Tumor-Derived Cyr61(CCN1) Promotes Stromal Matrix Metalloproteinase-1 Production and Protease-Activated Receptor 1–Dependent Migration of Breast Cancer Cells

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Abstract

Matrix metalloproteinases (MMPs) play a central role in remodeling the tumor-stromal microenvironment. We recently determined that stromal-derived MMP-1 also acts as a signaling molecule by cleaving protease-activated receptor 1 (PAR1) to cause breast cancer cell migration and invasion. Here, we show that ectopic PAR1 expression induces expression of the angiogenic factor Cyr61(CCN1) in breast cancer cells. The tumor-derived Cyr61 acts as an invasogenic signaling molecule that induces MMP-1 expression in adjacent stromal fibroblasts. Gene silencing of Cyr61 in breast cancer cells suppresses MMP-1 induction in stromal fibroblasts resulting in a major loss in migration of the cancer cells toward the fibroblasts. Cyr61-dependent loss of migration was complemented by exogenous MMP-1 and required the presence of the functional PAR1 receptor on the breast cancer cells. These results suggest that interrupting tumor-stromal cell communication by targeting Cyr61 may provide an alternative therapeutic approach for the treatment of invasive breast cancer. (Cancer Res 2006; 66(5): 2658-65)

Introduction

Matrix metalloproteinases (MMPs) are a large family of zinc-dependent enzymes that cleave extracellular matrix proteins during tissue remodeling processes, such as wound healing, angiogenesis, and tumor invasion. Among MMPs, the collagenase MMP-1 has been identified as one of the most highly up-regulated proteins in various cancers, including breast, esophageal, and colorectal carcinomas (1–3). Histologic studies of tumors derived from cancer patients revealed that the majority of the MMPs, including MMP-1, are produced by stromal cells rather than tumor cells (4). Stromal MMP-1 has recently been shown to cleave and activate a G-protein-coupled receptor, the protease-activated receptor 1 (PAR1), resulting in enhanced invasion and tumorigenesis of breast cancer cells (5, 6). Thus, MMP-1 mediates tumor-stromal interactions, and uncovering mechanisms that regulate MMP-1 expression may aid in blocking tumor invasion.

PAR1, an effector of MMP-1, has been identified as an oncogene (7–9) and is involved in the invasive and metastatic processes of breast cancer (10), pancreatic cancer (11), and melanoma (12–17). Early studies by Even-Ram et al. (10) showed that PAR1 expression levels were directly correlated with degree of invasiveness in both primary breast tissue specimens and established cancer cell lines. High levels of PAR1 mRNA were found in infiltrating ductal carcinomas and very low amounts in normal and premalignant atypical intraductal hyperplasia. PAR1 expression levels increased by up to 10-fold in 106 invasive ductal and 17 invasive lobular tumors compared with 6 normal breast specimens (18). Recent studies by our group (19) showed that the invasive MDA-MB-231 breast cancer cell line expresses very high levels of functional PAR, whereas minimally invasive MCF7 cells have no PAR1. Forced expression of PAR1 in MCF7 cells was sufficient to promote tumor growth and invasion in breast cancer xenografts (5). In the present study, we set out to identify whether tumor-derived Cyr61 could induce stromal MMP-1 production and mediate tumor cell migration and invasion through PAR1.

Cyr61, a member of the CCN family, is highly up-regulated during wound healing and induces the expression of a diverse array of genes, such as MMP-1, MMP-3, tissue inhibitor of metalloproteinase 1, urokinase-type plasminogen activator, and plasminogen activator inhibitor-1, and angiogenesis and lymphogenesis factors, such as vascular endothelial growth factor (VEGF)-A, VEGF-C, and interleukin (IL)-1β (20). Recent work suggests that the CCN family members may also play roles in contexts other than tissue repair, such as tumor growth, invasion, and metastasis. Interestingly, the human Cyr61 gene was mapped to the chromosome 1p region in which abnormal rearrangements have been shown previously to correlate with poor prognosis in breast cancer patients (21, 22). Up-regulated expression of Cyr61 has been detected in invasive and metastatic breast cancer cells and tumor biopsies (23). Ectopic expression of Cyr61 in MCF7 breast cancer cells supports tumor formation and neovascularization in mice (24–27). Similar to breast tumors, Cyr61 expression in U343 glioma cells increases tumorigenicity and vascularization (28). In addition to its role in vascularization, Cyr61 can enhance motility of fibroblasts and microvascular endothelial cells (24, 26); however, the mechanism of action of Cyr61 in tumor cell migration and invasion is not well understood.

In this study, we show that tumor-derived Cyr61 enhances PAR1-mediated migration of breast cancer cells toward stromal fibroblasts. We found that silencing Cyr61 in invasive breast cancer cells caused a major loss of MMP-1 induction from stromal fibroblasts. Conversely, silencing MMP-1 in stromal fibroblasts inhibited PAR1- and Cyr61-dependent migration. These results provide direct evidence for Cyr61 in paracrine regulation of fibroblast MMP-1 by tumor cells.

Materials and Methods

Cell culture and materials. The WI38 cell line was obtained from American Type Culture Collection (Manassas, VA), and breast cancer cell
Real-time PCR was conducted in 50 μl SYBR Green, 2.5 mM L cDNA, 25 mM primer, and 10 mM MgCl₂. cDNA was amplified in a final volume of 25 μl using MMP-1-specific primers or DNA constructs. Par1 was expressed for pCDEF3 (29). MCF7 cells were transfected with pCDEF3-Par1, and individual clones were selected as described previously (5). Small interfering RNAs (siRNA), directed against Cyre61 (5'-AATGATTTGTCGTTGAGTGA-3'), and firefly luciferase (5'-CTGACCGGATCATCTGA-3') luciferase-siRNA), were synthesized by Dharmaco (Lafayette, CO). MDA-MB-231 and MCF7-N55 cells were transfected with Oligofectamine using 20 μg RNA per 100-mm plate (800,000 cells per plate).

Breast cancer/fibroblast coculture for quantitative PCR. Fibroblasts (early passages 2-9) were plated on the top chamber of a Transwell plate (12-mm-diameter filter, 0.4-μm pore size; Costar, Corning, NY) at a density of 50,000 per well, cultured in complete medium, and starved in serum-free medium (RPMI 1640 with 0.1% BSA) 1 day before start of assay. Breast cancer cells were then seeded on the bottom well of a Transwell system and cocultured in serum-free medium for 5 to 40 hours. For some experiments, breast cancer cells were pretreated with siRNA for Cyre61, Par1, or luciferase control for 48 hours. Fibroblasts were harvested with PBS/1.5 mM/0.1 EDTA for RNA extraction.

Isolation of total RNA and quantitative PCR. Total RNA was extracted using the RNeasy mini kit (Qiagen, Valencia, CA). For semiquantitative PCR, 1 μg RNA was reverse transcribed using Moloney murine leukemia virus reverse transcriptase in the presence of deoxy nucleotide triphosphate (dNTP; 0.4 μM/l. Each), cDNA was amplified in a final volume of 25 μl supplemented with 1.5 mM/l Mglyc, dNTPs (0.2 μM/l. Each) using Taq polymerase (Roche Applied Science, Indianapolis, IN) and the following primers: hMMP-1 sense 5'-CGACTCTGAAACACAGGCAAGA-3' and antisense 5'-AAGGTTAGTCATCTCAGGCCTGCT-3'; mMMP-1 (MCOL-a) sense 5'-TCTTTATTCCTCTTCAGGGCCATGAA-3' and antisense 5'-CCCTCCTTCTCTAGGCGGGATCATA-3' and actin sense 5'-GCTCTCTTCCAGCTTCTCTCCT-3' and antisense 5'-CAAGAGTACTGGTGCGGAGG-3'. Real-time PCR was conducted in 50 μl volumes containing 1 μl cDNA, 25 μl SYBR Green, 2.5 μl of 5 mM/l. Each of the specific primers and the probe, and 19 μl RNA-free water. All of the reactions were done in triplicate in an iCycle iQ system (Bio-Rad, Hercules, CA). Sequence Detection System 1.9.1 alias program was used in detecting the fluorescent level of MMP-1 and contract for 40 cycles, and the thermal cycling conditions of the Qiagen system were as follows: 15 minutes at 95°C, 30 seconds at 94°C, 1 hour at 55°C, 30 seconds at 72°C, 15 seconds at 95°C, 20 seconds at 55°C, and 15 seconds at 95°C.

Specificity of human and mouse MMP-1 and Cyre61 primers. Total RNA was isolated from CRL-2076 (human) and NIH-3T3 (mouse) and reverse transcribed as described in Materials and Methods. Reverse-transcribed mRNA was PCR amplified using MMP-1-specific primers or actin and visualized by ethidium bromide agarose gel electrophoresis (Supplementary Fig. S1).

Isolation of primary human and mouse mammary fibroblasts. Mammary fat pads were removed from 3- to 6-week-old female wild-type C57BL/6 mice. Tissue was finely minced with a razor blade as described previously (30). Minced tissue was digested with 10 units/ml collagenase type I in RPMI 1640 for 20 minutes at 37°C. The suspension was centrifuged for 10 minutes at 200 × g and resuspended in 5 ml RPMI 1640 plus 20% FBS. The resultant culture was confirmed to be fibroblastic by a pathologist. Following approval by the Tufts-New England Medical Center (NEMC) Institutional Review Board, human mammary tissue was obtained from an unidentified female patient who underwent bilateral mastectomy at Tufts-NEMC for removal of an invasive breast adenocarcinoma. Stromal fibroblasts were isolated from human breast tissue as described above.

Western blot analysis. Cells were grown in 100-mm plates and disrupted by multiple passage through a 27-gauge needle in radioimmunoprecipitation assay (RIPA; 150 mM/l. NaCl, 50 mM/l. Tris (pH 8.0), 0.1% NP40, 0.5% deoxycholate, 1 mM/l. phenylmethylsulfonyl fluoride) and then incubated with heparin beads (Heparin Sepharose 6 Fast Flow, Amersham Pharmacia, Piscataway, NJ) at a ratio of 1:20 (slurry/beads) at 4°C for 1 hour. The beads were washed twice with RIPA containing 400 mM/l. NaCl and eluted with 800 mM/l. NaCl. The eluted lysate was loaded onto 12% polyacrylamide gels and transferred to polyvinylidene difluoride membranes (Immobilon-P; Millipore, Bedford, MA). Soluble Cyre61 from conditioned medium was concentrated 10-fold with Centricon-10 (Millipore). The membrane was incubated with the Cyre61 antibody (0.16 μg/ml) at 4°C for 1 hour. After washing with Tween 20 in TBS, the membrane was then incubated with anti-rabbit IgG-horseradish peroxidase antibody (Dako A/S, Glostrup, Denmark; 1:1000 dilution) for 1 hour at room temperature. Membrane proteins were detected by enhanced chemiluminescence (Amersham Pharmacia) and exposed on Hyperfilm (Amersham Pharmacia).

Tumor-stromal cell coculturing migration assays. Transwell plates (24-well), with cell culture inserts of 6.5 mm in diameter and 8.0-μm pore size, were used. Fibroblasts were seeded on the bottom wells (100,000 per well) of the Transwell apparatus 2 days before the experiment and starved 1 day before in serum-free medium (RPMI 1640 with 0.1% BSA). Before the experiment, breast tumor cells were resuspended in serum-free medium, plated on the top well (cell culture inserts) at a density of 50,000 (200 μl/well), and allowed to migrate to the underside of the top chamber for 40 hours. The migratory cells attached to the bottom surface of the membrane were stained with Hema 3 Fixative Solution, Hema 3 Solution I, and Hema 3 Solution II (Fisher, Hampton, NH) for 30 seconds at room temperature. Migratory cells in nine randomly selected migratory fields were counted using bright-field microscopy (19). Breast cancer cells or fibroblasts were treated with siRNA and then cocultured. For some experiments, coculture migration assays were also done in the presence of small-molecule ligand-based antagonist of PAR1, RWJ-36110 (31, 32), or aminophenylmercuric acetate–activated MMP-1 (5), which was added to the bottom chamber at the start of assay.

MMP-1 expression and collagenase assay. Collagenase activity was assayed in conditioned medium as described previously (5). Briefly, the cleavage of fluorescein-conjugated 10 μg DQ collagen (Molecular Probes, Carlsbad, CA) was assayed in 50 μl/l. Tris-HCl (pH 7.6), 150 mM/l. NaCl, 5 mM/l. CaCl₂, and Na₂F and cleavage was monitored continuously over time by a fluorescent microplate reader at 538 nm (excitation at 485 nm) at 25°C. Pro-MMP-1 concentration was determined using Quantikine ELISA (R&D Systems, Minneapolis, MN) according to the manufacturer’s instructions.
Statistics. All migration results are presented as mean ± SD. Comparisons were made with Student’s t test. Statistical significance was defined as \( P < 0.05 \). SYBR Green I–based quantitative PCR expression levels of target gene transcripts were quantified relative to internal β-actin expression level.

Results and Discussion

PAR1-mediated expression of Cyr61 in breast cancer cells.

To test the hypothesis that PAR1 modulates stromal MMP-1 expression through the angiogenic factor Cyr61 (Fig. 1A), we first determined whether PAR1 expression and activation induce Cyr61 expression in breast cancer cells. To conduct these studies, we used the PAR1-null breast carcinoma cell line MCF7, which is a poor migratory and nontumorigenic cell line (19) and expresses low basal levels of Cyr61 (26, 33). MCF7 cells were transiently transfected with PAR1 and activated with the strong PAR1 agonist, thrombin. Real-time reverse transcription-PCR (RT-PCR) analyses were used to quantify relative levels of Cyr61 mRNA. Because Cyr61 is an early response gene, we predicted that its expression would be rapidly up-regulated. Indeed, treatment of PAR1-expressing MCF7 cells with 1 nmol/L thrombin caused a 3.5-fold up-regulation of Cyr61 mRNA after 30 minutes with little effect on actin mRNA expression (Fig. 1B). Because the transfection frequency was 38%, the 3.5-fold up-regulation is probably an underestimate of PAR1 induction. As a positive control, 17β-estradiol (26, 33) gave a 3-fold induction in Cyr61 mRNA at the 5-hour time point (Fig. 1B).

Our next approach was to create a series of MCF7 cell lines that stably expressed PAR1 and determine whether PAR1 levels correlated with basal expression of Cyr61 because PAR1 is known to stimulate oncogenic pathways (7, 8) and angiogenesis (9). We generated 21 distinct clones that expressed differing levels of PAR1 on the cell surface. Clone N55 had the highest level of PAR1 expression that was similar to that of MDA-MB-231 cells (19). Clone N26 expressed intermediate levels of PAR1, whereas clone N41 had low or undetectable levels of PAR1 (Fig. 2A and C). Calcium flux measurements confirmed that PAR1 was activatable by thrombin. As shown in Fig. 2B, there was a direct correlation between PAR1 expression levels and the capacity of an individual clone to generate a thrombin-dependent Ca2+ signal. There was no thrombin Ca2+ signal in the parental MCF7 cells.

PAR1 expression levels have been shown to directly correlate with breast cancer invasion and migration in advanced tumors. We compared different levels of PAR1 expression in the MCF7 parental background and their ability to migrate toward conditioned medium from CRL-2076 fibroblasts. The N55 clone, which expressed the highest levels of PAR1, migrated ~20-fold higher than the parental MCF7 (Fig. 2C), which is comparable with the migration rate of the highly invasive MDA-MB-231 cells. Clones N26 and N29, which expressed intermediate levels of PAR1, migrated 10-fold higher than the MCF7 cells. Clones N41 and N50, with undetectable levels of PAR1, migrated at the level of the parental MCF7 cells.

Next, we measured the basal level of Cyr61 expression in each of the clones using Western blot analysis (34) and compared Cyr61 levels with the parental MCF7 cells and with the highly invasive MDA-MB-231 cells, which are known to express high levels of Cyr61 (26). We generated a polyclonal Cyr61 antibody against a peptide corresponding to the unique variable region of Cyr61 (GLLGKELGFDSAC). Western blot analysis with the affinity-purified Cyr61 antibody detected a specific band at the expected size of 42 kDa in MDA-MB-231 cells that was absent in MCF7 cells (Fig. 2D). Interestingly, basal expression of Cyr61 directly correlated with the level of PAR1 expression with high levels of Cyr61 in clone N55, low detectable levels of Cyr61 in clones N29 and N26, and undetectable in clones N50 and 41 (Fig. 2D). However, Cyr61 protein has been reported to form high molecular aggregates (35) that may be responsible for the high molecular weight material in lanes N26 and N29. Therefore, to more directly determine whether PAR and Cyr61 expression directly correlate in PAR1-MCF7-derived clones, we measured basal mRNA expression of Cyr61 and PAR1 (and actin) using quantitative real-time PCR analysis. We then tested whether PAR1 expression would have an effect on Cyr61 gene expression in the MCF7 clones. Surprisingly, we found a strong correlation (\( R = 0.99; P < 0.005 \)) between PAR1 and Cyr61 expression (Fig. 2D). Immunohistochemical staining confirmed that clone N55 expressed high levels of Cyr61 that was secreted into the pericellular space (36) in a pattern similar to that of MDA-MB-231 (Fig. 3A; green stain). By comparison, counterstaining for tumor-derived MMP-9 (5) revealed a punctate pattern (red stain) localizing to the cell surface.

Paracrine induction of fibroblast MMP-1 by breast cancer cells. In wound healing models, pure Cyr61 has been shown to

Figure 1. Activation of PAR1 induces Cyr61 mRNA expression in breast cancer cells. A, a model for tumor-derived Cyr61 induction of stromal MMP-1 that results in enhanced tumor cell migration. B, MCF7 cells transiently transfected with PAR1 were stimulated with thrombin (1 nmol/L) for 0.5 to 2 hours or 17β-estradiol (E2; 100 nmol/L) for 5 hours. Transfection efficiency was 38%. Expression levels of Cyr61 (CCN1) and actin were determined using SYBR Green I–based quantitative PCR. Similar conditions and total amount of RNA were used in each experiment. The relative Cyr61 gene expression was normalized to the mean value of nonstimulated MCF7 cells transiently transfected with PAR1 (noninduced control), and this value was set to 1. Each of the thrombin-induced time points was divided by the actin control conducted in the identical sample to generate the relative fold induction for Cyr61. Experiments were repeated twice. Points, mean; bars, SD.
Coculturing system, whereas MCF7 cells did not (Fig. 3A). The MDA-MB-231 or MCF7-N55 cells induced secretion of MMP-1 in fibroblasts (20). Thus, we hypothesized that tumor-derived Cyr61 may have an analogous function in stimulating MMP-1 production from adjacent stromal cells that may contribute to tumor invasion (5). To examine a potential paracrine role of Cyr61, we developed the coculturing model system shown in Fig. 3B. We compared the high-expressing Cyr61 (N11, N26, and N55) for their ability to induce MMP-1 from the WI38 fibroblasts. The MDA-MB-231 or MCF7-N55 cells induced MMP-1 mRNA expression in WI38 fibroblasts in the coculturing system, whereas MCF7 cells did not (Fig. 3C). Using quantitative RT-PCR, we showed that the presence of MDA-MB-231 cells caused a 3.5-fold induction of MMP-1 mRNA in WI38 cells compared with the WI38 cells grown in RPMI 1640 alone (Fig. 3C). There was no detectable change in actin mRNA levels. Furthermore, using Western blot analysis, we also confirmed MMP-1 induction at the protein level (Fig. 3D). We could detect the expression of both proform (~57/52 kDa) and active forms (42 kDa) of MMP-1 in conditioned medium from cocultured WI38 and MDA-MB-231 cells. There was no detectable MMP-1 in WI38 cells grown alone (Fig. 3D). Previously, we have shown that MDA-MB-231 cells do not express MMP-1 (5). We transiently induced Cyr61 expression in the MCF7 cells with 10 nmol/L 17β-estradiol and cocultured with WI38 cells. Estrogen stimulation of MCF7 cells caused an induction of MMP-1 from the WI38 compared with nonstimulated MCF7 control (Fig. 3C).

Previous studies (5) showed that mRNA and protein expression levels of MMP-1 were elevated in murine tissues from MCF7-N55 xenografts (7-week-old) compared with control mammary fat pads. To mimic breast cancer cell-stromal cell interactions in vitro, we prepared fresh primary mammary fibroblasts from mouse and human tissues. Fibroblasts were used within 7 days of tissue collection without propagation. First, we confirmed that neither the MDA-MB-231 and MCF7-N55 breast cancer cells nor cells from murine mammary fat pads constitutively express MMP-1 (5). Strikingly, there was a strong induction of MMP-1 from the mouse and human mammary stromal fibroblasts in the presence of MCF7-N55 or MDA-MB-231 cells (Fig. 4A and B). Together, these data indicate that breast cancer cells induce expression of MMP-1 in primary mammary stromal fibroblasts as occurred with WI38 fibroblasts. These data are consistent with previous observations, which showed that MMP-1 is largely expressed by the surrounding stromal cells recruited to the invasive front of the tumor (4, 37).

Effect of Cyr61 gene silencing on paracrine induction of fibroblast MMP-1. To directly show that breast cancer cells mediate their MMP-1-inducing effects through Cyr61, we silenced Cyr61 gene expression using Cyr61-siRNA. As shown in Fig. 5A, treatment of MDA-MB-231 or MCF7-N55 cells with Cyr61-siRNA...
caused a complete loss of Cyr61 expression by Western blot analysis and immunohistochemical staining compared with luciferase-siRNA control. Treatment of the MCF7-N55 cells with Cyr61-siRNA had little effect on expression of the closely homologous CCN family member connective tissue growth factor protein or PAR1 (Fig. 5B). Furthermore, treatment of the MCF7-N55 cells with PAR1-siRNA has little effect on expression of Cyr61 protein. Therefore, control of Cyr61 expression becomes independent of PAR1 at some point following the progression of PAR1 oncogenic transformation of MCF7 cells. This may suggest that transcriptional changes late after PAR1 oncogenic induction may result in a new epigenetic control of Cyr61 gene transcription.

Conversely, treatment of the MCF7-N55 cells with Cyr61-siRNA has no effect on PAR1 surface expression. The PAR1-siRNA reduces PAR1 surface expression by 80% at day 2 (these optimal conditions used here) in MDA-MB-231 cells with no effect on the expression of the closely related PAR4 gene (ref. 5; Fig. 1E). Furthermore, we showed previously that the PAR1-siRNA treatment did not affect migration of the MDA-MB-231 cells toward IL-8.

To show that tumor cell-derived Cyr61 mediates stromal MMP-1 induction, we cocultured Cyr61-siRNA- or luciferase-siRNA-treated breast cancer cells with WI38 fibroblasts. As shown in Fig. 5C, treatment of MCF7-N55 or MDA-MB-231 cells with Cyr61-siRNA

**Figure 3.** Breast cancer cells induce expression of MMP-1 in fibroblasts. A, expression of Cyr61 protein in extracellular matrix of breast tumor cells and fibroblasts. The expression levels of extracellular matrix (ECM)–bound Cyr61 protein were detected by immunoblotting analysis using polyclonal Cyr61 antibody. Cells grown on coverslips were stained with anti-human Cyr61 and MMP-9 antibodies, fluorescently labeled, and photographed. Magnification, ×400. Antibody against MMP-9 was used to stain for localization of tumor cells. B, schematic representation of coculturing assay for tumor-mediated induction of fibroblast-derived MMP-1. C, presence of MDA-MB-231 or MCF7-N55 tumor cells causes induction of MMP-1 mRNA expression in WI38 cells. RT-PCR of MMP-1 or actin product was generated using cDNA template isolated from WI38 cocultured with cancer cells. The amplification of actin was carried out to verify that a comparable amount of cDNA was analyzed. Quantitative real-time PCR analysis of MMP-1 expression in WI38 cells cocultured in the presence of MDA-MB-231 cells for 5 hours. MMP-1 mRNA expression levels were normalized to the reference gene β-actin to correct for variation in the amount of RNA amplified. Actin-normalized MMP-1 mRNA levels are plotted on Y axis as a function of RT-PCR cycle number. D, Western blot analysis of conditioned medium from WI38 cells alone or WI38/MDA-MB-231 coculture using MMP-1 antibody (AB806, Chemicon International, Inc., Temecula, CA).
caused a 75% to 80% loss of MMP-1 expression in WI38 cells compared with control cells treated with luciferase-siRNA. These data provide the first direct evidence for the involvement of cancer cell Cyr61 in paracrine regulation of fibroblast MMP-1.

Because MMPs are regulated at the protein level, next we measured MMP-1 levels using Western blot analysis or ELISA and MMP-1 activity using a collagenase assay. As shown in Fig. 3D, we found significant paracrine elevation of MMP-1 protein on

Figure 4. PAR1-expressing breast cancer cells promote MMP-1 production in primary mammary fibroblasts. Primary mouse (A) or human (B) mammary fibroblasts were cocultured for 20 hours with breast cancer cells MDA-MB-231 or MCF7-N55. MMP-1 mRNA expression levels in the three different fibroblast cells were normalized to the reference gene β-actin to correct for variation in the amount of RNA amplified between different cell types. Actin-normalized MMP-1 mRNA levels are plotted on Y axis as a function of RT-PCR cycle number.

Figure 5. Silencing of Cyr61 in breast cancer cells down-regulates MMP-1 expression levels in fibroblasts. A, effect of Cyr61-siRNA on Cyr61 protein expression in MDA-MB-231 or MCF7-N55 cells. Protein was purified using heparin beads, and Western blot analysis was done using the Cyr61 antibody. Immunohistochemical staining using the Cyr61 antibody of luciferase (luci)-siRNA- or Cyr61-siRNA-treated MCF7-N55 cells. B, effect of Cyr61- and PAR1-siRNA on protein expression of connective tissue growth factor or Cyr61. PAR1 expression profiles of MCF7-N55 treated with Cyr61- or luciferase-siRNA, labeled with SFLLRN antibody (1 μg/mL), and analyzed by flow cytometry. C, quantitative real-time PCR analysis of the MMP-1 expression in WI38 cells cocultured for 24 hours in the presence of MCF7-N55 or MDA-MB-231 cells treated with luciferase- or Cyr61-siRNA. The relative MMP-1 gene expression was normalized to the mean value of nonstimulated WI38 cells (noninduced control), and this value was set to 1. MMP-1 RT-PCR of each of the siRNA-treated N55 samples was divided by the actin control conducted in the identical sample to generate the relative fold induction for MMP-1. D, collagenase activity of conditioned medium from WI38 and MDA-MB-231 cocultures were treated with either luciferase-, PAR1-, or Cyr61-siRNA.
cocluring of WI38 fibroblasts with MDA-MB-231 cells. As expected, we detected expression of predominantly the proform (≈57/52 kDa) of MMP-1. As shown in Fig. 5D, treatment of MDA-MB-231 cells with Cyr61-siRNA results in ∼40% reduction of pro-MMP-1 compared with PAR1- or luciferase-siRNA (from 3.8 ± 0.2 and 4 ± 0.2 to 2.6 ± 0.1 ng/mL) by ELISA. Conversely, coculture with luciferase- or PAR1-treated MDA-MB-231 cells resulted in ∼36% increase of collagenase activity. After treatment of MDA-MB-231 cells with Cyr61-siRNA, collagenase activity dropped to basal levels (WI38 cells alone). Together, these data indicate that tumor-derived Cyr61 is directly involved in regulation of stromal MMP-1.

Effect of Cyr61-MMP-1 on migration of cancer cells toward stromal fibroblasts. We tested the ability of WI38 fibroblasts to induce PAR1-dependent chemotaxis of MDA-MB-231 cells. If the paracrine hypothesis is correct, one would predict that gene silencing of Cyr61 in coculturing experiments would give similar effects as silencing PAR1 alone. MDA-MB-231 cells were pretreated with siRNA directed against luciferase, Cyr61, or PAR1 and placed on the top well of the Boyden chamber with WI38 fibroblast cells on the bottom well. The ability of the MDA-MB-231 cells to migrate toward the WI38 cells was then assessed. As shown in Fig. 6A, treatment of MDA-MB-231 cells with PAR1-siRNA caused an 80% loss in migration toward WI38 cells. This is consistent with our notion that PAR1 is a major mediator of migration of breast cancer cells as they migrate toward the surrounding stromal fibroblasts (5). Likewise, treatment of MDA-MB-231 cells with Cyr61-siRNA led to a similar 90% loss of migration toward the WI38 fibroblasts compared with luciferase-siRNA control (Fig. 6A).

To probe the role of MMP-1 in this tumor-stromal paracrine system, we complemented Cyr61-siRNA-, PAR1-siRNA-, or luciferase-siRNA-treated cells with activated MMP-1. Pro-MMP-1 was first activated with aminophenylmercuric acetate and then dialyzed to remove the cytotoxic mercury compound. As shown in Fig. 6A and B, Cyr61-siRNA-treated cells, but not PAR1-siRNA-treated MDA-MB-231 or MCF7-N55 cells, could be complemented with 1 nmol/L MMP-1. Furthermore, the regain of migration was completely abrogated by pharmacologic blockade of PAR1 with 1 μmol/L RWJ-56110 (32). As expected, there was no MMP-1-dependent enhancement in migration of PAR1-siRNA-treated MDA-MB-231 or MCF7-N55 cells. Similar results were obtained using either WI38 or human primary mammary stromal fibroblasts (Fig. 6B). These data show that the effect of MMP-1 on MDA-MB-231 or MCF7-N55 cell migration toward fibroblasts is PAR1 dependent. Likewise, we showed that coculturing of Cyr61-positive tumor cells with fibroblasts or conditioned medium rescues the defect in migration of Cyr61-siRNA-treated tumor cells (Supplementary Fig. S2). However, tumor PAR1 is essential for this migration process and is not rescued by the Cyr61-positive cells. Together, these results strongly support a paracrine role for tumor-derived Cyr61 in induction of stromal cell MMP-1 that leads to PAR1-dependent cell migration.

Lastly, to confirm that stromal-derived MMP-1 was indeed mediating breast cancer cell migration, we knocked down MMP-1 expression in fibroblasts and cocultured the fibroblasts with breast cancer cells. MCF7-N55 cells were pretreated with siRNA directed against Cyr61, PAR1, or luciferase and placed on the top well of the Boyden chamber with MMP-1-siRNA-treated fibroblasts on the bottom well. As shown in Fig. 6C, treatment of fibroblasts with MMP-1-siRNA attenuated 50% chemotaxis of MCF7-PAR1/N55 cells compared with the luciferase-siRNA-treated fibroblasts. The level of inhibition achieved by MMP-1-siRNA treatment of the fibroblasts was similar to that of Cyr61- or PAR1-siRNA treatment of the MCF7-N55 cells. These results confirm that fibroblast-derived MMP-1 mediates migration of these MCF7- or PAR1-expressing cells.

Figure 6. Exogenous MMP-1 complements the Cyr61 defect in PAR1-mediated breast cancer cell migration. A and B, MDA-MB-231 and MCF7-N55 cells were treated for 48 hours with Cyr61-, PAR1-, or luciferase-siRNA and allowed to migrate toward fibroblasts grown in bottom chamber (100,000 per bottom well) of a Transwell plate in 600 μL RPMI 1640 with 0.1% BSA. Aminophenylmercuric acetate–activated MMP-1 (1 nmol/L) and/or RWJ-56110 (1 μmol/L) was added. C, MCF7-N55 (top well) and NIH-3T3 cells (bottom well) were treated for 48 hours with siRNA (Cyr61, PAR1, luciferase-1, or MMP-1). siRNA-treated MCF7-N55 cells (top chamber) were allowed to migrate toward luciferase-siRNA- or MMP-1-siRNA-treated NIH-3T3 cells (bottom chamber) in 600 μL RPMI 1640 with 0.1% BSA. The total number of cells that migrated >40-hour period was counted by microscopy. Columns, mean; bars, SD.
Conclusions

Cyr61 has been proposed previously to regulate cutaneous wound healing by induction of genes, such as MMPs (20). Matrix remodeling by the MMPs, including interstitial collagenase, MMP-1, is essential for the wound healing process. More recent studies have shown that MMP-1 is marker of poor prognosis in breast, colorectal, and esophageal cancers (1, 2, 38). The PAR1 receptor was identified as an effector molecule for MMP-1 and was found to promote breast cancer cell migration and invasion (5). Here, we present the first evidence that breast cancer cells use Cyr61 to instruct stromal fibroblasts to produce MMP-1, which in turn stimulates PAR1-dependent cancer cell migration. Interestingly, gain-of-constitutive Cyr61 expression was achieved by stably expressing PAR1 in MCF7 cells consistent with findings that PAR1 regulates expression of Cyr61 in fibroblasts (39). These Cyr61-expressing MCF7 cells form tumors that are highly invasive in nude mouse models (5). We show that gene silencing of Cyr61 results in nearly complete inhibition of migration and invasion of breast cancer cells toward stromal fibroblasts. Moreover, it has been shown that forced overexpression of Cyr61 results in a more invasive, tumorigenic MCF7 cell line in the absence of estrogen, and many aggressive breast cancers express high levels of Cyr61 (23, 26, 27). Thus, gain-of-constitutive Cyr61 expression may be an important event in the progression of breast cancer toward a more invasive phenotype.

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