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Kisspeptin Inhibits Colorectal Cancer Cell Invasiveness by Activating PKR and PP2A

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Abstract. *Background/Aim: The aim of the present study was to investigate the mechanism through which kisspeptin inhibits colorectal cancer metastasis. Materials and Methods: Colorectal cancer cells were treated with kisspeptin and then subjected to assays for cell viability, migration, invasion and anchorage-independent growth. Kisspeptin receptor (KISS1R) requirement was examined by siRNA-based gene silencing followed by western blot and invasion assays. Kisspeptin regulation of PKR and PP2A was examined by treating cells with inhibitors for PKR or PP2A. Results: Kisspeptin inhibited colorectal cancer cell invasiveness without affecting cell proliferation. Kisspeptin required activation of KISS1R and resulted in activation of PKR and PP2A. PKR inhibitor blocked kisspeptin-induced PP2A phosphorylation, while PP2A inhibitor failed to block kisspeptin-induced PKR phosphorylation. Conclusion: Kisspeptin-mediated activation of PKR-PP2A inhibited colorectal cancer cell invasiveness.*

While most kisspeptin studies focus on its physiological role in the brain (1, 2), its function was first identified in cancer (3, 4). Re-introduction of *KISS1* gene in metastatic breast cancer and melanoma cells repressed metastases, suggesting that a product of *KISS1* gene is a metastasis suppressor (3, 4). Kisspeptin is secreted from the cells and binds to its endogenous G-protein-coupled receptor KISS1R (also named GPR54) (5-8).

KISS1 gene expression is correlated with a favorable prognosis for patients with various cancer types, thus, it is probable that kisspeptin signaling is turned off when cancer metastasizes (9-13). Kisspeptin is known to generate its own intracellular signaling through KISS1R (9, 14). However, its signaling network that regulates phenotypes of cancer cells remains to be deciphered.

Colorectal cancer is one of the gastrointestinal cancers and its early detection improves treatment outcomes (15-17). However, colorectal cancer diagnosis is low and distant metastases are common and contribute to poor prognosis (18). Kisspeptin inhibits the invasiveness by reducing MMP9 expression in HCT-115 and HCT-119 colorectal cancer cell lines (19). Consistently, *KISS1* expression level is negatively correlated with MMP9 expression level in colorectal cancer tissues (10, 20). This relationship between kisspeptin and MMP9 was revealed in other cancer cell types (21, 22). Kisspeptin may reduce the expression of MMP9 by inhibiting phosphorylation of AKT in HCT-119 cells or ERK in HCT-115 cells (10, 19). Moreover, kisspeptin inhibits the invasiveness of LoVo colorectal cancer cells by inhibiting PKR activation (23). Nevertheless, kisspeptin-mediated intracellular signaling in colorectal cancer is still not fully understood.

The present study examined the mechanism by which kisspeptin inhibits the invasiveness of various colorectal cancer cells and may further our knowledge regarding the application of kisspeptin in treatments of colorectal cancer metastasis.

Materials and Methods

Cell culture and reagents. Colorectal cancer cell lines (CACO-2, COLO-205, HCT-116, HT-29, LoVo, LS174T, SNU0283, SNU1033, SW-620) were cultured in DMEM supplemented with 10% fetal bovine serum and 1% antibiotics. HCT-116 and HT-29 cells were obtained from ATCC while SNU-0283 and SNU-1033 cells were purchased from Seoul National Cell Line Bank. Other cell lines were obtained from Dr. Bharat Aggarwal (UT-MDA, TX, USA). PKR inhibitor C16 and control or KISS1R siRNA reagent system for gene silencing were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). PP2A inhibitor LB-100 was obtained from Selleckchem (Houston, TX, USA)., siRNA was. Anti-PKR, anti-p-PKR and anti-p-PP2A antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-Actin, anti-AKT and anti-ERK antibodies were purchased from Cell Signaling (Danvers, MA, USA). Anti-KISS1R antibody was purchased from Abcam (Cambridge, UK).

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Key Words: Kisspeptin, KISS1, KISS1R, PKR, PP2A, colorectal cancer.

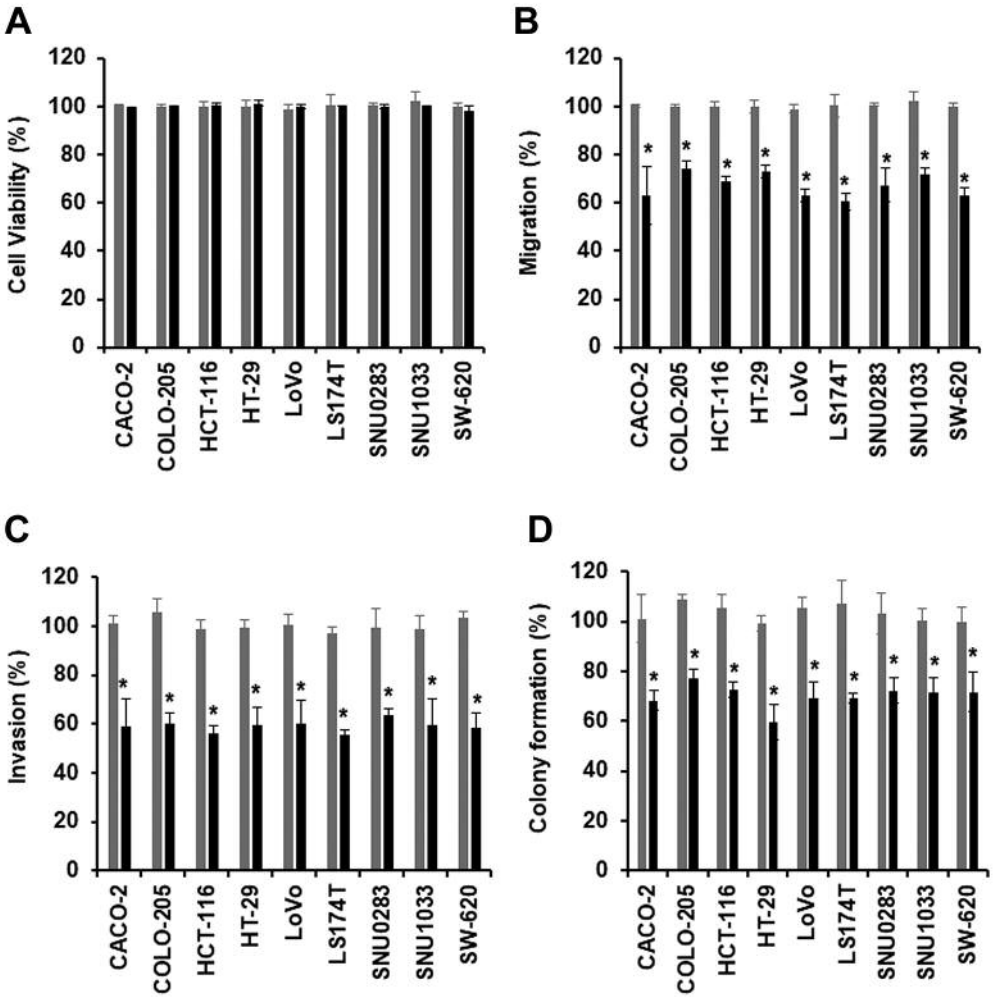

Figure 1. *Kisspeptin inhibits colorectal cancer cell invasiveness. (A) Kisspeptin effect on colorectal cancer cell viability. Cells were treated with 100 nM kisspeptin for 48 h and then subjected to WST assays. (B) Cells were scratched and then treated 100 nM with kisspeptin for 24 h. Migrated cells were counted. Experiments were performed in triplicate and repeated three times. *p<0.05. (C) Cells were cultured on the upper chamber precoated with matrigels and then treated with 100 nM kisspeptin for 18 h. Invaded cells were counted. *p<0.05. All data were presented as means±SD. (D) Cells were cultured in soft agars for 2 weeks and then colonies stained with crystal violet were counted. All experiments were repeated for three times. *p<0.05.*

Cell viability. 3×10^4 colorectal cancer cells were cultured onto 96-well plates and then treated with kisspeptin at 100 nM for 48 h. Cell viability was measured by EZ-CyTox WST-based cell viability and cytotoxicity assay kit (DoGen, Seoul, Korea). Experiments were performed in triplicate.

Western blot. Protein was extracted with RIPA buffer. 30μg of protein were loaded on 10% SDS-PAGE and transferred to PVDF membranes. Anti-PKR, anti-p-PKR and anti-p-PP2A antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-Actin, anti-AKT and anti-ERK antibodies were purchased from Cell Signaling (Danvers, MA, USA). Anti-KISS1R antibody was purchased from Abcam (Cambridge, UK). All primary antibodies were used in a 1:1,000 dilution. Horseradish peroxidase (HRP)-linked anti-rabbit IgG and anti-mouse IgG antibody as secondary antibodies were used in dilution 1:20,000. Western blots were detected using LumiGLO chemiluminescent reagent and peroxidase (Cell Signaling, Danvers, WI, USA).

Migration and invasion. 3×10^5 cells were cultured onto 6-well plates were scratched using 200 μl yellow tips and washed with PBS three times. Random cell migration was measured by counting cells migrated within the scratched regions. For invasion assays, 3×10^4 cells were seeded on top chambers precoated with matrigels. Top chambers were then filled with 1% serum-containing medium, and bottom chambers were filled with 10% serum-containing medium. 18 h after incubation, top chambers were carefully cleared with cotton swaps, and invaded cells stained with 1% crystal violet were counted. All experiments were performed in triplicate.

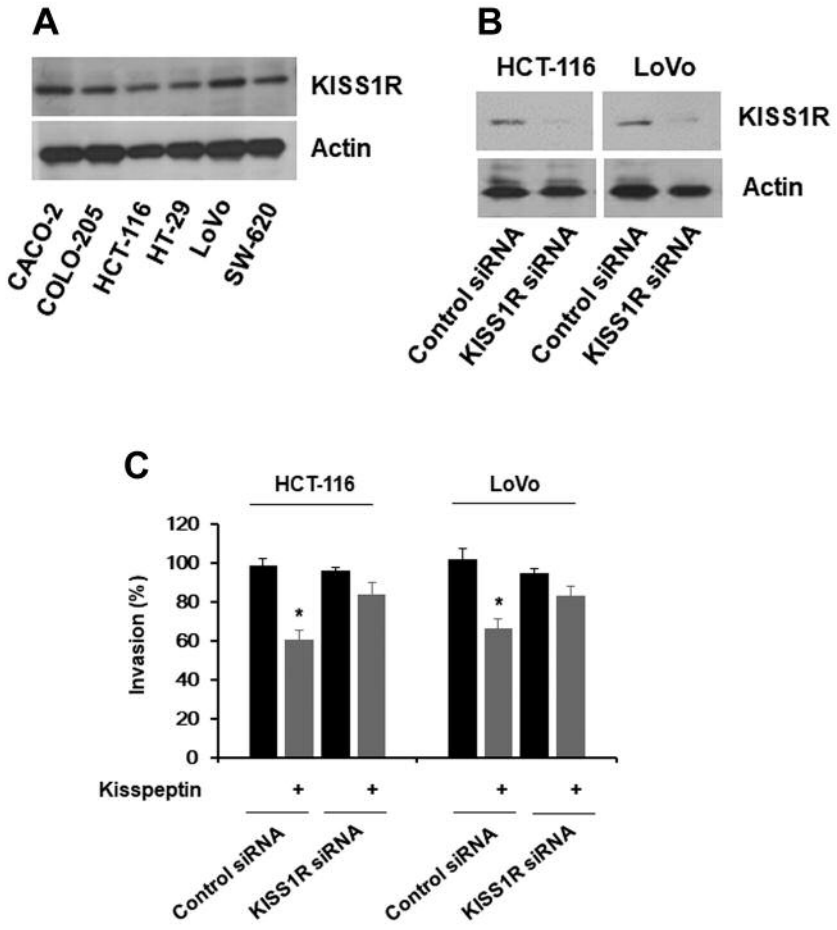

Figure 2. *Kisspeptin requires KISS1R to inhibit colorectal cancer cell invasiveness. (A) KISS1R expression in different colorectal cancer cells. Actin was used as a loading control. (B) Cells were transfected with either control siRNA or KISS1R siRNA for 24 h and then subjected to western blots. (C) KISS1R was silenced for 24 h and then cells were subjected to invasion assays. Cells were treated with 100 nM kisspeptin and then invasion were observed for 18 h. *p<0.05.*

Anchorage-independent growth. 3×10^4 cells were cultured in 0.35% soft agar for 2 weeks. Dishes were stained using 1% Crystal Violet and macroscopically visible colonies were counted. Experiments were conducted in triplicate.

Statistics. Statistical tests were performed using Student's *t*-test or one-way ANOVA. Results were expressed as means±SD or means±SE, and *p*-value less than 0.05 was considered statistically significant.

Results

Kisspeptin inhibited metastasis of colorectal cancer cells. To examine the effect of kisspeptin on colorectal cancer cell lines, different colorectal cancer cell lines (CACO-2, COLO-205, HCT-116, HT-29, LoVo, LS174T, SNU0283, SNU1033, SW-620) were treated with kisspeptin at different concentrations for 48 h and then subjected to cell proliferation assays. Kisspeptin treatment did not affect viability of the various colorectal cancer cell lines (Figure 1A). Consistently, we failed to detect both cleaved PARP and caspase-3 (data not shown), when cells were treated with kisspeptin for 24 h.

Next, we performed scratching assays to examine kisspeptin effect on colorectal cancer cell migration. Scratching assays indicated that treatment with kisspeptin for 24 h reduced colorectal cancer cell migration (Figure 1B). In addition, kisspeptin inhibited colorectal cancer cell invasiveness in two-chamber assays (Figure 1C). To confirm our findings on cell migration and invasion assays, soft agar assays were performed. Kisspeptin treatment inhibited anchorage-independent growth (Figure 1D). Collectively, our data indicate that kisspeptin inhibited metastatic abilities of colorectal cancer cells.

Kisspeptin inhibited metastasis of colorectal cancer cells via KISS1R. Next, the requirement of KISS1R for the kisspeptin

effects was investigated. Colorectal cancer cell lines (CACO-2, COLO-205, HCT-116, HT-29, LoVo, SW-620) expressed KISS1R (Figure 2A). In the absence of KISS1R expression, following its silencing in HCT-116 and LoVo cells using KISS1R siRNAs, kisspeptin had no effect on their invasiveness (Figure 2B and 2C). These findings indicate that kisspeptin required KISS1R for the inhibition of colorectal cancer cell invasiveness.

Kisspeptin inhibited colorectal cancer cell invasiveness via PKR. As PKR is involved in kisspeptin inhibition of cancer cell invasiveness (23), we examined kisspeptin-mediated PKR phosphorylation in HCT-116 and LoVo colorectal cancer cells. Kisspeptin increased PKR phosphorylation in both HCT-116 and LoVo cells (Figure 3A), and KISS1R silencing with KISS1R siRNAs in HCT-116 and LoVo cells blunted kisspeptin-induced PKR phosphorylation (Figure 3B).

To confirm that kisspeptin signaling requires PKR phosphorylation in the inhibition of colorectal cancer cell invasiveness, HCT-116 or LoVo cells were pretreated with the PKR inhibitor C16 for 5 min and then were incubated with kisspeptin for another 12 h. The invasion assays showed that PKR inhibition resulted in the rescue of kisspeptin-blocked invasiveness (Figure 3C). Thus, our data indicate that kisspeptin involved PKR in the inhibition of colorectal cancer cell invasiveness.

Kisspeptin activation of PP2A via PKR represses the invasiveness of colorectal cancer cells. Kisspeptin inhibited phosphorylation of ERK and AKT in colorectal cancer cells (10, 19). Thus, to examine whether kisspeptin affects phosphorylation of ERK and AKT *via* PKR, HCT-116 cells were pretreated with the PKR inhibitor C16 for 5 min and then incubated with kisspeptin for another 15 min. C16 blocked kisspeptin-mediated PKR phosphorylation, did not affect AKT phosphorylation but increased phosphorylation of ERK, while kisspeptin alone increased phosphorylation of both PKR and AKT but not ERK phosphorylation, and C16 alone increased phosphorylation of both AKT and ERK but inhibited PKR phosphorylation (Figure 4A). Therefore, our data indicate that kisspeptin-activated PKR may inhibit phosphorylation of both AKT and ERK.

PKR is known to inhibit AKT phosphorylation *via* PP2A activation (24, 25). PP2A activation also inhibits ERK phosphorylation (26). Moreover, PP2A activity is inversely correlated with colorectal cancer progression (27, 28). Therefore, to examine the role of PP2A, its inhibitor LB-100 was employed. PP2A inhibitor did not affect kisspeptin-mediated phosphorylation of PKR, while it increased phosphorylation of AKT and ERK (Figure 4B). Furthermore, PP2A inhibition rescued kisspeptin effect on the invasiveness of both HCT-116 and LoVo cells (Figure 4C). When HCT-116 cells were pretreated with either PKR or PP2A inhibitor for 5 min and then treated with kisspeptin for 15 min, PKR inhibition blocked PP2A phosphorylation, while PP2A inhibition did not affect PKR phosphorylation (Figure 4D). Therefore, our data indicate that kisspeptin repressed the invasiveness of colorectal cancer cells *via* PKR-PP2A signaling.

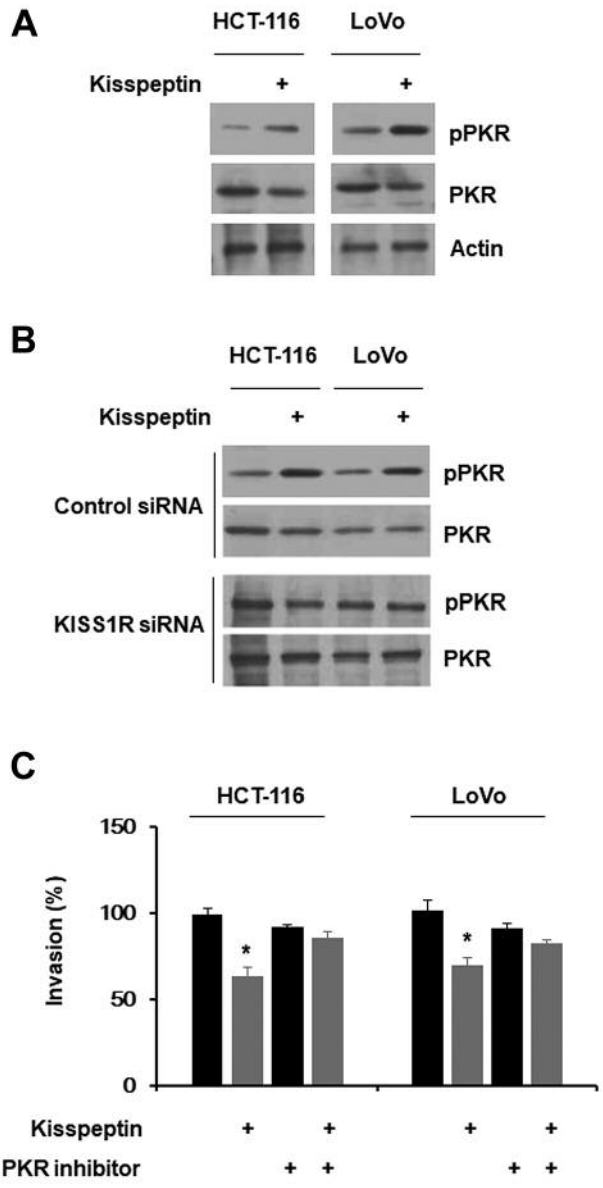

Figure 3. *Kisspeptin inhibits colorectal cancer cell invasiveness via PKR phosphorylation. (A) Cells were treated with 100 nM kisspeptin for 15 min and then cell extracts were subjected to western blots. (B) Cells were transfected with either control or KISS1R siRNA for 24 h and then treated with kisspeptin for 15 min. (C) Cells were pretreated with 200 nM PKR inhibitor for 5 min and then treated with kisspeptin for another 12 h. *p<0.05.*

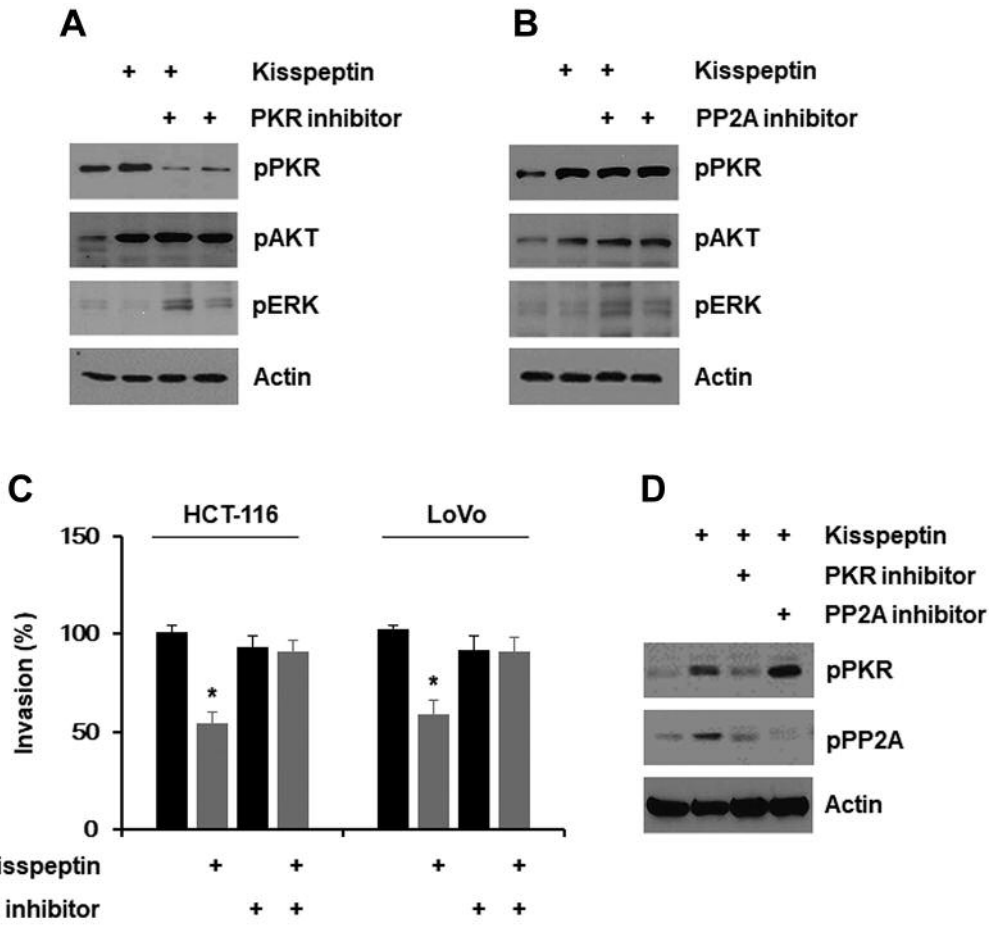

Figure 4. *Kisspeptin-PKR signaling requires PP2A to inhibit colorectal cancer cell invasiveness. (A) Cells were pretreated with 200 nM PKR inhibitor for 5 min and then treated with 100 nM kisspeptin for another 15 min. (B) Cells were pretreated with 3 μM PP2A inhibitor for 5 min and then treated with kisspeptin at 100 nM for another 15 min. (C) Cells were pretreated with 200 nM PKR inhibitor for 5 min and then treated with 100 nM kisspeptin for another 12 h. *p<0.05. (D) Cells were pretreated with either 200 nM PKR inhibitor or 3 μM PP2A inhibitor for 5 min and then treated with 100 nM kisspeptin for another 15 min.*

Discussion

Kisspeptin has been revealed to inhibit the invasiveness of colorectal cancer cells, while clinical data are controversial and required to be confirmed in depth (10, 19, 20, 23, 29-34). Kisspeptin is known to reduce MMP-9 expression through either AKT or ERK in colorectal cancer (10, 19). These findings from different groups indicated an inhibitory role of kisspeptin against colorectal cancer cells. Moreover, our *in vitro* and *in vivo* studies revealed that kisspeptin blocked distant metastasis of LoVo colorectal cancer cells through PKR activation (23). In this study, kisspeptin signaling in the inhibition of colorectal cancer cell invasiveness was further investigated. Our data showed that kisspeptin repressed the invasiveness of colorectal cancer cells *via* PKR and PP2A.

In clinical data, KISS1 expression level was lower in colorectal cancer tissues than in adjacent normal tissues, and negatively correlated with poor prognosis, which is supported by KISS1 promoter methylation status in colorectal cancer tissues (20, 31-33). However, other groups reported that KISS1 expression level was higher in colorectal cancer tissues than in adjacent normal tissues and that plasma kisspeptin level was also higher in colorectal cancer patients (30, 34). Kisspeptin treatment or KISS1 overexpression inhibited colorectal cancer cell migration and invasion by blocking MMP-9 expression, which was consistent with a negative correlation between KISS1 and MMP-9 expression levels in colorectal cancer patients (10, 19, 20). Our present data consistently showed that kisspeptin treatment selectively blocks colorectal cancer cell migration and invasion. Kisspeptin inhibition of MMP-9 expression

was achieved in part by inhibiting phosphorylation of either AKT or ERK. We have previously shown that kisspeptin inhibited pulmonary metastasis of LoVo colorectal cancer cells *via* PKR (23). Therefore, it is much plausible that kisspeptin represses the invasiveness of colorectal cancer cells. Higher kisspeptin levels in plasma samples of colorectal cancer patients or higher KISS1 expression levels in colorectal cancer tissues is likely to check colorectal cancer metastasis (30, 32). Therefore, colorectal cancer cells overcoming this kisspeptin-mediated metastasis checkpoint may metastasize to local tissues or distant organs. Kisspeptin regulation of epithelial-to-mesenchymal transition mechanism is one of putative mechanisms (35-37). Another possibility is that a role of kisspeptin signaling is dependent on different colorectal cancer types. Thus, we still require more research on the role of kisspeptin in colorectal cancer.

KISS1R requirement in kisspeptin-mediated inhibition of distant metastases is controversial. Welch and his colleagues showed that kisspeptin did not require KISS1R in melanoma metastases (38, 39). However, most studies reported kisspeptin repression *via* KISS1R (40-45). In colorectal cancer cells, our results showed that kisspeptin required KISS1R to inhibit the invasiveness. Meanwhile, we found that kisspeptin requires PKR and PP2A in the inhibition of colorectal cancer cell invasiveness. Our previous study revealed that kisspeptin regulated RhoA-PKR signaling (23). Here, we showed that the PKR inhibitor blocked kisspeptin-mediated phosphorylation of PKR but increased phosphorylation levels of both AKT and ERK. Thus, kisspeptin regulation of RhoA-PKR pathway is upstream of AKT and ERK. Moreover, kisspeptin activation of PKR inhibited phosphorylation of both AKT and ERK by increasing of PP2A activity. PKR regulation of PP2A has been revealed in various experimental sets (24, 25, 46). In addition, PP2A inhibition of either AKT or ERK has been reported (24, 47). A recent study revealed that KISS1R interacts with PP2A in yeast two-hybrid and *in vitro* affinity purification assays (48). Nevertheless, it is unclear how kisspeptin signals to PKR and PP2A *via* KISS1R. PP2A inhibitor did not affect kisspeptin-mediated PKR phosphorylation. Thus, kisspeptin signaling involves sequentially kisspeptin-KISS1R-RhoA-PKR-PP2A. Both PKR and PP2A are known as favorable prognosis markers in colorectal cancer patients (28, 49, 50). Therefore, kisspeptin signaling may prevent colorectal cancer malignancy.

Kisspeptin is a well-known metastasis suppressor that can be tested in the repression of colorectal cancer metastasis. Our study reports that kisspeptin represses the invasiveness of colorectal cancer cells *via* KISS1R/PKR/PP2A. While more research is still required to decipher kisspeptin-mediated intracellular signaling in colorectal cancer cells, our ongoing work will focus on defining its role in different subtypes of colorectal cancer. Understanding kisspeptin's role in cancer will increase our understanding of the process of metastasis and may allow its clinical application.

Conflicts of Interest

The Authors declare that they have no conflict of interest.

Acknowledgements

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