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Novel Regulation of Type IV Collagenase (Matrix Metalloproteinase-9 and -2) Activities by Transforming Growth Factor-β1 in Human Prostate Cancer Cell Lines

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The type IV collagenases/gelatinases matrix metalloproteinase-2 (MMP-2) and MMP-9 play a variety of important roles in both physiological and pathological processes and are regulated by various growth factors, including transforming growth factor-β1 (TGF-β1), in several cell types. Previous studies have suggested that cellular control of one or both collagenases can occur through direct transcriptional mechanisms and/or after secretion through proenzyme processing and interactions with metalloproteinase inhibitors. Using human prostate cancer cell lines, we have found that TGF-β1 induces the MMP-9 proenzyme; however, this induction does not result from direct effects on gene transcription but, instead, through a protein synthesis–requiring process leading to increased MMP-9 mRNA stability. In addition, we have examined levels of TGF-β1 regulation of MMP-2 in one prostate cancer cell line and found that TGF-β1 induces higher secreted levels of this collagenase through increased stability of the secreted 72-kDa proenzyme. These results identify two novel nontranscriptional pathways for the cellular regulation of MMP-9 and MMP-2 collagenase gene expression and activities.

INTRODUCTION

The family of matrix metalloproteinases (MMPs)[1] is an expanding group of zinc-dependent metallopeptidases consisting of at least 19 cloned members, including the type IV collagenases MMP-2 and MMP-9, interstitial collagenase, matrilysin, metalloelastase, stromelysin, and membrane-type MMPs (Bernhard, *et al.*, 1994; Murphy and Knauper, 1997). These matrix proteases target basement membrane constituents such as fibronectin, laminin, collagen, proteoglycans, and elastin (Matrisian, 1990; Bernhard *et al.*, 1994). They also act on a growing list of nonmatrix substrates, including insulin-like growth factor–binding protein-3, tumor necrosis factor-α (TNF-α), and fibroblast growth factor receptor 1, and angiogenic factors (Fowlkes *et al.*, 1994; Gearing *et al.*, 1994; Levi *et al.*, 1996; Patterson and Sang, 1997; Brooks *et al.*, 1998) and are involved in a wide array of biological activities such as wound healing, migration, apoptosis, differentiation, tumor invasion, angiogenesis, and growth factor modulation (Albini *et al.*, 1991; Sato and Seiki, 1993; Levi *et al.*, 1996; Gianluigi *et al.* 1997; Murphy and Knauper, 1997).

The type IV collagenases/gelatinases MMP-2 and MMP-9 are secreted as 72- and 92-kDa procollagenases, respectively, and can be subsequently activated by processing (Mazzieri *et al.*, 1997). These collagenases cleave type IV collagen as well as collagens I, III, V, and XI (Murphy and Knauper, 1997), disrupting the basement membrane during physiological processes such as angiogenesis and tissue morphogenesis (Overall *et al.*, 1991; Patterson and Sang, 1997; Brooks

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[1] Abbreviations used: CAT, chloramphenicol acetyltransferase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IL, interleukin; IFN, interferon; MMP, matrix metalloproteinase; PAI-1, plasminogen activator inhibitor type 1; TGF, transforming growth factor; TNF, tumor necrosis factor; TPA, 12-*O*-tetradecanoylphorbol 13-acetate; UTR, untranslated region.

et al., 1998) and pathological events such as arthritis (Koolwijk *et al.*, 1995; Ahrens *et al.*, 1996), glomerulonephritis (Marti *et al.*, 1994), and tumor invasion and metastasis (Liotta *et al.*, 1991; Bernhard *et al.*, 1994; Stearns and Stearns, 1996).

Collagenases are reported to be regulated during synthesis and then after secretion (Matrisian, 1990; Liotta *et al.*, 1991; Fridman *et al.*, 1995; Murphy and Knauper, 1997). MMP-2 is regulated by relatively few polypeptide factors (Sato and Seiki, 1993; Benbow and Brinckerhoff, 1997); however, expression of this collagenase is induced by transforming growth factor-β (TGF-β) in mesangial cells, melanomas, fibrosarcomas, and fibroblast cell lines (Brown *et al.*, 1990; Overall *et al.*, 1991; Marti *et al.*, 1994), by interferon-α (IFN-α) and IFN-γ in melanoma cells after short-term treatment (Hujanen *et al.*, 1994), and by interleukin-8 (IL-8) and IFN in some fibroblasts (Brown *et al.*, 1990; Hujanen *et al.*, 1994; Singh *et al.*, 1995). MMP-9 is induced by several polypeptides, including epidermal growth factor, TGF-α, amphiregulin, TNF-α, IL-1α, IL-1β, IFN-α, IFN-γ, and TGF-β (Okada *et al.*, 1990; Welch *et al.*, 1990; Samuel *et al.*, 1992; Lyons *et al.*, 1993; Hujanen *et al.*, 1994; Sehgal *et al.*, 1996; Kondapaka *et al.*, 1997), as well as by the oncogenes *ras*, *jun*, and v-*src* (Lyons *et al.*, 1993; Sato *et al.*, 1993; Gum *et al.*, 1996) and by phorbol ester stimulation. TNF-α, oncogene, and phorbol ester stimulation of MMP-9 results from increased transcriptional activation (Sato and Seiki 1993; Gum *et al.*, 1996); however, the mechanisms by which other growth factors mediate MMP-9 expression are largely unknown. MMP-2 has been reported to be induced by TGF-β1 through both transcription and increased mRNA stability in human gingival fibroblasts (Overall *et al.*, 1991) and through both changes in mRNA levels and extracellular processing in human fibrosarcomas (Brown *et al.*, 1990); however, detailed studies on TGF-β regulation of MMP-2 in epithelial cell types have not been reported.

We have previously observed that TGF-β1 selectively induced MMP-9 activity in a subset of metastatic but not primary mouse prostate tumors and implicated this TGF-β1–induced response as a potentially important selection step in the development of prostate cancer metastasis (Sehgal *et al.*, 1996). In addition, mRNA levels for both type IV collagenases are elevated in human prostate cancer cell line PC-3 M variants with higher metastatic potential than in those with lower potential (Greene *et al.*, 1997), and reduction of MMP-9 expression in a metastatic mouse prostate cancer cell line with an anti-MMP-9 ribozyme abrogates lung metastasis (Sehgal *et al.*, 1998). MMP-2 and MMP-9 RNA levels are also reported to be clinically increased in higher Gleason grade tumors and in tumors that are no longer organ confined (Stearns and Stearns, 1996; Wood *et al.*, 1997). Because TGF-β1 upregulation of type IV collagenase activities may play an important role in prostate cancer invasion and metastasis and could also influence a diverse set of other physiological and pathological cellular processes, we have investigated the potential levels for TGF-β regulation of MMP-9 and MMP-2 collagenases using human prostate cancer cell lines.

Although MMP-9 transcription is activated by other growth factor pathways, we have found that TGF-β1 does not stimulate transcription of this collagenase but instead appears to induce MMP-9 through increased mRNA stability. We further show that in one cancer cell line, TGF-β can regulate MMP-2–secreted protein levels. This novel level of regulation occurs through delayed decay of secreted enzyme activity rather than by altering MMP-2 transcription levels, mRNA stability, or translated intracellular protein levels. Collectively, these studies demonstrate the potential for additional levels of MMP regulation by growth factors and underscore the importance of understanding the complex interactions of gene activities under the direct and indirect control of TGF-β in malignancy (Ren *et al.*, 1998).

MATERIALS AND METHODS

Cell Culture

The human prostate cancer cell lines ND-1 (obtained from Dr. Perinchery Narayan, University of Florida, Gainesville, FL) and Tsu-Pr1 (obtained from Dr. Marco Marcelli, Baylor College of Medicine, Houston, TX) were subcultured in DMEM with 10% FCS, 100 U/ml sodium penicillin, and 100 μg/ml streptomycin. The DU145 cell line (obtained from the American Type Culture Collection, Gaithersburg, MD) was cultured in MEM with 10% FCS, penicillin, and streptomycin and 1.0 mM L-glutamine. Cell cultures were treated with TGF-β1 (obtained from R & D Systems, Minneapolis, MN) at 2 ng/ml, actinomycin D (Sigma, St. Louis, MO) at 10 μg/ml, and cycloheximide (Sigma) at 10 μg/ml. Actinomycin D and cycloheximide were added to cultures for 15 min and 1 h respectively, before addition of growth factors. For actinomycin D, this period of pretreatment effectively blocked >99% of transcriptional activity as assayed by [^{3}H]uridine uptake. 12-*O*-Tetradecanoylphobol 13-acetate (TPA) was used at a concentration of 50 ng/ml.

For the analysis of cell-free MMP activities and protein levels, Tsu-Pr1 cultures were stimulated with or without TGF-β1 for 48 h in DMEM, after which all medium was harvested and incubated without cells for 0, 1, or 2 additional days and then concentrated as described (Sehgal *et al.*, 1996).

Zymography

Type IV gelatinase/collagenase activity was assayed through acrylamide gel zymography as described (Sehgal *et al.*, 1996).

Immunoblotting

Conditioned Media. DMEM without additives was collected and centrifuged 1500 $\times$ *g* to remove particles and then concentrated to 650 μl (Centriprep-10 concentrator; Amicon, Beverly, MA). Medium concentrates were electrophoresed under nonreducing conditions and without heating through a 7.5% SDS-PAGE gel.

Cell Lysates. Culture plates were washed twice with PBS, and then cells were gently pelleted in PBS, followed by lysis as described

(Laiho *et al.*, 1990). Cytoplasmic protein samples (50 μg/ml) were electrophoresed under nonreducing conditions through a 7.5% SDS-polyacrylamide gel. After electrophoresis, both conditioned media and cell lysate samples were transferred to polyvinylidene difluoride nylon membranes as described (Sambrook *et al.*, 1989). Membranes were blocked for 2 h (5% nonfat dry milk in Tris-HCl, pH. 7.5, 140 mM NaCl) at room temperature and then incubated overnight at 4°C with each primary antibody. Antibodies and concentrations or dilutions used were anti-MMP-9 polyclonal antibody (pAB109; a generous gift from Dr. William G. Stetler-Stevenson, National Cancer Institute, Bethesda, MD), 1:5000 dilution; anti-MMP-2 monoclonal antibody (AB-3; Calbiochem, La Jolla, CA), 1 μg/ml; anti-TGF-β1 monoclonal antibody (R & D systems), 1 μg/ml; and anti-human plasminogen monoclonal antibody (American Diagnostica, Greenwich, CT) 1 μg/ml. Immunoblot bands were detected using enhanced chemiluminesence (Amersham, Dallas, TX).

Northern Blotting

MMP-9. Total RNA was extracted from all cell cultures using Ultraspec RNA reagent (Biotex Laboratories, Houston, TX) and then purified to poly(A^+) RNA (Oligotex mRNA midi kit; Qiagen, Hilden, Germany). Samples of poly(A^+) RNA were denatured and electrophoresed through formaldehyde/1.0% agarose gels (SeaKem; FMC Bioproducts, Chicago, IL) and transferred onto Zeta Probe membranes (Bio-Rad, Hercules, CA) as described (Ausubel *et al.*, 1989). Membranes were prehybridized, hybridized, and washed as according to the Zeta Probe membrane instructions. Hybridizations were performed using a riboprobe (in vitro transcription kit; Boeringer Mannheim, Indianapolis, IN) generated from a pBluescript KS vector containing a 2440-bp fragment of the 92-kDa collagenase (MMP-9) cDNA (a gift from Dr. Barry Marmer, Washington University, St. Louis, MO). Blots were cohybridized with a random-primed probe for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Egawa *et al.*, 1992).

MMP-2. Total RNA and poly(A^+) RNA were extracted as above and directly electrophoresed through formaldehyde/1.0% agarose gels. Random-primed probes were generated using a 3-kb fragment of 72-kDa collagenase (MMP-2) cDNA (a gift from Dr. Barry Marmer). MMP-9 mRNA used to calculate decay curves was collected at various time points from actinomycin D–treated cultures, and Northern blots from these samples were quantitated (normalized to equal levels of GAPDH) using a Bio-Rad 620 video densitometer and one-dimensional Analyst Macintosh data analysis software.

Nuclear Runoff

Adherent cells were washed twice with cold PBS, scrape collected into 15-ml conical tubes in PBS, and then pelleted and lysed using a lysis buffer containing 0.5% (vol/vol) NP-40, 10 mM Tris-HCl (pH 7.4), 3 mM $MgCl_2$, and 10 mM NaCl. After pelleting the nuclei for 5 min at 500 × *g*, 4°C, nuclear pellets were stored at −70°C in storage buffer containing 50 mM Tris-HCl (pH 8.3), 40% (vol/vol) glycerol, 5 mM $MgCl_2$, and 0.1 mM EDTA. Nuclear runoff transcriptions were carried out by adding 200 μl of 2× reaction buffer (10 mM Tris-HCl, pH 8.0, 5 mM $MgCl_2$, 0.3 M KCl, 1 mM ATP, CTP, and GTP, 5 mM DTT, 100 μCi [α-^{32}P]UTP, 3000 Ci/mmol) to 200 μl of thawed nuclei. After a 30-min incubation at 30°C, runoff samples were treated sequentially with 24 U of RNase-free DNase I in 0.5 M NaCl, 50 mM $MgCl_2$, 2 mM $CaCl_2$, and 10 mM Tris-HCl (pH 7.4), for 15 min at 37°C and then in 200 μg of proteinase K buffer (5% [wt/vol] SDS, 0.5 M Tris-HCl, pH 7.4, 0.125 M EDTA) at 42°C for 30 min. This portion of the runoff procedure is described in detail elsewhere (Ausubel *et al.*, 1989).

Samples were next extracted with 1 ml of 25:24:1 buffered phenol/chloroform/isoamyl alcohol, precipitated with 0.5 vol of 7.5 M NH_4CH_2COOH and 2.5 vol of ethanol, and redissolved in 40 mM Tris-HCl (pH 7.9), 10 mM NaCl, 6 mM $MgCl_2$, and 10 mM $CaCl_2$ with the addition of 200 U of RNase-free DNase I. After a second treatment with proteinase K buffer, each sample was reextracted with phenol/chloroform/isoamyl alcohol, precipitated, and dissolved in 100 μl of hybridization buffer consisting of 50 mM 1,4-piperazine-bis(ethanesulfonic acid) (pH 6.5), 100 mM NaCl, 50 mM NaH_2PO_4, 1 mM EDTA, and 5% SDS (Stevanovic *et al.*, 1997). Aliquots of each sample were counted, and all samples were normalized to yield 5×10^6 cpm/ml. This freshly transcribed RNA was added to membranes that had been previously slot blotted with 5 μg of linearized cDNA plasmid constructs and then prehybridized for 2 h at 65°C. After 3 d of hybridization, each membrane was washed once each with 2× SSC/0.1% SDS, 0.5× SSC/0.1% SDS, 2× SSC/10 μg/ml RNase H, and 2× SSC and exposed to film for 3 d.

Chloramphenical Acetyltransferase (CAT) Assays

Prostate cancer cell lines were transfected (LipofectAMINE; Life Technologies, Grand Island, NY) with a $CsCl_2$-purified plasmid containing the full-length human MMP-9 promoter fused to a CAT reporter gene (Sato and Seiki, 1993; Gum *et al.*, 1996). Additional plasmids (pBabeNeo, pBabe RasNeo, and pBabe TGF-β1Neo) used in transfections have been described previously (Timme *et al.*, 1996). CAT assays were performed as described (Seed and Sheen, 1988; Gum *et al.*, 1996) and normalized according to protein concentration and transfection efficiency based on expression of a cotransfected β-galactosidase vector.

RESULTS

TGF-β1 Induces MMP-9 in Human Prostate Cancer Cells

TGF-β1 stimulates MMP-9 (92 kDa)–secreted activity (Figure 1A) and immunoreactive protein levels (Figure 1B) in the conditioned media of human prostate cancer cell lines ND-1, DU145, and Tsu-Pr1. There was also a significant increase in the level of MMP-2 (72 kDa) after TGF-β1 stimulation in the Tsu-Pr1 line. To characterize the potential intracellular level(s) at which the TGF-β1-induced up-regulation of MMP-9 might occur, Northern blot analyses were performed, which revealed that TGF-β1 stimulated an increase in the levels of steady-state MMP-9 mRNA in each of the three lines (Figure 2).

TGF-β1 Induction of MMP-9 Does Not Involve Transcription

To investigate direct gene transcription as a potential mechanism for the TGF-β1–stimulated increased MMP-9 mRNA levels observed, the ND-1 cell line was chosen for further investigations. This cell line was transfected with a CAT reporter plasmid construct under the transcriptional control of the wild-type (670 nucleotides) MMP-9 promoter (Sato and Seiki, 1993). A positive control vector expressing Ha-*ras* (pBabe-RasNeo) induced promoter-mediated CAT activity; however, there was no significant activation of the MMP-9 promoter after either addition of exogenous TGF-β1 or cotransfection with a TGF-β1–containing plasmid construct (pBabe TGF-β1Neo; Figure 3). This lack of promoter activation was not cell line specific,

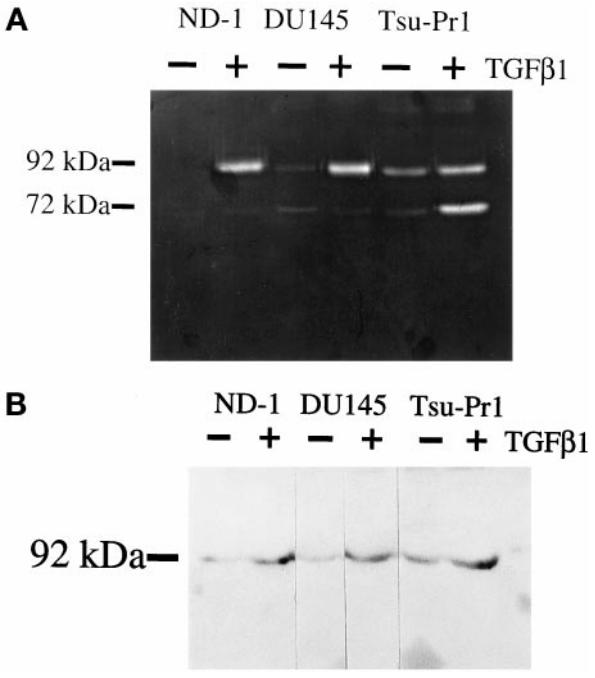

Figure 1. TGF-β1 induces MMP-9 in three human prostate cancer lines. ND-1 cells (lanes 1 and 2), DU145 cells (lanes 3 and 4), and TsuPr1 cells (lanes 5 and 6) were treated without (−) and with (+) TGF-β (2 ng/ml) for 2 d in DMEM. Conditioned media proteins were then collected, concentrated, and analyzed through gelatin zymography for secreted enzymatic activity (A) (the secreted proenzymes of human MMP-2 and MMP-9 are 72 and 92 kDa, respectively) and immunoblotting for MMP-9 protein secretion (B).

because both the DU145 and Tsu-Pr1 cell lines also failed to respond to TGF-β1 with any alteration of MMP-9 promoter activity. To verify that there was indeed growth factor present in these cultures, Western blot analysis was performed on the ND-1 cell conditioned media. These immunoblots (Figure 4A) showed the presence of some exogenous TGF-β1 (Figure 4A, lane 2) as well as high levels of secreted growth factor from pBabe TGF-β1Neo transfect cultures (Figure 4A, lane 3). A band of high-molecular-weight TGF-β1 is also evident, likely a result of monomeric pro-TGF-β1, which is present in conditioned media (Lyons *et al.*, 1990). In addition, MMP-9 enzymatic activity was still secreted into the ND-1 conditioned media after addition of exogenous or plasmid-generated TGF-β1 (Figure 4B).

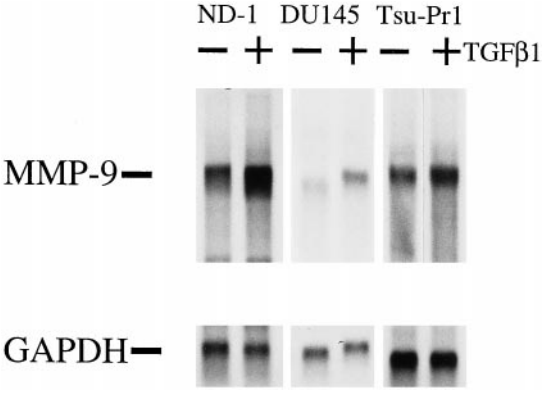

Figure 2. Northern Blot analysis of MMP-9 levels after TGF-β1 stimulation. Poly(A$^+$)-purified samples from untreated (lanes 1, 3, and 5) or TGF-β1–treated (lanes 2, 4, and 6) cells were subjected to Northern blot analysis. These blots were cohybridized with a GAPDH probe to assay loading conditions.

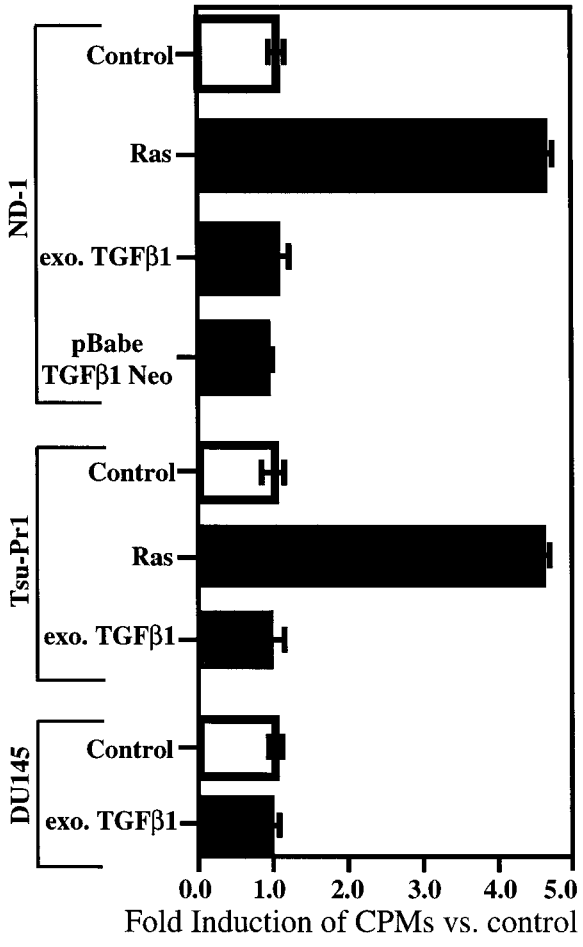

Figure 3. Analysis for potential transcriptional activation of the MMP-9 wild-type promoter by TGF-β1. ND-1, Tsu-Pr1, and DU145 cells transiently transfected with an MMP-9 wild-type (670 bp) human promoter CAT construct (MMP-9 CAT) were stimulated with Ha-*ras* cotransfection (for ND-1 and Tsu-Pr1 cells), exogenous TGF-β1 (for ND-1, Tsu-Pr1 and DU145 cells), or TGF-β1 cDNA cotransfection (ND-1 cells) and then assayed for CAT activity. Data are normalized to the untreated control values, which are equal to 1.0.

Promoter-driven CAT assays are a sensitive but indirect measure of transcriptional response; therefore, to directly examine the effects of TGF-β1 stimulation

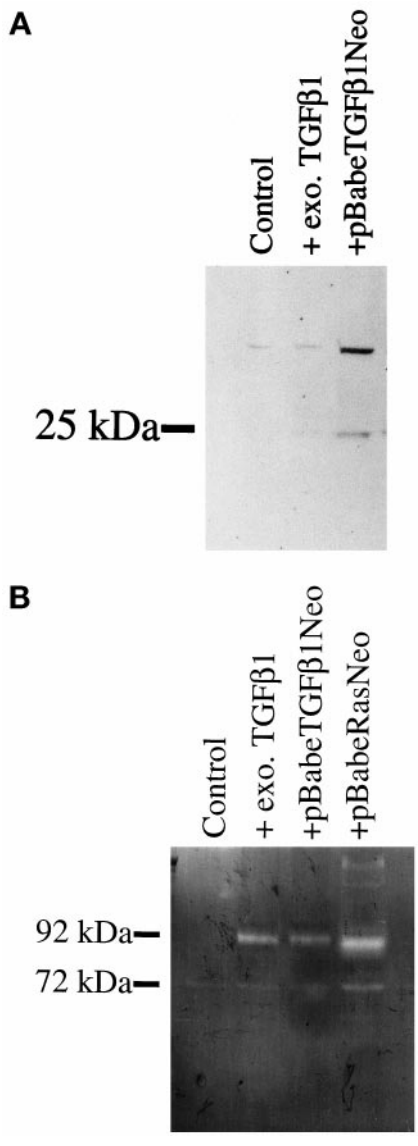

Figure 4. Medium from ND-1 cell cultures used in MMP-9 CAT assays (Figure 3) was further analyzed for TGF-β peptide levels (A) and type IV collagenase activity (B). Lane 1, control samples (transfected with pBabeNeo); lane 2, treated with exogenous TGF-β1; lane 3, transfected with pBabe TGF-β1Neo; lane 4, transfected with pBabeRasNeo (as positive control).

on MMP-9 transcription with the gene in its cellular context, a nuclear runoff assay was performed with ND-1 cell nuclei (Figure 5A). In these assays, TGF-β1 stimulated de novo RNA synthesis for plasminogen activator inhibitor type 1 (PAI-1), a gene known to be transcriptionally activated by TGF-β1 (Keeton *et al.*, 1991), at 12 and 24 h. However, there was no stimulation of MMP-9 RNA synthesis at time points that correlated with the up-regulation of mRNA levels or induction of secreted collagenase activity. Similar results were also obtained using Tsu-Pr1 nuclei collected at 12, 24, and 48 h after TGF-β1 stimulation (Figure 5B). In the Tsu-Pr1 cells, strong RNA synthesis for PAI-1 was noted at 24 h. These runoffs therefore verified our promoter–reporter gene data indicating that TGF-β1 does not induce steady-state mRNA levels of MMP-9 through activation of direct transcription.

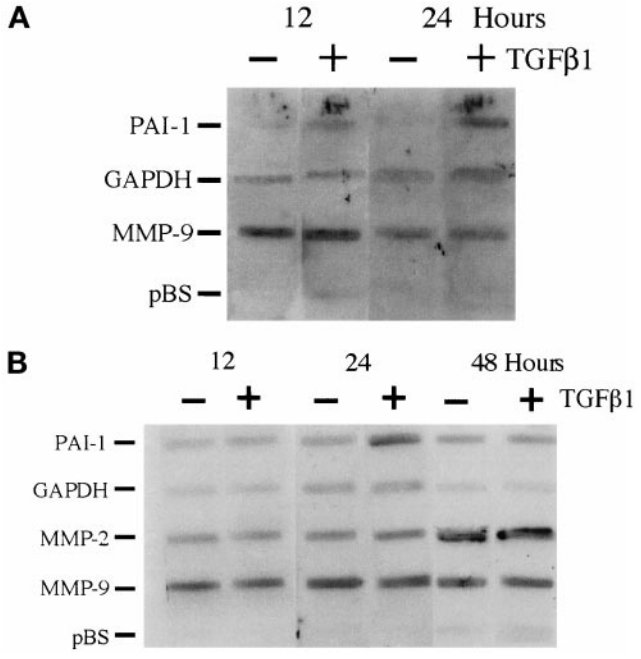

Figure 5. Nuclei were isolated from ND-1 (A) or Tsu-Pr1 (B) cultures and used for nuclear runoffs of transcription. Cultures were grown without (−) or with (+) TGF-β1 for the times indicated in hours. Note: pBluescript KS is a negative control because all plasmid constructs were cloned into a pBluescript background.

TGF-β1 Induces Cell-associated Protein Levels of MMP-9 in the Presence or Absence of Actinomycin D

Posttranscriptional mechanisms of MMP-9 regulation by TGF-β1 could involve changes in mRNA stability and/or alterations in the rate of protein translation, protein half-life, or secretion. To determine whether TGF-β1 up-regulates the level of MMP-9 before secretion, cell-associated levels of MMP-9 were assayed by immunoblot in ND-1 (Figure 6A) and Tsu-Pr1 (Figure 6B) cells. These analyses demonstrated that TGF-β1 stimulated an increase in MMP-9 levels before secretion and further showed that this up-regulation occurs with de novo RNA synthesis blocked by actinomycin D. As expected, cycloheximide addition prevented the increase in intracellular MMP-9 protein levels.

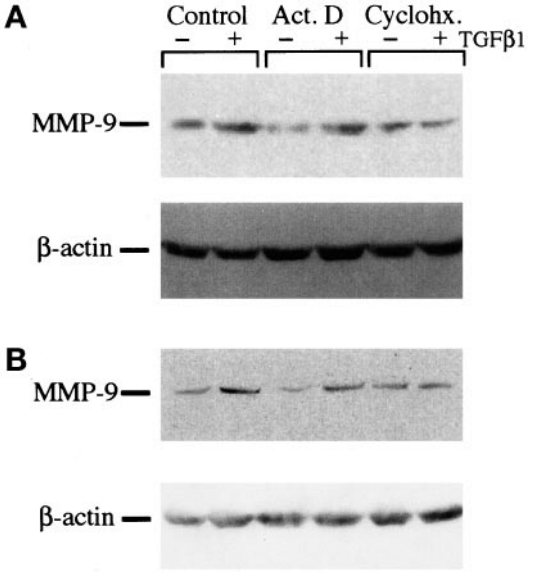

Figure 6. Western blot analysis of cell-associated MMP-9 levels in the absence and presence of actinomycin D or cycloheximide. ND-1 cell (A) and TsuPr1 cell (B) extracts were collected 24 h after treatment with TGF-β1, subjected to 7.5% SDS-PAGE, and immunoblotted using a polyclonal anti-MMP-9 antibody.

TGF-β1 Regulates MMP-9 Posttranscriptionally through Increases in mRNA Stability

The accumulation of mRNA for MMP-9 coupled with the induction of protein levels in the presence of inhibitory levels of actinomycin D strongly suggested that TGF-β1 regulates MMP-9 through increases in mRNA stability. To test this hypothesis in one cell line, MMP-9 mRNA levels in untreated and TGF-β1–treated ND-1 cells were compared in the presence and absence of actinomycin D. As shown in Figure 7A, levels of MMP-9 RNA from TGF-β1–stimulated cells were increased over those levels observed in unstimulated cells after 24 h. A time course of mRNA decay following actinomycin D treatment further demonstrated that in the ND-1 cell line, TGF-β1 increased the stability of MMP-9 RNA (Figure 7B). The time required for a 50% loss of RNA increased from ~19 to 33 h after TGF-β1 stimulation.

TGF-β1 Alteration of MMP-9 mRNA Stability Requires Protein Synthesis

To compare the TGF-β1-induced posttranscriptional regulation of MMP-9 RNA levels with a known transcriptional activator, the phorbol ester TPA was used in the presence and absence of actinomycin D and cycloheximide (Figure 8) in ND-1 cells. Both TGF-β1 and TPA stimulated increased MMP-9 RNA levels; however, the addition of cycloheximide to TGF-β1–treated cells blocked increases in mRNA levels,

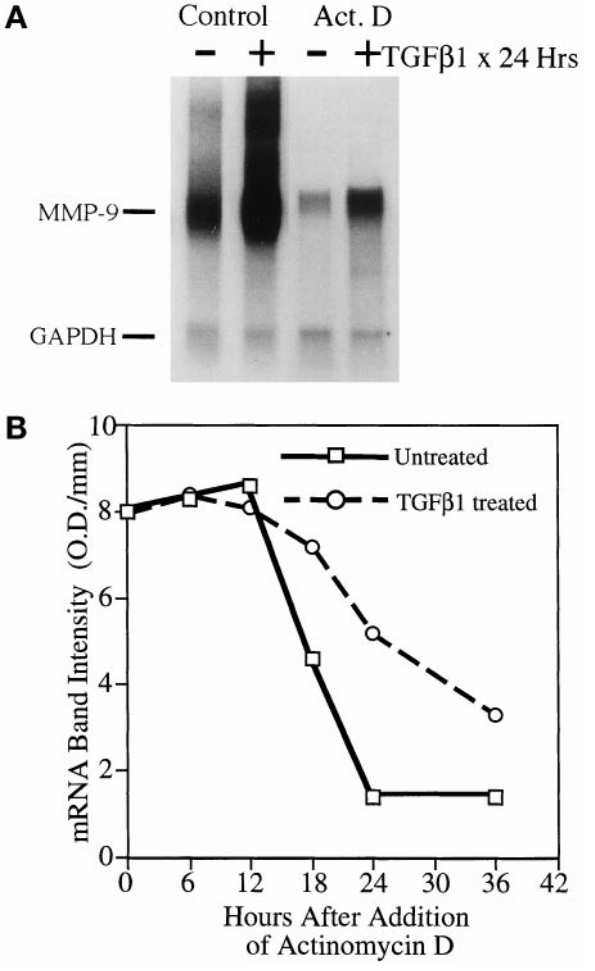

Figure 7. (A) Northern Blot analysis of MMP-9 mRNA levels after actinomycin D treatment and TGF-β1 stimulation for 24 h. (B) Decay time course of ND-1 MMP-9 mRNA levels in the absence or presence of TGF-β1. mRNA was collected at various periods after addition of actinomycin D.

whereas cycloheximide addition had no effect on induction by TPA. These data indicated that newly synthesized proteins are required for TGF-β1 stabilization of MMP-9 RNA, whereas preexisting AP-1 proteins can mediate the TPA transcriptional activity. In contrast to TGF-β1–mediated increases in steady-state RNA levels observed during transcriptional inhibition (Figure 7A), TPA-mediated increases in mRNA levels were completely blocked by the presence of actinomycin D (Figure 8).

TGF-β1 Also Induces MMP-2 through a Posttranscriptional Mechanism

In addition to up-regulation of MMP-9, TGF-β1 stimulation of the Tsu-Pr1 cell line led to a high level of secreted protein activity for MMP-2 72-kDa collage-

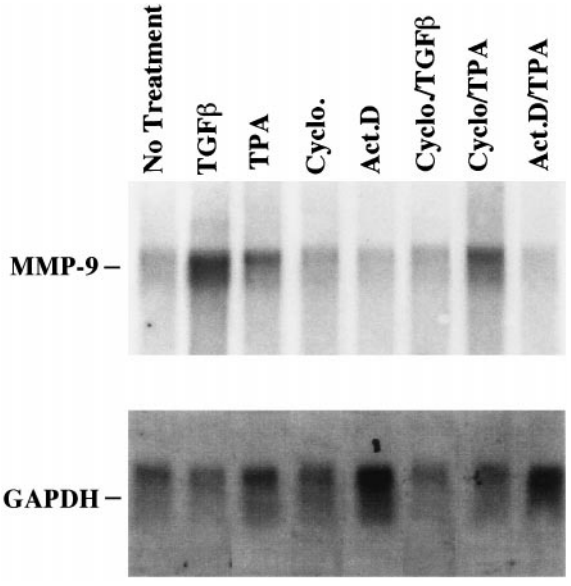

Figure 8. Comparison of MMP-9 mRNA regulation after treatment with TPA (50 ng/ml) or TGF-β1 (2 ng/ml). Upper panel, MMP-9; lower panel, GAPDH.

nase (Figure 1A); therefore, direct transcription and levels of steady-state MMP-2 mRNA were analyzed. As observed with MMP-9, TGF-β1 had no effect on new MMP-2 RNA synthesis (Figure 5B); however, in contrast to the TGF-β1–induced increases in MMP-9 steady-state RNA and intracellular protein, there was no change in either MMP-2 mRNA (Figure 9A) or cell-associated protein levels (Figure 9B) despite the presence of increased MMP-2 protein in the conditioned medium (Figure 9B). Thus, TGF-β1 appeared to modulate presecreted quantities of MMP-9 but not MMP-2. Because most MMPs are not stored before secretion (Woessner, 1991), we explored the possibility that TGF-β1 may regulate MMP-2 levels through an extracellular mechanism. Secreted collagenase activity in cell-free conditioned media was assayed by zymography over an extended period. These results revealed that medium from untreated cultures contained greatly reduced levels of detectable gelatinase activity after 2 d of incubation at 37°C (Figure 9C), whereas the medium collected from TGF-β1–treated cells retained gelatinase activity over this period.

Recent data have suggested the possibility of important biological interactions between the plasminogen activator systems and the metalloproteinase systems (Mazzieri *et al.*, 1997; Farina *et al.*, 1998). These studies have demonstrated that the serine proteinase plasmin can degrade soluble MMP-2 and MMP-9 under cell-free conditions similar to those we have used and that tissue inhibitor of metalloproteinase-2 can protect MMP-2 from such degradation. We have also shown in this study that TGF-β1 induced transcription of

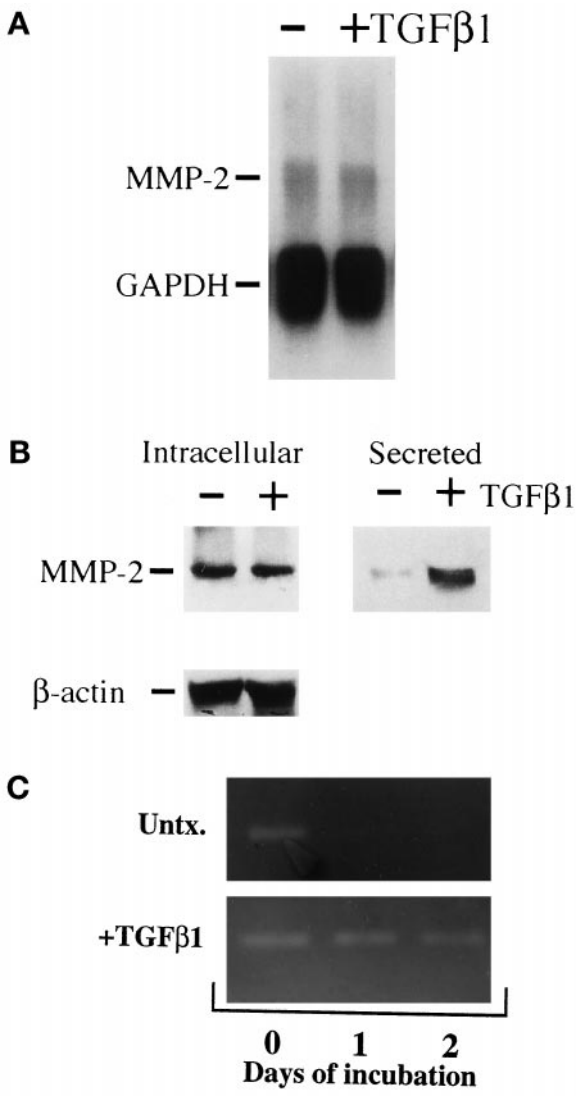

Figure 9. Analyses of MMP-2 regulation by TGF-β1 in the TsuPr1 cell line. MMP-2 levels were analyzed after TGF-β1 stimulation for 24 h by Northern blot (A), immunoblotting of intracellular and secreted proteins (B), or zymography of secreted 72-kDa gelatinase activity (C) present after 0, 1, or 2 d of cell-free incubation at 37°C.

PAI-1 in the Tsu-Pr1 cells (positive control for nuclear runoff assays), and therefore we investigated the potential for TGF-β1 regulation of MMP-2 through inhibition of one or more components of the plasmin cascade. We found no evidence of soluble plasmin and plasminogen protein levels in the medium conditioned by either control or TGF-β1–treated cultures (our unpublished results). Because biologically significant plasmin and plasminogen levels may be undetectable by immunoblot, we also attempted to block decay of MMP-2 in control cultures by addition of PAI-1 (to mimic potential up-regulation of PAI-1 in TGF-β1–treated cells) or neutralizing antibodies

against urokinase plasminogen activator. Neutralizing antibodies against PAI-1 were also added to TGF-β1–treated cultures to determine whether reduced soluble PAI-1 levels would enhance MMP-2 degradation. None of these studies revealed any modulation of MMP-2 decay (our unpublished results), indicating that stabilization of secreted MMP-2 is either mediated through inhibition of a non-plasmin or -plasminogen degradation pathway(s) or is not inhibitable through the antibody neutralization techniques we have used.

DISCUSSION

Increased understanding of the mechanisms through which growth factors mediate MMP activities can provide insight into a wide variety of both physiological and diseased states. Although transcriptional regulation of MMP-9 and other matrix proteinases by growth factors or growth factor pathways is well established (Edwards *et al.*, 1987; Matrisian, 1990; Sato *et al.*, 1993; Sato and Seiki, 1993; Gum *et al.*, 1996), our studies demonstrated no evidence of TGF-β1–stimulated promoter activation or gene transcription despite concurrent elevations in secreted MMP-9 (92 kDa) protein activities. Both the molecular mobility of secreted MMP-9 and -2 and immunoblot analyses indicated that the TGF-β1–stimulated elevations occurred through increased quantities of the native zymogen and not through processing to smaller activated forms or through the loss of binding to inhibitors. To investigate alternative levels for this MMP-9 induction, intracellular protein levels of MMP-9 were assayed in the presence and absence of the RNA synthesis inhibitor actinomycin D. These experiments indicated that TGF-β1 regulated MMP-9 protein levels before secretion and without a requirement for new RNA synthesis.

To broadly investigate posttranscriptional means for this MMP-9 up-regulation, cell-associated 92-kDa collagenase levels were determined in the presence and absence of the RNA synthesis inhibitor actinomycin D and the protein inhibitor cycloheximide. These experiments demonstrated that MMP-9 protein levels increased after TGF-β1 treatment without an absolute requirement for new RNA synthesis. A time course of MMP-9 mRNA levels in the presence of actinomycin D indicated that TGF-β1 treatment leads to increased stability of MMP-9 transcripts. TGF-β1 has also been previously reported to modulate some structural components of the extracellular matrix, in part through changes in mRNA stability (Penttinen *et al.*, 1988; Stefanovic *et al.*, 1997), and at least one other matrix-associated enzyme, urokinase plasminogen activator, is up-regulated through mRNA stability by calcitonin in renal porcine epithelial cells (Nagamine *et al.*, 1983; Altus and Nagamine, 1991). Although our current studies have focused on human prostate cancer cells, the novel mechanism we describe for TGF-β1 regulation of MMP-9 may also apply in other tumors, such as breast, which also induce MMP-9 activity in response to TGF-β1 (Welch *et al.*, 1990).

mRNA half-life is often regulated through sequences in the 3′-untranslated regions (UTRs) bound by protein complexes that stabilize or destabilize the mRNA product (Ross, 1995; Stefanovic *et al.*, 1997). TGF-β1 could enhance mRNA stability by either increasing levels of stabilizing proteins or by decreasing levels of destabilizing proteins, and cycloheximide treatment is often used in an effort to differentiate between these possibilities. Our observation that cycloheximide blocked the TGF-β1 induction of MMP-9 mRNA levels suggests that TGF-β1 action requires de novo synthesis of mRNA-stabilizing protein(s) rather than decreased levels of destabilizing binding peptides. Furthermore, the MMP-9 3′-UTR sequence lacks AU-rich sequence motifs identified as potential binding sites for destabilizing regulating proteins (Shaw and Kamen, 1986; Bohjanen *et al.*, 1992); it also lacks C-rich regions, which mediate RNA stability in collagen Iα (Stefanovic *et al.*, 1997), and therefore, unique stability regulatory sequence(s) within the MMP-9 3′-UTR remain to be determined.

Although these results demonstrate that increases in MMP-9 mRNA and secreted 92-kDa enzymatic activities are generated in part through increased half-life, we have not specifically ruled out additional potential levels for TGF-β1 regulation such as translation or secretion. However, it is unlikely that TGF-β regulates MMP-9 through secretion, because only one MMP, MMP-8 found in polymorphonuclear cells, is reported to be stored before secretion (Woessner, 1991; Benbow and Brinckerhoff, 1997).

In the Tsu-Pr1 cell line, TGF-β1 up-regulated secreted MMP-2 72-kDa collagenase levels in addition to MMP-9, providing a model to study mechanisms of MMP-2 regulation by TGF-β1 as well. Little is known about the regulation of MMP-2 by growth factors in human epithelial malignancies. To investigate the potential levels for TGF-β1 regulation of MMP-2 in the Tsu-Pr1 cell, we performed Northern blot and runoff analyses, which collectively demonstrated no evidence of either transcriptional activation or increased steady-state levels of mRNA after TGF-β1 stimulation. Unlike other MMP members, MMP-2 has no AP-1 or PEA3 sites within the promoter region, and it lacks the TATA binding sequence for RNA polymerase (Benbow and Brinckerhoff, 1997); therefore this collagenase could be a potential candidate for posttranscriptional regulation by growth factors. Further studies of cell-associated levels of MMP-2 protein demonstrated that, unlike MMP-9, TGF-β1 stimulation did not alter intracellular levels of MMP-2 mRNA or protein but led to large differences in both 72-kDa secreted activity and

protein levels, pointing toward an extracellular regulatory mechanism.

We next investigated MMP-2 degradation in conditioned media as a possible explanation for the TGF-β1 stimulation and observed a striking increase in the stability of MMP-2 protein in conditioned media collected from TGF-β1–treated cells over control cultures. Additional investigations will be necessary to elucidate the scope and mechanisms of this extracellular regulation.

Overall, these studies further demonstrate the importance of TGF-β1–regulated gene activities in prostate cancer (Ren *et al.*, 1998) by defining two novel and indirect mechanisms of regulation of type IV collagenase activity: increased stabilities of both mRNA and the extracellular secreted proenzyme. These data also add to the growing list of mechanisms by which growth factors may potentially mediate matrix proteases and raise the possibility that other growth factors or oncogenes may modulate type IV collagenases or other matrix proteases via increases in mRNA half-life or inhibition of enzyme degradation as well.

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REFERENCES

Ahrens, D., Koch, A.E., Pope, R.M., Stein-Picarella, M., and Niedbala, M.J. (1996). Expression of matrix metalloproteinase 9 (96-kd gelatinase B) in human rheumatoid arthritis. Arthritis Rheum. *39*, 1576–1587.

Albini, A., Melchiori, A., Santi, L., Liotta, L.A., Brown, P.D., and Stetler-Stevenson, W.G. (1991). Tumor cell invasion inhibited by TIMP-2. J. Natl. Cancer Inst. *83*, 775–779.

Altus, M.S., and Nagamine, Y. (1991). Protein synthesis inhibition stabilizes urokinase-type plasminogen activator mRNA. J. Biol. Chem. *266*, 21190–21196.

Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., and Struhl, K. (1989). Current Protocols in Molecular Biology, vol. 1, New York: Greene/Wiley-Interscience.

Benbow, U., and Brinckerhoff, C.E. (1997). The AP-1 site and MMP gene regulation: what is all the fuss about? Matrix Biol. *15*, 519–526.

Bernhard, E.J., Gruber, S.B., and Muschel, R.J. (1994). Direct evidence linking expression of matrix metalloproteinase 9 (92-kDa gelatinase/collagenase) to the metastatic phenotype in transformed rat embryo cells. Proc. Natl. Acad. Sci. USA *91*, 4293–4297.

Bohjanen, P.R., Petryniak, B., June, C.H., Thompson, C.B., and Lindsten, T. (1992). AU RNA-binding factors differ in their binding specificities and affinities. J. Biol. Chem. *267*, 6302–6309.

Brooks, P.C., Silletti, S., von Schalscha, T.L., Friedlander, M., and Cheresh, D.A. (1998). Disruption of angiogenesis by PEX, a noncatalytic metalloproteinase fragment with integrin binding activity. Cell *92*, 391–400.

Brown, P.D., Levy, A.T., Margulies, I.M.K., Liotta, L.A., and Stetler-Stevenson, W.G. (1990). Independent expression and cellular processing of Mr 72,000 type IV collagenase and interstitial collagenase in human tumorigenic cell lines. Cancer Res. *50*, 6184–6191.

Edwards, D.R., Murphy, G., Reynolds, J.J., Whitham, S.E., Docherty, A.J.P., Angel, P., and Heath, J.K. (1987). Transforming growth factor β modulates the expression of collagenase and metalloproteinase inhibitor. EMBO J. *6*, 1899–1904.

Egawa, S., Kadmon, D., Miller, G.J., Scardino, P.T., and Thompson, T.C. (1992). Alterations in mRNA levels for growth-related genes after transplantation into castrated hosts in oncogene-induced clonal mouse prostate carcinoma. Mol. Carcinog. *5*, 52–61.

Farina, A.R., Tacconelli, A., Teti, A., Gulino, A., and Mackay, A.R. (1998). Tissue inhibitor of metalloproteinase-2 protection of matrix metalloproteinase-2 from degradation by plasmin is reversed by divalent cation chelator EDTA and the bisphosphonate alendronate. Cancer Res. *58*, 2957–2960.

Fowlkes, J.L., Enghild, J.J., Suzuki, K., and Nagase, H. (1994). Matrix metalloproteinases degrade insulin-like growth factor-binding protein-3 in dermal fibroblast cultures. J. Biol. Chem. *269*, 25742–25746.

Fridman, R., Toth, M., Pena, D., and Mobashery, S. (1995). Activation of progelatinase B (MMP-9) by gelatinase A (MMP-2). Cancer Res. *55*, 2548–2555.

Gearing, A.J., *et al*. (1994). Processing of tumor necrosis factor-α precursor by metalloproteinases. Nature *370*, 555–557.

Gianluigi, G., Falk-Marzillier, J., Schiraldi, O., Stetler-Stevenson, W.G., and Quaranta, V. (1997). Induction of cell migration by matrix metalloprotease-2 cleavage of laminin-5. Science *277*, 225–228.

Greene, G.F., Kitadai, Y., Pettaway, C.A., von Eschenbach, A.C., Bucana, C.D., and Fidler, I.J. (1997). Correlation of metastasis-related gene expression with metastatic potential in human prostate carcinoma cells implanted in nude mice using an in situ mRNA hybridization technique. Am. J. Pathol. *150*, 1571–1582.

Gum, R., Lengyel, E., Juarez, J., Chen, J.H., Sato, H., Seiki, M., and Boyd, D. (1996). Stimulation of 92-kDa gelatinase B promoter activity by *ras* is mitogen-activated protein kinase kinase 1-independent and requires multiple transcription factor binding sites including closely spaced PEA3/*ets* and AP-1 sequences. J. Biol. Chem. *271*, 10672–10680.

Hujanen, E.S., Vaisanen, A., Zheng, A., Tryggvason, K., and Turpeenniemi-Hujanen, T. (1994). Modulation of Mr 72,000 and Mr 92,000 type-IV collagenase (gelatinase A and B) gene expression by interferons α and gamma in human melanoma. Int. J. Cancer *58*, 582–586.

Keeton, M.R., Curriden, S.A., van Zonneveld, A.-J., and Loskutoff, D.J. (1991). Identification of regulatory sequences in the type 1 plasminogen activator inhibitor gene responsive to transforming growth factor β. J. Biol. Chem. *266*, 23048–23052.

Kondapaka, S.B., Fridman, R., and Reddy, K.B. (1997). Epidermal growth factor and amphiregulin up-regulate matrix metalloproteinase-9 (MMP-9) in human breast cancer cells. Int. J. Cancer. *70*, 722–726.

Koolwijk, P., Miltenburg, M., van Erck, G., Oudshoorn, M., Niedbala, M.J., Breedveld, F.C., and van Hinsberg, V.W. (1995). Activated gelatinase-B (MMP-9) and urokinase-type plasminogen activator in synovial fluids of patients with arthritis. J. Rheumatol. *22*, 385–393.

Laiho, M., DeCaprio, J.A., Ludlow, J.W., Livingston, D.M., and Massague, J. (1990). Growth inhibition by TGF-β linked to suppression of retinoblastoma protein phosphorylation. Cell *62*, 175–185.

Levi, E., Fridman, R., Miao, H.-Q., Ma, Y.-S., Yayon, A., and Vlodavsky, I. (1996). Matrix metalloproteinase 2 releases active soluble ectodomain of fibroblast growth factor receptor 1. Proc. Natl. Acad. Sci. USA *93*, 7069–7074.

Liotta, L.A., Steeg, P.S., and Stetler-Stevenson, W.G. (1991). Cancer metastasis and angiogenesis: an imbalance of positive and negative regulation. Cell *64*, 327–336.

Lyons, J.G., Birkedal-Hansen, B., Pierson, M.C., Whitelock, J.M., and Birkedal-Hansen, H. (1993). Interleukin-1β and transforming growth factor-α/epidermal growth factor induce expression of Mr 95,000 type IV collagenase/gelatinase and interstitial fibroblast-type collagenase by rat mucosal keratinocytes. J. Biol. Chem. *268*, 19143–19151.

Lyons, R.M., Gentry, L.E., Purchio, A., and Moses, H.L. (1990). Mechanism of activation of latent recombinant transforming growth factor β1 by plasmin. J. Cell Biol. *110*, 1361–1367.

Marti, H.P., Lee, L., Kashgarian, M., and Lovett, D.H. (1994). Transforming growth factor-β1 stimulates glomerular mesangial cell synthesis of the 72-kd type IV collagenase. Am. J. Pathol. *144*, 82–94.

Matrisian, L.M. (1990). Metalloproteinases and their inhibitors in matrix remodeling. Trends Genet. *6*, 121–125.

Mazzieri, R., Masiero, L., Zanetta, L., Monea, S., Onisto, M., Garbisa, S., and 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*, 2319–2332.

Murphy, G., and Knauper, V. (1997). Relating matrix metalloproteinase structure to function: why the "hemopexin" domain? Matrix Biol. *15*, 511–518.

Nagamine, Y., Sudol, M., and Reich, E. (1983). Hormonal regulation of plasminogen activator mRNA production in porcine kidney cells. Cell *32*, 1181–1190.

Okada, Y., Tsuchiya, H., Shimizu, H., Tomita, K., Nakanishi, I., Sato, H., Seiki, M., Yamashita, K., and Hayakawa, T. (1990). Induction and stimulation of 92-kDa gelatinase/type IV collagenase production in osteosarcoma and fibrosarcoma cell lines by tumor necrosis factor α. Biochem Biophys. Res. Commun. *171*, 610–617.

Overall, C.M., Wrana, J.L., and Sodek, J. (1991). Transcriptional and posttranscriptional regulation of 72-kDa gelatinase/Type IV collagenase by transforming growth factor-β1 in human fibroblasts. J. Biol. Chem. *266*, 14064–14071.

Patterson, B.C., and Sang, Q.A. (1997). Angiostatin-converting enzyme activities of human matrilysin (MMP-7) and gelatinase B/type IV collagenase (MMP-9). J. Biol. Chem. *272*, 28823–28825.

Penttinen, R.P., Kobayashi, S., and Bornstein, P. (1988). Transforming growth factor β increases mRNA stability for matrix proteins in both the presence and in the absence of changes in mRNA stability. Proc. Natl. Acad. Sci. USA *85*, 1105–1108.

Ren, C., Yang, G., Timme, T.L., Wheeler, T.M., and Thompson, T.C. (1998). Reduced lysyl oxidase mRNA levels in experimental and human prostate cancer. Cancer Res. *58*, 1285–1290.

Ross, J. (1995). mRNA stability in mammalian cells. Microbiol. Rev. *59*, 423–450.

Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989). Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Samuel, S.K., Hurta, R A.R., Kondaiah, P., Khalil, N., Turley, E.A., Wright, J.A., and Greenberg, A.H. (1992). Autocrine induction of tumor protease production and invasion by a metallothionein-regulated TGF-β1 (Ser223, 225). EMBO J. *11*, 1599–1605.

Sato, H., Kita, M., and Seiki, M. (1993). v-Src activates the expression of 92-kDa type IV collagenase gene through the AP-1 Site and the GT box homologous to retinoblastoma control elements. J. Biol. Chem. *268*, 23460–23468.

Sato, H., and Seiki, M. (1993). Regulatory mechanism of 92 kDa type IV collagenase gene expression which is associated with invasiveness of tumor cells. Oncogene *8*, 395–405.

Seed, B., and Sheen, J.-Y. (1988). A simple phase-extraction assay for chloramphenicol acyltransferase activity. Gene *67*, 271–277.

Sehgal, G., Hua, J., Bernhard, E.J., Sehgal, I., Thompson, T.C., and Muschel, R.J. (1998). Requirement for matrix metalloproteinase-9 (gelatinase B) expression in metastasis by murine prostate carcinoma. Am. J. Pathol. *152*, 591–596.

Sehgal, I., Baley, P., and Thompson, T.C. (1996). Transforming growth factor β1 stimulates contrasting responses in metastatic *versus* primary mouse prostate cancer-derived cell lines *in vitro*. Cancer Res. *56*, 3359–3365.

Shaw, G., and Kamen, R. (1986). A conserved AU sequence from the 3′ untranslated region of GM-CSF mRNA mediates selective mRNA degradation. Cell *46*, 659–667.

Singh, R.K., Gutman, M., Reich, R., and Bar-Eli, M. (1995). UV B irradiation promotes tumorigenic and metastatic properties in primary cutaneous melanoma via induction of interleukin 8. Cancer Res. *55*, 3669–3674.

Stearns, M.E., and Stearns, M. (1996). Immunohistochemical studies of activated matrix metalloproteinase-2 (MMP-2a) expression in human prostate cancer. Oncol. Res. *8*, 63–67.

Stefanovic, B., Hellerbrand, C., Holcik, M., Briendl, M., Liebhaber, S., and Brenner, D.A. (1997). Posttranscriptional regulation of collagen α1(I) mRNA in hepatic stellate cells. Mol. Cell. Biol. *17*, 5201–5209.

Timme, T.L., Yang, G., Rogers, E., Kadmon, D., Morganstern, J.P., Park, S.H., and Thompson, T.C. (1996). Retroviral transduction of transforming growth factor-β1 induces pleiotropic benign prostatic growth abnormalities in mouse prostate reconstitutions. Lab. Invest. *74*, 747–760.

Welch, D.R., Fabra, A., and Nakajima, M. (1990). Transforming growth factor β stimulates mammary adenocarcinoma cell invasion and metastatic potential. Proc. Natl. Acad. Sci. USA *87*, 7678–7682.

Woessner, J.F., Jr. (1991). Matrix metalloproteinases and their inhibitors in connective tissue remodeling. FASEB J. *5*, 2145–2154.

Wood, M., Fudge, K., Mohler, J.L., Frost, A.R., Garcia, F., Wang, M., and Stearns, M.E. (1997). In situ hybridization studies of metalloproteinases 2 and 9 and TIMP-1 and TIMP-2 expression in human prostate cancer. Clin. Exp. Metastasis *15*, 246–258.