

Endothelialized tissue engineered 3D-construct for bone repair

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Thomas Meury
aus Basel, BS

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Genehmigt von der Philosophisch-Naturwissenschaftlichen Fakultät auf
Antrag von

Prof. Dr. Ueli Aebi

Prof. Dr. Michael Primig

Prof. Dr. Ranieri Cancedda

Prof. Dr. Mauro Alini

Basel, den 5. Juli 2005

Prof. Dr. Hans-Jakob Wirz
Dekan

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Chapter I

General Introduction

Bone tissue engineering – where we are – the problems – the future

Bone has an amazing self-healing capacity and is one of the very few tissues in the adult body that can heal itself without leaving scar tissue. One of the reasons why bone heals well by itself (i.e. compared to articular cartilage) is its high level of vascularization. Nevertheless, there are certain injuries where the normal healing capacity of bone is not sufficient, like in large fracture gaps, non-unions or after tumor resection. A promising way to heal these kinds of defects is by using tissue-engineered grafts. In bone tissue engineering, the tissue defects or gaps are filled with constructs (grafts) that will be integrated into the surrounding bone matrix and will eventually be replaced by newly formed bone through the process of bone remodeling. Vascularization of the bone graft is essential, as already proven by Cutting et al. in 1983 when he showed that vascularized autologous bone grafts were superior to unvascularized grafts [1]. In fact, according to a review by Brandi, the importance of the bone vascular system was already recognized by Albrecht von Haller in 1763 (though not in conjunction with bone repair), when he stated that “the origin of bone is the artery carrying the blood and in it the mineral element” [2]. Later, Trueta et al. showed that bone vascularization is not only important for calcium homeostasis, but also plays a pivotal role during bone tissue regeneration, mostly by supplying the damaged tissue with oxygen, nutrients, growth factors and precursor cells [3, 4]. Insufficient vascularization is one of the main problems encountered when dealing with critical-size full-thickness bone defects. The present methods to heal these kinds of large defects combine long-term (external or internal) fixation of the fracture with implantation of an autologous or allogenic bone graft. Since allografts bring up immunological as well as ethical problems, autologous bone grafting is to date the golden standard in orthopedic, oral- and plastic surgery. But despite the obvious advantages of autografts, like its capacity for osteoconduction as well as -induction and restricted immune reaction, there are also significant drawbacks, like its limited availability (largely depending on the amount and shape of the autograft), as well as the induction of a secondary defect at the donor site, followed by possible infection and morbidity at the donor site [5, 6]. Therefore alloplastic grafts are becoming more and more interesting as an alternative to autologous bone grafts. The obvious advantages are safety, virtually unlimited supply and optimal adaptation to a specific situation.

Common components of an alloplastic graft include a scaffold, cells and biological factors.

The scaffold

The scaffold principally provides the structural basis of a graft. But the demands on a scaffold that could replace autologous grafts go much further than that. An ideal scaffold should be biocompatible, biodegradable, porous, permeable to incoming cells, chemicals and blood vessels, as well as supportive, inert, non-immunogenic, and of course it should be easily available. Tissue engineered scaffolds should act as temporary matrices. Eventually, blood vessels will invade the scaffold, new tissue will be formed and the scaffold will slowly be resorbed by osteoclasts. The new tissue will be continuously remodeled and will ultimately be reorganized into normal, healthy tissue [7, 8]. Commonly used scaffolds are divided into natural scaffolds and synthetic (alloplastic) scaffolds. Natural scaffolds (i.e. based on collagen or hyaluronic acid) are biodegradable, biocompatible and do not result in harmful degradation products. However, some of them also have serious drawbacks like limited cell adhesion, possible immunogenic response, inhibition of endothelial proliferation (hyaluronic acid, HA) [9], structural weakness (fibrin glue) [10] or sub-optimal release of incorporated growth factors (alginate, agarose) [11, 12]. Examples of ways to overcome some of these problems at least partially include linkage of RGD-sequences to HA [13] or heparin addition to alginate, agarose and fibrin glue [10, 14]. Synthetic scaffolds that have been used in tissue engineering include ceramics (i.e. β -tricalcium phosphates, hydroxyapatites), polymers (i.e. polyurethanes PU, poly-L-lactic acids PLA, poly-D,L-lactic acids PGLA, poly-glycolic acids PGA) and combinations. Problems with these kinds of scaffolds include insufficient biodegradability (hydroxyapatite), hydrophobicity (PLA) [15], excessive leakage (PLGA) [16] or potential toxicity of degradation products [15].

Polyurethane scaffolds

Polyurethanes (PU) are polymers containing a characteristic $-\text{NH}-\text{CO}-\text{O}-$ linkage in the chain. PU consists of flexible soft- and stiff hard-segments. Stable PU have been used successfully in many medical devices, including many kinds of prostheses, tubings, catheters and blood bags [17]. Recently, efforts have been made to design non-toxic biodegradable PU to be used in tissue engineering by introducing labile units (non-aromatic compounds) into the stable PU chains [18]. The more elastic material properties of polyurethanes allow a mostly frictionless integration into the host tissue. The controllable degradation rate and hydrophilic/hydrophobic ratio allows for optimal adaptation to the situation [19]. More hydrophilic PU can be used as adhesion barriers, while more hydrophobic PU attract proteins and support the attachment and growth of cells. Those PU can be used as biodegradable scaffolds for bone tissue engineering. A recent study using polymers in the regeneration of bi-cortical defects of the iliac crest in sheep have shown promising results [20].

The cells

The main task of cells in tissue engineering is to produce a cell distribution and matrix composition similar to the healthy *in vivo* situation. Implantation of an empty osteoconductive scaffold will attract cells into the scaffold that eventually will form new bone tissue. This approach works quite well for smaller defects (up to 3cm), and can be drastically accelerated by seeding suitable cells into the scaffold before implantation [21-29]. In bone tissue engineering, one obvious possibility would be to preseed scaffolds with mature autologous culture-expanded osteoblasts. However, harvesting a piece of bone induces a severe secondary defect at the donor site. Furthermore, there are different types of bone (cortical, cancellous) containing different cell populations, which are at different maturation stages. A more promising strategy is the use of mesenchymal stem cells (MSC) which can be easily isolated from bone marrow aspirates using a much less invasive harvesting procedure. In addition, the use of mesenchymal stem cells would allow the recapitulation of natural bone formation and repair. Isolated MSC from bone marrow aspirates could be seeded directly into the scaffold or, if needed, be expanded *in vitro* before being seeded into the scaffold. Alternatively, one could induce a defined mature phenotype by stimulation of MSC with suitable differentiation factors *in vitro*, before seeding them into the scaffold.

However, the limited long-term viability of these cells after implantation of the graft is a limiting factor. The cells have to survive in an inhospitable environment until neo-vascularization takes place, which will enable oxygen and nutrient supply to reach them. This is the biggest problem of a large defect (>3cm). Therefore, accelerated vascularization of critical-size bone grafts would be of great clinical value. One approach to improve neo-vascularization could be to optimize the properties of the scaffold (i.e. chemical composition, linker proteins or sequences, pore size, pore linkage). Another approach could be to seed the scaffolds with an optimized cell-factor mixture prior to implantation, to promote vascular formation within the graft.

Mesenchymal stem cells (MSC) in vitro

Stem cells are defined through their self-renewability (their division-capacity) and their capacity to generate specialized cells. There are various types of stem cells, differing in the time-point of their appearance during the development of an organism and in their versatility. Pluripotent stem cells can give rise to all adult cell types. One example is embryonic stem cells (ES), which derive from very early embryos (inner layer of the blastocyst). These cells theoretically proliferate indefinitely in culture while retaining the potential to differentiate into virtually any cell type [30]. Stem cells collected from older embryos or even adult tissue are more restricted. They have limited proliferation capacity and generally differentiate within

their tissue of origin (i.e. within the mesenchymal or hematopoietic system). Recent studies have proposed trans-differentiation between different cell types of different germ layers, but this topic is very controversial and still highly under discussion [31, 32]. Due to the current legal and moral discussions about the usage of ES in clinical and therapeutic applications, adult stem cells are, at this time, the precursor cell pool of choice.

In the bone marrow, hematopoiesis is supported by the stromal system [33, 34], which consists of multipotent precursors for many mesenchymal cell types (called mesenchymal stem cells, MSC) as well as reticular endothelial cells and macrophages [35-39]. In *in vitro* experiments, marrow-derived cells were able to support and maintain hematopoietic cells when used as a feeder layer, which underlines the hematopoiesis-supporting task of the stromal component of bone marrow [33, 40]. The bone marrow stromal system has been shown to be highly regenerative after high doses of irradiation, chemotherapy or marrow ablation in young patients (<4yrs), although in older patients, the same treatments will result in serious damage [41]. This is one of the reasons why it is still under discussion whether the adult stromal system contains true stem cells, or just a very heterogeneous mixture of lineage-specific undifferentiated precursor cells. MSC are recruited to their tissue of origin through the blood circulation, where they differentiate into a specific mature cell type under the influence of the local microenvironment. An extensive transdifferentiation potential between mature cell types within the mesenchymal cell family has recently been shown, replacing the earlier belief that mesenchymal stem cells were restricted to a certain cell type once they have reached their tissue of fate and are differentiated into a mature cell [42].

In 1970, Friedenstein was the first to isolate MSC by plating bone marrow-derived cells on plastic, where the adherent fraction would form fibroblastic colonies (each colony originating from one single stromal cell clone). These colonies were termed colony-forming unit fibroblastic (CFU-F) [43]. Under the right culture conditions, they could give rise to osteoblasts (CFU-O) and chondrocytes (CFU-C) [44-49] as well as to other mesenchymal cell types including adipocytes [50] and tenocytes [51]. Aubin and others have shown that single CFU-F can be very heterogeneous in size, morphology and differentiation potential [37, 52, 53]. Studies looking at the three main mesenchymal lineages (osteogenic, chondrogenic, adipogenic) have shown that the MSC population consisted of tripotential (A30%), bipotential (osteo-chondrogenic (60-80%), but no osteo-adipogenic or chondro-adipogenic) and unipotential (osteogenic, no chondrogenic or adipogenic) precursors. Interestingly, their chondrogenic and adipogenic but not osteogenic potential was lost over culture time [50, 54]. This suggests that the adult stromal system consists of a very heterogeneous precursor population containing just a small fraction of possibly pluripotent stem cells, but a large fraction of pre-committed mesenchymal precursors of varying

differentiation potential, and that the default cell lineage of these precursors is the osteoblastic one. Another fact supporting the hypothesis that MSC contain no or very little real stem cells is that MSC can be expanded almost indefinitely (>260 population doublings) when transformed with the telomerase gene [55], but they reach senescence after 35-40 population doublings when untransformed [56]. Also, their progenitor properties are gradually lost with extensive culture expansion [57]. Addition of b-FGF to MSC cultures has been shown to enhance their proliferative potential while keeping the cells in a more undifferentiated state. This was demonstrated by an increased Stro-1 expression (a marker prominently expressed by CFU-F), a decreased number of ALP-positive clones, a longer preservation of the typical fibroblastic phenotype and the potential to differentiate into osteoblasts, chondrocytes or adipocytes even after extensive expansion [58-60].

Today, bone marrow aspirate is considered to be the most enriched and easily accessible source of mesenchymal precursors from which MSC can quickly be isolated, expanded and differentiated into various lines *in vitro*. Other possible sources of mesenchymal precursors include trabecular bone [61], blood [62], periosteum [63, 64], dermis [65], muscle [66, 67], fat [68] and the synovial membrane [69]. These alternative sources of MSC are, however, less used due to lower MSC concentrations than that found in the bone marrow, as well as to more complex and less standardized harvesting, expansion and differentiation methods.

The terms mesenchymal stem cells (MSC) and bone marrow stromal cells (BMSC) are generally used quite interchangeably even though BMSC and MSC are not exactly the same. Both cell pools are believed to derive from the same ancestor, but MSC appear more undifferentiated and show a more homogenous and fibroblastic cell phenotype, while BMSC are less homogenous and show fibroblastic as well as stromal (hematopoiesis-supporting) characteristics and might include endothelial cells and macrophages [40, 70, 71].

Blood vessels – endothelial cells

Blood vessels are part of the circulatory system, transporting blood to and from almost any part of the body. This is achieved by branching of the macrovasculature (arteries, veins) into microvasculature (arterioles, venules) and finally into capillaries. The capillaries serve to redistribute blood and its nutrients whilst lowering the pressure head, allowing blood to diffuse into the tissue and thereby allowing better nutrient distribution. The blood vessels found in bone are mainly microvascular vessels and capillaries. Capillaries have an inner lining of endothelial cells, which provides a surface that prevents blood cell attachment and thrombus formation. This endothelial layer is surrounded by pericytes that share a common basal lamina with the endothelial cells [72]. The basal lamina is approximately 40-50 nm thick and serves as a separation and isolation layer between the connective tissue and the

endothelium. It is composed of the lumina lucida (adjacent to the endothelial cells and made of the proteoglycan laminin and type IV collagen) and the lumina densa (made of type VII collagen). In addition to pericytes, microvascular vessels are surrounded by smooth muscle cells and fibroblasts. Pericytes and smooth muscle cells are responsible for expansion and contraction of the vessels. Pericytes have also been suggested to serve as osteoblastic precursors [73, 74].

Blood vessel formation can take place by three distinct processes: vasculogenesis, angiogenesis and arteriogenesis [75]. Vasculogenesis is the *de novo* vessel-forming process that takes place during early embryonic development. Endothelial cells differentiate from their precursors, the angioblasts, and proliferate within a previously avascular tissue to form a primitive tubular capillary network. Vasculogenesis is followed by angiogenesis, during which this initial vascular network is remodeled into more complex networks through vessel enlargement, sprouting, and bridging. Another (non-sprouting) angiogenic mechanism is the intussusceptive microvascular growth (IMG), during which existing vessel lumens are divided by formation of interstitial tissue folds. Arteriogenesis is the process of structural enlargement and remodeling of preexisting small arterioles into larger collateral vessels. There are many biological factors known to be involved in the molecular mechanisms of all these processes. Some of these factors have been known for a long time and are not specific for blood vessel formation (FGF, PDGF, TGF-) [75-78], while others are believed to be more specific and critical. Those include members of the vascular endothelial growth factor family (VEGF) and of the angiopoietin family [79-81]. During bone remodeling and repair, new microvascular vessels invade the injured site mainly through the process of angiogenesis.

Bone cell – endothelial cell interactions

Sufficient vascularization is very important for normal bone turnover and repair [82]. Endothelial cells are located inside the blood vessels forming a single layer, adhering tightly to the basement membrane of the vessel's wall, providing a non-thrombogenic surface to which platelets and other blood cells fail to adhere. Furthermore, they mediate the passage of nutrients and other solutes across the blood-tissue barrier. In bone development, the appearance of osteoblasts coincides with blood vessel invasion [83]. This suggests that endothelial cells could be a major source of modulators of bone development, turnover and repair. This hypothesis is also supported by the fact that the replacement of the cartilage during endochondral bone formation is accompanied by the capillary invasion from the mineralized cartilage, following the release of angiogenic factors from the hypertrophic chondrocytes [84, 85]. Guenther et al. were the first to report *in vitro* interactions between endothelial cells and bone cells by showing that bovine aortic endothelial cells produce a

mitogen for rat calvarial cells [86]. Several EC-produced factors that affect bone cells and *vice versa* have been discovered and several studies covering the topic of endothelial cell – osteoblastic cell interactions have been performed over the last few years [87-96]. Nevertheless, the outcomes of these studies were very diverse and experiments were often performed only over a rather short time period (for a more detailed discussion see chapter II), leaving many unknowns about the long-term effect of such interactions, especially in a 3D environment.

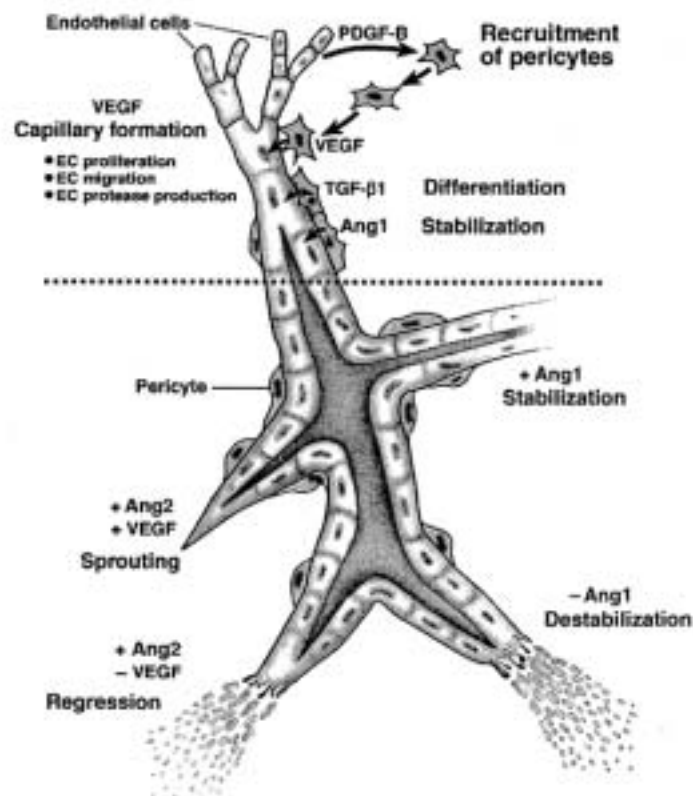
The biological factors

At a fracture site, one of the first elements to appear are platelets. This is followed by platelet activation, aggregation and clotting. Chemotactic factors are released attracting various types of cells. Neutrophilic granulocytes are usually the first cells to enter the wound site, followed by monocytic phagocytes (which differentiate into macrophages at the site of inflammation), then fibroblasts, osteoblasts, vascular endothelial cells and various precursor cells [97]. The factors that are produced by platelets during the very early phase of bone repair include FGF, PDGF, IGF, TGF- α , TGF- β and VEGF [98-101]. Local administration of exogenous TGF- β 1, TGF- β 2, PDGF-BB or b-FGF at fracture sites results in improved bone formation and/or chondrogenesis [102-109]. It is understood that the formation of new tissue is very dependent on the local dose and timing of these growth factors and cytokines.

VEGF and angiopoietin

VEGF is the best-characterized factor active during vascularization and angiogenesis. It induces hyperpermeability of vessels [110], degradation of the surrounding extracellular matrix and allows endothelial (precursor-) cell-recruitment, migration and reorganization into sprouting tubules (this complicated process involves also FGF, PDGF and TGF- β). Adult blood vessels are stabilized through angiopoietin-1 (Ang1) by binding to (and therefore activating) its receptor Tie2. During angiogenic remodeling, for example as a response to hypoxia [111] or tissue rupture, angiopoietin-2 (Ang2) is up-regulated and binds Tie2 receptor, thereby preventing Ang1 from binding. The action of Ang2 depends largely on VEGF. Ang2 mediated vessel destabilization can lead to endothelial cell death and vessel regression in the absence of VEGF, or to vessel sprouting in the presence of VEGF [112]. Matrix metalloproteinases also play a pivotal role in the matrix degradation during vessel sprouting [113]. The new vessel tubules are then remodeled and covered by pericytes, fibroblasts and potentially smooth muscle cells, which form a new extracellular matrix of basal lamina in response to TGF- β . The vessels then enter a quiescent state. Ephrins, even

though initially characterized in the nervous system, have recently been shown to also play a role during angiogenic remodeling. They are most probably involved in the establishment of the venous versus the arterial system [114].



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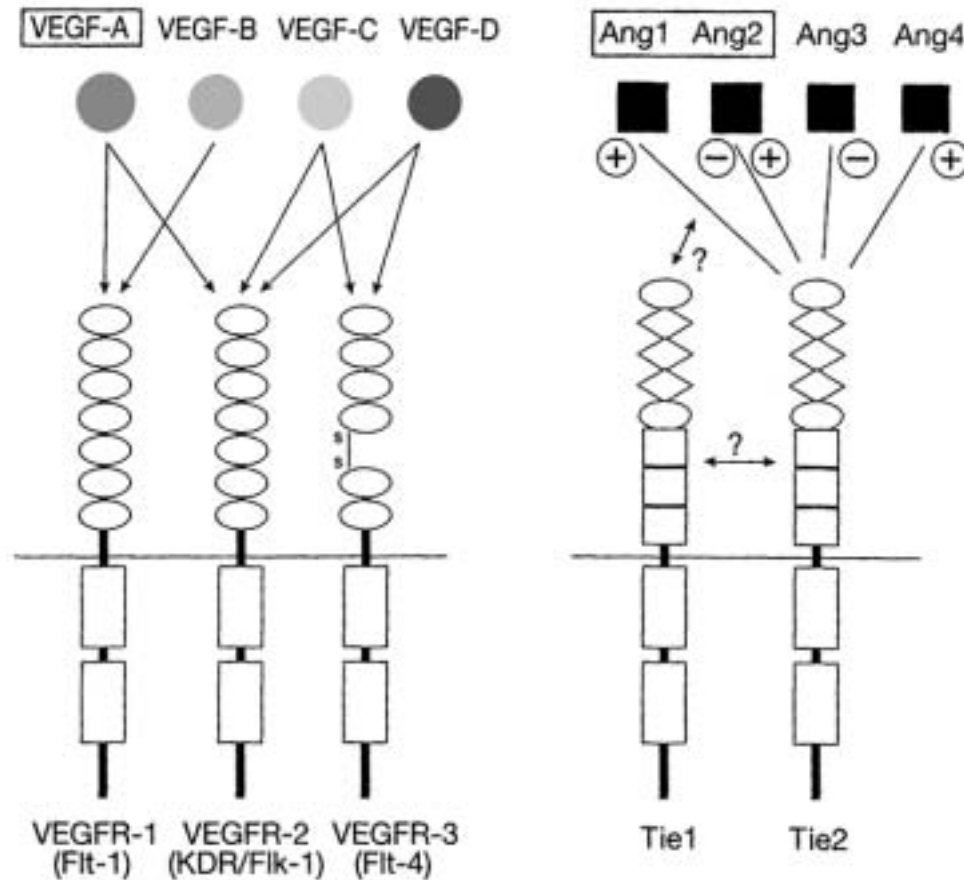
Ramsauer M, D'Amore P, Getting Tie(2)d up in angiogenesis, *J Clin Invest*, 110:1615-1617 (2002)

The factor generally (and therefore also in this thesis) referred to as VEGF is correctly named VEGF-A and is a member of a family also containing VEGF-B, -C, -D and placenta growth factor (PLGF). VEGF is by far the most studied and understood member of this family. VEGF-B seems to be involved in coronary vascularization and growth, but its role is not really understood yet [115]. VEGF-C and -D are believed to regulate lymphatic angiogenesis [116]. Through alternate mRNA splicing, four different isoforms of VEGF are produced: VEGF₁₂₁, VEGF₁₆₅, VEGF₁₈₉ and VEGF₂₀₆ with 121, 165, 189 and 206 amino acids after signal sequence cleavage [117]. VEGF₁₆₅ is the predominant isoform [118]. The 165, 189 and 206 splice variants have heparin-binding domains, which help to anchor them in the extracellular matrix (ECM). These domains are also responsible for the higher mitogenic activity of VEGF₁₆₅, VEGF₁₈₉ and VEGF₂₀₆ compared to the non heparin-binding isoform VEGF₁₂₁ [119]. VEGF₁₂₁ is freely diffusible, while VEGF₁₈₉ and VEGF₂₀₆ are almost

completely sequestered in the extracellular matrix. VEGF₁₆₅ has intermediate characteristics: A fraction of it is freely diffusible; the other fraction is bound to the cell surface and the extracellular matrix [120]. These observations suggest that VEGF₁₆₅ has an optimal combination of bioavailability and mitogenic potential and indeed, it is the main active VEGF during angiogenesis [117].

VEGF can bind to several different cell surface tyrosine kinase receptors. VEGF binds to VEGFR1 (formerly known as Flt-1) and VEGFR2 (Flk-1/KDR). VEGF-B and PLGF bind only to VEGFR-1. VEGF-C and -D bind to VEGFR-2 and -3 (Flt-4). In addition there are a number of accessory receptors such as the neuropilins, which are believed to be mainly involved in modulating the binding to the main VEGF receptors [121]. VEGFR2 seems to mediate the growth and permeability actions of VEGF whereas VEGFR1 is suggested to have a negative role (decoy receptor), but its exact function is still under debate [122]. Interestingly, VEGFR2 binds VEGF with lower affinity than VEGFR1 [123-125]. There is also a soluble splice variant of VEGFR1 (sFlt-1), which has been shown to compete with the binding of VEGF to VEGFR1 [126]. Mice lacking VEGFR2 fail to develop a vasculature and show low numbers of endothelial cells, while mice lacking VEGFR1 show excess formation of endothelial cells with abnormal tube formation [127, 128]. Another VEGF-receptor, VEGFR3, seems to be critical for the development of the lymphatic vessel system [129].

VEGF works in close relation with other factors, especially with angiopoietins. Angiopoietins are ligands for a family of receptor tyrosine kinases called Tie. The two most important angiopoietin family members are Ang1 and Ang2, both binding to Tie2. Knockout experiments have shown that mouse embryos lacking Ang1 or Tie2 developed a rather normal primary vasculature (unlike VEGF knockouts), but this vasculature failed to undergo further remodeling. Over-expression of Ang1 in skin resulted in a modest increase in vessel number, but in a significant increase in vessel size as well as in resistance to leak [130]. On the other hand, VEGF over-expression primarily increased the number of vessels, but these were leaky [131]. Over-expression of both factors resulted in an unprecedented hypervascularity resulting from increased vessel number and size. Interestingly, the vessels were still resistant to leak (induced by VEGF or inflammatory agents) [130]. Ang2 plays a very confusing role by being able to act as an agonist or antagonist to Tie2. The suggested role of Ang2 is a signal for destabilization of the vessel during the initiation of angiogenesis. It has been suggested that autocrine Ang2 has a different effect than paracrine Ang2 and that the effect of this factor depends largely on the presence of other factors including VEGF or Ang1 [132-134].



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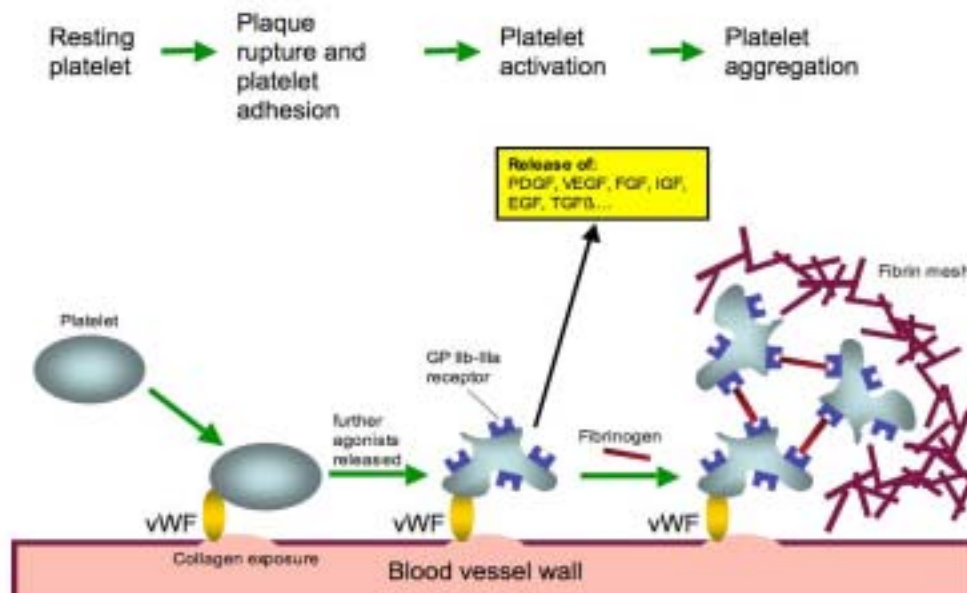
Yancopoulos et al., *Vascular-specific growth factors and blood vessel formation*, *Nature* 407:243 (2000)

The importance of VEGF during coupling of bone formation with cartilage resorption was demonstrated by Gerber et al., who showed that blocking VEGF results in suppression of blood vessel invasion, connected with impaired trabecular bone formation and expansion of the hypertrophic chondrocyte zone [135]. More recent *in vivo* data indicates that VEGF is not only important for bone vascularization, but also plays a role in the differentiation of hypertrophic chondrocytes, osteoblasts and osteoclasts [136, 137]. VEGF is secreted by many cell types including hypertrophic chondrocytes, osteoblasts and osteoblast-like cells and its expression is influenced by many cytokines, growth factors and hormones (i.e. IGF-1, PGE1, PGE2, Vit.D3, PTH, TGF- β , BMP-2, -4, -6) [93, 138-141]. Several groups have reported a direct effect of VEGF on osteoblastic cells (stimulation of migration, proliferation and differentiation) [93, 142, 143], however opposite findings suggest that further studies are necessary to resolve this controversy [91, 141]. Since angiopoietins have been discovered a few years later than VEGF, their exact role in angiogenesis and bone formation is less

understood. Ang1 and 2 have been detected in similar regions within bone as VEGF (cartilage-bone interface, remodeling sites). They are also expressed by various cell types including osteoblasts, osteoclasts and marrow space cells [144]. Angiopoietins do not seem to have a direct mitogenic effect on endothelial cells, even though the Tie2 receptor becomes activated.

Platelet-rich plasma (PRP)

Platelet-rich plasma (PRP) is autologous plasma with a platelet concentration around five times higher than that found in blood. It also contains an increased number of growth factors in their natural ratios upon platelet activation. Known growth factors released by activated PRP include PDGF-AA, PDGF-AB, PDGF-BB, TGF- β 1, TGF- β 2, VEGF and EGF [145]. The obvious advantage of PRP over the use of recombinant growth factors is the fact that it can be produced from the patient's blood intraoperatively. Recombinant human growth factors are usually synthesized by Chinese hamster ovarian cells transfected with human genes, and are delivered at non-physiological doses. In addition, while PRP forms a gel that is a prerequisite for growth factor delivery, recombinant growth factors are delivered using a synthetic carrier or a carrier processed from animal proteins. The PRP gel, in addition to containing the platelet released growth factors, will also be able to entrap cells, thus making PRP an easy and inexpensive source of autologous matrix and biological factors.



Platelet activation cascade: Platelets adhere to ruptured vessel walls by binding to von Willebrand factor and are activated upon exposure to collagen. The release of agonists like thrombin or ADP (generated at the sites of vascular trauma) results in a conformational shape change of the platelets, followed by degranulation, and the release of many growth factors and cytokines. The platelets then bind to circulating fibrinogen through their GP IIb-IIIa receptors to form large platelet-fibrin aggregates.

The key molecular players in osteogenesis

In a classic study, Urist described ectopic bone induction by intramuscular implantation of demineralized bone matrix (DBM) in rabbits and rats. This was a key discovery, which stimulated the search for a bone-inducing substance in the bone matrix. Subsequent investigations demonstrated that low-molecular weight proteins could be extracted from demineralized bone matrix [146]. These proteins showed more osteogenic activity than DBM, and were therefore termed bone morphogenetic proteins (BMPs) [147-150]. Today, several signaling molecules in addition to BMPs are known to play a role in controlling skeletal development, including other members of the transforming growth factor family (TGF- β), FGF, secreted factors of the Wnt-family and members of the hedgehog family [151-153]. The following paragraphs will describe the proteins and transcription factors involved in osteoblastic differentiation that will be investigated and evaluated in this thesis:

BMP: Around 20 genes coding for BMPs have been identified so far, and several recombinant BMPs have been shown to have a unique activity, which is to induce ectopic bone formation [147, 154-156]. Osteoblasts secrete BMP into their matrix during bone formation. BMPs (except for BMP-1 which is a peptidase) are members of the TGF- β superfamily, known to regulate cell proliferation, differentiation and death in various tissues [154, 157-160]. Many studies confirmed the hypothesis that BMPs are involved in the regulation of osteoblastic and chondrocytic differentiation by identifying skeletal abnormalities in individuals with mutations in BMP genes [161-167]. The mechanism of ectopic bone formation by recombinant BMPs was investigated using osteoblastic differentiation in *in vitro* experiments. Several BMPs enhance typical osteoblastic markers like ALP, type I collagen, osteocalcin and MMP-13 [168]. In addition, BMPs stimulate the formation of mineralized bone-like nodules *in vitro* [169].

Runx2: Runx2 (Cbfa1) is the α -subunit of a heterodimeric transcription factor consisting of Runx2 and the β -subunit Cbfa1. Runx2 is a structural homologue of the runt-gene product in *Drosophila*. It contains a conserved region (the Runt domain), which is required for dimerization with the β -subunit as well as for binding to OSE2 (osteoblast-specific cis-acting element), first discovered in the mouse osteocalcin gene 2 promoter (OG2) [170, 171]. There are two more α -subunits, Runx1 and Runx3, but none of them seems to be involved in osteoblastic differentiation and bone formation [172, 173]. In Runx2-null mice, the osteoblastic differentiation is completely blocked [174]. Cbfa1-null mice show much less deficiency in bone formation than Runx2-null mice, indicating that Runx2 can control bone formation to a limited degree in the absence of Cbfa1 [175, 176]. Runx2 is not osteoblast-specific; it also plays a role in the differentiation of hypertrophic chondrocytes [174, 177]. Over-expression of Runx2 in chondrocytes results in an accelerated endochondral

ossification [178]. Regulation of osteoblastic differentiation by members of the TGF-superfamily (i.e. TGF- 1 and BMP-2) is believed to act through interactions of Smads with Runx2 [179].

In summary, Runx2 is required for early osteoblastic differentiation as well as hypertrophic chondrocyte differentiation.

Osterix: Osterix (Osx, SP7) is a zinc-finger containing transcription factor highly specific for osteoblasts [180]. Osx-null mice show normal cartilage development but completely lack bone formation: A dense mesenchyme emerges in the zone of hypertrophic chondrocytes accompanied by blood vessels and osteoclasts. However, the mesenchymal cells are blocked in their differentiation. Arrested differentiation in these cells was shown by significantly reduced type I collagen expression, as well as undetectable osteoblastic markers including osteonectin, osteopontin, bone sialoprotein and osteocalcin. Interestingly, Runx2 expression levels were normal. This observation, together with the finding that Runx2-null mice showed no Osx transcripts suggests, that Osx and Runx2 are part of the same osteoblastic differentiation pathway and that Osx acts downstream of Runx2. This is also supported by studies comparing Runx2-null and Osx-null mice. In Runx2-null mutants, hypertrophic chondrocyte differentiation is inhibited and there is no invasion of the hypertrophic zone by blood vessels and osteoclasts. In Osx-null mutants though, hypertrophic chondrocyte differentiation is not impaired and the hypertrophic zone is invaded by blood vessels and osteoclasts, but osteoblastic differentiation does not occur. Interestingly, Runx2-null mutants show a significant decrease in VEGF expression by chondrocytes, which could explain the lack of blood vessel invasion into the hypertrophic cartilage zone of these mutants.

Based on the studies described above, a model for osteoblastic differentiation was proposed [181]: Mesenchymal progenitor cells differentiate first into preosteoblasts under the control of Runx2/Cbf . These preosteoblasts are bipotent, thus have the ability to differentiate into either chondrocytes or osteoblasts upon the right stimuli. However, when Osx is turned on, these bipotent cells are driven towards the osteoblastic pathway, while if Sox-9 is turned on they will differentiate into the chondrogenic lineage. Interestingly, studies comparing the effect of Sox9 inactivation before and after chondrogenic mesenchymal condensations have indicated that expression of Sox9 was required for the establishing of bipotential osteo-chondro-progenitor cells [182]. Once the chondrogenic mesenchymal condensation was completed, Sox9 was no longer needed for the osteoblast lineage, and Osx was suggested to be the negative regulator of Sox9. So Osx seems to be the regulator that segregates the osteoblastic from the chondrogenic differentiation pathway.

Dlx5: Dlx5 (distal-less homeobox 5) is a bone inducing transcription factor that is expressed in differentiating osteoblasts [183]. Dlx5-null mice show severe craniofacial abnormalities with delayed ossification and of the cranium and abnormal osteogenesis [184]. Interestingly, it has been shown that BMP-2 induction of Osterix expression is mediated by Dlx5 (and not by Runx2) [185].

Osteoblastic markers

Progression of osteoblastic differentiation is currently best described by the temporal expression of bone matrix proteins [186]. The relevant markers investigated in this thesis are quickly introduced below:

Collagen type I (COL1): Type I collagen is the major organic component of the bone matrix and is found in mineralized and non-mineralized areas. Interestingly, the collagen in mineralized tissue aligns differently from that in non-mineralized tissue to leave space for the apatite crystals [187, 188]. Type I collagen is secreted by osteoblasts prior to matrix mineralization [189] and is one of the early markers during osteoblastic differentiation [190].

Osteocalcin (OC): OC (earlier name bone GLA-protein) is the major non-collagenous protein in bone (10-20%). It's synthesized only by mature osteoblasts, therefore represents a late marker of osteogenesis [52, 190, 191]. OC is generally found in mineralized tissue and is a very useful marker to follow the osteoblastic differentiation in the rat model, although in human it is less useful [192] (see also our findings in chapter II).

Osteopontin (OP): OP is a phosphorylated glycoprotein expressed very early in bone cell differentiation and again later following mineralization. OP is highly abundant in bone, but can also be found in hypertrophic chondrocytes, kidney and in very small amounts in the brain and lungs. OP binds very firmly to hydroxyapatite and probably plays a role in the attachment and spreading of bone cells (it contains an RGD sequence) [52, 190, 193].

Osteonectin (ON): ON is a calcium and hydroxyapatite-binding glycoprotein that is also found in the lung, ovaries and other tissues. However, it is highly abundant in bone and a common marker of osteoblastogenesis [190, 191, 194].

Bone Sialoprotein II (BSP): BSP is a phosphorylated bone specific glycoprotein mainly found in mineralized tissue. It appears late during osteogenesis [52, 190, 191, 195, 196].

Collagenase 3 (MMP-13): The matrix metalloproteinases (MMPs) are a family of proteinases that are able to degrade extracellular matrix components (in particular collagens) [197]. MMP-13 was discovered in 1994 in connection with breast carcinomas [198] and was later found to be a major player during endochondral bone formation by efficiently degrading type II collagen, but also aggrecan and type I and IV collagens. MMP-13 is under the control

of the transcription factor Runx2 and is expressed as a late differentiation marker in osteoblasts [199].

ALP: Alkaline Phosphatase is a cell-surface ectoenzyme that hydrolyses monophosphate esters. Its precise physiological role is not completely clear yet. One important function is to cleave phosphate groups from OP, ON, OC and BSP to make them available for the process of matrix mineralization. ALP activity rises in early stages of culture, peaks before the onset of mineralization and decreases after mineralization (not expressed in osteocytes) [200, 201].

Matrix mineralization: Matrix mineralization is most often measured by $^{45}\text{Ca}^{2+}$ -isotope incorporation into the matrix and by Van Kossa staining [202]. Determination of calcium incorporation is a very powerful tool to judge osteoblastic differentiation not only because it is a very late event in osteogenesis but also because it measures the performance of an active “bone-forming” process.

Thesis aims

The overall goal of this PhD thesis was to define a construct to be used in bone tissue engineering that has the potential to improve *in vivo* vessel formation within the construct.

Bone healing is always in very close association with vessel formation and ingrowth [3, 4, 203-208]. It has even been shown, that bone formation is blocked, if endothelial invasion is inhibited [209]. We were therefore interested in the involvement of endothelial cells in bone formation.

- ~ **Our first aim was to study the interactions of endothelial cells (EC) and mesenchymal stem cells (MSC) to investigate the hypothesis that EC influence MSC differentiation into an osteoblastic phenotype, and that this effect is modulated by soluble factors and/or direct contact.**

One of the first events in a fracture is the formation of a blood clot to prevent further bleeding. The platelets in these clots then release factors and cytokines responsible for cell recruitment and other initial events during early fracture repair (see section “The biological factors”). Platelet-rich plasma (PRP) has been suggested to have the potential to improve bone healing.

- ~ **Our second aim was to test the usability of PRP as a source of autologous biological factors and if it could stimulate MSC differentiation into an osteoblastic phenotype.**

Bone defects of less than 3cm can usually be healed using autologous bone grafts. However, the grey zone is defects between 3 and 8cm, which are still a major clinical problem. Surgeons have tried to use scaffolds made of synthetic or natural materials that promote the migration, proliferation and differentiation of bone cells. However, these approaches have been shown to be only limited successful. Furthermore, the absence of an appropriate blood vessel network in such large defects is an additional concern.

- ~ **Therefore, our third and last aim was to develop a construct based on the outcome of aims 1 and 2, and to evaluate its potential to form vessel-like structures *in vitro*, as well as its capacity to induce MSC differentiation into osteoblasts.**

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Chapter II

Human endothelial cells inhibit human MSC differentiation into mature osteoblasts *in vitro* by interfering with Osterix expression

Meury TR, Alini M.

Our first experiments were based on the initial hypothesis of this PhD:

MSC differentiation into osteoblasts is modulated by interactions with EC

To investigate this hypothesis, we decided to use 2D co-culture systems for simplicity and reproducibility reasons. Two non-direct contact co-culture systems were chosen (trans-wells and conditioned medium). Direct contact co-cultures were left out due to the difficulty of culturing EC and osteoblastic cells in the same culture medium for long term without addition of growth factors or cytokines that enhance EC survival, and due to the difficulty in separating contributions of each cell type when analyzing markers.

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Introduction

Mesenchymal stem cells (MSC) derive from the adherent, mononucleated fraction of bone marrow [1]. It has been shown, that this cell pool contains precursors for several mesenchymal tissue cells including osteoblasts, fibroblasts, chondrocytes, adipocytes and myoblasts [2-4]. Bone marrow stroma is actually suggested to be the main source for osteoblastic precursors [3]. Recruitment, proliferation and differentiation of MSC into mature osteoblasts are regulated by many factors including cytokines, systemic hormones, growth factors and other regulators [5]. These factors are released by the osteoblastic cells themselves, but also by cells that are part of the tightly connected vascular system, such as endothelial cells [6-8] or pericytes [9]. Endothelial precursors can be isolated from bone marrow, as well as from peripheral blood [10;11]. During embryonic development, the major vascular network is formed by vasculogenesis, mediated mainly by factors belonging to the vascular endothelial growth factor (VEGF) family [12]. VEGFs bind to several receptors including Flt-1 (VEGFR-1) and KDR (VEGFR-2 or Flk-1) that have been identified on many cells types, including endothelial and osteoblastic cells [13-15]. VEGFs promote vascular endothelial cell proliferation and induce vascular leak and permeability, which allows the initial network to be remodeled by angiogenic sprouting [16;17], a process in which the immature and poorly functional vascular network is remodeled into a complex network of mature and stable blood vessels [18]. It is widely accepted that there must be communication between endothelial cells and osteoblastic cells in order to regulate blood vessel formation, osteoblast differentiation and bone turnover, processes that are all very tightly connected [19-24]. *In vivo* and *in vitro* experiments suggest a paracrine communication between endothelial cells and osteoblastic cells using gap junctions to mediate the angiogenic process required for bone formation and repair [25]. VEGF is believed to play an important role in these processes [6;9;26-29]. It was shown, in a mouse model, that angiogenesis, bone formation and callus mineralization in femoral fractures do not occur, if VEGF activity is inhibited [8]. *In vitro* studies have shown that VEGF has a stimulatory effect on osteoblastic differentiation [30], however it does not seem to have a direct effect on osteoblastic precursor cell proliferation [7;31]. VEGF is definitely not the only factor involved in these communication processes. There are several other factors that are expressed and secreted during bone healing and that can affect osteoblastic cells or endothelial cells, including PDGFs, TGFs, FGFs, angiopoietins and endothelins [32-35].

In vitro studies about the influence of endothelial cells on osteoblastic differentiation have shown a remarkable variety of results [6;7;9]. These differing results are partly due to the different cell (lines) and culture conditions used as well as due to different donor age and biopsy site. In addition, we hypothesize that the differentiation state of endothelial cells, following stimulation with different factors, could play an important role on how they influence MSC differentiation. In the present study, we have grown endothelial cells with or without VEGF stimulation and investigated their effect on MSC differentiation towards the expression of an osteogenic phenotype.

Materials and Methods

Origins of human cells

Bone marrow aspirates were taken from patients undergoing routine orthopedic surgery involving iliac crest exposure, after informed consent. Bone marrow aspirates (20mL) were harvested into CPDA-containing Sarstedt monovettes using a biopsy needle that was pushed through the cortical bone. Aspirates were processed within 12-24 hours after harvesting.

Isolation and expansion of human MSC

Bone marrow aspirates were homogenized by pushing them a few times through a syringe. The aspirate was then diluted 1:4 with IMDM (Gibco 42200-022) containing 5% (v/v) FBS (Gibco 10270-106) and centrifuged at 200g for 5min at room temperature (RT). The top layer (approx. 1cm) containing mostly fat tissue was removed. Per 1mL of undiluted sample, 2.6mL of Ficoll (Histopaque-1077, Sigma #1077-1) were pipetted into a 50mL Falcon tube and the aspirate was added carefully on top of the Ficoll. After centrifugation at 800g for 20min at RT, the mononucleated cells were collected at the interphase using a syringe. To 1mL of collected interphase solution, 5mL of IMDM/5%FBS were added, the tube was gently mixed and centrifuged at 400g for 15min at RT. The pellet was resuspended in the same amount of IMDM/5%FBS, centrifuged again and resuspended in IMDM/5%FBS. Cell number was determined using Methylene Blue in a hemocytometer. The cells were seeded at densities of $8-10 \times 10^6$ mononucleated cells per 150cm² T-flask in IMDM containing 10% FBS, nonessential aminoacids (Gibco 11140-035) and PenStrep (100U/mL, Gibco 15140-122). After 5 days, the monolayers were washed with Tyrode's balanced salt solution (TBSS) to remove non-adherent cells, and fresh medium containing 5ng/mL b-FGF (R&D 233-FB) was added as previously reported [36-38]. Medium was changed every 2-3 days and cells were subcultured 1:3 at subconfluence. The adherent cells after one subculture were termed mesenchymal stem cells (MSC). Only cells between passages 2-4 (approx. 12-18 populations doublings) were subsequently used [39].

Expansion of HUVEC

Primary Human Umbilical Vein Endothelial Cells (HUVEC) were obtained from Cascade Biologics (cat# C-003-5C). The cells were expanded in IMDM containing 10%FBS, nonessential aminoacids, PenStrep, 20ug/mL ECGS (Sigma E-

2759) and 90ug/mL heparin (Sigma H-3149). Only cells between passages 2-4 were subsequently used.

Stimulation of HUVEC with VEGF

HUVEC were expanded in IMDM containing 10% FBS, nonessential aminoacids, 20ug/mL ECGS, 90ug/mL heparin and PenStrep. At about 75% of confluency, 25ng/mL VEGF (Sigma V-7259) were added and the cells were stimulated for 3 days [8]. The HUVEC monolayers were then either used to produce HUVEC-conditioned medium (see *Conditioned medium (VEGF stimulated or non-stimulated) on MSC cultures*) or were trypsinized and used for indirect contact co-cultures.

MSC cultures

MSC were seeded in 24-well plates (Falcon BD 353504) at densities of 10'000 to 30'000 cells/well in 1mL of medium and were left to attach for 2-3 hours. Culture medium for the MSC culture systems contained IMDM, 10% FBS, nonessential aminoacids, 0.1mM ascorbic acid-2-phosphate (Sigma A-8960) and 10mM β -glycerophosphate (Sigma G-6251) with 10nM dexamethasone (osteogenic medium) or without dexamethasone (non-osteogenic medium). Dexamethasone was purchased at Sigma (D-2915). Media were changed twice a week.

Conditioned medium (VEGF stimulated or non-stimulated) on MSC cultures

HUVEC-conditioned medium was produced by washing the VEGF stimulated (see section "*Stimulation of HUVEC with VEGF*") or non-stimulated HUVEC monolayers 2x with phosphate buffered saline (PBS) and then culturing them for 24h in IMDM containing 10% FBS and PenStrep. After 24h, the medium (defined as VEGF-stimulated or non-stimulated HUVEC-conditioned medium) was removed, filtered (0.22 μ m), and stored at -20°C until used. This HUVEC-conditioned medium was added in a 1:10 mixture to MSC monolayers cultured in osteogenic or non-osteogenic medium in 24-well plates. Chondrocyte-conditioned medium (Chondro-CM) using primary bovine chondrocytes and fibroblast-conditioned medium (Fibro-CM) using the human hTERT-BJ1 fibroblast cell line were also similarly prepared and applied accordingly.

Indirect contact cultures of MSC and HUVEC (VEGF stimulated or non-stimulated)

To MSC monolayers, VEGF stimulated (see section "*Stimulation of HUVEC with VEGF*") or non-stimulated HUVEC were added in cell culture inserts (0.4 μ m

pore size, Falcon BD 353495) at a density of 10'000 cells/insert. Fresh HUVEC were added once a week to the system to provide a fresh supply of endothelial cell secreted factors. These experiments were also performed in osteogenic or non-osteogenic medium.

Recovery Experiment

MSC were grown in osteogenic (or non-osteogenic) medium supplemented 1:10 with non-stimulated HUVEC-conditioned medium, as described above. At different time points, medium supplementation with HUVEC-conditioned medium (non-stimulated) was stopped, and the MSC were grown in osteogenic (or non-osteogenic) medium. On day 27, the osteogenic differentiation stage of MSC was evaluated by determining matrix mineralization and OSX gene expression.

Inhibition experiment

MSC were grown in osteogenic medium (non-osteogenic medium as negative control). At different time points, the medium was switched to osteogenic medium containing non-stimulated HUVEC-conditioned medium (1:10). After 28 days, the osteogenic differentiation was evaluated by determining ALP activity and OSX gene expression.

DNA quantification

The protocol used for DNA quantification was based on the method described by Labarca et al. [40], which involves the binding of Hoechst 33258 to the minor groove of the DNA double helix resulting in a measurable enhancement in fluorescence. MSC were digested by adding 0.5mL proteinase K (0.5mg/mL proteinase K in phosphate buffer containing 3.36mg/mL disodium-EDTA) solution directly to the wells and incubating the plates at 56°C for 1h. Thereafter, the digested cells were transferred to Eppendorf tubes to avoid evaporation and were further digested overnight at 56°C. After appropriate dilution with Dulbecco's phosphate buffered saline (DPBS) containing 0.1% (v/v) H33258 (from 1mg/mL stock, Polysciences Inc, 09460), the samples were measured using a PE HTS 7000 Bio Assay Reader at 360nm excitation and 465nm emission wavelength.

Quantification of matrix mineralization using $^{45}\text{Ca}^{2+}$ isotope

1.25 $\mu\text{Ci/mL}$ of $^{45}\text{Ca}^{2+}$ isotope (Amersham CES3) were added to each well and the plates were incubated at 37°C for 6h [41]. The medium was removed and the monolayers were washed 3x with IMDM to remove unincorporated $^{45}\text{Ca}^{2+}$. Then

0.5mL of 70% formic acid were added to each well and the plates were incubated at 65°C for 1h. The formic acid solution was then transferred to 3.5mL of scintillation liquid (OptiPhase HiSafe'3 by Perkin Elmer) and the amount of radioactivity was measured using a Wallac 1414 WinSpectral liquid scintillation counter.

Quantification of alkaline phosphatase activity

The medium was completely removed and the monolayers were washed once with PBS. The cell layers were extracted by addition of 500µL of 0.1% Triton-X in 10mM Tris-HCl (pH 7.4) and incubation at 4°C on a gyratory shaker for 2h (See Sigma Technical Bulletin Procedure No.104). ALP activity was measured colorimetrically by measuring the p-nitrophenol production during 15min incubation at 37°C with p-nitrophenyl phosphate as substrate (Sigma Kit No.104) on a Perkin Elmer Bio Assay Reader HTS 7000.

Quantification of VEGF amounts in the culture supernatant

Culture medium was collected and VEGF_(165,121)-protein content was measured using a DuoSet ELISA Development System for human VEGF by R&D Systems (DY293) on a Perkin Elmer Bio Assay Reader HTS 7000.

RNA Isolation and Reverse Transcription

Monolayers were extracted using 500µL of TRI-Reagent (MRC Inc. TR-118) with 5ul/mL of Polyacryl-carrier (MRC Inc. PC-152) for 10min at RT and transferred to Eppendorf tubes. 150µL of 1-Bromo-3-Choro-Propane were added, the tubes vortexed for 15sec and then centrifuged at 12'000g for 15min at 4°C. The colorless layer on the top was transferred into a new tube and 750µL of isopropanol were added, then the tube was centrifuged at 12'000g for 10min at 4°C. Sample supernatants were removed and pellets were washed in 1mL of 75% EtOH by vortexing and centrifuging at 10'000g for 5min at 4°C. Pellets were air-dried and re-suspended in 30µL of DEPC-treated water. After 15min at 60°C, tubes were immediately transferred to ice. The total RNA amount and purity was assessed by measuring the absorbance at 260nm and 280nm. Reverse transcription was performed using 1µg of total RNA sample, which was mixed with 2µL of 10x TaqMan RT Buffer (500mM KCl, 100mM Tris-HCl, pH 8.3), 4.4µL of 25mM magnesium chloride, 4µL of dNTP mixture (2.5mM of each dNTP), 1µL of random hexamers (50µM), 0.4µL of RNase inhibitor (20U/mL) and 0.5µL of MultiScribe Reverse Transcriptase (50U/µL) (all from Applied Biosystems); DEPC-treated water was added to bring the final reaction volume to 20µL. Reverse transcription was

performed using a Thermal Cycler 9600 by Applied Biosystems. cDNA samples were appropriately diluted with Tris-EDTA buffer before being used for real-time RT PCR.

Real-time RT PCR

Oligonucleotide primers and TaqMan probes were designed using the Primer Express Oligo Design software (Ver. 1.5, Applied Biosystems). The nucleotide sequences were obtained from the GenBank database and the probes were designed to overlap an exon-exon junction in order to avoid amplification of genomic DNA (Table I). Primers and probes for amplification of 18S ribosomal RNA, used as endogenous control, were from Applied Biosystems. All other primers and labeled TaqMan probes were from Microsynth (Balgach, CH). TaqMan probes were labeled with the reporter dye molecule FAM (6-carboxyfluorescein) at the 5' end and with the quencher dye TAMRA (6-carboxy-N, N, N', N'-tetramethylrhodamine) at the 3' end. The PCR reaction mixture contained TaqMan Universal PCR master mix without AmpErase UNG (Applied Biosystems), 900nM primers (forward and reverse), 250nM TaqMan probe, and 2Al of cDNA sample for a total reaction volume of 25Al. PCR conditions were 95°C for 10min, followed by 42 cycles of amplification at 95°C for 15sec and 60°C for 1min using the GeneAmp 5700 Sequence Detection System (Applied Biosystems, Foster City, CA). Relative quantification of mRNA targets was performed according to the comparative C_T method with 18S ribosomal RNA as endogenous control (ABI PRISM 7700 Sequence Detector User Bulletin 2, PE Applied Biosystems 1997).

Van Kossa staining

MSC monolayers were rinsed with TBSS. A silver nitrate solution (5%) was added and the cells were exposed to strong light for 20min. After rinsing 3 times with distilled water, the cells were incubated in fresh 5% sodium thiosulfate for 10min. After rinsing 3 times with distilled water, 0.1% nuclear fast red solution was added and the cells were incubated for 10min, before being washed again with distilled water. Samples were left in distilled water and pictures were taken immediately.

Statistical analysis

The data are expressed as mean±SEM unless stated otherwise. Statistics were performed using the non-parametric Mann-Whitney U-test, which compares the medians of two independent distributions. P < 0.05 was considered statistically significant.

Table I. Primers and Probes for Real-Time RT-PCR

Target Gene	Sequence (5' ~ 3')
Collagen I	
Forw	CCC TGG AAA GAA TGG AGA TGA T
Rev	ACT GAA ACC TCT GTG TCC CTT CA
Probe	CGG GCA ATC CTC GAG CAC CCT
Osteonectin	
Forw	ATC TTC CCT GTA CAC TGG CAG TTC
Rev	CTC GGT GTG GGA GAG GTA CC
Probe	CAG CTG GAC CAG CAC CCC ATT GAC
MMP-13	
Forw	CGG CCA CTC CTT AGG TCT TG
Rev	TTT TGC CGG TGT AGG TGT AGA TAG
Probe	CTC CAA GGA CCC TGG AGC ACT CAT GT
BMP-2	
Forw	AAC ACT GTG CGC AGC TTC C
Rev	CTC CGG GTT GTT TTC CCA C
Probe	CCA TGA AGA ATC TTT GGA AGA ACT ACC AGA AAC TG
Osteocalcin	
Forw	AAG AGA CCC AGG CGC TAC CT
Rev	AAC TCG TCA CAG TCC GGA TTG
Probe	ATG GCT GGG AGC CCC AGT CCC
Osteopontin	
Forw	CTC AGG CCA GTT GCA GCC
Rev	CAA AAG CAA ATC ACT GCA ATT CTC
Probe	AAA CGC CGA CCA AGG AAA ACT CAC TAC C
Runx2	
Forw	AGC AAG GTT CAA CGA TCT GAG AT
Rev	TTT GTG AAG ACG GTT ATG GTC AA
Probe	TGA AAC TCT TGC CTC GTC CAC TCC G
BSP II	
Forw	TGC CTT GAG CCT GCT TCC
Rev	GCA AAA TTA AAG CAG TCT TCA TTT TG
Probe	CTC CAG GAC TGC CAG AGG AAG CAA TCA

Osx, Sox 9, Collagen II and PPAR!:

Primers and Probes: Assay-On-Demand by Applied Biosystems
(Assays: Hs00541729_m1, Hs00165814_m1, Hs00264051_m1,
Hs00234592_m1)

Probes were modified at the 5' end with the FAM fluorescent dye (6-carboxyfluorescein) and at the 3' end with the TAMRA fluorescent dye (6-carboxy-N,N,N',N'-tetramethylrhodamine). Assay-On-Demand probes had a non-fluorescent quencher at the 3' end.

Results

Bone marrow aspirates from seven human donors (age range 44-66 years) were obtained. Each experiment was done with cells from at least three different donors. Due to the high variability in the osteogenic potential of MSC between donors, some data are shown from one representative experiment, although, the same trends were observed in all three experiments, and they are summarized in Table II. The MSC used for the experiments were expanded using b-FGF to increase the cell number while keeping them in a tripotential state capable of osteogenic, chondrogenic and adipogenic differentiation [36-38].

Table II. Summary of the mean data observed in each single experiment

Col 1 gene expression	day 24 rel to MSC in OM		
Condition	A	B	C
MSC in osteogenic medium (OM)	1.00	1.00	1.00
MSC in OM with HUVEC	0.59	0.59	0.73
MSC in OM with HUVEC (VEGF)	0.49	0.54	0.52

ON gene expression	day 24 rel to day 1 and to MSC in OM		
Condition	A	B	C
MSC in osteogenic medium (OM)	1.00	1.00	1.00
MSC in OM with HUVEC	0.28	0.52	0.69
MSC in OM with HUVEC (VEGF)	0.25	0.20	0.65

BMP-2 gene expression	day 24 rel to day 1 and to MSC in OM		
Condition	A	B	C
MSC in osteogenic medium (OM)	1.00	1.00	1.00
MSC in OM with HUVEC	0.24	0.17	0.78
MSC in OM with HUVEC (VEGF)	0.16	not detectable	0.69

MMP-13 gene expression	day 24 rel to day 1 and to MSC in OM		
Condition	A	B	C
MSC in osteogenic medium (OM)	1.00	1.00	1.00
MSC in OM with HUVEC	0.55	0.01	0.99
MSC in OM with HUVEC (VEGF)	0.06	0.01	0.71

Indirect contact co-culture versus conditioned medium culture

Two different types of HUVEC-MSC culture systems were used; the first, using a trans-well system, allowed indirect 2-way communication between the two cells types; and the second, HUVEC-conditioned medium, allowed 1-way communication from HUVEC to MSC but not vice versa. Since no significant differences in the results could be observed between the two culture types, we will

not distinguish anymore between them, and we will generally discuss our results as: Effect of HUVEC on MSC differentiation.

Effect of HUVEC on MSC differentiation in non-osteogenic medium

In preliminary experiments, the effect of HUVEC on MSC differentiation when cultured in non-osteogenic medium was investigated. HUVEC did not show any effect on ALP activity or matrix mineralization of MSC compared to the control (no HUVEC). This suggested that HUVEC were not able to replace the positive effect of dexamethasone on MSC differentiation into osteoblasts in these *in vitro* culture systems (data not shown).

Effect of HUVEC on MSC differentiation in osteogenic medium (OM)

As a positive control, MSC were cultured in osteogenic medium without HUVEC. As expected, this led to an increase of several osteoblastic markers, such as matrix mineralization and ALP activity (Figures 1-3).

Furthermore, we observed an elevated expression of typical early and late osteoblastic marker genes including collagen 1 (COL1), osteonectin (ON), matrix metalloproteinase 13 (MMP-13), and bone morphogenetic protein 2 (BMP-2), when compared to MSC cultured in non-osteogenic medium (Figure 4a-d).

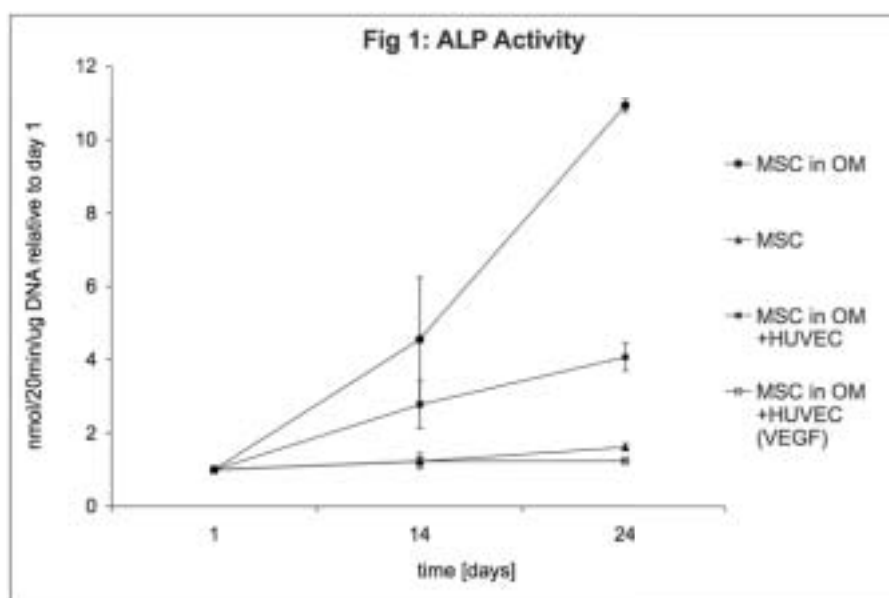


Figure 1: Representative graph of ALP activity of MSC: MSC grown in osteogenic medium (OM) showed a clear increase in ALP activity compared to MSC in non-osteogenic medium. HUVEC significantly ($P < 0.005$) reduced the increase in ALP activity of MSC. VEGF-stimulated HUVEC further reduced ALP activity ($P < 0.05$). Statistical analyses were performed for day 24 ($n=6$).

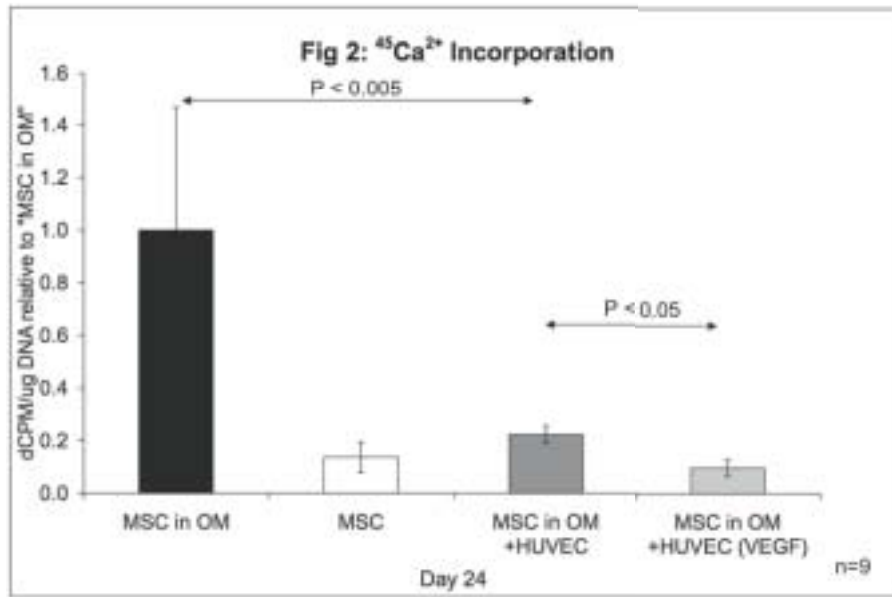


Figure 2: ⁴⁵Ca isotope incorporation of MSC at day 24 of culture (n=9): MSC grown in osteogenic medium (OM) showed high matrix mineralization, while MSC in non-osteogenic medium did not. Cultures of MSC and HUVEC showed a significant decrease in matrix mineralization when compared to MSC grown alone. This inhibitory effect was enhanced, when HUVEC were previously stimulated with VEGF.

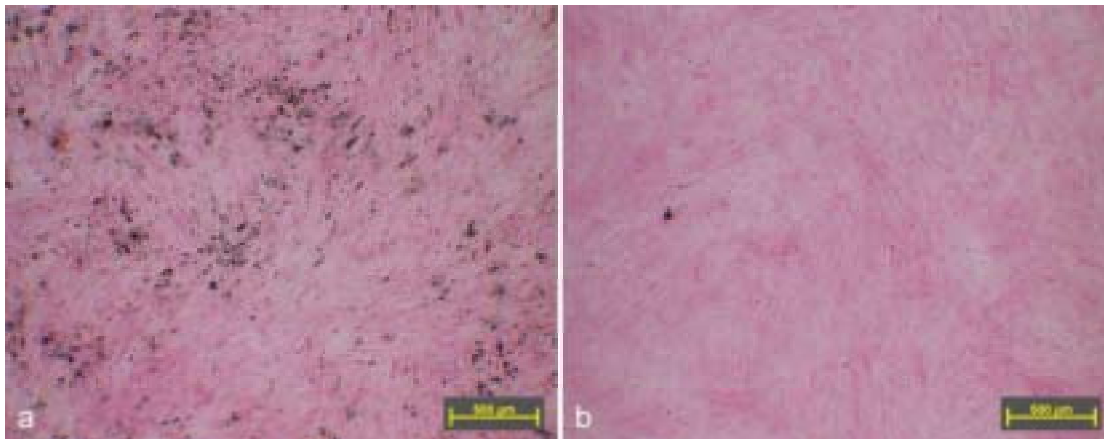
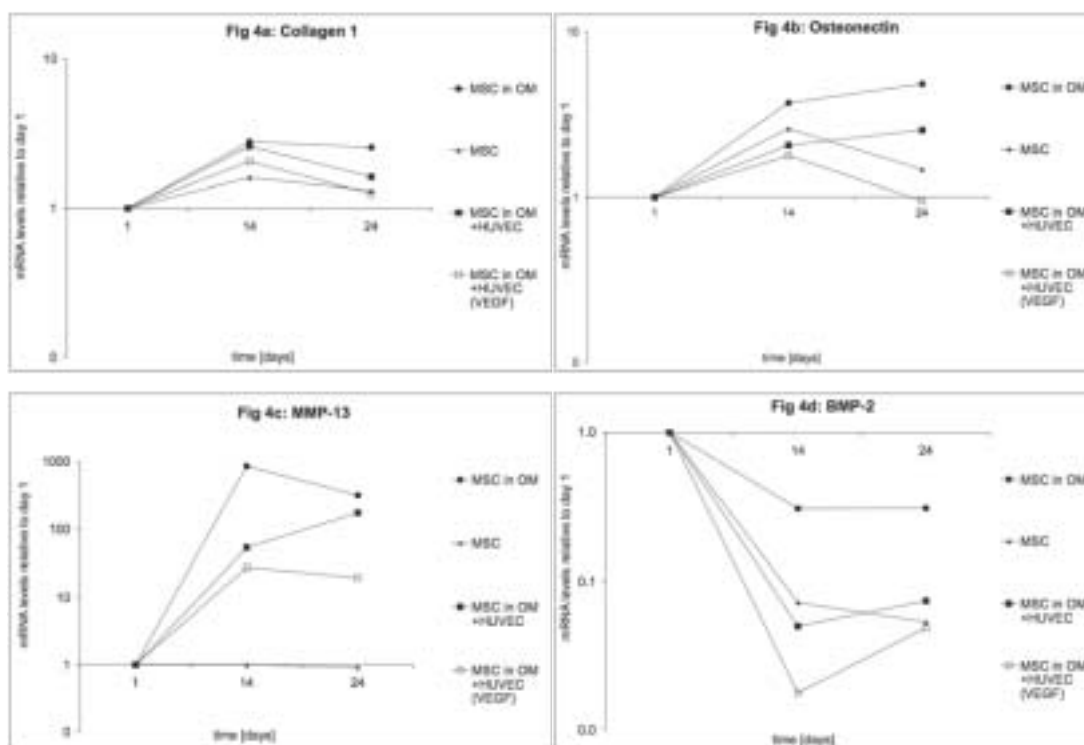


Figure 3: Van Kossa staining of MSC grown in osteogenic medium (a) and MSC grown in osteogenic medium supplemented with HUVEC-conditioned medium (b). Calcium deposition is clearly inhibited by the presence of HUVEC.

The addition of non-stimulated HUVEC to MSC cultures significantly decreased ALP activity (Figure 1), matrix mineralisation (Figures 2 and 3) and mRNA levels of COL1, ON, MMP-13 and BMP-2 (Figure 4). There was a slight increase in the DNA content of MSC upon addition of non-stimulated HUVEC, but this increase was not significant (data not shown).



Figures 4a-d: Representative graphs of mRNA levels of Collagen type I (a), Osteonectin (b), MMP-13 (c) and BMP-2 (d) measured by real-time RT-PCR: MSC cultured in osteogenic medium (OM) resulted in an upregulation of osteoblastic marker genes compared to MSC in non-osteogenic medium. Cultures with HUVEC decreased the expression of these marker genes significantly, an effect that was enhanced, when HUVEC were previously stimulated with VEGF. Day 24 data from all three experiments are summarized in Table II.

Osterix (OSX) was expressed at almost undetectable levels early in culture. After 24 days, MSC cultured in non-osteogenic medium still showed low OSX expression, while MSC in osteogenic medium expressed OSX prominently. HUVEC significantly decreased OSX gene expression of MSC, suggesting an inhibitory effect on MSC differentiation (Figure 5).

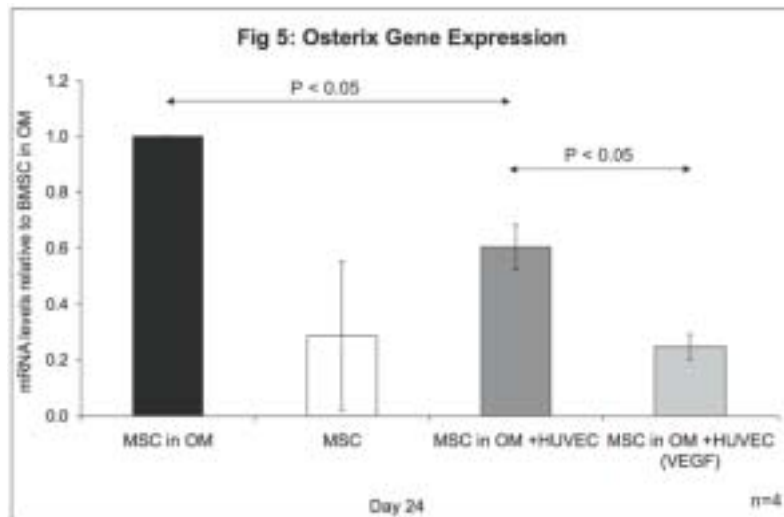


Figure 5: Osterix (OSX) mRNA levels relative to MSC in OM at day 24 (n=4): At day 1, OSX mRNA levels were almost undetectable in all cultures (data not shown). At day 24, MSC grown in non-osteogenic medium showed low OSX expression, while MSC grown in osteogenic medium (OM) showed elevated OSX expression. This expression was significantly down-regulated by HUVEC. Again, VEGF-stimulated HUVEC showed a more pronounced inhibitory effect.

Interestingly, Sox-9 expression levels dropped with culture time and reached almost undetectable levels in all cultures supplemented with osteogenic medium, including those with HUVEC (Figure 6). When VEGF-stimulated HUVEC were added to MSC, we observed an even more inhibitory effect on the measured markers than with non-stimulated HUVEC (Figures 1-6).

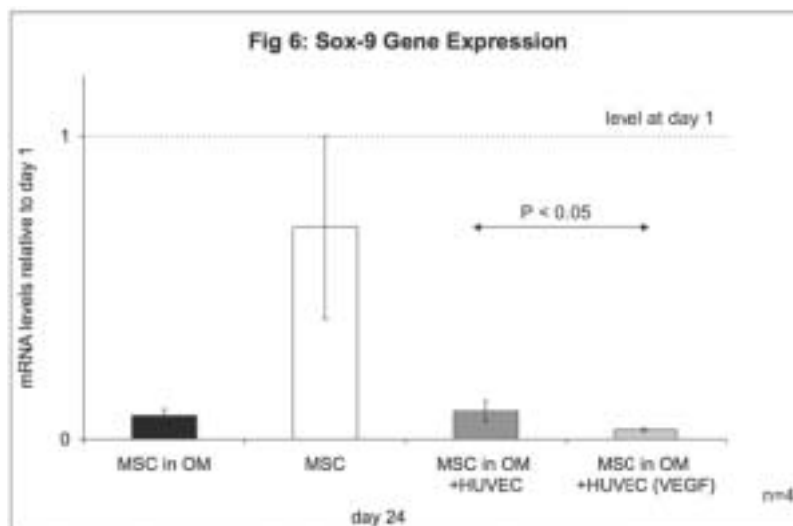


Figure 6: mRNA levels of the chondrocytic marker Sox-9 at day 24 relative to day 1 (n=4): The expression of Sox-9 dropped significantly after 24 days in all cultures, suggesting that none of these culture conditions propagates a chondrocytic phenotype. However, MSC cultured in osteogenic medium (OM) showed a more significant reduction of Sox-9 expression than MSC cultured in non-osteogenic medium. Unstimulated HUVEC had no effect on the inhibition of Sox-9 expression by OM, but VEGF-stimulated HUVEC significantly further decreased Sox-9 expression of MSC. Collagen 2 (COL2), another chondrocytic marker, was almost undetectable in all cultures during the whole experiment (data not shown).

We have also evaluated osteopontin (OP), osteocalcin (OC), bone sialoprotein II (BSP) and Runx2 gene expression. OP was highest at the very beginning of all cultures, then dropped and rose again with time in culture. Interestingly, MSC cultured in non-osteogenic medium showed higher OP expression than MSC in osteogenic medium (data not shown). When MSC were cultured with HUVEC, OP expression was further decreased. OC expression was constantly low in all culture systems, and almost undetectable in osteogenic medium. Addition of HUVEC didn't show any effect on OC (data not shown). Bone sialoprotein II (BSP) gene expression was decreased by HUVEC (data not shown). Runx2 expression was always higher in cultures with osteogenic medium and slightly increased in all cultures over time. HUVEC didn't change this pattern (data not shown).

Recovery Experiment

In order to evaluate whether the observed inhibitory effect of HUVEC on MSC differentiation was reversible, we cultured MSC in osteogenic medium with the addition of non-stimulated HUVEC-conditioned medium (1:10) and replaced it at different time points with only osteogenic medium. As expected, MSC that were exposed to osteogenic medium containing non-stimulated HUVEC-conditioned medium (1:10) during the whole experiment showed inhibition of $^{45}\text{Ca}^{+2}$ incorporation. Upon removal of non-stimulated HUVEC-conditioned medium, all these MSC cultures showed matrix mineralization similar to the positive control (Figure 7). Interestingly, MSC that were in contact with HUVEC-conditioned medium for a longer period of time (17 and 24 days) incorporated more calcium into their matrix upon HUVEC-conditioned medium removal, than MSC that were in contact with HUVEC-conditioned medium for a shorter period of time (3 and 10 days) (Figure 7). OSX gene expression was also analyzed and showed a similar picture (data not shown).

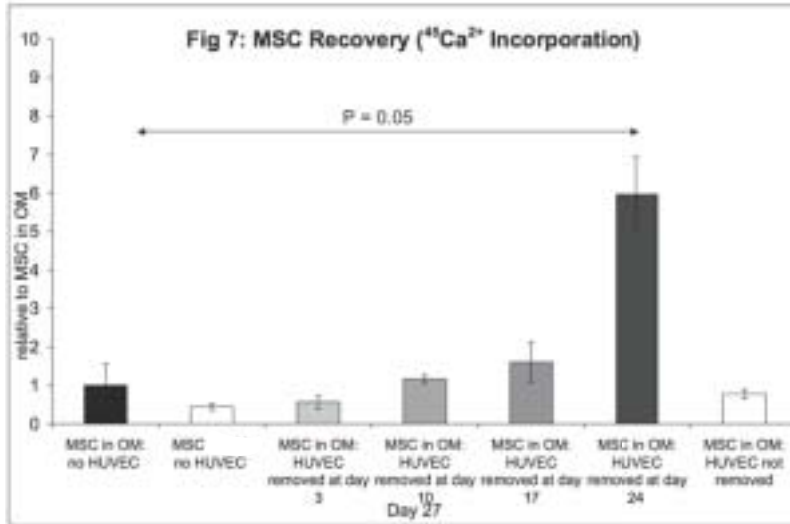


Figure 7: ⁴⁵Ca incorporation of MSC in osteogenic medium (OM) at day 27 after removal of HUVEC at different time points (n=4): Calcium deposition by MSC was inhibited by HUVEC, but upon removal of HUVEC, MSC showed again significant matrix mineralization. Interestingly, it seemed that the longer MSC were cultured with HUVEC-conditioned medium, the higher was the amount of ⁴⁵Ca incorporated into their extracellular matrix,

after removal of HUVEC-conditioned medium (a). OSX gene expression showed a similar response upon removal of HUVEC-conditioned medium (data not shown).

Inhibition experiment

To investigate at which times HUVEC could interfere with the MSC differentiation process, we cultured MSC in osteogenic medium and then replaced it with osteogenic medium containing non-stimulated HUVEC-conditioned medium (1:10) at different time points. The analysis of OSX gene expression showed, that osteoblastic differentiation was suppressed shortly after the medium was switched to medium containing non-stimulated HUVEC-conditioned medium (1:10). Even after 21 days of osteogenic stimulation, addition of non-stimulated HUVEC-conditioned medium resulted in a 4-5x decrease in OSX expression at day 28 compared to MSC in osteogenic medium (Figure 8b). ALP activity showed a similar pattern (Figure 8a).

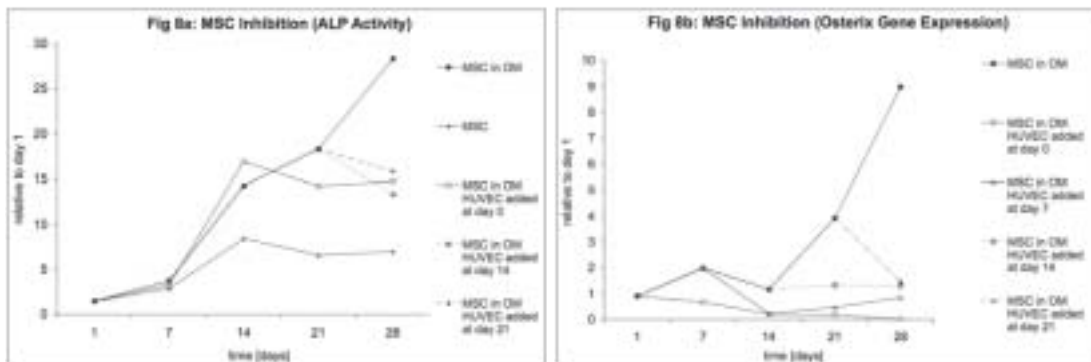
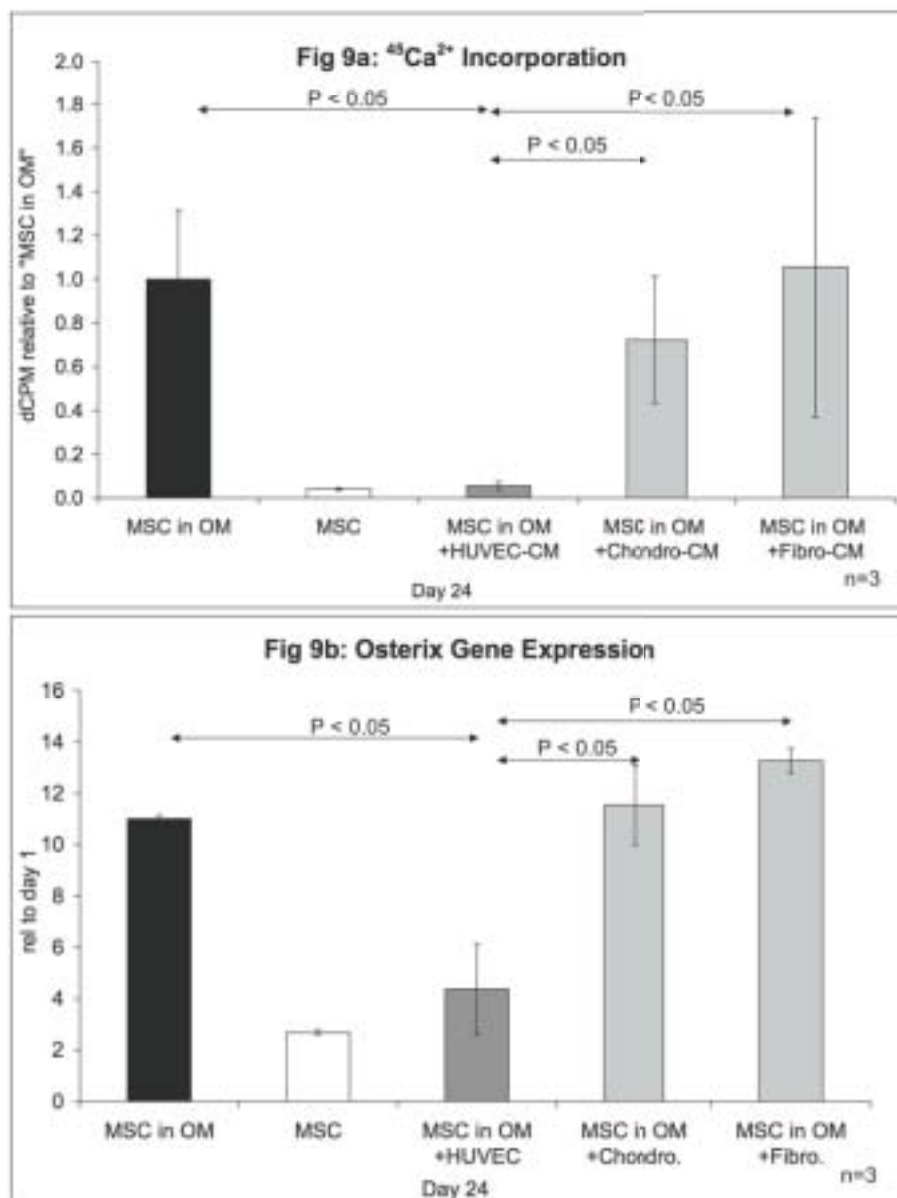


Figure 8a/b: Representative graphs of ALP activity (a) and OSX gene expression (b) of MSC: At different timepoints, the osteogenic medium (OM) was replaced by HUVEC-conditioned medium. The differentiation process was suppressed by the addition of HUVEC-conditioned medium, as judged by the inhibition of ALP activity (a) and suppression of OSX expression levels (b).

Chondro-CM and Fibro-CM on MSC

To investigate, if the inhibitory effect described in the previous sections was specific to HUVEC, we cultured MSC in osteogenic medium supplemented with chondrocyte-conditioned medium as well as with fibroblast-conditioned medium. Both Chondro-CM and Fibro-CM did not alter the calcium incorporation by MSC or the expression of osterix, while HUVEC-CM, in accordance with the results described above, was able to significantly down-regulate calcium incorporation as well as osterix gene expression (Figures 9a/b).



Figures 9a/b: ^{45}Ca incorporation (a) and OSX gene expression (b) of MSC in osteogenic medium supplemented with endothelial- (HUVEC-CM), chondrocyte- (Chondro-CM) or fibroblast-conditioned medium (Fibro-CM). ^{45}Ca -incorporation was significantly down regulated by HUVEC-CM, while Chondro-CM or Fibro-CM had no effect. Osterix gene expression showed the same pattern.

VEGF protein levels in culture media

We have measured the VEGF protein levels at several time points in the culture supernatant to investigate whether VEGF concentrations would be altered in the co-cultures compared to MSC alone. The highest amount of VEGF was observed in MSC cultured in non-osteogenic medium, while in osteogenic medium VEGF was down regulated. HUVEC had no effect on VEGF protein levels measured in the culture supernatant (data not shown).

Discussion

In the present study, we have investigated the effect of HUVEC on MSC differentiation towards the expression of an osteoblastic phenotype. The differentiation of MSC by osteogenic medium was inhibited following exposure to HUVEC. This was reflected by a significant decrease in matrix mineralization, ALP activity and in reduced expression of specific osteoblastic genes, including MMP-13, COL1, BMP-2 and ON. Interestingly, OC gene expression seems not to be an appropriate differentiation marker in human cells, as previously reported [42].

HUVEC were co-cultured with MSC for up to 28 days in two different types of culture systems: indirect contact and HUVEC-conditioned medium. The direct contact co-culture system was not used due to the difficulty of identifying specific markers of either HUVEC or MSC when cultured together, as well as to the lack of a suitable culture medium that could support the growth of MSC and HUVEC together for more than just a few days, without addition of growth factors (EGF, PDGF, VEGF, bFGF...). Interestingly, the observed inhibitory effect of HUVEC on MSC differentiation was seen in both co-culture systems, indicating that HUVEC secrete one or several factors that can delay MSC differentiation. Furthermore, it appears that this factor(s) acts by inhibiting OSX expression and therefore arresting MSC differentiation at a pre-osteoblastic stage (Figure 10). It has been shown that Runx2 plays a role in the commitment-step to osteo-chondro progenitor cells whereas OSX acts mainly on the terminal differentiation of osteoblasts and on distinguishing the osteogenic pathway from the chondrogenic one [43]. This might explain why Runx2 is not affected by HUVEC. The suppression of OSX expression by HUVEC could even be induced at later stages of MSC differentiation, just before mineralization of the extracellular matrix would have started. In addition, the reversibility of the HUVEC inhibitory effect on MSC differentiation was demonstrated by our recovery experiment, which showed that upon removal of HUVEC-conditioned medium, MSC could rapidly further differentiate towards the expression of an osteoblastic phenotype. Interestingly, this last experiment also indicates that MSC exposed to HUVEC for longer periods of time, seem to differentiate even faster towards an osteoblastic phenotype upon removal of HUVEC, when compared to MSC that have never been in contact with HUVEC (Figure 7).

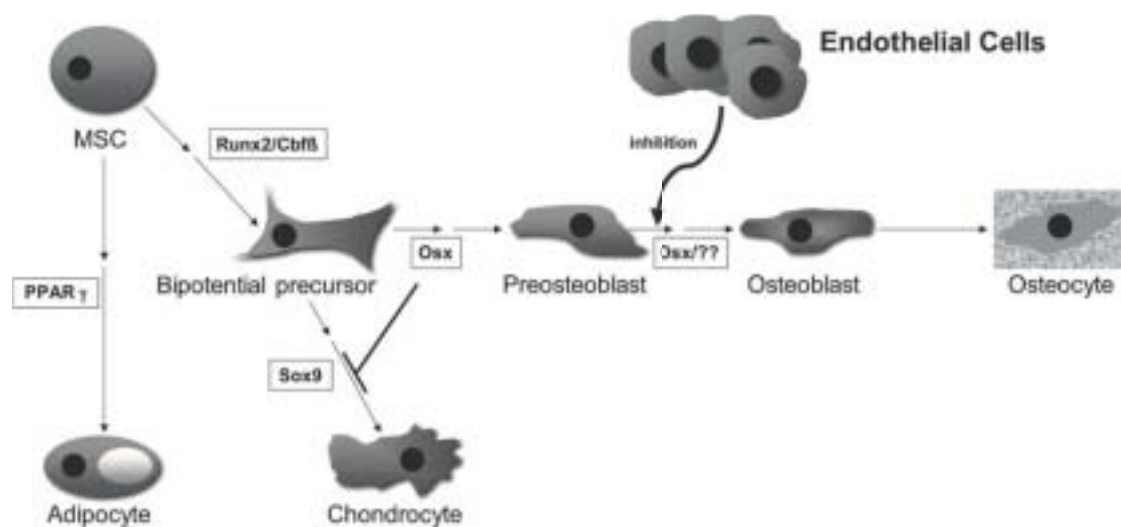


Figure 10: A simplified schematic representation of the different stages of the differentiation of MSC into osteoblasts: We postulate, that HUVEC may have the ability to arrest osteoblastic differentiation in a pre-osteoblastic stage by inhibiting OSX gene expression. The inhibitory effect of HUVEC does not result in an increase of chondrocytic or adipocytic markers. *In vivo*, this could lead to an accumulation of pre-osteoblasts at sites of bone turnover and repair.

It is also important to note that MSC exposed to HUVEC-CM showed no COL2 expression (data not shown) and extremely low expression of Sox-9, two typical markers of the chondrogenic phenotype (Figure 6). There was also no increase in the expression of the adipocytic marker PPAR-gamma over time (data not shown). These observations suggest that HUVEC-conditioned medium does not drive MSC towards the expression of a chondrogenic or an adipogenic phenotype. Together, our data from this *in vitro* system suggest that endothelial cells can increase the osteogenic potential of osteoprecursor cells while keeping them in a pre-osteoblast state (but at a later stage than the bipotential precursor stage for chondrocytes and osteoblasts, since Runx2 expression is unchanged by HUVEC). In addition, this effect is specific to HUVEC, since chondrocyte- as well as fibroblast-conditioned medium did not show this inhibitory effect on MSC. This would correlate with the *in vivo* potential of endothelial cells to recruit large numbers of osteogenic precursor cells at sites of bone modeling or remodeling. Further studies are necessary to verify this speculation.

Few studies have focused on the interactions of endothelial cells and osteoblastic cells in the past years, and the outcomes of these studies were somewhat divergent. While the positive influence of endothelial cells on osteoblastic cell proliferation has been reported quite consistently, their influence on osteoblastic cell differentiation seems rather controversial [6;7;9]. Wang et al. reported an increase in ALP activity in human osteoblast-like cells when cultured in HUVEC-conditioned medium [6], while

Jones et al. showed a decrease in ALP activity of MSC when cultured on EC feeder layers [9]. Villars et al. showed a decrease in ALP activity in all non-direct contact co-cultures, but an increase in ALP activity in direct contact co-cultures, but also a decrease in osteocalcin synthesis in direct contact co-cultures [7]. Kaigler et al. showed increased ALP activity as well as OC secretion only when MSC and EC were co-cultured in direct contact [44]. Guillotin et al. showed that different kinds of primary endothelial cells were able to induce ALP activity of osteoprogenitors when co-cultured in direct contact for up to 6 days, while transformed endothelial cells lines showed no effect [45]. Stahl et al. used EC and osteoblasts in an *in vitro* 3D spheroidal co-culture model. They described spontaneous organization of osteoblasts and endothelial cells into a core and a surface layer as well as changes in the gene expression patterns of both cell types, including an increase in ALP activity and down-regulation of VEGF after 48h, but did not distinguish between the two cell types during analysis [46;47]. Furthermore, all the studies mentioned above were carried out for a rather short period of time. Our preliminary studies showed no effect of EC on MSC differentiation in non-osteogenic medium (in no-direct contact co-cultures), which is in accordance with the above reports.

During the formation of a vascular network, endothelial progenitors originating from hemangioblasts differentiate into mature endothelial cells, which then participate in the processes of vasculogenesis and angiogenesis to form a mature vascular network. Especially in bone, this capillary network is constantly remodeled, which includes destabilization of vessels and sprouting of new branches, processes that are believed to require a change in the differentiation/maturation state of the vessel-covering endothelial cells. Therefore, this change in "maturation" of endothelial cells might influence their effect on MSC differentiation. These vessel remodeling processes are controlled by several factors, the most important one probably being VEGF [48]. Indeed, as observed in the present study, stimulation of HUVEC by VEGF further increases their inhibitory action on MSC differentiation. This inhibition could not be reproduced by VEGF itself. In fact, we could not detect any VEGF protein (results not shown) in the HUVEC-conditioned media (both VEGF-stimulated and non-stimulated) added to the MSC, as previously also reported by Villars et al. [7]. This conclusion is also supported by the observations that VEGF expression in MSC is decreased by glucocorticoids both *in vivo* and *in vitro*; that VEGF protein was detected in MSC cultures but its level did not show any changes upon addition of HUVEC-conditioned medium or HUVEC in indirect contact; and that Stahl et al. even measured a down-regulation of VEGF in their 3D direct contact co-culture model [46;47]. Furthermore, MSC cultured in non-osteogenic media (no dexamethasone)

showed higher levels of VEGF, again indicating that glucocorticoids suppress VEGF expression.

In conclusion, we have shown that HUVEC specifically have an inhibitory effect on dexamethasone-induced MSC differentiation *in vitro*. This effect is most probably due to a yet unknown HUVEC secreted factor(s), which suppresses OSX expression, therefore arresting MSC differentiation at a pre-osteoblastic stage. Furthermore, prior stimulation of HUVEC with VEGF resulted in a further increased inhibitory effect of HUVEC on MSC differentiation. A tempting speculation arising from these data is that HUVEC might regulate the rate at which MSC differentiate into osteoblasts, by initiating the recruitment of osteoprecursor cells at sites of bone remodeling and by keeping them in a pre-osteoblastic stage. This would avoid mineral deposition within vessels, but once these pre-osteoblastic cells extravasate out of the vessel and continue their homing to the remodeling/repair site, they will rapidly differentiate into mature osteoblasts and lay down new osteoid tissue.

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Chapter III

Effect of Ang1 and a combination of Ang1 and VEGF on osteoblastic differentiation of MSC

Meury TR, Alini M

After the interesting results obtained following VEGF-stimulation of HUVEC (see chapter II), we decided to investigate another factor involved in angiogenesis. While VEGF is mainly responsible to induce vascular leak, angiopoietin-1 (Ang1) is involved in the stabilization and maturation of blood vessels. We therefore decided to investigate the effect of Ang1 stimulated HUVEC on MSC differentiation.

This chapter is not planned as a publication in the current form.

Introduction

Vasculogenesis and angiogenesis are critical processes during bone development, turnover and repair [1-5]. *In vitro* and *in vivo* studies have shown, that endothelial cells interact closely with osteoblastic cells [6-13] (see also chapter II).

Angiopoietin-1 (Ang1) is probably the best-described angiogenic factor next to vascular endothelial growth factor (VEGF). VEGF is the most critical driver of vascularization and is mainly responsible for induction of vascular permeability and leak [14, 15]. Ang1 works in concert with VEGF [16, 17] but is restricted to later stages of angiogenesis where it plays a main role during the maturation and stabilization of blood vessels. This was illustrated by studies showing that mice lacking Ang1 develop a rather normal primary vasculature (unlike mice lacking VEGF), but the vasculature fails to undergo further remodeling and maturation [18]. Interestingly, recent studies have shown synergistic actions of VEGF and Ang1 during angiogenesis [19, 20], neovascularization [21, 22] and other *in vivo* assays [23, 24].

In chapter II, we were able to show that VEGF had a significant effect on how human umbilical vein endothelial cells (HUVEC) influence human mesenchymal stem cell (MSC) differentiation. Unstimulated HUVEC were able to down-regulate dexamethasone-induced osteogenic differentiation of MSC *in vitro*. This inhibitory effect was significantly enhanced when HUVEC were previously stimulated with VEGF (see chapter II).

The aim of the present study was to investigate the effect of HUVEC stimulated with Ang1 or a combination of VEGF and Ang1 (VEGF/Ang1) on MSC differentiation *in vitro*.

Materials and Methods

Origins of human cells

Bone marrow aspirates were taken from patients undergoing routine orthopedic surgery involving iliac crest exposure, after informed consent. Bone marrow aspirates (20mL) were harvested into CPDA-containing Sarstedt monovettes using a biopsy needle that was pushed through the cortical bone. Aspirates were processed within 12-24 hours after harvesting.

Isolation and expansion of human MSC

Bone marrow aspirates were homogenized by pushing them a few times through a syringe. The aspirate was then diluted 1:4 with IMDM (Gibco 42200-022) containing 5% (v/v) FBS (Gibco 10270-106) and centrifuged at 200g for 5min at room temperature (RT). The top layer (approx. 1cm) containing mostly fat tissue was removed. Per 1mL of undiluted sample, 2.6mL of Ficoll (Histopaque-1077, Sigma #1077-1) were pipetted into a 50mL Falcon tube and the aspirate was added carefully on top of the Ficoll. After centrifugation at 800g for 20min at RT, the mono-nucleated cells were collected at the interphase using a syringe. To 1mL of collected interphase solution, 5mL of IMDM/5%FBS were added, the tube was gently mixed and centrifuged at 400g for 15min at RT. The pellet was resuspended in the same amount of IMDM/5%FBS, centrifuged again and resuspended in IMDM/5%FBS. Cell number was determined using Methylene Blue in a hemocytometer. The cells were seeded at densities of $8-10 \times 10^6$ mono-nucleated cells per 150cm^2 T-flask in IMDM containing 10% FBS, nonessential amino acids (Gibco 11140-035) and PenStrep (100U/mL, Gibco 15140-122). After 5 days, the monolayers were washed with Tyrode's balanced salt solution (TBSS) to remove non-adherent cells, and fresh medium containing 5ng/mL b-FGF (R&D 233-FB) was added as previously reported [25-27]. Medium was changed every 2-3 days and cells were subcultured 1:3 at sub-confluence. The adherent cells after one subculture were termed Mesenchymal stem Cells (MSC). Only cells between passages 2-4 (approx. 12-18 populations doublings) were subsequently used [28].

Expansion of HUVEC

Primary Human Umbilical Vein Endothelial Cells (HUVEC) were purchased from Cascade Biologics (cat# C-003-5C). The cells were expanded in IMDM containing 10%FBS, nonessential amino acids, PenStrep, 20ug/mL ECGS (Sigma E-

2759) and 90ug/mL heparin (Sigma H-3149). Only cells between passages 2-4 were subsequently used.

Stimulation of HUVEC with Ang1 or VEGF/Ang1

HUVEC were expanded in IMDM containing 10% FBS, nonessential amino acids, 20ug/mL ECGS, 90ug/mL heparin and PenStrep. At about 75% of confluency, 100ng/mL Ang1 (R&D Systems 923-AN) or a combination of 100ng/mL Ang1 and 25ng/mL VEGF (Sigma V-7259) were added and the cells were stimulated for 3 days. The concentrations used in this study were chosen based on publications by Hayes et al. (Ang1) [29] and Street et al. (VEGF) [30]. The HUVEC monolayers were either used to produce HUVEC-conditioned medium (see Conditioned medium on MSC cultures)) or were trypsinized and used for indirect contact co-cultures.

MSC cultures

MSC were seeded in 24-well plates (Falcon BD 353504) at a density of 20'000 cells/well in 1mL of medium and were left to attach for 2-3 hours. Culture medium for the MSC culture systems contained IMDM, 10% FBS, nonessential amino acids, 0.1mM ascorbic acid-2-phosphate (Sigma A-8960) and 10mM - glycerophosphate (Sigma G-6251) with 10nM dexamethasone (osteogenic medium) or without dexamethasone (non-osteogenic medium). Dexamethasone was purchased at Sigma (D-2915). Media were changed twice a week.

Conditioned medium on MSC cultures

HUVEC-conditioned medium was produced by washing the VEGF/Ang1 stimulated (see section "*Stimulation of HUVEC with Ang1 or VEGF/Ang1*") or non-stimulated HUVEC monolayers twice with phosphate buffered saline (PBS) and then culturing them for 24h in IMDM containing 10% FBS and PenStrep. After 24h, the medium was removed, filtered (0.22µm), and stored at -20°C until used. This conditioned medium was added in a 1:10 mixture to MSC monolayers cultured in osteogenic or non-osteogenic medium in 24-well plates.

Indirect contact cultures of MSC and HUVEC (VEGF/Ang1 stimulated or non-stimulated)

To MSC monolayers, VEGF/Ang1 stimulated (see section "*Stimulation of HUVEC with Ang1 or VEGF/Ang1*") or non-stimulated HUVEC were added in cell culture inserts (0.4µm pore size, Falcon BD 353495) at a density of 10'000

cells/insert. Fresh HUVEC were added once a week to the system to provide a fresh supply of endothelial cell secreted factors. These experiments were also performed in osteogenic or non-osteogenic medium.

Quantification of matrix mineralization using $^{45}\text{Ca}^{2+}$ isotope

1.25 $\mu\text{Ci/mL}$ of $^{45}\text{Ca}^{2+}$ isotope (Amersham CES3) were added to each well and the plates were incubated at 37°C for 6h [31]. The medium was removed and the monolayers were washed 3x with IMDM to remove unincorporated $^{45}\text{Ca}^{2+}$. Then 0.5mL of 70% formic acid were added to each well and the plates were incubated at 65°C for 1h. The formic acid solution was transferred to 3.5mL of scintillation liquid (OptiPhase HiSafe'3 by Perkin Elmer) and the amount of radioactivity was measured using a Wallac 1414 WinSpectral liquid scintillation counter.

Quantification of alkaline phosphatase activity

The medium was completely removed and the monolayers were washed once with PBS. The cell layers were extracted by addition of 500 μL of 0.1% Triton-X in 10mM Tris-HCl (pH 7.4) and incubation at 4°C on a gyratory shaker for 2h (See Sigma Technical Bulletin Procedure No.104). ALP activity was measured colorimetrically by measuring the p-nitrophenol production during 15min incubation at 37°C with p-nitrophenyl phosphate as substrate (Sigma Kit Nr.104) on a Perkin Elmer Bio Assay Reader HTS 7000.

RNA Isolation and Reverse Transcription

Monolayers were extracted using 500 μL of TRI-Reagent (MRC Inc. TR-118) with 5 $\mu\text{L/mL}$ of Polyacryl-carrier (MRC Inc. PC-152) for 10min at RT and transferred to Eppendorf tubes. 150 μL of 1-Bromo-3-Chloro-Propane were added, the tubes vortexed for 15sec and then centrifuged at 12'000g for 15min at 4°C. The colorless layer on the top was transferred into a new tube and 750 μL of isopropanol were added, then the tube was centrifuged at 12'000g for 10min at 4°C. Sample supernatants were removed and pellets were washed in 1mL of 75% EtOH by vortexing and centrifuging at 10'000g for 5min at 4°C. Pellets were air-dried and re-suspended in 30 μL of DEPC-treated water. After 15min at 60°C, tubes were immediately transferred to ice. The total RNA amount and purity was assessed by measuring the absorbance at 260nm and 280nm. Reverse transcription was performed using 1 μg of total RNA sample, which was mixed with 2 μL of 10x TaqMan RT Buffer (500mM KCl, 100mM Tris-HCl, pH 8.3), 4.4 μL of 25mM magnesium

chloride, 4 μ L of dNTP mixture (2.5mM of each dNTP), 1 μ L of random hexamers (50 μ M), 0.4 μ L of RNase inhibitor (20U/mL) and 0.5 μ L of MultiScribe Reverse Transcriptase (50U/ μ L) (all from Applied Biosystems); DEPC-treated water was added to bring the final reaction volume to 20 μ L. Reverse transcription was performed using a Thermal Cycler 9600 by Applied Biosystems. cDNA samples were appropriately diluted with Tris-EDTA buffer before being used for real-time RT PCR.

Real-time RT PCR

Oligonucleotide primers and TaqMan probes were designed using the Primer Express Oligo Design software (Ver. 1.5, Applied Biosystems). The nucleotide sequences were obtained from the GenBank database and the probes were designed to overlap an exon-exon junction in order to avoid amplification of genomic DNA. Primers and probes for amplification of 18S ribosomal RNA, used as endogenous control, were from Applied Biosystems. All other primers and labeled TaqMan probes were from Microsynth (Balgach, CH). TaqMan probes were labeled with the reporter dye molecule FAM (6-carboxyfluorescein) at the 5' end and with the quencher dye TAMRA (6-carboxy-N, N, N', N'-tetramethylrhodamine) at the 3' end. The PCR reaction mixture contained TaqMan Universal PCR master mix without AmpErase UNG (Applied Biosystems), 900nM primers (forward and reverse), 250nM TaqMan probe, and 2 μ L of cDNA sample for a total reaction volume of 25 μ L. PCR conditions were 95°C for 10min, followed by 42 cycles of amplification at 95°C for 15sec and 60°C for 1min using the GeneAmp 5700 Sequence Detection System (Applied Biosystems, Foster City, CA). Relative quantification of mRNA targets was performed according to the comparative C_T method with 18S ribosomal RNA as endogenous control (ABI PRISM 7700 Sequence Detector User Bulletin (2), PE Applied Biosystems 1997). For a list of primers and probes see chapter II, Table I.

Statistical analysis

The data are expressed as mean \pm SEM. Statistics were performed using the non-parametric Mann-Whitney U-test, which compares the medians of two independent distributions. P < 0.05 was considered statistically significant.

Results

Experiments were performed with bone marrow aspirates from 3 different donors (age range 39-66). The MSC used during the experiments were expanded in standard medium supplemented with b-FGF to keep them in a tripotential state, capable of differentiating along the osteogenic, chondrogenic and adipogenic pathways [25, 26, 28].

In accordance to the results we obtained previously (see chapter II), there was no difference in the inhibitory effect of HUVEC on MSC differentiation between indirect co-cultures and HUVEC-conditioned medium cultures. The results presented in the following paragraphs therefore are combined data from all experiments, independent of the culture type (indirect co-cultures and HUVEC-conditioned medium).

Effect of (stimulated or unstimulated) HUVEC on MSC differentiation in non-osteogenic medium

In non-osteogenic medium, HUVEC had no effect on MSC differentiation. This pattern was not changed when HUVEC were previously stimulated with Ang1 or a combination of Ang1 and VEGF (Figures 1 and 2 left side).

Effect of (stimulated or unstimulated) HUVEC on MSC differentiation in osteogenic medium (OM)

As expected, osteogenic medium (OM) significantly up-regulated the expression of several osteoblastic markers like calcium incorporation, ALP activity and gene expression of *Osx*, *BSP11*, *MMP-13*, *ON* and *Col1* when compared to MSC in non-osteogenic medium (Figures 1-4). As shown before (see chapter II), the addition of unstimulated HUVEC to MSC in OM results in a significant down-regulation of osteoblastic markers, as prominently seen in significantly lowered calcium incorporation, ALP activity and gene expression (Figures 1-4).

When HUVEC were previously stimulated with Ang1, they showed a very comparable inhibitory effect on MSC differentiation as unstimulated HUVEC. The same observation was made for HUVEC that were stimulated with a combination of VEGF and Ang1 (VEGF/Ang1).

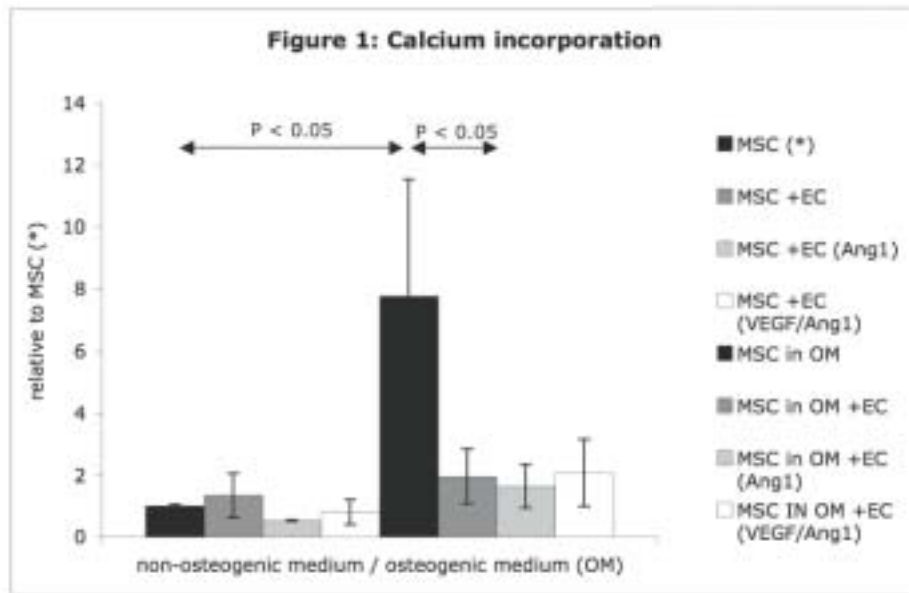


Figure 1: $^{45}\text{Ca}^{2+}$ incorporation by MSC at day 24: MSC in OM incorporated significantly more calcium into their matrix than MSC in non-osteogenic medium (*), as expected. While endothelial cells (EC) did not have an influence on MSC in non-osteogenic medium, they significantly down-regulated calcium incorporation of MSC in OM. Stimulation of HUVEC with Ang1 or VEGF/Ang1 did not show any further significant effect compared to unstimulated HUVEC (in non-osteogenic or osteogenic medium).

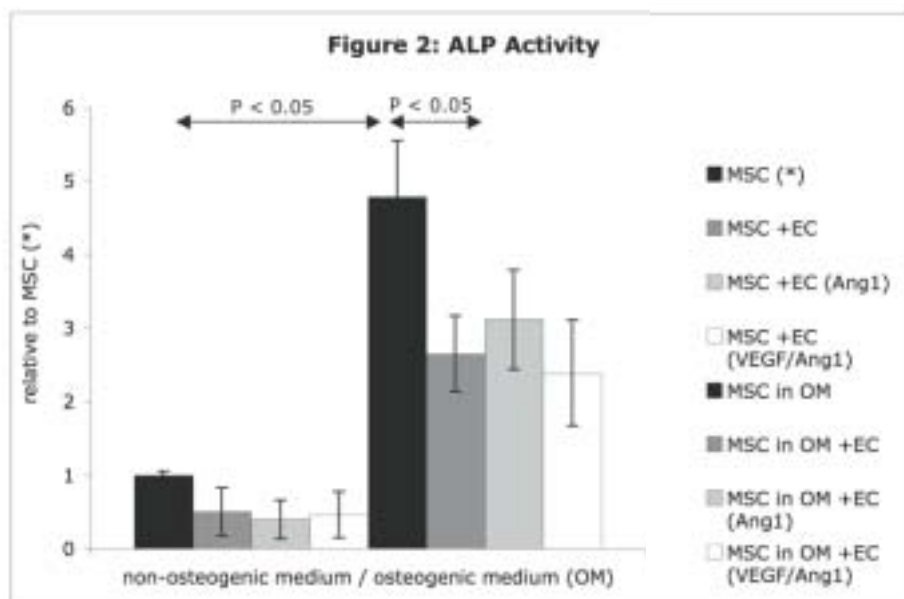


Figure 2: ALP activity of MSC at day 12: MSC in osteogenic medium (OM) showed significantly more ALP activity than MSC in non-osteogenic medium (*), as expected. In non-osteogenic medium EC did not show an effect, but in OM, ALP activity in MSC was significantly down-regulated by EC. Stimulation of HUVEC with Ang1 or VEGF/Ang1 did not show any further significant effect compared to unstimulated HUVEC (in non-osteogenic or osteogenic medium).

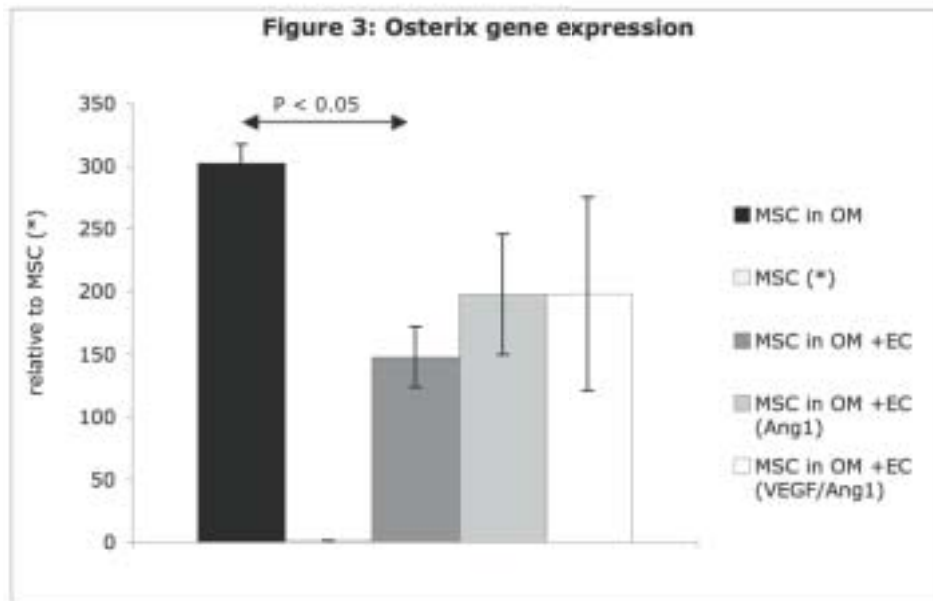


Figure 3: Expression of the Osterix gene by MSC at day 24: While MSC in OM expressed *Osx* prominently; MSC in non-osteogenic medium (*) did not. HUVEC significantly decreased *Osx* expression of MSC in OM. Stimulation of HUVEC with Ang1 or VEGF/Ang1 did not have a significant effect on *Osx* expression compared to unstimulated HUVEC.

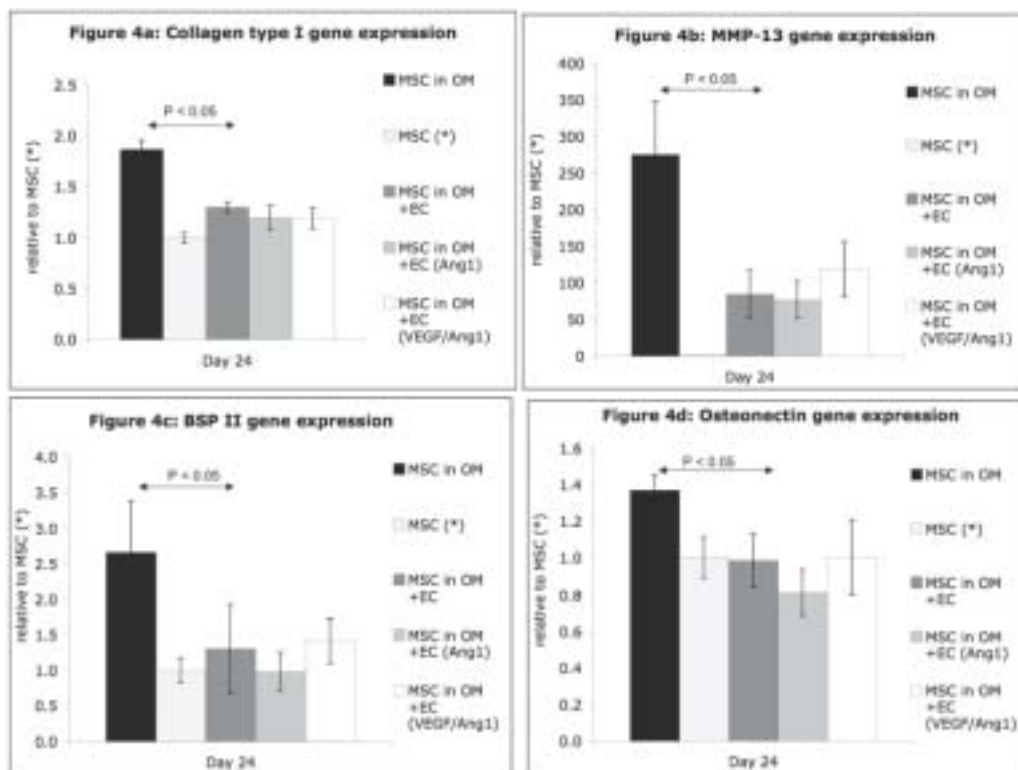


Figure 4a-d: Gene expression of Collagen type I, BSP II, MMP-13 and Osteonectin at day 24: MSC in OM expressed *Col1*, *BSP II*, *MMP-13* and *osteonectin* prominently, while MSC in non-osteogenic medium (*) showed significantly much lower levels. HUVEC significantly decreased the expression of *Col1*, *BSP II* and *MMP-13* by MSC in OM independent of being stimulated or not with Ang1 or VEGF/Ang1.

Discussion

Even though the generally accepted roles for VEGF and Ang1 during angiogenesis are rather oppositely (destabilization versus maturation of blood vessels), there are several recent *in vivo* studies that have shown Ang1 and VEGF also to act synergistically during angiogenesis and vasculogenesis [19-24]. There is very little literature about the *in vitro* effect of Ang1 on any cell type and its interactions with VEGF. Huang et al. showed that Ang1 alone had no effect on endothelial cell proliferation, but enhanced cell growth in the presence of VEGF [32]. Ang1 and VEGF have been shown to enhance endothelial cell migration and survival in a dose-dependent manner [33, 34]. However, Ang1 opposes the effect of VEGF on vascular permeability. In fact, Wang et al. have shown that VEGF-induced vascular leak required the translocation and activation of protein kinase C and that this pathway was efficiently blocked by Ang1 [35].

Since in an earlier study we were able to show that HUVEC had an inhibitory effect on MSC differentiation in OM, and that this inhibitory effect could be amplified by using VEGF-stimulated HUVEC (see chapter II), we were interested in investigating if a similar effect could be observed with Ang1-stimulated or VEGF/Ang1-stimulated HUVEC.

Our results showed, that Ang1-stimulated HUVEC were neither able to mimic the inhibitory observed with VEGF-stimulated HUVEC, nor did it have a stimulatory effect on osteogenic MSC differentiation. Interestingly, when MSC were exposed to VEGF/Ang1-stimulated HUVEC, there was also no change in the expression levels of osteoblastic markers compared to MSC exposed to unstimulated HUVEC. This indicates that Ang1 might override the inhibitory effect of VEGF-stimulated HUVEC on MSC differentiation.

Further studies will be necessary to evaluate whether higher concentrations of Ang1 might be necessary to induce a detectable effect on MSC differentiation [33, 34]. In addition, experiments involving Ang2 alone or in combination with VEGF should also be performed, since action of Ang2 is highly dependent on VEGF. Finally, based on the findings described in chapter V, it should be as well considered to repeat the 2D-experiments (including those with VEGF) in a 3D environment.

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Chapter IV

Definition of a PRP production protocol and the effect of PRP on osteoblastic differentiation of MSC *in vitro*

Meury TR, Kupcsik L, Heini P, Becker S, Stoll T, Alini M

In this chapter, we focused on another important component of a bone construct: The biological factors. We decided to investigate if platelet-rich plasma (PRP) due to its autologous origin and its highly concentrated factor mixture could influence MSC differentiation. In addition, due to its capacity to form a gel upon activation, PRP could also serve as a matrix for cells. Since there was no consistent routine protocol to produce PRP, we decided to first define a reliable PRP isolation and activation protocol. Then we studied the influence of PRP on MSC differentiation into bone forming cells.

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Introduction

Platelets play a pivotal role during wound healing. Upon being activated, they adhere to the exposed sub-endothelium and form a clot by binding to circulating fibrinogen molecules that cover the injured site and allow the healing process to begin. This clot is stabilized by a thick fibrin-mesh, which forms under the control of liver-produced thrombin. In addition, platelet activation and degranulation results in the release of a high number of biological factors, which attract more platelets to the site of injury, but also growth factors and cytokines that can target other cell types (including PDGF, VEGF, IGF, EGF, FGF and TGF) [1, 2]. Many of those factors have been known to play a direct role in normal bone turnover and in the events during early bone healing [3]. This led to the idea to use platelets in bone repair in the form of platelet-rich plasma (PRP) [4, 5]. PRP is autologous plasma that has a platelet concentration above baseline (~1Mio platelets/ μ L vs. ~0.2Mio platelets/ μ L), but a native fibrinogen concentration. A highly beneficial effect of PRP addition to bone grafts, eventually in combination with mesenchymal stem cells is suspected. However, the few studies performed using PRP in fracture repair resulted in only moderately successful outcomes. Some groups have observed an increased bone formation [4, 6-10], some an increased implant resorption [11], others have reported improved osseointegration [10, 12, 13] or increased vascularization [11]. Nevertheless, several studies have shown no beneficial influence of PRP on bone healing whatsoever [14-18].

The interpretation of all these studies is not simplified by the fact that they were performed using many different species, including dog [11, 12, 14], rat [8, 17], mini-pig [16, 19], goat [7], rabbit [6] and human [4, 9, 10, 15, 18] and many different defect sites and types. Furthermore, there is great inconsistency in the techniques used for PRP preparation. Some groups have used common protocols (1- or 2-step centrifugations using normal lab equipment) [8, 11, 12], while others used commercially available systems [19-21]. PRP preparations from these different systems can hardly be compared, since the resulting platelet concentrations as well as the growth factor concentrations released by the platelets are preparation dependent [4, 22, 23]. Furthermore, while some groups activated the PRP (mostly by addition of bovine Thrombin and CaCl_2) [9, 11, 12, 16], others did not. In addition, a few studies have shown, that PRP alone has a positive effect on bone healing, while others only report a beneficial effect in combination with autologous precursor cells [9, 11, 12].

Our aim was to define a simple and quick procedure to produce PRP using regular lab equipment. Additionally, we wanted to compare different activation methods and evaluate their efficiency and efficacy. Also, there is a lack of *in vitro* data concerning the influence of PRP on different cell types. A positive effect of PRP on the proliferation [22, 24-28] and migration [27, 29] of several cells types including MSC, stromal cells, osteoblastic cells or EC was reported quite consistently, but its effect on cell differentiation remains unclear. We therefore evaluated the potential effect of PRP on bone precursor cells in a controlled *in vitro* environment by investigating its effect on human mesenchymal stem cell differentiation into osteoblasts.

Materials and Methods

Origin of bone marrow and blood aspirates

Bone marrow (60mL) and blood (100mL) aspirates in CPDA-containing Sarstedt monovettes were received from patients undergoing routine orthopaedic surgery involving iliac crest exposure, after informed consent (KEK Bern 126/03). Blood aspirates were stored at RT under gentle agitation and bone marrow aspirates were stored at 4°C under gentle agitation until processed within 24 hours after harvesting. The platelets were counted using a Digitana Sysmex FS-3000.

MSC isolation and culture

Bone marrow aspirates were homogenized by pushing them a few times through a syringe. The aspirate was then diluted 1:4 with IMDM (Gibco 42200-022) containing 5% (v/v) FBS (Gibco 10270-106) and centrifuged at 200g for 5min at room temperature (RT). The top layer (approx. 1cm) containing mostly fat tissue was removed. To 1mL of undiluted sample, 2.6mL of Ficoll (Histopaque-1077, Sigma #1077-1) were pipetted into a 50mL Falcon tube and the aspirate was added carefully on top of the Ficoll. After centrifugation at 800g for 20min at RT, the mononucleated cells were collected at the interphase using a syringe. To 1mL of collected interphase solution 5mL of IMDM/5%FBS were added, the tube was gently mixed and centrifuged at 400g for 15min at RT. The pellet was resuspended in the same amount of IMDM/5%FBS, centrifuged again and resuspended in IMDM/5%FBS. Cell number was determined using Methylene Blue in a hemocytometer. The cells were seeded at densities of $8-10 \times 10^6$ mononucleated cells per 150cm^2 T-flask in IMDM containing 10% FBS, nonessential aminoacids (Gibco 11140-035) and PenStrep (100U/mL, Gibco 15140-122). After 5 days, the monolayers were washed with Tyrode's balanced salt solution (TBSS) to remove non-adherent cells, and fresh medium containing 5ng/mL basic-FGF (R&D 233-FB) was added as described previously [30-32]. Medium was changed every 3 days and cells were subcultured 1:3 at sub-confluence. The adherent cells after one subculture were termed mesenchymal stem cells (MSC). Cells between passages 2-4 were subsequently used.

PRP preparation

The blood aspirates were transferred from the CPDA-cuvettes into 15mL Falcon tubes, and were centrifuged at 200g for 30min at RT. The resulting plasma

supernatants were pooled, transferred into a new 15mL Falcon tube, and centrifuged at 2'000g for 5min at RT to get a platelet pellet. The resulting supernatant is called platelet-poor plasma (PPP). Platelet-rich plasma (PRP) was produced by resuspending the resulting pellet in the PPP (1/10th of the initial blood volume). Since PRP has a tendency to gel, even when added in low concentrations to culture medium, fibrinogen was removed by resuspending the platelet pellet in PBS (1/10th of the initial blood volume) instead of plasma supernatant. This platelets-containing PBS was termed platelet-released growth factors (PRGF). PRGF was stored at -20°C until used to supplement MSC cultures. PRP was used immediately to evaluate PRP activation efficiency.

PRP/PRGF activation

PRP or PRGF was activated using the following protocols:

Thrombin: 100U/mL of bovine Thrombin (Sigma T-4648) dissolved in PBS or 100mM CaCl₂ (10%) was added to the PRP or PRGF samples. The samples were kept at 37°C for 30-60min.

Freeze-Thaw cycles: PRP or PRGF in cryo-tubes (4.5mL, NUNC) were put in liquid N₂ for 1min to freeze "instantly", then the tubes were transferred to a 37°C water-bath to thaw for 5min. This procedure was repeated 5 times. After, the PRP or PRGF was kept at 37°C for 30-60min. To some samples, 100mM CaCl₂ (10%) was added before the freeze-thaw cycles.

Fridge: The PRP or PRGF was frozen at -20°C for 30min, then kept at 37°C for 30-60min. To some samples, 100mM CaCl₂ (10%) was added before freezing.

Sonication: The PRP or PRGF was sonicated using a SONOPLUS-Ultraschall Homogenisator (GM-70, UW-70, SH-70G) by Bandelin at 20 kHz for 10sec on ice, and then kept at 37°C for 30-60min. To some samples, 100mM CaCl₂ (10%) was added before sonication.

Platelet activation efficiency by ELISA

To estimate platelet activation efficiency of the methods described above, the release of PDGF-AB, -BB, and VEGF was determined. Following activation, the samples were centrifuged at 18'000g for 2min to pellet debris and the gel that formed after activation (only samples containing CaCl₂). The resulting supernatant was diluted 1:50 in PBS containing 0.1% BSA and human VEGF, PDGF-AB and -BB protein content was measured using a DuoSet ELISA Development System by R&D

Systems (PDGF-AB: DY222, PDGF-BB: DY220, VEGF: DY293B) on a Perkin Elmer Bio Assay Reader HTS 7000.

MSC culture supplementation with PRGF

MSC were trypsinized, washed, counted and seeded in 24-well (Falcon BD 353047) or 12-well plates (Falcon BD 353043) at densities of 10'000 to 15'000 cells/cm² and were left to attach over night in IMDM supplemented with 10% FBS. Culture medium during the experiments consisted of IMDM, 10% FBS, nonessential amino acids, 0.1mM ascorbic acid-2-phosphate (Sigma A-8960) and 10mM - glycerophosphate (Sigma G-6251). To some cultures (positive control), 10nM dexamethasone (Sigma D-2915) was added. Media were changed twice a week. Some cultures were supplemented with 1, 5, 10 or 20% activated PRGF (containing no CaCl₂).

⁴⁵Ca incorporation assay

1.25µCi/mL of ⁴⁵Ca²⁺ isotope (Amersham CES3) were added to each well and the plates were incubated at 37°C o/n. The medium was removed and the cell monolayers were washed 3x with IMDM to remove unincorporated ⁴⁵Ca²⁺. Then 0.5mL of 70% formic acid were added to each well and the plates were incubated at 65°C for 1h. The formic acid solution was transferred to a scintillation tube containing 3.5mL of scintillation liquid (OptiPhase HiSafe'3 by Perkin Elmer) and the amount of radioactivity was measured using a Wallac 1414 WinSpectral liquid scintillation counter.

Van Kossa staining

MSC monolayers were rinsed with TBSS. Fresh silver nitrate solution (5%) was added and the cells were exposed to strong light for 20min. After rinsing 3 times with distilled water, the cells were incubated in fresh 5% sodium thiosulfate for 10min, before being rinsed again 3 times with distilled water. Then the cells were incubated in 0.1% nuclear fast red solution for 10min, before being rinsed again with distilled water. Samples were left in distilled water and pictures were taken immediately.

ALP activity assay

The medium was completely removed and the MSC monolayers were washed once with PBS. The cell layers were extracted by addition of 500µL of

0.1% Triton-X in 10mM Tris-HCl (pH 7.4) and incubation at 4°C on a gyratory shaker for 3h (See Sigma Technical Bulletin Procedure No.104). ALP activity was measured colorimetrically by measuring the p-nitrophenol production during a 15min incubation at 37°C with p-nitrophenyl phosphate as substrate (Sigma Kit No.104) on a Perkin Elmer Bio Assay Reader HTS 7000.

RNA isolation and reverse transcription

Monolayers were extracted using 500µL of TRI-Reagent (MRC Inc. TR-118) with 5ul/mL of polyacryl-carrier (MRC Inc. PC-152) for 10min at RT and transferred to Eppendorf tubes. 150µL of 1-Bromo-3-Choro-Propane were added, the tubes vortexed for 15sec and then centrifuged at 12'000g for 15min at 4°C. The colorless layer on the top was transferred into a new tube and 750µL of isopropanol were added, then the tube was centrifuged at 12'000g for 10min at 4°C. Sample supernatants were removed and pellets were washed in 1mL of 75% EtOH by vortexing and centrifuging at 10'000g for 5min at 4°C. Pellets were air-dried and re-suspended in 30µL of DEPC-treated water. After 15min at 60°C, tubes were immediately transferred to ice. The total RNA amount and purity was assessed by measuring the absorbance at 260nm and 280nm. Reverse transcription was performed using 1µg of total RNA sample, which was mixed with 2µL of 10x TaqMan RT Buffer (500mM KCl, 100mM Tris-HCl, pH 8.3), 4.4µL of 25mM magnesium chloride, 4µL of dNTP mixture (2.5mM of each dNTP), 1µL of random hexamers (50µM), 0.4µL of RNase inhibitor (20U/mL) and 0.5µL of MultiScribe Reverse Transcriptase (50U/µL) (all from Applied Biosystems); DEPC-treated water was added to bring the final reaction volume to 20µL. Reverse transcription was performed using a Thermal Cycler 9600 (by Applied Biosystems). cDNA samples were appropriately diluted with Tris-EDTA buffer before being used for real-time RT PCR.

Real-time RT PCR

Oligonucleotide primers and TaqMan probes were designed using the Primer Express Oligo Design software (Ver. 1.5, Applied Biosystems). The nucleotide sequences were obtained from the GenBank database and the probes were designed to overlap an exon-exon junction in order to avoid amplification of genomic DNA. Primers and probes for amplification of 18S ribosomal RNA, used as endogenous control, were from Applied Biosystems. All other primers and labeled TaqMan probes were from Microsynth (Balgach, CH). TaqMan probes were labeled

with the reporter dye molecule FAM (6-carboxyfluorescein) at the 5' end and with the quencher dye TAMRA (6-carboxy-N, N, N', N'-tetramethylrhodamine) at the 3' end. The PCR reaction mixture contained TaqMan Universal PCR master mix without AmpErase UNG (Applied Biosystems), 900nM primers (forward and reverse), 250nM TaqMan probe, and 2 μ l of cDNA sample for a total reaction volume of 25 μ l. PCR conditions were 95°C for 10min, followed by 42 cycles of amplification at 95°C for 15sec and 60°C for 1min using the GeneAmp 5700 Sequence Detection System (Applied Biosystems, Foster City, CA). Relative quantification of mRNA targets was performed according to the comparative C_T method with 18S ribosomal RNA as endogenous control (ABI PRISM 7700 Sequence Detector User Bulletin (2), PE Applied Biosystems 1997). The sequences of the primer and probes used are shown in Table I.

Statistics

All results are shown as mean \pm SEM. Statistical analyses were performed using the non-parametric Mann-Whitney U-test, which compares the medians of two independent distributions. P < 0.05 was considered to be statistically significant.

Table I. Primers and Probes for Real-Time RT-PCR

Target Gene	Sequence (5' ~ 3')
Collagen I	
Forw	CCC TGG AAA GAA TGG AGA TGA T
Rev	ACT GAA ACC TCT GTG TCC CTT CA
Probe	CGG GCA ATC CTC GAG CAC CCT
Osteonectin	
Forw	ATC TTC CCT GTA CAC TGG CAG TTC
Rev	CTC GGT GTG GGA GAG GTA CC
Probe	CAG CTG GAC CAG CAC CCC ATT GAC
MMP-13	
Forw	CGG CCA CTC CTT AGG TCT TG
Rev	TTT TGC CGG TGT AGG TGT AGA TAG
Probe	CTC CAA GGA CCC TGG AGC ACT CAT GT
BMP-2	
Forw	AAC ACT GTG CGC AGC TTC C
Rev	CTC CGG GTT GTT TTC CCA C
Probe	CCA TGA AGA ATC TTT GGA AGA ACT ACC AGA AAC TG
Osteocalcin	
Forw	AAG AGA CCC AGG CGC TAC CT
Rev	AAC TCG TCA CAG TCC GGA TTG
Probe	ATG GCT GGG AGC CCC AGT CCC
Osteopontin	
Forw	CTC AGG CCA GTT GCA GCC
Rev	CAA AAG CAA ATC ACT GCA ATT CTC
Probe	AAA CGC CGA CCA AGG AAA ACT CAC TAC C
Runx2	
Forw	AGC AAG GTT CAA CGA TCT GAG AT
Rev	TTT GTG AAG ACG GTT ATG GTC AA
Probe	TGA AAC TCT TGC CTC GTC CAC TCC G
BSP II	
Forw	TGC CTT GAG CCT GCT TCC
Rev	GCA AAA TTA AAG CAG TCT TCA TTT TG
Probe	CTC CAG GAC TGC CAG AGG AAG CAA TCA

Osx and Dlx5:

Primers and Probes: Assays-On-Demand by Applied Biosystems

Osx: Hs00541729_m1

Dlx5: Hs00193291_m1

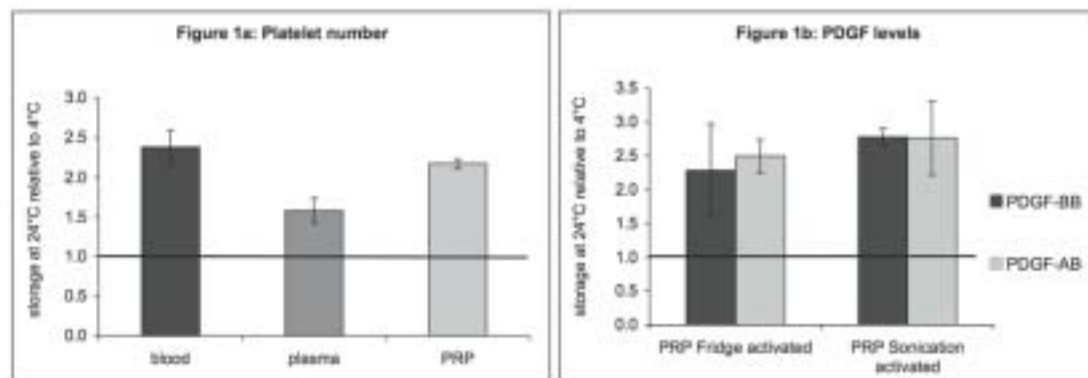
Probes were modified at the 5' end with the FAM fluorescent dye (6-carboxyfluorescein) and at the 3' end with the TAMRA fluorescent dye (6-carboxy-N,N,N',N'-tetramethylrhodamine). Assay-On-Demand probes had a non-fluorescent quencher at the 3' end.

Results

The average age of the patients in this study was 49 years (youngest 28, oldest 79). There were no obvious differences in the results obtained from the different donors (12 males, 4 females).

PRP production

Preparation: Two different common storage temperatures were tested for the blood aspirates, 4°C and room temperature (24°C). Aspirates that were stored at 24°C over night showed significantly higher intact platelet numbers in blood, plasma and PRP than aspirates that were stored at 4°C over night (Figure 1a). Also PDGF-AB and –BB protein levels were approximately 2.5x higher when the blood was stored at 24°C rather than at 4°C (Figure 1b).



Figures 1a/b: Comparison of storage temperatures for PRP (n=6): PRP stored at room temperature over night showed significantly higher ($P<0.05$) numbers of undamaged platelets than when stored at 4°C (a). Also, PDGF levels released by activated platelets that were stored at room temperature were significantly higher ($P<0.05$) than the levels released by platelets stored at 4°C (b).

Different combinations of centrifugation speeds were tested to obtain an optimal protocol for PRP preparation. A single-step protocol (150g, 10min) was compared to two 2-step protocols (30min at 200g followed by 5min at 200g and 30min at 200g followed by 5min at 2'000g). To better estimate the efficiency of the 2-step protocols, the number of the remaining platelets in PPP was also determined. A first centrifugation step at 200g for 30min at RT, followed by a second centrifugation step at 2'000g for 5min at RT showed the best platelet yield (Figure 2).

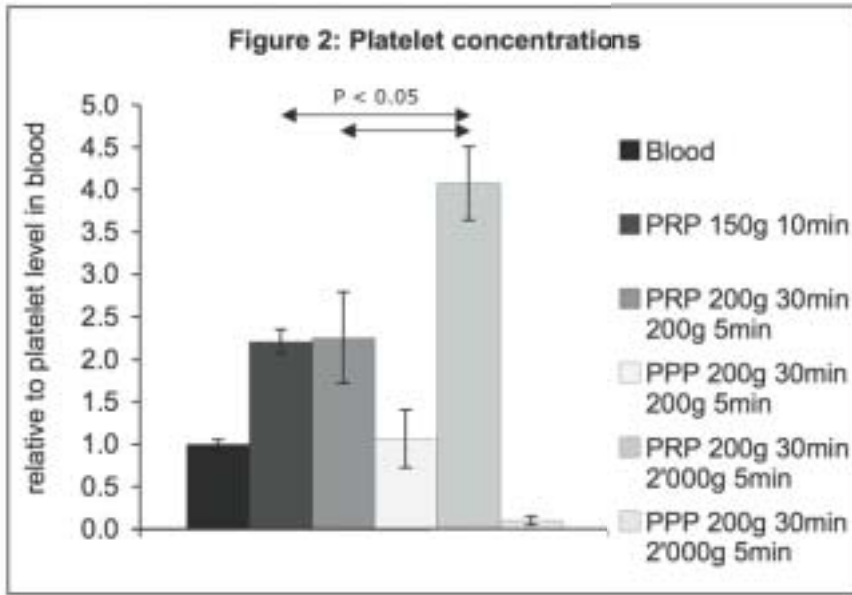


Figure 2: Comparison of different PRP-preparation protocols (n=6): Numbers of platelets were measured before and after different centrifugations. PRP-preparation using two consecutive centrifugation steps (200g for 30min followed by 2'000g for 5min) showed the highest platelet yield in PRP and almost no platelet leftovers in PPP.

PRP/PRGF Activation: Platelet activation was estimated by measuring the release of PDGF-AB, PDGF-BB and VEGF protein by the platelets upon appliance of different activation methods, without addition of CaCl₂ to avoid gel formation. The release of PDGF-AB and -BB upon activation was comparable for all activation methods (Figure 3). VEGF levels were extremely low and often not detectable at all (data not shown). When the same activation methods were used in the presence of CaCl₂, gels formed within 5-30min in all samples. The samples were then centrifuged at 18'000g for 3min to pellet the gel and the supernatant was used for the ELISA. Interestingly, PDGF levels were significantly lower in the presence of CaCl₂ than without (data not shown).

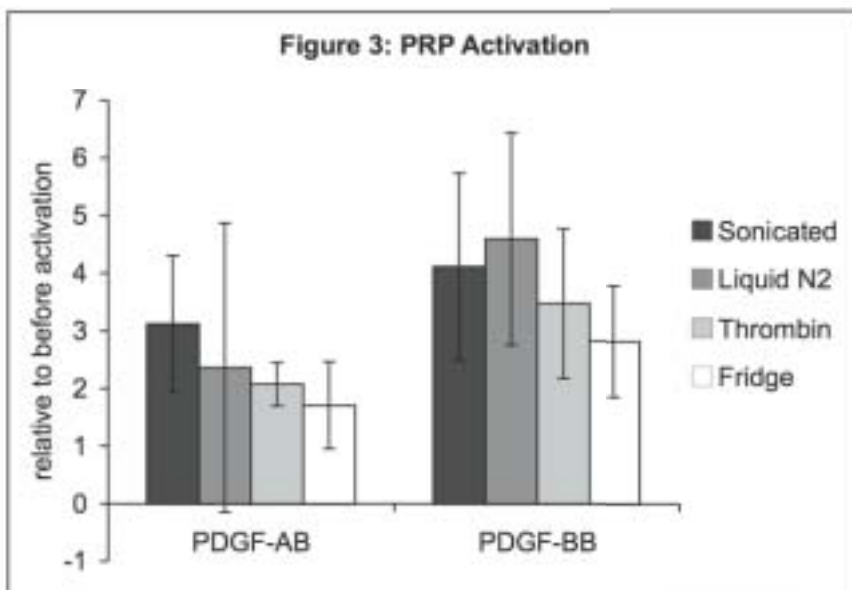


Figure 3: Comparison of different platelet activation methods (n=5): PRP activation efficiency was determined by the PDGF levels after activation. All tested methods resulted in similar PDGF levels, suggesting that all activation methods are equally effective.

There was no correlation between the platelet number in blood or PRP before activation and the resulting protein levels measured after activation (data not shown).

Effect on MSC

The activated PRP was centrifuged at 18'000g for 10min at RT to pellet the gel and the supernatant was used to supplement the culture medium at a 1:10 ratio. After the addition of PRP, the MSC culture medium showed again a strong tendency to coagulate after a few hours, even without CaCl₂ addition. We therefore supplemented MSC medium with PRGF (no fibrinogen), which did not show any coagulation. Comparison of the PDGF-AB and -BB levels in PRP and PRGF showed also very similar values, allowing us to conclude that the effect of PRGF on MSC should be analogous to that of PRP. So all experiments described below were carried out using PRGF (Figure 4).

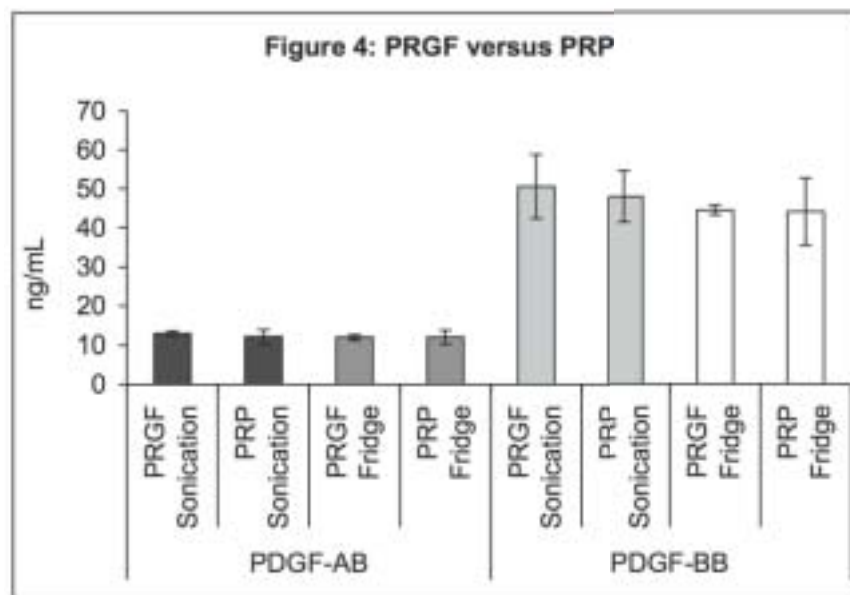


Figure 4: Comparison of PRP and PRGF protein levels upon activation (n=6): PRP and PRGF were activated using the sonication and the fridge methods and PDGF-AB and -BB levels were determined. There was no significant difference between PRP and PRGF. This justifies the replacement of PRP by PRGF as cell culture medium supplement.

Ca incorporation

The effect of PRGF on matrix mineralization by MSC was estimated by measuring ⁴⁵Ca²⁺ incorporation into the MSC extracellular matrix (Figure 5) and by Van Kossa staining (Figure 6). MSC grown in medium supplemented with PRGF showed an up-regulation of ⁴⁵Ca²⁺ incorporation by up to 400-fold compared to unsupplemented MSC. This effect was independent of the activation method used and was significantly higher than the effect of dexamethasone (Figure 5).

Van Kossa staining of MSC monolayers at day 14 showed slightly elevated calcium deposition in cultures supplemented with Dex (middle), compared to MSC grown without Dex (left). Supplementation with PRGF (right) resulted in a significant up-regulation of calcium deposition by MSC (Figure 6).

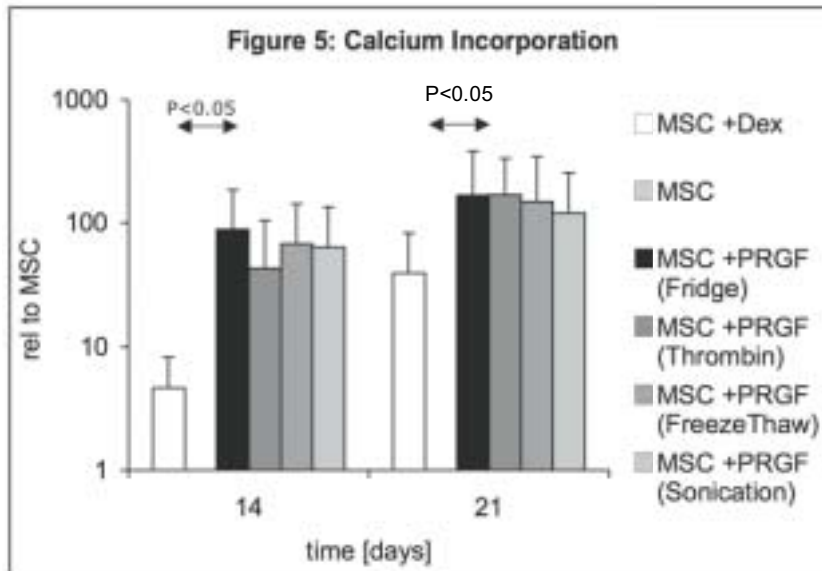


Figure 5: $^{45}\text{Ca}^{2+}$ incorporation by MSC at days 14 and 21, relative to unsupplemented (no PRGF, no Dex) MSC (n=9): MSC supplemented with Dex (MSC +Dex) served as a positive control. Supplementation of MSC with PRGF resulted in significantly higher calcium incorporation than MSC alone, even more than MSC +Dex. This result was independent of the PRGF activation method used.



Figure 6: Van Kossa staining of MSC monolayers at day 14: left: unsupplemented MSC, middle: MSC supplemented with Dex, right: MSC supplemented with PRGF.

ALP Activity

As expected, ALP activity in MSC supplemented with Dex was significantly higher than in MSC cultured without supplementation. Interestingly, PRGF did not show any effect on ALP activity of MSC (Figure 7).

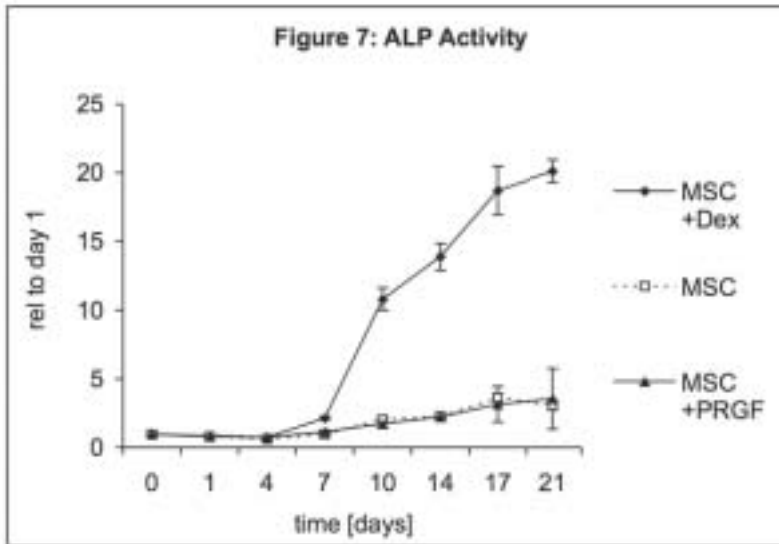


Figure 7: ALP activity of MSC (n=4): MSC supplemented with Dex (MSC +Dex) showed significantly elevated ALP activity compared to unsupplemented MSC (MSC). Supplementation of MSC with PRGF (MSC +PRGF) showed no effect of ALP activity.

Gene expression

The change in expression levels of several osteoblastic marker genes by MSC was analyzed. PRGF significantly increased the gene expression of Col1, BSP, ON, OP and BMP-2 in MSC at day 28 compared to MSC without PRGF (Figure 8). MMP-13 was expressed at very low levels in all cultures but was increased significantly by PRGF (data not shown). Interestingly, there was no significant effect of PRGF on the expression of the osteoblastic transcription markers Dlx5, Runx2 and Osterix (data not shown).

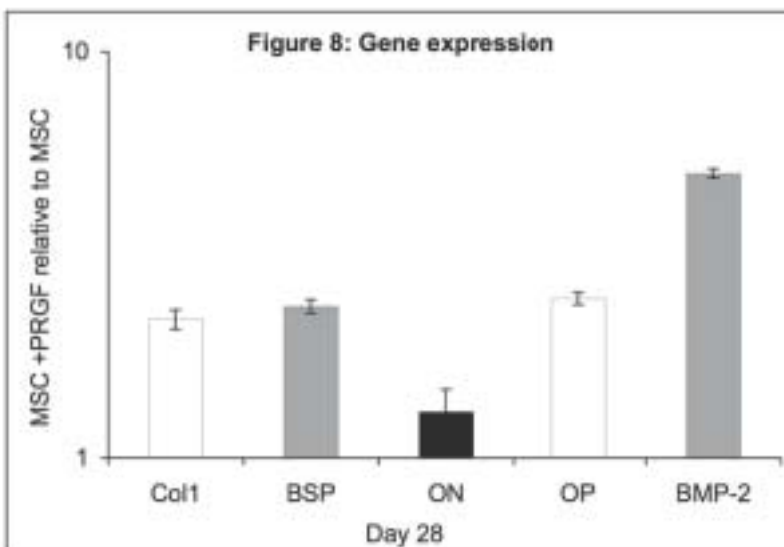


Figure 8: Gene expression of typical osteoblastic marker genes by MSC at day 28 (n=6): The type I collagen (Col1), bone sialoprotein (BSP), osteonectin (ON), osteopontin (OP) and bone morphogenetic protein 2 (BMP-2) were significantly ($P < 0.05$) up-regulated in MSC supplemented with PRGF. Data is expressed as mRNA levels in MSC+PRGF relative to unsupplemented MSC.

Dose dependency

We also evaluated how different concentrations of PRGF would affect MSC differentiation by measuring $^{45}\text{Ca}^{2+}$ incorporation. MSC culture media supplemented with 1% or 5% PRGF showed only a small increase (up to 2.6-fold) in $^{45}\text{Ca}^{2+}$ incorporation compared to the control. At 10% PRGF, a significant up-regulation (average of 288-fold) of $^{45}\text{Ca}^{2+}$ incorporation was observed, which did not show any further increase with 20% PRGF (Figure 9).

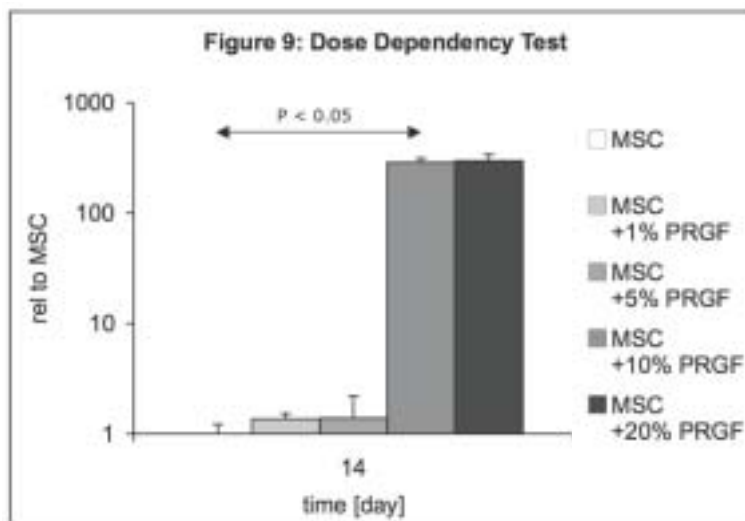


Figure 9:

Dose dependency assay using 1%, 5%, 10% and 20% (v/v) PRGF in MSC culture medium (n=3):

Supplementation of MSC culture medium with 1% and 5% PRGF did not result in a significant up-regulation of calcium incorporation.

However supplementation with 10% PRGF resulted in significant up-regulation of calcium incorporation.

Addition of 20% PRGF did not result in a further up-regulation.

Discussion

The aim of this study was to evaluate different protocols to isolate and activate platelet-rich plasma. Furthermore, we wanted to assess the possible influence of PRP on MSC differentiation *in vitro*. Regarding our first aim, our results showed that blood aspirates are best stored at room temperature until PRP is isolated. In fact, if PRP is conserved at 4°C for 24h, we observed a significant drop in platelet numbers and following activation, also lower PDGF protein levels. A possible explanation for this is that platelets have been shown to be activated upon exposure to 4°C [33-36] and that therefore the released factors could be lost during the subsequent PRP preparation. This also correlates with the common storage temperature of platelet concentrates at blood banks (20-24°C). PRP can easily be prepared by two consecutive centrifugation steps using 200g for 30min, followed by 2'000g for 5min, both at room temperature. It is very important to collect the entire plasma layer after the first centrifugation step, especially the lower parts, otherwise a large percentage of the platelets will be lost due to their close proximity to the leukocyte layer. The second centrifugation step should be rather short, 5min in our case, in order to facilitate the resuspension of the platelet pellet. Activation of the PRP is necessary to release the stored factors. This can be achieved by different methods, all of which resulted to be equivalent. The use of bovine thrombin for PRP activation is a widely used but rather controversial method due to its animal origin. On the other hand, the amount and the speed of PRP-gel formation can be very well controlled by this method. Repeated freeze-thaw cycles in liquid N₂ have the advantage of not using any debatable chemicals for activation, the disadvantage is that it is rather time consuming. Freezing the PRP at -20°C for >30min gives comparable results and is an easier PRP activation method, as is sonication. Overall, our activation method of choice was sonication, mainly due to the speed advantage. However, we realize that in a hospital setup, other methods might be preferred. Addition of CaCl₂ to the above activation methods leads to gelling of the PRP preparations in a controlled manner, normally within 30-60min. The use of CaCl₂ during platelet activation can have both advantages and disadvantages, depending on the application. In *in vivo* applications, the gelling effect is clearly useful. A PRP gel, implanted alone or in combination with cells seeded (or not) on a suitable scaffold, is not only a source of beneficial growth factors and cytokines, but it might also provide a matrix support (fibrin mesh) that could facilitate cell adhesion and cell-matrix interactions. However, for 2D *in vitro* studies, the gelling effect of PRP was a

major problem. We observed gelling of the PRP-supplemented MSC culture medium after less than 36h, even though the PRP preparation was activated without the addition of CaCl₂. We therefore had to supplement our culture media with platelet-released growth factors in PBS, thus avoiding the presence of fibrinogen and the subsequent gelling of the culture media. We validated this approach by showing that PRP and PRGF have comparable levels of PDGF-AB and -BB after activation by sonication and freezing at -20°C.

To estimate the effect that platelet released growth factors have on MSC differentiation towards the osteoblastic phenotype, we measured typical osteoblastic markers, including matrix mineralization, ALP activity and the expression of characteristic osteoblastic genes. The expression of the typical osteoblastic marker genes Col1, BSP, ON, OP and BMP-2 and MMP-13 was enhanced by PRGF suggesting that MSC were induced to differentiate into osteoblasts. As expected, the addition of the steroid dexamethasone also significantly increased calcium incorporation, but surprisingly to a lesser extent than PRGF. While calcium incorporation was greatly increased by the addition of PRGF after 21 days, PRGF had no effect on ALP activity. Studies have shown that BMP-2 and Dex highly increase matrix mineralization in human marrow-derived cells, but only Dex and not BMP-2 increases also ALP activity [37, 38]. We therefore suggest that PRGF is able to induce osteoblastic differentiation of MSC through a BMP-2 mediated pathway. This is also supported by highly up-regulated BMP-2 gene expression levels observed in PRGF-stimulated cultures.

In summary, we defined a quick, simple and yet efficient protocol for the production of platelet-rich plasma using common lab equipment. All the tested activation methods showed to be equally efficient, therefore the activation protocol can be chosen depending on the needs and on the available equipment. More importantly, we were able to show that PRP *in vitro* is able to induce MSC to differentiate into an osteoblastic phenotype. This osteoinductive potential of PRP might be useful to improve and accelerate bone healing, especially following infection, tumor resection, open fractures or non-unions, all of which are still major problems in today's orthopaedic clinics.

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Chapter V

***In vitro* evaluation of an endothelialized tissue engineered 3D-construct for bone repair**

Meury TR, Alini M

Finally, in the last experimental part of the thesis we combined all the observations and findings obtained and described in the previous chapters to develop a construct, which might serve as a bone graft for future *in vivo* experiments. By combining osteoblastic precursors, endothelial cells as well as PRP, we believe to have the necessary elements needed to accelerate the formation of blood vessels within the graft when implanted in an animal model of large bone defects, as well as the appropriate stimuli to induce MSC to differentiate into bone forming cells.

This chapter, following repetition of certain experiments, will lead to a manuscript to be submitted to *Tissue Engineering*.

Introduction

Autologous bone grafting is the current golden standard for the repair of large bone defects, despite its obvious drawbacks like limited availability of grafting material and donor site morbidity [1-4]. Possible alternatives are allografts or xenografts, but in addition to the problematic ethical aspects, these kinds of grafts also have serious limitations, like the risk of infections and possible immune reactions. Due to these problems with autografts, allografts and xenografts, researchers in the area of bone repair have explored alternative solutions. Calcium phosphate and hydroxyapatite based materials as well as polymer scaffolds have shown some interesting osteoconductive properties [5-10]. Nevertheless, the lack of osteoinductive potential very often prevents the perfect healing of the defect treated only with alloplastic materials. Many studies have shown that the lack of osteoinductive potential of these scaffolds can be partly overcome by seeding osteoblastic precursor cells into the scaffold prior to implantation. This approach resulted in a significant improvement of the healing process [6, 11-19]. However, not all problems have been solved yet and a major one remaining is the insufficient vascularization of the central part of such grafts when used in large bone defects [11].

We have therefore focused our studies on the interactions of endothelial cells and bone precursor cells. Our previous study showed that endothelial cells could modulate mesenchymal stem cell differentiation, by keeping them in a pre-osteoblastic state, while increasing their osteogenic potential (see chapter II). So the use of a combination of endothelial cells and bone precursor cells in an alloplastic bone construct could have a positive effect on the vascularization of the whole construct.

Early events during natural bone repair include the formation of a blood clot at the damaged site resulting in the attraction of many different cell types to the repair site. This initial recruitment is mainly mediated by factors released from the activated platelets present within the blood clot. In an earlier study (see chapter IV), we were able to show that activated platelet-rich plasma (PRP) has the potential to strongly promote osteoblastic differentiation of mesenchymal stem cells (MSC) in monolayer. This osteoblastogenesis-promoting property of PRP combined with its ability to form a gel upon activation as well as its richness in factors known to be involved in angiogenesis strongly suggest, that PRP could represent the ideal autologous biological stimuli necessary to improve angiogenesis and bone formation within a construct seeded with the appropriate cells.

We therefore studied the potential of a complex 3D construct composed of a polyurethane scaffold seeded with MSC and EC embedded in a PRP gel in a controlled *in vitro* environment.

Endothelial cell markers

During this study, we evaluated several markers related to angiogenesis and endothelial differentiation:

Von Willebrand factor (vWf, factor VIII-related antigen) is a cell surface glycoprotein necessary for adhesion of platelets to the sub-endothelium upon vascular rupture [20]. It also plays an important role in the interactions between endothelial cells and the surrounding matrix [21]. vWf is often used as an endothelial differentiation marker due to its very high specificity and to its increasing expression by endothelial cells during maturation of the microvasculature [22].

VE-cadherin (CDH5, CD144) is also an endothelial surface marker involved in cell-cell adhesion through intercellular junctions. It is specific for vascular endothelium and is suggested to be a later marker of endothelial cell differentiation *in vitro* [23].

EGFL7 (EGF-like domain 7, VE-statin) is expressed at high levels by endothelial cells during vascular remodeling and is down-regulated in mature vessels [24].

MMP-2 (72kDa-Gelatinase, Gelatinase A) and MMP-9 (92kDa-Gelatinase, Gelatinase B) are extracellular endopeptidases that are highly involved in angiogenesis. Together they are able to break down most extracellular matrix components including the collagen types I, II, IV, X, as well as gelatin, elastin, aggrecan, fibronectin, osteonectin and laminin [25-30]. They are suggested to play a major role in tissue remodeling and repair as well as in tumors metastases. MMP-2 has also been shown to be able to activate MMP-9 and MMP-13 [31, 32].

VEGF receptors-1 (Flt-1), -2 (Flk-1, KDR) and -3 (Flt-4) are all closely related receptor tyrosine kinases expressed in the vascular endothelium. VEGFR-2 seems to mediate the major actions of VEGF, whereas VEGFR-1 is suggested to have a negative role (either as a decoy receptor, or by suppressing signaling through VEGFR-2). VEGFR-3 is the least understood VEGF receptor and may be important during blood vessel development, but also seems to be critical for lymphatic vessel formation.

Tie1 and Tie2 (TEK) are receptors for angiopoietins (receptor tyrosine kinases). They are selectively expressed in the vascular endothelium [33, 34]. All known angiopoietins bind primarily to Tie2 while the role of Tie1 is still unclear. Both are highly expressed in EC during vessel formation and remodeling.

Platelet-derived growth factor receptor beta polypeptide (PDGFRB, CD140B) is a receptor for PDGF, which has been reported to promote angiogenesis.

Endothelin-1 (ET-1) is a potent vasoconstrictor known to be involved in the interactions of endothelial cells and osteoblastic cells. ET-1 has been shown to enhance proliferation and differentiation of osteoprogenitors cells [35].

Laminin: Laminin is an important component of the basal lamina. Laminin is linked to the vimentin protein located in the cytoskeleton of endothelial cells, which appears to play an important role in endothelial cell migration and in blood vessel morphogenesis [36].

CD31 (PECAM-1) is an integral membrane protein that mediates cell-to-cell adhesion. CD31 is expressed constitutively on the surface of adult and embryonic endothelial cells and is weakly expressed on platelets. CD31 mediated endothelial cell-cell interactions are involved in angiogenesis [37].

Materials and Methods

Origins of human cells

Aspirates of bone marrow and blood were obtained from patients undergoing hip surgery after informed consent (KEK Bern 126/03). Fifty milliliters of bone marrow and 100mL of blood aspirates were collected into CPDA-containing Sarstedt monovettes. Bone marrow aspirates were stored at 4°C and blood at room temperature (RT). Samples were processed within 24h after harvesting.

Isolation and expansion of human MSC

After homogenization of the bone marrow aspirates by pushing them a few times through a syringe, they were diluted 1:4 with IMDM (Gibco 42200-022) containing 5% (v/v) FBS (Gibco 10270-106) and were centrifuged at 200g for 5min at room temperature (RT). The fat-containing top layer was removed. Per 1mL of undiluted sample, 2.6mL of Ficoll (Histopaque-1077, Sigma #1077-1) were pipetted into a 50mL Falcon tube and the aspirate was added carefully on top of the Ficoll. After centrifugation at 800g for 20min at RT with lowest possible brake-settings, the mono-nucleated cells were collected at the interphase using a syringe. To 1mL of collected interphase solution, 5mL of IMDM/5%FBS were added, the tube was gently mixed and centrifuged at 400g for 15min at RT. The resulting pellet was resuspended in the same amount of IMDM/5%FBS, centrifuged again and resuspended in IMDM/5%FBS. Cell number was determined using Methylene Blue in a hemocytometer. The cells were seeded at densities of 15-20x10⁶ mono-nucleated cells per 300cm² T-flask in IMDM containing 10% FBS, nonessential amino acids (Gibco 11140-035) and PenStrep (100U/mL, Gibco 15140-122). After 5 days, the monolayers were washed with Tyrode's balanced salt solution (TBSS) to remove non-adherent cells, and fresh medium supplemented with 5ng/mL b-FGF (R&D 233-FB) was added [38-40]. Medium was changed twice a week and cells were subcultured 1:4 at sub-confluence. The adherent cells after one subculture were termed Mesenchymal stem Cells (MSC). Only cells between passages 2-5 (approx. 12-22 populations doublings) were subsequently used [41].

Expansion of HUVEC

Primary human umbilical vein endothelial cells (HUVEC) were purchased from Cascade Biologics (cat# C-003-5C). The cells were expanded in M200 (Cascade M200-500) supplemented with low serum growth supplement (S-003-10),

which contains fetal bovine serum, hydrocortisone, human epidermal growth factor, b-FGF and heparin. Cells were subcultured 1:4 at sub-confluence and passages 2-5 were subsequently used.

3D-constructs

Isosorbide polyurethane scaffolds (termed IsoK-40 and IsoK-43) were prepared using a combined salt leaching – phase-inverse technique. Salt crystals with particle sizes in the range of 200 – 600 μ m were used as a porogen. IsoK-40 and -43 were identical, except that IsoK-43 additionally contained 25% of nano-size hydroxyapatite with an average particle size of 20nm (Advanced Biomaterials, Inc., Berkeley, USA). The scaffolds had interconnected pores with average pore size of 500 μ m and pore to volume ratio of 90%. The presence of hydroxyapatite had no effect on the porous structure of the sponges. The scaffolds were gas-sterilized prior to be used.

PRP production

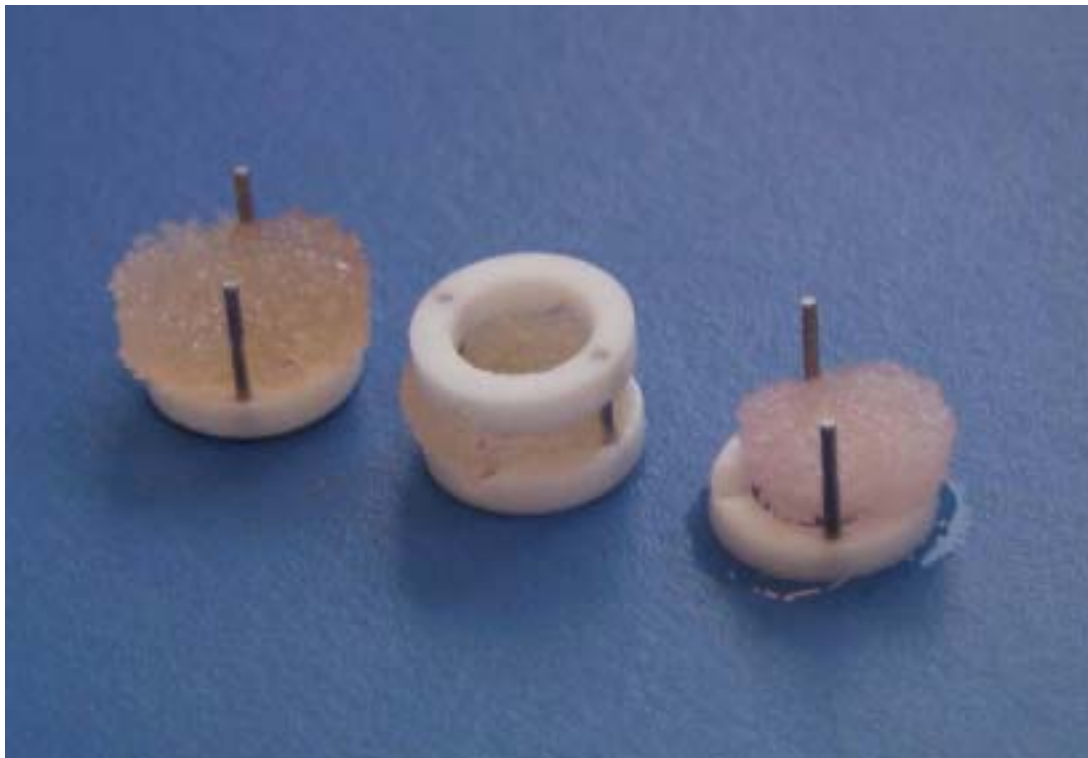
The blood aspirates were transferred from the CPDA-cuvettes into 15mL Falcon tubes, and were centrifuged at 200g for 30min at RT. The resulting plasma supernatants were pooled, transferred into a new 15mL Falcon tube, and centrifuged at 2'000g for 5min at RT to get a platelet pellet. Platelet-rich plasma (PRP) was produced by resuspending the resulting pellet in the remaining plasma supernatant (1/10th of the initial blood volume). PRP was used stored at -20°C until used.

Cell seeding on scaffolds

After sterilization, the scaffolds were degassed in IMDM under vacuum over night. The next day the liquid in the scaffolds was removed using vacuum and the scaffolds were ready to be loaded with cells:

MSC or HUVEC were trypsinized, washed twice (centrifuging at 380g for 10min at RT and resuspending in IMDM/FBS) and counted using Trypan Blue and a hemocytometer. After counting, the cells were centrifuged and resuspended in PRP (see “PRP production”) or fibrin sealant (Baxter Biosurgery, Vienna) at a concentration of 5x10⁶ cells/150 μ L. Fibrin sealant consisted of the fibrinogen component (diluted to final concentration 3.3mg/mL using proprietary dilution buffers provided by Baxter) and a thrombin component. Just before being loaded into the scaffolds, the cell/fibrin suspension was activated by addition of 15 μ L of the thrombin

component (final concentration 5U/mL). Cells in PRP were activated using 15 μ L of bovine thrombin (final concentration 5U/mL, Sigma T-4648) dissolved in PBS or 100mM CaCl₂ (10%). Quickly, the cell/fibrin or cell/PRP was pipetted into a sterile Eppendorf tube cap (cut off from the Eppendorf tube and turned upside down) and the scaffold was added on top of it. Since the inner part of the cap has a diameter just slightly larger than the scaffold, it provides a loading chamber that seals all sides of the scaffold but the top. So by carefully adding a bit of pressure to the top of the scaffold using forceps, the cell suspension located below the scaffold was sucked into the scaffold upon pressure release. Due to the thrombin component, the suspension gelatinized in the scaffold within a few minutes. The scaffolds were incubated in the Eppendorf tube caps for 30min at 37°C. The samples were then moved to 6-well plates and were cultured in IMDM containing 10% FBS, nonessential amino acids, PenStrep and 500KIU/mL Aprotinin from bovine lung (Fluka #10820) to avoid fibrin gel degradation [42]. Sandwich constructs were kept together using PTFE-rings (polytetrafluoroethylene = Teflon®).



Polyurethane scaffolds fixated by PTFE-rings

RNA Isolation and Reverse Transcription

The scaffolds containing cells in fibrin or PRP were pulverized in liquid nitrogen and total mRNA was extracted using 1mL of TRI-Reagent (MRC Inc. TR-118) with 5 μ L/mL of Polyacryl-carrier (MRC Inc. PC-152) in Eppendorf tubes. 150 μ L of 1-Bromo-3-Chloro-Propane were added, the tubes were vortexed for 15sec and then centrifuged at 12'000g for 15min at 4°C. The colorless layer on the top was transferred into a new tube and 750 μ L of isopropanol were added, then the tube was centrifuged at 12'000g for 10min at 4°C. Sample supernatants were removed and pellets were washed in 1mL of 75% EtOH by vortexing and centrifuging at 10'000g for 5min at 4°C. Pellets were air-dried and re-suspended in 30 μ L of DEPC-treated water. After 15min at 60°C, tubes were immediately transferred to ice. The total RNA amount and purity was assessed by measuring the absorbance at 260nm and 280nm. Reverse transcription was performed using 1 μ g of total RNA sample, which was mixed with 2 μ L of 10x TaqMan RT Buffer (500mM KCl, 100mM Tris-HCl, pH 8.3), 4.4 μ L of 25mM magnesium chloride, 4 μ L of dNTP mixture (2.5mM of each dNTP), 1 μ L of random hexamers (50 μ M), 0.4 μ L of RNase inhibitor (20U/mL) and 0.5 μ L of MultiScribe Reverse Transcriptase (50U/ μ L) (all from Applied Biosystems); DEPC-treated water was added to bring the final reaction volume to 20 μ L. Reverse transcription was performed using a Thermal Cycler 9600 by Applied Biosystems. cDNA samples were diluted to 250 μ L with Tris-EDTA buffer before being used for real-time RT PCR.

Real-time RT PCR

Oligonucleotide primers and TaqMan probes were either purchased from Applied Biosystems (Assay-On-Demand) or were designed using the Primer Express Oligo Design software (Ver. 1.5, Applied Biosystems). The nucleotide sequences were obtained from the GenBank database and the probes were designed to overlap an exon-exon junction in order to avoid amplification of genomic DNA. Primers and probes for amplification of 18S ribosomal RNA, used as endogenous control, were from Applied Biosystems. All other primers and labeled TaqMan probes were from Microsynth (Balgach, CH). TaqMan probes were labeled with the reporter dye molecule FAM (6-carboxyfluorescein) at the 5' end and with the quencher dye TAMRA (6-carboxy-N, N, N', N'-tetramethylrhodamine) at the 3' end. The PCR reaction mixture using self-designed primers and probes contained TaqMan Universal PCR master mix without AmpErase UNG (Applied Biosystems), 900nM primers (forward and reverse), 250nM TaqMan probe, and 2Al of cDNA sample for a

total reaction volume of 25 μ l. Assays-On-Demand oligonucleotides were mixed according to the manufacturers protocol. PCR conditions were 95°C for 10min, followed by 42 cycles of amplification at 95°C for 15sec and 60°C for 1min using the GeneAmp 5700 Sequence Detection System (Applied Biosystems, Foster City, CA). Relative quantification of mRNA targets was performed according to the comparative C_T method with 18S ribosomal RNA for MSC and p0 (large ribosomal protein) for HUVEC as endogenous controls (Table I).

Table I. Primers and Probes for Real-Time RT-PCR

see chapter II, Table I:

BMP-2

Collagen type I

MMP-13

Osteocalcin

Osteonectin

Osteopontin

Runx2

Assays-On-Demand by Applied Biosystems:

ALP: Hs00758162_m1

Ang1: Hs00181613_m1

Dlx5: Hs00193291_m1

EGFL7: Hs00211952_m1

MMP-2: Hs00234422_m1

MMP-9: Hs00234579_m1

Osx: Hs00541729_m1

p0: Hs99999902_m1

PDGFRB: Hs00182163_m1

Tie1: Hs00178500_m1

Tie2: Hs00176096_m1

VE-cadherin: Hs00174344_m1

VEGFR1: Hs00176573_m1

VEGFR2: Hs00176676_m1

VEGFR3: Hs00176607_m1

vWf: Hs00169795_m1

Self designed probes were modified at the 5' end with the FAM fluorescent dye (6-carboxyfluorescein) and at the 3' end with the TAMRA fluorescent quencher (6-carboxy-N,N,N',N'-tetramethylrhodamine). Assay-On-Demand probes had a non-fluorescent quencher at the 3'end.

Preparation of cryosections

The grafts were fixed in pure Methanol for one week at 4°C, were transferred to 5% Sucrose in PBS over night and were cryosectioned at 6-10µm using Microm embedding medium (Walldorf Switzerland, cat# 350100), a Cryostat-Microtome HM 560 OMV (Carl Zeiss AG, Zürich, CH) and SuperFrost Plus GOLD microscope slides (Menzel-Gläser, Braunschweig, De). The slides were left to dry at -20°C for at least 2 days.

Toluidene Blue and van Kossa staining

The dried slides were stained with Toluidene Blue (1% Toluidene Blue in 1% sodium tetraborate in ddH₂O, filtered) for 15min at RT, and then rinsed 2x in dH₂O. Van Kossa stainings were performed by incubating the slides in 5% Silver nitrate for 60min under natural light, rinsing with dH₂O, incubating in 5% sodium thiosulfate for 10min, rinsing with dH₂O and counterstaining with Nuclear Fast Red followed by rinsing with dH₂O. Coverslips were mounted using aqueous mounting solution (Hydromount HS-106 by National Diagnostics, Atlanta, GA, USA).

Immunohistochemistry

The dried sections were encircled using a hydrophobic DakoPen (Cat. No. S-2002, Dako, Glostrup, DK) and were left to rehydrate in PBS-Tween (0.32g/L NaH₂PO₄, 1.42g/L Na₂HPO₄, 9g/L NaCl, 0.1% Tween-20, pH 7.4) for 5min. Non-specific binding was reduced by incubating the samples in horse serum (diluted 1:20 in PBS, by Vector Labs, USA) for 60min at RT. The primary antibody (polyclonal mouse anti-human Laminin 2E8 by Developmental Studies Hybridoma Bank; polyclonal rabbit anti-human vWf: Dako A 0082) was added in the appropriate dilution (Laminin 1:5, vWf 1:400) and the samples were incubated for 3h at RT. PBS-Tween was used as control. The samples were washed 3x with PBS-Tween. The secondary antibody (Laminin: horse anti-mouse IgG antibody by Vectastain cat# PK6102; vWf: donkey anti-rabbit IgG antibody by RDI cat# RDI-711065152) was added at 1:200 dilution and the samples were incubated for 30min at RT, before being washed 3x with PBS-Tween again. Fresh ABC-complex (Vectastain ABC-kit Elite, cat# PK6102) was added and the samples were incubated for 30min at RT, before being washed 3x with PBS-Tween. DAB (Vector Laboratories cat# SK-4100) was added and the samples were incubated in the dark for 4min at RT. The samples were then counterstained in Mayer's haematoxylin for 3min (Merck 15-938) and were then

rinsed twice in water for 5min each. Coverslips were mounted using aqueous mounting solution (Hydromount HS-106 by National Diagnostics, Atlanta, GA, USA).

Immunofluorescence

The dried sections were encircled using a hydrophobic DakoPen and were left to rehydrate in PBS-Tween (0.32g/L NaH₂PO₄, 1.42g/L Na₂HPO₄, 9g/L NaCl, 0.1% Tween-20, pH 7.4) for 5min. Non-specific binding was reduced by incubating the samples in horse serum (diluted 1:20 in PBS, by Vector Labs, USA) for 60min at RT. The primary antibody (monoclonal mouse anti-human CD31 antibody by R&D cat#BBA7; polyclonal rabbit anti-human Osf2 by BioVendor cat#RD181045050) was added in the appropriate dilution (CD31 1:1000, Osf2 1:400) and the samples were incubated for 3h at RT. PBS-Tween was used as control. The samples were then washed 3x with PBS-Tween before being incubated with the fluorescent secondary antibody (CD31: CY3 sheep anti-mouse IgG antibody at 1:50 dilution by Sigma cat#C2181, Osf2: Alexa Fluor 488 goat anti-rabbit IgG antibody at 1:400 dilution by Molecular Probes cat#A11008) for 30min at RT. The samples were washed and coverslips were mounted using Prolong Gold Antifade reagent (Molecular Probes cat#P36930) and the samples were stored in the dark until being analyzed.

Cell viability stain

The culture medium was removed from the wells and the scaffolds were incubated in fresh IMDM containing 1 μ L/mL EthD1 (Fluka, cat# 46043), diluted from a stock solution of 1mg/ml EthD1 in 20% DMSO (Fluka cat# 46043) for 10min at 37°C. After washing with IMDM, the scaffolds were incubated in fresh IMDM containing 1 μ L/mL calcein AM (Fluka, cat# 11783), diluted from a stock solution of 1mg/ml calcein AM in 100% DMSO for 20min at 37°C. After washing, IMDM containing 5% FBS was added to the wells and the scaffolds were analyzed by fluorescence microscopy [43]. In this assay, living cells are distinguished from dead cells by the presence of ubiquitous intracellular esterase activity, determined by the enzymatic conversion of the non-fluorescent calcein AM to intensively fluorescent calcein. Calcein is well retained within living cells, producing an intense uniform green fluorescent light (ex/em ~495nm / ~515nm). Dead cells were stained using Ethidium homodimer-1 (EthD1), which only enters the cell with damaged cell membranes and undergoes a 40-fold enhancement of its fluorescence upon binding to nucleic acids, thereby producing a bright red fluorescence in dead cells (ex/em ~495nm / 635 nm).

Microscopical analysis

All histological sections were analyzed using a Zeiss Axioplan 2 Microscope, a Zeiss AxioCam camera and Zeiss Axiovision software. Zeiss filtersets 10, 15 and 25 were used for fluorescence analysis.

Statistics

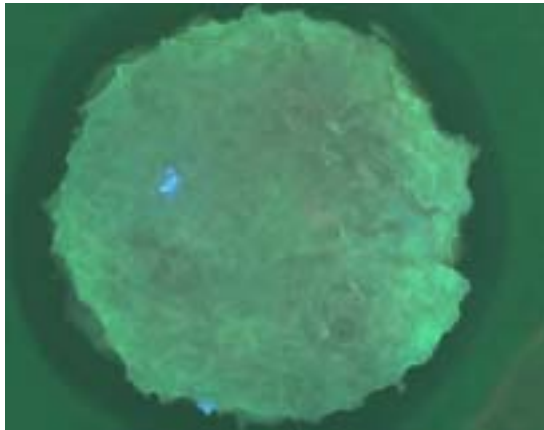
Results are shown as mean \pm SEM of triplicate samples of two independent experiments (n=6). Statistical analyses were performed using the non-parametric Mann-Whitney U-test, which compares the medians of two independent distributions. P<0.05 was considered to be statistically significant.

Results

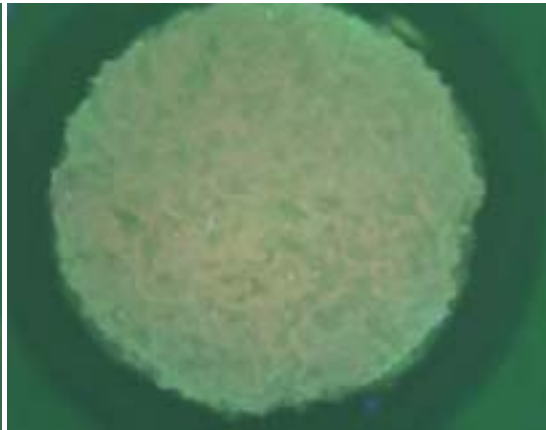
Choice of scaffold

Two types of polyurethane scaffolds were compared in order to select the better one for the 3D-experiments. IsoK-43 was of equal composition as IsoK-40 but contained an additional ceramic component. After culturing human MSC or HUVEC in the two different scaffolds for 7 days, cell viability was determined using EthD1/calcein staining. Microscopic analysis showed, that both scaffolds supported attachment of MSC and HUVEC. But while IsoK-40 was a clearly hospitable environment for both cell types after 7 days, the IsoK-43 environment induced cell death in a large fraction of the attached MSC and HUVEC. The IsoK-40 scaffold was therefore chosen for the following 3D-experiments.

Green cells are living, red cells are dead:



IsoK-40 seeded with MSC (1.25x)



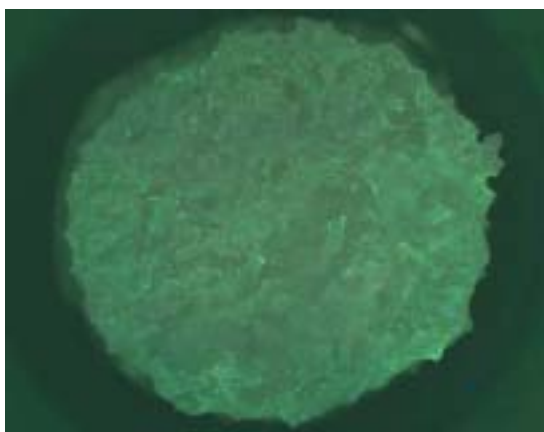
IsoK-43 seeded with MSC (1.25x)



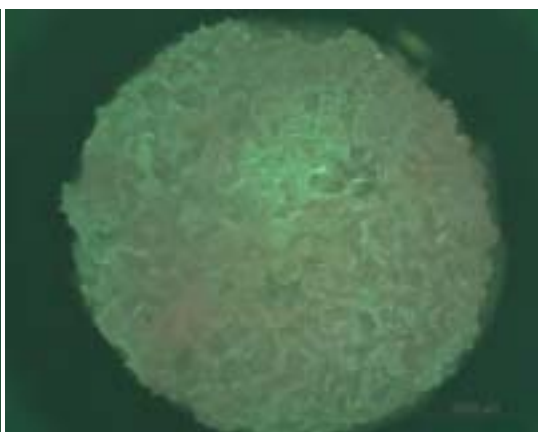
IsoK-40 seeded with MSC (2.5x)



IsoK-43 seeded with MSC (2.5x)



IsoK-40 seeded with HUVEC (1.25x)



IsoK-43 seeded with HUVEC (1.25x)



IsoK-40 seeded with HUVEC (2.5x)

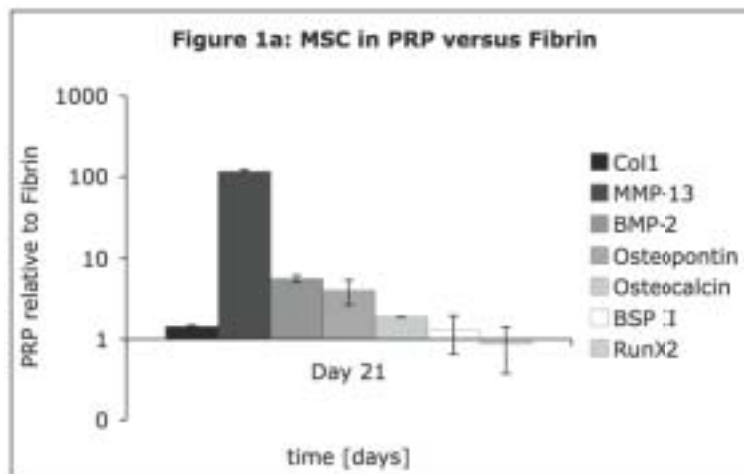


IsoK-43 seeded with HUVEC (2.5x)

PRP vs. Fibrin

A common method to seed cells into a scaffold and to retain them inside is the use of a fibrin sealant. Fibrin is mixed with cells and forms a gel within the scaffold upon thrombin addition, therefore keeping the cells from being washed out into the culture medium before their proper attachment to the scaffold. An easy-to-obtain alternative to fibrin sealant could be PRP, which would have the additional advantage of containing autologous growth factors and cytokines. We therefore evaluated the performance of PRP versus fibrin sealant as a carrier for MSC or HUVEC seeded into IsoK-40 scaffolds by measuring the expression of typical osteoblastic and endothelial genes using real-time RT-PCR.

MSC generally performed better in PRP than in fibrin, as shown by significantly elevated gene expression of Col1, MMP-13, BMP-2, OP and OC. BSP11 and Runx2 were expressed at similar levels in both PRP and Fibrin. Osterix expression by MSC could not be detected after 21 days culture time in PRP or fibrin (Figure 1a).

**Figure 1a:**

Gene expression of MSC seeded on IsoK-40 scaffolds using PRP or fibrin sealant: Typical osteoblastic marker genes (MMP-13, BMP-2, OP, OC) showed significantly ($P < 0.05$) elevated expression levels when MSC were cultured in PRP rather than in fibrin sealant.

HUVEC also performed better in PRP than in fibrin gel, as shown by the significantly increased expression of the endothelial markers vWf, VE-cadherin, VEGFR2, Tie1, Tie2, MMP-2 and BMP-2 (Figure 1b). MMP-9 could not be detected after 21 days of culture.

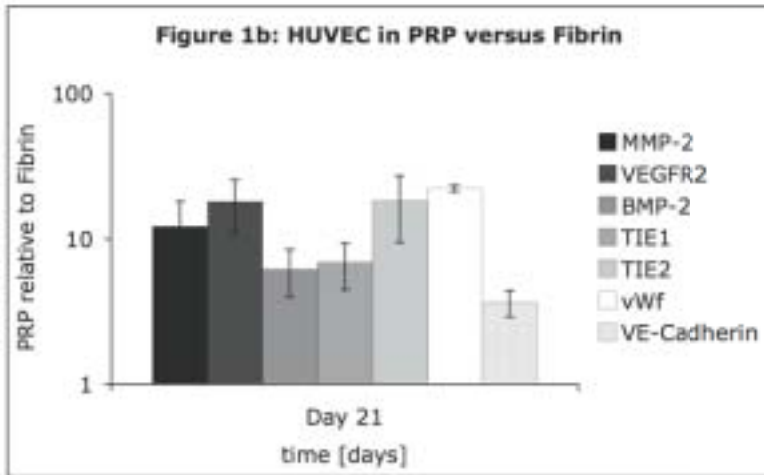
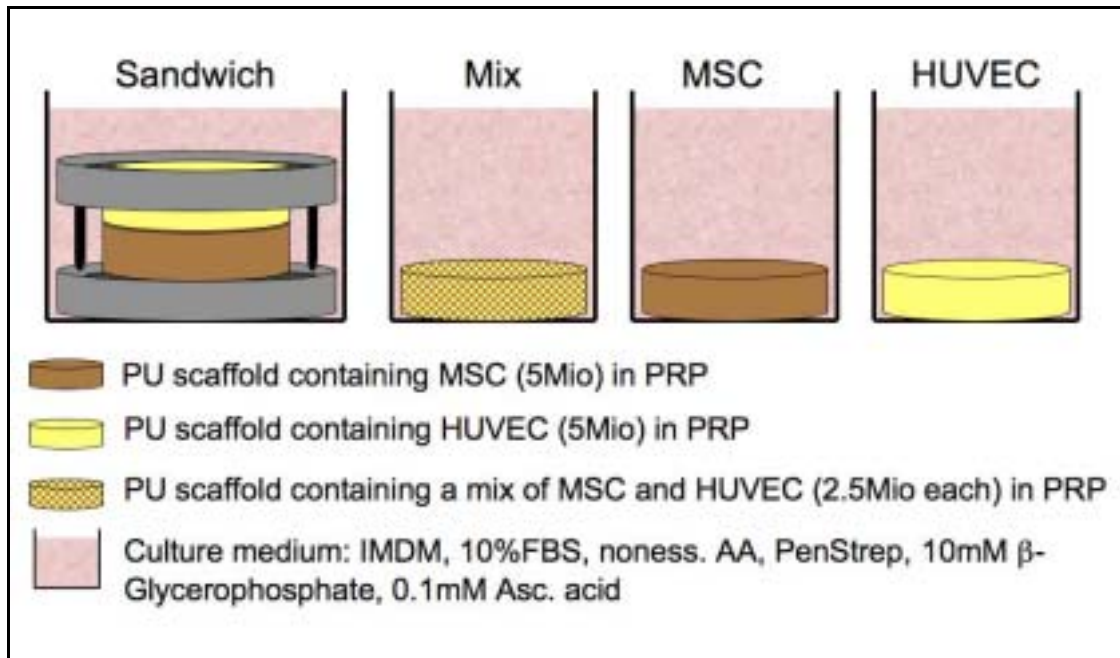


Figure 1b: Gene expression of HUVEC seeded on IsoK-40 scaffolds using PRP or fibrin sealant: All measured endothelial marker genes were expressed at significantly ($P < 0.05$) higher levels when HUVEC were cultured in PRP than in fibrin gel.

In summary, we were able to show, that PRP significantly up-regulated the expression of typical osteoblastic genes in MSC compared to fibrin gel. This would correlate with our earlier findings, in which PRP was able to enhance osteoblastic differentiation of MSC in monolayer (see chapter IV). Furthermore, PRP also significantly enhanced the expression of endothelial cell specific markers in EC when compared to EC in fibrin gel. It was therefore decided to use the polyurethane scaffold type IsoK-40 together with PRP as a carrier of cells and biological stimuli.

Experimental setup for different 3D grafts of MSC and HUVEC

Four 3D-constructs using two different kinds of co-cultures of MSC and HUVEC seeded on polyurethane scaffolds in combination with PRP were evaluated (see Scheme below). The indirect co-culture type was a sandwich construct with the two scaffolds stacked on top of each other and held in place by a PTFE-ring (SW-construct). The top scaffold was seeded with HUVEC in PRP, the bottom one with MSC in PRP. The direct contact co-culture type was a mixture of both cells types in PRP seeded together into one scaffold (Mix-construct). Scaffolds containing MSC or HUVEC alone were used as controls. Total cell numbers were equivalent in all constructs.



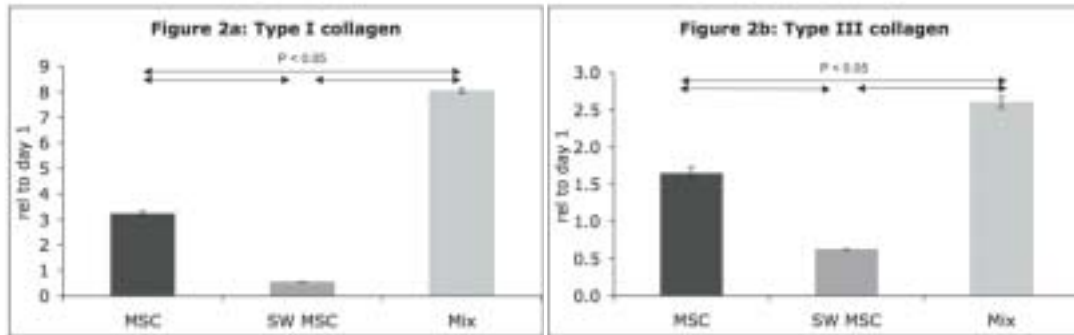
Scheme of experimental setup of the 3D co-cultures of MSC and HUVEC

Evaluation of gene expression of MSC and HUVEC in 3D constructs

In order to evaluate the different co-culture types, we compared the expression of typical genes involved in osteoblastic and endothelial cell differentiation, as well as in bone repair and angiogenesis.

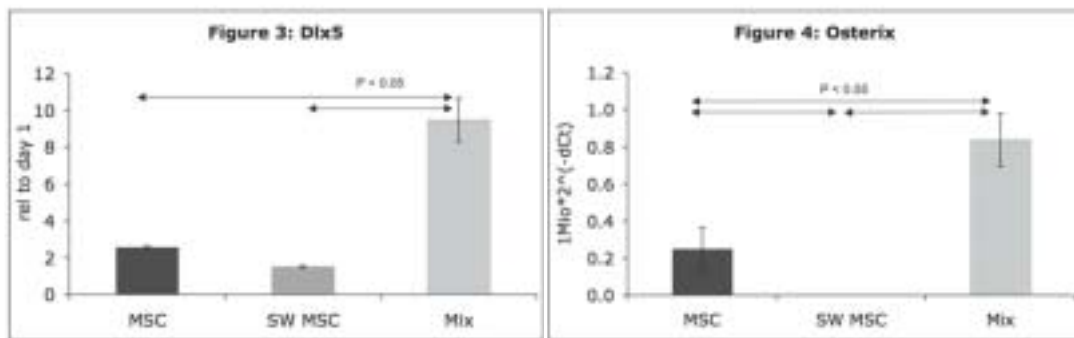
For the scaffolds containing MSC, the following genes were evaluated: Type I collagen (Col1), type III collagen (Col3), collagenase 3 (MMP-13), osteopontin (OP), osteocalcin (OC), bone sialoprotein II (BSP), alkaline phosphatase (ALP), distal-less homeobox 5 (Dlx5), runt-related transcription factor 2 (Runx2), Osterix (Osx), bone morphogenetic protein 2 (BMP-2) and vascular endothelial growth factor (VEGF).

Col1 and Col3 expression increased over time (35 days) in all cultures except in Sandwich-MSC, where Col1 and Col3 were down-regulated. However, in Mix-MSC both were significantly up-regulated compared to MSC (Figures 2a and 2b).



Figures 2a and 2b: Gene expression of types I and II collagen by MSC at day 35

Similar results were observed for the transcription factors Dlx5 and Osx: Down-regulation in Sandwich-MSC and significant up-regulation in Mix-MSC at day 35.



Figures 3 and 4: Gene expression of Dlx5 and Osterix by MSC at day 35: There was no Osx detectable at day 1, therefore Osx expressions is not normalized to day1.

At day 35, MMP-13 was similarly up-regulated in MSC and Mix-MSC compared to day 1. MMP-13 levels in Sandwich-MSC however were down-regulated to almost undetectable levels (Figure 5).

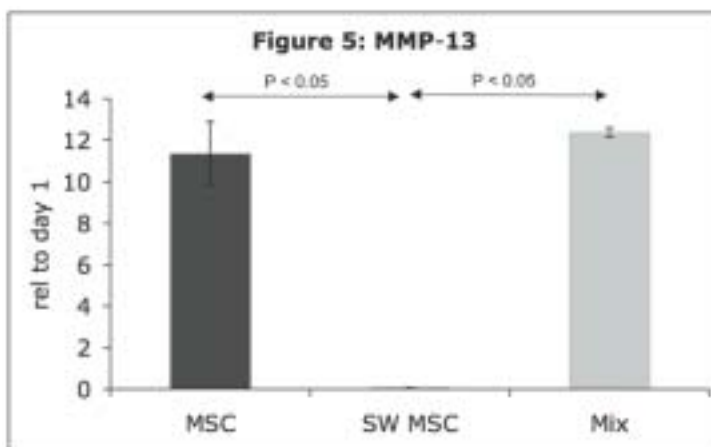


Figure 5: Gene expression of MMP-13 by MSC at day 35

Runx2 expression did not change over time in MSC and Sandwich-MSC, but was significantly up-regulated in Mix-MSC.

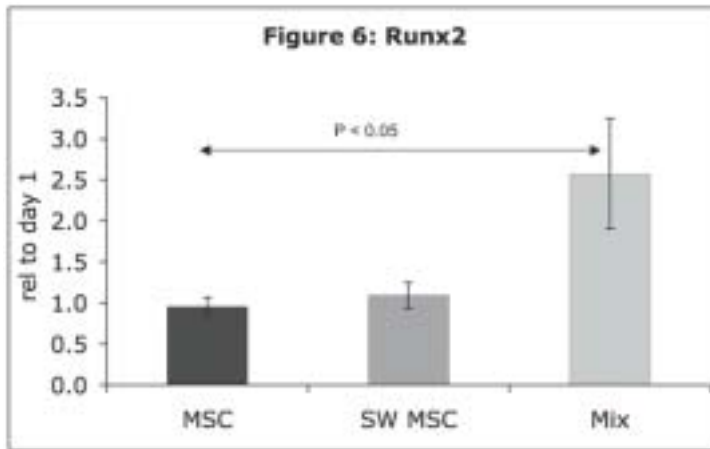


Figure 6:
Gene expression of Runx2 by MSC at day 35

BMP-2 expression decreased over time in both MSC and Sandwich-MSC cultures, but increased clearly in Mix-MSC.

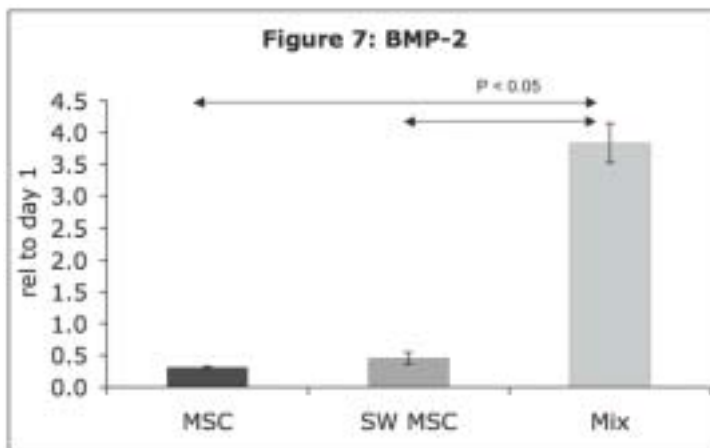


Figure 7:
Gene expression of BMP-2 by MSC at day 35

ALP gene expression increased in all cultures over time. However, while MSC and Sandwich-MSC showed a 3-4x increase at day 35 compared to day 1, Mix-MSC showed more than 14-fold increase.

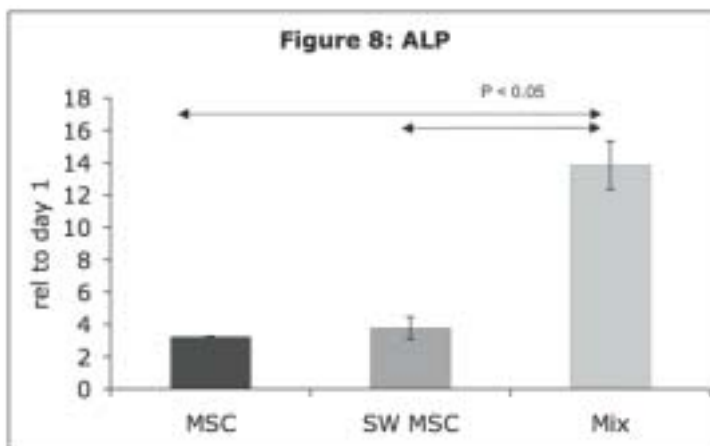


Figure 8:
Gene expression of alkaline phosphatase by MSC at day 35

Total VEGF expression decreased in all cultures over time except in Sandwich-MSc, where VEGF increased slightly compared to day 1.

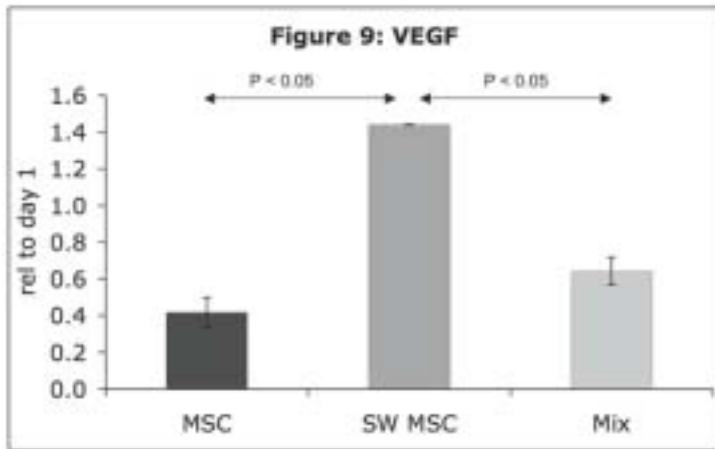
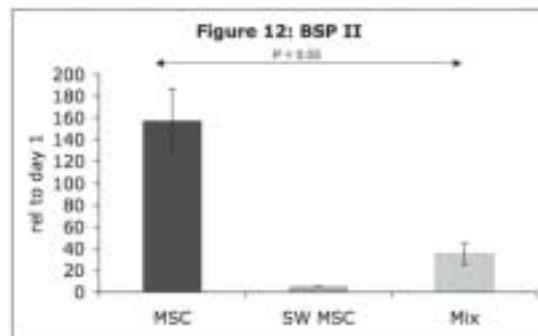
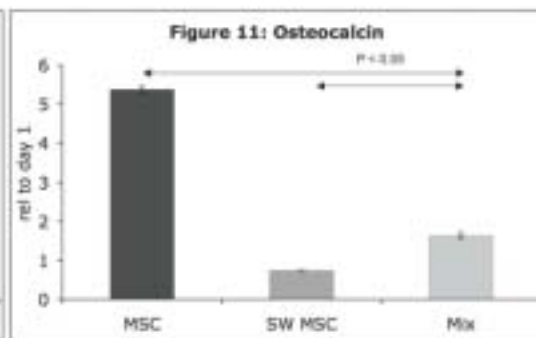
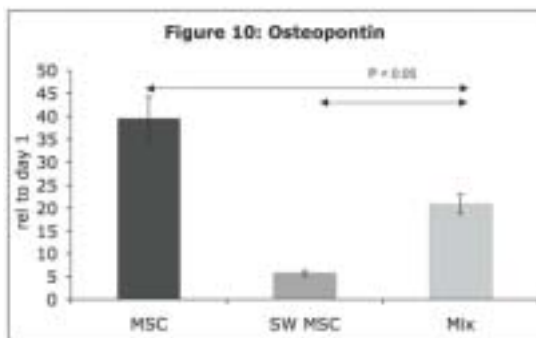


Figure 9: Gene expression of total VEGF by MSC at day 35

Osteopontin, osteocalcin and bone sialoprotein II all were significantly up-regulated in MSC cultures at day 35 compared to day 1. While OP, OC and BSP levels in Sandwich-MSc did not change significantly over time, they increased in Mix-MSc, but surprisingly not as much as in MSC.



Figures 10-12: Gene expression of OP, OC and BSP by MSC at day 35

The expression of osteocrin and Ang1 were also determined, but were at almost undetectable levels in all types of cultures at all times.

For the constructs containing HUVEC, the expression levels of the following genes were evaluated: Endothelin-1 (ET-1), VE-cadherin (CDH5), matrix metalloproteinase 2 (MMP-2), matrix metalloproteinase 9 (MMP-9), von Willebrand factor (vWf), EGF-like domain 7 (Egfl7), platelet-derived growth factor receptor β (PDGFRB), BMP-2, VEGF receptor 1 (Flt1, VEGFR1), VEGF receptor 2 (Flk1, KDR, VEGFR2), VEGF receptor 3 (Flt4, VEGFR3), Ang1, Tie1 and Tie2 (TEK).

The endothelial markers VE-cadherin and vWf showed both by far the highest expression levels in Mix-HUVEC, followed by Sandwich-HUVEC and HUVEC. While all VE-cadherin expression levels at day 35 were lower than at day 1, the expression levels of vWf by Mix-HUVEC was 3x higher than at day 1.

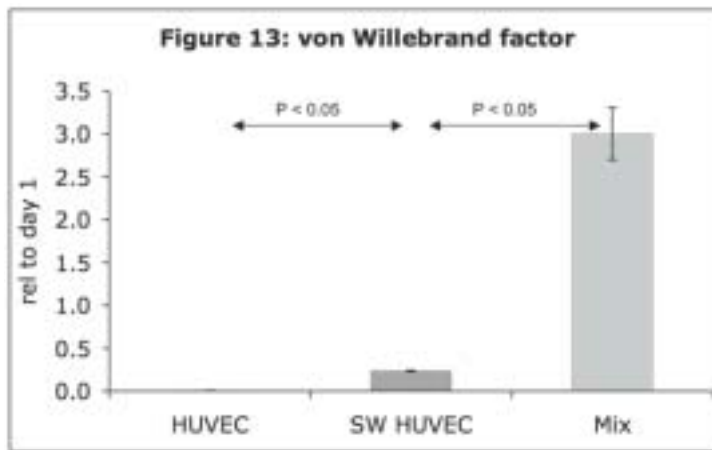


Figure 13:
Gene expression of vWf by HUVEC at day 35

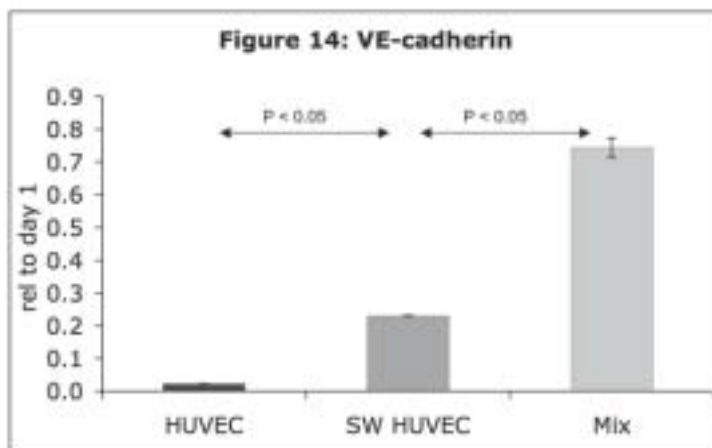


Figure 14:
Gene expression of VE-cadherin by HUVEC at day 35

Egfl7, MMP-2 and MMP-9, all factors known to be involved in vascular tissue development, remodeling and repair showed a similar picture. Egfl7 and MMP-2 showed rather low expression levels in HUVEC and Sandwich-HUVEC cultures, but were highly up-regulated in Mix-HUVEC. MMP-9 was not detectable at day 1 in any culture, but was highly expressed in Mix-HUVEC at day 35.

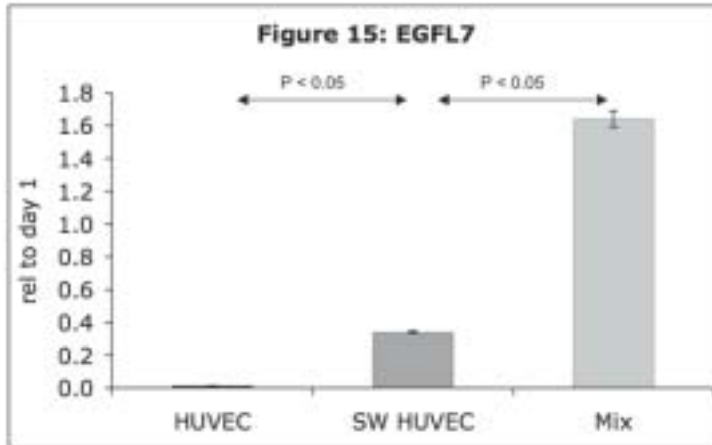


Figure 15:
Gene expression of EGF-like domain 7 by HUVEC at day 35

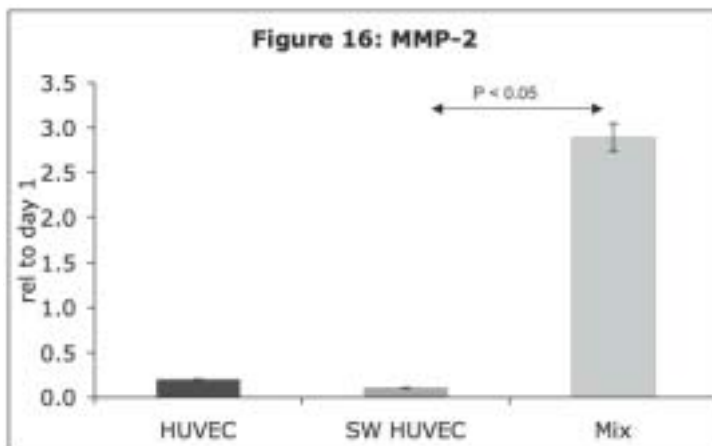


Figure 16:
Gene expression of MMP-2 (Gelatinase A) by HUVEC at day 35

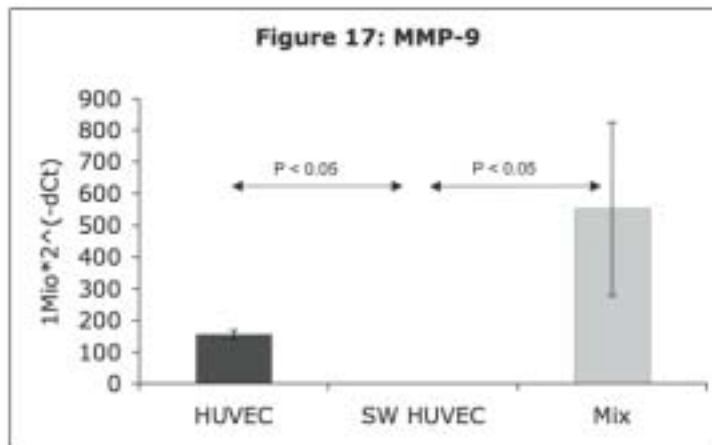
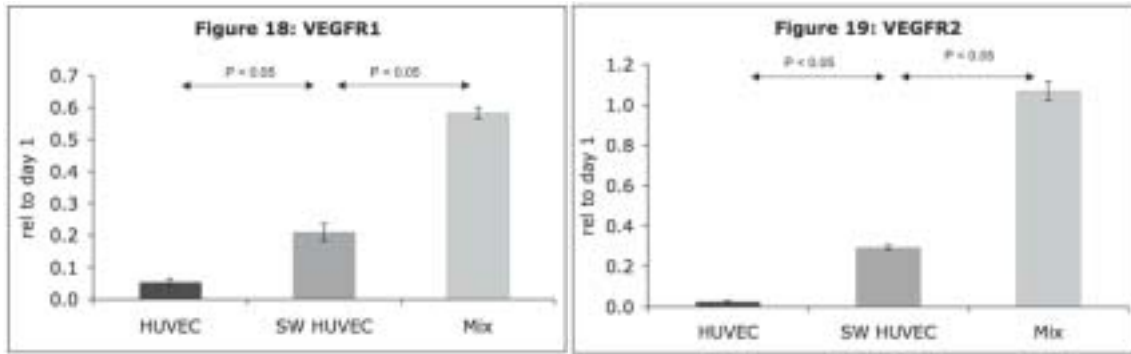
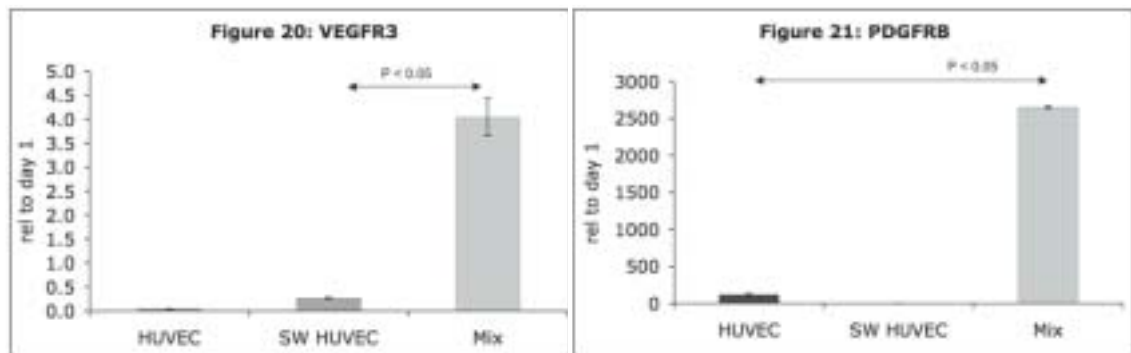


Figure 17:
Gene expression of MMP-9 (Gelatinase B) by HUVEC at day 35: There was no MMP-9 detectable at day 1, therefore MMP-9 expression is not normalized to day 1.

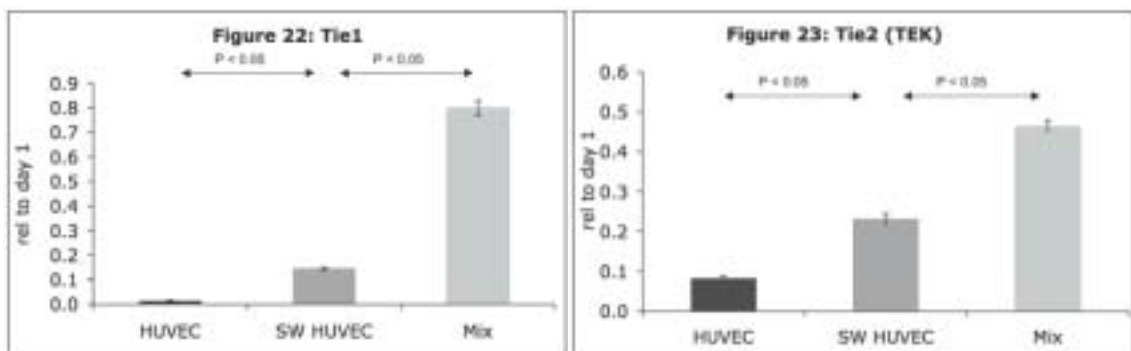
The expression of endothelial receptors VEGFR1, -2, -3, Tie1, -2, and PDGFRB were clearly highest in the Mix-HUVEC and lowest in the HUVEC cultures. Interestingly, the expression levels at day 35 compared to day 1 were only increased for VEGFR3 (> 4 times) and for PDGFRB (> 2500 times), while only a slight increase was observed for VEGFR2. The expression levels of all other receptors decreased with culture time.



Figures 18 and 19: Gene expression of VEGFR1 (Flt1) and -2 (Flk1, KDR) by HUVEC at day 35



Figures 20 and 21: Gene expression of VEGFR3 (Flt4) and PDGFRB by HUVEC at day 35



Figures 22 and 23: Gene expression of Tie1 and Tie2 (TEK) by HUVEC at day 35

BMP-2 and ET-1, both factors that are known to affect bone cells, were also most prominently expressed in Mix-HUVEC, followed by Sandwich-HUVEC and HUVEC cultures. ET-1 levels were generally lower at day 35 than at day 1, but BMP-2 significantly increased over time in Mix-HUVEC.

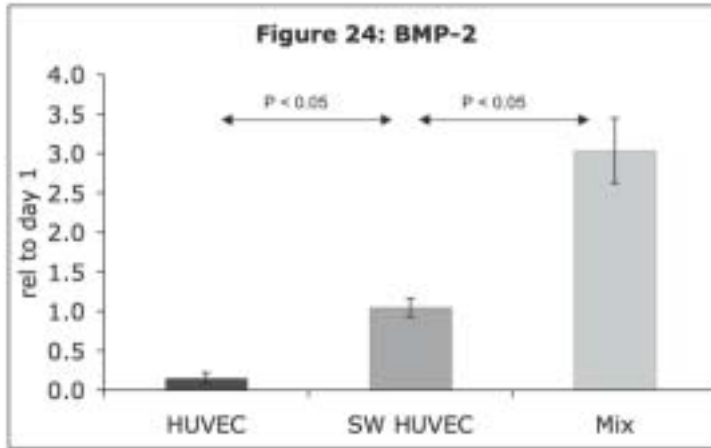


Figure 24:
Gene expression of bone morphogenetic protein 2 by HUVEC at day 35

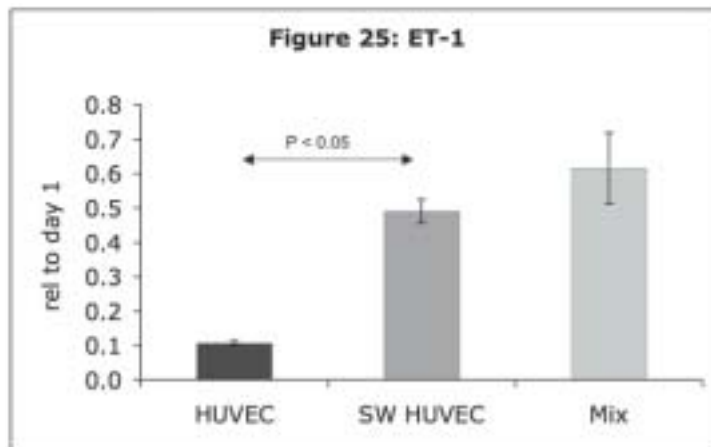


Figure 25:
Gene expression of endothelin-1 by HUVEC at day 35

Ang1 expression significantly increased over culture time to comparable levels in HUVEC and Mix-HUVEC but remained almost undetectable in Sandwich-MSC.

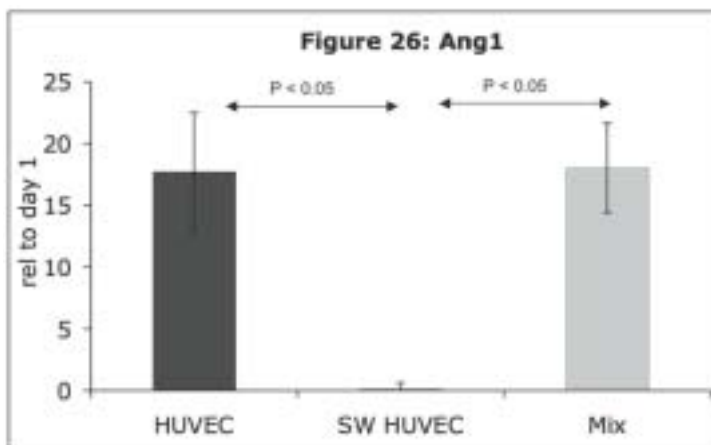


Figure 26:
Gene expression of Ang1 by HUVEC at day 35

Histological analysis of 3D-constructs

The gene expression pattern of constructs containing a mix of MSC and HUVEC (Mix-constructs) clearly showed an up-regulation of genes involved in angiogenesis and bone formation. To further explore this promising data, we performed histological analyses of constructs.

Van Kossa staining of Mix-constructs at day 21 showed mineral deposition in small distinct areas of the graft (Figure 27 right). There was no mineral deposition in MSC (Figure 27 left) or in SW-constructs (not shown).

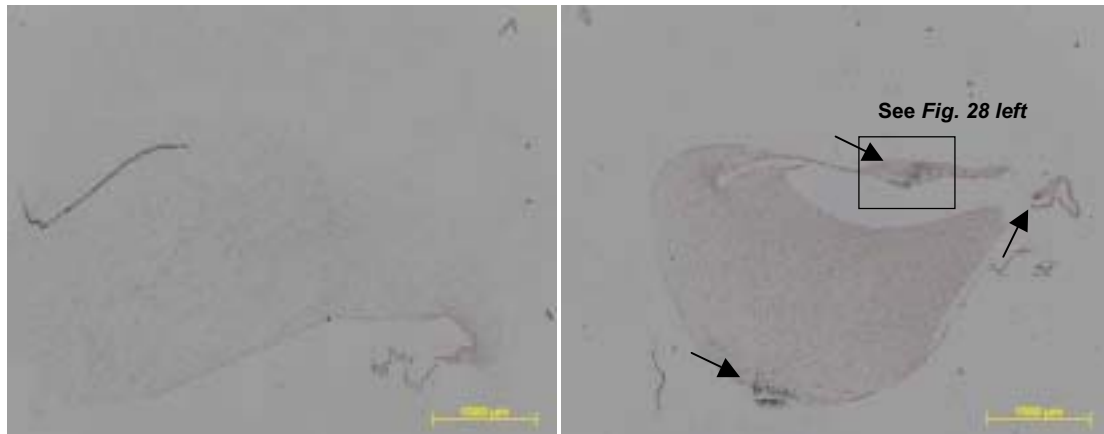


Figure 27: Van Kossa stain at day 21: Constructs containing MSC alone (left) showed no mineral deposition while in the constructs containing a mix of MSC and HUVEC (right), there was mineral deposition in distinct areas (arrows).

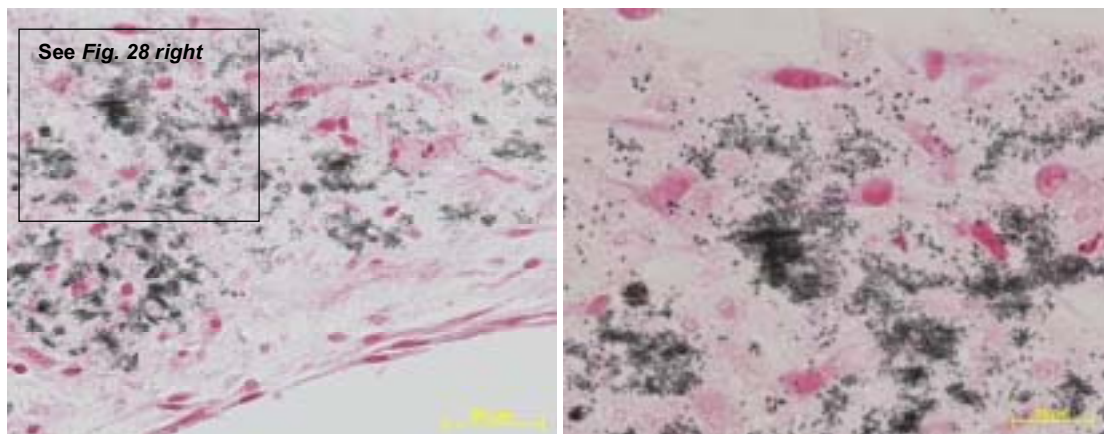


Figure 28: Van Kossa stain of Mix-constructs at day 21: The left picture shows elongated cells lining the outside of the construct while in the inside, calcium is deposited into the matrix.

Evaluation of cell morphology at day 21 using Toluidene Blue staining revealed interesting changes in cell shape. We observed the development of elongated cells in Mix-constructs (Figure 29). The fact that these elongated cells are always appearing in groups suggests that these cells might be starting to form a network. Nothing similar could be found in the other constructs (Figure 30).

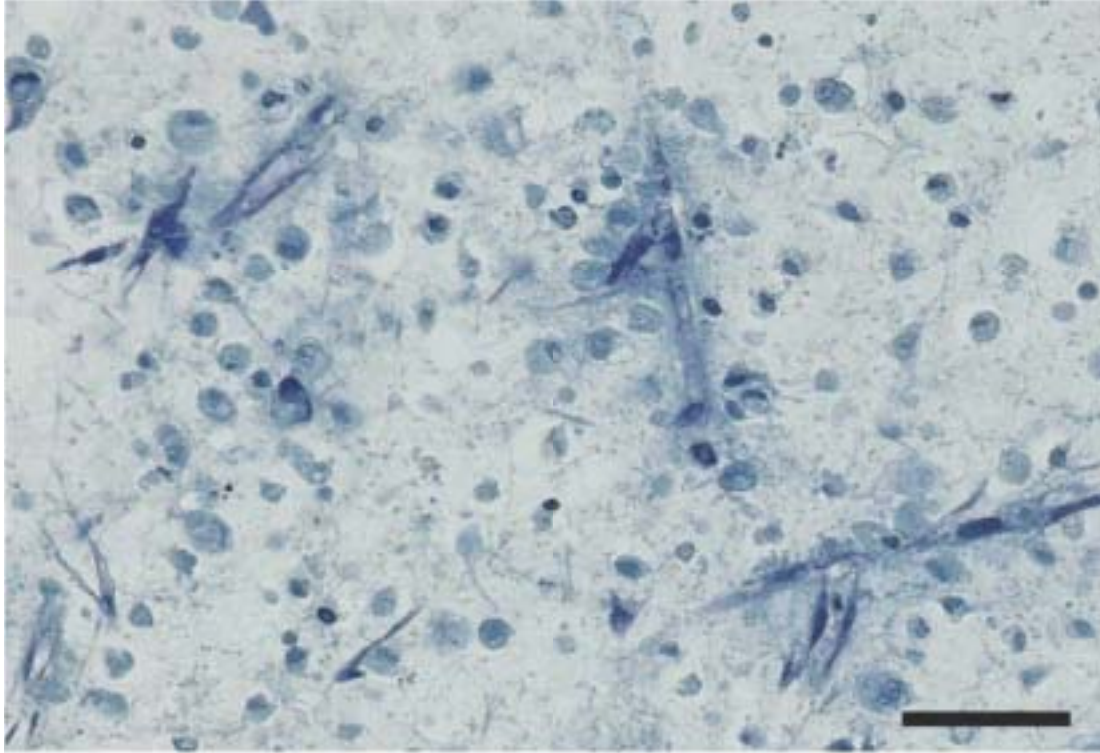


Figure 29: Toluidene Blue stain of Mix-constructs at day 21 revealed assemblies of elongated cells suggesting a possible start of network formation. Bar represents 50 μ m.

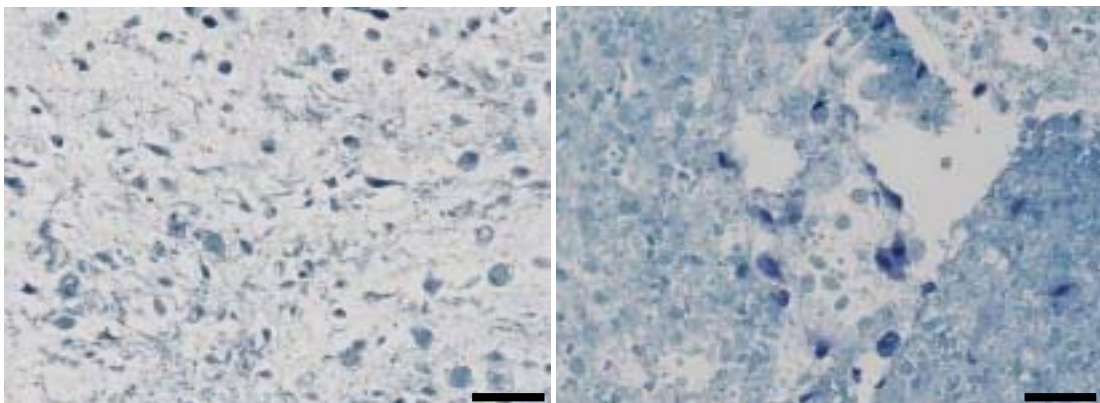


Figure 30: Toluidene Blue stain of constructs containing MSC (left) or HUVEC (right) at day 21 showed no remarkable morphological characteristics. Bar represents 50 μ m.

By using higher magnification in Mix-constructs, we observed cells forming an intracellular lumen (Figure 31 left and center) and also intercellular lumen in between two cells (Figure 31 right).

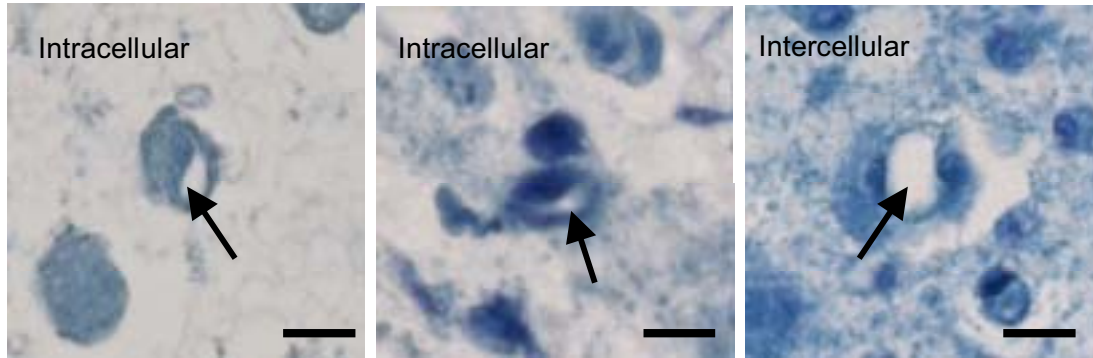


Figure 31: Toluidene Blue staining of Mix-constructs at day 21: Intracellular lumen formation (left, center) and extracellular lumen formation between two cells (right). Bar represents 20 μ m.

At day 35, Toluidene Blue staining revealed the formation of tube-like structures in Mix-constructs. Almost all cells were attached to these structures, with only very little cells left in-between these tubules (Figure 32).

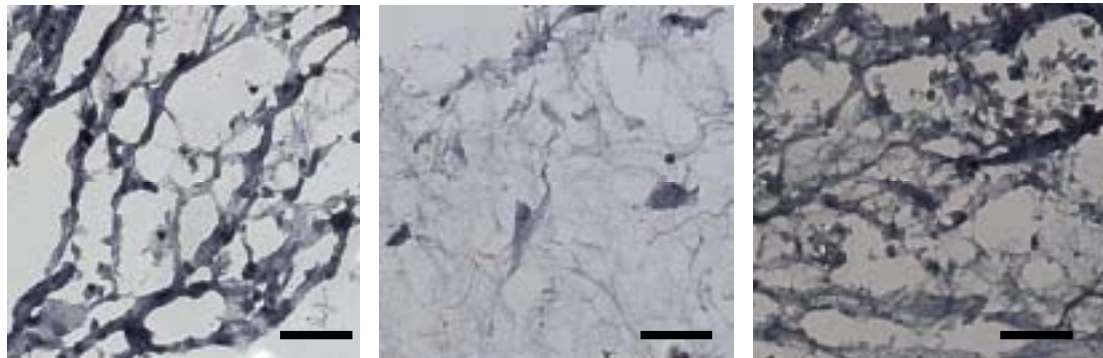


Figure 32: Toluidene Blue staining (bar represents 50 μ m) at day 35: Tube-like structures in Mix-constructs (left). Grafts containing only MSC did not show such structures (middle). Grafts containing only HUVEC showed similar, yet much less and only very short tubular-like structures (right).

We further explored these tubular structures by immunohistochemical analysis at day 35. Using an antibody against laminin, we were able to reveal possible tube-like structures in the Mix-constructs, consisting of a wall and a more or less continuous lumen (Figure 33). Those structures were exclusively found in Mix-constructs.

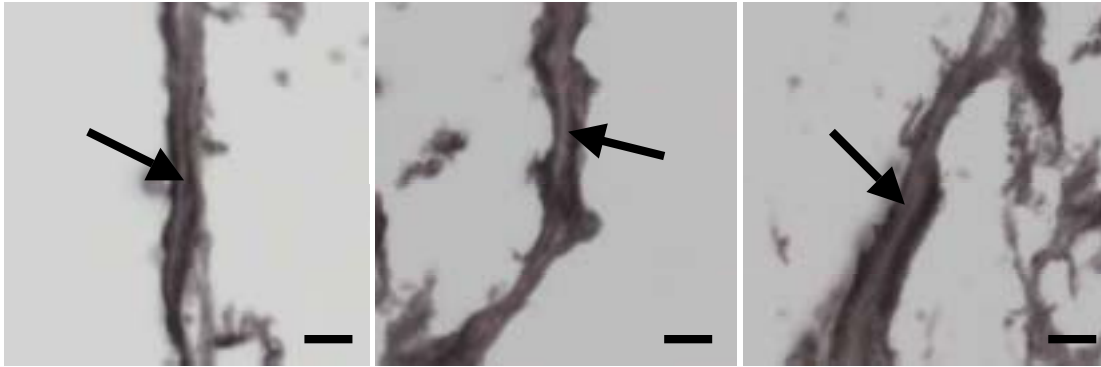


Figure 33: Immunostaining using an antibody against laminin on Mix-constructs at day 35: tubular structures containing extensive lumen (arrows). Bar represents 20uM.

Immunostaining of these tubular structures with an antibody to von Willebrand factor revealed that they were highly multicellular (Figure 34a). Interestingly, not only endothelial cells (smaller darker nuclei) were visible, but also cells featuring a larger unstained nucleus were participating in these tubular structures. This suggests that also MSC might contribute to these structures (Figure 34b).

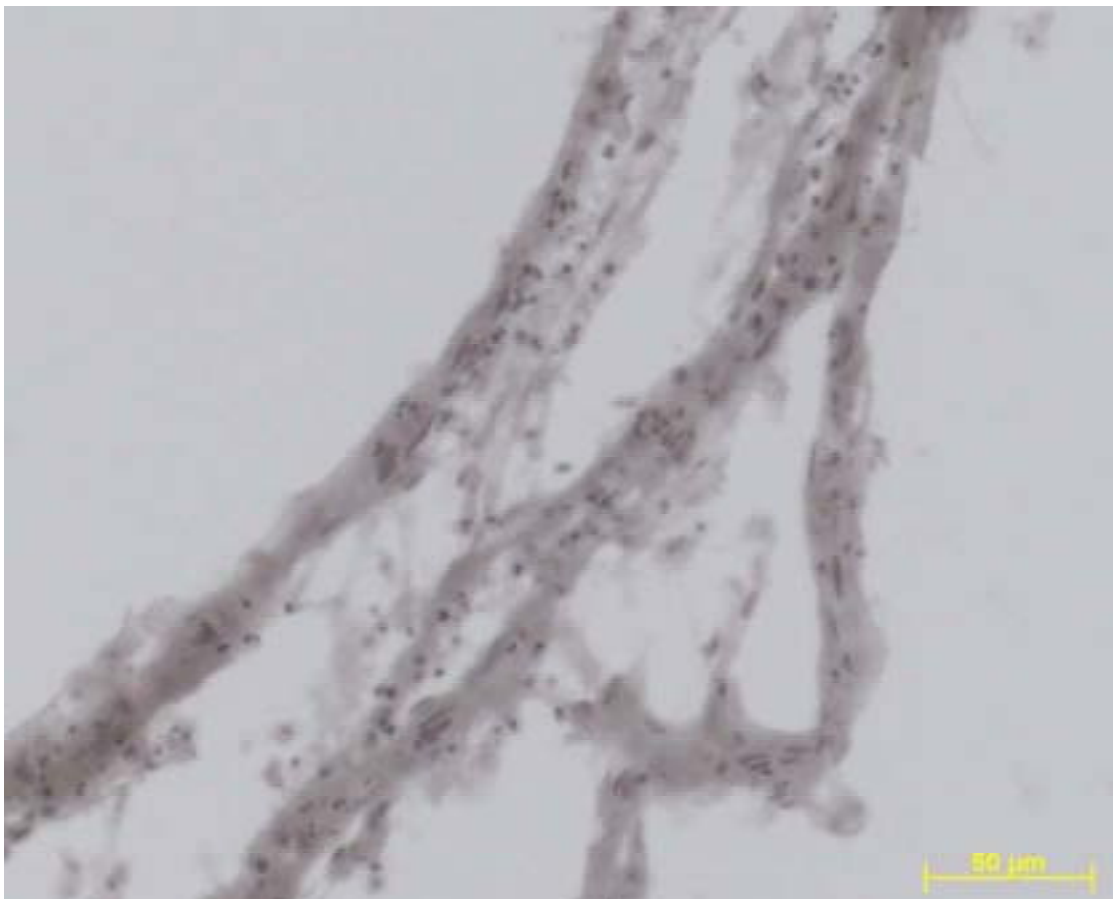


Figure 34a: vWf immunostaining of Mix-construct at day 35: Large multicellular tube-like structures

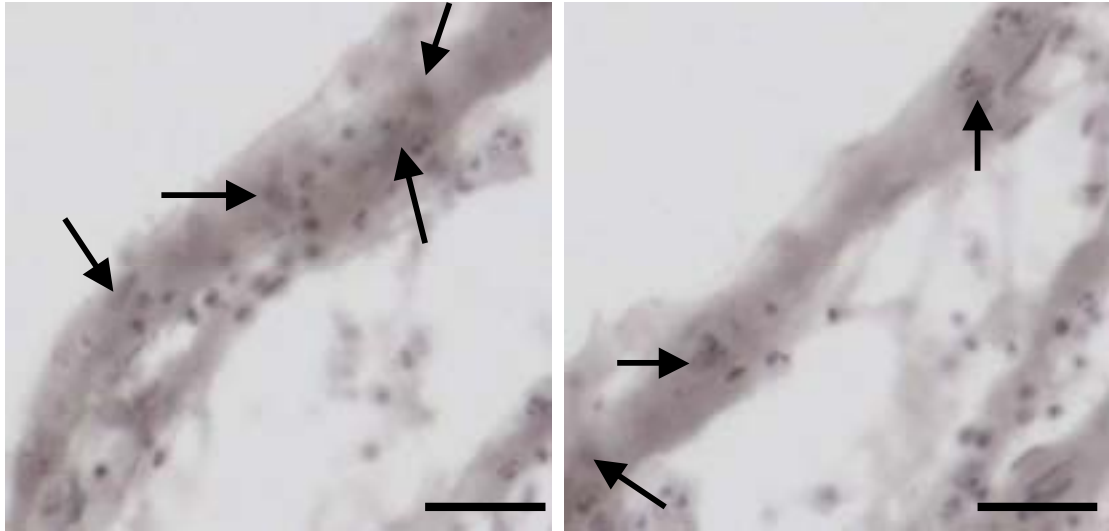


Figure 34b: vWf immunostaining of Mix-constructs at day 35: Large unstained nuclei (arrows) seem to contribute to the tubular structures. They possibly represent MSC. Bar represents 20 μ m.

To confirm, that also MSC contribute to these tubular structures, we performed an immunofluorescence analysis using an antibody against the osteoblastic transcription factor *Osf2* (gene product of *Runx2*). The presence of osteoblastic cells on the tubular structures was confirmed (Figure 35), even though our antibody produced considerable unspecific background-staining. Examples of specific bindings are marked (arrows).

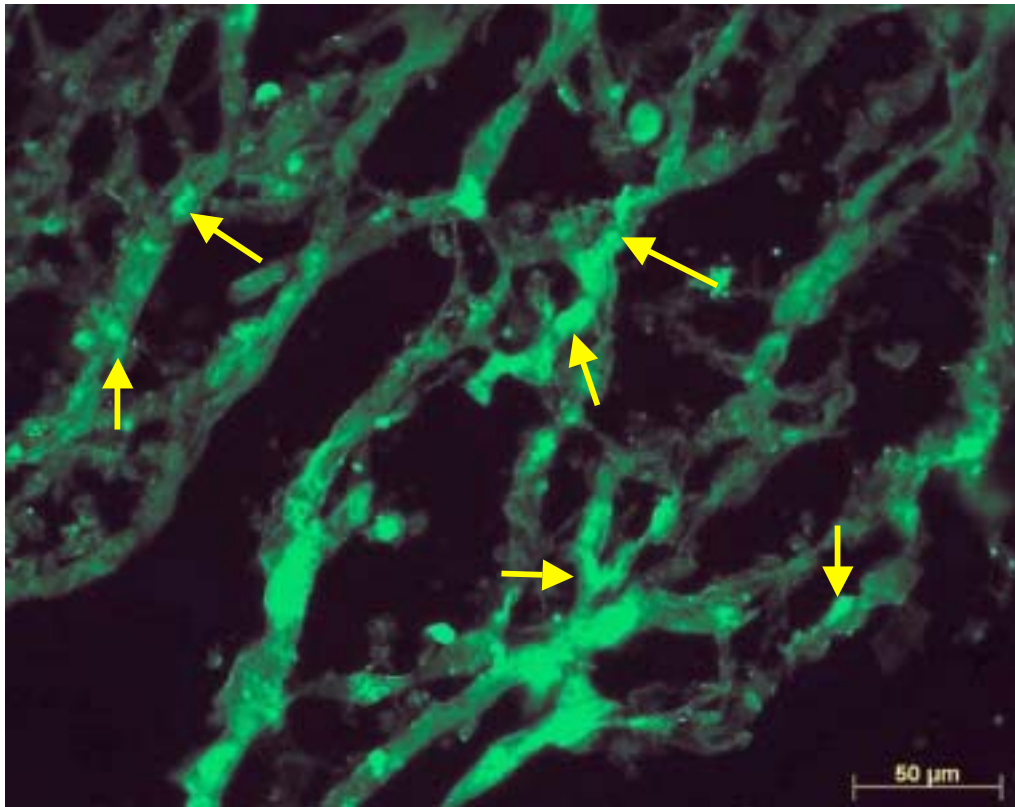


Figure 35: *Osf2* immunostaining of Mix-construct at day 35: Large areas of the tube-like structures show the presence of *Osf2*-positive cells, most probably differentiating MSC.

Immunofluorescent staining using an antibody to the EC surface marker CD31 confirmed the formation of tube-like structures by EC (Figure 36).

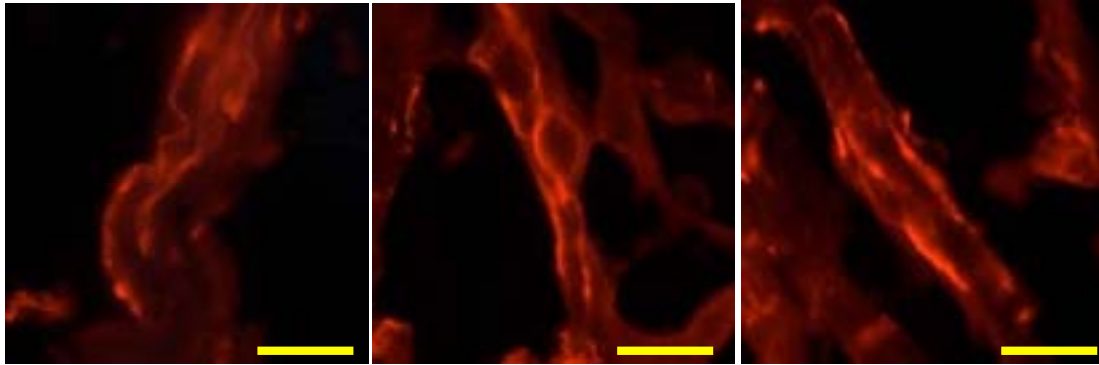
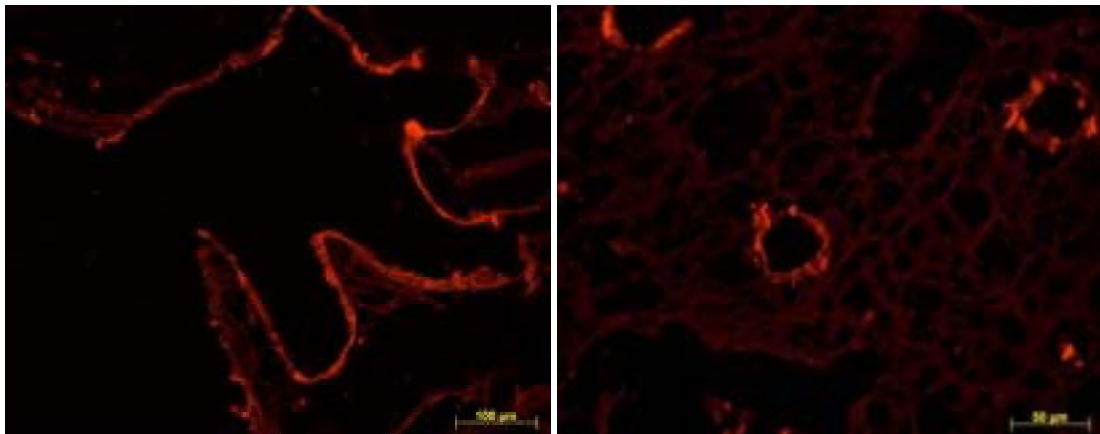


Figure 36: Immunofluorescence using an antibody to CD31 in Mix-constructs at day 35: Tubular structures as found only in Mix-constructs. Bar represents 20 μ m.

Endothelial cells were also found covering various surfaces, especially in Mix-constructs, further confirming their increased urge to fulfill their natural fate by building natural barriers. Figure 37a shows the outside of a graft covered by CD31-positive cells. Figure 37b shows CD31-positive cells forming circular structures within the PRP gel.



Figures 37a and b: Immunofluorescence of Mix-constructs at day 35: Endothelial cells (CD31+) covering the outside of a graft (left), and forming circular structures in PRP-gel (right).

In conclusion, we have shown significant up-regulation of osteoblastic markers in Mix-constructs. Also, angiogenic and endothelial markers expressed by HUVEC were significantly up-regulated in this construct type. This was in contrast to the much lower gene expression levels in non direct-contact co-cultures (Sandwich) and constructs seeded with MSC or HUVEC alone.

Furthermore, histological analysis revealed the formation of inter- and intracellular lumen as well as the presence of tubular and circular structures in Mix-constructs only.

Discussion

The aim of this study was to define an optimal scaffold-cell construct *in vitro* to serve as a potential alloplastic bone graft *in vivo* that could overcome the problem of insufficient vascularization in large bone defects. In addition, since during bone tissue repair *in vivo* fibrin clots are an important source of growth factors and cytokines, we evaluated the use of platelet-rich plasma (PRP) as a supplier of osteoinductive and angiogenic factors within the construct.

In previous studies (diplomawork), we have evaluated different scaffolds to be used in bone and cartilage tissue engineering using osteoblasts, chondrocytes and bone marrow aspirates. Based on these early results, we decided to use a biodegradable polyurethane scaffold for our endothelialized construct [44, 45]. Many studies have used calcium-phosphates (CaP) like β -tricalcium-phosphate (β TCP) or hydroxyapatite (HA) because of their “natural” composition as well as their superior mechanical strength. However, calcium phosphate based materials have also significant drawbacks, like the lack of macro-pores, resulting in impaired vessel ingrowth as well as their layered biodegradation (from the outside to the inside). In addition, due to the stiff properties of CaP, there is considerable friction at the bone-implant interface resulting in wear-debris, limited vessel ingrowth and implant integration. Biodegradable polyurethanes on the other side are very macro-porous and elastic, and can therefore be fitted very tightly into the site of injury, providing a more flexible tissue-implant interface that facilitates vessel ingrowth and implant integration. It has previously been shown that PU scaffolds can be seeded with chondrocytes up to 42 days without any apparent toxicity [46, 47]. In addition, similar polyurethane scaffolds were used in an iliac bone defect in a pilot experiment in sheep [48]. Therefore, we considered polyurethane as a potential scaffold for bone substitutes. Two different kinds of isosorbide polyurethane scaffolds were compared in this study. Both scaffolds were identical except that one contained a ceramic nano-phase. However, this ceramic component seemed to be harmful to our cells, we therefore decided to continue with the polyurethane scaffold that did not contain the mineral phase. The reason for this toxic effect is not clear. A possible explanation could be that the ceramic phase in the PU scaffold somehow hinders the removal of ethylene oxide after sterilization so that detoxification was not carried out long enough. Further experiments will be necessary to resolve this issue.

When seeding cells within a 3D structure with pore sizes between 200 to 500 microns, it is almost impossible to retain the cells within the scaffold structure. In addition, cell aggregates will occur, making cell distribution far from being

homogeneous [46]. A common way to avoid these cell-seeding problems is the use of fibrin gel [42]. Although fibrin is commonly used in surgery, its animal origin (bovine) raises some concerns about its safety. Therefore, autologous PRP would be a much better solution. As reported in chapter IV of this thesis, we were able to show that PRP had a strong osteogenic effect on MSC in monolayer culture. PRP contains a large amount of different growth factors and cytokines, including TGF- β , PDGF, FGF, IGF and EGF [49, 50].

We therefore have evaluated the effect of PRP versus fibrin gel as a carrier for MSC, HUVEC or a combination of both. Our findings showed that when MSC were cultured in PRP gels, a much higher expression of the typical osteoblastic genes was observed compared to MSC cultured in fibrin. This was most prominently seen in the expression of type I collagen, osteocalcin, osteopontin, MMP-13 and BMP-2. These results are in agreement with our earlier observations on the effect of PRP on MSC in monolayer (see chapter IV). However, the gene up-regulation observed in the 3D environment was generally higher than that measured in monolayer. The by far most up-regulated gene was MMP-13, an endopeptidase known to be involved in matrix turnover by its ability to cleave collagen triple helices, aggrecan core protein, fibronectin and other components of the extracellular matrix. MMP-13 and other MMPs (especially MMP-2 and -9) have been shown to be tightly connected to the process of angiogenesis and blood vessels ingrowth [31, 32]. Interestingly, also the expression of early and late bone matrix proteins such as Col1, OP and OC was up-regulated by PRP. This suggests that PRP also enhances the differentiation of precursor cells into mature osteoblasts.

We also measured the performance of PRP versus fibrin gel as a carrier for HUVEC by comparing the gene expression levels. The same picture as with MSC was observed: PRP greatly enhanced the expression of endothelial cell markers such as vWf, VE-cadherin and the endothelial receptors VEGFR2, Tie1 and Tie2. BMP-2 expression by HUVEC in PRP was also significantly up-regulated. We also had an up-regulation of MMP-2, which has been shown to be involved in angiogenesis. MMP-2 is the most abundant MMP produced by endothelial cells. MMP-2 is constitutively secreted as a zymogen and is believed to play a role during the initial steps of angiogenesis [51, 52]. Interestingly, MMP-9 (or 92kDa gelatinase), another MMP thought to be involved in angiogenesis was not detected after 21 days of culture, but was present after 35 days. From these experiments it was obvious that PRP was far superior to fibrin in inducing MSC differentiation into an osteogenic phenotype, as well as in stimulating typical EC markers on HUVEC.

We then focused on 3D co-culture experiments of MSC and HUVEC using PRP as a carrier. Two types of co-cultures were established. The first one was an indirect-contact co-culture, where HUVEC and MSC were seeded in two polyurethane scaffolds using PRP, and these two scaffolds were placed on top of each other (Sandwich). The second one was a direct contact co-culture, where MSC and HUVEC were seeded within the same polyurethane scaffold using PRP (Mix). Analysis of MSC gene transcription revealed, that Sandwich co-cultures resulted in a down-regulation of many osteoblastic genes, including Col1, Col3, OP, OC, BSP, Dlx5, Osx and MMP13. These results were in close agreement with our earlier indirect contact co-culture results, in which EC were shown to inhibit MSC differentiation (see chapter II). However, in Mix co-cultures (direct contact) a significant increase in a variety of osteoblastic markers, including Col1, Col3, Runx2, Dlx5, Osx, BMP-2, ALP and MMP13 was observed.

Type III collagen, which is generally much less abundant than type I collagen, is typically not found in bone, but is highly expressed around blood vessels. Thus, the up-regulation of Col3 in Mix co-cultures might suggest that the formation of blood vessels might be induced. Up-regulation of the early transcription factors Runx2 and Dlx5 as well as the most specific known osteoblastic transcription factor Osx in Mix-cultures clearly indicates that MSC are differentiating towards the expression of an osteoblastic phenotype. An interesting observation is that in Sandwich co-cultures, Runx2 expression is not altered compared to MSC alone, but Osx (and also Dlx5) is significantly down-regulated. This observation suggests again that a positive effect on MSC differentiation is observed only when MSC and EC are in direct contact. The bone matrix proteins OC, OP and BSP were also up-regulated in Mix co-culture at day 35 compared to day 1.

Analysis of the expression of the different EC genes revealed that most of these markers were significantly up-regulated by both co-culture types (Sandwich and Mix). However, in Mix co-cultures, up-regulation of EC markers was much higher than in Sandwich co-cultures. Interestingly, MMP-2 and -9 were highly up-regulated in Mix-cultures. Egfl7, VEGFR2, Tie1 and -2 as well as VE-cadherin are all EC markers appearing rather early in EC maturation events, while PDGFRB and vWf are later markers of vascular maturation [23] [22, 53]. Therefore, it is normal that VEGFR2, Tie1, Tie2 and VE-cadherin were expressed in all cultures containing EC. However, PDGFRB and vWf, were only prominently expressed in Mix-cultures, suggesting again that EC were able to undergo maturation only in direct contact co-cultures. Another important EC marker, endothelin-1, was up-regulated in both co-cultures (Mix higher than Sandwich) compared to EC alone. ET-1 has been shown to be a

major player in the signaling between EC and osteoblasts during bone development, remodeling and repair and is known to stimulate osteoblasts proliferation and differentiation [54, 55]. Another interesting observation was that ET-1 has been shown to down-regulate VEGF expression in long-term cultures of osteoblasts [55], a finding that we have also observed in the present study. In Sandwich-cultures, VEGF expression was high while ET-1 expression was low. In contrast, in Mix-cultures, VEGF expression was low and ET-1 was high. So the inhibitory effect of ET-1 on VEGF expression occurs only when MSC and EC are in direct contact, which might suggest a tight coupling between the angiogenic and bone formation processes. BMP-2, which has been shown to induce the expression of the osteogenic master transcription factors Runx2, Dlx5 and Osx in MSC [56, 57] was significantly up-regulated in Mix co-cultures. In addition, all those osteogenic master genes were, as expected, also significantly up-regulated in Mix-cultures.

These findings at the gene expression level were then confirmed by histological analyses. We were able to show, that only in Mix-cultures calcium deposition occurs. Furthermore we observed multicellular tube-like structures in Mix-cultures formed by HUVEC and possibly stabilized by differentiating MSC. We also detected cells forming intra- and intercellular lumen. Unfortunately, we were not able to study the formation of these structures over time, so we cannot conclude by which mechanisms these structures were formed. However, it seems clear that our constructs containing MSC and HUVEC in PRP promoted EC to form separating cell layers, which eventually might result in functional blood vessels.

In conclusion, constructs consisting of a mixture of MSC and EC seeded on a polyurethane scaffold within a PRP environment might be a very promising alternative to autologous bone grafting.

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Chapter VI

Isolation of CD34+ cells from blood aspirates and differentiation into endothelial cells

Meury TR, Alini M

Our aim was to define a 3D construct, which consisted of as many autologous components as possible. This chapter summarizes our attempts to isolate CD34+ cells from blood samples and to differentiate them into mature endothelial cells.

One of our aims was to develop a full construct made of autologous material. We succeeded by using MSC and PRP in our constructs, which can be easily obtained from the patient's bone marrow and blood respectively.

The initial plan was therefore also to obtain endothelial cells from an autologous source. Endothelial progenitor cells (EPC) and hematopoietic stem cells (HSC) share some surface molecules like VEGFR2, Tie2 and CD34. This led to the assumption, that these progenitors may have a common precursor cell present within the peripheral blood system [1, 2]. This hypothesis that blood might contain EPC was also supported by studies showing that the luminal side of artificial vascular grafts *in vivo* becomes eventually covered by EC [3, 4]. Asahara was the first to actually show that EPC can be isolated from peripheral blood and can be differentiated into endothelial-like cells [5]. A cell surface marker generally used for EPC isolation is CD34. It is not only expressed EPC but also on HSC and is lost during hematopoietic differentiation [6, 7], but not during EC differentiation and maturation [8-10]. Based on these studies, we decided to isolate CD34+ cells from human blood samples and differentiate them into endothelial cells, in order to obtain also EC from an autologous source.

From CD4-negative buffy-coat (kindly obtained from the local Asthma Research Institute SIAF in Davos), using the MiniMAC Magnetic Microbead System (by Miltenyi Biotec, Germany), we isolated CD34+/(CD4-) cells (MACS Direct CD34 Progenitor Isolation Kit, cat# 467-02) using the following procedure:

Buffy-coat was centrifuged at 1'500g for 10min at RT and the resulting pellet was resuspended in MiniMAC Buffer (2mM EDTA, 0.5% BSA in PBS, degassed) at a concentration of 10^8 cells/100 μ L. 100 μ L of FcR Blocking Reagent was added. The cells were then labeled by addition of 100 μ L of CD34 Microbeads per 10^8 total cells followed by incubation for 30min at 8°C under gentle agitation. After carefully washing with MiniMAC Buffer, the labeled cells were resuspended in 1mL of MiniMAC Buffer and were added to a pre-wetted separation column positioned in a magnetic stand. The separation column was washed 3x with MiniMAC Buffer, was removed from the stand and the CD34+ cells were eluted from the column into a fresh tube with MiniMAC Buffer. CD34+ cells were then centrifuged, resuspended in medium and seeded into flasks.

Different coatings of the culture flasks (no coating, gelatin, fibronectin) were tested. We also evaluated different initial seeding concentrations and most importantly, compared different media and supplements, including F-12K and IMDM in combination with serum, as well as several serum-free media like StemSpan (Stem Cell Technologies, cat# 09650) and StemPro-34 (Gibco, cat# 10639-011). Finally, we

settled on the serum-free StemPro-34 as EPC growth medium. In addition, various types of rather expensive commercial supplements and different combinations of cytokines and recombinant growth factors (including IL-1, IL-3, IL-6 and SCF, Flt-3, TPO and GM-CSF) for the expansion of CD34+ cells were evaluated. In the end, we were able to isolate CD34+ cells from buffy-coat and culture them in StemPro-34 medium, supplemented with IL-3, IL-6 and SCF (all at 50ng/mL). The concentrations of IL-3, IL-6 and SCF were based on a personal communication with Dr. Nicolai Ferrari [11].

The plan was then to check the phenotype of CD34+ cells after isolation by FACS analysis, to expand them for one or two weeks and then to recheck their phenotype by measuring the following surface markers: CD34, VEGFR2, CD133, CD45 and CD14. Depending on the results, a reselection of CD34+ and further expansion would have been evaluated. Then we would have differentiated the CD34+ cells into EC by stimulating them with a combination of ECGF and VEGF, both at 50-100µg/mL (again based on personal communication with Dr. Nicolai Ferrari) in different culture mediums like RPMI, F12K or M199.

While planning all these experiments we realized, that this sub-project was growing into a project itself. Especially the cell-characterization during differentiation of CD34+ cells into endothelial cells would have taken too much time and would have shifted our experimental aims too far away from the initial focus of this PhD thesis.

We therefore decided to continue our experiments using primary human umbilical vein endothelial cells (HUVEC) purchased from Cascade Biologics. We are of course aware that HUVEC are different from the microvascular EC found in the bone vasculature. However, we chose HUVEC since they are well characterized and easy to handle, compared to microvascular EC.

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Chapter VII

Final Thesis Conclusions and Remarks

One of the main problems arising when implanting a graft into a large bone gap is its insufficient vascularization in the central region. This results in impaired bone healing and often leads to necrosis in the graft. Vascularization and bone formation have been shown to be very closely connected [1-6]. In order to better understand the relationship between blood vessels and bone formation, we have studied the interactions between mesenchymal stem cells (MSC) and endothelial cells (EC). Since the blood vessel network undergoes dramatic remodeling during wound healing, a process that is connected with different endothelial cell maturational stages (reflected i.e. by different cell surface markers), we have also investigated the effect of growth factor-stimulated EC on MSC differentiation into bone forming cells. In addition, since during bone healing, one of the first events is the formation of a blood clot at the site of injury, we have evaluated the potential of platelet-rich plasma (PRP) as an osteoinductive substance by assessing its action on MSC differentiation. Finally, our last step was to combine all components into one autologous construct and to *in vitro* evaluate its potential to serve as a possible bone graft that could overcome the *in vivo* problem of limited vascularization generally occurring in large bone defects.

Aim 1:

Study the influence of EC on MSC differentiation into an osteoblastic phenotype

The first experiments of this PhD investigated the effect of EC on MSC differentiation towards an osteoblastic phenotype in monolayer cultures. At that time, *in vitro* communication between EC and osteoblastic cells had been reported in few publications with rather divergent outcomes [7-9]. The only generally accepted fact was that there must be some kind of communication between these two cells types, since the appearance of vessels precedes bone formation [5, 6, 10]. We showed that EC do not stimulate MSC differentiation into osteoblasts in non-direct contact co-cultures. On the contrary, EC significantly inhibited osteoblastic differentiation of MSC. We also demonstrated that this inhibitory effect by EC was increased following stimulation of EC with VEGF. From a mechanistic point of view, this inhibitory effect was due to the down-regulation of *Osx*. These findings observed in 2D cultures were confirmed in our 3D experiments. However, when MSC and EC were cultured together in direct contact co-cultures, MSC differentiation was induced, and *Osx* expression was up-regulated. These observations suggest that EC have a dual potential to influence osteoblastic differentiation that largely depends on the type of

communication between these two cell types as well as the maturational stage of EC. It seems that soluble factors released by EC inhibit, while direct contact with EC induces osteoblastic differentiation of MSC. One may therefore speculate that, depending on the developmental stage of the vascular system at a bone repair site, EC may induce either recruitment of osteoblastic precursor cells, or differentiation of these precursor cells into osteoblasts.

Aim 2:

Test the suitability of platelet-rich plasma (PRP) as a source of autologous biological factors in bone tissue engineering

The second part of the PhD focused on factors that could influence bone formation and angiogenesis in a potential bone graft. Although many studies at that time had focused on recombinant growth factors to improve bone healing (i.e. BMP or PDGF) [11-18], we were interested in a more autologous approach. Platelet-rich plasma (PRP) was a very new “material” that had been used by few people as an injectable substance or in combination with a scaffold, mainly in maxillofacial and dental surgery [19-21]. We wanted to use PRP in combination with MSC and have therefore explored the ability of PRP to induce osteoblastic differentiation in MSC. Since no standard protocol for the production of PRP was available (everyone used a different method), we first defined our own PRP preparation and activation method, which resulted in the filing of a patent for PRP activation by sonication (pending). We then tested the effect of PRP on MSC differentiation in monolayer as well as in 3D, and showed that PRP had the ability to greatly enhance osteoblastic differentiation of MSC. Furthermore, we showed that PRP also greatly enhanced the expression of many typical endothelial markers on EC in 3D-cultures.

In summary, we were able to isolate and activate PRP in an easy and quick way. We have also shown that PRP has the potential to induce osteogenic differentiation of MSC. Furthermore, because of the ability of PRP to form a gel upon activation, PRP has the capability to serve as an ideal carrier material for MSC and EC in a 3D construct.

Aim 3:

Define and evaluate an autologous 3D-construct in vitro with the potential to improve graft vascularization in vivo

The final part of this PhD work combined the results obtained in the other parts. Our constructs were designed using isosorbide single-phase polyurethane

scaffolds, seeded with combinations of cells in PRP. We showed that MSC and EC suspended in PRP could be cultured for at least 35 days in shared culture medium, without further addition of any growth factors or cytokines (they are probably present in the PRP). We then showed that direct contact co-cultures of EC and MSC in a 3D environment resulted in a significant up-regulation of osteoblastic markers in MSC, and endothelial markers in EC. Furthermore, histological analyses revealed tube-like structures and cells forming intra- and intercellular lumen in Mix-constructs. Interestingly, it seemed that MSC and EC both contributed to those tubular structures. We are aware, that this does not imply that vessels are about to form in this *in vitro* setting, but it shows that direct contact of MSC and HUVEC in a 3D environment containing PRP might have the potential to form a highly vascularized graft *in vivo*.

Future Perspectives

Overall, we believe to have contributed to a better understanding of the cellular interactions between MSC and EC. We have successfully used this knowledge to develop an endothelialized construct, ready to be used in *in vivo* experiments. Of course every answer raises another question.

- How would VEGF influence EC in direct contact co-cultures with MSC, or in 3D ?
- Which changes in the EC phenotype, induced by VEGF, are responsible for the effects described in chapter II ?
- The effect of angiopoietins on HUVEC-modulated MSC differentiation should be further explored, probably by using higher Ang1 concentrations and by including Ang2 (in combination with VEGF) into the studies. It is known that Ang2 action is largely dependent on the presence or absence of VEGF (see chapter I).
- Some of our 3D experiments (chapter V) will need to be repeated to confirm our results. The histological analyses should be expanded by using other markers, such as NG2, a pericyte cell surface marker. One might speculate that the MSC contributing to the tube-like structures differentiate into pericytes, fibroblasts or smooth muscle cells. This speculation should be explored.
- In order to be able to produce a completely autologous construct, we should reactivate the studies about generating endothelial cells from blood samples according to our protocols suggested in chapter VI.

- Finally it will be interesting to see how our construct performs *in vivo*, compared to empty scaffolds, or constructs missing the PRP (replace by fibrin gel) or the endothelial cells.

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Chapter VIII

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Publications

Publications based on this PhD thesis

- Stoll T, Maissen O, **Meury TR**, Becker S, “New aspects in osteoinduction”, *Mat Wiss Werkstofftech* 35,4:198-202
- **Meury TR**, Alini M, „Human endothelial cells inhibit human MSC differentiation into mature osteoblasts in vitro by interfering with Osterix expression“ *Submitted to J Cell Biochem*
- **Meury TR**, Kupcsik L, Heini P, Becker S, Stoll T, Alini M, „Definition of a PRP production protocol and the effect of PRP on osteoblastic differentiation of MSC in vitro.“ *Submitted to J Orthopaedic Research*
- **Meury TR**, Alini M, „In vitro evaluation of a tissue engineered 3D-construct for bone repair.“ *In preparation, to be submitted to Tissue Engineering*

Futher publications based on work done during this PhD thesis

- Becker, S, Maissen, O, Ponomarev, I, Stoll, T, **Meury, TR**, Alini, M, Wilke, I, “Osteopromotion with plasmatransglutaminase on a β -TCP ceramic: A pilot study.” *Submitted to Biomaterials*
- **Meury TR**, Gorna K, Alini M, Gogolewski S, “In vitro response of osteoblasts and chondrocytes to biodegradable polyurethane materials for tissue engineering.” *In preparation*
- Kupcsik L, **Meury TR**, Alini M, “Influence of statins on human bone marrow stromal cell differentiation.” *In preparation*

Curriculum Vitae

Thomas Meury was born in Basel, Switzerland on September 7th in 1973. He graduated high school in 1993 at the MNG (Mathematisch-Naturwissenschaftliches Gymnasium) in Basel. He started to study Biology in winter 1995 at ETH Zürich. In winter 1999-2000, he completed his diplomawork at the AO-Research Institute in Davos and received his diploma in "Naturwissenschaften" (Biotechnology) from ETH Zürich in spring 2000. He started his PhD in summer 2000 at the AO-Research Institute in Davos in the field of bone tissue engineering under the supervision of Dr. Mauro Alini. The faculty representative of the university of Basel was Prof. Dr. Ueli Aebi. The work done until spring 2005 resulted in this thesis, in a few submitted publications as well as in a few national and international presentations and posters. This thesis was defended successfully (*summa cum laude*) on the 20th of July 2005 in Basel in the presence of Prof. Dr. Michael Primig, Prof. Dr. Ranieri Cancedda, Prof. Dr. Mauro Alini and Prof. Dr. Ueli Aebi. Thomas Meury will continue his scientific career in the Genetics Unit of the Shriners Hospital in Montreal, Canada as a postdoctoral-research fellow starting August 2005.