# EGF promotes invasion by PANC-1 cells through Rac1/ROS-dependent secretion and activation of MMP-2

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Cancer metastasis involves tumor cells invading the surrounding tissue. Remodeling of tissue barriers depends on the ability of tumor cells to degrade the surrounding collagen matrix and then migrate through the matrix defects. Epidermal growth factor (EGF) has been shown to regulate tumor cell invasion through activation of matrix metalloproteinase-2 (MMP-2) in various tumor cell types. In the present study, we investigated the role of MMP-2 and the signaling pathway involved in EGF-promoted invasion by human pancreatic cancer cells PANC-1. Using specific inhibitors, we found that EGF stimulation of these tumor cells induced secretion and activation of the collagenase MMP-2, which was required for EGF-stimulated basement membrane degradation and cell invasion. Our results also indicate that signaling events downstream of EGF receptor involved PI3K- and Src-dependent activation of Rac1, which mediated the NADPH-generated reactive oxygen species responsible for MMP-2 secretion and activation.

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Cancer metastasis involves tumor cells invading the surrounding tissue. Invasive cells must traverse tissue barriers such as the basement membrane, comprised largely of type I collagen. Remodeling of these tissue barriers depends on the ability of tumor cells to degrade the surrounding collagen matrix and then migrate through the matrix defects. Several proteolytic enzymes, including matrix metalloproteinases (MMPs), seem to be implicated in this process. Among the MMP family, MMP-2 (gelatinase A, 72 kDa type-IV collagenase) is a main character in proteolytic degradation of basement membranes [1]. High expression and activation levels for this extracellular matrix-degrading proteinase have been found in various human cancer tissues [2–8]. In particular, high levels of activated MMP-2 showed to correlate with tumor invasion and metastasis in pancreatic carcinoma [5,6]. Although pre-clinical data supports the use of MMP inhibitors in cancer treatment, clinical trials involving these agents have rendered disappointing results, suggesting that the manipulation of MMPs to achieve tumor stasis may require altering the signal-transduction pathways that regulate the activity of MMPs rather than global inhibition [1].

Epidermal growth factor receptor (EGFR) signaling plays important roles in human cancers. Activation of EGFR enhances tumor growth, invasion, motility, tumor spreading and metastasis [9–11]. Remarkably, EGFR has been shown to regulate tumor cell invasion through activation of MMP-2 in various tumor cell types [12,13].

In the present study, we investigated the role of MMP-2 and the signaling pathway involved in EGF-promoted invasion by human pancreatic cancer cells PANC-1.

## Materials and methods

*Antibodies and reagents.* Mouse monoclonal anti-Rac1 was from Upstate (Chicago, IL). Dulbecco's modified Eagle's media (DMEM) and fetal bovine serum (FBS) were from GIBCO/Invitrogen (Carisbad, CA). Trans-well chambers (8 μm pore-size), Matrigel and protease inhibitor cocktail were from BD Biosciences (San Jose, CA). CM-$H_2$DCFDA was from Molecular Probes (Burlington, Ontario, Canada). NSC23766 was from Tocris (Bristol, UK). Human recombinant EGF and specific inhibitors of EGFR (AG1478), PI3 K (LY294002), NADPH (DPI), ROS (NAC) and MMP (Ilomastat), were from Sigma Chemical Co. (St. Louis, MO). Specific inhibitors of MEK1/2 (U0126), p38 MAPK (p38 MAPK inhibitor), PLC-γ (U-73122), PKC (Calphostin C) and Src (PP2) were from Calbiochem (San Diego, CA). GST-PAK-PBD-beads were from Cytoskeleton (Denver, CO).

*Cell culture.* PANC-1 human pancreatic adenocarcinoma cells (ATCC, Manassas, VA) were maintained in DMEM supplemented with 10% heat-inactivated FBS at 37 °C in a 5% $CO_2$ humidified atmosphere.

*Invasion assay.* The upper compartment of trans-well chambers was coated with Matrigel and placed in six-well plates. PANC-1 cells were washed with phosphate buffered saline (PBS) and then detached with 5 mM EDTA in PBS. Detached cells were washed once more in PBS, resuspended in serum-free DMEM and added to the upper compartment of the chamber ($1 \times 10^5$ cells/well). Conditioned medium was placed in the lower compartment of the chambers. To determine the effect of specific inhibitors, cells were pretreated with the inhibitor for 1 h before they were added to the chamber, and then fresh inhibitor was added to the well. After 24 h of incubation at 37 °C, the cells on the upper surface were completely removed by wiping with a cotton swab, and then the filter was fixed with methanol and stained with crystal violet solution. Cells that had migrated from the upper to the lower side of the filter were counted with a light microscope on 50 fields/filter.

*Gelatin zymography.* Equal number of PANC-1 cells was cultured in serum-free DMEM for 24 h in absence or in presence of 10 ng/ml EGF. To determine the effect of specific inhibitors, they were added to the cells 1 h before EGF, and they were maintained into the media along the experiment. Conditioned media obtained were mixed with substrate gel sample-buffer and loaded onto a 7.5% SDS–PAGE containing type-I-gelatin. After electrophoresis, the gel was soaked in Triton X-100, followed by rinse and incubation for 24 h at 37 °C in substrate buffer. After incubation, the gel was stained with Coomassie brilliant blue and destained with methanol–acetic acid in water. Activity of lytic bands was determined by densitometry employing the ImageJ software (NIH, Bethesda, MD).

*Rac1 activity assay.* Active Rac1 was determined by a pull-down assay as follow. Serum-starved PANC-1 cells were or were not stimulated for 3 min with EGF and then collected in 800 μl of ice-cold lysis-buffer. Lysates were centrifuged to remove cellu-lar debris. From each supernatant, 10 μl were removed to measure protein content using Protein Assay Kit (Bio-Rad, Hercules, CA), 20 μl were removed to determine total Rac1 in total lysate, and the rest of the volume was used for the pull-down assay. Lysates containing equal amount of proteins were then mixed with 15 μg of GST-PAK-PBD-beads. Samples for total Rac1 in total lysate and the pelleted beads were diluted in Laemmli sample-buffer and boiled. The proteins were separated using SDS–PAGE (12% gel). After transfer to nitrocellulose membranes (Bio-Rad), blots were blocked with bovine serum albumin, followed by incubation with Rac1 antibody. Binding of the antibody was visualized using peroxidase-coupled anti-mouse antibody, and enhanced chemiluminescence method (Amersham, Arlington Heights, IL). Equal loading was verified by reprobing membranes corresponding to total lysate with anti-tubulin antibody (not shown). To determine the effect of specific inhibitors, they were added to the cells 1 h before EGF, and they were maintained in the media along the experiment. Quantification was performed by densitometry using the ImageJ software.

*ROS production.* Free radical production was determined by incubating PANC-1 cells in the presence of 10 μM CM-H$_2$DCFDA. Fluorescence was measured in a stirred cuvette at 37 °C in a Hitachi F-2000 (Hitachi Ltd., Tokyo, Japan) spectrofluorometer with excitation at 488 nm and emission at 530 nm.

*Statistical analysis.* Data correspond to at least three independent experiments, each of which was done in triplicate. Results are presented as means ± standard error (SE). The data for each condition were subject to analysis of variance (ANOVA) followed by Dunnet post hoc test when comparing three or more conditions, or evaluated using Student's *t*-test when comparing only two conditions. Significant differences were considered with values of $p < 0.05$.
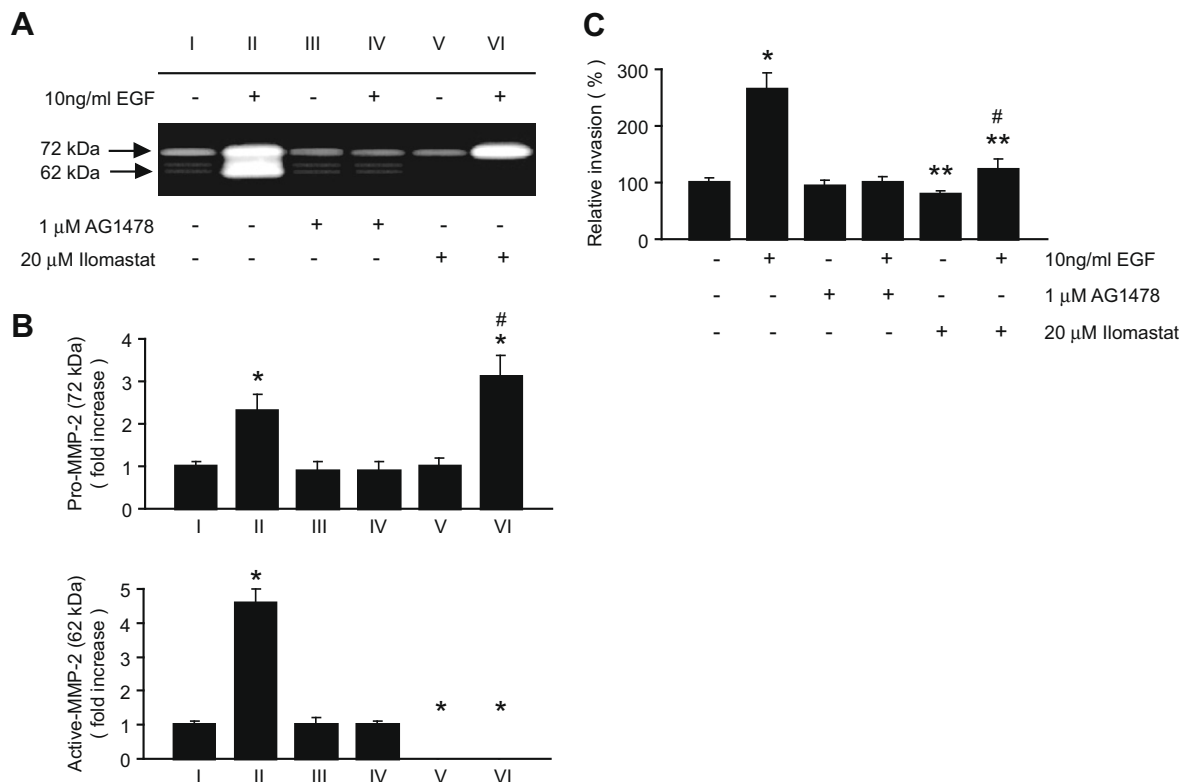


**Fig. 1.** MMP-2 activation is required for EGF-stimulated invasion by PANC-1 cells. (A) Representative zymography of conditioned media from PANC-1 cells. (B) Densitometric analysis of bands from gel zymograms as in (A). Results are expressed as fold-increase for gelatinolytic activity of unstimulated cells in the absence of inhibitors. (C) Cellular invasiveness through Matrigel by PANC-1 cells. Results are expressed as percent of invasive unstimulated cells in the absence of inhibitors. $^*p < 0.01$ and $^{**}p < 0.05$ compared to unstimulated cells in the absence of inhibitors, $^\#p < 0.05$ compared to EGF-stimulated cells in the absence of inhibitors.