Stem cell therapy in rat hind limb ischemic injury

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Preface

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Tiivistelmä: Mesenkymaalisia kantasolupopulaatioita (mesenchymal stem cells, MSC) on useimmissa ihmiskehon kudostyypeissä, vaikkakin niiden prosentuaalinen osuus on hyvin pieni. Ne ovat multipotentteja soluja, jotka toimivat luontaisesti kudosten uusiutumisessa ja vaurioiden korjautumisessa. Ihmisen sikiön kantasoluista johdetut mesenkymaaliset kantasolut (human embryonic stem cell derived mesenchymal stem cells, hESC MSC) on viimeaikoina kehitetty kantasolutyyppi, joka muistuttaa tavallisia MSC-soluja. Iskemia syntyy verisuonten tukkeutuessa, jolloin kudoksen ravinnon- ja hapensaanti estyy. Tämä johtaa nekroosin, apoptoosin ja tulehdusreaktion kautta tapahtuvaan kudostuhoon. Kudostuhon paranemiseen liittyy uudissuonitus eli uusien suonien muodostuminen vanhoista haarautumalla, mikä palauttaa verenkierron kudokseen. Uudissuonitusta säätelevät sekä kudoksen omat että tulehdussolut erittämällä useita signaalimolekyylejä. Sonic hedgehog (Shh) on tärkeä yksilönkehityksen aikana toimiva morfogeeni, jonka on todettu ottavan osaa verisuonten syntyyn myös aikuisessa kudoksessa.

Tämän tutkimuksen tarkoitus oli selvittää hESC MSC-solujen vaikutus rotan takajalan äkillisen iskemian korjaantumiseen. HESC MSC-solut leimattiin lentiviruksilla, joissa oli joko lusiferaasi- tai GFP-geeni. Luustolihasiskemia saatiin aikaan sulkemalla rottien oikea reisivaltimo kirurgisesti, ja 24 tuntia myöhemmin lihakseen injektoitiin leimattuja kantasoluja Shh:n kanssa ja ilman. Lusiferaasi-kantasoluja saaneet rotat kuvattiin 0, 6 ja 24 tuntia injektion jälkeen, ja GFP-kantasoluja saaneita rottia seurattiin kolme päivää. Kudosnäytteet analysoitiin immunohistokemiallisesti värjäämällä verisuonispesifinen proteiini sekä PCR-ajolla rotan genomisesta ja komplementaarisesta DNA:sta käyttäen ihmissekvenssin alukkeita. Värjätyt hiussuonet laskettiin ja analysoitiin tilastollisesti.

HESC MSC-solujen kasvatus ja lyhyt Shh-altistus eivät muuttaneet niiden ulkonäköä, mutta myöhemmässä vaiheessa pieniä muutoksia havaittiin *in vitro*. Solut myös leimaantuivat tehokkaasti lentiviruksilla. Bioluminesenssikuvaus paljasti siirrettyjen solujen määrän laskevan nopeasti ensimmäisten 24 tunnin aikana. Joitakin GFP-positiivisia soluja kuitenkin nähtiin fluoresenssimikroskoopilla ja PCR-tulokset vahvistivat ihmisen DNA:n ja lähetti-RNA:n olemassaolon rotan lihaksissa. Kontrolliin verrattuna hiussuonten lukumäärä oli tilastollisesti merkittävästi suurempi molemmissa hESC MSC-soluja saaneissa ryhmissä, mutta pelkkää Shh:a saaneessa ryhmässä ei, missä suonia oli vain hieman enemmän kuin kontrolliryhmässä. Lisäksi ero kantasoluryhmien ja Shh-ryhmän välillä oli merkittävä.

Bioluminesenssikuvauksen perusteella suurin osa kantasoluista kuoli siirtopaikalle vuorokauden kuluessa. Pieni osa soluista selviytyi seuranta-ajan loppuun, mutta varsinaista kudoksiin liittymistä ei havaittu. Positiivinen vaikutus uudissuonten kehittymiseen oli silti selvä. Näiden tulosten perusteella hESC MSC-solut näyttävät olevan tärkeitä uudissuonituksen alkuvaiheessa, jossa ne toimivat luultavasti vahvistamalla kudoksessa jo olevaa hapenpuutteesta johtuvaa tulehdusreaktiota. Tässä työssä pienellä Shh-morfogeeniannoksella ei ollut vaikutusta uudissuonituksen lisääntymiseen yksinään tai kantasolujen kanssa annettuna. Pro gradu-työ muodostaa suuren osan laajemmasta tutkimuksesta, joka keskittyy kantasolu-välitteiseen kudoksen uusiutumiseen iskeemisessä vauriossa. Tutkimusartikkeli, jossa kirjoittaja on toisena kirjoittajana, on lähetetty tarkastettavaksi alan lehteen.

Avainsanat: hESC MSC, MSC, iskemia, angiogeneesi, Shh, soluterapia

University of Jyväskylä
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Abstract of Master´s Thesis

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Abstract: Mesenchymal stem cells (MSC) are found in most tissue types throughout human body, though their percentage is very small. They are multipotent cells which function in tissue turnover and repair. Human embryonic stem cell derived mesenchymal stem cells (hESC MSC) have been developed recently and have the properties of regular MSCs. Ischemia occurs after occlusion of blood vessels hindering nutrient and oxygen supply to the respective tissue, and results in tissue damage including necrosis, apoptosis and inflammation. Regeneration of ischemic tissue involves angiogenesis, the formation of new capillaries through sprouting from existing vessels restoring the blood flow. It is regulated by a complex network of signaling molecules secreted by several types of tissue and inflammatory cells. Morphogen sonic hedgehog (Shh) is an important factor during embryogenesis and pattern formation. Additionally, it has been shown to enhance angiogenesis in adults.

This study was carried out to determine the effect of hESC MSC and Shh on the recovery of rat hind limb muscles from acute ischemia. Cells were labeled lentivirally with either luciferase or green fluorescent protein (GFP), and injected with or without Shh into muscles in which ischemia had been induced 24 hours earlier by ligating the proximal and distal ends of the femoral artery. Luciferase-hESC MSC transplanted animals were imaged for bioluminescence at 0, 6 and 24 hours, whereas the GFP-hESC MSC transplanted animals had three day follow-up period after transplantation. Tissue samples were analyzed with immunohistochemical staining for capillary marker and with PCR for genomic DNA and complementary DNA using human specific primers. Stained capillaries were calculated and analyzed statistically.

Expansion of hESC MSC and short-term incubation in Shh did not alter the stem cell morphology, although changes were observed at late passages. They were also efficiently labeled with lentivirus vector. Bioluminescence imaging showed a rapid decrease in the amount of transplanted cells during the first 24 hours. However, a few GFP positive cells were seen in the fluorescence microscope and PCR confirmed the presence of human DNA and messenger-RNA in rat tissues. Number of capillaries increased significantly in both hESC MSC groups as compared to control, but not in the group that received Shh alone, although there was a minor increase. In addition, the difference between both stem cell groups and Shh group was significant.

According to the bioluminescence imaging vast majority of the hESC MSC died at the transplantation site during the first 24 hours in host tissues. Some cells survived through the follow-up period but did not show any sign of engraftment. However, the angiogenic effect was clear. These results suggest that the hESC MSC are important in initiation of angiogenesis, and that they function possibly by promoting tissue inflammatory reaction first raised by hypoxia. In this study the small amount of morphogen Shh did not have an effect on capillary formation either alone or when administered with the hESC MSC. The thesis forms a major part of a study concentrating on stem cell mediated tissue regeneration in ischemic injury. A research paper in which the author places as the second author has been submitted to a journal covering the field.

Keywords: hESC MSC, MSC, ischemia, angiogenesis, Shh, cell therapy

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Abbreviations

a. = arteria
αMEM = α-Minimum Eagle's Medium
BLI = bioluminescence imaging
BM = bone marrow
bp, bps = base pair, base pairs
Dnase I = deoxyribonuclease I
EC = endothelial cell
ECM = extracellular matrix
EDTA = ethylenediaminetetraacetic acid
EF1-α = elongation factor 1α
FGF = fibroblast growth factor
Gag-pol = group antigen-polymerase
GFP = green fluorescent protein
hALU = human alu-element
HEK 293T = human embryonic kidney 293 cells with simian virus 40 large T antigen
hESC MSC = human embryonic stem cell derived mesenchymal stem cells
HRP = horseradish peroxidase
i.p. = intraperitoneal
IU = infective units
IVIS = *in vivo* imaging system
LB_{amp} = lysogeny broth with ampicillin
LV = lentivirus
MOI = multiplicity of infection
MSC = mesenchymal stem cell
P = passage
PCR, RT-PCR = polymerase chain reaction, reverse transcriptase-polymerase chain reaction
PDGF = platelet derived growth factor
Rnase = ribonuclease
RT = room temperature
s.c. = subcutaneous
SDS = sodium dodecyl sulphate
Shh = sonic hedgehog
SMC = smooth muscle cell
Tris = tris(hydroxymethyl)aminomethane
VEGF = vascular endothelial growth factor
Vsv-g = vesicular stomatitis virus envelope glycoprotein
vWF = von Willebrand factor

Introduction

Mesenchymal stem cells

Mesenchymal stem cells (MSC) were first described by Friedenstein and colleagues in 1974 as bone marrow stromal cells that maintain the suitable microenvironment for hematopoiesis to occur (Friedenstein et al., 1974; Friedenstein et al., 1976). Since then an increasing amount of research around the world has been committed to discover the nature and properties of these cells. The task has proven out more complicated than expected as the cell population seems to be highly variable and flexible *in vitro* therefore rendering the results from different groups controversial and difficult to compare. Additionally, due to these properties it has been nearly impossible to study the MSCs in their undifferentiated state and hence most of the current knowledge is based on research on cultured cells which are likely to differ from their *in vivo* counterparts.

The nomenclature of mesenchymal stem cells reflects their diverse properties. Many cell subpopulations of bone marrow and other tissues have names describing their *in vitro* behavior leading to confusion among scientists on their true identity. The International Society for Cellular Therapy (ISCT) has recently proposed (Horwitz et al., 2005) the name ‘mesenchymal stromal cell’ to be used instead of ‘mesenchymal stem cell’ in order to maintain scientific accuracy, and thus the term stem cell should only be used of cells that meet the accepted criteria. The acronym MSC can be used of both cell types as long as scientists clearly state in their publications which one is in question. A recently developed cell line used in this work, human embryonic stem cell derived mesenchymal stem cells (hESC MSC), has not been characterized for stem cell properties but for consistency their original name is used (Trivedi and Hematti, 2007).

Definition and properties on lab bench

Classical stem cell definition (Rosenthal, 2003) states that a stem cell is a multipotent cell originating from a previous stem cell, and capable of self-renewing. In other words, a single stem cell from homogenous population can produce daughter cells both similar to the original cell and several differentiated types. A true stem cell remains in an undifferentiated state in its niche, and as it proliferates through symmetric division the cell

population self-renews and expands. An asymmetric division accounts for the maintenance of stem cell pool and multipotency as one daughter cell is committed to differentiate. During embryogenesis the potency of stem cells decreases rapidly giving way to extensive differentiation and proliferation. Strictly, a stem cell should also functionally and robustly reconstitute tissue *in vivo* after transplantation but different stem cell populations seem to have variable capability for this, e.g. hematopoietic stem cells readily initiate blood formation in new bone marrow whereas MSCs engraft more occasionally. In 2006, the International Society for Cellular Therapy announced somewhat less strict criteria to be used when evaluating the stem cell properties of human MSCs (Dominici et al., 2006). In short, they should be characterized by trilineage differentiation capacity to osteoblasts, chondroblasts and adipocytes, plastic adherence, and a specific surface antigen pattern (figure 1).

The ISCT criteria are met in the numerous studies supporting multipotent differentiation abilities of MSCs. When cultured with proper components of extracellular matrix, growth factors, and cytokines they can differentiate into cells of mesenchymal tissue lineage such as osteoblasts, adipocytes, myocytes, endothelial cells and chondrocytes (Pittenger et al., 1999; Reyes et al., 2001). Also various chemical substances can induce similar differentiation (Grigoriadis et al., 1988). Furthermore, even the non-mesenchymal differentiation of MSCs into hepatocytes and neural cells has been reported (Lee et al., 2004; Woodbury et al., 2000). Their self-renewal is widely accepted as well since carefully cultured cells continue to produce similar cells over passaging by symmetric division. However, clonal assays have shown the populations to be heterogeneous separate colonies showing variable potency (Colter et al., 2000; Colter et al., 2001; Reyes et al., 2001) which indicates that a population consists of both primitive stem cells and committed progenitor cells (Zohar et al., 1997). Thus, to date the actual stem cell properties of adult MSCs are still under debate due to difficulties in gathering solid evidence.

Cultured MSCs are generally described as plastic-adherent cells with spindle-shaped morphology, although the latter changes according to the growth phase and confluence of the culture so that the cells become larger and flatter at high densities (Digirolamo et al., 1999; Sekiya et al., 2002). MSCs are capable of rapid proliferation in favorable *in vitro* conditions resulting in even more than 1 million-fold expansion (Colter et al., 2000) but

this and the overall lifespan of the cells depend on their host organism, tissue of origin, culture conditions such as plating density and medium composition, and sampling method (Digirolamo et al., 1999; Pittenger et al., 1999; da Silva Meirelles et al., 2006). As the cells are propagated the culture tends to become progressively homogenous and the multipotency is gradually lost, presumably driven by the microenvironment and ageing (Digirolamo et al., 1999; Majumdar et al., 1998; Muraglia et al., 2000). This may lead to reduction of multipotentiality if a lineage restricted subpopulation is inadvertently favored or the cells are induced to differentiate.

Cell surface markers of MSCs have been analyzed in a multitude of studies in order to find unique markers for their more efficient recognition and isolation. According to ISCT, cell populations that are positive for CD105, CD73, CD90, and negative for hematopoietic and endothelial markers CD45, CD34, CD14 or CD11b, CD79 or CD19, and HLA-DR along with the other requirements (figure 1) can be qualified as MSCs (Dominici et al., 2006). In addition, many other non-specific markers and CD antigens have been found but they vary between species and tissue of origin (Charbord et al., 2002). Among them are e.g. SH2, SH3, Sca-1, CD9, CD29, CD44, CD71, CD105 (Martin et al., 2002; Pittenger et al., 1999; Sun et al., 2003) and typical vascular smooth muscle cell marker α-smooth muscle actin (α-SMA) (da Silva Meirelles et al., 2006) which also denote the functions of MSCs *in vivo* as migratory, immunoregulatory and vascular support cells. Most uninduced MSC lines are also positive for MHC I and CD58 at low level but not for MHC II, CD40, CD54, CD80, or CD86 (Tse et al., 2003); a phenotype which is generally considered nonimmunogenic and offers an advantage in allogeneic transplantation therapy. So far the desired strictly MSC specific markers have not been identified.

The parameters mentioned in the context of expansion influence also MSC lineage commitment resulting in broad variation among adult cell lines (da Silva Meirelles et al., 2006). It is likely though, that MSCs from fetal tissue counterparts have higher potential for clonal expansion and plasticity, i.e. the capacity to convert from one cell type to another (see review by Moore and Quesenberry, 2003), than adult cells (Lee et al., 2006). However, stem cell plasticity requires careful evaluation since at least five mechanisms can cause false multipotential differentiation (see review by Pauwelyn and Verfaillie, 2006). First, tissue under examination can contain multiple stem cell populations with specific capacities. MSCs can also fuse with mature cells and thereby gain some of their

characteristics. When subjected to appropriate chemical conditions fully differentiated cells can transdifferentiate directly into another matured type, or they can sometimes take a step back, dedifferentiate, and then redifferentiate following the environmental cues. Finally, a pluripotent stem cell population can coexist in a given tissue, and when tissue-specific stem cells are purified they 'contaminate' the isolate.

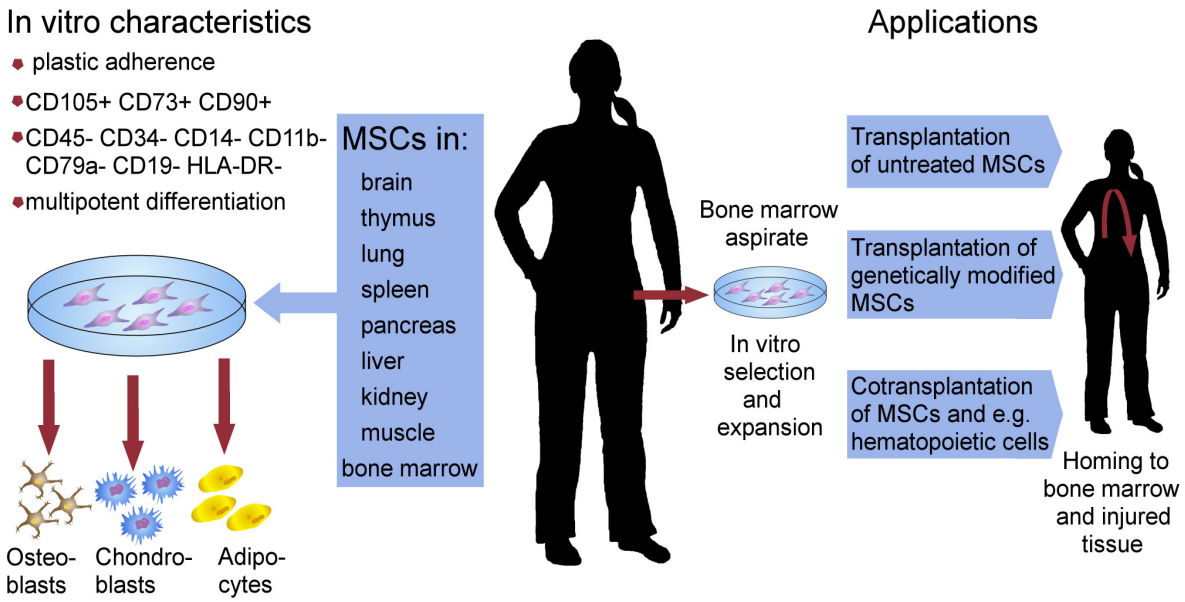


Figure 1. Mesenchymal stem cell (MSC) properties in vitro and their clinical applications. **On the left** are listed the in vitro requirements for the MSCs as declared by the International Society for Cellular Therapy. The MSCs should be plastic adherent, have specific cell surface antigens, and be able to differentiate along multiple lineages. To date, MSCs have been found in most mammalian tissues of mesoderm origin but also from ectoderm-derived tissues such as brain. **On the right** is an overview of the MSC pathway from donor to allogeneic recipient, although autologous transplantations are common as well. First, donor bone marrow aspirate is transferred into culture in vitro and consequently the plastic adherent MSCs are separated from the non-adherent hematopoietic cells. MSC population is then genetically modified if necessary and expanded until sufficient numbers of cells for transplantation are obtained. Lastly, the MSCs are transplanted alone or along with other cell types depending on the disease that is treated. In the recipient the cells frequently home to the bone marrow and the site of injury thus providing both long-term benefit and instantaneous recovery.

MSCs in natural environment

Mesenchymal stem cells are derived from the mesenchyme of an embryo but the early events that lead to their distribution into niches are poorly understood. A cell layer bearing stromal characteristics under the hematopoietic layer in fetal dorsal aorta in the aorto-gonadal-mesonephric (AGM) region has been suggested as the tissue of origin for MSCs (Minasi et al., 2002). Evidence for MSC migration from here has not been presented

but, interestingly, a large number of circulating stromal cells has been detected in fetuses from seven to twelve weeks' gestation after which they disappear (Campagnoli et al., 2001). This cell population closely resembled conventional MSCs except that it had wider and more long-lasting differentiation capacity. MSC-like fetal cells have been found also in maternal tissues supporting the circulation hypothesis (see review by Bianchi, 2000). Taken together the findings offer a plausible mechanism for MSCs to effectively spread during the early development into their ultimate niches, the bone marrow stroma and other tissues, from where they can operate throughout the life-span of an individual.

The actual niches accommodating the MSCs have long been subjects of keen research. Many groups have isolated MSC populations from a wide range of tissues other than bone marrow including adipose tissue (Zuk et al., 2001), skeletal muscle (Lee et al., 2000), and dental pulp (Gronthos et al., 2002). Similar cells have also been found in lungs, liver, pancreas, spleen, kidney, major blood vessels, and brain (figure 1) from both fetal and adult tissues (da Silva Meirelles et al., 2006; in 't Anker et al., 2003). Blood has been suggested as a source of circulating MSCs as well but as da Silva Meirelles et al. (2006) pointed out a contamination from detached vessel wall MSCs is very likely unless specific care is taken while obtaining a blood sample. However, even though research on MSC distribution in mammalian organ system is leaping forward their exact *in vivo* location on cellular level is still largely unknown.

Most tissues contain a population of MSCs but determining their niches has been complicated as they remain nearly invisible. Traditionally the fraction of MSCs in a bone marrow aspirate sample has been quantified using colony-forming unit fibroblast (CFU-F) assay in which adherent cells from the sample are plated in low density and the number of individual colonies assumed to derive from single cells is calculated (Digirolamo et al., 1999; Owen and Friedenstein, 1988). CFU-F assays with human and feline BM MSCs suggest they represent only 0.001% - 0.01% of all nucleated cells (Martin et al., 2002; Pittenger et al., 1999). Similarly, the number of MSCs in other tissues is likely to be very small. Their *in vivo* proliferation rate has also been difficult to determine. It is considered that in general MSCs are quiescent *in vivo*, and that they enter the mitotic phase only in specific conditions e.g. after exposure to activating signaling molecules or loss of contact inhibition. This is supported by many observations of rapid *in vitro* growth after low density plating (Colter et al., 2000; Sekiya et al., 2002).

During the lifetime of an individual its tissues need to be renewed, and larger scale regeneration may be necessary after severe tissue damage. Tissue resident stem cells in e.g. skin, gastrointestinal tract, and bone marrow have well-established roles in tissue turnover. Such populations have also been described in skeletal muscle and liver (Lee et al., 2000; Piscaglia et al., 2007), though BM-derived stem cells seem to participate actively in repair of most tissues. The regenerative capacity of tissues decreases throughout the post-natal life, presumably because the number of stem cells in BM and organs falls as the individual ages (Bellows et al., 2003). Moreover, many tissues, such as central nervous system and cardiac muscle, are known to lack this regenerative ability partially or completely. Thus, after early childhood healing of damaged tissues occurs mainly through scar tissue formation by fibroblast cells. The role of MSCs in natural turnover and repair of peripheral tissues remains therefore unknown until more efficient and reliable methods for *in vivo* tracking of MSCs are developed.

In general, very little is known about the functions of MSCs in their natural environment. They seem to be resident in distinct tissues which would explain their variable *in vitro* properties. Additionally, MSCs are thought to give rise to most of the several bone marrow cell types such as osteoblasts, pericytes, and myofibroblasts (Muguruma et al., 2006), thus being also responsible for hematopoietic stem cell development (Calvi et al., 2003; Majumdar et al., 1998; Zhang et al., 2003) and early maturation of T and B immune cells (Barda-Saad et al., 1999; Kurosaka et al., 1999). MSC mobilization from bone marrow has not been documented under natural conditions, i.e. without experimental intervention. Activated T cells may play a role as they secrete various cytokines and chemokines that may modulate MSC behavior indirectly (Burger et al., 1998) once they enter BM via circulation, but the actual factors inducing MSC activation and migration to the site of injury have not been determined.

Applied research on MSCs

Over the years of research on MSCs, developing clinical treatments for various pathologies has been one of the main goals. Stem cells offer a way to regenerate the defective tissues instead of merely alleviating or suppressing the symptoms by medication, irradiation or surgical operations. In this respect MSCs are considered especially valuable as they possess many clinically relevant characteristics (see review by Giordano et al.,

2007). First, MSCs can be readily isolated from bone marrow aspirates which, despite the seeming existence of these cells elsewhere in body, are their primary source. Second, their relatively high proliferation rate enables expansion in culture in order to obtain relevant numbers of cells for transplantations. Third, the ability to differentiate on such a wide scale renders this one cell type very versatile. Finally, MSCs have a remarkable ability to migrate to the sites of injury and suppress the host immune system. Researchers and physicians are working 'blindfolded', however, since the exact mechanisms behind the beneficial effects of MSCs are not known.

Most of the research on MSCs is still in preclinical phase, and only few MSC applications have reached a clinical trial phase. Before the stem cell treatments are ready for use in hospitals and clinics more basic knowledge about their biology and *in vivo* behavior need to be gathered. It is of outmost importance to understand and to be able to control the self-renewal and differentiation of stem cells for the safety of human patients. For instance, due to slower growth rate and lack of telomerase activity *in vitro* MSCs have considered less tumorigenic than ES cells but their immunosuppressive function can actually promote tumor growth (see review by Prockop and Olson, 2007). Two studies have reported MSC transformation either spontaneously (Rubio et al., 2005) or after genetic manipulation (Tolar et al., 2007) albeit such findings are rare. Moreover, calcification of myocardium presumably caused by transplanted BM cells or MSCs has been observed (Yoon et al., 2004). Undoubtedly, *in vivo* models are invaluable as similar *in vitro* settings are yet to be developed.

Engraftment of mesenchymal stem cells into new host is a popular topic in tissue regeneration research. Many investigators have recently shown how isolated, tagged, and culture expanded MSCs can home to bone marrow cavity after systemic infusion into healthy recipients or straight to the site of experimental injury (figure 1) in allo- and xenotransplantations to repair e.g. bone fracture (Devine et al., 2002), cerebral ischemia (Wang et al., 2002), infarcted heart (Makkar et al., 2005), and wounded skin (Fathke et al., 2004). Furthermore, after repopulating the BM the cells are able to migrate out to take part in normal tissue turnover of the body (Devine et al., 2003; Opalenik and Davidson, 2005). Arguments against MSC homing have been made, however, as they were found to lose their homing ability following transfer to culture (Rombouts and Ploemacher, 2003). If necessary, MSCs can also be targeted more accurately simply by implanting them to the

desired area. This has been the approach in numerous studies attempting to restore mesodermal tissues such as bone (Kon et al., 2000), cartilage (Fuchs et al., 2003), cardiac muscle (Strauer et al., 2002), as well as nervous tissue (Hofstetter et al., 2002) with success. However, the percentage of engrafted cells is usually less than 3% (Devine et al., 2003; Jackson et al., 2001) but much higher levels have been suggested (Direkze et al., 2003). Debate on incorporation has arisen, though, after a couple of studies disputed the engraftment of MSCs into newly formed blood vessels (Zentilin et al., 2006; Ziegelhoeffer et al., 2004).

In addition to direct repair of damaged tissues, allogeneic MSCs have shown a notable ability to evade and suppress host immune reactions. The finding is of particular value in therapeutic transplantations in which tissue rejection and graft versus host disease (GVHD) are severe threats. MSCs themselves do not seem to elicit immune responses as indicated by *in vitro* studies with mixed lymphocyte cultures (Bartholomew et al., 2002), and it is likely also *in vivo* since infused stem cells persist in recipient for weeks and even over a year (Azizi et al., 1998; Devine et al., 2003; Liechty et al., 2000). In addition, they can actively suppress immune cell proliferation and attenuate their cytolytic functions (Augello et al., 2005; Sotiropoulou et al., 2006). This has been exploited in cell and organ transplantations in which autologous or allogeneic MSCs delivered at the same time induce tolerance in host and thus protect the implant (Bartholomew et al., 2002). Nevertheless, in some occasions the MSCs have been rejected by the host to varying extent (Eliopoulos et al., 2005; Nauta et al., 2006), creating a need for more in-depth research on direct and indirect effects of MSCs on immune response.

Another interesting practice is to genetically modify mesenchymal stem cells and only then transfuse them for a therapeutic purpose (figure 1). Retro- or adenoviral transduction generally results in an efficient and long-term gene expression (Li et al., 1995) but since the virus vectors pose safety threats non-viral methods have been developed (Song et al., 2004). Favorable properties can be obtained depending on the introduced gene, e.g. prolonged stem cell lifespan by activation of telomerase catalytic subunit (Bodnar et al., 1998) or prosurvival protein Akt (Mangi et al., 2003). There has also been successful attempts to introduce secretion of therapeutic factors for protein deficiency disorders (Bartholomew et al., 2001), chemotherapy (Studeny et al., 2002), and enhanced organ repair (Moutsatsos et al., 2001). These can aid in expanding the cells *in*

vitro, in improving their post-transplantation viability, and even in maintaining their 'stemness'. Additional advantage in using MSCs arises from their ability to home to the site of injury, also a modifiable trait, allowing delivery into inaccessible tissues by simple infusion. Animal models have already shown interesting results in treatment of invasive tumors (Nakamura et al., 2004), cardiac infarction (Sun et al., 2007) and bone repair (Tsuchida et al., 2003).

The mechanisms by which mesenchymal stem cells attain the improvement of tissue functions are constantly under research. Attempts to treat various damaged tissues by cell transplantations have not shown robust evidence for wide scale tissue-specific differentiation or engraftment of MSCs, and yet improvement has been observed. Consequently, many studies have concentrated on alternative mechanisms. Differentiation to supporting cells such as endothelial or smooth muscle cells in cardiac muscle (Jackson et al., 2001; Tomita et al., 1999), myofibroblasts (Direkze et al., 2003), vascular supporting cells (Ziegelhoeffer et al., 2004), or hepatic stellate cells (Baba et al., 2004) which in turn contribute to healing have been observed. Moreover, paracrine secretion of factors which induce e.g. angiogenesis, or proliferation and differentiation of endogenous stem cells has gained attention recently. MSC conditioned growth medium has been shown to protect tissue cultures ex vivo and improve cardiac repair after ischemic injury (Iso et al., 2007; Kinnaird et al., 2004) but whether transplanted MSCs perform this *in vivo* has not been demonstrated.

Mesenchymal stem cells are slowly entering clinics as the number of phase I/II clinical trials is growing. Systematic preliminary trials on human patients began in the mid-1990's which showed that MSCs could be transplanted without unfavorable side-effects (Lazarus et al., 1995). Since then their abilities have been put to test in attempts to treat a wide variety of pathologies. Some trials have had promising outcomes in remedy of graft versus host disease (Le Blanc et al., 2004), hematological diseases (Lazarus et al., 2005), cardiac infarction (Strauer et al., 2002), osteogenesis imperfecta (Horwitz et al., 1999), and in recovery after cancer chemotherapy (Koc et al., 2000). Other studies on, for instance, neurological and inherited diseases have not directly shown positive results but nevertheless have provided the clinicians and investigators with information on which to built new techniques.

Ischemia

Blood circulation is a life supporting system responsible for the exchange of oxygen and nutrients to carbon dioxide and metabolic waste products. Blood also carries the chemical messages between individual tissues and evens differences in temperature, acidity and such thus contributing to homeostasis, the dynamic equilibrium of the body. Blood vessels consist of arteries which branch several times into smaller arterioles and finally into capillaries, which in turn fuse to form larger venules and veins leading back to heart. Two other types, collateral arteries and anastomoses, are also present in the vessel system. The first describes two parallel arteries with same target tissue, and the latter is a connective vessel joining two separate arteries. All these vessels operate in maintaining and adjusting blood pressure and tissue perfusion according to the metabolic needs at a given time. This is accomplished by contracting and dilating individual vessels and precapillary sphincters which regulate the blood flow to capillary bed. Generally, blood flows through larger vessels until exercise requires more efficient gas and fuel exchange raising the need to utilize the extensive capillary network.

Tissue growth, such as vertebrate development, reproduction and repair but also tumor growth, requires simultaneous development of vasculature because otherwise the new cells would be too far away from oxygen and nutrient supply (Folkman et al., 1971). Also, several pathological conditions result in narrowing the lumen of blood vessels or formation of circulating blood clots, and instantaneous traumas may cause vessels to be severed. When the blood supply to an organ or tissue is partially or completely hindered the oxygen and nutrient content decreases rapidly and ischemia occurs. Often collateral arteries and anastomoses can in part restore the blood flow to inflicted tissue (see review by Heil and Schaper, 2004) thereby attenuating the damage caused by hypoxia. Even short-term ischemia induces necrosis and apoptosis leading to inflammatory reaction. Accumulating macrophages and other inflammatory cells (Barbera-Guillem et al., 2002) together with e.g. fibroblasts and muscle cells (Gustafsson et al., 1999; Steinbrech et al., 1999) secrete vascular endothelial growth factor (VEGF) which is necessary for the initiation of healing process aiming at restoration of blood perfusion by constructing new blood vessels through arteriogenesis and angiogenesis.

Angiogenesis in response to ischemia

All blood vessels share similar structures but their differences reflect their specific functions. Arteries and smaller arterioles (figure 2) consist of an inner continuous layer of endothelial cells (EC) attached to a collagenous basement membrane, forming the intima. Smooth muscle cells (SMC) form the media which in turn is surrounded by fibroblasts and connective tissue, the adventitia. Veins and venules (figure 2) have essentially the same layer structure as arteries but are distinguished through thinner SMC layer and larger diameter since similar durability is not required. Capillaries (figure 2) are the smallest vessels with only basement membrane and a single layer of endothelial cells to minimize resistance for gas and nutrient exchange. The small vessels - precapillary arterioles, postcapillary venules, and capillaries - have an additional cell type, pericytes, embedded in their basement membrane (see review by Armulik et al., 2005). These cells are found in most tissues and do not appear as mere structural components but are involved in facilitating and coordinating communication between the other vascular cells through gap junctions. Pericytes may also have a more active role as sensors for varying extracellular conditions and may initiate appropriate signaling cascades in the vessel wall. Together pericytes and SMCs are often referred to as mural cells.

The adult vascular system remains quiescent until stimulated to form new vessels. These stimuli derive from hypoxia (Forsythe et al., 1996), changes in blood flow (see review by Heil and Schaper, 2004), and inflammation as the inflammatory cells and involved tissues secrete angiogenic growth factors (see review by Shireman, 2007). In ischemic injuries insufficient oxygen induces the expression of hypoxia-inducible factor 1 (HIF-1) which is a transcriptional activator of vascular endothelial growth factor (VEGF)-A (Forsythe et al., 1996), and thus is a key regulator of angiogenesis. VEGF-A and VEGF-D are considered the most important factors in early angiogenic response (Rissanen et al., 2003), but other members of the VEGF family, such as placental growth factor (PlGF), along with fibroblast growth factors (FGF), insulin-like growth factors (IGF), angiopoietins (Ang), and platelet-derived growth factors (PDGF) are required for efficient vessel remodeling, maturation, and stabilization (see review by Carmeliet, 2000). The functional network of these factors is a complex one and many molecular and cellular events remain still unknown.

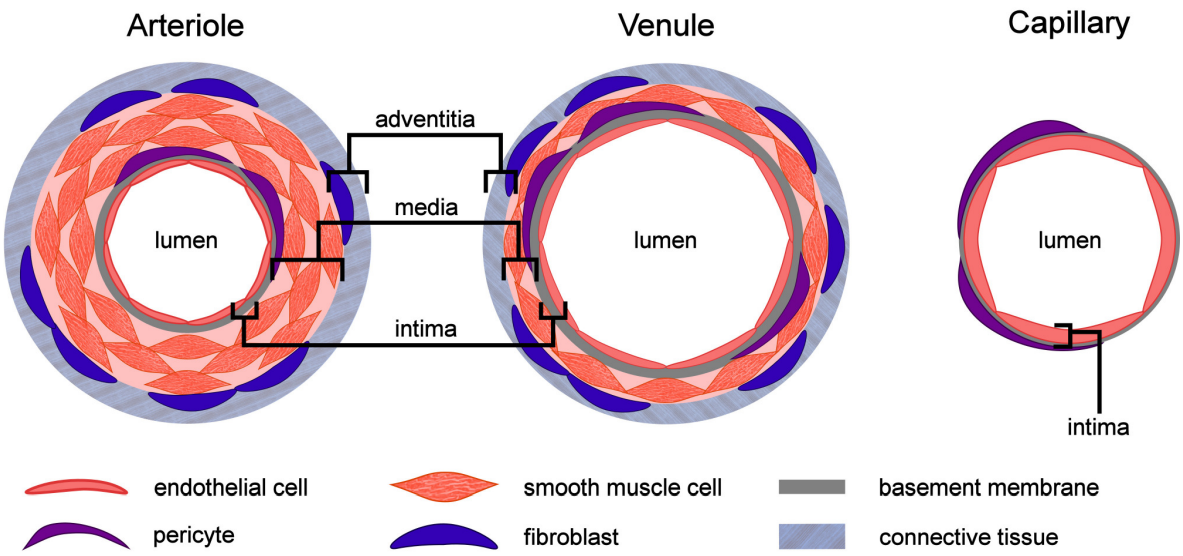


Figure 2. Blood vessel structure. Arterioles and venules have the same structural layers of the vessel wall, namely, intima, media and adventitia. **Arterioles** have a thick smooth muscle (SMC) cell layer and narrow lumen which maintain an adequate blood pressure. SMCs regulate blood pressure by contracting and relaxing according to stimuli. The pressure in **venules** is lower, and therefore thinner walls and wide lumen suffice. In these vessels fibroblasts produce collagenous connective tissue which supports the vessels. **Capillaries** lack adventitia and media, and their walls are very thin in order to enable efficient gas and nutrient exchange. In all vessel types endothelial cells and pericytes are closely connected through basement membrane which allows rapid communication about the environmental changes.

Sonic hedgehog (Shh) is a morphogen and transcriptional activator from the hedgehog family which functions in several key events of development and remains active in post-natal life (van den Brink et al., 2001). It has very versatile activities as suggested by a microarray analysis which revealed upregulation of e.g. cell cycle, adhesion and apoptosis related molecules (Ingram et al., 2002). Interestingly, Shh has also been implicated in ischemic injuries of skeletal muscles and myocardium (Kusano et al., 2005; Pola et al., 2003), although the molecular mechanism triggering Shh expression is still unknown. Both neoangiogenesis and collateral growth seem to depend to some extent on Shh stimulus (Kusano et al., 2005) but the effect is indirect as the target cells of Shh signaling are mainly fibroblasts while the ECs remain intact (Kusano et al., 2005; Pola et al., 2001). The Shh induced cells initiate a robust expression of three VEGF isoforms, namely $VEGF_{121}$, $VEGF_{165}$ and $VEGF_{189}$, and the angiopoietins 1 and 2 resulting in strong angiogenic response (Pola et al., 2001). However, a study by Kanda et al. (2003) has shown that Shh can induce ECs to begin capillary morphogenesis. In either case the true target cells and the intracellular signaling pathway remains elusive.

Blood vessel formation has been categorized into three partly overlapping subtypes, namely vasculogenesis, angiogenesis and arteriogenesis. Vasculogenesis refers to the de novo formation of primitive vascular network, the vascular plexus, from angioblasts in embryonic mesoderm (see review by Risau, 1997). After this period and during the postnatal life new vessels are formed mainly through angiogenesis, a process in which new capillaries sprout from existing ones (see review by Risau, 1997). It may also occur by intussusception or bridging, often involved in remodeling of newly formed vessels, in which a wall is formed in the lumen of a vessel by pericytes or endothelial cells, and the vessel is split into two (see review by Risau, 1997). Arteriogenesis becomes important during ischemia as it is responsible for the bulk blood flow to the tissues, though hypoxia is not a prerequisite for its initiation (see review by Schaper and Scholz, 2003). It is defined as the enlargement of anastomoses and small arterioles to form new collateral arteries that improve the blood supply to the inflicted tissue by circumventing the site of occlusion (see review by Schaper and Scholz, 2003).

Angiogenesis, whether physiological or pathological, is a multistep process involving coordinated functions of numerous growth factors and cell types. It initiates with upregulated HIF-1 expression from hypoxic cells of the tissue (Forsythe et al., 1996) and nitric oxide induced vasodilatation (figure 3A). HIF-1 induced VEGF-family expression enhances permeability of the vessel wall allowing leakage of plasma proteins into the surrounding tissues to form a scaffold for migrating endothelial cells (see review by Dvorak et al., 1995). VEGF also mobilizes bone marrow mononuclear cells which seem to have a role in enhancing vessel sprouting (Grunewald et al., 2006; Zentilin et al., 2006). Next, the intercellular junctions between pericytes, SMCs and ECs need to be loosened and the basement membrane disintegrated in order to enable EC and pericyte migration (figure 3B). This is accomplished by destabilizing growth factors, such as angiopoietin-2 (Maisonpierre et al., 1997), and several proteinases from different families which simultaneously release extracellular matrix (ECM)-bound growth factors such as FGF and VEGF (Houck et al., 1992; Saksela and Rifkin, 1990) (figure 3B). The process must be balanced to clear the way for migrating cells but to leave enough matrix to provide them with necessary molecular cues and support since too extensive ECM degradation can impair angiogenesis (Gutierrez et al., 2000).

The released growth factors stimulate the ECs, SMCs and pericytes to proliferate and migrate after they have been detached from the growth-inhibiting environment of a quiescent vessel wall (Hangai et al., 2002). The cells are guided by chemoattractants and perivascular cells such as pericytes (Nehls et al., 1994) as well as by the different components of the ECM scaffold such as collagen and elastin (Anderson et al., 2004; Hangai et al., 2002) (figure 3C). In addition, molecules mediating cell-cell and cell-matrix contacts, e.g. integrins (Davis and Camarillo, 1995), and other angiogenic factors, e.g. nitric oxide (Genis et al., 2007), are essential in spreading of ECs. Several other growth factors, hormones and cytokines are indicated in experimental angiogenesis but their roles *in vivo* remain to be determined. As the cells migrate they form solid cords that penetrate into surrounding tissue and fuse with the preexisting neighboring cords, possibly guided by the mechanical force created by mobile ECs on the ECM (Davis and Camarillo, 1995). Subsequently, lumen formation takes place as ECs in the middle of the cord undergo apoptosis and vacuolated ECs secrete their vacuoles (Meyer et al., 1997) (figure 3C). More ECs then settle into the vessel wall increasing the vessel diameter (Meyer et al., 1997).

The last phase of angiogenesis involves stabilization and maturation of the new vessels which would otherwise soon regress without sufficient support from the microenvironment. Recruitment of pericytes and SMCs is crucial for vessel maturation (figure 3D), and here PDGF apparently plays an important role since its absence disrupts the vessel development (Hellstrom et al., 2001; Lindahl et al., 1997). There is a short delay before pericytes cover the EC lined tubules during which the capillary density is matched to the oxygen supply (Benjamin et al., 1998). Connections between endothelial and mural cells are stabilized by e.g. angiopoietin-1 and TGFβ-1 which inhibit their proliferation and migration (Goumans et al., 2002; Suri et al., 1996). Moreover, the newly formed vessels acquire special characteristics such as fenestrae in kidney and liver capillaries, or other structures depending on their location in the body. After establishment of blood flow higher fluid shear stress and blood pressure against the vessel wall promote further remodeling of the cytoskeleton (Franke et al., 1984) but as soon as the forces are normalized the new vessels become quiescent until angiogenic stimuli are introduced again (figure 3D).

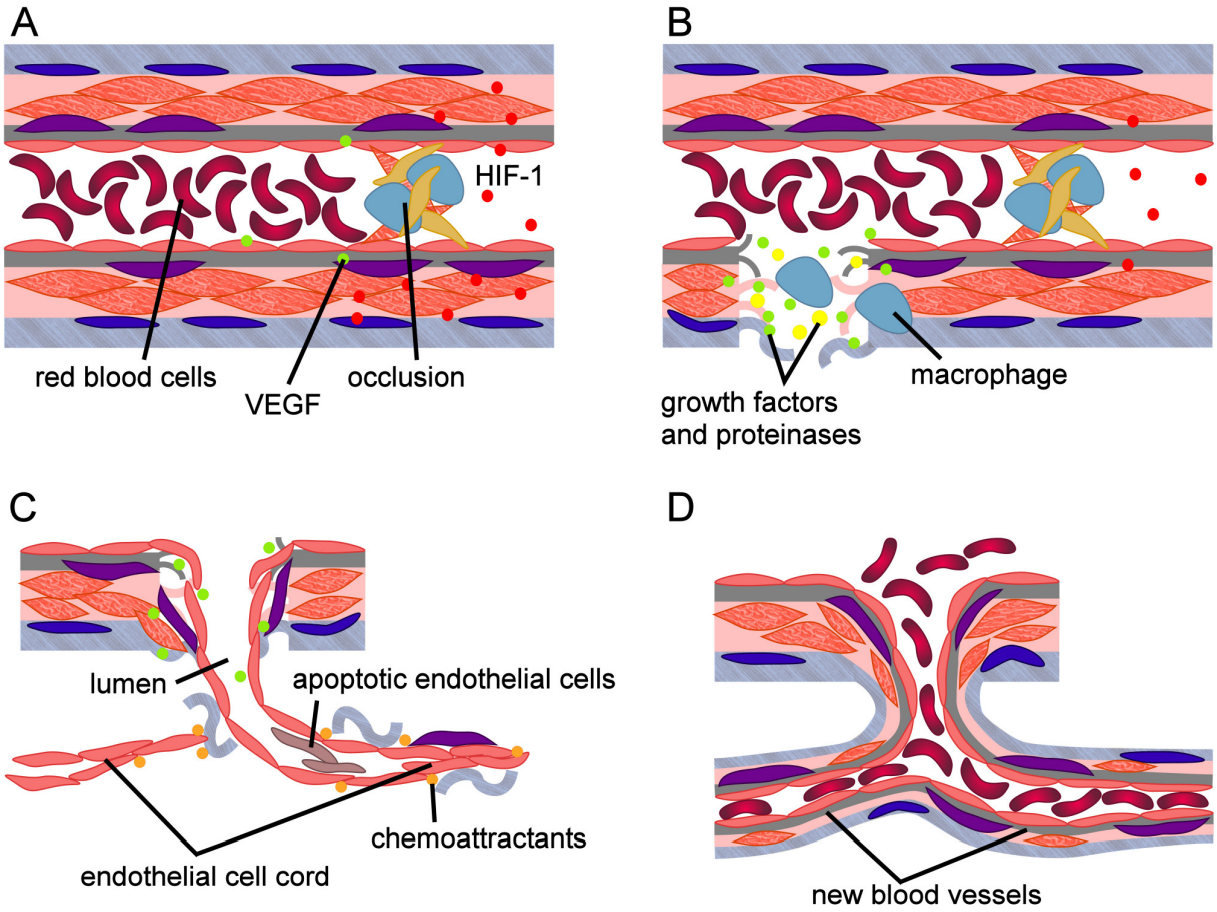


Figure 3. Overview of angiogenesis. **A** Hypoxia is developed downstream from the site of occlusion and induces HIF-1 expression (red dots) from hypoxic cells. HIF-1 further induces VEGF expression (green dots) which initiates the angiogenic events. **B** VEGF recruited mononuclear cells (e.g. macrophages) and nearby tissue cells produce several growth factors (green dots) and proteinases (yellow dots) which induce basement membrane and connective tissue degradation. More growth factors and cryptic binding sites are revealed from the connective tissue which aid in proliferation and migration of endothelial cells and pericytes. **C** Endothelial cells form cords as they migrate into the hypoxic tissue guided by chemoattractants and pericytes. These cords fuse together to form a network of primitive vessels. Later lumen is formed by e.g. apoptotic removal of the innermost endothelial cells of the cords. **D** Vessel network matures as pericytes and smooth muscle cells are recruited and other vessel structures are established. Blood flow induces shear stress forces that further remodel the new vessels until they enter a quiescent state.

Research models

The process of angiogenesis is a complex interplay of numerous tightly regulated molecular and cellular components. Moreover, it is dependent on the physical environment of the tissue which offers a scaffold and appropriate signals for the migrating cells. Since settings like this are difficult to establish *in vitro* most studies on angiogenesis must rely on animal models. One objective in research of angiogenesis is to find suitable therapeutic agents with which to promote or inhibit vessel growth according to the needs of pathological state. Induced brief ischemia and subsequent vessel growth also serve in

preconditioning an organ to be surgically operated (Murry et al., 1986) or transplanted (Torras et al., 2002). However, results from a single model can not be used to draw definitive conclusions because of the differences between e.g. species, target organs, tissue types i.e. embryonic or adult, and agent delivery routes. Thus additional *in vitro* studies and testing with models that more resemble human physiology are needed before preclinical trials can be commenced. A recent review on *in vivo* angiogenesis models by Norrby (2006) offers insight into assays currently in use.

Most angiogenesis assays developed can be used to study the angiogenic or anti-angiogenic effects of a test substance but many of them have more variability. The first assays to be introduced were the corneal micropocket assay in rodents and the chick chorioallantoic membrane (CAM) assay in 1974 by Folkman and associates (Auerbach et al., 1974; Gimbrone et al., 1974). They marked the beginning of systematic research but have been refined since, and over the years new ones have been created such as sponge implant and hollow fiber assays for studying tumor angiogenesis (Andrade et al., 1987; Casciari et al., 1994), disc angiogenesis system (DAS) for wound healing (Fajardo et al., 1988), and Matrigel plug assay for tissue regeneration (Passaniti et al., 1992). Imaging of tumor angiogenesis can be done in live animals using green fluorescent protein expressing tumor cells (Yang et al., 2001). Also non-mammalian systems utilizing zebrafish (Serbedzija et al., 1999) or xenopus (Levine et al., 2003) have proven functional. These assays along with those described in the review by Norrby (2006) overlap in terms of applicability allowing the same experiment to be carried out in another model for confirmation of the earlier results.

All animal models are not equal with respect to their similarity to human blood vessel anatomy and responses to angiogenesis modulating factors. Among the myocardial ischemia models domestic pig is the most popular due to the correct size and minimal amount of coronary collateral circulation of the heart although dogs, baboons, and small mammals have been used as well. Peripheral ischemia may occur in skeletal muscles and internal organs due to e.g. a disease such as diabetes, severe infection, or surgery. Here, rabbit hind limb model is the most frequently used and in different variations ischemia can be induced e.g. by excising the femoral artery (Pu et al., 1994) or by its gradual constriction (Baffour et al., 2000). Rats and mice are more economical but have naturally smaller blood vessels which may complicate the operation and interpretation of the results.

A pig model of collateral artery growth was introduced by Buschmann et al. (2003) and it offers a better temporal and size correlation to human. However, effective ischemia is more difficult to induce because of the more extensive branching of the femoral artery.

As already stated, there are numerous factors that may have a profound effect on the outcome of a study, and they should be taken into account when analyzing the collected data. Animal species and strain, gender, and age can influence the rate and extent of angiogenesis which are also subject to drug administration schedule, dosage and half-life in the circulation (see review by Norrby, 2006). In addition to this animal experiments are usually performed on healthy animals which are not as severely affected by ischemia as diseased human patients (Sprengel et al., 1995). When only the pro- or anti-angiogenic effect is tested the trauma caused by agent delivery should be minimized to avoid angiogenesis induced by local inflammation or wound healing that reduce specificity and sensitivity (see review by Norrby, 2006). Most assays requiring surgical operations are prone to such unfavorable reaction, e.g. the corneal micropocket assay and the CAM (Jakob et al., 1978; Wilting and Christ, 1992), but for instance the rodent mesentery angiogenesis assay could be used to circumvent this as no surgery is involved (Norrby et al., 1986). Lastly, performing an assay and interpreting the data usually requires a skilled person to ensure scientific accuracy.

Angiogenesis as clinical target

Increasing amount of research is dedicated to angiogenesis-related phenomena each year as new discoveries have shown its involvement in many more diseases than previously thought. Excessive, insufficient or otherwise dysregulated angiogenesis in human patients or experimental animals may appear in almost any organ of the body and take part in e.g. obesity, endometriosis, impaired bone formation, and gastric ulcerations, not to mention one of the most prominent causes of distress - cancer. A more complete list is provided in a review by Carmeliet (2003). Angiogenesis is therefore an appealing target for a multitude of therapies aiming at promotion or regression of vessel growth. The efforts in research are justified by the 500 million people estimated to benefit from these treatments in the coming decades (Niederhuber, 2006). Relatively few therapies in clinical use exist so far, however, as the intricate processes of angiogenesis and collateral growth

produce frequently unexpected outcomes. Thus, research is still mainly focused on clarifying these events in animal models.

A number of diseases derive from weakened angiogenesis or abnormally high vessel regression. One such group is the cardiovascular diseases plaguing the western world including atherosclerosis, hypertension and post-injury restenosis, which are characterized by attenuated collateral growth (Van Belle et al., 1997), decreased capillary density (Noon et al., 1997) and age-dependent impairment of endothelial cell growth (Gennaro et al., 2003), respectively. Other organ systems can suffer from e.g. defective cytokine or growth factor production, shown to be responsible for amyotrophic lateral sclerosis affecting the motor neurons, neonatal respiratory distress and uterine bleeding (see review by Carmeliet, 2003). In addition, certain pathogens, such as Helicobacter pylori in the gastro-intestinal tract, may up-regulate inhibitory cytokine expression (Jenkinson et al., 2002) and down-regulate angiogenic growth factor receptors (Kim et al., 2004) thereby preventing angiogenesis crucial for wound-healing. On the other hand, trauma-derived ischemia and tissue damage, for example after stroke, could be attenuated by proangiogenic therapy (Wang et al., 2004).

Both unrestrained and impaired angiogenesis can be detrimental to health of an individual. It is effectively a consequence of angiogenic factor overexpression by any of the cell components involved in the process. Often this is a result of genetic mutations which have been found behind many diseases such as cavernous hemangioma or vascular malformations (see review by Carmeliet, 2003), and cancer which hallmark is the angiogenic balance shifted to promote vessel growth, termed the angiogenic switch (Abdollahi et al., 2007). In most autoimmune and chronic inflammatory diseases vessel growth is a continuous vicious circle as cytokine-recruited leukocytes produce angiogenic factors which in turn lure more leukocytes thus supporting the inflammatory and angiogenic response (Bottomley et al., 2000). For example, arthritis and allograft vasculopathy are characterized by leukocyte infiltration (Uehara et al., 2006) but naturally other factors such as hypoxia play a role (Berse et al., 1999). Overexpression of proangiogenic mediators can also occur after viral infections as they express their own angiogenic genes (Meyer et al., 1999) or induce an angiogenic cascade in the host leading to tumor growth (Samols et al., 2007).

Considering the relatively clear role of cytokines, growth factors and other molecules in the course of angiogenesis, therapy based on their inhibition, stimulation or enhanced expression seems an effective approach. However, the perplexity of the system gives rise to multiple difficulties that need to be overcome. Successful pro-angiogenic therapy requires vessel maturation, and thus inadequate perfusion leading to lack of fluid shear stress, or deficient mural cell recruitment could be detrimental (Lindahl et al., 1997). Anti-angiogenic cancer therapy with only one or a few therapeutic substances is often complicated by the numerous tumor cell-derived angiogenesis factors (Taylor et al., 2002). Common problems are also targeting of the substances into the desired location, and unpredictable effects of the therapeutic agent in various tissues and with endogenous angiogenic factors. The former is more of a technical question yet under keen investigation (Richardson et al., 2001) whereas the latter indicates that much still remains unknown about the interconnections of angiogenic factors as well as the effect of age, gender and individual metabolism of the test subject on angiogenesis.

Accumulating knowledge from both basic and applied angiogenesis-related research continues to produce new approaches and to elaborate the old ones in treating the various pathologies of the field. Probably the most popular means to modulate vessel growth is to introduce specific stimulatory cytokines and growth factors, or their inhibitors. It has become clear, though, that a combination of several factors (Richardson et al., 2001) or a continuous low-dose medication, termed metronomic therapy, yield better results (Kamat et al., 2007). Moreover, anti-inflammatory agents can suppress angiogenesis and T-cell proliferation in diseases which involve cyclo-oxygenase-2-mediated inflammation such as arthritis and tumors (Muthian et al., 2006). Essentially, any substance delivered in therapeutic purpose influences the cells of the vascular and immune system either killing or attenuating them, or stimulating them to carry out the desired task. If molecular therapy and the body's resources are not enough the angiogenic abilities of endothelial progenitor cells (Rehman et al., 2003) or other stem cells can be exploited.

Mesenchymal stem cells have become a popular research subject on many fields in the recent years, and their abilities in correcting various types of ischemic injuries have already been tested in a myriad of studies. The faith in their applicability is based on observations that speak for their central role in postnatal neovascularization as bone marrow derived cells seem to contribute extensively to the development of endothelium

(Murayama et al., 2002). MSCs have been implicated in improving the functions of several tissue types after induced ischemia, especially by participating in the blood vessel formation. Much debate and suggestions have been brought up on the actual mechanism, however. Some studies state the effect is paracrine referring to angiogenic cytokines secreted by the stem cells result in vessel growth (Kinnaird et al., 2004), while others show physiological engraftment as vascular supporting cells (Ziegelhoeffer et al., 2004) or even as endothelial cells (Yan et al., 2007). Evidently, mesenchymal stem cell therapy with all its promises is worth investigating but patience and consideration is needed as these cells still have many tricks as well as treats.

Aim of the Study

Numerous studies have shown that mesenchymal stem cells participate at some level to neoangiogenesis enhancing the recovery of the ischemic tissues involved. This study was carried out to determine the suitability of recently developed hESC MSC cell line to the treatment of acute ischemic injury in a rat hind limb model. The specific aims are as follows:

1. To analyze the efficiency of hESC MSC transplantation and engraftment to the site of ischemic injury.
2. To elucidate the faith of hESC MSC after transplantation.
3. To test the effect of morphogen Shh to the transplantation efficiency.
4. To determine the effect of hESC MSC and Shh on tissue recovery.

Materials and Methods

Plasmid production and purification

Lentiviral expression vectors pWPXLd (GFP with EF1-α promoter), psPAX2 (gag-pol), and pMD2.G (vsv-g) were acquired from Addgene (Massachusetts, USA) and transformed into either Stbl3 or DH5α E. coli. Each bacterial stock was expanded in standard LB_{amp} (20 µg/ml) medium for plasmid production and pelleted for purification. Low copy number Shh plasmid was introduced into competent NEB 5α E. coli (New England Biolabs) with heat shock following the manufacturer's High Efficiency Transformation Protocol with minor exceptions: after the heat shock the bacteria were suspended in 300 µl SOC medium of which 15 µl and 285 µl were streaked on their respective plates without diluting since by experience this does not affect the growth of bacteria significantly. A single colony was then inoculated into a standard LB_{amp} (20 µg/ml) culture. When sufficient culture density was reached chloramphenicol (25 µg/ml) was added to increase plasmid copy number for an overnight period and after that the bacteria were pelleted for purification.

All the plasmids were purified with High Purity Plasmid Maxiprep System (Marligen Biosciences Inc.) according to the manufacturer's instructions with some exceptions. Firstly, to increase GFP+ lentivirus yield 12 ml each of cell suspension buffer, cell lysis buffer and neutralization buffer were used. Cell lysates were then centrifuged with 18 000 x g to remove excess cell debris, and an additional filtration with gauze was included to prevent column clogging before loading the plasmid solutions into columns. Plasmid preps were then precipitated with the less toxic 3M sodium acetate-ethanol method after which the DNA pellets were gently rinsed with 70% ethanol and air dried. Finally, plasmids were resuspended in sterile water instead of the suspension liquid provided to prevent deoxyribonuclease (DNAse) contamination. The plasmid concentrations and purities (A_{260}/A_{280} ratio) were determined with Ultrospec 2100 *pro* Uv/Visible Spectrophotometer.

Cell culture of HEK 293T

HEK 293T human renal epithelial cells were used for lentivirus (LV) and Shh protein production. The cells were cultured on ∅10 cm cell culture plates in Dulbecco's Modified Eagle's Medium (DMEM, Sigma) supplemented with 10% fetal bovine serum (Sigma), 1% penicillin-streptomycin (Sigma), and 1% L-glutamine (Sigma) in 5% CO_2 at +37°C. Plates were trypsinized (Sigma) and split every three to four days before reaching full confluence. The day before transfection the cells were split on poly-D-lysine coated (1 µg/ml, Sigma) plates to obtain suitable cell density.

Lentivirus production

Lentiviruses containing GFP (LV-GFP) but incapable of replication were produced by cotransfection of the plasmids pWPXLd, psPAX2, and pMD2.G. Only plasmids with A_{260}/A_{280} ratio over 1.80 were used. Transfections were carried out with a calcium-phosphate-mediated transfection protocol described in the Molecular Cloning – A Laboratory Manual, 3rd edition, Vol. 3, pages 16.19-16.20 (Sambrook&Russell, 2001). DMEM was replaced with 6 ml of prewarmed αMEM 15 hrs post transfection, and three LV-αMEM collections (6 ml per plate) were done with 24 h intervals starting 24 hrs after changing the medium. Each batch was pooled and stored at -70°C for later use. First batch of the collected media was tested for the presence of virus by incubating HEK 293T cells for 48 hrs in the medium along with hexadimethrine bromide (1 µg/ml, Sigma), after which fluorescence was visualized with a fluorescence microscope.

Lentivirus-GFP titer

In order to determine the LV-GFP titer, virus medium from the first batch was serially diluted 1:1, 1:10, 1:100, 1:1000 and 1:10 000. On the previous day, HEK 293T cells were seeded onto a six-well plate $1e10^5$ cells per well, and at the time of transduction the cells from one well were counted. The transduction was carried out as described in the previous chapter. The cells were allowed to grow for 72 hrs, after which they were trypsinized and fixed in 2% bovine serum albumin (BSA) /1% formaldehyde for FACS analysis (Flow Cytometer LSR II, BD Biosciences).

Based on the results number of infective units per milliliter of medium (IU/ml) and multiplicity of infection (MOI) were calculated. The titer in IU/ml is calculated from the

dilution which gives 10-20% GFP positive (GFP+) cells since it can then be assumed that a single virus has infected the cell, though pseudotyping allows several of them to infect an individual cell. The formulae used here were as follows:

1. percentage of GFP+ cells x number of cells at the time of transduction / 100 = number of GFP+ cells in the dilution
2. number of GFP+ cells in the dilution x dilution factor = IU/ml

MOI indicates the number of virus particles per cell in the transduction medium. In general, MOI higher than one with pseudotyped viruses should result in more effective labeling.

3. (milliliters of virus medium x IU/ml) / number of cells on the plate = MOI

Shh production and purification

Transfection of HEK 293T cells with the Shh plasmid was done with PolyFect (Qiagen) reagent following an appropriate protocol of the manufacturer. However, after optimization of the constituents 6 µg of plasmid DNA and 300 µl serum free DMEM together with 30 µl of PolyFect reagent were used. A_{260}/A_{280} ratio of the plasmid was over 1.80. Medium was replaced with 8 ml αMEM 12-16 hrs post transfection to ensure removal of excess DNA-PolyFect complexes from medium collections. Two Shh-αMEM batches were collected as LV-αMEM mentioned above, syringe filtrated with 0.2 µm filter units (Schleicher-Schuell) and stored at -70°C.

Part of the Shh-αMEM was taken separate to purify Shh from growth medium with 5 ml HiTrap Desalting column (Amersham Biosciences). Purification was performed following the manufacturer's instructions. Sample size was the maximum of 1.5 ml which was eluted with 5 ml 25 mM NaCl in PBS in 500 µl fractions. Total protein concentration in the fractions was determined with absorbance measurement and a standard curve, and the presence of Shh in each fraction with a western blot.

Cell culture of hESC MSC

A vial of passage 4 human embryonic stem cell derived mesenchymal stem cells (hESC MSC, received from Peiman Hematti, Wisconsin, USA) was thawed in +37°C water bath and suspended in warm αMEM. Traces of dimethyl sulphoxide (DMSO) were

removed by centrifugation with 250 x g 3 min at RT after which the cells were gently resuspended in αMEM and plated on a gelatinized (10 ml of 1 mg/ml solution, 4h) ∅10 cm cell culture plate (BD Falcon) in 10 ml αMEM. The cells were maintained by changing 8 ml of the medium every three days until near confluent. Plate was then washed twice with PBS (pH 7.4) prior to trypsinization with 0.05% Trypsin-EDTA (Gibco), and centrifuged and split in 1:5 on gelatinized plates as mentioned above. Originally two centrifugations were recommended during splitting a plate but here the cells were centrifuged only once to avoid causing excess stress.

HESC MSC cultures derived from the original vial were maintained for characterization of the cells and for the transplantation purposes. Normal and GFP labeled cells were passaged until their growth ceased. Part of the Shh medium was used for hESC MSC incubation to find out its effect on the cells. The cells were incubated in Shh either overnight or continuously. All cultures were examined daily with a microscope, and all procedures and observations were recorded.

Treatment of hESC MSC for transplantation

For bioluminescence imaging the hESC MSCs were labeled at passage 6 with luciferase containing lentivirus provided by Jari Heikkilä with titer of approx. $1x10^7$ IU/ml. HESC MSCs were incubated in 5 ml fresh αMEM in which LV-luciferase with multiplicity of infection (MOI) of approx. 20 was added with 40 µl hexadimethrine bromide (1 mg/ml) for 11 hrs. Fresh αMEM was then added on the cells, and luciferase expression was confirmed with IVIS 48 hrs after labeling. Before transplantation the cells were split to passage 7 and when near confluent trypsinized, centrifuged with 170 x g for 4 min, resuspended in 150 µl PBS per plate, and transplanted into rats.

Unlabeled hESC MSCs were split to passage 7 a day before labeling them with the GFP positive lentiviruses to obtain suitable cell density. Labeling was done by incubating each plate in 7 ml LV-αMEM (MOI approx. 3) from the first collected batch overnight with 56 µl of hexadimethrine bromide (1 mg/ml) to aid transduction of the virus. Batch was chosen after testing the medium with HEK 293T cells. After the incubation the LV medium was replaced with fresh αMEM, and the cells were allowed to recover and start

GFP expression for 48 hrs before transplantation. Untreated cells were then handled as mentioned above with the exception of resuspending them in 300 µl PBS per plate.

Some of the GFP+ plates were incubated in fresh Shh-αMEM for 3 hrs shortly before the transplantation. Cells were then trypsinized as mentioned above but resuspended in 300 µl of purified Shh in 25 mM NaCl-PBS per plate, and injected into rats.

Animal work

All animal experiments were conducted under the license 1547/05 permitted by the Experimental Animal Committee of Turku University, and followed the European Union legislation. Male Fisher 344 rats (n=15, 107-159 g) were anesthetized with 150 µl i.p. injections of fentanyl fluanisone (Hypnorm, fentanyl citrate 0.315 mg/ml and fluanisone 10 mg/ml, Vetapharma) and midazolame (Dormicum 1 mg/ml, Roche) diluted 1:1:4 in sterile water, and when necessary additional injections of 30-50 µl were given during the operation. Ischemia in the right hind limb was induced by surgically ligating the proximal end of femoral artery (a. femoralis) and its branch a. circumflexa femoris lateralis right below their bifurcation point, and the distal end above the branches a. saphena and a. poplitea. Operations were performed by Juha Laurila. The wound was sutured and the animals were injected with 50 µl naloxone (Narcanti 0.4 mg/ml, Bristol-Myers Squibb) i.p. to antagonize anesthesia.

All animals received 1.5 mg (10 mg/kg) cyclosporine A (Fluka) in PBS s.c. at the operation (day 1) and then once daily (days 2-4) total follow-up time being four days. Group 1 rats (n=4) were transplanted with hESC MSC at day 2 each animal receiving approx. 500 000 cells in 150 µl PBS at several locations i.m. in the ischemic limb. Group 2 and 3 rats (both n=4) were similarly injected with the Shh incubated hESC MSC or with 150 µl HiTrap purified Shh protein, respectively. Control rats (group 4, n=3) did not receive stem cells or Shh protein. All injections were given after 15-20 s sedation with O_2/high CO_2.

Immediately after killing (day 5) tissue samples were taken from both ischemic and normal muscle. The histological samples were freshly frozen in 2-methylbutane cooled with liquid nitrogen, and the tissue samples were frozen in cryotubes in liquid nitrogen. Histological samples were sectioned promptly and the rest stored at -70°C.

Bioluminescence imaging

The same femoral artery ligation operation was performed on two male rats by Juha Laurila, and the animals received similar cyclosporine A injections during the follow-up period as mentioned above. Luciferase-labeled hESC MSC, approx. $1x10^6$ cells, were transplanted in both rats 24 hrs after vessel ligation in 150 µl PBS on O_2/high CO_2 sedation. One rat was anesthetized as before for transplantation and injected i.p. with 200 µl D-luciferin (150 mg/kg, Synchem OHG) in PBS. After 16 minutes the wound was opened and the rat was imaged with Xenogen IVIS 50 Imaging System for bioluminescence. A second image was taken at six-hour time point 16 min after similar injection of D-luciferin. For imaging the rat was killed and the skin removed from the trunk to get a stronger signal. The other rat was imaged in the same manner at 24-hour time point.

RT-PCR and PCR

Genomic DNA was isolated from muscle samples of two rats from both group 1 and group 2 along with hESC MSC and normal rat tissue without stem cells. First, tissue was lysed with with 5µl proteinase K (20 mg/ml, Finnzymes) in 5 ml lysis buffer (10 mM Tris, 1 mM EDTA, 300 mM sodium acetate, 1% SDS, autoclaved) overnight at +37°C shaking. The initial amount of the proteinase K was not enough to disaggregate tissue and therefore 50 µl proteinase solution was added and the incubation continued at +55°C shaking until tissue was completely dissolved. DNA was extracted by adding 5 ml phenol and 3 ml chloroform, and then centrifuging with 2000 x g for 20 min. Top layer was transferred into a new tube for further two extractions with 6 ml chloroform and similar centrifugations. DNA was precipitated with 3M sodium acetate-ethanol overnight at -20°C and pelleted by centrifuging 17 000 x g for 30 min at +4°C. Pellets were resuspended in sterile water pooling the samples from both rats of the same group, and let stand for 2 hrs at RT and overnight at +4°C to allow complete resuspension. Concentrations were determined with Ultrospec 2100 *pro* Uv/Visible Spectrophotometer and the isolates stored at -20°C.

Total RNA of three rats from group 1 and from a control animal was isolated by first homogenizing the tissue samples with 1 ml Tri Reagent (Sigma). RNA was extracted by adding 200 µl chloroform to the homogenate and centrifuging with 17 000 x g for 15 min. Top layer containing the RNA was then precipitated with 3M sodium acetate-ethanol 30

min at -20°C, centrifuged with 20 000 x g for 30 min at +4°C and stored at -70°C. For reverse transcriptase (RT)-PCR messenger-RNA (mRNA) was purified with GenElute mRNA Miniprep Kit (Sigma) following the manufacturer's instructions. Before complementary DNA (cDNA) synthesis purified mRNA was treated with ribonuclease (RNase) inhibitor (Fermentas) and DNaseI (Fermentas) according to the manufacturer's instructions with 14 µl mRNA solution, 1.6 µl DNaseI buffer and 1 µl DNaseI.

RT-PCR was performed with Revertaid M-MuLV (Fermentas) reverse transcriptase following the manufacturer's instructions with the exception of mixing 1.6 µl random hexamer primers (Promega), 20 µl M-MuLV buffer (Fermentas), 8 µl dNTPs (Finnzymes) and 1 µl RNase inhibitor (Fermentas) before dividing it on the four DNaseI treated mRNA samples. Additionally, the first short incubation at RT was not included. Reaction products were stored at -20°C until amplification with PCR.

PCR for the prepared genomic DNA and cDNA with appropriate controls was done with human ALU (hALU) primers (Oligomer) which recognize an approximately 100 basepair (bp) long sequence. Amplification reaction volume was ~25 µl in each tube consisting of 10.5 µl water, 12.5 µl JumpStart REDTaq ReadyMix (Sigma), 1 µl both forward and reverse hALU primers, and either ~7 ng genomic DNA template or 1 µl cDNA template. In a 25-cycle reaction steps were as follows: 96°C 1 min, 60°C 1 min and 72°C 1 min with initial 4 min at 96°C and final 10 min at 72°C. The products were then prepared for agarose gel electrophoresis. From each sample 10 µl was mixed with 6x loading dye (Fermentas) and run on 2.5% gel with Generuler 100 bp DNA ladder (Fermentas). Gel was stained 3 min with ethidium bromide (0.5 µg/ml), rinsed with tap water and photographed (BioRad Gel Doc 2000).

Immunohistochemistry

Frozen muscle tissue was sectioned with a cryostat (Microm HM 500 M) into 10 µm thin sections on glass slides and kept at -20°C. Right after sectioning five slides from each group 1 and 2 animal were scanned with a fluorescence microscope (Olympus BX60) for the presence of GFP positive transplanted cells. For von Willebrand factor (vWF) staining of blood vessel endothelium endogenous peroxidase of the cryosections was blocked with 3% H_2O_2 diluted 1:10 in methanol for 30 min. Slides were rinsed briefly in PBS and blocked 30 min with FcR Blocking Reagent (MACS, Miltenyi Biotec) diluted 1:50 in 2%

BSA/TBS. Rabbit polyclonal primary antibody (Abcam) for vWF was diluted 1:800 in TBS and the slides stained for 1 hour at RT. Additional immunocontrol was incubated in TBS. Slides were washed twice in TBS for 30 min prior to incubation with HRP-anti-rabbit (Dakocytomation) secondary antibody for 30 min at RT. After two 10 min washes in PBS staining was visualized with 3,3-diaminobentzidine (DAB) diluted 1:50 in DAB substrate buffer (Dakocytomation). DAB reaction was allowed to proceed for 3 min. Slides were then rinsed briefly in milli-Q water, counterstained with hematoxylin-eosin, and mounted with Permount (Fisher Scientific) under coverslips.

Statistics

The vWF stained histological slides from each animal were blinded for two investigators and capillaries were counted from twenty randomly chosen 0.066 mm^2 rectangles from one section per animal using light microscope (Leica Aristoplan) with 500x (12.5x40) magnification. In each group, average number of capillaries per mm^2 was calculated, and the significances as compared to the control and Shh group were analyzed with two-tailed Student's t-test. Values of $p<0.05$ (*) and $p<0.01$ (**) were considered significant.

Results

Expansion of hESC MSC

The hESC MSC at passage 4 (P4) obtained from Peiman Hematti (University of Wisconsin, Madison WI, USA) were maintained in order to specify and supplement the data in the recently published work of Trivedi and Hematti (2007).

In culture the cells remained in a typical mesenchymal spindle-shaped phenotype with small filopodia and lamellipodia up until the passage 12, and were unaffected by one freeze-thaw cycle (figure 4A). When the cells were nearly confluent they oriented into wavy formations (figure 4A). However, at passage 13 the cells obtained a more neuronal cell-like morphology (figure 4B), with an axon-like protrusion and a wide dendrite-like lamella forming in the opposite ends. A small proportion of cells grew radial filopodia. Additionally, the size of the cells increased and they lost orientation at confluence but remained in good condition.

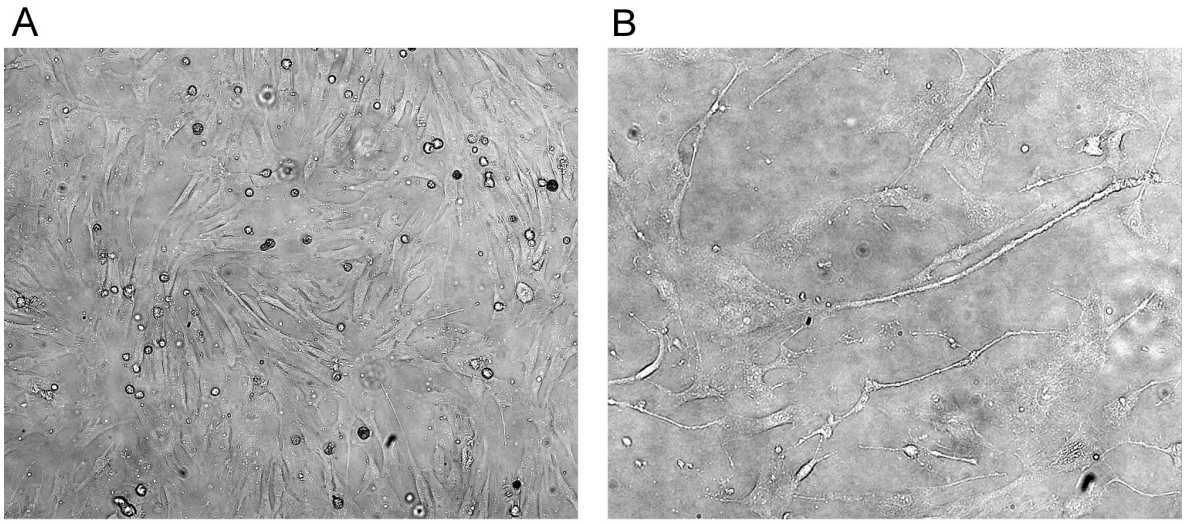


Figure 4. HESC MSC morphology at early and late passages. **A** At early passage the hESC MSC had recognizable spindle-like morphology. Round spots are detached cells. **B** Towards the end of their lifespan most hESC MSC developed a neuron-like polarized morphology.

The growth rate of the hESC MSCs differed in the course of culturing based on the visual observations and the frequency of splitting. The first ten passages up to P15 grew slightly more rapidly than the last few, which is in concordance with the experiments of Trivedi and Hematti (2007). The cells were also frozen and thawed once during this period,

but it did not have an apparent effect on the growth rate. Ultimately the hESC MSC reached passage 17, after which they became stagnant and died.

Shh production and its effect on hESC MSC in vitro

Shh protein was purified with a commercial kit for injection into the injured hind limb with and without hESC MSCs. The absorbance measurement at 280 nm (A_{280}) with spectrophotometer showed most of the protein in the fractions IV-VII (figure 5A). Protein concentration was determined with a standard curve and majority of isolated protein (0.13-0.17 µg/µl) was in the fractions III-V (figure 5B). Finally, a western blot made from all protein purification fractions confirmed the presence of Shh in the second fraction (figure 5C). The Shh protein concentration in fraction II was 0.073 µg/µl.

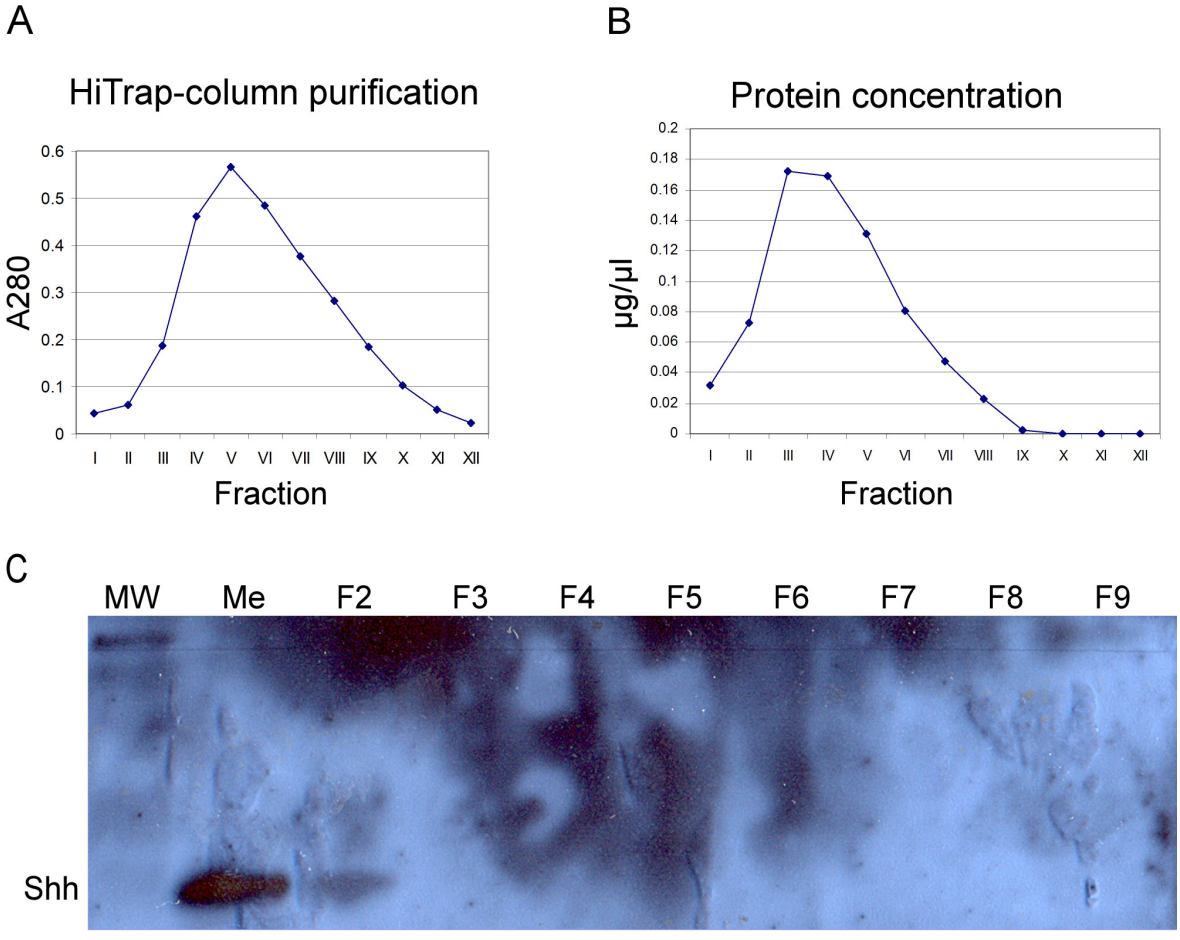


Figure 5. Shh protein purification. **A** Absorbance readings from the twelve fractions after HiTrap desalting column purification. **B** Protein concentrations from the same fractions as in (A) calculated from the standard curve. **C** Western blot made from the Shh containing medium (Me) and the fractions 2 to 9 (F2-F9). MW = molecular weight marker.

In order to find out the effect of Shh on the hESC MSC they were incubated in medium containing Shh. Either of the single overnight or long-term incubations did not cause changes in the growth or appearance of the cells. Their lifespan was similar to that of untreated cells with respect to growth rate and age.

LV-GFP production and transduction of hESC MSC

Lentivirus-GFP titer

By the first medium collection majority of the HEK 293T cells producing lentiviruses with GFP (LV-GFP) were fluorescent indicating a successful transfection (figure 6) but the presence of virus was further confirmed by running a transduction test and by determining the titer with HEK 293T cells. Majority of the cells on the test transduction plate were GFP positive two days after virus addition. LV-GFP titer was calculated from the result of the fluorescence activated cell sorting analysis using the 1:10 dilution which gave 3.2% fluorescent cells:

1. 3.2% GFP+ cells * 275 000 cells / 100 = 8800 GFP+ cells in 100 µl medium
2. 8800 GFP+ cells in 100 µl medium * 10 = $0.88e10^5$ IU/ml

Multiplicity of infection was determined for the 7 ml of transduction medium used for GFP labeling of the hESC MSC when the number of cells on the plate was approx. $2e10^5$:

3. (7 ml LV-medium * $0.88e10^5$ IU/ml) / 2* 10^5 = 3.08 ~ 3 MOI

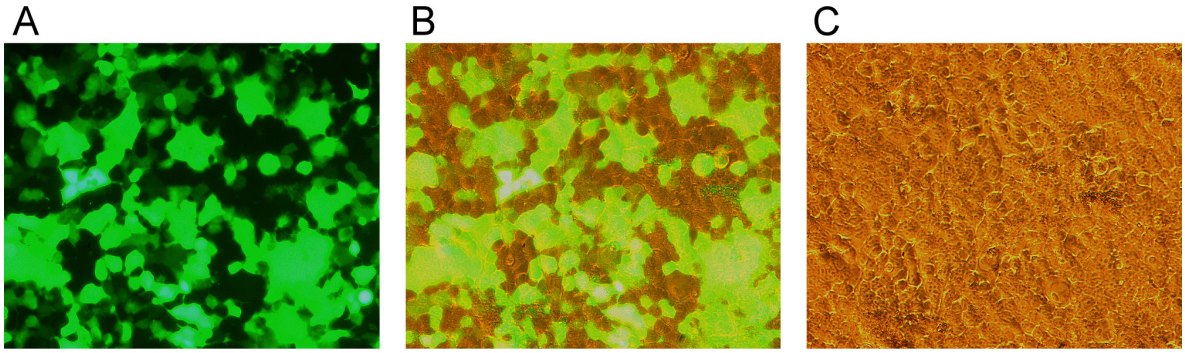


Figure 6. GFP+ lentivirus production with HEK 293T cells. **A** A fluorescent microscope image of transfected HEK 293T cells. **B** Merged ultraviolet and visible light images. **C** Visible light image of the cells in (A) and (B).

Transduction of hESC MSC turned out effective

HESC MSCs were labeled with luciferase-lentivirus to be used in bioluminescence imaging (BLI) with Xenogen IVIS 50 Imaging System after transplantation. The cells were labeled with multiplicity of infection (MOI) of approx. 20, and BLI 48 hrs after transduction showed even luminescence on the plate, average value being $1.54*10^{10}$ p/sec/cm^2/sr (figure 7A). The cells remained in good condition during the labeling procedure and until the transplantation.

HESC MSCs were also labeled with GFP for visual detection from the rat muscle tissue sections. MOI on the labeling was approx. 3, and incubation in LV-αMEM was highly efficient. By the time of transplantation nearly 100% of the cells appeared fluorescent in the microscope (figures 7B-C). However, due to the LV-αMEM being somewhat depleted of nutrients by the virus producing HEK 293T cells and the viral infection itself the condition of the stem cells weakened, although not critically. After changing fresh αMEM medium for the next 48 hrs prior to transplantation the cells recovered and displayed normal morphology.

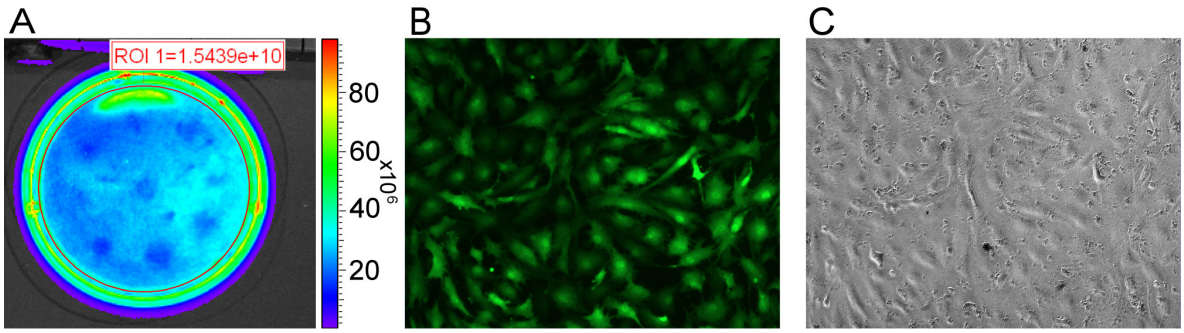


Figure 7. Luciferase and GFP labeling of the hESC MSC. **A** Bioluminescence image of the luciferase+ hESC MSC on ∅10 cm plate prior to transplantation. **B** Fluorescence microscope image of the GFP+ hESC MSC on ∅10 cm plate prior to transplantation. **C** Visible light image of the cells in (B).

The lentiviral transduction of the GFP and luciferase plasmid into the hESC MSC resulted in a strongly positive fluorescent and luminescent phenotype, respectively, after a single exposure to the virus. The cells retained this trait even after freezing and thawing rendering the method a very efficient one. However, the first signs of alterations in the morphology of GFP-labeled cells were observed already at P10 as the cells developed neuron-like, and shortly after senescent traits. The life span of GFP+ hESC MSC was

similar to that of the GFP negative cells in respect of passage number but the growth rate was continuously faster.

Stem cell transplantation and hESC MSC tracking

Amount of hESC MSC decreased rapidly at the transplantation site

Two animals were imaged for bioluminescence at three time points after luciferase-hESC MSC transplantation to elucidate their fate in rat tissues (figure 8A). Initial signal from the transplanted cells was clear and centered at the site of injections (figure 8B). After six hours the signal had decreased to 28% of the original while scattering of the cells was not observed (figure 8C). By the 24 hour time point the signal intensity had weakened further being only 1.5% of the original (figure 8D). The location of the signal had not changed, and no new luminescent spots had appeared elsewhere indicating that the cells had died in situ.

A few scattered GFP+ cells were seen in cryosections

Histological sections of the two transplantation groups were visually scanned for GFP fluorescence with a fluorescence microscope immediately after sectioning. Fluorescent spots presumed to be the hESC MSC were seen on the slides from both groups scattered on the whole area of the sections which indicates that after three days the cells were still present in rat tissues (figure 9A). Localization to the proximity of recognizable structures such as blood vessels was not observed indicating that the cells did not incorporate into the tissue, though present at the site of injury.

Human DNA and mRNA were found in the rat tissues three days post-transplantation

PCR products of both genomic DNA and mRNA isolated from the rat muscle tissue were run in the agarose gel to determine the presence of human nucleic acids. Positive control, i.e. the genomic DNA of hESC MSCs, appeared as a bright band on both rows on the gel, and the size of the fragment was the expected 100 bps according to the ladder. Fragments of the same size were seen on the sample lanes containing the genomic or the complementary DNA (cDNA). The band for genomic DNA was clear but less intense than the positive control indicating the presence of hESC MSCs in the rat hind limb tissues

three days after the transplantation (figure 9B). The cDNA band was very faint but in all visible suggesting that at least some hESC MSCs were still alive at the end of the follow-up period since otherwise mRNA would have been rapidly destroyed (figure 9C).

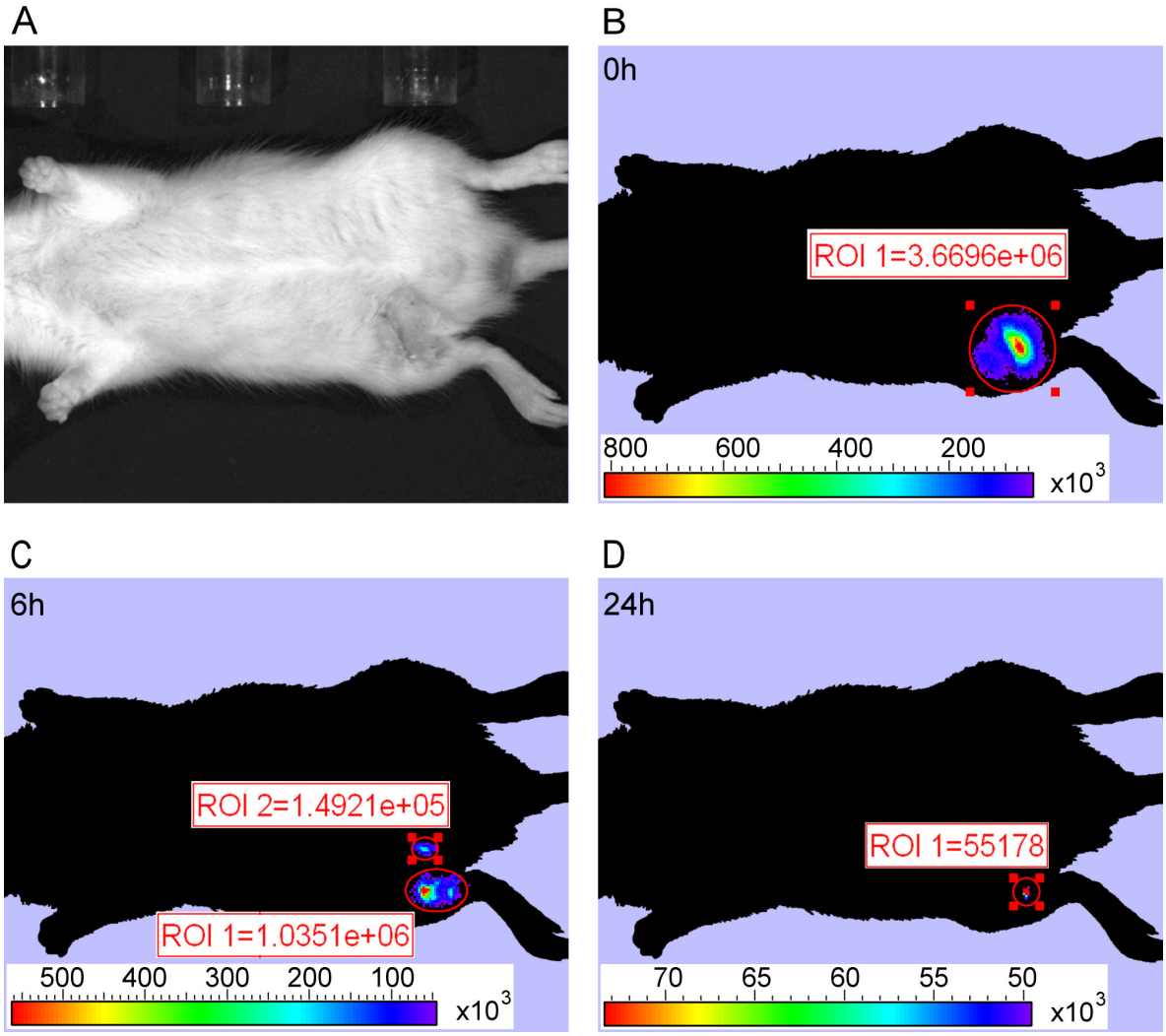


Figure 8. Reduction of locally injected hESC MSC during the first 24 hours after transplantation. **A** The rats were photographed from the ventral side. **B** Bioluminescence at the time of transplantation (0 hours). **C** Bioluminescence 6 hours after transplantation was 28% of the initial luminescence. The small luminescent area is likely an artifact from the preparation procedure. **D** Bioluminescence 24 hours after transplantation was 1.5% of the initial luminescence. ROI = region of interest. Units at ROI as p/sec/cm^2/sr.

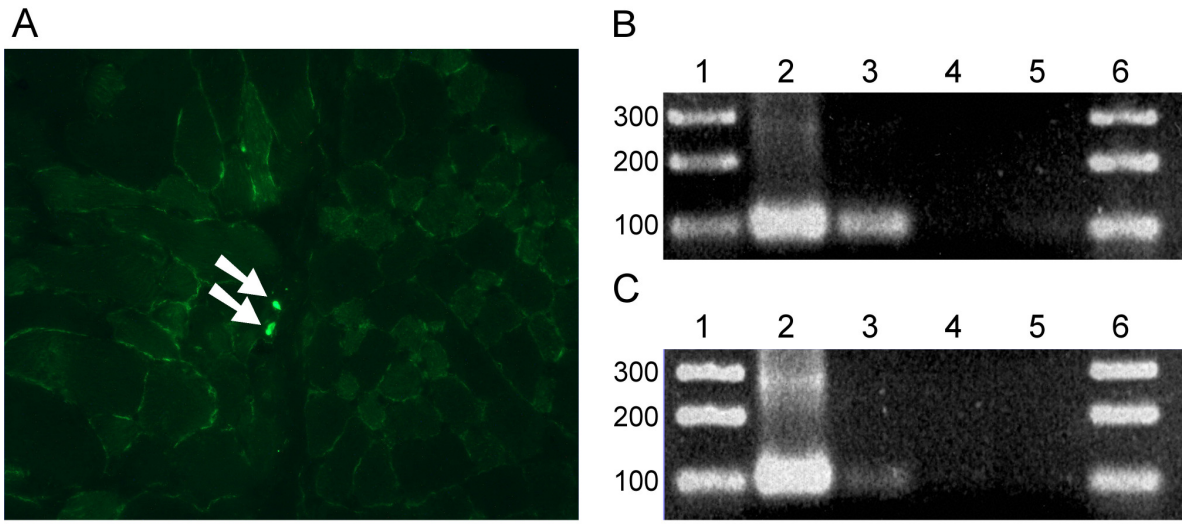
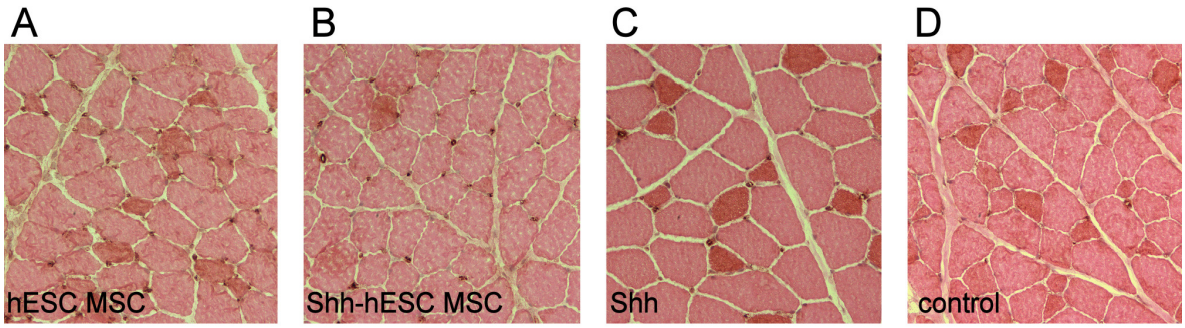


Figure 9. HESC MSC presence in the rat tissues 72 hours after transplantation. **A** Rare fluorescent spots were detected from the unstained cryosections (arrows). Background fluorescence from the muscle tissue was weak. **B** PCR amplification of the genomic DNA revealed a small amount of human DNA present in rat tissues. **C** PCR amplification of the complementary DNA (cDNA) confirmed the presence of human mRNA in rat tissues. Lanes: 1) and 6) Molecular weight marker (kb). 2) Positive control (hESC MSC). 3) Sample. 4) Negative control (untransplanted rat). 5) Water control.

Effect of transplantation on angiogenesis

Experimental groups were injected with either hESC MSC, Shh incubated hESC MSC or Shh protein alone, and the number of capillaries per mm^2 was counted from each animal (figures 10A-D). In both stem cell groups the capillary density was 48% higher (on average 311 capillaries/mm^2) than in the control group (approx. 210 capillaries/mm^2). Number of capillaries in the Shh group was 14% higher than in the control group (approx. 239 capillaries/ mm^2). Capillary density was significantly higher in hESC MSC and Shh-hESC MSC groups ($p<0.014$ and $p<0.017$, respectively) as compared to control whereas in the Shh group it was not (figure 10E). Additionally, the capillary number increase in stem cell groups was significant when compared to Shh group ($p<0.0012$ for hESC MSC and $p<0.002$ for Shh-hESC MSC, figure 10E). These results indicate that the observed improvement in angiogenesis was not attributable to Shh administration but to the hESC MSCs.



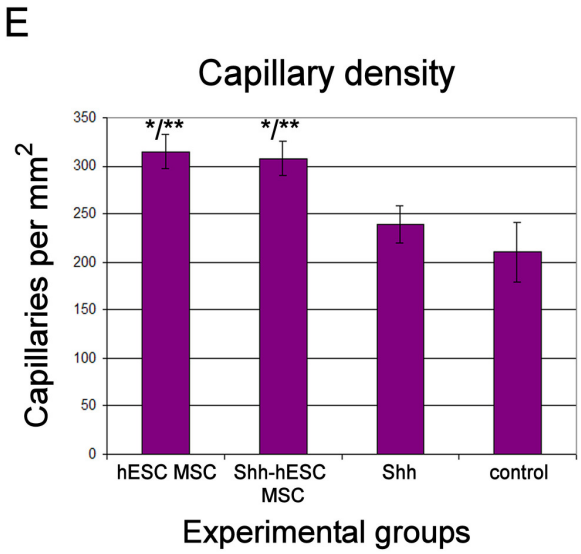


Figure 10. Capillary formation in the rat muscle. In **A** hESC MSC group and **B** Shh-hESC MSC group the number of capillaries was higher than in the group that received **C** only Shh protein or **D** in control group. Horseradish peroxidase stained capillaries are visible as brown spots on thin sections. **E** Statistical analysis showed similar significant increase in both cell transplantation groups as compared to control (*) and Shh group (**). The difference between Shh group and control group was not significant.

Discussion

Human embryonic stem cell derived mesenchymal stem cells (hESC MSC) constitute a new cell line that has gained interest in the recent years in terms of their derivation and possible clinical applications. This is justified by the benefits they have over more traditional stem cell types, such as the practically endless source material, resistance to malignant transformation (Barberi et al., 2007), and multilineage differentiation (Trivedi and Hematti, 2007). These characteristics provide a good starting point for a range of therapies. This study focused on the ability of hESC MSC to repair tissue damage after induced ischemia and following inflammation in a rat hind limb model. The injury model used here offers a rather simple and reliable way to generate acute ischemia, and thus it was considered suitable in investigating the course of events after transplantation.

According to our observations the hESC MSC resemble normal bone marrow derived mesenchymal stem cells (Sekiya et al., 2002). The cells were readily labeled with lentiviral GFP and luciferase, and the label was stable through passaging and freeze-thaw cycles. Additionally, the cell line was found to be finite with similar passage numbers as described in the paper of Trivedi and Hematti (2007). Malignant transformation in stem cells is always a concern when conducting research with clinical perspectives, but a promising study suggested that this type of cells did not form tumors during six month surveillance after transplantation into mice (Barberi et al., 2007). Here spontaneous transformation was not detected *in vitro* but the follow-up time was too short to establish this *in vivo*. However, alterations in morphology *in vitro* at late passages may indicate that the hESC MSC loose their ‘stemness’, although it has been shown that differentiation potential is preserved even in prolonged culturing (Barberi et al., 2007). In this study, transplanted cells were at passage 7 whereas the hESC MSC have been found to be multipotent still at passage 9 (Trivedi and Hematti, 2007).

Ischemic environment in skeletal muscle is characterized by massive cell death and high amounts of free oxygen radicals, cytokines and inflammatory cells responding to acute hypoxia. Consequently, apoptosis-inducing and cytolytic factors from necrotic cells, and infiltrating monocyte-macrophages and granulocytes create harsh conditions for the transplanted cells. Apoptosis or necrosis in situ has probably been the fate of most transplanted hESC MSC since any signal from possible target organs of stem cell

migration was not detected. The presence of live hESC MSC was initially indicated by the few scattered fluorescing dots in the histological sections and faint bioluminescence obtained with the IVIS at 24 hour time point. Three day time point was not considered necessary since bioluminescence had already decreased to 1.5% of the original 24 hours after transplantation. Our RT-PCR and PCR results showed presence of human mRNA and genomic DNA confirming that some hESC MSC were indeed alive after three days in the host tissues. It is well known that free DNA and RNA are rapidly destroyed once the cell has ruptured necessitating the presence of live cells in our experimental conditions.

Presence of a number of different signaling molecules in the ischemic site where the hESC MSC were transplanted could possibly promote their differentiation. Multipotent mesenchymal stem cells have a wide differentiation capacity from adipocytes and myoblasts (Pittenger et al., 1999) even beyond mesenchymal borders to e.g. neuronal cells (Woodbury et al., 2000). In our model, extensive tissue damage and inflammation would likely promote differentiation of the stem cells towards fibroblast-like cells and result in scar tissue formation (Yan et al., 2007). Other possibilities include myoblasts and pericytes, which are naturally of mesenchymal origin and have been observed to arise from transplanted autologous MSCs in ischemic muscle tissue (Tang et al., 2006). Our three day follow-up time was far too short for the stem cells to fully differentiate since it usually requires at least a few cell divisions. Additionally, the random distribution of the cells and rapid decline in bioluminescence implies that the hESC MSC were not even engrafted into host tissue which could be due to the unfavorable environment and *in vitro* culturing (Rombouts and Ploemacher, 2003).

Neoangiogenesis is an absolute requirement for tissue healing, and it is regulated by a complex signaling network of growth factors e.g. VEGF, FGF, and cytokines produced by the participating cells such as endothelial and smooth muscle cells, monocyte-macrophages, and fibroblasts. As a sign of active angiogenesis we observed significantly increased capillary formation in both test groups that received hESC MSC. However, Shh alone did not have any major effect. These results suggest a more important role for the stem cells in tissue recovery, although Shh has been shown to take part in the angiogenetic events (Kusano et al., 2005). Possible causes for our observations could be the low amount of Shh in medium, its inefficient purification or inactivation of the purified protein during

the short storage in -70°C. These would also account for the seemingly ineffective *in vitro* incubation of the hESC MSC in Shh medium.

Another substance that has been observed to affect angiogenesis is cyclosporine A. Despite the side effects cyclosporine A has been used for long to suppress the host immune system against a transplanted organ and its effect is based on the downregulation of the T cell response. Administered alone it has been shown to inhibit VEGF-mediated angiogenetic signaling pathway *in vitro* (Rafiee et al., 2004) but in our study the hESC MSCs seemed to exert an antagonizing effect. Cyclosporine A has also been shown to be important in enhancing MSC graft survival after transplantation into rat spinal cord (Swanger et al., 2005); however, we did not see any sign of actual engraftment. Whether the cyclosporine A affected the rapid clearance of the hESC MSCs from rat tissues in unclear but, altogether, our results suggest an alternative mechanism for the MSC-induced tissue recovery.

Results of mesenchymal stem cell transplantations vary immensely from study to study suggesting either high engraftment efficiency (Direkze et al., 2003) or none at all (Zentilin et al., 2006), or something between, although their therapeutic effect remains undisputed. We did not observe engraftment but the contribution of hESC MSC to tissue recovery was evident and therefore other mechanisms must underlie the positive effect. As shown by several studies, the paracrine effect by the MSCs can improve cardiac muscle recovery from ischemia (Iso et al., 2007; Kinnaird et al., 2004) but here the dead hESC MSCs clearly outnumbered the live ones very rapidly rendering this mechanism unlikely or at least highly ineffective. A more feasible explanation would be that the cell debris and released cell contents activated resident macrophages and other tissue cells, thus stimulating the expression of angiogenic factors as a result of inflammatory reaction first initiated by hypoxia; however, we have not confirmed the activation of the immune system. In the future, a thorough investigation of the signaling events and interactions between the hESC MSC and host tissue are needed to estimate the potential of this cell population in clinical settings.

In summary, we have demonstrated the ability of hESC MSCs to improve tissue recovery through angiogenesis in ischemic muscle tissue of rat hind limb. Our data does not support stem cell engraftment into host tissues after transplantation as shown by

bioluminescence imaging and rare GFP+ cells in the tissues, although a small number of them had survived according to the results of PCR amplification using human specific primers on rat tissue DNA and mRNA. The actual mechanism behind the significantly enhanced angiogenesis and tissue healing still needs to be elucidated, and safety studies regarding their tumorigenicity are required.

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