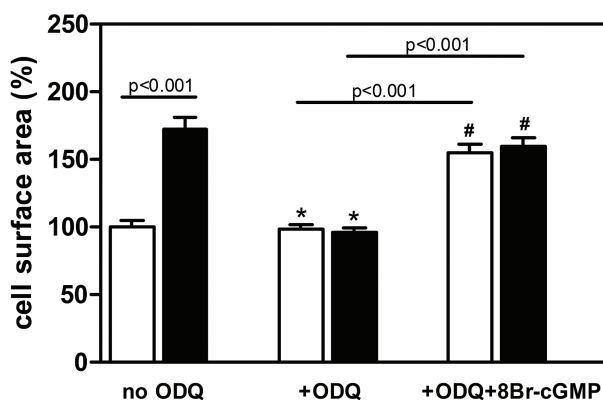


Figure 4.4: Mean CSAs ($\pm$ SEM) of NRCMs treated without GRGDS (white bars) and with GRGDS ($300 \mu\text{g/mL}$) (black bars) for 24 h in the absence and presence of ODQ ($10 \mu\text{mol/L}$) without or with 8-Br-cGMP (0.5 mmol/L). All bars are normalized to the average CSA of NRCMs incubated without GRGDS, ODQ and 8-Br-cGMP. Numbers of cells analyzed are, from left to right, 23, 23, 22, 18, 19 and 21. * n.s. vs. "no GRGDS, no ODQ". # n.s. vs. "+ GRGDS, no ODQ".

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NO-responsive system comprising sGC and cGMP is crucial for the integrin stimulation-dependent induction of hypertrophy in NRCMs.

4.3.6 Integrin stimulation-induced NRCM hypertrophy does not require SFK activity

Incubation of NRCMs with the specific SFK inhibitor PP1 ($30 \mu\text{mol/L}$) influenced neither the cell size of NRCMs incubated in the absence of GRGDS ($108.9 \pm 18.0\%$ with PP1 versus $100.0 \pm 20.0\%$ without PP1; $p=\text{n.s.}$) nor the hypertrophy induced by GRGDS (CSA compared to control cells: $153.8 \pm 35.5\%$ with PP1 and $146.9 \pm 39.5\%$ without PP1; difference between GRGDS-treated NRCM cultures with and without PP1: n.s.) (mean $\pm$ standard deviation [SD]).

4.3.7 Involvement of PI3K/AKT signaling in integrin stimulation-induced NRCM hypertrophy

While activation of receptor tyrosine kinases and the PI3K/AKT signaling cascade are strongly linked with physiological hypertrophy of cardiomyocytes, G-protein-coupled receptors and the calcineurin/nuclear factor of activated T-cells (NFAT) pathway are key players in pathological cardiac hypertrophy. Investigating the P-AKT/AKT ratio in GRGDS-treated NRCMs and the sensitivity of integrin stimulation-induced hypertrophy in these cells to PI3K inhibition should thus provide information about the nature of the hypertrophic process. Integrin stimulation of NRCMs by GRGDS caused a 3-4 fold increase in P-AKT/AKT ratio, which increase was reduced to <2-fold by the presence of L-NAME ($100 \mu\text{mol/L}$) as analyzed by western blotting (fig. 5A). The P-AKT/AKT ratio as deter-

mined by western blotting correlated quite well with that determined by immunocytology (fig. 5B). Thus, integrin stimulation by GRGDS elicits an NO-dependent signaling pathway that includes AKT. Upstream of AKT is PI3K that can be inhibited by wortmannin. Using wortmannin at various concentrations we noticed that the GRGDS-dependent increase in CSA of NRCMs is sensitive to wortmannin at concentrations ≥ 50 nmol/L (fig. 5D), whereas the GRGDS-independent CSA is not affected by wortmannin treatment (fig. 5C).

AKT activity is inhibited by SH-6 which is a cell permeable, reversible and substrate-competitive phosphatidylinositol analogue that inhibits the activation of AKT. Administration of SH-6 (20 μ mol/L) had no significant effect on the CSA of NRCMs that were incubated without GRGDS for 24 h, but completely inhibited GRGDS-induced hypertrophy of NRCMs (fig. 6A).

AKT activation stimulates phosphorylation (by mTOR complex 1) of the 70-kDa S6K at threonine residue 389.³¹ The resulting P-S6K protein stimulates protein synthesis through the phosphorylation of, amongst others, ribosomal protein S6 (rpS6), eukaryotic translation initiation factor 4B and eukaryotic elongation factor-2 kinase. We thus investigated by immunocytology, the effect of integrin stimulation and AKT inhibition on the level of P-S6K in NRCMs. Incubation of NRCMs with GRGDS caused an increase in P-S6K-associated fluorescence of $17.0 \pm 4.7\%$ ($p=0.005$ compared to NRCMs exposed to neither GRGDS nor SH-6) whereas coadministration of SH-6 reduced the P-S6K signal by $34.8 \pm 5.6\%$ in comparison to GRGDS-treated cells not incubated with SH-6 ($p<0.001$) and by $17.8 \pm 4.3\%$ compared to NRCMs exposed to neither GRGDS nor SH-6 ($p=0.003$) (fig. 6B).

Besides through pharmacological interventions, we also studied the involvement of AKT in integrin stimulation-induced cardiomyocyte hypertrophy by genetic means. To this purpose, we employed SIN-LVs encoding either a dominant-negative or constitutively active version of AKT1. Western blot analysis of protein lysates from NRCMs transduced with these SIN-LVs confirmed their functionality with LV.CMV.DN-AKT1.IRES.eGFP directing the synthesis of a 60 kDa protein species indistinguishable from wild-type AKT1 and LV.CMV.iCA-AKT1.IRES.eGFP specifying an AKT1 fusion protein of 105 kDa (fig. 6C).

Consistent with the results of the SH-6 treatment, overexpression of DN-AKT1 did not change the mean CSA of NRCMs incubated without GRGDS, but markedly reduced the GRGDS-induced hypertrophy (from $55.8 \pm 8.3\%$ to $27.8 \pm 8.4\%$; $p=0.047$) (fig. 6D).

Finally, overexpression of iCA-AKT1 induced hypertrophy of NRCMs (by $54.6 \pm 8.3\%$) that was only partially inhibited (to $29.4 \pm 8.5\%$; $p=0.052$) by 10 nmol/L rapamycin, an inhibitor of especially mTOR complex 1, which is downstream from AKT (fig. 6E).

4.3.8 LPS-induced NOS2 activity is equally efficient in promoting NRCM hypertrophy as integrin-stimulation dependent NOS1 activity

To study whether induction of NO signaling by other means than via integrin stimulation or exogenous NO donor administration could also induce cardiomyocyte hypertrophy, NRCMs were exposed to LPS, which triggers NOS2 expression via an NF- κ B-dependent pathway³² (fig. 7B-E). Induction of NOS2 resulted in an increase of mean CSA from

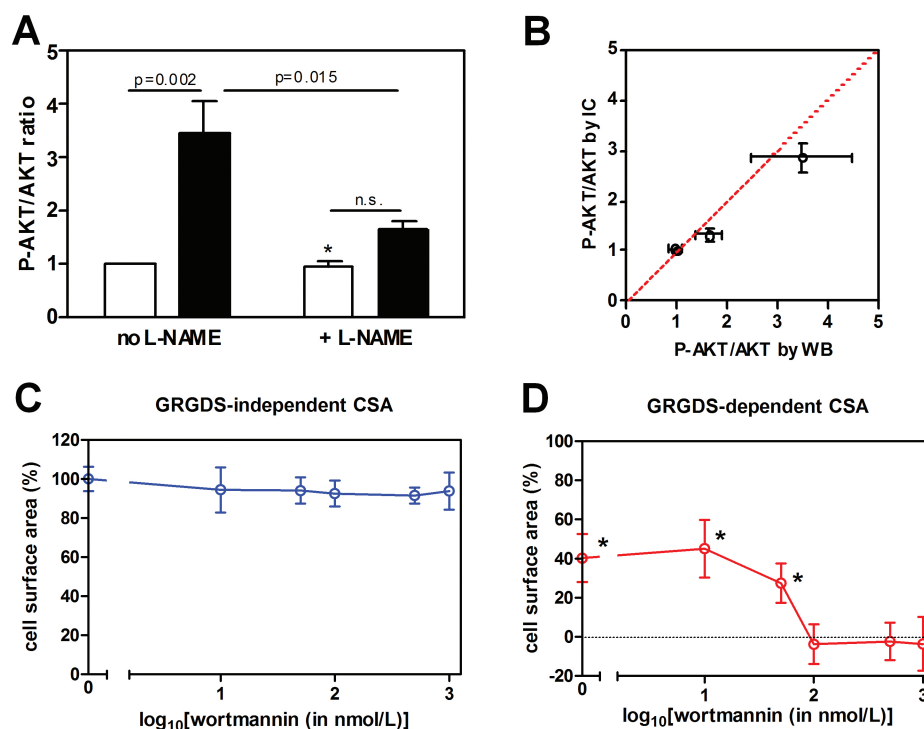


Figure 4.5: **A.** P-AKT/AKT ratio in NRCMs incubated without GRGDS (white bars) or with 300 $\mu\text{g/mL}$ GRGDS (black bars) in the absence or presence of 100 $\mu\text{mol/L}$ L-NAME as analyzed by western blotting. All bars are normalized to the average P-AKT/AKT ratio of NRCMs incubated without GRGDS and L-NAME. Numbers of cultures analyzed are, from left to right, 6, 10, 7 and 8. * n.s. vs. "no GRGDS, no L-NAME". **B.** P-AKT/AKT ratios (mean $\pm$ SD) were determined by immunocytochemistry (IC; 6-10 cells per data point) and western blotting (WB; 3 cultures per data point) in NRCMs incubated with or without GRGDS (300 $\mu\text{g/mL}$) in the absence and presence of 100 $\mu\text{mol/L}$ L-NAME. The red striped line represents the line of identity. **C.** For wortmannin doses >0 nmol/L, GRGDS-independent NRCM surface area is plotted against the common logarithm of the wortmannin concentration in the culture medium. All CSA values are normalized to the average CSA value of NRCMs incubated without GRGDS and wortmannin. Numbers of cells per data point are 7-14. **D.** For wortmannin doses >0 nmol/L, GRGDS-dependent NRCM surface area is plotted against the common logarithm of the wortmannin concentration in the culture medium. All CSA values are normalized to the average CSA value of NRCMs incubated without GRGDS and wortmannin. Numbers of cells per data point are 7-13. * $p < 0.05$ compared to an increase in CSA of 0%.

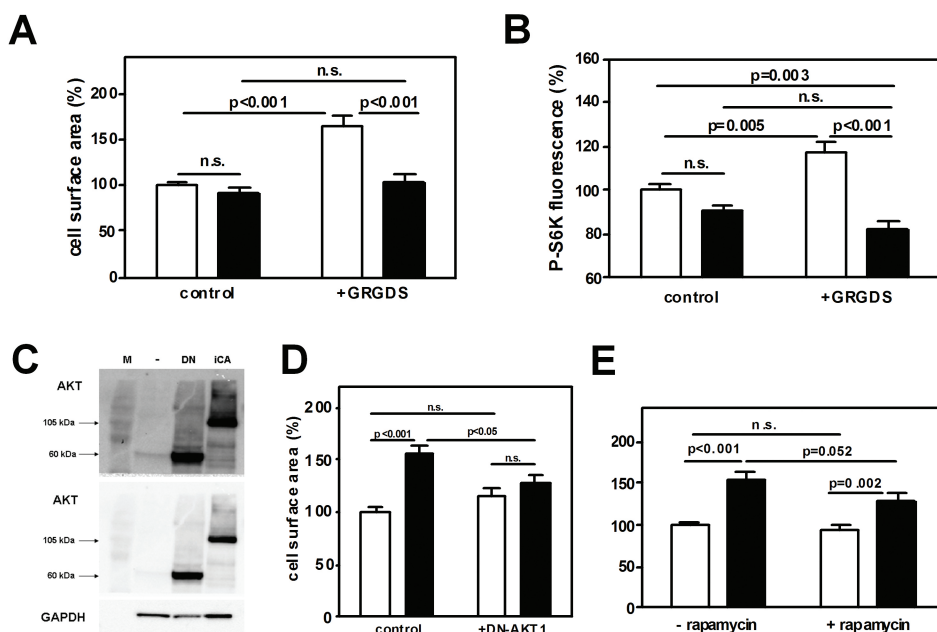


Figure 4.6: **A.** Mean CSAs ($\pm$ SEM) of NRCMs treated without SH-6 (white bars) and with SH-6 ($20 \mu\text{mol/L}$) (black bars) for 24 h in the absence and presence of GRGDS ($300 \mu\text{g/mL}$). All bars are normalized to the average CSA of NRCMs incubated without GRGDS and SH-6. Numbers of cells analyzed are, from left to right, 25, 25, 20 and 22. **B.** Mean P-S6K immunofluorescence signal ($\pm$ SEM) of NRCMs treated without SH-6 (white bars) and with SH-6 ($20 \mu\text{mol/L}$) (black bars) for 24 h in the absence and presence of GRGDS ($300 \mu\text{g/mL}$). All bars are normalized to the average fluorescence of P-S6K of NRCMs incubated without GRGDS and SH-6. Numbers of cells analyzed are, from left to right, 12, 10, 10 and 10. **C.** Western blot analysis of AKT and GAPDH levels in untransduced NRCMs (-), NRCMs transduced with a SIN-LV encoding DN-AKT1 (DN) and in NRCMs transduced with a SIN-LV encoding iCA-AKT1 (iCA). The two upper panels represent photographs of the same blot at different exposure times. M, molecular weight marker. **D.** Mean CSA ($\pm$ SEM) of NRCMs transduced with a dominant-negative version of mouse AKT1 (DN-AKT1) or transduced with a LV expressing only the eGFP gene (control) incubated for 24 h in the absence (white bars) and presence (black bars) of GRGDS ($300 \mu\text{g/mL}$). Numbers of cells analyzed are, from left to right, 24, 25, 26 and 22. **E.** Mean CSA ($\pm$ SEM) of NRCMs transduced with LV.CMV.IRES.eGFP (white bars) and of NRCMs transduced with LV.CMV.iCA-AKT1.IRES.eGFP (black bars) treated without and with rapamycin (10 nmol/L). All bars are normalized to the average CSA of NRCMs transduced with LV.CMV.IRES.eGFP not incubated with rapamycin. Numbers of cells analyzed are, from left to right, 29, 23, 25 and 27.

100±29.8% (mean±SD) in the absence of LPS to 125±25.2% after incubation with LPS (10 µg/mL) for 48 h. The NOS2 concentration in the NRCMs, as quantified by immunocytochemistry, increased from 26.6±7.2 AUs to 42.7±10.7 AUs ($p<0.001$) by the LPS treatment. The CSA of NOS2-expressing NRCMs positively correlated with NOS2 signal intensity ($r=0.48$, $p<0.005$) (fig. 7A). The presence of L-NAME (100 µmol/L) during the last 24 h of incubation with LPS completely abolished NOS2-dependent NRCM hypertrophy with the consequential loss of the positive correlation between CSA and NOS level ($r=-0.08$, $p=n.s.$) (fig. 7A). ODQ (10 µmol/L) blocked the LPS-induced hypertrophy almost completely; the mean CSA went down to 111.3±31.1% which was not significantly different from NRCMs incubated without LPS and without ODQ. However, coadministration of 8-Br-cGMP (0.5 mmol/L) prevented this ODQ-induced inhibition of LPS-induced hypertrophy, and the average CSA went up to 193.2±35.8% being higher than observed in NRCMs incubated with LPS only ($p=0.024$) (fig. 7F).

Intense NOS2-specific staining was invariably observed at sites of cell-to-cell contact, implying the association of a fraction of the NOS2 molecules with the sarcolemma (fig. 7G).

4.4 Discussion

The present study shows that integrin stimulation of NRCMs leads to hypertrophy via a mechanism involving NO production, sGC activation by NO and cGMP production by activated sGC. This conclusion is based on the finding that complete inhibition of GRGDS-induced NRCM hypertrophy was accomplished using 100 µmol/L of the general NOS inhibitor L-NAME (fig. 1A) while administration of the NO donor SNP at a dose ≥ 20 µg/mL to NRCMs pretreated by L-NAME (100 µmol/L) resulted in almost total ($>80\%$) rescue of integrin stimulation-induced NRCM hypertrophy (fig. 1B). Also, at a dose of 10 µmol/L the sGC inhibitor ODQ completely blocked the hypertrophic response of NRCMs to GRGDS treatment, which block could be overcome by concurrent incubation of the cells with the cGMP analogue 8-Br-cGMP (fig. 4).

Since previous research has demonstrated that NRCMs express all three NOS genes,³³ each of the three NOS proteins could potentially be involved in the production of NO in response to integrin stimulation. Based on the fact that treatment of NRCMs with GRGDS peptides led to an increase in the amount of NOS1 molecules activated by phosphorylation at serine residue 1446,¹ we focused on the role of NOS1 in integrin stimulation-dependent cardiomyocyte hypertrophy. Accordingly, NRCMs were transduced with a SIN-LV encoding rat PIN. This resulted in a PIN concentration-dependent inhibition of the GRGDS-induced increase in NRCM surface area, which was complete in cells containing high PIN levels (fig. 2B). In the first study on PIN, the protein was shown to interact *in vitro* with NOS1 but not with NOS2 or NOS3 using lysates of 293T cells individually transfected with recombinant versions of each of the NOS genes.¹² Consistently, in lysates of HMC-1 cells, PIN associates with endogenous NOS1 but not with endogenous NOS3.³⁴ Furthermore, the fact that the PIN-binding site in NOS1 has been located in a region not present in NOS2 or NOS3 makes inhibition of these enzymes by PIN very unlikely.³⁵ Collectively, these findings implicate NOS1 as the major producer of NO in



Figure 4.7: A. The CSA of NRCMs incubated with LPS (10 ng/mL) to induce NOS2 activity is positively correlated to NOS2 signal intensity (red line and confidence limits, n=33 cells), but in the presence of L-NAME (100 μ mol/L) this correlation is lost (blue line and confidence limits, n=36). All CSA values are normalized to the average CSA value of NRCMs incubated without LPS and without L-NAME. **B-E.** Fluoromicrographs of representative NRCMs showing NOS2 (green fluorescence) and sarcomeric α -actinin (red fluorescence) after 48 h of mock treatment (B, D) or after incubation for 48 h with LPS (10 ng/mL) (C, E). **F.** Mean CSAs ($\pm$ SEM) of NRCMs treated without LPS (white bars) and with LPS (10 ng/mL) (black bars) for 24 h in the absence and presence of ODQ (10 μ mol/L) without or with 8-Br-cGMP (0.5 mmol/L). All bars are normalized to the average CSA of NRCMs incubated without LPS, ODQ and 8-Br-cGMP. Numbers of cells analyzed are, from left to right, 14, 14, 13, 14, 11 and 14. **G.** Intense NOS2 staining was generally observed at the boundaries of LPS-treated NRCMs demonstrating its clustering underneath the plasma membrane, and particularly at sites adhering to neighboring cells. Insert shows the results of the immunostaining for sarcomeric α -actinin, which indicates that both cells are cardiomyocytes.

response to integrin stimulation. The suppressive effect of a shRNA specifically targeting rat *NOS1* transcripts on integrin ligation-dependent NRCM hypertrophy corroborates the key role of NOS1 in GRGDS-induced hypertrophic signaling (fig. 3). However, induction of NOS2 by LPS also resulted in hypertrophic growth of NRCMs which was dependent of NOS2 level. Although LPS will have many other effects on NRCMs, such as induction of contractile dysfunction via Toll-like receptor 4,³⁶ the observation that 100 $\mu\text{mol/L}$ L-NAME fully blocked the LPS-induced hypertrophy points to an important role for NOS2-derived NO in stimulating cardiomyocyte growth in response to endotoxin treatment (fig. 7A).

Our results further identified PI3K and AKT as important mediators of integrin stimulation-dependent cardiomyocyte hypertrophy acting downstream of cGMP. However, the observation that treatment of NRCMs with L-NAME at a concentration that fully blocked cardiomyocyte hypertrophy (fig. 1A) did not completely normalize the P-AKT/AKT ratio (fig. 5A), indicates that integrin stimulation of NRCMs results in the phosphorylation of AKT at the serine residue in the regulatory domain not only via an NO-dependent pathway.

The present study did not detect a suppressive role of SFK inhibition by PP1 analogue on hypertrophic growth of NRCMs in response to integrin signaling even though our research group previously showed an increase in phosphorylation of FAK at the tyrosine residue in the SFK docking site (Y³⁹⁷ in human FAK) following GRGDS treatment of NRCMs.¹⁰ Consistently, Clemente *et al.* recently found that transgenic mice with cardiomyocyte-specific overexpression of focal adhesion kinase (FAK) displayed concentric cardiac hypertrophy that was associated with enhanced signaling through PI3K, AKT, mTOR, S6K and rpS6, but did not involve the Src/ERK1/2 pathway.³⁷ Thus, NRCMs should possess an integrin stimulation-dependent but SFK-independent mechanism to activate NOS1.

4.4.1 Role of NOS1 in myocardial hypertrophy

NOS1 may exist in different forms as a result of (i) alternative RNA splicing, (ii) complex formation with itself (dimeric versus monomeric state) or with other proteins such as calmodulin and (iii) posttranslational modifications including phosphorylation at various sites.³⁸⁻⁴¹ In cardiomyocytes, a large fraction of NOS1 colocalizes with ryanodine receptor 2 (RyR2)⁴² and regulates the open probability (P_o) of this sarcoplasmic reticulum (SR) Ca^{2+} release channel via S-nitrosylation.^{43,44} Our research group recently showed that integrin stimulation of NRCMs in culture by an RGD motif-containing peptide is associated with RyR2-dependent intracellular Ca^{2+} -release, NO production and hypertrophy.^{1,10} On the basis of these findings we proposed an important role for SR-associated NOS1 in integrin stimulation-induced cardiomyocyte hypertrophy. In the present study, we demonstrated that shRNA-mediated inhibition of *NOS1* expression blocked GRGDS-induced hypertrophy of NRCMs in an shRNA dose-dependent manner (fig. 3A). In another study, specific depletion of *NOS1* transcripts by RNA interference was associated with inhibition of phenylephrine-induced (*i.e.* pathological) hypertrophy of NRCMs.⁴⁵ In mice overexpressing *NOS1* in cardiomyocytes, aortic constriction caused more left ventricular hypertrophy (LVH) than in wild-type mice that underwent the same treatment.⁴⁶ The pressure overload-induced LVH appeared to be of the physiological type as (i) myocardial ex-

pression of the genes encoding atrial natriuretic peptide (*NPPA*), brain natriuretic peptide (*NPPB*), β -myosin heavy chain (*MYH7*) and α -skeletal-actin (*ACTA1*) was barely upregulated, (ii) left ventricular remodeling hardly occurred, (iii) Ca^{2+} transients were preserved or had increased amplitude and (iv) myocardial inotropy and lusitropy had increased, whereas wild-type mice showed pronounced left ventricular dilatation and impaired contractility in response to pressure overload.⁴⁶ Adaptive cardiac hypertrophy is not only induced by cardiomyocyte-specific *NOS1* overexpression⁴⁶ but also by insulin-like growth factor-1 (IGF1).⁴⁷ The signaling pathway activated by IGF1 receptor stimulation⁴⁸ resembles the signaling cascade initiated by stretch⁴⁹ in many aspects, such as the involvement of signaling proteins like FAK, Shc, Grb2, Sos, Ras and Raf. Although the aforementioned results provide a clear link between increased *NOS1* activity and myocardial hypertrophy, the fact that *NOS1*^{-/-} mice develop cardiac hypertrophy and show increased premature mortality⁵⁰ indicates that *NOS1* also plays a role in limiting myocardial hypertrophy. This suggests that the effects of *NOS1*-derived NO on cardiomyocyte size are context- and probably also dose-dependent.

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4.4.2 Role of *NOS2* in myocardial hypertrophy

NOS2 is absent in the healthy heart but its expression is induced by pro-inflammatory mediators as occurs during myocardial infarction and in heart failure,⁵¹ and references herein]. In neonatal mouse cardiomyocyte cultures stimulated with 10 $\mu\text{g/mL}$ LPS, *NOS2* expression was detectable from 6 h onwards.¹¹ In the present study induction of *NOS2* by LPS treatment of NRCMs for 48 h resulted in an increase in CSA up to 40%, dependent on the *NOS2* level (fig. 7A). Although *NOS2* was predominantly present in the cytosol, we noticed (sub)sarcolemmal accumulation of *NOS2*, especially at contact sites between NRCMs (fig. 7G) as has been described before.^{33,52,53} Whether the localization of *NOS2* immediately underneath the sarcolemma of NRCMs has any functional consequences remains to be investigated.

In a rat model of septic cardiomyopathy, induction of *NOS2* mRNA was detected as early as 30 min following injection of endotoxin (*i.e.* LPS), resulting in a peak of *NOS2* activity after 6 h.⁵⁴ It is generally believed that inflammation-induced *NOS2* expression produces high levels of NO that may be toxic. Mice with myocardial *NOS2* overexpression suffered from cardiac fibrosis, myocardial hypertrophy (presumably of the pathological type) and ventricular dilatation, and displayed a high cardiomyocyte death rate.⁵⁵ Consistently, in *NOS2*^{-/-} mice, aortic constriction caused less hypertrophy, dilatation, fibrosis and dysfunction of the left ventricle than in wild-type mice.⁵⁶ However, the idea that *NOS2*-derived NO is a major contributor to the development of heart failure has been challenged by Heger and colleagues, who did not observe any deleterious effects of myocardial *NOS2* overexpression in hearts of transgenic mice.⁵⁷

Role of *NOS3* in myocardial hypertrophy Myocardial stretch leads to cardiomyocyte hypertrophy⁴⁹ and is believed to underlie the hypertrophic response of endurance exercise, aortic constriction and hypertension. Stretch-induced cardiomyocyte hypertrophy is coordinated by FAK/Src signaling,⁵⁸ in close connection with integrin stimulation.^{6,7} In a recent study, Cheng and coworkers found that in NRCMs subjected to mechanical stretch *NOS3* expression and *NOS3* activity are upregulated.⁵⁹ However, in the presence of L-

NAME, stretch-induced [^3H]-leucine incorporation into proteins was increased, whereas incubation of the cells with the NO donor S-nitroso-N-acetyl-DL-penicillamine inhibited stretch-induced protein synthesis. The authors hence concluded that NOS3 has a negative role in mechanical stretch-induced cardiomyocyte hypertrophy.⁵⁹ In keeping with the results of Cheng *et al.*, studies in *in vivo* models of myocardial infarction has led to the suggestion that cardiomyocyte-specific NOS3 overexpression can limit compensatory hypertrophy in remote myocardium and preserve left ventricular performance.⁶⁰ Like NOS1^{-/-} also NOS3^{-/-} mice spontaneously develop cardiac hypertrophy, while combined NOS1 and NOS3 deficiency leads to even more pronounced myocardial hypertrophy.⁵⁰ On the basis of these results, Kempf and Wollert concluded that apparently the relatively low quantities of NO produced by the constitutive NOS isoforms under normal conditions protect from maladaptive (*i.e.* pathological) cardiac hypertrophy, such as induced by angiotensin-II, endothelin-1 and phenylephrine, which leads to adverse left ventricular remodeling and early death.

After having shown in this and previous studies^{1,10} that signaling pathways downstream of stimulated integrins involve RyR2-associated NOS1, it is tempting to propose that this form of signaling leads to physiological hypertrophy. Evidence in favor of this view is the finding that (i) integrin stimulation-induced cardiomyocyte hypertrophy is associated with AKT phosphorylation (fig. 5A), (ii) AKT phosphorylation following treatment of NRCMs with an RGD motif-containing pentapeptide is reduced by L-NAME (fig. 5A) and (iii) wortmannin, an inhibitor of PI3K, inhibits dose-dependently the increase in NRCM surface area resulting from GRGDS-mediated integrin stimulation (fig. 5D). AKT is known to control phosphorylation of mTOR, S6K and glycogen synthase kinase-3 β , three serine/threonine kinases involved in increased protein synthesis and cardiomyocyte hypertrophy.⁶¹ AKT inhibition by SH-6, as well as transduction of NRCMs with a gene encoding a dominant-negative version of mouse AKT1, blocked GRGDS-induced NRCM hypertrophy which was associated with a decrease of phosphorylated S6K (fig. 6B). In addition, constitutive AKT signaling using iCA-AKT1 induced hypertrophy of NRCMs that was $\approx 50\%$ inhibited by rapamycin, an inhibitor of mTOR which is a signaling protein downstream of AKT (fig. 6E).

4.4.3 Conclusion

The present study demonstrates that GRGDS-induced hypertrophy of NRCM is inhibited by (i) PIN, an endogenous inhibitor of NOS1, (ii) shRNA-mediated inhibition of NOS1 expression and (iii) L-NAME, a general inhibitor of all NOS isoforms. However, this blockade can be rescued by administration of an exogenous NO donor, like SNP. In NRCMs, NO bioavailability thus is a regulator of GRGDS-induced hypertrophy and this regulatory pathway includes PI3K/AKT, suggesting that the process mimics physiological instead of pathological cardiac hypertrophy. Collectively, our results fit the model shown in fig. 8.

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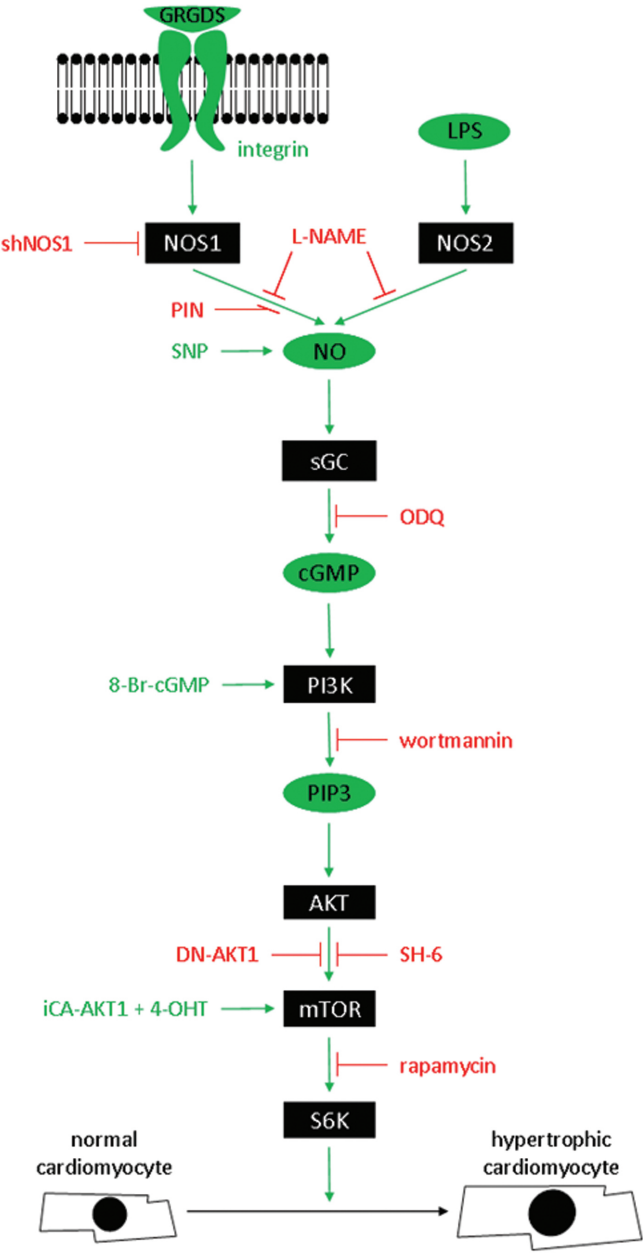


Figure 4.8: Model representation of the NO-dependent pathway leading to cardiomyocyte hypertrophy after integrin stimulation of NRCMs in culture. Only those factors that were directly or indirectly investigated in this study are indicated. Inhibitory and stimulatory interventions are indicated in red and green, respectively, and enzymes are indicated by black boxes.

4.5 Acknowledgements

The technical support from Minka Bax, Cindy Schutte, Lizet van der Valk and Margreet de Jong is gratefully acknowledged. We also thank Martine Moester for constructing the SIN-LV shuttle plasmid pLV.CMV.iCA-AKT1.IRES.eGFP.

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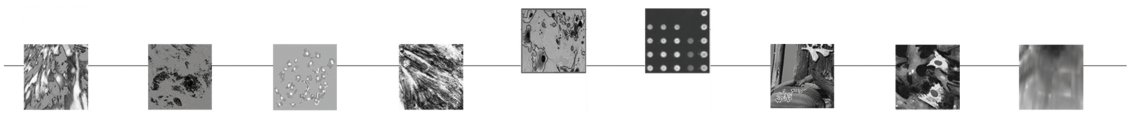
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Prelude to chapters 5 and 6

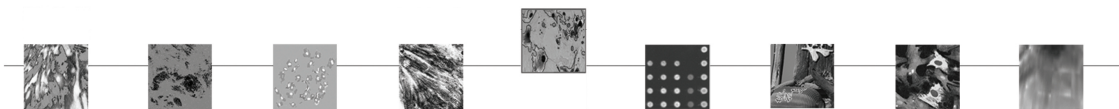
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Connexins and gap junctional coupling

Gap junctional coupling between CMCs is vital for normal cardiac conduction. Gap junction channels are formed by the interaction of two connexons from adjacent cells. The gap junctions are primarily located at the intercalated disks and thereby modulate uni-directional conduction. Each connexon is made up of six connexin (Cx) proteins and creates a pore between the cells, allowing molecules up to 1 kDa to travel between cells.¹ Cx40, Cx43 and Cx45 are the main connexins in the heart. In the ventricles Cx43 is the predominant connexin, whereas both Cx40 and Cx43 are highly expressed in the atria. Cx45 is mainly found in the cardiac conduction system.² In mice a fourth connexin, Cx30.2, is found in the cardiac conduction system³, but its human orthologue (*i.e.* Cx31.9) appears absent in the human heart.⁴ Besides being expressed by CMCs, connexins are also found in cardiac fibroblasts.⁵ In addition, it was discovered that myofibroblasts and CMCs from rabbit can couple via gap junctions.⁶ Although the coupling between fibroblasts and CMCs was shown to be poor⁷, Cx43 expression in fibroblasts and heterocellular coupling were increased upon cardiac injury.^{8,9} Recent research has shown that excessive fibroblasts proliferation in co-culture with nrCMCs induced reentrant arrhythmias.¹⁰ Together these results indicate a role for gap junctions in the occurrence of these arrhythmias, probably as a result of a less negative resting membrane potential of the CMCs.⁸ Gap junctions are also required for normal cardiac development, and connexin deficiency was shown to lead to cardiac malformations in mouse models.^{11,12} A recent study has provided evidence that the gap junctional transfer of microRNAs, may be involved in inducing cardiomyogenesis.¹³ Gap junctional coupling could therefore be important for the differentiation of stem cells to CMCs. The finding that human mesenchymal stem cells (hMSCs) that express Cx43 abundantly were able to differentiate towards CMCs, whereas hMSCs expressing a low level of Cx43 were unable to undergo cardiomyogenesis, is consistent with this hypothesis.¹⁴ Nonetheless, to what extent and by what mechanism gap junctional coupling is responsible for the cardiomyogenic differentiation of stem cells remains to be investigated.

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Chapter 5

Targeting of Heterocellular Coupling in Fibrotic Neonatal Rat Myocardial Cell Cultures by Lentiviral Connexin43 Knockdown in Myofibroblasts is Anti-Arrhythmic.

5

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* Equal contribution

Abstract

Aims: Cardiac fibrosis is associated with an increased risk of cardiac arrhythmias. However, the underlying proarrhythmic substrate remains poorly understood and the efficacy and curative potential of antiarrhythmic therapies are limited. This study aims to test the hypothesis that myofibroblasts (MFB) alter cardiomyocyte (CMC) excitability by electrotonic coupling via gap junctions leading to arrhythmias and that therefore connexin43 (Cx43) knockdown in MFBs, through lentiviral vector transduction would lower the incidence of such arrhythmias.

Methods and Results: Co-cultures of CMCs and MFBs in a 1:1 ratio as a model of cardiac fibrosis, and purified cultures of CMCs were studied for arrhythmogeneity at day 9 of culture by optical mapping using a voltage-sensitive dye. Cultures were also studied for electrophysiological properties, gap junctional coupling and protein expression. Heterocellular coupling in MFB-CMC co-cultures was reduced by transducing MFBs with a lentiviral vector encoding shRNA targeting Cx43 prior to co-culturing. In control groups, MFBs were transduced with a lentiviral vector encoding a shRNA targeting pLuc.

Conduction velocity (CV) was significantly lower in co-cultures compared to purified CMC cultures (10.9 ± 2.4 cm/s vs. 21.6 ± 3.6 cm/s $p < 0.0001$). Of the former cultures ($n=30$ total) 30.0% showed spontaneous reentrant tachyarrhythmias compared to 3.2% in the latter cultures ($n=63$ in total). CMC membrane potential was significantly more depolarized in fibrotic cultures, and was associated with lower rate of rise of depolarization (by 42.3%, $p < 0.0001$), compared to CMC cultures. Silencing of Cx43 expression in MFBs, however, increased CV by 37.5% ($p < 0.001$), reduced depolarization of adjacent CMCs and improved CMC excitability, thereby reducing the incidence of reentry by 41.1%, compared to co-cultures containing pLuc-silenced MFBs.

Conclusion: Electrotonic coupling of MFBs to CMCs in myocardial cell cultures gives rise to spontaneous reentrant tachyarrhythmias. However, targeting of this heterocellular coupling, by reducing the expression of Cx43 in MFBs, limits conduction slowing and ectopic activity, and thereby inhibits such arrhythmias. These results provide a rationale for the development of substrate-oriented anti-arrhythmic therapies.

5.1 Introduction

Acute cardiac injury such as myocardial infarction and chronic cardiac injury as seen in chronic pressure overload or in the ageing heart generally lead to cardiac fibrosis.^{1,2} Besides the detrimental consequences for the mechanical function of the myocardium, fibrosis is also closely associated with an increased risk of potentially lethal cardiac tachyarrhythmias.^{3,4} Currently, no truly curative therapeutic option is available for such fibrosis-associated arrhythmias.^{5,6} This has raised a strong incentive to identify novel therapeutic targets of such arrhythmias.

One of the hallmarks of cardiac fibrosis is a substantial increase in the relative number of myofibroblasts (MFBs) that actively remodel the myocardium.^{7,8} These MFBs increase structural heterogeneity and play a dominant role in ectopic activity and conduction slowing, which culminates in an increased propensity towards reentrant tachyarrhythmias.^{9,10} These observations could implicate MFBs as (part of) the pro-arrhythmic substrate in fibrosis-associated arrhythmias. The underlying mechanisms are based on secretion of paracrine factors or heterocellular coupling between MFBs and cardiomyocytes (CMCs), which would depolarize the CMC's resting membrane potential and would modulate ion channel availability and concomitant excitability.⁹⁻¹³ Recently, we showed that limitation of such paracrine factors and depolarization by preventing MFB proliferation has very strong anti-arrhythmic effects.¹¹ To further define the arrhythmic substrate and provide new insights in potential anti-arrhythmic targets, we investigated the effect of direct inhibition of MFB-CMC coupling on development of arrhythmias in fibrotic myocardial cell cultures by selective downregulation of Cx43 expression in MFBs. Our results demonstrate that inhibition of heterocellular coupling limits (1) MFB-induced depolarization, (2) ectopic activity and (3) conduction slowing, and consequently (4) development of spontaneous reentrant arrhythmias. This data highlight MFB-CMC coupling as a potential novel target for anti-arrhythmic therapies.

5.2 Materials and methods

All animal experiments were approved by the Animal Experiments Committee of the Leiden University Medical Center and conform to the Guide for the Care and Use of Laboratory Animals as stated by the US National Institutes of Health.

5.2.1 Cell isolation and culture

Primary neonatal rat CMCs and cardiac fibroblasts were isolated and cultured according to methods adopted from Pijnappels *et al.*¹⁴ After isolation, cell-aggregates were removed by filtering cell-suspensions through a 20 μ m cell-strainer. Next, cells were counted and plated out on fibronectin-coated round glass coverslips (15 mm) at a cell density in the range of 1×10^5 to 8×10^5 cells/well in 24-well plates (Corning Life Sciences, Amsterdam, the Netherlands), depending on the experiment.

For fibrotic co-cultures, primary CMCs and MFBs obtained from an earlier isolation were counted and mixed in a 1:1 ratio before plating. To maintain the 1:1 MFB-CMC ratio, all co-cultures were treated with the anti-proliferative agent mitomycin-C (Sigma-Aldrich,

St. Louis, MO, USA) at day 1 of culture as described previously.¹¹ In addition, purified CMC cultures were prepared and treated with mitomycin-C at day 1, which prevented the culture from becoming fibrotic. All cultures were refreshed daily with DMEM/Ham's F10 in a 1:1 mixture with 5% added horse serum (HS)(all from Invitrogen, Breda, the Netherlands) and cultured in a humidified incubator at 37°C and 5% CO₂.

5.2.2 Characterization of cultures

Immunocytochemical analyses Following mapping experiments at day 9 of culture, cultures were stained for proteins of interest, or cultures were stained for various markers parallel to mapping experiments as described in earlier studies.¹¹ Cultures were stained for α -smooth muscle actin (α -SMA) and vimentin expression to study fibroblasts phenotype (antibodies from Sigma-Aldrich), collagen-I expression to quantify MFB numbers (antibody from Abcam, Cambridge, MA, USA), and α -actinin as CMC-specific marker (antibody from Sigma-Aldrich). In addition, cultures were also stained for connexin 43 (Cx43) (antibody from Sigma-Aldrich) to study gap junction formation between CMCs and MFBs. Corresponding AlexaFluor-568 conjugated donkey-anti-mouse IgG and AlexaFluor-488 conjugated donkey-anti-rabbit IgG secondary antibodies (Invitrogen) were used at a dilution of 1:400. Nuclei were stained with Hoechst 33342 (10 μ g/mL; Invitrogen). A fluorescent microscope equipped with a digital camera (Nikon Eclipse, Nikon Europe, Badhoevedorp, the Netherlands) and dedicated software (Image-Pro Plus, version 4.1.0.0, Media Cybernetics, Silver Spring, MD, USA) was used to analyze the stained cultures. All proteins of interest were studied in at least 6 different cultures from a specific group, from which at least 20 representative images were acquired at various magnifications (10, 40, 100 $\times$). All cultures were stained using the same solutions and captured using equal exposure times for the protein of interest.

5.2.3 SIN-LV production

The monocistronic SIN-LV LV.hUbiC.TurboFP635.WHVPRE, which codes for a far red-shifted mutant of the red fluorescent protein of the sea anemone *Entacmaea quadricolor* (designated TurboFP635 or Katushka¹⁵), was made with the aid of shuttle construct pLV.hUbiC.TurboFP635.WHVPRE. The shuttle plasmid pLV.hUbiC.TurboFP635.WHVPRE contains the human *ubiquitin C* gene promoter coupled to the coding sequence of TurboFP635 and the woodchuck hepatitis virus post-transcriptional regulatory element. pLV.hUbiC.TurboFP635.WHVPRE was generated by ligation of the 743-bp BstUI fragment from pTurboFP635-N (Evrogen, Moscow, Russia) to the 9.1-kb Klenow polymerase-treated XbaI fragment of pFugW.¹⁶

To suppress Cx43 expression, MFBs were transduced by lentiviral vectors carrying a Cx43-specific shRNA (Open Biosystems, Huntsville, AL, U.S.A.). The lentiviral vectors LV.SM2C.Cx43.hPGK.eGFP targeting Cx43 and LV.SM2C.pLuc.hPGK.eGFP targeting firefly luciferase (control) have been described previously.¹⁷

Vesicular stomatitis virus G-protein-pseudotyped self-inactivating human immunodeficiency virus type I vectors (SIN-LVs.) were produced by seeding six 175 cm² culture flasks with 1×10^5 293T cells per cm² in DMEM (Invitrogen) supplemented with 10% fe-

tal bovine serum (FBS, Invitrogen) and 10 $\mu\text{mol/L}$ cholesterol (Sigma-Aldrich). The next day, these producer cells were transfected with either one of the lentiviral vector plasmids together with psPAX2 (Addgene, Cambridge, MA, USA) and pLP/VSVG (Invitrogen) at a 2:1:1 molar ratio using a total of 200 ng DNA/cm² and 3 ng polyethyleneimine (Polysciences, Warrington, USA) per ng DNA as transfection agent. Sixteen hours later, the transfection medium was replaced by DMEM containing 5% FBS, 10 mM HEPES-NaOH (pH 7.4) and 10 $\mu\text{mol/L}$ cholesterol. At 64 h post-transfection, the culture fluid was collected and freed of cellular debris by centrifugation at room temperature for 10 min at 825 $\times$ g and filtration through a 0.45 μm pore-sized cellulose acetate filter (Pall Corporation, East Hills, NY, USA). To concentrate the lentivirus vector particles, a 5 mL cushion of 20% sucrose in phosphate-buffered saline (PBS) was loaded under 30 ml of the cleared culture medium, which was then centrifuged for 90 min at 15,000 rpm and 10°C in an SW28 rotor (Beckman Coulter, Fullerton, CA, USA). Next, the supernatant was discarded and the pellets with the vector particles were suspended in 400 μL of PBS containing 1% bovine serum albumin (BSA) by gentle rocking overnight at 4°C.

The gene transfer activity of the vector stocks was determined by end-point titration on HeLa indicator cells using flow-cytometric analysis of *eGFP* or *Katushka* expression as read-out. The titers of the SIN-LV preparations are thus expressed in HeLa cell-transducing units (HTUs) per mL.

MFBs were transduced with SIN-LV particles at a multiplicity of infection (MOI) of 32 HTUs per cell in culture medium containing 20 $\mu\text{g/mL}$ diethylaminoethyl-dextran sulfate (GE Healthcare, Diegem, Belgium). After 4 h, the cultures were washed three times with PBS and supplied with fresh culture medium. MFBs were passaged twice before application in experiments.

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5.2.4 Western blot analyses

MFB cultures were transduced with LV.SM2C.Cx43.hPGK.eGFP or LV.SM2C.pLuc.hPGK.eGFP parallel to mapping experiments. At day 9 of culture, transduced and mock-transduced cultures were rinsed in ice-cold PBS and homogenized in RIPA-buffer consisting of 50 mmol/L Tris-HCl at pH 8.0, 150 mmol/L NaCl, 1% NP-40, 0.5% sodiumdeoxycholate, and 0.1% SDS. Homogenates were size-fractionated on NuPage 12% Tris-Acetate NuPage gels (Invitrogen) before transfer to Hybond PVDF membranes (GE Healthcare). These membranes were incubated with an antibody against Cx43 (Sigma-Aldrich) for 1 h followed by incubation with corresponding HRP-conjugated secondary antibody (Santa Cruz Biotechnologies, Santa Cruz, CA, USA). To check for equal protein loading, β -tubulin (antibody from Millipore, Billerica, MA, USA) expression was determined. ECL Advance Detection reagents (GE Healthcare) were used to induce chemiluminescence which was subsequently caught on Hyperfilm ECL (GE Healthcare). The intensity of Cx43 and β -tubulin bands was quantified by Scion Image analysis software (Scion Corporation, Frederick, MD, USA).

5.2.5 Optical mapping

Action potential propagation patterns of purified CMC cultures and MFB-CMC co-cultures (8×10^5 cells/well in a 24-wells format) were studied by optical mapping with the voltage-sensitive dye di-4-ANEPPS (Invitrogen). Structurally heterogenous cultures ($<5\%$, determined by light microscopy or electrophysiological mapping) were all excluded for reasons of standardization and reproducibility. Only spontaneously active cultures ($>60\%$) were included for further analyses. On day 9 of culture, cells were incubated for 15 ± 5 min with culture medium (DMEM/Ham's F10 + 5% HS) containing $16 \mu\text{mol/L}$ di-4-ANEPPS. After incubation, cultures were refreshed with DMEM/Ham's F12 (37°C) and subsequently mapped. Epi-illumination excitation light ($\lambda_{\text{ex}} = 525 \pm 25 \text{ nm}$) was delivered by a halogen arc-lamp (MHAB-150W, Moritex Corporation, San Jose, CA, USA). To limit phototoxic effects of the mapping protocol on the cultures, the same culture was never exposed to excitation light for longer than 40s and mapping experiments in a 24-well plate typically did not exceed 30 min. Fluorescent emission light ($\lambda_{\text{em}} > 590 \text{ nm}$) passed through a camera lens ($1 \times$ Plan-Apo, $\text{WD} = 15 \text{ mm}$; Leica, Wetzlar, Germany) and a dichroic mirror, after which it was focused onto a 100 by 100 pixels (100 mm^2) CMOS camera (Ultima-L, SciMedia, Costa Mesa, CA, USA) by a $1.6 \times$ converging lens, resulting in a total field of view of 256 mm^2 and a spatial resolution of $160 \mu\text{m}/\text{pixel}$. During measurements, cultures were kept at 37°C . Electrical activation was recorded for at least 4 s at a rate of 500 or 167 frames/s, high-pass filtered and analyzed using Brain Vision Analyze 1008 (Brainvision Inc, Tokyo, Japan). For each pixel signals were averaged with 8 of its nearest neighboring pixels. Time points at which the rate of rise of fluorescence signal (dF/dt) was maximal were defined as the activation time point. Conduction velocity (CV) in cultures with a uniform activation pattern was calculated between two 3×3 pixel grids, typically spaced 2-8 mm apart, and perpendicular to the activation wavefront. For cultures showing reentrant conduction wavefronts, CV was determined similarly at half the maximal distance from the core perpendicular to the wavefront over a length of 2-4 mm. Per culture, CV was determined in 6-fold and averaged for further comparisons. Reentrant cycle length was calculated from 3 separate cycles per culture and averaged. As this study focuses on reentry occurrence and prevention of such arrhythmias and CV is a valuable parameter in determining therapeutic effects *in vitro*, all CV values for reentry and non reentry cultures were pooled within each group, unless stated otherwise.

5.2.6 Assessment of ectopic activity

All cultures underwent programmed stimulation with a unipolar electrode at 6 V for 4 s using an electrical stimulus module with corresponding software (Multichannel Systems, Reutlingen, Germany) to eliminate reentry, after which spontaneous activity could resume. Spontaneous electrical activity was recorded by optical mapping for 24 s after the programmed stimulus. Ectopic activity was defined as the presence of multiple pace-maker sites.

5.2.7 Whole-cell patch-clamp

Whole-cell patch-clamp measurements were performed in co-cultures of CMCs and MFBs at day 7-9 of culture. CMCs were identified by phase contrast microscopy and lack of fluorescence signal. Next, maximal diastolic potentials in CMCs were recorded in current-clamp at 25°C. Whole-cell recordings were performed using a L/M-PC patch-clamp amplifier (3 kHz filtering) (List-Medical, Darmstadt, Germany). The pipette solution contained (in mmol/L) 10 Na₂ATP, 115 KCl, 1 MgCl₂, 5 EGTA, 10 HEPES/KOH (pH 7.4). Tip and seal resistance were 2.0-2.5 MΩ and >1 GΩ, respectively. The bath solution contained (in mmol/L) 137 NaCl, 4 KCl, 1.8 CaCl₂, 1 MgCl₂, and 10 HEPES (pH 7.4). For data acquisition and analysis, pClamp/Clampex8 software (Axon Instruments, Molecular Devices, Sunnyvale, CA, USA) was used.

5.2.8 Dye transfer

To investigate functional coupling between MFBs and CMCs, MFBs were labeled with LV.hUbiC.TurboFP635.WHVPRE (MOI 20). MFBs were kept in culture for 2 weeks and at least passaged twice before they were used in experiments. CMC cultures with a density of 1.0×10^5 cells/well were treated with 10 μg/ml mitomycin-C to prevent endogenous MFB overgrowth. At day 4, these cultures (n=12) were loaded for 7 min with calcein-AM (Invitrogen) diluted in HEPES-buffered salt solution (HBSS, Gibco, Grand Island, USA), which once internalized is hydrolyzed to the green fluorescent dye calcein. Cells were rinsed twice with PBS and kept on culture medium containing 2.5 mmol/L probenecid (Invitrogen) to block calcein leakage from the cells. Katushka-labeled MFBs were subsequently plated out in a 1:1 ratio with the calcein-loaded CMCs. Bright-field and fluorescent images (at least 10 per culture) were acquired after 7 h of co-culture. To determine the functional effect of Cx43 downregulation in MFBs, dye transfer experiments were performed using MFBs transduced with LV.SM2C.Cx43.hPGK.eGFP or LV.SM2C.pLuc.hPGK.eGFP, and CMCs loaded with 10 μg/mL Calcein Red-Orange-AM (Invitrogen). These experiments were subsequently conducted identically to the dye-transfer experiments described above.

5.2.9 Statistical analysis

Statistical analyses were performed using SPSS11.0 for Windows (SPSS Inc., Chicago, IL, USA). Data were compared with one-way or two-factor mixed ANOVA test with Bonferroni's *post-hoc* correction when appropriate, and expressed as mean±SD. Comparison between two groups was performed using Student's *t*-test. Differences were considered statistically significant if $p < 0.05$.

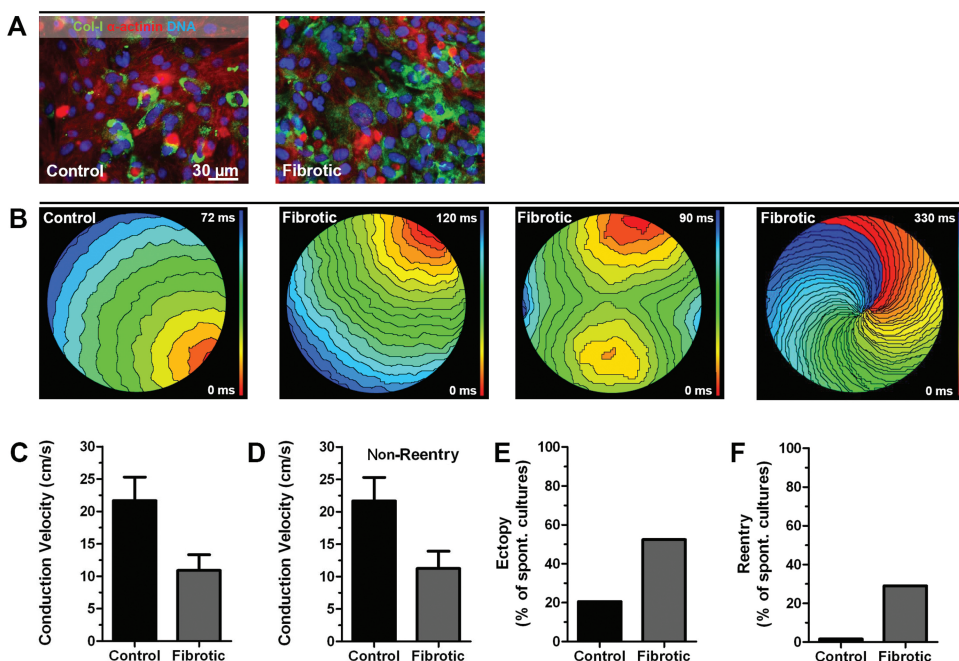


Figure 5.1: Fibrotic cultures show pro-arrhythmic tendencies. (A) Typical example of a collagen I (green) and α -actinin (red) double staining showing more MFBs in fibrotic cultures compared to control cultures. Nuclei are stained blue. (B) From left to right: typical activation map (6 ms isochrones) of purified CMC cultures showing fast propagation; typical activation map (6 ms isochrones) of slowed conduction, ectopic activity and reentry in MFB-CMC co-cultures. (C) CV in general was slowed in fibrotic cultures also when (D) reentry was excluded from analysis *: $p < 0.001$. (E) Incidence of ectopic activity was significantly higher in the fibrotic group, as was the case for (F) spontaneous reentry.

5.3 Results

5.3.1 MFBs induce ectopy and cause spontaneous reentrant tachyarrhythmias

In all cultures, $>95\%$ of all fibroblasts expressed α -SMA and collagen-I and were therefore considered as MFBs as described previously.¹¹ In fibrotic cultures, relative MFB numbers were $53.0 \pm 0.6\%$ at day 9, whereas purified CMC cultures contained $18.7 \pm 1.2\%$ MFBs, as judged by quantification of collagen I/ α -actinin staining (Figure 1A).

To assess the arrhythmogenic potential of MFBs, fibrotic co-cultures of MFBs and CMCs and purified CMC cultures were assessed for several electrophysiological parameters using optical mapping. CV was significantly lower in fibrotic cultures compared to controls (10.9 ± 2.4 cm/s vs. 21.6 ± 3.6 cm/s; $p < 0.0001$ $n=30$ and $n=60$) (Figure 1B, 1C). To correct for conduction slowing attributable to reentrant conduction, CV was also

exclusively determined in non-reentry cultures. CV in these cultures was still significantly lower in fibrotic cultures than in CMC cultures (11.2 ± 2.6 cm/s vs. 21.9 ± 3.28 cm/s; $p < 0.0001$) (Figure 1D). In addition to lowered CV, fibrotic cultures were prone to develop ectopic activity (Figure 1B, second picture from the right). Of all fibrotic cultures (total $n=30$) 52.3% showed ectopic activity as opposed to 19.6% in CMC cultures (total $n=60$) (Figure 1E). Moreover the incidence of reentrant tachyarrhythmias (Figure 1B, right) was considerably higher in fibrotic cultures when compared to CMC cultures (30.0% vs. 3.2%) (Figure 1F). Together these results illustrate the arrhythmogenic potential of MFBs in cardiac cultures.

5.3.2 Electrotonic interaction between MFBs and CMCs alters CMC excitability

To study intercellular coupling between CMCs and MFBs, MFB-CMC co-cultures were stained for the gap junctional protein Cx43. Immunocytochemical staining revealed that Cx43 expression was located between adjacent MFBs, CMCs and MFBs adjacent to CMCs, suggesting the ability of MFBs to form homo- and heterocellular gap junctions (Figure 2A and B). As to the functionality of these gap junctions, we studied calcein dye transfer from CMCs to adjacent MFBs. Figure 2C shows a representative image of adjacent calcein-positive MFBs and CMCs, confirming this ability. Next, electrophysiological consequences of heterocellular coupling were assessed. The presence of MFBs in fibrotic cultures made the membrane potential of CMCs significantly more positive (-49.4 ± 6.1 mV vs. $-64.8.4 \pm 5.9$ mV; $p < 0.001$ $n=7$ and $n=8$) (Figure 2D). In agreement with the previous finding, $dF/dt_{\max}$ was significantly lower in fibrotic cultures than in CMC cultures (Figure 2E and F), which is consistent with the lower CV in these cultures.

5.3.3 shRNA-mediated Cx43 silencing by viral transduction

At MOI 32 we found maximal expression of eGFP-tag in pure MFB cultures. After transduction with LV.SM2C.Cx43.hPGK.eGFP, Cx43 expression in MFBs was 53% lower than in LV.SM2C.pLuc.hPGK.eGFP-transduced MFBs as judged by western blot analysis (Figure 3A). Uniform eGFP expression was consistently confirmed by fluorescence microscopy (Figure 3B). To exclude the possibility of solely silencing the cytoplasmic or compartmentalized Cx43, Cx43 knockdown was confirmed by immunocytochemical analysis. After transduction with LV.SM2C.Cx43.hPGK.eGFP, Cx43 expression in MFB-MFB junctions was markedly lower (74%) after transduction with Cx43 silencing (Figure 3B, C).

5.3.4 Silencing Cx43 expression in MFBs reduces functional heterocellular coupling

The effect of Cx43 knockdown on functional heterocellular coupling was first assessed by calcein dye transfer experiments. The functional effect of Cx43 knockdown on intercellular dye transfer between MFBs and CMCs was a lower percentage of calcein-positive LV.SM2C.Cx43.hPGK.eGFP-transduced MFBs in co-cultures when compared to

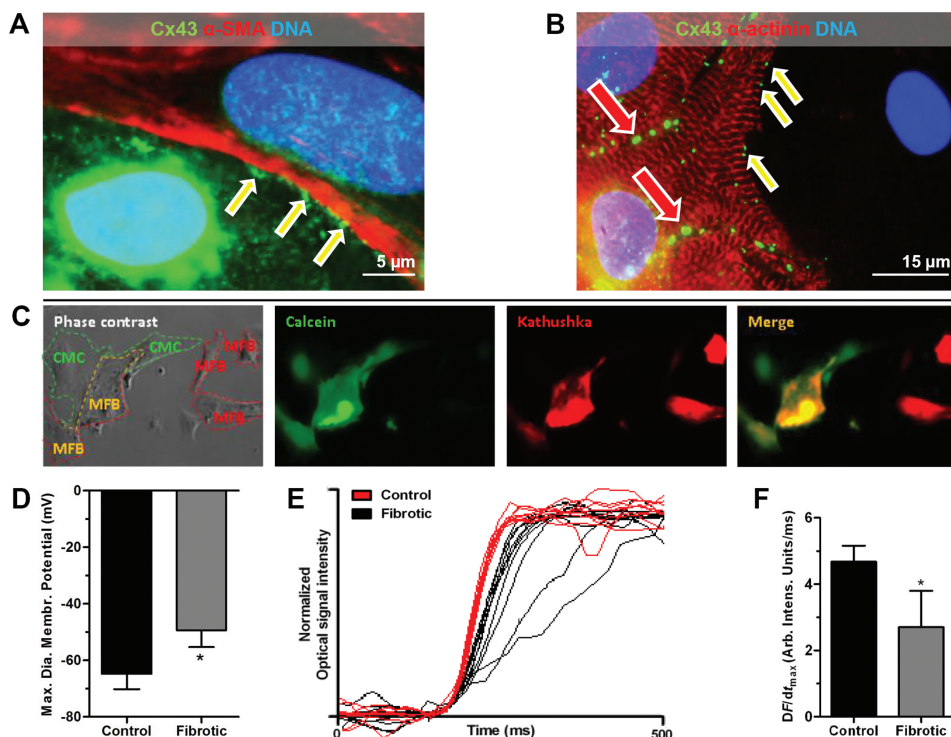


Figure 5.2: Heterocellular MFB-CMC coupling alters cardiomyocyte excitability. (A, B) Cx43 expression was present at homo- and heterocellular junctions. Red arrows indicate homocellular, CMC-CMC junctions. Yellow arrows indicate heterocellular MFB-CMC junctions. (C) Dye transfer assay shows that Katushka-labeled MFBs in direct contact with calcein-loaded CMCs exhibit transfer of the calcein, whereas isolated MFBs showed no such transfer. (D) Maximal diastolic potentials obtained from whole-cell current-clamp recordings revealed that CMCs in fibrotic cultures are significantly more depolarized as compared to CMC cultures (control). *: $p < 0.001$. (E) Upstroke traces from 10 amplitude normalized optical signals show less steep slopes of the action potential upstrokes in fibrotic cultures, indicating lowered excitability of CMCs in these cultures. (F) Quantification of the maximal upstroke velocity shows significantly lower excitability in fibrotic cultures than in CMC cultures (control). *: $p < 0.001$.

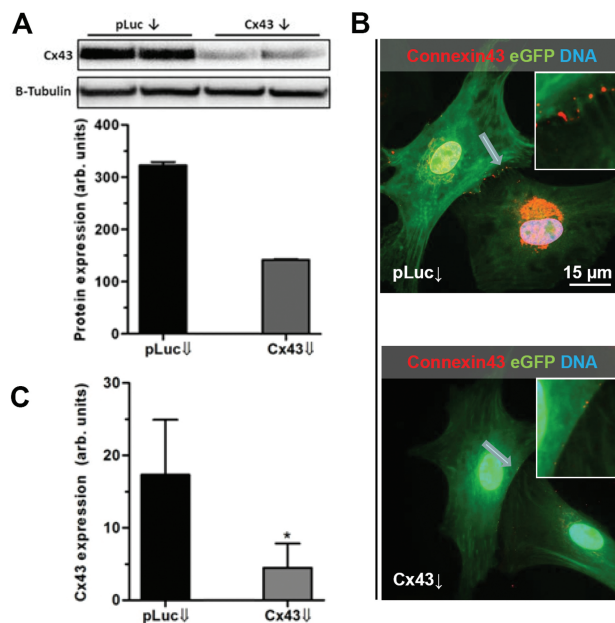


Figure 5.3: shRNA-mediated Cx43-silencing by lentiviral vectors decreases Cx43 expression in infected MFBs. (A) Typical example and quantification of Cx43 expression, normalized for β -tubulin expression from Western blot analysis showing significantly lower expression of Cx43 in Cx43-silenced MFBs compared to control ($p < 0.001$). (B) Typical example of an immunocytochemical staining, showing less intercellular expression of Cx43 between Cx43-silenced MFBs compared to pLuc-silenced MFBs (control). (C) Quantification of intercellular Cx43 expression. *: $p < 0.001$ vs. pLuc silencing (control).

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Lv.SM2C.pLuc.hPGK.eGFP-transduced MFBs in co-cultures ($22.4 \pm 6.5\%$ vs. $40.8 \pm 10.6\%$; $p < 0.01$ $n=50$ cell pairs across 7 photos per group) (Figure 4A and B). In addition, fluorescence intensity of the transferred dye was significantly lower in the Cx43 knockdown group than in the pLuc-silenced MFBs in co-cultures, also demonstrating decreased heterocellular coupling (Figure 4C). Next, CMCs in the co-cultures were analysed by whole-cell patch-clamp (Figure 4D). As a result of depressed heterocellular coupling, resting membrane potential was preserved in co-cultures with LV.SM2C.Cx43.hPGK.eGFP-transduced MFBs compared to co-cultures containing pLuc-silenced MFBs (61.4 ± 5.7 mV vs. 51.6 ± 4.0 mV; $p < 0.05$) and not significantly different from the resting membrane potential in purified CMC cultures (67.5 ± 3.6 mV; n.s.) (Figure 4E and F). Relative MFB numbers, distribution and density did not differ between co-cultures with Cx43-silenced and pLuc-silenced MFBs (Figure 5A). However, intercellular Cx43 expression at heterocellular junctions was 68% lower in co-cultures with Cx43-silenced MFBs than in co-cultures with pLuc-silenced MFBs (2.7 ± 1.3 arb. units vs. 8.5 ± 3.8 arb. units; $p < 0.001$) (Figure 5B).

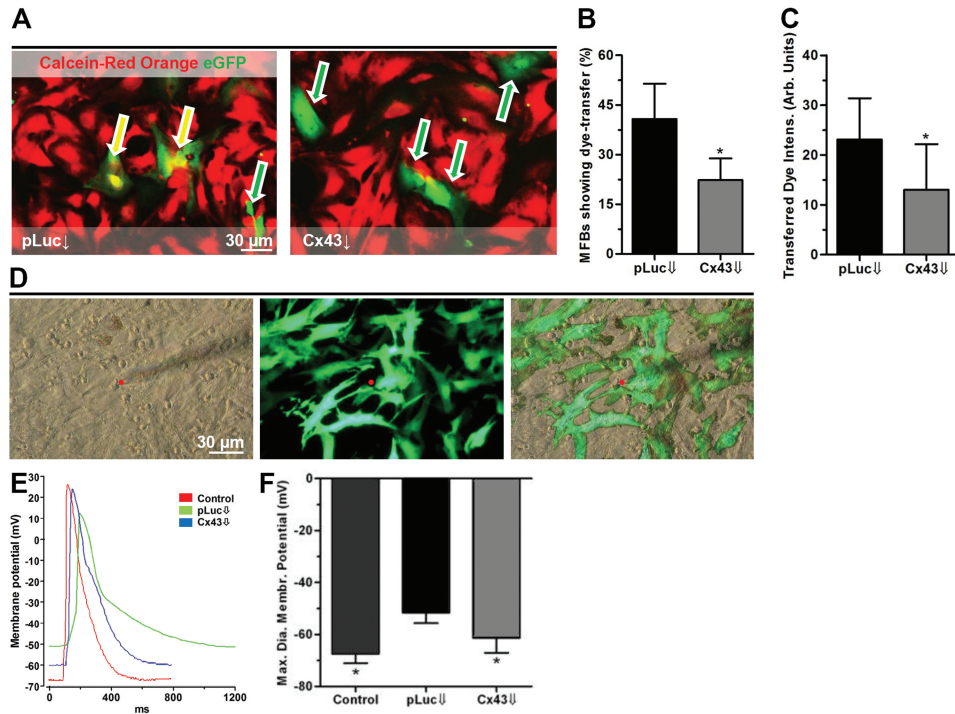


Figure 5.4: Functional consequences of MFB Cx43 silencing in CMC-MFB co-cultures. (A) Typical examples of calcein Red-Orange loaded CMCs in co-culture with eGFP expressing, Cx43-silenced MFBs or eGFP expressing, pLuc silenced MFBs. Yellow arrows indicate MFBs that show transfer after 7 h of co-culture, whereas green arrows indicate MFBs that do not receive calcein from neighboring CMCs. (B) Quantification of GFP-positive MFBs showing transfer of calcein Red-Orange per image shows significantly less cells positive for the gap junctional permeable calcein. *: $p < 0.001$ (C) Quantification of fluorescence intensity of the transferred dye shows significantly lower fluorescent signal in Cx43-silenced MFBs than in pLuc-silenced MFBs. *: $p < 0.001$. (D) Typical example of co-cultures under patch-clamp conditions showing selective targeting of CMCs surrounded by eGFP positive MFBs. Red dot signifies the tip of the electrode. (E) Typical examples of whole-cell patch-clamp recordings of CMCs in different myocardial cultures. (F) In fibrotic cultures with Cx43-silenced MFBs, the resting membrane potential of CMCs is significantly more negative as compared to CMCs in co-culture with pLuc-silenced MFBs, although not as negative as the resting membrane potential of CMCs in purified CMC cultures (control). *: $p < 0.05$ vs. pLuc silencing.

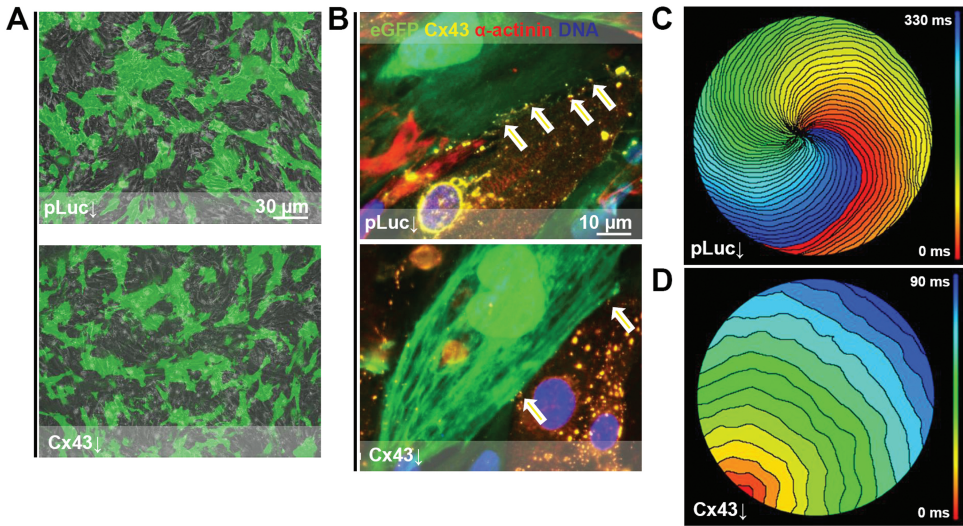


Figure 5.5: Heterocellular Cx43 expression is diminished in co-cultures with Cx43 silenced MFBs. (A) Typical example of a merged image of phase-contrast and eGFP fluorescence showing equal distribution and relative numbers of Cx43-silenced or pLuc-silenced green MFBs in co-cultures. (B) Typical example of Cx43/α-actinin staining showing decreased intercellular Cx43 expression in co-cultures with Cx43-silenced MFBs compared to co-cultures with pLuc-silenced MFBs (control). (C) Typical activation map of reentry in a co-culture with pLuc-silenced MFBs (6 ms isochrones). (D) Typical activation map of uniform propagation in a co-culture with Cx43-silenced MFBs (6 ms isochrones).

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5.3.5 Cx43 knockdown in MFBs prevents reentrant tachyarrhythmias

The anti-arrhythmogenic potential of Cx43 downregulation in MFBs was assessed by optical mapping of co-cultures. CV was significantly higher in co-cultures with Cx43-silenced MFBs than in co-cultures with pLuc-silenced MFBs (16.0 ± 3.2 cm/s vs. 10.6 ± 4.3 cm/s; $p < 0.001$) (Figure 6A, C). Furthermore, the incidence of ectopic activity in co-cultures was significantly decreased by Cx43 downregulation in MFBs. Of all co-cultures with LV.SM2C.Cx43.hPGK.eGFP-transduced MFBs 7.9% (total $n=38$) showed ectopy compared to 40.7% in co-cultures with pLuc-silenced MFBs (total $n=27$) (Figure 6D). Foremost, the incidence of reentrant tachycardias in co-cultures was markedly decreased by 44.1% ($n=42$) by Cx43 knockdown in MFBs compared to co-cultures with pLuc-silenced MFBs (Figure 5C, 5D and 6E). And if a co-culture with Cx43-silenced MFBs exhibited a reentrant tachycardia, its reentrant cycle length was shorter than observed in a co-culture with pLuc-silenced MFBs (Figure 6F). Together, these results demonstrate that reduction of heterocellular coupling in fibrotic myocardial cell cultures through Cx43 silencing in MFBs resulted in considerable anti-arrhythmic effects.

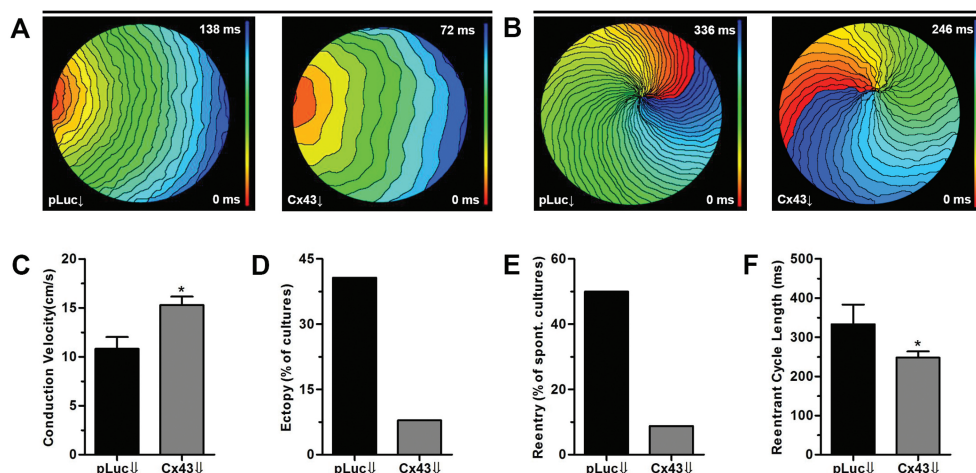


Figure 5.6: Cx43-silencing in MFBs protects the co-culture against ectopic activity and arrhythmias in fibrotic myocardial cultures. (A) Typical activation maps of uniform propagation in co-cultures with Cx43-silenced MFBs and pLuc-silenced MFBs (6 ms isochrones). (B) Typical activation maps of reentrant conduction in co-cultures with Cx43-silenced MFBs and in co-cultures with pLuc-silenced MFBs (control). (C) CV was significantly higher for co-cultures with Cx43-silenced MFBs than in co-cultures with pLuc-silenced MFBs. *: $p < 0.001$ vs. fibrotic cultures with pLuc-silenced MFBs. (D) Co-cultures with Cx43-silenced MFBs showed less ectopic activity and (E) less spontaneous reentrant activity compared to co-cultures with pLuc-silenced MFBs (control). (F) Reentrant arrhythmias were significantly faster in co-fibrotic cultures with Cx43-silenced MFBs than in co-cultures with pLuc-silenced MFBs. *: $p < 0.001$ vs. co-cultures with pLuc silenced MFBs.

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5.4 Discussion

The key findings of this study are (1) MFBs are functionally coupled to CMCs through gap junctions and are thereby able to depolarize the CMC membrane potential through electrotonic interaction, which diminishes CMC excitability and increases the incidence of ectopic activity and spontaneous tachyarrhythmias and (2) reduction in Cx43 expression in MFBs through lentiviral vector-mediated shRNA transfer significantly decreases heterocellular coupling, leading to improvement of CMC's resting membrane potential and excitability which reduce the incidence of ectopic activity and spontaneous tachyarrhythmias.

5.4.1 MFBs and cardiac arrhythmogeneity

Cardiac fibrosis, for a long time known to impair myocardial mechanics, is currently known to be pro-arrhythmic, both *in vivo* and *in vitro*.¹⁸ Unfortunately, the exact pro-arrhythmic mechanisms are still incompletely understood which hinders the development of new anti-arrhythmic strategies. The mechanism by which cardiac fibrosis was thought to be pro-arrhythmic was that of extracellular matrix-mediated CMC detachment, which might

lead to anatomical conduction block.^{19,20} However, in recent years several studies have demonstrated that MFBs, independently of matrix deposition, could play a more crucial pro-arrhythmic role than initially thought. Among the suggested mechanisms is the secretion of paracrine factors that alter CMC excitability.¹³ In addition, electrotonic interaction between MFBs and CMCs has been shown to slow conduction, increase ectopic activity, and increase the propensity to arrhythmias,^{9,10,12,21,22} as was also confirmed by the present study. By targeting MFB proliferation, all these pro-arrhythmic aspects of the MFB can be effectively targeted at once.¹¹ However, as MFB activation and proliferation typically occur after myocardial damage, as seen for instance after myocardial infarction to secure structural integrity of the myocardium, targeting of more specific mechanisms is preferable to prevent adverse effects on the cellular make-up and myocardial structure. Therefore, we investigated whether targeting of heterocellular coupling in fibrotic myocardial cell cultures diminished arrhythmias in these cultures. Our results imply a method to prevent fibrosis-associated arrhythmias while leaving the MFBs and structural integrity of the myocardium intact.

5.4.2 Gap junctional coupling and cardiac action potential conduction

Gap junctions are intercellular channels that allow electrical communication between adjacent cells carried by ions. In the myocardium, gap junctions are indispensable for mediating rapid propagation of the electrical impulse, thereby synchronizing mechanical activity.³ Gap junctional conductance can be altered through gap junctional remodeling associated with several acquired heart diseases leading to arrhythmias. This marks gap junctions as a major player in cardiac arrhythmogeneity.²³ Gap junctions are composed of hemichannel-forming connexons, which in turn are composed of connexins. In the heart, Cx43, Cx40 and Cx45 are prevalent, with Cx43 being most prominent in ventricular myocytes.^{3,23,24} Several *in vitro* studies have suggested that Cx43 upregulation in CMCs might be an anti-arrhythmic strategy, as this could counteract gap junction remodeling and prevent consequential conduction slowing and arrhythmias.^{25,26} However, overexpression of Cx43 in CMCs might pose some difficulties in fibrotic tissue. MFBs can depolarize CMCs through gap junctional coupling as their resting membrane potential is more positive than in CMCs. As heterocellular coupling depolarizes the membrane potential, CMC's excitability may become drastically altered by coupling to MFBs which slows action potential propagation. Moreover, a substantial upstroke velocity is required as the driving force of rapid propagation and would remain diminished in a fibrotic syncytium with increased coupling. The notion that an increased heterocellular coupling might in fact amplify the depolarizing effect of MFBs on CMCs precludes an increase of Cx43 expression in fibrotic myocardium as therapeutic option. In contrast, an *in vitro* study by Kizana *et al.* suggested downregulation of Cx43 expression in CMCs as a possible anti-arrhythmic strategy, as it would focally interrupt macro-reentrant arrhythmias.²⁷ However, several *in vivo* studies of mouse models with various levels of cardiac Cx43 knockdown have revealed significantly slower impulse conduction and an increased vulnerability to arrhythmias compared to wild-type mice.²⁸⁻³⁰ In addition, CMCs show a relative redundancy of Cx43 expression, illustrated by several studies performed with heterozygous knockout mice which showed that 50% or less reduction in Cx43 expression did not result

in reduction of conduction velocity.^{31,32} Taken together, these observations demonstrate the complex nature of targeting Cx43 expression in CMCs, and suggest that further refinement is necessary for gap junction modulation to become a feasible anti-arrhythmic strategy. However, the present study shows that specific targeting of Cx43 expression in MFBs can reduce the depolarizing effect of MFBs on CMCs and act therefore anti-arrhythmic and without impairing gap junctional conductance between CMCs. Technical refinement of gene transfer, for instance by using MFB-specific promotor sequences, may improve the applicability of this approach.

5.4.3 MFB dependent depolarization of CMCs and arrhythmias

Several *in vitro* studies indicate that MFB-CMC coupling could contribute to arrhythmogenesis by altering electrophysiological tissue characteristics. Functional heterocellular coupling was first observed by Rook *et al.*, and was later shown to cause slow conduction through electrotonic interaction.³³ In addition, heterocellular coupling might account for the mechanism by which cardiac excitability is affected in fibrotic tissue.^{7,9,14} These results have been confirmed in our present study showing dye transfer between MFBs and CMCs, and depressed action potentials in CMCs of MFB-CMC co-cultures, which can be attenuated by silencing Cx43 expression in MFBs. Furthermore Vasques *et al.* have demonstrated that after cardiac injury heterocellular interactions are enhanced as a consequence of a phenotypic switch of fibroblasts to MFBs.³⁴ In addition, Lau *et al.* have shown that overexpression of skeletal muscle Na⁺-channel Skm1 in depolarized myocardial infarct border zones suppresses ventricular arrhythmias. These ventricular arrhythmias are attributed to the depolarized resting membrane potential of CMCs in the infarct borderzone leading to decreased CMC excitability and lowering of CV. As Skm1 operates more effectively at a depolarized membrane potential, arrhythmias are prevented.³⁵ These studies, therefore, imply a role for targeting depolarization of CMC's resting membrane potential, possibly through MFB-CMC coupling, to prevent cardiac arrhythmias. However targeting depolarization-induced lowering of CMC excitability by overexpression of Skm1 only targets one factor altered by depolarization namely I_{Na} . In the present study we show a way of targeting depolarization of the CMC membrane potential itself, as opposed to focusing on its consequences. This was achieved by targeting of Cx43 expression in MFBs which thereby prevents the incidence of reentrant tachyarrhythmias. MFB Cx43 silencing could therefore be a promising approach in the treatment of fibrosis-associated arrhythmias.

5.4.4 Study limitations

The use of adult human CMCs and MFBs may have been more clinically relevant, but these CMCs cannot be kept in culture for longer periods and the proliferation rate of such MFBs *in vitro* does not allow a study like this within a reasonable time-frame. Furthermore Cx43 was targeted to reduce heterocellular coupling between CMCs and MFBs. However, in addition to Cx43, MFBs also express Cx40 and Cx45.¹⁶ Heterocellular gap junctions through Cx40 and 45 are not inhibited by this intervention and could therefore still contribute to MFB-induced depolarization of adjacent CMCs. However, considering

the relative expression of Cx43 opposed to Cx40 and Cx45 in CMCs as well as in MFBs, and the marked effect of Cx43 silencing on dye transfer suggests that the roles of Cx40 and Cx45 on electrical coupling of MFBs with CMCs are of minor importance.

5.4.5 Conclusions

MFBs are able to form functional heterocellular gap junctions with CMCs and are therefore able to (1) diminish CMC excitability, (2) reduce CV in cardiac cultures, (3) induce ectopic activity, and (4) give rise to spontaneous reentrant tachyarrhythmias in fibrotic myocardial cell cultures. MFB-CMC coupling can be attenuated by selective silencing of Cx43 expression in MFBs using lentiviral vector mediated shRNA technology. Moreover, targeting heterocellular gap junctional coupling preserves CMC excitability, limits conduction slowing and the incidence of ectopic activity and thereby prevents reentrant tachyarrhythmias. Thus, this study provides a rationale for new therapies of cardiac arrhythmias, based on a selective, substrate-oriented approach.

5.4.6 Funding

This work was supported by the Dutch Heart Foundation (2008/B119). D.A.P. is the recipient of the Netherlands Organisation for Scientific Research (NWO) VENI grant.

5.5 Acknowledgements

We thank Dr. Wilbert P.M. van Meerwijk for constructive discussions, and Huybert J.F. van der Stadt for excellent technical support.

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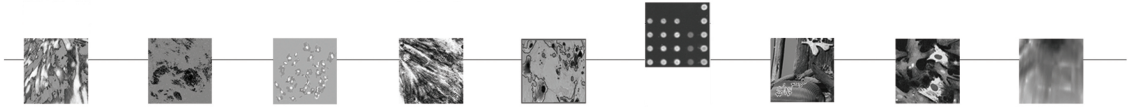
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Chapter 6

Gap junctional coupling with cultured neonatal rat cardiomyocytes induces the cardiomyogenic differentiation of fetal human mesenchymal stem cells

6

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Abstract

Rationale: Gap junctional coupling is important for functional integration of transplanted cells with host myocardium. However, the role of gap junctions in cardiomyogenic differentiation of such cells has not been directly investigated.

Objective: To study the role of Cx43 in cardiomyogenic differentiation of human mesenchymal stem cells (hMSCs).

Methods: Knockdown of Cx43 was established in Cx43-rich fetal amniotic membrane hMSCs (Cx43↓ fetal AM hMSCs), while Cx43 was overexpressed in Cx43-poor adult adipose tissue hMSCs (Cx43↑ adult AT hMSCs). The hMSCs were exposed to cardiomyogenic stimuli by co-incubation with neonatal rat cardiomyocytes (nrCMCs) for 10 days. Differentiation was assessed by immunostaining and whole-cell current-clamping. To establish whether the effects of Cx43 knockdown could be reversed, Cx45 was overexpressed in Cx43↓ fetal AM hMSCs.

Results: Ten days after co-incubation not a single Cx43↓ fetal AM hMSC or adult AT MSC expressed α -actinin, while control fetal AM hMSCs did ($2.18 \pm 0.39\%$, $n=5,000$). Moreover, functional cardiomyogenic differentiation, based on action potential recordings, occurred only in control fetal AM hMSCs. Cx45 overexpression in Cx43↓ fetal AM hMSCs restored their ability to undergo cardiomyogenesis.

Conclusion: Cx43 and in their absence Cx45 is required for transdifferentiation of fetal AM hMSCs into functional cardiomyocytes after co-incubation with nrCMCs. However, Cx43 overexpression does not endow adult AT hMSCs with the capacity to differentiate into cardiomyocytes in co-culture with nrCMCs. Heterocellular gap junctional coupling thus plays an important role in the transfer of cardiomyogenic signals from nrCMCs to hMSCs but is not sufficient to induce cardiomyogenic differentiation in adult AT hMSCs.

6.1 Introduction

Gap junctional coupling is essential in establishing electrochemical communication between cardiac muscle cells.¹ Such coupling of cytoplasmic compartments is mediated by gap junctions, which consist of two hexameric assemblies of connexin (Cx) proteins embedded in the plasma membranes of neighboring cells that form so-called hemichannels or connexons. Gap junctions permit the bidirectional passage of small molecules and ions between cells and are thus able to regulate both physiological and pathophysiological processes.

During embryonic development, spreading of signals across tissues through gap junctions contributes to the migration and specialization of cells.² In the developing heart, Cx40, Cx43 and Cx45 deficiency was shown to result in serious cardiac malformation.³⁻⁷ Besides being important for proper cardiac development, gap junctional communication has also been shown to affect the therapeutic and hazardous potential of cardiac cell therapy.⁸⁻¹⁰ However, the role of gap junctional coupling in cardiomyogenic differentiation of stem cells remains unclear, although gap junction-mediated processes, such as spread of miRNAs and hyperpolarization, have been implicated in the induction of cardiomyogenesis.^{11,12}

Mesenchymal stem cells (MSCs) are a population of mononuclear stromal cells that can be harvested from a wide variety of tissues, are easily expandable *in vitro*, have immunomodulatory properties, secrete paracrine factors that stimulate tissue regeneration and can differentiate into various types of mesodermal and non-mesodermal cells.^{13,14} In addition, these cells were shown to improve cardiac function upon transplantation in the diseased rodent, pig and human heart.¹⁵ However, to what extent MSCs can undergo cardiac differentiation and which factors are involved in this process is still unclear.¹⁶⁻¹⁸ Although donor age and heterocellular interactions were found to affect the cardiomyogenic differentiation potential of MSCs¹⁹ more research is needed into their biological properties and the modification thereof to improve the therapeutic outcome of MSC-based cardiac cell therapy.

Accordingly, in this study, we investigated the role of Cx43, the major gap junction protein of the working myocardium, in the cardiomyogenic differentiation of fetal Cx43-rich and adult Cx43-poor human MSCs¹⁹ in co-cultures with neonatal rat ventricular cardiomyocytes (nrCMCs). To this end, Cx43 expression levels in the hMSCs were either downregulated by short hairpin RNA (shRNA)-mediated RNA interference (RNAi) or upregulated by recombinant human Cx43 (hCx43) gene delivery. To control for possible off-target effects of the hCx43-specific shRNAs, an RNAi rescue experiment based on the forced expression of hCx45 in the Cx43 knockdown cells was included. The consequences of these genetic interventions on the cardiomyogenic differentiation capacity of the hMSCs were assessed using immunocytological and patch-clamp analyses.

The present study shows that gap junctions are directly involved in the transfer of cardiomyogenic signals from nrCMCs to fetal hMSCs. Downregulation of Cx43 gene expression in these hMSCs prevented their cardiomyogenic differentiation in co-cultures with nrCMCs. Overexpression of hCx45 in the Cx43 knockdown cells restored their ability to respond to the cardiomyogenic cues provided by neighboring nrCMCs. However, overexpression of hCx43 in adult hMSCs did not lead to their cardiomyogenic differentiation

in co-cultures with nrCMCs.

6.2 Materials and methods

The role of gap junctional coupling in the transfer of cardiomyogenic signals from nrCMCs to hMSCs was investigated by suppressing Cx43 expression in fetal amniotic membrane (AM) hMSCs using two lentiviral vectors (LVs) encoding different hCx43-specific shRNAs (Cx43↓(1) fetal AM hMSCs and (Cx43↓(2) fetal AM hMSCs) and by LV-mediated over-expression of hCx43 in adult adipose tissue (AT) hMSCs (Cx43↑ adult AT hMSCs). To facilitate the identification of genetically modified cells, the LVs also directed the synthesis of version I (shRNA-encoding LVs) or II (hCx43-encoding LV) of the green fluorescent protein (GFP) of *Renilla reniformis* (hrGFPI and hrGFPII, respectively). Fetal AM hMSCs transduced with an LV encoding an shRNA directed against the *Aequorea victoria* enhanced green fluorescent protein (eGFP) gene and adult AT hMSCs transduced with an LV coding for hrGFPII alone served as control cells (control fetal AM hMSCs and control adult AT hMSCs, respectively). Immunocytological analysis, quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) and western blot analysis were applied to determine Cx43 expression levels, while dye transfer assays and whole-cell current-clamp recordings were used to assess the level of functional coupling. To study cardiomyogenic differentiation, hMSCs were co-incubated with nrCMCs for 10 days. Differentiation was assessed by immunocytological staining and whole-cell current-clamping. LV-encoded hCx45 was overexpressed in Cx43↓(1) fetal AM hMSCs (Cx43↓(1)+Cx45↑ fetal AM hMSCs) to establish whether the effects of Cx43 knockdown could be reversed using the techniques described above. Cx43↓(1) fetal AM hMSCs transduced with an LV encoding eGFP were used as control cells (Cx43↓(1)+eGFP fetal AM hMSCs) in these experiments. For a detailed description of the materials and methods the reader is referred to the Supplement Material and to several previous papers of our research group.^{16,19-21}

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6.3 Results

6.3.1 Characterization of fetal AM hMSCs and adult AT hMSCs

Both the fetal AM hMSCs and the adult AT hMSCs were characterized according to established criteria. To this purpose, their cell surface marker profile and adipogenic and osteogenic differentiation capacity were studied. Both types of hMSCs were negative for CD31 (endothelial cell marker), CD34 and CD45 (both hematopoietic cell markers), whereas they were positive for CD73, CD90 and CD105 (mesenchymal stem cell markers) (Figure 1A-C). *in vitro* differentiation assays confirmed that the two types of hMSCs were able to differentiate into adipocytes (Figure 1D1-D2) and osteoblasts (Figure 1E1-E2), thus confirming their multipotency.

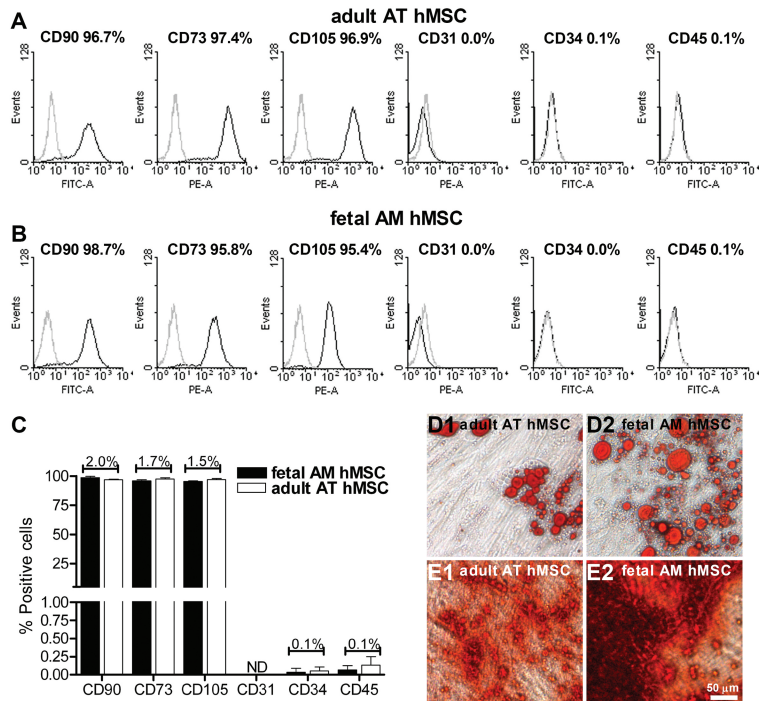


Figure 6.1: Characterization of fetal AM hMSCs and adult AT hMSCs. (A-B) Flow cytometric analyses showed for both types of hMSCs abundant surface expression of the MSC markers, CD90, CD105 and CD73 and hardly any surface expression of the hematopoietic markers CD34 and CD45 or the endothelial marker CD31 (black lines). Isotype-matched control antibodies were included to determine background fluorescence levels (gray lines). Percentages are means of ≥ 4 measurements. (C) Only minor differences in the expression levels of the surface markers were present between fetal AM hMSCs and adult AT hMSCs. ND is not detected. (D1-D2) Adipogenic differentiation was visualized by the presence of Oil Red O-stained fat vacuoles. (E1-E2) Calcium depositions after osteogenic differentiation were visualized by Alizarin Red S staining.

6.3.2 Evaluation of Cx43 expression levels

The impact of the different genetic interventions on the Cx43 expression levels in fetal AM hMSCs and adult AT hMSCs was investigated by three methods. Immunocytological analysis showed that Cx43 $\uparrow$ adult AT hMSCs (Figure 2A2) contained higher levels of Cx43 than control adult AT hMSCs (Figure 2A1), while the protein was also more abundant in control fetal AM hMSCs (Figure 2B1) than in Cx43 $\downarrow$ (1) fetal AM hMSCs (Figure 2B2) or in Cx43 $\downarrow$ (2) fetal AM hMSCs (Figure 2B3) (for each genetic modification $n=4$ isolates of each hMSC type were assessed). These results were validated by western blot analysis ($n=4$ hMSC isolates per experimental group), which showed that Cx43 $\uparrow$ adult AT hMSCs contained $1797.7 \pm 145.5\%$ more Cx43 protein than control adult AT hMSCs ($P < 0.001$) (Figure 2C1 and C3). Western blot analysis also confirmed the presence of

significantly less Cx43 in Cx43↓(1) fetal AM hMSCs (by $79.4 \pm 3.0\%$) and in Cx43↓(2) fetal AM hMSCs (by $77.4 \pm 2.6\%$) than in control fetal AM hMSCs ($P < 0.001$) (Figure 2C2 and C4). In agreement with these results, qRT-PCR revealed that Cx43↑ adult AT hMSCs contained 9.77 ± 0.63 fold ($P < 0.001$) (Figure 2D1) more Cx43 transcripts than control adult AT hMSCs while the amount of Cx43 RNA in Cx43↓(1) fetal AM hMSCs and in Cx43↓(2) fetal AM hMSCs was, respectively, 3.67 ± 0.14 and 3.94 ± 0.25 fold ($P < 0.001$) lower than in control fetal AM hMSCs (Figure 2D2).

6.3.3 Assessment of functional heterocellular gap junctional coupling via Cx43

The effects of modulating Cx43 expression levels in fetal AM hMSCs and in adult AT hMSCs on their functional heterocellular coupling with nrCMCs were investigated by dye transfer experiments using the gap junction-permeable fluorochrome calcein red-orange AM (calcein). Cx43↑ adult AT hMSCs showed a significantly higher dye intensity than control adult AT hMSCs ($67.6 \pm 4.7\%$ vs. $8.43 \pm 1.6\%$ of that in adjacent nrCMCs) ($P < 0.001$) (Figure 3A1-A2 and C1). Furthermore, the fraction of hMSCs that took up calcein from neighboring nrCMCs was much higher for the Cx43↑ adult AT hMSCs than for the control adult AT hMSCs ($71.6 \pm 7.6\%$ vs. $28.3 \pm 4.6\%$) ($P < 0.001$) (Figure 3C3). The relative dye intensity in control fetal AM hMSCs ($63.2 \pm 3.8\%$) was higher than in Cx43↓(1) fetal AM hMSCs ($5.94 \pm 1.4\%$) and in Cx43↓(2) fetal AM hMSCs ($6.00 \pm 1.9\%$) ($P < 0.001$) (Figure 3B1-B3 and C2). Moreover, significantly more of the control fetal AM hMSCs ($75.9 \pm 9.2\%$) had taken up calcein than of the Cx43↓(1) fetal AM hMSCs ($13.2 \pm 2.4\%$) or of the Cx43↓(2) fetal AM hMSCs ($13.0 \pm 2.1\%$) ($P < 0.001$) (Figure 3C4).

To further assess functional coupling between hMSCs and nrCMCs, maximal diastolic membrane potentials (MDPs) were measured in each group of hMSCs in the absence and presence of adjacent nrCMCs. Similar average MDPs were found in hMSC monocultures ($n=6$) (Figure 3D, black bars). However, MDPs, on average, became more negative when nrCMCs were adjoining the hMSCs, although the degree of hyperpolarization differed between the various groups ($n=8$) (Figure 3D, white bars). Cx43↑ adult AT hMSCs and control fetal AM hMSCs showed the most negative MDPs (-38 ± 4 mV and -43 ± 5 mV, respectively). On the other hand, control adult AT hMSCs, Cx43↓(1) fetal AM hMSCs and Cx43↓(2) fetal AM hMSCs, which all contained low levels of Cx43, showed less negative MDPs.

6.3.4 Assessment of cardiomyogenic differentiation ability of hMSCs with genetically altered Cx43 levels

Human-specific immunocytological evaluation. At day 10 of co-culture with nrCMCs, $2.27 \pm 0.4\%$ GFP/human lamin A/C-positive control fetal AM hMSCs were positive for the sarcomeric protein α -actinin with a cross-striated staining pattern like that of the nrCMCs (Figure 4A1-A2 and C). However, GFP/human lamin A/C-positive Cx43↓(1) fetal AM hMSCs and Cx43↓(2) fetal AM hMSCs did not stain positive for α -actinin (Figure 4A3-A4 and C). Furthermore, neither Cx43↑ adult AT hMSCs nor control adult AT hMSCs stained positive for α -actinin (Figure 4B1-B3 and C).

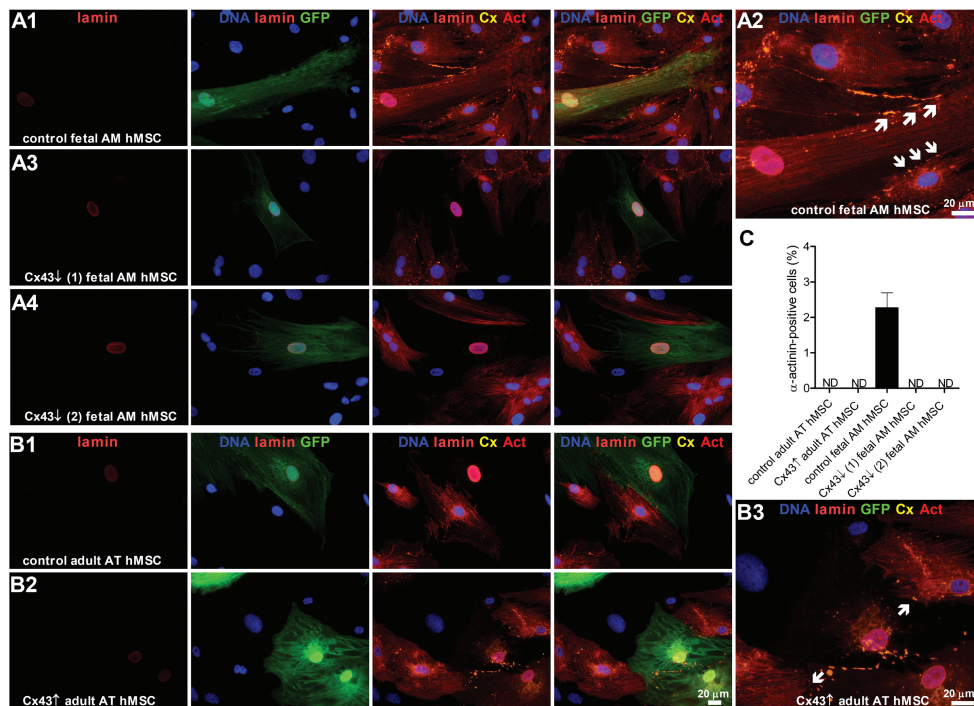


Figure 6.2: Analysis by immunofluorescence microscopy, western blotting and qRT-PCR of Cx43 expression in monocultures of genetically modified fetal AM hMSCs and adult AT hMSCs. (A1-A2) Immunocytochemical analyses revealed low Cx43 levels in control adult AT hMSCs, while high levels of Cx43 were detected in Cx43↑ adult AT hMSCs. (B1) High Cx43 levels were present in control fetal AM hMSCs. (B2-B3) After knockdown of Cx43 gene expression, low Cx43 levels were detected in Cx43↓(1) fetal AM hMSCs and in Cx43↓(2) fetal AM hMSCs. Nuclei were stained with the DNA-binding fluorochrome Hoechst 33342. (C1-C2) Pictures of representative western blots showing that Cx43↑ adult AT hMSCs have large amounts of Cx43 in contrast to control adult AT hMSCs and that control fetal AM hMSCs contain large amounts of Cx43 in contrast to Cx43↓(1) fetal AM hMSCs and Cx43↓(2) fetal AM hMSCs. The GAPDH-specific immunostaining was included for normalization purposes. (C3-C4) Bar graphs of the quantification by western blotting of Cx43 levels in the different populations of hMSCs corrected for differences in GAPDH expression. *P<0.001 vs. Cx43↑ adult AT hMSCs or vs. Cx43↓(1) fetal AM hMSCs and Cx43↓(2) fetal AM hMSCs. (D1-D2) Bar graphs of the assessment by qRT-PCR of Cx43 mRNA levels in control adult AT hMSCs, Cx43↑ adult AT hMSCs and in control fetal AM hMSCs, Cx43↓(1) fetal AM hMSCs and Cx43↓(2) fetal AM hMSCs. *P<0.001 vs. control adult AT hMSCs or vs. Cx43↓(1) fetal AM hMSCs and Cx43↓(2) fetal AM hMSCs.

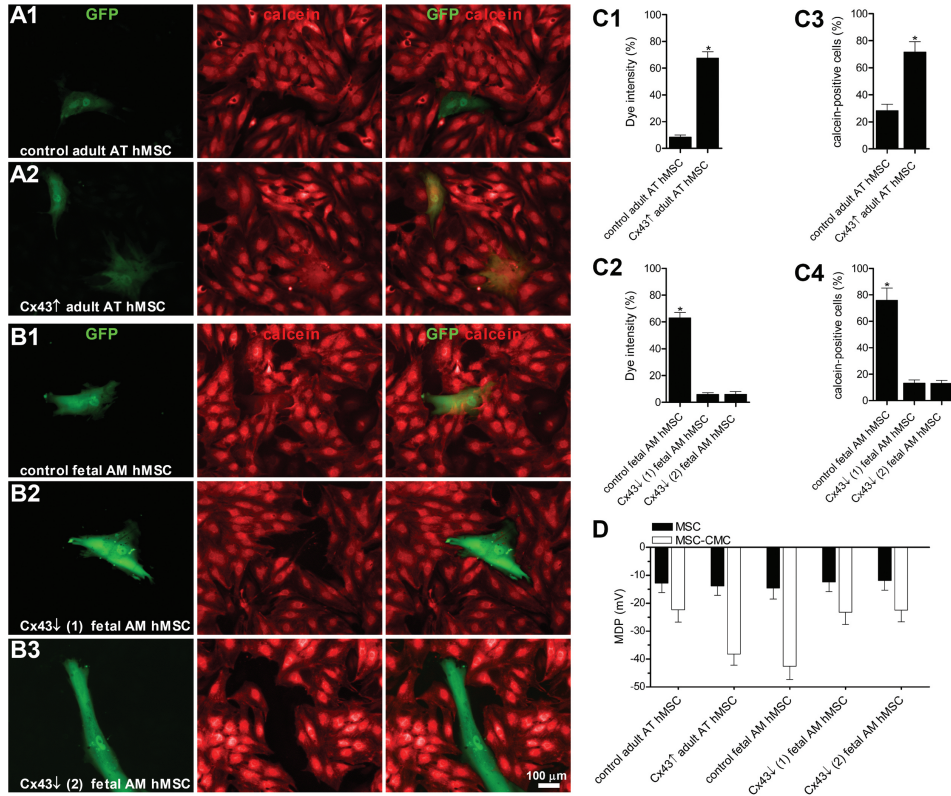


Figure 6.3: Study of the influence of Cx43 levels in hMSCs on their functional coupling with nrCMCs. (A1-A2) Calcein red/orange (indicated as calcein) transfer from nrCMCs to Cx43[↑] adult AT hMSCs was much more efficient than to control adult AT hMSCs. (B1-B3) Dye transfer from nrCMCs to fetal AM hMSCs was strongly inhibited by shRNA-mediated downregulation of Cx43 gene expression in the hMSCs. (C1-C2) Bar graphs of the quantification of dye intensity in GFP-positive hMSCs. Dye intensity in the GFP-positive hMSCs was expressed as percentage of the dye intensity in the surrounding nrCMCs. (C3-C4) Bar graphs of the assessment of the percentage of GFP-positive hMSCs that had taken up calcein from neighboring nrCMCs. *P<0.001 vs. control adult AT hMSC or vs. Cx43[↓](1) fetal AM hMSCs and Cx43[↓](2) fetal AM hMSCs. (D) Average MDPs in hMSC monocultures (indicated as MSC) as measured by whole-cell current-clamping were similar for the different experimental groups. However, the average MDP in hMSCs became more negative in the presence of adjoining nrCMCs (indicated as MSC-CMC), with Cx43[↑] adult AT hMSCs and control fetal AM hMSCs reaching the most negative values.

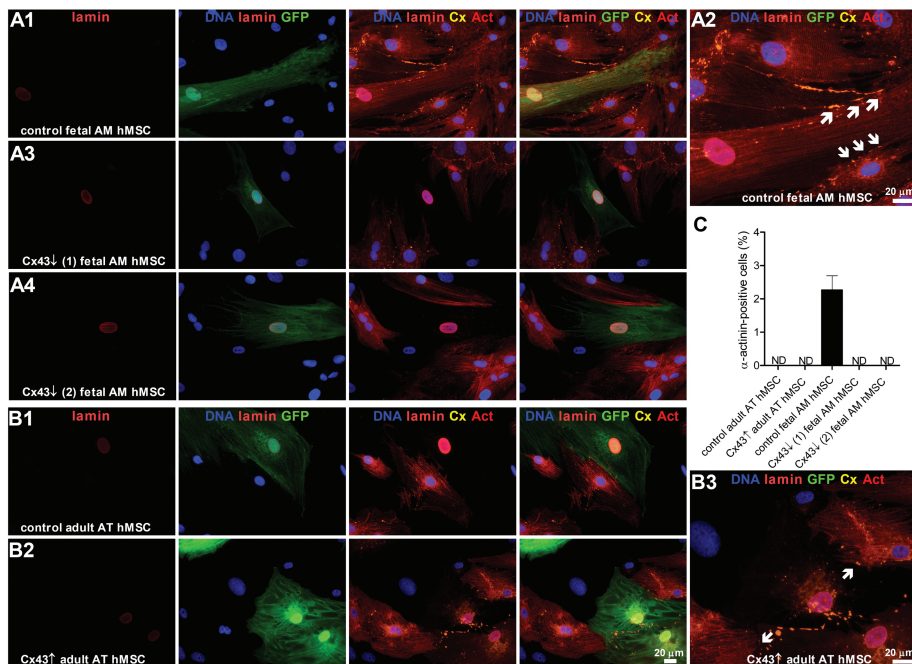


Figure 6.4: Immunocytological assessment of cardiomyogenic differentiation of genetically modified adult AT hMSCs and fetal AM hMSCs after 10 days of co-culture with nrCMCs. (A1) Upon co-culture with nrCMCs, a fraction of the GFP- and human-specific lamin A/C-positive control fetal AM hMSCs became positive for sarcomeric α -actinin (indicated as Act). **(A2)** Intense punctate Cx43 (indicated as Cx) immunostaining of the interfaces between a control fetal AM hMSC and two adjacent nrCMCs (white arrows). **(A3-A4, B1-B2)** GFP-labeled Cx43⁺(1) fetal AM hMSCs, Cx43⁺(2) fetal AM hMSCs, Cx43⁺(adult AT hMSCs and control adult AT hMSCs in co-culture with nrCMCs did not stain positive for α -actinin. **(B3)** Presence of Cx43 plaques at the interfaces between a Cx43⁺ adult AT hMSCs and two bordering nrCMCs (white arrows). **(C)** Quantitative analysis of the cardiomyogenic differentiation ability of the genetically modified hMSCs using immunopositivity for sarcomeric α -actinin as read-out. The graph is based on a minimum of 5,000 cells analyzed from 4 separate hMSC isolates per experimental group. ND is not detected.

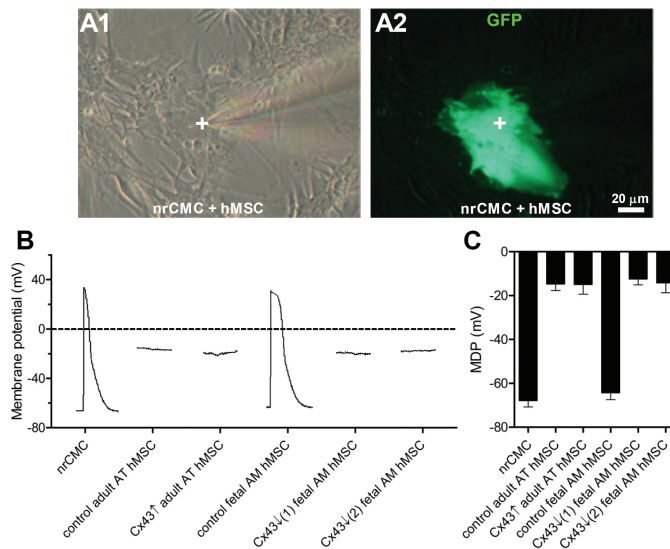


Figure 6.5: Electrophysiological assessment, after gap junctional uncoupling, of cardiomyogenic differentiation of hMSCs. (A1-A2) Bright field (A1) and fluorescence (A2) image of a GFP-positive hMSC with adjacent nrCMCs and patch-clamp electrode. (B) Current-clamp recordings in nrCMCs and in GFP-positive hMSCs from the different experimental groups. Action potentials were measured in nrCMCs and some control fetal AM hMSCs, while the other cells displayed stable membrane potentials. (C) Average MDPs of nrCMCs and in the different groups of GFP-positive hMSCs. For the control fetal AM hMSCs, only the average MDP of cells showing action potentials is presented.

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hMSC-specific intracellular electrophysiological measurements. To assess cardiomyogenic differentiation at a functional level, the ability of GFP-positive hMSCs to generate spontaneous action potentials after gap junction uncoupling was studied (Figure 5A-B). A fraction of the control fetal AM hMSCs showed spontaneous action potentials ($n=6$), with MDPs similar to those of native CMCs ($n=9$) (Figure 5B-C). In contrast, both Cx43[↑] adult AT hMSCs and control adult AT hMSCs showed a steady membrane potential ($n=7$) and more depolarized MDPs of $\sim -15.0 \pm 4$ mV and $\sim -14.8 \pm 3$ mV, respectively. Also, Cx43[↑] adult AT hMSCs remained inexcitable ($n=5$). Importantly, knockdown of Cx43 in fetal AM hMSCs rendered these cells incapable of generating spontaneous action potentials after 10 days of co-culture with nrCMCs ($n=9$ for both shRNAs) and left them with an MDP comparable to those of adult AT hMSCs (~ -64 mV vs. ~ -12 and ~ -14 mV for Cx43[↓](1) fetal AM hMSCs and for Cx43[↓](2) fetal AM hMSCs, respectively).

6.3.5 Evaluation of Cx45 expression levels

To check whether the inability of the Cx43[↓] fetal AM hMSCs to undergo cardiomyogenic differentiation in co-cultures with nrCMCs was not caused by some off-target effect(s) of the hCx43-specific shRNAs, a rescue experiment was carried out. To this end, the

Cx43↓(1) fetal AM hMSCs were transduced with an LV encoding hCx45 or with a control LV directing the synthesis of eGFP. Immunocytological analysis of the resulting cell populations showed that Cx45 was much more abundant in Cx43↓(1)+Cx45↑ fetal AM hMSCs than in Cx43↓(1)+eGFP fetal AM hMSCs (Figure 6A1-A2). Western blot analysis confirmed these results by showing $1162.8 \pm 57.0\%$ higher levels of Cx45 in Cx43↓(1)+Cx45↑ fetal AM hMSCs than in Cx43↓(1)+eGFP fetal AM hMSCs ($n=4$ for both sample types) ($P<0.001$) (Figure 6B1-B2). Consistently, qRT-PCR showed that Cx45 transcript levels were 12.5 ± 1.9 fold higher in Cx43↓(1)+Cx45↑ fetal AM hMSCs than in Cx43↓(1)+eGFP fetal AM hMSCs ($P<0.01$) (Figure 6C).

6.3.6 Assessment of functional heterocellular gap junctional coupling via Cx45

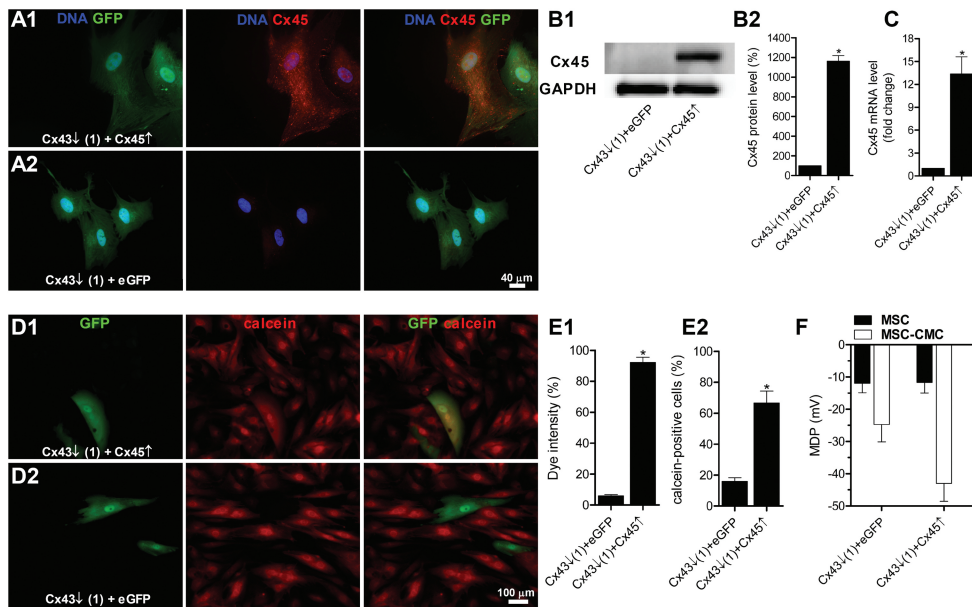
The extent of functional heterocellular coupling between nrCMCs and Cx43↓(1)+Cx45↑ fetal AM hMSCs or Cx43↓(1)+eGFP fetal AM hMSCs was determined in dye transfer assays. The dye intensity relative to that in neighboring nrCMCs was much higher in Cx43↓(1)+Cx45↑ fetal AM hMSCs ($92.3 \pm 3.2\%$) than in Cx43↓(1)+eGFP fetal AM hMSCs ($5.96 \pm 0.8\%$) ($P<0.001$) (Figure 6D1-D2 and E1). Also, the proportion of hMSCs that took up calcein from adjacent nrCMCs was ~4-fold higher for the Cx43↓(1)+Cx45↑ fetal AM hMSCs than for the Cx43↓(1)+eGFP fetal AM hMSCs ($66.7 \pm 7.6\%$ vs. $16.0 \pm 2.3\%$) ($P<0.001$) (Figure 6E2).

To study the impact of Cx45 overexpression on the electrophysiological properties of Cx43↓ fetal AM hMSCs, whole-cell current-clamp measurements were performed. Cx45 overexpression did not significantly alter the average MDP of Cx43↓(1) fetal AM hMSCs in monoculture ($n=6$) (Figure 6F). However, in co-culture with nrCMCs, Cx43↓(1)+Cx45↑ fetal AM hMSCs ($n=6$) showed more negative MDPs as compared to Cx43↓(1)+eGFP fetal AM hMSCs (-24.8 ± 5 mV and -43 ± 5 mV, respectively) (Figure 6F), indicating that electrical coupling of Cx43↓ fetal AM hMSCs with neighboring nrCMCs was enhanced by Cx45 overexpression.

6.3.7 Assessment of cardiomyogenic differentiation capacity of fetal AM hMSCs after rescue of Cx43 knockdown by Cx45 overexpression

Human-specific Immunocytological evaluation Ten days after co-incubation with nrCMCs, Cx43↓(1)+Cx45↑ fetal AM hMSCs and Cx43↓(1)+eGFP fetal AM hMSCs were analyzed for α -actinin positivity. While Cx43↓(1)+eGFP fetal AM hMSCs did not contain detectable amounts of α -actinin, $1.57 \pm 0.4\%$ GFP/human lamin A/C-positive Cx43↓(1)+Cx45↑ fetal AM hMSCs stained positive for this sarcomeric protein indicating that these human cells had transdifferentiated into cardiomyocytes (Figure 7A1-A2 and B).

hMSC-specific intracellular electrophysiological measurements To further study the cardiomyogenic differentiation capacity of Cx43↓(1)+Cx45↑ fetal AM hMSCs, they were subjected to whole-cell current-clamp measurements following pharmacological uncoupling. Cx43↓+eGFP fetal AM hMSCs ($n=5$) showed steady membrane potentials, while some of the Cx43↓(1)+Cx45↑ fetal AM hMSCs ($n=5$) produced spontaneous action potentials with MDPs comparable to those of nrCMCs ($n=7$) (-65 ± 4 mV and -69 ± 5 mV,



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Figure 6.6: Assessment of Cx45 expression and functionality. (A1-A2) Immunocytochemical analyses showed abundant Cx45 expression in monocultures of Cx43↓(1)+Cx45↑ fetal AM hMSCs but not of Cx43↓(1)+eGFP fetal AM hMSCs. A part of the Cx45 signal was detected at the interfaces between Cx43↓(1)+Cx45↑ fetal AM hMSCs. Nuclei were stained with Hoechst 33342. (B1) Representative picture of a western blot confirming high-level expression of Cx45 in Cx43↓(1)+Cx45↑ fetal AM hMSCs and the presence of very low amounts of Cx45 in Cx43↓(1)+eGFP fetal AM hMSCs. The GAPDH-specific immunostaining was included for normalization purposes. (B2) Bar graph of the assessment by western blotting of Cx45 levels in Cx43↓(1)+Cx45↑ fetal AM hMSCs and in Cx43↓(1)+eGFP fetal AM hMSCs corrected for differences in GAPDH expression. *P<0.001 vs. Cx43↓(1)+eGFP fetal AM hMSCs. (C) Bar graph of the quantification by qRT-PCR of Cx45 mRNA levels in Cx43↓(1)+Cx45↑ fetal AM hMSCs and in Cx43↓(1)+eGFP fetal AM hMSCs. *P<0.01 vs. Cx43↓(1)+eGFP fetal AM hMSCs. (D1-D2) Cx43↓(1)+Cx45↑ fetal AM hMSCs much more readily take up calcein from adjacent nrCMCs than Cx43↓(1)+eGFP fetal AM hMSCs. (E1) Quantitative analysis of the dye intensity in Cx43↓(1)+Cx45↑ fetal AM hMSCs and Cx43↓(1)+eGFP fetal AM hMSCs. Dye intensity in GFP-positive hMSCs was expressed as percentage of the dye intensity in the surrounding nrCMCs. (E2) Bar graph of the assessment of the percentage GFP-positive hMSCs that had taken up calcein from neighboring nrCMCs. *P<0.001 vs. Cx43↓(1)+eGFP fetal AM hMSCs. (F) The average MDPs of Cx43↓(1)+Cx45↑ fetal AM hMSCs and of Cx43↓(1)+eGFP fetal AM hMSCs in monocultures are less negative than in co-cultures with nrCMCs. The effect of adjacent nrCMCs on the average MDP was bigger for fetal AM hMSCs in which the knockdown of Cx43 expression was compensated by Cx45 overexpression than for Cx43↓(1)+eGFP fetal AM hMSCs.

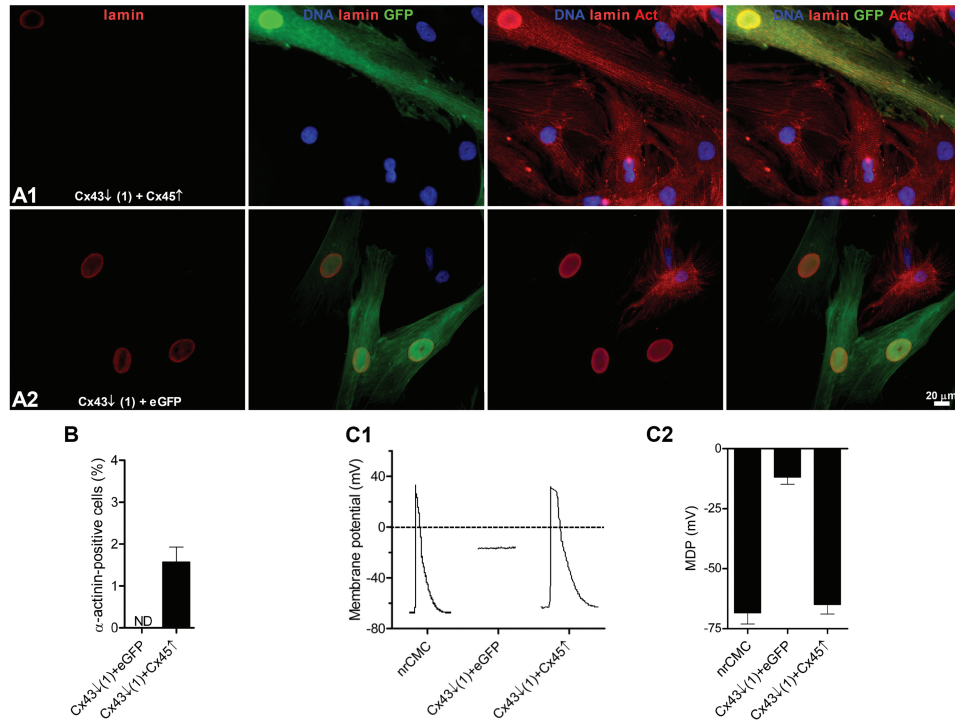


Figure 6.7: Investigation by immunocytochemical and patch-clamp analysis of cardiomyogenic differentiation of fetal AM hMSCs in co-culture with nrCMCs after rescue of Cx43 knockdown by Cx45 overexpression. In the presence of nrCMCs, a fraction of the GFP- and human-specific lamin A/C-positive Cx43↓(1)+Cx45↑ fetal AM hMSCs (**A1**) expressed α-actinin (indicated as Act) in a striated pattern typical of sarcomeric proteins, while Cx43↓(1)+eGFP fetal AM hMSCs (**A2**) did not. (**B**) Quantitative analysis of the cardiomyogenic differentiation capacity of Cx43↓(1)+Cx45↑ fetal AM hMSCs and of Cx43↓(1)+eGFP fetal AM hMSCs using immunopositivity for sarcomeric α-actinin as read-out. The graph is based on a minimum of 5,000 cells analyzed from 4 separate hMSC isolates per experimental group. ND is not detected. (**C1**) Current-clamp recordings in pharmacologically uncoupled co-cultures of nrCMCs and Cx43↓(1)+eGFP fetal AM hMSCs or Cx43↓(1)+Cx45↑ fetal AM hMSCs. Fetal AM hMSCs that had lost the capacity to produce action potentials due to knockdown of Cx43 expression regained this ability following forced Cx45 expression but not after eGFP overexpression. (**C2**) Average MDPs of nrCMCs, Cx43↓(1)+eGFP fetal AM hMSCs and Cx43↓(1)+Cx45↑ fetal AM hMSCs. For the Cx43↓(1)+Cx45↑ fetal AM hMSCs, only the average MDP of cells showing action potentials is presented.

respectively) (Figure 7C1-C2). Thus, the loss of excitability in Cx43↓ fetal AM hMSCs could be overcome by Cx45 overexpression.

6.4 Discussion

The key findings of this study are (1) Fetal hMSCs expressing Cx43 at high levels efficiently communicate with adjacent nrCMCs via gap junctions and can differentiate into functional cardiomyocytes when co-cultured with nrCMCs. (2) Adult hMSCs containing low amounts of Cx43 form few functional gap junctions with adjacent nrCMCs and do not undergo cardiomyogenic differentiation in co-culture with nrCMCs. (3) Cardiomyogenic differentiation of fetal hMSCs in co-culture with nrCMCs is inhibited by Cx43 knockdown, but is rescued by concurrent overexpression of Cx45. (4) Overexpression of Cx43 in adult hMSCs does not lead to cardiomyogenic differentiation of these cells upon co-culture with nrCMCs.

6.4.1 Gap junctions and hMSCs

The expression of the three major cardiac Cxs by hMSCs has previously been demonstrated.^{22,23} Also, the formation of functional gap junctions, important for integration with the surrounding myocardium after cardiac stem cell therapy, has been shown to occur.^{9,22,23} Recently, our research group has shown that hMSCs derived from fetal tissues contain much higher levels of Cx43 than those derived from adult tissues.¹⁹ In addition, evidence was provided that hMSCs of embryonic or fetal origin can undergo cardiomyogenic differentiation in co-culture with nrCMCs while hMSCs derived from adult sources fail to do so. In the present study, Cx43 expression was successfully downregulated in fetal AM hMSCs and upregulated in adult AT hMSCs through LV gene transfer. This resulted in a strong reduction in the gap junctional communication between fetal AM hMSCs and nrCMCs and led to a considerable increase in the gap junctional coupling between adult AT hMSCs and nrCMCs. These results enabled the investigation of the role of gap junctional signaling in the cardiomyogenic differentiation of hMSCs. To this purpose, it was also investigated whether the effects of Cx43 knockdown could be reversed by Cx45 overexpression as it has been previously shown that Cx45 is able to form functional gap junctions.²⁴ In the present study, it was indeed established that transduction with Cx45 led to Cx45 expression at a protein and mRNA level and that the capacity to form functional gap junctions with nrCMCs was restored.

6.4.2 Role of gap junctional communication in cardiomyogenic differentiation

Cxs have been reported to be involved in differentiation processes, such as osteogenesis, neural differentiation and hematopoiesis.²⁵⁻²⁹ However, very little is known about the role of gap junctional communication in cardiomyogenic differentiation. Recently, our research group demonstrated that fetal AM hMSCs express Cx43 in high levels in contrast to adult AT hMSCs. Interestingly, these fetal AM hMSCs were able to transdifferentiate, in co-culture with nrCMCs, into functional CMCs while adult AT hMSCs could not.¹⁹

Various other groups that have used co-cultures with CMCs to induce cardiac differentiation of stem cells have also stated that intercellular communication through gap junctions might be important in this differentiation process.^{30,31} However, the role of gap junctional communication was not directly investigated. Also, Hosada *et al.* recently showed that gap junctional transfer of microRNA-499 promoted the cardiomyogenic differentiation of human cardiac stem cells.¹¹ In the present study, the role of Cx43 in cardiomyogenic differentiation was further elucidated. Fetal AM hMSCs lost their ability to transdifferentiate into functional CMCs after knockdown of Cx43 gene expression. However, not only did overexpression of Cx45 restore the capacity of these hMSCs to form functional gap junctions with nrCMCs, but more importantly, it also rescued the cardiomyogenic differentiation potential of these fetal hMSCs during co-incubation with nrCMCs. In contrast to these findings, adult AT hMSCs did not gain the ability to differentiate into functional CMCs after overexpression of Cx43. The inability of adult AT hMSCs to undergo cardiomyogenesis in co-culture with nrCMCs could have an epigenetic explanation.³²⁻³⁴ According to this hypothesis, genes important for cardiomyogenic differentiation could, in adult AT hMSCs, exist in a transcriptionally repressive state imposed by specific DNA methylation patterns and/or chromatin signatures. Also, qualitative and/or quantitative differences in the repertoire of transcription factors and miRNAs expressed by fetal AM hMSCs and by adult AT hMSCs might contribute to their differential responsiveness to cardiomyogenic signals produced by neighboring nrCMCs.^{35,36}

Nonetheless, heterocellular gap junction coupling seems to play an important role in the transfer of cardiomyogenic signals from nrCMCs to hMSCs and the subsequent transdifferentiation of the latter cells into functional CMCs. Since gap junctions enable intercellular communication, induction of cardiomyogenesis after co-incubation with nrCMCs may be caused by exchange of signals between nrCMCs and fetal hMSCs. Stimulation of cardiomyogenic differentiation by microRNA-499, as previously described, has been demonstrated to be a gap junction-mediated process.¹¹ Moreover, intercellular communication allows calcium oscillations, which has been shown to increase the regenerative potential of human cardiac progenitor cells in a mouse myocardial infarction model.³⁶ Consistent with these findings are the observations by Muller-Borer *et al.* who describe that after co-culture with CMCs, signals are transmitted from these CMCs into liver stem cells through gap junction channels, which induce calcium oscillations in these stem cells. These calcium oscillations in the liver stem cells ultimately lead to acquisition of a cardiac phenotype.³¹ The recent observation that hyperpolarization is sufficient to induce the differentiation of human cardiomyocyte progenitor cells underlines the importance of gap junctional coupling in this process.¹² In the current study, fetal AM hMSCs in co-culture with nrCMCs were hyperpolarized in contrast to those that were transduced with hCx43-specific shRNAs. More importantly, the latter cells were also hyperpolarized in co-culture with nrCMCs after overexpression of Cx45. This could explain why knockdown of Cx43 leads to an inability of fetal AM hMSCs to differentiate towards functional CMCs, while after Cx45 overexpression their cardiomyogenic differentiation potential was restored. However, the percentage of α -actinin-positive cells in the Cx43 $\downarrow$ (1)+Cx45 $\uparrow$ fetal AM hMSCs was somewhat but not significantly lower than in the control fetal AM hMSCs ($2.27 \pm 0.4\%$ vs. $1.57 \pm 0.4\%$) ($P > 0.05$). This could be the consequence of different properties of Cx43 connexons compared to those of Cx45 connexons.³⁸ nrCMCs express Cx40, Cx43 and

Cx45, and Cx45 can be engaged in the formation of homotypic as well as heterotypic gap junctions composed of homomeric or heteromeric connexins.³⁹⁻⁴¹ So while the precise composition of the gap junctions involved in the restoration of the cardiomyogenic differentiation capacity upon overexpression of Cx45 in Cx43↓(1) fetal hMSCs remains elusive, the average MDPs of control fetal AM hMSCs and Cx43↓(1)+Cx45↑ fetal AM hMSCs are quite similar and more importantly the factor(s) that exert their cardiomyogenic effects on fetal AM hMSCs via gap junctions can pass Cx45-containing channels in high enough amounts to set off the cardiomyogenic differentiation of these cells.

6.4.3 Conclusions

Collectively, our data indicate that efficient gap junctional coupling with adjacent CMCs is necessary to induce cardiomyogenic differentiation of fetal hMSCs in co-culture with nrCMCs. However, adult AT hMSCs that express low levels of Cx43 and do not differentiate into CMCs do not gain the ability to transdifferentiate into functional CMCs after overexpression of Cx43.

6.4.4 Limitation

It would have been interesting to conduct the Cx43 knockdown experiments using a cell type with a higher propensity to differentiate into functional cardiomyocytes than fetal hMSCs. However, such a cell type was not available to us.

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6.4.5 Funding

This research forms part of Project P1.04 SMARTCARE of the BioMedical Materials (BMM) program, which is co-funded by the Dutch Ministry of Economic Affairs, Agriculture and Innovation. The financial contribution of the Dutch Heart Foundation (NHS) is gratefully acknowledged. D.E.A. is funded by the Smart Mix Program of the Netherlands Ministry of Economic Affairs and the Netherlands Ministry of Education, Culture and Science. D.A.P. is recipient of Veni grant 91611070 from the Netherlands Organisation for Scientific Research (NWO).

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6.6 Expanded materials and methods

6.6.1 Isolation, culture and characterization of human mesenchymal stem cells (hMSCs)

All investigations with human-derived tissues conformed to the Declaration of Helsinki and were approved by the Medical Ethics Committee of the Leiden University Medical Centre (LUMC). Written informed consent was obtained from all subjects or, in case of the fetal material, from the parents of the fetuses.

Human fetal amniotic membranes (gestational age 17-22 weeks) were collected through legal interventions by the Department of Obstetrics. The membranes were washed twice with phosphate-buffered saline (PBS) and finely minced into 1-2 mm fragments using scissors and scalpels. Cells were dissociated by treatment with 0.1% collagenase type I (Worthington, Lakewood, NJ, USA) for 3 h at 37°C in a humidified 95% air/5% CO₂ atmosphere. Thereafter, 10 mL of Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 µg/mL streptomycin (all from Invitrogen, Breda, the Netherlands; hereinafter referred to as MSC medium) was added. The cell suspension was transferred to a 25-cm² culture flask (Becton Dickinson, Franklin Lakes, NJ, USA) and incubated for 3-4 days at 37°C in a humidified atmosphere of 95% air/5% CO₂ to allow the cells to adhere. The MSC medium was replaced twice a week until the primary cultures had reached ±80% confluency, after which the fetal amniotic membrane (AM) hMSCs were amplified by serial passage using a buffered 0.05% trypsin-0.02% EDTA solution (TE; Lonza Vervier, Vervier, Belgium) for cell detachment.

Adult adipose tissue (AT) hMSCs were derived from subcutaneous abdominal fat tissue (n=4 donors, mean donor age 42.5±5.4 yrs). Tissue samples were washed twice with PBS containing 100 U/mL penicillin and 100 µg/mL streptomycin and finely minced with a scissor. For tissue disruption 0.1% collagenase type I solution was added and the samples were incubated for 1 h at 37°C in a humidified incubator containing 95% air and 5% CO₂. Collagenase type I activity was quenched by adding MSC medium. Samples were then centrifuged at 330×g for 10 min. After centrifugation the top layer of primary adipocytes was removed and the collagenase type I-containing solution was aspirated. The cell pellet was suspended in MSC medium, passed through a nylon cell strainer with a mesh pore size of 70 µm (Becton Dickinson) and the cells were once again collected by centrifugation. The latter procedure was repeated twice, after which the cell pellet was resuspended in 8 ml MSC medium. The resulting cell suspension was transferred to a 75-cm² culture flask (Becton Dickinson) and the adult AT hMSCs were expanded by serial passage using standard methods.

The isolated cells were characterized according to generally accepted criteria using flow cytometry for the detection of surface antigens and adipogenic and osteogenic differentiation assays to establish multipotency. Surface marker expression was examined after culturing the cells for at least 3 passages. Thereafter, the hMSCs were detached using TE, suspended in PBS containing 1% bovine serum albumin fraction V (BSA; Sigma-Aldrich Chemie, Zwijndrecht, the Netherlands) and divided in aliquots of 10⁵ cells. Cells were then incubated for 30 min at 4°C with fluorescein isothiocyanate or phycoerythrin-conjugated monoclonal antibodies (MAbs) directed against human CD105 (Ancell, Bayport, MN, USA), CD90, CD73, CD45, CD34 or CD31 (all from Becton Dickinson). Labeled cells were washed three times with PBS containing 1% BSA and analyzed using a BD LSR II flow cytometer (Becton Dickinson). Isotype-matched control MAbs (Becton Dickinson) were used to determine background fluorescence. At least 10⁴ cells per sample were acquired and data were processed using FACSDiva software (Becton Dickinson).

Established differentiation assays were used to determine the adipogenic and osteogenic differentiation ability of the cells.¹ Briefly, 5×10³ hMSCs per well were plated in a 12-well culture plate

(Corning Life Sciences, Amsterdam, the Netherlands) and exposed to adipogenic or osteogenic differentiation medium. Adipogenic differentiation medium consisted of MEM-plus (*i.e.* α -minimum essential medium [Invitrogen]) containing 10% FBS, 100 U/mL penicillin and 100 μ g/mL streptomycin supplemented with insulin, dexamethason, indomethacin and 3-isobutyl-1-methylxanthine (all from Sigma-Aldrich) to final concentrations of 5 μ g/mL, 1 μ mol/L, 50 μ mol/L and 0.5 μ mol/L, respectively, and was refreshed every 3-4 days for a period of 3 weeks. Lipid accumulation was assessed by Oil Red O (Sigma-Aldrich) staining of the cultures (15 mg of Oil Red O per mL of 60% 2-propanol). Osteogenic differentiation medium consisted of MEM-plus containing 10 mmol/L β -glycerophosphate, 50 μ g/mL ascorbic acid and 10 nmol/L dexamethason (all from Sigma-Aldrich) and was refreshed every 3-4 days for a period of 3 weeks. Afterwards, the cells were washed with PBS and calcium deposits were visualized by staining the cells for 5 min with 2% Alizarin Red S (Sigma-Aldrich) in 0.5% NH_4OH (pH 5.5).

6.6.2 Isolation and culture of neonatal rat cardiomyocytes and cardiac fibroblasts

All animal experiments were approved by the Animal Ethics Committee of the LUMC and conformed to the Guide for the Care and Use of Laboratory Animals, as stated by the US National Institutes of Health (permit number: 10236).²

Primary myocardial cells were dissociated from ventricles of 2-day old male Wistar rats. Neonatal rat cardiomyocytes (nrCMCs) and cardiac fibroblasts (nrCFBs) were separated from each other by differential plating and maintained in a 1:1 (v/v) mixture of DMEM and Ham's F-10 medium (MP Biomedicals, Solon, OH, USA) containing 5% horse serum (Invitrogen), 100 U/mL penicillin and 100 μ g/mL streptomycin as previously described.³

Myocardial cells were plated on fibronectin (Sigma-Aldrich)-coated coverslips (15 mm $\varnothing$) at a density of 2×10^5 cells per well in 12-well plates and incubated at 37°C in a humidified incubator containing 95% air and 5% CO_2 . Proliferation of residual nrCFBs in nrCMC cultures was inhibited by incubating the cells with 10 μ g/mL mitomycin-C (Sigma-Aldrich) in PBS for 1 h.⁴

6.6.3 Production of lentiviral vectors

Two self-inactivating (SIN) lentiviral vector (LV) shuttle plasmids encoding short hairpin (sh) RNAs targeting the human connexin 43 (hCx43) gene (*i.e.* TRCN0000059773 and TRCN0000059775) were selected from the Mission Library (Sigma-Aldrich). The shRNAs encoded by these constructs efficiently inhibit hCx43 expression in hMSCs as assessed by western blotting and contain sense strands that differ at 3 positions from the coding sequence of the rat Cx43 gene. As negative control, a SIN-LV shuttle plasmid directing the synthesis of an enhanced green fluorescent protein (eGFP) gene-specific shRNA (SHC005; Sigma-Aldrich) was used. To allow for the easy assessment of transduction efficiencies, the puromycin N-acetyl transferase-coding sequence in each of these constructs was replaced by that of version I of the green fluorescent protein of *Renilla reniformis* (hrGFPI) as previously described.⁵ The resulting SIN-LV shuttle plasmids were designated pLV.shRNA-hCx43.1.hPGK1.hrGFPI, pLV.shRNA-hCx43.2.hPGK1.hrGFPI and pLV.shRNA.eGFP.hPGK1.hrGFPI, respectively.

Overexpression of hCx43 or hCx45 in hMSCs was accomplished using bicistronic SIN-LVs. The shuttle plasmids for generating these SIN-LVs were made as follows. First, pIRES.hrGFPII (Stratagene/Agilent Technologies, Santa Clara, CA, USA) was digested with HincII and XbaI and the 2.1-kb fragment containing the human cytomegalovirus immediate-early gene promoter, the internal ribosome entry site of encephalomyocarditis virus and the hrGFP version II open reading frame

was combined with the 6.1-kb *Sma*×*Xba*I fragment of pLV.MCS.WHVPRE (GenBank accession number: JN622008) to create pLV.hCMV-IE.IRES.hrGFPII.WHVPRE. Next, the hCx43- and hCx45-coding sequences were released from Mammalian Gene Collection clones IRATp970C0822D and IRAMP995E152Q (Source BioScience LifeSciences, Nottingham, United Kingdom) by restriction enzyme digestion using *Eco*RI (hCx45) or *Cfr*I and *Mun*I (hCx43). After treatment with the Klenow fragment of DNA polymerase I, the resulting hCx43- and hCx45-coding fragments of 1.3 and 1.2 kb, respectively, were combined with the 8.3-kb *Eco*32I fragment of pLV.hCMV.IRES.hrGFPII.WHVPRE using T4 DNA ligase to create pLV.hCMV-IE.hCx43.IRES.hrGFPII.WHVPRE and pLV.hCMV-IE.hCx45.IRES.hrGFPII.WHVPRE. As negative control the original lentiviral vector plasmid pLV.hCMV-IE.IRES.hrGFPII.WHVPRE was used. All restriction and DNA modifying enzymes were obtained from Fermentas (St. Leon-Rot, Germany).

Ligation mixtures were introduced in chemocompetent GeneHogs cells (Invitrogen) and large stocks of the correct plasmids were prepared with the aid of the JETSTAR 2.0. Plasmid Maxiprep Kit (Genomed, Löhne, Germany).

Vesicular stomatitis virus G-protein-pseudotyped SIN human immunodeficiency virus type I vectors were produced in 175-cm² culture flasks (Greiner Bio-One, Alphen a/d Rijn, the Netherlands) seeded with 10⁵ 293T cells per cm² in DMEM supplemented with 10% FBS and 0.01 mmol/L cholesterol. The next day, the producer cells in each flask were transfected with a total of 35 µg of DNA at a 2:1:1 molar ratio of (1) one of the SIN-LV shuttle plasmids, (2) psPAX2 (Addgene, Cambridge, MA, USA) and (3) pLP/VSVG (Invitrogen) using 3 µg of polyethylenimine (Polysciences Europe, Eppelheim, Germany) per µg of DNA as transfection agent. Sixteen hours later, the transfection medium in each flask was replaced by 15 mL of DMEM containing 5% FBS, 10 mmol/L HEPES-NaOH (pH 7.4) and 0.01 mmol/L cholesterol. At 64 h post-transfection, the culture fluid was collected and freed of cellular debris by centrifugation at room temperature for 10 min at 825×g and filtration through a 0.45 µm pore-size cellulose acetate filter (Pall Corporation, East Hills, NY, USA). To concentrate the SIN-LV particles, 5 mL of 20% (w/v) sucrose in PBS was carefully layered under 30 mL of the cleared culture medium, which was then centrifuged for 2 h at 15,000 rpm and 10°C in an SW28 rotor (Beckman Coulter, Fullerton, CA, USA). Next, the supernatant was discarded and the pellet containing the SIN-LV particles was suspended in 500 µL of PBS-1% BSA by gentle rocking overnight at 4°C.

The gene transfer activity of the SIN-LV stocks was determined by end-point titration on HeLa indicator cells using flow cytometric analysis of hrGFPI or hrGFPII expression as read-out. The titers of the SIN-LV preparations are thus expressed in HeLa cell-transducing units (HTUs) per mL.

6.6.4 Transduction of hMSCs

hMSCs were seeded at a density of 2×10⁴ cells per well in 6-well plates (Corning) and incubated overnight in a humidified 95% air/5% CO₂ atmosphere. Next, the culture fluid was replaced by 1 ml per well of fresh MSC medium supplemented with 10 µg/mL diethylaminoethyl-dextran sulfate (GE Healthcare, Leiderdorp, the Netherlands) and 20 HTUs per cell of SIN-LV. After an incubation period of 4 h at 37°C in a humidified atmosphere of 5% CO₂ in air, the cells were washed with PBS, cultured in MSC medium and passaged ≥2 times before being used in any of the co-culture experiments. AT hMSCs were transduced with LV.hCMV-IE.hCx43.IRES.hrGFPII.WHVPRE to overexpress Cx43 (Cx43[↑] adult AT hMSCs), while cells exposed to LV.hCMV-IE.IRES.hrGFPII.WHVPRE served as control (control adult AT hMSCs). To suppress hCx43 gene expression, fetal AM hMSCs were transduced with LV.shRNA-hCx43.1.hPGK1.hrGFPI (Cx43[↓][1] fetal AM hMSCs) or with LV.shRNA-hCx43.2.hPGK1.hrGFPI (Cx43[↓][2] fetal AM hMSCs). Fetal AM hMSCs used as control cells (control fetal AM hMSCs) were incubated with LV.shRNA-eGFP.hPGK1.hrGFPI. Cx43[↓](1) fetal AM hMSCs were transduced with LV.hCMV-IE.hCx45.IRES.hrGFPII.WHVPRE to overexpress

Cx45 (Cx43↓(1)+Cx45↑ fetal AM hMSCs) in these cells. Cx43↓(1) fetal AM hMSCs transduced with the eGFP-encoding SIN-LV LV.hCMV-IE.IRES.eGFP.HBVPRE (previously designated LV-CMV-IRES-eGFP⁶) were used as control cells (Cx43↓(1)+eGFP fetal AM hMSCs).

6.6.5 Analysis of Cx43 and Cx45 expression

The distribution of Cx43 and Cx45 was studied by immunocytochemical stainings as described previously.⁷ In short, cells were fixed on ice with 4% paraformaldehyde in PBS for 30 min, washed with PBS, permeabilized with 0.1% Triton X-100 in PBS for 5 min at 4°C and rinsed again with PBS. To decrease non-specific binding of the primary antibodies the cells were incubated with 1% donkey serum (Sigma Aldrich) in PBS for 30 min. Thereafter, cells were incubated overnight at 4°C with Cx43-specific rabbit polyclonal antibodies (PABs) (C6219; Sigma Aldrich) or with Cx45-specific goat PABs (C-19; Santa Cruz, CA) diluted 1:200 and 1:100, respectively, in PBS containing 0.1% donkey serum. Binding of these primary antibodies to their target antigen was visualized using Alexa 568-conjugated donkey anti-rabbit IgG or anti-goat IgG (both from Invitrogen) at dilutions of 1:200. Nuclei were stained by incubating the cells for 10 min at room temperature with 10 µg/mL Hoechst 33342 (Invitrogen) in PBS. Cells that went through the entire staining procedure but were not exposed to primary antibodies served as negative controls. A fluorescence microscope equipped with a digital color camera (Nikon Eclipse 80i, Nikon Europe, Badhoevedorp, the Netherlands) and dedicated software (NIS Elements, Nikon) were used to analyze the data.

Quantification of Cx43 and Cx45 protein levels was done by western blot analysis. Lysates were made from ≥4 different isolates of hMSCs per experimental group. After determining the protein concentration in each sample using the BCA Protein Assay Reagent (Thermo Fisher Scientific, Etten-Leur, the Netherlands), equal amounts of protein per slot was size-fractionated in a 12% NuPage Bis-Tris gel (Invitrogen) and transferred to a Hybond-P polyvinylidene difluoride membrane (GE Healthcare) using a wet blotting system. This membrane was blocked for 1 h at room temperature with 2% blocking buffer (ECL Advance blocking agent, GE Healthcare) in Tris Buffered Saline-Tween (TBST) solution composed of 10 mmol/L Tris-HCl, 0.05% Tween-20, 150 mmol/L NaCl, pH 7.6. Thereafter, the membrane was incubated for 1 h at room temperature with the PABs directed against Cx43 or Cx45 diluted 1:15,000 and 1:2,000 in blocking buffer, respectively. After multiple washing steps in TBST, the membrane was incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit or donkey anti-goat secondary antibodies (both from Santa Cruz) diluted 1:15,000. For normalization purposes, a mouse MAb recognizing the housekeeping protein glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Chemicon International, Temecula, CA; clone 6C5) was used, which was detected by HRP-conjugated goat anti-mouse secondary antibody (Santa Cruz; 1:15,000). Chemiluminescence was induced with the aid of the ECL Advance Western Blotting Detection Kit and was captured on Hyperfilm ECL (both from GE Healthcare). The intensity of the Cx43-, Cx45- and GAPDH-specific signals was quantified using Image J software (version 1.43; National Institutes of Health, Bethesda, MD, USA). For each sample, the ratios between the GAPDH signal intensity and that of the Cx43 or Cx45 protein were taken as measure of the absolute amounts of both these gap junction proteins in the different hMSC cultures. The Cx43 or Cx45 levels in the experimental samples were expressed as percentage of those of the corresponding control samples, which were set to 100%.

hCx43 and hCx45 transcript levels were determined by quantitative reverse transcription-polymerase chain reaction (qRT-PCR). To this purpose, total cellular RNA was extracted from ≥4 samples for each group of hMSCs using the RNeasy Mini Kit (QIAGEN Benelux, Venlo, the Netherlands). cDNA was synthesized in 20-µL volumes using 2 µg of RNA, 0.25 µg of random hexanucleotides, 25 nmol of dNTPs and 500 U of Superscript III RNase H⁻ reverse transcriptase (all from Invitrogen). The resultant cDNA was amplified by PCR using the primer pairs QT00012684 and

QT00239659 (both from QIAGEN), which are specific for the hCx43 (official name: human gap junction protein, alpha 1 [GJA1]) and hCx45 (official name: human gap junction protein, gamma 1 [GJC1]) gene, respectively. The annealing temperature for these primer combinations was 55°C. The expression of the genes of interest was normalized to that of the housekeeping gene GAPDH using primer set QT01192646 (QIAGEN) also at an annealing temperature of 55°C. The resultant cDNA was used for PCR amplification using SYBR Green (QuantiTect SYBR Green PCR kit; Qiagen, Valencia, CA, USA) following the recommendations of the supplier. Agarose gel electrophoresis and melting curve analysis were carried out to verify that each primer pair yielded a single PCR product of the expected size. PCR amplifications carried out with human right atrium-specific cDNA or without cDNA served as positive and negative controls, respectively. Data were analyzed using the δ Ct method.⁸

6.6.6 Assessment of functional heterocellular gap junctional

Dye transfer Dye transfer assays were used to directly determine functional heterocellular coupling between nrCMCs and the GFP-positive hMSCs. Four days after cell isolation, nrCMC cultures with a density of 2×10^5 cells per well were loaded with dye by incubation for 15 min with 4 μ mol/L calcein red-orange AM (calcein; Invitrogen) in Hank's Balanced Salt Solution (Invitrogen). Thereafter, the cells were rinsed three times with PBS and were kept in the incubator in nrCMC culture medium supplemented with 2.5 mmol/L probenecid (Invitrogen) for ≥ 30 min before 2×10^4 GFP-positive hMSCs were added. Fluorescent images (≥ 30 per group) were captured after 10 h and evaluated in a blinded manner. In all experimental groups, GFP-positive hMSCs surrounded by the same number of nrCMCs were analyzed. ImageJ software was used to determine the intensity of the calcein-associated fluorescence in several randomly-chosen, equally-sized subcellular regions for both the GFP-positive hMSCs and the adjoining nrCMCs. To correct for possible variations in calcein loading efficiency, the dye intensity in the GFP-positive hMSCs was expressed as a percentage of that in the surrounding nrCMCs. The percentage of calcein-positive cells among the GFP-labeled hMSCs was also determined by counting these cells in ≥ 60 fields of view per group.

Intracellular measurements Whole-cell patch-clamp measurements were performed in co-cultures of 2×10^6 nrCMCs and 5×10^4 GFP-positive hMSCs. Typically, 4-6 nrCMCs were adjacent to a single hMSC. Four days after culture initiation, current-clamp recordings were performed at 25°C using an L/M-PC patch-clamp amplifier (List-Medical, Darmstadt, Germany; 3 kHz filtering).⁷ Tip and seal resistance were 2.0-2.5 M Ω and >1 G Ω , respectively. The pipette solution contained (in mmol/L) 10 Na₂ATP, 115 KCl, 1 MgCl₂, 5 EGTA and 10 HEPES/KOH (pH 7.4) and the bath solution consisted of (in mmol/L) 137 NaCl, 4 KCl, 1.8 CaCl₂, 1 MgCl₂ and 10 HEPES (pH 7.4) in water. pClamp/Clampex8 software (Axon Instruments, Molecular Devices, Sunnyvale, CA, USA) was used for data acquisition and analysis. All patch-clamp measurements were conducted in a blinded manner.

6.6.7 Assessment of cardiomyogenic differentiation

To investigate their cardiomyogenic differentiation ability, 2×10^4 hMSCs from each experimental group were co-incubated for 10 days with 2×10^5 nrCMCs. The GFP-positive hMSCs were added to the nrCMC cultures 2 days after isolation of the nrCMCs.

Human-specific immunocytochemical analysis of cardiomyogenic differentiation potential Immunocytochemical stainings were conducted as previously described.³ On day 10 after culture initiation, the co-cultures of nrCMCs and GFP-positive hMSCs were fixed and stained with a mouse MAb recognizing the sarcomeric protein α -actinin (clone EA53; Sigma-Aldrich; dilution 1:400) and with the Cx43-specific PAb Cx43 described above. The primary antibodies were visualized using

Alexa 568-coupled donkey anti-mouse IgG and Alexa 532-coupled goat anti-rabbit IgG secondary antibodies at dilutions of 1:200. Besides through their green fluorescence, the hMSCs in the co-cultures were identified by labeling with a human lamin A/C-specific murine MAb (clone 636; Vector Laboratories, Burlingame, CA; dilution 1:200). Lamin A/C staining was visualized with Qdot 655-streptavidin conjugates (Invitrogen; dilution 1:200) after incubation of the cells with biotinylated goat anti-mouse IgG2b secondary antibodies (Santa Cruz; dilution 1:200). Nuclei were stained using a 10 μ g/mL solution of Hoechst 33342 in PBS. The percentage of GFP/lamin A/C-positive cells expressing α -actinin was determined by microscopic analysis, at 40 $\times$ magnification, of 25 cultures (200 cells per culture) of a total of 4 MSC isolates per experimental group. A fluorescence microscope equipped with a digital color camera (Nikon Eclipse 80i, Nikon Europe, Badhoevedorp, the Netherlands) and dedicated software (NIS Elements, Nikon) were used to analyze the data. All co-cultures were treated equally using the same antibody dilutions and exposure times.

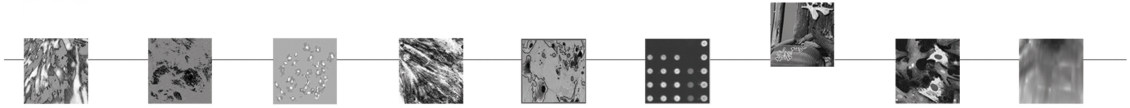
Electrophysiological measurements in co-cultures of hMSCs and nrCMCs after pharmacological uncoupling Cells were plated, cultured and studied under the same conditions as described above. Whole-cell current-clamp recordings were performed 10 days after culture initiation. Prior to the start of the measurements, the cells were treated for 15 min with 180 μ mol/L of the pharmacological gap junctional uncoupler 2-aminoethoxydiphenyl borate (2-APB; Tocris, Ballwin, MO, USA) as previously described.⁷ All measurements were conducted in a blinded manner.

6.6.8 Statistics

Experimental results were expressed as mean $\pm$ standard deviation for a given number (n) of observations. Data was analyzed by Student's *t*-test for direct comparisons. Analysis of variance followed by appropriate *post-hoc* analysis was performed for multiple comparisons. Statistical analysis was carried out using SPSS 16.0 for Windows (SPSS, Chicago, IL, USA). Differences were considered statistically significant at $P < 0.05$.

6.6.9 References

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Chapter 7

Development of adeno-associated virus vectors for the transduction of myocardial scar fibroblasts

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Abstract

Introduction Scar formation after myocardial infarction is an important cause of cardiac dysfunction. This makes myocardial scar fibroblasts (MSFs) interesting targets for therapeutic intervention. One way of modulating the properties of MSFs is by genetic modification with viral vectors. Among the currently used viral gene delivery vehicles, adeno-associated virus (AAV) vectors stand out for their *in vivo* safety and ability to spread easily through tissues. The aim of this study was to compare the transduction of neonatal rat cardiomyocytes (nrCMCs) and of human, mouse and rat MSFs by AAV vectors with different capsids and with transgenes driven by different promoters.

Methods and results AAV vector shuttle plasmids containing a *LacZ* gene driven by 9 different promoters including the human, mouse and rat cytomegalovirus *immediate early* gene (CMV-IE) promoter were constructed. The AAV vector genomes specified by these plasmids were packaged in AAV serotype 2 (AAV2) capsids. β -galactosidase assays revealed that of all CMV-IE promoters, the human CMV-IE promoter outperformed the rodent CMV-IE promoters in human MSFs while the rodent CMV-IE promoters were more active in murine and rat MSFs. Furthermore, of all nine promoters tested the human *eukaryotic translation elongation factor 1 alpha 1* gene (hEF1 α) promoter was most active in rodent MSFs while the Rous sarcoma virus (RSV) promoter gave the highest transgene expression in nrCMCs. A comparison of the transduction efficiency of rat MSFs and nrCMCs with AAV2 vectors carrying capsids of AAV serotypes 2, 5, 6, 8 and 9 showed that transduction of rat MSFs was most efficient with AAV2/2 vectors whereas AAV2/9 best transduced the nrCMCs.

Conclusion The highest transgene expression in rat MSFs is obtained by transduction with an AAV2/2 vector containing an hEF1 α promoter-driven transgene while transgene expression in nrCMC is highest after transduction with an AAV2/9 vector in which foreign gene expression is controlled by the RSV promoter.

7.1 Introduction

After myocardial infarction (MI), cardiomyocytes in the affected region die and are replaced by scar tissue consisting of fibroblasts and dense extracellular matrix (ECM). The scar tissue contributes to the stiffening of the myocardium, reducing the overall contractile performance of the heart.¹ Furthermore, the scar tissue prevents proper action potential propagation due to (i) poor electrical coupling of scar fibroblasts with each other and with adjacent cardiomyocytes and (ii) high electrical resistance of the ECM.²

To overcome the adverse mechanical and electrical effects of scar tissue on cardiac performance, several approaches have been tested. First of all, transplantation of unmodified autologous bone marrow-derived stem cells (BMSCs) has been attempted to regenerate the damaged myocardium.³ Some minor improvement of cardiac function was observed in some studies,⁴⁻⁶ with the therapeutic effect being predominantly attributable to paracrine factors released by the donor cells rather than to the differentiation of the injected BMSCs into cardiomyocytes.⁷ To increase their therapeutic effect, it has been proposed to endow BMSCs with genes that can induce their cardiomyogenic differentiation before injecting them into the heart.⁸ However, numerous issues remain with cardiac cell therapy, including the inefficient retention of the donor cells at the site of injection, the poor survival of the injected cells, and the low propensity of the surviving BMSCs to differentiate into cardiomyocytes.

A possible strategy to bypass at least some of the limitations associated with cell therapy is to directly target the resident myocardial scar fibroblasts (MSFs) using *in vivo* gene therapy with the aim to endow them with more favorable biosynthetic, contractile and/or electric properties. Via this approach, the number of cells available for therapeutic intervention would be much higher than by the injection of stem cells, provided that the MSFs can be efficiently transduced *in vivo* with the gene construct of choice. Of the various gene delivery vehicles available for cardiac gene therapy, adeno-associated virus (AAV) vectors constitute an attractive option due to their excellent *in vivo* safety record and small size (≈ 20 nm in diameter) allowing them to penetrate dense tissues better than other commonly used viral vectors. Other advantages of AAV vectors are their low immunogenicity and the long-term persistence of their genomes in target cells mainly as episomes leading to prolonged transgene expression with a low chance of insertional mutagenesis.⁹

Vectors based on recombinant AAV serotype 2 (AAV2) genomes packaged in capsids of AAV serotypes 1 through 9 (*i.e.* AAV2/1 through AAV2/9 vectors) have been tested in healthy rodent hearts.¹⁰⁻¹³ Unfortunately, in none of these *in vivo* experiments the identity of the transduced cardiac cells was investigated making it impossible to determine the contribution of the four major cell types in the non-diseased heart (*i.e.* cardiac fibroblasts, cardiomyocytes, vascular smooth muscle cells and endothelial cells) to the observed transgene expression. However, Qi *et al.* did test the ability of AAV2/2, AAV2/5, AAV2/7, AAV2/8 and AAV2/9 vectors to transduce cardiac fibroblasts and cardiomyocytes of neonatal rats in culture¹² but thus far the transduction of MSFs by different AAV vector pseudotypes has not been investigated.

Depending on the therapeutic strategy that is being pursued to treat infarcted hearts by gene therapy, it may be important to develop AAV vectors that efficiently transduce

MSFs and induce high-level therapeutic expression in these cells but do not cause appreciable transgene expression in cardiomyocytes. In an attempt to generate an AAV vector meeting these requirements, five different AAV vector pseudotypes were tested for the efficiency by which they transduce MSFs as compared to cardiomyocytes. These experiments revealed that AAV2/6 and AAV2/2 vectors best transduce neonatal rat cardiomyocytes (nrCMCs) and rat MSFs (rMSFs), respectively. In addition, MSFs of man, mouse and rat and nrCMCs were exposed to AAV vectors carrying nine different promoters, to determine which of these promoters is most specific for either of these cell types. Of all promoters tested, the human *eukaryotic translation elongation factor 1 alpha 1* gene (hEF1 α) promoter was the most active in rodent MSFs while the human cytomegalovirus *immediate early* gene (hCMV-IE) promoter and Rous sarcoma virus (RSV) promoter performed best in human MSFs (hMSFs) and nrCMCs, respectively. We also generated two new helper plasmids based on the widely used AAV packaging construct pDG¹⁴ for the production of AAV2/8 and AAV2/9 vectors in a two-plasmid system. Use of these new helper plasmids resulted in AAV vector yields similar to those obtained with commonly applied AAV2/8 and AAV2/9 vector production systems based on the transfection of host cells with three different plasmids.

7.2 Materials and Methods

7.2.1 Primary cell cultures

All animals were treated in accordance with national guidelines and with permission of the Animal Experiments Committee of the Leiden University Medical Center.

rMSFs were obtained from myocardial scar tissue of 16-week-old Wistar Kyoto rats 2 weeks after MI induction by ligation of the left anterior descending artery (LAD). The white scar tissue was dissected from the heart and cut into small pieces. Each of these pieces was then placed in a gelatin-coated 10-cm² cell culture dish (Greiner Bio-One, Alphen a/d Rijn, the Netherlands) containing Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% fetal bovine serum (FBS), 100 U/mL penicillin and 100 μ g/mL streptomycin (all from Invitrogen, Breda, the Netherlands) and covered with a glass coverslip. After 7 days, the scar tissue was removed and the adherent fibroblasts that had grown out from the tissue were passaged. rMSFs were further propagated in DMEM supplemented with 20% FBS, 100 U/mL penicillin and 100 μ g/mL streptomycin. mMSFs were obtained from myocardial scar tissue of 10 week old NOD/Scid mice 6 days after MI induction by ligation of the LAD. The white scar tissue was dissected from the heart and used to obtain mMSFs following the same protocol as for the isolation of the rMSFs.

Cultures of nrCMCs and neonatal rat cardiac fibroblasts (nrCFBs) were prepared from 2-day-old rats as described before.¹⁵ nrCMCs were cultured in a 1:1 mixture of DMEM and Ham's F10 (Invitrogen) supplemented with 5% HS, (Invitrogen) 100 U/mL penicillin and 100 μ g/mL streptomycin. In order to determine the fraction of nrCFBs present in purified nrCMC cultures, these cultures were immunostained for the cardiomyocyte marker sarcomeric α -actinin and for the fibroblast marker collagen type I. To this end, the cells were incubated overnight at 4°C with mouse IgG1-anti-sarcomeric α -actinin antibody (clone EA-53; Sigma-Aldrich, St. Louis, MO) and with collagen type I-specific rabbit IgG

(ab292; Abcam, Cambridge, MA) which were diluted 1:400 in phosphate-buffered saline (PBS) + 5% FBS. The next day, the cells were washed once with PBS and then incubated for 1 hour at 4°C with Alexa Fluor 568-conjugated goat-anti-mouse IgG and Alexa Fluor 488-conjugated goat-anti-rabbit IgG antibodies (both from Invitrogen) diluted 1:400 in PBS + 5% FBS. The percentage of nrCFBs in purified nrCMC cultures ranged from 5 to 10% of the total cell number. nrCFBs were cultured in DMEM supplemented with 10% FBS, 100 U/mL penicillin and 100 µg/mL streptomycin.

hMSFs were obtained and cultured as described before.¹⁶ All primary cells were cultured at 37°C in an atmosphere of 10% CO₂ in air saturated with water vapour.

7.2.2 Cell lines

293T cells¹⁷ [DuBridge RB, 1987] were cultured in DMEM supplemented with 10% FBS at 37°C and 10% CO₂. 911E4 cells [He TC, 1998]¹⁸ were grown in DMEM supplemented with 10% FBS, 0.4 mg/mL geneticin (Invitrogen) and 0.1 mg/mL hygromycin B (Invitrogen) at 37°C and 10% CO₂. Upon seeding the 911E4 cells for end-point titration of AAV vector stocks, the cells were cultured in DMEM supplemented with 10% FBS, 0.4 mg/mL geneticin, 0.1 mg/mL hygromycin B and 100 ng/mL of doxycyclin (Sigma-Aldrich).

7.2.3 Plasmid constructs

Helper plasmids for making AAV2/8 and AAV2/9 vectors in a two-plasmid production system were generated by replacing the AAV2 *cap* gene in pDG by the *cap* gene of AAV serotype 8 (AAV8) and AAV serotype 9 (AAV9), respectively. To this end, pDG (obtained from Jürgen Kleinschmidt¹⁴) was digested with Bsu15I, treated with the Klenow fragment of DNA polymerase I and subsequently digested with Sall. The AAV8 and AAV9 *cap* genes were released from pAAV2/8 or pAAV2/9 (both obtained from James Wilson; Penn Vector Core, Philadelphia, PA), respectively, by digesting these plasmids with MssI and Sall. The resulting 3.1-kb fragments containing the *cap* gene of either AAV8 or AAV9 were combined with the 18.7-kb Bsu15I×Sall fragment of pDG to create pDP8 and pDP9, respectively.

The various AAV vector shuttle plasmids encoding *Escherichia coli* β-galactosidase were constructed as follows. The AAV vector shuttle constructs pDD1, pDD2 and pDD345 containing RSV, hCMV-IE and the hybrid, chicken β-actin gene-based CAG promoter, a multiple cloning site and the simian virus 40 (SV40) polyadenylation signal (pA) flanked by the AAV2 inverted terminal repeats (ITRs) were a gift of Dongsheng Duan.¹⁹ First, the 3.1-kb XhoI×XbaI fragment from pCMVβ.LacZnls12co (Marker Gene Technologies, Eugene, OR) containing a *LacZ* gene that is codon-optimised for expression in mammalian cells and encodes a nuclear-targeted version of β-galactosidase (LacZnls12co) was ligated to the vector backbone of XhoI- and XbaI-digested pDD345 using T4 DNA ligase to create pDD.CAG.nlsLacZco.SV40pA. Next, a 46-bp synthetic polyadenylation (synpA) signal²⁰ flanked by BamHI and NotI restriction enzyme recognition sequences was created by hybridizing two oligonucleotides (sense strand: 5'-GGCCGCAATAAAATATCTTTATTTTCAT TACATCTGTGTGTTGTTTTTTGTGTG-3'; antisense strand: 5'-GATCCACACAAAAA CCAACACACAGATGTAATGAAAATAAGATATTTTATTGC-3'). The synpA site was sub-

sequently cloned into BamHI- and NotI-digested pDD.CAG.nlsLacZco.SV40pA to make pDD.CAG.nlsLacZco.synpA. The latter construct was incubated with BamHI and HindIII and the 3.2-kb fragment containing the *LacZ* gene and synpA was combined with the 4.7-kb fragment of BamHI- and HindIII-digested pDD2 to produce pDD.hCMV-IE.nlsLacZco.synpA.

The hybrid, SV40 *early* gene-based $SR\alpha$ promoter was excised from plasmid pMX1 (R&D Systems, Abingdon, UK) using XbaI and BglII and inserted in XbaI- and BamHI-digested pBluescriptSK(-) (Stratagene, Palo Alto, CA) to generate pBluescriptSK(-). $SR\alpha$. The $SR\alpha$ promoter was subsequently excised from pBluescriptSK(-). $SR\alpha$ using EcoRV and inserted in reverse orientation in EcoRV-digested pBluescriptSK(-) to generate pBluescriptSK(-). $SR\alpha$ (-). The latter construct was treated with BclI and HindIII and the resulting 0.7-kb fragment containing the $SR\alpha$ promoter was combined with the 7.2-kb BclI×HindIII fragment of pDD.CAG.nlsLacZco.synpA using T4 DNA ligase. This led to the construction of AAV vector shuttle plasmid pDD. $SR\alpha$.nlsLacZco.synpA.

The RSV promoter was obtained by digestion of pDD1 with XhoI and HincII and cloned into EcoRV- and SalI-digested pBluescriptSK(-) yielding pBluescriptSK(-).RSV. Next, the 0.7-kb BclI×HindIII fragment from pBluescriptSK(-).RSV containing the RSV promoter was combined with the 7.2-kb BclI×HindIII fragment of pDD.CAG.nlsLacZco.synpA yielding pDD.RSV.nlsLacZco.synpA.

The mouse *S100 calcium binding protein A4* gene (mFSP1) promoter,²¹ rat *lysyl oxidase* (rLOX) promoter,²² hEF1 α promoter, mouse *CMV-IE* gene (mCMV-IE) promoter and rat *CMV-IE* gene (rCMV-IE) promoter sequences were obtained by polymerase chain reaction (PCR). The mFSP1 and rLOX promoters were amplified from chromosomal DNA, whereas the hEF1 α , rCMV-IE and mCMV-IE promoters were amplified from pLVET-KRAB,²³ pSSHK3²⁴ and pMH5 (Microbix Biosystems, Mississauga, Ontario, Canada), respectively. The PCRs were performed in a total volume of 50 μ L using 200 ng (for genomic DNA) or 10 ng (for plasmid DNA) template, 0.5 μ mol/L of both forward and reverse primer (mFSP1: 5'-GCTGATATCGAAGCCTGGTTCCTCCAGAC-3' and 5'-CTGGATATCACCTTTCTGTGCCTCTCA-3'; rLOX: 5'-GCCGATATCCTGGGGTTTATTCGCTG-3' and 5'-GAAGATATCGATGATCTCCCGGCTCGTC-3'; hEF1 α : 5'-GCTGATATCGGCTCCGGTGC CCGTCAG-3' and 5'-CTGGATATCTCACGACACCTGAAATGG-3'; mCMV-IE: 5'-CTAACT AGTGGGGTGGTGA CTCAATGGCC-3' and 5'-GATAAGCTTAGTACCGACGCTGGTGC GC-3'; rCMV-IE: 5'-GTACTAGTTCCCATATTAAGAATGAGTTATAG-3' and 5'-GTAAAGCTT CCGATCCGAAGACTGTGACTG-3'), 200 μ mol/L of each dNTP, 1× Phusion HF buffer (Finnzymes, Espoo, Finland) and 1 U Phusion high-fidelity DNA polymerase (Finnzymes). The PCRs were performed according to the protocol provided with the DNA polymerase using an annealing temperature of 60°C and 35 amplification cycles. The 1.1-kb PCR fragment containing the mFSP1 promoter, the rLOX promoter PCR fragment of 0.8 kb and the 1.2-kb PCR fragment containing the hEF1 α promoter were digested with EcoRV, purified from agarose gel using the JETSORB gel extraction kit (GENOMED, Löhne, Germany) and cloned into EcoRV-digested pBluescriptSK(-) to produce pBluescriptSK(-).mFSP1, pBluescriptSK(-).rLOX and pBluescriptSK(-).hEF1 α , respectively. The mFSP1 promoter was released from pBluescriptSK(-).mFSP1 using EcoRV and combined with the 7.2-kb EcoRV fragment of pDD. $SR\alpha$.nlsLacZco.synpA to generate pDD.mFSP1.nlsLacZco.synpA. The rLOX and hEF1 α promoters were excised from pBluescriptSK(-).rLOX

and pBluescriptSK(-).hEF1 α , respectively, using BclI and HindIII and combined with the 7.2-kb BclI $\times$ HindIII fragment of pDD.CAG.nlsLacZco.synpA to produce pDD.rLOX.nlsLacZco.synpA and pDD.hEF1 α .nlsLacZco.synpA. The 1.1-kb PCR fragments containing the mCMV-IE and rCMV-IE promoter were digested with BclI and HindIII, purified from agarose gel and ligated to the 7.2-kb BclI $\times$ HindIII fragment of pDD.CAG.nlsLacZco.synpA yielding pDD.mCMV-IE.nlsLacZco.synpA and pDD.rCMV-IE.nlsLacZco.synpA, respectively. The correctness of the cloned PCR fragments was verified by nucleotide sequence analysis using the BigDye Terminator v3.1 cycle sequencing kit, the BigDye X Terminator purification kit and the 3730xl DNA analyzer (all from Applied Biosystems Life Technologies, Carlsbad, CA).

To create an AAV vector containing an hEF1 α promoter-driven *Aequorea victoria* enhanced green fluorescent protein (eGFP) gene, the AAV vector shuttle plasmid pTR.hCMV-IE.eGFP.WHVPRE.bGHpA, a derivative of pTR-CMVgfp containing the woodchuck hepatitis virus posttranscriptional regulatory element (WHVPRE)²⁵ was digested with BamHI and KpnI. Next, the 5'- and 3'-recessed ends were blunted using T4 DNA polymerase and the 1.6-kb fragment containing the eGFP gene and the WHVPRE was ligated to the 5.2-kb Klenow DNA polymerase-treated HincII $\times$ HindIII fragment of pDD.hEF1 α .nlsLacZco.synpA to create pDD.hEF1 α .eGFP.WHVPRE.synpA. To obtain a similar AAV vector encoding the near-infrared fluorescent protein TurboFP650 the AAV vector shuttle plasmid pDD.RSV.FP650.WHVPRE.synpA was constructed. First, pTurboFP650-N (Evrogen, Moscow, Russia) was digested with BamHI and DraI and the 0.8-kb fragment containing the TurboFP650-coding sequence was combined with the 4.8-kb BstZ17I $\times$ BamHI fragment of pTR.hCMV-IE.eGFP.WHVPRE.bGHpA to create pTR.hCMV-IE.FP650.WPRE.SV40pA. This plasmid and pDD.RSV.nlsLacZ.co.synpA were then used to make pDD.RSV.FP650.WHVPRE.synpA applying the same strategy as for the construction of pDD.hEF1 α .eGFP.WHVPRE.synpA. All restriction and DNA modifying enzymes as well as the dNTPs were purchased from Fermentas (St. Leon-Rot, Germany) and the oligodeoxyribonucleotides were provided by Eurofins MWG Operon (Ebersberg, Germany).

7.2.4 AAV vector production, purification and quantification

AAV vector particles were produced in 293T cells. Per AAV vector production, 10^6 (small scale) or 7×10^7 (intermediate scale) cells were seeded at a density of 10^5 cells/cm² and cultured in DMEM supplemented with 10% FBS 1 day before transfection. Two hours prior to transfection, the cell culture medium was refreshed with DMEM + 10% FBS. The cells were transfected with 71 ng AAV vector shuttle plasmid and 214 pDG,¹⁴ pDF5, pDG6,²⁶ pDP8 or pDP9 per cm² of cells (two-plasmids production system) or 71 ng AAV vector shuttle plasmid, 71 ng pAAV2/8 or AAV2/9 and 143 ng pAd. Δ F6 (provided by James Wilson; Penn Vector Core)²⁷ per cm² of cells (three-plasmids production system) using 3 ng linear 25-kDa polyethylenimine (Polysciences Europe, Eppelheim, Germany) per ng DNA as transfection agent. After 16 hours, the medium was replaced by DMEM + 5% FBS. Sixty-four hours after transfection, the 293T cells from four 175 cm² flasks were collected in 10 mL PBS + 1 mM CaCl₂ + 1 mM MgCl₂. The cells were then subjected to 3 cycles of snap freezing in liquid nitrogen and thawing in a water bath of 37°C to release the AAV

vector particles from the cells. The AAV vector preparation was then treated for 30 min with 100 U of OmniCleave Endonuclease (EPICENTRE Biotechnologies, Madison, WI) per mL sample at 37°C. Next, the cell debris was pelleted at 250×g and the supernatant was collected.

The AAV vector particles were purified using an iodixanol block gradient. To this end, 17.5 mL of the cleared vector suspension was loaded into a 39-mL polyallomer tube (Quick-Seal, 25×89 mm; Beckman Coulter, Fullerton, CA, USA). Next, 9 mL of 15%, 5 mL of 25%, 5 mL of 40% and 3 mL of 60% iodixanol solution (OptiPrep, Sigma-Aldrich) in 1 mM MgCl₂, 2.5 mM KCl was sequentially layered under the AAV vector preparation, which was then centrifuged for 70 min at 69,000 revolutions per min in a 70 Ti rotor (Beckman Coulter). Thereafter, the bottom of the centrifuge tube was punctured and the AAV vector particles at the 40-60% iodixanol interface were collected.

Finally, the AAV vector particles were washed with PBS + 5% sucrose and concentrated in three steps to approximately 200 μ L per AAV vector in an Amicon Ultra-15 filter unit with a nominal molecular weight limit of 100 kDa (Millipore, Amsterdam, the Netherlands) following the instructions of the filter supplier. AAV vector stocks were stored in 50 μ L aliquots at -80°C.

7.2.5 Titration of AAV vector stocks

The titers of AAV vector stocks were determined by dot-blot assay and are based on DNase-resistant AAV genome copies (GC). Two μ L of each AAV vector stock was treated for 30 min at 37°C with 10 U OmniCleave in 30 μ L 50 mM Tris-HCl (pH 8.0), 1 mM MgCl₂ to digest any unpackaged DNA. Then, 10 μ L proteinase K solution (50 mM Tris-HCl [pH 8.0], 8 M urea, 4 mM CaCl₂ and 1 μ g/ μ L proteinase K [Fermentas]) was added and the sample was incubated at 37°C for 1 hour to release the AAV vector genomes. Next, the samples was boiled for 5 min in a water bath and immediately chilled on ice to denature the vector DNA. The sample was subsequently diluted 1:1 with 20× SSC buffer (3 M NaCl, 300 mM trisodium citrate [pH 7-8]) and blotted onto a Hybond-N+ nylon membrane (GE Healthcare, Diegem, Belgium) using a Bio-Dot microfiltration apparatus (Bio-Rad Laboratories, Veenendaal, the Netherlands). The vector DNA was then fixed to the membrane by incubation for 2 hours at 80°C. The AlkPhos direct labelling and detection system with CDP-*Star* (GE Healthcare) was used to quantify the DNA. As a probe either a 501-bp fragment specific for *nlsLacZco* (primers: 5'-CGTGACCGTGTCCCTGTGGC-3' and 5'-GGCACCATGCCGTGGGTCTC-3') or a 438-bp fragment binding to the WHVPRE (primers: 5'-TCAGGCAACGTGGCGTGGTGTG-3' and 5'-AAGACCAAGCAACACGGAC CGG-3') was obtained by PCR and isolated from agarose gel. The membrane was prehybridized for 30 min at 55°C after which 150 ng of the thermostable alkaline phosphatase-labelled probe was added to hybridize overnight at 55°C. Stringency washes and signal detection were performed following the recommendations of GE Healthcare. The Chemi-Doc XRS+ system and Quantity One software (both from Bio-Rad Laboratories) were used to quantify the DNA. The titers typically ranged from 10¹¹ to 10¹² GC/mL.

To compare the amount of pTR.hCMV-IE.eGFP.WHVPRE.bGHpA-based AAV vectors produced with the two- and three-plasmid AAV2/8 and AAV 2/9 vector production systems, unconcentrated vector preparations were subjected to end-point titration in 911E4

cells using flow cytometric analysis of *eGFP* expression at 6 days post transduction as readout. Vector yields were expressed in transducing units (TU)/producer cell.

7.2.6 AAV transductions

To test the ability of different AAV vector pseudotypes to transduce MSFs and nrCMCs and to compare the activity of the different promoters in MSFs and nrCMCs, cells were seeded in fibronectin (Sigma-Aldrich)-coated cell culture dishes at a density of 6.25×10^4 cells/cm². For measuring β -galactosidase activity by chemiluminescence assay, cells were cultured in 96-well plates (Corning Life Sciences, Amsterdam, the Netherlands). Chromogenic detection of β -galactosidase activity was done with cells maintained in 24-well plates (Greiner Bio-One). To test the ability of the AAV vectors AAV2/2.hEF1 α .eGFP.WHVPRE.synpA and AAV2/9.RSV.FP650.WHVPRE.synpA to achieve cell type-specific transgene expression in rMSFs and nrCMCs, cells were seeded on fibronectin-coated round glass coverslips (14 mm $\varnothing$) in 24-well cell culture plates.

All nrCMC cultures were treated with the anti-proliferative agent mitomycin C (10 μ g/mL; Sigma-Aldrich) at day 1 of culture for 2 hours to inhibit the proliferation of the remaining nrCFBs in these cultures. The next day, the cells were transduced with 100,000 GC of AAV vector per cell in 500 μ L (2-cm² well) or 100 μ L (0.32-cm² well) of a 1:1 mixture of DMEM and Ham's F10 without FBS. After 2 hours, an equal volume of 1:1 mixture of DMEM and Ham's F10 supplemented with 10% HS, 200 U/mL penicillin and 200 μ g/mL streptomycin was added to the cells. The MSFs were transduced at day 1 of culture with 100,000 GC of AAV vector per cell in 500 μ L (2-cm² well) or 100 μ L (0.32-cm² well) DMEM without FBS. After 2 hours, an equal volume of DMEM + 20% FBS, 200 U/mL penicillin and 200 μ g/mL streptomycin was added to the cells.

At 7 days post-transduction, the cultures were either lysed and the lysates stored at -80°C for future β -galactosidase activity measurements using a chemiluminescence assay or the cells were fixed for subsequent cytochemical visualisation of β -galactosidase activity.

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7.2.7 β -galactosidase activity assays

To quantify β -galactosidase levels in whole cell cultures, cells in a single well of a 96-well plate were lysed in 50 μ L lysis buffer (25 mM Tris-HCl [pH 7.8], 2 mM 1,2-diaminocyclohexanetetraacetic acid, 2 mM dithiothreitol, 10% glycerol and 1% Triton X-100). The Galacto-Light β -galactosidase reporter gene assay system (Applied Biosystems Life Technologies) was used to quantify β -galactosidase activity using a Wallac 1420 VICTOR 3 multilabel plate reader (Perkin Elmer Nederland, Groningen, the Netherlands). These experiments were performed in triplicate to allow for statistical analysis.

To visualize β -galactosidase in individual cells, cell cultures were incubated for 10 min with 0.25% (v/v) glutaraldehyde (grade II; Sigma-Aldrich) in PBS. The cells were then washed twice with PBS to remove the fixative and incubated overnight with 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-gal) solution (1 mg/mL X-gal [Sigma-Aldrich], 100 mM Na₂HPO₄/NaH₂PO₄ [pH 7.0], 2 mM MgCl₂, 5 mM K₃Fe(CN)₆, 5 mM K₄Fe(CN)₆) at 37°C. After two 5-min washes with PBS to remove the X-gal solution, the cells were

viewed using an inverse microscope (IX51; Olympus, Zoeterwoude, the Netherlands) equipped with a Peltier-cooled digital camera (XC30) and CellF software (both from Olympus) for image capture and processing/analysis, respectively.

7.2.8 Immunofluorescent staining

Cell cultures exposed to AAV2/2.hEF1 α .eGFP.WHVPRE.synpA or AAV2/2.RSV.FP650.WHVPRE.synpA were fixed with phosphate-buffered 4% formaldehyde (Mallinckrodt Baker, Phillipsburg, NJ) for 30 min 7 days after transduction. After fixation, the cells were washed 3 times with PBS containing 10 mM glycine (PBSG) and permeabilized with PBS containing 0.1% Triton X-100 for 10 min. After 3 more washes with PBSG, the cells were incubated for 1 hour with 5% 0.22- μ m filter-sterilized FBS in PBS to block potential aspecific antibody binding sites. To distinguish nrCMCs from rMSFs, the nrCMC cultures were stained for the cardiomyocyte marker sarcomeric α -actinin or for the fibroblast marker collagen type I as described above except for the use of 100-fold diluted Alexa Fluor 350-conjugated donkey-anti-mouse IgG or Alexa Fluor 350-conjugated donkey-anti-rabbit IgG antibodies (both from Invitrogen) as secondary antibodies. After washing the cells 4 times for 5 min with PBS, the staining results were analyzed with the aid of the aforementioned Olympus IX51 inverse fluorescence microscope. The intensity of the fluorescent signals was determined using ImageJ software (<http://rsb.info.nih.gov/ij/index.html>).

7.2.9 Statistics

The significance of differences between means was calculated by one-way analysis of variance followed by the Bonferroni's *post hoc* test whenever appropriate using Graph Pad Prism 4 (Graph Pad Software, La Jolla, CA). Comparison between two groups was performed using Student's *t*-test. Differences were considered significant if $p < 0.05$. Results are expressed as mean $\pm$ standard deviation.

7.3 Results

7.3.1 Comparison of two- and three-plasmid AAV vector production systems

AAV vector production protocols that do not require infection of the producer cells with a helper virus have improved the safety of AAV vectors for *in vivo* use. In addition, the development of the AAV helper plasmids pDG, pSH3 and pSH5 encoding not only the AAV Rep and Cap proteins but also adenoviral helper functions has reduced efforts and costs to produce AAV2 vector particles compared to three- or four-plasmid based production systems.^{14,28} Two-plasmid-based systems for the production of AAV2 vectors pseudotyped with capsids from AAV serotypes 1, 3, 4, 5 and 6 have also been generated²⁶ but are not yet available for generating other AAV vector pseudotypes. Since AAV vectors carrying capsids of AAV8 or AAV9 have been shown to efficiently transduce the hearts of rodents,^{10-12,29} we set out to generate pDG-based helper plasmids for the production of

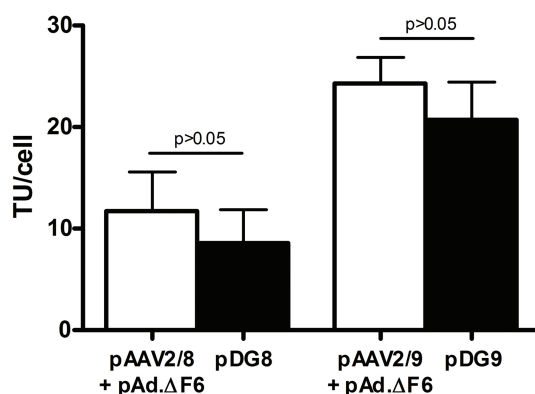


Figure 7.1: Titers of small-scale AAV2/8 and AAV2/9 vector stocks generated with the aid of two- or three-plasmid AAV vector production systems. AAV vectors were produced using the eGFP-encoding AAV vector shuttle plasmid pTR.hCMV-IE.eGFP.WHVPRE.bGHpA and titered by serial dilution in 911E4 cells. Titers are based on the number of eGFP-positive cells as assessed by flow cytometry at 6 days post infection and given as the number of TU obtained per producer cell. The titers of AAV vector stocks made with the two-plasmid method (pDG8 or pDG9) did not differ significantly from those produced with the corresponding three-plasmid approach (pAd.ΔF6 and pAAV2/8 or pAAV2/9). Pseudotransduction did not contribute to the observed gene transfer activities as eGFP-positive 911E4 cells were undetectable when the AAV vector productions were carried out with helper plasmids lacking the AAV *cap* gene (data not shown).

AAV2/8 and AAV2/9 vectors. To this end, the AAV2 *cap* gene in pDG was replaced by the *cap* gene of AAV8 or of AAV9, resulting in construction of pDP8 and pDP9, respectively.

To compare the AAV vector yields obtained with these new helper plasmids to those achieved with conventional three-plasmid-based AAV2/8 and AAV2/9 vector production methods, crude AAV2/8.hCMV-IE.eGFP.WHVPRE.bGHpA and AAV2/9.hCMV-IE.eGFP.WHVPRE.bGHpA vector preparations were made using both production systems. Limiting dilution assays in 911E4 cells revealed that the amounts of functional AAV vector particles generated with pDP8 and pDP9 did not differ significantly from those obtained with a combination of pAd.ΔF6 and either pAAV2/8 or pAAV2/9 (8.60 ± 3.28 TU/cell vs. 11.75 ± 3.81 TU/cell, $p > 0.05$, and 20.70 ± 3.72 TU/cell vs. 24.18 ± 2.68 TU/cell, $p > 0.05$, respectively; $n = 6$; Figure 1). These results show that the new helper plasmids pDP8 and pDP9 can be used to produce high-titered stocks of functional AAV2/8 and AAV2/9 vector particles.

7.3.2 Comparison of promoter activity in MSFs and nrCMCs

To compare the efficiency by which MSFs of man, mouse and rat can be transduced by AAV2/2 vectors, cultures of these cells were exposed to equal doses of AAV2/2.hCMV-IE.nlsLacZco.synpA, AAV2/2.mCMV-IE.nlsLacZco.synpA or AAV2/2.rCMV-IE.nlsLacZco.synpA. X-gal staining at 7 days post infection revealed that hMSFs were transduced with a higher efficiency than mMSFs and rMSFs (Figure 2). Furthermore, in hMSFs *LacZ*

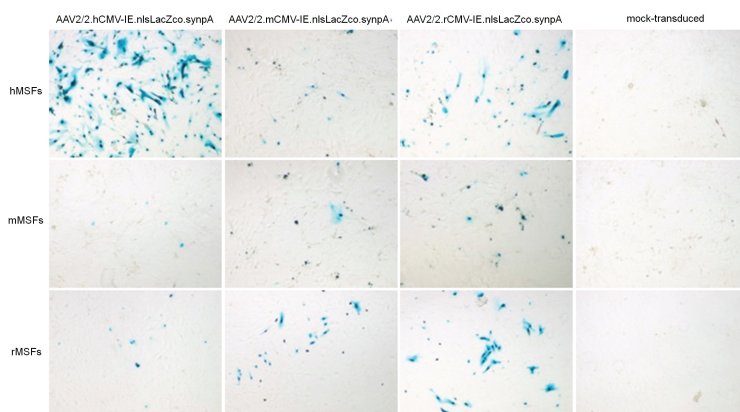


Figure 7.2: β -galactosidase staining of AAV2/2 vector- or mock-transduced MSFs. The hCMV-IE promoter induces high-level transgene expression in hMSFs but is hardly active in mMSFs or rMSFs. The mCMV-IE and rCMV-IE promoters are more active in mMSFs and rMSFs than the hCMV-IE promoter and give similar low levels of *LacZ* expression in hMSFs.

expression was highest when driven by the hCMV-IE promoter, while in mMSFs and rMSFs the *immediate early* gene promoters of the rodent cytomegaloviruses were more active. These results show that there is some level of species specificity in the activity of CMV-IE promoters.

Next, the activity of the three CMV-IE promoters in MSFs was compared with that of other ubiquitous promoters and of two fibroblast-specific promoters (Table 1). For this purpose, MSF cultures were transduced in quadruplicate by AAV2/2 vectors carrying the *nlsLacZco* gene driven by one of nine different promoters. After 7 days, the cells were lysed and the β -galactosidase activity in the cell lysates was measured to assess the transcription rate of each promoter. The results showed that in hMSFs the hCMV-IE promoter displays the highest transcriptional activity, resulting in significantly higher β -galactosidase levels compared to those found with the other promoters ($p < 0.001$; Figure 3A). Furthermore, in mMSFs, rMSFs and nrCFBs the hEF1 α promoter showed the highest transcription rate, which resulted in significantly higher β -galactosidase levels than were obtained with the other promoters ($p < 0.001$; Figure 3B, C and D).

To achieve the selective transduction of myocardial scar tissue, a promoter should ideally not only direct high levels of transgene expression in MSFs, but also be show low or no activity in cardiomyocytes. Thus, the activity of the nine promoters was also determined in purified nrCMC cultures. Quantification of β -galactosidase levels in each cell culture showed that the RSV promoter had a significantly higher transcriptional activity in nrCMCs than the other promoters ($p < 0.001$), whereas the hEF1 α promoter was only weakly active in these cells (3E). Together, these results indicate that of the promoters tested, the hEF1 α promoter is preferred when targeting myocardial scar tissue in mouse or rat models, since it causes high-level transgene expression in MSFs but is relatively weakly active in nrCMCs.

promoter	promoter length (in nts)	AAV genome length (in nts)
Rous sarcoma virus long terminal repeat (RSV)	522	4284
Simian virus 40 early + R-U5 from human T cell leukemia virus I long terminal repeat (SRA)	616	4354
Human cytomegalovirus immediate-early (hCMV-IE)	741	4368
Mouse cytomegalovirus immediate-early (mCMV-IE)	1099	4711
Rat cytomegalovirus immediate-early (rCMV-IE)	1126	4733
hCMV-IE enhancer + chicken β -actin core promoter + rabbit β -globin intron (CAG)	715	4503
Human elongation factor 1 α (hEF1 α)	1177	4824
Mouse fibroblast-specific protein 1 (mFSP1)	1110	4774
Rat lysyl oxidase (rLO)	803	4484

Table 7.1: Overview of the promoters used to drive *LacZ* expression in the AAV vectors.

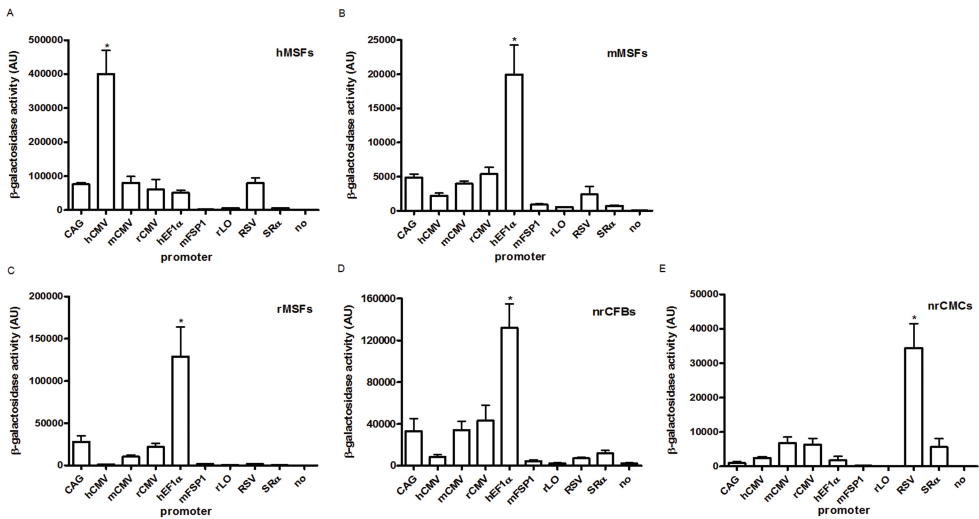


Figure 7.3: Luminometric quantification of β -galactosidase activity at 7 days after transduction in hMSFs (A), mMSFs (B), rMSFs (C) and nrCMCs (D) transduced with AAV2/2 vectors carrying a *LacZ* expression module driven by nine different promoters. Please note the vertical axes of the graphs have different scales. No: no transduction; * $p < 0.001$ compared to the other samples in the same graph.

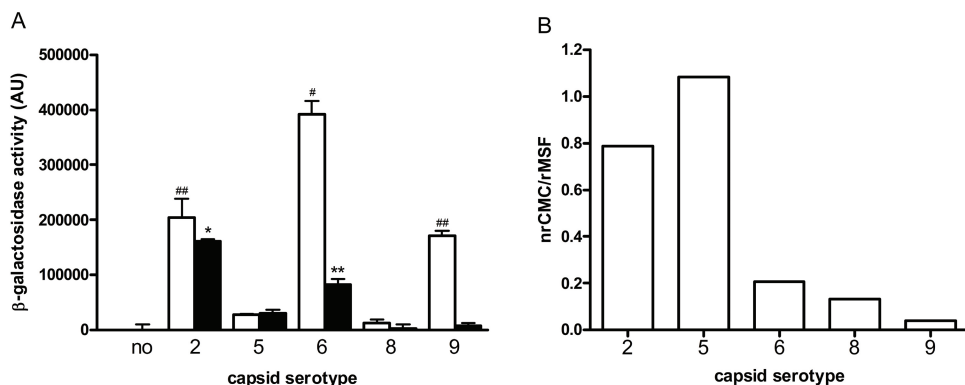


Figure 7.4: Comparison of the ability of five different AAV vector pseudotypes to transduce nrCMCs (white bars) or rMSFs (black bars). (A) Transduction efficiencies were determined by luminometric quantification of β -galactosidase levels in lysates of each sample prepared at 7 days after infection ($n=6$ samples per cell type and per AAV vector pseudotype). The nrCMCs were transduced with AAV vectors carrying LacZ driven by the RSV promoter while the rMSFs were transduced by AAV vectors in which the β -galactosidase-coding sequence was preceded by the hEF1 α promoter. No: no transduction; # $p<0.001$ vs. all other AAV vector pseudotypes in nrCMCs; ## $p<0.001$ vs. AAV2/5 and AAV2/8 vectors in nrCMCs; * $p<0.001$ vs. all other AAV vector pseudotypes in rMSFs; ** $p<0.001$ vs. AAV2/8 and AAV2/9 vector pseudotypes, $p<0.01$ vs. AAV2/5 vector pseudotypes in rMSFs. (B) Ratio's of the transduction efficiencies of the different serotypes in nrCMCs and rMSFs.

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7.3.3 Comparison of the gene transfer activity of five different AAV vector pseudotypes in rMSFs and nrCMCs

Beside through transcriptional targeting, cell type-specific transgene expression in MSFs may also be accomplished by transductional targeting. To determine which AAV vector pseudotype transduces MSFs most specifically, rMSF and purified nrCMC cultures were exposed to AAV2 vectors with capsids of AAV serotypes 2, 5, 6, 8 or 9. To achieve the highest possible transgene expression levels, MSFs were transduced with vectors carrying an hEF1 α promoter-driven *nlsLacZco* expression unit, whereas the cardiomyocytes received vectors in which the β -galactosidase-coding sequence was preceded by the RSV promoter.

In rMSFs, transduction with AAV2/2 vectors resulted in higher β -galactosidase levels than those obtained with the other AAV vector pseudotypes ($p<0.001$; $n=6$; Figure 4A). In addition, AAV2/6 vectors showed superior transduction of rMSFs compared to AAV2 vectors with capsids of AAV serotypes 5, 8 and 9 ($p<0.001$ vs. AAV2/8 and AAV2/9 vectors, $p<0.01$ vs. AAV2/5 vectors). In contrast, nrCMCs were most efficiently transduced by AAV2/6 vectors ($p<0.001$ vs. the other AAV vector pseudotypes), followed by AAV2/2 and AAV2/9 vectors ($p<0.001$ vs. AAV2/5 and AAV2/8; Figure 4A).

Based on these results, rMSFs are best transduced with AAV2/2 vectors while AAV2/9 vectors are a good candidate for the selective transduction of nrCMCs since they induce

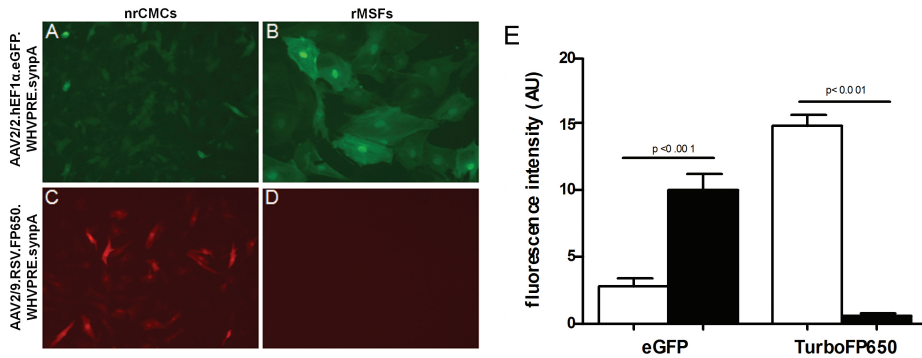


Figure 7.5: A-D. Representative fluoromicrographs of nrCMCs (A, C) or rMSFs (B, D) transduced with either AAV2/2.hEF1 α .eGFP.WHVPRE.synpA (A, B) or with AAV2/9.RSV.FP650.WHVPRE.synpA (C, D). E. Quantification of the intensity of the fluorescent signals in transduced nrCMCs (white bars) and in gene-modified rMSFs (black bars).

high-level transgene expression in these cells and poorly transduce MSFs, in contrast to AAV2/2 and AAV2/6 vectors (Figure 4B).

7.3.4 Transduction of nrCMCs and rMSFs with optimized AAV vectors

Two different AAV vector shuttle plasmids were generated to test whether optimal combinations of promoter and AAV vector pseudotype would result in rMSFs- or nrCMCs-specific transgene expression. For the preferential transduction of rMSFs, an AAV2/2 vector expressing *eGFP* under control of the hEF1 α promoter was made, while for the transduction of nrCMCs, an AAV2/9 vector in which *TurboFP650* expression is driven by the RSV promoter was generated. Cultures of either rMSFs or purified nrCMCs were separately transduced with these AAV vectors and after 7 days the intensity of the fluorescent signals in the genetically modified cells was measured. On average, rMSFs transduced with AAV2/2.hEF1 α .eGFP.WHVPRE.synpA were brighter green than nrCMCs transduced with the same AAV vector (9.99 ± 10.76 arbitrary units [AU] vs. 2.44 ± 4.32 AU, $p < 0.001$; $n = 89$ and 34 , respectively; Figure 5A, B and E). Conversely, transduction with AAV2/9.RSV.FP650.WHVPRE.synpA resulted in higher fluorescent intensity in nrCMCs compared to rMSFs (15.93 ± 10.47 AU vs. 0.94 ± 1.57 AU, $p < 0.001$; $n = 36$ and 100 ; Figure 5C, D and E). These results demonstrate that through rational design it is possible to make AAV vectors for the preferential transduction of MSFs or cardiomyocytes and provide a basis for determining the transduction profiles of AAV2/2.hEF1 α .eGFP.WHVPRE.synpA and AAV2/9.RSV.FP650.WHVPRE.synpA in infarcted rat hearts.

7.4 Discussion

The aim of this study was to develop AAV vectors conferring MSF-specific transgene expression. To this purpose, nine different promoters were tested for their activity in MSFs

and cardiomyocytes. Furthermore, five distinct AAV vector pseudotypes were assessed for the efficiency with which they transduce MSFs and cardiomyocytes. The use of AAV vectors with capsids of AAV2 and a transgene driven by the hEF1 α promoter resulted in most specific transgene expression in rMSFs compared to nrCMCs. In addition, we found that the combination of AAV vectors with AAV9 capsids and the RSV promoter was least specific for rMSFs and would therefore be ideal to use when nrCMCs but not rat cardiac (myo)fibroblasts are the preferential target.

Although recombinant baculovirus-based AAV vector production systems are rapidly gaining popularity,³⁰ co-transfection of 293 cells or derivatives of these cells with an AAV vector shuttle plasmid containing the AAV ITRs, a construct encoding AAV Rep and Cap proteins and a plasmid providing adenovirus helper functions (e.g. pAd. Δ F6 or pAd Helper 4.1)^{31,32} still represents the most common means of producing AAV vectors. The production of AAV vectors by plasmid transfections has been simplified by the development of packaging constructs such as pDG and later pDP1 and pDP3 to pDP6, in which the AAV *rep* and *cap* genes are combined with the *E2A*, *E4orf6* and *VAI* and *VAII* genes of adenovirus to provide in one plasmid all *trans*-acting functions that are required for efficient AAV vector production and that are not encoded by the producer cells. Similar packaging plasmids have thus far not been reported for the production of AAV vectors with capsids of more recently discovered serotypes. Since several studies comparing different AAV vector pseudotypes for their capability to transduce rodent hearts have shown that AAV2/8 and AAV2/9 vectors are superior compared to the other AAV vector pseudotypes that were tested,^{10-12,29} we constructed two pDG-based helper plasmids for the production of AAV2 vectors with capsids of AAV8 or AAV9. The yields of AAV vectors produced with these new helper plasmids are similar to those of AAV vectors produced using pDG or one of its previously constructed derivatives.²⁶ pDG8 and pDG9 may thus be useful to facilitate the production of AAV2/8 and AAV2/9 vectors in quantities sufficient for animal experiments.

7

Cardiac fibrosis or scar formation after MI negatively affect cardiac function and predispose the heart to the development of arrhythmias.³³ To treat the effects of cardiac fibrosis, the electrical properties of the MSFs may be genetically altered to either improve the conduction through these cells or to functionally uncouple them from the surrounding cardiomyocytes thus reducing the risk of early afterdepolarizations. Since coupling between cardiac fibroblasts and cardiomyocytes results in the partial depolarization of the latter cells, it may also be beneficial to increase the resting membrane potential of the former cells. Alternatively, it may be possible to endow the MSFs with properties of cardiomyocytes by their genetic reprogramming with cardiac transcription factor genes. However, cardiac (myo)fibroblast-directed gene therapy may have a detrimental effect on the function of neighboring healthy cardiomyocytes when these cells become transduced in parallel. The gene delivery vectors to be used for this purpose should therefore ideally transduce cardiac (myo)fibroblasts with high specificity. Thus far, in papers about the transduction of the heart by AAV vectors, no distinction was made between cardiomyocytes and cardiac (myo)fibroblasts. In this study, we aimed to generate an AAV vector for the preferential transduction of MSFs in comparison to cardiomyocytes. Five distinct

AAV vector pseudotypes were compared to determine which of them is most suited for the selective transduction of MSFs. Also, nine different promoters were tested for their transcriptional activity in MSFs versus nrCMCs. In rMSFs and mMSFs, the hEF1 α promoter was superior to the other promoters tested, whereas in hMSFs the hCMV-IE promoter was most active. The high transcriptional activity of the hEF1 α promoter in the rodent cells is in concurrence with a study by Smolenski *et al.*, who showed by serial analysis of gene expression in mouse cardiac fibroblasts that the hEF1 α transcript was the most abundant of all RNA polymerase II transcripts.³⁴ It is conceivable that the rat or mouse EF1 α promoter may even be more active in the rodent MSFs than the hEF1 α promoter. This will, however, only be beneficial if these rodent promoters not also display an undesirable increase in transgene expression in the nrCMCs. The high and low activity of the RSV promoter in nrCMCs and rat cardiac (myo)fibroblasts, respectively, is consistent with previous findings³⁵ and suggests that this promoter is very useful to induce high-level transgene expression in cardiomyocytes but not in cardiac (myo)fibroblasts. The fibroblast-specific promoters that were tested displayed only very low activity in the rodent MSFs. Possibly, crucial cis-acting signals and enhancer sequences are missing from the promoters we used due to size limitations. A challenge for future research would therefore be to construct a small but strong fibroblast-specific promoter.

Comparison of five different AAV vector pseudotypes showed that only AAV2/2 vectors transduce rMSFs efficiently, whereas cardiomyocytes are best transduced by AAV2/2, AAV2/6 and AAV2/9 vectors. The only other study that investigated the transduction by AAV vectors of cardiac fibroblasts and cardiomyocytes separately yielded similar results, although AAV2/6 vectors were not tested in that study.¹² Since the goal of the aforementioned study was to identify an AAV vector pseudotype that displayed a preferential tropism for cardiomyocytes, they did not investigate other ways to achieve cardiomyocyte-specific transgene expression. However, our findings and those of Qi *et al.* show that AAV2/2 vectors, whilst being the only option to efficiently transduce MSFs, also targets nrCMCs. Thus, in order to obtain specific transgene expression in MSFs multiple targeting strategies need to be combined. The combination of transductional and transcriptional targeting to preferentially transduce either rMSFs or nrCMCs was tested with AAV2/2 vectors expressing *eGFP* under control of the hEF1 α promoter and with AAV2/9 vectors expressing *TurboFP650* under control of the RSV promoter. The results indeed show some degree of specificity for rMSF of the AAV2/2.hEF1 α .eGFP.WHVPRE.synpA vector that can be attributed to the differential promoter activity. The vector AAV2/9.RSV.FP650.WHVPRE.synpA, which was designed for the selective transduction of cardiomyocytes, showed a more pronounced difference in transgene expression between rMSFs and nrCMCs, which is due to the combination of both transductional and transcriptional targeting. Ideally, we would like to target the rMSFs with the same level of specificity as the nrCMCs. This may be achieved by incorporating cardiomyocyte-specific microRNA target sites into the untranslated region(s) of the transcripts encoded by the transgene whose expression should occur in cardiac (myo)fibroblasts but not in cardiomyocytes.³⁶ This method exploits the naturally occurring microRNA-based gene regulatory mechanisms of cells to post-transcriptionally silence expression of genes that contain the target sequence of such a microRNA in their mRNAs. Selective suppression of transgene ex-

pression in cardiac muscle cells depends on the identification of cardiomyocyte-specific microRNAs. MicroRNA-1, -133, -206 and -208 may all qualify for this purpose as they have been shown to be (cardiac) muscle cell-specific.^{37,38}

Another interesting finding of this study is the difference in the capability of AAV vectors to transduce MSFs from different species. This may be caused by many factors including differences in receptor availability or amino acid sequence or differences in the fate of the vector particles after cellular uptake. In a recent study, Bhargava and Trempe showed that nuclear entry of AAV2 particles is impaired in murine fibroblasts as compared to that in human cervical carcinoma cells.³⁹ Furthermore, it was shown that tyrosine-mediated ubiquitination and subsequent proteasomal degradation of internalized AAV particles limits the transduction of cells by AAV2 vectors.⁴⁰ Mutation of three tyrosines in the AAV2 capsid into phenylalanines greatly reduced the susceptibility of AAV2/2 vectors to proteasomal degradation and caused a 130-fold increase in transgene expression in mouse fibroblasts transduced with these vectors.⁴¹ These results indicate that it might be beneficial to equip the MSF-specific AAV2/2 vectors with AAV2 capsids containing the three tyrosine-to-phenylalanine substitutions. The performance of these vectors should be compared with that of wild-type AAV2/2 vectors in both rMSFs and nrCMCs to determine whether the capsid mutations would also improve the specificity of AAV2/2 vectors for rMSFs.

In conclusion, this study showed that in order to efficiently transduce rMSFs with AAV vectors, an AAV2/2 vector containing a transgene driven by the hEF1 α promoter should be used. In addition, in nrCMCs the highest transgene levels are obtained using an AAV2/9 vector that has the RSV promoter to drive the transgene. In the absence of a strong, small-sized fibroblast-specific promoter, some level of specificity for MSFs can be obtained by a combination of transductional and transcriptional targeting, but other (e.g. microRNA-based) targeting principles should be added to achieve truly MSF-specific transgene expression in rMSFs. Our optimized AAV vectors should next be tested *in vivo* to confirm our *in vitro* findings.

7.5 Acknowledgments

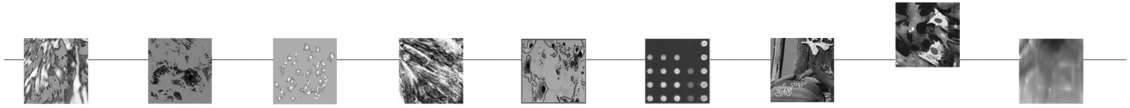
We thank Joost Verhaagen and Erich Ehlert for sharing their knowledge on the production of AAV vectors.

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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 stimulation⁵, 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 established⁶, but its role in macropinocytosis is new. Since macropinocytosis is independent of receptor molecules⁷, 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 α_1 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

AKT¹⁵, 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 down-regulated 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-associated 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 activity³⁶, 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 questioned³⁹, 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, ~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 whole⁴²⁻⁴⁵, 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 EF1 α 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.

8

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.

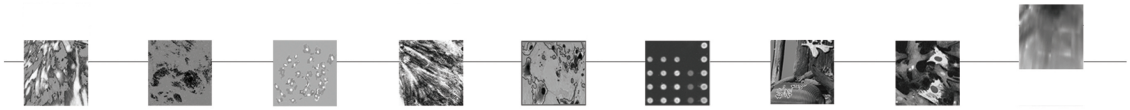
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Chapter 9

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.

9.3 Samenvatting en conclusies

De structuur en functie van cellen worden voor een groot gedeelte bepaald door het repertoire van genen dat in deze cellen wordt geëxprimeerd. Voor de behandeling van diverse cardiale aandoeningen geldt het modificeren van genexpressie, om de expressie van een bepaald gen te verhogen of te verlagen, als mogelijke therapie. Als methode om het gewenste gen in de hartcel te brengen, hebben virale vectoren de beste resultaten opgeleverd. Dit proefschrift beschrijft een aantal studies die onderzoeken hoe virale vectoren kunnen worden benut om op een efficiënte manier hartcellen te beïnvloeden en wat de effecten van bepaalde genetische interventies zijn op de (patho)fysiologie van hartcellen.

Hoofdstuk 1 geeft een overzicht van de huidige virale vector technologie en hoe deze vectoren gebruikt worden in basaal cardiaal onderzoek en als nieuwe alternatieven voor de behandeling van hartziekten. De drie meest gebruikte vector systemen voor *in vitro* en *in vivo* genoverdracht worden geïntroduceerd, gevolgd door verschillende methoden die kunnen worden gebruikt om virale vectoren aan te passen ten einde specifiek het hart te modificeren. Vervolgens worden verscheidene manieren van interventie, zoals overexpressie en inhibitie van endogene gen expressie, bediscussieerd. De laatste delen van dit hoofdstuk zijn gericht op de toepassing van virale vector technologie in basaal cardiaal onderzoek en de mogelijke toepassing van virale vector-gemedieerde genoverdracht in het hart om diverse hartziekten te behandelen.

Hoofdstuk 2 beschrijft hoe expressie van de skeletspier-specifieke transcriptie factor MyoD in humane mesenchymale stamcellen (hMSCs) het potentieel van deze cellen om het dystrofine gen over te brengen naar Duchenne spierdystrofie (DMD) myoblasten door middel van celfusie vergroot. Met behulp van immunofluorescente kleuring en reverse transcriptie PCR werd vastgesteld dat transductie van hMSCs met een lentivirale vector coderend voor MyoD leidde tot de expressie van verschillende vroege en late skeletspiermarkers. Ook was het percentage MyoD-expresserende hMSCs die gefuseerd waren met myoblasten tot myotubes hoger dan het percentage gefuseerde hMSCs die geen MyoD expresseerden. Vervolgens werden MyoD-expresserende hMSCs getransduceerd met een adenovirale vector coderend voor het dystrofine eiwit om te testen of deze hMSCs in staat waren dystrofine expressie in DMD myoblasten te herstellen. Immunofluorescente kleuring van dystrofine liet zien dat dystrofine slechts aanwezig was in myotubes die gefuseerd waren met hMSCs. Bovendien was dystrofine-expressie correct gelokaliseerd langs het plasmamembraan. Expressie van β -dystroglycan, onderdeel van het dystrofine-geassocieerde glycoproteïne complex, was sterker in myoblast-hMSC myotubes dan in myotubes die bestonden uit alleen myoblasten, wat er op wijst dat het overbrengen van dystrofine naar DMD myotubes de formatie van dit complex kan herstellen. Deze resultaten laten zien dat het overbrengen van transgenen door middel van celfusie mogelijk is en dit verschaft mogelijk een nieuwe behandeling van DMD.

Hoofdstuk 3 onderzoekt of stimulatie van integrines op neonatale ratten cardiomyocyten (nrCMCs) de opname van macromoleculen door deze cellen bevordert. Daartoe werd de opname van Texas Red-gemerkt ovalbumine (OTR) of Texas Red-gemerkt

dextran (DTR) gekwantificeerd. Integrine stimulatie door het pentapeptide GRGDS, dat het RGD-motief bevat, resulteerde in een verhoogde opname van zowel OTR als DTR afhankelijk van hun concentratie in het medium. Daarnaast correleerde de opname van macromoleculen met de GRGDS concentratie en incubatietijd. De opname van OTR als gevolg van stimulatie met GRGDS werd bewerkstelligd door macropinocytose, aangezien werd aangetoond dat wortmannin, een remmer van dit proces, de OTR opname na GRGDS stimulatie verminderde. Hierna werd de rol van dysferline in macromoleculopname onderzocht. Immunofluorescente kleuring van dysferline toonde aan dat de grootte van dysferlineplekken in het celmembraan van nrCMCs was toegenomen na GRGDS stimulatie vergeleken met ongestimuleerde cellen. Deze toename in de grootte van dysferlineplekken werd echter niet waargenomen wanneer het contraheren van de nrCMCs werd onderdrukt, wat er op wijst dat het optreden van membraanscheurtjes betrokken is bij macromoleculopname. Remming van dysferline-expressie met behulp van lentivirale vectoren coderend voor short hairpin (sh)RNAs tegen het dysferline mRNA resulteerde in een afname van de door GRGDS geïnduceerde OTR opname, wat aanduidt dat dysferline vereist is voor macropinocytose. Aangezien het uitrekken van de cellen het voorkomen van membraanscheurtjes deed toenemen, lieten nrCMCs die werden uitgerekt meer OTR opname zien dan niet-gerekte nrCMCs en deze toename was afhankelijk van de mate van rek. Samenvattend laat dit onderzoek zien dat integrine stimulatie tot opname van macromoleculen via macropinocytose leidt. Dysferline dat betrokken is bij het herstel van membraanscheurtjes lijkt betrokken te zijn bij dit proces.

Hoofdstuk 4 beschrijft een onderzoek waarin het effect van veranderingen in de beschikbaarheid van stikstofmonoxide (NO) op door integrine stimulatie geïnduceerde hypertrofie in nrCMCs werd getest. Daartoe werd in GRGDS-gestimuleerde nrCMCs NO-synthase 1 (NOS1) geremd door incubatie met L-NAME of door transductie met een lentivirale vector coderend voor de proteïne inhibitor van NOS1 (PIN) of coderend voor een shRNA gericht tegen het NOS1 mRNA. Elk van deze interventies leidde tot een vermindering van het hypertrofe effect en de mate van de afname was afhankelijk van de beschikbaarheid van NO. Daarnaast bleek dat exogene toediening van NO de NOS1 remming kon omzeilen, wat leidde tot hypertrofie in GRGDS gestimuleerde nrCMCs. Inductie van NOS2 door lipopolysaccharide (LPS) resulteerde in hypertrofie, welke afhankelijk was van het NOS2 expressie niveau. Hypertrophy werd volledig teniet gedaan door ODQ, een remmer van guanylaat cyclase (sGC), maar de hypertrofe reactie werd hersteld na gelijktijdige toediening van 6-Br-cGMP, een analoog van cGMP dat het product is van de door sGC gekatalyseerde reactie. Aangezien sGC op NO reageert, geven deze resultaten het belang van NO-signaal transductie in de hypertrofe respons op integrine stimulatie aan. Vervolgens werd onderzocht of de signaalcascade die leidt tot hypertrofe groei via Akt1 loopt. Activatie van Akt1 gebeurt door fosforylering van Akt1. De P-Akt1/Akt1 ratio was verhoogd na GRGDS stimulatie en was significant verlaagd na toediening van L-NAME. Daarnaast resulteerde remming van PI3K voor Akt1 in de signaalcascade door wortmannin in het volledig uitblijven van de door GRGDS geïnduceerde hypertrofie. Overexpressie van constitutief-actief Akt1 met behulp van een lentivirale vector induceerde hypertrofie in nrCMCs, terwijl lentivirale vector gemedieerde expressie van een dominant-negatieve versie van Akt1 de door GRGDS geïnduceerde hypertrofie remde. Samenvattend impliceren deze resultaten dat de PI3K/Akt1 signaalcascade be-

trokken is bij de door integrine stimulatie geïnduceerde hypertrofie in nrCMCs. Hierbij wordt een model voorgesteld waarin integrine stimulatie, NO en de PI3K/Akt1 signaal cascade koppelen aan het hypertrofische proces in nrCMCs.

In Hoofdstuk 5 wordt de hypothese getest dat inhibitie van de koppeling tussen myofibroblasten (MFBs) en CMCs de ontwikkeling van aritmieën in fibrotische kweken vermindert. In fibrotische kweken bleek dat de geleidingssnelheid significant langzamer was dan in controle kweken. Bovendien was de frequentie van ectopische activiteit en re-entry tachyarritmieën hoger in fibrotische kweken dan in controle kweken. Immunofluorescente kleuring liet zien dat Cx43 aanwezig was tussen aangrenzende MFBs en CMCs. De rust membraanpotentiaal van de CMCs in de fibrotische kweken was significant minder negatief dan die van CMCs in controle kweken, en dit verhoogde de kans op re-entry in fibrotische kweken. Om onze hypothese te testen werden MFBs getransduceerd met een lentivirale vector coderend voor een shRNA gericht tegen Cx43. In MFBs die de Cx43-shRNA tot expressie brachten, was de Cx43 expressie verminderd met 53% en werd significant minder kleurstofoverdracht door de gap junctions waargenomen. De rust-membraanpotentiaal van CMCs in fibrotische kweken die MFBs met geremde Cx43 expressie bevatten was significant lager dan de rust-membraanpotentiaal van CMCs in kweken met MFBs die een controle shRNA hadden ontvangen. In kweken met MFBs waarin Cx43 was geremd was de geleidingssnelheid significant hoger dan in kweken met MFBs die een controle shRNA hadden ontvangen en ectopische activiteit en re-entry tachyarritmieën waren verminderd met respectievelijk 32.8% en 44.1%. Samenvattend laten deze resultaten zien dat functionele ont koppeling van MFBs en CMCs door middel van het remmen van Cx43 expressie het optreden van aritmieën in fibrotische culturen kan verhinderen. Deze resultaten wijzen er op dat myofibroblasten in het myocard een nieuw richtpunt kunnen zijn voor anti-aritmische therapie.

Hoofdstuk 6 beschrijft de rol van gap junction koppeling bij de transdifferentiatie van hMSCs naar CMCs. In tegenstelling tot volwassen hMSCs kunnen foetale hMSCs differentiëren tot CMCs, wanneer deze cellen samen met CMCs worden gekweekt. De bevinding dat foetale hMSCs een hoog Cx43 expressie niveau hebben, wat er voor zorgt dat deze cellen kunnen koppelen met de CMCs, terwijl volwassen hMSCs bijna geen Cx43 tot expressie brengen, zou dit verschil kunnen verklaren. Om dit verder uit te zoeken werd Cx43 expressie geremd in foetale hMSCs en Cx43 tot expressie gebracht in volwassen hMSCs met behulp van lentivirale vectoren. Kleurstofoverdracht metingen met calceïne lieten zien dat een hoger percentage van de volwassen Cx43 expresserende hMSCs calceïne positief was en dat de intensiteit van de kleuring sterker was vergeleken met controle volwassen hMSCs. In foetale hMSCs met geremde Cx43 expressie werd daarentegen het omgekeerde effect waargenomen. Immunofluorescente kleuring van α -actinin werd gebruikt om de differentiatie van hMSCs naar CMCs te bepalen. Hoewel ongeveer 2,2% van de controle foetale hMSCs positief was voor α -actinin, was geen van de foetale hMSCs, waarin Cx43 was geremd, positief voor α -actinin, wat er op wijst dat de door Cx43 gemedieerde koppeling met omliggende CMCs nodig is voor de differentiatie van hMSCs tot CMCs. Differentiatie van volwassen hMSCs werd echter niet vastgesteld, zelfs niet wanneer Cx43 tot overexpressie was gebracht. Om te bepalen of de expressie van een ander gap junction eiwit Cx45 het potentieel van foetale hMSCs met geremde Cx43 expressie om te differentiëren tot CMCs, werden genoemde

cellen getransduceerd met een lentivirale vector coderend voor Cx45. Zowel het percentage calceïne-positieve cellen als de intensiteit van de kleurstof was duidelijk verhoogd. Bovendien bleken ongeveer 1,6% van de cellen α -actinin positief. Deze resultaten laten zien dat koppeling van foetale hMSCs en CMCs door middel van gap junctions nodig is voor de differentiatie van hMSCs naar CMCs. Waarschijnlijk zijn kleine moleculen in staat om door deze gap junctions te bewegen en zo differentiatie te induceren. Volwassen hMSCs, daarentegen, bleken niet in staat om te differentiëren naar CMCs, waarschijnlijk omdat deze minder primitieve cellen hun differentiatiecapaciteit hebben verloren door veranderingen in hun epigenetische opmaak.

Hoofdstuk 7 beschrijft een onderzoek dat tot doel had adeno-geassocieerd virus (AAV) vectoren te ontwikkelen voor de transductie van myocardiale litteken fibroblasten (MSFs). Een voorwaarde voor AAV vectoren als genoverdracht vector in littekenweefsel is dat het therapeutische transgen alleen in litteken fibroblasten tot expressie dient te komen en niet in de overlevende CMCs. Daarom beoogden wij te bepalen welke de optimale promotor en AAV serotype zijn die resulteren in een hoge transgenexpressie in MSFs maar niet in CMCs. Negen verschillende promotors werden getest op hun activiteit in humane, muizen en ratten MSFs evenals in CMCs. In humans MSFs was de humane CMV promotor meest actief, terwijl in muizen en ratten MSFs de humane EF1 α promotor de hoogste activiteit vertoonde. De RSV promotor gaf de hoogste transgen expressie in nrCMCs. Vervolgens testten we vijf verschillende serotypen om te bepalen welk serotype ratten MSFs of nrCMCs het meest efficiënt zou transduceren. Deze vergelijking liet zien dat AAV serotype 2 ratten MSFs het meest efficiënt transduceerden en AAV serotype 9 het meest specifiek nrCMCs transduceerden. Deze resultaten laten zien dat de meest specifieke transgenexpressie in ratten MSFs kan worden bereikt door transductie met een AAV vector met een serotype 2 capside en een transgen dat wordt aangedreven door de EF1 α promotor, terwijl nrCMCs het best kunnen worden getransduceerd met een AAV serotype 9 vector met de RSV promotor die transgenexpressie stuurt. Om deze te resultaten te bevestigen werden geoptimaliseerde AAV vectoren coderend voor GFP voor ratten MSFs of het rood fluorescerende eiwit FP650 voor nrCMCs gemaakt. Zoals verwacht was de intensiteit van het fluorescente signaal van cellen getransduceerd met de AAV2.EF1 α .GFP vector 4 keer hoger in ratten MSF kweken dan in nrCMC kweken. Transductie van ratten MSFs met de AAV9.RSV.FP650 vector resulteerde niet in significante transgenexpressie, ofschoon hoge expressieniveaus werden gemeten in nrCMCs. Deze resultaten bevestigen inderdaad dat onze geoptimaliseerde AAV vectoren in staat zijn om specifiek MSFs of CMCs te transduceren in de rat. Of dezelfde resultaten ook *in vivo* kunnen worden gevonden vereist meer experimenten.

De onderzoeken die in dit proefschrift worden beschreven benadrukken het enorme potentieel van gentherapie om verscheidene ziekten te behandelen met behulp van virale vectoren. Aandoeningen die zijn bestudeerd omvatten Duchenne spierdystrofie en harttritmestoornissen als gevolg van cardiale fibrose. Verder is genetische modificatie met behulp van virale vectoren een belangrijk hulpstuk bij het ontrafelen van (patho)fysiologische mechanismen, zoals integrine fysiologie van CMCs, de rol van NO in de hypertrofie van CMCs en de rol van cel-cel contact in stamcel differentiatie. Om virale vectoren ook *in vivo* te kunnen toepassen, moeten vraagstukken als de veiligheid van virale vec-

toren, efficiënte targeting van het hart en de duur van genexpressie verder onderzocht worden.

9.4 Vervolgonderzoek

Het beperkte herstellend vermogen van het hart na schade en de bevinding dat cellen die worden toegediend aan het hart als celtherapie niet differentiëren tot CMCs zijn de aanleiding geweest tot onderzoek naar nieuwe manieren om primitieve cellen *in vitro* te differentiëren tot CMCs. Humane MSCs die de skeletspier-specifieke transcriptie factor MyoD hadden ontvangen brachten een aantal skeletspiermarkers tot expressie en waren meer geneigd tot fusie met myoblasten in een gemengde kweek om myotubes te vormen dan controle cellen. Deze resultaten laten het potentieel zien van genetisch gemodificeerde stamcellen om te differentiëren richting spiercellen. Hoewel geen van de bekende cardiomyogene transcriptiefactoren in staat is om volledige differentiatie van stamcellen te bewerkstelligen, bleken combinaties van transcriptie factoren wel voldoende om muriene cellen te differentiëren tot CMCs. Vervolgonderzoek naar kweken met humane MSCs moet duidelijk maken welke combinatie van factoren nodig is om deze cellen te differentiëren naar CMCs. De bevinding dat meer primitieve stamcellen, d.w.z. foetale MSCs, gemakkelijker differentiëren tot CMCs dan volwassen MSCs spreekt voor het gebruik van de foetale cellen in deze experimenten. Behalve het gebruik van cardiomyogene transcriptiefactoren zou verbeterde koppeling door middel van gap junctions tussen MSCs en de omliggende CMCs kunnen leiden tot verbeterde differentiatie van deze MSCs. Foetale MSCs in kweken met nrCMCs waren in staat om te differentiëren tot CMCs, maar dit vermogen werd te niet gedaan wanneer Cx43 expressie werd geremd. Het wordt verondersteld dat gap junctions de overdracht van cardiomyogene signalen van de CMCs naar de MSCs mogelijk maken en daardoor differentiatie van MSCs induceren. Het zou interessant zijn om te onderzoeken wat deze signalen zijn en welke mechanismen verantwoordelijk zijn voor dit proces. Geforceerde expressie van Cx43 in getransplanteerde cellen bij celtherapie zouden dus de differentiatie van ingenestelde cellen kunnen verbeteren, hoewel enkele valkuilen in acht moeten worden genomen. Ten eerste moeten de getransplanteerde cellen in staat zijn om te differentiëren tot CMCs, aangezien het is aangetoond dat gap junction-gemedieerde koppeling van nrCMCs met ongedifferentieerde cellen de membraanpotentiaal van CMCs doet afnemen, wat leidt tot ectopische activiteit en re-entry tachyarritmieën. Toediening van MSCs die Cx43 tot expressie brengen, maar niet differentiëren tot CMCs, zouden daarom een nadelig effect op de hartfunctie kunnen hebben. Deze resultaten benadrukken opnieuw de noodzaak om nieuwe methoden te ontwikkelen om de het potentieel van autologe MSCs om te differentiëren tot CMCs te verbeteren voordat deze cellen worden geïnjecteerd in het hart van de patiënt.

Afgezien van het gebruik van gentherapie om cardiale celtherapie te verbeteren, zou genetische modificatie van fibroblasten uit myocardiaal littekenweefsel in patiënten die een hartinfarct hebben gehad de hartfunctie kunnen verbeteren. De littekenfibroblasten en de extracellulaire matrix die door deze cellen wordt uitgescheiden vormen een anatomische geleidingsblokkade, die leidt tot dyssynchronie en mogelijke aritmieën. Genetische modificatie van de littekenfibroblasten door middel van de introductie van

een virale vector die ionkanaal eiwitten, zoals Cx43, tot expressie brengt, zou de geleiding door het litteken kunnen verbeteren en daardoor de dyssynchronie kunnen verminderen. Een geschikte virale vector is nodig om de efficiëntie van het overbrengen van het therapeutische gen naar het litteken te verhogen en tegelijkertijd de nadelige effecten te verkleinen. Vooral van AAV vectoren is de laatste jaren aangetoond dat deze vectoren uitermate geschikt zijn als vector systeem om het hart te bereiken. Aangezien het onwenselijk is om gezonde CMCs te transduceren met de therapeutische vector, omdat dit zou kunnen leiden tot suprafysiologische transgen niveaus in deze cellen, beschrijft dit proefschrift de ontwikkeling van een AAV vector die specifiek fibroblasten target. De bruikbaarheid van deze vector *in vivo* moet worden onderzocht in een hartinfarct model in de rat. Toekomstige experimenten zouden het effect van de introductie van ionkanaal genen in littekenfibroblasten moeten onderzoeken. Daarnaast moeten AAV vectoren verder geoptimaliseerd worden om de littekenfibroblasten beter te targeten.

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Dankwoord

Het in dit proefschrift beschreven onderzoek is het resultaat van een samenwerking tussen de afdelingen hartziekten en moleculaire celbiologie. Hoewel ik mijn praktische werk in het lab van MCB uitvoerde, werkte ik nauw samen met het laboratorium van hartziekten. Tijdens mijn promotie heb ik daardoor gebruik kunnen maken van "the best of both worlds" en ik ben dan ook vele mensen dank verschuldigd voor hun hulp bij het tot stand komen van dit proefschrift.

Van de medewerkers van het laboratorium Hartziekten bedank ik Daniël, Cindy, Minka, Ton, Lizet, Margreet, Wilbert, Arti, Saïd, Brian, Zeinab, Vanessa, Melina, Niek, Cheryl en Soban voor alle hulp tijdens mijn onderzoek. Hoewel ik niet vaak in het lab Hartziekten te vinden was, waren jullie altijd onmiddellijk bereid om mij te helpen als ik iets nodig had of iets niet kon vinden. Arti, Saïd en Brian, onze samenwerking heeft geleid tot een aantal mooie publicaties, waarvoor mijn dank. Ik wens jullie veel succes met de afronding van jullie promotie. Verder wil ik het secretariaat van de afdeling hartziekten bedanken voor alle hulp die het AIO-leven een stuk makkelijker maakte. De collega's uit "de tuin" dank voor de gezelligheid tijdens de congressen.

Mijn studenten Kondababu, Aysegül, Martijn en Conrad, bedankt voor jullie inzet en enthousiasme tijdens jullie stages. Veel succes in jullie verdere carrière.

De meeste tijd heb ik doorgebracht bij de virus- en stamcelbiologie sectie binnen het lab van Moleculaire Celbiologie. Manuel, Shosh, Ietje, Harald, Dirk, Gijs, Maarten, Kim, Jin, Kit, Tineke, Hester, Lianne, Anabel, Laetitia en Selina, dank jullie wel voor de gezellige tijd in het lab en zeker ook daar buiten. Shosh en Ietje, bij jullie ben ik als student begonnen. Niet alleen heb ik in die tijd veel geleerd, maar het interessante werk is ook een reden geweest om bij lab van Moleculaire Celbiologie mijn promotie te beginnen. Manuel, ook jij bedankt voor de hulp bij de celfusie proeven. John, jij was bijna klaar toen ik met mijn promotie begon, bedankt voor de alle hulp die je mij destijds hebt gegeven. Maarten, dank je dat je mijn paranimf wil zijn.

Ook de andere leden van de groep: Steve, Rob, Danijela, Françoise, Arnoud, Martijn, Diana, Jeroen, Taco, Iris en Sanne, bedankt voor alle hulp en gezelligheid binnen en buiten het lab.

Jeroen, Nathalie en Paula, bedankt voor die vriendschap tijdens en na onze studie. We

zijn inmiddels elk (letterlijk en figuurlijk) een andere weg op gegaan. Hoewel het daarvoor niet altijd makkelijk was om elkaar weer eens te zien, hebben we toch altijd goed contact gehouden. Jeroen, ik ben blij dat je mijn paranimf wil zijn.

Lieve ouders, bedankt voor de niet aflatende interesse in mijn promotieonderzoek. Jullie steun door de jaren heen heeft het voor mij makkelijk gemaakt om zover te komen.

Tot slot gaat mijn grootste dank uit naar Huining. Lieve Huining, het is een klein wonder dat jij bij mij in het lab terecht bent gekomen. Hoewel jij door je ervaring in het lab het klappen van de zweep wel kende, was het toch wennen dat werk vaak voor ging. Ik ben heel erg trots op jou aanpassingsvermogen en kan je nooit genoeg bedanken voor alles dat je voor mij betekent.

Curriculum Vitae

The author of this thesis was born in Rotterdam, the Netherlands, on the 3rd of October 1982. After receiving his gymnasium diploma from the Rotterdams Montessori Lyceum in Rotterdam in 2001, he started studying biomedical sciences at the University of Leiden, the Netherlands. As part of his study, he performed research at the Department of Infectious Diseases of the Leiden University Medical Center. A second training period at the Department of Molecular Cell Biology of the Leiden University Medical Center focused on the differentiation of mesenchymal stem cells to skeletal muscle cells and resulted in chapter 2 of this thesis. He obtained his master's degree in biomedical sciences in 2006. In April 2007, he started his PhD training at the Leiden University Medical Center in a collaboration between the Departments of Cardiology and Molecular Cell Biology on the use of viral vector-mediated gene transfer for fundamental and applied cardiovascular research. The results of this research are presented in this thesis.