

**CELL LINEAGE SPECIFIC
EXPRESSION OF MATRIX
METALLOPROTEINASES -2
AND -9 IN TRANSGENIC MICE**

**TUIRE
SALONURMI**

Biocenter Oulu,
Department of Biochemistry,
University of Oulu

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Supervised by
Professor Karl Tryggvason

Reviewed by
Professor Leena Alhonen
Professor Jorma Keski-Oja

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Biocenter Oulu, University of Oulu, P.O.Box 5000, FIN-90014 University of Oulu, Finland,
Department of Biochemistry, University of Oulu, P.O.Box 3000, FIN-90014 University of Oulu,
Finland

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Abstract

Mammalian extracellular matrix metalloproteinases, MMPs, are a family of enzymes capable of degrading components of the connective tissue. The *in vivo* regulation of the cell lineage-specific expression of MMPs, however, is not well known. This study used transgenic mice to identify cell-specific elements in the upstream regulatory regions of MMP-2 and MMP-9. Transgenic mice were generated by pronuclear microinjections into fertilised oocytes using *lacZ* as a reporter gene. The reporter gene constructs containing varying lengths of the MMP-9 5'-upstream region revealed an area that allowed for expression in osteoclasts and migrating keratinocytes, the cells that also express MMP-9 *in vivo*. The sequence driving the cell specific expression included the nucleotides from -2722 to -7745.

When the same upstream regulatory fragment of MMP-9 was used to drive the expression of the human tissue specific inhibitor of MMPs, TIMP-1, instead of *lacZ*, the transgenic mice developed normally and the animals were fertile with normal post-embryonic growth. However, cutaneous wound healing was remarkably retarded, but not totally prevented, and the migration of keratinocytes over the wound was slow. The mice expressed the human TIMP-1 in keratinocytes during wound healing and *in situ* zymography revealed a total blockage of the gelatinolytic activity of MMP-2 and MMP-9, the main gelatinases active in the healing wound tissues.

By using a sequence of 6500 base pairs from the 5'-upstream regulatory region of the MMP-2 gene it was possible to drive the expression of *lacZ* in mesenchymal cells of the developing transgenic mouse embryo. The expression pattern was similar to that found in previous *in situ* hybridization studies, following the different stages of tissue morphogenesis and being present in the areas of basement membrane degradation and epithelial cell invasion. Computer analyses of the sequence revealed three regulatory upstream regions conserved between human, mouse, and rat, and possibly responsible for the cell-and tissue specificity. New transgene constructs containing fragments of the conserved regions will provide a more detailed profile of the *in vivo* MMP-2 regulation in the future.

This study defined a fragment in the upstream regulatory region of MMP-9 that is essential for expression in osteoclasts and migrating keratinocytes. Furthermore, the keratinocyte derived MMPs, including MMP-9, were found to play important role in epithelial cell migration in the area of the healing wound.

Keywords: gelatinases, mesenchyme, mice; transgenic, osteoclasts, promoter regions, regulatory sequences, TIMP-1

To my family

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This work was carried out at the Department of Biochemistry, Biocenter Oulu, and Institute of Dentistry, University of Oulu.

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Abbreviations

μg	microgram
AEC	Aminoethyl carbazole
AP-1	activator protein 1
APMA	<i>p</i> -aminophenylmercuric acetate
ATP	adenosine-triphosphate
BM	basement membrane
bp	base pair
DAB	3,3-diaminobenzidine tetrahydrochloride
DNA	deoxyribonucleic acid
ECM	extracellular matrix
EGF	epidermal growth factor
EMSA	electrophoretic mobility shift assay
ER	estrogen receptor
FN	fibronectin
GelA, B	gelatinases A, B
HPV16	human papillomavirus type 16
HRP	horseradish peroxidase
ISZ-buffer	<i>in situ</i> zymography -buffer
kb	kilobase
<i>lacZ</i>	gene encoding β -galactosidase protein
LCR	locus control region
LM	laminin
MMP	matrix metalloproteinase
MMTV-LTR	mouse mammary tumour virus long terminal repeat-promoter
mRNA	messenger RNA
NGF	nerve growth factor
p.c	post coitus
RPA	RNase protection analysis
RT	room temperature
SDS	sodium dodecyl sulphate
TGF- α	transforming growth factor- α

TGF- β	transforming growth factor- β
TIMP	tissue inhibitor of matrix metalloproteinase
TNF- α	tumour necrosis factor- α
t-PA	tissue-type plasminogen activator
TSP1	thrombospondin 1
u-PA	urokinase-type plasminogen activator
VEGF	vascular endothelial growth factor
X-gal	5-bromo-4-chloro-3-indolyl- β -galactopyronoside

List of original articles

This thesis is based on the following articles, which are referred to in the text by their Roman numerals:

- I Munaut C*, Salonurmi T*, Kontusaari S, Reponen P, Morita T, Foidart J-M & Tryggvason K (1999) Murine matrix metalloproteinase 9 gene: 5'-upstream region contains *cis*-acting elements for expression in osteoclasts and migrating keratinocytes in transgenic mice. *J. Biol. Chem* 274: 5588-5596.
*equal contribution
- II Salonurmi T, Parikka M, Kontusaari S, Pirilä E, Munaut C, Salo T & Tryggvason K (2004) Overexpression of TIMP-1 under the MMP-9 promoter interferes with wound healing in transgenic mice. *Cell Tissue Res*, 315: 27-37.
- III Salonurmi T, Kontusaari S & Tryggvason K (2004) The 5'-upstream region of murine matrix metalloproteinase 2 gene drives embryonic *lacZ*-expression in mesenchymal cells in transgenic mice. Unpublished.

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1 Introduction

Matrix metalloproteinases (MMPs) form a group of enzymes that share common structural elements such as a conserved Zn^{2+} binding catalytic site, similar activation mechanisms, and inhibition by tissue specific inhibitors of matrix metalloproteinases, TIMPs. While MMPs have some substrate similarities because they degrade most of the proteins of the connective tissue, each member of this group also has a high substrate specificity *in vivo*. This specificity is related to the role of the enzyme in certain developmental and pathological processes, such as bone tissue degradation or trophoblast invasion. MMPs that degrade the denatured type IV collagen, MMP-2 (gelatinase-A) and MMP-9 (gelatinase-B), have also been found to cleave several other proteins, including some that are not extracellular matrix components. The role of MMP-2 and MMP-9 in pathogenesis has been studied extensively and they are known to be involved in cancer invasion, inflammation, bone degradation and wound healing. This multifunctionality requires regulation of the synthesis of these two MMPs at several different levels both inside and outside of the cell

In this work, transgenic mice were used as a model to study the regulatory network governing the expression of MMP-2 and MMP-9. The upstream regulatory region was examined to find the elements driving the expression of MMP-2 and MMP-9 during embryonic development and in pathogenesis. In addition, regulation at the protein level was included in these studies by utilising the overexpression of TIMP-1. The expression of this inhibitor was driven by the upstream regulatory region of MMP-9. Expression of the transgenes and the results of TIMP-1 overexpression were studied during embryonic development and in adult mice.

2 Review of the literature

2.1 Regulation of gene transcription

The regulation of gene transcription occurs at the levels of chromatin and DNA-strands. Within the DNA-sequence at least three different kinds of regulatory groups are known: regulatory switches, such as enhancer and silencer sequences, regulatory limiting or restricting signals, also referred to as A-elements, and locus control regions (LCR) that activate the genetic locus prior to transcription. The elements that affect the transcription within the same DNA-molecule, are called *cis*-acting elements and, correspondingly, *trans*-acting factors affect gene regulation from outside of the DNA-segment. Usually, *trans*-acting factors bind to *cis*-elements that are recognized by their sequence. To function properly and in a tissue specific manner, genes often need numerous different regulatory factors flanking both 5'- and 3'-ends of the coding region, and possibly also inside the transcriptional unit. (Sippel *et al.* 1987, Bonifer *et al.* 1990, Grewal *et al.* 1992)

The *trans*-acting factors binding to DNA can be structurally divided into several groups, such as the TAAT-sequence recognizing a homeodomain together with a POU-domain (Qian *et al.* 1989, Kissinger *et al.* 1990), hormone receptors with a zinc-finger structure (Arriza *et al.* 1987) and a large and heterogeneous group of leucine-zipper containing protein families (Landschulz *et al.* 1989). The sequence specific DNA-binding factors are connected to the transcriptional machinery by so-called coactivators and corepressors, complexes with two distinct roles: first, to interact or be part of the transcriptional machinery and second, to remodel or remodel chromatin (Näär *et al.* 2001). Transcriptional regulation by the estrogen receptor (ER) can be presented as an example of diversity in gene regulation (reviewed by Sanchez *et al.* 2002). In short, hormone binding releases the ER from inactive complexes heat-shock proteins and immunophilins (Ylikomi *et al.* 1998) and induces dimerisation. Dimerisation is necessary for the sequence-specific binding of the ER to the regulatory regions of the target genes. Furthermore, when ERs are DNA bound, they modulate the expression of their target genes by interacting with coactivators. These complexes of ERs with coactivators can modulate chromatin structure and favour the interaction between the transcriptional

machinery and DNA (Xu *et al.* 1999, Robyr *et al.* 2000). Interestingly, ERs can also regulate gene expression in the absence of direct interaction with DNA by protein-protein interactions with other transcription factors or by modulating the activity of upstream signalling components.

The mechanism of transcriptional control can be studied in both *in vitro* and *in vivo* systems. This kind of work has been significantly facilitated by the availability of complete genome sequences and bioinformatics. The *cis*-regulatory DNA-elements, either enhancers or silencers, can be scattered over large regions of non-coding sequence. The effect of a single *cis*-regulatory element on transcription varies depending on its location and on the other flanking regulatory sites. Detection of these elements can be highly time-consuming and laborious, as the number of different elements is growing constantly. Identification and characterisation of *cis*-elements can be performed using conventional methods such as DNase I-hypersensitivity assays, footprinting and the electrophoretic mobility shift assay (EMSA). Cell transfection studies using promoter/reporter gene constructs are a common method for studying promoter regions and sequences of crucial *cis*-elements. However, these methods provide only a very restricted amount of information about the *in vivo* regulation. Use of bioinformatics for comparison of promoter and other gene sequences between different species can reveal functional homologies, a method referred to as phylogenetic footprinting (reviewed by Müller *et al.* 2002). This is a useful tool when combined with the analysis of whole organisms as soon as conserved regions of high homology have been identified. Numerous different programs and databases are also available nowadays for analysing the *cis*-regulatory elements of the DNA sequence. Transgenic fish models, such as zebrafish and medaka (Lin 2000, Tanaka *et al.* 2001), have received growing interest recently, their advantage relying mostly on fast and relatively inexpensive technology, but the sequence of the entire genome is not available for those species yet. The full sequence is necessary for sequence comparison between such distant species as zebrafish and human. Transgenic mice are a crucial and well established method providing multilevel information when used as a model system in regulatory studies. In fact, transgenic mice produced by microinjecting reporter gene constructs into pronuclei of fertilised oocytes (Gordon *et al.* 1980) are still the method of choice for identification and characterisation of enhancer elements driving cell lineage/tissue specific gene expression. The reporter gene can be, for example, *lacZ* or *luc*, encoding β -galactosidase or luciferase enzymes, respectively, and its expression is driven by the regulatory region of interest. Transcription of the reporter genes can be detected both during embryonic development and in adult animals after treating the tissues for the enzymatic colour formation or luminescence (*luc*). Correct gene expression is usually a summary of functions of several different regulatory elements, and longer promoter constructs, containing also more distant *cis*-regulatory elements, are often more resistant to the effects of the random integration site in the genome.

2.2 Matrix metalloproteinases

MMP-protein studies began in 1962, when Gross and Lapiere (1962) found a factor (now referred to as MMP-1) necessary for the metamorphosis of the tadpole. However, it was not until 1980 that a second collagenolytic activity (MMP-2) was identified and subsequently purified from cancer cells and tumours (Liotta *et al.* 1980, Salo *et al.* 1983). Since then, 26 additional MMPs have been identified, either as enzyme proteins or based on genome analyses (Overall & Lopez-Otin 2002). The MMPs were first found to degrade collagens, but it is now known, that they can degrade most proteins of the extracellular matrix (ECM). The MMPs participate in the turnover of the ECM during embryonic development, reproduction and in the different processes of tissue resorption and remodelling such as in pathological states like cancer and inflammation. MMPs participate also in the inactivation or activation of cytokines and growth factors, degrade serpins, and hydrolyse cell receptors (Woessner & Taplin 1988, Hornebeck *et al.* 2002).

The similarities within the family of MMPs include their production as latent preproenzymes, a structure with common functional domains, homology in their amino acid sequence and activity dependent on Ca^{2+} and Zn^{2+} in addition to neutral pH. MMPs can be activated by chaotropic agents or by proteolytic cleavage of the propeptide, and they are inhibited by tissue inhibitors of metalloproteinases, the TIMPs. (Nagase & Woessner 1999)

2.2.1 MMP protein family

The structures of MMPs share some common elements that are used to further classify the 28 homologous members of the gene family into four subgroups. The domain structures of different subgroups are presented in Figure 1. The group of nine archetypal secreted MMPs contain a signal peptide, an amino-terminal propeptide, a zinc-binding catalytic domain and a hemopexin C-terminal domain. The carboxy-terminal domain is apparently required for determining the substrate specificity, and it participates in the formation of a complex with TIMPs. The signal peptide is required for secretion. (Birkedal-Hansen *et al.* 1993, Overall 2002)

The minimal domain MMPs, matrilysins, lack the hemopexin C-terminal domain (Uria & Lopez-Otin 2000), but otherwise do not differ in domain structure. The group of membrane type MMPs (MT-MMPs) has a transmembrane segment or a glycosylphosphatidylinositol (GPI) to bind them to the plasma membrane (Seiki 1999, Kang *et al.* 2001).

Other small units can also give more diversity among MMPs. MMP-2 and MMP-9 have three fibronectin type II-like modules in their catalytic domain, forming a compact collagen-binding domain (Morgunova *et al.* 1999). There are several MMPs that have a basic insert in their propeptide that is cleaved by furin-like proprotein convertase proteases, (BOX 2) (Steiner 1998). Additionally, MMP-23A and -23B are encoded by two distinct genes, but have the same amino-acid sequence (Gururajan *et al.* 1998, Velasco *et al.* 1999).

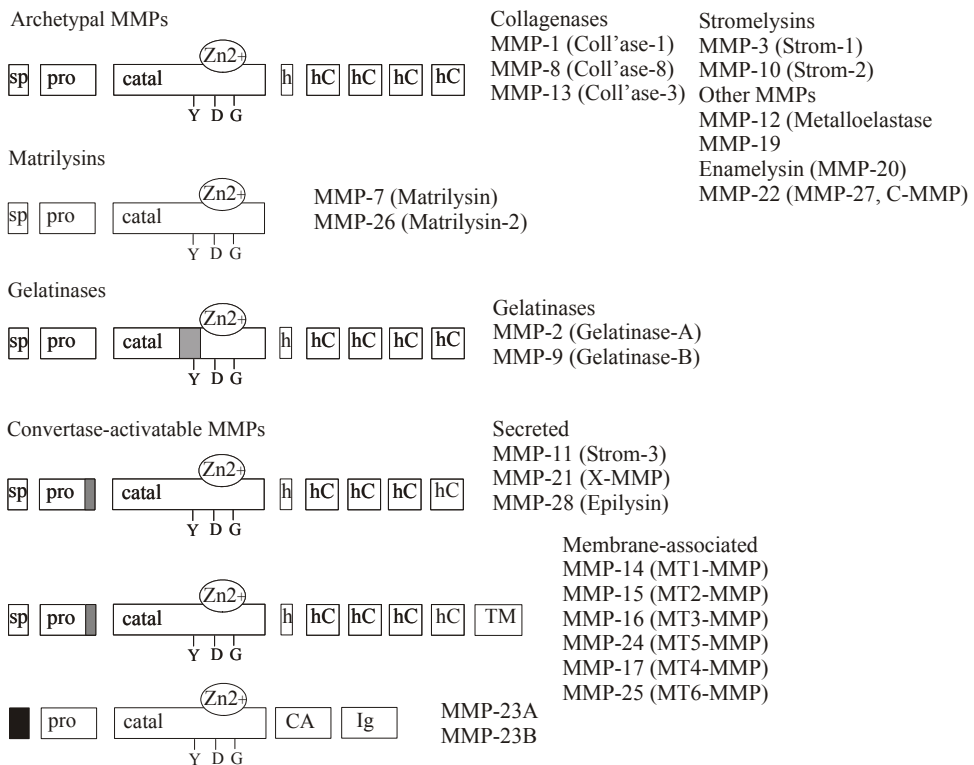


Fig. 1. The structural family of human MMPs. Abbreviations: sp=signal peptide, pro=propeptide, catal=catalytic domain, h = hinge/linker, hC = hemopexin C-terminal domain, TM=Transmembrane sequence, CA = cysteine array, Ig = immunoglobulin-like domain. The grey box in the catalytic domain of gelatinases indicates three fibronectin type II modules. The grey box in the propeptide of the convertase activatable MMPs indicates a basic insert that is a convertase cleavage site. MMPs 14-16 and MMP-24 contain a membrane linker, a type I transmembrane segment (TMI) and a cytoplasmic tail within their transmembrane segment. MMPs 17 and 25 have a membrane linker fused to a glycosylphosphatidylinositol (GPI) and no TMI or cytoplasmic tail. A black box replacing the signal peptide is a transmembrane type II segment. (Modified from Overall & Lopéz-Otín, 2002, and Birkedal-Hansen, 1995)

2.2.2 MMP-2 and MMP-9

MMP-2 and MMP-9 are two distinct homologous enzymes, initially referred to as 72 kDa and 92 kDa type IV collagenases, respectively, due to their cleavage of basement membrane (type IV) collagen (Salo *et al.* 1983, Fessler *et al.* 1984, Murphy *et al.* 1989). Both enzymes were first cloned by Goldberg's group (Collier *et al.* 1988, Wilhelm *et al.* 1989). Later, the two enzymes were shown to be identical to enzymes that degrade denatured collagen (gelatin). They had been previously identified as 70 kDa and 90 kDa

gelatin-binding proteins (Vartio & Vaheri 1981, Vartio *et al.* 1982). As a result, MMP-2 and MMP-9 have also been termed gelatinase A and B, respectively. Both of these enzymes can degrade most basement membrane proteins as well as some other ECM proteins. The substrate specificity of MMPs has mostly been studied *in vitro*, and only a few potential *in vivo* substrates have been characterised. Both MMP-2 and MMP-9 can generate an initial single cleavage of soluble type IV collagen molecules into about $\frac{1}{4}$ and $\frac{3}{4}$ size fragments, after which the collagen is further degraded (Fessler *et al.* 1984, Murphy *et al.* 1989). Several cleavage sites for MMP-2 have been identified, such as the peptide bonds Gly-Val, Gly-Leu, Gly-Glu, Gly-Asn, and Gly-Ser in denatured collagen (Seltzer *et al.* 1981, Seltzer *et al.* 1990). MMP-2 and MMP-9 have a number of well known matrix substrates (Table 1), but MMPs are known to be much more diverse in their substrate binding as reviewed by Overall (2002). As an example, such bioactive substrates as chemokines are bound by the hemopexin C domain of MMP-2, cleaved and therefore inactivated efficiently (McQuibban *et al.* 2001). This substrate-binding of MMP-2 has revealed a more detailed role for MMPs in the regulation of the inflammatory response.

Table 1. Extracellular matrix substrates of MMP-2 and MMP-9.

Name	Substrate	Reference
MMP-2 (Gelatinase A)	Collagen I, IV, V, VII, X, Gelatins, FN, LM, aggrecan, elastin, proGel B	(Matrisian 1992, Birkedal- Hansen <i>et al.</i> 1993, Sang & Douglas 1996)
	Fibrillin	(Ashworth <i>et al.</i> 1999)
	proColl-3	(Knauper <i>et al.</i> 1996)
	Laminin-5	(Giannelli <i>et al.</i> 1997, Koshikawa <i>et al.</i> 2000)
	SPARC (BM-40, osteonectin)	(Sasaki <i>et al.</i> 1997)
	Collagen IX	(Brown <i>et al.</i> 1996)
	Vitronectin	(Imai <i>et al.</i> 1995)
MMP-9 (Gelatinase B)	Collagen III	(Okada <i>et al.</i> 1992)
	Collagen IV, V, gelatin, elastin, entactin, aggrecan	(Matrisian 1992, Birkedal- Hansen <i>et al.</i> 1993, Sang & Douglas 1996)
	Collagen XI	(Pourmotabbed <i>et al.</i> 1994)
	Collagen XIV	(Sires <i>et al.</i> 1995)
	Fibrillin	(Ashworth <i>et al.</i> 1999)
	Vitronectin	(Imai <i>et al.</i> 1995)

Other proteins, such as myelin basic protein, galactoside-binding proteins (CBP30, 35), α_1 -antitrypsin, α_1 -antichymotrypsin, substance P and amyloid β peptide have also been found to serve as substrates for MMP-9 (Desrochers *et al.* 1992, Gijbels *et al.* 1993, Proost *et al.* 1993, Mehul *et al.* 1994, Ochieng *et al.* 1994, Sires *et al.* 1994, Backstrom & Tokes 1995, Backstrom *et al.* 1996, Ito *et al.* 1996).

2.3 Regulation of MMP activity

The regulation of MMP activity occurs at several different levels, including transcription, RNA-stabilization, translation, secretion, enzymatic and nonenzymatic activation and endogenous tissue specific inhibition (Figure 2).

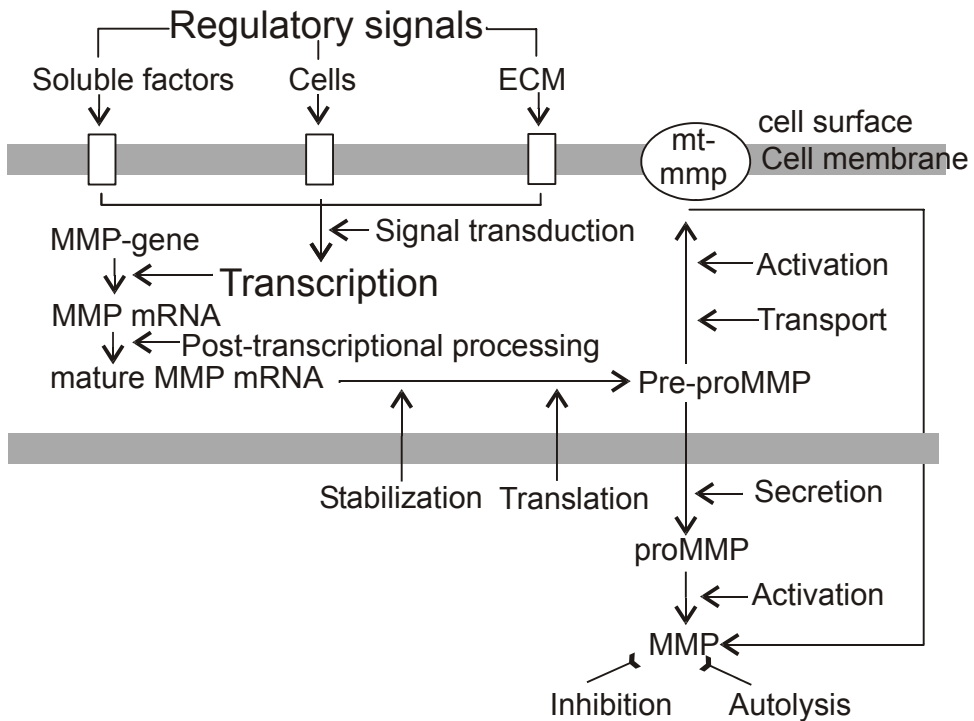


Fig. 2. Multilevel regulation of MMPs. Different regulatory signals interact with specific receptors at the cell surface and trigger a cascade of events leading to the generation of functional MMPs. MT-MMPs are localised to the cell surface and pro-MMPs are secreted into the extracellular medium where they are activated by later processes. (Modified from Overall & Lopéz-Otín 2002)

2.3.1 Transcriptional regulation of MMP-2 and MMP-9

The various MMPs have different expression profiles *in vivo*, which suggests different biological roles. Currently, there is very little known about the regulation of these enzymes *in vivo*, but MMP transcription has been studied quite extensively in cultured cells transfected with promoter-reporter gene expression constructs. Such studies have indicated that promoter regulation of the MMPs is quite similar. However, MMP-2 is an exception, as in many cultured cells it is expressed constitutively and its regulation appears to be subjected to a multilevel mechanism of enzyme activation (Strongin *et al.* 1995, Ellerbroek *et al.* 2001). Regulation of MMP-9 has been shown to be complicated in different cell lines, its expression being induced with different growth factors and cytokines. Thus, interleukin-1 β (IL-1 β), transforming growth factor- α (TGF- α) and epidermal growth factor (EGF) induce MMP-9 expression in rat mucosal keratinocytes (Lyons *et al.* 1993), whereas in organ cultured human skin, tumour necrosis factor- α (TNF- α) and transforming growth factor- β (TGF- β) together can induce expression of this gene (Han *et al.* 2001). MMP-9 expression can also be induced by ECM molecules, cell-cell and cell-ECM adhesion molecules, and agents that alter cell shape (review in Vu & Verb, 2000). The proximal promoters of the human MMP-2 and MMP-9 have been quite extensively characterised in cell transfection studies, as well as by using promoter mapping with an electrophoretic mobility shift assay (EMSA) to define regions for DNA/protein complex formation (Figure 3).

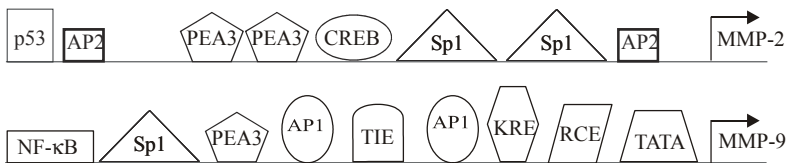


Fig. 3. Regulatory elements in the proximal promoters of the human MMP-2 and MMP-9 genes. The regulatory region is shown in the 5'-3' direction, with an arrow indicating the transcription start site. The shaped boxes stand for the binding sites of the transcription factors that have been tested for functional activity by cell transfection studies with different lengths promoter constructs of MMP-2 and MMP-9. The relative positions of the elements are not to scale. Abbreviations for transcription factor binding sites: the activator proteins (AP)-1 and -2, stimulating protein (SP)-1, the polyomavirus enhancer-A binding protein (PEA3), the cyclic AMP response-element binding protein (CREB), the TATA-box (TATA), the retinoblastoma control element (RCE), the keratinocyte differentiation -factor responsive element (KRE), the transforming growth factor- β inhibitory element (TIE) and the nuclear factor of κ B. (Modified from Overall & Lopéz-Otín, 2002)

In addition to the regulatory elements in the proximal promoter of MMP-2, elements such as a functional p53-binding site have been identified in the MMP-2 gene promoter at -1649- -1630 bp upstream from the transcription start site in the human sarcoma line (HT1080) (Bian & Sun 1997). However, this possible p53/r2 element was not functional in astrogloma cells (Qin *et al.* 1999). In the same study by Qin and co-authors (1999), two distal silencer elements, S-1629 and S-1612, were found to negatively affect the constitutive MMP-2 promoter activity.

When a panel of 32 individuals was scanned for genetic variants in the area spanning from the 2-kilobase promoter region over the 13 exons of the human MMP-2 gene, one common C to T transition at position -1306 was found to disrupt a Sp1-type box (Price *et al.* 2001). This disruption caused remarkably reduced promoter activity due to abolished Sp1 binding and may be linked in future tests to such pathologies as atherogenesis and tumorigenesis. Later, Harendza and co-authors (2003) have been able to link the -1306 site to another common polymorphism, a G to A transition at bp -1575. This polymorphism affects the binding of the estrogen receptor- α to the site and the estrogen receptor was shown to be necessary for enhancing activity in MCF-7 cells that are estrogen receptor-positive. MMP-2 is a well known estrogen-responsive gene both *in vitro* and *in vivo*. This kind of loss of function polymorphism was also associated with a decrease in genetic fitness.

A region with a functional polymorphism has also been described 1562 base pairs upstream from the human MMP-9 promoter. This region, named C-1562T, affects the promoter activity, and it may alter the severity of coronary atherosclerosis (Zhang *et al.* 1999). Another study using multiple logistic analysis of several polymorphisms in a group of 131 patients with stable angina, did not find any relation between the C-1562T transition and the severity of atherosclerosis in the coronary artery or in stent restenosis (Kim *et al.* 2002), whereas Cho and co-authors (2002) found a C/C homozygosity of this allele to protect against coronary artery disease. A study revealing an A21 allele of the MMP-9 promoter region showed that a polymorphism in the promoter region can protect against the development and progression of diabetic nephropathy (Maeda *et al.* 2001).

The extensive variation in tissue expression profiles between MMPs indicates that the transcription of these enzymes is tightly regulated by cell lineage specific enhancer elements. For example, during embryo- and morphogenesis, MMP-2 is widely expressed in fibroblast-like cells, but after birth its expression decreases significantly in most organs. This is contrary to the findings in cultured fibroblasts, where the expression of MMP-2 is constitutive. Expression of MMP-9 is normally mainly observed *in vivo* in osteoclasts, macrophages, leukocytes, invading trophoblasts and migrating keratinocytes. Thus, both these enzymes must have specific enhancers that drive cell/tissue specific expression *in vivo*. However, the characterisation of such elements is very limited.

2.3.2 Post-transcriptional regulation and secretion

The mRNA of MMP-2 is processed for post-transcriptional stabilization (Overall *et al.* 1991), and as the AU-rich sequences in the 3'-untranslated region of MMP1 have been observed to regulate its turnover (Vincenti 2001), it is very likely that similar kinds of sequences also regulate the stability of other MMP transcripts.

Most MMPs are constitutively secreted after translation, but some control of the secretion exists. For example, MMP-9 is synthesized by differentiating granulocytes in the bone marrow and stored in the granules of circulating neutrophils until released after neutrophil activation by inflammatory factors (Hasty *et al.* 1990).

2.3.3 MMP activation

Most MMPs are produced as zymogens, inactive enzymes that need to be further processed to become active proteases. The latency is due to an unpaired cysteine sulphhydryl group near the C-terminal end of the propeptide domain with the length of 80 amino acids, in a conserved region of PRCG(V/N)PD. The sulphhydryl group is a ligand for the zinc ion, and the bond between cysteine and zinc has to be cleaved and replaced by a water molecule for the activation of MMPs (Van Wart & Birkedal-Hansen 1990, Becker *et al.* 1995). The water molecule is needed for cutting peptide bonds of the target molecules. In contrast to all other MMPs, MMP-23 lacks the conserved cysteine that is required for its latency (Gururajan *et al.* 1998).

All MMPs seem to require proteases of other groups to be present for activation to occur. The effect of the accompanying proteases for the activation process is either direct or indirect (Overall & Lopez-Otin 2002). The site of MMP activation is usually the pericellular space, where the enzyme precursors can be bound with high affinity, as in the case of MT1-MMP or MT2-MMP mediated activation of proMMP-2 (Sato *et al.* 1994, Morrison *et al.* 2001). In addition to extracellular and pericellular mechanisms of proMMP activation, there exists another mechanism used by MT-MMPs, MMP-11, MMP-23 and MMP-28. These proteases are activated intracellularly by furin-like pro-protein convertases (Pei & Weiss 1995, Seiki 1999, Kang *et al.* 2002). Moreover, it is possible that furin can also function at the cell surface for activation of MT-MMPs.

In addition to the MMP-activation mechanisms studied *in vivo*, it is well known that the activation of MMPs *in vitro* can also be achieved by various chemical agents and proteases. Chemical agents interact directly with the cysteine switch or they change the conformation of the propeptide. Agents that convert a sulphhydryl group to a species that cannot serve as a ligand for the zinc atom, are, for example, sulphhydryl alkylating agents, chaotropic agents (NaSCN), surfactants (SDS), organomercurials (Hg(II), Au(II)), disulphides and oxygen-derived free radicals (Sorsa *et al.* 1989, Stetler-Stevenson *et al.* 1989b, Springman *et al.* 1990). The activation mechanism of organomercurials (such as APMA), as an example, is based on the conformational changes in the propeptide without a loss of molecular weight. In contrast to chemical activation, some proteases can also activate MMPs, and this activation results in the loss of molecular mass of the target MMP as a consequence of the cleavage of the amino terminal fragment. For example, trypsins (Birkedal-Hansen *et al.* 1976, Morodomi *et al.* 1992, Sorsa *et al.* 1997), u-PA (Keski-Oja *et al.* 1992, Mazzieri *et al.* 1997), plasmin (Monea *et al.* 2002) and mast cell tryptase (Lohi *et al.* 1992) activate MMP-2 and MMP-9.

2.3.3.1 Activation of proMMP-2

Activation of proMMP-2 requires the presence of active MT1-MMP and the binding of TIMP-2 (Strongin *et al.* 1995, Butler *et al.* 1998, Kinoshita *et al.* 1998). In this process, TIMP-2 is thought to bind first to the hemopexin domain of pro-MMP-2 after which this complex is targeted to bind to the surface bound MT1-MMP through TIMP-2. The crystal structure of the proMMP-2/TIMP-2 complex provides support for this (Morgunova *et al.*

1999). The role of TIMP-2 in this complex is to bind to the hemopexin domain of proMMP-2 by its C-terminal domain. This localizes the active MMP-2 more proximal to the active MT1-MMP (Butler *et al.* 1998). The *in vivo* evidence for the importance of the MT1-MMP in the activation process of MMP-2 is found from the results of the MT1-MMP –null mice studies. The transgenic mice were found to have an impaired activation of pro-MMP-2, when studied by using the gelatinolytic assay, whereas the activation of pro-MMP-9 was not affected. (Holmbeck *et al.* 1999, Zhou *et al.* 2000)

Moreover, it has been found *in vitro* by recombinant MMP-2 proteins that the activation can be autolytic (Bergmann *et al.* 1995). The autocatalytic activation is made by another active MMP-2 (Overall *et al.* 2000, Lehti *et al.* 2002). MMP-2 has a deep pocket that can “accommodate” Tyr81, therefore a second active MMP-2 can carry out activation *in trans*. As mentioned before, plasmin can also activate proMMP-2. This occurs through the cell surface-associated uPA/plasmin system, where proMMP-2 is surface bound (Mazzieri *et al.* 1997, Monea *et al.* 2002). If plasmin is in a soluble form, its role turns from activation into degradation, which is somewhat comparable to the dual role of TIMP-2 as an activator and inhibitor of MMP-2.

In addition to TIMP-2 dependent activation of MMP-2, Morrison and co-authors (2001) have published another, TIMP-2 independent pathway, which is MT2-MMP driven at the cell surface and inhibited by TIMP-2 and TIMP-4.

2.3.3.2 Activation of proMMP-9

The profile of the activation mechanisms for MMP-9 has been examined quite extensively, showing that MMP-9 activation from the 92 kDa pro-form into the 82 kDa active form can be promoted by TNF- α , which is found to be associated with down-regulation of TIMP-1. The proteolytic activation of MMP-9 was simultaneously mediated by a tissue-associated chymotrypsin-like proteinase, pro-MMP-9 activator (pM9A). (Han *et al.* 2002)

TIMP-1 can form a complex with Pro-MMP-9 and therefore inhibits not only its gelatinase activity but also its conversion from zymogen to an active proteinase (Wilhelm *et al.* 1989, O'Connell *et al.* 1994). Binding of MMP-9 to its substrate can also induce its activity as a proteinase (Bannikov *et al.* 2002). This process requires that the propeptide is intact.

2.3.4 Inactivation of MMPs

2.3.4.1 Tissue inhibitors of MMPs

The family of TIMPs consists of four members, namely TIMPs 1-4 (Docherty *et al.* 1985, Stetler-Stevenson *et al.* 1989a, Apte *et al.* 1995, Greene *et al.* 1996), which have a homology of 41 to 52 % inside the protein family (Douglas *et al.* 1997). They inhibit the MMPs reversibly in a 1:1 stoichiometric ratio. The theoretical molecular mass of TIMP-1

is 28.5 kDa and TIMP-2 is 21 kDa (Stricklin & Welgus 1983, Stetler-Stevenson *et al.* 1989a, Stetler-Stevenson *et al.* 1989b). However, the mass of TIMP-1 varies depending on the glycosylation, ranging from 30 to 34 kDa (Williamson *et al.* 1990, Tolley *et al.* 1993). The 21 kDa molecule of TIMP-3 has also been found to have an N-terminal glycosylation site (Wilde *et al.* 1994). The most recently found TIMP-4 is 22 kDa (Greene *et al.* 1996).

TIMPs are expressed in many different tissues and cell types, and they can also be detected in body fluids. TIMP-1 and TIMP-2 are secreted in a soluble form, whereas TIMP-3 associates as an insoluble protein with the ECM (Stricklin & Welgus 1983, Leco *et al.* 1994). It binds via its C-terminal domain to heparan sulfate proteoglycans within the ECM and, as a consequence, the concentration of TIMP-3 rises at focal regions within tissues and basement membranes (Langton *et al.* 1998). The primary structure for TIMPs is well characterised (review in (Bode *et al.* 1999).

TIMP-1 and -2 bind tightly in a 1:1 ratio to all MMPs, the only exception being TIMP-1 binding rather weakly to MT1-MMP and MT2-MMP (Sato *et al.* 1996, Butler *et al.* 1997). In addition to the activated forms of MMPs, TIMP-1 can bind to proMMP-9, and TIMP-2 can bind to proMMP-2 (Strongin *et al.* 1995). The role of TIMP-2 in this case is activating rather than inactivating, as it binds to proMMP-2 through its C-terminal domain (Morgunova *et al.* 1999) and participates in the cell surface activation process of proMMP-2 by MT1-MMP. As soon as MMP-2 is activated, it can be also inhibited, by the action of membrane-bound TIMP-2 (Itoh *et al.* 1998b). When TIMP-1 forms a complex with proMMP-9, they can also inhibit active MMP-9 and form a more stable and active ternary complex MMP-9/TIMP-1/MMP (stromelysin-1) (Kolkenbrock *et al.* 1995). Studies with the truncated N-terminal domains of TIMP-1 and -2 have shown that their inhibitory activity is due to this end of the protein (Murphy *et al.* 1991, Williamson *et al.* 1996). The C-terminus of MMPs is not necessary for the inhibition (Williamson *et al.* 1993). The inhibitory activity of TIMP-3 is targeted to MMP-9 more than by any other TIMPs (Sternlicht & Werb 2001).

TIMP-1 does not only function as an inhibitor of MMPs (pro-MMP-9, active MMPs) but has also activity as a growth factor, stimulates gonadal steroidogenesis, changes cell morphology, inhibits angiogenesis and participates in tissue remodelling (Gomez *et al.* 1997). TIMP-2 expression is mostly constitutive (Overall 1994), TIMP-3 promotes the detachment of transformed cells from the ECM and accelerates oncogenic cell transformation (Yang & Hawkes 1992). TIMP-4 presents a highly restricted expression pattern and has been found so far only in human and murine heart and murine ovary tissue (Greene *et al.* 1996, Rahkonen *et al.* 2002).

2.3.4.2 Other inhibitors of MMPs

In addition to TIMPs, there are also other endogenous inhibitors of the MMPs. The plasma protein, α 2-macroglobulin, has been found to be the major endogenous inhibitor (Sottrup-Jensen & Birkedal-Hansen 1989), preferably in tissue fluids, whereas TIMPs act more locally (Sternlicht & Werb 2001). As TIMPs are known to inhibit MMPs reversibly, α 2-macroglobulin binds MMP-molecules irreversibly and the complex is further

endocytosed together by the plasma-membrane-bound LDL-receptor-related protein (LRP), which is a scavenger-receptor (Zhang *et al.* 1998, Herz & Strickland 2001). Several protein fragments have been found, mainly protein subdomains, that are structurally homologous to TIMPs, such as procollagen C-terminal proteinase enhancer protein (PCPE) (Mott *et al.* 2000) that can release a C-terminal fragment similar to the inhibitory domain of TIMPs. A non-collagenous NC1 domain of type IV collagen (Netzer *et al.* 1998) is an MMP inhibitor that can inhibit also angiogenesis and tumour growth (Petitclerc *et al.* 2000). Another cell surface MMP inhibitor that can also regulate angiogenesis is RECK, reversion-inducing-cysteine-rich protein with Kazai motifs (Oh *et al.* 2001). TFPI2 (tissue-factor pathway-inhibitor-2) is both a serine proteinase inhibitor and an MMP-inhibitor (Herman *et al.* 2001). The laminin-binding domain of agrin also has structural similarities to TIMPs (Stetefeld *et al.* 2001). It is obvious that there are numerous potential inhibitors for MMPs, but their physiological targets are unclear, as their activity for example against MMP2 is lower than with TIMPs (Netzer *et al.* 1998, Mott *et al.* 2000).

In addition to the endogenous inhibitors of MMPs listed above, thrombospondin 1 (TSP1) can prevent the activation process of proMMP-9 (Rodriguez-Manzanique *et al.* 2001), as TSP2 has been shown to regulate the availability of active MMP-2 (Yang *et al.* 2001). This process is not inhibition of MMP-activity, but very likely related to LRP-binding and endocytosis (Hornebeck *et al.* 2002).

2.4 Physiological roles of MMP-2 and -9 expression

The MMPs have a broad role in development and tissue formation. They participate in tissue turnover through ECM degradation and remodeling, and their effect on the microenvironment may lead to changes in cellular behaviour, such as angiogenesis. They also regulate numerous biologically active molecules by directly cleaving and releasing them from storage sites or by altering the activity of their inhibitors. The developmental effects of MMPs can vary, depending on the site of action, salivary glands and kidney are examples. In the salivary glands the branching mechanism differs from that in kidney. The inhibition of MMPs leads to higher branching *in vivo* in the salivary gland, whereas in the kidney the effect is controversial. (reviewed by Vu and Werb 2000)

2.4.1 Expression of MMP-2 during implantation and embryogenesis

The *in situ*-studies have revealed the expression of MMP-2 during murine embryo implantation. The MMP-2 transcript was detected only in the uterine stromal mucosal cells that were not in the decidualized area during implantation (days 5.5-7.5 p.c). In addition, a layer of myometrial smooth muscle cells and some parts in the non-pregnant uterine stroma were positive. The mRNA of mouse TIMP-2 was not detected in those areas during the same gestational days. (Reponen *et al.* 1995)

The continuous expression of TIMP-2 and MMP-2 (5.5-9.5 days p.c.) has been reported by Alexander and his colleagues (1996) in the mouse embryonic implantation site while the expression of MMP-2 declined by 6.5 days p.c. MMP-2 mRNA was

detected by *in situ* hybridization in the undifferentiated decidual zones of the implanting embryo, and TIMP-2 appeared to be constitutively expressed in the same areas.

MMP-2 mRNA was also expressed at later stages of mouse embryonic development (10-15 p.c. and also 9 days p.p), mainly by mesenchymal cells, with the exception of the epithelial cells of salivary glands and the central epithelial cells of hair buds. MMP-2 expression was detected especially in the head branchial arches, upper and lower mandibular regions, kidney, lung and limb mesenchyme. (Reponen *et al.* 1992) The expression at the mandibular region is explained mainly by the process of tooth development in the presence of MMP-2 *in vivo* (Sahlberg *et al.* 1992). During rat kidney development, MMP-2 is expressed in the undifferentiated mesenchyme of the embryonic kidney, while the fully differentiated kidney did not produce any transcripts of either MMP-2 or MT1-MMP. Immunohistochemical analysis of the same tissues show the MMP-2 protein to be produced in those nephron structures where differentiation of the epithelium occurs. (Tanney *et al.* 1998) Several other studies describe MMP-2 expression during differentiation of tissues and organs, including, ductal branching morphogenesis of murine mammary glands, embryonic avian heart valves and osteogenesis (Witty *et al.* 1995, Alexander *et al.* 1997, Filanti *et al.* 2000).

2.4.2 Expression of MMP-9 during implantation and embryogenesis

In contrast to the expression of MMP-2 in many different cells and tissues during development, MMP-9 is expressed in a more restricted pattern. Although it is expressed only in few cell lines *in vivo*, its expression *in vitro* can be induced by many different agents in several cell lines.

In situ hybridization analyses of MMP-9 expression during mouse development indicate the gene is strictly regulated. At the time of implantation, MMP-9 is found in invading trophoblasts (Reponen *et al.* 1995, Alexander *et al.* 1996), but expression is not detected in the embryo until day 12 p.c., when strong expression is found in the mesenchymal cells of the developing limbs. Transgenic mouse studies using Ets-2 null mice (Yamamoto *et al.* 1998) strongly suggest that MMP-9 plays an important role during implantation. Ets-2 null mice die at embryonic day 8.5, which indicates serious problems during early trophoblast invasion. MMP-9 is a known target of Ets-2 at the transcriptional level.

Several studies have reported MMP-9 is expressed in osteoclasts (Hill *et al.* 1994, Reponen *et al.* 1994, Wucherpfennig *et al.* 1994, Blavier & Delaisse 1995, Okada *et al.* 1995). At day 13 p.c. the cells surrounding the incisor tooth primordia express MMP-9 in a manner resembling the expression in the limbs (Reponen *et al.* 1994). Strong signals are restricted to the mesenchymal areas surrounding the developing teeth. However, these areas are not TRAP-positive, as would be expected of osteoclasts.

Furthermore, a study by Canete-Soler and colleagues (1995) reported MMP-9 expression in other organs, including the yolk sac during implantation, the central nervous system at day 11 p.c., and liver, bronchial epithelium of the lungs, primordial alveoli, epithelium of the thyroid gland, cells in the thymus, bone and neural cells at day 15 p.c.

Many studies indicate that MMP-9 is expressed during kidney development and it has been assumed that MMP-9 is necessary for the proper kidney branching morphogenesis. This hypothesis was supported both by *in vitro* studies where MMP-9 was found to be required for renal differentiation (Lelongt *et al.* 1997), and by immunohistochemical, western and laser confocal microscopy studies (Legallicier *et al.* 2001) that localized MMP-9 to the apical cells of both embryonic and adult mouse renal tubules. However, no kidney failure or defects in kidney tubular differentiation were observed in MMP-9 knock out mice (Andrews *et al.* 2000). This may be due to the presence of MT1-MMP, which has affects on kidney branching morphogenesis similar to those of MMP-9. Antisense oligonucleotides against MT1-MMP decrease branching of the ureteric bud in embryonic kidney cultures and, as an opposite reaction, anti-sense oligos against TIMP-2 increase and rescue the branching (Kanwar *et al.* 1999).

2.4.3 Expression of MMP-2 and -9 after embryogenesis

MMP-2 and MMP-9, like other MMPs and TIMPs, are found in adult tissues during normal cellular processes requiring tissue remodelling, such as ovulation, skeletal growth and remodelling, post partum involution and branching processes in the mammary gland preceding lactation (Birkedal-Hansen *et al.* 1993, Nelson *et al.* 2000, Steffensen *et al.* 2001). The branching of the mammary gland is a good example of the processes seen also in other ductal tissues with branching morphogenesis.

The formation of the mammary glands starts during embryogenesis by the budding of an epithelial tube into the mammary fat pad. The morphogenic process from this point through the steps of growth and branching of the ducts into the terminal lobular-alveolar units at the ductal ends occurs during embryogenesis (Hennighausen & Robinson 1998). The alveoli of the mammary gland develop during pregnancy and lactation and they involute during weaning. This continuous ductal morphogenesis includes several MMPs in the process of side-branching (Wiseman & Werb 2002). Transgenic mice expressing autoactivated isoforms of stromelysin-1 (Sympson *et al.* 1994, Witty *et al.* 1995) have morphological defects in their mammary glands. In transgenic mice expressing TIMP-1 anti-sense RNA in their mammary glands, increased ductal branching was found (Fata *et al.* 1999). Interestingly, an extracellular protein directing epithelial morphogenesis, epimorphin, increases the expression levels of MMP-2, -3 and -9, when studied in mammary epithelial cells (Simian *et al.* 2001). In the same study, the expression of MMPs was shown to be necessary for morphogenic activity, because branching of mammary organoids was entirely blocked with MMP inhibitors, but not with serine, cysteine or aspartate proteinase inhibitors. It is evident that MMP-remodeling of the stroma is also needed to make space for and support the growing epithelial structures in other corresponding processes (Vu & Werb 2000).

2.4.3.1 Bone resorption

New bone formation results from the action of osteoblasts, and the resorption of bone is carried out by osteoclasts. Osteoclasts are multinucleated bone cells that differentiate from the monocyte precursors of haematopoietic origin. Osteoblasts, on the other hand, are cells of mesenchymal origin and secrete bone collagen, osteoid, that is further mineralized to form bone. The mature bone is under a continuous process of resorption and ossification that ensures the maintenance of the structure and strength of living bone tissue and its proper growth. These processes are strictly regulated by different factors, such as growth factors and cytokines, and any imbalance between them leads very easily to a pathological state. Bone maintains also the concentration of phosphate and calcium ions constant in the extracellular fluids. (Baron *et al.* 1985, Baron *et al.* 1986, Nijweide *et al.* 1986, Delaisse *et al.* 2000)

The most abundant collagen of bone, type I collagen, is degraded by two types of proteinases; cysteine proteinases and MMPs (Everts *et al.* 1992). Cathepsin K as a member of the cysteine proteinases is able to cleave the native triple helix of collagen at several sites causing the unwinding and availability of the triple helix to proteinases with gelatinolytic activity, such as cathepsin K itself, MMP-2 and MMP-9 (Bromme *et al.* 1996, Garnero *et al.* 1998). Cathepsin K knock-out mice have been found to develop osteopetrosis as a consequence of the impaired osteoclastic collagen degradation (I and II, presumably) (Saftig *et al.* 1998, Gowen *et al.* 1999). A detailed profile of the MMPs responsible for collagen degradation during ossification is still partly lacking, although the *in situ* hybridization and immunolocalization studies have shown that MMP-9 is found in the osteoclasts of human, mouse and rabbit (Reponen *et al.* 1994, Tezuka *et al.* 1994, Okada *et al.* 1995). Furthermore, MMP-9 knock-out mice exhibit abnormal skeletal growth plate vascularization and ossification (Vu *et al.* 1998) which is mostly due to improper osteoclast and endothelial cell invasion into the hypertrophic cartilage (Engsig *et al.* 2000). Another function for MMP-9 during ossification is to retrieve vascular endothelial growth factor (VEGF) from the ECM (Bergers *et al.* 2000). The function of VEGF is to chemically attract osteoclasts, and its function is necessary for growth plate invasion (Gerber *et al.* 1999) and diaphysis invasion (Engsig *et al.* 2000). Interestingly, the role of MMP-9 is minor at the secondary ossification center of the epiphysis where VEGF is absent (Vu *et al.* 1998). The activation of TGF- β possibly resembles that of VEGF, as it is activated by MMP-9, when CD44 acts as a docking receptor in normal keratinocytes (Yu & Stamenkovic 2000). This hypothesis is supported by studies with TGF- β knockout mice that have smaller tibial growth plates, longitudinal growth retardation and a diminished number of hypertrophic chondrocytes (Geiser *et al.* 1998).

2.5 MMP-2 and MMP-9 in pathological conditions

The presence of MMP-2 and MMP-9 has been implicated in many pathological processes, such as wound healing and tissue inflammation, including arthritis and periodontal disease (Matrisian 1992, Sodek & Overall 1992, Birkedal-Hansen *et al.*

1993), tumour invasion (Tryggvason *et al.* 1993, Coussens *et al.* 2002) and aortic aneurysm (Newman *et al.* 1994, Tamarina *et al.* 1997). The list of diseases where these MMPs are involved is still increasing, indicating that a loss of balance between disruption and regeneration of the basement membrane plays a fundamental role in the induction of pathogenesis.

2.5.1 Cutaneous wound healing

The process of wound healing is dependent upon the type of tissue wounded and the extent of tissue damage (especially whether basement membrane is injured or not). When a wound involves cutaneous tissue damage with a broken layer of basement membrane major events take place at several sites immediately after tissue damage. The phases of healing partly overlap, starting from early and late phase inflammation, continuing with reepithelialization and granulation tissue formation and ending with matrix formation and remodelling (Table 1) (Clark 1996)

Table 2. Wound repair as divided into phases (according to Clark. 1996)

Phase of healing	Time after injury	Description	Processes during the phase
I. Inflammation	Early (0-3 days)	Neutrophil-rich	Blood coagulation Platelet adhesion Neutrophil-invasion
II. Tissue formation	Late (0.3-10 days)	Mononuclear-cell -rich	Monocyte-invasion
	3-18 (First within hours)	Re-epithelialization	Migration of keratinocytes Phenotypic alteration of keratinocytes Proliferation of keratinocytes Basement membrane protein production
III. Tissue remodeling	4 days	Granulation tissue formation	Cytokine production Blood vessel formation Loose connective tissue Fibroblast and macrophage accumulation
	8 days -	Neovascularization/angiogenesis	Blood vessel formation
		ECM-remodelling	Fibrin clot deposition Fibronectin, collagen III and I deposition Hyaluronan replaced by proteoglycans, → deposition of proteoglycans and collagen Formation of larger collagen bundles
	Cell maturation	Fibroblast transition: migratory – profibrotic (type I and III collagen production) – myofibroblast (wound contraction)	
	Cell apoptosis	Endothelial cells Myofibroblasts	

A fibrin clot forms in most skin wounds after blood vessel damage and leakage. The clot protects the wounded tissue at the beginning and at the same time provides a primary matrix for the cells migrating to the area of repair. The clot contains platelets in the network of fibrin fibers, smaller amounts of fibronectin, vitronectin and thrombospondin (Clark 1996). The first signalling molecules that initiate the wound healing process, cytokines and growth factors, are released from these activated platelets. These signals attract the first inflammatory cells, start the reepithelialization and contraction of connective tissue and induce angiogenesis at the wound site. The inflammatory cells responding to the chemotactic signal from the damaged tissue include neutrophils and monocytes from the blood. The neutrophil invasion starts normally within minutes of wounding and ceases after a couple of days when tissue macrophages phagocytose the remaining neutrophils. Macrophages are necessary at the wound area especially for this phagocytic function: they also ingest pathogenic organisms and other cell and matrix debris. In addition, they release growth factors and cytokines therefore reinforcing the signals from neutrophils and platelets. (Martin 1997)

At this stage epithelial movement by migrating keratinocytes is induced. Amoeboid-like movement takes place at the epithelial front to eventually cover the wounded tissue with a thin epithelial layer (Figure 4.). The initial signal for migration is chemotactic and the direction is controlled by the concentration (Garrett 1998). The migratory action occurs also at the sites of possible intact hair follicles in the wound area that supply the surrounding tissue with epithelial stem cells (Rochat *et al.* 1994). In addition, the suprabasal epithelial cells have the capacity to travel over basal cells and thus accelerate the healing process (Garlick & Taichman 1994). Reepithelialization is facilitated by the connective tissue contracting beneath the wound. This highly regulated process starts when proliferating fibroblasts migrate and produce a collagen-rich matrix within the wound clot (Eckes 1996). Furthermore, some of the fibroblasts transform into myofibroblasts that have the ability to contract in a manner resembling smooth muscle cells (Desmoulière & Gabbiani 1996).

When a monolayer of keratinocytes covers the wound surface, a new stratified epidermis and basal lamina are built starting from the sides of the wound and proceeding toward the center as indicated by the appearance of new hemidesmosomes and anchoring fibril collagen (Gipson *et al.* 1988). Anchoring fibrils apparently finalize the reepithelialization as they link the basal lamina to the connective tissue underneath (Compton *et al.* 1989). This final processing of the healing tissue can take from weeks to several years, depending on the extent of the damage.

Angiogenesis at the wound site is promoted by growth factors, such as fibroblast growth factor-2 (FGF-2) and vascular endothelial growth factor (VEGF), and neuronal recovery at least by nerve growth factor (NGF) and possibly indirectly by TGF- β (Martin 1997). As with all other cell migrations, the migration at a wound site also requires tightly regulated proteolysis of the ECM during the invasion phases. Many of the MMPs are responsible for this proteolysis

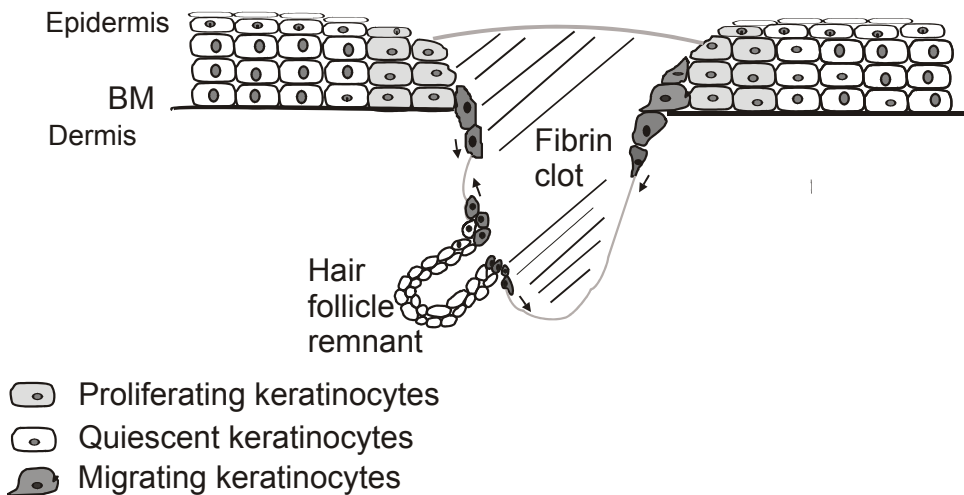


Fig. 4. The healing of a skin wound. The wound is temporarily plugged with a fibrin clot. Epidermal healing starts from the edges of the wound and from the cut hair follicle remnants by the migration of keratinocytes of the leading edge. The cells crawl beyond the cut basal lamina and over provisional matrix and healthy dermis. Cell division occurs behind the leading edge in the proliferating keratinocytes. (Modified from Martin P. 1997)

2.5.1.1 Migrating keratinocytes in wound healing

After injury, the undamaged keratinocytes of the epithelium migrate off the basement membrane and onto the dermal matrix once the initial signal is received. Migration of keratinocytes is an early event in wound healing (Table 2). The keratinocytes that are at the edge of the wound site, start migrating within 24 to 48 hours. The first migrating cells cover the wound and do not actively proliferate. The keratinocytes at the leading edge of the migrating epithelial tip phagocytose wound debris and some parts of the provisional wound matrix and use the provisional matrix containing mainly fibronectin and fibrin for support (Clark *et al.* 1982). The direction of the cell movement is defined by the chemotactic signal. Migration is compared to a cycling process in which the first step is the extension of a protrusion, followed by formation of stable attachments near the leading edge of the protrusion, movement of the cell body forward, release of adhesions and finally retraction at the cell rear (Lee *et al.* 1993, Sheetz 1994, Lauffenburger & Horwitz 1996).

The keratinocytes travel from several cut sites of the wound bed so that eventually small epidermal islands are found all over the wound bed, and these contribute equally to the reepithelialization process (Martin 1997). In other words, they are said to follow the so-called “Free edge effect” which means viable epithelial cells that have lost their neighbours stimulate epithelial cell migration (Garrett 1998).

As a part of the cell movement, migrating keratinocytes break cell-cell and cell-matrix contacts and temporarily form new contacts at the wound edge. Constitutively expressed integrins such as $\alpha 2\beta 1$, which are localised basolaterally in intact epidermis, are found

at the basal surface of the migrating tip at the wound edge. In contrast, a hemidesmosomal integrin, $\alpha 6\beta 4$, can be found on the basal surface in intact skin cells, but after wounding, it is present all over the surface of those cells at the migratory tip. Moreover, such integrins as $\alpha 5\beta 1$ and $\alpha v\beta 3$ are induced basally in intact epidermis and also on the basal surface of the keratinocytes of the wound edge. (Pilcher *et al.* 1997)

2.5.1.2 Involvement of MMPs and TIMPs in wound healing

Wound healing, as a process, shares similarities with embryonic development. It requires cell migration, ECM degradation and tissue reorganization. Healing requires the presence of MMPs as shown by studies on mice treated with an MMP inhibitor (Lund *et al.* 1999), and studies on MMP-3 –null mice that failed to upregulate a multicellular contractile ring of actin in dermal fibroblasts (Bullard *et al.* 1999). In both cases wound healing was retarded.

When tested *in vitro* using primary human keratinocytes, MMP -9 expression is induced, in parallel with the migratory phenotype, when type I collagen is available (Sarret *et al.* 1992, McCawley *et al.* 1998). At the same time, the catalytic activity of collagenase-1 is necessary for the keratinocyte migration (Pilcher *et al.* 1997), when the native fibrillar type I collagen is an elementary stimulus for collagenase-1 production. Additionally, when studied with wounded HaCat-keratinocytes, it appeared that MMP-9 expression did not correlate with migration (Mäkela *et al.* 1999). The synthesis and secretion of MMP-2 also takes place in primary human keratinocytes in the presence of either type I or type IV collagen (Petersen *et al.* 1990). In other studies, MMP-2 expression was induced in the keratinocytes and dermal fibroblasts only if both cell types are present (Sarret *et al.* 1992, Kratz *et al.* 1995, Zeigler *et al.* 1996).

In the study by Saarialho-Kere and colleagues (1996), the expression of collagenase-1 and stromelysin-2 was detected in the migrating keratinocytes of punch wounds made into human skin. In the same study, hyperproliferative basal keratinocytes following the epithelial front expressed stromelysin-1. In *in vivo* studies of human oral mucosa, the MMP-9 expression during wound healing was localized to the migrating epithelial sheet, to its basal cell layer, and after seven days of healing, also to granulation tissue (Salo *et al.* 1994b). Furthermore, MMP-2 was expressed in the fibroblasts of the oral mucosa. The specific role of MMP-9 during re-epithelialization is still unclear. It has been suggested that during wound healing MMP-9 could cleave type IV collagen, the fibrin-fibronectin clot, denatured collagen, and be involved in processing proteins of the regenerating basement membrane (Steffensen *et al.* 2001).

When cutaneous wound healing was studied *in vivo* in mice, several MMPs and TIMPs were expressed. Based on RNase protection analysis (RPA), only MMP-2, MT1-MMP, TIMP-2 and TIMP-3 were expressed in intact skin, while TIMP-1 was not induced. One day after wounding, murine collagenase (the assumed orthologue of human MMP-13), MMP-2, MMP-3 (stromelysin-1), MMP-9, MMP-10 (stromelysin-2), MMP-12 (MME) MT1-MMP and TIMP-1 were expressed. TIMP-1 is expressed in stromal tissue under the migrating edge of the epithelium, whereas the expression of MMP-9 was initially epithelial and later restricted to the area of the migrating tip. By day 5 MMP-9

was also found in granulation tissue. (Madlener 1998) The expression profile for murine MMPs during cutaneous acute wound healing is presented in figure 6. The expression of MMP-9 is found also in the migrating rat epithelial cells of acute skin wounds (Okada *et al.* 1997) and corneal wounds made by excimer laser keratectomy (Ye & Azar 1998).

In the corneal wound model using MMP-9 deficient mice, the MMP-9 deficiency accelerated wound closure and the inflammatory response (Mohan *et al.* 2002). The faster wound closure may have been partially due to enhanced cell replication and/or defects in the processing of the fibrin/fibrinogen matrix. MMP-9 –deficient mice produced a multilayered matrix compared to the epithelial monolayer sheets in the cornea of their normal counterparts.

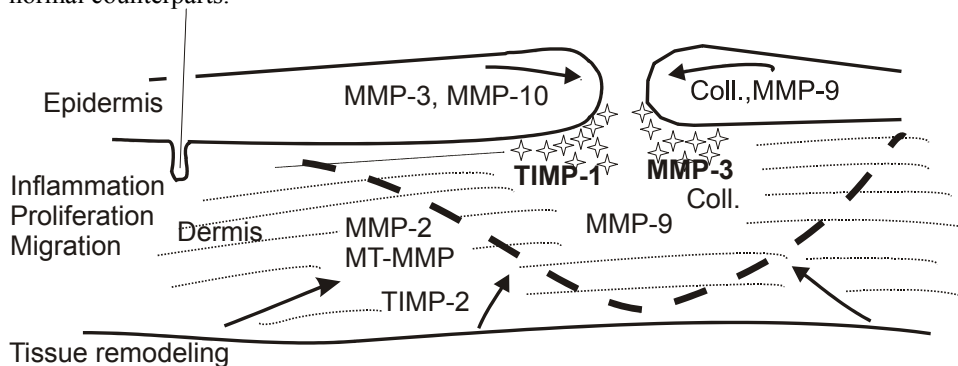


Fig. 5. Expression profile of MMPs and TIMPs during re-epithelialization of a cutaneous wound. Various soluble members of the MMP family are produced by separate subpopulations of the epidermis. Some MMPs are also secreted by mesenchymal cells. In granulation tissue, MMP-2 and MT1-MMP transcripts are the most abundant, but later, during tissue remodeling, MME shows a distinct expression profile. (Modified from Madlener 1998)

The healing of a chronic wound differs from that of the acute cutaneous wound. Chronic human wounds are known to have higher collagenase/MMP -activity, such as MMP-8 (Nwomeh *et al.* 1999), and the activation profile during the time-course of the healing is altered (reviewed by Kähäri and Saarialho-Kere 1997). Diabetes is known to be one common cause of poorly or non-healing wounds. Diabetic human foot ulcers have a higher expression of MMP-2 when compared to normally healing cutaneous wounds (Lobmann *et al.* 2002). On the other hand, Ashcroft with colleagues (1997) have studied the effect of ageing in wound healing, and found the MMPs previously associated with chronic wound healing to be up-regulated in the acute cutaneous wounds of aged people.

2.5.2 Cancer

Tumour progression requires enzymes that degrade the surrounding matrix barrier and allow release of metastasizing cells into the blood circulation and further to other distant organs. The MMPs can fulfil this requirement and many of them were originally cloned from tumours or tumour cell lines (reviewed by Coussens *et al.* 2002). *In situ* hybridisation studies and immunohistochemical staining have localised MMP-9 to the

stromal macrophages surrounding invasive human tumour cells (Pyke *et al.* 1992, Pyke *et al.* 1993, Tryggvason *et al.* 1993). Later it has been confirmed in many studies that most of the proteolytic potential of tumours originate from the cells of the stroma (Nelson *et al.* 2000, Bissell & Radisky 2001). What is now known to be true in other types of tissues is recognized also in the case of tumour tissue: MMPs not only dissolve the matrix barrier, but also solubilize cell surface and matrix-bound factors that can further affect such cellular processes as growth, death and migration.

The role of MMP-2 in tumour progression has been studied using MMP-2 –deficient mice. These animals have reduced angiogenesis, experimental tumour growth and metastasis as a consequence of host-derived response to B16-BL6 melanoma cells or Lewis lung carcinoma cells (Itoh *et al.* 1998a). When MMP-9 –knock-out mice were challenged with human papilloma virus-16-induced squamous cell carcinomas, the rate of tumourigenesis was diminished (Coussens *et al.* 2002). The tumour growth and invasiveness were restored by transplanted wild-type bone marrow cells. In parallel with these results are the studies by Kruger with colleagues (1998) with TIMP-1 overexpressing mice that showed 75% higher resistance to experimental metastasis of fibrosarcoma in brains.

It has been shown, that CD44 localizes the MMP-9 protein on the cell surface and this correlates with tumour cell invasiveness, growth and angiogenesis both *in vitro* and *in vivo* (Yu & Stamenkovic 1999). One link between MMP-9 and tumour growth and invasion depends on TGF- β –activation, which is mediated by proteolytically active MMP-9 and MMP-2 (Yu & Stamenkovic 2000). Soluble MMP-9 does not have this effect. The cell surface bound activation of TGF- β by MMP-9 can be found in normal keratinocytes and in invasive cancer cells. Furthermore, when studied in RIP1-Tag2 –transgenic mice, MMP-9 was found to be involved in the angiogenesis of pancreatic tumours that developed in these animals (Bergers *et al.* 2000). The role of MMP-9, as expressed in a few cells proximal to the vasculature, was to make vascular endothelial growth factor (VEGF) available to its receptors. In the same study by Bergers and co-authors (2000), MMP-2-null mice crossed with RIP1-Tag2 mice had smaller tumours without an effect on tumour number. This led to speculation that the role of MMP-2 during carcinogenesis is related to tumour growth rather than invasion. It is a theory that needs to be further considered in the light of data collected from different types of tumours. In addition, it is known, that MMP-2 can proteolytically expose an epitope of type IV collagen, HUIV26, on the vascular basement membrane so that it correlates also with tumour angiogenesis (Xu *et al.* 2001). Interestingly, enzymatic activation of proMMP-9 is inhibited by thrombospondin-1 (TSP-1) and it has been shown that TSP-1 suppresses the accumulation of VEGF, VEGFR2 and active MMP-9 in the tumours, when studied with mammary tumour-prone mice crossed with transgenic mice with altered expression of TSP-1 (Rodriguez-Manzanque *et al.* 2001). These results suggest that the use of more specific inhibitors for MMP-2 and MMP-9 may hold some promise for cancer treatment. Against all expectations, however, the therapeutic use of the MMP-inhibitors for cancer treatment has faced several setbacks after phase III clinical trials. These are partly due to differences between the human versus animal tests. As an example, in the mouse models, such as RIP1-Tag2 (Hanahan 1985) treated with the synthetic MMP-inhibitor batimastat (Bergers *et al.* 1999), the rate of tumour progression decreased. The inhibitor was administered to mice at early and intermediate stages of the

cancer, whereas human patients in clinical trials received the treatment at late stages of the disease. The role of different MMPs and TIMPs at different stages of tumour development *in vivo* has to be considered when planning new therapies based on MMP – inhibitors (Coussens *et al.* 2002). The results from different cancer cell studies often disagree with those from transgenic mouse studies, and, in addition, the effects of different levels of TIMPs do not always correspond in the two types of studies. As reviewed by Sternlicht and Werb (2001), it is very probable that TIMPs can either block or promote cancer, depending on their target metalloproteinase and may have other effects on cells that are independent of their inhibition of MMPs.

2.6 Transgenic mouse models in MMP research

Several reports in which transgenic mice were used to study MMP regulation, including TIMPs, have been published during recent years. One of the very first studies involving MMP was by (Carmeliet *et al.* 1994, Carmeliet *et al.* 1997). Evidence for a urokinase-type plasminogen activator (u-PA) dependent proMMP activation was found by studying atherosclerotic aorta in mice with a deficiency of apolipoprotein E (ApoE^{-/-}) both with or without a deficiency of tissue-type plasminogen activator (t-PA) or of u-PA. A deficiency of u-PA was shown to protect transgenic mice against ECM destruction and aneurysm formation possibly due to the reduced plasmin-dependent activation of pro-MMPs, such as pro-MMP-9.

TIMP-1 overexpression studies have interested several researchers, partly because of the broad group of MMPs that TIMP-1 inhibits and the possible therapeutic use of TIMP-1. During mouse embryonic implantation, TIMP-1 overexpression did not significantly affect the decidual processes. The placenta was functional, and the embryos were viable (Alexander *et al.* 1996). In these studies, the overexpression of TIMP-1 was driven by the β -actin promoter. Many other TIMP-1 overexpression studies with transgenic mice involved the growth of various carcinomas and tumour invasion, often indicating the inhibitory effect of TIMP-1 in these processes (Martin *et al.* 1996, Kruger *et al.* 1998, de Lorenzo *et al.* 2003, Ikenaka *et al.* 2003). Additional studies by Soloway and colleagues (1996) found the effect of TIMP-1 to be dependent on tumour type. Furthermore, the influence of TIMP-1 on lung tumor invasion *in vivo* was said to be only tumor TIMP-1 genotype dependent, not host dependent. However, another study using TIMP-1 transgenic mice with intestinal tumours obtained similar results (Goss *et al.* 1998). The knock-out of TIMP-1 had a very mild effect on the phenotype, providing only resistance to *Pseudomonas* induced infections in the cornea of the transgenic mice (Osiewicz *et al.* 1999). Similarly, the TIMP-2 ^{-/-} phenotype is normal with only weakly defective MMP-2 activation (Wang *et al.* 2000).

A mouse mammary tumour virus long terminal repeat-promoter (MMTV-LTR) has been used to target the overexpression of MT1-MMP to the mammary glands of transgenic mice (Ha *et al.* 2001). This increased the activation of MMP-2, as MT1-MMP is known to be an activator of pro-MMP-2. As a consequence, 82% of the mammary glands of the female mice developed abnormalities, many of them directly related to carcinogenesis. Previous studies using MMP-2 knock-out mice had shown that when

non-transgenic tumour cells where implanted intradermally into the transgenic mice, the rate of tumour angiogenesis and growth as well as metastasis were reduced (Itoh *et al.* 1998a). The same mice were found to develop normally but with a 15% delay in their growth and a delay in mammary gland development (Itoh *et al.* 1997, Alexander *et al.* 2001).

The MMP-9 –null mutations in mice were originally generated by Vu and co-authors (1998) to study the ossification and angiogenesis in the bones. The lack of MMP-9 was shown to cause delayed endochondral ossification and inefficient vascularization of the hypertrophic cartilage. The implantation and osteopetrosis of the animals was normal. A more detailed list of non-malignant phenotypes found in MMP-9-null mice was prepared by Sternlicht and Werb (2001). Transgenic mice expressing the human papillomavirus type 16 (HPV16) early region genes in basal keratinocytes (K14-HPV16 –mice) have high rates of invasive squamous cell carcinoma (SCC) of the epidermis (Arbeit *et al.* 1994, Coussens *et al.* 1996). When crossed with MMP-9 *-/-* mice the phenotype was milder but the carcinomas were more malignant (Coussens *et al.* 2000). The original invasive SCC phenotype was restored by supplying K14-HPV16/MMP-9 *-/-* mice with wild type bone marrow cells that serve as a source of neutrophils, macrophages and mast cells. Those cells populated and expressed MMP-9 in the stroma surrounding the SCC. The role of MMP-9 as an angiogenic switch was shown by crossing the same MMP-9 knock-out mice with Rip1-Tag2 mice that develop islet tumours of the pancreas by 12-14 weeks of age (Bergers *et al.* 2000).

As mentioned earlier with the wound healing studies, Mohan and co-authors (2002) have used MMP-9 knock-out mice to elucidate the role of MMP-9 in corneal wound healing. This study together with a study using a kidney disease model, anti-glomerular basement membrane (GBM) nephritis (Lelongt *et al.* 2001) found the accumulation of fibrin in the MMP-9 null mice. Because of the accumulation of fibrin in anti-GBM nephritis mice, the renal disease was more severe with the MMP-9 *-/-* phenotype when compared to the wild type. As mentioned earlier, the kidneys of the MMP-9 *-/-* mice are normal (Andrews *et al.* 2000). Finally, the deficiency of MMP-9 is also shown *in vivo* to cause defects in smooth muscle cell migration as MMP-9 together with CD44 might function to connect the cell surface to the underlying matrix (Johnson & Galis 2004).

While several studies use either the overexpression or knock-out of the entire MMP-2 or MMP-9 gene, only a few report the use of transgenic mice as a model to study the promoter region of those MMPs. A rabbit MMP-9 promoter region with a length of 541 bp is reported to induce the expression of the *lac-Z*-reporter gene *in vivo* as an endogenous promoter in one mouse line and later this mouse line was used in tumour invasion studies (Mohan *et al.* 1998, Kupferman *et al.* 2000). In tumour studies MMP-9 expression was inducible in this one transgenic mouse line in invasive forms of carcinomas but not in the alveolar macrophages (Kupferman *et al.* 2000).

3 Outlines of the present study

MMP-2 and MMP-9, also known as gelatinases or type IV collagenases, are homologous proteins that degrade gelatin, type IV collagen and several other ECM proteins. Both enzymes have an important role in ECM turnover during embryo- and morphogenesis, and they are also involved in pathological processes, such as cancer invasion, inflammation, wound healing and atherosclerosis. Although these enzymes have similar substrate specificities, their expression *in vivo* differs extensively. However, the regulatory mechanisms of their cell and tissue specific gene expression are unknown. The overall goals of this thesis project were to analyse tissue specific regulation of the expression of the MMP-2 and MMP-9 genes, and whether such regulatory elements can be used to modify tissue turnover when TIMP-1 is overexpressed in cells normally expressing MMP-9. The specific goals were:

1. To define the upstream regulatory region(s) required for the tissue specific expression of MMP-2 and MMP-9 by using reporter gene constructs in transgenic mice.
2. To identify the upstream region of the MMP-9 gene containing regulatory elements driving expression of the protein in the bone osteoclasts and migrating keratinocytes.
3. To use the upstream regulatory region of the MMP-9 gene to overexpress TIMP-1 in cells normally expressing MMP-9 and study the effects of TIMP-1 overexpression on wound healing and bone ossification in transgenic mice.

4 Materials and methods

4.1 Isolation and characterization of the mouse MMP-9 Gene (I), sequencing of the DNA (I) (III) and primer extension assay (I)

Mouse genomic libraries cloned in the cosmid pWE15 (Stratagene, 95303) and λ Fix phage (Stratagene, 946309) were screened by using a human MMP-9 cDNA probe (pHG1)(Huhtala *et al.* 1991) labelled with ^{32}P by random priming (I). The clones were isolated and purified utilising standard procedures and mapped using restriction endonucleases. The nucleotide sequence was determined by the dideoxynucleotide chain termination procedure (Sanger *et al.* 1977) using Sequenase or TAQuence DNA sequencing kits (United States Biochemical Corp.) and M13 universal primers or specific oligonucleotide primers. Both strands of the gene and its promoter were sequenced following subcloning into pBluescript II SK +/- (Stratagene).

For the sequence of the hMMP-2 upstream region (III), M13 universal primers and specific oligonucleotide primers were also used after subcloning into an M13-plasmid.

For the primer extension assay, total RNA from a 7-day-old mouse skull was isolated by the acid guanidium thiocyanate phenol chloroform extraction method (Chomczynski & Sacchi 1987)(I). Primer extension was performed by hybridising 20 μg of total RNA with an anti-sense nucleotide primer annealing at positions 117-144 in the cDNA (Reponen *et al.* 1994). The primer was end-labelled with $[\gamma\text{-}^{32}\text{P}]$ ATP using T4 polynucleotide kinase (Ausubel 1989). The reverse transcription reaction was carried out under standard conditions and the primer-extended products were analysed on a sequencing gel along with sequencing reactions from the mouse MMP-9 gene using the same oligonucleotide as in the primer extension assay.

4.2 Plasmid constructs (I, II, III)

Promoter-*lacZ* reporter gene constructs (I,II,III) were prepared by using the pKK2480 vector (provided by Mikkel Rohde, University of Copenhagen, Denmark). The plasmid contained a multiple cloning site immediately upstream of the *lacZ* gene. Different length fragments of the 5'-flanking region, as well as the 5'-end of the MMP-9 gene containing the first exon and intron were inserted for the construction of mMMP-9 promoter-*lacZ* fusion genes (I). For the TIMP-1 overexpression studies the construct was made by replacing the *lacZ* gene with the human TIMP-1 cDNA in the 7700ExIn-construct (II).

For the MMP-2 regulation studies (III), the length of the hMMP-2 upstream region was 6.5 kb from the 5'-end of the gene. The figure 6 summarises the constructs that were injected to produce transgenic mice for these studies.

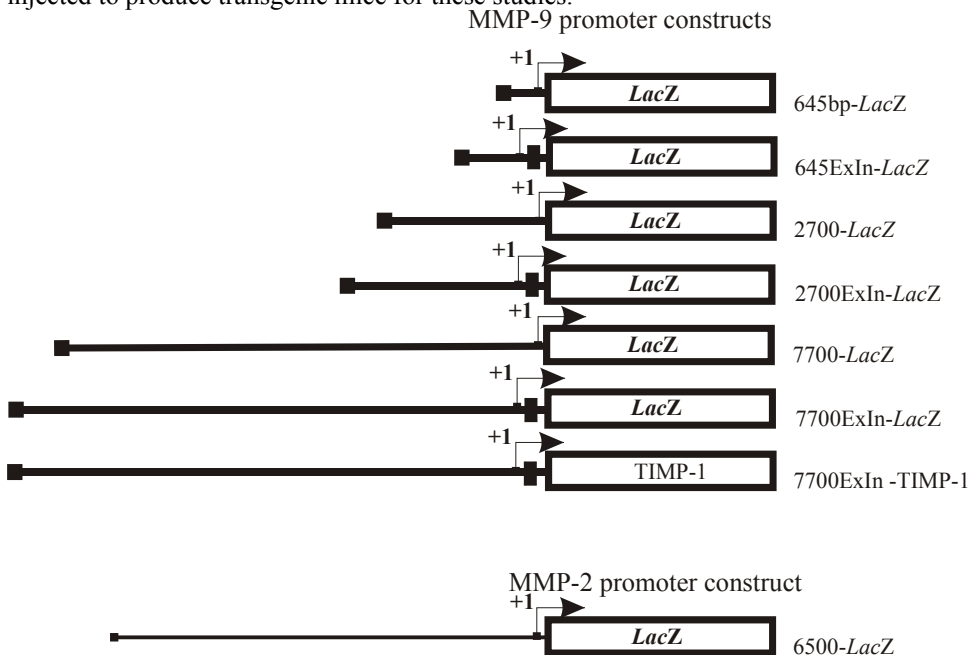


Fig. 6. Schematic illustration of the constructs used for the studies of MMP-2 and MMP-9 regulation and overexpression of their tissue specific inhibitors. The numbers after the constructs stand for the length of the promoter/regulatory region fragment, boxes are the transcribed genes and the arrows with +1 mark the transcription start site. Small black boxes indicate the first exon and first intron of the MMP-9 gene. The mMMP-9 promoter constructs with the first exon and first intron have the ATG codon mutated to ATC so that the ATG codon of *lacZ* gene is the translation start site.

4.3 Generation and genotypic analysis of transgenic mice (I, II, III)

Transgenic mice were generated by pronuclear microinjection of linearized fusion gene constructs (I, II, III) into fertilised mouse oocytes C57BL/6 x DBA/2 F1 (Hogan 1986). Microinjected eggs (15-20) were transferred into the oviduct of a recipient, pseudopregnant female of either the CD1 (II, III) or NMRI (I, III) strain, and the mice were allowed to develop to term. Three weeks after the pups were born, their tail DNA was isolated (Drews *et al.* 1994) and the transgene was detected by PCR analysis. Two internal primers were used for the *lacZ* gene (Hanley & Merlie 1991) (I,III) or hTIMP-1 (II). The primers for hTIMP-1 were TIMP1-370 (5'-CACAAACCGCAGCGAGGAGTTT-3') and TIMP1-732rev (5'-CACTGTGCAGGCTTCAGCTTC-3'). The annealing temperature for the reactions was 60°C and the product was 362 bp long.

4.4 Wound tests (I, II)

All experiments involving mice were approved by the Animal Use and Care Committee at the University of Oulu before commencing the studies. The mice for the wound tests were anesthetized prior to wounding. Incision wounds of 1 cm were made into the dorsal skin of the *lacZ*-expressing mice and sutured with a pair of stitches to bring the wound edges together (I). For pain treatment, the mice were injected twice subcutaneously with 1.5 µg of buprenorphine hydrochloride at 12 h intervals following the anesthesia (I, II).

Punch wounds (II) were also made during the anesthesia, but no stitches were used and the area of the wound site was shaved before wounding. A punch wound of 3 mm of diameter was made into the dorsal skin of both transgenic and control mice. The wounds were allowed to recover for one to 14 days, after which the wound outlines were traced on object glasses. The outlines were then scanned, and the areas calculated by using analySIST™ software (Soft Imaging System GmbH). The wound tissue was collected with a 6 mm punch after the animal was sacrificed by carbon dioxide inhalation. For histology, immunohistochemistry and *in situ* hybridisation studies, a total amount of 20 transgenic wound samples and 8 control wound samples of non-transgenic littermates were analysed. In addition, 24 punch biopsies of non-wounded transgenic mouse skin and 7 control skin samples of non-transgenic mice were studied.

4.5 Histological and immunohistochemical analyses (I, II, III)

Tissues taken from transgenic mice were fixed for 2 h or overnight at 4 °C in 4 % paraformaldehyde - 0.2 % glutaraldehyde in PBS. Wound samples from *lacZ*-positive mice were stained with X-gal (5-bromo-4-chloro-3-indolyl-β-galactopyronoside) as described previously (Behringer *et al.* 1993). All embryos and other tissues were rinsed several times in PBS, dehydrated and embedded in paraffin. Sections of 5-8 µm were stained either by hematoxylin and eosin (Bancroft 1990) (I, III), hematoxylin (Zymed

laboratories Inc., San Francisco, CA) (II) or with safranin (I). Mounting was done by Mountex (Histolab products Ab, Sweden)

Immunohistochemical staining of paraffin sections (5-10 μm) from skin wounds expressing *lacZ* (I) (II) was carried out by using either the ABCComplex HRP kit (DAKO) or the TSA kit (NEN Life Science). Deparaffinized sections were treated 5 min. with 0.4 % pepsin in 0.01 M HCl at 37 °C. After blockage of endogenous peroxidase activity the sections were incubated with the antiserum raised against cytokeratin (rabbit pan-cytokeratin, ZYMED) for 1.5 h at RT, or MMP-9 (rabbit polyclonal antibody kindly provided by P. Carmeliet) (I) overnight at RT. For the anti-cytokeratin immunostaining, biotinylated swine anti-rabbit IgG (Boehringer-Mannheim) (1:400 dilution) was applied for 30 min at RT. Peroxidase activity was revealed by incubation with the chromogen substrate DAB (3,3-diaminobenzidine tetrahydrochloride) or AEC (Aminoethyl carbazole) chromogen substrate (DAKO). For the MMP-9 immunostaining, peroxidase swine anti-rabbit IgG (DAKO) was applied and followed by tyramide signal amplification (TSA kit, NEN Life Science). The AEC chromogen substrate was used to detect peroxidase activity.

The anti-laminin $\gamma 2$ -chain staining (II) was performed on paraffin sections (5-10 μm) of either wounded or non-wounded transgenic mouse skin expressing hTIMP-1. Deparaffinized sections were boiled 4 minutes in 10 mM citrate buffer (pH 6.0) in a microwave oven for the retrieval of the antigens. After the endogenous peroxidase activity and nonspecific binding of the primary antibody were blocked, the sections were incubated overnight at 4 °C with rabbit antisera raised against the laminin $\gamma 2$ -chain. Biotinylated swine anti-rabbit IgG (DAKO A/S, Denmark) (1:300 dilution) was applied for 30 min at RT. DAB was used as a chromogen substrate.

Immunostaining with the cell proliferation marker Ki-67 (II) was performed on 6 μm cryosections fixed with ethanol. The sections were incubated 1 h at RT with the 1:50 diluted rat polyclonal antibody against mouse Ki-67 (DAKO) followed by the secondary antibody, rabbit anti-rat (DAKO), with 1:500 dilution (30 min at RT).

4.6 RNA-purification and RT-PCR (II)(III)

The tissue samples from transgenic mice were homogenised in a Ultra-turrax -type of homogeniser preceding the RNA-purification by Trizol (Gibco™, Invitrogen Corporation, Carlsbad, CA) (II, III). Additionally, bone samples of adult mice were frozen in liquid nitrogen and pulverized before Trizol was added. Prior to the RT-PCR (II) the RNA-samples were treated with DNaseI (Invitrogen) to prevent false positive signals caused by traces of DNA remaining after the purification process. An RNase inhibitor (Invitrogen) was used to prevent degradation of RNA. The RT-PCR for hTIMP-1 RNA detection was performed by using QIAGEN® OneStep RT-PCR Kit according to manufacturer's instructions. The primers used for the PCR were TIMP1-139 (5'-CCACAGACGGCCTTCTGCAA-3') and TIMP1-482REV (5'-ACAGTGTAGGTCTTGGTGAA-3'); the annealing temperature was 60°C

4.7 *In Situ* hybridization (I) (II)

The RNA probe (I) for the mMMP-9 gene was prepared from the cDNA-fragment cut by SmaI and EcoRI as described elsewhere (Reponen *et al.* 1994). The radioactively labelled RNA probes were transcribed by a transcription kit (Promega) and the radioactive *in situ* hybridisation for the mouse embryos was carried out according to Wilkinson and Green (1990). The mouse embryos were stained with X-gal, embedded in paraffin, and sectioned before the hybridisation. The same mMMP-9 cDNA fragment was used also for preparing the probe for the skin sections of hTIMP-1 expressing mice (II). The expression of the TIMP-1 transgene in mouse skin sections was detected by using the BamHI-HindIII –restriction fragment of human TIMP-1 as a probe (Hurskainen *et al.* 1996). The length of the fragment was 626 bp. *In situ* hybridization was carried out as described earlier (Parikka *et al.* 2001). The antisense or sense probe was diluted to 500 ng/ml and hybridised on the sections overnight at 58°C.

4.8 *In situ* zymography (II)

In situ zymography (ISZ) was performed as previously described (Pirilä *et al.* 2001). Briefly, 10 mm thick serial frozen sections of cutaneous wounds from the skin of control and transgenic mice were thawed at room temperature and preincubated for 30 minutes at 37 °C with either (ISZ)-buffer (50 mM Tris-HCl, pH 7.4; 1 mM CaCl₂), 500 mM CTT-peptide in ISZ-buffer or 500 mM control peptide in ISZ-buffer. Following the preincubation, the solutions were changed to a 1:1 mixture of 1 mg/ml Oregon Green 488-conjugated gelatin (Molecular Probes, Inc., Eugene, OR) and 1% low melting temperature agarose (Sigma, St. Louis, MO) with or without 500 mM CTT-peptide or 500 mM control peptide. After the mixture was congealed under the coverslip (1 hour at RT) samples were incubated 7 hours at 37 °C in a dark, humidified chamber. Gelatinolytic activity was seen as dark areas in the otherwise fluorescing substrate layer.

5 Results

5.1 Structure of the 5' flanking region of mMMP-9, generation of transgenic mice with the reporter gene constructs and the embryonic expression of the transgene (I)

The structure of the murine MMP-9-gene was characterised and transcription was designed to start from the double start site -19 and -20 bp upstream of the translation initiation site. Several common promoter elements, including a TATA box like motif, GC boxes possibly binding Sp1, AP-1-like binding sites, one AP-2 site and several conserved sequences reminiscent of the polyoma virus enhancer A binding protein-3 (PEA3) were found within the 2800 bp flanking the 5' region. In addition, several microsatellite segments of alternating CA residues and one NF kappaB motif were also present. During the course of this work characterization of the MMP-9 gene from mouse (Masure *et al.* 1993) and rabbit (Fini *et al.* 1994) were published.

In order to study the regulation of the mouse MMP-9 gene, transgenic mice were generated by microinjection of six different promoter-reporter gene constructs into fertilised oocytes. The gene encoding β -galactosidase was used as a reporter gene. Intron 1 was included in some of the constructs as it has been shown to contain enhancer elements in other genes such as the gene for the $\alpha 1$ chain of type I collagen (Rossi & de Crombrughe 1987). Three to eight mouse lines were generated with each construct to ensure that the expression pattern obtained with each construct was reproducible. PCR and Southern analyses were carried out to establish integration of the inserts into the genome and histochemical analyses with X-gal revealed the cell specific expression patterns of the transgene.

The introduction of constructs containing the minimum promoter, 645-*lacZ*, and a longer one, 2700-*lacZ*, containing 2.7 kb of the upstream region, did not result in expression of the *lacZ* gene in transgenic mice or mouse embryos in cells that normally are found to express MMP-9. Ectopic expression was observed in some lines with both constructs, but its pattern was neither uniform nor reproducible in four founder lines.

Addition of the 5' end of the MMP-9 gene, including intron 1, to constructs 645-*lacZ* and 2700-*lacZ*, i.e. the 5' UTR, and first exon and intron, did not alter the expression pattern. Consequently, it could be concluded that the first intron does not contain cis-acting elements conferring tissue-specific expression on the endogenous MMP-9 gene.

In contrast to the shorter constructs, mouse embryos harbouring 7700-*lacZ* revealed expression of the *lacZ* gene in bones of 14.5 - 16.5 day old embryos. For example, at E-15.5 distinct expression could be observed in the scapule, long bones of fore and hind limbs, ribs and the lower jaw. Additionally, expression was observed in hair follicles in several mouse lines made with this construct. Construct 7700ExIn-*lacZ*, also containing the first exon and intron, yielded an expression pattern similar to that of 7700-*lacZ* when analysed in whole X-gal stained embryos, except that no expression was present in hair follicles.

5.2 The -2722 to - 7745 upstream region of the MMP-9 gene confers expression to osteoclasts (I)

Only transgenic mice with constructs containing 7.7 kb of the 5' flanking region of the MMP-9 gene yielded expression of the *lacZ* gene in bones. In order to assess the expression pattern at the cellular level, microscopic histochemical analysis was carried out, partially combined with *in situ* hybridization to establish whether the *lacZ* expression corresponded to that of the endogenous gene. Staining with X-gal showed expression of the transgene in single cells located at the site of endochondral ossification in the diaphysis of embryonic long bones, beneath hypertrophic chondrocytes of the epiphysis. This result was identical to that previously shown for the endogenous gene by *in situ* hybridization (Reponen *et al.* 1994). In that report the endogenous gene expression was colocalized specifically to cells that were shown to be osteoclasts by histochemical staining with tartrate resistant acid phosphatase (TRAP). In this study, in order to demonstrate that the cells expressing 7700-*lacZ* were indeed osteoclasts, *in situ* hybridization of X-gal stained tissues was carried out and these experiments showed the signals to be present in cells positive for the blue colour produced by β -galactosidase. These experiments demonstrated that expression of the 7700-*lacZ* construct was confined to osteoclasts in developing bone. Transgenic mice harbouring the insert 7700ExIn-*lacZ* showed exactly the same expression pattern as construct 7700-*lacZ*, demonstrating that the upstream segment -2722 to -7745 includes the *cis*-regulatory element(s) required for osteoclast expression.

5.3 Localization of 7700-*lacZ* expression to migrating keratinocytes (I)

To determine whether any of the MMP-9 promoter-*lacZ* gene constructs were expressed in epithelial cells, tissues from the transgenic mice were analysed for expression of β -galactosidase. In general, we did not observe expression of the transgene in the epithelia of organs such as skin, lung, or gastro-intestinal tract with any of the six constructs made in this study.

Keratinocytes of healing incisional skin wounds were also examined for expression of the constructs. When pieces of whole recovering wounds were stained with X-gal one to seven days after wounding, expression of *lacZ* could be followed in mice harbouring constructs containing 7.7 kb of the 5' flanking region of the MMP-9 gene. When the surface of a wound was stained with X-gal, one could macroscopically observe cells expressing β -galactosidase at the wound edges. Histologically, a strong positive reaction was detected in keratinocytes migrating in under the fibrin clot. Double staining of sections with X-gal and anti-MMP-9 antibodies demonstrated that most migrating keratinocytes expressing X-gal also costained with the MMP-9 antibody. Furthermore, scattered cells beneath the wound contained the protein and some of them also expressed the *lacZ* reporter gene. The fact that all cells and their immediate surroundings stained with the MMP-9 antibody, but not X-gal, can be due to secretion of the MMP-9 enzyme.

Identification of β -galactosidase expressing cells as keratinocytes was carried out by counterstaining with cytokeratin antibodies. Keratinocytes resting on the normal basement membrane adjacent to the wound did not show any staining reaction. At day 7 the reepithelialization process was complete. The new epidermis was thicker and the presence of fibrotic tissue was apparent, but expression of β -galactosidase by keratinocytes had ceased. In all founder lines, cells at wound edges expressed β -galactosidase.

5.4 Phenotype, histology and hTIMP-1 expression in the bone and skin of the transgenic mice containing a MMP-9 promoter/hTIMP-1 construct (II)

To explore the overexpression of TIMP-1 in MMP-9 expressing cells and its influences on wound healing, transgenic mice containing the construct 7700ExIn-hTIMP1 were generated. The mice expressed hTIMP-1 in cells that normally express MMP-9. Integration of the transgene was confirmed by PCR (not shown), and expression of the transgene was verified by RT-PCR and *in situ*-hybridization. To examine for expression of hTIMP-1 under the MMP-9 promoter, RNA from adult and newborn transgenic mouse bone samples was isolated and RT-PCR was performed with hTIMP-1 primers. By this method the expression of the transgene in the bone was demonstrated. The expression was more prominent in newborn and young adult mice, and had diminished significantly

at the age of two years. The transgenic mice appeared normal at birth, their postnatal growth rate was normal, and there were no apparent defects in their long bones or skin. However, during the aging of second generation mice, they started to present with manifestations in their skin. By age 6-7 months, mice not living alone in their cage developed crust in the skin of their back, neck and head, poorly healing scratches and ragged ears. In particular, aggressive and fighting males were more prone to this phenotype than females that were slower to develop the same problems. Mice living alone in their cage did not develop such problems.

Histology and expression of the hTIMP-1 transgene was studied in 6 mm punch skin biopsies from control and transgenic mice carrying the MMP-9-promoter/hTIMP-1 – construct. Histological examination revealed that the skin of transgenic mice was clearly different from that in control mice, with regional epithelium thickenings, several cyst-like structures and generally damaged areas that did not properly recover. In the control mice no such thickenings or cysts were observed. To verify expression of hTIMP-1 in transgenic mice, *in situ* hybridisation analyses of skin biopsies were carried out. It was possible to distinguish between the endogenous and transgenic TIMP-1 expression by using a human TIMP-1 –specific probe that only detects expression of the transgene (the sequence identity of human and mouse TIMP-1 sequences is 78.4%). The biopsies were taken from dorsal skin. Normal, non-transgenic littermates were used as a control. A strong signal was detected in the epithelial cells and in the cells under the tissue with an erosive appearance.

5.5 Delayed wound healing in mice expressing hTIMP-1 in migrating keratinocytes (II)

To explore the effects of hTIMP-1 overexpression on wound healing, the healing process was studied by making punch wounds 3 mm in diameter into the dorsal skin of control and transgenic mice. The wounds were allowed to recover from 1 to 14 days before they were biopsied for analysis. After 7 days of healing there was a remarkable difference in the healing of the wounds in transgenic mice compared to those in control mice. By 7 days post-wounding, the re-epithelialization was nearly complete in the control mice, whereas in transgenic mice the wound area had diminished only slightly. The thickened epithelial tips had remained almost static at the 7-day-old wound margins and had not migrated to cover the wound bed, as in the control wounds. Even after a two week period of healing, the keratinocytes were not covering the entire wound area. When the 7 days old punch wounds were scanned and the area was measured, a significant retardation in the healing process was revealed in the transgenic mice. Immunostaining of wounds with the Ki-67 proliferation marker did not reveal increased cellular proliferation in the wounds of transgenic mice, indicating that the thickening may be due to accumulation of cells caused by retarded migration.

In situ hybridization studies of the biopsies with the hTIMP-1 specific probe showed strong expression of hTIMP-1 in the wound area of the transgenic mice. The cells positive for hTIMP-1 were positioned in the area of migrating keratinocytes. Expression

of hTIMP-1 was detectable from the first day of wound recovery, and it could be observed 2 weeks after the wound was made, colocalising with the endogenous expression of MMP-9 at the migrating epithelial tip. Pan-cytokeratin antibody was used to mark all keratinocytes at the wound area and anti-Laminin $\gamma 2$ chain staining was a marker for the migrating keratinocytes and regenerating BM. Only a few anti-cytokeratin positive cells were found in the central area of the 5-day-old transgenic wound, colocalising with hTIMP-1 expression and anti-Laminin $\gamma 2$ chain staining. The $\gamma 2$ antibody staining was weaker with less positive migrating epithelial cells in the transgenic sample, and it was not continuous as in the control.

5.6 *In situ* gelatin zymography of the wounds of MMP-9 promoter/hTIMP-1 transgenic mice (II)

In situ gelatin zymography is a method used for localization of gelatinolytic proteinases in the tissue (Galis *et al.* 1995). This method was combined with the use of MMP-2 and -9 -specific inhibitor, the synthetic CTTHWGFTLC (CTT)-peptide (Koivunen *et al.* 1999). In cutaneous wounds from control mice, intense gelatinolytic activity was detected at the epithelial edge of the healing wound. Most of the gelatinolytic activity was abolished when incubated with the CTT-peptide. However, slight activity could be detected around the hair follicles, indicating that some enzyme(s) other than MMP-2 or MMP-9 is also active in this area. In cutaneous wounds of transgenic mice, no gelatinolytic activity could be detected, indicating that MMP-9 is the major gelatinase active during wound healing. Incubation with a control peptide did not affect the gelatinolytic activity of control mouse.

5.7 Localisation of mMMP-2/*lacZ*-transgene expression in mesenchymal cells during embryonic development (III)

The MMP-2 promoter/reporter gene construct for generating transgenic mice contained a 6.5 kb fragment from the upstream region of the hMMP-2 gene. Six mouse lines were found to carry the transgene and express the β -galactosidase during embryonic development. All the lines had a very similar expression pattern, differing mainly with regard to intensity of the staining. Strong *lacZ* expression was observed especially in mesenchymal cells of developing limbs, but also in developing mammary glands, head and lower mandibular area, as well as in different areas of the embryonic body. Histological examination of the transgenic embryos at different developmental stages confirmed that mesenchymal cells continued the expression throughout development. X-Gal -staining in the limbs of the *lacZ*-positive embryos was specific for the stromal cells, while the epithelial cells on the surface of the embryo were negative. Also the mesenchymal cells surrounding the epithelium of the developing mammary gland expressed *lacZ*.

During tooth development, mesenchymal cells beneath the ectoderm or surrounding the epithelium of the developing tooth were found to express *lacZ*. Also, the mesenchyme of the developing lower jaw expressed *lacZ* widely at different time points, but by varying intensity. At early stages of tooth development (E11,5), the expression of the transgene in the mandibular area was weaker in some individuals or not detected except in the mesenchyme underlying the dental lamina. Already in the bud stage (E12,5) the stain was visible in all the lines studied. At developmental stages E13-14, the stain was more intense and extended to the more distant areas of the upper and lower jaws. The epithelial cells of the developing teeth were negative in all the lines studied.

5.8 Analysis of the 6.5 kb 5'-flanking sequence of mMMP2 (III)

The 6500 base pair upstream region of the human MMP-2 gene appeared to contain regulatory elements necessary for driving *lacZ* expression in a manner similar to the endogenous expression. Therefore, the entire region was sequenced and compared with the corresponding sequence in mice and rats. Sequence comparison between human, mouse and rat revealed three regions with high homology between the species. Homologous region I spanned from position -1749 to -1118 base pairs upstream from the transcription start site. This region is 64.4 % homologous between human, mouse and rat. Immediately downstream from that is another, 697 bp long region referred to as homologous region II that is about 50% homologous between mouse and human. This region has 69% homology in the regions containing the previously reported conserved consensus sites for p53 and AP-2 sites, or r2 (Frisch & Morisaki 1990, Somasundaram *et al.* 1996, Bian & Sun 1997). These elements were found to promote transcription in cell transfection studies. The third homologous region III, located in the proximal promoter region, contains consensus sequences for transcription factors AP-2 and Sp1. The homology in this 204 bp region was approximately 71% between mouse, human and rat. In addition to these previously described regions located within the first 2000 basepairs upstream of the transcription start site, we identified additional more distal sequences that are potential binding sites for transcription factors as determined by computer analysis. Two possible NF- κ B -binding sites, were localised at positions -1811 and -1188. Furthermore, two AP-1 consensus binding sites were detected at -3949 and -5482, and one CREB-motif at position -4911 in the upstream sequence.

6 Discussion

Some of the members of the large family of MMPs exhibit highly restricted temporal and spatial expression patterns *in vitro*, indicating tissue-specific roles for these enzymes in extracellular matrix turnover. The expression of MMP-2 is known to be mesenchymal and MMP-9 found mainly in osteoclasts during development, yet not much information is available regarding the regulatory mechanisms driving these cell lineage specific expression patterns *in vivo*. Furthermore, tissue remodelling during wound healing requires a delicate balance of synthesis and degradation of extracellular matrix molecules. In these processes, the specific role of MMP-9 and TIMP-1 is not well characterised and the consequences of misregulation are still poorly known.

6.1 The *in vivo* expression of *lacZ* and hTIMP-1 under the 5' upstream fragments of the murine MMP-9 gene during embryonic development and in adult mouse bone (I) (II)

At the cellular level both the 7700-*lacZ* and 7700ExIn-*lacZ* constructs were shown to yield highly specific expression in osteoclasts of developing bone, cells that normally strongly express MMP-9 (Reponen *et al.* 1994). Coexpression of β -galactosidase with that of the endogenous gene was verified by *in situ* hybridisation analysis of the same tissue sections with an MMP-9 probe. The experiments with different promoter/*lacZ* reporter gene constructs in transgenic mice showed that expression of the MMP-9 gene in osteoclasts requires the region between -2722 and -7745. Constructs containing this segment yielded strong expression in osteoclasts. It has previously been shown that MMP-9 is expressed in invading trophoblasts of the implanting embryo (33,34), as well as by macrophages infiltrating invasive breast and colon cancers, while the actual cancer cells do not express the enzyme (Tryggvason *et al.* 1993, Nielsen *et al.* 1996). When studied in these transgenic mice, it was noticed that trophoblasts of implanting embryos of mice transgenic for constructs 7700-*lacZ* and 7700ExIn-*lacZ* express the reporter gene, as do macrophages located around invading exogenous carcinoma cells. This suggests that the -2795 to -7745 segment also contains element(s) necessary for induction of

expression of the gene in both trophoblasts and macrophages. These results are especially interesting when compared with a study by Kupferman and co-authors (2000) where MMP-9 promoter/*lacZ*-transgenic mouse line was crossbred with transgenic mice that have a high incidence of mammary tumours. The length of the MMP-9 promoter used in that study was 541 bp, and it was very likely too short to mimic the full scale endogenous expression, as evidenced by the lack of expression, for example, in alveolar macrophages, although expression during embryonic bone ossification and wound healing was reported for this one mouse line (Mohan *et al.* 1998).

The effects of *in vivo* overexpression of TIMP-1 in cells normally expressing MMP-9 were studied by generating transgenic mice expressing human TIMP-1 under the murine MMP-9 promoter and enhancers. The results demonstrated that transgenic mice having the same expression patterns for human TIMP-1 and mouse MMP-9 are viable, fertile, and exhibit normal growth and general development, including that of the bones. It was unexpected to observe no defects at all with regard to bone development in the MMP-9/TIMP-1 transgenic mice. During normal ossification of cartilage and remodelling and growth of bone, osteoclasts express MMP-9 intensively (Reponen *et al.* 1994), but also other proteinases such as cathepsin K (Inaoka *et al.* 1995) and MT1-MMP (Okada *et al.* 1997). Studies with MMP-9 deficient mice have revealed that MMP-9 alone is not crucial for bone growth, as the only bone defects observed in those mice were rather minor abnormalities in the growth of metatarsals, tibia and femur (Vu *et al.* 1998), but it has been postulated that the MMP-9 function normally exerted by osteoclasts can partially be replaced in such mice by other MMPs such as collagenase-3 (MMP-13), stromelysin-1 (MMP-3) and gelatinase A (MMP-2). However, the present results indicate that other osteoclast MMPs may not be so important either, since overexpression of TIMP-1 that inhibits MMP activity quite broadly does not seem to have much effect on bone development. Therefore, it is likely that other types of proteinases, such as cathepsins (Ohsawa *et al.* 1993, Inaoka *et al.* 1995, Rantakokko *et al.* 1996) and the plasminogen activator/plasmin system (Daci *et al.* 1999) can, at least partially, take over the role of osteoclast MMPs.

6.2 The *in vivo* expression of *lacZ* and hTIMP-1 under the 5' upstream fragments of the murine MMP-9 gene in mouse skin and during cutaneous wound healing (I) (II)

Expression of MMP-9 has been shown to occur in the migrating keratinocytes of healing human wounds (Salo *et al.* 1994b). In this study both constructs, 7700-*lacZ* and 7700ExIn-*lacZ*, were expressed in the corresponding keratinocytes of transgenic mice, and an extensive codistribution of the MMP-9 protein and *lacZ* expression was shown by double staining. Furthermore, as in the *in vivo* situation for the endogenous MMP-9 gene, keratinocytes resting on a normal mature basement membrane did not express the reporter gene in transgenic mice or contain the MMP-9 protein, as determined by immunohistochemical staining. Following complete healing and reepithelialization of the skin wound at day 7, expression of the reporter gene ceased, essentially as has been

shown for the endogenous gene (Salo *et al.* 1994b). As verified with osteoclast specific expression of the promoter/*lacZ* reporter gene constructs in transgenic mice, the MMP-9 gene expression in the migrating keratinocytes requires the region between -2722 and -7745. Constructs containing this segment yielded strong expression in migrating keratinocytes of a healing wound. Therefore, cis-regulatory element(s) for this expression pattern must also be present in the same sequence. The results also demonstrated that the first intron does not contain an enhancer, as it does in several extracellular matrix genes (Rossi & de Crombrughe 1987, Metsäranta *et al.* 1995). However, this intron may be important for restricting ectopic expression, as the 7700-*lacZ* construct yielded ectopic expression in epithelial hair follicle cells of the developing embryo, while mice expressing construct 7700ExIn-*lacZ* only exhibited expression in cells normally expressing MMP-9.

When MMP-9/TIMP-1 expressing mice were examined under normal unchallenging circumstances, such as when kept alone in a cage, the transgenic mice did not develop any detectable defects in their skin. *In situ* hybridization did not reveal any expression of the transgene in normally developed skin. However, upon skin damage the wound healing of the transgenic mice was retarded. Thus, when living two or more together in a cage, most transgenic mice gradually started to exhibit visible skin alterations in the form of crust, poorly healing wounds and ragged ears. This was presumably provoked by internal “fighting” of the animals, especially by more aggressive males. Histological analysis of the skin revealed poorly healing wounds and expression of TIMP-1 in epithelial cells. The keratinocytes that migrate over the wound during healing and that normally express MMP-9 were affected by the TIMP-1 overexpression.

These results as combined with previous studies have demonstrated a central role for keratinocyte-derived MMP-9 in the remodeling of extracellular matrix during wound healing (Salo *et al.* 1994a, Madlener *et al.* 1998, Agren 1999). When applying GM6001, a synthetic broad-spectrum inhibitor of MMPs, topically to human wounds, it has been found to prevent epidermal regeneration (Agren *et al.* 2001), leading to delayed wound healing. The epidermic cell proliferation was not affected by a broad MMP inhibitor, GM6001. In this study, observation of the epithelium proximal to the wounded site revealed multiple layers of epithelial cells, while the migratory tip of the epithelium did not move actively towards the wound bed. It appeared that the cells were capable of proliferating, but not moving properly. However, overexpression of TIMP-1 did not totally prevent the migration of keratinocytes and wound healing. A reasonable explanation is that in addition to MMP-9, inhibition of other MMPs secreted in acute mouse wound tissue during the healing process, MMP-2, -3, -10 and -13 (Madlener 1998), also contributed to the decreased cellular migration. In human keratinocytes, the activity of MMP-1 (corresponding to mouse MMP-13) is necessary for migration *in vitro* (Pilcher *et al.* 1997). Furthermore, when collagenase-resistant mutant mice were studied during a 70 d period of wound healing, they had more than a one week delay in early wound healing due to the inability of MMP-13 to degrade collagen I (Beare *et al.* 2003). The role of the recently discovered mouse MMP-1 (Balbin *et al.* 2001) in keratinocyte migration is not yet known. Neither is any *in vivo* data available supporting the *in vitro* model published by Mäkelä and colleagues (1999) for the participation of MMP-2 in keratinocyte migration. TIMP-1 is normally not found in the microenvironment of the

migrating keratinocytes during wound healing but it is more abundant at the border between proliferative keratinocytes and underlying granulation tissue (Madlener 1998).

A previous study by Mignatti and Rifkin (1996) suggested that two types of proteolytic systems, MMPs and plasminogen/plasmin primarily control the extracellular proteolysis used for keratinocyte migration during wound healing. Lund with colleagues (1999) have found that mouse wound healing is retarded, but not prevented by a broad MMP inhibitor, GM6001. When the same inhibitor was used on transgenic mice with a deficiency for plasminogen, wound healing was totally blocked and keratinocyte coverage prevented during a 100-day follow up period. Clearly these two groups of enzymes are the crucial proteinases in wound healing.

Conflicting results were obtained from MMP-9 null mice with corneal wounds (Mohan *et al.* 2002). The mice showed increased epithelial cellular proliferation in the wounds and accelerated healing. One reason for these conflicting results may be the wound model chosen.

The results of the *in situ* gelatin zymography demonstrated the presence of intense gelatinolytic activity in migrating keratinocytes at the wound edge in wild type animals. This confirmed the results obtained from *in situ* hybridization of wound tissues. The gelatin *in situ* zymography in combination with the MMP-2 and MMP-9 -specific synthetic inhibitor (CTT-peptide) on samples from mice overexpressing TIMP-1 showed a total blockage of gelatinolytic activity. As MMP-2 has also been proposed to be involved in keratinocyte-related proteolysis (Mäkela *et al.* 1999), but no gelatinolytic activity could be detected in the wounds of mice overexpressing TIMP-1, this shows that MMP-2 did not compensate for MMP-9 activity. The gelatinolytic activity was not compensated by any other proteinases either, but as the wounds were finally able to close, it can be concluded again, that other MMPs also participate the non-gelatinolytic degradation required for the migration of the keratinocytes.

6.3 The *in vivo* expression of mMMP-2/*lacZ* during embryogenesis and analysis of the 5'-sequence of hMMP-2

The studies with MMP-9 promoter constructs supported the strategy of starting with longer constructs to mimic the endogenous expression, therefore the length of the hMMP-2 5'-flanking region for the transgene construct was 6500 basepairs. The well-studied human β -globin regulatory region has been shown to contain an enhancer as much as 30000 base pairs downstream from the last exon (Bulger & Groudine 1999), indicating that regulatory elements can be located far away from the coding region, or even within the gene.

The expression of the reporter gene was observed in stromal cells of loose mesenchyme surrounding epithelial cells of early developing breast and teeth. The *lacZ* expression mimicked completely the developmental expression pattern of the endogenous mouse gene as previously described (Reponen *et al.* 1992, Sahlberg *et al.* 1999). Therefore, the results strongly suggest that most, if not all the necessary *cis*-acting elements are located in the 6500 base pair upstream fragment of the human MMP-2 gene.

Vertebrate embryonic tooth development including the earliest stages of these ectodermal appendages is morphologically and molecularly very similar to such processes as hair and gland development. Most of the paracrine signal molecules regulating tooth morphogenesis, especially the interaction between the ectoderm and mesenchyme, belong to the conserved families of transforming growth factor β (TGF- β), fibroblast growth factor (FGF), Hedgehog and Wnt (Thesleff 2003). As the results from the hMMP-2/*lacZ* mice also evidenced, the role of MMP-2 is central in tooth morphogenesis and one can expect that its transcriptional regulation will also be linked to these families. The elements in the regulatory region of human MMP-2 for tooth morphogenesis undoubtedly form a complicated cascade and their analysis will require careful sequential *in vivo* analysis.

The sequence analysis of the 6500 base pair upstream region of the human *MMP-2* and its comparison with corresponding regions revealed several conserved consensus sequences for known transcription factors. Additionally, there were three upstream regions with high homologies between human, mouse and rat sequences. Two previously described conserved AP-2 and Sp-1 sites were located in the proximal promoter, whereas a second AP-2 site and a p53-binding site were found further upstream (positions -1685 and -1649, respectively) in a conserved region, inside the third homologous region. When the entire 6500 base pair region was analysed by computer, several more distal possible binding sites were recognized, but their function *in vivo* will need to be tested in future regulatory studies with new constructs. Thus, we found at least two possible NF- κ B binding segments, both situated in the areas of high homology between human, mouse, and rat. Interestingly, the osteopontin-stimulated nuclear factor (NF)- κ B has been found to mediate induction of MT1-MMP that activates pro-MMP-2 in a murine melanoma cell line (Philip *et al.* 2001). However, recently Vayalil and his colleagues found, that when the NF- κ B pathway was prevented in human prostate carcinoma DU145 cells, the expression of MMP-2 and MMP-9 was concomitantly inhibited. In addition to the consensus sequences found in this study, it is very likely that some other, yet unidentified sequences and *trans*-acting factors are involved in tissue specific regulation of the gene. The two distal upstream regions homologous between human, mouse and rat are good candidates for being areas containing such regulatory elements. Those regions would be also the prime sequences to use in constructs for generating transgenic mice with varying size upstream regions and the proximal promoter fused to a reporter gene.

7 Conclusions

Matrix metalloproteinases are a group of enzymes capable of degrading proteins of the extracellular matrix. However, only a very limited amount of information is available regarding the *in vivo* regulation of their cell lineage-specific expression. This study focused on two members of this group, the 72 kDa and 92 kDa type IV collagenases, MMP-2 and MMP-9, and on one of the tissue specific inhibitors of MMPs, TIMP-1. The regulation of MMP-2 and MMP-9 was studied in transgenic mice.

Constructs containing varying lengths of the upstream region of MMP-9 promoting a *lacZ* reporter gene were used to identify cell- and tissue specific regulatory elements. The expression of the gene constructs was studied in transgenic mice generated by pronuclear microinjections into fertilised mouse oocytes. When the transgenic mice were analysed during embryonic development and wound healing, it was found, that the sequences between -2722 and -7745 upstream from the transcription start site were responsible for the expression of the reporter gene in osteoclasts and migrating keratinocytes. Furthermore, the results of this study together with previous studies confirmed that MMP-9 is also expressed by those cells *in vivo*. This study represents a first step in the identification of the detailed mechanisms driving the cell specific expression of MMP-9. The ongoing work will focus on narrowing down the region and finally identifying the actual nucleotide sequences responsible for these activities.

When human TIMP-1 was expressed instead of *lacZ* under the 5'-flanking region of MMP-9 in transgenic mice, the animals were found to be viable and fertile with normal growth and general development. The expression of hTIMP-1 was induced during wound healing in migrating keratinocytes, in a pattern similar to that of *lacZ*, and the healing of cutaneous punch wounds was remarkably retarded with slow migration of keratinocytes at the wound edges of the transgenic mice. Total blockage of gelatinolytic activity was revealed when *in situ* zymography was carried out on wound tissues. When a MMP-9 specific synthetic inhibitor, CTT-peptide, was used on the control wound tissue for studying the gelatinolytic activity, MMP-9 was found to be the major gelatinase active during wound healing, and MMP-2 could not compensate for this activity. This study confirmed the important role of MMP-9 and other MMPs that are derived from keratinocytes during wound healing and inhibited by TIMP-1. Although the development of MMP-9/TIMP-1 transgenic mice was not affected, they can be useful tools in future

studies revealing the role of MMP-9 in challenging situations, such as with osteoclasts during bone fracture recovery and with macrophages during growth of exogenous tumours.

In the study using human MMP-2 promoter/*lacZ*-mice, it was found that a 6500 base pair fragment upstream from the 5'-flanking region of the transcription start site was sufficient for expression equivalent to that of endogenous MMP-2. Active β -galactosidase was detected in the mesenchymal cells of the transgenic mice during embryogenesis following the different stages of tissue morphogenesis especially in the areas of basement membrane degradation and epithelial cell invasion. Analysis of the sequence that was included in the transgene construct revealed three homologous regions between human, mouse, and rat. Two of the regions were located distal to the transcription start site, whereas one, shorter area was proximal and contained previously characterised *cis*-acting factors. In the more distal homologous regions, two possible NF- κ B-, two AP-1-binding sites, and one CREB-motif were detected. The homologous regions will be good candidates to include in future transgenic mouse studies with constructs of varying lengths. The study with these MMP-2/*lacZ*-mice represents the first step toward defining elements necessary for mesenchymal expression.

References

- Agren MS (1999) Matrix metalloproteinases (MMPs) are required for re-epithelialization of cutaneous wounds. *Arch Dermatol Res* 291(11): 583-90.
- Agren MS, Mirastschijski U, Karlsmark T & Saarialho-Kere UK (2001) Topical synthetic inhibitor of matrix metalloproteinases delays epidermal regeneration of human wounds. *Exp Dermatol* 10(5): 337-48.
- Alexander CM, Hansell EJ, Behrendtsen O, Flannery ML, Kishnani NS, Hawkes SP & Werb Z (1996) Expression and function of matrix metalloproteinases and their inhibitors at the maternal-embryonic boundary during mouse embryo implantation. *Development* 122(6): 1723-36.
- Alexander CM, Selvarajan S, Mudgett J & Werb Z (2001) Stromelysin-1 regulates adipogenesis during mammary gland involution. *J Cell Biol* 152(4): 693-703.
- Alexander SM, Jackson KJ, Bushnell KM & McGuire PG (1997) Spatial and temporal expression of the 72-kDa type IV collagenase (MMP-2) correlates with development and differentiation of valves in the embryonic avian heart. *Dev Dyn* 209(3): 261-8.
- Andrews KL, Betsuyaku T, Rogers S, Shipley JM, Senior RM & Miner JH (2000) Gelatinase B (MMP-9) is not essential in the normal kidney and does not influence progression of renal disease in a mouse model of Alport syndrome. *Am J Pathol* 157(1): 303-11.
- Apte SS, Olsen BR & Murphy G (1995) The gene structure of tissue inhibitor of metalloproteinases (TIMP)-3 and its inhibitory activities define the distinct TIMP gene family. *J Biol Chem* 270(24): 14313-8.
- Arbeit JM, Munger K, Howley PM & Hanahan D (1994) Progressive squamous epithelial neoplasia in K14-human papillomavirus type 16 transgenic mice. *J Virol* 68(7): 4358-68.
- Arriza JL, Weinberger C, Cerelli G, Glaser TM, Handelin BL, Housman DE & Evans RM (1987) Cloning of human mineralocorticoid receptor complementary DNA:

- structural and functional kinship with the glucocorticoid receptor. *Science* 237(4812): 268-75.
- Ashcroft GS, Horan MA, Herrick SE, Tarnuzzer RW, Schultz GS & Ferguson MW (1997) Age-related differences in the temporal and spatial regulation of matrix metalloproteinases (MMPs) in normal skin and acute cutaneous wounds of healthy humans. *Cell Tissue Res* 290(3): 581-91.
- Ashworth JL, Murphy G, Rock MJ, Sherratt MJ, Shapiro SD, Shuttleworth CA & Kielty CM (1999) Fibrillin degradation by matrix metalloproteinases: implications for connective tissue remodelling. *Biochem J* 340: 171-81.
- Ausubel F, Brent, R., Kingston, R., Moore, D., Seidman, J., Smith, J., and Struhl, A. (1989) *Current protocols in molecular biology*. John Wiley & Sons, Inc., New York.
- Backstrom JR, Lim GP, Cullen MJ & Tokes ZA (1996) Matrix metalloproteinase-9 (MMP-9) is synthesized in neurons of the human hippocampus and is capable of degrading the amyloid-beta peptide (1-40). *J Neurosci* 16(24): 7910-9.
- Backstrom JR & Tokes ZA (1995) The 84-kDa form of human matrix metalloproteinase-9 degrades substance P and gelatin. *J Neurochem* 64(3): 1312-8.
- Balbin M, Fueyo A, Knauper V, Lopez JM, Alvarez J, Sanchez LM, Quesada V, Bordallo J, Murphy G & Lopez-Otin C (2001) Identification and enzymatic characterization of two diverging murine counterparts of human interstitial collagenase (MMP-1) expressed at sites of embryo implantation. *J Biol Chem* 276(13): 10253-62.
- Bancroft JD, Stevens, A (ed) (1990). *Theory and practice of histological techniques*. Churchill Livingstone, Edinburgh.
- Bannikov GA, Karelina TV, Collier IE, Marmer BL & Goldberg GI (2002) Substrate binding of gelatinase B induces its enzymatic activity in the presence of intact propeptide. *J Biol Chem* 277(18): 16022-7.
- Baron R, Neff L, Louvard D & Courtoy PJ (1985) Cell-mediated extracellular acidification and bone resorption: evidence for a low pH in resorbing lacunae and localization of a 100-kD lysosomal membrane protein at the osteoclast ruffled border. *J Cell Biol* 101(6): 2210-22.
- Baron R, Neff L, Tran Van P, Nefussi JR & Vignery A (1986) Kinetic and cytochemical identification of osteoclast precursors and their differentiation into multinucleated osteoclasts. *Am J Pathol* 122(2): 363-78.
- Beare AH, O'Kane S, Krane SM & Ferguson MW (2003) Severely impaired wound healing in the collagenase-resistant mouse. *J Invest Dermatol* 120(1): 153-63.
- Becker JW, Marcy AI, Rokosz LL, Axel MG, Burbaum JJ, Fitzgerald PM, Cameron PM, Esser CK, Hagmann WK, Hermes JD & et al. (1995) Stromelysin-1: three-dimensional structure of the inhibited catalytic domain and of the C-truncated proenzyme. *Protein Sci* 4(10): 1966-76.
- Behringer RR, Crotty DA, Tennyson VM, Brinster RL, Palmiter RD & Wolgemuth DJ (1993) Sequences 5' of the homeobox of the Hox-1.4 gene direct tissue-specific expression of lacZ during mouse development. *Development* 117(3): 823-33.
- Bergers G, Brekken R, McMahon G, Vu TH, Itoh T, Tamaki K, Tanzawa K, Thorpe P, Itohara S, Werb Z & Hanahan D (2000) Matrix metalloproteinase-9 triggers the angiogenic switch during carcinogenesis. *Nat Cell Biol* 2(10): 737-44.

- Bergers G, Javaherian K, Lo KM, Folkman J & Hanahan D (1999) Effects of angiogenesis inhibitors on multistage carcinogenesis in mice. *Science* 284(5415): 808-12.
- Bergmann U, Tuuttila A, Stetler-Stevenson WG & Tryggvason K (1995) Autolytic activation of recombinant human 72 kilodalton type IV collagenase. *Biochemistry* 34(9): 2819-25.
- Bian J & Sun Y (1997) Transcriptional activation by p53 of the human type IV collagenase (gelatinase A or matrix metalloproteinase 2) promoter. *Mol Cell Biol* 17(11): 6330-8.
- Birkedal-Hansen H, Cobb CM, Taylor RE & Fullmer HM (1976) Synthesis and release of procollagenase by cultured fibroblasts. *J Biol Chem* 251(10): 3162-8.
- Birkedal-Hansen H, Moore WG, Bodden MK, Windsor LJ, Birkedal-Hansen B, DeCarlo A & Engler JA (1993) Matrix metalloproteinases: a review. *Crit Rev Oral Biol Med* 4(2): 197-250.
- Bissell MJ & Radisky D (2001) Putting tumours in context. *Nat Rev Cancer* 1(1): 46-54.
- Blavier L & Delaisse JM (1995) Matrix metalloproteinases are obligatory for the migration of preosteoclasts to the developing marrow cavity of primitive long bones. *J Cell Sci* 108: 3649-59.
- Bode W, Fernandez-Catalan C, Grams F, Gomis-Ruth FX, Nagase H, Tschesche H & Maskos K (1999) Insights into MMP-TIMP interactions. *Ann N Y Acad Sci* 878: 73-91.
- Bonifer C, Vidal M, Grosveld F & Sippel AE (1990) Tissue specific and position independent expression of the complete gene domain for chicken lysozyme in transgenic mice. *Embo J* 9(9): 2843-8.
- Bromme D, Bonneau PR, Purisima E, Lachance P, Hajnik S, Thomas DY & Storer AC (1996) Contribution to activity of histidine-aromatic, amide-aromatic, and aromatic-aromatic interactions in the extended catalytic site of cysteine proteinases. *Biochemistry* 35(13): 3970-9.
- Brown DJ, Bishop P, Hamdi H & Kenney MC (1996) Cleavage of structural components of mammalian vitreous by endogenous matrix metalloproteinase-2. *Curr Eye Res* 15(4): 439-45.
- Bulger M & Groudine M (1999) Looping versus linking: toward a model for long-distance gene activation. *Genes Dev* 13(19): 2465-77.
- Bullard KM, Lund L, Mudgett JS, Mellin TN, Hunt TK, Murphy B, Ronan J, Werb Z & Banda MJ (1999) Impaired wound contraction in stromelysin-1-deficient mice. *Ann Surg* 230(2): 260-5.
- Butler GS, Butler MJ, Atkinson SJ, Will H, Tamura T, van Westrum SS, Crabbe T, Clements J, d'Ortho MP & Murphy G (1998) The TIMP2 membrane type 1 metalloproteinase "receptor" regulates the concentration and efficient activation of progelatinase A. A kinetic study. *J Biol Chem* 273(2): 871-80.
- Butler GS, Will H, Atkinson SJ & Murphy G (1997) Membrane-type-2 matrix metalloproteinase can initiate the processing of progelatinase A and is regulated by the tissue inhibitors of metalloproteinases. *Eur J Biochem* 244(2): 653-7.
- Canete-Soler R, Gui YH, Linask KK & Muschel RJ (1995) Developmental expression of MMP-9 (gelatinase B) mRNA in mouse embryos. *Dev Dyn* 204(1): 30-40.

- Carmeliet P, Moons L, Lijnen R, Baes M, Lemaitre V, Tipping P, Drew A, Eeckhout Y, Shapiro S, Lupu F & Collen D (1997) Urokinase-generated plasmin activates matrix metalloproteinases during aneurysm formation. *Nat Genet* 17(4): 439-44.
- Carmeliet P, Schoonjans L, Kieckens L, Ream B, Degen J, Bronson R, De Vos R, van den Oord JJ, Collen D & Mulligan RC (1994) Physiological consequences of loss of plasminogen activator gene function in mice. *Nature* 368(6470): 419-24.
- Cho HJ, Chae IH, Park KW, Ju JR, Oh S, Lee MM & Park YB (2002) Functional polymorphism in the promoter region of the gelatinase B gene in relation to coronary artery disease and restenosis after percutaneous coronary intervention. *J Hum Genet* 47(2): 88-91.
- Chomczynski P & Sacchi N (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 162(1): 156-9.
- Clark RA (ed) (1996). The molecular and cellular biology of wound repair. Wound repair: Overview and general considerations. Plenum Press, New York.
- Clark RA, Lanigan JM, DellaPelle P, Manseau E, Dvorak HF & Colvin RB (1982) Fibronectin and fibrin provide a provisional matrix for epidermal cell migration during wound reepithelialization. *J Invest Dermatol* 79(5): 264-9.
- Collier IE, Wilhelm SM, Eisen AZ, Marmer BL, Grant GA, Seltzer JL, Kronberger A, He CS, Bauer EA & Goldberg GI (1988) H-ras oncogene-transformed human bronchial epithelial cells (TBE-1) secrete a single metalloprotease capable of degrading basement membrane collagen. *J Biol Chem* 263(14): 6579-87.
- Compton CC, Gill JM, Bradford DA, Regauer S, Gallico GG & O'Connor NE (1989) Skin regenerated from cultured epithelial autografts on full-thickness burn wounds from 6 days to 5 years after grafting. A light, electron microscopic and immunohistochemical study. *Lab Invest* 60(5): 600-12.
- Coussens LM, Fingleton B & Matrisian LM (2002) Matrix metalloproteinase inhibitors and cancer: trials and tribulations. *Science* 295(5564): 2387-92.
- Coussens LM, Hanahan D & Arbeit JM (1996) Genetic predisposition and parameters of malignant progression in K14-HPV16 transgenic mice. *Am J Pathol* 149(6): 1899-917.
- Coussens LM, Tinkle CL, Hanahan D & Werb Z (2000) MMP-9 supplied by bone marrow-derived cells contributes to skin carcinogenesis. *Cell* 103(3): 481-90.
- Daci E, Udagawa N, Martin TJ, Bouillon R & Carmeliet G (1999) The role of the plasminogen system in bone resorption in vitro. *J Bone Miner Res* 14(6): 946-52.
- de Lorenzo MS, Ripoll GV, Yoshiji H, Yamazaki M, Thorgeirsson UP, Alonso DF & Gomez DE (2003) Altered tumor angiogenesis and metastasis of B16 melanoma in transgenic mice overexpressing tissue inhibitor of metalloproteinases-1. *In Vivo* 17(1): 45-50.
- Delaisse JM, Engsig MT, Everts V, del Carmen Ovejero M, Ferreras M, Lund L, Vu TH, Werb Z, Winding B, Lochter A, Karsdal MA, Troen T, Kirkegaard T, Lenhard T, Heegaard AM, Neff L, Baron R & Foged NT (2000) Proteinases in bone resorption: obvious and less obvious roles. *Clin Chim Acta* 291(2): 223-34.

- Desmoulière A & Gabbiani G (1996) The role of myofibroblast in wound healing and fibrocontractive diseases. In: R. A. Clark (ed) *The molecular and cellular biology of wound repair*. New York, Plenum Press, p 391-423.
- Desrochers PE, Mookhtiar K, Van Wart HE, Hasty KA & Weiss SJ (1992) Proteolytic inactivation of alpha 1-proteinase inhibitor and alpha 1-antichymotrypsin by oxidatively activated human neutrophil metalloproteinases. *J Biol Chem* 267(7): 5005-12.
- Docherty AJ, Lyons A, Smith BJ, Wright EM, Stephens PE, Harris TJ, Murphy G & Reynolds JJ (1985) Sequence of human tissue inhibitor of metalloproteinases and its identity to erythroid-potentiating activity. *Nature* 318(6041): 66-9.
- Douglas DA, Shi YE & Sang QA (1997) Computational sequence analysis of the tissue inhibitor of metalloproteinase family. *J Protein Chem* 16(4): 237-55.
- Drews R, Drohan WN & Lubon H (1994) Transgene detection in mouse tail digests. *Biotechniques* 17(5): 866-7.
- Eckes B, Aumailley, M, and Krieg, T (1996) Collagens and the reestablishment of dermal integrity. In: R. A. Clark (ed) *The molecular and cellular biology of wound repair*. New York, Plenum Press, p 493-560.
- Ellerbroek SM, Wu YI & Stack MS (2001) Type I collagen stabilization of matrix metalloproteinase-2. *Arch Biochem Biophys* 390(1): 51-6.
- Engsig MT, Chen QJ, Vu TH, Pedersen AC, Therkildsen B, Lund LR, Henriksen K, Lenhard T, Foged NT, Werb Z & Delaisse JM (2000) Matrix metalloproteinase 9 and vascular endothelial growth factor are essential for osteoclast recruitment into developing long bones. *J Cell Biol* 151(4): 879-89.
- Everts V, Delaisse JM, Korper W, Niehof A, Vaes G & Beertsen W (1992) Degradation of collagen in the bone-resorbing compartment underlying the osteoclast involves both cysteine-proteinases and matrix metalloproteinases. *J Cell Physiol* 150(2): 221-31.
- Fata JE, Leco KJ, Moorehead RA, Martin DC & Khokha R (1999) Timp-1 is important for epithelial proliferation and branching morphogenesis during mouse mammary development. *Dev Biol* 211(2): 238-54.
- Fessler LI, Duncan KG, Fessler JH, Salo T & Tryggvason K (1984) Characterization of the procollagen IV cleavage products produced by a specific tumor collagenase. *J Biol Chem* 259(15): 9783-9.
- Filanti C, Dickson GR, Di Martino D, Ulivi V, Sanguineti C, Romano P, Palermo C & Manduca P (2000) The expression of metalloproteinase-2, -9, and -14 and of tissue inhibitors-1 and -2 is developmentally modulated during osteogenesis in vitro, the mature osteoblastic phenotype expressing metalloproteinase-14. *J Bone Miner Res* 15(11): 2154-68.
- Fini ME, Bartlett JD, Matsubara M, Rinehart WB, Mody MK, Girard MT & Rainville M (1994) The rabbit gene for 92-kDa matrix metalloproteinase. Role of AP1 and AP2 in cell type-specific transcription. *J Biol Chem* 269(46): 28620-8.
- Frisch SM & Morisaki JH (1990) Positive and negative transcriptional elements of the human type IV collagenase gene. *Mol Cell Biol* 10(12): 6524-32.
- Galis ZS, Sukhova GK & Libby P (1995) Microscopic localization of active proteases by in situ zymography: detection of matrix metalloproteinase activity in vascular tissue. *Faseb J* 9(10): 974-80.

- Garlick JA & Taichman LB (1994) Fate of human keratinocytes during reepithelialization in an organotypic culture model. *Lab Invest* 70(6): 916-24.
- Garnero P, Borel O, Byrjalsen I, Ferreras M, Drake FH, McQueney MS, Foged NT, Delmas PD & Delaisse JM (1998) The collagenolytic activity of cathepsin K is unique among mammalian proteinases. *J Biol Chem* 273(48): 32347-52.
- Garrett B (1998) Re-epithelialisation. *J Wound Care* 7(7): 358-9.
- Geiser AG, Zeng QQ, Sato M, Helvering LM, Hirano T & Turner CH (1998) Decreased bone mass and bone elasticity in mice lacking the transforming growth factor-beta1 gene. *Bone* 23(2): 87-93.
- Gerber HP, Vu TH, Ryan AM, Kowalski J, Werb Z & Ferrara N (1999) VEGF couples hypertrophic cartilage remodeling, ossification and angiogenesis during endochondral bone formation. *Nat Med* 5(6): 623-8.
- Giannelli G, Falk-Marzillier J, Schiraldi O, Stetler-Stevenson WG & Quaranta V (1997) Induction of cell migration by matrix metalloproteinase-2 cleavage of laminin-5. *Science* 277(5323): 225-8.
- Gijbels K, Proost P, Masure S, Carton H, Billiau A & Opdenakker G (1993) Gelatinase B is present in the cerebrospinal fluid during experimental autoimmune encephalomyelitis and cleaves myelin basic protein. *J Neurosci Res* 36(4): 432-40.
- Gipson IK, Spurr-Michaud SJ & Tisdale AS (1988) Hemidesmosomes and anchoring fibril collagen appear synchronously during development and wound healing. *Dev Biol* 126(2): 253-62.
- Gomez DE, Alonso DF, Yoshiji H & Thorgeirsson UP (1997) Tissue inhibitors of metalloproteinases: structure, regulation and biological functions. *Eur J Cell Biol* 74(2): 111-22.
- Gordon JW, Scangos GA, Plotkin DJ, Barbosa JA & Ruddle FH (1980) Genetic transformation of mouse embryos by microinjection of purified DNA. *Proc Natl Acad Sci U S A* 77(12): 7380-4.
- Goss KJ, Brown PD & Matrisian LM (1998) Differing effects of endogenous and synthetic inhibitors of metalloproteinases on intestinal tumorigenesis. *Int J Cancer* 78(5): 629-35.
- Gowen M, Lazner F, Dodds R, Kapadia R, Feild J, Tavarria M, Bertoncello I, Drake F, Zavarselk S, Tellis I, Hertzog P, Debouck C & Kola I (1999) Cathepsin K knockout mice develop osteopetrosis due to a deficit in matrix degradation but not demineralization. *J Bone Miner Res* 14(10): 1654-63.
- Greene J, Wang M, Liu YE, Raymond LA, Rosen C & Shi YE (1996) Molecular cloning and characterization of human tissue inhibitor of metalloproteinase 4. *J Biol Chem* 271(48): 30375-80.
- Grewal T, Theisen M, Borgmeyer U, Grussenmeyer T, Rupp RA, Stief A, Qian F, Hecht A & Sippel AE (1992) The -6.1-kilobase chicken lysozyme enhancer is a multifactorial complex containing several cell-type-specific elements. *Mol Cell Biol* 12(5): 2339-50.
- Gross J & Lapiere CM (1962) Collagenolytic activity in amphibian tissues: a tissue culture assay. *Proc Natl Acad Sci U S A* 48: 1014-22.

- Gururajan R, Grenet J, Lahti JM & Kidd VJ (1998) Isolation and characterization of two novel metalloproteinase genes linked to the Cdc2L locus on human chromosome 1p36.3. *Genomics* 52(1): 101-6.
- Ha HY, Moon HB, Nam MS, Lee JW, Ryoo ZY, Lee TH, Lee KK, So BJ, Sato H, Seiki M & Yu DY (2001) Overexpression of membrane-type matrix metalloproteinase-1 gene induces mammary gland abnormalities and adenocarcinoma in transgenic mice. *Cancer Res* 61(3): 984-90.
- Han YP, Nien YD & Garner WL (2002) Tumor necrosis factor-alpha-induced proteolytic activation of pro-matrix metalloproteinase-9 by human skin is controlled by down-regulating tissue inhibitor of metalloproteinase-1 and mediated by tissue-associated chymotrypsin-like proteinase. *J Biol Chem* 277(30): 27319-27.
- Han YP, Tuan TL, Hughes M, Wu H & Garner WL (2001) Transforming growth factor-beta - and tumor necrosis factor-alpha -mediated induction and proteolytic activation of MMP-9 in human skin. *J Biol Chem* 276(25): 22341-50.
- Hanahan D (1985) Heritable formation of pancreatic beta-cell tumours in transgenic mice expressing recombinant insulin/simian virus 40 oncogenes. *Nature* 315(6015): 115-22.
- Hanley T & Merlie JP (1991) Transgene detection in unpurified mouse tail DNA by polymerase chain reaction. *Biotechniques* 10(1): 56.
- Harendza S, Lovett DH, Panzer U, Lukacs Z, Kuhl P & Stahl RA (2003) Linked common polymorphisms in the gelatinase a promoter are associated with diminished transcriptional response to estrogen and genetic fitness. *J Biol Chem* 278(23): 20490-9.
- Hasty KA, Pourmotabbed TF, Goldberg GI, Thompson JP, Spinella DG, Stevens RM & Mainardi CL (1990) Human neutrophil collagenase. A distinct gene product with homology to other matrix metalloproteinases. *J Biol Chem* 265(20): 11421-4.
- Hennighausen L & Robinson GW (1998) Think globally, act locally: the making of a mouse mammary gland. *Genes Dev* 12(4): 449-55.
- Herman MP, Sukhova GK, Kisiel W, Foster D, Kehry MR, Libby P & Schonbeck U (2001) Tissue factor pathway inhibitor-2 is a novel inhibitor of matrix metalloproteinases with implications for atherosclerosis. *J Clin Invest* 107(9): 1117-26.
- Herz J & Strickland DK (2001) LRP: a multifunctional scavenger and signaling receptor. *J Clin Invest* 108(6): 779-84.
- Hill PA, Murphy G, Docherty AJ, Hembry RM, Millican TA, Reynolds JJ & Meikle MC (1994) The effects of selective inhibitors of matrix metalloproteinases (MMPs) on bone resorption and the identification of MMPs and TIMP-1 in isolated osteoclasts. *J Cell Sci* 107 (Pt 11): 3055-64.
- Hogan B, Beddington, R., Constantini, F., and Lacy, E. (1986) Manipulating the mouse embryo: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
- Holmbeck K, Bianco P, Caterina J, Yamada S, Kromer M, Kuznetsov SA, Mankani M, Robey PG, Poole AR, Pidoux I, Ward JM & Birkedal-Hansen H (1999) MT1-MMP-deficient mice develop dwarfism, osteopenia, arthritis, and connective tissue disease due to inadequate collagen turnover. *Cell* 99(1): 81-92.

- Hornebeck W, Emonard H, Monboisse JC & Bellon G (2002) Matrix-directed regulation of pericellular proteolysis and tumor progression. *Semin Cancer Biol* 12(3): 231-41.
- Huhtala P, Tuuttila A, Chow LT, Lohi J, Keski-Oja J & Tryggvason K (1991) Complete structure of the human gene for 92-kDa type IV collagenase. Divergent regulation of expression for the 92- and 72-kilodalton enzyme genes in HT-1080 cells. *J Biol Chem* 266(25): 16485-90.
- Hurskainen T, Hoyhtya M, Tuuttila A, Oikarinen A & Autio-Harmainen H (1996) mRNA expressions of TIMP-1, -2, and -3 and 92-KD type IV collagenase in early human placenta and decidual membrane as studied by in situ hybridization. *J Histochem Cytochem* 44(12): 1379-88.
- Ikenaka Y, Yoshiji H, Kuriyama S, Yoshii J, Noguchi R, Tsujinoue H, Yanase K, Namisaki T, Imazu H, Masaki T & Fukui H (2003) Tissue inhibitor of metalloproteinases-1 (TIMP-1) inhibits tumor growth and angiogenesis in the TIMP-1 transgenic mouse model. *Int J Cancer* 105(3): 340-6.
- Imai K, Shikata H & Okada Y (1995) Degradation of vitronectin by matrix metalloproteinases-1, -2, -3, -7 and -9. *FEBS Lett* 369(2-3): 249-51.
- Inaoka T, Bilbe G, Ishibashi O, Tezuka K, Kumegawa M & Kokubo T (1995) Molecular cloning of human cDNA for cathepsin K: novel cysteine proteinase predominantly expressed in bone. *Biochem Biophys Res Commun* 206(1): 89-96.
- Ito A, Mukaiyama A, Itoh Y, Nagase H, Thogersen IB, Enghild JJ, Sasaguri Y & Mori Y (1996) Degradation of interleukin 1beta by matrix metalloproteinases. *J Biol Chem* 271(25): 14657-60.
- Itoh T, Ikeda T, Gomi H, Nakao S, Suzuki T & Itohara S (1997) Unaltered secretion of beta-amyloid precursor protein in gelatinase A (matrix metalloproteinase 2)-deficient mice. *J Biol Chem* 272(36): 22389-92.
- Itoh T, Tanioka M, Yoshida H, Yoshioka T, Nishimoto H & Itohara S (1998a) Reduced angiogenesis and tumor progression in gelatinase A-deficient mice. *Cancer Res* 58(5): 1048-51.
- Itoh Y, Ito A, Iwata K, Tanzawa K, Mori Y & Nagase H (1998b) Plasma membrane-bound tissue inhibitor of metalloproteinases (TIMP)-2 specifically inhibits matrix metalloproteinase 2 (gelatinase A) activated on the cell surface. *J Biol Chem* 273(38): 24360-7.
- Johnson C & Galis ZS (2004) Matrix metalloproteinase-2 and -9 differentially regulate smooth muscle cell migration and cell-mediated collagen organization. *Arterioscler Thromb Vasc Biol* 24(1): 54-60.
- Kang T, Nagase H & Pei D (2002) Activation of membrane-type matrix metalloproteinase 3 zymogen by the proprotein convertase furin in the trans-Golgi network. *Cancer Res* 62(3): 675-81.
- Kang T, Yi J, Guo A, Wang X, Overall CM, Jiang W, Elde R, Borregaard N & Pei D (2001) Subcellular distribution and cytokine- and chemokine-regulated secretion of leukolysin/MT6-MMP/MMP-25 in neutrophils. *J Biol Chem* 276(24): 21960-8.

- Kanwar YS, Ota K, Yang Q, Wada J, Kashihara N, Tian Y & Wallner EI (1999) Role of membrane-type matrix metalloproteinase 1 (MT1-MMP), MMP-2, and its inhibitor in nephrogenesis. *Am J Physiol* 277(6 Pt 2): F934-47.
- Keski-Oja J, Lohi J, Tuuttila A, Tryggvason K & Vartio T (1992) Proteolytic processing of the 72,000-Da type IV collagenase by urokinase plasminogen activator. *Exp Cell Res* 202(2): 471-6.
- Kim JS, Park HY, Kwon JH, Im EK, Choi DH, Jang YS & Cho SY (2002) The roles of stromelysin-1 and the gelatinase B gene polymorphism in stable angina. *Yonsei Med J* 43(4): 473-81.
- Kinoshita T, Sato H, Okada A, Ohuchi E, Imai K, Okada Y & Seiki M (1998) TIMP-2 promotes activation of progelatinase A by membrane-type 1 matrix metalloproteinase immobilized on agarose beads. *J Biol Chem* 273(26): 16098-103.
- Kissinger CR, Liu BS, Martin-Blanco E, Kornberg TB & Pabo CO (1990) Crystal structure of an engrailed homeodomain-DNA complex at 2.8 Å resolution: a framework for understanding homeodomain-DNA interactions. *Cell* 63(3): 579-90.
- Knauper V, Will H, Lopez-Otin C, Smith B, Atkinson SJ, Stanton H, Hembry RM & Murphy G (1996) Cellular mechanisms for human procollagenase-3 (MMP-13) activation. Evidence that MT1-MMP (MMP-14) and gelatinase a (MMP-2) are able to generate active enzyme. *J Biol Chem* 271(29): 17124-31.
- Koivunen E, Arap W, Valtanen H, Rainisalo A, Medina OP, Heikkilä P, Kantor C, Gahmberg CG, Salo T, Konttinen YT, Sorsa T, Ruoslahti E & Pasqualini R (1999) Tumor targeting with a selective gelatinase inhibitor. *Nat Biotechnol* 17(8): 768-74.
- Kolkenbrock H, Orgel D, Hecker-Kia A, Zimmermann J & Ulbrich N (1995) Generation and activity of the ternary gelatinase B/TIMP-1/LMW-stromelysin-1 complex. *Biol Chem Hoppe Seyler* 376(8): 495-500.
- Koshikawa N, Giannelli G, Cirulli V, Miyazaki K & Quaranta V (2000) Role of cell surface metalloprotease MT1-MMP in epithelial cell migration over laminin-5. *J Cell Biol* 148(3): 615-24.
- Kratz G, Jansson K, Gidlund M & Haegerstrand A (1995) Keratinocyte conditioned medium stimulates type IV collagenase synthesis in cultured human keratinocytes and fibroblasts. *Br J Dermatol* 133(6): 842-6.
- Kruger A, Sanchez-Sweetman OH, Martin DC, Fata JE, Ho AT, Orr FW, Ruther U & Khokha R (1998) Host TIMP-1 overexpression confers resistance to experimental brain metastasis of a fibrosarcoma cell line. *Oncogene* 16(18): 2419-23.
- Kupferman ME, Fini ME, Muller WJ, Weber R, Cheng Y & Muschel RJ (2000) Matrix metalloproteinase 9 promoter activity is induced coincident with invasion during tumor progression. *Am J Pathol* 157(6): 1777-83.
- Kähäri VM & Saarialho-Kere U (1997) Matrix metalloproteinases in skin. *Exp Dermatol* 6(5): 199-213.
- Landschulz WH, Johnson PF & McKnight SL (1989) The DNA binding domain of the rat liver nuclear protein C/EBP is bipartite. *Science* 243(4899): 1681-8.

- Langton KP, Barker MD & McKie N (1998) Localization of the functional domains of human tissue inhibitor of metalloproteinases-3 and the effects of a Sorsby's fundus dystrophy mutation. *J Biol Chem* 273(27): 16778-81.
- Lauffenburger DA & Horwitz AF (1996) Cell migration: a physically integrated molecular process. *Cell* 84(3): 359-69.
- Leco KJ, Khokha R, Pavloff N, Hawkes SP & Edwards DR (1994) Tissue inhibitor of metalloproteinases-3 (TIMP-3) is an extracellular matrix-associated protein with a distinctive pattern of expression in mouse cells and tissues. *J Biol Chem* 269(12): 9352-60.
- Lee J, Ishihara A, Theriot JA & Jacobson K (1993) Principles of locomotion for simple-shaped cells. *Nature* 362(6416): 167-71.
- Legallicier B, Trugnan G, Murphy G, Lelongt B & Ronco P (2001) Expression of the type IV collagenase system during mouse kidney development and tubule segmentation. *J Am Soc Nephrol* 12(11): 2358-69.
- Lehti K, Lohi J, Juntunen MM, Pei D & Keski-Oja J (2002) Oligomerization through hemopexin and cytoplasmic domains regulates the activity and turnover of membrane-type 1 matrix metalloproteinase. *J Biol Chem* 277(10): 8440-8.
- Lelongt B, Bengatta S, Delauche M, Lund LR, Werb Z & Ronco PM (2001) Matrix metalloproteinase 9 protects mice from anti-glomerular basement membrane nephritis through its fibrinolytic activity. *J Exp Med* 193(7): 793-802.
- Lelongt B, Trugnan G, Murphy G & Ronco PM (1997) Matrix metalloproteinases MMP2 and MMP9 are produced in early stages of kidney morphogenesis but only MMP9 is required for renal organogenesis in vitro. *J Cell Biol* 136(6): 1363-73.
- Lin S (2000) Transgenic zebrafish. *Methods Mol Biol* 136: 375-83.
- Liotta LA, Tryggvason K, Garbisa S, Hart I, Foltz CM & Shafie S (1980) Metastatic potential correlates with enzymatic degradation of basement membrane collagen. *Nature* 284(5751): 67-8.
- Lobmann R, Ambrosch A, Schultz G, Waldmann K, Schiweck S & Lehnert H (2002) Expression of matrix-metalloproteinases and their inhibitors in the wounds of diabetic and non-diabetic patients. *Diabetologia* 45(7): 1011-6.
- Lohi J, Harvima I & Keski-Oja J (1992) Pericellular substrates of human mast cell tryptase: 72,000 dalton gelatinase and fibronectin. *J Cell Biochem* 50(4): 337-49.
- Lund LR, Romer J, Bugge TH, Nielsen BS, Frandsen TL, Degen JL, Stephens RW & Dano K (1999) Functional overlap between two classes of matrix-degrading proteases in wound healing. *Embo J* 18(17): 4645-56.
- Lyons JG, Birkedal-Hansen B, Pierson MC, Whitelock JM & Birkedal-Hansen H (1993) Interleukin-1 beta and transforming growth factor-alpha/epidermal growth factor induce expression of M(r) 95,000 type IV collagenase/gelatinase and interstitial fibroblast-type collagenase by rat mucosal keratinocytes. *J Biol Chem* 268(25): 19143-51.
- Madlener M (1998) Differential expression of matrix metalloproteinases and their physiological inhibitors in acute murine skin wounds. *Arch Dermatol Res* 290 Suppl: S24-9.

- Madlener M, Parks WC & Werner S (1998) Matrix metalloproteinases (MMPs) and their physiological inhibitors (TIMPs) are differentially expressed during excisional skin wound repair. *Exp Cell Res* 242(1): 201-10.
- Maeda S, Haneda M, Guo B, Koya D, Hayashi K, Sugimoto T, Isshiki K, Yasuda H, Kashiwagi A & Kikkawa R (2001) Dinucleotide repeat polymorphism of matrix metalloproteinase-9 gene is associated with diabetic nephropathy. *Kidney Int* 60(4): 1428-34.
- Martin DC, Ruther U, Sanchez-Sweatman OH, Orr FW & Khokha R (1996) Inhibition of SV40 T antigen-induced hepatocellular carcinoma in TIMP-1 transgenic mice. *Oncogene* 13(3): 569-76.
- Martin P (1997) Wound healing--aiming for perfect skin regeneration. *Science* 276(5309): 75-81.
- Masure S, Nys G, Fiten P, Van Damme J & Opdenakker G (1993) Mouse gelatinase B. cDNA cloning, regulation of expression and glycosylation in WEHI-3 macrophages and gene organisation. *Eur J Biochem* 218(1): 129-41.
- Matrisian LM (1992) The matrix-degrading metalloproteinases. *Bioessays* 14(7): 455-63.
- Mazzieri R, Masiero L, Zanetta L, Monea S, Onisto M, Garbisa S & Mignatti P (1997) Control of type IV collagenase activity by components of the urokinase-plasmin system: a regulatory mechanism with cell-bound reactants. *Embo J* 16(9): 2319-32.
- McCawley LJ, O'Brien P & Hudson LG (1998) Epidermal growth factor (EGF)- and scatter factor/hepatocyte growth factor (SF/HGF)- mediated keratinocyte migration is coincident with induction of matrix metalloproteinase (MMP)-9. *J Cell Physiol* 176(2): 255-65.
- McQuibban GA, Butler GS, Gong JH, Bendall L, Power C, Clark-Lewis I & Overall CM (2001) Matrix metalloproteinase activity inactivates the CXC chemokine stromal cell-derived factor-1. *J Biol Chem* 276(47): 43503-8.
- Mehul B, Bawumia S, Martin SR & Hughes RC (1994) Structure of baby hamster kidney carbohydrate-binding protein CBP30, an S-type animal lectin. *J Biol Chem* 269(27): 18250-8.
- Metsäranta M, Garofalo S, Smith C, Niederreither K, de Crombrughe B & Vuorio E (1995) Developmental expression of a type II collagen/beta-galactosidase fusion gene in transgenic mice. *Dev Dyn* 204(2): 202-10.
- Mignatti P & Rifkin DB (1996) Plasminogen activators and matrix metalloproteinases in angiogenesis. *Enzyme Protein* 49(1-3): 117-37.
- Mohan R, Chintala SK, Jung JC, Villar WV, McCabe F, Russo LA, Lee Y, McCarthy BE, Wollenberg KR, Jester JV, Wang M, Welgus HG, Shipley JM, Senior RM & Fini ME (2002) Matrix metalloproteinase gelatinase B (MMP-9) coordinates and effects epithelial regeneration. *J Biol Chem* 277(3): 2065-72.
- Mohan R, Rinehart WB, Bargagna-Mohan P & Fini ME (1998) Gelatinase B/lacZ transgenic mice, a model for mapping gelatinase B expression during developmental and injury-related tissue remodeling. *J Biol Chem* 273(40): 25903-14.
- Monea S, Lehti K, Keski-Oja J & Mignatti P (2002) Plasmin activates pro-matrix metalloproteinase-2 with a membrane-type 1 matrix metalloproteinase-dependent mechanism. *J Cell Physiol* 192(2): 160-70.

- Morgunova E, Tuuttila A, Bergmann U, Isupov M, Lindqvist Y, Schneider G & Tryggvason K (1999) Structure of human pro-matrix metalloproteinase-2: activation mechanism revealed. *Science* 284(5420): 1667-70.
- Morodomi T, Ogata Y, Sasaguri Y, Morimatsu M & Nagase H (1992) Purification and characterization of matrix metalloproteinase 9 from U937 monocytic leukaemia and HT1080 fibrosarcoma cells. *Biochem J* 285 (Pt 2): 603-11.
- Morrison CJ, Butler GS, Bigg HF, Roberts CR, Soloway PD & Overall CM (2001) Cellular activation of MMP-2 (gelatinase A) by MT2-MMP occurs via a TIMP-2-independent pathway. *J Biol Chem* 276(50): 47402-10.
- Mott JD, Thomas CL, Rosenbach MT, Takahara K, Greenspan DS & Banda MJ (2000) Post-translational proteolytic processing of procollagen C-terminal proteinase enhancer releases a metalloproteinase inhibitor. *J Biol Chem* 275(2): 1384-90.
- Murphy G, Houbrechts A, Cockett MI, Williamson RA, O'Shea M & Docherty AJ (1991) The N-terminal domain of tissue inhibitor of metalloproteinases retains metalloproteinase inhibitory activity. *Biochemistry* 30(33): 8097-102.
- Murphy G, Ward R, Hembry RM, Reynolds JJ, Kuhn K & Tryggvason K (1989) Characterization of gelatinase from pig polymorphonuclear leucocytes. A metalloproteinase resembling tumour type IV collagenase. *Biochem J* 258(2): 463-72.
- Müller F, Blader P & Strahle U (2002) Search for enhancers: teleost models in comparative genomic and transgenic analysis of cis regulatory elements. *Bioessays* 24(6): 564-72.
- Mäkela M, Larjava H, Pirilä E, Maisi P, Salo T, Sorsa T & Uitto VJ (1999) Matrix metalloproteinase 2 (gelatinase A) is related to migration of keratinocytes. *Exp Cell Res* 251(1): 67-78.
- Nagase H & Woessner JF, Jr. (1999) Matrix metalloproteinases. *J Biol Chem* 274(31): 21491-4.
- Nelson AR, Fingleton B, Rothenberg ML & Matrisian LM (2000) Matrix metalloproteinases: biologic activity and clinical implications. *J Clin Oncol* 18(5): 1135-49.
- Netzer KO, Suzuki K, Itoh Y, Hudson BG & Khalifah RG (1998) Comparative analysis of the noncollagenous NC1 domain of type IV collagen: identification of structural features important for assembly, function, and pathogenesis. *Protein Sci* 7(6): 1340-51.
- Newman KM, Malon AM, Shin RD, Scholes JV, Ramey WG & Tilson MD (1994) Matrix metalloproteinases in abdominal aortic aneurysm: characterization, purification, and their possible sources. *Connect Tissue Res* 30(4): 265-76.
- Nielsen BS, Timshel S, Kjeldsen L, Sehested M, Pyke C, Borregaard N & Dano K (1996) 92 kDa type IV collagenase (MMP-9) is expressed in neutrophils and macrophages but not in malignant epithelial cells in human colon cancer. *Int J Cancer* 65(1): 57-62.
- Nijweide PJ, Burger EH & Feyen JH (1986) Cells of bone: proliferation, differentiation, and hormonal regulation. *Physiol Rev* 66(4): 855-86.
- Nwomeh BC, Liang HX, Cohen IK & Yager DR (1999) MMP-8 is the predominant collagenase in healing wounds and nonhealing ulcers. *J Surg Res* 81(2): 189-95.

- Näär AM, Lemon BD & Tjian R (2001) Transcriptional coactivator complexes. *Annu Rev Biochem* 70: 475-501.
- Ochieng J, Fridman R, Nangia-Makker P, Kleiner DE, Liotta LA, Stetler-Stevenson WG & Raz A (1994) Galectin-3 is a novel substrate for human matrix metalloproteinases-2 and -9. *Biochemistry* 33(47): 14109-14.
- O'Connell JP, Willenbrock F, Docherty AJ, Eaton D & Murphy G (1994) Analysis of the role of the COOH-terminal domain in the activation, proteolytic activity, and tissue inhibitor of metalloproteinase interactions of gelatinase B. *J Biol Chem* 269(21): 14967-73.
- Oh J, Takahashi R, Kondo S, Mizoguchi A, Adachi E, Sasahara RM, Nishimura S, Imamura Y, Kitayama H, Alexander DB, Ide C, Horan TP, Arakawa T, Yoshida H, Nishikawa S, Itoh Y, Seiki M, Itohara S, Takahashi C & Noda M (2001) The membrane-anchored MMP inhibitor RECK is a key regulator of extracellular matrix integrity and angiogenesis. *Cell* 107(6): 789-800.
- Ohsawa Y, Nitatori T, Higuchi S, Kominami E & Uchiyama Y (1993) Lysosomal cysteine and aspartic proteinases, acid phosphatase, and an endogenous cysteine proteinase inhibitor, cystatin-beta, in rat osteoclasts. *J Histochem Cytochem* 41(7): 1075-83.
- Okada A, Tomasetto C, Lutz Y, Bellocq JP, Rio MC & Basset P (1997) Expression of matrix metalloproteinases during rat skin wound healing: evidence that membrane type-1 matrix metalloproteinase is a stromal activator of pro-gelatinase A. *J Cell Biol* 137(1): 67-77.
- Okada Y, Gonoji Y, Naka K, Tomita K, Nakanishi I, Iwata K, Yamashita K & Hayakawa T (1992) Matrix metalloproteinase 9 (92-kDa gelatinase/type IV collagenase) from HT 1080 human fibrosarcoma cells. Purification and activation of the precursor and enzymic properties. *J Biol Chem* 267(30): 21712-9.
- Okada Y, Naka K, Kawamura K, Matsumoto T, Nakanishi I, Fujimoto N, Sato H & Seiki M (1995) Localization of matrix metalloproteinase 9 (92-kilodalton gelatinase/type IV collagenase = gelatinase B) in osteoclasts: implications for bone resorption. *Lab Invest* 72(3): 311-22.
- Osiewicz K, McGarry M & Soloway PD (1999) Hyper-resistance to infection in TIMP-1-deficient mice is neutrophil dependent but not immune cell autonomous. *Ann N Y Acad Sci* 878: 494-6.
- Overall CM (1994) Regulation of tissue inhibitor of matrix metalloproteinase expression. *Ann N Y Acad Sci* 732: 51-64.
- Overall CM (2002) Molecular determinants of metalloproteinase substrate specificity: matrix metalloproteinase substrate binding domains, modules, and exosites. *Mol Biotechnol* 22(1): 51-86.
- Overall CM & Lopez-Otin C (2002) Strategies for MMP inhibition in cancer: innovations for the post-trial era. *Nat Rev Cancer* 2(9): 657-72.
- Overall CM, Tam E, McQuibban GA, Morrison C, Wallon UM, Bigg HF, King AE & Roberts CR (2000) Domain interactions in the gelatinase A.TIMP-2.MT1-MMP activation complex. The ectodomain of the 44-kDa form of membrane type-1 matrix metalloproteinase does not modulate gelatinase A activation. *J Biol Chem* 275(50): 39497-506.

- Overall CM, Wrana JL & Sodek J (1991) Transcriptional and post-transcriptional regulation of 72-kDa gelatinase/type IV collagenase by transforming growth factor-beta 1 in human fibroblasts. Comparisons with collagenase and tissue inhibitor of matrix metalloproteinase gene expression. *J Biol Chem* 266(21): 14064-71.
- Parikka M, Kainulainen T, Tasanen K, Bruckner-Tuderman L & Salo T (2001) Altered expression of collagen XVII in ameloblastomas and basal cell carcinomas. *J Oral Pathol Med* 30(10): 589-95.
- Pei D & Weiss SJ (1995) Furin-dependent intracellular activation of the human stromelysin-3 zymogen. *Nature* 375(6528): 244-7.
- Petersen MJ, Woodley DT, Stricklin GP & O'Keefe EJ (1990) Enhanced synthesis of collagenase by human keratinocytes cultured on type I or type IV collagen. *J Invest Dermatol* 94(3): 341-6.
- Petitclerc E, Boutaud A, Prestayko A, Xu J, Sado Y, Ninomiya Y, Sarras MP, Jr., Hudson BG & Brooks PC (2000) New functions for non-collagenous domains of human collagen type IV. Novel integrin ligands inhibiting angiogenesis and tumor growth in vivo. *J Biol Chem* 275(11): 8051-61.
- Philip S, Bulbule A & Kundu GC (2001) Osteopontin stimulates tumor growth and activation of promatrix metalloproteinase-2 through nuclear factor-kappa B-mediated induction of membrane type 1 matrix metalloproteinase in murine melanoma cells. *J Biol Chem* 276(48): 44926-35.
- Pilcher BK, Dumin JA, Sudbeck BD, Krane SM, Welgus HG & Parks WC (1997) The activity of collagenase-1 is required for keratinocyte migration on a type I collagen matrix. *J Cell Biol* 137(6): 1445-57.
- Pirilä E, Maisi P, Salo T, Koivunen E & Sorsa T (2001) In vivo localization of gelatinases (MMP-2 and -9) by in situ zymography with a selective gelatinase inhibitor. *Biochem Biophys Res Commun* 287(3): 766-74.
- Pourmotabbed T, Solomon TL, Hasty KA & Mainardi CL (1994) Characteristics of 92 kDa type IV collagenase/gelatinase produced by granulocytic leukemia cells: structure, expression of cDNA in *E. coli* and enzymic properties. *Biochim Biophys Acta* 1204(1): 97-107.
- Price SJ, Greaves DR & Watkins H (2001) Identification of novel, functional genetic variants in the human matrix metalloproteinase-2 gene: role of Sp1 in allele-specific transcriptional regulation. *J Biol Chem* 276(10): 7549-58.
- Proost P, Van Damme J & Opdenakker G (1993) Leukocyte gelatinase B cleavage releases encephalitogens from human myelin basic protein. *Biochem Biophys Res Commun* 192(3): 1175-81.
- Pyke C, Ralfkiaer E, Huhtala P, Hurskainen T, Dano K & Tryggvason K (1992) Localization of messenger RNA for Mr 72,000 and 92,000 type IV collagenases in human skin cancers by in situ hybridization. *Cancer Res* 52(5): 1336-41.
- Pyke C, Ralfkiaer E, Tryggvason K & Dano K (1993) Messenger RNA for two type IV collagenases is located in stromal cells in human colon cancer. *Am J Pathol* 142(2): 359-65.
- Qian YQ, Billeter M, Otting G, Muller M, Gehring WJ & Wuthrich K (1989) The structure of the Antennapedia homeodomain determined by NMR spectroscopy in solution: comparison with prokaryotic repressors. *Cell* 59(3): 573-80.

- Qin H, Sun Y & Benveniste EN (1999) The transcription factors Sp1, Sp3, and AP-2 are required for constitutive matrix metalloproteinase-2 gene expression in astrogloma cells. *J Biol Chem* 274(41): 29130-7.
- Rahkonen OP, Koskivirta IM, Oksjoki SM, Jokinen E & Vuorio EI (2002) Characterization of the murine Timp4 gene, localization within intron 5 of the synapsin 2 gene and tissue distribution of the mRNA. *Biochim Biophys Acta* 1577(1): 45-52.
- Rantakokko J, Aro HT, Savontaus M & Vuorio E (1996) Mouse cathepsin K: cDNA cloning and predominant expression of the gene in osteoclasts, and in some hypertrophying chondrocytes during mouse development. *FEBS Lett* 393(2-3): 307-13.
- Reponen P, Leivo I, Sahlberg C, Apte SS, Olsen BR, Thesleff I & Tryggvason K (1995) 92-kDa type IV collagenase and TIMP-3, but not 72-kDa type IV collagenase or TIMP-1 or TIMP-2, are highly expressed during mouse embryo implantation. *Dev Dyn* 202(4): 388-96.
- Reponen P, Sahlberg C, Huhtala P, Hurskainen T, Thesleff I & Tryggvason K (1992) Molecular cloning of murine 72-kDa type IV collagenase and its expression during mouse development. *J Biol Chem* 267(11): 7856-62.
- Reponen P, Sahlberg C, Munaut C, Thesleff I & Tryggvason K (1994) High expression of 92-kD type IV collagenase (gelatinase B) in the osteoclast lineage during mouse development. *J Cell Biol* 124(6): 1091-1102.
- Robyr D, Wolffe AP & Wahli W (2000) Nuclear hormone receptor coregulators in action: diversity for shared tasks. *Mol Endocrinol* 14(3): 329-47.
- Rochat A, Kobayashi K & Barrandon Y (1994) Location of stem cells of human hair follicles by clonal analysis. *Cell* 76(6): 1063-73.
- Rodriguez-Manzaneque JC, Lane TF, Ortega MA, Hynes RO, Lawler J & Iruela-Arispe ML (2001) Thrombospondin-1 suppresses spontaneous tumor growth and inhibits activation of matrix metalloproteinase-9 and mobilization of vascular endothelial growth factor. *Proc Natl Acad Sci U S A* 98(22): 12485-90.
- Rossi P & de Crombrughe B (1987) Identification of a cell-specific transcriptional enhancer in the first intron of the mouse alpha 2 (type I) collagen gene. *Proc Natl Acad Sci U S A* 84(16): 5590-4.
- Saarialho-Kere UK, Vaalamo M, Puolakkainen P, Airola K, Parks WC & Karjalainen-Lindsberg ML (1996) Enhanced expression of matrilysin, collagenase, and stromelysin-1 in gastrointestinal ulcers. *Am J Pathol* 148(2): 519-26.
- Saftig P, Hunziker E, Wehmeyer O, Jones S, Boyde A, Rommerskirch W, Moritz JD, Schu P & von Figura K (1998) Impaired osteoclastic bone resorption leads to osteopetrosis in cathepsin-K-deficient mice. *Proc Natl Acad Sci U S A* 95(23): 13453-8.
- Sahlberg C, Reponen P, Tryggvason K & Thesleff I (1992) Association between the expression of murine 72 kDa type IV collagenase by odontoblasts and basement membrane degradation during mouse tooth development. *Arch Oral Biol* 37(12): 1021-30.
- Sahlberg C, Reponen P, Tryggvason K & Thesleff I (1999) Timp-1, -2 and -3 show coexpression with gelatinases A and B during mouse tooth morphogenesis. *Eur J Oral Sci* 107(2): 121-30.

- Salo T, Liotta LA & Tryggvason K (1983) Purification and characterization of a murine basement membrane collagen-degrading enzyme secreted by metastatic tumor cells. *J Biol Chem* 258(5): 3058-63.
- Salo T, Makela M, Kylmäniemi M, Autio-Harminen H & Larjava H (1994a) Expression of matrix metalloproteinase-2 and -9 during early human wound healing. *Lab Invest* 70(2): 176-82.
- Salo T, Mäkelä M, Kylmäniemi M, Autio-Harminen H & Larjava H (1994b) Expression of matrix metalloproteinase-2 and -9 during early human wound healing. *Lab Invest* 70(2): 176-82.
- Sanchez R, Nguyen D, Rocha W, White JH & Mader S (2002) Diversity in the mechanisms of gene regulation by estrogen receptors. *Bioessays* 24(3): 244-54.
- Sang QA & Douglas DA (1996) Computational sequence analysis of matrix metalloproteinases. *J Protein Chem* 15(2): 137-60.
- Sanger F, Nicklen S & Coulson AR (1977) DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci U S A* 74(12): 5463-7.
- Sarret Y, Woodley DT, Goldberg GS, Kronberger A & Wynn KC (1992) Constitutive synthesis of a 92-kDa keratinocyte-derived type IV collagenase is enhanced by type I collagen and decreased by type IV collagen matrices. *J Invest Dermatol* 99(6): 836-41.
- Sasaki T, Gohring W, Mann K, Maurer P, Hohenester E, Knauper V, Murphy G & Timpl R (1997) Limited cleavage of extracellular matrix protein BM-40 by matrix metalloproteinases increases its affinity for collagens. *J Biol Chem* 272(14): 9237-43.
- Sato H, Kinoshita T, Takino T, Nakayama K & Seiki M (1996) Activation of a recombinant membrane type 1-matrix metalloproteinase (MT1-MMP) by furin and its interaction with tissue inhibitor of metalloproteinases (TIMP)-2. *FEBS Lett* 393(1): 101-4.
- Sato H, Takino T, Okada Y, Cao J, Shinagawa A, Yamamoto E & Seiki M (1994) A matrix metalloproteinase expressed on the surface of invasive tumour cells. *Nature* 370(6484): 61-5.
- Seiki M (1999) Membrane-type matrix metalloproteinases. *Apmis* 107(1): 137-43.
- Seltzer JL, Adams SA, Grant GA & Eisen AZ (1981) Purification and properties of a gelatin-specific neutral protease from human skin. *J Biol Chem* 256(9): 4662-8.
- Seltzer JL, Akers KT, Weingarten H, Grant GA, McCourt DW & Eisen AZ (1990) Cleavage specificity of human skin type IV collagenase (gelatinase). Identification of cleavage sites in type I gelatin, with confirmation using synthetic peptides. *J Biol Chem* 265(33): 20409-13.
- Sheetz MP (1994) Cell migration by graded attachment to substrates and contraction. *Semin Cell Biol* 5(3): 149-55.
- Simian M, Hirai Y, Navre M, Werb Z, Lochter A & Bissell MJ (2001) The interplay of matrix metalloproteinases, morphogens and growth factors is necessary for branching of mammary epithelial cells. *Development* 128(16): 3117-31.
- Sippel AE, Borgmeyer U, Puschel AW, Rupp RA, Stief A, Strech-Jurk U & Theisen M (1987) Multiple nonhistone protein-DNA complexes in chromatin regulate the cell- and stage-specific activity of an eukaryotic gene. *Results Probl Cell Differ* 14: 255-69.

- Sires UI, Dublet B, Aubert-Foucher E, van der Rest M & Welgus HG (1995) Degradation of the COL1 domain of type XIV collagen by 92-kDa gelatinase. *J Biol Chem* 270(3): 1062-7.
- Sires UI, Murphy G, Baragi VM, Fliszar CJ, Welgus HG & Senior RM (1994) Matrilysin is much more efficient than other matrix metalloproteinases in the proteolytic inactivation of alpha 1-antitrypsin. *Biochem Biophys Res Commun* 204(2): 613-20.
- Sodek J & Overall CM (1992) Matrix metalloproteinases in periodontal tissue remodelling. *Matrix Suppl* 1: 352-62.
- Soloway PD, Alexander CM, Werb Z & Jaenisch R (1996) Targeted mutagenesis of Timp-1 reveals that lung tumor invasion is influenced by Timp-1 genotype of the tumor but not by that of the host. *Oncogene* 13(11): 2307-14.
- Somasundaram K, Jayaraman G, Williams T, Moran E, Frisch S & Thimmapaya B (1996) Repression of a matrix metalloprotease gene by E1A correlates with its ability to bind to cell type-specific transcription factor AP-2. *Proc Natl Acad Sci U S A* 93(7): 3088-93.
- Sorsa T, Saari H, Konttinen YT, Suomalainen K, Lindy S & Uitto VJ (1989) Non-proteolytic activation of latent human neutrophil collagenase and its role in matrix destruction in periodontal diseases. *Int J Tissue React* 11(4): 153-9.
- Sorsa T, Salo T, Koivunen E, Tyynela J, Konttinen YT, Bergmann U, Tuuttila A, Niemi E, Teronen O, Heikkila P, Tschesche H, Leinonen J, Osman S & Stenman UH (1997) Activation of type IV procollagenases by human tumor-associated trypsin-2. *J Biol Chem* 272(34): 21067-74.
- Sottrup-Jensen L & Birkedal-Hansen H (1989) Human fibroblast collagenase-alpha-macroglobulin interactions. Localization of cleavage sites in the bait regions of five mammalian alpha-macroglobulins. *J Biol Chem* 264(1): 393-401.
- Springman EB, Angleton EL, Birkedal-Hansen H & Van Wart HE (1990) Multiple modes of activation of latent human fibroblast collagenase: evidence for the role of a Cys73 active-site zinc complex in latency and a "cysteine switch" mechanism for activation. *Proc Natl Acad Sci U S A* 87(1): 364-8.
- Steffensen B, Hakkinen L & Larjava H (2001) Proteolytic events of wound-healing--coordinated interactions among matrix metalloproteinases (MMPs), integrins, and extracellular matrix molecules. *Crit Rev Oral Biol Med* 12(5): 373-98.
- Steiner DF (1998) The proprotein convertases. *Curr Opin Chem Biol* 2(1): 31-9.
- Sternlicht MD & Werb Z (2001) How matrix metalloproteinases regulate cell behavior. *Annu Rev Cell Dev Biol* 17: 463-516.
- Stetefeld J, Jenny M, Schulthess T, Landwehr R, Schumacher B, Frank S, Ruegg MA, Engel J & Kammerer RA (2001) The laminin-binding domain of agrin is structurally related to N-TIMP-1. *Nat Struct Biol* 8(8): 705-9.
- Stetler-Stevenson WG, Kruttsch HC & Liotta LA (1989a) Tissue inhibitor of metalloproteinase (TIMP-2). A new member of the metalloproteinase inhibitor family. *J Biol Chem* 264(29): 17374-8.
- Stetler-Stevenson WG, Kruttsch HC, Wacher MP, Margulies IM & Liotta LA (1989b) The activation of human type IV collagenase proenzyme. Sequence identification of the major conversion product following organomercurial activation. *J Biol Chem* 264(3): 1353-6.

- Stricklin GP & Welgus HG (1983) Human skin fibroblast collagenase inhibitor. Purification and biochemical characterization. *J Biol Chem* 258(20): 12252-8.
- Strongin AY, Collier I, Bannikov G, Marmer BL, Grant GA & Goldberg GI (1995) Mechanism of cell surface activation of 72-kDa type IV collagenase. Isolation of the activated form of the membrane metalloprotease. *J Biol Chem* 270(10): 5331-8.
- Sympton CJ, Talhouk RS, Alexander CM, Chin JR, Clift SM, Bissell MJ & Werb Z (1994) Targeted expression of stromelysin-1 in mammary gland provides evidence for a role of proteinases in branching morphogenesis and the requirement for an intact basement membrane for tissue-specific gene expression. *J Cell Biol* 125(3): 681-93.
- Tamarina NA, McMillan WD, Shively VP & Pearce WH (1997) Expression of matrix metalloproteinases and their inhibitors in aneurysms and normal aorta. *Surgery* 122(2): 264-71.
- Tanaka M, Kinoshita M, Kobayashi D & Nagahama Y (2001) Establishment of medaka (*Oryzias latipes*) transgenic lines with the expression of green fluorescent protein fluorescence exclusively in germ cells: a useful model to monitor germ cells in a live vertebrate. *Proc Natl Acad Sci U S A* 98(5): 2544-9.
- Tanney DC, Feng L, Pollock AS & Lovett DH (1998) Regulated expression of matrix metalloproteinases and TIMP in nephrogenesis. *Dev Dyn* 213(1): 121-9.
- Tezuka K, Nemoto K, Tezuka Y, Sato T, Ikeda Y, Kobori M, Kawashima H, Eguchi H, Hakeda Y & Kumegawa M (1994) Identification of matrix metalloproteinase 9 in rabbit osteoclasts. *J Biol Chem* 269(21): 15006-9.
- Thesleff I (2003) Epithelial-mesenchymal signalling regulating tooth morphogenesis. *J Cell Sci* 116(Pt 9): 1647-8.
- Tolley SP, Davies GJ, O'Shea M, Cockett MI, Docherty AJ & Murphy G (1993) Crystallization and preliminary X-ray analysis of nonglycosylated tissue inhibitor of metalloproteinases-1, N30QN78Q TIMP-1. *Proteins* 17(4): 435-7.
- Tryggvason K, Hoyhtya M & Pyke C (1993) Type IV collagenases in invasive tumors. *Breast Cancer Res Treat* 24(3): 209-18.
- Uria JA & Lopez-Otin C (2000) Matrilysin-2, a new matrix metalloproteinase expressed in human tumors and showing the minimal domain organization required for secretion, latency, and activity. *Cancer Res* 60(17): 4745-51.
- Van Wart HE & Birkedal-Hansen H (1990) The cysteine switch: a principle of regulation of metalloproteinase activity with potential applicability to the entire matrix metalloproteinase gene family. *Proc Natl Acad Sci U S A* 87(14): 5578-82.
- Wang Z, Juttermann R & Soloway PD (2000) TIMP-2 is required for efficient activation of proMMP-2 in vivo. *J Biol Chem* 275(34): 26411-5.
- Vartio T, Hovi T & Vaheri A (1982) Human macrophages synthesize and secrete a major 95,000-dalton gelatin-binding protein distinct from fibronectin. *J Biol Chem* 257(15): 8862-6.
- Vartio T & Vaheri A (1981) A gelatin-binding 70,000-dalton glycoprotein synthesized distinctly from fibronectin by normal and malignant adherent cells. *J Biol Chem* 256(24): 13085-90.
- Vayalil PK, Mittal A & Katiyar SK (2004) Proanthocyanidins from grape seeds inhibit expression of matrix metalloproteinases in human prostate carcinoma cells

- which is associated with the inhibition of activation of MAPK and NF{ κ }B. Carcinogenesis. E-pub ahead of printing.
- Velasco G, Pendas AM, Fueyo A, Knauper V, Murphy G & Lopez-Otin C (1999) Cloning and characterization of human MMP-23, a new matrix metalloproteinase predominantly expressed in reproductive tissues and lacking conserved domains in other family members. *J Biol Chem* 274(8): 4570-6.
- Wilde CG, Hawkins PR, Coleman RT, Levine WB, Delegeane AM, Okamoto PM, Ito LY, Scott RW & Seilhamer JJ (1994) Cloning and characterization of human tissue inhibitor of metalloproteinases-3. *DNA Cell Biol* 13(7): 711-8.
- Wilhelm SM, Collier IE, Marmer BL, Eisen AZ, Grant GA & Goldberg GI (1989) SV40-transformed human lung fibroblasts secrete a 92-kDa type IV collagenase which is identical to that secreted by normal human macrophages. *J Biol Chem* 264(29): 17213-21.
- Wilkinson DG & Green J (1990) *Postimplantation Mammalian Embryos*. Oxford University Press, Oxford.
- Williamson RA, Marston FA, Angal S, Koklitis P, Panico M, Morris HR, Carne AF, Smith BJ, Harris TJ & Freedman RB (1990) Disulphide bond assignment in human tissue inhibitor of metalloproteinases (TIMP). *Biochem J* 268(2): 267-74.
- Williamson RA, Natalia D, Gee CK, Murphy G, Carr MD & Freedman RB (1996) Chemically and conformationally authentic active domain of human tissue inhibitor of metalloproteinases-2 refolded from bacterial inclusion bodies. *Eur J Biochem* 241(2): 476-83.
- Williamson RA, Smith BJ, Angal S, Murphy G & Freedman RB (1993) Structural analysis of tissue inhibitor of metalloproteinases-1 (TIMP-1) by tryptic peptide mapping. *Biochim Biophys Acta* 1164(1): 8-16.
- Vincenti MP (2001) The matrix metalloproteinase (MMP) and tissue inhibitor of metalloproteinase (TIMP) genes. Transcriptional and posttranscriptional regulation, signal transduction and cell-type-specific expression. *Methods Mol Biol* 151: 121-48.
- Wiseman BS & Werb Z (2002) Stromal effects on mammary gland development and breast cancer. *Science* 296(5570): 1046-9.
- Witty JP, Wright JH & Matrisian LM (1995) Matrix metalloproteinases are expressed during ductal and alveolar mammary morphogenesis, and misregulation of stromelysin-1 in transgenic mice induces unscheduled alveolar development. *Mol Biol Cell* 6(10): 1287-303.
- Woessner JF, Jr. & Taplin CJ (1988) Purification and properties of a small latent matrix metalloproteinase of the rat uterus. *J Biol Chem* 263(32): 16918-25.
- Vu TH, Shipley JM, Bergers G, Berger JE, Helms JA, Hanahan D, Shapiro SD, Senior RM & Werb Z (1998) MMP-9/gelatinase B is a key regulator of growth plate angiogenesis and apoptosis of hypertrophic chondrocytes. *Cell* 93(3): 411-22.
- Vu TH & Werb Z (2000) Matrix metalloproteinases: effectors of development and normal physiology. *Genes Dev* 14(17): 2123-33.
- Wucherpennig AL, Li YP, Stetler-Stevenson WG, Rosenberg AE & Stashenko P (1994) Expression of 92 kD type IV collagenase/gelatinase B in human osteoclasts. *J Bone Miner Res* 9(4): 549-56.

- Xu J, Rodriguez D, Petitelerc E, Kim JJ, Hangai M, Moon YS, Davis GE, Brooks PC & Yuen SM (2001) Proteolytic exposure of a cryptic site within collagen type IV is required for angiogenesis and tumor growth in vivo. *J Cell Biol* 154(5): 1069-79.
- Xu L, Glass CK & Rosenfeld MG (1999) Coactivator and corepressor complexes in nuclear receptor function. *Curr Opin Genet Dev* 9(2): 140-7.
- Yamamoto H, Flannery ML, Kupriyanov S, Pearce J, McKercher SR, Henkel GW, Maki RA, Werb Z & Oshima RG (1998) Defective trophoblast function in mice with a targeted mutation of *Ets2*. *Genes Dev* 12(9): 1315-26.
- Yang TT & Hawkes SP (1992) Role of the 21-kDa protein TIMP-3 in oncogenic transformation of cultured chicken embryo fibroblasts. *Proc Natl Acad Sci U S A* 89(22): 10676-80.
- Yang Z, Strickland DK & Bornstein P (2001) Extracellular matrix metalloproteinase 2 levels are regulated by the low density lipoprotein-related scavenger receptor and thrombospondin 2. *J Biol Chem* 276(11): 8403-8.
- Ye HQ & Azar DT (1998) Expression of gelatinases A and B, and TIMPs 1 and 2 during corneal wound healing. *Invest Ophthalmol Vis Sci* 39(6): 913-21.
- Ylikomi T, Wurtz JM, Syväälä H, Passinen S, Pekki A, Haverinen M, Blauer M, Tuohimaa P & Gronemeyer H (1998) Reappraisal of the role of heat shock proteins as regulators of steroid receptor activity. *Crit Rev Biochem Mol Biol* 33(6): 437-66.
- Yu Q & Stamenkovic I (1999) Localization of matrix metalloproteinase 9 to the cell surface provides a mechanism for CD44-mediated tumor invasion. *Genes Dev* 13(1): 35-48.
- Yu Q & Stamenkovic I (2000) Cell surface-localized matrix metalloproteinase-9 proteolytically activates TGF-beta and promotes tumor invasion and angiogenesis. *Genes Dev* 14(2): 163-76.
- Zeigler ME, Dutcheshen NT, Gibbs DF & Varani J (1996) Growth factor-induced epidermal invasion of the dermis in human skin organ culture: expression and role of matrix metalloproteinases. *Invasion Metastasis* 16(1): 11-8.
- Zhang B, Ye S, Herrmann SM, Eriksson P, de Maat M, Evans A, Arveiler D, Luc G, Cambien F, Hamsten A, Watkins H & Henney AM (1999) Functional polymorphism in the regulatory region of gelatinase B gene in relation to severity of coronary atherosclerosis. *Circulation* 99(14): 1788-94.
- Zhang JC, Sakthivel R, Kniss D, Graham CH, Strickland DK & McCrae KR (1998) The low density lipoprotein receptor-related protein/alpha2-macroglobulin receptor regulates cell surface plasminogen activator activity on human trophoblast cells. *J Biol Chem* 273(48): 32273-80.
- Zhou Z, Apte SS, Soininen R, Cao R, Baaklini GY, Rauser RW, Wang J, Cao Y & Tryggvason K (2000) Impaired endochondral ossification and angiogenesis in mice deficient in membrane-type matrix metalloproteinase I. *Proc Natl Acad Sci U S A* 97(8): 4052-7.