Cell-based Cardiac Repair

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Cell-based cardiac repair
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Cell-based Cardiac Repair

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1 Introduction

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CLINICAL BACKGROUND

Each year approximately seven million people die of ischemic heart disease, making it one of the leading causes of mortality worldwide[1]. With increasing incidence of cardiac risk factors such as obesity, hypertension and diabetes, especially in developing countries, ischemic heart disease and its consequences will form a growing burden on society.
A major contributor to ischemic heart disease is myocardial infarction (MI). The erosion or rupture of a coronary atherosclerotic plaque can result in the acute obstruction of blood flow which in turn leads to a cascade of events in the ischemic area[2] (Figure 1). Deprivation of oxygen will cause cardiomyocytes to die via apoptosis or necrosis. Circulating inflammatory cells are drawn to the injured region due to the release of cytokines (including stromal-cell derived factor (SDF)-1α and monocyte chemoattractant protein 3[3]) and contribute to proteolysis and phagocytosis of cell debris. Subsequently, myofibroblasts invade the area and extracellular matrix is formed in the border zone and infarcted region, increasing the tensile strength of the infarct and thereby preventing rupture. New blood vessels start to appear in the border zone derived from pre-existing blood vessels and from migrated cells from the circulation[2]. Finally, since mammalian adult cardiomyocytes are unable to replace the large number of lost cardiomyocytes in the heart, a scar with a definitive nature is formed (Figure 1).

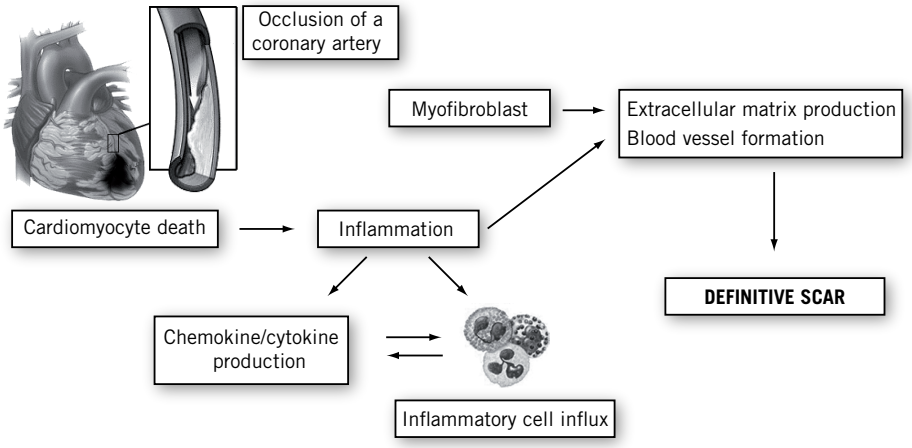

Figure 1: Myocardial infarction leads to a cascade of events that culminate in a definitive scar.

Important advances have been made in prevention and treatment of acute complications after MI and the extent of the infarct can be limited by thrombolysis and percutaneous coronary intervention (PCI). As a result, the number of patients that acutely die from MI

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has been reduced. However, patients that survive are prone to develop heart failure, a state that cannot be halted, it will gradually progress into a severely impaired cardiac function, and death is generally inevitable. The only therapy for heart failure that addresses the fundamental problem of cardiomyocyte loss is heart transplantation. However, this is restricted by donor availability and transplant rejection. If it were possible to reconstruct the myocardium by replacing dead cardiomyocytes and blood vessels through cell-based therapy, it would provide a powerful approach to treat cardiovascular disease.

SOURCES FOR CELL-BASED CARDIAC THERAPY

The ideal cell population for cardiac cell-therapy should be able to generate cardiomyocytes, as well as endothelial and smooth muscle cells to form new blood vessels. Newly formed cardiomyocytes have to integrate, couple electrically and metabolically to surviving host tissue, and beat in synchrony to prevent arrhythmias. Many different cell sources have been explored, and their differentiation characteristics *in vitro* and *in vivo* are extensively discussed in Chapter 2.

The most obvious cell-source to generate new heart muscle is healthy mature cardiomyocytes. However, replacing cardiomyocytes through *in situ* proliferation or transplantation of mature cardiomyocytes is unattainable due to their post-mitotic state and failure to survive ectopic transplantation[5]. In contrast, fetal cardiomyocytes still posses the ability to undergo several rounds of cell division. Multiple studies have shown that fetal cardiomyocytes can form grafts in the healthy mouse[6] and infarcted rat heart[5]. However, the use of human fetal cells is surrounded by ethical, political, and practical constraints.

The first progenitor cell strategy explored in an early stage of cell-based cardiac regeneration was the injection of autologous skeletal myoblasts into ischemic myocardium[7]. Myoblasts represent an appealing option for cardiac repair; they are resistant to ischemia, have a high proliferative capacity and could therefore be used in an autologous fashion[7]. Myoblasts differentiate into myotubes (but not cardiomyocytes) *in vivo*[8] and their capacity to improve ventricular function in animal experiments has been documented[9,10]. Unfortunately, the injection of skeletal myoblast in human clinical trials induced arrhythmias in some cases[11,12], which likely resulted from the inability of myotubes to couple electrically to surviving cardiomyocytes[9].

The field of cardiac regeneration was fueled by the finding of a cell population that appeared to differentiate into cardiomyocytes after injection into the infarcted heart[13]. In 2001, Orlic et al. showed that the injection of an eGFP-labeled subpopulation of bone marrow cells (lineage$^-$, c-kit$^+$), occupied over 60% of the infarcted mouse heart and restored cardiac function. EGFP cells were shown to express cardiac and vascular pro-

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teins, suggesting (trans)differentiation. Unfortunately in subsequent years, these spectacular results could not be confirmed[14-16]. Nevertheless, the use of bone marrow cells after acute MI was immediately translated to the clinic with an unprecedented speed. Although the results from the placebo controlled trials at 5-year follow-up show that the use of bone marrow-derived cells is safe, the effect on cardiac function has been small[17].

Ultimately, the best source of cells to repair the damaged myocardium may come from the heart itself. The finding of populations of adult cardiac-tissue derived progenitors (which will collectively be referred to as cardiovascular progenitor cells) located in the interstitial spaces in the myocardium[18] generated great enthusiasm. Cardiovascular progenitor cells with the ability to differentiate into cardiomyocytes, endothelial and smooth muscle cells have been isolated from the human heart, by 1) cardiac explant culture and subsequent formation of cardiospheres[19,20], 2) identification of the transcription factor islet-1[21], or 3) based on surface antigen expression of c-Kit[22] or a still unidentified protein cross-reacting with the mouse stem cell antigen-1 (Sca-1)[23-25]. It remains unclear if these populations are distinct cell populations or if they represent different developmental stages of the same precursor cell.

Unfortunately, at this point, the isolation and expansion to obtain sufficient numbers of cells takes too long to use these cells autologously in an acute clinical setting. Nevertheless, the finding that cardiovascular progenitor cells can differentiate into all required cell-types to reconstitute the heart *in vitro* and *in vivo*, and the fact that they can easily be isolated from small cardiac biopsies holds great promise for the future of cell-based cardiac therapy.

CONSIDERATIONS FOR CELL-BASED THERAPY

Several important questions need to be addressed before cell-based cardiac therapy becomes a reality and these are summarized in figure 2. The most obvious, as explained in the previous section, is the question which type of stem- or progenitor cell is the best candidate for therapy[26]. Myoblasts do not become cardiomyocytes and are arrhythmogenic, and the differentiation potential of bone-marrow derived cells is still uncertain. Embryonic stem (ES) cells are capable of forming all cell types in the adult body including cardiomyocytes[27-29], but the injection of undifferentiated ES cells leads to the formation of teratomas[30]. Moreover, the use of human ES cells is still heavily debated. These ethical issues may be overcome by the recently identified induced pluripotent stem (iPS) cells, a new method by which ES-like cells can be generated by reprogramming human adult fibroblasts[31,32]. Finally, human cardiovascular progenitor cell isolation and culture procedures are in the early development stages, but appear encouraging.

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A second challenge is determining the optimal delivery of the cells into the damaged area, with regard to the best time, method and location (Figure 2). Retention of cells is highly dependent on the delivery strategy: if cells are injected intramyocardially during open-chest surgery, many cells are lost through the vasculature[7], and when using intracoronary injections only few cells ultimately engraft[26]. Direct injection of cells in or near the infarcted tissue exposes them to an extremely hostile environment that is low in oxygen and highly inflamed, which may compromise cell survival (Figure 2).
Other approaches to distribute new cells in the damaged heart that are under investigation include intravenous injection. Subsequent to MI, the damaged myocardium produces chemoattracting factors (Figure 1) that can induce the migration of cells into the infarcted region. Using these intrinsic signals to recruit intravenously injected cardiovascular progenitor cells from the circulation to the injured myocardium may provide a promising alternative approach to intramyocardial transplantation. Importantly, since cells are carried to sites where the delivery of oxygen and nutrients still persists, it enhances the chance of survival of progenitor cells in the heart.
The final challenges in cell-based therapy will include the long-term engraftment and electromechanical integration into the host tissue, which will be essential for cardiac regeneration (Figure 2).

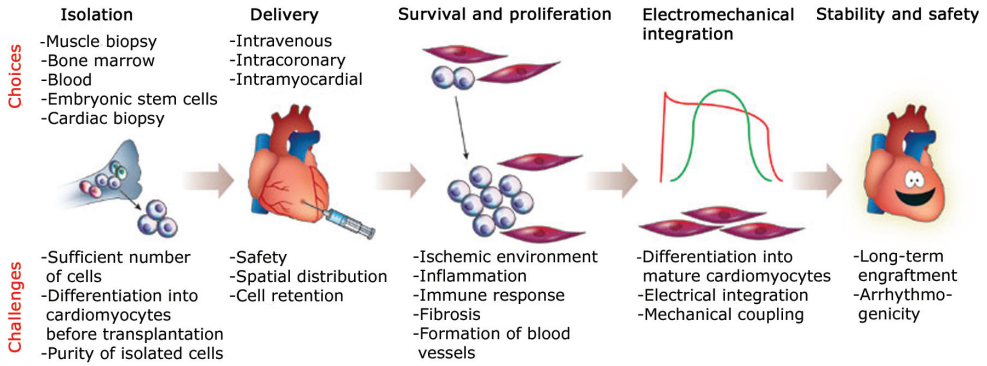

Figure 2: Summary of challenges for cell-based therapy (adapted from Segers et al.[33])

Generally, optimizing cell-based therapy in experimental models has focused on repairing the heart by providing new cardiomyocytes via cardiovascular progenitor cell delivery. However, the introduction of cells into the damaged area may also have other benefits on the surrounding myocardium. New cells may exert a paracrine effect which affects the surrounding tissue by altering the inflammatory or remodeling response[33]. Additionally, cells may induce angiogenesis which favors the survival of host myocardium, or activate

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endogenous stem cells[33]. For all these reasons, it is expected that cell-based therapy will have great potential in cardiac repair.

SCOPE OF THIS THESIS

The following chapters will describe several aspects of cell-based therapy to repair the injured heart. In **Chapter 2** an overview is provided of different types of stem- and progenitor cells and their characteristics. In **Chapter 3** a cardiovascular progenitor cell population isolated from the adult and fetal heart that we have termed cardiomyocyte progenitor cells (CMPCs) is described. Insight into the characteristics of CMPCs *in vitro* is given, combined with a detailed method for their isolation, culture and differentiation into cardiomyocytes. **Chapter 4** compares the CMPCs and CMPC-derived cardiomyocytes in their ability to form new cardiac tissue, and restore cardiac function.
An important aspect of healing after MI is the homing of mononuclear cells (MNCs) to the injured region, where they participate in vessel repair and the inflammatory response. Patients suffering from Hereditary Hemorrhagic Telangiectasia type 1 (HHT1) have vascular problems that may result from a decreased homing of MNCs. In **Chapter 5** we show that MNCs from HHT1 patients do not respond adequately to the homing signal SDF-1α. This is due to high levels of CD26, an inhibiting peptidase present on the surface of MNCs and other cells. Inactivating CD26 increased the recruitment of cells towards the ischemic myocardium and normalized the behavior of HHT1 MNCs *in vivo*.
In **Chapter 6**, we investigated if CD26 inhibition can be used to increase the response of CMPCs to SDF-1α, and thereby improve the recruitment of intravenously delivered CMPCs to the damaged heart.
One of the limitations of analyzing differentiation of cells *in vitro* is that the interaction between different cell types cannot be investigated. In **Chapter 7** a new *in vitro* model is presented that enables the analysis of differentiation of cells into a particular cell type, and determine the contribution of this cell-type to the (differentiation) process via cell-specific ablation. In **Chapter 8** we report the progress of transforming the *in vitro* system described in Chapter 7 to *in vivo* transgenic mouse models.
Finally, in **Chapter 9** the results and conclusions of the previous chapters are discussed, together with their future implications.

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REFERENCES

1. World Health Organization. The Atlas of Heart Disease and Stroke 2007;48-49.
2. Cleutjens JP, Blankesteijn WM, Daemen MJ, Smits JF. The infarcted myocardium: simply dead tissue, or a lively target for therapeutic interventions. Cardiovasc Res 1999;44(2):232-241.
3. Penn MS, Mangi AA. Genetic enhancement of stem cell engraftment, survival, and efficacy. Circ Res 2008;102(12):1471-1482.
4. Smits AM, van Vliet P, Hassink RJ, Goumans MJ, Doevendans PA. The role of stem cells in cardiac regeneration. J Cell Mol Med 2005;9(1):25-36.
5. Reinecke H, Zhang M, Bartosek T, Murry CE. Survival, integration, and differentiation of cardiomyocyte grafts: a study in normal and injured rat hearts. Circulation 1999;100(2):193-202.
6. Soonpaa MH, Koh GY, Klug MG, Field LJ. Formation of nascent intercalated disks between grafted fetal cardiomyocytes and host myocardium. Science 1994;264(5155):98-101.
7. Menasche P. Skeletal myoblasts as a therapeutic agent. Prog Cardiovasc Dis 2007;50(1):7-17.
8. Hagege AA, Carrion C, Menasche P, Vilquin JT, Duboc D, Marolleau JP, Desnos M, Bruneval P. Viability and differentiation of autologous skeletal myoblast grafts in ischaemic cardiomyopathy. Lancet 2003;361(9356):491-492.
9. Leobon B, Garcin I, Menasche P, Vilquin JT, Audinat E, Charpak S. Myoblasts transplanted into rat infarcted myocardium are functionally isolated from their host. Proc Natl Acad Sci U S A 2003;100(13):7808-7811.
10. Guo C, Haider HK, Shim WS, Tan RS, Ye L, Jiang S, Law PK, Wong P, Sim EK. Myoblast-based cardiac repair: xenomyoblast versus allomyoblast transplantation. J Thorac Cardiovasc Surg 2007;134(5):1332-1339.
11. Menasche P, Hagege AA, Vilquin JT, Desnos M, Abergel E, Pouzet B, Bel A, Sarateanu S, Scorsin M, Schwartz K, Bruneval P, Benbunan M, Marolleau JP, Duboc D. Autologous skeletal myoblast transplantation for severe postinfarction left ventricular dysfunction. J Am Coll Cardiol 2003;41(7):1078-1083.
12. Siminiak T, Kalawski R, Fiszer D, Jerzykowska O, Rzezniczak J, Rozwadowska N, Kurpisz M. Autologous skeletal myoblast transplantation for the treatment of postinfarction myocardial injury: phase I clinical study with 12 months of follow-up. Am Heart J 2004;148(3):531-537.
13. Orlic D, Kajstura J, Chimenti S, Jakoniuk I, Anderson SM, Li B, Pickel J, McKay R, Nadal-Ginard B, Bodine DM, Leri A, Anversa P. Bone marrow cells regenerate infarcted myocardium. Nature 2001;410(6829):701-705.
14. Balsam LB, Wagers AJ, Christensen JL, Kofidis T, Weissman IL, Robbins RC. Haematopoietic stem cells adopt mature haematopoietic fates in ischaemic myocardium. Nature 2004;428(6983):668-673.
15. Murry CE, Soonpaa MH, Reinecke H, Nakajima H, Nakajima HO, Rubart M, Pasumarthi KB, Virag JI, Bartelmez SH, Poppa V, Bradford G, Dowell JD, Williams DA, Field LJ. Haematopoietic stem cells do not transdifferentiate into cardiac myocytes in myocardial infarcts. Nature 2004;428(6983):664-668.
16. Nygren JM, Jovinge S, Breitbach M, Sawen P, Roll W, Hescheler J, Taneera J, Fleischmann BK, Jacobsen SE. Bone marrow-derived hematopoietic cells generate cardiomyocytes at a low frequency through cell fusion, but not transdifferentiation. Nat Med 2004;10(5):494-501.
17. Lipinski MJ, Biondi-Zoccai GG, Abbate A, Khianey R, Sheiban I, Bartunek J, Vanderheyden M, Kim HS, Kang HJ, Strauer BE, Vetrovec GW. Impact of intracoronary cell therapy on left ventricular function in the setting of acute myocardial infarction: a collaborative systematic review and meta-analysis of controlled clinical trials. J Am Coll Cardiol 2007;50(18):1761-1767.
18. Beltrami AP, Barlucchi L, Torella D, Baker M, Limana F, Chimenti S, Kasahara H, Rota M, Musso E, Urbanek K, Leri A, Kajstura J, Nadal-Ginard B, Anversa P. Adult cardiac stem cells are multipotent and support myocardial regeneration. Cell 2003;114(6):763-776.
19. Smith RR, Barile L, Cho HC, Leppo MK, Hare JM, Messina E, Giacomello A, Abraham MR, Marban E.

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Regenerative potential of cardiosphere-derived cells expanded from percutaneous endomyocardial biopsy specimens. Circulation 2007;115(7):896-908.
20. Messina E, De Angelis L, Frati G, Morrone S, Chimenti S, Fiordaliso F, Salio M, Battaglia M, Latronico MV, Coletta M, Vivarelli E, Frati L, Cossu G, Giacomello A. Isolation and expansion of adult cardiac stem cells from human and murine heart. Circ Res 2004;95(9):911-921.
21. Laugwitz KL, Moretti A, Lam J, Gruber P, Chen Y, Woodard S, Lin LZ, Cai CL, Lu MM, Reth M, Platoshyn O, Yuan JX, Evans S, Chien KR. Postnatal isl1+ cardioblasts enter fully differentiated cardiomyocyte lineages. Nature 2005;433(7026):647-653.
22. Bearzi C, Rota M, Hosoda T, Tillmanns J, Nascimbene A, De Angelis A, Yasuzawa-Amano S, Trofimova I, Siggins RW, Lecapitaine N, Cascapera S, Beltrami AP, D'Alessandro DA, Zias E, Quaini F, Urbanek K, Michler RE, Bolli R, Kajstura J, Leri A, Anversa P. Human cardiac stem cells. Proc Natl Acad Sci U S A 2007;104(35):14068-14073.
23. Smits AM, van Vliet P, Metz CH, Korfage T, Sluijter JP, Doevendans PA, Goumans MJ. Human cardiomyocyte progenitor cells differentiate into functional mature cardiomycoytes: an *in vitro* model for studying human cardiac physiology and pathophysiology. Nat Protoc 2009;4(2):232-243.
24. van Vliet P, Roccio M, Smits AM, van Oorschot AA, Metz CH, van Veen TA, Sluijter JP, Doevendans PA, Goumans MJ. Progenitor cells isolated from the human heart: a potential cell source for regenerative therapy. Neth Heart J 2008;16(5):163-169.
25. Goumans MJ, de Boer TP, Smits AM, van Laake LW, van Vliet P., Metz CH, Korfage TH, Kats KP, Hochstenbach R, Pasterkamp G, Verhaar MC, van der Heyden MA, de KD, Mummery CL, van Veen TA, Sluijter JP, Doevendans PA. TGF-beta1 induces efficient differentiation of human cardiomyocyte progenitor cells into functional cardiomyocytes *in vitro*. Stem Cell Res 2007;1(2):138-149.
26. Wollert KC, Drexler H. Clinical applications of stem cells for the heart. Circ Res 2005;96(2):151-163.
27. Xu C, Police S, Rao N, Carpenter MK. Characterization and enrichment of cardiomyocytes derived from human embryonic stem cells. Circ Res 2002;91(6):501-508.
28. Kehat I, Kenyagin-Karsenti D, Snir M, Segev H, Amit M, Gepstein A, Livne E, Binah O, Itskovitz-Eldor J, Gepstein L. Human embryonic stem cells can differentiate into myocytes with structural and functional properties of cardiomyocytes. J Clin Invest 2001;108(3):407-414.
29. Mummery C, Ward-van Oostwaard D., Doevendans P, Spijker R, van den Brink S, Hassink R, van der Heyden M, Opthof T, Pera M, Brutel de la Riviere A, Passier R, Tertoolen L. Differentiation of human embryonic stem cells to cardiomyocytes: role of coculture with visceral endoderm-like cells. Circulation 2003;107(21):2733-2740.
30. Nussbaum J, Minami E, Laflamme MA, Virag JA, Ware CB, Masino A, Muskheli V, Pabon L, Reinecke H, Murry CE. Transplantation of undifferentiated murine embryonic stem cells in the heart: teratoma formation and immune response. FASEB J 2007;21(7):1345-1357.
31. Takahashi K, Tanabe K, Ohnuki M, Narita M, Ichisaka T, Tomoda K, Yamanaka S. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell 2007;131(5):861-872.
32. Yu J, Vodyanik MA, Smuga-Otto K, Antosiewicz-Bourget J, Frane JL, Tian S, Nie J, Jonsdottir GA, Ruotti V, Stewart R, Slukvin II, Thomson JA. Induced pluripotent stem cell lines derived from human somatic cells. Science 2007;318(5858):1917-1920.
33. Segers VF, Lee RT. Stem-cell therapy for cardiac disease. Nature 2008;451(7181):937-942.

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2 The role of stem cells in cardiac regeneration

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ABSTRACT

After myocardial infarction, injured cardiomyocytes are replaced by fibrotic tissue, thereby promoting the development of heart failure. Cell transplantation has emerged as a potential therapy and stem cells may be an important and powerful cellular source. Embryonic stem cells can differentiate into true cardiomyocytes, making them in principle an unlimited source of transplantable cells for cardiac repair, although there are immunological and ethical constraints. Somatic stem cells are an attractive option to explore for transplantation as they can be used autologous, but their differentiation potential is more restricted than embryonic stem cells. Besides the use of skeletal myoblasts to replace damaged tissue, the other major sources of somatic stem cells used for basic research and in clinical trials originate from the bone marrow. The differentiation capacity of different populations of bone marrow-derived stem cells into cardiomyocytes has been studied intensively. The results are rather confusing and difficult to compare, since various isolation and identification methods have been used to determine the cell population studied. To date, only mesenchymal stem cells seem to form cardiomyocytes, and only a small percentage of this population will do so *in vitro* or *in vivo*. A newly identified cell population isolated from cardiac tissue, called cardiovascular progenitor cells, holds great potential for cardiac regeneration. Here we discuss the potential of the different cell populations and their usefulness in stem cell based therapy to repair the damaged heart.

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Stem cells

Cardiovascular disease is one of the leading causes of mortality in the western world[1]. Important contributing factors in the progression of heart failure are the loss of cardiomyocytes after myocardial infarction (MI) combined with the absence of an adequate endogenous repair mechanism. The injured myocardium is replaced by a fibrous scar resulting in progressive loss of ventricular function and ultimately, heart transplantation is the only effective treatment. However recently, stem cell based therapy has become a promising option to replace damaged cardiomyocytes. Stem cells hold a great potential for regenerative medicine, especially in replacing cells in tissues that hardly have any intrinsic renewal capacity, including the heart[2,3]. Over the past few years, several promising results have been reported, but many hurdles remain before stem cells can actually be used to treat patients with a damaged heart. Questions remain on which cell type to use, if cells need to be differentiated into cardioblasts or cardiomyocytes before transplantation, and how this can be achieved. Eventually, questions need to be answered concerning the best methods to deliver these cells to the patients, at what time-point after injury and if allogenic cells can be used.

Embryonic Stem Cells

Since the first isolation of embryonic stem (ES) cells of human origin, their characteristics, differentiation potential, and possible suitability for clinical therapy have been studied and discussed. ES cells are derived from the inner cell mass of blastocyst stage embryos. One important characteristic of ES cells *in vitro* is their capacity to replicate indefinitely. This makes it feasible to culture them on a large scale, a prerequisite for cell transplantation therapy. Moreover, ES cells are pluripotent and have the ability to differentiate into all cells of the mammalian body. Their pluripotency was demonstrated by injection of undifferentiated human embryonic stem (hES) cells into immunodeficient mice. This resulted in benign teratocarcinomas harboring cells of all three germ layers[4]. The ultimate goal of investigating ES cell-characteristics and their growth potential is to use these cells or their derivatives as stem cell transplants in regenerative medicine. For application in cardiac repair, ES cells need to be able to differentiate into cardiomyocytes. *In vitro* this differentiation can be achieved by growing ES cells in cell aggregates termed embryoid bodies (EBs)[5], which are multilayered spheres of differentiating cells. Interestingly, after culturing EBs for several days, clusters of cells within the EBs can be identified that start to contract spontaneously. These cell clusters harbor a panel of myocardial phenotypes based on their expression of cardiac-specific transcription factors, genes, and proteins[6,7]. It is also possible to induce differentiation of ES cells into cardiomyocytes in a monolayer culture, by co-culturing ES cells with either mature cardiomyocytes or endodermal cells or using stimulating agents like 5-azacytidine, retinoic and ascorbic

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acid or growth factors, like members of the TGFβ, FGF, IGF, and PDGF families[7,8].
Murine ES cells have been tested for their *in vivo* capacity to replace damaged cardiomyocytes in an experimental model for MI. Murine ES cells, engineered to express enhanced cyan fluorescent protein (ECFP) by a cardiomyocyte-specific promoter, were injected into rats 8 weeks after an MI was induced. Newly formed ES cell-derived cardiomyocytes had integrated into the injured host myocardium and revealed a normal architecture. A functional benefit was already observed 3 weeks after injection[9]. HES cell-derived cardiomyocytes have also been tested *in vivo*. In a porcine model of conduction block it was demonstrated that hES cells electromechanically integrated into the myocardium and revealed pacemaker activity[10]. In another study, hES-derived cardiomyocytes were injected into the infarcted mouse heart, and compared to the hES-derived non-cardiomyocyte population[11]. At mid-term follow-up the cardiomyocytes had a more positive effect on cardiac function than the non-cardiomyocyte fraction, but there was no longer a significant difference at 12 weeks post-surgery. Although there was no additional value of cardiomyocytes compared to other cell-types, it is important to note that both cell-receiving groups performed better than the vehicle-receiving control group with regard to cardiac function. These results indicate that ES cells can contribute to an enhanced cardiac function, but it remains unclear if this is through the formation of new viable cardiac tissue.
There are some obstacles to be taken before ES cells become a realistic option for cell based therapy. First of all, although ES cells have the potential to replicate indefinitely, such conditions and examples are rare and no large scale production of hES cells is yet possible. Secondly, the use of hES cells is still a topic of intense ethical debate. Thirdly, hES cells are mainly derived from *in vitro* fertilization surplus embryos meaning that they are of allogenic origin, which may result in rejection by the recipient's immune system. We would need many different ES cell lines to be able to match every recipient. This problem may be overcome by the use of the recent breakthrough in generation of induced pluripotent stem (iPS) cells. In 2006, two research groups described the reprogramming of somatic cells to a pluripotent state by retrovirally expressing four transcription factors (Oct3/4, Sox2 combined with c-Myc and Klf4[12] or NANOG and LIN28[13]). IPS cells behaved similarly to human ES cells, forming cell types of all three germ layers *in vitro* and in teratomas. Several studies have since shown that both human and mouse iPS cells can form functional cardiomyocytes[14,15]. IPS cells provide the possibility of using an autologous cell source for cell-based therapy, but one major obstacle that prevented the clinical applicability of iPS cells has been the use retroviruses that integrated into the host genome. However, very recently several laboratories described the induction of iPS cells by using virus-free systems[16,17], which could quicken the application of iPS cells in the future.

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Somatic Stem Cells

In various tissues of the adult body, multipotent stem cells exist that can give rise to new stem cells (self-renewal) and differentiated cells, necessary for maintenance or for the restoration of damaged tissue. Somatic stem cells were generally believed to differentiate only into cells characteristic of the tissue wherein they reside. However, recent experiments have suggested that somatic stem cells can differentiate into cells other than those of their tissue of origin. Even though their exact origin remains debatable, adult bone marrow, fat, liver, skin, brain, skeletal muscle, pancreas, lung, heart, and peripheral blood may possess stem or progenitor cells with the capacity to transdifferentiate[6]. Due to this developmental plasticity, somatic stem cells may have great potential in autologous regenerative medicine, circumventing problems like rejection and the ethically challenged use of ES cells[18].

The most widely studied types of somatic stem cells are the bone marrow-derived hematopoietic stem cells (HSCs) and mesenchymal stem cells (MSCs) (Table 1). HSCs give rise to cells of the blood- and immune system and can also be found in umbilical and peripheral blood and in the fetal hematopoietic system. HSCs are well characterized, highly plastic, relatively easy to isolate, and have been used for decades in bone marrow transplantations to treat patients with inherited blood disorders or blood-related diseases like leukemia[19].

In bone marrow, the HSCs are surrounded by stromal cells, which contain the MSCs. These cells represent only a small fraction of the nucleated cells in the bone marrow, and are almost 10-fold less abundant than HSCs. MSCs are multipotent cells that are easily isolated from the bone marrow using their capacity to adhere to culture-plastic. These adhering fibroblast-like cells from the bone marrow form a heterogeneous population that can differentiate into osteoblasts, chrondoblasts, adipocytes and myocytes *in vitro* and *in vivo*. This plasticity of MSCs suggests their suitability for cell therapy. MSCs have already been tested in the clinic for treatment of bone disorders like osteogenesis imperfecta and other diseases[20].

A more primitive adherent stem cell, the multipotent adult progenitor cell (MAPC) or mesodermal progenitor cell, co-purifies with MSCs. This subpopulation of the heterogeneous MSCs, holds multipotent potential as well. Human and rodent MAPCs have been shown to differentiate into ectodermal, mesodermal, and endodermal cell types *in vitro* and *in vivo* even from a single cell[21]. However, MAPCs emerge only after prolonged culture of MSCs, after which an increased growth speed is observed, suggesting that the existence of these cells may be cell culture-related. It is not yet known whether MAPC exist as such *in vivo* or if they are the result of dedifferentiation of an MSC-like cell into a cell with greater potential. So far, the isolation and culture protocol makes it difficult to use these cells for stem cell-based therapy.

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Both HSCs and MSCs comprise different cell populations, some of which have been proven to be more multipotent than others. Their ability to differentiate into skeletal and cardiac muscle, brain, liver, kidney, gastro-intestinal tract, lung, or skin cells both *in vitro* and/or *in vivo* makes them ideal candidates in the search for cells that can contribute to the restoration of cardiac muscle[22]. In this review we will discuss experiments in which somatic stem cells have been observed to differentiate into cardiomyocytes both *in vitro* and *in vivo*, which factors influence their development, what is already known from clinical trials in which stem cell-derivatives have been used, and which stem cells, in our opinion, have the greatest potential to be used for myocardial repair.

In vitro differentiation of somatic stem cells

To understand the process of somatic stem cells differentiating into cardiomyocytes, numerous *in vitro* experiments have been performed to determine which cell population has the potential to become cardiac myocytes, and to elucidate which factors and techniques influence this differentiation (Table 1).

Of all populations of bone marrow-derived cells, only MSCs have been shown to differentiate into cardiomyocytes *in vitro*[8]. Isolation of MSCs is mostly accomplished by using their ability to adhere to plastic surfaces, a feature that other populations isolated from the bone marrow do not possess. In culture, MSCs have a fibroblast-like morphology and retain the potential to differentiate into several mesenchymal cell lineages. Many different cell surface markers have been observed, but generally, MSCs are negative for the hematopoietic markers CD34 and CD45[20].

To establish an *in vitro* model for studying cardiomyocyte differentiation, a cardiomyogenic (CMG) cell line was isolated. An immortalized murine marrow stromal cell line was treated *in vitro* with 5-azacytidine[23]. Subclones containing spontaneously beating cells were isolated and studied for cardiomyogenic characteristics. Before treatment, the cells had a fibroblast-like morphology, but after 5-azacytidine administration the cells began to beat and showed myotube-like structures and myofilaments. They stained positive for the cardiomyocyte-specific markers sarcomeric myosin, desmin, and actinin and cardiomyocyte-specific gene and transcription factor expression was detected. During electrophysiological studies, differentiated cells revealed cardiomyocyte-like action potentials, thereby indicating that functional cardiomyocytes could be derived from MSCs *in vitro*[23]. Since adrenergic and muscarinic receptors are involved in mammalian cardiac function, the CMG cell line was tested for expression of these receptors. Both up- and downregulation of several receptors before or after 5-azacytidine treatment was observed in the CMG cells and administration of specific stimulants and/or inhibitors modified their downstream signaling and beating rate[24].

The differentiation of MSCs into cardiomyocytes *in vitro* has not only been achieved with

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MSCs isolated form murine bone marrow, but also with MSCs from rabbit[25] and human fatty tissue[26], and with rat[27,28] and human[29] bone marrow. As in experiments with murine MSCs, the human bone marrow-derived MSCs were thoroughly screened for surface antigen expression and exposed to 5-azacytidine. The differentiated cells stained positive for several cardiomyocyte-specific markers and showed a myogenic morphology and functionality[29].
Another way of differentiating cells into cardiomyocytes is by simulating the cardiac environment *in vitro*. This can be achieved by co-culturing (stem) cells of interest with adult cardiomyocytes. Co-culture of rat bone marrow-derived stromal cells with rat neonatal cardiomyocytes induced cardiac-specific marker expression and spontaneous beating in some of the stromal cells[30]. This suggested that cell-cell interaction, electrical and/or mechanical stimulation, and/or undetermined growth factors from cardiomyocytes may stimulate differentiation in the rat stromal cells. However, cell fusion, resulting in chimeric cells containing characteristics of both cell types, was not ruled out.
The *in vitro* experiments with MSCs suggest that these cells have the capacity to become cardiomyocytes, and thereby may contribute to myocardial repair. However, it is important to determine the *in vivo* behavior of MSCs before they can be considered to be used as a therapeutic means.

Somatic stem cells and their *in vivo* differentiation capacity

Bone marrow

The cardiomyogenic properties of bone marrow-derived cells *in vivo* were observed for the first time by Bittner *et al*. After a sex-mismatched bone marrow transplantation in female dystrophic *mdx* mice suffering from cardiac muscle degeneration, Y-chromosome containing cardiomyocytes had integrated into the myocardium. This indicates that circulating bone marrow-derived cells can be recruited to the injured heart and differentiate into cardiomyocytes[31].
To further understand the capacity of bone marrow cells to differentiate into cardiomyocytes and repair the injured myocardium, stem cells were instantly delivered to the demanding area by injecting cells directly into the myocardium or coronary arteries. Murine GFP-tagged bone marrow cells were injected into the myocardium subsequent to coronary artery ligation. After 28 days, GFP^+ cells expressing hematopoietic markers were detected in the cardiac muscle but no differentiation into cardiomyocytes was observed[32]. Few animal studies have been performed to investigate the possibilities of injecting crude bone marrow into the myocardium (Table 1). In most cases, the bone marrow mononuclear cell fraction, harboring most of the stem and progenitor cells, was cultured *in vitro* before injection. This will inevitably lead to the selection of either HSCs or MSCs. The population of cells with cardiomyogenic properties likely represents only a small fraction of total bone marrow.

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Hematopoietic stem cells

Bone marrow-derived HSCs have been investigated for their differentiation potential *in vivo* (Table 1). The first indication that HSCs may participate in cardiac regeneration came from Jackson *et al.*. They isolated a specific HSC population called the side population, and transplanted these cells into lethally irradiated mice. The donor cells were $CD34^{-}$/low, c-Kit^{+}, Sca-1^{+} and tagged with LacZ. Subsequently, the transplanted mice were used in a myocardial ischemia-reperfusion model, and hearts were analyzed after 2 and 4 weeks. Although their prevalence was not very high, donor-derived cardiomyocytes were found, primarily in the peri-infarct zone, demonstrating the cardiomyogenic ability of circulating HSCs[33]. Direct injection of HSCs into the infarcted myocardium has also been investigated by Orlic and colleagues. After ligating the coronary artery, a population of GFP-tagged Lin^{-}, c-Kit^{+} cells was injected into the contracting wall bordering the infarcted area. After 9 days, 40% of the mice showed regeneration of the cardiac muscle. Approximately 68% of the infarcted area was occupied by newly formed myocardium. Donor cells were shown not only to differentiate into cardiomyocytes but also to form endothelial cells and fibroblasts. Evidence for the restoration of the myocardium was further supported by a prolonged survival of the mice and a recovery of cardiac function[34].

Although these studies demonstrate that different populations of HSCs appear to have a very high capacity both in homing to and regeneration of the damaged myocardium, some groups argue otherwise. Several investigators have tried to repeat these promising results and injected GFP labeled Lin^{-}, c-Kit^{+} cells into the ischemic myocardium as well[32,35]. Others used a genetic approach injecting HSCs containing LacZ under control of a cardiac specific promoter[36]. Hearts were investigated relatively short after MI (+/- 10 days) or at later time points (30 days to 6 weeks). All these studies detected donor cells in the heart, but these cells had not differentiated into cardiomyocytes. Instead, at all time-points, the donor cells expressed hematopoietic markers. It should be noted that Balsam et al. found a small but significant increase in cardiac function 6 weeks after MI[35]. Therefore, it remains unclear what the potential morphological and physiological contribution of HSCs is to the regeneration of the myocardium.

Mesenchymal stem cells

MSCs have been studied extensively for their *in vivo* cardiomyogenic potential, especially since they have the capacity to differentiate into cardiomyocytes *in vitro* (Table 1). In the majority of *in vivo* experiments MSCs were selected based on their ability to adhere to plastic and not on the basis of specific markers, making it difficult to directly compare the results. Wang and colleagues show that murine MSCs participate in the formation of new cardiomyocytes in the normal, uninjured heart. Starting 4 weeks after the injection of *in vitro*-

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expanded, labeled MSCs into the healthy heart, donor cells expressing cardiac markers were detected. These cells also expressed connexin 43, suggesting they coupled with surrounding cells[37]. The same *in vivo* potential has been demonstrated for human MSCs, which were injected into the heart of SCID mice. Although the human LacZ-tagged cells were only present in a small percentage (0.44%), the engrafted cells did express cardiac markers[38].

In addition to the use of healthy animals, MSCs have also been injected into the myocardium of experimental models for cardiac damage. Autologous MSCs labeled with BrdU were injected into the left ventricle (LV) of rats 3 weeks after myocardial cryoinjury. Transplanted MSCs were identified in all animals 8 weeks after injury. Immunohistochemistry revealed muscle-like BrdU positive cells expressing troponin I and myosin heavy chain. Moreover, injection of MSCs pre-treated with 5-azacytidine lead to a decreased scar area and a thicker LV free wall. The animals injected with pre-treated cells also had a decreased LV chamber size/body weight and improved cardiac function compared to controls[28].

Interestingly, rat MSCs transduced with a virus encoding for Akt, an anti-apoptotic gene prolonging cell survival, prevented the pathological remodeling of the rat LV after infarction. Approximately 80% of the injured myocardium regenerated and cardiac function was completely normalized[39], demonstrating the great potential of these MSCs.

Mobilization of bone marrow-derived stem cells

Another possibility to deliver stem cells to the heart besides injection may be by increasing the number of circulating endogenous stem cells, assuming they will migrate and home to the injured area.

The first indication that there is a circulating pool of stem cells that participate in the regeneration of cardiac muscle came from analyzing post-transplant organs of sex-mismatched heart transplantations. Examination of the female donor hearts revealed cardiomyocytes and vascular cells derived from the male recipients. These cells presumably derived from the bone marrow or peripheral blood of the heart transplantation recipient, and may embody a population of endogenous circulating stem cells. Additionally, Y-chromosome containing primitive cells, expressing Sca-1, MDR-1 and c-Kit were sporadically observed[40]. These cells could represent a population of bone marrow-derived undifferentiated stem cells residing in the heart. Although the investigators claimed that in heart transplantation patients 9% of the cardiomyocytes were recipient-derived, others have not been able to confirm these numbers and revealed a much lower contribution of recipient-derived stem cells to the cardiomyocyte population (0.016%)[41]. These reports did however raise the interest for studying endogenous stem cell mobilization in general but also after myocardial damage.

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Mobilizing stem cells can be achieved by administering cytokines like granulocyte-colony stimulating factor (G-CSF) and stem cell factor (SCF). G-CSF and SCF cause an increase in the release of stem cells from the bone marrow into the peripheral blood circulation. If these circulating stem cells can home to the myocardium and differentiate into cardiomyocytes, the administration of G-CSF and/or SCF could contribute to myocardial regeneration. Therefore, it was determined whether increasing the number of circulating stem cells by administering these cytokines led to more regeneration after MI. Orlic *et al*. injected G-CSF and SCF into mice starting 5 days prior to the induction of MI and continued until 3 days after. At 27 days, the infarct size was decreased by 40%, new cardiomyocytes were formed and cardiac function increased; this was combined with an increased survival of the mice[42]. These positive results readily led to assessment of cytokine-treatment in primates. Norol et al. ligated the circumflex coronary artery in baboons. Treatment with SCF and G-CSF was initiated either before or after the myocardial damage. Although the infarct size remained unchanged, the treatment did enhance the perfusion of the infarcted area, indicating a contribution of circulating stem cells to vessel formation, but not to myocardial repair[43]. The reason for this variation in results is unclear but may be a consequence of an interspecies difference in reaction to the administered drugs.

In the mobilization experiments described, it has been impossible to determine retrospectively which population of bone marrow-derived cells is responsible for the observed effects. Recently, Kawada *et al*. showed that this population is mainly the MSCs[44]. Mice were lethally irradiated and either EGFP-tagged crude BM, single HSCs that were $CD34^-$, c-Kit^+, and Sca-1^+, or clonal MSCs from the CMG cell-line described above were transplanted. MI was induced and mice were treated with G-CSF. After eight weeks, $EGFP^+$-$actinin^+$ cells were present in the hearts of the crude BM group; these cells were also found in the MSCs but not in the HSC transplanted animals.

Cardiac regeneration in non-ischemic cardiac disease

The majority of the *in vivo* experiments have focused on the capacity of stem cells to restore regional ischemic injury in the heart after, for example, ligating the coronary artery or inducing cryoinjury. However, chronic heart failure can be caused not only by ischemic injury but also by diffuse cardiomyopathy. In contrast to ischemic forms of cardiac disease, which are amendable by procedures like revascularization and remodeling operations, non-ischemic cardiomyopathies can ultimately only be treated by heart transplantation. Therefore, the delivery of new viable cardiomyocytes through stem cell therapy could offer a less invasive therapeutic option.

By using a doxorubicin (DOX) induced model for cardiomyopathy in rats[45], the potential

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role of stem cells has been investigated. DOX is an anthracycline antibiotic and antineoplastic agent used in chemotherapy. One familiar side effect of prolonged DOX-therapy is the development of congestive heart failure, which is attributed to cardiomyocyte apoptosis due to increased oxidative stress[46]. A beneficial effect on this diffuse form of cardiac injury has been reported after the injection of allogenic unpurified bone marrow cells[47], injection of bone marrow mononuclear cells[48], and after the early administration of G-CSF[49]. Positive effects were observed on cardiac function, cardiomyocyte replacement, and survival rate, making stem cell transplantation an interesting option for treatment.

Cardiovascular progenitor cells

Until recently the heart has been considered a terminally differentiated organ, excluding the need to search for a tissue specific cardiac stem cell. The major response to myocardial damage was thought to be hypertrophy of still viable cardiomyocytes. This dogma has been challenged by recent findings of cycling myocytes undergoing mitosis and cytokinesis under both physiological and pathological conditions[50]. That would imply that there is a population of stem cells or cardiovascular progenitor cells (CPCs) either resident in the mammalian heart or recruited from the circulation from which new myocytes can be derived. Identifying this cardiomyogenic cell population is the subject of ongoing investigation.

Hierlihy and colleagues presented the first evidence for the existence of a putative CPC population in the adult heart. They showed that a specific cell type, the side-population, could be isolated from the adult rat heart and cultured *in vitro*. The cells showed stem cell-like behavior and they were restricted to differentiate into the cardiomyocyte lineage (Table 1), suggestive of CPCs[51]. Further support for the existence of CPCs, came from recent studies where small cell clusters were observed in the interstitium of the adult rat heart. These clusters contained Lin^-, c-Kit^+ cells and many of these cells expressed markers for cell proliferation, and transcription factors associated with early cardiac development like GATA-4 and Nkx2.5. When isolated, these cells showed *in vitro* properties of stem cells. They were self-renewing, clonogenic, and multipotent, giving rise to myocytes, smooth muscle, and vascular cells, but were unable to contract *in vitro*. Furthermore, when these cells were injected into the border zone of an MI, they were able to forming a large area of new myocardium, containing CPC-derived myocytes and blood vessels[52].

The same potential has been shown for Sca-1^+ cells isolated from murine adult heart, which had an expression profile comparable to c-Kit^+ cells. Upon oxytocin[53] or 5-azacytidine[54] stimulation *in vitro*, the cells started to express cardiac transcription factors, showed sarcomeric structures and formed spontaneously beating clusters. Various ago-

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nists and antagonists could affect the beating rate of the differentiated cells[53]. When freshly isolated murine Sca-1$^+$ cells were injected intravenously into mice after ischemia-reperfusion, they were shown to target the border-zone of the injured myocardium and differentiate into cardiomyocytes, with and without fusing with host cells[54].

In the human adult heart, clusters of progenitor cells have been identified as well. These cells expressed stem cell markers like, c-Kit and MDR-1 and Sca-1 –like protein and harbored telomerase activity, which is only present in replicating cells. The *in vivo* relevance of these CPCs was emphasized by the fact that their number increased more than 13-fold in the hypertrophied myocardium of aortic stenosis patients[55]. The cell clusters revealed stem cell markers on cells in different stages of cardiomyocyte differentiation, which was determined by both early and late cardiac markers. This suggests the existence of a lineage commitment of stem cells towards becoming cardioblast and then cardiomyocytes in the heart. Very recently it has become evident that CPCs can be isolated from very small tissue samples of human myocardium. These cells were expanded many-fold *in vitro* without losing their differentiation potential. Cells were shown to differentiate into cardiomyocytes and vascular cells[56], providing new options for autologous transplantation of CPCs.

The origin of these primitive cells is still unclear, they can be either cells that have resided there from fetal life onwards or they could come from the bone marrow or circulation. As explained earlier, sex-mismatched heart transplantation patients reveal recipient-derived Y-chromosomes in the nuclei of some cardiomyocytes[40]. If the CPCs as discussed above reached the myocardium through the circulation, then they have been resident in the myocardium long enough to adjust their phenotype to the cardiac specific environment, since up to date, no cells have been isolated from the circulation with CPC specific characteristics and capacity.

Clinical Trials

The encouraging results of tissue regeneration by stem cells in animal models for cardiac disease led to the first clinical studies (Table 1; for a detailed overview see [57]). In the TOPCARE-AMI study patients were randomized to receive intracoronary infusion of either circulating progenitor cells, which mainly comprises endothelial progenitor cells, or crude bone marrow cells. At one-year follow-up an increase in LV ejection fraction, a decrease in end-systolic volumes, and a reduced infarct size was observed, without any differences between the two cell groups. During the follow-up, practically no cardiovascular events had occurred, demonstrating that the procedure of intracoronary infusion of cells is safe[58].

Another large, long-term trial has been performed by Perin *et al.*. MI Patients were given

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Table 1: Differentiation of somatic stem cells into cardiomyocytes.

Type of induction		Key references on cardiomyocyte differentiation experiments			
		BMC	HSC	MSC	Cardiac derived cells
In vitro	Spontaneous				Beltrami *et al.*[52] Messina et al.[56 $]
	Stimulation with 5-azacytidine/ oxytocin			Makino *et al.*[23] Hakuno *et al.*[24] Rangappa *et al.*[25] Xu *et al.*[29] Liu *et al.*[65 †]	Matsuura *et al.*[53] Oh *et al.*[54]
	Co-culturing		Hierlihy *et al.*[51]	Fukuhara *et al.*[30]	Messina *et al.*[56]
In vivo	Direct injection of undifferentiated cells into myocardial injury	Ishida *et al.*[48] Nygren *et al.*[32]	Nygren *et al.*[32] Orlic *et al.*[34] Balsam *et al.*[35 #] Murry *et al.*[36 #]	Wang *et al.*[37] Toma *et al.*[38] Mangi *et al.*[39]	Beltrami *et al.*[52] Messina *et al.*[56 $]
	Direct injection of stimulated cells into infarct area	Tomita *et al.*[28]			
	Integration of circulating cells	Bittner *et al.*[31] Orlic *et al.*[42] Agbulut *et al.*[47] Tomita *et al.*[49] Balsam *et al.*[35 #]	Jackson *et al.*[33] Nygren *et al.*[32] Kawada *et al.*[44]	Kawada *et al.*[44]	Oh *et al.*[54]
Clinical *	Direct injection into myocardial injury	Perin *et al.*[59] Assmus *et al.*[66] Wollert *et al.*[60]			
	Integration of circulating cells	Kang *et al.*[61] Quaini *et al.*[40] Laflamme *et al.*[41]			

† Rat MSCs did not differentiate into cardiomyocytes by 5-azacytidine treatment.
# Donor-derived cells did not differentiate into cardiomyocytes.
$ Clusters of cells, but not independently grown cells, showed cardiomyocyte differentiation.
* Although improvement of cardiac function was shown, donor-derived cardiomyocytes could not be detected.

an injection of bone marrow cells into ischemic viable myocardium and controls were treated equally except for the actual injection. The cardiac characteristics were monitored after 2, 6 and 12 months. There was a significant improvement in myocardial perfusion and functional capacity in treated patients compared to controls, and at 12 months there was a sustained improvement in exercise capacity[59].
One of the first randomized, controlled trials was performed by Wollert and colleagues.

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Sixty patients with acute MI and successful primary intervention were randomized to receive either autologous bone marrow cells by intracoronary infusion or optimal medical therapy. At 6 months follow-up patients that had received a bone marrow cell infusion showed a significant improvement of left ventricular function compared to controls[60]. We should, however, be cautious to directly translate the positive effects observed in animals to patients. In a study were G-CSF was administered to patients with MI who underwent coronary stenting, investigators noted an unexpectedly high rate of in-stent restenosis and stopped the trial prematurely[61]. This emphasizes the need for more research before the safety of stem cell-based therapy can be guaranteed.

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CONCLUSION

Stem cell based therapy for treating cardiac disease is a potentially powerful therapy. Animal and clinical studies clearly show that stem cells may improve cardiac function after a heart attack, but we still not have identified the optimal population of cells to use. Although the first beneficial effects of stem cell transplantation have been attributed to HSCs, accumulating evidence suggests that, although cardiac function seems to improve in some of the studies, these cells do not differentiate into cardiomyocytes. MSCs, on the other hand appear to possess cardiomyogenic properties both *in vitro* and *in vivo*. Besides improving cardiac function, MSCs were shown to be able to increase the ventricular wall mass. CPCs, however, may prove to be a very useful population in cardiac regeneration, especially if these cells can also be induced to proliferate and differentiate *in vivo*.

A more profound understanding of stem cells and CPCs, their exact developmental origin and characteristics will demonstrate the potential of cell-based therapy. Investigation of gene expression profiles will be necessary to manipulate genetic pathways that influence cell survival, renewal, differentiation, and migration. Cardiomyocyte precursor cells have been isolated from the embryonic mouse heart at different developmental stages and have been subjected to microarray analysis. This resulted in the identification of sets of cardiac development stage-specific and stage-independent gene expression patterns[62]. This transcriptional profile will help understanding the necessary molecular regulation of cardiac differentiation pathways in stem and progenitor cells and predict their potential in regenerative medicine.

Furthermore, the mathematics of stem cell transplantation would suggest that differentiation maybe not be the key mechanism explaining the beneficial effects observed after stem cell injections. Potentially, cytokines and growth factors from stem cells or activated upon cell injection could be more relevant[63,64]. Finally, complete regeneration of the cardiac muscle can not be achieved by solely replacing cardiomyocytes, it is also necessary to create new blood vessels. Therefore, it may prove useful to either co-transplant endothelial progenitor cells or to transplant a cell population that can differentiate into both cardiomyocytes and endothelial cell.

Although the step to the clinic has been taken rather quickly, it is shown that cell transplantation can have a positive effect on cardiac function. More research is needed to determine the long-term outcome and assess the possible associated risks.

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REFERENCES

1. American Heart Association. American Heart Association-Heart Disease and Stroke Statistics - 2004 Update. 2003.
2. Hassink RJ, Dowell JD, Brutel de la Riviere A, Doevendans PA, Field LJ. Stem cell therapy for ischemic heart disease. Trends Mol Med 2003;9(10):436-441.
3. Zwaginga JJ, Doevendans P. Stem cell-derived angiogenic/vasculogenic cells: possible therapies for tissue repair and tissue engineering. Clin Exp Pharmacol Physiol 2003;30(11):900-908.
4. Thomson JA, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, Marshall VS, Jones JM. Embryonic stem cell lines derived from human blastocysts. Science 1998;282(5391):1145-1147.
5. Doevendans PA, Kubalak SW, An RH, Becker DK, Chien KR, Kass RS. Differentiation of cardiomyocytes in floating embryoid bodies is comparable to fetal cardiomyocytes. J Mol Cell Cardiol 2000;32(5):839-851.
6. Passier R, Mummery C. Origin and use of embryonic and adult stem cells in differentiation and tissue repair. Cardiovasc Res 2003;58(2):324-335.
7. Gepstein L. Derivation and potential applications of human embryonic stem cells. Circ Res 2002;91(10):866-876.
8. Heng BC, Haider HK, Sim EK, Cao T, Ng SC. Strategies for directing the differentiation of stem cells into the cardiomyogenic lineage in vitro. Cardiovasc Res 2004;62(1):34-42.
9. Hodgson DM, Behfar A, Zingman LV, Kane GC, Perez-Terzic C, Alekseev AE, Puceat M, Terzic A. Stable benefit of embryonic stem cell therapy in myocardial infarction. Am J Physiol Heart Circ Physiol 2004;287(2):H471-H479.
10. Kehat I, Kenyagin-Karsenti D, Snir M, Segev H, Amit M, Gepstein A, Livne E, Binah O, Itskovitz-Eldor J, Gepstein L. Human embryonic stem cells can differentiate into myocytes with structural and functional properties of cardiomyocytes. J Clin Invest 2001;108(3):407-414.
11. van Laake LW, Passier R, Monshouwer-Kloots J, Verkleij AJ, Lips DJ, Freund C, den Ouden K, Ward-van Oostwaard D, Korving J, Tertoolen LG, van Echteld CJ, Doevendans PA, Mummery CL. Human Embryonic Stem Cell-Derived Cardiomyocytes Survive and Mature in the Mouse Heart and Transiently Improve Function after Myocardial Infarction. Stem Cell Research 2007;1(1):9-24.
12. Takahashi K, Tanabe K, Ohnuki M, Narita M, Ichisaka T, Tomoda K, Yamanaka S. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell 2007;131(5):861-872.
13. Yu J, Vodyanik MA, Smuga-Otto K, Antosiewicz-Bourget J, Frane JL, Tian S, Nie J, Jonsdottir GA, Ruotti V, Stewart R, Slukvin II, Thomson JA. Induced pluripotent stem cell lines derived from human somatic cells. Science 2007;318(5858):1917-1920.
14. Zhang J, Wilson GF, Soerens AG, Koonce CH, Yu J, Palecek SP, Thomson JA, Kamp TJ. Functional cardiomyocytes derived from human induced pluripotent stem cells. Circ Res 2009;104(4):e30-e41.
15. Mauritz C, Schwanke K, Reppel M, Neef S, Katsirntaki K, Maier LS, Nguemo F, Menke S, Haustein M, Hescheler J, Hasenfuss G, Martin U. Generation of functional murine cardiac myocytes from induced pluripotent stem cells. Circulation 2008;118(5):507-517.
16. Woltjen K, Michael IP, Mohseni P, Desai R, Mileikovsky M, Hamalainen R, Cowling R, Wang W, Liu P, Gertsenstein M, Kaji K, Sung HK, Nagy A. piggyBac transposition reprograms fibroblasts to induced pluripotent stem cells. Nature 2009;458(7239):766-770.
17. Kaji K, Norrby K, Paca A, Mileikovsky M, Mohseni P, Woltjen K. Virus-free induction of pluripotency and subsequent excision of reprogramming factors. Nature 2009;458(7239):771-775.
18. Wingard JR, Vogelsang GB, Deeg HJ. Stem cell transplantation: supportive care and long-term complications. Hematology (Am Soc Hematol Educ Program) 2002;422-444.
19. Bonnet D. Haematopoietic stem cells. J Pathol 2002;197(4):430-440.
20. Pittenger MF, Martin BJ. Mesenchymal stem cells and their potential as cardiac therapeutics. Circ Res 2004;95(1):9-20.

proefschrift Anke.indd 30 15-07-2009 12:00:34

21. Jiang Y, Jahagirdar BN, Reinhardt RL, Schwartz RE, Keene CD, X.R., Reyes M, Lenvik T, Lund T, Blackstad M, Du J, Aldrich S, Lisberg A, Low WC, Largaespada DA, Verfaillie CM. Pluripotency of mesenchymal stem cells derived from adult marrow. Nature 2002;418(6893):41-49.
22. Krause DS. Plasticity of marrow-derived stem cells. Gene Ther 2002;9(11):754-758.
23. Makino S, Fukuda K, Miyoshi S, Konishi F, Kodama H, Pan J, Sano M, Takahashi T, Hori S, Abe H, Hata J, Umezawa A, Ogawa S. Cardiomyocytes can be generated from marrow stromal cells in vitro. J Clin Invest 1999;103(5):697-705.
24. Hakuno D, Fukuda K, Makino S, Konishi F, Tomita Y, Manabe T, Suzuki Y, Umezawa A, Ogawa S. Bone marrow-derived regenerated cardiomyocytes (CMG Cells) express functional adrenergic and muscarinic receptors. Circulation 2002;105(3):380-386.
25. Rangappa S, Fen C, Lee EH, Bongso A, Wei EK. Transformation of adult mesenchymal stem cells isolated from the fatty tissue into cardiomyocytes. Ann Thorac Surg 2003;75(3):775-779.
26. Gaustad KG, Boquest AC, Anderson BE, Gerdes AM, Collas P. Differentiation of human adipose tissue stem cells using extracts of rat cardiomyocytes. Biochem Biophys Res Commun 2004;314(2):420-427.
27. Wakitani S, Saito T, Caplan AI. Myogenic cells derived from rat bone marrow mesenchymal stem cells exposed to 5-azacytidine. Muscle Nerve 1995;18(12):1417-1426.
28. Tomita S, Li RK, Weisel RD, Mickle DA, Kim EJ, Sakai T, Jia ZQ. Autologous transplantation of bone marrow cells improves damaged heart function. Circulation 1999;100(19 Suppl):II247-II256.
29. Xu W, Zhang X, Qian H, Zhu W, Sun X, Hu J, Zhou H, Chen Y. Mesenchymal stem cells from adult human bone marrow differentiate into a cardiomyocyte phenotype in vitro. Exp Biol Med (Maywood) 2004;229(7):623-631.
30. Fukuhara S, Tomita S, Yamashiro S, Morisaki T, Yutani C, Kitamura S, Nakatani T. Direct cell-cell interaction of cardiomyocytes is key for bone marrow stromal cells to go into cardiac lineage in vitro. J Thorac Cardiovasc Surg 2003;125(6):1470-1480.
31. Bittner RE, Schofer C, Weipoltshammer K, Ivanova S, Streubel B, Hauser E, Freilinger M, Hoger H, Elbe-Burger A, Wachtler F. Recruitment of bone-marrow-derived cells by skeletal and cardiac muscle in adult dystrophic mdx mice. Anat Embryol (Berl) 1999;199(5):391-396.
32. Nygren JM, Jovinge S, Breitbach M, Sawen P, Roll W, Hescheler J, Taneera J, Fleischmann BK, Jacobsen SE. Bone marrow-derived hematopoietic cells generate cardiomyocytes at a low frequency through cell fusion, but not transdifferentiation. Nat Med 2004;10(5):494-501.
33. Jackson KA, Majka SM, Wang H, Pocius J, Hartley CJ, Majesky MW, Entman ML, Michael LH, Hirschi KK, Goodell MA. Regeneration of ischemic cardiac muscle and vascular endothelium by adult stem cells. J Clin Invest 2001;107(11):1395-1402.
34. Orlic D, Kajstura J, Chimenti S, Bodine DM, Leri A, Anversa P. Bone marrow stem cells regenerate infarcted myocardium. Pediatr Transplant 2003;7 Suppl 3:86-88.
35. Balsam LB, Wagers AJ, Christensen JL, Kofidis T, Weissman IL, Robbins RC. Haematopoietic stem cells adopt mature haematopoietic fates in ischaemic myocardium. Nature 2004;428(6983):668-673.
36. Murry CE, Soonpaa MH, Reinecke H, Nakajima H, Nakajima HO, Rubart M, Pasumarthi KB, Virag JI, Bartelmez SH, Poppa V, Bradford G, Dowell JD, Williams DA, Field LJ. Haematopoietic stem cells do not transdifferentiate into cardiac myocytes in myocardial infarcts. Nature 2004;428(6983):664-668.
37. Wang JS, Shum-Tim D, Galipeau J, Chedrawy E, Eliopoulos N, Chiu RC. Marrow stromal cells for cellular cardiomyoplasty: feasibility and potential clinical advantages. J Thorac Cardiovasc Surg 2000;120(5):999-1005.
38. Toma C, Pittenger MF, Cahill KS, Byrne BJ, Kessler PD. Human mesenchymal stem cells differentiate to a cardiomyocyte phenotype in the adult murine heart. Circulation 2002;105(1):93-98.
39. Mangi AA, Noiseux N, Kong D, He H, Rezvani M, Ingwall JS, Dzau VJ. Mesenchymal stem cells modified with Akt prevent remodeling and restore performance of infarcted hearts. Nat Med 2003;9(9):1195-1201.
40. Quaini F, Urbanek K, Beltrami AP, Finato N, Beltrami CA, Nadal-Ginard B, Kajstura J, Leri A, Anversa P.

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Chimerism of the transplanted heart. N Engl J Med 2002;346(1):5-15.
41. Laflamme MA, Myerson D, Saffitz JE, Murry CE. Evidence for cardiomyocyte repopulation by extracardiac progenitors in transplanted human hearts. Circ Res 2002;90(6):634-640.
42. Orlic D, Kajstura J, Chimenti S, Limana F, Jakoniuk I, Quaini F, Nadal-Ginard B, Bodine DM, Leri A, Anversa P. Mobilized bone marrow cells repair the infarcted heart, improving function and survival. Proc Natl Acad Sci U S A 2001;98(18):10344-10349.
43. Norol F, Merlet P, Isnard R, Sebillon P, Bonnet N, Cailliot C, Carrion C, Ribeiro M, Charlotte F, Pradeau P, Mayol JF, Peinnequin A, Drouet M, Safsafi K, Vernant JP, Herodin F. Influence of mobilized stem cells on myocardial infarct repair in a nonhuman primate model. Blood 2003;102(13):4361-4368.
44. Kawada H, Fujita J, Kinjo K, Matsuzaki Y, Tsuma M, Miyatake H, Muguruma Y, Tsuboi K, Itabashi Y, Ikeda Y, Ogawa S, Okano H, Hotta T, Ando K, Fukuda K. Non-hematopoietic mesenchymal stem cells can be mobilized and differentiate into cardiomyocytes after myocardial infarction. Blood 2004;[Epub ahead of print].
45. Schwarz ER, Pollick C, Dow J, Patterson M, Birnbaum Y, Kloner RA. A small animal model of non-ischemic cardiomyopathy and its evaluation by transthoracic echocardiography. Cardiovasc Res 1998;39(1):216-223.
46. Kalyanaraman B, Joseph J, Kalivendi S, Wang S, Konorev E, Kotamraju S. Doxorubicin-induced apoptosis: implications in cardiotoxicity. Mol Cell Biochem 2002;234-235(1-2):119-124.
47. Agbulut O, Menot ML, Li Z, Marotte F, Paulin D, Hagege AA, Chomienne C, Samuel JL, Menasche P. Temporal patterns of bone marrow cell differentiation following transplantation in doxorubicin-induced cardiomyopathy. Cardiovasc Res 2003;58(2):451-459.
48. Ishida M, Tomita S, Nakatani T, Fukuhara S, Hamamoto M, Nagaya N, Ohtsu Y, Suga M, Yutani C, Yagihara T, Yamada K, Kitamura S. Bone marrow mononuclear cell transplantation had beneficial effects on doxorubicin-induced cardiomyopathy. J Heart Lung Transplant 2004;23(4):436-445.
49. Tomita S, Ishida M, Nakatani T, Fukuhara S, Hisashi Y, Ohtsu Y, Suga M, Yutani C, Yagihara T, Yamada K, Kitamura S. Bone marrow is a source of regenerated cardiomyocytes in doxorubicin-induced cardiomyopathy and granulocyte colony-stimulating factor enhances migration of bone marrow cells and attenuates cardiotoxicity of doxorubicin under electron microscopy. J Heart Lung Transplant 2004;23(5):577-584.
50. Anversa P, Nadal-Ginard B. Myocyte renewal and ventricular remodelling. Nature 2002;415(6868):240-243.
51. Hierlihy AM, Seale P, Lobe CG, Rudnicki MA, Megeney LA. The post-natal heart contains a myocardial stem cell population. FEBS Lett 2002;530(1-3):239-243.
52. Beltrami AP, Barlucchi L, Torella D, Baker M, Limana F, Chimenti S, Kasahara H, Rota M, Musso E, Urbanek K, Leri A, Kajstura J, Nadal-Ginard B, Anversa P. Adult cardiac stem cells are multipotent and support myocardial regeneration. Cell 2003;114(6):763-776.
53. Matsuura K, Nagai T, Nishigaki N, Oyama T, Nishi J, Wada H, Sano M, Toko H, Akazawa H, Sato T, Nakaya H, Kasanuki H, Komuro I. Adult cardiac Sca-1-positive cells differentiate into beating cardiomyocytes. J Biol Chem 2004;279(12):11384-11391.
54. Oh H, Bradfute SB, Gallardo TD, Nakamura T, Gaussin V, Mishina Y, Pocius J, Michael LH, Behringer RR, Garry DJ, Entman ML, Schneider MD. Cardiac progenitor cells from adult myocardium: homing, differentiation, and fusion after infarction. Proc Natl Acad Sci U S A 2003;100(21):12313-12318.
55. Urbanek K, Quaini F, Tasca G, Torella D, Castaldo C, Nadal-Ginard B, Leri A, Kajstura J, Quaini E, Anversa P. Intense myocyte formation from cardiac stem cells in human cardiac hypertrophy. Proc Natl Acad Sci U S A 2003;100(18):10440-10445.
56. Messina E, De Angelis L, Frati G, Morrone S, Chimenti S, Fiordaliso F, Salio M, Battaglia M, Latronico MV, Coletta M, Vivarelli E, Frati L, Cossu G, Giacomello A. Isolation and expansion of adult cardiac stem cells from human and murine heart. Circ Res 2004;95(9):911-921.
57. Lee MS, Lill M, Makkar RR. Stem cell transplantation in myocardial infarction. Rev Cardiovasc Med 2004;5(2):82-98.

proefschrift Anke.indd 32 15-07-2009 12:00:34

58. Schachinger V, Assmus B, Britten MB, Honold J, Lehmann R, Teupe C, Abolmaali ND, Vogl TJ, Hofmann WK, Martin H, Dimmeler S, Zeiher AM. Transplantation of progenitor cells and regeneration enhancement in acute myocardial infarction: final one-year results of the TOPCARE-AMI Trial. J Am Coll Cardiol JID - 8301365 2004;44(8):1690-1699.
59. Perin EC, Dohmann HF, Borojevic R, Silva SA, Sousa AL, Silva GV, Mesquita CT, Belem L, Vaughn WK, Rangel FO, Assad JA, Carvalho AC, Branco RV, Rossi MI, Dohmann HJ, Willerson JT. Improved exercise capacity and ischemia 6 and 12 months after transendocardial injection of autologous bone marrow mononuclear cells for ischemic cardiomyopathy. Circulation 2004;110(11 Suppl 1):II213-II218.
60. Wollert KC, Meyer GP, Lotz J, Ringes-Lichtenberg S, Lippolt P, Breidenbach C, Fichtner S, Korte T, Hornig B, Messinger D, Arseniev L, Hertenstein B, Ganser A, Drexler H. Intracoronary autologous bone-marrow cell transfer after myocardial infarction: the BOOST randomised controlled clinical trial. Lancet 2004;364(9429):141-148.
61. Kang HJ, Kim HS, Zhang SY, Park KW, Cho HJ, Koo BK, Kim YJ, Soo LD, Sohn DW, Han KS, Oh BH, Lee MM, Park YB. Effects of intracoronary infusion of peripheral blood stem-cells mobilised with granulocyte-colony stimulating factor on left ventricular systolic function and restenosis after coronary stenting in myocardial infarction: the MAGIC cell randomised clinical trial. Lancet 2004;363(9411):751-756.
62. Masino AM, Gallardo TD, Wilcox CA, Olson EN, Williams RS, Garry DJ. Transcriptional regulation of cardiac progenitor cell populations. Circ Res 2004;95(4):389-397.
63. Doevendans PA, Chien KR, Mummery C. Towards Stem Cell Therapy. In: Chien KR, ed. The Molecular Basis of Cardiovascular Disease.Philadelphia: Ssaunders; 2004. p. 107-22.
64. Hassink RJ, Passier R, Goumans MJ, Mummery CL, Doevendans PA. New and viable cells to replace lost and malfunctioning myocardial tissue. Minerva Cardioangiol 2004;52(5):433-445.
65. Liu Y, Song J, Liu W, Wan Y, Chen X, Hu C. Growth and differentiation of rat bone marrow stromal cells: does 5-azacytidine trigger their cardiomyogenic differentiation? Cardiovasc Res 2003;58(2):460-468.
66. Assmus B, Schachinger V, Teupe C, Britten M, Lehmann R, Dobert N, Grunwald F, Aicher A, Urbich C, Martin H, Hoelzer D, Dimmeler S, Zeiher AM. Transplantation of Progenitor Cells and Regeneration Enhancement in Acute Myocardial Infarction (TOPCARE-AMI). Circulation 2002;106(24):3009-3017.

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3

Human cardiomyocyte progenitor cells differentiate into functional mature cardiomyocytes; an *in vitro* model for studying human cardiac physiology and pathophysiology

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ABSTRACT

To date, there is no suitable *in vitro* model to study human adult cardiac cell biology. While embryonic stem cells are able to differentiate into cardiomyocytes *in vitro*, the efficiency of this process is very low. Other methods to differentiate progenitor cells into beating cardiomyocytes rely on co-culturing with rat neonatal cardiomyocytes, making it difficult to study human cardiomyocyte differentiation and (patho)physiology. Here we have developed a method for efficient isolation and expansion of human cardiomyocyte progenitor cells (CMPCs) from cardiac surgical waste or alternatively, from fetal heart tissue. Furthermore, we provide a detailed *in vitro* protocol for efficient differentiation of CMPCs into cardiomyocytes with great efficiency (80-90% of differentiation). Once CMPCs are rapidly dividing (approximately 1 month after isolation), differentiation can be achieved in 3-4 weeks.

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INTRODUCTION

Until recently, the heart was considered a terminally differentiated organ, with a very low intrinsic renewal capacity. Although the finding of resident cardiomyocyte progenitor cells (CMPCs) has questioned this dogma (reviewed in[1,2]), it remains a challenge to restore the damaged myocardium, for example after a myocardial infarction. Insight into the processes regulating the expansion and differentiation of CMPCs is crucial for the development of new therapies in cardiac disease. Furthermore, CMPCs may provide an *in vitro* model to investigate congenital heart disease and genetic defects.

Human models to study cardiomyocytes

To date, there are no suitable human models to study adult cardiac biology. Cardiomyocytes isolated directly from heart tissue have been studied[3], but their applicability is hampered by the fact that the cardiomyocytes do not divide in culture, and can only be maintained for a relatively short time-period[3]. Although electrophysiological characteristics can be analyzed, cultured cardiomyocytes do not provide a system to investigate the development of cardiomyocytes. Human embryonic stem (hES) cells have the ability to differentiate into cardiomyocytes *in vitro*[4], however, their culture is very labor intensive. Although culture and differentiation techniques have improved dramatically[5,6], it remains challenging to obtain cardiomyocyte-enriched cultures. We have recently introduced a new model that allows the study of the differentiation process and cardiac cell biology, by using human CMPCs isolated from human cardiac surgical waste material or fetal hearts[7,8].

Isolating progenitor cells

Human cardiomyocyte progenitor cells have been isolated previously using different methods. First, when placing small cardiac tissue explants in culture, a population of progenitors migrates out of the explant. These spontaneously shed cells form aggregates in culture, designated cardiospheres, consisting of proliferating c-kit$^+$ cells[9]. However, these cardiospheres may consist of a heterogeneous population of cells as their collection is not defined. Therefore, characterization of the isolated cells by, e.g., flow cytometry remains necessary after each isolation. Secondly, progenitors have been identified for isolation based on their expression of surface stem cell markers (e.g., c-kit[10, 11], MDR-1 or Sca-1 (sca-1-reactive protein[11])), or the ability to efflux Hoechst dye (side-population cells[12]), allowing the use of flow cytometry or magnetic cell sorting (MACS) for their collection. Thirdly, cells can be isolated on the basis of the expression of progenitor specific transcription factors (e.g. Islet-1[13]). However, it remains unclear how these previously identified populations of progenitor cells are related to each other. This will need extensive collaboration between research groups.

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In this protocol, we provide a method to isolate cells from adult human heart tissue (the auricle: appendix of the atrium) that is removed during heart surgery. If such material is not available, a similar result can be achieved by pooling 5 needle biopsies. Alternatively, we are also able to isolate cells from fetal heart tissue after elective abortion under informed consent. This latter tissue source provides many more CMPCs and the expansion of the cells is much faster and efficient. However we realize that this tissue source is not available for most researchers, and therefore we have focused on the isolation from cardiac surgical waste.

We provide two methods to isolate cells; by clonal expansion or alternatively by MACS isolation using an epitope that is recognized by a Sca-1 antibody. Although the human equivalent of murine Sca-1 is not yet known, the antibody may cross-react with an unknown protein, still leading to a homogenous cell population. Using MACS, CMPCs can be obtained much faster than using the clonogenic method. In both cases, after an initial lag in growth, the CMPCs can be easily expanded *ex vivo* by many folds without loss of the thereby providing high numbers of progenitor cells in culture[7].

Differentiating progenitors into cardiomyocytes

To gain further insight into the processes that regulate the formation of new cardiac cells, it is important to generate an *in vitro* model which can achieve differentiation into a homogenous population of cardiomyocytes to a reproducible degree. Inducing the differentiation of human cardiomyocyte progenitor cell populations into cardiomyocytes has been described previously[2,14]. Until now it has been necessary to co-culture these cells with neonatal (rat) cardiomyocytes to achieve differentiation into spontaneously beating cardiomyocytes. Furthermore, the efficiency of differentiation has been low.

In this protocol, we describe a differentiation method for CMPCs that excludes the need for co-culture with neonatal rat cardiomyocytes, or other types of feeder layers.

When stimulated *in vitro* with 5-azacytidine and growth factors, the CMPCs very efficiently differentiate into cardiomyocytes[7]. This is evident by a shift in gene expression. In an undifferentiated state, stem cell specific genes are expressed (isl-1, c-kit), as well as transcription factors that are indicative of a cardiomyocyte predisposition (e.g. Nkx-2.5, MEF2C and GATA-4). However, after induction of differentiation, CMPCs start to express genes that are necessary to form the complex sarcomeric structures in the cardiomyocyte (e.g. β-MHC, MLC2V, α-actinin) and for electrophysiological function (for example SCN5a and Ryr2; unpublished results). CMPC-derived cardiomyocytes reveal organized sarcomeric structures, contract spontaneously, and are responsive to adrenergic stimulation[7]. The cells show excitation-contraction coupling, and action potentials that resemble ventricular cells. They couple functionally and metabolically via gap junctions[7], there-

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by resembling the *in vivo* myocardium, which consists of large syncytia.
For these reasons, CMPCs provide an excellent model to study cardiomyocyte function and differentiation. By examining cells from cardiac surgery patients with congenital heart disease or genetic defects, more information on how particular mutations can affect the differentiation of progenitors as well as the integrity of the newly formed cardiomyocytes can be obtained. In addition, CMPCs can provide a tool for pharmacological studies, for example to investigate the influence of drugs on the differentiation of stem cells, contractile function, action potential duration (QT prolongation), and gene expression. The possibility of introducing gene overexpression and/or reporter plasmids by standard calcium phosphate transfection offers opportunities to study signaling pathways and drug screening.
This protocol intends to provide a method to study cardiogenesis and cardiomyocyte differentiation *in vitro*. As there are many controversies in the field of stem cell biology, providing a detailed protocol for *in vitro* isolation and differentiation will help to establish a more unified approach to the use of cardiac progenitor cells. The limitation of this protocol is that isolation and upscaling of cells, as well as the differentiation into cardiomyocytes will take several weeks from the start of the protocol, and obtaining reproducible results requires some degree of experience with the behavior of the cells in culture. We did not intend to provide a protocol for cardiomyocytes that are ready to be transplanted in the clinical setting. 5-Azacytidine treatment used for the differentiation of CMPCs may not be suitable for their use in patients, as the effects of demethylation may be random.
In the future, CMPCs may provide a source for tissue engineered cardiac grafts or cell-based transplantation therapy as we have recently found that CMPCs can differentiate into cardiac tissue *in vivo* (unpublished results). Clinical efficacy, however, still needs to be established, and it has to be noted that the current protocol for cell isolation does not reach the high Good Clinical or Manufacturing Practice requirements, but this protocol may provide a framework for clinical application.
In summary, human cardiomyocyte progenitor cells will provide researchers with a tool to study cardiomyogenic development and differentiation as well as normal progenitor cell or cardiomyocyte function and signaling.

EXPERIMENTAL DESIGN

Source of cardiac material and preparation

Various sources for cardiac tissue can be used as starting material in this protocol. In general, we use human heart auricle which is routinely removed during e.g., valve

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replacement surgery. This surgical waste, that is approximately 2-4 cm^3, can provide an excellent source for CMPCs. Another source of cardiac tissue is human (total) fetal hearts, obtained after elective abortion. Furthermore, CMPCs can be isolated from catheter-based small biopsy specimens by pooling at least 5 needle biopsies. This allows isolation of cells from patients with, for example, genetic defects not undergoing invasive surgery. Although the number of cells that can be isolated from these biopsies is limited, they have the advantage that it may provide a good research tool to gain insight into effects of these mutations on the generation and integrity of cardiomyocytes. The preparation The preparation of cardiac material includes keeping the tissue sterile during collection, and transferring the sample to ice-cold medium containing antibiotics as quickly as possible.

Clonogenic expansion versus MACS using Sca-1

We provide two different protocols to isolate CMPCs from cardiac tissue. First, we describe a method to clonally isolate CMPCs by seeding the cells at a density of 1 cell per well in growth medium. Most differentiated cells types can not withstand single cell culture; thereby a selection for progenitors occurs. Approximately 75% of the cells that survive and expand will be CMPCs based on morphology and surface protein expression.
Secondly, we describe an isolation method by means of magnetic cell sorting using an iron-labeled mouse anti-Sca-1 antibody. Although the human epitope recognized by this antibody has not yet been identified, we found that using the Sca-1 antibody, a population of cells is isolated every time that expands efficiently and is similar to clonogenically isolated cells on the basis of morphology and surface marker expression. Therefore, the MACS isolation protocol is more efficient resulting in higher cell numbers in a shorter time course.

Number of obtained cells

These different isolation procedures as well as the starting material will lead to a variation in the number of CMPCs that are initially isolated (ranging from 10 to 5,000 cells when using MACS isolation). In culture, after an initial lag phase, the cells will double every day, for at least 75 times, thereby high numbers of cells can be obtained.

Use of 5-azacytidine

In this protocol for differentiation, we use a combination of 5-azacytidine treatment, followed by Transforming Growth Factor (TGF)-β and vitamin C stimulation. 5-Azacytidine is a general demethylating agent, leading to unmasking of genes that are not expressed due to promoter hypermethylation. It is not specific for cardiac genes but has previously been described in P19 embryonic stem cell[15] and bone marrow stromal cell[16] differentiation towards cardiomyocytes. We have tested other reagents to circumvent the use of

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5-azacytidine and used oxytocin, TGFβ alone, or a combination of TGFβ and BMP6[7]. Although differentiation into cardiomyocytes was observed, this was not as efficient as the combination of 5-azacytidine and TGFβ[7]. This protocol was designed to study cardiogenic processes and cardiomyocyte differentiation *in vitro*. For these experiments, a high differentiation efficiency of CMPCs is crucial, which to date can be achieved only by addition of 5-azacytidine.

Generated cardiomyocytes

To confirm the differentiation into cardiomyocytes multiple characteristics need to be addressed. First of all, the expression of cardiac genes such as cardiac troponin–T, β-Myosin heavy chain and cardiac actin, need to be analyzed. As a negative control RNA isolated from CMPCs, cultured in differentiation medium until 90% confluence, is used. As a positive control RNA from cardiomyocytes or total cardiac tissue is a good source.
Secondly, it is crucial to stain the cells with antibodies directed at the sarcomeric structures in the cells, for instance using α-actinin or troponin–I, as a well-organized striated pattern is indispensible for cardiomyocytes. To do so, cells needs to be differentiated on coverglasses to allow immunofluorescent staining for sarcomeric proteins. Quantification of differentiation is performed by counting the number of α-actinin or troponin-I-positive cells, relative to the total number of nucleated cells microscope field.
Beating cells can be observed in many cases. Approximately 80% of the beating cells are able to beat individually. The average beating rate of the cells is around 40 beats per minute, and they are responsive to β-adrenergic stimulation[7]. Differentiated CMPC-derived cardiomyocytes will continue to beat up to 10 weeks. Interestingly, cells that do not beat spontaneously can still be paced *in vitro*.

MATERIALS

Reagents:

- PBS (pH 7.4) without calcium and magnesium (Gibco, cat. No. 10010-056 or home made and sterilized).
- EDTA (Sigma cat. no. E4884)
- Trypan Blue (Sigma, T8154)
- Trypsin/EDTA (Sigma, cat. no.T4904)
- Collagenase A (Roche cat no 10103578001) CAUTION: Irritant; wear protective goggles, clothing and gloves as appropriate.
- Gelatin Type A (Sigma, cat. no. G6144)
- 5-Azacytidine (Sigma, A2385) CAUTION: carcinogenic, wear protective goggles, cloth-

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ing and gloves as appropriate.
- L-Ascorbic Acid (Vitamin C, Sigma A4403)
- TGF-β1 (PeproTech, 100-21C)
- Fibroblast Growth Factor-Basic human (bFGF, Sigma F0291),
- Triton X-100 (Sigma, cat. no. T8787)
- Paraformaldehyde (PFA; Sigma cat. no. 158127) CAUTION: Irritant; wear protective goggles, clothing and gloves as appropriate
- Mowiol (Calbiochem cat. no. 475904)
- Tween-20 (Merck cat. no. 8.22184.0500)
- Hoechst 33342, (Invitrogen cat. no. H1399) CAUTION: carcinogenic, wear protective goggles, clothing and gloves as appropriate.

Equipment
- Delicate scissors (Aesculap)
- Forceps (Aesculap)
- 15 ml centrifuge tubes (Corning, cat. no. 430791)
- 50 ml centrifuge tubes (Corning cat. no. 430829)
- Cell culture dish, 100mm x 20 mm (Corning, cat. no. 430293)
- 5 ml pipette (Corning cat. no. CLS4050)
- Waterbath, circulator type E100 (Lauda)
- Roller bench (e.g. ROCK 'N ROLLER, Model 34201m, Snijders).
- Cellstrainer 40µm (Falcon cat. no. 103578)
- 5 ml Syringe (BD, Bioscience)
- Centrifuge, type 5810R (Eppendorf)
- 1.5 ml microcentrifuge tubes (Eppendorf)
- Test-tube rotator, model 34528 (Snijders) (optional)
- Counting chamber (e.g. Bürker-Türk Bright-Line; 0,100mm; 0,0025 mm2)
- Inverted light microscope
- MiniMACS™ Separator (order no. 130-090-312) (optional)
- MiniMACS columns, type MS plus (cat. no. 130-041-301) (optional)
- 24 well cell culture plate (Corning cat. no. 3524)
- 6 well cell culture plate (Corning cat no.3506)
- 96 well cell culture plate (Corning cat. no. 3596)
- Microscope cover glasses 12mm Ø
- Microscope slides (Menzel-Gläser, cat. no. AA00000102E)
- For cell culture: see equipment setup
- CO2 incubator (CO2 at 5% vol/vol; humidified, T=37 ºC)
- Anti-sca-1 microbeads mouse, (Miltenyi Biotech cat. No. 130-091-176) (optional).

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Reagent setup

Stock solutions:

Collagenase A: dissolve a vial of collagenase A (100mg) in 4 ml of sterile PBS, yielding a concentration of 25 mg/ml. Filter sterilize and divide in aliquots of 100µl (= 2.5 mg) per vial and store at -20ºC. The stock can be stored for at least 9 months.

5-Azacytidine: weigh an appropriate amount on an analytical scale, and dissolve in DMEM at a concentration of 250 µM. Filter sterilize, divide in aliquots, store at -20ºC, and keep protected from light. The stock can be stored for at least 9 months, and can be thawed only once.

TGF-β: Dissolve to a concentration of 1µg/ml in sterile acidified H_2O (4mM HCl): this is a 1000x stock. Divide in aliquots and store at 4ºC. The stock can be stored for at least 6 months.

Gelatin (1% wt/vol): dissolve 10 g of gelatin A in 1000 ml of distilled H_2O. Autoclave to sterilize. Store at 4ºC. The stock can be stored for at least 1 year.
CRITICAL: Note that the gelatin will not dissolve completely prior to autoclaving the solution.

Ascorbic Acid: for a 1000x stock, dissolve 0.88 gram in 50 ml of distilled H_2O, yielding a 10^{-1} M solution. Filter sterilize and store at 4ºC. The stock can be used until the solution turns yellow.

EDTA (200mM): dissolve 74.45 mg in 1000 ml distilled H_2O. Set pH to 7.4. Autoclave to sterilize. The stock can be stored at 4°C for at least 1 year.

bFGF: Prepare a concentration 25 µg/ml: dissolve 1 vial of bFGF (25µg) in 1 ml of sterile PBS. Divide in aliquots and store at -20ºC. The stock can be stored for at least 9 months.

PFA for cell fixation: 4% PFA (wt/vol) in PBS. Dissolve on a magnetic stirrer at 50 ºC. 4% PFA can be stored in aliquots at -20 ºC for at least 1 year.
CAUTION: PFA is toxic, wear gloves when handling and use a safety biohazard hood.

Hoechst: prepare a 1000x stock: dissolve 1 mg of Hoechst in 5 ml distilled water (or an equivalent). The solution can be stored in the dark in aliquots at 4ºC for several weeks. When stored dark at -20°C, the stock can be stored for at least 1 year.

Medium for collecting tissue

DMEM containing 10% FBS and 1x penicillin/ streptomycin (50x). The medium can be stored at 4°C in the dark for 3 months. Alternatively, aliquots can be stored at -20°C for at least one year.

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Buffers for cell isolation

M-buffer: add 10 ml of 200mM EDTA and 10 ml FBS to 1 liter of PBS. Store at 4ºC for 6 months, and keep on ice during use.

Collagenase solution: PBS containing 1 mg/ml collagenase A. One vial of stock solution per 2.5 ml PBS. Always prepare fresh before use.

Cell culture media and solutions

0.1% gelatin: dilute 10ml of 1% gelatin solution in 90 ml of sterile PBS. Store at 4ºC for at least 6 months.

Prepare EGM-2: remove 15 ml from 500ml of EBM-2 and add EGM-2 Single Quots. Store at 4ºC for up to 6 months.

Growth medium: add 125 ml of EGM-2 to 375 ml of M199. Discard 65 ml. Add 50 ml FBS, 1x (10ml) penicillin/streptomycin (from 50x stock) and 1x (5 ml) MEM non-essential amino acids (from 100x stock). Store at 4ºC for up to 6 months. Alternatively, aliquots can be stored at -20 ºC for at least a year.

Differentiation medium: combine 235 ml IMDM and 235 ml Ham's F12 nutrient mixture with GlutaMAX-I. Add 10 ml horse serum, 1x (5ml) MEM non-essential amino acids (from 100x stock), 1x (5ml) Insulin-transferrin-selenium (from 100x stock), 1x (10 ml) penicillin/streptomycin (from 50x stock). Store at 4ºC for up to 6 months. Alternatively, aliquots can be stored at -20 ºC for at least a year.

Antibodies and dilutions

Monoclonal mouse-anti-α Actinin (sarcomeric) clone EA-53 (Sigma, cat. no. A7811) 1:800. polyclonal Rabbit anti-Troponin I (Chemicon cat. no. AB1627) 1:100. The diluted antibody solutions can be stored at 4°C for up to 6 weeks, or at -20°C for 6 months, but it can only be thawed once.

As secondary antibodies use Alexa Fluor 488 goat anti mouse IgG (Invitrogen cat. No. A-11001) 1:400 and Alexa Fluor 555 goat anti rabbit IgG (Invitrogen cat. no. A-21428) 1:400. The diluted antibody solutions can be stored at 4°C in the dark for up to 6 weeks.

Hoechst: as a nuclear counterstain dilute the stock solution 1:1,000 in PBS. The diluted Hoechst solutions can be stored at 4°C in the dark for at least 6 months.

Blocking reagent: prepare 4% (vol/vol) normal goat serum (Vector Laboratories Cat. no.6-S-1000) in PBS. The diluted solutions can be stored at 4°C for up to 6 months.

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Buffers for staining:

0.1% Triton X-100: Dilute 50 µl Triton in 50 ml PBS. Store at room temperature (RT) for at least 1 year.

0.05% PBS-Tween: Dilute 500 µl Tween-20 in 1 liter of PBS. Store at RT for at least 1 year.

Equipment setup

Cell Culture

For all culture procedures described here, tissue culture, reagent preparation and sterilizing facilities are necessary. Class II Biological Flow Hoods and laminar flow horizontal draft hoods are used.

PROCEDURE

Preparation of single-cell suspension TIMING 4h

1 Receive human tissue on ice in medium for tissue collection (Figure 1a). Under these conditions, tissue can be kept at 4ºC for up to 3 hours, but should be processed as soon as possible.
CRITICAL STEP Keep all tissue and reagents sterile, and work in the flow cabinet.

2 Wash the tissue twice in cold M-buffer to remove residual blood

3 Place the tissue in a 100mm cell culture dish in cold M-buffer and remove any fat or scar tissue using sterile small scissors and forceps (Figure 1b).

4 Cut the tissue into small clumps of ~ 1mm^3 (Figure 1c-e). The clumps should be small enough to enter a 5 ml pipette.

5 Transfer the tissue clumps into a 15 ml centrifuge tube, and let the tissue sink to the bottom.

6 Remove the M-buffer, by pipetting or careful aspiration and wash twice in cold PBS, each time allowing the tissue to sink to the bottom before removing the solution.
CRITICAL STEP It is crucial to wash the tissue twice in PBS without serum. In the subsequent steps, collagenase A is used and the activity of this enzyme is inhibited by serum.

7 Remove the PBS, by pipetting or aspiration, and estimate the volume of the tissue. If the starting material was a human heart auricle of approximately 2-4 cm^3, a volume of 2 ml is expected (Figure 1a,f). Add the collagenase solution in the proportion 1:2.5 of tissue volume versus collagenase solution. For 4 cm^3 tissue with a 2 ml volume 5 ml collagenase solution is added with a concentration of 1 mg/ml (two vials of collagenase stock solution in 5 ml of PBS).

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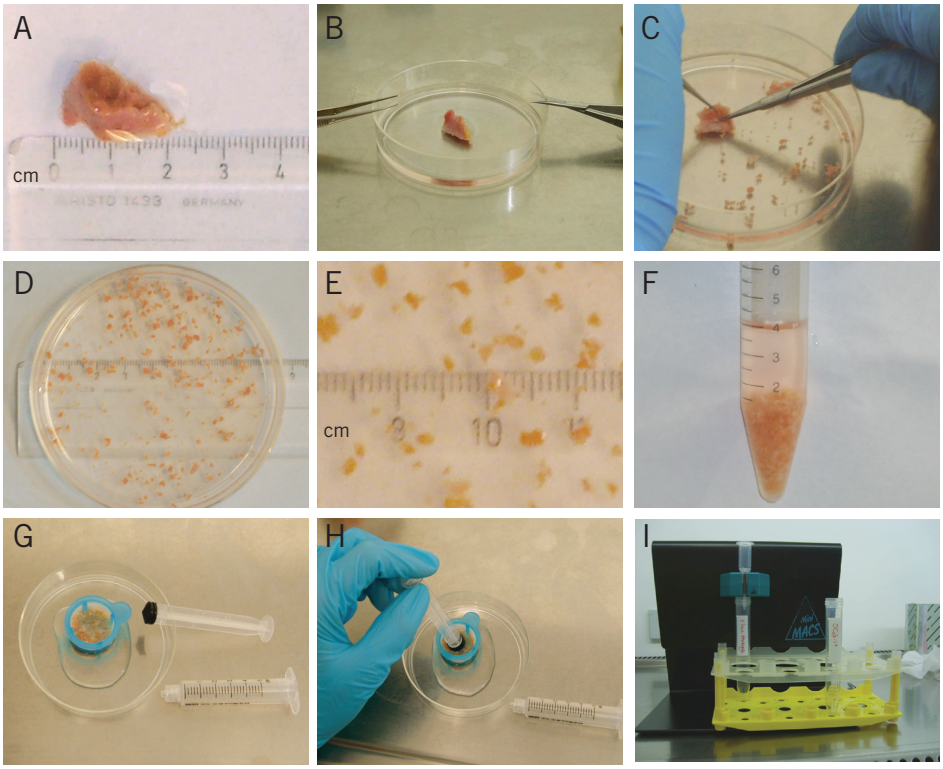

Figure 1 Isolating CMPCs from human atrial tissue
(a) The size of the auricle of the left atrium that is removed during surgery is approximately 2-4 cm^3. (b) Tissue is placed in a cell culture dish in M-buffer and cut into small pieces (c) of approximately 1-2 mm^2 (d, close up in e). (f) Volume of tissue obtained from auricle. (g) After collagenase incubation, the tissue is poured into a cell strainer placed in a cell culture dish. (h) The tissue is grinded through the mesh using the plunger of a 5 ml syringe. (i) MACS magnet and holder setup.

8 Incubate the solution at 37°C for 2 hours in a waterbath. Mix the solution from time-to-time

PAUSE POINT Alternatively, the solution can be placed on a roller bench and incubated overnight at 4°C.

9 Continue in the flow cabinet, pipette the solution gently up and down using a 5 ml pipette and an automated pipette.

CRITICAL STEP Note that most, but not all of the tissue will be dissociated.

TROUBLESHOOTING

10 Place a 40 µm cell strainer in a 100 mm cell culture dish, and take out the plunger from a 5 ml syringe (Figure 1g).

11 Transfer the tissue in collagenase solution into the cell strainer (Figure 1g) and grind the tissue through the strainer using the plunger (Figure 1h).

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12 After grinding thoroughly, transfer the fluid that has passed through the strainer into a 50ml falcon tube and keep on ice.
CRITICAL STEP Note that not all of the tissue will pass through the strainer.
13 Wash the cell strainer 5 times with 5 ml of cold M-buffer and collect the flow-through in the 50ml tube.
14 Centrifuge the cell suspension for at 300 g for 5 min at RT.
15 Remove the supernatant and resuspend the cells in 5 ml cold M-buffer.
16 Centrifuge the cell suspension for 5 minutes at 300 g at RT.
PAUSE POINT The cell suspension can be resuspended in 10 ml DMEM containing 10% FBS and 1x Penicillin/Streptomycin and kept overnight at 4°C.

Isolation of CMPCs

17 From this point forward, there are two options to isolate CMPCs; clonogenic isolation of CMPCs (option A); or MACS isolation of CMPCs (option B).

A. Clonogenic isolation of CMPCs **TIMING 60 min**

i Remove supernatant by aspiration and resuspend the cells in 5 ml of PBS, and let the cardiomyocytes sink to the bottom of a 15 ml tube for 5 minutes.
ii By pipetting, collect the supernatant in a new 15 ml tube and centrifuge at 300 g, for 5 min at RT.
iii Resuspend the cells in 1 ml of PBS.
iv Add 20 µl Trypan blue to 20 µl cell suspension in a 1.5 ml eppendorf tube, mix by pipetting up and down, and count the viable (i.e. round white) cells in a counting chamber.
v Dilute the cells to a concentration of 5 cells/ml in growth medium
vi Coat a 96-well plate with 0.1% gelatin; incubate the plate for at least 15 minutes at 37°C before removing the gelatin solution.
vii Add 100 µl of cell solution to each well
CRITICAL STEP The dilution is prepared in such a way that it contains an average of 1 cell per 2 wells, increasing the chance of clonogenic growth.

B. MACS isolation of CMPCs **TIMING 2-4 h, depending on the flow rate of the column**

i Remove the supernatant, by aspiration and resuspend the cell pellet in 450 µl cold M-buffer, and transfer the solution to a 1.5 ml eppendorf tube.
ii Add 50 µl anti Sca-1 microbeads, and incubate under rotation, in a test-tube rotator, for 1 hour at 4°C.
PAUSE POINT Incubation can be prolonged to 3 hours.
iii During the incubation, disinfect the MiniMACS magnet and holder with 70% ethanol and place them in the flow cabinet.

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iv For each sample: click a separation column into the magnet, and label two 15 ml tubes, one for the flow through, one for the CMPCs (Figure 1i).

v Place the flow-through tube under the column, and pre-wet the column with 1 ml cold M-buffer.

vi Coat four wells of a 24-wells cell culture plate with 0.1% gelatin in PBS. Place the plate in a 37°C incubator for at least 15 minutes, before removing the gelatin.

vii After incubating with the beads, transfer the solution from Step 17Bii to a 15-ml tube and centrifuge for 5 minutes at 300g at RT then remove the supernatant by pipetting and carefully resuspend the pellet in 1 ml of cold M-Buffer, ensuring that there are no clumps.

viii Run the cell solution through a 40 µm cell strainer that is placed on a 50 ml centrifuge tube, to remove clumps that may clog the column. Wash the strainer with 1 ml cold M-buffer, while leaving it on the 50 ml tube, and collect all the flow-through into the same tube. This is the filtered cell solution.

ix Transfer the filtered cell solution onto the column (Step 17Biv), 1 ml at a time, and collect the flow-through in the designated tube (Step 17Biv).

TROUBLESHOOTING

x Wash the column 3 times with 500 µl cold M-buffer and collect flow-through; again in the designated tube.

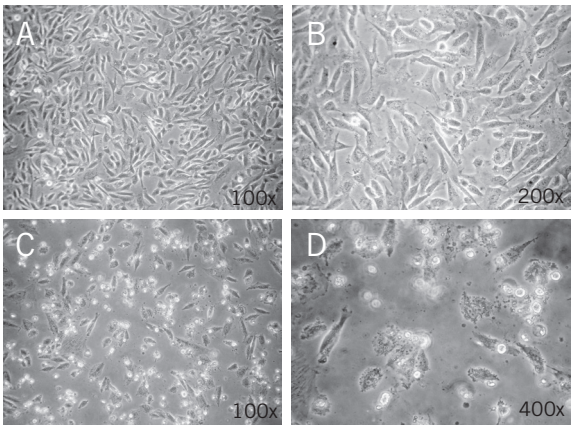

Figure 2. CMPC culture conditions
(a,b) Appearance of CMPCs in culture at 80% confluence. Under normal culture conditions, the CMPCs need to be passaged. Alternatively, when starting a differentiation experiment, the cells are seeded at this density. (c) In some cases, cell death occurs after treatment with 5-azacytidine. (d) Small dark vacuoles are observed as well as light sphere-like-structures consisting of dead cells

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CRITICAL STEP Do not remove the column from the magnet during these steps!

TROUBLESHOOTING

xi When all the fluid has run through, place the tube for CMPCs (step 17Biv) underneath the column. Take out the column, hang it in the tube and tap it a couple of times to loosen the cells.

CRITICAL STEP The first droplets contain most of the cells; ensure that when the column is taken from the magnet it is placed directly above the tube to avoid spilling of fluid.

TROUBLESHOOTING

xii Collect the CMPC fraction by washing the column with 500 µl cold M-buffer 2 times.

TROUBLESHOOTING

xiii Flush out the remaining buffer using the plunger that is supplied with the column.

TROUBLESHOOTING

xiv Centrifuge flow-through and the CMPCs tubes obtained in steps 17Bix/x and 17Bxi at 300 g for 3 min at RT and discard the supernatant by careful suction or pipetting.

xv Dissolve the CMPC pellet in 500 µl growth medium.

CRITICAL STEP Note that the flow-through pellet (with unbound cells obtained during step 17Bx can be resuspended in 1.5 ml growth medium and divided over three wells. However, there will be contamination with CMPCs that have not bound to the column. Therefore, the flow-through should not be considered a negative fraction.

TROUBLESHOOTING

xvi Remove the gelatin from the 24-well plates from Step 17Biv.

xvii Apply 500 µl of the cell suspension in growth medium from Step 17Bxv to the coated well. Add bFGF (10 ng/ml) to the growth medium to stimulate the growth of the cells.

Culturing CMPCs TIMING Splitting of cells: 30 minutes; culture until first differentiation: 6-7 weeks using clonogenic isolation, 3-4 weeks using MACS isolation.

18 After 3 d, remove the medium and wash the attached cells twice with PBS. Add 1 ml of growth medium to the cells, supplemented with 10 ng/ml bFGF. Optional: place the old medium (containing debris) in a new gelatin coated well and add 1:1 growth medium. The cells that are still not attached may adhere better in a new well.

CRITICAL STEP Cells should be given at least 3 days to attach before refreshing the medium.

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TROUBLESHOOTING

19 Let the cells grow to 80-90% confluence (Figure 2a,b), remove the culture medium, and wash the cells with PBS containing 2mM EDTA.

20 Remove the PBS-EDTA and add 100 µl trypsin-EDTA.

CRITICAL STEP The cells need to be washed with PBS to remove all traces of serum, which interferes with the enzyme activity of trypsin.

21 Incubate in a 37°C stove for 2-5 minutes. Check under the microscope if the cells are detached. Then add 2 ml of growth medium containing bFGF (10 ng/ml) and gently resuspend the cells to a single cell solution.

CRITICAL STEP Prolonged exposure to trypsin-EDTA may influence the cell integrity.

CRITICAL STEP Ensure that there is at least a 10x larger volume of medium containing 10% serum to abrogate the trypsinization process.

Box 1. Validation and test induction of differentiation

An easy way to validate the procedure and time course of cardiac differentiation is to perform real-time reverse transcription polymerase chain reaction (RT-qPCR) as described by Nolan et al.[18]. Typically, you need 4 time points and 1 negative control as described below:

1 Coat five 60 mm dishes with 0.1% gelatin.

2 Seed 10,000 cells/cm^2 in growth medium.

3 Proceed with the differentiation protocol (Steps 33-38) but leave the negative control in differentiation medium without additives. Since these cells will keep proliferating, isolate RNA when they are at 90% confluence.

4 Isolate RNA[18] after step 35, and 1, 2 and 3 weeks after seeding the cells.

5 Make cDNA[18] (we use iScript from BioRad, cat no. 170-8891)

6 Perform RT-qPCR using e.g. the following primers for MEF2C, GATA-4 and Nkx-2.5 as early cardiac transcription factors, and Troponin T, βMHC and cardiac-Actin as sarcomeric proteins:

Gene	Primers	Annealing temperature
MEF2C	5'-agatacccacaacacaccacgcgcc-3'	59°C
	5'-atccttcagagagtcgcatgcgctt-3'	
GATA-4	5'-gacaatctggttaggggaagc-3'	55°C
	5'-accagcagcagcgaggagat-3'	
Nkx-2.5	5'-cgccgctccagttcatag-3'	55°C
	5'-ggtggagctggagaagacaga-3'	
TropT	5'-gtgggaagaggcagactgag-3'	62°C
	5'-atagatgctctgccacagc-3'	
βMHC	5'-gaagcccagcacatcaaaag-3'	60°C
	5'-gatcaccaacaacccctacg-3'	
cActin	5'-tcctgatgcgcatttttattc-3'	60°C
	5'-aacaccactgctctagccacg-3'	

The expression of MEF2C, GATA-4 and Nkx-2.5 is expected after the first week of differentiation followed by Troponin T, βMHC and cardiac-Actin after 2-3 weeks.

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TROUBLESHOOTING

22 In general, the cells can be split 1:5 (e.g. from a 24 well (2 cm^2) to a 6 well (10cm^2)). Transfer the 2 ml of cell solution to one gelatin coated well on a 6 well-plate.

CRITICAL STEP Especially in the beginning of the culture period, the cells will grow in small, very dense clusters. When cells are growing in clusters instead of spread through the well, the cells can be split 1:2 (from a 24 well to a 12 well), this way the cells are spread more evenly, and they will reach 80% confluence faster.

23 Passage the cells whenever they reach 70-90% confluence (Figure 2a,b). When the cells grow on a large surface e.g. larger than 10 cm^2, the cells can be cultured in growth medium without adding extra bFGF. Cells can be kept in culture for at least 25 passages. On average, a T75 flask at 90% confluence will harbor approximately 4 million cells.

CRITICAL STEP Do not culture the cells at densities covering less than 50% or more than 90% of the surface of the dish, as this will affect their growth rate and differentiation capacity.

PAUSE POINT Note that cells can be stored in liquid nitrogen, preferentially at an early passage (below passage 10). Cells can be frozen in culture medium containing 10% DMSO, and stored in liquid nitrogen for many years.

Differentiation into cardiomyocytes **TIMING Seeding cells for differentiation: 45 min; differentiation into cardiomyocytes: 3-4 weeks**

24 Coat a 6-well plate or 60 mm dishes with 0.1% gelatin. Per condition that is tested, (e.g. TGFβ stimulated versus non-TGFβ stimulated), prepare at least two wells on a 6 well-plate or two 60 mm dishes. In one of the two wells, place 2 or 3 cover glasses. The coverglasses will be used to determine the degree of differentiation.

CRITICAL STEP When using the differentiation protocol for the first time, it is important to determine the degree of differentiation in time. Coat five extra wells with gelatin (see Box 1) that will be used to check the expression of cardiac genes. Seed the same number of cells and treat them according to the differentiation protocol described below. RNA will be isolated at different time points during the differentiation protocol and cardiac differentiation can be determined by RT-PCR (as described in detail in Box 1).

25 Wash the cultured CMPCs with PBS

26 Add trypsin-EDTA to the flask (e.g., 1 ml to a T75 flask with 90% confluence). Incubate for 2-5 minutes at 37°C.

TROUBLESHOOTING

27 Resuspend the cells in 10 ml of growth medium

28 Add 50 µl of Trypan blue to 50 µl of cell suspension and count the live cells by using a counting chamber.

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29 Centrifuge the single cell suspension for at 300g for 5 min at RT.

30 Resuspend the cells in growth medium, at a concentration of 1 million cells per ml

31 Seed the cells on the gelatin coated surface (from Step 24) at a density of ± 10,000 cells per cm^2 (for example 100 µl in a 6-well plate well) (Figure 3). The cells should be 80-90% confluent the next morning (Figure 2a,b).

TROUBLESHOOTING

32 Add growth medium to the appropriate level (2 ml in 6-well plates or 3 ml in 60 mm dish) and incubate the cells overnight at 37°C

33 The next morning, (if the cells are >80% confluent) change the medium to differentiation medium (2 ml per 6-well).

CRITICAL STEP if cells are under- or overconfluent, stop and plate new cells.

34 After 6-8 hours, change the medium and add differentiation medium containing 5 µM 5-azacytidine (40 µl 5-azacytidine from the stock solution in 2 ml medium, 1:50) (Figure 3).

CAUTION 5-azacytidine is light sensitive, and has a short half-life. When adding the substance to the culture turn off the light in the flow cabinet, and afterwards place the cells in the back of the stove to prevent excessive exposure to light.

CRITICAL STEP 5-azacytidine may cause cell death (Figure 2c,d).

TROUBLESHOOTING

35 In the next 2 d, add 40 µl 5-azacytidine directly to the medium at the end of the day (Figure 3) and refresh differentiation medium at day 4.

36 6 days after the start of the differentiation assay (2 days after finishing the 5-aza stimulation) add 1x Ascorbic Acid (1000x stock, 10^{-4}M), and 1 ng/ml TGF-β to the medium (e.g. 1 µl of stock to 1 ml of medium) (Figure 2).

37 From this point forward, ascorbic acid should be added every 2 d, and TGF-β twice weekly. Both reagents can be added directly to the cells, or changed with medium (Figure 3).

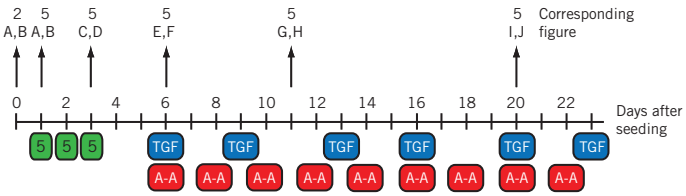

Figure 3 Timeline of the differentiation procedure

The gray line indicates the number of days after plating. The 3 consecutive days when 5-azacytidine is added to the culture are labeled by 5 in the green boxes. TGF-β is added twice weekly, shown by TGF in the blue boxes. Ascorbic Acid is added every two days and indicated by A-A in the red boxes. The time points to refresh the medium are not indicated since they depend on the color of the medium, but in general should be performed once every 3 days. The arrows indicate the corresponding figures showing the cell culture morphology at that point

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38 Refresh medium every 2-3 days, depending on pH of the medium. When refreshing medium, wash the cells twice with PBS to remove all cell debris.
CRITICAL STEP Regularly check the color of the medium. When pH drops too fast and medium turns yellow, refresh medium
TROUBLESHOOTING

Determining the state of differentiation

39 Check regularly for beating cells and make sure that the whole well is investigated; in some cases beating is observed in small clusters, starting between 1.5 and 3 weeks. In time, beating will include most of the cells. The presence of beating cells is considered a successful differentiation, however the degree of differentiation is quantified by staining.
TROUBLESHOOTING

Quantify the degree of differentiation by staining for sarcomeric proteins TIMING 4h

40 Carefully remove the cover glasses from the well or dish, and place them in a 24-well plate, cells facing upwards.
CRITICAL STEP The remaining cells in the well can for example be used for RNA and protein isolation.
TROUBLESHOOTING

41 Gently wash the cells with PBS, aspirate and fixate cells with 500 µl 4% PFA for 15 min at RT.
CRITICAL STEP Never pipette directly onto the cover glass

42 Wash 3 times with 1 ml PBS

43 Add 300 µl 0.1% triton X-100 per well and incubate for 8 min at RT

44 Wash three times with 1 ml PBS

45 Incubate 60 min with 250 µl 4% NGS (diluted in PBS) per well at RT.

46 Meanwhile prepare 50 µl primary antibody solution per sample. Both primary antibodies can be combined. Dilute Troponin-I 1:100 and α-actinin 1:800 in PBS. Pipette 50 µl primary antibody solution onto parafilm. Take the cover glass carefully from the plate with forceps, and place the cover glass (cells down) on the droplet of antibody on the parafilm.

47 Incubate for 60 min at RT

48 Place the cover glasses back into a 24 well plate and wash 3 times with 1 ml of 0.05% PBS-Tween.

49 During last wash step prepare the secondary antibody solution by diluting Alexa fluor 488 goat anti-mouse and Alexa fluor 555 in 1:400 dilution of 4% NGS solution. Again 50 µl for each sample is used.

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CRITICAL STEP While working with secondary antibodies keep samples protected from light as much as possible.

50 Pipette 50 µl secondary antibody solution onto parafilm and place the cover glass on the droplet, cells facing downwards. Incubate during 1 h at RT in the dark.

51 After incubation place cover-glasses back into 24-well plate and wash 3 times with 1ml PBS

52 Add 300 µl/well Hoechst solution. Incubate during 5-10 minutes at RT in the dark
CRITICAL STEP Hoechst is light-sensitive, keep the samples protected from light as much as possible

53 Wash microscope cover glasses 2 times with 1ml PBS, and once with 1 ml distilled water.

54 Pipette 5 µl of mowiol onto a microscope slide per cover glass. Approximately 5 cover slips can be placed on 1 microscope slide.

55 Take cover glasses out of 24 well plate and dip them gently into water and tap of the excess water on a tissue and place them upside down (cells faced down) into mowiol.

56 Analyze on fluorescent microscope with suitable filters. To quantify the degree of differentiation, determine the ratio of nuclei within (double) positively stained cells compared to unstained cells (Figure 4 a-d).
PAUSE POINT The samples can be stored at 4°C in the dark for 2-3 weeks before analyzing.

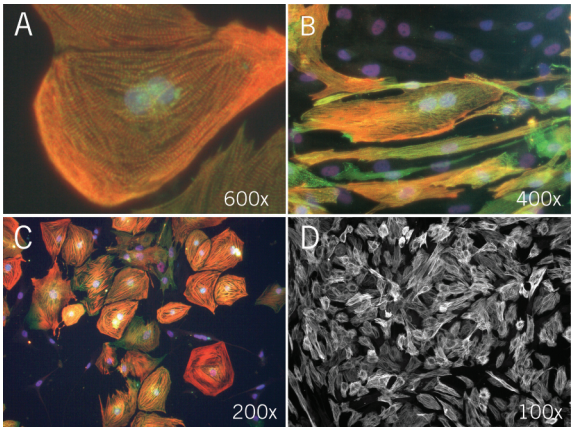

Figure 4. Cardiomyocyte differentiation indicated by staining sarcomeric structures
(a) At high magnification, cross-striations can be observed consisting of troponin–I (red) and α-actinin (green, nuclei in blue). (b) Cells with an adult cardiomyocyte phenotype have an elongated structure. (c) Cardiomyocytes with a more fetal phenotype are rounded, but still display a sarcomeric pattern. (d) High levels of differentiation can be obtained, reaching approximately 90%, indicated by α-actinin staining (in white).

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TIMING

Step 1-16: obtaining a single cell suspension: ~4 h
Step 17Ai-vii: clonogenic isolation: 45-60 min
Step 17Bi-xvii: MACS isolation: can range from 2 to 4 h, depending on flow rate
Step 18-23: culturing CMPCs: after MACS isolation it will take up to 4 weeks to obtain sufficient CMPCs for a differentiation experiment. Using the clonogenic approach, it can take 7-8 weeks
Step 24-38: differentiation procedure: the differentiation procedure takes 3-4 weeks, but this is donor and cell isolation specific.
Steps 39-56: assessment of differentiation: 4 h

TROUBLESHOOTING

Troubleshooting advice can be found in TABLE 1

ANTICIPATED RESULTS

Isolating the CMPCs (steps 1- 17Bxvii).

The number of CMPCs that are obtained depends on the source and size of the human tissue sample used for isolation. When applying the clonogenic isolation approach, approximately 5-10% of the wells contain cells that will start dividing and fill the well after approximately 1 week. Of the dividing clones, ±75% are CMPCs, as determined by RT-PCR (see Box 1), or by their surface protein pattern (Table 2) using flow cytometry as described in[7] or[17]. However, these cell clones need to be tested individually for growth and differentiation potential using the differentiation procedure described above.
When using the MACS isolation approach, expect to have low cell numbers (100 to 5000), especially in the beginning. This number varies per isolation.

Culturing CMPCs

When the cells are growing at a stable rate, expect to passage the cells twice weekly at a surface density of 1:5 – 1:10. If high numbers of cells are necessary they can be passaged 1:5, 3 times per week. This process can be maintained for at least 25 passages.
At an average rate of 1 population doubling per day, this means that at least 75 doublings can be achieved. After this point cells will still grow, but their differentiation capacity diminishes.

Differentiation process

An overview of the differentiation method is given in Figure 3 and the corresponding pho-

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Table 1: Troubleshooting

Step	Problem	Possible reason	Solution
9	Tissue has not dissociated	Concentration of collagenase A too low.	Check the concentration used. If too low, prepare new collagenase stock, add, and re-incubate
		Collagenase stock thawed too many times	Prepare new collagenase stock, add, and re-incubate.
B, ix-xiii	Column is obstructed	Many cardiomyocytes were present in the cell solution	Let the cardiomyocytes sink to the bottom longer.
		Clumps were present in the solution	Carefully pipette the solution up and down in the column. Apply slight pressure by pressing on the top of the column. Remove the fluid and use a 70 µm cell strainer to filter the cell solution before applying the solution to a new column. Pool samples afterwards.
		Cell number was very high	Carefully pipette the solution up and down in the column. Apply slight pressure by pressing on the top of the column. Remove the fluid from the column and dilute in cold M-buffer and apply on multiple columns.
B, xv	No pellet visible in CMPC fraction	Pellet is very small, and is easily overlooked.	Continue protocol, refreshing the wells twice weekly for at least 2 weeks before concluding the isolation has failed.
18	No cells are visible	Cells divide slowly in the beginning	Keep refreshing the wells twice weekly for at least 2 weeks before concluding the isolation has failed
		Concentration of cell suspension too low (clonogenic).	Check if all wells are empty after two weeks. Stop experiment.
		Cells are relatively transparent when growing sparsely, and located at the edges of the well.	Check wells after 1 or 2 washes with PBS. Check the wells more carefully.
21, 26	Cells do not detach after 5 min of trypsin incubation	Trypsin is old	Tap the plate, if cells do not detach, replace trypsin.
		Cells were not washed with PBS prior to trypsinization	Collect trypsinized cells in 15 ml tube, wash the cells with PBS. Collect the PBS in the same tube. Add new trypsin to the cells. Incubate until detached, collect trypsinized cells. Spin tube at 300 g and resuspend the pellet.
31	The well is not 80-90% confluent the day after seeding	Differences between cell isolations in growth rate	If the cells are over/under confluent, stop the experiment. Prepare a test. Seed the cells at different densities and determine the correct cell number to reach 80-90% confluence the next day. Restart the experiment.

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Step	Problem	Possible reason	Solution
33	Cell death after 5-azacytidine treatment	Concentration too high Cell isolations may react different to treatment.	Check stock concentration and added volume. If too many cells die (Figs. 2c,d), the 5-azacytidine treatment can be performed in growth medium. Change to differentiation medium after 3 days of 5-azacytidine treatment. Note: if cells survive in the differentiation medium, this will achieve higher differentiation efficiency. Instead of adding 5 µM once daily, add 3 µM twice daily (morning and evening) to prevent overexposure to 5-azacytidine
38	Medium turns yellow within 2 days.	Change in medium pH. Cells proliferate instead of differentiate	Refresh medium and additives. A change in pH can interfere with the differentiation process. Refresh medium and additives. A change in pH can interfere with the differentiation process. Add more medium (up to 3 ml in a 6-wells-well) when refreshing to create a larger buffer capacity.
39	No beating cells	Old medium (3 days) can inhibit the beating. Cold medium (e.g. cells under the microscope too long) will inhibit the beating. No differentiation	Refresh the medium and reheat to 37°C. Reheat the cells to 37°C. Check the next days.
40	Cells detach from coverglass	Cells may grow differently on coated glass surface	Coat cover glasses with gelatine in a 24 wells plate. Detach the differentiated CMPCs with 1 mg/ml collagenase for approx 10 min, and add 1:6 diluted trypsin for additional 5 min at 37ºC. Let the cells attach to the coated glasses for 1 or 2 days. Note: this will lead to a dying of fraction of the differentiated cardiomyocytes and will give an underestimation of the degree of differentiation
24-38	No differentiation	Serum batch specific Cells lost differentiation capacity	If no differentiation is observed, test different serum batches, since differentiating capacity can be batch specific. Use lower passage of cells (preferably <20 passages)
24-38	Cells detach during differentiation process	Cells grow too confluent Wash more carefully	Determine the optimal plating density. Instead of two times, wash only 1 time with PBS when refreshing.

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Table 2: Surface marker expression profile on undifferentiated CMPCs

Surface marker	presence
CD45	-
CD14	-
CD34	-
CD133	-
CD105	+
CD31	+
Sca-1(-like)	+
c-Kit	+/-

tos of the cell culture are indicated. The expected appearance of the cells is provided in Figure 5. After the addition of 5-azacytidine, one can note the appearance of small black extrusions at cell membrane border and increased black dots in the cytosol, indicating that the 5-azacytidine stimulation works (Figure 5a,b). After addition of 5-azacytidine for 3 d, the cells return to their normal morphology (Figure 5c,d). If cells continue to be stressed or massive cell death has taken place, stop the experiment. After the treatment with TGF-β for 1 d, cells are slightly enlarged and the culture has reached 100% confluence. Additionally, cells should align in circular patterns (Figure 5e,f). When the cells have been treated with TGF-β three times, the cells start to grow in multiple layers with different cell alignments (Figure 5g,h). The areas that may start to beat can be recognized as clusters of cells with a high density (Figure 5i,j). The beating often starts in these small clusters, but if the differentiation has been performed optimally, the whole well can beat synchronously. An average rate of 40 beats per minute can be measured[7]; however this depends on the temperature and state of both the medium and cells. Beating can continue for at least 10 weeks. As the cells no longer divide at this point, it is sufficient to refresh the medium twice weekly.

Quantification of differentiation

The staining of the sarcomeric proteins should lead to striated patterns (Figure 4a) co-localizing with nuclei. The cells can have an elongated morphology, resembling a more adult phenotype (Figure 4b), as well as a rounded structure which is more comparable to fetal cardiomyocytes (Figure 4c). When differentiation has been successful, almost 90% of the cover glass contains cardiomyocytes, based on the number of nucleated cells that are positive for Troponin or Actinin (Figure 4d).

The number of cardiomyocytes obtained from one differentiation experiment can reach 80% of the starting population, since not all cells will differentiate and a percentage of cells will die after 5-azacytdine treatment.

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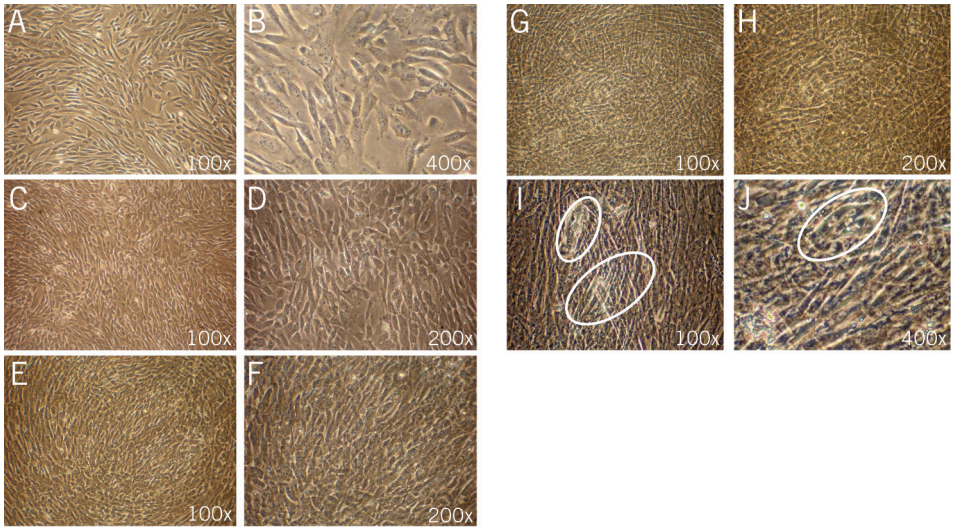

Figure 5. Morphological characteristics of CMPCs during the differentiation procedure
(a) One day after the addition of 5-azacytidine, the general morphology of the cells is intact but there is an increase in small dark spots in the cytosol (b, indicated by arrows). (c,d) Treating the cells with 5-azacytidine three times; the cells grow more dense and have a normal morphology. (e,f) After the first treatment with TGFβ, the cells may reach 100% confluence, and start to form circular patterns. (g,h) When the cells have been treated with TGFβ 3 times, the cells grow in multiple layers and in different directions. (i,j) Beating typically starts in dense cell clusters, indicated by the white circles.

ACKNOWLEDGEMENTS

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REFERENCES

1. Smits AM, van Vliet P., Hassink RJ, Goumans MJ, Doevendans PA. The role of stem cells in cardiac regeneration. J Cell Mol Med 2005;9(1):25-36.
2. van Vliet P, Sluijter JP, Doevendans PA, Goumans MJ. Isolation and expansion of resident cardiac progenitor cells. Expert Rev Cardiovasc Ther 2007;5(1):33-43.
3. Mitcheson JS, Hancox JC, Levi AJ. Cultured adult cardiac myocytes: future applications, culture methods, morphological and electrophysiological properties. Cardiovasc Res 1998;39(2):280-300.
4. Passier R, Mummery C. Cardiomyocyte differentiation from embryonic and adult stem cells. Curr Opin Biotechnol 2005;16(5):498-502.
5. Passier R, Oostwaard DW, Snapper J, Kloots J, Hassink RJ, Kuijk E, Roelen B, Brutel de la Riviere A, Mummery C. Increased cardiomyocyte differentiation from human embryonic stem cells in serum-free cultures. Stem Cells 2005;23(6):772-780.
6. Braam SR, Denning C, van den Brink S, Kats P, Hochstenbach R, Passier R, Mummery CL. Improved genetic manipulation of human embryonic stem cells. Nat Methods 2008;5(5):389-392.
7. Goumans MJ, de Boer TP, Smits AM, van Laake LW, van Vliet P., Metz CH, Korfage TH, Kats KP, Hochstenbach R, Pasterkamp G, Verhaar MC, van der Heyden MA, de Kleijn D, Mummery CL, van Veen TA, Sluijter JP, Doevendans PA. TGF-beta1 induces efficient differentiation of human cardiomyocyte progenitor cells into functional cardiomyocytes in vitro. Stem Cell Res 2007;1(2):138-149.
8. van Vliet P, Roccio M, Smits AM, van Oorschot AA, Metz CH, van Veen TA, Sluijter JP, Doevendans PA, Goumans MJ. Progenitor cells isolated from the human heart: a potential cell source for regenerative therapy. Neth Heart J 2008;16(5):163-169.
9. Messina E, De Angelis L, Frati G, Morrone S, Chimenti S, Fiordaliso F, Salio M, Battaglia M, Latronico MV, Coletta M, Vivarelli E, Frati L, Cossu G, Giacomello A. Isolation and expansion of adult cardiac stem cells from human and murine heart. Circ Res 2004;95(9):911-921.
10. Bearzi C, Rota M, Hosoda T, Tillmanns J, Nascimbene A, De Angelis A, Yasuzawa-Amano S, Trofimova I, Siggins RW, Lecapitaine N, Cascapera S, Beltrami AP, D'Alessandro DA, Zias E, Quaini F, Urbanek K, Michler RE, Bolli R, Kajstura J, Leri A, Anversa P. Human cardiac stem cells. Proc Natl Acad Sci U S A 2007;104(35):14068-14073.
11. Urbanek K, Quaini F, Tasca G, Torella D, Castaldo C, Nadal-Ginard B, Leri A, Kajstura J, Quaini E, Anversa P. Intense myocyte formation from cardiac stem cells in human cardiac hypertrophy. Proc Natl Acad Sci U S A 2003;100(18):10440-10445.
12. Martin CM, Meeson AP, Robertson SM, Hawke TJ, Richardson JA, Bates S, Goetsch SC, Gallardo TD, Garry DJ. Persistent expression of the ATP-binding cassette transporter, Abcg2, identifies cardiac SP cells in the developing and adult heart. Dev Biol 2004;265(1):262-275.
13. Laugwitz KL, Moretti A, Lam J, Gruber P, Chen Y, Woodard S, Lin LZ, Cai CL, Lu MM, Reth M, Platoshyn O, Yuan JX, Evans S, Chien KR. Postnatal isl1+ cardioblasts enter fully differentiated cardiomyocyte lineages. Nature 2005;433(7026):647-653.
14. Barile L, Messina E, Giacomello A, Marban E. Endogenous cardiac stem cells. Prog Cardiovasc Dis 2007;50(1):31-48.
15. Choi SC, Yoon J, Shim WJ, Ro YM, Lim DS. 5-azacytidine induces cardiac differentiation of P19 embryonic stem cells. Exp Mol Med 2004;36(6):515-523.
16. Makino S, Fukuda K, Miyoshi S, Konishi F, Kodama H, Pan J, Sano M, Takahashi T, Hori S, Abe H, Hata J, Umezawa A, Ogawa S. Cardiomyocytes can be generated from marrow stromal cells *in vitro*. J Clin Invest 1999;103(5):697-705.
17. Duda DG, Cohen KS, Scadden DT, Jain RK. A protocol for phenotypic detection and enumeration of circulating endothelial cells and circulating progenitor cells in human blood. Nat Protoc 2007;2(4):805-810.
18. Nolan T, Hands RE, Bustin SA. Quantification of mRNA using real-time RT-PCR. Nat Protoc 2006;1(3):1559-1582.

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Human cardiomyocyte progenitor cell transplantation preserves long-term function of the infarcted mouse myocardium

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ABSTRACT

Aims

Recent clinical studies revealed that positive results of cell transplantation on cardiac function are limited to the short and mid-term restoration phase following myocardial infarction (MI) emphasizing the need for long-term follow-up. These transient effects may depend on the transplanted cell-type or its differentiation state. We have identified a population of cardiomyocyte progenitor cells (CMPCs) capable of differentiating efficiently into beating cardiomyocytes, endothelial cells and smooth muscle cells *in vitro*. We investigated whether cardiomyocyte progenitor cells (CMPCs) or pre-differentiated CMPC-derived cardiomyocytes (CMPC-CM) are able to restore the injured myocardium after MI in mice.

Methods

MI was induced in immunodeficient mice, followed by intra-myocardial injection of CMPCs, CMPC-CM or vehicle. Cardiac function was measured longitudinally up to 3 months post-MI using 9.4 Tesla MRI. The fate of the human cells was determined by immunohistochemistry.

Results

Transplantation of CMPCs or CMPC-CM resulted in a higher ejection fraction and reduced the extent of left ventricular remodeling up to three months after MI compared to vehicle-injected animals. CMPCs and CMPC-CM generated new cardiac tissue consisting of human cardiomyocytes and blood vessels. Fusion of human nuclei with murine nuclei was not observed.

Conclusions

CMPCs differentiated into the same cell-types *in situ* as can be obtained *in vitro*. This excludes the need for *in vitro* pre-differentiation, making CMPCs a promising source for (autologous) cell-based therapy.

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INTRODUCTION

Cardiovascular disease is the leading cause of death in the western world[1]. Following myocardial infarction (MI), massive cell death in the myocardium initiates fibrosis and scarring. This negatively affects heart function and can ultimately lead to heart failure and sudden cardiac death. Repairing the damaged tissue with healthy myocytes provides an attractive therapeutic option to restore cardiac function and structure after MI[2]. However, replacing cardiomyocytes through *in situ* proliferation or transplantation of mature cardiomyocytes is compromised owing to the post-mitotic state of adult cardiomyocytes and their failure to survive ectopic transplantation[3]. Other cell-types have therefore been investigated as sources of transplantable cardiomyocytes[4].

Several clinical trials have been launched where bone marrow-derived cells were injected into the injured myocardium[5]. At mid-term follow-up most trials showed positive effects on cardiac function, but in some cases this effect was merely transient[6,7]. These findings demonstrate the requirement for long-term analysis of cardiac function before robust conclusions on effectiveness of transplanting a particular cell population on cardiac function can be made.

The heart itself contains pools of progenitor cells that are committed to cardiac cell lineages[8-11]. Recently, we described a population of cardiomyocyte progenitor cells (CMPCs) isolated from the fetal and adult human heart[12,13]. CMPCs differentiate efficiently and robustly into spontaneously beating cardiomyocytes *in vitro* after stimulation with 5-azacytidine and TGF-β without the need for co-culture with cardiac fibroblasts or myocytes. CMPC-derived cardiomyocytes (CMPC-CM) have an electrophysiological profile similar to fetal ventricular myocytes, indicating their potential to survive transplantation and integrate into the myocardium[3]. Additionally, *in vitro* exposure of CMPCs to vascular endothelial growth factor (VEGF) resulted in their differentiation into endothelial cells and smooth muscle cells[12].

Since in culture CMPCs are able to form all cell-types required to restore the damaged myocardium, we questioned whether intra-myocardial transplantation of the undifferentiated CMPCs would be sufficient to form new cardiac tissue. For the first time, we have compared the potential of (undifferentiated) human CMPCs and their differentiated offspring (CMPC-CM) to regenerate the infarcted mouse heart and affect cardiac function in long-term follow-up.

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METHODS

CMPC isolation, culture and differentiation to CMPC-CM

Approval by the Medical Ethics committee of the University Medical Center Utrecht for these experiments was obtained. Human fetal hearts were collected after elective abortion based on individual informed consent. The investigation conforms to the principles outlined in the Declaration of Helsinki. A single cell suspension was obtained by Langendorff perfusion with Tyrode's solution containing collagenase and protease. After cardiomyocyte depletion, CMPCs were isolated from suspension by magnetic cell sorting, using magnetic Sca-1 coupled beads (Miltenyi Biotech Inc.)[13]. Cells were cultured on gelatin coated dishes in growth medium[13] for continued propagation or on coverslips for immunofluorescent staining. Differentiation towards cardiomyocytes was induced by treating the cells with 5 μM 5-azacytidine for 72 hours in differentiation medium[12,13] followed by stimulation with 1 ng/ml TGFβ1 (PeproTech). The cells started beating 2-3 weeks after stimulation, and were designated CMPC-CM. For transplantation experiments, CMPCs (passage 7) of 5 different isolations were used. Transplanted CMPC-CM were beating in culture for at least 2 weeks. Cells were trypsinized, washed twice in PBS and counted. Cells were kept at room-temperature prior to transplantation. CMPCs and CMPC-CM were used to generate conditioned medium. The cells were cultured in 1:1 Dulbecco's modified eagle medium (DMEM, Gibco) and M199 (Gibco) containing 0.5% fetal bovine serum for 2 days. Medium was collected and used in a human VEGF ELISA (Peprotech) according to the manufacturer's protocol. Absorbance was measured at 415 nm.

Real-time PCR

RNA was isolated from cultured cells using Tripure isolation reagent (Roche Applied Science) according to the manufacturer's protocol. cDNA was synthesized with the iScript cDNA synthesis kit (Bio-Rad Laboratories). Quantitative realtime-PCR was performed with Sybr green mastermix (Bio-Rad Laboratories). Values were normalized for β-Actin expression and the relative expression compared to CMPCs was calculated by ΔΔCT. Primer sequences are available upon request.

Immunofluorescent staining on cultured cells

Cells were fixed in 2% paraformaldehyde, washed with PBS and stained as described previously[14] with antibodies directed against human integrin-β1 (a gift from A. Sonnenberg, Netherlands Cancer Institute) troponin-I (1:500, rabbit, Chemicon AB1627). Cy3-labeled (1:250, Jackson ImmunoResearch) and Alexa488-labeled (1:400, Invitrogen) secondary antibodies were used.

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Animals

All experiments were carried out in accordance with the Guide for the Care and Use of Laboratory Animals, prepared by the institute of Laboratory Animal Resources and with prior approval by the Animal Ethical Experimentation Committee, Utrecht University.

Myocardial infarction and cell transplantation

In male NOD-SCID mice (Charles River Laboratories), aged 10-12 weeks, MI was induced by ligation of the left coronary artery under isoflurane anesthesia, as described previously[15]. Immediately after MI, 10µl PBS containing $0.5x10^6$ CMPCs (n=20) or CMPC-CM (n=10) was divided over two injection-sites in the infarct-borderzone. As negative control, 10 µl vehicle (PBS) was injected (n=18). All surgical procedures and injections were performed by a blinded investigator. To determine if surgical procedures were performed uniformly, mice that received CMPC injection (n=9) or PBS (n=9) were sacrificed 2 days after MI. Hearts were flushed with ice-cold PBS, weighed and cut into 1 to 1.5 mm slices. Each slice was weighed and incubated in 1% Triphenyl tetrazolium chloride (TTC, Sigma) at 37°C for 20 minutes. After washing in 10% formalin for 3 hours, the slices were scanned on both sides. The percentage of infarcted (white) surface area was analyzed using ImageJ, and subsequently the weight of the infarct compared to the total heart weight was calculated.

MRI measurements

Cardiac parameters were determined 2 days, 4 weeks and 12 weeks post-MI. End-diastolic volume, end-systolic volume and ejection fraction were measured by magnetic resonance imaging (MRI) on a 9.4 Tesla scanner (Bruker Biospin) as described previously[15,16]. Analysis was performed using Q-mass for mice digital imaging software (Medis) by a blinded investigator.

Infarct size was estimated as the a- or dyskinetic proportion of the circumference of the left ventricle. Wall thickness was determined at the level of the papillary muscles, and 3 axial slices apical-wards. The circumference of the LV was divided into 6 equal regions. The inferior septal part was used to determine the septal thickness, the lateral, anterolateral and inferior lateral values were pooled to represent the infarct thickness. The anterolateral septal value combined with the inferior value were pooled to give the borderzone value.

Characterization of grafts by immunofluorescence

Mice were sacrificed 3 months post-MI, the hearts were isolated and fixed as described previously[17] and embedded in Tissue Tec (Sakura Finetek). Hearts were cut into 7 µm cryosections, which were stained as described[15] using the following antibodies and dilu-

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tions: human integrin-β1 (undiluted) (mouse, a gift from A. Sonnenberg, Netherlands Cancer Institute), troponin–I (1:500, rabbit, Chemicon), myosin light chain (MLC)2A (1:100, mouse, Synaptic Systems), connexin-43 (1:200, rabbit, Zymed), smooth muscle actin (1:100, rabbit, Abcam), human CD31 (1:300, mouse, DAKO), Ki-67 (1:100, rabbit, Abcam), human mitochondria (1:500, mouse, Chemicon, MAB1273), desmoplakin (undiluted, mouse, Progen Biotechnik). Isotype controls (DAKO) were performed for each class of antibody used. Cy-3 labeled secondary antibodies were obtained from Jackson ImmunoResearch Laboratories and Alexa 488 labeled secondary antibodies from Invitrogen. DAPI, dissolved in Vectashield mounting medium (Vector Laboratories) was used for nuclear counterstaining. Confocal laser scanning microscopy was performed on a Leica TCS SPE confocal on a DMI4000B microscope using LAS-AF software (Leica application suite- advanced fluorescence). For quantification of phenotype markers, all human β1 integrin positive cells in at least 6 evenly distributed sections of each heart (n=5 per group) were scored by two blinded investigators. The number of cells in the graft was calculated based on the distance between sections containing positive cells and the average size of a cell.

Fluorescent in situ Hybridization of human and mouse chromosomes

After fluorescent images were obtained, fluorescent in situ hybridization was performed as described previously[18]. Briefly, coverglasses were removed and sections were washed with SSC, treated with RNaseI, and pepsin before applying fluorescently labeled probes. Slides and probes were denatured simultaneously and hybridized for 16 hours hybridization at 37°C followed by post hybridization washes in 50% formamide, 2xSSC at 44°C and 0.1xSCC at 60°C. Finally slides were embedded in Citifluor AF1D (Citifluor Ltd.) solution containing 4',6-diamidino-2-phenylindole (DAPI) (400 ng/ml). As probes for detecting mouse nuclei, murine whole chromosome painting probes for chromosome 1 and 12 were labeled with RGreen-ULS (Kreatech, The Netherlands), and for detecting human nuclei human whole chromosome painting probes chromosomes 1 and 12 were labeled with Cy3-ULS (Kreatech, The Netherlands) precipitated using both mouse and human Cot-1 DNA (both from Invitrogen). Using re-localization technique, images were obtained at the previously documented locations of the grafts. Images were obtained on a Leica epifluorescence microscope equipped with a Quantix, CCD digital camera (Photometrix).

Immunohistochemical staining

For quantification of vessel density, cryosections were post-fixed with acetone, and blocked for endogenous peroxidase activity. After blocking with 1% bovine serum albumin (BSA), the sections were incubated overnight at 4°C with mouse CD31 (1:20, rat, Cell Signaling). A second blocking step was performed using TNB (Perkin Elmer TSA-

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kit). Subsequently, the sections were incubated with a secondary rat antibody (1:250, rabbit, DAKO). Rabbit Powervision-HRP (Immunologic) incubation was followed by incubation with diamino benzidine (DAB) substrate to visualize CD31. Nuclei were counterstained with hematoxylin before dehydrating and mounting with Pertex. Vessel density was counted in 6 hearts per group from 20 fields per heart divided over 3 to 5 sections at standardized locations (infarct, borderzone and healthy tissue) along the long axis of the infarct area using an Olympus-BH2 microscope with AnalySIS software. The number of small vessels in the borderzone and infarct region was counted and calculated as vessels per square millimeter.

Statistical analysis

Statistical significance was evaluated with SPSS 14.0 for Windows using ANOVA (with Bonferroni correction for multiple-group comparisons) or Mann-Whitney U test, as applicable. Survival was analyzed by Kaplan-Meier test. Results are expressed as mean ± SEM. A value of $p \leq 0.05$ was considered statistically significant.

RESULTS

Characterization of transplantation sources

Human CMPCs were isolated and cultured for at least 4 passages before differentiation was initiated. Cardiomyogenic differentiation was evident by the presence of beating cells 2-3 weeks after 5-azacytidine stimulation. Gene expression analysis of CMPCs and CMPC-CM generated from the same donor heart was carried out to confirm their differentiation into cardiomyocytes. The cardiac genes MLC-2V, β-MHC, cardiac-actin and troponin-T were highly expressed in CMPC-CM (supplemental Figure 1A). Protein expression of troponin-I in CMPC-CM was confirmed by immunohistochemistry on cells cultured on cover slides (supplemental Figure 1B to 1G). The percentage of nucleated cells that showed striated sarcomeric troponin–I patterns was determined as the degree of differentiation. Clear striated sarcomeres were present in more than 70% of the CMPC-CM culture (not shown). troponin-I positive cells were never observed on cover slips carrying CMPCs (supplemental Figure 1C and 1F). Therefore we conclude that CMPC-CM represent the differentiated cardiomyocyte-cell fraction of the CMPCs.

General health and survival

After induction of MI, mice were followed longitudinally for 12 weeks. During this period there was no significant difference in survival between the groups (Figure 1A). Starting two days after surgery, the average body weight increased in all animals (Figure 1B).

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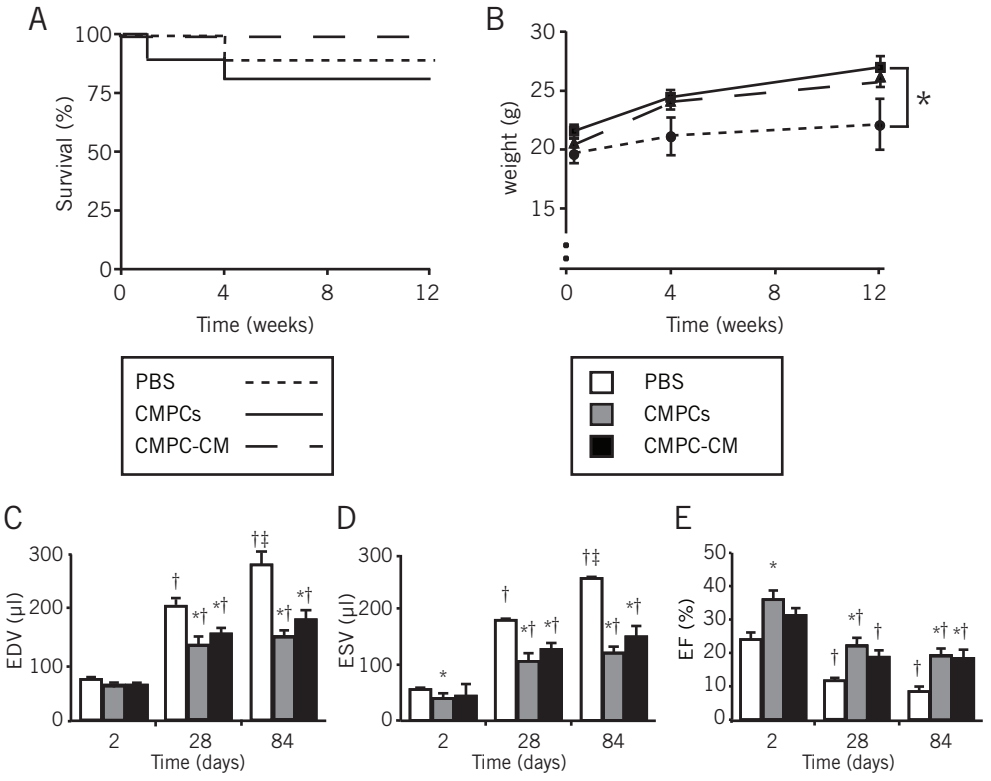

Figure 1. General health and cardiac function

(A) Survival curve of all mice during the course of the experiment. At the start groups consisted of PBS n=9, CMPC n=11, CMPC-CM n=10. (B) Body weight was significantly lower in the vehicle group compared to CMPC treated group after 12 weeks: * p<0.05. (C) End-diastolic volume, (D) end-systolic volume and (E) ejection fraction were determined 2 days, 4 weeks and twelve weeks post-surgery by high resolution MRI. In cell injected animals less deterioration of cardiac function in observed in time. * p<0.05 compared to PBS, † p<0.05 compared to two days measurement of the same group, ‡ p<0.05 compared to 4 weeks measurement of the same group.

After 3 months, the PBS-injected control animals weighed significantly less than the CMPC-treated animals (27 ± 2g versus 31 ± 1g, p=0.049), indicating the animals were less healthy. However, this did not affect the survival of the animals.

LV function

Cardiac performance was monitored by MRI during 3 months post-MI. Two days after surgery the EDV was comparable between the three groups (Figure 1C). The ESV was slightly lower in the CMPC-transplanted group compared to control animals (39 ± 4µl versus 55 ± 4µl respectively, p= 0.036, Figure 3B). Four and twelve weeks post-MI the EDV and ESV in both cell-injected groups were significantly lower compared to PBS recipients (Figure 1C and 1D). Whereas after 4 weeks the volumes continued to rise

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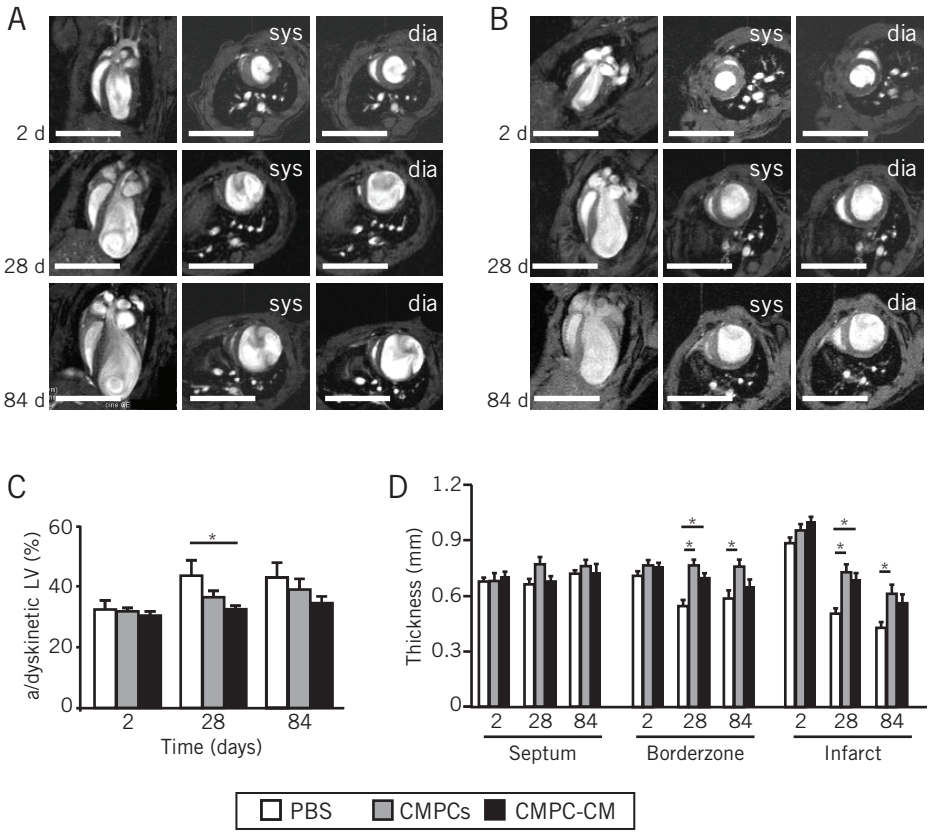

Figure 2. LV remodeling
Series of MRI images showing the same PBS (A) or CMPC (B) receiving animal in time. A four-chamber view (left) is provided for each time-point as well as the axial view in systole (sys) and diastole (dia). (C) Infarct size represented as the percentage of the circumference of the LV that was a- or dyskinetic shows no differences at the start of the experiment. (D) Wall-thickness was measured at three time-points post-surgery in 3 different regions: septum, borderzone and infarct. Wall thinning in time was observed mostly in PBS-injected animal. Scale bars: 1 cm, * $p<0.05$.

in the control group, these values remained stable in the cell treated animals, implying that cell transplantation prevents dilatation of the LV. The functional benefit of cell transplantation on cardiac function was evident from the ejection fraction which, after a decrease directly after MI, stabilized in the cell-treated animals from 4 weeks post-MI onwards (Figure 1E). Overall, cell transplantation of either cell-type had a positive effect on cardiac performance.

LV Remodeling

Detailed investigation of the MRI data revealed that cell-injection prevents progression of adverse LV-remodeling. Following MI an increase in the size of the heart was observed

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(Figure 2A and 2B) which was more pronounced in controls (Figure 2A) than in the cell-transplantation animals (Figure 2B). We excluded the possibility that variation in surgical procedures confounded the observations by analyzing the infarct size in two ways. Two days post-MI hearts of cell receiving (n=9) or PBS receiving (n=9) animals were excised and used for TTC staining to analyze the size of the infarct. We did not find any differ-

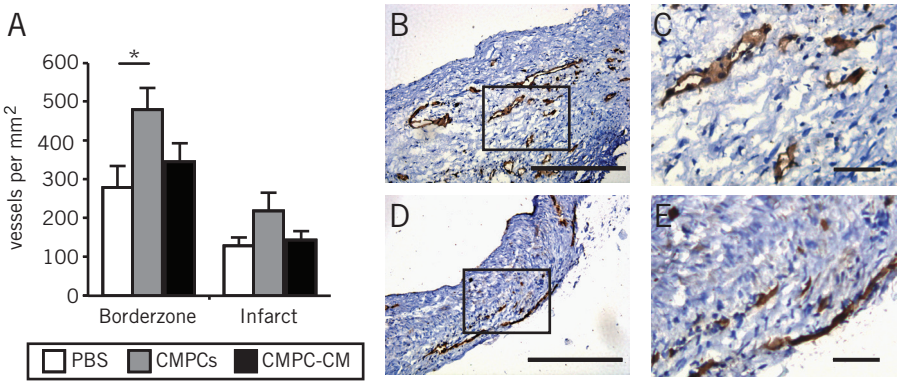

Figure 3. Vessel density
The vascular density in the borderzone and the infarct region was assessed by counting the number of CD31⁺ small vessels per mm² (A) Representative pictures of the CD31 staining are shown for the borderzone (B, inset is shown in C), and the infarcted region (D, inset in E). Scale bars: (B and D) 500 µm, (C and E) 90 µm. * p<0.05.

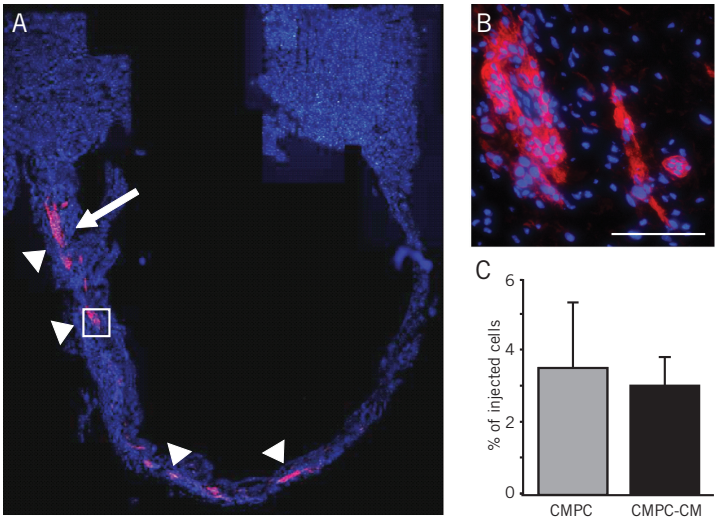

Figure 4. Graft location and size
An overview of integrin-β1⁺ human cells (red) in the infarcted heart, reconstructed from single images. Arrow indicates the site of injection. Arrow heads point at patches of human grafts. The white square is enlarged in (B). The total number of cells found in the heart is represented as the percentage of the total injected cell population (C). Scale bar: 100µm.

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ence in the percentage of infarcted heart between these groups (supplemental Figure 2). Alternatively we determined the percentage of a- or dyskinetic myocardium, which can be measured throughout the experiment and here we also found that the infarct was similar among groups (Figure 2C). After 4 weeks, the infarcted region in the PBS group was larger compared to the CMPC-CM injected animals (43.38% ± 0.05 and 32.29% ± 0.01 of the LV circumference, p=0.03) (Figure 2C), again indicating the prevention of adverse remodeling by cell injection. Preservation of cardiac structure is likely to play an important role in preventing further remodeling. Indeed, the LV-wall in the cell-treated animals was thicker than in controls at 4 weeks post- MI (Figure 2D). After 12 weeks the LV-wall remained thicker in CMPC-injected animals, compared to vehicle.

Vessel density

Cell injection may impose a paracrine effect on the surrounding heart tissue, therefore the density of small murine blood vessels in the border zone and infarcted myocardium was quantified (Figure 3A to 3C). Interestingly, only transplantation of CMPCs led to more vessels in the borderzone compared to vehicle controls (478 ± 56 versus 278 ± 57 vessels per mm^2 p=0.041), and showed the same trend in the infarcted region. However, this effect was not observed in the CMPC-CM receiving group. Since the difference in vessel density may represent a change in cytokine excretion by the different cell-types, we performed an ELISA for human VEGF on conditioned medium of CMPC and CMPC-CM. CMPC conditioned medium contained 223 ± 9 pg/ml while CMPC-CM did not produce any detectable VEGF (supplemental figure 3).

Graft location and size

The transplanted CMPCs and CMPC-CM were traced with an antibody recognizing human integrin-β1, which is expressed constitutively on the human cell membrane. The transplanted cells formed patches of viable tissue in the borderzone as well as in the infarcted region (Figure 4A and 4B). Grafts were occasionally detected in the apex (Figure 4A), indicating that cells were not confined to the site of injection. We identified 3.5 ± 1.8% and 3.0 ± 0.8% of the initially injected CMPCs or CMPC-CM respectively (Figure 4C) and there was no significant difference between the cell-types.

***In vivo* differentiation of CMPCs**

While CMPCs did not express any sarcomeric proteins at the time of transplantation (supplemental Figure 1D) intra-cardiac grafts contained troponin-I^+ human cells (Figure 5A). Likewise, CMPC-CM derived grafts consisted predominantly of troponin-I^+ cells (Figure 5B). In contrast to murine ventricular myocardium, nearly all human cardiomyocytes expressed MLC2A, in accordance with a fetal-like-phenotype[19] (Figure 5C and 5D). Both

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in vivo differentiated CMPCs (Figure 5E and 5F), and *in vitro* differentiated CMPC-CM (not shown) in grafts had clearly distinguishable sarcomeres. To assess coupling of the donor cardiomyocytes, we looked for the presence of desmosomal and gap junction

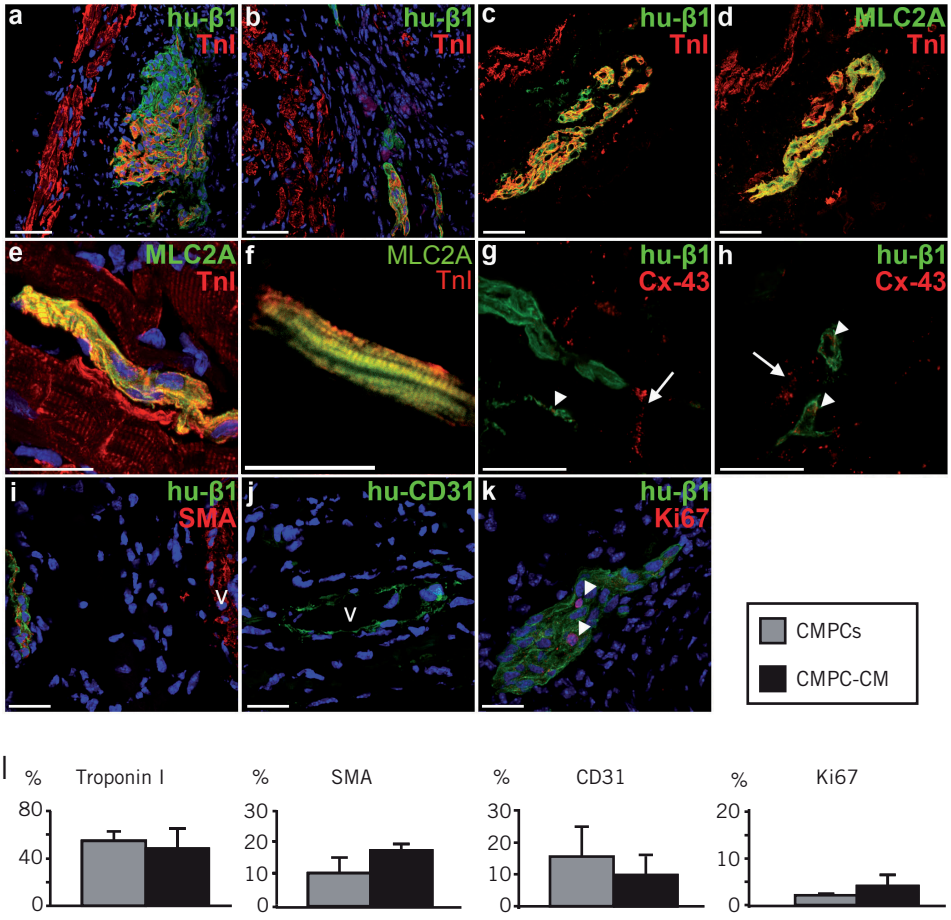

Figure 5. Differentiation of human cells

(A and B) Staining for human integrin-β1 and troponin-I in hearts, of CMPC- (A) or CMPC-CM- (B) receiving animals 12 weeks post-surgery shows the presence of human derived cardiomyocytes. (C and D) Two consecutive tissue sections indicating that human cells (C) expressing troponin-I (C and D) are also positive for MLC2A (D). (E and F) Sarcomeric striated patterns of MLC2A and troponin-I are present in CMPCs. (G and H) Connexin-43 is less present between human cells (arrowheads), than between mouse cells (arrows); (G) image from CMPC receiving animal, (H) from CMPC-CM receiving animal. (I) Staining for human integrin-β1 and smooth muscle actin shows human-derived smooth muscle cells. (J) Human specific CD31 staining indicates human-derived endothelial cells arranged in a vessel-like structure (v). (K) Cell-cycle activity in human cells is visualized with Ki67 staining. (L) Quantification of phenotypes expressed as percentage of total human cells found in the graft. Scale bars (A to D) 50 μm, (E to K) 25 μm. Blue: nuclei (DAPI). Hu-β1= human integrin-β1, TnI = troponin I; MLC2A = myosin light chain-2A; Cx-43 = connexin-43; SMA = smooth muscle actin

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proteins. Desmoplakin was abundant in the graft area (not shown); connexin-43 was present at gap junctions between the human cardiomyocytes, but was found in higher quantities between adult mouse cardiomyocytes (Figure 5G and 5H). Connexin-43 was not observed between human and mouse cardiomyocytes.

Composition of human grafts

Troponin-I^{+} cardiomyocytes represented the major cell-type present in the human grafts, accounting for 54.8 ± 7.7% of the total human cells in the CMPC- and 48.0 ± 17.2% in the CMPC-CM grafts (Figure 5L). A proportion of the human cells had differentiated into smooth muscle cells (Figure 5I); of the total human cells 10.6 ± 4.9% in the CMPC and 17.7 ± 2.3% in the CMPC-CM group were positive for smooth muscle actin (Figure 5L). Human endothelial cells formed vessel-like structures (Figure 5J). CD31 was present on 15.5 ± 9.3% (CMPC) and 9.7 ± 6.4% (CMPC-CM) of the human cells (Figure 5L). Furthermore cell cycle activity, indicated by positive nuclear staining for Ki-67, was present in a small fraction of the donor cells (2.08 ± 0.3% in CMPC and 4.13 ± 2.4% in CMPC-CM grafts)(Figure 5K and 5L). Quantification of cardiomyocyte, smooth muscle and endothelial cells in CMPC- and CMPC-CM derived grafts revealed no differences between the groups (Figure 5L). Thus, *in vivo* transplantation of CMPCs resulted in formation of the same cell-types that were identified when transplanting pre-differentiated cells.

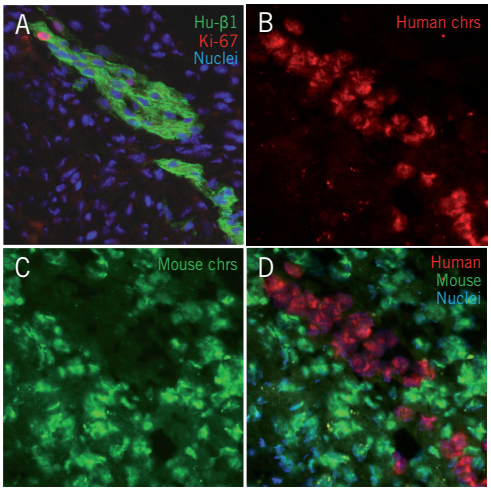

Figure 6. Analyzing fusion

(A) Shape of a human graft is demarcated by integrin-β1 staining; Ki67 staining shows the ability to stain nuclei, nuclei in blue. (B to D) The same sample as in (A) stained for (B) human chromosomes 1 and 12 (green) and (C) mouse chromosomes 1 and 12 (red). (D) Overlay of D and E shows no colocalization of chromosomes. Chrs = chromosomes; Hu-β1= human integrin-β1

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Analysis of fusion between human and mouse nuclei

We investigated the possibility that newly formed human cardiac tissue was the result of fusion of surviving mouse myocardium with transplanted human cells. Fluorescent in situ hybridization was performed on tissue sections in which the shape and location of the human graft had been documented (Figure 6A). We re-stained these samples with probes labeled red for human chromosomes (Figure 6B), and green recognizing mouse chromosomes (Figure 6C). In the overlay (Figure 6D) it is clear that there are no nuclei double-positive for both human and mouse chromosomes. The shape of the human graft (Figure 6D) corresponds with the human antibody staining (Figure 6A). We analyzed multiple sections and found no nuclear fusion events. Furthermore, we found no evidence for cellular fusion based on a human cell having a mouse and a human nucleus.

DISCUSSION

This is the first study that directly compares the potential of undifferentiated and differentiated offspring of the same progenitor cell source, and investigates their long-term effects of intra-myocardial transplantation on cardiac function. The principal questions addressed were: (i) can CMPCs or their cardiomyocyte derivatives restore damaged myocardium, and (ii) is it necessary to pre-differentiate CMPCs? CMPCs may have greater potency and can therefore be more efficacious, while differentiated cells might be safer with regard to the formation of unwanted cell-types including the formation of teratoma, which is observed when transplanting undifferentiated embryonic stem cells[20]. Differentiating CMPCs into cardiomyocytes is labor intensive and takes several weeks[13], increasing the risk of introducing mutations in the cells. In the present study we show that there is no advantage using pre-differentiated CMPC for cell transplantation post MI.

To address our research questions, we directly compared the CMPC to CMPC-CM transplanted animals in the functional analysis. Both CMPCs and CMPC-CM were able to prevent progression of cardiac dilatation and maintain EF compared to vehicle. This was not accompanied by a decreased survival rate in the PBS receiving mice. However, they did show a significantly lower body weight at three months post-MI.

The long-term cardiac improvement is likely cell-type specific. Recent studies from van der Bogt et al.[21] as well as Smith and co-workers[22], show that inert cell-types like fibroblasts are not able to improve cardiac function when compared to cardiac derived cells[22] or mononuclear cells[21]. Fibroblast injection resulted in the same deterioration of cardiac function as observed with PBS injection[21,22], arguing that CMPCs have an additive value. Moreover, the enhanced cardiac function was sustained compared to control animals for 3 months, similar to our previous study injecting human embryonic stem cell-derived

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cardiomyocytes[23], which is important since several clinical cell transplantation studies have reported positive effects on cardiac performance at short-term follow-up that were not always sustained[6,7].

A small difference in ejection fraction was observed in the cell-injected groups at two days after MI that could not be explained by differences in infarct size. All surgical procedures as well as MRI analysis were performed by blinded investigators. TTC staining after 2 days, as well as MRI data on kinesis showed no difference in infarcted myocardium at two days. Active contribution to contraction of the transplanted cells seems unlikely since no connexin-43 was found between mouse and human cardiomyocytes. This was previously reported when transplanting human embryonic stem cell derived cardiomyocytes into the mouse heart[23].

Early paracrine effects on infarct size were reported using medium conditioned by cultured cells[24]. Paracrine stimulation can enhance neovascularization, which contributes to preservation of cardiac function after MI[16]. Interestingly, CMPCs but not CMPC-CM increased the vessel density in the borderzone. CMPCs may thus secrete more or other growth factors than their differentiated derivatives. In fact, an ELISA for human VEGF using conditioned medium revealed that only CMPCs secrete high levels of VEGF, but the differentiated counterparts do not. This is in agreement with a microarray analysis comparing mRNA from CMPCs and CMPC-CM, which revealed that CMPC produce higher levels of VEGF, Angiopoietin1, and Platelet Derived Growth Factor mRNA (unpublished data). These angiogenic factors may explain the observed increased vascularization. However, both groups revealed an increased cardiac performance compared to vehicle, this indicates that the generation of vessels is not the only process responsible for the preservation of cardiac function after MI. Therefore, it is conceivable that the cells may also provide passive support to the infarcted region or that CMPC-CM excrete other (non-angiogenic) factors that can influence the surrounding tissue or survival of the transplanted cells.

While very different at the time of transplantation, CMPCs and CMPC-CM gave similar results in terms of engraftment and phenotype of transplanted cells, confirming that human heart-derived progenitor cell populations can realize myocardial regeneration[22,25,26], albeit with low engraftment efficiency. Fusion has been mentioned as a possible mechanism of survival and engraftment of human cells in the infarcted mouse heart[9]. In our experiments we found no evidence of nuclear fusion or cellular fusion. If fusion does occur, it is a rare event and will not represent a major contributor to the process of engraftment.

New cardiac tissue was formed in both cell-treated animal groups. An extremely interesting finding is that comparable numbers of new cardiac (~50%), endothelial (15.5%, 9.7%) and smooth muscle cells (10.6, 17.7%) were formed in both groups, showing that CMPCs differentiate *in vivo* into the same cell-types that could be achieved *in vitro*[12]. This is probably a consequence of the abundance of growth factors and cytokines in

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the ischemic tissue, for example TGFβ[27]. Given the differentiation response of CMPCs to this growth factor *in vitro*[12], it is conceivable that TGFβ stimulation *in vivo* is a major contributor to the efficient differentiation of CMPCs. Interestingly, we also observed endothelial cells and smooth muscle cells in the pre-differentiated CMPC-CM group. At the time of transplantation, the CMPC-CM had differentiated into cardiomyocytes for 70%. The endothelial and smooth muscle cells may therefore be derived from the remaining 30% non-cardiomyocyte fraction. One explanation could be that undifferentiated cells selectively survive in the heart and differentiate to form all the observed cell types. However, using real-time PCR we were unable to detect progenitor cell markers in the differentiated cultures indicating that no CMPCs were present. Another possibility may be that CMPCs have differentiated into a cardiovascular precursor, no longer expressing progenitor cells markers, and not yet expressing definitive cardiomyocyte, endothelial or smooth muscle cell markers. To date, we do not know what marker to use for identifying such a precursor cell. Therefore this hypothesis can not be analyzed in the current study. Importantly, there was no indication of tumor formation in the cell injected hearts, or any other organ. This is in agreement with the lack of pluripotency markers Oct-4 and NANOG in undifferentiated CMPCs (unpublished data).

In conclusion, we provide clear evidence that CMPCs are an excellent source for transplantation, and there is no advantage in pre-differentiating these cells. CMPCs can form all necessary cell-types without the need of *in vitro* differentiation and are able to survive the hostile ischemic environment in the infarcted area. In the current study we used fetal-heart derived cells, which may form a limitation for clinical application. Interestingly, we have found that adult-heart derived CMPCs can form the same cell-types as fetal CMPCs *in vitro*[12,13]. Therefore, CMPCs represent a very promising candidate cell source for future (autologous) clinical application.

FUNDING

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REFERENCES

1. Rosamond W, Flegal K, Furie K, Go A, Greenlund K, Haase N, Hailpern SM, Ho M, Howard V, Kissela B, Kittner S, Lloyd-Jones D, McDermott M, Meigs J, Moy C, Nichol G, O'Donnell C, Roger V, Sorlie P, Steinberger J, Thom T, Wilson M, Hong Y. Heart Disease and Stroke Statistics 2008 Update. A Report From the American Heart Association Statistics Committee and Stroke Statistics Subcommittee. Circulation 2007;e25-e146.
2. Evans SM, Mummery C, Doevendans PA. Progenitor cells for cardiac repair. Semin Cell Dev Biol 2007;18(1):153-160.
3. Reinecke H, Zhang M, Bartosek T, Murry CE. Survival, integration, and differentiation of cardiomyocyte grafts: a study in normal and injured rat hearts. Circulation 1999;100(2):193-202.
4. Guan K, Hasenfuss G. Do stem cells in the heart truly differentiate into cardiomyocytes? J Mol Cell Cardiol 2007;43(4):377-387.
5. Murry CE, Field LJ, Menasche P. Cell-based cardiac repair: reflections at the 10-year point. Circulation 2005;112(20):3174-3183.
6. Lipinski MJ, Biondi-Zoccai GG, Abbate A, Khianey R, Sheiban I, Bartunek J, Vanderheyden M, Kim HS, Kang HJ, Strauer BE, Vetrovec GW. Impact of intracoronary cell therapy on left ventricular function in the setting of acute myocardial infarction: a collaborative systematic review and meta-analysis of controlled clinical trials. J Am Coll Cardiol 2007;50(18):1761-1767.
7. Rosenzweig A. Cardiac cell therapy--mixed results from mixed cells. N Engl J Med 2006;355(12):1274-1277.
8. Beltrami AP, Barlucchi L, Torella D, Baker M, Limana F, Chimenti S, Kasahara H, Rota M, Musso E, Urbanek K, Leri A, Kajstura J, Nadal-Ginard B, Anversa P. Adult cardiac stem cells are multipotent and support myocardial regeneration. Cell 2003;114(6):763-776.
9. Oh H, Bradfute SB, Gallardo TD, Nakamura T, Gaussin V, Mishina Y, Pocius J, Michael LH, Behringer RR, Garry DJ, Entman ML, Schneider MD. Cardiac progenitor cells from adult myocardium: homing, differentiation, and fusion after infarction. Proc Natl Acad Sci U S A 2003;100(21):12313-12318.
10. Laugwitz KL, Moretti A, Lam J, Gruber P, Chen Y, Woodard S, Lin LZ, Cai CL, Lu MM, Reth M, Platoshyn O, Yuan JX, Evans S, Chien KR. Postnatal isl1+ cardioblasts enter fully differentiated cardiomyocyte lineages. Nature 2005;433(7026):647-653.
11. Wu SM, Fujiwara Y, Cibulsky SM, Clapham DE, Lien CL, Schultheiss TM, Orkin SH. Developmental origin of a bipotential myocardial and smooth muscle cell precursor in the mammalian heart. Cell 2006;127(6):1137-1150.
12. Goumans MJ, de Boer TP, Smits AM, van Laake LW, van Vliet P, Metz CH, Korfage TH, Kats KP, Hochstenbach R, Pasterkamp G, Verhaar MC, van der Heyden MA, de KD, Mummery CL, van Veen TA, Sluijter JP, Doevendans PA. TGF-beta1 induces efficient differentiation of human cardiomyocyte progenitor cells into functional cardiomyocytes *in vitro*. Stem Cell Res 2007;1(2):138-149.
13. Smits AM, van Vliet P, Metz CH, Korfage T, Sluijter JP, Doevendans PA, Goumans MJ. Human cardiomyocyte progenitor cells differentiate into functional mature cardiomycoytes: an *in vitro* model for studying human cardiac physiology and pathophysiology. Nat Protoc 2009;4(2):232-243.
14. Passier R, Oostwaard DW, Snapper J, Kloots J, Hassink RJ, Kuijk E, Roelen B, Brutel de la Riviere A, Mummery C. Increased cardiomyocyte differentiation from human embryonic stem cells in serum-free cultures. Stem Cells 2005;23(6):772-780.
15. van Laake LW, Passier R, Monshouwer-Kloots J, Nederhoff MG, Ward-vanOostwaard D, Field LJ, van Echteld CJ, Doevendans PA, Mummery CL. Monitoring of cell therapy and assessment of cardiac function using magnetic resonance imaging in a mouse model of myocardial infarction. Nat Protoc 2007;2(10):2551-2567.
16. van Laake LW, van den Driesche S, Post S, Feijen A, Jansen MA, Driessens MH, Mager JJ, Snijder RJ, West-

proefschrift Anke.indd 77 15-07-2009 12:00:41

ermann CJ, Doevendans PA, van Echteld CJ, ten Dijke P, Arthur HM, Goumans MJ, Lebrin F, Mummery CL. Endoglin has a crucial role in blood cell-mediated vascular repair. Circulation 2006;114(21):2288-2297.
17. Bajanca F, Luz M, Duxson MJ, Thorsteinsdottir S. Integrins in the mouse myotome: developmental changes and differences between the epaxial and hypaxial lineage. Dev Dyn 2004;231(2):402-415.
18. Szuhai K, Tanke HJ. COBRA: combined binary ratio labeling of nucleic-acid probes for multi-color fluorescence in situ hybridization karyotyping. Nat Protoc 2006;1(1):264-275.
19. Chuva de Sousa Lopes SM, Hassink RJ, Feijen A, van Rooijen MA, Doevendans PA, Tertoolen L, Brutel de la Riviere A, Mummery CL. Patterning the heart, a template for human cardiomyocyte development. Dev Dyn 2006;235(7):1994-2002.
20. Nussbaum J, Minami E, Laflamme MA, Virag JA, Ware CB, Masino A, Muskheli V, Pabon L, Reinecke H, Murry CE. Transplantation of undifferentiated murine embryonic stem cells in the heart: teratoma formation and immune response. FASEB J 2007;21(7):1345-1357.
21. van der Bogt KE, Sheikh AY, Schrepfer S, Hoyt G, Cao F, Ransohoff KJ, Swijnenburg RJ, Pearl J, Lee A, Fischbein M, Contag CH, Robbins RC, Wu JC. Comparison of different adult stem cell types for treatment of myocardial ischemia. Circulation 2008;118(14 Suppl):S121-S129.
22. Smith RR, Barile L, Cho HC, Leppo MK, Hare JM, Messina E, Giacomello A, Abraham MR, Marban E. Regenerative potential of cardiosphere-derived cells expanded from percutaneous endomyocardial biopsy specimens. Circulation 2007;115(7):896-908.
23. van Laake LW, Passier R, Monshouwer-Kloots J, Verkleij AJ, Lips DJ, Freund C, den Ouden K, Ward-van Oostwaard D, Korving J, Tertoolen LG, van Echteld CJ, Doevendans PA, Mummery CL. Human Embryonic Stem Cell-Derived Cardiomyocytes Survive and Mature in the Mouse Heart and Transiently Improve Function after Myocardial Infarction. Stem Cell Research 2007;1(1):9-24.
24. Timmers L, Lim SK, Arslan F, Armstrong JS, Hoefer IE, Doevendans PA, Piek JJ, El Oakley RM, Choo A, Lee CN, Pasterkamp G, de Kleijn D. Reduction of myocardial infarct size by human mesenchymal stem cell conditioned medium. Stem Cell Research 2007;1(2):129-137.
25. Bearzi C, Rota M, Hosoda T, Tillmanns J, Nascimbene A, De Angelis A, Yasuzawa-Amano S, Trofimova I, Siggins RW, Lecapitaine N, Cascapera S, Beltrami AP, D'Alessandro DA, Zias E, Quaini F, Urbanek K, Michler RE, Bolli R, Kajstura J, Leri A, Anversa P. Human cardiac stem cells. Proc Natl Acad Sci U S A 2007;104(35):14068-14073.
26. Messina E, De Angelis L, Frati G, Morrone S, Chimenti S, Fiordaliso F, Salio M, Battaglia M, Latronico MV, Coletta M, Vivarelli E, Frati L, Cossu G, Giacomello A. Isolation and expansion of adult cardiac stem cells from human and murine heart. Circ Res 2004;95(9):911-921.
27. Chuva de Sousa Lopes SM, Feijen A, Korving J, Korchynskyi O, Larsson J, Karlsson S, ten Dijke P, Lyons KM, Goldschmeding R, Doevendans P, Mummery CL. Connective tissue growth factor expression and Smad signaling during mouse heart development and myocardial infarction. Dev Dyn 2004;231(3):542-550.

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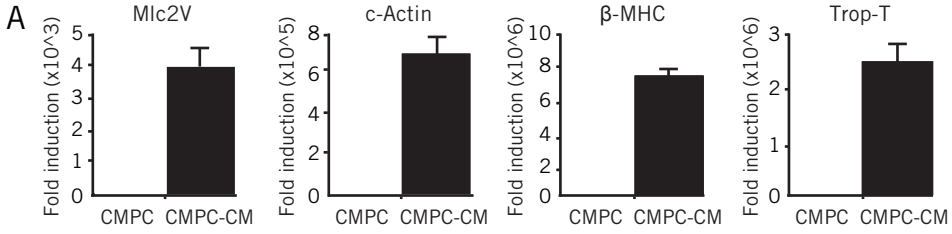
SUPPLEMENTAL FIGURES



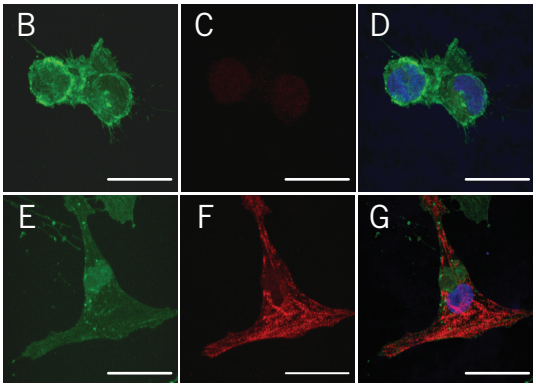


Figure 1. Characteristics of cell populations
(A) Relative expression of MLC2V, c-Actin, β-Myosin Heavy Chain (β-MHC), and troponin-T in CMPC-CM, compared tot CMPCs was determined by quantitative Real time-PCR. Confocal microscopy images of cultured CMPCs (B to D) and CMPC-CM (E-G) stained with integrin-β1 (green) as a marker for human cells, troponin-I (red) for sarcomeres and nuclei in blue. (D and G) represent overlays. Values are mean ± SEM, scale bars: 25 μm. CMPC=cardiomyocyte progenitor cell; CMPC-CM= cardiomyocyte progenitor cell-derived-cardiomyocyte; MLC2V= myosin light chain-2V; β-MHC= β myosin heavy chain.

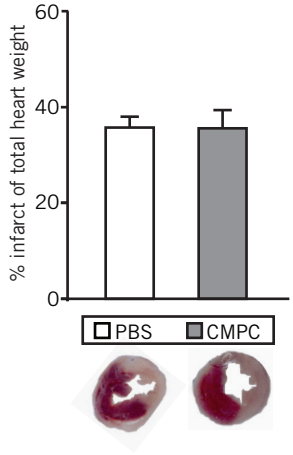


Figure 2. Infarct size after two days.
To confirm that the infarct size was comparable between PBS or CMPC receiving animals after induction of MI, mice receiving PBS (n=9) or CMPC (n=9) were sacrificed 2 days after the ligation and transplantation. Hearts were cut into 1-1.5 mm slices and stained with TTC (bottom). The infarcted surface (white) was determined and the weight of infarct was calculated as a percentage of the total heart weight. There was no difference in infarct size between PBS or CMPC receiving animals.

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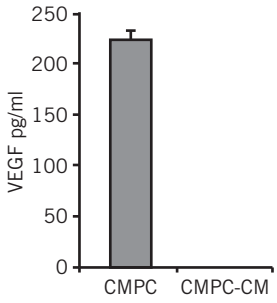

Figure 3. CMPCs secrete VEGF
To analyze the excretion of one very well known angiogenic cytokine, an ELISA for human VEGF was performed on medium conditioned by CMPCs or CMPC-CM. Cells were cultured in low growth factor and serum containing medium for two days. In CMPC-CM conditioned medium, no VEGF was present, while the CMPC conditioned medium contained VEGF.

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5

Impaired recruitment of HHT-1 mononuclear cells to the ischemic heart is due to an altered CXCR4/CD26 balance

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ABSTRACT

Aim

Mononuclear cells (MNCs) from patients with hereditary hemorrhagic telangiectasia type 1 (HHT1), a genetic disorder caused by mutations in endoglin, show a reduced ability to home to infarcted mouse myocardium. Stromal-cell derived factor-1α (SDF-1α) and the chemokine receptor CXCR4 are crucial for homing, and negatively influenced by CD26. The aim of this study was to gain insight into the impaired homing of HHT1-MNCs.

Methods

CXCR4 and CD26 expression on MNCs was determined by flow cytometry. Transwell migration to SDF-1α was used to analyze *in vitro* migration. Experimentally induced myocardial infarction in mice, followed by tail vein injection of MNCs, was utilized to study homing *in vivo*.

Results

HHT1 MNCs expressed elevated levels of CXCR4, but this was counterbalanced by high levels of CD26, resulting in decreased migration towards an SDF-1α gradient *in vitro*. Migration was enhanced by inhibiting CD26 with Diprotin-A. While MNCs from healthy controls responded to TGF-β stimulation by increasing CXCR4 and lowering CD26 expression levels, HHT1-MNCs did not react as efficiently, in particular CD26 expression remained high. Inhibiting CD26 on MNCs increased the homing of human cells into the infarcted mouse heart. Interestingly, the defect in homing of HHT1-MNCs was restored by pre-incubating the HHT1-MNCs with Diprotin-A before injection into the tail vein.

Conclusions

We show that a decreased homing of HHT1-MNCs is caused by an impaired ability of the cells to respond to SDF-1α. Our results suggest that modulating CD26 levels using inhibitors like Diprotin-A can restore homing in cases where increased expression of CD26 contributes to the underlying pathological mechanism.

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INTRODUCTION

Hereditary hemorrhagic telangiectasia (HHT, also known as Rendu-Osler-Weber disease) is an autosomal dominant disorder with an estimated prevalence of approximately 1 in 10,000. Several different types have been described for this disease, but characteristically all classes are associated with mutations in components of the transforming growth factor-beta (TGFβ) signaling pathway. The underlying cause of HHT type 1 (HHT1) are mutations in endoglin (CD105), an accessory TGFβ type III receptor. Endoglin is primarily expressed on proliferating endothelial cells *in vitro* and angiogenic blood vessels *in vivo* but also on other cells types, like circulating blood mononuclear cells (MNCs), although to a lesser extent.

Clinically, HHT1 is characterized by multi organ vascular dysplasia, telangiectasias and epistaxis[1,2]. With age, the incidence and severity of bleedings increases. Because endoglin is crucial for the formation of new vessels[3], the higher number of hemorrhages in the endoglin haplo-insufficient HHT1 patients may result from a reduced ability to restore the injured vasculature. Vascular repair is mediated by activation of endothelial cells lining the vessel wall to replace the damaged cells, but it has become evident that circulating MNCs also have the ability to restore damaged vessels[4].

Repairing the injured heart for example after a myocardial infarction (MI) requires the influx of MNCs, including monocytes and lymphocytes. MNCs promote healing of the damaged heart via stimulation of myofibroblast proliferation, deposition of collagen and the induction of angiogenesis[5]. Recently, we demonstrated that the recruitment of human MNCs to the infarcted murine heart and subsequent vessel formation is severely impaired when using HHT1-derived MNCs compared to healthy MNCs[6].

Homing and trafficking of cells is regulated to a large extent by the chemokine stromal cell-derived factor-1α (SDF-1α or CXCL12) and its receptor CXCR4. SDF-1α is upregulated in ischemic myocardium shortly after MI, resulting in the recruitment of (CXCR4 expressing) cells from the circulation to the damaged area[7-9]. Besides SDF-1α, the expression of transforming growth factor β (TGFβ) is increased after MI[10]. TGFβ is a growth factor that controls the proliferation, adhesion, apoptosis, homing and migration of many cell types[11]. TGFβ was shown to positively influence the SDF-1α/CXCR4 axis by increasing the expression of CXCR4 on circulating blood cells and different cancer cells[12-15]. Furthermore, TGFβ reduced the levels of the homing-inhibiting peptidase CD26 on these cell types [12-15].

CD26 is a surface serine di-peptidylpeptidase (also known as DPP-IV) that cleaves the amino-terminal dipeptide from SDF-1α, known to interact with the extracellular portion of CXCR4. Additionally CD26 can co-internalize with CXCR4[16,17]. CD26 therefore interferes with the SDF-1α/CXCR4 axis by preventing the recruitment of cells to SDF-1α.

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Many different cell types carry CD26 on their surface, which thereby influences their migratory capacity[17,18]. Inhibiting CD26 using Diprotin-A has been shown to increase the homing and migration of cells *in vitro*[19], and *in vivo*[20,21], and enhanced their engraftment in the bone marrow of lethally irradiated recipient mice[22].
Since all mutations in the endoglin gene reported to date result in reduced functional cell- surface protein and deregulation of TGFβ signaling, we hypothesized that this changes the balance between CXCR4 and CD26 on HHT-MNCs. This would lead to an impaired ability of MNCs to respond to SDF-1α expression *in vitro* and *in vivo*.

MATERIALS AND METHODS

Patients and blood samples

Venous blood samples from healthy human volunteers and HHT1 patients, age and gender matched, were collected in potassium/EDTA tubes (Vacuette, Greiner Bio-One). Peripheral blood MNCs were isolated by density gradient centrifugation using Ficoll Paque Plus (Amersham Biosciences), according to the manufacturer's protocol. Isolated MNCs were washed twice with PBS supplemented with 2 mM EDTA, and counted on a hemocytometer. All procedures were approved by the medical ethics committee of the St. Antonius Hospital Nieuwegein, the Netherlands. The investigation conforms to the principles outlined in the Declaration of Helsinki.

Flow cytometry

Flow cytometric analysis was performed using 100µL of whole blood or 3*10E5 MNCs in PBS. Whole blood was stained with anti-CD14-PE (DakoCytomation), anti-Endoglin-Fluorescein (R&D Systems), anti-CD34-FITC (BD Pharmingen) and anti-VEGFR2-PE (R&D Systems). MNCs were stained with anti-CD14-ECD (Immunotech), anti-CD26-FITC (Serotec) and anti-CXCR4-PE (BD Pharmingen). Isotype-matched fluorochrome-conjugated antibodies were used as controls. Red blood cells were lyzed before measuring fluorescence on a flow cytometer (Cytomics FC500, Beckman Coulter). Analysis was performed using CXP software (Beckman Coulter). The number of positive cells is expressed as absolute cell number per ml of whole blood, or as percentage of positive cells within a cell fraction. The Mean-Fluorescent Intensity (MFI) is presented for cell populations of interest.

MNC migration

Migration of freshly isolated MNCs was assessed in a transwell system using polycarbonate filters with 5 µm pores (Corning). Prior to migration, MNCs were incubated for 1

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hour in RPMI 1640 Glutamax medium, supplemented with 10% FBS at 37°C. If applicable, MNCs were stained with Calcein AM (Invitrogen). MNCs were pretreated at room temperature for 15 minutes with 5 mM Diprotin-A (Sigma-Alderich) to inhibit CD26, or for 30 minutes at room temperature with 5ug/mL AMD3100 (Sigma) to block CXCR4. One-hundred thousand MNCs were applied to the upper well, and in the lower well medium with or without 200ng/ml SDF-1α (PeproTech), was added. The cells were allowed to migrate for 3 hours at 37°C. After migration, cells were collected and 75,000 PeakFlowtm carmine flow cytometry reference beads (6 µm, Invitrogen) were added. The number of MNCs per 10.000 beads was assessed by flow cytometry. The migration percentage was calculated from the number of cells migrated to SDF-1α compared to the number of cells migrated in the absence of SDF-1α.

MNC stimulation

Freshly isolated MNCs were stimulated for 24 hours at 37°C with 2ng/mL TGFβ_1 (PeproTech) in RPMI medium supplemented with 1% FBS. Stimulated MNCs were stained with anti-CD14-ECD, anti-CD26-Fluorescein and anti-CXCR4-PE. Expression levels of CXCR4 and CD26 were determined by measuring MFI. Expression changes were calculated by dividing the MFI after 24 hour TGFβ_1 stimulation by the MFI before stimulation and presented as fold induction.

Animals

All experiments were carried out in accordance with the *Guide for the Care and Use of Laboratory Animals*, prepared by the institute of Laboratory Animal Resources and with prior approval by the Animal Ethical Experimentation Committee, Utrecht University.

Isolation of LacZ$^+$ bone marrow

Bone marrow MNCs were isolated from the femur and humerus bones of six male ROSA26 mice using Ficoll as described above.

Myocardial infarction

In BALB/cOlaHsd or C57/BL6 mice (Harlan), aged 10-12 weeks, a myocardial infarction (MI) was induced as described previously[23]. Briefly, mice were intubated and ventilated with 2% isoflurane/98% oxygen. A left thoracotomy was performed and the left anterior descending coronary artery (LAD) was visualized. The LAD was permanently occluded by placing a 7-0 prolene suture.

Intravenous injection of human or murine MNCs

One day after induction of MI, BALB/c mice received 5*10^6 human MNCs (HHT1 or

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control, n=6 per group) via tail vein injection. C57/Bl6 mice were injected with $5*10^6$ ROSA 26 bone –marrow-derived $LacZ^+$ MNCs. Prior to intravenous injection, MNCs were incubated for 15 minutes with or without 5 mM Diprotin-A at room temperature. Mice receiving human cells were immunosuppressed by subcutaneous injection of tacrolimus (5 mg/kg/day) for four days.

Tissue collection

Five days after MI mice were euthanized. The hearts were flushed with 5 mL of PBS via the right ventricle and dissected. The tissue was processed for cryosectioning in OCT compound (Sakura). Alternatively, hearts of mice receiving $LacZ^+$ MNCs were cut longitudinally into two, through the infarcted region. One part was used for whole mount X-gal staining the other halve was embedded in OCT compound (Sakura) for cryosectioning.

Whole mount staining

Tissues were fixed in 2% paraformaldehyde/0.2% glutaraldehyde in PBS on ice. After washing in PBS twice, tissue was placed in staining solution containing 5mM ferrocyanide, 5mM ferricyanide, 2mM $MgCl_2$ and 1 mg/ml X-Gal in dimethylformamide overnight at 37°C. After washing away the X-gal the samples were postfixed in 4% paraformaldehyde, and kept in 70% EtOH. Tissue was embedded into paraffin, sectioned at 10 µm and stained with hematoxylin.

Immunohistochemistry

Frozen longitudinal 7 µm thick sections of the whole ventricle were stained using a mouse anti-human nuclei antibody (Chemicon) for the identification of human cells. Briefly, sections were fixed in acetone, air dried and rehydrated in PBS. Endogenous peroxidase activity was blocked, followed by incubation with avidin and biotin, respectively. The tissue was permeabilized in 0.2% Triton X-100 in PBS and blocked with 3% BSA in PBS for 30 minutes. During this incubation, the anti-human nuclei antibody was biotinylated 1:50 using the Dako-ARK for mouse primary antibodies (Dako) according to the manufacturer's protocol. Sections were incubated with α-human nuclei, PBS or mouse IgG1 (Dako) as an isotype control overnight at 4°C. After washing, slides were incubated with ABC peroxidase complex (DAKOcytomation, Dako) at room temperature for 30 minutes. Peroxidase activity was detected using AEC substrate. After counterstaining with hematoxylin sections were mounted in gelatin/glycerin.

Semi-quantitative polymerase chain reaction for human Alu sequences or LacZ

Genomic DNA was isolated by pooling 10-50 crysections (10 µm) followed by incubation

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in 500 µl lysis buffer (0.1 M Tris-HCl pH 8.5, 5 mM EDTA, 0.2% SDS, 0.2 M NaCl). Genomic DNA was extracted using phenol-chloroform, and precipitated with ethanol. The polymerase chain reaction (PCR) primers were described previously and are specific for human Alu sequences, resulting in a product of 224 basepairs[24]. For the quantification of the LacZ gene we used 5' PCR primers: Forward 5'- CCTGAGGCCGATACTGTCGT-3', Reverse 5'- TTGGTGTAGATGGGCGCAT-3', leading to a product of 70 bp. PCR was carried out using 50 ng of total DNA under the following conditions: 95°C for 10 minutes, followed by x cycles of 95°C for 30 seconds, 58°C for 45 seconds and 72°C for 45 seconds; and 72°C for 10 minutes. Amplification of DNA isolated from mice receiving Diprotin-A treated or untreated human MNCs was optimized at 23 and 27 cycles, respectively, and for LacZ all samples were run for 25 cycles. The samples were run on a 10% polyacrylamide gel and quantified using Quantity One software (Biorad).

Statistics

Statistical significance was evaluated using the Mann-Whitney U test for comparison between two independent samples, Wilcoxon Signed Ranks test for two related samples, and Spearman's rho for correlation calculations using SPSS v11.0 for Windows. Results are expressed as mean ± standard error of the mean (SEM). A value of $P<0.05$ was considered statistically significant.

RESULTS

Circulating cell populations

To establish whether the attenuated response of HHT1 to ischemic injury[6] is due to fewer circulating cells capable of participating in restoration of vascular damage, we compared peripheral blood samples of HHT1 patients to age and gender matched healthy controls (see supplementary Table 1 for HHT1 patient characteristics). Monocytes (defined as $CD14^+$) are circulating cells that are able to home to damaged tissue and are known to express endoglin. We found no difference in the number of circulating $CD14^+$ cells between HHT1 and control samples (Figure 1A,B) but, as expected, the HHT1 $CD14^+$ monocytes did show decreased endoglin expression on their cell surface (not shown).
Other cells that can contribute to vascular repair are those within the $CD34^+$ cell fraction. Endothelial progenitor cells (EPCs) are part of this $CD34^+$ population, and one way to identify EPCs may be as $CD34^+$-$VEGFR2^+$ cells. In contrast to our expectation, HHT1 patients show a significantly higher number of $CD34^+$ cells (Figure 1C,D), but the number of $CD34^+$-$VEGFR2^+$ cells is comparable between HHT1 patients and controls (Figure 1E,F).

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Distribution of CXCR4 and CD26

The CXCR4/SDF-1α axis is particularly important for cell homing, chemotaxis, engraftment and retention of cells in ischemic tissues. Analyzing the distribution of CXCR4 on MNCs (Figure 2A) showed that, although the number of cells in the MNC population expressing CXCR4 was not different between groups (Figure 2B), the mean expression level of CXCR4 on the cell surface (represented by the MFI) was significantly higher on HHT1-MNCs (Figure 2C).

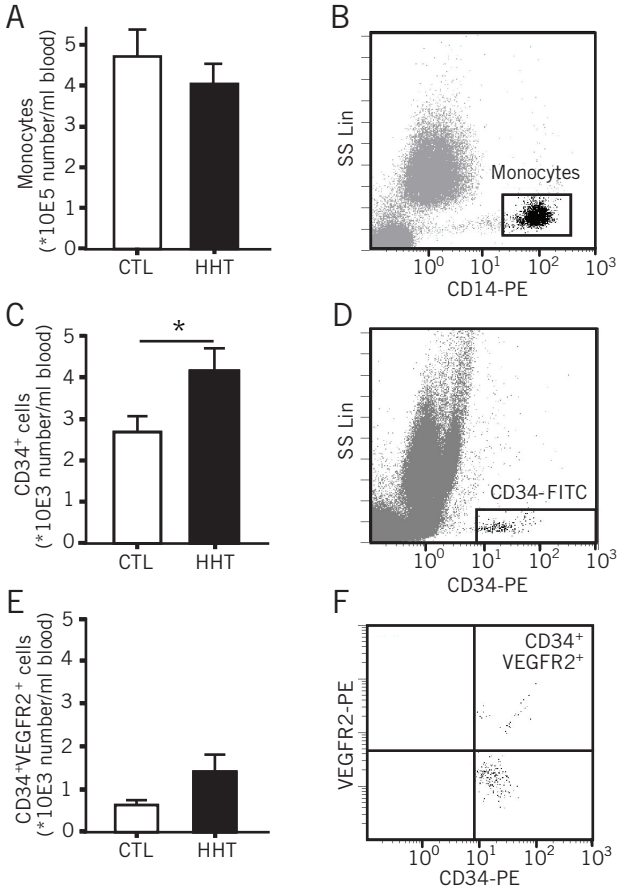

Figure 1. Composition of peripheral blood

(A) Flow cytometric analysis of showed no difference in the number of $CD14^+$ monocytes in peripheral blood obtained from HHT1 patients (HHT) or controls (CTL). (C) HHT1 patients have an increased number of $CD34^+$ cells compared to controls. (E) There is no significant difference in $CD34^+VEGFR2^+$ (EPCs) between the groups. Representative flow cytometry plots are shown in B, D and F. *P<0.05, Bars show mean ± SEM.

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CD26 is a negative regulator of the SDF1/CXCR4 axis. The HHT1-MNC population showed a significant increase in the expression level of CD26 per cell but no difference in the total number of CD26 positive cells (Figure 2E,F).
Additionally, within the MNC subpopulations, lymphocytes and monocytes demonstrated similar patterns in the expression of CXCR4 and CD26 (Supplementary Figure 1A,B).

HHT1-MNCs show impaired migratory response in vitro

CD26 can regulate SDF-1α/CXCR4 mediated chemotaxis; therefore we performed a migration assay using SDF-1α as a chemo-attractant. HHT1-MNCs exhibited decreased migration towards SDF-1α compared to control MNCs (Figure 3A). Interestingly, while pretreatment with the CXCR4 inhibitor AMD3100 completely blocked the migration of

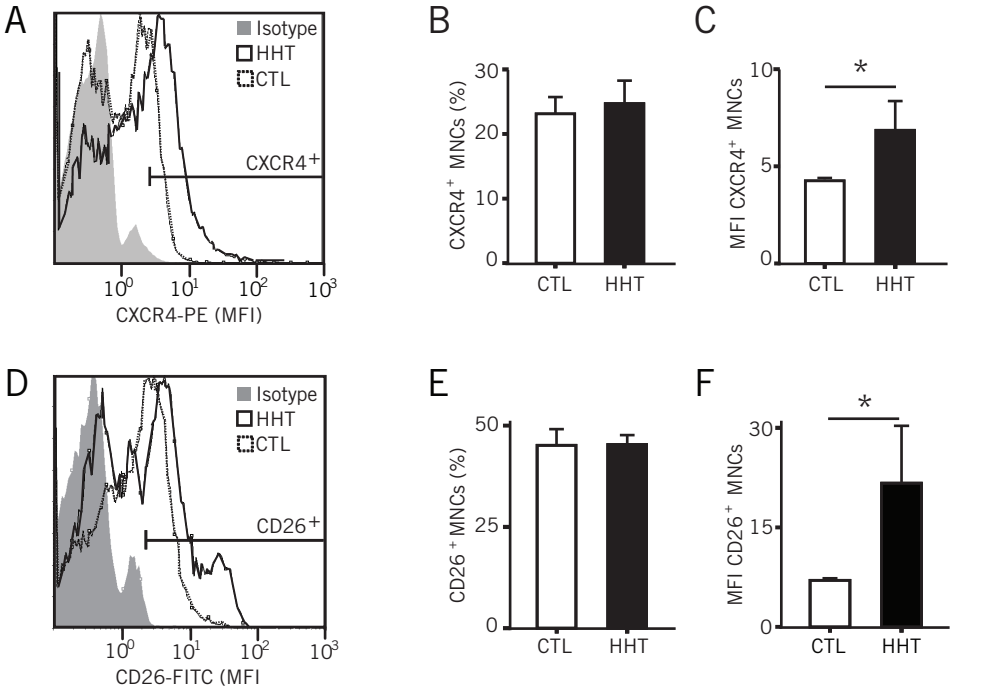

Figure 2. CXCR4 and CD26 expression on circulating cells

(A) Representative flow cytometry plot for CXCR4 on control (CTL) and HHT1-MNCs (HHT) and isotype control. (B) There is no difference in the number of CXCR4+ cells between groups. (C) The mean surface expression of CXCR4 within the CXCR4+ population is higher on HHT1-MNCs. (D) Representative graph of MFI analysis showing the CD26 levels versus the isotype control. (E) The HHT1-MNC population contains the same number of CD26+ cells, but the mean surface expression of CD26 is significantly higher on HHT1 CD26+ MNCs (F). CTL n=13, HHT n=14. *$P<0.05$, bars show mean ± SEM.

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both control and HHT1-MNCs, pretreatment with the CD26 inhibitor Diprotin-A only significantly improved the migration of the HHT1-MNCs compared to untreated MNCs (Figure 3B).

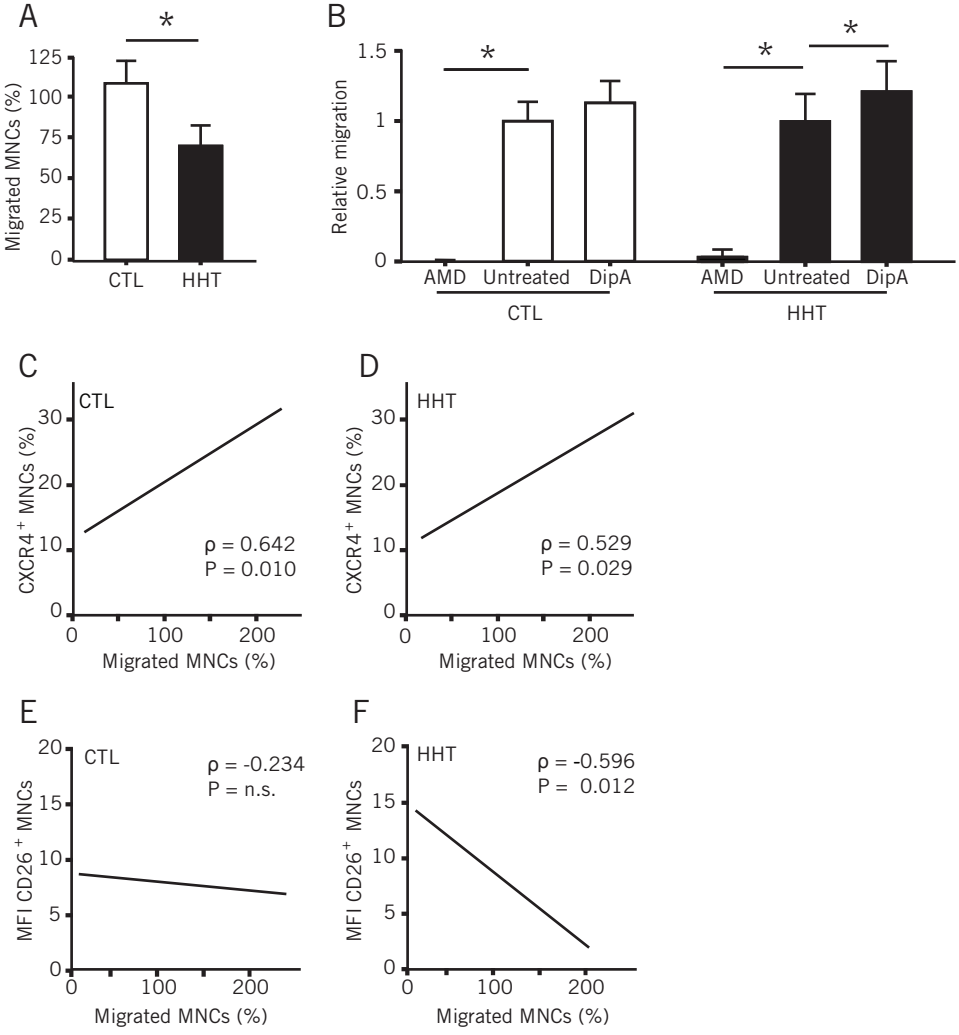

Figure 3. Migration of MNCs to SDF-1α in vitro

(A) In a transwell assay using SDF-1α as a chemoattractant, HHT1-MNCs show a decreased migration capacity compared to healthy controls. Shown is the result of the calculation (+SDF-1α/-SDF-1α) -1 represented as a percentage. (B) The effect on migration of CXCR4 inhibition (AMD) or CD26 inhibition (DipA). Depicted is the relative migration capacity towards SDF-1α compared to untreated cells (set as 1 in each MNC source). (C and D) MNC migration positively correlates with the percentage of CXCR4+ cells in the MNCs population as shown by a positive Spearman's rho. (E and F) In HHT1-MNCs, a higher level of CD26 per cell negatively correlates with the migration towards SDF-1α. CTL n=17, HHT1 n=17; AMD: CTL n=5, HHT1 n=4; Correlations: CTL n=15, HHT1 n=17. *P<0.05. Bars show mean ± SEM.

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Analyzing the CXCR4 and CD26 expression profile on migrated cells revealed that the number of CXCR4+-MNCs positively correlated with the migration capacity for both control as well as HHT1-MNCs (Figure 3C,D). Interestingly, the mean CD26 expression on HHT1-MNCs was negatively correlated with their migratory capacity (Figure 3F), while the migration of control MNC did not correlate with CD26 levels (Figure 3E).

MNC response to TGFβ stimulation

After MI, TGFβ levels are increased in the ischemic area. TGFβ can play a role in the homing and migration of cells to damaged tissue. Since in HHT1 patients the TGFβ signaling pathway is disturbed[25] and their homing capacity is abrogated, we investigated the effect that TGFβ stimulation has on the CXCR4 and CD26 expression levels on MNCs. Although flow cytometric analysis of TGFβ stimulated MNCs revealed a pronounced increase in total percentage of CXCR4 expressing cells as well as the mean CXCR4 expression level per cell on both HHT1 and control MNCs (Figure 4A,B), the relative induction

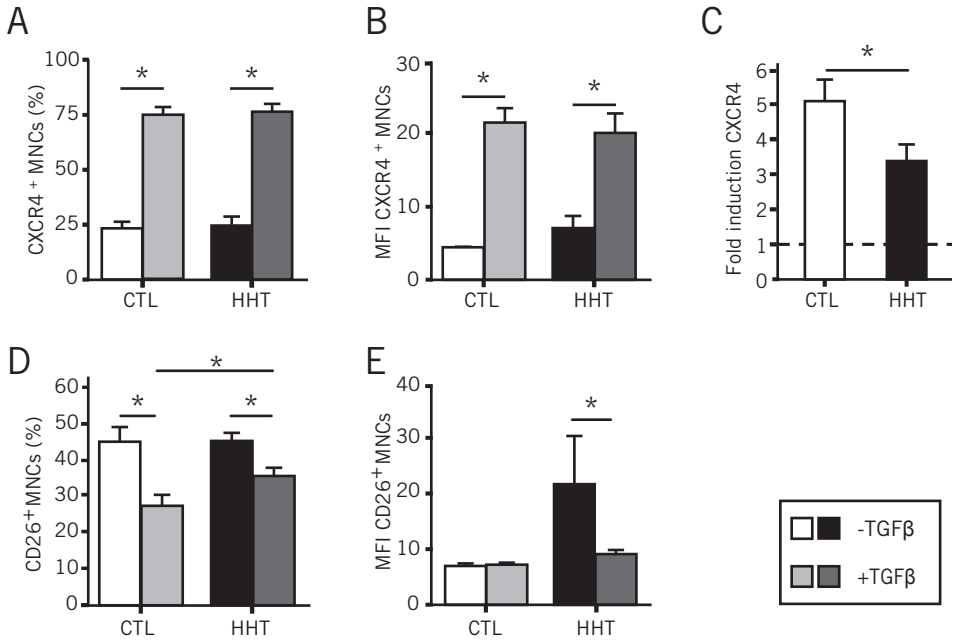

Figure 4. The effect of TGFβ stimulation on CXCR4 and CD26 expression

(A,B) Both HHT1 and control MNCs are able to up regulate CXCR4 expression after TGFβ stimulation, thereby increasing the number of CXCR4+ MNCs (A) and the level of CXCR4 per cell (B). (C) The relative induction of the receptor was significantly lower on HHT1-MNCs compared to controls. (D and E) CD26 is downregulated in response to TGFβ stimulation on HHT1 and control MNCs. (D) The number of CD26+ cells within HHT1-MNCs after TGFβ stimulation remains significantly higher compared to controls. CTL n=13, HHT1 n=14. *P<0.05; bars show mean ± SEM.

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of the receptor was less on HHT1-MNCs than on control MNCs (3.4 fold for HHT1 versus 5.1 fold for control MNCs, Figure 4C). After TGFβ stimulation, the total number of CD26 expressing cells in both control and HHT1-MNCs was reduced, as well as the overall mean expression level (Figure 4D,E). Importantly, the percentage of CD26 positive cells remained higher within HHT1-MNCs than within control MNCs, even after TGFβ stimulation (Figure 4D,E). These experiments show that endoglin haplo-insufficiency results in decreased TGFβ responsiveness. Although HHT1-MNCs are able to respond to TGFβ stimulation by increasing CXCR4 and down regulating CD26 expression, this occurred to a lesser extent and the net result is a decreased migration capacity to SDF-1α compared to controls.

CD26 inhibition increases homing towards the infarcted myocardium

To determine the role of CD26 in homing and migration of cells in an *in vivo* injury setting, we used LacZ$^+$ bone marrow-derived MNCs isolated from ROSA26 mice in a mouse model for MI. After the onset of ischemia SDF-1α increases as part of the stress response. SDF-1α reaches a maximum after one day, hereafter SDF-1α gradually decreases[7]. Therefore, MNCs, either untreated, or pre-treated with DiprotinA were introduced in to the circulation via tail vein injection, one day after inducing MI. Four days later, mice were sacrificed and the location of MNCs was determined via whole mount staining. LacZ$^+$ cells were found throughout the infarcted left ventricle (Figure 5A, overview and close-up), but not in sham operated animals (not shown). Since the number of cells in the heart was too high to quantify microscopically, we isolated genomic DNA from frozen sections. Semi-quantitative PCR for the LacZ gene was performed and revealed

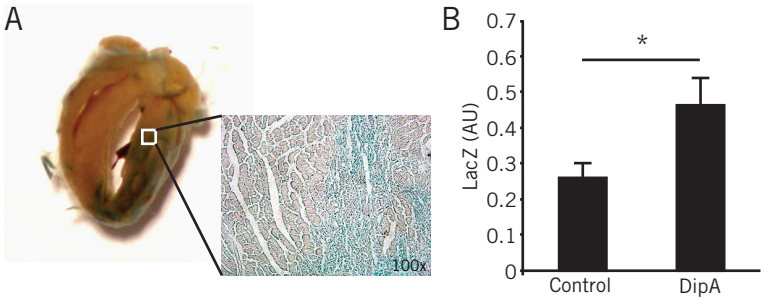

Figure 5. Inhibiting CD26 by DiprotinA increases homing of MNCs after MI

The effect of CD26 inhibition on homing *in vivo* was assessed by injecting LacZ$^+$ bone-marrow-derived MNCs into the tail vein of mice that underwent MI the previous day. (A) Five days after MI, blue cells are found in the infarcted area as shown by whole mount staining, and a hematoxylin counterstained section of the border zone. (B) Quantification of LacZ$^+$ cell by semi-quantitative PCR showed a significantly higher number of LacZ$^+$ cells when MNCs were pretreated with DipA prior to injection. Control n=6, DipA n=6, shown is mean ± SEM, $p<0.05$)

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that DiprotinA treated MNCs home more efficiently to the infarcted heart compared to non-treated MNCs (Figure 5B).

Analyzing homing of HHT1 and control MNCs in vivo

To evaluate the importance of the increased expression of CD26 on MNCs in HHT1 patients, for homing to damaged tissue, we used the mouse MI model. Mice underwent a

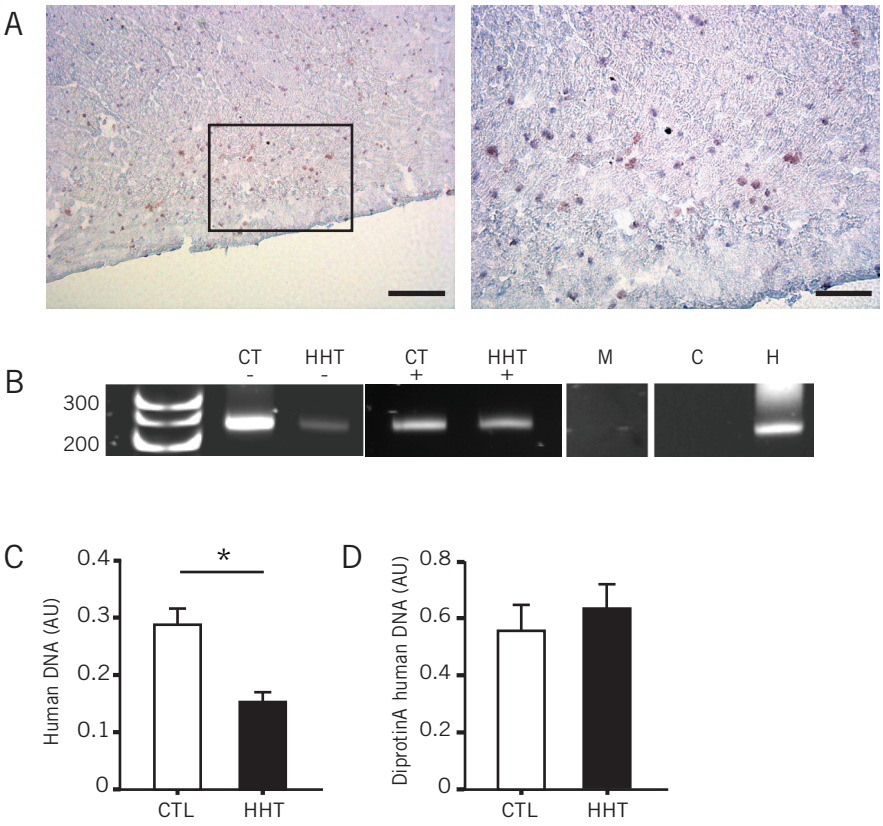

Figure 6. Improved homing of MNCs *in vivo* by CD26 inhibition

Mice were injected intravenously with 5*10E6 human MNCs one day post MI. (A) Five days after infarction, anti-human nuclei positive cells (red) are found scattered throughout the infarct border zone in the left ventricle. Left: scale bar 100µm, right: magnification of boxed area, scale bar: 50 µm. (B) The amount of human DNA was determined by semi-quantitative PCR. Polyacrylamide gel showing an example of the Human Alu repeats PCR. CT- and HHT-: DNA samples of mice receiving untreated MNCs from controls and HHT1 patients respectively. CT+ and HHT+: DNA samples of mice receiving MNCs from controls and HHT1 patients after DipA treatment. M: wildtype mouse DNA; H: human genomic DNA; C: H_2O control. (C) Quantification of DNA isolated from hearts of mice injected with control MNCs (n=5) or HHT1 MNCs (n=7). (D) Quantified DNA isolated from hearts of mice injected with Diprotin-A pretreated control (n=6) or HHT1 (n=5) MNCs. *P<0.05. Bars show mean ± SEM.

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permanent ligation of the LAD; one day after MI the mice received an intravenous injection of human MNCs, which were pretreated or not with the CD26 inhibitor Diprotin-A. Mice were sacrificed five days after MI to assess MNC homing.
Immunohistochemical staining using an anti-human nuclei antibody revealed human cells scattered throughout the injured myocardium five days after infarction (Figure 6A) comparable to what we observed with murine LacZ-expressing MNCs. The number of human cells found in the myocardium was quantified by semi-quantitative PCR for human specific Alu repeats (Figure 6B).
Five days post-MI, a significantly lower amount of human DNA was found in the myocardium of mice injected with HHT1-MNCs as compared to those receiving untreated control MNCs (Figure 6C, 0.15 ± 0.05 and 0.29 ± 0.06 arbitrary units respectively). Strikingly, when human MNCs were pre-treated with Diprotin-A, there was no longer a difference in the homing and engraftment into the infarcted heart between HHT1 patients and controls (Figure 6D, 0.64 ± 0.18 versus 0.55 ± 0.22 for HHT1 and controls respectively). These results underline the importance of CD26 expression for the homing and migratory capacity of MNCs.

DISCUSSION

One of the hallmarks of HHT1 is an increasing frequency of hemorrhages with age. This can be the result of impaired vascular repair. Restoration of vasculature is initiated by a local rapid increase in chemotactic chemokines followed by homing of cells to the site of injury. Previously, we showed that the HHT1-MNC population had a reduced ability to accumulate and induce vessel formation in the infarcted region of the heart[6]. Here we show that this observed defect in homing is not explained by changes in the numbers of cells with angiogenic properties within the heterogeneous MNC population known to participate in tissue repair. Analysis of the composition of the MNC fraction using flow cytometry did not show a significant difference in the number of circulating $CD14^+$-monocytes or $CD34^+VEGFR2^+$ cells. However, HHT-1 patients have a significant higher number of $CD34^+$ cells. The increase in $CD34^+$ cell fraction may be the result of diffuse vascular damage in HHT-1 patient, resulting in increased $CD34^+$ progenitor cell mobilization. Similarly, increases in CD34+ cell count were found in patients after myocardial infarction[27]. Progenitor cell numbers do not seem to be the cause of the HHT-1 MNC dysfunction. Therefore, an alternative explanation for the reduced cell numbers in the ischemic heart[6,28] may be an impaired chemotactic response of the circulating cells in HHT1 patients rather than reduced circulating MNC numbers.
Myocardial wound healing is a tightly controlled process and has been divided into dis-

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tinct phases[29]. One recognizable phase is the infiltration of MNCs into the infarct area, inducing blood vessels growth, myofibroblast proliferation and ECM production. An important mechanism for cell recruitment to ischemic areas is the formation of an SDF-1α gradient, resulting in the mobilization of circulating cells expressing its cognate receptor CXCR4. Binding of SDF-1α to CXCR4 was shown to be essential for mobilization and migration of different cell types e.g. hematopoietic stem cell (HSC)[20,30], monocytes/mesothelial cells[31] and tumor metastasis[21,32]. Since SDF-1α expression is increased as early as 1 hour after induction of hypoxia in the myocardium, it is believed to play a role in the initiation of tissue repair. More importantly, modulating the SDF-1α/CXCR4 axis, either positively by delivering a protease-resistant SDF-1α[33] or negatively, by inhibiting SDF-1α binding to its receptor using the CXCR4 antagonist AMD3100, was shown to influence the recruitment and engraftment of cells in infarcted myocardium as well as myocardial repair[8]. Furthermore, the expression of SDF-1α serves as a retention signal and is crucial for the engraftment and maintenance of pro-angiogenic CXCR4 expressing cells within the tissue[34]. The lower number of HHT1-MNC within the infarcted myocardium might be explained by reduced numbers of CXCR4 positive cells or reduced CXCR4 expression per cell. However, we found no difference between HHT1 patients and controls. In HHT1 patients, the mean expression of CXCR4 on the cell surface was even higher than control MNCs. Therefore, the reduced number of HHT1- MNCs found in the injured heart cannot be explained by a reduction in CXCR4.

Although CXCR4 expression levels are known to be important for migration, our data imply that this alone does not predict the migratory behavior of cells to SDF-1α. SDF-1α mediated chemotaxis is also regulated by the cell surface peptidase CD26, which cleaves the amino-terminus of SDF-1α[18]. This N-terminal cleavage will block its binding to CXCR4 and limit the effectiveness of SDF-1α as a chemotactant in the inflammatory environment of infarcted myocardium. CD26 has been reported to be expressed by several cell types within the MNC fraction, including $CD14^+$ monocytes and $CD34^+$ cells[35]. Analysis of CD26 indicated that the HHT1-MNCs have a higher mean expression level of CD26 per cell. These increased CD26 levels suggest that, although the high CXCR4 levels on HHT1-MNCs would imply increased recruitment of cells to ischemic tissue, their homing capacity is negatively influenced by the high CD26 expression levels. Using a transwell migration assay, we indeed found a decreased chemotactic response of the HHT1-MNCs towards SDF-1α as compared to healthy controls. Furthermore, the migration of HHT1 cells to SDF-1α was significantly improved when the activity of CD26 was blocked by Diprotin-A; a tri-peptide which was previously shown to increase to homing of embryonic stem cells36 and HSCs to SDF-1α[20,37]. Additionally, we show that the effect of CD26 cell migration is negatively correlated with its expression level. The inability to influence the migration of control MNCs by Diprotin-is probably due to the low

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CD26 levels observed on these cells that did not correlate with their migratory behavior capacity. Our results clearly demonstrate that the balance of CD26 levels in relation to CXCR4 levels is of great importance to predict the chemotactic response of MNCs. The increased number of CD26 expressing cells in the HHT1 population, causing a misbalance between CD26 and CXCR4, may have a major impact on their total homing and retention capacity.

As a consequence of the endoglin mutations underlying HHT1, the TGFβ signaling is impaired[2,25]. TGFβ has previously been reported to influence the SDF-1α/CXCR4 axis, by increasing the expression of CXCR4 and decreasing the levels CD26 on tumor and mesothelial cells[15,31]. We investigated the response of the MNCs to TGFβ stimulation. TGFβ stimulation increased the surface expression of CXCR4 on control MNCs, whereas HHT1-MNCs show impaired induction of expression and impaired reduction in CD26 levels. These observations suggest that the imbalance in the CXCR4/CD26 axis we observe in these cells might be caused by an impaired response of HHT1-MNCs to TGFβ stimulation. Therefore, when HHT1-MNCs are exposed to stress signals after myocardial infarction, like TGFβ, their capacity to shift the balance between CXCR4 and CD26 is disturbed, thereby rendering the cells less capable for migration and homing. The relatively high CXCR4 expression observed on unstimulated HHT1-MNCs might be a compensatory mechanism to deal with the high CD26 expression on these cells.

Since *in vitro* analysis of the HHT1-MNCs indicated CD26 imbalance as an explanation for impaired homing, we interfered with its expression. Previous studies have shown that pretreatment of HSCs with Diprotin-A greatly enhanced their homing capacity towards the bone marrow[22,37]. Therefore, we investigated whether altering the CXCR4/CD26 balance, by inhibiting CD26 with Diprotin-A, also increased their homing *in vivo*. When untreated MNCs were injected one day after MI, we found significantly fewer HHT1-MNCs in the infarcted area 5 days after MI, compared to healthy controls. This confirmed the impaired homing capacity we reported previously[6], as well as, the *in vitro* data presented in this study. Strikingly, pretreatment with Diprotin-A completely normalized the homing of HHT1-MNCs to the infarcted heart so that it was comparable with controls. This indicates the significance of CD26 for homing not only for cells towards the bone marrow but also for MNCs to ischemic tissue.

In conclusion, the SDF-1α/CXCR4 axis and its negative regulator CD26 are crucial for the homing of MNCs to the ischemic myocardium. In HHT1 patients, the balance of CXCR4 and CD26 on HHT1-MNCs is skewed. Restoring the balance in this axis, via CD26 inhibition resulted in "normal" homing capacities *in vitro* and *in vivo*, providing an explanation for the dysfunctional homing of HHT1-MNCs. Interestingly, CD26 inhibition might also be of importance for studies aiming at regeneration of ischemic areas, by improving homing and engraftment of circulating cells into the injured organ.

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REFERENCES

1. Abdalla SA, Letarte M. Hereditary haemorrhagic telangiectasia: current views on genetics and mechanisms of disease. J Med Genet 2006;43(2):97-110.
2. van den Driesche S., Mummery CL, Westermann CJ. Hereditary hemorrhagic telangiectasia: an update on transforming growth factor beta signaling in vasculogenesis and angiogenesis. Cardiovasc Res 2003;58(1):20-31.
3. Lebrin F, Goumans MJ, Jonker L, Carvalho RL, Valdimarsdottir G, Thorikay M, Mummery C, Arthur HM, ten DijkeP. Endoglin promotes endothelial cell proliferation and TGF-beta/ALK1 signal transduction. EMBO J 2004;23(20):4018-28.
4. Isner JM, Kalka C, Kawamoto A, Asahara T. Bone marrow as a source of endothelial cells for natural and iatrogenic vascular repair. Ann N Y Acad Sci 2001;953:75-84.
5. Nahrendorf M, Swirski FK, Aikawa E, Stangenberg L, Wurdinger T, Figueiredo JL, Libby P, Weissleder R, Pittet MJ. The healing myocardium sequentially mobilizes two monocyte subsets with divergent and complementary functions. J Exp Med 2007;204(12):3037-47.
6. van Laake LW, van den Driesche S, Post S, Feijen A, Jansen MA, Driessens MH, Mager JJ, Snijder RJ, Westermann CJ, Doevendans PA, van Echteld CJ, ten Dijke P, Arthur HM, Goumans MJ, Lebrin F, Mummery CL. Endoglin has a crucial role in blood cell-mediated vascular repair. Circulation 2006;114(21):2288-97.
7. Ma J, Ge J, Zhang S, Sun A, Shen J, Chen L, Wang K, Zou Y. Time course of myocardial stromal cell-derived factor 1 expression and beneficial effects of intravenously administered bone marrow stem cells in rats with experimental myocardial infarction. Basic Res Cardiol 2005;100(3):217-23.
8. Abbott JD, Huang Y, Liu D, Hickey R, Krause DS, Giordano FJ. Stromal cell-derived factor-1alpha plays a critical role in stem cell recruitment to the heart after myocardial infarction but is not sufficient to induce homing in the absence of injury. Circulation 2004;110(21):3300-5.
9. Askari AT, Unzek S, Popovic ZB, Goldman CK, Forudi F, Kiedrowski M, Rovner A, Ellis SG, Thomas JD, DiCorleto PE, Topol EJ, Penn MS. Effect of stromal-cell-derived factor 1 on stem-cell homing and tissue regeneration in ischaemic cardiomyopathy. Lancet 2003;362(9385):697-703.
10. Chuva de Sousa Lopes SM, Feijen A, Korving J, Korchynskyi O, Larsson J, Karlsson S, ten Dijke P, Lyons KM, Goldschmeding R, Doevendans P, Mummery CL. Connective tissue growth factor expression and Smad signaling during mouse heart development and myocardial infarction. Dev Dyn 2004;231(3):542-50.
11. Roberts AB, Flanders KC, Kondaiah P, Thompson NL, Van Obberghen-Schilling E, Wakefield L, Rossi P, de Crombrugghe B, Heine U, Sporn MB. Transforming growth factor beta: biochemistry and roles in embryogenesis, tissue repair and remodeling, and carcinogenesis. Recent Prog Horm Res 1988;44:157-97.
12. Ao M, Franco OE, Park D, Raman D, Williams K, Hayward SW. Cross-talk between paracrine-acting cytokine and chemokine pathways promotes malignancy in benign human prostatic epithelium. Cancer Res 2007;67(9):4244-53.
13. Franitza S, Kollet O, Brill A, Vaday GG, Petit I, Lapidot T, Alon R, Lider O. TGF-beta1 enhances SDF-1alpha-induced chemotaxis and homing of naive T cells by up-regulating CXCR4 expression and downstream cytoskeletal effector molecules. Eur J Immunol 2002;32(1):193-202.
14. Chen S, Tuttle DL, Oshier JT, Knot HJ, Streit WJ, Goodenow MM, Harrison JK. Transforming growth factor-beta1 increases CXCR4 expression, stromal-derived factor-1alpha-stimulated signalling and human immunodeficiency virus-1 entry in human monocyte-derived macrophages. Immunology 2005;114(4):565-74.
15. Uematsu T, Tanaka H, Yamaoka M, Furusawa K. Effects of oral squamous cell carcinoma-derived TGF-beta1 on CD26/DPPIV expression in T cells. Anticancer Res 2004;24(2B):619-24.
16. Vanhoof G, Goossens F, De Meester, I, Hendriks D, Scharpe S. Proline motifs in peptides and their biologi-

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cal processing. FASEB J 1995;9(9):736-44.
17. Herrera C, Morimoto C, Blanco J, Mallol J, Arenzana F, Lluis C, Franco R. Comodulation of CXCR4 and CD26 in human lymphocytes. J Biol Chem 2001;276(22):19532-9.
18. Proost P, Struyf S, Schols D, Durinx C, Wuyts A, Lenaerts JP, DeClercq E, De Meester I, Van Damme J. Processing by CD26/dipeptidyl-peptidase IV reduces the chemotactic and anti-HIV-1 activity of stromal-cell-derived factor-1alpha. FEBS Lett 1998;432(1-2):73-6.
19. Christopherson KW, Hangoc G, Broxmeyer HE. Cell surface peptidase CD26/dipeptidylpeptidase IV regulates CXCL12/stromal cell-derived factor-1 alpha-mediated chemotaxis of human cord blood CD34+ progenitor cells. J Immunol 2002;169(12):7000-8.
20. Christopherson KW, Hangoc G, Mantel CR, Broxmeyer HE. Modulation of hematopoietic stem cell homing and engraftment by CD26. Science 2004;305(5686):1000-3.
21. Narducci MG, Scala E, Bresin A, Caprini E, Picchio MC, Remotti D, Ragone G, Nasorri F, Frontani M, Arcelli D, Volinia S, Lombardo GA, Baliva G, Napolitano M, Russo G. Skin homing of Sezary cells involves SDF-1-CXCR4 signaling and down-regulation of CD26/dipeptidylpeptidase IV. Blood 2006;107(3):1108-15.
22. Campbell TB, Hangoc G, Liu Y, Pollok K, Broxmeyer HE. Inhibition of CD26 in human cord blood CD34+ cells enhances their engraftment of nonobese diabetic/severe combined immunodeficiency mice. Stem Cells Dev 2007;16(3):347-54.
23. De Celle T, Cleutjens JP, Blankesteijn WM, Debets JJ, Smits JF, Janssen BJ. Long-term structural and functional consequences of cardiac ischaemia-reperfusion injury in vivo in mice. Exp Physiol 2004;89(5):605-15.
24. Dekel B, Shezen E, Even-Tov-Friedman S, Katchman H, Margalit R, Nagler A, Reisner Y. Transplantation of human hematopoietic stem cells into ischemic and growing kidneys suggests a role in vasculogenesis but not tubulogenesis. Stem Cells 2006;24(5):1185-93.
25. Fernandez L, Sanz-Rodriguez F, Blanco FJ, Bernabeu C, Botella LM. Hereditary hemorrhagic telangiectasia, a vascular dysplasia affecting the TGF-beta signaling pathway. Clin Med Res 2006;4(1):66-78.
26. Rehman J, Li J, Orschell CM, March KL. Peripheral blood "endothelial progenitor cells" are derived from monocyte/macrophages and secrete angiogenic growth factors. Circulation 2003;107(8):1164-9.
27. Shintani S, Murohara T, Ikeda H, Ueno T, Honma T, Katoh A, Sasaki K, Shimada T, Oike Y, Imaizumi T. Mobilization of endothelial progenitor cells in patients with acute myocardial infarction. Circulation 2001;103(23):2776-9.
28. Fernandez L, Sanz-Rodriguez F, Zarrabeitia R, Perez-Molino A, Hebbel RP, Nguyen J, Bernabeu C, Botella LM. Blood outgrowth endothelial cells from Hereditary Haemorrhagic Telangiectasia patients reveal abnormalities compatible with vascular lesions. Cardiovasc Res 2005;68(2):235-48.
29. Cleutjens JP, Blankesteijn WM, Daemen MJ, Smits JF. The infarcted myocardium: simply dead tissue, or a lively target for therapeutic interventions. Cardiovasc Res 1999;44(2):232-41.
30. Mohle R, Bautz F, Rafii S, Moore MA, Brugger W, Kanz L. The chemokine receptor CXCR-4 is expressed on CD34+ hematopoietic progenitors and leukemic cells and mediates transendothelial migration induced by stromal cell-derived factor-1. Blood 1998;91(12):4523-30.
31. Kajiyama H, Shibata K, Ino K, Nawa A, Mizutani S, Kikkawa F. Possible involvement of SDF-1alpha/CXCR4-DPPIV axis in TGF-beta1-induced enhancement of migratory potential in human peritoneal mesothelial cells. Cell Tissue Res 2007;330(2):221-9.
32. Lee BC, Lee TH, Avraham S, Avraham HK. Involvement of the chemokine receptor CXCR4 and its ligand stromal cell-derived factor 1alpha in breast cancer cell migration through human brain microvascular endothelial cells. Mol Cancer Res 2004;2(6):327-38.
33. Segers VF, Tokunou T, Higgins LJ, MacGillivray C, Gannon J, Lee RT. Local delivery of protease-resistant stromal cell derived factor-1 for stem cell recruitment after myocardial infarction. Circulation 2007;116(15):1683-92.
34. Jin DK, Shido K, Kopp HG, Petit I, Shmelkov SV, Young LM, Hooper AT, Amano H, Avecilla ST, Heis-

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sig B, Hattori K, Zhang F, Hicklin DJ, Wu Y, Zhu Z, Dunn A, Salari H, Werb Z, Hackett NR, Crystal RG, Lyden D, Rafii S. Cytokine-mediated deployment of SDF-1 induces revascularization through recruitment of CXCR4+ hemangiocytes. Nat Med 2006;12(5):557-67.

35. Wang CH, Verma S, Hsieh IC, Chen YJ, Kuo LT, Yang NI, Wang SY, Wu MY, Hsu CM, Cheng CW, Cherng WJ. Enalapril increases ischemia-induced endothelial progenitor cell mobilization through manipulation of the CD26 system. J Mol Cell Cardiol 2006;41(1):34-43.
36. Guo Y, Hangoc G, Bian H, Pelus LM, Broxmeyer HE. SDF-1/CXCL12 enhances survival and chemotaxis of murine embryonic stem cells and production of primitive and definitive hematopoietic progenitor cells. Stem Cells 2005;23(9):1324-32.
37. Christopherson KW, Uralil SE, Porecha NK, Zabriskie RC, Kidd SM, Ramin SM. G-CSF- and GM-CSF-induced upregulation of CD26 peptidase downregulates the functional chemotactic response of CD34+. Exp Hematol 2006;34(8):1060-8.

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SUPPLEMENTAL DATA

Exon	Mutation	Gender	Age	Exon	Mutation	Gender	Age
3	Gln77stop	M	23	8	1117insT	M	48
3	Gln77stop	F	48	8	1117insT	F	16
3	Gln77stop	M	64	8	1117insT	M	50
3	Glu83stop	F	65	8	1122delAG	M	70
3	Glu83stop	M	15	9	1255delA	M	55
3	Glu83stop	F	50	9	1255delA	F	53
3	Gln83stop	M	39	9	1310delG	F	19
3	Gln83stop	F	42	9	Arg437Arg	M	20
3	Glu83stop	M	15	9	Arg437Arg	F	42
6	trp261Arg	M	37	9	Arg437Arg	M	36
7	887-918delins870-880	M	56	9	Arg437Arg	M	51
7	887ins18bp	F	36	9	Arg437Arg	M	23
7	887ins18bp	M	28	9	Arg437Arg	F	63
7	Gly331Ser	F	32	9	Arg437Arg	F	23
7	Gly331Ser	M	60	9	Gly413Val	F	36
8	1083delAA	F	57	10	1346delCT	M	35

Table 1. Patients characteristics
Location of the endoglin mutation, gender, and age per HHT-1 patient.

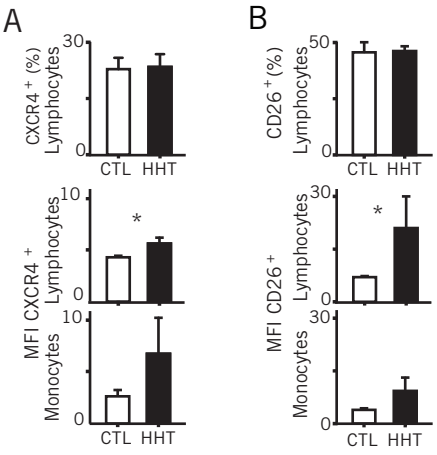

Figure 1. CXCR4 and CD26 expression profiles on lymphocytes and monocytes show similar trends as total MNCs
Percentage of CXCR4 (A) and CD26 positive (B) cells within lymphocyte and monocyte cell-fractions. (A and B) The mean expression of CXCR4 and CD26 per cell on positive lymphocytes and monocytes show similar expression profiles as the total MNC population. *P<0.05; bars show mean ± SEM.

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The effect of CD26 inhibition on homing and migration of cardiomyocyte progenitor cells

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In Preparation

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ABSTRACT

Aim

One of the major challenges of cell-based cardiac repair after myocardial infarction (MI) is to efficiently deliver sufficient numbers of cells to the damaged area. Mostly, cells are delivered via intramyocardial or intracoronary delivery, but these options are invasive and result in loss of cells. Intravenous injection and subsequent homing of cells towards damaged tissue provides an attractive less invasive alternative. In this study we investigated the ability of cardiomyocytes progenitor cells (CMPCs), to respond to stress signals generated by the infarcted myocardium.

Methods

Stromal-cell derived factor (SDF)-1α and its receptor are important for recruitment of cells. CD26, a surface peptidase is a negative regulator of this process. We analyzed surface expression of CXCR4 and CD26 on CMPCs. Their ability to respond to SDF-1α, and the effect on this process of CD26 inhibition was determined in a transwell system. Experimentally induced MI in mice, followed by tail vein injection of CD26 inhibited or untreated CMPCs, was used to study homing *in vivo*. Cardiac function was measured by ultrasound at 2 days, one week and four weeks after MI.

Results

CMPCs carry CXCR4 on their surface, but also very high levels of the inhibitor CD26. Homing of CMPCs towards SDF-1α is low, but inhibiting CD26 using RNAi, Sitagliptin, or a combination increases the homing response dramatically. Injecting CMPCs via the tail vein two days after MI did not result in enhanced cardiac function, and CD26 inhibition prior to injection did not alter this. CMPCs were found in the lungs one day after injection. Their size likely causes CMPCs to be trapped in the capillary lung bed.

Conclusion

Although CMPCs possess the proper tools to respond to SDF-1α cues, high levels of CD26 prevent this. *In vivo*, the effects remain unclear since human CMPCs are trapped in the pulmonary vasculature.

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INTRODUCTION

Myocardial infarction (MI) results in massive cell death in the cardiac muscle. Dead tissue is replaced by a fibrotic scar, which further impairs the pump function of the heart and can ultimately lead to heart failure. Current therapies are palliative and fail to repair the loss of contracting cells. Therefore, cell-based therapy after MI, which aims at preventing scar formation or replacing the scar by new cardiac tissue would be a possible new approach[1]. Since adult cardiomyocytes do not survive ectopic transplantation[2], focus has turned to stem or progenitor cells with the capacity to differentiate into all the cell types necessary to generate heart tissue[3]. We have previously shown that a population of cardiomyocyte progenitor cells (CMPCs) exists in human adult and fetal hearts. These CMPCs have the ability to form spontaneously beating cardiomyocytes, endothelial cells and vascular smooth muscle cells *in vitro*[4]. Moreover, we have shown that the injection of human undifferentiated CMPCs or differentiated CMPC-derived cardiomyocytes (CMPC-CM) into the murine infarcted heart can lead to the formation of human cardiac tissue[5] (chapter 4). After three months, approximately 3% of the originally injected cell population was found back in the heart. This low engraftment may result from backwash during the injection or the immediate exposure of cells to a hostile environment with very low oxygen[6] and nutrient levels, causing acute cell death. Therefore, other methods for delivering cells may be more suitable.

The accepted route for cell delivery in many animal models has been via intramyocardial injection into the infarct region or the borderzone. While this method has the advantage of localized delivery, it has several limitations that prevent instant translation towards a clinical setting[7]. First, intramyocardial injection is an invasive procedure, requiring open chest surgery or catheter insertion. Secondly, direct injection can induce arrhythmias in patients that already have a compromised cardiac function. Potentially, a more suitable route of cell delivery would be intravenous injection. This method is flexible with regard to timing of delivery and allows for repeated injections and the possibility of using (*in vitro*) expanded autologous cells. Furthermore, the cells will home to sites in the heart were delivery of nutrients and oxygen persists, thereby improving their chance of survival.

Intravenous delivery requires homing of cells from the circulation towards the damaged region. One of the most important systems involved in cell homing, chemotaxis, and retention of circulating cells in ischemic myocardium is the interaction between Stromal-cell derived factor-1α (SDF-1α) and its receptor CXCR4[8-10]. SDF1α is expressed in the infarcted region and border zone in the first days after MI[8], attracting cells that carry CXCR4 on their surface. The CXCR4–SDF1α axis is negatively influenced by CD26 (also known as Dipeptidyl peptidase IV, DPPIV). CD26 cleaves the amino-terminal di-peptide of SDF-1α, thereby preventing interaction with the extracellular part of CXCR4[11,12]. An-

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other study suggests that CD26 can co-internalize CXCR4, again disturbing the CXCR4-SDF1α interaction[13].

Previously, we found that blood-derived mononuclear cells (MNCs) carry CD26 on their surface. When CD26 was inhibited, MNC homing towards SDF-1α was greatly enhanced *in vitro*. The homing of intravenously injected bone marrow-derived MNCs towards the ischemic mouse heart increased approximately two-fold when the cells were treated with a CD26 inhibitor prior to injection (chapter 5); for human blood-derived MNCs this effect was even more pronounced (five-fold increase, not shown). Additionally, we found that MNCs isolated from Hereditary Hemorrhagic Telangiectasia-1 (HHT-1) patients are poorly recruited to the damaged murine myocardium[14]. HHT-1 MNCs carry higher levels of CD26 on their surface compared to healthy controls and inhibiting this peptidase prior to intravenous injection led to a normalization of the homing response of HHT-MNCs towards the ischemic heart (chapter 5).

However, even though enhanced homing and integration of MNCs may improve the function of the heart via paracrine stimulation of angiogenesis[14] or decreased recruitment of inflammatory cells[15], MNCs do not regenerate the cardiac muscle.

Therefore in the present study, we questioned if CMPCs can be used for intravenous applications. We investigated if manipulation of the SDF-1α/CXCR4 axis by altering CD26 expression can lead to an improvement of CMPCs' response to SDF-1α *in vitro* and *in vivo*, and if this is translated to an enhanced cardiac function after MI.

MATERIAL AND METHODS

Isolation and culture of CMPCs

CMPCs were isolated from human fetal hearts as described previously[16]. Cells were cultured for at least eight passages in growth medium before experiments were initiated.

CD26 knockdown

Two different clones were used for RNAi-mediated CD26 knock-down or transduction with a scrambled sequence, obtained from the MISSION-library (Sigma). Cells were transduced with the shRNA lentivirus at an MOI of 1 in the presence of 4 μg/ml polybrene for 6 hours. Cells were washed and after 24 hours, 1 μg/ml puromycin was added for 2 days, after which the cells were used for the indicated experiments.

PCR analysis

RNA was isolated using Tripure (Roche) and reverse transcribed using oligo-dT Superscript 3 (Invitrogen). The PCR reactions started with 5 min at 94 °C, followed by 35

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cycles of: 94°C for 15 seconds, 56°C for 30 seconds and 72°C for 45 seconds. Products were analyzed on ethidium bromide-stained 1% agarose gel. GAPDH was used as input control. Primer sequences were:
CD26: For 5'-GTGGGTCACATGGTCACCAG, Rev 5'-ACAGAGCAGAGTAGGCACTGA.
GAPDH: For 5'-ATCACTGCCACCCAGAAGAC, Rev 5'- ATGAGGTCCACCACCCTGTT.

FACS analysis
Surface expression levels of CXCR4 and CD26 were analyzed by flow cytometric analysis using $2*10^5$ CMPCs in 100µl PBS. CMPCs were stained with anti-CD26-FITC (Serotec) and anti-CXCR4-PE (BD Pharmingen) antibodies. Isotype-matched fluorochrome-conjugated antibodies were used as controls. Fluorescence was measured on a flow cytometer (BD LSR-II). Analysis was performed using BD FACS Diva software.

Migration assay
Migration of CMPCs was assessed in a transwell system using polycarbonate filters with 8 µm pores (Corning). The filter was coated with 0.1% gelatin. Untreated CMPCs, CMPCs treated with CD26 RNAi, or CMPCs treated with with off-target RNAi were incubated at room temperature for 15 minutes with 5 mM DiprotinA (DipA, Sigma-Alderich) or 10 mM Sitagliptin (Januvia, Merck). Fifty thousand CMPCs in DMEM/M199 medium supplemented with 0.5% FCS were applied to the upper well. In the lower well, medium without or with 200ng/ml SDF-1α (PeproTech) was added. The cells were allowed to migrate for 3 hours at 37°C. After migration, cells were washed and the upper surface was wiped to remove the non-migrating cells. The membranes were fixed in methanol, washed with water, stained with 0.2% crystal violet, and lyzed using 10% acetic acid. The OD was measured at 595 nm. The migration ratio was calculated as the number of cells migrated to SDF-1α in comparison to the number of cells migrated in the absence of SDF-1α.

Cell size
The diameter of CMPCs in suspension was determined on a Casy cell counter and analyzer (model TT), after trypsinizing the cells.

Animals
All experiments were carried out in accordance with the *Guide for the Care and Use of Laboratory Animals*, with prior approval by the Animal Ethical Experimentation Committee, Leiden University.

Myocardial infarction and cell transplantation
MI was induced by ligation of the left coronary artery in male 10-12 weeks old NOD-SCID

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mice (Charles River Laboratories) under isoflurane anesthesia, as described previously[17]. In sham-operated animals, the surgery was performed identically, except for the actual ligation. Two days after surgery, after ultrasound measurement a total of $0.5*10^6$ CMPCs in 100-150 µl PBS was injected via the tail vein. Eight MI mice received untreated CMPCs, 10 MI mice received CMPCs that were incubated with 10 mM Sitagliptin, and four MI mice were injected with the vehicle (PBS). In the sham operated group, 3 mice received Sitagliptin treated CMPCs and three mice were injected with PBS.
One day after i.v. injection, 2 MI mice that received CMPCs pretreated with Sitagliptin were sacrificed. Four weeks after MI the remaining animals were sacrificed. The heart was flushed with 2 ml cold PBS via the right ventricle before excision. The lungs, spleen, and liver were removed and stored at -80°C. The hearts were treated as previously described 18 and embedded in OCT compound (Sakura).

Cardiac function measurement

Cardiac function was determined by ultrasound at 2 days, 1 week, and 4 weeks after induction of MI. Mice were anesthetized with 2% isoflurane through a facemask and were laid on a platform in the supine position with all legs taped to ECG electrodes. Body temperature was monitored via a rectal thermometer and maintained at 36–38°C using a heating pad and lamp. Excess hair was removed from the chest using a chemical hair remover (Veet). A preheated ultrasound gel (Aquasonic 100) was spread over the chest. Imaging was performed using a high-resolution, micro-ultrasound imaging system (Vevo 770, VisualSonics). The transducer had a central frequency of 30 MHz and a focal length of 12.7 mm. The repetition rate for M-mode recording was 1,000 Hz. An EKV (ECG-based kilohertz visualization) image was obtained of the parasternal long axis and short axis. The parasternal long axis EKV was used to determine the volumes of the heart in diastole and systole, and ejection fraction was calculated using Visualsonics software.

Staining

The hearts were cut into 7 µm cryosections. Per series 15 consecutive sections were mounted on 5 glass slides, and 25 sections were collected in a vial. At least 10 series per heart were cut. One slide from each series was used for immunostaining to identify human cells. The sections were air-dried and fixed in cold acetone for 5 minutes. The tissue was incubated with 0.2% Triton in PBS for 10 minutes. After washing, the sections were blocked in 1% BSA in PBS for 30 minutes and subsequently incubated with mouse anti-human integrin-β1 antibody (Santa Cruz) 1:50 or PECAM-1 antibody (Santa Cruz) 1:200 directed against mouse as a positive control in blocking reagent overnight at 4°C. Negative controls were incubated with blocking reagent or isotype control. As a

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secondary antibody, anti-mouse Alexa 555 or anti-Rabbit Alexa 555 was used 1:400 in PBS for 30 minutes. After washing, slides were mounted with Vectashield containing DAPI (Vector Laboratories).

PCR for human specific genomic DNA

Genomic DNA was isolated by pooling 50 cryosections (7µm) followed by incubation in 500 µl lysis buffer (0.1 M Tris-HCl pH 8.5, 5 mM EDTA, 0.2% SDS, 0.2 M NaCl and proteinase K). Genomic DNA was extracted using phenol-chloroform and precipitated with ethanol. The polymerase chain reaction (PCR) primers were described previously and are specific for human Alu sequences, resulting in a product of 224 basepairs[19]. PCR was carried out using 100 ng of total DNA under the following conditions: 95°C for 10 minutes, followed by 25 cycles of 95°C for 30 seconds, 58°C for 45 seconds and 72°C for 45 seconds; and 72°C for 10 minutes. The samples were run on a 1% agarose gel.

Statistical analysis

Statistical significance was evaluated with SPSS v16.0 for Windows using ANOVA (with Bonferroni correction for multiple-group comparisons) or Mann-Whitney U test, as applicable. Results are expressed as mean ± SEM. A value of $p \leq 0.05$ was considered statistically significant.

RESULTS

CMPCs express CXCR4 and CD26 on their surface

Using flow cytometric analysis, the expression of the receptor CXCR4 and the peptidase CD26 on CMPCs was determined. CMPCs clearly carry CXCR4 on their surface (Figure 1A). High levels of CD26 were observed on CMPCs (Figure 1B) and this expression was confirmed by PCR (Figure 1C).

Inhibition of CD26 greatly enhances the migration of CMPCs to SDF1α

Knock-down of CD26 expression by lentiviral RNAi was analyzed by PCR (Figure 2A). RNAi- or Sitagliptin-mediated downregulation of CD26 surface expression was confirmed by flow cytometry (Figure 2B). Treatment of CMPCs with DipA also lowered the surface expression of CD26, but not as potently as Sitagliptin (not shown).

The role of CD26 in migration of CMPCs was assessed in a transwell assay using SDF-1α as a chemoattractant. In off-target RNAi-treated CMPCs, SDF-1α increased the migration ± 2.7 fold (Figure 2C,D). When CD26 was inhibited by RNAi, the migration of the cells to SDF-1α increased five-fold. The difference was even more pronounced when

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Sitagliptin was used to inhibit CD26 (7-fold increase: Figure 2C,D). These results show that CMPCs are able to respond to stress signals like SDF-1α, but their response may be compromised due to high CD26 levels.

In vivo homing of CMPCs after intravenous injection

To investigate whether manipulation of the CXCR4-SDF-1α axis can enhance the migration of CMPCs towards the damaged heart and thereby enhance cardiac function, immunodeficient mice underwent MI and 2 days later received an intravenous injection of vehicle, or untreated or Sitagliptin-treated CMPCs. Ultrasound measurements were performed at 2 days (cells were injected immediately after this measurement), 7 days and 4 weeks post-MI. We did not observe a difference in cardiac function between the MI groups at any time-point based on end-diastolic, end-systolic volumes and ejection fraction (Figure 3).

Four weeks after induction of MI, hearts were excised and processed for sectioning. As a positive control for the tissue embedding, we used a mouse specific anti-PECAM antibody (Figure 4A). As positive control for human cells, a slide was stained from a previous experiment[5] (chapter 4) from which we knew human cells were present

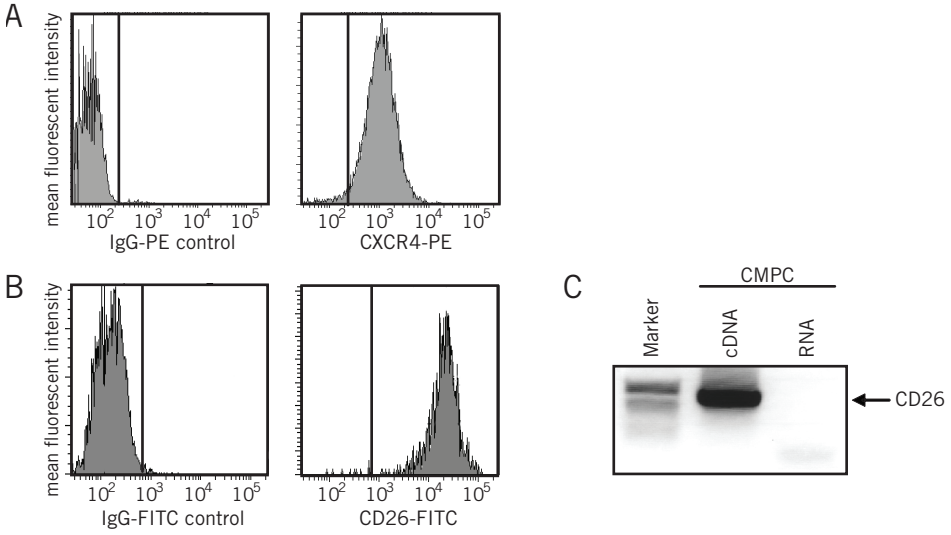

Figure 1. CMPCs express CXCR4 and CD26

(A, B) Flow cytometric analysis shows surface expression of CXCR4 and CD26 on CMPCs, mean fluorescent intensity (MFI) is depicted. Left plots show isotype controls. (C) CD26 expression in CMPCs was confirmed by PCR, the corresponding RNA sample (not reverse transcribed) was used as a negative control.

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(Figure 4B). We very sporadically detected human integrin-β1-positive cells in hearts of intravenously injected mice in both the Sitagliptin treated group (n=2) or untreated CMPC group (n=2). Per heart a minimum of 10 sections was analyzed, at intervals of approximately 210μm. In 4 out of a total of 40 sections analyzed, small foci of human cells were observed in the border zone and infarcted region (Figure 4C). To further analyze the presence of human cells, genomic DNA was isolated from collected heart

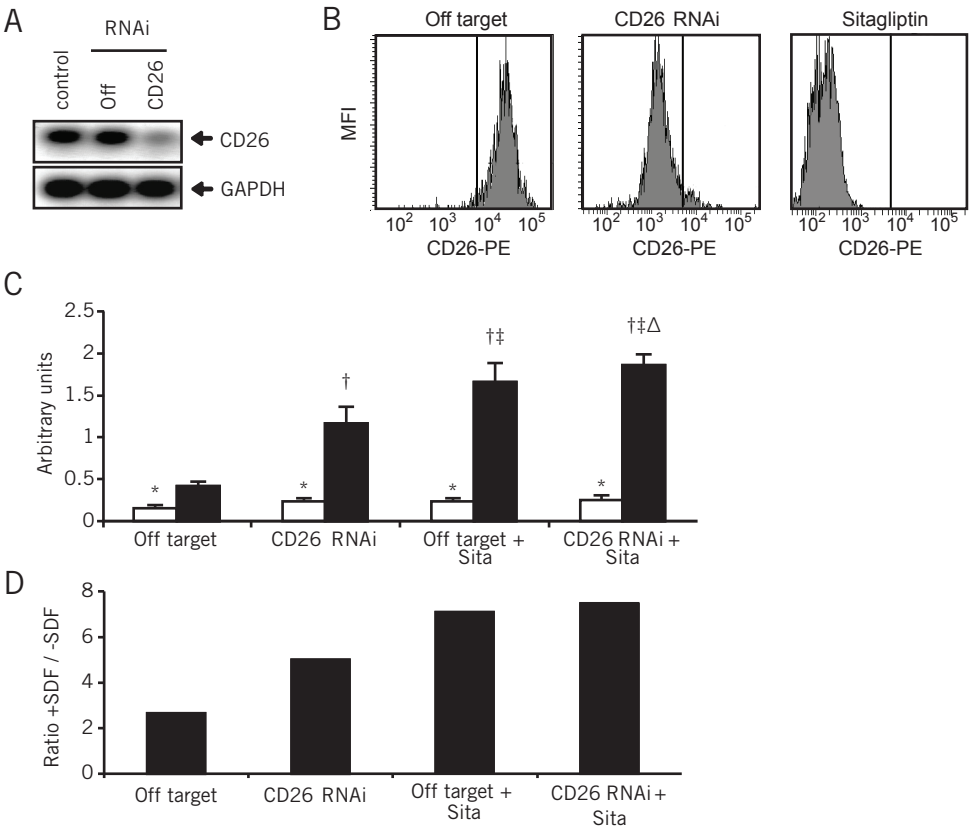

Figure 2. Effect of CD26 inhibition on SDF-1α induced migration

(A) CMPCs were treated with lentiviral off-target (Off) or CD26 RNAi. Knock-down of CD26 expression was validated by PCR. Control lane represents non-transduced CMPCs. (B) The CD26 surface expression was measured flow cytometrically on off-target treated CMPCs (left). After inhibition with CD26 RNAi or Sitagliptin CMPCs show only low-levels of CD26 on their surface (middle and right). (C) The migration capacity of control (off-target) or 3 conditions of CD26 inhibited cells towards SDF-1α (black bars) and control medium (white bars) was determined in a transwell system. CMPC migration is shown in arbitrary units (C) or as the ratio of SDF-1+/SDF-1- migrated cells (D).

* P<0.05 compared to SDF-1+ condition of the same condition. † p<0.05 compared to off-target SDF-1+, ‡ p<0.05 compared to CD26 RNAi SDF-1+, Δ p<0.05 compared to off-target + Sitagliptin.

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tissue cryosections. A PCR using primers specific for human Alu repeats was performed to identify human chromosomal DNA. Only very low traces of human Alu DNA were present (Figure 4D).

Since no substantial numbers of human CMPCs could be found in the mouse hearts, we analyzed the DNA of other collected organs. DNA was isolated from spleen, liver, and lung of CMPC-receiving animals. Based on PCR for human Alu repeats we were able to detect only very low levels of human DNA in these tissues (Figure 4E). In an attempt to identify human cells in tissues, sections taken from lung, spleen and liver were stained for human integrin-β1, but no human cells were observed.

Short-term analysis of homing

Likely, after four weeks, human cells that have been captured in capillary beds have died and are scavenged, making it impossible to identify them by antibody staining.

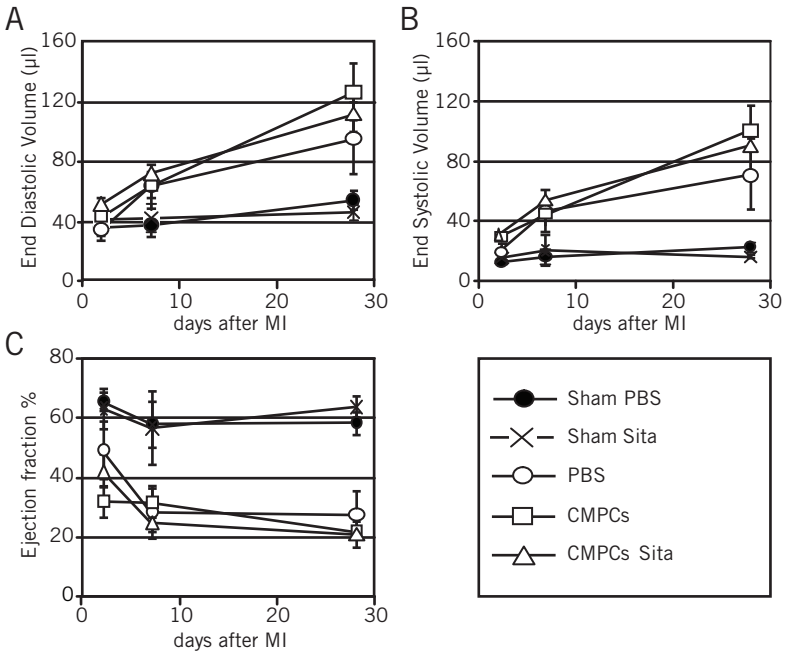

Figure 3. Cardiac function after intravenous injection of CMPCs

To determine the effect of intravenous injection of CMPCs on cardiac function after MI, ultrasound analysis was performed 2, 7 and 28 days after MI. (A) The end-diastolic and (B) end-systolic volume were measured. (C) The ejection fraction was calculated with visualsonics software. There was no statically significant difference in any of the measured parameters in the MI animals. However, cardiac function was decreased in MI animals in general, as can be observed in the lower EF and higher EDV and ESV compared to sham operated animals.

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To determine where human CMPCs are found shortly after injection, we sacrificed two mice one day after receiving human CMPCs intravenously. Heart, lung, spleen, and liver were isolated and processed for cryosections. Integrin-β1-positive cells were identified in the lungs of transplanted mice (Figure 5A,B), low numbers of cells were found in the liver (not shown).

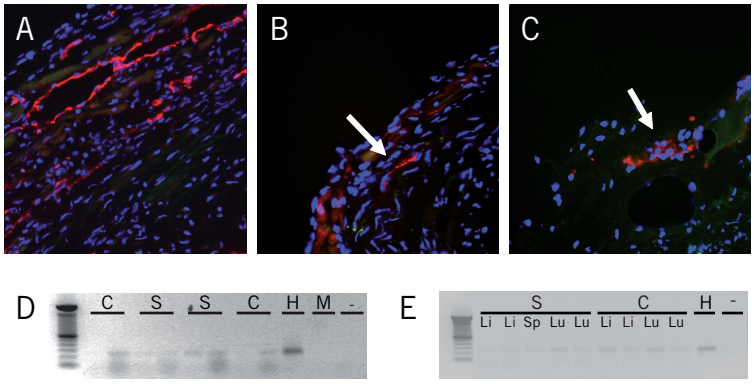

Figure 4 Detection of human CMPCs
(A) As a positive control for staining procedures a mouse specific endothelial staining was performed (PECAM-1 in red, nuclei in blue 250x). (B) Staining for human specific integrin-β1 showing human CMPCs in mouse heart after intramyocardial injection. Arrow indicates a small patch of human cells (400x). (C) In intravenously injected animals, very small foci of human cells could be observed in the infarcted regions in the heart (arrow) in a minority of tissue sections (human cells in red, nuclei in blue, 400x). (D) Genomic DNA was extracted from heart tissue, and a PCR for human Alu repeats was performed in duplo. Only very low levels of human DNA were detected. Mice receiving untreated CMPCs are labeled **C**, Sitagliptin pretreated cells **S**, human heart-derived genomic DNA **H** as a positive control, **M** mouse genomic DNA, and - is water. (E) Genomic DNA was extracted from other organs: Liver (Li), Spleen (Sp) and Lung (Lu) but only very low traces of human DNA were detected.

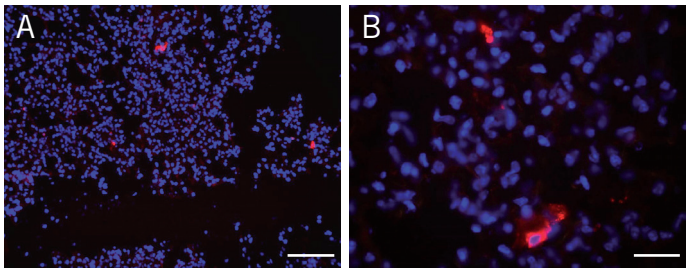

Figure 5 Human cells in the lung after 2 days
(A, B) Lungs were excised and stained for human specific integrin β1 antibody (red, nuclei in blue) 1 day after CMPC injection. A few human cells can be observed. Scale bars: A 250 μm, B 50 μm.

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As this may indicate that the cells may be trapped in the lung capillary bed, the size of CMPCs in suspension was measured. The average diameter of most CMPCs was 17-20 μm, This is in contrast to MNCs which are reported to have a diameter of 8 μm[20]. This could explain why CMPCs were hardly found beyond the lung circulation.

DISCUSSION

Cell-based therapy is a promising treatment for patients suffering from cardiac disease. However, the major obstacle is to deliver sufficient cells that remain in the damaged tissue to induce repair. Intramyocardial injection has the advantage of targeted delivery, ensuring that the cells are released in the ventricular wall. However, it remains an invasive procedure and the damaged area is a very hostile environment. Moreover, injections need to take place before large scar formation has occurred, which shortens the time-window for treatment opportunities in a clinical setting. Intravenous delivery of cells has the benefit of being less invasive and may prolong the time for treatment. A major advantage of i.v. delivery is the fact that cells will arrive in the heart via the circulation at a destination where the delivery of oxygen and nutrients is still present, enhancing their chances of survival. Previous studies using mesenchymal stem cells (MSCs) have shown that intravenous injection of cells can lead to an improvement of cardiac left ventricular function and a smaller infarct size[21,22], but it remains debatable if MSCs differentiate *in vivo* into new cardiac tissue or if they exert their effects via paracrine stimulation[23]. CMPCs can differentiate into cardiomyocytes, endothelial cells, and smooth muscle cells when injected intramyocardially[5] (chapter 4), making them a promising source for cell-based therapy. In this study we set out to determine if CMPCs can be delivered intravenously and are able to home to the heart by responding to the signals provided by the damaged myocardium.

After MI, the heart induces the expression of several chemokines cytokines, and growth factors that are involved in the repair process after damage (reviewed in [24]). Besides promoting cell survival and differentiation, signals are expressed that regulate trafficking of cells. Especially SDF-1α appears to play a central role in the recruitment of cells towards the injured tissue[9]. SDF-1α is upregulated after MI, and peaks around 24 hours post-MI[8]. MSCs that were intravenously delivered were found in the damaged heart at the highest levels when transplanted 24 hours after MI8. Importantly, in the absence of injury, homing of bone marrow cells to the heart does not occur[9]. Delivering SDF-1α to the myocardium via cell-based gene therapy[10], direct injection of SDF-1α[25] or protease-resistant SDF-1α[26] induced recruitment of cells towards the injured heart. Similarly, overexpression of the cognate receptor CXCR4 on the surface of injected MSCs also in-

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creases the homing of cells to the infarcted myocardium[27]. Although CXCR4 expression levels are known to be important for migration, our previous data (chapter 5) imply that the migratory behavior of (mononuclear) cells is also regulated by the cell surface peptidase CD26[11]. When inhibiting CD26 on the surface of HSCs a dramatic increase in the engraftment of cells in the bone marrow was observed[28], a process that is also controlled by SDF-1/CXCR4. We have shown in chapter 5 that inhibiting CD26 on the surface of MNCs greatly enhanced the homing of cells towards SDF-1α *in vitro* and towards the ischemic myocardium *in vivo*. In this study we show that CXCR4 is present on CMPCs, which should make them responsive to SDF-1α cues. However, we also observed high levels of CD26 on the CMPC surface, which may explain its low responsiveness to SDF-1α stimulation *in vitro*.

We were able to increase the response of CMPCs to SDF-1α *in vitro* by inhibiting CD26 expression with RNAi, DipA, or Sitagliptin. Blocking CXCR4 on CMPCs by the CXCR4 antagonist AMD3100 incubation abolished the migration towards SDF-1α (not shown). These data indicate that CMPCs have the proper tools to home towards damaged tissue if necessary.

To test CMPC applicability for intravenous injection *in vivo*, we induced MI in immunodeficient mice and delivered Sitagliptin-treated or untreated CMPCs into the circulation via the tail vein. Unfortunately, we did not observe any effect on cardiac function, which can be explained by the low numbers of human CMPCs in the heart 4 weeks post-MI. Since we were able to identify human MNCs in the heart under similar experimental conditions in a previous study (chapter 5), we do not expect this lack of CMPCs in the heart to be due to intra-species differences. Conceivably, CMPCs did not pass the pulmonary system. Multiple studies have shown that intravenous delivery of human stem or progenitor cells into rodents does not lead to high numbers of cells reaching the organ of interest[29,30]. Instead, most cells are trapped in the lungs. A study in rats compared intravenous infusion of multiple types of human stem cell sources including MSCs, neural stem cells, and bone marrow MNCs[20]. The pulmonary passage of MNCs is 30-fold higher compared to that of MSCs, due to different cell sizes: rat MNCs have a diameter of 7 μm, while MSCs have a diameter of 18 μm. Human MNCs have a diameter of ± 8 μm and were shown to reach the mouse heart. We measured the average size of human CMPCs, which is approximately 18 μm, strongly suggesting that the CMPCs were too large for pulmonary passage. Indeed, one day after intravenous injection of CMPCs following MI, cells were found the lung. Thus, even though CD26 inhibition provided a stronger attraction between the CXCR4 on CMPCs and the SDF-1α stimulation *in vitro*, expression of SDF1α in the heart *in vivo* was not sufficient to force the cells back into the circulation.

Although the current study does not provide an alternative to the invasive intramyocardial injection procedure, it does show that CMPCs can respond to stress signals. Future

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studies should be aimed at delivering cells directly into the left ventricular cavity infusion, which circumvents the first passage effects in the lungs, and was proven more effective in the case of MSCs[29]. Another important aspect of the SDF-1α-CXCR4 axis is that it also plays a role in retention of cells in tissue (reviewed in[31]). It is possible that inhibiting CD26 on the surface of CMPCs prior to intramyocardial injection will lead to the formation of larger cell grafts; this will be investigated in the future.

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REFERENCES

1. Evans SM, Mummery C, Doevendans PA. Progenitor cells for cardiac repair. Semin Cell Dev Biol 2007;18(1):153-160.
2. Reinecke H, Zhang M, Bartosek T, Murry CE. Survival, integration, and differentiation of cardiomyocyte grafts: a study in normal and injured rat hearts. Circulation 1999;100(2):193-202.
3. Smits AM, van Vliet P., Hassink RJ, Goumans MJ, Doevendans PA. The role of stem cells in cardiac regeneration. J Cell Mol Med 2005;9(1):25-36.
4. Goumans MJ, de Boer TP, Smits AM, van Laake LW, van Vliet P, Metz CH, Korfage TH, Kats KP, Hochstenbach R, Pasterkamp G, Verhaar MC, van der Heyden MA, de KD, Mummery CL, van Veen TA, Sluijter JP, Doevendans PA. TGF-beta1 induces efficient differentiation of human cardiomyocyte progenitor cells into functional cardiomyocytes in vitro. Stem Cell Res 2007;1(2):138-149.
5. Smits AM, van Laake LW, denOuden K, Schreurs C, Szuhai K, van Echteld CJ, Mummery CL, Doevendans PA, Goumans MJ. Human cardiomyocyte progenitor cell transplantation preserves long-term function of the infarcted mouse myocardium. Cardiovasc Res 2009.
6. Muller-Ehmsen J, Krausgrill B, Burst V, Schenk K, Neisen UC, Fries JW, Fleischmann BK, Hescheler J, Schwinger RH. Effective engraftment but poor mid-term persistence of mononuclear and mesenchymal bone marrow cells in acute and chronic rat myocardial infarction. J Mol Cell Cardiol 2006;41(5):876-884.
7. Wollert KC, Drexler H. Clinical applications of stem cells for the heart. Circ Res 2005;96(2):151-163.
8. Ma J, Ge J, Zhang S, Sun A, Shen J, Chen L, Wang K, Zou Y. Time course of myocardial stromal cell-derived factor 1 expression and beneficial effects of intravenously administered bone marrow stem cells in rats with experimental myocardial infarction. Basic Res Cardiol 2005;100(3):217-223.
9. Abbott JD, Huang Y, Liu D, Hickey R, Krause DS, Giordano FJ. Stromal cell-derived factor-1alpha plays a critical role in stem cell recruitment to the heart after myocardial infarction but is not sufficient to induce homing in the absence of injury. Circulation 2004;110(21):3300-3305.
10. Askari AT, Unzek S, Popovic ZB, Goldman CK, Forudi F, Kiedrowski M, Rovner A, Ellis SG, Thomas JD, DiCorleto PE, Topol EJ, Penn MS. Effect of stromal-cell-derived factor 1 on stem-cell homing and tissue regeneration in ischaemic cardiomyopathy. Lancet 2003;362(9385):697-703.
11. Proost P, Struyf S, Schols D, Durinx C, Wuyts A, Lenaerts JP, DeClercq E, De Meester I, Van Damme J. Processing by CD26/dipeptidyl-peptidase IV reduces the chemotactic and anti-HIV-1 activity of stromal-cell-derived factor-1alpha. FEBS Lett 1998;432(1-2):73-76.
12. Vanhoof G, Goossens F, De Meester I, Hendriks D, Scharpe S. Proline motifs in peptides and their biological processing. FASEB J 1995;9(9):736-744.
13. Herrera C, Morimoto C, Blanco J, Mallol J, Arenzana F, Lluis C, Franco R. Comodulation of CXCR4 and CD26 in human lymphocytes. J Biol Chem 2001;276(22):19532-19539.
14. van Laake LW, van den Driesche S, Post S, Feijen A, Jansen MA, Driessens MH, Mager JJ, Snijder RJ, Westermann CJ, Doevendans PA, van Echteld CJ, ten Dijke P, Arthur HM, Goumans MJ, Lebrin F, Mummery CL. Endoglin has a crucial role in blood cell-mediated vascular repair. Circulation 2006;114(21):2288-2297.
15. Burchfield JS, Iwasaki M, Koyanagi M, Urbich C, Rosenthal N, Zeiher AM, Dimmeler S. Interleukin-10 from transplanted bone marrow mononuclear cells contributes to cardiac protection after myocardial infarction. Circ Res 2008;103(2):203-211.
16. Smits AM, van Vliet P, Metz CH, Korfage T, Sluijter JP, Doevendans PA, Goumans MJ. Human cardiomyocyte progenitor cells differentiate into functional mature cardiomycoytes: an in vitro model for studying human cardiac physiology and pathophysiology. Nat Protoc 2009;4(2):232-243.
17. van Laake LW, Passier R, Monshouwer-Kloots J, Nederhoff MG, Ward-vanOostwaard D, Field LJ, van Echteld CJ, Doevendans PA, Mummery CL. Monitoring of cell therapy and assessment of cardiac function using magnetic resonance imaging in a mouse model of myocardial infarction. Nat Protoc 2007;2(10):2551-

proefschrift Anke.indd 117 15-07-2009 12:00:47

2567.
18. Bajanca F, Luz M, Duxson MJ, Thorsteinsdottir S. Integrins in the mouse myotome: developmental changes and differences between the epaxial and hypaxial lineage. Dev Dyn 2004;231(2):402-415.
19. Dekel B, Shezen E, Even-Tov-Friedman S, Katchman H, Margalit R, Nagler A, Reisner Y. Transplantation of human hematopoietic stem cells into ischemic and growing kidneys suggests a role in vasculogenesis but not tubulogenesis. Stem Cells 2006;24(5):1185-1193.
20. Fischer UM, Harting MT, Jimenez F, Monzon-Posadas WO, Xue H, Savitz SI, Laine GA, Cox CS. Pulmonary passage is a major obstacle for intravenous stem cell delivery: The pulmonary first pass effect. Stem Cells Dev 2008.
21. Jiang W, Ma A, Wang T, Han K, Liu Y, Zhang Y, Dong A, Du Y, Huang X, Wang J, Lei X, Zheng X. Homing and differentiation of mesenchymal stem cells delivered intravenously to ischemic myocardium in vivo: a time-series study. Pflugers Arch 2006;453(1):43-52.
22. Nagaya N, Fujii T, Iwase T, Ohgushi H, Itoh T, Uematsu M, Yamagishi M, Mori H, Kangawa K, Kitamura S. Intravenous administration of mesenchymal stem cells improves cardiac function in rats with acute myocardial infarction through angiogenesis and myogenesis. Am J Physiol Heart Circ Physiol 2004;287(6):H2670-H2676.
23. Penn MS, Mangi AA. Genetic enhancement of stem cell engraftment, survival, and efficacy. Circ Res 2008;102(12):1471-1482.
24. Vandervelde S, van Luyn MJ, Tio RA, Harmsen MC. Signaling factors in stem cell-mediated repair of infarcted myocardium. J Mol Cell Cardiol 2005;39(2):363-376.
25. Zhuang Y, Chen X, Xu M, Zhang LY, Xiang F. Chemokine stromal cell-derived factor 1/CXCL12 increases homing of mesenchymal stem cells to injured myocardium and neovascularization following myocardial infarction. Chin Med J (Engl) 2009;122(2):183-187.
26. Segers VF, Tokunou T, Higgins LJ, MacGillivray C, Gannon J, Lee RT. Local delivery of protease-resistant stromal cell derived factor-1 for stem cell recruitment after myocardial infarction. Circulation 2007;116(15):1683-1692.
27. Cheng Z, Ou L, Zhou X, Li F, Jia X, Zhang Y, Liu X, Li Y, Ward CA, Melo LG, Kong D. Targeted migration of mesenchymal stem cells modified with CXCR4 gene to infarcted myocardium improves cardiac performance. Mol Ther 2008;16(3):571-579.
28. Christopherson KW, Hangoc G, Mantel CR, Broxmeyer HE. Modulation of hematopoietic stem cell homing and engraftment by CD26. Science 2004;305(5686):1000-1003.
29. Barbash IM, Chouraqui P, Baron J, Feinberg MS, Etzion S, Tessone A, Miller L, Guetta E, Zipori D, Kedes LH, Kloner RA, Leor J. Systemic delivery of bone marrow-derived mesenchymal stem cells to the infarcted myocardium: feasibility, cell migration, and body distribution. Circulation 2003;108(7):863-868.
30. Gao J, Dennis JE, Muzic RF, Lundberg M, Caplan AI. The dynamic in vivo distribution of bone marrow-derived mesenchymal stem cells after infusion. Cells Tissues Organs 2001;169(1):12-20.
31. Petit I, Jin D, Rafii S. The SDF-1-CXCR4 signaling pathway: a molecular hub modulating neo-angiogenesis. Trends Immunol 2007;28(7):299-307.

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7

A new *in vitro* model for stem cell differentiation and interaction

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ABSTRACT

Development involves an interplay between various cell types from their birth to their disappearance by differentiation, migration, or death. Analyzing these interactions provides insights into their roles during the formation of a new organism. As a study tool for these interactions, we have created a model based on embryoid bodies (EBs) generated from mouse embryonic stem (mES) cells, which can be used to visualize the differentiation of mES cells into specific cell types while at the same time allowing controlled removal of this same cell population using an enzyme–prodrug approach. Cell-specific expression of Cre induces a switch of EGFP expression to LacZ. Furthermore, it leads to the expression of nitroreductase (NTR), which in combination with the prodrug CB1954 induces apoptosis. Here, we validate this model by showing expression of LacZ and NTR after Cre-mediated recombination. Additionally we show, as an example, that we can target the endothelial cells in EBs using the Tie-2 promoter driving Cre. Ablating Cre-expressing cells by adding CB1954 to the culture led to an abrogated vascular formation.
This system can easily be adapted to determine the fate and interaction of many different cell types provided that there is a cell-type-specific promoter available.

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INTRODUCTION

Embryonic development involves a dynamic interplay between different progenitor cells populations, their differentiated progeny and adjacent neighbors to generate a living organism. Embryonic stem (ES) cell-derived embryoid bodies (EBs) resemble the post-implantation egg-cylinder stage embryo and provide an *in vitro* model to study differentiation during early development[1]. For example, vascular development within EBs follows an orderly sequence of events that recapitulates vasculogenesis *in vivo*[2]. When cultured in a collagen I matrix, EBs develop a network of branching endothelial cell outgrowths that are covered with pericytes, thus constituting a valuable model to study angiogenesis[3].

To study the differentiation and interaction of cells during development we have designed a model to target a specific cell type within the EB, allowing us to visualize the differentiation into a specified cell type and determine its contribution to development by selective ablation.

A constitutively active promoter drives the expression of EGFP. After Cre-mediated excision, EGFP is replaced by nitroreductase (NTR) and β-galactosidase expression. The NTR enzyme catalyzes the reduction of nitro-groups into hydroxylamine in the presence of NADH or NAD(P)H. NTR can metabolize the pro-drug CB1954 into an alkylating agent that cross-links DNA, resulting in apoptosis[4]. This thereby acts as an inducible ablation system. Previously, NTR has been reported as an option for gene-directed enzyme pro-drug therapy (GDEPT) in patients[5].

Furthermore, the LacZ gene allows visualization of Cre-expressing (and thus NTR expressing) cells. By placing Cre under control of a cell-type specific promoter, a specific population of cells can be targeted, and the time-point at which this population appears can be investigated. In this report we validate this system, and show as an example the result of targeting endothelial cells in EBs.

MATERIAL AND METHODS

Constructs

The ActENIL construct (± 14 Kb) was generated using pCAG as a backbone, which contains a modified chicken actin promoter, polyA sequence and a hygromycin resistance gene. An EGFP-lox-stop cassette, NTR (obtained from CMV-NTR), and an IRES-LacZ cassette (isolated from pGT1.8IresBgeo) were cloned into pCAG. The ActENIL construct was linearized using AhdI.

The Tie2-Cre plasmid used in this model was generated by inserting the Tie-2 promoter (obtained from PGL3-Tie2[6]) and Cre (derived from pCAGGS) into pBS.

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Mouse ES (mES) cell culture

IB10-mES cells were cultured under feeder-free conditions on gelatin coated dishes in Buffalo Rat Liver cell conditioned medium (BRL-CM) as described previously[7]. The ActENIL plasmid was electroporated into mES cells, which were subsequently placed on Hygromycin selection. Surviving clones were cultured separately in BRL-CM.
To determine the characteristics of the model, ActENIL harboring cells were stably transfected with CMV-Cre (pCAGGS) or Tie-2-Cre.

Flow cytometry

Flow cytometric analysis was performed using a Cytomics FC500 (Beckman Coulter) to determine EGFP expression. The activity of NTR was determined by incubating cells with 1μM CytoCy5S (GE-Healthcare) for 3 hours prior to measuring red fluorescence.

MTT assay

To determine the sensitivity mES cell lines to CB1954, cell survival was measured by MTT assay at different concentrations of CB1954 ,as described previously[8]. Each measurement was carried out in triplicate and each condition at least in duplicate. Cell viability was calculated relative to untreated cells.

Endothelial sprouting assay

EBs were generated using hanging drops as described previously[9] in DM (DM: Dulbecco's modified Eagle's Medium (DMEM) supplemented with 2 mM L-glutamin, non-essential amino acids, 20% FCS, β-mercaptoethanol, 50 ng/ml VEGF (Sigma) and 25 ng/ml bFGF (Sigma)). After 2 days, EBs were transferred to ultra-low attachment dishes (Corning) and grown in suspension for 7 days. To induce sprouting, EBs were placed in a collagen matrix containing 0.8mg/ml rat collagen I (Roche) in DM[3]. CB1954 was added to the collagen matrix as required.

Cellular staining

To visualize endothelial cells and Cre recombinase, collagen matrices were mounted on glass microscope glasses as described[3]. EBs were fixed in IHC Zinc Fixative (BD Biosciences) and stained with anti-mouse PECAM (BD PharMingen) or anti-Cre (Novagen), Alexa Fluor 555 goat anti-mouse or anti-rabbit (Invitrogen) and Hoechst (Invitrogen). For LacZ staining: cells were fixed in 1% formaldehyde, 0.2% glutaraldehyde and 0.02% NP40. β-galactosidase activity was visualized by incubating cells with 1mg/ml X-gal, 5mM $K_3Fe(CN)_6$, 5mM $K_4Fe(CN)_6$ and 2mM $MgCl_2$.

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Sprout characteristics

Sprouting was indexed as: non-sprouting, intermediate sprouts and sprouting. The latter is characterized by extensive sprouting around the whole circumference of the EB. Sprout length was measured on PECAM stained EBs using Angioquant, the average length was determined on 8 EBs per condition.

Statistics

Statistical significance was evaluated using ANOVA (with Bonferroni correction for multiple-group comparisons) or Mann-Whitney U test, as applicable. Results are expressed as mean ± SEM. A value of $p \leq 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

To generate an inducible ablation system, the ActENIL expression construct (Figure 1A) was stably transfected into mouse ES (mES) cells, and hygromycin resistant, EGFP-expressing clones were selected. To analyze the effects of Cre-mediated recombination, CMV-Cre plasmid was transfected into the ActENIL harboring mES lines (ActENIL+Cre). Flow cytometric analysis confirmed that ActENIL mES cells were EGFP positive, and that this expression was lost after Cre-mediated recombination (Figure 1B). Additionally, ActENIL+Cre mES cells expressed β-galactosidase, which was not present in the ActENIL mES cells (Figure 1C).

To confirm the expression and activity of NTR, cells were incubated with CytoCy5S which is a quenched fluorescent substrate, converted by NTR into a red fluorescent dye. Although the IB10 (wild type cells) and ActENIL cell lines show a shift in red fluorescent background when CytoCy5S is added, the ActENIL+Cre cells reveal a highly red fluorescent population, indicating NTR activity (Figure 2A).

Next, the effect of CB1954 in culture was determined by incubating ActENIL and ActENIL+Cre cells with different concentrations of CB1954 for 3 days. MTT assay showed that the ActENIL+Cre cell line was indeed more sensitive to CB1954 treatment than ActENIL cells (LD_{50} approximately 2 μM versus 20 μM respectively, Figure 2B). In the non-transfected cells, cell death also occurs, but at higher concentrations of CB1954. Cell death due to CB1954 has been described for other cell types as well: e.g. adipocytes[10], SKOV3[11], and 3T3 cells[5]. Since at high concentration CB1954 may also induce necrosis[11], we chose a concentration of 10μM CB1954 which shows cell death in the CMV-Cre targeted cells, while little effect is seen in the non-targeted cells. When incubating cells with a concentration of 10 μM CB1954 for 1 day, we found that the number of $EGFP^+$ cells remained unaffected in the ActENIL cell line, while the contribu-

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tion of EGFP$^+$ cells in the ActENIL+Cre line became higher, demonstrating that CB1954 kills the NTR/LacZ$^+$ cells, while leaving the EGFP$^+$ cell population intact (Figure 2C).
To confirm ablation of a specific cell population in this system, we chose to target the endothelial cell. After stable transfection of Tie2-Cre into the ActENIL cell line (ActENIL+T2-Cre), the mES cells were differentiated as EBs using the hanging drop method. The EBs were then grown in collagen matrix to induce the formation of sprouts. After 15 days of aggregation we found that LacZ$^+$ sprouts had arisen from the ActENIL+T2-Cre EBs (Figure 3A). Furthermore, endothelial sprouts were positive Cre recombinase (Figure 3B). Next, the effect of removal of endothelial cells on sprouting was assessed by adding CB1954 to the collagen matrix. Three categories of EBs were observed, non-sprouting (Figure 3C), small sprouts on only a minor fraction of the EBs (Figure 3D), and strong sprouting (Figure 3E). The CB1954 did not affect sprouting of EBs generated from the IB10 or the ActENIL mES cells (Figure 3F); there was no difference in the percentage of non-spouting or small sprouting EBs in either group. In the ActENIL+T2-Cre cells the number of sprouting EBs without CB1954 treatment was similar to ActENIL EBs.

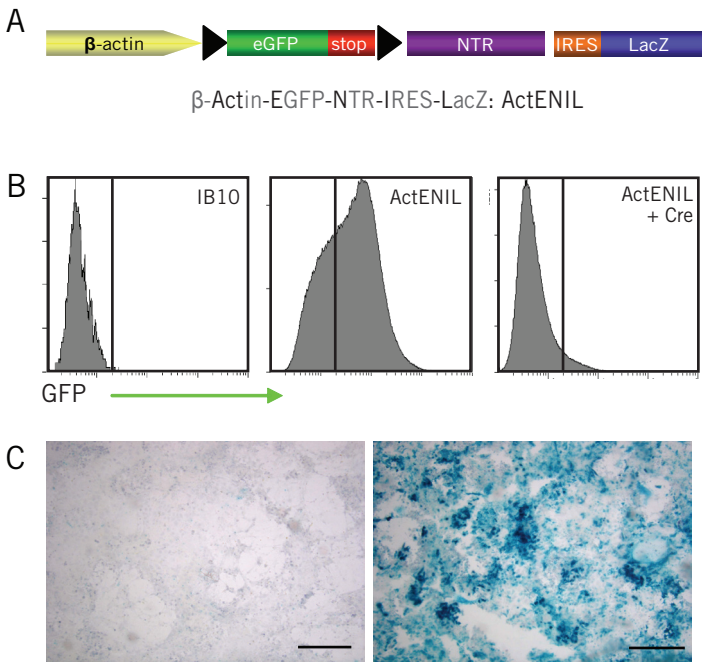

Figure 1. Characteristics of ActENIL mES cells.
(A) Structure of the ActENIL construct containing a β-actin promoter, a floxed EFGP-stop cassette, NTR and IRES-LacZ. (B) Flow cytometric analysis of EGFP expression in the IB10, ActENIL and ActENIL+Cre mES cells. (C) X-gal staining of ActENIL (left) and ActENIL+Cre (right) cells shows LacZ expression after Cre excision.

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However, when CB1954 is present, the percentage of non-sprouting EBs increased with increasing concentration of CB1954.
PECAM staining confirmed that in the presence of 10μM CB1954 the sprouting in the ActENIL cells is unaffected, while sprouting was abrogated in the ActENIL+T2-Cre EBs (Figure 4A-D). To quantify this effect the average sprout length in the different groups of

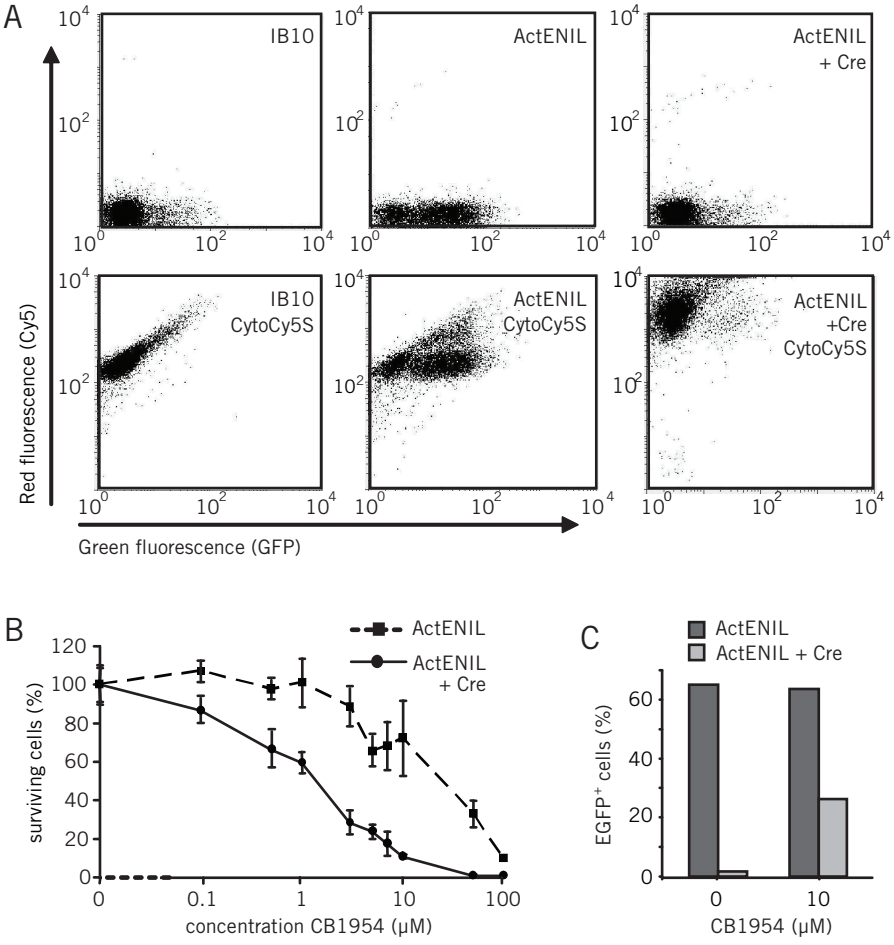

Figure 2. NTR expression and sensitivity to CB1954
(A) Flow cytometric analysis of NTR enzyme activity. The quenched CytoCy5S is converted into a red fluorescent dye by NTR. The 3 top panels show controls, the 3 lower panels the CytoCy5S incubated cells. (B) The sensitivity of ActENIL and ActENIL+Cre cells to CB1954 determined by MTT assay. Mean ± SEM. (C) Percentage of EGFP+ cells in the presence of 10 μM CB9154 in the ActENIL and ActENIL+Cre cell line, determined by flow cytometry (representative graph).

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EBs was measured. This revealed that the average sprout length in the IB10-derived EBs was unaffected when incubated with CB1954 (Figure 4D). A minor effect was observed on ActENIL EBs, which could be a consequence of background toxicity of the CB1954. However, the effect of CB1954 treatment on ActENIL+T2-Cre sprouting was significantly affected by CB1954 incubation, leading to no or very short sprouts (Figure 4D).These

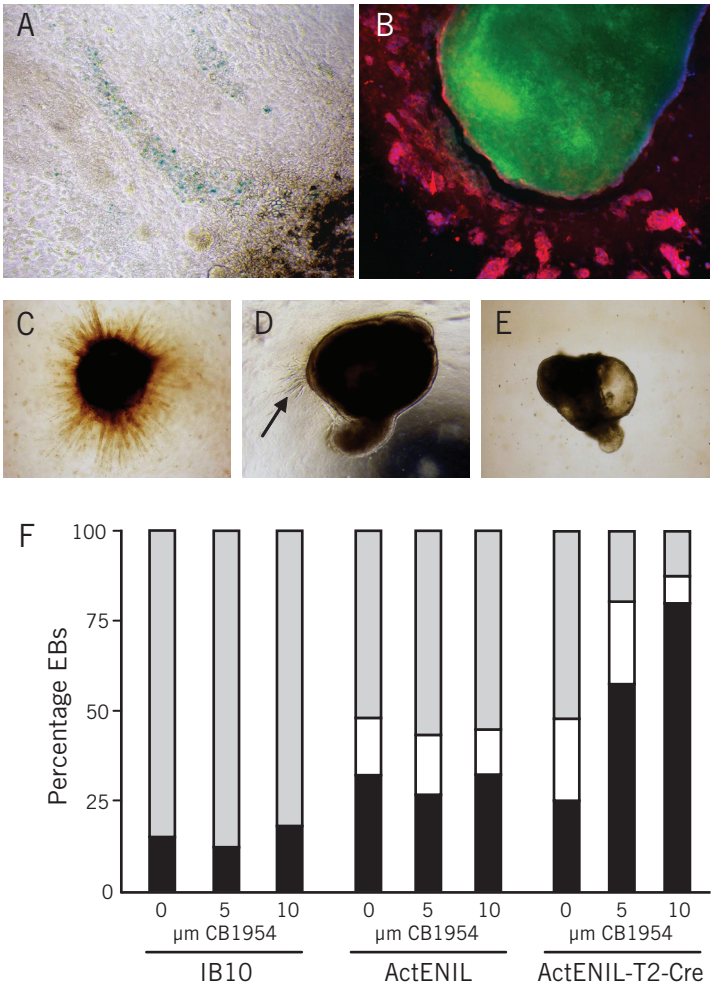

Figure 3. Endothelial sprouting
(A) LacZ^{+} sprout in the ActENIL+Tie2-Cre cells grown in collagen, (B) Cre expression in sprouts of ActENIL+Tie-2 EBs Cre in red. (C) Sprouting EB (D) intermediate sprouting EB, arrow indicates sprouts. (E) non-sprouting EB. (F) The percentage of non- (black), intermediate- (white) and sprouting (gray) EBs incubated with 0, 5 or 10 µM CB1954 is shown. (Representative graph of 3 independent experiments).

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data confirm that we can specifically identify and ablate endothelial cells thereby affecting the development of vascular sprouts.
This system has been generated to provide a flexible and easy method to identify specific cell populations and determine their role in tissue differentiation. Provided that the desired cells harbor a cell-type specific promoter that can be coupled to Cre, this population can be targeted by stably transfecting the Cre plasmid into the mES ActENIL cell line.
Furthermore, this ablation model can provide an important tool to determine the interaction between specific cell-types as it is possible to remove one cell from the system at any given time point by the addition of CB1954, leaving non-Cre expressing cells alive. Recently a similar ablation model was reported using diphtheria toxin upon cell-type specific Cre expression[12]. Although diphtheria toxin-induced cell death is comparable to the NTR/CB1954 system, our model has the advantage of temporal regulation of target cell ablation by addition of CB1954. Many different cell-types have been detected in

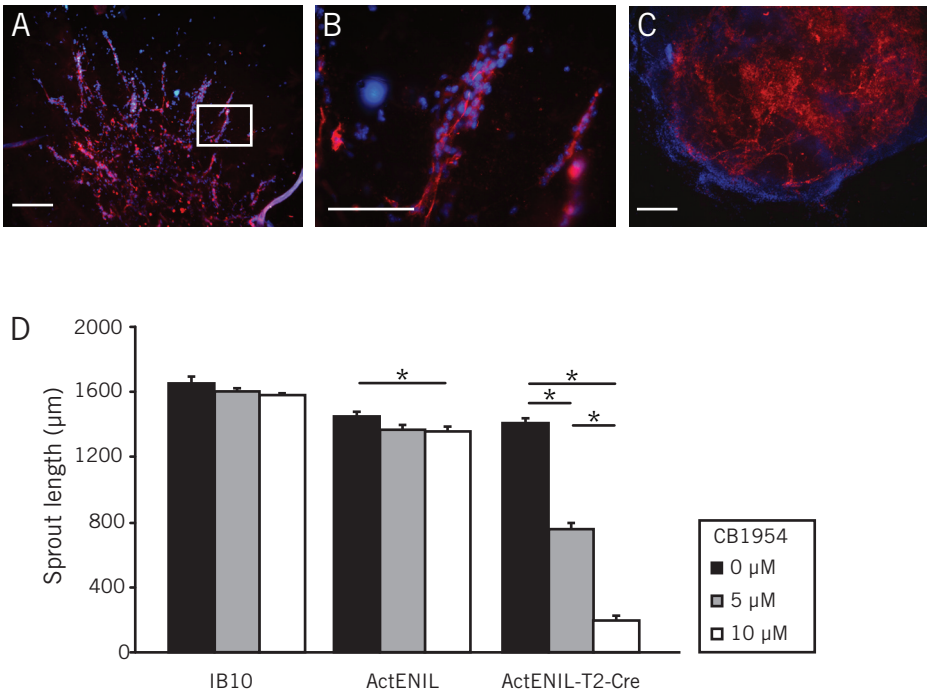

Figure 4. Endothelial sprouting is affected by CB1954
(A) PECAM staining (red) shows that the sprouting is unaffected in the ActENIL EBs incubated with 10 µM CB1954 (enlargement in B). (C) In the ActENIL+T2-Cre incubated with 10 µM CB1954 sprouting is abolished. (D) The length of sprouts determined for the IB10, ActENIL and ActENIL+T2-Cre cells after incubation with 0, 5 or 10 µM CB1954. Mean ± SEM, * $p<0.05$, determined by ANOVA for multiple group comparison. Scale bars: 250µm

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EBs. These include cardiomyocytes[13], motorneurons[14] and kidney derived cell-types like glomerular podocytes and renal-distal tubule epithelial cells[15] providing an almost unlimited potential for studying differentiation and cellular interaction. As future applications, one can for example consider using this model to screen compounds or small molecules for their effect on cell-type specific differentiation. Furthermore, the embryonic stem cells generated in this study were used to generate an *in vivo* mouse model for cell ablation.

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REFERENCES

1. Doetschman TC, Eistetter H, Katz M, Schmidt W, Kemler R. The *in vitro* development of blastocyst-derived embryonic stem cell lines: formation of visceral yolk sac, blood islands and myocardium. J Embryol Exp Morphol 1985;87:27-45.
2. Vittet D, Prandini MH, Berthier R, Schweitzer A, Martin-Sisteron H, Uzan G, Dejana E. Embryonic stem cells differentiate *in vitro* to endothelial cells through successive maturation steps. Blood 1996;88(9):3424-3431.
3. Feraud O, Cao Y, Vittet D. Embryonic stem cell-derived embryoid bodies development in collagen gels recapitulates sprouting angiogenesis. Lab Invest 2001;81(12):1669-1681.
4. Cui W, Gusterson B, Clark AJ. Nitroreductase-mediated cell ablation is very rapid and mediated by a p53-independent apoptotic pathway. Gene Ther 1999;6(5):764-770.
5. Bridgewater JA, Springer CJ, Knox RJ, Minton NP, Michael NP, Collins MK. Expression of the bacterial nitroreductase enzyme in mammalian cells renders them selectively sensitive to killing by the prodrug CB1954. Eur J Cancer 1995;31A(13-14):2362-2370.
6. Dube A, Akbarali Y, Sato TN, Libermann TA, Oettgen P. Role of the Ets transcription factors in the regulation of the vascular-specific Tie2 gene. Circ Res 1999;84(10):1177-1185.
7. Mummery CL, Feyen A, Freund E, Shen S. Characteristics of embryonic stem cell differentiation: a comparison with two embryonal carcinoma cell lines. Cell Differ Dev 1990;30(3):195-206.
8. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 1983;65(1-2):55-63.
9. Slager HG, van Inzen W, Freund E, van den Eijnden-Van Raaij AJ, Mummery CL. Transforming growth factor-beta in the early mouse embryo: implications for the regulation of muscle formation and implantation. Dev Genet 1993;14(3):212-224.
10. Felmer RN, Clark JA. The gene suicide system Ntr/CB1954 causes ablation of differentiated 3T3L1 adipocytes by apoptosis. Biol Res 2004;37(3):449-460.
11. Palmer DH, Milner AE, Kerr DJ, Young LS. Mechanism of cell death induced by the novel enzyme-prodrug combination, nitroreductase/CB1954, and identification of synergism with 5-fluorouracil. Br J Cancer 2003;89(5):944-950.
12. Wu S, Wu Y, Capecchi MR. Motoneurons and oligodendrocytes are sequentially generated from neural stem cells but do not appear to share common lineage-restricted progenitors in vivo. Development 2006;133(4):581-590.
13. Maltsev VA, Rohwedel J, Hescheler J, Wobus AM. Embryonic stem cells differentiate *in vitro* into cardiomyocytes representing sinusnodal, atrial and ventricular cell types. Mech Dev 1993;44(1):41-50.
14. Soundararajan P, Miles GB, Rubin LL, Brownstone RM, Rafuse VF. Motoneurons derived from embryonic stem cells express transcription factors and develop phenotypes characteristic of medial motor column neurons. J Neurosci 2006;26(12):3256-3268.
15. Kramer J, Steinhoff J, Klinger M, Fricke L, Rohwedel J. Cells differentiated from mouse embryonic stem cells via embryoid bodies express renal marker molecules. Differentiation 2006;74(2-3):91-104.

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8

An *in vivo* system to investigate progenitor cells in physiological and pathophysiological models

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ABSTRACT

Introduction

Cardiomyocyte progenitor cells have been identified in the adult heart. Their origin and potential in cardiac regeneration after myocardial infarction (MI) remains unclear. The aim of this project is to study to what extend CMPCs recruited from the circulation or resident in the heart, contribute to myocardial repair after damage, and what their contribution in normal turnover is.

Methods

We generated new mouse models that can be used to visualize the differentiation of stem or progenitor cells into a specific cell type, while at the same time allowing controlled removal of this same cell population using an enzyme pro-drug approach. A β-actin-promoter drives the expression of a floxed eGFP-stop cassette, nitroreductase (NTR) and an internal IRES-LacZ cassette. This system is designated ActENIL. All cells will initially express eGFP, but upon Cre expression, eGFP will be exchanged for LacZ and NTR. NTR in itself is harmless, but it converts the pro-drug CB1954 into a DNA crosslinking agent, thereby inducing apoptosis.

By for example placing a tamoxifen inducible Cre-recombinase under control of an early cardiac enhancer element, the expression of LacZ and NTR can be targeted to CMPCs or early developing cardiomyocytes. LacZ expression will function as a reporter of differentiation of a progenitor cell into a cardiomyocyte, while NTR acts as a suicide gene when the prodrug CB1954 is administered.

Results

ActENIL mES cells were used to generate chimeric mice, and subsequent germline transmission was confirmed. Additionally, nkx-enhancer element (NEE)-Cre and tamoxifen inducible NEE-mER-Cre-mER mice have been generated to target early developing cardiomyocytes. We are validating our newly generated mouse lines with regard to the presence of eGFP, and Cre mediated expression of LacZ and NTR. Furthermore, we are determining the time-frame of the NEE-promoter activity during embryonic development.

Ultimately, these newly generated mouse lines will provide a system to observe the contribution of CMPCs to cardiac repair after MI, as well as a method to directly compare the effects of the absence or presence of these endogenous progenitor cells on the healing process. Since the setup is very flexible, it can easily be adapted to study other diseases and/or other cell-types provided that there is a cell-type specific promoter available.

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INTRODUCTION

Recent evidence shows that cardiovascular progenitor cells are present in the adult human heart[1,2] When these cells are isolated, expanded and reintroduced into injured myocardium, they are able to form new cardiac tissue (chapter 4 and Smith et al.[2]). Although endogenous progenitor cells exist, it is clear that their participation in cardiac repair after for example infarction is insufficient to reduce the scar size. Several important questions regarding the *in vivo* function of cardiovascular progenitor cells remain unanswered. For example, are cardiomyocytes continuously replaced during life by the endogenous progenitor cells present in the heart? Are these progenitor cells a remainder of embryonic heart development, or do they originate from other sources e.g., the bone marrow and home to specified cardiac niches? Do cardiovascular progenitor cells provide new viable tissue after injury? Gaining more insight into the actual contribution of resident progenitor cells to cardiac repair and maintenance may lead to new approaches to stimulate the endogenous progenitor cell pool and regenerate the heart more efficiently without the need for invasive transplantation procedures.

To investigate the origin, as well as the contribution of endogenous cells in various systems, we designed a transgenic mouse model in which the differentiation of a progenitor cell into a certain cell type can be monitored, as well as its contribution to (cardiac) repair by using an inducible suicide gene approach.

Previously, we have shown that the ActENIL construct in combination with cell-type specific Cre-recombinase expression can be used to identify differentiating cells based on the expression of β-galactosidase[3] and chapter 7. Furthermore, Cre-expressing cells also produced nitroreductase (NTR) which sensitizes them to prodrug CB1954-mediated apoptosis[4]. In theory, any cell that carries a cell-type specific promoter can be targeted via Cre/lox recombination, making it a very flexible system. *In vitro* we have used this approach to target endothelial cells during embryoid body (EB) formation[3]. In the present study we set out to determine the *in vivo* applicability of this inducible cell type-specific ablation system.

The generated ActENIL plasmid (Figure 1A) is based on a constitutively active β-actin promoter containing an internal enhancer element driving the expression of a floxed eGFP-stop cassette, followed by E. Coli-derived nitroreductase (NTR), and an internal ribosomal entry site (IRES) coupled to LacZ to regulate the translation of two separate proteins from one single mRNA molecule[5]. The expression of Cre in this system will lead to excision of the eGFP-stop, placing NTR-LacZ under transcriptional control of the β-actin promoter (Figure 1A). Expression of LacZ allows the identification of Cre-expressing cells. NTR in itself is harmless, but upon interaction with CB1954 [5-(aziridin-1-yl)-2,4-dinitrobenzamide] it is converted into an alkylating agent that crosslinks

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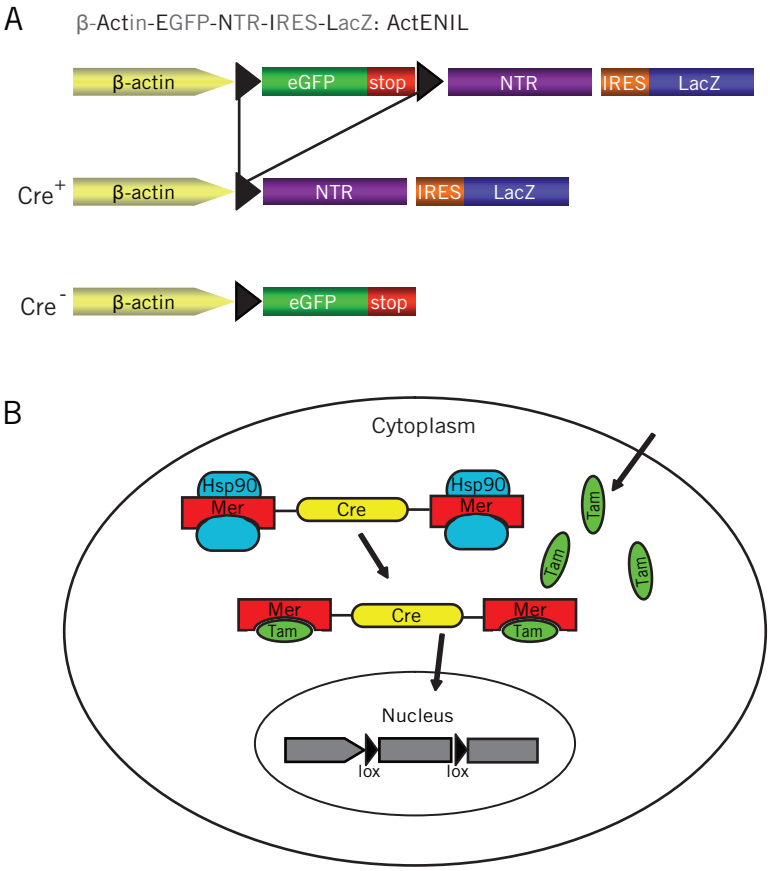

Figure 1A: Properties of the ActENIL construct
(Top) A β-actin promoter carrying an internal enhancer element is followed by an eGFP cassette ending in a stopcodon. eGFP-stop is flanked by two loxP sites (black triangles) that are oriented in the same direction. Behind this cassette is a gene encoding E. Coli derived nitroreductase (NTR). An internal ribosomal entry site (IRES), preceding LacZ should ensure the translation of 2 separate proteins from 1 mRNA molecule. (Middle) When cells express Cre, the loxP sites are recombined and eGFP-stop is removed from the construct. This leads to expression of NTR and LacZ. (Bottom) However when Cre is not present, β-actin promoter activity will lead to eGFP expression in the cell.
Figure 1B: Temporal control of recombination using MerCreMer
Under normal circumstances, Cre is sequestered in the cytoplasm by Mer binding to Hsp90 (blue), unable to recombine the lox sites in the nucleus. After administration of tamoxifen (Tam, green), it competes with Hsp90 and enables MerCreMer to enter the nucleus where it mediates recombination of loxP sequences. Tamoxifen administration therefore gives temporal control of Cre recombination.

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DNA leading to cell-death mainly by apoptosis[6]. Targeting this system to cardiovascular progenitor cells will give us the unique opportunity to analyze cardiovascular progenitor cells endogenously, and gain insight into their origin, and their contribution in ageing and injury (see examples in figure 2). Importantly, since differentiated cells can be removed by administering CB1954, the situation with or without the newly formed cells can be compared directly, providing clues about the functional involvement of the endogenous progenitor cell pool (Figure 2).

To specifically target cardiovascular progenitor cells, a promoter is needed that is active in the early stages of cardiomyogenesis, and that is not active in adult cardiomyocytes. To drive transcription of LacZ and NTR in early cardiomyocytes we used an Nkx2-5 enhancer element (NEE) coupled to the hsp68 basal promoter[7]. This enhancer element was previously described by Lien et al. as fragment 5, which spans the region 9700-3050 nucleotides upstream of the Nkx2-5 gene[7]. NEE was shown to be active at E7.5 when heart development is initiated. At E8.0 the fragment showed activity in the linear heart tube and expression remained high during heart looping (E8.5-9.5). Although NEE recapitulated the expression of the Nkx2-5 gene up to this point, it became restricted to the right ventricular region from day E10.5[7]. Since the activity of the enhancer element was not investigated in the adult heart, and the expression did not completely mimic the Nkx2-5 gene expression, we need to investigate if activity will be low to zero in the adult heart.

This promoter coupled to Cre should lead to expression of LacZ and NTR in cells starting to differentiate into the cardiomyogenic lineage and once the cell is labeled it will remain so during the whole experiment. To achieve temporal regulation of the system, Cre flanked by two mutated estrogen receptors (Mer) called MerCreMer[8] can be used. MerCreMer is sequestered in the cytoplasm unless the estrogen antagonist tamoxifen is present; this results in translocation of Cre to the nucleus where it mediates recombination of loxP sequences (Figure 1B). Since MerCreMer will be cleared from the cytoplasm within several days, the addition of tamoxifen will lead to Cre-mediated recombination only in those cells where the promoter is - or was recently - active. This is in contrast with regular (non-tamoxifen inducible) Cre that will immediately translocate to the nucleus, leading to irreversible labeling of all cells with an active promoter driving Cre expression at any time-point. The tamoxifen inducible Cre activity provides the possibility of lineage tracing progenitors, and observing their role in normal ageing (Figure 2).

In this chapter we show the progress that has been achieved in generating these mouse models for lineage specific cell ablation.

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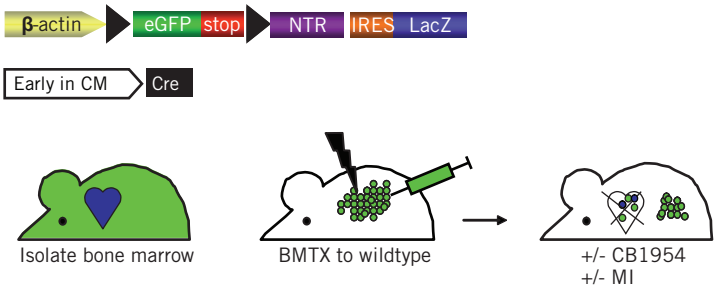
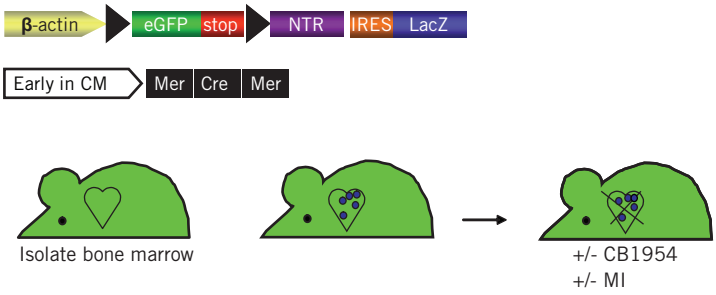


- Ageing: do circulating cells home to niches in heart?
- Is there homing of circulating cells after MI (green cells)?
- Do homed cells differentiate into CM (blue cells)?
- Addition of CB1954 removes newly formed CM: effect on cardiac repair?

B



- Where are progenitor cells located, and how many?
- Ageing/turnover: do progenitors become adult cardiomyocytes?
- Is there an increase in progenitor cell contribution to repair after MI?
- Addition of CB1954 removes progenitors: effect on cardiac repair?

Figure 2: Example experiments
In the ActENIL construct the expression of eGFP is exchanged for LacZ and NTR when Cre is expressed. (A) Combining ActENIL with Cre under control of a promoter expressed in young or early cardiomyocytes (CM), yields a "green mouse" with a "blue heart" since all cardiomyocytes have once turned on this promoter during their development. When the (green) bone marrow of this double transgenic is transplanted (BMTX) into a healthy wildtype mouse, one can study the homing, differentiation and contribution of bone marrow cells in different cardiac disease models. Addition of CB1954 leads to removal of differentiated cells. Comparing this to the situation where the blue cells are still present provides data on the importance of newly formed cells.
(B) Crossing the ActENIL mouse with a transgenic mouse carrying MerCreMer under control of the early cardiomyocyte-specific promoter that is not active in adult cardiomyocytes, enables the labeling of the progenitor cell pool by administration of tamoxifen at different time-points in development or adulthood. Pulse-labeling the cells with tamoxifen and letting the mice age will confirm any contribution of progenitors to cardiomyocyte turn-over. Inducing MI or other cardiac injury models shows the participation of the endogenous progenitor cell pool. Addition of CB1954 reveals the effects of eliminating this cell-contribution to cardiac repair.

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MATERIAL AND METHODS

NEE-Cre and NEE-MerCreMer vector assembly

The Nkx2-5 enhancer element coupled to the basal Hsp68 promoter was derived from plasmid Hsp106 which contained fragment 5 (-9700/-3050) published by Lien et. al[7], which was a kind gift from Dr. R. Passier. The NEE fragment was isolated by digestion with SalI and NcoI yielding a fragment of approximately 6.5 kb. Cre was removed from pCAGGS by digestion with EcoRI, and the 1.2 kb fragment was subcloned into the EcoRI site of a modified Bluescript (BS)-SK that contained an NcoI site introduced into HinDIII (BS^{NCO}). The NEE-fragment was ligated into the SalI and NcoI sites in the BS vector containing Cre.

MerCreMer was isolated from pANMerCreMer[8], a kind gift from dr. M. Reth by digestion with HinDIII. MerCreMer was subcloned into a modified BS vector containing a HinDIII site flanked by two NotI sites, allowing the excision of MerCreMer from this vector by NotI. The NEE-fragment (NcoI-SalI) was ligated into BS^{NCO} and MerCreMer was subsequently cloned into the NotI site. Correct ligation of all fragments was confirmed by restriction and subsequent sequence analysis.

Assembly and testing of the ActENIL vector

The generation of the ActENIL vector has been described previously[3]. The construct was electroporated into IB10 derived mouse embryonic stem (mES) cells. Correct integration was confirmed by restriction and sequence analysis. The efficacy of several derived cell lines was tested previously[3].

Testing NEE-Cre in vitro

ActENIL mES cells were maintained in Buffalo Rat Liver cell conditioned medium on 0.1% gelatin coated dishes as previously described[9]. The cells were co-transfected with NEE-Cre and pGK-NEO using lipofectamine according to the manufacturer's protocol. After selection with G418 (150 µg/µl), the cells were used in embryoid body (EB) assays. EBs were generated using the hanging drop method[10]. One-thousand cells were suspended in 30 µl Dulbecco's modified Eagle's Medium (DMEM) supplemented with 2 mM L-glutamin, non-essential amino acids, 20% fetal calf serum, β-mercapto-ethanol, 50 ng/ml VEGF (Sigma) and 25 ng/ml bFGF (Sigma). Medium was refreshed every two days, EBs were collected and stained whole mount.

Animals

All experiments were carried out in accordance with the *Guide for the Care and Use of Laboratory Animals*, with prior approval by the Animal Ethical Experimentation Commit-

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tee, Utrecht University. Additionally, permits were obtained for the generation of new genetically modified mouse lines from the Dutch Ministry of Agriculture, Nature and Food Quality (LNV No. RBD0184).

Generation of NEE transgenic mice

Fertilized eggs from pregnant C57BL/6J (Bl6) females (Harlan) were collected for pronuclear injection. A total of seven foster mothers for each construct implanted with injected eggs. For genotyping stable transgenic lines, tails of 2-week-old pups were cut for DNA isolation and subsequent PCR using previously described Cre primers[11]. Of each line, two founders were obtained. These founder lines were bred with wildtype Bl6 mice.

ActENIL transgenic mouse

Two mES cell-lines that previously showed correct integration of the ActENIL construct and were the most effective *in vitro* were used for blastocyst injection. Host blastocysts were derived from a Bl6 background. ES cell-injected blastocyst were implanted into Bl6 foster mothers. Chimeric mice were identified based on coat color. The obtained chimeric male was crossed with Bl6 females to determine germ-line transmission. Tail snips were used for genomic DNA isolation, and the presence of the ActENIL construct was confirmed by performing a PCR for the LacZ gene, using the primer set LacZ 5': For 5'-CCTGAGGCCGATACTGTCGT, Rev 5'- TTGGTGTAGATGGGCGCAT

Testing NEE-Cre and NEE-MerCreMer founderlines

To test efficacy and tissue-specificity of Cre mediated excision, NEE-Cre and Nee-MerCreMer heterozygous mice were crossed with the Rosa26R Cre reporter line (R26R)[12]. NEE-Cre pregnant females were terminated at E9.5 through E13.5. NEE-MerCreMer pregnant females were injected intraperitoneally once with 2 mg Tamoxifen (Sigma, T5648) diluted in 10% EtOH and sunflower oil at E8.5 or E9.5 and embryos were isolated at E10.5-13.5. Yolk sacs were used for genotyping.

ActENIL transgenic mouse

Previously reported Cre lines were used to test the expression of LacZ and NTR in the ActENIL transgenic mouse line. Female heterozygous ActENIL mice were cross-bred with heterozygous Tie-2-Cre males (a gift from A. de Bruin, Utrecht University). Embryos were harvested at E12.5, and yolk sacs were used for genotyping. Double transgenic offspring was sacrificed to determine LacZ expression in adult tissues.

To ascertain NTR activity in double transgenic embryos, we injected the pregnant mother intraperitoneally with 40 mg/kg CB1954 diluted in 10% acetone in sunflower oil.

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β-Galactosidase staining and histology

EBs, embryos or adult tissues were fixed in 2% paraformaldehyde/0.2% glutaraldehyde in PBS on ice. After washing in PBS twice, the tissues were placed in staining solution containing 5mM ferrocyanide, 5mM ferricyanide, 2mM $MgCl_2$ and 1 mg/ml X-Gal in dimethylformamide overnight at room temperature. After washing away the X-gal the samples were post-fixed in 4% paraformaldehyde, and kept in 70% EtOH. Tissue was embedded into paraffin, sectioned at 7-10 µm and stained with hematoxillin or Nuclear Fast Red.

RESULTS

Cloning of NEE-Cre and NEE-MerCreMer

After successful excision of the NEE fragment from the original plasmid it was ligated to Cre or MerCreMer. The correct orientation was confirmed by restriction analysis and sequencing (not shown). To test the efficacy of the NEE-Cre construct, it was stably transfected into mES cells harboring ActENIL. Subsequently EBs were formed to drive the differentiation of these mES into different cell-types including cardiomyocytes. NEE-Cre EBs clearly contained large areas of LacZ positive cells (Figure 3A,B), which corresponded to beating regions (not shown). Although low levels of background were observed in the non-transfected ActENIL (Figure 3C) and IB10 wildtype EBs (Figure 3D), staining was more scattered throughout the EB, which may be caused by senescent cells.

Generating NEE founderlines

The NEE-Cre construct was used for pronucleus injection. In total 14 pups were born, two of which carried the Cre transgene as detected by PCR on genomic DNA. These founderlines were designated NEE-Cre-1 and NEE-Cre-2. Transgenic mice were backcrossed with C57/Bl6 mice for more than eight generations.

To determine the performance of the introduced Cre recombinase, female NEE-Cre mice were crossed with R26R Cre reporter males. The R26R mouse carries a floxed stop sequence followed by LacZ, therefore Cre mediated excision leads to β-galactosidase expression[12]. Timed matings were carried out and embryos were isolated at different time-points during development. After whole mount staining, the presence of blue cells in the embryos was analyzed. LacZ positive cells were observed in the hearts of embryos aged E9.5 to E13.5 derived from the NEE-Cre-2 line (Figure 4 A-D). Cells were not only observed in the heart, but also in the mouth which is in accordance with the report by Lien et al.[7] . As expected in the case of NEE-Cre, $LacZ^+$ cells were still present in the adult heart.

NEE-MerCreMer-construct injection into the pronucleus also led to the generation of two

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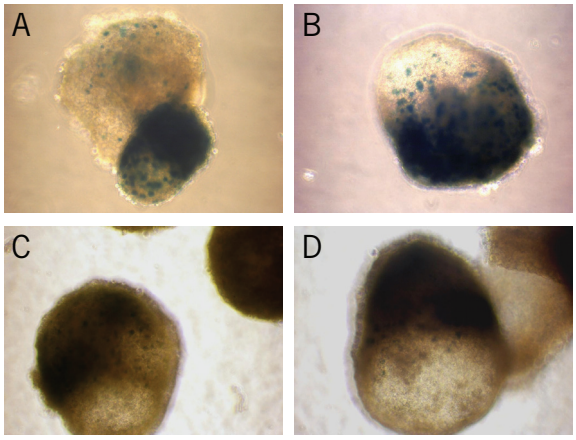

Figure 3. LacZ whole mount stained EBs
(A, B) NEE-Cre transfected ActENIL expressing mES cells, differentiated into cardiomyocytes show clear blue regions, which corresponded to beating regions (not shown). (C) ActENIL expressing mES cells, as well as (D) IB10 wildtype mES cells show low levels of background staining.

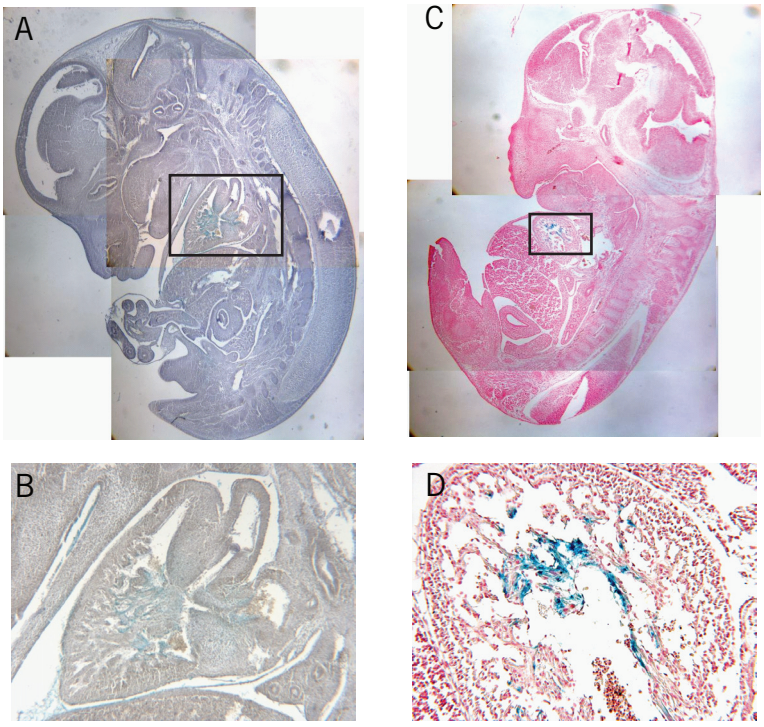

Figure 4. Expression pattern of NEE-Cre
After crossbreeding NEE-Cre1 (A,B) and NEE-Cre2 (C,D) females with R26R males, whole mount LacZ staining indicated the presence of blue cells in offspring at E 13.5. A and C are composed overviews of a hematoxylin (A), and Nuclear fast red (C) staining, respectively. (B) shows blue cells in the heart (100x), (D) shows a close up of the heart (200x).

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founder lines: NEE-MCM-1 and NEE-MCM2. Heterozygous females were crossed with Rosa26R males. Pregnant mice were injected with Tamoxifen at different moments after fertilization. Embryos were isolated at multiple stages, as well as two week old offspring. Blue hearts were observed in the case of NEE-MCM-1 (Figure 5A-D), but no LacZ expression was observed in the 10 double transgenic NEE-MCM-2 embryos we analyzed (not shown).
Since it remains unclear when the promoter is active and how strong the activity is, the true efficiency of both Cre lines needs to be investigated further.

The ActENIL-transgenic mouse

The ActENIL construct was electroporated into mouse embryonic stem cells, and tested *in vitro* for correct integration of the transgenes and their activity[3]. Two of the cell-lines that performed correctly were used for blastocyst injection. Since the IB10 mES cells (a subclone of E14 derived from 129/Ola mice with an agouti coat color) were injected into a B6-derived blastocyst (black coat color), we were able to identify correctly injected animals based on chimeric coat color (Figure 6A). One chimeric mouse was obtained and cross-bred with B6 females to determine germline transmission. In the offspring, two

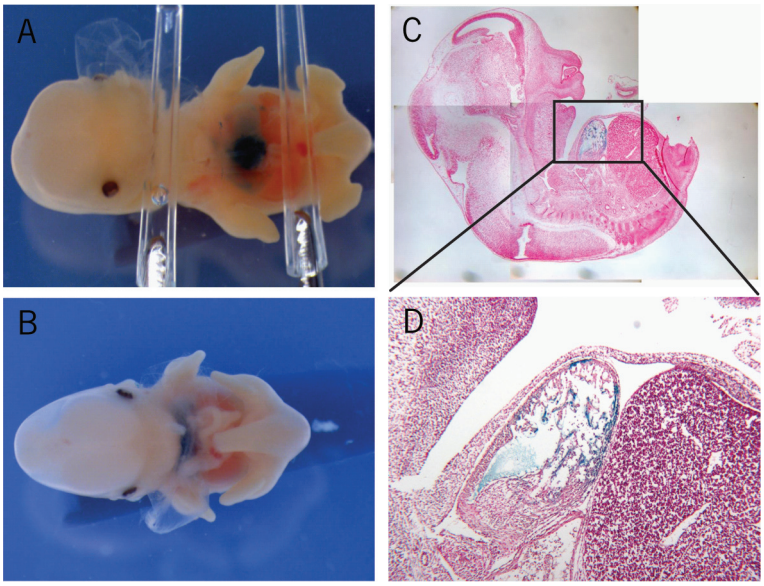

Figure 5. NEE MerCreMer expression
(A-D) NEE-MerCreMer founder females were crossed with R26R males, and pregnant females were injected intraperitoneally with 2 mg of tamoxifen at E8.5. The embryos were isolated at E11.5 and whole mount X-Gal staining was performed. (A,B) Whole mount staining revealed blue staining in the heart. (C) An overview picture was composed of a section of the embryo shown in A, LacZ activity was observed in the heart (inset D, 100x).

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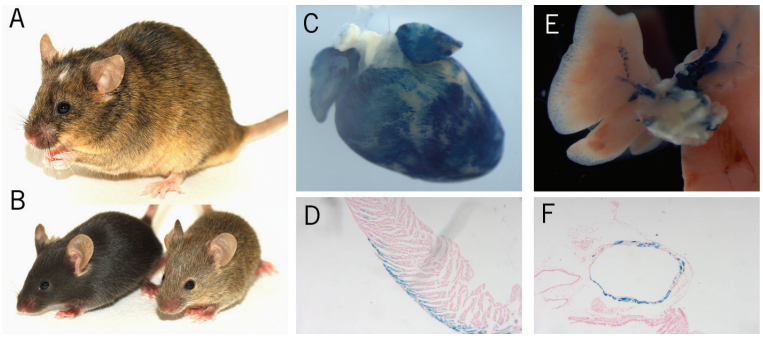

Figure 6. The ActENIL transgenic mouse
(A) ActENIL harboring mES cells for blastocyst injection yielded a chimeric mouse. (B) Germline transmission was confirmed based on the presence of two different coat colors in the offspring (black coat, left and agouti coat right). Preliminary analysis crossing ActENIL$^{+/-}$ mice with Tie2Cre$^{+/-}$ indicates LacZ activity in the heart (C-D) and lung vessels (E-F) of adult double-heterozygous 2 week-old offspring.

coat colors were observed (Figure 6B) indicating that germ cells were generated from the wild type, as well as the newly introduced mES cells.
To test whether the ActENIL system is working *in vivo*, ActENIL heterozygous animals were cross-bred with a previously reported endothelial specific Tie-2 Cre line[13]. We were able to identify blue vessels in the adult mouse lung (Figure 6E,F). Surprisingly, the surface of the heart also stained blue (Figure 6C,D), it is unclear if this is background activity since it does not match the known Tie2 expression patterns.
To confirm NTR activity in the embryos, pregnant females (ActENILxTie2Cre) were injected with CB1954. In theory, this should kill all double transgenic embryos, since the removal of endothelial cells during embryogenesis would abrogate further development. Therefore, only single-transgenic (carrying the ActENIL or Cre transgene) or wildtype embryos should survive. Indeed we found a high survival of single transgenic embryos (mothers n=3), and no double transgenic mice (data not shown). Due to ethical policies and animal welfare, injecting ActENIL/Tie2Cre with CB1954 can only be performed during pregnancy, and not during adult life, since it would lead to progressive degeneration of the blood vessels, and subsequent death.

DISCUSSION

Studying the contribution of endogenous stem cells has been hampered by the lack of efficient *in vivo* model systems. In this chapter we describe the generation of several transgenic mouse lines that will help understand the behavior of endogenous stem cells in physiological and pathopysiological conditions in the future.

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Progenitor cell contribution in adult hearts

The role of endogenous progenitor cells has been studied previously by other groups. Very recently, the group of Frisén elegantly showed that cardiomyocytes are renewed in the adult human[14]. By measuring the levels of carbon-14 (^{14}C) from nuclear bomb tests in cardiomyocyte nuclear DNA extracts, they were able determine the exact age of the cells. They calculated that cardiomyocytes are renewed at a rate of ~1% a year up to the age of 25 after which the renewal decreases to ~0.45% at age 75. This would mean that during life, 45% of the total cardiomyocytes have been renewed. However, it remained unclear whether these younger cardiomyocytes derive from progenitors or from dividing cardiomyocytes.

Hsieh and colleagues tried to investigate the contribution of progenitor cells to normal turnover of cardiac tissue during ageing as well as the role of progenitors during injury in the mouse heart[15]. To this end they designed an experiment in which adult cardiomyocytes were pulse-labeled via tamoxifen injection. In these specific experiments they used the Z/EG reporter mouse in which cells change from LacZ into EGFP expression upon cre mediated recombination[16]. The Z/EG reporter was crossed with an adult cardiomyocyte specific α-Myosin heavy chain-MerCreMer mouse. They hypothesized that after giving a tamoxifen pulse at a certain moment in adult life, all the existing cardiomyocytes are labeled with EGFP. If normal turnover occurs from a non-cardiomyocyte cell pool, the percentage of $EGFP^+$ cells should decrease with time. They did not observe any contribution of non-EGFP cells during normal ageing, but when injury was inflicted via an infarction or pressure overload to induce hypertrophy, EGFP-negative cells appeared. Although they claimed that these cells must have arisen from progenitors, such a statement needs to be substantiated using a more forward approach, by directly investigating progenitor cells, instead of their postulated progeny.

To provide a more direct approach to determine the participation of progenitor cells, a similar protocol to that of Hsieh et al. could be used, applying a pulse-chase method with tamoxifen. This requires that MerCreMer (or other tamoxifen inducible Cre's like $CreER^T$ [17]) expression is driven by a promoter that is specifically expressed during the early stages of cardiomyocyte development, and inactive in the adult cardiomyocyte. Adult inactivity is crucial, since a tamoxifen pulse should only label the progenitors and not adult cardiomyocytes.

Markers for cardiovascular progenitor cells

Such a promoter can be identified in the embryo at stages when the hearts starts to develop. In the current chapter we chose to investigate a cardiac enhancer element that is upstream of Nkx2.5 and was shown to be active in the cardiac crescent[7], which harbors cardiovascular progenitor cells and is an early site of cardiac development[18]. From previ-

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ous studies, it remains unclear if this enhancer element is active in the adult mouse. Although NEE-hsp68 coupled to LacZ recapitulated the expression of Nkx2.5 in the heart up to E9.5, it became restricted to the developing right ventricular region from day E10.5 onward. Unfortunately we have not yet been able to conclusively examine the behavior of this enhancer in the adult mouse. Future experiments will include different time points of tamoxifen induction in the NEE-MerCreMer mouse that is crossbred with the R26R Cre reporter line ranging from newborn to adult developmental stages. Even if NEE-Cre is active in adult cardiomyocytes it can still be a valuable tool, since it can serve to identify early differentiation into cardiomyocytes of e.g. bone marrow cells (Figure 2A).
Other previously reported promoters that could be used are Islet-1 (Isl-1)[19], xMLC2[20] or mef2C[21], all of which are active during very early heart development. But it is likely that these promoters are also active during adult life. Conversely, cardiovascular progenitors in the adult heart have progressed to a stage when the early promoter is no longer active. For example, isl-1 is a transcription factor and marks a population of cells that substantially contributes to the development of the heart[19]. Laugwitz et al. used the isl-1 promoter driving the expression of Cre or MerCreMer to identify cardiovascular progenitor cells[22]. Interestingly, some positive isl-1 cells can be isolated from the human or rodent postnatal heart and differentiate into cardiomyocytes *in vitro*[22]. However, these isl-1$^+$ cells were only found in very young specimens and not in adult hearts older than 5 days (in mice), therefore it remains unclear if isl-1 is the correct marker to trace cardiovascular progenitor cells in the adult heart. More effort has to be put into finding suitable promoters.

Reporter and ablation mouse models

The ActENIL transgenic mouse can be used to identify Cre expressing cells. In this regard it is similar to the Z/EG[16] or Z/AP[23] mice in which Cre expression exchanges LacZ expression for EGFP or alkaline phosphatase respectively. However, the advantage of the ActENIL mouse model is that it can also be used to selectively kill Cre expressing cells. We have applied the NTR/CB1954 system to target cells in an "inducible suicide gene" like approach. In cells the NTR enzyme is reduced by nicotinamide adenine dinucleotide (NADH) or NADPH. Upon binding, CB1954 is reduced by NTR and thereby converted into a potent DNA interstrand cross-linking agent, causing cell death[24]. Other inducible enzyme-prodrug systems have been developed; like Thymidine Kinase (TK)/Ganciclovir[25]. Although this system relies on a similar principle, the converted toxic metabolites act by inhibiting DNA polymerase, and therefore the activity is limited to proliferating cells. NTR/CB1954 is cell-cycle independent, and can therefore be used to target all cell populations.
Another well-known ablation system was recently described by Wu et al. were the cells expressed Diptheria toxin upon Cre mediated recombination[26]. Although Diptheria toxin-

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induced cell death is similar to that of NTR/CB1954, there is no temporal regulation of target cell ablation. Timing the moment of inducing cell death is a major advantage to our system. A disadvantage of NTR/CB1954 is that some "bystander effect" was reported when using the system in certain cell types. This is due to the fact that the toxic CB1954 metabolite is cell permeable[27]; therefore it can also affect neighboring cells. However, there are also reports that the bystander effects in a mammary gland targeting system *in vivo* were low[28], leaving the surrounding non-targeted cells intact. In our own *in vitro* model using NTR to target endothelial cells, we did not observe a bystander effect[3], suggesting that the bystander effect may have different effects in different cell types. Recently, an ablation model in zebrafish was published were the authors used NTR in combination with another compound; Metronidazole (Mtz)[29]. The authors showed that the Mtz metabolite did not lead to any bystander effects. In our system, Mtz can easily replace CB1954, and in the future this compound will be tested in our models.

CONCLUSION

Although we cannot show any conclusive data yet, preliminary results indicate that the system works. In the near future we will need to optimize staining procedures to identify Cre recombinase in the embryo. Furthermore, other approaches to stain LacZ may prove to be more specific than whole mount staining. Certainly, crossing Nee-Cre with ActENIL is one of the most exciting experiments that needs to be performed. Especially the ActENIL mouse model possesses important features that will allow the analysis of aspects of regenerative medicine that until now not been possible. Because the ActENIL mouse can easily be crossed with other Cre expressing lines, it can be used for many other applications (see supplemental figure for an overview). We anticipate that the ActENIL mouse will be a useful addition to the mouse geneticists' toolbox.

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REFERENCES

1. Goumans MJ, de Boer TP, Smits AM, van Laake LW, van Vliet P., Metz CH, Korfage TH, Kats KP, Hochstenbach R, Pasterkamp G, Verhaar MC, van der Heyden MA, de KD, Mummery CL, van Veen TA, Sluijter JP, Doevendans PA. TGF-beta1 induces efficient differentiation of human cardiomyocyte progenitor cells into functional cardiomyocytes in vitro. Stem Cell Res 2007;1(2):138-149.
2. Smith RR, Barile L, Cho HC, Leppo MK, Hare JM, Messina E, Giacomello A, Abraham MR, Marban E. Regenerative potential of cardiosphere-derived cells expanded from percutaneous endomyocardial biopsy specimens. Circulation 2007;115(7):896-908.
3. Smits AM, van den Hengel LG, van den Brink S, Metz CH, Doevendans PA, Goumans MJ. A new in vitro model for stem cell differentiation and interaction. Stem Cell Research 2009;2(2):108-112.
4. Bridgewater JA, Springer CJ, Knox RJ, Minton NP, Michael NP, Collins MK. Expression of the bacterial nitroreductase enzyme in mammalian cells renders them selectively sensitive to killing by the prodrug CB1954. Eur J Cancer 1995;31A(13-14):2362-2370.
5. Mountford P, Zevnik B, Duwel A, Nichols J, Li M, Dani C, Robertson M, Chambers I, Smith A. Dicistronic targeting constructs: reporters and modifiers of mammalian gene expression. Proc Natl Acad Sci U S A 1994;91(10):4303-4307.
6. Cui W, Gusterson B, Clark AJ. Nitroreductase-mediated cell ablation is very rapid and mediated by a p53-independent apoptotic pathway. Gene Ther 1999;6(5):764-770.
7. Lien CL, Wu C, Mercer B, Webb R, Richardson JA, Olson EN. Control of early cardiac-specific transcription of Nkx2-5 by a GATA-dependent enhancer. Development 1999;126(1):75-84.
8. Verrou C, Zhang Y, Zurn C, Schamel WW, Reth M. Comparison of the tamoxifen regulated chimeric Cre recombinases MerCreMer and CreMer. Biol Chem 1999;380(12):1435-1438.
9. Mummery CL, Feyen A, Freund E, Shen S. Characteristics of embryonic stem cell differentiation: a comparison with two embryonal carcinoma cell lines. Cell Differ Dev 1990;30(3):195-206.
10. Slager HG, van Inzen W, Freund E, van den Eijnden-Van Raaij AJ, Mummery CL. Transforming growth factor-beta in the early mouse embryo: implications for the regulation of muscle formation and implantation. Dev Genet 1993;14(3):212-224.
11. Luque RM, Amargo G, Ishii S, Lobe C, Franks R, Kiyokawa H, Kineman RD. Reporter expression, induced by a growth hormone promoter-driven Cre recombinase (rGHp-Cre) transgene, questions the developmental relationship between somatotropes and lactotropes in the adult mouse pituitary gland. Endocrinology 2007;148(5):1946-1953.
12. Soriano P. Generalized lacZ expression with the ROSA26 Cre reporter strain. Nat Genet 1999;21(1):70-71.
13. Kisanuki YY, Hammer RE, Miyazaki J, Williams SC, Richardson JA, Yanagisawa M. Tie2-Cre transgenic mice: a new model for endothelial cell-lineage analysis in vivo. Dev Biol 2001;230(2):230-242.
14. Bergmann O, Bhardwaj RD, Bernard S, Zdunek S, Barnabe-Heider F, Walsh S, Zupicich J, Alkass K, Buchholz BA, Druid H, Jovinge S, Frisen J. Evidence for cardiomyocyte renewal in humans. Science 2009;324(5923):98-102.
15. Hsieh PC, Segers VF, Davis ME, MacGillivray C, Gannon J, Molkentin JD, Robbins J, Lee RT. Evidence from a genetic fate-mapping study that stem cells refresh adult mammalian cardiomyocytes after injury. Nat Med 2007;13(8):970-974.
16. Novak A, Guo C, Yang W, Nagy A, Lobe CG. Z/EG, a double reporter mouse line that expresses enhanced green fluorescent protein upon Cre-mediated excision. Genesis 2000;28(3-4):147-155.
17. Feil R, Brocard J, Mascrez B, LeMeur M, Metzger D, Chambon P. Ligand-activated site-specific recombination in mice. Proc Natl Acad Sci U S A 1996;93(20):10887-10890.
18. Moorman AF, Christoffels VM, Anderson RH, van den Hoff MJ. The heart-forming fields: one or multiple? Philos Trans R Soc Lond B Biol Sci 2007;362(1484):1257-1265.

proefschrift Anke.indd 146 15-07-2009 12:00:52

19. Cai CL, Liang X, Shi Y, Chu PH, Pfaff SL, Chen J, Evans S. Isl1 identifies a cardiac progenitor population that proliferates prior to differentiation and contributes a majority of cells to the heart. Dev Cell 2003;5(6):877-889.
20. Breckenridge R, Kotecha S, Towers N, Bennett M, Mohun T. Pan-myocardial expression of Cre recombinase throughout mouse development. Genesis 2007;45(3):135-144.
21. Dodou E, Verzi MP, Anderson JP, Xu SM, Black BL. Mef2c is a direct transcriptional target of ISL1 and GATA factors in the anterior heart field during mouse embryonic development. Development 2004;131(16):3931-3942.
22. Laugwitz KL, Moretti A, Lam J, Gruber P, Chen Y, Woodard S, Lin LZ, Cai CL, Lu MM, Reth M, Platoshyn O, Yuan JX, Evans S, Chien KR. Postnatal isl1+ cardioblasts enter fully differentiated cardiomyocyte lineages. Nature 2005;433(7026):647-653.
23. Lobe CG, Koop KE, Kreppner W, Lomeli H, Gertsenstein M, Nagy A. Z/AP, a double reporter for cre-mediated recombination. Dev Biol 1999;208(2):281-292.
24. Anlezark GM, Melton RG, Sherwood RF, Coles B, Friedlos F, Knox RJ. The bioactivation of 5-(aziridin-1-yl)-2,4-dinitrobenzamide (CB1954)--I. Purification and properties of a nitroreductase enzyme from Escherichia coli--a potential enzyme for antibody-directed enzyme prodrug therapy (ADEPT). Biochem Pharmacol 1992;44(12):2289-2295.
25. Heyman RA, Borrelli E, Lesley J, Anderson D, Richman DD, Baird SM, Hyman R, Evans RM. Thymidine kinase obliteration: creation of transgenic mice with controlled immune deficiency. Proc Natl Acad Sci U S A 1989;86(8):2698-2702.
26. Wu S, Wu Y, Capecchi MR. Motoneurons and oligodendrocytes are sequentially generated from neural stem cells but do not appear to share common lineage-restricted progenitors in vivo. Development 2006;133(4):581-590.
27. Bridgewater JA, Knox RJ, Pitts JD, Collins MK, Springer CJ. The bystander effect of the nitroreductase/CB1954 enzyme/prodrug system is due to a cell-permeable metabolite. Hum Gene Ther 1997;8(6):709-717.
28. Clark AJ, Iwobi M, Cui W, Crompton M, Harold G, Hobbs S, Kamalati T, Knox R, Neil C, Yull F, Gusterson B. Selective cell ablation in transgenic mice expression E. coli nitroreductase. Gene Ther 1997;4(2):101-110.
29. Curado S, Anderson RM, Jungblut B, Mumm J, Schroeter E, Stainier DY. Conditional targeted cell ablation in zebrafish: a new tool for regeneration studies. Dev Dyn 2007;236(4):1025-1035.

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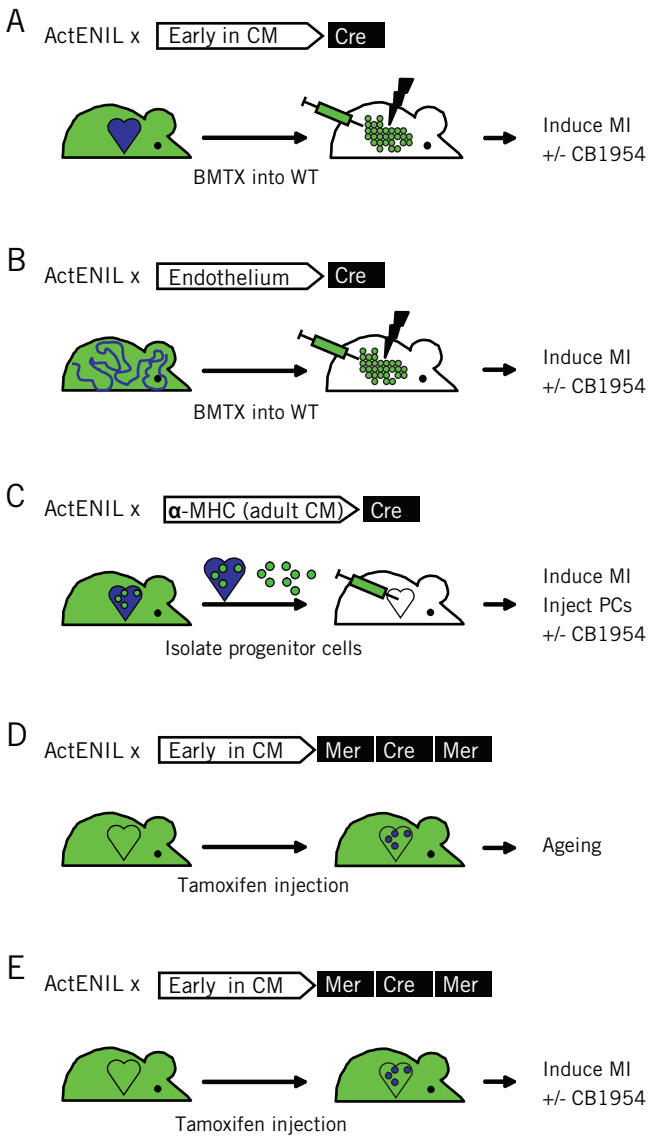

Supplemental Figure1. Overview of possibilities with the ActENIL transgenic mouse
In the ActENIL construct the expression of EGFP (green) is exchanged for LacZ (blue) and NTR when Cre is expressed. **A)** Combining ActENIL with a Cre that is expressed under control of a promoter expressed in young or early cardiomyocytes yields a "green mouse" with a "blue heart" since all cardiomyocytes have once turned on this promoter during their development. When the green bone marrow of this double transgenic is transplanted (BMTX) into a healthy wildtype (WT) mouse, one can study the homing, differentiation and contribution of bone marrow cells in different cardiac disease models, for example MI.
B) When cross-breeding the ActENIL mouse with an endothelial cell specific Cre line (Tie1-Cre, Tie2-Cre), all blood

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vessels are labeled. Performing a BMTX with double transgenic bone marrow gives the possibility to study the role of bone marrow derived cells in angiogenesis in e.g. MI.

C) Using a transgenic mouse that expresses Cre under control of a promoter that is expressed only in adult cardiomyocytes (α-Myosin Heavy Chain) provides the opportunity to isolate GFP^+ progenitors from the double transgenic mouse heart. Injecting these into WT heart after MI is a means of tracing the differentiation of these cells, and determining their contribution to repair by removing differentiated cells from the system by adding CB1954.

D) Crossing the ActENIL mouse with a transgenic carrying MerCreMer under control of an early cardiomyocyte specific promoter that is not active in adult cells, enables the labeling of the progenitor cell pool with tamoxifen at different time-points in development, providing clues about the role of this population. Pulse-labeling the cells with tamoxifen and letting the mice age shows the contribution of progenitors to cardiomyocyte turn-over.

E) Using the same approach as in D, but combined with an MI and the addition of CB1954 shows the contribution of progenitors to repair of the heart after injury

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9 General Discussion

9

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Although the development of cell-based therapy is rapidly evolving, many challenges remain to be addressed before this approach can be implemented in the clinic. The most important questions that need to be answered include: what cell type is most efficient to repair the heart, how do we deliver these cells efficiently, and do the cells survive and proliferate after transplantation[1]? Subsequently, successful integration into the host myocardium and long-term stability need to be investigated[1]. In this thesis, several aspects of these challenges have been explored.

CELL SOURCES FOR CARDIAC REPAIR

To achieve repair after cardiac injury via cell-based therapy, a source of cells is required that provides new cardiomyocytes, and preferably also generates or induces the formation of blood vessels. Several cell sources for cardiac repair have been mentioned in Chapter 2, including embryonic stem (ES) cells, and bone marrow-derived mesenchymal stem cells (MSCs), which at the time appeared to be the most promising with regard to their cardiomyogenic differentiation capacity[2]. However, over the last few years it has become clear that heart-derived cardiovascular progenitor cells meet several important prerequisites for their use in cell-based cardiac repair[3].

Cardiovascular progenitor cells

The historical paradigm that the heart is a terminally differentiated organ with little or no intrinsic regenerative capacity was challenged by the finding of cycling cells in the heart[4]. Subsequent studies revealed that the myocardium contained small clusters of cells interspersed in the interstitial space, carrying stem cell markers like c-kit and sca-1[5]. The possible relevance of these cells was emphasized by the fact that their number increased more than 10-fold in the hypertrophic myocardium of aortic stenosis patients[5].

Several groups reported the isolation of cardiovascular progenitor cells from human heart tissue using different techniques, including cardiac explant culture followed by cardiosphere formation[6,7] or isolation based on the expression of cell-surface stem cell markers like c-kit[8] or transcription factor islet-1 (isl-1)[9]. In chapter 3 the isolation of a cardiovascular progenitor cell population from the fetal and adult human heart is described that we termed cardiomyocyte progenitor cells (CMPCs)[10,11]. Isolation was based either on clonal (single cell) culture or by binding of cells to a mouse monoclonal anti-Sca-1 antibody, which was previously shown to bind cardiovascular progenitor cells in the mouse[12,13] and human heart[5,10,11]. Although the human homologue of the Sca-1 epitope has not been identified[14], the antibody may bind or cross-react with a yet unidentified protein on the surface of these cells. Nevertheless, CMPCs obtained by both methods

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consistently show a stem cell-like morphology (small cytoplasm to nucleus ratio). CMPCs do not express hematopoietic markers on their surface, like CD34, CD14, and CD45, which reduces the possibility that the cells are derived from blood cells that were present in the heart tissue sample. Furthermore, isolated CMPCs have telomerase activity[10] and express transcription factors typical for "young" cardiomyocytes, but no mature sarcomeric proteins[10,11], indicating their cardiovascular progenitor cell state[10,11].
It remains difficult to understand how an organ with such limited regenerative capacity can carry so many different progenitor cell populations. Therefore, investigators within the cardiovascular progenitor cell field are trying to determine whether these populations are truly different, or if they represent other developmental stages of one common ancestor cell population[15,16]. Where our CMPC will fit within this putative hierarchy remains to be determined.

Differentiation into cardiomyocytes

Generating cardiomyocytes from cardiovascular progenitor cells *in vitro* requires co-culture with mature cardiomyocytes[6], and/or stimulation with growth factors or compounds[8]. For CMPCs we show that the optimal protocol to differentiate them into mature cardiomyocytes is a combination of 5-azacytidine (5-aza) treatment followed by stimulation with transforming growth factor (TGF)-β (chapter 3, Smits et al.[17]). Incubation with 5-aza alone can also induce differentiation of CMPCs into cardiomyocytes[11], but less efficient than in combination with TGF-β[10]. 5-Aza is a general demethylating agent that unmasks epigenetically silenced genes, and was previously shown to induce cardiac differentiation in p19 embryonic carcinoma cells[18], ES cells[19] , MSCs[20] and mouse Sca-1^{+} cardiovascular progenitor cells[12]. Additionally, stimulating CMPCs solely with TGF-β induced cardiomyogenic differentiation in CMPCs[10], as was also reported for p19[21], ES[22] and bone marrow stem cells[23].
The combination of 5-aza and TGF-β induces the strongest differentiation response in CMPCs, but their synergistic effect is yet unknown. Breast cancer cells treated with 5-aza, were shown to increase the TGF-β receptors I and II[24]. Conversely, 5-aza stimulation in chondrocytes led to a downregulation of SMAD3 regulated TGF-β response, and caused a shift in signaling dominance from TGF-β to bone morphogenetic proteins (BMPs)[25]. In CMPCs we have observed similar effects on TGF-β responsiveness. Preliminary data show that 5-aza treatment leads to downregulation of the SMAD3 response, but how this translates to cardiac differentiation in CMPCs needs to be investigated further.
Injection of CMPCs into the infarcted mouse myocardium induced spontaneous differentiation into cardiomyocytes, endothelial cells and smooth muscle cells *in vivo* (chapter 4), indicating that the infarcted heart itself provides the necessary signals to induce its repair. Interestingly, since TGF-β is one of the factors expressed acutely after MI[26]; it is

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conceivable that TGF-β stimulation *in vivo* is a major contributor to the efficient differentiation of CMPCs. Identifying the exact factors that induce an optimal differentiation are subject of future investigation

The biological role of cardiovascular progenitor cells

Although the heart was shown to contain cardiovascular progenitor cells that are able to differentiate into cardiomyocytes, endothelial cells and smooth muscle cells *in vitro*, and *in vivo* when reintroduced into the tissue, the organ is unable to regenerate itself after damage. Therefore the question arises what the biological function of progenitor cells is. Probably, these cells participate in tissue homeostasis and regeneration but their role in repairing the heart is inefficient.

Ever since the finding of dividing cells in the adult heart[4], the role of cardiovascular progenitor cells in tissue homeostasis has been investigated. Pulse-labeling adult cardiomyocytes with eGFP in a transgenic mouse model, revealed that cardiomyocyte turn-over during ageing is very low to non-existent[27]. However, in these experiments, the contribution of cardiomyocyte division could not be determined. Recently, Bergmann et al. were able to retrospectively determine the birth date of single cardiomyocytes by measuring ^{14}C from nuclear bomb tests, in genomic DNA of human cells. They calculated that 45% of cardiomyocytes are replaced during adult life[28], but whether newly formed cells are derived from progenitors or dividing cardiomyocytes can not be established in this analysis. The discrepancy between rodents and humans may be due to interspecies differences; it may be that humans, who live much longer, have a different requirement for cardiomyocyte replacement. Alternatively, discrepancies result from the differences in experimental models. For instance, in the mouse pulse-chase experiments not all cells were labeled, even after high dose tamoxifen[27], which could for instance be a feature specific for young cardiomyocytes, leading to misinterpretation of the data.

To determine the contribution of cardiovascular progenitor cells to normal turn-over requires transgenic mouse models that allow direct lineage tracing of progenitor cells. In chapter 8 such a model is proposed in which progenitor cells can be labeled during any phase of cardiac development by injecting tamoxifen.

The participation of cardiovascular progenitor cells after injury has been investigated as well. In human post-MI hearts, a 70-fold increase in mitotic cells in the borderzone was noted[29], indicating activity in the injured area. In the mouse, a population of cardiovascular progenitor cells (termed side population (SP) cells) is identified by its ability to efflux Hoechst dye. By enzymatically dissociating hearts and performing flow cytometric analysis, it was shown that the pool of SP cells in the heart rapidly diminished in the first days after MI, and was restored again at seven days post-MI[30]. However, what happened to the cells that lost their progenitor status remained unclear. Hsieh et al. were able to show

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that in the borderzone of the infarcted mouse heart, new cardiomyocytes were generated from a non-cardiomyocyte pool of cells[27], although they claim these were cardiovascular progenitor cells the origin of these cells could not be confirmed. Again, more forward approaches using conditional labeling of progenitor cells and performing lineage tracing experiments like those suggested in chapter 8 should provide more direct evidence for the role in turn-over and in cardiac repair.

The origin of cardiovascular progenitor cells

There are two possibilities for the origin of cardiovascular progenitor cells present in the heart. Either, they are a remainder of embryonic or fetal development, or the pool of progenitors is continuously replenished by extra-cardiac sources of uncommitted cells. The best established source for stem cells in the adult body is the bone marrow, which hosts several types of stem cells including hematopoietic stem cells (HSCs), mesenchymal stem cells (MSCs) and SP cells. Sex-mismatched cardiac transplants in humans have shown that female hearts in a male host contained y-chromosome$^+$ cardiomyocytes, indicating their extra-cardiac origin[31]. In mice, Mouquet et al. showed that after MI the depleted SP cell population was partially (~25%) replenished by bone marrow derived cells[30].

Conversely, during embryonic development, cells expressing the LIM-homeodomain transcription factor isl-1 substantially contribute to heart formation[32]. After the heart is formed, undifferentiated precursors remain embedded in the cardiac tissue that *in vitro* posses the ability to differentiate into cardiomyocytes[9]. Their number decreases during fetal life until only low numbers of embryonic derived isl-1$^+$ cells remain postnatally; their role in tissue regeneration and homeostasis remains speculative.

Decisive answers on the origin of cardiovascular progenitor cells may be found using a pulse-chase method by labeling progenitor cells either in the heart or in the bone marrow, as suggested in chapter 8. The difficulty in labeling cardiovascular progenitor cells will be finding a gene that is active only in progenitor cells, but not in adult cardiomyocytes, and is carried by most if not all the different subpopulations that have been identified up to date. We expect that these new transgenic mouse models will add valuable information to the field of cardiovascular progenitor cells in the future.

OPTIMIZING CELL DELIVERY

Besides decisions on the cell-source and-type, another major challenge for cell-based cardiac repair is finding the optimal delivery strategy after MI. Options include intramyocardial, intracoronary or intravenous injection[33] and in this thesis, two of these were explored. Intramyocardial injection into the border zone of the infarct was shown to be an

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efficient delivery strategy for CMPCs resulting in a beneficial effect on cardiac function (Chapter 4); however the size of the grafts was small. After 3 months, three percent of the total injected population could be found in the heart. This low engraftment may result from loss of cells during the injection[34,35], or by rapid death of the cells as a response to the hypoxic and inflamed conditions that exist in the infarcted heart. In humans, controlled delivery of cells into the myocardium can be facilitated using guided catheter injections combined with cardiac mapping[33]. However, using this approach to deliver CMPCs into the heart will need to be tested in large animals instead of small rodents.

In contrast to intramyocardial injection, we tested intravenous delivery because it could have several important advantages: intravenous injection is non-invasive and cells arrive in the border zone of the ischemic tissue were nutrients are still present, which may increase their survival chances. However, this approach requires the injected cells to home from the circulation to the injured area. Recruitment of cells from the circulation after MI is mediated by several factors, but stromal-cell derived factor (SDF)-1α interaction with its receptor CXCR4 plays a prominent role[36,37]. Following MI, SDF-1α gene therapy enhanced the recruitment of HSCs to the heart[38]. Conversely, blocking CXCR4 on infused cells, or SDF-1 in the host, resulted in a dramatic reduction in progenitor cell homing to ischemic tissue and decreased neovascularization and blood flow[39]. When systemically delivering MSCs 3 hours after MI, positive effects on cardiac function were observed, as well as a reduction in infarct size[40,41]. Approximately 3% of the injected cells were found in the heart, 24 hours after MI[40].

The SDF-1/CXCR4 axis is inhibited by CD26 (dipeptidylpeptidase IV)[42] in different ways: CD26 cleaves the N-terminus of SDF-1 leading to its inactivation[43,44], but also internalizes the CXCR4 receptor[45]. The inhibitory effect of CD26 on the surface of cells and their ability to respond to SDF-1α is shown in chapter 5. Mononuclear cells (MNCs) isolated from hereditary hemorrhagic telangiectasia type 1 (HHT1) patients have high levels of CD26 and reveal a decreased responsiveness to SDF-1α gradients *in vitro* and *in vivo*. Inhibiting CD26 on MNCs resulted in normalization of the migratory response *in vitro*, and homing of cells towards the infarcted myocardium (Chapter 5).

Using CD26 inhibition to enhance cell homing and migration was translated to CMPC delivery in Chapter 6. CMPCs carried high levels of CD26 on their surface, and correspondingly, their migration towards SDF-1α *in vitro* was low. Inhibiting CD26 indeed resulted in an increase in homing *in vitro*. Using this system, combined with intravenous injection as an alternative delivery strategy for CMPCs after MI failed to restore the injured heart, likely because cells are captured in the lung capillary bed, or by the liver or spleen.

A previous study shows that when intravenously delivering different populations of stem cells into healthy rats, MSCs were trapped mainly in the lungs[46], which was attributed to their size. Performing a second bolus injection decreased the pulmonary first pass effect

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resulting in a higher number of circulating cells. Reducing the expression of adhesion molecules (like VLA-4) increased the number of circulating MSCs[46]. Alternatively, cells could be injected directly into the ventricular cavity, thereby circumventing the pulmonary circulation. These strategies remain to be explored for CMPC intravenous delivery. CD26 inhibition may provide a powerful approach to deliver cells to the infarcted myocardium, but autologous or at least interspecies experiments will need to be performed before conclusions of intravenous injection as a delivery option are drawn.

SURVIVAL AND (LONG-TERM) INTEGRATION

Another important aspect that needs to be considered when choosing the best possible approach to cell-based therapy is the survival of cells in the myocardium, and their integration into the host tissue.

Regarding CMPCs as a cell source, we questioned if cell-survival and integration would benefit from pre-differentiating the cells *in vitro* prior to transplantation, or if undifferentiated cells would give better results. For undifferentiated ES cells, it is known that injection can lead to the formation of teratomas in the heart[47]. However, this risk is low when using undifferentiated CMPCs, since they do not express any markers of pluripotency like NANOG or OCT4[10]. Furthermore, pre-differentiating CMPCs is a time consuming process[17] (Chapter 3) that can take several weeks to complete. Importantly differentiated CMPCs loose their proliferative potential[17]. These aspects make CMPC-derived cardiomyocyte (CMPC-CM) transplantation a less favorable option. We established that transplanting either human fetal CMPCs or CMPC-CM into the infarct border zone did not matter for survival, or for the CMPC derivatives that were found. This excludes the need for pre-differentiation of CMPCs *in vitro*. After intramyocardial injection, three percent of the injected CMPCs survived in the heart for three months. This does not take into account the number of cells that are generated by cell division. Additionally, the number of cells present two days after injection was higher, which may indicate that a part of the cells died during the three month follow-up.

Although graft size was small, we found significant effects on cardiac function up to three months after injection. Previous studies using human ES cells have shown that ES cell-derived cardiomyocytes can enhance long-term cardiac function compared to vehicle[48]. However, when comparing the cardiomyocyte fraction to other differentiated ES-cell types, there was no sustained functional advantage of ES cell derived cardiomyocyte injection after three months follow-up[48]. This suggests that cell injection can have other (positive) effects on cardiac function besides the formation of new contractile components (Figure 1). In fact, CMPC as well as ES cell grafts were found in clusters,

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isolated from the host tissue. No connexins between mouse and human cells were observed, making their electrical coupling unlikely (Chapter 4 and van Laake et al.[48]). The presence of cells in the scar can induce structural support to the heart, which should be a cell-type independent effect. However, previous studies comparing human heart-derived cardiovascular progenitor cells to skin fibroblasts showed that fibroblasts did not enhance cardiac function to the same degree as progenitor cells[7,49]. Passive support can therefore only partially explain the favorable effects.

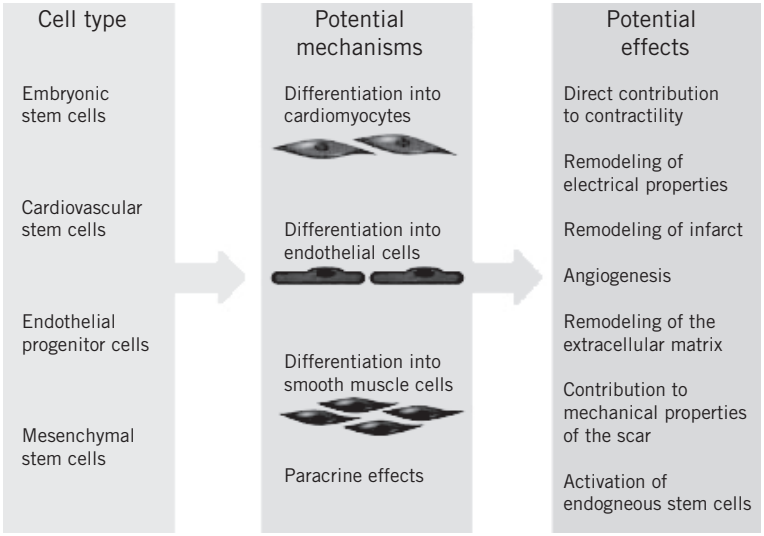

Figure 1. How cell-based therapy can contribute to cardiac repair

Cell injection may lead to the secretion of paracrine factors that influence the surviving host myocardium by inducing angiogenesis, remodeling the extracellular matrix, or activating the endogenous stem cell pools (Figure 1). The importance of paracrine factors has been underlined by studies injecting stem cell-conditioned medium into the infarcted heart, leading to a reduction in infarct size[50]. This approach can lead to an improvement in cardiac function shortly after MI. However, the presence of cells that continue to produce factors may be required for a long-term effect. A recent study by Ziebart et al. showed that removal of transplanted bone marrow cells that had incorporated into the infarcted mouse heart, immediately reversed any beneficial effect on cardiac function[51].

In chapter 4 is shown that CMPCs induce the formation of vessels in the borderzone, possibly via secretion of VEGF. Furthermore, secreted factors may increase the homing of circulating cells towards the infarcted region. For example, MNC invasion into the

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injured region is important for restoring cardiac function after MI, by increasing the vessel density in the infarct[52].

In conclusion the transplantation of CMPCs may have many consequences that ultimately result in improved cardiac function. However, effects on arrythmogenicity and electrical and metabolic integration remain to be established before CMPCs can be clinically applied in cell-based therapy.

FUTURE PERSPECTIVES

In this thesis we have investigated several of the challenges associated with cell-based cardiac repair.

In theory the ideal cell-source for tissue repair is the ES cell, since it can differentiate into all the cells of the body. However, the ethical issues regarding the use of these pluripotent cells are still a topic of intense public debate. This issue may be overcome by the recent breakthrough in generation of induced pluripotent stem (iPS) cells. In 2006, two research groups described the reprogramming of human fibroblasts to a pluripotent state by retrovirally expressing four transcription factors (Oct3/4, Sox2 combined with c-Myc and Klf4[53] or NANOG and LIN28[54]). IPS cells behaved similarly to ES cells, forming cell types of all three germ layers *in vitro* and in teratomas. Several studies have since shown that both human and mouse iPS cells can form functional cardiomyocytes[55,56]. One major obstacle that prevented the clinical applicability of iPS cells was the need for using retroviruses that randomly integrated into the host genome. However, very recently, several laboratories described the induction of iPS cells by using virus-free systems[57,58], which could quicken their route towards the clinic. One needs to bear in mind that the use of iPS cells in an undifferentiated state has an identical risk of teratoma formation as with ES cells. Therefore, iPS will require pre-differentiation into cardiomyocytes before implantation. In chapter 4 it is shown that undifferentiated CMPCs provide a better source for transplantation than their differentiated counterparts, the same may be the case with iPS or ES cells. Perhaps differentiating iPS cells into a cardiac progenitor cell state and isolating and purifying these progenitors using cell surface markers will prove to be an efficient strategy.

Currently, CMPCs provide a very promising source for transplantation after cardiac injury. CMPCs are easily isolated from the human adult and fetal heart, and differentiate into spontaneously beating cells *in vitro* (Chapter 3). When injected into the infarcted mouse myocardium, CMPCs form new cardiac tissue and prevent deterioration of cardiac function (Chapter 4). However, the size of the grafts and the influence on cardiac function can still be improved. Interestingly, we found that co-transplanting adult CMPCs

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together with epicardium derived cells increased the cardiac function after MI more than the separate cell types (EM Winter, unpublished results). Transplanting a mixture of cell sources, thereby combining their strengths, may provide a powerful strategy.

To increase the efficiency of CMPCs after transplantation, pre-treatment with TGF-β may be useful. Using c-kit$^+$ bone marrow derived cells, the revascularization as well as cardiac function were improved when cells were pretreated with TGF-β 24 hours before injection[59]. Another way of improving cardiac function with CMPCs may be through tissue engineering heart muscle[60]. Preliminary tests have shown that the formation of engineered heart tissue can be achieved using CMPCs as a cell source.

To improve the delivery of cells, we found that the SDF1/CXCR4 axis can be influenced by interfering with CD26 present on the cell surface. Using HHT1 patients' MNCs we observed that inhibiting the surface peptidase normalized the homing of MNCs to sites of damage. Interestingly, based on these results a clinical trial has been initiated in which HHT1 patients with very severe nose-bleeds are receiving a clinically approved CD26 inhibitor (Januvia) to explore if increasing the homing capacity of circulating cells can decrease the number of bleedings. Although the CD26 inhibition approach did not yet prove useful to CMPC recruitment in a setting of mouse MI, we are currently investigating if the retention of cells after intramyocardial transplantation can be optimized by CD26 inhibition.

Before CMPCs can be used in a clinical setting many hurdles need to be taken. For example, CMPC transplantation will need to be performed in an autologous fashion. Previous experiments in pigs have shown that CMPCs are immunogenic, and will be rejected by the heart (unpublished data). This is in contrast to MSCs which were shown to escape immune recognition[61]. The *in vivo* experiments described in this thesis have been performed using fetal heart derived CMPCs, and the ability of adult heart derived CMPCs to form new cardiac tissue is currently under investigation. Given the equal differentiation capacity of fetal and adult CMPCs *in vitro*, their *in vivo* potential is likely comparable. Other important aspects that need attention before any patient should be treated through injections with CMPCs, is that the isolation and expansion of CMPCs need to be performed according to Good Clinical Practice and Manufacturing codes. Furthermore, the electrical properties of injected CMPCs need to be assessed to rule out the possibility of inducing arrhythmias, as is the case with skeletal muscle derived myoblasts[34]. In conclusion, cell-based cardiac repair represents a promising approach to treat cardiovascular diseases, but it will take time and effort before patients can truly benefit from these procedures.

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REFERENCES

1. Segers VF, Lee RT. Stem-cell therapy for cardiac disease. Nature 2008;451(7181):937-942.
2. Smits AM, van Vliet P., Hassink RJ, Goumans MJ, Doevendans PA. The role of stem cells in cardiac regeneration. J Cell Mol Med 2005;9(1):25-36.
3. Barile L, Messina E, Giacomello A, Marban E. Endogenous cardiac stem cells. Prog Cardiovasc Dis 2007;50(1):31-48.
4. Anversa P, Nadal-Ginard B. Myocyte renewal and ventricular remodelling. Nature 2002;415(6868):240-243.
5. Urbanek K, Quaini F, Tasca G, Torella D, Castaldo C, Nadal-Ginard B, Leri A, Kajstura J, Quaini E, Anversa P. Intense myocyte formation from cardiac stem cells in human cardiac hypertrophy. Proc Natl Acad Sci U S A 2003;100(18):10440-10445.
6. Messina E, De Angelis L, Frati G, Morrone S, Chimenti S, Fiordaliso F, Salio M, Battaglia M, Latronico MV, Coletta M, Vivarelli E, Frati L, Cossu G, Giacomello A. Isolation and expansion of adult cardiac stem cells from human and murine heart. Circ Res 2004;95(9):911-921.
7. Smith RR, Barile L, Cho HC, Leppo MK, Hare JM, Messina E, Giacomello A, Abraham MR, Marban E. Regenerative potential of cardiosphere-derived cells expanded from percutaneous endomyocardial biopsy specimens. Circulation 2007;115(7):896-908.
8. Bearzi C, Rota M, Hosoda T, Tillmanns J, Nascimbene A, De Angelis A, Yasuzawa-Amano S, Trofimova I, Siggins RW, Lecapitaine N, Cascapera S, Beltrami AP, D'Alessandro DA, Zias E, Quaini F, Urbanek K, Michler RE, Bolli R, Kajstura J, Leri A, Anversa P. Human cardiac stem cells. Proc Natl Acad Sci U S A 2007;104(35):14068-14073.
9. Laugwitz KL, Moretti A, Lam J, Gruber P, Chen Y, Woodard S, Lin LZ, Cai CL, Lu MM, Reth M, Platoshyn O, Yuan JX, Evans S, Chien KR. Postnatal isl1+ cardioblasts enter fully differentiated cardiomyocyte lineages. Nature 2005;433(7026):647-653.
10. Goumans MJ, de Boer TP, Smits AM, van Laake LW, van Vliet P, Metz CH, Korfage TH, Kats KP, Hochstenbach R, Pasterkamp G, Verhaar MC, van der Heyden MA, de KD, Mummery CL, van Veen TA, Sluijter JP, Doevendans PA. TGF-beta1 induces efficient differentiation of human cardiomyocyte progenitor cells into functional cardiomyocytes in vitro. Stem Cell Res 2007;1(2):138-149.
11. van Vliet P, Roccio M, Smits AM, van Oorschot AA, Metz CH, van Veen TA, Sluijter JP, Doevendans PA, Goumans MJ. Progenitor cells isolated from the human heart: a potential cell source for regenerative therapy. Neth Heart J 2008;16(5):163-169.
12. Oh H, Bradfute SB, Gallardo TD, Nakamura T, Gaussin V, Mishina Y, Pocius J, Michael LH, Behringer RR, Garry DJ, Entman ML, Schneider MD. Cardiac progenitor cells from adult myocardium: homing, differentiation, and fusion after infarction. Proc Natl Acad Sci U S A 2003;100(21):12313-12318.
13. Matsuura K, Nagai T, Nishigaki N, Oyama T, Nishi J, Wada H, Sano M, Toko H, Akazawa H, Sato T, Nakaya H, Kasanuki H, Komuro I. Adult cardiac Sca-1-positive cells differentiate into beating cardiomyocytes. J Biol Chem 2004;279(12):11384-11391.
14. Holmes C, Stanford WL. Concise review: stem cell antigen-1: expression, function, and enigma. Stem Cells 2007;25(6):1339-1347.
15. Kattman SJ, Adler ED, Keller GM. Specification of multipotential cardiovascular progenitor cells during embryonic stem cell differentiation and embryonic development. Trends Cardiovasc Med 2007;17(7):240-246.
16. Laugwitz KL, Moretti A, Caron L, Nakano A, Chien KR. Islet1 cardiovascular progenitors: a single source for heart lineages? Development 2008;135(2):193-205.
17. Smits AM, van Vliet P, Metz CH, Korfage T, Sluijter JP, Doevendans PA, Goumans MJ. Human cardiomyocyte progenitor cells differentiate into functional mature cardiomycoytes: an in vitro model for studying human cardiac physiology and pathophysiology. Nat Protoc 2009;4(2):232-243.

proefschrift Anke.indd 161 15-07-2009 12:00:54

18. Choi SC, Yoon J, Shim WJ, Ro YM, Lim DS. 5-azacytidine induces cardiac differentiation of P19 embryonic stem cells. Exp Mol Med 2004;36(6):515-523.
19. Xu C, Police S, Rao N, Carpenter MK. Characterization and enrichment of cardiomyocytes derived from human embryonic stem cells. Circ Res 2002;91(6):501-508.
20. Xu W, Zhang X, Qian H, Zhu W, Sun X, Hu J, Zhou H, Chen Y. Mesenchymal stem cells from adult human bone marrow differentiate into a cardiomyocyte phenotype in vitro. Exp Biol Med (Maywood) 2004;229(7):623-631.
21. Lim JY, Kim WH, Kim J, Park SI. Involvement of TGF-beta1 signaling in cardiomyocyte differentiation from P19CL6 cells. Mol Cells 2007;24(3):431-436.
22. Kumar A, Rogers T, Maizel A, Sharma S. Loss of transforming growth factor beta 1 receptors and its effects on the growth of EBV-transformed human B cells. J Immunol 1991;147(3):998-1006.
23. Li TS, Komota T, Ohshima M, Qin SL, Kubo M, Ueda K, Hamano K. TGF-beta induces the differentiation of bone marrow stem cells into immature cardiomyocytes. Biochem Biophys Res Commun 2008;366(4):1074-1080.
24. Ammanamanchi S, Brattain MG. 5-azaC treatment enhances expression of transforming growth factor-beta receptors through down-regulation of Sp3. J Biol Chem 2001;276(35):32854-32859.
25. Zuscik MJ, Baden JF, Wu Q, Sheu TJ, Schwarz EM, Drissi H, O'Keefe RJ, Puzas JE, Rosier RN. 5-azacytidine alters TGF-beta and BMP signaling and induces maturation in articular chondrocytes. J Cell Biochem 2004;92(2):316-331.
26. Chuva de Sousa Lopes SM, Feijen A, Korving J, Korchynskyi O, Larsson J, Karlsson S, ten Dijke P, Lyons KM, Goldschmeding R, Doevendans P, Mummery CL. Connective tissue growth factor expression and Smad signaling during mouse heart development and myocardial infarction. Dev Dyn 2004;231(3):542-550.
27. Hsieh PC, Segers VF, Davis ME, MacGillivray C, Gannon J, Molkentin JD, Robbins J, Lee RT. Evidence from a genetic fate-mapping study that stem cells refresh adult mammalian cardiomyocytes after injury. Nat Med 2007;13(8):970-974.
28. Bergmann O, Bhardwaj RD, Bernard S, Zdunek S, Barnabe-Heider F, Walsh S, Zupicich J, Alkass K, Buchholz BA, Druid H, Jovinge S, Frisen J. Evidence for cardiomyocyte renewal in humans. Science 2009;324(5923):98-102.
29. Beltrami AP, Urbanek K, Kajstura J, Yan SM, Finato N, Bussani R, Nadal-Ginard B, Silvestri F, Leri A, Beltrami CA, Anversa P. Evidence that human cardiac myocytes divide after myocardial infarction. N Engl J Med 2001;344(23):1750-1757.
30. Mouquet F, Pfister O, Jain M, Oikonomopoulos A, Ngoy S, Summer R, Fine A, Liao R. Restoration of cardiac progenitor cells after myocardial infarction by self-proliferation and selective homing of bone marrow-derived stem cells. Circ Res 2005;97(11):1090-1092.
31. Quaini F, Urbanek K, Beltrami AP, Finato N, Beltrami CA, Nadal-Ginard B, Kajstura J, Leri A, Anversa P. Chimerism of the transplanted heart. N Engl J Med 2002;346(1):5-15.
32. Cai CL, Liang X, Shi Y, Chu PH, Pfaff SL, Chen J, Evans S. Isl1 identifies a cardiac progenitor population that proliferates prior to differentiation and contributes a majority of cells to the heart. Dev Cell 2003;5(6):877-889.
33. Coletta JE, Rosenthal N, Costa MA. Cardiac mapping and stem cell delivery for the damaged myocardium. Expert Rev Cardiovasc Ther 2008;6(9):1181-1190.
34. Menasche P. Skeletal myoblasts as a therapeutic agent. Prog Cardiovasc Dis 2007;50(1):7-17.
35. Teng CJ, Luo J, Chiu RC, Shum-Tim D. Massive mechanical loss of microspheres with direct intramyocardial injection in the beating heart: implications for cellular cardiomyoplasty. J Thorac Cardiovasc Surg 2006;132(3):628-632.
36. Askari AT, Unzek S, Popovic ZB, Goldman CK, Forudi F, Kiedrowski M, Rovner A, Ellis SG, Thomas JD, DiCorleto PE, Topol EJ, Penn MS. Effect of stromal-cell-derived factor 1 on stem-cell homing and tissue regeneration in ischaemic cardiomyopathy. Lancet 2003;362(9385):697-703.

proefschrift Anke.indd 162 15-07-2009 12:00:54

37. Abbott JD, Huang Y, Liu D, Hickey R, Krause DS, Giordano FJ. Stromal cell-derived factor-1alpha plays a critical role in stem cell recruitment to the heart after myocardial infarction but is not sufficient to induce homing in the absence of injury. Circulation 2004;110(21):3300-3305.
38. Tang YL, Qian K, Zhang YC, Shen L, Phillips MI. Mobilizing of haematopoietic stem cells to ischemic myocardium by plasmid mediated stromal-cell-derived factor-1alpha (SDF-1alpha) treatment. Regul Pept 2005;125(1-3):1-8.
39. Ceradini DJ, Kulkarni AR, Callaghan MJ, Tepper OM, Bastidas N, Kleinman ME, Capla JM, Galiano RD, Levine JP, Gurtner GC. Progenitor cell trafficking is regulated by hypoxic gradients through HIF-1 induction of SDF-1. Nat Med 2004;10(8):858-864.
40. Nagaya N, Fujii T, Iwase T, Ohgushi H, Itoh T, Uematsu M, Yamagishi M, Mori H, Kangawa K, Kitamura S. Intravenous administration of mesenchymal stem cells improves cardiac function in rats with acute myocardial infarction through angiogenesis and myogenesis. Am J Physiol Heart Circ Physiol 2004;287(6):H2670-H2676.
41. Jiang W, Ma A, Wang T, Han K, Liu Y, Zhang Y, Dong A, Du Y, Huang X, Wang J, Lei X, Zheng X. Homing and differentiation of mesenchymal stem cells delivered intravenously to ischemic myocardium in vivo: a time-series study. Pflugers Arch 2006;453(1):43-52.
42. Christopherson KW, Hangoc G, Broxmeyer HE. Cell surface peptidase CD26/dipeptidylpeptidase IV regulates CXCL12/stromal cell-derived factor-1 alpha-mediated chemotaxis of human cord blood CD34+ progenitor cells. J Immunol 2002;169(12):7000-7008.
43. Vanhoof G, Goossens F, De Meester I, Hendriks D, Scharpe S. Proline motifs in peptides and their biological processing. FASEB J 1995;9(9):736-744.
44. Proost P, Struyf S, Schols D, Durinx C, Wuyts A, Lenaerts JP, DeClercq E, De Meester I, Van Damme J. Processing by CD26/dipeptidyl-peptidase IV reduces the chemotactic and anti-HIV-1 activity of stromal-cell-derived factor-1alpha. FEBS Lett 1998;432(1-2):73-76.
45. Herrera C, Morimoto C, Blanco J, Mallol J, Arenzana F, Lluis C, Franco R. Comodulation of CXCR4 and CD26 in human lymphocytes. J Biol Chem 2001;276(22):19532-19539.
46. Fischer UM, Harting MT, Jimenez F, Monzon-Posadas WO, Xue H, Savitz SI, Laine GA, Cox CS. Pulmonary passage is a major obstacle for intravenous stem cell delivery: The pulmonary first pass effect. Stem Cells Dev 2008.
47. Nussbaum J, Minami E, Laflamme MA, Virag JA, Ware CB, Masino A, Muskheli V, Pabon L, Reinecke H, Murry CE. Transplantation of undifferentiated murine embryonic stem cells in the heart: teratoma formation and immune response. FASEB J 2007;21(7):1345-1357.
48. van Laake LW, Passier R, Monshouwer-Kloots J, Verkleij AJ, Lips DJ, Freund C, den Ouden K, Ward-van Oostwaard D, Korving J, Tertoolen LG, van Echteld CJ, Doevendans PA, Mummery CL. Human Embryonic Stem Cell-Derived Cardiomyocytes Survive and Mature in the Mouse Heart and Transiently Improve Function after Myocardial Infarction. Stem Cell Research 2007;1(1):9-24.
49. van der Bogt KE, Sheikh AY, Schrepfer S, Hoyt G, Cao F, Ransohoff KJ, Swijnenburg RJ, Pearl J, Lee A, Fischbein M, Contag CH, Robbins RC, Wu JC. Comparison of different adult stem cell types for treatment of myocardial ischemia. Circulation 2008;118(14 Suppl):S121-S129.
50. Timmers L, Lim SK, Arslan F, Armstrong JS, Hoefer IE, Doevendans PA, Piek JJ, El Oakley RM, Choo A, Lee CN, Pasterkamp G, de Kleijn D. Reduction of myocardial infarct size by human mesenchymal stem cell conditioned medium. Stem Cell Research 2007;1(2):129-137.
51. Ziebart T, Yoon CH, Trepels T, Wietelmann A, Braun T, Kiessling F, Stein S, Grez M, Ihling C, Muhly-Reinholz M, Carmona G, Urbich C, Zeiher AM, Dimmeler S. Sustained persistence of transplanted proangiogenic cells contributes to neovascularization and cardiac function after ischemia. Circ Res 2008;103(11):1327-1334.
52. van Laake LW, van den Driesche S, Post S, Feijen A, Jansen MA, Driessens MH, Mager JJ, Snijder RJ, Westermann CJ, Doevendans PA, van Echteld CJ, ten Dijke P, Arthur HM, Goumans MJ, Lebrin F, Mummery CL. Endoglin has a crucial role in blood cell-mediated vascular repair. Circulation 2006;114(21):2288-

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2297.
53. Takahashi K, Tanabe K, Ohnuki M, Narita M, Ichisaka T, Tomoda K, Yamanaka S. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell 2007;131(5):861-872.
54. Yu J, Vodyanik MA, Smuga-Otto K, Antosiewicz-Bourget J, Frane JL, Tian S, Nie J, Jonsdottir GA, Ruotti V, Stewart R, Slukvin II, Thomson JA. Induced pluripotent stem cell lines derived from human somatic cells. Science 2007;318(5858):1917-1920.
55. Zhang J, Wilson GF, Soerens AG, Koonce CH, Yu J, Palecek SP, Thomson JA, Kamp TJ. Functional cardiomyocytes derived from human induced pluripotent stem cells. Circ Res 2009;104(4):e30-e41.
56. Mauritz C, Schwanke K, Reppel M, Neef S, Katsirntaki K, Maier LS, Nguemo F, Menke S, Haustein M, Hescheler J, Hasenfuss G, Martin U. Generation of functional murine cardiac myocytes from induced pluripotent stem cells. Circulation 2008;118(5):507-517.
57. Woltjen K, Michael IP, Mohseni P, Desai R, Mileikovsky M, Hamalainen R, Cowling R, Wang W, Liu P, Gertsenstein M, Kaji K, Sung HK, Nagy A. piggyBac transposition reprograms fibroblasts to induced pluripotent stem cells. Nature 2009;458(7239):766-770.
58. Kaji K, Norrby K, Paca A, Mileikovsky M, Mohseni P, Woltjen K. Virus-free induction of pluripotency and subsequent excision of reprogramming factors. Nature 2009;458(7239):771-775.
59. Li TS, Hayashi M, Ito H, Furutani A, Murata T, Matsuzaki M, Hamano K. Regeneration of infarcted myocardium by intramyocardial implantation of ex vivo transforming growth factor-beta-preprogrammed bone marrow stem cells. Circulation 2005;111(19):2438-2445.
60. Zimmermann WH, Melnychenko I, Wasmeier G, Didie M, Naito H, Nixdorff U, Hess A, Budinsky L, Brune K, Michaelis B, Dhein S, Schwoerer A, Ehmke H, Eschenhagen T. Engineered heart tissue grafts improve systolic and diastolic function in infarcted rat hearts. Nat Med 2006;12(4):452-458.
61. Atoui R, Shum-Tim D, Chiu RC. Myocardial regenerative therapy: immunologic basis for the potential "universal donor cells". Ann Thorac Surg 2008;86(1):327-334.

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Nederlandse samenvatting

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Hart- en vaatziekten zijn de belangrijkste oorzaak van ziekte en sterfte in de westerse wereld. Vooral de gevolgen van een hartinfarct leveren hieraan een belangrijke bijdrage. Een hartinfarct ontstaat door een tekort aan bloedtoevoer in een deel van de hartspier. Meestal is dit het resultaat van aderverkalking in een van de kransslagaderen (de bloedvaten die de hartspier van bloed voorzien). Een deel van het weefsel krijgt dan geen zuurstof en voedingsstoffen aangeleverd, waardoor spiercellen in het getroffen gebied afsterven.
Als gevolg van de schade in het hart geven de overlevende cellen signaalstoffen af die ontstekingscellen (witte bloedcellen) vanuit het bloed aantrekken. De ontstekingscellen ruimen de dode cellen op en vervangen deze door een taai litteken waarmee voorkomen wordt dat het hart scheurt door een gebrek aan stevigheid. De combinatie van een verminderd aantal spiercellen en de aanwezigheid van een litteken zorgt er echter voor dat het hart minder efficiënt kan samentrekken. In de loop van de tijd wordt het voor het hart steeds zwaarder om voldoende bloed rond te pompen en kan de patiënt uiteindelijk sterven aan hartfalen.
De medicatie die wordt voorgeschreven aan hartinfarctpatiënten zorgt voor minder belasting van het hart, maar het onderliggende probleem (het verlies hartspiercellen en dus van spierkracht) wordt niet behandeld. Het lichaam is niet in staat voldoende nieuwe hartspiercellen te genereren en de overlevende spiercellen kunnen zelf niet delen. Daarom worden alternatieve manieren gezocht om nieuwe hartspiercellen aan het hart te leveren. Een van de mogelijke therapieën die wordt bestudeerd is (stam)celtherapie.
Een stamcel is een cel die zichzelf kan vernieuwen door deling en kan veranderen (differentiëren) in één of meer andere celtypes. Het idee achter stamceltherapie is dat cellen worden getransplanteerd in het beschadigde hart en dat deze stamcellen zich vervolgens ter plekke ontwikkelen tot het benodigde hartweefsel. Voordat celtherapie daadwerkelijk kan worden toegepast in patiënten dienen er echter een aantal belangrijke aspecten onderzocht te worden.
Ten eerste moet er een bron van stamcellen geïdentificeerd worden die in staat is nieuw hartweefsel te vormen dat bestaat uit zowel hartspiercellen als bloedvaten. Ten tweede moet onderzocht worden wat de beste manier is om cellen aan het hart toe te dienen. Deze toediening is bijvoorbeeld mogelijk via directe transplantatie in de hartspier of door injectie in de bloedcirculatie. Ten derde moeten de cellen kunnen overleven onder zware omstandigheden; in het infarctgebied waarin ze terecht komen is namelijk weinig zuurstof en voedsel aanwezig. En ten vierde moeten de cellen ter plekke integreren in het bestaande weefsel en koppelen met omringende hartspiercellen zodat ze in hetzelfde ritme gaan kloppen.
In dit proefschrift komen verschillende aspecten van celtherapie aan bod en is een aantal van bovenstaande criteria onderzocht.

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In **Hoofdstuk 2** wordt een literatuuronderzoek beschreven waarin verschillende cel- en weefselbronnen voor celtherapie zijn vergeleken. Onderzocht is in hoeverre, en onder welke omstandigheden, verschillende stamceltypen in staat zijn te differentiëren naar hartspiercellen. Vooral embryonale stamcellen lijken een goede optie voor weefselherstel. Deze cellen worden geïsoleerd uit de ontwikkelende bevruchte eicel en kunnen daarom nog alle celtypen worden die voorkomen in het lichaam, dus ook hartspiercellen. Het gebruik van embryonale stamcellen is vanuit ethisch oogpunt echter nog steeds omstreden. Daarom heeft men gezocht naar (stam)celpopulaties die uit het volwassen lichaam geïsoleerd kunnen worden. Een bekende bron hiervoor is het beenmerg, dat verschillende soorten stamcellen bevat die normaliter betrokken zijn bij de vorming van bloedcellen. Na transplantatie van beenmergstamcellen in het hart van infarctpatiënten dragen beenmerg stamcellen bij aan een licht herstel van de hartfunctie. Tot op heden lijken ze echter niet in staat daadwerkelijk nieuwe hartspiercellen te vormen. Andere weefsels waaruit stamcellen kunnen worden gehaald zijn bijvoorbeeld skeletspieren en het hart zelf.

Tijdens dit promotieonderzoek is gezocht naar cellen die afkomstig zijn uit het orgaan dat men probeert te herstellen: het hart. In voorgaande jaren is in het menselijke hart een populatie cellen gevonden die in kweek in het laboratorium zichzelf vele malen kan vermenigvuldigen. Met behulp van de juiste stimulatie kunnen deze cellen differentiëren naar hartspiercellen of bloedvaten. Omdat ze zich lijken te beperken tot de vorming van celtypen die in hartweefsel voorkomen, worden ze hartspiervoorloper cellen genoemd, ofwel cardiomyocyte progenitor cells (CMPCs). In **Hoofdstuk 3** wordt uitgebreid beschreven hoe CMPCs uit een klein stukje menselijk hartweefsel geïsoleerd kunnen worden en hoe CMPCs gestimuleerd moeten worden om ze in kweek te veranderen in kloppende hartspiercellen.

CMPCs kunnen nieuwe hartspiercellen en bloedvaten maken en bezitten daarom de juiste eigenschappen om zich te specialiseren naar hartweefsel. Daarom is onderzocht of CMPCs kunnen bijdragen aan de reparatie van een hartinfarct. In **Hoofdstuk 4** is bepaald wat het effect is van celtransplantatie van ongedifferentieerde CMPCs en uit CMPC gekweekte hartspiercellen op het herstel van het hart. In muizen is een infarct geïnduceerd door één van de kransslagaders af te binden, vervolgens zijn de cellen ingespoten op de rand van het afstervende weefsel. Door de aanwezigheid van CMPCs of uit CMPC gekweekte hartspiercellen bleef de pompfunctie van het muizenhart grotendeels behouden, terwijl deze achteruitging in dieren die geen celtransplantatie hadden gekregen. Daarnaast hebben we aangetoond dat CMPCs in het beschadigde hart nieuw hartweefsel kunnen vormen, hoewel dit beperkt blijft tot kleine gebiedjes. CMPCs lijken

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dus een goede bron van cellen om te gebruiken voor celtherapie, maar de hoeveelheid hartweefsel dat wordt gevormd moet geoptimaliseerd worden.

In **Hoofdstuk 5** is een ander aspect van hartherstel onderzocht. De signaalstoffen die vrijkomen na een hartinfarct trekken witte bloedcellen aan. Deze ontstekingscellen zorgen voor het opruimen van dood weefsel, maar ook voor de vorming van nieuwe bloedvaten. Indien witte bloedcellen niet goed migreren naar het beschadigde hartweefsel herstelt het hart slechter na infarct. In Hoofdstuk 5 is een populatie patiënten onderzocht met de zeldzame erfelijke ziekte Hereditary Hemorrhagic Telangiectasia type-I (HHT-1), ook bekend als de ziekte van Rendu-Osler-Weber. HHT-1 patiënten vertonen afwijkingen aan het vaatstelsel en hebben een slecht herstel van beschadigde bloedvaten. Deze patiënten hebben bijvoorbeeld regelmatig last van onophoudelijke bloedneuzen. Het verslechterde vaatherstel komt deels voort uit slecht functionerende ontstekingscellen van HHT-1 patiënten die niet in staat zijn te migreren naar gebieden van (vaat)schade. De afwijkende migratiecapaciteit van cellen van HHT-1 patiënten is een gevolg van hoge concentraties van het eiwit CD26 op het oppervlak van deze cellen. Omdat CD26 de door het beschadigde weefsel uitgezonden signaalstoffen afbreekt reageren HHT-1 cellen nauwelijks op deze signalen. De cellen migreren daardoor niet meer naar de juiste plaats. Door CD26 te verwijderen van het celoppervlak verbeterd de reactie van HHT-1 bloedcellen op schade. Dit hebben we aangetoond in celkweeksystemen waarin de schadesignalen worden nagebootst, en in muizen met een hartinfarct.

Zoals in Hoofdstuk 4 aangetoond zijn CMPCs een goede bron voor celtherapie maar blijven ze slechts in kleine aantallen in het hartinfarct na transplantatie. In **Hoofdstuk 6** is daarom onderzocht of er een efficiëntere methode is om CMPCs toe te dienen. Er is onderzocht of CMPCs na een injectie in de bloedbaan in de richting van de schade gestuurd kunnen worden door gebruik te maken van de schadesignalen die het hart produceert. Omdat in Hoofdstuk 5 duidelijk is geworden dat CD26 verhindert dat cellen reageren op schade, is het niveau van dit eiwit op CMPCs geanalyseerd. CMPCs blijken hoge concentraties CD26 op het oppervlak te hebben, terwijl ze verder alle onderdelen bezitten om op schade te kunnen reageren. Door CD26 van het oppervlak van CMPCs te verwijderen reageren de cellen in kweek zeer efficiënt op schadesignalen. Deze CD26-loze CMPCs zijn vervolgens geïnjecteerd in de staartvene van een muis met een hartinfarct. De verwachting was dat deze CMPCs vanuit het bloed naar het beschadigde hart migreren en daar bij dragen aan herstel van de pompfunctie. Drie weken na injectie waren er echter geen CMPCs aanwezig in het hart. Het grootste deel van de cellen werd teruggevonden in andere organen. Helaas hebben we nog geen definitieve verklaring gevonden voor deze effecten.

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In **Hoofdstukken 7 en 8** wordt een nieuw model beschreven om celtypes te identificeren, dat zowel in kweek als in muizen kan worden toegepast. Door gebruik te maken van moderne transgene technieken hebben we een systeem ontworpen waarin we de differentiatie van stamcellen naar een zelfgekozen celtype (bijvoorbeeld hartspiercel of bloedvatcel) kunnen visualiseren. In het celtype dat vanuit stamcellen is ontstaan treedt een kleurverandering op waardoor het onderscheiden kan worden van andere aanwezige celtypes. Daarnaast gaan de cellen een enzym aanmaken dat het normaal onschuldige CB1954 omzet in een giftige stof. Het toevoegen van CB1954 schakelt dus alleen het gekozen celtype uit, terwijl de andere celtypes intact blijven.
In **Hoofdstuk 7** wordt een voorbeeld gegeven van de mogelijkheden van dit systeem. In een celkweekmodel worden bloedvatcellen geïdentificeerd en vervolgens uitgeschakeld door toevoegen van CB1954.
Hoofdstuk 8 wordt het model toegepast om de voorlopercellen in het muizenhart te analyseren. Hiermee kan onderzocht worden of voorlopercellen die aanwezig zijn in het hart lokaal kunnen bijdragen aan het herstel na een hartinfarct. De voorlopercellen ondergaan een kleurverandering wanneer ze differentiëren naar hartspiercellen waardoor het aantal nieuw gevormde spiercellen geteld kan worden. Door toediening van CB1954 aan de muis worden de nieuwe hartspiercellen verwijderd. De verwachting is dat zonder de nieuwe cellen het herstel na infarct slechter zal zijn. De relatieve bijdrage van de cellen aan het herstel van hartfunctie kan dus worden gemeten met dit model. Uiteindelijk is er dan een systeem beschikbaar om de voorlopercellen “van binnen uit” te analyseren. Hierna kan gezocht worden naar methodes om de populatie te stimuleren zonder dat daar transplantaties aan te pas hoeven komen.

CONCLUSIE

Tot een aantal jaren geleden werd gedacht dat het hart helemaal geen mogelijkheden had om zichzelf te herstellen. Immers, men dacht afgestorven hartspiercellen niet werden vervangen door nieuwe. De bevinding dat het hart voorlopercellen bevat die in staat zijn nieuw hartweefsel te vormen is erg belangrijk en heeft het bestaande dogma veranderd. Hoewel het duidelijk is dat deze voorlopercellen in het volwassen hart niet voldoende bijdragen aan herstel (het infarct lost niet vanzelf op), bieden deze cellen wel degelijk nieuwe mogelijkheden.
Wanneer voorlopercellen uit het hart worden geïsoleerd, in kweek worden vermenigvuldigd, en vervolgens aan de patiënt zelf worden teruggegeven via injectie in de hartpier of de bloedbaan, kunnen ze wellicht wel bijdragen aan de vorming van nieuw hartweefsel. Daarnaast kan meer inzicht in de oorsprong en rol van de voorlopercellen in het hart

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tot de ontwikkeling van nieuwe behandelingsmethoden leiden, waarbij bijvoorbeeld de in het hart aanwezige voorloper cellen van binnenuit worden gestimuleerd zonder dat daar tijdrovende isolaties en transplantaties voor nodig zijn. Dit proefschrift levert een bijdrage aan het begrip van voorlopercellen in het hart en aan de mogelijkheden voor herstel van het hart na schade. De toekomst zal uitwijzen wat de beste strategie is voor regeneratie van het hart.

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Dankwoord

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DANKWOORD

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Tom, mijn niet-meer-zo-kleine broertje. Ik vind het leuk dat je een heel stuk dichterbij komt wonen. Ik weet zeker dat je het naar je zin zult hebben in Amsterdam. Heel veel succes!

Lieve papa en mama, jullie geven een goed voorbeeld hoe je samen kunt genieten van

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het leven. Ik kom nog steeds met heel veel plezier naar “huis”. Het is fijn dat jullie zo goed begrijpen waar ik het de afgelopen jaren zo druk mee heb gehad. Bedankt voor jullie onvoorwaardelijke steun en liefde.

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LIST OF PUBLICATIONS

1. **Smits AM***, van Laake LW*, den Ouden K, Schreurs C, Szuhai K, van Echteld CJ, Mummery CL, Doevendans PA, Goumans MJ. Human cardiomyocyte progenitor cell transplantation preserves long-term function of the infarcted mouse myocardium. *Cardiovascular Research 2009; 83(3):527-535*.

2. **Smits AM**, van den Hengel LG, van den Brink S, Metz CH, Doevendans PA, Goumans MJ. A new in vitro model for stem cell differentiation and interaction. Stem Cell Research 2009;2(2):108-112.

3. Liu J, Sluijter JP, Goumans MJ, **Smits AM**, van der Spoel T, Nathoe H, Doevendans PA. Cell therapy for myocardial regeneration. *Current Molecular Medicine 2009;9(3):287-298*.

4. **Smits AM**, van Vliet P, Metz CH, Korfage T, Sluijter JP, Doevendans PA, Goumans MJ. Human cardiomyocyte progenitor cells differentiate into functional mature cardiomyocytes: an in vitro model for studying human cardiac physiology and pathophysiology. *Nature Protocols 2009;4(2):232-243*.

5. Bot PT, Hoefer IE, Sluijter JP, van Vliet P, **Smits AM**, Lebrin F, Moll F, de Vries JP, Doevendans P, Piek JJ, Pasterkamp G, Goumans MJ. Increased expression of the transforming growth factor-beta signaling pathway, endoglin, and early growth response-1 in stable plaques. *Stroke 2009;40(2):439-447*.

6. van Vliet P, Roccio M, **Smits AM**, van Oorschot AA, Metz CH, van Veen TA, Sluijter JP, Doevendans PA, Goumans MJ. Progenitor cells isolated from the human heart: a potential cell source for regenerative therapy. *Netherlands Heart Journal 2008;16(5):163-169*.

7. Goumans MJ, de Boer TP, **Smits AM**, van Laake LW, van Vliet P, Metz CH, Korfage TH, Kats KP, Hochstenbach R, Pasterkamp G, Verhaar MC, van der Heyden MA, de Kleijn D, Mummery CL, van Veen TA, Sluijter JP, Doevendans PA. TGF-beta1 induces efficient differentiation of human cardiomyocyte progenitor cells into functional cardiomyocytes in vitro. *Stem Cell Research 2007;1(2):138-149*.

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8. van Maarseveen NM, Huigen MC, de Jong D, **Smits AM**, Boucher CA, Nijhuis M. A novel real-time PCR assay to determine relative replication capacity for HIV-1 protease variants and/or reverse transcriptase variants.
Journal of Virological Methods 2006;133(2):185-194.

9. van Laake LW*, **Smits AM***. Molecular Mechanisms of Cardiac Disease and Regeneration.
Netherlands Heart Journal 2006;(14):5-195.

10. **Smits AM**, Doevendans PA. Stamcellen als therapie na hartinfarct.
Tromnibus 2005;33(1):13-14.

11. **Smits AM**, van Vliet P, Hassink RJ, Goumans MJ, Doevendans PA. The role of stem cells in cardiac regeneration.
Journal of Cellular and Molecular Medicine 2005;9(1):25-36.

12. **Smits AM**, Smits JF. Ischemic heart disease: models of myocardial hypertrophy and infarction.
Drug Discovery today: disease models 2004;1(3):273-278.

13. Rookmaaker MB*, **Smits AM***, Tolboom H, Van 't Wout K, Martens AC, Goldschmeding R, Joles JA, Van Zonneveld AJ, Grone HJ, Rabelink TJ, Verhaar MC.
Bone-marrow-derived cells contribute to glomerular endothelial repair in experimental glomerulonephritis.
American Journal of Pathology 2003;163(2):553-562.

14. van Vliet P, van Oorschot AAM, **Smits AM**, Liu Z, Sluijter JPG, Hoefer IE, Doevendans PA, Goumans MJ. Survivin increases cardiomyocyte progenitor cell proliferation and survival.
Submitted.

15. van Vliet P, **Smits AM**, de Boer TP, Korfage T, Metz CH, Roccio M, van der Heyden MA, van Veen TA, Sluijter JP, Doevendans PA, Goumans MJ. Fetal and adult cardiomyocyte progenitor cells have different developmental potential.
Submitted.

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16. Tian F, Zhou X, **Smits AM**, Larsson E, Goumans MJ, Heldin CH, Borén J, Akyürek LM Endothelial cells are activated during hypoxia via endoglin/ALK-1/SMAD1/5 signaling in vivo and in vitro.
Submitted.

17. Post S*, **Smits AM***, van den Broek AJ, Sluijter JP, Hoefer IE, Janssen BJ, Snijder RJ, Mager JJ, Pasterkamp G, Mummery CL, Doevendans PA, Goumans MJ. Impaired recruitment of HHT-1 mononuclear cells to the ischemic heart is due to an altered CXCR4/CD26 balance.
Submitted.

18. Winter EM, Hogers B, Lie-Venema H, Swildens J, Nauerth A, van der Graaf LM, Dorjee AL, Bax NAM, Salvatori D, **Smits AM**, Klautz RJM, van der Weerd L, de Vries AAT, Doevendans PA, Poelmann RE, Gittenberger-de Groot AC. The potential of epicardium in the infarcted heart.
Submitted.

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CURRICULUM VITAE

Anke Smits was born on November 17th, 1980 in Geleen. She graduated from secondary school (Jeanne d'Arc College, Maastricht) in 1998. In that same year she moved to Utrecht to study Medical Biology at the University of Utrecht. During this period she became interested in the role of stem cells in tissue repair. She performed her first internship at the department of Vascular Medicine, analyzing the role of bone-marrow derived cells in kidney disease. However, as a second internship she chose to study a very different subject. For six months, she investigated the viral fitness of HIV variants at the Eijkman Winkler Institute. In October 2003 she obtained her Master's degree in Biomedical Sciences.

After graduation, Anke started her PhD at the department of Cardiology at the UMC in Utrecht, under supervision of dr. Marie-José Goumans and prof. Pieter Doevendans. The results of her research are described in this thesis. In March 2008, she moved together with Marie-José to the department of Molecular Cell Biology at the LUMC in Leiden, where they integrated in the group of prof. Peter ten Dijke. Since July 2009, Anke is working as a post-doc at the department of Molecular Cell Biology at the LUMC.

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