The role of KiSS1 gene on the tumor growth and migration of prostate cancer and the underlying molecular mechanisms

Cho-Won Kim  
Chungbuk National University

Hong Kyu Lee  
Chungbuk National University

Min-Woo Nam  
Chungbuk National University

Gabsang Lee  
Johns Hopkins University

Kyung-Chul Choi  (kchoi@cbu.ac.kr)  
Chungbuk National University

Research

Keywords: KiSS1, Kisspeptin, Prostate cancer, EMT, Migration, Angiogenesis, Metastasis

Posted Date: June 9th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1615647/v1

License: This work is licensed under a Creative Commons Attribution 4.0 International License. Read Full License
Abstract

**Background** Prostate cancers have a very high mortality rate, with no improvement in the survival rate being achieved over the past few decades. KiSS1 was originally identified as the metastasis suppressor gene in metastatic melanoma breast cancer, but its role in prostate cancer has been contradictory. This study was therefore undertaken to investigate the effects of KiSS1 overexpression on the growth and migration of human metastatic prostate cancer cells.

**Methods** DU145 human metastatic prostate cancer cells were infected with the culture medium of 293T cells, which produce lentivirus particles containing KiSS1. The tumor spheroid formation assay, qPCR and immunoblotting analysis, migration and invasion assays were performed in this study following KiSS1 introduction. A xenograft mouse model was employed to investigate the overexpression of KiSS1 in prostate cancer in vivo.

**Results** A significant increase in proliferation of KiSS1-overexpressing cancer cells was observed, and these cells formed tumor spheroids larger than the vector control group. qPCR and immunoblotting revealed the association between increased cell growth and regulation of the PI3K/Akt and cell cycle genes, and also that increases in β-catenin and CD133 contribute to tumor aggregation. KiSS1 overexpression resulted in upregulation of the β-arrestin1/2 and Raf-MEK-ERK-NF-κB pathways via KISS1R. Moreover, the migration and invasion of KiSS1-overexpressing cells were determined to be faster than the control group, along with increased metastatic colonization of the KiSS1-overexpressing cancer cells. These were associated to the regulation of EMT gene expressions, such as E-cadherin and N-cadherin, and the upregulation of MMP9. In the xenograft mouse model, KiSS1 significantly increased the tumor growth, with upregulation of PCNA and Ki-67 in the tumor tissues. In addition, KiSS1 increased the angiogenic capacity by upregulating VEGF-A and CD31, both in vitro and in vivo.

**Conclusion** These results indicate that KiSS1 not only induces prostate cancer proliferation, but also promotes metastasis by increasing the migration, invasion, and angiogenesis of malignant cells.

**Background**

Metastasis is the process through which cancer cells disseminate to different areas of the body [1]. Morphological transformation is required for primary cancer cells to initiate migration to other tissues, via a process known as epithelial-mesenchymal transition (EMT) [2, 3]. The transdifferentiated cancer cells invade nearby normal tissue and, after intravasation, migrate to distant organs via blood vessels or lymphatic vessels [4]. At a distant location, the cancer cells break out of the blood vessels through extravasation, and imbed and grow in the surrounding tissue until a tiny tumor forms (colonization) [5]. Creation of new blood vessels supplies blood to maintain the growth of metastatic tumors [6].

Prostate cancer is the second leading cause of death among malignancies in men, accounting for 15% of all cancer diagnoses [7, 8]. The incidence of prostate cancer is globally increasing, with no significant improvement in the survival rate over the past few decades [8, 9]. Due to the numerous blood vessels
KiSS1 is a gene encoding the KISS1 (known as kisspeptin) protein that acts as a ligand for the G-protein coupled receptor, KISS1 receptor (KISS1R) [19, 20]. KiSS1 was originally identified as a metastasis suppressor gene in metastatic melanoma breast cancer, and reports indicate that activation of this gene suppresses tumor growth [21]. Thereafter, numerous researchers have investigated the effect of KiSS1 in different carcinomas, and it was found that KiSS1 plays a role as a metastasis suppressor in various malignancies including pancreatic cancer, colorectal cancer, bladder cancer, ovarian cancer, and lung cancer [22–26].

It is now clear that KiSS1 regulates the development and progression of several cancers, but interestingly, some researchers have raised the possibility that KiSS1 may act as an oncogene and a dual-action mechanism. For the first time, one study reported increased expression of KiSS1 in estrogen receptor (ER)α-negative breast cancer as compared to ERα-positive breast cancer, and breast tumors that metastasized to lymph nodes showed higher KiSS1 levels than lymph node negative tumors [27]. Tamoxifen-treated ERα-positive MCF7 and T47D breast cancer cells had down-regulated kisspeptin/KISS1R levels, and increased expressions of KISS1R correlated more with metastasis than tumor growth [28, 29]. In addition, KISS1R signaling increased the expressions of Akt strain transforming (Akt), extracellular-signal-regulated kinase (ERK), and survivin, and stimulated the invasion of triple negative breast cancer (TNBC) cells via β-arrestin2 and ERK [28, 30]. Moreover, the in vivo evidence that haploinsufficiency in KISS1R inhibited the development and metastasis of breast cancer tissue raised the possibility of the role of KiSS1 as a metastasis promoter [31]. Consistent with these findings in breast cancer, increased KiSS1 mRNA and protein levels were observed in tumor tissues of liver cancer patients, which correlated with adverse prognosis and survival rates [32, 33].

To date, few studies have investigated the role of KiSS1 in prostate cancer, and the findings have been contradictory. In particular, researches investigating the functional and mechanistic roles related to the development and metastasis of prostate cancer by regulating the expression of KiSS1 gene and identifying their correlations are very limited. The expression of kisspeptin was low in tumor or prostate
tissue samples from prostate cancer patients, and reports indicated a negative correlation with clinical staging [34]. In contrast, another study observed no significant differences in plasma kisspeptin levels in 92 prostate cancer patients [35]. Signal transduction systems in prostate cancer cells are very complex, interconnected, and have various heterogeneities, highlighting the need for further examination. Furthermore, since the effects of KiSS1 revealed to date may be dependent on the carcinoma, microenvironment and steroid receptors, efforts are needed to determine the exact role of this gene in prostate cancer.

Based on these interesting findings, we speculated that KiSS1 may have multiple unexplained signaling pathways in prostate cancer metastasis. This study therefore undertook to investigate the roles and molecular mechanisms of KiSS1 on cell growth and migration in human metastatic prostate cancer cells.

**Materials And Methods**

**Cell culture and medium preparation**

DU145 human metastatic prostate cancer cell line was purchased from Korean Cell Line Bank (KCLB; Seoul, Republic of Korea). 293tsA1609neo (293T) cells line was purchased from American Type Culture Collection (ATCC; Manassas, VA, USA). These cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% heat inactivated fetal bovine serum (FBS; Biowest Nuailléa, France), 1% antibiotic-antimycotic solution (Welgene, Gyeongsan, Republic of Korea) and 0.01 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (Welgene) at 37°C in the humidified CO₂ incubator. When cells reached approximately 70–80% confluence, they were trypsinized with 0.1% Trypsin-ethylenediaminetetraacetic acid (EDTA) (Invitrogen Life Technologies Inc., Carlsbad, CA, USA) for 5 min at 37°C, then subcultured at a split ratio of 1:8 (DU145 cells) and 1:10 (DU145-KiSS1 cells and 293T cells).

Human umbilical vein endothelial cells (HUVECs) were purchased from PromoCell GmbH (Heidelberg, Germany) and cultured in endothelial cell growth medium (PromoCell GmbH). Referring to our previous study [36, 37], HUVECs were immortalized by simian virus 40 (SV40) large T antigen in the second passage, and an SV40-HUVEC line was established. The SV40-HUVECs were also cultured in endothelial cell growth medium, when cells reached approximately 70–80% confluence, they were trypsinized with 0.05% Trypsin-EDTA for 3 min at 37°C, then subcultured at a split ratio of 1:3.

**Establishment of stable cell line overexpressing KiSS1 gene**

Transduction of the KiSS1 gene into 293T cells was performed according to the manufacturer's manual (Origene, Rockville, MD, USA). For transformation, we made 293T cells that stably expressed the lentiviral vector pLenti-C-mGFP-P2A-Puro (Fig. 2A) as a lentivirus packaging. 293T cells were plated at a cell density of 5×10⁵ cells/well in 6-well plate. In a 1.5 mL tube, 1 µg of KiSS1 cDNA clone (pLenti-ORF expression construct), packaging plasmids and 6.6 µL of TurboFectin transfection reagent (Origene) were diluted in 250 µL of Opti-MEM (Gibco, Waltham, MA, USA), and the transfection mixture was gently mixed completely. The transfection mixture was incubated for 15 min at room temperature. The transfection
mixture was added to the 293T cells and incubate for 18 h at 37°C in the humidified CO₂ incubator. The lentivirus-containing medium was collected, passed through a 0.22 µm low protein binding filter (Millipore, Darmstadt, Germany), at the half-confluent condition then, transferred to DU145 cells (50–60% confluence) with polybrene (10 µg/mL) (Sigma-Aldrich Inc., Saint Louis, USA) used to increase efficiency of lentivirus infection. After infection for 18 h, the lentivirus-containing medium was replaced with fresh cell culture medium and further incubated for 2 days. Transfectants were selected with antibiotics free-medium (DMEM supplemented with 10% FBS) containing puromycin (10 µg/mL; Sigma-Aldrich Inc.) for 48 h, and was selected 3 times. In this study, the DU145 cell line overexpressing the KiSS1 gene was named DU145-KiSS1 cell. For vector control group, mock transfection was performed without adding only KiSS1 cDNA clone.

**Cell counting**

Cell proliferation was measured by counting cells. The cells were seeded at a cell density of 1×10⁵ cells/well in 6-well plates (SPL Life Sciences Co., Ltd., Pocheon, Republic of Korea) and incubated at 37°C in the humidified CO₂ incubator. After 4 days (96 h), the number of cells was measured using EVE™ Automatic Cell Counter (NanoEntek, Seoul, Republic of Korea). The experiments were conducted independently three times, and the results all showed similar trends.

**Tumor spheroid formation**

The cells were seeded at a cell density of 2×10³ cells/well with 200 µL of cell culture medium in ultra-low attachment plate (96-well type, round bottom clear; Corning Inc., Corning, NY, USA). To minimize evaporation of the culture medium, the edge wells of the plate were filled with Dulbecco's phosphate buffered saline (DPBS; Welgene). At the center of the round bottom, the cells were aggregated and formed tumor spheroids. After 1, 3, and 7 days, the tumor spheroids were photographed using a phase-contrast microscope (IX73, Olympus, Tokyo, Japan). The area of tumor spheroids was quantified by cellSens Dimension software (ver. 1.18; Olympus). The experiments were conducted independently three times, and the results all showed similar trends.

**Transwell migration assay**

To assess the migratory ability of the cells, migration assay was used as previously described, with slight modification [38, 39]. To prevent changes in the number of cells by cell division, the cells were treated with 12.5 µg/mL of Mitomycin C (MMC) for 2 h. After staining with 10 µg/mL of Hoechst 33342 for 10 min, the cells were seeded at a cell density of 2×10⁴ cells/well in the upper chamber of a transwell insert (8 µm pore; BD Biosciences, Franklin Lakes, NJ, USA) in a total of 200 µL of DMEM media containing 10% FBS. The lower chamber was added with 500 µL of DMEM media containing 15% FBS. The cells were then incubated at 37°C with 5% CO₂ for 72 h. Blue fluorescence of the cells that migrated from the upper chamber to the bottom chamber were photographed using a fluorescence microscope (IX73, Olympus) at ×100 magnification. The experiments were conducted independently three times, and the results all showed similar trends.
Transwell invasion assay

To assess the invasive ability of the cells, transwell invasion assay was used with slight modification [38, 39]. To prevent changes in the number of cells by cell division, the cells were treated with 12.5 µg/mL of MMC for 2 h. The cells were then stained with 10 µg/mL of Hoechst 33342 for 10 min. After 50 µL of fibronectin (250 µg/mL; Sigma-Aldrich Inc.) pre-coating of the bottom of a transwell (8 µm pore, BD Biosciences), the cells were seeded at a cell density of 2×10^4 cells/well in the upper chamber of a transwell insert in a total of 200 µl of DMEM media containing 10% FBS. Fibronectin was used as extracellular matrix (ECM). The lower chamber was added with 500 µL of DMEM media containing 15% FBS. The cells were then incubated at 37°C with 5% CO₂ for 72 h. After invading the fibronectin layer from the upper chamber, the blue fluorescence of cells that migrated to the bottom chamber were photographed using a fluorescence microscope (IX73, Olympus) at ×100 magnification.

Collagen invasion assay

To test the ability of tumor spheroids to penetrate the ECM, a collagen invasion assay was performed with slight modifications referring to previous study [40]. MMC-treated cells were seeded at a cell density of 5×10^3 cells/200 µL/well in ultra-low attachment plates (96-well type, round bottom clear; Corning Inc.) and tumor spheroids were formed for 72 h. Native collagen derived from bovine dermis (KOKEN; Tokyo, Japan) was mixed with cell culture media at a ratio of 2:1, and 50 µL of collagen mixture placed in a round bottom 96-well plate (SPL Life Sciences Co., Ltd.) was solidified in 37°C incubator for 2 h. One spheroid per well was transferred to a round bottom 96-well plate coated with collagen, and 200 µL of cell culture medium was added. To minimize evaporation of the culture medium, the edge wells of the plate were filled with DPBS (Welgene). Images of tumor spheroids were taken using a phase-contrast microscope (IX73, Olympus) every 24 h and for up to 96 h. Invasion area of tumor spheroids was quantified by cellSens Dimension software (ver. 1.18; Olympus), and the invasion area was normalized as initial area.

In vitro metastasis assay

To evaluate the ability of primary cancer cells to metastasize and then form secondary tumors at metastatic site, we developed an in vitro metastasis assay. After coating the outer bottom surface of the 96-well type transwell insert (Corning Inc.) with 12.5 µg/50 µL of fibronectin, the transwell inserts were mounted one by one on an ultra-low attachment plate (round bottom clear; Corning Inc.) (Fig. 1A). To minimize evaporation of the culture medium, the edge wells of the plate were filled with DPBS (Welgene). MMC-treated cells were seeded in the upper chamber of the transwell at a density of 2×10^4 cells/100 µL/well, and 180 µL of cell culture medium was added to the bottom chamber (Fig. 1B). The spheroids formed in the bottom chamber were photographed using a phase-contrast microscope (IX73, Olympus) every 24 h and for up to 96 h. The area of spheroids was quantified by cellSens Dimension software (ver. 1.18; Olympus).

Tube formation assay
To evaluate the ability of KiSS1 overexpressing cancer cells for angiogenesis, a tube formation assay was used using SV40-HUVECs with slight modifications based on previous study [41]. DU145 cells (vector control) or DU145-KiSS1 cells were seeded in a 6-well plate (SPL Life Sciences Co., Ltd.) at a density of $5 \times 10^5$ cells/well, and the cell culture medium was replaced with an endothelial cell growth medium after 2 h of cell adhesion. After 24 h of incubation with endothelial cell growth medium, the medium was harvested after 0.22 µm filtration (Millipore). Two hundred-fifty µl mixture of cold media and Matrigel (Corning Inc.) (1:4 ratio) was dispensed in each well of 24-well plates (Corning Inc.), and hardened for 30 min in 37°C incubator. SV40-HUVECs were stained with 2 µg/ml of Calcein-AM (Invitrogen Life Technologies), and the cells were seeded at a density of $7.5 \times 10^4$ cells/100 µL/well with the cancer cell culture medium in the Matrigel coated plates. After 3 h, SV40-HUVECs formed tubes, observed using a fluorescence microscope (IX73, Olympus) at ×100 magnification.

**Mouse line and husbandry**

Five-week-old athymic nude mice ($\text{Foxn1}^{\text{nu}}$) were purchased from KOATECH (Pyeongtaek, Republic of Korea), and maintained at the Laboratory Animal Research Center of Chungbuk National University under specific pathogen-free conditions. The mice were housed in a temperature- and humidity-controlled environment under a 12-h light-dark cycle, and all animal experiments were performed during the light cycle. All animal experiments were approved by Institutional Animal Care and Use Committee (IACUC) at Chungbuk National University (CBNUA-1565-21-01).

**Establishment of xenograft mouse model**

After a one-week acclimatization, DU145 cells (vector control) or DU145-KiSS1 cells were mixed 1:1 in Matrigel, and 200 µL of the cell mix was inoculated subcutaneously in right upper flank of mice ($3 \times 10^6$ cells/mouse; $n = 6$ per group). Tumor volume was measured using a digital caliper (CD-15APX; Mitutoyo Korea, Gunpo, Republic of Korea) every 2–3 days from 5 days after cancer cell inoculation, and calculated by shortest diameter$^2 \times$longest diameter$ \times 0.5236$ (mm$^3$). Body weight of mice was also measured on the same day. On day 19 post-inoculation, all mice were sacrificed, and tumor tissues were weighed and fixed in 4% paraformaldehyde solution (GeneAll Biotechnology, Seoul, Republic of Korea) for histological analysis. The representative organs (lung, liver, spleen and kidney) of mice were weighed immediately after sacrifice.

**Histological analysis (IHC and H&E staining)**

Immunohistochemistry (IHC) was performed with some modifications based on our previous work [42]. The fixed tumor tissues were embedded in paraffin and cut into 4 mm sections. Tumor tissue sections deparaffinized in xylene (OCI, Seoul, Republic of Korea) were rehydrated according to the ethanol (OCI) concentration gradient. Antigen retrieval was achieved by rehydrating the slides, followed by incubation with 10 mM sodium citrate buffer (pH 6.0; Sigma-Aldrich Inc.) at 100°C for 10 min. After removal of endogenous peroxidase with 3% hydrogen peroxide (Sigma-Aldrich Inc.), blocking was performed with 5% bovine serum albumin (BSA; RMBIO, Missoula, MT, USA). The tissue sections were subsequently incubated 18 h with primary antibodies against proliferating cell nuclear antigen (PCNA; Biolegend, San
Diego, CA, USA), Ki-67 (Biolegend), vascular endothelial growth factor-A (VEGF-A; Bioss Inc., Woburn, MA, USA) or cluster of differentiation 31 (CD31; Santa Cruz Biotechnology, Dallas, TX, USA) at 1:50, followed by incubation with biotinylated goat anti-mouse or anti rabbit IgG antibody (H + L) at 1:100 for 1 h. Then, the tissue sections were reacted with Avidin-biotin peroxidase complexes (Vector Labs, Burlingame, CA, USA) for 30 min. A 3,3-diaminobenzidine (DAB) kit (Vector Labs) was used to visualize the peroxidase, and a counterstain was performed with hematoxylin (Sigma-Aldrich Inc.).

Hematoxylin and eosin (H&E) stain was performed for pathological analysis of tumor tissue. The tumor tissue sections that had undergone the above-mentioned rehydrating process were stained with hematoxylin and eosin (Sigma-Aldrich Inc.). All slides were observed under a bright microscope (IX73, Olympus), and representative images were selected.

**Total RNA extraction and cDNA synthesis**

DU145 cells (vector control) or DU145-KiSS1 cells were seeded 100 mm cell culture dish. When cells reached approximately 80–90% confluence, total RNA was isolated from cells using TRIzol reagent (Invitrogen Life Technologies), following the manufacturer’s instructions. A RNA pellet was dissolved in 50–100 µL of UltraPure™ DNase/RNase-Free Distilled Water (Gibco). Total RNA concentration was measured with a microreader (BioTek Instruments Inc.) at 260/280 nm. cDNA synthesis was done using an PrimeScript RT Master Mix (Takara Bio Inc., Kusatsu, Japan), following their instructions. cDNA synthesis was performed at 37°C for 15 min, and the enzyme was inactivated at 95°C for 5 sec.

**Quantitative reverse transcription polymerase chain reaction (RT-qPCR)**

For quantitative analysis on the mRNA expression, the cDNA was amplified using 10 pmole/µL of each forward, reverse primer (Bioneer Co., Dae-Jeon, Republic of Korea) and TB Green Premix Ex Taq II (Takara Bio Inc.). qPCR was carried out for 40 cycles of 95°C for 15 s, 58°C for 60 s using QuantStudio 3 Real-Time PCR System (Applied Biosystems, Waltham, MA, USA). Table 1 shows the sequence of each primer used in this study. The housekeeping gene GAPDH was used as the internal control. The ΔCt was calculated using the following the formula: ΔCt = Ct (target gene) – Ct (GAPDH). Then, the ΔΔCt was calculated using the following the formula: ΔΔCt = ΔCt (transfected group) – ΔCt (vector control). Finally, expression of each sample as $2^{-\Delta\Delta C_t}$ was calculated.
<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequences (5’→3’)</th>
</tr>
</thead>
</table>
| **GHPDH** | Forward: GGTTTCCATAGGACCTGCTG  
Reverse: TCTTGGGTGTCTCGTCTTCT |
| **KiSS1** | Forward: AGCAGCTAGAATCCCTGGG  
Reverse: GTTCCAGTTTGTAGTTCCGCA |
| **Akt3** | Forward: TAGATGGGTAGGATGGCTGG  
Reverse: CTCAAATTTGGCCGTGTGAC |
| **PIK3R1** | Forward: ATGGTTGGTTTCCCTGTCC  
Reverse: AGGCAGGAATTTGTGAAGCA |
| **HRAS** | Forward: AGCAGATCAAACGGGTGAAG  
Reverse: CATCAGGAGGGTTCCAGCTTC |
| **NRAS** | Forward: CTTGAGGTTTTCTGCTGTG  
Reverse: TGGTCTCTCATGGCACTGTA |
| **B-RAF** | Forward: GAGTCTTCTGCCCACAAAC  
Reverse: TCCAACACTTCCACATGCAA |
| **CCNE1** | Forward: GCACTTTTCTTGAGCAACACC  
Reverse: TCCTCAAGTTTGCTGCAAT |
| **CDKN2A** | Forward: CCCCACTACCGTTAATGTCC  
Reverse: CAAGAGAAGCCAGTAACCCC |
| **PTEN** | Forward: AGATGGCACCATTCCCCGTTT  
Reverse: TCAGAAACCTCTCTTACAGCA |
| **CXCR4** | Forward: CCTGCTGATTTTGTCTATCC  
Reverse: CTCGACATCCACCCCTTGG |
| **CTNNB1** | Forward: TGCAGTTATGTCATCCAGC  
Reverse: CCTCAAGTGATGGAAGG |
| **EZH2** | Forward: GACCACAGTGGTTACAGCAT  
Reverse: TTTCACTCCCTGCTTCCTTA |
To visualize the amplified KiSS1 genes, PCR products in which the KiSS1 gene was amplified were separated in a 1.5% agarose gel and the gels were scanned using Lumino Graph 2 (ATTO Corporation, Tokyo, Japan).

Automated capillary-based western immunoblot

The proteins were extracted using radio immunoprecipitation assay (RIPA) buffer (ATTO corporation) from the cells. Protein concentration was determined by a bicinchoninic acid (Sigma-Aldrich Inc.) assay at 562 nm. Immunoblotting was conducted on a JESS™ Simple Western automated nano-immunoassay system (ProteinSimple, San Jose, CA, USA), and a capillary based size separation of proteins was used with an internal system control. The protein that extracted from the cells were processed according to the manufacturer’s standard method for the 12–230-kDa Jess separation module. Briefly, a mixture of proteins, fluorescent 5× master mix, 400 mM dithiothreitol (ProteinSimple) and biotinylated molecular weight markers was prepared, and then denatured at 95 °C for 5 min. Primary antibodies were diluted with antibody diluent, Table 2 shows the information of the antibodies used in this study. HRP-conjugated anti-mouse and rabbit secondary antibodies included in this kit were used. For chemiluminescence detection, luminol-peroxide mix was prepared. Assay protocol was as follows: 25 min for separation time, 375 V for separation voltage, 20 min for blocking time, 45 min for primary antibody incubation, 45 min for secondary antibody incubation. The Compass Simple Western software (version 6.0.0, ProteinSimple) was used to capture the digital image of the capillary chemiluminescence. Band intensity was quantified with CSAnalyzer4 software (ATTO Corporation). All protein expression levels were normalized to the levels of GAPDH protein expression in each band. Statistical analysis was performed only for blots with a sample number of 3 or greater.
<table>
<thead>
<tr>
<th>Antigen</th>
<th>Type</th>
<th>Dilution</th>
<th>Manufacturer</th>
<th>Catalog number</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>MM</td>
<td>1:100</td>
<td>Abcam</td>
<td>ab8245</td>
</tr>
<tr>
<td>Cyclin E1</td>
<td>MM</td>
<td>1:100</td>
<td>Abcam</td>
<td>ab3927</td>
</tr>
<tr>
<td>p21</td>
<td>MM</td>
<td>1:100</td>
<td>Cell Signaling Technology</td>
<td>2946</td>
</tr>
<tr>
<td>p27</td>
<td>RM</td>
<td>1:100</td>
<td>Abcam</td>
<td>ab32034</td>
</tr>
<tr>
<td>PCNA</td>
<td>MM</td>
<td>1:100</td>
<td>Biolegend</td>
<td>307901</td>
</tr>
<tr>
<td>p85α</td>
<td>MM</td>
<td>1:20</td>
<td>Santacruz</td>
<td>sc-1637</td>
</tr>
<tr>
<td>p-Akt\textsuperscript{Ser473}</td>
<td>RM</td>
<td>1:100</td>
<td>Cell Signaling Technology</td>
<td>4060T</td>
</tr>
<tr>
<td>Akt 1/2/3</td>
<td>RM</td>
<td>1:100</td>
<td>Abcam</td>
<td>ab179463</td>
</tr>
<tr>
<td>β-catenin</td>
<td>MM</td>
<td>1:100</td>
<td>Biolegend</td>
<td>862602</td>
</tr>
<tr>
<td>CD133</td>
<td>MM</td>
<td>1:100</td>
<td>Biolegend</td>
<td>372802</td>
</tr>
<tr>
<td>KiSS1R</td>
<td>RM</td>
<td>1:20</td>
<td>Cell Signaling Technology</td>
<td>13776</td>
</tr>
<tr>
<td>β-arrestin1/2</td>
<td>MM</td>
<td>1:100</td>
<td>Santacruz</td>
<td>sc-74591</td>
</tr>
<tr>
<td>p-Raf\textsuperscript{Ser445}</td>
<td>RP</td>
<td>1:100</td>
<td>Cell Signaling Technology</td>
<td>2696T</td>
</tr>
<tr>
<td>B-Raf</td>
<td>RM</td>
<td>1:100</td>
<td>Cell Signaling Technology</td>
<td>14814</td>
</tr>
<tr>
<td>p-MEK1/2\textsuperscript{Ser217/221}</td>
<td>RM</td>
<td>1:20</td>
<td>Cell Signaling Technology</td>
<td>9154T</td>
</tr>
<tr>
<td>MEK1/2</td>
<td>MM</td>
<td>1:100</td>
<td>Cell Signaling Technology</td>
<td>4694</td>
</tr>
<tr>
<td>p-ERK1/2\textsuperscript{Thr202/Tyr204}</td>
<td>MM</td>
<td>1:100</td>
<td>Biolegend</td>
<td>369501</td>
</tr>
<tr>
<td>ERK1/2</td>
<td>RM</td>
<td>1:100</td>
<td>Cell Signaling Technology</td>
<td>4695T</td>
</tr>
<tr>
<td>p50</td>
<td>MM</td>
<td>1:100</td>
<td>Biolegend</td>
<td>616701</td>
</tr>
<tr>
<td>p65</td>
<td>MM</td>
<td>1:20</td>
<td>Biolegend</td>
<td>653001</td>
</tr>
<tr>
<td>E-cadherin</td>
<td>RP</td>
<td>1:20</td>
<td>Abcam</td>
<td>ab15148</td>
</tr>
<tr>
<td>N-cadherin</td>
<td>MM</td>
<td>1:100</td>
<td>Biolegend</td>
<td>844702</td>
</tr>
<tr>
<td>Slug</td>
<td>MM</td>
<td>1:100</td>
<td>Santacruz</td>
<td>sc-166476</td>
</tr>
<tr>
<td>MMP9</td>
<td>RM</td>
<td>1:100</td>
<td>Abcam</td>
<td>ab76003</td>
</tr>
<tr>
<td>VEGF-A</td>
<td>RP</td>
<td>1:100</td>
<td>Bioss</td>
<td>bs-0279R</td>
</tr>
</tbody>
</table>

MM: Mouse monoclonal; RM: Rabbit monoclonal; RP: Rabbit polyclonal
<table>
<thead>
<tr>
<th>Antigen</th>
<th>Type</th>
<th>Dilution</th>
<th>Manufacturer</th>
<th>Catalog number</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD31</td>
<td>MM</td>
<td>1:100</td>
<td>Santacruz</td>
<td>sc-376764</td>
</tr>
</tbody>
</table>

MM: Mouse monoclonal; RM: Rabbit monoclonal; RP: Rabbit polyclonal

**Statistical analysis**

All experiments were run at least three times, and all data presented as means ± standard error of the mean (SEM). Data from the experiments were statistically analyzed by Student’s *t*-test, or multiple *t*-test using the GraphPad prism 7.0 software (GraphPad Software Inc., San Diego, CA, US). Statistical significance was presented at * (p < 0.05), ** (p < 0.01) or *** (p < 0.001).

**Results**

**Overexpression of KiSS1 in metastatic prostate cancer cells and characterization**

To investigate the role of *KiSS1* in metastatic prostate cancer, we first established the DU145-KiSS1 cell line overexpressing *KiSS1* in the DU145 human metastatic prostate cancer cell line (Fig. 2). DU145-KiSS1 cells expressed the *KiSS1* gene 2,485 times higher than the control vector-inserted cells (Fig. 2B). Moreover, the *KiSS1* overexpressing cells showed mesenchymal morphology, as compared to the vector control (Fig. 2C). Based on this morphological alteration of the cells by *KiSS1* overexpression, we questioned whether *KiSS1* overexpression affects the metastatic capacity of prostate cancer cells. To address this question, we investigated the effects of *KiSS1* on cell proliferation, followed by examination of the effects on the migratory and invasive ability.

**KiSS1 increases the proliferation of prostate cancer cells**

The effects of *KiSS1* were first investigated on cell proliferation and tumor spheroid formation (Fig. 3). We observed a 2.5-fold increase in the proliferation of DU145-KiSS1 compared to the vector control (Fig. 3A), which correlated with the expressions of genes that regulate the cell cycle (*CCNE1* and *CDKN2A*). Immunoblotting revealed that *KiSS1* overexpression also upregulates the protein levels of Cyclin E1 and PCNA, and downregulates the protein expressions of p21 and p27 (Fig. 3B and D). In addition, *KiSS1* increased the gene expressions of the phosphoinositide 3-kinase (PI3K) regulatory subunits p85α and Akt3, increased the gene expressions of the Harvey rat sarcoma oncogene homolog (HRAS) and neuroblastoma rat sarcoma viral oncogene homolog (NRAS) among the rat sarcoma (RAS) gene family, and also increased the expression of the B-BAF gene (Fig. 3C). Changes in the mRNA levels of p85α and Akt induced alterations in the translation level and increased protein levels of p85α and total Akt (Fig. 3E). Interestingly, *KiSS1* not only increased the total-form of Akt, but also phosphorylation of the serine 473 site (Fig. 3E). Moreover, a significant increase was obtained in the p-Akt/Akt ratio, suggesting that *KiSS1* increases not only the protein level of Akt, but also signal transduction through phosphorylation (Fig. 3E).
These results indicate that *KiSS1* overexpression increases the proliferation of prostate cancer cells by increasing the expressions of cell cycle-related genes and PI3K/Akt activity.

We further observed that the ability of prostate cancer cells to aggregate and form spheroids is significantly promoted by *KiSS1* in a time-dependent manner (Fig. 3F). The vector control cells start to aggregate on the 4th day after seeding, whereas the *KiSS1* overexpressing cells aggregate and form spheroids just one day after seeding (Fig. 3F). Based on the previous study reports that cancer stem cells promote tumor formation [43–45], we attempted to determine whether the expression of cancer stem cell markers is altered in the DU145-*KiSS1* cells (Fig. 3G and H), and observed that *KiSS1* overexpression significantly increases the expressions of β-catenin and cluster of differentiation 133 (CD133) (Fig. 3H). Particularly, both mRNA and protein expressions were increased for β-catenin (Fig. 3G and H). We therefore speculated that increased tumor spheroid formation by *KiSS1* is associated with increased cancer stem cell markers.

**KiSS1 regulates KISS1R-mediated downstream signaling**

Given that *KiSS1* is translated into kisspeptin (a ligand of KISS1R), we investigated changes in the protein levels involved in the KISS1R-mediated signaling pathways (Fig. 4). There was a 2-fold increase in the protein level of KISS1R (Fig. 4A), and the total protein levels of β-arrestin1 and β-arrestin2 (downstream proteins of KISS1R) were significantly increased in DU145-*KiSS1* cells (Fig. 4B). Examination of the Ras-Raf-MEK-ERK pathway with *KiSS1* overexpression revealed that both, the protein levels and phosphorylation of Rapidly Accelerated Fibrosarcoma (Raf), Mitogen-activated protein kinase (MEK) and ERK, were increased in DU145-*KiSS1* cells (Fig. 4C-E). Therefore, similar to the results obtained in Fig. 3E, we considered that *KiSS1* promotes signal transduction by increasing both the translation and phosphorylation of Raf, MEK, and ERK. Furthermore, we observed that both p65 and p50, which are subunits of nuclear factor kappa light chain enhancer of activated B cells (NF-κB), were significantly increased via the NF-κB signaling pathway (Fig. 4F and G). These results imply that overexpression of *KiSS1* activates KISS1R and increases the expression of NF-κB via the β-arrestin1/2 and Raf-MEK-ERK pathways.

**KiSS1 increases metastatic capacity by increasing migratory and invasive ability of prostate cancer cells**

Next, we investigated whether *KiSS1* affects the migratory and invasive ability of metastatic prostate cancer cells (Fig. 5). We observed that DU145-*KiSS1* cells migrated faster than vector control cells, along with a relatively increased invasive ability (Fig. 5A). The collagen invasion assay performed to evaluate the ability of prostate cancer cells to penetrate the ECM (Fig. 5B and C) revealed that the spheroids formed from DU145-*KiSS1* cells penetrated the collagen relatively faster in all directions (Fig. 5B), and this invasion area increased significantly in a time-dependent manner (Fig. 5C). In general, since metastasis involves the dissemination of cancer cells and results in metastatic colonization, the metastatic ability of cancer cells can therefore be predicted by evaluating the ability to form spheroids at
metastatic sites. As presented in Fig. 5D, the DU145-KiSS1 cells which migrated from the upper chamber to the bottom chamber (metastatic sites) aggregated relatively faster and rapidly formed spheroids. In contrast, vector control cells had relatively slower spheroid formation, due to lesser aggregation and wider distribution in the bottom chamber (Fig. 5D). We determined a statistically significant increase in the metastasized area of DU145-KiSS1 cells (Fig. 5E).

To further investigate the mechanism of the migratory and invasive ability of prostate cancer cells increased by \textit{KiSS1} overexpression, the expressions of EMT markers and matrix metallopeptidase 9 (MMP9) were further determined (Fig. 5F-J). Our results revealed that the expression of epithelial cadherin (E-cadherin), an epithelial marker, decreases at both the mRNA and protein levels (Fig. 5F and G), whereas the mRNA and protein expressions of neural cadherin (N-cadherin), a mesenchymal marker, were increased (Fig. 5F and H), and slug expression was increased only at the mRNA level (Fig. 5F and I). In addition, the protein level of MMP9, a type IV collagenase that degrades ECM, was determined to be significantly increased (Fig. 5J). These results indicate that \textit{KiSS1} increases the migratory and invasive ability by inducing expressions of the EMT genes and proteins, and the protein expression of MMP9, thereby consequently increasing the metastatic capacity.

**\textbf{KiSS1 increases prostate tumor growth in vivo}**

To investigate the effect of \textit{KiSS1} on tumor growth, vector control cells and DU145-KiSS1 cells were inoculated into athymic mice, and the tumor growth was monitored for 19 days (Fig. 6). We observed that compared to the control, \textit{KiSS1} overexpression induces a statistically significant increase in tumor growth from 5 days after inoculation (p < 0.05, 2.7-fold), and after 19 days, the tumor volume was increased approximately 25-fold (Fig. 6A-B). Similar to the data of tumor volume, macroscopic observation revealed that \textit{KiSS1} overexpression significantly increases the tumor weight (Fig. 6C), and induces a remarkable difference in size (Fig. 6D). However, no significant differences were obtained for body weight or weight of organs such as lung, liver, spleen and kidney (Fig. 6E-G). Macroscopic examination of all organs by necropsy revealed no metastasis of the subcutaneously xenografted cancer cells (data not shown). Histological analysis of the specimens revealed higher expressions of the tumor proliferation index markers, Ki-67 and PCNA, in the DU145-KiSS1 group (Fig. 6H). Interestingly, higher PCNA expression was observed around the blood vessels (Fig. 6H). These results indicate that \textit{KiSS1} increases the expressions of Ki-67 and VEGF, which subsequently induces increased tumor growth.

**\textbf{KiSS1 increases angiogenesis in prostate cancer cells}**

Since it has been documented that angiogenesis is essential for cancer cells to receive sufficient nutrients from the host and to secure a metastatic pathway \cite{46, 47}, we questioned whether \textit{KiSS1} overexpression affects the angiogenic ability of metastatic prostate cancer cells. We therefore investigated the role of \textit{KiSS1} on angiogenesis in cellular and animal models (Fig. 7). Compared with vector control cells, the medium cultured with DU145-KiSS1 cells promoted tube formation of SV40-HUVECs, indicating that \textit{KiSS1} increases the angiogenic ability of vascular endothelial cells (Fig. 6A). We further determined that \textit{KiSS1} increases the VEGF and CD31 expressions at the cellular level, with no alteration in the mRNA level
of VEGF-A (Fig. 7B-E). It is noteworthy that overexpression of KiSS1 induces more than 4-fold increase in the CD31 protein level (Fig. 7E). Furthermore, histopathological analysis of xenografted tumors stained by H&E showed remarkable evidence of blood vessels in the DU145-KiSS1-tumor tissue (marked with black arrows) (Fig. 7F). In addition, the expressions of VEGF and CD31 were significantly increased in the tumor tissue overexpressing KiSS1, as compared to the vector control (Fig. 7G). These results indicate that KiSS1 promotes angiogenesis in prostate cancer by regulating the expressions of VEGF and CD31.

**Discussion**

Depending on the cancer type, the KiSS1 gene is reported to play a role as either a tumor suppressor or oncogene, and therefore it is very important to understand the exact role of this gene in each cancer type [48]. Results of a previous study indicate that low expression of kisspeptin detected in prostate tissue samples from patients with prostate cancer negatively correlates with patient prognosis and clinical staging [34]. However, another study reported no significant differences in plasma kisspeptin levels in prostate cancer patients, as compared to healthy subjects [35]. Therefore, the role and mechanism of KiSS1 in metastatic prostate cancer remains unanswered. In the current study, we determined that KiSS1 potentially acts as an oncogene and promotes metastasis by increasing the growth, migratory and invasive ability, and angiogenic ability of metastatic prostate cancer, and eventually promoting the metastasis of prostate cancer.

In this report, we focus on our study on the effect of KiSS1 on cancer growth and migration in metastatic prostate cancer. We determined that overexpression of KiSS1 results in increased cell proliferation and tumor growth by regulating the expression of cell cycle genes involved in the G1/S phase, and up-regulation of PI3K/Akt. The cell cycle is also upregulated by altering the expression of p21 by phosphorylation of Akt and suppressing the glycogen synthase kinase 3 beta (GSK-3β) activity, or by repressing the expression of p27 via forkhead [49, 50]. This supports our hypothesis that increased expression of PI3K/Akt by KiSS1 promotes the proliferation of prostate cancer cells by suppressing expression of the cell cycle arrest gene involved in the G1/S phase. In addition, it is reported that the PI3K-Akt signaling pathway increases cell survival by indirectly activating NF-κB through phosphorylation of IκB kinase; thus, the Akt increased by KiSS1 overexpression probably upregulates NF-κB expression and increases prostate cancer cell survival [50, 51]. Increased PCNA by KiSS1 induces the activity of cyclin-dependent kinases by decreasing the expression of p21, which subsequently inhibits the G1/S phase and increases the proliferation of prostate cancer cells [52]. Taken together, our results indicate that KiSS1 increases the activity of PI3K/Akt, which induces the growth of prostate cancer by regulating the expressions of cell cycle genes, and increases cell survival through NF-κB.

We further showed that KiSS1 overexpression increases the formation of tumor spheroids, which are clusters of cancer cells aggregated and adherent to each other, mimicking tumorigenesis [53, 54]. In addition, tumor spheroids were identified to have characteristics similar to cancer stem cells [55]. Therefore, increase in the size of tumor spheroids by KiSS1 indicates the potential to promote tumor growth. One possible explanation for this observation can be speculated from our results that CD133 and
β-catenin are relatively upregulated in DU145-KiSS1 cells. It has previously been reported that CD133, a cancer stem cell marker, induces tumorigenesis in various carcinomas including prostate cancer, by directly promoting tumorigenesis, or upregulating the expressions of other critical genes that drive tumorigenesis [56–59]. Emerging observations have suggested that CD133 interacts with the PI3K regulatory subunit p85 to activate the PI3K/Akt pathway and activate the Wnt/β-catenin signaling pathway through Akt [60–62]. This suggests that the KiSS1-induced CD133 contributes to increased cell proliferation and promotes tumor growth by upregulating PI3K and Akt in prostate cancer cells. Furthermore, based on the results of a previous study that β-catenin mediates the enrichment of cancer stem cells in tumors and increases the growth of tumor spheroids and tumors, KiSS1 probably contributes to prostate cancer tumor growth by upregulating β-catenin [55].

In the current study, we further established that KiSS1 overexpression results in mesenchymal morphological changes in DU145 prostate cancer. EMT occurs when epithelial cells undergo long-term morphological and molecular changes as a result of trans-differentiation to a mesenchymal cell type [63]. This EMT is related to carcinogenesis, and confers metastatic properties to cancer cells by enhancing the mobility and invasion [4, 64]. Before examining the effect of KiSS1 overexpression on the migration and invasion of prostate cancer cells, we first investigated alterations in the EMT-related markers. Our results of qPCR and immunoblotting showed up-regulated N-cadherin and down-regulated E-cadherin, and mesenchymal changes were therefore attributed to changes in the EMT protein expressions. A previous study reported that the activity of KISS1R induces the acquisition of the mesenchymal phenotype by acting on EMT genes such as snail, slug, vimentin, N-cadherin, and E-cadherin [64]. Our results indicate that prostate cancer acquires metastatic properties by regulating the expressions of N-cadherin and E-cadherin via KISS1R. Since Wnt/β-catenin signaling activation up-regulates EMT-related genes, the mesenchymal morphology of prostate cancer cells transformed by KiSS1 may indirectly be due to increased β-catenin expression [60]. Meanwhile, KISS1R, also called G-protein-coupled receptor 54 (GPR54), is known to regulate ERK1/2 activity via β-arrestins [65]. β-arrestins play a role in various pathological processes, including GPCR desensitization and sequestration. β-arrestin2 potentiates GPR54 signaling to ERK, whereas β-arrestin1 inhibits this signaling [65–67]. It is interesting to note that KiSS1 overexpression results in increased total level of β-arrestin1/2, with a concurrent increase in the protein level and phosphorylation of ERK1/2, which is downstream of β-arrestins. Similar to the results of previous studies, we found increased ERK activity via phosphorylation of Raf-MEK1/2, and increased MMP9 expression by promoting transcription of NF-κB subunits (p65/p50) [68, 69]. Moreover, KISS1R also induced increased expression of MMP9 by indirectly interacting with the NF-κB protein via I-κB [68]. We therefore propose that KiSS1 increases the mobility and invasiveness of prostate cancer via the above signaling pathways.

Primary tumors which have undergone EMT disseminate into other tissues, initiating metastatic colonization at distant sites [70]. The prognosis and clinical outcomes of prostate cancer are highly dependent on the occurrence of metastases, and most recurrences are caused by disseminated tumor cells that have already colonized in other tissues [71]. Examining metastatic colonization requires systematic and macroscopic observation using animal models of tumor metastasis, but this is time-
consuming and labor intensive. Therefore, we established a cellular model that can predict the ability to colonize at metastatic sites. This experimental approach has the advantage of being able to evaluate metastatic colonization that cannot be reproduced in conventional transwell invasion assays, and can be applied to evaluate metastatic colonization after intravasation or extravasation of a primary tumor by seeding endothelial cells on the opposite side of the transwell insert. Plating tumor spheroids instead of trypsinized cells in the upper chamber conserves the characteristics of the primary tumor, and allows the evaluation of EMT and its reverse process, MET. Based on our results, the in vitro metastasis assay developed in this study provides the first clue that KiSS1 has the ability to increase metastatic colonization at distant sites.

Progression of malignant tumors depends on their ability to promote host responses to angiogenesis and lymphangiogenesis in a phase of rapid growth [6, 72, 73]. In the current study, KiSS1 overexpression resulted in increased angiogenesis and increased levels of VEGF and CD31 in xenografted metastatic prostate tumors. VEGF is known as a key mediator of angiogenesis in metastatic prostate cancer, and it has been reported that CD31 interacts with VEGF to result in higher levels of angiogenesis in prostate cancer [74–76]. Therefore, KiSS1 may induce angiogenesis in prostate cancer by upregulating VEGF and CD31. In addition, CD31 upregulates the MMP9 expression to increase cancer cell migration and invasion, indicating that it helps facilitate the invasion of vascular endothelial cells into tissues, which in turn increases angiogenesis [77, 78].

Prostate cancer cells injected with the control vector had relatively smaller and slow growing tumors during the observation period. Given that the absence of nutrient supply via blood vessels can lead to tumor necrosis or even apoptosis [79], it is reasonable to postulate from our findings that prostate tumors with relatively low KiSS1 expression are smaller in size. In other words, KiSS1 promotes tumor growth by stimulating angiogenesis, an important factor in cancer progression [6, 80]. However, simply increasing the activity of angiogenic factors is insufficient for tumor angiogenesis, and there needs to be accompanying downregulation of negative regulators or inhibitors of vessel growth [72, 81]. Thus, subsequent studies are required to investigate the effect of KiSS1 on inhibitors of angiogenesis. Meanwhile, one study has reported that CD133 promotes angiogenesis by interacting with CD31 or VEGF, thereby supporting our findings that KiSS1 overexpression induces a cancer stem cell marker to increase neovascularization and tube formation [60].

**Conclusions**

Given these observations, we conclude that KiSS1 not only induces prostate cancer proliferation, but also facilitates metastasis by increasing the migration, invasion, and angiogenesis of metastatic prostate cancer. Based on the interactions between proteins involved in signal transduction related to cancer cell growth and migration reported in previous studies, and the changes in the levels of genes and proteins by KiSS1 overexpression found in this study, Fig. 8 proposes a molecular mechanism by which KiSS1 regulates the development and metastasis of metastatic prostate cancer. It is still unclear whether KiSS1 can serve as a biomarker in specific malignant cancer cells, and whether regulation of KiSS1 gene
expression via a viral vector system can be applied for therapeutic purposes in malignant tumors. Further studies are required to investigate whether the KiSS1-knock out/knock down model using the RNAi system exhibits an effect reversal to the results of this study. In addition, it is necessary to investigate the role of KiSS1 in PC-3 and LNCaP cell lines, which are the more widely used prostate adenocarcinomas for prostate research. Nevertheless, we expect that this study provides a strong foundation for delineating the underlying molecular mechanisms for the role of KiSS1 in tumor growth and metastasis of prostate cancer, thereby enhancing its clinical value for prostate treatment.

**Abbreviations**

CD: Cluster of differentiation

ECM: Extracellular matrix

EMT: Epithelial-mesenchymal transition

ER: Estrogen receptor

IHC: Immuno-histological analysis

GSK-3β: glycogen synthase kinase 3 beta

GESTECs: Genetically engineered stem cells

HRAS: Harvey rat sarcoma oncogene homolog

H&E: Hematoxylin and eosin

HUVECs: Human umbilical vein endothelial cells

KISS1R: KISS1 receptor

MAPK: Mitogen-activated protein kinase

NRAS: Neuroblastoma rat sarcoma viral oncogene homolog

PI3K: Phosphoinositide 3-kinase

RAS: rat sarcoma

Raf: Rapidly accelerated fibrosarcoma

RT-qPCR: Quantitative reverse transcription polymerase chain reaction

SV40: Simian virus 40
TNBC: Triple negative breast cancer

VEGF: Vascular endothelial growth factor

Declarations

Ethics approval

All animal experiments were approved by Institutional Animal Care and Use Committee (IACUC) at Chungbuk National University (CBNUA-1565-21-01).

Consent for publication

Not applicable

Availability of data and material

All data generated or analyzed during this study are included in this published article. The materials used in this study are available to any qualified researcher upon reasonable request addressed to K.C.C.

Competing interests

The authors do not have any conflicts of interest to declare.

Funding

This work was supported by the Basic Science Research Program (2020R1A2C2006060) and the Global Research and Development Center (GRDC) Program (2017K1A4A3014959) through the National Research Foundation (NRF) of Korea, funded by the Ministry of Science and ICT. This work was also supported by the Sejong Fellowship of National Research Foundation of Korea (NRF) funded by the Ministry of Science and ICT (2021R1C1C2093998).

Author's contributions

K.C.C. conceived an idea of the manuscript and held the research funding for this study. C.W.K., H.K.L, and M.W.N. performed the experiments and statistical analysis. C.W.K. made the first draft of the manuscript and H.K.L. and K.C.C. edited and revised the manuscript based on the concept. C.W.K., H.K.L. and M.W.N. provided essential techniques for this study.

References


60. Behrooz AB, Syahir A. Could We Address the Interplay Between CD133, Wnt/beta-Catenin, and TERT Signaling Pathways as a Potential Target for Glioblastoma Therapy? Front Oncol. 2021;11:642719.


14. Roberts E, Cossigny DA, Quan GM. The role of vascular endothelial growth factor in metastatic prostate cancer to the skeleton. Prostate Cancer. 2013;2013:418340.


Figures

Figure 1

Schematic diagrams of the in vitro metastasis assay. After coating the outer bottom surface of the 96-well type transwell insert with fibronectin, (A) the transwell inserts were mounted on an ultra-low attachment plate. (B) MMC-treated cells were seeded in the upper chamber of the transwell at a density of $2 \times 10^4$ cells/well; spheroids formed in the bottom chamber were photographed using a phase-contrast microscope, every 24 h up to 96 h.
**Figure 2**

*KiSS1 gene insertion for overexpression and characterization.* DU145 (vector control) cells or DU145-KiSS1 cells stably overexpressing KiSS1 were established using a 293T packaging cell line transfected with a lentiviral vector designed to express the KiSS1 gene. (A) Plasmid map for KiSS1 gene transfection. (B) Comparison of relative expression levels of KiSS1 gene in the two cell lines. Gene levels were quantified through RT-qPCR, and the genes amplified through RT-qPCR were visualized through electrophoresis. The bar graph is expressed logarithmically (log10), and data in the graph were obtained from at least three repeated experiments. The PCR data were statistically analyzed by Student's *t*-test, and are presented as the means ± SEM. Significance is presented at *** (*p*<0.001). (C) Changes in morphology of DU145 cell line before and after lentiviral infection. Scale bar = 100 μm.
**Increased cell proliferation after KiSS1 overexpression.** (A) Cell proliferation was measured by counting the cells. Briefly, cells were seeded at a cell density of $1 \times 10^5$ cells/well in 6-well plates; after 4 days, the number of cells was measured using the EVE™ Automatic Cell Counter. The value of vehicle control was set as 100%. (B, C and G) mRNA levels were measured by RT-qPCR, and (D, E and H) protein levels were measured using automated capillary-based western immunoblot. (B) mRNA expressions of cell cycle genes and proliferation-related genes, (C) PI3K/Akt signaling genes, Ras family and Raf, (D) Protein expressions of cell cycle proteins, and (E) PI3K/Akt signaling proteins. (F) Tumor spheroid formation: cells were seeded at a density of $2 \times 10^3$ cells/well in ultra-low attachment plate, and cells were allowed to form tumor spheroids. After 1, 4, and 7 days, the tumor spheroids were photographed using a microscope. Scale bar = 200 μm. (G) mRNA expressions of cancer stem cell-related genes. (H) Protein expressions of cancer stem cell-related proteins. Data in the graphs were obtained from at least three repeated experiments, statistically analyzed by multiple t-test, and are presented as the mean ± SEM. Significance is presented at * ($p<0.05$), ** ($p<0.01$) or *** ($p<0.001$). AU: arbitrary unit.
Effects of KiSS1 overexpression on the KiSS1R down-stream pathway. Cell proteins were extracted using RIPA buffer, and automated capillary-based western immunoblot was performed to investigate the changes in protein levels after overexpression of the KiSS1 gene. The protein levels were quantified using the Compass Simple Western software, and all protein expression levels were normalized to the levels of GAPDH protein expression in each band. Protein expressions in DU145 (vector control) and DU145-KiSS1 cells: (A) KiSS1R, (B) β-arrestin1/2, (C) p-Raf^{Ser445}/Raf ratio, (D) p-MEK1/2^{Ser217/221}/MEK1/2 ratio, (E) p-ERK1/2^{Thr202/Tyr204}/ERK1/2 ratio, (F) p65, and (G) p50. Data in the graph were obtained from at least three repeated experiments, statistically analyzed by Student’s t-test, and are presented as the means ± SEM. Significance is presented at * (p<0.05), ** (p<0.01) or *** (p<0.001).
**Figure 5**

**Effects of KiSS1 overexpression on cancer cell migration.** (A) Transwell migration assay and transwell invasion assay. MMC-treated and Hoechst 33342 pre-stained cells were seeded in the upper chamber of a transwell insert with (invasion assay) or without (migration assay) fibronectin coating; 72 h later, the blue fluorescence of cells that migrated to the bottom chamber was photographed using a fluorescence microscope. Scale bar = 200 μm. (B) Collagen invasion assay and (C) quantitative graph. MMC-treated tumor spheroids were seeded in a 96-well plate coated with collagen, and the tumor spheroids were photographed using a phase-contrast microscope every 24 h, for up to 96 h. Scale bar = 200 μm. (D) *In vitro* metastasis assay and (E) quantitative graph. MMC-treated cells were seeded in the upper chamber containing a combination of a fibronectin-coated transwell insert and ultra-low attachment plate, and the spheroids formed in the bottom chamber were photographed using a phase-contrast microscope every 24 h, for up to 96 h. (F) mRNA expressions of EMT genes (RT-qPCR). Protein expressions of (G) E-cadherin, (H) N-cadherin, (I) Slug, and (J) MMP9 (immunoblotting). Data in the graph were obtained from at least three repeated experiments, statistically analyzed by Student’s *t*-test or multiple *t*-test, and are presented as the means ± SEM. Significance is presented at * (p<0.05), ** (p<0.01) or *** (p<0.001). AU: arbitrary unit.
Effects of KiSS1 overexpression on tumor growth in the xenograft mouse model. (A) Tumor volumes were measured by a caliper every 2-3 days from day 5 after cancer cell inoculation, and calculated by the equation: shortest diameter$^2$$\times$longest diameter$\times$0.5236 (mm$^3$). (B) Area under curve (AUC) of (A). (C) Tumor weights were measured at study termination. (D) Tumor images were obtained at study termination (day 19). (E) Body and (G) organ weights were measured at study termination. (F) AUC of (E). Data are presented as the means ± SEM obtained from six mice per group. Significance is presented at * ($p<0.05$), ** ($p<0.01$) or *** ($p<0.001$). (H) Ki-67 and PCNA expressions in tumor sections.
Figure 7

Effects of KISS1 overexpression on angiogenesis. (A) HUVEC tube formation assay. SV40-HUVECs were stained with Calcein-AM, and DU145 or DU145-KiSS1 cells were seeded in the cancer cell culture medium in Matrigel coated plates. Tube formation in SV40-HUVECs was observed after 3 h, using a fluorescence microscope. mRNA expressions of (B) VEGF-A and (C) CD31 (RT-qPCR). Protein expressions of (D) VEGF-A and (E) CD31 (immunoblotting). Data in the graph were obtained from at least three repeated experiments, statistically analyzed by Student’s t-test, and are presented as the means ± SEM. Significance is presented at * (p<0.05), ** (p<0.01) or *** (p<0.001). (F) H&E staining in tumor sections of the xenograft mouse model. DU145 cells (vector control) or DU145-KiSS1 cells were subcutaneously inoculated in mice (n=6 per group). H&E was performed for pathological analysis of angiogenesis. In the ×40 magnification images, the rectangle indicates a ×200 magnification image below. Blood vessels are denoted by black arrows. Scale bar for the ×40 image = 500 μm; Scale bar for the ×200 image = 100 μm. (G) IHC was performed to determine the expressions of VEGF-A and CD31 proteins in tumor tissues. Scale bar =100 μm.
Figure 8

**Proposed model for KiSS1-mediated signaling pathway in metastatic prostate cancer.** The kisspeptin translated from the *KiSS1* gene binds to KISS1R, a G-protein receptor located in the cell membrane, and initiates signal transduction. Phosphorylation of Akt increases the growth of prostate cancer by regulating the cell cycle gene expressions, and increases cell survival through NF-κB. Upregulation of CD133 and β-catenin induces tumorigenesis, which is accelerated by increased cell growth. MMP9 is upregulated by either NF-κB or β-arrestin1/2, and β-arrestin1/2 interacts with the phosphorylated Raf-MEK1/2-ERK1/2 axis to induce upregulation of the MMP9 protein by transcribing the *NF-κB* gene, which in turn induces migration and invasion of prostate cancer cells. The up-regulation of N-cadherin or down-regulation of E-cadherin by KISS1R induces EMT in cancer cells, thereby contributing to cell migration. Up-regulated expressions of CD31 and VEGF by *KiSS1* affects angiogenesis, and contributes to prostate cancer metastasis.