

# Expression of Matrix Metalloproteinases during Rat Skin Wound Healing: Evidence that Membrane Type-1 Matrix Metalloproteinase Is a Stromal Activator of Pro-Gelatinase A

Akiko Okada, Catherine Tomasetto, Yves Lutz, Jean-Pierre Bellocq,\* Marie-Christine Rio, and Paul Basset

Institut de Génétique et de Biologie Moléculaire et Cellulaire, Centre National de la Recherche Scientifique/Institut National de la Santé et de la Recherche Médicale/Université Louis Pasteur, BP 163, 67404 Illkirch Cedex, C.U. de Strasbourg, France; and \*Service d'Anatomie Pathologique Générale, Hôpitaux Universitaires, Hôpital de Hautepierre, 67098 Strasbourg Cedex, France

**Abstract.** Skin wound healing depends on cell migration and extracellular matrix remodeling. Both processes, which are necessary for reepithelization and restoration of the underlying connective tissue, are believed to involve the action of extracellular proteinases. We screened cDNA libraries and we found that six matrix metalloproteinase genes were highly expressed during rat skin wound healing. They were namely those of stromelysin 1, stromelysin 3, collagenase 3, gelatinase A (GelA), gelatinase B, and membrane type-1 matrix metalloproteinase (MT1-MMP). The expression kinetics of these *MMP* genes, the tissue distribution of their transcripts, the results of cotransfection experiments in COS-1 cells, and zymographic analyses performed using microdissected rat wound tissues support the possibility that during cutaneous wound healing pro-GelA and pro-gelatinase B are activated by MT1-MMP and stromelysin 1, respectively.

Since MT1-MMP has been demonstrated to be a membrane-associated protein (Sato, H., T. Takino, Y. Okada, J. Cao, A. Shinagawa, E. Yamamoto, and M. Seiki. 1994. *Nature (Lond.)*. 370: 61–65), our finding that *GelA* and *MT1-MMP* transcripts were expressed in stromal cells exhibiting a similar tissue distribution suggests that MT1-MMP activates pro-GelA at the stromal cell surface. This possibility is further supported by our observation that the processing of pro-GelA to its mature form correlated to the detection of MT1-MMP in cell membranes of rat fibroblasts expressing the *MT1-MMP* and *GelA* genes. These observations, together with the detection of high levels of the mature GelA form in the granulation tissue but not in the regenerating epidermis, suggest that MT1-MMP and GelA contribute to the restoration of connective tissue during rat skin wound healing.

**S**KIN wound healing is a complex process characterized by reepithelization and restoration of the underlying connective tissue. During this process, keratinocytes, endothelial cells, fibroblasts, and inflammatory cells proliferate and/or migrate to the site of injury, interacting with each other and with extracellular matrices (ECMs)<sup>1</sup> (Gailit and Clark, 1994).

Address all correspondence to Paul Basset, Institut de Génétique et de Biologie Moléculaire et Cellulaire, Centre National de la Recherche Scientifique/Institut National de la Santé et de la Recherche Médicale/Université Louis Pasteur, BP 163, 67404 Illkirch Cedex, France. Tel.: 33 3 88 65 34 25. Fax: 33 3 88 65 32 01. E-Mail: basset@igbmc.u-strasbg.fr

The current address of A. Okada is the Department of Cancer Cell Research, Institute of Medical Science, University of Tokyo, Tokyo 108, Japan.

1. *Abbreviations used in this paper:* Col1, interstitial collagenase; Col2, neutrophil collagenase; Col3, collagenase 3; ECM, extracellular matrix; GelA and GelB, gelatinase A and B; MMP, matrix metalloproteinase; MT1-MMP, MT2-MMP, MT3-MMP, and MT4-MMP, membrane-type-1, -2, -3, and -4 MMP; ST1, ST2, and ST3, stromelysin 1, 2, and 3; TIMP1, 2, and 3, tissue inhibitor of metalloproteinase type-1, -2, and -3.

The migration of cells and the remodeling of tissues during wound healing require the controlled degradation of the ECM and the activation or release of growth factors (Vassalli and Saurat, 1996). These processes are achieved by extracellular proteases, particularly those belonging to the serine protease and matrix metalloproteinase (MMP) families. It has been demonstrated that urokinase-deficient mice develop nonhealing skin ulcerations (Carmeliet and Collen, 1995) and that plasminogen-deficient mice have a marked defect in the closure of skin wounds (Rømer et al., 1996). However, the exact contribution of MMPs to wound healing has not been well established yet. The expression of gelatinase A (GelA) was localized in the connective tissue and that of gelatinase B (GelB) in the migrating epithelial sheet during human skin wound healing (Salo et al., 1994). In healing human burn wounds, *interstitial collagenase* (*Col1*) RNA has been detected in epithelial cells, hair follicles, and eccrine sweat structures (Stricklin et al., 1993). Furthermore, *in vitro* studies indicate that Col1 may play a role in the formation of new blood vessels (Fisher et al., 1994).

When the present study was initiated, ten human MMPs were known (Col1-3, stromelysin1-3 [ST1-3], GelA, GelB, matrilysin, and the macrophage metalloelastase; reviewed in Birkedal-Hansen, 1995), while only four rat MMPs (ST1, Matrisian et al., 1986a; ST2, Breathnach et al., 1987; Col3 [According to Knäuper et al. (1996), the rat collagenase identified by Quinn et al. (1990) should be considered as the rat counterpart of collagenase 3], Quinn et al., 1990; GelA, Marti et al., 1993) had been identified. To gain insight into the contribution of MMPs to wound healing, we designed a two-step procedure for screening cDNA libraries, which allowed the identification of MMPs expressed highly during rat skin wound healing. We thus identified six rat MMP cDNAs, including *ST1*, *ST3*, *Col3*, *GelA*, *GelB*, and a novel member of the MMP family. The latter one was found to exhibit a high level of homology with a human MMP cloned by Sato et al. (1994), and now known as membrane-type-1 MMP (MT1-MMP; Sato and Seiki, 1996).

MT1-MMP is the first member of a new MMP subgroup characterized by its localization at the cell surface, where it is anchored through a transmembrane domain located at the COOH-terminal portion of the molecule. Three additional MT-MMPs (MT2-MMP, Will and Hinzmann, 1995; MT3-MMP [Although the MT-MMP described by Takino et al. (1995) was initially termed MT2-MMP, this MMP should be now referred as MT3-MMP and that found by Will and Hinzmann (1995) as MT2-MMP] Takino et al., 1995; MT4-MMP, Puente et al., 1996) have so far been described. While the function of MT4-MMP is still unknown, the three other MT-MMPs have been shown to be cell surface activators of pro-GelA. MT1-MMP was predominantly detected in malignant cells of lung and gastric carcinomas (Sato et al., 1994; Nomura et al., 1995; Tokuraku et al., 1995). This has led to the proposition that MT1-MMP activates pro-gelA at the cancer cell surface (Sato and Seiki, 1996). However, *MT1-MMP* transcripts were predominantly detected in fibroblastic cells of colon, breast, and head and neck carcinomas (Okada et al., 1995a; Heppner et al., 1996). These observations suggest that pro-GelA activation by MT1-MMP also occurs at the stromal cell surface, which is supported by the specific expression of MT1-MMP in microglial cells of brain tissues (Yamada et al., 1995). Furthermore, *MT1-MMP* gene expression has been recently demonstrated in mesenchymal cells of mouse embryos (Kinoh et al., 1996), indicating that this MMP could contribute to pro-GelA activation in physiological conditions.

In the present study, we show that *MT1-MMP* and *GelA* RNAs are both expressed in stromal cells of rat skin wounds, and that high levels of the mature GelA form are detected in the wound stroma, but not in the regenerating epithelium. These findings suggest that during rat skin wound healing, GelA is activated at the stromal cell surface by MT1-MMP and contributes to the remodeling processes implicated in the restoration of the connective tissue.

## Materials and Methods

### Animals

Pathogen- and virus-free 10-wk-old female Wistar rats were obtained from IFFA-CREDO (L'Arbresle, France). Animals were maintained under the guidelines of the Institut de Génétique et de Biologie Moléculaire

et Cellulaire (IGBMC). Rats were fed with "D 04" (Usine Alimentation Rationnelle, Epinay-sur-Orge, France) and tap water ad libitum. Rat dorsal skin was shaved and cleaned with isopropyl alcohol under anesthesia, and the skin was linearly incised (3-cm long) up to the level of the subcutaneous adipose tissue. Incisions were made on either side of the midline using a #10 sterile surgical scalpel. A sterile dressing was then placed circumferentially around the trunk in order to protect against infection. On days 1, 3, 5, 7, 10, and 14 after wounding, the entire dorsal skin encompassing healing wounds was excised. For RNA and protein extractions, wound tissues were immediately frozen in liquid nitrogen. For RNA in situ hybridization and histopathological examination, the samples were embedded in OCT compound (Miles, Elkhart, IN), and frozen in liquid nitrogen. Adequate sampling was ensured by obtaining wound specimen from at least three separate animals for each time point.

### Cell Lines

COS-1 cells (Amer. Type Culture Collection, Rockville, MD), and RAT1 and FR3T3 rat fibroblasts (Matrisian et al., 1985) were grown and maintained in DMEM supplemented with 5% or 10% FCS, or 10% calf serum, respectively. Con A (Sigma Chem. Co., St. Louis, MO) treatment of rat fibroblasts in culture was carried out in serum-free DMEM supplemented with 50 µg/ml Con A for 24 h (Overall and Sodek, 1990). After treatment, conditioned media and cells were harvested for protein analysis and RNA extraction.

### RNA Extraction

Tissues were homogenized in 4 M guanidinium thiocyanate (Merck, Darmstadt, Germany). Total RNA was purified by ultracentrifugation (SW60 rotor, Beckman Instrs., Fullerton, CA, 35,000 rpm) through a 2.5-ml cushion of 5.7 M cesium chloride. Cells in culture were washed with PBS, and RNA was extracted using the single-step method of Chomczynski and Sacchi (1987).

### Construction of a Rat Skin Wound cDNA Library and Cloning of cDNAs Encoding MT1-MMP

Poly(A)<sup>+</sup>RNA from rat skin wounds was purified using the Pharmacia purification kit (Uppsala, Sweden). The RNA thus isolated was used to construct a cDNA library with the Uni-ZAP<sup>TM</sup>XR Vector kit (Stratagene, La Jolla, CA). This library was screened with size-selected (~350–550 bp) RT-PCR products. For the RT-PCR experiments, poly(A)<sup>+</sup>RNA from rat skin wounds was reverse-transcribed using an oligo (dT) primer, and the resulting cDNA was amplified using a set of degenerate primers corresponding to the cysteine-switch and zinc-binding domains of rat MMPs (5'-C(CT)N(AC)GNTGTGGN(AG)(AT)NCCNGA, and 5'-(CT)C(AG)A(AG)(CT)TC (AG)TGNGC(AT)GC(AC)AC, respectively). Additional screening was conducted using cDNA probes (see Results) encoding rat GelA (nucleotides 482-2306; accession number No. U65656), GelB (nucleotides 517-2392), ST1 (nucleotides 418-1771), ST3 (nucleotides 644-1810), and Col3 (nucleotides 1387-2606). The clones which were only recognized by the RT-PCR probe, were plaque-purified and used for plasmid rescue. After cross-hybridization experiments, all independent clones were sequenced from their 5'-terminus. The full nucleotide sequence of rat *MT1-MMP* cDNA was determined on both strands as previously described (Okada et al., 1995a). Nucleotide and amino acid sequences were analyzed using the sequence analysis software of the Wisconsin program package.

### Cloning of cDNAs Encoding Rat TIMP2 and TIMP3

Rat tissue inhibitor of metalloproteinase type-2 (*TIMP2*) (Santoro et al., 1994) and *TIMP3* (Wu and Moses, 1996) cDNA fragments containing the complete open reading frame sequence were obtained by PCR amplification from a previously described skin wound library (Okada et al., 1994) and a 13.5-d placenta library in the λ ZAPII vector, respectively. The PCR products were cloned into the pBluescript vector and sequenced.

### RNA Analyses

For Northern blot analysis, 10 µg of total RNA from rat tissues was electrophoresed on formaldehyde-agarose (1%) gels and transferred to Hybond-N membranes (Amersham, Buckinghamshire, UK), according to the supplier's instructions. Hybridizations were carried out with <sup>32</sup>P-labeled cDNA probes in 50% formamide at 42°C. The cDNA probes used for these hybridizations were identical to those used for the differential

screening of the skin wound cDNA library. An additional probe used for hybridization was rat *MT1-MMP* cDNA (nucleotides 911-2410).

For in situ hybridization, tissue sections from rat normal skin and skin wounds were collected as described above. Sections were cut perpendicularly to the cutaneous incision. <sup>35</sup>S-labeled RNA sense and antisense probes were synthesized from DNA templates identical to those used for Northern blot analyses, using either the T3 or T7 polymerase. Probe length was reduced to an average size of 150 nucleotides by limited alkaline hydrolysis before hybridization as previously described (Okada et al., 1995a). Frozen sections were fixed with 4% paraformaldehyde, dehydrated, and hybridized with <sup>35</sup>S-labeled RNA probes. After washing, autoradiography was performed using NTB2 emulsion (Kodak, Rochester, NY). Slides were then developed and counterstained with toluidine blue.

### Construction of Expression Plasmids for Rat MMPs and TIMPs

To express rat MT1-MMP in *E. coli*, a cDNA fragment encoding the putative catalytic domain and the hemopexin-like domain (amino acids 112-508) was inserted into the pET15b vector containing an NH<sub>2</sub>-terminal 6-histidine tag (Novagen, Madison, WI). For expression in COS-1 cells, cDNA fragments containing the complete open reading frame sequence of rat MT1-MMP, GelA, GelB, ST1, ST3, TIMP1 (Okada et al., 1994), TIMP2, and TIMP3, were inserted into the pTL1 expression vector as previously described (Okada et al., 1995b). The resulting plasmids, pET15bMT1-MMP (MT1-MMP, amino acids 112-508), pTL1MT1-MMP (pro-MT1-MMP), pTL1GelA (pro-GelA), pTL1GelB (pro-GelB), pTL1ST1 (pro-ST1), pTL1ST3 (pro-ST3), pTL1TIMP1 (TIMP1), pTL1TIMP2 (TIMP2), and pTL1TIMP3 (TIMP3) were verified by restriction enzyme digestion and DNA sequencing.

### Transient Transfection into COS-1 Cells

pTL1 expression plasmids for rat MMPs and TIMPs, and the control pTL1 vector alone were transiently transfected into COS-1 cells using the calcium phosphate procedure.  $5 \times 10^5$  COS-1 cells per 10-cm diameter culture dish were grown in DMEM supplemented with 5% FCS for 16 h, and transfected with 3  $\mu$ g of each expression plasmid and 12  $\mu$ g of carrier DNA (pBluescript vector) in fresh culture medium. After a 1-d incubation, cells were washed with DMEM and incubated in 3 ml of serum-free DMEM for another day.

### Gelatin Zymography

Zymography was performed as previously described (Okada et al., 1995b), using conditioned media or rat tissues. 20  $\mu$ l of each conditioned medium was mixed with a same volume of Laemmli's 2 $\times$  SDS sample buffer in the absence of a reducing agent, and incubated for 30 min at 22°C. The samples were electrophoresed on 8%-polyacrylamide gels containing 0.2% gelatin. After electrophoresis, gels were soaked in 50 mM Tris-HCl (pH 7.6) containing 2.5% Triton X-100 for 30 min, and incubated with 50 mM Tris-HCl (pH 7.6) containing 150 mM NaCl, 10 mM CaCl<sub>2</sub>, and 0.02% Brij-35, at 37°C for 16 h. Gels were subsequently stained with 0.5% Coomassie brilliant blue R-250. Protein molecular standards were purchased from Bio-Rad (Hercules, CA).

Normal rat skin epidermis and dermis, and proliferative epidermis or granulation tissue from rat skin wounds were carefully microdissected under a light microscope from 30- $\mu$ m thick frozen sections. After microdissection, individual samples were suspended in Laemmli's 2 $\times$  SDS sample buffer (40  $\mu$ l/mg of fresh tissue). After a 30-min incubation at 22°C, the extracted proteins were analyzed by gelatin zymography as described above.

### Western Blot Analysis

Anti-rat MT1-MMP mAb (1MMP-1C1) was obtained by immunizing Balb/c mice with recombinant rat MT1-MMP (amino acids 112-508) purified from *E. coli* inclusion bodies. The antigen was purified by Ni<sup>2+</sup>-NTA-Agarose (Qiagen Inc., Chatsworth, CA) chromatography according to the manufacturer's instruction. The specificity of mAb 1MMP-1C1 for MT1-MMP was demonstrated by immunocytochemical analysis of COS-1 cells transfected with cDNAs for *MT1-MMP*, *MT2-MMP* (Will and Hinzmann, 1995), or *MT3-MMP* (Takino et al., 1995).

Subconfluent transfected COS-1 cells, and RAT1 and FR3T3 rat fibroblasts incubated with serum-free DMEM in the absence or presence of 50  $\mu$ g/ml Con A for 24 h, were scraped and lysed in PBS containing 1% NP-

40 and 5 mM EDTA (100  $\mu$ l/ 10-cm diam culture plate). After centrifugation at 4,000 g for 15 min, supernatants corresponding to crude cell membrane extracts were collected and used for Western blot analysis (Okada et al., 1997). Proteins were separated by electrophoresis performed on 10%-polyacrylamide gels under reducing conditions. The proteins were then electrophoretically transferred to a nylon membrane (Immobilon-P, Millipore, Bedford, MA). After treatment with a blocking solution, the nylon membrane was incubated with anti-rat MT1-MMP mouse mAb 1MMP-1C1, diluted 1/1,000 in PBS containing 2% BSA. The nylon membrane was washed and incubated with peroxidase-conjugated goat anti-mouse IgG antibody (Jackson ImmunoResearch Laboratory, West Grove, PA). After washing in PBS containing 0.05% Tween-20, proteins were detected using a chemiluminescence procedure (Renaissance, DuPont, Boston, MA).

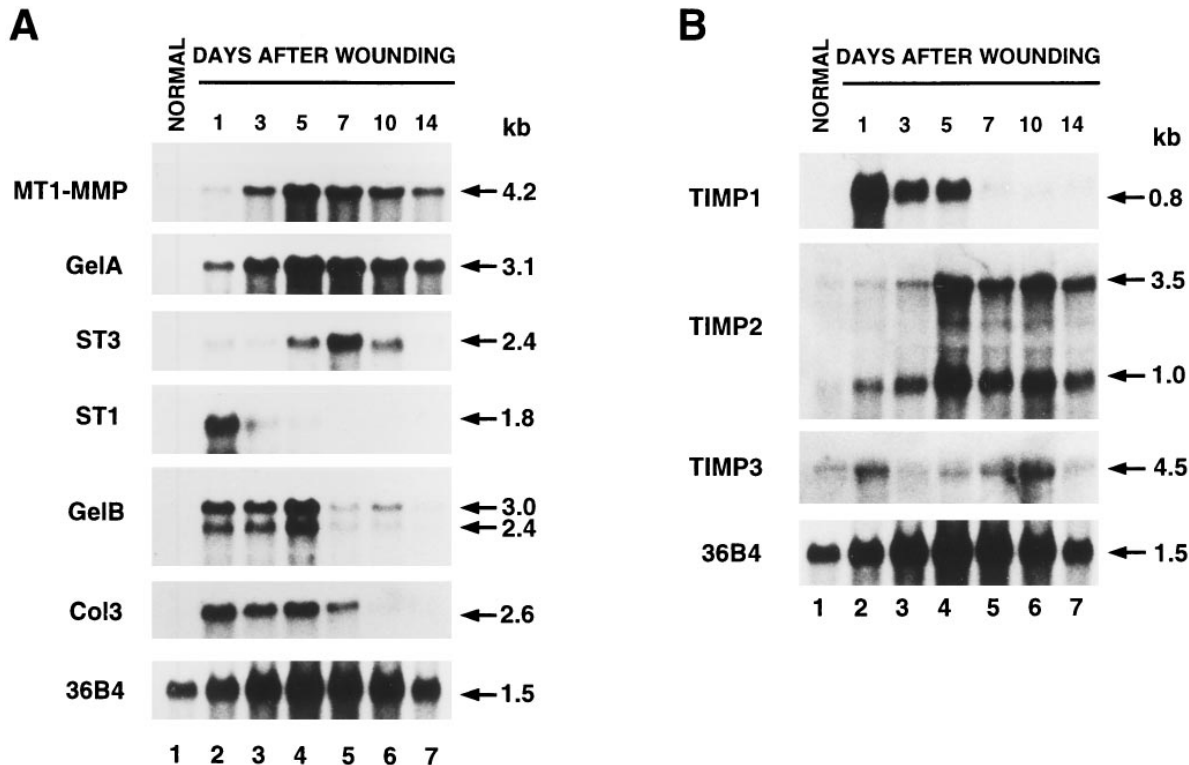
## Results

### Cloning of Rat MT1-MMP from a Skin Wound cDNA Library

To investigate the contribution of MMPs to skin wound healing, we devised a two-step procedure to identify *MMP* genes expressed during this process. As a first approach, screening of a 5-d rat skin wound library constructed in the  $\lambda$ gt10 vector (Okada et al., 1994) with six human *MMP* cDNA probes (*ST2*, *ST3*, *Coll*, *GelA*, *GelB*, and *matrilysin*), led to the identification of four rat *MMP* cDNAs (*ST1*, *ST3*, *GelA*, and *GelB*) (Okada et al., 1995b, 1997, and Okada, A., unpublished results). However, *ST2*, *matrilysin*, and the rat homologue of human *Coll* were not found in the library. A nearly full-length cDNA for rat *Col3* (nucleotides 145-2583) was obtained by screening the library with a partial rat *Col3* cDNA fragment (nucleotides 865-1262, Quinn et al., 1990).

The next step was to determine whether other MMPs were expressed during rat skin wound healing. We constructed a second cDNA library in the Uni-ZAP<sup>TM</sup>XR vector (Stratagene, La Jolla, CA) using RNA prepared from healing wounds on days 3 and 5 after cutaneous incision, which corresponded to the period of concomitant expression of the *MMP* genes already identified (Fig. 1 A). For screening this second library, a probe was designed in such a way that any known or novel members of the *MMP* family would be identified. We amplified 3- and 5-d rat skin wound RNA by RT-PCR, using a set of degenerate primers derived from nucleotide sequences corresponding to the highly conserved cysteine-switch and zinc-binding domains of the six rat MMPs known at that time (*ST3*, *GelA*, *GelB*, *ST1*, *ST2*, and *Col3*) (Fig. 2).

Sixty thousand plaques of the ZAP library were screened with the RT-PCR probe. After dehybridization, the same membranes were hybridized with the five rat *MMP* cDNA probes (*ST3*, *GelA*, *GelB*, *ST1*, and *Col3*) previously isolated. Among the clones that were detected selectively by the RT-PCR probe and not by the *MMP* cDNA probes, we identified 10 cDNAs which had been previously described that did not belong to the *MMP* family, and 11 novel cDNA sequences (data not shown). One of them was found to correspond to a putative novel member of the *MMP* family, and by sequence homology we proposed that it represented the rat homologue of human MT1-MMP (Sato et al., 1994; Okada et al., 1995a). Although three other human MMPs containing a transmembrane domain have since been described (Will and Hinzmann, 1995; Takino et al., 1995; Puente et al., 1996), the rat *MMP*



**Figure 1.** Northern blot analysis of *MMP* and *TIMP* RNAs during rat skin wound healing. Total RNA (10  $\mu$ g) from normal skin (lanes 1) and skin wounds on days 1, 3, 5, 7, 10, and 14 after cutaneous incision (lanes 2–7), were electrophoresed, transferred to nylon membranes, and hybridized with  $^{32}$ P-labeled cDNA probes for rat *MT1-MMP*, *GelA*, *ST3*, *ST1*, *GelB*, *Col3* (A); *TIMP1*, *TIMP2*, *TIMP3* (B). Blots were reprobbed with the *36B4* cDNA used as a loading control (Masiakowski et al., 1982).

that we have identified exhibits the highest sequence homology with human *MT1-MMP* until now (data not shown).

#### Expression of *MMP* RNAs during Skin Wound Healing

*MT1-MMP* RNA was found to be expressed in most normal adult rat tissues (data not shown). However, higher levels of *MT1-MMP* RNA were detected during rat skin wound healing. *MT1-MMP* RNA expression was maximal on day 5 after cutaneous incision (Fig. 1 A). This corresponded to a period of intense stromal activity, filling of granulation tissue in the incisional space, maximal neovascularization, and when collagen fibers were present at the incision margins where the scar tissue was being formed. The expression kinetics of *GelA* RNA was identical to that of *MT1-MMP* (Fig. 1 A). However, *ST3* RNA was highly expressed only on day 5 and reached a peak on day 7. The maximal expression of *ST1* RNA was detected on day 1 after cutaneous incision. *GelB* and *Col3* RNAs were also highly expressed on day 1, and maintained high expression levels until day 5.

We then examined *MMP* transcript distribution by *in situ* hybridization on normal skin sections and skin wound sections from days 1–14 after cutaneous incision. Each wound was examined at its extreme and median portions. The results of analyses performed on days 3 and 5 are presented in Fig. 3. On day 3, various stages of granulation tissue and immature capillary lumens were observed, together with spurs of epithelial cells migrating from both

edges of wounds beneath the surface scab (Fig. 3 A and data not shown). *MT1-MMP* transcripts were detected in cells of the granulation tissue and those of the superficial dermis juxtaposed to the proliferative epithelial cell layer (Fig. 3 B). The transcripts were also observed in stromal cells surrounding hair follicles and especially in the dermal papilla. Most cells expressing *MT1-MMP* transcripts were of the fibroblastic type, thus likely corresponding to fibroblasts or myofibroblasts. However, some cells were round or oval, suggesting that other types of stromal cells, including endothelial cells or macrophages, may also express the *MT1-MMP* gene. While it is clear that additional studies are required to better define the precise nature of these stromal cells, *MT1-MMP* transcripts could not be detected in any epithelial cell. The localization of *GelA* transcripts was similar to that observed for *MT1-MMP*, although the expression levels were higher for *GelA* than for *MT1-MMP* (Fig. 3 C). *ST3* transcripts were undetectable, except in a few stromal cells surrounding hair follicles (Fig. 3 D). *ST1* transcripts were detected in some proliferative epithelial basal cells, and in a few stromal cells juxtaposed to the proliferative epithelial layer (Fig. 3 E). *GelB* and *Col3* RNAs were highly expressed in the epithelial basal cell layer, but not in the dermis (Fig. 3, F and G). On day 5, though granulation tissue was still observed in the median portion of incision (data not shown), reepithelization had been completed at the wound extremities, where thickened epidermis, scar tissue and tissue contraction were observed (Fig. 3 H). *MT1-MMP*, *GelA*, and *ST3* transcripts

MT1-MMP	-----MSPAPRPSRLLPLLTGLTGLASLWGAQ-----SSNFSPFAWLLQVQVYLPFGDLRHTQSRPQSLASAAIAAMQRFYGLQVTGKADSDTKMAMR- <u>RP</u> -RCGVNP	97
ST3	-----MARAACLRAISRALLPLP-----LILLLLLLLPQLMARARFPENHRR-----FVKRVQLLPAALNSLPSVPAHWVGPASSSRPLRCGVNP	88
GelA	MEARLVWGLVGLPRLVCLLGHAAIAPSP-----IIKFPGDVSFK-----TDKELAVQYLNTFYGCPKESCN-----LFLVKDTLKKMKQFFGLQCTGLDQNTLETMR- <u>RP</u> -RCGVNP	106
GelB	-----MNPWQPLLLVLLALGYSFAHPHQRTYVVFPRDLKTSNLTDTQLAEDVLYRYGYTRAQMMGE-----KQSLRFPALLMLQKLSLPTQGLDSETLKAIK- <u>RP</u> -RCGVNP	104
ST1	-----MKGLPVLWLTCAVSSYPLHGSE-----EDAGMEVLQKYLENYGLEKDKVQFTKKKDDSSPVVKKIQEMQKFLGLMKTGLKDSNTMELMH- <u>RP</u> -RCGVNP	94
ST2	-----MEPLAILVLLCFPICSAYLPHGAVR-----Q-----DHSMTDLAQQLYKLYNFRKNEKQFFKRKDDSSPVVKKIEMQKFLGLEMTGLKDSNTVEMMH- <u>RP</u> -RCGVNP	96
Col3	-----ATFLLSWTHCWSLPLPYGDD-----DDDLSEEDLEFAEHLKSYHYE-VTLAGLKKSTVTSVDRLEMQSFFGLDVTGKLDPTLDMR- <u>RP</u> -RCGVNP	95
Matrilysin	-----MAAMRILTFRICVLLPGLCALPLSQEAG-----EVTALQWEQQAQYRKLRLHDSKTKK-----ATSADVLRKEMQKFFGLPETGKLSFRVMEIQ- <u>RP</u> -RCGVNP	94
	* * * * *	
MT1-MMP	KFGTEIKANVRKRYAIQG--LKWQHNEITFCIQNYTPKVEYATFAIRKAFRVMSATPLFRFREVYAIYREGHEKQADIMILFAEGFHGDSPTFFDGGGLAHAYFPGNIGDTHF	215
ST3	FPD-VLNAARNRQKRFVLSGGR--WEKTDLYRILRFWQVLRQVQRTVAEARLWVSEVITPLTFTEVH-----EGRADIMIDFTRYVHGDNLFFDGGGILAHAFPKTRREGDVHF	197
GelA	VAN-----YNFPRKPKMKDQKITRYIIIGYTPDLDPETVDDAFARALKVMSDVITPLRFSRIH-----DGEADIMINFRGWEHGDGYFFDGGGLLAHAFAGTGVGSDGSHF	207
GelB	VGK-----PQTFFGLDMLKHHHNTIYVIQSYTEDLPRVDVDDSPARAFVMSAVTFLTFTRVY-----GLEADIVIQGVARHGDGYFFDGGGLLAHAFPGPGIQDAHF	205
ST1	VGG-----PSTFFPGSPKMKKNIHSYRIVNYTLDLPRVSDSAIERALKVMSDVITPLRFSRIH-----EGEADIMISFAVERHGDGYFFDGGGLLAHAFAGTGVGSDGSHF	195
ST2	VGG-----PSTFFPGSPKMKKNIHSYRIVNYTLDLPRVSDSAIERALKVMSDVITPLRFSRIH-----EGEADIMISFAVERHGDGYFFDGGGLLAHAFAGTGVGSDGSHF	196
Col3	VGV-----YNVFPRFLKWSQNTLYRIVNYTLDLPRVSDSAIERALKVMSDVITPLRFSRIH-----EGEADIMISFAVERHGDGYFFDGGGLLAHAFAGTGVGSDGSHF	197
Matrilysin	VAE-----FSLMNPNSPKHSRTVYRIVSYTDLRPLRVDQIVKRALRMSMQIPLNFKRVS-----WGTADIIIGFARGDHDNFFDGGGLLAHAFAGTGVGSDGSHF	195
	* * * * *	
MT1-MMP	DSAEPTWVQNE-----	227
ST3	DYDETWTIG-----	207
GelA	DDDELWLTGEGVVRVRYKGNADGEYCKPFLFNGREYSCTDTRGSDGLKWCSTTYNFEKDKGVGFCHEALFTMGGGNGDQCKPFFRFQCTSYNSCTEGRTDGYRWCCTTEDYDRDK	327
GelB	DDDELWLTGEGVVRVRYKGNADGEYCKPFLFNGREYSCTDTRGSDGLKWCSTTYNFEKDKGVGFCHEALFTMGGGNGDQCKPFFRFQCTSYNSCTEGRTDGYRWCCTTEDYDRDK	325
ST1	DDDERWTD-----	205
ST2	DDDEKWSLG-----	207
Col3	DDDETWTSS-----	206
Matrilysin	DKDEWTDGE-----	206
	* * * * *	
MT1-MMP	-----DLNGNDIPLVAVHELGHALGLEHSDPSAIMAPPYQWMDTEN-FVLPDDDRRGIQ	281
ST3	-----DKGTDLLEVAAEHFGHVLGQHTIAAKALMSPFYTF--RYPLSLSPDDRRIQ	258
GelA	KYGFPCPETAMSTVGG--NSEGAPCVFFPTFLGNKYESCTSAGRSDDKQVWCATTNYDDRRKWSFCDDQGYSLLEVAAEHFGHAGLGHSDPSAIMAPPYQWMDTEN-FVLPDDDRRGIQ	442
GelB	LYGFCPTRADVTVTGGNSAGEMCVFFVFLGKQYSTCTGEGRSDDGLWCATTSNFDADKKWGFCDQGYSLLEVAAEHFGHAGLGHSDPSAIMAPPYQWMDTEN-FVLPDDDRRGIQ	443
ST1	-----VTGNTLPLVAAEHGHSLGLFHSNAEALMYPVYKSTDLARPHLSQDDVDGQIS	259
ST2	-----PSGTNLPLVAAEHGHSLGLFHSNKEKSLMYPVYKSTDLARPHLSQDDVDGQIS	261
Col3	-----SKGYNLPLVAAEHGHSLGLDHSKDPGALMFPYIYTGKSHFMLPDDVDGQIS	259
Matrilysin	-----DSGVNPLVAVHELGHSLGLHSDPSVSSVMYPTQYQ--DHSDFSLTKDIAGTQ	259
	* * * * *	
MT1-MMP	LYG-----SKSGS-PTKMPQPR-----TTSRSPVDPKFRNPTVYGNICDG-----NFDVTAMLRGEMFVKERWFRVRNQG--VMVGQYF	355
ST3	LYGR-PQLTPTSPPTLSSQAGTDTN-----ETALQEFVPP--EVCETS-----FNAVSTIRGELFFPKAGFVWLRSGG--LQGVYFA	333
GelA	LYG-----PSPDADTDTGTG-----TPTLGGVPTG-----EICKD-----IVFDGIAQIRGEIFFKDRFIRWTVTPR--DKTGTPL	509
GelB	LYGRGSKPDRPAPATAAEPQPTAPPTMCPATPAPMAYTGGTVAFTGAPSPGPTG-----PTAGSEAPTESSTFVDFNCPNDVFDATADIQGLHFFKDRYKWFNSHSGSQGGPF	557
ST1	LYG-----PPTSEFDLVVPTKSN-----SLDFETL-----PNCSSALS-----FNAVTLRGEVLFKDRHFRKSLRT--PEPGFY	325
ST2	LYG-----ARPS--DATVAVPVS-----SKPETP-----VKCDPALS-----FNAVTLRGEVLFKDRHFRKSLRT--PEPEFH	326
Col3	LYG-----FGDED--PNPK-----HFKTP-----EKCDPALS-----LDAITSLRGETMIFKDRFFWRLHPQQ--VEPEFH	316
Matrilysin	LYG-----FGDED--PNPK-----HFKTP-----EKCDPALS-----LDAITSLRGETMIFKDRFFWRLHPQQ--VEPEFH	267
	* * * * *	
MT1-MMP	PIQGFWRGLPASINTAYER--KDGKVFVFKGDKHWVDEASLEPGYFKHIELGRGLPTDKIDAAFLWM--PNGKTYFRRGNKYRNFNEFRAVDSYVKNK--VWBGIPESPRGSMFGSDE	472
ST3	LASRHWQLPSPVDAAFED--AQQIWFQGAQYVWVYDGEKPVLG--PAPLSKLGQ--SPVHAALVWGPENKIYFRRGGDYWRPHRPTQRVDNVPVRRRT--DWRGVPEIDAAFQD--AE	447
GelA	LVATWPELPEIKDAIYVBAPOBEKAVFAGNEVWVYSASTLGERGVFKLTLGLPLPDDVQVQVDAAFNWS--KNKTYIFSGDKFWRVYVKKKMDPQFKLIADSNWAIPTNLDVAVDLQGG	628
GelB	LIARTWPALPAKLNQAFEDPQSKIFFFGRKRWVYVYTGQSVLQ--FRSLDKLGLGSEVTLVTLGLR--RGGKALLISRERIKWFDLKSQKVDQSVTRLDNEFSGVFNWVHVFYQDK	673
ST1	LISFVWPLPSNMDAAVEVNTDVTVEILKGNQIWARIGHEELAGYFKSIHTLGLPETVQKIDAAISLK--DQKTYFVVDKFWRDEKQKSMDEPFPRKIAENPFQIGTKVDAVFEA--F	442
ST2	LISAFWPLPSGLDAAYEANKDRVLIIFKGSQFVAVRGNVEVQAGYFKRIHTLGLPETVQKIDAAVFEK--EKKTYFVVDKFWRDETRQLMDKGFRLITDDPFIQFQVDAVLA--F	443
Col3	LTKSFWPELPHNDAAVEHFSRDLMIIFRGRKFWALNGYDIMEGYFKISDLGFPKVEKRLSAAVHFE--DTGKTLFSGHNVWSDANQTMKDKYPRLIEEFPFIQDKVDAVYK--N	433
	* * * * *	
MT1-MMP	VFTYFYKGNKYWFKNQKLVKVEPYGPKSALRDMWGCPSGGRFDECTEETEVIIIEVDEEGSGAVSAAAVLPLVLLLLVLAAGLVAFFRRHGTPKRLLYQCRSLDKV	582
ST3	GYAYFLRHGLYKFDVVKVLESEFRPIGDFDCAEPAFTFR	491
GelA	GHSYFFKGAAYLKLQNSLKSVKFGSISKS--DWLGC	662
GelB	AYFCHDKYFWRVGFHNRVWQVDHVAIVTY--DLLQCP	708
ST1	GFLYFFGSSQLQEFENAGKVTHTLKS--SWFNC	475
ST2	GFYFFGSSQFEDFNARTVTHLKS--SWLCC	476
Col3	GYIYFNGPIQFEYSIWSNRIVRVMPTN--SLLWC	466

**Figure 2.** Amino acid sequence alignments of rat MMPs. Amino acids are represented using the one-letter code. The predicted amino acid sequences of MT1-MMP, ST3, GelA, GelB, and ST1 were derived from cDNAs cloned from two distinct rat skin wound libraries (Okada et al., 1994, and the present study). One amino acid residue of ST1 (isoleucine 189) differs from the corresponding residue (threonine) in the sequence reported by Matrisian et al. (1985). The sequences of ST2, Col3, and matrilysin were obtained from the PIR-protein data bank. These sequences were aligned using the CLUSTAL program of the Wisconsin program package. Identical amino acid residues in all MMPs are indicated by asterisks below the sequences. The cysteine-switch region (P-LRCGV/NPD), and the zinc-binding domain (XVAXHXHEL/FGHXL/MGLXHS/T) are in the boxed regions. The two regions selected for designing the degenerated oligonucleotides used for RT-PCR amplification are indicated by arrows above the sequences. The putative transmembrane segment (amino acid 539-563) of rat MT1-MMP is overlined.

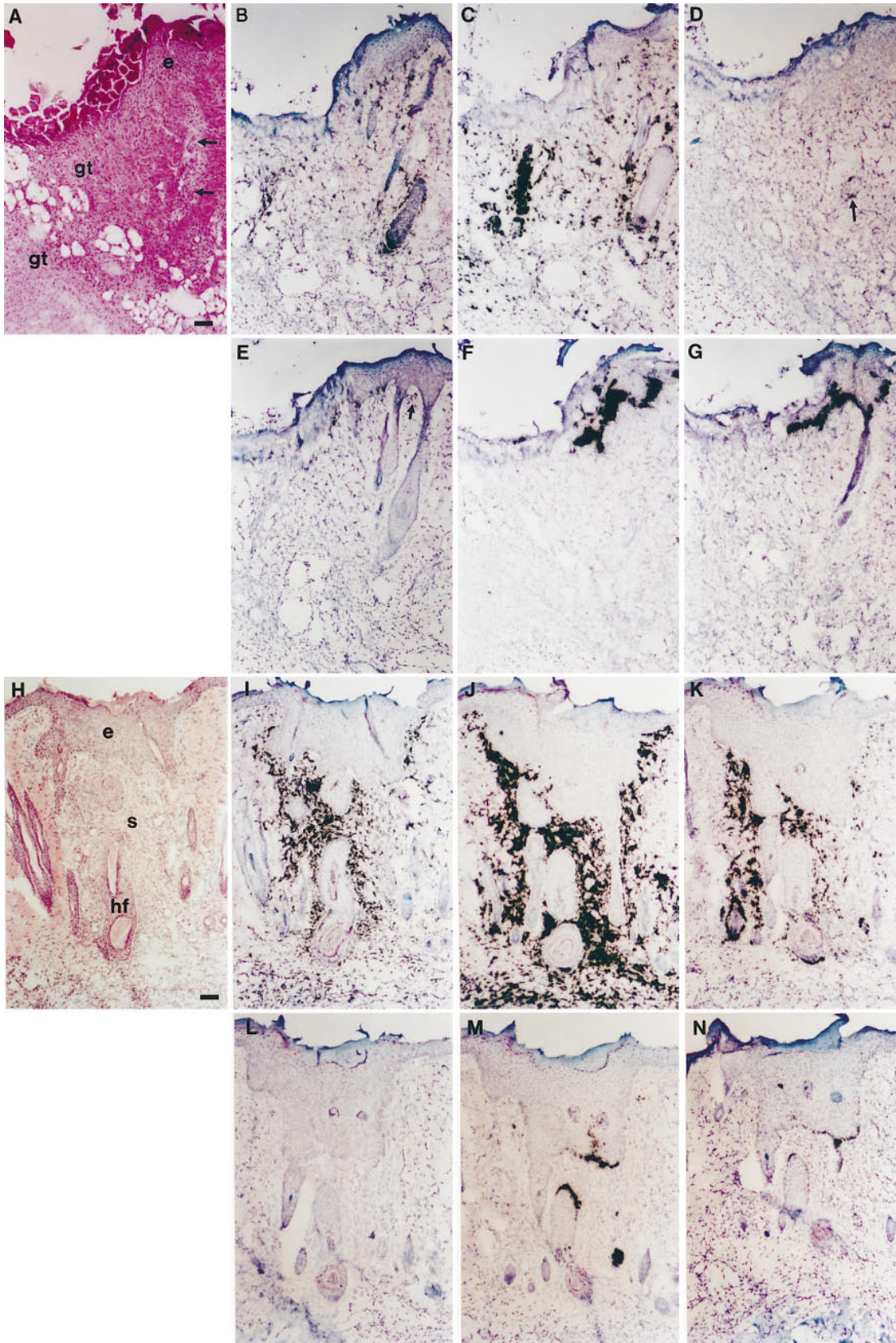
were detected in cells of the scar tissue, below the thickened epithelial cell layer (Fig. 3, I-K). The distribution of *MT1-MMP* transcripts (Fig. 3 I) was more narrow than that of *GelA* (Fig. 3 J), but broader than that of *ST3* (Fig. 3 K). *MT1-MMP*, *GelA*, and *ST3* transcripts were not detected in epithelial cells. *ST1* transcripts were not detected (Fig. 3 L). *GelB* and *Col3* transcripts were intensely expressed in some basal cells of the thickened epidermis, and in some cells of the hair follicle sheath (Fig. 3, M and N).

### MMP Interactions during Skin Wound Healing

Expression vectors encoding rat MT1-MMP, GelA, GelB, ST1, and ST3, were transiently transfected into COS-1 cells, which do not produce any detectable levels of these MMPs endogenously (Okada et al., 1995b, 1997, and data not shown). The expression plasmids were transfected in

pairs into COS-1 cells. Conditioned media were analyzed by gelatin zymography for GelA and GelB production. As shown in Fig. 4 A, pro-GelA was selectively activated by MT1-MMP, and pro-GelB by ST1 only. Other combinations which could not be analyzed by gelatin zymography, were investigated by Western blot analyses and immunoprecipitation, using specific antibodies against ST1 (Matrisian et al., 1986b), ST3 (mAb 5ST-4C10, Santavicca et al., 1995), and MT1-MMP (mAb 1MMP-1C1). None of the other pairs led to activation of the MMP proform (data not shown). Furthermore, MT1-MMP was found exclusively in crude cell membrane extracts and could not be detected in conditioned media (data not shown).

The possibility that MT1-MMP interacts with GelA, and ST1 with GelB, respectively, during rat skin wound healing, appears consistent both with the expression kinetics of their RNAs (Fig. 1 A) and their expression patterns in wound



**Figure 3.** In situ hybridization of rat *MMP* RNAs in cutaneous wounds. Serial frozen sections of rat skin wounds on day 3 (A–G), or day 5 (H–N) after cutaneous incision, were hybridized with  $^{35}\text{S}$ -labeled antisense RNA probes derived from cDNA templates for rat MT1-MMP (B and I), GelA (C and J), ST3 (D and K), ST1 (E and L), GelB (F and M), Col3 (G and N), or stained with hematoxylin and eosin (A and H). Note that the hair follicle which is observed on sections B and C, is only partially visible on A (arrows). In D and E, arrows indicate transcripts expressed in stromal cells surrounding a hair follicle and juxtaposed to the proliferative epithelial cell layer, respectively. No signal above background could be detected with the corresponding  $^{35}\text{S}$ -labeled sense RNA probes (data not shown). *gt*, granulation tissue; *e*, epithelial layer; *hf*, hair follicle; *s*, scar tissue. Bar, 100  $\mu\text{m}$ .

tissues (Fig. 3). Thus, *MT1-MMP* and *GelA* transcripts were detected in stromal cells exhibiting a similar tissue distribution (Fig. 3, *B*, *C*, *I*, and *J*). In the case of *ST1* and *GelB*, the expression patterns of their transcripts were not superimposable on day 5 after cutaneous incision (Fig. 3, *L* and *M*). However, *ST1* and *GelB* transcripts were co-expressed in the proliferative epithelial basal cell layer on day 1 (data not shown) and to some extent on day 3 (Fig. 3, *E* and *F*), when the mature form of *GelB* was detected in wound tissues (see below). We also note that the *GelB* and *Col3* genes exhibited comparable expression kinetics (Fig. 1 *A*) and distribution of transcripts in tissues (Fig. 3, *F*, *G*, *M*, and *N*), suggesting that *GelB* and *Col3* could also interact during rat skin wound healing. However, we could not further evaluate this possibility in the present study, since we could not obtain any full-length rat *Col3* cDNA for protein expression.

### **Pro-GelA Activation by MT1-MMP Is Regulated by TIMP2 and TIMP3**

Activity of mature MMP forms and activation of proforms are regulated by their physiological inhibitors, the TIMPs (Stetler-Stevenson et al., 1996). In particular, TIMP2 has been shown to be both a more efficient inhibitor than TIMP1 in pro-GelA activation (Atkinson et al., 1995) and an active agent in promoting pro-GelA binding to MT1-MMP (Strongin et al., 1995). The effect of TIMP3 on the activation of pro-GelA has not been reported yet. As shown in Fig. 1 *B*, *TIMP1* RNA was not expressed in normal skin, while *TIMP2* and *TIMP3* RNAs were weakly expressed. The induction kinetics of *TIMP1* RNA after cutaneous incision was comparable to that of *ST1*, while that of *TIMP2* was identical to that of *MT1-MMP* and *GelA* (compare Fig. 1, *A* and *B*). However, the induction kinetics of *TIMP3* RNA was different from that of the other *TIMPs* and the six *MMPs* examined. *TIMP3* kinetics exhibited a biphasic pattern, with the highest RNA levels on day 1 after cutaneous incision, which corresponds to the onset of epithelial and mesenchymal cell migrations, and on day 10, when cell migration terminates.

To test the influence of TIMPs on pro-GelA activation by MT1-MMP, we performed triple transfection experiments in COS-1 cells. Different TIMPs and pro-GelA/MT1-MMP expression plasmids were used for these triple transfections. Pro-GelA activation by MT1-MMP was not affected by the presence of TIMP1 (Fig. 4 *B*, lane 3) and only partially prevented by TIMP2 (Fig. 4 *B*, lane 4). However, TIMP3 was found to completely inhibit pro-GelA activation by MT1-MMP (Fig. 4 *B*, lane 5). In contrast to the observations made for *GelA* and MT1-MMP, pro-GelB activation by *ST1* was similarly prevented by all the three TIMPs (Fig. 4 *B*, lanes 7–10). These observations strongly suggest that TIMP3 may be a physiological inhibitor of pro-GelA activation by MT1-MMP.

### **Pro-GelA Is Activated by MT1-MMP-Expressing Rat Fibroblasts**

During rat skin wound healing, *MT1-MMP* and *GelA* transcripts were specifically found in wound stromal cells (Fig. 3, *B*, *C*, *I*, and *J*). This finding prompted us to examine *MT1-MMP* and *GelA* gene expression in two different rat

fibroblast cell lines, RAT1 and FR3T3. Both cell lines were found to express high levels of *MT1-MMP* and *GelA* transcripts (Fig. 5 *A*, lanes 1 and 3). However, the MT1-MMP protein could not be detected either in crude cell membrane extracts or in cytosolic fractions by Western blot analysis (Fig. 5 *B*, lanes 1 and 3, and data not shown). Consistently, media conditioned by these cells contained mainly pro-GelA, as shown by gelatin zymography (Fig. 5 *C*, lanes 3 and 5). Since pro-GelA activation has been demonstrated to be induced by Con A treatment in human fibroblasts (Overall and Sodek, 1990), we then examined whether treatment with con A of these rat fibroblasts was associated with pro-GelA activation. Although both cell lines stimulated by Con A showed only a weak increase in *MT1-MMP* and *GelA* transcript levels (Fig. 5 *A*, lanes 2 and 4), the MT1-MMP protein could now be easily detected by Western blot analysis of crude cell membrane fractions prepared from the Con A-treated cells (Fig. 5 *B*, lanes 2 and 4). Furthermore, as shown by gelatin zymography, the mature form of *GelA* was found to be the major *GelA* species represented in media conditioned by Con A-treated cells (Fig. 5 *C*, lanes 4 and 6).

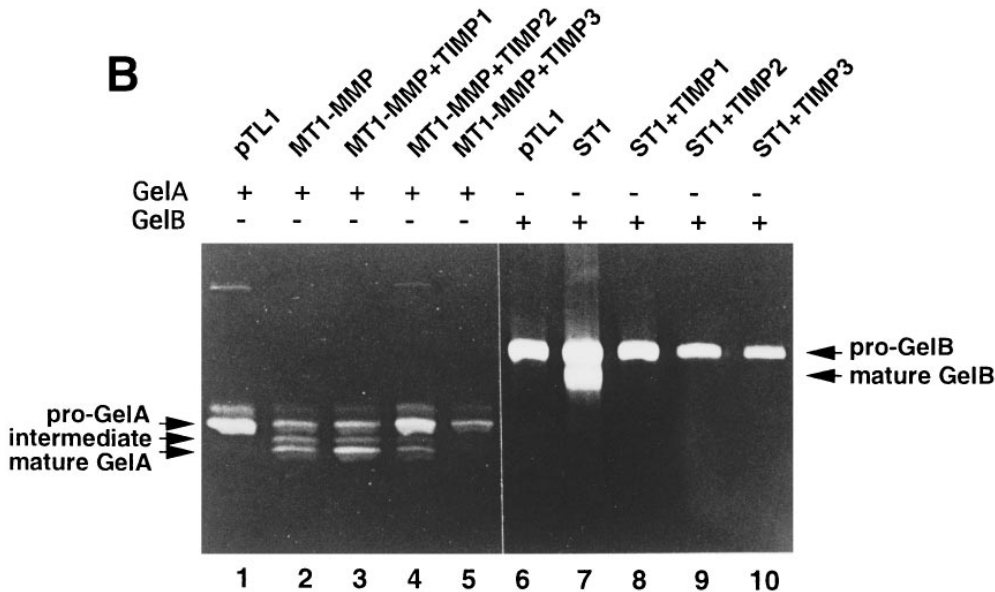
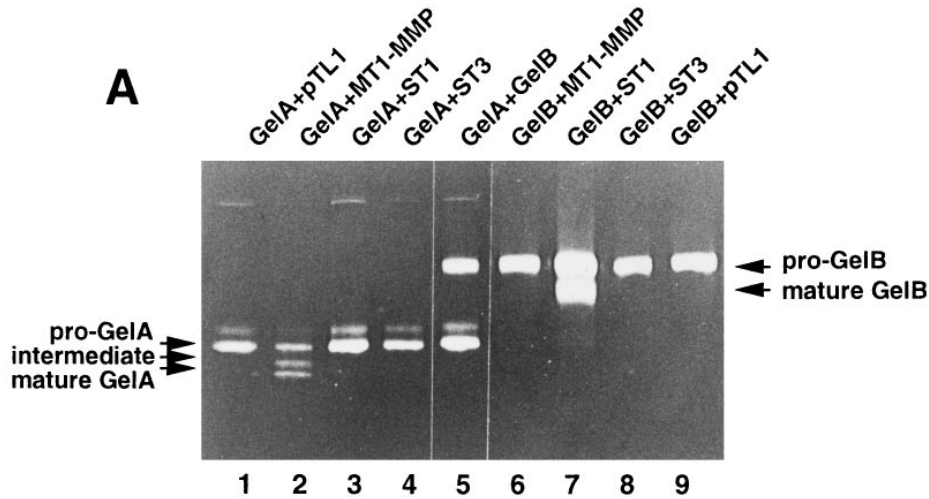
### **Detection of High Levels of GelA and GelB Activities in the Granulation Tissue**

To directly evaluate pro-GelA activation during rat skin wound healing, we analyzed microdissected tissue samples from frozen sections by gelatin zymography. These samples were collected at different times after cutaneous incision. In wound tissues containing both the stroma and epithelium, protein species corresponding to pro-GelA/*GelA* were detected from days 1–14, and pro-GelB or pro-GelB/*GelB* were detected from days 1–7 (Fig. 6 *A*). The level of pro-GelA was maximal on day 5, when the granulation tissue was most active, and pro-GelB expression was elevated all through from days 1–5. However, the mature form of *GelB* was only detected on days 1 and 3, while the mature form of *GelA* was observed, along with its corresponding pro-form, in all the tissue samples examined (Fig. 6). When the granulation tissue and the proliferative epithelial cell layer from healing wounds on days 3 and 5 were analyzed separately, pro-GelA/*GelA* was almost exclusively detected in the granulation tissue (Fig. 6 *B*). Pro-GelB/*GelB* was also predominantly detected in the granulation tissue, with only low levels associated with the proliferative epithelial cell layer. These observations indicate that the wound stroma is the predominant site of action for both *GelA* and *GelB* during rat skin wound healing.

## **Discussion**

### **MMP Gene Expression during Rat Skin Wound Healing**

In the present study, we found that six *MMP* (*ST1*, *ST3*, *GelA*, *GelB*, *Col3*, *MT1-MMP*) genes were expressed during rat skin wound healing. However, we could not detect rat *ST2* and *matrilysin* cDNAs, nor the rat homologue of human *Coll* cDNA in the skin wound cDNA libraries used for our screening. Furthermore, we could detect neither *MT2-MMP* nor *MT3-MMP* RNA in wound tissues by Northern blot analysis (Okada, A., unpublished results).



**Figure 4.** Pro-GelA and pro-GelB activation by MT1-MMP and ST1, in the absence or presence of TIMPs. (A) COS-1 cells were transiently cotransfected with expression plasmids for MMPs whose transcripts were found to be expressed during rat skin wound healing (Figs. 1 and 3), according to the following combinations: pTL1GelA and pTL1 (lane 1) or pTL1MT1-MMP (lane 2) or pTL1ST1 (lane 3) or pTL1ST3 (lane 4) or pTL1GelB (lane 5); pTL1GelB and pTL1MT1-MMP (lane 6) or pTL1ST1 (lane 7) or pTL1ST3 (lane 8) or pTL1 (lane 9). (B) COS-1 cells were transiently cotransfected with pTL1GelA and pTL1 (lane 1), or pTL1GelA and pTL1MT1-MMP (lanes 2–5), or pTL1GelB and pTL1 (lane 6), or pTL1GelB and pTL1ST1 (lanes 7–10), in the absence (lanes 1, 2, 6, and 7) or presence of one of the TIMP expression plasmids: pTL1TIMP1 (lanes 3 and 8), pTL1TIMP2 (lanes 4 and 9), pTL1TIMP3 (lanes 5 and 10). In A and B, 20  $\mu$ l of each conditioned medium was analyzed by gelatin zymography.

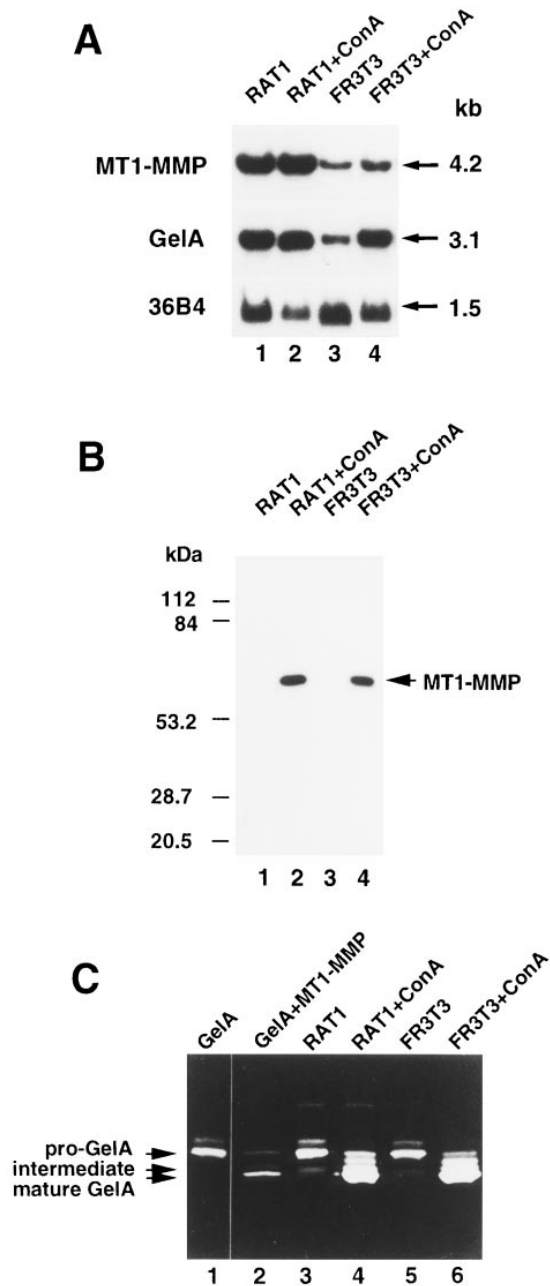
Among the six MMP genes expressed during rat skin wound healing, *ST1*, *GelB*, and *Col3* were mainly expressed between days 1 and 5 after cutaneous incision, while the three others, *MT1-MMP*, *GelA*, and *ST3*, continued to be expressed at high levels at least until day 7. Interestingly, the transcripts corresponding to the early genes were found predominantly in migrating epithelial cells while those corresponding to the late genes were specifically detected in wound stromal cells. However, *MT1-MMP* and *GelA* transcripts were both found in granulation and scar tissues, while *ST3* expression was not detected in granulation tissue.

#### Pro-GelB Activation by ST1

Ogata et al. (1992) have demonstrated that human pro-

GelB was processed to its mature form by human ST1 when both enzymes were incubated together in vitro. In agreement with this observation, we found that rat pro-GelB was processed to its mature form by rat ST1 when the two cDNAs corresponding to GelB and ST1 were transiently transfected into COS-1 cells. The expression kinetics of *GelB* and *ST1* genes, the tissue distribution of cells expressing their transcripts, and the detection of mature GelB protein in wound tissue from days 1 to 3 after cutaneous incision all together support the possibility that pro-GelB is activated by ST1 during rat skin wound healing. The most convincing argument is that while *GelB* transcripts and protein were detected at similar high levels from days 1 to 5 after cutaneous incision, the active form of GelB was only detected from days 1 to 3, consistent with maximal *ST1* gene expression on day 1. Finally, we





**Figure 5.** MT1-MMP gene expression in rat fibroblasts and pro-GelA activation in the presence of Con A. (A) Northern blot analysis. 10  $\mu$ g of total RNA obtained from RAT1 (lanes 1 and 2) or FR3T3 (lanes 3 and 4) rat fibroblasts cultured in the absence (lanes 1 and 3) or presence (lanes 2 and 4) of Con A (50  $\mu$ g/ml) were used in each lane. Blots were hybridized with  $^{32}$ P-labeled rat *MT1-MMP* and *Gela* cDNA probes. Blots were reprobated with the *36B4* cDNA used as a loading control (Masiakowski et al., 1982). (B) Western blot analysis. Crude cell membrane extracts corresponding to half a culture plate of subconfluent RAT1 (lanes 1 and 2) or FR3T3 (lanes 3 and 4) fibroblasts cultured in the absence (lanes 1 and 3) or presence (lanes 2 and 4) of Con A (50  $\mu$ g/ml) were analyzed. Proteins were detected using anti-rat MT1-MMP mouse monoclonal antibody, 1MMP-1C1. (C) Gelatin zymography. 20  $\mu$ l of medium conditioned by COS-1 cells transiently transfected with pTL1Gela (lane 1), or pTL1Gela and pTL1MT1-MMP (lane 2), and 20  $\mu$ l of medium conditioned by RAT1 (lanes 3 and 4) and FR3T3 (lanes 5 and 6) rat fibroblasts cultured in the absence (lanes 3 and 5) or presence (lanes 4 and 6) of Con A (50  $\mu$ g/ml), were used.

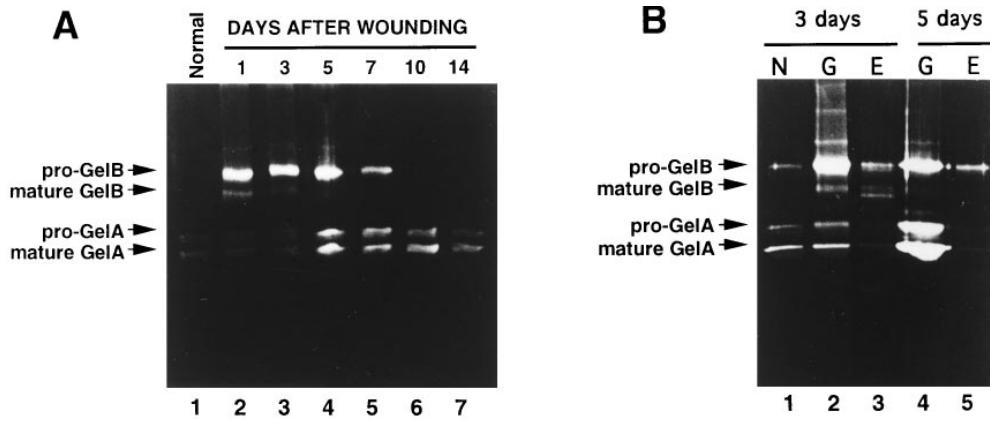
observed that pro-GelB/GelB were predominantly detected in wound stroma, although their transcripts were predominantly expressed in the epithelial basal cell layer. Taken together, these observations support the proposition of Salo et al. (1994) that GelB plays a role in epithelial cell migration, assuming that the substrate for GelB is found in the wound stroma immediately surrounding the migrating epithelial cells.

#### *Pro-GelA Activation by MT1-MMP*

Our finding that pro-GelA was processed to its corresponding mature form when rat *MT1-MMP* and *Gela* cDNAs were transiently expressed in COS-1 cells, is consistent with the previous observation of Sato et al. (1994) made with the human enzymes. However, GelB, ST1, or ST3 could not activate pro-GelA. The possibility that pro-GelA activation by MT1-MMP also occurs during rat skin wound healing is supported by some of our observations. First, *MT1-MMP* and *Gela* transcripts were found to be expressed by wound stromal cells exhibiting a similar tissue distribution. Second, the expression kinetics after cutaneous incision were identical for both genes. Third, high levels of the mature form of Gela were observed in the wound stroma, particularly on day 5 after cutaneous incision when both the *MT1-MMP* and *Gela* RNAs attained their highest expression levels. However, the ratios between the amounts of mature Gela and pro-GelA were found to be similar in all the wound tissue samples examined, and they did not significantly differ from that observed in normal skin. In this respect, pro-GelA activation by MT1-MMP during rat skin wound healing differs from that of pro-GelB by ST1, although both MMPs are predominantly found in the wound stroma. Another difference observed between Gela and GelB, is that the cells expressing *Gela* transcripts are not found specifically at the stromal-epithelial interface. The *Gela* transcripts are in fact distributed throughout the wound stroma, both in the granulation tissue and in the scar tissue. This observation suggests that Gela activated by MT1-MMP contributes to the remodeling processes implicated in the restoration of connective tissue.

#### *MT1-MMP as a Stromal Cell Surface Activator of Pro-GelA*

MT1-MMP is believed to correspond to a transmembrane proteinase whose active site is orientated extracellularly (Sato et al., 1994). Our finding that the *MT1-MMP* and *Gela* genes are coexpressed in stromal cells, suggests that MT1-MMP activates pro-GelA at the stromal cell surface during rat skin wound healing. Consistent with this suggestion, we observed that the induction of MT1-MMP by Con A treatment in two different rat fibroblast cell lines, RAT1 and FR3T3, resulted in pro-GelA activation. We note that similar observations have been made in human skin fibroblasts, where the processing of pro-GelA was coincident with MT1-MMP expression induced by Con A treatment (Atkinson et al., 1995). Furthermore, we have previously shown that *MT1-MMP* and *Gela* transcripts were specifically detected in fibroblast-like cells of breast, colon, and head and neck carcinomas (Okada et al., 1995a). More recently, *MT1-MMP* transcripts and the MT1-MMP protein



**Figure 6.** Gelatinolytic activities during rat skin wound healing. Tissues were microdissected from cryostat sections, incubated with SDS sample buffer, and subjected to gelatin zymography. (A) Normal skin containing both epidermis and dermis (lane 1), and healing skin wounds at days 1, 3, 5, 7, 10, and 14 after cutaneous incision (lanes 2–7). (B) Normal skin adjacent to the wound tissue and containing both epidermis and dermis (N) (lane 1), and granulation tissue (G)

(lanes 2 and 4) or proliferative epithelial layer (E) (lanes 3 and 5) of healing skin wounds at days 3 (lanes 2 and 3) and 5 (lanes 4 and 5) after cutaneous incision. In A and B, proteins extracted from ~1 mg of fresh tissue were analyzed in each lane.

have been shown to be expressed by mesenchymal cells during embryonic development (Kinoh et al., 1996). It remains to be seen, however, whether GelA acts at the same cell surface where it has been activated by MT1-MMP, or on the surface of other cells, or maybe after binding to the ECM. The possibility that GelA binds to cell surface sites distinct from the extracellular domain of MT1-MMP is supported by a recent observation that active forms of GelA bind to integrin  $\alpha v \beta 3$  on the surface of angiogenic blood vessels (Brooks et al., 1996).

Further studies are required to precisely define the sites where GelA is functionally active in remodeling tissues, and to determine whether MT1-MMP and molecules such as integrin  $\alpha v \beta 3$  cooperate in targeting active GelA to the cell surface. The expression of integrin  $\alpha v \beta 3$  and/or molecules with similar GelA-binding function at the stromal cell surface during wound healing would be consistent with our finding that the mature form of GelA is specifically found in the stroma. In this case, by activating pro-GelA, MT1-MMP would contribute to the migration of stromal cells and ECM remodeling implicated in the restoration of the connective tissue during wound healing.

We thank P. Chambon for his support, J.M. Garnier for helpful discussions, R. Kannan for critical reading, I. Stoll for protein purification, S. Vicaire for DNA sequencing, and G. Duval for animal care. We are grateful to Dr. Matrisian (Vanderbilt University, Nashville, TN) for anti-ST1 antibody, to Dr. Will (InViTek GmbH, Berlin-Buch, Germany) for human *MT2-MMP* cDNA, and to Dr. Seiki (Kanazawa University, Kanazawa, Japan) for human *MT3-MMP* cDNA.

This work was supported by funds from the Institut National de la Santé et de la Recherche Médicale, the Centre National de la Recherche Scientifique, the Centre Hospitalier Universitaire Régional, the Bristol-Myers Squibb Pharmaceutical Research Institute, the Association pour la Recherche sur le Cancer, the Ligue Nationale Française contre le Cancer and the Comité du Haut-Rhin, the Fondation de France, the Programme Hospitalier de Recherche Clinique 1995, the BIOMED 2 (contract no BMH4CT96-0017) and BIOTECH 2 (contract no ERBBIO4CT96-0464) Programmes, and a grant to P. Chambon from the Fondation Jeantet. A.Okada was a recipient of fellowships from the Ministère des Affaires Étrangères and the Fondation pour la Recherche Médicale Française.

Received for publication 14 August 1996 and in revised form 4 January 1997.

## References

- Atkinson, S.J., T. Crabbe, S. Cowell, R.V. Ward, M.J. Butler, H. Sato, M. Seiki, J.J. Reynolds, and G. Murphy. 1995. Intermolecular autolytic cleavage can contribute to the activation of progelatinase A by cell membranes. *J. Biol. Chem.* 270:30479–30485.
- Birkedal-Hansen, H. 1995. Proteolytic remodeling of extracellular matrix. *Curr. Opin. Cell Biol.* 7:728–735.
- Breathnach, R., L.M. Matrisian, M.C. Gesnel, A. Staub, and P. Leroy. 1987. Sequences coding for part of oncogene-induced transin are highly conserved in a related rat gene. *Nucleic Acids Res.* 15:1139–1151.
- Brooks, P.C., S. Strömblad, L.C. Sanders, T.L. von Schalscha, R.T. Aimes, W.G. Stetler-Stevenson, J.P. Quigley, and D.A. Cheresh. 1996. Localization of matrix metalloproteinase MMP-2 to the surface of invasive cells by interaction with integrin  $\alpha v \beta 3$ . *Cell.* 85:683–693.
- Carmeliet, P., and D. Collen. 1995. Gene targeting and gene transfer studies of the plasminogen/plasmin system: implications in thrombosis, hemostasis, neointima formation, and atherosclerosis. *FASEB (Fed. Am. Soc. Exp. Biol.) J.* 9:934–938.
- Chomczynski, P., and N. Sacchi. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate phenol chloroform extraction. *Anal. Biochem.* 162:156–159.
- Fisher, C., S. Gilbertson-Beadling, E.A. Powers, G. Petzold, R. Poorman, and M.A. Mitchell. 1994. Interstitial collagenase is required for angiogenesis in vitro. *Dev. Biol.* 162:499–510.
- Gailit, J., and R.A.F. Clark. 1994. Wound repair in the context of extracellular matrix. *Curr. Opin. Cell Biol.* 6:717–725.
- Heppner, K.J., L.M. Matrisian, R.A. Jensen, and W.H. Rodgers. 1996. Expression of most matrix metalloproteinase family members in breast cancer represents a tumor-induced host response. *Am. J. Pathol.* 149:273–282.
- Kinoh, H., H. Sato, Y. Tsunozuka, T. Takino, A. Kawashima, Y. Okada, and M. Seiki. 1996. MT-MMP, the cell surface activator of proMMP-2 (pro-gelatinase A), is expressed with its substrate in mouse tissue during embryogenesis. *J. Cell. Sci.* 109:953–959.
- Knäuper, V., C. López-Otin, B. Smith, G. Knight, and G. Murphy. 1996. Biochemical characterization of human collagenase-3. *J. Biol. Chem.* 271:1544–1550.
- Marti, H.P., L. McNeil, M. Davies, J. Martin, and D.H. Lovett. 1993. Homology cloning of rat 72 kDa type IV collagenase: cytokine and second-messenger inducibility in glomerular mesangial cells. *Biochem. J.* 291:441–446.
- Masiakowski, P., R. Breathnach, J. Bloch, F. Gannon, A. Krust, and P. Chambon. 1982. Cloning of cDNA sequence of hormone-regulated genes from the MCF-7 human breast cancer cell line. *Nucleic Acids Res.* 10:7895–7903.
- Matrisian, L.M., N. Glaichenhaus, M.C. Gesnel, and R. Breathnach. 1985. Epidermal growth factor and oncogenes induce transcription of the same cellular mRNA in rat fibroblasts. *EMBO (Eur. Mol. Biol. Organ.) J.* 4:1435–1440.
- Matrisian, L.M., P. Leroy, C. Ruhlmann, M.C. Gesnel, and R. Breathnach. 1986a. Isolation of the oncogene and epidermal growth factor-induced transin gene: complex control in rat fibroblasts. *Mol. Cell Biol.* 6:1679–1686.
- Matrisian, L.M., G.T. Bowden, P. Krieg, G. Fürstenberger, J.P. Briand, P. Leroy, and R. Breathnach. 1986b. The mRNA coding for the secreted protease transin is expressed more abundantly in malignant than in benign tumors. *Proc. Natl. Acad. Sci. USA.* 83:9413–9417.
- Nomura, H., H. Sato, M. Seiki, M. Mai, and Y. Okada. 1995. Expression of membrane-type matrix metalloproteinase in human gastric carcinomas. *Cancer Res.* 55:3263–3266.

- Ogata, Y., J.J. Enghild, and H. Nagase. 1992. Matrix metalloproteinase 3 (stromelysin) activates the precursor for the human matrix metalloproteinase 9. *J. Biol. Chem.* 267:3581-3584.
- Okada, A., J.M. Garnier, S. Vicaire, and P. Basset. 1994. Cloning of the cDNA encoding rat tissue inhibitor of metalloproteinase 1 (TIMP-1), amino acid comparison with other TIMPs, and gene expression in rat tissues. *Gene (Amst.)* 147:301-302.
- Okada, A., J.P. Belloq, N. Rouyer, M.P. Chenard, M.C. Rio, P. Chambon, and P. Basset. 1995a. Membrane-type matrix metalloproteinase (MT-MMP) gene is expressed in stromal cells of human colon, breast, and head and neck carcinomas. *Proc. Natl. Acad. Sci. USA* 92:2730-2734.
- Okada, A., M. Santavica, and P. Basset. 1995b. The cDNA cloning and expression of the gene encoding rat gelatinase B. *Gene (Amst.)* 164:317-321.
- Okada, A., S. Saez, Y. Misumi, and P. Basset. 1997. Rat stromelysin3: cDNA cloning from healing skin wound, activation by furin and expression in rat tissues. *Gene (Amst.)* 185:187-193.
- Overall, C.M., and J. Sodek. 1990. Concanavalin A produces a matrix-degradative phenotype in human fibroblasts. Induction and endogenous activation of collagenase, 72-kDa gelatinase, and Pump-1 is accompanied by the suppression of the tissue inhibitor of matrix metalloproteinases. *J. Biol. Chem.* 265:21141-21151.
- Puente, X.S., A.M. Pendás, E. Llano, G. Velasco, and C. López-Otín. 1996. Molecular cloning of a novel membrane-type matrix metalloproteinase from a human breast carcinoma. *Cancer Res.* 56:944-949.
- Quinn, C.O., D.K. Scott, C.E. Brinckerhoff, L.M. Matrisian, J.J. Jeffrey, and N.C. Partridge. 1990. Rat collagenase. Cloning, amino acid sequence comparison, and parathyroid hormone regulation in osteoblastic cells. *J. Biol. Chem.* 265:22342-22347.
- Rømer, J., T.H. Bugge, C. Pyke, L.R. Lund, M.J. Flick, J.L. Degen, and K. Danø. 1996. Impaired wound healing in mice with a disrupted plasminogen gene. *Nature Med.* 2:287-292.
- Salo, T., M. Mäkelä, M. Kylmäniemi, H. Autio-Harmanen, and H. Larjava. 1994. Expression of matrix metalloproteinase-2 and -9 during early human wound healing. *Lab. Invest.* 70:176-182.
- Santavica, M., A. Noël, M.P. Chenard, Y. Lutz, I. Stoll, J.P. Segain, N. Rouyer, M.C. Rio, C. Wolf, J.P. Belloq, and P. Basset. 1995. Characterization of monoclonal antibodies against stromelysin-3 and their use to evaluate stromelysin-3 levels in breast carcinoma by semi-quantitative immunohistochemistry. *Int. J. Cancer.* 64:336-341.
- Santoro, M., C. Battaglia, L. Zhang, F. Carluomagno, M.L. Martelli, D. Salvatore, and A. Fusco. 1994. Cloning of the rat tissue inhibitor of metalloproteinases type 2 (TIMP-2) gene: analysis of its expression in normal and transformed thyroid cells. *Exp. Cell Res.* 213:398-403.
- Sato, H., and M. Seiki. 1996. Membrane-type matrix metalloproteinases (MT-MMPs) in tumor metastasis. *J. Biochem.* 119:209-215.
- Sato, H., T. Takino, Y. Okada, J. Cao, A. Shinagawa, E. Yamamoto, and M. Seiki. 1994. A matrix metalloproteinase expressed on the surface of invasive tumour cells. *Nature (Lond.)* 370:61-65.
- Stetler-Stevenson, W.G., R. Hewitt, and M. Corcoran. 1996. Matrix metalloproteinases and tumor invasion: from correlation and causality to the clinic. *Semin. Cancer Biol.* 7:147-154.
- Stricklin, G.P., L. Li, V. Jancic, B.A. Wenczak, and L.B. Nanney. 1993. Localization of mRNAs representing collagenase and TIMP in sections of healing human burn wounds. *Am. J. Pathol.* 143:1657-1666.
- Strongin, A.Y., I. Collier, G. Bannikov, B.L. Marmer, G.A. Grant, and G.I. Goldberg. 1995. Mechanism of cell surface activation of 72-kDa type IV collagenase. Isolation of the activated form of the membrane metalloprotease. *J. Biol. Chem.* 270:5331-5338.
- Takino, T., H. Sato, A. Shinagawa, and M. Seiki. 1995. Identification of the second membrane-type matrix metalloproteinase (MT-MMP-2) gene from a human placenta cDNA library. MT-MMPs form a unique membrane-type subclass in the MMP family. *J. Biol. Chem.* 270:23013-23020.
- Tokuraku, M., H. Sato, S. Murakami, Y. Okada, Y. Watanabe, and M. Seiki. 1995. Activation of the precursor of gelatinase A/72 kDa type IV collagenase/MMP-2 in lung carcinomas correlates with the expression of membrane-type matrix metalloproteinase (MT-MMP) and with lymph node metastasis. *Int. J. Cancer.* 64:355-359.
- Vassalli, J.D., and J.H. Saurat. 1996. Cuts and scrapes? Plasmin heals! *Nature Med.* 2:284-285.
- Will, H., and B. Hinzmann. 1995. cDNA sequence and mRNA tissue distribution of a novel human matrix metalloproteinase with a potential transmembrane segment. *Eur. J. Biochem.* 231:602-608.
- Wu, I.M., and M.A. Moses. 1996. Cloning and expression of the cDNA encoding rat tissue inhibitor of metalloproteinase 3 (TIMP-3). *Gene (Amst.)* 168:243-246.
- Yamada, T., U. Yoshiyama, H. Sato, M. Seiki, A. Shinagawa, and M. Takahashi. 1995. White matter microglia produce membrane-type matrix metalloprotease, an activator of gelatinase A, in human brain tissues. *Acta Neuropathol.* 90:421-424.