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CHAPTER VIII

SUMMARY, CONCLUSIONS, DISCUSSIONS, AND FUTURE PERSPECTIVES
SAMENVATTING EN CONCLUSIES

Summary

The general introduction of this thesis, **Chapter I**, describes the origins and typical characteristics of mesenchymal stem cells (MSCs). Next, the capacity of MSCs to undergo cardiac differentiation *in vitro* and *in vivo* is discussed with emphasis on the criteria and the methods used to determine differentiation into cardiac cell types. Finally, an overview is presented of the clinical studies using MSCs to treat cardiac diseases, including outcomes and (potential) hurdles.

The aim of this thesis was to study the integration and cardiac differentiation of MSCs in cardiac tissue using dedicated *in vitro* models and molecular interventions.

In **Chapter II** the cardiac differentiation potential of MSCs derived from human (h) embryonic, fetal and adult tissues in co-cultures with neonatal rat ventricular cardiomyocytes (nrCMCs) was evaluated. It was shown that the capacity of hMSCs to differentiate towards cardiomyocytes, smooth muscle cells and endothelial cells declined with the age of the MSC donor. Cardiomyogenic differentiation was most efficient in embryonic hMSCs, occurred at a lower frequency in fetal hMSCs and could not be detected in hMSCs from adults.

In **Chapter III** the effects of alignment of neonatal rat MSCs on electrical integration in nrCMC cultures was studied using multi-electrode arrays. During 9 days of co-culture a portion of these MSCs differentiated towards cardiac muscle cells and depending on the predefined direction of alignment the degree of electrical integration of the MSCs with surrounding cardiomyocytes differed significantly.

The role of gap junctional coupling between cultured cardiomyocytes and hMSCs in inducing cardiac differentiation of the hMSCs was studied in **Chapter IV**. Connexin 43 downregulation and subsequent connexin 45 upregulation in fetal hMSCs co-cultured with nrCMCs decreased and restored, respectively, their ability to differentiate into cardiomyocyte-like cells that could produce action potentials.

In **Chapter V**, co-cultures between lentiviral vector-modified hMSCs and nrCMCs were used to investigate secondary transduction of cardiomyocytes with vector particles originating from primary transduced. Secondary transduction of native cardiomyocytes under these conditions is a frequent event, thereby seriously disturbing the interpretation of experiments in which cardiac differentiation of MSCs is inferred from the co-expression of specific cardiac features together with a virally introduced marker gene. However, with some additional measures the percentage of falsely labeled cells can be reduced to zero.

Chapter VI shows the results of a study focusing on the role of hMSC engraftment patterns on arrhythmicity in nrCMC cultures. Not only the percentage of engrafted cells affected arrhythmia incidence and characteristics but also the pattern in which these cells engrafted (clustered *versus* diffuse distribution). Electrical

coupling between hMSCs and cardiomyocytes as well as secretion of certain freely diffusible paracrine factors contributed to the pro-arrhythmic effects observed in these co-cultures.

Besides the pro-arrhythmic effects of transplanting unexcitable cells in cardiac tissue, also the role of endogenous, unexcitable myofibroblasts in arrhythmogenesis was studied. Accordingly, in **Chapter VII** nrCMC cultures were treated with cytostatic agents (*i.e.* mitomycin-c and paclitaxel) to inhibit the proliferation of the (myo)fibroblasts in these cultures. This strongly reduced the incidence of reentrant arrhythmias by preserving tissue excitability.

In conclusion, the cardiac differentiation potential of hMSCs negatively correlates with donor age, which in its own turn shows a negative relationship with connexin 43 levels in these cells. However, a causal relationship between connexin 43 expression levels and cardiomyogenic differentiation potential only exists for hMSCs from prenatal sources, *i.e.* while knockdown of connexin 43 expression in fetal hMSCs inhibits their ability to differentiate into cardiomyocytes, connexin 43 overexpression in adult hMSCs does not endow them with cardiomyogenic differentiation capacity. In addition, co-culture studies showed that the alignment and distribution of MSCs affect their electrical integration into host myocardium and are major determinants of their pro-arrhythmic risk. The mechanisms underlying the pro-arrhythmic effects of hMSCs are to some extent comparable to those of cardiac myofibroblasts, cells that are found in fibrotic myocardium.

DISCUSSION & FUTURE PERSPECTIVES

Whether and to which extent mesenchymal stem cells (MSCs) are intrinsically able, or can be forced, to undergo cardiac differentiation, especially with regard to formation of functional cardiomyocytes (CMCs), has been subject of intense investigation.[1] The incentive for these studies was probably based on the apparent advantageous properties of MSCs following transplantation in damaged myocardium. Their ease with which MSCs can be isolation from a variety of relatively easily accessible tissues (*e.g.* bone marrow and adipose tissue) and subsequently expanded *in vitro*, their favorable immunological effects and indications that these cells could differentiate towards cardiac cells contributed to a rapid transition from bench to bed.[2] However, the initial enthusiasm for MSC therapy to actually regenerate myocardial tissue after ischemic damage is now replaced with contemplation, while new strategies are being developed to overcome the encountered challenges related to cardiac cell therapy.[3-5]
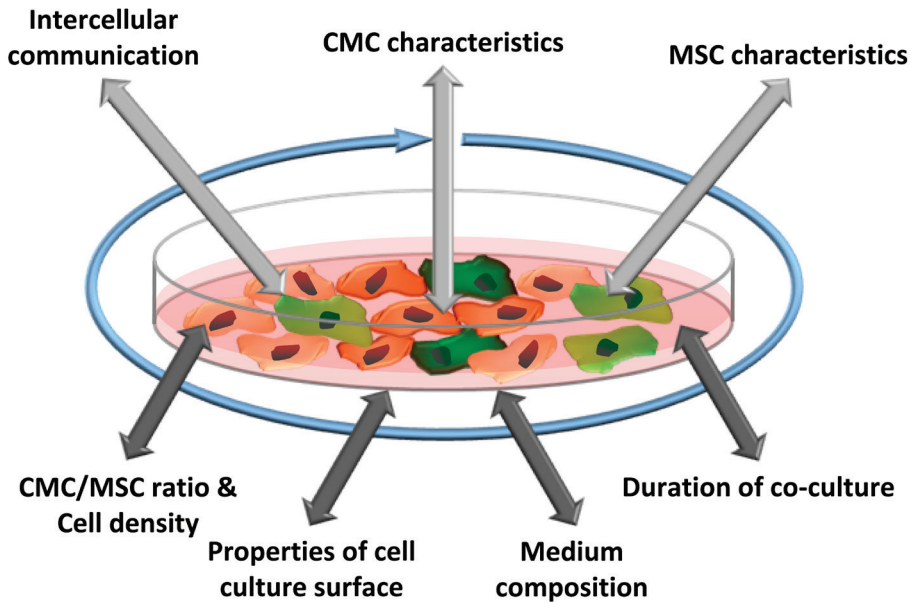
Figure 1. Schematic overview of factors that might affect the outcome of studies on cardio-myogenic differentiation of MSCs using co-culture models. Intrinsic and extrinsic factors are indicated by light and dark grey arrows, respectively. These factors probably also affect each other, thereby creating a highly complex and dynamic setting in which the outcome is determined by the interplay between the various factors.

Before stem cells can undergo cardiomyogenic differentiation specific transcriptional pathways need to be activated. A method from which it is believed to deliver the triggers needed to initiate this process involves co-culture with CMCs. However, the exact co-culture conditions needed to induce (complete) cardiomyogenic differentiation in MSCs, if possible at all, are not fully understood.[6] It is therefore no surprise that conflicting results have been reported about the potential of MSCs to undergo cardiomyogenesis as most of these studies relied on co-cultures between MSCs and CMCs.[7-9] In fact, there is still little known about the triggers for cardiac differentiation in any stem cell type using any model, especially when it comes to guiding differentiation towards a specific cardiac cell type.[10] In CMC-MSC co-culture models a number of factors and mechanisms have been identified which seem to play a role in inducing cardiomyogenic differentiation. However, the degree to which these factors contribute to the underlying mechanism of cardiomyogenic differentiation is directly influenced by the experimental setup and conditions. Basically these factors can be divided into two groups, intrinsic and extrinsic factors (Figure 1).

Intercellular communication between co-cultured cells could be one of these intrinsic factors. It has been shown that MSCs derived from human or rodent tissue express certain proteins called connexins (Cx).[11] These proteins form hemichannels called connexons which consist of a ring of 6 connexin molecules with a central pore. When connexons in 2 neighboring cells associate with each other, gap junctions are formed. These intercellular channels comprise relatively aselective low-conductance conduits allowing transmission of electrical and chemical signals between MSCs.[12] MSCs can also form functional gap junctions with adjacent CMCs, thereby establishing heterocellular communication.[13] The degree and nature of gap junctional communication is determined by several factors, including the extent of physical contact between cells, the surface levels of Cxs, the spreading of gap junctions across the plasma membrane, the specific Cx isoforms incorporated in the gap junctions and the conductive properties of the connexons, which are regulated by chemical, electrical and mechanical signals as well as by posttranslational modifications (*e.g.* phosphorylation).[14] In this thesis it has been shown that functional gap junctional coupling between neonatal rat ventricular CMCs (nrCMCs) and co-cultured adult human MSCs is important for the induction of cardiomyogenic differentiation in these stem cells (Chapter IV). Therefore the degree of intercellular communication, determined by the factors named above, could directly affect the efficiency and extent to which MSCs differentiate into CMCs.

The *characteristics of CMCs* used in co-culture experiments might also affect the propensity of MSCs to acquire a cardiomyogenic phenotype. For practical reasons nrCMCs are the primary choice for CMC-MSC co-culture experiments. Such CMCs can be kept in culture for many days without losing excitability and contraction.[15] These key properties of CMCs have been linked to induction of cardiomyogenic differentiation.[16,17] However, during the isolation procedure CMCs might become damaged, thereby affecting their characteristics in terms of excitation and contraction. Additionally, CMCs are mostly isolated from 2-day old rats, but at this stage the heart is still under development with changing CMC characteristics,[18] and a seemingly insignificant difference in age (12 h or 1 day difference for example) might therefore affect the outcome of co-culture experiments. Importantly, although breeding of rats can be standardized to some degree, the actual time point of conception and parturition could create a significant variety in age when different nests are put together to create a sufficient supply. Also, intercellular communication between hMSCs and non-human CMCs could be suboptimal, but obtaining hCMCs in large quantities and keeping them in culture long enough to conduct such co-culture experiments does not seem feasible.

Besides culture-related aspects, one also needs to consider that healthy, nrCMCs are very different from those found in the target tissue of cardiac cell therapy, being a diseased human heart.[19] In such a heart, and especially near the border

zone of myocardial infarction, CMCs are often remodeled, exposed to pro-apop-totic/necrotic signals, inflammatory stimuli and changes in pH, redox balance and oxygen tension. Therefore, even if the conditions are fully standardized in CMC-MSC co-culture experiments and cardiac differentiation can indeed be studied in a robust fashion, the data derived from these experiments should always be inter-preted and presented with great care.

Logically, also the *characteristics of MSCs* might have an impact on the outcome of cardiac differentiation experiments in co-cultures of MSCs and CMCs. Human adult MSCs are usually derived from tissues like bone marrow or fat, while human MSCs can also be derived from fetal tissues and neonatal sources like amnion, placenta and umbilical cords.[1] Although some studies suggest that the source of MSC could determine their potential to differentiate towards certain cell types,[20] it is now recognized that donor age of MSCs certainly affects their differentiation potential, including their ability to form cardiac cells.[21,22] This phenomenon can be explained by the fact that cellular plasticity in general declines with age.[23] In this thesis it is shown that Cx43 expression levels in hMSCs also decline with age, which is accompanied by a loss of cardiomyogenic differentiation. However, while Cx43 knockdown fully eliminated the cardiomyogenic differentiation potential of fetal human MSCs in co-culture with CMCs, overexpression of Cx43 in adult human MSCs did not cause them to become CMCs when coupled to nrCMCs. This shows that the potential of stem cells to differentiate into various cell types is constrained by other factors and mechanisms besides those related to gap junctions. Impor-tantly, the recent developments in genetic reprogramming of cells, including those involving direct cardiac reprogramming of fibroblasts have further uncovered the requirements for cardiomyogenic differentiation.[24,25]

One of the extrinsic factors in these co-culture experiments that could affect the outcome is the *CMC to MSC ratio and density* at which the cells are plated, as this determines the extent of intercellular communication through gap junctions, ligand-receptor interactions and vesicle release/uptake as well as the cumulative strength of cardiomyogenic cues. Consequently, a relatively high number of adja-cent CMCs will probably have a greater influence on a MSC than a lower num-ber of CMCs thereby increasing the likelihood that a suprathreshold stimulus for cardiomyogenic differentiation is achieved. Although this thesis (Chapter 4) does not provide direct evidence for a role of paracrine factors in inducing cardiac dif-ferentiation, in other situations and under other conditions such factors could be of importance.[26] Increasing the supply of paracrine factors could result in a higher local concentration of these factors and thereby have a stronger effect. This may, for instance, hold true for hypoxia-induced myocardial angiogenesis, which is often suggested to represent the major beneficial effect in cardiac cell therapy.[27]

It is known that the *characteristics of the cell culture supports and coatings* used in cardiac differentiation experiments can have a strong effect on the induction of differentiation.[28,29] However, the underlying mechanisms of these *in vitro* findings remain poorly understood. Coatings applied in culture dishes interact with their surface and create a substrate on which cells can attach, survive and grow. Such coatings are usually based on extracellular matrix components and act as ligand for transmembrane receptors called integrins, which ensure attachment. Apparently, the degree of attachment, and thereby flattening and spreading of cells, could have an effect on the outcome of cardiac differentiation experiments. In the developing heart, stem and progenitor cells attach to adjacent cells and extracellular structures, however, whether inhibition of this process also leads to suboptimal growth of heart muscle, as a consequence of disturbed cardiac differentiation, is still not fully understood.[30]

The conditions to which cultured cells are exposed largely depend on *the composition of the culture medium* applied onto the cells. Especially serum, which is a key ingredient of most culture media and derived from species like bovine and equine, is known to affect the outcome of cardiac differentiation experiments, especially when using embryonic stem cells.[31,32] Serum is rich in proteins, including various growth factors. How these or other factors in serum modulate the propensity of stem cells to differentiate into cardiac cells is incompletely understood. However, it is known that depending on source, concentration and pretreatment *e.g.* heat treatment to inactivate complement. of serum, the proliferation rate, growth and viability of stem cells is changed.[33] These changes may have a direct or indirect effect on the susceptibility to certain cardiac differentiation-inducing stimuli.

Once stem cells and CMCs are put together in culture and have attached to the underlying surface, the *duration of co-culture* is likely to determine to which extent cardiomyogenic differentiation can take place. Based on other cardiac differentiation protocols, a 10-day incubation time seems sufficient to allow formation of cardiac cells.[34] Apparently, during this period stem cells are exposed to the appropriate stimuli of sufficient duration and (cumulative) strength to activate cardiac transcriptional pathways. However, if other requirements for cardiomyogenic differentiation, which are listed above, are not met, obviously, MSC-derived CMCs will not be obtained.

Parallel to extensive *in vitro* studies, also numerous pre-clinical *in vivo* studies,[1,35,36] as well as clinical studies have been performed to assess the therapeutic potential of cell transplantation in the diseased heart.[4,37-39] Although a significant number of these clinical trials have studied the effect of transplanted bone marrow cells in damaged myocardium, few clinical studies have investigated the effects of MSC transplantation on cardiac function.[40-42] Nevertheless, most of these studies showed a beneficial effect. This effect is often associated with enhanced

myocardial perfusion,[43] although such trials obviously do not allow investigation of the therapeutic mechanisms. As these mechanisms remain poorly understood, there is only a very small basis for the development of strategies to enhance the therapeutic effects. As another result, the therapeutic window of cardiac MSC therapy in general and the possible (dose-dependent) hazardous effects of MSC transplantation are still unknown. While the optimal therapeutic effect could be achieved by trial-and-error, such an approach bears the danger of going beyond the therapeutic window, resulting in detrimental effects on cardiac function,[44] a danger that is typical for any new intervention. In this thesis it was shown that the alignment of transplanted MSCs determines their degree of spatial and functional integration with adjacent CMC fields, which was reflected as changes in conduction velocity. In the intact heart, the extracellular matrix is the main determinant of cell alignment.[45] However, in the damaged heart, particularly after myocardium infarction, this matrix undergoes extensive changes/remodellng and cellular spatial arrangement is disturbed especially in the border zones of the infarcted areas.[46] In most stem cell studies focusing on myocardial infarction, cells have been injected in the border zone and could therefore not be properly guided in their alignment. As a result structural and functional heterogeneity may increase locally leading to different conduction velocities in specific directions and areas, which could increase the pro-arrhythmic risk if the number of misaligned cells is high enough.[47] Additional guidance in the alignment of transplanted cells or tissue engineering techniques might overcome these potential dangers.[48] Furthermore, in chapter 6 it was shown that the number and engraftment patterns of transplanted MSCs affect pro-arrhythmic risk by two different mechanisms. While electrotonic interaction between MSCs and CMCs lead to partial depolarization of the CMCs, secretion of paracrine factors from MSCs results in prolongation of the cardiac action potential duration. Depending on the extent of these effects, which is directly related to the number and engraftment pattern of MSCs, the incidence of arrhythmias was strongly increased in MSC-CMC co-cultures. Although *in vivo* studies are needed to proof the relevance of these mechanisms in the intact organism, *in vitro* studies like the ones described in this thesis could help us in developing strategies to minimize the adverse effects related to MSC therapy for the heart and may also help to guide the development/improvement of cardiac cell therapy involving other (stem) cell types.

Nevertheless, virtually all current interventions in cardiovascular medicine target either symptoms or certain disease pathways, but do not actually heal the damaged heart. Despite the existing deficiencies in our knowledge and understanding of stem cells and their therapeutic potential, there is no denying in the positive impact regenerative medicine could have on patients suffering from cardiovascular diseases in either the near or distant future.

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SAMENVATTING

De algemene introductie van dit proefschrift, **Hoofdstuk I**, geeft de eigenschappen en de oorsprong van mesenchymale stamcellen (MSCs) weer. Tevens wordt de *in vitro* en *in vivo* capaciteit van MSCs om te differentiëren naar hartspiercellen beschreven. Hierbij wordt specifiek ingegaan op hoe differentiatie naar hartspiercel gedefiniëerd wordt en op de methoden die gebruikt worden om het proces van differentiatie in stamcellen te onderzoeken. Tenslotte, wordt er een overzicht gegeven van de klinische studies die gebruikmaken van MSCs om hartziekten te behandelen. Hierbij worden tevens de uitkomsten en conclusies van deze studies beschreven.

Het hoofddoel van dit proefschrift was dan ook de integratie van MSCs in hartspierweefsel en de differentiatie van MSCs naar hartspiercellen in deze omgeving kritisch te onderzoeken.

In **Hoofdstuk II** werd het differentiatie vermogen van MSCs die afkomstig waren uit humane (h) embryonale, foetale en volwassen bronnen, met elkaar vergeleken. Specifiek werd het vermogen om te differentiëren naar hartspiercel, gladde spiercel en endotheelcel onderzocht. Aangetoond werd dat de capaciteit van hMSCs om te differentiëren naar deze drie celtypen, direct gerelateerd was aan de leeftijd van de weefseldonor, waarbij het differentiatie vermogen van embryonale MSCs superieur was ten opzichte van de andere MSCs.

Hoofdstuk III evalueerde de effecten van ruimtelijke oriëntatie van getransplanteerde stamcellen op de functionele integratie met gekweekte hartspiercellen waarbij gebruik gemaakt werd van micro-electrode arrays. Gedurende 9 dagen co-cultuur met de hartspiercellen, differentiëerde een deel van de MSCs naar hartspiercel en afhankelijk van de vooraf gedefiniëerde ruimtelijke oriëntatie was de mate van electrische integratie significant verschillend.

De rol van gap junctie koppeling tussen gekweekte hartspiercellen en hMSCs bij het induceren van differentiatie naar hartspiercel van deze hMSCs werd bestudeerd in **Hoofdstuk IV**. Het genetisch blokkeren van de expressie van het gap junctie eiwit connexine 43 in hMSCs resulteerde in het verlies van vermogen om te differentiëren naar hartspiercel. Wanneer vervolgens de expressie van het connexine 45 eiwit in deze hMSCs verhoogd werd, keerde het differentiatie vermogen naar hartspiercel van deze cellen terug.

In **Hoofdstuk V** werd secondaire transductie van neonatale rat hartspiercellen onderzocht door deze te kweken in co-cultuur met humane cellen die getransduceerd werden met een lentivirale vector die codeerde voor een fluorescent eiwit. De resultaten toonden dat secondaire transductie in dit soort modellen frequent voorkomt. Hierdoor wordt de interpretatie van dit soort experimenten, waarbij slechts expressie van een fluorescent eiwit door een stamcel gebruikt wordt ter identificatie

van deze cel, sterk verstoord. In deze studie worden echter meerdere mogelijkheden beschreven om het plaatsvinden van secondaire transductie te voorkomen.

Hoofdstuk VI beschrijft de resultaten van een studie die zich gericht heeft op de rol van integratie patronen van hMSCs op de incidentie en eigenschappen van aritmieën in hartspiercelkweken. Niet alleen het percentage geïntegreerde hMSCs beïnvloedt het pro-aritmisch vermogen van de hartspiercelkweken, maar ook het integratiepatroon (geclusterd versus diffuus). Electrische koppeling tussen hMSCs en hartspiercellen en de secretie van bepaalde paracriene factoren dragen bij aan de pro-aritmische effecten die geobserveerd werden in deze co-culturen.

Naast de pro-aritmische effecten van getransplanteerde niet-exciteerbare cellen in hartspierweefsel werd bestudeerd maar ook de rol van endogene, niet-exciteerbare myofibroblasten. In **Hoofdstuk VII** werd bestudeerd of en op welke manier anti-proliferatieve behandeling van myofibroblasten in hartspiercelkweken het ontstaan van aritmieën kon voorkomen. De incubatie met cytostatica (mitomycine-c en paclitaxel) reduceerde de incidentie van re-entry aritmieën sterk door weefsel exciteerbaarheid in stand te houden.

Concluderend, het vermogen van hMSCs te differentiëren tot een hartspiercel neemt af met de donorleeftijd, dat op zichzelf ook een negatieve relatie heeft met het Cx43 expressie niveau. De expressie van Cx43 is essentieel bij het differentiatie proces van MSCs die afkomstig zijn van jonge donoren, maar het is niet een sturende factor bij cardiale differentiatie van MSCs afkomstig uit volwassen donoren. Naast de bovenstaande factoren, beïnvloeden ruimtelijke oriëntatie en integratie van MSCs de electrische integratie van deze cellen in het myocardiale weefsel. Dit kan leiden tot pro-aritmische effecten na stamcel therapie. De onderliggende pro-aritmische mechanismen zijn deels vergelijkbaar met die van cardiale myofibroblasten, die aanwezig zijn in het fibrotische hartspierweefsel.