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Kisspeptin Promotes Glioblastoma Cell Invasiveness *Via* the Gq-PLC-PKC Pathway

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Abstract. *Background/Aim: Kisspeptin produced from the KISS1 gene is secreted from the living cells, binds to endogenous receptor KISS1R (also called G protein-coupled receptor 54, GPR54), and has various functions in normal physiological conditions. Although an anti-metastatic role of kisspeptin in cancer is well known in several cancer types, its role in brain tumors is not yet understood. Herein, we investigated a the role of kisspeptin in glioblastoma cells. Materials and Methods: Glioblastoma cells were treated with kisspeptin and subjected to proliferation, migration, and invasion assays. KISS1R dependency was tested by KISS1R silencing with KISS1R siRNAs. Results: Kisspeptin inhibited migratory and invasive abilities of U87-MG, U-251-MG and U373-MG glioblastoma cells with no effect on cell viability. KISS1R gene silencing with KISS1R siRNAs blocked kisspeptin-induced glioblastoma cell invasiveness. Moreover, chemical inhibitors against Gq, PLC or PKC blocked kisspeptin-induced glioblastoma cell invasiveness. Conclusion: Kisspeptin induces glioblastoma cell invasiveness via the KISS1R-Gq-PLC-PKC signaling pathway.*

Glioblastoma, type IV astrocytoma or glioblastoma multiforme, occurs in the brain or spinal cord, and originates from abnormal astrocytes (1-4). Although surgery, radiotherapy and and drug treatment are applied to treat glioblastoma, high-grade glioblastomas are not well treated and supportive care is rather considered (2, 5-7). For example, type IV glioblastoma multiforme is resistant to temozolomide, while type III glioblastoma is sensitive to it (6, 8-12).

Kisspeptin produced from the *KISS1* gene is secreted from cells, binds to endogenous receptor KISS1R (also called G protein-coupled receptor 54, GPR54), and plays multiple roles in normal physiological conditions, thereby being applied for treating abnormalities (13-16). It has been well known to regulate the onset of puberty and inhibit cancer metastasis (16-19). Moreover, kisspeptin-mediated signaling has been revealed to regulate circadian rhythm, appetite and reproduction (13, 15, 19-23). Kisspeptin is known to regulate KISS1R-Gq/11-PLC-PKC pathway in the brain, although we still need more knowledge for kisspeptin-mediated signaling networks (17, 18, 20, 22-24). Likewise, kisspeptin regulates this KISS1R-Gq/11-PLC-PKC signaling pathway in the inhibition of cancer metastasis (17, 18, 20).

Kisspeptin is on clinical trials to solve problems in either fertility or puberty (13, 15-17). A therapeutic drug design is also considered on the basis of its anti-metastatic role (25-27). Kisspeptin inhibits cancer cell proliferation, migration and invasion in dependence of cancer cell types (17, 20). Moreover, kisspeptin blocks VEGF-induced tumor angiogenesis by inhibiting FAK in endothelial cells (28). However, kisspeptin-mediated signaling is likely to be required in the *in vivo* breast cancer model systems (29). Consistently, kisspeptin was reported to accelerate the invasiveness of highly metastatic triple-negative breast cancer cells (30, 31). Therefore, understanding its biological role in cancer is urgent in order to decide if such treatment schedules are applicable in the clinic (13, 21, 25-27, 32, 33).

Knowledge for an anti-metastatic role of kisspeptin in cancer is well introduced in different cancer types, although its role in brain tumor is yet reported (17, 18, 34). Considering its anti-metastatic role, we focused on its role in highly aggressive type IV glioblastoma multiforme. Interestingly, we found that kisspeptin accelerates the metastatic ability of glioblastoma cells representing type IV glioblastoma multiforme *via* its well-known G protein-coupled receptor (GPCR) pathway. Brain tumor cells do not express endogenous kisspeptin. As exogenous kisspeptin stimulated endogenous KISS1R-mediated intracellular signaling pathways in brain tumor cells, we could assume that endogenous kisspeptin from other regions of the brain could activate tumoral KISS1R and regulates brain tumor invasiveness.

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Materials and Methods

Cell culture and reagents. U87-MG, U-251-MG and U373-MG cells were kindly received from Dr. Sun-Ha Paik at Seoul National University Medical Center, Seoul, Korea. All cells were cultured in DMEM supplemented with 10% FBS and 1% Pen/Strep antibiotics. Kisspeptin-10 (YNWNSFGLRF-NH_2) was purchased from Anaspec (Fremont, CA, USA). Gq inhibitor YM-254890 was obtained from Focus biomolecules (Plymouth Meeting, PA, USA). PLC inhibitors D609 and U73122 were purchased from Tocris (Minneapolis, MN, USA). PKC inhibitor bisindolylmaleimide III was purchased from Cayman (Ann Arbor, MI, USA).

Cell studies. A total of 1×10^4 cells were cultured on 96 well plates and subjected to WST assays (Dogen, Seoul, Republic of Korea). In addition, we conducted trypan blue assays to confirm cell growth by manually counting viable cells. Each experiment was conducted in triplicate and independently repeated three times. For cell migration, 1×10^5 cells were cultured on 6 well plates, followed by scratching using yellow tips and washing with PBS twice. After a 24-h scratching, cells which migrated onto scratched regions were counted. Experiments were duplicated and independently conducted in triplicate. For cell invasion, 1×10^4 cells were settled in chambers with matrigel-precoated 8-μm pore polyester membrane (Corning, NY, USA) and cultured in no serum medium. Twenty-four-well plates at bottom were filled with 10% serum medium. Cells invaded into matrigel were stained with 0.5% crystal violet solution and counted manually. Experiments were conducted in triplicate. *KISS1R* siRNAs for *KISS1R* gene silencing and control siRNAs were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). *KISS1R* gene silencing was conducted by manufacturer's instruction. In brief, 1×10^6 cells were transfected with siRNAs for 12 h and then subjected to the appropriate experiments.

Western blots. A total of 3×10^6 cells were lysed with radioimmunoprecipitation buffer for 30 min on ice and centrifuged at 20,000 × *g* for 10 min at 4˚C. Protein concentration was measured with the Pierce BCA protein assay kit (Thermo Fisher Scientific, Rockford, IL, USA) according to the manufacturer's protocol. A total of 30 μg of protein was loaded on 10-12% SDS-PAGE and transferred to polyvinylidene difluoride membrane (GE Healthcare Life Sciences, Piscataway, NJ, USA). Membranes were blocked with 5% milk for 1 h at room temperature and incubated with appropriate antibodies for 1 h at room temperature. Protein bands were detected with LumiGLO chemiluminescent reagent and peroxidase (Cell Signaling Technology, Danvers, MA, USA). Antibodies for KISS1 and KISS1R were purchased from Abcam (Cambridge, UK). Antibodies for PKCα/β and actin were purchased from Cell Signaling Technology (Danvers, MA, USA). Horseradish peroxidase (HRP)-linked anti-rabbit IgG or anti-mouse IgG antibody was used as a secondary antibody in dilution 1:10,000.

Statistical analysis. Unpaired Student's *t*-test or one-way analysis of variance with a *post-hoc* Tukey's test was performed to calculate the statistical significance of the results. Data were expressed as the mean±standard deviation, and $p<0.05$ was considered to indicate a statistically significant difference. Calculations were performed using SPSS version 22 software (IBM Corp., Armonk, NY, USA).

Results

Kisspeptin induces glioblastoma cell invasiveness in vitro. To investigate kisspeptin effect on highly aggressive grade IV glioblastoma, we examined kisspeptin effect on glioblastoma cell migration. When U87-MG, U-251-MG or U373-MG cells were scratched and then treated with kisspeptin at 100 nM for 24 h, kisspeptin increased cell migration by approximately 65%, 80% and 45%, respectively (Figure 1A). Furthermore, kisspeptin increased migrated cell numbers even under no serum condition by about 35~60% (Figure 1B).

Accordingly, we examined kisspeptin effect on glioblastoma cell invasiveness. When kisspeptin at 100 nM was added on the upper chamber where the cells were cultured, kisspeptin treatment for 16 h increased the invaded cell number by approximately 30~55% (Figure 1C). However, the invasion was not promoted when kisspeptin was added in the bottom chambers (Figure 1C), indicating that kisspeptin did not induce the invasion of glioblastoma cells by chemotactic attraction.

Next, when U87-MG, U-251-MG and U373-MG cells were treated with kisspeptin for 48 h, kisspeptin did not significantly affect the viability of those cell lines (Figure 1D). Moreover, we failed to find its synergistic effect with temozolomide, when cells were co-treated with kisspeptin and temozolomide (data not shown).

Kisspeptin induces metastatic abilities of glioblastoma cells through KISS1 receptor, KISS1R. Kisspeptin, encoded from the *KISS1* gene and released from cells by post-translational modifications, is known as an endogenous ligand for G-protein-coupled receptor 54 (GPR54; also named KISS1 receptor, KISS1R) (35-37). To test whether kisspeptin requires its receptor for the induction of metastatic abilities, we examined protein expression levels of both KISS1 and KISS1R in U87-MG, U-251-MG or U373-MG cells. All glioblastoma cell lines only expressed KISS1R (Figure 2A).

Next, U87-MG, U-251-MG or U373-MG cells were transfected with *KISS1R* siRNAs or control siRNAs, to answer kisspeptin dependency of KISS1R. Silencing of *KISS1R* gene expression was confirmed by KISS1R blotting (Figure 2B), and the cells were subjected to the invasion assays. *KISS1R* gene silencing blocked kisspeptin-induced cell invasion (Figure 2C). Thus, our data indicate that kisspeptin requires KISS1R in the induction of glioblastoma cell invasiveness.

Kisspeptin induces metastatic abilities of glioblastoma cells by activating the Gq-PLC pathway. It has been known that kisspeptin-activated KISS1R mediates Gq activation (36-38). To examine whether kisspeptin-induced glioblastoma cell invasiveness requires Gq activation, U87-MG cells were pre-

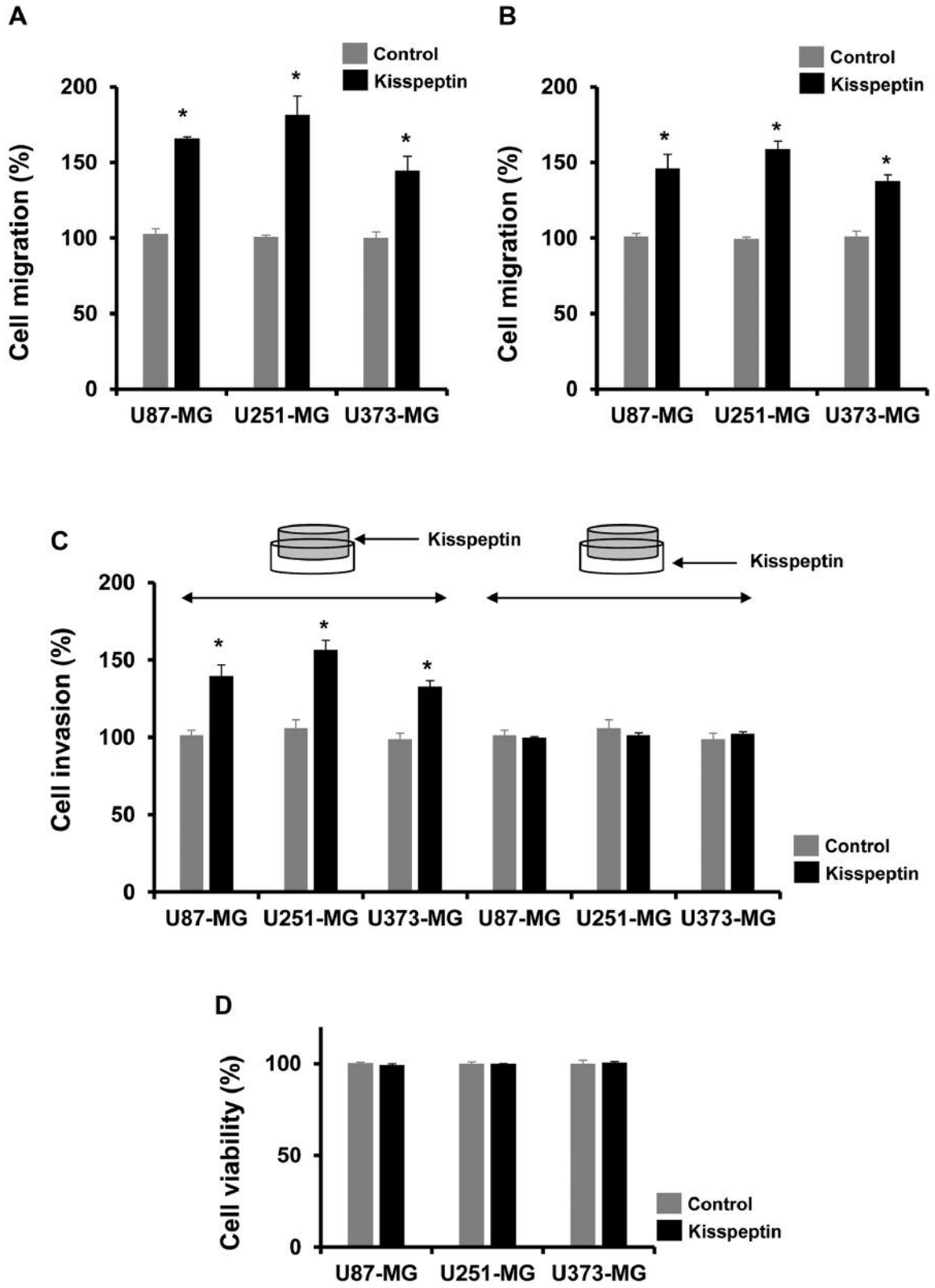

Figure 1. *Kisspeptin induces migratory and invasive abilities of brain tumor cells. (A) Kisspeptin-induced migration of brain tumor cells in complete medium. (B) Kisspeptin-induced migration of brain tumor cells in serum-depleted medium. (C) Kisspeptin-induced invasion of brain tumor cells. (D) Kisspeptin did not affect brain tumor cell viability. *p<0.05.*

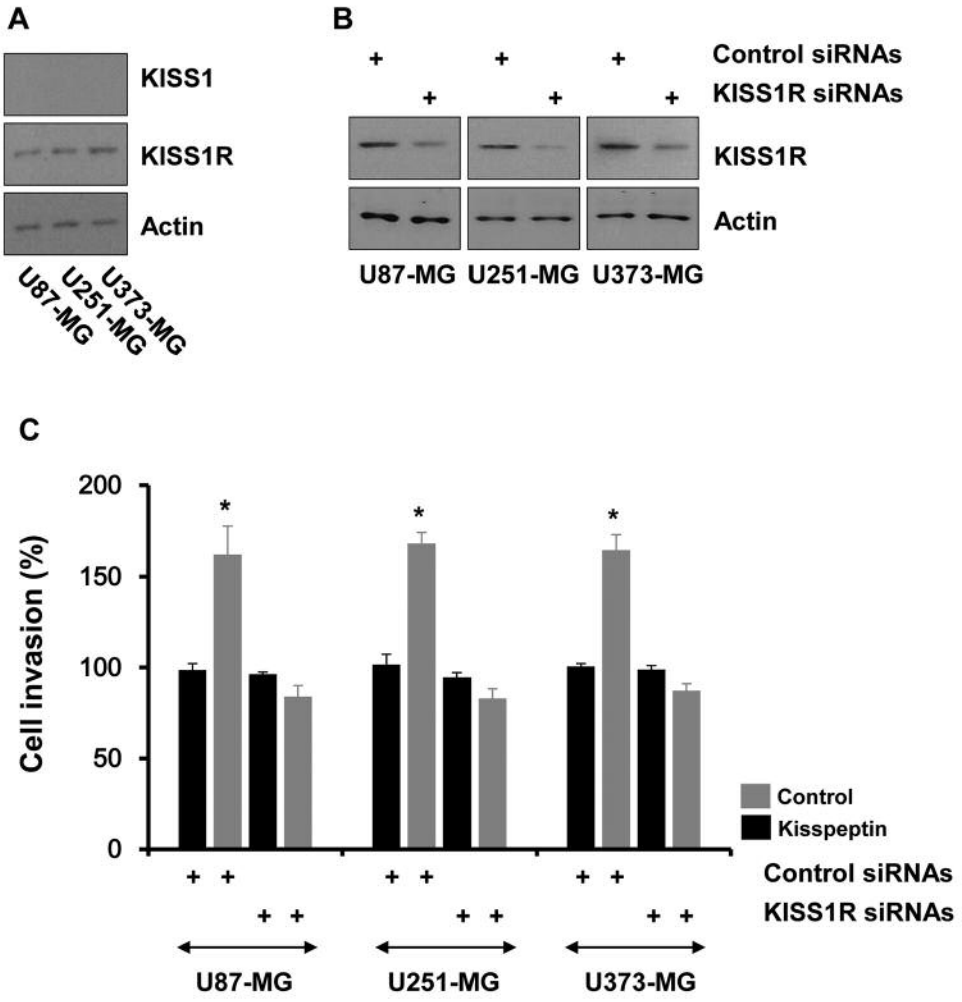

Figure 2. *Kisspeptin induces brain tumor cell invasion via KISS1R. (A) Protein levels of KISS1 and KISS1R in brain tumor cells. Actin was used as an internal control. (B) KISS1R silencing with KISS1R siRNAs was confirmed by KISS1R western blotting. (C) KISS1R gene silencing inhibits brain tumor cell invasion (D). *p<0.05.*

treated with Gq inhibitor, YM-254890, and then treated with kisspeptin. Gq inhibition repressed kisspeptin-induced invasiveness of glioblastoma cells (Figure 3A).

Kisspeptin is known to increase IP3 production *via* KISS1R-Gq-PLC pathway (38). To examine whether kisspeptin-activated KISS1R requires phospholipase C (PLC) in the invasiveness, U87-MG cells were pre-treated with PLC inhibitor D609 or U73122, and then treated with kisspeptin. PLC inhibition blocked kisspeptin-induced invasiveness (Figure 3B). Therefore, our data indicate that kisspeptin activation of KISS1R-Gq-PLC pathway promotes glioblastoma cell invasiveness.

Kisspeptin induces metastatic abilities of glioblastoma cells by activating PKC. PKC activation promotes glioblastoma cell invasiveness (39, 40). Because kisspeptin activates PKC, we next examined whether kisspeptin activation of PKC leads to metastatic abilities of glioblastoma cells. When cells were pre-treated with PKC inhibitor, bisindolylmaleimide III at 20 nM for 1 h and then treated with kisspeptin for another 23 h, PKC inhibition blocked kisspeptin-induced invasiveness (Figure 4A). Next, PKCα/β phosphorylation status was examined. Kisspeptin increased PKCα/β phosphorylation, when cells were serum-starved for 3 h and then treated with kisspeptin for another 15 min (Figure 4B). Moreover, kisspeptin increased PKCα/β phosphorylation even when cells were cultured in serum-contained medium and treated with kisspeptin for 15 min (Figure 4B). Thus, kisspeptin activates PKCα/β, although serum factors may attenuate kisspeptin effect on PKCα/β phosphorylation. In addition, bisindolylmaleimide III blocked kisspeptin activation of PKCα/β phosphorylation (Figure 4C).

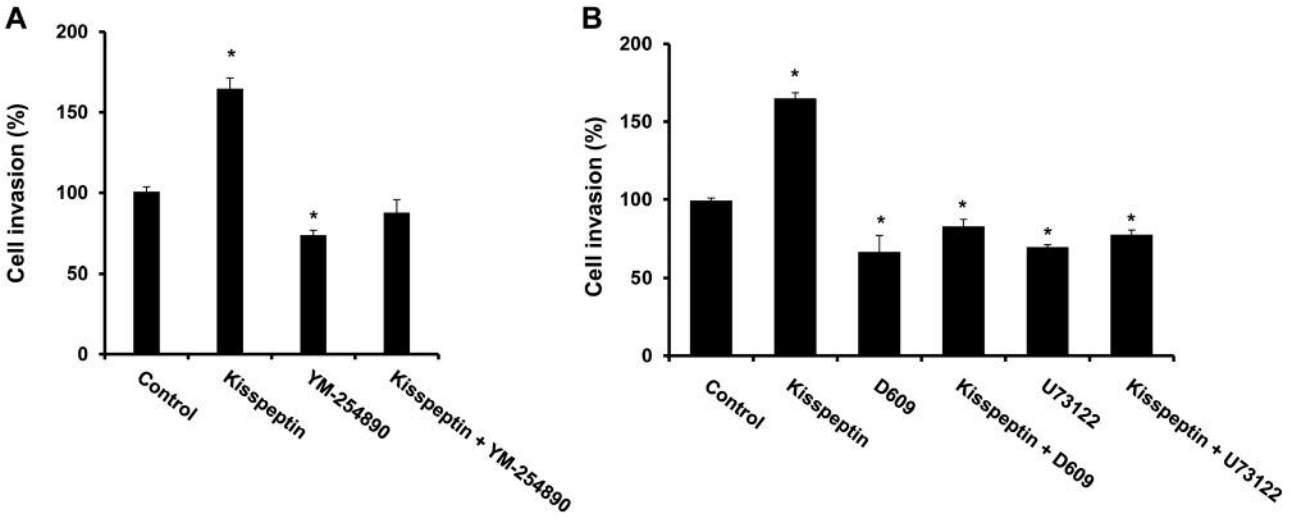

Figure 3. *Kisspeptin induces brain tumor cell invasiveness via the Gq-PLC pathway. (A) Gq inhibitor, YM-254890 blocked kisspeptin-induced brain tumor cell invasion. (B) PLC inhibitors, D609 and U73122 blocked kisspeptin-induced brain tumor cell invasion. *p<0.05.*

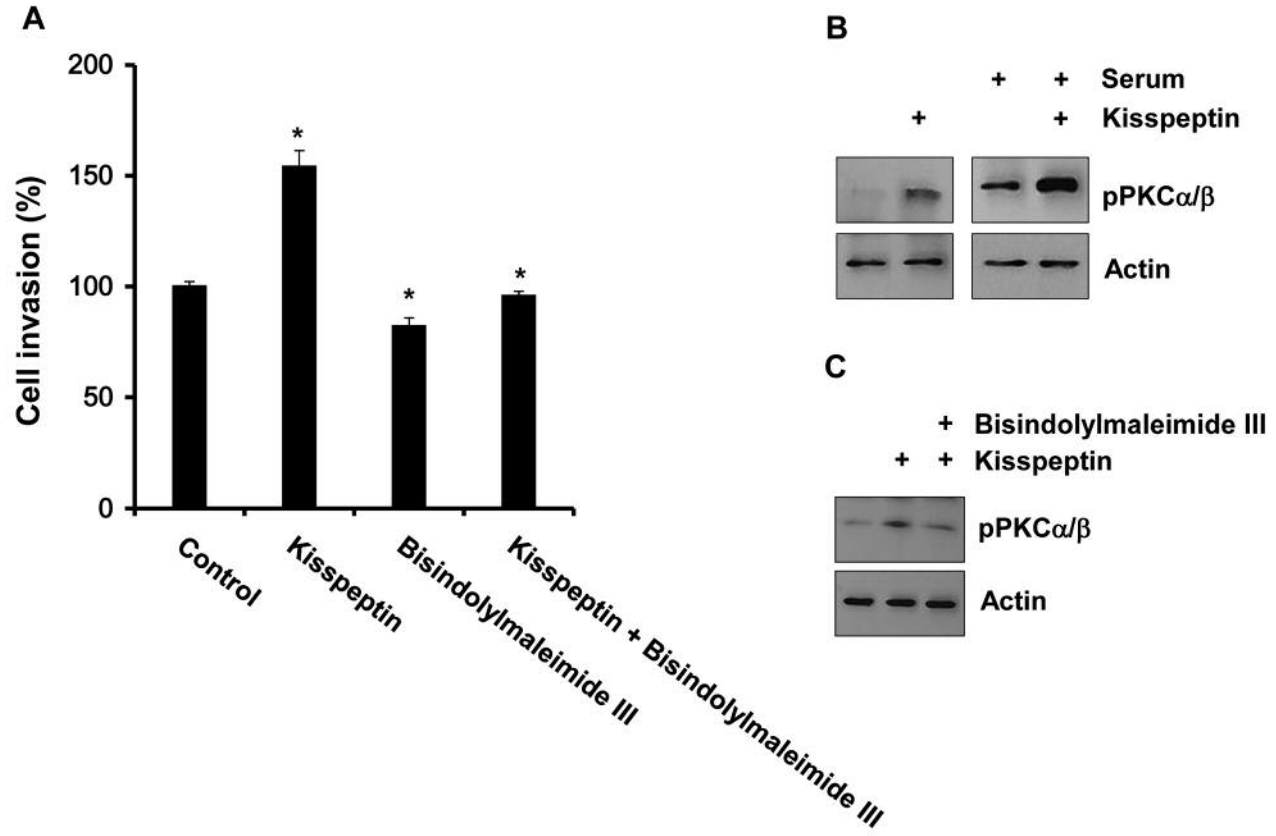

Figure 4. *Kisspeptin-induced brain tumor cell invasion requires PKC activation. (A) PKC inhibitor, bisindolylmaleimide III blocked kisspeptin-induced brain tumor cell invasion. *p<0.05. (B) Kisspeptin induced PKCα/β phosphorylation in both serum-starved and serum-contained conditions. (C) PKC inhibitor, bisindolylmaleimide III inhibited kisspeptin-induced PKCα/β phosphorylation.*

Thus, kisspeptin activation of PKC is crucial for the invasiveness of glioblastoma cells.

Discussion

This study is the first study to report a role of kisspeptin in brain tumor. Our data showed that kisspeptin promotes invasiveness of glioblastoma cells by inducing KISS1R-Gq-PLC-PKC signaling.

Glioblastoma cell lines conventionally used for *in vitro* studies do not express endogenous kisspeptin while expressing its endogeneous receptor, KISS1R. Kisspeptin expression in glioblastoma cell lines may be shut down by epigenetic mechanisms or mutations. Epigenetic regulation of either KISS1 or KISS1R has been reported in various biological conditions (41, 42). Meanwhile, TCGA and cBioPortal show no mutations of KISS1 in glioblastoma. However, it is still unknown whether *KISS1* gene expression is epigenetically repressed in glioblastoma. Therefore, it is expected that future studies will address an epigenetic regulation of *KISS1* gene expression.

Our study aiming to investigate the role of kisspeptin in glioblastoma, explored a conventional KISS1R-mediated intracellular signaling pathway. In glioblastoma cells, kisspeptin activated Gq-PLC pathway *via* KISS1R. Moreover, glioblastoma cells required PKC activation in kisspeptin-promoted invasiveness. This finding is in line with a recent report that Gq-PLC-PKC promotes cancer cell invasiveness (17, 39, 40).

Our data showed that exogenous kisspeptin promotes the invasiveness of glioblastoma cells. This finding raises a question of whether endogenous kisspeptin affects local metastasis of glioblastoma. If kisspeptin is expressed in cells near glioma cells, it may promote the invasiveness of glioma cells. In addition, circadian rhythm and/or dietary conditions may alter glioma cell invasiveness *via* endogenous kisspeptin. Circadian rhythm is known to promote glioblastoma cell proliferation and migration (43, 44). Thus, it is important to find kisspeptin-expressing cells in microenvironment of glioblastoma.

Kisspeptin has been known to repress invasiveness of various cancer cell types (17, 18, 20). In this study, we presented opposite results; kisspeptin was shown to induce glioblastoma invasiveness. We still do not know how kisspeptin has two different roles in different cancer cell types. Future studies will address whether a blockade of kisspeptin-KISS1R signaling inhibits the invasiveness of glioblastoma cells. These studies may help elucidate how the tumor microenvironment regulates glioblastoma metastasis and potentially provide a therapeutic target.

Conflicts of Interest

The Authors declare no potential conflicts of interest.

Authors' Contributions

Kim TH conducted experiments and analysed data. Yoon JH performed experiments. Cho SG designed experiments and wrote the manuscript.

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