Chapter 7

Summary, conclusions and future perspectives

Samenvatting, conclusies en toekomstperspectieven

Summary

The general introduction of this thesis, **chapter I**, starts with a brief comparison of the pathological features of skeletal muscle disorders and cardiac diseases. Furthermore, the lack of (effective) therapies for many cardiac and skeletal muscle diseases is discussed. This paucity in adequate treatment options for (cardio)myopathies is partially explained by the limited knowledge about the precise mechanisms underlying skeletal and cardiac muscle diseases and, related to this, by the non-specific action of many therapeutic interventions. Therefore, the aim of this thesis was to establish cellular models and employ viral vector technology to identify potential targets for future therapeutic interventions in skeletal and cardiac muscle diseases.

Chapter II describes the development of a bipartite lentivirus vector (LV)-based assay to quantify cell fusion in which the cellular fusion partners are transduced with a *Gaussia princeps luciferase* (*GpLuc*) expression unit (acceptor cells) or with a recombinant gene encoding FLPe, a nuclear-targeted and molecularly evolved version of flippase (donor cells). GpLuc is a secretory protein allowing repeated analysis of the same study object, a great advantage over cell fusion assays using *Photinus pyralis luciferase* (*PpLuc*), which is not a secretory protein precluding consecutive analysis of the same study object. To investigate whether the spread of $FLPe^{NLS+}$ in myotubes is limited due to its nuclear localization signal (NLS), myoblasts were transduced with LVs encoding either $FLPe^{NLS+}$ or an NLS-less version of FLPe ($FLPe^{NLS-}$) and subsequently co-cultured in different ratios with myoblasts containing the FLPe-activatable *GpLuc* expression cassette. At different times after induction of cell-to-cell fusion the GpLuc activity in the culture medium was determined. In general, *GpLuc* expression increased with increasing fractions of *GpLuc*-transduced myoblasts and both $FLPe^{NLS+}$ and $FLPe^{NLS-}$ activated the latent *GpLuc* gene but when the percentage of *FLPe*-expressing myoblasts was limiting, $FLPe^{NLS+}$ generally yielded slightly higher signals than $FLPe^{NLS-}$ while at low acceptor-to-donor cell ratios $FLPe^{NLS-}$ was usually superior. Thus, it was shown that NLS does not limit the ability of $FLPe^{NLS+}$ to spread through myofibers and to induce reporter gene expression. However, at high fraction of *FLPe*-expressing myoblasts the presence of the NLS negatively affected reporter gene expression. These results

show that a rapid and simple chemiluminescence assay for quantifying cell-to-cell fusion progression based on GpLuc has been developed.

In **chapter III** different properties of Gelatin/Siloxane/Hydroxyapatite (GS-Hyd) scaffolds are investigated such as *in vivo* biodegradability, cytotoxic effects and ability to support cell adhesion. Mesenchymal stem cells (MSCs) were treated with different volumes of the scaffold suspension for evaluation of its cytotoxic effects. MSCs were also cultured on the scaffold for 2 weeks to evaluate the ability of the scaffold to promote cell adhesion and growth. The GS-Hyd scaffold did not exert noticeable cytotoxic effects on the MSCs and these cells could adhere to the scaffold, expand their elongations and form colonies. To study its biodegradability the GS-Hyd scaffolds were implanted in thigh muscle, testicle and liver of Wistar rats. At different times after implantation, scaffolds were excised and their dry weight was measured. The largest reduction in scaffold weight occurred during the first days after implantation and varied from 53% in liver to 71% in thigh muscle at 3 days post implantation. Subsequently, scaffold degradation slowed down with 30%, 25% and 18% of the initial scaffold remaining at 3 weeks after implantation in liver, testis and thigh muscle, respectively. Scanning electron microscopy (SEM) indicated obvious morphological changes on the surface of the scaffold and in the diameter of the pores after 21 days of implantation. In conclusion, the GS-Hyd scaffold seems to be a promising tool for cell-based therapeutic interventions, but additional research is needed to assess its clinical utility.

Chapter IV is dedicated to the development of an *in vitro* model for studying the contribution of pathological cardiac hypertrophy (PCH) per se to arrhythmia independent of fibrosis or other PCH-related processes. Treatment of neonatal rat ventricular cardiomyocyte (nr-vCMC) monolayers with phorbol 12-myristate 13-acetate (PMA) for two times 24 hours, led to increases in cell surface area (CSA) and protein content of the cardiomyocytes. Assessment of the electrophysiological properties of PMA-treated and control nr-vCMC monolayers by optical mapping at day 9 of culture, showed a decrease in conduction velocity (CV) and increases in action potential duration (APD) and APD dispersion upon PMA treatment. It also caused a 32% reduction in sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase 2 level and an increase in natriuretic peptide A (42%) and α1-skeletal muscle actin (34%) levels indicating that the hypertrophic response induced by PMA was pathological in nature. Upon local 1-Hz stimulation, 54% of the PMA-treated cultures showed focal

tachyarrhythmias based on triggered activity, while the rate of tachyarrhythmias was only 4% in control nr-vCMC cultures. PMA-treated nr-vCMC cultures may thus represent a well-controllable *in vitro* model for testing new therapeutic interventions targeting specific aspects of hypertrophy-associated arrhythmias.

Chapter V describes the development of *in vitro* models of post-myocardial infarction (MI) compact and patchy fibrosis and the assessment of their pro-arrhythmic features by optical mapping. To this end, either a single large circular anatomical obstruction or multiple smaller circular anatomical obstructions were made in the center of confluent nr-vCMC monolayers by forcefully pressing tailor-made plexiglass stamps onto the cells. To gain mechanistic insight into arrhythmias associated with post-MI scars several parameters such as rate of inducibility, cycle length and maintenance of reentry were compared between these two types of anatomical obstructions. In patchy cultures, reentry was slightly less easily inducible (41% vs 52%) and, when it occurred, had a shorter cycle length (234±52 vs 288±38 ms) than in compact cultures. Sustained reentry was less often observed in patchy cultures (40% vs 88% in compact cultures) while the percentage of complex arrhythmias was higher (31% vs 11%). Meandering of phase singularities and gradients of excitability during reentrant arrhythmias were only detectable in patchy cultures. Reentry could be terminated more easily in compact cultures (82% vs 20% in patchy cultures). Taken together, this *in vitro* model of patchy and compact obstructions reproduced arrhythmic features similar to those observed after early- and non-reperfused MIs, respectively, and may hence provide mechanistic insights into the efficacy of anti-arrhythmic interventions in infarcted hearts with different anatomical substrates.

In **chapter VI** the effects of constitutive acetylcholine-regulated K^+ current ($I_{K,Ach\text{-}c}$), which flows through Kir3.1 and Kir3.4 channels, on atrial fibrillation is investigated. Neonatal rat atrial CMC cultures and intact atria were burst paced to induce reentry. Treatment of these cultures with tertiapin prolonged APD, indicating the presence of $I_{K,ACh\text{-}c}$. Furthermore, tertiapin decreased rotor frequency and complexity. Reduction of Kir3.1 or 3.4 expression through transduction of cells with LVs encoding Kcnj3- or Kcnj5-specific shRNAs gave similar results. Tertiapin prevented/terminated reentry by prolonging APD and changing APD and CV restitution slopes, thereby lowering the probability of APD alternans and inducing rotor destabilization. Whole-heart mapping experiments confirmed key findings (*e.g.* >50% reduction in atrial fibrillation

inducibility after $I_{K,ACh-c}$ blockade). This study provided new molecular and mechanistic insights into atrial tachyarrhythmias and identified Kir3.1 and Kir3.4 as promising atrium-specific targets for anti-arrhythmic strategies.

In conclusion, the experiments described in this thesis emphasize the importance of cellular models for (i) elucidating the mechanisms underlying skeletal and cardiac muscle diseases and (ii) identification of novel therapeutic targets. This thesis also underlines the usefulness of viral vector-mediated gene transfer technology for the development of biological assays and the evaluation of therapeutic targets.

Future perspectives

Without a thorough understanding of the mechanisms underlying cardiac and skeletal muscle diseases, their treatment by pharmacological or genetic interventions involve a lot of trial and error. Cellular models are important tools to gain mechanistic insights into skeletal muscle degeneration and cardiac arrhythmias mechanisms allowing the rational design of new treatments. This relates at least in part to their relative simplicity, providing the possibility to investigate a specific pathological feature independent of other complications. Despite of having this and some other advantages, *in vitro* cellular models often do not mimic well enough the *in vivo* situation, which may lead to poor *in vitro-in vivo* correlations. Accordingly, future research should focus on the further improvement of these cellular models in such a way that they better resemble/represent *in vivo* pathological states reducing the need for preclinical and clinical studies. For example, in the case of *in vitro* models of cardiac hypertrophy it is essential to determine which stage in the transition from PCH to heart failure the hypertrophy model represents as this will affect the therapeutic approach. Besides by checking the extent of electrophysiological changes and incidence and nature of arrhythmias, measuring of the contractile force-generating capacity and comparative transcriptome analyses will provide useful information on this issue.

An important hurdle in the development of *in vitro* cellular models arises from the low expansion capability of differentiated cells. For example, postnatal CMCs display very limited proliferation capacity which demands for repetitive isolation and culture of these cells for *in vitro* studies. Therefore, immortalization of atrial or ventricular CMCs from animal or human would circumvent this obstacle. This could be achieved through viral vector-mediated transfer, into these cells, of genes such as those

encoding simian virus 40 large T antigen, B cell-specific Moloney murine leukemia virus integration site 1 (BMI1) or telomerase (TERT) reverse transcriptase.

Genetic manipulations through viral vectors (*e.g.* overexpression of certain genes or RNA interference) may also help to gain better mechanistic insights into skeletal and cardiac muscle disorders and to further optimize current therapies for these diseases.

Regarding to skeletal muscle wasting, optimization of cell-based therapies may be achieved through enhancement of the fusion of donor cells which each other and with recipient myocytes and by promoting their myogenic differentiation. For instance, forced and controlled expression of fusion-enhancing proteins through transduction of (non-muscle) stem cells with viral vectors encoding these proteins may be beneficial as could be the regulated expression in these cells of myogenic transcription factor genes.

Genetic intervention may also be employed for therapeutic purposes in cardiac arrhythmias. For example, the forced expression of connexins like connexin43 in (myo)fibroblasts present in infarct scars may improve conduction and reduce arrhythmia incidence. This approach could, however, also be deleterious as coupling of CMCs with (myo)fibroblasts would depolarize CMCs and make the tissue susceptible to reentrant tachycardias. The latter problem may be overcome by simultaneously endowing (myo)fibroblasts with a gene like *KCNJ2* whose product is involved in setting the resting membrane potential.

Genetic interventions (overexpression or knockdown) affecting the expression of the genes encoding for ion channels involved in the repolarization phase of the action potential (AP) may also have anti-arrhythmic effects. For example, manipulation of the expression of genes encoding for those ion channels that exert their effect on repolarization in the AP phases at which early afterdepolarizations occur (*e.g.* adenosine triphosphate (ATP)-sensitive K^+ channels [K_{ATP} channels]) could provide novel insights into pro- and anti-arrhythmic mechanisms but could also have therapeutic potential.

Effective therapeutic application of viral vectors *in vivo* often requires uniform and near-quantitative transduction of the target tissue and transgene expression at a specific level. This is of particular importance when developing gene therapy for arrhythmias as heterogeneous transduction and inappropriate transgene expression levels may be pro-arrhythmic.

Hence, efficient delivery and high target cell specificity of viral vectors and precise control of transgene expression are of vital importance and deserve further study. Other important topics of future gene therapy research are the further improvement of the safety and reduction of immunogenicity of viral vectors.