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KiSS1 suppresses TNF α -induced breast cancer cell invasion via an inhibition of RhoA-mediated NF- κ B activation

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Abstract

Tumor necrosis factor- α (TNF α) induces cancer development and metastasis, which is prominently achieved by nuclear factor- κ B (NF- κ B) activation. TNF α -induced NF- κ B activation enhances cellular mechanisms including proliferation, migration, and invasion. KiSS1, a key regulator of puberty, was initially discovered as a tumor metastasis suppressor. The expression of KiSS1 was lost or down-regulated in different metastatic tumors. However, it is unclear whether KiSS1 regulates TNF α -induced NF- κ B activation and further tumor cell migration. In this study, we demonstrate that KiSS1 suppresses the migration of breast cancer cells by inhibiting TNF α -induced NF- κ B pathway and RhoA activation. Both KiSS1 overexpression and KP10 (kisspeptin-10) stimulation inhibited TNF α -induced NF- κ B activity, suppressed TNF α -induced cell migration and cell attachment to fibronectin in breast cancer cells while KP10 has little effect on cancer cell proliferation. Furthermore, KP10 inhibited TNF α -induced cell migration and RhoA GTPase activation. Therefore, our data demonstrate that KiSS1 inhibits TNF α -induced NF- κ B activation via downregulation of RhoA activation and suppression of breast cancer cell migration and invasion.

Keywords

KiSS1; Gpr54; TNF α ; RhoA; NF- κ B

Introduction

Increasing evidence indicates that TNF α is important for cancer malignancy [Balkwill, 2006; Hacker and Karin, 2006; Karin, 2006]. The TNF α signaling cascade includes TNF α receptors, which promotes a recruitment of multiple adaptor proteins such as TRADD and TRAF [Balkwill, 2006; Hacker and Karin, 2006; Karin, 2006]. Chronically produced TNF α induces tumor cell motility and invasion via the activation of NF- κ B and its target genes, resulting in cancer progression and metastasis [Balkwill, 2006; Hacker and Karin, 2006; Karin, 2006]. Abnormally and constitutively active NF- κ B is implicated in cancer progression and metastasis [Balkwill, 2006; Hacker and Karin, 2006; Karin, 2006]. NF- κ B

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consists of five subunits named RelA (p65), RelB, cRel (Rel), p50 (NF- κ B1), and p52 (NF- κ B2) that form homo- or heterodimers and tightly bind to their inhibitors, I κ Bs at the cytoplasm in the resting state [Balkwill, 2006; Hacker and Karin, 2006; Karin, 2006]. Upon diverse stimulation, IKKs (I κ B kinases) phosphorylates I κ Bs, inducing the 26S proteasomal degradation of I κ Bs and liberating NF- κ B to translocate into the nucleus and function as a transcriptional factor [Balkwill, 2006; Hacker and Karin, 2006; Karin, 2006]. Given the importance of TNF α induced NF- κ B pathway in cancer development and metastasis, discovery of agents (peptides or chemical compounds) against TNF α -induced NF- κ B pathway is crucial for cancer treatment [Balkwill, 2006; Hacker and Karin, 2006; Karin, 2006].

KiSS1, initially identified as an anti-metastatic gene, produces truncated forms of peptides named kisspeptins (KP), including KP10, KP13, KP14, and KP54 [Fernandez-Fernandez et al., 2006; Lee et al., 1996; Lee and Welch, 1997; Mead et al., 2007b; Nash et al., 2007; Nash and Welch, 2006; Ohtaki et al., 2001; Popa et al., 2008; Stafford et al., 2002]. Recent studies have provided various functions of KiSS1 in normal and disease conditions [Fernandez-Fernandez et al., 2006; Mead et al., 2007b; Nash and Welch, 2006; Popa et al., 2008]. In normal physiology, KiSS1 is implicated in the regulation of puberty, reproduction, and homeostasis [Fernandez-Fernandez et al., 2006; Mead et al., 2007b; Popa et al., 2008]. In tumorigenesis, the function of KiSS1 as one of metastasis suppressor genes was supported by both experimental and clinical data [Mead et al., 2007b; Nash and Welch, 2006]. Furthermore, KiSS1 gene products target atherosclerotic vessels, indicating its function in cardiovascular system [Mead et al., 2007a; Mead et al., 2007b]. However, a mechanism by which KiSS1 regulates cancer progression and metastasis remains yet to be resolved although the role of KiSS1 in cancer biology was first revealed and confirmed [Fernandez-Fernandez et al., 2006; Lee et al., 1996; Lee and Welch, 1997; Mead et al., 2007b; Nash et al., 2007; Nash and Welch, 2006; Ohtaki et al., 2001; Popa et al., 2008; Stafford et al., 2002]. Kisspeptins transiently stimulate the formation of focal adhesion complex and stress fiber in CHO cells overexpressing GPR54, the endogenous KiSS1 receptor [Ohtaki et al., 2001]. KiSS1 overexpression also suppresses migration and invasion in MDA-MB-435 breast cancer cells [Lee and Welch, 1997] and inhibits MMP-9 expression via downregulating NF- κ B activation in HT-1080 fibrosarcoma cells [Yan et al., 2001]. Inversely, MMPs suppress the function of kisspeptins by cutting kisspeptin's C-terminus [Takino et al., 2003].

In different cancer cells, active RhoA promotes cell migration, invasion, and metastasis [Brantley-Sieders et al., 2008; Fromigue et al., 2008; Gadea et al., 2007; Gumireddy et al., 2007; Joshi et al., 2008; Lee et al., 2008; Loberg et al., 2007; Martin et al., 2007; Sun et al., 2007; Zhu et al., 2007]. Furthermore, RhoA induces NF- κ B activation, which is required for tumor progression [Benitah et al., 2003; Debidda et al., 2005; Perona et al., 1997]. However, it is still unclear whether KiSS1 regulates cell migration and invasion by regulating RhoA activation and its downstream signaling pathways. In this study, we demonstrate that KiSS1 suppresses breast cancer cell migration and invasion by suppressing RhoA-mediated TNF α -induced NF- κ B activation.

Materials and methods

Cell culture and reagents

MDA-MB-231, MCF-7, and 293T cells were cultured in DMEM supplemented with 10% FBS and 1% antibiotics. C-terminal amidated Kisspeptin-10 (KP10) was prepared in distilled water with 0.005% DMSO, stored at -20°C and then diluted as needed in the cell culture medium. TNF α (kindly provided by Dr. Aggarwal) was stocked in distilled water with 0.01% DMSO.

Cell proliferation assay

Cell proliferation was monitored using CellTiter 96® Aqueous Non-Radioactive Cell Proliferation Assay kit according to the manufacturer's protocol (Promega, Madison, WI).

Flow cytometry analysis

3×10^6 cells were fixed with 70% ethanol, stained with propidium iodide (PI), and then subjected to flow cytometry analysis. 5000 cells were gated and PI-stained cells were counted. Experiment was performed in triplicate.

Electrophoretic mobility shift assay

Nuclear extract sampled from MDA-MB-231 cells were incubated with ^{32}P -end-labeled 45mer double-stranded oligonucleotide (10 μg of protein with 5fmol of DNA), 5'-TTGTTACAAGGGACTTTCCGCTGGGGACTTTCCAGGGAGGCGTGC-3' (the bold indicates NF- κB binding sites), for 10 min at RT. The DNA-protein complex formed was separated from free oligonucleotide on 6% native polyacrylamide gels.

Chromatin immunoprecipitation (ChIP) assay

MDA-MB-231 cells were cross-linked with formaldehyde and then nuclear extracts were sonicated. After washed three times, the chromatin was immunoprecipitated with 5 μg of NF- κB p65 antibody and protein A/G beads and then heated to reverse the cross-linking. DNA was purified and analyzed by PCR using the primers specific for the NF- κB binding site of MMP-9 promoter. Primers used were as following: 5'-GACCAAGGGATGGGGATC-3' and 5'-CTTGACAGGCAAGTGCTGAC-3'. DNA purified from the sonicated nuclear fraction was directly analyzed by general PCR using the same primers and then used for the input control.

Western blot

30 μg of total protein was resolved on SDS-PAGE, transferred to PVDF membranes and probed with specific antibodies. The blots were washed, exposed to HRP-conjugated secondary antibodies (Pierce, Rockford, IL), and detected by chemiluminescence reagents. Antibodies against pIKK α/β , IKK α , NF- κB p65, and I $\kappa\text{B}\alpha$ were kindly provided by Dr. Aggarwal. Antibody against RhoA, Rac1, or Cdc42 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Rho GTPases activity assay

For detecting active RhoA, GST pull-down was performed. In brief, Rhotekin-RBD (Rho binding domain) purified from *E. Coli* was incubated with 20 μl of 50% Glutathione-bead slurry and 500 μg of whole cell lysates for 12hr at 4°C. Active RhoA binding to RhoA-RBD was detected using RhoA antibody. For detecting active Rac1 or Cdc42, protein samples mixed with GST-PAK PBD (Pak binding domain) were pulled-down.

Luciferase assay

Cells were transiently transfected with pNF- κB -luc (Stratagene, La Jolla, CA) and/or plasmids shown in figures. 5hr after transfection, fresh medium was added; cells were treated with KP10 for another 20hr, and then harvested for luciferase assay. To obtain the basal level of NF- κB activity, cells were incubated with medium containing 0.2% serum for 20hr, and then assay. Data was normalized using β -gal assays.

Chemotactic cell migration assay

Cells were cultured in the upper chamber of the Boyden Chamber precoated with gelatin, and the bottom chamber precoated with gelatin was filled with medium containing TNF α to induce chemotactic migration. To examine KiSS1 effect, different concentrations of KP10 were added in the upper chamber. 24hr after cell culture, cells attached on the bottom were stained with crystal violet (2% v/v) and counted. To verify cell counting, crystal violet was eluted and read using a microplate reader at 490nm. To visualize and confirm cell migration, scratching assay was performed. Cells were cultured on 6 well plates and scratched using a 1000 μ l tip, washed three times with PBS, and cultured for 24hr after treatment with KP10 and/or TNF α . Images were obtained using a Nikon invert microscope. Experiments were performed four times and data was analyzed by the two-tailed student t-test.

Cell attachment assay

Cells were trypsinized with 0.1% Trypsin/EDTA, washed with PBS, incubated in medium containing 0.01% DMSO or KP10 for 30 min. at 37°C, and then poured into fibronectin-precoated wells. After 10 min, cells were washed with PBS and stained with 0.2% crystal violet. Cells that were attached were eluted with 1% SDS and then an absorbance of elutes was counted using a spectrometer plate reader at 490nm.

Immunofluorescence

Cells were cultured on coverslips precoated with poly-L-lysine and then treated with the molecules indicated in figures. Cells were fixed with 3.7% formaldehyde for 15min and then permeabilized with 0.1% Triton X-100 for 10min, and blocked with 1% bovine serum albumin (BSA) for 30 min. For p65 intracellular localization, cells were incubated with anti-p65 antibody for 1hr and then incubated with Texas red-conjugated secondary rabbit antibody for 30 min. Images were acquired using Nikon microscope.

Results

KiSS1 suppresses TNF α -induced tumor cell migration, invasion, and matrix-attachment

Since TNF α is highly expressed in the tumor microenvironment and induces cancer malignancy [Balkwill, 2006; Hacker and Karin, 2006; Karin, 2006], we investigate the roles of KiSS1 on breast cancer cell behavior induced by TNF α treatment. To determine how KiSS1 affects TNF α -activated tumor cell migration and invasion, we performed wound healing assays. As shown in Figure 1A, TNF α increased the migration of MDA-MB-231 cells by ~61% compared with none-treated cells (Figure 1A). Addition of KP10 at 1 μ M significantly inhibited TNF α -induced cell migration in MDA-MB-231 cells by 40% (Figure 1A). Similar data were obtained for the inhibition of TNF α -induced invasion in both MCF7 and MDA-MB-231 cells by KP10 using Boyden chamber assays. TNF α increased an invasiveness of both MCF7 and MDA-MB-231 cells by ~40 % (Figure 1B). However, 1 μ M of KP10 blocked TNF α -induced cell invasion (Figure 1B).

To examine whether KiSS1 affects TNF α -induced cell attachment to extracellular matrices, MDA-MB-231 cells were plated on fibronectin-coated culture dishes and then TNF α -induced cell attachment to the matrix was measured by spectrometer at 490nm. As shown in Figure 1C, incubation of 1 μ M of KP10 with breast cancer cells for 30 min significantly inhibited TNF α induced MDA-MB-231 cell attachment to fibronectin (Figure 1C). Together, our data indicate that KiSS1 inhibited TNF α -mediated breast cancer cell migration and matrix-attachment, suggesting a possible inhibitory function of KiSS1 against TNF α -enriched tumor microenvironment.

Since TNF α -induced NF- κ B pathway regulates tumor cell proliferation and survival [Balkwill, 2006; Hacker and Karin, 2006; Karin, 2006], we further examined how KiSS1 regulated tumor cell proliferation in presence of TNF α . KP10 did not affect MDA-MB-231 (Figure 1D) or MCF7 (data not shown) cell proliferation in the presence or absence of TNF α , when we performed the cell proliferation assays. On the other hand, KiSS1 has been revealed to arrest MDA-MB-436S cells at G2/M phase [Becker et al., 2005]. However, KP10 did not affect cell cycle in the presence or absence of TNF α , when MDA-MB-231 and MCF7 cells were subjected to the flow cytometry (Figure 1E). In sum, KiSS1 affects TNF α -mediated cell motility but not proliferation.

KiSS1 blocks NF- κ B transcriptional activity by inhibiting DNA binding of NF- κ B

To determine whether KiSS1 inhibits TNF α -induced NF- κ B activation in breast cancer cells, we first examined the transcriptional activation of NF- κ B in MDA-MB-231 cells either overexpressed with KiSS1 or treated with KP10. When MDA-MB-231 cells were treated with different concentration of TNF α (0.1 to 10nM), NF- κ B transcriptional activity was increased with the increase of TNF α in a dose-dependent manner (Figure 2A). Overexpression of KiSS1 gene in the cell inhibited TNF α -induced NF- κ B activation even at 10nM concentration of TNF α (Figure 2A), suggesting a role of KiSS1 in TNF α -mediated NF- κ B activation. Furthermore, we examine whether KP10, a 10-amino acid peptide derived from KiSS1, can inhibit TNF α -induced NF- κ B transcriptional activation. As shown in figure 2B, KP10 at 1 to 10 μ M also decreased TNF α -induced NF- κ B activation in MDA-MB-231 cells stimulated with TNF α . However, the inhibitory effect of KP10 on TNF α -induced NF- κ B activation was weaker than that induced by KiSS1 overexpression (Figure 2B).

To confirm our observation that KiSS1 and KP10 inhibit TNF α -induced NF- κ B activation, we examined the DNA binding activity of NF- κ B using electrophoresis gel mobility-shift assays (EMSA). As shown in figure 2C, KP10 at 0.1 μ M significantly inhibited TNF α induced DNA binding of NF- κ B in MDA-MB-231 cells (Figure 2C). To further investigate the KiSS1-inhibitory function in TNF α -induced DNA binding of NF- κ B *in vivo*, we examined whether KiSS1 affects DNA binding of NF- κ B on MMP-9 promoter in TNF α -induced MDA-MB-231 cells using chromatin immunoprecipitation (ChIP) assays. DNAs from MDA-MB-231 cells treated with control buffer, TNF α alone, or TNF α plus KP10, or from cells overexpressing KiSS1 and treated with TNF α were extracted, precipitated with anti-NF- κ B p65 antibody, and then subjected to PCR using MMP-9 promoter specific promoters in the chromatin immunoprecipitation (ChIP) assays. As shown in figure 2D, KiSS1 gene overexpression or treatment of KP10 peptide strongly suppressed TNF α -induced DNA binding of NF- κ B on MMP-9 promoter (Fig. 2D, lane 3 and lane 4, respectively). Therefore, our data consistently demonstrate that KiSS1 inhibits TNF α -induced DNA binding of NF- κ B.

KiSS1 blocks TNF α -induced NF- κ B nuclear translocation and I κ B α degradation

To understand the mechanism of KiSS1 inhibition of NF- κ B activation, we examined whether KP10 inhibits TNF α -induced NF- κ B nuclear translocation, an upstream event of NF- κ B DNA binding [Balkwill, 2006; Hacker and Karin, 2006; Karin, 2006]. As shown in figure 3A, addition of TNF α induced the nuclear translocation of p65 NF- κ B subunit in nuclear extracts of MDA-MB-231 cells (Fig. 3A). However, treatment of the cells by KP10 at 1 μ M blocked the nuclear translocation of p65 NF- κ B subunit (Figure 3A).

To confirm the inhibition of NF- κ B nuclear translocation by KP10, we performed immunofluorescence assays using the anti-NF- κ B p65 antibody. In MDA-MB-231 cells, p65 NF- κ B subunit was located at the cytosol without stimulation (Fig. 3B, left top panel, none). 1 μ M KP10 alone did not alter the cytosol localization of p65 NF- κ B in the resting status

(Fig. 3B, left bottom panel, KP10). When the cells were stimulated with TNF α , p65 was activated and translocated into the nucleus (Fig. 3B, right top panel). Addition of 1 μ M KP10 significantly blocked TNF α -induced nuclear translocation of p65 NF- κ B (Fig. 3B, right bottom panel). Together, these data suggest that KP10 inhibit TNF α -induced NF- κ B activation by blocking the nuclear translocation of NF- κ B.

We next examined whether KP10 affects TNF α -induced I κ B α degradation, as I κ B α degradation liberates NF- κ B from I κ B α /NF- κ B complex, resulting in NF- κ B nuclear translocation [Balkwill, 2006; Hacker and Karin, 2006; Karin, 2006]. MDA-MB-231 cells were stimulated with TNF α in the presence or absence of KP10 for different time points, and then the cytosolic fraction was subjected for the analysis of I κ B α degradation. In MDA-MB-231 cells, TNF α induced I κ B α degradation at 5 min, which was completed at 15 min (Fig. 3C). Treatment of I κ B α degradation KP10 inhibited TNF α -induced I κ B α degradation when cytosolic I κ B α was probed for 60 min (Fig. 3C).

KiSS1 blocks NF- κ B transcriptional activity by inhibiting IKK phosphorylation

I κ B α degradation and NF- κ B nuclear translocation resulted from IKK phosphorylation of I κ B α , which is sequentially promoted by phosphorylation of IKKs [Balkwill, 2006; Hacker and Karin, 2006; Karin, 2006]. To further understand the mechanism of KiSS1 inhibition on TNF α -induced NF- κ B, we examined whether KP10 inhibits IKK phosphorylation. In MDA-MB-231 cells, TNF α induced phosphorylation of IKK α / β at 15 min, which was prolonged for 30 min (Fig. 4A). However, treatment of the cells by 1 μ M KP10 suppressed TNF α -induced IKK α / β phosphorylation (Fig. 4A), suggesting that KP10 inhibit TNF α -mediated NF- κ B activation by regulating IKK α / β phosphorylation.

In the TNF α -induced NF- κ B pathway, TNF α receptor signaling complex mediates phosphorylation of IKKs [Balkwill, 2006; Hacker and Karin, 2006; Karin, 2006]. To investigate KP10-target molecules upstream of IKKs in TNF α -induced NF- κ B pathway, MDA-MB-231 cells were transfected with NF- κ B reporter gene combined with TNFR1, TRADD, TRAF2, NIK, IKK α , and p65, respectively. The cells were then stimulated with KP10 for 24hr, and subjected to the luciferase assays. As shown in figure 4B, KP10 blocked NF- κ B transcriptional activation induced by molecules including TNFR1, TRADD, TRAF2, NIK, and IKK α , but not p65.

KiSS1 inhibition of RhoA activation results in the inhibition of TNF α -induced cell migration

RhoA drives cell motility in terms of dynamics of Rho GTPases and is known to regulate TNF α -induced NF- κ B activation [Debidda et al., 2005; Hodge et al., 2003; Perona et al., 1997; Urbinati et al., 2005]. To understand the mechanism of KiSS1 inhibition of TNF α -induced cell migration, we examined RhoA activation in MDA-MB-231 cells treated with TNF α alone or TNF α plus KP10. As shown in figure 5A, TNF α -induced RhoA activation was blocked by KP10 at 0.1 to 10 μ M (Fig. 5A). Thus, our data indicate that KP10 target RhoA in TNF α -induced NF- κ B activation.

Since RhoA regulates tumor cell migration [Brantley-Sieders et al., 2008; Fromiguet et al., 2008; Gadea et al., 2007; Gumireddy et al., 2007; Joshi et al., 2008; Lee et al., 2008; Loberg et al., 2007; Martin et al., 2007; Sun et al., 2007; Zhu et al., 2007], we next examined whether KP10 inhibits RhoA-dependent migration. MDA-MB-231 cells were transfected with dominant active RhoA (RhoA G14V) and then examined cell migration in the presence or absence of KP10. Compared with cells transfected with empty vector, cells overexpressing RhoA G14V showed increased cell migration (Fig. 5B). However, KP10 treatment inhibited RhoA-enhanced cell migration (Fig. 5B). To confirm RhoA-induced cell migration and KP10 inhibition of RhoA-mediated cell migration, MDA-MB-231 cells were

transfected with dominant negative RhoA (RhoA T19N), and then examined for cell migration in the presence or absence of TNF α . Cells overexpressing RhoA T19N migrated slightly slower than cells transfected with empty vector. However, TNF α -induced MDA-MB-231 cell migration was significantly inhibited by RhoA T19N (Fig. 5B, fourth and fifth bars). Thus, our data indicate that TNF α -induced cell migration requires RhoA activity and KP10 targets RhoA in cell migration.

KiSS1 inhibits TNF α -induced NF- κ B activation through RhoA GTPase

Next, we examined whether RhoA is KP10-target molecule in the KP10 inhibition of TNF α -induced NF- κ B activation. To examine RhoA requirement in TNF α -induced NF- κ B activation, MDA-MB-231 cells were transfected with wild type RhoA, stimulated with TNF α , and then subjected to the luciferase assays to monitor NF- κ B reporter gene activity. Wild type RhoA induced NF- κ B transcriptional activation, which was enhanced by TNF α (Figure 6A, left three bars). To confirm RhoA activation of NF- κ B transcriptional activity, we transfected the cells with active RhoA G14V or inactive RhoA T19N, and then performed the same luciferase assays. Consistent with our experiments, RhoA G14V induced NF- κ B transcriptional activity while RhoA T19N inhibited NF- κ B activity (Fig. 6A, fifth and eighth bars, respectively).

To further investigate the role of RhoA in TNF α -induced NF- κ B pathway, MDA-MB-231 cells were transfected with RhoA T19N combined with TNFR1, TRADD, TRAF2, and IKK α , respectively. RhoA T19N repressed NF- κ B transcriptional activity induced by TNFR1, TRADD, TRAF2 (Figure 6A, tenth to twelfth bars). However, RhoA T19N partially repressed IKK α -induced NF- κ B transcriptional activation (Figure 6A, ninth bar), suggesting RhoA is upstream of IKKs in TNF α -induced NF- κ B activation.

We next analyzed whether KiSS1 inhibits RhoA-mediated NF- κ B activation upon TNF α stimulation. Compared with NF- κ B transcriptional activity in cells transfected with RhoA wild type and stimulated with TNF α , the activity in cells co-transfected with KiSS1 and RhoA wild type, and stimulated with TNF α , was significantly reduced (Figure 6A, third and fourth bars). In addition, NF- κ B transcriptional activity was reduced in cells co-transfected with KiSS1 and RhoA G14V when compared to that in cells transfected with RhoA G14V alone (Figure 6A, fifth and sixth bars), suggesting KiSS1 inhibit RhoA-mediated NF- κ B activation.

To confirm KiSS1 inhibition of RhoA-mediated NF- κ B activation, we performed EMSA to examine DNA binding of NF- κ B in MDA-MB-231 cells overexpressing RhoA G14V in the presence of different concentrations of KP10. RhoA G14V induced DNA binding of NF- κ B, which was significantly inhibited by KP10 at 1 μ M (Figure 6B). Furthermore, we performed ChIP assays to confirm KiSS1 inhibition of RhoA-mediated DNA binding of NF- κ B *in vivo*. In MDA-MB-231 cells overexpressing RhoA G14V, DNA binding of NF- κ B on MMP-9 promoter was enhanced. However, KP10 inhibited the active RhoA-induced DNA binding of NF- κ B on MMP-9 promoter in a dose-dependent manner (Figure 6C). Together, our data demonstrate that KiSS1 suppresses breast cancer cell migration and invasion by targeting RhoA and inhibiting TNF α -induced NF- κ B transcriptional activation.

Discussion

TNF α -induced NF- κ B pathway is one of the well known molecular targets for cancer treatment [Balkwill, 2006; Hacker and Karin, 2006; Karin, 2006; Naugler and Karin, 2008]. In tumor microenvironment, the active NF- κ B in immune cells highly produces TNF α , resulting in the NF- κ B-dependent expression of MMPs in tumor cells [Deryugina and Quigley, 2006; Nyberg et al., 2008]. In this study, we demonstrate that KiSS1 inhibits breast

cancer cell migration by suppressing RhoA activation and TNF α -induced NF- κ B activation (Fig. 7).

We found that KiSS1 inhibits TNF α -induced breast cancer cell migration. Recently, two groups found that KiSS1 is highly expressed in breast tumors compared with normal breast tissues [Marot et al., 2007; Martin et al., 2005], although a loss of KiSS1 expression in invasive breast cancer cell lines was proven [Lee et al., 1996; Lee and Welch, 1997; Mitchell et al., 2006]. Thus, it is possible that KiSS1 has an autocrine role in non-invasive breast tumor cells and a paracrine path of kisspeptins blocks a motility of invasive breast cancer cells in TNF α -enriched tumor environment, as our data show that KiSS1 targets both MCF7 and MDA-MB-231 breast cancer cells. However, we cannot exclude another paracrine path of kisspeptins in tumor environment as suggested by Nash et al. in tumor microenvironment [Nash et al., 2007]. Our lab previously reported that KiSS1 inhibits the proliferation and migration of NIH3T3 fibroblast overexpressing GPR54 [Stafford et al., 2002]. In addition, Mead et al. found that KP10 targets atherosclerotic vessels [Mead et al., 2007a]. Therefore, the exact role of KiSS1 in tumor microenvironment needs to be elucidated *in vivo*.

RhoA activity is implicated in cancer cell migration, invasion, and cytoskeletal re-organization [Brantley-Sieders et al., 2008; Fromigue et al., 2008; Gadea et al., 2007; Gumireddy et al., 2007; Joshi et al., 2008; Lee et al., 2008; Loberg et al., 2007; Martin et al., 2007; Sun et al., 2007; Zhu et al., 2007]. In TNF α -stimulated cells, RhoA activation mediates NF- κ B activation and further induces cell migration [Debidia et al., 2005; Hodge et al., 2003; Perona et al., 1997; Urbinati et al., 2005]. Our study demonstrates KiSS1 inhibited TNF α -induced RhoA activation and further RhoA-mediated tumor cell migration. Moreover, we also found that KiSS1 blocked RhoA-mediated NF- κ B activation. However, KiSS1 alone induced stress fiber formation, which was mediated by RhoA in ACHN and Caki-1 renal carcinoma cell lines [Shoji et al., 2008]. Considering studies by Shoji et al. and us, KiSS1-targeted RhoA appears to determine cell fate [Gumireddy et al., 2007; Shoji et al., 2008]. Thus, KiSS1-mediated signaling may be pleiotropic in different biological conditions. This is consistent with recent studies that KP10 inhibits CXCL12/CXCR4-induced calcium mobilization [Navenot et al., 2005] but KP10 alone increases intracellular calcium level [Bilban et al., 2004; Brailoiu et al., 2005]. Therefore, it will be interesting to understand a functional relationship between KiSS1-induced GPR54 signaling and the TNF α -TNFR-mediated proinflammatory cytokine-mediated signaling in our future research.

Direct GPCR regulation of NF- κ B pathway is also crucial for normal and disease conditions [Ye, 2001]. Intracellular signaling mediators such as PKCs and CARMAs are important for GPCR activation of NF- κ B pathway [Wegener and Krappmann, 2007]. KiSS1-induced GPR54 activates G α q/11 and PLC β , and affects phosphorylation of PKCs and the intracellular calcium level [Jiang et al., 2005; Nash et al., 2007; Niida et al., 2006; Stafford et al., 2002]. In HT-1080 cells, KiSS1 overexpression induced I κ B α expression, resulting in the inhibition of NF- κ B activation [Yan et al., 2001]. Likewise, KP10 stimulation also induced NF- κ B-dependent I κ B α expression in MDA-MB-436S cells in the DNA microarray [Becker et al., 2005]. In ovarian cancer cells, KiSS1 increased intracellular Ca²⁺ level, induced NFAT-dependent expression of MCIP1 (myocyte-enriched calcineurin interacting protein 1; an inhibitor of calcineurin), and then inhibited NFAT nuclear translocation [Stathatos et al., 2005]. MCIP1-dependent negative feedback mechanism in the intracellular Ca²⁺-mediated signaling pathway is implicated in cancer biology and vascular biology [Arron et al., 2006; Rothermel et al., 2003; Ryeom et al., 2008; Stathatos et al., 2005; Vega et al., 2003]. However, KiSS1 inhibited PKC α , the upstream molecule of NFAT, in ovarian cancer cells [Jiang et al., 2005]. Thus, together with our study, KiSS1-induced GPR54 signaling seems to gain complexity under different biological conditions.

In conclusion, our study demonstrates that KiSS1 suppresses the activation of TNF α -RhoA-NF- κ B signaling, resulting in the suppression of NF- κ B-mediated gene transcription, cell migration and invasion (Fig. 7). Therefore, a loss of KiSS1 expression in invasive breast cancer cells fails to suppress TNF α -RhoA-NF- κ B activation, resulting in an increase of tumor cell invasiveness [Mead et al., 2007b; Nash and Welch, 2006]. However, both direct molecular targets of KiSS1 in TNF α -stimulated tumor cells and KiSS1-target cell types in tumor microenvironment remain to be determined.

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References

- Arron JR, Winslow MM, Polleri A, Chang CP, Wu H, Gao X, Neilson JR, Chen L, Heit JJ, Kim SK, Yamasaki N, Miyakawa T, Francke U, Graef IA, Crabtree GR. NFAT dysregulation by increased dosage of DSCR1 and DYRK1A on chromosome 21. *Nature*. 2006; 441:595–600. [PubMed: 16554754]
- Balkwill F. TNF-alpha in promotion and progression of cancer. *Cancer Metastasis Rev*. 2006; 25:409–16. [PubMed: 16951987]
- Becker JA, Mirjolet JF, Bernard J, Burgeon E, Simons MJ, Vassart G, Parmentier M, Libert F. Activation of GPR54 promotes cell cycle arrest and apoptosis of human tumor cells through a specific transcriptional program not shared by other Gq-coupled receptors. *Biochem Biophys Res Commun*. 2005; 326:677–86. [PubMed: 15596153]
- Benitah SA, Valeron PF, Lacal JC. ROCK and nuclear factor-kappaB-dependent activation of cyclooxygenase-2 by Rho GTPases: effects on tumor growth and therapeutic consequences. *Mol Biol Cell*. 2003; 14:3041–54. [PubMed: 12857884]
- Bilban M, Ghaffari-Tabrizi N, Hintermann E, Bauer S, Molzer S, Zoratti C, Malli R, Sharabi A, Hiden U, Graier W, Knofler M, Andreae F, Wagner O, Quaranta V, Desoye G. Kisspeptin-10, a KiSS-1/metastatin-derived decapeptide, is a physiological invasion inhibitor of primary human trophoblasts. *J Cell Sci*. 2004; 117:1319–28. [PubMed: 15020672]
- Brailoiu GC, Dun SL, Ohsawa M, Yin D, Yang J, Chang JK, Brailoiu E, Dun NJ. KiSS-1 expression and metastatin-like immunoreactivity in the rat brain. *J Comp Neurol*. 2005; 481:314–29. [PubMed: 15593369]
- Brantley-Sieders DM, Zhuang G, Hicks D, Fang WB, Hwang Y, Cates JM, Coffman K, Jackson D, Bruckheimer E, Muraoka-Cook RS, Chen J. The receptor tyrosine kinase EphA2 promotes mammary adenocarcinoma tumorigenesis and metastatic progression in mice by amplifying ErbB2 signaling. *J Clin Invest*. 2008; 118:64–78. [PubMed: 18079969]
- Debidda M, Wang L, Zang H, Poli V, Zheng Y. A role of STAT3 in Rho GTPase-regulated cell migration and proliferation. *J Biol Chem*. 2005; 280:17275–85. [PubMed: 15705584]
- Deryugina EI, Quigley JP. Matrix metalloproteinases and tumor metastasis. *Cancer Metastasis Rev*. 2006; 25:9–34. [PubMed: 16680569]
- Fernandez-Fernandez R, Martini AC, Navarro VM, Castellano JM, Dieguez C, Aguilar E, Pinilla L, Tena-Sempere M. Novel signals for the integration of energy balance and reproduction. *Mol Cell Endocrinol*. 2006; 254-255:127–32. [PubMed: 16759792]
- Fromiguet O, Hamidouche Z, Marie PJ. Blockade of the RhoA-JNK-c-Jun-MMP2 cascade by atorvastatin reduces osteosarcoma cell invasion. *J Biol Chem*. 2008; 283:30549–56. [PubMed: 18757369]
- Gadea G, de Toledo M, Anguille C, Roux P. Loss of p53 promotes RhoA-ROCK-dependent cell migration and invasion in 3D matrices. *J Cell Biol*. 2007; 178:23–30. [PubMed: 17606864]
- Gumireddy K, Sun F, Klein-Szanto AJ, Gibbins JM, Gimotty PA, Saunders AJ, Schultz PG, Huang Q. In vivo selection for metastasis promoting genes in the mouse. *Proc Natl Acad Sci U S A*. 2007; 104:6696–701. [PubMed: 17420453]

- Hacker H, Karin M. Regulation and function of IKK and IKK-related kinases. *Sci STKE*. 2006; 2006:re13. [PubMed: 17047224]
- Hodge JC, Bub J, Kaul S, Kajdacsy-Balla A, Lindholm PF. Requirement of RhoA activity for increased nuclear factor kappaB activity and PC-3 human prostate cancer cell invasion. *Cancer Res*. 2003; 63:1359–64. [PubMed: 12649199]
- Jiang Y, Berk M, Singh LS, Tan H, Yin L, Powell CT, Xu Y. KiSS1 suppresses metastasis in human ovarian cancer via inhibition of protein kinase C alpha. *Clin Exp Metastasis*. 2005; 22:369–76. [PubMed: 16283480]
- Joshi B, Strugnell SS, Goetz JG, Kojic LD, Cox ME, Griffith OL, Chan SK, Jones SJ, Leung SP, Masoudi H, Leung S, Wiseman SM, Nabi IR. Phosphorylated caveolin-1 regulates Rho/ROCK-dependent focal adhesion dynamics and tumor cell migration and invasion. *Cancer Res*. 2008; 68:8210–20. [PubMed: 18922892]
- Karin M. Nuclear factor-kappaB in cancer development and progression. *Nature*. 2006; 441:431–6. [PubMed: 16724054]
- Lee JH, Miele ME, Hicks DJ, Phillips KK, Trent JM, Weissman BE, Welch DR. KiSS-1, a novel human malignant melanoma metastasis-suppressor gene. *J Natl Cancer Inst*. 1996; 88:1731–7. [PubMed: 8944003]
- Lee JH, Welch DR. Suppression of metastasis in human breast carcinoma MDA-MB-435 cells after transfection with the metastasis suppressor gene, KiSS-1. *Cancer Res*. 1997; 57:2384–7. [PubMed: 9192814]
- Lee MJ, Jeon ES, Lee JS, Cho M, Suh DS, Chang CL, Kim JH. Lysophosphatidic acid in malignant ascites stimulates migration of human mesenchymal stem cells. *J Cell Biochem*. 2008; 104:499–510. [PubMed: 18027882]
- Loberg RD, Tantivejkul K, Craig M, Neeley CK, Pienta KJ. PAR1-mediated RhoA activation facilitates CCL2-induced chemotaxis in PC-3 cells. *J Cell Biochem*. 2007; 101:1292–300. [PubMed: 17492768]
- Marot D, Bieche I, Aumas C, Esselin S, Bouquet C, Vacher S, Lazenne G, Perricaudet M, Kuttent F, Lidereau R, de Roux N. High tumoral levels of Kiss1 and G-protein-coupled receptor 54 expression are correlated with poor prognosis of estrogen receptor-positive breast tumors. *Endocr Relat Cancer*. 2007; 14:691–702. [PubMed: 17914099]
- Martin MJ, Tanos T, Garcia AB, Martin D, Gutkind JS, Coso OA, Marinissen MJ. The Galphai2/13 family of heterotrimeric G proteins and the small GTPase RhoA link the Kaposi sarcoma-associated herpes virus G protein-coupled receptor to heme oxygenase-1 expression and tumorigenesis. *J Biol Chem*. 2007; 282:34510–24. [PubMed: 17881360]
- Martin TA, Watkins G, Jiang WG. KiSS-1 expression in human breast cancer. *Clin Exp Metastasis*. 2005; 22:503–11. [PubMed: 16320113]
- Mead EJ, Maguire JJ, Kuc RE, Davenport AP. Kisspeptins are novel potent vasoconstrictors in humans, with a discrete localization of their receptor, G protein-coupled receptor 54, to atherosclerosis-prone vessels. *Endocrinology*. 2007a; 148:140–7. [PubMed: 17023533]
- Mead EJ, Maguire JJ, Kuc RE, Davenport AP. Kisspeptins: a multifunctional peptide system with a role in reproduction, cancer and the cardiovascular system. *Br J Pharmacol*. 2007b; 151:1143–53. [PubMed: 17519946]
- Mitchell DC, Abdelrahim M, Weng J, Stafford LJ, Safe S, Bar-Eli M, Liu M. Regulation of KiSS-1 metastasis suppressor gene expression in breast cancer cells by direct interaction of transcription factors activator protein-2alpha and specificity protein-1. *J Biol Chem*. 2006; 281:51–8. [PubMed: 16260418]
- Nash KT, Phadke PA, Navenot JM, Hurst DR, Accavitti-Loper MA, Sztul E, Vaidya KS, Frost AR, Kappes JC, Peiper SC, Welch DR. Requirement of KISS1 secretion for multiple organ metastasis suppression and maintenance of tumor dormancy. *J Natl Cancer Inst*. 2007; 99:309–21. [PubMed: 17312308]
- Nash KT, Welch DR. The KISS1 metastasis suppressor: mechanistic insights and clinical utility. *Front Biosci*. 2006; 11:647–59. [PubMed: 16146758]
- Naugler WE, Karin M. NF-kappaB and cancer-identifying targets and mechanisms. *Curr Opin Genet Dev*. 2008; 18:19–26. [PubMed: 18440219]

- Navenot JM, Wang Z, Chopin M, Fujii N, Peiper SC. Kisspeptin-10-induced signaling of GPR54 negatively regulates chemotactic responses mediated by CXCR4: a potential mechanism for the metastasis suppressor activity of kisspeptins. *Cancer Res.* 2005; 65:10450–6. [PubMed: 16288036]
- Niida A, Wang Z, Tomita K, Oishi S, Tamamura H, Otaka A, Navenot JM, Broach JR, Peiper SC, Fujii N. Design and synthesis of downsized metastin (45-54) analogs with maintenance of high GPR54 agonistic activity. *Bioorg Med Chem Lett.* 2006; 16:134–7. [PubMed: 16242330]
- Nyberg P, Salo T, Kalluri R. Tumor microenvironment and angiogenesis. *Front Biosci.* 2008; 13:6537–53. [PubMed: 18508679]
- Ohtaki T, Shintani Y, Honda S, Matsumoto H, Hori A, Kanehashi K, Terao Y, Kumano S, Takatsu Y, Masuda Y, Ishibashi Y, Watanabe T, Asada M, Yamada T, Suenaga M, Kitada C, Usuki S, Kurokawa T, Onda H, Nishimura O, Fujino M. Metastasis suppressor gene KiSS-1 encodes peptide ligand of a G-protein-coupled receptor. *Nature.* 2001; 411:613–7. [PubMed: 11385580]
- Perona R, Montaner S, Saniger L, Sanchez-Perez I, Bravo R, Lacal JC. Activation of the nuclear factor-kappaB by Rho, CDC42, and Rac-1 proteins. *Genes Dev.* 1997; 11:463–75. [PubMed: 9042860]
- Popa SM, Clifton DK, Steiner RA. The role of kisspeptins and GPR54 in the neuroendocrine regulation of reproduction. *Annu Rev Physiol.* 2008; 70:213–38. [PubMed: 17988212]
- Rothermel BA, Vega RB, Williams RS. The role of modulatory calcineurin-interacting proteins in calcineurin signaling. *Trends Cardiovasc Med.* 2003; 13:15–21. [PubMed: 12554096]
- Ryeom S, Baek KH, Rieth MJ, Lynch RC, Zaslavsky A, Birsner A, Yoon SS, McKeon F. Targeted deletion of the calcineurin inhibitor DSCR1 suppresses tumor growth. *Cancer Cell.* 2008; 13:420–31. [PubMed: 18455125]
- Shoji S, Tang XY, Umemura S, Itoh J, Takekoshi S, Shima M, Usui Y, Nagata Y, Uchida T, Osamura RY, Terachi T. Metastin Inhibits Migration and Invasion of Renal Cell Carcinoma with Overexpression of Metastin Receptor. *Eur Urol.* 2008
- Stafford LJ, Xia C, Ma W, Cai Y, Liu M. Identification and characterization of mouse metastasis-suppressor KiSS1 and its G-protein-coupled receptor. *Cancer Res.* 2002; 62:5399–404. [PubMed: 12359743]
- Stathatos N, Bourdeau I, Espinosa AV, Saji M, Vasko VV, Burman KD, Stratakis CA, Ringel MD. KiSS-1/G protein-coupled receptor 54 metastasis suppressor pathway increases myocyte-enriched calcineurin interacting protein 1 expression and chronically inhibits calcineurin activity. *J Clin Endocrinol Metab.* 2005; 90:5432–40. [PubMed: 15998767]
- Sun HW, Tong SL, He J, Wang Q, Zou L, Ma SJ, Tan HY, Luo JF, Wu HX. RhoA and RhoC -siRNA inhibit the proliferation and invasiveness activity of human gastric carcinoma by Rho/PI3K/Akt pathway. *World J Gastroenterol.* 2007; 13:3517–22. [PubMed: 17659701]
- Takino T, Koshikawa N, Miyamori H, Tanaka M, Sasaki T, Okada Y, Seiki M, Sato H. Cleavage of metastasis suppressor gene product KiSS-1 protein/metastin by matrix metalloproteinases. *Oncogene.* 2003; 22:4617–26. [PubMed: 12879005]
- Urbinati C, Bugatti A, Giacca M, Schlaepfer D, Presta M, Rusnati M. alpha(v)beta3-integrin-dependent activation of focal adhesion kinase mediates NF-kappaB activation and mitogenic activity by HIV-1 Tat in endothelial cells. *J Cell Sci.* 2005; 118:3949–58. [PubMed: 16105876]
- Vega RB, Rothermel BA, Weinheimer CJ, Kovacs A, Naseem RH, Bassel-Duby R, Williams RS, Olson EN. Dual roles of modulatory calcineurin-interacting protein 1 in cardiac hypertrophy. *Proc Natl Acad Sci U S A.* 2003; 100:669–74. [PubMed: 12515860]
- Wegener E, Krappmann D. CARD-Bcl10-Malt1 signalosomes: missing link to NF-kappaB. *Sci STKE.* 2007; 2007:pe21. [PubMed: 17473310]
- Yan C, Wang H, Boyd DD. KiSS-1 represses 92-kDa type IV collagenase expression by down-regulating NF-kappa B binding to the promoter as a consequence of Ikappa Balpha -induced block of p65/p50 nuclear translocation. *J Biol Chem.* 2001; 276:1164–72. [PubMed: 11060311]
- Ye RD. Regulation of nuclear factor kappaB activation by G-protein-coupled receptors. *J Leukoc Biol.* 2001; 70:839–48. [PubMed: 11739545]
- Zhu Q, Krakowski AR, Dunham EE, Wang L, Bandyopadhyay A, Berdeaux R, Martin GS, Sun L, Luo K. Dual role of SnoN in mammalian tumorigenesis. *Mol Cell Biol.* 2007; 27:324–39. [PubMed: 17074815]

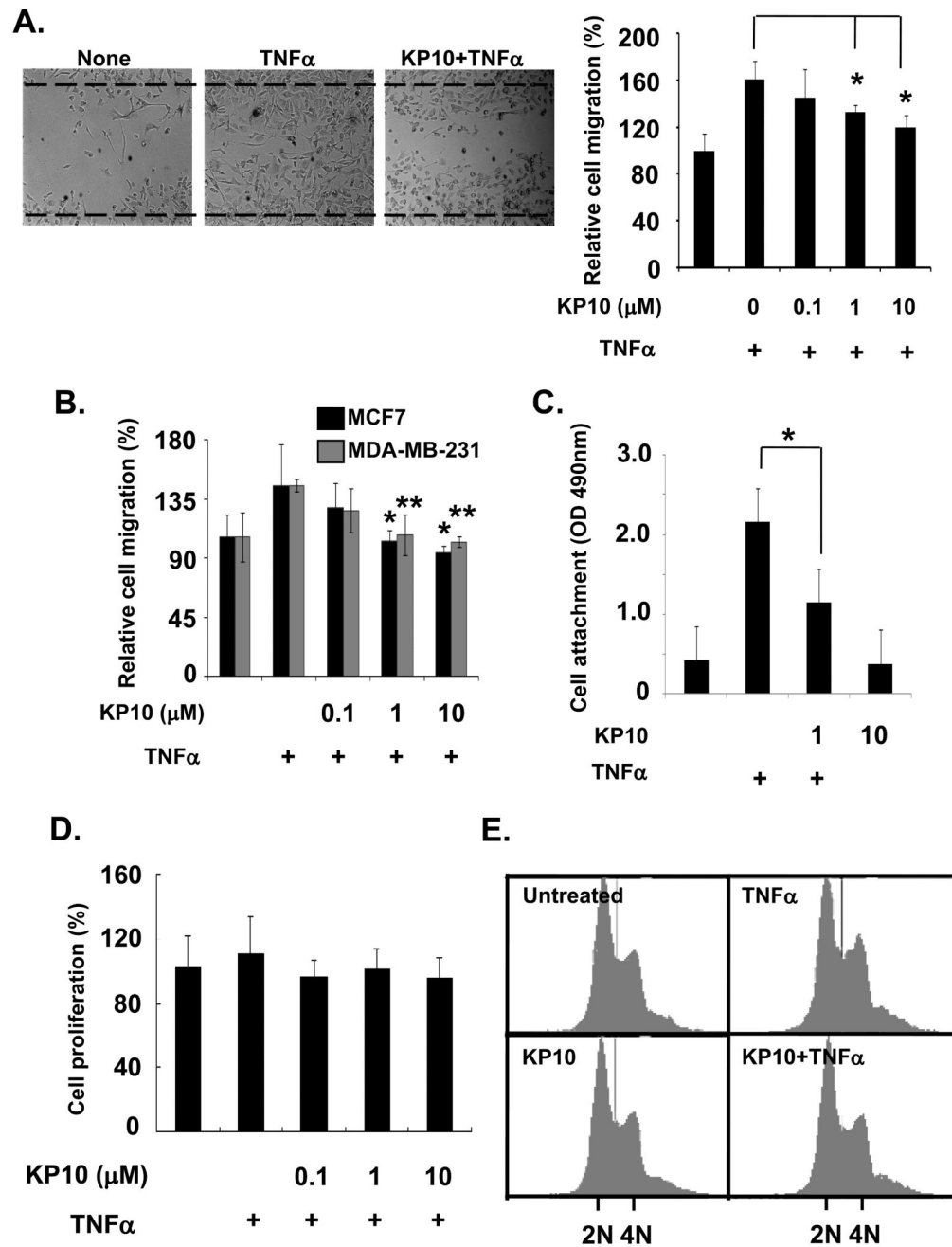


Figure 1. KiSS1 inhibits breast cancer cell migration and attachment but not proliferation. (A) Cell migration was determined using scratching assays. TNFα-induced migration of MDA-MB-231 cells was downregulated by KP10 at 1 to 10μM. Mean and standard deviations represent data from triplicate experiments (*, $p < 0.01$ vs. TNFα alone). (B) Cell invasion was examined using Boyden chamber assays. In MCF7 and MDA-MB-231 cells, TNFα-stimulated cell invasiveness was inhibited by KP10 at 1 to 10μM. Mean and standard deviations represent data from eight independent experiments (*, $p < 0.01$ vs. TNFα alone). (C) TNFα-induced cell attachment to fibronectin was inhibited by KP10 treatment (1μM of KP10). Mean and standard deviations represent data from triplicate experiments (*, $p < 0.05$ vs. TNFα-treated).

(D) Cell proliferation was not affected by KP10, when cells were stimulated by KP10 or TNF α for 24hr. (E) Cell cycle was analyzed using flow cytometry. KP10 and/or TNF α did not affect cell cycle at 24hr after stimulation.

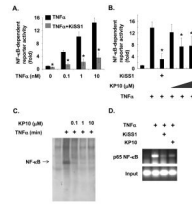


Figure 2.

Inhibition of NF- κ B activation by KiSS1 and KP10. (A) KiSS1 overexpression blocks a basal and TNF α -induced NF- κ B activation. Mean and standard deviations represent data from triplicate experiments (*, $p < 0.05$ vs. TNF α -treated samples). (B) TNF α -induced NF- κ B promoter activity is downregulated by KiSS1 overexpression and KP10 (at 1 to 10 μ M). Mean and standard deviations represent data from triplicate experiments (*, $p < 0.05$ vs. TNF α -treated samples). (C) KiSS1 blocks TNF α -induced DNA binding activity of NF- κ B in EMSA assays. Cells were treated with 1 μ M of KP10 for 24hr and then stimulated by 10nM of TNF α for 6hr. (D) KiSS1 inhibits TNF α -induced DNA binding activity of NF- κ B on MMP-9 promoter in ChIP assays. DNA from cells stimulated with the indicated agents was cross-linked with formaldehyde, isolated, and then precipitated with anti-NF- κ B p65 antibody. Immunoprecipitates were analyzed with PCR using primers specific for MMP-9 promoter region. For the input control, DNA purified from sonicated chromatin was directly subjected to the PCR using primers used.

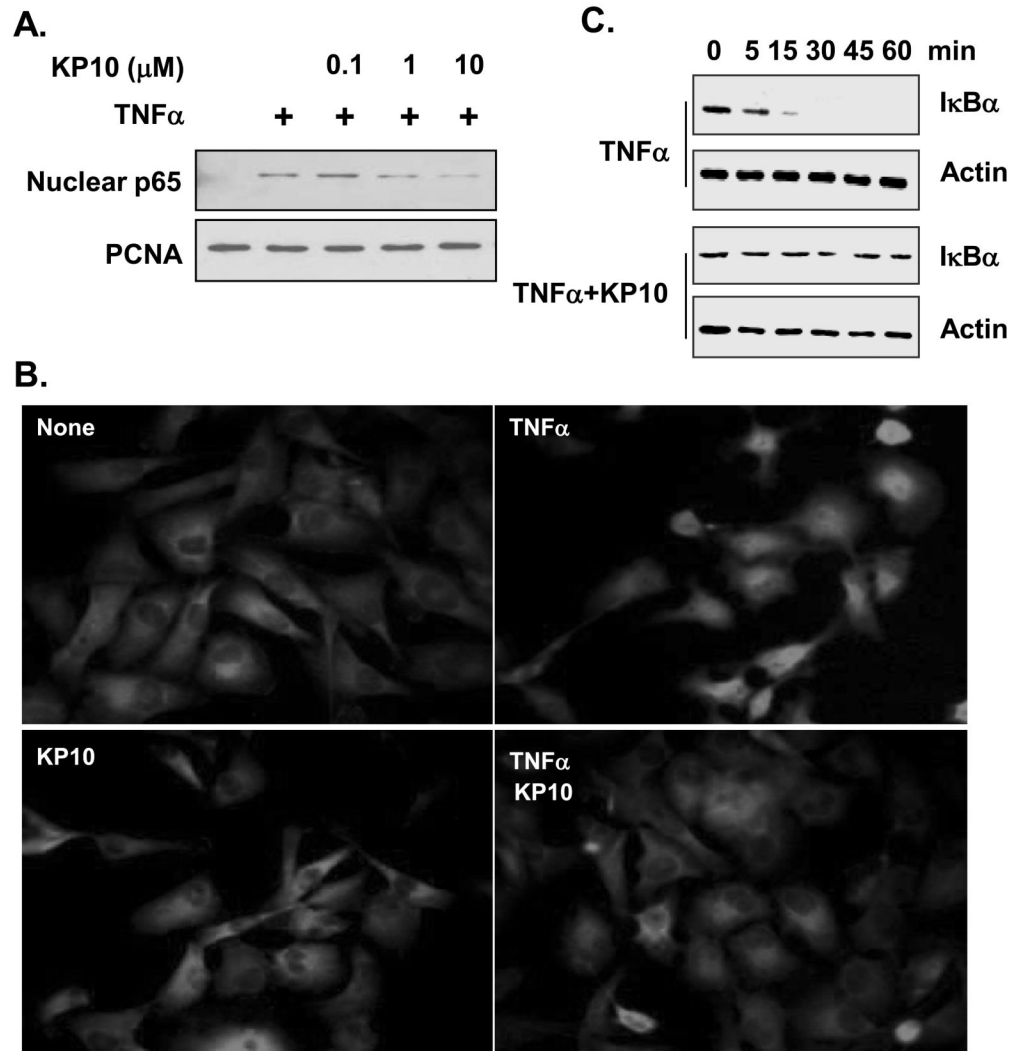


Figure 3. KiSS1 inhibits NF- κ B nuclear translocation by blocking I κ B α degradation. (A) KP10 (1 to 10 μ M) inhibits nuclear translocation of p65. Nuclear fraction of cell extracts was used to examine nuclear population of p65. PCNA was used as a loading control. (B) p65 translocation was confirmed by immunofluorescence. KP10 blocked TNF α -induced p65 nuclear translocation. (C) KP10 blocked I κ B α degradation that is required for the release of NF- κ B. Cytosolic fraction of cell extracts was used for examination of I κ B α degradation. Actin was used for a loading control.

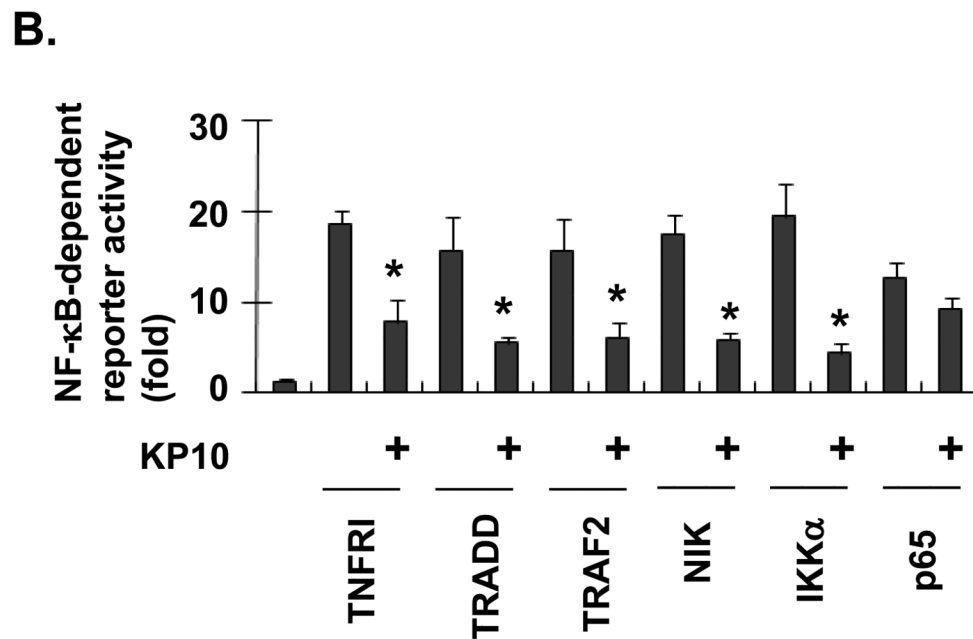
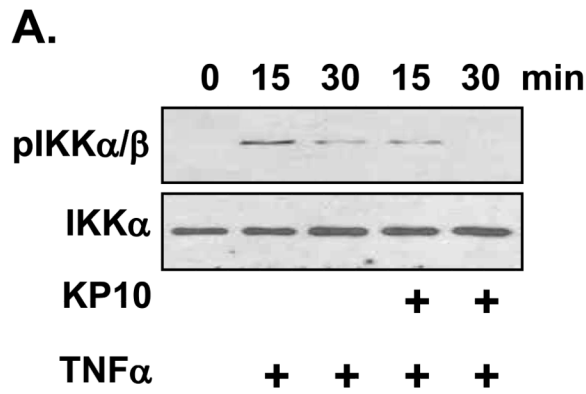


Figure 4. KiSS1 inhibits NF- κ B transcriptional activation by inhibiting IKK phosphorylation. (A) KP10 inhibited IKK phosphorylation. Cells treated with KP10 and/or TNF α were sampled for Western blot at the times indicated. IKK phosphorylation was examined with anti-IKK α/β antibody. (B) KiSS1 downregulates NF- κ B activation mediated by TNFR1, TRADD, TRAF2, NIK, or IKK α . Mean and standard deviations represent data from triplicate samples (*, $p < 0.05$ vs. KP10-untreated).

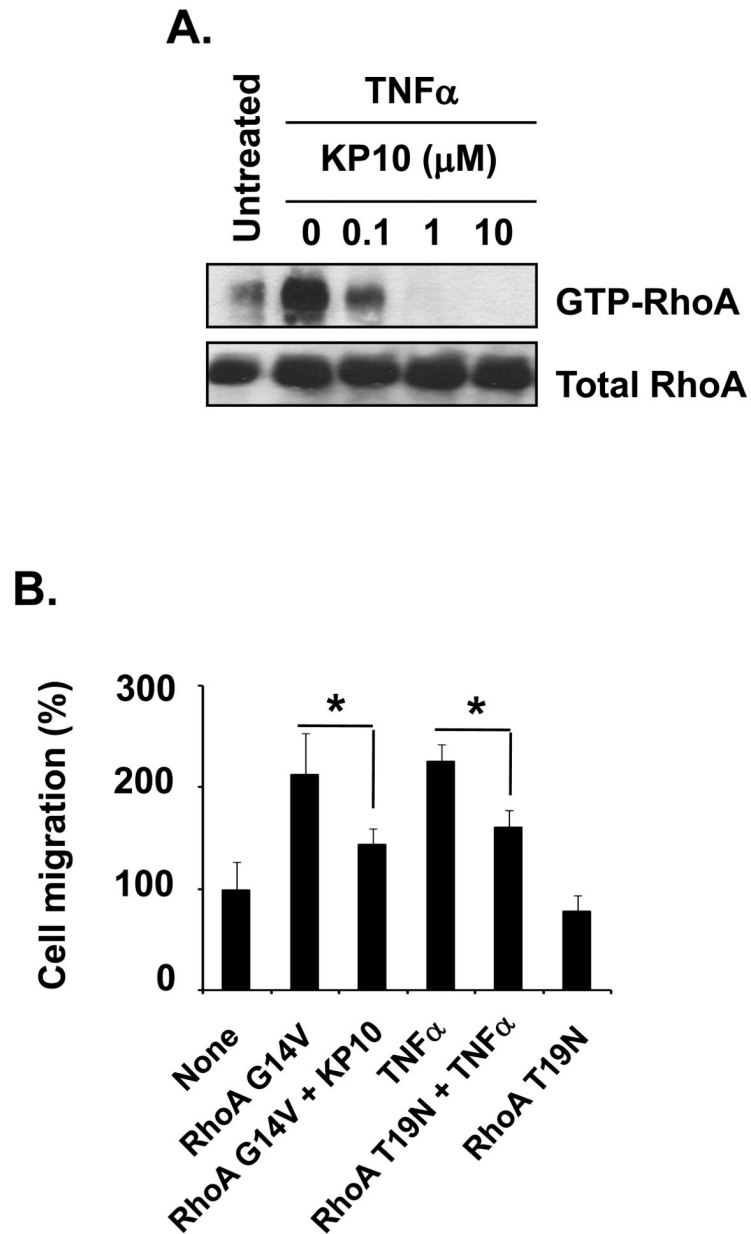


Figure 5. KiSS1 inhibited RhoA activation (GTP-bound RhoA) and RhoA-mediated cell migration. (A) Cells treated with KP10 and/or TNF α for 24hr and then subjected to the RhoA activity assays. TNF α -induced RhoA activity (GTP-bound RhoA) was inhibited by different concentrations of KP10. (B) Cells were transfected with empty vector, RhoA G14V, or RhoA T19N for 24hr, transferred to the upper chamber precoated with gelatin, and then stimulated with control peptide or KP10 for another 24hr. TNF α was added in the bottom chamber for 24hr in order for the chemotactic attractance. Mean and standard deviations represent data from triplicate experiments (*, $p < 0.05$).

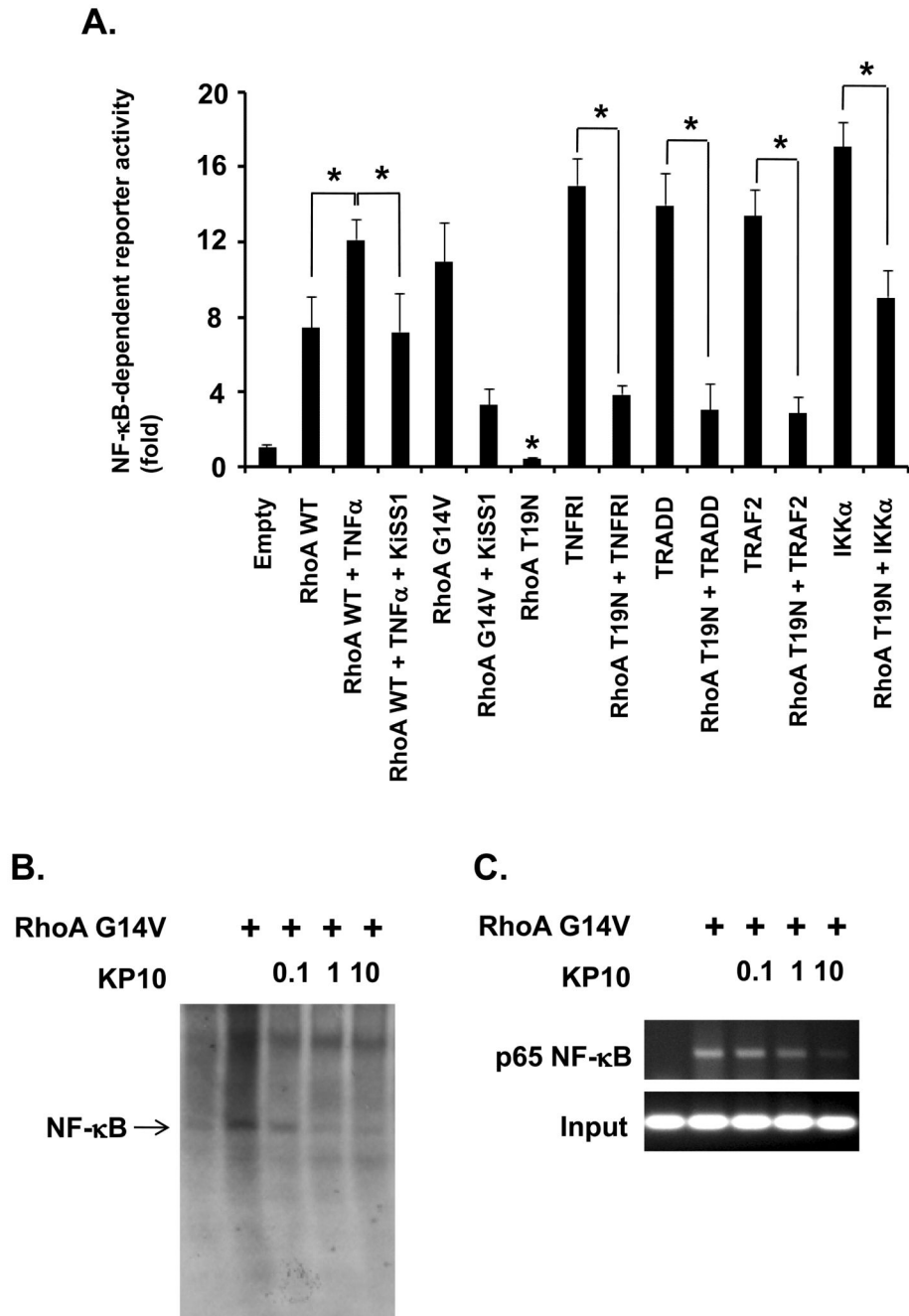


Figure 6. KiSS1 inhibited RhoA-mediated NF-κB activation. (A) NF-κB transcriptional activity was examined in cells transfected with the indicated plasmids. Luciferase assays were performed at 36hr after transfection. In the case of TNFα, cells were sampled 6hr after treatment. Mean and standard deviations represent data from triplicate experiments (*, $p < 0.05$). (B) DNA binding activity of NF-κB was examined in the conditions as indicated in EMSA. A dominant active RhoA (Rho G14V) enhanced NF-κB binding onto DNA, but was reduced by different concentrations of KP10. (C) KiSS1 inhibits RhoA-induced DNA binding of NF-κB on MMP-9 promoter. DNA from cells stimulated with the indicatives was isolated, and then precipitated with anti- NF-κB p65 antibody. Immunoprecipitates were subjected to the

PCR using primers specific for MMP-9 promoter region. For the input control, DNA purified from sonicated chromatin was directly subjected to the PCR using primers used.

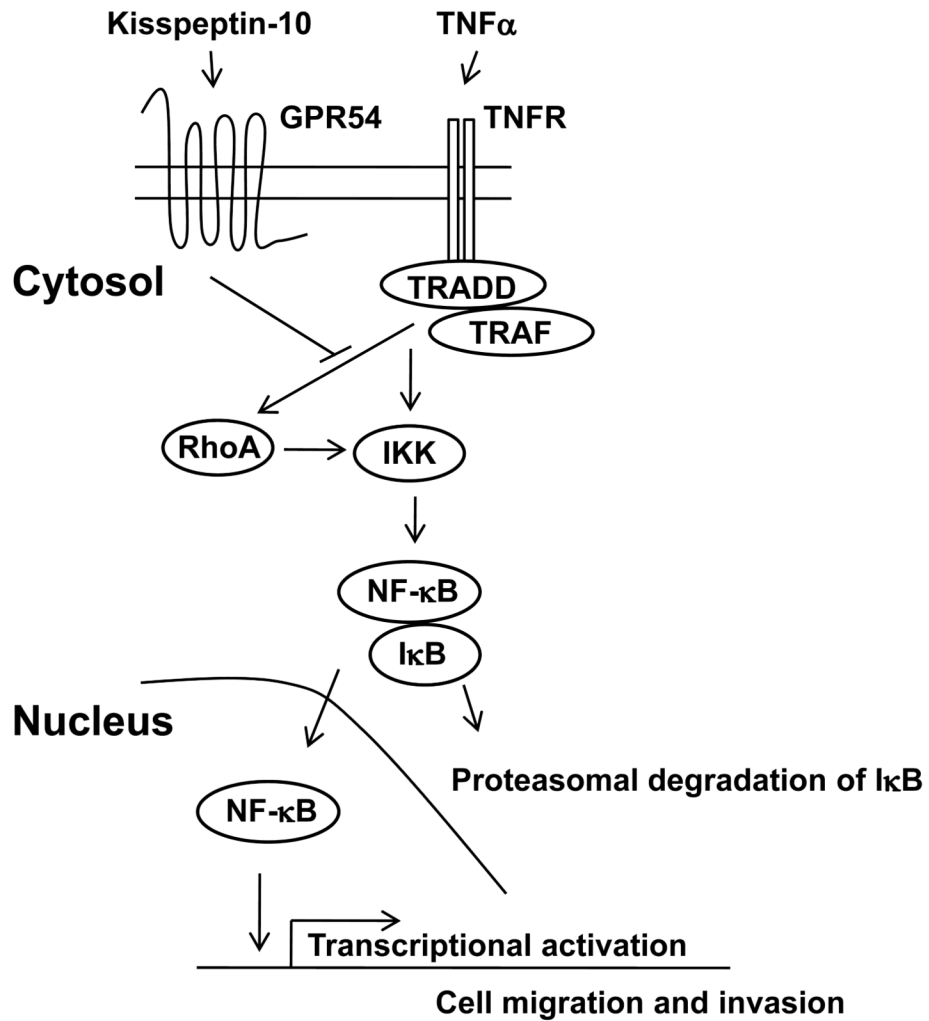


Figure 7. Schematic model for KiSS1 inhibition of TNF α -mediated NF- κ B activation. TNF α -activated TNFR complex leads to the activation of NF- κ B via IKK activation. In addition, TNF α -activated TNFR complex activates RhoA, which also induces IKK-mediated NF- κ B activation. Upon kisspeptin-10 stimulation, KISS1 signaling via GPR54 inhibits TNF α -induced RhoA activation, which results in the suppression of TNF α -induced NF- κ B activation.