VEGF-expressing mesenchymal stem cells for improved angiogenesis in regenerative medicine

- a bone tissue engineering approach -

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auf Antrag von

Prof. Dr. Markus Affolter
Dr. Andrea Banfi
Prof. Dr. Michael Heberer

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Prof. Dr. Martin Spiess
(Dekan)

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„Damals war heute noch Zukunft."
(Christoph Sonntag)

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1 INTRODUCTION

1.1 BONE BIOLOGY

The Oxford Dictionary defines bone as "any of the pieces of hard whitish tissue making up the skeleton in humans and other vertebrates. The substance of bones is formed by specialized cells (osteoblasts) which secrete around themselves a material containing calcium salts (which provide hardness and strength in compression) and collagen fibers (which provide tensile strength)".

More precisely, bone is a dynamic, highly vascular and mineralized connective tissue which, together with cartilage, builds up the skeletal system. Its main functions are to provide mechanical support, protection of vital organs and a site of muscle attachment for locomotion. In addition, bone contains bone marrow and is therefore the primary site for the synthesis of blood cells. Furthermore, bone tissue serves as a reserve of calcium and phosphate, which is used for the maintenance of serum homeostasis (1).

Bone consists of approximately 70% inorganic and 30% organic composites. The inorganic component contains mainly spindle- or plate-shaped crystals of carbonated hydroxyapatite (bone apatite), an insoluble calcium and phosphorus salt that precipitates on the protein matrix. This hydroxyl-deficient, but carbonate-rich substance is similar to hydroxyapatite but it is not a direct analog (2).

Only 2% of the organic fraction is made up by cells, growth factors and cytokines, such as fibroblast growth factors (FGFs), platelet-derived growth factors (PDGFs), transforming growth factor-beta (TGFβ) and bone morphogenic proteins (BMPs). Ninety percent of the so called organic extracellular matrix is formed by collagen type-I fibers. Proteoglycans and non-collagenous proteins, as for instance Osteocalcin (OC), Osteopontin (= Bone Sialoprotein I, OP), and Bone Sialoprotein II (BSP), account for the remaining 8% (1, 3).

Skeletal elements are initially formed as soft tissue templates. During this process, which in humans is completed by the end of the embryonic development, the basic shape of all bones is preformed. Thereafter the soft tissue template undergoes gradual ossification and growth, leading to an increase in size with relatively little changes in shape. This phase of bone formation lasts from the early stages of fetal life until adulthood.

Two distinct mechanisms are responsible for bone formation: Intramembranous ossification, giving rise to flat bones (e.g. scull bones, scapula, mandible and ilium) and endochondral ossification, creating long bones (e.g. tibia, femur, humerus and vertebrae).

Both types of ossification involve initial condensation of mesenchyme followed by the formation of calcified bone. Whereas intramembranous bone formation accomplishes this directly, endochondral ossification involves an intermediate step in which a cartilaginous template regulates the growth and patterning of the developing skeletal element. Endochondral bone formation initiates with the condensation of loose mesenchyme, resulting in the differentiation into cartilage tissue. The cartilaginous center subsequently becomes hypertrophic and a center of ossification forms by vascular invasion. The secretion and mineralization of extracellular matrix is mediated by osteoblasts that are associated with the newly developed vasculature. As bones grow, this center of ossification expands and the inside of the shaft region is resorbed (Figure 1.1) (4, 5).

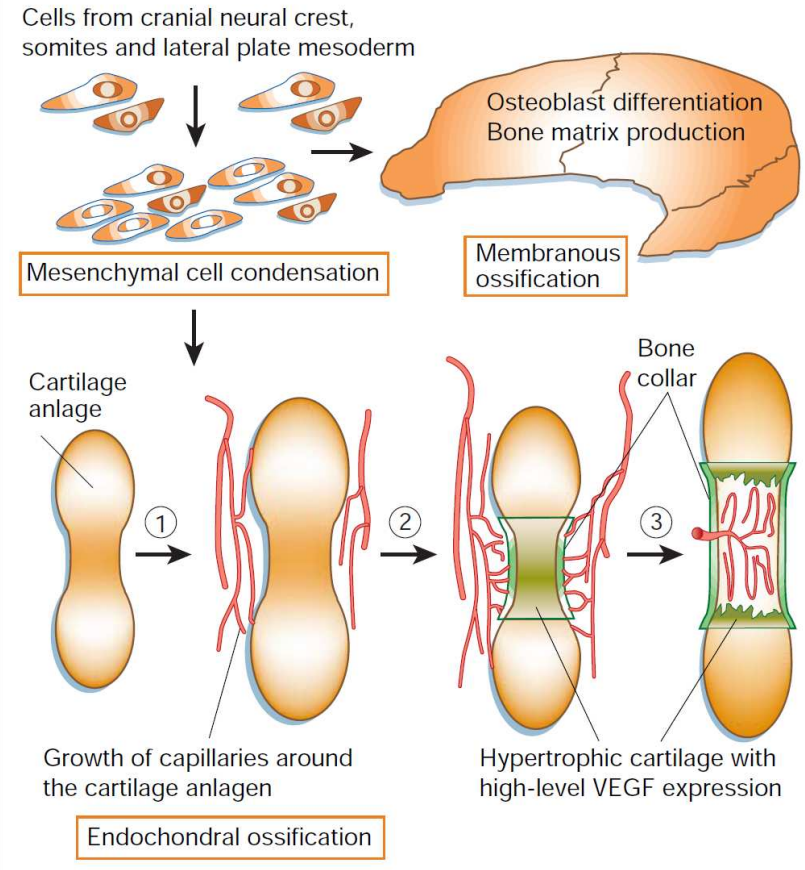


Figure 1.1 Intramembranous and endochondral ossification. While in membranous ossification, differentiation of mesenchymal cells to osteoblasts and bone matrix production occur directly, in endochondral ossification, differentiation to chondrocytes and formation of a cartilaginous template (anlagen) occurs first, followed by replacement of the models by bone through vascular invasion. (Source (6))

The bone surface consists of compact cortical bone (cortex), a thick and dense protective layer of calcified tissue which has a high resistance to bending and torsion. It shows an increase in thickness in mechanically demanding regions, such as the shafts of long bones. The interior of bones, such as within the distal ends of long bones, in short bones, in the inner surfaces of flat bones and in irregular bones (e.g. vertebrae), contains thin calcified trabeculae, called cancellous or trabecular bone. Spaces enclosed by trabecular bone are filled with bone marrow. Due to its more elastic properties, trabecular bone contributes to the mechanical support of the skeleton. While cortical bone makes up 80% of the skeletal mass, trabecular bone represents only 20% of the skeleton by mass, but, due to its structure, 80% of the bone surface (3).

The external surface of all bones is covered with the periosteum, a fibrous membrane which contains the blood vessels and nerves that provide nourishment and sensation to the bone. It also plays an important role in osteogenesis, as it is colonized by bone cells (7). Also the internal bone surface is lined by a thin layer of osteogenic and other cells (endosteum).

In addition to external vascular supply through the peri- and the endosteum, cortical bone itself is perfused by blood vessels. These are located in special canals, which also accommodate nerves and lymphatic vessels (Haversian canals), and are essential for homeostasis of cells embedded within the bone matrix (Figure 1.2).

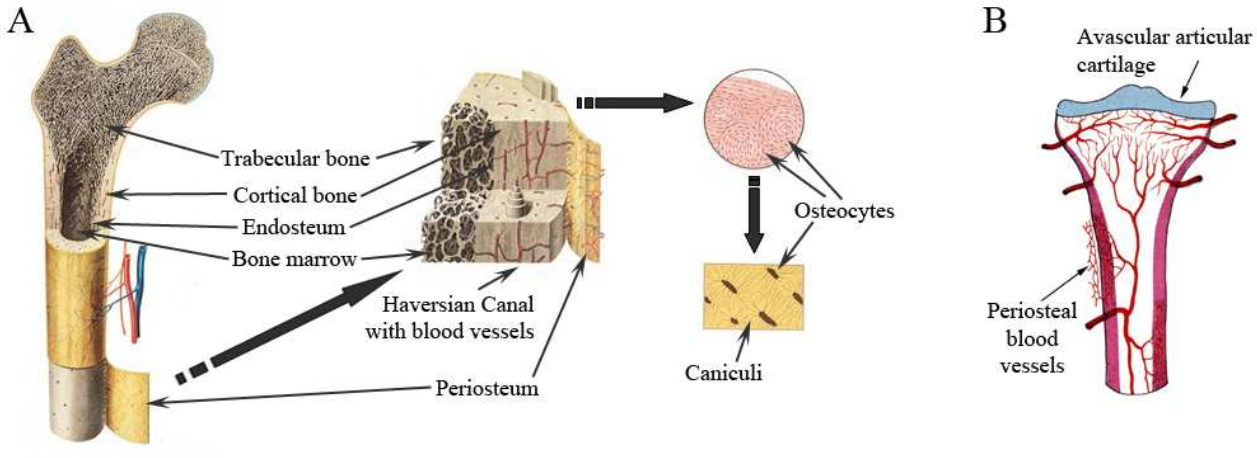


Figure 1.2 Schematic drawings of (A) bone tissue and (B) bone blood supply. (Adopted from (8, 9))

Osteoblasts, osteocytes and osteoclasts are the major types of bone cells. Osteoblasts are bone forming cells. They originate from local pluripotent mesenchymal stem cells, either bone marrow stromal cells residing in the endosteum or connective tissue mesenchymal stromal cells from the periosteum. Lining the bone surface, they are gradually trapped in the bone matrix which they produce and that calcifies. As a result those cells dramatically decrease their metabolic activity, now becoming fully differentiated mature bone cells (osteocytes) enclosed in small lacunae. The older they are and the deeper they are located within the calcified bone the more they show a decrease in cell volume and organelles. Osteocytes are the most abundant cell type in bone tissue. In order to communicate with other osteocytes and bone lining cells they extend their cytoplasmic processes into the bone matrix and, in this way, constitute a well-developed canalicular network. (1, 10).

In contrast to osteoblasts and osteocytes, osteoclasts are giant multinucleated bone resorbing cells, which differentiate from hematopoietic progenitors of the monocyte/macrophage lineage. Like osteoblasts they are situated on the bone surface (11). The correct interplay between those cell types is essential in bone homeostasis, remodeling and repair.

The process of bone remodeling is a turnover mechanism which permits old bone replacement as well as changes in shape, architecture or density of the skeleton. It involves three consecutive phases: resorption, during which osteoclasts digest old mineralized bone; reversal, when mononuclear cells appear on the bone surface; and formation, when osteoblasts lay down new bone until the resorbed bone is completely replaced (Figure 1.3) (12).

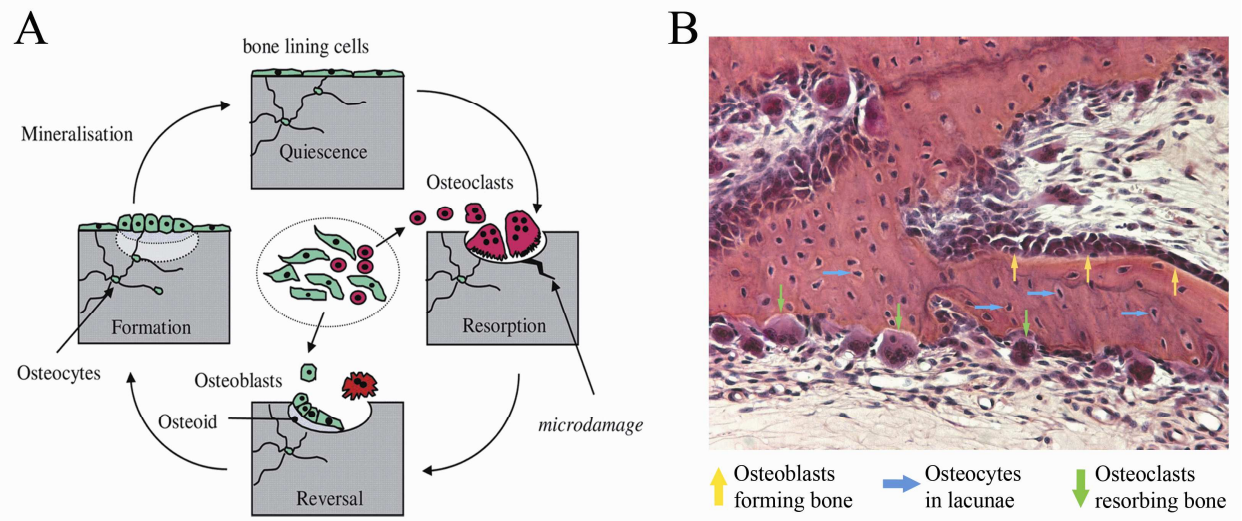


Figure 1.3 Bone remodeling. (A) Bone is formed through the synthesis of bone matrix by osteoblasts (formation). As osteoblasts are integrated in the matrix they produce, they become osteocytes or resting bone lining cells on the bone surface (quiescence). Due to specific stimulatory factors, preosteoclasts are activated to mature to osteoclasts which digest the mineral matrix (resorption). As soon as bone resorption finalizes, osteoblasts are recruited and the cycle restarts (reversal). (Source (13)) (B) Histologic image of remodeling bone. (Adopted from (14))

Bone healing is initiated by hematoma formation and inflammation at the fracture site. The inflammatory phase is a critical period characterized by low oxygen tension, impaired perfusion, and the migration of a wide array of growth factors. Systemwide inflammatory conditions also modulate the primary processes of fracture management. Osteoprogenitor cells, mesenchymal cells, osteoblasts and chondrocytes contribute to the healing and inflammatory response at the bone level. As a second step, bone is formed through endochondral or intramembranous ossification very rapidly in a provisional manner. During the following remodeling process it is progressively replaced by mature bone (15).

1.2 BONE DAMAGE AND RECONSTRUCTION

1.2.1 BONE LOSS AND STANDARD PROCEDURES FOR BONE REPLACEMENT

As bone remodeling is strictly regulated within a tightly coupled system, any local or systemic perturbation leading to uncoupling of the osteoclastic and osteoblastic balance may substantially change bone mass. Bone turnover is perturbed in many diseases, each characterized by a specific pathophysiology.

An example for such a metabolic bone diseases is osteoporosis, a chronic progressive disorder, in which decreasing bone mass and micro architectural deterioration leads to increased bone fragility and fracture risk. Also loss of bone blood supply in ostoenecrosis has serious consequences, as it results in death and eventually collapsing of bone tissue. External factors well known to cause altered bone turnover are for instance use of corticosteroids, immobilization, as well as calcium and vitamin D deficiency (3).

Apart from those degenerative and metabolic forms, bone loss may also be caused by trauma. For various reasons, which might be associated with the host, the surgical technique, inadequacy of the vasculature and infections, fracture healing can be disturbed. In the case of critical-sized bone defects, the osseous damage can be up to several centimeters and will not reach complete healing spontaneously (16, 17).

In contrast to those bone degenerative disorders, bone tumours are characterized by an excessive cell growth within the bone which may replace healthy tissue with abnormal tissue, thus weakening the bone and causing it to break. Often the tumours need to be removed.

In order to replace bone two different sources are currently used in the clinics. Bone taken from the iliac crest, the distal radius, the tibia or the fibula allows autologous transfer, but is limited by the amount of tissue that can be removed and the high donor-site morbidity. Acellular allografts and synthetic bone substitutes do not show these disadvantages, but, on the other hand, do not posses the same physiological properties as natural bone.

Several different approaches to improve therapeutic options, including the refinement of synthetic bone grafts or tissue engineering, are at present under investigation.

1.2.2 BONE TISSUE ENGINEERING

As defined by Langer and Vacanti, "tissue engineering is an interdisciplinary field of research that applies the principles of engineering and the life sciences towards the development of biological substitutes that restore, maintain, or improve tissue function" (18). There are three main approaches to engineer biological bone grafts: (i) to use isolated cells, (ii) to use acellular, three-dimensional biomaterials capable of inducing tissue regeneration, and (iii) to use a combination of cells and materials (typically in the form of scaffolds) (Figure 1.4) (19). In practice, this comprises the following main areas:

1) Matrices: including biomaterials that are designed to direct the organization, growth, and differentiation of cells in the process of forming functional bone tissue by providing both physical and chemical cues. A large number of various materials are currently under investigation or commercially available. The ideal scaffold should be: osteoconductive (bone growth on the surface), osteoinductive (induction of osteogenesis), biocompatible, bio-resorbable and have similar strength as well as, at the same time, a similar modulus of elasticity to that of bone. In addition, it should be easy to use and cost-effective. Osteoinductive scaffolds can be devided in natural biomaterials, like demineralised bone matrix or collagen based matrices, and synthetic bone substitutes, including ceramics (e. g. tricalcium phosphate, hydroxyapatite, …) and composite grafts. More recently, also glass- and metal-based scaffolds have been developed. As natural biomaterials provide only minimal structural support, synthetics are mainly used for the regeneration of large bone defects. (20, 21)
The osteoinductive material used in the experimental work presented in this thesis is a degradable, synthetic product based on hydroxyapatite (Actifuse™ Microgranules from Apatech®). An additional silicate substitution changes the surface charge of the material

attracting more of the proteins that bind osteoblasts and therefore accelerates bone formation. In addition, an optimised structure with interconnected porosity encourages and supports rapid bone formation.

2) Cells: including enabling methodologies for the proliferation without loss of progenitor cell capacities and osteogenic differentiation of cells, as well as acquiring the appropriate source of cells. Based on many reasons presented in chapter 1.3, mesenchymal stem cells (MSC) are the most widely used for the purpose of bone tissue engineering.

3) Biomolecules: including growth and differentiation factors. Fibroblast growth factor-2 (FGF-2) is effective in MSC *in vitro* expansion while preserving their osteogenic potential (22, 23), whereas a cocktail containing dexamethasone, β-glycerophosphate and ascorbic acid-2-phosphate is typically used to induce osteogenic differentiation of MSC (24).

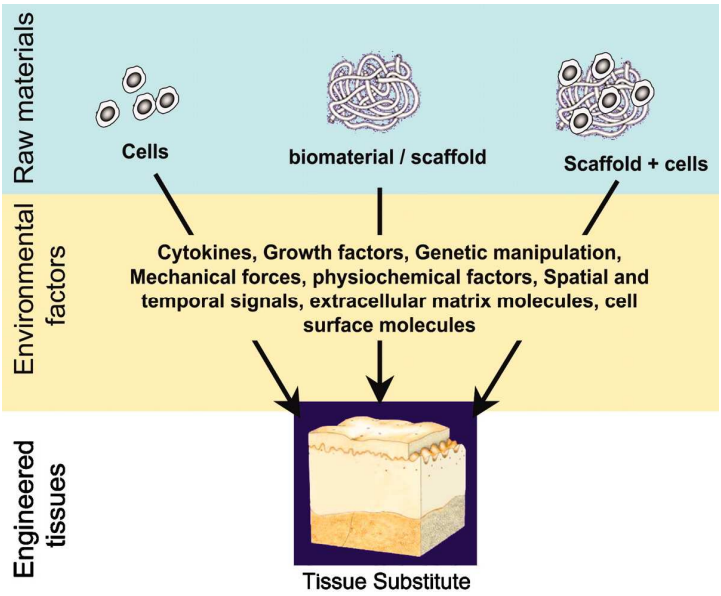


Figure 1.4 Tissue engineering approaches. Tissue engineering approaches are classified into three categories: (i) cells alone, (ii) cells with scaffolds, and (iii) scaffolds alone. Each one of these approaches can be enhanced by in vitro microenvironmental factors before application as a tissue substitute. (Source (19))

In addition to having a therapeutic application, where the tissue is either grown in a patient or outside the patient and transplanted, tissue engineering can have diagnostic applications where the tissue is made in vitro and used for testing drug metabolism and uptake, toxicity, and pathogenicity (25).

1.3 MESENCHYMAL STEM CELLS

Cells used for bone tissue engineering need to fulfill certain requirements. Apart from the prerequisite of having the consistent potential to differentiate towards the osteogenic lineage, the ideal cells should be easily accessible by a minimally invasive procedure and be highly proliferative *in vitro* without being mutagenic *in vivo*. Other points to be avoided are immunorejection and graft-versus-host disease. These negative side effects, as well as contamination with diseases such as hepatitis and HIV can be prevented when using autologous instead of allogeneic or even xenogeneic transplants.

Even though autologous osteoblasts are the cell source being most connatural with fully differentiated bone tissue, their use is constrained by their invasive and destructive method of isolation. Moreover, osteoblasts are not capable of extensive proliferation to gain sufficient cells for tissue engineering purposes.

Pluripotent stem cells, as embryonic stem cells or induced pluripotent stem cells, on the other hand, are easily expandable and can commit into a large number of cell types including bone cells, but are prone to forming teratomas (26-29). In addition, the use of embryonic stem cells always carries ethical concerns.

Therefore, most tissue engineering approaches focus on the use of autologous adult Mesenchymal Stem Cells (MSC). These multipotent cells are capable of *in vitro* differentiation into mesodermal cell lineages, like osteoblasts, chondrocytes and adipocytes. Their *in vitro* differentiation capacity towards other lineages, including cardiomyocytes, endothelial cells, pericytes, neurons and astrocytes, has also been proposed by several groups, but is still controversial (30-34). *In vivo*, expanded MSC demonstrated tissue engraftment and differentiation into lineages as cardiomyocytes (32), endothelial cells (35-37), vascular smooth muscle cells (36), pericytes (35) and bone (38).

Nowadays MSC have been found to reside in many adult tissues such as liver, muscle, lung, spleen, aorta and thymus (30), where they represent a rare population localized in small niches, specialized local microenvironments that protect the stem cells from differentiation and apoptotic stimuli or uncontrolled proliferation (39).

Right after tissue harvest and cell isolation, MSC are still a minor fraction within a very heterogeneous cell population, which gradually becomes more homogeneous throughout cell expansion on tissue culture plastics in the appropriate media and culture conditions. MSC are thought to home to damaged tissues, where they support wound healing and hematopoiesis, but many of the native functions of MSC remain poorly understood. When engrafted at sites of tissue injury, MSC differentiate into connective tissue elements, support vasculogenesis and secrete cytokines and growth factors that facilitate healing (40). In addition, MSC have been shown to be hypoimmunogenic, immunosuppressive and immunoregulatory (41).

MSC fate is influenced by a multitude of factors. Not only growth factors, but also biophysiological and mechanical stimuli, such as pH, temperature, oxygen and nutrient delivery as well as matrix elasticity or fluid shear provide lineage-specific stimulation in a spatially and temporally controlled manner (42). Even though MSC are expandable *in vitro*, they gradually loose their differentiation potential and finally undergo senescence (43, 44). More importantly, they can develop karyotype abnormalities *in vitro* due to long-term culture (45).

As *ex vivo* expansion is necessary for genetic modification and to obtain a sufficient cell number for tissue engineering of grafts with a clinically relevant size, the culture duration needs to be minimized and the conditions of culture must be optimal.

As the field of MSC research expands rapidly, investigators developed different methods of isolation and expansion and different approaches to characterize these cells. To address this issue, the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy (ISCT) has proposed minimal criteria to define human MSC: 1) MSC must be plastic-adherent when maintained in standard culture conditions; 2) MSC must express CD105, CD73 and CD90, and lack expression of CD45, CD34, CD14 or CD11b, CD79a or CD19 and HLA-DR surface molecules; and 3) MSC must be able to differentiate to osteoblasts, adipocytes and chondroblasts *in vitro* (46).

The most commonly used and characterized MSC are derived from bone marrow (BMSC) (47) and the stroma vascular fraction of adipose tissue (ASC) (48).

The idea that bone marrow contains osteogenic precursor cells was first demonstrated in the mid-20th century, when several research groups showed that bone marrow *in*

vitro or implanted *in vivo* gave rise to bone tissue (49-51). The similarity between the mesenchymal stem cell properties of ASC and BMSC, including their osteogenic potential *in vitro,* was discovered only recently (31, 52). Also the confirmation that ASC can differentiate into bone tissue *in vivo* is relatively novel, as it was first observed in the last few years (53, 54).

Cells from both sources share many biological characteristics and are very promising for regenerative medicine approaches. Nevertheless, each of them has pros and cons in comparison to the other. Adipose tissue, for instance, is more easily accessible and more abundant than bone marrow and also contains higher numbers of colony-forming (clonogenic) cells, which is an advantage for clinical applications (55-57). Furthermore, it has been shown that the yield of clonogenic cells obtained from bone marrow, but not from adipose tissue, decreases with the age of the patient (58). Schmuck et al. recently discovered that aging even increases the abundance of ASC, although this may be due to an accumulation of non-replicative ASC (59).

A study comparing the yield of adherent cells, growth kinetics, cell senescence, *in vitro* adipo-, osteo- and chondrogenic differentiation capacity and gene transduction efficiency of ASC and BMSC derived from the same patients by De Ugarte et al. revealed no significant differences between the two kinds of MSC (55). On the other hand the *in vivo* osteogenic capacity of ASC is still controversial and seems to be less reliable then that of BMSC. While some research groups claim no difference in *in vivo* osteogenic potential comparing ASC and BMSC (54), others were not able to reproduce those results (60-62). Although studies carried out in our lab indicate an *in vivo* osteogenic potential of ASC (17), BMSC appear to produce bone tissue more easily and reproducibly.

1.4 GENETIC MODIFICATION OF MSC

As described in the previous chapter, MSC are an attractive cell source for tissue engineering. In many cases, however, it is challenging to achieve the desired outcome with MSC alone. Breitbach et al., for instance, showed the risk of bone formation after BMSC injection into the infarcted heart (63). Similar problems faced De Bari et al. when implanting *in vitro* pre-differentiated MSC which de- and re-differentiated from a chondrogenic towards a myogenic phenotype and finally were cleared (64). However, adding specific factors, either directly as proteins or via gene transfer, often leads to better results (65, 66). Wojtowicz et al. recently reported that overexpression of the osteogenic transcription factor Runx2 in MSCs accelerated healing of critical-sized defects compared to unmodified MSCs (67). Similar results were described when implanting MSC overexpressing BMP4. These BMP4-MSC showed a better *in vivo* bone formation capacity than control cells (68).

Gene therapy is 'the use of genes as medicine' involving the transfer of a therapeutic, or working, copy of a gene into patient's cells instead of using drugs or surgery. The advantage of gene therapy over simple addition of the protein of interest is that the delivered gene leads to continuous production of the therapeutic factor and thus allows long-term treatment (69).

Various gene delivery strategies can be used to transfer genes of interest into damaged tissues. *In vivo* applications involve the direct injection of viral or DNA vectors either systemically into the host or locally into the target tissue. As the required vector concentration to achieve therapeutic effects needs to be very high, those methods are associated with possible risks of toxic effects.

A different approach constitutes the genetic modification of progenitor cells *ex vivo*. Here, gene delivery takes place while cells are in culture. Advantages of this technique are that cell transduction, expansion, cell population selection, external delivery of growth factors and differentiation signals, as well as other chemical or physical alterations to the culture environment can be achieved prior to transplantation into patients. Afterwards, the genetically modified cells can be delivered either directly, in an encapsulated form, or as tissue-engineered constructs comprised of cells seeded into

scaffolds. With this approach the patients' safety can be improved, as the host immune or inflammatory response to viral particles, or toxic effects from transfection reagents, are avoided (70).

1.4.1 GENE THERAPY VECTORS

In order to channel the specific nucleotide sequences inside host cells, non-viral and viral vectors can be used.

Although novel non-viral systems have continued to emerge as innovative vehicles for controlled gene delivery, viral vectors remain the most efficient means by which exogenous genes can be introduced and expressed by cells.

Viral vectors can be divided into integrating and non-integrating vectors. The latter comprise adeno-, adeno-associated- and herpesviral vectors, which do not replicate with the host genome and are rapidly lost with cell proliferation. Therefore, they lead to transient gene expression unless they are used to transduce post-mitotic cell types. Adenoviral vectors lead to transient expression in any case, since they are highly immunogenic and are cleared by the host immune system within 2 weeks of delivery (71).

Retroviral vectors, in contrast, are the most commonly used vehicles for stable gene transfer into various cell types. These vectors can integrate into the host genome to provide long-term transgene expression in the target cells and are passed on to daughter cells after cell division (72). Like all other viral vectors, they are derived from wild-type viruses by exploiting some of their biological features , like infectivity, integration, strong overexpression, on the one hand and by eliminating pathogenic characteristics, such as replication in the infected cells, on the other hand. The generation of a retroviral vector from a wild-type retrovirus is described here in more detail as a general example.

Wild-type retroviruses are a class of enveloped viruses that contain a single-stranded RNA molecule as their genome. The principle feature of this family is its replicative

strategy, which includes reverse transcription of the viral RNA into linear double stranded DNA and the subsequent integration of this dsDNA into the genome of the host cell. RNA polymerase II from the host cell later transcribes the viral genome into mRNA, based on which the corresponding proteins will be produced and new viral particles can be assembled. As they bud from the host cell they are finally covered by a lipid bilayer derived from the cell membrane of the host cell containing virus-encoded surface and transmembrane glycoproteins (70).

Retroviral vectors are engineered to carry a transgene of interest into a target cell, without being replication-competent in order to avoid pathogenic effects. Generally, the retroviral vectors are composed of the therapeutic transgene and the cis-acting elements of the viral genome: attachment sites (att), 5'- and 3'-Long Terminal Repeats (LTR, made of the U3, R and U5 sequences), the polypurine track (PPT), the primer-binding site (PBS) and the packaging signal (ψ). As demonstrated by Choulika et al., the elimination of any of the viral cis-acting sequences leads to a nonfunctional retroviral system (73). The attachment sites, for instance, allow efficient integration of the viral DNA into the host genome through interactions with the enzyme integrase. The packaging signal is necessary for the encapsulation of the retroviral genome into viral particles. While PBS, PPT, and the LTR R sequence are required for its reverse transcription and transgene expression, the LTR U3 and U5 sequences are necessary for the integration of the reverse-transcribed retroviral material. In addition, the 5' LTR acts as an RNA pol II promoter. The trans-acting viral genes that code for the viral core protein (gag), for the reverse transcriptase and integrase (pol), as well as for the surface and transmembrane components of the viral envelope proteins (env), are removed (Figure 1.5). Those three genes, which are necessary for the generation of functional retroviral particles, are co-expressed in so-called packaging cells through independent helper plasmids, so that infective, but non-replicating, viral vector particles can be produced (70).

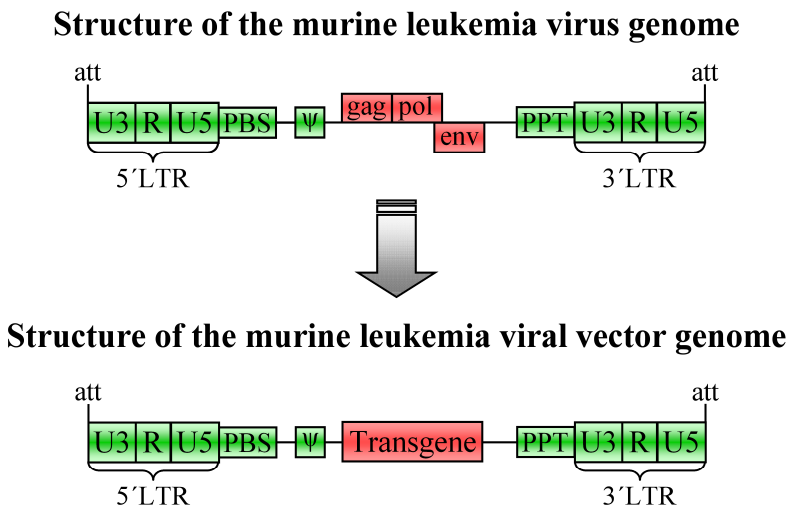


Figure 1.5 Structures of the genomes of MLV and an MLV-derived viral vector. The trans-acting viral genes "gag", "pol" and "env" are replaced by the transgene. Therefore retroviral vectors are replication-incompetent but allow high-level expression of therapeutic genes.

There are three subfamilies of retroviruses from which retroviral vectors can be derived: oncogenic retroviruses, lentiviruses and spumaviruses. The latter two are so called complex viruses. While lentiviral vectors possess the advantages that they are capable of transducing non-dividing cells, spumaviral vectors are rather unconventional as many features in their replication cycle are different from those of onco- and lentiviral vectors. In addition they can be toxic to cultured cells (74).

Oncogenic retroviruses however are classified as simple retroviruses, as their genome is less complex. It is limited to transduction of dividing cells, as it requires breakdown of the nuclear membrane before it can integrate into the host genome. Murine leukemia virus (MLV)-based oncogenic retroviral vectors are one of the most frequently used gene delivery vehicles in marking studies, as well as in clinical investigations (68, 72).

1.5 ANGIOGENESIS

1.5.1 VASCULARIZATION IN BONE TISSUE ENGINEERING

Induction of angiogenesis is a crucial aspect in the development of regenerative medicine approaches that require rapid vascularisation of tissue-engineered grafts.
As any tissue that is more than about 200 microns thick needs a vascular supply in order to ensure cell survival and function, also tissue engineered constructs with a clinically relevant size have to be connected to the host vasculature. A non-vascularized graft is nourished by simple diffusion of oxygen and nutrients. However, the survival of tissue-engineered constructs *in vivo* depends on the provision of an adequate blood supply to the tissue as well as the capacity of the engineered microcirculation to connect with the existing recipient circulation. Lack of vascularization in the centre of large cell-containing constructs invariably leads to ischemia followed by necrosis within the first week after implantation (Figure 1.6) (75).

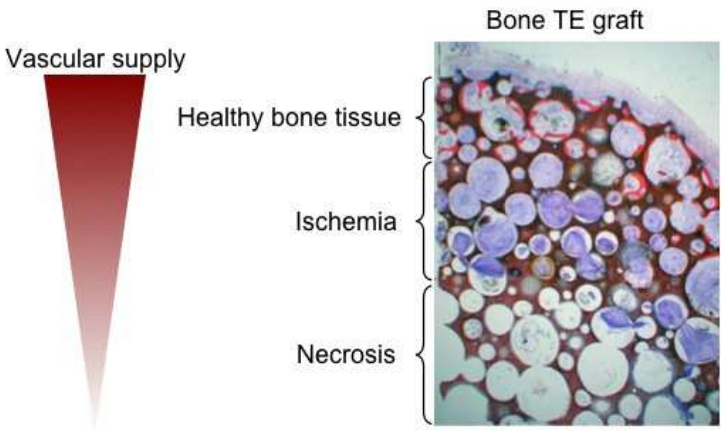


Figure 1.6 Histological sections of large-size osteogenic grafts stained with McNeal tetrachrome 12 weeks after implantation. Constructs displayed three distinct zones according to the vascular supply: an outer one including newly formed bone tissue (red stain); a middle zone formed by a fibrous connective tissue (violet stain); and a central zone containing only empty pores. The non-degraded scaffold is stained brown. (Adapted from (76))

The supply of nutrients and oxygen to the cells and the clearance from waste products are not the only functions of blood vessels. They also allow the recruitment and extravasation of cells and growth factors, which may improve tissue regeneration and formation. In bone it has been shown that vascularization establishes the conduit for a series of highly specialized cells involved in the regulation of osteogenesis, such as

osteoblasts and osteoclasts (77). In particular, bone endothelium has been found to actively recruit circulating cells and to direct hematopoietic cell homing to the bone marrow. Moreover, consistent with their putative roles in regulating osteogenesis, osteoclastogenesis, and bone remodelling, endothelial cells synthesize and display or secrete many factors known to exert major effects in controlling the differentiation, metabolism, survival and function of bone cells. Conversely, bone endothelial cells are also capable of responding to bone modulators (78).

In fracture repair both osteoblasts and osteoclasts express VEGF, thereby contributing to local angiogenesis. In addition to endothelial cells also BMSC, osteoblasts and osteoclasts possess VEGF receptors and are therefore influenced by this angiogenic factor (78-81).

In summary, vasculature plays a major role in the generation and homeostasis of bone, without which bone tissue repair or rejuvenation would be impossible. In addition, modifications in the blood supply correlate with numerous skeletal pathologies, including osteoporosis or osteopetrosis, inflammatory bone loss and tumor-associated osteolysis (78).

1.5.2 STRATEGIES TO ACCELERATE THE ESTABLISHMENT OF A VASCULAR NETWORK IN BONE GRAFTS

Different strategies have been investigated to establish blood perfusion in osteogenic grafts. In surgical techniques, like the flap- or arterio-venous loop- fabrication, the bone graft is wrapped in vascularized host tissue, or an arterio-venous loop is placed in close proximity of the construct (e.g. placed in a groove within the scaffold) in order to favor vascular ingrowth. The donor site morbidity, however, is still a problem when applying these techniques. Moreover, flap pre-fabrication has the major clinical disadvantage of requiring a two-step surgery, the cost and complexity of which could limit its potential for future clinical applications (17, 82, 83).

Biomaterial based methods to engineer vascularized bone tissue are diverse. One approach is the design of nano-/micro-fiber combined scaffolds, which provide

mechanical support for bone repair and angiogenesis at the same time, and scaffold microfabrication aiming on the inclusion of a network with a vascular geometry in a biocompatible polymer. Microfabrication is mainly applied to the establishment of a complex branching vascular tree in soft organs. A hard tissue such as bone has an increased level of complexity, which demands microfabrication methods to be adapted in order to simultaneously address design and mechanical issues (83). The loading of scaffolds with pro-angiogenic factors is another variant in biomaterial-based approaches (84). Even though angiogenesis can be enhanced using this strategy, the release of growth factors with optimal kinetics and the achievement of local concentrations that are both safe and therapeutic remain unresolved challenges. In addition, the instability of the factors *in vivo* and the required high doses of recombinant protein cause an escalation in therapy costs (83). These problems can be overcome using genetically modified cells for the delivery of angiogenic factors (see chapter 1.4).

On the other hand, a closer approximation to the physiological *in vivo* situation can be created by the co-culture of osteogenic and vasculogenic precursor cells. Although normally endothelial cells and osteoblasts are mainly isolated from different sources (85, 86), from the clinical point of view it is more convenient to obtain the two cell populations from a common cell source, as has been described for adipose tissue and bone marrow (87, 88).

Although many strategies are currently under investigation, the need to achieve rapid vascularization of large-size grafts still remains a major challenge in the field of tissue engineering and a bottleneck for the development of clinical applications.

1.5.3 MOLECULAR REGULATION OF ANGIOGENESIS

Blood vessels provide efficient and simultaneous transport of gases, liquids, nutrients, signaling molecules and circulating cells between tissues and organs and insufficient blood supply causes tissue ischemia, leading to necrosis.

In the human body oxygenated blood is pumped from the heart through arteries into arterioles and finally reaches an extensive network of capillaries. Because of their wall structure and the large surface-area-to-volume ratio, these vessels form the main site of exchange of nutrients between blood and tissue. Afterwards, the oxygen-depleted blood passes through venules and veins back to the heart (Figure 1.7). From there, blood is then transported to the lungs, where it is replenished with oxygen.

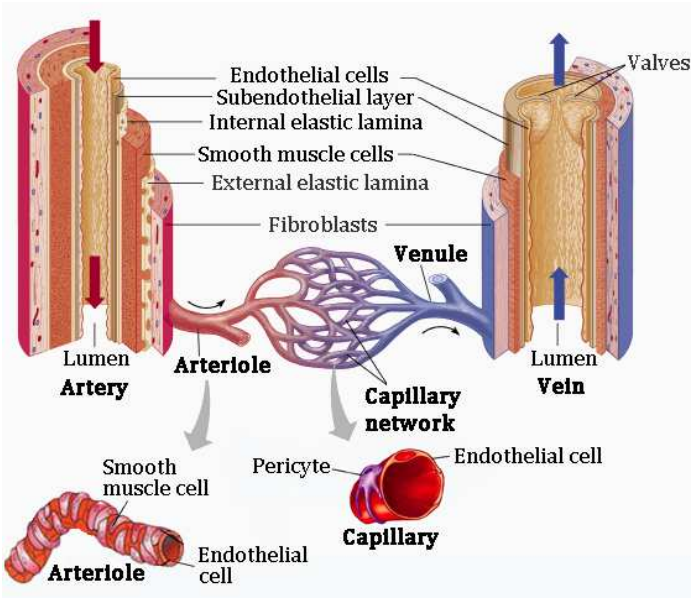


Figure 1.7 vessel composition. Blood flows from the heart through arteries into arterioles and finally into a branched capillary network where an exchange between the blood stream and the surrounding tissue occurs. The blood is thereafter returned through venules and veins to the heart. All vessels consist of a tube of endothelial cells. In addition, capillaries are covered and stabilized by pericytes, while arterioles and venules are completely invested by vascular smooth muscle cells. The walls of arteries consist of vascular smooth muscle cells packed into an internal and external elastic lamina and an additional fibroblastic layer. (Adapted from (90))

All these different vessel types are based on a tube of endothelial cells (EC). Dependent on their biological function, they are equipped with additional structures. Capillaries, for instance, consist of endothelial cells surrounded by a basement membrane and a sparse layer of pericytes embedded within the endothelial cell basement membrane, while arterioles and venules have an increased coverage of mural cells. Here, endothelial cells are overlaid with a basement membrane and an internal elastic lamina which is completely invested with vascular smooth muscle cells that form their own basement membrane and are circumferentially arranged, closely packed and tightly associated with the endothelium. The walls of larger vessels (arteries) consist of three specialized

layers: an intima composed of endothelial cells, a media of smooth muscle cells and an adventitia of fibroblasts, together with matrix and elastic laminae. Smooth muscle cells and elastic laminae contribute to the vessel tone and mediate the control of vessel diameter and blood flow (Figure 1.7) (89).

New blood vessels form by different mechanisms, including de novo assembly by endothelial precursor cells or angioblasts (vasculogenesis), splitting of existing vessels (intussusception), and enlargement of the vasculature through sprouting, proliferation and remodeling (angiogenesis). Vasculogenesis appears to be largely confined to the formation of the first primitive vascular structures as well as the large axial vessels in the early embryo. Conversely, angiogenesis appears to be the main process for the formation of the vast majority of blood vessels during development, tissue repair, or disease processes. In response to insufficient local supply of oxygen (hypoxia), tissues up-regulate the expression of proangiogenic growth factors. These signals activate receptors in the endothelium and lead to phenotypic and functional changes in some ECs. As they extend long philopodia, these are called tip cells are able to sense gradients of growth factors, become motile, invasive, and initiate sprouting from the basal (outer) surface of the blood vessel tubules. Other ECs form the stalk of the growing sprout and stay behind to maintain tissue perfusion. Due to substantial stalk cell proliferation angiogenic sprouts extend, until they meet with other sprouts or capillaries, and are finally converted into new blood-carrying capillaries. Later steps of vascular morphogenesis involve the pruning and remodeling of the newly formed and initially dense vessel network (Figure 1.8). Finally, tip cells recruit pericytes to the new sprouts. The physical contact between ECs and pericytes induces a quiescent, non-sprouting phenotype and therefore stabilizes blood vessels and promotes a mature, non-angiogenic state of the vasculature, independent of further angiogenic stimuli. Pericytes provide survival signals to endothelial cells and stabilize new vessels by regulating endothelial function and differentiation (91, 92). Previous results from Dor et al. and Ozawa et al. identified that the expression of the proangiogenic factor VEGF must be sustained for approximately 4 weeks in order to stabilize the newly formed vessels *in vivo* (93, 94).

As mentioned, angiogenesis is stimulated by a variety of growth factors. The most potent and specific pro-angiogenic factor is vascular endothelial growth factor-A (VEGF-A). VEGF-A binds and activates the receptor tyrosine kinases VEGFR1 and VEGFR2 on the EC surface and is an essential positive regulator of blood vessel growth. Recently, VEGF has been shown to control angiogenesis not only by directly stimulating endothelial cells, but also through the recruitment and activation a heterogeneous mix of myeloid cells recruited from the circulation. These cells are trapped at the sites of angiogenesis and are pro-angiogenic through the actions of paracrine factors (95).

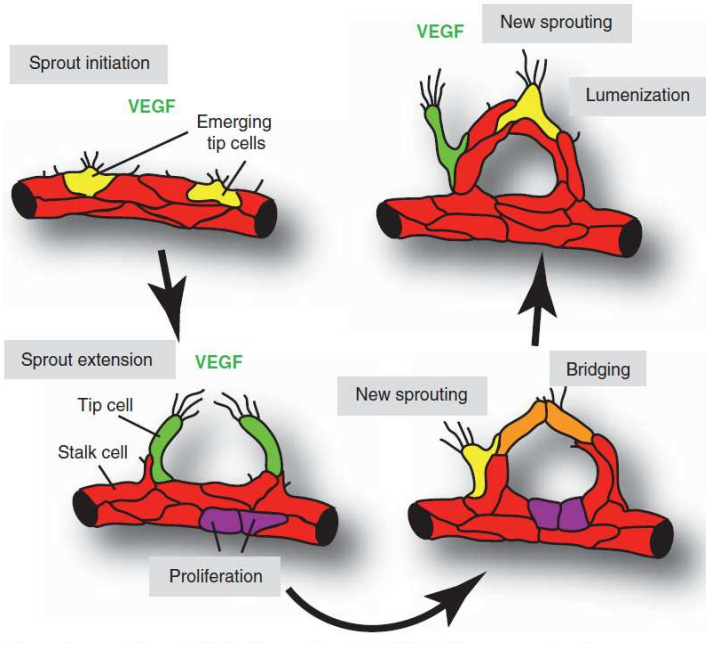


Figure 1.8 Angiogenic sprouting of blood vessels. Pro-angiogenic factors (e.g. VEGF) promote a fraction of endothelial cells to develop into tip cells (yellow) with long philopodia, which lead the growing sprout. Other ECs form the sprout stalk by extensive proliferation or stay behind to maintain tissue perfusion (red). When tip cells contact other tip cells or vessels, they establish new connections via cell bridges (orange) and finally form new perfused vessels. New sprouting is initiated at other sites and additional ECs are generated by proliferation (purple). (Source (91))

VEGF-A is a heparin-binding, disulfide-linked dimeric glycoprotein that exists as numerous splice variant isoforms. Longer isoforms, as $VEGF_{183}$, $VEGF_{189}$ or $VEGF_{206}$, possess strong heparin-binding domains and are therefore strongly attached to the extracellular matrix of secreting cells. Short isoforms, in contrast, as $VEGF_{121}$ or $VEGF_{145}$ lack these sequences and are therefore diffusible. The most biologically active VEGF-A is $VEGF_{165}$ ($VEGF_{164}$ in rodents), which binds heparin with an intermediate affinity and thus sticks to the extracellular matrix with a frequency of 50-70% (96, 97). Subsequently $VEGF_{165/164}$ remains localized in the microenvironment around its

producing cell and does not distribute uniformly in the tissue. As a consequence, sustained over-expression of heterogeneous levels by different cells do not average each other and even rare areas of high expression, which form VEGF-hotspots, are sufficient to induce aberrant angiogenesis marked by the growth of hemangioma-like vascular tumors. As shown in figure 1.9, angiomas are aberrant bulbous vessels characterized by a loss of normal pericyte coverage and its substitution with smooth-muscle-like mural cells. They do not stabilize and remain VEGF dependent, thus growing into large, blood-filled vascular sacs, which eventually bleed and cause the animal death (93, 98).

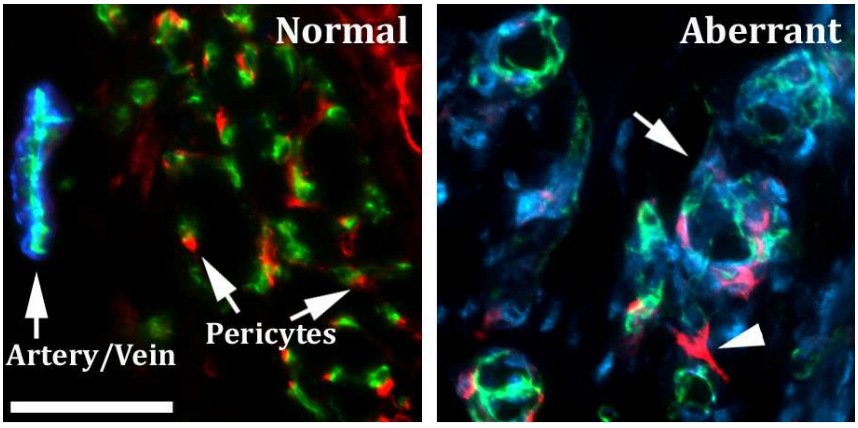
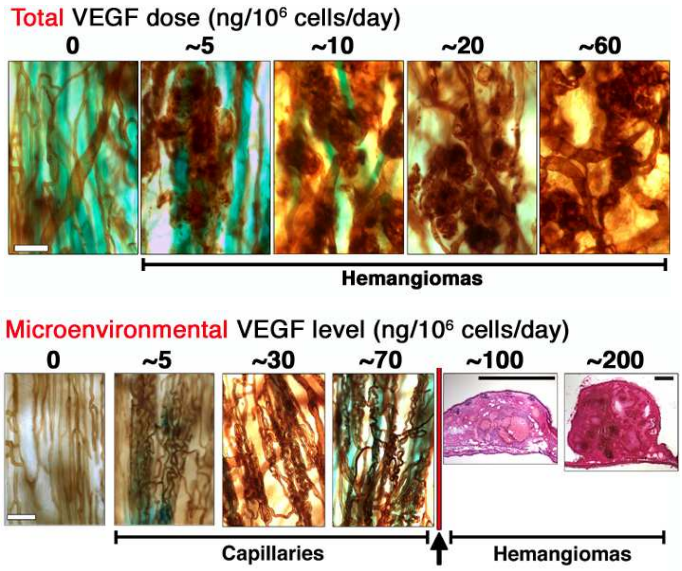


Figure 1.9 Normal versus aberrant angiogenesis. Endothelial cells are stained in green, pericytes in red and smooth muscle cells in blue. Normal capillaries are stabilized by pericytes, while angioma-like structures retain only few and dysfunctional pericytes (arrowhead), but are associated with smooth muscle actin-positive mural cells (arrow). Normally, only arteries and veins are covered by smooth muscle cells. Scale bar: 50μm. (Adapted from (93))

Our group previously found that the induction of normal and stable angiogenesis or aberrant angioma growth depends on the amount of VEGF in the microenvironment around each producing cell, and not on the total dose of the factor in the tissue (93, 98). In fact, whenever the microenvironmental VEGF doses can be expressed homogeneously through the tissue, normal, stable and functional angiogenesis is induced over a wide range of VEGF expression levels, up to a discrete threshold, above which angioma growth occurs (Figure 1.10).



Figue 1.10 Total versus micro-environmentally controlled VEGF expression level in angiogenesis. Manipulation of the total dose of VEGF gene delivery by dilution of a heterogeneous population of VEGF-overexpressing cells with untreated cells cannot prevent the development of aberrant vasculature. If the microenvironmental distribution of expression level is controlled by the use of clonal populations, in which every cell expresses the same amount of VEGF, normal angiogenesis can be induced over a wide range of doses. However, above a dose-dependent threshold normal angiogenesis switches to an aberrant phenotype. (Adapted from (93))

Control over the microenvironmental distribution of VEGF doses for therapeutic purposes can be achieved via the genetic manipulation and selection of progenitor cells *ex vivo* before re-implantation. Implantation of clonal populations, in which every cell has the same number of copies and site of integrated retroviral vectors, and therefore expresses the same VEGF dose, provided a clear proof-of-concept of the functional and therapeutic implications of VEGF dose control at the total or microenvironmental level (93, 98). However, this approach requires excessive time, costs and cell expansion, making it unsuitable for a clinical application (99). Therefore, in order to translate this biological concept into a clinically applicable therapeutic strategy, we recently developed a Fluorescence-Activated Cell Sorting (FACS)-based technology to predict the level of VEGF expression in single cells. VEGF expression was quantitatively linked to that of a FACS-quantifiable syngenic cell surface marker, i.e. a truncated version of CD8a (Figure 1.11). In this way, high-throughput purification of cells homogeneously expressing specific levels from a heterogeneous progenitor population could be achieved rapidly, without the need to expand and screen individual clones (100). Rapidly purified populations induced only normal, mature and functional microvascular

networks, and avoided completely angioma growth, both in normal (100) and chronically ischemic muscle tissue (101).

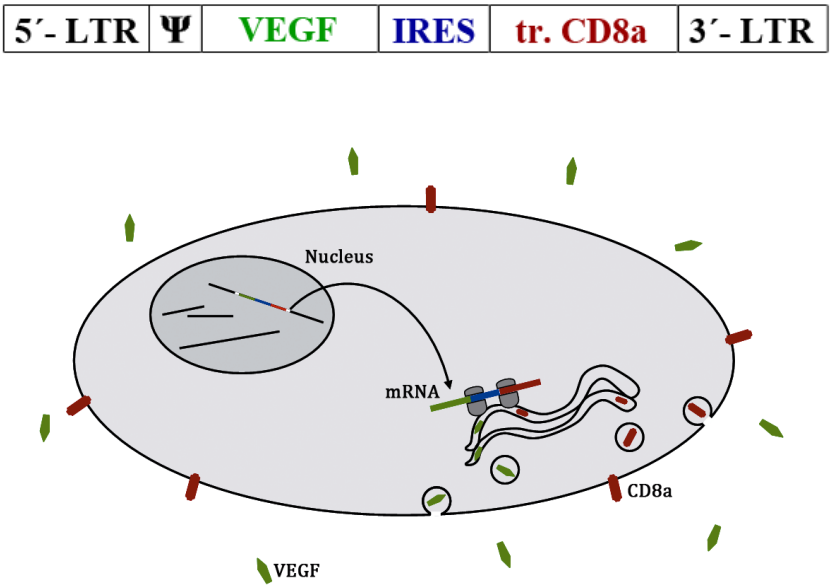


Figure 1.11 Viral vector genome coding for VEGF-IRES-truncated CD8a, therefore allowing VEGF-secretion to be correlated with CD8 accumulation on the cell surface. Top: The coding sequences for $VEGF_{164}$ and truncated CD8a (tr. CD8a) were linked through an Internal Ribosomal Entry Site (IRES) sequence in a bicistronic retroviral vector construct (pAMFG-rVICD8). Bottom: Schematic representation showing co-expression of both genes at a fixed ratio from the bicistronic mRNA, so that the amount of VEGF secreted (green molecules) is correlated to that of tr. CD8a retained on the cell surface (red molecules), which can be quantified.

AIMS OF THE THESIS

Rapid vascularization of tissue-engineered grafts is a major bottleneck in the development of regenerative medicine approaches. In order to overcome this limitation, we aimed to develop a bone tissue engineering strategy combining cell therapy with pro-angiogenic gene therapy. To this end, we sought to determine the potential of genetically modified MSC derived from bone marrow and adipose tissue overexpressing VEGF. To develop a MSC-based platform for bone tissue engineering endowed with an increased vascularization capacity, two facts need to be considered:

1) It is well known that MSC rapidly lose their differentiation potential after isolation and during *in vitro* expansion (43, 44). Therefore, in order to preserve their progenitor properties, it is crucial that genetic modification takes place both, with high efficiency, so as to minimize the need for cell selection, and with minimal expansion of progenitors.

2) VEGF is the master regulator of angiogenesis. However, as described in the previous sections (chapter 1.5), excessive doses can cause deleterious effects and it is desirable to control its expression in each producing cell, since it remains localized in the microenvironment after secretion and different levels do not average each other in tissue.

In order to overcome both of these limitations, we took advantage of a high-throughput technology we recently developed (100) to rapidly purify populations of genetically engineered myoblasts secreting a desired level of a transgene based on fluorescence-activated cell sorting (FACS).

As a first step (**Chapter 2**), we sought to develop an optimized technique to realize high-efficiency retroviral vector transduction of both primary bone marrow and adipose tissue derived MSC in the initial culture stages, before the first passage. This is crucial in order to achieve genetic modification while avoiding a prolongation of *in vitro* culture. Furthermore, we sought to rapidly purify specific sub-populations of the transduced MSC expressing different VEGF levels already at the time of the first passage.

Using these genetically modified MSC as a tool, in **Chapter 3** we investigated the potential of VEGF-expressing human bone marrow-derived mesenchymal progenitors:

1) to increase the vascularization of osteogenic grafts and

2) to efficiently generate bone tissue.

In order to minimize variables and to rigorously determine whether transduction, FACS-purification or VEGF-expression affected the progenitor properties of genetically modified MSC, non-critical size grafts were implanted ectopically in a well-established nude rat model.

Finally, the thesis is summarized and discussed in **Chapter 4**. This chapter includes perspectives of ongoing projects introducing the potential of genetically modified MSC to control angiogenesis, both positively and negatively, in other fields of regenerative medicine, including heart revascularization after infarction and cartilage regeneration.

2 GENERATION OF HUMAN MSC EXPRESSING DEFINED VEGF LEVELS BY OPTIMIZED TRANSDUCTION AND FLOW CYTOMETRY PURIFICATION

Submitted for publication

Uta Helmrich, Anna Marsano, Ludovic Melly, Thomas Wolff, Liliane Christ, Michael Heberer, Arnaud Scherberich, Ivan Martin and Andrea Banfi

2.1 ABSTRACT

Adult mesenchymal stem/stromal cells (MSC) are a valuable source of multipotent progenitors for tissue engineering and regenerative medicine, but may require to be genetically modified to widen their efficacy in therapeutic applications. For example, over-expression of the angiogenic factor vascular endothelial growth factor (VEGF) at controlled levels is an attractive strategy to overcome the crucial bottleneck of graft vascularization and to avoid aberrant vascular growth. Since the regenerative potential of MSC is rapidly lost during *in vitro* expansion, we sought to develop an optimized technique to achieve high-efficiency retroviral vector transduction of both adipose tissue- and bone marrow-derived MSC (ASC and BMSC) and rapidly select cells expressing desired levels of VEGF with minimal *in vitro* expansion.

The proliferative peak of freshly isolated human ASC and BMSC was reached 4 and 6 days after plating, respectively. By performing retroviral vector transduction at this time-point, >90% efficiency was routinely achieved before the first passage. MSC were transduced with vectors expressing VEGF quantitatively linked to a syngenic cell-surface marker (truncated CD8). Retroviral transduction and VEGF expression did not affect MSC phenotype nor impair their *in vitro* proliferation and differentiation potential. Transgene expression was also maintained during *in vitro* differentiation. Furthermore, three sub-populations of transduced BMSC homogeneously producing specific low, medium and high VEGF doses could be prospectively isolated by flow cytometry based on the intensity of their CD8 expression already at the first passage.

In conclusion, this optimized platform allowed the generation of populations of genetically modified MSC, expressing specific levels of a therapeutic transgene, already at the first passage, thereby minimizing *in vitro* expansion and loss of regenerative potential.

2.2 INTRODUCTION

Adult mesenchymal stem/stromal cells (MSC) are a population of multipotent progenitors, capable of generating bone, cartilage, fat and possibly other mesodermal tissues and represent a fundamental tool in regenerative medicine (1, 2). MSC have been described in many tissues as a pericyte-like population in close association with blood vessels (3), raising the intriguing possibility that they may reside in the vascularized stroma of every tissue. However, the most commonly used and characterized MSC are derived from bone marrow (BMSC) and adipose tissue (ASC), due to their abundance and ease of harvesting (4, 5).

Despite their potential, it may be desirable to genetically modify MSC in order to increase their survival and/or differentiation in therapeutic applications. For example, spontaneous vascularization of tissue-engineered grafts *in vivo* is too slow to allow survival of progenitors in constructs larger than a few millimeters. In order to overcome this bottleneck in the generation of clinical-size grafts, it is necessary to increase their ability to rapidly attract a vascular supply from the host, e.g. by over-expressing an angiogenic factor from the implanted progenitors (6-8).

Vascular endothelail growth factor (VEGF) is the master regulator of vascular growth both in embryonic development and adult tissues (9). When expressed at the appropriate dose, VEGF can start the complex cascade of events leading to the formation of stable and functional new blood vessels (10). However, sustained expression is required for about 4 weeks in order to avoid regression of newly induced unstable vessels (11, 12).

Non-integrating gene therapy vectors are progressively lost during cell expansion and lead to short-term and variable expression. Gene expression is thus less controllable, making it challenging to achieve a desired therapeutic effect. Integrating vectors, such as retroviral vectors on the other hand, replicate with the host genome and ensure constant expression throughout cell expansion (13, 14).

MSC have been shown to rapidly lose their differentiation potential during *in vitro* expansion (15, 16). Therefore it is crucial that genetic modification takes place both with

high efficiency, in order to minimize the need for cell selection, and with minimal *in vitro* manipulation of progenitors.

Therefore, here we sought to develop an optimized technique to achieve rapid and high-efficiency transduction of primary MSC from both bone marrow and adipose tissue with minimal *in vitro* expansion, together with high-throughput purification of the progenitor populations expressing specific transgene levels based on fluorescence-activated cell sorting (FACS). Greater than 90% transduction efficiency of freshly isolated BMSC and ASC could be routinely achieved and FACS-purification was possible already at the time of the first passage, while no loss of *in vitro* proliferation and differentiation potential was caused by either the genetic modification or the FACS-sorting.

2.3 MATERIALS AND METHODS

MSC isolation and culture

Human primary ASC and BMSC were isolated from liposuction and bone marrow aspirates, respectively. The aspirates were obtained from healthy donors as previously described, after informed consent by the patients and approval by the local ethical committee. Briefly, subcutaneous adipose tissue was digested in 0.075% type-II collagenase (Worthington) in PBS and plated at a density of 5.5 x 10^3 nucleated cells/cm^2 (17). Bone marrow was subjected to red blood cell lysis (18) and plated at a density of 10^5 nucleated cells/cm^2. Both ASC and BMSC were cultured in α-MEM supplemented with 10% FBS (Gibco) and 5 ng/ml FGF-2 (BD Biosciences). Cells were passaged when 80% confluent and replated at a density of 3 x 10^3 cells/cm^2.
Colony forming efficiency (CFE) assays were performed on freshly isolated cells as described (19). The proliferation rate was determined by counting the number of cells at every passage with a Neubauer chamber (Roth), determining the number of population doublings from the previous passage (19) and plotting the cumulative doublings against the time in culture.

Myoblast culture

Primary C57Bl/6 mouse myoblasts were cultured on collagen-coated dishes in medium consisting of 40% F10, 40% low-glucose DMEM (both Sigma), supplemented with 20% FBS and 2.5 ng/ml FGF-2, as previously described (20).

Cell cycle analysis

The proportion of actively cycling cells was determined by measuring their nuclear DNA content by flow cytometry after staining with propidium iodide as described (21). The data were analyzed using the cell cycle analysis tool from FlowJo Software (Tree Star) using the Watson model (22). ASC (n=4 donors) and BMSC (n=3 donors) from duplicate dishes were analyzed at each time-point.

Retroviral transduction

The optimization experiments were performed with frozen aliquots of a pooled stock of retroviral vector supernatant, to ensure the same titer in all conditions. Subsequent experiments were performed with fresh viral vector supernatants. Primary MSC were transduced with previously described retroviral vectors (23) according to a high-efficiency protocol (24). Briefly, MSC were cultured in 60-mm dishes and were incubated with retroviral vector supernatants supplemented with 8μg/ml polybrene (Sigma-Aldrich) for 5 minutes at 37°C and centrifuged at 1100g for 30 minutes at room temperature in the dishes, followed by fresh medium replacement.

Flow cytometry

CD8a expression was assessed by staining with a FITC-conjugated anti-rat CD8a antibody (clone OX-8; BD Pharmingen), using previously optimized staining conditions (23), i.e. 2μg of antibody/10^6 cells in 200μl of PBS with 5% BSA.

Expression of MSC surface markers was assessed by staining with specific antibodies against: CD31 (clone WM59, BD Pharmingen), CD34 (clone 581, BD Pharmingen), KDR/VEGFR2 (clone 89106, R&D Systems), CD73 (clone AD2, BD Pharmingen), CD90 (clone 5E10, BD Pharmingen), CD105 (clone SN6, AbD Serotec). All antibodies were used at a 1:20 dilution except for CD90 (1:100). Aspecific binding was measured by staining with appropriate isotype control antibodies. Data were acquired with a FACSCalibur flow cytometer (Becton Dickinson) and analyzed using FlowJo software (Tree Star). Cell sorting was performed with the Influx cell sorter (Becton Dickinson).

MSC immunostaining

50%-confluent MSC cultures were fixed with 4% formalin, blocked for 1 hour with 10% calf serum in PBS and incubated with an antibody against rat CD8a (clone OX-8, BD Pharmingen; 1:200), followed by an Alexa 488-conjugated chicken anti-mouse IgG (Invitrogen; 1:500). Nuclei were stained with DAPI (Invitrogen).

In vitro differentiation

Adipogenic differentiation was induced as previously described (17). Briefly, subconfluent cell layers were treated with 10µg/ml insulin, 10^{-5}M dexamethasone, 100µM indomethacin and 500µM 3-isobutyl-1-methyl xanthine for 72 hours and subsequently with 10µg/ml insulin for 24 hours, repeating this 96-hour treatment cycle four times. Lipid droplets were detected with Oil Red-O staining.

Osteogenic differentiation was induced by culturing MSC with α-MEM medium supplemented with 10% fetal bovine serum, 100nM dexamethasone, 10mM β-glycerophosphate and 0.05mM ascorbic acid-2-phosphate for 3 weeks, as previously described (17). Calcium deposits were detected with Alizarin Red staining.

Quantitative real time RT-PCR

Total mRNA was isolated using the RNeasy Mini Kit (Qiagen) and cDNA was synthesized with the Superscript III Reverse Transcriptase (Invitrogen).

Quantitative real-time reverse transcriptase polymerase chain reaction (qRT-PCR) was performed on a 7300 AB thermocycler (Applied Biosystem) to quantify the expression of the following genes: human cbfa-1/Runx2 (Forward: CGTTACCCGCCATGACAGTA; Reverse: GCCTTCAAGGTGGTAGCCC; Probe: CCACAGTCCCATCTGGTACCTCTCCG; primers=300 nM and probe=100 nM) (25); PPARγ (Applied Biosystems assay Hs00234592_m1; primers=900 nM and probe=250 nM). To quantify expression of the retroviral cassette, a specific primers-and-probe set was designed with the Primer Express 3 software (Applied Biosystems) to recognize the Internal Ribosomal Entry Sequence (IRES) (Forward: GCTCTCCTCAAGCGTATTCAACA; Reverse: CCCCAGATCAGATCCCATACA; Probe: CTGAAGGATGCCCAGAAGGTACCCCA; primers=400 nM and probe=400 nM). GAPDH was used as a housekeeping gene (Forward: TAAAAGCAGCCCTGGTGACC; Reverse: ATGGGGAAGGTGAAGGTCG; Probe: CGCCCAATACGACCAAATCCGTTGAC; primers=300 nM and probe=150 nM) (26). Two independent cDNA samples/condition/donor were analyzed in triplicate with the following cycling parameters: 50°C for 2 minutes, followed by 95°C for 10 min and 45 cycles of denaturation at 95°C for 15 seconds and annealing/extension at 60°C for 1 minute.

ELISA

VEGF production was quantified in cell culture supernatants using a Quantikine rat VEGF immunoassay ELISA kit (R&D Systems). One ml of fresh medium was incubated on MSC cultured in 60-mm dishes in duplicate for 4 hours, filtered and frozen. Results were normalized by the number of cells in each dish and the time of incubation.

Statistics

Data are presented as means ± standard error. The significance of differences in multiple comparisons was evaluated using one-way analysis of variance (ANOVA) followed by the Bonferroni test. $P<0.05$ was considered statistically significant.

2.4 RESULTS

Optimization of MSC transduction

Since retroviral vectors efficiently transduce only dividing cells, we first sought to determine the earliest time after isolation and plating when ASC and BMSC enter the cell cycle and proliferation is at its peak. Cell cycle analysis was performed on samples from 3 independent donors every day from the initial plating to the time of first confluence, which was reached consistently by day 12 and 14 for ASC and BMSC, respectively. ASC reproducibly reached a proliferation peak 4 days after isolation (52.2 ± 5.0% of cycling cells) that was maintained until day 7, after which replication declined with increasing confluence (Figure 2.1A). BMSC displayed a higher variability in the first days, but their growth trend was similar, proliferation reached its maximum on day 6 (54.7 ± 5.5%) and declined after day 10 (Figure 2.1B).

Based on these results, we determined the optimal time to start transduction to be on day 4 after isolation for ASC and day 6 for BMSC. Cells were transduced twice per day up to 6 times with a retroviral vector expressing the cell surface marker CD8 and the transduction efficiency was evaluated after each round by FACS quantification of the CD8-positive cells. The majority of the increase in the number of transduced cells took place during the first 4 rounds for both ASC and BMSC (Figure 2.1C-D), while the last 2 rounds only contributed marginal improvement. Therefore, it was determined that 4 rounds of transduction would provide a suitable balance between efficacy and cell manipulation and this protocol was used to generate all cell populations for further analysis.

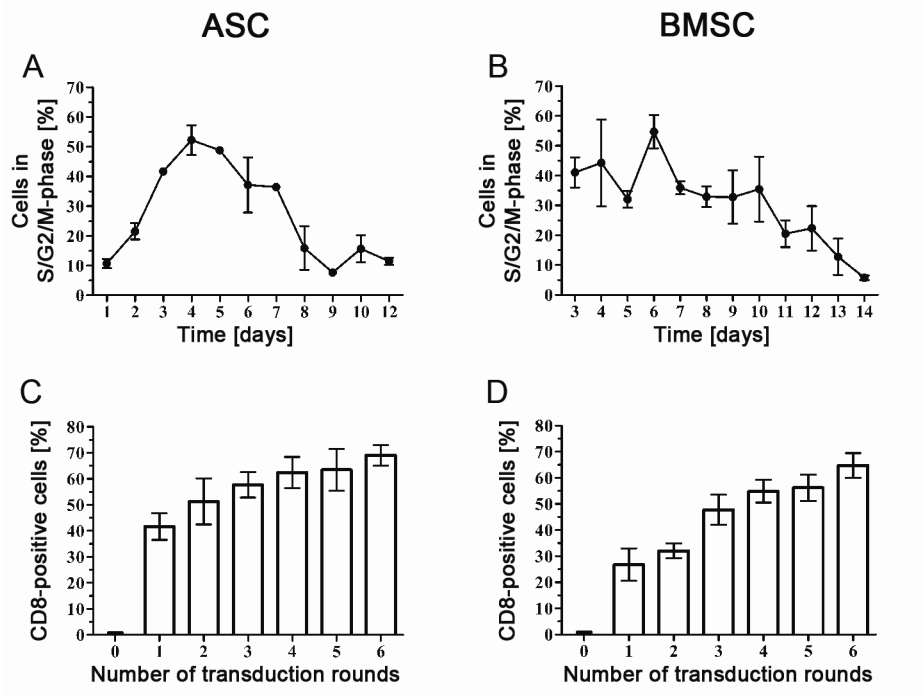


Figure 2.1 Optimization of MSC retroviral vector transduction. The proportion of ASC (A) and BMSC (B) in active proliferation (S/G2/M phases) was assessed by flow-cytometry analysis of nuclear DNA content after staining with Propidium Iodide during the first 12 to 14 days after initial plating (n=3 each). The transduction efficiency of ASC (C) and BMSC (D) was assessed as a function of infection rounds (n=3 each). The amount of CD8-positive cells was determined by FACS after up to 6 rounds of transduction with a CD8-expressing retroviral vector. Naïve MSC were used as negative control (0 rounds).

Generation of FACS-sortable MSC

Freshly isolated ASC and BMSC were transduced according to the optimized protocol with a bicistronic retroviral vector co-expressing rat $VEGF_{164}$ (VEGF) and a truncated version of rat CD8a (CD8) as a FACS-quantifiable cell-surface marker, joined through an internal ribosomal entry sequence (IRES) that allows the translation of two proteins from the same mRNA at a fixed ratio (27) (Figure 2.2). A retroviral vector expressing only CD8 was used to generate control cells. These genetically modified populations were used for further analysis (Figure 2.2).

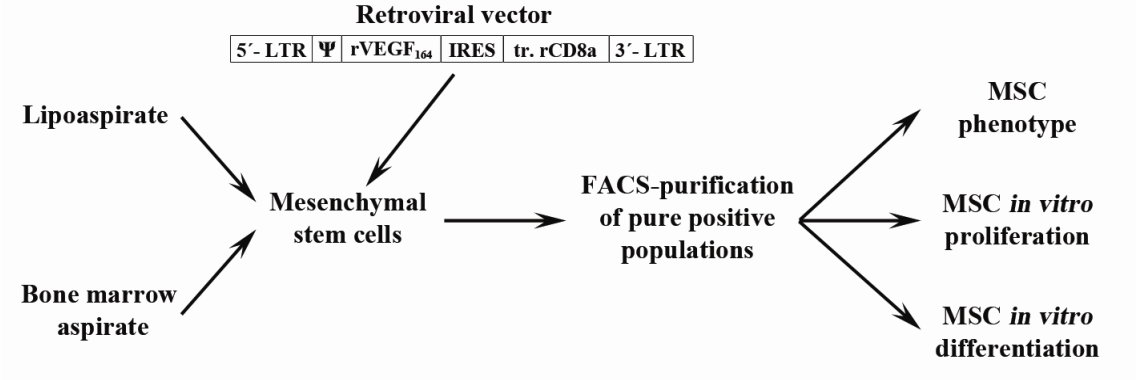


Figure 2.2 Study design. ASC and BMSC were isolated and transduced with a bicistronic retroviral vector co-expressing rat $VEGF_{164}$ ($rVEGF_{164}$) and a truncated version of rat CD8a (tr. rCD8a). LTR = retroviral Long Terminal Repeats; ψ = packaging signal; IRES = Internal Ribosomal Entry Sequence. CD8-positive MSC were FACS-sorted to generate pure populations of transduced cells and were analyzed to determine the effects of retroviral transduction and transgene expression on their phenotype, as well as their *in vitro* proliferation and differentiation potential.

Using freshly produced viral vector supernatants with the optimized protocol described above, a transduction efficiency >90% was routinely achieved (Figure 2.3A-B). Upon reaching the first confluence, MSC were FACS-purified in order to remove non-transduced cells and to yield pure CD8-positive populations (Figure 2.3A-B), which were replated for all subsequent experiments. Neither sorting nor transduction affected the morphology of ASC and BMSC, which exhibited a spindle-shaped phenotype similar to that of the corresponding naïve cells (Figure 2.3C-D, brightfield). Detection of membrane CD8 by immunofluorescent staining confirmed the high efficiency of transduction (Figure 2.3C-D, Unsorted) and the lack of non-transduced cells after sorting (Figure 2.3C-D, Sorted).

We further investigated whether transduction or VEGF expression could change the phenotype of MSC populations or cause the expansion of endothelial sub-populations. For this purpose the expression of surface markers specific for mesenchymal (CD105, CD90 and CD73) and endothelial (CD31, CD34 and VEGFR2) cells was analysed by flow cytometry. Naïve ASC and BMSC were compared with mock-transduced cells (centrifuged only) and with CD8- or VEGF-expressing transduced and sorted cells. As shown in Table 1, first-passage naïve ASC and BMSC were almost uniformly positive for

all the analyzed mesenchymal markers and contained less than 0.5% of endothelial cells. No significant changes in the mesenchymal phenotype of manipulated MSC were caused by transduction and sorting or by VEGF expression, nor was the ratio of endothelial cells increased in any condition.

TABLE 1: EXPRESSION OF ENDOTHELIAL AND MESENCHYMAL MARKERS BY NAÏVE AND TRANSDUCED MSC

		% of positive cells in ASC populations			
		Naive	*Mock*	*CD8*	*VEGF*
Endothelial markers	CD31+/CD34+	0.2 ± 0.1	0.2 ± 0.1	0.3 ± 0.2	0.1 ± 0.1
	VEGFR2+	0.0 ± 0.0	0.1 ± 0.1	0.5 ± 0.2	0.0 ± 0.0
Mesenchymal markers	CD73+	94.4 ± 5.0	95.7 ± 3.5	91.2 ± 8.4	91.6 ± 8.1
	CD90+	94.4 ± 5.2	94.9 ± 4.7	90.6 ± 8.9	89.3 ± 10.1
	CD105+	93.6 ± 4.7	91.9 ± 3.9	87.7 ± 6.5	86.2 ± 6.9
		% of positive cells in BMSC populations			
		Naive	*Mock*	*CD8*	*VEGF*
Endothelial markers	CD31+/CD34+	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	VEGFR2+	0.0 ± 0.0	0.5 ± 0.4	0.0 ± 0.0	0.1 ± 0.1
Mesenchymal markers	CD73+	99.6 ± 0.1	99.8 ± 0.1	99.7 ± 0.1	99.7 ± 0.1
	CD90+	89.2 ± 3.5	89.0 ± 3.2	91.8 ± 3.1	93.1 ± 1.5
	CD105+	98.9 ± 0.4	98.3 ± 1.4	99.2 ± 0.3	99.0 ± 0.5

Values are expressed as the mean ± standard error of the mean (n = 3); p = n.s. for each marker expression between all groups.

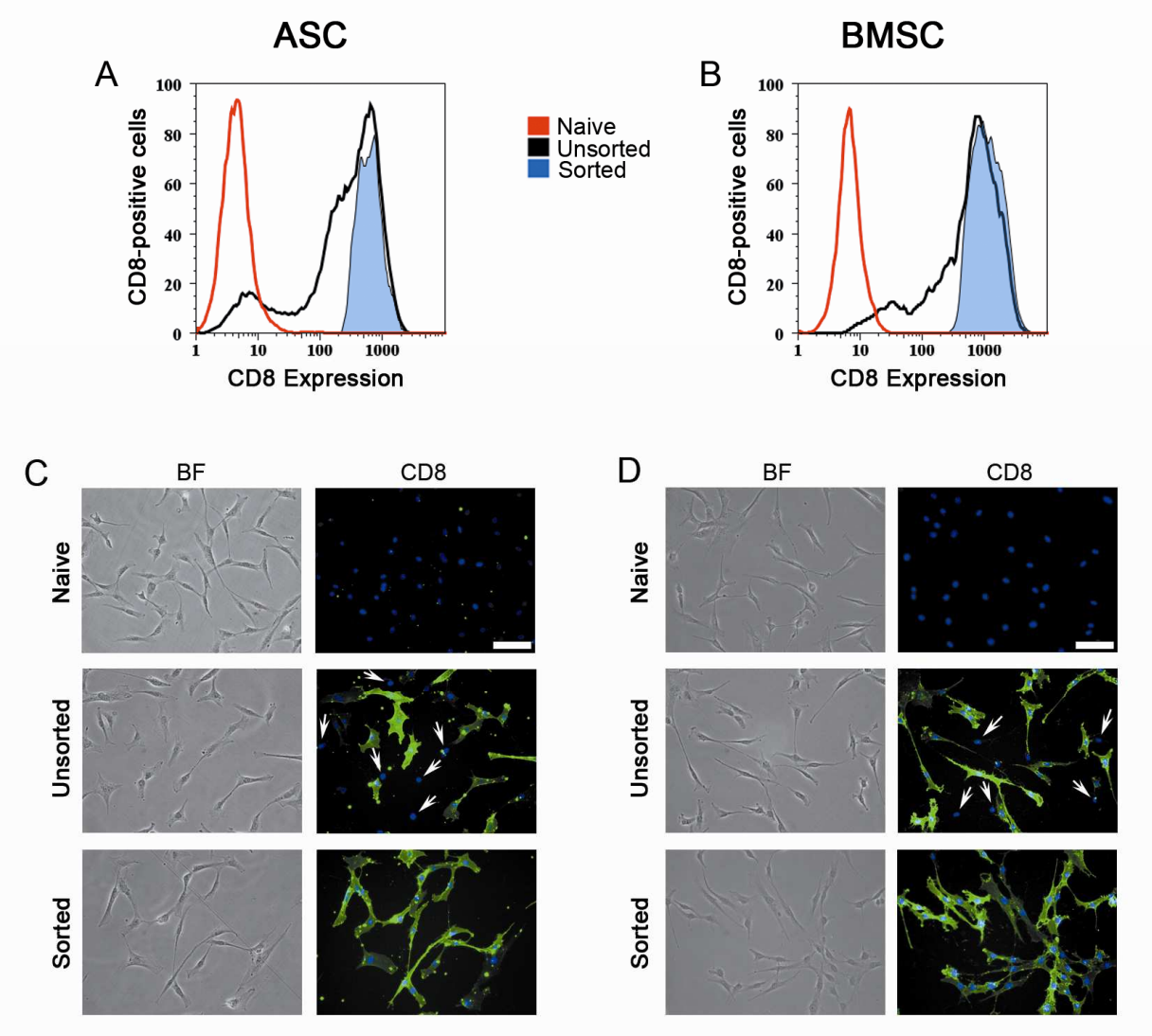


Figure 2.3 FACS-purification of transduced MSC. Representative FACS plots show that both ASC (A) and BMSC (B) were transduced with >90% efficiency (black plots) and that pure CD8-positive populations were obtained after sorting (tinted blue plots). Control naïve cells = red plots. Microscopic images of naïve or VEGF-expressing ASC (C) and BMSC (D), either before (Unsorted) or after (Sorted) FACS-purification. BF = brightfield images; CD8 = cells immunostained with an anti-ratCD8 antibody (green) and DAPI (nuclei, in blue). White arrows indicate the nuclei of CD8-negative cells in the unsorted populations (n=3 each). Size bar = 100µm.

In vitro proliferation and differentiation of genetically modified MSC

The effects of transduction and FACS-purification on MSC proliferative potential were assessed by measuring the cumulative population doublings of CD8- and VEGF-expressing ASC and BMSC for 8 and 9 passages after sorting, respectively. These were compared to naïve cells and mock-transduced cells that were subjected to the physical manipulation involved in the transduction protocol, i.e. centrifugation for 30' in the dish, but in the absence of viral vector particles. Both transduced ASC and BMSC populations proliferated more slowly than the naïve and centrifuged MSC during the first 2 passages after sorting (Figure 2.4). However, this effect was temporary and growth of transduced MSC resumed at the same rate as control cells approximately 10 days after the sorting (Figure 2.4). Therefore, transduction and sorting did not affect the intrinsic proliferative potential of MSC.

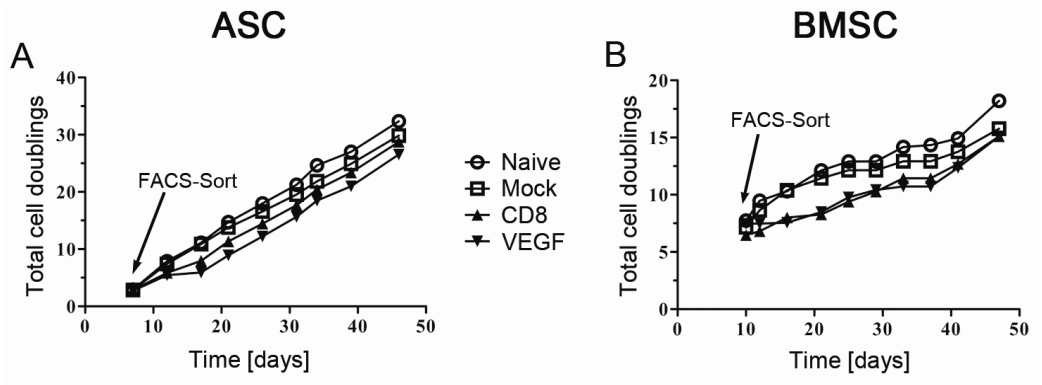


Figure 2.4 In vitro proliferation of transduced MSC. The in vitro proliferative potential of CD8- and VEGF-expressing ASC (A) and BMSC (B) was not impaired compared to that of naïve and mock-transduced cells over 9 passages after FACS sorting (n=3 each).

The differentiation potential of the same ASC and BMSC populations towards the adipogenic and osteogenic lineages was determined in vitro after the first passage and FACS-purification. Mock-transduced, CD8- and VEGF-expressing ASC (Figure 2.5A) and BMSC (Figure 2.5C) were able to differentiate into both lineages similarly to the naïve MSC. Interestingly, in ASC from one donor with poor intrinsic osteogenic differentiation potential (Figure 2.5B), VEGF expression actually increased osteogenic differentiation compared to naïve, mock-transduced and CD8-expressing cells (Figure 2.5B).

Differentiation was confirmed by quantifying the expression of transcription factors that are responsible for initiating adipogenesis (PPAR-γ, Figure 2.5D-E) and osteogenesis (Runx2, Figure 2.5F-G) by qRT-PCR. The results confirmed that ASC and BMSC differentiation along both lineages was not impaired by retroviral transduction, FACS-purification or VEGF expression.

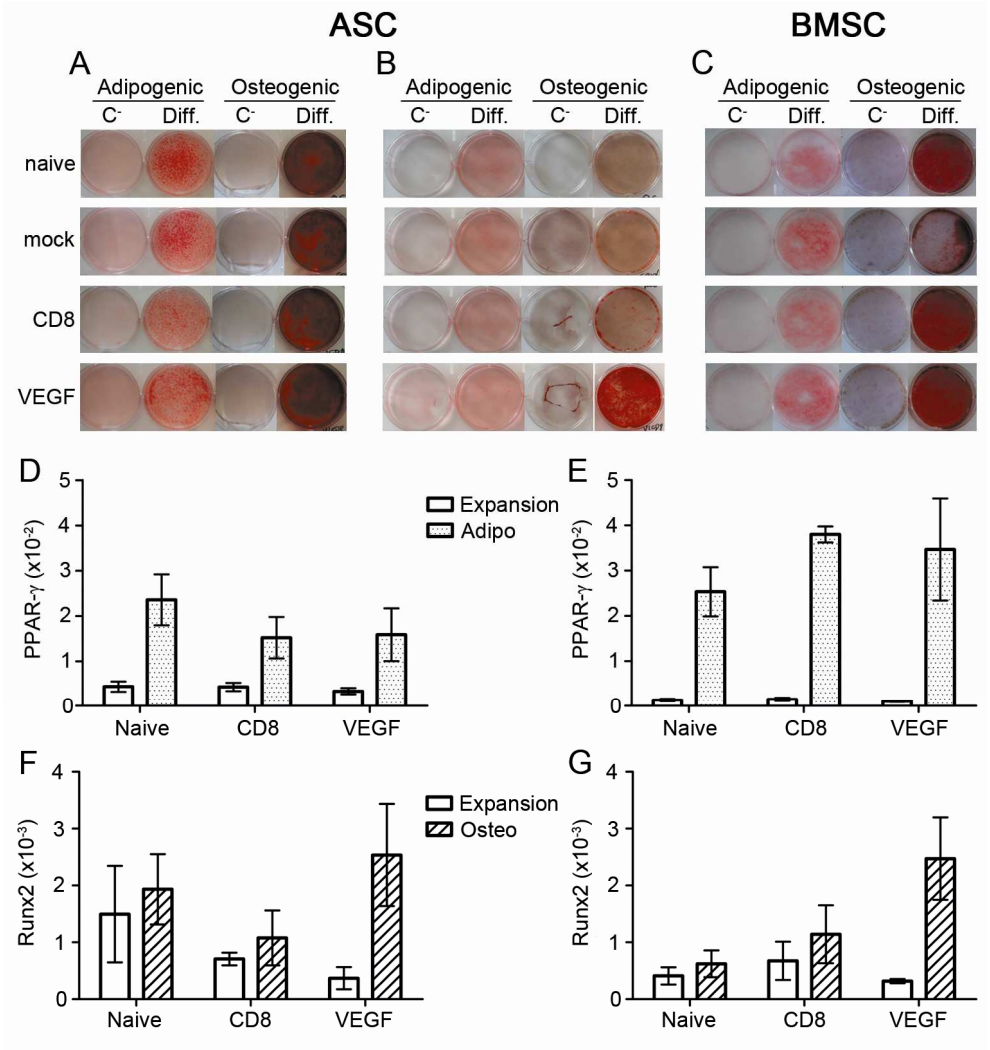


Figure 2.5 *In vitro* differentiation of transduced MSC. Naïve, mock-transduced, CD8- and VEGF-expressing were differentiated in vitro towards the adipogenic and osteogenic lineages. (A-C) Oil Red-O staining (adipogenic) and Alizarin Red staining (osteogenic) of control (C-) or differentiated (Diff.) ASC (A-B, from two different donors) and BMSC (C) (n=4 each). Differentiation was confirmed by quantifying the expression of PPAR-γ (adipogenesis, D-E) and Runx2 (osteogenesis, F-G) in expansion and differentiation conditions by quantitative real-time PCR (n=3 each). The graphs show relative expression compared to that of the housekeeping gene GAPDH.

Transgene expression after cell differentiation

Retroviral vectors integrate in the host genome and their level of expression can be affected by remodeling of the surrounding chromatin that accompanies gene activation and repression during differentiation (28). Therefore, we assessed the stability of transgene expression by transduced MSC during differentiation.

Transgene mRNA levels were quantified by qRT-PCR of the IRES sequence, which is common to the expression cassettes in both retroviral vectors used (CD8 and VEGF), so that the use of a single pair of primers allowed comparisons between all cell populations (Figure 2.6A-B). VEGF protein was measured by ELISA (Figure 2.6C-D). Both assays indicated that transgene expression did not decline during in vitro adipogenic or osteogenic differentiation compared to cells in expansion medium, and that in some instances it actually increased.

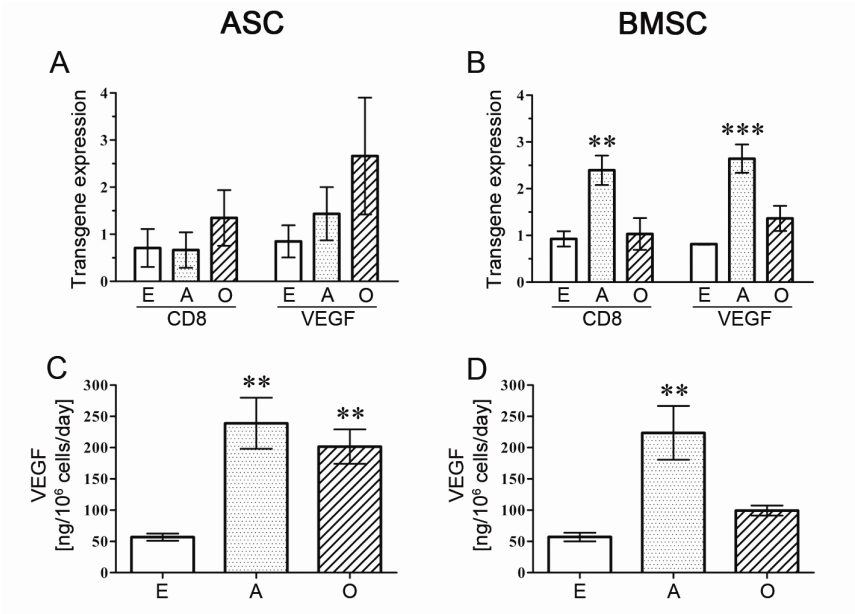


Figure 2.6 Transgene expression after *in vitro* differentiation. Transgene expression was quantified in expansion culture (E) or after adipogenic (A) and osteogenic (O) *in vitro* differentiation of CD8- and VEGF-expressing ASC (A, C) and BMSC (B, D). Expression of the transgene mRNA levels were quantified by qRT-PCR (A-B). VEGF protein in the supernatants was measured by ELISA (C-D). ** $p \leq 0.01$ and *** $p \leq 0.001$ vs. cells cultured in expansion medium (n=3 each).

FACS-purification of MSC expressing specific VEGF levels

In order to rapidly purify homogeneous populations expressing desired levels of VEGF, we employed a technology we recently developed with transduced skeletal myoblasts (23, 29). Previously established clonal populations of skeletal myoblasts transduced with the same ratVEGF-IRES-ratCD8 retrovirus and homogeneously expressing specific VEGF levels (23) were used as reference populations to FACS-sort transduced MSC expressing similar CD8 levels on the cell surface (Figure 2.7A). After FACS-purification, low-, medium- and high-level sorted populations produced about 12, 79 and 154 ng/10^6 cells/day, respectively. As shown in Figure 2.7B, these VEGF amounts were remarkably similar to those produced by the corresponding reference clonal populations (13, 53 and 155 ng/10^6 cells/day, respectively). VEGF secretion by the FACS-purified populations was stable also 5 and 18 days after sorting (data not shown).

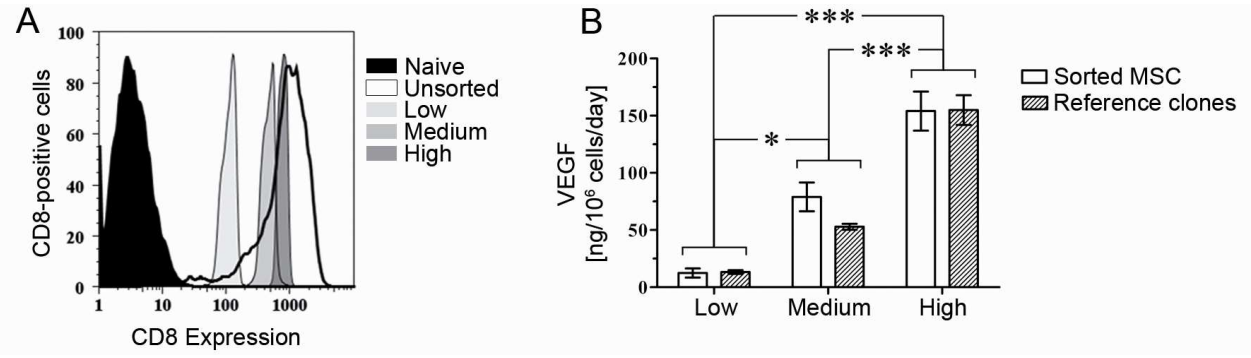


Figure 2.7 FACS-purification of transduced BMSC expressing specific VEGF levels. Clonal populations of mouse skeletal myoblasts transduced with the same retroviral vector expressing rat VEGF and rat CD8, that produced specific low, medium and high VEGF levels, were used as reference populations to FACS-purify VEGF-producing BMSC that expressed similar levels of CD8 on the cell surface (A). Quantification of rat $VEGF_{164}$ by ELISA (B) shows that FACS sorting was successful in rapidly purifying BMSC populations homogeneously producing the same VEGF amounts as the corresponding reference populations. * $p \leq 0.05$ and *** $p \leq 0.001$.

2.5 DISCUSSION

In this study we developed a tool to genetically modify primary human MSC derived from bone marrow and adipose tissue with high efficiency and rapidly select cells expressing desired levels of VEGF with minimal *in vitro* manipulation, so as to preserve their progenitor properties. This was achieved by combining an optimized transduction protocol with a high-throughput FACS-based purification technology we recently developed (29). Neither retroviral vector transduction, nor FACS-purification, nor expression of VEGF and the CD8 marker impaired MSC proliferation and differentiation potential *in vitro*. Transgene expression was sustained during *in vitro* differentiation.

Genetic modification with integrating vectors, such as retroviruses, ensures consistent long-term expression of factors despite cell expansion (14). However, current methods for MSC transduction require that standardized proliferating cultures are established by replating MSC at specific densities after the first confluence is reached (30-32), so that until now a primary genetically modified population could not be generated before the second passage. In addition, high transduction efficiency is desirable, so as to minimize the need for selection of the expressing cells and for further expansion to reach the required quantity. Therefore, by rigorously determining the exact time at which freshly isolated MSC reach the highest proliferative activity after first plating, we were able to target retroviral vector transduction at the most efficient window and achieve a transduction efficiency of up to 95% before first confluence was reached. It should be noted that initial experiments to determine the optimal conditions led to efficiencies of only approximately 70% because they were performed with frozen aliquots of pooled viral vector supernatants in order to ensure standardized conditions. However, fresh supernatants, which have higher titers, allowed the routine achievement of >90% efficiency with the same protocol.

A crucial aspect of the technique we developed is the possibility to rapidly and prospectively purify sub-populations of transduced cells expressing specific desired levels of the transgene. Currently used selection methods are designed to simply eliminate non-transduced cells and cannot distinguish cells expressing different levels of e.g. antibiotic resistance (33), or can do so only after retrospective analysis of each

isolated population by e.g. GFP co-expression (34). In fact, although FACS is intrinsically quantitative, the measured fluorescence intensity values cannot be directly translated into absolute amounts of transgene production. Here we overcame this limitation by taking advantage of a technique we recently developed to predict the absolute amount of expression by individual transduced skeletal myoblasts in a population by FACS, based on the combined use of a syngenic non-functional cell-surface marker (truncated CD8a) with that of immortal clonal populations as a reference (29).

The possibility to control the expressed dose of a transgene has important therapeutic implications. In fact, in a heterogeneous population, high levels can be toxic and low levels inefficient, leading to a waste of therapeutic potential (10). For example, VEGF over-expression is an attractive strategy to induce therapeutic angiogenesis in ischemic conditions (35), as well as to increase and accelerate vascularization of clinical-size tissue-engineered grafts (36). However, VEGF can induce either normal and therapeutic angiogenesis, or aberrant angioma-like vascular growth depending on its concentration in the microenvironment around each producing cell *in vivo*, and not on its total dose (11, 37) as it remains bound to the extracellular matrix after secretion. Here we showed that MSC sub-populations homogeneously producing specific desired VEGF doses can be rapidly and prospectively purified from the initial transduced population already at the first passage, with no loss of their proliferation and differentiation potential, by combining the optimized transduction protocol and high-throughput FACS-based purification technology we developed.

The platform described here is expected to increase the feasibility of therapeutic approaches based on genetically modified MSC, by ensuring both high efficiency and minimal *in vitro* manipulation. Furthermore, it should prove useful also to study the dose-dependent effects of genes of therapeutic interest on early-passage, primary MSC. In fact, the results obtained studying heterogeneous transduced populations usually reflect the effects of the highest levels of expression, which may obscure those of the lower and more physiological levels. Uniform expression of different transgene levels could so far only be studied in clonal populations, which are disadvantageous, as their generation requires extensive in vitro culture, which is known to rapdidly change the biological properties of mesenchymal progenitors.

ACKNOWLEDGEMENTS

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3 VEGF EXPRESSION BY HUMAN MESENCHYMAL STEM CELLS PROMOTES ANGIOGENESIS, BUT STIMULATES OSTEOCLAST RECRUITMENT AND REDUCES BONE FORMATION

Uta Helmrich, Sinan Güven, Elena Groppa, Francine Wolf, Michael Heberer, Arnaud Scherberich, Ivan Martin and Andrea Banfi

3.1 INTRODUCTION

Tissue engineering is a promising strategy for the replacement of large bone defects. In recent years, bone marrow-derived mesenchymal stromal/stem cells (BMSC) have been extensively used for this purpose, as they are rich in osteoprogenitors (42). However, one of the current limitations of bone tissue engineering is the inability to provide sufficient blood supply. The lack of vascularization in the centre of large cell-containing constructs invariably leads to ischemia followed by necrosis (75, 76).

Apart from the supply of nutrients and oxygen to the cells and the clearance from waste products, which are necessary for the survival, growth and differentiation of engrafted cells, blood vessels also allow the recruitment and extravasation of highly specialised cells, as mesenchymal stem cells, hematopoietic stem cells, pre-osteoblasts and pre-osteoclasts, which may improve tissue regeneration and formation (77, 78).

Many different approaches to rapidly increase angiogenesis in bone tissue engineering are under investigation, such as surgical techniques, like the flap- or arteriovenous loop-fabrication (17, 82, 83), biomaterial-based methods (83, 84) or the co-culture of osteogenic and vasculogenic precursor cells (85, 86, 102). Another strategy is the supplementation with pro-angiogenic factors (84). Among these, Vascular Endothelial Growth Factor (VEGF) is the master regulator of vascular growth both in normal and pathological angiogenesis and therefore is the most attractive and well-characterized factor for inducing the therapeutic growth of new blood vessels (96, 97). On one hand VEGF has a very short half-life *in vivo*, but on the other its expression needs to be sustained for approximately 4 weeks in order to allow the stabilization and persistence of newly formed vessels (93, 94). Therefore, the use of recombinant protein is not an effective therapeutic strategy, whereas the continuous secretion of the factor would be required. Stable long-term production in the tissue is best achieved by gene therapy, i.e. the delivery of the genetic sequence to allow continuous secretion of the factor by the target cells (103).

In this study, we sought to use a cell-based gene therapy approach to generate bone grafts with an increased vascularization potential *in vivo*, by genetically modifying human BMSC to achieve sustained VEGF expression. *In vivo* bone formation and

vascularization potential by genetically modified BMSC were investigated in a well-established nude rat model of osteogenic graft ectopic implantation. We found that VEGF over-expression by hBMSC caused significantly increased angiogenesis in the osteogenic constructs. However, this was accompanied by increased osteoclast recruitment and decreased bone formation specifically in the VEGF–expressing grafts.

3.2 MATERIALS AND METHODS

BMSC isolation and culture

Human primary BMSC were isolated from bone marrow aspirates. The aspirates were obtained from the iliac crest of healthy donors during routine orthopaedic surgical procedures according to established protocols, after informed consent by the patients and following protocol approval by the local ethical committee (EKBB, Ref. 78/07). Cells were isolated and cultured as described (104). Briefly, after centrifugation, the pellet was washed in PBS (GIBCO), resuspended in α-MEM medium (GIBCO) containing 10% bovine serum and 5 ng/ml FGF-2 (BD Biosciences) and plated at a density of 10^5 nucleated cells/cm^2. hBMSC were cultured in 5% CO2 at 37°C. When 80 - 90% confluency was reached, cells were replated at a density of 3000 cells/cm^2.

MSC genetic modification

Primary hBMSC were transduced according to the high-efficiency protocol described in Chapter 2 (Helmrich et al., manuscript submitted). Briefly, starting on day 6 after plating, cells were transduced twice a day for a total of 4 rounds with retroviral vectors carrying the gene for rat $VEGF_{164}$ (rVEGF) linked through an internal ribosomal entry site (IRES) to a truncated version of the FACS-quantifiable cell-surface marker rat CD8a (101).

Flow cytometry and cell-sorting

CD8a expression was assessed by staining with a FITC-conjugated anti-rat CD8a antibody (clone OX-8; BD Pharmingen), using previously optimized staining conditions (101), i.e. 2 µg of antibody/10^6 cells in 200µl of PBS with 5% BSA. Staining was performed for 20 minutes on ice. Data were acquired with a FACSCalibur flow cytometer (Becton Dickinson and Company) and analyzed using FlowJo software (Tree Star). Cell sorting was performed with an Influx cell sorter (Becton Dickinson and Company) after staining for CD8a according to the same protocol.

Osteogenic graft implantation in vivo

Osteogenic grafts were generated as previously described (105). Briefly, 60 mm^3 of 1-2 mm silicated apatite granules (Actifuse®, Apatech) where mixed with at least $5x10^5$ cells embedded in a fibrin gel, with a final concentration of 20 mg Fibrinogen/ml and 6 IU of Thrombin/ml. The resulting pellets were implanted subcutaneously in nude rats (RNu/Nu Charles-River, Germany) as previously described (87). After 8 weeks, the animals were sacrificed by inhalation of CO2. Animals were treated in agreement with Swiss legislation and according to a protocol approved by the veterinary office of Canton Basel- Stadt (permission #2167).

Sample processing

Explanted constructs were washed with PBS and fixed for 4 hours with freshly prepared 1% paraformaldehyde (Sigma) in PBS. Subsequently, the samples were decalcified according to an established protocol (Kusser and Randall, 2003). Briefly, granules were transferred into a PBS-based solution containing 7% w/v EDTA (Fluka) and 10% w/v sucrose (Sigma-Aldrich) and incubated at 37°C on an orbital shaker. The solution was renewed daily for 5-21 days, until the samples were fully decalcified, as estimated by the degree of sample stiffness. Finally the samples were embedded in OCT compound (Sakura Finetek), snap-frozen and 10 µm-thick sections were obtained with a cryotome.

Analysis of angiogenesis and bone formation

To visualize blood vessels, immunofluorescence staining was performed with the following primary antibodies and dilutions: mouse-anti-rat CD31 (Clone TLD-3A12; AbD Serotec, 1:100), rabbit-anti-mouse NG2 (Millipore, 1:200) and mouse anti-Smooth Muscle Actin (Clone 1A4; MPBiomedicals, 1:400). Since these antibodies cross-react with the corresponding human proteins, the contribution of human (donor) or rat (host) cells to blood vessel formation could not be distinguished. Fluorescently labeled secondary antibodies (Invitrogen) were used at a concentration of 1:200.

The amount of blood vessels was quantified on images taken from CD31-stained sections. Three representative fluorescent images per sample (2 replicates for each of 3

donors) were acquired with an Olympus BX61 microscope (Münster, Germany). Vessel length density (VLD) was measured with Cell^P software (Olympus) by tracing the total length of vessels in the fields and by normalizing it to the area of the fields.
Bone tissue was detected by staining sections with hematoxylin and eosin (H&E) and microscopic observation both under transmitted and fluorescent light as previously described (106, 107).

Assessment of cell engraftment

Human Alu repeat sequences were detected by *in situ* hybridization, according to a protocol adapted from a previously described procedure (108). A digoxigenin-labeled probe for human-specific Alu repetitive sequence was prepared by PCR containing 1x PCR buffer (Promega), 1.5mM MgCl2, 0.1mM dATP, 0.1mM dCTP, 0.1mM dGTP, 0.065mM dTTP, 0.035mM digoxigenin-11-dUTP (Roche), 0.2µM of Alu-specific primers, 3.75 U Platinum Taq DNA Polymerase (Invitrogen) and 50 ng of human genomic DNA. The following primers were used: forward 5'-cgaggcgggtggatcatgaggt-3', reverse 5'-ttttttgagacggagtctcgc-3' (108). PCR was performed with the GeneAmp® PCR System 9600 (Perkin Elmer). After an initial denaturation step at 94°C for 5 minutes, the reaction was carried out with 40 cycles consisting of a denaturation step at 95°C for 15s, an annealing step at 65°C for 30s and an elongation step at 72°C for 30s. The PCR product was purified using the QIAquick PCR Purification Kit (Quiagen) according to the manufacturer's protocol.
Sections were first treated with 0.5 mg/ml Pepsin in 0.01N HCl for 10 min at 37°C and incubated in 0.1M TEAC (SIGMA) for 10 min at room temperature. Subsequently they were pre-hybridized for 30 min at 42°C in pre-hybridization buffer containing 4x SSC, 50% deionized formamide, 1x Denhardt's solution, 5% dextrane sulphate and 0.2µM bovine DNA. Afterwards the pre-hybridization buffer was replaced by fresh buffer containing 0.2 ng/µl digoxigenin-labeled probe. Following a denaturation step at 95°C for 3min and immediate cooling at 4°C for 3min, hybridization was carried out for 16 h at 42°C in a wet chamber. Slides were washed twice for 5 min in 2x SSC at room temperature and twice for 10 min at 42°C in 0.1x SSC. Finally, signals were detected by immunohistochemistry using anti-digoxigenin alkaline phosphatase-conjugated Fab

fragments (Roche) and NBT/BCIP (Roche) as substrate. The sections were counterstained with nuclear fast red (Sigma).

TRAP-assay

Sections were stained for tartrate-resistant acid phosphatase (TRAP) activity using the leukocyte acid phosphatase kit (Sigma-Aldrich). Briefly, after rinsing with water, the slides were stained with 0.3 mg/ml Fast Red Violet LB dissolved in TRAP buffer (0.1 M sodium acetate, 0.1 M acetic acid, 1 mg/ml naphthol AS-MX phosphate, pH 5.0) for 1 hour at 37°C. After TRAP staining, nuclear staining was performed with haematoxylin for 1 min at room temperature.

Statistics

Data were analyzed with the software Prism 4.0a (GraphPad) and are presented as means ± standard error. The significance of differences was evaluated by analysis of variance (ANOVA) followed by a Bonferroni test. $P < 0.05$ was considered statistically significant.

3.3 RESULTS

Generation of VEGF-expressing BMSC

Primary human BMSC were transduced to express rat VEGF linked to the FACS-detectable cell-surface marker truncated CD8a (VEGF-BMSC). Naïve BMSC and BMSC transduced with a retroviral vector expressing only the truncated CD8a marker (CD8-BMSC) were used as controls. Transduction efficiencies of >90% were routinely achieved by the first confluence. At the first passage, the genetically modified BMSC were FACS-purified to eliminate non-transduced cells and to yield a pure CD8-positive population.

VEGF-expressing BMSC improve graft vascularization despite extensive expansion

For many clinical applications, BMSC need to be significantly expanded *in vitro* after isolation in order to generate critical-size osteogenic grafts. Therefore, we first asked whether genetic modification endowed transduced BMSC with a stable potential to effectively improve graft vascularization despite extensive expansion. BMSC derived from 2 independent donors were cultured until p7 after transduction and FACS-purification, reaching a total of about 30-35 population doublings. The cells were then seeded onto hydroxyapatite granules and implanted subcutaneously in nude rats for 8 weeks. Analysis of vascularization in the explanted tissues by immunofluorescence staining showed that the pellets loaded with both naïve or CD8-expressing BMSC contained mostly large-caliber regular vessels, covered with α-SMA-positive smooth muscle cells and only a few NG2-positive pericytes, similar to arterioles (Figure 3.1A-B). On the other hand, VEGF over-expression caused the appearance of large numbers of small-diameter vessels, which were associated with NG2-positive pericytes and displayed the morphology of normal and stable capillaries (Figure 3.1C). Among these, a few smooth muscle-covered arterioles of larger diameter were also visible (Figure 3.1C), showing the formation of a hierarchically structured vascular bed, with both conductance vessels for efficient flow and microvascular networks for metabolic exchange. As shown in Figure 3.1D, vessel quantification showed that VEGF-expressing

BMSC caused a 2- to 3-fold increase in vessel length density, compared to both naïve and CD8-BMSC (13.25 ± 0.8 vs. 4.5 ± 0.3 and 6.6 ± 0.8 mm/mm^2, respectively, p<0.0001).

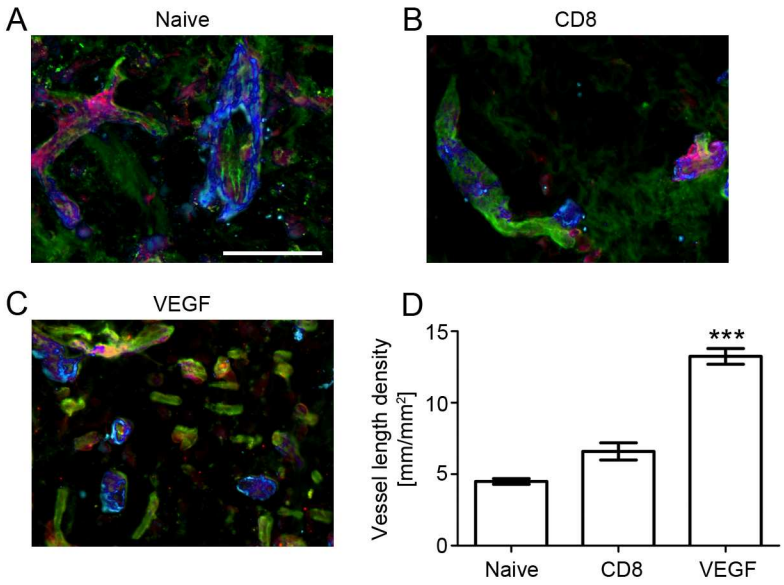

Figure 3.1 *In vivo* angiogenesis after long-term expansion. (A-C) Immunofluorescence staining for endothelium (CD31, green), pericytes (NG2, red) and smooth muscle cells (SMA, cyan) in sections of silicated apatite granules seeded with naïve BMSC (A), control CD8-BMSC (B) or VEGF-BMSC (C) 8 weeks after subcutaneous implantation in nude rats (Scale bar = 50 µm). (D) The amount of angiogenesis was quantified by measuring vessel length density, expressed as millimeters of vessel length per square millimeter of tissue area [mm/mm2]. Data represent mean values ± SEM (n = 2 independent donors); ***p < 0.0001 compared to naïve cells.

On the other hand, H&E staining and analysis under fluorescent light showed that only fibrous tissue, but no bone, was detectable in all constructs implanted with either control or VEGF-expressing BMSC (Figure 3.2), as expected with osteoprogenitors that had been expanded *in vitro* for more than 30 population doublings.

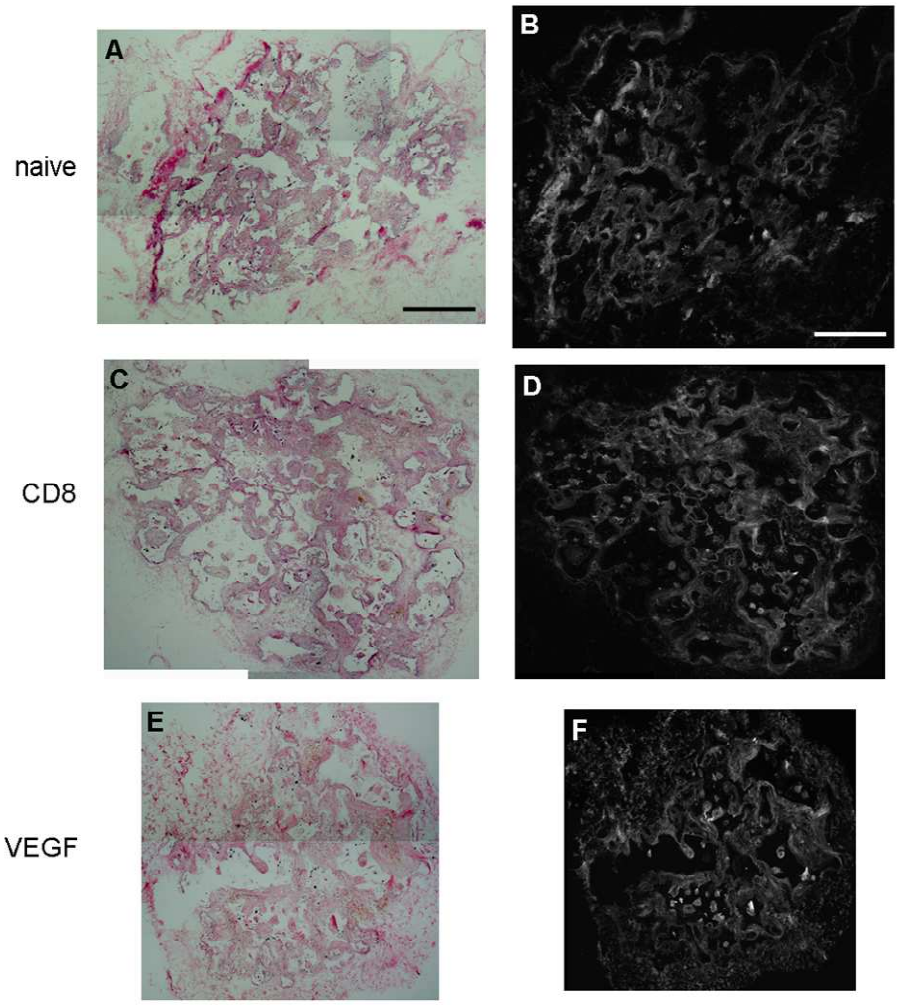

Figure 3.2 *In vivo* bone formation after long-term expansion. Composite images of entire sections 8 weeks after subcutaneous implantation of silicated apatite granule pellets seeded with naïve BMSC (A-B), CD8-BMSC (C-D) or VEGF-BMSC (E-F) in nude rats. After staining with hematoxylin and eosin, sections were viewed under transmitted (A, C, E), as well as fluorescent light (B, D, E). Scale bar = 1 mm; n = 2 independent donors.

VEGF-BMSC improve graft vascularization, but reduce bone formation after minimal expansion

In order to determine the effects of VEGF over-expression on bone formation by transduced osteoprogenitors, BMSC were minimally expanded, so that their intrinsic osteogenic capacity was not impaired by extensive *in vitro* culture. As shown in Figure 3.3, two conditions were investigated: 1) populations implanted at the earliest possible

time, i.e. upon reaching the first confluence, which contained <10% non-transduced cells (p1); and 2) FACS-purification at the first passage to eliminate the few non-transduced cells, followed by implantation at the second passage (p2).

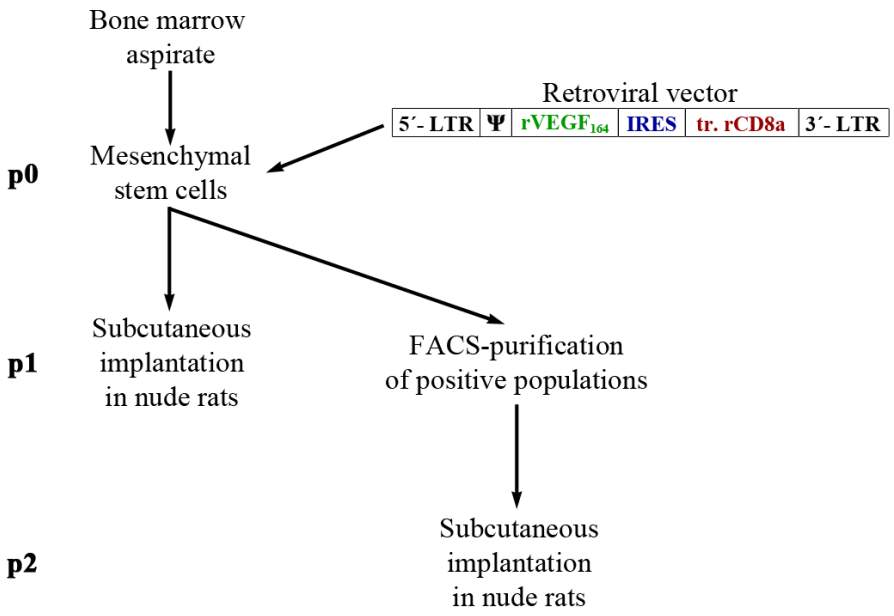

Figure 3.3 Study design for short-term expansion experiments. BMSC were isolated and transduced with a bicistronic retroviral vector co-expressing rat VEGF164 (rVEGF164) and a truncated version of rat CD8a (tr.rCD8a). Implantation was performed either immediately after transduction, when cells reached the first confluency (p1), or after FACS-purification to eliminate the few non-transduced cells, which required one more passage (p2). LTR = retroviral Long Terminal Repeats; ψ = packaging signal; IRES = Internal Ribosomal Entry Sequence.

Grafts were explanted 8 weeks after implantation to evaluate both blood vessel growth and bone tissue formation. Similarly to the results described with extensively expanded cells, grafts generated both by p1 and p2 naïve and CD8-BMSC were vascularized by sparse vessels displaying characteristics of normal arterioles, with a regular smooth muscle coating, and only few pericyte-covered capillaries (Figure 3.4A-D). Both p1 and p2 VEGF-expressing BMSC, on the other hand, caused a large increase in the amount of pericyte-covered microvascular networks, while preserving the presence of a few arterioles (Figure 3.4E-F). As shown in Figure 3.4G, quantification of vessel length

density showed that VEGF-expressing BMSC caused a 2- to 3-fold increase in graft vascularization (p1 = 13.3 ± 1.1 and p2 = 13.1 ± 1.3 mm/mm^2; p<0.001 for all comparisons) compared to naïve (p1 = 5.7 ± 0.7 and p2 = 4.7 ± 0.6 mm/mm^2) and CD8-BMSC (p1 = 6.0 ± 0.5 and p2 = 4.5 ± 0.4 mm/mm^2). No difference was observed in the amount of vascular growth induced by either p1 or p2 cells.

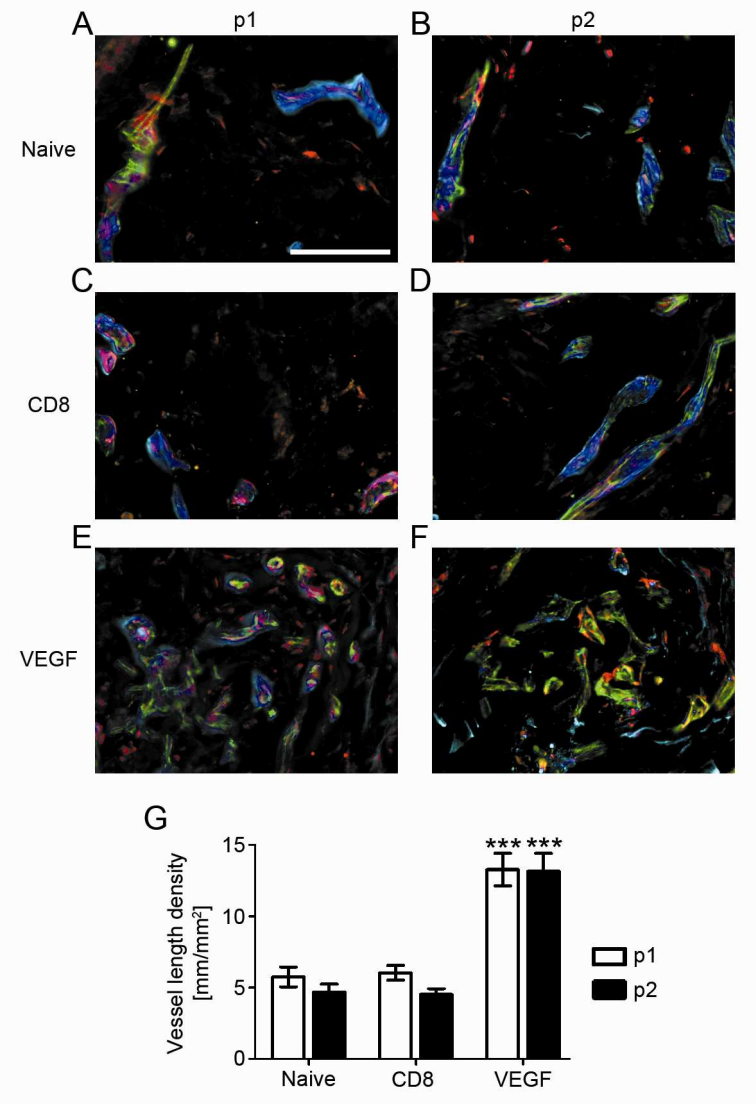

Figure 3.4 *In vivo* angiogenesis after short-term expansion. (A-F) Immunofluorescence staining for endothelium (CD31, green), pericytes (NG2, red) and smooth muscle cells (SMA, cyan) in sections of silicated apatite granules seeded with naïve BMSC (A-B), CD8-BMSC (C-D) or VEGF-BMSC (E-F), expanded for either 1 (p1) or 2 passages (p2), 8 weeks after subcutaneous implantation in nude rats. Scale bar = 50 µm. (G) The amount of angiogenesis was quantified by measuring vessel length density, expressed as millimeters of vessel length per square millimeter of tissue area [mm/mm2]. Data represent mean values ± SEM (n = 3 independent donors); **p<0.001, ***p < 0.0001.

Bone tissue was identified by analysis of H&E-stained sections under fluorescent light, in which conditions the packed parallel arrangement of collagen fibers specifically present in bone lamellae gives rise to autofluorescence (107). All constructs seeded with minimally expanded naïve- or CD8-BMSC displayed abundant formation of bone tissue, both with p1 and p2 cells (Figure 3.5A-D and 3.5G-J). This tissue was characterized by clear osteocyte lacunae, embedded in a very dense lamellar matrix, typical of mature bone structure (Figure 3.5M-N). However, constructs seeded with VEGF-BMSC contained a severely reduced amount of bone compared to the naïve or CD8-BMSC (Figure 3.5E-F and 3.5K-L). Qualitatively, on the other hand, the bone tissue that was present was similarly mature and well formed as that induced in the control conditions (Figure 3.5O-P).

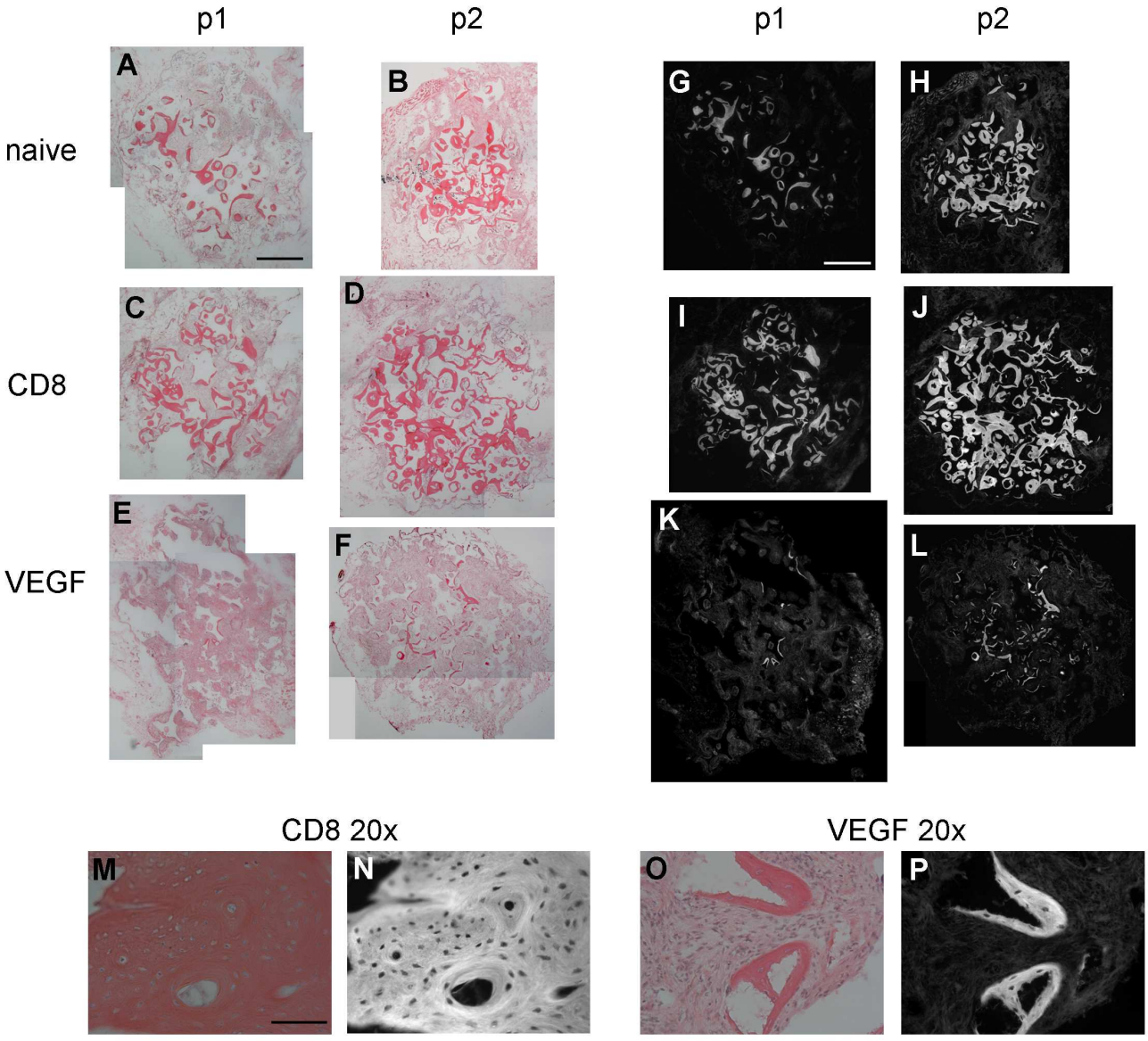

Figure 3.5 *In vivo* bone formation after short-term expansion. Composite images of entire sections 8 weeks after subcutaneous implantation in nude rats of silicated apatite granule pellets seeded with naïve BMSC (A-B, G-H), CD8-BMSC (C-D, I-J) or VEGF-BMSC (E-F, K-L), expanded for either 1 (p1) or 2 passages (p2). After staining with hematoxylin and eosin, sections were viewed under transmitted (A-F), as well as fluorescent light (G-L). Both duplicates of one donor are shown for each condition. Scale bar = 1 mm; n = 3 independent donors. (M-P) High-magnification detail of dense lamellar bone matrix in a sample seeded with control CD8-BMSC (M-N) or VEGF-BMSC (O-P) viewed under transmitted (M, O) and fluorescent (N, P) light, taken from panels (C, I) and (E, K), respectively. Scale bar = 100 µm.

Stable engraftment of implanted BMSC

In order to determine whether reduced bone formation in the VEGF-expressing constructs might be due to impaired survival of the implanted osteoprogenitors, human BMSC were tracked in histological sections by *in situ* hybridization with a probe for the human-specific Alu repeat sequence in nuclear DNA. Constructs seeded with naïve and CD8-BMSC showed a homogenous mixture of positive and negative staining within the bone and the surrounding soft tissue, indicating the contribution of both the implanted BMSC and host-derived osteoprogenitors to the new bone formation (Figure 3.6A-D). Human cells were also abundantly present in the VEGF-expressing constructs, both within the mature bone tissue and in the surrounding soft tissue (Figure 3.6E-F), suggesting that no impairment of BMSC engraftment had occurred compared to the control conditions. Interestingly, the human cells at the interface between bone and surrounding tissue displayed a different morphology between the VEGF-expressing and the control conditions. In fact, some areas of bone, formed by both naïve and CD8-BMSC, were covered by a single layer of flattened Alu-positive cells, similar to osteoblasts, or bone-lining cells (Figure 3.6A and 3.6D). In the VEGF-expressing condition, instead, the human cells at the bone surface often displayed many thin cytoplasmic protrusions stretching into the surrounding space (Figure 3.6E-F), which sometimes made contact with each other (arrow in Figure 3.6F), similar to the morphology of differentiated osteocytes.

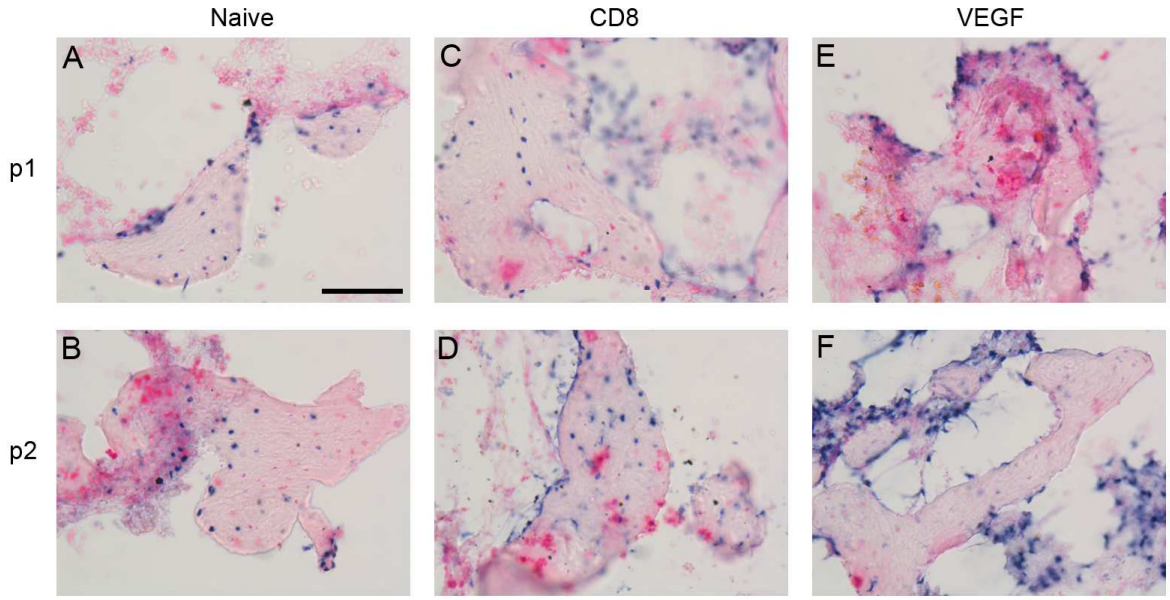

Figure 3.6 *In vivo* cell survival. *In situ* hybridization for the human-specific Alu repetitive sequence (blue) and counterstaining with nuclear fast red (red) were performed on sections of silicated apatite granule pellets seeded with naïve BMSC (A-B), CD8-BMSC (C-D) or VEGF-BMSC (E-F), expanded for either 1 (p1) or 2 passages (p2), 8 weeks after subcutaneous implantation in nude rats. Scale bar = 100 µm; n = 3 independent donors.

VEGF-BMSC stimulate osteoclast recruitment

In order to analyse bone resorption, tartrate-resistant acid phosphatase positive (TRAP+) osteoclastic cells were visualized. In naïve- and CD8-BMSC grafts, few TRAP+ cells were detected in contact or in close proximity to bone matrix (Figure 3.7A-D). However, constructs containing VEGF-overexpressing MSC showed a highly increased density of TRAP+ cells (Figure 3.7E-F).

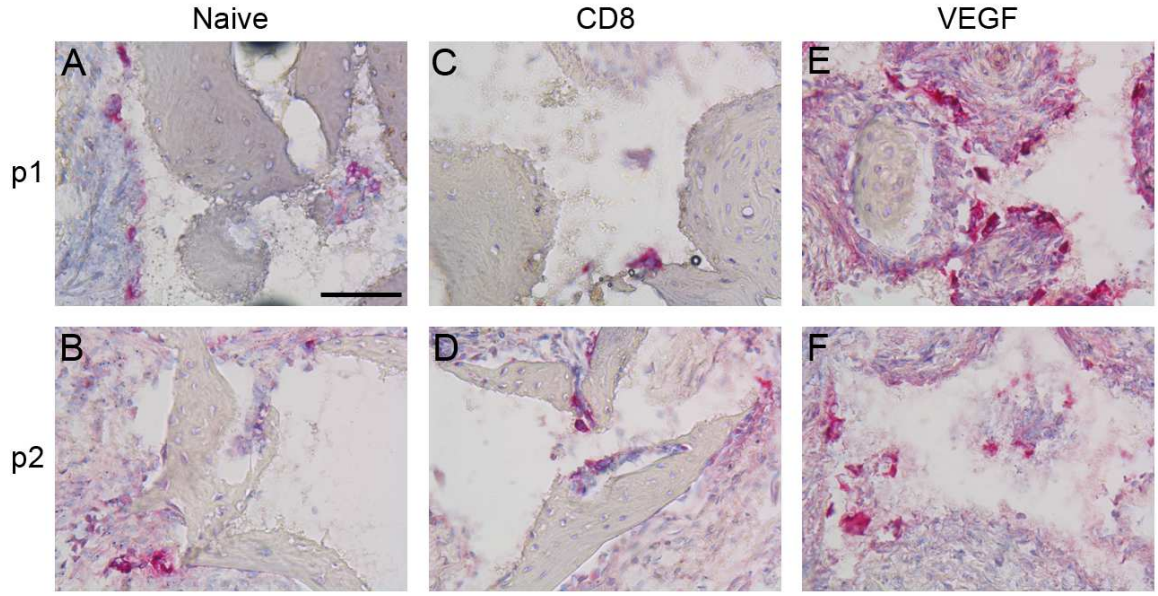

Figure 3.7 Osteoclast activity. Histochemical stain for TRAP activity (red) and counterstaining with haematoxylin (blue) were performed on sections of silicated apatite granule pellets seeded with naïve BMSC (A-B), CD8-BMSC (C-D) or VEGF-BMSC (E-F), expanded for either 1 (p1) or 2 passages (p2), 8 weeks after subcutaneous implantation in nude rats. Scale bar = 100 µm; n = 3 independent donors.

3.4 DISCUSSION

In this study we aimed at improving vascularization of tissue-engineered bone grafts by genetically modifying human BMSC to achieve sustained over-expression of VEGF. Our results confirmed that angiogenesis was significantly increased by this approach, leading to a 2- to 3-fold increase in vessel length density compared to naïve cells.

Interestingly, VEGF over-expression caused not only an increase in the number of vessels, but also a qualitatively different composition of the vascular supply. In fact, grafts seeded with control cells contained mainly small arteries, which efficiently carry blood flow (conductance vessels), but whose thick smooth muscle coating prevents metabolic exchanges with the tissue. VEGF-BMSC, on the other hand, generated physiologically structured vascular networks, composed of a few arterioles feeding into a large number of pericyte-covered capillaries, whose thin walls allow efficient exchange of respiratory gases and nutrients. Only this second kind of vascular bed is optimal to sustain a high metabolic demand by growing tissue.

The improvement in vascularization was not diminished by extensive *in vitro* expansion of the transduced BMSC up to 35 population doublings, showing that genetic modification conferred a stable angiogenic potential. As expected, these expanded BMSC lost their osteogenic potential (43, 44). However, their sustained capacity to induce vascularization could be useful in other applications, where effective expansion of the vascular bed is required, but not progenitor differentiation, such as in cell-based approaches for therapeutic angiogenesis in peripheral or coronary artery diseases.

By minimizing cell expansion in order to maintain the osteogenic differentiation potential of BMSC, we observed a strong reduction in bone formation specifically when VEGF was over-expressed. In fact, CD8-BMSC, which underwent similar retroviral vector transduction and FACS-purification, generated bone tissue as efficiently as naïve BMSC. A reduced amount of bone may result from decreased bone formation, either by impairment of BMSC engraftment or of their osteogenic differentiation potential, or from increased resorption.

In situ hybridization for the human specific Alu sequence demonstrated that VEGF-MSC survived similarly to naïve cells and that implanted cells could be found embedded in

the bone matrix in osteocyte position in all conditions. Previous results by our group showed that VEGF over-expression did not reduce osteoblastic differentiation of retrovirally transduced human BMSC *in vitro* (109). Furthermore, an impairment of BMSC osteogenicity would likely lead to abortive bone formation, with areas of dense matrix that fails to mineralize. However, the bone generated by VEGF-BMSC was fully mature and similar to that formed by control cells, only reduced in quantity. On the other hand, VEGF over-expression specifically caused a greatly increased density of TRAP-positive osteoclasts, which could be found at the surface of bone areas. Taken together, these results suggest that VEGF over-expression caused a reduction in the amount of bone tissue rather through increased resorption than by impaired cell engraftment or differentiation.

Bone formation and bone homeostasis are highly connected with angiogenesis. VEGF upregulation is crucially required in osteochondral bone formation (6, 110). In addition, it has been shown that sustained VEGF over-expression promotes an increase in bone mass through direct stimulation of osteoblasts (81). A large number of studies found a positive effect of VEGF on bone regeneration (111-113), while others did not observe any alteration in the osteogenic capacity of rabbit BMSC over-expressing VEGF after AAV transduction (114). On the other hand, Schonmeyr et al. recently showed that VEGF can inhibit BMP2 expression by rat BMSC in an autocrine and paracrine manner (115). Furthermore, VEGF is a chemo-attractant for osteoclast progenitors in developing bone, supporting *in vitro* osteoclast differentiation and enhancing osteoclast survival and bone resorption (116, 117). In light of the sometime contradictory results reported in literature, the relationship between VEGF expression, vascularization, bone formation and bone resorption is certainly complex and likely to be context-dependent. In the model of ectopic implantation used in the present study, which allows the study of the intrinsic bone-forming capacity of the implanted cells without the influence of an osteogenic environment, our results suggest that VEGF over-expression impairs bone formation by unbalancing bone homeostasis towards disproportionate resorption. Further investigations, for example in osteoclast-deficient models like the op/op mouse (118), will be needed to conclusively prove this hypothesis.

It should be considered that some specific features of the model we used could affect the relationship between VEGF expression and osteogenicity and these could provide directions to explore in order to preserve the positive effect on vascularization while avoiding the negative effect on bone formation. These parameters are the duration of VEGF expression, its dose and the presence of an osteogenic environment.

VEGF expression is required only for about 4 weeks in order to generate persistent vessels (93, 94) and it is possible that continued expression beyond this time, as it happens from the constitutive retroviral promoter that was used here, is not necessary for vascularization, but only provides a continued stimulation of osteoclast recruitment. The use of inducible promoters, which can be switched off by systemic treatment with a drug (119), could address this point.

Further, the transduced BMSC we used expressed heterogeneous levels of VEGF, as a result of the random integration of retroviral vectors in genomic areas with different transcriptional activities. As normal angiogenesis can be induced over a wide range of VEGF levels up to a defined threshold dose (93), controlled expression at moderate levels might induce effective vascularization while limiting osteoclast recruitment. This could be achieved by using BMSC populations FACS-purified to homogeneously express specific VEGF levels, as we recently showed (109, 120).

Lastly, subcutaneous implantation in an ectopic environment is a well-controlled experimental model. However, in physiological bone homeostasis mechanical load provides crucial stimulation of anabolic osteoblastic activity and, in fact, lack of muscle activity causes severe bone rarefaction and osteoporosis (121). Therefore, it is possible that implantation in an orthotopic model, where the graft is subjected to the same mechanical load as the natural bone, the balance between bone formation and resorption might be restored.

Bone tissue engineering using bone marrow-derived stromal/stem cells is a promising technique for the treatment of bone defects. The lack of rapid angiogenesis, however, constitutes a major obstacle in the engineering of tissues of clinically relevant size. Therefore, several approaches to increase neovascularisation, including the supply of VEGF, are currently under investigation (37, 113). Our data suggest that an equilibrium

exists between VEGF-triggered angiogenesis, osteogenesis and bone resorption, which needs to be fully understood and controlled for a clinical application.

4 SUMMARY & FUTURE PERSPECTIVES

Bone transplantation is an absolute requirement in several pathological conditions. An ideal bone substitute in comparison to allografts, xenografts, synthetic materials or autografts would be tissue engineered by autologous progenitors. Due to their ease of procurement, *in vitro* expandability and multi-lineage differentiation potential, patient-derived MSC are an attractive autologous cell source for regenerative medicine. However, any tissue which is more than 200 µm thick needs a vascular supply (75). This also applies to the field of tissue engineering and other regenerative medicine approaches, where the lack of rapid vascularization is a major obstacle, leading to ischemia followed by necrosis in the centre of the grafts.

Vascular Endothelial Growth Factor (VEGF) is the master regulator of physiological vascular growth and is commonly used as a therapeutic transgene for the induction of angiogenesis. However, uncontrolled and high levels of VEGF expression can lead to aberrant vascular growth (93, 98). To achieve controlled expression *in vivo*, a high-throughput flow cytometry-based method has previously been developed in our group. Linking the VEGF cDNA to a cell-surface marker (a truncated version of CD8a) in a bicistronic construct enabled the rapid purification of genetically-modified myoblasts secreting a desired VEGF level, using FACS sorting based on the intensity of CD8 expression in each cell (100, 101).

The aims of this thesis were to adapt this method to human adipose tissue- and bone marrow-derived mesenchymal stromal/stem cells (ASC and BMSC), and to apply these in a bone tissue engineering approach to increase the vascularization potential of osteogenic grafts.

As MSC gradually loose their regenerative potential during *in vitro* expansion, we first optimized our genetic engineering method for MSC, so as to enable high transduction efficiency and FACS-purification with minimal *in vitro* manipulation. **Chapter 2** describes the generation of an optimized protocol allowing routine transduction efficiencies of > 90% of primary human ASC and BMSC already during the first plating, as well as flow cytometry purification of transduced cells at the time of the first passage. In addition we demonstrated that it was possible to FACS-purify specific sub-populations of transduced MSC homogeneously producing desired VEGF doses. Neither

retroviral vector transduction, FACS-purification, nor the expression of the transgenes VEGF and CD8 impaired MSC proliferation and *in vitro* differentiation potential. Transgene expression was not lost during *in vitro* differentiation.
Taken together, these data demonstrate that the technique previously developed to generate myoblast populations expressing specific VEGF levels was successfully adapted to primary human MSC derived from adipose tissue and bone marrow, thereby allowing *in vitro* manipulation to be minimized. Hence, we developed a platform, which could be employed for a broad range of regenerative medicine approaches.

In **Chapter 3**, proof-of-principle was obtained by applying this platform to a bone tissue engineering approach. Human BMSC, transduced and rapidly FACS-purified to eliminate non-expressing cells, were seeded onto hydroxyapatite granules to generate non-critically sized constructs, and were implanted subcutaneously in nude rats. *In vivo* vascularization potential was significantly increased in VEGF-expressing BMSC. Although VEGF expression was heterogeneous, no aberrant angiogenesis was observed. Indeed, orderly vascular beds were induced, with flow-conducting arterioles feeding into extensive capillary networks, where metabolic exchanges can take place efficiently.
Thanks to our optimized transduction technique and rapid FACS purification, constructs could be generated with minimal expansion of the genetically modified BMSC for no more than 2 passages. In these conditions, we observed a severe reduction in the amount of produced bone specifically in consequence of VEGF over-expression, and not of retroviral transduction or FACS purification. This correlated with an increased recruitment of TRAP-positive osteoclasts specifically in VEGF-expressing constructs.

These data suggest that VEGF over-expression might impair bone formation by disrupting the balance between bone formation and resorption towards excessive degradation. To fully understand the underlying mechanism, further experiments will be needed.

4.1 PERSPECTIVES IN BONE REPAIR

4.1.1 IDENTIFICATION OF A SUITABLE VEGF DOSE

Since the platform we developed allows FACS-purification of MSC expressing specific VEGF levels, the effect of different VEGF concentrations on *in vivo* bone formation can be studied without changing the number of VEGF-expressing cells. In this way the extent of the increase in angiogenesis could be controlled and the effect of microenvironmental VEGF dose on bone formation could be investigated. Thus such tests could reveal either a linear relationship between increase in VEGF expression and decrease in bone formation, or identify a threshold between normal and reduced bone formation due to specific VEGF levels. If bone formation was reduced only at VEGF levels higher than those needed to induce angiogenesis, increased vascularization could be obtained without impaired osteogenicity. Varying the number of VEGF-expression hotspots by co-seeding naïve and VEGF-BMSC at different ratios, therefore manipulating the total VEGF dose, could discriminate the role of paracrine and autocrine actions of VEGF within the bone graft (115).

A 3D *in vitro* bone organ model previously developed by a collaborator allows the process of bone turnover to be mimicked (122). This osteoblastic-osteoclastic-endothelial cell co-culture system, which has been used together with non-invasive monitoring techniques, could be used to identify the coupling of vascularisation with the stage of osteoblastic differentiation of the progenitor cells and osteoclastic activity *in vitro*.

4.1.2 SCALE-UP OF THE GRAFT SIZE

Sample analysis at different time-points during the *in vivo* graft ossification could give insight into the dynamics of ongoing processes, including angiogenesis, bone formation and osteoclast recruitment/bone resorption, as well as their chronological relationship. Furthermore, an inducible expression system (119) would allow the switching-off of

VEGF expression at different time-points, so that long-term consequences of short-term VEGF-expression could be analysed. For this experimental setting, it should be taken into account that the process of vessel ingrowth needs to take place as rapidly as possible after graft implantation in order to protect the centre of the construct from ischemia and necrosis. Therefore, it would be fundamental to analyse the speed and depth of vascular invasion into constructs with a size of clinical relevance (e.g. $1cm^3$), as well as the depth of cell survival and bone formation inside those grafts, and to determine their dependency on the duration of VEGF expression. A minimum of 4 weeks of VEGF expression is required in order to induce stable persistent vessels in other tissues (93, 94) and it would be useful to determine whether this process may be faster in bone.

4.1.3 EXTENSION TO AN ORTHOTOPIC AND IMMUNOCOMPETENT ANIMAL MODEL

The experiments described here were performed in an ectopic implantation model, which allows the most controlled conditions to investigate the intrinsic potential of the implanted cells without the influence of the bone environment of orthotopic models. However, due to the presence of crucial stimulation by mechanical load, as well as of a more osteogenic and inflammatory environment, the implantation of the same grafts directly into the bone defect might favour osteoblastic differentiation and change the balance in bone homeostasis, possibly restoring bone formation (110, 123, 124).

An additional element that has to be noted is the use of a nude rat model, which lacks T-lymphocytes and therefore has a suppressed immune system, in order to avoid rejection of the human cells. As T-lymphocytes regulate osteoclastogenesis and recruit osteoblasts, this might have relevant consequences (123). On the other hand, it has been shown that several immunological surface antigens important for B-cell and T-cell recognition, including MHC-II, CD40, CD40L, CD80 and CD86, are not expressed on the surface of human MSC and that they have immunosuppressive actions (41). Therefore, the use of a xenogenic immunocompetent animal model would be an option to test human cells, even though current literature presents controversial results (125). Using

autologous cells and species-specific transgenes in an immunocompetent animal model, however, would come closest to the setting of a potential clinical application.

Based on these results, some of the questions mentioned above are being addressed in a recently initiated project in our group, aimed at increasing vascularization and bone formation in critical-size bone grafts in an ectopic rabbit model by syngenic VEGF-expressing autologous BMSC. To further stimulate vascular growth, bone tissue engineering using MSC overexpressing heterogenous VEGF-levels and flap pre-fabrication were combined by wrapping the constructs in the highly vascularised panniculus carnosus muscle. Kinetic studies on construct perfusion 1, 4 and 8 weeks after implantation indicate that VEGF expression accelerated perfusion of critical-size bone grafts and reduced the size of the ischemic central core. Complete vessel quantification and histological analysis for bone formation in the explanted grafts are currently ongoing.

4.2 PERSPECTIVES IN OTHER FIELDS OF REGENERATIVE MEDICINE

The method described in chapter 2 provides a general platform to generate populations of genetically modified MSC, expressing specific levels of a therapeutic transgene, already at the time of the first passage. Therefore, it has the potential to be applied in other fields of regenerative medicine, beyond bone tissue engineering. Here we briefly describe two recently initiated projects, based on the results described in this thesis, which aim at either promoting or inhibiting angiogenesis in order to improve cardiac function after myocardial infarction, or cartilage tissue formation, respectively.

In order to investigate cell-based delivery of controlled VEGF levels for safe and robust angiogenesis in the heart, human adipose tissue-derived stem cells (ASC) were transduced with the same retroviral vector construct described in Chapters 2 and 3 VEGF-expressing progenitors were then FACS-purified to generate populations producing either a specific (SPEC) or heterogeneous (ALL) VEGF levels. In a first trial, healthy nude rats underwent intramyocardial cell injection. Histological analysis 4 weeks post implantation revealed that both VEGF-producing groups induced robust angiogenesis, with a 2-3-fold increase in vessel density compared to control cells, despite very limited stable engraftment of injected cells. Nevertheless, heterogeneous VEGF levels caused numerous angioma-like structures, while the homogeneous expression of a specific VEGF dose generated only normal and stable angiogenesis. Following those proof-of-principle experiments, the study was extended to a nude rat model of myocardial infarction. A control group which received only PBS was added. After 4 weeks, the left ventricular ejection fraction (EF) was preserved by the treatment with SPEC VEGF cells (EF=+1.1%), but significantly worsened by treatment with ALL VEGF cells (-13.2%), control cells (-6.4%) and PBS injection (-8.3%). Vessel density was increased by 2- to 3-fold with both VEGF-producing cell groups. However, ALL VEGF cells caused the development of numerous aberrant angioma-like structures, while they were completely prevented by SPEC VEGF cells, which induced only normal and stable angiogenesis also in ischemic myocardium.

These results show that controlled VEGF delivery by FACS-purified ASC is effective to reliably induce only normal vascular growth and functional improvement in ischemic myocardium, opening up the exciting possibility of its clinical application to achieve safe and therapeutic angiogenesis to treat cardiac ischemia.

Unlike in bone and cardiac tissue, VEGF expression is suppressed during hyaline cartilage formation and maintenance, and vascular invasion actually causes destruction of the cartilagineous tissue. Therefore, in order to favour chondrogenic differentiation of mesenchymal progenitors *in vivo*, we hypothesized that it could be beneficial to block endogenous VEGF signalling with a soluble form of VEGF receptor-2 (sFlk-1). Human chondrocytes and BMSC, which are promising cell sources for cartilage regeneration, were transduced to express sFlk-1 using the protocol described in chapter 2. Chondrogenic differentiation capacity was investigated in a pellet culture system *in vitro*. *In vivo* chondrogenesis and the control of angiogenesis were analyzed after seeding on collagen-based scaffolds and ectopic implantation in nude mice.
In vitro, sFlk-1 appeared to have no effect on chondrogenic differentiation of either chondrocytes or BMSC. *In vivo*, blood vessel ingrowth was significantly reduced in the engineered tissues formed by sFlk-1-expressing cells, compared to naïve and control cells tranduced with an empty vector. Blocking of angiogenesis significantly enhanced chondrogenesis *in vivo* by human chondrocytes, but was found to be actually critical to allow any cartilage formation by BMSC *in vivo*. In fact, no chondrogenesis was observed in any condition except in the sFlk-1-expressing constructs.

In conclusion, this work proposes a technological platform to control vascularization in several tissue engineering and regenerative medicine approaches, either to stimulate or to inhibit angiogenesis. The thesis also provides an exemplification of this approach in the context of bone repair and discusses further extensions in different therapeutic areas.

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Curriculum Vitæ : Uta Helmrich

Flachsländerstr. 11
CH-4057 Basel
Tel: 0041 787082218
helmrichu@uhbs.ch

Date of birth: 09/11/1982
Nationality: German

EDUCATION

August 2007 - present	**PhD thesis** Laboratry of Cell and Gene Therapy (Dr. Andrea Banfi) Institute for surgical research and hospital management (ICFS) Thesis title : "VEGF-expressing mesenchymal stem cells for controlled angiogenesis in regenerative medicine – a bone tissue engineering approach " **University of Basel (Switzerland)**
October 2002 - March 2007	**Diploma in Biology (corresponding to Master of Biology)** • Main subject: Human Molecular and Clinical Genetics • 1st minor subject: Immunology • 2nd minor subject: Human Physiology Diploma thesis Laboratry of Dr. Jacques Mallet Laboratoire de Génétique Moléculaire de la Neurotransmission et des Processus Neurodégénéraifs (LGN/CNRS-UMR 7091) Thesis title: "Inhibition of neovascularisation through RNA interference as a gene therapeutic approach to treat the exudative form of age releated macular degeneration"
July 2006 – March 2007	**grade: very good** **Eberhard Karls Universität Tübingen (Germany)/Paris (France)**
July 2001 – June 2002	**"Cambridge Certificate in Advanced English"** **Dublin (Ireland)**
June 2001	**A-levels** with the main subjects Biology/German **Staatliches Gymnasium "Leuchtenburg" Kahla (Germany)**

EMPLOYMENT AND PRACTICAL TRAININGS

September – December 2009	**Tutor**, "Introduction into Biology" for 1st year Biology students University of Basel (Devision of Biochemestry) **Basel (Switzerland)**
September - November 2004 and March - July 2005	**Teaching assistant**, "Biology for medical students" Eberhard Karls Universität Tübingen **Tübingen (Germany)**
August 2004	"Forensic Entomology **Training**" guided by Mark Benecke **Cologne (Germany)**
July - September 2002	**Internship** in leucemia diagnostics Institute for Genetics - Campus Charité **Berlin (Germany)**

ORAL PRESENTATIONS AT CONGRESSES AND MEETINGS

June 2011	Helmrich U, Melly L, Christ L, Güven S, Scherberich A, Heberer M, Martin I, Banfi A. VEGF-expressing MSC for rapid vascularization of tissue-engineered bone grafts. TERMIS EU Conference 2011, Granada (Spain)
April 2011	Melly L, Marsano A, Helmrich U, Heberer M, Eckstein F, Carrel T, Giraud MN, Tevaearai H, Banfi A. Controlled angiogenesis in the heart. ISHLT 31st Annual Meeting and Scientific Sessions, San Diego (CA, USA)
December 2010	Melly L, Marsano A, Helmrich U, Heberer M, Eckstein F, Carrel T, Giraud MN, Tevaearai H, Banfi A. Safe and efficient angiogenesis in the heart by controlled VEGF expression**.** 2nd EACTS Meeting on Cardiac and Pulmonary Regeneration , Vienna (Austria)
June 2010	Marsano A, Helmrich U, Bernegger P, Schaeren S, Martin I, Banfi A. VEGF blockade by soluble VEGF Receptor-2 improves the *in vitro* chondrogenic capacity of human mesenchymal stem cells and nasal chondrocytes. TERMIS EU Conference 2010, Galway (Ireland)
October 2008	Helmrich U, Wolff T, Scherberich A, Müller A, Heberer M, Martin I, Banfi A. Controlled angiogenesis by FACS-purifiable engineered progenitors expressing specific VEGF levels. 14th Cardiovascular Biology and Clinical Implications Meeting, Muntelier (Switzerland)
June 2008	Misteli H, Fueglistaler P, Wolff T, Gianni'Barrera R, Helmrich U, Gurke L, Heberer M, Banfi A. Controlled angiogenesis by rapid FACS-purification of transduced progenitors expressing defined VEGF levels. TERMIS EU Conference 2008, Porto (Portugal)
October 2007	Helmrich U, Wolff T, Scherberich A, Müller A, Martin I, Banfi A. Controlled angiogenesis by rapid FACS-purification of progenitors expressing defined VEGF levels. 13th Cardiovascular Biology and Clinical Implications Meeting, Muntelier (Switzerland)

PUBLICATIONS

In preparation	Helmrich U, Marsano A, Güven S, Groppa E, Heberer M, Scherberich A, Martin I, Banfi A. VEGF-expressing mesenchymal stem cells for rapid vascularization of tissue-engineered bone grafts.
Submitted	Helmrich U, Marsano A, Melly L, Wolff T, Christ L, Heberer M, Scherberich A, Martin I, Banfi A. Generation of human MSC expressing defined VEGF levels by optimized transduction and flow cytometry purification. *Tissue Engineering*
Submitted	Melly L, Marsano A, Helmrich U, Heberer M, Eckstein F, Carrel TP, Giraud MN, Tevaearaic HT and Banfi A. Controlled angiogenesis in the heart by cell-based expression of specific VEGF levels. *Cardiovascular Research*
March 2011	Wolff T, Mujagic E, Gianni-Barrera R, Fueglistaler P, Helmrich U, Misteli H, Gurke L, Heberer M, Banfi A. FACS-purified myoblasts producing controlled VEGF-levels induce safe and stable angiogenesis in chronic hind limb ischemia. *Journal of Cellular and Molecular Medicine*