

KiSS1 Metastasis Suppressor Gene Product Induces Suppression of Tyrosine Kinase Receptor Signaling to Akt, Tumor Necrosis Factor Family Ligand Expression, and Apoptosis^[S]

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ABSTRACT

The powerful metastasis suppressor function of *KiSS1* gene products has been demonstrated in both clinical studies and experimental models, but its mechanism is still incompletely understood. Studies on the antimetastatic function of *KiSS1* and GPR54 largely focused on the autocrine inhibition of cell motility, despite experimental evidence of an alternative post-migratory effect. We showed previously that the activation of its cognate receptor GPR54 by kisspeptin-10 suppressed the capacity of the prometastatic chemokine receptor CXCR4 to induce chemotaxis in response to stromal cell derived factor 1 and abolished the activation of Akt by CXCR4. We demonstrate here that activation of GPR54 can also abolish the activation of Akt by the tyrosine kinase receptors for epidermal growth factor and insulin. The signaling of GPR54 was sufficient to trigger

apoptosis in epithelial and lymphoid cell lines. Surprisingly, this phenomenon depended largely on the activation of extracellular signal-regulated kinase (ERK) rather than the inhibition of Akt. Activation of GPR54 resulted in the ERK-dependent expression of tumor necrosis factor- α and FasL in a lymphoid cell line, the latter being the main trigger of apoptosis. These data provide novel mechanisms relevant to a potential autocrine metastasis suppression effect of *KiSS1* on GPR54-positive tumor cells. More importantly, they also establish an experimental basis for a paracrine mode of action by which kisspeptins suppress the metastatic potential of tumor cells lacking expression of the receptor, as observed in several animal models of metastasis. The action on stromal cells significantly broadens the clinical relevance of this metastasis suppressor.

Metastasis is a complex process that requires tumor cells to acquire multiple characteristics that their normal and nonmetastatic counterparts lack (Gupta and Massagué, 2006). Biological selection experiments have revealed genes associated with the metastatic phenotype to common target

organs (Kang et al., 2003; Minn et al., 2005), including the chemokine receptor CXCR4 that can direct tumor cells to home into organs expressing its ligand stromal cell derived factor 1 (SDF-1, CXCL12). An antithetical group of genes encodes molecules that specifically inhibit the spread of cancer cells but not tumorigenicity by targeting multiple steps of the metastatic process (Stafford et al., 2008). One of these metastasis suppressors, *KiSS1*, was initially identified in experiments in which the introduction of chromosome 6 in metastatic melanoma cells suppressed metastasis to the lungs and lymph nodes (Welch et al., 1994; Lee et al., 1996). The gene was later localized to chromosome 1q32, suggesting

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ABBREVIATIONS: SDF-1, stromal-derived factor 1; BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle's medium; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; ERK, extracellular signal-regulated kinase; FBS, fetal bovine serum; GPCR, G-protein-coupled receptor; JNK, c-jun N-terminal kinase; Kp, kisspeptin; MAPK, mitogen-activated protein kinase; PI, propidium iodide; PI3K, phosphatidylinositol-3 kinase; PKC, protein kinase C; PLC, phospholipase C; RTK, receptor tyrosine kinase; TNF- α , tumor necrosis factor α ; HEK, human embryonic kidney; PBS, phosphate-buffered saline; PARP, poly(ADP-ribose) polymerase; MEK, mitogen-activated protein kinase kinase; ELISA, enzyme-linked immunosorbent assay; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; U73122, 1-[6-[[17 β -methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1*H*-pyrrole-2,5-dione; U0126, 1,4-diamino-2,3-dicyano-1,4-bis(methylthio)butadiene; PD98059, 2'-amino-3'-methoxyflavone; LY294002, 2-(4-morpholinyl)-8-phenyl-1(4*H*)-benzopyran-4-one hydrochloride; SB202190, 4-(4-fluorophenyl)-2-(4-hydroxyphenyl)-5-(4-pyridyl)-1*H*-imidazole; SB203580, 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)-1*H*-imidazole; SP600125, anthra(1,9-*cd*)pyrazol-6(2*H*)-one 1,9-pyrazoloanthrone.

that genes located on chromosome 6 regulate its expression (West et al., 1998; Shirasaki et al., 2001), a fact that was confirmed more recently (Goldberg et al., 2003; Mitchell et al., 2007).

Besides melanoma, the metastasis-suppressing function of *KiSS1* has been demonstrated in breast cancer (Lee and Welch, 1997b) and ovarian carcinoma models (Jiang et al., 2005). In addition, in clinical studies, the loss of *KiSS1* expression has been linked to poor prognosis in several malignancies, including ovarian cancer (Hata et al., 2007; Prentice et al., 2007), melanoma (Shirasaki et al., 2001; Martins et al., 2008), and carcinomas of the stomach (Dhar et al., 2004), urinary bladder (Sanchez-Carbajo et al., 2003), and esophagus (Ikeguchi et al., 2004).

The *KiSS1* protein is proteolytically processed to polypeptides ranging from 54 to 10 amino acids known as kisspeptins (Kp), which are secreted (Kotani et al., 2001; Muir et al., 2001; Ohtaki et al., 2001). A G-protein-coupled receptor (GPCR), GPR54 (also known as AXOR12 and hOT7T175) has been identified as the receptor for kisspeptins (Kotani et al., 2001; Muir et al., 2001; Ohtaki et al., 2001). Kp binding to GPR54 activates $G\alpha_q$ and results in the inhibition of chemotaxis to FBS, activation of ERK MAPK, formation of stress fibers, phosphorylation of focal adhesion complexes, decreased matrix metalloproteinase activity, and reduced cell proliferation in receptor transfectants (Kotani et al., 2001; Ohtaki et al., 2001). We have shown previously that activation of GPR54 by Kp-10 abrogated cellular responses to SDF-1, including chemotaxis, calcium mobilization, and activation of Akt/protein kinase B by CXCR4. (Navenot et al., 2005).

The PI3 kinase (PI3K)-Akt pathway plays a major role in several key cellular processes, including survival and apoptosis, glucose metabolism, cell cycle progression, and gene expression (Manning and Cantley, 2007). Both GPCRs and receptor tyrosine kinases (RTKs) can activate Akt, although through different isoforms of PI3K (Carpenter and Cantley, 1996). Because we demonstrated previously that GPR54 signaling did not activate Akt and induced a negative cross-talk with CXCR4, resulting in the inactivation of Akt, we investigated whether activation of GPR54 could also suppress RTK signaling through Akt, whether cell viability would be compromised, and what signaling mechanism would be involved.

The nature of the cells expressing Kp and GPR54 still remains to be determined. In fact, if transcripts of GPR54 have been shown to be increased in some primary cancer cells relative to normal tissues (Ohtaki et al., 2001), several of the cell lines shown to have decreased metastatic behavior when expressing Kp did not express GPR54 (Lee and Welch, 1997; Becker et al., 2005; Nash et al., 2007). This suggests that the antimetastatic effect of Kp, at least in some malignancies, may target nontumor cells in the microenvironment rather than the tumor cells themselves in an autocrine loop. This hypothesis is supported by the fact that a number of normal organs and cell types (such as peripheral blood lymphocytes) have been shown to express transcripts of GPR54 (Kotani et al., 2001; Ohtaki et al., 2001). We elected to use HEK-293 and Jurkat cells transfected with GPR54 to investigate the signaling events resulting from GPR54 activation that could be relevant to a paracrine and an autocrine mode of action of Kp.

Materials and Methods

Cell Lines and Reagents. The human cell line HEK-293T was modified to express GPR54 with an N-terminal Myc epitope tag by transfection with a pcDNA3.1 construct (Invitrogen, Carlsbad, CA) as described previously (Navenot et al., 2005). Jurkat cells were transfected with an Myc-tagged GPR54 in a pME vector (a gift from Dr Makio Iwashima, Medical College of Georgia, Augusta, GA). After selection either in G418 (293) or puromycin (Jurkat), transfected cells were selected for expression of the Myc tag by magnetic sorting (Miltenyi Biotec, Auburn, CA) using the 9E10 antibody (Santa Cruz Biotechnology, Santa Cruz, CA). YWNVSFGLRF-NH₂ (Kp-10) was synthesized at the Kyoto University (Kyoto, Japan). Inhibitors of protein kinase A (4-cyano-3-methylisoquinoline), PKC (bis-indolylmaleimide-1), phospholipase C (PLC; U73122), MEK1/2 (U0126 and PD98059), PI3K (LY294002 and wortmannin), p38 MAPK (SB202190 and SB203580), and JNK (SP600125, JNK inhibitor II) were from Calbiochem (La Jolla, CA). All antibodies used for Western blots were from Cell Signaling Technology (Danvers, MA). The antibody to CXCR4 12G5 was a gift from Dr. James Hoxie (University of Pennsylvania, Philadelphia, PA).

Activation of ERK1/2 and Akt by Western Blot. 293 and 293-GPR54 cells (2×10^5) were seeded in 35-mm dishes and grown for 24 h in complete growth medium (DMEM containing 10% FBS) before an overnight starvation (DMEM containing 0.25% BSA). After stimulation for 5 min at 37°C with Kp-10 (100 nM), epidermal growth factor (EGF, 10 ng/ml; PeproTech, Rocky Hill, NJ) or insulin (100 nM; Sigma, St. Louis, MO), the cells were washed with PBS before being solubilized in 200 μ l of sample buffer containing SDS and dithiothreitol. Jurkat and Jurkat-GPR54 cells (1×10^6) in serum-free medium (RPMI 1640 containing 0.25% BSA) were exposed to Kp-10 (100 nM) or the PI3K inhibitors LY294002 (50 μ M) or wortmannin (100 nM) before being washed in PBS. Whole-cell lysates were prepared in SDS sample buffer for 5 min at 100°C. The relative amount of proteins in the samples was determined after SDS-PAGE and Coomassie blue staining of 10 μ l of each sample and analysis with a LAS-3000 digital imaging system (Fuji, Stamford, CT). Identical amounts of proteins of all samples were then analyzed by Western blotting, the polyvinylidene difluoride membranes (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK) being incubated with antibodies specific for total ERK1/2, phospho-ERK1/2, total Akt, phospho-Akt Ser⁴⁷³, and phospho-Akt Thr³⁰⁸. After incubation with the appropriate horseradish peroxidase-labeled antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) followed by the ECL Plus substrate (GE Healthcare), blots were analyzed with the LAS-3000 system, and the intensity of the specific bands was quantified using the Multi Gauge software (Fuji).

Analysis of Apoptosis by Western Blot. 293 cells were seeded at 5×10^4 cells/35-mm dish in complete growth medium. After 24 h, the medium was replaced with serum-free medium containing 0.25% BSA. For inhibition experiments, the medium contained one of the following inhibitors: protein kinase A (10 μ M), PKC (1 μ M), PLC (2 μ M), PI3K (50 μ M LY294002 or 100 nM wortmannin), MEK1/2 (10 μ M U0126 or 50 μ M PD98059), p38 MAPK (10 μ M SB202190 or 20 μ M SB203580), or JNK MAPK (10 μ M). After 1 h at 37°C, Kp-10 (100 nM) was added, and the cells were grown for another 48 to 72 h. The cells in the supernatant were collected and washed in ice-cold PBS. The cells adhering to the dishes were also washed in ice-cold PBS. Detached and adherent cells were pooled for each sample and solubilized in SDS, reducing sample buffer for 5 min at 100°C. Samples were processed for Western blotting as described above. Blots were incubated with antibodies to caspase 9, cleaved caspase 9, caspase 7, cleaved caspase 7, caspase 3, cleaved caspase 3, caspase 8 (detects both uncleaved and cleaved fragments, including the 18-kDa catalytically active fragment), PARP, and cleaved PARP. Incubations and detection were conducted as described above.

Measurement of Cell Viability. 293-GPR54 cells were seeded in triplicate in 12-well plates (2×10^4 cells/well) and treated as de-

scribed above. Cell morphology was assessed from pictures of representative fields from an inverted microscope (TE-2000E; Nikon, Melville, NY) equipped with a 10 \times phase contrast objective and a charge-coupled device camera (CoolSnap HQ; Roper, Pleasanton, CA), using Metamorph (Molecular Devices, Sunnyvale, CA). Supernatants were collected and transferred to 5-ml tubes. Adherent cells in the plates were washed with PBS, detached with trypsin, and resuspended in their medium supplemented with propidium iodide (PI; Sigma) to a final concentration of 5 μ g/ml. Live cells were counted for 1 min by flow cytometry (LSR II; BD Biosciences, San Jose, CA) based on their morphology (forward versus side scatter) and their capacity to exclude PI. For Jurkat cells, the protocol was similar except that cells were resuspended in complete growth medium and exposed to Kp-10 for 16 to 30 h before being resuspended in their own medium supplemented with PI and counted by flow cytometry.

Staining with Annexin-V and Detection of Mitochondrial Potential. Expression of phosphatidyl-serine on the cell surface was detected with annexin-V combined, in Jurkat cells, with a measurement of the loss of mitochondrial potential. For 293 cells, cells were grown on glass-bottomed 35-mm dishes (MatTek, Ashland, MA) and treated as described above. After 48 h of exposure to Kp-10, cells were washed in annexin-V binding buffer (10 mM HEPES, 140 mM NaCl, and 2.5 mM CaCl₂, pH 7.4) and incubated with 5 μ l of annexin-V Alexa Fluor 488 (Invitrogen) for 15 min at room temperature. Cells were then washed twice in binding buffer, covered with buffer containing 10 μ g/ml Hoechst 33342 (Invitrogen), and observed with an inverted microscope equipped with a 60 \times objective. Images of representative fields were captured for differential interference contrast, fluorescence of annexin-V and fluorescence of Hoechst. Jurkat cells were resuspended at 5 \times 10⁵ cells/ml in complete growth medium and exposed to Kp-10, LY294002, or staurosporine for 12 h at 37°C. The mitochondrial membrane potential indicator 1,1',3,3,3',3'-hexamethylindodicarbo-cyanine iodide (Invitrogen) was added for 30 min at 37°C at a final concentration of 50 nM. After 1 wash in PBS, cells were resuspended in 100 μ l of annexin-V binding buffer and 5 μ l of annexin-V Alexa Fluor 488 for 15 min at room temperature. Cells were finally diluted in 400 μ l of binding buffer with 5 μ g/ml PI and analyzed by flow cytometry.

Measurement of Secreted TNF- α by ELISA. Jurkat cells in complete medium were placed in 24-well plates at 1 \times 10⁶ cells/ml and exposed to Kp-10 or SDF-1 (100 nM) for intervals ranging from 30 min to 6 h. Cells were preincubated with MAPK inhibitors for 1 h before the addition of Kp-10 when indicated. Samples were collected in 1.5-ml microtubes, centrifuged at low speed (2 min at 1,500 rpm) to remove cells, and then at high speed (5 min at 13,000 rpm) to remove any debris. 293-GPR54 cells were plated in complete medium in 35-mm dishes (2.5 \times 10⁵ cells/dish) and grown for 24 h. Medium was then replaced with 1 ml of serum-free medium, and the cells were exposed or not to Kp-10 for 6 h (duplicates for each condition). Supernatants were collected and centrifuged (5 min at 13,000 rpm) to remove debris and frozen at -80°C until use. ELISA for TNF- α was performed using the Quantikine kit (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions.

Detection of FasL on the Cell Surface. Jurkat-GPR54 cells (5 \times 10⁵) were exposed to Kp-10 (100 nM) in complete medium for 2,

4, or 6 h at 37°C. Cells were then washed in cold PBS containing 0.1% BSA and incubated on ice for 1 h with 10 μ g/ml of control mouse IgG1 or mouse monoclonal antibody to human FasL (MAB126; R&D Systems). After two washes in PBS-BSA, cells were incubated with an R-phycoerythrin-labeled secondary antibody at 10 μ g/ml (Jackson ImmunoResearch) for 1 h on ice. After two washes, cells were analyzed by flow cytometry.

Inhibition of Apoptosis with Neutralizing Antibodies to TNF- α , FasL, and TRAIL. Jurkat-GPR54 and 293-GPR54 cells were processed for induction of apoptosis as described above. Neutralizing antibodies to TNF- α (mouse monoclonal antibody MAB210 and goat polyclonal antibody AF-210-NA, both from R&D Systems) were pooled and added to Jurkat-GPR54 at concentrations ranging from 5 ng/ml to 5 μ g/ml and from 1 ng/ml to 1 μ g/ml, respectively. Kp-10 (100 nM) was then added, and the cells were incubated for 16 h at 37°C. Live cells were counted by flow cytometry as indicated above. 293-GPR54 cells were incubated for 48 h with the highest concentrations of antibodies only and apoptosis was assessed by microscopy based on morphological changes. Inhibition of apoptosis was also studied with neutralizing antibodies specific for FasL (concentrations from 0.1 to 10 μ g/ml, MAB126 and mouse IgG2b; R&D Systems) and TRAIL (concentrations from 0.05 to 5 μ g/ml, goat IgG and AF375; R&D Systems). Mouse IgG2b at 10 μ g/ml (Southern Biotechnology, Birmingham, AL) was used as a control.

Statistical Analyses. Averages of quantitative data were compared by Student's *t* test. Two-tailed *p* values are expressed as *p* < 0.05, *p* < 0.01, and *p* < 0.001.

Results

Activation of GPR54 Abolishes the Activation of Akt by EGFR and the Insulin Receptor. The consequences of GPR54 signaling were investigated in 293-GPR54 and Jurkat-GPR54 obtained by stable transfection. Limited rounds of selection of GPR54-positive cells by magnetic sorting were performed to keep the expression of the receptor to a physiological level and avoid the bias of an oligoclonal selection. Immunofluorescence and flow cytometric analysis show that the expression level of GPR54 obtained in both 293 and Jurkat cells is similar to the endogenous expression of CXCR4 in Jurkat cells (Fig. 1).

The exposure of 293-GPR54 cells to EGF or insulin resulted in a strong phosphorylation of Akt (Fig. 2, A and B). Kp-10 induced phosphorylation of ERK, but not of Akt, in 293-GPR54 cells. In addition, basal phosphorylation of Akt was reduced (Fig. 2). Pre-exposure to Kp-10 abolished Akt phosphorylation in response to EGF or insulin (Fig. 2). Thus, the negative cross-talk from GPR54 signaling also affects Akt activation by RTK. These effects of Kp-10 were reproduced by the PI3K inhibitor LY294002 and were independent of the order of addition of the ligands (data not shown). Exposure of parental 293 cells to Kp-10 had no effect on ERK and Akt phosphorylation (Fig. 2).

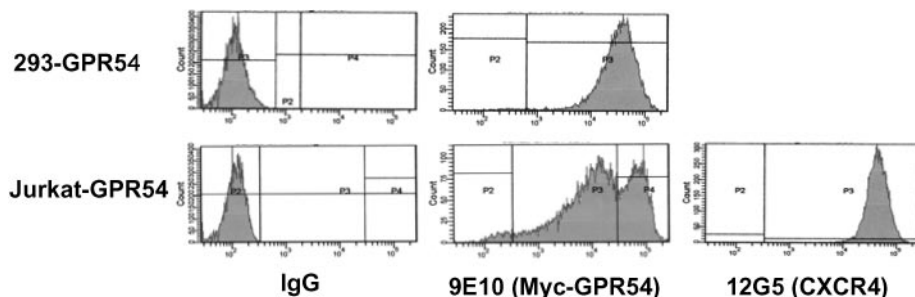


Fig. 1. Expression of GPR54 and CXCR4 by flow cytometry. 293-GPR54 and Jurkat-GPR54 were stained with a saturating concentration of anti-Myc antibody 9E10 and phosphatidylethanolamine-labeled secondary antibody to measure the surface expression of GPR54. The expression level of endogenous CXCR4 was similarly measured on Jurkat-GPR54 by incubating the cells with a saturating concentration of the anti-CXCR4 antibody.

Kp-10 Mediates GPR54-Dependent Cell Death in 293 and Jurkat Cells. Cell-rounding consistent with an apoptotic phenotype was detected after 24 h of exposure of 293-GPR54 cells to Kp-10 (Fig. 3A). The phenomenon intensified after 48 and 72 h, at which point the number of live cells present among GPR54-positive cells was significantly reduced compared with control cells, which remained unaffected by Kp-10 (Fig. 3B). Increasing concentrations of FBS up to 10% only partially reduced the effect (Fig. 3A). 293 and 293-GPR54 cells exhibited identical morphologies in the absence of Kp-10, irrespective of the FBS concentration (data not shown). Likewise, the number of live cells after 16 to 30 h was severely reduced in Jurkat-GPR54 cells but not in control cells (Fig. 3C). Cell death was proportional to the dose of Kp-10 between 3 and 100 nM (Fig. 3D).

Activation of GPR54 Triggers Apoptosis. Exposure of Jurkat-GPR54 cells to Kp-10 resulted into the emergence of a population with a reduced mitochondrial potential but no annexin-V staining (cells in early apoptosis) and a large increase of the annexin-V positive population (mid to late apoptosis) compared with the untreated control (Fig. 4A), indicative of the involvement of the mitochondrial pathway of apoptosis. The PI3K inhibitor LY294002 reproduced this effect. In control Jurkat cells, only LY294002 and staurosporine, but not Kp-10, could trigger apoptosis (Fig. 4A). Dose-response of Kp-10 (Fig. 4B) and kinetics experiments (Fig. 4C) with annexin-V staining confirmed that apoptosis increased between 3 and 100 nM Kp-10 and from 6 h to 24 h of

treatment. In 293-GPR54, most fields were devoid of apoptotic cells in unstimulated samples, but a large fraction of the cells treated with Kp-10 for 48 to 72 h were positive for

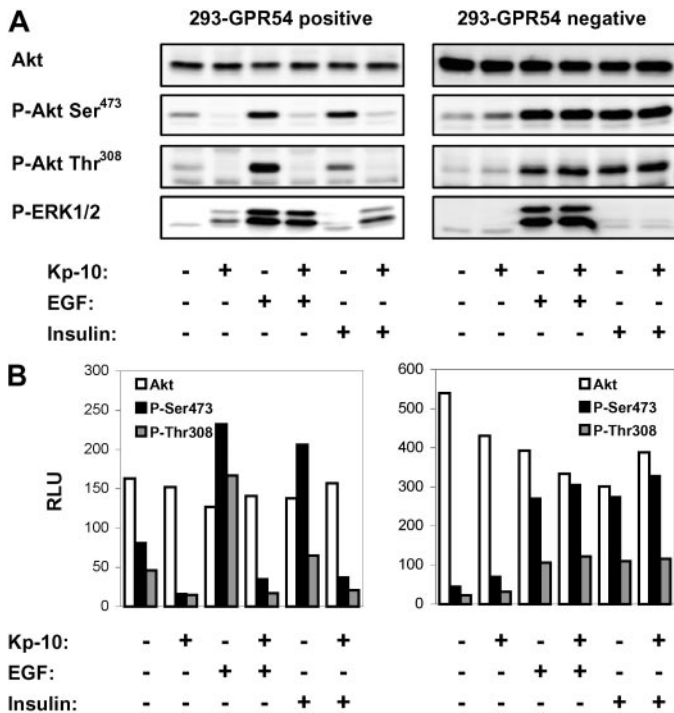


Fig. 2. Activation of GPR54 abolishes the activation of Akt by RTKs EGFR and insulin receptor. **A**, HEK-293 cells transfected with GPR54 or CXCR4 (GPR54-negative) were stimulated with Kp-10 100 nM for 7 min, EGF 10 ng/ml, or insulin 100 nM for 5 min. For combined stimulations, cells were first exposed to Kp-10 for 2 min before the addition of EGF or insulin for an additional 5 min. Whole-cell SDS lysates were analyzed by Western blots with antibodies to total Akt, phospho-Akt Ser473 and Thr308, and phospho-ERK1/2. The results are representative of at least four independent experiments. **B**, quantitative analysis of experiment in Fig. 2A.

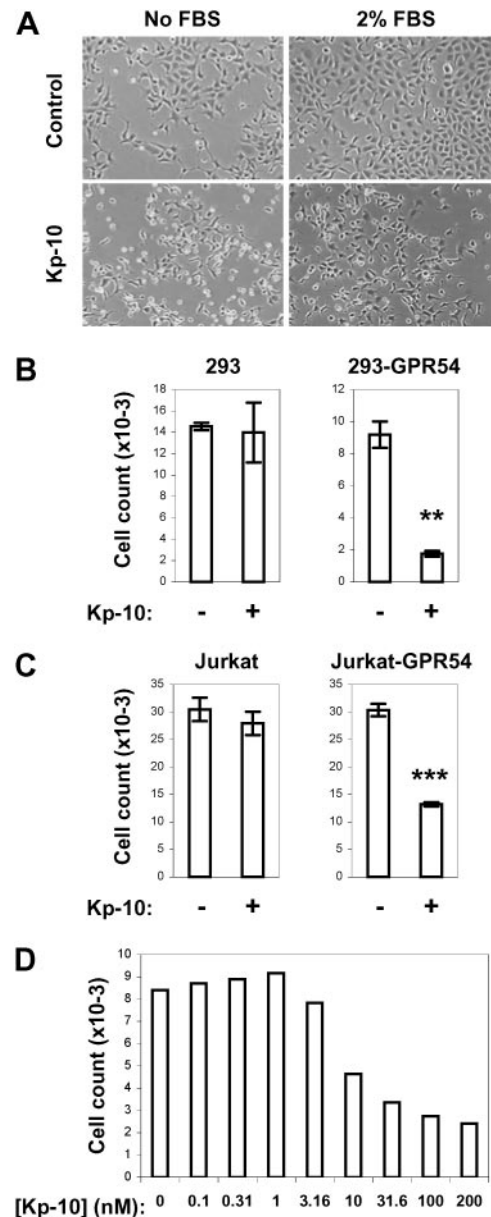


Fig. 3. Kp-10 induces GPR54-dependent cell death in 293 and Jurkat cells. **A**, 293-GPR54 cells were plated for 24 h in complete growth medium. The medium was then replaced with serum-free medium or medium containing from 2% FBS, complemented or not with 100 nM Kp-10. After 48 h, cells were observed by phase-contrast microscopy (10 \times magnification). **B**, 293 cells were plated in 12-well plates for 24 h and then exposed to 100 nM Kp-10 in DMEM-BSA. After 72 h, cells were detached, and live cells, defined by morphological gating and PI exclusion, were counted by flow cytometry. Graphs show the average cell count (\pm S.D.) of three identical samples for each condition (**, $p < 0.01$ for Kp-10 versus control). The results are representative of three independent experiments. **C**, Jurkat or Jurkat-GPR54 cells were incubated with 100 nM Kp-10 for 24 h. The cells were then resuspended and live cells were counted by flow cytometry for 1 min after the addition of PI. Graphs show the number (average \pm S.D.) of live cells in four identical samples for each condition obtained in one experiment representative of three independent experiments. *** $p < 0.001$ for Kp-10 versus control. **D**, dose-response of Kp-10 in Jurkat-GPR54. Cells were exposed to concentration of Kp-10 ranging from 0.1 to 200 nM for 24 h. Live cells were then counted by flow cytometry. Results are representative of two independent experiments.

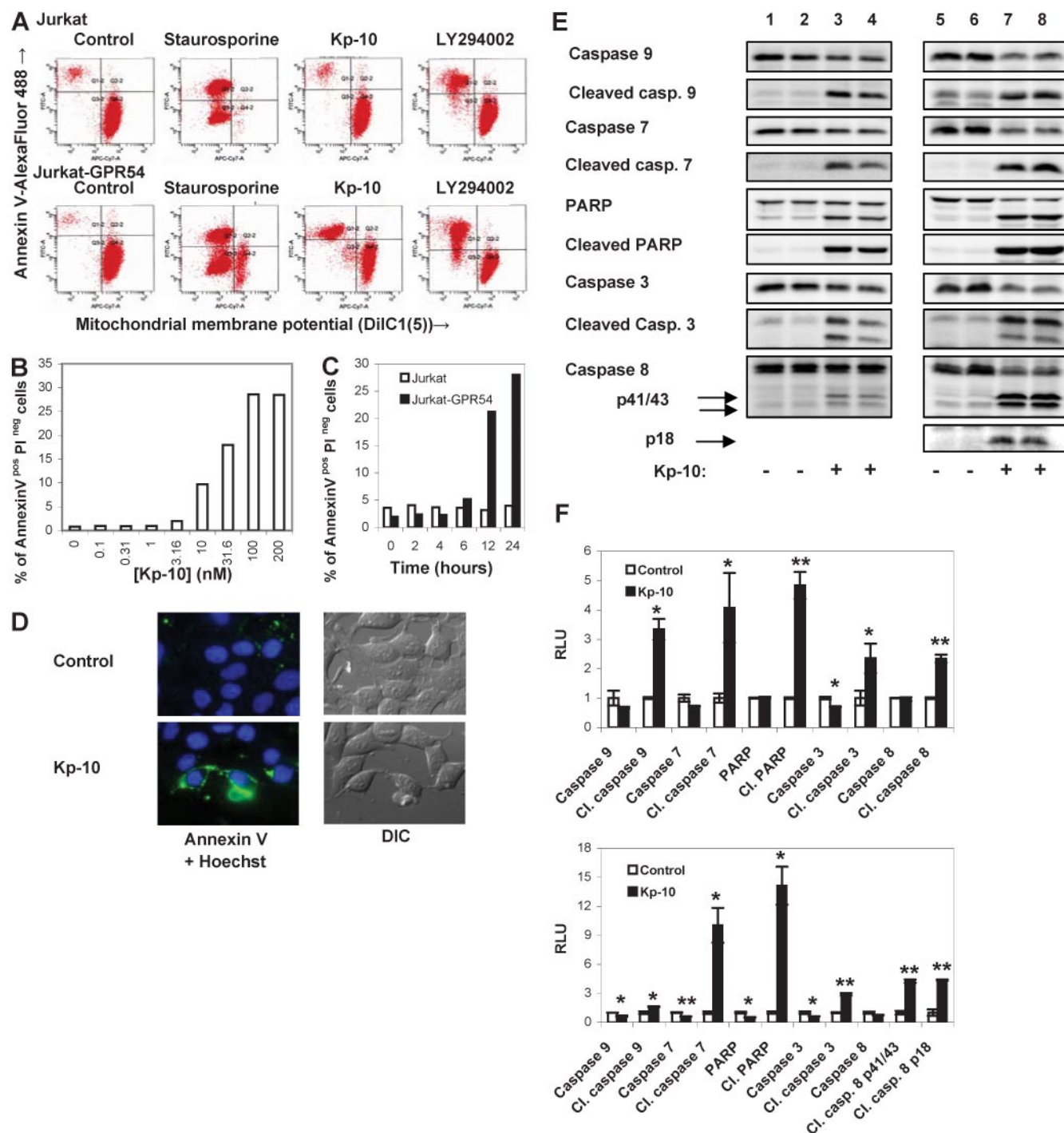


Fig. 4. Kp-10 induces apoptosis in Jurkat and 293 cells expressing GPR54. **A**, Kp-10 induced loss of mitochondrial potential and expression of phosphatidyl serine on the plasma membrane of Jurkat-GPR54. Cells were exposed to 100 nM Kp-10, 50 mM LY294002, or 100 nM staurosporine for 16 h before being stained with 1,1',3,3',3'-hexamethylindodicarbo-cyanine iodide for mitochondrial membrane potential and annexin-V Alexa Fluor 488 for expression of phosphatidylserine before being analyzed by flow cytometry. Early apoptotic cells had decreased mitochondrial membrane potential but remained largely negative for annexin-V staining (bottom left quadrant). Cells in later-stage apoptosis had both decreased mitochondrial potential and positive staining with annexin-V (top left quadrant). **B** and **C**, dose-response of Kp-10 (**B**) and time course of apoptosis (**C**) determined by annexin-V staining in Jurkat-GPR54. For the dose-response, cells were incubated with the indicated concentrations of Kp-10 for 16 h. The percentage of apoptotic cells (defined by annexin-V positivity and PI exclusion) over the entire cell population is indicated. For the time course, Jurkat-GPR54 and control Jurkat cells were incubated with 100 nM Kp-10 for the indicated times. The results are representative of two independent experiments. **D**, induction of apoptosis in 293-GPR54 cells after 48 h of incubation with 100 nM Kp-10. Cells were then stained with annexin-V Alexa Fluor 488 (green) and Hoechst 33342 (blue) and analyzed by fluorescence microscopy and differential interference contrast. The results are representative of two independent experiments performed in duplicate. **E**, activation of caspases by Kp-10 in 293 and Jurkat cells transfected with GPR54. For 293 cells (samples 1–4), cells were plated for 24 h before being exposed to 100 nM Kp-10 for 48 h. For Jurkat cells (samples 5–8), cells in complete growth medium were treated with 100 nM Kp-10 for 16 h. Duplicate samples were prepared for each experimental condition. Samples were analyzed by Western blots with antibodies specific for whole proteins or cleaved (active) fragments of caspase 9, caspase 7, caspase 3, and PARP, as well as an antibody to caspase 8 capable of binding the whole protein as well as the intermediate fragments p43 and p41 and the active fragment p18. The results are representative of three independent experiments. **F**, quantitative analysis of experiment shown in **E**. Statistically significant differences between control and Kp-10-treated samples are indicated. *, $p < 0.05$; **, $p < 0.01$.

annexin-V (Fig. 4D), suggesting that apoptosis was also responsible for cell death in that cell line.

In both 293-GPR54 and Jurkat-GPR54, addition of Kp-10 resulted in the activation of caspases and the resulting cleavage of PARP (Fig. 4, E and F). This indicates that the activation of GPR54 alone had the capacity to trigger a complete array of proapoptotic signals. Similar treatment had no effect on control cells (data not shown). Interestingly, caspase 8 seemed to be extensively cleaved to its active 18-kDa fragment in Jurkat-GPR54, suggesting the involvement of the extrinsic pathway, but not in 293-GPR54, in which only a very small amount of intermediate fragments (p41 and p43) and no p18 could be detected (Fig. 4E).

Inhibition of Akt Activity Alone Is Not Sufficient for GPR54-Mediated Apoptosis. We sought to establish a connection between loss of phosphorylation of Akt and apoptosis by first testing whether the pharmacological inhibition of PI3K resulted in apoptosis. Whereas Jurkat-GPR54 cells were equally sensitive to the treatment with the PI3K inhibitor LY294002 and Kp-10 (Fig. 4A), treatment of 293-GPR54 cells with LY294002 or wortmannin resulted into a reduced growth rate but not apoptosis (Fig. 5A). Furthermore, pretreatment with PI3K inhibitors did not alter the capacity of Kp-10 to trigger apoptosis (Fig. 5A). This strongly suggests that inhibition of Akt is not the main mechanism for induction of apoptosis by GPR54. Additional evidence came from experiments performed in Jurkat cells that have high basal phospho-Akt resulting from a constitutive defect in expression of the lipid phosphatases phosphatase and tensin homolog and inositol polyphosphate 5' phosphatase-1. Stimulation of Jurkat-GPR54 cells with Kp-10 did not decrease Akt phosphorylation, although ERK was phosphorylated (Fig. 5, B and C). A parallel treatment with LY294002 or wortmannin resulted in a rapid decrease in Akt phosphorylation, mediated by PP2A, which demonstrates the short half-life of phospho-Akt. This confirms that the negative cross-talk between activated GPR54 and Akt is not the only mechanism by which this GPCR triggers apoptosis.

ERK MAPK Plays an Essential Role in GPR54-Mediated Apoptosis. We and others have observed previously that the activation of GPR54 resulted in the phosphorylation of ERK, p38, and JNK MAPK. Because p38 and JNK activation can contribute to apoptosis, we tested whether the inhibition of MAPK could influence the capacity of GPR54 to trigger apoptosis in Jurkat and 293 cells. Because this receptor is known to signal through G_q , a direct activator of PLC, we also determined whether PLC and PKC inhibitors reduced apoptosis. Inhibition of PLC or PKC had no effect on Kp-10-mediated apoptosis or suppression of Akt phosphorylation in 293-GPR54 (data not shown). Prolonged inhibition of JNK itself induced extensive cell death in 293 cells during the course of the experiment. Cell death was also important in Jurkat-GPR54 cells with JNK inhibition alone. Decreasing the concentration of inhibitor to reduce toxicity showed that Kp-10 could further decrease the number of live cells, indicating that activation of JNK by GPR54 did not seem required for GPR54-mediated apoptosis (Supplemental Fig. 1A). Inhibition of p38 had no effect on apoptosis in 293-GPR54 (Supplemental Fig. 1, B and C) but elicited a partial inhibitory effect in Jurkat-GPR54 (Supplemental Fig. 1D). In contrast, inhibition of the MEK-ERK pathway significantly reduced the apoptotic effect of Kp-10 as assessed by cell

morphology (data not shown) and cell survival (Fig. 6, A and B), as well as the induction of cleavage of caspases and PARP in 293-GPR54 and Jurkat-GPR54 (Fig. 6, C and D).

Thus, the activation of ERK seems to be a major component of the proapoptotic signaling of GPR54. We confirmed that the role of ERK in GPR54-mediated apoptosis was independent of the capacity of the receptor to suppress Akt activity by showing that a treatment with an MEK inhibitor that inhibits both apoptosis and ERK1/2 phosphorylation did not alter the ability of Kp-10 to abrogate basal and EGF-induced phosphorylation of Akt in 293-GPR54 (Supplemental Fig. 2).

Activation of GPR54 Induces ERK-Dependent Expression of Proinflammatory and Proapoptotic Ligands in Jurkat Cells. Because we noted that Kp-10 induced extensive activation of caspase 8 in Jurkat-GPR54 cells, we investigated the possible role of the expression of

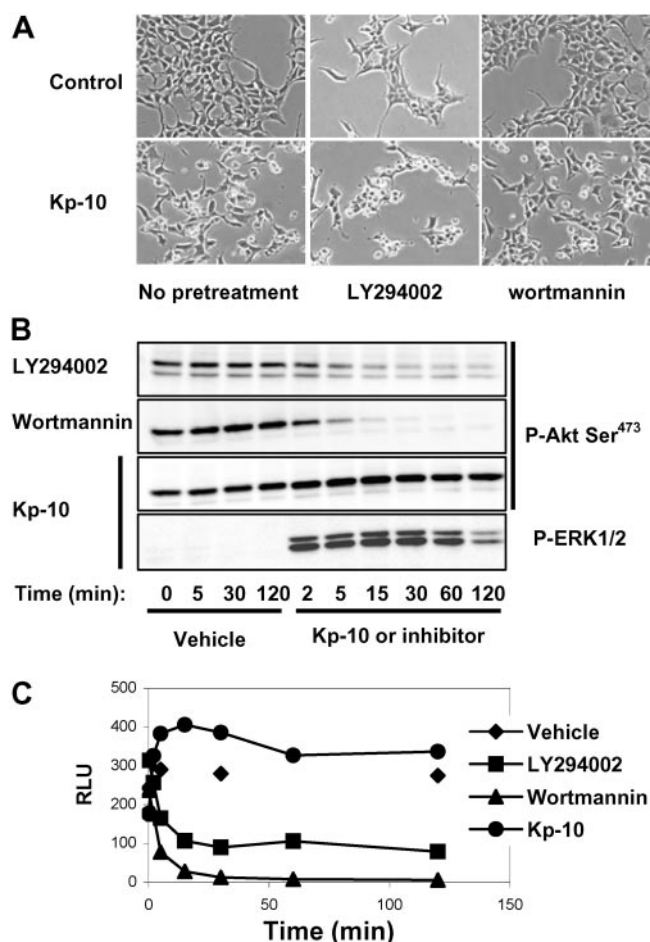


Fig. 5. Induction of apoptosis by Kp-10 does not correlate with Akt inhibition. A, 293-GPR54 cells resisted chemical inhibition of PI3K by LY294002 and wortmannin but were sensitive to treatment with Kp-10. Cells were plated and grown for 24 h in complete medium before being pretreated with LY294002 (50 μ M) or wortmannin (10 μ M) in DMEM-BSA for 1 h. Kp-10 (100 nM) was then added for 48 h. Representative fields observed by phase contrast are shown. The results are representative of two independent experiments. B, Kp-10 does not reduce the high constitutive phosphorylation of Akt in Jurkat-GPR54. Cells were treated for the indicated time with LY294002 (50 μ M), wortmannin (100 nM), Kp-10 (100 nM), or vehicle. Whole-cell lysates were analyzed by Western blotting for phospho-Akt Ser473 or phospho-ERK1/2. The results are representative of two independent experiments. C, quantitative analysis of experiment in Fig. 5B.

death receptor ligands, TNF- α , TRAIL, and FasL, in Kp-10-induced apoptosis. Exposure of Jurkat-GPR54 cells to Kp-10 revealed a rapid and abundant secretion of TNF- α into culture supernatants (Fig. 7A). In contrast, exposure of Jurkat-GPR54 cells to SDF-1 and parental Jurkat cells to Kp-10 had no effect on TNF- α secretion (Fig. 7A). The amount of TNF- α secreted by 293-GPR54 cells exposed to Kp-10 was 12 pg/ml, which is at the lower limit of detection of the assay. Inhibition of the ERK pathway by U0126 significantly reduced the secretion of TNF- α from 77 to 89% in three independent experiments (Fig. 7B). Inhibition of p38 and JNK had more limited effects (Fig. 7B). Because the activation of TNF- α receptors can induce either proapoptotic or prosurvival signals, the role of TNF- α secretion in apoptosis in this system was determined with neutralizing antibodies. Neutralization of TNF- α had no effect on the induction of apoptosis by Kp-10 (Supplemental Fig. 3). Likewise, neutralization of TRAIL did not inhibit the effect of Kp-10 on cell viability in Jurkat-GPR54 and 293-GPR54 cells (data not shown). In contrast, neutralization of FasL inhibited apoptosis in Jurkat-GPR54 cells exposed to Kp-10 in a dose-dependent fashion (Fig. 7C). The maximum efficacy was similar to that obtained by inhibition of MEK. Neutralization of FasL also decreased the cleavage of PARP and caspases in Jurkat-GPR54 (Fig. 7, D and E) but did not reduce apoptosis in 293-GPR54 cells (data not shown). Because the induction of apoptosis is most effective with membrane-bound FasL, expression of FasL on the surface of Jurkat-GPR54 cells was deter-

mined after stimulation with Kp-10. FasL was detected at 2 h after the addition of Kp-10, was maximum at 4 h, and started to decrease at 6 h (Fig. 7F), at which time apoptosis could be detected, as shown in Fig. 4C. Pretreatment with U0126 inhibited the up-regulation of FasL (Fig. 7F), indicating that ERK regulates GPR54-induced apoptosis in Jurkat cells by modulating the expression of FasL.

Discussion

Among the approximately 23 genes classified so far as metastasis suppressors (Stafford et al., 2008), KiSS1 is the only ligand of a GPCR. Although several effects of the expression of KiSS1 and the activation of GPR54 by Kps have been described *in vitro*, the mechanism(s) for the antimetastatic action of KiSS1 and the exact nature of the cells expressing the ligand and its receptor are still largely unknown. We provide here evidence that Kp signaling through GPR54 induces functional negative cross-talk with mechanisms involved in metastatic cell homing and survival and can induce the secretion of soluble factors capable of altering the support to tumor cells from the microenvironment.

Whereas multiple GPCRs contribute to tumor growth, angiogenesis, and metastasis (Dorsam and Gutkind, 2007), Kps and GPR54 exert opposing functions that suppress migration and survival. We previously reported the cross-talk between GPR54 and the chemokine receptor CXCR4 evidenced by the suppression of migration and Akt phosphorylation induced by SDF-1

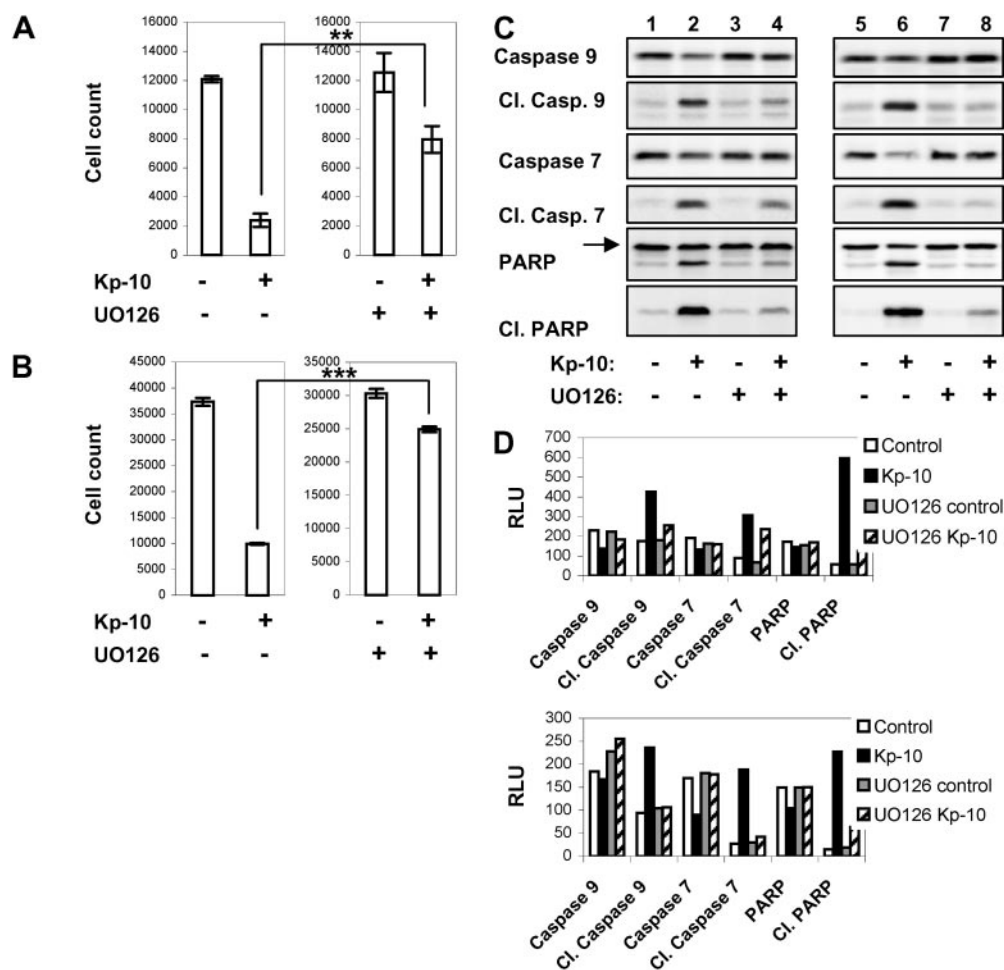


Fig. 6. Inhibition of the MEK-ERK pathway inhibits Kp-10-induced apoptosis in 293 and Jurkat cells. **A**, inhibition of MEK inhibits apoptosis by Kp-10 in 293-GPR54. 293-GPR54 cells were pretreated with the MEK inhibitor U0126 (10 μ M) for 1 h before the addition of Kp-10 (100 nM) for 72 h and counting by flow cytometry. Results represent the average \pm S.D. of duplicate samples for each condition in one experiment representative of four independent experiments. **, $p < 0.01$. **B**, inhibition of MEK significantly reduced the induction of apoptosis of Jurkat-GPR54 cells by Kp-10. Cells were pretreated with U0126 (10 μ M) before the addition of Kp-10 (100 nM) for 24 h. Live cells were counted by flow cytometry. Graphs represent the results (average \pm S.D.) of triplicate samples of one experiment representative of three independent experiments. ***, $p < 0.01$. **C**, 293-GPR54 cells (samples 1–4) were pretreated with U0126 (10 μ M) for 1 h before being exposed to 100 nM Kp-10 for 48 h. Whole-cell lysates were analyzed by Western blotting for cleavage of caspases and PARP (arrow, uncleaved PARP). Jurkat-GPR54 cells (samples 5–8) were pretreated with U0126 for 1 h before the addition of Kp-10 for 24 h. Whole cell SDS lysates were analyzed by Western blotting. The results are representative of two independent experiments. **D**, quantitative analysis of experiment in Fig. 6C for 293-GPR54 cells (top) and Jurkat-GPR54 cells (bottom).

(Navenot et al., 2005). Here, we demonstrate that GPR54 efficiently suppressed the activation of Akt by RTKs, including EGFR involved in the proliferation of multiple tumors types. This raised the possibility that Kps could exert a broad-spectrum negative regulation of this pathway. Other G_q -coupled GPCRs, like the angiotensin II receptor, have been shown to partially reproduce that effect (Folli et al., 1997), but this effect is not universal for G_q -coupled receptors.

Previous studies reported that cell lines transfected with

GPR54 and exposed to Kps exhibited reduced cell growth (Hori et al., 2001; Kotani et al., 2001; Becker et al., 2005) but not apoptosis (Kotani et al., 2001). Exposure of mammary carcinoma cells MDA-MB-435S programmed to express GPR54 to Kp-10 resulted in nuclear condensation consistent with apoptosis (Becker et al., 2005). We show here that Kp-10 induced proapoptotic signals and cell death in two common cell lines programmed to express GPR54 but not in the parental cells. Although it is unclear what expression level of

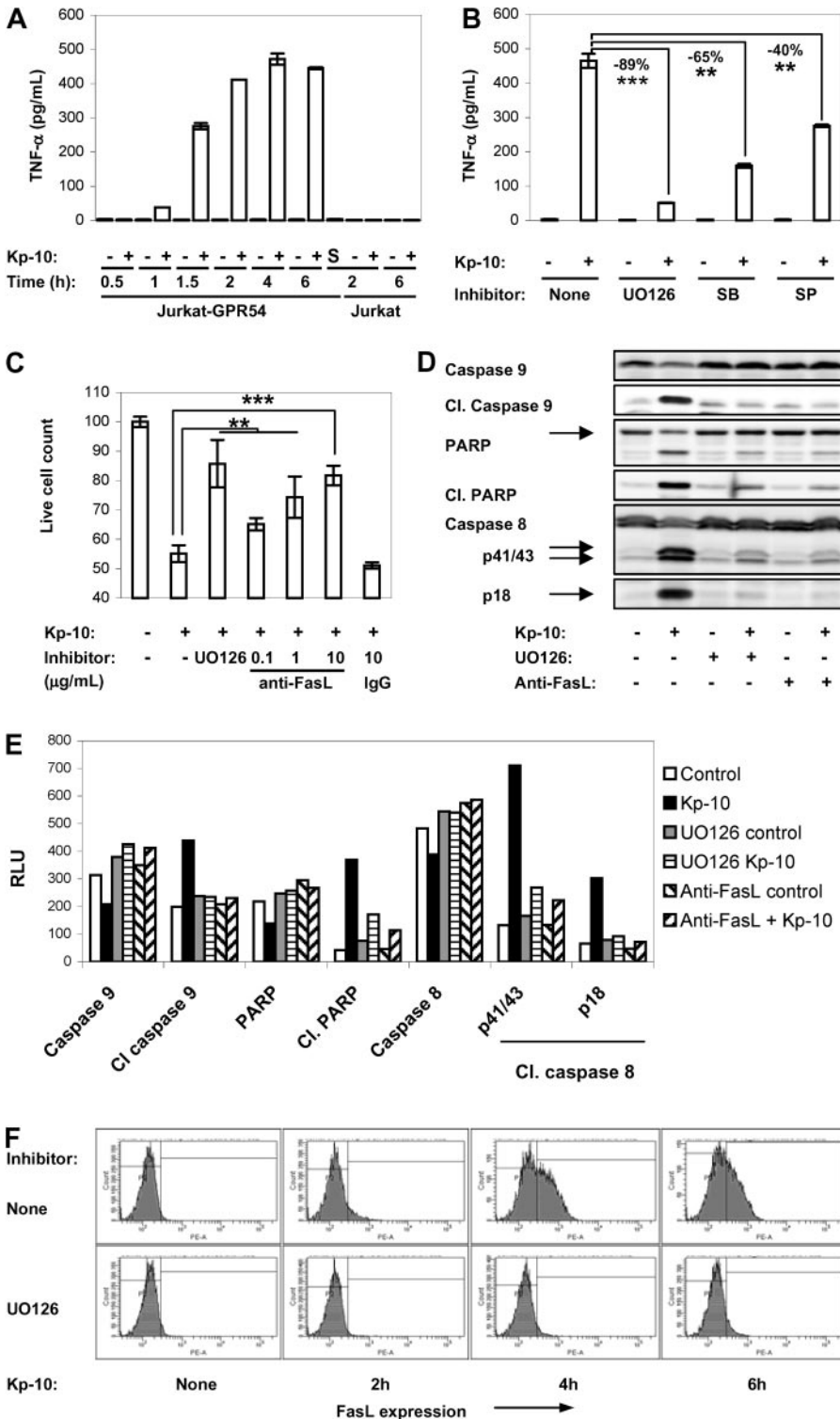


Fig. 7. Activation of GPR54 promotes ERK-dependent expression of TNF- α and FasL and FasL-mediated apoptosis in Jurkat-GPR54 cells. A, Kp-10 induces expression of TNF- α by Jurkat-GPR54. Cells were exposed to 100 nM Kp-10 or SDF-1 (S) for the indicated times (from 0.5 to 6 h), and then supernatant was collected and analyzed for TNF- α content by ELISA. Data are representative of three identical experiments in which each histogram represents duplicate samples. B, inhibition of TNF- α secretion by inhibitors of MEK (UO126), p38 (SB), and JNK. Cells were pretreated with each inhibitor (10 μ M) for 1 h before being exposed to 100 nM Kp-10 for 4 h. Data are representative of three experiments performed with duplicate samples for each condition. **, $p < 0.01$; ***, $p < 0.001$. C and D, neutralization of FasL inhibits apoptosis by Kp-10 in Jurkat-GPR54 similarly to MEK inhibition. A neutralizing mouse monoclonal antibody was added to the culture medium at the indicated concentration before the addition of Kp-10 (100 nM). After 16 h of incubation, live cells were counted by flow cytometry (C). Data representative of five experiments performed with duplicate samples for each condition. For assessment of caspase activation (D), cells were treated with UO126 (10 μ M) or a neutralizing antibody to FasL (10 μ g/ml) before the addition of Kp-10 (100 nM) for 16 h. Samples were analyzed by Western blotting with the indicated antibodies. The results are representative of two independent experiments. E, quantitative analysis of experiment in D. F, membrane expression of FasL is promoted by Kp-10 and prevented by ERK inhibition. Jurkat-GPR54, with (bottom) or without (top) pretreatment with MEK inhibitor UO126, were stimulated with 100 nM Kp-10 for the indicated time before being stained with an antibody specific for FasL as described in *Materials and Methods*. Cell fluorescence representative of FasL expression was analyzed by flow cytometry. The results are representative of two independent experiments.

GPR54 is necessary, apoptosis was triggered by concentrations of Kp-10 in the low nanomolar range, which corresponds to the EC₅₀ value for ligand binding. In addition, the conditioned medium of tumor cells programmed to secrete the 54-residue Kp designated metastatin (Nash et al., 2007) was capable of inducing apoptosis with an efficiency similar to that of Kp-10 (data not shown).

The inhibition of Akt did not seem to play a major role in GPR54-mediated apoptosis despite the prosurvival function of this kinase. Because it is central to multiple essential signaling pathways, negative regulation of Akt could position Kp-10 as an agent to sensitize tumor cells or stromal cells in the microenvironment to chemotherapeutic agents. The major role the ERK1/2 pathway played in Kp-10-mediated apoptosis seems surprising in regard to the largely recognized role of this pathway in cancer cell survival and proliferation (Roberts and Der, 2007; Sebolt-Leopold, 2008) and contradicts the finding that up-regulation of a subset of genes involved in apoptosis induced by GPR54 activation was suppressed by inhibition of PKC and PLC but not ERK (Becker et al., 2005). In Jurkat cells, ERK activation was necessary for the expression of FasL and TNF- α . Several studies have shown the role of ERK in TNF- α up-regulation (Dumitru et al., 2000; Häcker and Karin, 2006; Skinner et al., 2008). Based on kinetics and neutralization data, we can conclude that the activation of ERK by GPR54 led to the expression of FasL which, in turn, bound Fas on the cell surface to assemble a death-inducing signaling complex and activate caspase 8. This mechanism is reminiscent of the one underlying activation-induced cell death of T cells (van den Brink et al., 1999; Zhu et al., 1999), in which activation of ERK controls the expression of FasL. To our knowledge, this is the first description of such a mechanism triggered by a GPCR. The effector(s) of ERK signaling in Kp-10-mediated apoptosis of 293-GPR54 cells was not identified. It can be postulated that either the activation of ERK occurs in conjunction with other critical signaling events or GPR54 activates ERK in a specific fashion. For instance, it may induce the assembly of a specific signaling complex not reproduced by other receptors, such as the complexes constituted after recruitment of β -arrestins (Luttrell et al., 2001; Lefkowitz and Whalen, 2004; Kolch, 2005).

KiSS1, being a metastasis suppressor and not a tumor suppressor, may not induce apoptotic death of tumor cells at the primary site. Rather, secreted Kps could target subpopulations of GPR54-expressing metastatic cancer cells in an autocrine fashion by inhibiting their motility and/or compromising their survival by activating proapoptotic signals and blocking prosurvival signals from RTKs. In addition, induction of FasL expression by Fas-negative tumor cells was reported to promote tumor rejection in vivo in mice, induce inflammation, recruit neutrophils, and favor tumor-specific immunity (Seino et al., 1997; Shimizu et al., 1999, 2005). Besides this potential autocrine model, the observation that the activation of GPR54 could promote the expression of ligands like TNF- α and FasL also suggests the possibility of a paracrine mode of action for Kp-10 in which Kps secreted by tumor cells would activate GPR54 expressed by cells present in the microenvironment and stimulate them to express membrane-bound or soluble proinflammatory mediators. These soluble factors could then in turn target the metastatic tumor cells either directly or indirectly by altering compo-

nents of their microenvironment (such as carcinoma-associated fibroblasts) and depriving them from a nurturing support. The combined effects of kisspeptins on tumor cells and stromal cells (disruption of cell recruitment, survival, response to local growth factors) could ultimately prevent the formation of a functional supportive niche for metastatic cells. Several lines of evidence provide direct support for that model. First, we have demonstrated that endogenously produced and secreted Kps exert a potent antimetastatic effect on the human melanoma cell line C8161.9 despite the absence of GPR54 expression or response to Kp-10 in vitro (Nash et al., 2007). Likewise, it was shown that the human breast cancer cell line MDA-MB-435, which also lacks GPR54 expression, lost its metastatic potential when programmed to express KiSS1 (Lee and Welch, 1997b). However, an in vitro response to Kp-10 was achieved only after GPR54 transfection (Becker et al., 2005). The nature of the GPR54-positive cells present in the microenvironment in that model remains to be determined. Whether the mechanism of action of Kps is predominantly autocrine or paracrine is likely to be tumor-specific. The data presented here provides new mechanistic insights into the remarkable efficiency of KiSS1 by demonstrating effects that are most relevant to isolated metastatic cells (micrometastasis) and could contribute to the phenomenon of dormancy.

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