THE ROLE OF MATRIX METALLOPROTEINASES AND INFLAMMATORY CYTOKINES ON HUMAN MESENCHYMAL STEM CELL INVASIVENESS AND DIFFERENTIATION CAPACITY

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Dedicado a mis padres

PROLOGUE

Una niña muy sencilla
con zapatos de tacón,
pues solo con eso brilla
y sobresale del montón.

Sus ojos son dos luceros
que imponen claridad,
son sus minimos deseos
una única verdad.

Su pelo negro rizado
es signo de distinción
y su perfecto acabado
por todo causa impresión.

La dentadura que tiene
es por gracia de Dios,
se sonrie cuando quiere
y provoca sensación.

Pues era niña bonita
que la miras y te extraña,
con eso se justifica,
¡que ha nacido en España!

Emilia de la Torre

CONTENTS

ABBREVIATIONS

ASC	adult stem cell
BMSC	bone marrow derived stem cell
BMP-2	bone morphogenic protein-2
cDNA	complementary deoxyribonucleic acid
CNS	central nervous system
CXCR4	chemokine (C-X-C motif) receptor 4
DMEM	dulbecco's modified eagle medium
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
dNTP	3´-deoxynucleotide-5´-triphosphate
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
ERK	extracellular signal-regulated kinase
ESC	embryonic stem cell
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
GFAP	glial fribrillary acidic protein
hMSC	human mesenchymal stem cell
HUVEC	human umbilical vein endothelial cells
IL-1β	interleukin-1beta
JNK	C-Jun N-terminal kinase
kDa	kilodalton
LIF	leukemic inhibitory factor
MAP-2	microtubullary acidic protein-2
MAPK	mitogen-activated protein kinase
MEK	MAPK/ERK kinase
mRNA	messenger ribonucleic acid
MMP	matrix metalloproteinase
MT1-MMP	membrane type 1- matrix metalloproteinase
NFκβ	nuclear factor kappa beta
NSC	neural stem cell
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
RNA	ribonucleic acid
RNAi	RNA interference

RPMI	“Roosewelt Park Memorial Institute”
RT-PCR	reverse transcriptase-polymerase chain reaction
SD	standard deviation
SDF-1α	stroma cell-derived factor-1alpha
SDS	sodium dodecyl sulphate
siRNA	silencing RNA
SOX-2	SRY-box containing gene-2
TF	transcription factor
TGF-1β	transforming growth factor-1beta
TEMED	n,n,n,n´-tetramethylendiamine
TIMP	tissue inhibitor of matrix metalloproteinase
TNF-α	tumor necrosis factor-alpha
TRIS	tris hydroxymethylaminoethane
v/v	volume per volume

The abbreviations and symbols are listed according to the recommendations of the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (IUBMB) and the Joint Commission on Biochemical Nomenclature. All approved human gene symbols can be found in the database of the Human Gene Nomenclature Committee (HGNC): *(http://www.genenames.org/guidelines.html).*

A SUMMARY

Tissue repair requires a well orchestrated integration of various cellular and molecular events such as cell migration, proliferation, differentiation, and remodelling of extracellular matrix. Recent studies provide evidence that bone marrow-derived stem cells including human mesenchymal stem cells (hMSC) may be involved in these processes. Endogenous hMSC are thought to be mobilized from bone marrow to migrate to sites of injury and inflammation, where they participate in the repair of damaged areas. Furthermore, transplantation of hMSC into injured tissues was shown to improve the regeneration process through differentiation of hMSC into tissue types such as bone, fat, and cartilage involving the release of various paracrine factors from these cells. Therefore, an enhanced understanding of the mechanisms controlling hMSC functions is required for the development of novel cell-based therapies.

At the beginning of this study almost nothing was known about the molecular requirements that direct hMSC movement through extracellular matrix (ECM) barriers such as basement membranes finally enabling tissue repair at the impaired body site. Since degradation of ECM mediated by specific proteolytic enzymes, in particular by matrix metalloproteinases (MMPs), is an important prerequisite for cell migration, we were primarily interested to study the role of MMPs and related factors for hMSC migration.

Consequently, the first aim of this study was to analyze the expression and functional role of MMPs and the endogenous tissue inhibitors of metalloproteinases (TIMPs) in the capacity of hMSC for transmigrating through human reconstituted basement membranes. This was achieved by use of various molecular biological, biochemical and cellular methods including qRT-PCR, RNA interference (RNAi), Western blotting, and the establishment of a modified Transwell-invasion assay solely applying human components.

Thereby we were able to demonstrate that hMSC exhibit a strong constitutive expression and synthesis of MMP-2 and membrane type 1 (MT1)-MMP as well as of their inhibitors TIMP-1 and TIMP-2. hMSC were shown to traverse reconstituted human extracellular matrix (hECM) which was effectively blocked by addition of synthetic MMP inhibitors. Detailed studies applying RNAi revealed that gene knock-down of MMP-2, MT1-MMP, or TIMP-2 substantially impaired hMSC invasion capacity, whereas silencing of TIMP-1 enhanced cell migration, indicating opposing roles of both TIMPs in this process.

Moreover, for the first time preliminary data provide evidence for the existence of a novel crosslink between the endogeneous TIMP-1 and the Wnt signalling pathway, repressing hMSC proliferation, differentiation, and invasion capacity.

Since inflammatory cytokines and chemokines are known to mobilize immune cells and leukocytes to tissue sites of injury, a potential importance of these factors in MMP/TIMP-mediated invasiveness of hMSC was investigated. The inflammatory cytokines TGF-β1, Il-1β, and TNF-α were found to upregulate MMP-2, MT1-MMP, and/or MMP-9 production in hMSC which resulted in a strong stimulation of chemotactic migration through ECM, whereas the chemokine SDF-1α exhibited minor effects on MMP/TIMP expression and cell invasiveness.

One interesting feature of hMSC is their capacity to transdifferentiate across the mesodermal lineages into non-mesodermal cell types including glial- and neuronal-like cells. This plasticity renders hMSC as a valuable candidate for autologous replacement of damaged tissues in neurodegenerative disorders. However, little is known about the molecular mechanisms which regulate the hMSC plasticity.

In this context, we were able to demonstrate for the first time that the inflammatory cytokine TNF-α induces neural properties in hMSC. After incubation of hMSC for 14-28 days with TNF-α, the cells acquired neuroglial-like morphology without change in cell proliferation. In particular, they gained the ability to form neurosphere-like structures as an attribute characteristic of neural progenitor cells. In addition, TNF-α significantly upregulated the expression of numerous genes such as *LIF*, *BMP2*, *SOX2*, *GFAP* and *MAP2* which are important to neural cell growth and function, whereas *NES* transcription ceased, suggesting a neuroglial-like character in these cells.

Moreover, studies on intracellular MAPK signalling revealed that inhibition of ERK1/2 activity but not of p38 and JNK abolished the TNF-α-mediated regulation of neural genes, indicating a pivotal role of ERK1/2 in neural transdifferentiation of hMSC. Strikingly, TNF-α significantly enhanced expression of the chemokine receptor CXCR4 in hMSC during 14 days of incubation which was shown to facilitate chemotactic migration of these cells towards SDF-1α, a chemokine known to be augmented in injured brain tissues.

Taken together, in this dissertation it has been elucidated for the first time in detail that hMSC constitutively express particular MMPs and TIMPs which are upregulated by inflammatory cytokines promoting the directed migration of hMSC across human reconstituted basement membranes *in vitro.* Interestingly, endogenous TIMP-1 may act as a repressor of hMSC invasion, proliferation, and differentiation. Moreover, it has been demonstrated that *in vitro* incubation of hMSC with TNF-α triggers neuroglial gene expression and enhances CXCR4-mediated chemotactic invasiveness towards SDF-1. These results indicate a potential mechanism for the recruitment and extravasation of hMSC into injured tissues *in vivo* and may provide a base for the use of autologous hMSC for the treatment of neurological disorders.

B INTRODUCTION

One of the most exciting expectations of the scientific community lays in the biology of stem cells due to their ability to renew themselves through symmetric cell division and differentiate via asymmetric division into a diverse range of specialized cell types.[1] Therefore, it is to be hoped that stem cells will play a crucial role in the treatment of a large number of so far incurable diseases.

Recent studies have shown that various tissues accommodate adult stem cells that are involved in specific tissue homeostasis and tissue repair upon injury. Yet, on the other hand, increasing evidence suggest that stem cells may also be a source of cancer formation.[2] Hence, detailed understanding of the regulatory mechanisms controlling stem cell functions is one of the most important challenges for future science.

In contrast to adult stem cells with restricted differentiation capacity embryonic stem cells are totipotent cells which can give rise to any cell of the body. However, the isolation of human embryonic stem cells, at least with methods available now, necessarily involves the sacrifice of an embryo. This critical step in the procurement of stem cells has stimulated intense discussion at all levels of academia, government, and society in general.[3-5] Fortunately, over the past few years an alternative to this ethical dilemma developed, as evidence accumulated indicating that adult stem cells from bone marrow might have pluripotent properties similar in some respect to that of embryonic stem cells.[6]

B.1 Human mesenchymal stem cells (hMSC)

Adult stem cells are defined as multipotent cells which can produce a limited number of different cell types.[7,8] Among all types of adult stem cells, bone marrow-derived stem cells (BMSC) are unique with regard to their abundance and their role in the continuous lifelong physiological replenishment of numerous different cell species. Traditionally, BMSC are thought to develop into haematopoietic and mesenchymal stem cells. Human mesenchymal stem cells (hMSC) contribute to the regeneration of mesenchymal tissues such as bone, fat, and cartilage.[9] In addition, hMSC are essential in providing support for the growth and differentiation of primitive haematopoietic cells within the bone marrow microenvironment.

Although adult stem cells may not be as 'powerful' and diverse as embryonic stem cells, accumulating evidence indicates that particularly hMSC might have a broader differentiation potential than previously thought.[10,11] Moreover, hMSC offer many advantages for the development of cellular therapeutics due to their ease of isolation and expansion, stable phenotype, and compatibility with different delivery methods. Thus, hMSC provide a substantial opportunity in the development of innovative therapeutical applications.

B.1.1 Isolation and characterization of hMSC

hMSC are present in various tissues such as calvaria, periosteum, and muscle of adult persons.[12,13] Due to its accessibility the bone marrow (BM) is the primary source for isolation of hMSC.

Purification of hMSC from bone marrow aspirates is achieved according to a protocol by Friedenstein et al.[14] based on the adherence of the cells to the plastic surface of cell culture plates, whereas haematopoietic cells remain in solution.[15,16] Purified hMSC represent a heterogeneous cell population consisting of three major cell types described as spindle-shaped cells, large cuboidal or flattened cells, and rapidly self-renewing cells.[17,18] Despite their heterogeneity, hMSC populations from different sources show high reproducibility in their biological functions[19] especially with respect to their mesodermal differentiation capacity.

Meanwhile, the presence or absence of a variety of cells markers have turned out to be valuable tools for hMSC charazterization as mentioned next.

B.1.1.1 Positive protein markers

hMSC expressing Stro-1 were shown to differentiate into adipocytes, osteoblasts, chondrocytes, smooth muscle cells, and fibroblasts supporting haematopoietic stem cells.[20] However, Stro-1 is unlikely to be a general MSC marker because its expression is not exclusively seen in MSCs and it is lost during culture expansion.[21] This limits the single use of Stro-1 for MSC identification and isolation. However, a combination of CD105, CD166, CD29 and CD44 has proven to be useful in the molecular characterization of hMSC.[22]

B.1.1.2 Negative protein markers

There is a consensus that hMSC do not express CD11b (an immune cell marker), CD14 (a macrophage cell marker), glycophorin-A (an erythroid lineage marker), or CD45 (a marker of all haematopoietic cells). CD34 (a marker of primitive haematopoietic stem cells) is rarely expressed in hMSC, although being present in mouse MSCs. CD31 (expressed on endothelial and haematopoietic cells) and CD117 (a haematopoietic stem/progenitor cell marker) are normally absent in hMSC.[22]

B.1.2 Self-renewal and differentiation of hMSC

A characteristic feature of stem cells is their ability for asymmetric cell division, i.e the generation of identical cell copies by proliferation ("self-renewal") as well as differentiation towards specified cell types.

As graphically depicted in figure 1, genomic array analyses have identified molecular signatures maintaining stemness in stem cells like hMSC.[23] Growth factors and cytokines such as leukemia inhibitory factor (LIF)[24,25] or fibroblast growth factors (FGFs),[26,27] as well as Wnt proteins[28,29] were shown to be implicated in the control of MSC self-renewal. Nevertheless, the identification of specific signalling networks and "master" regulatory genes that govern the unique MSC differentiation lineages still remain a challenge. Detailed knowledge about biological effectors which maintain a desired differentiation program and prevent unwanted maturation of hMSC is a prerequisite for effective clinical application of these cells.

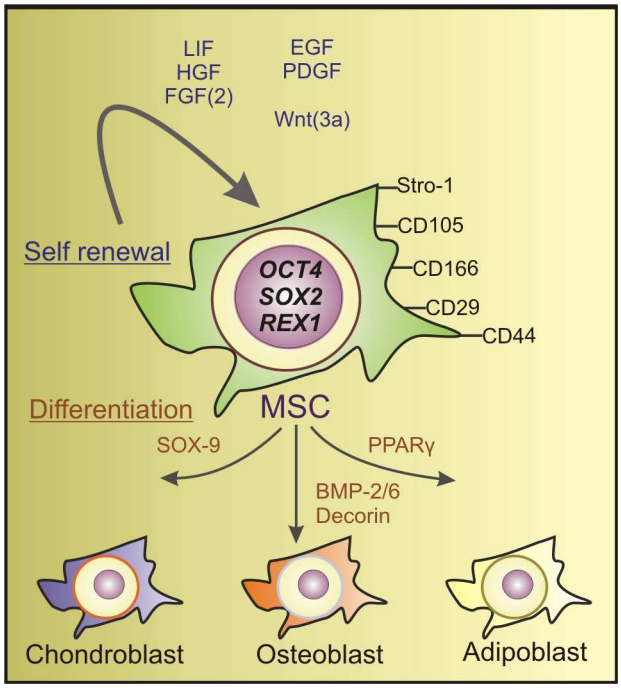

Fig. 1*:* Genes and factors involved in hMSC self-renewal and mesodermal differentiation.

Extracellular signalling factors including growth factors, and cytokines, and Wnt proteins have been demonstrated to maintain mesenchymal stem cell (MSC) self-renewal or induce mesodermal differentiation *in vitro*. (For details see text).

A further major characteristic of hMSC is their capacity for differentiation into the three mesodermal lineages of chondrocytes, osteocytes, and adipocytes (Fig.1). However, the identification of molecular mechanisms controlling the maturation of hMSC still remains a great challenge.[9]

B.1.2.1 Chondrogenesis

Chondrogenic differentiation of MSCs *in vitro* mimics that of cartilage development *in vivo*.[30] TGF-β3, dexamethasone, and thyroxine are known to efficiently promote the chondrogenesis of hMSC *in vitro* which can be examined by detection of anionic sulfated proteoglycans deposition in the extracellular space using Safranin O staining and toluidine blue.[30]

Several expression markers associated with chondrogenesis have been positively characterized in MSC-derived chondrocytes, including various transcription factors such as Sox-9 and components of the extracellular matrix (ECM) like the collagen types II and IX, aggrecan, biglycan, and cartilage oligomeric matrix protein.[31,32] However, specific signalling pathways that induce the expression of these and other chondrogenic genes are still unknown.

B.1.2.2 Osteogenesis

Differentiation of hMSC into osteoblastic cells can be induced *in vitro* by a combination of dexamethasone, *β*-glycerophosphate, and L-ascorbic acid 2-phosphate. After osteogenic differentiation, calcium depositon can be demonstrated by Alizarin-Red staining of intracellular mineralized nodules.

Several molecular markers are well characterised for osteogenic differentiation. For example, decorin, bone morphogenic protein (BMP)-2, and BMP-6 were shown to promote osteogenesis in hMSC by acetylation of the RUNX2 gene.[33]

B.1.2.3 Adipogenesis

A combination of L-glutamine, dexamethasone, indomethacine, insulin, and 3-isobutyl-1-methylxanthine is used to induce adipogenic differentiation in hMSC, which can be verified by staining of intracellular fat vacuoles with Oil-Red-O.

One strong marker for the adipogenic differentiation is the peroxisome proliferator-activated receptor γ (PPARγ), a nuclear hormone receptor which is critical in promoting adipogenesis in hMSC while repressing osteogenesis.[34] The binding of PPARγ to various ligands, including long-chain fatty acids and thiazolidinedione compounds, induces the transactivation and transrepression of PPARγ. Recently the bipotent coregulator tafazzin (TAZ) was discovered to function as a coactivator of RUNX2 and as a corepressor of PPARγ, thus promoting osteogenesis while blocking adipogenesis.[35]

B.1.3 Transdifferentiation of hMSC (plasticity)

Bone marrow-derived human mesenchymal stem cells (hMSC) were previously thought to be lineage-restricted and only able to differentiate into mesodermal cell types such as adipocytes, chondrocytes, and osteocytes.[9] Accumulating data from the past years, however, provide evidence that hMSC can transdifferentiate across lineage barriers ("plasticity") and adopt expression profiles as well as functional phenotypes of non-mesodermal types such as neuroectodermal, endodermal, and visceral mesodermal cells.[10,11,36]

One of the most striking properties of hMSC in this context is their potential for transdifferentiation into neural cells of both glial and neuronal lineages. This has been shown *in vitro* [36-40] and *in vivo*[41,42] after transplantation of hMSC into the brain and spinal cord. Despite these multiple potentials of hMSC, little is known about the molecular mechanisms which regulate hMSC transdifferentiation into neural-like cells. Nevertheless, the clinical findings made hMSC valuable candidates for cell-based therapies in patients with developmental and neurodegenerative disorders of the central nervous system (CNS) including Parkinson´s disease, Alzheimer´s disease, Huntington´s disease, and amyotrophic lateral sklerosis.[43-46]

B.1.4 Tissue repair and regeneration: Clinical applications of hMSC

Therapeutical concepts based on the application of embryonic and neural stem cells are limited by logistic and ethical problems. The benefit of hMSC in clinical use is supported by easy accessibility and isolation from bone marrow, *in vitro* expansion in cell culture, and possibility for retransplantation into the same patient without the drawbacks associated with allotransplantation and xenotransplantation. *In vivo* studies showed that MSCs after peripheral injection can cross the blood-brain barrier and migrate to damaged areas in the brain, where they improve functional recovery in e.g. patients with ischemic stroke.[47,48]

Another interesting observation is that systemically delivered hMSC are mobilized to and integrate into brain tumor tissues [49,50] which is similar to that in neural stem cells.[51] After intravascular or local delivery, hMSC were shown to specifically integrate into glioma tissue attracted by the release of cytokines and growth factors, suggesting hMSC as delivery vehicles in brain tumor therapy.[49,50,52]

A remarkable but less understood finding is the importance of hMSC in physiological process of tissue repair and regeneration of hMSC. These cells are mobilized to travel from bone marrow or peripheral blood into damaged tissues for re-colonization and differentiation into new cell types. Transplantation experiments in animals and patients demonstrated that MSCs migrate to sites of injury, where they enhance wound healing,[53] support tissue regeneration following myocardial infarction,[54] home to and promote the restoration of bone marrow microenvironment after damage by myeloablative chemotherapy,[55] or help to overcome the molecular defect in children with osteogenesis imperfecta.[56]

B.1.5 hMSC migration and invasion through ECM

In haematopoetic stem cells detailed studies have demonstrated that homing from blood into bone marrow as well as their mobilization from bone marrow into blood and tissues is mainly controlled by cytokines/chemokines, adhesion molecules, and proteolytic enzymes.[57,58] However, the molecular mechanisms regulating cell movement across extracellular matrix (ECM), relocalization, and specific differentiation in hMSC are not clarified in detail, so far.

The ECM is a dynamic fibrillar protein meshwork comprising collagens, proteoglycans, fibronectin, and laminin which form barriers between tissue compartments and provide a structural scaffold for tissue support.[59] Basement membranes represent a specialized form of ECM that separate epithelium or endothelium from stroma by a dense layer of ECM. Moreover, ECM serves as a reservoir for growth factors that are released during degradation and remodeling. ECM turnover also leads to the generation of fragments capable of mediating signals through their interactions with cell surface adhesion/receptor proteins.

A key requirement for migrating cells to reach distant target sites is the ability to overcome ECM barriers. These processes are mediated by specific proteolytic enzymes including metallo-, serine-, and cysteine proteinases. Since matrix metalloproteinases are supposed to be the main ECM-degrading proteinases, a short review of these enzymes and their regulatory endogenous inhibitors is given in the following chapter.

B.2 Matrix metalloproteinases (MMPs) and their endogenous inhibitors

The MMP family comprises 23 Zn^{2+}-dependent endopeptidases capable of degrading almost all ECM components. Consequently, MMPs are found to be involved in various physiological and pathological processes.[60] All MMPs are produced in a zymogen form, which requires activation of the proenzymes by removal of their N-terminal propeptide (Fig. 2).

The MMPs can be divided according to their preferential substrates in gelatinases (MMP-2, MMP-9), collagenases (e.g. MMP-1), and stromelysins (e.g. MMP-3). Another classification is based on their domain structure as depicted in Fig. 2. With respect to the prominent role of the gelatinases MMP-2 and MMP-9, and the membrane type 1 (MT1)-MMP in cell migration, these particular MMPs are described in more detail.

B.2.1 The gelatinases MMP-2 and MMP-9

Gelatinase A (MMP-2) and gelatinase B (MMP-9) form a MMP-subgroup due to their ability to preferentially cleave denatured collagens (gelatin), laminin, and collagen type IV as the major constituent of basement membranes.[59,60] Additionaly, MMP-2 digests the collagens type I, II, and III.[61,62] Biosynthesis and activity of the gelatinases are associated with the invasive capacities of various cell types such as leukocytes, endothelial cells, and metastasizing tumour cells.[63] These enzymes have three repeats of a type II fibronectin domain inserted in the catalytic domain, which mediates binding to gelatin.

MMP-2 seems to be especially important for osteogenesis in humans,[64] since mutations in the MMP-2 gene resulting in the absence of active enzyme are linked with an autosomal recessive form of multicentric osteolysis, a rare genetic disorder that causes destruction and resorption of the affected bones.[64] In mice, knock-out of MMP-2 does not cause any apparent abnormality,[65] suggesting redundancy in function among the MMP-group.

The two gelatinases MMP-2 and MMP-9 are secreted from the cells as latent zymogens which are rapidly complexed by the specific endogenous tissue inhibitors of metalloproteinases TIMP-2 and TIMP-1, respectively. Like all MMPs, they require activation by proteolytic removal of the N-terminal proenzyme domain. Whereas proMMP-9 is converted into its active form by cleavage through soluble proteinases such as MMP-3 and plasmin, proMMP-2 is activated on the cell surface by a unique mechanism implicating TIMP-2 as well as membrane-type 1 (MT1)-MMP.[66]

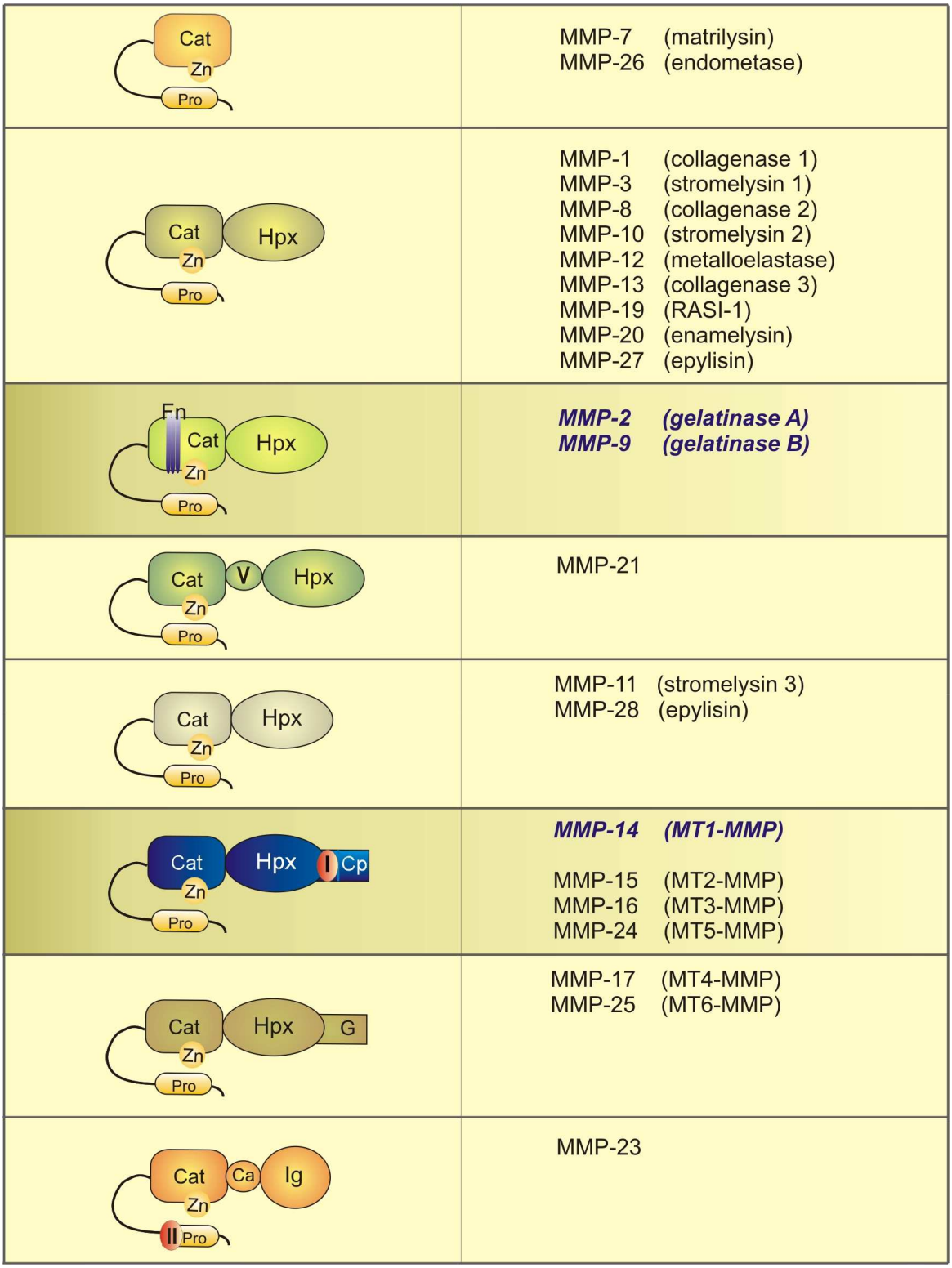

Fig. 2*:* Domain structure of MMPs.

The domain organization of MMPs is as indicated:

Pro, propeptide; **Cat**, catalytic domain; **Zn**, active-site Zn^{2+}; **Hpx**, hemopexin domain; **Fn**, fibronectin domain; **V**, vitronectin insert; **I**, type I transmembrane domain; **II**, type II transmembrane domain; **G**, GPI anchor; **Cp**, cytoplasmatic domain; **Ca**, cysteine array region; and **Ig**, IgG-like domain.

(Figure taken from Visse et al. [60] with some modifications).

B.2.2 The membrane-type 1 (MT1)-MMP and activation of MMP-2

Another MMP-subgroup is represented by specialised MMPs, the so-called MT-MMPs which are integrated into the plasma membrane of the cell. This is mediated via a transmembrane domain, either type I or type II with a GPI anchor (Fig. 2). MT-MMPs can digest a variety of ECM molecules.

The zymogen form of MT1-MMP (MMP-14) is intracellularly activated during the secretory pathway by furin or furin-like convertases.[67] MT1-MMP has collagenolytic activity by cleaving collagens type I, II, and III which explains that mice lacking the MT1-MMP gene exhibit skeletal abnormalities during postnatal development.[68] MT1-MMP also plays an important role in angiogenesis by mediating the activation of proMMP-2.[69]

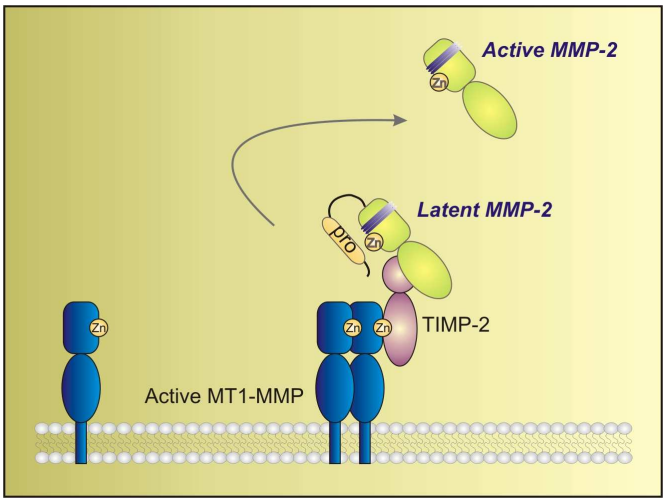

Fig. 3: Current model of proMMP-2 activation by MT1-MMP in the presence of TIMP-2.

For details see text.

ProMMP-2 activation (Fig. 3) has been extensively studied, because it is an important step for cancer cells to invade ECM barriers. The activation process is not a simple interaction of proMMP-2 and MT1-MMP but involves the endogenous inhibitor TIMP-2 as well.[70] MT1-MMP on the cell surface forms a complex with TIMP-2 through the catalytic domain of the enzyme and the N-terminal inhibitory domain of TIMP-2. Then the C-terminal domain of TIMP-2 binds to the hemopexin domain of proMMP-2, which results in the formation of a ternary complex consisting of MT1-MMP, TIMP-2, and proMMP-2.[70] The interaction of these molecules is essential and proMMP-2 activation does not occur in the absence oft TIMP-2.[71,72] Since MT1-MMP in this system is inhibited by TIMP-2, another MT1-MMP free of TIMP-2 is required to carry out the activation of proMMP-2. This is achieved by homo-dimerization of two MT1-MMP molecules via binding of their hemopexin and/or transmembrane/cytoplasmic domains.[73,74] In this complex one of the MT1-MMP molecules acts as a receptor and the other one acts as an activator. MT1-MMP homodimerization is crucial in proMMP-2 activation because their separation effectively inhibits this process.[74]

B.2.3 Regulation of MMP activity

In addition to degrading numerous ECM substrates, MMPs were also demonstrated to cleave (activate/inactivate) various non-matrix proteins such as cytokines, chemokines, and their receptors.[75] Thus, MMPs should not be viewed solely as proteinases for matrix catalysis, but rather as extracellular processing enzymes involved in cell-cell and cell-matrix signalling events.[76-78]

As for all secreted proteinases, the catalytic activity of MMPs is primarily regulated by proenzyme activation and inhibition of active MMPs.

B.2.3.1 Activation of MMPs via the cysteine-switch

After biosynthesis in the cell, proMMPs are kept in a catalytically inactive state by interaction between the thiol group of the cysteine residue present in the prodomain and the zinc ion in the catalytic site. To become catalytically active, the thiol-Zn^{2+} interaction in the proMMP must be disrupted ("cysteine-switch").[79] Basically, this can occur by three mechanisms (Fig.4):

(I) *Non-proteolytic way*: modification of the thiol by physiological oxidants, disulfides, electrophiles, or by non-physiologic compounds such as alkylating agents or heavy metal ions.[80]

(II) *Proteolytic way*: direct cleavage of the prodomain by another proteinase.

(III) *Autocatalytic way*: chemical or allosteric perturbation of the zymogen for the induction of an inter- or intra-molecular autocatalytic cleavage of the prodomain.

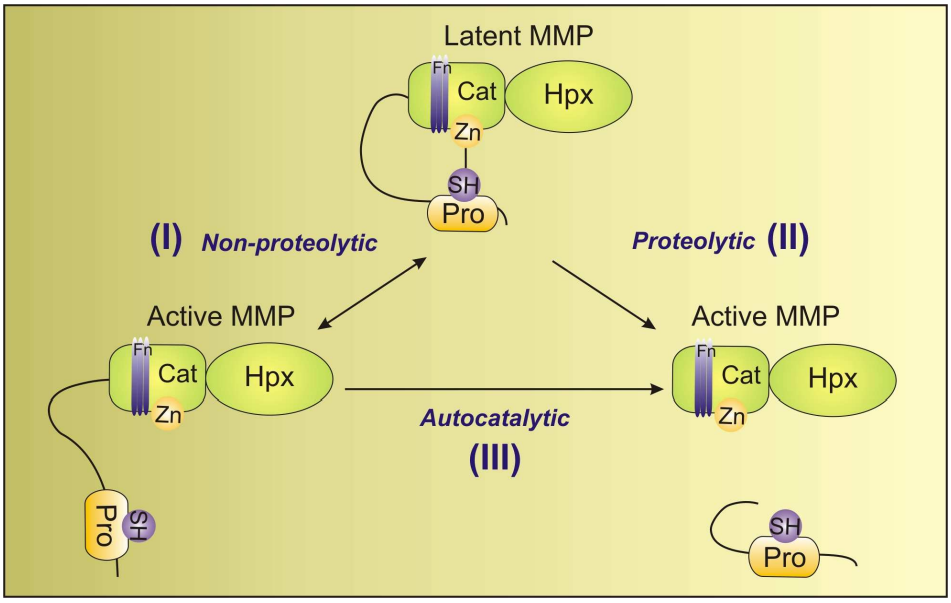

Fig. 4*:* Mechanisms of proMMP activation.
For details see text.

B.2.3.2 Inhibition by tissue inhibitors of metalloproteinases (TIMPs)

The TIMPs are specific endogenous inhibitors that bind to MMPs in a 1:1 stoichiometry. Four TIMPs (TIMP-1, TIMP-2, TIMP-3, and TIMP-4) have been identified in vertebrates[81] and were shown to play important roles in the development and remodelling of tissues.

TIMPs have a two-domain structure with N- and C-terminal regions that each contain six conserved cysteine residues forming three disulfide bonds.[82,83] The N-terminal domain comprises a region of higher homology among the four TIMPs and is sufficient for inhibition of MMP activity.[82] This domain holds residues that interact with the Zn^{2+}-binding pocket of active MMPs. The C-terminal domain of TIMPs is important for protein-protein interactions and binding to proMMPs, thereby regulating the MMP activation process.[84]

TIMP-1 and TIMP-2 can bind proMMP-9 and proMMP-2, respectively, through the C-terminal hemopexin-like domain present in the two gelatinases.[85] Although, TIMP-2 and TIMP-4 can bind to proMMP-2 and inhibit MMP-2 and MT1-MMP activities, only TIMP-2 but not TIMP-4 is able to form a trimolecular complex with proMMP-2 and MT1-MMP as a prerequisite of proMMP-2 activation.[86] The C-terminal domain of TIMP-3 also mediates unique protein-protein interactions by its binding to ECM components, whereas the other TIMPs are only found in soluble forms.[87]

B.2.4 TIMPs as signalling molecules

Interestingly, distinct from their MMP inhibitory activity, accumulating evidence indicates that TIMPs also act as signalling molecules.[88,89] This notion was further supported by recent findings of the cell binding partners of TIMP-2 and -3, the integrin α3β1 and vascular endothelial growth factor (VEGF) receptor-2, respectively.[88,89] Another discovery considers CD63, a member of the tetraspanin family of membrane proteins, as a TIMP-1 interacting protein which also substantiates the role of TIMP-1 as a signalling molecule.[90]

By now, the intracellular signalling pathways involved in these new roles of TIMPs are not clear. Furthermore, nothing is known, wether the processes are MMP-dependent or independent.

B.3 The inflammatory cytokines

Cytokines are proteins and peptides which mediate intercellular communication. They are produced by a variety of cell types and play central roles in the immune system being involved in immunological as well as inflammatory diseases. Inflammatory cytokines such as transforming growth factor (TGF)-β1, interleukin (Il)-1β, and tumor necrosis factor (TNF)-α,[91-94] are especially increased in damaged and inflamed tissues[95] thereby activating inflammatory cells to release detrimental substances as for example proteases and oxygen radicals. Further, chemokines, like stromal-derived factor (SDF)-1α represent small chemotactic peptides that direct the movement of circulating leukocytes to sites of inflammation and injury.[96]

TNF-α is a pleiotropic cytokine playing important albeit often contradictory roles in numerous physiological and pathophysiological processes including immunity and inflammation.[97,98] Following cerebral ischemic injury, TNF-α is induced during a period time from minutes up to a few hours and persists in the damaged tissue during the following days.[99] TNF-α is present at elevated levels and implicated in various neurodegenerative disorders such as Alzheimer´s disease, Parkinson´s disease, amyotrophic lateral sklerosis, and stroke.[100,101] On the other hand, TNF-α is reported to play a neuroprotective role and is believed to contribute to repair and recovery after stroke.[102,103] The biological and cellular effects of TNF-α are mediated through two cell surface receptors, the TNF receptor type 1 (TNF-R1) and type 2 (TNF-R2). TNF-R1 is stimulated by soluble TNF-α, while TNF-R2 is activated by the membrane-bound precursor preTNF-α.[104] Shedding of preTNF-α from the cell surface is accomplished by the membrane-associated TNF-α converting enzyme (TACE).[105] Intracellular transmission of TNF-α signalling has been reported to occur via several different pathways including that of NFκβ and the mitogen-activated protein kinases (MAPKs).[101]

We speculate that the presence of certain cytokines, chemokines, and/or growth factors at diseased or damaged tissues might mobilize hMSC from bone marrow, peripheral blood, or surrounding tissues into the defective sites. This process may be enabled via upregulation of MMP activity in these cells. This concept is supported by own recent findings showing that Wnt3a enhances MT1-MMP-dependent migration of bone marrow-derived hMSC through human reconstituted basement membranes.[106] Moreover, previous studies of others had demonstrated that SDF-1 and hepatocyte growth factor (HGF) stimulates chemoinvasion of hMSC across Matrigel implicating MT1-MMP activity.[107] Nevertheless, detailled analysis on the role of inflammatory cytokines in hMSC invasion and identification of particular intracellular signal transduction pathways such as the MAPK-pathways or the Wnt/β-catenin pathway involved in this process has not been performed, so far.

B.4 The mitogen-activated protein kinase (MAPK) signalling pathways

As mentioned before, MAPKs play a crucial role in the signalling of the inflammatory cytokine TNF-α, but the MAPK signalling cascade can be also activated by a wide variety of other stimuli. With respect to these stimuli three major cascade pathways can be differentiated (Fig. 5). In general, ERK1 and ERK2 are preferentially activated in response to growth factors and phorbol esters, while the JNK and p38 kinases are more responsive to stress stimuli ranging from osmotic shock and ionizing radiation to cytokine stimulation. [108]

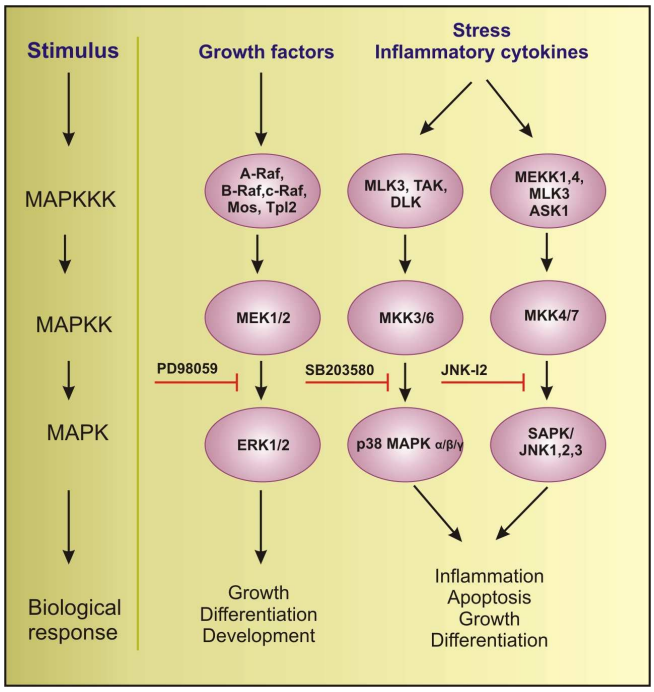

Fig. 5: Overview of MAPK signalling pathways.

Specific inhibitors of the pathway are

PD98059 (MEK1/2 inhibitor), SB203580 (MKK3/6 inhibitor) and JNK-I1 (MKK4/7 inhibitor).

For further details see text.

(Figure taken from Cell Signalling Technology with some modifications).

Although each MAPK cascade has unique characteristics, a number of features are shared by the different MAPK pathways. Each cascade of MAPKs is composed of a set of three evolutionarily conserved, sequentially acting kinases: a MAPK, a MAPK kinase (MAPKK), and a MAPKK kinase (MAPKKK). The MAPKKKs, which are serine/threonine kinases, are often activated through phosphorylation and/or as a result of their interaction with a small GTP-binding protein of the Ras/Rho family in response to extracellular stimuli. MAPKKK activation leads to the phosphorylation and activation of a MAPKK, which then stimulates MAPK activity through dual phosphorylation on threonine and tyrosine residues located in the activation loop of kinase subdomain VIII. Phosphorylation of the MAPKs results in their translocation to the nucleus, where they modulate gene expression by phosphorylating targets such as downstream kinases and transcription factors.[108]

B.5 The Wnt signalling pathway in hMSC biology

The canonical Wnt cascade is also one of the major signal transduction pathways associated with both stem cell functions and tumour cell development (Fig. 6). In some tissues, where stem cell attributes are under the control of the Wnt signalling cascade, aberrant activation of this pathway results in tumour formation.

Wnts regulate self-renewal of mesenchymal, haematopoietic, neural, intestinal, and skin stem cells.[28,29] Evidences from our laboratory suggest that treatment with Wnt3a also increases hMSC proliferation and invasion.[106] However, discerning the exact involvement of Wnts is complicated by their pleiotropic effects. Examples of canonical Wnt functions include the promotion of long-term culture expansion of stem cells, the increased *in vivo* reconstitution of haematopoietic lineages, and the Wnt3a-specific maintenance of skin and intestinal stem cell populations.[28]

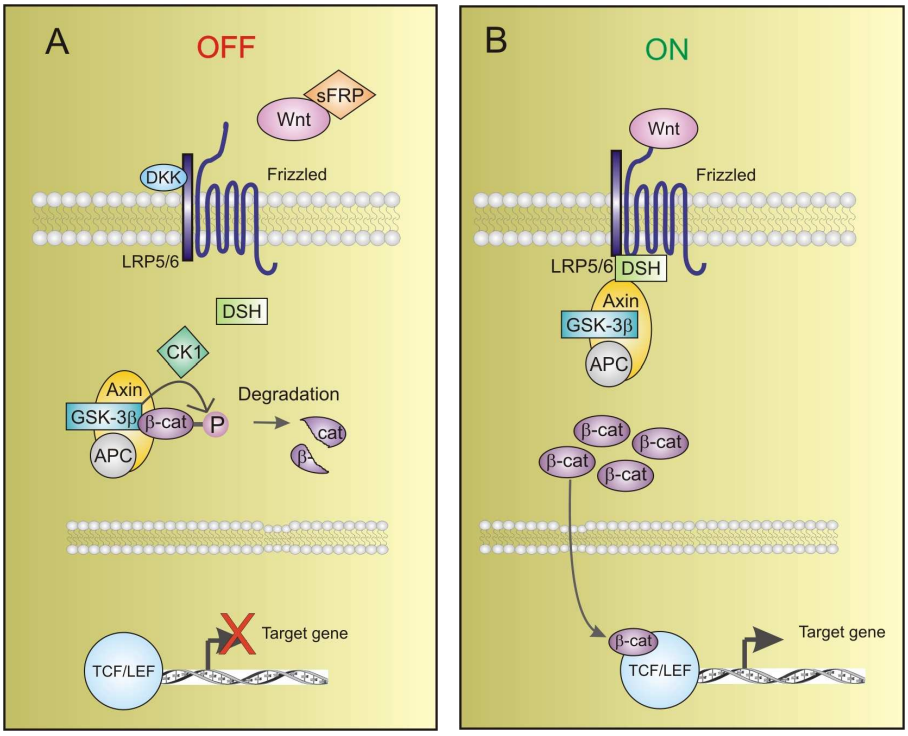

Fig. 6: The canonical Wnt signalling pathway.

(A) In the absence of active Wnt ligands ("off state"), β-catenin is retained in a destruction complex composed of Axin, APC, and GSK-3β. Subsequently, β-catenin gets phosphorylated and after ubiquitination it is degraded by the proteasome. Under these conditions, Wnt target genes are kept in a repressed state.

(B) Upon binding of Wnt ligands to the frizzled receptor ("on state"), β-catenin is uncoupled from the degradation complex and translocates into the nucleus where it binds to TCF/LEF-family transcription factors and activates Wnt target gene transcription.

APC (adenomatous polyposis coli); β-cat (β-catenin); CK1 (casein kinase 1); DKK (Dickkopf); DSH (Dishevelled); GSK-3β (glycogen synthase kinase-3 β); LEF (lymphoid enhancer-related protein); P (phosporylation); sFRP (secreted Frizzled-related protein); TCF (T-cell factor).

C AIMS OF THE STUDY

hMSC represent promising tools in various clinical applications including the regeneration of injured tissues by endogenous or transplanted hMSC. However, the molecular mechanisms that control hMSC mobilisation and homing, which require invasion through extracellular matrix (ECM) barriers, are almost unknown.

According to our working hypothesis as depicted in figure 7, hMSC residing in their original bone marrow niche are mobilised by gradients of cytokines/chemokines released at sites of tissue injury and inflammation. This requires hMSC to migrate through ECM barriers such as basement membranes by the involvement of specific proteolytic systems such as matrix metalloproteinases (MMPs) and their inhibitors (TIMPs) in order to reach the diseased tissues (target niche), where they differentiate into functional cell types.

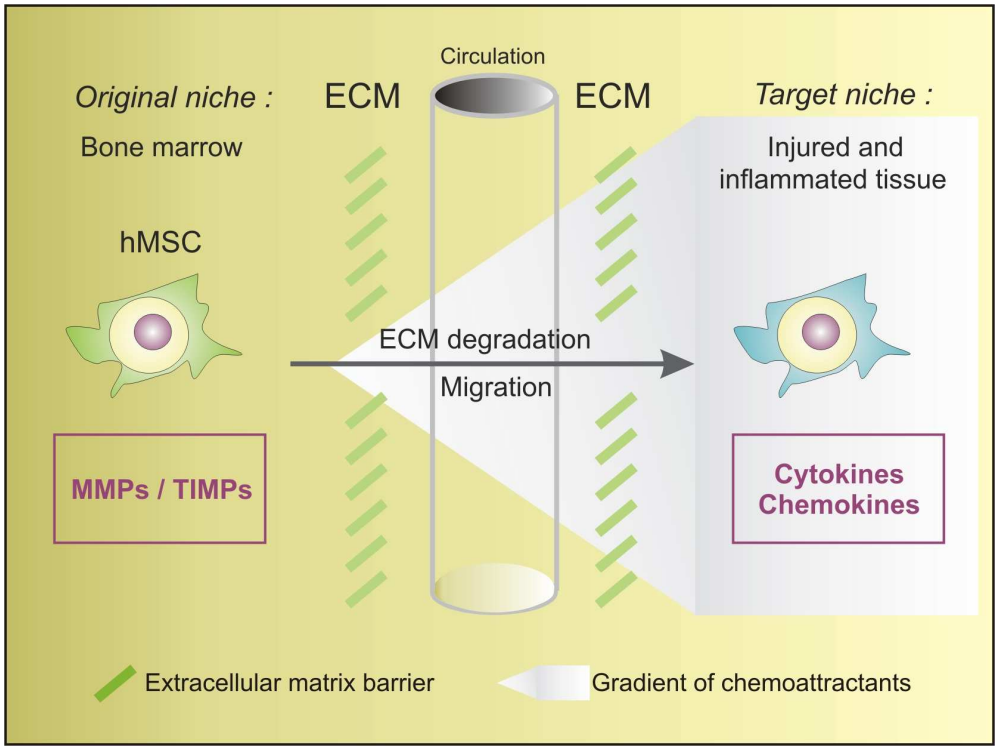

Fig. 7: Working model for the present studies.

For details see text.

To further elucidate the underlying molecular mechanisms, the major goals of this study were as follows:

1. Analysis of the basal expression of MMPs and TIMPs in hMSC

For this aim qRT-PCR, zymography and Western blotting were intended to be applied.

2. Establishment of an in vitro assay to study hMSC invasiveness

For this propose, establishment of a Transwell-assay using solely biological material of human source was planned in order to closer match the *in vivo* situation of hMSC migration.

3. Investigation of the role of MMPs and TIMPs in hMSC invasiveness

Application of synthetic MMP inhibitors as well as RNA interference (RNAi) technology was attempted to be employed for the elucidation of the specific role of particular MMPs and TIMPs in hMSC invasiveness.

4. Studies on the role of inflammatory cytokines and chemokines on hMSC invasiveness

This aim was intended to study, wether a selection of inflammatory cytokines and chemokines was able to regulate MMP/TIMP expression in hMSC and act as chemoattractants for these cells.

Moreover, the following aims should be also addressed:

5. Evaluation of TIMP-1 as a regulator of hMSC functions

To clarify the specific role of TIMP-1 on cell proliferation and differentiation of hMSC, a knock-down of TIMP-1 expression was planned using RNAi technology. Furthermore, a preliminary analysis of gene expression presumably regulated by TIMP-1 was purposed.

6. Examination of TNF-α effects on hMSC functions

For this aim, we intended long-term incubation of hMSC with TNF-α and subsequent analysis of differentiation and invasion capacities as well as of effects of TNF-α on specific gene expression and intracellular signal transduction factors.

D MATERIAL AND METHODS

D.1 Material

D.1.1 Equipment

Apparatus	Source
Autoclave	
3850	Systec GmbH, Wettenberg, Germany
VST 500-C 12D	Zirbus, Bad Grund, Germany
Balance	
Analytic Balance, A 120 S (0.001-12 g range)	Sartorius, Göttingen, Germany
Analytic Balance, 3716MP (0.001-250 g range)	Sartorius, Göttingen, Germany
Biophotometer	
Biophotometer	Eppendorf, Hamburg, Germany
Thermal printer DPU-414	Seiko Instruments, Neu Isenburg, Germany
Cell Incubator	
Type BB16 EC-CO2	Heareus Sepatech, München, Germany
Centrifuge	
Kontron, Centrikon H-401 with rotor A8.24	Kontron Instruments, Eching, Germany
Heraeus, Varifuge 3.2 RS Heraeus	
Heraeus, Sepatech Biofuge A (rotor 1230)	Heraeus Sepatech, München, Germany
Heraeus, Sepatech Biofuge A (rotor 3042)	Heraeus Sepatech, München, Germany
ELISA-Reader	
DigiScan 400	ASYS Hitech GmbH, Austria
MPP 3408	ASYS Hitech GmbH, Austria
Electrophorese	
XCell SureLock™ Mini-Cell	Invitrogen, Karlsruhe, Germany
Power Supply EPS 301, EPS 601	Amersham Bioscience, München, Germany
Incubator Unihood	Uniequip, Planegg, Germany
Fluorescence Microscope	
Olympus IX70, IX50	Olympus, Feldkirchen-Westerham, Germany
PCR	
Thermomixer Comfort	Eppendorf, Hamburg, Germany
Lightcycler™ II	Roche, Mannheim, Germany

Apparatus	Source
Protein Transfer	
XCell SureLock™ Blot Module	Invitrogen, Karlsruhe, Germany
Power Supply EPS 301, EPS 601	GE Healthcare, Freiburg, Germany
Roller RM5	Uniequip, Planegg, Germany
Biomax Cassette	Eastman Kodak Co., New York, USA

Equipment for the cell culture	Source
Flasks	
Nunclon™ with Filter Caps (T25, T75)	NUNC, Wiesbaden, Germany
Plates	
Multidishes Nunclon™ (6, 12, 24, 48, 96-well)	NUNC, Wiesbaden, Germany
Others	
Cell Scrapers Nunclon™	NUNC, Wiesbaden, Germany
Falcon™ Conical Centrifuge Tubes (15, 50 ml)	BD Bioscience, San Jose, USA
Microscope Slides 25 x 75 mm	NUNC, Wiesbaden, Germany
Poly-D-lysine Coated Cellware	BD Biosciences, San Jose, USA
Serological Pipettes (5, 10, 25 ml)	NUNC, Wiesbaden, Germany
Transwell Chamber System (24-well, 8 µm Pore)	Costar, Pleasanta, CA, USA

D.1.2 Chemicals

D.1.2.1 Cell culture

Chemical	Source
Dulbecco´s Modified Eagle´s Medium (DMEM)	PAA Laboratories, Pasching, Austria
Phosphate Buffered Saline (PBS)	Sigma, Hamburg, Germany
EDTA	Biochrom KG, Berlin, Germany
Human Extracellular Matrix (hECM)	BD Bioscience, San Jose, USA
Fetal Calf Serum (FCS)	PAN Biotech, Aidenbach, Germany
Glucose	Merck, Darmstadt, Germany
Heparin	Sigma, Hamburg, Germany
Human Serum (HS)	PAA Laboratories, Coelbe, Germany
Diff Quick	Dade Diagnostika, München, Germany
Nutridoma SP	Roche, Mannheim, Germany
IL-1β	Peprotech, London, England

Chemical	Source
L- Glutamine	PAN Biotech, Aidenbach, Germany
Mesenchymal Stem Cell Basal/Growth Medium (MSCBM), (MSCGM)	Lonza, Wuppertal, Germany
MMP-14 Biotrak Activity Assay	Calbiochem, Schwalbach, Germany
Penicillin/Streptomycin	Biochrom KG, Berlin, Germany
Ro 206-0222	Roche Diagnostics, Penzberg, Germany
GM6001	Calbiochem, Schwalbach, Germany
Lipofectamine 2000	Invitrogen, Karlsruhe, Germany
WST-1 Cell Proliferation Assay	Roche, Mannheim, Germany
CyQuant Proliferation Assay	Invitrogen, Karlsruhe, Germany
AMD3100	Sigma, Hamburg, Germany
Dimethyl Sulphoxide (DMSO)	Merck, Darmstadt, Germany
Ethanol	Merck, Darmstadt, Germany
Normal Goat Serum (NGS)	Sigma, Hamburg, Germany
RPMI	PAA Laboratories, Coelbe, Germany
SDF-1α	Peprotech, London, England
TGF-β	Peprotech, London, England
TNF-α	Peprotech, London, england
Trypan Blue	Sigma, Hamburg, Germany
Trypsin for hMSC	Lonza, Wuppertal, Germany

D.1.2.2 Protein biochemistry

Chemical	Source
Hyperfilm-MB	GE Healthcare, Freiburg, Germany
Immobilon-P	Milipore Corporation, Bedford, USA
Novex 10 % Zymogram (Gelatin) Gel	Invitrogen, Karlsruhe, Germany
ProLong®Gold Antifade Reagent with DAPI	Molecular Probes,Oregon,USA
NuPAGE™ Novex 4-12 % Bis Tris Gel	Invitrogen, Karlsruhe, Germany
NuPAGE™ LDS sample buffer	Invitrogen, Karlsruhe, Germany
Whatman Filterpaper Nr.1	Whatman, Ammerbuch, Germany

D.1.2.3 Antibodies

Primary antibody[a]	Experimental application	Dilution	Source
	MMP detection		
ms anti MMP-2		1:100	Calbiochem, Schwalbach, Germany
ms anti MMP-9		1:100	Calbiochem, Schwalbach, Germany
rb anti MT1-MMP		1:100	Sigma, Hamburg, Germany
	TIMP detection		
rb anti TIMP-1		1:400	Sigma, Hamburg, Germany
ms anti TIMP-2		1:100	Chemicon, Massachusetts, USA
	Neural Markers		
ms anti β-III Tubulin	early postmitotic neuron[109]	1:100	Sigma, Hamburg, Germany
ms anti MAP-2	mature neuron[110]	1:100	Sigma, Hamburg, Germany
ms anti αGFAP	mature glia[111]	1:300	Sigma, Hamburg, Germany
ms anti αGal C	mature oligodendrocyte[112]	1:300	Sigma, Hamburg, Germany
ms anti CXCR4		1:400	
	Control		
gt anti β-actin		1:400	Santa Cruz, California, USA

[a] ms=mouse; rb=rabbit; gt=goat; sh=sheep; dk= donkey

Secondary antibody[a]	Dilution	Source
gt anti ms Fluorescein (FITC)	1:1000	Molecular Probes, Oregon, USA
gt anti ms Rhodamine Red (RHOX)	1:1000	Molecular Probes, Oregon, USA
gt anti rb Rhodamine Red (RHOX)	1:1000	Molecular Probes, Oregon, USA
gt anti sh Fluorescein (FITC)	1:1000	Molecular Probes, Oregon, USA
dk anti rb IgG	1:1500	GE Healthcare, Freiburg, Germany
Peroxidase-labelled anti rb	1:1000	GE Healthcare, Freiburg, Germany
Peroxidase-labelled anti ms	1:1000	GE Healthcare, Freiburg, Germany

[a] ms=mouse; rb=rabbit; gt=goat; sh=sheep; dk= donkey

D.1.3 Molecular biochemistry

D.1.3.1 Interfering siRNA molecules

Gene	Accession Number	Sequence
MMP-2	NM_004530	**Target sequence** 5´- AAGGAGAGCTGCAACCTGTTT -3´ **siRNA sense** 5´- GGAGAGCUGCAACCUGUUU -3´ **siRNA antisense** 5´- AAACAGGUUGCAGCUCUCC -3´
MT1-MMP	NM_004995	**Target sequence** 5´- AACCAGAAGCTGAAGGTAGAA -3´ **siRNA sense** 5´- CCAGAAGCUGAAGGUAGAA -3´ **siRNA antisense** 5´- UUCUACCUUCAGCUUCUGG -3´
TIMP-1	NM_003254	**Target sequence** 5´- AATCAACCAGACCACCTTATA -3´ **siRNA sense** 5´- UCAACCAGACCACCUUAUA -3´ **siRNA antisense** 5´- UAUAAGGUGGUCUGGUUGA -3´
TIMP-2	NM_003255	**Target sequence** 5´- AAGGATCCAGTATGAGATCAA -3´ **siRNA sense** 5´- GGAUCCAGUAUGAGAUCAA -3´ **siRNA antisense** 5´- UUGAUCUCAUACUGGAUCC -3´
Negative control		**siRNA sense** 5´- UUCUCCGAACGUGUCACGU -3´ **siRNA antisense** 5´- ACGUGACACGUUCGGAGAA -3´

D.1.3.2 Chemicals and kits

Chemical/ Kits	Source
1st Strand cDNA Synthesis-Kit	Roche, Mannheim, Germany
AmpoLabeling-LRP-Kit	Superarray, Frederick, USA
Biotin-16-dUTP	Roche, Mannheim, Germany
GEArray® Q Serie Signaltransduktion Pathfinder	Superarray, Frederick, USA
LightCycler FastStart DNA Master SYBR Green I	Roche, Mannheim, Germany
QIAprep Spin Miniprep-Kit	Qiagen, Hilden, Germany
QIAshredder	Qiagen, Hilden, Germany
RNeasy MiniElute-Kit	Qiagen, Hilden, Germany

D.1.3.3 Solutions and buffers

Reagent	Application	Source
Denaturing Buffer (Zymography)	50 mM Tris, pH 7.5 200 mM NaCl 5 mM $CaCl_2$ 0.2 % Brij35	Invitrogen, Karlsruhe, Germany
Loading buffer (Zymography samples)	126 mM Tris-HCl 20 % glycerol 4 % SDS 0.005 % bromophenol blue	Invitrogen, Karlsruhe, Germany
PFA (4 %, 100 ml)	4 g paraformaldehyde 10 N NaOH 10 ml 10 x PBS	Sigma, Hamburg, Germany
Preservation buffer (Zymography)	5 % glycerol 20 % EtOH	Invitrogen, Karlsruhe, Germany
Renaturing buffer (Zymography)	2.7 % Triton X-100	Invitrogen, Karlsruhe, Germany
Trypan Blue	PBS pH 7,2 0.5 % BSA 2 mM EDTA 0.5 % (v/v) trypan blue	Sigma, Hamburg, Germany
Zymography stain	0.002 % Coomassie Blue R-350 30 % methanol 10 % acetic acid PhastGel-Blue-R Tablets	Invitrogen, Karlsruhe, Germany Roche, Mannheim, Germany

D.1.4 Cells

Cell type		Source
hMSC	(human mesenchymal stem cells)	Lonza, Wuppertal, Germany
THP-1	(acute monocytic leukaemia cells)	DSMZ, Braunschweig, Germany
HT1080	(fibrosarcoma cells)	DSMZ, Braunschweig, Germany
HMEC	(human endothelial cells)	DSMZ, Braunschweig, Germany
II4RT	(metastatic breast cancer cells)	Prof. Fusenig, Freiburg, Germany
3T3	(mouse fibroblasts)	DSMZ, Braunschweig, Germany
A5	(non-metastatic breast cancer cells)	Prof. Fusenig, Freiburg, Germany

D.1.4.1 Features of hMSC donors

hMSC Lot #	Age	Race	Sex	Supplier
1F1061	21	Caucasian	Female	Lonza, Wuppertal, Germany
3F0664	19	Black	Female	Lonza, Wuppertal, Germany
3F1451	23	Black	Female	Lonza, Wuppertal, Germany
4F0591	32	Caucasian	Male	Lonza, Wuppertal, Germany
4F1127	23	Black	Male	Lonza, Wuppertal, Germany
4F1560	23	Black	Female	Lonza, Wuppertal, Germany

D.1.5 Software

Program	Source
ArrayVision 8.0	Imaging Research, Braunschweig, Germany
CorelDRAW 12	Microsoft, Unterschleissheim, Germany
ImageMaster-1D Elite	GE Healthcare, Freiburg, Germany
Image-Pro® PLUS	Media Cybernetics, Silver Spring, USA
LightCycler Software 3.5	Roche, Mannheim, Germany
Magic Scan V4.4	GE Healthcare, Freiburg, Germany
Microsoft Office EXCEL 2003	Microsoft, Unterschleissheim, Germany
Origin 7.5	OriginLab, Massachusetts, USA
XFluor4 Safire2	Tecan, Crailsheim, Germany

D.2 Methods

D.2.1 Cell culture

The experiments shown in this study were performed with hMSC which had been isolated from bone marrow of different healthy persons (see D.1.4.1). The hMSC lots had been tested by the providing company for purity by flow cytometry analysis. The cells were positive for CD105, CD166, CD29, and CD44. The absence of haematopoetic cell contamination was ensured by controlling cells for negative expression of CD14, CD34, and CD45.[9] The cells were also checked for their ability to differentiate into the osteogenic, chondrogenic, and adipogenic lineage.

Cultivation of hMSC in our laboratory was performed using the MSCGM BulletKit according to the supplier´s instructions at 37°C in a humidified air atmosphere containing 5 % CO_2. Cells were passaged at a confluency of ~90 % using Trypsin-EDTA.

For experiments under serum-free conditions, hMSC were washed with serum-free medium and incubated in DMEM supplemented with 1% Nutridoma SP in the absence or presence of TGF-β (100 ng/ml), Il-1β (50 ng/ml), TNF-α (50 ng/ml), or SDF-1α (100 ng/ml). hMSC proliferation did not significantly change during exposition to these agents for 48 hours as determined by use of the CyQuant proliferation assay (Invitrogen). All experiments were carried out with hMSC of the 5^{th} or 6^{th} passage which exhibited an average cell doubling time of 96 hours. Cells were tested by us for their ability to differentiate into the adipogenic and osteogenic lineage as described in D.2.2.

For long term incubation studies of hMSC with TNF-α, the cells were seeded at a concentration of $1.5x10^3$ cells/cm² on tissue culture flasks (Nalgen Nunc) and cultured in MSCGM supplemented with 50 ng/ml of recombinant TNF-α for up to four weeks. The medium was changed once a week and the cells were dissociated with PBS containing 0.05 % Trypsin and 0.04 % EDTA and replated after three weeks.

For comparative analysis additional cell types were used such as THP-1, HT1080, HMEC, II4RT, 3T3 and A5 which were purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ) (see D.1.4). Cells were grown in RPMI-1640 supplemented with 10 % (v/v) heat-inactivated fetal calf serum, 2 mM glutamine, 100 units/ml penicillin and 100 µg/ml streptomycin. Incubation was performed at 37 °C in a humidified air atmosphere in the presence of 5 % CO_2. Cells were passaged twice a week. For all further experiments cells were washed two times with serum-free medium and maintained under serum-free conditions in RPMI-1640 supplemented with 1 % (v/v) Nutridoma SP.

D.2.2 Mesodermal differentiation of hMSC

For osteogenic and adipogenic differentiation hMSC were cultured until confluency.

Differentiation into osteoblastic cells was induced by using hMSC medium supplemented with 100 nM dexamethasone, 10 mM β-glycerophosphate, and 50 µM L-ascorbic acid 2-phosphate. The medium was changed twice a week.

Differentiation of hMSC into adipocytes was achieved by adding DMEM supplemented with 10 % (v/v) FCS, 40 IU/ml Pen/Strep, 4 mM L-glutamine, 1 µM dexamethasone, 0.2 mM indomethacine, 0.01 mg/ml insulin, 0.5 mM 3-isobutyl-1-methylxanthine to the cells.

Three cycles of induction for 4, 5, and 6 days were carried out, interrupted by two periods of 4 and 2 days of maintainance of the cells in DMEM supplemented with 10 % (v/v) FCS, 40 IU/ml penicillin/streptomycin, 4 mM L-glutamine, 0.01 mg/ml insulin.

The differentiation capacity towards the two different mesodermal lineages was verified by typical changes at the morphological level as well as by the induction of mRNA expression of characteristic markers decorin for osteogenic and PPAR-γ for adipogenic cells.

Additionally, after osteogenic differentation intracellular deposition of calcium was demonstrated by Alizarin-Red staning (Fig. 8A). For detection of the mineralized nodules, cells were fixed with 4 % paraformaldehyde and stained with a 1 % Alizarin-Red staining solution in water for 10 minutes. Adipogenic differentiation was verified by Oil-Red-O staining of intracellular vacuoles (Fig. 8B). The Paraformaldehyde-fixed cells were covered with 3 mg/ml Oil-Red-O dissolved in 60 % (v/v) isopropanol for 10 minutes.

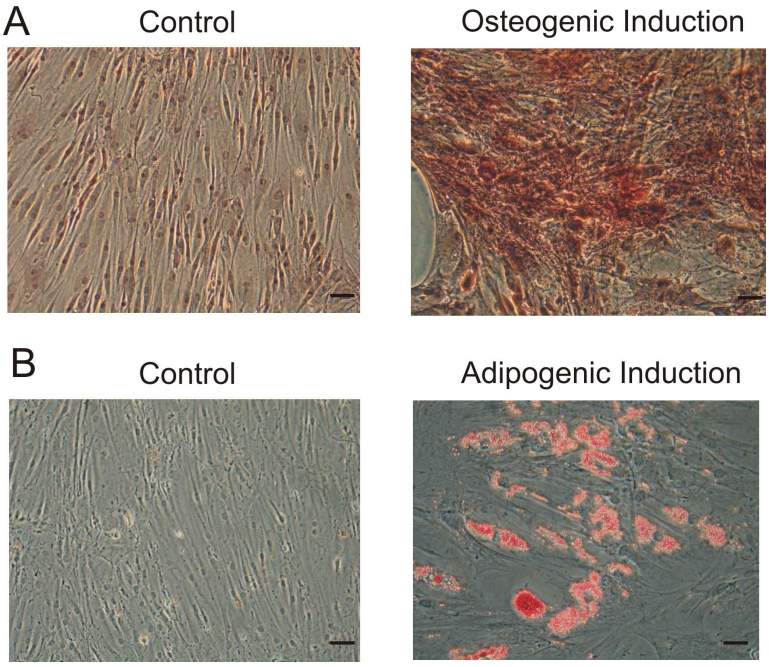

Fig. 8: Microscopic analysis of hMSC morphology and differentiation.

(A) hMSC were incubated for 14 days in medium inducing osteogenic differentiation, and were subsequently stained with Alizarin Red for calcium deposition.

(B) hMSC were incubated for 14 days without and with adipogenic differentiation medium and then assayed by Oil-Red-O for the accumulation of lipid droplets staining.

All scale bars indicate 50 µm.

D.2.3 Quantitative real time-polymerase chain reaction (qRT-PCR)

Isolation of total RNA from hMSC was performed using the RNeasy Mini Kit and an on-column DNase digestion with the RNase-free DNase-set according to the manufacturer´s protocols. The cDNA synthesis was completed following the instructions of the First Strand cDNA Synthesis Kit for RT- PCR (AMV). Therefore, 500-1000 ng of purified total RNA was used and priming with oligo dT. qRT-PCR was carried out on a LightCycler applying the LightCycler-FastStart DNA Master SYBR Green I Kit.

For amplification of specific transcripts the LightCycler Primer Sets were used according the manufacturers instructions. PCR was performed with 1 µl cDNA (see above), 2 µl primer mix, 2 µl LC-FastStart DNA Master SYBR Green I mix, and water to a final volume of 20 µl. Amplification occurred in a three-step cycle procedure initiated by denaturation at 95°C for 10 min to activate the polymerase followed by 45 cycles of heating at 95°C for 10 sec, annealing at 68°C for 10 sec, and extension at 72°C for 16 sec. The final PCR cycle was followed by a melting curve analysis to confirm PCR product indentity and to differentiate it from nonspecific products. The concentration of each sample was calculated automatically by reference to the respective standard curve as generated during PCR according to the protocol of the manufacturer. The amplified products were also checked by electrophoresis on ethidium-bromide stained agarose gels.

D.2.4 Microarray analysis

Gene expression was analysed by the *Stem Cell* and *Human Signal Transduction Pathway-Finder GEArray* (Superarray Bioscience Corporation, MD, USA) applying 1µg of total RNA according to the manufacturer´s protocol. The microarray data were quantified with the Array-Vision software.

D.2.5 Transfection of hMSC with small interfering RNA (siRNA)

RNAi technology was employed to generate specific knock-downs of MMP- and TIMP-mRNA transcription in hMSC. siRNAs targeted against human mRNAs of MMP-2, MT1-MMP, TIMP-1, and TIMP- 2 were designed in our laboratory according to the protocol of Reynolds et al.[113] Sense and antisense oligonucleotides were synthesized by Qiagen. Non-specific siRNA which has no target in the human transcriptome was used as a negative control and was also purchased from Qiagen. The respective sequences are as depicted in D.1.3.1.

One day before transfection, hMSC were plated at a density of $5x10^3$ cells/cm^2 in MSCGM into 6-well dishes and allowed to reach 30 % confluency after 24 hours of incubation. siRNA at a final concentration of 25 nM was combined with 10 µl of Lipofectamine 2000 (Invitrogen) in a total volume of 500 µl DMEM and allowed to complex by incubation for 20 minutes at room temperature. The transfection mixture was then applied to the hMSC and incubated for 6 hours at 37°C in a humidified atmosphere containing 5 % CO2. Subsequently, cells were washed with PBS and maintained in MSCG-medium for 24 hours before application in the experiments.

Cell viability and the capacity for differentiation along the mesodermal lineage (see D.2.2) were not affected under these conditions.

D.2.6 Immunocytochemistry

For immunocytochemical analysis hMSC were seeded onto culture slides. The cells were fixed in 4 % paraformaldehyde in PBS. Subsequently, the culture slides were washed in PBS and blocked with 0.5 % Triton X-100 and 10 % NGS during 30 minutes at room temperature. The culture slides were then incubated within the respective antibodies (see D.1.2.3) in a solution containing 0.5 % Triton X-100 and 10 % NGS for 2 hours at 37°C. After several washes in PBS the culture slides were incubated in the secondary antibody for 45 minutes at room temperature. Secondary subclass-specific FITC- or TRIC-coupled antisera were applied at a dilution of 1:50. After three further washes, the culture slides were mounted and stained in ProLongGold antifade reagent with DAPI.

To rule out any unspecific binding of the secondary antisera, control experiments were performed by using a primary antibody against a non-expressed antigen (e.g. CD34).

D.2.7 Zymography and MMP activity assay

Cell culture supernatants were analysed for the presence of gelatinolytic enzymes by zymography using precast 10 % polyacrylamide minigels containing 0.1 % gelatin as substrate. The samples were mixed 1:1 with a nonreducing buffer containing 126 mM Tris-HCl, 20 % glycerol, 4 % SDS and 0.005 % bromophenol blue.

After electrophoresis, the gels were washed twice for 15 minutes in 2.7 % Triton X-100 on a rotary shaker to remove SDS and to allow proteins to renature. The gels were then incubated in a buffer containing 50 mM Tris, pH 7.5, 200 mM NaCl, 5 mM $CaCl_2$ and 0.2 % Brij35 for 18 hours at 37°C. The zymograms were stained for 90 min with 0.02 % Coomassie Blue R-350 in a 30 % methanol/10 % acetic acid solution using PhastGel-Blue-R tablets. Zymograms were scanned using a Umax ImageScanner driven by MagicScan software.

As a marker for electrophoretic mobility of gelatinases in zymograms, we used conditioned medium from PMA-treated HT1080 fibrosarcoma cells containing the latent form of proMMP-9 (~92 kDa), the latent form of MMP-2 (~72 kDa) and the activated form of MMP-2 (~66 kDa).[114]

For determination of MT1-MMP activity the *"MMP-14 Biotrak Activity Assay"* (Calbiochem, Schwalbach, Germany) was used following the instructions of the manufactures.

D.2.8 Preparation of cell extracts and Western blotting

For detection of proteins in hMSC, Western blotting was applied to cell lysates. Lysis and protein extraction in hMSC were performed by using a buffer containing 50 mM Tris pH 7.4, 150 mM NaCl, 1 % Triton X-100, and a mixture of small-molecular-weight inhibitors of metallo-, serine-, and cysteine-proteinases (Complete-Mini) which was then added to sub-confluently grown cells. Thereafter, the cell lysate was vortexed and incubated for 30 minutes at 4°C. Subsequently, the supernatants containing extracted proteins were collected by centrifugation at 16,000 x g and stored at –20°C.

SDS-PAGE gel electrophoresis was performed under reducing conditions in precast 4-12 % minigels applying the NuPAGE Bis-Tris-buffer system. After electrophoresis the separated proteins in the gel were electroblotted on polyvinyl difluoride (PVDF) membranes. The membranes were blocked in 10 % non-fat milk and then incubated with polyclonal rabbit antibodies against MT1-MMP (1 µg/ml) and TIMP-1 (0.4 µg/ml), or polyclonal goat antibody against actin (0.5 µg/ml) or 1 µg/ml monoclonal mouse antibodies against MMP-2 or MMP-9 as listed in D.1.2.3.

D.2.9 Cell invasion assay

Studies on chemoattractant-induced invasion capacity of hMSC were performed using the 24-well Costar Transwell chamber system (Fig.9). Membrane filters with a pore size of 8 µm and a diameter of 6.5 mm were coated with 10 µg of human ECM, which is mainly comprised of laminin, collagen type IV, and proteoglycans providing a composition similar to that of human basement membranes.

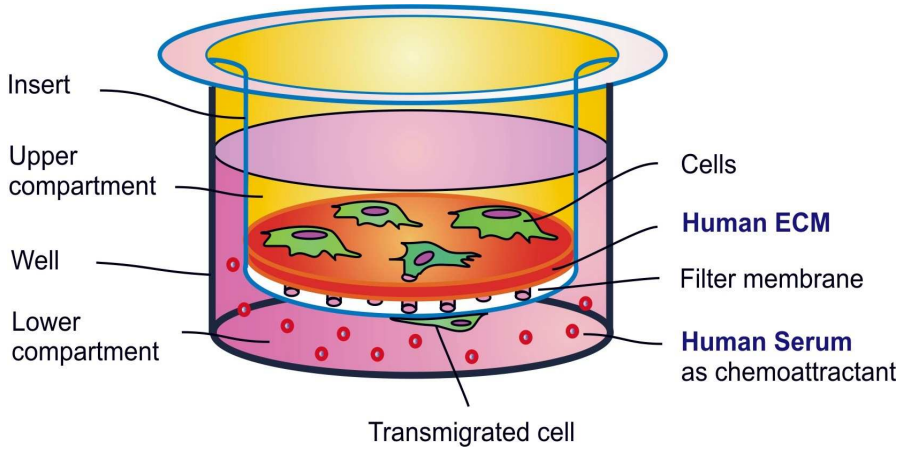

Fig. 9: Schematic representation of the modified Transwell assay system for determination of cell invasion.

For details see text.

The coated filters were dried overnight at room temperature under sterile conditions. Prior to the experiment, they were reconstituted with serum-free medium for 2 hours. The lower compartment of the invasion chamber was filled with 600 µl of DMEM containing 10 % human serum or cytokines/chemokines as a source of chemoattractants. Then the coated filter inserts were placed into the wells forming the upper compartment. hMSC ($5x10^3$) either untreated or transfected with the respective siRNA (see D.1.3.1) were suspended in 200 µl of serum-free medium and seeded into the upper compartment of the invasion chamber. The invasion chambers were incubated for 48 hours at 37°C in a humidified air atmosphere with 5 % CO_2.

After incubation cells and ECM on the top surfaces of the filters were wipped off with cotton swabs. Cells that had migrated into the lower compartment and attached to the lower surface of the filter were counted after staining with Diff Quick (Fig. 10).

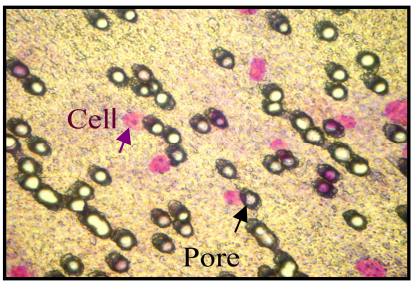

Fig. 10: Invaded cells after Diff Quick staining.

Cells that had migrated through the pores (8 µm) and attached onto the lower surface of the filter were fixed and stained with Diff Quick (violet colour).

Cell viability was assessed by trypan blue staining. The invasion rate was calculated from the ratio of the number of cells recovered from the lower compartment to the total number of cells loaded in the upper compartment. Each invasion experiment was performed in triplicate.

For inhibition of cell migration hMSC were pre-incubated for 30 minutes with 10 µg/ml GM6001 (a synthetic broad-spectrum MMP inhibitor[115]), 10 µg/ml AMD3100 (a highly specific antagonist of the chemokine receptor CXCR4[116]), or with 10 µg/ml of Ro 206-0222 (a specific inhibitor for MMP-2, MMP-9, and MT1-MMP) before being transferred into the upper compartment. The respective inhibitors were also added to the medium in the upper and lower compartment at the same concentrations of 10 µg/ml.

Preceding measurements had shown that incubation of hMSC with the inhibitors at a concentration of 10 µg/ml for 48 hours resulted in maximal inhibition of cell migration without substantially affecting viability and proliferation.

D.2.10 Neurosphere formation

hMSC which had been pre-incubated for two weeks in the absence and presence of 50 ng/ml TNF-α were seeded at a concentration of 1.5 x 10^4 cells/cm^2 onto Cellware dishes coated with poly-D-lysine and further cultivated in the absence and presence of TNF-α until cell spheroid formation became visible.

D.2.11 Data analysis

Statistical significance was assessed by comparing mean (±SD) values of triplicate experiments with the Student´s *t*-test for independent groups. Significance was assumed for $p<0.05$. Statistical analysis was performed using the Origin 7.5 software.

E RESULTS

E.1 Analysis of MMP and TIMP expression in hMSC

At the beginning of these studies almost nothing was known about the expression of MMPs and TIMPs in hMSC. Therefore, a detailed analysis of mRNA expression of MMP-2, MMP-9, MT1-MMP, TIMP-1 and TIMP-2 as well as of the protein synthesis of these factors in hMSC was performed.

Determination of mRNA expression in hMSC by qRT-PCR revealed a pronounced transcription of MMP-2, MT1-MMP, TIMP-1, and TIMP-2 when compared to that of GAPDH, whereas MMP-9 mRNA was only slightly detectable (Fig. 11).

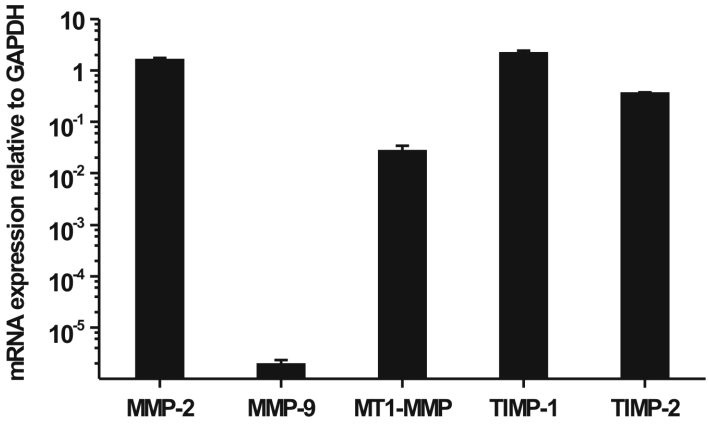

Fig. 11: Constitutive mRNA expression of MMPs and TIMPs in hMSC.

qRT-PCR analysis of MMP-2, MMP-9, MT1-MMP, TIMP-1, and TIMP-2 gene transcription was carried out in hMSC cultivated in MSCG medium. The results are mean values ±SD of mRNA expression of the target factors relative to GAPDH (set as 1) from a triplicate measurement representative for three independent experiments with three different hMSC lots.

To verify these findings on protein level, Western blotting analysis of cell-associated MMPs and TIMPs was performed using hMSC lysates. In addition, conditioned medium obtained from hMSC cultivated for 72 hours under serum-free conditions was examined for the presence of secreted MMPs and TIMPs. In accordance with the mRNA data, the cell extracts contained proMMP-2 (72-kDa), MT1-MMP (in form of its 58 kDa active species), TIMP-1 (30 kDa), and TIMP-2 (21 kDa) (Fig. 12A). Furthermore, hMSC released substantial amounts of proMMP-2 (72-kDa) into culture supernatants together with a smaller portion of its activated 64-kDa form as well as TIMP-1 and TIMP-2 (Fig. 12A). MT1-MMP was not detected in the culture supernatants corresponding to its nature as a membrane-anchored proteinase. MMP-9 protein was missing in both hMSC lysates and conditioned medium (Fig. 12A), which was in accordance with its extremely low mRNA expression level.

The findings on gelatinase secretion were confirmed by a 72 hour-time-course analysis of hMSC culture supernatants applying zymography which approved the accumulation of released proMMP-2 as well as of its fully activated species, whereas MMP-9 was not detectable (Fig. 12B).

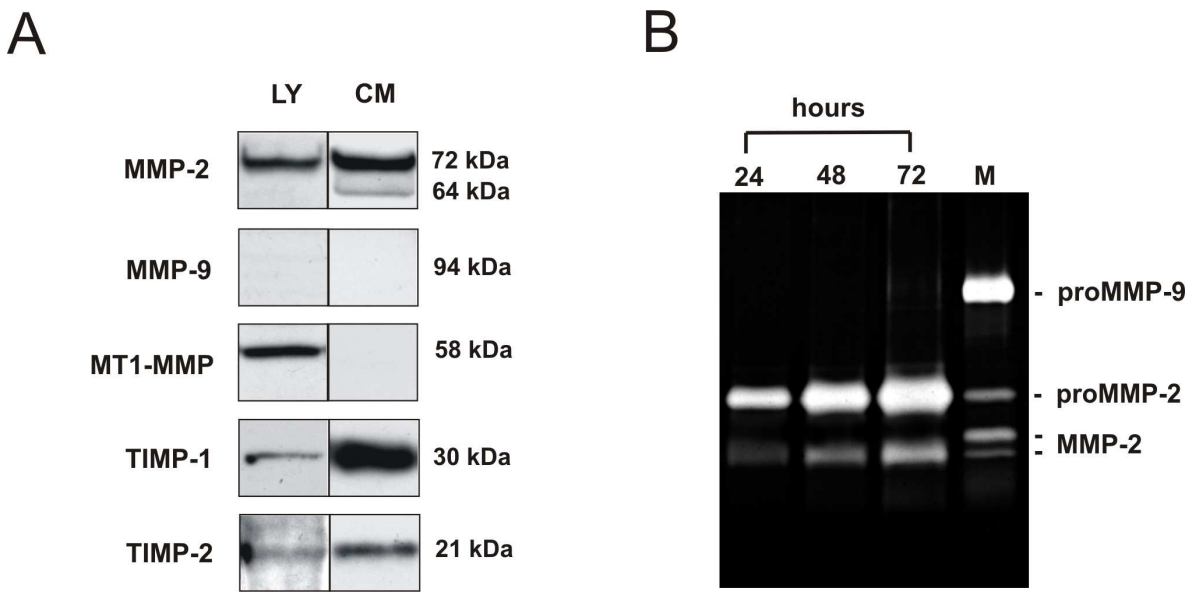

Fig. 12: Constitutive protein expression of MMPs and TIMPs in hMSC.

(A) Western blot detection of MMP-2, MMP-9, MT1-MMP, TIMP-1, and TIMP-2 in cell lysates (LY) and conditioned medium (CM) of hMSC cultivated for 72 hours under serum-free conditions. 30-µl aliquots standardized by protein content were separated by SDS-PAGE under reducing conditions, blotted, and probed with the specific antibodies.

(B) 10-µl aliquots of culture supernatants taken at different time points during a 72 hour cultivation period of hMSC in serum-free medium were analysed by zymography.
HT1080 conditioned medium containing proMMP-9, proMMP-2 and active forms of MMP-2 was used as a marker (M).

E.2 Establishment of an *in vitro* assay to study hMSC invasiveness

The ability of cells, particularly of tumour cells, to migrate through ECM barriers is typically examined by *in vitro* methods based on the modified Boyden chamber adopting Matrigel-coated inserts and FCS.[117] To closer match the *in vivo* situation of hMSC migration, we optimized the assay by solely using biological material of human origin. Instead of Matrigel that represents ECM extracted from mouse tumor tissue,[118,119] we applied commercially available human extracellular matrix (hECM) from healthy tissue. The composition of this hECM is similar to that of basement membranes. In addition, human serum instead of fetal claf serum was utilized as a source of unspecified chemoattractants. A schematic representation of the Transwell assay is given in Fig.9.

E.2.1 Human extracellular matrix as cell migration barrier

Different amounts of hECM were coated onto the transwell inserts. The homogeneous distribution and complete coverage of the filter pores with the reconstituted human basement membrane was examined using Comassie Blue staining of proteins on the inserts. A concentration of 10 µg per insert was found to provide an appropriate cell migration barrier (data not shown) and was used for further experiments.

E.2.2 Human serum as chemoattractant

Various dilutions of human serum were added to the lower compartment of the Transwell assay. hMSC placed into the upper compartment were able to traverse the reconstituted hECM by migration towards the diluted human serum. The rate of invaded cells exhibited a maximum towards a gradient of 10 % human serum and showed no further augmentation by an increase of the serum concentration in the lower compartment of the Transwell system (Fig 13).

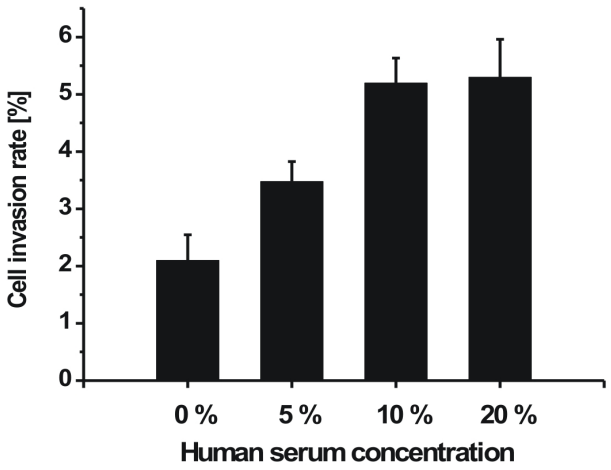

Fig. 13: Induction of hMSC invasiveness by human serum gradients.

hMSC ($5x10^3$) were placed onto filters coated with hECM and incubated for 48 hours in the presence of different concentrations of human serum in the lower compartment.

Absolute cell invasion rate was determined in percent calculated from the ratio of the number of cells recovered from the lower compartment to the total number of cells loaded in the upper compartment. Data are presented as mean ±SD of one triplicate experiment representative of three independent measurements.

E.2.3 Time course analysis

hMSC invasion capacity was assayed for different time intervals applying 10 µg hECM/insert and 10 % human serum as a chemoattractant. The rate of invaded cells exhibited a maximum after 48 hours of incubation and showed no further increase by elongation of the incubation period (Fig. 14).

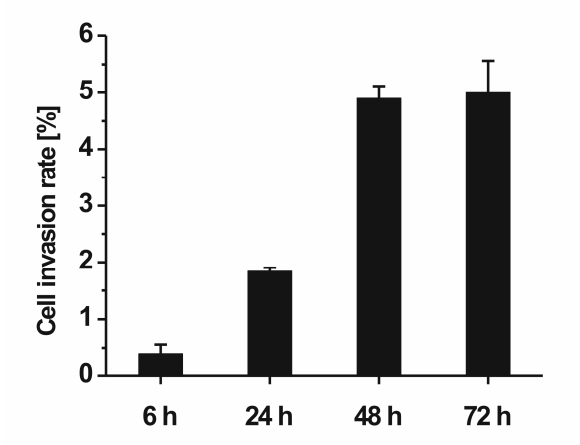

Fig. 14: Time course analysis of hMSC invasiveness under standardised conditions.

hMSC ($5x10^3$) were placed onto filters coated with 10 µg hECM and incubated for different time intervals in the presence of 10 % human serum in the lower compartment.

Absolute cell invasion rate was determined in percent calculated from the ratio of the number of cells recovered from the lower compartment to the total number of cells loaded in the upper compartment. Data are presented as mean ±SD of one triplicate experiment representative of three independent measurements.

E.2.4 Invasion potential of different cell types

Applying the "standard human invasion assay conditions" as determined before with hMSC, comparative analysis with various cell types of known invasive capacities was performed. As shown in Fig. 15, the highest invasion rates were observed for THP-1 (human leukemic cells) and HT1080 (human fibrosarcoma cells). Furthermore, II4RT (metastatic breast cancer cells) exhibited a strong invasive potential, whereas A5 (non-metastatic breast cancer cells) were nearly unable to transmigrate hECM, which is consistent with the physiological properties of both cell types. The hMSC invasion rate was similar to that of II4RT and human endothelial cells (HMEC) indicating a pronounced invasive potential of these cells, whereas mouse fibroblasts (3T3) showed only weak abilities for directed migration through hECM towards human serum (Fig.15).

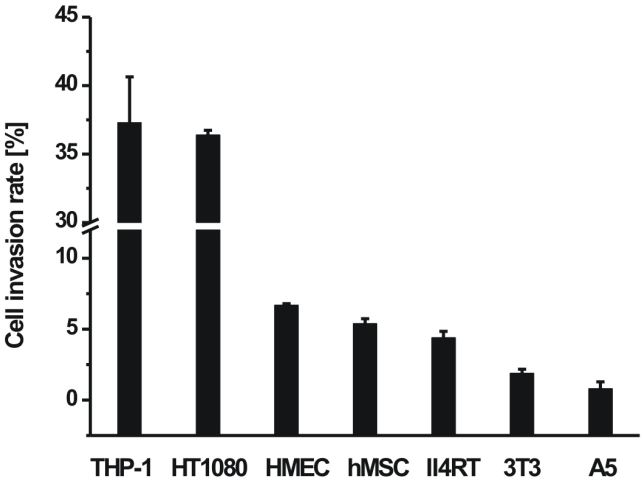

Fig. 15: Comparison of the invasion potential of different cell types.

THP-1 ($1.5x10^5$), HT1080 ($1x10^4$), HMEC ($5x10^3$), hMSC ($5x10^3$), II4RT ($5x10^3$), 3T3 ($5x10^3$), and A5 ($5x10^3$) were placed onto Transwell filters coated with 10 µg of hECM and incubated for 48 hours.

Absolute cell invasion rate towards 10 % HS was determined in percent relative to control. Data are presented as mean ±SD of one triplicate experiment representative of three independent measurements.

E.3 Role of MMPs and TIMPs in hMSC invasiveness

To reach damaged tissues migrating hMSC must be able to invade the dense network of extracellular matrix (ECM) which is thought to be facilitated by proteolytic activity.

E.3.1 Effect of synthetic MMP inhibitors on hMSC invasiveness

In order to investigate the involvement of MMPs in the ability of hMSC to traverse human reconstituted basement membrane, the invasion assay was performed in the presence and absence of synthetic MMP inhibitors.

Addition of GM6001 representing a broad-spectrum inhibitor of MMP activity significantly reduced the transmigration rate of hMSC compared to untreated control cells (Fig. 16). Likewise, Ro 206-0222, a highly specific inhibitor of MMP-2, MMP-9, and MT1-MMP activity, impaired the invasive capacity of hMSC to a similar extent (Fig. 16). Cell viability was not affected by either of the two inhibitors at the concentration used to achieve maximal migratory inhibition (data not shown).

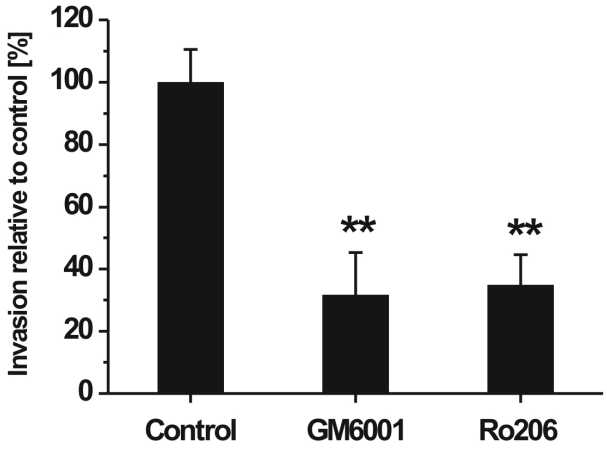

Fig. 16: Influence of MMP inhibitors on hMSC invasiveness through a barrier of reconstituted human basement membrane.

hMSC were placed onto Transwell filters coated with human ECM and incubated for 48 hours in the absence (control) or presence of the broad-spectrum MMP inhibitor GM6001 (10 µg/ml) or Ro 206-0222 (10 µg/ml), a highly specific inhibitor of MMP-2, MMP-9, and MT1-MMP. Cell invasion rate was determined in percent relative to control cells (set as 100 %). Data are presented as mean ±SD of one triplicate experiment representative of three independent measurements. **$p<0.01$.

From these data it can be concluded that MMPs play a major role in the directed traversal of hMSC through human ECM barriers.

E.3.2 Selective knock-down of MMP and TIMP expression by siRNA

To clarify the individual contribution of constitutively expressed MMPs and TIMPs to the cellular invasion capacity, conditions to specifically silence the gene transcription of MMP-2, MT1-MMP, TIMP-1, and TIMP-2 in hMSC by RNAi were established.

As determined by qRT-PCR 24 hours after siRNA transfection, we achieved knock-down efficiencies of 92-98 % for those MMPs and TIMPs when compared to control cells transfected with non-target-directed siRNA (Fig. 17). The downregulation of mRNA expression was still effective with levels between 70-98 %, when determined 72 hours after treatment with the respective siRNAs (Fig. 17).

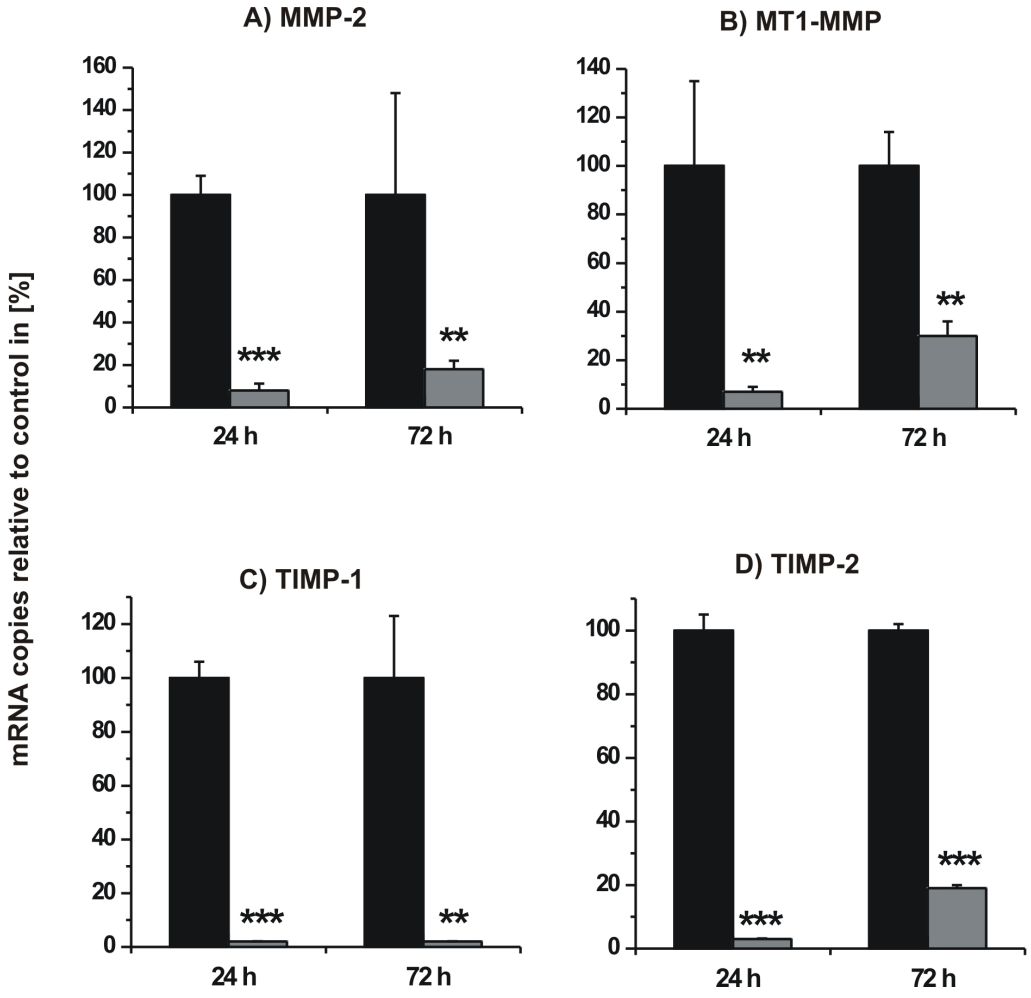

Fig. 17: Knock-down-efficiencies on mRNA levels of MMPs/TIMPs.

hMSC were transfected with siRNAs targeting the gene expression of MMP-2 **(A)**, MT1-MMP **(B)**, TIMP-1 **(C)**, or TIMP-2 **(D)**.

Control cells were transfected with non-target-directed siRNA (set as 100 %). Transcription of specific mRNAs was quantified by qRT-PCR 24 and 72 hours after siRNA transfection. Data represent the mean ±SD of a triplicate measurement representative for five transfection experiments. ***$p<0.001$; **$p<0.01$.

In addition, the mRNA-results could also be confirmed on protein level. Time course analysis of culture supernatants from hMSC transfected with siRNA against MMP-2 revealed a pronounced decline of secreted proMMP-2 as determined by zymography (Fig. 18A). Likewise, successful blockage of MT1-MMP production as well as of the release of TIMP-1 and TIMP-2 in hMSC for 72 hours was demonstrated by Western blotting analysis (Fig. 18B).

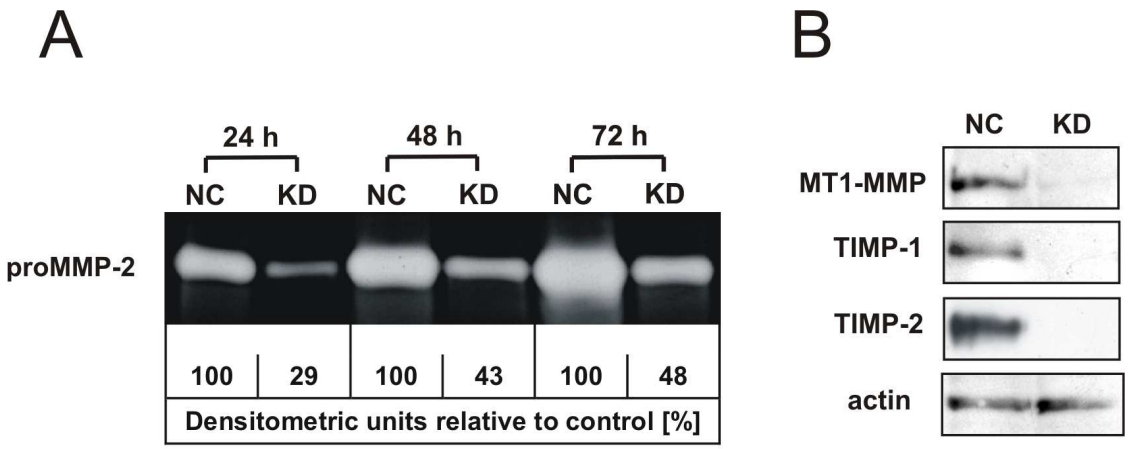

Fig. 18: Effect of MMP/TIMP knock-downs on protein level

(A) hMSC transfected with non-target-directed control siRNA (NC) or with siRNA against MMP-2 (KD) were cultivated under serum-free conditions and analysed for secreted proMMP-2 after different time intervals by zymography. For densitometric quantification, enzyme release from control cells transfected with non-target-directed siRNA was set as 100 % at each time point.

(B) Protein extracts obtained from hMSC 72 hours after transfection with control siRNA (NC) or with siRNA against MT1-MMP (KD) were examined. Secretion of TIMP-1 and TIMP-2 from hMSC carrying the respective knock-downs (KD) or control siRNA (NC) was examined by Western blotting of 72 hour-culture supernatants. B-actin was detected on the same blot to control for application of equal amounts of protein in each lane. Protein data are representative of three independent experiments with similar results.

Hence, the RNAi technology represents an effective tool for studying the functions of MMPs and TIMPs in hMSC.

E.3.3 Invasion capacity of hMSC after knock-down of MMPs/TIMPs

In a next step, RNAi was applied to elucidate the individual role of constitutively expressed MMP-2, MT1-MMP, TIMP-1, and TIMP-2 in the invasive capability of hMSC. For this intention, hMSC carrying specific knock-downs of MMPs and TIMPs were analysed in the Transwell invasion assay.

Down-regulation of MMP-2, MT1-MMP, and TIMP-2 were found to significantly impair the migration of hMSC through the reconstituted basement membranes by 72 %, 75 %, and 65 %, respectively, when compared to control cells carrying a non-target-directed siRNA (Fig. 19).

In contrast, blockage of TIMP-1 raised the invasive behavior of hMSC (Fig. 19).

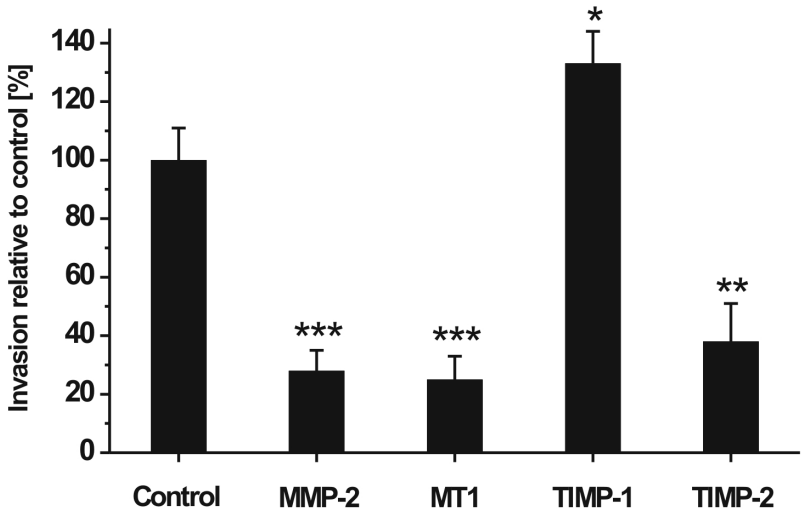

Fig. 19: Effect of knock-down of MMPs and TIMPs on hMSC invasiveness.

hMSC carrying knock-downs for the expression of MMP-2, MT1-MMP (MT1), TIMP-1, and TIMP-2 were assessed for their ability to traverse human ECM. The hMSC were applied in the Transwell invasion assay 24 hours after their transfection and incubated for 48 hours. Thereafter, cells that had migrated to the lower chamber were counted. Control cells transfected with non-target-directed siRNA were set as 100 %. Data are presented as mean ±SD of one triplicate experiment representative of three independent measurements. ***$p<0.001$. **$p<0.01$. *$p<0.05$.

These findings together indicate that the expression of MMP-2 and MT1-MMP as well as of TIMP-2 enable hMSC to migrate across ECM, whereas the production of TIMP-1 exhibited a repressive effect on this process.

Continuative analyses of TIMP-1 functions in hMSC biology are given in chapter E5.

E.3.4 Validation of results in hMSC from different donors

Our findings on the expression of MMPs and TIMPs and their importance for hMSC invasion capacity, which had been obtained in a single hMSC lot, could be confirmed by similar data obtained in hMSC from additional healthy donors (Fig. 20).

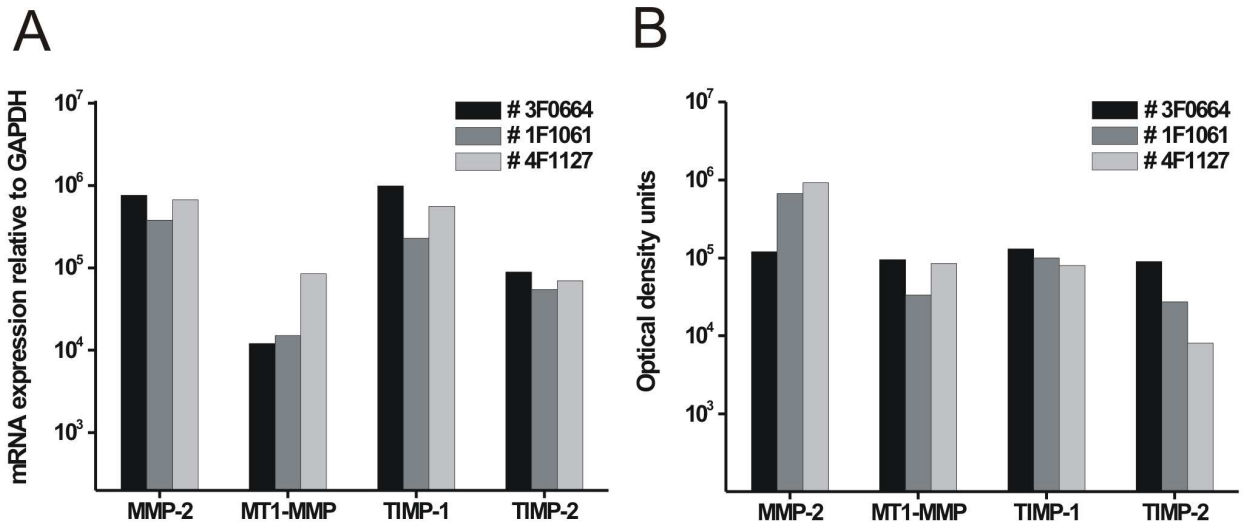

Fig. 20: Comparative analysis expression of MMPs and TIMPs in hMSC from different donors.

(A) mRNA expression of MMPs and TIMPs in cell lysates was examined by qRT-PCR. hMSC isolated from three different healthy donors (lots #3F0664, #1F1061, and #4F1127) were grown at the 5th or 6th passage for 72 hours under serum-free conditions and examined for mRNA expression of MMPs and TIMPs.
Data from a representative experiment are given as mRNA expression relative to GAPDH (mean of triplicate measurement).

(B) hMSC were grown for 72 hours under serum-free conditions. Secretion of MMP-2, TIMP-1 and TIMP-2 were determined by zymography or Western blot analysis of conditioned media. Production of MT1-MMP was evaluated by Western blotting of cell extracts.
The respective protein bands were quantified by densitometry. Results are given as optical density units (mean of two determinations).

All hMSC specimens analysed in our laboratory showed comparable results with respect to constitutive transcription (Fig 20A) and protein synthesis (Fig 20B) of MMP-2, MT1-MMP, TIMP-1 and TIMP-2.

As outlined in table 1, the invasion rates of the different hMSC samples after knock-down of MMP-2, MT1-MMP, TIMP-1 or TIMP-2 correlated with the transfection efficiency in different hMSC lots.

siRNA	hMSC donor	Expression 24 h		Expression 72 h		Invasion
		mRNA	Protein	mRNA	Protein	
MMP-2	3F0664	9	32	12	48	25
	1F1061	25	55	30	58	47
	4F1127	37	57	40	57	41
MT1-MMP	3F0664	7	23	36	36	39
	1F1061	10	19	24	21	45
	4F1127	25	22	26	35	57
TIMP-1	3F0664	12	ND	22	34	111
	4F0591	22	ND	7	15	117
	4F1127	9	ND	6	22	120
TIMP-2	3F0664	26	ND	30	54	56
	4F0591	31	ND	26	39	43
	4F1127	22	ND	29	24	49

Table 1: Efficiencies of knock-down of MMPs/TIMPs in hMSC from three different donors and influence on hMSC invasiveness.

hMSC were transfected with siRNA against MMP-2, MT1-MMP, TIMP-1 or TIMP-2. 24 h and 72 h later cells were examined by qRT-PCR and Western blot for residual mRNA and protein synthesis.

The ability to traverse through human ECM was assayed 24 h after transfection of the cells using the Transwell invasion assay for a 48 h incubation period.

All data obtained in knock-down cells are presented as residual mRNA expression, protein production and invasion capacity in percent compared to hMSC transfected with non-target directed control siRNA (set as 100 %). ND (not determined).

The results show a prominent decrease of the invasion capacity due to the reduced expression of the MMPs and TIMP-2, whereas the knock-down of TIMP-1 was associated with an obvious increase in the hMSC invasiveness (Table 1).

Thus, the data strongly confirms that MMP-2, MT1-MMP, and TIMP-2 are essential for hMSC traversal through human ECM, whereas TIMP-1 inhibits this process.

E.4 Role of inflammatory cytokines/chemokine in hMSC invasiveness

A variety of physiological stimuli has been described to induce or enhance MMP gene expression in diverse cell types. These factors include inflammatory cytokines such as TGF-β1, Il-1β, and TNF-α as well as the chemokine SDF-1α. Since these factors are also found to be increased in damaged and inflamed tissues, the following experiments were designed to elucidate a potential influence of these cytokines and the chemokine on the MMP-mediated invasion capacity of hMSC.

E.4.1 Effects of TGF-β1, Il-1β, TNF-α, and SDF-1α on MMP/TIMP expression

To find appropriate cytokine/chemokine concentrations, hMSC were incubated with increasing amounts of TGF-β1, Il-1β, TNF-α, or SDF-1α, and analysed for mRNA expression of MMP-2, MT1-MMP, MMP-9, TIMP-1, and TIMP-2 by qRT-PCR after different intervals.

Concentrations of 100 ng/ml for TGF-β and SDF-1α and 50 ng/ml for IL-1β and TNF-α were determined as being most effective in the regulation of MMP/TIMP expression (Table 2) without affecting cell vitality (data not shown) and were, therefore, used for further experiments.

	Concentration [ng/ml]	MMP-2		MMP-9		MT1-MMP		TIMP-1		TIMP-2	
		1 d	3 d	1 d	3 d	1 d	3 d	1 d	3 d	1 d	3 d
TGF-β1	10	0.64	2.28	1.40	1.13	1.34	0.40	1.02	0.68	0.75	0.70
	50	0.30	2.20	0.79	0.99	1.44	0.99	0.74	0.70	0.77	0.71
	100	0.27	2.83	0.68	1.34	1.14	1.93	0.98	0.93	0.80	0.81
IL-1β	10	1.30	0.89	2.27	4.40	6.51	2.32	0.88	0.77	0.98	0.88
	50	1.32	1.37	2.86	5.60	7.79	2.98	1.01	0.98	0.88	0.82
	100	1.29	0.98	3.20	3.48	7.90	2.52	1.03	0.99	0.107	1.03
TNF-α	10	0.64	0.29	7.15	64.16	4.56	1.78	1.08	0.90	0.86	0.81
	50	0.29	0.39	7.85	92.04	2.01	2.18	1.02	0.89	1.10	0.98
	100	0.27	0.28	12.16	60.42	1.77	1.26	0.72	0.86	0.78	1.17
SDF-1α	10	0.86	0.31	1.20	1.39	1.00	1.68	1.01	0.92	0.97	1.15
	50	0.86	0.23	1.55	3.60	0.71	2.58	1.04	1.46	1.39	2.34
	100	0.39	0.21	0.94	1.46	0.35	1.38	0.80	0.89	0.64	0.80

Table 2: Cytokine/chemokine dose finding experiments.

hMSC were incubated with 10, 50, 100 ng/ml of TGF-β1, IL-1β, TNF-α and SDF-1α. After 1 (1d) and 3 days (3d) cells were examined for mRNA synthesis of MMP-2, MMP-9, MT1-MMP, TIMP-1, and TIMP-2. The data are shown as x-fold change in mRNA expression (GAPDH corrected) relative to non-treated hMSC (set as 1). Values are given as mean of triplicate determinations.

More detailed studies revealed that incubation of hMSC with TGF-β1 (100 ng/ml) induced the mRNA expression of MMP-2 and MT1-MMP about 3-fold and 2-fold, respectively, when compared to untreated control cells, whereas that of MMP-9 and the TIMPs remained almost unaltered (Fig. 21A). Addition of Il-1β (50 ng/ml) significantly stimulated the transcription of MT1-MMP (~8-fold) and MMP-9 (~5-fold) but did not strongly alter the mRNA levels of MMP-2 and the TIMPs (Fig. 21B). Exposure of hMSC to TNF-α (50 ng/ml) was found to halve the MMP-2 transcription level and concurrently duplicate that of MT1-MMP (Fig. 21C). Remarkably, TNF-α exhibited a strong increase in MMP-9 mRNA synthesis with values of 80-fold and 90-fold after 24 and 72 hours of incubation, respectively, whereas TIMP-1 and TIMP-2 expression was poorly affected (Fig. 21C). Addition of the chemokine SDF-1α (100 ng/ml) to hMSC clearly diminished MMP-2 expression and also reduced that of TIMP-2 and TIMP-1, yet to a minor degree. Interestingly, the mRNA levels of MT1-MMP and MMP-9 declined 24 hours after exposure to SDF-1α but showed some elevation above basal transcription after 72 hours of incubation (Fig. 21D).

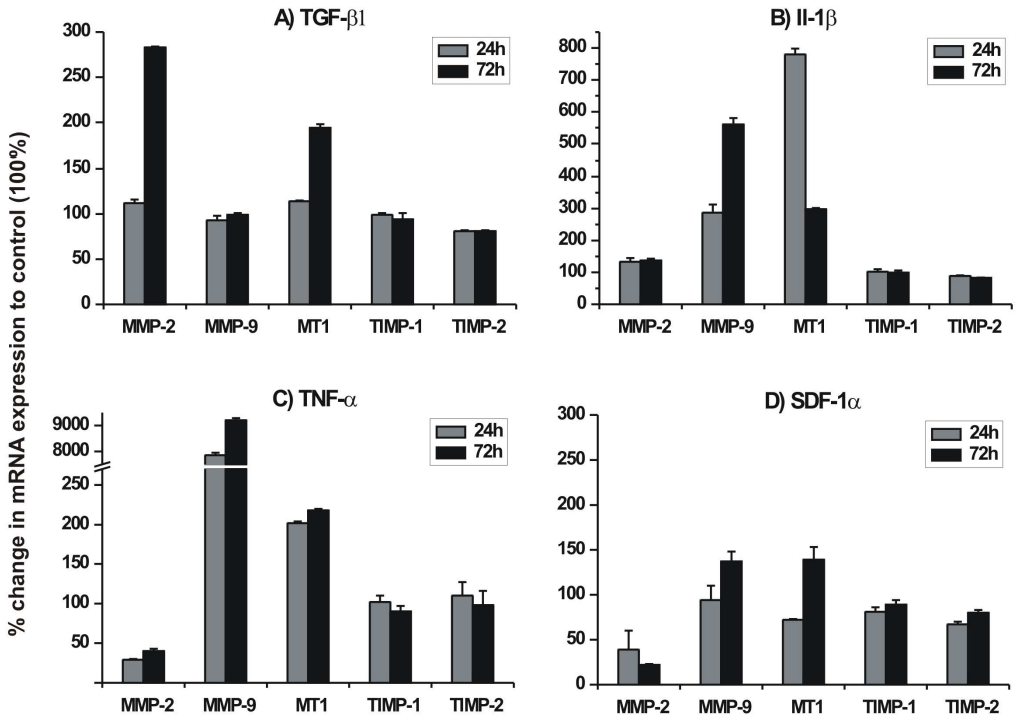

Fig. 21: Influence of cytokines/chemokine on mRNA of MMPs/TIMPs in hMSC.

hMSC were incubated with TGF-β1 (100 ng/ml) **(A)**, Il-1β (50 ng/ml) **(B)**, TNF-α (50 ng/ml) **(C)**, SDF-1α (100 ng/ml) **(D)** or left untreated (control) and cultivated under serum-free conditions for 24 and 72 hours. mRNA expression of MMP-2, MMP-9, MT1-MMP (MT1), TIMP-1, and TIMP-2 were quantified by qRT-PCR. Results are given as percent change in mRNA expression relative to untreated cells set as 100%. Values represent the mean ±SD of one triplicate experiment of two independent measurements.

In correspondence with the mRNA data, TGF-β1 and Il-1β induced an augmentation in secretion of proMMP-2 and its active forms from hMSC, whereas TNF-α and SDF- 1α evoked a reduction in the release of these enzymes as determined by zymographic analysis of diluted culture supernatants (Fig. 22A). TNF-α also stimulated hMSC to produce proMMP-9 and its active form which however became detectable only if non-diluted supernatants were analysed (Fig. 22B).

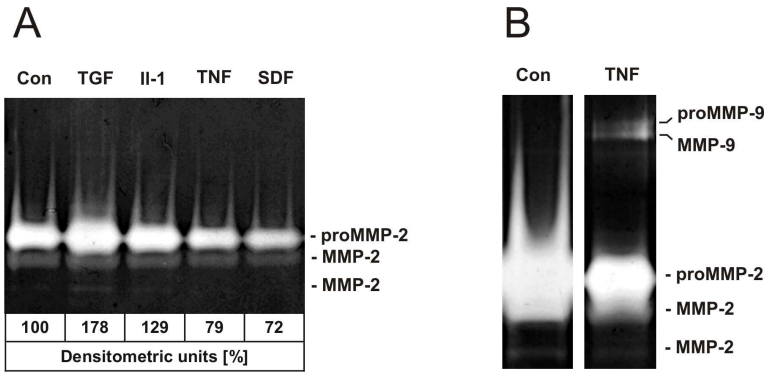

Fig. 22: Zymographic analysis of hMSC supernatants.

hMSC were incubated with TGF-β1 (100 ng/ml), Il-1β (50 ng/ml), TNF-α (50 ng/ml), SDF-1α (100 ng/ml) or left untreated (control) and cultivated under serum-free conditions for 24 and 72 hours. Aliquots of 72 hour-culture supernatants were subjected to zymography. Medium samples were applied in a 1:4 dilution to allow densitometric quantification of proMMP-2 **(A)** or undiluted to achieve higher sensitivity for the detection of proMMP-9 **(B)**.

In addition, protein synthesis of cell membrane-bound MT1-MMP in hMSC was quantified by the use of an assay measuring the biologically active form of this enzyme which is characteristically produced immediately after its synthesis in the cell. In agreement with the mRNA data, basal MT1-MMP activity was clearly enhanced upon incubation of hMSC with TGF-β1 (~5-fold), Il-1β (~9-fold), TNF-α (~7-fold), and SDF-1α (~2-fold) (Fig. 23).

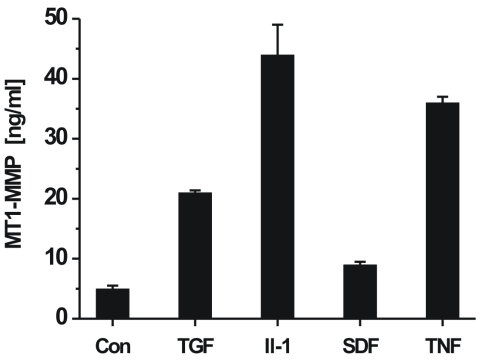

Fig. 23: Quantification of MT1-MMP synthesis in hMSC.

hMSC were incubated with TGF-β1 (100 ng/ml), Il-1β (50 ng/ml), TNF-α (50 ng/ml), SDF-1α (100 ng/ml) or left untreated (control) and cultivated under serum-free conditions for 72 hours**.**

MT1-MMP protein was determined in cell lysates of hMSC incubated for 72 hours with or without cytokines/chemokine using the "MMP-14 Biotrak Activity Assay". Data are shown as mean ±SD of one of two triplicate experiments.

Summarizing our findings, TGF-β1, Il-1β, TNF-α, and SDF-1α differentially regulate the mRNA expression and protein synthesis of MMP-2, MMP-9 and MT1-MMP in hMSC, whereas that of TIMP-1 and TIMP-2 are less affected by these cytokines or chemokine.

E.4.2 Invasion capacity of hMSC towards gradients of cytokines/chemokine

To further elucidate a potential chemoattractive role on hMSC, the cytokines TGF-β1 (100 ng/ml), Il-1β (50 ng/ml), and TNF-α (50 ng/ml), as well as the chemokine SDF-1α (100 ng/ml) were added instead of human serum as chemoattractant into the lower compartment of the Transwell chamber in presence or absence of Ro 206-0222 (10 µg/ml), the specific inhibitor of MMP-2, MT1-MMP, and MMP-9 activity.

Determination of hMSC migration rates after a 48 hour-incubation period revealed highly increased cell invasiveness stimulated by TGF-β1 (~7-fold), Il-1β (~6-fold), TNF-α (~3-fold), and SDF-1α (~2-fold) relative to the serum-free medium alone (control) (Fig. 24). By comparison, addition of 10 % human serum used as a chemoattractant under standard assay conditions increased spontaneous hMSC migration by 14-fold (data not shown).

The strong chemotactic responses of hMSC in trafficking through the ECM barrier towards gradients of TGF-β1, Il-1β, and TNF-α, respectively, were clearly abrogated or even diminished below the level of spontaneous cell migration by the addition of Ro 206-0222, whereas the SDF-1α-stimulated invasion was only poorly attenuated in the presence of the MMP-inhibitor (Fig. 24).

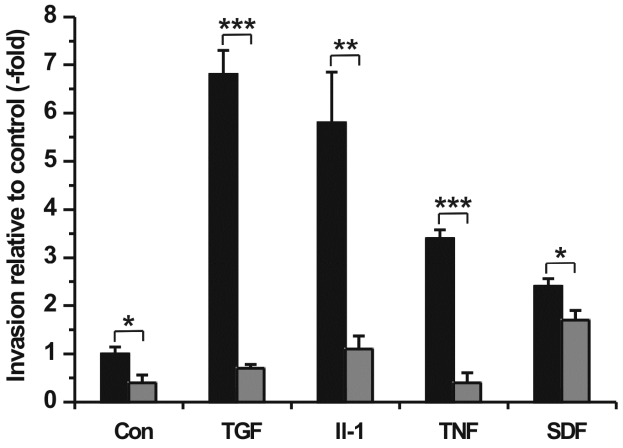

Fig. 24: Chemotactic effects of cytokines/chemokine on hMSC invasion capacity and the implication of MMPs.

hMSC were seeded in the upper compartment of Transwell invasion chambers. The lower compartment was filled with DMEM containing TGF-β (100 ng/ml), Il-1α (50 ng/ml), TNF-α (50 ng/ml), or SDF-1α (100 ng/ml) as chemoattractants. Control wells contained DMEM medium only (Con). Both, upper and lower compartments were provided without (black bars) and with Ro 206-0222 (10 µg/ml) to inhibit MMP-2, MMP-9, and MT1-MMP activity (grey bars). After a 48-hour period of incubation the amount of migrated cells was quantified. Results are given as x-fold increase relative to spontaneous cell migration in control wells. All experiments were performed in triplicate. The mean values ±SD of one of two equivalent separate experiments are shown. *p<0.05. **p<0.01. ***p<0.001.

Taken together, these findings indicate that (in contrast to the chemokine SDF-1α) the inflammatory cytokines TGF-β1, Il-1β, and TNF-α act as strong chemoattractants for hMSC and enable their directed traversal through basement membranes which is dependent on the activity of specific MMPs.

E.5 TIMP-1 as a regulator of hMSC functions

As shown in chapter E1, hMSC constitutively express and secrete high amounts of TIMP-1, whereas MMP-9, the major target protease of TIMP-1, is not produced in the cells. Moreover, knock-down studies have demonstrated that TIMP-1 exhibits only weak inhibitory impact on hMSC invasiveness (see chapter E.3.3). These observations led to the speculation that strong basal expression of TIMP-1 might serve additional purposes in hMSC function. Therefore, endogeneous TIMP-1 was investigated for its influence on two important hMSC characteristics, the cell proliferation and differentiation including related effects on specific gene expression.

E.5.1 Influence of TIMP-1 on hMSC proliferation

The role of TIMP-1 expression in hMSC was silenced by RNAi and cellular proliferation was determined after 3 and 7 days of incubation. Significantly higher numbers of viable hMSC were detected after kock-down of TIMP-1 compared to control cells transfected with non-target directed siRNA (Fig. 25). These data indicate that endogenously produced TIMP-1 represses the proliferative capacities of hMSC.

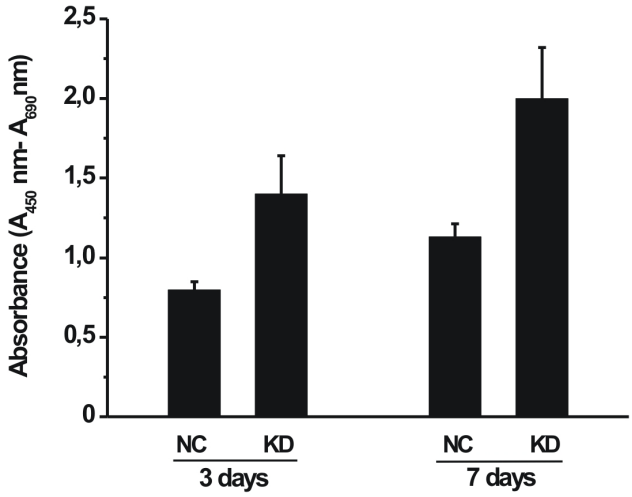

Fig. 25: Cell proliferation after knock-down of TIMP-1.

hMSC transfected with control siRNA (NC) or siRNA against TIMP-1 (KD) were seeded in 96-well plates at 5 x 10^3 cells / well containing 100 µl of serum-free culture medium. After 3 and 7 days of incubation cell proliferation was determined using the WST-1 assay. Absorbance at 450 nm was measured in a multiwell-plate-reader. Values represent the mean ±SD of one triplicate experiment from two independent measurements.

E.5.2 Influence of TIMP-1 on hMSC differentiation

A characteristic feature of hMSC is their ability to differentiate into osteogenic and adipogenic cells upon *in vitro* incubation with specific differentiation media. To investigate the role of endogeneous TIMP-1 in these processes, osteogenic and adipogenic differentiation was induced in hMSC 3 days after their transfection with TIMP-1 siRNA or control siRNA.

E.5.2.1 Osteogenic differentiation of hMSC after knock-down of TIMP-1

After 2 weeks of incubation in osteogenic differentiation medium, hMSC both with and without knock-down of TIMP-1 showed characteristic osteogenic morphological changes with a typical cobble-stone arrangement of cells. Comparative analysis of calcium deposition as a marker for osteoblastic cells using Alizarin-Red staining indicated that TIMP-1-knock-down cells produce a stronger red coloration than control cells (Fig. 26A) suggesting enhanced osteogenic differentiation in hMSC after downregulation of TIMP-1.

This finding was confirmed by mRNA expression analysis of decorin, an additional characteristic marker for osteogenic differentiation. Decorin transcription was up-regulated by 50 % in knock-down cells compared to control cells (Fig. 26B). Potential TIMP-1-mediated interferences with cell proliferation were excluded by standardizing the qRT-PCR determination of decorin against that of GAPDH.

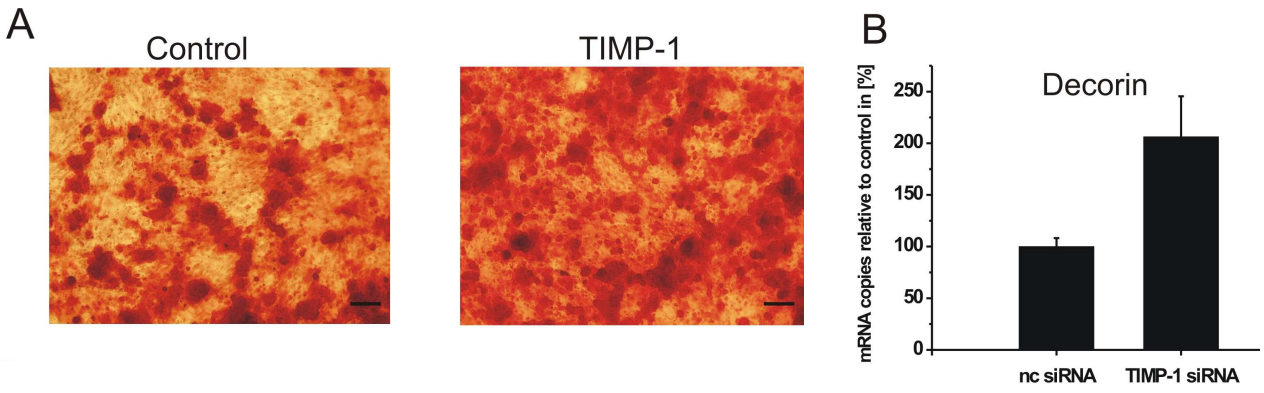

Fig. 26: Osteogenic differentiation of hMSC after knock-down of TIMP-1.

hMSC were transfected with control siRNA (nc) or siRNA against TIMP-1. Thereafter, the cells were incubated for 14 days in medium inducing osteogenic differentiation.

(A) Microscopic analysis of calcium deposition by Alizarin-Red staining. All scale bars indicate 50 µm.

(B) qRT-PCR analysis of decorin, a marker of osteogenic differentiation. The results are given as mean values ±SD of mRNA expression relative to GAPDH from a triplicate measurement representative for three independent experiments.

These data indicate that constitutive TIMP-1 production in hMSC negatively influences the capacity of these cells for osteogenic differentiation.

E.5.2.2 Adipogenic differentiation of hMSC after knock-down of TIMP-1

To induce adipogenic differentiation, hMSC were incubated for 2 weeks in specific differentiation medium. After this time interval, the ability of cells to form intracellular lipid droplets was assayed using Oil-Red-O staining. Microscopical analysis revealed a higher number of lipid droplets in hMSC with knock-down of TIMP-1 as compared to control cells (Fig. 27A). This was confirmed by quantification of lipid formation in cells using Oil-Red-O staining which revealed higher levels in cells with knock-down of TIMP-1 compared to controls (Fig. 27B).

In addition, the mRNA expression of the adipogenic marker PPAR-γ was shown to be upregulated 2-fold in hMSC lacking TIMP-1 transcription (Fig. 27C).

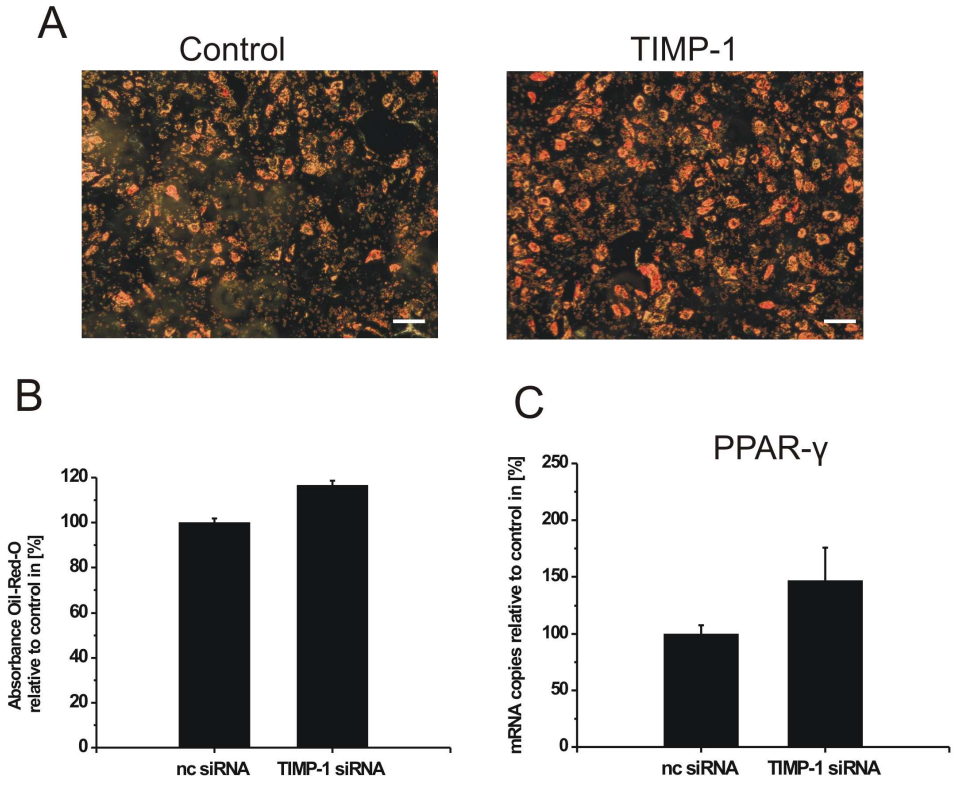

Fig. 27: Adipogenic differentiation of hMSC after knock-down of TIMP-1.

hMSC were transfected with control siRNA or siRNA (nc) against TIMP-1. Thereafter the cells were incubated for 14 days in medium inducing adipogenic differentiation.

(A) Microscopic analysis of intracellular lipid formation by Oil-Red O staining. All scale bars indicate 50 µm.

(B) Quantification of Oil-Red-O staining after lysis of cells and measurement of absorbance at 450 nm.

(C) qRT-PCR analysis of PPAR-γ, a marker of adipogenic differentiation. The results are given as mean values ±SD of mRNA expression relative to GAPDH from a triplicate measurement representative for three independent experiments.

Taken together, the data indicate a repressive role of endogeneous TIMP-1 in the ability of hMSC for adipogenic differentiation.

E.5.3 Microarray analysis of genes regulated by TIMP-1

To identify genes involved in the intracellular signalling of TIMP-1 in hMSC, we applied the mRNA array *"Human Signal Transduction PathwayFinder"* (Superarray Bioscience Corporation). Blockage of TIMP-1 transcription in hMSC by RNAi evoked upregulation (≥ 2 fold) of 15 genes, the majority of which are known to be involved in the Wnt signalling pathway. They include *BIRC5, JUN, MYC, PPARG* which are components of the Wnt pathway or represent typical target genes such as *TCF7, WISP1, WISP2* and *WISP3*.

Two gene transcripts, *BMP2* and *BMP4*, were found to be decreased (≥ 2 fold) in cells with the knock-down of TIMP-1 compared to control cells (Table 3).

Accession Number	Symbol	Name	Modulation
NM_000633	BCL2	B-cell CLL/lymphoma 2	UP
NM_138578	BCL2L1	BCL2-like 1	UP
NM_001168	BIRC5 *	Baculoviral IAP repeat-containing 5 (survivin)	UP
NM_001200	BMP-2	Bone morphogenetic protein 2	DOWN
NM_130851	BMP-4	Bone morphogenetic protein 4	DOWN
NM_002228	JUN *	Jun oncogene	UP
NM_002467	MYC *	V-myc myelocytomatosis viral oncogene homolog (avian)	UP
NM_003998	NFKβ1	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 (p105)	UP
NM_020529	NFKβ1A	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	UP
NM_015869	PPARG *	Peroxisome proliferator-activated receptor gamma	UP
NM_015869	PRKCA	Peroxisome proliferator-activated receptor gamma	UP
NM_004180	TANK	TRAF family member-associated NFKB activator	UP
NM_003202	TCF7 *	Transcription factor 7 (T-cell specific, HMG-box)	UP
NM_003234	TRFC	Transferrin receptor (p90, CD71)	UP
NM_003882	WISP1 *	WNT1 inducible signaling pathway protein 1	UP
NM_003881	WISP2 *	WNT1 inducible signaling pathway protein 2	UP
NM_003880	WISP3 *	WNT1 inducible signaling pathway protein 3	UP

Table 3: Genes modulated in hMSC by TIMP-1

hMSC were transfected with TIMP-1 siRNA and maintained in culture for 3 days until TIMP-1 protein expression was abrogated. Then cDNA microarray analysis was performed. Results are shown for genes modulated ≥ 2-fold in knock-down cells as compared to cells transfected with control siRNA. Genes involved in the Wnt signalling pathway are marked with (*).

Taken together, these findings clearly suggest the implication of the Wnt pathway in the transmission of cellular effects mediated by endogenous TIMP-1 in hMSC.

E.5.4 mRNA analysis of Wnt target genes in hMSC after knock-down of TIMP-1

To verify a potential interaction between TIMP-1 and the Wnt signalling transduction pathway as suggested by microarray analysis, we next examined the regulation of typical Wnt target genes[120] by TIMP-1. For this purpose mRNA expression of the cell cycle regulator cyclin D1 and the proteolytic enzyme MT1-MMP was monitored in hMSC with silenced and non-silenced TIMP-1 transcription. Cells were analysed at day 7 after treatment without and with Wnt3a (150 ng/ml).

As to be expected, Wnt3a increased the expression of its target genes cyclin D1 in hMSC transfected with siRNA (Fig. 28A). The stimulatory effect of Wnt3a on the expression of cyclin D1 was significantly stronger in hMSC if TIMP-1 production was abolished. This increase in susceptibility to Wnt3a stimulation in cells with knock-down of TIMP-1 was dramatically reduced upon addition of recombinant TIMP-1 to the cells (Fig. 28A). Similar results, albeit with less obvious effects, were observed in the expression of MT1-MMP, another Wnt target gene (Fig. 28B).

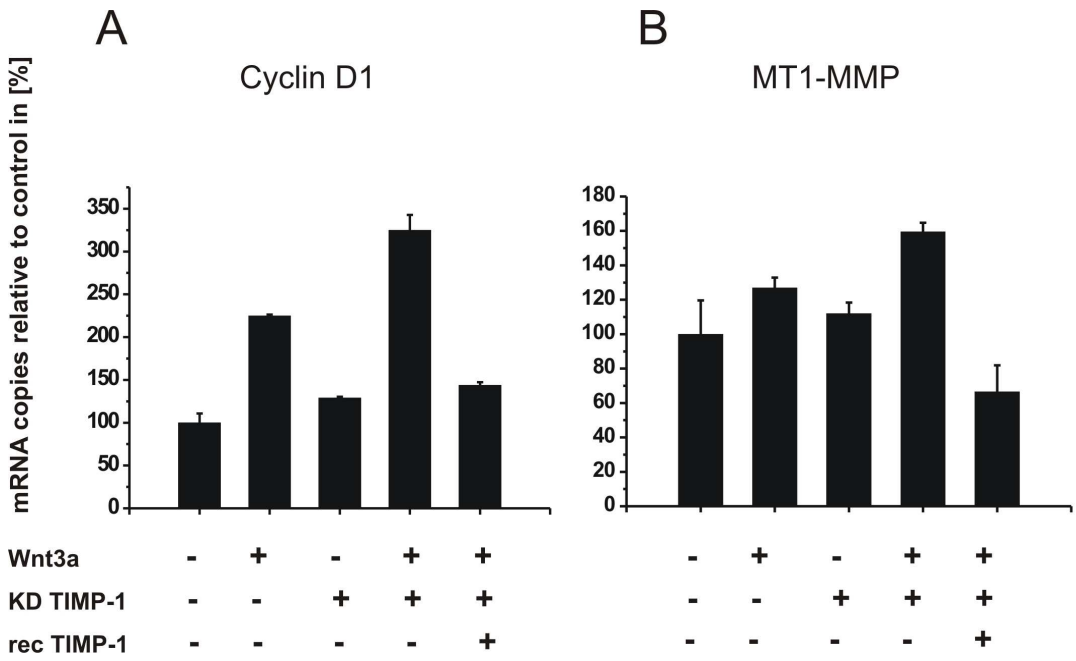

Fig. 28: Regulation of Wnt target genes by TIMP-1.

qRT-PCR analysis of the Wnt target genes cyclin D1 **(A)** and MT1-MMP **(B)** after 7 days of incubation in presence or absence of 150 ng/ml Wnt3a in hMSC transfected with control siRNA or TIMP-1 siRNA. The results are mean values ±SD of mRNA expression from a triplicate measurement representative for three independent experiments.

KD TIMP-1 (hMSC transfected with TIMP-1 siRNA) NC (hMSC transfected with negative control siRNA), W (stimulation with Wnt3a), rec TIMP-1 (recombinant TIMP-1).

Taken together, these results strongly implicate that TIMP-1 exhibits a repressive function on the induction of typical Wnt target genes by Wnt3a in hMSC.

E.6 TNF-α as an inductor of hMSC differentiation

As specified above, TNF-α stimulates migration and modulates MMP expression of hMSC during a 3 day-incubation period. Moreover, preliminary treatment of hMSC with TNF-α for an extended period of time (1-4 weeks) revealed prominent changes in cell morphology, which was investigated in more detail as outlined below.

E.6.1 Influence of TNF-α on hMSC functions

To analyse the influence of TNF-α on morphlogy and diverse cell functions, a long-term incubation of hMSC with TNF-α was performed.

E.6.1.1 Influence of TNF-α on cell morphology

Incubation of hMSC in the presence of TNF-α (50 ng/ml) for 14-28 days induced remarkable changes in cell morphology. After 14 days of treatment with TNF-α, hMSC had mostly lost their typical flat spindle-shaped morphology and displayed a more elongated and contracted cell contour in comparison with untreated cells (Fig. 29A, B). Upon 28 days of exposition to TNF-α the majority of cells had developed spherical refractile cell bodies which prevalently showed radial and/or branched cellular extensions (Fig. 29C). This morphology is similar to that of neuroglial cells such as astrocytes.

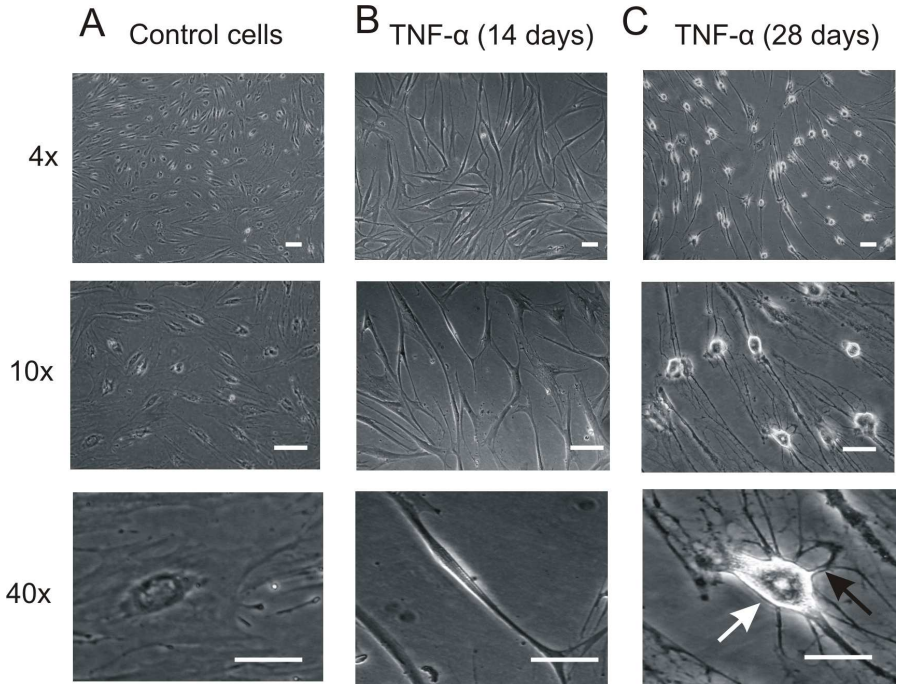

Fig. 29: Morphological changes of hMSC after long-term treatment with TNF-α.

hMSC were grown in the absence **(A)** and presence of TNF-α (50 ng/ml) **(B, C)**. Changes in cellular morphology were monitored by microscopy after 14 days **(B)** and 28 days **(C)** of treatment with TNF-α. Branching cellular extensions (black arrow) and spherical cell bodies (white arrow) became overt. All scale bars indicate 50 µm. Magnification: 4x, 10x, 40x.

E.6.1.2 Influence of TNF-α on cell proliferation

To investigate the influence of TNF-α on hMSC proliferation, cells were incubated for 14 days with different concentrations of TNF-α. At low concentrations (1-25 ng/ml) of TNF-α hMSC proliferation was enhanced with a maximum of 2-fold at 5 ng/ml TNF-α (Fig. 30). At the higher dosage (50 ng/ml) of TNF-α, the cell division rate did not differ significantly from that of untreated control cells, whereas hMSC grown in the presence of 100 ng/ml TNF-α showed a reduced proliferative capacity (Fig. 30).

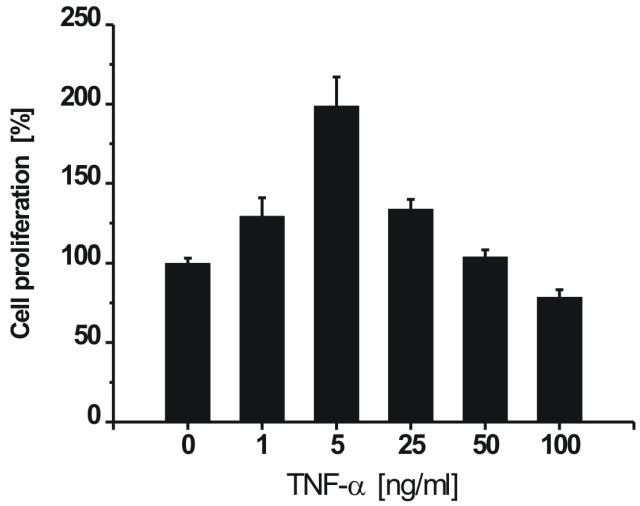

Fig. 30: hMSC proliferation after 14 days of treatment with TNF-α.

hMSC were incubated with different concentrations of TNF-α for 14 days and then analysed using the WST-1 cell proliferation assay. The results are mean values ±SD of triplicate deter-minations on cellular proliferation relative to untreated control cells (set as 100 %).

E.6.1.3 Invasive capacity of hMSC after pretreatment with TNF-α

Next, we investigated whether or not pretreatment of hMSC with TNF-α affected their capacity for transmigration through human reconstituted extracellular matrix (ECM) using the Transwell assay. After pre-incubation for 14 days with increasing concentrations of TNF-α hMSC showed a dose-dependent impairment of their invasion capacity when applying human serum as a chemoattractant (Fig. 31).

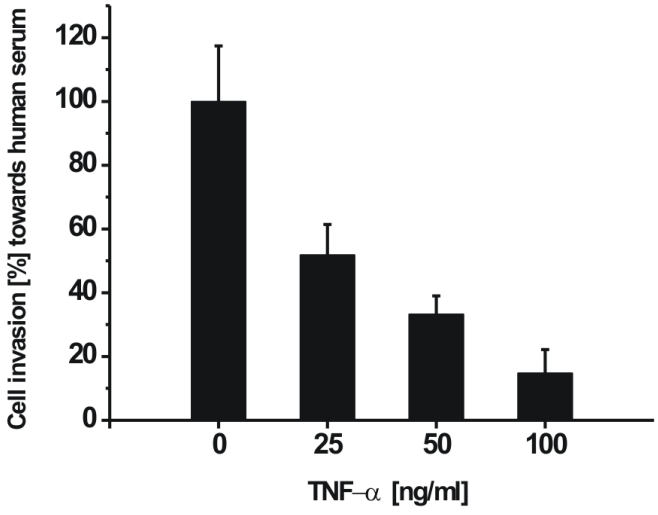

Fig. 31: Invasive capacity of hMSC preincubated with TNF-α.

hMSC were pretreated with different concentrations of TNF-α for 14 days. The cells were then analysed for their potential to migrate through hECM towards human serum using the Transwell-invasion assay. The invasion rate of non-treated hMSC was set as 100%. The results are mean values ±SD of a triplicate measurement.

E.6.1.4 Microarray analysis of TNF-α-modulated gene expression in hMSC

The influence of TNF-α on hMSC gene expression was investigated in detail, using the GEArray Human Stem Cell Gene Array (Superarray Bioscience Corporation) which allows determination of the transcription of marker genes known to be important for identification, growth, and differentiation of various types of (stem) cells. The microarray analysis was carried out in hMSC after a 14 day incubation period in the absence or presence of TNF-α (50 ng/ml).

This study revealed that TNF-α upregulated the transcription of 40 genes in hMSC and downregulated 6 genes by at least 2-fold compared to untreated control cells (Table 4). Many of these genes including *GFAP, MAP2, LIF, BMP2, SOX2, NES, NCAM2, CDH2, S100B* and *CXCR4* are characteristically expressed in neural (stem) cells and known for their relevance in the development of these cells.

Strikingly, a number of genes which were found to be modulated in hMSC by TNF-α are involved in the Wnt signalling pathway such as *CTNNA1*, *CTNNB1*, *FDZ3*, *FDZ4*, *WNT3*, *WNT5A* and *WNT7B* (Table 4).

E.6.1.5 Validation of neural gene expression in hMSC treated with TNF-α

To approve the data obtained by the microarray studies, mRNA analysis of selected neural marker genes was performed in hMSC cultivated without or with TNF-α (50 ng/ml) over a time course of 14 days using qRT-PCR.

The transcription of LIF and BMP2 was upregulated about 11-fold and 13-fold, respectively, as compared to untreated hMSC when determined 1 day after stimulation with TNF-α and declined during prolonged cultivation (Fig. 32). Expression of SOX2 was constantly elevated 2-3-fold during 1-14 days, while that of MAP2 began to increase after 3 days reaching 3-fold augmentation after 14 days of exposition to TNF-α. In contrast, basal transcription of NES was dramatically diminished 1 day after treatment with TNF-α and further persisted on a low level over 14 days of incubation (Fig. 32).

Thus, qRT-PCR analysis in hMSC confirmed the microarray data demonstrating TNF-α to stimulate expression of LIF, BMP2, SOX2 and MAP2, whereas that of NES is blocked.

Accession Number	Symbol	Name	Modulation
NM_001200	BMP2	Bone morphogenetic protein	UP
NM_001202	BMP4	Bone morphogenetic protein 4	DOWN
NM_004329	BMPR1A	Bone morphogenetic receptor, type 1A	UP
NM_001203	BMPR1B	Bone morphogenetic receptor, type 1B	UP
NM_001204	BMPR2	Bone morphogenetic protein receptor, type II	UP
NM_019895	C3orf4	Chromosome 3 open reading frame	UP
M34064	CDH2	N-Cadherin	UP
U26727	CDKN2A	Cyclin dependent kinase inhibitor 2A	UP
NM_001903	CTNNA1	Catenin alpha 1	UP
NM_001904	CTNNB1	Catenin beta 1	UP
NM_003467	CXCR4	Chemokine receptor 4	UP
NM_004412	DNMT2	DNA (cytosine-5-)methyltransferase 2	DOWN
NM_017412	FDZ3	Frizzled homolog 3	UP
NM_012193	FDZ4	Frizzled homolog 4	UP
X51943	FGF1	Fibroblast growth factor	UP
NM_002009	FGF7	Fibroblast growth factor 7	UP
M55614	FGFR2	Fibroblast growth factor receptor 2	DOWN
NM_003923	FOXH1	FOXH1 forkhead box H1	UP
NM_002015	FOXO1A	FOXO1A forkhead box O1A	UP
NM_002055	GFAP	Glial fibrillary acidic protein	UP
NM_000876	IGFR2	Insulin-like growth 2 factor receptor	UP
XM_167711	ITGA8	Integrin alpha 8	UP
NM_000526	KRT14	Keratin 14	DOWN
X13967	LIF	Leukemia inhibitory factor	UP
NM_002310	LIFR	Homo sapiens leukaemia inhibitory factor receptor (LIFR) mRNA	UP
NM_031846	MAP2	Microtubule-associated protein 2	UP
Z12020	MDM2	P53 binding protein	UP
NM_004540	NCAM2	Neural cell adhesion molecule 2	UP
X65964	NES	Human nestin	DOWN
NM_013957	NGR1	Neuregulin 1	UP
NM_005450	NOG	Noggin	UP
NM_002530	NTRK3	Neurotrophic tyrosine kinase receptor type 3	UP
NM_002609	PDFGFRB	Platelet-derived growth factor receptor, beta polypeptide	UP
NM_000442	PECAM1	Paleted endothelial cell adhesion molecule (CD31 antigen)	UP
U96180	PTEN	Phosphatase and tensin homolog	UP
NM_006272	S100B	S100 calcium binding protein, beta	UP
NM_006942	SOX15	SRY box 15	UP
NM_022454	SOX17	SRY box 17	UP
BC013923	SOX2	SRY box 2	UP
NM_003107	SOX4	SRY box 4	UP
NM_017489	TERF1	Telomeric repeat binding factor 1	UP
D50683	TGFBR2	Transforming growth factor, beta receptor 2	UP
NM_003380	VIM	Vimentin	DOWN
NM_030753	WNT3	Wingless-type MMTV integration site family,member 3	UP
NM_003392	WNT5A	Wingless-type MMTV integration site family,member 5A	UP
NM_058238	WNT7B	Wingless-type MMTV integration site family,member 7B	UP

Table 4: Genes modulated in hMSC after treatment with TNF-α.

hMSC were incubated without and with TNF-α (50 ng/ml) for 14 days. 1 µg RNA was isolated and microarray analysis was performed using the "*Stem Cell cDNA GEArray"* (Superarray Bioscience Corporation). Results are shown for genes modulated ≥ 2-fold in the cells treated with TNF-α when compared to non-treated cells (set as 1). The expression of the genes was normalised to cyclophilin B.

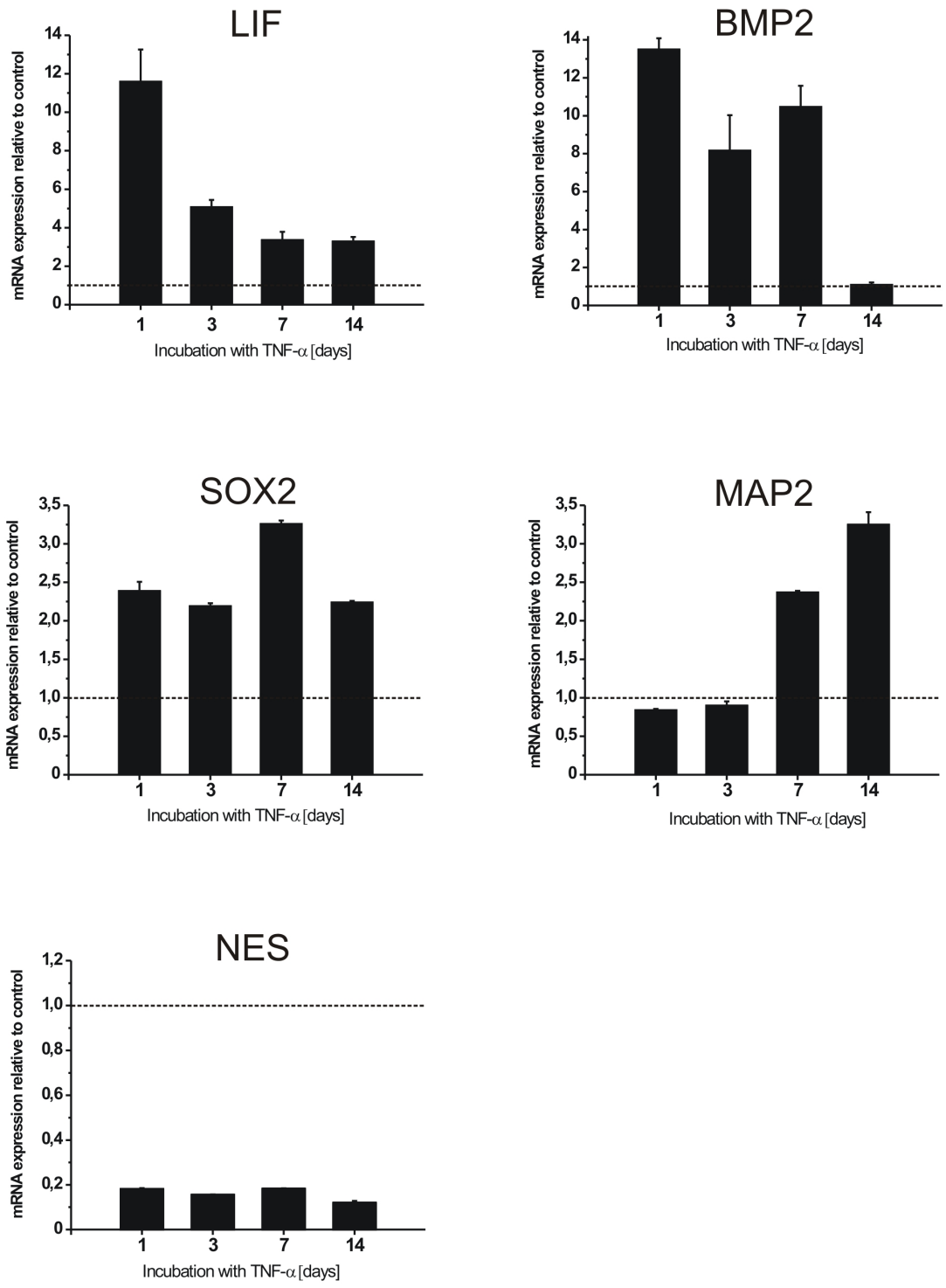

Fig. 32: mRNA expression analysis of neural markers in hMSC treated with TNF-α.

hMSC were incubated without or with TNF-α (50 ng/ml) for different time intervals. mRNA expression of typical neural genes was examined by qRT-PCR analysis. Results are given as change in mRNA expression normalized against cyclophilin B (CPB) relative to untreated cells set as 1. Values represent the mean ±SD of one triplicate experiment from two independent measurements.

LIF (leukemia inhibitory factor), BMP2 (bone morphogenetic protein), SOX2 (SRY box 2), MAP2 (microtubule-associated protein 2), NES (human nestin).

E.6.1.6 Detection of neural proteins in hMSC pretreated with TNF-α

On protein level, TNF-α was shown to induce *de novo*-synthesis of the neural marker MAP-2 and augmented the production of GFAP in hMSC as determined by immunocytochemistry (Fig. 33) which was in agreement with the mRNA data. Interestingly, while GFAP production was homogeneously distributed within the hMSC population, MAP-2 became only detectable in a minority of cells upon incubation with TNF-α for 28 days.

The neural marker proteins β-tubulin III and GalC were not detected by immunocytochemistry, neither in untreated nor in TNF-α-treated hMSC (data not shown).

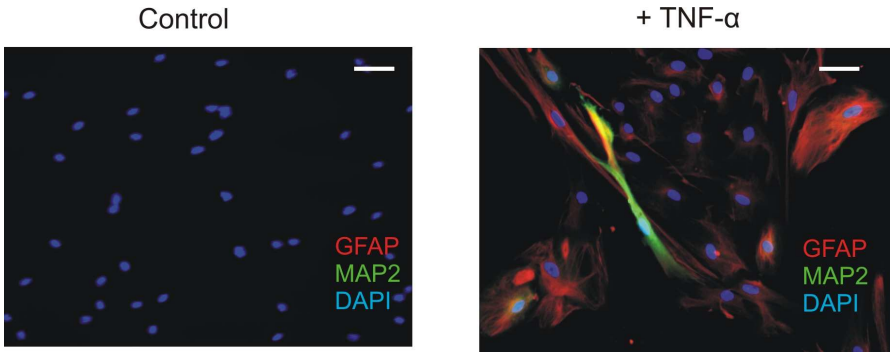

Fig. 33: Immunostaining analysis of hMSC treated with TNF-α.

Immunocytochemical staining of neural marker proteins in hMSC treated without (control) or with TNF-α (50 ng/ml) for 28 days. GFAP and MAP-2 were stained in red and green, respectively. Cell nuclei were counterstained with DAPI (blue). Scale bars indicate 50 µm.

E.6.1.7 Neurosphere formation in hMSC pretreated with TNF-α

A characteristic feature of neural progenitor cells is their ability to build spheroids, the so-called neurospheres. When hMSC were pretreated with TNF-α (50 ng/ml) for 14 days and then further cultivated with TNF-α on poly-D-lysine coated dishes, they formed neurosphere-like structures (Fig. 34), whereas untreated hMSC did not.

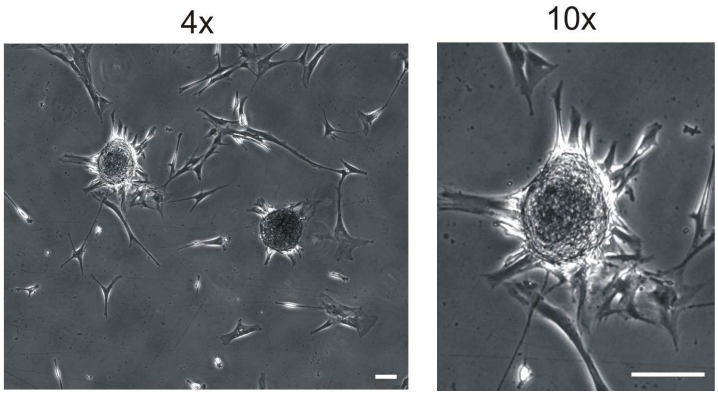

Fig. 34: Neurosphere formation of hMSC treated with TNF-α.

hMSC were incubated with TNF-α (50 ng/ml) for 14 days and then cultivated for another 2 days on dishes coated with poly-D-lysin. Formation of neurosphere like structures was assayed under the microscope.

Scale bars indicate 50 µm.

E.6.2 Influence of MAPK-inhibitors on the expression of neural genes

Next, we were interested in the intracellular signalling mechanisms controlling the TNF-α-mediated neural differentiation in hMSC. To explore the involvement of MAPKs in this process, inhibitors which block the signalling pathways of ERK1/2, p38, and JNK were added to cultures of hMSC grown in the presence of TNF-α.

After 1 and 7 days of incubation, cells were analysed for mRNA expression of neural markers using qRT-PCR. Application of the ERK1/2 inhibitor PD98059 (20 µM) prevented the TNF-α-induced mRNA upregulation of leukemia inhibitory factor (LIF), bone morphogenetic protein-2 (BMP2), SRY box 2 (SOX2), and microtubule-associated protein 2 (MAP2). Furthermore, downregulation of human nestin (NES) was circumvented (Fig. 36).

In contrast, neither the addition of SB203580, an inhibitor of p38 activity, nor the application of JNK-I2 which blocks JNK activity had significant influence on mRNA expression of these genes (data not shown).

As shown exemplarily for GFAP, the protein synthesis of this neural marker in hMSC treated with TNF-α was abrogated in the presence of PD98059 (Fig. 35).

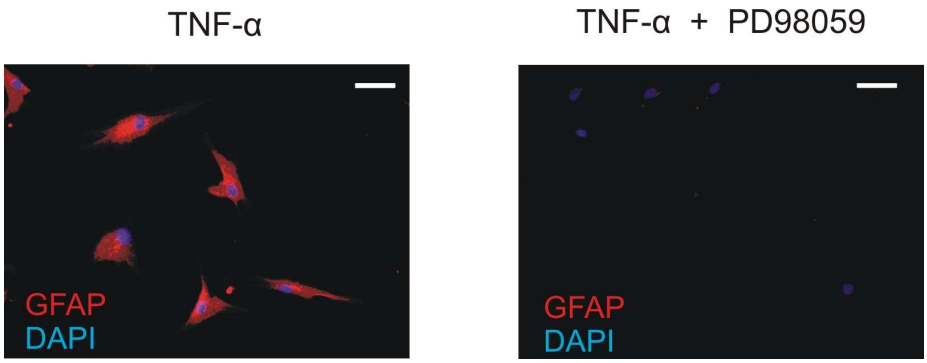

Fig. 35: Immunofluorescence analysis of GFAP protein expression.

hMSC were incubated for 14 days in presence of TNF-α (50 ng/ml) without and with the addition of PD98059 (20 µM) and were stained for GFAP in green (FITC) and cell nuclei in blue (DAPI).
Scale bars indicate 50 µm.

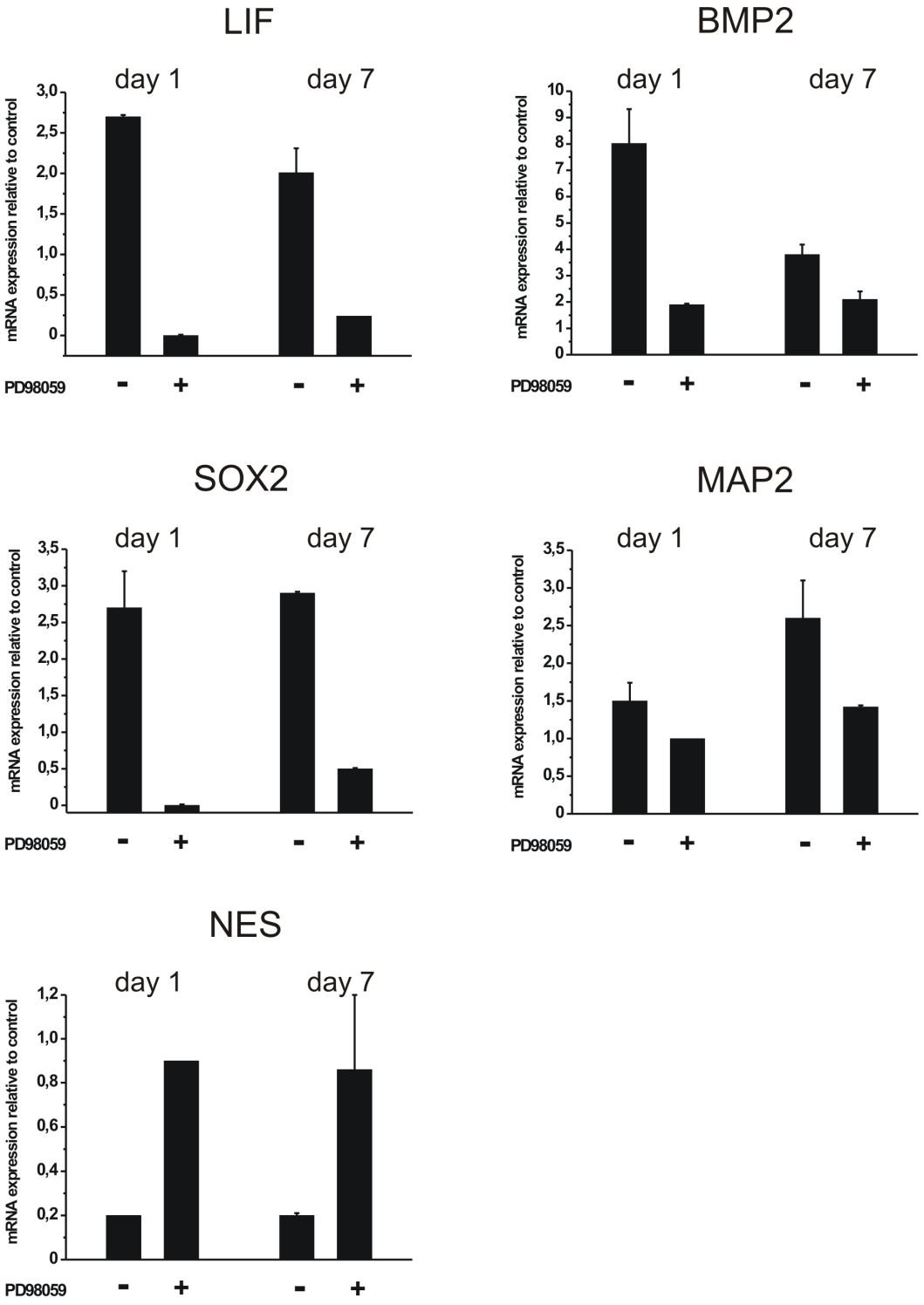

Fig. 36: Role of ERK1/2 MAP kinase in TNF-α-induced neural differentiation.

hMSC were cultivated in the presence of TNF-α (50 ng/ml) without and with addition of PD98059 (20 µM), a specific inhibitor of ERK1/2 phosphorylation. mRNA expression of neural marker genes was measured by qRT-PCR after 1 and 7 days of incubation. Results indicate change in mRNA expression after normalization against cyclophilin B (CPB) relative to untreated hMSC set as 1. Values represent mean ±SD of one triplicate experiment representative for two independent measurements.

E.6.3 Influence of TNF-α on ERK1/2 phosphorylation

The detailed examination of ERK1/2 phosphorylation by Western blotting revealed that TNF-α strongly stimulated intracellular formation of phosphorylated ERK1/2 after 7-14 days of incubation. Interestingly, TNF-α also increased total ERK1/2 protein detectable in the cell extracts (Fig. 37A).

TNF-α-induced ERK1/2 phosphorylation was prevented upon administration of PD98059 (Fig. 37B), an inhibitor of MEK1/2 (upstream kinase of ERK1/2).

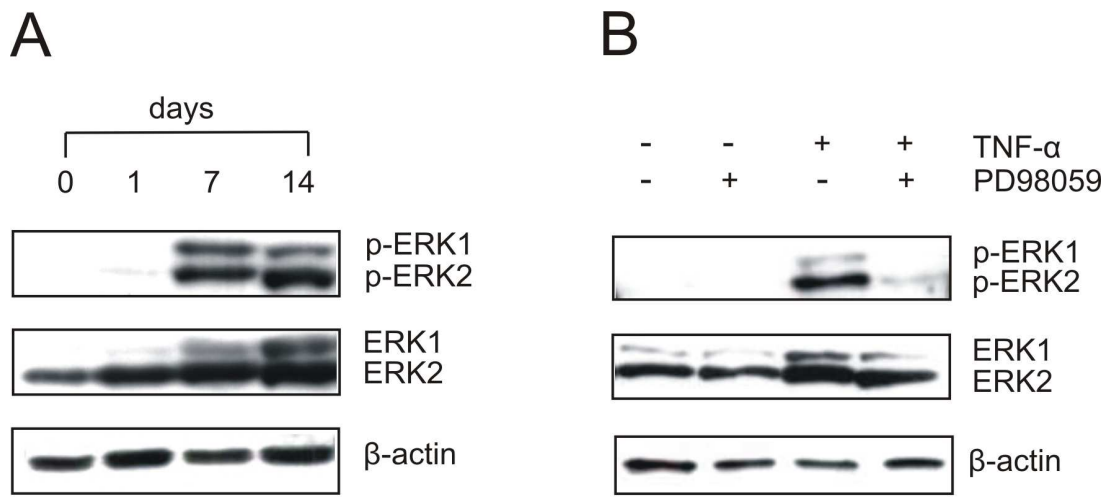

Fig. 37: Western blot analysis of ERK1/2 in hMSC treated with TNF-α.

(A) Western blot analysis of phosphorylated forms (p-ERK1/2) and total amount of ERK1/2 in lysates of hMSC after cultivation in the absence or presence of TNF-α (50 ng/ml) after different time intervals.

(B) Western blot detection of phosphorylated (p-ERK1/2) and total ERK1/2 after 14 days of co-incubation with or without PD98059 (20 µM).

β-actin was detected on the same blots to control for application of equal amounts of protein present on each lane.

Taken together, these results provide evidence that TNF-α-mediated differentiation of hMSC is primarily regulated via the ERK1/2 signalling pathway.

E.6.4 Role of CXCR4 in the invasiveness of hMSC pretretaed with TNF-α

Microarray analysis had indicated that mRNA expression of the chemokine receptor CXCR4 was elevated in hMSC after stimulation with TNF-α (Table 4).

This finding was confirmed by a time course study using qRT-PCR which demonstrated that TNF-α induced a dramatic upregulation of CXCR4 transcription by more than 20-fold on day 1 and about 35-fold on days 3, 7 and 14 (Fig. 38A).

Consistently, TNF-α augmented CXCR4 protein present in cell extracts as determined by Western blotting (Fig. 38B).

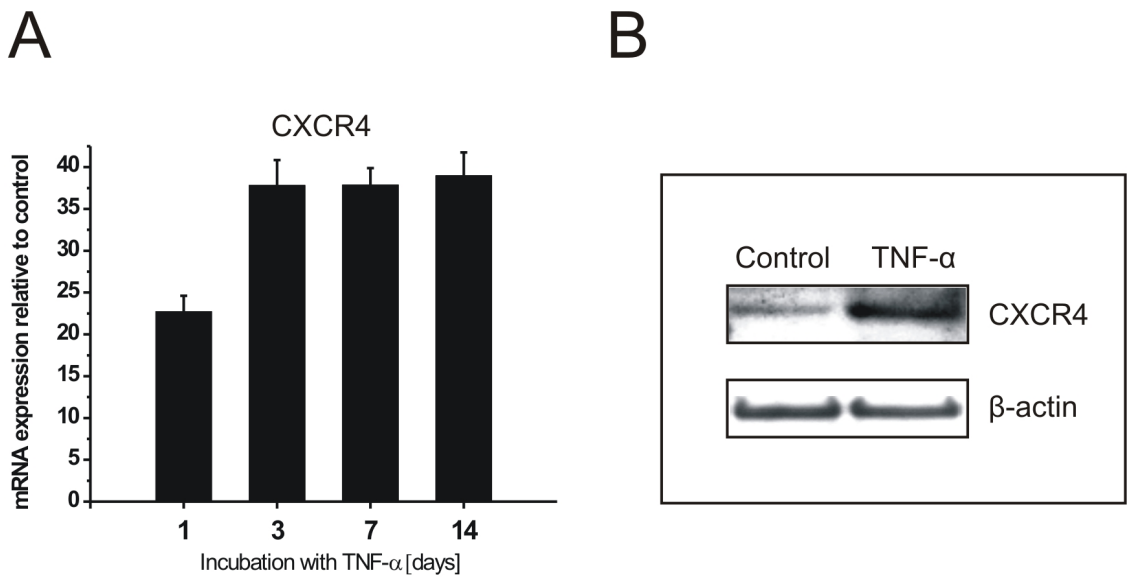

Fig. 38: mRNA and protein expression of CXCR4 in hMSC.

hMSC were incubated without (control) and with TNF-α (50 ng/ml) for different time intervals.

(A) qRT-PCR analysis of CXCR4 mRNA expression. Results are given as x-fold change in expression normalized against CPB.

(B) Western blot analysis using monoclonal antibodies against 42 kDa CXCR4 in protein extracts from hMSC after 3 on incubation.

β-actin was detected on the same blot to control for application of equal amounts of protein present on each lane.

Taking a possible involvement of CXCR4 in cell migration into account, we investigated next whether or not pretreatment of hMSC with TNF-α affected their capacity for transmigration through human reconstituted basement membranes (ECM) using SDF-1α as a chemoattractant in the Transwell assay.

Pretreatment with TNF-α exhibited an improved potential of hMSC for transmigration of ECM transmigration (Fig. 39). This may be a consequence of enhanced CXCR4 expression since application of AMD3100 (10 µg/ml), a specific antagonist of binding of SDF-1α to CXCR4, efficiently blocked the ECM transmigration capacity towards SDF-1α in TNF-α-pre-treated and untreated hMSC as well.

Similarly, addition of the broad-spectrum MMP-inhibitor GM6001 (10 µg/ml) caused a dramatic reduction in chemotactic invasion by hMSC either treated or not with TNF-α (Fig. 39), indicating the requirement of MMPs in this process.

Cell viability was not affected by AMD3100 and GM6001 at the concentrations used to achieve maximal migration inhibition.

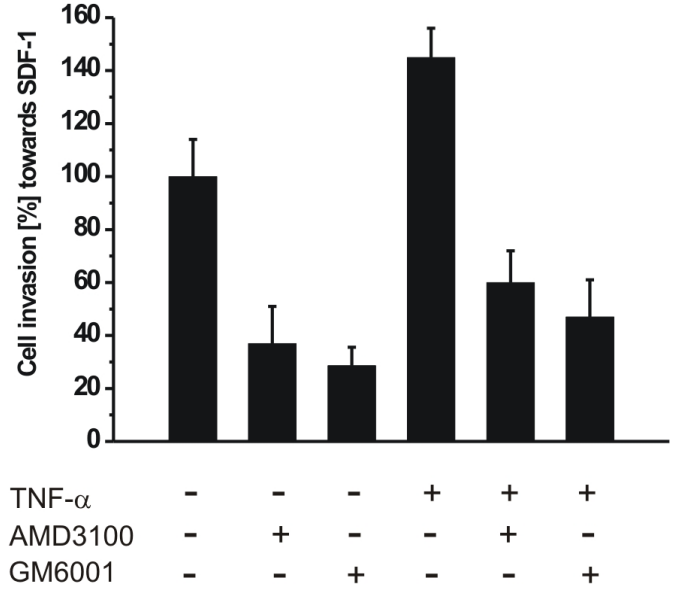

Fig. 39: CXCR4 invasion of hMSC pretreated with TNF-α.

hMSC were incubated in absence (control) or presence of TNF-α (50 ng/ml) for 14 days and then applied in the Transwell invasion assay without and with addition of 10 µg/ml AMD3100 (a specific antagonist of SDF-1 binding to CXCR4) or 10 µg/ml GM6001 (a broad-spectrum MMP-inhibitor). Chemotactic invasion was induced by SDF-1α and cell invasion rate was determined in percent relative to control.

Results are presented as mean ±SD of one triplicate experiment representative of three measurements.

Summing up, these data suggest that incubation of hMSC with TNF-α upregulates CXCR4 expression which results in an increase of cellular susceptibility to SDF-1α-directed trans-migration of ECM involving MMP activity.

F DISCUSSION

Great efforts have been made in the past few years to comprehend the molecular mechanisms involved in hMSC self-renewal and differentiation.[9,10,31] The regulatory network, however, that allows hMSC to migrate from distant sources such as bone marrow, peripheral blood, or surrounding tissues to areas of injury, where they participate in tissue repair and regeneration, are still unclear. Therefore, identification of factors that control and enable the migratory capability of hMSC represents an essential goal that would lead to a better understanding of hMSC function and possibly enhance their application in therapeutic strategies.

F.1 Expression of MMP and TIMP in hMSC

In order to participate in tissue repair and regeneration processes, endogenous hMSC need to migrate from bone marrow into the blood circulation with subsequent extravasation into target tissues.[13] This concept is supported by the presence of hMSC in peripheral blood and multiple tissue types.[13,121,122] Since MMP-2 and MMP-9 are known to contribute to the proteolysis of ECM enabling the migration of various cell types,[63,123-125] we hypothesized that the two gelatinases may also facilitate this process in hMSC.

Our data show for the first time that hMSC constitutively express and secrete MMP-2 as well as its specific inhibitor TIMP-2 which is known to control both MMP-2 activity and activation.[126] Analysis of hMSC culture supernatants revealed the presence of MMP-2 not only in its zymogen form but also as a fully activated protease. This observation is in concordance with the detection of MT1-MMP and TIMP-2 production in these cells, since both factors are required for MMP-2 proenzyme activation. According to the current understanding of this process, the secreted proMMP-2 re-associates in a first step with the cell surface by building a trimolecular complex with TIMP-2 and MT1-MMP, the latter being anchored in the plasma membrane. In a second step, MT1-MMP performs N-terminal cleavage of proMMP-2, giving rise to an intermediate active form which is further converted into the fully active species of MMP-2.[72,127] The active MMP-2 may then dissociate from the cell surface or remain bound and thereby facilitate collagen degradation and cellular invasion.[128]

In contrast to MMP-2, MMP-9 protein was absent in hMSC culture supernatants and only barely detectable on the transcriptional level. Interestingly, a remarkably strong expression of the MMP-9 inhibitor TIMP-1 was measured in hMSC suggesting complete abrogation of the MMP-9 activity in the cells under standard cultivation conditions. Our results showing striking differences in *MMP-2* and *MMP-9* gene expression are explainable by their distinct promoter structures [91,129] and agree with data reported from other mesodermal cells such as fibroblasts and endothelial cells which constitutively secrete MMP-2 but synthesize MMP-9 only upon stimulation.[124,130]

F.2 Role of MMPs and TIMPs in hMSC invasiveness

Since it was of essential interest whether the continuous release of MMPs may allow hMSC to migrate through ECM barriers, we exerted a method based on the modified Boyden chamber assay which has already been well established for *in vitro* studies on tumour cell invasion.[117] To closer match the *in vivo* situation of hMSC migration, the assay was optimized using biological material of human origin. Instead of Matrigel that represents ECM extracted from mouse tumor tissue,[118,119] we applied commercially available human ECM from healthy tissue which is primarily comprised of laminin, collagen type IV, and heparan sulfate proteoglycan, the major components of basement membranes (BM). In addition, human serum instead of fetal calf serum was utilized as a source of unspecified chemoattractants. By means of this assay, hMSC were clearly demonstrated to exhibit the potential for targeted migration through the barrier of human reconstituted ECM which we also showed by comparative analysis of hMSC with different other cell types.

Because both unspecific and gelatinase-specific inhibitors of MMPs largely blocked the transmigration process, while MMP-9 secretion was not detectable in hMSC, we assumed MMP-2 and MT1-MMP to play a critical role in the invasive capacity of these cells. To validate this hypothesis, specific knock-downs of mRNA expression of MMPs and TIMPs were accomplished in hMSC by RNAi technology. Applying siRNA transfection of hMSC,[131] a highly efficient blockage of mRNA expression and protein synthesis of MMPs and TIMPs was achieved in the cells. Using these cells in the invasion assay it became clear that the constitutive expression of MMP-2, MT1-MMP, and TIMP-2 but not of TIMP-1 essentially contributes to the ability of bone marrow-derived hMSC to traverse human reconstituted extracellular matrix.

The fact that complete abolishment of hMSC invasiveness by RNA interference against MMP-2, MT1-MMP and TIMP-2 could not be obtained may be explained by the residual protein synthesis in transfected cells and by the involvement of additional so far not further characterized proteinases in the degradation of ECM by hMSC. Moreover, the differential knock-down efficiencies for the expression of MMPs and TIMPs as observed in at least three hMSC lots analysed in our study correlated well with the invasion rates of the cells, thus again confirming the relevance of MMP-2, MT1-MMP and TIMP-2 in the invasion process. The variations in transfection efficiency as becoming overt in the three hMSC lots investigated may be due to the heterogeneity in cellular composition which is a characteristic feature of hMSC[17] and thus may vary to some extent in hMSC isolated from distinct individuals.

Taken together, we have shown for the first time that hMSC possess the capability for invading human ECM and demonstrate that this process is strongly dependent on expression of MMP-2, MT1-MMP, and TIMP-2 in these cells.

F.3 Role of inflammatory cytokines/chemokine in hMSC invasiveness

Based on the hypothesis that signalling molecules overexpressed in damaged or inflamed tissues might trigger chemotactic migration of hMSC *in vivo,*[122] the influence of distinctive cytokines/chemokine on the MMP-dependent invasion capacity of these cells was investigated *in vitro*.

TGF-β1 has been reported to be produced at elevated levels in wounds, where it exhibits various functions including the stimulation of fibroblast and leukocyte migration. Our study has shown a TGF-β1-induced upregulation of MMP-2 in hMSC which is in agreement with similar findings in other cell types such as tumour cells and keratinocytes.[91,94] In contrast to MMP-2, expression of MT1-MMP is normally under minor influence of cytokines and growth factors.[132] However, the TGF-β1-dependent induction of MT1-MMP expression in hMSC may be explained by a unique crosstalk mechanism between the TGF-β1 and Wnt signalling pathways recently described to initiate TGF-β1-evoked effects on hMSC proliferation and differentiation via accumulation of nuclear β-catenin.[133] Based on this finding and our recent results showing that gene expression of MT1-MMP is controlled by nuclear β-catenin levels in hMSC,[106] we postulate that the TGF-β1-induced upregulation of MT1-MMP in hMSC may be mediated via β-catenin. Since TGF-β1 did not considerably influence the expression of TIMP-1 and TIMP-2 in hMSC, its stimulatory effect on hMSC trafficking through human ECM appears to be mainly facilitated by induction of MMP-2 and MT1-MMP, as we have demonstrated by the use of a specific inhibitor against these MMPs.

For a better understanding of the potential interaction between TGF-β1 and Wnt signalling and its impact on expression and activity of MMPs the presumable crosstalk is depicted in Fig.40 with a detailed explanation given in the figure legend.

In wounds and inflamed tissues, the proinflammatory and chemotactic cytokines Il-1β and TNF-α are highly expressed and play multiple roles during the early phase of healing and repair.[95] Both cytokines have also been reported to influence the expression of MMP-9 and other MMPs or TIMPs in different cell types.[93,94,134,135]

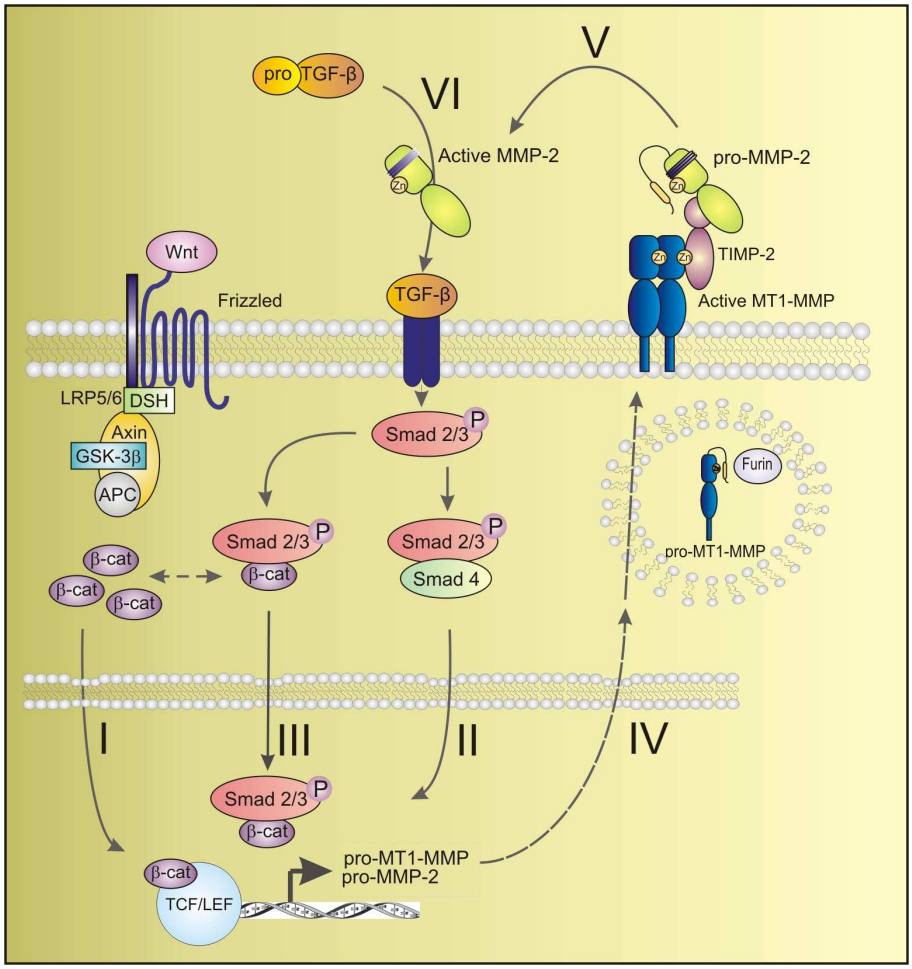

Fig. 40: Proposed model for a potential cross-talk between Wnt and TGF-β1 signalling and its influence on the expression and activity of MMPs in hMSC.

(I) Basal activity of the Wnt pathway in unstimulated hMSC generates a constitutive level of free β- catenin which is responsible for the expression of e.g. MT1-MMP.[106]

(II) TGF-β1 binds and activates the membrane-anchored type II and type I receptor Ser/Thr kinases, which phosphorylate the effectors Smad2 and Smad3. Subsequently, phosphorylated Smad2/Smad3 form complexes with Smad4 which allows translocation into the nucleus, where they activate or repress target genes.

(III) A unique cross-talk between the TGF-β and Wnt pathway present in hMSC results in complex formation between Smad2/3 and β-catenin. Thus, two sets of partners, the β-catenin/Smad2/3 and β-catenin/TCF/LEF, may then regulate the expression of different target genes.[133]

(IV) Addition of exogenous active TGF-β1 increased MT1-MMP production as well as proMMP-2 secretion, possibly as a result of the interaction between the Wnt and the TGF-β1 pathway via β-catenin.[106]

(V) Under these conditions, proMMP-2 is activated after binding to TIMP-2 and MT1-MMP at the cell surface.

(VI) Moreover, MMP-2 can activate latent proTGF-β[136] which then binds to its receptors driving a positive feedback loop. Both MMP-2 and MT1-MMP efficiently degrade ECM components which promotes the migratory and invasive potential of hMSC.[106] In that way, the TGF-β1 and Wnt signalling pathways may act together, resulting in the amplification of hMSC invasiveness.

(Figure taken from Neth et al.[120] with some modifications).

In our study it has been described for the first time that hMSC respond with high sensitivity to Il-1β and TNF-α by strong upregulation of MMP-9 as well as of MT1-MMP transcription in these cells. Very recent research has revealed a role of MT1-MMP not only on the activation of MMP-2, but also on an independent degradation of ECM components such as fibronectin, vitronectin, laminin, and collagen type I.[137] Thus, enhanced MT1-MMP activity in hMSC may also directly increase the cleavage of ECM, thereby contributing to increased cell invasiveness. This agrees with the fact that MT1-MMP has been shown to be essential in monocytic transmigration through TNF-α-activated endothelium [138] demonstrating the importance of this enzyme in cellular extravasation.

Despite of the fact that TNF-α dramatically upregulated the extremely low basal level of the mRNA expression of MMP-9 in hMSC, only little amount of this enzyme was secreted within three days of incubation. This suggests that MMP-9 may play a minor role in TNF-α-promoted invasion capacity of hMSC. Within the incubation period, MMP-2 expression was also decreased under the influence of this cytokine. Nevertheless, the early and simultaneous increase of MT1-MMP and MMP-9 might be efficient and enough in facilitating chemoattractive invasion of hMSC towards a gradient of TNF-α produced in inflamed tissues.

The chemokine SDF-1α and its receptor CXCR4 have been intensively studied concerning the migration, growth, and differentiation of various cell types including hematopoetic $CD34^+$ stem cells. Exemplarily, modulations of SDF-1/CXCR4 levels contribute to the recruitment of leukocytes from bone marrow to injured and inflamed tissues.[96,139] In our study, SDF-1α stimulated chemotactic migration of hMSC across human ECM, although its influence was relatively weak and barely dependent on MMP activity. This limited cellular response towards SDF- 1α may be explained by the fact that only a small subpopulation of hMSC has been shown to express functional CXCR4 receptors.[140] Another explanation could be the potential cleavage and inactivation of SDF-1α by MMP-2 and MT1-MMP constitutively expressed in hMSC which may be similar to the mechanism already shown in $CD34^+$ cells.[141] Other authors have described very recently that SDF-1α stimulates transmigration through a Matrigel barrier by bone marrow- and cord blood-derived hMSC cultivated for up to 18 passages.[107] The authors provide evidence for the involvement of MT1-MMP in this process, which is confirmed by our finding that SDF-1α slightly upregulated the activity of MT1-MMP in bone marrow-derived hMSC. Moreover, the exposure of the cells to SDF-1α decreased transcription and release of MMP-2 and also reduced the mRNA levels of TIMP-1 and TIMP-2 in these cells, which indicates differential regulatory influence of SDF-1α on MMP/TIMP expression in hMSC.

Thus, we have demonstrated that hMSC are capable of migrating through human reconstituted extracellular matrix. For this purpose, hMSC utilize constitutively expressed MMP-2, MT1-MMP, and TIMP-2. The essential contribution of these gene products to hMSC invasiveness has been clearly shown by the use of synthetic inhibitors of MMPs and a detailed RNAi approach. Moreover, the inflammatory cytokines TGF-β1, Il-1β, and TNF-α rather than the chemokine SDF-1α showed significant chemoattractive potential on hMSC and induced cellular trafficking across human ECM barriers via upregulation of MMP-2, MT1-MMP, and/or MMP-9. Therefore, hMSC fulfill essential requirements for homing, extravasation, and migration into tissues in response to inflammatory stimuli.

Taken together, these data may be of fundamental relevance in clinical applications such as the mobilization of endogenous hMSC from bone marrow into blood and to sites of injury by cytokine administration, or the treatment of *ex vivo* expanded hMSC with cytokines to increase their migration / homing potential after transplantation into patients.[142]

F.4 Importance of TIMP-1 in the regulation of hMSC functions

TIMP-1 is best known for its capacity as an endogenous high affinity inhibitor of MMP-9 thereby efficiently controlling the invasive behaviour of various different cell types including leukocytes and tumour cells.[143,144] For this reason, TIMP-1 is usually found to be co-expressed with MMP-9 in many cell types. Strinkingly, we could demonstrate that hMSC release massive amounts of TIMP-1 but no MMP-9, suggesting roles for TIMP-1 distinct from its pure MMP-inhibitory function during the cell invasion process.

Interestingly, endogeneous TIMP-1 in hMSC appears to act as a repressor of proliferation and differentiation into osteogenic and adipogenic cells as demonstrated in this study by analyses of TIMP-1 knock-down. Inhibition of proliferation by TIMP-1 is also documented in mammary epithelial cells.[145] In contrast, abundant data have shown that TIMP-1 can stimulate cell proliferation in a wide range of cells.[146,147] This is in agreement with the discovery of TIMP-1, where it was found to be identical to a protein named erythroid-potentiating activity (EPA) due to its ability to stimulate growth of murine erythroid precursors and human leukemic cells.[143,144] Therefore, the divergent findings with respect to the influence of TIMP-1 on cell proliferation may depend on the different cell types being studied.

For the first time, our data indicate an influence of TIMP-1 on cellular differentiation of hMSC which may be mediated via negative interference with the Wnt signalling pathway. Yet, further investigations are required to clarify if this effect is conveyed through blockage of MMP activity or appears to be MMP-independent. This may be achieved by direct interaction of TIMP-1 with Wnt-receptors on the cell surface or by influence on intracellular Wnt-regulated factors like β-catenin. Although CD63 has recently been recognised as a receptor of TIMP-1,[148] the mechanisms of specific signalling triggered by TIMP-1 still need to be elucidated.

F.5 Influence of TNF-α on hMSC differentiation

TNF-α is secreted by various cell types including activated macrophages and T lymphocytes playing a central role in both innate and specific acquired immunity. TNF-α exhibits pleiotropic properties, including its ability to cause apoptosis in tumour-associated endothelial cells that can result in the complete destruction of the tumour vasculature.[7,98,149]

In neurophysiology, results obtained during the past decade describe that TNF-α mediates oligodendroglial cell death,[150,151] microglia/astroglia reactivity and proliferation,[150,152] as well as demyelination.[151] In contradiction, TNF-α has also been reported to protect neurons from injury, [102,153] again denoting the multifaceted roles of this cytokine. After incubation of hMSC with TNF-α, the majority of the cells showed morphological characteristics similar to that of astrocytes. The appearance of this neuroglial phenotype was simultaneously accompanied by enhanced or *de novo* synthesis of several gene products typically produced in neural cells.

Autocrine release of LIF and BMP-2 has been shown to synergistically induce astrocyte differentiation from neuroepithelial cells *in vitro* and also promote the differentiation of neural progenitor cells into astrocytes.[154] Alike, we found that TNF-α strongly upregulates both factors in hMSC, suggesting potential similarities with an astrocytic cell phenotype.

SOX-2, which is enhanced in hMSC by stimulation with TNF-α, represents a marker which is highly expressed in the neuroepithelium of the developing central nervous system (CNS).[155] SOX-2 is also important in the maintenance of neurons and in the proliferation of neural stem cells.[156]

MAP-2 is typically produced in post-mitotic neurons.[157,158] Surprisingly, we found hMSC to express MAP-2 mRNA when treated with TNF-α, again suggesting a neuronal character of these cells. A more detailed analysis on protein level revealed that MAP-2 was synthesized in a subpopulation of hMSC incubated with TNF-α. MAP-2 usually indicates mitotically terminated neurons. Since we did not detect expression of β-tubulin III (marker for immature neurons),[159] it is likely that hMSC treated with TNF-α did not represent functional neurons but rather exhibit premature transcription of MAP-2, suggesting at least the commitment of hMSC for neural-like features.

After treatment with TNF-α the majority of hMSC expressed GFAP, a cytoskeletal intermediate filament that is characteristically present in mature astrocytes and their precursors.[160] Strong expression of GFAP in the absence of nestin and vimentin is typically found in astrocytes.

Nestin was discovered as an early marker of neuroepithelial cell development, being required for filament formation by coassembling with vimentin.[161] In accordance to these observations, we found down-regulation of nestin together with vimentin upon treatment of hMSC with TNF-α.

Furthermore, hMSC pretreated with TNF-α were able to build neurospheres which is a characteristic property of neural progenitor cells.[39] Although, neurosphere formation without functional validation is not sufficient for a reliable determination of cell type and different status, it is an additional evidence for a neural nature of these cells.

Because TNF-α exhibited strong morphological and molecular changes in hMSC, we were interested in the intracellular mechanisms controlling these processes. We identified the MAP kinase ERK1/2 to be persistently activated after treatment with TNF-α and to be responsible for the regulation of neural gene expression in hMSC. These data correlate with those reported from bone marrow stromal cells describing the requirement of ERK for neural differentiation in these cells.[162]

Adult stem cells in general and hMSC in particular have been of great interest as a resource of autologous stem cells for cell replacement therapy of neurodegenerative disorders and traumatic injuries. One major advantage in therapeutic use of hMSC is their presumable ability to differentiate into non-mesodermal lineages including neuroectodermal cells as shown e.g. in our *in vitro* studies. Such *in vitro*-predifferentiation of hMSC into more restricted cells could enhance their ability to integrate into brain tissue after transplantation.

In the past years, several reports describe *in vitro* conditions for differentiating hMSC derived from multiple mammalian species into neurons or at least into cells that exhibit neuronal phenotypes. Some protocols include simple chemicals,[163,164] while others use mixtures of various growth factors [39,40] or apply transgenic expression of particular genes.[46] By now, these methods for hMSC differentiation remained not successful after application in animal model systems due to the limited viability of *in vitro* generated neurons.

According to our data, pretreatment of hMSC with TNF-α triggers their development into neural-like cells which are fully mitotic and viable. Thus, similar to the use of neural stem cells, TNF-α-committed hMSC may provide the opportunity for re-transplantation into areas of neural tissue damage.

Moreover, we have shown that hMSC express high levels of the chemokine receptor CXCR4 after treatment with TNF-α. Consistenly, neural progenitors are also known to express CXCR4.[165-167] Several studies using rodent neural progenitors have suggested the potential involvement of chemokines in directing the migration of neural progenitors during CNS development.[168,169] In particular, the interaction of CXCR4 and its ligand SDF-1α has been

strongly proposed to be important in neuronal patterning of cerebellum and hippocampus as well as astrocyte development in the rat brain.[167,170]

Interestingly, after TNF-α-induced differentiation into neural-like cells, hMSC displayed an enhanced sensitivity and invasion capacity towards a gradient of SDF-1α similar to that required for neural progenitor cells during brain development. This enhanced invasion capacity was inhibited by either blocking CXCR4 or the activity of MMPs which indicates the importance of both the CXCR4/SDF-1α axis and the proteolysis mediated by MMPs in the process of directed hMSC migration.

Growing evidence over the last few years suggests that hMSC might be an alternative to neural stem cells as cellular vectors due to their unique tropism for malignant tumours of the brain.[49,159] As shown in animal models, engineering hMSC into cells producing interferon-ß or interleukin-2 before engraftment into the glioma leads to a significantly increased survival as demonstrated in animal models.[49,50,171] Until now, the molecular mechanisms controlling migration of hMSC towards glioma are far from being clear. In normal brain tissue, low levels of SDF-1α are observed in astrocytes, neurons, and in the phagocytic cells around vessels. However, in tumours, SDF-1α expression is strongly enhanced with increasing tumour grade.[172] Own studies[i] using glioma cell spheroids as an *in vitro* tumour model demonstrated that enhanced CXCR4 expression in hMSC pretreated with TNF-α improved infiltration of these cells into the glioma spheroid. This indicates the importance of the CXCR4/SDF-1α axis in the attraction of hMSC towards brain tumours (unpublished data, manuscript V. Egea et al., submitted 2008). In *vivo* studies using glioma animal models are required to validate potentially enhanced tumour infiltrating properties of hMSC pretreated with TNF-α.

Taken together, for the first time our data demonstrate that TNF-α as a single cytokine induces neural differentiation in hMSC which is largely controlled via the ERK1/2 cascade. In contrast to the protocols designed for induction of neural transdifferentiation,[173] TNF-α did not affect the viability of hMSC. Therefore, in the next future, we intend to address with reliable *in vitro* and *in vivo* studies the question, whether hMSC pretreated with TNF-α can develop into mature neurons or astrocytes, possibly by direct cell-to-cell interaction with differentiated neural cells as suggested by Jiang et al.[10] Thus, our findings may be helpful in the development of innovative therapies using hMSC for the treatment of neurodegenerative diseases and malignant gliomas.

[i] Performed in collaboration with Prof. Goldbrunner (Department of Neurosurgery, LMU Munich, Germany).

F.6 Conclusions

The results obtained in the present *in vitro* study as summarized and schematically depicted in Fig. 41, provides novel insights in the biology of hMSC. Our findings deliver relevant information on so far unknown molecular mechanisms which may be involved in the control of hMSC invasiveness, proliferation, and differentiation *in vivo*, and thereby facilitate the clinical application of hMSC e.g. on autologous therapies for the treatment of disorders like neurodegenerative diseases and brain tumours.

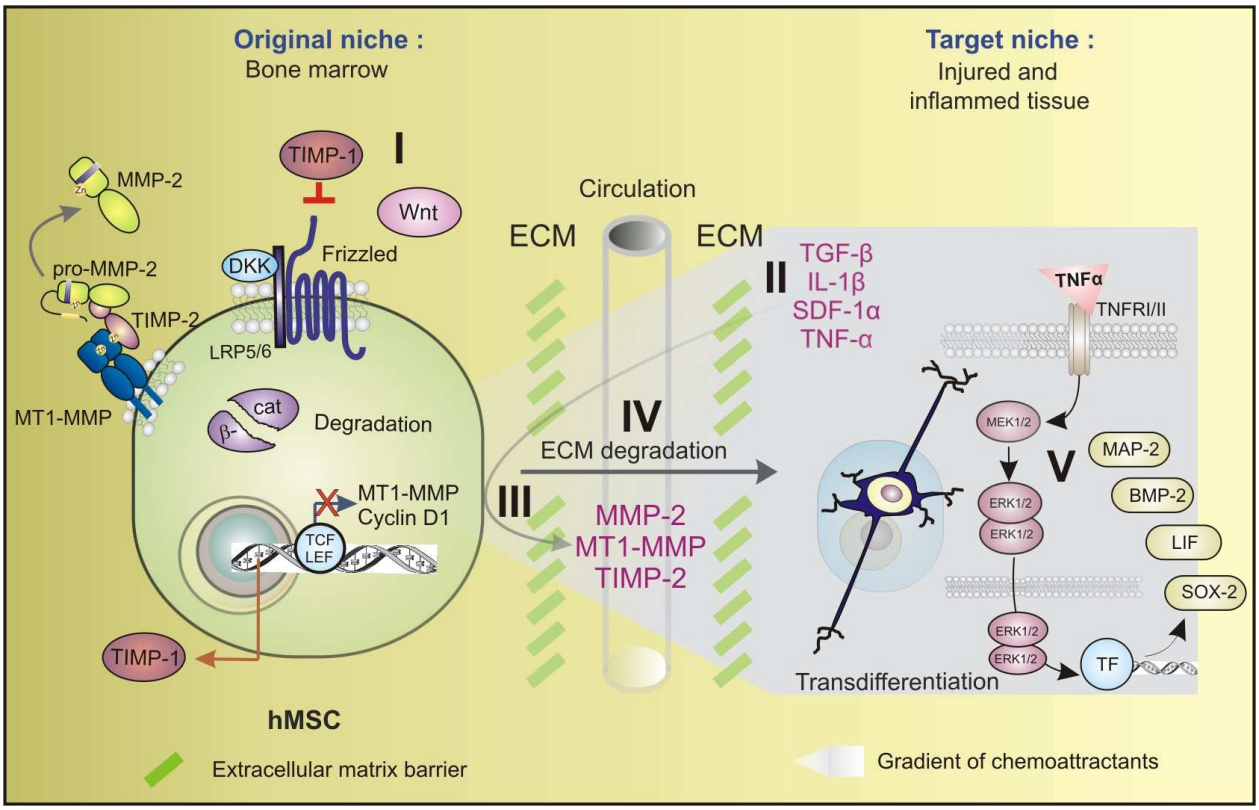

Fig. 41: Illustration of results gained in this study.

hMSC residing in the bone marrow secrete TIMP-1 which keeps cells in a quiescence state by repressing proliferation, differentiation and invasion (I). Inflammatory factors like TGF-β, IL-1β, TNF-α and SDF-1α released at sites of tissue injury induce mobilization of hMSC (II) by upregulation of MMP-2, MT1-MMP and TIMP-2 in these cells (III) allowing their traversal through ECM barriers (IV). Continuous stimulation by TNF-α induces differentiation into neural-like precursor cells via enhanced ERK1/2 actvity (V).

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"This is not the end. It is not even the beginning of the end.
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Wnt-Signalling in mouse mesenchymal stem cells: impact on proliferation, invasion and MMP-expression.

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TNF-α regulation of ERK1/2 induces neural transdifferentiation in human mesenchymal stem cells and promotes CXCR4/SDF-1 mediated invasion.

[Manuscript in preparation]