# AUTOCRINE AND PARACRINE EFFECTS OF MECHANICALLY REGULATED MATRIX METALLOPROTEASES IN HUMAN MESENCHYMAL STEM CELLS

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## List of abbreviations

| | |
|---|---|
| Ang | angiopoietin |
| AP-1 | activator protein-1 |
| AP | alkaline phosphatase |
| AR | alizarin red |
| BFA | brefeldin A |
| bFGF | basic fibroblast growth factor |
| BMPs | bone morphogenic proteins |
| $Ca^{2+}$ | calcium ion |
| cDNA | complementary DNA |
| CEA | carcinoembryonic antigen-related adhesion molecule |
| CM | conditioned medium |
| Col | collagenase |
| DCC | deleted in colorectal carcinoma |
| DMEM | Dulbecco's modified essential medium |
| DmMMP | drosophila melanogaster MMPs |
| DNA | deoxyribonucleic acid |
| ECM | extracellular matrix |
| ECs | endothelial cells |
| EDTA | ethylene-diamine-tetra-acetic acid |
| EGF | epidermal growth factor |
| Egr | early growth response protein |
| ELAM-1/E-selectin | endothelial leukocyte adhesion molecule |
| ELISA | enzyme-linked immunosorbent assay |
| ERK | extracellular signal-regulated kinases |
| FACS | fluorescent-activated cell sorting |
| FCS | foetal calf serum |
| FGF | fibroblast growth factor |
| FGFR | fibroblast growth factor receptor |
| *fur* | furin gene |
| GAPDH | glyceraldehyde 3-phosphate dehydrogenase |
| GPI | glycosylphosphatidylinositol |
| G-protein | guanine nucleotide-binding protein |
| GSCs | drosophila germ stem cells |
| HMEC | human dermal microvascular epithelial cell |
| HMG | high mobility group |
| HSCs | haematopoietic stem cells |
| ICAM | intracellular adhesion molecule |
| ICSM | intracellular signalling molecules |
| IGF | insulin-like growth factor |
| IGF-BP | insulin-like growth factor binding protein |
| IgG | immunoglobulin G |
| IL | interleukin |
| JAK | janus kinase |
| JNK | c-jun-N-terminal kinase |
| $K^+$ | potassium ion |
| MAPK | mitogen-activated protein kinase |
| MMPs | matrix metalloproteases |
| MSCs | mesenchymal stem cells |

| | |
|---|---|
| MT-MMP | membrane type-MMP |
| $Na^+$ | sodium ion |
| NCAM | neural cell adhesion molecule |
| NF-κB | nuclear factor-kappa B |
| NRCAM | neuronal cell adhesion molecule |
| o/n | overnight |
| PAI | plasminogen activator inhibitor |
| PBS | phosphate-buffered saline |
| PDGF | platelet-derived growth factor |
| PEA | polyoma enhancer A-binding protein |
| PECAM | platelet/endothelial cell adhesion molecule |
| PPARγ | peroxisome proliferator-activated receptor gamma |
| mRNA | messenger ribonucleic acid |
| RS cells | recycling stem cells |
| RTK | receptor tyrosine kinase |
| Runx | runt-related transcription factor |
| SAC | stretch-activated ion channels |
| SDF | stromal cell-derived factor |
| SDS | sodium dodecyl sulfate |
| Sox | sry-related HMG box |
| SPRE | stromelysin platelet-derived growth factor-responsive element |
| STAT | signal transducers and activator of transcription |
| SV | simian vacuolating virus |
| TGF-β | transforming growth factor-beta |
| TIMPs | tissue inhibitors of metalloproteases |
| TNF-α | tumour necrosis factor-alpha |
| tPA | plasminogen activator, tissue |
| Tris-HCl | tris(hydroxymethyl)aminomethane-hydrochloride |
| uPA | plasminogen activator, urokinase |
| uPAR | urokinase-type plasminogen activator receptor |
| UTP | uridine triphosphate |
| VCAM | vascular cell adhesion molecule |
| VE-cadherin | vascular endothelial-cadherin |
| VEGF | vascular endothelial growth factor |

# 1 General introduction

This chapter provides a comprehensive review of the literature relevant to the presented work. Firstly, the anatomy and physiology of bone as well as the bone healing process are described. This is followed by detailed information about angiogenesis and mesenchymal stem cells (MSCs) - key players in bone regeneration processes - and their cellular microenvironment. Next, the role of matrix metalloproteases (MMPs) in the regulation of matrix remodelling and their impact on paracrine and autocrine signalling processes are discussed, along with the mechanism for MMP mechano-regulation. It is then summarised, how mechanical loading known to occur during bone regeneration is transmitted to the cells and thereby affects the gene/protein expression of MSCs. Finally, the problem and hypothesis as well as the aims and outline of the thesis are presented.

## 1.1 Structure and function of bone

The skeleton is a highly specialised form of connective tissue. It has mechanical and protective, but also metabolic functions. Thereby, bone serves as the main store and supply of inorganic ions, such as calcium and phosphorus in form of hydroxyapatite, which in turn impact metabolic processes in the whole body[1].

Morphologically, two types of bone need to be distinguished. Cortical bone is characterised by densely packed concentric layers, which impart mechanical strength, due to their inhomogenous orientation. Trabecular bone has a loosely organised porous matrix[2]. Its structure and strength align with the principle stress trajectories. This adaptive response of trabecular bone is generally referred to as Wolff's law established in 1892[3]. In long bones, such as the human femur, trabecular bone is primarily found in the epiphyseal and metaphyseal region, whereas cortical bone is present in the diaphysis (Figure 1-1)[4].

Histologically, bone is divided into woven bone and lamellar bone. Woven bone, which is randomly arranged without relation to lines of stress, and irregular and variable in thickness, is chiefly present during bone development and regeneration. In lamellar bone, mineralized bone matrix is deposited in concentric layers (lamellae) around a central vascular channel. Both bone types harbour a network of tiny channels pervaded by blood vessels and nerves, allowing for nutrition and cell-to-cell signalling[5, 6].

The principal constituents of bone are organic matrix comprising 95% type I collagen, 5% proteoglycans and non-collagenous proteins as well as various cell types, including osteoblasts, osteoclasts, osteocytes and bone lining cells. A key role of these cells is bone remodelling the well-ordered resorption and deposition of bone matrix[6]. For example, osteoblasts regulate the mineralization and osteoclasts the resorption of bone. Osteocytes are located within the concentric lamellae and have a role in calcium and phosphorus homoeostasis[6].

The external surface of diaphyseal bone is covered by the periosteum and the inner by the endosteum. The periosteum consists of an outer fibrous layer and an inner cambial layer. The outer fibrous layer is composed of fibroblasts, collagen and elastin fibers that partly form the insertions of tendons, ligaments and muscles. The inner cambial layer is highly cellular containing mesenchymal stem cells (MSCs), osteoprogenitor cells, osteoblasts, fibroblasts, microvasculature and sympathetic nerves and is involved in bone formation[7, 8].

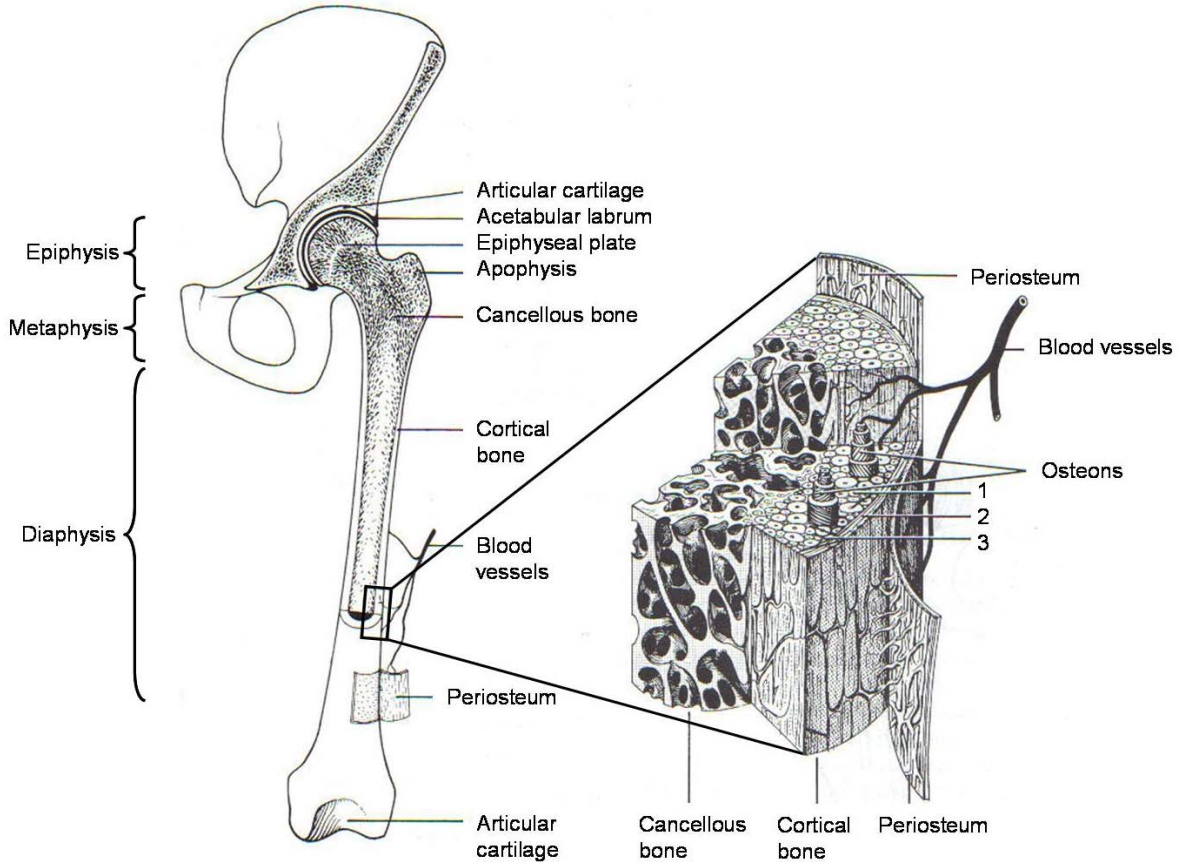



Figure 1-1 *Overall composition of long bone.* Left: Anatomic terms are displayed. Right: Section of diaphyseal bone and appropriate terms is shown. 1: haversian canal, 2: outer general lamellae, 3: intermediate lamellae (reproduced and modified from Rohen, 1998[4]).

The endosteum lines the inner cavity of bone and contains osteoblasts as well as stromal cells that provide stem cells with essential biological factors[9].

The bone marrow, which fills the cavity, harbours blood vessels and is a habitat for haematopoietic stem cells (HSCs) and MSCs. As such, it functions as a primary site for haematopoiesis the process of blood cell production, including the differentiation of HSCs to all classes of blood cells[10].

## 1.2 Course of bone healing

Bone regeneration is characterised by extensive matrix synthesis and resorption including the re-establishment of vascular supply, formation of woven bone and its remodelling into lamellar bone[11-13]. These processes are both spatially and temporally regulated and recapitulate aspects of embryonic skeletal development[14]. Four partly overlapping stages can be distinguished[12, 14]:

**Inflammatory Phase**

Injury of the bone results in a disruption of bone matrix, blood vessels and surrounding soft tissues (Figure 1-2 A)[11]. Bleeding of these tissues into the fracture gap and release of bone marrow give rise to the initial haematoma[11, 12]. The disruption of blood vessels creates a hypoxic state around the fracture gap[11]. Following haematoma formation, inflammation leads to the secretion of a variety of cytokines, growth factors and extracellular matrix (ECM) proteins, which in turn stimulate the recruitment and proliferation of cells that are essential to the repair process[11, 15, 16]. For example, platelets are deposited at the site of injury and secrete, among other proteins, platelet-derived growth factor (PDGF) and transforming growth factor-beta (TGF-β), which induce chemotaxis of acute inflammatory cells and MSCs[11, 16]. Interleukin-1 and -6 (IL-1, IL-6) and tumour necrosis factor-alpha (TNF-α), secreted by inflammatory cells, have a chemotactic effect on other cells in inflammation and on the recruitment of mesenchymal cells[16]. Furthermore, the release of pro-angiogenic factors such as angiopoietin (Ang) stimulate angiogenesis, the establishment of new blood vessels from existing vasculature[17]. Subsequent to these processes, MSCs differentiate into functional mesenchymal cells including chondrocytes and osteoblasts (Figure 1-2 B)[12, 15].

**Endochondral Ossification**

In mechanically less stable regions, endochondral bone formation occurs, involving the formation of a cartilage template. Specifically, differentiation of progenitor cells into chondrocytes and subsequent proliferation and secretion of biological factors by these chondrocytes results in the production of an abundant cartilagenous matrix, including collagen II[16]. This soft callus spans the fracture gap[18]. Similarly to the processes known to take place in the growth plate during development, chondrocytes undergo hypertrophy and chondrocyte-mediated mineralization[12, 19]. As vasculature begins to invade, the hypertrophic chondrocytes are removed and woven bone formation occurs after recruitment of osteoprogenitor cells (Figure 1-2 C)[16, 19].

**Intramembranous Ossification**

A few millimetres proximal and distal from the fracture site, a hard callus of fully mineralized woven bone is formed[12, 16]. To accomplish this, osteoblasts from the cortical bone and osteoprogenitors derived from the periosteum proliferate and deposit new bone matrix onto existing bone surfaces[16].

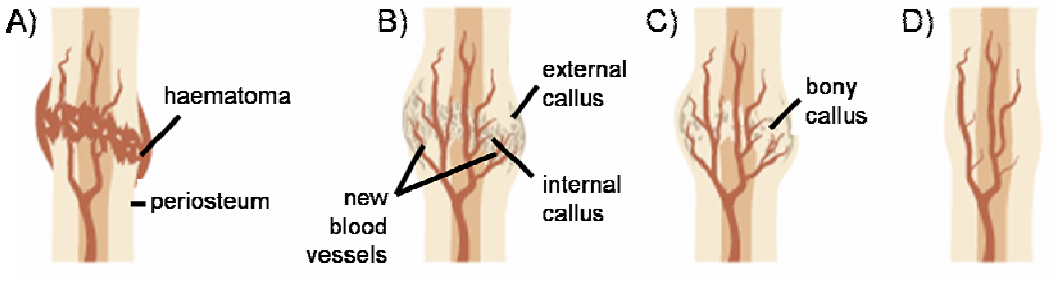



Figure 1-2 *The course of bone healing.* A) Disruption of the bone matrix, blood vessels and surrounding soft tissues results in the formation of the haematoma. B) Secretion of a variety of cytokines, growth factors and ECM proteins stimulates the recruitment and proliferation of cells involved in the repair process and angiogenesis. Thereafter, progenitor cells differentiate into cells of the mesenchymal lineage. C) Subsequent to the differentiation of MSCs, soft callus and hard callus are formed. D) Newly formed woven bone is remodelled to lamellar bone and refined in thickness (reproduced and modified from Carano and Filvaroff, 2003[20]).

**Remodelling phase**

During this stage, newly formed woven bone is converted to lamellar bone (Figure 1-2 D). Firstly, osteoclasts begin to erode a cavity, referred to as cutting cone. Osteoblasts migrate into this cone and deposit a layer of bone matrix in apposition to the existing surface. These mechanisms restore the original structure and biomechanical competence of the injured bone[6].

## 1.3 Pathophysiology of delayed and non healing

Non healing (also non-union) of a fracture is defined as the cessation of bone repair processes without bone union and is generally declared between 6 and 8 months following injury[21]. Delayed healing (also delayed union), as a diagnosis using distinct clinical and radiological signs, implies that the restorative process for a specific fracture was not completed within the interval expected for the repair[21, 22].

Numerous factors have an impact on the rate and quality of the healing process, including mechanical parameters, but also the patient's biological competence to heal. Inadequate immobilisation has been among the first factors implicated in delayed healing[22]. For example, an excessive interfragmentary compression of 50% strain results in compromised healing compared to smaller movements in a sheep model, due to the disruption of newly formed blood vessels[23, 24]. In contrast, improved healing was associated with the application of a controlled movement of approximately 30% between the bone fragments[23]. Limited motion at the fracture site can be achieved by the application of treatments including plaster casts, external fixators, intramedullary nailing or plate fixation[22, 25]. Particularly, the external fixator allows for adjustment of the motion between the fracture ends by alteration of the fixator

stiffness, the diameter and number of bone screws and the distance between the fixator connecting rod and the bone[25]. However, in the case of a failure in the patient's activation of local cellular cascades, e.g. due to systemic factors, such as age, controlled micro-movement at the defect site might be not sufficient to stimulate bone healing[22]. To augment biological processes, bone marrow grafts or implants containing recombinant bone morphogenic proteins (BMPs) are applied and gene therapies, including cell-based gene therapies (cells transfected with the target gene) are being investigated[6, 26, 27]. Autologous bone grafts obtained from illiac crest are currently the gold standard for the treatment of bone defects[28]. However, they are only successful in small defects and have been associated with extended operative time, increased pain and donor site morbidity[29, 30]. Application of BMP-2 is an alternative, since it has been shown to result in improved healing, less pain and fewer infections compared to bone grafts[29, 30]. In case of recombinant BMP-2 delivery, large doses of this protein are required to augment healing and there are still risks of overdose[31-33]. In this respect, application of recombinant BMP-2 to stimulate spinal fusion in humans has been shown to lead to several adverse effects, such as edema, ectopic bone formation and bone resorption in the graft area[32, 33]. To improve the delivery of these proteins, gene- and cell-based therapies, as well as cell-based gene therapies are under investigation[30, 31]. Indeed, there is evidence that cell-mediated gene therapy is more efficient than recombinant BMPs[30, 31]. In contrast to gene therapies, applications of cell-based therapies and cell-based gene therapies benefit from the cellular characteristics of MSCs, e.g. differentiation, homing and immunosuppression potential[30, 34]. However, gene and cell-based gene therapies are not jet established as standards in the clinic, due to concerns over potential risks, such as mutagenesis or immune response, and costs, as well as a deficit of knowledge regarding the cells' functional behaviour after application[30, 35].

## 1.4 Angiogenesis

Angiogenesis describes new vessel formation from existing blood vessels that either sprout capillary buds (sprouting), become divided by periendothelial cells (intussusception) or are separated by transendothelial cell bridges (bridging)[36]. Of these, sprouting is the most common.

The process of angiogenic sprouting involves proteolytic degradation of the ECM, endothelial cell (EC) proliferation, migration, lumen formation and vessel maturation (Figure 1-3)[37].

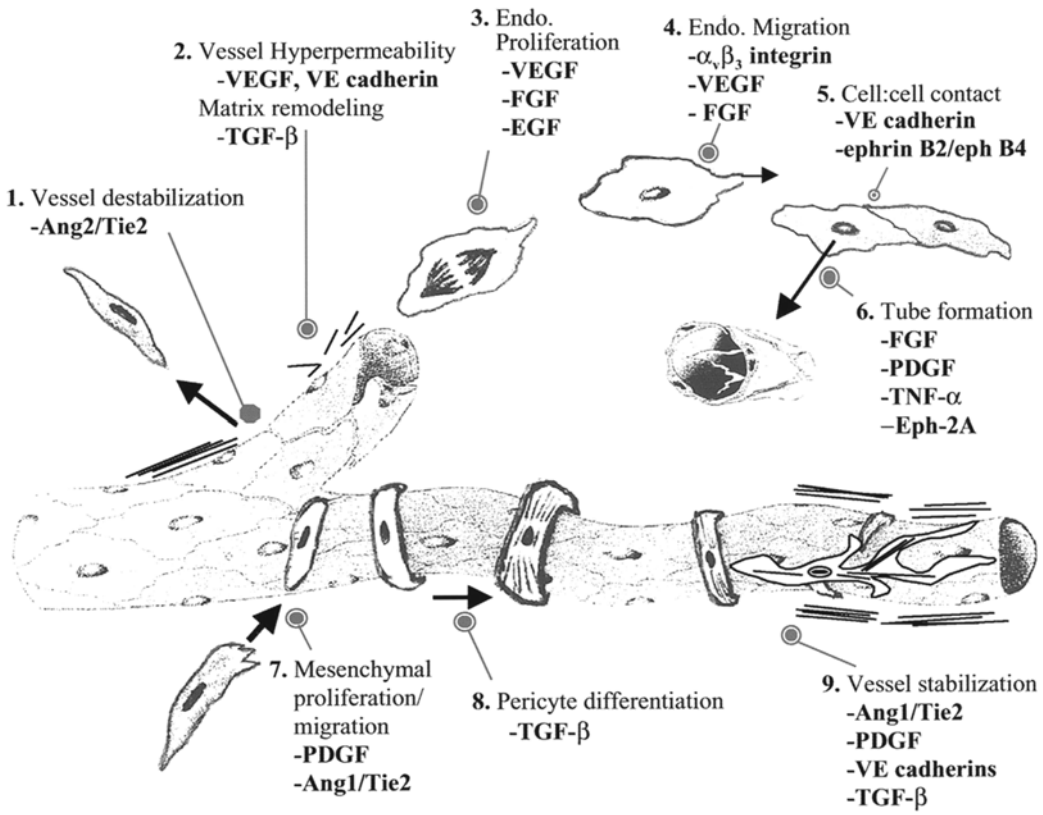



Figure 1-3 *Molecular regulation of angiogenesis.* Removal of pericytes from the endothelium and destabilisation (1) is followed by vessel hyperpermeability and degradation of the EC basement membrane (2). ECs proliferate (3), migrate (4), arrest in a monolayer (5) and form tube-like structures through which blood can flow (6). Progenitor cells proliferate and migrate along the new vessel (7) and differentiate into mature pericytes (8). Establishment of EC quiescence, strengthening of cell-cell contacts and elaboration of new matrix stabilise the new vessel (9) (reproduced from Papetti and Herman, 2002[38]).

Sprouting is controlled by the balance between pro-angiogenic signals, such as the vascular endothelial growth factor (VEGF), and factors that promote quiescence, including certain ECM molecules or VEGF inhibitors such as angiostatin[37, 39].

In conditions that favour angiogenesis such as hypoxia, pericytes detach from the endothelium and vessels become destabilised by angiogenic factors such as Ang-2[38, 39]. Furthermore, vasodilation (relaxation of the muscular vessel wall) and vascular permeability increase in response to VEGF, allowing the extravasion of ECM proteins[38, 39].

This is followed by degradation of the EC basement membrane and remodelling of the ECM, accomplished by proteases such as matrix metalloproteases (MMPs)[37, 39, 40]. ECM remodelling contributes to the release of growth factors including basic fibroblast growth factor (bFGF) that foster the migration and proliferation of ECs[38, 39]. After this, ECs arrest in a monolayer and form a tube-like structure, which involves the fusion of vacuoles[37, 38]. Thinning of ECs and fusion of vessels allow an increase in tube diameter and length[39]. The newly formed

blood vessels are stabilised by pericytes that proliferate and migrate along the new vessel[38]. Interaction between ECs and smooth muscle cells further contributes to vessel stabilisation, a process for which TGF-β and Ang-1 are needed[38].

## 1.5 Mesenchymal stem cells

MSCs were first described in 1968 by Friedenstein and colleagues, who showed that bone marrow stroma contains cells that have significant proliferative capacity and are able to form bone[41]. The name MSC was introduced by Caplan and colleagues in 1990 and is based on the MSCs' developmental origin in the mesenchyme[42]. However, it has been demonstrated that MSCs are not only able to differentiate into mesenchymal cells, such as osteoblasts, adipocytes and chondrocytes, but also into non-mesenchymal cells including ECs and neural cells[15, 43, 44].

For *in vitro* cultivation, MSCs are typically isolated from the mononuclear layer of the bone marrow after separation by density gradient centrifugation and subsequently by adherence to cell culture plastic[45]. However, these cells have been also obtained from sources such as muscle, fat, umbilical cord blood, liver and spleen[46-48]. The resulting cell population is morphologically heterogeneous, containing a major population of large and moderately granular cells, referred to as mature MSCs, and a minor population of small and agranular cells, referred to as recycling stem cells or RS-1 cells[45]. After short-term cultivation, RS-1 cells give rise to a new population of rapidly growing small and densely granular cells (RS-2). These cells decrease in numbers during passaging until they have disappeared. At this point in time, mature MSCs and RS-1 cells rapidly expand[45]. To distinguish MSCs from non-MSCs, their potential to differentiate into the osteogenic, adipogenic and chondrogenic lineage and their cell surface marker expression pattern is investigated[46, 48, 49]. This pattern includes the cell surface markers CD105 (SH2), CD73 (SH3/4), CD44 and CD90 (Thy-1), as well as the adhesion molecule CD106 (VCAM-1)[50], none of which is by itself specific for MSCs. Furthermore, the absence of the haematopoetic markers CD45 and CD34 is used to characterise MSCs[50].

The function of MSCs is chiefly influenced by their surrounding ECM[51]. The ECM maintains the tissue architecture, acts as a ligand for cellular adhesion receptors such as integrins, and provides signalling molecules, including growth factors and growth factor-binding proteins, to control cellular behaviour[51, 52]. Interaction of cells with the ECM modulates signalling cascades that control cell growth, differentiation, survival and morphogenesis, and therefore

changes in the microenvironment are able to affect these processes[53]. For example, differentiation can be initiated by binding of growth factors, such as TGF-β, to transmembrane serine/threonine kinase receptors that signal via the TGF-β/Smad pathway, which consequently regulates genes expression in the nucleus[54]. Transcription factors known to be essential to MSC differentiation are runt-related transcription factor-2 (Runx-2) for osteogenesis, peroxisome proliferator-activated receptor gamma2 (PPARγ2) for adipogenesis and Sry-related high mobility group (HMG) box9 (Sox9) for chondrogenesis[47, 55, 56].

MSCs are thought to contribute crucially to bone regeneration processes, since they do not only differentiate into various mesenchymal cells, but also home to injury sites and secrete bioactive factors[15, 16, 57]. Secretion of proteins by MSCs might be particulary important for the re-establishment of the vascular supply during musculoskeletal regeneration[12, 14, 58]. For example, MSCs facilitate wound healing through stimulation of angiogenesis, with a concurrent up-regulation of the pro-angiogenic factors VEGF and Ang-1[59]. Moreover, in response to mechanical loading, the pro-angiogenic capacity of MSCs is even enhanced and levels of secreted bFGF and TGF-β are elevated[60].

Until tissue regeneration or natural physiological turnover, stem cells in general are thought to reside in special tissue microenvironments, or niches, which keep them in a quiescent state[61, 62]. These niches have been extensively studied for drosophila germ stem cells (GSCs) and HSCs[61-63]. Results proved that direct physical interaction with non-stem cell neighbours, such as osteoblasts, contributes to the regulation of stem cell activity[61, 62]. The location and lineage commitment of MSCs are much less characterised[49, 62]. Supported by the knowledge that osteoblasts provide biological factors and adhesive properties to maintain HSC viability, a recent study suggested that MSCs, as osteoblast progenitors, reside in close proximity to the HSC niche[49, 64]. Furthermore, the existence of a perivascular niche in all tissues was recently suggested; this would allow the rapid release of MSCs when required for regeneration[57]. However, direct evidence for the existence of an MSC niche remains scarce to date.

Due to their healing-promoting properties and their great expansion potential *in vitro*, MSCs are an attractive cell source for cell therapy and tissue engineering[65, 66]. Indeed, the use of the mononuclear fraction of the bone marrow, containing MSCs, has been investigated in clinical trials[27, 67, 68]. For example, implantation of autologous bone marrow cells into an osteonecrotic femoral head significantly reduced the necrotic area compared to the untreated group[67]. Furthermore, transplantation of allogenic bone marrow into children with osteogenesis

imperfecta, via intravenous infusion, led to an increase in total body mineral content and reduced frequencies of bone fracture[69].

## 1.6 Matrix metalloproteases

MMPs belong to a family of 25 endoproteinases and are classified according to their partly overlapping substrate specifities, their sequence similarities and domain organisation (Table 1, Figure 1-4 A)[70, 71].

Table 1: *Overview of MMP family members and their ECM substrate specificities* (reproduced from Vincenti et al., 2001[71]).

| MMP family member | Common names | Substrates |
|---|---|---|
| MMP-1 | Collagenase-1 | Collagens I, II, III, VI, X, gelatins, aggrecan, entactin |
| MMP-2 | 72 kDa Gelatinase, Gelatinase A | Gelatins, collagens I,IV, V, VII, X, XI, fibronectin, laminin, aggrecan, elastin, large tenascin C, vitronectin, ß-amyloid protein precursor |
| MMP-3 | Stromelysin-1 | Aggrecan, gelatins, fibronectin, laminin, collagen III, IV, IX, X, large tenascin C, vitronectin |
| MMP-7 | Matrilysin, Pump | Aggrecan, fibronectin, laminin, collagen IV, elastin, entactin, small tenascin C, vitronectin |
| MMP-8 | Collagenase-2, Neutrophil Collagenase | Collagens I, II, III, aggrecan |
| MMP-9 | 92 kDa Gelatinase, Gelatinase B | Gelatins, collagens IV, V, XIV, aggrecan, elastin, entactin, vitronectin |
| MMP-10 | Stromelysin-2 | Aggrecan, fibronectin, laminin, collagen IV |
| MMP-11 | Stromelysin-3 | Fibronectin, laminin, collagen IV, aggrecan, gelatins |
| MMP-12 | Metalloelastase | Elastin |
| MMP-13 | Collagenase-3 | Collagens I, II, III |
| MMP-14 | Membrane-type-1-MMP | Collagens I, II, III, fibronectin, laminin, vitronectin, proteoglycan, ProMMP-2, ProMMP-13 |
| MMP-15 | Membrane-type-2-MMP | Not known |
| MMP-16 | Membrane-type-3-MMP | ProMMP-2 |
| MMP-17 | Membrane-type-4-MMP | Not known |
| MMP-18 | Collagenase-4, *Xenopus* Collagenase | Collagen I |
| MMP-19 | | Not Known |
| MMP-20 | Enamelysin | Amelogenin (The major tooth enamel matrix protein) |

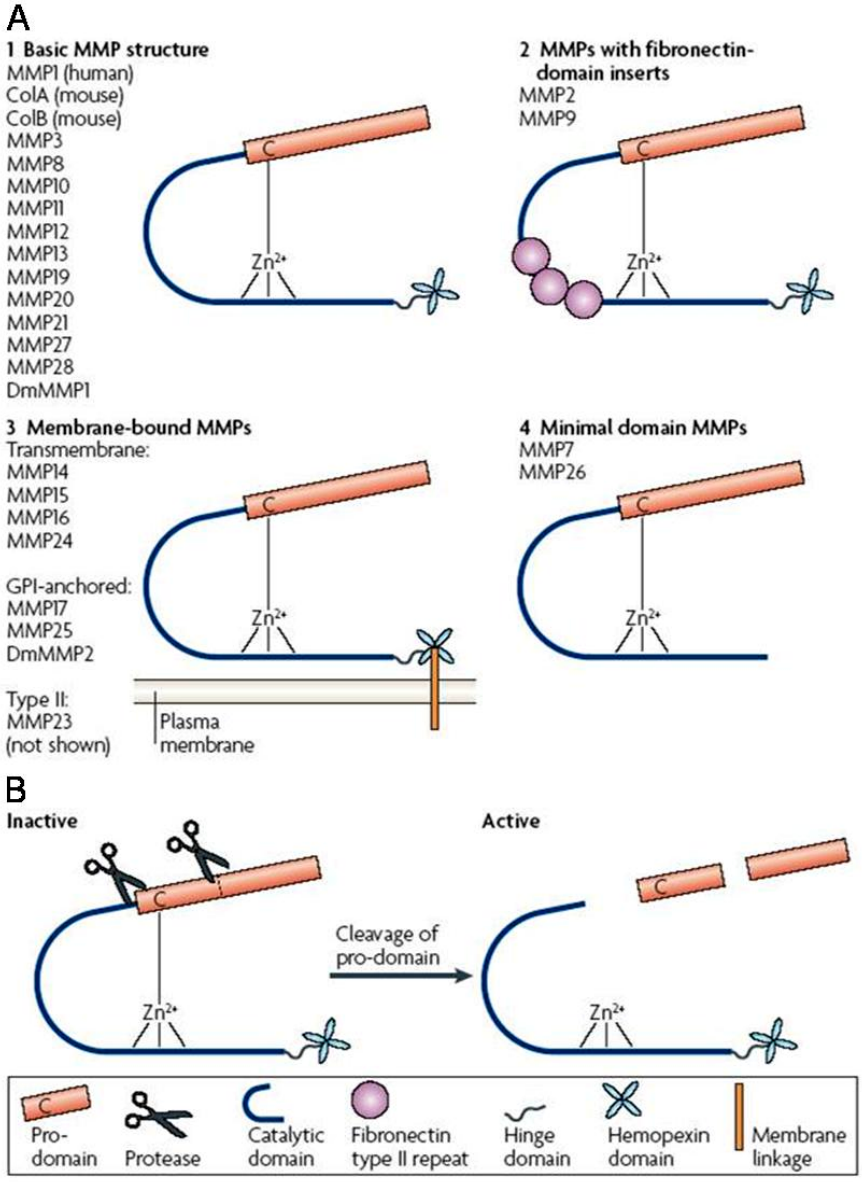



Figure 1-4 *Schematic structure of MMPs*. A) The basic structure of most MMPs consists of a pro-peptide, catalytic domain, hinge region and hemopexin domain. Pro-domain: coordinates the zinc-ion in the catalytic domain; catalytic domain: is needed for substrate binding and cleavage; hinge region: connects catalytic domain and hemopexin domain; hemopexin domain: interacts with the C-terminal domain of TIMPs and accomplishes the binding of certain substrates; fibronectin type II repeats: important for an effective cleavage of type IV collagen, elastin and gelatinase (known in MMP-2 and -9); membrane linkage: enables binding to the cell membrane (known in e.g. MMP-14). B) Removal of the pro-domain is accomplished by cleavage within the domain and between the pro-domain and the catalytic domain, allowing a water molecule to bind to the zinc atom. Col: collagenase; GPI: glycosylphosphatidylinositol; DmMMP: drosophila melanogaster MMP (reproduced from Page-McCaw et al., 2007[72]).

MMPs are synthesised with an autoinhibitory pro-domain that coordinates the zinc ion bound to the catalytic domain, preventing its involvement in catalysis (Figure 1-4 B)[72-74]. Cleavage of the pro-domain results in MMP activation.

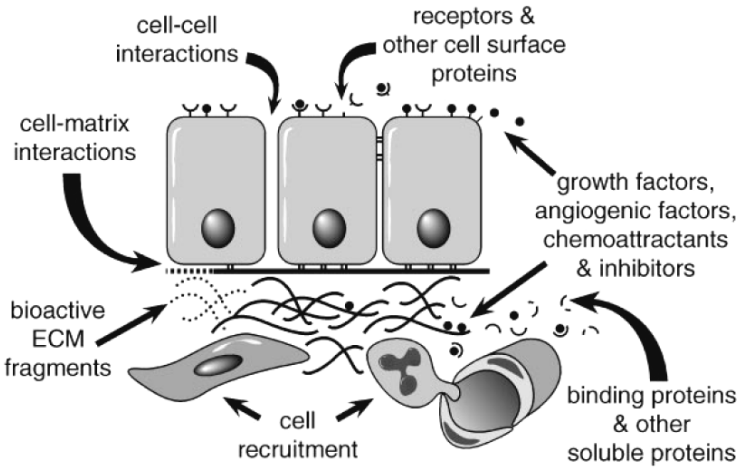



Figure 1-5 *Potential mechanisms of MMP-mediated cellular signalling.* MMPs are able to release ECM fragments (e.g. collagen), disrupt cell-matrix interactions or release growth factors (e.g. TGF-β) and growth factor-binding proteins (e.g. insulin-like growth factor binding protein) from the ECM. Furthermore, cell-cell adhesion proteins (e.g. E-cadherin), and cell surface receptors (e.g. fibroblast growth factor receptor-1 (FGFR-1)) are MMP substrates and can therefore also be involved in the regulation of the cellular microenvironment (reproduced from Sternlicht and Werb, 2001[75]).

MMPs are able to cleave virtually all components of the ECM, and also other substrates, such as growth factor-binding proteins or latent growth factors. Thereby, MMPs regulate ECM composition and cell-matrix interactions, and consequently cell functions such as morphology, movement, growth, differentiation and survival[75] (Figure 1-5). For example, collagen IV cleavage by MMP-2 and -9 exposes a cryptic epitope that is known to stimulate angiogenesis. Release of growth factors such as VEGF and TGF-β by MMPs including MMP-2 and MMP-9 is known to stimulate both cell proliferation and angiogenesis[76, 77].

The cleavage site specificity of MMPs is primarily defined by a structure of the catalytic domain close to the zinc atom, the so-called "S1"-pocket, differing in size and shape among MMPs[70, 74]. The S1-pocket interacts with the cleavage site of MMP substrates, which is in general a peptide bond before a residue with a hydrophobic side chain (e.g. leucine, methionine or tyrosine). However, other substrate contact sites, such as the hemopexin domain, are also involved in the cleavage site specificity[70].

Since uncontrolled proteolysis can generate pathological conditions, MMP function must be spatially and temporally regulated. This can be accomplished at transcriptional and post-transcriptional levels, as well as post-translationally, via MMP controlled activation, inhibition and cell surface localisation (Figure 1-6).

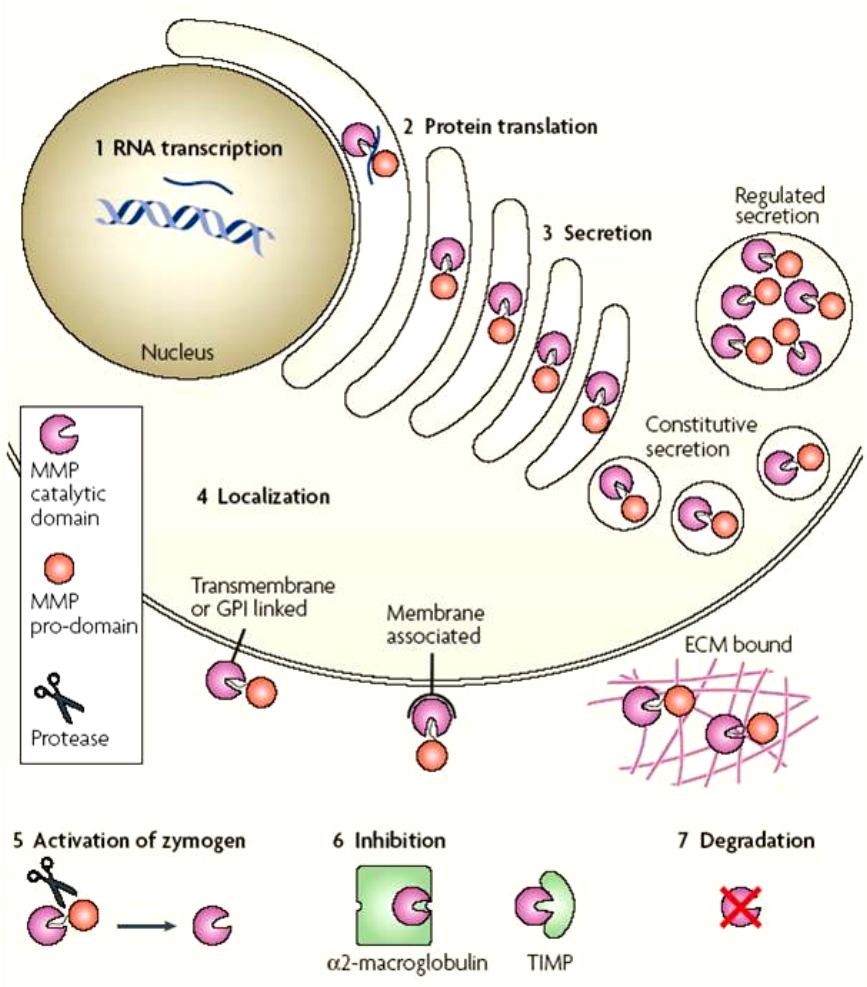



Figure 1-6 *Regulation of extracellular proteolysis.* Besides regulation of RNA transcription (1) and protein translation (2), MMPs can be regulated by an altered secretion (3), their subcellular or extracellular localisation (4) and by activation of the zymogen (5). Extracellularly, suppression by inhibitors such as TIMPs or α2-macroglobulin (6) and protease degradation (7) also regulate the action of MMPs (reproduced from Page-McCaw et al., 2007[72]).

For example, activated MMPs are inhibited by tissue inhibitors of metalloproteases (TIMP 1-4), which are a family of glycoproteins that reversibly inhibit MMPs in a 1:1 stoichiometric manner[75]. The inhibitory activity of TIMPs seems to reside in their N-terminal domain slotting into the active site of MMPs, whereas the C-terminus influences enzyme-inhibitor binding[74, 75]. Besides locally acting TIMPs also other endogenous MMP inhibitors have been found, such as the plasma protein, α2-macroglobulin. This protein seems to be the major inhibitor of MMPs in tissue fluids[75].

MMP/TIMP transcription is regulated by extracellular signals including growth factors, matrix proteins or cytokines[71, 78]. Signalling pathways known to be involved in transcriptional activation of MMPs/TIMPs are the nuclear factor-kappa B (NF-κB) pathway, leading to an activation of the NF-κB transcription factor. Also, mitogen-activated protein kinases (MAPK) can be activated in response to growth factor binding. In particular, the extracellular signal-regulated kinases (ERK) pathway activates Jun/Fos transcription factors that bind activator protein-1 (AP-1) and phosphorylate ETS family transcription factors, which in turn target polyoma enhancer A-binding protein (PEA-3) sites in MMP promotors. Moreover, the c-jun-N-terminal kinase (JNK) pathway can be activated and initiates MMP transcription by phosphorylation of AP-1 subunits. Finally, the janus-kinase-signal transducers and activator of transcription (JAK-STAT) pathway is involved in MMP transcription in response to growth factors[71]. However, the binding sites typically present in the promoters of inducible MMPs and TIMPs, such as AP-1 or PEA-3 sites, are also able to interact with the promoter elements of other transcription factors[71].

Less well-characterised are the mechanisms of post-transcriptional or post-translational regulation of MMPs. Several studies have provided evidence for the involvement of growth factors (e.g. TGF-β or PDGF) in post-transcriptional regulation, leading to a stabilisation or destabilisation of MMP transcripts[71].

Post-translational mechanisms of MMP regulation can involve the modulation of the secretory pathway or of protein stability[79, 80]. Depending on the cell type, this could be mediated by specialised secretory granules known to occur in granulocytes (cells of the immune system) or by secretory lysosomes, which serve as both a degradative and a secretory compartment and occur, amongst other cells, in osteoblasts[81]. For example, MMP-8 and -9, synthesised by granulocytes in the bone marrow, are stored in circulating neutrophils. These MMPs are released following neutrophil activation by inflammatory mediators[82]. Also, localising cell-MMP interactions, such as binding of MMPs to cell surface proteins, might regulate MMP activity. Pro-enzyme activation or substrate binding to proteases can thereby be altered[73].

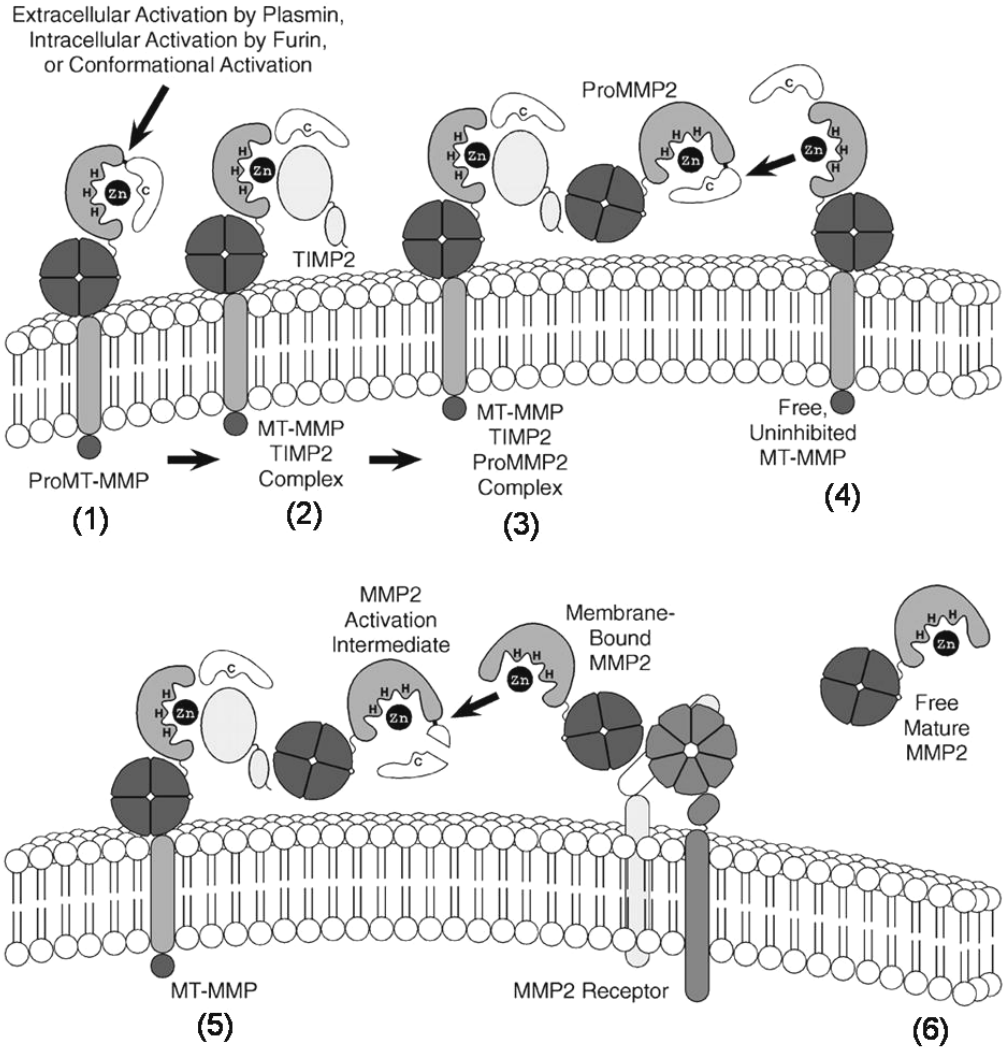



Figure 1-7 *Cell surface activation of MMP-2.* At the cell surface, previously activated MMP-14 (MT-MMP) binds TIMP-2 and thereby inhibits its activity (1-2). Binding of secreted pro-MMP-2 to the C-terminal domain of TIMP-2 and formation of a trimolecular complex enables MMP-14 to partially activate proMMP-2 (3-5). Full activation of MMP-2 is accomplished by a separate MMP-2 molecule that acts on the cell surface (6) (reproduced and modified from Sternlicht and Werb, 2001[75]).

A well-described mechanism of MMP regulation is MMP-2 activation involving MMP-14 and TIMP-2 (Figure 1-7)[70, 75]. Here, an extracellular ternary complex is assembled, in which TIMP-2 is presumed to localise pro-MMP-2 adjacent to MMP-14[83]. Subsequently, MMP-14 cleaves pro-MMP-2, resulting in the release and activation of MMP-2[84, 85]. Active MMP-14 is in turn generated intracellularly by the endoprotease furin[75, 86].

The regulation of MMPs, allowing for controlled ECM remodelling, suggests their functional involvement in bone development and regeneration processes. Indeed, a stage-specific expression of several MMPs, such as MMP-2, -9 and -13, has been demonstrated during bone healing[14, 87]. Furthermore, effects on bone morphology have been described for MMP-9, -13 and -14 knock out mice and, for MMP-2 and -13, in human individuals with loss of function mutations[87-90]. For example, deletion of the MMP-9 gene in mice led to fractures with delayed healing or non-union, caused by the persistent production of cartilage at the site of injury[91]. These complications seem to be due to the limited bioavailability of VEGF caused by the lack of MMP-9 activity[72]. Additionally, the involvement of MMPs in the regulation of stem cells was, for example, demonstrated in MMP-9 knock out mice displaying an impaired release of the Kit-Ligand, as well as impaired HSC motility[92].

## 1.7 Cellular mechanotransduction

As previously mentioned, bone regeneration is not only affected by biochemical stimuli, but also by mechanical stimuli. Particularly, the early phase of bone healing is highly dependent upon the surrounding mechanical environment[93-96]. In this respect, it has been shown that the gene expression and function of MSCs is affected by mechanical signals such as compressive loading[97-99]. For example, MSCs respond with an altered gene expression pattern for ECM proteins involving MMPs[98, 99]. Furthermore, mechanical loading of MSCs seems to affect processes such as osteogenic differentiation and paracrine angiogenesis stimulation[60, 100-102].

Various mechanisms have been proposed for how these mechano-sensitive cells detect mechanical loading and convert it into biochemical signals (Figure 1-8). Stimuli can be transduced via stretch-activated ion channels, cell adhesion molecules (e.g. integrins or cadherins), and also by guanine nucleotide-binding protein-(G-protein-)coupled receptors and growth factor receptor tyrosine kinases (RTK)[103]. For example, stretch-activated ion-channels are able to induce a rapid internal ion transient, e.g. of $Ca^{2+}$, which in turn regulate cellular processes such as growth or differentiation[103]. In many cases, a connection from a membrane-spanning receptor to the cytoskeleton and nucleus in a tensegrity unit may transmit the signal[104]. Furthermore, transmembrane-spanning integrin subunits are known to unite the ECM with actin-binding proteins, including talin and the focal adhesion kinases.

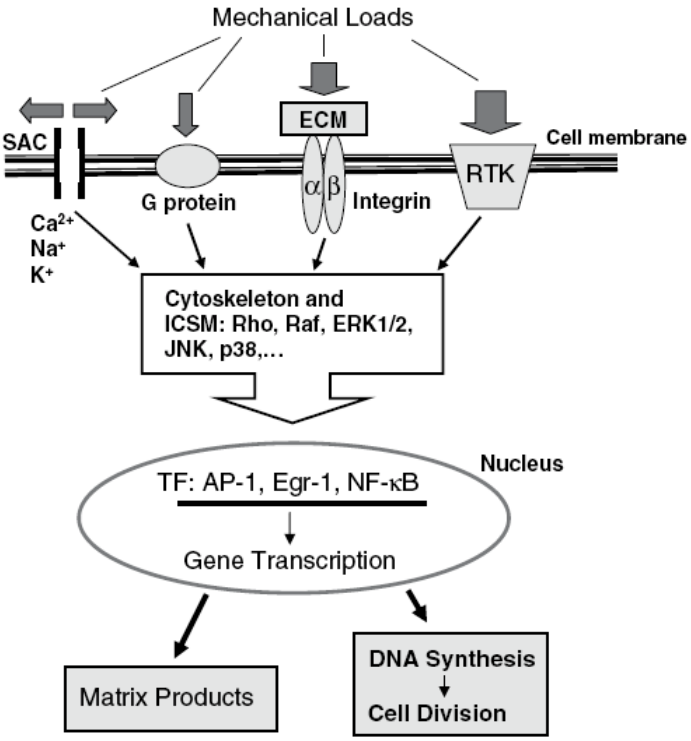



Figure 1-8 *Mechanisms of cellular mechanotransduction.* Major cellular components involved include the cytoskeleton, stretch-activated ion channels (SAC), G proteins, integrins and RTK. Activation of intracellular signalling molecules (ICSM) leads to the rapid transmission of signals into the nucleus and to the activation of specific promoter elements. Transcription of downstream genes results, for example, in cell proliferation or protein secretion (reproduced from Wang et al., 2001[104]).

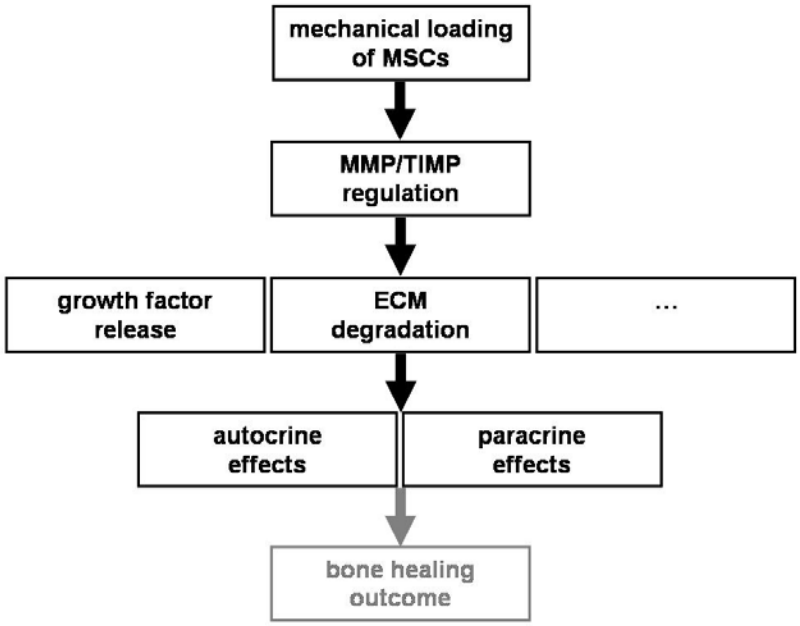
This induces an assembly of actin filaments and higher order structures such as stress fibers and focal adhesions. Activation of these complexes initiates subsequent signalling cascades including ERK, JNK or JAK-STAT, known to contribute also to MMP gene transcription[71, 103]. Furthermore, since there is evidence for a transactivation between integrins and other receptors (e.g. RTK), loading might act synergistically with ligand-activated receptors to drive certain pathways[105].

## 1.8 Problem and hypothesis

The increasing knowledge of the molecular biology of bone regeneration has demonstrated that delayed bone healing is not only a result of inappropriate stability, but also of impeded cellular responses to biochemical and mechanical stimuli; hence, approaches for biological augmentation are being investigated. This may involve local and systemic application of biologically active agents, but also cell-based therapies[26, 27, 30]. Although mesenchymal stem cells (MSCs) are, due to their contribution to the healing outcome and their easy expansion *in vitro,* a promising tool for bone defect treatments, the application of MSCs in bone is to date limited[34, 35].

To expand/optimise the application of MSCs for bone defects, it is of interest to gain further insights into their functional behaviour in response to healing-supportive mechanical conditions, and to identify proteins that might regulate the transduction of mechanical signals into the biological processes of healing. Since matrix metalloproteases (MMPs) and tissue inhibitors of metalloproteases (TIMPs) are critically involved in matrix remodelling, such as by ECM degradation and growth factor release, and are mechanically regulated in MSCs, these proteins might provide a link between mechanical signals and MSC function.

Therefore, it is hypothesised that mechanical regulation of MMPs/TIMPs in MSCs stimulates processes important for bone regeneration in an autocrine and paracrine manner. Results of this study could form the basis for the rational design of MSC-based therapies.



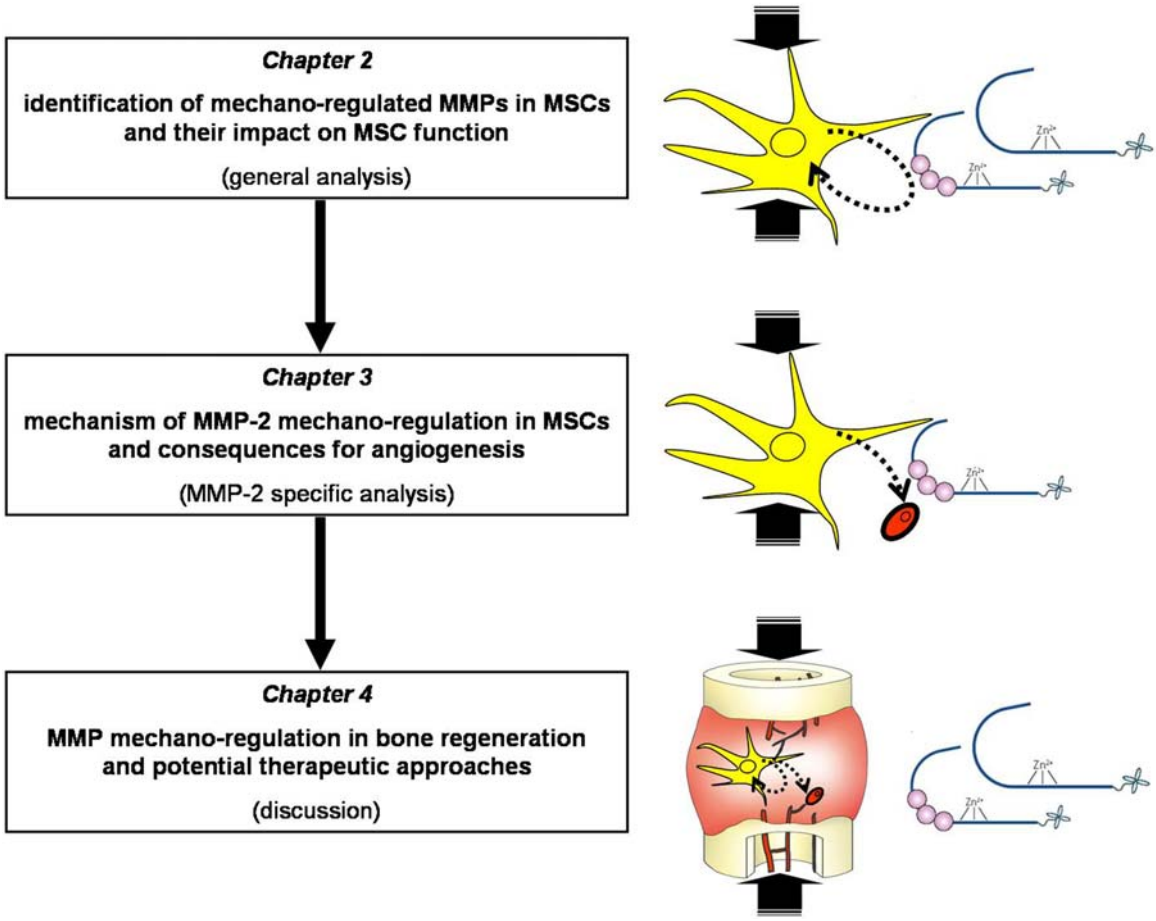
## 1.9 Aims and outline of the thesis

The aims of this project are therefore

to investigate whether mechanical loading of MSCs stimulates MMP gene/protein expression and activity, and whether these mechanically regulated MMPs impact the function of MSCs *(Chapter 2)*.

to analyse one of the MMP candidates identified in *Chapter 2* in more detail for its mechano-regulation *in vitro*, the underlying mechanism of mechano-regulation as well as its paracrine impact on angiogenesis stimulation *(Chapter 3)*.

to bring together the knowledge of MMP mechano-regulation presented in *Chapters 2 and 3* and to discuss its potential pathways, along with consequences of MMPs for cells and processes involved in bone regeneration. Based on the results obtained within this study, potential strategies for the development of therapeutic approaches will further be discussed *(Chapter 4).*



# 2 MMP activity links mechanical stimulus and MSC behaviour

In this chapter, the contribution of MMPs to MSC behaviour is shown by conducting migration, proliferation and differentiation assays in the presence of broad spectrum MMP inhibitors. Furthermore, the expression profile of MMPs/TIMPs at mRNA/protein level in MSCs is shown. The effect of mechanical loading on MMP protein expression levels and activity in MSCs is demonstrated by employing a bioreactor system that simulates the early phase of bone healing. Finally, the contribution of the mechanically up-regulated MMPs to MSC function is again evidenced by addition of specific MMP inhibitors to the functional assays.

## *Original article:*

## *"Matrix metalloprotease activity is an essential link between mechanical stimulus and mesenchymal stem cell behavior"*

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# 3 MMP-2 in angiogenesis stimulation by mechanically loaded MSCs

In this chapter, MMP-2 is investigated in detail for its mechano-regulation in MSCs, as well as potential consequences of MMP-2 enhancement for angiogenesis. Initially, the effect of small and large interfragmentary movements on MMP-2 activity is shown in early haematomas. This is followed by an analysis of MMP-2 mechano-regulation in MSCs *in vitro*, as well as investigation of the underlying mechanism. Finally, an MMP-2 blocking antibody and recombinant MMP-2 and TIMP-2 are examined in tube formation assays, to evidence the paracine impact of increased MMP-2 levels on angiogenesis.

***partially submitted as***

***Control of pro-MMP-2 levels and activation of MMP-2 via the furin/MMP-14/TIMP-2 axis contribute concurrently to the pro-angiogenic effects of mechanically stimulated mesenchymal stem cells, 2008***

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## 3.1 Introduction

Angiogenesis is a critical step for bone regeneration and responsible for re-establishing the blood supply, resorption of necrotic tissue and mobilisation of different cell types including mesenchymal cells[134-136]. During bone regeneration, angiogenesis is strongly influenced by mechanical loading[24, 96, 137]. In this regard, limited micro-movement of the fracture ends is crucial for uneventful healing, whereas exessive loading disrupts newly formed vessels[23, 24].

MSCs are likely to play a crucial role in angiogenesis during musculoskeletal regeneration, due to their ability to home to sites of injury and to generate a pro-angiogenic microenvironment[12, 14, 58]. Indeed, the healing-promoting properties of MSCs were recently attributed to their paracrine effects, such as those on ECs, rather than merely to their ability to differentiate into functional mesenchymal cells[58, 59, 138, 139].

Several members of MMP protein family are well-established regulators of angiogenesis[72]. Particularly, secreted MMP-2 and cell membrane-anchored MMP-14 are expressed by MSCs and have pro-angiogenic properties[72, 140, 141]. Proteolysis of the ECM by these MMPs, for example, results in the release of pro-angiogenic factors such as VEGF and TGF-β, which in turn are able to induce EC migration and proliferation[142]. MMP-14 and -2 also show phenotypes related to bone remodelling in knock out mice and in humans with loss of function mutations, respectively[89, 90]. For example, mice deficient in MMP-14 develop multiple bone abnormalities after birth and die by 3-12 weeks, due to an ablation of collagenolytic activity in osteoblastic cells essential for modelling of the skeletal system[143]. Humans having *MMP-2* mutations suffer from osteolysis and arthritis[90]. Furthermore, the mechano-regulation of MMP-2 in MSCs indicates a potential role of this protease in translating mechanical signals into the regulation of angiogenesis in bone[140]. For full function of MMP-2, this protease needs to be activated by its ternary complex, consisting of active-MMP-14, TIMP-2 and pro-MMP-2[83-85, 144]. Active MMP-14, however, is intracellularly generated via proteolysis by furin[75, 86].

To date, it remains unclear whether the activation complex of MMP-2 is sensitive to mechanical signals and whether pro- and/or active-MMP-2 are involved in the paracrine stimulation of angiogenesis by mechanically loaded MSCs. To answer these questions, the study presented in this chapter aimed to identify MMP-2-inducing loading conditions for MSCs. Subsequently, the mechanism of MMP-2 mechano-regulation is examined in detail by inhibitor application. Finally, the relevance of mechanically regulated MMP-2 to angiogenesis stimulation is investigated.

## 3.2 Materials and methods

### 3.2.1 Animal bone defect model for haematoma sampling

A previously described animal model was used[145]. Briefly, the left femur of 12 female Sprague-Dawley rats (aged 12 months) was osteotomised and distracted to an osteotomy gap of 1.5mm. Semi-rigid and rigid fixations were obtained by varying the offset of the external fixation.

Haematoma samples were collected 7 days post surgery from each group and frozen at -20°C for a maximum of 2 months. For investigation, each haematoma was dispersed in 30µl phosphate-buffered saline (PBS) and the supernatant was analysed. Total protein content was determined by the DC Protein assay according to the manufacturer's instructions (Biorad, Germany).

### 3.2.2 Cell culture

MSCs were isolated, characterised and cultured as stated in 2.2.1. Passages 3-5 were used for experiments. The mean donor age was 63 years (ranging from 18 to 86 years). For angiogenesis assays, simian vacuolating virus (SV)40 immortalised human dermal microvascular ECs (HMEC-1) were employed and cultured in MCDB-131 (Invitrogen, Germany) supplemented with 1µg/ml hydrocortisone, 2mM L-glutamine, 5% FCS (Biochrom, Germany) and 100U/ml penicillin + 100µg/ml streptomycin (EC culture media) [146].

### 3.2.3 Bioreactor

The bioreactor was employed as specified in 2.2.3. Levels of compression, frequency and duration of loading were adjusted as stated in the corresponding experiments. CM were sampled and frozen at -20°C for a maximum of three months. For application of the Golgi disturbing agent Brefeldin A (BFA) (2.8µg/ml) (Sigma-Aldrich, Germany)[147], constructs were transferred into 24-well culture plate containing MSC medium without FCS. After 8h incubation CM was harvested. Furin inhibitor I (Calbiochem, Germany) was added to the MSC medium prior to loading at 4µg/ml. Equal cell numbers in constructs exposed to mechanical loading and in unloaded control constructs were verified by the Cell Titer 96® AQueous test (Promega, Germany). Cells were recovered from the constructs by the addition of 1ml trypsin solution (225U/ml) (Serva, Germany) and incubation for 15min at 37°C. Trypsinised cells were repeatedly transferred into a tube containing 5ml MSC medium und

2ml Trasylol® (Bayer, Germany) until complete digestion of the construct. Cell vitality was after digestion of the cell/fibrin construct determined by the CASY® cell counter system (Schärfe System, Germany)[148]. Furthermore, cell vitality was analysed by acridine orange staining (acridine orange, ethidium bromide, Sigma-Aldrich, Germany) by means of a counting chamber (Neubauer, Germany).

### 3.2.4 ELISA, zymography and MMP-2/-14 activity assay

Total-MMP-2 ELISA and gelatine zymograms were performed as stated in 2.2.5. Reverse zymography was conducted using a 12% polyacrylamide, 0.4% sodium dodecyl sulfate (SDS) gel containing porcine gelatine (Sigma-Aldrich, Germany) (2.3mg/ml) and recombinant pro-MMP-2 (Calbiochem, Germany) (0.4mg/ml). This approach was performed according to the manufacturer's protocol of the Novex® system with either 20µl of CM or 60µg of total protein from haematoma samples. Analysis of digital images was carried out using the NIH ImageJ software package (http://rsb.info.nih.gov/ij/). MMP-2 and MMP-14 activity were determined using the biotrak activity assay (GE healthcare) according to manufacturer's protocol employing 100µl of sample. To measure levels of MMP-2, CM was diluted in a ratio of 1:10 and to determine MMP-14, cells were removed from the construct, $2.5x10^5$ cells were incubated in 125µl lysis buffer for 15min, resuspended and the supernatant was used as 1:2 dilution.

### 3.2.5 Tube formation assay

24-well plates were coated with 50µl matrigel (10mg/ml, Invitrogen, Germany) and allowed to gel for 30min at 37°C. Afterwards, $4x10^4$ HMEC-1 cells were seeded in 100µl EC culture medium per well and 500µl CM was added. The assay was incubated for 17h before results were visualised by light microscopy. The cumulative length of the capillary network was analysed for each well. Quantification was performed using NIH ImageJ. Recombinant active-MMP-2 (150ng/ml) (Calbiochem, Germany) TIMP-2 (13ng/ml) (Calbiochem, Germany) and anti-MMP-2 pro-form blocking antibody, clone CA-4001 (4µg/ml) (Millipore, Germany) were used.

### 3.2.6 Statistics

A Student´s t-test was employed for statistical evaluation. Analysis of rat haematomas was accomplished by an unpaired test. For the evaluation of the effect of bioreactor samples, a paired test was applied. All tests were two sided, and at a significance level of $p<0.05$. In the presented figure legends, the number of experiments is displayed and in the figures, statistical significance is indicated by a star.

## 3.3 Results

### 3.3.1 Mechano-regulation of MMP-2 *in vivo*

To investigate whether the *in vitro* described mechano-regulation of MMP-2 also occurs *in vivo*[140], haematomas from bone defects with different interfragmentary movements were analysed for their MMP-2 activity. Indeed, MMP-2 was significantly elevated in haematomas from semi-rigidly stabilised fractures 7 days post surgery compared to those with a rigid fixator (factor: 1.6, p=0.017, Figure 3-1).

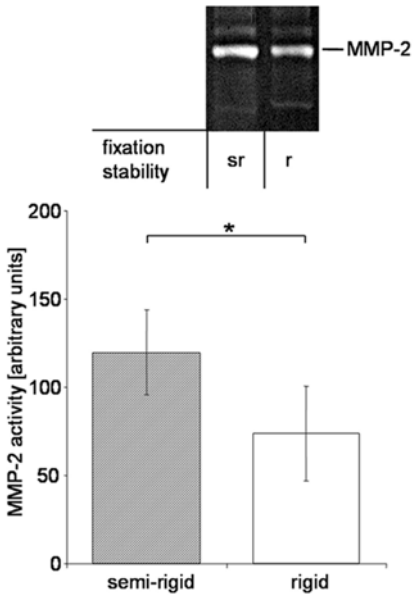



Figure 3-1 *Up-regulation of MMP-2 by mechanical stimulation during fracture healing.* Representative zymogram showing the gelatinolytic activity at the migration height of MMP-2 in semi-rigidly and rigidly fixated rat haematomas sampled at day 7. Absolute densities at the migration height of pro-MMP-2 were quantified. sr: semi-rigidly fixated; r: rigidly fixated (n=6) (*: $p<0.05$).

### 3.3.2 Influence of loading parameters on MMP-2 levels

To optimise the *in vitro* conditions for MMP-2 induction in response to mechanical stimulation, loading parameters affecting cellular processes of bone healing were individually varied. Firstly, compression levels were investigated[149]. 10%, 30%, 50% and 60% compression were applied at a frequency of 1Hz for 72h. 30% compression significantly enhanced MMP-2 levels compared to unloaded controls ($mean_{total\text{-}MMP\text{-}2\ amount}$: 114%, p=0.015; $mean_{pro\text{-}MMP\text{-}2\ activity}$: 174%, p=0.026, Figure 3-2 A, B).

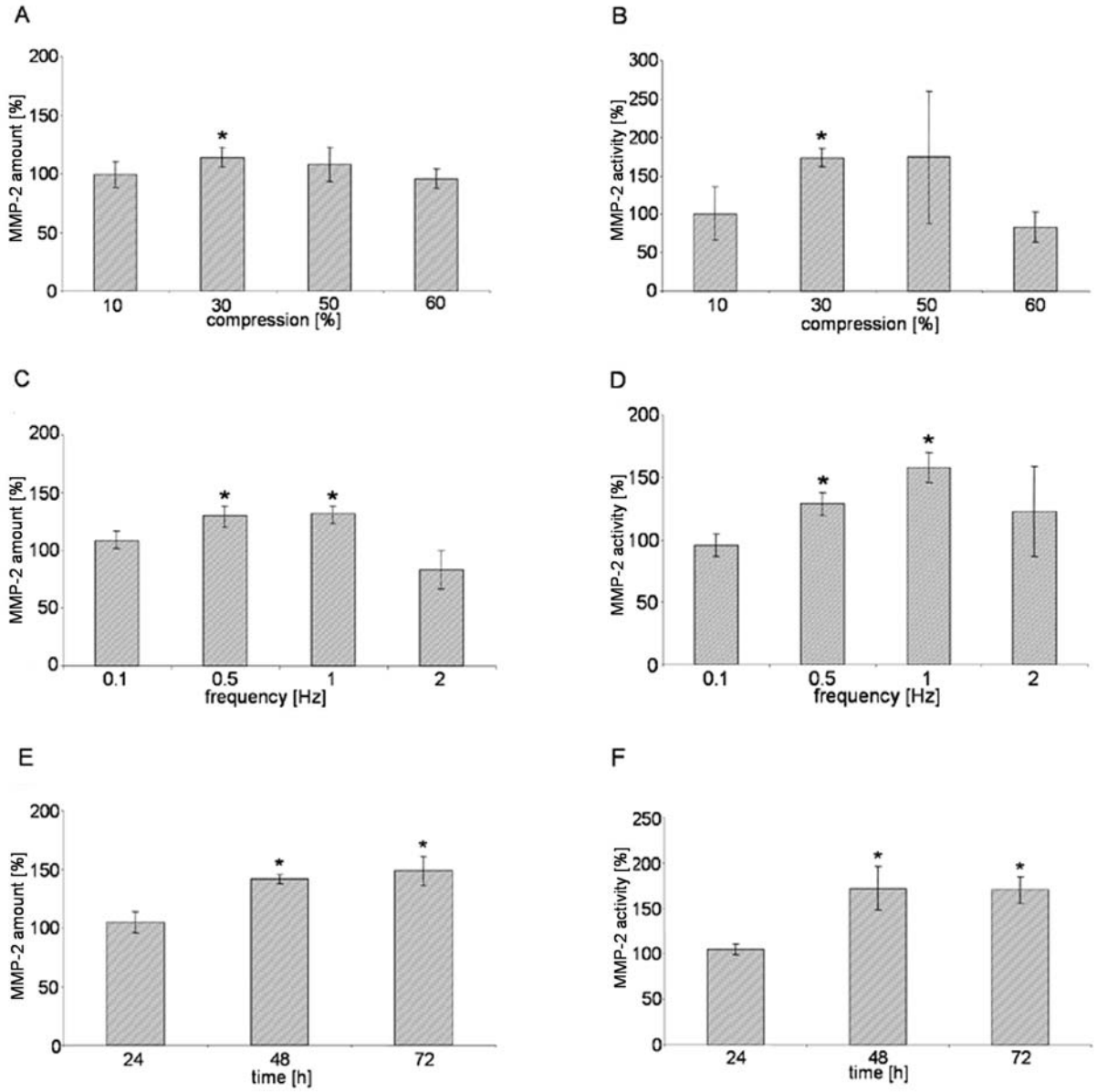



Figure 3-2 *MMP-2 levels increase at 30% compression and 0.5/1Hz, as well as 48h/72h of stimulation.* Total-MMP-2 amounts were determined by ELISA and pro-MMP-2 activities by quantification of zymograms. A-F) MMP-2 levels versus loading conditions. All data were normalized to the unloaded controls (n=3) (*: $p<0.05$).

10%, 50% and 60% compression had no impact on MMP-2 concentration or activity. Cell vitality was reduced in constructs loaded at 50% and 60% compared to unloaded controls ($mean_{50\%}$: 92%, $p$=0.001; $mean_{60\%}$: 90%, $p<0.001$, Figure 3-3 A, B).

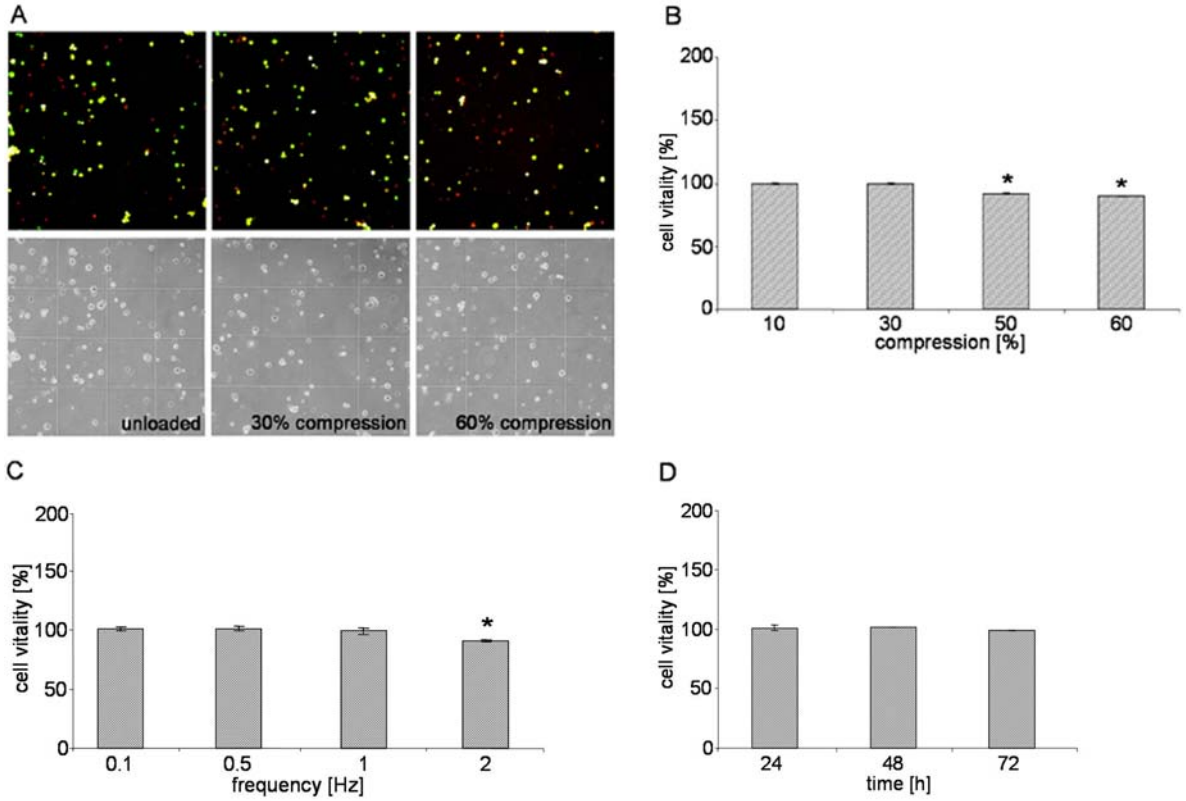



Figure 3-3 *Cell vitality is diminished at 50/60% compression and 2Hz.* A) Representative pictures of cell vitality of MSCs recovered from constructs with and without loading. Top: acridine-orange staining (green: living cells, red: dead cells). Bottom: Light microscopy B-D) Cell vitality determined by means of a cell counter system. All data were normalized to the unloaded controls (n=3) (*: $p<0.05$).

A frequency range from 0.1 to 2Hz was additionally tested for its effect on MMP-2 levels; this is considered to include the range of normal human walking[150]. Load cycles were maintained at 30% for a duration of 72h. 0.5Hz and 1Hz resulted in a significant up-regulation of total-MMP-2 protein concentration compared to unloaded controls ($mean_{0.5Hz}$: 130%, p=0.028; $mean_{1Hz}$: 131%, p=0.005, Figure 3-2 C). Furthermore, pro-MMP-2 activities were elevated at these frequencies ($mean_{0.5Hz}$: 129%, p=0.014; $mean_{1Hz}$: 157%, p=0.005, Figure 3-2 D). Frequencies of 0.1Hz and 2Hz had little or no effect on MMP-2 concentration or activity (Figure 3-2 C, D). Cell vitality was reduced at 2Hz (mean: 91%, p=0.004), whereas loading frequencies of 0.1Hz, 0.5Hz and 1Hz had no effect on cell vitality compared to unloaded controls (Figure 3-3 C).

The duration of stimulation was then examined at 1Hz and 30% compression. Total-MMP-2 amounts were increased at 48h and 72h, but not at 24h compared to unloaded controls ($mean_{48h}$: 141%, p=0.022; $mean_{72h}$: 148%, p=0.012, Figure 3-2E). Comparable results were obtained for pro-MMP-2 activity ($mean_{48h}$: 172%, p=0.037; $mean_{72h}$: 170%, p=0.013, Figure 3-2 F). MSC vitality was not altered in loaded and unloaded constructs (Figure 3-3 D).
From these experiments, inducing loading conditions in respect to MMP-2 expression and activity were concluded to be 1Hz and 30% compression over a time period of 72h and non-inducing to be 0.1Hz and 10%.

### 3.3.3 Mechanism of MMP-2 mechano-regulation

Having established conditions for enhanced MMP-2 production, the mechanism of its mechano-regulation was examined under the inducing conditions.
To investigate whether regulation occurs intracellularly, the secretion inhibitor BFA was added. This hindered the elevation of total-MMP-2 amounts in response to mechanical loading ($mean_{BFA}$: 104%, $mean_{control}$: 148%, p=0.012, Fig. 3-4 A).
Subsequently, extracellular activities of the activation complex components were determined. TIMP-2 activity was increased ($mean_{TIMP-2}$: 200%, p=0.014, Fig. 3-4 B), and MMP-14 activity showed a trend of up-regulation ($mean_{MMP-14}$: 150%, p=0.104, Figure 3-4 C). Moreover, the concentration of active-MMP-2 was elevated under mechanical stimulation ($mean_{active-MMP-2}$: 153%, p=0.006, Figure 3-4 D).
Inhibition of furin, which is responsible for intracellular MMP-14 activation, suppressed the mechanical up-regulation of MMP-14, as well as that of active-MMP-2 (Figure 3-4 C, D).

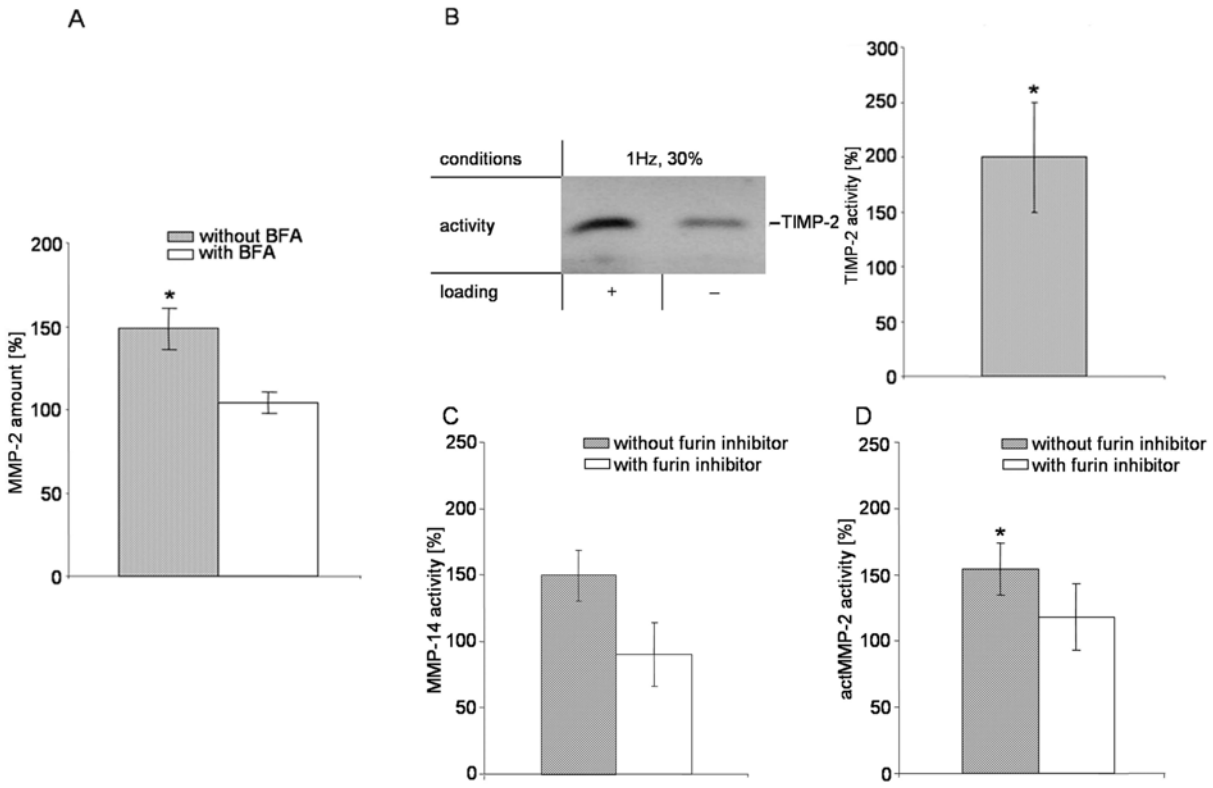



Figure 3-4 *Regulation of MMP-2 production and activation by mechanical loading.* A) Diagram displays relative amounts of total-MMP-2 in the presence and absence of BFA. B) Representative pictures of reverse zymograms at the migration height of TIMP-2 in CM of loaded and unloaded MSCs. Diagram displays quantified TIMP-2 (n=3). C) MMP-14 (n=4) and D) active-MMP-2 activities (n=5) in the presence and absence of a furin inhibitor. All data were determined from mechanical stimulation at 1Hz and 30% compression and normalized to unloaded controls (*: $p<0.05$).

### 3.3.4 Mechano-regulated MMP-2 in angiogenesis stimulation

To assess the role of MMP-2 mechano-regulation in the paracrine stimulus of angiogenesis, CM from MSC, which were loaded at inducing or non-inducing conditions, or unloaded, were tested for levels of MMP-2 concentrations and angiogenic responses.

Total-MMP-2 expression was significantly up-regulated at the inducing conditions compared to non-inducing conditions and unloaded samples (δOD450: $mean_{1Hz,30\%}$: 2.4; $mean_{0.1Hz,10\%}$: 1.5; $mean_{unloaded}$: 1.5, $p_{1Hz,30\% \text{ vs.} 0.1Hz,10\%}=0.026$; $p_{1Hz,30\% \text{ vs. unloaded}}=0.002$) (Figure 3-5 A).

Angiogenic responses followed this pattern, showing increased tube formation at the inducing conditions compared to non-inducing conditions and unloaded controls (cumulative tube length [x$10^5$ pixel]: $mean_{1Hz,30\%}$: 203.6; $mean_{0.1Hz,10\%}$: 86.3; $mean_{unloaded}$: 81.5, $p_{1Hz,30\% \text{ vs. } 0.1Hz,10\%}$=0.025; $p_{1Hz,30\% \text{ vs. unloaded}}$=0.026, Figure 3-5 B, C). Controls using CM from equivalent experiments without cells in the bioreactor construct showed no increase in tube formation.

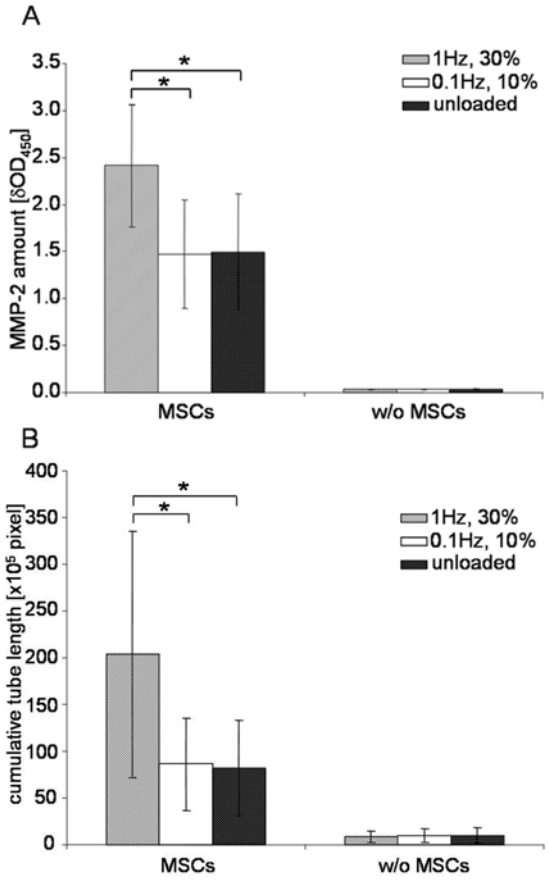
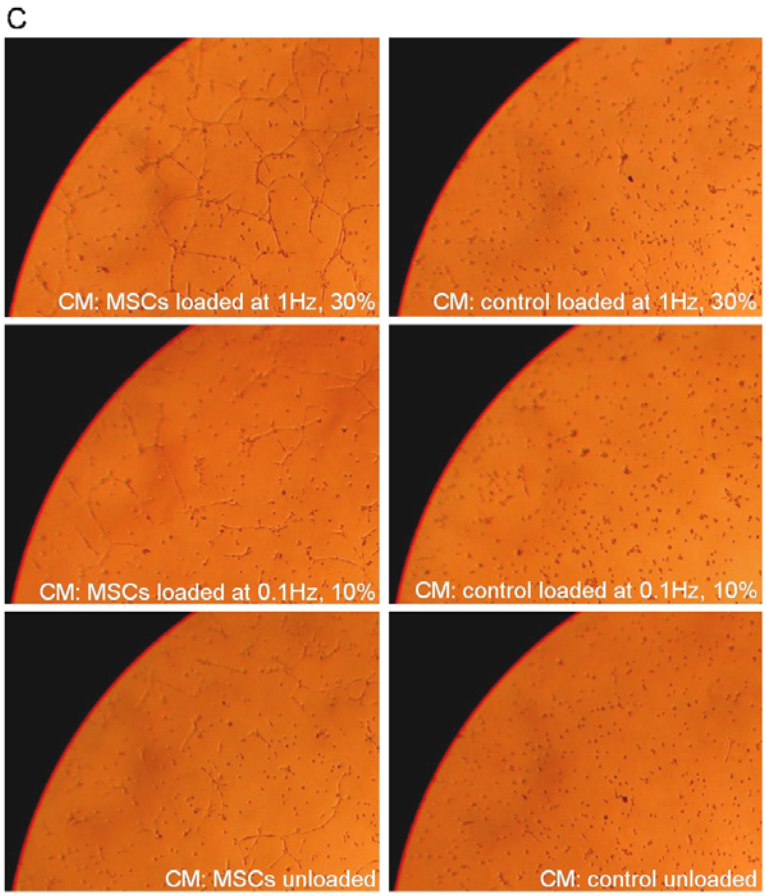



Figure 3-5 *CM from loaded MSCs shows similar increases in MMP-2 concentration and tube formation.* A) MMP-2 amounts in CM from different loading conditions. B) Cumulative tube length in response to the different CM. CM from inducing conditions (1Hz, 30% compression), non-inducing conditions (0.1Hz, 10% compression), unloaded MSCs and controls (CM from equivalent experiments without MSCs in the bioreactor construct) were tested (n=6) (*: $p<0.05$).




C) Representative photographs of the tubular network formed under each loading condition.

To further examine the involvement of MMP-2 in angiogenesis stimulation by loaded MSCs, TIMP-2 as natural MMP-2 inhibitor, and recombinant active-MMP-2 were delivered in concentrations within the range measured after mechanical loading of MSCs[140] (Figure 3-6). Furthermore, a blocking antibody preventing MMP-2 activation was tested. MMP-2 blocking reduced the pro-angiogenic effect of CM from MSCs at inducing loading, whereas no reduction was detected at non-inducing conditions (cumulative tube length [$x10^5$ pixel]: $mean_{1Hz,30\%}$: 114.3; $mean_{0.1Hz,10\%}$: 103.7). An almost complete blockage of tube formation was demonstrated after supplementation with recombinant TIMP-2[151]. The level of remaining angiogenesis was similar between inducing and non-inducing conditions (cumulative tube length [$x10^5$ pixel]: $mean_{1Hz,30\%}$: 84.0; $mean_{0.1Hz,10\%}$: 82.7). Supplementation of CM from non-inducing conditions with recombinant active-MMP-2 led to an elevation of the angiogenic response, whereas in CM from inducing conditions, no further stimulation was achieved (cumulative tube length [$x10^5$ pixel]: $mean_{1Hz,30\%}$: 149.3; $mean_{0.1Hz,10\%}$: 120.8).

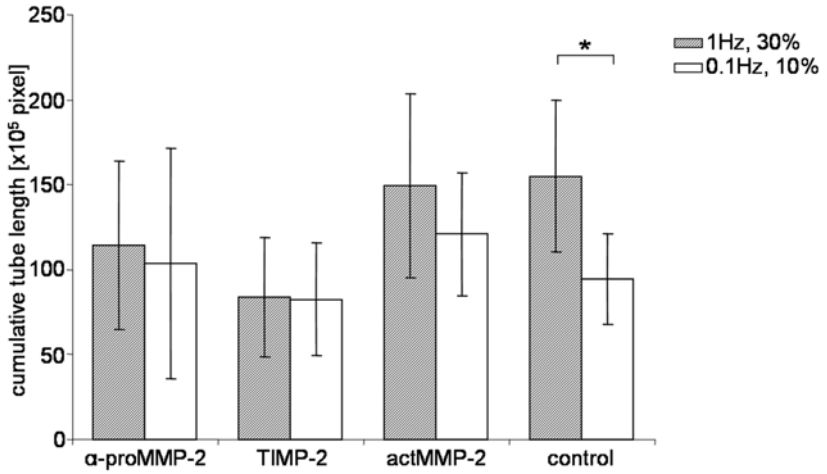



Figure 3-6 *MMP-2 activity influences angiogenesis stimulation.* Diagram displays cumulative tube length in CM from MSCs loaded at inducing (1Hz, 30% compression) and non-inducing (0.1Hz, 10% compression) conditions. Tube formation assays were investigated in the presence of pro-MMP-2 antibody (α-proMMP-2), recombinant TIMP-2 (TIMP-2), recombinant active-MMP-2 (actMMP-2), and without supplementation (control) (n=3) (*: $p<0.05$).

In control experiments without supplements, tube formation was significantly enhanced in response to inducing loading compared to non-inducing stimulation (cumulative tube length [$x10^5$ pixel]: $mean_{1Hz,30\%}$: 154.9; $mean_{0.1Hz,10\%}$: 94.6, $p_{1Hz,30\% \text{ vs.} 0.1Hz,10\%}$=0.028).

## 3.4 Discussion

### 3.4.1 Mechanical parameters

Mechanical loading for 72h and 48h, but not 24h stimulated the MMP-2 production by MSCs. This is consistent with a study showing that mechanical stimulation of human fibroblasts leads to an enhanced generation of active-MMP-2 at 48h, but not at 24h[152]. Regarding frequencies, 0.5Hz and 1Hz resulted in an up-regulation of MMP-2 protein and activity, whereas 0.1Hz and 2Hz had little or no effect. These results agree with those demonstrating that mechanical stimulation of 1Hz influences mRNA and protein expression by MSCs[153-155]. Compressive deformation of 30% caused an increase in MMP-2 concentration and activity, but not 10%, 50% or 60%. These results imply that 1Hz and 30% strain are in the range of optimal mechanical stimulation of MSCs in regard to MMP-2 production. Parameters both above and below these values result in a reduced response. This might be due to unresponsiveness of the cells at parameters outside the identified range, but may be also due to decreased cell vitalities, which were demonstrated especially at higher values of loading, such as 2Hz and 50% compression.

Remarkably, the MMP-2-inducing parameters determined (1Hz and 30% compression) correspond to *in vivo* conditions measured during uneventful bone healing[23, 150]. An axial displacement of approximately 1mm in a 3mm fracture gap (approximately 30% compression) was measured in fracture models showing successful healing[23]. Regarding frequencies, rat tibias respond with increased bone formation at loading above 0.5 Hz, but not below[156]. Furthermore, 0.5-1Hz corresponds to the rate of human walking[23, 150]. Thus, it could be hypothesised that *in vivo* mechanical conditions for fast bone regeneration are associated with a stimulation of MMP-2. This proposal is strengthened by the observed up-regulation of MMP-2 activity in haematomas from less-rigidly stabilised fractures, which exhibited improved healing[145].

### 3.4.2 Regulation of MMP-2 levels

The up-regulation of pro- and total-MMP-2 by mechanical stimuli was not observed before 48h and could be blocked by the Golgi disturbing agent, BFA. Furthermore, only protein and not mRNA levels appear to be affected[140]. Thus, the mechano-regulation of intracellar pro-MMP-2 seems to be mediated via an indirect mechanism, occuring after transcription and prior to protein secretion. This mechanism might be based on an increase in protein stability

and/or alteration of the secretory pathway, since both possibilities have been shown to regulate MMP-2 protein levels[79, 157].

In addition to pro-/total-MMP-2, active-MMP-2 concentrations were enhanced by mechanical loading. This enhancement corresponded to the mechanical regulation of the other members of the ternary MMP-2 activation complex, evidenced by a significant elevation of TIMP-2 and a trend towards increased MMP-14. Based on previous results, the up-regulation of TIMP-2 activity corresponds to an elevation of TIMP-2 protein concentration[140]. Furthermore, furin activity was required for the enhancement of MMP-14 and active-MMP-2 by mechanical loading. This suggests that the mechano-regulation of active-MMP-2 is mediated by a stimulation of the furin/ternary complex axis[75, 86]. In experiments comparable to the present study, a simultaneous up-regulation of pro- and active-MMP-2, TIMP-2 and MMP-14, and the involvement of furin in enhanced active-MMP-2 and -14 activity, was demonstrated in cardiac fibroblasts exposed to collagen I[158]. Hence, the concurrent regulation of pro-MMP-2 levels, as well as of the MMP-2 activation machinery, might not only occur in response to mechanical stimuli, but also to biochemical signals.

### 3.4.3 Angiogenesis stimulation

Inhibition experiments demonstrated that both modes of MMP-2 mechano-regulation the elevation of pro-MMP-2 levels, as well as the enhancement of active-MMP-2 seem to independently mediate the pro-angiogenic impact of mechanically loaded MSCs. Since the angiogenesis experiments were performed in the absence of contact with MSCs, the effect of pro-MMP-2 is likely to be caused by a subsequent MMP-2 activation, e.g. by EC-associated MMP-14[159]. Thereby, enhanced levels of active-MMP-2 would be available in the microenvironment for angiogenesis stimulation (*for more details see 4.3.2*).

# 4 General Discussion

In this section, the findings described in Chapters 2 and 3 are summarised and discussed with respect to their consequences for the bone healing outcome. Then pathways of MMP/TIMP induction in MSCs in response to mechanical conditions known from uneventful bone healing are discussed. Furthermore, consequences of the altered MMP/TIMP balance for the MSC microenvironment during healing are hypothesised. Finally, the potential value of the project's results for the rational design of new MSC-based therapies for bone regeneration is discussed, and the conclusions of the thesis are presented.

## 4.1 Summary of the findings

The early phase of bone healing and especially angiogenesis are known to be influenced by mechanical conditions. Although MSCs, present in the early haematoma, are thought to stimulate bone regeneration by production of proteins that act as autocrine and paracrine signals, the interplay between mechanical stimuli,and molecular and cellular responses of MSCs, as well as their effect on angiogenesis, remain poorly understood.

Results presented in chapter 2 showed an up-regulation of MMP-2, -3, -13 and TIMP-2 protein levels, but not of RNA levels. Furthermore, MMP-13 was demonstrated to be involved in MSC differentiation and MMP-2 to likely contribute to the migratory behaviour of MSCs. These findings indicate that alterations in the MMP/TIMP balance might mediate the translation of mechanical stimuli into the cellular response of MSCs. In chapter 3, it was shown that mechano-regulation of MMP-2 occurs at two levels in MSCs: via an increase in pro-MMP-2 production and enhanced activation via the ternary MMP-2 activation complex including MMP-14 and TIMP-2. Furthermore, it was demonstrated that both pro- and active-MMP-2 are involved in the paracrine stimulation of angiogenesis by mechanically loaded MSCs.

## 4.2 Mechano-regulation of MMPs in MSCs

Mechanical loading of MSCs resulted in an enhancement of MMP-2, -3, -13 and TIMP-2 proteins, whereas mRNA levels remained constant. Furthermore, detailed investigation of MMP-2 demonstrated an enhancement of this protease by an intracellular mechanism, since addition of BFA hindered the up-regulation of secreted MMP-2 levels. Due to the lack of a difference in mRNA amounts between loaded and unloaded samples, the regulation of MMPs in response to mechanical stimulation seems to occur via a post-transcriptional mechanism[79, 80].

Several studies provide evidence for a regulation of MMPs through modulations of the secretory pathway or of protein stability. However, non of these studies specified the underlying mechanism for these effects[79, 80, 157]. One option is an alteration of MMP protein secretion by secretory lysosomes that are known to exist in osteoblasts[81]. Interestingly, it has been shown that these lysosomes are sensitive to intracellular $Ca^{2+}$ levels and could thereby be affected by mechanical stimulation of the cells via stretch-activated ion-channels[81, 103].

Extracellular MMP levels might be also regulated by shedding of matrix vesicles, membrane-enclosed microstructures released from the plasma membrane, which occur in ECs, but also in

differentiating chondrocytes and osteoblasts[160, 161]. Various pro- and active-MMPs have been shown to be associated with these vesicles, including pro- and active-MMP-2, MMP-13, -14 and TIMP-2, which overlap with the mechano-regulated MMPs identified within this study[160, 162]. Previous results provide evidence for shedding of these vesicles in response to growth factors, including VEGF and bFGF[160]. Therefore, the enhancement of MMP levels in response to loading could be an indirect result of growth factor up-regulation, as seen for bFGF and TGF-β in MSCs in response to mechanical stimulation[60].

Results from this study further indicate a superior activation of MMP-2 on the MSC-cell surface in response to loading, which was evidenced by an up-regulation of all members of the ternary complex, as well as by the dependency of active-MMP-2 and MMP-14 activities on the endoprotease furin. Notably, furin is also stimulated by TGF-β1 via enhanced transactivation of the *fur* gene[163]. This could explain increased levels of MMP-14 and active-MMP-2 protein, and also of MMP-13, since MMP-14 and MMP-2 were shown to be able to activate this protease[78].

One or several of the listed mechanisms could account for the up-regulation of MMP-2, MMP-3 and MMP-13 in response to mechanical stimulation reported in the present study.

Nevertheless, other investigations using different experimental settings have provided evidence for the regulation of MMP mRNA by mechanical loading. For example, an up-regulation of MMP-1, -2, -3 and MMP-9 mRNA has been observed after cyclic tensile loading in cultured chondrocytes and MMP-13 up-regulation has been described after stretching of murine osteoblasts[106, 164]. These data indicate the existence of additional regulatory mechanisms on mRNA levels in other mesenchymal cells or under different stimulatory conditions. Furthermore, the nature of the applied stress seems to be important for the regulation of matrix remodelling by MSCs, since a recent study demonstrated differential effects in response to equiaxial and uniaxial strain[99, 165]. This strain-specific regulation might also account for MMPs, since stromelysin mRNA was shown to be downregulated by uniaxial strain, whereas in this study using uniaxial loading, no alterations of these mRNA levels were found.

## 4.3 Consequences of altered MMP/TIMP balance

### 4.3.1 Consequences for MSCs

The mechano-regulation of MMP-2, -3, -13 and TIMP-2 indicates an involvement of these MMPs in the autocrine regulation of MSCs.

Indeed, data from this study showed enhanced MMP-2 activities are likely to contribute to superior MSC migration. As previously discussed (*see 2.4.2*), this effect might occur via mechanisms including ECM degradation, VEGF release or alteration of the IGF-BP-3/IGF interactions[75, 77, 128-130]. In addition, adipogenic differentiation could also be altered by MMP-2 up-regulation, since levels of this protease have been shown to increase during the differentiation process, and MMP-2 inhibition leads to a decrease in adipogenesis[166]. One potential link is the ability of MMP-2 to cleave the fibroblast growth factor (FGF) receptor 1 resulting in the release of a soluble fragment that may indirectly influence bFGF availability, which in turn enhances PPARγ ligand-induced adipogenesis in MSCs[167, 168].

Up-regulated MMP-3 was shown not to be associated with altered MSC osteogenic differentiation, proliferation or migration. However, a negative regulatory role of MMP-3 in adipogenic differentiation of MSCs has been previously demonstrated[169, 170].

MMP-13 up-regulation was demonstrated within this study to be involved in osteogenic differentiation, and this may be explained by an interaction with the SDF-1/CXCR4 axis[121, 126] (*see 2.4.2*). In addition, enhanced levels of MMP-13 could stimulate chondrogenic differentiation, due to its ability to cleave ECM fragments, such as collagen II, or activate latent TGF-β, both of which are critically involved in chondrogenesis[162, 171, 172]. For example, matrix vesicle-associated MMP-13 obtained from growth plate cartilage has been shown to activate latent TGF-β and might therefore play a role in chondrocyte-mediated mineralization[162]. Interestingly, mechanical loading of bovine bone marrow stromal cells results in an increase in Smad2/3 phosphorylation, which is known to be critical to TGF-β-induced chondrogenic differentiation[154]. Based on the results of this study, it is possible that the enhanced chondrogenesis results from an increase in TGF-β activation by up-regulated MMPs, e.g. MMP-13.

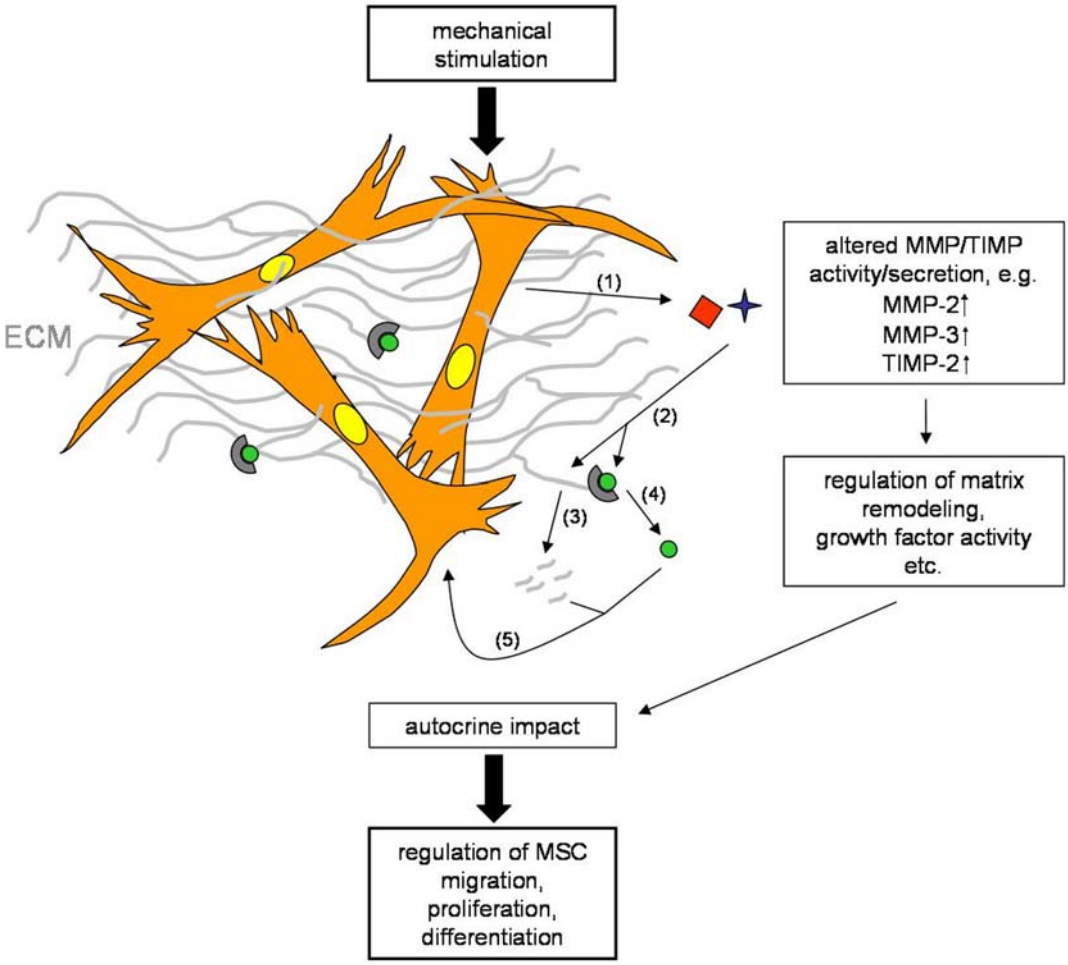



Figure 4-1 *MMP/TIMP balance as a potential transducer of mechanical stimuli into MSC functions.* Mechanical loading of MSCs leads to enhanced secretion and potential activation of MMPs/TIMPs (1), which might mediate various regulatory processes (2), such as degradation of the ECM (3) or release of latent growth factors (4). Interaction of ECM fragments or activated growth factors with cell surface receptors (5) might result in altered MSC migration, proliferation and differentiation in an autocrine manner.

The effects of increased MMP-14 levels in MSCs on cell function were not investigated within this study. However, it is likely that this protease contributes not only indirectly via its MMP-2-activating properties, but also directly to alterations in the functional behaviour of these cells[75, 132]. In this respect, MMP-14 might be involved in MSC proliferation, differentiation, migration and adhesion[132, 133, 173]. The contribution of MMP-14 to proliferation has been shown by maintenance of osteoblast and also osteocyte viability, which is potentially mediated by increased activation of latent TGF-β[132, 133]. Perhaps, MMP-14 is required for differentiation, and particularly for osteocytogenesis in bone[173]. This effect could be accomplished by cleavage of various MMP-14 substrates, such as collagen I/II, growth factors (e.g. SDF-1 or bFGF) or indirectly via the action of MMP-2[75, 127, 174]. Cleavage of cell surface

tissue transglutaminase (tTG) by MMP-14 could also affect MSC migration and adhesion, since over-expression of this protease was shown to inhibit migration and adhesion of tumor cells[175 176].

Likewise, the enhancement of TIMP-2 by mechanical loading could have MMP-2-independent effects on MSCs, due to its ability to stimulate proliferation in a wide range of human cells[177, 178].

However, functional analyses conducted within this study did not discover all potential actions of mechano-regulated MMPs on MSCs. It is furthermore most likely that the activities of the MMPs studied are not independent from other family members, since they can act synergistically as been observed in MMP-9/MMP-13 knock out animals[88]. Moreover, other MMP family members could compensate for lost activity, since most substrates are recognised by more than one of these proteases. However, it is likely that alterations in the MMP/TIMP balance due to mechanical loading create a microenvironment for MSCs that determines their functional behaviour, such as migration, proliferation and differentiation (Figure 4-1).

### 4.3.2 Consequences for angiogenesis

This study provided evidence for the involvement of mechanically induced MMP-2 in the augmentation of angiogenesis. Furthermore, angiogenesis stimulation by mechanically loaded MSCs has been shown to be dependent upon FGF receptor 1 and VEGF receptor, but not upon VEGF levels[60]. However, although FGF receptor 1 is an MMP-2 substrate, a stimulation of angiogenesis after receptor cleavage is unlikely, since it leads to a loss of the receptor's signalling capacity[167, 179, 180]. Therefore, it may be hypothesised that angiogenesis stimulation by CM from mechanically loaded MSCs is rather a result of other mechanisms. For example, degradation of ECM components may facilitate EC migration by removing physical barriers[72, 76]. Also, modulation of angiogenic signals by MMP-2, including the exposure of a cryptic epitope of collagen IV, or cleavage of pro-TGF-β, could affect EC migration and proliferation[76, 77, 181]. MMP-2 might further activate other enzymes such as pro-angiogenic MMP-9, as expressed by ECs[78, 182]. The important role of MMP-9 in the release of ECM-bound VEGF could thereby potentiate the pro-angiogenic stimuli[60, 77].

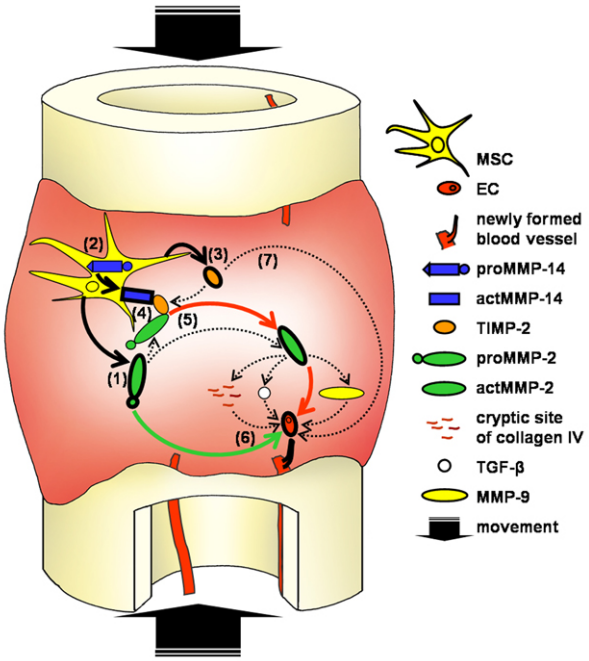



Figure 4-2 *Hypothetical mechanism of angiogenesis stimulation by mechanically loaded MSCs.* Mechanical loading of MSCs at inducing conditions results in (1) enhanced production of pro-MMP-2, (2) increased levels of active MMP-14 involving the intracellular activity of furin, and (3) up-regulation of TIMP-2. (4) This increased availability of the members of the MMP-2 activation complex seem to enhance assembly/action of the ternary complex, (5) leading to elevated MMP-2 activation. (6) Higher levels of both, pro- and already-activated MMP-2 appear to affect angiogenesis. Mechanisms of MMP-2 action on ECs may be an increased disruption of structural barriers, exposure/release of pro-angiogenic factors (e.g. cryptic epitope of collagen IV, TGF-β) or activation of further MMPs such as MMP-9 secreted by ECs. (7) Elevated levels of TIMP-2 might also directly affect angiogenesis. Bold lines: pathway was shown within this study, red: MMP-2 activation cascade, green: pro-MMP-2 cascade.

In addition to these putative MMP-2-mediated effects, the up-regulation of TIMP-2 in response to mechanical loading might also contribute to the regulation of blood vessel formation by mechanical signals, since TIMP-2 inhibits angiogenesis by an MMP-independent mechanism, which requires α3β1 integrin-mediated binding of TIMP-2 to ECs[183, 184].

Also, pro-angiogenic MMP-14 showed a trend of up-regulation and might therefore be involved in the observed EC responses. An overlap in most of the ECM substrates and growth factor substrates of MMP-14 and -2, including TGF-β and VEGF, suggests similar actions of both MMPs on ECs[74, 75]. A particular role of MMP-14 seems to be the degradation of fibrin barriers, thus facilitating EC migration and invasion[185]. However, the results of this study indicate MMP-2 to be the critical component for angiogenesis stimulation in response to loading of MSCs. Consequently, a hypothetical mechanism of angiogenesis stimulation by mechanically regulated MMP-2/TIMP-2 in MSCs is proposed (Figure 4-2).

### 4.3.3 Consequences for bone regeneration

Enhanced MMP levels were demonstrated within this thesis to stimulate the function of MSCs themselves, but also to augment angiogenesis. Both processes are crucially involved in early bone healing, including the phases of inflammation and endochondral ossification[16]. Therefore, it is likely that altered MMP levels have an impact on the healing outcome.

In particular, enhancement of MMP-13 was shown to result in improved osteogenic and chondrogenic differentiation of MSCs, indicating an important role of this protease during endochondral ossification. This is supported by the strong expression of MMP-13 mRNA during this phase in murine fracture repair models[14]. Moreover, the role of MMP-13 in bone remodelling was evidenced by knock out mice displaying an abnormal growth plate development and by human individuals having mutations in the MMP-13 gene[88, 186] For example, MMP-13 knock out mice showed an abnormal growth plate development due to a lack of collagen type II and aggrecan proteolysis, which impedes chondrocyte remodelling[88].

Enhanced MMP-2 activity in mechanically loaded MSCs potentially stimulates MSC migration and also angiogenesis. Therefore, it is hypothesised that increased levels of MMP-2 play an important role during the inflammatory phase, which is thought to involve the recruitment of MSCs to the fracture site and blood vessel stimulation[12, 16]. Evidence has been provided by MMP-2 gene mutations resulting in severe bone defects[90]. However, there is a lack of evidence of the contribution of MMP-2 to bone remodelling in mice and MMP-2/MMP-14 double knock out mice die at birth[187]. Therefore, it is likely that in MMP-2 knock out mice the deficiency of this protease is compensated by other proteases, including potentially MMP-14.

Moreover, MMP-9 seems to be crucially involved in bone regeneration by a regulation of progenitor differentiation and angiogenesis. This was shown in MMP-9 knock out mice having vascular defects as well as impeded chondrogenic and osteogenic differentiation[91]. However, these effects seem not to be a result of MMP-9 mechano-regulation in MSCs, since the fracture stability had no influence, and MMP-9 is not expressed at protein level in MSCs, as demonstrated in this study. This indicates that, besides MSCs, other cells present in early haematomas, e.g. ECs or inflammatory cells, might also contribute to bone healing processes by a regulation of MMP-9 levels.

## 4.4 Potential therapeutic approaches

Data from this study indicate that loading at conditions known from uneventful bone healing results in an up-regulation of MMPs in MSCs, which contributes to MSC differentiation, migration and paracine angiogenesis stimulation. Moreover, MMP-2 activity was shown to be up-regulated in fracture haematomas under loading conditions that support bone healing. Therefore, MMPs might be considered as candidates for the design of new gene and cell therapies to treat delayed or failed healing of bone. In this respect, it is interesting that osteoinductive BMP-2, which is already available in the clinic for non-healing bone and open tibial fractures, is also up-regulated in response to loading in mesenchymal cells[188]. These findings support the importance of mechano-regulation in the identification of genes/proteins relevant for bone healing.

In particular, data presented in this thesis indicate mechano-regulated MMP-2 to be involved in MSC migration and in augmentation of angiogenesis. Therefore, this protease might be interesting for a treatment of patients suffering from insufficient blood vessel regeneration in bone (e.g. atrophic pseudarthrosis), which may result from risk factors including age, diabetes and alcohol abuse[13]. The ability of MMP-2 to release a variety of pro-angiogenic factors in an orchestered manner makes this protease even more attractive, since a combination of pro-angiogenic factors has been shown to be more effective than application of single factors[189, 190]. For example, a combination of recombinant VEGF-A and bFGF has been reported to have potent synergistic effects on neovascular formation in experimental conditions *in vivo* and *in vitro*[189]. In addition to the MMP-2-mediated effects, mechano-regulated MMP-13 has been demonstrated in this and other studies to stimulate osteogenic and chondrogenic differentiation of MSCs. Thus, this MMP could be considered as an alternative/complement to BMP-2 treatment for a recovery of bone function, and applied via binding to scaffolds, e.g. adsorbable collagen sponges, or via viral or non-viral gene delivery; e.g. adenoviral systems or nucleofection[30].

The response of MSCs to loading conditions by an expression of functionally relevant MMP-2 and -13 could also be supportive for engineering of vascularised tissues. At present, vascularisation is critical for the size of these engineered tissues, containing for example MSCs and ECs[191]. The results obtained within this study suggest that limited compressive loading of such MSC constructs might stimulate blood vessel formation by enhanced secretion of pro-angiogenic MMPs.

Genetic engineering of MSCs to overexpress the MMP candidates using viral/non-viral systems could further potentiate the effect of MSCs in tissue engineering approaches or cell-based therapies. Evidence for the efficacy of MMP transfection for regeneration *in vivo* was recently provided by the restoration of the vascular network in aged murine dystrophic muscle in response to application of tendon fibroblasts transduced with lentiviral vectors encoding placental growth factor (PlGF) and MMP-9[192]. Based on the results of the present study, MMP-2 should be considered as candidate gene for design of genetically modified MSCs to restore the vescular system in compromised healing.

Since pro-angiogenic MMPs, such as MMP-2, have been also implicated as agonists in tumour invasion, one has to be aware of this potential risk[75]. However, innovative delivery systems using vectors with exonenously regulated gene expression could enable for a tight spatial and temporal control of MMP-2 activity and therefore make this risk manageable[30].

The healing-supportive properties of mechano-regulated MMPs presented in this study highlight these proteases as valuable candidates for therapies. Their application might be especially interesting for bone treatments in elderly patients, since they display not only age-related decreases in regeneration potential; there are also hints for the influence of age on the effects of the mechanical environment on bone defect healing, which might be a result of diminished cell responses to loading[13, 145].

## 4.5 Conclusion

Mechanical stimuli have been shown to influence mesenchymal cell characteristics. From the results of this thesis, it seems likely that these stimuli also regulate autocrine and paracrine effects of MSCs including angiogenesis stimulation, which might in turn be mediated by the regulation of MMPs. Whether these mechanisms also occur *in vivo* and whether they are important for bone healing outcomes can only be speculated. However, there are promising hints that this might be the case, since the range of *in vitro* mechano-induction of MMPs resembles the parameters occurring *in vivo* during uneventful bone healing. Furthermore, MMP-2 activity was increased in fracture haematomas subjected to mechanical conditions that have been associated with superior healing. This mechano-regulation of MMPs could be important for activating MSCs in the case of injury, e.g. in response to mechanical forces present at the fracture site. However, to understand the physiological coordination of progenitor cell differentiation, angiogenesis and tissue regeneration *in vivo*, further insight into the influence of mechanical conditions on cell interactions is essential. Therefore, mechano-regulation of MMPs should be validated in MSC co-culture systems with, for example, ECs and immune competent cells. In this respect, the effect of loading on MMP levels in human haematomas is currently under investigation in the Julius Wolff Institute. Since various MMPs and TIMP-2 have been shown to be influenced by mechanical stimuli in MSCs, the effect of complex alterations in the MMP/TIMP balance need to be prospectively investigated in detail. Understanding the mechanisms of MMP involvement in MSC biology and regeneration/developmental processes might be important for the design of novel treatment strategies in bone healing, and also for assessing unintentional side effects of MMP activity.

## Summary (English)

Mechanical conditions have an impact on the early phase of bone healing and in particular on angiogenesis. Mesenchymal stem cells (MSCs) play a key role, due to their ability to home to the defect site, to differentiate into functional bone cells and to secrete pro-angiogenic factors. However, it is yet unknown how MSCs respond to the loading conditions during defect healing, and which proteins transduce the mechanical stimulus into the control of MSC function. Matrix metalloproteases (MMPs) and the tissue inhibitors of metalloproteases (TIMPs) are mechanically regulated in mesenchymal cells and are able to affect cell functions in an autocrine and paracrine manner.

This project investigated MMP/TIMP levels in MSCs in response to mechanical loading, as well as the consequences for MSC function. Furthermore, the pro-angiogenic capacity of mechano-regulated MMP-2 was determined and the underlying mechanism of MMP-2 regulation in MSCs examined.

The involvement of MMPs in migration, proliferation and osteogenic differentiation was evidenced using broad spectrum MMP inhibitors. Expression analysis detected MMP-2, -3, -10, -11, -13, -14 and TIMP-2 in MSCs at the mRNA and protein levels. Mechanical stimulation of MSC/fibrin constructs in a bioreactor system simulating conditions of early bone healing (30% compression at a frequency of 1Hz) resulted in increased concentrations of MMP-2, -3, -13 and TIMP-2 proteins, as well as enhanced gelatinolytic activities of MMP-2 and MMP-13. However, mRNA expression levels of MMPs/TIMPs showed no changes in response to mechanical stimulation. Specific inhibition of mechano-regulated MMP-2, -3 and -13 proved MMP-13 to be involved in osteogenic differentiation. Furthermore, MMP-2 appeared to alter MSC migration, although statistical significance was not reached.

Detailed investigation of MMP-2 demonstrated enhanced gelatinolytic activities in early haematomas under large versus small interfragmentary movements. The measurement of MMP-2 from MSCs at varying loading conditions confirmed loading at 1Hz and 30% compression to be MMP-2-inducing and demonstrated 0.1Hz and 10% compression to be non-inducing. Supplementation with the Golgi disturbing agent, brefeldin A, prevented the mechanical up-regulation of MMP-2. In addition to the enhancement of pro-MMP-2 levels, also the activities of active-MMP-2 and TIMP-2 were elevated, while MMP-14 activity showed a trend of up-regulation. Furin inhibition suppressed this mechano-regulation of MMP-14 and active-MMP-2. A pro-angiogenic effect, seen for conditioned medium (CM) from MSCs loaded at inducing conditions, was reduced by blocking pro-MMP-2 activation,

as well as by inhibiting active-MMP-2. Furthermore, addition of recombinant active-MMP-2 to CM partially compensated for ineffective loading.

The results of this thesis suggest that MSC function is controlled by MMP/TIMP activity, which in turn is regulated by mechanical stimulation of these cells under conditions of uneventful healing. In the case of MMP-2, mechanical loading seems not only to stimulate levels of the secreted pro-enzyme, but also its activation cascade. Both mechanisms appear to contribute to the paracrine augmentation of angiogenesis in response to loading. Thus, a failure to induce cascades of MMP regulation might contribute to the impaired clinical outcome in suboptimally stabilised bone defects.

## Summary (German)

Mechanische Bedingungen beeinflussen die frühe Phase der Knochenheilung und insbesondere die Angiogenese. Mesenchymale Stammzellen (MSCs) sind aufgrund ihrer Fähigkeit, zum Defektort zu migrieren, in Knochenzellen zu differenzieren und pro-angiogene Faktoren zu sezernieren, essentiell für den Heilungsverlauf. Bis heute ist jedoch unklar, wie MSCs auf die relevanten Belastungsbedingungen reagieren und welche Proteine die Übertragung des mechanischen Stimulus in ein verändertes Zellverhalten vermitteln. Matrix-Metalloproteasen (MMPs) und ihre Inhibitoren, die TIMPs, werden durch mechanische Stimulation in mesenchymalen Zellen reguliert und beeinflussen Zellfunktionen autokrin sowie parakrin.

In dieser Arbeit wurde untersucht, ob mechanische Belastung von MSCs zu einer Regulation der MMP/TIMP Expressionen/Aktivitäten führt, und ob dies Auswirkungen auf die Funktion der MSCs hat. Weiterhin wurde mechanisch-reguliertes MMP-2 in Bezug auf seine Regulation in MSCs sowie seine pro-angiogenen Eigenschaften überprüft.

Dass MMPs in die Migration, Proliferation und osteogene Differenzierung involviert sind, wurde anhand von MMP Breitspektruminhibitoren gezeigt. Expressionsanalysen von MMPs detektierten MMP-2, -3, -10,- 11, -13, -14 sowie TIMP-2 in MSCs auf mRNA und Protein-Niveau. Mechanische Stimulation von MSC/Fibrin-Konstrukten in einem Bioreaktor, welcher die Bedingungen der frühen Knochenheilung simuliert (30% Kompression bei 1Hz), führte zu einer Erhöhung der MMP-2, -3, -13 und TIMP-2 Proteinexpressionen sowie erhöhten gelatinolytischen Aktivitäten von MMP-2 und -13. Es wurde hingegen keine Veränderung in der Genexpression festgestellt. Durch die spezifische Inhibierung von mechanisch-reguliertem MMP-2, -3 und -13 konnte ein Einfluss von MMP-13 auf die osteogene Differenzierung gezeigt werden. Darüber hinaus scheint MMP-2 die Migration zu beeinflussen.

Detaillierte Untersuchungen von MMP-2 zeigten erhöhte gelatinolytische Aktivitäten in frühen Hämatomen, die großen interfragmentären Bewegungen ausgesetzt waren. Expressions- und Aktivitätsanalysen der mechanischen Regulation von MMP-2 in MSCs unter unterschiedlichen Belastungsbedingungen zeigten, dass 30% Kompression bei 1Hz MMP-2-induzierend und 10% Kompression bei 0,1Hz nicht-induzierend wirken. Neben einer Erhöhung von pro-MMP-2, wurden auch erhöhte Aktivitäten von aktivem MMP-2 und TIMP-2 detektiert. Zudem waren MMP-14 Aktivitäten tendenziell erhöht. Die Inhibierung von Furin unterdrückte diese Stimulation von MMP-14 und aktivem MMP-2. Eine pro-angiogene Wirkung des konditionierten Mediums (KM) von MSCs, die

MMP-2-induzierender Belastung ausgesetzt waren, konnte durch Blockierung der pro-MMP-2 Aktivierung sowie Inhibierung von aktivem MMP-2 reduziert werden. Zugabe von aktivem MMP-2 zum KM kompensierte hingegen teilweise eine ineffektive Belastung.
Diese Ergebnisse weisen darauf hin, dass die Funktion von MSCs durch MMP-/TIMP-Aktivitäten reguliert wird, welche wiederum durch mechanische Stimulation dieser Zellen unter Bedingungen normaler Heilung aktiviert werden. Im Falle von MMP-2 scheinen das Pro-Enzym sowie aktiviertes MMP-2 zu einer parakrinen Stimulation der Angiogenese durch mechanische Belastung beizutragen. Infolgedessen könnte eine Störung in der Induktion der MMP-Regulation zur Beeinträchtigung des Heilungserfolges in suboptimal-stabilisierten Knochendefekten beitragen.

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# Appendix

Table 2. Gene expression analysis of mechanically loaded MSCs (> 0.05 of GAPDH)

| Gene name | unloaded | | | loaded | | | Ratio (loaded/unloaded) | | |
|---|---|---|---|---|---|---|---|---|---|
| | Median | Min | Max | Median | Min | Max | Median | Min | Max |
| Meth 1 | 0.5 | 0.17 | 0.71 | 0.32 | 0.15 | 0.56 | 1.06 | 0.21 | 1.47 |
| Meth 2 | n.d. | n.d. | n.d. | $0.07^{1/4}$ | 0.07 | 0.07 | n.d. | n.d. | n.d. |
| Caspase 8 | n.d. | n.d. | n.d. | $0.21^{2/4}$ | 0.13 | 0.28 | n.d. | n.d. | n.d. |
| Caspase 9 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| Caveolin 1 | $0.24^{2/4}$ | 0.19 | 0.29 | $0.13^{2/4}$ | 0.08 | 0.18 | n.d. | n.d. | n.d. |
| CD44 antigen | 0.91 | 0.11 | 0.29 | 0.98 | 0.84 | 1.00 | 1.04 | 0.93 | 1.20 |
| E-cadherin | 0.23 | 0.11 | 0.34 | 0.26 | 0.08 | 0.46 | 0.81 | 0.70 | 2.74 |
| CEA | 0.63 | 0.05 | 0.84 | 0.70 | 0.47 | 0.76 | 0.93 | 0.87 | 1.74 |
| Contactin 1 | $0.07^{3/4}$ | 0.05 | 0.14 | $0.05^{1/4}$ | 0.05 | 0.05 | n.d. | n.d. | n.d. |
| Endostatin | $0.11^{2/4}$ | 0.07 | 0.14 | $0.12^{2/4}$ | 0.07 | 0.18 | n.d. | n.d. | n.d. |
| Collagen type I, α1 | 0.16 | 0.06 | 0.22 | $0.23^{3/4}$ | 0.10 | 0.24 | n.d. | n.d. | n.d. |
| Collagen type IV, α2 | $0.06^{1/4}$ | 0.06 | 0.06 | $0.08^{1/4}$ | 0.08 | 0.08 | n.d. | n.d. | n.d. |
| Cystatin C | 0.90 | 0.80 | 0.98 | 0.95 | 0.79 | 1.07 | 1.06 | 0.99 | 1.10 |
| Catenin α 1 | n.d. | n.d. | n.d. | $0.08^{1/4}$ | 0.08 | 0.08 | n.d. | n.d. | n.d. |
| Catenin α -like 1 | $0.11^{3/4}$ | 0.08 | 0.16 | $0.12^{2/4}$ | 0.07 | 0.16 | n.d. | n.d. | n.d. |
| Catenin β1 | 1.03 | 0.45 | 1.11 | 1.06 | 0.98 | 1.16 | 1.06 | 0.91 | 1.86 |
| Catenin β 1 | 0.60 | 0.05 | 0.74 | 0.59 | 0.53 | 0.75 | 1.07 | 0.85 | 1.18 |
| Catenin δ 2 | $0.05^{2/4}$ | 0.05 | 0.06 | $0.06^{1/4}$ | 0.06 | 0.06 | n.d. | n.d. | n.d. |
| Cathepsin B | $0.07^{3/4}$ | 0.07 | 0.08 | $0.05^{2/4}$ | 0.05 | 0.05 | n.d. | n.d. | n.d. |
| Cathepsin D | 0.20 | 0.06 | 0.42 | 0.29 | 0.21 | 0.49 | 1.14 | 0.79 | 2.62 |
| Cathepsin G | $0.07^{3/4}$ | 0.06 | 0.07 | $0.08^{3/4}$ | 0.08 | 0.20 | n.d. | n.d. | n.d. |
| Cathepsin L | 0.91 | 0.85 | 1.06 | 1.04 | 0.87 | 1.17 | 1.07 | 1.02 | 1.17 |
| DCC | n.d. | n.d. | n.d. | $0.08^{1/4}$ | 0.08 | 0.08 | n.d. | n.d. | n.d. |
| ECM protein 1 | 0.15 | 0.12 | 0.18 | 0.12 | 0.07 | 0.18 | 0.77 | 0.45 | 1.40 |
| Fibrinogen β | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| Fibronectin 1 | 1.08 | 1.02 | 1.10 | 1.05 | 0.98 | 1.15 | 0.96 | 0.91 | 1.13 |
| Heparanase | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| ICAM-1 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| Integrin α 1 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| Integrin α 10 | $0.10^{1/4}$ | 0.10 | 0.10 | $0.06^{2/4}$ | 0.06 | 0.06 | n.d. | n.d. | n.d. |
| Integrin α 11 | 0.95 | 0.91 | 1.00 | 0.97 | 0.96 | 1.00 | 1.04 | 0.95 | 1.06 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Integrin α 2 | n.d. | n.d. | n.d. | $0.08^{2/4}$ | 0.07 | 0.08 | n.d. | n.d. | n.d. |
| Integrin α 2b | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| Integrin α 3 | $0.12^{2/4}$ | 0.11 | 0.13 | $0.06^{3/4}$ | 0.05 | 0.15 | n.d. | n.d. | n.d. |
| Integrin α 4 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| Integrin α 5 | 0.37 | 0.24 | 0.61 | 0.38 | 0.26 | 0.53 | 1.12 | 0.42 | 2.05 |
| Integrin α 6 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| Integrin α 7 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| Integrin α 8 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| Integrin α 9 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| Integrin α L | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| Integrin α M | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| Integrin α V | $0.05^{1/4}$ | 0.05 | 0.05 | $0.06^{1/4}$ | 0.06 | 0.06 | n.d. | n.d. | n.d. |
| Integrin α X | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| Integrin β 1 | 0.26 | 0.20 | 0.66 | 0.27 | 0.23 | 0.57 | 1.00 | 0.87 | 1.19 |
| Integrin β 2 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| Integrin β 3 | 0.08 | 0.06 | 0.14 | 0.11 | 0.07 | 0.18 | 1.46 | 0.50 | 2.70 |
| Integrin β 4 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| Integrin β 5 | 0.56 | 0.25 | 0.78 | 0.57 | 0.43 | 0.69 | 1.10 | 0.80 | 1.72 |
| Integrin β 6 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| Integrin β 7 | $0.09^{1/4}$ | 0.07 | 0.09 | $0.19^{1/4}$ | 0.19 | 0.19 | n.d. | n.d. | n.d. |
| Integrin β 8 | $0.11^{2/4}$ | 0.07 | 0.15 | $0.09^{1/4}$ | 0.09 | 0.09 | n.d. | n.d. | n.d. |
| Laminin B1 | 0.19 | 0.17 | 0.60 | 0.23 | 0.15 | 0.51 | 1.04 | 0.84 | 1.26 |
| Laminin B2 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| Hyaluronidase | 0.60 | 0.06 | 0.88 | 0.81 | 0.50 | 0.88 | 1.17 | 1.01 | 2.18 |
| MUC-18 | $0.06^{1/4}$ | 0.06 | 0.06 | $0.12^{1/4}$ | 0.12 | 0.12 | n.d. | n.d. | n.d. |
| MMP-1 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| MMP-10 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| MMP-11 | $0.10^{2/4}$ | 0.06 | 0.15 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| MMP-12 | n.d. | n.d. | n.d. | $0.06^{1/4}$ | 0.06 | 0.06 | n.d. | n.d. | n.d. |
| MMP-13 | 0.54 | 0.05 | 0.84 | 0.50 | 0.20 | 0.92 | 0.97 | 0.79 | 1.21 |
| MMP-14 | $0.10^{2/4}$ | 0.05 | 0.14 | 0.08 | 0.06 | 0.17 | n.d. | n.d. | n.d. |
| MMP-15 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| MMP-16 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| MMP-17 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| MMP-2 | 0.80 | 0.06 | 0.96 | 0.75 | 0.38 | 1.04 | 1.04 | 0.39 | 1.35 |
| MMP-20 | $0.06^{1/4}$ | 0.06 | 0.06 | $0.10^{1/4}$ | 0.10 | 0.10 | n.d. | n.d. | n.d. |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| MMP-24 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| MMP-26 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| MMP-3 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| MMP-7 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| MMP-8 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| MMP-9 | 0.16 1/4 | 0.16 | 0.16 | 0.05 1/4 | 0.05 | 0.05 | n.d. | n.d. | n.d. |
| NCAM | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| NRCAM | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| PECAM-1 | 0.20 | 0.06 | 0.51 | 0.31 | 0.29 | 0.60 | 3.02 | 0.94 | 5.13 |
| tPA | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| uPA | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| uPAR | 0.07 1/4 | 0.07 | 0.07 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| ELAM-1/E-selectin | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| Selectin L | 0.06 2/4 | 0.06 | 0.07 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| Selectin P | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| PAI-2 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| Maspin | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| PAI-1 | 0.14 1/4 | 0.09 | 0.14 | 0.13 3/4 | 0.09 | 0.15 | n.d. | n.d. | n.d. |
| Osteonectin | 0.09 | 0.09 | 0.67 | 0.13 3/4 | 0.11 | 0.50 | n.d. | n.d. | n.d. |
| Osteopontin | 0.95 | 0.37 | 1.00 | 1.01 | 0.95 | 1.04 | 1.05 | 1.01 | 1.08 |
| Thrombospondin 1 | 0.75 | 0.37 | 0.92 | 0.45 | 0.1 | 0.76 | 0.64 | 0.11 | 1.30 |
| Thrombospondin 1 | 1.04 | 0.17 | 1.08 | 1.02 | 0.97 | 1.08 | 1.00 | 0.96 | 1.02 |
| Thrombospondin 1 | 0.17 1/4 | 0.17 | 0.17 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| TIMP-1 | 0.17 1/4 | 0.17 | 0.17 | 0.06 1/4 | 0.06 | 0.06 | n.d. | n.d. | n.d. |
| TIMP-2 | 0.34 | 0.08 | 0.57 | 0.36 | 0.3 | 0.54 | 1.38 | 0.63 | 2.74 |
| TIMP-3 | 0.08 1/4 | 0.08 | 0.08 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| TMPRSS4 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| VCAM1 | 0.57 | 0.08 | 0.86 | 0.58 | 0.16 | 0.84 | 0.77 | 0.46 | 1.85 |
| Vitronectin | 0.10 3/4 | 0.08 | 0.51 | 0.12 3/4 | 0.06 | 0.30 | n.d. | n.d. | n.d. |
| Cyclophilin A | 0.21 | 0.13 | 0.32 | 0.27 | 0.13 | 0.30 | 1.34 | 0.45 | 2.40 |
| Ribos. protein L13a | 0.46 1/4 | 0.07 | 0.46 | 0.07 1/4 | 0.07 | 0.07 | n.d. | n.d. | n.d. |
| β-actin | 0.12 | 0.07 | 0.19 | 0.09 | 0.06 | 0.15 | 0.73 | 0.50 | 1.68 |

| | |
|---|---|
| 1/4 | detected in 1 out of 4 donors |
| 2/4 | detected in 2 out of 4 donors |
| 3/4 | detected in 3 out of 4 donors |
| lack of exponent | detected in 4 out of 4 donors |
| n.d. | not determined |

*Anteils-Erklärung meiner Person an in diese Dissertation eingegangene Publikationen*

Kasper G†, Glaeser JD†, Geissler S, Ode A, Tuischer J, Matziolis G, Perka C, and Duda GN, MMP activity is an essential link between mechanical stimulus and mesenchymal stem cell behaviour, Stem Cells, 2007, 25:1985-1994, †: authors contributed equally

- Konzeption: das Konzept dieser Arbeit wurde von meiner Betreuerin Dr.-Ing. Grit Kasper erstellt.

- Durchführung: Die Charaktierisierung der MSCs mittels FACS-Analyse erfolgte durch Dipl.-Ing. Miriam Tschirschmann, TU-Berlin. Bei der Durchführung der funktionellen Assays wurde ich teilweise von unserer technischen Assistentin Marzena Princ unterstützt. Alle weiteren Versuche wurden von mir durchgeführt.

- Berichtsabfassung: das Manuskript wurde zu gleichen Teilen von Frau Dr.-Ing. Grit Kasper und mir verfasst.

Glaeser JD†, Geissler S†, Strube P, Schipp CJ, Matziolis G, Taylor WR, Knaus P, Perka C, Duda GN and Kasper G, Control of pro-MMP-2 levels and activation of MMP-2 via the furin/MMP-14/TIMP-2 axis contribute concurrently to the pro-angiogenic effects of mechanically stimulated mesenchymal stem cells, submitted, 2008, †: authors contributed equally

- Konzeption: das Konzept dieser Arbeit wurde von mir erstellt.

- Durchführung: Die Optimierung der einzelnen Belastungsparameter erfolgte durch Dipl.-Ing. Sven Geissler (FH) unter meiner Anleitung im Rahmen seiner Masterarbeit, die er 2006-2007 am Julius Wolff Institut anfertigte. Alle weiteren Versuche wurden von mir durchgeführt.

- Berichtsabfassung: das Manuskript wurde von mir verfasst.

Kasper G, Dankert N, Tuischer J, Hoeft M, Gaber T, Glaeser JD, Zander D, Tschirschmann M, Thompson M, Matziolis G and Duda GN, Mesenchymal stem cells regulate angiogenesis according to their mechanical environment, Stem Cells, 2007, 25:903-910

- Konzeption: das Konzept dieser Arbeit wurde von meiner Betreuerin Dr.-Ing. Grit Kasper erstellt.

- Durchführung: Die Zymogramm-Daten bezüglich MMP-2 wurden von mir beigesteuert.

- Berichtsabfassung: das Manuskript wurde von Frau Dr.-Ing. Grit Kasper verfasst.

*Eidesstattliche Erklärung*

Hiermit erkläre ich, Juliane Dagmar Gläser, an Eides statt, dass ich die vorgelegte Dissertation selbst verfasst habe und keine anderen als die angegebenen Quellen und Hilfsmittel verwendet habe. Außerdem erkläre ich, dass ich an keiner anderen Stelle ein Promotionsverfahren beantragt habe.

Berlin den _____ _____

Juliane Gläser