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## KISS1 gene suppresses metastasis of nasopharyngeal cancer via activation of the ERK1/2 pathway

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Nasopharyngeal cancer (NPC) is one of the most common head and neck cancers in the world. The failure of the treatment of NPC is mainly caused by the metastasis of the cancer cells. There is a great need to study the mechanism of metastasis for accurate diagnosis, prognosis and efficient therapeutic strategy. The *KISS1* gene has already been reported as a metastasis suppressor in numerous cancers. In this study, by integrating the Transwell assay, western blotting and real-time reverse transcriptase polymerase chain reaction (RT-PCR) analysis, we found that the expression of *KISS1* and its receptor gene (hOT7T175, *KISS1R*) negatively related with the metastasis of NPC cells. Overexpression of the *KISS1* and *KISS1R* genes reduced migration and invasion of the SUNE-1-5-8F cells that are a cell line of NPC. Additionally, overexpression of these genes significantly increased the phosphorylation of focal adhesion kinase (FAK) and decreased the expression of ezrin (EZR), indicated by western blotting. Further study showed the metastasis suppression role of *KISS1* and *KISS1R* was mediated through phosphorylation of the ERK1/2 pathway for that blocking the phosphorylation of the pathway with inhibitor (PD98059) reversed the metastasis suppression and phosphorylation of FAK as well as up-regulation of EZR simultaneously. Together, all of these results suggested for the first time that *KISS1* and *KISS1R* suppress the metastasis of NPC cells by phosphorylation of FAK and decreasing EZR through phosphorylation of the ERK1/2 pathway.

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## Introduction

Nasopharyngeal cancer (NPC), as one of the most common head and neck cancers,<sup>1</sup> is vastly more common in certain regions of East Asia and Africa than elsewhere around the world.<sup>2,3</sup> According to previous reports, about 80% percent of the NPC cases occur in Asia and 50 000 deaths occur from it annually.<sup>4</sup> Based on the characteristics, NPC cases are mainly divided into three groups: keratinizing squamous cell carcinoma, differentiated cell carcinoma and undifferentiated non-keratinizing cell carcinoma.<sup>5</sup> In clinical practice, the most effective treatment for NPC has been a combination of radiotherapy and chemotherapy. Cisplatin and 5-fluorouracil are frequently used as chemotherapeutics.<sup>6,7</sup> At one year, the survival rate with radio-chemotherapy is 100%, while at three years and at five years it is 94% and 73% respectively.<sup>8</sup> The failure of the treatment is

mainly caused by surgery failure and the metastasis of cancer cells. Considering the side effects and high rates of failure with the conventional treatment strategies, there is a great need to develop new therapeutic approaches or medicines. The suppression of metastasis is an ideal candidate target for treatment.

Numerous studies have found that tobacco smoke, Epstein-Barr virus, nitrosamines consumption, genetic predisposition and racial patterns were associated with the development of NPC.<sup>9,10</sup> Also, molecular substrates, such as Major Histocompatibility Complex Class I, A2, B14 and B16 play important role in the development of NPC. Additionally, cell adhesion/migration genes like matrix metalloproteinase 2 (MMP2) and ezrin (EZR), cell cycle control genes like mouse double minute 2 human homolog (MDM2) and tumor protein p53 (TP53), as well as the ERBB-PI3K pathway and autophagy activation are reported regulating the development of NPC.<sup>11,12</sup> However, the mechanism underlying the metastasis of NPC cells has not been clearly elucidated.

The *KISS1* gene was first described by Lee *et al.*,<sup>13</sup> works as a metastasis suppressor gene in metastatic melanoma. *KISS1* encodes a 145 amino acids protein, which is then cleaved into various peptides, namely kisspeptin-10, kisspeptin-13, kisspeptin-14 and kisspeptin-54 that ultimately bind to the G protein-coupled receptor.<sup>14,15</sup> It is well established that *KISS1*

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and *KISS1R* can suppress the metastasis of various cancer cells, such as melanoma, breast cancer,<sup>16</sup> gastric cancer,<sup>17</sup> esophageal carcinoma<sup>18</sup> and pancreatic cancer<sup>19</sup> and the loss of *KISS1* and *KISS1R* expression also has been reported.<sup>18</sup> In addition, it has been reported that the phosphorylation of the ERK1/2 pathway and phosphorylation of the FAK, formation of stress fibers and focal adhesion were also induced after expression of the *KISS1* gene, which subsequently led to the suppression of the migration and invasion of cancer cells.<sup>20</sup> Additionally, overexpression of *KISS1* was reported to decrease the expression of matrix metalloproteinase 9 (MMP9) and subsequently reduced the invasion.<sup>21</sup> However, the roles of *KISS1* and *KISS1R* during the development of NPC has not been reported to date. Based on previous studies, we want to determine the mechanism of metastasis suppress function of *KISS1* and *KISS1R* in this study. Using RT-PCR and western blot, we found that the expression of *KISS1* and *KISS1R* are negatively related with metastasis of NPC cells. In addition, using the Transwell assay, we found that overexpression of *KISS1* and *KISS1R*, separately or together, significantly suppressed the migration and invasion of NPC cells. Moreover, the phosphorylation of the FAK was increased, while the actin-binding protein EZR was decreased. Further study showed the ERK1/2 pathway was activated though phosphorylation. Additionally, blocking the phosphorylation of this pathway with inhibitor (PD98059) reversed the suppression of the migration and invasion of the NPC cells, as well as the phosphorylation of the FAK and down-regulation of EZR caused by the overexpression of *KISS1* and *KISS1R*.

## Materials and methods

### Cell culture and chemicals

The subtypes of the NPC cell line, namely SUNE-1-5-8F with high metastasis and SUNE-1-6-10B with low metastasis were purchased (Guangzhou Genesee Biotech. Co., Guangzhou, China). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco, Grand Island, NY, USA), supplemented with 2 mM L-glutamine, 10% heat-inactivated fetal bovine serum (FBS) and antibiotics (100 U mL<sup>-1</sup> penicillin and 100 µg mL<sup>-1</sup> streptomycin). The cells were cultured at a density of 5 × 10<sup>4</sup> cells per mL in an incubator with an atmosphere of 5% CO<sub>2</sub> and 95% O<sub>2</sub>. The culture medium was exchanged every 2–3 days and cells were sub-cultured twice a week. The antibodies for *KISS1*, ERK1/2, FAK, phosphorylation of FAK (tyr397) and EZR were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The inhibitor of the ERK1/2 pathway (PD98059) was purchased from Sigma (Saint Louis, MO, USA).

### Overexpression of the *KISS1* and *KISS1R* genes in SUNE-1-5-8F cells

The cell line that stably expressing the *KISS1R* gene was constructed with the pSIREN-RetroQ retroviral vector (Clontech, Mountain View, CA, USA). First, the pSIREN-RetroQ retroviral vector containing the complementary DNA (cDNA) encoding the polypeptide of the *KISS1R* and the vectors encoding GFP alone were used to transfect 293T cells and the supernatant was

collected 48 h later. The supernatants were applied to SUNE-5-8F cells with 5 µg mL<sup>-1</sup> polybrene followed by the addition of 1 µg mL<sup>-1</sup> puromycin 24 h later for selection of the infected cells (referred as SUNE-1R and SUNE-GFP cells). Subsequently, the cells were selected and proliferated. For the overexpression of *KISS1*, 4 µg of the plasmid containing the *KISS1* cDNA sequence or a negative control were mixed with 8 µg Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) and used to transfect the SUNE-1R cells or the SUNE-GFP cells for 24 h according to the manufacturer's instructions. Each experiment was replicated four times. After 24 h of the transfection, the cells were sampled for the experiments.

### *KISS-1* interference with siRNA

The specific siRNA (5'-GCCGAACUACAACUGGA ACTT-3') with GFP tags for *KISS-1* gene and negative control (NC) that constructed in lentivirus were bought (Genechem Biotech. Co., Shanghai, China). The SUNE-1-5-8F cells were transfected according to the instruction. Briefly, the cells were adjusted to 1 × 10<sup>4</sup> cells per mL in one of 24-well plate before transfection. The most effective lentivirus (10 µL, 2 × 10<sup>7</sup> TU mL<sup>-1</sup> and 90 µL medium) determined by multiplicity of infection (MOI) was used to transfect the cells with Lipofectamine 2000 and then the transfected cells were selected with 50 µg mL<sup>-1</sup> puromycin. The metastasis (migration and invasion) and phosphorylation of ERK1/2 was detected 96 h after transfection.

### Real time polymerase chain reaction (PCR) analysis

For the RT-PCR analysis, the total RNA was extracted from the cells using the TRIZOL (Sangon Biotech., Shanghai, China) reagent according to manufacturer's instructions. The purity and concentration of the isolated RNA was determined by UV spectrophotometry. Then, the cDNA was synthesized using a RT-PCR kit (Sangon Biotech.) and amplified in a reaction mixture containing 10 µL of 2 × SYBR Green Master Mix, 0.5 µL of each primer of *KISS1* (forward: 5'-AGCCGCCAGATCCCGCA-3'; reverse: 5'-GCGAA GGAGTTCCAGTTGTAGTT-3'), 1 µL cDNA template, and 8 µL ddH<sub>2</sub>O. In this experiment, the GAPDH gene was used as reference. The relative gene expression was analyzed with the Data Assist software version 3.0 (Applied Biosystems, Foster City, CA, USA).

### Western blot analysis

The proteins of interest in this study were analyzed by Western blot analysis. After transfection of SUNE-5-8F cells with *KISS1* or negative control for 48 h, the obtained SUNE-1R or SUNE-GFP cells, were washed 3 times with PBS and then lysed in 100 µL RIPA buffer (Sangon Biotech.). Subsequently, the supernatant of the lysates were collected after centrifugation (12 000g) at 4 °C for 20 min. The concentration of the protein samples were determined with the BCA assay kit (Beyotime, China), using GAPDH as reference. Afterwards, 20 µg of the prepared proteins were separated using 10% sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and subsequently transferred onto polyvinylidene difluoride (PVDF; Millipore, Billerica, MA, USA) membranes



at 15 V for 30 min. The membranes were blocked with 5% non-fat milk for 1 h at room temperature and then washed three times for 5 min with TBST. The membranes were incubated with the corresponding primary antibodies at room temperature for 1 h and subsequently incubated with the appropriate secondary HRP antibodies for 40 min. Finally, the membranes were washed three times with TBST before detection of the bands using a Gel Imaging System (Fusion Solo, VILBER, France).

### Migration and invasion analysis by Transwell assay

The migration and invasion of SUNE-1-5-8F cells overexpressing *KISS1* and its receptor *KISS1R* or not were analyzed using the Transwell assay (Corning Inc., Corning, NY, USA). Briefly, after expression of the transfected genes for 24 h, the cells were washed twice with PBS and detached by digestion with 0.25% trypsin. Then, the cells were centrifuged for 5 min and re-suspended at  $1 \times 10^5$  cells per mL using DMEM without serum. The Transwell membranes with 8  $\mu$ m pore size (Corning Inc.) were placed in the wells of the 24-well plates containing 600  $\mu$ L DMEM with 10% FBS. Next, the membranes were seeded with  $1 \times 10^5$  cells in 200  $\mu$ L culture medium and incubated for 24 h in 5% CO<sub>2</sub> incubator. Finally, after fixing the cells on the Transwell membranes with 4% polyformaldehyde, images were captured using a Leica DM2500 (Leica Microsystems GmbH, Wetzlar, Germany) microscope and the cells were counted using ImageJ (NIH, Bethesda, MD, USA). For the invasion assay, the polyethylene terephthalate membranes were coated with Matrigel (Sigma) and then the cells were seeded on the Matrigel. After culturing for 24 h, the cells attached to membrane were fixed and counted.

### Wound healing assay

For the wound healing assay, the cells were seeded on the six-well plates with  $1 \times 10^5$  cells per well and allowed to grow to a confluent monolayer in the incubator at 37 °C before wounding the monolayer by scratching the surface with 1 mL pipette tip. Then, the cells were washed for 3  $\times$  5 min and incubated for 24 h, followed by fixing with 4% paraformaldehyde dissolved in phosphate buffer. The images were captured with a Leica DM2500 (Leica Microsystems GmbH) microscope.

### Statistical analysis

All the data in this study are presented as the mean  $\pm$  SEM. The unpaired Student's *t*-test or ANOVA (one-way or two-way) test was used to statistically analyse the experimental data between different groups, to determine if differences among groups are significant. All the analyses were performed using the SPSS version 19.0 (IBM Corp., Armonk, NY, USA) or GraphPad Prism 6 (GraphPad Inc., La Jolla, CA, USA). *P* values  $<0.05$  was considered statistically significant.

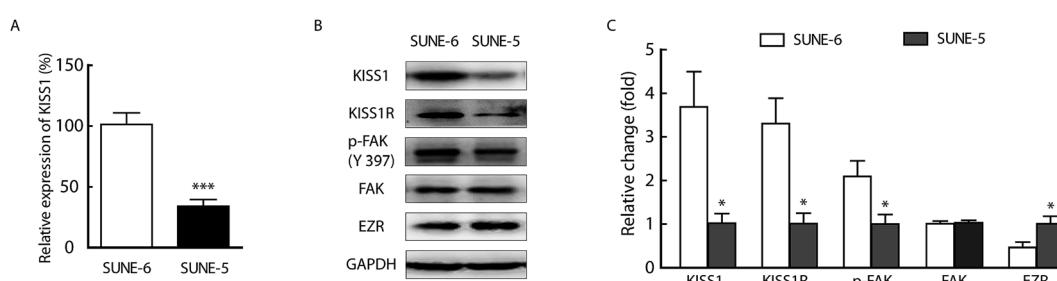
## Results

### *KISS1* is highly expressed in SUNE-1-6-10B cells and lowly expressed in SUNE-1-5-8F cells

First, we determined whether the metastasis of human NPC cells was associated with the expression of *KISS1*. The protein and mRNA expression of *KISS1* were analysed in SUNE-1-6-10B and SUNE-1-5-8F cells which are poorly metastatic and highly metastatic, respectively. The RT-PCR expression analysis revealed that the mRNA of *KISS1* is highly expressed in the SUNE-1-6-10B cells compared with the SUNE-1-5-8F cells (Fig. 1A). The Western blot protein expression analysis revealed that *KISS1* and its receptor *KISS1R* were also highly expressed in SUNE-1-6-10B cells compared with SUNE-1-5-8F cells (Fig. 1B and C), which is consistent with the RT-PCR analysis results. Meanwhile, the expression of the metastasis related phosphorylation of FAK was lower in SUNE-5-8F cells than in SUNE-1-6-10B cells and the EZR expression was the opposite. While the expression of FAK was unchanged (Fig. 1B and C). According to these data, we can surmise that the expression of *KISS1* was negatively associated with the metastasis of human NPC cells.

### Overexpression of *KISS1* inhibited the migration of SUNE-1-5-8F cells

To further evaluate of the role of *KISS1* and *KISS1R* in the metastasis of human NPC cells separately or together, these genes were overexpressed in SUNE-1-5-8F cells. The SUNE-1R and SUNE-GFP cell lines that stably express the *KISS1R* or GFP alone in the SUNE-5-8F cells were generated (Fig. 2A and B). Then, the cells were transfected with the plasmid expressing the *KISS1* gene. Compared with the SUNE-GFP cells expressing GFP



**Fig. 1** Expression of the *KISS1* gene negatively associated with metastasis of human nasopharyngeal cancer cells. (A) RT-PCR analysis shows relative expression of *KISS1* in SUNE-1-5-8F compared with SUNE-1-6-10B. (B) Representative bands of *KISS1R*, *KISS1*, phosphorylated FAK (p-FAK), FAK and EZR expressed in SUNE-1-6-10B and SUNE-1-5-8F cells. (C) Statistical data of the relative expression of *KISS1R*, *KISS1*, p-FAK, FAK and EZR expressed in SUNE-1-6-10B and SUNE-1-5-8F. Unpaired *t* test. Data are presented as the mean  $\pm$  SEM, \**P* < 0.05, \*\*\**P* < 0.001.

alone, the migration of the SUNE-1R was slightly inhibited as measured by the wound healing assay (Fig. 2C and D). In addition, the migration was significantly inhibited after the *KISS1* gene was expressed in the SUNE-GFP (SUNE-KISS1) or in SUNE-1R (SUNE-1R-KISS1) (Fig. 2C and D). We also utilized the Transwell assay to analyze the migration of the cells. The results of the Transwell assay, in line with the wound healing assay, revealed that the migration was slightly and significantly suppressed after the receptor *KISS1R* and *KISS1* were overexpressed separately (Fig. 3A and C). Furthermore, the migration was inhibited more intensely when *KISS1* and *KISS1R* were both expressed simultaneously, but no statistical difference was detected when compared with expression of *KISS1* alone (Fig. 3B and D).

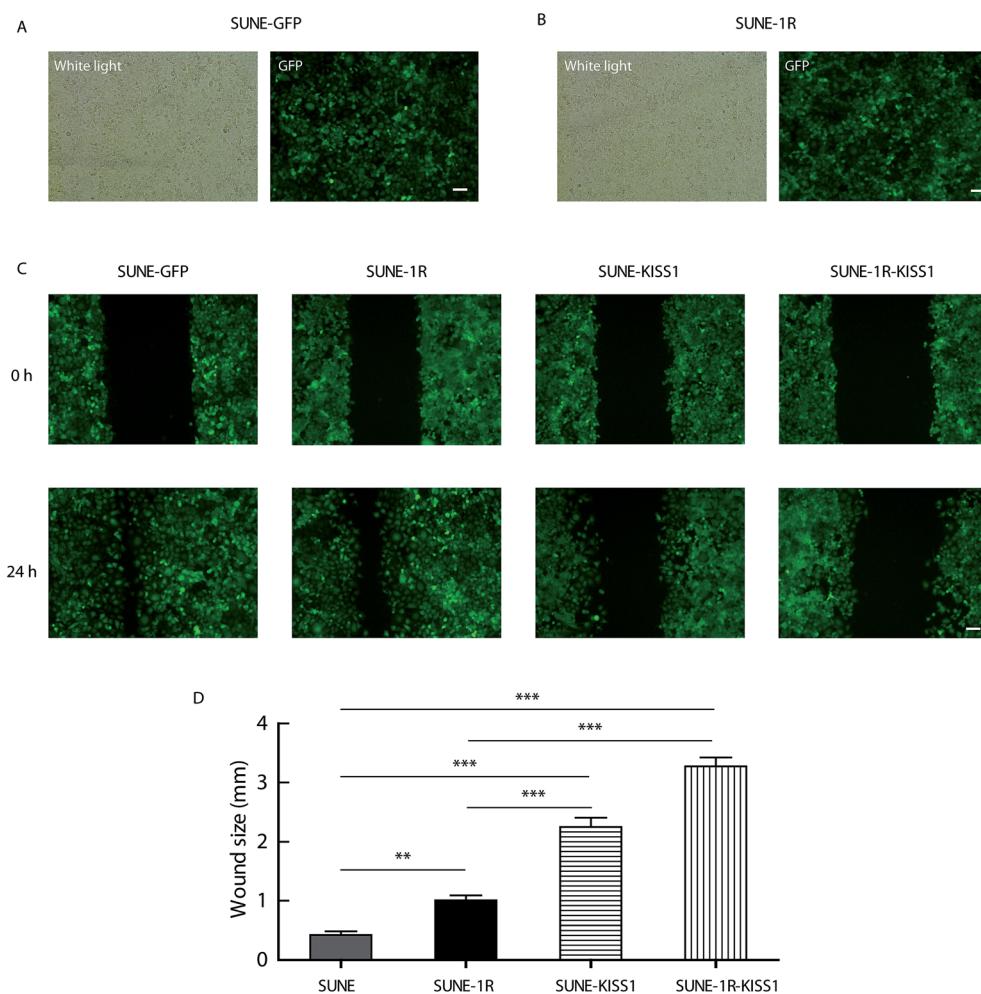
#### Overexpression of *KISS1* inhibited the invasion of SUNE-1-5-8F cells

For further evaluation of the metastasis suppression function of *KISS1* and *KISS1R*, the impact on invasion were assayed with the

Transwell assay. The invasion was slightly and intensely suppressed in SUNE-1R and SUNE-KISS1 cells respectively (Fig. 3B and D). The invasion was greatly inhibited when *KISS1* and *KISS1R* were simultaneously overexpressed, however no significant statistical difference was observed when compared with the invasion of SUNE-KISS1 cells expressing the *KISS1* gene alone (Fig. 3B and D).

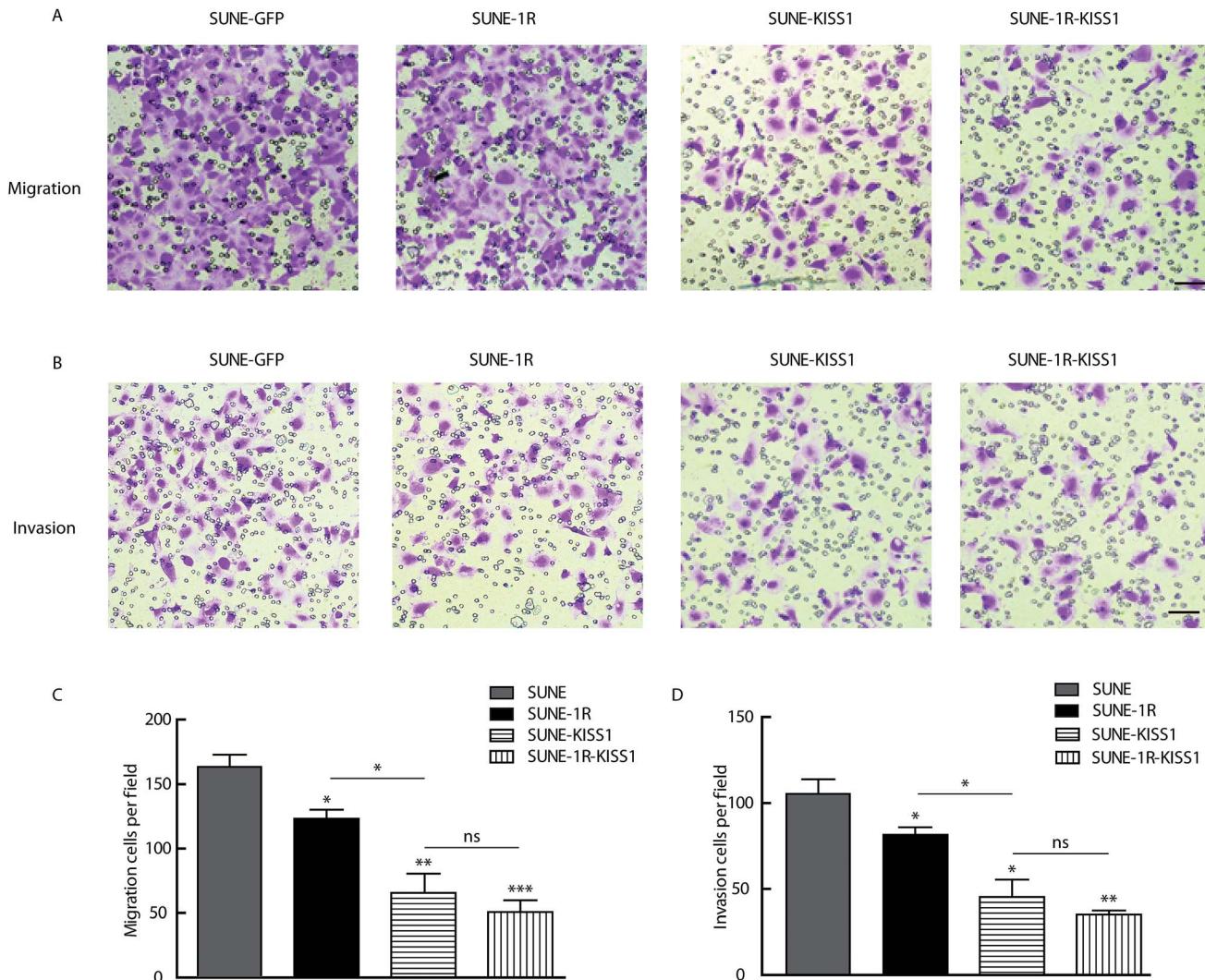
#### Overexpression of *KISS1* increases the p-FAK and decreases expression of EZR

To further investigate the underlying mechanism, the p-FAK and expression of EZR was analysed for that the functions of these protein in migration and invasion have been reported.<sup>14,22,23</sup> The *KISS1* and *KISS1R* were successfully expressed in SUNE-1-5-8F cells as indicated by the significantly increased expression of *KISS1R* in SUNE-1R and *KISS1* in SUNE-KISS1 cells (Fig. 4A-C). In addition, *KISS1* was up-regulated after *KISS1R* was overexpressed (Fig. 4C). We also evaluated the expression of EZR and phosphorylation of FAK (tyr 397) after the



**Fig. 2** Expression of *KISS1* inhibited the migration of NPC cells detected by wound healing assay. (A and B) Typical images of GFP alone (A) and *KISS1R*-GFP (B) stably expressed in SUNE-1-5-8F cells. Scale bar, 500  $\mu$ m. (C) Representative images of the wound healing assay of indicated groups (SUNE-GFP, SUNE-1R, SUNE-KISS1, SUNE-1R-KISS1) after *KISS1* and *KISS1R* expressed together or separately for 24 h. (D) Statistical data of the wound healing assay. Data are presented as the mean  $\pm$  SEM, \*\* $P$  < 0.01, \*\*\* $P$  < 0.001.





**Fig. 3** Overexpression of *KISS1* suppressed the migration and invasion of NPC cells. (A and B) Images of the Transwell assay showed the migration and invasion of indicated groups (SUNE-GFP, SUNE-1R, SUNE-KISS1, SUNE-1R-KISS1). Scale bar 50  $\mu$ m. (C and D) Summary data of the migration (C) and invasion (D) of different groups (SUNE-GFP, SUNE-1R, SUNE-KISS1, SUNE-1R-KISS1) that expressed *KISS1* and *KISS1R* separately or together. One-way ANOVA test. Data are presented as the mean  $\pm$  SEM. \* $P$  < 0.05; \*\* $P$  < 0.01.

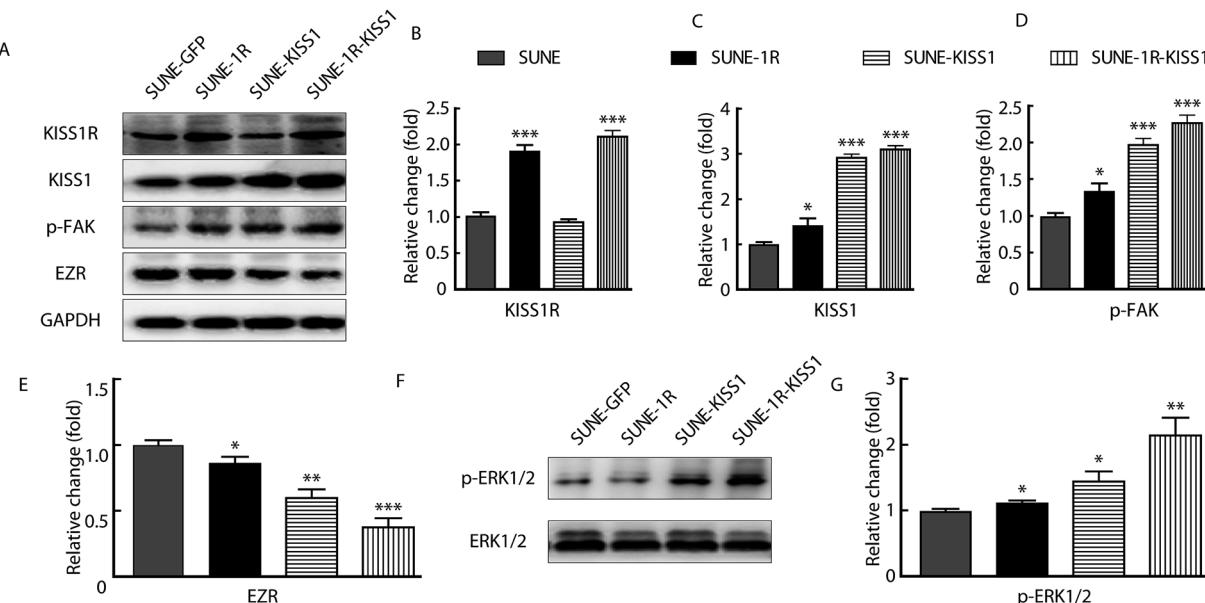
overexpression of *KISS1* and *KISS1R* alone or together. We found that the phosphorylation of FAK was significantly up-regulated, while the expression of EZR was significantly down-regulated when *KISS1* and *KISS1R* were expressed alone or together compared with SUNE-GFP cells (Fig. 4A, D and E). No significant statistical difference between the SUNE-1R-KISS1 cells and the SUNE-KISS1 cells was detected.

#### *KISS1* increased phosphorylation of ERK1/2

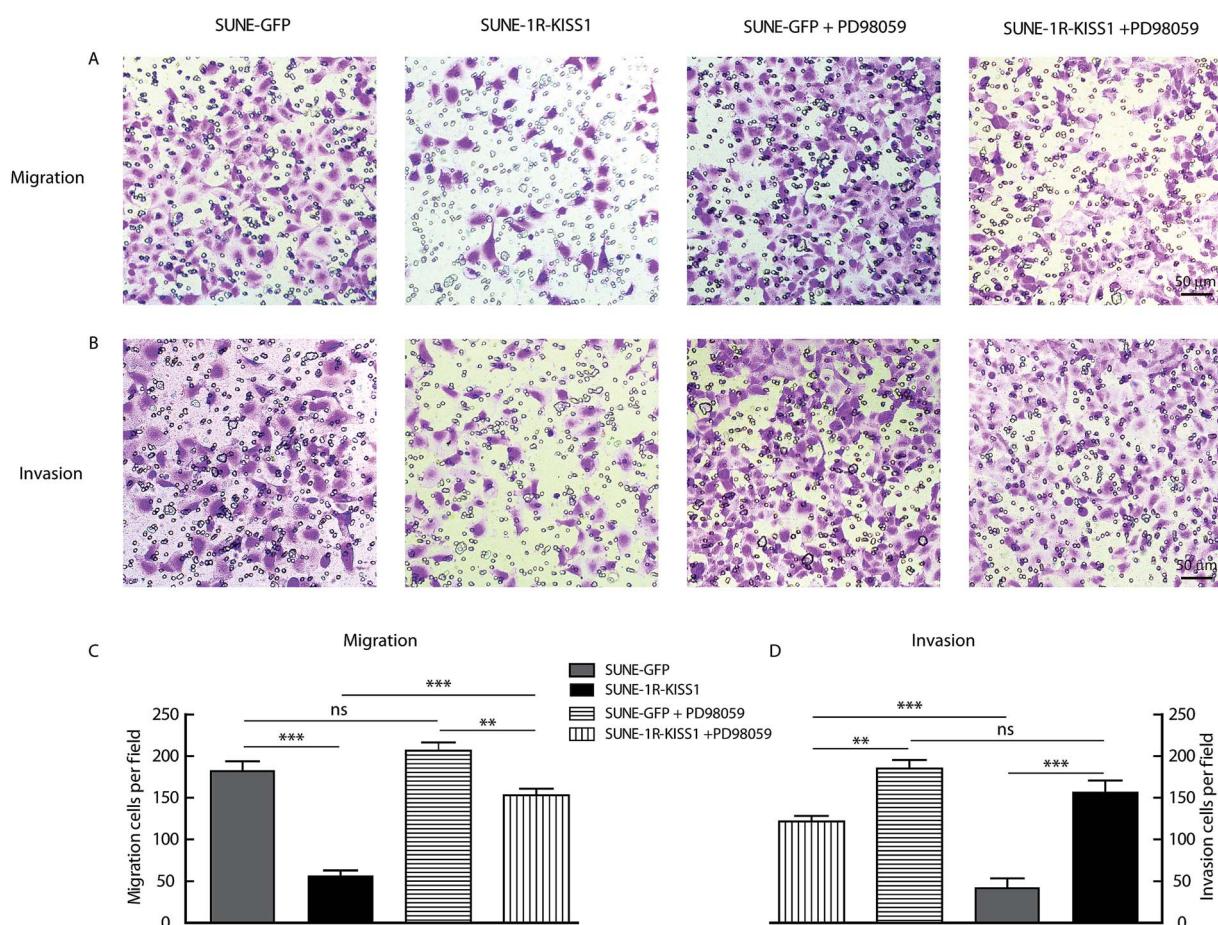
To determine how the expression of EZR and phosphorylation of FAK were regulated, the phosphorylation of the ERK1/2 pathway was assayed for the activation of the pathway was described in breast carcinoma and papillary thyroid cancer.<sup>20,24</sup> After overexpression of *KISS1* and *KISS1R*, separately or together, the phosphorylation of the ERK1/2 was variously increased (Fig. 4F and G).

#### Inhibition of the ERK1/2 pathway reversed the metastasis suppression caused by expression of *KISS1*

In order to determine whether the phosphorylation of ERK1/2 pathway was necessary and sufficient for the metastasis suppression of *KISS1* and *KISS1R*, the pathway was blocked with the inhibitor PD98059 (25  $\mu$ M). We found the migration and invasion suppression induced by overexpression of *KISS1* and *KISS1R* were reversed after PD98059 was added to the cells for 24 h during the Transwell assay (Fig. 5A–D). The phosphorylation of the ERK1/2 pathway in the SUNE-1R and SUNE-1R-KISS1 cells was decreased (Fig. 6A and B). The Western blot analysis results showed that the phosphorylation of FAK and the down-regulation of EZR were also reversed (Fig. 6C and D). Taking all these data together, we can conclude that *KISS1* and its receptor *KISS1R* gene suppress metastasis of NPC cells by phosphorylation of FAK and the down-regulation of EZR through phosphorylation of the ERK1/2 pathway.

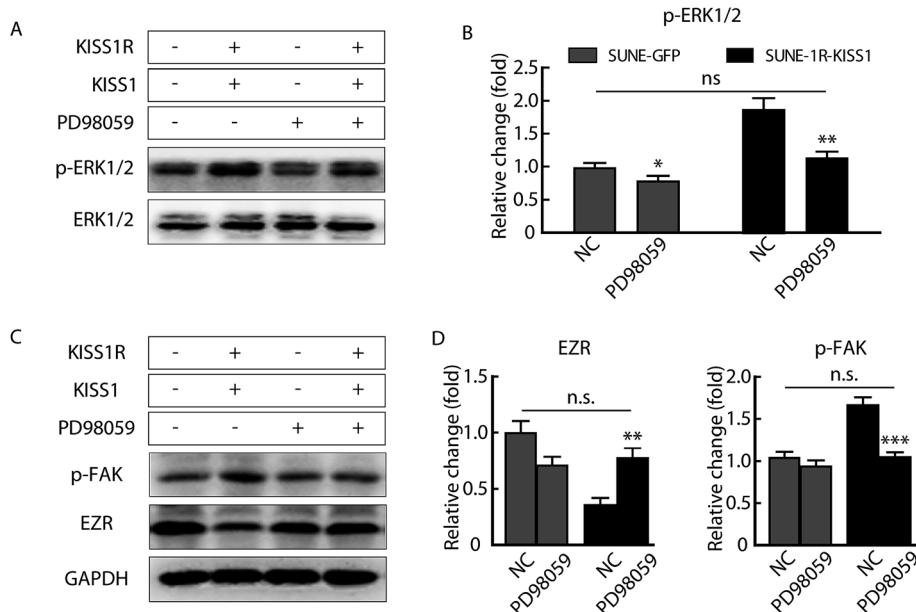


**Fig. 4** Overexpression of *KISS1* increased phosphorylation of FAK, ERK1/2 and decreased expression of EZR. (A) Representative bands that show the expression of KISS1R, KISS1, p-FAK, and EZR in SUNE, SUNE-1R, SUNE-KISS1 and SUNE-1R-KISS1 cells. (B–E) Statistical data of the expression of KISS1R, KISS1, p-FAK, FAK and EZR in indicated groups. (F) Bands of phosphorylated ERK1/2 (p-ERK1/2) in cells that overexpressed *KISS1* and *KISS1R* separately or together. (G) Histogram showing that the phosphorylation of ERK1/2 pathway in indicated groups. Unpaired Student's *t* test was used to analyse the difference between different groups. Data are presented as the mean  $\pm$  SEM. \**P* < 0.05; \*\**P* < 0.01.



**Fig. 5** Blocking the phosphorylation of ERK1/2 pathway reversed the suppressive effects induced by overexpression of *KISS1* and *KISS1R* on the migration and invasion. (A and B) Images of the Transwell assay show that the migration (A) and invasion (B) of indicated groups with PD98059 or not. (C and D) Histogram shows summary data of the migration and invasion of indicated groups. Unpaired Student's *t* test was used to analyze the difference between different groups. Data are presented as the mean  $\pm$  SEM. \**P* < 0.05; \*\**P* < 0.01. n.s. no significant.





**Fig. 6** Inhibition of the ERK1/2 pathway reversed the phosphorylation of the FAK, ERK1/2 and decrease of EZR that induced by overexpression of *KISS1* and *KISS1R*. (A) Representative bands of the phosphorylation of ERK1/2 in indicated groups with PD98059 or not. (B) Summary data of the phosphorylation of the ERK1/2 pathway in indicated groups with PD98059 or not. NC, negative control. Unpaired Student's *t* test. (C) Typical bands of p-FAK and EZR expression after overexpression of *KISS1* and *KISS1R* in the presence or absence of PD98059. (D) Statistical data of EZR and p-FAK in indicated groups with PD98059 or not. Unpaired Student's *t* test. Data are presented as the mean  $\pm$  SEM. \**P* < 0.05; \*\**P* < 0.01; \*\*\**P* < 0.001. n.s. no significant.

### Interference of *KISS1* increased and metastasis of NPC by decreasing phosphorylation of ERK1/2 pathway

In order to validate the critical function of *KISS1* in NPC, the *KISS1* was suppressed with siRNA as previously reported.<sup>25</sup> As showed in Fig. 7, the migration and invasion were significantly increased (Fig. 7B–E). Meanwhile, the phosphorylation of ERK1/2 pathway was significantly decreased (Fig. 7F and G). All these data in line with the results of overexpression of *KISS1* and clinical research.<sup>18</sup>

## Discussion

In clinical practice, the invasion and metastasis of NPC cells represent a challenge for the treatment of this cancer and is the leading reason for the failure of the treatment and death. Accordingly, there is a great need to investigate the underlying mechanism of the metastasis of NPC cells for the accurate diagnosis, prognosis and development of efficient therapeutic strategy. Even though numerous factors have been reported regulating the development of NPC, the underlying mechanism remains unclear.

The invasion and metastasis of the cancer cells are lethal characteristics and mainly depend on the adhesion with surrounding tissues. Phosphorylation of the FAK has been found to participate in the regulation of cell migration, proliferation and survival.<sup>26,27</sup> Additionally, it also plays a critical role in the metastasis of cancer cells.<sup>28–30</sup> It is also well established that EZR, as a member of the ERM (ezrin–radixin–moesin) family of cytoskeletal proteins, also plays a crucial role in cell

adhesion, migration and organization. Increased expression of EZR has been reported in various cancers, such as NPC, breast carcinoma and squamous cell carcinoma.<sup>31</sup>

*KISS1* was first reported by Lee JH *et al.*, in 1996 as a metastasis-suppressor gene in numerous cancers, such as colorectal cancer, melanoma, breast cancer, gastric cancer, esophageal carcinoma and pancreatic cancer. Besides, *KISS1R* was also reported to play an important role in esophageal squamous cell carcinoma.<sup>18</sup> However, few studies have paid attention to the role of *KISS1* and *KISS1R* in NPC metastasis and the underlying mechanism. In this study, the metastasis suppressor function of *KISS1* and *KISS1R* in NPC cells metastasis is described for the first time.

In this study, using RT-QPCR and western blot analyses, we found that the expression of *KISS1* and *KISS1R* were significantly increased in SUNE-1-6-10B cells that are less metastatic than SUNE-1-5-8F cells, which are highly metastatic. These data suggested that the metastasis of NPC cells may be negatively related with the expression of *KISS1* and *KISS1R*. The negative relation of expression of *KISS1* and *KISS1R* miRNA with cancers has already reported in pancreatic cancer. This means the RNA interference of *KISS1* and *KISS1R* should increases the metastasis of cancer cells. Moreover, the migration and invasion of NPC cells were significantly suppressed after *KISS1* and its receptor *KISS1R* gene overexpressed separately and together in UNE-1-5-8F cells. By Western blot analysis, we found that the phosphorylation of the FAK was significantly increased and the expression of EZR was dramatically decreased after overexpression of *KISS1* and *KISS1R*. Additional phosphorylation

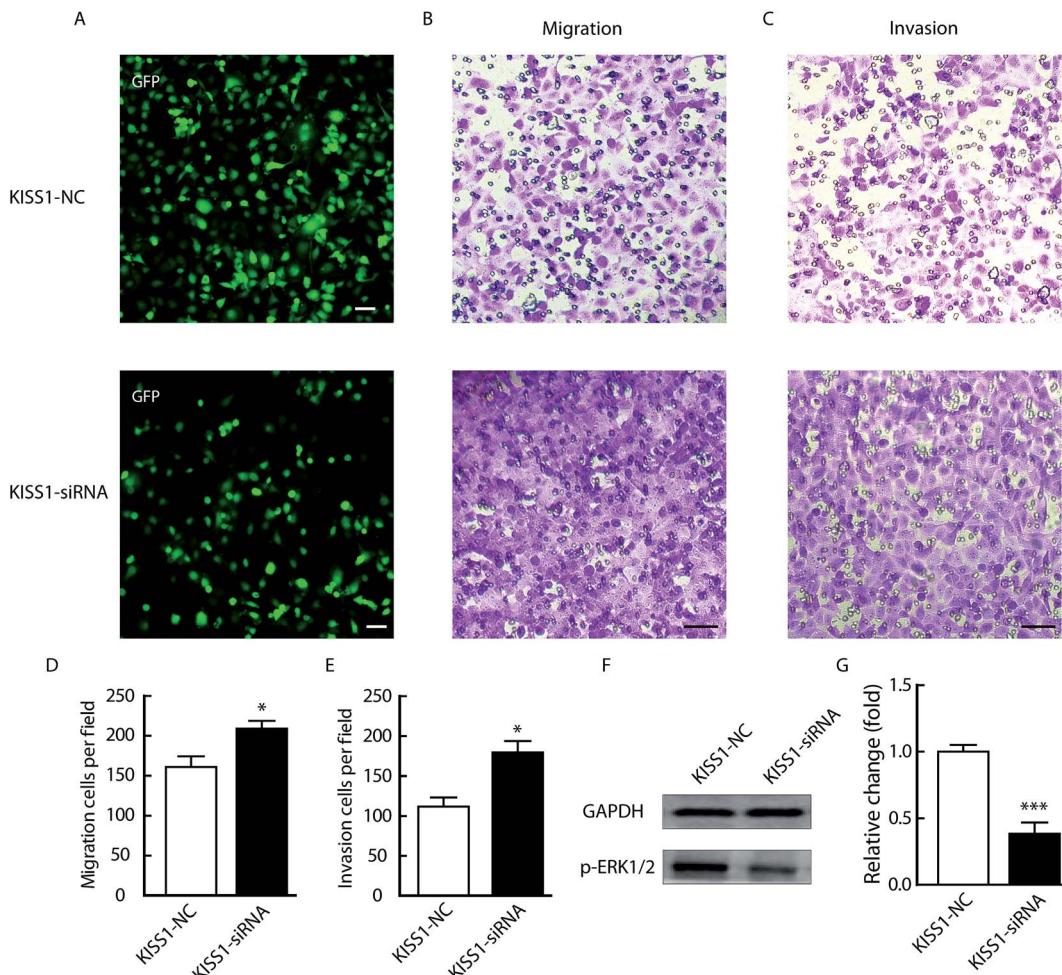


Fig. 7 Suppression of KISS1 increased and metastasis of NPC by decreasing phosphorylation of ERK1/2 pathway. (A) Representative images SUNE-1-5-8F that transfected with siRNA of KISS1 and negative control. Scale bar, 500  $\mu$ m. (B and C) Representative images of migration (B) and invasion (C) of indicated groups. Scale bar, 50  $\mu$ m. (D and E) Statistical data of migration (D) and invasion (E) of indicated groups. (F and G) Representative bands of phosphorylation of indicated groups (F) and the statistical data (G). Unpaired Student's *t* test. Data are presented as the mean  $\pm$  SEM. \* $P$  < 0.05; \*\*\* $P$  < 0.001.

analysis studies further showed that the ERK1/2 pathway was phosphorylated and inhibition of the ERK1/2 pathway with the inhibitor reversed the changed expression of EZR and phosphorylation of the FAK caused by overexpression of *KISS1*. However, the statistical difference between metastasis suppression in SUNE-1R and SUNE-KISS1 cells (Fig. 3C and D), as well as the difference between SUNE-GFP and SUNE-1R-*KISS1* after treatment with PD98059 (Fig. 5C and D) indicated that the *KISS1* gene may work through another receptor or pathway in NPC cells. The critical function of the *KISS1* and *KISS1R* genes in NPC cells reported in this study provides a new insight regarding the underlying mechanism of the metastasis of NPC cells and reveals a new target for the development of a novel treatment strategy and for the diagnosis of NPC in clinical practice. According to clinical studies and our results in this study, we partly revealed the mechanism of occurrence of cancers and the expression of *KISS1* and *KISS1R* could be an indicator for diagnosis.

## Conflicts of interest

There are no conflicts to declare.

## Acknowledgements

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