Tissue Vascularization with Endothelial-like Mesenchymal Stromal Cells

Karolina Janeczek Portalska

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2014

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TISSUE VASCULARIZATION WITH ENDOTHELIAL-LIKE MESENCHYMAL STROMAL CELLS

Karolina Janeczek Portalska

PhD Thesis, University of Twente, Enschede, The Netherlands

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TISSUE VASCULARIZATION WITH ENDOTHELIAL-LIKE MESENCHYMAL STROMAL CELLS

DISSERTATION

to obtain
the degree of doctor at the University of Twente,
on the authority of the rector magnificus,
Prof. Dr. H. Brinksma
on account of the decision of the graduation committee,
to be publicly defended
on Thursday, April 10th, 2014, at 12.45 hrs

by

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Summary

Although most tissues in the human body have self-renewal capabilities, there are defects, e.g. caused by trauma or disease, which are beyond regenerative potential. Tissue engineering offers a possibility to heal such defects without the necessity of finding a suitable graft donor. While a number of *in vivo* studies concerning tissue engineering for tissues replacement have been successfully performed, a need occurred to introduce vascular networks in newly formed tissues in order to guarantee their survival and correct functionality. Therefore, tissue engineering of blood vessels and capillaries is a widely investigated aspect necessary for various applications.

This thesis describes the *in vitro* and *in vivo* biological evaluation of the potential of human mesenchymal stromal cells (hMSC) to differentiate towards endothelial-like cells (EL-MSC). The success of regenerative strategies where EL-MSCs are applied relies on the following requirements: the differentiation protocol should be simple and robust, the issue of donor-related variability needs to be addressed, clinically approved ways of delivery must to be provided and finally, the usefulness of EL-MSCs in certain applications ought to be proven. Since hMSCs are already widely used in various therapies, EL-MSCs are likely to fulfil all these requirements.

Chapter 1 summarizes the content of this thesis. In chapter 2, a general review of literature is given, with the particular emphasis on emerging strategies for blood vessel engineering. Various cell types, biomaterials and approaches to generate functional vessels are described. Chapter 3 contains the evaluation of hMSC endothelial differentiation. The robustness of the presented protocol is assessed in chapter 4 together with the estimation of hMSC *in vitro* expansion potential when endothelial differentiation is required. Several molecular markers are also suggested for predicting whether cells obtained from a particular donor can be used in therapies that require EL-MSC application. Chapters 5 and 6 contain the description of two potential strategies to deliver EL-MSC for vascularization purposes. Modular tissue engineering approaches based on collagen modules and injectable in-situ-forming dextran-based hydrogel applications are presented. Two strategies are described to improve biological performance of EL-MSCs: creation of 3-dimensional structures allowing for easier vessel in-growth and alternatively, applying growth factor stimulation to induce angiogenesis from host vessels. The potential applications of EL-MSCs for tissue engineering purposes are described in chapter 7 and 8. In chapter 7 the beneficial influence of EL-MSCs on bone formation is presented while chapter 8 shows how EL-MSCs can be used for

improving vascularization of islets of Langerhans. Finally, chapter 9 summarizes and discusses all the results presented in this thesis.

Overall, this thesis presents a multifactorial approach towards improving vascularization of both *in vitro* and *in vivo* engineered tissues and also suggests future developments and applications of endothelial-like cells derived from MSCs .

Samenvatting

Alhoewel de meeste weefsels in het menselijk lichaam zelf-vernieuwend capaciteiten hebben, zijn er defecten, bijvoorbeeld veroorzaakt door trauma of ziekte, die verder gaan dan de regeneratieve mogelijkheden. Weefselregeneratie biedt de mogelijkheid om zulke defecten te genezen zonder dat het zoeken naar een geschikte weefseldonor nodig is. Sinds een aantal *in vivo* weefselregeneratiestudies voor weefselvervanging succesvol uitgevoerd zijn, is er vraag naar het introduceren van vasculaire netwerken in nieuw gevormde weefsels om de overleving en functionaliteit te verbeteren. Om die reden is de weefselregeneratie van bloedvaten en capillairen een veel onderzocht aspect dat waardevol is voor verschillende toepassingen.

Dit proefschrift beschrijft de *in vitro* en *in vivo* biologische evaluatie van de mogelijkheid van humane mesenchymale stromale cellen (hMSC) om te differentiëren naar endotheel-achtige cellen (EL-MSC). Het succes van regeneratieve strategieën met behulp van EL-MSC berust op de volgende eisen: het differentiatieprotocol moet simpel en robuust zijn; er moet gekeken worden naar het probleem van donor-gerelateerde verschillen; er moeten klinisch goedgekeurde methoden zijn voor aflevering in het lichaam en tot slot moet de bruikbaarheid van EL-MSC in bepaalde toepassingen bewezen zijn. Omdat hMSC al veel gebruikt worden in verschillende therapieën, is er een grote kans dat EL-MSC aan deze eisen zullen voldoen.

Hoofdstuk 1 vat de inhoud van dit proefschrift samen. In hoofdstuk 2 wordt een algemeen overzicht gegeven van de literatuur, met de nadruk op de opkomende strategieën voor bloedvatregeneratie. Verschillende celtypen, biomaterialen en methoden om functioneel weefsel te regenereren zijn beschreven. Hoofdstuk 3 bevat de evaluatie van hMSC endotheeldifferentiatie. De kracht van het beschreven protocol wordt bekeken in hoofdstuk 4 samen met de bepaling van het hMSC *in vitro* expansieprotocol voor endotheeldifferentiatie. Verschillende moleculaire markers worden gesuggereerd voor het voorspellen of cellen afkomstig van een bepaalde donor geschikt zijn in therapieën waar EL-MSC benodigd zijn. Hoofdstukken 5 en 6 bevatten de beschrijving van twee mogelijke strategieën om EL-MSC in het lichaam af te leveren voor vascularisatie doeleinden. Modulaire weefselregeneratiemethoden gebaseerd op collageenmodules en injecteerbare in-situ-vormende dextraan-gebaseerde hydrogeltoepassingen worden beschreven. Twee strategieën om de biologische functie van EL-MSC te verbeteren worden beschreven: het creëren van driedimensionale structuren die ingroei van vaten makkelijker maken en het toevoegen van groeifactoren voor het stimuleren van

angiogenese van gastheervaten. De mogelijke toepassingen van EL-MSC voor weefselregeneratie doeleinden worden beschreven in hoofdstuk 7 en 8. In hoofdstuk 7 wordt de positieve invloed van EL-MSC op botvorming beschreven en in hoofdstuk 8 wordt beschreven hoe EL-MSC gebruikt kunnen worden voor de verbetering van vascularisatie van eilandjes van Langerhans. Tot slot worden in hoofdstuk 9 de resultaten uit dit proefschrift samengevat en bediscussieerd.

Kort samengevat beschrijft dit proefschrift een multifactoriële aanpak voor de verbetering van vascularisatie van zowel *in vitro* als *in vivo* geregenereerde weefsels en suggereert toekomstontwikkelingen en toepassingen van endotheel-achtige cellen afkomstig van MSC.

Streszczenie

Wiele tkanek ludzkiego organizmu wykazuje całkowitą lub częściową zdolność do regeneracji, jednak niektóre ubytki, spowodowane urazem lub chorobą są zbyt rozległe aby ten proces zaszedł samoistnie. Inżynieria tkankowa daje możliwość naprawy takich ubytków bez konieczności przeszczepu tkanek pobranych od obcego dawcy. Liczne doświadczenia *in vivo* dotyczące wykorzystania inżynierii tkankowej do uzupełniania poważnych ubytków wykazały konieczność unaczynienia nowo powstałych tkanek aby zapewnić ich pełną funkcjonalność. Tym samym badania dotyczące inżynierii tkankowej naczyń krwionośnych stanowią obecnie ważny aspekt przy wprowadzaniu nowych terapii.

Niniejsza praca przedstawia badania *in vitro* i *in vivo* dotyczące biologicznego potencjału ludzkich mezenchymalnych komórek macierzystych (human mesenchyma stromal cells, hMSC) zróżnicowanych w kierunku komórek podobnych do komórek endotelialnych (endothelial-like mesenchymal stromal cells, EL-MSC). Powodzenie terapii opartej na wykorzystaniu tych komórek zależy od kilku czynników: różnicowanie powinno być nieskomplikowane i wydajne, jak również proces ten nie powinien być zależny od źródła pochodzenia komórek (odporny na zmiany związane z różnorodnością hMSC od różnych dawców). Konieczne jest także opracowanie metod wprowadzania EL-MSC do organizmu pacjenta oraz potwierdzenie ich korzystnego wpływu na przebieg terapii. Ponieważ niezróżnicowane MSC są obecnie szeroko stosowane w medycynie regeneracyjnej, istnieje duża szansa, iż powyższe warunki mogą być spełnione także w przypadku EL-MSC.

Pierwszy rozdział prezentowanej pracy streszcza ogólne założenia i wyniki badań przedstawione w kolejnych rozdziałach. Rozdział drugi podsumowuje dotychczasowe osiągnięcia medycyny regeneracyjnej w zakresie inżynierii tkankowej naczyń krwionośnych z naciskiem na praktyczne zastosowanie prowadzonych badań. Omówione zostały zarówno typy komórek i biomateriałów jak również metody konstruowania i wprowadzania nowo zbudowanych żył w kontekście różnych terapii. W rozdziale trzecim opisane jest różnicowanie MSC w kierunku EL-MSC z uwzględnieniem metod oceny wydajności tego procesu. Rozdział czwarty przedstawia porównanie wydajności opisanego różnicowania w przypadku zastosowania komórek pochodzących od różnych dawców. Przedstawione są również badania dotyczące wyodrębnienia markerów molekularnych pozwalających przewidzieć, czy komórki wyizolowane od danego dawcy będą mogły zostać wykorzystane do terapii wymagających konstruowania naczyń

krwionośnych. Rozdziały piąty i szósty zawierają opis dwóch różnych metod wprowadzania EL-MSC do organizmu pacjenta. Rozdział piąty omawia podejście oparte na modularnej inżynierii tkankowej opartej na modułach kolagenowych, podczas gdy w rozdziale szóstym opisane jest zastosowanie opartych na dekstranie żeli formowanych in situ. Metody te zakładają ulepszenie wydajności w tworzeniu naczyń krwionośnych przez EL-MSC dwiema różnymi drogami: poprzez zapewnienie odpowiednich struktur trójwymiarowych oraz poprzez dostarczenie stymulacji czynnikami wzrostowymi. W rozdziałach siódmym i ósmym przedstawione zostały propozycje wykorzystania EL-MSC we współcześnie stosowanych terapiach. W rozdziale siódmym opisany został korzystny wpływ obecności EL-MSC na regenerację kości, rozdział ósmy dotyczy natomiast unaczyniania wysp Langerhansa przy przeszczepach stosowanych w terapii pacjentów z cukrzycą. Na koniec, w rozdziale dziewiątym, omówione zostały wszystkie rezultaty przedstawione w tej pracy w kontekście możliwego wykorzystania ich w medycynie regeneracyjnej.

Podsumowując, niniejsza praca prezentuje wielokierunkowe podejście do doskonalenia metod unaczyniania tkanek i przedstawia możliwości dalszego rozwoju tej dziedziny z wykorzystaniem EL-MSC.

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Chapter 1

General introduction and thesis outline

1.1 Regenerative medicine

Regenerative medicine is a biomedical research area which investigates the possibility of stimulating the body's natural ability to repair damaged tissue and organs or to imitate such repair in order to guarantee functional recovery. This field emerged as an answer to the growing need for healing serious diseases concerning various tissues, due to the increase in average human lifespan. Currently, the main goal of regenerative medicine is to preserve human independence and productivity but it also has the potential to solve the problem of the shortage of organs available from donations compared to the number of patients that require life-saving organ transplantation. Due to such a broad scope, regenerative medicine is based on both basic scientific disciplines such as biology, immunology, chemistry and physics and on more application-oriented disciplines such as tissue engineering and biomaterials research. The current challenge in regenerative medicine is to combine state-of-the-art knowledge into a new therapy paradigm. Since some tissues are unable to heal themselves even after application of various stimulants, it is necessary to design a strategy that will allow to manufacture healthy tissue without relying on the regenerative potential of patient's own tissues.

1.2 Tissue Engineering

Tissue engineering has developed as a multidisciplinary scientific field that implements the principles of biology, medicine and engineering to obtain tissue substitutes for restoration, maintenance and improvement of diseased or damaged tissues [1, 2]. For many years the only possible form of repairing damaged tissues was transplantation of biological material collected from a donor (e.g. blood transfusion or bone grafting). The best results were obtained when autologous grafts were applied [3], even though in such cases there are always issues of donor site morbidity and the need for additional surgery inducing risk of infection at the harvest site [4]. Unfortunately, autologous

sources are limited and not suitable in case of several diseases such as autoimmune diseases or osteoporosis [5–7]. On the other hand, the main problem with allogenic transplantations is the risk of immune rejection and disease transfer. Despite these problems, allografts represent approximately 30% of all bone grafts used in the clinic. Nevertheless, there are tissues where allogenic transplantation cannot be applied, e.g. endothelial cells obtained from allogenic sources were shown to induce a chronic immune reaction in the patients [8] and therefore their application for clinical purposes is not an option. An alternative method to tissue grafting is tissue engineering where three main strategies can be distinguished [9]. The simplest strategy is implantation of freshly isolated or cultured cells in the damaged tissue. This approach includes only very small interference in the isolated material (e.g. stem cells are reported to be able to replace damaged heart muscle cells and establish new blood vessels to supply them with nutrients [10, 11]). The other two methods are more complex. A complete three-dimensional tissue graft can be generated *in vitro* from cells and artificial matrices and implanted afterwards, or the artificial matrix could be implanted directly into the injured tissue to stimulate the body’s own cells to promote local tissue repair (see Figure 1.1).

Regardless of the chosen method, latest developments in the field show that very often there is another issue that needs to be tackled. Providing proper vascularization of newly formed or regenerated tissue has been found to be crucial to maintain tissue homeostasis. Most of the tissues within the human body (except e.g. cartilage) are highly vascularized and rely on the blood vessels to supply nutrients and oxygen to the

Figure 1.1: Basic tissue engineering strategies

individual cells. Since the diffusion limit of oxygen is around 100-200 μm , proper blood vessel density within the tissue is required. The same is true for newly engineered tissues. Therefore, tissue engineering of blood vessels and methods of introducing them into *in vitro* or *in vivo* formed tissue is a widely investigated aspect necessary for various applications.

So far, most studies in tissue engineering relied on blood vessel in-growth from the host after implantation. Several strategies have been designed to improve this process, such as introduction of pro-angiogenic growth factors or cells with high paracrine activity in the construct. Such methods were shown to improve the vascularization of newly formed tissue but were not sufficient to maintain tissue homeostasis in case of larger constructs. Therefore, graft vascularization prior to or upon implantation became a widely investigated topic [12]. Particularly, introduction of cells that could create a vascular network within the implant came to be an issue of interest. These cells will be described in details in chapter two of this thesis, together with our candidate for an ideal cell source for tissue vascularization described further in chapter three.

1.3 Aim and outline of this thesis

Although a remarkable amount of research has been performed on the most efficient ways of promoting tissue vascularization for applications in regenerative medicine, there are still aspects that need to be improved. The general goal of this thesis is to evaluate the potential of human mesenchymal stromal cells (hMSC), differentiated towards endothelial-like cells, in improving angiogenesis and vasculogenesis.

Chapter 2 provides a general overview of the current state-of-the-art in vascular engineering. An overall description of cell types and biomaterials that are used in this field is provided together with the evaluation of emerging strategies.

In **Chapter 3** the ability of hMSCs to differentiate towards endothelial-like cells is evaluated. A protocol for hMSC differentiation is presented which yields cells capable of replacing endothelial cells in various applications. hMSCs are shown to acquire specific endothelial cell properties, both phenotypically and functionally. The potential consequences of this phenomenon are described together with the possible applications of endothelial-like MCSs in various therapies.

Chapter 4 describes the donor variability among hMSCs as far as endothelial differentiation is concerned. The ability to differentiate towards an endothelial phenotype of hMSCs obtained from 20 donors is assessed and the robustness of the applied differentiation protocol is demonstrated. In addition, the effect of prolonged *in vitro* expansion on the multipotency of hMSCs is examined and its limited influence on endothelial differentiation is described. Moreover, the correlation between endothelial differentiation of hMSCs and the gene expression profile of the whole genome in the undifferentiated state of hMSCs is examined. Based on that, several candidates for

molecular markers are provided that can be used to predict the potential of hMSCs to differentiate into endothelial-like cells.

In **Chapter 5**, we demonstrate how modular tissue engineering can be used in the context of MSC delivery for vascularization purposes. Clinically relevant modules created from bovine collagen are shown to be suitable to deliver endothelial-like MSCs in the place where vascularization is needed. We describe the possibility to adjust this method such that it becomes fully injectable and thus minimally invasive.

Chapter 6 demonstrates another way of enhancing angiogenesis and vasculogenesis in tissue engineered constructs. The use of endothelial-like MSCs in combination with injectable dextran-hyaluronic acid hydrogels (Dex-g-HA) in vascularization is investigated. Several combinations of cell types and hydrogel modifications together with reciprocal influences between cells and matrixes are examined for selecting the best one for vascular tissue engineering. This chapter proves that Dex-g-HA is an efficient delivery vehicle for VEGF, and that functional neo-vascularization can be achieved *in vitro* and *in vivo* with the combination of Dex-g-HA and endothelial-like MSCs.

Chapter 7 deals with the possible applications of endothelial-like MSCs in bone tissue engineering. We show that human MSCs can be used a single cell source for both endothelial-like cells and bone forming osteoblasts *in vivo*. Addition of EL-MSCs cells to a previously developed bone-forming system had a positive effect on the amount of obtained bone in all but one case. We hypothesize that this effect is induced by endothelial-like MSCs via their trophic effect. Studies performed *in vitro* reveal that endothelial-like MSCs did not influence the expression of osteogenic and angiogenic markers in the construct. Due to the limited amount of samples we were not able to conclude whether EL-MSCs significantly increase the amount of obtained bone nor what could be the mechanism of their influence. Therefore, further studies are required to explore the potential of these cells in the field of bone tissue engineering.

In **Chapter 8**, endothelial-like MSCs are shown to improve vascularization of islets of Langerhans. Introducing dense vascular network is of crucial importance in islets transplantation for diabetic patients. Only then the islets can survive and the insulin can be efficiently delivered into the blood stream. Previous studies with clinically non-applicable endothelial cells demonstrated that this improvement speeds up vessel in-growth in the islets and improves their survival. This *in vitro* study shows that coating islets of Langerhans by endothelial-like MSCs improves their sprouting ability without hampering their functionality.

Chapter 9 provides a collective overview of obtained results and presents future perspectives of endothelial-like MSC application in tissue engineering.

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Chapter 2

Tissue engineering of blood vessel: from large vessels towards capillaries

Karolina Janeczek Portalska, Hugo Fernandes, Daniel Saris, Clemens van Blitterswijk and Jan de Boer

Abstract

Vascular grafts are required to treat various disorders such as myocardial infarction and ischemic diseases. In addition, introduction of a vascular network is necessary for many tissue engineering strategies. Due to the limited number of autologous vessels that can be surgically harvested, an alternative source of vascular graft material needs to be developed. Cell-free synthetic grafts have been developed, combining desired mechanical properties of the implanted materials with the capability of long-term survival within the body without development of thrombosis, calcification or immune-rejection. The addition of cells can accelerate the formation of vascular grafts and provide enhanced biological properties when compared to cell-free grafts. In this manuscript, we describe the progress in tissue engineering of blood vessels and discuss the source of cells, biomaterials and combination thereof. We also describe the specific challenges in fabricating small vessels and capillaries. This is crucial when treating peripheral ischemic diseases and implanting tissue constructs where a new vascular network must be developed within the graft shortly after implantation. Special emphasis is given to the challenge of bringing these strategies to the clinic.

2.1 Blood vessels and vascularization

Blood vessels transport blood and thus nutrients and waste products throughout the body. There are three types of vessels: the macrovessels (veins and arteries), which then branch into microvessels (venules and arterioles) which subsequently branch into

capillaries. The capillaries provide the cells in the body with nutrients and remove the waste products produced by the cellular metabolism.

Figure 2.1: Schematic representation of a vessel.

Macro vessels and micro vessels are made of three layers (Figure 2.1): an inner lining composed of endothelial cells followed by a middle layer of a basal lamina and pericytes (a specialized smooth muscle cell) and then a layer of smooth muscle cells (SMC) and fibroblasts. Endothelial cells (EC) provide a selective permeable surface that prevents blood components from attaching and forming thrombus [1]. This layer is also responsible for controlling vessel tone and leukocyte adhesion. Pericytes participate in vessel stabilization and maturation [2, 3], blood flow regulation [3] and are thought to be a candidate cell source for tissue regeneration [4, 5]. SMCs and fibroblasts provide integrity to the vessels and are mostly responsible for their characteristic mechanical properties [6]. Capillaries, due to their function, do not require the outermost layer of SMCs and fibroblasts.

2.2 Blood vessel failure

The proper functioning of the circulatory system is crucial to maintain homeostasis of the body. All tissues need to receive sufficient levels of oxygen and nutrients, and efficient removal of waste products is paramount to maintain local homeostasis. Blood vessel pathologies such as myocardial infarction and ischemic diseases have

lately become a leading cause of death. For example, cardiovascular diseases alone are estimated to be the cause of death of over 23 million people by 2030 [7]. Several reasons for blood vessel failure are known. Arteriosclerosis, which is responsible for the buildup of plaques in the inner layer of the blood vessels results in a reduction of the lumen which, if not treated, can lead to vessel rupture, clot formation or even total obstruction of the vessel [8]. These effects pose a major risk for heart attacks and amputation of limbs due to the concomitant lack of oxygen followed by cell death. Puncturing can also result in vessel pathologies, and are common in patients that require regular dialysis due to kidney diseases. Regular puncturing of the vessel leads to vessel hardening, healing problems and, concomitantly, vessel necrosis [9].

Surgically harvested autologous grafts have been widely applied in such situations but frequently, limitations and complications are encountered [10]. Although autologous material remains the preferable source for grafted vessels, this source is often seriously limited or not available, for instance when a patient only has a low number of good quality vessels which can be isolated without collateral damage [11].

Last but not least, necrosis of capillaries is commonly observed in diseases such as peripheral arterial disease and diabetes, hampering oxygen delivery to the limbs and hence reducing the healing capacity of the tissues. As a consequence, gangrene and tissue necrosis are prevalent in these patients and, if left untreated, limb amputation is the only option available. Even more importantly, transplantation is not a method of choice in illnesses connected with capillary degeneration, because it is impossible to reproduce the complexity of the capillary system [12]. Proper vascularization is also of crucial importance in tissue engineering of large tissue constructs. Without a strategy to speed up the process of vascularization of the graft, ischemia will occur in implanted tissues leading to graft failure. It is widely recognized that generation of a functional vasculature is crucial to allow clinical application of many *in vitro* engineered tissues. So far, only a few engineered tissues and organs (e.g. bladder, skin and cartilage [13–15]) achieved clinical success.

2.3 Large vessel engineering

The classical tissue engineering approach has been used to generate large vessels, in which various types of cells have been used in combination with a suitable scaffold. After implantation at the injury site, the scaffold is meant to degrade and be replaced by extracellular matrix (ECM) produced by cells and the cells should organize into native tissue. Below, we will review cell sources, scaffold types and techniques to combine them.

2.3.1 Cell sources

The ideal cell source for vessel engineering should be non-immunogenic, easy to isolate and expand in culture and capable of differentiating into the different cell types comprising a vessel. Similar to other fields of tissue engineering, many studies in blood

vessel tissue engineering have focused on autologous cells. For instance, tissue engineered blood vessels have been engineered within roughly 10 weeks using ECs and SMCs isolated from healthy patients [16]. However, because most candidates for vessel transplantation entail patients with underlying cardiac conditions, one can anticipate problems with the proposed approach. Alternatives are therefore needed to shorten the time required to obtain a functional vessel and improve its clinical performance. Moreover, ECs and SMCs have poor proliferative capacity, [17, 18]. Allogenic cells have been proposed as an alternative, after careful consideration of the possible immune response elicited by these cells. Whereas the use of allogenic SMCs may be possible, the same cannot be said for allogenic ECs, since a chronic immune reaction was observed after their implantation [19, 20].

Endothelial and smooth muscle progenitor cells (EPC and SMPC) have been investigated as cell source for blood vessel TE as well, because they have the ability to differentiate towards their respective lineages [21–24]. EPCs can readily be isolated from peripheral blood or umbilical cord blood based on their CD34+KDR+AC133+ phenotype [25, 26]. Convincing evidence showing improvements in vessel functionality upon EPC implantation was observed [27–29], but recent results in animal studies raised questions about their mechanism of action [30]. The increase in neovascularization observed in the presence of EPCs may be based on their secretion of pro-angiogenic factors rather than a direct contribution to the formation of new vessels [31]. On the other hand, evidence exists showing that EPCs can contribute to vessel walls [32]. Unfortunately, the number of circulating EPCs as well as their functionality is severely affected in several cardiac-related diseases despite the fact that a small pool of EPCs remains functional in these patients [33]. Prospective isolation of this fraction remains an appealing alternative for TE applications. Similarly to ECs, SMCs can also be obtained from the recently described smooth muscle progenitor cell population (SMPC) present in peripheral blood, bone marrow and the vascular wall [34]. Further studies are needed to unequivocally show that the SMPC population is a suitable source of unlimited and functionally active SMCs.

Another autologous source of progenitor cells are mesenchymal stromal or stem cells (MSCs). There are several sources from which MSCs can be obtained and initially it was thought that these cells can only differentiate towards the phenotypes present in originating tissues [35]. With increased research it became clear that at least for certain MSC populations this is not the case. For instance, the ability of MSCs to differentiate following a vascular smooth muscle differentiation pathway was already described in 1993 by Galmiche *et al.* [36]. Recent *in vivo* studies presented by Au *et al.* and Melero-Martin *et al.* revealed that MSC-derived cells can replace perivascular cells [37, 38] and stabilize neo-vessels upon implantation. On the other hand, several studies revealed the possibility of differentiating MSCs towards an endothelial phenotype [39–44]. Oswald *et al.*, Cao *et al.*, Fisher *et al.* and Rouwkema *et al.* characterized MSC-derived ECs and demonstrated that *in vitro* such cells express typical endothelial markers: KDR and von Willebrand Factor, as well as formed characteristic capillary-like structures.

The need to obtain a sufficient number of cells is paramount for any clinical application of tissue engineered constructs. Instead of isolating different progenitors such as EPCs and SMPCs one can use a pool of pluripotent stem cells, grow them until reaching sufficient numbers are reached and subsequently differentiate those cells into the desired cell types. For instance, Levenberg *et al.* showed that upon formation of embryonic bodies, embryonic stem cells (ESC) spontaneously differentiated into ECs. PECAM⁺ cells comprise 2% of the total number of cultured ESCs within embryonic bodies and the functionality of the obtained fraction resembles ECs [45]. Several protocols for endothelial differentiation of ESCs were described and optimized for efficiency [46] but still, the risk of tumor formation hinders the deployment of this technology into the clinic. Since one single undifferentiated cell in the population is sufficient to induce the formation of tumors, protocols to obtain purified populations are in much need [47, 48].

Induced pluripotent stem cells (iPSC) are another interesting cell source, because they can be produced from the patients' own somatic cells, which solves both the ethical issues surrounding the use of ESCs and the immune rejection associated with them. They provide an unprecedented opportunity for developing novel approaches for regenerative therapy based on immuno-compatible cells and their pluripotency, including differentiation towards EC, has been demonstrated [49]. Recent work provides the field with yet another alternative: instead of reprogramming the cells to an embryonic state one can reprogram the cells directly to a new lineage via so-called partial-iPSCs (PiPS) [50, 51]. The advantage of this approach is that PiPS cells do not form tumors when implanted *in vivo* and still retain the capacity to differentiate into ECs upon exposure to defined media. The PiPS-derived ECs were fully functional and improved neovascularization in a hindlimb ischemic model [52]. Recent work by Ginsberg et al showed that using EC-specific transcription factors one can also reprogram amniotic cells directly to ECs without transiting through a pluripotent state [53]. These latest developments surely open the possibility to use the obtained cells in clinical applications where the function of ECs is impaired. It seems a matter of time until similar approaches are deployed to generate the other cell types needed to assemble a fully mature vessel. Once that is feasible, the next step would be to implement the technology *in situ*.

2.3.2 Scaffolds

The second key factor to engineer a vessel is a scaffold that will provide mechanical support and a suitable template for cell growth, migration and differentiation [54–56]. General requirements for scaffolds in vessel engineering are similar to the ones in other TE fields, i.e. being biocompatible and biodegradable. Additionally, in vessel engineering, due to the direct contact with blood, they should not be thrombogenic and should exhibit elasticity in order to allow contraction. Several materials, including decellularized matrixes, natural proteins and synthetic polymeric scaffolds have been investigated for vessel engineering.

Decellularization is a process in which all cellular content is removed from a tis-

sue while preserving the extra-cellular tissue components (mostly ECM) and most importantly, preserving the original tissue architecture [57]. Decellularization is typically accomplished by treating tissues with a combination of detergents, enzyme inhibitors and buffers [58]. Decellularized vessels are fully biocompatible since they are engineered based on natural tissues. Such matrixes can fulfill all the requirements concerning mechanical properties and chemical composition. Several trials were done with animal-derived (mostly porcine) vessels to assess the biological and mechanical properties [59, 60]. However, for some people using porcine vessels as a base is not a choice e.g. due to their religious beliefs. Using vessels of human origin would be therefore the preferred solution but their limited availability hampers their extensive use [61].

Another strategy to fabricate scaffolds for vessel engineering is to use proteins isolated from ECM such as collagens, elastin or fibronectin [62, 63]. Scaffolds based on such proteins will be, as in the case of decellularized vessels, fully biocompatible and biodegradable. There are several approaches to create 3D structures from natural proteins. One currently investigated method to reproduce the physical and mechanical properties of blood vessels with natural-protein scaffolds is known as electrospinning [64]. A novel hybrid approach combining elastin scaffolds isolated and purified from porcine carotids and then combined with type I collagen provided increased strength compared with collagen-only constructs [65]. Lovett *et al.* [66] showed that microtubes obtained from silk fibroin provided not only a scaffold suitable for EC culture but can be also downscaled towards microvascular scale (<6 mm inner diameter), which is difficult to achieve with synthetic grafts.

Due to the cost and difficulties with natural protein isolation and purification there is a need for synthetic replacements. Synthetic polymers can also be more precisely modified to design desired biological and mechanical properties. Several synthetic polymers have been tested for their suitability in blood vessel engineering. Among them, poly(L-lactid-co- ϵ -caprolactone) displayed very favorable properties with SMC interactions resembling the interactions in normal vessels [67]. This polymer was also tested in combination with collagen. He *et al.* [68] showed that this combination mimics the natural ECM both morphologically and chemically. Collagen-blended polymer nanofibers enhanced viability, spreading and attachment of endothelial cells and, moreover, preserved the EC phenotype in culture. Additionally, Ma *et al.* [69] used non-woven polyethylene terephthalate nanofiber mats that were surface-modified to mimic the fibrous proteins in native ECM, and were grafted with gelatin. As in the previous case, such surfaces were suitable for EC culture allowing both spreading and proliferation while preserving their phenotype.

A completely different approach towards vessel engineering was presented by L'Heureux *et al.* in 1998 [70]. In this case, the authors produced tissue-engineered blood vessels exclusively using cultured human cells. Using cell sheets of SMCs and fibroblasts they managed to construct tubular structures that, after seeding with ECs, exhibited a well-defined, three-layered organization. Such structures contained also numerous extracellular matrix proteins including elastin and supported both SMC

and EC phenotype. More importantly, the engineered vessel had a burst strength comparable to that of human vessels and displayed functional endothelium after implantation in canine models. Further study of this method proved that the cell-based approach allowed for fabrication of vessels that are biologically and clinically relevant and can be assembled exclusively from the patient's own cells [16].

2.4 From large vessel engineering towards capillaries

Capillaries are the anatomical structures where water, oxygen, carbon dioxide and other nutrients are exchanged between blood and adjacent tissues. Therefore, their damage or degeneration seriously affects tissue homeostasis. Moreover, capillary engineering is of crucial importance while implanting tissue grafts, where new a vascular network must be developed within the implant [71]. Cell/biomaterial constructs described above for blood vessel engineering cannot easily be scaled down to build capillaries. The main reason for that is the size of these structures (5-10 μm), the way they are built (single cell layer) and their unique properties concerning permeability. Furthermore, to work properly, capillary networks have to provide a level of spatial complexity adequate for the needs of the tissue in question. Blood vessels must be within 150-200 μm from metabolically active cells in order to provide sufficient oxygen and nutrient level [72, 73]. A major challenge in tissue engineering is to achieve microcirculation rapidly after implantation in order to prevent cell starvation and death. There are two main physiological approaches to induce vascular networks within tissues: vasculogenesis and angiogenesis.

2.4.1 Vasculogenesis and angiogenesis

Blood vessels can arise through the aggregation of angioblasts into a vascular plexus. This process is called vasculogenesis and is dominant during embryogenesis. Recent studies demonstrate that vasculogenesis can also occur in adults, when endothelial progenitor and bone marrow-derived cells are recruited [74]. Alternatively, angiogenesis is the formation of new vessels by sprouting of existing capillaries and is dominant post-natally [75]. Being able to control and direct both processes opens the possibility to create capillary networks both within tissues as well as in *in vitro* engineered constructs.

Initially, most studies on angiogenesis were aimed to stop this process, e.g. to control tumor growth [76]. However, information about growth factors and cytokines capable of inducing physiological blood vessel formation has been used to achieve the reverse goal. The possibility of inducing vasculogenesis or angiogenesis within tissues was first tested with the simple injection of pro-angiogenic growth factors such as basic fibroblast growth factor (FGF-2) and vascular endothelial growth factor (VEGF) [77, 78]. It was quickly recognized that this method is expensive and not very efficient due to the high concentration of growth factors required. Following on this concept however, materials were chemically and mechanically tailored to transport and release bioactive molecules. In particular, hydrogels and synthetic polymer-based

scaffolds, within which growth factors can be entrapped, are widely investigated for this purpose [79–81]. Richardson *et al.* demonstrated that dual delivery of VEGF and platelet-derived growth factor (PDGF), each with distinct kinetics, resulted in the rapid formation of a mature vascular network [82].

Figure 2.2: Cell therapy for neovascularization. De-novo vessel formation via self-organization into vessels or via induction of angiogenesis.

Another approach to controlled drug delivery into ischemic tissues is based on the concept of “living factories”. The transplanted cells can either create new vessels *de novo* or attract vessel in-growth sending pro-angiogenic signals (Figure 2.2). Several cell types are able to produce growth factors and cytokines that improve angiogenesis in the place of delivery. Cells such as EPCs [83–85] or MSCs [86] were injected directly in the place of damage or systemically in a large vessel. The assumption was that the cells would reach the destination with blood flow. Those treatments showed limited improvement of the vessel network but the results were used as proof of principle and fueled further research in this area.

One possibility that offers good perspectives in improving abovementioned methods and making them clinically relevant is to precondition MSCs such that they secrete more pro-angiogenic factors. For instance, MSCs under hypoxic conditions secrete more VEGF [87, 88]. Moreover, it was shown recently that the same subset of hypoxia-induced genes can be activated in hMSCs by exposing them to small molecules treatment which mimic the hypoxia pathway [89, 90]. The added benefit

of the small molecule approach is that controlled release strategies can be devised to enhance angiogenesis *in vivo*. For instance, we have recently shown that treatment of MSCs with the small molecule phenanthroline results not only in enhanced VEGF secretion but also enhanced vessel formation *in vivo* [91].

MSCs and other stem and progenitor cells can also directly contribute to creating a capillary network through vasculogenesis. Levenberg *et al.* described the induction of capillary networks in engineered tissue constructs using a three-dimensional system consisting of myoblasts, embryonic fibroblasts and endothelial cells co-seeded on porous polymer scaffolds [92]. They showed that induction and stabilization of the vessels *in vitro* improved the survival and vascularization of the engineered implants *in vivo*. Liu *et al.* showed that implantation of MSC-derived ECs results in vascularized collagen plugs and that MSC-derived ECs were incorporated in the vessel walls [93]. Based on these studies we investigated whether endothelial-like MSCs could replace embryonic fibroblasts and endothelial cells while maintaining their functionality. We observed that endothelial-like MSCs express endothelial markers and are incorporated in neo-vessel walls, while significantly increasing the capillary network in implanted constructs (Figure 2.3) [43].

Figure 2.3: Endothelial-like MSCs in tissue engineering. Endothelial-like MSCs form capillary like structures on Matrigel (A) and express CD31 marker when implanted *in vivo* (B). For Matrigel assay cells were seeded and cultured for 24 hours before picture acquisition [43]. *In vivo* data were obtained 6 weeks after implantation, cells were seeded within dextran-based hydrogels [94]. Immunohistochemistry was used to stain for human CD31. CD31 positive cells are brown, CD31 positive lumen is indicated by black arrow.

To prevent cell migration from the place of delivery and provide efficient therapeutic influence, the strategy of delivering cells is of crucial importance. Modular tissue engineering [95, 96] allows us to combine hydrogels with growth factors and cells in order to create ready-to-use channels for in-growing capillaries upon implantation. This approach allows us to modulate the cell environment and by doing so

boosts tissue vascularization. We have shown recently that modular tissue engineering is suitable for applications including endothelial-like MSCs allowing us to combine a novel three-dimensional approach with clinically relevant cell types [97].

However, despite all the proof of principle described above, the ability to create a stable vascular network that can offer functionality to an ischemic organ or tissue implant remains a major challenge. The focus of research in this area should move to validate that the engineered strategies are functionally beneficial *in vivo*. As in the case of large vessel engineering, also for capillaries engineering, long-term studies are necessary to prove their safety and stability. The research and clinical trials described herein contribute to the broad set of clinical applications in the field of vascular tissue engineering. In addition, the cost of therapies that are currently based on complicated protocols including extensive scaffold material processing combined with the need for various cell types required to build functional vessels is a crucial issue that remains to be addressed. Thus, there are two main challenges for vascular researchers: first, to provide techniques that will allow for fast and efficient tissue engineering of vessels and capillaries and second, to establish the bridge between science and the market, allowing for introduction of vascular engineering into clinic.

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Chapter 3

Endothelial differentiation of mesenchymal stromal cells

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Abstract

Human mesenchymal stromal cells (hMSCs) are increasingly used in regenerative medicine for restoring worn-out or damaged tissue. Newly engineered tissues need to be properly vascularized and current candidates for *in vitro* tissue pre-vascularization are endothelial cells and endothelial progenitor cells. However, their use in therapy is hampered by their limited expansion capacity and lack of autologous sources. Our approach to engineering large grafts is to use hMSCs both as a source of cells for regeneration of targeted tissue and at the same time as the source of endothelial cells. Here we investigate how different stimuli influence endothelial differentiation of hMSCs. Although growth supplements together with shear force were not sufficient to differentiate hMSCs with respect to expression of endothelial markers such as CD31 and KDR, these conditions did prime the cells to differentiate into cells with an endothelial gene expression profile and morphology when seeded on Matrigel. In addition, we show that endothelial-like hMSCs are able to create a capillary network in 3D culture both *in vitro* and *in vivo* conditions. The expansion phase in the presence of growth supplements was crucial for the stability of the capillaries formed *in vitro*. To conclude, we established a robust protocol for endothelial differentiation of hMSCs, including an immortalized MSC line (iMSCs) which allows for reproducible *in vitro* analysis in further studies.

3.1 Introduction

Human mesenchymal stromal cells (hMSCs), also referred to as colony forming unit-fibroblasts (CFU-F), mesenchymal stem cells, or mesenchymal progenitor cells were

first identified as a subpopulation of bone marrow cells by Friedenstein [1]. Their abundance among other bone marrow cells was estimated to be 0.01% to 0.001% [1–3]. Later research showed that MSCs can be isolated from other types of tissues as well, including adipose tissue, placenta, periosteum, trabecular bone and femur [4–7]. MSCs can be characterized based on their fibroblast-like morphology and ability to differentiate into various cell type [8]. To induce adipogenic differentiation, stimulation with insulin, dexamethasone and IBMX is typically applied [9]. MSCs cultured in dexamethasone differentiate towards osteoblasts and can participate in new bone formation after implantation in critical size bone defects [10–12]. TGF- β stimulation, especially when combined with BMP-2 treatment, can trigger chondrogenic differentiation of MSCs [13]. While MSC differentiation towards adipo-, osteo- and chondrogenic lineages is widely investigated, some studies have also shown that MSCs can differentiate towards muscular and neural phenotype, but those are less documented and the differentiation protocols are not yet widely applied. For instance, myoblasts can be obtained from MSCs after applying basic fibroblast growth factor (bFGF) and forskolin [14–16]. On the other hand, platelet-derived growth factor (PDGF) together with forskolin and glial growth factor (GGF-2) stimulation results in differentiation of MSCs into cells with a Schwann cell-like phenotype [17]. Continuous trials to obtain neural cells have resulted in several studies demonstrating the possibility of obtaining MSC-derived cells that can support the regeneration of nerves and participate among others in therapy of erectile dysfunction, multiple sclerosis and spinal cord injury [18, 19]. MSCs are also capable of suppressing allo-responses and appear to be non-immunogenic [20]. Therefore, hMSCs are increasingly used in regenerative medicine as a source of cells for restoring worn-out or damaged tissues such as cartilage, cardiac muscle or bone. In the field of bone tissue engineering there are a total of 9 human clinical trials performed to date [21]. Other clinical trials with MSCs are performed to improve cardiac functions after myocardial infarction [22, 23] and to restore liver and kidney function after failure [24, 25].

The standard approach in regenerative medicine when MSCs are used is to identify the cell type necessary for the therapy and then differentiate MSCs towards this phenotype. Differentiated cells are then used in animal models and when the therapy is successful, clinical trials are performed. There is, however, a clear need for endothelial cells (EC) in this approach. ECs are needed to line artificial vessels and to restore vascularization of ischemic tissues. This is a crucial point in therapy of peripheral vascular diseases [26] which is a growing medical problem in Western societies and manifests itself in obstruction of large arteries. This leads to retraction of small arteries and capillaries followed by acute or chronic ischemia in surrounding tissues. Only the recovery of the vascular network in such tissues can restore blood flow and prevent limb amputation [27]. Another difficulty that may be solved by providing a good source of endothelial cells is the maintenance of cell survival after graft implantation. The key parameter in this problem is the supply of nutrients and oxygen in which diffusion is a limiting factor. Metabolically active cells must be situated within 150-200 μm distance from blood vessels in order to maintain proper functions [28, 29]. To maintain cell survival in large grafts, a vascular structure needs

to be introduced within the graft which can rapidly connect to the host's blood vessels upon implantation. Without that, cell death and lack of new tissue formation in the interior of the implant will occur [30]. The proof of principle for the application of ECs in the abovementioned conditions has been achieved using endothelial progenitor cells (EPCs) [31] and endothelial cells. EPCs are immature cells capable of differentiating into mature endothelial cells *in vitro* and *in vivo* [32]. EPCs are cells with some self-renewing capacity present both in the bone marrow and in circulation [33]. Recently, the vasculogenic potential of different EPCs was investigated and compared [34, 35]. EPCs adhere to gelatin and fibronectin, take up acetylated LDL, bind lectins from *Ulex Europaeus*, and express marker proteins of the EC lineage (e.g. CD31, KDR, vWF). However, they either lose their potential after prolonged expansion or their expansion capacity is not enough to provide sufficient numbers of cells for therapeutic applications [36, 37]. Mature ECs isolated from umbilical vein or aorta are considered as another cell source for graft vascularization [38]. These cells can be expanded *in vitro* and perform well in creating vascular networks *in vivo* [39, 40]. Autologous isolation is however only possible by sacrificing a current vessel of the patient. Since bone marrow derived MSCs were shown to differentiate into adipogenic, osteogenic and chondrogenic lineages [41] they can also be considered as a promising source for obtaining endothelial cells that are able to create vascular networks. There are several factors reported to influence endothelial differentiation and maintain endothelial potential *in vitro* while using embryonic stem cells or EPCs. Specifically, the effects of endothelial growth supplements [42–44], shear forces [45–49] and composition of extracellular matrix [44, 50] are important factors in EC culture. These factors influence the efficiency of capillary-like structure formation on Matrigel, endothelial markers expression (CD31, vWF, KDR), the ability to take up acetylated LDL and their *in vivo* performance. In the last 10 years, much effort was put into establishing protocols for endothelial differentiation of mesenchymal stromal cells. In particular, cells isolated from adipose tissue were reported to respond positively for endothelial differentiation [51, 52]. In the case of MSCs isolated from bone marrow (BM-MSCs) several studies were conducted with various outcomes. The work of Oswald *et al.* shows that BM-MSCs can acquire *in vitro* phenotypic and functional features of ECs [53]. Silva *et al.* demonstrated that MSCs injected into ischemic myocardium can differentiate into smooth muscle cells and endothelial cells *in vivo*, resulting in increased vascularity and improved cardiac functions [54]. Differentiation of MSCs into endothelial cells *in vitro* did not improve their performance *in vivo* [55], which could be due to a sub-optimal differentiation protocol, similar to previous attempts to improve ectopic bone formation by hMSCs. Only after precise adjustment of several parameters a protocol was obtained that resulted in an improvement *in vivo* [56]. In this manuscript we demonstrate a robust and efficient endothelial differentiation protocol for MSCs, which describes the isolation, expansion and differentiation of BM-MSCs and their potential in tissue engineering as endothelial-like cells.

3.2 Materials and methods

3.2.1 Isolation and culture

Human mesenchymal stromal cells (hMSCs) were isolated from human bone marrow from donors with written informed consent [57]. Aspirates were resuspended using a 20G needle and plated at a density of 0.5 million mono-nucleated cells per cm^2 . Cells were grown in MSC proliferation medium which contains minimal essential medium (alfa-MEM, GIBCO) supplemented with 10% fetal bovine serum (FBS, Lonza), 100 U/ml penicillin (GIBCO), 10 $\mu\text{g}/\text{ml}$ streptomycin (GIBCO), 2 mM L-glutamin (GIBCO), 0.2 mM L-ascorbic acid 2-phosphate magnesium salt (ASAp, Sigma-Aldrich) and 1ng/ml bFGF (Fisher Scientific) at 37°C in a humid atmosphere with 5% CO_2 . Cells were expanded up to passage 2. For further experiments hMSCs from two different donors and one immortalized clone (iMSCs, courtesy of Ola Myklebost, University of Oslo, Norway) were cultured in basic medium (alfa-MEM supplemented with 10% FBS, 100 U/ml penicillin, 10 $\mu\text{g}/\text{ml}$ streptomycin, 2 mM L-glutamin and 0.2 mM ASAp). Human umbilical vein endothelial cells (HUVEC, Lonza) were cultured in endothelial growth medium (EGM-2, Lonza). Mouse skeletal myoblast cells (C2C12) and mouse embryonic fibroblasts (MEF) (Cell Essentials) were cultured in Dulbecco's Modified Eagle's Medium (DMEM, GIBCO) supplemented with 10% FBS, 100 U/ml penicillin and 10 $\mu\text{g}/\text{ml}$ streptomycin.

3.2.2 Endothelial induction of MSCs

hMSCs from passage 2 and iMSCs from passage 25 were used for the endothelial induction protocol. Cells were seeded at a density of 3,000 cells/ cm^2 on tissue culture plastic in EGM-2 and cultured for 10 days. After one day in static culture shear force was applied using an XYZ shaker (3D shaker). Cultures were rotated at a rate of 20 rpm. Cells that were cultured according to this protocol will be referred to as pre-differentiated MSCs.

Phalloidin and DAPI stainings were used for cell size and shape analysis. Pictures were taken with a BD PathwayTM Bioimager and analyzed using Attovision software. A minimal number of 300 cells per condition were analyzed.

For induction on Matrigel, wells of 6-well plates were covered with 1 ml of growth factor reduced Matrigel (BD Bioscience) diluted 1:1 in EGM-2 without growth factors. Cells were seeded at a density of 30,000 cells/ cm^2 and cultured in a humid atmosphere with 5% CO_2 for 24 hours. The formation of capillary-like structures (CLS) was observed over time using an inverted microscope (Nikon Eclipse TE300). Pictures were taken at different time points using a Nikon DS-L2 camera.

3.2.3 Wound healing assay

iMSCs were cultured for 10 days in EGM-2 on an XYZ shaker and were then trypsinized and seeded in 6-well plates at a density of 15,000 cells/ cm^2 . iMSCs expanded in basic

medium were used as a negative control. After 24h of culture, cells reached 90% confluency. The cell monolayers were gently scratched using a pipette tip across the entire diameter of the well. Cells were washed twice with PBS to remove cellular debris and then cultured in basic medium to avoid differential growth factor stimulation during the assay. The size of the wounds directly after making the scratch and following wound closure was observed over time using an inverted microscope (Nikon Eclipse TE300). Pictures were taken at each time-point using a NikonDS-L2 camera. The wound size was determined using TScratch software (CSElab) as percentage of the picture area that was not occupied by cells.

3.2.4 TubeCount

Custom image recognition and analysis software has been implemented in C++ programming language with the aid of dlib library (<http://dlib.net/>) used for image processing and graphical user interface, Anti-Grain Geometry library (<http://www.antigrain.com/>) as vector graphics engine and FFTW library (<http://www.fftw.org/>) for calculating Fourier transforms. The purpose of the software is to quantify tube formation efficiency which is performed in two stages. The first stage is a fully automated segmentation routine which partitions the image into background region (insignificant to further analysis) and foreground region (containing cells). Segmentation is based on the assumed characteristics of cell images obtained with phase contrast microscopy (Figure 3.1A) and primarily relies on the existence of well-defined cell edges and on low pixel intensity (dark shades) within the cells as compared to image background. To extract regions which fulfill these criteria the image is first enhanced using generic image quality improvement methods such as homomorphic filtering, median-based noise removal and contrast stretching (Figure 3.1B). Such enhanced images are then treated with a localized intensity thresholding algorithm extracting pixels which have lower-than-average intensity with the average intensity calculated over a window of a specified size (Figure 3.1C). The algorithm is repeated for multiple window sizes and the final result is an intersection of the results obtained in all iterations. Thus, the pixels which show below-average intensity regardless of averaging window size are classified as potential foreground pixels. On the other hand, the enhanced cell image is also processed using a Sobel edge detector in order to find cell boundaries (Figure 3.1D). The detected edges are processed using a fill / connect algorithm which connects neighboring edges resulting in a uniform binary bitmap containing pixels corresponding to objects with distinct edges (Figure 3.1E). An intersection of this binary bitmap with the one obtained with intensity thresholding is assumed to be a correct foreground mask (Figure 3.1F).

The second stage consists of manual tube identification where the software user depicts beginning and ending points of individual tubes on the processed image. As result of this process a tube topology graph is formed which, in combination with the information obtained in the segmentation stage, enables to gather valuable statistics such as total and average tube length, average tube width, number of tube branching points and total tube area (Figure 3.1G).

Figure 3.1: Image segmentation steps. Original image (A), quality enhancement (B), intensity thresholding (C), edge detection (D), edge connection / filling (E), final segmentation result (F). Screenshot of the tube topology analysis (G).

3.2.5 EL-MSC characterization

RNA isolation and quantitative PCR

Total RNA was isolated using TRIZOL reagent according to the manufacturer's protocol. Briefly, 1 ml of Trizol reagent was added per T25 flask (cells cultured in basic medium) or per well (cells cultured on Matrigel in 6-well plates). Samples were incubated for 5 min at room temperature to allow complete dissociation. Phase separation was performed by adding chloroform, and then samples were shaken vigorously for 15 seconds and incubated for 3 min at room temperature. After that samples were centrifuged at 12,000 x g for 15 min. RNA was precipitated by mixing the aqueous phase with isopropyl alcohol followed by 10 min incubation at room temperature. Samples were centrifuged again and the remaining RNA pellet was washed with 75% ethanol. The obtained samples were dissolved in water. The quantity and quality of RNA was analyzed using spectrophotometry (ND-1000 spectrophotometer).

For first strand cDNA synthesis, 500 ng of RNA was used in combination with Superscript II (Invitrogen) according to the manufacturer's protocol. One μ l of 3x diluted cDNA was used for further gene amplification. PCR was performed in a Light Cycler real time PCR machine (BioRad). Data was analyzed using Bio-Rad iQ5 software. Expression of endothelial genes was calculated relative to GAPDH levels by the comparative Δ CT method. Primers used in the study are listed in Table 3.1.

CD31 (Platelet Endothelial Cell Adhesion Molecule-1)	F 5' TCTATGACCTCGCCCTCCACAAA 3' R 5' GAACGGTGTCTTCAGGTTGGTATTTCA 3'
KDR (VEGF receptor 2)	F 5' ACTTTGGAAGACAGAACCAAATTATCTC 3' R 5' TGGGCACCATTCCACCA 3'
vWF (von Willebrand factor)	F 5' TGCTGACACCAGAAAAGTGC 3' R 5' AGTCCCCAATGGACTCACAG 3'
GAPDH	F 5' CGCTCTCTGCTCCTCCTGTT 3' R 5' CCATGGTGTCTGAGCGATGT 3'

Table 3.1: Primers used for qPCR.

Immunostaining

Cells for immunostaining were fixed with 70% ethanol and permeabilized with 0.01% Triton-X. To block non-specific background staining, cells were incubated with 5% BSA (Sigma- Aldrich) in PBS for 30 min. Cells were then incubated with mouse anti-human CD31 antibody (Dako) or with rabbit anti-human VEGFR2 (Cell Signaling) for 2 hours. Cells were washed in PBS with 1% BSA and subsequently incubated with the secondary antibody (AlexaFluor 488 conjugated goat anti mouse or AlexaFluor 594 conjugated goat anti rabbit, Invitrogen) for 1 hour. Cells were washed and counterstained with DAPI and photographed with a BD PathwayTM Bioimager.

Acetylated low-density lipoprotein (ac-LDL) uptake assay

iMSCs were cultured in EGM-2 on an XYZ shaker for 10 days, then transferred to Matrigel and cultured for another 24 hours. Cells were then recovered from Matrigel by 30 min incubation in a 1:1 mixture of 0.25% trypsin and dispase (BD Bioscience). Recovered cells were seeded in 96-well plates and cultured in EGM-2 for one more day. Subsequently, cells were incubated for 4 hours in EGM-2 supplemented with 10 $\mu\text{g}/\text{ml}$ Dil-labeled ac-LDL (Invitrogen) at 37°C in a humid atmosphere. Finally, cells were washed with PBS 3 times.

3.2.6 Biodegradable scaffold preparation

Porous scaffolds composed of a 50/50 blend of poly-(l-lactic acid) (PLLA) and polylactic-glycolic acid (PLGA) were fabricated by a salt-leaching process. PLLA (Polysciences) and PLGA (Sigma-Aldrich) were dissolved 1:1 in chloroform (Fisher Scientific) with a final concentration of 5% (wt/vol). Two ml of this solution were poured into Teflon containers (Savillex) with a diameter of 50 mm and homogenized with 3.4 g of sodium chloride particles with an average grain size of 425 μm . The solvent was evaporated overnight under nitrogen flow. To leach the salt, the obtained polymer films were immersed in distilled water for 6 hours (changed every hour). The leached films with a thickness of 500 μm and a pore size of $200 \pm 60 \mu\text{m}$ were lyophilized overnight and subsequently cut in circular disks with a diameter of 5 mm. Before culture the scaffolds were sterilized in 70% ethanol for 2 days, washed three times with PBS and incubated in culture medium overnight.

3.2.7 PLLA / PLGL construct preparation and implantation

Constructs for implantation were prepared as described before [58]. Briefly, 500,000 cells were pooled and resuspended in 20 μl of a 1:1 mixture of EGM-2 medium without growth factors and growth factor reduced Matrigel. This suspension was applied onto the scaffold and allowed to be absorbed and solidify for 30 min at 37°C in a humid atmosphere with 5% CO₂. Culture medium (EGM-2) was then added; scaffolds were detached from the wells and cultured further on a shaker at 37°C in a humid atmosphere with 5% CO₂. Medium was changed every other day. After 10 days of culture samples were fixed in 10% formalin or implanted into mice.

Male 6-week old NMRI-nu mice (Harlan) were anesthetized with a mixture of isoflurane and oxygen after which constructs were subcutaneously implanted in four pockets. Animals were housed at the Central Laboratory Animal facility (Utrecht University, Utrecht, The Netherlands), and experiments were approved by the local animal care and use committee. Two weeks after implantation mice were sacrificed and implants were recovered. Samples were fixed in 10% formalin, embedded in paraffin and sectioned at 5 μm before staining.

3.2.8 Histochemical staining and image analysis

Hematoxylin (Sigma-Aldrich) and eosin (Sigma-Aldrich) staining as well as Masson's trichrome (Merck Chemicals) staining were performed according to manufacturers' protocols. Samples were photographed using a Nikon Eclipse E600 microscope. Based on Masson's trichrome stainings vessels were counted manually by 4 observers blinded to the sample composition. Three areas of each sample (10 samples per condition) were used for this quantification.

For detecting endothelium of human origin CD31 staining was performed. Antigen retrieval was achieved with IHC-Tek Epitope Retrieval Solution (IHC World). Sections were then incubated with mouse-anti-human CD31 primary antibody (Dako), which does not cross-react with mouse tissue. Following this, biotinylated horse anti-mouse secondary antibody (Antibodies-online.com) was applied. Slides were developed with Labeled Streptavidin Biotin (LSAB) with DAB Chromogen (IHC World) and weakly counterstained with Mayer's hematoxylin (Sigma-Aldrich).

3.2.9 Statistics

Each experiment was performed in triplicate. The data was analyzed using Student's t-test at $p < 0.05$. Data that required multiple comparison test was analyzed in SPSS (PASW statistics) using one-way Anova followed by Tukey's multiple comparison test ($P < 0.05$). Error bars on graphs represent standard deviation or 95% confidence interval as indicated in the graph legends.

3.2.10 Ethics statement

Human mesenchymal stromal cells (hMSCs) were isolated from human bone marrow from donors with written informed consent. This study was carried out in strict accordance with the recommendations of Medisch Ethische Toetsings Commissie Twente (Medical Ethical Research Committee Twente) and was approved by this Committee.

The animal study reported on in this manuscript was ethically assessed a priori by an animal ethics committee 2010-III-10-125 DEC-Utrecht. Animals were housed at the Central Laboratory Animal facility (Utrecht University, Utrecht, The Netherlands), and experiments were approved by the local animal care and use committee Dierexperimentencommissie Academisch Biomedisch Centrum (DEC-ABC). All surgery was performed under isoflurane/oxygen anesthesia, and all efforts were made to minimize suffering.

3.3 Results

3.3.1 Endothelial differentiation medium effects cell shape

Because EGM-2 induces endothelial differentiation in human amniotic fluid-derived stem (AFS) cells [46], we decided to use this medium to differentiate MSCs towards

an endothelial-like phenotype. First, we analyzed whether MSCs cultured in EGM-2 acquired an endothelial-like phenotype. HUVECs were used as positive control for expression of endothelial markers. Cytoskeleton staining and qPCR study showed that the observed change of shape and size of pre-differentiated MSCs was not followed by expression of endothelial specific markers such as CD31, KDR or vWF (data not shown). Additionally, MSCs grown in abovementioned conditions did not take up ac-LDL (data not shown). We did observe a difference in cell shape between MSCs cultured in EGM-2 and basic medium. Cells cultured in EGM-2 were clearly more elongated than MSCs cultured in basic medium but exact measurements of cell shape and area were difficult due to the high cell density. To quantify this phenomenon, sub-confluent cell culture was necessary. Cells were trypsinised and seeded at lower density and further growth was allowed for 2 more days to ensure cell spreading. Morphology of naïve MSCs showed a typical fibroblast-like shape in contrast to cells grown in EGM-2 (Figure 3.2A,B). The average size of MSCs cultured in basic medium was approximately $100 \mu\text{m}^2$ and the average cell circularity was 3 (circularity of 1 represents round objects, the higher the coefficient is, the less round object it describes). The shape of pre-differentiated MSCs was significantly altered: the area increased to approximately $200 \mu\text{m}^2$ and the circularity coefficient was close to 5, suggesting that cells grown in EGM2 were more elongated than naïve MSCs (Figure 3.2C-D). The observed phenomenon was opposite to what was expected because MSCs from other sources [59] acquired a cobblestone-like morphology typical for endothelial cells.

3.3.2 Wound healing assay

The wound-healing assay is a simple method to study directional cell migration *in vitro* [60]. Migration of vascular endothelial cells plays an important role in vasculogenesis and angiogenesis [61]. We used the scratch wound healing assay of tissue-culture cell monolayers to assess the migration potential of predifferentiated MSCs. The wound recovery increased over time and the rate of this was taken as a measure of cell migration (Figure 3.3A). We observed that 10 days culture in EGM-2 significantly increased the migration rate of MSCs (Figure 3.3B). The size of the wound in EGM-2-cultured MSC monolayer was reduced by 40% after 8 hours and the wound closed completely after 24 hours. Naïve MSCs needed 24 hours for a 45% reduction in wound size and 48 hour to close the wound completely. This assay showed that MSCs cultured in EGM-2 migrate faster than naïve MSCs.

3.3.3 Capillary-like structure formation

The angiogenic capability of various cell types was assessed using an *in vitro* capillary formation assay on Matrigel. Three cell types were used for this study: HUVEC which served as positive control, bone-marrow derived hMSCs and iMSCs. We decided to test whether iMSCs react in a similar way to hMSCs to serve as a cell source for future studies without problems associated with donor variation. As shown in Figure 3.4A, cells from each cell type performed with similar efficiency. Measurement of total tube length (Figure 3.4B) revealed significant but not critical differences between iMSCs

Figure 3.2: Predifferentiation of iMSCs. Cell shape after 10 days of culture in basic medium (A) and in EGM-2 (B). Average cell area (C) was compared. Cell shape was characterized based on circularity coefficient (D). Error bars represents 95% confidence interval, * denotes statistical significance ($P < 0.05$).

and hMSCs and HUVECs. Total tube area was similar in all three cases and the number of branching points did not reveal significant differences between iMSCs, hMSCs and HUVECs. Comparison of all those parameters allowed us to use iMSCs as a model of MSCs for further study.

Figure 3.3: Wound healing assay. Pictures were taken directly after making the wound, 12 and 24 hours later (A). Quantification of wound recovery was performed with TScratch software and presented on the graph (B). Error bars represent standard deviation, * denotes statistical significance ($P < 0.05$).

As we described above, MSCs expanded in endothelial medium undergo significant changes in shape. We hypothesized that these changes might influence the results of the Matrigel assay. In order to confirm this, we performed a Matrigel assay with naïve MSCs and MSCs expanded in EGM-2 (pre-differentiated MSCs). Both naïve MSCs

A **B**

Figure 3.4: Capillary-like structures formation on Matrigel. EL-hMSCs, EL-iMSCs and HUVECs were cultured on Matrigel for 24h in EGM-2 medium (A). Total tube length, total tube area and number of branching points were compared (B). * and ** denote statistical significance ($P < 0.05$).

as well as pre-differentiated MSCs were seeded onto Matrigel in basic medium or in EGM-2. We observed cell behavior on Matrigel in a time course study (Figure 3.5) where we found that naïve MSCs seeded in basic medium start to form capillaries on Matrigel at an earlier time point than pre-differentiated MSCs but those capillaries were very unstable and disrupted after 24 hours. A similar situation was observed when naïve MSCs were seeded on Matrigel in EGM-2. In contrast, pre-differentiated MSCs started to form capillaries with a 20 hours delay compared to naïve MSCs. However, those capillaries grew thicker and created a more complex network during the next 7 days. This showed that the expansion phase in EGM-2 is crucial for the stability of capillary structures.

Figure 3.5: Capillary-like structures formation on Matrigel. Time course study. iMSCs were cultured for 10 days in basic or EGM-2 medium and then seeded on Matrigel in basic or EGM-2 medium. Dynamics of capillaries formation was observed for following 7 days.

Quantification of the capillaries obtained in different conditions (Figure 3.6) demonstrated a significant increase in the total tube length, total tube area, number of tubes and branching points when cells were expanded in EGM-2 even when the process of tube formation was performed in basic medium. This means that cells expanded in EGM-2 behave more like endothelial cells even when used in less promoting conditions (basic medium is not supplemented with growth factors as much as EGM-2).

This is interesting when taking into account that endothelial cells need well supplemented medium to survive prolonged culture: HUVECs seeded in basic medium did not survive (data not shown).

3.3.4 Endothelial induction of MSCs

Since we did not observe endothelial marker expression in MSCs when growth factors and shear stress were applied we decided to test whether introduction of extracellular matrix could trigger an endothelial phenotype in MSCs. Cells cultured for 10 days in EGM-2 on a XYZ shaker were reseeded on Matrigel for another 24 hours. This short period of Matrigel culture allows for endothelial induction without the risk of MSCs modifying the gel itself [62]. Cells obtained in this way will be called endothelial-like MSCs (EL-MSCs).

3.3.5 EL-MSCs express endothelial markers

To characterize the phenotype of EL-MSCs, the expression of several endothelial genes, CD31, KDR and vWF, was assessed. hMSCs from 2 donors and iMSCs were used in this study. In all three cases the expression of CD31 and KDR was significantly higher in EL-MSCs than in naïve MSCs from the same source. CD31 expression was up-regulated between 15-60 times and KDR expression between 80-1000 times (Figure 3.7A). In the case of vWF the expression was 5-6 times higher in hMSCs from both donors but not in case of the immortalized clone (Figure 3.7A). To confirm the qPCR data, immunostaining was performed. iMSCs expanded in EGM-2, seeded on Matrigel for 24h and recovered with dispase/trypsin solution were stained for CD31 and KDR. Staining directly on Matrigel was not possible due to the very high background signal generated by unspecific antibody binding to Matrigel. HUVECs and naïve iMSCs served as positive and negative control respectively. We observed cells positive for tested markers among EL-iMSCs whereas naïve iMSCs were negative (Figure 3.7B). The specificity of the antibody for both markers was confirmed by HUVECs staining – cells recovered from Matrigel maintained round shape and did not spread on tissue culture plastic, which made the observation of characteristic staining on the border of neighboring cells very difficult.

The last characteristic of endothelial cells we tested was the ability of EL-MSCs to take up ac-LDL. iMSCs that were expanded in EGM-2 for 10 days and seeded on Matrigel for 24 hours were then recovered from the gel and reseeded on tissue culture plastic. Twenty-four hour later cells were incubated with ac-LDL for 4 hour, washed and imaged. Most of the cells were lost during this process. The cells that survived returned to a fibroblast-like morphology but were still able to take up ac-LDL. This uptake was however limited in the view of the number of positive cells when compared to HUVECs but was not observed at all in naïve MSCs (Figure 3.7B).

Figure 3.6: Quantification of capillary-like structures on Matrigel. Quantification of tube formation by cells cultured in 3 different conditions: cells predifferentiated in EGM-2 and seeded on Matrigel in the same medium (EE), cells predifferentiated in EGM-2 and seeded on Matrigel in basic medium (EB) and naïve MSCs expanded in basic medium and seeded on Matrigel in basic medium (BB). Graphs show total tube length, average tube length and average tube width, total tube area, number of tubes and number of branching points per picture. Error bars represent standard deviation, * and ** denotes statistical significance ($P < 0.05$) towards all other bars.

Figure 3.7: Endothelial marker expression in differentiated MSCs. Gene expression profiles of endothelial markers in EGM-2 and Matrigel culture (EE) (A). Expression is indicated as fold induction compared to cells grown in basic medium (BB) on plastic and normalized to GAPDH RNA. hMSCs from donors 55 and 56 and iMSCs were used in this experiment. Error bars represent standard deviation, * denotes statistical significance ($P < 0.05$). Staining for endothelial markers (B).

3.3.6 PLLA / PLGL construct

We previously reported the induction of vessel networks in engineered tissue constructs using a three-dimensional coculture system consisting of cells seeded on porous and

biodegradable polymer scaffolds [58]. The scaffolds were composed of 50% PLLA and 50% PLGL, with a pore size of $200 \pm 60 \mu\text{m}$ (Figure 3.8A and 3.8B). We have previously vascularized muscle tissue with tissue engineered constructs combining PLLA/PLGL scaffolds, Matrigel and HUVECs [58]. To assess the angiogenic potential of EL-MSCs we have compared their ability to improve construct vascularization with that of HUVECs and naïve MSCs. C2C12 monoculture was used as negative control for angiogenic potential of constructs seeded with C2C12 with HUVECs, MSCs or EL-MSCs in 1:1 ratio.

We showed that scaffolds filled with Matrigel without cells and cultured for 10 days in EGM-2 kept their structure (Figure 3.8C). Hematoxylin/eosin staining performed on cross sections of constructs 10 days after cell seeding indicates that cells attached to and grew on the scaffolds in all four culture systems (Figure 3.8D-G), no differences in cell density were detected. Elongated cells were observed in all used schemes, indicating cell-matrix interactions.

To observe the therapeutic potential of EL-MSCs we subcutaneously implanted tissue engineered constructs in immune-deficient mice (NMRI-nu, Harlan). The constructs were permeated with host blood vessels (Figure 3.9) and the number of ingrown vessels were quantified. There were no significant differences between constructs seeded with C2C12 cells alone or in coculture with HUVECs or with naïve MSCs (Figure 3.10A). Only in constructs seeded with C2C12 cells in coculture with EL-MSCs the number of vessels was significantly higher compared to all other tested conditions. These *in vivo* results showed that introduction of EL-MSCs in the implant improve construct vascularization and by that can promote cell survival in large grafts.

Staining of implants with anti-human specific endothelial antibody (anti-CD31) demonstrated that in the constructs seeded with EL-MSCs vessels lined with human cells were present (Figure 3.10B). Moreover, these vessels contained intraluminal red blood cells suggesting that the vessels had anastomosed with the host vasculature.

3.4 Discussion

Proper vascularization is essential for maintaining tissue well-being and functionality. It is also crucial for engineering of bone graft constructs, liver and many other tissues used for transplantation [63–65]. To provide extensive tissue or graft vascularization, a source of endothelial cells must be found. Those cells have to be available in large quantities and be able to create a vascular network within the tissue. Furthermore, this network should be both structurally and functionally appropriate.

Since BM-MSCs are widely used for bone tissue engineering, we decided to investigate whether we can use the same cell source for vascular network formation. In this study we used a number of techniques including immune-fluorescent imaging, qPCR and quantitative image analysis to examine endothelial differentiation of MSCs at the cellular and protein level. This study is the first to fully describe differentiation of MSCs into endothelial-like cells that perform in an *in vivo* study better than undifferentiated (naïve) MSCs.

Figure 3.8: *In vitro* vascularization of polymeric construct combined with Matrigel and cells. PLLA/PLGL scaffold before cell seeding (A). Scanning electron microscope (SEM) pictures of scaffold taken after gold coating (B). Eosin / hematoxylin staining of tissue sections taken from constructs after 10 days of *in vitro* culture without cells (C), with C2C12 (D), with C2C12 and MSCs (E), with C2C12 and HUVECs (F), with C2C12 and EL-MSCs (G).

BM-MSCs can be obtained in large quantities and further expanded *in vitro*. For most *in vivo* applications, a minimal number of cells, in the range of $10^8 - 10^9$, is necessary [66] and this can be easily obtained from MSCs after only a few passages. We did not observe any serious loss of endothelial potential in cells expanded up to such

Figure 3.9: *In vitro* vascularization of polymeric construct combined with Matrigel and cells. PLLA/PLGL scaffold before cell seeding (A). Scanning electron microscope (SEM) pictures of scaffold taken after gold coating (B). Eosin / hematoxylin staining of tissue sections taken from constructs after 10 days of *in vitro* culture without cells (C), with C2C12 (D), with C2C12 and MSCs (E), with C2C12 and HUVECs (F), with C2C12 and EL-MSCs (G).

a level. In the differentiation protocol MSCs were first cultured for 10 days in EGM-2 medium on an orbital shaker. The effect of this step was limited to changes in morphology and ability to perform in the Matrigel assay. After this culture period we did not observe a change either in marker expression or in the ability to take up ac-LDL.

Figure 3.10: Quantitative analysis of vessels in polymeric constructs.

Number of vessels per sample was quantified by four people blinded for the conditions (A). ** denotes statistical significance ($P < 0.01$), *** denotes statistical significance ($P < 0.001$). Tissue construct sections were stained with anti-human CD31 antibody (brown, indicated by black arrows) and counterstained with hematoxylin (blue) (B).

Nevertheless, this step was crucial for further performance in functional assays and differentiation. Functional properties of EGM-2-cultured MSCs were demonstrated using the Matrigel assay. MSCs that underwent 10 days of culture in EGM-2 medium performed with similar efficiency to endothelial cells (HUVECs) in Matrigel sprouting

assay and much better than naïve MSCs. Interestingly, capillaries formed by MSCs were more stable than the ones formed by HUVECs – when applying prolonged culture, capillaries formed by HUVECs get disorganized whereas capillaries formed by MSCs remain stable. This can indicate that while forming capillaries MSCs can play both the role of endothelial cells that create vessels as well as the role of pericytes that stabilize those vessels [67]. The fact that MSCs can take the role of pericytes in various engineered constructs has already been shown by Chamberlain et al [68]. They reported that in co-culture with HUVECs on collagen modules MSCs became smooth muscle actin positive and migrated to surround the EC layer of the vessel (location typical for pericytes).

Pre-differentiated MSCs were also able to acquire endothelial characteristic (endothelial markers expression and ability to take up ac-LDL) after 24h culture on Matrigel which was not the case when using naïve MSCs. This showed that growth supplements combined with shear force play an important role in triggering endothelial differentiation of MSCs, though this effect cannot be concluded based on marker expression only. Although the molecular mechanisms of angiogenesis and vasculogenesis are currently not fully understood, there is evidence strongly supporting the crucial role of VEGF in both processes [69]. The expression of the main receptor for VEGF, KDR, did not increase in hMSCs after culture in EGM-2, but other studies showed that many key events in VEGF signaling occur inside endothelial cells and are regulated by endosomal receptor trafficking [70]. According to this model even basally expressed KDR can propagate the VEGF signaling. The VEGF-KDR complex is endocytosed directly after signal transduction and then proteolytic cleavage takes place, releasing the cytoplasmic KDR dimmer and making it available to form new signaling complexes. In our work we did not check for expression of Neuropilins and Ephryns that play a crucial role in both VEGF and KDR endocytosis [71, 72] but we hypothesize that this can be the mechanism that triggers MSC differentiation towards endothelial lineages. This would be similar to the processes that occur in the embryo when the multipotent mesodermal progenitor cells differentiate in situ into endothelial cells during early stages of vascular development. VEGF, together with bFGF, is known to play a critical role in these events [73]. The input of Matrigel on pre-differentiated MSCs that leads to further differentiation towards endothelial cell phenotype can be explained based on the work of Lozito *et al.* [62]. They showed that change in the composition and crosslinking level of the matrix influences MSC differentiation towards endothelial and smooth muscle phenotype. Osawa *et al.* [74] showed that CD31 can be the mechanoresponsive molecule in case of endothelial cells, but we did not observe an increase in CD31 expression in MSCs before Matrigel stimulation. Therefore, two possible explanations of the effect of Matrigel on MSCs can be suggested. There can be another molecule on MSCs that acts as mechanoreceptor or there is a positive feedback loop between the ECM stimulation and CD31 expression in MSCs. Culture on Matrigel also induced KDR expression in MSCs which can further stimulate endothelial differentiation by increasing MSC sensitivity to VEGF present in culture medium. These results are similar to the ones obtained by Gu *et al.* [75] while studying murine embryonic stem cells. They showed that increased ex-

pression of endothelial markers is induced by extracellular matrix via CEACAM1, a glycoprotein involved in cell-cell adhesion. Therefore we can hypothesize that the role of Matrigel in our differentiation system is mainly to provide the environment in which MSCs can create cell-cell contact promoting endothelial differentiation. Nevertheless, those results suggest that careful examination of the MSC differentiation protocols is necessary as our knowledge concerning their signaling pathways is still limited.

CD31 staining performed on the constructs with EL-MSCs revealed the presence of human cells lining the walls of vessels. These vessels were fully functional as demonstrated by the presence of erythrocytes in the vessel lumens. This is a proof that EL-MSCs actively participated in building of these vessels. Nevertheless, there were also vessels present that were not human CD31 positive. This can suggest that the EL-MSC influence on vascularization is also due to their trophic effect on surrounding tissues. There is also the possibility that EL-MSCs create a network within the sample that is then gradually replaced by in-growing vessels. In that case the input of EL-MSCs can be limited to providing the route for host endothelial cells.

Although the primary purpose of this study was to differentiate hMSCs into endothelial-like cells, the results also serve as proof of concept for using bone marrow-derived hMSCs to create a vascularised graft. Our *in vivo* study showed that introduction of EL-MSCs in the engineered construct doubles the number of vessels in-growing in the construct 2 weeks after implantation. The fact that HUVECs did not induce vessel in-growth when compared to C2C12 or C2C12/MSC seeded constructs indicates that these endothelial cells are not able to create stable vascular structures without the support of other cells, pericytes or smooth muscle cells, which will stabilize newly formed blood vessels. Similar results were obtained by Levenberg *et al.* [58] where they have shown that the addition of embryonic fibroblasts promotes stable vessel formation. According to our results, EL-MSCs do not require the presence of an additional cell type to improve construct vascularization. Increased level of vascularization is crucial for the implantation of large grafts and the possibility to use the patient's own cells to stimulate such an effect is a promising finding for a number of different applications. Further studies are required to verify whether differentiation of hMSCs prior to application can further improve performance of these cells in peripheral vascular disease (PVD) treatment as our data suggest.

This study also presents the opportunity of using iMSCs as model cells for studying the endothelial differentiation of MSC. This can be a useful tool for further study since it provides cells that are not affected by potential donor variation and isolation procedures.

In summary, hMSCs derived from bone marrow acquire several endothelial-like characteristics when cultured in endothelial cell growth supplement and exposed to shear force and extracellular matrix stimuli. These features include both phenotypical as well as functional characteristics. Furthermore, when applied *in vivo*, EL-MSCs show greater angiogenic potential than both naïve MSCs as well as truly endothelial cells, HUVECs, that are generally used as golden standard for vascularization studies.

This study presents a facile protocol for MSC preconditioning that improves *in vivo* performance of these cells with respect to attracting neovascularization. We believe that this approach has potential applications in tissue engineering and provides a tool for various clinical studies where improved vascularization is desired.

To conclude, training MSCs *in vitro* can be an efficient way to manipulate the fate of these cells *in vivo*.

3.5 Bibliography

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Chapter 4

The effect of donor variation and senescence on endothelial differentiation of human mesenchymal stromal cells

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Abstract

Application of autologous cells is considered for a broad range of regenerative therapies because it is not surrounded by the immunological and ethical issues of allo- or xenogenic cells. However, isolation, expansion and application of autologous cells does suffer from variability in therapeutic efficacy due to donor to donor differences and due to prolonged culture. One important source of autologous cells are mesenchymal stromal cells (MSCs), which can differentiate towards endothelial-like cells, thus making them an ideal candidate as cell source for tissue vascularization. Here, we screened MSCs from 20 donors for their endothelial differentiation capacity and correlated it with the gene expression profile of the whole genome in the undifferentiated state. Cells of all donors were able to form tubes on Matrigel and induced the expression of endothelial genes, although with quantitative differences. In addition, we analyzed the effect of prolonged *in vitro* expansion on the multipotency of hMSCs and found that endothelial differentiation is only mildly sensitive to expansion-induced loss of differentiation as compared to osteogenic and adipogenic differentiation. Our results show the robustness of the endothelial differentiation protocol and the gene expression data give insight in the differences in endothelial differentiation between donors.

4.1 Introduction

The use of allo- and xenogenic cells in regenerative therapies will always entail the possibility of graft rejection and the necessity of applying immunosuppressive therapy, which as a result will be a burden for patients. Therefore, most investigators in the field currently consider autologous cells as the most logical choice for study.

Mesenchymal stromal cells (MSC), a much used source of autologous cells, can be isolated relatively easily from multiple sources including adipose tissue, tibia, femur, lumbar spine and trabecular bone [1–4]. They can be then expanded *in vitro* and are characterized by their multipotency. Among others they can differentiate into the adipogenic, osteogenic and chondrogenic lineages [5, 6]. What is less well documented is the ability of MSCs to differentiate towards other cell types such as skeletal muscle cells and neural cells [7–9]. Moreover, it was shown lately that MSCs can also be a source of endothelial-like cells [10–12], which would qualify them as candidate cells for therapies aimed at improved tissue vascularization, such as treatment of ischemic tissues and various strategies concerning large graft engineering.

We and others have shown that when human MSCs are grown in endothelial differentiation medium, they express endothelial markers such as CD31, von Willebrand factor and VE-cadherin, both at the mRNA and protein level [10–16]. In addition, the actively take up acidic LDL, another hallmark of endothelial cells. When grown on Matrigel, the cells will form tube-like structures. Moreover, when these cells are implanted in immune-deficient animals, CD31 positive human cells can be seen in the vessel wall of perfused blood vessels, demonstrating that human cells can functionally contribute to blood vessels. Nevertheless, further characterization of the cells is needed, and for that reason we refer to the cells as “endothelial-like cells”.

In order to apply MSC-derived endothelial-like cells, it is essential to establish a stable protocol for MSC isolation, culture and differentiation, because it will affect the therapeutic effect of the cells, as demonstrated previously for the application of MSCs in bone tissue engineering [17–19]. We have recently described a robust protocol to induce endothelial-like cells from bone marrow-derived MSCs *in vitro* and demonstrated their ability to contribute to the vasculature upon implantation in a mouse model. What remains to be addressed in order to bring endothelial-like MSCs to the clinic is both the large inter-donor variation in multi-lineage potential [1, 20] and the phenomenon of loss of multipotency upon culture expansion of MSCs. We and others have observed striking differences between hMSCs of different donors with regard to growth rate, expression of both lineage-specific and non-specific markers such as ALP and STRO-1 and their response to *in vitro* differentiation and ectopic bone formation [1, 20–22]. Similar situation can occur upon endothelial differentiation which can explain the controversy concerning the ability of MSCs to acquire endothelial characteristics. A complicating fact is that therapeutic efficacy of MSCs is often not directly linked to marker gene or protein expression *in vitro* [23–25].

Because the yield of hMSCs upon isolation is very low, expansion in culture is an essential step in their application. Unfortunately, this is associated with culture-

induced loss of multipotency, as described by several researchers [1, 26]. It was demonstrated that *in vitro* expanded MSCs acquire a phenotype characterized by loss of multipotency already at early passages [26–28] followed by replicative senescence at later stages of expansion. It appears that a hierarchy exists among the different lineages with respect to the number of population doublings at which loss of the particular differentiation route comes in effect [1, 29].

Although donor variability and loss of multipotency has been well-described for differentiation into the osteo- and adipogenic lineages, no such data is available for endothelial-like differentiation. To address this deficiency we have created a bank of MSCs isolated from 62 donors (patients that were undergoing orthopedic surgery) and collected data about their *in vitro* expansion and differentiation capacity as well as their gene expression profiles. Using microarray study we have identified a diagnostic bone-forming classifier [22] capable of indicating the *in vivo* bone-forming capacity of hMSCs from different donors (unpublished data). In this manuscript, we have selected 20 donors of this bank and evaluated their propensity to differentiate into endothelial-like cells in order to sketch inter-donor variability and to evaluate whether gene expression in undifferentiated hMSCs correlated to it. Moreover, we have studied loss of endothelial-like differentiation potential during culture expansion and compared it to loss of adipo- and osteogenic differentiation.

4.2 Materials and methods

4.2.1 Isolation and culture

Human mesenchymal stromal cells (hMSCs) were isolated from human bone marrow from donors who provided us with written informed consent [30]. Aspirates were resuspended using a 20G needle and plated at a density 0.5 million mononucleated cells/cm². Cells were grown in MSC proliferation medium which contains minimal essential medium (alfa-MEM, GIBCO) supplemented with 10% fetal bovine serum (FBS, Lonza), 100 U/ml penicillin (GIBCO), 10 µg/ml streptomycin (GIBCO), 2 mM L-glutamin (GIBCO), 0.2 mM L-ascorbic acid 2-phosphate magnesium salt (ASAp, Sigma Aldrich) and 1ng/ml basic fibroblast growth factor (bFGF, Instruchemie) at 37°C in a humid atmosphere with 5% CO₂. Cells were expanded up to passage 2. For further experiments hMSCs from 23 different donors and one immortalized clone (iMSCs, courtesy of Ola Myklebost) were cultured in basic medium (alfa-MEM supplemented with 10% FBS, 100 U/ml penicillin, 10 µg/ml streptomycin, 2 mM L-glutamin, 0.2 mM ASAp). Human umbilical vein endothelial cells (HUVEC, Lonza) were cultured in endothelial growth medium (EGM-2, Lonza).

4.2.2 ALP analysis

In order to analyze the activity of alkaline phosphatase, hMSCs from one donor were seeded at a density of 5,000 cells/cm² in 6-well plates. Osteogenic medium (alfa-MEM, 10% FBS, 100 U/ml penicillin, 10 µg/ml streptomycin, 2 mM L-glutamine,

0.2 mM ASAp, 0.01 M BGP, 10⁻⁸ M dexamethasone) was changed twice a week and cells were cultured for 1 week. ALP activity was analyzed using the Sigma kit #85 per manufacturer's instructions. Briefly, the culture medium was aspirated, cells were washed twice with calcium and magnesium free PBS and fixed in acetone/citrate. After washing in deionized water cells were stained with Fast Blue RR/naphthol. As a result naphthol AS-MX is liberated and immediately coupled with a diazonium salt forming an insoluble, visible pigment at sites of phosphatase activity. Cells were photographed using a Nikon SMZ 10A camera.

To confirm the results, an ALP biochemical assay (CDP-Star, Roche) was performed on selected samples. Briefly, cells were washed with PBS and lysed with lysis buffer (100mM potassium phosphate, pH 7.8, 0.2% Triton-X-100) for 10 min at room temperature. 10 μ l of the lysate was put in an optiplate together with 40 μ l of CDP-Star, incubated for 30 min in darkness at room temperature and then measured with a Victor Light Luminescence Plate Reader (Perkin Elmer).

4.2.3 Adipogenesis

Adipogenic differentiation capacity of hMSCs was determined as described previously [31]. Briefly, cells from one donor were cultured for three weeks in adipogenic medium (DMEM, 10% FBS, 100 U/ml penicillin, 10 μ g/ml streptomycin, 0.5 mM IBMX, 1 μ M dexamethasone, 10 μ M insulin, 200 μ M indomethacin). After that, lipid formation was visualized by staining with Oil red O. Cells were photographed using a Nikon Eclipse TE300 and the adipocytes were counted in the picture frame. At least 3 different locations of each well were included in the quantification.

4.2.4 Endothelial induction of MSCs

hMSCs from passage 3 and iMSCs from passage 25 were used for endothelial induction protocol as described previously [10]. Briefly, cells were seeded at a density of 3,000 cells/cm² on tissue culture plastic in EGM-2 and cultured for 10 days. After one day in static culture, shear force was applied using an orbital shaker. For induction on Matrigel, wells on 6-well plates were covered with 1 ml of growth factor reduced Matrigel (BD Bioscience) diluted 1:1 in EGM-2 without growth factors. Cells were seeded at the density of 30,000 cells per cm² (60,000 cells per cm² in case of HUVECs due to their smaller size) and cultured in a humid atmosphere with 5% CO₂. After 24 hours of culture on Matrigel hMSCs start to express endothelial markers and are referred to as endothelial-like MSCs (EL-MSCs).

4.2.5 Matrigel assay

The assay was performed as described above. The formation of capillary-like structures (CLS) on Matrigel was observed over time using an inverted microscope (Nikon Eclipse TE300). Cells from all 20 donors were used in this study. Pictures were taken at different time points (4, 8, 16, 24, 48, and 96 hours after seeding) using a Nikon DS-L2 camera. The experiment was performed in triplicate.

4.2.6 TubeCount

Capillary-like structure formation was quantified based on images taken at a 24-hour time point using TubeCount software, as described previously [10]. As a result we gathered valuable statistics such as total and average tube length, average tube width, number of tube branching points and total tube area. A minimal number of 3 pictures per condition were analyzed.

4.2.7 RNA isolation and quantitative PCR

Total RNA was isolated using TRIZOL reagent according to manufacturer's protocol. In short, 1 ml of Trizol reagent was added per T25 flask (cells cultured in basic medium) or per well (cells cultured on Matrigel in 6-well plates). Samples were incubated for 5 min at room temperature to allow complete dissociation, and phase separation was performed by adding chloroform. After that samples were centrifuged at 12,000 x g for 15 min. RNA was precipitated by mixing the aqueous phase with isopropyl alcohol followed by 10 min incubation at room temperature. Samples were centrifuged again and the remaining RNA pellet was washed with 75% ethanol. The obtained samples were dissolved in water, and after that the quantity and quality of RNA was analyzed using spectrophotometry (ND-1000 spectrophotometer). OD 260/280 nm ratios >1.8 were observed for all samples indicating high purity.

500 ng of RNA was used for first strand cDNA synthesis using Superscript II (Invitrogen) according to the manufacturer's protocol. One μ l of 3x diluted cDNA was used for PCRs performed in a Light Cycler real time PCR machine (BioRad). Data was analyzed using Bio-Rad iQ5 software and expression of endothelial genes was calculated relative to GAPDH levels by the comparative Δ CT method [32]. Primers used in the study are: Platelet Endothelial Cell Adhesion Molecule-1 (CD31) F 5' TCTATGACCTCGCCCTCCACAAA 3', R 5' GAACGGTGTCTTCAGGTGGTATTTCA 3'; VEGF receptor 2 (KDR) F 5' ACTTTGGAAGACAGAACCAAATTATCTC 3', R 5' TGGGCACCATTCACCA 3'; von Willebrand factor (vWF) F 5' TGCTGACACCAGAAAAGTGC 3', R 5' AGTCCCCAATGGACTCACAG 3'; GAPDH F 5' CGCTCTCTGCTCCTCCTGTT 3', R 5' CCATGGTGTCTGAGCGATGT 3'.

4.2.8 Microarray analysis

To analyse the gene expression profile of hMSCs, microarray analysis was used. RNA was hybridized to the Human Genome U133A 2.0 Array (Affymetrix) and scanned with a GeneChip G3000 scanner (Affymetrix). To normalize the measurements, we used a normalization method which removes hybridization, amplification and array location based technical effects. Further data analysis and statistical testing were performed using R and Bioconductor statistical software (<http://www.bioconductor.org/>). A linear modelling approach with empirical Bayesian methods, as implemented in Limma package [33], was used to determine differential gene expression. To analyse the donor variation in terms of endothelial differentiation ability, we scored the different donor-derived MSCs based on their CD31 expression

on one hand and KDR expression on the other hand. Subsequently, a list of genes ranked on fold change between the best and poorest endothelial differentiating donors was generated.

4.2.9 Statistics

Each experiment was performed in triplicate. Data that required multiple comparison test was analyzed in SPSS (PASW statistics) using one-way Anova followed by Tukey's multiple comparison test ($P < 0.05$). Error bars on graphs represent standard deviation.

4.2.10 Ethics statement

Human mesenchymal stromal cells (hMSCs) were isolated from human bone marrow from donors with written informed consent. This study was carried out in strict accordance with the recommendations of the Medisch Ethische Toetsings Commissie Twente (Medical Ethical Research Committee Twente) and was approved by this Committee.

4.3 Results

4.3.1 hMSC characterization

hMSC from our bank of 62 donors have been characterized previously (unpublished data) according to the set of standards proposed by the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy [34]. In this manuscript, we have randomly selected 20 donors from the donor bank [1]. After defrosting and brief expansion, a subsection of cells of all donors were subjected to osteogenic and adipogenic differentiation. As shown in Figure 4.1, MSCs from all donors readily acquired properties of adipocytes or osteoblast respectively, demonstrating their multipotency.

4.3.2 Effect of culture expansion on multipotency of hMSCs

We previously showed [1] that efficient differentiation of hMSCs is limited after prolonged expansion *in vitro*. To confirm these results, osteogenic and adipogenic differentiation of hMSCs from one donor was evaluated over 10 passages. Similar to previous observations, changes in cell morphology occurred during prolonged *in vitro* expansion (Figure 4.2A). hMSCs between passage 7 and 10 lost their fibroblast-like shape and acquired a more spread morphology. The cells in culture became larger, with irregular and heterogeneous contours. Moreover, hMSCs expanded *in vitro* lost their proliferation capacity over time, being unable to reach confluency even after prolonged culture (data not shown).

To document osteogenesis, we measured dexamethasone-induced activity of the bone-maker alkaline phosphatase (ALP) [1]. Cells from passage 1 showed a 3 times higher activity of ALP than cells from passage 2 to 5 (Figure 4.2B) and ALP activity

A **B**

Figure 4.1: Pluripotency of hMSCs. Adipogenic differentiation (A) demonstrated by Oil red O staining and osteogenic differentiation (B) demonstrated by ALP staining.

declined again three-fold in cells from passage 6 and higher (Figure 4.2B). A similar trend was observed for adipogenic differentiation (Figure 4.3A), where cells from passage 1 differentiated more efficiently than cells from passage 2 to 7. A gradual loss of adipogenic potential was observed (Figure 4.3B). Next, we evaluated whether the endothelial differentiation capacity of hMSCs was lost as well upon culture expansion. In this case, we expanded cells from 2 donors (D42, D56) and first looked at their capacity to form tubes on Matrigel (Figure 4.4A). Overall, MSCs did not lose their capacity to form tubes, up to passage 7. At higher passages, we were unable to retrieve sufficient number of cells to perform the assay. In the case of donor 42, prolonged expansion led to shorter capillary-like structures, although the number of branching points seemed to increase. With cells from donor 56, we observed the opposite, with

Figure 4.2: Expansion and osteogenic potential of serially passaged hMSCs. Cell morphology (A). CDP-Star assay was used to quantify ALP activity (B). Error bars represent standard deviation, * and ** denote statistical significance towards other groups ($p < 0.05$).

longer capillary like structure and fewer branching point at high passages. As a second readout of endothelial differentiation, we measured the expression of the endothelial marker CD31 in hMSCs exposed to proliferation medium and endothelial differentiation medium of three donors at various passage numbers. At passage 3, we observed induction of CD31 in all three donors, but this was completely lost in donor 57. Surprisingly, the cells of the other two donors were still able to induce CD31 expression at passage 7, and even with a higher fold induction than at passage 3 (Figure 4.4B).

Our data show that endothelial differentiation is not as sensitive to culture-induced loss of differentiation capacity as adipogenic and osteogenic differentiation are.

Figure 4.3: Adipogenic potential of serially passaged hMSCs. Adipogenic differentiation was visualized by staining with Oil red O (A) and the number of adipocytes was quantified (B). Error bars represent standard deviation, * and ** denote statistical significance towards other groups ($p < 0.05$).

Figure 4.4: Endothelial potential of serially passaged hMSCs. Capillary-like structure formation was observed on Matrigel (A). CD31 expression was compared using qPCR (B). Error bars represent standard deviation.

4.3.3 Endothelial differentiation of hMSCs

To investigate whether hMSC source (donor) influences endothelial differentiation, capillary-like structure formation was observed on Matrigel. Tube formation was recorded by phase contrast microscopy and pictures were used for further study. As shown in Figure 4.5A, cells from different donors performed with very different outcome. The quantification of CLS formation was performed using in-house developed TubeCount software. HUVECs were used as positive control in this assay and had a total tube length per analyzed area of 30 mm and more than 70 branching points.

Measurement of these parameters with MSCs of 20 donors (Figure 4.5B) revealed that cells from all donors were able to form tube-like structures although substantial inter-donor variation was seen, ranging from equal efficiency as HUVECS to donors of whom the cells produced five-fold less tubes. It also occurred to us that cells from some donors started to form capillary-like structures at a later time point than most, but in the end reached the same level of CLS formation (data not shown). Overall, the total tube length and the number of branching points correlated to each other. Since all results were taken at an arbitrarily chosen time point (24h) identical in case of all analyzed samples, this variability is present in the final quantification. Taken together, our data shows that cells from all donors responded to the Matrigel stimulation but with different efficiency. Yet, there was not a single donor from whom the cells did not respond at all.

4.3.4 Endothelial gene expression profile in MSCs

For further classification of EL-MSCs from different donors we analyzed endothelial-specific genes expression. As previously, cells were cultured for 10 days in EGM-2 on an orbital shaker and were then reseeded on Matrigel for another 24 hours. This relative short time of culture was chosen because in some cases, the Matrigel coating was completely resorbed at later time points, indicating donor specific matrix degrading activity. We performed qPCR on platelet endothelial cell adhesion molecule (CD31), vascular endothelial growth factor receptor 2 (KDR), von Willebrand factor (vWF) and vascular endothelial Cadherin (VE-Cadherin) and expressed it relative to GAPDH. We found a great variability of endothelial marker induction in cells from different donors. Cells from all donors responded to the differentiation protocol with increased CD31 expression (Figure 4.6A), with a fold induction between 3 and 78. In fifteen donors out of twenty we observed increased KDR expression (fold induction between 16 and 1000) and twelve of the 20 donors showed increased vWF expression (fold induction between 2 and 18) (Figure 4.6B and 4.6C). Only six donors responded with significantly increased VE-Cadherin expression (fold induction between 32 and 88) (Figure 4.6D). Not in all donors VE-cadherin expression was matched by other markers and we did not observe a robust correlation between total tube length or number of branching points and endothelial marker expression. The expression levels of all tested endothelial markers except KDR did not reach the level of its expression in HUVECs, however, we confirmed in our previous study with immunostainings [10], that these expression levels were relevant.

For further analysis we divided twenty donors into four groups, scoring their endothelial differentiation capability based on changes in CD31 and KDR expression: 25% of worst performing donors received grade 1, next 25% grade 2, third group grade 3 and the best performing 25% donors received grade 4 (Table 4.1). These groups of samples allowed to discriminate between bone marrow-derived hMSCs able to differentiate efficiently into the endothelial lineage (grade 4) from hMSCs that do not have this differentiation capacity (grade 1) and were further used to find differences in gene expression of hMSCs at proliferating state.

Figure 4.5: Tube assay on Matrigel. EL-hMSCs from different donors were cultured on Matrigel for 24h in EGM-2 medium (A). Total tube length and number of branching points were compared (B).

4.3.5 Correlation between gene expression profile and ability to show endothelial gene expression

We investigated whether the differential expression of marker genes in endothelial-like cells is reflected in the gene expression repertoire of their undifferentiated ancestors, in other words we tried to find genes predictive of the efficacy of induction of endothelial

Figure 4.6: Donor variation in endothelial gene expression. Data are represented as fold induction compared to gene expression level of undifferentiated MSCs from the same donor.

donor	CD31	donor	KDR
57	4	71	4
64	4	64	4
49	4	59	4
55	4	57	4
51	4	47	4
67	4	27	4
47	3	74	3
40	3	55	3
65	3	49	3
43	3	40	3
71	2	67	2
59	2	65	2
56	2	60	2
60	2	56	2
78	2	51	2
27	1	78	1
74	1	75	1
38	1	58	1
58	1	43	1
75	1	38	1

Table 4.1: Donor comparison based on qPCR data. Grades were calculated based on the data distribution: 25% of worst performing donors received grade 1, next 25% grade 2, third group grade 3 and best 25% donors received grade 4.

marker gene expression. To this end, we compared the expression profiles of MSCs between the four different groups defined above. The top 30 genes from the resulting list were ranked on p value (Table 4.2). Interestingly, among the listed genes we found three that are directly involved in VEGF signaling pathway: CRYAB, EGR3, IL7R; four that are typical for endothelial cells: GFPT2, EGR3, ILR7, MEIS2; four involved in the proliferation and migration of cancer cells: SULF1, CRYZ, CA12, PHLDA1 and six involved in cardiovascular system development and diseases: ELN, CRYAB, SLC5A3, CA12, PHLDA1, FHL1 (Table 4.3).

4.4 Discussion

Human mesenchymal stromal cells are multipotent cells with well acknowledged capacity to differentiate into cell types from several lineages [5–7, 35, 36] and have therefore been extensively tested in various tissue engineering applications. One of the important questions that appear concerning the application of differentiated MSCs is the true nature of cells obtained upon various differentiation protocols. MSCs have been shown to differentiate towards various cell types based both on the expression

Gene name	Gene Symbol	Fold Change
genes identified based on CD31 expression		
elastin	ELN	-2.05
sulfatase 1	SULF1	-1.77
crystallin, zeta (quinone reductase)	CRYZ	1.62
crystallin, alpha B	CRYAB	-1.54
chromosome 1 open reading frame 54	C1orf54	1.46
solute carrier family 5 (sodium/myo-inositol cotransporter), member 3	SLC5A3	1.45
carbonic anhydrase XII	CA12	1.45
glutamine-fructose-6-phosphate transaminase 2	GFPT2	1.44
pleckstrin homology-like domain, family A, member 1	PHLDA1	1.44
early growth response 3	EGR3	1.44
carbonic anhydrase XII	CA12	1.37
elastin	ELN	-1.33
solute carrier family 16, member 4 (monocarboxylic acid transporter 5)	SLC16A4	1.31
interleukin 7 receptor	IL7R	-1.26
adenosine deaminase, RNA-specific, B1 (RED1 homolog rat)	ADARB1	-1.26
stomatin	STOM	-1.25
haloacid dehalogenase-like hydrolase domain containing 1A	HDHD1A	-1.23
serine incorporator 5	SERINC5	1.23
iduronate 2-sulfatase	IDS	1.21
kazrin	KAZ	-1.21
genes identified based on KDR expression		
four and a half LIM domains 1	FHL1	-1.79
four and a half LIM domains 1	FHL1	-1.74
keratin 19	KRT19	1.69
KIAA1199	KIAA1199	-1.45
ferritin, heavy polypeptide 1	FTH1	-1.40
glutaminase	GLS	-1.28
Meis homeobox 2	MEIS2	1.28
ATPase, class V, type 10D	ATP10D	-1.21
endothelin receptor type A	EDNRA	1.18
structural maintenance of chromosomes 2	SMC2	1.18
(-) in the fold change value denotes down-regulation when comparing donors with good and poor endothelial differentiation capabilities		

Table 4.2: Differentially expressed genes as identified by whole genome expression analysis. Donors were grouped based on their ability to differentiate into endothelial-like cells. Fold change between the best and poorest endothelial differentiating donors (based on CD31 and KDR expression) is specified in the table. Repeated gene names that appear in the table correspond to various probes for the same gene.

of characteristic markers *in vitro* as well as on their ability to regenerate specific tissues *in vivo*. Still, the mechanism lying behind the ability of implanted MSCs to regenerate or improve regeneration of various tissues is not fully explored [37]. In our previous work [10], we demonstrated that MSCs can differentiate towards endothelial-like cells. We based this conclusion on both the increased expression of endothelial markers in EL-MSCs, confirmed additionally by immunostainings, and on the effect

on construct vascularization when EL-MSCs were implanted. Nonetheless, we did not claim to obtain endothelial cells from MSCs but rather cells that are able to replace them in therapeutic applications. To prove the usefulness of the developed protocol, its robustness must be showed with respect to overpowering the variability among cells obtained from different donors.

In this study we isolated and characterized hMSCs from 20 independent donors. First we analyzed cell performance in a Matrigel assay where we found that the efficiency in tube formation was not the same between different donors. According to our observations at various time points (data not shown), some of the differences in results could be caused by the chosen observation time that was set for 24 hours after seeding. Since cells from different donors started to form CLSs at different time points, the results were not fully reflecting the absolute potential of hMSCs to form CLSs. The differences between donors occurred rather as a result of differences in the speed of attachment and migration. CLS network formation on Matrigel occurs as an effect of cells attaching to the gel surface, spreading and getting in contact with each other and with extracellular matrix (ECM). Since the cells used in this assay were freshly trypsinized differences were likely to occur at the level of molecules responsible for ECM binding on the cell surface. Therefore, it was to be expected that the speed of attachment may vary depending on the sample. Another reason for the differences in the speed of cell attachment and migration can be explained by the age of the donors, as Kasper *et al.* [67] demonstrated that those parameters are strongly correlated. Although donor variation in CLS formation was observed, CLS maturity was reached in all cases within 24 hours. Endothelial cells of all origins are able to form tubules spontaneously [68] but the exact time of this event can be different for various cells of this type [69]. Thus, since EL-MSCs from all investigated donors responded to Matrigel stimulation, the differences in time of response does not mean that those cells did not undergo endothelial differentiation.

Next we analyzed the gene expression profile of differentiated hMSCs with respect to expression of endothelial markers: CD31, KDR, vWF and VE-Cadherin. Here we found great variability of endothelial marker induction in cells from different donors. Up-regulation in the expression of one selected gene was not necessarily followed by up-regulation of the others. This phenomenon could be explained by the single time point that we have chosen for analysis. Cells from various donors were most likely at different level of differentiation after 24 hours of Matrigel culture. As demonstrated by Levenberg *et al.* [70], the expression of endothelial specific genes in differentiating cells is not correlated with each other in time. Therefore, for microarray studies we decided to focus on these two genes, namely CD31 and KDR, which are commonly used as endothelial markers and for which up-regulation occurred in most of the donors.

Based on the performed microarray study we selected 30 genes that were differentially expressed. Among those we identified 17 genes whose expression is involved in VEGF signaling, expression of markers typical for endothelial cells, development and diseases of cardiovascular system as well as cell proliferation and migration in malignant cancers. hMSCs from various donors have in general a similar gene expres-

Gene Symbol	Gene name and function	References
VEGF signaling		
CRYAB ↓	Crystallin alpha B, protects VEGF from proteolytic degradation, knockdown of CRYAB had an inhibitory effect on endothelial cells survival, mutations in the Cryab gene are associated with a broad variety of neurological, cardiac and muscular disorders	[38, 39]
EGR3 ↑	Early growth response 3, plays a role in endothelial cell growth and migration, mediates VEGF activation in endothelial cells	[40, 41]
IL7R ↓	Interleukin 7 receptor, present on human microvascular endothelial cells, activation by IL7 enhances endothelial cell growth, migration and generation of lymphatic tubules via upregulating the expression of the lymphangiogenic growth factor and vascular endothelial growth factor-D, IL-7 regulates also VEGF-D in lung cancer cell lines, plays a role in rheumatoid arthritis angiogenesis, and can mediate rheumatoid arthritis pathogenesis	[42–46]
Characteristic for endothelial cells		
GFPT2 ↑	Glutamine-fructose-6-phosphate transaminase 2, highly active in all endothelial cells, expressed in most tissues involved in the development of diabetic late complications	[47, 48]
EGR3 ↑	See above	
IL7R ↓	See above	
MEIS2 ↑	Meis homeobox 2, differentially expressed in placental microvascular endothelial cells compared with macrovascular cells, possible role in the development of cleft palate and cardiac septum	[49, 50]
Cell survival, proliferation, migration and cancer malignancy		
SULF1 ↓	Sulfatase 1, selectively removes 6-O-sulfate groups from heparan sulfate proteoglycans and by that alters binding sites for various cytokines and growth factors	[51–53]
CRYZ ↑	Crystallin zeta (quinone reductase), enzyme with NADPH-dependent quinone reductase activity, involved in antiapoptotic bcl-2 overexpression	[39, 54]
CA12 ↑	Carbonic anhydrase XII, zinc metalloenzyme that catalyzes the reversible hydration of carbon dioxide, expressed at high levels on various types of cancer cells including cancer stem cells, involved in tumour progression, additionally contributes to various other diseases like glaucoma and arteriosclerotic plaques, abundantly expressed in normal human corneal endothelium	[55–58]
PHLDA1 ↑	Pleckstrin homology-like domain, family A, member 1, Apoptosis-associated nuclear protein, contributes to migration and proliferation in colon cancer cells, contributes to the development of atherosclerosis observed in hyperhomocysteinemia	[59, 60]
Cardiovascular system development and diseases		
ELN ↓	Elastin, components of elastic fibers, disrubtion leads to various vascular diseases	[61, 62]
CRYAB ↓	See above	
SLC5A3 ↑	Solute carrier family 5 (sodium/myo-inositol cotransporter), member 3, essential for osteogenesis and bone formation, additive effects of multiple genetic variants of SLC5A3 on the risk of coronary artery disease	[63, 64]
CA12 ↑	See above	
PHLDA1 ↑	See above	
FHL1 ↓	Four and a half LIM domains 1, heavily expressed in skeletal and cardiac muscles, plays a role in cardiovascular development, hypertrophy, atherosclerosis, and angiogenesis	[65, 66]
↑ denotes up-regulation, ↓ denotes down-regulation when comparing donors with good and poor endothelial differentiation capabilities		

Table 4.3: Identity and function of differentially expressed genes as identified by whole genome expression analysis. Donors were grouped based on their ability to differentiate into endothelial-like cells.

sion profile in their undifferentiated state. Therefore, many differentially expressed genes connected with endothelial cells might give the possibility to predict the po-

tential of a given hMSC for endothelial differentiation. We consider that from genes involved in VEGF signaling the up-regulation of expression of EGR3 in proliferating hMSCs is of major importance. EGR3 is a transcription factor that plays a positive role in VEGFR1 transcription [40]. The abundance of EGR3 in hMSCs might make them more responsive to VEGF present in culture media and by that more prone to become EL-MSCs. Additionally EGR3 is a critical determinant of VEGF signaling in activated endothelial cells where the EGR3 expression level influences VEGF-mediated proliferation, migration, and tube formation [41]. It is possible that EGR3 plays a similar role in differentiating hMSCs. The observed down-regulation of two other genes involved in VEGF signaling that we found as differentially expressed in MSCs is probably connected to the fact that both CRYAB and IL7R are playing a role in VEGF signaling mostly in cases where cancer cells are involved [38, 42, 43].

Among genes that are specific to endothelial cells and were differentially expressed in proliferating hMSCs the expression of three was up-regulated (GFPT2, EGR3 and MEIS2) while one, IL7R, typical for microvascular cells, was down-regulated. Since GFPT2 [47] and EGR3 are typical for all endothelial cells, MEIS2 is a marker of macrovascular endothelial cells and is not highly expressed in microvascular endothelial cells [49] and IL7R is present on microvascular endothelial cells [44] we suggest that hMSCs might be more susceptible to differentiate towards endothelial cells that are different in their characteristic from microvascular endothelial cells.

Our results do not allow us to draw firm conclusions about hMSC potential to differentiate towards endothelial cells based on the differentially expressed genes involved in cell survival, proliferation and migration or the one involved in cardiovascular system development and diseases. Further studies are necessary to conclude whether differences in these genes' expression are anyhow important for their application as therapeutic cells.

The applicability of hMSCs in regenerative medicine depends largely on their ability to expand *in vitro* without losing their potential to differentiate before reaching clinically relevant cell number. Since EL-MSCs are required in rather large quantities to be used in TE applications, we performed a series of experiments to check whether extended proliferation will influence multipotency of hMSCs. To compare our results with the ones previously obtained [1, 71], we evaluated osteogenic and adipogenic differentiation along with endothelial differentiation on cells from all 10 passages. Our results concerning the osteogenic and adipogenic potential of serially passaged hMSCs were consistent with those obtained earlier. Interestingly, the endothelial differentiation was not influenced by a prolonged expansion phase, as showed both in the Matrigel assay as well as in the gene expression studies. In the Matrigel assay we observed that the parameters of structures formed after prolonged expansion are donor-dependent, therefore no conclusions about the influence of passage number can be made. According to our unpublished results we can conclude that in this assay it is rather the number of seeded cells that is crucial for the final outcome, therefore even cells from late passages that did not lose their ability to attach, spread and migrate could efficiently form CLSs. Cells from one investigated donor stopped proliferating

at passage 6 which excluded them from the endothelial senescence study but in all other cases no negative effect of *in vitro* expansion on endothelial potential was observed. To our knowledge, this is the first observation of such phenomenon in hMSCs. From this study we conclude that hMSCs can be expanded *in vitro* at least up to passage 6 while keeping endothelial potential for effective use in tissue regeneration applications.

To summarize, our report provides several candidates for molecular markers that can be used for prediction of hMSC potential to differentiate into endothelial-like cells. We also showed that this potential is not affected by prolonged *in vitro* expansion as long as the cells keep proliferating. Altogether, our results indicate that hMSCs from various donors perform well enough to be considered in potential clinical applications.

4.5 Bibliography

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Chapter 5

Collagen modules for *in situ* delivery of mesenchymal stromal cell-derived endothelial cells for improved angiogenesis

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Abstract

Modular tissue engineering is a strategy to create scalable, self-assembling, three-dimensional tissue constructs. This strategy was used to deliver endothelial-like cells derived from bone marrow mesenchymal stromal cells (EL-MSCs) to locally induce vascularization. First, tissue engineered modules were formed, comprising EL-MSCs and collagen-based cylinders. Seven days of module culture in a microfluidic chamber under continuous flow resulted in the formation of interstices, formed by random packing of the modules, which served as channels and were lined by the EL-MSCs. We observed maintenance of the endothelial phenotype of the EL-MSCs as demonstrated by CD31 staining and the cells proliferated well. Next, collagen modules covered with EL-MSCs, with or without embedded MSCs, were implanted subcutaneously in immune-compromised SCID/Bg mice. After 7 days, CD31 positive vessels were observed in the samples. This data demonstrates the feasibility of EL-MSCs coated collagen module as a strategy to locally stimulate angiogenesis and vasculogenesis.

5.1 Introduction

There is a great need for technology to enhance the formation of blood vessels, such as in peripheral vascular disease (PVD) or tissue engineering of large three-dimensional

tissues. As such, engineering of small diameter blood vessels is one of the major challenges in current tissue engineering and revascularization strategies. Numerous trials have been performed to discover the optimal way of restoring or introducing vascular networks in the tissues that require such treatment. Delivery of cells of endothelial nature *in situ*, either in the tissue engineered construct or in the human body, has been one of these strategies. There are various types of endothelial cells (EC) which are able to improve tissue vascularization, either by providing signals that induce angiogenesis [1] or by contributing to the new vessel system by self-organization into capillaries that then get connected to the surrounding vascular network [2]. The usage of those cells in therapy is unfortunately hampered due to problems with their isolation, expansion capacity and the lack of autologous sources [3–5]. As an alternative, we and others have investigated the possibility of using another cell type, i.e. mesenchymal stromal cells (MSCs), in therapies that require vessel regeneration. MSCs are adult cells that can easily be isolated from many sources including adipose tissue, tibia, femur, lumbar spine, and trabecular bone [6–9]. MSCs can be expanded *in vitro* and have the capacity to differentiate into the adipogenic, osteogenic and chondrogenic lineages [10] as well as towards endothelial cells, skeletal muscle cells and neural cells [11–13]. The differentiation of MSCs is influenced by soluble factors [14], oxygen tension [15] and mechanical stimuli such as flow or shear stress [16]. Another important feature of MSCs is that they secrete various growth factors and cytokines by which they can influence both cells within tissue engineered constructs as well as cells from surrounding host tissues. It was shown that by this trophic effect, MSCs can among others inhibit apoptosis, fibrosis and stimulate angiogenesis [17], which is beneficial for the survival of cells within the graft.

The first experiments in which MSCs were applied in vascular therapies were performed by simple injection of the cells into the vein [18]. The assumption was that injected MSCs would reach the tissue where vascularization is needed through the regular blood flow. The observed improvement of vascularization was very limited, but was seen as proof of principle and it encouraged further research in this area. It was hypothesized, that simple injection of cells might not be the best way of delivering them at the place of injury. The potential of MSCs in vascular network formation could be improved by providing a matrix support that will direct these cells towards formation of capillaries being able to connect with the recipient's vascular network.

One of the approaches to create scalable, self-assembling, three-dimensional tissue constructs is modular tissue engineering. This technique allows fabricating tissue building blocks with specific features and then using these modular units to engineer biological tissues bottom up [19]. This is of crucial importance because the tissues to be engineered (e.g. fragments of bone) are often in the centimeter range and thus, too large to be generated *in vitro* in one piece. There are several ways to create modular blocks, including assembling of hydrogel layers [20], aggregation of microtissues [21], direct gel printing [22] and using cell sheets [23]. In order to build larger tissues, such blocks are then assembled by random packaging [24–26] or layer stacking [23]. Such systems can be used, among others, to improve vascular network formation within

an engineered construct by providing channels that can serve as a route for both implanted cells and in-growing host vessels.

Cooper *et al.* showed that coating collagen modules with HUVECs prevented loss of shape of these modules and allowed for vessel in-growth in contrast to the situation when uncoated modules were implanted; the latter led to creation of an indistinguishable mass of collagen and limited vessel penetration [27]. Chamberlain *et al.* [28] showed that endothelial cell coated collagen constructs with embedded MSCs can be used for the formation of functional blood vessels. They used MSCs and ECs isolated from Sprague-Dawley rats (ECs from aorta and MSCs from bone marrow). To obtain clinically relevant data we decided to use human MSCs and ECs in the following experiments.

In our previous work [13, 29], we showed that human MSCs can be differentiated into endothelial-like cells (EL-MSCs), which can be used to create vascular networks within grafts. We showed that EL-MSCs express characteristic endothelial markers and perform well in in vitro functional tests like Ac-LDL uptake and capillary-like structure formation on Matrigel and will contribute to the vasculature upon implantation in immune-deficient mice. Importantly, MSCs used for obtaining EL-MSCs can be autologous to the patients. In our previous study [30] we have observed donor variability among MSCs obtained from various donors. All cells that we tested responded for applied differentiation protocol, showing endothelial phenotype but with varying efficiency. Therefore, to be able to compare obtained data with the one obtained previously or after, we decided to use the immortalized clone which we tested before also for this study.

We have also tested several ways of delivering those cells *in vivo*, employing either a gel (Matrigel) plug or a PLLA /PLGL scaffold [13]. In this manuscript, we will describe how EL-MSCs can be combined with collagen modules, in which a clinically approved biomaterial is used, is easy to obtain and is cheaper than Matrigel, but also can be formed in vitro, which would enable *in vivo* formation of channels lined with cells. We hypothesize, that EL-MSCs in modules are useful for the pre-formation of functional vasculature in tissue engineered constructs.

5.2 Materials and methods

5.2.1 Isolation and culture

An immortalized clone of human mesenchymal stromal cells (referred to as iMSCs, a kind gift of Prof. Ola Myklebost) was used in these studies. Cells were grown in MSC proliferation medium which contains minimal essential medium (alfa-MEM, GIBCO) supplemented with 10% fetal bovine serum (FBS, Lonza), 100 U/ml penicillin (GIBCO), 10 μ g/ml streptomycin (GIBCO), 2 mM L-glutamin (GIBCO), 0.2 mM L-ascorbic acid 2-phosphate magnesium salt (ASAp, Sigma Aldrich) and 1ng/ml basic fibroblast growth factor (bFGF, Fisher Scientific) at 37°C in a humid atmo-

sphere at 5% CO₂. Human umbilical vein endothelial cells (HUVEC, Lonza) were cultured in endothelial growth medium (EGM-2, Lonza).

5.2.2 Endothelial induction of MSCs

iMSCs at passage 25 were used for endothelial induction, as described previously [13]. Cells were seeded at a density of 3,000 cells per cm² on tissue culture plastic in EGM-2 and cultured for 10 days. For induction on Matrigel, wells of 6-well plates were coated with 1 ml of growth factor reduced Matrigel (BD Bioscience) diluted 1:1 in EGM-2 without growth factors. Cells were seeded at a density of 30,000 cells per cm² and cultured in a humid atmosphere with 5% CO₂ for 24 hours. Cells that were cultured according to this protocol will be referred to as EL-MSCs.

5.2.3 Collagen module fabrication

Modular collagen cylinders were fabricated as described before [25]. In brief, Purcol acidified collagen (type I, bovine dermal, 3 mg/ml, Cedarlane) was mixed with 10x minimum essential medium (Invitrogen); 128 μ l of 10x medium per ml of collagen, and neutralized with 0.8 M NaHCO₃ (Sigma-Aldrich). Pelleted iMSCs were mixed with the neutralized collagen (1 x 10⁶ cells per ml), and the solution was drawn into the lumen of an ethylene oxide gas-sterilized PE tube (0.76 mm inner diameter x 1.22 mm outer diameter) connected to a syringe at one end. One hour incubation at 37°C was applied to allow collagen gelation. The gel-filled tubes were cut into fragments of 1.5 mm using a custom-built automated cutter (FCS Technology). Sections were vortexed gently in cell culture medium to recover the gel modules from the tubing lumen. The collagen-cell modules were allowed to settle, separated from the PE tubing, and cultured for 24 hours in Petri dishes under static conditions. Collagen-only modules were fabricated identically (same collagen concentration) without the addition of the cells.

5.2.4 Cell-seeding

HUVECs (5 x 10⁶ cells per pack of modules; 1 ml collagen) or EL-MSCs (2.5 x 10⁶ cells per pack of modules; 1 ml collagen) were added to modules with or without encapsulated MSCs and incubated for 1 hour with gentle shaking. Modules were transferred into Petri dishes and cultured for another 24 hours. Four module systems were used for in vitro study: surface seeded EL-MSCs only, surface seeded EL-MSCs with embedded MSCs, surface seeded HUVECs with embedded MSCs and embedded MSCs only. The conditions “surface seeded EL-MSCs” and “surface seeded EL-MSCs with embedded MSCs” were also used for *in vivo* studies.

5.2.5 Chamber assembly and flow circuit construction

Modules were loaded in chambers (3D KUBE, Kiyatec) on 0.45 μ m polyethersulfone membranes (Figure 5.1A). These chambers were connected to a multichannel peristaltic pump and EGM-2 medium was circulated through the chamber in a closed

loop as described before [31, 32]. Medium was pumped using a Masterflex L/S Digital standard drive with a 4-channel L/S multichannel pump head (Cole-Parmer, Vernon Hills, IL). 50 mL BD FalconTM polystyrene conical tubes (Becton Dickinson) were used as a reservoir for cell culture medium and tubing was a combination of L/S 13 Tygon® lab tubing and L/S 13 Tygon® 2-stop tubing with connections made via luer lock connectors (Cole-Parmer). Flow dampeners (fabricated from Nalgene Cryogenic Vials) were used to reduce pulsation and remove air bubbles from the system (Figure 5.1B-C). 35 ml of EGM-2 was circulated from separate reservoirs with a flow rate of 0.1 ml per minute and was changed twice a week. Samples were maintained for 7 days at 37°C and 5% CO₂. After 7 days, remodeling chambers were perfused with 5% formalin for 30 min. Chambers were opened, samples recovered and embedded in blocks of 5% agarose (Roche Diagnostics), and then fixed again in 10% formalin. Samples were embedded in paraffin and sectioned at 4 μm (two level sections, 50 μm apart) before analysis.

5.2.6 Module implantation

Male 6-week old Fox Chase severe combined immunodeficient / beige mice (SCID/Bg, Charles River) were anesthetized with a mixture of isoflurane and oxygen. Approximately 200 modules in 0.2 ml PBS were injected subcutaneously, one injection per mouse. After implantation, mice were individually housed. One and three weeks after implantation mice were sacrificed and implants were recovered. Samples were fixed in 10% formalin, embedded in paraffin and sectioned at 4 μm (two levels sections, 50 μm apart) before staining. The study was approved by the University of Toronto Animal Care Committee.

5.2.7 Histochemical analysis

Hematoxylin (Sigma-Aldrich) and eosin (Sigma-Aldrich) staining as well as Masson's trichrome (Merck Chemicals) stainings were performed according to the manufacturers' protocols. Immunohistochemical analyses were used to detect and characterize the endothelium. Sheep anti-vWF (Dako), rabbit anti-CD31 (Santa Cruz Biotechnology), mouse anti-desmin (Dako) and rabbit anti-SMA (Vector Laboratories) antibodies were used. All primary antibodies recognize both mouse and human antigens. All secondary antibodies were obtained from Vector Laboratories. To detect endothelium of human origin, biotinylated Ulex europaeus agglutinin 1 (UEA-1) supplied by Vector Laboratories was used, after which anti-biotin secondary antibody (Vector Laboratories) was applied. All slides were developed with DAB Chromogen and weakly counterstained with Mayer's hematoxylin (Sigma-Aldrich). Normal mouse and human tissues served as positive controls. As described before [13], vessels were counted manually (without considering their size) based on Masson's trichrome staining by 3 observers blinded to the sample composition. Three areas of each sample (3 samples per condition) were used for this quantification.

Figure 5.1: Experimental setup. Microfluidic chamber before and after assembly (A). Flow circuit (B). Scheme of medium flow through the chamber (C). Scale bar 1 cm.

5.2.8 Statistics

Each experiment was performed in triplicate. Data that required multiple comparison test was analyzed in SPSS (PASW statistics) using one-way Anova followed by Tukey's multiple comparison test ($P < 0.05$).

5.3 Results

5.3.1 Collagen module assembly

To examine EL-MSCs behavior under flow conditions and relate those observations to our previous results [13], four types of modular constructs were assembled. We used collagen modules with surface seeded EL-MSCs only, surface seeded EL-MSCs with

embedded MSCs, surface seeded HUVECs with embedded MSCs and embedded MSCs alone. The last two conditions were nearly the same as those used by Khan *et al.* [32] with, in our case, human instead of rat cells and were used as positive control for the system. The first two conditions were chosen to show EL-MSC potential in replacing endothelial cells. Collagen module preparation (Figure 5.2A) and the efficiency of coating with cells was assessed 24h after cell seeding. As expected, MSCs within the modules were uniformly distributed (Figure 5.2B). The size of collagen modules with or without embedded MSCs was similar, with a diameter of around 0.7 mm and a length of 1.5 mm (estimation based on microscopic observations). Coating with HUVECs resulted in an approximately two-fold shrinkage of the modules (Figure 5.2C), whereas coating with EL-MSCs induced even more shrinkage, both with empty and MSC-embedded modules, with a four-fold size reduction (Figure 5.2C-D). Twenty four hours after cell seeding all modules were uniformly coated with cells.

Figure 5.2: Collagen modules 24 hours after assembly: empty collagen modules (A), modules with embedded MSCs (B), modules with embedded MSCs and surface-seeded HUVECs (C), modules with surface-seeded EL-MSCs (D), modules with embedded MSCs and surface-seeded EL-MSCs (E). Scale bar 500 μm .

5.3.2 Cell characterization in microfluidic chamber

Cell survival under flow conditions was assessed after 7 days. Collagen modules with embedded MSCs formed a single mass of collagen over that time period (Figure 5.3A). MSCs could be easily observed within the mass, however we did not witness evidence of proliferation (only single cells were observed with lack of cell colonies within the collagen). Similarly, MSC proliferation was also not identified in modules with embedded MSCs coated additionally with HUVECs (Figure 5.3B). As shown before for rat endothelial cells [32], the endothelial coating of the collagen modules was still intact after 7 days of culture of HUVECs, but also here no increase in the amount of cells was observed. In contrast, we observed a large increase in the amount of EL-MSCs on the surface, but not in the interior of the modules (Figure 5.3C-D). Several markers were examined to characterize the phenotype of MSCs and EL-MSCs under flow conditions. First, we stained the modules for the endothelial marker, CD31. No staining was observed in samples with embedded MSCs only (Figure 5.4A), whereas staining was observed in the HUVEC-coated samples (Figure 5.4B). In samples with EL-MSC coating (Figure 5.4C-D), the cells present in the inter-modular region were CD31 positive, which indicates that EL-MSCs kept their phenotype acquired after Matrigel induction [13]. UEA-1 positive cells were found uniformly distributed throughout the whole sample only in constructs containing HUVECs (Figure 5.4F). In samples with EL-MSC coating but without embedded MSCs, several regions were found, in which cells were UEA-1 positive, but those regions were not uniformly spread through the sample (Figure 5.4G). No UEA-1 positive cells were observed in modules with embedded MSCs, both in case of modules without coating as well as modules with EL-MSC coating (Figure 5.4E and H).

To observe whether differentiation towards smooth muscle cells occurred in the system as described before [32], we performed smooth muscle actin (SMA) and desmin staining as smooth muscle cell and pericyte markers respectively (Figure 5.5), however no staining was observed. Therefore we can conclude that culture in EGM-2 supported endothelial phenotype of EL-MSCs, whereas did not induce MSC or EL-MSC differentiation towards a smooth muscle phenotype.

5.3.3 *In situ* delivery of endothelial cells

To evaluate whether the collagen module system can be used to deliver EL-MSCs in the place of interest and whether a vascular network will be created at the place of administration, one package of modules was injected subcutaneously per immunocompromised SCID/Bg mouse. We implanted collagen modules coated with EL-MSCs with or without embedded MSCs. Embedded MSCs were included in this experiment, since these cells were required to support HMECs in the Butler *et al.* study [33]. Samples were explanted at 7 and 21 days after implantation. Both after 7 and 21 days, we observed that the collagen mass was separated by layers of cells (Figure 5.6A and B) and the collagen modules were uniformly infiltrated by host blood vessels. Quantification based on Masson's trichrome staining (Figure 5.6AC) showed that 7 days after injection, modules with and without embedded MSCs were penetrated by simi-

Figure 5.3: Cell migration and proliferation over time. Masson's trichrome staining was performed on samples subjected to flow conditions for 7 days. Collagen appeared blue while nuclei appeared dark brown. Modules with embedded MSCs (A), modules with embedded MSCs and surface-seeded HUVECs (B), modules with surface-seeded EL-MSCs (C), modules with embedded MSCs and surface-seeded EL-MSCs (D). In samples with embedded MSCs, flow did not cause a migration of cells from the interior of the modules towards the outer surfaces and the intermodular regions. EL-MSC coated modules showed a greater number of cells in the intermodular regions and a higher number of total cells, as compared to uncoated modules and modules coated with HUVECs. Scale bar 100 μm .

lar amounts of vessels. Results were different when the quantification was performed on samples explanted 21 days after injection. In case of collagen modules coated with EL-MSCs without embedded MSCs, the number of vessels was the same as at day 7, but in samples with embedded MSCs, a significant, two-fold decrease in vessel number was observed.

To confirm our results and further study the maturity of in-growing vessels, we performed several additional stainings including markers like CD31, UEA-1, SMA and desmin (Figure 5.7). Our results showed that the vessels were CD31 and SMA positive, which indicates mature blood vessels with a smooth muscle cell layer. Surprisingly,

Figure 5.4: CD31 and UEA-1 staining. Staining was performed on samples subjected to flow conditions for 7 days. Cells were stained for CD31 (brown) and nuclei appeared blue. Modules with embedded MSCs (A), modules with embedded MSCs and surface-seeded HUVECs (B), modules with surface-seeded EL-MSCs (C), modules with embedded MSCs and surface-seeded EL-MSCs (D). MSCs did not stain positive for CD31. In samples containing HUVECs or EL-MSCs, CD31 positive cells can be observed. Cells were stained for UEA-1 (brown) and nuclei appeared blue. Modules with embedded MSCs (E), modules with embedded MSCs and surface-seeded HUVECs (F), modules with surface-seeded EL-MSCs (G), modules with embedded MSCs and surface-seeded EL-MSCs (H). MSCs did not stain positive for UEA-1. In samples containing HUVECs or EL-MSCs without embedded MSCs, UEA-1 positive cells can be observed. Scale bar 100 μm .

we did not observe desmin-positive cells in the samples. The vessels present in the samples were also UEA-1 negative.

Figure 5.5: SMA and desmin staining. Staining was performed on samples subjected to flow conditions for 7 days. No SMA staining (brown) was observed in any modules, indicating the lack of cells that differentiated towards smooth muscle cells. Modules with embedded MSCs (A), modules with embedded MSCs and surface-seeded HUVECs (B), modules with surface-seeded EL-MSCs (C), modules with embedded MSCs and surface-seeded EL-MSCs (D). Similarly to the SMA staining, no desmin staining (brown) was observed in any modules, indicating the lack of cells that differentiated towards smooth muscle cells. Modules with embedded MSCs (E), modules with embedded MSCs and surface-seeded HUVECs (F), modules with surface-seeded EL-MSCs (G), modules with embedded MSCs and surface-seeded EL-MSCs (H). Scale bar 100 μm .

5.4 Discussion

Modular tissue engineering allows for the formation of three-dimensional tissue constructs that can be scalable and have self-assembly abilities. Such systems can be used

Figure 5.6: Blood vessel formation *in vivo*. Masson's trichrome staining of modules with embedded MSCs and surface-seeded EL-MSCs (A) and modules with surface-seeded EL-MSCs (B) after 7 and 21 days *in vivo*. Collagen appeared blue, erythrocytes are bright red and nuclei appeared brown. Presence of erythrocytes indicated that vessels that within the sample are functional. Scale bar 100 μm . Quantitative analysis of vessels in polymeric constructs (C). Seven days after injection, modules with and without embedded MSCs were penetrated by similar amounts of vessels. Twenty one days after injection, the number of vessels within samples coated with EL-MSCs without embedded MSCs was the same as at day 7, but in samples with embedded MSCs, a significant, two-fold decrease in vessel number was observed. Number of vessels per sample was quantified by three people blinded for the conditions. ** denotes statistical significance ($P < 0.01$).

among others to improve vascularization of implanted tissues by providing a route for host vessel in-growth [25]. It can also be implemented as a system to guide angiogenesis in the host ischemic tissues by the supply of cells that will secrete pro-angiogenic factors.

Figure 5.7: Immunohistochemistry on blood vessels. Modules with surface-seeded EL-MSCs were recovered after 21 days of *in vivo* culture and following stainings were performed: Masson's trichrome, H&E, CD31, SMA, desmin and UEA-1. Observed vessels were CD31 and SMA positive, which indicates mature blood vessels with a smooth muscle cell layer. Scale bar 200 μm .

Several *in vitro* and *in vivo* studies have shown the potential of the modular tissue engineering concept in enhancing modular construct remodeling upon implantation [27, 33, 34], which enables faster tissue and vessel in-growth. In this paper we describe modular tissue constructs with various cell types that were assembled and subjected to flow for 7 days to observe cell self-organization with the focus on the performance of EL-MSCs. We examined whether these cells can be used in pre-vascularization strategies. Additionally, the aspect of de-differentiation of EL-MSCs was analyzed. As observed previously, cell coating influences the size of collagen modules, possibly by traction forces, and this effect is dependent on the cell type [32, 35].

Interestingly, covering the modules with EL-MSCs caused higher shrinkage than HUVEC coating which is most likely due to the faster spreading of EL-MSCs on the surface of the modules. Faster spreading resulted in an increased area subjected to the forces influencing the contraction of the modules [36]. Cells embedded within the modules did not cause any change in the module volume, as the 24 hours period was too short a time for the embedded MSCs to conduct significant matrix remodeling.

After 7 days of culture in the microfluidic chamber the effect of flow on cell behavior and phenotype was assessed. First of all, contrary to previous study with MSCs isolated from rat bone marrow [32], flow did not noticeably affect the differentiation of the embedded MSCs. We did not observe MSC migration towards the surface of modules, or differentiation towards SMCs or endothelial cells. This was unexpected considering the fact that many previous reports showed that growth factors and cytokines present in the medium together with applied mechanical forces can stimulate MSC differentiation [16, 37]. A possible explanation is that in our system, MSCs were not directly exposed to shear forces. Also, the flow rate was lower than previously tested by Khan et al [32]. Another observed difference was the absence of MSC migration from the interior of individual modules towards the perimeter, and lack of SMA expression, indicating that MSCs did not differentiate towards a smooth muscle cell phenotype. MSCs embedded in the modules also did not show any expression of endothelial-specific markers. These results confirm our previous findings, where Matrigel stimulation was necessary for induction of endothelial differentiation of MSCs [13].

EL-MSCs which were subjected to flow conditions for 7 days increased in number both in the samples with and without embedded MSCs. This was not observed with HUVEC coated modules or in the study of Khan et al [32] that used rat aortic endothelial cells. It remains to be assessed whether this is due to the difference in the type of cells used, the differentiation protocol to which the EL-MSCs have been exposed or whether the immortalized nature of the EL-MSCs plays a role. Proliferating EL-MSCs were expressing the endothelial marker CD31, which indicates that even 7 days after Matrigel induction, these cells maintained their endothelial phenotype. This clearly shows that no dedifferentiation occurs in the tested conditions and we speculate that EL-MSCs, upon implantation *in vivo*, are able to maintain their endothelial phenotype in these conditions as well. Since one of the current challenges in applying MSCs in various therapies is to lock them in the desired differentiation stage [38], our study provides a good example of a differentiation protocol that allows for obtaining a stable phenotype of MSCs. The lack of SMA and desmin expression indicates that EL-MSCs did not differentiate into pericytes.

Remodeling is a critical and still not fully understood parameter determining the fate of tissue engineered constructs *in vivo*. Generally, implanted constructs are spontaneously invaded by host vasculature as a response to hypoxia signals released by the cells within the construct [39]. This process, when occurring spontaneously, is too slow to provide a proper level of oxygen and nutrients to allow cell survival in large grafts. In addition, for some patients, spontaneous vascularization will not be

progressing well due to the patient's age or other factors compromising normal angiogenesis such as PVD [40]. Our *in vitro* results indicate that collagen modules covered with EL-MSCs can be valuable for various therapeutic applications. As shown in this *in vitro* study, EL-MSCs can proliferate on the surface of collagen modules. Increased number of cells in the place of implantation, which is hypoxic, will result in increased signals that are pro-angiogenic and, therefore, attract more vessel in-growth. We also tested whether the presence of embedded MSCs is beneficial for the EL-MSCs performance. We used Masson's trichrome staining for this purpose, since this method has been considered as the best for visualizing functional vessels connected with host vasculature network (presence of erythrocytes). The addition of MSCs did not influence the number of vessels within the sample 7 days after implantation. The situation changed after 21 days, where significantly fewer vessels within the sample were observed. We hypothesize that this phenomenon is related to the normal healing process enhanced by the modulatory effect on the immune-system by MSCs [41]. Inflammation caused by the presence of the engineered construct and by the injection itself can have pro-angiogenic effects, because proteases released by recruited inflammatory cells can release reactive ECM-fragments and also activate ECM-bound pro-angiogenic growth factors [42]. Since bone marrow-derived MSCs can limit the inflammation, their presence in the construct can result in decreased density of vessels. This hypothesis can be supported by the reduced number of interstitial cells within construct with embedded MSCs. Such situation is typical for the tissue healing where vessel regression to the normal level is caused by apoptosis of endothelial and fibroblast cells. However, this assumption needs to be further investigated.

In conclusion, this paper presents an effective way of EL-MSC delivery *in vivo* which can be used to improve tissue vascularization. The method is simple and can be used for several applications including tissue engineered scaffold vascularization and PVD treatment.

5.5 Bibliography

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Chapter 6

Boosting angiogenesis and functional vascularization in injectable dextran-hyaluronic acid hydrogels by endothelial-like mesenchymal stromal cells

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Abstract

Angiogenesis and neo-vascularization are fundamental for the success of clinically relevant-sized tissue engineered (TE) constructs. The next generation of TE constructs relies on providing instructive materials combined with the delivery of angiogenic growth factors and cells to avoid tissue ischemia. However, the majority of materials and cell types screened so far show limited clinical relevance, either due to insufficient number of cells or due to the use of animal-derived matrixes. Here we investigated whether endothelial-like cells derived from mesenchymal stromal cells (EL-MSC) can be used for vascular TE in combination with injectable dextran-hyaluronic acid hydrogels (Dex-g-HA). These hydrogels can be easily modified, as demonstrated by the incorporation of vascular endothelial growth factor (VEGF). We examined *in vitro* the reciprocal influences between cells and matrix. Dex-g-HA enabled higher EL-MSC metabolic rates associated with optimal cell sprouting *in vitro*, compared to HUVECs. *In vivo* evaluation demonstrated absence of an acute inflammatory response and EL-MSCs incorporated within Dex-g-HA formed a functional vascular network integrated with the host vascular system. This work demonstrates that Dex-g-HA is an efficient delivery method of VEGF to induce angiogenesis. Additionally,

functional neo-vascularization can be achieved *in vitro* and *in vivo* by the combination of Dex-g-HA with EL-MSCs.

6.1 Introduction

The survival of tissue engineered constructs of clinically relevant sizes is critically dependent on angiogenesis and neo-vascularization. Cell survival within large scaffolds upon implantation is largely hampered due to limited diffusion of oxygen and nutrients ultimately leading to tissue necrosis [1–3]. Vascular tissue engineering aims at establishing a vascular network in the tissue engineered construct which can rapidly connect to the host vasculature. New vessel formation can be enhanced by designing instructive materials containing supplying angiogenic growth factors [4] or by the inclusion of endothelial cells [5]. Combining both strategies would be highly beneficial for the survival of tissue engineered constructs.

Endothelial progenitor cells (EPC) are established cell candidates for vascularization in tissue engineering [6, 7]. These cells are reported to be able to differentiate into mature endothelial cells both *in vitro* and *in vivo*. Yet their expansion potential appears to be insufficient for therapeutic application, since it is difficult to maintain endothelial characteristics of EPCs during prolonged expansion *in vitro* [8, 9]. However, the cell main sources: umbilical-cord veins and aortas are not autologous to the patient. The clinical use of allogenic ECs is not possible due to chronic immune reactions [10, 11]. Consequently, there is great need for a novel vascularising cell source. We have recently developed a procedure for efficient differentiation of mesenchymal stromal cells (MSC) into endothelial-like MSCs (EL-MSCs) [12]. Unlike EPCs and ECs, MSCs can easily be isolated and are capable of being further expanded. EL-MSCs are shown to induce functional vessel formation within engineered constructs [12]. Unfortunately, Matrigel Basement Membrane Matrix was necessary during the initial stages of the procedure. This prevents clinical translation as Matrigel is of a sarcoma origin it, is composed of a not fully defined composition and has a risk of pathogen transfer [13]. Therefore, another gel is needed to act as ECM for EL-MSCs.

Hydrogels are easily tunable to facilitate the formation of vasculature networks by providing instructive elements. We have recently addressed the enhancement of the biological properties of injectable enzymatically crosslinkable dextran-tyramine (Dex-TA) hydrogels by incorporating biofunctional polymers or by growth factor enrichment [14, 15]. To further promote tissue infiltration and cell-mediated hydrogel degradation, we generated hybrid biomimetic hydrogels by engrafting Dex-TA on hyaluronic acid (HA) [16]. HA is a main player in several biological functions such as cell proliferation and migration, morphogenesis, healing, inflammation and angiogenesis. Moreover, HA oligosaccharides are involved in the stimulation of cytokine secretion and endothelial cell proliferation [17, 18].

Herein we evaluate the angiogenic potential of this biomimetic hydrogel scaffold in combination with vascularising cells. This combinatorial approach maximized the therapeutic potential of MSCs and EL-MSCs by inducing functional neo-vascularization.

6.2 Materials and Method

6.2.1 Cell isolation and culture

An immortalized clone of human mesenchymal stromal cells (iMSCs, courtesy of Dr. Ola Myklebost, University of Oslo, Norway) and human umbilical vein endothelial cells (HUVEC, Lonza) were used. Cells were expanded as previously described [12].

6.2.2 Endothelial induction of MSCs

iMSCs (passage 25) were used for our endothelial induction protocol, as described previously [12]. Cells were seeded at a density of 3,000 cells per cm^2 on tissue culture plastic in EGM-2 and cultured for 10 days. After one day in static culture, shear force was applied using an orbital shaker (20 rpm). Cells that were cultured according to this protocol will be referred to as EL-MSCs.

6.2.3 Synthesis of HA grafted with Dex-TA

Copolymers of HA grafted with Dex-TA (Dextran-Tyramine), denoted as Dex-g-TA, were synthesized by a coupling reaction of Dex-TA-NH₂ (with primary amines) with HA using EDAC/NHS (Carbodiimide/N-Hydroxysuccinimide Ester) as coupling reagent, as previously described [16]. Briefly, amine-terminated dextran-tyramine conjugates (denoted as Dex-TA-NH₂) were synthesized by a two-step procedure. Dex-TA conjugates were first reacted with N-Boc-1,4-diaminobutane and sodium cyanoborohydride to end functionalize the dextran. In the second step, Dex-TA-NH-Boc (4.4 g) was dissolved in 110 ml of deionized water and after addition of 4.4 ml of TFA, the mixture was stirred overnight under nitrogen. Afterwards, sodium hyaluronate (1 g) was dissolved in 50 ml of MES (0.1 M), to which EDAC (9.4 mmol) and NHS (9.4 mmol) were added. After 30 minutes, a Dex-TA-NH₂ solution was added and the mixture was stirred under nitrogen for 3 days. The number of grafted Dex-TA chains per HA molecule was determined using ¹H NMR by comparing integrals of signals at δ 2.0 (acetamide methyl protons of HA) and δ 5.0 (dextran anomeric proton) and calculated to be 15. Enzymatic crosslinking was induced by HRP (Horseradish peroxidase) and H₂O₂ (hydrogen peroxide), using 0.25 mg HRP per mmol phenol groups and a H₂O₂/phenol molar ratio of 0.2. The final concentration of Dex-g-HA hydrogels was 10 wt%.

6.2.4 Chorioallantoic membrane (CAM) model

Fertilized eggs (local farm) were incubated at 37°C with 75% humidity. After 3 days, the eggs were punctured to remove 3 ml of liquid. A rectangle was sawed off the eggshell, the window covered with tape and the viable eggs with a developing membrane were placed back into the incubator. On day 7, 100 μ l of Matrigel (BD Biosciences) or Dex-g-HA hydrogels, with and without VEGF, were placed in contact with the CAM. Eggs without hydrogels were considered the controls (n=3 to 6). Four days after placing the hydrogels in contact with the membrane, images were collected.

Additionally, after sacrificing the embryos by ethanol injection, the CAM with the hydrogel, fixated in 10% formalin, was processed for further visualization.

6.2.5 *In vivo* inflammatory response study

The *in vivo* inflammatory response study was approved by the local animal ethical committee. Animal surgery and husbandry were performed in compliance with the regulations of the Central Laboratory Animal Institute (GDL), Utrecht University (Netherlands). The mice were anesthetized with an isoflurane/O₂ mixture. Hydrogel samples (100 μ l) were implanted subcutaneously in 8-weeks old wild-type male mice (Balb-c, Harlan). The same volume of polymer solution, without the addition of HRP and H₂O₂, was considered as the control. 1 week (n=6) and 4 weeks (n=6) post-implantation, the mice were euthanized via CO₂ asphyxiation and samples with adjacent skin were harvested, fixed with 10% formalin and processed for histology.

6.2.6 VEGF release profile

Dex-g-HA hydrogels (100 μ l) were loaded with human VEGF (100 ng/ml, R&D Systems), prior to gelation. After gelation, PBS (100 μ l) was added to each sample and incubated at 37°C, under agitation. The supernatant was collected and the PBS refreshed at every time point, for 21 days. Release of VEGF-A isoform was quantified by ELISA, according to manufacturer's instructions (human DuoSet ELISA, calibrated against a highly purified Sf 21-expressed recombinant human VEGF₁₆₅, R&D Systems). The cumulative release of VEGF is expressed as function of time.

6.2.7 Cell encapsulation and culture

Dex-g-HA polymer (10% wt) was dissolved in PBS. HUVECs, iMSCs, EL-MCSs or co-cultures of iMSCs+EL-MCSs or iMSCs+HUVECs were incorporated in Dex-g-HA hydrogels at 10 million cells/ml. The cells were resuspended in the polymer solution and crosslinking was induced by the addition of HRP and H₂O₂ (constructs' final volume was μ l). Equal volumes of Matrigel with EL-MSCs and cells on tissue culture plastic (TCP) cultured in 2D served as controls. 100 ng/ml of VEGF was mixed in the polymer solution prior to gelation. The constructs were cultured in basic or EGM-2 medium. Visualization and image acquisition were performed using Nikon Eclipse TE300. Pictures were taken at various time intervals (NikonDS-L2 camera).

6.2.8 Cell viability, DNA quantification and metabolic activity

A viability study on cells incorporated in Dex-g-HA hydrogels was performed with a live-dead assay and metabolic activity was assessed with the MTT assay. Data were normalized to the DNA content. For the Live/Dead assay, at day 14, the hydrogel constructs were stained with calcein AM/ethidium homodimer (Invitrogen), according to the manufacturer's instructions [19]. Hydrogel/cell constructs were visualized using fluorescence microscopy (Zeiss). The MTT staining was performed at day 14 (10% MTT solution (5 mg/ml, Gibco), 2 hours incubation, 37°C). Hydrogel/cell constructs

were then visualized under a microscope (Nikon SMZ-10A). To quantify metabolic activity of the cells encapsulated in the hydrogels, the constructs were incubated with 10% MTT solution (4 hours, 37°C), the MTT/culture medium solution was removed and the formazan was extracted with DMSO. The quantification of formazan was performed with a microplate reader (Bio-TEK Instruments). Values were corrected for DNA content (CyQuant DNA kit), according to the manufacturer's description (Molecular Probes). The assays were performed after 1 and 14 days in culture.

6.2.9 Histological evaluation *in vitro*

Dex-g-HA-MSCs-constructs were cultured in basic medium, basic medium supplemented with 50ng/ml VEGF and in EGM-2 medium for 21 days, fixed in 10% formalin, embedded in cryomatrix (Cryomatrix, Shandon), cut in 10 μ m sections (cryomicrotome, Leica) and stained with either phalloidin conjugated with AlexaFluor 488 and counterstained with DAPI or hematoxylin and eosin (H&E, Sigma-Aldrich) according to manufacturers' protocols. Samples were photographed using a Nikon Elipse E600 microscope.

6.2.10 RNA isolation and quantitative PCR

Total RNA was isolated (Nucleospin RNA II kit, Bioke) according to the manufacturer's protocol. First strand cDNA was synthesized (iScript, Invitrogen) according to the manufacturer's protocol. qPCR was performed in a MyiQTM single-color real-time PCR detection system (BioRad). Expression of genes was calculated relative to major histocompatibility complex (B2M) levels by the comparative Δ CT method. Primers used in the study are: CD31 F:5'-TCTATGACCTCGCCCTCCACAAA-3', R:5'-GACGGTGTCTTCAGGTTGGTATTTC-3'; VEGF receptor 2 (KDR) F:5'-ACTTTGGAAGACAGAACCAAATTATCTC-3', R:5'-TGGGCACCATTCACCA-3'; von Willebrand factor (vWF) F:5'-TGCTGACACCAGAAAAGTGC-3', R:5'-AGTCCC CAATGGACTCACAG-3'; B2M F:5'-GACTTGTCTTTCAGCAAGGA-3', R:5'-ACAAAGTCACATGGTTCACA-3'.

6.2.11 Construct implantation

Male 6-week old NMRI-nu mice (Harlan) were anesthetized with a mixture of isoflurane/oxygen, after which constructs were subcutaneously implanted in four pockets. Dex-g-HA gels without cells, with embedded HUVECs, with HUVECs together with MSCs, with MSCs alone, with combination of MSCs and EL-MSCs and with EL-MSCs alone were implanted. Two weeks after implantation, the mice were sacrificed and implants were recovered (n=6), fixed in 10% formalin, embedded in paraffin and sectioned at 5 μ m before staining.

6.2.12 Histochemical analysis of implanted constructs

H&E staining and Masson's trichrome (Merck Chemicals) staining were performed according to manufacturers' protocols. Samples were photographed using a Nikon

Elipse E600 microscope. Based on Masson's trichrome staining vessels were counted manually by 3 people blinded to the sample composition. Six areas of each sample (6 samples per condition) were used per quantification.

For CD31 staining antigen retrieval was performed with IHC-Tek Epitope Retrieval Solution (IHC World). Sections were then incubated with mouse-anti-human CD31 primary antibody (Dako), which does not cross-react with mouse tissue. Afterwards, biotinylated horse anti-mouse secondary antibody (Antibodies-online.com) was applied. Slides were developed with the Labeled Streptavidin Biotin (LSAB) method with DAB Chromogen (IHC World) and weakly counterstained with Mayer's hematoxylin (Sigma-Aldrich).

6.2.13 Statistics

Each experiment was performed in triplicate. Data requiring multiple comparison test was analysed in SPSS (PASW statistics) using one-way Anova followed by Tukey's multiple comparison test ($P < 0.05$).

6.3 Results

6.3.1 Hydrogel integration in vascular network and inflammatory response

First, we evaluated the interaction of Dex-g-HA hydrogels with a developing vasculature system using the chick CAM model [20]. Dex-g-HA with and without VEGF was compared to a negative control without any implant and to Matrigel, commonly used matrix for EC culture and encapsulation (Figure 6.1A). We observed that the Matrigel samples did not adhere to the chick membrane resulting in no interaction with the forming vasculature. In contrast, Dex-g-HA showed integration with the membrane by attachment and orientation of the forming blood vessels. Intricate vasculature formation was particularly visible around Dex-g-HA hydrogel loaded with VEGF.

We evaluated the immune response towards Dex-g-HA by subcutaneous implantation in mice. The inflammatory response for both hydrogels and polymer precursor (Dex-g-HA without HRP and H_2O_2) was assessed based on the thickness of fibrous tissue layer and the abundance of extravasated immune cells, 7 and 28 days post-implantation (Figure 6.1B). After 7 days, capsule formation was detected around the hydrogels, whereas no reaction occurred when the polymer alone was injected. At day 28, the capsule detected earlier showed a clear reduction in thickness. This is a typical sign of a foreign body response and not of an acute inflammatory response.

6.3.2 VEGF incorporation in Dex-g-HA

To enhance angiogenesis, VEGF was incorporated in the Dex-g-HA hydrogel network prior to gelation. Quantification of the VEGF released from the hydrogel was assessed

Figure 6.1: Dex-g-HA hydrogel characterization. CAM assay of dextran gel engrafted with HA with or without VEGF and Matrigel (A). Gels were placed in open window in viable eggs and incubated for 4 days. After that representative pictures were taken and vessel ingrowth in the sample was compared. Black arrows indicate gel position. Dextran *in vivo* characterization: inflammatory response (B). Dex-g-HA and polymer solution (without the addition of HRP and H_2O_2) was implanted for 1 week and 4 weeks ($n=6$), after that samples were recovered and examined. Dex-g-HA gel is marked with a *, black line indicates the thickness of fibrous tissue.

by ELISA and the cumulative release profile was determined up to 21 days (Figure 6.2). VEGF concentration of 100 ng/ml was chosen based on previous studies sug-

gesting that such concentration was likely to enhance ECs' mitogenic and sprouting potential [21]. Two distinct phases can be identified in the release profile, consisting of an initial burst release of the VEGF occurring in the first 100 hours, followed by a continuous yet low release until the last day of assessment. At day 21, 65% of the incorporated VEGF was released. Remaining 35% was still entrapped in the gel or could not be detected.

Figure 6.2: Release profile of VEGF from Dex-g-HA hydrogel. Cumulative release of VEGF from Dex-g-HA hydrogels monitored over 21 days. Initial concentration of VEGF was 100 ng/ml. Indicated values represent mean ($n=3$).

6.3.3 Cell viability and metabolic activity within Dex-g-HA

To confirm the absence of cytotoxicity of Dex-g-TA upon cell encapsulation and culture, we assessed the viability and metabolic activity of HUVECs, MSCs and EL-MSCs. We showed that no cytotoxicity was induced after 14 days in culture (Figure 6.3A). Only a limited amount of dead cells (red) could be detected when HUVECs were cultured for 14 days. The MSCs and EL-MSCs were fully viable (green) and uniformly distributed throughout the Dex-g-HA hydrogel.

Cells' metabolic activity was initially assessed using MTT staining. After 14 days of culture, both MSCs and EL-MSCs were more metabolically active than HUVECs (Figure 6.3B). The quantification of this phenomenon (Figure 6.3C) demonstrated that the metabolic activity of HUVECs per cell is initially twice as high as the activity of MSCs and EL-MSCs. At day 14, we observed a significant decrease in metabolic activity of HUVECs. In contrast, the metabolic activity of both MSCs and EL-MSCs increased significantly from day 1 to day 14. This increase was more pronounced with

Figure 6.3: Cells in Dex-g-HA hydrogel. *In vitro* characterization two weeks after seeding: live dead assay after 14 days in culture (A), MTT staining after 14 days in culture (B). Metabolic activity of cells in Dex-g-HA 1 and 14 days after seeding corrected for cell number (C). Scale bar 400 μ m. Indicated values represent mean (n=3) with standard deviation, *** denotes statistical significance ($P < 0.001$).

EL-MSCs, which suggests that they have a superior metabolic performance compared to MSCs when incorporated in Dex-g-HA for prolonged culture.

6.3.4 Capillary network assembly in Dex-g-HA

As next step, we evaluated the influence of Dex-g-HA on the behaviour of cells grown within it. Consistent with the results of MTT assay, we did not find any evidence of HUVEC activity within Dex-g-HA. ECM remodeling was not detected in gel samples with HUVECs as indicated by lack of sprouting and capsule formation. Interestingly, this was not the case when gels containing MSCs and EL-MSCs were examined. On day 21 of *in vitro* culture tubulogenesis was observed in the form of sprouting and branching (Figure 6.4A).

We applied different media to determine major factors influencing capillary network formation. Our results suggest that the level of FBS in the medium is of major importance. Cells cultured in EGM-2 with 2% FBS content sprouted less efficiently than cells cultured in basic medium with 10% FBS. Moreover, addition of VEGF to the gel clearly boosted sprouting (Figure 6.4B). Nevertheless, even in conditions without additional growth factors, capillaries with lumens were observed, proving the potential of Dex-g-HA hydrogels to support cell sprouting.

6.3.5 Molecular profile and vascular morphogenesis of cells

We characterized the gene expression profile of cells seeded in Dex-g-HA hydrogels. At day 7 of *in vitro* culture, we observed that expression of endothelial markers CD31, KDR and vWF in HUVECs seeded in the gel is lower than in HUVECs seeded on TCP (considered as control). Nevertheless, at day 14 the expression level of these markers within the gels matched the control and kept on increasing (day 21), reaching 8 fold induction (day 28) (Figure 6.5).

Very promising results were also obtained after endothelial induction of MSCs within Dex-g-HA hydrogels. Endothelial gene expression in both MSCs and in EL-MSCs was presented as fold induction compared to cells cultured on TCP. Our previous study [12] showed that in TCP culture neither MSCs nor EL-MSCs express any of the studied markers, while Matrigel stimulation can induce the cells towards an endothelial-like phenotype. Our results show that Dex-g-HA can substitute Matrigel. For both MSCs and EL-MSCs, the expression of CD31 and KDR was increased after 21 days of culture within Dex-g-HA gels. Consistent with our previous results [12], the MSCs used in this study did not show any induction in vWF expression neither in Matrigel nor in Dex-g-HA (Figure 6.5).

6.3.6 Functional vascular network formation within dex-g-HA hydrogels

To determine whether cells embedded in Dex-g-HA hydrogels can stimulate vascular network formation, we implanted into nude mice Dex-g-HA gels in six variants, namely without cells, with embedded HUVECs, with both HUVECs and MSCs, with MSCs alone, with a combination of MSCs and EL-MSCs and with EL-MSCs alone. Two weeks after implantation hardly any vessels were observed within empty gels, as well

Figure 6.4: Cells in Dex-g-HA hydrogels after 21 days of *in vitro* culture. Phase-contrast microscopy microphotograph of cell monoculture in Dex-g-HA hydrogel (A). Sprouting cells forming capillary-like structures are indicated by black arrows. Influence of culture conditions for MSC morphology after 21 days of *in vitro* culture (B). The effect of introduction of VEGF in Dex-g-HA hydrogel was accessed to determine its contribution to the formation of the capillary-like structures. Lumens are indicated by arrows. Scale bar 100 μm .

as within constructs with embedded HUVECs and HUVECs/MSCs. On the other hand, in constructs with embedded MSCs, MSCs/EL-MSCs and EL-MSCs, vessels were present in the gels in the periphery and in the middle of the sample (Figure 6.6A).

Quantification of vascular network confirmed these observations. No significant differences were found between samples with empty gel, gel with HUVECs or HU-

Figure 6.5: Endothelial marker expression in cells cultured in Dex-g-HA. Gene expression is represented as fold induction compared to cells seeded on tissue culture plastic (HUVECs and MSCs). CD31, KDR and vWF expression were observed at four time points in case of HUVEC culture (7, 14, 21 and 28 days) and at one time point (21 days) in case of MSC and EL-MSC culture. VEGF was incorporated in the Dex-g-HA hydrogel to determine its influence on the endothelial marker expression. Indicated values represent mean ($n=3$) with standard deviation. * denotes statistical significance ($P<0.05$), ** denotes statistical significance ($P<0.01$), *** denotes statistical significance ($P<0.001$).

Figure 6.6: *In vivo* vascularization of Dex-g-HA construct combined with cells. Vessel in-growth *in vivo* in 3D constructs. Tissue construct sections were stained with Masson's trichrome staining (blue for collagen, red for erythrocytes) and counterstained with hematoxylin (brown) (A). Quantitative analysis of vessels in Dex-g-HA constructs (B). Number of vessels per sample was quantified by three people blinded for the conditions. ** denotes statistical significance ($P < 0.01$), *** denotes statistical significance ($P < 0.001$).

VECs/MSCs. Embedded MSCs significantly stimulated host vessel formation in the construct and the effect was further improved by addition of EL-MSCs. Compared to other conditions, the highest number of vessels was observed in gels with embedded EL-MSCs (Figure 6.6B).

To determine whether the implanted cells participated in vessel formation, human CD31 staining was performed. CD31 positive cells were observed in constructs seeded with all cell types (Figure 6.7). CD31 positive lumens were observed only in three of the tested conditions, namely in samples with HUVECs+MSCs, with EL-MSCs and with EL-MSCs+MSCs. The majority of vessels in the gels did not stain positive for human CD31. This suggested that these vessels were derived from ingrowing mouse endothelial cells, which was mostly stimulated in gels loaded with EL-MSCs.

Figure 6.7: CD31 staining in Dex-g-HA constructs. Immunohistochemistry was used to stain for human CD31. Dex-g-HA is stained blue (*), CD31 positive cells are brown, aggregates of HUVEC are indicated by red arrows, CD31 positive lumens are indicated by black arrows.

6.4 Discussion

Tissue engineered constructs require proper vascularization for a successful therapeutic use. Since the contribution of ECM components in regulation of vascularization is widely recognized [22, 23], there is a need to provide clinically relevant matrixes which accelerate vessel in-growth and, improve cell survival. In this study, we exploited an injectable HA-based biomimetic hydrogel to encapsulate different cell types, ultimately determining the cell-biomaterial combination that showed superior angiogenic potential.

The biomaterial reported herein is a hybrid of HA, a key ECM instructive component directing proliferation, migration and angiogenesis [24], and dextran, recently

reported as a promising biomaterial to enhance neo-vascularization [25, 26]. We have also entrapped VEGF in Dex-g-HA hydrogel further boosting cell survival and tissue formation. We demonstrated that Dex-g-HA, with and without incorporation of VEGF, enabled the formation of blood vessels. Evaluation of the immune response triggered by Dex-g-HA revealed an absence of an acute inflammatory response. These highly favourable results are strongly indicative of the biocompatibility of the biomaterial chosen, as shown previously [24, 26].

An important finding in our study was the possibility to incorporate VEGF in the Dex-g-HA. It has been previously reported that VEGF incorporated in HA-containing hydrogels induces the production of metalloproteinases [27], which actively contribute to the ECM remodelling. The presence of VEGF causes the reduction of ECM mechanical resistance, allowing cells to migrate and reorganize. We used VEGF for proof-of-principle, demonstrating that it is achievable to incorporate pro-angiogenic factors in this matrix system. The achieved pattern of first bursty followed by steady VEGF release is highly desirable, since a high VEGF concentration is particularly important during the initial stages of scaffold implantation to trigger angiogenesis and neo-vasculature formation [21].

Another issue underlying construct vascularization is the selection of cells to create vessel network. Widely investigated endothelial or endothelial progenitor cells so far did not meet expectations due to: a) problems with isolation of large quantities of autologous cells; b) immune-rejection of allogenic cells; and c) expansion of these cells without losing their differentiation capacity in both cases [28]. Therefore, we propose another cell type, namely MSCs, whose pro-angiogenic paracrine effect and potential to become endothelial-like cells has been shown lately by several groups including our own [12, 29–31]. We have explored the potential of MSCs in two ways: triggering the commitment into the endothelial lineage and as a powerful enhancer of angiogenesis in a co-culture environment.

Although many studies focus on the effect of the biomaterial on cell viability, none of them compares the metabolic activity per cell using different cell types. Our results showed that all analysed cell types: MSCs, HUVECs and EL-MSCs, incorporated in Dex-g-HA and cultured *in vitro* were fully viable. Interestingly, HUVECs showed very low metabolic activity rates at later time points, likely due to their inability to remodel the surrounding matrix or lack of stimulation to interact with it. The opposite observation was made for EL-MSCs, which showed the highest metabolic rate after 14 days in culture. This significant increase strongly suggested that EL-MSCs are able to interact well with the Dex-g-HA. This is highly advantageous, as metabolically active cells are expected to interact more intensively with the surrounding matrix, by enhanced proliferation, migration and/or matrix remodelling [32, 33]. More active cells are likely to contribute more to the development of a vascular network via for example by higher secretion of pro-angiogenic factors. As the vascular inductive effect of the Dex-g-HA gel represents one of the most innovative aspect of the present work, we propose two types of mechanisms that might justify the observed results: a) unique

hypoxic conditions created within this gel type, and/or b) the degradation products of hyaluronic acid are strongly triggering a pro-angiogenic response.

To generate a gel suitable for use in vascular tissue engineering, several modifications such as integrin binding domain or growth factor introduction have been studied [34, 35]. In our work we demonstrate that MSCs and EL-MSCs do not require complicated gel modifications to interact with the hydrogel, sprout, and ultimately form a blood vessel. Additional introduction of VEGF in the system further improved the ability of those cells to form capillary-like structures. This result showed higher capabilities of both naïve and differentiated MSCs for to interaction with matrix as compared to endothelial cells like HUVECs. Furthermore, we showed that Dex-g-HA-induced changes in the molecular profile of MSCs and EL-MSCs were similar to the ones induced by Matrigel. Chemically defined Dex-g-HA can, therefore, replace undefined Matrigel in endothelial differentiation of MSCs. Additionally, although HUVECs within the Dex-g-HA did not sprout, constant increase in endothelial marker expression proved that these cells were not senescent.

Once implanted *in vivo*, Dex-g-HA constructs were stable and did not show morphological signs of fast degradation in the host tissue. In constructs without cells or seeded with HUVECs host tissue and vessels did not grow into the gel. This effect is in line with the *in vitro* data, where we showed that HUVECs have a low metabolic activity within Dex-g-HA, probably followed by decreased amount of released pro-angiogenic factors. In constructs with MSCs or EL-MSCs, the trophic effect of incorporated cells was much higher, boosting angiogenesis from host blood vessels. As expected, EL-MSCs were most efficient in attracting vessel ingrowth. Nonetheless, MSC influence on the number of vessels within the construct was also beneficial as compared to both empty and HUVEC-seeded gels. MSCs are known to secrete a wide range of cytokines and growth factors, which have both immunomodulatory and regenerative properties [31]. It is likely that the trophic effect of EL-MSCs is even larger, since these cells were cultured in growth factor-rich medium that can induce additional pro-angiogenic molecule secretion. The fact that constructs seeded with a combination of HUVECs and MSCs were not penetrated by host tissue and vessels is probably a result of decreased MSC number in the construct. Since HUVECs alone were shown to not have any effect on construct vascularization, decreasing the number of MSCs was likely to reduce their beneficial influence. Human CD31 staining was performed to determine whether seeded cells played more than a trophic role in attracting vessel in-growth. This led to the observation that HUVECs within the Dex-g-HA tended to form aggregates. In samples seeded only with HUVECs, no human CD31 positive lumens were observed. Similar results were obtained with constructs seeded only with MSCs, as we found CD31 positive cells, which did not form lumens. Opposite results were obtained when HUVECs and MSCs were seeded together. In these conditions, both CD31 positive aggregates and CD31 positive lumens were observed. These results may have two possible explanations: a) the presence of MSCs caused sufficient matrix remodeling to allow HUVECs to spread and form lumens, or b) MSCs benefited from the influence of HUVECs. The second hypothesis can be supported by previous study of Rouwkema *et al.* [36], reporting that MSCs cultured

in the presence of HUVECs started to express endothelial markers. This effect was suggested to be caused both by cell-to-cell contact and by trophic effects. When EL-MSCs were present, both single CD31 positive cells as well as CD31 positive lumens were observed. This allows us to conclude that EL-MSCs can be used without any supporting cells to create vessel network within tissue engineered constructs.

In conclusion, Dex-g-HA injectable hydrogels are capable of sustaining neo-vasculature formation both *in vitro* and *in vivo*, and can be used as an effective delivery method of VEGF to induce angiogenesis. Additionally, combining Dex-g-HA with EL-MSCs enables higher cell metabolic rates. This effect leads to increased secretion of pro-angiogenic factors associated with optimal cell sprouting *in vitro*, ultimately leading to improved neo-vasculature formation *in vivo*. The boost in angiogenesis and vessel ingrowth in EL-MSCs loaded hydrogels is due to 1) endothelial differentiation of these cells and 2) due to increased trophic potential. This strategy can be applied in combination with other scaffolds or by itself to avoid or reverse tissue ischemia.

6.5 Bibliography

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Chapter 7

Human mesenchymal stromal cells as single cell source for both bone and vessel engineering

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Abstract

Current bone tissue engineering approaches need further optimization on the amount of bone deposited by the grafted osteoprogenitors and the rate at which the graft is vascularised. We have recently described a modular bone tissue engineering system, comprising spheroids of human mesenchymal stromal cells (hMSCs), calcium phosphate microparticles and in-situ-setting autologous fibrin thrombin gel as carrier. Its modular nature allowed optimization of critical parameters such as material properties, handling and culture conditions. In this manuscript, we investigate the possibility to use MSCs as the single source for both bone and endothelial engineering, in order to create a more physiological bone graft and improve vascularization. To this end, we differentiated hMSCs towards endothelial-like cells. Here we combine these developments and demonstrate that endothelial-like cells can improve the bone forming capacity of hMSCs. We also discuss possible explanations for observed improvements and propose further system modifications that could lead to development of clinically relevant bone grafts.

7.1 Introduction

Even though bone has amazing self-healing capacity, in several cases such as large defects, non-unions or tumor-resections, the healing capabilities of bone is not sufficient [1]. In addition, osteoporosis is an epidemic disorder that increases with advanc-

ing age. In these patients fractures often occur in which compression of the cancellous bone causes a defect that has to be filled with bone or bone substitutes.

The current golden standard for treatment of bone defects is harvesting of autologous bone from the patient's body (e.g. the iliac crest) and transplantation into the defect. There are no issues with immune-rejection or pathogen-transfer, but the downside is the limited availability of bone, additional pain caused by second wound and risk of infection during acquisition [2]. Several other strategies for bone defect grafting have been developed and are currently used in the clinic, each with their own advantages and limitations. Allogeneic bone grafting can result in satisfactory but limited outcome compared to autografting, since allografts are devitalized to avoid an immunological response from the host. Such grafts are also associated with risk of transmitting diseases. In case of synthetic grafts, two major problems typically occurring are unsatisfactory mechanical strength (e.g. in the case of calcium phosphate ceramics) or poor integration with surrounding tissue (e.g. metal grafts) [3]. Furthermore, metal implants are not biodegradable and thus do not allow full tissue regeneration.

Due to unsatisfactory results of bone replacement with artificial materials another approach based on tissue engineering principles was suggested and developed [4]. The concept of combining scaffolds materials with cells that will deposit new bone tissue and allow for good integration with surrounding tissue was widely investigated and was shown to offer excellent results in numerous animal models [5–7], including critical sized defects. Several clinical trials on humans have been performed with such various outcomes [4, 8] demonstrating that standardization and improvement of existing protocols is crucial. Several issues need to be addressed to achieve this, including the type and number of used cells, culture conditions and applied material.

Cells used in bone tissue engineering (BTE) can be isolated from several sources, each associated with specific advantages and drawbacks. Various types of both mature cell types including osteoblasts and osteoprogenitor cells together with several types of stem cells, including embryonic stem cells, induced pluripotent stem cells and adult stem cells were investigated as potential source for BTE. Due to problems with acquisition of relevant number of osteoblast and osteoprogenitor cells [9] and the teratoma formation associated with the use of embryonic and pluripotent stem cells [10], adult stem cells are currently one of the most interesting cell types for BTE applications.

Mesenchymal stromal cells (MSC), often referred to as mesenchymal stem cells, were first described by Friedenstein *et al.* [11], and are adult stromal cells that can be isolated from various tissues including bone marrow, adipose tissue, trabecular bone, placenta, periosteum, dental pulp and femur [12–15]. They have an excellent proliferation capacity and were shown to be able to differentiate among others into osteogenic, adipogenic, chondrogenic, muscle and endothelial lineages [16–19]. It is relatively easy to obtain sufficient quantities of MSCs from adults and by that, they can be used as an autologous source of therapeutic cells. Several studies were performed to optimize MSC performance in bone tissue engineering including solving

the problems with donor variation [20] and necessity of *in vitro* expansion [21]. Still, the main issue is the total amount of newly deposited bone *in vivo*, where human cells do not perform as well as previously investigated animal-derived MSCs. Therefore, many strategies aim at further improving the efficiency of BTE. Changes in the culture conditions of MSCs *in vitro* were applied, including 3D culture and culture in bioreactors [22], pre-differentiation towards osteogenic lineages [23] and combining these cells with various scaffold materials [24].

We have recently developed a system that allows for efficient and reproducible bone formation [21]. It consists of aggregated hMSCs combined with calcium phosphate micro particles [21], and contains an in-situ-setting autologous fibrin thrombin gel as carrier. In house, we refer to it as the micro-aggregate system. The system requires little time to generate, since there is no need for *in vitro* culture and additionally, all components used are clinically approved. Furthermore, since the delivery can be optimized to be injectable, the method is minimally invasive to the patient.

Rouwkema *et al.* [25] demonstrated that a trophic effect of endothelial cells can improve the bone forming capacity of hMSCs. To further improve our system without the necessity of applying clinically irrelevant cells, we decided to combine it with yet another relevant cell type: endothelial-like MSCs (EL-MSC). These cells can easily be obtained from hMSCs [19] from all tested donors [26] and were shown to possess several endothelial characteristics. The use of a single source of progenitor cells reduces the complexity of the system and allows for easier clinical implementation. It also allows to obtain cells for therapy during one single surgery.

This work presents the beneficial influence of EL-MSCs on the amount of engineered bone and discusses the possible explanation for the observed improvement.

7.2 Materials and methods

7.2.1 Isolation and culture

Human mesenchymal stromal cells (hMSCs) were isolated from human bone marrow from donors with written informed consent [27]. Aspirates were resuspended using a 20G needle and plated at a density of 0.5 million mono-nucleated cells per cm^2 . Cells were grown in MSC proliferation medium which contains minimal essential medium (alfa-MEM, GIBCO) supplemented with 10% fetal bovine serum (FBS, Lonza), 100 U/ml penicillin (GIBCO), 10 $\mu\text{g}/\text{ml}$ streptomycin (GIBCO), 2 mM L-glutamin (GIBCO), 0.2 mM L-ascorbic acid 2-phosphate magnesium salt (ASAp, Sigma-Aldrich) and 1ng/ml basic fibroblast growth factor (bFGF, Fisher Scientific) at 37°C in a humid atmosphere with 5% CO_2 . hMSCs used in this study have been characterized previously (unpublished data) according to the set of standards proposed by the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy [28]. We also have data concerning the osteogenic differentiation of these cells which allows us to select donors with varying bone-forming potential for further study. Here we used MSCs that were shown before to express

osteogenic markers upon differentiation and form bone *in vivo* (unpublished data). Cells were expanded up to passage 2. For further experiments hMSCs were cultured in basic medium (alfa-MEM supplemented with 10% FBS, 100 U/ml penicillin, 10 μ g/ml streptomycin, 2 mM L-glutamin and 0.2 mM ASAp).

7.2.2 Endothelial induction of MSCs

hMSCs from passage 2 were used for endothelial induction as described previously [19]. Briefly, cells were seeded at a density of 3,000 cells/cm² on tissue culture plastic in endothelial growth medium (EGM-2, Lonza) and cultured for 10 days. After one day in static culture, shear force was applied using an orbital shaker at a rate of 20 rpm. Cells that were cultured according to this protocol will be referred to as EL-MSCs.

7.2.3 Capillary-like structures formation on Matrigel

Wells of 6-well plates were covered with 1 ml of growth factor reduced Matrigel (BD Bioscience) diluted 1:1 in EGM-2 without growth factors. Cells were seeded at a density of 30,000 cells/cm² and cultured in a humid atmosphere with 5% CO₂ for 24 hours. The formation of capillary-like structures (CLS) was observed after 24 hours using an inverted microscope (Nikon Eclipse TE300). Pictures were taken using a Nikon DS-L2 camera.

7.2.4 RNA isolation and quantitative PCR

Total RNA was isolated using TRIZOL reagent according to the manufacturer's protocol. Briefly, 1 ml of Trizol reagent was added per T25 flask (cells cultured in basic medium) or per well (cells cultured on Matrigel in 6-well plates and cells cultured in micro-aggregate system). Samples were incubated for 5 min at room temperature to allow complete dissociation. Phase separation was performed by adding chloroform, and then samples were shaken vigorously for 15 seconds and incubated for 3 min at room temperature. After that samples were centrifuged at 12,000 x g for 15 min. RNA was precipitated by mixing the aqueous phase with isopropyl alcohol followed by 10 min incubation at room temperature. Samples were centrifuged again and the remaining RNA pellet was washed with 75% ethanol. The obtained samples were dissolved in water. The quantity and quality of RNA was analyzed using spectrophotometry (ND-1000 spectrophotometer).

For first strand cDNA synthesis, 500 ng of RNA was used in combination with Superscript II (Invitrogen) according to the manufacturer's protocol. One μ l of 3x diluted cDNA was used for further gene amplification. PCR was performed in real time PCR machine (BioRad). Data was analyzed using Bio-Rad iQ5 software. Expression of endothelial genes was calculated relative to major histocompatibility complex (B2M) levels by the comparative Δ CT method. Primers used in the study are listed in Table 7.1.

CD31 (Platelet Endothelial Cell Adhesion Molecule-1)	F 5' TCTATGACCTCGCCCTCCACAAA 3' R 5' GAACGGTGTCTTCAGGTTGGTATTTCA 3'
VEGF (Vascular Endothelial Growth Factor)	Commercially bought from SA Biosciences
ALP (alkaline phosphatase)	F 5' GACCCTTGACCCCCACAAT 3' R 5' GCTCGTACTGCATGTCCCCT 3'
BMP2 (bone morphogenetic protein 2)	Commercially bought from SA Biosciences
BSP (bone sialoprotein)	F 5' TGCCTTGAGCCTGCCTGCTTCC 3' R 5' CAAAATTAAAGCAGTCTTCATT 3'
OP (osteopontin)	F 5' CCAAGTAAGTCCAACGAAAG 3' R 5' GGTGATGTCCTCGTCTGTA 3'
OC (osteocalcin)	F 5' GGCAGCGAGGTAGTGAAGAG 3' R 5' GATGTGGTCAGCCAACTCGT 3'
B2M	F 5' GACTTGTCTTTTCAGCAAGGA 3' R 5' ACAAAGTCACATGGTTCACA 3'

Table 7.1: Primers used for qPCR.

7.2.5 Generation of cell aggregates

Microwells in agarose chips were prepared as described previously [21, 29, 30]. Briefly, PDMS (poly-dimethylsiloxane) stamps (each to prepare 1400 wells of 400 μm in diameter and 200 μm in depth) were disinfected in 70% ethanol, placed in the wells of 6-well plates and covered with 3% agarose (Ultra-pure agarose, Invitrogen). Upon solidification, the agarose chips were placed in 12-well plates and wetted with culture medium. 1 ml of cell suspension containing 1.5 million hMSCs was uniformly dispersed over the wells on each chip. The chips were then shortly centrifuged at 1500 rpm to facilitate settling down of the cells. Cell aggregates spontaneously formed within the following 24 hours of culture in basic medium.

7.2.6 Platelet gel acquisition and preparation

Platelet gel was obtained and prepared as described before [21]. Briefly, a standard thrombocyte apheresis procedure using a Terumo BCT Spectra/Trima apheresis unit was performed on healthy donors with written consent (Institute für Klinische Transfusionsmedizin, Braunschweig, Germany). Samples were stored at -80°C . At the time of the experiment, samples were heated to 37°C to lyse the cryopreserved platelets, release growth factors and form platelet lysate. 235 μl of 1 M calcium chloride was added per 10 ml of platelet lysate and mixed at 37°C for 10 minutes. As a result, the lysate separated into gel containing fibrin and liquid containing thrombin. To solidify the gel, remaining platelet lysate was mixed again with thrombin (at 1:1 ratio) which activated the clotting pathway and resulted in platelet gel formation.

7.2.7 Generation of the micro-aggregate system

Biphasic calcium phosphate (BSP) ceramic particles (average size of 53-63 μm) were produced as described before [31]. 20 μg of ceramic micro particles were put within each 10 ml tube. The cell aggregates were gently flushed from the agarose chips with basic medium and transferred to the tubes. Emptied chips were then examined for the possibility of remaining aggregates and in such a case flushed again to provide equal number of aggregates per sample. This procedure did not result in any changes in the shape or size of the aggregates [21]. Next, 10% or 50% (0.15 or 0.75 million) of hMSCs or EL-MSCs in suspension were added to each tube. The 10 ml tubes containing micro particles, cell aggregates and cells in suspension were gently centrifuged and the supernatant was removed. After that, 300 μl of the prepared thrombin was added to each tube followed by the addition of 300 μl of platelet lysate. The content of the tube was then gently mixed and pipetted into a well of a non-tissue culture treated, 12-well plate. Resulting constructs were then maintained in culture in micro-aggregate medium (DMEM (GIBCO) supplemented with 100 U/ml penicillin, 10 $\mu\text{g}/\text{ml}$ streptomycin, 0.2 mM ASAp, ITS, proline, 100 mM 11 mg/ml sodium pyruvate and dexamethasone) or EGM-2 medium at 37°C for one week (*in vitro* study) or proceeded to implantation after first 24 hours in culture in micro-aggregate medium.

7.2.8 Construct implantation and histochemical analysis

Male 6-week old NMRI-nu mice (Harlan) were anesthetized with a mixture of isoflurane and oxygen after which constructs were implanted in four subcutaneous pockets ($n=10$). Ceramic particles with 1.5 million of hMSCs in aggregates and 10% hMSC addition in suspension, ceramic particles with 1.5 million of hMSCs in aggregates and 10% EL-MSC addition in suspension, ceramic particles with 1.5 million of hMSCs in aggregates and 50% MSC addition in suspension and ceramic particles with 1.5 million of hMSCs in aggregates and 50% EL-MSC addition in suspension were implanted. Six weeks after implantation ($n=7$, due to sample loss caused by exposure to air), the mice were sacrificed and implants were recovered. Samples were fixed in 10% formalin, embedded in methacrylate and sectioned at 300 μm on a histological diamond saw (Leica saw microtome cutting system). A minimum of 6 sections were made from each sample. Sections were stained with basic Fuchsin and methylene blue to visualize bone formation. Quantitative histomorphometry was performed on the middle (4th) section of each sample of which high resolution digital pictures were obtained. Whole sample area and bone areas were semi-automatically selected based on color hue in GIMP 2 software (Select by Color Tool: Selects regions with similar colors) and percentage of bone area per sample was calculated.

7.2.9 Statistics

Each experiment was performed in triplicate. Data which required multiple comparison tests was analysed in SPSS (PASW statistics) using one-way Anova followed by Tukey's multiple comparison test ($P<0.05$). In case where comparisons between two groups were made, a Student's paired t-test was performed.

7.2.10 Ethics statement

Human mesenchymal stromal cells (hMSCs) were isolated from human bone marrow from donors with written informed consent. This study was carried out in strict accordance with the recommendations of Medisch Ethische ToetsingsCommissie Twente (Medical Ethical Research Committee Twente) and was approved by this Committee.

The animal study reported on in this manuscript was ethically assessed a priori by animal ethics committee 2010-III-10-125 DEC-Utrecht as required by Dutch law. Animals were housed at the Central Laboratory Animal facility (Utrecht University, Utrecht, The Netherlands), and experiments were approved by the local animal care and user committee Dierexperimentencommissie Academisch Biomedisch Centrum (DEC-ABC). All surgery was performed under isoflurane/oxygen anesthesia, and all efforts were made to minimize suffering. Animals were fed *ad libitum*.

7.3 Results and Discussion

7.3.1 Endothelial nature of EL-MSCs

To confirm the endothelial nature of differentiated hMSCs from donor 240, a Matrigel assay was performed and their gene expression profile was assessed. As described previously [19, 26], differentiated hMSCs formed capillary-like structures on Matrigel (Figure 7.1A). EL-MSCs obtained from the analyzed donor, after 24 hour culture on Matrigel, also expressed the endothelial marker CD31 (Figure 7.1B). These results demonstrated that the hMSCs isolated from donor 240 acquired endothelial characteristics, both phenotypical and functional. Based on our previous results (unpublished data) we can expect that donor 240-derived EL-MSCs, when implanted, would be able to attract in-growing host vessel and also contribute to their walls.

7.3.2 Micro-aggregate system preparation

Ceramic particles, cell spheroids, single cells and platelet gel were mixed together to generate tissue engineered constructs. The cells and scaffolds were rapidly encapsulated within the fibrin thrombin components of platelet gel (Figure 7.2A). Resulting constructs were cultured for 24 hours in micro-aggregate medium (for *in vivo* study) or one week in micro-aggregate or EGM-2 medium (for *in vitro* study). Already after 24 hours of culture the samples shrank significantly, but after that their shape did not change for one week (Figure 7.2A). Due to the presence of ceramic particles in the construct we were not able to observe whether MSCs or EL-MSCs formed capillary-like structures within the sample. Upon histological evaluation of the explants after 6 weeks large area of newly formed bone can be seen in which we observed osteocytes embedded in a mineralized extracellular matrix (Figure 7.2B). The presence of bone marrow areas indicated functional bone.

Figure 7.1: EL-MSC characterization. Matrigel assay with EL-MSCs (A). CD31 expression in MSCs and EL-MSCs obtained from donor 240 (B). *** denotes statistical significance ($P < 0.001$).

7.3.3 Effect of EL-MSCs on *in vivo* bone formation

To observe the influence of introduction of single-cell suspension on the amount of obtained bone, four conditions were analyzed, each including ceramic particles with 1.5 million hMSCs in aggregates combined with 10% hMSCs, 10% EL-MSCs, 50% hMSCs or 50% EL-MSCs added in suspension. The 10% hMSC group was used as benchmark. Histochemical analyses were performed on the explants after 6 weeks *in vivo*. Staining with basic Fuchsin revealed the presence of newly formed bone

Figure 7.2: Micro-aggregate system 30 min, 24 hours and 1 week after assembly. Cell aggregates (*), ceramic particles (black arrow) and cells in suspension (red arrow) can be observed (A). Bone formation within micro-aggregate system *in vivo*. Methylen blue and basic Fuchsin stained section shows large areas of mature bone (dark pink), osteocytes embedded in matrix (yellow arrow) and scaffold (white arrow). Six weeks after implantation hardly any osteoid (unmineralized matrix indicated by yellow star) can be seen.

within the samples (Figure 7.3A). Quantitative histomorphometry showed differences between samples with MSCs and EL-MSCs (Figure 7.3B). Due to the limited number of samples per condition, the performed Anova analysis did not detect any significant differences in the amount of bone detected in these samples. However, when we looked

at the individual samples within each mouse separately, in all but one case, addition of 10% EL-MSCs led to a higher bone area in the sample as compared to addition of 10% hMSCs (Figure 7.3C). In case of 50% EL-MSC addition the same was true in all but two cases. Addition of 50% hMSCs seemed to have random effect on the bone area when compared to 10% hMSCs (more bone in 4 cases, less bone in 3). Based on our previous results [32] we can thus conclude, that the increased bone formation in the samples with 50% more cells was simply due to the increased number of cells in the construct. We showed this effect with MSCs [32] and we assume that the same happened with the addition of EL-MSCs. Contrary to this, the effect caused by the presence of 10% additional EL-MSCs was likely not only due to the increased cell number, since the increased amount of bone was not observed in reference conditions (10% MSCs addition). Therefore we decided to look for another explanation behind EL-MSCs' influence on bone formation in implanted constructs.

7.3.4 Osteogenic and endothelial gene expression in micro-aggregate system

To determine whether addition of EL-MSCs to the system has any effect on the expression of the osteogenic genes, we performed qPCR using a panel of commonly used osteogenic genes on two types of constructs (with 10% hMSC or 10% EL-MSC addition) after 1 week of *in vitro* culture. The panel of osteogenic genes included alkaline phosphatase (ALP), bone morphogenetic protein 2 (BMP2), bone sialoprotein (BSP), osteopontin (OP) and osteocalcin (OC). We used two types of medium (micro-aggregate medium and EGM-2) to eliminate the risk that the potential EL-MSC influence would be obscured by strong osteogenic influence of the components of micro-aggregate medium. Neither in micro-aggregate nor in EGM-2 medium did we find any significant changes in osteogenic marker expression (Figure 7.4A) though the absolute value is higher in the samples with the addition of EL-MSCs. Our results clearly showed that introduction of EL-MSCs in the micro-aggregate system did not influence osteogenic marker expression when compared to the introduction of hMSCs. Interestingly, there were also no significant differences between these marker expressions when comparing the two culture media which were used. This indicated that the osteogenic influence of the system itself (BCP ceramic particles combined with platelet gel) was independent of the applied medium. Additionally, these results indicate that the EL-MSCs did not cause the increased bone formation in micro-aggregates by enhancing the expression of osteogenic genes in MSCs.

Another possibility is that EL-MSCs implanted in micro-aggregate system improved the vascularization of the constructs and by that assured better oxygen and nutrients supply. EL-MSCs could cause such effect in two ways, by expressing pro-angiogenic factors and attracting the in-growth of host blood vessels or by directly contributing to the vascular network, as demonstrated previously [19, 33, 34]. In order to determine which of the situations occurred, we have tested whether addition of EL-MSCs to the system has any effect on the expression of the endothelial and pro-angiogenic marker genes. We performed qPCR for human CD31 as a marker of

Figure 7.3: *In vivo* bone formation on porous ceramic scaffolds. Representative histological section of tissue engineered constructs (A). Newly formed bone (dark pink), and ceramic particles (black) can be distinguished. Bar graph comparing the amount of bone formed per scaffold area within samples seeded with 10% or 50% addition of MSCs or EL-MSCs (B). Graph comparing the amount of bone formed per scaffold area within samples seeded with 10% addition of MSCs or EL-MSCs, grouped per mouse, n=7 (C). Error bars represent standard deviation.

endothelial cells and for VEGF to check for a possible trophic effect. Similar to the osteogenic genes, two types of constructs (with 10% hMSC or 10% EL-MSC addition) after 1 week of *in vitro* culture were used in this study. Neither in micro-aggregate

Figure 7.4: Osteogenic (A) and endothelial (B) gene expression in micro-aggregate system. The expression of alkaline phosphatase (ALP), bone morphogenetic protein 2 (BMP2), bone sialoprotein (BSP), osteopontin (OP), osteocalcin (OC), CD31 and VEGF was assessed after 7 days of *in vitro* culture in micro-aggregate or EGM-2 medium.

nor in EGM-2 medium did we find any significant changes in CD31 or VEGF expression (Figure 7.4B). Since we know that EL-MSCs express more CD31 as compared to MSCs, the lack of significant differences in CD31 expression in two tested conditions is probably caused by the abundance of MSCs in the micro-aggregate system. Possible mechanisms in which EL-MSCs can contribute to bone formation are discussed next. First, both the pro-angiogenic and the vasculogenic influence of EL-MSCs can be too subtle to be detected *in vitro*, whereas *in vivo* it can be enough to improve bone formation. Moreover, EL-MSCs present in the sample could influence not only the cells implanted in the micro-aggregate system but also host cells. To confirm such possibility, additional studies are required that will check for osteogenic gene expression

within the implanted samples and their surroundings and not only within constructs cultured *in vitro*. Furthermore, we have tested for only one pro-angiogenic growth factor, namely VEGF. Since we did not find any increase in VEGF expression as compared to control samples, it is therefore likely, that the trophic effect of EL-MSCs is enforced via other growth factors. There is also a possibility that the quantified expression level of VEGF does not reflect the actual amount of released VEGF since it depends strongly on various post-translational control mechanisms [35]. Finally, we have tested the expression levels at one time point only, whereas the beneficial effect of EL-MSC in tested constructs could be related to speeding up of the expression of osteogenic or pro-angiogenic genes at earlier or later time points.

7.4 Conclusions

We have confirmed the ability of MSCs to differentiate into endothelial-like cells. We have also shown that the addition of single cells to the previously developed bone-forming system based on platelet gel, cell spheroids and ceramic micro particles, did not negatively affect the bone-forming capacity of this system. The most important result presented in this study is that a relatively small (10%) addition of EL-MSCs in the bone forming system increased the amount of obtained bone in all but one case. We tested for two possible explanations of this phenomenon. Unfortunately, we did not find any influence of EL-MSCs on the osteogenic or endothelial gene expression in the micro-aggregate system *in vitro*. Still, the possibility that this is the mechanism of their influence cannot be excluded, since we have tested the expression of these genes at one time point only, moreover, the expression levels *in vitro* might not reflect the levels *in vivo*.

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Chapter 8

Endothelial-like mesenchymal stromal cells to promote islets revascularization

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Abstract

Proper tissue vascularization is necessary to maintain tissue homeostasis since it ensures an appropriate level of oxygen and nutrients. Therefore, one of the major challenges in regenerative medicine and transplantology is to enforce vascular engraftment of the newly formed or implanted tissues and, by that, assure its survival and success of the therapy. The time necessary to provide adequate vascularization is particularly important when implanted tissue has a high metabolic rate or needs to respond quickly to external signals, like in the case of transplantation of pancreatic islets. Such transplantation is considered as a possible alternative for whole pancreas transplantation for patients with diabetes type 1, but the vascularization of these structures needs to be improved to allow for successful diabetes treatment. One of the widely investigated methods to improve islet vascularization is combining the islets with cells that will improve angiogenesis and vasculogenesis of the graft. In this study we show that clinically relevant mesenchymal stem cell-derived endothelial cells (EL-MSC) improve the sprouting of islets *in vitro* without hampering their functionality. We also show that covering the islets with EL-MSCs ensures better vasculogenic potential as compared to previously tested endothelial and mesenchymal stem cell-based strategies.

8.1 Introduction

Recent clinical trials have demonstrated the ability of allogeneic islet transplants to regulate blood glucose levels in patients with type 1 diabetes [1–3]. The main benefit

of this procedure compared to whole organ transplantation, is the significant reduction of glycemic fluctuations while having reduced postoperative trauma and complication rates. One of the most restricting factors of this therapy though, is the large number of islets necessary to achieve long-term normoglycemia after transplantation. Islets from at least two donors are needed to cure one patient. Besides, insulin independence lasts for only a few years, due to progressive islet loss in the post-transplantation period [1, 3, 4].

A factor considered to be detrimental for islet survival and function is inadequate revascularization of the transplanted islets. When islets are isolated from a donor pancreas, their vascular connections are disrupted and it takes several days *in vivo* before an initial vascular supply is established [5, 6]. Therefore, immediately after transplantation, islets solely depend on oxygen and nutrient transport by diffusion. Although new blood vessels are formed within transplanted islets, the resulting vascular density is chronically lower compared with that of native islets [7, 8], resulting in a 95 percent reduction in blood perfusion in mice [9]. Since the vascular network is important to maintain the islets' oxygen-dependent nutrient metabolism and their ability to quickly secrete insulin in response to changes in blood glucose levels, a reduction in blood perfusion will affect both islet survival and function.

Hence, various attempts have been made to improve vascularization of islets after transplantation. One of these attempts is to increase the action of pro-angiogenic factors or to inhibit anti-angiogenic factors in order to stimulate the proliferation, migration, and maturation of endothelial cells [10–12]. Precise control of timing, dose and duration of factor administration remains the challenge in order to get mature, functional blood vessels within islets. Another approach is to directly use endothelial cells, endothelial progenitor cells, or mesenchymal stromal cells (MSCs).

Johansson *et al.* [13] have shown that coating human islets with endothelial cells initiates the formation of vessel-like structures *in vitro*, without impairing islet functionality. The sprouting capacity of endothelial cell-coated islets was further improved by the addition of MSCs. Other studies have shown that co-transplanting islets with stromal cells derived from various sources can induce neovascularization, resulting in enhanced islet revascularization [14–17] and better function [14–18], regardless whether the implantation site was under the kidney capsule [14, 15, 17–19], in the liver [17] or subcutaneously in a fibrin gel [15]. In most of these studies, islets were co-injected with undifferentiated adipose-derived [15] or bone-marrow derived MSCs [15, 17, 19], while Oh *et al.* co-transplanted islets with murine bone-marrow derived endothelial progenitor cells [14]. A direct comparison of the efficiency of revascularization between the various studies using different cell populations is difficult though, because of differences in species, transplantation sites, detection methods, and in amount of transplanted cells.

Recently, we have demonstrated that we are able to obtain MSCs with specific endothelial cell properties by culturing them in endothelial growth medium in the presence of shear force and extracellular matrix stimuli [20–22]. These endothelial-like MSCs (EL-MSC) were shown to acquire specific endothelial cell properties, both

phenotypical and functional. EL-MSCs 1) express typical endothelial markers CD31 and KDR, 2) form capillary-like structures on Matrigel and 3) improve vascularization of implanted constructs, both via trophic pro-angiogenic effects and via contributing to the in-growing vessel walls [20–22]. Therefore, we hypothesize that EL-MSCs promote better islet revascularization compared to naïve MSCs, and thereby improve islet survival and function. The advantage of EL-MSCs over endothelial cells (ECs) is that they can be easily isolated in substantial quantities, which is relevant for therapeutic applications. We have also demonstrated that their endothelial properties are independent of the donor and can be therefore autologous to the patient.

To prove the utility of these cells in islet revascularization, we have coated human islets of Langerhans with EL-MSCs and checked whether in such a combination islet vascularization improves. In this manuscript, we present an efficient and reproducible technique for the formation of EL-MSC-covered islets. Our data demonstrates that replacing previously described MSC and EC-MSC coatings with EL-MSC coatings enhances the sprouting capacity of the composite without compromising islet functionality.

8.2 Research design and methods

8.2.1 Islet of Langerhans isolation and culture

Human islets of Langerhans were obtained from the Human Islet Isolation Laboratory at the Leiden University Medical Center (Leiden, The Netherlands), which has permission from the Dutch government to isolate human islets for clinical and research purposes. Human islets that were not eligible for clinical transplantation were used in these experiments, in accordance with Dutch law and institutional requirements. Islets were cultured in islet medium (CMRL-1066, Mediatech, Cellgro, Herndon, USA), supplemented with 10% FBS (Lonza), 100 U/ml penicillin (GIBCO), and 10 μ g/ml streptomycin (GIBCO).

8.2.2 Cell isolation and culture

Human mesenchymal stromal cells (MSCs) were isolated from human bone marrow from donors with written informed consent [23]. Aspirates were resuspended using a 20G needle and plated at a density of 0.5 million mono-nucleated cells per cm^2 . Cells were grown in MSC proliferation medium (alfa-MEM (GIBCO), supplemented with 10% FBS (Lonza), 100 U/ml penicillin (GIBCO), 10 μ g/ml streptomycin (GIBCO), 2 mM L-glutamin (GIBCO), 0.2 mM L-ascorbic acid 2-phosphate magnesium salt (Sigma-Aldrich) and 1ng/ml bFGF (Fisher Scientific) at 37°C in a humid atmosphere with 5% CO_2 . Cells were expanded up to passage 2. For further experiments MSCs from different donors and one immortalized clone (iMSCs, courtesy of Prof. Ola Myklebost, University of Oslo, Norway) were cultured in basic medium (alfa-MEM supplemented with 10% FBS, 100 U/ml penicillin, 10 μ g/ml streptomycin, 2 mM L-glutamin and 0.2 mM ASAp). Human umbilical vein endothelial cells (HUVECs, Lonza) were cultured in endothelial growth medium (EGM-2, Lonza).

8.2.3 Endothelial induction of MSCs

iMSCs (passage 25) and MSCs from 5 different donors (passage 1-2) were used for an endothelial induction protocol, as described previously [20]. Cells were seeded at a density of 3,000 cells per cm^2 on tissue culture plastic in EGM-2 and cultured for 10 days. After one day in static culture, shear force was applied using an orbital shaker (20 rpm). Cells that were cultured according to this protocol will be referred to as EL-MSCs.

8.2.4 Cell labelling

When indicated, MSCs (both naïve and EL-MSCs) and HUVECs were labelled using DiI (red) and/or DiO (green), respectively, according to the manufacturer's protocol (Life Technologies).

8.2.5 Islet coating

Agarose chips containing microwells were prepared by replica moulding as described previously [24]. Briefly, replicates of patterned PDMS (poly-dimethylsiloxane) stamps, each containing 130 pillars with a diameter of 400 μm and a height of 200 μm , were prepared using 3% agarose solution (Ultra-pure agarose, Invitrogen). Before cell seeding, the agarose chips were incubated o/n in islet culture medium. After uniformly seeding the islets of Langerhans in the microwells of each chip, a single cell suspension of naïve MSCs, a mixture of naïve MSCs and HUVECs, or EL-MSCs was added. Different cell concentrations (300, 625, and 1250 cells/islet) were tested in order to obtain uniform islet coating. The chips were then shortly centrifuged at 1500 rpm. Cells attached to the surface of the islets within o/n culture in EGM-2 medium. Coating efficiency was estimated based on pictures taken with Nikon Elipse E600 microscope.

8.2.6 Glucose-induced insulin secretion test

To assess islet functionality, a glucose-induced insulin secretion test was performed at day 1 and day 5 after islet coating. Per condition per islet donor, 100 islets were handpicked, seeded in the agarose chips, and coated o/n with naïve MSCs (1250 cells/islet), a mixture of naïve MSCs (625 cells/islet) and HUVECs (1250 cells/islet), or EL-MSCs (1250 cells/islet). As a control, uncoated islets were cultured o/n in agarose chips. Islet coating was performed in EGM-2 medium, after which the islets were cultured for 5 days in islet medium, with a medium change every day. For the glucose-induced insulin secretion test, per condition 30 islets (in triplicate) were incubated for 90 minutes in a modified Krebs Ringer Bicarbonate (KRBH) buffer (115 mM NaCl, 5 mM KCl, 24 mM NaHCO_3 and 2.2 mM CaCl_2 , pH 7.4), 20 mM HEPES, and 2 mg/ml human serum albumin (Cealb, Sanquinm The Netherlands). Islets were successively incubated for 1 hour in KRBH buffer with 1.7 mM and 16.7 mM D-glucose at 37°C. Insulin concentration was determined in the supernatants by ELISA (Mercodia, Sweden). The experiment was performed separately for three different islet donors, each time coated with MSCs from another donor.

8.2.7 Sprouting assay on Matrigel and fibrin gel

For the Matrigel sprouting assay, control (uncoated), naïve MSC, HUVEC/ naïve MSC, and EL-MSC coated islets were placed between 2 layers of growth factor reduced Matrigel (BD Biosciences) diluted 1:1 in EGM-2 medium. For fibrin gel assay, control (uncoated), naïve MSC, HUVEC/ naïve MSC, and EL-MSC islets were placed between 2 layers of fibrin gel (2.5 mg/ml fibrinogen and 2 μ g/ml thrombin; Sigma-Aldrich). For each gel layer 1 ml of gel was used. After polymerization, islets were cultured for 96 hours in EGM-2 medium. Islet sprouting was observed over time using an inverted microscope (Nikon Eclipse TE300). Pictures were taken at different time points (24, 48 and 96 hours) using a Nikon DS-L2 camera. Matrigel assay was performed with islets obtained from 6 different islet donors, while fibrin assay with islets from 4 islet donors. hMCSs from 5 different donors and one clone of iMSCs were used in this study. Sprouts (independent of their length) were counted manually by a single person (blinded to the composition of the islet preparation), and a minimum of 10 islets were counted per group.

8.2.8 Statistics

Each experiment was performed in triplicate. Data that required multiple comparison test was analysed in SPSS (PASW statistics) using one-way Anova followed by Tukey's multiple comparison test ($P < 0.05$).

8.3 Results

8.3.1 Optimal islet coating using agarose microwell chips

Since EL-MSCs were observed to create big clumps when incubated in microcentrifuge tubes (data not shown) we modified the coating protocol published by Johansson *et al.* [13]. To fabricate aggregates of islets and single cells, we used a nonadherent agarose microwell culture platform which has been developed in our group for cell and cell cluster aggregation [25]. To determine the cell concentration for optimal islet coating, three different cell concentrations of DiI labelled MSCs were applied to human islets. As can be observed in Figure 8.1, MSCs tend to aggregate and attach as a cell cluster to the islets within 24 hours. When increasing the cell density up to 1250 cells per, the coating was more uniform and the majority of the islets' surface was covered with MSCs.

8.3.2 Coating islets with EL-MSCs does not affect islet functionality

To assess whether the applied coatings affect islet functionality, the ability of the islets to respond to a glucose challenge with adequate levels of insulin, was measured. Regardless whether the islets were coated with cells or not, a stimulation with 16.7 mM glucose buffer led to a two to threefold increase in insulin secretion compared to basal insulin release levels (Figure 8.2). Both at day 1 and 5 after islet coating, no

Figure 8.1: Efficiency of islets coating with various cell types. Islets were centrifuged into microwells after what various amount of cells (stained with DiI) was added. Scale bar 1mm.

significant differences in relative insulin secretion levels could be observed between coated and uncoated islets. When applying the low glucose buffer of 1.7 mM, insulin secretion of all conditions decreased to basal insulin levels again. This implies that the cell coatings did not affect the islets' secretory capacity upon glucose stimulation.

8.3.3 Matrigel sprouting assay

To evaluate the effect of cell coating on islet sprouting, uncoated (control) and coated (MSC, EL-MSC, HUVEC/MSC and HUVEC/EL-MSC) islets were cultured between two layers of Matrigel (Figure 8.3A). We have chosen for Matrigel in the first place as a sprouting matrix since we have previously performed sprouting assays within this gel and fully functional EL-MSCs were obtained only after contact with this matrix. To determine which cell type contributes to the sprouts in case of co-culture, DiI and DiO cell labelling was applied. In the case of islets covered with two different cell types, sprouts were formed by both MSCs / EL-MSCs (red) and HUVECs (green). The close

Figure 8.2: Islets functionality after coating with various cell types.

The insulin production was measured in low and high glucose buffer, 1 and 5 days after coating with cells. Control islets (C), HUVEC-MSC islets (HM), MSC islets (M) and EL-MSC islets (EL). Bars on the graph represent mean of insulin secretion normalized towards insulin secretion in islets incubated in low glucose buffer $\pm$ standard deviation, * denotes statistical significance ($p < 0.05$), ** denotes statistical significance ($p < 0.01$), *** denotes statistical significance ($p < 0.001$).

contact between MSCs and HUVECs within one sprout can be observed in Figure 8.3B. Sprout quantification revealed that already after 24 hours the average number of sprouts per islet was significantly higher in case of islets covered with EL-MSCs and HUVECs/EL-MSCs than in case of islets covered with MSCs or HUVECs/MSCs (Figure 8.3C). This advantage remained unchanged after 48 and 96 hours. Uncoated islets obtained from all 6 donors did not sprout in Matrigel even after 96 hours from seeding. Islets coated with HUVECs alone were used for the initial study (data not shown) and sprouting observed in this group was very inefficient, as shown previously by Johansson *et al.* [13]. Therefore, we have excluded this group from further study.

Figure 8.3: Sprout formation in Matrigel gel. Phase contrast microscope images taken 24hours after seeding: control (uncoated) islets, HUVEC-MSC islets, MSC islets and EL-MSC islets (A). Fluorescent microscope images taken 48h after seeding (B). HUVECs were stained with DiO (green), MSCs and EL-MSCs with DiI (red). Sprouts built of MSCs and HUVECs in close contact can be observed (white arrows). The number of sprouts per islet after 24, 48 and 96 hours in culture (C). Mean of total sprout count $\pm$ standard deviation, * denotes statistical significance ($p < 0.01$), ** denotes statistical significance ($p < 0.01$), *** denotes statistical significance ($p < 0.001$). Uncoated islets did not sprout in Matrigel.

8.3.4 Fibrin gel sprouting assay

We have also compared our results with the ones previously described [13] by assessing the sprouting of islets within fibrin gel. Uncoated (control) and coated (MSC, EL-MSC and HUVEC/MSC) islets were cultured between two layers of fibrin gel (Figure 8.4A). EL-MSC / HUVEC coating was not included in this study since Matrigel assay did not show any additional sprouting improvement when compared with islets coated with EL-MSCs only. As shown previously [13], uncoated islets have the ability to sprout in fibrin gel. Nevertheless, the average number of sprouts per islet was significantly improved in case of each coating. The effect was most striking in case of EL-MSC coating, where the average number of sprouts per islet was 15, 10 and 6 times higher after 24, 48 and 96 hours, respectively. Other coatings were significantly less efficient in inducing islet sprouting, leading to average 7, 6 and 4 fold increase (time points as above) in the number of sprouts per islet.

8.4 Discussion

To ensure that implanted islets of Langerhans exert their therapeutic effect to diabetic patients, insulin and glucagon produced by the these micro organs needs to be secreted directly into the blood flow. Therefore, islets need to be surrounded by a dense capillary network upon implantation as soon as possible. During the process of islet isolation prior to transplantation, the capillaries in the islets loose the connection with external vessels and decay [26]. Thus, recovering of capillary network within the islets is also an important part of the treatment. Various tissue engineering techniques were applied to improve the process of islet vascularization upon implantation, but none of the previously described solutions guarantee a clinically sufficient vascularization level. Furthermore, some of these studies were based on cell types, e.g. HUVECs, that cannot be used for therapy.

Bone-marrow-derived MSCs are ideal cell candidates for tissue engineering in regenerative medicine. They can easily be obtained in quantities sufficient for application in various therapies [27]. Moreover, there is no controversy concerning the safety of applying these cells in therapy, since they were successfully used for numerous clinical studies in the past [28]. We have shown lately that EL-MSCs derived from MSCs do not lose endothelial characteristics after implantation and are able to improve construct vascularization [20, 21]. We have also demonstrated that EL-MSCs surpass truly endothelial cells as far as the ability for surrounding matrix remodelling is compared [24]. This feature is extremely important since the formation of new capillaries during revascularization is a complex process that involves tissue digestion to provide space for new vasculature, much needed when dense organs such as islets are involved [29]. The in-growth of host blood vessels is also faster in the presence of cells able to remodel extracellular matrix [21, 30].

In this study we demonstrated that EL-MSCs can improve islet vascularization. We showed that EL-MSC-coated islet constructs were able to show fast and efficient

Figure 8.4: Sprout formation in fibrin gel. Phase contrast microscope images taken 48 hours after seeding of control (uncoated) islets, HUVEC-MSC islets, MSC islets and EL-MSC islets (A). The number of sprouts per islet after 24, 48 and 96 hours in culture (B). Mean of total sprout count $\pm$ standard deviation, * denotes statistical significance ($p < 0.05$), ** denotes statistical significance ($p < 0.01$), *** denotes statistical significance ($p < 0.001$).

sprouting through surrounding matrixes. This offers a possibility of significant improvement in the speed of capillary network formation when EL-MSC-covered islets are implanted. As demonstrated in our previous study [27], implanted EL-MSCs do not only prepare a route for in-growing of host vessels but also participate in vessel formation. Additionally, even undifferentiated MSCs are known to secrete a wide

range of cytokines and growth factors, which have both immunomodulatory and pro-angiogenic properties [31, 32]. It is likely that the trophic effect of EL-MSCs is even larger, since these cells were cultured in growth-factor-rich medium that can induce additional pro-angiogenic molecule secretion. The most compelling evidence for a trophic role of EL-MSCs in attracting vessel in-growth comes from our previous work in which MSCs and EL-MSCs were transplanted in collagen modules as delivery vehicles. In-growth of vessels was increased in modules loaded with both cell types while collagen modules themselves did not support vessel ingrowth [24]. In this study EL-MSCs provided more stable capillary structure within the implanted construct than MSCs. Furthermore, MSCs were reported to have an ability to limit the progress of the graft-versus-host disease [33, 34]. It is possible that EL-MSCs keep this capability and therefore their introduction can have an additional positive effect on the therapy when transplantation of islets is required.

To show the potential of EL-MSCs in improving islets vascularization we used well established models of angiogenesis, namely Matrigel and fibrin assay. We have shown that induction of sprout formation within both gels significantly improved in the presence of EL-MSCs as compared to previously reported HUVEC/MSC complexes. Additionally, the introduction of MSCs did not enhance this effect which signifies that EL-MSCs were able to take both the function of endothelial cells as well as the function of MSCs. Reducing the number of cell types involved in preparation of the engineered construct not only simplifies the development itself but also enables faster clinical introduction, especially due to the fact that MSCs are already allowed in clinical studies provided that isolation and *in vitro* maintenance is performed according to guidelines. To introduce any other cell type for improving islet vascularization, numerous additional studies need to be performed. First, the cells themselves must be proven safe for patients. Such research is very time consuming and expensive due to the necessity of animal studies in various animal models followed by clinical studies with human patients. Moreover, isolation methods and media used for *in vitro* maintenance of each cell type need to be described and FDA-approved. Therefore, choosing cells that are already tested greatly reduces the amount of studies necessary for bench to bedside transition.

The study described herein also showed that the effect of improved angiogenic potential of islets covered by EL-MSCs is not donor-dependent. As described previously [35], MSCs obtained from all tested donors were able to differentiate into EL-MSCs. The present study extends this statement from phenotypical characteristics only towards the functional potential that was shown before with cells obtained from a limited number of donors [20].

Moreover, no negative influence on islet functionality has been observed after the coating, as demonstrated in the test where insulin secretion was measured after challenging the islets with various glucose levels.

Finally, we also demonstrated the possibility of using iMSCs as model cells for studying not only phenotypical changes of MSCs after differentiation as described

previously [20], but also to study the interactions between islets of Langerhans and MSCs. This simplifies the realization of future *in vivo* experiments that are necessary to prove the utility of our design for diabetes therapy. We are currently working on the best delivery method of the EL-MSC-coated islets into patients. Based on our previous experience with collagen [21] and dextran [22] gels as well as our research concerning PEOT/PBT block copolymers [36], we are looking for optimal ways of construct preparation that would host the necessary number of islets allowing for fast vascularization and proper islet functioning.

To conclude, promising results obtained with EL-MSC-islet constructs strongly suggest that such constructs can contribute to the success of implantations strategies in diabetes therapy.

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Chapter 9

General discussion

9.1 Tissue engineering of blood vessels

Regenerative medicine emerged as an answer to the necessity of healing large tissue damage and defects which surpass the self-regeneration potential of these tissues. Since the amount of organs available from donations is much too low to serve all the patients in need, other solutions must be found to fill this gap.

Tissues are capable of repairing themselves in a variety of ways. Some of them contain a population of cells that undergo constant asymmetric divisions, giving rise to cells that keep proliferating and to cells that differentiate. This situation is common in tissues that are exposed to constant wear or renewal, such as skin, blood and intestines. Other tissues that in principle do not require constant cell replacement, usually include some specialized cells that can start proliferation and give rise to cells able to repair small tissue damage after injury. An example of such cells are for instance satellite cells found in muscle tissue.

In 1970 Friedenstein *et al.* described a population of cells that can be found in various tissues [1]. These cells, currently called human mesenchymal stromal cells (hMSC), were able to adhere to plastic, proliferate and form colonies. It was demonstrated that in some tissues (e.g. skin) MSC are the base for regeneration in adult body by giving rise to progenitor cells that can differentiate and replace damaged tissues [2, 3]. Nevertheless, some defects are too large to be regenerated only by residual hMSCs, which appear in the tissue in rather low density. Therefore, since these cells can be easily isolated from various tissues [4], it is possible to graft them back in the patient's body in the required quantity. It was also demonstrated that hMSCs possess multilineage potential and by proper treatment *in vitro* can be differentiated towards desired precursor and adult cells (e.g. osteoblasts). Several studies have shown that hMSCs can also be used to repair tissues that in normal conditions are not regenerated via residual hMSCs, such as kidney tissue [5]. This ability of hMSCs allows nowadays to engineer *in vitro* pieces of tissue that can be then implanted in order to replace defective tissue.

Currently, several aspects are hampering broader usage of cell-based therapies for tissue regeneration, one of which is the difficulty in maintaining the survival and functionality of newly formed tissue after implantation. This is of particular importance for large tissue engineered constructs containing living cells that require a proper level of nutrients and oxygen to perform their function. Without providing robust and efficient vascularization of such constructs, cells used for their assembly will die which leads to the failure of the applied therapy. Therefore, vascularization is nowadays recognized as one of the main hurdles that have to be overcome to translate tissue engineering research to clinical applications on a broad scale [6–8].

To date, most approaches to tissue vascularization are based on stimulating the in-growth of blood vessels from the host. This strategy can be very successful when small grafts are implanted but is not good enough to satisfy the requirements of larger constructs. The main problem with vascularization of larger grafts is the time needed for the in-growing vessels to create a network that will be dense enough to cover the tissue's needs for nutrients and oxygen. For most tissues the requirement surmounts to a blood capillary every 200 μm [9, 10], which obviously takes some time to grow after implantation. Studies showed that the speed of vascularization can be increased by the delivery of pro-angiogenic growth factors like VEGF or bFGF [11, 12] immobilized in the construct. Still, further improvement of this process is necessary. Lately, a lot of studies concentrate either on improving tissue regeneration strategies by introducing vascular networks in the engineered constructs prior to tissue implantation or by providing a way to guarantee fast vascularization by introducing cells that will create such a network upon transplantation. In this thesis, we describe hMSC-derived, endothelial-like cells (EL-MSC) as ideal candidate cells for building functional vascular networks prior to or upon transplantation. The necessity of connecting created networks with the host network together with the possibility of increasing the speed of host vessel in-growth is also investigated.

9.2 Factors influencing EL-MSC applications in therapy

9.2.1 Differentiation protocol

In chapter 3 of this thesis a protocol for endothelial differentiation of hMSCs is described. Obtaining cells that can be used to create vascular networks in engineered constructs from cells that can be isolated in large quantities circumvents problems related to the long time necessary for endothelial cell expansion. Since hMSCs can be autologous to every patient, problems with immune rejection can also be omitted. These cells are also approved to be used in the clinic when isolation and *in vitro* maintenance is performed according to guidelines. The presented protocol for endothelial differentiation is simple and robust; nevertheless, there are several issues that need to be addressed before using it for therapies. First of all, medium used for cell differentiation must be tested and FDA approved. Since EGM-2 is a commercially available medium with unspecified quantity of added growth factors, joined efforts between scientists and Lonza company are needed to prove that this medium is safe to be

used for maintaining *in vitro* cells for *in vivo* applications [13]. Secondly, replacement for Matrigel induction is necessary before clinical applications are considered. Since Matrigel is a gel derived from murine sarcoma, the use of it prompts controversy, even when a growth-factor-reduced version is applied [14]. Not only is Matrigel not likely to ever be FDA approved but there is also controversy to its specific angiogenic effect, e.g. it was shown that this gel can promote adipogenesis as well [15]. Our results presented in chapter 6 show, that dextran-based hydrogels are sufficient to direct hMSCs towards endothelial-like cells. Because these gels are already approved for clinical use, the possibility to introduce EL-MSCs into therapy is now real.

9.2.2 Donor variation

One of the challenges of clinical application of MSCs and other MSC-derived cells is connected with the huge variability often observed between cells obtained from different donors [16, 17]. There are some applications where donor variability among MSCs was shown to have little influence on the therapeutic outcome, such as in the treatment of graft versus host disease (GVHD) [18]. The study of le Blanc *et al.* revealed that infusion of MSCs can be an effective therapy for patients with GVHD, irrespective of MSC donor. Nevertheless, cells applied in this study were not differentiated and therefore the therapy was likely to be less sensitive to donor variation. When the variability in response to differentiation procedures occurs [16], differentiation protocols applied on cells designated for the therapies must take this in to account and should be carefully examined for robustness and repeatability prior to application. To truly control differentiation, quality control mechanisms should be implemented. For instance, in cartilage tissue engineering, Dell’Accio *et al.* described a panel of markers that allow prediction of the capability of *in vitro* expanded adult human articular chondrocyte to generate stable cartilage *in vivo* [19]. Although, as shown in chapter 4, hMSCs obtained from different donors all differentiated into endothelial-like cells, quantitative differences occurred that should and most likely can be overcome after precise adjustments in culture conditions. Such adjustments require deeper insight in the differentiation mechanism combined with identification of proper markers that will allow for more personalised protocol optimization. Our research in this area, presented in chapter 4, can be considered as a starting point towards this goal.

9.2.3 EL-MSC introduction in vivo

As described above, one hurdle towards clinical application of EL-MSCs is the method of delivery of the cells. In this thesis we present three ways of delivering EL-MSCs that can be considered as clinically relevant: on the surface of collagen modules (chapter 5), within dextran-based gels (chapter 6) or combined with platelet gel (chapter 7). All these strategies worked well in the animal models used, nevertheless, each of these methods should be carefully adjusted for the specific applications. An approach that will consider not only the vascularization issue but also the type of tissue that needs to be regenerated needs to be developed. Therefore, more studies are required to find the optimal carrier for every regenerative application separately.

9.2.4 EL-MSC application in regenerative medicine

Another issue that should be addressed before EL-MSCs will be widely introduced in regenerative medicine is proving their value in particular applications. This topic is addressed in chapters 7, where we found out that EL-MSCs can be delivered in a bone forming construct (chapter 7). Further studies are required to determine the mechanism of improved bone formation before clinical trials can be performed. Also for treatment of diabetic patients more research is necessary to achieve optimal methods to transplant the islets. The implantation place that is currently used (kidney capsule) was selected, among others, based on its well-developed capillary network that was supposed to compensate for the destroyed capillaries surrounding the islets. Since EL-MSCs can improve the vascular status of the islets, this opens new possibilities with respect to selection of implantation site. Therefore, the results described in this thesis can be considered as proof of principle for application of EL-MSCs in various therapies.

9.3 Concluding remarks and future perspectives

The *in vitro* research and animal experiments described herein contribute to the broad set of clinical applications of endothelial-like cells in the field of tissue engineering. It is important to demonstrate on a large sample the additive effect of EL-MSCs on tissue regeneration. Until now, we have demonstrated that the differentiation potential of these cells is robust and donor-independent *in-vitro*, but not in all *in vivo* studies we were able to show significant improvement of new tissue formation or vascularization. Our bone study (chapter 7) was performed with a limited amount of samples and did not yield statistically significant data. Still, we were able to show an increased amount of bone in 6 out of 7 samples. Further study is necessary to confirm this phenomenon. At the moment of writing, an *in vivo* experiment investigating the effect of EL-MSCs on islets of Langerhans functionality is running. No matter which application is chosen, from a translational point of view, pre-clinical studies of constructs containing EL-MSCs need to be carried out in large immunocompetent animal models, and long-term outcomes should be followed up. This is necessary to finally prove that such therapies will be safe for patients. For such experiments, development of an immunologically humanized animal is crucial. Additional research is also required that will allow to determine the fate of implanted EL-MSCs. Although our *in vitro* experiments (chapter 5) demonstrated that EL-MSCs did not undergo dedifferentiation, it is still necessary to find out what exactly is happening with these cells *in vivo*.

In our studies we did not compare the use of EL-MSCs for vascularization purposes with other clinically relevant cells like endothelial progenitor cells (EPC). Such study might reveal complimentary results of EL-MSC and EPC application and, likely, open a possibility of combining these cell types for better vascularization. A similar possibility lies in the combination of EL-MSCs and pro-angiogenic growth factors in the engineered construct. In chapter 6 we show that VEGF incorporation has a beneficial effect on the sprouting of MSCs and EL-MSCs within dextran-based hydrogel. Nev-

ertheless, further research is necessary to determine the optimal combination, way of introduction and amount of applied growth factors.

The influence of EL-MSCs on tissue engineered constructs should also be examined from one more angle. Although our study did not show any negative impact of EL-MSCs on other cell types within constructs (e.g. the bone forming capacity of hMSCs was not affected), there is always the possibility that EL-MSCs will disturb the expected performance of other cells. An example of such risk was demonstrated in chapter 5 of this thesis. MSC addition to collagen-based modular constructs had long-term negative effect on the vascularization of these constructs. Therefore, all possible interactions between various cell types need to be carefully examined before application and in case any undesirable influence is discovered, alternative protocols, e.g. separation of various cell types in the construct, both special and temporal, should be considered.

Next, the work described in this thesis is restricted to one strategy of vascularization, that is by introducing cells which are able to organize themselves and create a vascular network within the construct. This approach still requires in-growth of host blood vessels at least in the outer region of the implant. The in-growing vessels need to connect to the engineered vessel network. In case of large implants this process might still be too slow. The solution to that problem could be the introduction, within the construct, of engineered vessels that are large enough (microvessels) to be surgically anastomosed with host blood vessels. Future research should therefore be focused on a bi-directional approach that will provide both capillaries and microvessels within tissue engineered graft.

Finally, we have tested EL-MSCs only as cell source for construct vascularization. Still, endothelial-like cells derived from MSCs are likely to be useful in other applications that up to now required endothelial cells. Both MSCs and EL-MSCs in prolonged *in vitro* expansion create cell sheets that are strong and easily detachable even when cultured on standard tissue culture plastic. It will be very interesting to check, whether EL-MSCs can replace endothelial cells in cell-sheet-based large vessel engineering, as described by L'Heureux *et al.* [20]. Thus, the full potential of EL-MSCs in various applications remains yet to be explored.

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List of Publications

Hugo Fernandes, Koen Dechering, Eugene Van Someren, Ilse Steeghs, Marion Apotheker, Anouk Leusink, Ruud Bank, **Karolina Janeczek**, Clemens Van Blitterswijk, and Jan de Boer (2009) The role of collagen crosslinking in differentiation of human mesenchymal stem cells and MC3T3-E1 cells. *Tissue Eng Part A* 15: 3857-3867

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Karolina Janeczek Portalska, Nathalie Groen, Guido Krenning, Nicole Georgi, Anouk Mentink, Martin C. Harmsen, Clemens van Blitterswijk and Jan de Boer (2013) The Effect of Donor Variation and Senescence on Endothelial Differentiation of Human Mesenchymal Stromal Cells. *Tissue Eng Part A*.

Karolina Janeczek Portalska, M. Dean Chamberlain, Chuen Lo, Clemens van Blitterswijk, Michael V Sefton and Jan de Boer (2013) Collagen modules for in situ delivery of mesenchymal stromal cell-derived endothelial cells for improved angiogenesis. *J Tissue Eng Regen Med*.

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Karolina Janeczek Portalska, Hugo Fernandes, Daniel Saris, Clemens van Blitterswijk, Jan de Boer. Tissue engineering of blood vessel: from large vessels towards capillaries. Submitted

*shared first co-authorship

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*shared first co-authorship

Curriculum Vitae

Karolina Janeczek Portalska was born on the 23rd of February 1984 in Kraków, Poland. In October 2003 she started her study of Biotechnology at the Jagiellonian University, Kraków, Poland. During her study she performed several traineeships, including a summer assignment in the Laboratory for Stem Cell Research, Aalborg University, Denmark, an Erasmus project in the Institute for Human Genetics, George August University, Goettingen, Germany and a master assignment in the Group of Tissue Regeneration at the University of Twente, Enschede, the Netherlands. In June 2003 she graduated and obtained her Master of Science in Medical Biotechnology degree. Her master project was performed under the supervision of Dr. Justyna Drukała and Prof. Dr. Jan de Boer and her master thesis was entitled “Relation Between Human Mesenchymal Stem Cells Differentiation, Cell Shape and Focal Adhesion Assembly”. In July 2008 she started her PhD at the department of Tissue Regeneration, MIRA Institute for Biomedical Technology and Technical Medicine at the University of Twente under the supervision of Prof. Dr. Jan de Boer. For part of the work described in this thesis Karolina received a research grant to be used for working abroad as guest researcher in the Donnelly Centre for Cellular and Biomolecular Research, Toronto, Canada. This project was performed in close collaboration with Prof. Dr. Michael Sefton and Dr. Dean Chamberlain. During the past 5 years Karolina explored the potential of mesenchymal stromal cells for vessel engineering, the results of which are described in this thesis.

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