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

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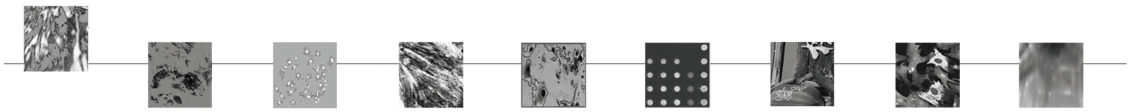

Chapter 1

Introduction

1.1 Background

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 tot 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 output[14], 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
In vivo 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.

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 cells[29] 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 systems[33]) or three (for third-generation packaging systems[34]) 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

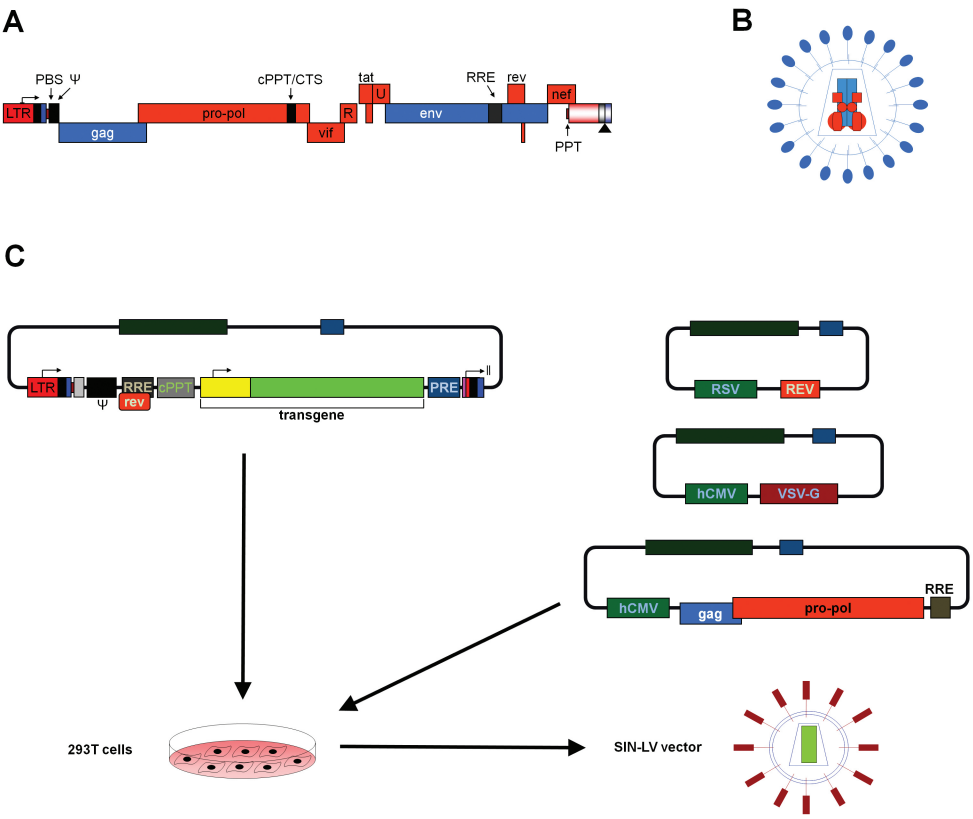

Figure 1.1: A. Schematic drawing of the HIV-1 genome. Blue boxes indicate genes encoding structural proteins, red boxes indicate non-structural proteins. ψ, packaging signal; LTR, long terminal repeat; cPPT-CTS, complementary polypurine tract-central termination site. **B.** Schematic drawing of a HIV-1 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 293[45], human embryonic retinoblasts (HER) 911[46] 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-deficient[49] and can only be produced in cell

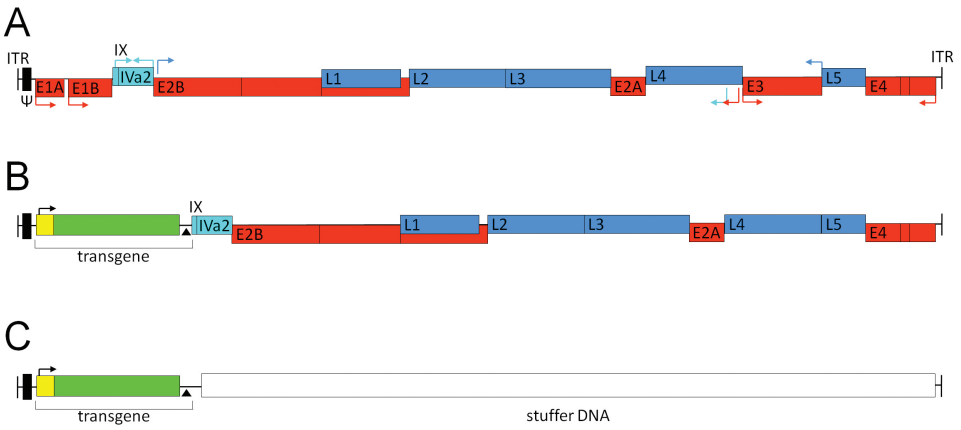

Figure 1.2: A. Schematic drawing of the HAdV-5 genome. The early (in red), intermediate (in light blue) and late (in dark blue) transcriptional units are indicated. ψ, packaging signal; ITR, inverted terminal repeat. **B.** Schematic drawing of a first-generation HAdV-5 vector genome lacking both the 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 antibodies[60], 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 19[61] 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

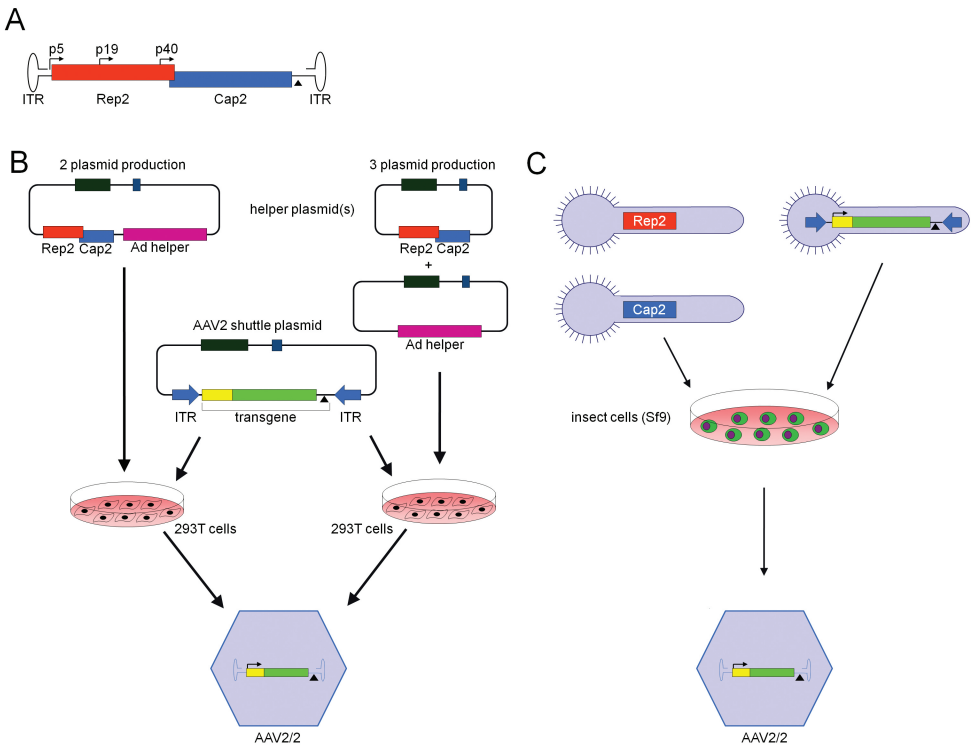

Figure 1.3: A. drawing of the AAV-2 genome. ITR, inverted terminal repeat. **B.** Overview of the two- plasmid or three-plasmid production of AAV vector in 293T cells. **C.** Production of AAV vector in insect cells using baculoviral vectors.

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. Dephophorylation 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^+,Ca^{2+}-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 levels[93-94], 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 general[120], 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 an 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 methylation[130] 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.

1.7.1 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 lineages[148], 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 iPSs 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 IPSs 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 SERCA2a[167] 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-1α), 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^+, Ca^{2+} 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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