Chapter 1

General Introduction

Background

Skeletal and cardiac muscle disorders are associated with substantial morbidity and mortality. Collectively, these diseases affect millions of people worldwide and enormous amount of time, effort and money have been spent to identify and improve recuperative strategies for prevention and treatment of such disorders. Although many striated muscle diseases manifest themselves only in the heart or skeletal musculature, others affect both tissues although the moment of onset, progression, severity of disease and specific disease symptoms may differ between skeletal and cardiac muscle tissue.[1] The existence of a large number of disorders with both skeletal and cardiac muscle involvement is a direct consequence of the large overlap in gene expression profile between skeletal and cardiac myocytes, which relates to commonalities in their contractile systems. In many striated muscle diseases loss of functional myocytes eventually exceeds the tissue's regenerative capacity. This causes gradual replacement of these parenchymal cells by adipocytes and/or (myo)fibroblasts leading to progressive wasting and pathological remodelling of skeletal and cardiac muscle tissue, respectively.

Skeletal muscle wasting

A common feature of many skeletal muscle disorders is gradual muscle degeneration leading to impairment or loss of mobility. In healthy individuals, skeletal muscle damage triggers the activation of a population of muscle-resident stem cells called satellite cells. Following their activation, satellite cells start to proliferate giving rise to myoblasts, which are responsible for skeletal muscle repair and regeneration by fusion with injured myofibers or formation of new myofibers. Hence, fusion plays a key role in the regeneration process.[2-4] In muscular dystrophies, because of the repetitive cycles of degeneration and regeneration the myoregenerative potential of skeletal muscle tissue becomes progressively exhausted. As a consequence, damaged myofibers are gradually replaced by fibroblasts and adipocytes. One of the therapeutic options to overcome this problem is cell transplantation. The success rate of transplantation is highly dependent on the ability of the donor cells to fuse with each other and/or with the recipient's skeletal myocytes in order to produce new myofibers and repair existing ones, respectively.[5] Mechanistic studies on (myogenic)

cell fusion may thus be of great help to further optimize cell-based therapies for degenerative skeletal muscle diseases.

Pathological cardiac remodelling

Cardiac diseases are usually associated with distinct alterations of the expression and function of ion channels, Ca^{2+}-handling proteins, metabolic enzymes and structural proteins including sarcomeric components, and intercellular adhesion molecules. These changes are often accompanied by pathological cardiac hypertrophy (PCH) and fibrosis leading to electrophysiological and structural remodelling, which have been identified to increase pro-arrhythmic risk.[6-8]

An increase in cardiac demand due to physiological or pathological changes in hemodynamics makes the heart respond in several ways, including by the enlargement of cardiomyocytes. Such cardiac hypertrophy is essentially a beneficial compensatory process as it decreases wall stress, while increasing cardiac output.[9] This adaption by growth occurs under physiological conditions like exercise and pregnancy, but also in response to myocardial infarction and other cardiac pathologies (*e.g.* hypertension, aortic stenosis, aortic coarctation, valvular regurgitation, septal defects and arteriovenous fistulae).[10] Physiological hypertrophy typically is a reversible process to fulfill a temporary need for additional cardiac output. Pathological hypertrophy, on the other hand, is essentially an irreversible process involving permanent changes in cardiac structure and function that initially secure but subsequently reduce cardiac output. Besides an increase in cardiomyocyte size, diseased hearts usually also display a decrease in cardiomyocyte number together with a (compensatory) fibrotic response. The changes in cardiac geometry, myocardial tissue composition and cardiomyocyte biology increase the risk of cardiac arrhythmias and thus both directly and indirectly contribute to a reduction in the pumping capacity of the heart, which may ultimately lead to heart failure.[11,12] The exact pro-arrhythmic mechanisms of PCH are not well understood, partly because of the concurrent presence of fibrosis which is a pro-arrhythmic feature by itself.

Adult heart displays limited regenerative capacity.[13] In case of cardiac injury, fibroblasts get activated, proliferate and form myofibroblasts, which secrete large

amounts of extracellular matrix.[14] This process, which is called fibrosis, helps to maintain the heart's integrity; but negatively affects heart function due to the replacement of contractile by non-contractile tissue, an increase in myocardial stiffness and disturbed impulse generation and propagation. The disturbances in the heart's electrical activity are caused by the (electrical) isolation of cardiomyocytes by newly deposited extracellular matrix resulting in slowing or even block of conduction. Coupling of cardiomyocytes with (myo)fibroblasts may also add to disturbed impulse generation and propagation in fibrotic hearts. Taken together, structural and electrical remodelling in the heart could provide both the substrates and triggers for cardiac arrhythmias.[15,16]

Cardiac arrhythmias

Proper electrical cardiac function relies on coordinated and well-timed generation of electrical impulses (*i.e.* action potentials) by cardiomyocytes and propagation of these impulses from cell to cell through gap junctions. Disturbances in electrical impulse generation and propagation could contribute to cardiac arrhythmias.[17] Such heart rhythm disturbances concern any type of condition in which the atrial and/or ventricular rhythm is irregular, slower than normal (bradycardia) or faster than normal (tachycardia). In general, tachyarrhythmias are maintained by reentrant electrical activity or high-frequency electrical signals originating from focal sources. The most dangerous types of cardiac arrhythmias are those which are maintained by fibrillatory conduction (*i.e.* by chaotic activation patterns) especially when this happens in ventricular myocardium. Although our understanding of heart rhythm disturbances has surely improved over time, more insight, especially related to the underlying molecular and cellular mechanisms, is needed in order to improve the diagnosis and treatment of these disorders.

Focal tachyarrhythmias

Disturbances in repolarization can lead to prolongation of action potential duration (APD) and, when occurring at the earlier phases of repolarization (between -40 and 0 mV), may favor formation of aberrant electrical signals, referred to as early afterdepolarizations (EADs). These EADs, in combination with other pro-arrhythmic

conditions could give rise to focal or reentrant tachyarrhythmias. EADs are mainly Ca^{2+}-dependent and can be induced by anachronistic reactivation of the L-type Ca^{2+} channel, sarcoplasmic Ca^{2+} release and inward Na^{+}/Ca^{2+} exchanger activity.[18-20] EADs can occur during phase 2 (*i.e.* the plateau phase) or phase 3 of the cardiac action potential. The mechanisms involved in the generation of phase-2 and phase-3 EADs are not the same as reflected by their different responsiveness to pharmacological inhibitors of ion channels. Since at the depolarized membrane voltages of phase 2, most Na^{+} channels are inactivated, the L-type Ca^{2+} current (I_{CaL}) and the Na^{+}/Ca^{2+} exchanger current (I_{NCX}) are the main currents responsible for phase-2 EADs. During the plateau phase of the action potential, L-type Ca^{2+} channels can undergo transitions between closed and open states. An increase in I_{CaL} in this phase can provide enough depolarizing force for EAD formation. At the same time, the cardiac Na^{+}/Ca^{2+} exchanger generates a net inward current by coupling the export of a single Ca^{2+} ion to the import of three Na^{+} ions, thereby resisting repolarization. The increase in the I_{CaL} further increases the inward current of the Na^{+}/Ca^{2+} exchanger, and thereby may increase the probability of an EAD-triggered action potential.[21] Forward mode of Na^{+}/Ca^{2+} exchanger activity and possibly I_{Na} can promote phase-3 EAD generation. Finally, recent evidences suggest that electrotonic current flow in response to large voltage gradients resulting from heterogeneous repolarization are an important cause of phase-3 EADs.[21,22]

Reentrant arrhythmias

Reentrant arrhythmias are those electrical impulses which are propagated in self-sustaining circuits that do not follow the normal cardiac conduction pattern, in which action potentials generated in the sinoatrial node move through the atrial myocardium and to the atrioventricular node and, after some delay, via the bundle of His and Purkinje fibers through the ventricular myocardium. Under normal conditions, impulses disappear automatically after the entire heart has been activated because the duration of the refractory period exceeds that of the excitation wave. However, if the heart contains an area of inexcitable tissue causing local conduction block and at the same time the tissue around this area shows large heterogeneity in repolarization or conduction, unidirectional block may develop forcing the wavefront of excitation to move in one direction which based on the timing may reenter the original site of excitation, establishing a reentrant circuit.

Reentry can occur in the presence (anatomical reentry),[23] or absence (functional reentry)[24] of an anatomical obstruction (*e.g.* myocardial scar tissue) or can be the result of both structural and functional disturbances in electrical impulse propagation.[25,26] While anatomical reentry in many cases leads to reentrant circuits of constant wavelength and position, rotors caused by functional reentry often meander throughout the tissue.[27,28]

Challenges in skeletal and cardiac muscle research

Research in the field of skeletal and cardiac muscle diseases mainly focuses on unravelling the underlying pathogenic mechanisms and on developing (better) therapeutic interventions. Despite many improvements in the medical and surgical management of skeletal and cardiac muscle disorders, development of effective and durable treatments has proven to be challenging. In cardiac muscle disorders, device therapies and interventional procedures such as catheter ablation have multiple limitations and are associated with a risk of complications.[29,30] Pharmacological therapies for skeletal and cardiac muscle diseases are largely directed toward palliation of the symptoms of the disease rather than targeting the underlying causes.[31-33] Improvement in therapeutic modalities requires better understanding of molecular pathways involved in the initiation and progression of these diseases. Much of the available information about the underlying mechanisms of skeletal and cardiac muscles disorders is obtained from *in vivo* studies. These studies are, however, complicated by the complexity of three-dimensional (3D) tissues, the occurrence of disease symptoms secondary to the primary condition, primary causes directly affecting other organs besides the heart and skeletal muscles and the interplay between different organ systems. For example, Duchenne muscular dystrophy (DMD), the most common inherited myopathy affects different skeletal muscles to a different extent but may also impair cardiac and brain function to various degrees depending on the specific mutation involved.[34-37] Also, the coincident presence of *e.g.* inflammation, hypoxia and fibrosis in PCH makes it very hard to determine its specific/mechanistic contribution to the occurrence of arrhythmias.[38,39]

The development and use of dedicated cellular experimental models to study the mechanisms underlying skeletal and cardiac muscle diseases in combination with

genetic interventions to investigate the role of specific factors, may resolve these limitations and lay the basis for the development of novel treatment options.

Cellular models

Cultures of skeletal myoblasts or cardiomyocytes offer the possibility to analyse cellular functions and molecular pathways in a highly specific and controllable manner, which is difficult to accomplish *in vivo.* Given the ease with which *in vitro* models can be established and manipulated to mimic physiological or pathological conditions, they are ideally suited for proof-of-concept studies and testing new therapeutic interventions targeting specific aspects of disease. Indeed, cellular models have greatly contributed to our current understanding of skeletal and cardiac muscle diseases.[40,41] Although their relative simplicity facilitates data interpretation, cells in culture are not subjected to the complex regulatory systems controlling organ function *in vivo*. Accordingly, results obtained in *in vitro* experiments will always need to be validated in clinical studies. In spite of their shortcomings, *in vitro* models keep on being highly useful for mechanistic and therapy-directed skeletal and cardiac muscle research. This is particularly true when cell culture models are combined with genetic interventions to investigate the involvement of specific genes in physiological and pathophysiological processes.

Genetic interventions

Recently, there has been considerable interest in the application of gene therapy in the field of skeletal and cardiac muscle diseases. Many of these genetic interventions have focussed on counteracting the pathological processes in failing myocytes either directly by correction of the underlying genetic defect if applicable or indirectly by inhibition of pathogenic mechanisms or stimulation of physiological processes. Gene therapy has, for example, been used to complement gene mutations causing various types of muscular dystrophy including DMD as well as defects in several sarcomeric protein genes linked to PCH.[42,43] Genetic intervention can be also employed for overexpression of a protein like sarcoplasmic reticulum Ca^{2+}-ATPase pump (Serca2a) to improve cardiac function[44] or downregulation of a protein like the acetylcholine-acticated K^+ channel Kir3.4 to terminate atrial fibrillation (AF).[45] Clinical and preclinical studies have shown beneficial effects of myocardial

gene transfer in neovascularization of ischemic myocardium, increasing myocardial contractility, induction of cardiac repair and reduction of AF and ventricular tachycardia vulnerability. [46-51] Gene therapy has also demonstrated improvements in skeletal muscle disorders.[52-54] The safe and successful delivery of a gene is very important in order to gain high therapeutic efficacy. A large number of gene delivery methods have been developed using both viral and non-viral vectors, each of which have their own pros and cons. Non-viral methods include using naked DNA alone or in combination with cationic liposomes or polymers.[55,56] Non-viral vectors are typically easy to synthesize and can be used for the transfer of genetic material of all kind of different sizes. Additional advantages of non-viral vectors are their safety and the ease with which they can be modified *e.g.* to alter their cell tropism. By applying non-viral gene delivery one can avoid disadvantages intrinsic to the use of viral vectors such as limited genetic payloads and expensive/laborious production methods. Also, nonviral vectors are generally less immunogenic and have a lower risk of insertional oncogenesis than viral vectors.[57] Major limitations of non-viral vectors are their very low *in vivo* gene transfer activity and their difficulty to efficiently transduce differentiated cells both *in vitro* and *in vivo*. Viral vectors are much better suited for this purpose especially when high transduction rates and transgene expression levels are required.[58]

Viral vectors

Viral vectors are commonly used for the genetic modification of skeletal and cardiac muscle cells and tissues because they transfer genes much more efficiently than any of the non-viral vectors. The most commonly used viral vectors are derived from retroviruses (including lentiviruses), adenoviruses and adeno-associated virus, which belongs to the parvoviruses. These potentially harmful viruses are converted into innocuous viral vectors by the replacement of one or more essential viral genes by heterologous gene expression units, which renders the viral vectors replication-deficient but still allows introduction and expression of their genetic cargo into target cells. The production of these vectors requires the missing viral genes to be provided *in trans* through packaging plasmids, complementing cell lines or helper viruses.

Retroviral vectors

The first retroviral vectors were derived in the 1980s from gammaretroviruses. Since these vectors require cell division for efficient transduction, they are not suitable for the genetic modification of differentiated cell types like skeletal and cardiac myocytes. Unlike gammaretroviral vectors, lentivirus vectors (LVs) do not depend on cell division for efficient transduction of target cells. Following target cell entry, the LV genome, which consists of a positive-sense, single-stranded linear RNA molecule, is reverse transcripted into cDNA and subsequently integrated into the host cell genome. LV vectors can accommodate a fair amount of foreign DNA due to the absence of a strict upper packaging limit. There is, however, an inverse relationship between LV yields and genome lengths[59] which practically restricts insert sizes to ± 5 kb. LVs are generally produced in human embryonic kidney (HEK) 293T cells by a simple transfection procedure involved a LV shuttle plasmid and two or three so-called helper or packaging plasmids. These features and the ease with which LVs can be generated may explain why LVs have become such popular gene delivery vehicles for the permanent *ex vivo* genetic modification of both differentiated and proliferating cell populations.[60,61] LVs are much less suitable for *in vivo* gene therapies due to their large diameter, which hampers their spreading through tissues, and the preferential integration of LV genomes into transcriptionally active chromosome loci imposing a certain risk of insertional oncogenesis.[62,63] Currently, much effort is put in the improvement of lentiviral vector design to reduce as much as possible the risk of insertional oncogenesis.[64,65]

Adenoviral vectors

Adenoviral vectors (AdVs) have the capacity to carry large DNA molecules (± 37 kb for human adenovirus type 5), can be produced in very large quantities and very efficiently transduce all kinds of cells, irrespective of their cell cycle status. AdV genomes are linear double-stranded DNA molecules covalently linked at their 5' ends to the so-called terminal protein.[66] These genomes normally do not integrated to the host chromosomal DNA but reside in the nucleus of the target cells as nonreplicating episomes, which causes transient expression especially in dividing cell populations but simultaneously limits concerns related to oncogene activation. The main disadvantages of AdVs are their large size limiting their dissemination through tissue and their relatively high immunogenicity, which may result in the *in*

vivo elimination of transduced cells by cell-mediated immune responses. This latter problem especially applies to first- and second-generation AdVs, which in contrast to their third-generation counterparts still contain adenoviral genes that are express at low levels in target cells. As a consequence, severe inflammation leading to toxicity and even, in very rare cases, organ failure has been reported following *in vivo* administration of early-generation AdVs.[67] In recent year, AdV vector development has mainly focussed on lowering the immunogenicity of the adenovirus particle, reducing unintended interactions with host proteins and increasing target cell specificity by chemical or genetic modification of the adenoviral capsid/coat proteins.[68,69]

Adeno-associated virus vectors

Adeno-associated virus vectors (AAVs) carry a single-stranded DNA genome with a T-shaped hairpin at both termini. Following their delivery in the target cell nucleus most AAV DNA is converted into highly stable double-strand circular monomers and concatemers. Moreover, AAVs are significantly less immunogenic than AdVs, which is partially explained by the absence in the vector genome of parvoviral genes. Because of these property AAVs can mediate long-term albeit not permanent gene expression.[70] Another advantage of AAVs is their small diameter, which allows them to easily penetrate tissues. The downside of their small size is their limited packaging capacity, which does not allow incorporation of transgene > 4.7 kb. Other disadvantages of AAVs include the time-consuming and expensive procedures needed for their production and purification and their relatively low gene transfer activity requiring high amount of AAV particles to achieve efficient transduction.[71] In addition, due to the slow onset of transgene expression AAVs cannot be used for studies with a short time course. Still, at present AAVs are the only viral vectors that can tranduce entire organs, including the heart.[72] Therefore, currently, much effort is put in improving AAV production methods and in modifying the vector genome and capsid to increase the specific gene transfer activity of AAVs and to overcome their limited packaging capacity.[73]

Aim an outline of the thesis

The limited suitability of existing experimental models for acquiring a thorough understanding of the mechanisms underlying skeletal and cardiac muscle diseases and the lack of efficiency and specificity of many of the currently available therapeutic interventions have made efficient treatment of these diseases challenging. Therefore, the aim of this thesis is to establish dedicated cellular models and use viral vector systems to study the biology of skeletal and cardiac muscles in healthy and diseased states and thereby identify potential targets for future therapeutic interventions.

Chapter I of this thesis explains the common pathological features of skeletal and cardiac muscle diseases, the limitations of current therapies and advantages of cellular models and genetic interventions in improved treatment of these diseases.

The successful deployment of cell transplantation in skeletal muscle disorders depends on the potential of the donor cells to engage in myotube formation (myogenesis), which is amongst others determined by the ability of the transplanted cells to fuse with cells present in the host tissue.[5,74] Cell fusion also plays an important role in fertilization, syncytiotrophoblast production, bone remodelling, eye lens development and certain forms of tissue repair.[75] Different methods can be used for monitoring cell fusion activity. These methods and a newly developed non-destructive quantitative cell fusion assay are described in **chapter II**.

Efficient engraftment of transplanted cells is another factor which determines the success rate of transplantation. Scaffolds provide a framework for cells to attach, proliferate, and form extracellular matrix *in vivo*. The scaffolds may also serve as carriers for cells, growth factors, and/or other biomolecular signals. Ideally, scaffolds should be degraded *in vivo* at an appropriate rate to allow its gradual replacement by regenerated host tissue. Therefore, the *in vivo* biodegradability of Gelatin/Siloxane/Hydroxyapatite scaffolds and their ability to support adhesion and proliferation of rat bone marrow mesenchymal stem cells have been studied in **chapter III**.

Employment of 2D cell culture models makes it possible to study the contribution of PCH per se to arrhythmogenicity, which cannot be easily done *in vivo* due to the simultaneous presence of other pro-arrhythmic features. Induction of hypertrophy-related pathological changes in cardiomyocyte cultures can be achieved by exposing

the cells to a variety of different peptide and non-peptide hormones and growth factors. It has been shown that phorbol 12-myristate 13-acetate (PMA), which activates protein kinases C and D, induces a gene expression program in cardiac muscle cells resembling that of cardiomyocytes in pathologically hypertrophied hearts.[76-78] In **chapter IV**, the use of the PMA to establish an *in vitro* model of PCH based on ventricular neonatal rat cardiomyocytes is described and its pro-arrhythmic features are studied.

Early- and no-reperfusion after myocardial infarction (MI) leads to formation of patchy and compact scars, respectively. These post MI scars facilitate circular conduction of the impulses in the heart. How scar composition affects arrhythmogenicity and arrhythmic phenotype has been investigated in an *in vitro* model in **chapter V**.

Atrium-selective drugs and interventions with higher efficacy in AF rhythm control but fewer side effects such as ventricular pro-arrhythmia are paramount needs in AF treatment. In **chapter VI**, the role of acetylcholine-activated K^+ channels, whose expression in mammalian hearts is largely restricted to the atria, has been studied in AF initiation, dynamics and termination in a cell culture and whole-heart model of AF.

Finally, **chapter VII** summarizes the findings of this thesis and provides future perspectives based on the conclusions drawn from each study.

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