Breast cancer cells induce stromal fibroblasts to express MMP-9 via secretion of TNF-α and TGF-β

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Summary

We used 2D-cocultures employing fibroblasts of different genetic backgrounds and MCF10A-derived human breast epithelial cells of increasingly malignant potential to investigate tumor-stroma interactions in breast cancer and to identify possible signaling pathways involved. Tumor cells induced expression of matrix-metalloproteinase 9 (MMP-9) in fibroblasts in a pattern dependent on the degree of their malignancy. In-situ zymography localized the main gelatinolytic activity around stromal cells in cocultures and xenografted tumors. Use of Smad3 knockout fibroblasts, small molecule inhibitors, and neutralizing antibodies showed that MMP-9 expression was induced by tumor cell-derived TNF-α and TGF-β, dependent on Smad-, Ras-, and PI3-kinase-signaling, and likewise modulated by subsequent HGF- and EGF-signaling. Together, our results indicate that MMP-9 levels in tumor fibroblasts are regulated by a complex tumor-stroma cross-talk, involving multiple ligands and cellular signaling pathways.

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Key words: Breast cancer, Coculture, MMP-9, TGF-β, TNF-α

Introduction

Although tumors are typically defined by their uncontrolled and invasive growth, it is now appreciated that the tumor phenotype is regulated in a complex fashion based on interactions of the malignant cells with the tumor stroma including the resident immune system, the vasculature and components of the extracellular matrix (Elenbaas and Weinberg, 2001; Noel and Foidart, 1998; Radisky and Bissell, 2004). This system has such intricate interactions that it can be addressed as a 'new organ' (Bissell and Radisky, 2001). Tumor cells typically differ from their epithelial precursors by modified cell signaling, enzyme expression, proliferation, apoptosis and invasive, migratory activity. It is also recognized that the stroma is altered in the vicinity of tumors and often supports, if not promotes, the growth of the tumor (Liotta and Kohn, 2001). As an example, prostate tumor stroma is known to promote epithelial-mesenchymal transition of SV40-transformed prostate epithelial cell lines (Olumi et al., 1999). Moreover, fibroblasts derived from normal breast tissue but not fibroblasts from breast tumors have been shown to inhibit the growth of breast cancer cells (Dong-Le Bourhis et al., 1997).

A variety of cytokines and growth factors, such as transforming growth factor-β (TGF-β), hepatocyte growth factor (HGF), epidermal growth factor (EGF) or tumor necrosis factor-α (TNF-α), have been implicated in tumor-stroma cross-talk. It has been shown in a breast cancer model using humanized stroma that overexpression of TGF-β and HGF by fibroblasts promotes hyperplastic or tumorigenic outgrowth of normal breast epithelium, whereas normal stromal cells repress such outgrowth (Kuperwasser et al., 2004). Further implicating the involvement of these two growth factors, loss of TGF-β-signaling and subsequent activation of HGF-signaling in fibroblasts were shown to result in prostate intraepithelial neoplasia (Bhowmick et al., 2004).

The TGF-β-pathway is one of the major pathways altered in tumors, including breast cancer (Roberts and Wakefield, 2003; Wakefield and Roberts, 2002). Binding of TGF-β to the TGF-β receptor II (TβRII) activates the signal-transducing TGF-β receptor I (TβRI) that phosphorylates latent cytoplasmic transcriptional activators, Smad2 and Smad3. They then, together with Smad4, translocate into the nucleus where they regulate TGF-β-dependent gene transcription (Heldin et al., 1997; Massague, 2000; Wakefield and Roberts, 2002; Wrana et al., 1994). In breast cancer, TGF-β-signaling was shown to reduce growth of the primary tumor but also to promote metastasis, indicating that the apparent effect of TGF-β depends on its cellular context (Muraoka et al., 2003; Siegel et al., 2003; Tang et al., 2003; Tian et al., 2003). Insight into this dual activity of TGF-β on tumorigenesis has come from studies based on a breast cancer cell system derived from MCF10A (10A) cells, immortal breast epithelial cells that do not survive in immunodeficient mice (Dawson et al., 1996). Ras-transformants of these cells, MCF10At.1k (At.1k) cells, form small nodules in nude mice that rarely develop into carcinomas, whereas derivatives of At.1k cells, MCF10CA1h (CA1h) cells and MCF10CA1a (CA1a) cells, form xenograft tumors and lung metastases in mouse tail vein assays, respectively (Dawson et al., 1996; Heppner et al., 2000; Miller

et al., 2000; Santner et al., 2001; Tang et al., 2003; Tian et al., 2003). While the different derivatives of MCF10 cells are *ras*-transformed they nevertheless originate from the same genetic background and represent different, well-defined stages of breast cancer and thus are a valuable tool to investigate the biology of epithelial tumor progression.

TNF-α, which often antagonizes effects of TGF-β, signals through TNF-α receptor II and TNF-α receptor I resulting in activation of downstream effectors including JunN-terminal kinase (JNK), extracellular signal-regulated kinases (Erks), p38 and NFκB (Idriss and Naismith, 2000; Locksley et al., 2001; Szlosarek and Balkwill, 2003). TNF-α has been implicated in skin carcinogenesis and in metastatic tumor spread of a variety of carcinomas and sarcomas. It can induce tumor necrosis by affecting tumor vascularization and initiating apoptotic cell death, but paradoxically it also can promote cell proliferation (Idriss and Naismith, 2000; Szlosarek and Balkwill, 2003). Thus, TNF-α, like TGF-β, displays characteristics of both a tumor promoter and a tumor suppressor.

Differing cytokine expression, cell signaling and protein expression cause malignant epithelial tumors to grow beyond the basement membrane, a process involving proteolytic degradation mediated by matrix metalloproteinases (MMPs), a family of Zn^{2+}- and Ca^{2+}-dependent endopeptidases that are capable of degrading almost all extracellular matrix proteins. MMPs are synthesized as proenzymes and typically activated by proteolytic removal of the propeptide (Jones et al., 2003a). Gelatinase A (matrix metalloproteinase 2, MMP-2) and gelatinase B (matrix metalloproteinase 9, MMP-9) degrade components of the basement membrane, particularly collagen IV and also a variety of other pericellular substances, such as laminins and fibronectin. Additionally, gelatinases are involved in the degradation of fibrillar collagens after their initial degradation by collagenases and also target non-matrix substrates such as TGF-β and TNF-α (Lynch and Matrisian, 2002; Sternlicht and Werb, 2001). In contrast to MMP-2, which is constitutively expressed, MMP-9 levels are usually low and enzyme expression is induced, for example by TGF-β, EGF, HGF or TNF-α. Elevated serum and tissue levels of MMP-9 are found to be associated with tumors and correlate with cancer invasion and metastasis (Nabeshima et al., 2002). However, the contribution of particular cell types to MMP-9 expression in breast tumors is still controversial as MMP-9 has been detected in vascular pericytes (Nielsen et al., 1997), tumor cells and stromal cells (Giannelli et al., 2002; Scorilas et al., 2001).

Here, we used a 2D-coculture system to mimic tumor-stroma interactions in a xenograft model. We cultured mouse fibroblasts together with MCF10-derived human breast cancer cells of varying degrees of malignancy to study tumor-stroma interactions in vitro and to analyze induction and localization of MMP-9 expression in this breast cancer model. We show that MMP-9 is secreted predominantly by fibroblasts in response to signals from the tumor cells, presumably including TNF-α and TGF-β, and that the level of induction depends both on multiple signaling pathways in the fibroblasts and the degree of malignancy of the tumor cells.

Materials and Methods
Cell cultures
Dermal fibroblasts (DFs) were isolated from newborn Smad3 wild type and Smad3 knockout mice as described previously (Flanders et al., 2002). MMP9- and MMP2-knockout mouse embryonic fibroblasts (MMP9KO, MMP2KO, respectively) as well as the corresponding wild-type fibroblasts (MMP9WT, MMP2WT) were a kind gift from Zena Werb (University of California San Francisco, San Francisco, CA). Mouse mammary fibroblasts were a kind gift from Zhijun Du (NCI, Bethesda, MD). Human embryonic lung fibroblasts (WI-38) were a kind gift from Kristin Bauer (NCI, Bethesda, MD). All fibroblast cultures were maintained in Dulbecco's modified Eagle's Medium (DMEM, Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Gemini Bio-Products, Woodland, CA) (DMEM compl.) at 37°C, 5% CO_2 in a humidified atmosphere. MCF10CA1a and MCF10CA1h cells were grown in DMEM/F12 (Invitrogen) supplemented with 5% horse serum (Gemini Bio-Products) (DMEM/F12 compl.); MCF10A and MCF10At1k.cl2 cells were cultured in DMEM/F12 supplemented with 5% horse serum, 10 µg/ml insulin (Biofluids, Rockville, MD), 20 ng/ml EGF (Biofluids, Rockville, MD), 0.5 µg/ml hydrocortisone and 100 ng/ml cholera toxin (both from Sigma, St Louis, MO) at 37°C, 5% CO_2 in a humidified atmosphere.

Labeling of cells
Fibroblasts were labeled with 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI) (Molecular Probes, Eugene, OR) according to the instructions of the manufacturer.

Cocultures of breast epithelial cells and fibroblasts
Confluent fibroblast and epithelial cell cultures were trypsinized and 0.5×10^6 cells of each type were plated together in at 100-mm tissue culture dishes containing 10 ml coculture medium [50% DMEM compl. and 50% DMEM/F12 compl., a mixture that had proven to be suitable for the growth of fibroblasts, epithelial cells, and cocultures (data not shown)]. Cocultures were incubated at 37°C, 5% CO_2 in a humidified atmosphere. Homotypic cultures of only one cell type served as controls. Sterile coverslips were placed in dishes before plating the cells to obtain specimens for immunohistochemistry, immunofluorescence, and in-situ zymography.

Generation of conditioned media
1.5×10^6 cells of each cell type were plated in a 150-mm tissue culture dish and cultured in 15 ml coculture medium at 37°C, 5% CO_2 in a humidified atmosphere for 4 days. All experiments also included a 150-mm tissue culture dish containing coculture medium without cells to generate a negative control for effects of the 4-day incubation on the coculture medium itself. At day 4 the supernatants were collected and cells were removed by centrifugation (420 *g*, 5 minutes). The cell-free conditioned media was then used either directly to investigate MMP activities or diluted with fresh coculture medium (1:1) and used for further experiments.

Generation of CA1a cell lysates
Lysates were obtained by washing CA1a cells with Dulbecco's PBS (DPBS) twice, then freeze-thawing the pellet three times, resuspending the disrupted cells in DPBS (10^6 cells/ml), and storing lysates at –20°C until further use.

Inhibition of MMP-expression
Fibroblasts were plated in 24-well plates (2.5×10^4 cells/well) and allowed to grow for 48 hours. Cells were then serum starved as indicated for 24 hours. Thirty minutes after the addition of inhibitors or equal amounts of DMSO cells were stimulated with either 10^4 CA1a cells/well or lysates of 10^4 CA1a cells/well. The following

inhibitors were used: PD98059, 25 μM; SB203580, 10 μM; SP600125, 10 μM; LY294002, 20 μM; manumycin, 1.25 μM (all from Calbiochem, San Diego, CA); SB431542, 5 μM; staurosporine, 10 nM; cycloheximide, 10 ng/ml; actinomycin D, 10 ng/ml (all from Sigma). Growth of fibroblasts plated as described above had previously been shown to be similar by tritium incorporation (data not shown). To ensure that cells remained vital during experiments involving inhibitors, plates were fixed and stained using the solutions for protocols 1, 2 and 3 (Fisher Scientific) according to the manufacturer's instructions, and evaluated microscopically to ensure similar growth of cells in all wells of a plate.

The following antibodies and cytokines were used: anti-TNF-α antibody, anti-HGF antibody, human TGF-β1, human EGF (all from R&D Systems Inc., Minneapolis, MN), human TNF-α (PeproTech, Rocky Hill, NJ) human HGF (kind gift from Don Bottaro, NCI, Bethesda, MD). Plates were incubated for 24 hours at 37°C in a humidified atmosphere, supernatants were collected, centrifuged at 400 g for 5 minutes, and MMP activity of the cell free supernatant was investigated using gelatin zymography.

Gelatin zymography

Cell culture supernatants were collected and centrifuged at 400 g, 5 minutes. The cell free supernatant was mixed with 2× sample buffer (Invitrogen) and zymography was performed using precast gels (10% polyacrylamide, 0.1% gelatin), renaturing buffer and developing buffer (all Invitrogen), according to the manufacturer's instructions. Gelatinase activities were visualized by staining zymograms with Coomassie Brilliant Blue G250 [0.25% Coomassie Brilliant Blue G250, acetic acid 30%, methanol 10% (all from Sigma)] and destained in acetic acid-methanol-dH$_2$O (1:3:6). Recombinant pro-MMP-2 and pro-MMP-9 standards were prepared as previously described (Fridman et al., 1992; Fridman et al., 1993).

In-situ zymography

In-situ zymography was performed as described by Mook et al., (Mook et al., 2003). In brief, tissue culture specimens were washed twice with DPBS, overlaid with a solution of 50 μg/ml fluorescein-labeled gelatin (DQ gelatin; Molecular Probes), 1% (w/v) low-melting agarose (BME, Rockland, ME), and 5 μg/ml DAPI (Molecular Probes) in DPBS (Invitrogen), and incubated on ice for 15 minutes followed by incubation in a humidified chamber at room temperature for 1 hour. Specimens were fixed with neutral buffered formalin (Fisher Scientific, Fair Lawn, NJ) and gelatin degradation was visualized by confocal microscopy

Cryosections were thawed, fixed in acetone (–20°C) for 10 minutes, air dried, rehydrated with DPBS for 10 minutes, and then underwent the described protocol.

Generation of xenograft tumors

Xenografts were derived as described earlier (Tian et al., 2003).

Immunofluorescence

To obtain immunostaining of cytokeratin, smooth muscle α-actin, and prolyl-4-hydroxylase, cryosections were thawed at ambient temperature, fixed with acetone (–20°C) for 10 minutes, and air dried. Specimens were rehydrated in TBST [25 mM Tris/HCl pH 7.5, 100 mM NaCl (both from Biofluids, Rockville, MD), 0.1% (v/v) Tween 20 (Sigma)], incubated with the primary antibody appropriately diluted in DPBS (see supplementary material, Table1) overnight at room temperature, followed by incubation with the secondary antibody in DPBS for 60 minutes at 37°C.

Tissue culture specimens stained for TGF-β1 were fixed with 2% formaldehyde in DPBS pH 7.4, washed with 0.1% (w/v) glycine (ICN,

Aurora OH) in DPBS, blocked 1 hour at room temperature with 0.5% (w/v) casein (Sigma) in DPBS, and incubated with the primary antibody overnight at 4°C, followed by incubation with the secondary antibody for 60 minutes at room temperature. Specimens were mounted using DAPI containing mounting medium (Vector Laboratories, Burlingame, CA), and immunoreactivity of the specimens was visualized by confocal microscopy.

Confocal microscopy

Confocal microscopy was performed using a Leica DM IRBE confocal microscope and the supplied software (Leica Confocal Software Version 2.00). DAPI: Excitation UV, emission 420-470 nm; Alexa488, fluorescein: excitation 488 nm (argon), emission 510-540 nm; DiI, TRITC: excitation 568 nm (krypton), emission 590-666 nm. Objectives and numeric apertures were 25×, 0.75; 40×, 1.25; 63×, 1.32; 100×, 1.40.

Images were analysed using Metamorph and Microsoft Excel XP.

Immunocytochemistry

Specimens were thawed at ambient temperature, fixed in a solution of 2% paraformaldehyde in PBS for 8 minutes at room temperature, and endogenous peroxidase was blocked by incubation in 0.3% H$_2$O$_2$ in methanol in DPBS (20 minutes, room temperature). Cells were permeabilized using 0.1% saponin (Sigma) for 20 minutes, incubated with the primary antibody diluted in DPBS (see supplementary material, Table1) overnight at room temperature, followed by incubation with the secondary antibody (anti-mouse IgG biotin) and ABC-complex according to the manufacturer's instruction (Vector Laboratories). Immunoreactivity of the specimens was visualized with a 3′,3′-aminobenzidine (DAB)-based staining solution according to the manufacturer's instructions (BioGenex, San Ramos, CA). Specimens were viewed and images captured using an Axioplan microscope (Carl Zeiss AG, Goettingen, Germany) and Image Pro Plus Version 4.5 software.

Immunoprecipitation

Eight microliters of antisera raised in rabbit against human MMP9 or against human MMP2 (Brown et al., 1990) were added to 4 ml cell-culture supernatant and incubated on a rocking platform for 48 hours and 24 hours, respectively. Protein A-Sepharose (100 μl, Amersham, Piscataway, NJ) was added for a further 24 hours, and beads were pelleted by centrifugation (420 g, 2 minutes, 4°C). The supernatant was collected and the pellet washed four times with serum-free medium to remove unbound enzyme. Finally, the beads were suspended in 2× sample buffer (Invitrogen) and underwent zymography. Supernatants were used to control effective removal of MMP-9 or MMP-2 from the medium.

Results

Coculturing fibroblasts and breast cancer cells induces MMP-9 secretion

Cocultures of breast cancer and stromal cells were made employing mouse DFs and human metastasizing breast cancer cells CA1a. The cultures organized quickly, typically showing spindle shaped fibroblasts surrounding tumor-cell islets after 2 days, and becoming confluent after 4 days (Fig. 1A). Gelatin zymography of the culture-supernatant showed that a gelatinolytic enzyme of an apparent molecular weight about 105 kD was detected in cocultures but not in homotypic cultures after 4 days. Enzyme induction was also observed in cocultures employing Millipore chambers to prevent cell-cell
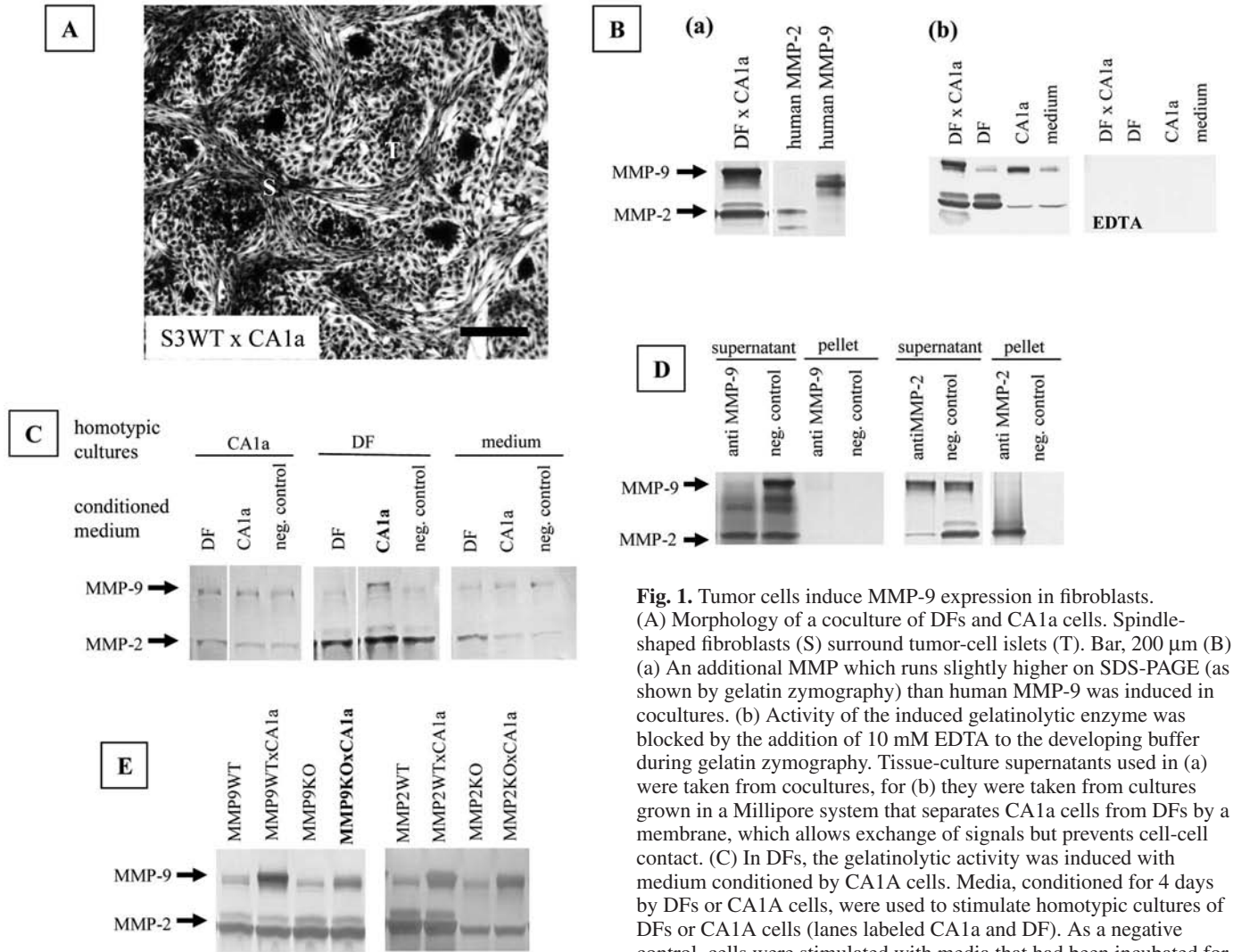
Fig. 1. Tumor cells induce MMP-9 expression in fibroblasts. (A) Morphology of a coculture of DFs and CA1a cells. Spindle-shaped fibroblasts (S) surround tumor-cell islets (T). Bar, 200 µm (B) (a) An additional MMP which runs slightly higher on SDS-PAGE (as shown by gelatin zymography) than human MMP-9 was induced in cocultures. (b) Activity of the induced gelatinolytic enzyme was blocked by the addition of 10 mM EDTA to the developing buffer during gelatin zymography. Tissue-culture supernatants used in (a) were taken from cocultures, for (b) they were taken from cultures grown in a Millipore system that separates CA1a cells from DFs by a membrane, which allows exchange of signals but prevents cell-cell contact. (C) In DFs, the gelatinolytic activity was induced with medium conditioned by CA1A cells. Media, conditioned for 4 days by DFs or CA1A cells, were used to stimulate homotypic cultures of DFs or CA1A cells (lanes labeled CA1a and DF). As a negative control, cells were stimulated with media that had been incubated for 4 days in an empty dish to distinguish between the influence of CA1A cells and DFs on the media and the age of the medium (4-day incubation at 37°C) (lanes labeled medium). (D) Gelatin zymography of the tissue-culture supernatant and the pellet obtained by immunoprecipitation. The induced enzyme was removed from the supernatant by immunoprecipitation with an anti-MMP9 antibody but not an anti-MMP2 antibody. (E) The gelatinolytic enzyme was not induced in the medium of CA1A and MMP9KO cells cocultured for 4 days, but was induced in cocultures of CA1A and MMP2KO cells and also in cocultures of CA1A cells and the corresponding wild-type fibroblasts MMP9WT or MMP2WT. Homotypic cultures and also medium kept in empty dishes served as controls.

contacts between fibroblasts and tumor cells (Fig. 1B, left panel). Notice that, the band induced in the cocultures is of slightly higher molecular weight than that seen in supernatants of CA1a cells alone or human MMP-9 used as a standard, which is indicative of the mouse MMP-9 (Fig. 1B, lane 1 versus lane 3, left panel). The enzyme activity was abolished by addition of EDTA (Fig. 1B) or the MMP-inhibitor Galardin to the developing buffer whereas phenylmethylsulfonylfluoride (PMSF), a serine protease inhibitor, did not affect enzyme activity (data not shown). These results showed that the induced enzyme belonged to the MMP-family.

Whereas medium conditioned by CA1a cells induced expression of this enzyme in DFs, it was not induced in CA1a cells cultured in medium conditioned by either homotypic cultures or cocultures. This indicates the fibroblast origin of the enzyme (Fig. 1C). The gelatinolytic activity could be removed from the medium by MMP-9 antibodies bound to sepharose beads but not by MMP-2 antibodies, again suggesting that this enzyme is mouse MMP-9 (Fig. 1D). To verify this, we used mouse embryonic MMP9KO and MMP2KO fibroblasts, and found that the putative MMP-9 was not induced in cocultures of CA1a cells and MMP9KO fibroblasts, but was still induced, although to a lesser extent, in cocultures of CA1a cells with

MMP2KO fibroblasts or with the corresponding wild-type fibroblasts (Fig. 1E).

Localization of gelatinolytic and collagenolytic enzymes in cocultures

To localize gelatinolytic and collagenolytic enzymes in the cocultures, we carried out in-situ zymography with DQ gelatin where labeling is so strong that the fluorescence of the uncleaved substrate is quenched. Gelatinolytic products were mainly localized around fibroblasts and a trend towards
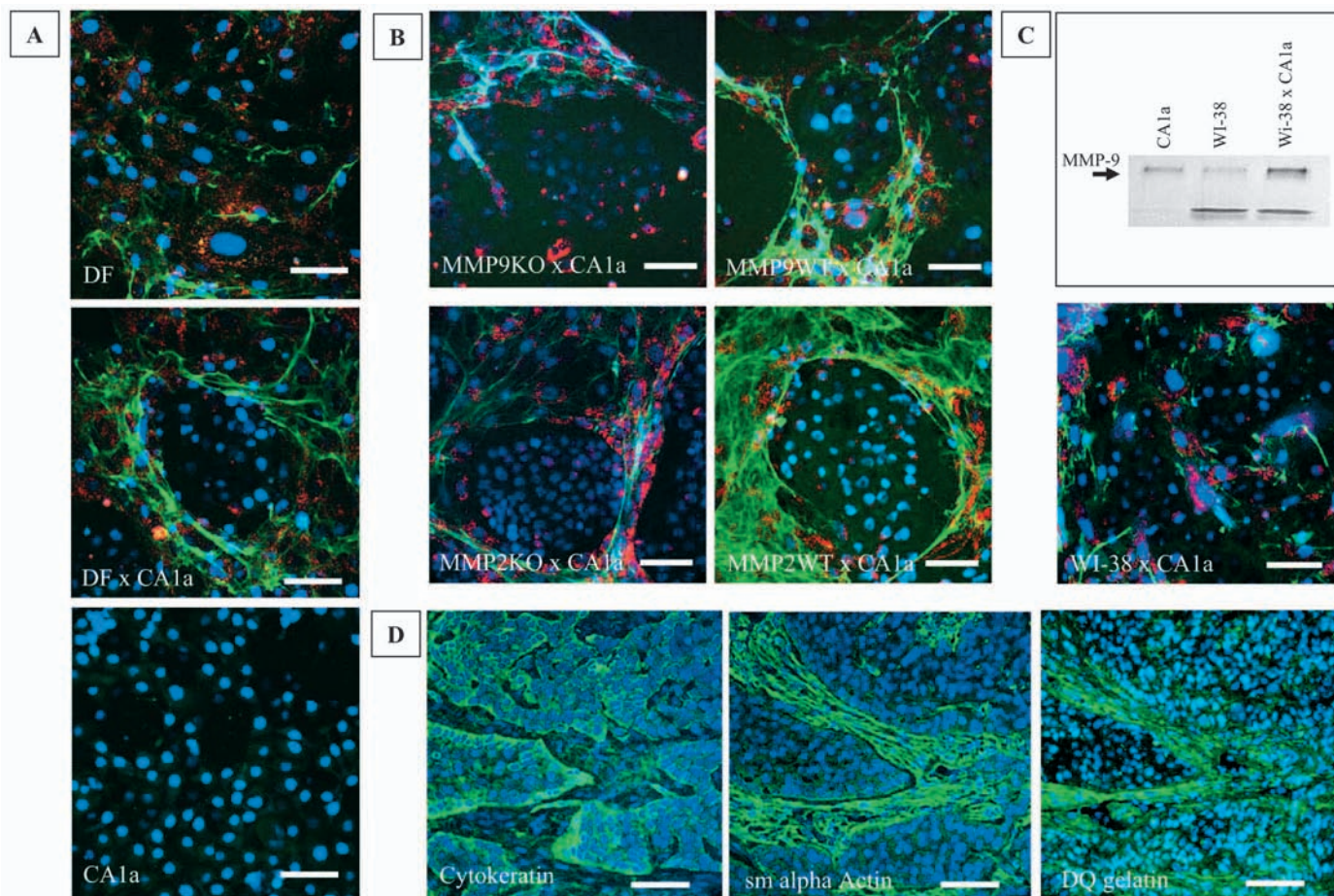
Fig. 2. The main gelatinolytic enzyme activity is localized to the stromal partner in cocultures of fibroblasts and CA1a cells and also in xenograft tumors, and contains significant levels of MMP-9 and MMP-2 activity. (A-C) Fibroblasts were labeled with DiI (red) before plating, fluorescein-labeled gelatin was used as a substrate (green). Nuclei were stained with DAPI (blue). The instrument settings of the microscope were kept constant for each experiment to allow comparison of the collected fluorescence signals of the different cultures. (A) In situ zymography of homotypic cultures and cocultures of DFs and CA1a cells. (B) Cocultures of MMP2KO or MMP9KO with CA1a cells showed significantly less enzyme activity than cocultures employing the corresponding wild-type fibroblasts. (C) Cocultures of human embryonic-lung fibroblasts (WI-38) and CA1a cells showed a growth pattern similar to the one in the heterologous cultures; however, presumably because of the slow growth of WI-38 cells, there were fewer fibroblasts. Gelatin zymography of culture supernatants of 4-day homotypic and heterotypic cultures showed an induction of MMP-9 in the coculture supernatant. (D) In situ zymography with DQ gelatin on serial sections of a xenograft tumor, formed by CA1A cells in nude mice, shows the main gelatinolyic activity (green) of the specimen localized in tissue that is also α-smooth muscle actin-positive, thus exhibiting characteristics of stromal tissue. Tumor cells are cytokeratin-positive and show only a low gelatinolytic activity. Cytokeratin and α-smooth muscle actin are detected with a secondary FITC-labeled antibody (green) by indirect immunofluorescence; nuclei are stained with DAPI (blue). Bars, 80 μm.

higher gelatinolysis was observed in cocultures compared with homotypic fibroblast cultures (Fig. 2A). Similar results were obtained when fluorescein-labeled collagen IV (DQ collagen IV, Molecular Pobes) was used as a substrate (supplementary material, Fig. S1 A). Noticeably less substrate was cleaved in cocultures of CA1a cells with MMP9KO or with MMP2KO fibroblasts compared with the corresponding wild-type fibroblasts (Fig. 2C, supplementary material, Fig. S1 B). To further explore this effect we quantified the intensity of fluorescence derived from gelatin-degradation products. The average intensity, after subtraction of the background, was significantly higher with wild-type fibroblasts than with knockout fibroblasts (relative units: MMP9WT 54.48±7.79 (*n*=5), MMP9KO 27.53±6.16 (*n*=5), *P*=0.0003; MMP2WT 62.35±7.69 (*n*=3), MMP2KO

30.4±11.47 (*n*=5), *P*=0.0033; mean ± standard deviation, two-tailed Student's *t*-test). These data support the conclusion that in our coculture system, the fibroblasts secreted the components with the main gelatinolytic and collagenolytic activity.

Heterologous and homologous cocultures and also xenografted tumors show similar patterns of MMP-9 induction and localization of main gelatinolytic activities

Having identified the enzyme as mouse MMP-9, we investigated whether its induction was specifically owing to the heterogeneous mouse-human culture-system used. We found that cocultures of WI-38 and CA1a cells (human-human) grew in a pattern similar to that of mouse-human cocultures; they

secreted MMP-9 optimally in coculture compared with corresponding homotypic cultures (Fig. 2C, top panel). In-situ zymography showed that the main gelatinolytic activity, again, was localized around fibroblasts, with the tumor cells exhibiting only low levels of gelatinase activity (Fig. 2C, bottom panel).

To investigate whether gelatinolytic enzyme activity is similarly localized to the stroma in tumors, we performed in-situ zymography using DQ gelatin on cryosections of xenografted tumors formed by CA1a cells in nude mice. To identify the histological structures, serial sections were stained for cytokeratin (tumor cells), and smooth muscle α-actin

(stromal cells, Fig. 2D). Degradation of DQ gelatin again was mainly localized to stromal elements, showing that our results in the 2D-coculture-system are representative of the interactions of mesenchyme and tumor cells in vivo and not a tissue-culture artifact.

The time course of tumor cell-induced stromal MMP-9 expression depends on the malignancy of the tumor cell line

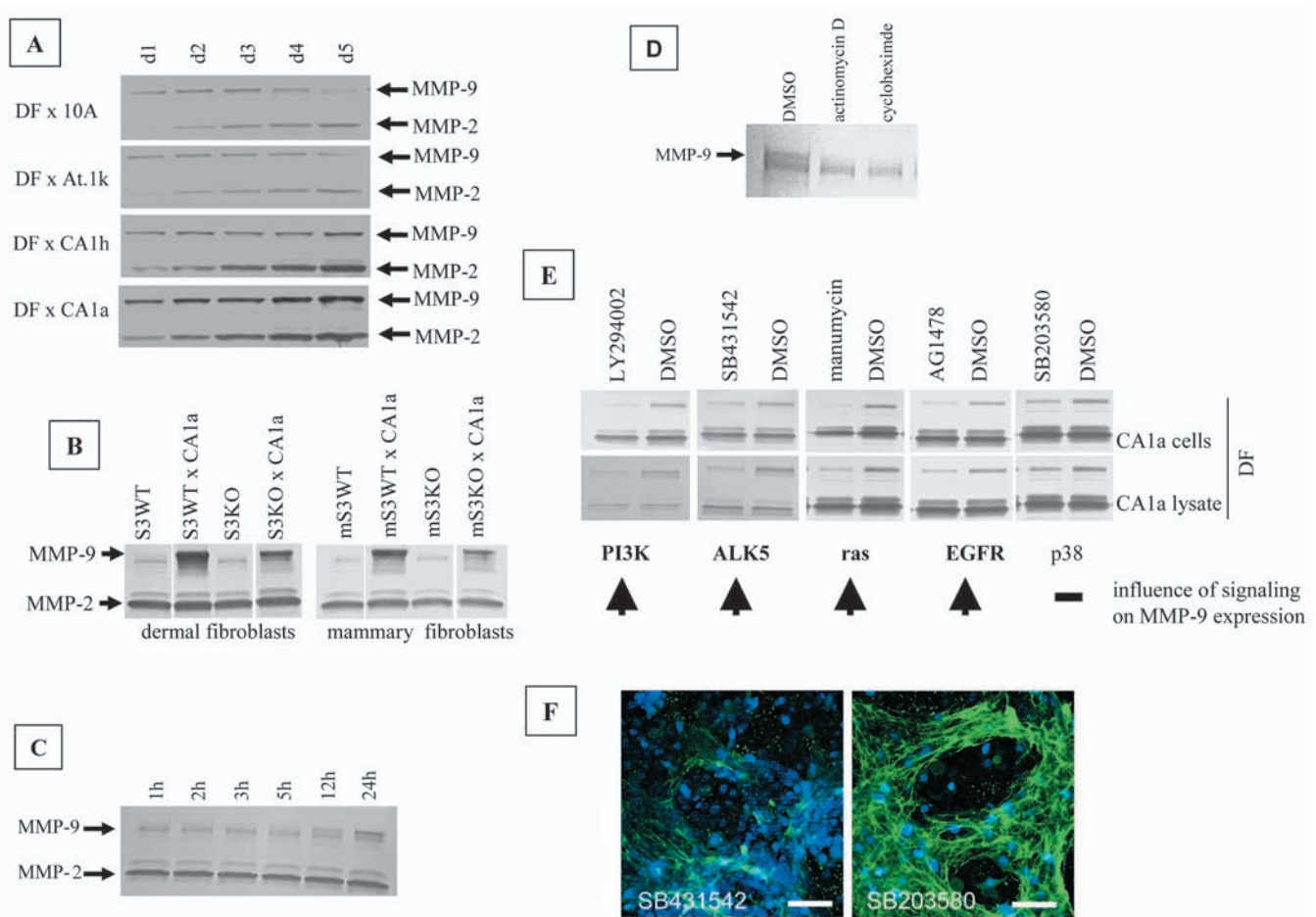To investigate the influence of the tumor cell line on MMP-

Fig. 3. In fibroblasts, MMP-9 induction by tumor cells depends on the malignancy of the tumor cell line and on the integrity of TGF-β-, EGFR- and MAPK-signaling. (A) MMP-9 was induced in cocultures of DFs and 10A, At.1k, CA1h or CA1a cells as early as at day 1. The level of its activity depends on the cell-type and also on the age of the culture. (B) Compared with S3WT and CA1a cells cultured together, the expression of MMP-9 is reduced in cocultures of S3KO and CA1a cells. Similar results were obtained for mammary fibroblasts (mS3KO, mS3WT). Notice, MMP-9-forms of a lower molecular weight, suggesting an activation by proteolytic processing in the cocultures. (C) DFs grown to 80% confluence in 24-well plates with DMEM compl. stimulated with CA1a cells (10,000/well) for 24 hours. The culture-supernatant was analyzed by gelatin zymography. Expression of MMP-9 was induced by CA1a cells within 12 hours. (D) Thirty minutes before addition of CA1a cells, DFs were treated with actinomycin D (10 ng/ml) or cycloheximide (10 ng/ml). Both substances blocked the induction of MMP-9 expression, indicating that it depends on an intact RNA- and protein synthesis. Experimental design as in C. (E) Induction of MMP-9 in serum-starved (0% serum) DFs by CA1A cells or CA1a lysate is modulated by several small-molecule inhibitors. DFs were incubated with inhibitors (LY294002, 20 μM; SB431542, 5 μM; manumycin, 5 μM; AG1478, 10 μM; or SB203580, 10 μM) or DMSO (negative control) for 30 minutes, and stimulated for 24 hours with CA1A cells (10,000/well) or lysates of the same number of cells. The culture-supernatant was analyzed by gelatin zymography. Zymograms shown here were optimized to show the effect of inhibitors on MMP-9 induction and show representative results of five to ten experiments. Notice, whereas MMP-9 levels are influenced by stimulation with inhibitors, MMP-2 levels remain constant. This indicates equal growth of fibroblasts, which was confirmed by fibroblast-monolayer staining as described in Materials and Methods. (F) SB431542 inhibits gelatinolysis in cocultures as shown by in-situ zymography. Cultures were treated with SB431542 (5 μM) or SB203580 (10 μM) for a 24-hour period and then again 1 hour before in-situ zymography. Media used for in-situ zymography were supplemented with SB431542 (5 μm) or SB203580 (10 μM). Bar, 80 μm.

9 induction we employed less malignant cell lines of the MCF10A cell series: MCF10A cells, MCF10At.1k cells and MCF10CA1h cells. Cocultures of these cell lines grew similarly to that observed for MCF10CA1a cells and DFs (supplementary material, Fig. S1 C). Whereas all epithelial cell types induced expression of MMP-9 in DFs within 1 day, the enzyme activity typically declined after 3 days when non-tumorigenic MCF10A and *ras*-transformed MCF10At.1k cells (Fig. 3A) were used, but increased continuously over 5 days with tumorigenic MCF10CA1h or metastatic CA1a cells. Importantly, MMP-9 expression levels in cocultures correlated inversely with the estimated number and growth area of fibroblasts, because the width of the fibroblast cords decreased with increasing malignancy of the tumor cell line (Fig. 3A, supplementary material Fig. S1 C). By contrast, MMP-2 levels in the same cultures increased continuously over the culture period, regardless of the degree of malignancy (Fig. 3A). We observed a trend of increasing levels of gelatinolysis around DFs with increasing degree of malignancy of the tumor cells. Low levels of gelatinolysis were seen in cocultures with 10A cells, with the strongest levels occurring with CA1a cells (supplementary material Fig. S1 D)

MMP-9 induction by tumor cells is regulated through Smad-, MAPK- and PI3-kinase-signaling

Next, we attempted to identify signaling pathways involved in tumor cell-induced MMP-9 expression in fibroblasts. MMP-9 expression is known to be affected by a variety of cytokines, including TGF-β, EGF, TNF-α and HGF, which signal through both distinct and overlapping pathways. As a first step we used mouse dermal Smad3 knockout fibroblasts (S3KO) and small-molecule inhibitors in an attempt to identify involved intracellular pathways.

Although MMP-9 induction was reduced in cocultures using S3KO and mammary Smad3 knockout fibroblasts (mS3KO) compared with the corresponding wild-type fibroblasts (S3WT, mS3WT, respectively), levels were nevertheless still detectable after 4 days of culture (Fig. 3B), indicating that Smad-signaling was not the only pathway involved in our model.

To identify other signaling pathways involved in the induction of MMP-9 expression by CA1a cells, serum-starved DFs (0% or 05% serum) were stimulated by addition of CA1a cells onto fibroblast monolayers to which small-molecule inhibitors had been added 30 minutes earlier. Preliminary experiments showed that, MMP-9 levels were detectable within 12 hours and measurable after 24 hours (Fig. 3C). This
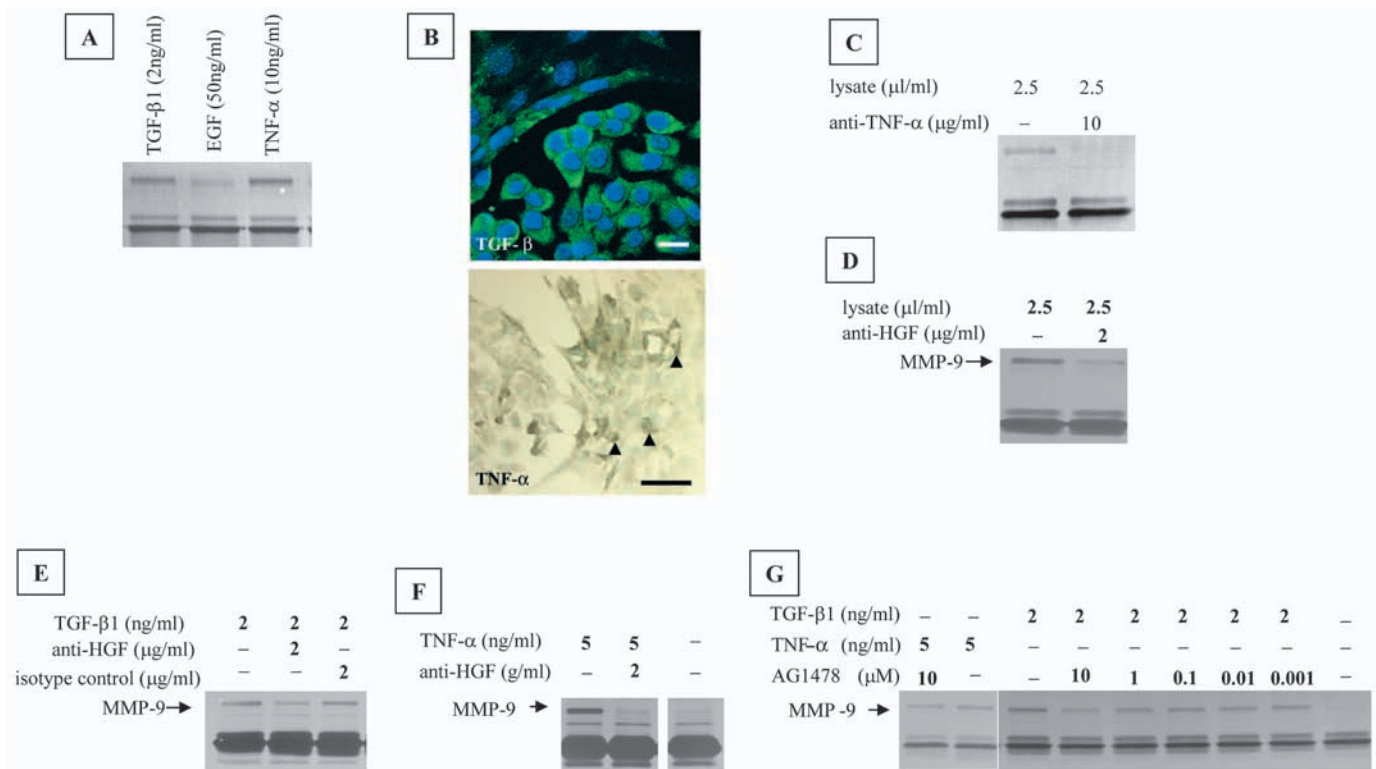

Fig. 4. In fibroblasts, TGF-β, TNF-α, EGF and HGF are involved in the induction of MMP-9 by tumor cells. (A) TGF-β1, TNF-α and, to a lesser extent, EGF induce the expression of MMP-9 in serum-starved (0.5% serum) DFs as shown by gelatin zymography (experimental set up as described in Fig. 3). (B) Indirect immunofluorescence and immunohistochemistry of cocultures of DFs and CA1a shows expression of TGF-β mainly in tumor cells. The highest immunoreactivity for TNF-α is observed in CA1a cells, migrating on the fibroblast monolayer (arrow heads). Bars, 32 μm (TGF-β) and 100 mm (TNF-α). (C,D) Neutralizing TNF-α or HGF antibody significantly decreases MMP-9 levels induced by CA1a lysate. CA1a lysate and serum-starved (0.5% FCS), 80% confluent DFs were separately preincubated for 30 minutes with the antibodies, before combining and incubating them for an additional 24 hours. Cell-free culture-supernatants were analyzed by gelatin zymography. (E) Neutralizing HGF antibody slightly reduces levels of MMP-9 induced by TGF-β1 (experimental set-up as in C). (F) MMP-9 induction by TNF-α is decreased by neutralizing HGF antibody (experimental set-up as in C). (H) The EGF-receptor inhibitor AG1478 interfers with TGF-β- but not TNFα–induced MMP-9 induction (experimental set up as in Fig. 3E).
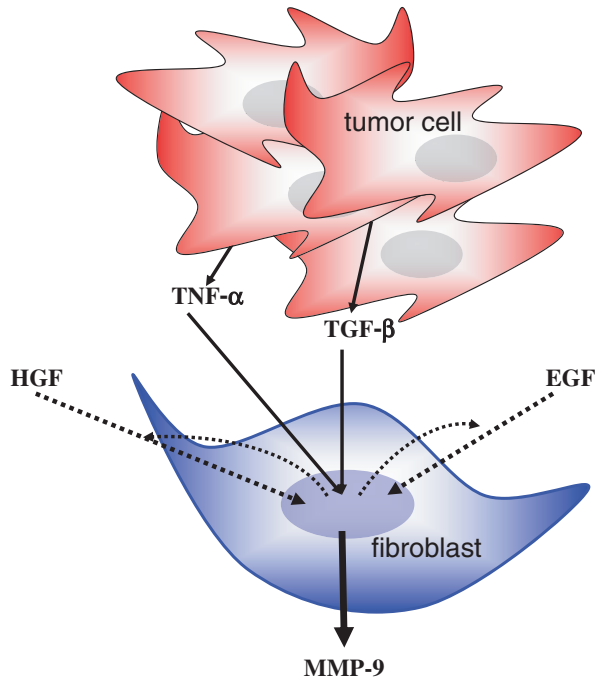
Fig. 5. Model of MMP-9 induction in fibroblasts by breast-cancer cells. Tumor-cell-derived TGF-β and TNF-α directly induce expression of MMP-9, which is augmented indirectly by the effects of these cytokines on HGF- and EGF-signaling.

induction was abolished by actinomycin and cycloheximide (12-hour incubation, Fig. 3D), suggesting a de-novo synthesis of MMP-9. Induction of MMP-9 was inhibited by the ALK5 inhibitor SB431542 (Laping et al., 2002), the phosphoinositide 3-kinase (PI3-kinase) inhibitor LY294002, the EGF receptor kinase (EGFR kinase) inhibitor AG1478, and the Ras inhibitor manumycin, whereas the p38 inhibitor SB203580 did not significantly affect MMP-9 expression (Fig. 3E). Slight decreases in MMP-9 expression were observed with the MEK/ERK inhibitor PD98059; slight increases in expression were seen following treatment with the JNK inhibitor SP600125 and also the protein kinase C (PKC) inhibitor staurosporine (data not shown). A similar pattern of inhibited MMP-9 induction was obtained when lysates of CA1a cells were used instead of vital cells (Fig. 3E). With the exception of SB431542, which directly affects the Smad3 pathway, effects of the inhibitors on MMP-9 expression were similar whether DFs or S3KO cells were used, suggesting that effects of these other pathways on MMP-9 induction are independent of Smad3 (data not shown). Whereas SB431542 not only reduced MMP-9 activity [as seen in zymography (Fig. 3E)] but also gelatinolysis in cocultures [as seen by in-situ zymography (Fig. 3E,F)], the structurally related SB203580 had no effect . Together, these results demonstrate that data obtained from supernatants of treated fibroblast- cultures reflect the effect of the inhibitors on the coculture. Overall, our data imply that the induction of MMP-9 expression in fibroblasts is regulated in a complex pattern and that impaired signaling via Smad-, Ras-MAPK-, and PI3-kinase-pathways attenuates tumor-cell induced MMP-9 expression, consistent with putative roles of TGF-β, EGF, or TNF-α. Moreover, because lysates of CA1a

cells induce MMP-9 expression of fibroblasts, it can be concluded that the mediators necessary for MMP-9 induction already exist in, or are on, naive CA1a cells.

TGF-β, TNF-α and EGF cooperate in tumor cell-induced MMP-9 expression by stromal cells

Based on these results we investigated the role of TGF-β, TNF-α, EGF and HGF in MMP-9 induction in DFs. EGF induced MMP-9 only minimally compared with induction by human TGF-β1 and human TNF-α (Fig. 4A). Nonetheless, EGF augmented the effect of TGF-β and TNF-α (data not shown). Importantly, treatment of DFs with TGF-β, TNF-α or EGF enhanced the expression of MMP-9 without affecting levels of MMP-2 (Fig. 4A). Immunohistochemical analysis showed that TNF-α and TGF-β were present in cocultures and mainly localized in and around CA1a cells (Fig. 4B). To test the involvement of these and other endogenous ligands we used specific neutralizing antibodies and found that the induction of MMP-9 expression in response to CA1a lysate was inhibited by addition of a TNF-α-neutralizing antibody (Fig. 4C) and also, surprisingly, an HGF-neutralizing antibody (Fig. 4D). This was despite the fact that HGF up to 80 ng/ml had no detectable effect on MMP-9 expression by DFs (data not shown). Moreover, anti-HGF decreased MMP-9 induction by both TGF-β and TNF-α (Fig. 4E,F). Induction of MMP-9 was not influenced by an unspecific antibody used as isotype control (Fig. 4E). We therefore suggest that HGF acts downstream of TGF-β and TNF-α, but that it is not sufficient by itself to induce expression of MMP-9 in this system.

Similarly, the different effects on MMP-9 induction by the EGF-receptor inhibitor AG1478, EGF itself and EGF with TGF-β1 or TNF-α together (suppressing effect, no effect and additive effect, respectively), let to our hypothesis that EGF acts downstream of TGF-β- and TNF-α. Interestingly, AG1478 inhibited the induction of MMP-9 by TGF-β, but did not negatively affect its expression induced by TNF-α- (Fig. 4G).

These data on HGF and the EGF receptor suggest that, TGF-β and TNF-α cause either the secretion of HGF or facilitate HGF-signaling (subsequently contributing to increased MMP-9 expression), and TGF-β additionally influences the expression of MMP-9 through EGF-receptor-signaling in our system.

Discussion

We have described a 2D-tissue-culture system to investigate tumor-stroma interactions in which the cells quickly organize into a stromal and a tumor component, and in which tumor cells induced expression of MMP-9 in the fibroblasts, depending on the malignancy of the tumor cell line used. MMP-9 is known to play an important role in the context of tumorigenesis and metastasis because it degrades collagen IV and weakens the basement membrane (Liotta and Stetler-Stevenson, 1990; Stetler-Stevenson, 1990). Degradation products of extracellular matrix, including fragments of collagen IV, can act as signaling substances regulating cell motility (Xu et al., 2001).

The level of proteolytic acticity of MMP-9 is controlled by a complex balance between activation, which typically requires enzymatic removal of the inhibitory propeptide, and inhibition

by the tissue inhibitors of metalloproteinases (TIMPs). Recently, an atypical activation of pro MMP-9 that is independent of proteolytic cleavage and removal of the profragment has also been described (Fedarko et al., 2004). It is still controversial which cells secrete MMP-9 and which cells regulate its activation in breast cancer. MMP-9 mRNA expression and MMP-9 immunoreactivity, neither of which is synonymous with MMP-9 activity, have been localized in a variety of cell types in breast tumors (Baker et al., 2002; Giannelli et al., 2002; Monteagudo et al., 1990; Scorilas et al., 2001) and also to both partners in cocultures of breast cancer cells and fibroblasts (Singer et al., 2002; Wang et al., 2002). Furthermore, depending on the culture-system employed, coculturing tumor-derived or normal fibroblasts with breast cancer cells resulted in an increased expression of MMP-2 by either partners (Petersen et al., 2003; Saad et al., 2000). In the 2D-coculture system we describe here, and also in xenografted tumors in nude mice, the main gelatinolytic activity (as visualized by in-situ zymography) was localized around the fibroblasts. It also was dependent on the degree of tumorigenicity of the tumor cells, paralleling the results obtained by gelatin zymography where we found increased MMP-9 secretion by fibroblasts. In contrast to other methods of visualizing enzyme localization, such as immunohistochemistry, in-situ zymography has the advantage of detecting only activated MMPs. Gelatinolytic activity was dramatically reduced by the substitution of wild-type fibroblasts with MMP9KO or MMP2KO fibroblasts, consistent with the known ability of MMP-2 to activate MMP-9 (Lynch and Matrisian, 2002; Sternlicht and Werb, 2001). These results also suggest that, although MMP-9 and MMP-2 have almost identical substrates, MMP-2 does not fully compensate for the absence of MMP-9 and vice versa. Although, MMP-9 and MMP-2 could be detected in the conditioned media of tumor cells, the gelatinolytic enzyme activity localized to tumor cells in-situ was negligible compared with the activity localized around fibroblasts, possibly because of the coexpression of TIMPs.

Compared with benign breast tumors, malignant breast tumors have increased MMP-9 activity (Hanemaaijer et al., 2000) and there is a trend towards increasing production and activation of MMP-9 in later stages of breast cancer (Davies et al., 1993; Rha et al., 1997). Nevertheless, MMP-9 expression has also been described as a positive prognostic marker in node-negative breast cancer (Scorilas et al., 2001). In another study, higher MMP-9 expression in tumor cells was associated with less lymphovascular invasion and lower tumor grade (Baker et al., 2002). Thus, MMP-9 expression is associated with both inhibition and stimulation of tumor growth and progression, probably because not all of these studies have measured actual MMP-9 activity.

Expression of MMP-9 is regulated at transcription level and can be induced by classic mediators such as TNF-α (Yang et al., 2004), TGF-β (Rundhaug et al., 1997), EGF (Menashi et al., 2003) or HGF (Park et al., 2003). In our experiments, induction of MMP-9 did not require cell-cell contacts or viability of CA1a cells, indicating that it depends on one or more soluble factors already present in naive CA1a cells. We found that TNF-α and TGF-β induced MMP-9 expression more strongly than EGF, whereas HGF had no detectable activity. Because inhibition of the TGF-β type I (ALK5)

receptor or the use of S3KO lowered MMP9 expression, we suggest that TGF-β contributes to the activity of the tumor-cell lysate but it is not the only factor regulating MMP-9 induction by tumor cells. The involvement of other than the TGF-β pathway is supported by data showing the regulation of MMP-9 expression in the Smad4 negative breast cancer cell MDA-MB468 that lacks signaling via the Smad pathway (Jones et al., 2003b). Indeed, the use of inhibitors suggested involvement of several other signaling pathways in MMP-9 induction by breast cancer cells in cocultures, including EGFR-, Ras- and PI3-kinase, most of which can be activated by TNF-α (Argast et al., 2004; Marchetti et al., 2004; Sizemore et al., 2004; Szlosarek and Balkwill, 2003). TNF-α has already been shown to be involved in cell-cell communication, and in a coculture of breast cancer cells and tumor-associated macrophages, macrophage-derived TNF-α increased the levels of MMP-9 in tumor cells (Hagemann et al., 2004). Both TGF-β and TNF-α were present in cocultures and homotypic CA1a cultures as shown by immunohistochemistry. Our data suggest that, tumor-cell-derived TNF-α and TGF-β cooperate in the regulation of MMP-9-induction in fibroblasts and the tumor stroma by signaling via MAPK- and Smad pathways, because the ALK5 inhibitor and a TNF-α neutralizing antibody each significantly reduced MMP-9 induction by tumor cells.

Although MMP-9 expression in fibroblasts was not induced by ectopic HGF, an HGF neutralizing antibody reduced the induction of MMP-9 by tumor cell lysates. This same antibody interfered with the induction of MMP-9 by TGF-β and TNF-α, suggesting that HGF-signaling is involved in the induction of MMP-9 downstream of these two cytokines. By contrast, TGF-β- but not TNF-α-induced expression of MMP-9, was decreased by an EGFR inhibitor. However, it cannot be determined from our data, whether these results are owing to an increased expression of HGF and EGF by the fibroblasts, an enhanced sensitivity to HGF and EGF, expression of their respective receptors (Met and EGFR) at higher levels or, because of facilitated downstream signaling. TGF-β-induced transactivation of the EGF receptor has shown to be relevant for the expression of another component of extracellular matrix, fibronectin (Uchiyama-Tanaka et al., 2002). Activation of the MMP-9 promotor by EGF has previously been shown to require Ets-1 (Watabe et al., 1998). Interestingly, this same transcription factor together with MMP-9 is upregulated in the stroma of pre-invasive breast cancer (Behrens et al., 2001).

TGF-β- and TNF-α-induced expression of HGF have also been described previously (Lewis et al., 2004; Rosen et al., 1994). A recent study again described an interaction between TGF-β- and HGF-dependent pathways in tumors of mice whose TβRII was selectively knocked-out in the mesenchyme but there, HGF secretion and c-Met phosphorylation were increased (Bhowmick et al., 2004).

In conclusion, we used cocultures of tumor cells and fibroblasts to study signaling pathways that are involved in the cross-talk between tumor- and stroma-regulating expression of MMP-9. We have shown that this cell-culture-system appropriately models the induction of MMP-9 as seen in xenografted tumors of human breast cancer cells in vivo. In both cases the main in vivo gelatinase activity is localized to the tumor stroma, emphasizing the important contribution of mesenchyme in the immediate vicinity of tumor cells to the tumor phenotype. Most importantly, we clearly show for the

first time that it is not a single cytokine but an intricate cytokine network that contributes to tumor-cell-induced expression of MMP-9 in fibroblasts. This requires not only the activation of multiple signaling pathways but also includes TGF-β and TNF-α as crucial signaling components, and HGF- and EGF-signaling as secondary contributors (Fig. 8). This in-vitro coculture system can now be taken to further explore the complex bi-directional signaling between tumor cells and stromal elements that are involved in modulating the tumor phenotype.

References

Argast, G. M., Campbell, J. S., Brooling, J. T. and Fausto, N. (2004). Epidermal growth factor receptor transactivation mediates tumor necrosis factor-induced hepatocyte replication. *J. Biol. Chem.* **279**, 34530-34536.

Baker, E. A., Stephenson, T. J., Reed, M. W. and Brown, N. J. (2002). Expression of proteinases and inhibitors in human breast cancer progression and survival. *Mol. Pathol.* **55**, 300-304.

Behrens, P., Rothe, M., Wellmann, A., Krischler, J. and Wernert, N. (2001). The Ets-1 transcription factor is up-regulated together with MMP 1 and MMP 9 in the stroma of pre-invasive breast cancer. *J. Pathol.* **194**, 43-50.

Bhowmick, N. A., Chytil, A., Plieth, D., Gorska, A. E., Dumont, N., Shappell, S., Washington, M. K., Neilson, E. G. and Moses, H. L. (2004). TGF-beta signaling in fibroblasts modulates the oncogenic potential of adjacent epithelia. *Science* **303**, 848-851.

Bissell, M. J. and Radisky, D. (2001). Putting tumours in context. *Natl. Rev. Cancer* **1**, 46-54.

Brown, P. D., Levy, A. T., Margulies, I. M., 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.

Davies, B., Miles, D. W., Happerfield, L. C., Naylor, M. S., Bobrow, L. G., Rubens, R. D. and Balkwill, F. R. (1993). Activity of type IV collagenases in benign and malignant breast disease. *Brit. J. Cancer* **67**, 1126-1131.

Dawson, P. J., Wolman, S. R., Tait, L., Heppner, G. H. and Miller, F. R. (1996). MCF10AT: a model for the evolution of cancer from proliferative breast disease. *Am. J. Pathol.* **148**, 313-319.

Dong-Le Bourhis, X., Berthois, Y., Millot, G., Degeorges, A., Sylvi, M., Martin, P. M. and Calvo, F. (1997). Effect of stromal and epithelial cells derived from normal and tumorous breast tissue on the proliferation of human breast cancer cell lines in co-culture. *Int. J. Cancer* **71**, 42-48.

Elenbaas, B. and Weinberg, R. A. (2001). Heterotypic signaling between epithelial tumor cells and fibroblasts in carcinoma formation. *Exp. Cell Res.* **264**, 169-184.

Fedarko, N. S., Jain, A., Karadag, A. and Fisher, L. W. (2004). Three small integrin binding ligand N-linked glycoproteins (SIBLINGs) bind and activate specific matrix metalloproteinases. *FASEB J.* **18**, 734-736.

Flanders, K. C., Sullivan, C. D., Fujii, M., Sowers, A., Anzano, M. A., Arabshahi, A., Major, C., Deng, C., Russo, A., Mitchell, J. B. et al. (2002). Mice lacking Smad3 are protected against cutaneous injury induced by ionizing radiation. *Am. J. Pathol.* **160**, 1057-1068.

Fridman, R., Fuerst, T. R., Bird, R. E., Hoyhtya, M., Oelkuct, M., Kraus, S., Komarek, D., Liotta, L. A., Berman, M. L. and Stetler-Stevenson, W. G. (1992). Domain structure of human 72-kDa gelatinase/type IV collagenase. Characterization of proteolytic activity and identification of the tissue inhibitor of metalloproteinase-2 (TIMP-2) binding regions. *J. Biol. Chem.* **267**, 15398-15405.

Fridman, R., Bird, R. E., Hoyhtya, M., Oelkuct, M., Komarek, D., Liang, C. M., Berman, M. L., Liotta, L. A., Stetler-Stevenson, W. G. and Fuerst, T. R. (1993). Expression of human recombinant 72 kDa gelatinase

and tissue inhibitor of metalloproteinase-2 (TIMP-2): characterization of complex and free enzyme. *Biochem. J.* **289 (Pt 2)**, 411-416.

Giannelli, G., Fransvea, E., Marinosci, F., Bergamini, C., Daniele, A., Colucci, S., Paradiso, A., Quaranta, M. and Antonaci, S. (2002). Gelatinase levels in male and female breast cancer. *Biochem. Biophys. Res. Commun.* **292**, 161-166.

Hagemann, T., Robinson, S. C., Schulz, M., Trumper, L., Balkwill, F. R. and Binder, C. (2004). Enhanced invasiveness of breast cancer cell lines upon co-cultivation with macrophages is due to TNF-(R) dependent upregulation of matrix metalloproteases. *Carcinogenesis* **25**, 1543-1549.

Hanemaaijer, R., Verheijen, J. H., Maguire, T. M., Visser, H., Toet, K., McDermott, E., O'Higgins, N. and Duffy, M. J. (2000). Increased gelatinase-A and gelatinase-B activities in malignant vs. benign breast tumors. *Int. J. Cancer* **86**, 204-207.

Heldin, C. H., Miyazono, K. and ten Dijke, P. (1997). TGF-beta signalling from cell membrane to nucleus through SMAD proteins. *Nature* **390**, 465-471.

Heppner, G. H., Miller, F. R. and Shekhar, P. M. (2000). Nontransgenic models of breast cancer. *Breast Cancer Res.* **2**, 331-334.

Idriss, H. T. and Naismith, J. H. (2000). TNF alpha and the TNF receptor superfamily: structure-function relationship(s). *Microsc. Res. Tech.* **50**, 184-195.

Jones, C. B., Sane, D. C. and Herrington, D. M. (2003a). Matrix metalloproteinases: a review of their structure and role in acute coronary syndrome. *Cardiovasc. Res.* **59**, 812-823.

Jones, J. L., Shaw, J. A., Pringle, J. H. and Walker, R. A. (2003b). Primary breast myoepithelial cells exert an invasion-suppressor effect on breast cancer cells via paracrine down-regulation of MMP expression in fibroblasts and tumour cells. *J. Pathol.* **201**, 562-572.

Kuperwasser, C., Chavarria, T., Wu, M., Magrane, G., Gray, J. W., Carey, L., Richardson, A. and Weinberg, R. A. (2004). Reconstruction of functionally normal and malignant human breast tissues in mice. *Proc. Natl. Acad. Sci. USA* **101**, 4966-4971.

Laping, N. J., Grygielko, E., Mathur, A., Butter, S., Bomberger, J., Tweed, C., Martin, W., Fornwald, J., Lehr, R., Harling, J. et al. (2002). Inhibition of transforming growth factor (TGF)-beta1-induced extracellular matrix with a novel inhibitor of the TGF-beta type I receptor kinase activity: SB-431542. *Mol. Pharmacol.* **62**, 58-64.

Lewis, M. P., Lygoe, K. A., Nystrom, M. L., Anderson, W. P., Speight, P. M., Marshall, J. F. and Thomas, G. J. (2004). Tumour-derived TGF-beta1 modulates myofibroblast differentiation and promotes HGF/SF-dependent invasion of squamous carcinoma cells. *Brit. J. Cancer* **90**, 822-832.

Liotta, L. A. and Stetler-Stevenson, W. G. (1990). Metalloproteinases and cancer invasion. *Semin. Cancer Biol.* **1**, 99-106.

Liotta, L. A. and Kohn, E. C. (2001). The microenvironment of the tumour-host interface. *Nature* **411**, 375-379.

Locksley, R. M., Killeen, N. and Lenardo, M. J. (2001). The TNF and TNF receptor superfamilies: integrating mammalian biology. *Cell* **104**, 487-501.

Lynch, C. C. and Matrisian, L. M. (2002). Matrix metalloproteinases in tumor-host cell communication. *Differentiation* **70**, 561-573.

Marchetti, L., Klein, M., Schlett, K., Pfizenmaier, K. and Eisel, U. L. (2004). TNF mediated neuroprotection against glutamate induced excitotoxicity is enhanced by NMDA receptor activation: Essential role of a TNF receptor 2 mediated, PI3 kinase dependent NF-kB pathway. *J. Biol. Chem.* **279**, 32869-32881.

Massague, J. (2000). How cells read TGF-beta signals. *Nat. Rev. Mol. Cell Biol.* **1**, 169-178.

Menashi, S., Serova, M., Ma, L., Vignot, S., Mourah, S. and Calvo, F. (2003). Regulation of extracellular matrix metalloproteinase inducer and matrix metalloproteinase expression by amphiregulin in transformed human breast epithelial cells. *Cancer Res.* **63**, 7575-7580.

Miller, F. R., Santner, S. J., Tait, L. and Dawson, P. J. (2000). MCF10DCIS.com xenograft model of human comedo ductal carcinoma in situ. *J. Natl. Cancer Inst.* **92**, 1185-1186.

Monteagudo, C., Merino, M. J., San Juan, J., Liotta, L. A. and Stetler-Stevenson, W. G. (1990). Immunohistochemical distribution of type IV collagenase in normal, benign, and malignant breast tissue. *Am. J. Pathol.* **136**, 585-592.

Mook, O. R., van Overbeek, C., Ackema, E. G., van Maldegem, F. and Frederiks, W. M. (2003). In situ localization of gelatinolytic activity in the extracellular matrix of metastases of colon cancer in rat liver using quenched fluorogenic DQ-gelatin. *J. Histochem. Cytochem.* **51**, 821-829.

Muraoka, R. S., Koh, Y., Roebuck, L. R., Sanders, M. E., Brantley-Sieders, D., Gorska, A. E., Moses, H. L. and Arteaga, C. L. (2003). Increased

malignancy of Neu-induced mammary tumors overexpressing active transforming growth factor beta1. *Mol. Cell Biol.* **23**, 8691-8703.

Nabeshima, K., Inoue, T., Shimao, Y. and Sameshima, T. (2002). Matrix metalloproteinases in tumor invasion: role for cell migration. *Pathol. Int.* **52**, 255-264.

Nielsen, B. S., Sehested, M., Kjeldsen, L., Borregaard, N., Rygaard, J. and Dano, K. (1997). Expression of matrix metalloprotease-9 in vascular pericytes in human breast cancer. *Lab. Invest.* **77**, 345-355.

Noel, A. and Foidart, J. M. (1998). The role of stroma in breast carcinoma growth in vivo. *J. Mammary Gland. Biol.* **3**, 215-225.

Olumi, A. F., Grossfeld, G. D., Hayward, S. W., Carroll, P. R., Tlsty, T. D. and Cunha, G. R. (1999). Carcinoma-associated fibroblasts direct tumor progression of initiated human prostatic epithelium. *Cancer Res.* **59**, 5002-5011.

Park, Y. H., Ryu, H. S., Choi, D. S., Chang, K. H., Park, D. W. and Min, C. K. (2003). Effects of hepatocyte growth factor on the expression of matrix metalloproteinases and their tissue inhibitors during the endometrial cancer invasion in a three-dimensional coculture. *Int. J. Gynecol. Cancer* **13**, 53-60.

Petersen, O. W., Nielsen, H. L., Gudjonsson, T., Villadsen, R., Rank, F., Niebuhr, E., Bissell, M. J. and Ronnov-Jessen, L. (2003). Epithelial to mesenchymal transition in human breast cancer can provide a nonmalignant stroma. *Am. J. Pathol.* **162**, 391-402.

Radisky, D. C. and Bissell, M. J. (2004). Cancer. Respect thy neighbor! *Science* **303**, 775-777.

Rha, S. Y., Kim, J. H., Roh, J. K., Lee, K. S., Min, J. S., Kim, B. S. and Chung, H. C. (1997). Sequential production and activation of matrix-metalloproteinase-9 (MMP-9) with breast cancer progression. *Breast Cancer Res. Tr.* **43**, 175-181.

Roberts, A. B. and Wakefield, L. M. (2003). The two faces of transforming growth factor beta in carcinogenesis. *Proc. Natl. Acad. Sci. USA* **100**, 8621-8623.

Rosen, E. M., Joseph, A., Jin, L., Rockwell, S., Elias, J. A., Knesel, J., Wines, J., McClellan, J., Kluger, M. J., Goldberg, I. D. et al. (1994). Regulation of scatter factor production via a soluble inducing factor. *J. Cell Biol.* **127**, 225-234.

Rundhaug, J. E., Park, J., Pavone, A., Opdenakker, G. and Fischer, S. M. (1997). Opposite effect of stable transfection of bioactive transforming growth factor-beta 1 (TGF beta 1) versus exogenous TGF beta 1 treatment on expression of 92-kDa type IV collagenase in mouse skin squamous cell carcinoma CH72 cells. *Mol. Carcinogen.* **19**, 122-136.

Saad, S., Bendall, L. J., James, A., Gottlieb, D. J. and Bradstock, K. F. (2000). Induction of matrix metalloproteinases MMP-1 and MMP-2 by co-culture of breast cancer cells and bone marrow fibroblasts. *Breast Cancer Res. Tr.* **63**, 105-115.

Saad, S., Gottlieb, D. J., Bradstock, K. F., Overall, C. M. and Bendall, L. J. (2002). Cancer cell-associated fibronectin induces release of matrix metalloproteinase-2 from normal fibroblasts. *Cancer Res.* **62**, 283-289.

Santner, S. J., Dawson, P. J., Tait, L., Soule, H. D., Eliason, J., Mohamed, A. N., Wolman, S. R., Heppner, G. H. and Miller, F. R. (2001). Malignant MCF10CA1 cell lines derived from premalignant human breast epithelial MCF10AT cells. *Breast Cancer Res. Tr.* **65**, 101-10.

Scorilas, A., Karameris, A., Arnogiannaki, N., Ardavanis, A., Bassilopoulos, P., Trangas, T. and Talieri, M. (2001). Overexpression of

matrix-metalloproteinase-9 in human breast cancer: a potential favourable indicator in node-negative patients. *Brit. J. Cancer* **84**, 1488-1496.

Siegel, P. M., Shu, W., Cardiff, R. D., Muller, W. J. and Massague, J. (2003). Transforming growth factor beta signaling impairs Neu-induced mammary tumorigenesis while promoting pulmonary metastasis. *Proc. Natl. Acad. Sci. USA* **100**, 8430-8435.

Singer, C. F., Kronsteiner, N., Marton, E., Kubista, M., Cullen, K. J., Hirtenlehner, K., Seifert, M. and Kubista, E. (2002). MMP-2 and MMP-9 expression in breast cancer-derived human fibroblasts is differentially regulated by stromal-epithelial interactions. *Breast Cancer Res. Tr.* **72**, 69-77.

Sizemore, N., Agarwal, A., Das, K., Lerner, N., Sulak, M., Rani, S., Ransohoff, R., Shultz, D. and Stark, G. R. (2004). Inhibitor of kappaB kinase is required to activate a subset of interferon gamma-stimulated genes. *Proc. Natl. Acad. Sci. USA* **101**, 7994-7998.

Sternlicht, M. D. and Werb, Z. (2001). How matrix metalloproteinases regulate cell behavior. *Annu. Rev. Cell Dev. Biol.* **17**, 463-516.

Stetler-Stevenson, W. G. (1990). Type IV collagenases in tumor invasion and metastasis. *Cancer Metastasis Rev.* **9**, 289-303.

Szlosarek, P. W. and Balkwill, F. R. (2003). Tumour necrosis factor alpha: a potential target for the therapy of solid tumours. *Lancet Oncol.* **4**, 565-573.

Tang, B., Vu, M., Booker, T., Santner, S. J., Miller, F. R., Anver, M. R. and Wakefield, L. M. (2003). TGF-beta switches from tumor suppressor to prometastatic factor in a model of breast cancer progression. *J. Clin. Invest* **112**, 1116-1124.

Tian, F., DaCosta, B. S., Parks, W. T., Yoo, S., Felici, A., Tang, B., Piek, E., Wakefield, L. M. and Roberts, A. B. (2003). Reduction in smad2/3 signaling enhances tumorigenesis but suppresses metastasis of breast cancer cell lines. *Cancer Res.* **63**, 8284-8292.

Uchiyama-Tanaka, Y., Matsubara, H., Mori, Y., Kosaki, A., Kishimoto, N., Amano, K., Higashiyama, S. and Iwasaka, T. (2002). Involvement of HB-EGF and EGF receptor transactivation in TGF-beta-mediated fibronectin expression in mesangial cells. *Kidney Int.* **62**, 799-808.

Wakefield, L. M. and Roberts, A. B. (2002). TGF-beta signaling: positive and negative effects on tumorigenesis. *Curr. Opin. Genet. Dev.* **12**, 22-29.

Wang, T. N., Albo, D. and Tuszynski, G. P. (2002). Fibroblasts promote breast cancer cell invasion by upregulating tumor matrix metalloproteinase-9 production. *Surgery* **132**, 220-225.

Watabe, T., Yoshida, K., Shindoh, M., Kaya, M., Fujikawa, K., Sato, H., Seiki, M., Ishii, S. and Fujinaga, K. (1998). The Ets-1 and Ets-2 transcription factors activate the promoters for invasion-associated urokinase and collagenase genes in response to epidermal growth factor. *Int. J. Cancer* **77**, 128-137.

Wrana, J. L., Attisano, L., Wieser, R., Ventura, F. and Massague, J. (1994). Mechanism of activation of the TGF-beta receptor. *Nature* **370**, 341-347.

Xu, J., Rodriguez, D., Petitclerc, E., Kim, J. J., Hangai, M., Moon, Y. S., Davis, G. E., Brooks, P. C. and Yuen, S. M. (2001). Proteolytic exposure of a cryptic site within collagen type IV is required for angiogenesis and tumor growth in vivo. *J. Cell Biol.* **154**, 1069-1079.

Yang, W. L., Godwin, A. K. and Xu, X. X. (2004). Tumor necrosis factor-alpha-induced matrix proteolytic enzyme production and basement membrane remodeling by human ovarian surface epithelial cells: molecular basis linking ovulation and cancer risk. *Cancer Res.* **64**, 1534-1540.