Paracrine stimulation of endothelial cells and mesenchymal stem cells by the mechanically loaded human fracture haematoma

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Eidesstattliche Erklärung

Hiermit erkläre ich an Eides statt, dass ich die Dissertation selbständig verfasst habe; die von mir benutzten Hilfsmittel und Quellen sind aufgeführt und die Arbeit ist nicht in Zusammenarbeit mit anderen Wissenschaftlern oder Wissenschaftlerinnen erstellt worden.
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Seuzach, den 29. Oktober 2010

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Zusammenfassung

Obwohl die meisten Knochenfrakturen komplikationslos verheilen, zeigen manche eine verzögerte Heilung oder Pseudoarthrose. Angiogenese wird als essentieller Prozess betrachtet, da ihre Hemmung die Heilung verzögern oder inhibieren kann. Sowohl die frühe Phase der Knochenheilung als auch die Blutgefäßbildung werden durch mechanische Stimulation angeregt, die Mechanismen sind jedoch unklar. Die Ziele dieser *in vitro* Studie waren erstens, zu untersuchen, wie das ganze, frische Hämatom, sowie dessen mechanische Stimulation, Endothelzellen (EC, primäre Zellen und Zelllinie) und mesenchymale Stammzellen parakrin stimuliert. Zweitens wurden die involvierten Faktoren identifiziert. Drittens wurde eine Methode zur parakrinen Stimulation der Angiogenese im Hämatom etabliert.

Mit frühen Frakturhämatomen von gesunden Patienten konnte gezeigt werden, dass die Hämatome pro-angiogen auf primäre Endothelzellen und auf eine Endothelzelllinie wirken, dass dieses Potential durch mechanische Stimulation erhöht ist und mindestens 24 Stunden anhält. Hämatome setzten die Regulatoren der Angiogenese MMP-1, -2, -3, -9, TIMP-2, VEGF, TGF-β1 und Ang-1 ins konditionierte Medium (CM) frei. Durch mechanische Stimulation werden die Konzentrationen von MMP-1, -2, -9 und TIMP-2 bei allen Patienten, und VEGF bei jungen, aber nicht bei älteren Patienten oder gemischten Populationen im CM erhöht. Im Gegensatz zu den ECs, wurde das funktionelle Verhalten der MSCs nicht von den CMs beeinflusst. Drug delivery Systeme wurden getestet. Die Transfektion von MSCs wurde durch PEI beschichteten SPIONs und ein Lipofektionskit getestet, aber erfolglos. Nukleofektion wurde nicht weiterverfolgt, wegen der niedrigen Überlebensrate der MSCs.

Applikation von rekombinanten VEGF ins CM hemmte die Netzwerkbildung der primären EC und der EC-Linie. Trotzdem wurde die Netzwerkbildung auch unter Hemmung von VEGFR2 reduziert. Die Netzwerkbildung wurde durch Hinzufügen von MMP-$1_{1x\ regulierte\ Menge}$, MMP-$1_{10x\ regulierte\ Menge}$ und MMP-$2_{1x\ regulierte\ Menge}$ im CM erhöht. Frische Hämatome stimulierten die Tubebildung, und dieses Potential konnte durch Applikation von MMP-1_{10x} in 1:5 verdünnten Hämatomen mit serumhaltigem (FCS) Medium erhöht werden. Das Hinzufügen in serumfreiem Medium konnte die Netzwerkbildung nicht stimulieren.

Diese Studie bestätigt die parakrine, pro-angiogene Stimulation von ECs durch das humane Frakturhämatom *in vitro*, und dessen Erhöhung durch eine angepasste mechanische Belastung. Dies wurde durch eine erhöhte Konzentration von Regulatoren der Angiogenese begleitet, wie GFs und MMPs. Das angiogene Potential von frischen humanen Hämatomen wurde durch Hinzufügen von mechano-regulierten MMP-1 und Serum, wahrscheinlich durch Aktivierung und / oder Freisetzung eines GFs des FCS stimuliert.

Abstract

While most bone fractures heal without complications, some of them develop a delayed union or pseudoarthrosis. Angiogenesis has been shown to be an essential process as its blocking delays or inhibits the bone healing process. Both the early phase of bone healing and blood vessel formation are known to respond to mechanical stimulation, however through unclear mechanisms. The aims of this *in vitro* study were first to investigate the paracrine stimulation by the whole, fresh haematoma and its mechanical loading, on endothelial cells (ECs, primary human cells and a cell line) and human mesenchymal stem cells (MSCs). In a second part, the factors involved were identified. Finally, a method for the paracrine stimulation of angiogenesis in the haematoma was established.

By analysing early fracture haematomas of healthy patients, this study demonstrated that these haematomas are pro-angiogenic on both an EC line and primary ECs, that this pro-angiogenic potential is increased by mechanical stimulation, and persisted for at least 24h. Haematomas released the angiogenesis regulators MMP-1, -2, -3, -9, TIMP-2, VEGF, TGF-β1 and Ang-1 in the conditioned medium (CM). Mechanical stimulation increased the CM concentration of MMP-1, -2, -9 and TIMP-2 in all patients, as well as VEGF in young patients, but not in old patients or mixed populations. In contrast to ECs, the CMs did not significantly influence the functional behaviour of MSCs. Drug delivery systems targeting MSCs were tested. MSC transfection was tested as a delivery method for plasmid DNA, however unsuccessfully through both the PEI-coated SPIONs system and a lipofection kit. Nucleofection was inadequate due to the low survival rate of the MSCs.

The application of recombinant VEGF in CM decreased tube formation on both the EC line and primary ECs. However, a VEGFR2 inhibitor also reduced the tube formation. Tube formation was enhanced by addition of recombinant MMP-$1_{1x\ \text{regulated concentration}}$, MMP-$1_{10x\ \text{regulated concentration}}$ and MMP-$2_{1x\ \text{regulated concentration}}$ in the CM. Fresh haematomas stimulated the EC tube formation, and this could be increased by the supplementation of MMP-1_{10x} in haematomas diluted 1:5 in medium containing foetal calf serum (FCS). The supplementations did not influence the tube formation in absence of FCS.

In summary, this project confirmed the paracrine pro-angiogenic stimulation of ECs by the human fracture haematoma *in vitro*, and its response to an adequate mechanical stimulation. This response was accompanied by an increased concentration of angiogenesis mediators, such as GFs and MMPs. The angiogenic potential of the fresh human haematoma was increased by supplementation of the mechano-regulated MMP-1 in presence of serum, probably due to the activation and / or release of GF from the FCS.

Index

Chapter 1: Introduction

This part will present the most important characteristics of bone homeostasis and fracture healing, and show the cases of the uneventful and abnormal healing. Bone fracture healing is strongly dependent on the mechanical microenvironment in the fracture gap from the first days post trauma. Fractures disrupt the blood vessels network and its reconstruction, called angiogenesis is one of the key steps in the healing process, and also reacts to the local mechanical stimulation. Blood vessels formation mechanisms and regulators will be presented, before an overview of different in vitro *models of angiogenesis. On the cellular level, the bone regeneration process is regulated by mesenchymal stem cells, which response to mechanical stimulation and pro-angiogenic properties will be discussed. However mostly successful and scarless, bone fractures need therapeutic improvements for some risk patients and thus, some drug delivery methods as treatment strategies will be presented. After this presentation of the clinical problems, the aims of this study will be described.*

1. Structure, functions and properties of bone

Bones form an important part of the human skeleton. They function to move, support, and protect the various organs of the body, produce red and white blood cells and store minerals, amongst others [2]. Macroscopically, bones can be categorized according to their shape: for example flat bones, like the ones of the skull, protect organs while long bones contain bone marrow, where for example stem cells niche (mesenchymal and haematopoietic stem cells), that can be recruited in case of a trauma. The typical architecture of long bones is presented in the figure 1. The extremities are called epiphysis, and the shaft diaphysis. The outer shell of the bones is either made of cartilage in regions of articulations, or periosteum, a highly cellularized membrane with a nourishing role. Going further towards the centre of the bone, the cortical, compact bone resists to mechanical stresses, with a complex architecture of Haversian canals. The inner part of the bone is the medullary cavity (in the diaphysis) or spongy, woven bone (in the epiphysis) both containing bone marrow [2].

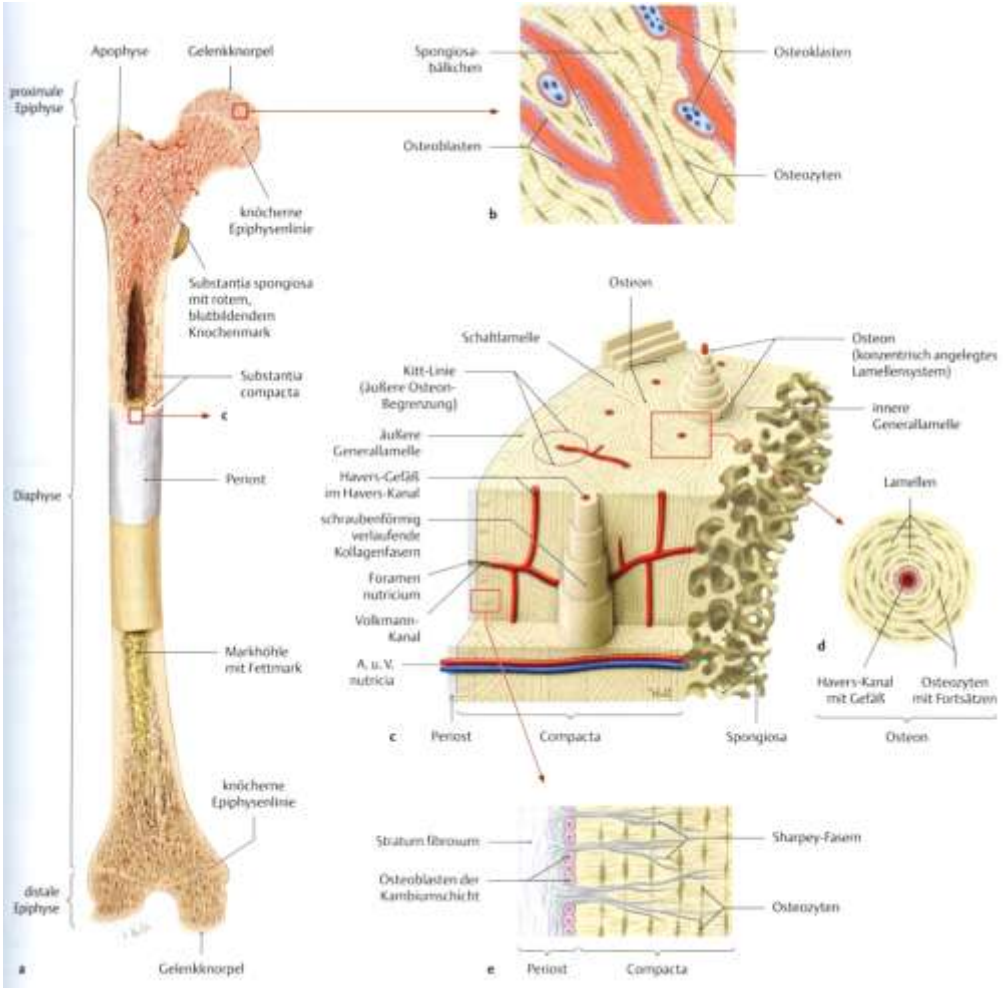

Figure 1 : Architecture of long bones (here a human, adult femur). Section a: frontal cut of the proximal and distal sections of the bone, showing the cortical bone around the spongious bone, containing the bone marrow. Bone is surrounded by a protecting, nourishing layer, the periosteum. Section b: detailed scheme of lamellar

architecture of the spongy bone. Section c: detailed scheme of the compact bone, showing the osteons around the Haversian channels, connecting the osteons with the Volkmanns' channels, connecting the periosteum to the bone marrow. Section d: detailed scheme of an osteon with their concentric layers consisting of osteocytes and ECM. Section e: detailed scheme of the periosteum. The figure was adapted from [3].

The hollow macrostructure provides the best combination of stability and lightness, combined with the characteristics of the woven bone, adapting to mechanical conditions (Wolff's law), (Figure 2) [4].

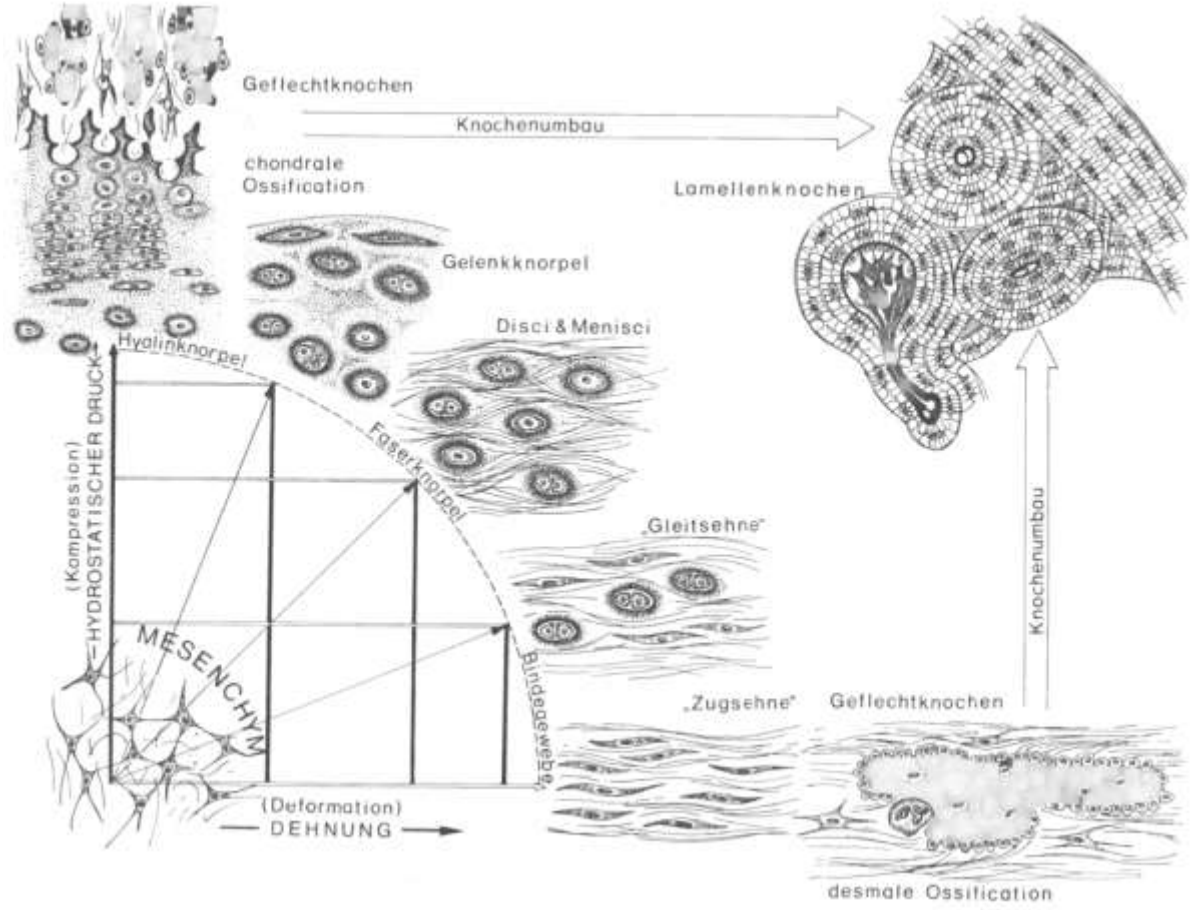

Figure 2 : Pauwels' diagram of tissue differentiation in response to the mechanical stimulation. The mesenchymal tissue differentiates to the different lineages according to the mechanical stimulation. Stretching of the mesenchyme leads to the formation of connective tissue, and cartilage under hydrostatic pressure. The different transition tissues are formed under mixed mechanical conditions of stretching and hydrostatic pressure. Woven bone may arise through two mechanisms: the endochondral ossification (through pressure) and the desmale ossification (through stretching). Both woven bone types may be remodelled into spongious bone. The figure was adapted from [4].

Bone is continuously adapted to the local mechanical stimulation, mainly by the interplay of ostoblasts and osteoclasts (homeostasis). Osteoclasts are multinucleated cells of hematopoietic origin differentiated from monocytes. Osteoclasts cut tunnels in the osteoid, the extracellular matrix (ECM) laid around the osteocytes, the fully differentiated bone cells. Osteoid fragments are then degraded by macrophages. These tunnels left by the osteoclasts are then covered by preosteoblasts, differentiated from mesenchymal stem cells (MSCs). Preosteoblasts differentiate into osteoblasts and become osteocytes by secreting new osteoid matrix, filling the gap (Figure 3). Bone turnover occurs continuously through the lifespan of an adult.

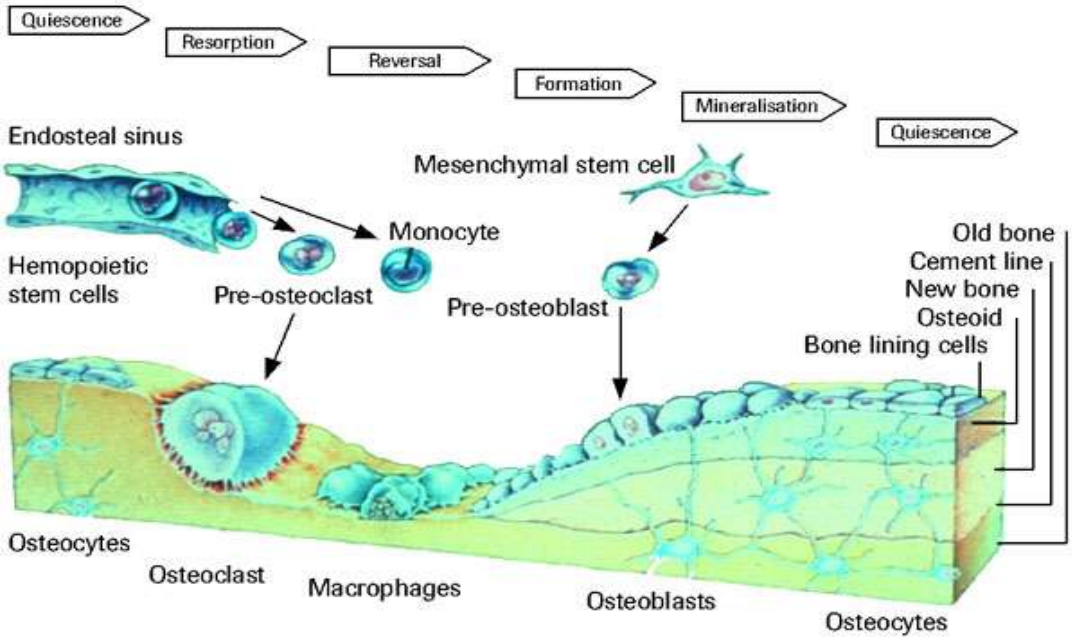

Figure 3 : Bone turnover. The remodelling of the bone tissue (osteocytes) begins with the recruitment of osteoclasts, dissolving the matrix and macrophages, removing the debris. Then preosteoblasts, differentiated from MSCs, adhere to the tunnel formed by the osteoclasts and further differentiate into osteoblasts and then, through the mineralization of the matrix, to osteocytes. The figure was adapted from http://www.roche.com/pages/facets/11/bone_remodelling2.jpg.

To summarize, bones play capital roles in the body, have a complex architecture and react to mechanical stimulations. However, bone fractures occur and, even if most patients heal without presenting complications, for some of them non-union will occur and will need additional treatments.

2. Bone fracture healing

Bones possess remarkable healing capacities. While soft tissues always heal by creating a scar, bone has the potential to fully regenerate, until the fracture site cannot be distinguished anymore. However, bone fracture is a clinically important problem, with increasing cases per year [5]. While most patients heal uneventfully, healing is complicated in 10 to 20% of the cases, for a cost around three times higher to the normal fracture healing cost [5, 6]. The healing outcome is also influenced by the extent of trauma in the surrounding soft tissues. Moreover, in healthy elder women, the clinical vertebral fractures and hip fractures are associated with a substantial increase in mortality, while other locations of fractures do not influence the mortality [7]. Thus, additional therapies are needed to treat bone fractures, and for it, the mechanisms of healing need to be better understood.

2.1. The four phases of fracture healing

The fracture healing process can be separated in four phases: 1) the inflammation, where ruptured blood vessels create a haematoma; 2) the formation of a soft callus, where new blood vessels are created and the haematoma is changed into a soft, fibrocartilaginous callus; 3) the building of a hard callus, by mineralization of the soft callus into woven bone; and 4) the remodelling of the callus, changing back the hard callus into the usual architecture of the bone. These phases overlap during healing and are distributed unevenly through the fracture area [8]. The four phases of fracture healing are presented in Figure 4 and are described in the next sections, with a focus on the inflammation phase.

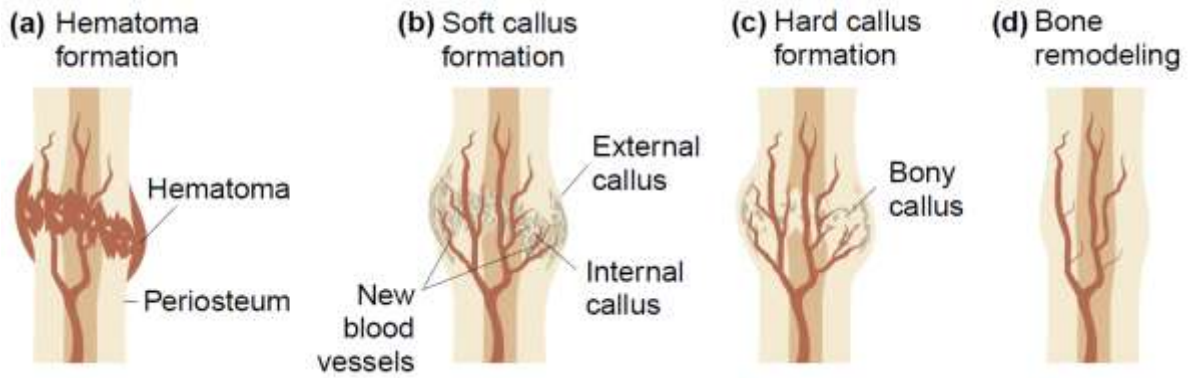

Figure 4 : The four phases of fracture healing. After the fracture, a blood clot, the haematoma, is formed between the fracture ends. Inflammatory cells and MSCs migrate to the site of injury and remodel the haematoma into a soft callus through formation of new blood vessels, the angiogenesis process. The soft callus is further remodelled into a hard callus, where the fibrocartilaginous tissue of the soft callus becomes mineralized. Finally, the bone fracture site is remodelled to meet the shape and characteristics of the native bone. The figure was adapted from [9].

Phase I: inflammation

The inflammatory phase of the fracture spans for around 3 days [9, 10].

Description

Because of a fracture, bones' blood vessels are disrupted, leading to extravasation into the fracture gap, retained by the surrounding tissues. The blood coagulates and a haematoma is formed. The absence of blood supply locally leads to hypoxia and acidosis, activating the platelets to release alpha-granules into the fibrin-rich ECM [11]. This mobilizes inflammatory cells - in particular, macrophages, granulocytes, lymphocytes and monocytes - invading the haematoma to fight the infection, secreting cytokines and growth factors and remove bone splitters [12, 13]. Blood vessels sprout into the blood clot, transformed into a granulation tissue within 24-36h post trauma [11]. MSCs, key to bone regeneration [11], are recruited to the haematoma about three days after fracture [10, 13]. Removal of the fracture haematoma impairs repair, and its transplantation leads to the formation of new bone [9].

Molecular level

Many cytokines are secreted during the inflammation phase. Pro-inflammatory cytokines, like IL-1, IL-6 and TNF-α, are secreted by macrophages, inflammatory cells and mesenchymal cells to initiate the healing cascade and recruit other inflammatory and mesenchymal cells [14]. These cytokines peak around 24h post trauma [9, 14, 15].

Growth factors (GF) have been thoroughly investigated and an important number of them were detected during the early phase of fracture healing [9]. Platelet-derived growth factor (PDGF) is released from platelets and is an important chemotactic stimulation for inflammatory cells and MSCs [14]. Furthermore, MSCs' proliferation is stimulated by this factor [14].

Fibroblast growth factors (FGFs) were detected during this phase [9, 14]. FGFs are secreted by monocytes, macrophages and mesenchymal cells in the haematoma, and stimulate the proliferation and differentiation of many cells and stimulate the angiogenesis [14]. GFs from the transforming growth factor-β (TGF-β) superfamily were described in the haematoma [14]. TGF-β is released from platelets during the inflammatory phase and can stimulate the proliferation of MSCs, osteogenic differentiation and angiogenesis [16]. BMPs, secreted by mesenchymal cells, osteoprogenitors, osteoblasts and osteoclasts, play capital roles in the fracture healing process by stimulation of the chondro-osteogenesis through the recruitment and differentiation of mesenchymal cells and osteoprogenitors. Also, the synthesis of ECM is enhanced by bone morphogenic proteins (BMPs) [14].

Angiogenic factors and metalloproteinases play capital roles in the haematoma [14]: without the blood vessel formation the constructive phase of bone healing will not occur [11]. For example, vascular endothelial growth factor (VEGF) is an essential mediator of angiogenesis, acting as a mitogen for endothelial cells (ECs) [17]. VEGF expression is induced by BMPs in osteoblasts and osteoblast-like cells [9, 17] and was investigated in the haematoma [18]. Participation of angiopoietins (Ang-1 and -2), implicated in the formation of bigger blood vessels and separations, is speculated in the process of bone fracture healing [14, 17].

Besides these molecular and cellular factors, mechanics play a role in the early phase of fracture healing [19] [20], as will be discussed later (p. 21).

Phase II: soft callus formation

In the case of a mechanically instable callus, the haematoma is transformed into a soft callus. This phase spans from around three to seven days [21], during which blood vessels are formed in two separate calluses: the external callus, where intramembranous ossification will occur, and the internal callus, in the medullary channel, mostly formed by fibrocartilage [9]. This phase is dominated by chondrocytes and fibroblasts [12]. A semi-rigid, soft callus of cartilage is formed, bringing some mechanical stability to the fracture gap, before the chondrocytes hypertrophy, mineralize the matrix and undergo apoptosis. This soft callus serves as a template for the next phase, the hard callus [9].

Phase III: hard callus formation

The third phase of fracture healing is the hard callus, spanning up to 4 months [21]. The mineralization of the soft callus, leads to the formation of a hard callus of woven bone by osteoblasts, beginning in the most stable areas of the callus, and replacing it. At the same time the callus is revascularized, stimulating the differentiation to osteoblasts, recruited from the periosteum and bone marrow [9].

Phase IV: remodelling

Bone union ends with the remodelling of the bony callus between three to six months post trauma into cortical and woven bone back to the normal anatomical shape. The blood supply also returns to normal levels. As during homeostasis, osteoblasts and osteoclasts act in a controlled manner, as osteoclasts remove old matrix, creating tunnels that will be covered by

preosteoblasts. These cells will terminate their differentiation into osteoblasts and then osteocytes, secreting bone matrix, the osteoid [9].

In summary, bone fracture healing is a complex and long process, implying the participation of many cell types and proteins. However, this process may be disturbed and lead to a delayed union or non-union.

2.2. Pathology of fracture healing

The normal consolidation time of fractures is 6 weeks. At three months post-trauma, non-healed fractures are divided on the base of radiographs into delayed unions or non-union, and also atrophic (no tissue formed) or hypertrophic pseudoarthrosis. Many factors may influence the healing time [22].

As the patient's age increases, not only the fracture incidence increases, but also the duration of the healing time, and thus the hospital stay. The morbidity rate is also higher for older than younger patients. Thus, the capacity of bone regeneration after a fracture slows with advancing age. With increasing age, temporal progression through the healing stages (inflammation, soft and hard callus, remodelling) is protracted and constituent elements of ECM and cells diminish through time [11]. For women, this bone mass loss and slowed homeostasis begins around the age of 45. These observations have been further tested in numerous animal models. For example, 6-week old rats heal completely within 4 weeks, while 26- and 52-week old rats need 10 weeks and 6 months, respectively [23]. An impaired angiogenesis is one of the cellular factors of delayed bone healing in the aged, where a decrease of ECs, GFs and their receptors is observed. Also, alterations in the structural and regulatory components of the matrix contiguous to forming vessels on aged tissues could influence bone healing in elderly patients [11]. Impaired vasodilation, enhanced thrombotic potential, inhibited EC migration, alterations of matrix synthesis and turnover and decreased proliferation of mature and progenitor ECs were also postulated as causes for this decreased angiogenesis [24]. In a rat model of ageing, the older animals were shown to be less responsive to mechanical stimulation than younger, delaying bone union [25] and in an *in vitro* study of MSCs of young and aged animals MSCs numbers were stable but the differentiation potential and proliferation were reduced with age [26].

Moreover, the patient's health status and muscular mass may affect bone healing. Diseases like diabetes mellitus or osteoporosis increase the union time. Insufficient nutritional apports (protein, calcium, phosphorus, Vitamin D) affect the callus mechanical strength. Also non-steroidal anti-inflammatory drugs (NSAIDs) consumption reduces the osteoblastic activity and the synthesis of prostaglandin, delaying bone union. Smoking increases the risk of

delayed fracture healing and pseudoarthrosis by decreased vascularisation. Alcohol abuse inhibits new bone formation, thus delaying the bony callus formation. The local status of the fracture, like site, trauma energy, and infection, also influence healing [27, 28].
However, with any non-risk patient, the healing may also be delkayed because of an inadequate mechanical stimulation of the haematoma and the callus.

2.3. Response of bone fracture to mechanical stimulation

The effects of mechanical stimulation on the bone fracture healing were investigated both *in vivo* and *in vitro*.

In vivo

Bone fracture healing is sensitive to mechanical stimulation, inside a therapeutic window [19, 29]. A loose fixation frame, leading to an excessive mechanical stimulation, leads to hypertrophic callus formation, due to a too intense interfragmentary motion breaking the new formed tissues. This can even lead to hypertrophic pseudoarthrosis [30, 31]. In ovine bone healing, excessive interfragmentary movement inhibits or delays revascularisation, and results in the impaired restoration of the tissue's mechanical properties; about 20% compression seems to be an acceptable stimulation [32-34]. Lack of, or too low mechanical stimulation, for example due to a too stable fixation, may impair callus formation and lead to oligotrophic or atrophic non-union [31]. Conversely, increased blood flow and tissue stretching have been shown to stimulate angiogenesis [35-37], and mechanical stimulation of pro-angiogenic responses has been reported *in vitro* [38].

Mechanical microenvironment affects both the callus size and its mineralization: as appropriate mechanical stimulation promotes callus formation and increases its mechanical stability, larger loading increases the callus size but decreases its mechanical properties [39]. However, the sensitivity to mechanical stimulation of an individual depends on its age, as it affects its ability to heal in time: a study in the rat osteotomy model showed the interdependence of the age of the animal and the fixation stability on the callus stiffness [25].

Angiogenesis is sensitive to mechanical stimulation [8, 33] and compromised angiogenesis is considered as the major risk factor for healing [40]. In a murine model of distraction osteogenesis (DO), many pro-angiogenic GFs and matrix metalloproteinases (MMPs) were differentially expressed after the distraction phases. VEGF, Hypoxia-inducible factor 1 (HIF-1α) and Neuropilin-1 (Nrp1) were significantly induced after each distraction cycle,

suggesting that these factors are early mediators that are produced by distraction and contribute towards the processes that promote bone formation [41].

In vitro

The exact mechanical conditions present in the fracture gap are complex, a mix of compression, bending, tension and fluid shear stresses [4] that cannot be applied *in vitro*. Furthermore, different cell types may be involved in the healing process, which response to mechanical stimulation should be investigated: MSCs, fibroblasts, chondrocytes and osteoblasts [42]. Thus, the *in vitro* investigation (both two- and tri-dimensional) of the cellular response to mechanical stimulation already simplifies on two sides. As this is a very bright field, the mechanical stimulation of MSCs will be described later as an example (p. 30).

In summary, mechanical stimulation is a key parameter during bone healing, in particular the early phases, as seen with its influence on the healing rate and angiogenesis. However, the modulation mechanisms of the angiogenic stimulation by mechanical loading are far from being solved.

3. Angiogenesis

Angiogenesis is the formation of new blood vessels from pre-existing ones. Animal models demonstrated the importance of angiogenesis in bone healing: inhibition of angiogenesis delays bone healing, as shown in Figure 5. In this study, Fang et al. used a mandibular DO in rats to test the effects of the inhibition of angiogenesis. The rats were osteotomized and distracted gradually with or without application of a systemic angiogenesis inhibitor (TNP-470) for 14 days and a 28 days consolidation phase. Administration of TNP-470 prevented normal osteogenesis and resulted in a fibrous non-union. Histologically, a lack of platelet cell adhesion molecules was observed in the angiogenesis inhibitor group. However, TNP-470 did not affect rat osteoblasts *in vitro* [43]. Hausman et al. also showed that the treatment of fractures with angiogenesis inhibitors led to formation of defective granulation tissue, absence of cartilage differentiation and subsequent endochondral ossification [44].

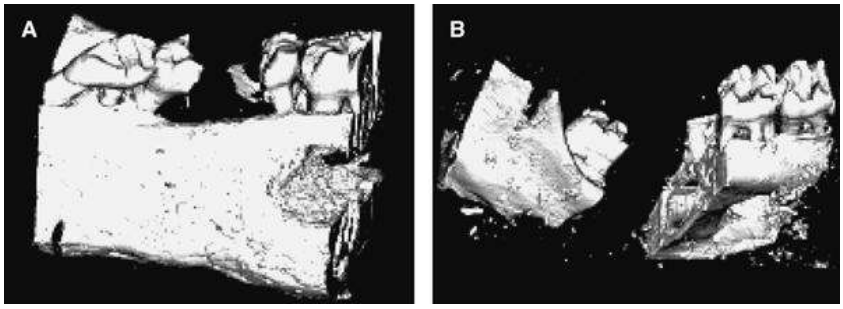

Figure 5 : Inhibition of angiogenesis delays bone healing. µCT pictures of the distraction osteogenesis of a rat's jaw. After osteotomy, the rats were treated with an angiogenesis inhibitor (TNP-470). A: control animals, osteotomized but not treated with the inhibitor, healed normally; B: animals treated with the inhibitor lost their ability to mineralize the osteotomy gap. Microarray analysis revealed distinct patterns of expression of genes associated with osteogenesis, amongst others. Figure from the reference [43].

The early stage of fracture healing was already investigated by Street et al. They found that the VEGF level in human fracture haematomas was high enough to induce tube formation *in vitro*; however, ECs proliferation and tube formation were inhibited *in vitro*, due to the high, cytotoxic potassium levels in the haematoma. However, the implantation of haematoma *in vivo* increased the local angiogenic response through the mediation of VEGF [18].
So, the lack of angiogenesis inhibits the bone union in animal experiments and the early human fracture haematoma shows pro-angiogenic. Thus, the mechanisms of angiogenesis and the proteins involved in its regulation should be described in more details.

3.1. Mechanisms of angiogenesis

Three mechanisms of angiogenesis are known. New blood vessels either sprout from pre-existing postcapillary venules (sprouting angiogenesis), which is the most frequent mechanism. New blood vessels can also be formed by the separation of pre-existing, enlarged capillaries by pillars of peripheric ECs (intussusception) or by transendothelial cell bridges (bridging) (see Figure 6) [17].

Sprouting angiogenesis happens in several steps: vessels are dilated, become permeable, EC proliferate and migrate, a lumen is formed and the vessel is finally differentiated. Once formed, blood vessels are stable for long periods of time [17]. These steps are further described below.

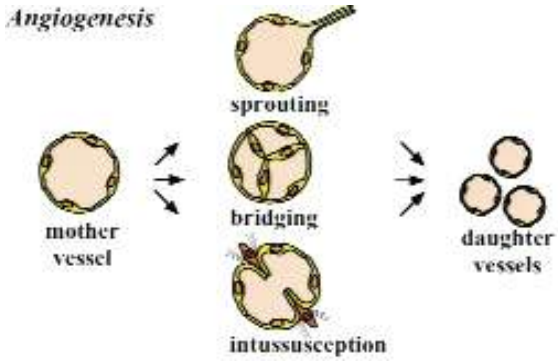

Figure 6 : Mechanisms of angiogenesis. The separation of the mother vessel into daughter vessels can happen through several mechanisms, amongst which sprouting, bridging and intussusception. Figure adapted from reference [17].

Vasodilation, endothelial permeability, and periendothelial support

Angiogenesis is initiated by vasodilation. VEGF increases the vascular permeability, allowing the extravasation of plasma proteins forming a scaffold for the later migration of ECs, and the formation of fenestrations.

Ang-2 may detach smooth muscle cells (SMCs) and loosen the matrix, destabilizing the mature vessels, whereas Ang-1 is an inhibitor of vascular permeability, tightening pre-existing vessels. Amongst other molecules, MMPs can stimulate angiogenesis by degrading the ECM and activating or releasing GFs, in particular bFGF, VEGF and the insulin-like growth factor-1 (IGF-1) [17].

Endothelial cell proliferation and migration

Once the MMPs, in collaboration with other proteinases, created clearances in the structure, proliferating ECs migrate to the formed fenestrations. Ang-1 is chemotactic for ECs, but in contrast to VEGF, Ang-1 does not initiate endothelial network organization, but stabilizes networks initiated by VEGF, presumably by stimulating the interaction between endothelial and periendothelial cells. So, Ang-1 may act at later stages than VEGF. Ang-2 is angiogenic in presence of VEGF. Members of FGF and PDGF families affect angiogenesis at this stage, probably by the stimulation of the recruitment of mesenchymal and inflammatory cells [17] (see Figure 7). PDGF stimulates the proliferation of ECs and the angiogenesis *in vitro* [45].

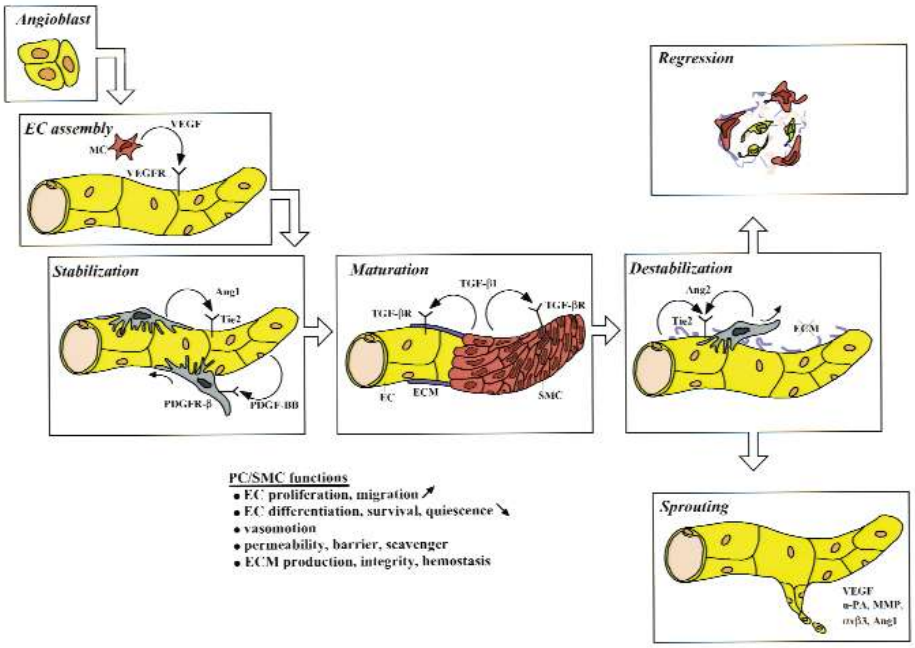

Figure 7: Mechanisms of sprouting angiogenesis. Figure adapted from reference [17].

For a balanced process, some molecules like angiostatin or endostatin inhibit the proliferation and migration of ECs. However, some molecules like TGF-β1 and tumor necrosis factor-α (TNF-α) can both stimulate or inhibit angiogenesis [17].

Lumen formation

A lumen is formed as ECs assemble as solid cords. VEGF isoforms can increase or decrease the lumen diameter and Ang-1 increases the lumen diameter in combination with VEGF [17].

Endothelial differentiation

In a final step, blood vessels are further differentiated to be adapted to their function. For example, specialized vessels in the endocrine glands for the exchange of particles become fenestrated and discontinuous, by the action of VEGF. The formed blood vessels become quiescent and can survive without modifications for years providing the angiogenic stimulation does not change. In this case, Ang-1 promotes, while Ang-2 suppresses the ECs survival. Hemodynamic forces are essential for endothelial maintenance [17].

3.2. Regulation of angiogenesis

Hypoxia, mechanical stimulation, cell-to-cell interactions and components of the ECM stimulate angiogenesis. Moreover, numerous diffusible factors are associated with the regulation of angiogenesis in a time- and concentration-dependent manner [17]. The most important of them are described below.

Diffusible growth factors

The most potent diffusible GF is VEGF-A. VEGF-A (also called VEGF) belongs to the VEGF-family and can bind to the cell surface receptors VEGFR1 and VEGFR2. However, only VEGFR2 is implicated in angiogenesis. VEGF induces EC proliferation, sprouting and tube formation [46, 47]. Deletion of VEGF is lethal in mice, leading to vascular defects and cardiovascular abnormalities [46].

The Ang-1 and -2 are involved in the blood vessels maintenance, growth and stabilization. Angiopoietins can bind to the cell surface receptors tie1 and tie2. However, only tie2 is involved in angiogenesis. Tie2 deficient mice die before birth, and show a reduction of ECs in vessels and abnormal vasculature with lack of branching and sprouting vessels [46].

FGFs (both aFGF and bFGF) are involved in angiogenesis by stimulating EC proliferation and migration. FGFs induce sprouting angiogenesis in the chick chorioallantoic membrane and cornea assays [46].

PDGF is also involved in angiogenesis, by the recruitment, and proliferation of pericytes, and the proliferation of smooth muscle cells and MSCs. Capillary ECs also express the PDGF-β receptor, and react to PDGF by forming sprouts and chords *in vitro* [46, 48].

TGF-β1 displays both pro- and anti-angiogenic properties by affecting several cell types. At low doses, TGF-β1 stimulates angiogenesis by up-regulating stimulating factors and proteinases. Higher doses of TGF-β1 inhibit EC growth, promote basement membrane reformation and stimulate smooth muscle cells recruitment and differentiation [46, 49].

Matrix metalloproteinases

MMPs belong to a family of zinc-dependent endopeptidases not only able to degrade all ECM components, but also releasing and / or activating GFs from the matrix, exposing cryptic sites and neo-epitopes from the ECM [50, 51] and thus, affect cell behaviour *in vivo* and *in vitro* [52, 53]. Involvement of MMPs in angiogenesis is well established [50, 54] and their activity is amongst the key rate-limiting steps in ECM degradation [55-57], and thus bone healing and angiogenesis [58], as the recruitment and invasion of cells involve

proteolytic activities [54, 59, 60]. MMPs are necessary for EC migration and tube formation, as well as the invasion of fibrin barriers [50]. Moreover, MMPs, in particular MMP-2, are involved in the angiogenic switch [61] and tumour growth [50, 62]. MMP-9 is involved in the recruitment of endothelial progenitor cells [54], in osteoporosis [63] and in the tumour angiogenic switch [50, 64]. However, MMPs can also inhibit angiogenesis by generating anti-angiogenic ECM fragments, like endostatin [54]. All MMP-knockout mice survive birth, possibly because of the functional overlapping of the different MMPs [50]. However, MMP-9 knockout mice show deficient endochondral ossification and thus delay the apoptotic loss of hypertrophic chondrocytes, possibly by delaying the release of angiogenic factors from the ECM and thus vascular invasion [52].

Degradation of the extracellular matrix

Six classes of human MMPs are known, according to their structure and preferred substrate: the collagenases (MMP-1, 8, and 13, able to cleave interstitial collagens I, II and III), gelatinases (MMP-2 and -9, digesting denatured collagen, gelatin), stromelysins (MMP-3, -10, -11, digesting some ECM components), matrilysins (MMP-7 and MMP-26, able to degrade most ECM structures), membrane-type MMPs (MT-MMPs: MMP-14, -15, -16, -17, -24, -25, anchored to the plasma membrane and also able to digest numerous ECM molecules) and others [65, 66].

MMP-1 is the only enzyme able to initiate breakdown of the interstitial collagens, collagen type I, collagen type II, and collagen type III [67, 68]. Activated MMP-2 can bind to the cell membrane on $\alpha_v\beta_3$ sites [52, 54], while MMP-9 can bind to the membrane via CD44. MMP-3, but not MMP-2, is able to degrade fibrin from a blood clot [69]. MT1-MMP (MMP-14) and urokinase can form multiprotein complexes in the lamellopodia and focal adhesions of migrating cells, facilitating proteolysis and supporting migration and survival of ECs. MT1-MMP co-localizes with β1-integrins in cell-cell contacts [54].

Activation and inhibition of matrix metalloproteinases

MMP expression is regulated by several cytokines and GFs [52]. Soluble MMPs are secreted as inactive zymogens, activated extracellularly by other MMPs [54]. For example, MT1-MMP is involved in the activation of MMP-2 with TIMP-2 [52] and MMP-3 activates several MMPs, for example pro-MMP-1 to fully active MMP-1 [66]. Membrane-bound MMPs are activated intracellularly by furin-like enzymes and anchored to the membrane [54].

In tissues, MMPs are inhibited by the tissue inhibitors of metalliproteinases (TIMPs) in a 1:1 stoichiometry and reversible manner [52], but in the plasma the general protease inhibitor α2-

globulin is the predominantly MMP inhibitor [50]. Four TIMPs are known, showing many basic similarities. However, there are distinct structural features, biochemical properties and expression patterns [70]; TIMP-1, -2 and -4 are soluble, while TIMP-3 is bound to the matrix [70, 71]. All four TIMPs can inhibit the active form of all known MMPs [70]. However, the dominant *in vivo* function of TIMP-2 is the activation of MMP-2. The activation of MMP-2 dominates at low to moderate TIMP-2 concentrations, but higher levels tend to inhibit MMP-2 [52]. The inhibition of TIMP-1 stimulates angiogenesis and fibroblasts migration [72].
Next to their function on MMPs, effects of TIMPs on the cell behaviour are contradictory. While some stimulate the tumour growth or act anti-apoptotic, some inhibit its formation or lead to programmed cell death [70, 73].

Release and activation of growth factors

Many GFs are released and / or activated by MMPs. For example, MMP-1, -3, -7 and -13 can dissociate the most common form of VEGF (165 amino acids) from the complex formed with the connective tissue growth factor (CTGF) [50]. MMP-2 and MMP-9 release VEGF from the proteoglycan matrix [46, 52]. MMP-2, -3 and -7 degrade decorin to release the latent TGF-β1, further activated by MMP-2 and -9 through the cleavage of the latency-associated peptide [50, 52]. As TGF-β promotes osteoblastic activity, lack of MMP-2 or MMP-9 might hinder the bone deposition and thus, unfavour bone remodelling [52]. Both MMP-1 and MMP-3 degrade perlecan in EC-basement membranes to release bFGF [50, 74].
MMP-1 may participate in the regulation of IGF by cleavage of its binding proteins [74]. MMP-2 can cleave the ectodomain of the fibroblast growth factor receptor 1 (FGFR1), which, still able to bind to FGF, is unable to signal, and thus, MMP-2 also has anti-angiogenic activity [50].

In summary, MMPs are involved in the degradation of the totality of ECM components. Furthermore, they are able to release or activate most GFs and thus, have a central role in many biological processes like angiogenesis.

3.3. *In vitro* models of angiogenesis

The ethical questions linked to the use of animal models stimulated the development of in vitro assays for quick testing.

The aortic ring assay is a bridge between *in vivo* and *in vitro* assays. Aortic ring-like segments from rats or mice are embedded in a soft matrix (fibrin, collagen or Matrigel), and cultivated, typically during one week, while branching microvessels are generated from the ring. The length and / or branching of the formed networks may be quantified [75]. This assay displays several advantages, as all the key steps of angiogenesis are tested by the interaction of multiple, physiologically relevant cell types. However, no microvessels can be used for size reasons, and the three-dimensional quantification is challenging [76].

The three-dimensional sprouting assay may be the most relevant *in vitro* angiogenesis assay, where ECs are embedded as a bead in a soft matrix and the tubular sprouts are quantified [77]. However quite quick and relevant, the method is hard to quantify reliably, which compromises the relevance of the obtained results.

However, the two-dimensional capillary-like structures formation on basement membrane, like Matrigel or collagen, is reasonably quick and easy to quantify. Even if the assay does not imply the sprouting of the vessels into a matrix, the formed vessels contain a lumen and tight cell-cell contact. Most factors tested on this assay confirmed their effect *in vivo* and thus, this assay is mostly considered as relevant [78, 79].

After the description of the mechanisms of angiogenesis and the molecular players involved in this key step for bone healing, the MSCs will be described, as these cells are considered as the main cellular players during bone healing.

4. Mesenchymal stem cells

Mesenchymal stem cells (MSCs) are considered as the main cellular players for the reconstructive phases of bone regeneration, and are recruited to the haematoma around day three [1, 11, 14, 80-83], possibly through PDGF-bb into the site of injury [84]. These cells can differentiate into the adipogenic, chondrogenic and osteogenic, but not the hematopoietic lineage [1, 85] (see Figure 8). They have the ability of self-renewal [86] and are able to escape allogenic rejection [85, 87, 88]. Moreover, MSCs can be easily isolated by gradient centrifugation and plastic adherence from bone marrow aspirates, but also fat and muscle tissue for example [1, 89].

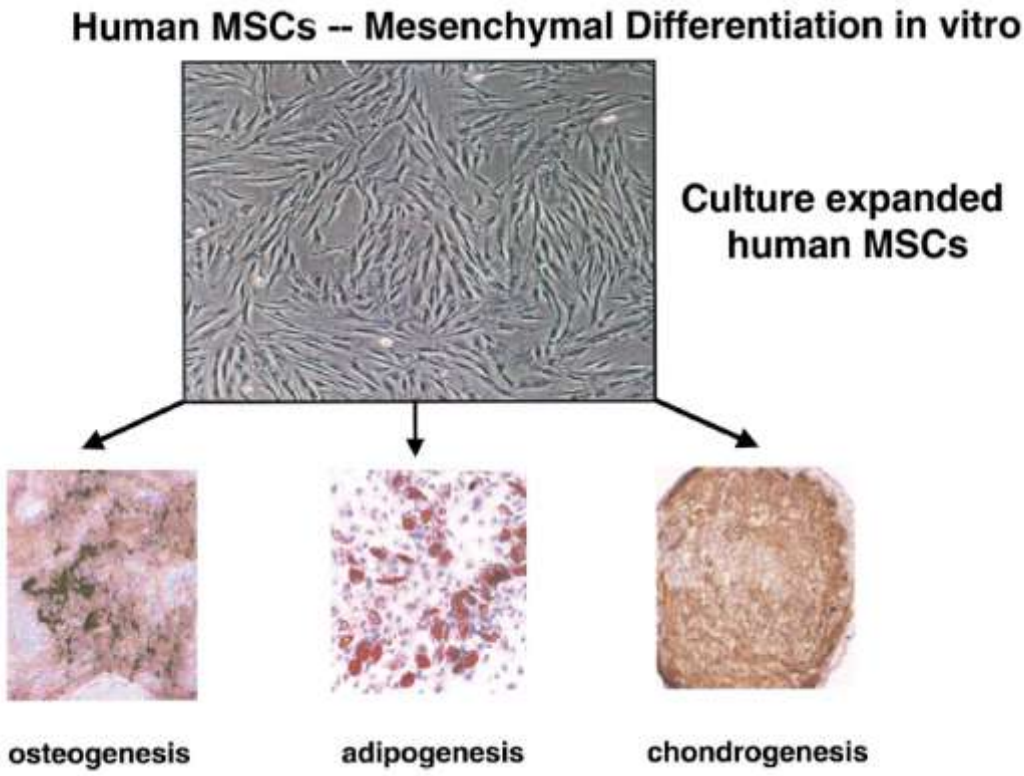

Figure 8: Differentiation capacity of mesenchymal stem cells. MSCs are able to differentiate *in vitro* into the osteogenic, adipogenic and chondrogenic lineages under chemical induction. Figure adapted from reference [1].

MSCs are sensitive to mechanical stimulation. In an *in vitro* model of cyclic mechanical loading of human MSCs, an adequate stimulation increased the secretion of pro-angiogenic factors, amongst them MMP-2, and TIMP-2, which increased the paracrine stimulation of tube formation in vitro [90, 91]. MSCs are able to stimulate migration and formation of tubular structures of ECs from capillaries and large vessels [92]. However, these cells' response to the bone fracture haematoma stays unclear.

Because of these characteristics, MSC-based therapies have become attractive in the field of regeneration of injured tissues, in particular bone [85, 93]. For this, the development of drug delivery methods is important.

4.1. Drug delivery

The problem of bone loss, for example through fractures, may be solved by engineering viable bone grafts or the enhancing of cellular material from the patient [94, 95]. Moreover, as systemic drug administration may lead to unwanted side-effects, the locally and timely-defined application of molecules to the body is preferable.

Three different application methods may be considered: application of proteins, RNA by viruses (transduction), or DNA by plasmids (transfection). Clearance of recombinant proteins disadvantage their application compared to the others strategies, but the handling is relatively easy and safe, and present advantages, at least for the first steps of the development of a therapy. Transfection and transduction show the advantage of a local, timely well defined application of factors. Transduction leads to high efficiency rates (50-100% depending on the cell type [94, 96, 97]) but the potential risk of mutagenesis, carcinogenesis and the immune response to the infection involve special security measures [94, 97-99]. Thus, viruses will not be considered as an option in this study. Transfection is a challenging field, combining the locally and timely control of the over-expression of the desired protein by the target cells with relative safety. Here, the interaction with the surface proteins and ECM should be avoided, to protect the DNA material, as well as the aggregation of the transfection complexes. Furthermore, the complexes should allow endolysosomal release once entered the cells by endocytosis [94]. Numerous transfection kits are available, involving many methods: electroporation or nucleofection (consisting of a depolarisation of the plasma membrane, allowing the entrance of DNA material [97], for example in the Amaxa system), or the application of a lipidic of cationic solution [97] (for example in the Lipofectamine or Primefect kits). However, the use of nanoparticles offers the possibility to timely and locally control the application and the follow-up of the magnetic nanoparticles by magnetic resonance imaging (MRI) [100].

Cationic polymers (typically polyethylenimine, PEI, see Figure 9) complexed with DNA, can enter the cells via endocytosis. PEI can also prevent the degradation of the genetic material before arrival in the nucleus. During endocytosis, the pH is drastically reduced by the entry of ions H^+ and Cl^- into the endosome, in order to destroy the foreign DNA and RNA. The amino

groups of the polymer can be protonated ($NH_2 \rightarrow NH_3^+$) and act as a proton buffer, known as the proton sponge mechanism [101, 102].

To improve efficacy of transfection and improve the control of the local delivery of the plasmid, the PEI-DNA complex can be complexed to superparamagnetic iron oxide nanoparticles (SPIONs[1]), the magnetofection. SPIONs show a very small size (~9nm) and a tight size distribution. The coating with PEI results in colloidally stable suspension with a tight size distribution [103]. The coating of SPIONs by PEI for DNA delivery was tested on the HeLa cell line with high transfection efficiency and survival rate.

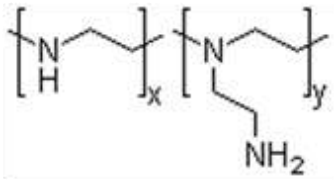

Figure 9: The repetitive unit of PEI

The surface charge of SPION-PEI-DNA complexes can be regulated by the atomic Nitrogen-to-Phosphorus (N/P) ratio of the PEI and DNA [104]. The most efficient N/P ratio for transfection of HeLa cells is 7.6 [96]. Kamau et al further describe the enhanced transfection efficiency by application of a static magnetic field, and the greater efficiency by application of a pulsed magnetic field in primary, non-human cells by PEI-coated magnetic nanoparticles [96, 105]. Cell toxicity of SPION-PEI-DNA complexes is low [106]. However, the transfection of human MSCs by PEI-coated SPIONs was not tested so far.

[1] In collaboration with Prof. H. Hofmann from the Laboratory of Powder Technology (LTP) of the EPFL, Lausanne, Switzerland

5. Aims of the study

A successful bone fracture healing involves the interplay of mechanical stimulation, angiogenesis and MSCs, however through so far unsolved mechanisms. Thus, the aims of this study were to investigate the functional effects of ECs and MSCs by the human haematoma, with and without application of mechanical loading on the haematoma, as well as the factors possibly involved in the different functional effect. Furthermore, the detected factors were used to increase the pro-angiogenic potential of the haematoma. For this, delivery methods were tested on the MSCs and fresh human haematomas *in vitro*.

In particular, the following aims were to be achieved:

- investigation of the paracrine effect of the haematoma on ECs and MSCs,
- evaluation of the influence of the haematoma's mechanical loading on this paracrine effect,
- quantification of angiogenic GFs, MMPs and TIMPs, released by the haematoma with and without mechanical stimulation,
- testing virus-free transfection methods for MSCs as potential carriers for identified factors
- testing of the effects of identified mechano-regulated factors on the haematoma in an *in vitro* angiogenesis assay with and without the presence of serum.

For this purpose, human long bones fracture haematomas from patients undergoing surgery within 72h post trauma were to be used. Bioreactor experiments were to be conducted with these haematomas, and the conditioned media were to be applied on ECs (cell line and primary cells) to quantify the tube formation, and on MSCs to evaluate their functional behaviour. The relevant factors' concentrations were to be determined by enzyme-linked immunosorbent assays (ELISAs). After the testing of different drug delivery methods and selection of the most adapted to the *in vitro* system, the most efficient factors and concentrations detected were to be applied on fresh haematomas. This should allow the selection of a factor, which could be tested in animal assays to investigate the stimulation of angiogenesis during bone healing.

Chapter 2: Materials and Methods

This section firstly lists the materials and chemicals used during this project. The collection of the haematomas from the clinics and the cultivation in bioreactors is described. The cell culture is described for the ECs (both the cell line and the primary cells), MSCs and HeLa cells. Then, the in vitro *tube formation assay on Matrigel is described for fresh haematomas and the conditioned medium produced in bioreactor. The methods regarding the functional assays of MSCs, the characterization of the proliferation, migration and differentiations are presented. Specific ELISAs were used to quantify the GFs, MMPs and TIMPs detected by zymography. Transfection methods are presented, using PEI-coated SPIONs, lipofection and nucleofection. Finally, the statistical analysis methods of the data are presented.*

1. Materials

Table 1: List of materials used

Product	*Supplier*	*Product number*
Alcian Blue	Sigma	8GX
2-propanol	Roth	1.096.342.511
A. Dest	Millipore	(-)
Acetic acid	Merck	1000562500
Agar-Agar	Roth	2266.1
Alamar Blue	AbD Serotech	BUF 012B
Alizarinred-S	Sigma	A5533
Alkaline Phosphatase Kit	Vector Laboratories	AK 5200
Ammonium persulfate	Sigma	A3678
Ampuwa	Fresenius Kabi	B23067A
Aquatex	Merck	1.08562.0050
Bacto-tryptone	Roth	8952.1
Beta-glycerophosphate	Calbiochem	35675
Biotinylated anti-mouse IgG (H + L)	Vector Laboratories	BA-2001
BSA	Sigma	A1933
CellTiter 96 Non-Radioactive Cell test	Promega	G5430
Cetylpyridinium chloride monohydrate	Sigma	C0732
Collagen type II antibody	DSHB	II-II6B3-s
Dako Cytomation Antibody Diluent	Dako	S0809
DetachKit-125: HepesBSS, T/E, TNS	PromoCell	C-41210
Dexamethasone	Sigma	D4902
DMEM	Gibco	41965-039
DMSO	Sigma	D2650
EC growth medium II	PromoCell	C-22011
EDTA	Sigma	E6511
ELISA kits (Quantikine):		
Human Angiopoietin-1 Immunoassay	R&D Systems	DANG10
Human Angiopoietin-2 Immunoassay	R&D Systems	DANG20
Human FGF basic Immunoassay	R&D Systems	DFB50
Human pro-MMP-1 Immunoassay	R&D Systems	DMP100
Human/Mouse/Rat MMP-2 (total)	R&D Systems	DMP200

Product	*Supplier*	*Product number*
Immunoassay		
Human MMP-3 (total) Immunoassay	R&D Systems	DMP300
Human MMP-9 (total) Immunoassay	R&D Systems	DMP900
Human pro-MMP-13 Immunoassay	R&D Systems	DM1300
Human PDGF-BB Immunoassay	R&D Systems	DBB00
Human TGF-β1 Immunoassay	R&D Systems	DB100B
Human TIMP-2 Immunoassay	R&D Systems	DTM200
Human VEGF Immunoassay	R&D Systems	DVE00
Eosin Y	Sigma	HT110232
Ethanol (DNA isolation)	Roth	5054,1
Ethanol (Histology)	Herbeta	200-578-6
Fetal calf serum	Biochrom	S0113, Charge 1038K
$Fe(NO_3)_3$	Sigma	31233
$FeCl_2 \cdot 4H_2O$	Sigma	205082500
$FeCl_3 \cdot {}_6H_2O$	Fluka	44944
Fluoromount-G	SouthernBiotech	0100-01
Full-Range Rainbow Molecular Weight Markers	GE Healthcare	RPN800E
Glycine	Merck	4169
Hematoxylin (Meyer´s Hämalaun)	Merck	1.09249
HCl	Sigma	H1758
Histopaque 1077	Sigma Aldrich	10771
HNO_3	Sigma	364576
Hoechst 33342	Molecular Probes	H3570
Human Umbilical Vein Endothelial Cells, pooled	PromoCell	C-12203
Hydrocortisone	Invitrogen	H0888
IBMX	Sigma	6634
Indomethacin	Fluka	57413
Insulin	Sigma	6634
ITS	Sigma	I1884
Kanamycin	Roth	T832.1
KCl	Roth	6781.3
L-Ascorbic acid 2-phosphate	Sigma	A8960
L-Glutamine 200mM	Gibco	250-30-024
Linolenic acid	Sigma	L1012
Matrigel	BD Bioscience	354234

Product	*Supplier*	*Product number*
MCDB-131	Gibco	10372019
$MgCl_2$	Sigma	449164
hMMP-1pro	Calbiochem	444208
rhMMP-2 active	Calbiochem	PF023
rhMMP-9 active	Calbiochem	PF140
N,N,N′,N′-Tetramethylethylenediamine	Sigma	T9281
Na_2EDTA	Sigma	E6511
NaCl	Roth	9265.1
NH3 conc. p.a. ≥65%	Sigma	7697-37-2
Normal horse serum	Vector Laboratories	S-2000
Novex® 10% Zymogram (Gelatin) Gel 1.0 mm	Invitrogen	EC61752BOX
Novex® Zymogram Developing Buffer (10X)	Invitrogen	LC2671
Novex® Zymogram Renaturing Buffer (10X)	Invitrogen	LC2670
NuPAGE® LDS 4X LDS Sample Buffer	Invitrogen	NP0008
Nuclear-fast-red Alumin. Sulpha.	Chroma	2E012
Nukleofector® (Human MSC Nucleofector® Kit)	Amaxa	VPE-1001
Oil Red O	Sigma	75087
One shot competent cells	Invitrogen	C4040-10
Paraffin	McCormick Scientific	502004
Paraformaldehyde	Merck	1.040.051.000
Phosphate buffer saline	PAA	H15-002
Polyethylenimine (25 kDa)	Sigma	408727
Penicillin/Streptomycin (10.000 U/10.000 µg/ml)	Biochrom	BCR A2213
Pepsin	Sigma	P-7012
Paraformaldehyde	Merck	1.040.051.000
pNPP Kit	Sigma	COIPD
PrimeFect Diluent Transfection Reagent	Lonza	PA-3271
Primefect II DNA Transfection Reagent	Lonza	PA-3268
Proline	Sigma	P5607
Purecol (3,0 mg/ml) (type I collagen)	Inamed Biomaterials	5409
Pyruvate	Roth	8793.1
QIAGEN® Plasmid Purification (Maxiprep)	Qiagen	12162
Rotiphorese Gel 30 (37,5:1)	Roth	3029.2
Spongious bone chips	Charité	(-)
TGFβ-1	Sigma	T1654

Product	*Supplier*	*Product number*
rhTIMP-2	Calbiochem	PF021
Tissucol-kit 2,0 Immuno	Baxter	2546654
Trasylol (Aprotinin solution, 10.000KIE/ml)	Bayer	34579.00.00
Tris	Biochrom	108.382
Triton X	Sigma	T8787
Trizma	Sigma	T1503
Trypsin (for HMEC-1 and MSC culture)	Serva	37290
Tween	Sigma	P1379
Vectastain Universal ABC-AP Kit	Vector Laboratories	AK 5200
Vector Red Alkaline Phosphatase Substrate Kit	Vector Laboratories	SK-5100
rhVEGF	Promokine	64420
VEGFR2 Kinase Inhibitor VI, Ki8751	Calbiochem	676484
Vitro-Clud	R.Langenbrinck	04-0011
Xylene	Sigma	184829
Yeast extract	Roth	2363.1

2. Methods

2.1. Haematomas

Collection of haematomas

Bone fracture haematomas were collected from patients undergoing surgery, within 72 hours after trauma (n=36, median age: 59±21y) in the Klinik für Unfall- und Wiederherstellungschirurgie der Charité (Berlin). The patient cohort characteristics are given in Table 2. Haematomas were either used for the bioreactor experiments (paracrine stimulation of ECs and MSCs by the haematoma and its mechanical stimulation), or for its angiogenic stimulation. Patients suffering from open fractures, concomitant disorders, drug addiction, as well as pregnant and breast feeding women, were excluded from the study. The ethics commission approved the study (ethics application number EA2/123/07). All patients gave informed consent. Samples were placed in sterile syringes and kept at 4°C (<12h) until experimental investigation.

Table 2 : Patients cohort characteristics

Characteristics		Number of cases
age	young (<45y)	n=10
	female	*n=3*
	male	*n=7*
	aged (>45y)	n=26
	female	*n=19*
	male	*n=7*
fracture sites	femur	n=9
	humerus	n=9
	tibia	n=5
	radius	n=4
	ankle	n=2
	patella	n=1
	(unknown)	n=6
surgery time after trauma	0-24h	n=22
	25-48h	n=8
	49-72h	n=6

Mechanical stimulation

For 3D construct preparation, 100mg of haematoma were diluted in 250µl PBS and mixed with 150µl fibrinogen solution (Tissucol-kit 2.0 Immuno) and 150µl bioreactor medium (consisting of DMEM supplemented by 10% FCS, 100U/ml penicillin, 100µg/ml streptomycin, and 2.5% Trasylol), then mixed with 50µl thrombin S (Tissucol-kit 2.0 Immuno) diluted 1:1 in bioreactor medium. The matrices were allowed to gel at 37°C for 30min and placed between two cancellous bone chips. These constructs were cyclically compressed at 1Hz to 20% for three days [90], in a previously studied bioreactor [107] containing 25ml bioreactor medium, shown in Figure 10. Haematoma-free control matrices contained phosphate buffered saline (PBS) instead of the haematoma. Unloaded controls consisted of haematoma-containing constructs cultivated in the bioreactor without mechanical stimulation. Conditioned medium (CM) was collected after experiments and frozen at -80°C for further analysis. Bioreactors were washed with tap water and rinsed with A. dest. after each assay and disinfected using 70% ethanol for 2h before each assay. The bioreactors were additionally sterilized using plasma each month.

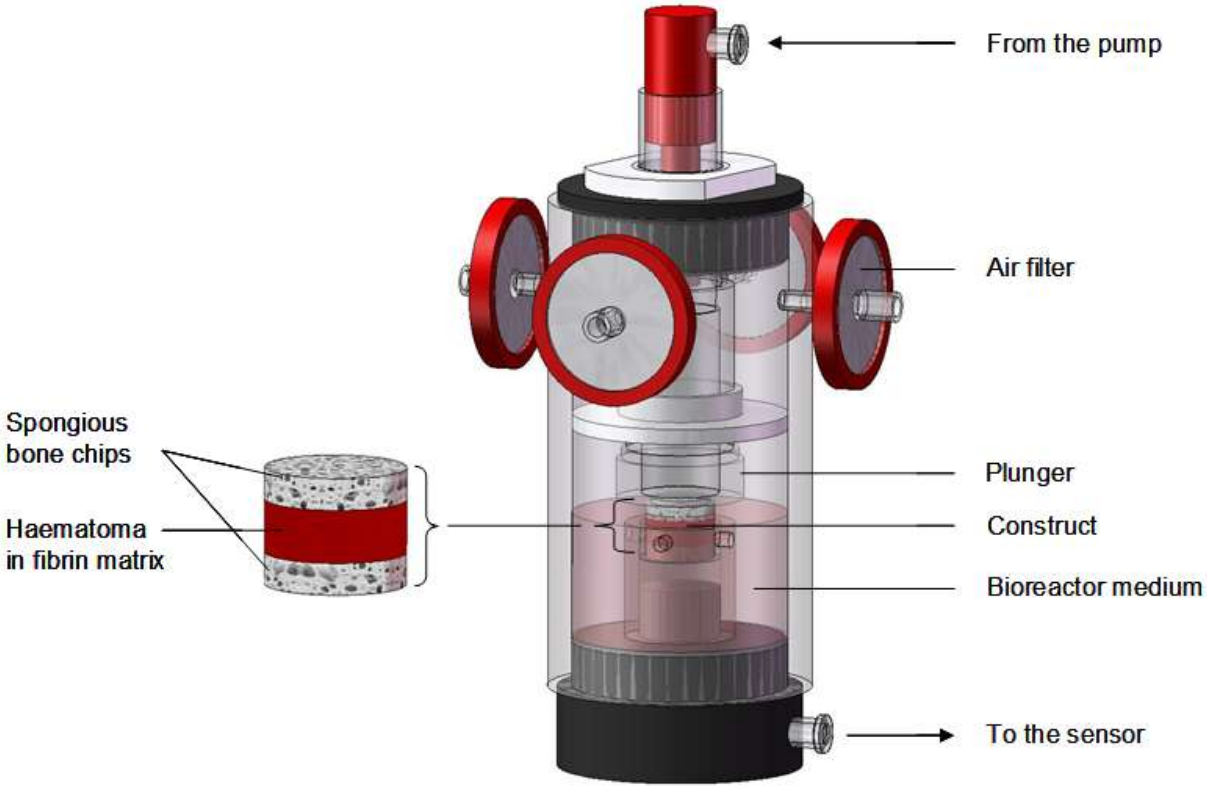

Figure 10 : Schematic representation of the bioreactor and the construct. The haematoma is embedded in a fibrin matrix and placed between cancellous bone chips in a cyclic compression bioreactor. The construct was mechanically compressed to 20% at a frequency of 1Hz for three days. 25ml medium are contained in the bioreactor. Figure was adapted from J.-E. Hofmann, JWI.

Persistence assay

In order to evaluate the duration of loading-induced effects, the constructs were transferred from the bioreactor into 24-well culture plates containing 1.3ml of fresh bioreactor medium and incubated for another 24h. The so conditioned medium was frozen at -80°C and then tested for tube formation on HMECs. At the end of the 24h-incubation, cell activity within the constructs was evaluated using the CellTiter 96 Aqueous test (MTS-test), following the manufacturer's instructions.

Histological analysis [2]

After loading experiments, the constructs were fixed in 4% Paraformaldehyde (PFA) overnight at 4°C. Then the samples were dehydrated by an ethanol series at RT, on shaker (1h 70% ethanol, 1h 80% ethanol, 2h 96% ethanol, 1h 100% ethanol), completed by 1h in xylene (room temperature, RT) and 1h in paraffin (60°C), and then embedded in paraffin. The paraffin blocks were cut by a microtome to 4µm thickness, and then placed on microscope slides.

The cell distribution was evaluated by staining of the slides using the haematoxillin-eosin staining (HE). For this, the slides were rehydrated by sequential ethanol dilutions (100%, 96%, 80%, 70%, A. dest), then soaked in haematoxylin (Harris) for 7min, washed 3x in A. dest, and shortly in HCl-Ethanol (0.25% HCl), and finally 10min in tap water. The cells were stained in eosin for 2min. Stained slides were dehydrated by soaking twice in 96% ethanol, twice in 100% ethanol, shortly in xylene, before mounting.

2.2. Cell culture

Human mesenchymal stem cells

Human MSCs were isolated from bone marrow aspirates from patients undergoing total hip prosthesis replacement. The local ethics committee accepted the study (ethics number EA2/126/07) and all patients gave informed consent.

MSCs were isolated by gradient centrifugation. Briefly, 8 ml of bone marrow (if necessary diluted with PBS) were centrifuged at 350×*g* over 5 ml Histopaque 1077 for 20min in 15 ml Falcon tubes. The fat was aspirated and discarded, before the buffy coat was transferred into a 75cm² cell culture flask containing 15 ml expansion medium (EM: DMEM, supplemented

[2] Microtomy, HE stainings and microscopy were accomplished by Morgan R. Hunter during an internship, under the supervision of Aline Groothuis.

with 10% FCS, 100U/ml penicillin and 100µg/ml streptomycin). The first medium change was 48h after isolation.

The cells were further cultivated in EM with medium change every 3-4 days. The cells were passaged when reaching 80% confluence. For this, the medium was discarded and the cells were washed twice with PBS, before trypsinization (13µl trypsin/cm², 1-2min, 37°C), blocking with serum-containing medium (80µl/cm²) and counting. 2500 cells were seeded in 200µl EM/cm².

Such isolated cells present the typical cell surface markers for MSCs (CD73+, CD90+, CD105+, CD34-, CD4- and CD45- [90, 108].) and are able to differentiate into the osteogenic, chondrogenic and adipogenic lineage [90].

HeLa cell line

The HeLa cell line was kindly provided by Dipl. Biol. W. Seifert from the Institute for Medical Genetics, Charité-Universitätsmedizin, Berlin. The cells were cultivated in EM, with medium change every 3-4 days. Cells were passaged when reaching 80% confluence, following the same protocol as for the MSCs.

HMEC-1 cell line

The human microvascular endothelial cell line, HMEC-1, was kindly provided by Prof. G. Schönfelder (Institut für Klinische Pharmakologie und Toxikologie, Charité-Universitätsmedizin Berlin, Germany) and cultivated in EC culture medium (EC-medium) - MCDB-131, supplemented with 5% FCS, 2mM L-glutamine, 100U/ml penicillin, 100µg/ml streptomycin and 1µg/ml hydrocortisone, an inhibitor of tube formation *in vitro* [77]. Cells were passaged when reaching 80% confluence, following the same protocol as for the MSCs, and seeded at 3000 cells/cm².

HUVECs

Human umbilical vein endothelial cells were supplied by Invitrogen, and cultivated following their protocols and kits. Cells were passaged using the DetachKit-125 when reaching 80% confluence, and seeded at 5000 cells/cm² in endothelial cells Growth Medium II (ECGM2).

2.3. Tube formation assays

Fresh haematomas

In vitro tube formation assays were conducted in 24-well plates with samples in duplicate. The wells were coated with 50µl Matrigel™ diluted 5:1 with EC-medium. The coated plates were allowed to gel at 37°C for 30min. The ECs were detached accounting to the relevant protocol, counted and centrifuged (350×*g*, 10min), then resuspended in EC-medium at a concentration of 5.5×10^5 HMEC-1/ml or 4×10^5 HUVEC/ml. 5.5×10^4 HMECs or 4×10^4 HUVECs were seeded per well. If necessary, the haematoma was supplemented with the relevant recombinant proteins and centrifuged (10min, 4°C, 300×*g*). The supernatant was placed on the ECs, and the cellular pellet was placed on a 1µm filter, allowing the diffusion of the factors, but not of the cells, as shown in Figure 11. Dilution of the haematoma was tested with FCS (i. e. EC-Medium) or without (i. e. MCBD131 + 1% P/S + 2mM L-Glutamine) as shown in Figure 11.

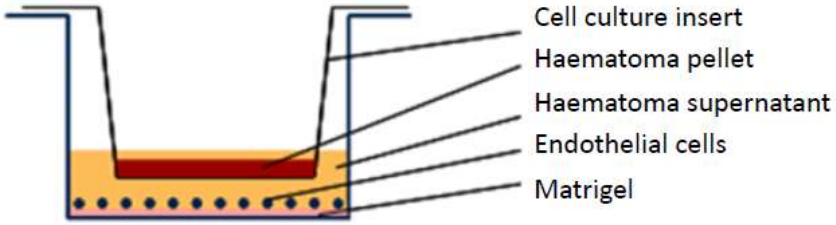

Figure 11 : Schematic representation of the tube formation assay setup for fresh haematomas. The ECs were seeded on a Matrigel-coated microtiter plate. In parallel, the fresh haematoma was diluted (or not) and centrifuged. The cellular pellet was placed on a cell culture insert (1µm pore diameter) and the supernatant of the diluted haematoma was placed on the ECs.

The plates were incubated for 17h at 37°C, 5% CO_2, before being photographed at 3x objective magnification. Evaluation was performed by measurement of the total network length in a central, representative picture using NIH ImageJ (version 1.39u, 2007, W.S. Rasband, U.S. National Institutes of Health, Bethesda, Maryland, USA, http://rsbweb.nih.gov/ij/). A section of a representative picture of tube formation assay with the reference medium is shown in Figure 12.

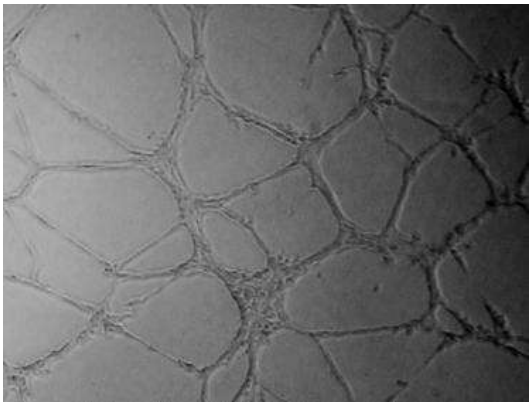

Figure 12: Representative picture of tube formation assay with the reference medium

EC numbers were measured by MTS test according to the manufacturer's instructions. All results were normalized to those obtained with the control medium (EC-medium, without hydrocortisone).

Conditioned medium

The process was the same as described earlier, but replacing the haematoma by 500µl CM, supplemented by recombinant factors or inhibitors if indicated, as shown in Figure 13.

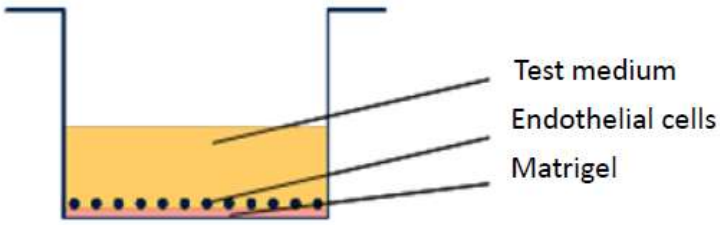

Figure 13 : Schematic representation of the tube formation assay setup for CM. The ECs were seeded on a Matrigel-coated microtiter plate, and stimulated by the conditioned medium or a reference medium.

MMP-1, 2, 9 and TIMP-2 were supplemented at 0.1, 0.5, 1, 2 and 10 times the mechanically up-regulated concentration. VEGF was applied at the up-regulated concentration and 15ng/ml as positive control and the VEGFR2 kinase inhibitor VI at 10nM [109], whereas the solvent of the inhibitor (Dimethylsulphoxide, DMSO) was diluted the same way to serve as a negative control of the inhibitor.

2.4. Proliferation of MSCs[3]

The proliferation of MSCs stimulated with CM was quantified by MTS-test in a 96-well plate format. On day one, 1.3×10³ MSCs were seeded in EM in two plates: one as reference and one for the assay. On day two, cellular activity on the reference plate was measured using MTS, while the assay plate was stimulated with 100µl CM per well, with or without addition of FCS at concentrations of 0.1, 0.5 and 1%. On day five, the cellular activity was evaluated on the test plate using MTS. The MSC's EM was used as positive control, and cell-free wells were used as blanks. The test was run in triplicate and the median of the optical densities at 490nm (OD) of the replicates was used in the evaluation.

The proliferation index was calculated as follows:

$$index = \frac{(OD_{CM}^{day\,5} - OD_{blank}^{day\,5}) - (OD_{CM}^{day\,2} - OD_{blank}^{day\,2})}{(OD_{EM}^{day\,5} - OD_{blank}^{day\,5}) - (OD_{EM}^{day\,2} - OD_{blank}^{day\,2})}$$

2.5. Migration assays[4]

Both MSC and HMEC -1 were tested for migration in a modified Boyden chamber. The filters were either coated with Matrigel (migration of HMEC-1), Collagen type I (MSCs) or left uncoated (MSCs). Assays were run in duplicates.

Matrigel was diluted in EC Medium on ice at a concentration of 1mg proteins/ml. 50µl Matrigel were spread on top of the filters, and incubated for 1h at 37°C before being washed twice with PBS. HMECs were detached, counted, centrifuged (350×*g*, 10min) and resuspended in EC medium at a concentration of 2×10^5cells/ml. 2×10^4cells were given on top of the filter. 500µl CM were given on and under the filter membrane. Filters were incubated for 1h at 37°C before overnight fixation of the cells with 500µl 4% PFA on and under the filter at 4°C.

Collagen type I was diluted using EM to a concentration of 0.01mg/ml, and 50µl were spread on top of the filters. The coated filters were incubated at 37°C for 1h and washed twice with PBS. MSCs were detached, counted and centrifuged (350×*g*, 10min). MSCs were resuspended in EM at 3×10^5 cells/ml and 3×10^4 cells per filter were seeded. 500µl CM was

[3] Work accomplished by Beatrice Woltmann during her Master thesis, under the supervision of Aline Groothuis.

[4] Work accomplished by Beatrice Woltmann during her Master thesis, under the supervision of Aline Groothuis.

given on and under the filter membrane. Filters were incubated for 5h at 37°C before overnight fixation with 500µl 4% PFA on and under the filter at 4°C.

The fixed cells (HMECs and MSCs) were washed with PBS twice and the non-migrated cells were scrapped from the top of the filters. The membranes were washed with PBS and the migrated cells, on the bottom part of the filter were stained with 500µl Hoechst (diluted 1:1000 in A. dest.) for 20min in darkness. The filters were washed with PBS twice. The membranes were cut out of the inserts, dipped in distilled water, and finally mounted on an object holder on Fluoromount.

The membranes were observed with fluorescence microscopy and five microscopic fields per filter were photographed. Positively stained cells were counted using NIH ImageJ.

2.6. Differentiation assays of MSCs[5]

Osteogenic differentiation

For osteogenic differentiation, $1x10^4$ MSCs/cm^2 were seeded in a 48-well plate format and grown until reaching confluence before stimulation with CMs and control media (day 0).

Both CMs and EM were tested with and without osteogenic additives (0.01mM dexamethasone, 7mM β-glycerophosphate, and 0.2mM L-ascorbic acid 2-phosphate.) The cells were cultivated until day 7 for the measurement of the alkaline phosphatase (AP) activity, and until day 14 for the matrix mineralization with Alizarin red (AR). The media were changed twice a week.

Alkaline Phosphatase activity

The AP activity was measured following the manufacturer's instructions at day 7. Briefly, the wells were washed once with 500µl PBS and once with 500µl AP-buffer (50mM glycine, 0.1M Tris, 1mM $MgCl_2$, pH 10.5). 1.3mg of p-nitrophenyl phosphate was added to 1ml of AP substrate. Cells were stained with 271.5µl/well of AP substrate and incubated for 30min under humidified conditions. The optical density (OD) was measured at 405nm. Cell-free wells were treated as well and used as blanks. A second plate was used for the evaluation of the cellular activity with MTS according to the manufacturer's instructions. The AP index was calculated as follows:

[5] Work accomplished by Beatrice Woltmann during her Master thesis, under the supervision of Aline Groothuis.

$$index = \frac{\frac{OD_{well}^{AP} - OD_{blank}^{AP}}{OD_{well}^{MTS} - OD_{blank}^{MTS}} - \frac{OD_{EM}^{AP} - OD_{blank}^{AP}}{OD_{EM}^{MTS} - OD_{blank}^{MTS}}}{\frac{OD_{OM}^{AP} - OD_{blank}^{AP}}{OD_{OM}^{MTS} - OD_{blank}^{MTS}} - \frac{OD_{EM}^{AP} - OD_{blank}^{AP}}{OD_{OM}^{MTS} - OD_{blank}^{MTS}}}$$

Alizarin red staining

The AR staining was performed to visualize the mineralization of the matrix at day 14. Briefly, the wells were washed with PBS, fixed with 4% PFA (10min). The wells were washed once with PBS and twice with A. dest., before staining with a 0.5% AR solution. The dye was dissolved using 120µl cetylpyridinium chloride for 20min. The OD of 100µl of the resultant solution was measured at 570nm. Cell-free wells were also stained and measured as blanks. A second plate was used for the evaluation of the cellular activity with MTS according to the manufacturer's instructions. The median of the ODs for a condition were used for the evaluation, as follows:

$$index = \frac{\frac{OD_{well}^{AR} - OD_{blank}^{AR}}{OD_{well}^{MTS} - OD_{blank}^{MTS}} - \frac{OD_{EM}^{AR} - OD_{blank}^{AR}}{OD_{EM}^{MTS} - OD_{blank}^{MTS}}}{\frac{OD_{OM}^{AR} - OD_{blank}^{AR}}{OD_{OM}^{MTS} - OD_{blank}^{MTS}} - \frac{OD_{EM}^{AR} - OD_{blank}^{AR}}{OD_{OM}^{MTS} - OD_{blank}^{MTS}}}$$

Chondrogenic differentiation

Chondrogenic differentiation was performed according to Liu et al [110]. For this, 3×10^5 MSCs were centrifuged (300x*g*, 5min) in a 15-ml conical tube. The cell pellet was washed once with PBS and centrifuged (300x*g*, 3min), before the test media were added (CM and EM, with and without additives: 0.0001mM dexamethasone, 0.2mM L-ascorbic acid 2-phosphate, 40µg/ml proline, 100µg/ml pyruvate, 6.25µg/ml ITS, 1.25mg/ml BSA, 5.35mg/ml linolenic acid, and 10ng/ml TGFβ-1). Medium was changed twice a week. The cell pellets were cultivated for 21 days. A negative control was produced with EM and cultivated for one day only.

The pellets were then fixed with 500µl 4% PFA (2h) and washed twice with PBS (5min). The pellets were dehydrated in 70% ethanol (30s), and 80% ethanol (30min), before the cells were stained with Eosin (30s). Pellets were further dehydrated in 96% ethanol (30min, twice), 100% ethanol (30min, twice) and xylene (15min). The pellets were embedded in paraffin and cut in 4µm sections.

Alcian blue staining

For alcian blue staining, the sections were deparafinized in xylene (10min, twice), 100% ethanol (2 min, twice), 96% ethanol (2min), 80% ethanol (2min), 70% ethanol (2min). The slides were pretreated in 1% acetic acid (3min) before staining with 1% alcian blue in acetic acid (pH 2.5, 30min). The slides were then washed in 3% acetic acid and in A. dest., before the nuclei were counterstained with Nuclear-fast-Red Aluminium sulphate (5min). The slides were rinsed in A. dest. and dehydrated again in 70% ethanol (2min), 80% ethanol (2min), 96% ethanol (2min), 100% ethanol (2min) and in xylene (10min, twice) before covering in Vitro-clud. The slides were qualitatively observed under the microscope.

Collagen II staining

The pellets were stained with a Collagen type II antibody (II6B3-s). For this, the slides were dewaxed in xylene (10min, twice), 100% ethanol (2 min, twice), 96% ethanol (2min), 80% ethanol (2min), 70% ethanol (2min) and 2 min in A. dest. The slides were then washed twice in PBS (5min) and pretreated in 0.1% pepsin (300 mg freshly thawed pepsin in 3ml 1N HCl and 297ml A.dest.) in a moisture chamber at 37°C (30min). The slides were washed in PBS (5min, twice) followed by blocking with normal horse serum (1% in PBS) for 20min in moisture chamber. The primary antibody (diluted 1:5 in Dako Cytomation Antibody Diluent) was applied (1h) in the moisture chamber, before being washed with PBS (5min, twice). The secondary antibody (biotinylated anti-mouse IgG [H+L], 1:2 diluted in 1:50 normal horse serum in PBS) was given in the moisture chamber (30min), before being washed with PBS (5min, twice). The AB-complex (1% reagent A and 1% reagent B [Alkaline Phosphatase Kit, AK 5200] in PBS) was given on the slides (50min) in the moisture chamber, before being washed with PBS (5min, twice). The chromogen buffer (3.96g HCl, 0.54 g Trizma® Base, 2.63g NaCl in 300ml A.dest, pH8.2) was added to the cells (5min, twice) in the moisture chamber, before the alkaline phosphatase substrate (50µl reagent 1-3 [Vector Red Alkaline Phosphatase Substrate Kit] in 2.5ml of chromogen buffer) was added (7min). The slides were washed with PBS (5min, twice) and shortly in A. dest. Nuclei were counterstained in Nuclear-fast-red (1min) and washed in A. dest., and covered using Aquatex. The slides were qualitatively observed under the microscope.
Slides of a rat femur were used as a positive control. The primary antibody was omitted for a negative control of the staining.

Adipogenic differentiation

For the adipogenic differentiation, $1x10^4$ MSCs/cm^2 were seeded in a 48-well plate format and grown until reaching confluence before stimulation with CMs and control media (day one). CMs and EM were tested with and without adipogenic additives (0.001mM dexamethasone, 0.002mM insulin, 500mM IBMX, 0.2mM indomethacin). The cells were cultivated until day twelve for the evaluation of the lipid droplets. The media were changed twice a week.

The cell viability was first measured using the Alamar Blue test (10% Alamar Blue in EM) for 3h in the incubator. The cells were then washed with PBS and fixed with 200µl 4% PFA per well (5min). The cells were then dried by washing with 200µl of 60% 2-propanol, and exposed to air. The wells were stained with 200µl Red Oil Red (3.5mg/ml in 99% 2-propanol, stand for 48h, then mixed 60% - 40% with A. dest., stand for 20min) (10min) and washed with A. dest (4 times). The dye was dissolved in 200µl 2-propanol (10min). The OD of 100µl of the obtained solution was measured at 540nm in 96-well plate. Cell-free wells were stained as blanks. The median of the obtained optical densities were used for the evaluation as follows:

$$index = \frac{\frac{OD^{ROR}_{well} - OD^{ROR}_{blank}}{OD^{AB}_{well} - OD^{AB}_{blank}} - \frac{OD^{ROR}_{EM} - OD^{ROR}_{blank}}{OD^{AB}_{EM} - OD^{AB}_{blank}}}{\frac{OD^{ROR}_{OM} - OD^{ROR}_{blank}}{OD^{AB}_{OM} - OD^{AB}_{blank}} - \frac{OD^{ROR}_{EM} - OD^{ROR}_{blank}}{OD^{AB}_{OM} - OD^{AB}_{blank}}}$$

2.7. Quantification of proteins

GFs, MMPs and TIMP-2 (detected in the CM by gelatine zymography (Novex system, data not shown) were quantified in the CM using ELISA kits according to the producer's instructions. CM was used undiluted in the cases of TGF-β1 and angiopoietins (Ang-1 and Ang-2), or concentrated by centrifugation through filters (5kDa), 10x in the case of VEGF, and 30x for bFGF and PDGF-bb. The CM was applied undiluted in the case of MMP-3, 10x concentrated for TIMP-2 quantification, and 30x concentrated by centrifugation over filters in the case of pro-MMP-1, MMP-2 and MMP-13. MMP-9 was measured with a dilution of 1:20.

2.8. Transfections

Plasmid propagation and isolation

The plasmid pEGFP-C2 was kindly provided by the laboratory of powder technology, EPFL, Lausanne (Dr. B. Steitz) and propagated following the manufacturer's protocol (Invitrogen). Briefly, 2µg plasmid was added to 50µl One shot competent cells and incubated 30min on ice, then 30sec at 42°C. 250µl of SOC medium (2g Bacto-tryptone, 0.5g Bacto-yeast extract, 0.05g NaCl, 250µl 1M KCl dissolved into 100ml A. dest, and autoclaved) was added to the cells on ice, then incubated for 1h at 37°C under shaking. The cells were then plated on kanamycin sulphate (Kan) -containing petri dishes overnight (2% Agar-Agar dissolved in hot LB Medium [5g Bacto-tryptone, 2.5g yeast extract, 5g NaCl in 500ml A. dest, autoclaved] cooled down to ~37°, then 100µg/ml Kan mixed, plated and cooled down to RT). Per tube, 5ml LB medium, 30µg/ml Kan and one bacteria colony were placed in 37°C under shaking for 24h. 1ml of bacteria culture was added to 500ml LB medium containing 30µg/ml Kan and cultivated for 24h under shaking at 37°C.

The plasmid was isolated using the Maxiprep kit following the manufacturer's instructions. The concentration was measured using a biophotometer.

The PEI-SPIONs system

PEI-SPIONs were prepared in two steps: first the production of a SPION batch, and then the coating of the SPIONs by PEI.

SPIONs preparation

The SPIONs preparation was performed as previously described [111]. Briefly, $FeCl_3 \cdot 6H_2O$ and $FeCl_2 \cdot 4H_2O$ were dissolved in ultrapure water, then 30ml concentrated NH_3 were added to the iron solution under vigorous mixing. The particles were then separated magnetically from the supernatant and rinsed twice with A dest. Then the suspension was centrifuged for 5 min at 5000×*g*, and the pellet was transferred into a two-neck round bottom flask with 60ml 0.27M $Fe(NO_3)_3$ and 40ml 2M HNO_3 to the mixture and heated by reflux in an 110°C oil bath (1h), before cooling down to RT. The suspension was magnetically separated and resuspended in 100ml ultrapure water. The suspension was dialyzed two days in 10^{-2}M HNO_3, exchanging the liquid twice a day. The suspension was finally centrifuged 15min at 30'000×*g*, then transferred into plastic containers and kept at 4°C. These particles, now designated SPIONs, were described elsewhere in terms of size distribution, iron content and colloidal stability [106].

PEI coating of the SPIONs

The SPIONs were coated using PEI as described elsewhere [106]. Briefly, PEI was diluted to 20% (w/w) using ultrapure water. 100µl of 20% PEI were mixed with the corresponding volume of SPION containing 8.5 mg iron under vigorous stirring. The PEI coated SPIONS (PEI-SPIONs) were incubated overnight at RT before experiments.

Transfection

The transfection procedure was followed according to a master thesis about transfection of human cells with SPIONs. The transfection procedure took three days. On day one, the cells were passaged, counted and seeded in 6-wells plates in 2ml EM to reach 80-90% confluence on the next day (MSCs: 1×10^5cells/well, HeLa: 3×10^5 cells/well).

On day two, the plasmids were diluted in TE buffer (10 mM Tris-HCl, 1mM Na_2EDTA, pH 8,0) to 50µg/ml and the PEI-SPIONs 1:800 in PBS. The DNA : PEI-SPIONs complexes were prepared at a 0.8 : 1 volume ratio and mixed by pipetting, followed by a 30min incubation at RT. This volume ratio is consistent with a N/P ratio of 7.6, supposed to be the most efficient for the transfection of human primary cells [96]. The appropriate volume of DNA-PEI-SPIONs (MSCs: 76µl, HeLa: 228µl) was added to 1ml of DMEM and mixed by pipetting up and down. The cells were washed once with PBS and the nanoparticles-containing medium was added to the cells under continuous shaking of the plate. The wells were incubated on magnets (static or sinusoidal magnetic field) in the incubator for 20 min, and then the plate was carefully removed from the magnets before further 4h incubation (37°C). The medium was then carefully changed into EM (2ml/well) and the cells were further incubated overnight.

On day three, the cells were observed for fluorescence and random pictures were taken at 3x objective magnification under transmitted and fluorescent light. The transfection rate was estimated by the number of transfected (fluorescing) cells, normalized to the total number of cells, in three microscopic fields.

Lipofection

The Primefect II transfection was conducted following the manufacturer's instructions in the 6-well plate format. On day one, MSCs were passaged, counted, and 1×10^5 cells were seeded per well in DMEM + 10% FCS + 1% P/S to reach approximately 80% confluence on day two. On day two, the medium was changed for fresh EM. 250µl transfection diluent and 3, 4, 6 or 12µl Primefect transfection reagent were mixed in centrifugation tubes, mixed by flicking and incubated for 10min at RT. 2µg plasmid DNA were added, then mixed by flicking

and incubated for 20min at RT. Cells were incubated overnight and analysed under fluorescent light on day three for positive transfected cells in three microscopic fields.

Nucleofection

The nucleofection was conducted according to the manufacturer's instructions. On day one, MSCs were passaged and counted. 5×10^5 cells were washed twice with PBS, then resuspended in 100µl Nukleofector solution und 4µg plasmid. The cell suspension was electroporated in the Amaxa device and immediately transferred into pre-warmed cell culture medium and placed in an incubator.

On day two, the cells were photographed under the microscope and fluorescence light. Transfected cells were counted in three microscopic fields.

2.9. Statistics

Statistical analysis was performed employing the SPSS software package (version 14.0, SPSS Inc., USA). The non-parametric Wilcoxon test was used for all comparisons except for the maturation of the haematoma which was analysed by the Kruskal-Wallis test, and the migration and differentiations which were analysed using the T-test because of the low sampling number. Correlation analysis was done using the Pearson's test. All tests were two-sided, with a level of significance set at $p\leq0.050$. Data are displayed in box plots presenting the median as a bar, the quartiles 0.25 and 0.75 as a box. * indicates statistical significance.

Chapter 3: Results

This section will present the results of this study. First, the cultivation of the haematoma in the bioreactor is described. Then the paracrine effects of the haematoma by the CM on different cell types are described: an endothelial cell line and primary endothelial cells are used to evaluate the pro-angiogenic potential of conditioned media. The functional behaviour of mesenchymal stem cells was investigated by evaluating their proliferation, migration and differentiation capacities in contact with conditioned medium. The regulation of factors in the CM is presented, followed by the application of these regulated factors into the haematoma to stimulate angiogenesis. Finally, the results of the transfection of MSCs are presented.

1. Cultivation of haematomas in a compression bioreactor

Haematomas were cultivated in bioreactors in fibrin matrix, with and without application of cyclic mechanical loading. The haematoma matrices were first investigated for the cell distribution and viability after bioreactor stimulation and 24h incubation in medium. After three days in the bioreactor, the cells were evenly distributed in the fibrin and no relevant formation of clumps was observed (see Figure 14).

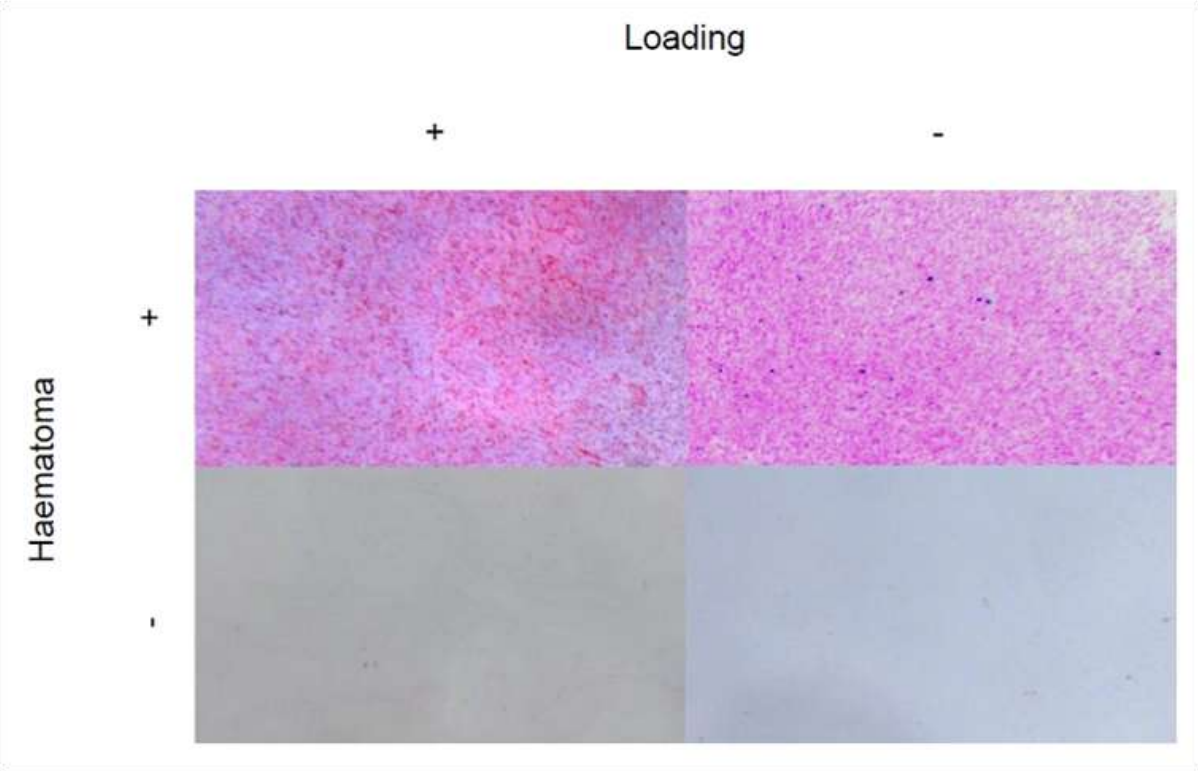

Figure 14 : **Cell distribution within the matrices.** The sections of the dehydrated constructs were stained with Haematoxyllin-Eosin to visualize the cell distribution. Cells were homogeneously distributed through the loaded and unloaded haematoma constructs.

The cellular activity, as measured by MTS assay within the haematoma constructs, were not affected by mechanical stimulation ($ratio_{loaded\ /\ unloaded\ haematoma}$=0.98, n=6, p=0.753, Figure 15), excluding important cell damage eventually induced by mechanical loading, or mechanically driven cell proliferation in the bioreactor. Therefore, potentially different paracrine effects cannot be due to different cell activity in the loaded versus unloaded constructs. However, an eventual counterbalancing effect of proliferation and apoptosis may not be detected.

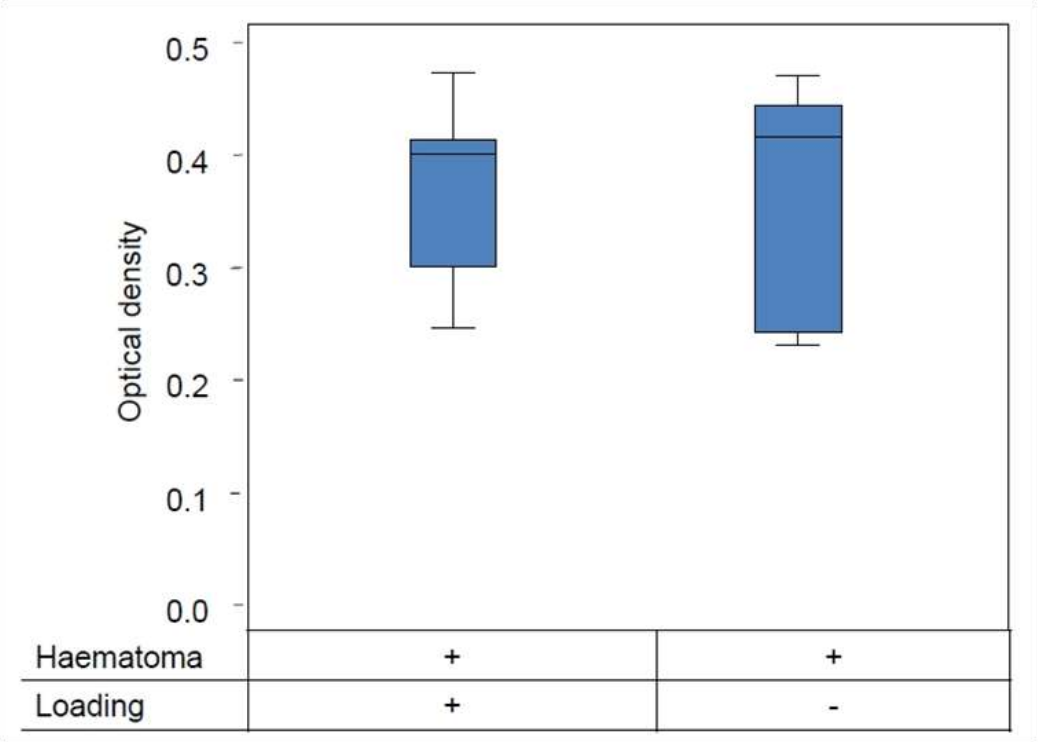

Figure 15: Cellular activity within the haematoma matrices is not affected by mechanical stimulation. The matrices were incubated in medium after the bioreactor stimulation to measure the cell viability. The OD of loaded matrices was not significantly different than unloaded.

2. The paracrine stimulation of ECs by conditioned media

The influence of the haematoma and its mechanical stimulation on angiogenesis was investigated. Thus, the haematomas were cultivated in bioreactors with or without mechanical stimulation for three days before the CM was tested on ECs for tube formation and cellular activity. The paracrine angiogenic effect of CM from haematomas and their mechanical loading was first investigated on a cell line (HMEC-1), and then some of the main results were tested for confirmation on primary cells (HUVECs). The potential correlation of the angiogenic potential to the age and gender of the patients was tested, as well as the maturation of the haematoma.

2.1. Endothelial cell line: HMEC-1

Paracrine stimulation by the haematoma

CM from haematomas and negative controls were applied to HMEC-1 to quantify the paracrine stimulation of tube formation. EC numbers were evaluated after the assay.

CM from haematomas stimulate tube formation

Application of the CM from the haematoma significantly increased the tube formation, compared to the unloaded, negative control (fibrin only) ($ratio_{haematoma/without\ haematoma}$=2.13, n=20, p<0.001, Figure 16). The presented data are normalized to that obtained from the reference medium.

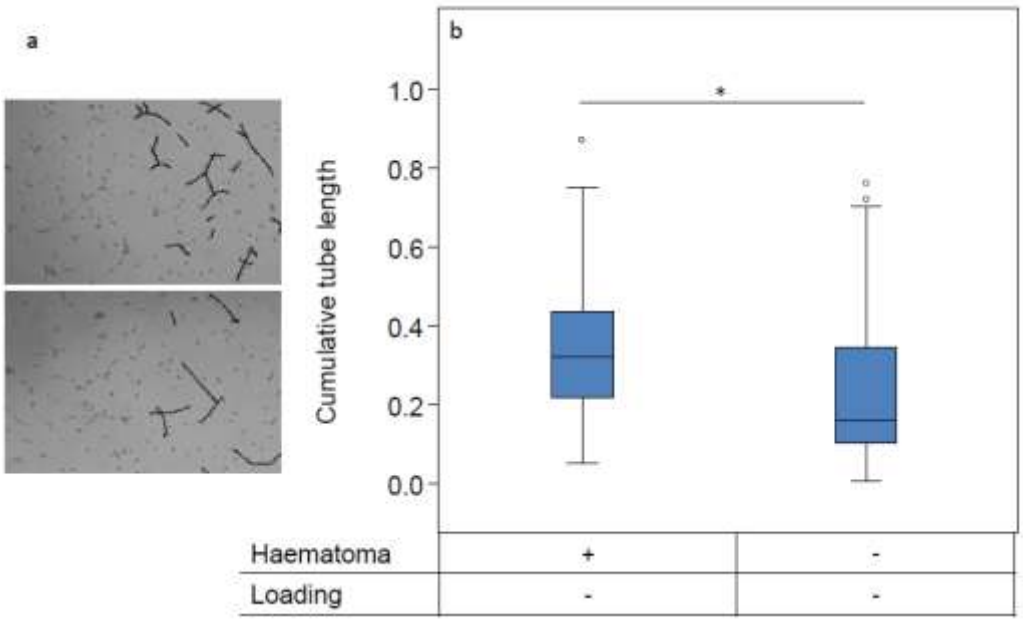

Figure 16: Tube formation by HMECs. Representative pictures of tube formation assay of CM from the haematoma (section a, top) and CM from the negative control (section a, bottom picture) after 17j incubation. The tubes are highlighted as evaluated. Quantification of the tube length showed a significant increase in tube formation by HMECs in CM from haematomas compared to controls (section b). Data are displayed normalized to the reference medium. * indicate statistical significance

This broad distribution of the cumulative tube length was then separated between the characteristics of the patients and the time of surgery. Neither the age nor the gender of the patients influenced the tube formation from the CM of haematomas (young patients: ratio=1.82, n=7, aged patients: ratio=2.13, n=13, p=0.968; male patients: ratio=2.13, n=11, female patients: ratio=2.20, n=9, p=0.790), Figure **17**).

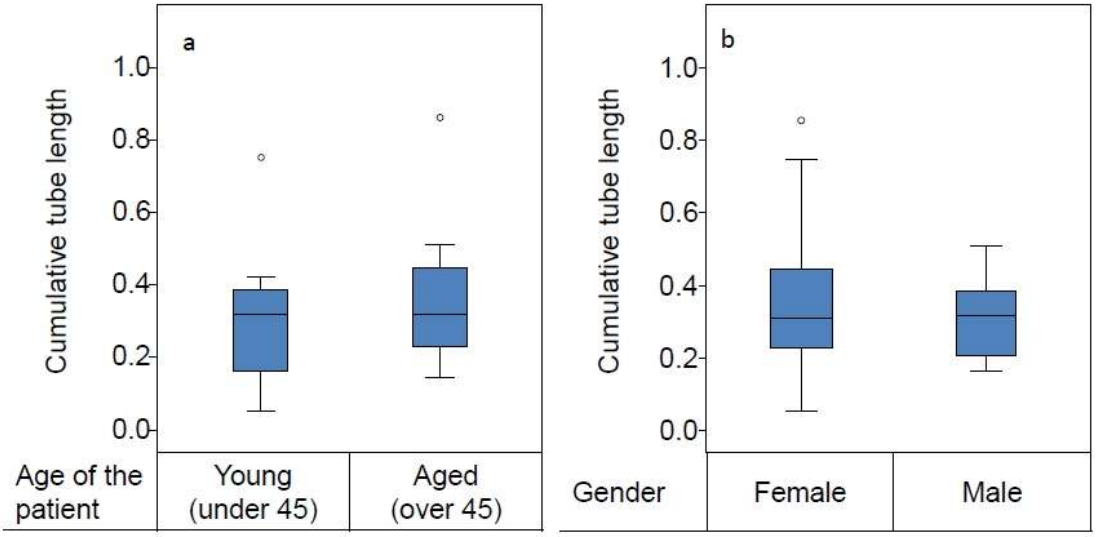

Figure 17**: Tube formation by HMECs with CM from haematomas is neither influenced by the age nor the gender of the patients.** The haematomas were separated according to the age category and gender of the patient. Neither the age nor the gender of the patient influenced the angiogenic potential of the early haematoma. Data are displayed normalized to the reference medium.

The separation of the haematomas according to the time between trauma and surgery tended towards an increased angiogenic potential of the haematoma with longer maturation of the haematoma (see Figure 18); however, this result was not significant probably because of the low number of haematomas that could be collected in the time span of 25 to 72h after trauma ($ratio_{haematoma/no\ haematoma}$ up to 24h: 1.95; 25-48h: 2.23; 49-72h: 2.08; p=0.238, Figure 18).

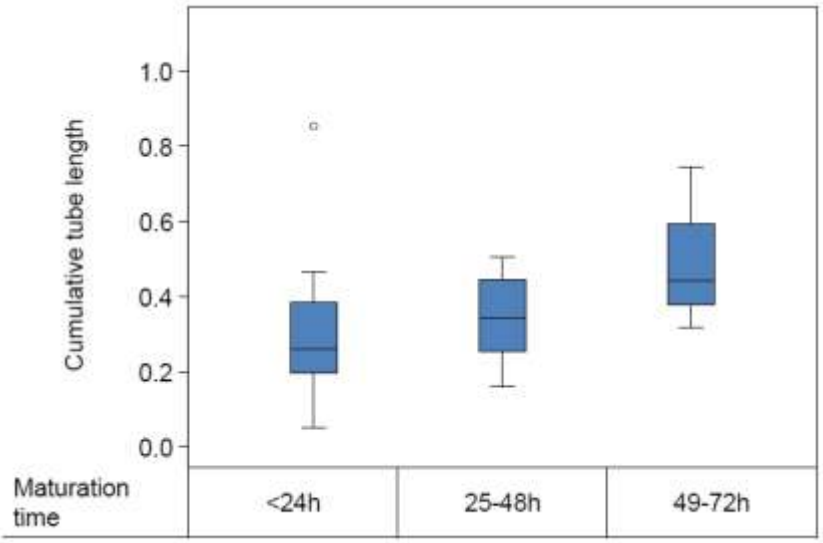

Figure 18: Tube formation tended to increase with longer maturation of the haematomas. The haematomas were categorized according to the maturation time in the patient. A longer maturation tends to increase the angiogenic potential of the haematoma. Data are displayed normalized to the reference medium.

EC numbers are unaffected by the CM from haematomas

HMEC numbers were estimated using the MTS assay after the tube formation assay. HMEC numbers were not affected by CM from haematomas compared to the controls ($ratio_{haematoma/no\ haematoma}$=0.97, n=27, p=0.370, Figure 19), and were not affected by the age and gender of the patients, or the haematoma maturation time (data not shown).

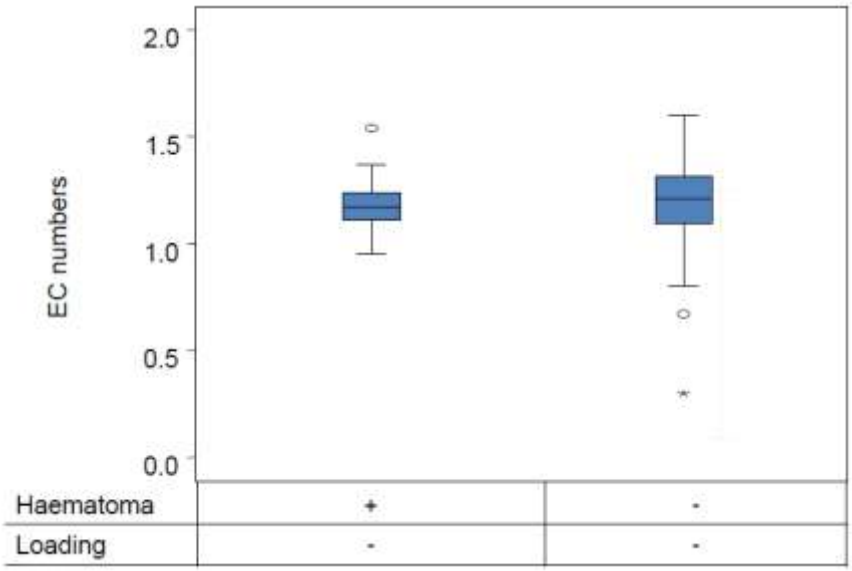

Figure 19: EC numbers are not affected by the CM from the haematoma or the negative control. The EC numbers were evaluated after the stimulation with the CM of haematomas and controls. The cell numbers were not significantly changed by the CM from haematomas. Data are normalized to the reference medium.

EC migration is unaffected by the CM from haematomas

The migration potential of the cells, as measured after migration through a filter in the CMs, was not affected by the haematoma CM compared to the control CM (median $ratio_{haematoma/no\ haematoma}$=1.21, n=3, p=0.289, Figure 20).

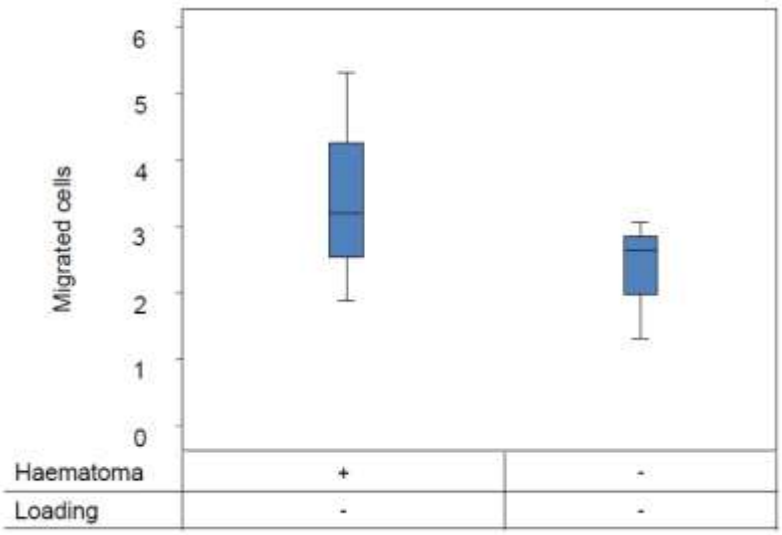

Figure 20: HMEC migration is not significantly increased by the haematoma CM, compared to the negative control. The cells were allowed to migrate through membranes under stimulation of CM. The migrated cells were not statistically different in CM from haematomas and controls. Data are normalized to the reference medium.

Modulation of the paracrine stimulation by mechanical loading of the haematoma

CM from mechanically loaded haematomas stimulate tube formation more than unloaded

Since mechanical loading stimulates angiogenesis *in vivo* [8], the dependency of the paracrine stimulation of the haematoma with a physiologically relevant *in vitro* mechanical loading was investigated.

Loaded haematomas stimulated tube formation *in vitro* significantly more than unloaded ones ($ratio_{loaded/unloaded}$=1.49, n=27, p<0.001, Figure 21). No increased stimulation of tube formation by mechanical stimulation of the negative control matrixes was observed ($ratio_{loaded/unloaded}$=1.13, n=27, p=0.151, Figure 22).

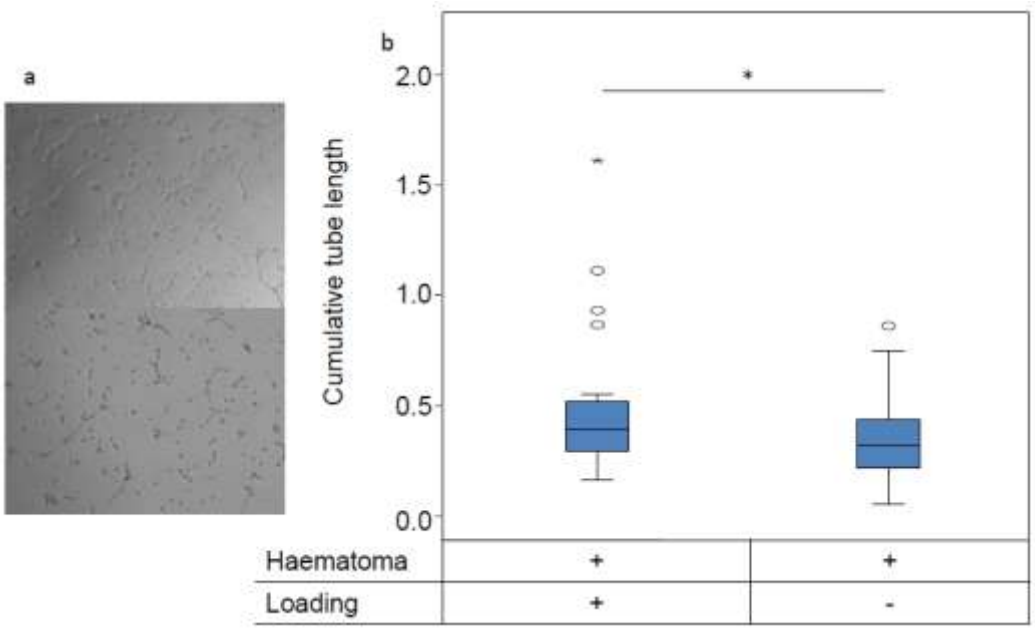

Figure 21: Increased tube formation stimulation by HMEC-1 in CM from mechanically stimulated haematomas. Representative pictures of tube formation assay of CM from the loaded (section a, top) and unloaded haematoma (section a, bottom picture). The tubes are highlighted as evaluated. Quantification of the tube length showed a significant increase in tube formation by HMECs in CM from loaded haematomas versus unloaded ones (section b). Data are displayed normalized to the reference medium. * indicates statistical significance

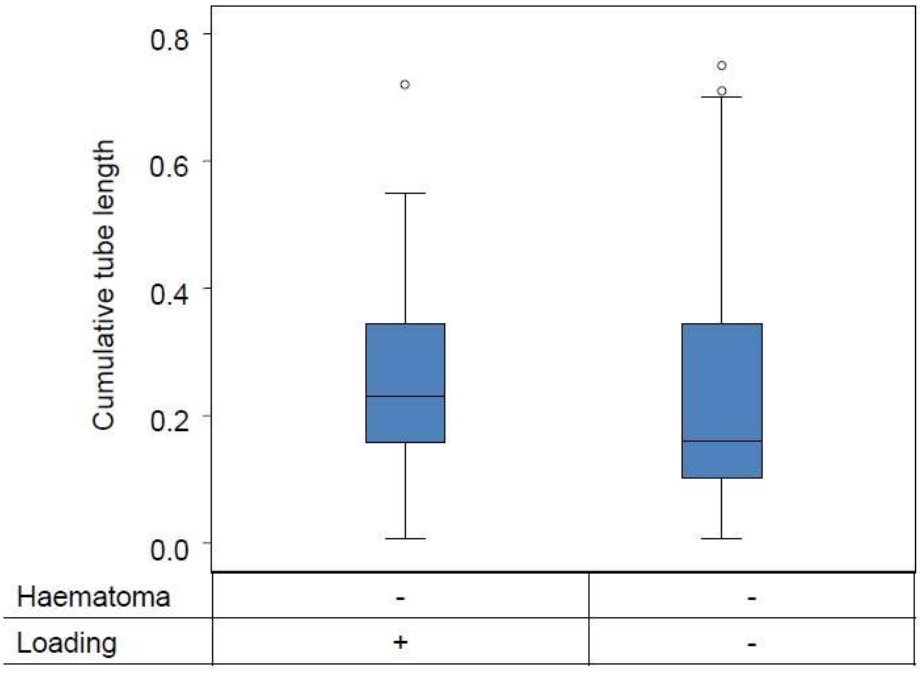

Figure 22: Tube formation of HMEC-1 in CM of haematoma-free matrices is independent on their mechanical stimulation. The mechanical stimulation of the control matrices did not significantly affect the paracrine stimulation of tube formation. Data were normalized to those obtained with the reference medium.

The increased angiogenic potential of mechanically loaded haematomas was independent on the gender (female patients: median $ratio_{loaded/unloaded}$=1.43, n=14, male patients=1.53, n=13, p=0.755, Figure 23a), or the age of the studied patients (young patients: ratio=1.73, n=10, aged patients: ratio=1.43, n=17, p=0.152, Figure 23b).

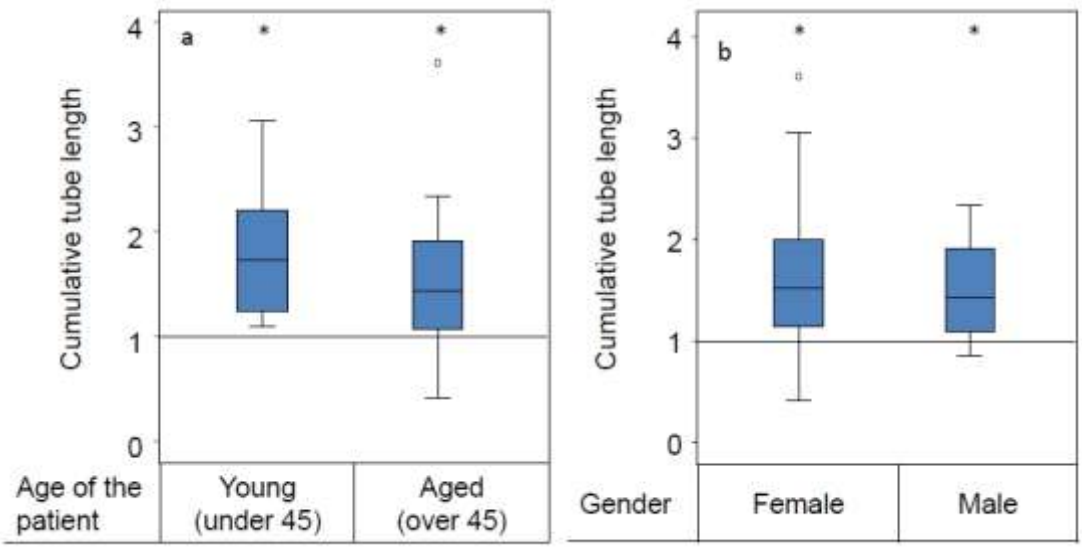

Figure 23: Tube formation by HMECs with CM from loaded over unloaded haematomas is neither influenced by the age nor the gender of the patients. The haematomas were separated on the basis of the age category (section a) and the gender (section b) of the patient. Neither the age nor the gender of the patient influenced the angiogenic potential of the haematoma. Data are displayed normalized to the reference medium. * indicates statistical significance

The maturation of the haematoma did not affect its pro-angiogenic reactivity to mechanical stimulation. (<24h: median $ratio_{loaded/unloaded}$=1.66, 25-48h: ratio=1.13, 49-72h: ratio=1.43, p=0.653, Figure 24).

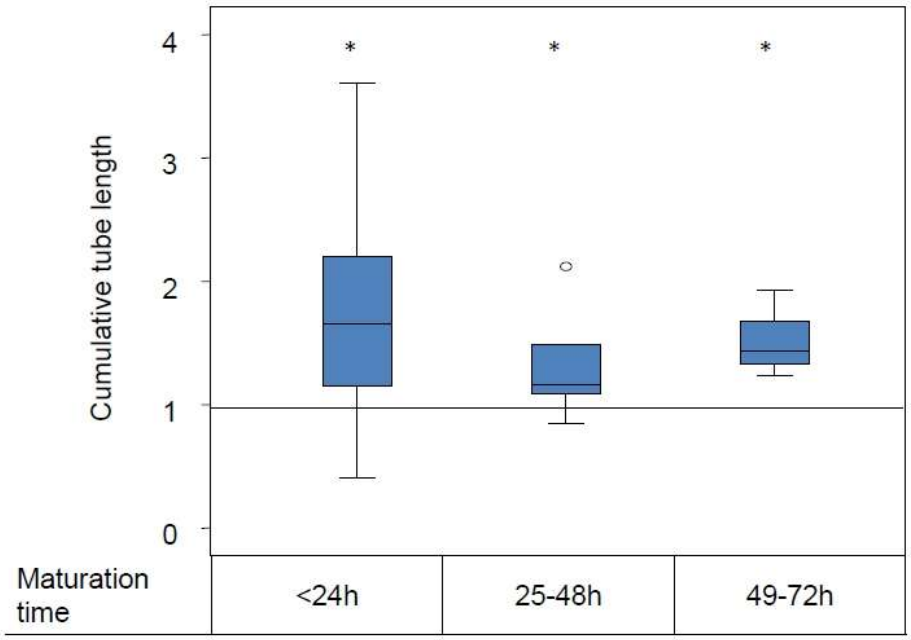

Figure 24: The reactivity of tube formation to mechanical stimulation was independent on the maturation of the haematomas in the patient. The haematomas were categorized according to the maturation time in the patient. A longer maturation did not influence the pro-angiogenic reaction of the haematoma under mechanical stimulation. Data are displayed normalized to the reference medium. * indicates statistical significance

Cell number is unchanged by the mechanical stimulation

Since the increased paracrine stimulation of tube formation by mechanical loading of the haematoma could be due to the stimulation of the EC numbers, the cellular activity was evaluated directly after the tube formation assays using the MTS assay.

The increased tube formation in CM from loaded haematomas was not followed by an increased cell number ($ratio_{loaded/unloaded}$=1.02, n=23, p=0.688, Figure 25) and not affected by the patients' age, gender or the maturation of the haematoma (data not shown).

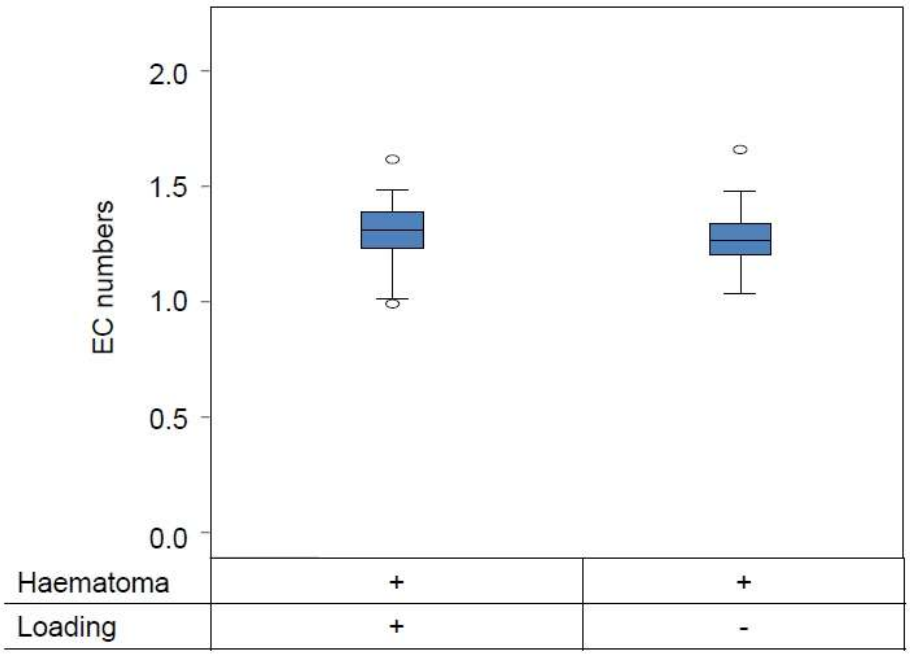

Figure 25: Unchanged HMEC-1 numbers in CM from mechanically stimulated vs. unstimulated haematomas. The EC numbers were evaluated after the stimulation with the CM of loaded and unloaded haematomas. The cell numbers were not significantly changed by the mechanical stimulation of the haematomas. Data are normalized to the reference medium.

So, the mechanically loaded haematoma stimulated the tube formation on the HMEC-1 cell line. The bright distribution of the angiogenic potential between patients did not depend on the haematomas' characteristics (age and gender of the patient, maturation of the haematoma).

Persistence of the pro-angiogenic mechanical stimulation over 24h

To test whether the bioreactor matrices retained their increased pro-angiogenic potential after the mechanical stimulation, the matrices were conditioned for 24h in fresh medium after the 72h cultivation in bioreactors. The pro-angiogenic effects of the mechanical stimulation could be retained in the matrices for this time, as shown on HMEC-1 ($ratio_{loaded/unloaded}$=1.54, n=6, p=0.043, Figure 26). The HMEC cell numbers were similar under both conditions ($ratio_{loaded/unloaded}$=1.04, n=6, p=0.753).

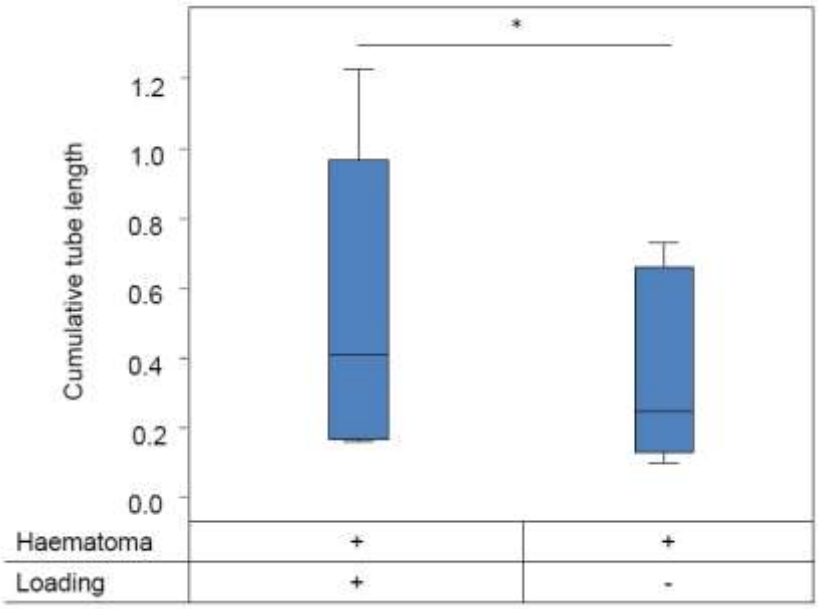

Figure 26: Pro-angiogenic response to mechanical stimulation is retained for at least 24h. Quantification of tube formation assays of 24h-conditioned medium of loaded and unloaded haematoma matrices showed the persistence of the increased pro-angiogenic potential of the haematoma by mechanical stimulation. Data are displayed normalized to the reference medium. * indicates statistical significance

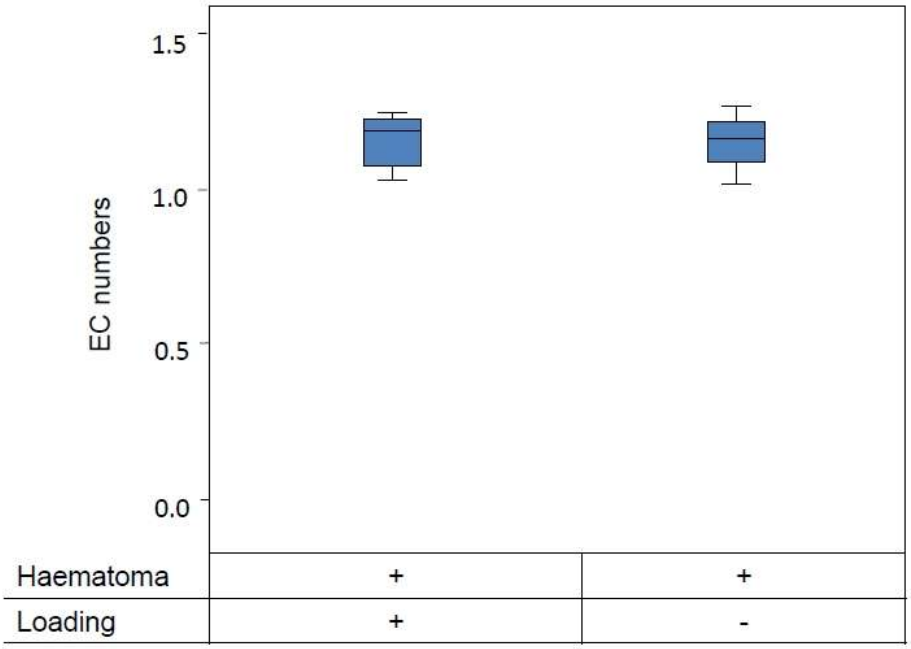

Figure 27: The persistence of the pro-angiogenic potential of the haematoma did not affect the EC numbers. EC numbers were evaluated after the tube formation assay and were independent of the mechanical stimulation of the haematoma (section c). Data are displayed normalized to the reference medium.

EC migration potential is unchanged by the mechanical stimulation of the haematomas

The migration potential of the cells stimulated by mechanically loaded haematoma in the bioreactor was not changed compared to the CM of unloaded haematomas (median $ratio_{loaded/unloaded}$=0.913, n=3, p=0.163, Figure 28).

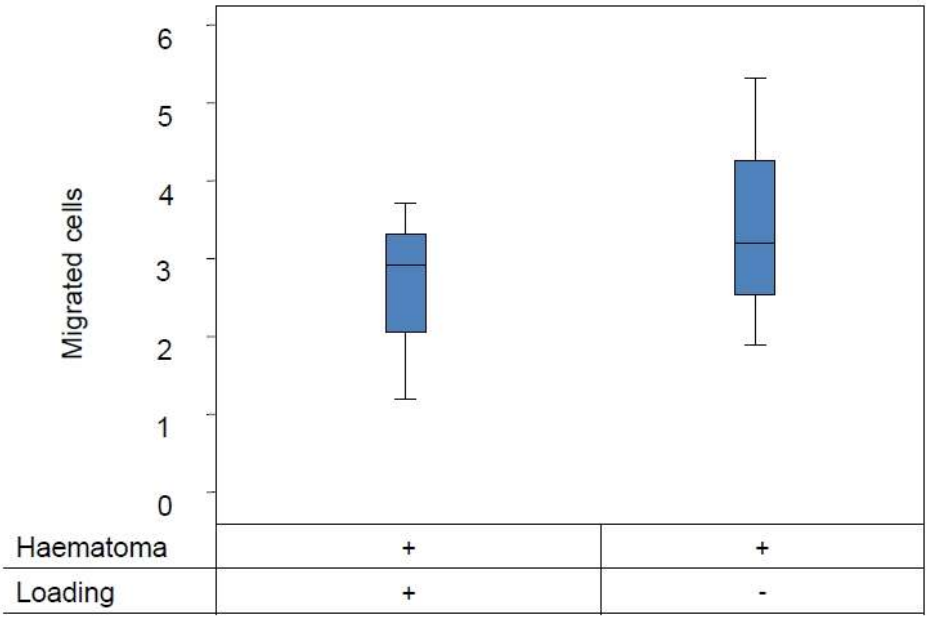

Figure 28: HMEC migration is unchanged by the CM from loaded versus unloaded haematomas. The cells were allowed to migrate through membranes under stimulation of CM. The migrated cells were not statistically different in CM from loaded and unloaded haematomas. Data are normalized to the reference medium.

To summarize, HMECs' tube formation is stimulated by the CM of haematomas, and even more by mechanically loaded haematomas. The angiogenic potential and its reaction to mechanical stimulation were independent of the tested patients' characteristics. HMECs cells numbers are not differently affected by the CMs. The increased tube formation is not correlated to an increased migration of the cells *in vitro*.

2.2. Primary endothelial cells: HUVEC

The main results obtained on the EC line (HMEC-1) were tested on primary cells for validation.

Paracrine stimulation of HUVECs by CM from haematomas

CM from haematomas stimulate tube formation more than the control CM

The CMs were applied on HUVECs to test the tube formation and cellular activity. Like on HMEC-1 cells, the haematoma induced a statistically significant increased paracrine stimulation of the tube formation of HUVECs, compared to the unloaded control ($ratio_{haematoma/control}$=1.74, n=5, p=0.004, Figure 29).

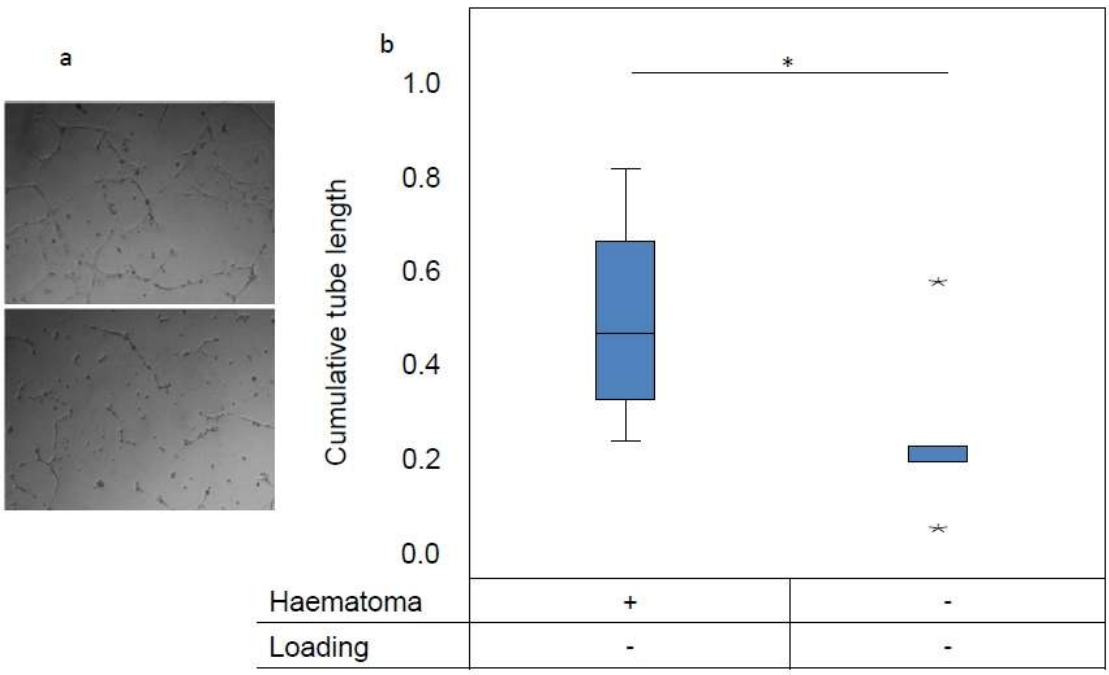

Figure 29: Tube formation by HUVECs. Representative pictures of tube formation assay of CM from the haematoma (section a, top) and CM from the negative control (section a, bottom picture). Quantification of the tube length showed a significant increase in tube formation by HUVECs in CM from haematomas compared to controls (section b). Data are displayed normalized to the reference medium. * indicates statistical significance

Cells numbers

HUVECs numbers after tube formation tended to be increased by CM from unloaded haematomas, compared to unloaded controls, without reaching statistical significance ($ratio_{haematoma/control}$=1.12 n=5, p=0.068, Figure 30).

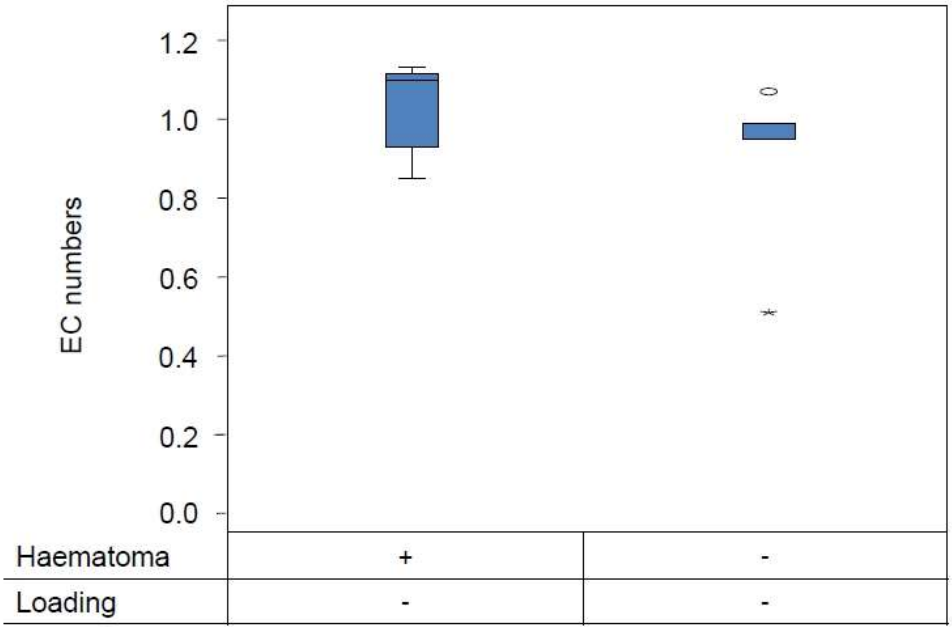

Figure 30: HUVEC numbers. HUVEC numbers tend to be increased by application of the CM from the haematoma compared to the control, without reaching statistical significance. Data are normalized to the reference medium.

Stimulation by mechanically loaded haematomas

The angiogenic potential of HUVECs was further increased by application of the CM from mechanically stimulated haematoma ($ratio_{loaded/unloaded}$=1.31, n=6, p=0.028, Figure 31). Mechanical loading of the control matrices did not influence the tube formation ($ratio_{loaded/unloaded}$=1.05, n=5, p=0.245, Figure 32).

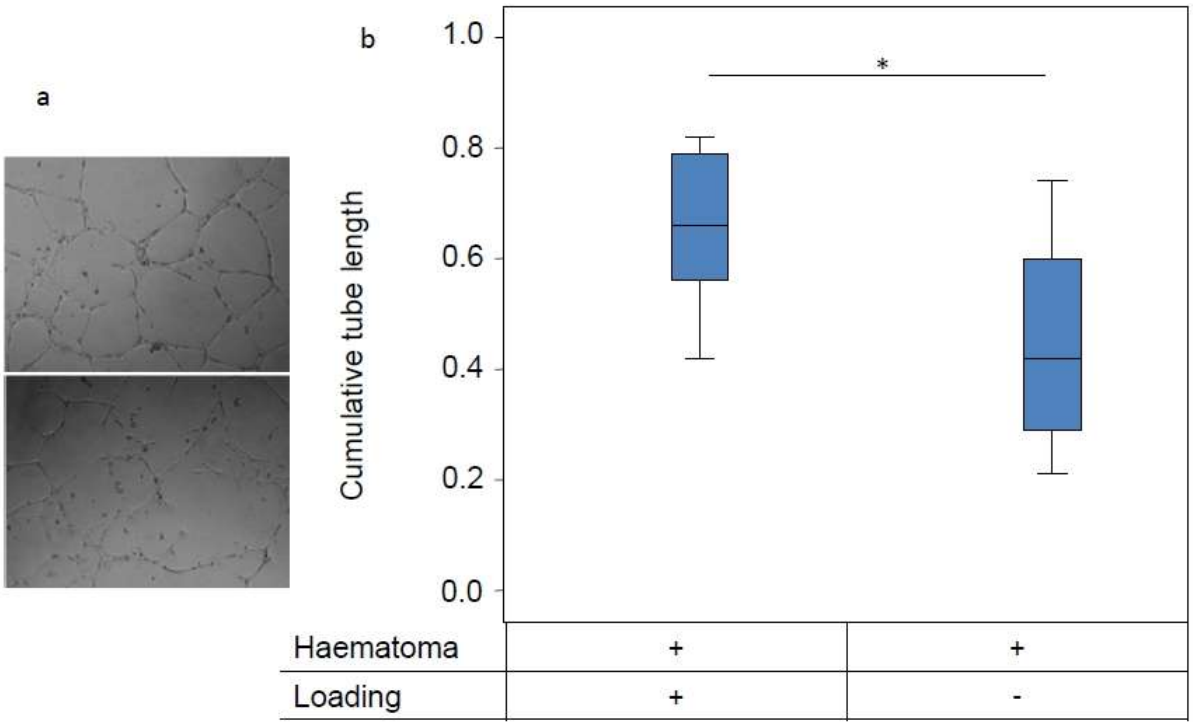

Figure 31: Tube formation of HUVECs. Representative pictures of tube formation assays (section a, top picture: loaded, bottom picture: unloaded haematoma) are displayed. Quantification showed the statistically significant increased tube formation by the CM from mechanically loaded haematomas, compared to unloaded (section b). The mechanical stimulation of haematoma-free matrices did not affect their paracrine stimulation of tube formation (section c). Data are normalized to the reference medium. * indicates statistical significance

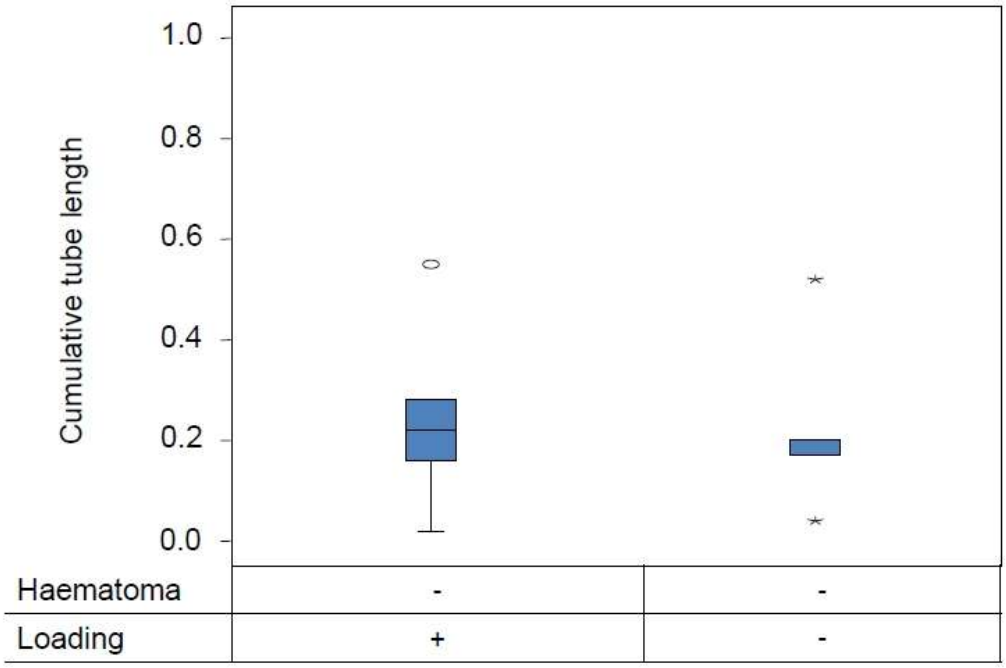

Figure 32: Tube formation of HUVECs. Tube formation of HUVECs is unaffected by the mechanical stimulation of the control matrices. Data are displayed normalized to the reference medium.

Cell numbers

CM of the loaded haematoma increased the paracrine tube formation without affecting the cell numbers ($ratio_{lo\ aded/unloaded}$=1.02, n=6, p=0.973, Figure 33).

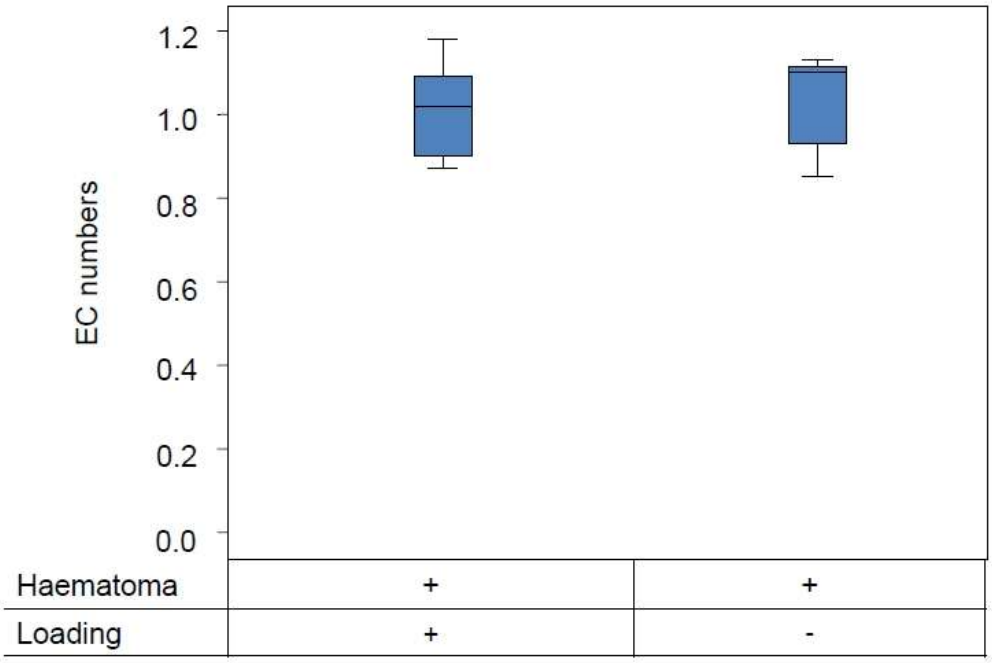

Figure 33: HUVECs numbers. HUVEC numbers are not affected by the CM from mechanically loaded haematoma compared to unloaded. Data are normalized to the reference medium.

To summarize, the results obtained with the human EC line (HMEC-1) could be confirmed with human primary cells (HUVEC). Even if the tube formation of HUVECs was higher compared to HMECs, the cell line will further be used for the stimulation of angiogenesis because of the flexibility compared to the haematomas isolation in the clinics.

3. Mechano-regulation of soluble proteins

Molecular mediators, which might be responsible for the paracrine regulation of the pro-angiogenic potential of the haematoma, were analysed in the CM. For this, the concentrations of relevant pro-angiogenic GF, MMPs and TIMPs were measured in the CM.

3.1. Angiogenic factors released by the haematoma

The concentrations of relevant GFs, MMPs and TIMPs, potentially responsible for ECs stimulation were measured in the CM using ELISAs. VEGF (increased concentration $\Delta_{haematoma\text{-}control}$= 15pg/ml, n=20, p=0.001, Figure 34a) and TGF-β1 ($\Delta_{haematoma\text{-}control}$=41pg/ml, n=8, p=0.017, Figure 34b) were released in the CM by haematoma samples, while Ang-1 levels ($\Delta_{haematoma\text{-}control}$=33pg/ml, n=8, p=0.063, Figure 34c) tended to be higher in the CM of haematomas than controls. Ang-2 concentration was not elevated by the haematoma ($\Delta_{haematoma\text{-}control}$=0pg/ml, n=8, p=0.994, Figure 34d). PDGF-bb (n=3) and bFGF (n=3) were not present in the CMs.

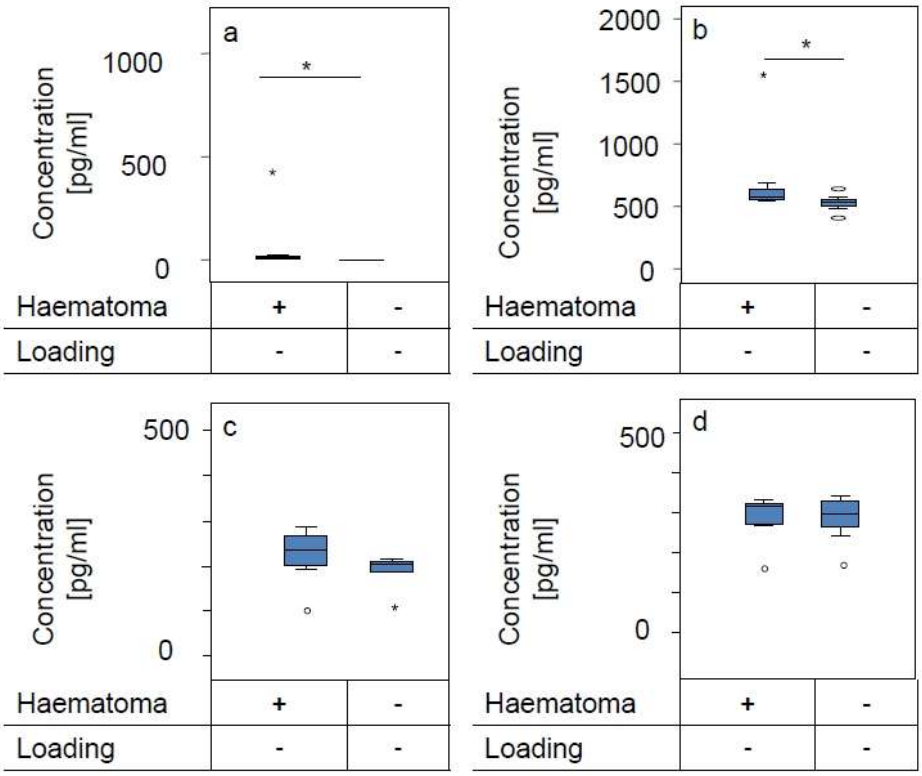

Figure 34: Concentration of the detected GFs in the CM of unloaded haematomas and controls. VEGF (a) and TGF-β1 (b) were elevated in the CM from the haematoma, while Ang-1 (c) tended to be increased. Ang-2 (d) levels were unaffected by the haematoma. Data are displayed in pg/ml. * indicates statistical significance

MMP-1 (increased concentration $\Delta_{haematoma-control}$=253pg/ml, n=10, p=0.005, Figure 35a), MMP-2 ($\Delta_{haematoma-control}$=350pg/ml, n=7, p=0.018, Figure 35b), MMP-3 ($\Delta_{haematoma-control}$=0.082ng/ml, n=8, p=0.012, Figure 35c), MMP-9 ($\Delta_{haematoma-control}$=6.871ng/ml, n=10, p=0.005, Figure 35d) and TIMP-2 ($\Delta_{haematoma-control}$=0.187ng/ml, n=8, p=0.012, Figure 35e) levels were higher in the haematoma CM than the negative controls. MMP-13 was not present in CMs (n=3).

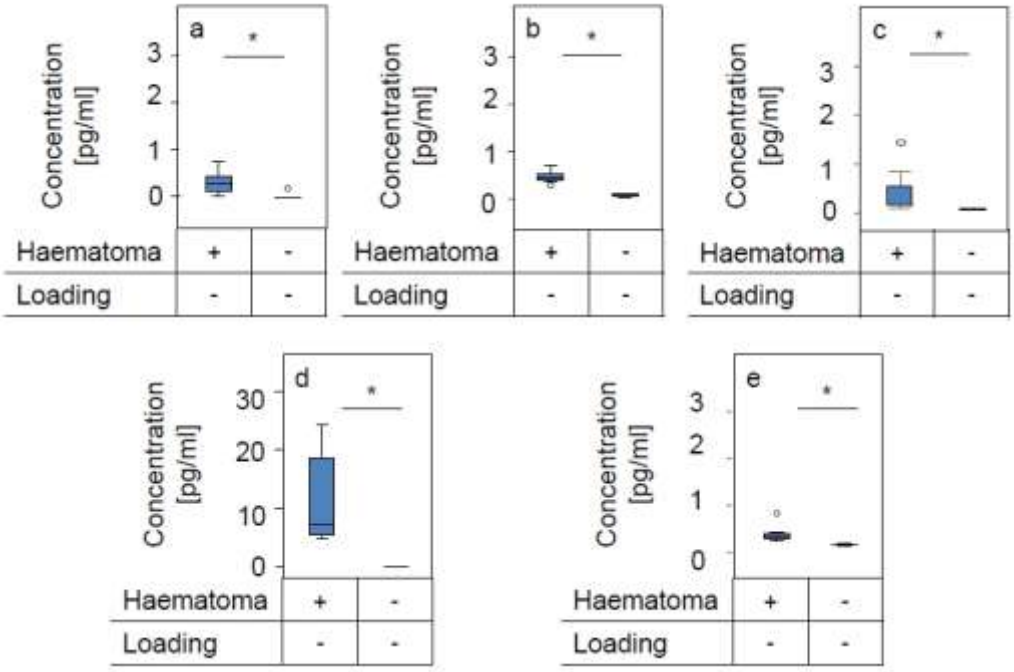

Figure 35: Concentration of the detected MMPs and TIMP-2 in the CM of unloaded haematomas and controls. MMP-1 (a), -2 (b), -3 (c), -9 (d) and TIMP-2 (e) were elevated in the CM from the haematoma. Data are displayed in pg/ml. * indicates statistical significance

3.2. Mechano-regulated angiogenic factors from the haematoma

As mechanical loading of the haematoma increased the paracrine stimulation of tube formation, the concentration of the angiogenesis regulators detected in the previous section was measured in the CM from loaded haematomas. Mechanical stimulation of the haematomas tended to increase the concentration of VEGF in the CM (ratio $_{loaded/unloaded}$=1.10, p=0.136, n=20) with a large distribution of the ratio $_{loaded/unloaded}$ over the whole patient cohort (Figure 36a). However, the increased concentration of VEGF was significant in the CM from haematomas of young patients (ratio $_{loaded/unloaded}$=1.23, n=10, p=0.013), but not aged patients (ratio $_{loaded/unloaded}$=0.97, p=0.553, n=10, Figure 36b).

Mechanical stimulation of the haematomas did not increase the concentration of TGF-β1 (ratio $_{loaded/unloaded}$=1.02, n=8, p=0.499) or Ang-1 (ratio $_{loaded/unloaded}$=1.05, n=8, p=0.327) in the CM (Figure 36a).

Mechanical stimulation of the haematomas led to an increased concentration of MMP-1 (ratio$_{loaded/unloaded}$=2.25, n=7, p=0.028, Figure 37), MMP-2 (ratio$_{loaded/unloaded}$=1.19, n=12, p=0.043, Figure 37), MMP-9 (ratio$_{loaded/unloaded}$=1.53, n=12, p=0.005, Figure 37) and TIMP-2 (ratio $_{loaded/unloaded}$=1.14, n=8, p=0.020, Figure 37) in the CMs. MMP-3 concentrations did not react to mechanical stimulation (ratio$_{loaded/unloaded}$=1.04, n=7, p=0.161, Figure 37).

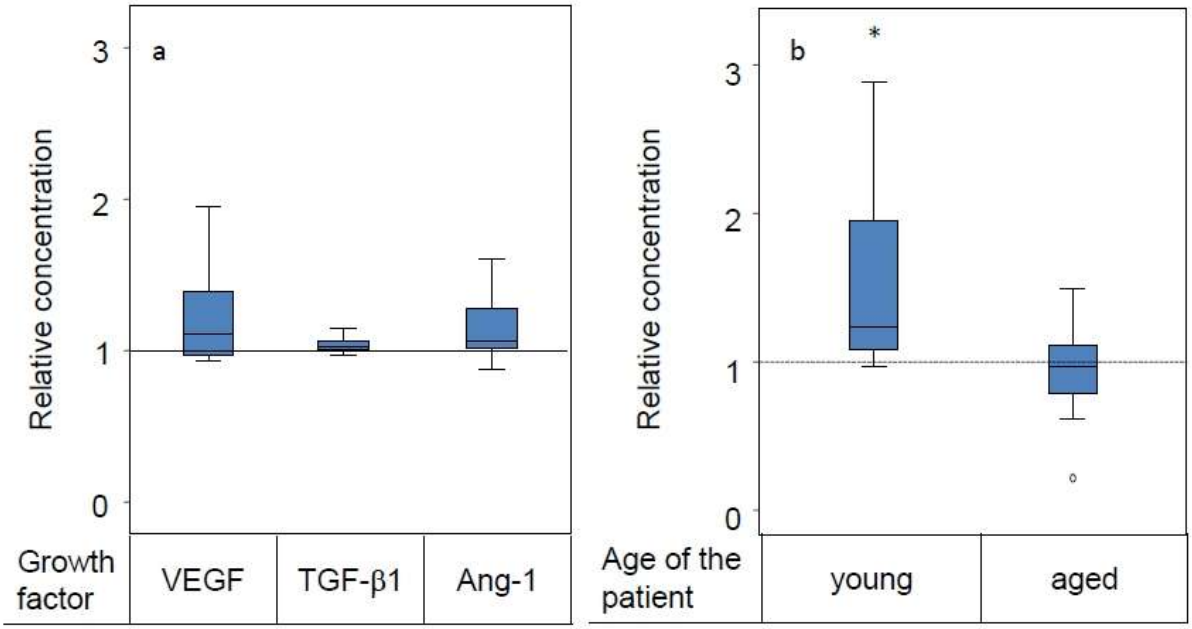

Figure 36: Mechanical regulation of the detected GFs in the CM. VEGF tended to be increased by mechanical loading of the haematoma, while TGF-β1 and Ang-1 levels were independent of loading (section a). However, young patients were able to regulate VEGF, while older patients could not (section b). Data are displayed normalized to the CM from unloaded haematomas. * indicates statistical significance

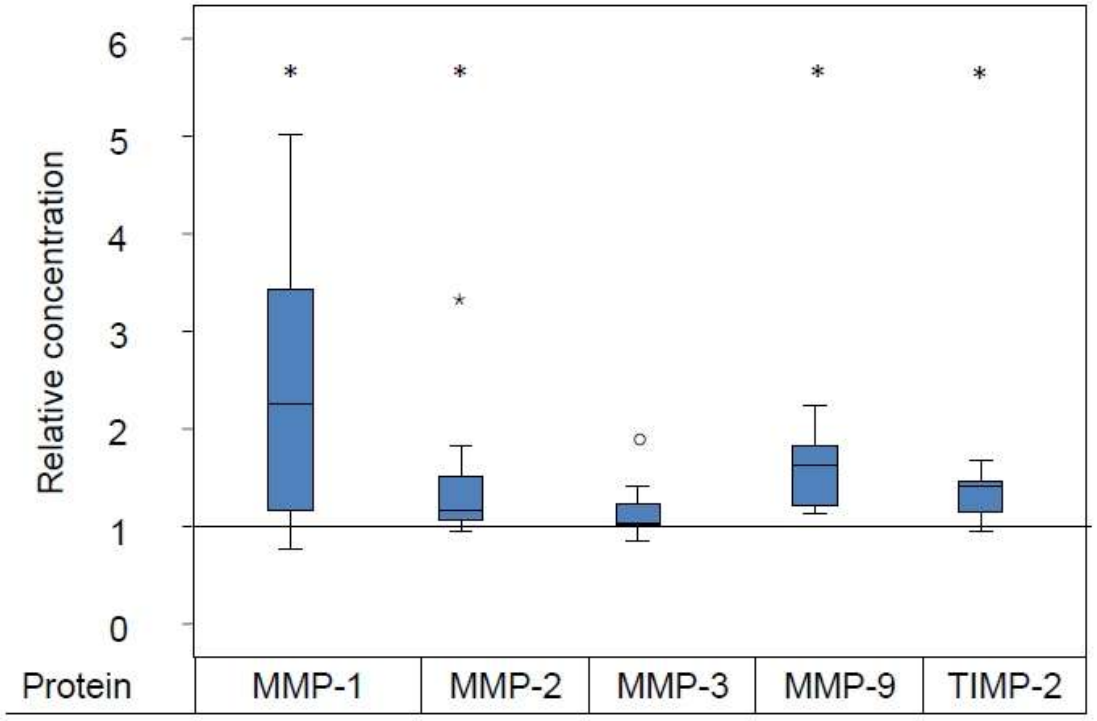

Figure 37: Mechanical regulation of the detected MMPs and TIMP-2 in the CM. Mechanical loading of the haematoma led to an increased concentration of MMP-1, -2 and -9, as well as TIMP-2, in the CM. However, MMP-3 levels were not increased. Data are displayed normalized to the CM from unloaded haematomas. * indicates statistical significance

To summarize, VEGF, TGF-β1, MMP-1, -2, -3, -9, and TIMP-2 are increased in the presence of the haematoma in the bioreactor, and MMP-1, -2 and -9 and TIMP-2 are further increased in CM by mechanical stimulation of the haematoma matrices. VEGF was mechanically regulated in an age-dependent manner. The varying concentrations of GF, MMPs and TIMP-2 in the CM could be responsible of the changed functional behaviour of ECs *in vitro*. MSCs' functional behaviour might be affected by these differentially concentrated regulators, and this will be presented in the next section.

4. The paracrine stimulation of mesenchymal stem cells by conditioned media[6]

MSCs are recruited into the injury site and contribute to the healing process. However, the interaction of the haematoma and the MSCs are unclear yet. The proliferation and migration potential of the MSCs in contact with CMs were evaluated in contact to the haematoma and in response to its mechanical stimulation, as well as their differentiation potential in the osteogenic, adipogenic and chondrogenic lineages, with and without lineage-specific chemical induction.

4.1. Proliferation

Exposure of MSC to haematoma CM did not affect MSC proliferation, compared to the exposure to CM from negative controls (median ratio $_{\text{haematoma / no haematoma}}$=0.91, n=7, p=0.585, data not shown). However, mechanical loading of the haematoma tended to decrease the proliferation index of the MSCs *in vitro* (ratio$_{\text{loaded/unloaded}}$=0.86, n=7, p=0.056, Figure 38a), whereas negative controls showed no influence (ratio $_{\text{loaded/unloaded}}$=0.92, n=7, p=0.477, Figure 38b). The addition of 0.1, 0.5 and 1% FCS in the CM did not change the proliferation index of the MSCs (data not shown).

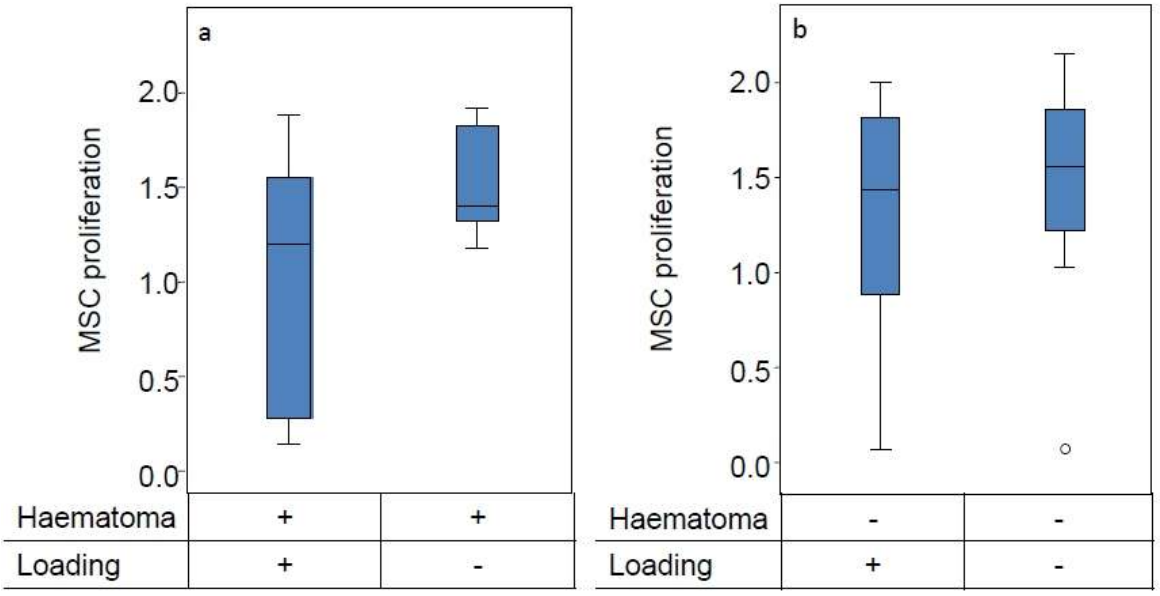

Figure 38: Proliferation of MSCs. MSCs tended to proliferate less in CM from loaded haematomas compared to Cm from unloaded haematomas (section a), which was due to the mechanical loading of the fibrin (section b). Data are normalized to the reference medium.

[6] Work accomplished by Beatrice Woltmann during her master thesis, under the supervision of Aline Groothuis

4.2. Migration

MSCs' migration was tested through collagen I-coated and uncoated filters. The number of cells that migrated through collagen I-coated filters was higher than for uncoated ones, but the CM from haematomas or controls stimulated the migration of MSCs to the same extent (coated filters: median ratio $_{\text{haematoma / without haematoma}}$=1.16, n=3, p=0.323; uncoated filters: median ratio $_{\text{haematoma / without haematoma}}$=1.17, n=3, p =0.171, data not shown).
Mechanical stimulation of the haematoma did not affect the paracrine stimulation of migration of MSCs through both types of filters (coated: median ratio $_{\text{loaded / unloaded}}$ =1.01, n=3, p= 0.436; uncoated: median ratio $_{\text{loaded / unloaded}}$=1.08, n=3, p =0.532, data not shown).

4.3. Differentiation

The three clinically most relevant differentiation lineages of MSCs were tested: chondrogenic, osteogenic and adipogenic lineages. CMs were tested with and without chemical induction of differentiation. EM was used as control (with chemical induction: positive control – without induction: negative control).

A considerable amount of ECM was observed in the positive control for chondrogenic differentiation and a low amount in the negative control. Neither the haematoma nor mechanical loading induced a differentiation, while the addition of lineage-specific additives did. Representative pictures of the stained pellets are shown in the Annex I.
No differentiation into the osteogenic lineage was shown after AP staining if no differentiation additives were added to the CM and EM. Supplemented CMs with additives led to similar AP values like the positive controls. AR showed the same tendency. No significant differences were observed between the CMs (data not shown).
For the adipogenic differentiation, the positive control (EM with additives) showed positive ROR staining and intracellular formation of lipid droplets. The supplemented CMs showed a similar differentiation, like the CM of unloaded haematomas. Mechanically stimulated, unsupplemented haematomas showed less differentiation than the unloaded haematomas, but the results were more pronounced than the unloaded and loaded controls (data not shown)

To summarize, MSCs functional behaviour, such as the proliferation, migration and differentiation potential, was not significantly affected by the haematoma and its mechanical stimulation. Thus, this project will further focus on ECs and the stimulation of angiogenesis.

5. Stimulation of the endothelial cells by supplementation of regulated factors

This section will present the establishment of a supplementation strategy to enhance the pro-angiogenic potential of the haematoma. For this, the potential factors will first be tested in the CM of haematomas and then on fresh, diluted haematomas. Then, further delivery strategies by transfection of MSCs were tested, by magnetofection, lipofection and nucleofection.

5.1. Supplementation of recombinant factors in solution

The supplementation of mechano-regulated factors was tested with recombinant factors, first in the CM of unloaded haematomas, then in fresh haematomas. MMP-1, -2, -9 and TIMP-2 concentrations were significantly increased by mechanical stimulation. Even if VEGF only tended to be increased by mechanical stimulation, this factor was still tested because of its relevance for the stimulation of angiogenesis (VEGF).

On the conditioned medium

VEGF

Mechanical stimulation of the haematoma tended to increase the VEGF concentration in the CM. However, the supplementation of the mechano-regulated concentration of rhVEGF in the CM of unloaded haematomas of mixed patients populations decreased the tube formation of HMEC-1 cells *in vitro* ($ratio_{supplemented\ /\ not\ supplemented}$=0.31, n=5, p=0.014, Figure 39), without effects on the cell numbers ($ratio_{supplemented\ /\ not\ supplemented}$ =0.98, n=5, p=0.191, Figure 40). Also, the tube stimulation of HUVECs with rhVEGF showed the same reduced tube formation ($ratio_{supplemented\ /\ not\ supplemented}$ =0.83, n=5, p=0.031, Figure 42a), however HUVECs numbers tended to be decreased by the application of VEGF ($ratio_{supplemented\ /\ not\ supplemented}$=0.96, n=5, p=0.068, Figure 42b).

The inhibition of the VEGF pathway by the inhibition of VEGFR2 (10nM) decreased the tube formation by mechanically stimulated haematoma on HMECs ($ratio_{inhibited\ /\ not\ inhibited}$=0.60, n=5, p=0.008, Figure 41), without affecting the cell numbers ($ratio_{inhibited\ /\ not\ inhibited}$=0.95, n=5, p=0.128, Figure 43).

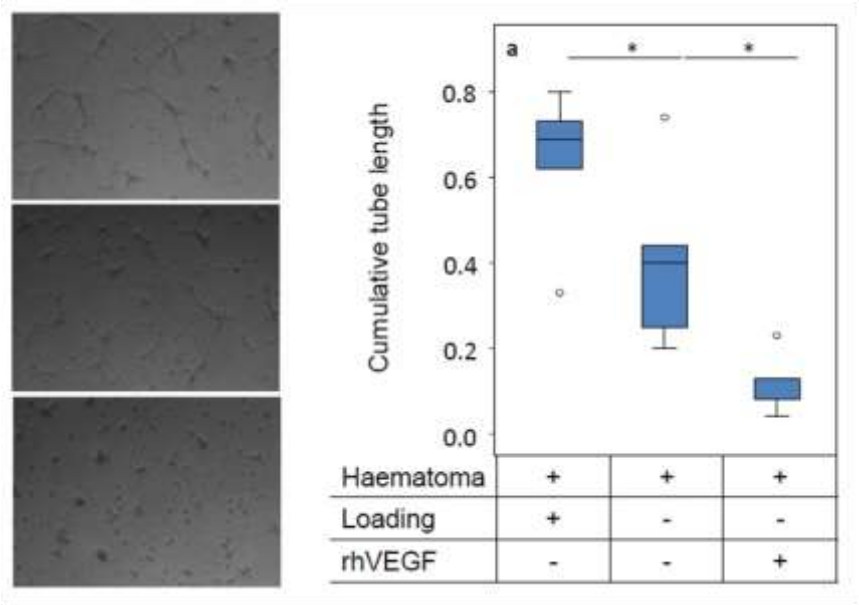

Figure 39: Supplementation of rhVEGF on HMECs: tube formation. Representative pictures of tube formation assays (section a, top picture: loaded haematoma, middle picture: unloaded haematoma, bottom picture: unloaded haematoma, supplemented with rhVEGF) are displayed. Quantification showed the statistically significant decreased tube formation by the supplementation of rhVEGF in the CM from unloaded haematomas (section b). Data are normalized to the reference medium. * indicates statistical significance

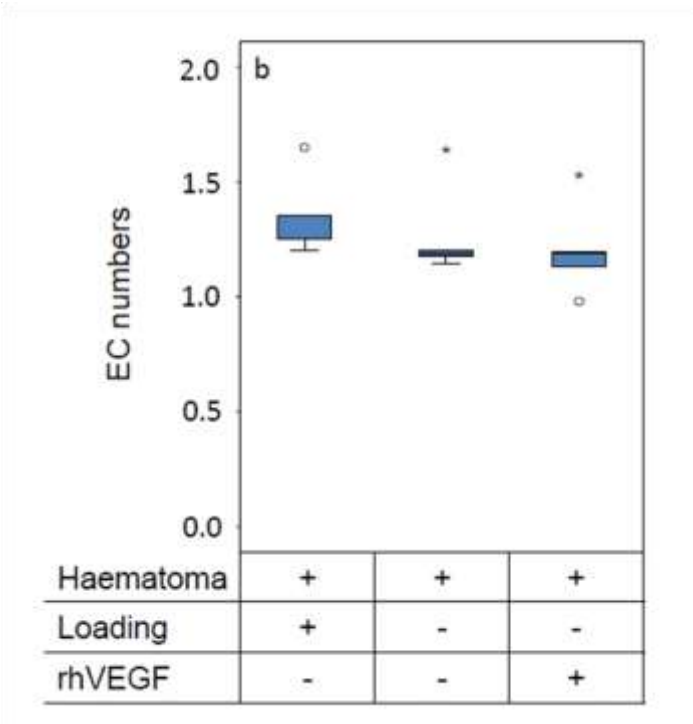

Figure 40: EC numbers. HMEC numbers are not affected by the supplementation of rhVEGF in the CM of unloaded haematomas. Data are normalized to the referenced medium.

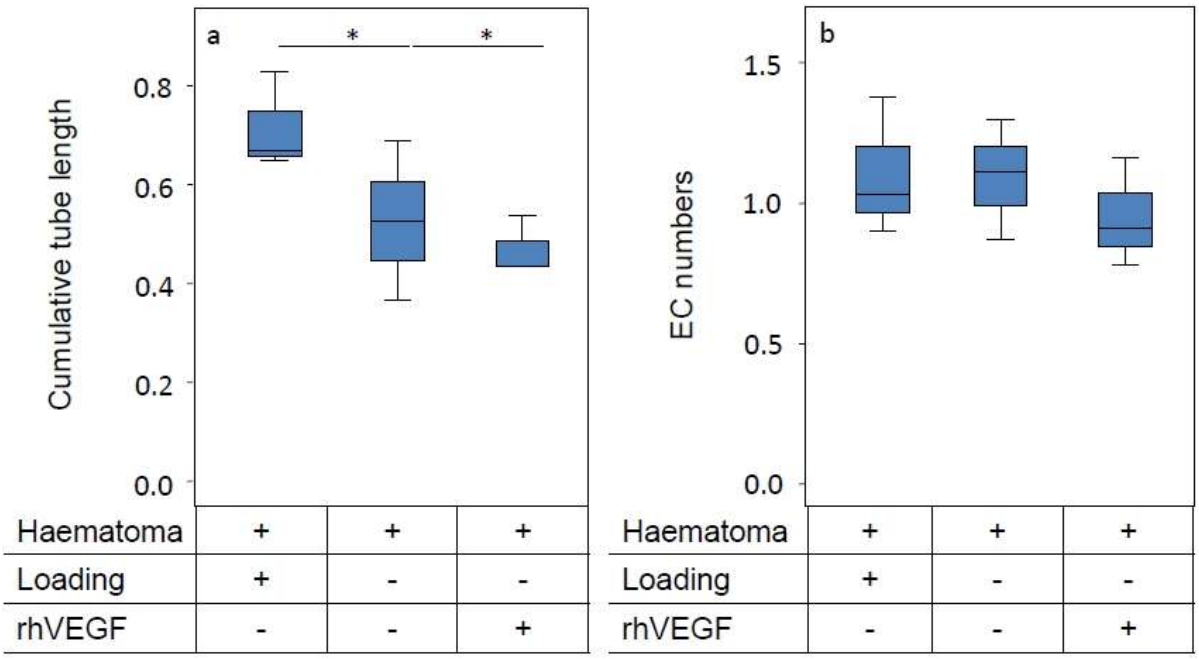

Figure 42: Supplementation of rhVEGF on HUVECs. Tube formation was decreased by application of VEGF in the CM of unloaded haematomas (section a), while the cell numbers tended to be decreased (section b). * indicates statistical significance

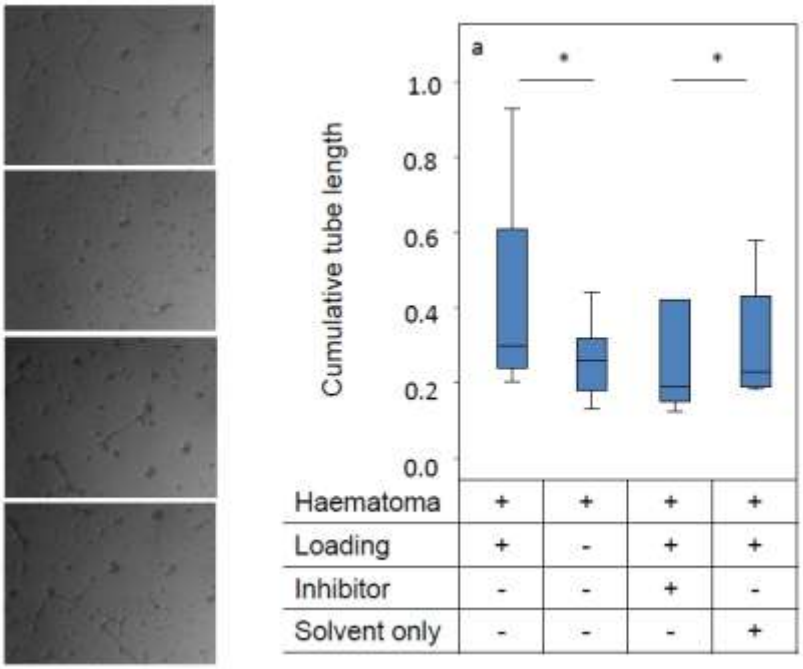

Figure 41: VEGFR2 inhibition. The inhibition of VEGFR2 in HMECs, stimulated with the CM from loaded haematomas, decreased the tube formation of HMEC-1 cells. The stimulation with the solvent only (DMSO) did not influence the tube formation (section a). EC numbers were not affected by the inhibition (section b). * indicates statistical significance

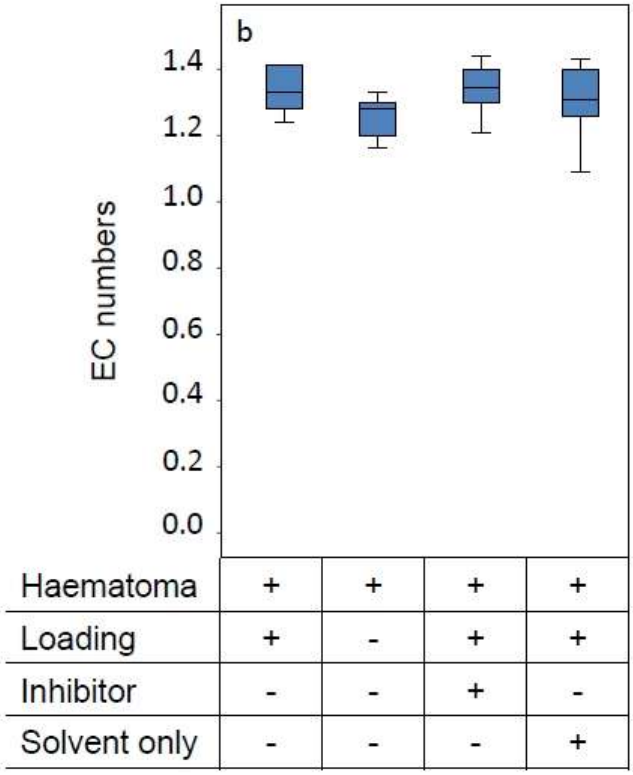

Figure 43: EC numbers. HMEC numbers are not affected by the inhibition of VEGFR2 in the CM of loaded haematomas. Data are displayed normalized to the reference medium.

The increased paracrine stimulation of tube formation by mechanical loading of the haematoma is dependent on the VEGFR2-signalling of HMEC-1 cells, but surprisingly, the addition of VEGF is detrimental to the tube formation on Matrigel.

MMPs, TIMPs

MMP-1, -2, -9 and TIMP-2 were tested for stimulation of tube formation in the CM of unloaded haematomas in the regulated concentration and a dilution series thereof. MMP-1 stimulated the tube formation when applied in the mechano-regulated concentration (MMP-1_{1x}: $ratio_{MMP\text{-}1,\ 1x\ /unsupplemented}$=1.32, n=5, p=0.068, Figure 44) but without reaching statistical significance, and 10x this concentration (MMP-1_{10x}: $ratio_{MMP\text{-}1,\ 10x\ /unsupplemented}$=1.25, n=5, p=0.030, Figure 44). Supplementation of the CM from unloaded haematomas with the mechanically regulated concentration of MMP-2 tended to increase the tube formation ($ratio_{MMP\text{-}2,\ 1x\ /unsupplemented}$=1.17, n=5, p=0.080, Figure 46) also without reaching statistical significance. Other tested concentrations of MMP-1 and MMP-2 were either inefficient to

influence the tube formation, or showed a inhibitive effect. Supplementation of MMP-9 and TIMP-2 decreased tube formation in all tested concentrations (Figure 45 and Figure 47). All ratios $_{\text{supplemented / unsupplemented}}$ and p-values are presented in the Annex II.

MMP supplementation did not affect EC numbers, until the supplementation of MMP-1 ($\text{ratio}_{\text{MMP-1, 2x /unsupplemented}}$=1.04, p=0.043), MMP-9 ($\text{ratio}_{\text{MMP-9, 2x /unsupplemented}}$=0.95, p=0.043), and TIMP-2 ($\text{ratio}_{\text{MMP-1, 2x /unsupplemented}}$=0.93, p=0.043). All the ratios $_{\text{supplemented / unsupplemented}}$ and p-values are presented in the Annex II.

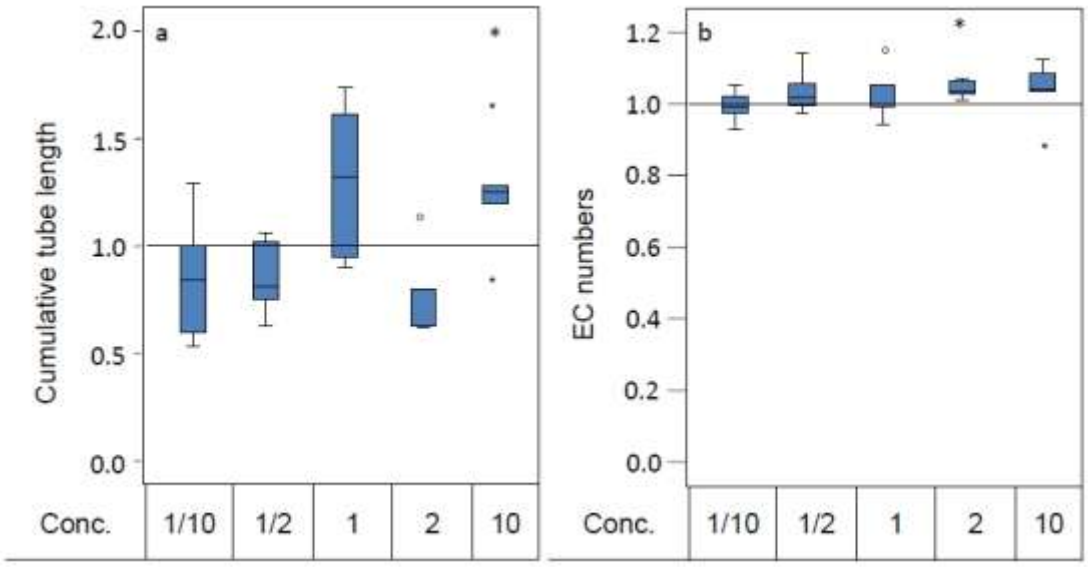

Figure 44: Supplementation of MMP-1 in the CM of unloaded haematomas. CM from unloaded haematomas was supplemented with MMP-1 in the mechanically upregulated concentration and a dilution series thereof. Application of MMP-1_{1x} tended to increase the tube formation, and the application of MMP-1_{10x} increased the tube formation. EC numbers were increased by the application of MMP-1_{2x}. Data were normalized to unsupplemented. * indicates statistical significance

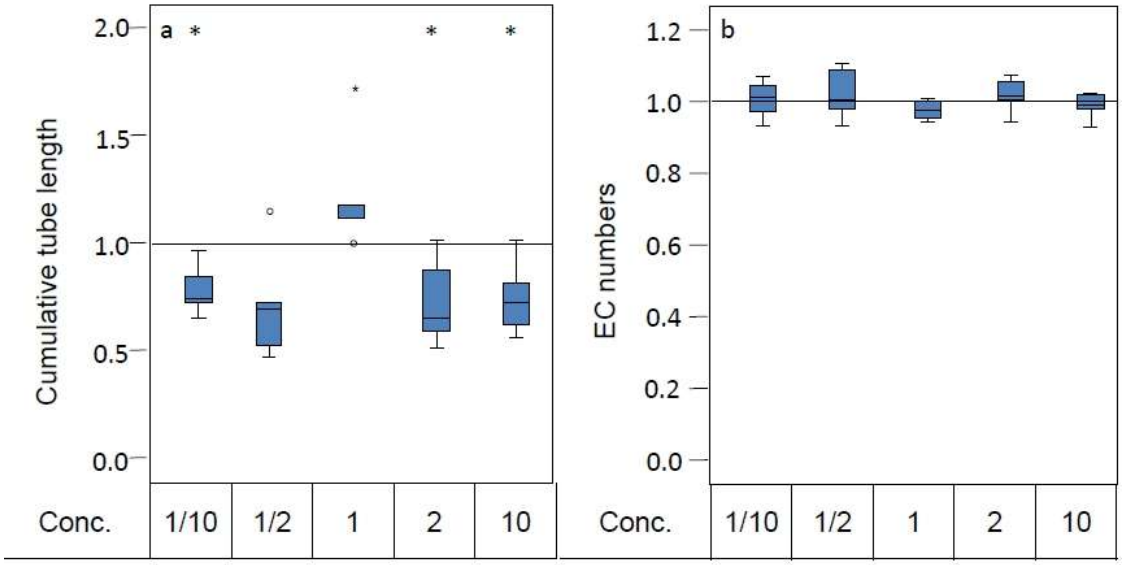

Figure 46: Supplementation of MMP-2 in the CM of unloaded haematomas. CM from unloaded haematomas was supplemented with MMP-2 in the mechanically upregulated concentration and a dilution series thereof. Application of MMP-2 $_{1/10x,\ 2x}$ and $_{10x}$ decreased the tube formation, and the application of MMP-2_{1x} tended to increase the tube formation. EC numbers were unaffected by the stimulation by MMP-2. Data were normalized to unsupplemented. * indicates statistical significance

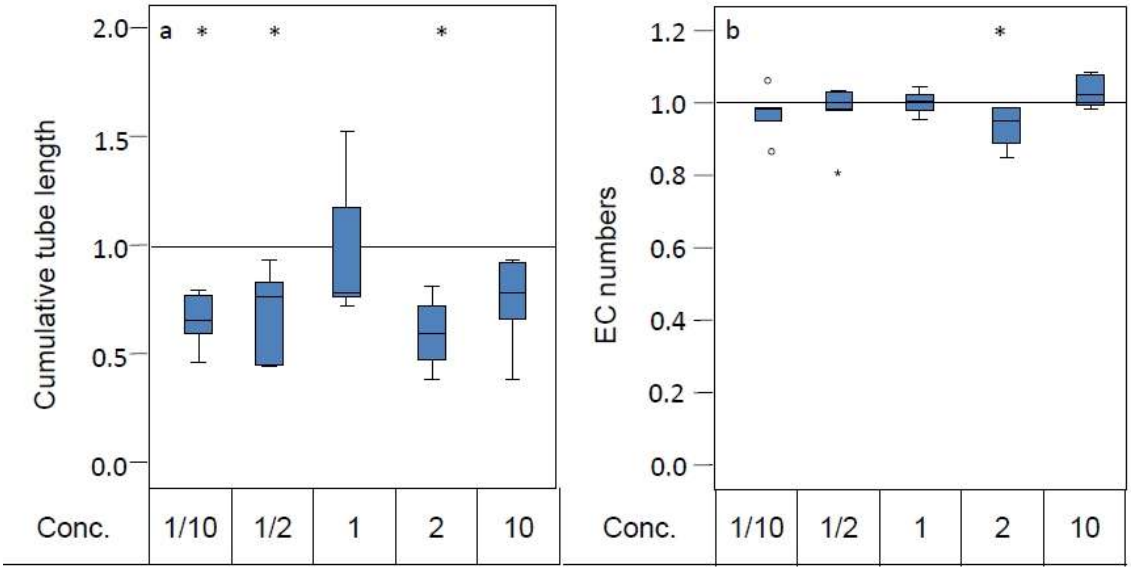

Figure 45: Supplementation of MMP-9 in the CM of unloaded haematomas. CM from unloaded haematomas was supplemented with MMP-9 in the mechanically upregulated concentration and a dilution series thereof. Application of MMP-9 $_{1/10x,\ 1/2}$ and $_{2x}$ decreased the tube formation. EC numbers were decreased by the application of MMP-9 $_{2x}$. Data were normalized to unsupplemented. * indicates statistical significance

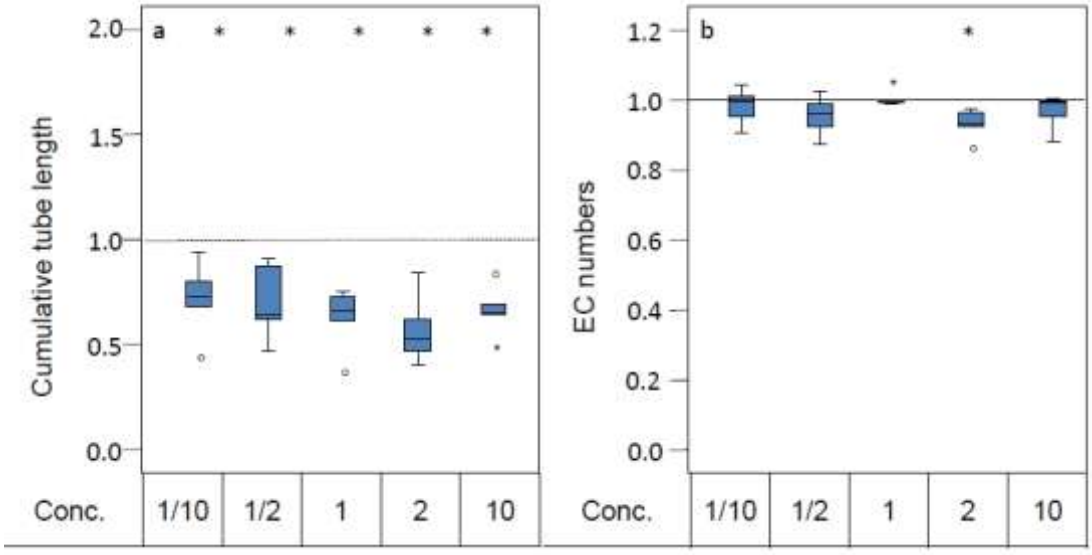

Figure 47: Supplementation of TIMP-2 in the CM of unloaded haematomas. CM from unloaded haematomas was supplemented with TIMP-2 in the mechanically upregulated concentration and a dilution series thereof. Application of TIMP-2 decreased the tube formation. EC numbers were decreased by the application of MMP-9 $_{2x}$. Data were normalized to unsupplemented. * indicates statistical significance

Dilution series of the mechanically regulated factors showed the positive stimulation of tube formation by supplementation of MMP-1 (1 and 10x the regulated concentration) and MMP-2 (1x the regulated concentration). MMP-1 and -2 and their combinations will be tested on fresh haematomas for tube formation. Other concentrations of MMP-1 and -2, as well as MMP-9 and TIMP-2, will not be further tested.

On fresh haematomas

MMP-1 and -2 and their combinations were applied in fresh haematomas to evaluate their stimulation of tube formation.

Fresh haematomas stimulate tube formation and the EC proliferation

The application of the haematoma on HMECs stimulated tube formation in a concentration-dependent manner (see Figure 48).

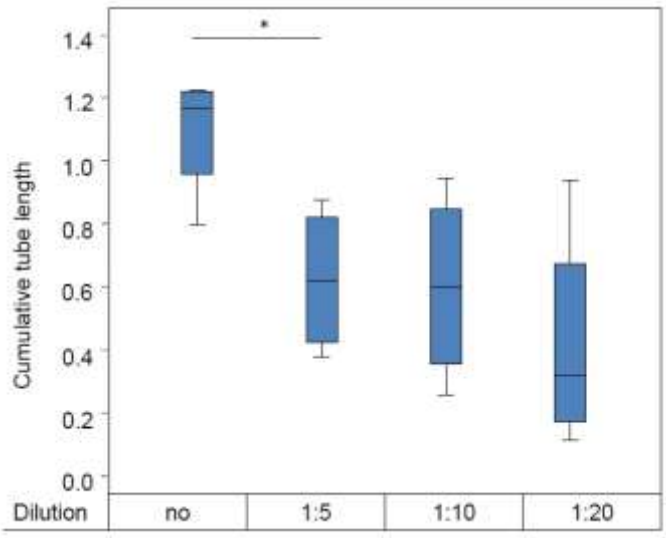

Figure 48: Paracrine stimulation of the angiogenesis is decreased by the dilution of fresh haematomas. Data are normalized to the reference medium, used for the dilution. * indicates statistical significance

Evaluation of the cell number after tube formation assay showed a decreased cell numbers with a higher dilution of the haematoma (Figure 49). The data are normalized to the reference medium, used to dilute the haematoma.

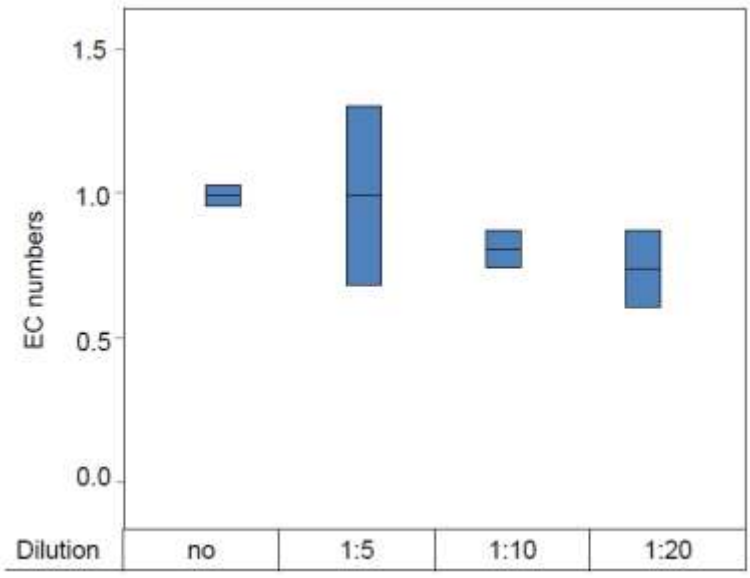

Figure 49: EC numbers are decreased during serial dilution of the haematoma with the reference medium. Data are displayed normalized to the reference medium.

Supplementation of MMPs in fresh haematomas

To identify the factors capable of angiogenic stimulation in the haematoma microenvironment, the on CM selected MMP-1 and -2 were applied on the diluted haematoma. For this, fresh haematomas were diluted in MCDB-131 (i. e. serum-free medium, Figure 50) or in EC-Medium (i. e. containing 5% FCS, Figure 51) and supplemented by MMP-1 ($_{1x}$ or $_{10x}$) and/or MMP-2 ($_{1x}$) before application on HMECs. Addition of 10x the mechanically up-regulated concentration of MMP-1 into the haematoma significantly increased the tube formation on Matrigel, when the haematoma was diluted with serum-containing medium, but no influence of MMP-1 was shown in the case of the haematoma diluted in serum-free medium (with serum: ratio $_{\text{MMP1, 10x/unsupplemented}}$=1.21, n=5, p=0.043, without serum: ratio$_{\text{MMP1, 10x/unsupplemented}}$=1.03, n=5, p=0.893). The mechano-regulated concentration of MMP-1 and -2, as well as their combinations, failed to increase the tube formation, in both the dilution mediums. All ratios $_{\text{supplemented / unsupplemented}}$ and p-values are given in the Annex III.

EC numbers were not affected by the supplementations (Figure 50 and Figure 51). All ratios $_{\text{supplemented / unsupplemented}}$ and the p-values are given in the Annex III.

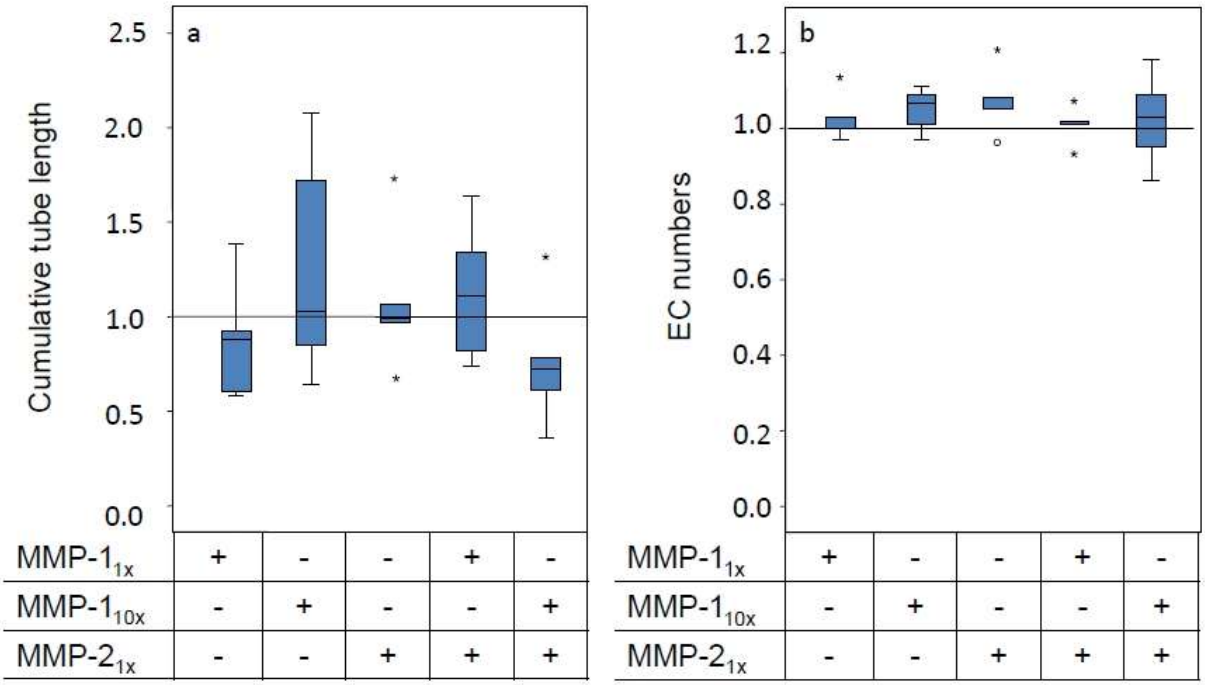

Figure 50: Tube formation of supplemented haematomas with MMP-1 and -2 without adjunction of serum. Supplementation of the diluted haematoma without serum and MMPs did not affect the tube formation. EC numbers were unchanged by the supplementations. Data were normalized to the unsupplemented haematoma.

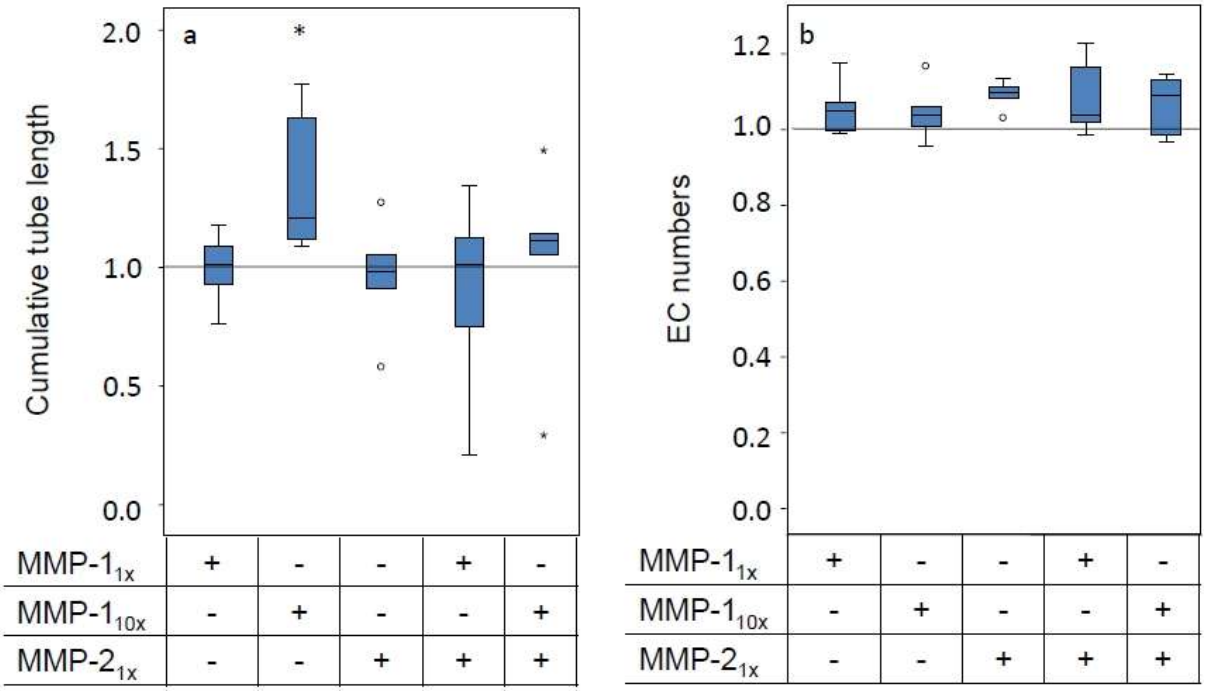

Figure 51: Tube formation after supplementation of the haematoma with MMP-1 and -2 with adjunction of serum. Supplementation of the diluted haematoma with serum and MMP-1_{10x} increased the tube formation. EC numbers were unchanged by the supplementations. Data were normalized to the unsupplemented haematoma. * indicates statistical significance

Thus, the supplementation of the haematoma with MMP-1 (10x the mechano-regulated concentration) increased the tube formation of HMEC-1 in presence of serum in the dilution medium. The supplementation of MMP-1 and MMP-2 (both 1x the mechano-regulated concentration), both separately or in combination with each other did not stimulate the tube formation. The application of the most stimulating, mechano-regulated factor (MMP-1, 10x the mechano-regulated concentration) will be tested by different drug transfection methods on MSCs.

5.2. Transfection of MSCs

Three transfection methods were tested on MSCs in order to over-express MMP-1 on the site of injury. Magnetofection, with the PEI-SPION system, was tested on MSCs. Moreover, two kinds of transfections were tried using kits for primary cells: lipofection and nucleofection.

5.2.1. Magnetofection

Colloidal stability

The PEI- coated SPIONs were diluted in PBS and the colloidal stability was evaluated by the size of the particles in suspension. The coated particles seem to not aggregate in PBS, as shown in Figure 52, which is a requisite for an adequate complexation with the DNA, and a further transfection.

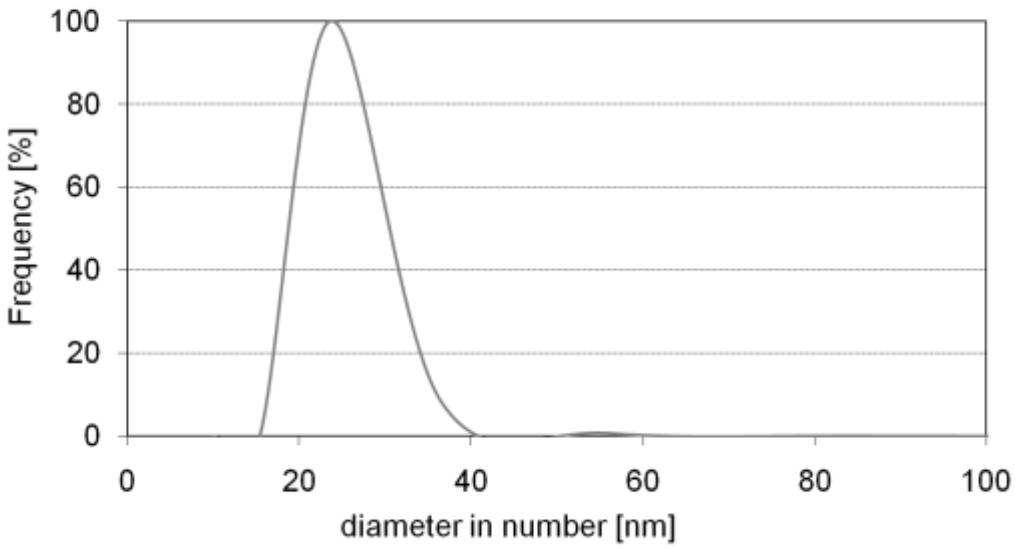

Figure 52: Size distribution of PEI coated SPIONs. The size distribution of the PEI-coated SPIONs in PBS was measured to evaluate the aggregation of the system.

Transfections

The followed transfection procedure did not produce transfected MSCs, however at a high survival rate of the cells (Figure 53a). However, the positive control, HeLa cells, showed a transfection rate of about 40% with a high cell survival rate, indicating the suitability of the technique used to bring the nanoparticles to the cell surface, but not working for the MSCs (Figure 53).Further trials with MSCs using different N/P ratio (2, 4, 6, 8, 10), an increased concentration of nanoparticles in the medium (up to 3 orders of magnitude), as well as the use of a variable magnet, did not increase the transfection rate of MSCs, with positive transfection rates of HeLa cells (10-50% transfected cells, data not shown). The clinical relevance of HeLa cells being low, the assays were not further conducted or evaluated more precisely.

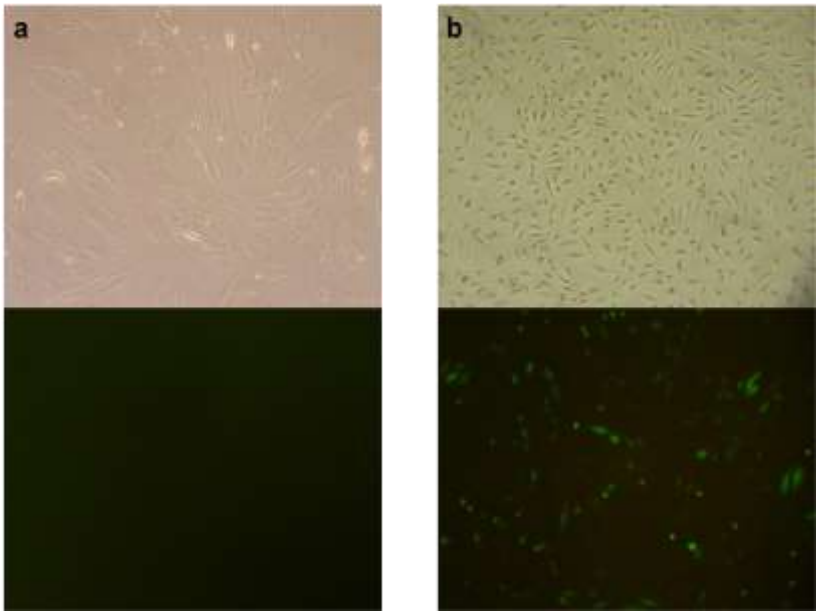

Figure 53: Typical pictures of magnetofection. Transmitted light pictures (top) and fluorescent light pictures (bottom) of magnetofection assays of MSCs (section a) and HeLa cells (section b). Magnetofection of HeLa cells, but not MSCs, lead to the expression of the GFP-plasmid (green).

5.2.2. Lipofection

No positive transfection was observed following the use of the Primefect II kit (data not shown). Variations of the protocol (surface charge, concentration, presence of FCS in the medium) were unable to change the result.

5.2.3. Nucleofection

Transfection rates of ~50% were achieved using nucleofection, however with a 90% decrease in the total cells numbers (Figure 54). As MSCs are “rare and precious”, this survival rate, if typical for Amaxa systems, was not acceptable for a possible clinical application. The low survival rate also increases the risk of selection of the cells and so may alter the functional behaviour of such transfected MSCs.

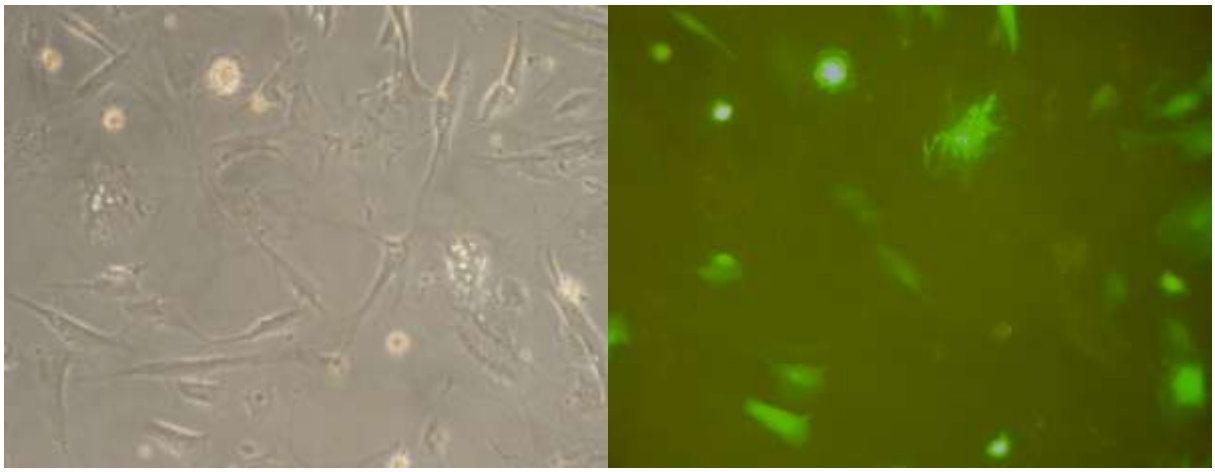

Figure 54: Nucleofection of MSCs. Typical pictures of nucleofection of MSCs. Left picture: transmitted light, right picture: fluorescent light picture.

In summary, the magnetofection by the PEI-SPIONs system did not successfully transfect MSCs, but the control cells (HeLa). Also, the use of a lipofection kit did not transfect MSCs. However, the nucleofection showed positive results, but a too low survival rate of the MSCs. Thus, the transfection methods were not further considered as drug delivery methods in this study.

Chapter 4: Discussion and conclusions

In this chapter, the results will be briefly summarized and then discussed. The technical limitations of the bioreactor system used will be described. The most relevant results from previous studies of human MSCs and their co-cultures with T-lymphocytes will be presented. Then, the results obtained from the present study will be discussed: the paracrine stimulation of the haematoma on ECs and MSCs, the proteins potentially involved in these different stimulations and the stimulation methods for the pro-angiogenic stimulation of the haematoma on ECs. Finally, some conclusions of the study will be drawn.

1. Summary of the results

While most factures heal uneventfully, some of them present a delayed healing or pseudoarthrosis. Adequate mechanical stimulation and angiogenesis stimulate healing, however through unknown mechanisms. This study aimed to investigate the paracrine stimulation of the haematoma on endothelial cells (ECs) and mesenchymal stem cells (MSCs), its response to mechanical stimulation and the key molecular players in this system. Furthermore, a pro-angiogenic stimulation method was to be established in the haematoma microenvironment.

This study demonstrated the pro-angiogenic potential of the haematoma on ECs *in vitro* and its increase by mechanical stimulation, which persisted for at least 24h. The functional behaviour of MSCs, such as proliferation, migration or differentiation, was unaffected. Haematomas increased the MMP-1, -2, -3, -9, and TIMP-2, VEGF, TGF-β1 and Ang-1 concentrations in the CM, and mechanical stimulation led to an increased concentration in the CM of MMP-1, -2, -9 and TIMP-2, as well as VEGF in an age-dependent manner. The application of recombinant VEGF led to a decreased tube formation on both the EC line and primary ECs, however depending on the VEGFR2 pathway. The supplementation of mechano-regulated MMPs and TIMP-2 led to an enhanced tube formation by MMP-1 (1x and 10x the regulated concentration) and MMP-2 (1x the regulated concentration), but not by MMP-9 and TIMP-2 or other concentration of MMP-1 and -2. The supplementation of recombinant factors was tested in 1:5 diluted haematomas. Supplementation of the haematoma with MMP-1_{10x} in presence of serum (FCS) increased the tube formation, probably by activation of certain factor in the FCS; however after the supplementation of other MMPs and in serum-free medium diluted haematoma, the tube formation remained unaffected. Transfection of MSCs was tested, however unsuccessfully, both by PEI-coated SPIONs and lipofection. The survival rate of MSCs after nucleofection was too low to be considered, although this method gave a positive transfection result.

In summary, this project confirmed the angiogenic potential of the haematoma as showed by tube formation assay, which was further increased by mechanical loading. The angiogenic potential of the fresh haematoma was increased by application of the mechano-regulated MMP-1 and dilution in serum-containing medium, possibly through the activation and / or release of GF from the serum.

2. Discussion

2.1. Technical limitations

The cultivation of the haematoma in the bioreactor seemed to maintain the number of viable cells in the matrices unaffected by mechanical stimulation. Thus, the fibrin matrices and the loading parameters seem to be relevant for this study.

No PDGF-bb, bFGF and MMP-13 were detected in the CM, but in the haematomas (data not shown). This may be due to an anchorage of the factors to the ECM or to a complexation with binding proteins, making them unmeasurable in the CM. Possible reasons for the absence of these factors will be discussed later on. However, it may be that the choice of another matrix material would have been more suitable for this study. Moreover, the viscoelastic deformation of the matrix under the repeated loading may influence the behaviour of the haematoma's cells. Another relatively stiff and elastic hydrogel might have led to more reliable results.

While the effects of the haematoma and its mechanical stimulation were easily quantified on ECs, application the CM did not influence the tested MSCs functions. This may be due to an insufficient ratio of quantity of haematoma (100mg) cultivated in the bioreactor (25ml) and applied to MSCs (200µl medium /cm^2 confluent cells).

The choice of another test for angiogenesis, like the sprouting assay, could also have led to more reliable results, but it would have brought further complications. Hypoxia may be a factor to take into account in the tube formation assays from fresh haematomas. However, this supplementary factor seems to have been avoided by the further dilution of the haematoma, which led to unchanged tube formation stimulation with further dilutions. The surprising non-reaction of the EC cell line to VEGF may be linked to a lack of VE-Cadherin [112], interacting with VEGF on the cell membrane [112], thus might not have been the optimal cells for this study. Microvascular cells would have been more adapted, ideally as a cell line for the disponibility. However, HUVECs, if described positive to VE-Cadherin [113], were also not reactive to VEGF, thus the reason may be linked to the assay, for example to the Matrigel.

2.2. Mechanical loading of MSCs and their co-culture with T-lymphocytes

Mechanical stimulation of human MSCs and their co-cultures with T-lymphocytes from aged patients was investigated in the same bioreactor as used for the present study. Their paracrine stimulation of tube formation and the regulation of proteins were evaluated *in vitro*. Mechanical stimulation of MSCs alone [90] and in co-cultures with T-lymphocytes increased the paracrine stimulation of tube formation *in vitro*. This was correlated with the up-regulation of MMP-2 by MSCs and their co-culture with T-lymphocytes, but not by T-lymphocytes alone [90, 114]. However, the paracrine stimulation of tube formation of T-lymphocytes alone after 17h incubation with CM was independent of mechanical loading *in vitro*. However, many agglomerates of cells were observed and might indicate the earlier formation and collapse of a tubular network [114].

While MSCs are necessary for the mechano-regulation of angiogenesis, their co-culture with T-lymphocytes had an effect on the expression and mechanical regulation of proteins. Thus, TIMP-2 was mechanically regulated by MSCs [90], but not by T-lymphocytes and their co-culture, as well as the MSC-only control of the co-culture study [114]. Also, VEGF was mechanically under-regulated by co-cultures of MSCs and T-lymphocytes, but not regulated by MSCs alone [90], and not expressed by T-lymphocytes [114]. Interestingly, TGF-β1 was mechanically regulated by MSCs [90], but not by T-lymphocytes or their co-culture [114]. The same was observed in the mechanically loaded haematoma [90].

With these results, the regulation of proteins by the haematoma seems very similar to the regulation pattern of co-cultures of MSCs and T-lymphocytes *in vitro*, so that this co-culture seems a good model for the haematoma.

2.3. Cellular activity is unchanged by mechanical loading

The unchanged cellular activity within the haematoma matrices indicates that the haematoma cell viability is unchanged by mechanical loading, and thus, that an adequate mechanical loading was applied. The application of a too intense mechanical loading could lead to the death of the embedded cells, and so, a decreased cellular activity within the matrices [115]. The hypothesis of a different proliferation rate under mechanical stimulation could also be rejected: the application of mechanical stimulation does not affect the cellular activity in the haematoma. So, a different paracrine stimulation of the haematoma is not to be linked to different cell numbers in the matrices. However, the hypothesis of a counterbalanced apoptosis and proliferation may not be excluded.

2.4. Paracrine stimulation of endothelial cells

Effects of the haematoma

Presented data showed that the soluble fraction of the haematoma stimulates tube formation without affecting the EC numbers *in vitro*. A study of Street et al. has already shown the angiogenic potential of the early haematoma (collected maximally 24h after fracture), but concludes on the toxicity of the haematoma through a cytotoxic potassium level in the haematoma, and this toxicity was not observed in this study. However, the angiogenic potential was confirmed *in vivo* without toxicity where the haematoma was implanted in Matrigel in a mouse model [18]. Here, the effect of the high levels of potassium could be deleted by the dilution of the haematoma in the bioreactor and thus revealing the pro-angiogenic effects of growth factors present in the haematoma and possibly acting physiologically after the potassium levels are normalized. However, our observations of the fresh haematoma on the EC line did not show toxicity. Furthermore, the cultivation of the ECs on Matrigel could bring additional protection of the cells in a more *in vivo*-like environment in the present study. The EC line might also be less sensitive than the HUVECs used by Street et al.

The patients' age and gender did not influence the pro-angiogenic potential of its CM as measured by tube formation. So, age and gender may not be the reason for different healing for different patients groups *in vivo*. However, the older patients are known to present more complications and a longer healing time than younger ones. The age limit was placed at 45 years old to avoid all effects due to the hormonal changes from menopause in the younger patients' group. This was in response to some authors' description of a very pronounced bone mineral density decrease in the years before the beginning of the menopause [116-119]. This would indicate a changed bone homeostasis, and the possibility of a changed bone fracture response. However, the angiogenic potential of fracture haematomas was shown to be conserved in elderly patients [120].

A longer maturation of the haematoma in the patient tended to increase its pro-angiogenic potential. MSCs, known as angiogenesis regulators even in absence of mechanical stimulation [90], are expected to migrate into the fracture area around day three [10]. However, the low number of MSCs present in the haematoma at this stage may not significantly influence the response to mechanical stimulation and the pro-angiogenic potential is probably due to inflammatory cells and to the peak of cytokines around 24h after fracture [9, 14, 15]. However, the reduced number of haematoma samples isolated between 25 and 72h post trauma may have masked a potential correlation.

Effects of the mechanical loading

The application of a cyclic compressive load resembling physiological conditions increased the pro-angiogenic potential of the haematoma samples. Animal experiments have given indirect evidence that mechanical stimulation, angiogenesis and healing outcomes are associated [31, 33, 44, 121, 122]. But in this study, a direct relationship between mechanical stimulation of early components of fracture healing and angiogenesis was demonstrated in an *in vitro* model, and most importantly, with human material. Because the pro-angiogenic signal is paracrine, it may stimulate revascularisation from the surrounding tissues, even though the immediate mechanical environment within the fracture gap may be disruptive.

The mechanically enhanced pro-angiogenic capacity was maintained after removing the mechanical stimulus, indicating persistent cellular changes, such as on a translational or transcriptional level. Thus, a single initial or repetitive intermittent mechanical loading *in vivo* might be sufficient for an angiogenesis augmentation and hence, a possible stimulation of regeneration, in case the patient is able to respond.

Mechanosensitivity of the pro-angiogenic response of the haematoma was not gender-dependant. The higher risk of developing healing problems in risk group patients (aged women) might be either due to other processes than angiogenesis, such as recruitment of progenitor cells and/or changes in their differentiation potential. Also inflammatory response and its regulation (possibly by progenitor cells) might be altered in these groups [26]. However, at least the initial signals seem to be similar between gender and age groups. Therefore, the mechanical loading-associated angiogenesis might not be the key factor in the different healing rates seen for ages and genders in the clinic. Systemic factors, as hormone levels, osteoporosis or general health condition may play a bigger role in the differences in bone healing than the angiogenesis stimulation under mechanical loading.

Aged patients showed a similar increase of pro-angiogenic potential by mechanical stimulation of the haematoma, even if a slight tendency to reduced response to mechanical stimulation was observed in samples from older patients. Aged patients are known to have an increased healing time and higher morbidity compared to younger individuals. One of the possible causes for this is decreased blood vessel formation [11]. Interestingly, a study of bone defect healing in rats proved that age and mechanics act dependently on each other on healing outcome. It appeared that aged animals are unable to adequately respond to mechanical stimuli [25] and show a delayed maturation *in vivo* [123]. Such a dependency was already postulated in former studies [124]. A possible reason of the lack of correlation of

angiogenic stimulation to gender and age could be the timing of investigation, since haematomas were collected during the first hours after trauma, when the phase of inflammation and recruitment of progenitor cells is going on. Although it is assumed that this phase lies the basis for later angiogenesis stimulation by the tremendous release of bioactive molecules, e. g. from platelets after clot formation, recruited cells or activated ECs / progenitor cells, it cannot be ruled out that later phases associated with angiogenesis are actually more relevant for age/gender specific bone regeneration. For example, the responsiveness of ECs to regulators might be altered [24], which is not detected in the used system, as a cell line and HUVECs were used for all the patients.

A longer maturation of the haematoma in the patient did not affect its response to mechanical stimulation. MSCs are expected to migrate into the fracture area around day three [10]. The cyclical mechanical compression of MSCs increased their paracrine stimulation of tube formation *in vitro* [90]. However, the low number of MSCs present in the haematoma at this stage seems to not significantly influence the response of the haematoma to mechanical stimulation.

2.5. Paracrine stimulation of mesenchymal stem cells

The stimulation of ECs by haematomas and their mechanical stimulation were also tested on MSCs, a cell type also present in the haematoma and playing essential roles in fracture repair. Proliferation, migration and differentiation capacities were investigated.

MSC proliferation was higher with the CMs than the expansion medium, but not affected by the haematoma, and tended to be decreased by its mechanical stimulation. The supplementation with FCS did not affect the MSC proliferation, indicating the sufficient amount of FCS from the CM, containing 10% FCS before performance of the bioreactor experiment. The combination of detected GFs and MMPs does not seem to affect the MSC proliferation too much, but the higher concentrations after mechanical loading may have stressed the MSCs too much, leading to the loss of the cells. For example TGF-β1, increased by the haematoma but not mechanically regulated, is known as a proliferation stimulator for MSCs in a dose dependent manner [10, 49, 125].

Next to the proliferation, the paracrine stimulation of the migration of MSCs was tested by application of CMs. MSCs were seeded on filters and stimulated with CMs. MSCs migrated independently of the CM, but more cells migrated through the collagen coated filters than

through uncoated ones. The presence of the collagen, simulating an ECM, might have provided adhesion points that could have enhanced the migration. While MMPs influence the migration of MSCs *in vitro* [91], the mechano-regulated factors seem not to have influenced the MSC migration, potentially because of their insufficient concentration in the CM or a cross-talk between proteins.

One of the major properties of MSCs is their ability to differentiate into the osteogenic, chondrogenic and adipogenic lineages. The influence of the CMs on MSC differentiation was investigated both supplemented or not with the lineage-specific chemicals additions. The detected up-regulation of factors, in particular TGF-β1 (osteogenic [10, 14, 125] and chondrogenic lineage [126]) from the haematoma, VEGF from the haematoma and its tendency to mechanical stimulation (osteogenic lineage [127]) and MMP-2 and -9 (adipogenic lineage [128]), elevated from the haematoma and its mechanical stimulation, were expected to stimulate the differentiations. However, the chondrogenic differentiation was similar for all CMs tested. The pellets cultivated in CM without supplementation showed a lower chondrogenic differentiation than with supplementation. This spontaneous differentiation may be due to the fibrin, but also to the hypoxic conditions in the assay: hypoxia is a major element in the chondrogenic differentiation of MSCs, by secretion of HIF1-α in the medium, stimulating ECM production [129]. Additional collagen X staining could further have characterized the cells as prehypertrophic, an earlier stadium in the chondrogenic differentiation [129, 130].

Unlike the chondrogenic differentiation, unsupplemented CMs did not induce osteogenic differentiation of the MSCs. Apparently, the regulated factors were not present in an high enough concentration to affect the osteogenic differentiation. FGF is essential for osteoblast activation [49] but, as undetectable in the CMs, may have been degraded or retained in the fibrin matrix, and may have not been content of the CM.

Adipogenic differentiation was important with supplementation of the additives, but only the CM from mechanically stimulated haematomas induced an adipogenic differentiation without supplementation. This could be linked to the increased concentration of MMP-2 and MMP-9 in the CM of loaded haematomas.

Thus, differentiation of MSCs was not affected by the CMs of the haematomas and their mechanical stimulation, possibly through a dilution effect of the factors from the haematoma into the CMs, or the too early sampling of the haematomas to induce differentiation *in vitro*.

2.6. Proteins detected in the conditioned medium

The pro-angiogenic effect of the haematoma and its mechanical regulation could be linked to an increased concentration of some angiogenesis regulators measured in the CM.

Growth factors regulation

VEGF was elevated in the CM of haematomas, and tended to be increased by mechanical stimulation in the whole cohort of patients. However, VEGF was regulated in an age-dependent manner, with an unsignificant response for elderly patients, correlated with a slight tendency to a lower angiogenic mechano-response.

VEGF is one of the most potent inducers of angiogenesis, stimulating the proliferation and migration of ECs [131], as well as promoting the mobilization and recruitment of endothelial progenitor cells [132]. VEGF controls a variety of EC functions involved in angiogenesis and protects EC from apoptosis. Multiple stimuli, including hypoxia, cytokines and GFs (in particular bFGF and TGF-β1) control VEGF expression.

Potential cellular sources for mechanoregulated VEGF in the haematoma could be granulocytes, lymphocytes, monocytes [12, 133] or MSCs [90] which were shown to express VEGF and are present in the haematoma [12]. However, MSCs from older patients loaded under the same experimental setting did not change VEGF concentrations [90]. Also, co-cultures of MSCs and T-lymphocytes from older patients down-regulated VEGF concentrations under mechanical stimulation. However, MSCs and T-lymphocytes isolated from younger patients were not investigated.

Our data are in line with Street et al. showing that VEGF is present in excess in the supernatant of human haematomas [18]. These discrepancies between VEGF regulation and angiogenesis induction might be due to different sensitivities and resolutions of the applied assays, but also suggest the involvement of other factors, independently on the patients' age. This lower regulation of VEGF and angiogenesis stimulation could be correlated with the more frequent clinical incidence of delayed fracture healing or pseudoarthrosis in elder patients. VEGF level was already shown to be lower in the plasma of postmenopausal women [134]. The effect of elevated VEGF levels in young, mobilized patients could induce an additional recruitment of endothelial progenitor cells [132]. Interestingly, a study of bone defect healing in rats showed an inadequate response to mechanical stimuli in aged animals [25]. Also, bone formation in older individuals is only induced by greater mechanical stimuli, whereas younger individuals already respond to lower levels [124]. Therefore, it may be that haematoma samples from elderly patients would actually up-regulate VEGF at mechanical conditions greater than those determined to be optimal in *in vivo* studies, which are preferentially performed on young animals.

TGF-β1 was elevated in the CM from haematomas, but independent of the mechanical stimulation. Although TGF-β1 is mostly assumed to be an anti-angiogenic factor by promoting vessel maturation, TGF-β1 concentrations seem to be critical for its effect on ECs. For example, high concentrations (above 1ng/ml) can inhibit EC proliferation and tube formation ability [135, 136], while lower concentrations appear to promote angiogenesis *in vitro* [135]. Indeed, local application of TGF-β1, in combination with IGF-1 in a rat osteotomy model, increased the healing rate and mechanical properties of treated fractures [137]. However, the dual effect of TGF-β1 on EC behaviour possibly causes the contradictory *in vivo* results, as it was also shown to have inhibitory effects on the development of peripheral callus in the presence of axial movements [138]. Besides its role in the angiogenesis regulation, TGF-β1 also shows osteogenic properties [16] and the TGF-β family plays capital roles in the bone fracture healing process [139]. Measured TGF-β1 levels in CM ranged from 373 to 695pg/ml, so that TGF-β1 could act pro-angiogenic and thus contribute to the baseline angiogenic capacity of human haematoma, but does not participate to the increased angiogenesis under mechanical stimulation as not mechanically regulated.
TGF-β1 is expressed by MSCs and increased by mechanical stimulation. However, in co-culture of MSCs with T-lymphocytes, supposed to simulate an early fracture haematoma, TGF-β1 is not regulated by mechanical loading. Thus, the discrepancy between the MSCs alone and the haematoma might be explained. The osteogenic properties of TGF-β1 [16], could further explain its presence in the haematoma.

The haematomas released Ang-1 in the CM, but mechanical stimulation did not influence Ang-1 concentrations. Ang-1 is involved in the maturation of blood vessels and the tightening of cell-cell junctions and in the recruitment of bone-marrow-derived circulating endothelial progenitor cells [132]. The detection of Ang-1 in the haematoma so early after trauma (<72h) is surprising, considering its role in the maturation of blood vessels. However, its role in the recruitment of endothelial progenitor cells may explain its presence in such an early haematoma.
Ang-2 levels were not increased in the CM by the haematoma. Ang-2 can induce sprouting of capillaries in association with VEGF [140], also detected, and may detach smooth muscle cells and loosen the matrix [17]. Ang-2 was detected in the mouse during the whole process of fracture healing on the mRNA level [141]. However, the absence of Ang-2 in the CM of haematomas may be due to a too early time point for the collection of the haematomas [9, 10].

PDGF-bb could not be detected in the CM. However, the median concentration of PDGF-bb measured in fresh haematomas was 2.3ng/ml, above the detection limits of the ELISA (data not shown). The presence of PDGF-bb in the haematoma is supposed to be essential, since it is involved in the recruitment and proliferation of pericytes, and the proliferation of smooth muscle cells [46]. It is also an important chemoattractant for inflammatory cells and MSCs, and haematomas were collected during the period of recruitment of MSCs into the haematoma [142]. The early haematoma is firstly a blood clot and thus, an important amount of platelets are to be expected. PDGF-bb may be released from the haematoma but kept in the fibrin matrix or bound to a protein to make it undetectable by ELISA in the CM.

bFGF was not detected in any CM. However, this GF was still detected in the fresh haematoma at a median concentration of 850pg/ml (data not shown), which is consistent with the literature [17]. FGFs are secreted by monocytes, macrophages and mesenchymal cells in the haematoma, amongst others, and stimulate the proliferation and differentiation of many cells [46], including MSCs. Also, bFGF is released from the EC-basement membranes by MMP-1 and MMP-3 [50]. As in the case of PDGF-bb, bFGF may be released from the haematoma but kept in the fibrin matrix or covalently linked to a bFGF-binding protein [143], possibly making it undetectable in the CM by ELISA.

MMPs and TIMP-2 regulation

MMP-1 was elevated in the haematoma CM and increased by mechanical stimulation. While MMP-1 is undetectable in normal resting tissues, it is expressed by fibroblasts, monocytes and macrophages, but not MSCs [144], during physiological and pathological tissue remodelling *in vivo* [74]. These MMP-1 secreting cells were described in the haematoma [12]. The mechanically induced up-regulation of MMP-1 by early human haematomas may result in an increased degradation of collagen fibrils under mechanical stimulation. MMP-1 is also involved in the release and activation of bFGF, which is an important factor during angiogenesis, and present in the haematoma, as well as in the FCS added during the preparation of bioreactor medium.

MMP-2 was released into the haematoma CM, and elevated by mechanical stimulation. MMP-2 is a key MMP involved in migration, angiogenesis and metastasis [52, 145]. MMP-2 binds to integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$ to loosen the cells from the extracellular ECM [146]. Moreover, MMP-2 is involved in the release and activation of numerous GFs, like VEGF and TGF-β [52]. When ECs are exposed to thrombin, like in the fracture haematoma, MMP-2 activation is induced [50]. Upregulation of MMP-2 by MSCs is observed under mechanical stimulation and co-culture of MSCs and T-lymphocytes [114]. Both kinds of cells are expected in the

haematoma [12]. The presence of MMP-2 in the haematoma and its responsiveness to mechanical stimulation are consistent to the increased tube formation observed because of its role in the cleavage of the ECM and the activation of GFs. Moreover, the MMP-2 could activate ECs through thrombin, present in the haematoma to increase angiogenesis *in vivo*.

MMP-3 was released by haemtomas, but not increased under mechanical stimulation. MMP-3 is involved both in the release of bFGF by the degradation of perlecan from the EC-basement membrane and in the activation of pro-MMP-1 to the fully active form [66] to stimulate angiogenesis, both possible roles in this case. However, mechanical stimulation of the haematoma increased the concentration of MMP-1 in the CM, which may indicate further activation mechanisms of MMP-1 *in vivo*.

MMP-9 levels were increased by the haematoma, and further concentrated after mechanical stimulation. MMP-9 is not expressed by MSCs, but secreted by macrophages [144, 147], which have been described in the human fracture haematoma [12]. MMP-9 is a key MMP in angiogenesis and bone fracture repair. MMP-9 is involved in the activation of MMP-2 and the release and activation of some GFs like VEGF, and enhances ECs growth [50]. Mmp9-/- mice present abnormal skeletal growth plate angiogenesis [50] and fractures non-unions and delayed unions [148]. So, the mechanical stimulation, leading to higher levels of MMP-9, may induce a more successful fracture healing.

MMP-13 was not detected in any CM. However, MMP-13 is expressed by MSCs [91], and could be present in the haematoma. MMP-13 is described later in the bone fracture healing process, involved in the matrix remodelling during the endochondral ossification [149-151]. The role of MMP-13 in the early haematoma is unclear, but seems to be linked to hypoxia: the release of nitric oxide by inflammatory cells induces the up-regulation and activation of MMP-13 by ECs [50]. The possibility of an anchorage of MMP-13 in the matrix seems unlikely, as it could be measured in MSCs cultivated in the same matrix [91]. The investigated haematomas may have been isolated too early after fracture to contain MMP-13.

TIMP-2 was increased by the haematoma, and further enhanced under mechanical stimulation. TIMP-2 is not only a MMP inhibitor; it is also involved in the activation of MMP-2 [52]. TIMP-2 was mechanically regulated by MSCs [52], but not by T-lymphocytes and their co-culture [114]. TIMP-2 inhibits bFGF signaling pathways through association with integrin $\alpha_3\beta_1$ and inhibition of p42/44(MAPK) signaling, thus suppressing the bFGF-stimulated EC

mitogenesis [152] and proliferation [153], and angiogenesis *in vivo*, independently of MMP inhibition [153].

In summary, MMP-1, -2, -9 and TIMP-2, and, for younger patients, VEGF were regulated by mechanical loading. MMPs and TIMP-2 are involved in the activation and / or release of relevant GFs for angiogenesis, for example. This was concomitant to the changed potential of tube formation by mechanical stimulation of the haematoma, thus these factors were tested to stimulate the angiogenic potential of the haematoma.

2.7. Stimulation of the angiogenic potential of the haematoma

The mechano-regulated pro-angiogenic factors were further tested for their potential as angiogenesis stimulators in the haematoma. For this, the appropriate factors and concentrations were tested for tube formation first in CM, then in fresh haematomas. Finally, transfection methods were tested on hMSCs to overexpress the most promising factors.

Preliminary tests to define the most stimulating factors (CM)

VEGF

Application of the active, recombinant human VEGF to the CM of unloaded haematomas decreased the tube formation, both at the regulated concentration and the "standard" concentration for angiogenesis stimulation (15ng/ml). HMEC-1 cells lack the VE-cadherin, interacting with VEGF and a potential explication for a lack of stimulation, but not an inhibition. Two batches of VEG were tested, minimizing the possibility of a degraded VEGF, but the inactivation / degradation of VEGF by some factor in the CM is still possible. The concentration applied on ECs, added to the factors already present in the CM, may be irrelevant to stimulate angiogenesis. Besides, a too high stimulation of EC migration by the applied VEGF might have disturbed the formed / forming networks and thus, result in a decreased tube formation after the 17 hours incubation.
The inhibiting effect of the addition of VEGF in the CM of unloaded haematomas may be linked to the unchanged tube formation with ageing of the patients, while haematomas from younger patients regulate VEGF under mechanical stimulation.

While the supplementation of VEGF in the CM of unloaded haematoma failed to enhance tube formation, the increased tube formation by CM of mechanically loaded haematomas still depends on the VEGFR2 (flk-1) pathway, the angiogenesis signalling receptor for VEGF [146], as shown in the experiments. The relevance of VEGFR2 signalling is not only known

for angiogenesis but also for fracture healing as shown in *in vivo* studies, where the VEGFR2 blockage leads to a decreased blood flow of the fracture ends and non-unions. As VEGFR2 is not only involved in angiogenesis but also in EC migration [47, 131], it may impair the tube formation ability of the ECs.

MMPs and TIMP-2

The supplementation of recombinant MMP-1 and -2 and serial dilutions and concentrations thereof in the CM of unloaded haematomas increased the tube formation, when applied at the up-regulated concentration for MMP-1 ($MMP\text{-}1_{1x}$) and MMP-2 ($MMP\text{-}2_{1x}$), and 10x this concentration for MMP-1 ($MMP\text{-}1_{10x}$), indicating them as relevant factors in the increased tube formation, and leading to a "rescue" of unloaded samples to the pro-angiogenic level of loaded ones. MMP-1 and -2 are known to release and activate many GFs, like bFGF and TGF-β, that also stimulate tube formation. Moreover, matrigel is isolated from a mouse sarcoma, rich in ECM proteins, mainly laminin. MMP-2 and MMP-14 mediate the cleavage of the γ2 chain of laminin 5 and expose a cryptic promigratory site and promotes the migration of normal breast epithelial cells [66], possibly ECs as well.
Surprisingly, supplementation of MMP-9 failed to increase tube formation *in vitro*. However, MMP-9 is not only a pro-angiogenic factor, it also has anti-angiogenic properties, for example by the generation of angiostatin from circulating plasminogen involved in the fibrinolysis [146, 154], and so, present in blood clots [155] like the haematoma, and inhibiting the EC migration on the Matrigel. Furthermore, MMP-9 is involved in the activation of VEGF, which was shown earlier to have detrimental effects on tube formation in Matrigel.
The supplementation of the CM by TIMP-2 inhibited tube formation, probably by inhibition of MMP-2. The increased concentration of TIMP-2 in the CM by mechanical loading seems to have inhibiting effects, and not MMP-2-activating, as hypothesized earlier.

In summary, the supplementation of the CM from unloaded haematomas with MMP-1 and MMP-2 stimulated tube formation of HMECs. Thus, these MMPs in the relevant concentrations will be applied on HMECs in fresh haematomas to quantify their pro-angiogenic stimulation.

Stimulation of fresh haematomas

Pro-angiogenic potential of the fresh haematoma

Tube formation and EC numbers decreased with the concentration of haematoma. However, the EC medium, used as reference and as dilution medium, stimulated the tube formation to a similar extent as the undiluted haematoma. This apparent paradox may be due to hypoxia; the undiluted haematoma actually formed a thick cellular layer on the filter, possibly blocking the pores and so, allowing diffusion of the oxygen along the radius of the well only, leading to a reduced oxygen availability in the centre of the well, under the filter. The dilution of the haematoma thinned the cellular layer on the filters, and then, may have allowed an easier diffusion of the oxygen to the cells. ECs react strongly to hypoxia [17]. The pro-angiogenic factors from the haematoma were simultaneously diluted away, and so stimulate the tube formation and proliferation of the ECs less. A dilution of 1:5 was chosen as a compromise between the risk of hypoxia-induced angiogenesis and the too diluted factors.

This result is in contrast to a study from Street et al., showing a decreased angiogenesis in response to the supernatant of human haematomas (<24h after trauma) and conclude on an inhibitive effect of the haematoma on primary ECs due to elevated potassium levels [18]. In this study the whole haematoma - supernatant and cellular pellet - were applied to an EC line. The different time point and the application of the cellular part of the haematoma to the cells may explain this difference. Also, the haematoma was applied on the ECs on Matrigel, before measuring the cell number, and the Matrigel could have protected the ECs by providing ECM attachment [156]. However, the immortalized ECs could also be less sensitive to stresses.

Stimulation of the pro-angiogenic potential

Fresh haematomas were supplemented with the most stimulating concentrations of MMP-1 and -2 and combinations thereof. While the supplementation of the MMPs and their combination in the serum-free haematoma did not affect the tube formation, the presence of 5% FCS in the dilution medium permitted the pro-angiogenic stimulation by MMP-1_{10x}, possibly due to GFs from the FCS. MMP-1 is involved in the release and activation of bFGF, strongly promoting angiogenesis *in vivo* [50, 74, 157], so one could speculate the implication of the release and activation of bFGF in the shown increased tube formation.

Addition of MMP-2 either decreased or did not affect the tube formation. MMP-2 is involved in the release of VEGF [50]. However, this putative lack of an effect of MMP-2 on the

haematoma through VEGF is not surprising, as the supplementation of recombinant, active VEGF in the CM of unloaded haematoma decreased its pro-angiogenic properties when applied on ECs in the Matrigel assay. MMP-2 is also involved in the release and activation of TGF-β1 [52]. However, TGF-β1 has a concentration-related angiogenic properties with higher concentrations of it inhibiting tube formation. Moreover, MMP-2 can show anti-angiogenic activity as well, cleaving the ectodomain of FGFR1, which, still able to bind to FGF, impairs its function [50]. Thus, MMP-2 effects are mostly pro-angiogenic, but it may act anti-angiogenic as well under certain conditions.

Drug delivery of detected factors

The magnetofection of MSCs presents the advantage to monitor the fate of the cells. SPIONs applied to MSCs resulted in a very low transfection rate only, while the cell line (HeLa) showed high transfection rates. This could prove that the plasmid was at least delivered on the cell surface under the conditions used. The variation of the surface charge of the complexes (N/P ratio) and the concentration of the complexes in the medium did not enhance the transfection rate, thus the method was abandoned. These coated nanoparticles could transfect many cell lines *in vitro*, but primary cells remain challenging.

A well-established and published method for transfection of MSCs is the nucleofection (electroporation). However, the cell survival rate is very low in comparison to the other methods. MSCs are rare cells in the human body, appearing at around 0.001-0.01 % of the bone marrow cells [1], and their role during fracture repair is essential. Thus, the nucleofection method, while successful, showed a too low survival rate *in vitro* to be considered. The loss of 80-90% of the applied MSCs, even if previously expanded *ex vivo*, could impair the bone healing process and the advantage of the application of nucleofected cells could be shed by the low survival rate. Moreover, a very large amount of cells need to be applied in animal studies to show an effect. The potential selection of the cells through the nucleofection method could lead to unwanted side-effects. Furthermore, the application of freshly transfected cells into the fracture gap, a hypoxic, hypoglycaemic and inflamed environment could stress the cells even more and also distort the results by showing side-effects.

To avoid the problems linked to nucleofection and magnetofection methods, a lipofection kit was used on MSCs, but again unsuccessfully. Variations of the amount and concentration of

transfection complexes and presence of FCS did not change the result. Moreover, successful transfections of MSCs using this product were not reported in the literature.

After having ruled out the possibilities of delivery methods, the application of recombinant factors was kept as the *in vitro* proof-of-concept. For possible *in vivo* testings, further delivery methods or an optimized magnetofection should be considered to avoid the major disadvantages of recombinant proteins: the lack of control of their fate, their rapid clearance in the body, and their price. However, transfection would also bring new complications and challenges for further *in vivo* testing, firstly because the method is not FDA-approved.

3. Conclusions

Mechanical stimulation and angiogenesis are linked to the improvement of the healing outcome. With the results presented here, mechanical loading of the haematoma was linked to stimulated angiogenesis through elevated VEGF, MMP-1, -2, -9 and TIMP-2 levels. Furthermore, this project established a method for the stimulation of the pro-angiogenic potential of fresh haematomas, without the application of cyclic mechanical loading. This may improve the healing outcome of immobilized patients for example due to multiple traumas. However, the transfection of MSCs for over-expression of stimulating factors was unsuccessful and recombinant factors were used instead. The pro-angiogenic potential of fresh haematomas could be increased by the application of MMP-1 in presence of FCS, possibly through the activation of bFGF from the serum.

It is commonly accepted that MSCs are the key players in fracture healing. However, the results obtained in this project, different from the MSCs', indicate that other cell types than MSCs are also implied in angiogenesis regulation by the haematoma, and thus one could speculate that healing necessitates the collaboration of other cell types. Co-cultures of T-lymphocytes and MSCs from older patients showed similarities with the response of whole haematomas, but further knowledge from younger patients should be collected.

Mechanical stimulation seems to show different VEGF responses in different age categories of the patient and so, from a therapeutic point of view, there may be age-specific needs for fixation systems to better support bone regeneration in elderly patients.

Chapter 5: Perspectives

In this project, the pro-angiogenic potential of the haematoma and its response to mechanical stimulation were analyzed and increased *in vitro* through the application of the mechano-regulated MMP-1. However, some points remained unclear and would deserve further investigation.

On the side of the CM composition, the quantification of the fragments of the ECM released by the haematoma stimulated by MMP-1 may improve the knowledge of the mechanisms of the increased angiogenesis in the haematoma, and potentially bring further investigation directions in the haematoma. COMP and Hydroxyproline may be ECM fragments present in the CM.

On the cellular side, this project used an EC line and HUVECs, but the response of physiologically more relevant ECs to the haematoma should be investigated. Currently, the isolation and culture of human microvascular ECs from human fat tissue are established in the Cell Therapy group of the Julius Wolff Institute and their paracrine response to the haematoma would be very interesting.

Co-cultures of cells present in the early haematoma may increase the understanding of the mechanisms of this increased angiogenic stimulation by mechanical stimulation, and possibly, improve the knowledge of the regulated factors. As already mentioned, a project implying a co-culture of T-lymphocytes and MSCs under mechanical stimulation showed an increased paracrine pro-angiogenic stimulation *in vitro* [114]. RNA analysis of such cultivated cells may give clues about the regulators that would be relevant to be tested on fresh haematomas *in vitro*.

The role of MSCs in the haematomas should be further investigated. For this, a co-culture of MSCs with fresh haematomas, or the stimulation of MSCs with mechano-regulated factors, may deliver further answers. A further study with later haematomas may optimize the efficacy of therapy during the inflammation phase, a destructive environment by a timely more adequate stimulation of the haematoma. However, inflammation plays an essential role in the bone healing.

More comprehensive data about the way nanoparticles can enter the cells may improve the opportunities to transfect primary cells, and in particular human MSCs and then, permit the use of SPIONs the transfection of the patient's isolated and expanded MSCs, to overcome the disadvantages of the use of recombinant factors: rapid clearance, probable cleavage of the factor, and high costs. However, MSC transfection remains a long-term goal of the

project, as the FDA has not approved the use of SPIONs yet. The MSCs could also be transduced using lentiviral vectors to over-express relevant factors, and possibly test their functional behaviour. In a short term perspective, the use of recombinant proteins, possibly in association with biomaterials, could permit the establishment of a delivery system. Then, a small animal study to enhance the fracture healing outcome could be contemplated, possibly in the rat model, with the possibility to control the mechanical stresses, where animals are known to heal differently with advancing age [25, 158].

Once the key regulating factor(s) (MMP-1, bFGF), the relevant delivery method and time window are identified, the relationship between a potentially increased angiogenesis and an improved fracture healing should be assessed on human material, but more importantly, as GFs and MMPs are linked to the growth of tumors, the absence of cancer formation should be checked. Moreover, the different response of the aged patients compared to younger ones should be kept in mind and considered further in basic research and from a therapeutic view.

Annexes

Annex I: Paracrine stimulation of the chondrogenic differentiation of MSCs by haematomas and their mechanical stimulation

Collagen II stainings

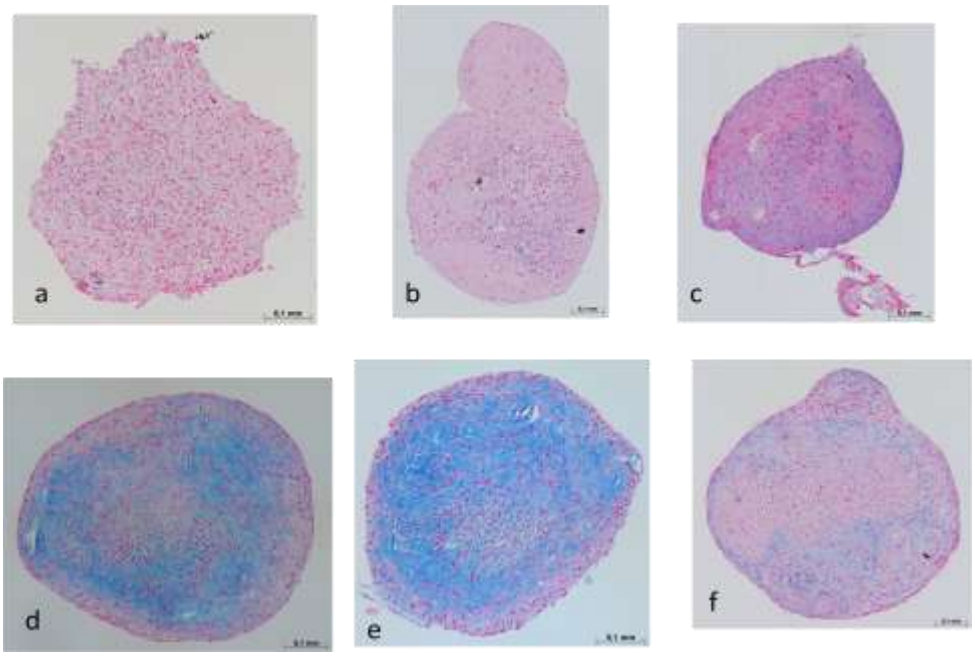

Figure 55: Alcian Blue staining of the pellets stimulated with unloaded haematomas. a: unloaded haematoma, b: unloaded control, c: negative control. d: supplemented unloaded haematoma, e: supplemented unloaded control, f: positive control.

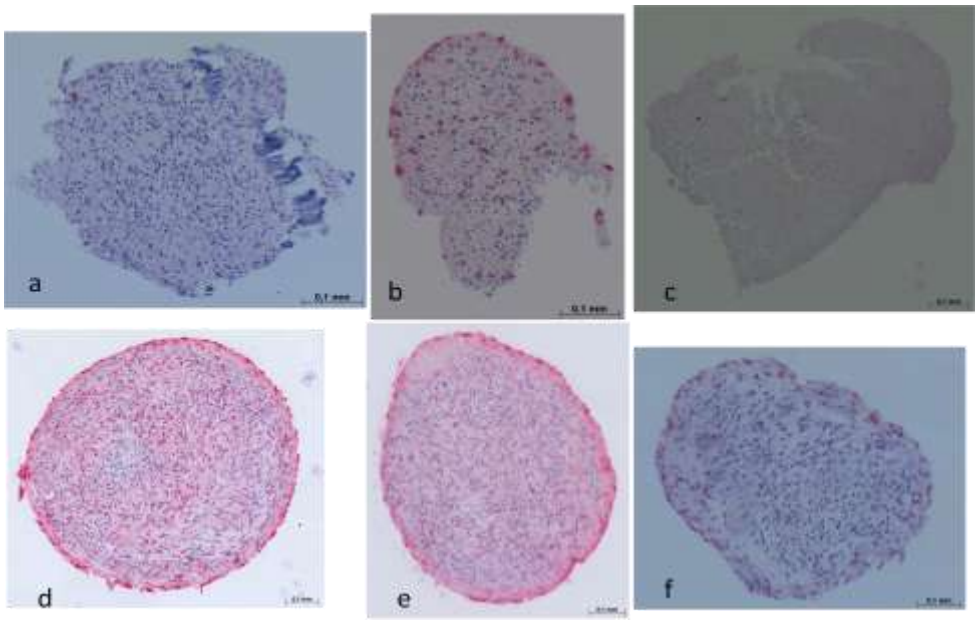

Figure 56: Collagen II staining of the pellets stimulated with unloaded haematomas. a: unloaded haematoma, b: unloaded control, c: negative control. d: supplemented unloaded haematoma, e: supplemented unloaded control, f: positive control.

Alcian Blue

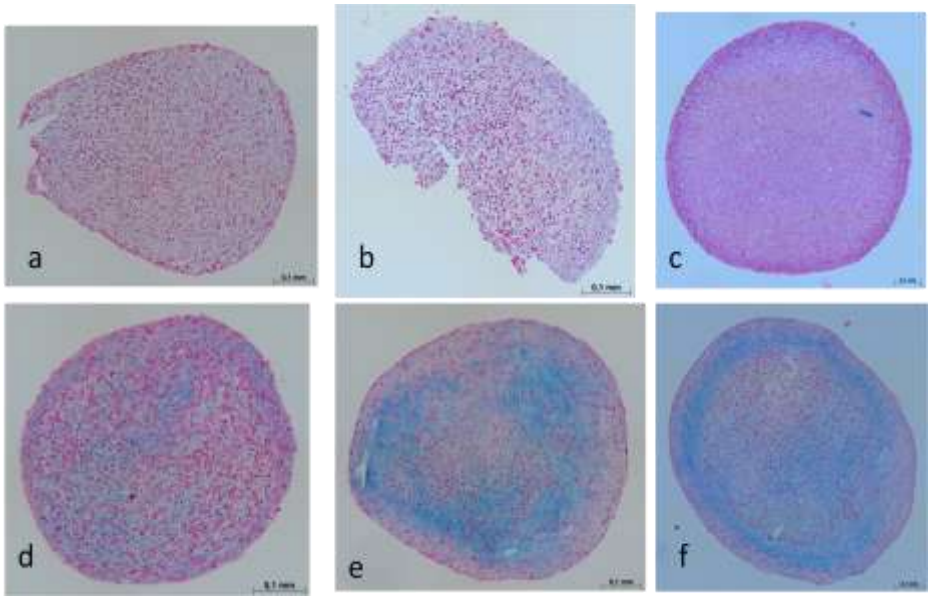

Figure 57: Alcian Blue staining of the pellets stimulated with loaded and unloaded haematomas. a: loaded haematoma, b: unloaded haematoma, c: negative control. d: supplemented loaded haematoma, e: supplemented unloaded haematoma, f: positive control.

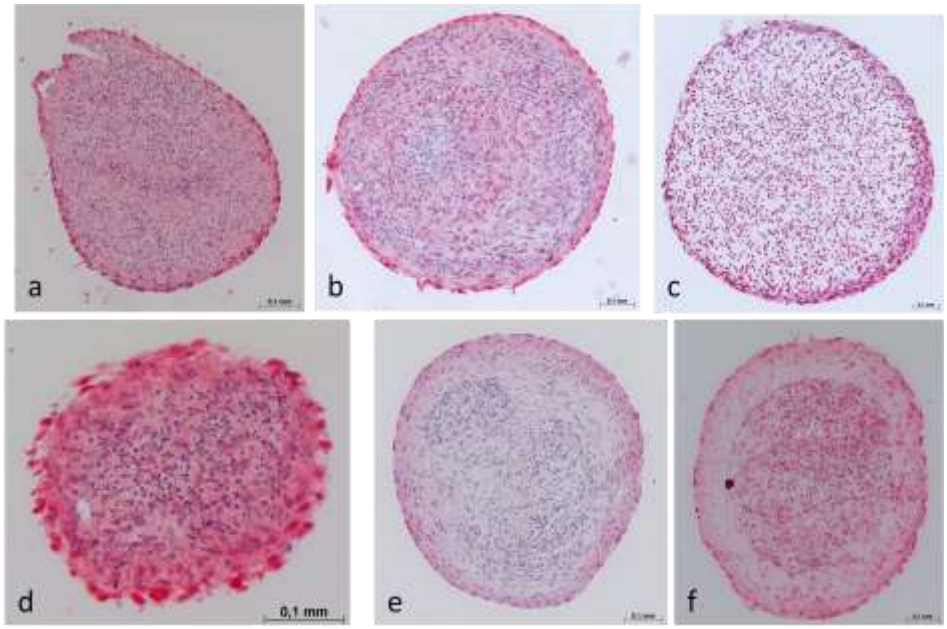

Figure 58: Collagen II staining of the pellets stimulated with loaded and unloaded haematomas. a: loaded haematoma, b: unloaded haematoma, c: negative control. d: supplemented loaded haematoma, e: supplemented unloaded haematoma, f: positive control.

Annex II: Stimulation of the tube formation of HMECs by supplementation of MMPs and TIMP-2 in the CM of unloaded haematomas

		Tube formation		EC numbers	
		$ratio_{supplemented / unsupplemented}$	p-value	$ratio_{supplemented / unsupplemented}$	p-value
MMP-1	1/10	0.84	0.343	0.99	0.893
	1/2	0.81	0.143	1.02	0.345
	1	1.32	0.068	0.99	0.893
	2	0.80	0.092	1.04	0.043
	10	1.25	0.030	1.04	0.500
MMP-2	1/10	0.74	0.015	1.01	0.893
	1/2	0.69	0.069	1.00	0.500
	1	1.17	0.080	0.98	0.225
	2	0.65	0.041	1.02	0.345
	10	0.72	0.032	0.99	0.686
MMP-9	1/10	0.65	0.004	0.98	0.686
	1/2	0.76	0.035	0.98	0.500
	1	0.79	0.981	1.00	0.893
	2	0.59	0.007	0.95	0.043
	10	0.72	0.072	1.02	0.225
TIMP-2	1/10	0.73	0.027	0.99	0.500
	1/2	0.64	0.023	0.96	0.138
	1	0.66	0.006	0.99	0.500
	2	0.53	0.005	0.93	0.043
	10	0.65	0.004	0.99	0.068

Annex III: Stimulation of the tube formation of HMECs by supplementation of MMPs and TIMP-2 in the fresh haematomas

With adjunction of serum

			Tube formation		EC numbers	
Serum	MMP-1	MMP-2	ratio supplemented / unsupplemented	p-value	ratio supplemented / unsupplemented	p-value
+	1x	0	1.01	0.892	1.00	1.000
+	10x	0	1.21	0.043	0.99	0.715
+	0	1x	0.98	0.416	1.05	0.138
+	1x	1x	1.01	0.893	0.99	0.686
+	10x	1x	1.11	1.000	1.04	0.588

Without adjunction of serum

			Tube formation		EC numbers	
Serum	MMP-1	MMP-2	ratio supplemented / unsupplemented	p-value	ratio supplemented / unsupplemented	p-value
-	1x	0	0.88	0.225	0.88	0.343
-	10x	0	1.03	0.893	1.03	0.138
-	0	1x	0.99	0.893	0.99	0.080
-	1x	1x	1.11	0.225	1.11	0.345
-	10x	1x	0.72	0.080	0.72	0.786

Annex IV: Abbreviations

aFGF	Acidic fibroblast growth factor
Ang	Angiopoietin
AB	Alamar Blue
AP	Alkaline phosphatase
AR	Alizarin red
bFGF	Basic fibroblast growth factor
BMP	Bone morphogenic protein
BSA	Bovine serum albumine
CM	Conditioned medium
CTGF	Connective tissue growth factor
DMEM	Dulbecco's modified essential medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DO	Distraction osteotomy
EC	Endothelial cell
ECM	Extracellular matrix
ECGM2	Endothelial cells growth medium 2
EDTA	Ethylene-diamine-tetra-acetic acid
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescent-activated cell sorting
FCS	Foetal calf serum
FGF	Fibroblast growth factor
FGFR1	Fibroblast growth factor receptor 1
GF	Growth factor
h	Human
HE	Haematoxillin - Eosin
HIF-1α	Hypoxia-inducible factor 1, alpha subunit
HMEC -1	Human microvascular endothelial cell line-1
HSC	Hematopoietic stem cell
HUVEC	Human umbillical cord vascular endothelial cells
IBMX	3-Isobutyl-1-methy xanthine
IGF	Insulin-like growth factor
ITS	Insulin-transferrin-sodium selenite
Kan	Kanamycine sulfate
MSC	Mesenchymal stem cell

Mmp	Gene encoding for MMP
MMP	Matrix metalloprotease
MRI	Magnetic resonance imaging
mRNA	Messenger ribonucleic acid
MSC	Mesenchymal stem cell
MT-MMP	Membrane type-MMP
Nrp1	Neuropilin-1
NSAIDs	Non-steroidal anti-inflammatory drugs
OD	Optical density
P/S	Penicillin / Streptomycin
PBS	Phosphate-buffered saline
PDGF	Platelet-derived growth factor
PFA	Paraformaldehyde
PEI	Polyethylenimine
R	Recombinant
RNA	Ribonucleic acid
ROR	Red Oil Red
RT	Room temperature
SDS	Sodium dodecyl sulfate
SMC	Smooth muscle cell
SPIONs	Superparamagnetic iron oxide nanoparticles
TGF	Transforming growth factor
TGF-β	Transforming growth factor-beta
TIMP	Tissue inhibitor of metalloprotease
TNF-α	Tumor necrosis factor-α
VEGF	Vascular endothelial growth factor
VEGFR2	VEGF receptor 2

Annex V: Figures list

Annex VI: Tables list

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