Tumorigenesis and Neoplastic Progression

Protease-Activated Receptor-2 Activation in Gastric Cancer Cells Promotes Epidermal Growth Factor Receptor *Trans*-Activation and Proliferation

Roberta Caruso,* Francesco Pallone,*
Daniele Fina,* Valentina Gioia,* Ilaria Peluso,*
Flavio Caprioli,* Carmine Stolfi,*
Alessandra Perfetti,[†] Luigi Giusto Spagnoli,[†]
Giampiero Palmieri,[†] Thomas T. MacDonald,[‡] and
Giovanni Monteleone*

From the Department of Internal Medicine and the Centre of Excellence for Genomic Risk Assessment in Multifactorial and Complex Diseases* and the Pathological Anatomy Unit,[†] University "Tor Vergata" of Rome, Rome, Italy; and the Institute of Cell and Molecular Science,[‡] Barts and the London School of Medicine and Dentistry, London, United Kingdom

Dysregulated epidermal growth factor receptor (EGFR) signaling is involved in gastric cancer (GC) cell growth. However, the mechanism that sustains EGFR signaling in GC remains unknown. Since protease-activated receptor-2 (PAR-2), a G protein-coupled receptor, has been shown to *trans*-activate EGFR in several cell types, we examined the role of PAR-2 in GC. We show here that *in vitro* activation of PAR-2 enhances the growth of two GC cell lines, AGS and MKN28. In both these cell lines, PAR-2 *trans*-activated EGFR and inhibition of EGFR tyrosine kinase activity by AG1478 or specific EGFR siRNA completely prevented PAR-2-driven proliferation. Antibody blockade of EGF-like ligands to EGFR did not modify EGFR signaling or cell growth induced by PAR-2 activation. In contrast, PAR-2 promoted Src activation and interaction of this kinase with EGFR. In support of this, inhibition of Src kinase activity by PP1 or siRNA blocked PAR-2-induced EGFR signaling cascade and cell growth. Finally, PAR-2 was detectable in both normal and GC specimens, but its expression was more pronounced in GC than controls and correlated with activated EGFR. These data show that PAR-2 is overexpressed in GC and suggest a role of PAR-2 in EGFR *trans*-activation and cell growth. (*Am J Pathol* 2006, 169:268–278; DOI: 10.2353/ajpath.2006.050841)

Gastric cancer (GC) is one of the most common malignant tumors in the world, and it is the second largest cause of cancer-related death, responsible for ~10% of all deaths from cancer worldwide.¹ It is now assumed that, in genetically predisposed individuals, microenvironment factors, such as dietary carcinogens and *Helicobacter pylori* (Hp)-related infection, produce genotypic and phenotypic changes that ultimately progress to malignant transformation.¹ In this context, overexpression of epidermal growth factor receptor (EGFR) has been documented in GC,² and activation of EGFR is thought to trigger a coordinate sequence of molecular events that sustain GC growth and metastasis. Consistent with this, patients with EGFR-positive GC have a worse prognosis than those with EGFR-negative GC, and blocking the EGFR signaling pathway has been successfully used to inhibit the growth of human GC xenografts.^{2–4} The exact molecular mechanisms that cause activation of EGFR within the GC microenvironment are not fully understood.

EGFR can be directly activated by members of the EGF family, including EGF, transforming growth factor (TGF)- α , and amphiregulin, all produced in excess in GC tissue.³ Studies in other systems have also revealed that, during neoplastic transformation and/or progression, EGFR can be transactivated by various extracellular stimuli, unrelated to EGFR ligands, such as cytokines, and agonists of the G protein-coupled receptor, such as proteases-activated receptors (PARs).^{5–7} PARs are seven transmembrane-spanning domain G protein-coupled receptors, comprising four receptors: PAR-1, PAR-2, PAR-3, and PAR-4. Activation of PARs is an irreversible phenomenon in which the protease binds to and cleaves the amino-terminal exodomain of the receptor. The cleavage generates a new amino-terminal sequence that binds to the core receptor and serves as a tethered ligand.⁸ Whereas PAR-1, -3, and -4 are activated by

Supported by the Fondazione Umberto Di Mario, Rome, Italy.

Accepted for publication April 6, 2006.

Address reprint requests to Giovanni Monteleone, Cattedra di Gastroenterologia, Dipartimento di Medicina Interna, Università Tor Vergata, Via Montpellier, 1, 00133 Rome, Italy. E-mail: Gi.Monteleone@Med.uniroma2.it.

thrombin, PAR-2 is activated by multiple trypsin-like enzymes, such as trypsin itself and mast cell tryptase.^{9,10} Evidence has been accumulated to show that trypsin is produced in excess in many cancers of the digestive tract, including GC, and it is supposed to contribute to the growth and diffusion of cancer cells.¹¹ In line with this, overexpression of exogenous trypsinogen cDNA in human gastric cancer cells has been reported to increase their tumorigenicity in nude mice.¹² Whether the ability of trypsin to enhance GC tumorigenesis relies on PAR-2 activation remains unknown, however. These observations together with the demonstration that PAR-2 has been involved in the growth of epithelial cancer¹³ prompted us to explore the role of PAR-2 in human GC. To this end, we first used AGS and MKN28 gastric cancer cell lines as a model of GC to examine whether PAR-2 activation results in enhanced EGFR signaling and cell growth. Second, we dissected the molecular mechanism by which PAR-2 regulates EGFR activation. Finally, the expression of PAR-2 in human gastric cancer specimens was evaluated.

Materials and Methods

Human Samples

GC specimens were taken from 15 patients undergoing subtotal gastrectomy. No patient had received preoperative chemotherapy. Seven GCs were of intestinal type, whereas the remaining were signet-ring cell carcinomas (diffuse), according to the Lauren classification. Additionally gastric biopsies were taken from eight patients with Hp-related gastritis and 12 Hp-negative patients (controls). All specimens were taken from the antrum.

Cell Culture and Proliferation

The gastric cancer cell lines AGS and MKN28 (kindly provided by Prof. Marco Romano, Dipartimento di Internistica Clinica e Sperimentale-Gastroenterologia, II University of Naples, Italy) were cultured in 25-cm² plastic flasks and maintained at 37°C in a humidified atmosphere of 5% CO₂ in Dulbecco's modified Eagle's and RPMI 1640 media (both from Sigma-Aldrich, Milan, Italy), respectively, supplemented with 10% inactivated fetal bovine serum (FBS, Sigma-Aldrich). To assess cell proliferation, AGS and MKN28 cells were starved in serum-free medium for 24 hours, then 3000 to 5000 cells/well were seeded in 96-well culture dishes in medium supplemented with 0.1% of bovine serum albumin (Sigma-Aldrich), allowed to adhere for 4 hours, and then stimulated with the PAR-2-activating peptide (SLIGKV-NH₂) or -inactivating peptide (VKGILS-NH₂, both used at a final concentration of 20 μmol/L; Sigma-Aldrich) for 48 hours. In parallel, cells were preincubated with the EGFR tyrosine kinase inhibitor, AG1478 (20 μmol/L) or the Src tyrosine kinases inhibitor, PP1 (20 μmol/L; both from Inalco, Milan, Italy) or dimethylsulfoxide (DMSO, vehicle) for 60 minutes before adding the PAR-2-activating peptide. The optimal concentration of both AG1478 and PP1

was selected on the basis of data obtained in preliminary experiments. To confirm the role of EGFR on PAR-2-mediated cell growth, AGS cells were transfected with EGFR or control small interference RNA (siRNA) according to the manufacturer's instructions (Santa Cruz Biotechnology, Santa Cruz, CA). Cells were then cultured in complete medium for 48 hours. At the end, an aliquot of cells was used to examine EGFR, whereas the remaining was used to examine whether silencing of EGFR reduced the PAR-2-mediated cell growth. For this purpose, both control and EGFR siRNA-treated AGS cells were cultured in the presence or absence of PAR-2 peptide (PAR-2 P) or 10% FBS (used as a positive control of proliferation) as indicated above. To examine whether the mitogenic properties of PAR-2 were related to the ability of PAR-2 to enhance the activity/secretion of EGFR ligands, cells were preincubated with a neutralizing EGFR antibody that prevents binding of EGF-like ligands to EGFR (Upstate Biotechnology, Lake Placid, NY) or control IgG for 1 hour before adding the PAR-2-activating peptide. Bromodeoxyuridine (BrdU) was added to the cell during the last 4 hours of incubation, and the level of BrdU-positive cells was assessed by a colorimetric kit (Roche Diagnostics, Monza, Italy).

To assess the effect of PAR-2 activation on EGFR, extracellular signal-regulated kinase (ERK), and Src activation, AGS and MKN28 cells were starved in serum-free medium for 24 hours, then stimulated with PAR-2-activating or -inactivating peptide (20 μmol/L) for 2 to 120 minutes. In parallel, cells were preincubated with AG1478 (20 μmol/L) or PP1 (20 μmol/L) or DMSO (vehicle) for 60 minutes before adding the PAR-2-activating peptide or EGF (200 ng/ml; Peprotech, London, UK). To evaluate further the role of Src on PAR-2-mediated EGFR activation, AGS cells were transfected with Src or control siRNA according to the manufacturer's instructions (Diagnostic Broker Associated, Milan, Italy). Cells were then cultured in complete medium for 48 hours. At the end, an aliquot of cells was used to examine Src, whereas the remaining was used to examine whether silencing of Src prevented the effect of PAR-2 on EGFR activation. To this end, both control and Src siRNA-treated AGS cells were cultured and stimulated with the PAR-2 peptide as indicated above.

To examine whether the effect of PAR-2 on EGFR activation was dependent on the activity of EGFR ligands, cells were preincubated with graded doses (0–20 μg/ml) of the neutralizing EGFR antibody or control IgG for 1 hour and then stimulated with PAR-2 peptide. In parallel, cell cultures were added of EGF (200 ng/ml) or TGF-α (100 ng/ml, Peprotech) to confirm that the neutralizing EGFR antibody was effective in preventing the action of EGFR ligands. To examine whether the effect of PAR-2 on EGFR *trans*-activation was secondary to the activity of matrix metalloproteinases (MMPs), which could promote the cleavage and secretion of EGF-like ligands into the culture medium,⁶ cells were preincubated with two general inhibitors of MMPs, 1,10-phenanthroline (300 μmol/L; Sigma-Aldrich) and GM6001 (5 μmol/L; Inalco) for 1 hour before adding the PAR-2 peptide.

Activation of EGFR was also evaluated in AGS cells either left unstimulated or stimulated with trypsin (10 or 100 $\mu\text{mol/L}$; Sigma-Aldrich) for 5 to 15 minutes.

Finally, we examined the effect of PAR-2 signaling on EGFR activation in primary gastric epithelial cells. To this end, gastric mucosal specimens were taken from three patients undergoing gastric resection for GC. Mucosal strips were taken from macroscopically and microscopically unaffected areas and washed once in phosphate-buffered saline (PBS). Epithelial cells were then isolated by two consecutive washes in PBS containing 1 mmol/L ethylenediamine tetraacetic acid (Sigma-Aldrich) for 15 minutes each. Purity and viability of the isolated epithelial cells were >93% and 90%, respectively. Epithelial cells were then cultured in RPMI 1640 in the absence of FBS with or without the initial addition of PAR-2-activating peptide for 5 to 30 minutes. At the end, cells were harvested and used for analyzing EGFR activation by Western blotting.

Immunohistochemistry

Serial tissue sections were cut, deparaffinized, and dehydrated through xylene and ethanol. For antigen retrieval, slides were incubated in the microwave oven for 10 minutes in 0.01 mol/L citrate buffer, pH 6 (Sigma-Aldrich). To block endogenous peroxidase, slides were then incubated in 2% H_2O_2 for 20 minutes at room temperature. Incubation with human monoclonal PAR-2 (1:25 final dilution; Santa Cruz Biotechnology) or human monoclonal activated EGFR antibody (1:10 final dilution; US Biological, Swampscott, MA) was performed at room temperature for 1 hour. After rinsing in Tris-buffered saline (Sigma-Aldrich), slides were incubated with a rabbit anti-mouse IgG antibody conjugated to horseradish peroxidase (1:100 final dilution; DAKO, Milan, Italy) for 30 minutes at room temperature. Immunoreactive cells were visualized by addition of diaminobenzidine (Sigma-Aldrich) as substrate and lightly counterstained with hematoxylin. Isotype control sections were prepared under identical immunohistochemical conditions, as described above, replacing the primary PAR-2 or activated EGFR antibodies with a purified, normal IgG control antibody (R&D Systems, Minneapolis, MN).

Flow Cytometry

To examine whether PAR-2 is expressed by gastric cancer cell lines, AGS and MKN cells were incubated with the above indicated PAR-2 (1:100 final dilution in PBS) or isotype control antibody (1:100 dilution; BD Biosciences, Milan, Italy) at 4°C for 45 minutes. Cells were then washed, resuspended in PBS, and incubated with phycoerythrin-goat anti-mouse antibody (1:100 dilution; BD Biosciences) at 4°C for 30 minutes. Finally cells were washed, resuspended in PBS, and analyzed by flow cytometry.

Immunoprecipitation and Western Blotting

For the detection of phosphorylated EGFR (p-EGFR), blots were incubated with a mouse anti-human monoclo-

nal EGFR antibody (0.2 $\mu\text{g/ml}$; Inalco) that specifically recognizes phosphorylation of EGFR on tyrosine residue 1173. Phosphorylation of this residue reflects EGFR activation. After analysis of p-EGFR, blots were stripped and incubated with an antibody recognizing total EGFR (1:500 final dilution; Santa Cruz Biotechnology), followed by a goat anti-rabbit antibody conjugated to horseradish peroxidase (1:20,000 final dilution). To further confirm the effect of PAR-2 activation on p-EGFR, total extracts were immunoprecipitated using an anti-human monoclonal EGFR antibody (sc 120, 2 $\mu\text{g/sample}$; Santa Cruz Biotechnology) or control isotype antibody for 2 hours, followed by incubation with protein A/G-agarose beads overnight. The resulting immunoprecipitates were washed thoroughly with cold lysis buffer, separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and then immunoblotted with a human phosphotyrosine antibody (1:1000 final dilution; Santa Cruz Biotechnology). After analysis of p-EGFR, blots were stripped and incubated with a second monoclonal anti-human EGFR antibody (Inalco). For the analysis of ERK, total proteins (50 $\mu\text{g/sample}$) were separated on a 10% gel of sodium dodecyl sulfate-polyacrylamide gel electrophoresis and incubated with a mouse anti-human p-ERK1/2 antibody (1:500 final dilution; Santa Cruz Biotechnology) followed by a rabbit anti-mouse antibody conjugated to horseradish peroxidase (1:20,000 dilution). After detection of p-ERK 1/2, blots were stripped and subsequently incubated with a rabbit anti-human total ERK1/2 antibody (1:500 final dilution; Santa Cruz Biotechnology), followed by a goat anti-rabbit antibody conjugated to horseradish peroxidase (1:20,000 dilution; Dako). Src activation was evaluated by using an antibody that specifically recognizes phosphorylated Src (p-Src) on tyrosine residue 418. To this end, total proteins were immunoblotted and incubated with a specific rabbit anti-human p-Src antibody (0.1 $\mu\text{g/ml}$; Sigma-Aldrich) followed by a goat anti-rabbit antibody conjugated to horseradish peroxidase (1:50,000 dilution).

To examine whether PAR-2 activation promotes the interaction of Src with EGFR, total extracts were immunoprecipitated using a monoclonal anti-human Src (2 $\mu\text{g/sample}$; Santa Cruz Biotechnology) or control isotype antibody as indicated above and then the membrane was incubated with the antibody recognizing p-EGFR. After analysis of Src-bound p-EGFR, blots were stripped and incubated with a second monoclonal anti-human Src antibody (Santa Cruz Biotechnology) to confirm the equivalent loading of the lanes. To confirm further the interaction between Src and EGFR in response to PAR-2 activation, total proteins were immunoprecipitated using a monoclonal anti-human EGFR (2 $\mu\text{g/sample}$; Santa Cruz Biotechnology) or control isotype antibody as indicated above, and then the membrane was incubated with an antibody recognizing p-Src on tyrosine residue 418. After analysis of p-Src-bound EGFR, blots were stripped and incubated with a second monoclonal anti-EGFR antibody (Santa Cruz Biotechnology) to confirm the equivalent loading of the lanes. Computer-assisted scanning densitometry (Total lab; AB.EL Sience-Ware Srl, Rome,

Italy) was used to analyze the intensity of the immunoreactive bands.

Statistical Analysis

Data were tested for normality of distribution by means of the Kolmogorov-Smirnov test and then indicated as mean values $\pm$ SD. Student's *t*-test was used to compare normal variables. One-way analysis of variance with Bonferroni correction was used for multiple comparisons.

Results

PAR-2 Enhances Gastric Cancer Cell Proliferation

AGS and MKN28 cells were used as *in vitro* model of GC to examine the effect of PAR-2 activation on GC cell growth. To this end, we performed a flow cytometry analysis to examine whether both these cell lines constitutively express PAR-2. Data in Figure 1A show that both AGS and MKN28 cells express PAR-2. To assess the effect of PAR-2 activation on GC cell growth, serum-starved AGS and MKN28 cells were either left untreated or treated with PAR-2-activating or -inactivating peptide, and the rate of proliferating cells was evaluated after 48 hours of culture. As shown in Figure 1B the addition of PAR-2-activating but not -inactivating peptide to the cell cultures significantly increased the percentage of BrdU-positive cells, thus confirming and expanding on previous data showing the ability of PAR-2 signaling to enhance the growth of other cancer cells.¹³

PAR-2 Stimulates GC Cell Proliferation via the EGFR Pathway

It is known that PAR-2 transactivates EGFR in other cell systems,¹³ and activated EGFR is thought to trigger mitogenic signals in GC cells²; so we then explored the possibility that the effect of PAR-2 on the GC cell proliferation was mediated by EGFR. To this end, we first examined whether stimulation of serum-starved GC cells with PAR-2 peptide resulted in EGFR *trans*-activation. As shown in Figure 2A, stimulation of AGS cells with PAR-2-activating but not -inactivating peptide enhanced phosphorylation of EGFR on tyrosine residue 1173. Time-course studies revealed that the induction of p-EGFR in AGS cells occurred as early as 5 minutes after PAR-2-activating peptide exposure, and this effect persisted over the time course (Figure 2A). No induction in p-EGFR was seen in GC cells treated with PAR-2-activating peptide for a time shorter than 5 minutes, independently on the cell type used (not shown). Importantly, the effect of PAR-2 on EGFR *trans*-activation was confirmed also when the overall level of p-EGFR was analyzed by immunoprecipitation and immunoblotting (Figure 2B). Similar results were obtained when MKN28 cells were used (not shown). The functional relevance of this finding was confirmed by the demonstration that PAR-2-activating but not -inactivating peptide also enhanced the phosphorylation of

Figure 1. A: Representative flow cytometry histograms showing the expression of PAR-2 in AGS and MKN28 cells. Cell phenotype analysis was carried out after incubation of these two cell lines with a monoclonal PAR-2 or isotype control antibody at 4°C for 45 minutes. Numbers above the lines indicate the percentage of positive cells within the designed gates. **B:** Stimulation of both AGS and MKN28 with PAR-2-activating peptide results in enhanced cell growth. Cells were cultured in the presence or absence of PAR-2-activating (PAR-2 AP, 20 μ mol/L) or -inactivating (PAR-2 IP, 20 μ mol/L) peptide for 48 hours, and BrdU was added during the last 4 hours of culture. PAR-2 AP significantly enhances the growth of both AGS ($P = 0.002$) and MKN28 ($P = 0.04$) cells. Data are expressed in arbitrary units and indicate mean $\pm$ SD of four separate experiments.

ERK1/2, two downstream targets of EGFR tyrosine kinase (Figure 2C). This effect was also seen in MKN28 cells (not shown). Additionally, p-EGFR was activated in AGS cells by trypsin (Figure 2D), a physiological inducer of PAR-2 activation.⁹ Finally, the ability of PAR-2 to activate EGFR was confirmed using primary gastric epithelial cell cultures, in which the addition of PAR-2-activating peptide resulted in a marked induction of p-EGFR (Figure 2E).

Figure 2. PAR-2 P stimulates EGFR *trans*-activation in AGS cells. **A:** Representative expression of p-EGFR (upper blot) and total EGFR (lower blot) protein in AGS cells cultured in the presence or absence of PAR-2-activating or -inactivating peptide (20 μ mol/L) for the indicated time points. p-EGFR was examined by Western blotting using an antibody that recognizes phosphorylation of the receptor on tyrosine residue 1173. One of four representative blots is shown. **B:** Total extracts of AGS cells cultured with or without the initial addition of PAR-2 AP for 1 hour were immunoprecipitated using a monoclonal anti-human EGFR antibody and then incubated with a p-Tyr antibody. No band was seen when proteins were immunoprecipitated using a control isotype antibody (ve-). After detection of p-EGFR, blots were stripped and incubated with a second anti-EGFR to ascertain equivalent loading of the lanes (lower blot). **C:** Representative Western blots showing p-ERK1/2 (upper blot) and total ERK1/2 (lower blot) in AGS cells stimulated with PAR-2 activating or inactivating peptide (20 μ mol/L) for the indicated time points. One of four representative experiments is shown. **D:** Representative Western blot showing p-EGFR (upper blot) and total EGFR (lower blot) in AGS cells cultured in the presence or absence of trypsin (TRYP (10 and 100 μ mol/L) for the indicated time points. **E:** Effect of PAR-2P on p-EGFR in primary gastric epithelial cells. Cells were isolated from normal mucosal specimens and cultured in the presence or absence of PAR-2 AP for the indicated time points. At the end, total proteins were prepared and analyzed by Western blotting. One of three representative experiments is shown.

We then assessed the effect of inhibition of EGFR tyrosine kinase activity on EGFR signaling and cell growth induced by PAR-2 activation. To this end, GC cells were preincubated with the EGFR kinase inhibitor tyrphostin AG1478, then stimulated with PAR-2-activating peptide. Finally both EGFR and ERK1/2 phosphorylation and cell growth were analyzed. To show that AG1478 specifically inhibits p-EGFR, GC cells were also stimulated with EGF. Data in Figure 3A clearly indicate that pretreatment of AGS cells with AG1478 completely prevented the EGF-induced p-EGFR. Importantly, AG1478 also abrogated the effects of PAR-2-activating peptide on the phosphorylation of EGFR and ERK1/2 (Figure 3B) and growth of both AGS and MKN28 cells (Figure 3C). To confirm further that PAR-2-mediated GC cell growth relies on EGFR activation, we specifically inhibited EGFR in AGS by siRNA (Figure 3D) and then evaluated the effect of PAR-2 peptide on cell growth. Data in Figure 3E clearly indicate that PAR-2-acti-

vating peptide failed to enhance the growth of AGS cells with targeted suppression of EGFR. Importantly, the same cells, however, were able to proliferate in response to FBS (Figure 3E), suggesting that EGFR inhibition prevents the PAR-2-mediated cell growth but does not block the ability of these cell to proliferate in response to additional stimuli. Together these data support the involvement of EGFR tyrosine kinase activity in the proliferative response induced by PAR-2 in GC cells.

Blockade of the EGFR Ligand Binding Domain Does Not Inhibit the PAR-2-Mediated Effects on EGFR Phosphorylation and Proliferation of GC Cells

Studies in other cell systems have shown that *trans*-activation of EGFR by several stimuli can occur via an

Figure 3. A: The tyrosinase AG1478, an inhibitor of EGFR tyrosine kinase activity, suppresses EGF-induced phosphorylation of EGFR in AGS cells. Representative Western blots showing p-EGFR (upper blot) and total EGFR (lower blot) protein in AGS cells preincubated with AG1478 (20 μ mol/L) or DMSO (vehicle) for 1 hour, and then either left unstimulated or stimulated with EGF (200 ng/ml) for 10 minutes. **B:** AG1478 inhibits PAR-2 P-stimulated p-EGFR and p-ERK1/2. Representative Western blots showing p- and total EGFR, and p- and total ERK1/2 protein in AGS cells preincubated with AG1478 (20 μ mol/L) or medium for 1 hour and then either left unstimulated or stimulated with PAR-2 P (20 μ mol/L) for an additional hour. **C:** AG1478 suppresses the PAR-2-driven GC cell growth. AGS and MKN28 cells were preincubated with AG1478 (20 μ mol/L) or DMSO for 1 hour and then stimulated with PAR-2 AP (20 μ mol/L) for a further 48 hours, and cell proliferation was assessed as indicated in Material and Methods. Data are expressed as mean $\pm$ SD of four separate experiments and indicate the maximal response of growth of cells cultured in the presence of PAR-2 P with or without AG1478 or DMSO. **D:** Representative Western blot showing EGFR (upper blot) and β -actin (lower blot) in AGS cells transfected with either EGFR or control siRNA for 48 hours. **E:** Suppression of EGFR by siRNA prevents the PAR-2P-mediated growth of GC cells. Cells were transfected with either EGFR or control siRNA for 48 hours, then washed, and cultured in 96-well plates in the presence or absence of PAR-2AP or FBS, as indicated in Material and Methods, for 48 hours. BrdU was added during the last 4 hours of culture. PAR-2 P significantly enhances the growth of AGS transfected with control siRNA ($P = 0.03$) but not the proliferation of EGFR siRNA-treated cells. In contrast, FBS significantly enhances the growth of cells treated with either EGFR or control siRNA ($P < 0.001$). Data are expressed in arbitrary units and indicate mean $\pm$ SD of three experiments.

autocrine/paracrine mechanism involving the release of soluble EGF-like ligands.^{5,6} For example, this mechanism has been described in colonic cancer cells after *in vitro* activation of PAR-2.¹³ To determine whether PAR-2 transactivates EGFR in GC cells through a similar mechanism, we designed experiments to block the binding of EGFR ligands to the extracellular domain of the receptor using a specific monoclonal antibody directed against the extracellular portion of EGFR. To test the efficiency of the

antibody we first showed that it dose-dependently inhibited the phosphorylation of EGFR induced in AGS cells by both EGF and TGF- α , two EGFR ligands (Figure 4, A and B). However, preincubation of AGS cells with the same antibody, at a dose that completely prevented the induction of p-EGFR by both EGF and TGF- α , did not inhibit PAR-2-mediated p-EGFR expression (Figure 4C). The neutralizing EGFR antibody did not interfere with the ability of PAR-2 peptide to promote GC cell growth (Figure

Figure 4. PAR-2 P-induced EGFR *trans*-activation is not mediated by EGF-like ligands. **A** and **B**: Representative expression of p-EGFR (upper blot) and total EGFR (lower blot) protein in AGS cells preincubated with graded doses of a neutralizing EGFR (aEGFR) or control (IgG) antibody for 1 hour and then stimulated or not with EGF (200 ng/ml [**A**]) or TGF- α (100 ng/ml [**B**]) for a further 10 minutes. **C**: The aEGFR is not effective in neutralizing the effect of PAR-2 P on p-EGFR. Representative Western blots showing p-EGFR and total EGFR in AGS cells preincubated with aEGFR for 1 hour and then stimulated with PAR-2 AP for further 60 minutes. **D**: Pretreatment of GC cells with aEGFR does not affect the PAR-2-driven cell growth. AGS cells were preincubated with aEGFR or control IgG for 1 hour and then stimulated with PAR-2 AP (20 μ mol/L) for further 48 hours, and cell proliferation was assessed as indicated in Material and Methods. Data are expressed as mean $\pm$ SD of four separate experiments and indicate the maximal response of growth of cells cultured in the presence of PAR-2 P with or without aEGFR or IgG. **E**: Neither 1,10-phenanthroline nor GM6001 inhibit the PAR-2 P-induced p-EGFR. Representative Western blots showing p-EGFR and total EGFR in AGS cells preincubated with 1,10-phenanthroline (300 μ mol/L) or GM6001 (5 μ mol/L) for 1 hour and then stimulated with PAR-2 AP for a further 60 minutes. One of two separate experiments is shown.

4D). As studies in other cell systems have shown that *trans*-activation of EGFR by PAR-2 relies on the activity of MMPs, which cleave and favor the release of EGFR ligands,¹³ we then tested the effect of two general inhibitors of MMPs, 1,10-phenanthroline and GM6001, in our model. As shown in Figure 4E, neither 1,10-phenanthroline nor GM6001 prevented the PAR-2-stimulated p-EGFR.

Src Activity Is Crucial in the PAR-2-Induced EGFR Trans-Activation

Nonreceptor tyrosine kinases, such as Src, can be activated by G protein-coupled receptors.¹⁴ In addition, there is evidence that, in response to specific stimuli, Src can promote EGFR activation.¹⁵ Therefore, we examined the involvement of Src in PAR-2-mediated response in GC cells. To this end we first determined whether PAR-2

signaling caused the activation of Src. As shown in Figure 5A, stimulation of AGS cells with PAR-2-activating but not -inactivating peptide caused a rapid and sustained activation of Src, as evidenced by phosphorylation of the protein on tyrosine residue 418. Importantly, activation of Src in AGS cells occurred as early as 2 minutes after PAR-2 peptide stimulation (Figure 5A) and preceded the phosphorylation of EGFR, which was evident only after 5 minutes of stimulation (Figure 2A). As the direct activation of EGFR by Src would require an interaction between these proteins, we then evaluated whether PAR-2 activation promoted the interaction of Src with EGFR. For this purpose, we immunoprecipitated total proteins extracted from either unstimulated or PAR-2-activating peptide-stimulated GC cells with a monoclonal Src or control IgG (ve-) antibody and then probed the membrane with a p-EGFR antibody. As shown in Figure 5B, a high content of Src-bound p-EGFR was seen in PAR-2 peptide-stimu-

Figure 5. **A:** Representative expression of p-Src (upper blot) and β -actin (lower blot) protein in AGS cells cultured in the presence or absence of PAR-2 activating peptide (PAR-2 AP) or inactivating peptide (PAR-2 IP) (20 μ mol/L) for the indicated time points. p-Src was examined by Western blotting using an antibody that recognizes phosphorylation of Src on tyrosine residue 418. **Lower inset:** quantitative data of p-Src/ β -actin as measured by densitometry scanning of the representative Western blot. Values are expressed in arbitrary units (a.u.). **B:** Total extracts of AGS cells cultured with or without the initial addition of PAR-2 AP for 1 hour were immunoprecipitated using a monoclonal anti-human Src antibody and then incubated with a p-EGFR antibody. No band was seen when proteins were immunoprecipitated using a control isotype antibody (ve-). After detection of Src-bound p-EGFR, blots were stripped and incubated with a second anti-Src to ascertain equivalent loading of the lanes (lower blot). **C:** Total extracts of AGS cells, cultured with or without the initial addition of PAR-2 AP for the indicated time points, were immunoprecipitated using a monoclonal anti-human EGFR antibody and then incubated with a p-Src antibody. No band was seen when proteins were immunoprecipitated using a control isotype antibody (ve-). After detection of p-Src-bound EGFR, blots were stripped and incubated with a second anti-EGFR to ascertain equivalent loading of the lanes (lower blot).

lated cells. This effect was seen both in AGS and MKN28 cells (not shown). In additional experiments, this interaction was confirmed by immunoprecipitating total proteins with an EGFR antibody and then probing the membrane with a p-Src antibody (Figure 5C).

To determine the involvement of Src on the PAR-2-mediated EGFR transactivation, we used the Src tyrosine

kinase inhibitor PP1. To examine whether such an inhibitor was effective in blocking Src activation, we first treated AGS cells with PP1 or DMSO for 1 hour and then monitored p-Src expression by Western blotting. As expected, PP1 but not DMSO completely inhibited p-Src in AGS cells (Figure 6A). Moreover, preincubation of GC cells with PP1 but not DMSO markedly inhibited the PAR-2-induced phosphorylation of both EGFR and ERK1/2 (Figure 6B). This was evident in both GC cell lines (not shown) and was paralleled by a significant inhibition of the cell growth (Figure 6C). The role of Src in promoting PAR-2-mediated p-EGFR was independently confirmed by studies showing that targeted inhibition of Src by siRNA abrogated the ability of PAR-2-activating peptide to enhance p-EGFR in AGS cells (Figure 6D).

PAR-2 Is Overexpressed in Gastric Cancer Cells

These findings suggest that PAR-2 activation may play a decisive role in sustaining GC cell growth. Therefore, we extended our analysis by first examining PAR-2 in human GC tissue. To this end we performed an immunohistochemical analysis of GC and control sections using monoclonal anti-human PAR-2 antibody. All gastric sections contained PAR-2-positive cells, even though staining was more intense and diffuse in GC in comparison to control samples (Figure 7). In GC sections, PAR-2 was particularly evident in cancer cells (Figure 7, A and B), and this occurred independently on the histological type of cancer analyzed (not shown). Among controls, PAR-2 was weakly expressed by both epithelial and lamina propria mononuclear cells (Figure 7C), with no obvious difference between Hp-positive and -negative samples (not shown). The specificity of these findings was confirmed using a non-relevant isotype control antibody (Figure 7D). To examine whether PAR-2 and activated EGFR colocalize in GC cells, serial GC section were stained with monoclonal anti-human PAR-2 and activated EGFR antibodies. As shown in Figure 7 (E–H) most of GC cells were positive for both PAR-2 and activated EGFR.

Discussion

In the present study we have analyzed the role of PAR-2 in human GC. PAR-2 is a G protein-coupled receptor that is constitutively expressed in a variety of tissues, particularly in the gut.^{16–20} Analysis of RNA expression has revealed that PAR-2 is highly expressed in the pancreas, small intestine, colon, and liver, and several functional studies have convincingly shown that activation of PAR-2 plays an important role in gut physiology and pathology.^{21,22} For example, PAR-2 activation triggers mitogenic signals in colonic and pancreatic cancers.^{23–27} Consistent with this, we show here that activation of PAR-2 in GC cells by a specific, synthetic, peptide results in enhanced cell proliferation. This effect seems to rely on the ability of PAR-2 to trigger intracellular signals that ultimately lead to the *trans*-activation of EGFR, a crucial step in the growth and diffusion of several epithelial cancer cells.^{2,28–31} Indeed, we show that both inhibition of the

Figure 6. **A:** Representative Western blots showing p-Src and β -actin in total extracts from AGS cells cultured with or without the Src kinases inhibitor, PP1 (20 μ mol/L) or DMSO (vehicle) for 1 hour. **B:** Pretreatment of AGS cells with PP1 prevents the PAR-2 P-induced phosphorylation of EGFR and ERK1/2. AGS cell cultures were preincubated with, medium, PP1, or DMSO for 1 hour before adding PAR-2 AP for an additional hour. One of three representative Western blots is shown. **C:** Treatment of GC cells with PP1 prevents the PAR-2-driven growth. AGS and MKN28 cells were preincubated with PP1 or DMSO for 1 hour and then stimulated with PAR-2 AP (20 μ mol/L) for a further 48 hours, and cell proliferation was assessed as indicated in Material and Methods. Data are expressed as mean $\pm$ SD of three separate experiments and indicate the maximal response of growth of cells cultured in the presence of PAR-2 P with or without PP1 or DMSO. **D:** Representative Western blots showing Src (upper blot), p-EGFR (middle blot), and β -actin (lower blot) in AGS either transfected with either Src or control siRNA for 48 hours and then cultured in the presence or absence of PAR-2AP for an additional hour. One of three separate experiments is shown.

EGFR tyrosine kinase activity by AG1478 or targeted suppression of EGFR by siRNA is sufficient to prevent the PAR-2-induced GC cell growth.

To elucidate the mechanism whereby PAR-2 induces EGFR *trans*-activation, we initially explored the possibility that PAR-2 could promote EGFR *trans*-activation through the action of EGF-like ligands, whose cleavage and release is mediated by MMPs.¹³ However, preincubation of GC cells with a neutralizing EGFR antibody, which blocks binding of EGF-like ligands to EGFR, failed to prevent both the PAR-2-mediated p-EGFR and cell growth. Importantly, the same antibody dose-dependently inhibited the phosphorylation of EGFR induced by both exogenous EGF and TGF- α , thus confirming that it was active in our experiments. Additionally, the PAR-2-induced p-EGFR was not prevented by pretreatment of GC cells with 1,10-phenanthroline or GM6001, two powerful and general inhibitors of MMPs. This would seem therefore to suggest that PAR-2-induced EGFR *trans*-activation in GC cells is

mediated through a cell type-specific mechanism that is at least in part distinct from that documented in other cell types.¹³ In this context, it is noteworthy, however, that Prenzel and colleagues⁶ as well as Darmoul et al¹³ have implicated MMP activity in EGFR transactivation using batimastat, an inhibitor of MMPs that is not commercially available.

In contrast, some observations made in this study strongly suggest that the PAR-2-mediated EGFR activation can rely on the activity of Src. First we show that in GC cells PAR-2-activating peptide enhances the phosphorylation of Src on tyrosine residue 418, thus confirming and expanding on data of previous studies indicating that this tyrosine kinase may be a target of G protein-coupled-receptor proteins.¹⁴ By immunoprecipitation and immunoblotting, we also show that PAR-2 signaling promotes the interaction between active Src and EGFR. Second, suppression of Src tyrosine kinase activation by pretreatment of AGS cells with PP1 prevents the phos-

Figure 7. An immunohistochemical staining for PAR-2 in paraffin-embedded sections from GC and control tissue sections. A marked accumulation of PAR-2 is seen in GC cells (original magnification, $\times 400$ [A], $\times 40$ [B]). C: PAR-2 in gastric mucosa of a patient with Hp infection (original magnification, $\times 200$). In D, control immunohistochemical reactions using an isotype IgG that corresponds to the sections in B are shown (original magnification, $\times 40$). E-H: PAR-2 and activated EGFR in serial gastric sections taken from a patient with GC. (original magnification, $\times 200$ [E-G]; original magnification $\times 40$ [F-H]). The example is representative of four separate experiments, in which biopsies taken from 15 patients with GC, eight patients with Hp-related gastritis, and 12 Hp-negative subjects were analyzed.

phorylation of EGFR and ERK1/2 induced by PAR-2 peptide. These data were independently confirmed by studies in which targeted suppression of Src by siRNA inhibited the PAR-2-mediated p-EGFR induction. Finally, we provide evidence that PP1 also inhibits the PAR-2-driven GC cell proliferation. It remains unknown whether, in PAR-2 peptide-treated GC cells, the activation of Src and the Src-mediated EGFR phosphorylation require the involvement of additional kinases/adaptors. Studies are now in progress to examine the connection between PAR-2 and Src in GC cells.

Our immunohistochemical data indicate that PAR-2 is strongly up-regulated in human GC tissue and that cancer cells are the major source of PAR-2 within the GC microenvironment. As expected, most PAR-2 was located at the membrane level, even if some cells exhibited cytosolic staining, likely reflecting the internalization of the receptor. Additionally, in GC tissue, PAR-2-positive cells also expressed activated EGFR.

The increased expression of PAR-2 in GC tissue and the efficient and potent action of PAR-2 on EGFR signaling and GC cell growth raise the question of which factors regulate PAR-2 in human GC cells. A possibility is that PAR-2 may be induced by Hp, because Hp-associated gastric atrophy and intestinal metaplasia are considered as precancerous lesions of the stomach.^{32,33} Although this study was not undertaken to examine the role of Hp infection in the induction and activation of PAR-2, PAR-2 was barely detectable in the stomach of Hp-infected patients by immunohistochemistry. In this context, it is also noteworthy that up-regulation of PAR-2 is seen in both histological types of GC (ie, intestinal and diffuse), the latter not being associated with Hp infection.³³ Another possibility is that PAR-2 may be positively regulated by locally released molecules, such as cytokines and growth factors. Finally, it is possible that PAR-2 up-regulation relies on posttranslational modifications that enhance protein stability. Indeed it was recently shown that PAR-2 protein can be ubiquitinated and that this modification is necessary for translocation of the receptor from early endosomes to late endosomes and lysosomes, where it is degraded.³⁴ A defective ubiquitination of PAR-2 could thus result in a diminished turnover of the receptor, thereby enhancing its expression.

PAR-2-mediated signaling is triggered by the activation of the receptor, a process that requires the cleavage of the amino-terminal exodomain of PAR-2, and generation of a new amino-terminal sequence that binds to the core receptor and serves as a tethered ligand.⁸ PAR-2 can be activated by multiple enzymes, including trypsin and mast cell tryptase. As trypsin is produced in excess within the GC tissue and overexpression of trypsin is associated with enhanced gastric tumorigenicity in xenograft models,^{3,11} it is likely that trypsin, and probably other serine protease acting via PAR-2, could represent important signaling proteins in the control of GC growth. It is thus conceivable that the use of potent trypsin/serine protease inhibitors may be helpful in limiting or suppressing gastric carcinogenesis as suggested for other cancers.³⁵ In this context, our data also suggest that inhibitors of PAR-2 synthesis/activation could represent a new and promising way to contain EGFR signaling and therefore the growth of GC cells. However, selective PAR-2 antagonists have not yet been described.

References

1. Hohenberger P, Gretscher S: Gastric cancer. *Lancet* 2003; 362:305-315
2. Tokunaga A, Onda M, Okuda T, Teramoto T, Fujita I, Mizutani T, Kiyama T, Yoshiyuki T, Nishi K, Matsukura N: Clinical significance of epidermal growth factor (EGF), EGF receptor, and c-erbB-2 in human gastric cancer. *Cancer* 1995; 75(suppl):1418-1425
3. Naef M, Yokoyama M, Friess H, Büchler MW, Korc M: Co-expression of heparin-binding EGF-like growth factor and related peptides in human gastric carcinoma. *Int J Cancer* 1996; 66:315-321
4. Seaki T, Salomon D, Normanno N, Johnson G, Gullick WJ, Mandai K, Moriawaki S, Takashima S, Kuniyasu M, Tahara E, Kawami H, Nishiyama M, Toge T: Immunohistochemical detection of c-erbB-1, amphiregulin and transforming growth factor alpha in human gastric carcinomas and intestinal metaplasias. *Int J Oncol* 1994; 5:215-223

5. Daub H, Weiss U, Wallasch C, Ullrich A: Role of transactivation of the EGF receptor in signalling by G-protein-coupled receptors. *Nature* 1996, 379:557–560
6. Prenzel N, Zwick E, Daub H, Leserer M, Abraham R, Wallasch C, Ullrich A: EGF receptor transactivation by G-protein-coupled receptors requires metalloproteinase cleavage of proHB-EGF. *Nature* 1999, 402:884–888
7. Pai R, Soreghan B, Szabo IL, Pavelka M, Baatar D, Tarnawski AS: Prostaglandin E2 transactivates EGF receptor: a novel mechanism for promoting colon cancer growth and gastrointestinal hypertrophy. *Nat Med* 2002, 8:289–293
8. Coughlin SR, Camerer E: PARTICIPATION in inflammation. *J Clin Invest* 2003, 111:25–27
9. Macfarlane SR, Seatter MJ, Kanke T, Hunter GD, Plevin R: Proteinase-activated receptors. *Pharmacol Rev* 2001, 53:245–282
10. Kato Y, Nagashima Y, Koshikawa N, Miyagi Y, Yasumitsu H, Miyazaki K: Production of trypsins by human gastric cancer cells correlates with their malignant phenotype. *Eur J Cancer* 1998, 34:1117–1123
11. Compton SJ, Sandhu S, Wijesuriya SJ, Hollenberg MD: Glycosylation of human proteinase-activated receptor-2 (hPAR2): role in cell surface expression and signaling. *Biochem J* 2002, 368:495–505
12. Miyata S, Miyagi Y, Koshikawa N, Nagashima Y, Kato Y, Yasumitsu H, Hirahara F, Misugi K, Miyazaki K: Stimulation of cellular growth and adhesion to fibronectin and vitronectin in culture and tumorigenicity in nude mice by overexpression of trypsinogen in human gastric cancer cells. *Clin Exp Metastasis* 1998, 16:613–622
13. Darmoul D, Gratio V, Devaud H, Laburthe M: Protease-activated receptor 2 in colon cancer. *J Biol Chem* 2004, 279:20927–20934
14. Luttrell LM, Hawes BE, Van Biesent T, Luttrell DK, Lansing TJ, Lefkowitz RJ: Role of c-Src tyrosine kinase in G protein-coupled receptor and G $\beta\gamma$ subunit-mediated activation of mitogen-activated protein kinases. *J Biol Chem* 1996, 271:19443–19450
15. Luttrell LM, Della Rocca GJ, Van Biesent T, Luttrell DK, Lefkowitz RJ: G $\beta\gamma$ subunits mediate Src-dependent phosphorylation of the epidermal growth factor receptor. *J Biol Chem* 1997, 272:4637–4644
16. D'Andrea MR, Derian CK, Leturcq D, Baker SM, Brunmark A, Ling P, Darrow AL, Santulli RJ, Brass LF, Andrade-Gordon P: Characterization of protease-activated receptor-2 immunoreactivity in normal human tissues. *J Histochem Cytochem* 1998, 46:157–164
17. D'Andrea MR, Derian CK, Santulli RJ, Andrade-Gordon P: Differential expression of protease-activated receptor-1 and -2 in stromal fibroblasts of normal, benign, and malignant human tissues. *Am J Pathol* 2001, 158:2031–2041
18. Vergnolle N: Proteinase-activated receptors-novel signals for gastrointestinal pathophysiology. *Aliment Pharmacol Ther* 2000, 14:257–266
19. Vergnolle N: Clinical relevance of proteinase activated receptors (PARs) in the gut. *Gut* 2005, 54:867–874
20. Nysted S, Emilsson K, Larsson AK, Strömbeck B, Sundelin J: Molecular cloning and functional expression of the gene encoding the human proteinase-activated receptor 2. *Eur J Biochem* 1995, 232:84–89
21. MacNaughton W: Epithelial effects of proteinase-activated receptors in the gastrointestinal tract. *Mem Inst Oswaldo Cruz* 2005, 100(suppl):211–215
22. Shibata K, Yada K, Matsumoto T, Sasaki A, Ohta M, Kitano S: Protease-activating-receptor-2 is frequently expressed in papillary adenocarcinoma of the gallbladder. *Oncol Rep* 2004, 12:1013–1016
23. Darmoul D, Marie JC, Devaud H, Gratio V, Laburthe M: Initiation of human colon cancer cell proliferation by trypsin acting at protease-activated receptor 2. *Br J Cancer* 2001, 85:772–779
24. Jikuhara A, Yoshii M, Iwagaki H, Mori S, Nishibori M, Tanaka N: MAP kinase-mediated proliferation of DLD1 carcinoma by the stimulation of protease-activated receptor-2. *Life Sci* 2003, 73:2817–2829
25. Shimamoto R, Sawada T, Uchima Y, Inoue M, Kimura K, Yamashita Y, Yamada N, Nishihara T, Ohira M, Hirakawa K: A role for protease-activated receptor-2 in pancreatic cancer cell proliferation. *Int J Oncol* 2004, 24:1401–1406
26. Ohta T, Shimizu K, Yi S, Takamura H, Amaya K, Kitagawa H, Kaya-hara M, Ninomiya I, Fushida S, Fujimura T, Nishimura G, Miwa K: Protease-activated receptor-2 expression and the role of trypsin in cell proliferation in human pancreatic cancers. *Int J Oncol* 2003, 23:61–66
27. Ikada O, Egami H, Ishiko T, Ishikawa S, Kamohara H, Hidaka H, Mita S, Ogawa M: Expression of proteinase-activated receptor-2 in human pancreatic cancer: a possible relation of cancer invasion and induction of fibrosis. *Int J Oncol* 2003, 22:295–300
28. Hynes NE, Lane HA: ERBB receptors and cancer: the complexity of targeted inhibitors. *Nat Rev Cancer* 2005, 5:341–354
29. Marciniak DJ, Moragoda L, Mohammad RM, Yu Y, Nagothu KK, Aboukameel A, Sarkar FH, Adsay VN, Rishi AK, Majumdar AP: Epidermal growth factor receptor-related protein: a potential therapeutic agent for colorectal cancer. *Gastroenterology* 2003, 124:1337–1347
30. Wiseman SM, Makretsov N, Nielsen TO, Gilks B, Yorlida E, Cheang M, Turbin D, Gelmon K, Huntsman DG: Coexpression of the type1 growth factor receptor family members HER-1, HER-2, and HER-3 has a synergistic negative prognostic effect on breast carcinoma survival. *Cancer* 2005, 103:1770–1777
31. Amann J, Kalyankrishna S, Massion PP, Ohm JE, Girard L, Shigematsu H, Peyton M, Juroske D, Huang Y, Stuart Salmon J, Kim YH, Pollack JR, Yanagisawa K, Gazdar A, Minna JD, Kurie JM, Carbone DP: Aberrant epidermal growth factor receptor signaling and enhanced sensitivity to EGFR inhibitors in lung cancer. *Cancer Res* 2005, 65:226–235
32. Konturek PC, Bielanski W, Konturek SJ, Hahn EG: Helicobacter pylori associated gastric pathology. *J Physiol Pharmacol* 1999, 50:695–710
33. Kato M, Asaka M, Shimizu Y, Nobuta A, Takeda H, Sugiyama T, Multi-Center Study Group: Relation between Helicobacter pylori infection and the prevalence, site and histological type of gastric cancer. *Aliment Pharmacol Ther* 2004, 20(suppl. 1):85–89
34. Jacob C, Cottrell GC, Gehringer D, Schmidlin F, Grady EF, Bunnett NW: c-Cbl mediates ubiquitination, degradation and down-regulation of human protease-activated receptor 2 (PAR2). *J Biol Chem* 2005, 280:16076–16087
35. Paris S, Sesboue R, Delpech B, Chauzy C, Thiberville L, Martin JP, Frebourg T, Diarra-Mehrpour M: Inhibition of tumour growth and metastatic spreading by overexpression of inter-alpha-trypsin inhibitor family chains. *Int J Cancer* 2002, 97:615–620