Haploinsufficiency in the pro-metastasis Kiss1 receptor Gpr54 delays breast tumor initiation, progression and lung metastasis

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Running title: Kiss1r (Gpr54) heterozygosity delays breast cancer progression

PRECIS: Using a knockout model for the pro-metastatic receptor Kiss1R, this study established its role and signaling functions in initiation as well as progression in well-established models of breast cancer.

Keywords: Kiss1, Kiss1r, GPR54, GEFT/p63RhoGEF, RhoA, tumor progression, metastasis

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Abstract

Activation of KISS1 receptor (KISS1R or GPR54) by its ligands (kisspeptins) regulates a diverse function both in normal physiology and pathophysiology. In cancer, KISS1R has been implicated in tumor angiogenesis and metastasis, but a broader evaluation of KISS1R in tumorigenesis and tumor progression has yet to be conducted. In this study, we used mouse models of Kiss1r gene knockout and MMTV-PyMT induced breast cancer to conduct such an evaluation. Kiss1r heterozygosity in MMTV-PyMT mice was sufficient to attenuate breast cancer initiation, growth, latency, multiplicity and lung metastasis. To confirm these effects and assess possible contributions of endogenous ligands, we isolated primary tumor cells from PyMT/Kiss1r+/+ and PyMT/Kiss1r+/- mice and compared their phenotypes using in vitro and in vivo assays. Kiss1r loss attenuated in vitro tumorigenic properties as well as tumor growth in vivo in immunocompromised NOD.SCID/NCr mice. Kiss1r activation in these cells resulting from the addition of its ligand Kisspeptin-10 resulted in RhoA activation and RhoA-dependent gene expression through the Gαq-p63RhoGEF signaling pathway. Anchorage-independent growth was tightly linked to dose-dependent regulation of RhoA by Kiss1r. In support of these results, siRNA-mediated knockdown of KISS1R or inactivation of RhoA in human MCF10A breast epithelial cells overexpressing H-RasV12 was sufficient to reduce Ras-induced anchorage-independent growth. In summary, we concluded that Kiss1r attenuation was sufficient to delay breast tumor initiation, progression and metastasis through inhibitory effects on the downstream Gαq-p63RhoGEF-RhoA signaling pathway.
Introduction

KISS1 receptor (KISS1R, also named GPR54) coupled to its endogenous ligands, kisspeptins (KISS1 gene product), has been revealed to play a pivotal role for the onset of puberty and to suppress cancer metastasis (1-7). Kisspeptins regulate cell proliferation, migration, and invasion in different cell lines via KISS1 receptor/GPR54 (8-10). In pubertal development, mouse Kiss1 was revealed as a phenocopy of Kiss1r, since in knockout mouse models where Kiss1 or Kiss1r was deleted, similar phenotypes were observed in both Kiss1 and Kiss1r knockout mice (1, 3, 11). In cancer, various experimental and clinical studies have shown that kisspeptins could suppress cancer metastasis (12). We recently found that genes located at chromosome 6q21-32, which was often lost in metastatic breast cancer and melanoma, regulated KISS1 gene at chromosome 1q32 (9, 13). Notably, genomic studies have revealed a gain of chromosome 1q32 in primary breast cancer and loss of chromosome 6q21-32 in aggressive breast cancer (14-21). Those genomic studies with recent findings suggest that KISS1 and KISS1R expression may be increased at the early stage of tumor development. Earlier studies focused on KISS1 function in cancer metastasis on the basis of KISS1 loss in metastatic cancer, and revealed a role of kisspeptin-activated KISS1R signaling (hereafter, KISS1/KISS1R signaling) for metastasis suppression. However, a role of endogenous KISS1/KISS1R signaling in early stages of cancer progression is still unclear, while it has been shown that the expression of KISS1 and KISS1R was higher in non-aggressive cancer than in normal and/or metastatic cancer (9, 13, 22-24).

MMTV (mouse mammary tumor virus)-PyMT (polyoma virus middle T antigen) mouse model is widely used to investigate a relationship between human and mouse breast cancer development and metastasis (25, 26). The MMTV-PyMT mouse model is time-saving for investigating tumor progression, since PyMT-induced hyperplasia is usually detected as early as the onset of puberty at 3 weeks and aggressive carcinoma with lung metastasis is found at 11 weeks (25, 26). An absence of mammary gland development in Kiss1- or Kiss1r-deficient female
mice was closely linked to the absence of central Kiss1/Kiss1r signaling for the onset of puberty (1, 3, 11). However, the heterozygous mice for Kiss1 or Kiss1r did not cause any defects in pubertal development, including postnatal mammary gland development (1, 3, 11), suggesting that Kiss1r heterozygous condition in MMTV-PyMT mouse could be a good model to understand breast-restricted Kiss1/Kiss1r signaling in the early stage of breast cancer development.

In this study, we found that Kiss1r heterozygosity delayed PyMT-induced breast cancer development and metastasis. Notably, Kiss1r heterozygosity (Kiss1r+/−) attenuated breast tumor initiation, tumor growth, latency, multiplicity and metastasis induced in MMTV-PyMT/Kiss1r mouse models. Kiss1r silencing in pubertal breast epithelia confirmed that Kiss1r signaling in breast epithelial cells was necessary for breast hyperplasia. To limit any effect of endogenous hormones, we isolated mouse primary breast cancer cells from MMTV-PyMT/Kiss1r+/+ and MMTV-PyMT/Kiss1r+/- mice and examined the tumorigenesis in vitro and in vivo. We found that Kiss1r heterozygosity (Kiss1r+/−) attenuated breast tumor growth when tumor cells were orthotopically injected into NOD.SCID/NCr mice. To understand the molecular mechanism of Kiss1r regulation of tumorigenesis, we further determined that dosage-dependent regulation of RhoA activity by KISS1R was critical for Ras-induced tumorigeneity. In summary, our study suggests that KISS1/KISS1R signaling regulates breast tumor initiation, progression, and metastasis by activating Goq-p63RhoGEF-RhoA signaling pathway.
Materials and methods

Animal Studies

Transgenic FVB/N mice expressing the polyoma middle T antigen under the control of MMTV long terminal repeat promoter (MMTV-PyMT) were received from Dr. Jeffrey M. Rosen and Dr. Jianming Xu at Baylor College of Medicine (Houston, TX). All matings were performed with male mice heterozygous for the PyMT transgene and female C57BL/6 Kiss1r+/- mice received from Dr. Eric L. Gustafson at Schering-Plough Research Institute (Kenilworth, NJ). Genotyping for the PyMT transgene was performed according to the protocol of Jackson Laboratories (Bar Harbor, ME). All mice analyzed in this study were virgin females. Animal protocols used for this study were approved by the IACUC, Texas A&M Health Science Center.

In vivo studies

Mice were observed three times a week for mammary tumors by eye examination and finger palpation. Tumor length (L) and width (W) were measured once a week by a caliper and tumor volume was estimated by the formula, \((W^2 \times L) / 2\). Mice were euthanized at different stages of mammary tumorigenesis, and their mammary glands and tumors were collected for morphologic and biochemical analyses. For the whole-mount staining, the fourth inguinal mammary fat pads were excised, fixed with ethanol, and then stained with carmine alum. For histological study, tissues were formalin fixed for 24 hrs, and embedded in paraffin. Tissues were cut at 5μm in width, stained with hematoxylin and eosin, and evaluated by three different observers. Analyses and descriptions were followed in accordance with the guidelines in the NIH mammary gland pathology. Anti-Kiss1 and –Kiss1r antibodies from Santa Cruz Biotechnology (Santa Cruz, CA) were used for the immunohistochemistry. HistoStain® Plus Broad Spectrum (DAB) (Invitrogen, Camarillo, CA) and Vectorstain ABC kit with Vector® NovaRED™ Substrate kit (Vector Laboratories, Burlingame, CA) were used. Lentiviral plasmids were obtained from Open Biosystems (Huntsville, AL). At two weeks of age, 4th mammary glands were injected with
lentiviruses through the nipple. At 5 week, mice were sacrificed, and mammary glands were stained and counted for hyperplastic nodules or lyzed for Western blot. Primary tumors cells were isolated from breast tumors in 11 week-old PyMT/Kiss1r+/+ and PyMT/Kiss1r+/- female mice. For the orthotopic injection of primary tumor cells, 1x10^6 cells were mixed with 0.1% matrigel and then injected into the left 4th mammary fat pad of NOD.SCID/NCr mouse (NCI-Frederick).

**In vitro studies**

Primary tumor cells were isolated from the tumor mice and cultured in DMEM/F12 medium supplemented with 5% horse serum, 1% streptomycin/penicillin, 20ng/ml EGF, 0.5mg/ml hydrocortisone, 100ng/ml cholera toxin, 10µg/ml insulin at first day, and then in DMEM medium. To examine cell proliferation, cells were manually counted every day until 12 days. In order to examine cell migration, Boyden chamber assays were used. For colony formation assays, cells were cultured in 0.35% soft agar for 2 weeks and then colonies were stained with crystal violet. MCF10A cells were obtained from American Type Culture Collection (ATCC, Manassas, VA) and cultured in DMEM/F12 medium supplemented with 5% horse serum, 1% streptomycin/penicillin, 20ng/ml EGF, 0.5mg/ml hydrocortisone, 100ng/ml cholera toxin, 10µg/ml insulin. For RNA quantification, real-time PCR analyses were performed using SYBR green reagents in Prism 7300 real-time PCR machine (Applied Biosystems, Foster City, CA), and ΔΔCt values were calculated. *Gapdh* as a reference was used for normalization. Anti-KiSS1, KiSS1R, RhoA, Cdc42, Rac1, Ras, and Actin antibodies were used for the Western blot (Santa Cruz Biotechnology, Santa Cruz, CA). For the Rho GTPases activity was performed using pull-down assays with GST-PAK PBD or GST-Rhotekin RBD, respectively. To detect active Rho GTPases, the appropriate antibody was used. For the luciferase assay, luciferase assay kit was used according to the manufacture’s protocol (Promega, Madison, MI). Cells were transfected with pSRF-luc or pSRE-luc for 6hrs to 24hrs and then subjected to the luciferase activity.
Statistics

Statistics were done using MedCalc (Medcalc Software, Mariakerke, Belgium). All statistics are two-sided. One-way analysis of variance and student’s t test were appropriately done. Tumor-free survival curves were performed by Kaplan-Meier method and compared with log-rank test. All data were expressed as means with 95% confidential intervals. A P value < .05 was considered as statistical significance.
Results

*Kiss1r heterozygosity delays mouse breast tumor development and lung metastasis*

To investigate the roles of Kiss1 receptor (Kiss1r) in breast tumor development and metastasis *in vivo*, we generated the MMTV-PyMT/Kiss1r mouse models. As described in the materials and methods section, we obtained PyMT/Kiss1r wild type (PyMT/Kiss1r\(^{+/+}\)), heterozygous (PyMT/Kiss1r\(^{+/−}\)), and Kiss1r null (PyMT/Kiss1r\(^{−/−}\)) mice for our analysis of Kiss1r in tumor progression and metastasis. For breast tumor development, we examined palpable tumors in different groups of mouse models, including PyMT/Kiss1r\(^{+/+}\) (n = 30), PyMT/Kiss1r\(^{+/−}\) (n = 50), and PyMT/Kiss1r\(^{−/−}\) mice (n = 20) until 16 weeks. PyMT/Kiss1r\(^{+/−}\) mice in comparison with PyMT/Kiss1r\(^{+/+}\) mice showed a delay of breast tumor development as measured by the percentage of tumor-free mice in the Kaplan-Meier plot analysis (Fig. 1A). In PyMT/Kiss1r\(^{+/−}\) mice, 50% of mice developed tumors in 11 weeks compared to 7 weeks in PyMT/Kiss1r\(^{+/+}\) mice (P < .0001), whereas PyMT/Kiss1r\(^{−/−}\) mice generated no palpable tumor (Fig. 1A). Thus, our data indicated that Kiss1r heterozygosity affected tumor latency and Kiss1r might be essential for breast tumor development.

Meanwhile, pubertal phenotypes such as vaginal opening at the precise time schedule showed no differences between PyMT/Kiss1r\(^{+/+}\) and PyMT/Kiss1r\(^{+/−}\) mice, which was consistent with previous reports that Kiss1r (as well as Kiss1) heterozygous condition did not significantly alter the level of sexual hormones and not affect pubertal development (1, 11). Lack of tumor development in PyMT/Kiss1r\(^{+/−}\) mice suggests that hypothalamic Kiss1/Kiss1r signaling regulates postnatal mammary development, which is consistent with previous findings that Kiss1r knockout mice did not have postnatal mammary gland development and that PyMT-induced tumors arose at the time of pubertal mammary gland development (1, 11, 26). Altogether, Kiss1r heterozygosity resulted in the haploinsufficiency for PyMT-induced mammary tumor development, which was not due to the pubertal defect of mammary gland development.
We next counted the number of mammary tumors in different mammary fat pads of each mouse at 11 weeks. Kiss1r heterozygosity (Kiss1r\(^{+/−}\)) reduced the number of tumors per mouse (Fig. 1B; \(n = 20\) per group, \(P < .00001\)). Thus, Kiss1r heterozygosity further affected mammary tumor multiplicity. We then measured tumor burden at 15 weeks to analyze whether Kiss1r heterozygosity further affected tumor growth. Tumor volume in PyMT/Kiss1r\(^{++}\) mice was smaller than that in PyMT/Kiss1r\(^{+/−}\) mice (Fig. 1C; \(n = 10\) per group, \(P = .029\)). Therefore, our data indicated that Kiss1r haploinsufficiency attenuated breast tumor latency and multiplicity, and further affected tumor growth.

PyMT-induced breast tumors primarily metastasize to lung, which is normally detected at 11 weeks of age (25, 26). To investigate whether Kiss1r heterozygosity affected breast tumor metastatic growth at lung, we prepared lung tissue sections and counted metastatic tumor foci in PyMT/Kiss1r\(^{+/+}\) and PyMT/Kiss1r\(^{+/−}\) mice. At 11 weeks of age, the number of tumor foci found in PyMT/Kiss1r\(^{+/+}\) lung was significantly higher than that in PyMT/Kiss1r\(^{+/−}\) lung (Fig. 1D; \(n = 10\) per group, \(P < .0001\)). Thus, our data indicated that Kiss1r haploinsufficiency further affected breast tumor lung metastasis.

**Kiss1r heterozygosity delays the incidence of mouse breast hyperplasia, tumor formation, and malignancy**

As Kiss1r heterozygosity affected tumor latency, multiplicity and growth, we next examined whether those results were due to a delay of tumor incidence. To analyze mammary tumor incidence, we prepared whole-mounting analysis on the fourth inguinal mammary fat pads at the age of 5 to 9 weeks since whole mounting preparations were preferred to address premalignant lesions of mammary glands, termed mammary intraepithelial neoplasia (MIN) or hyperplastic atypia that results in mammary tumors. When hyperplastic nodules in the 4\(^{th}\) inguinal mammary fat pads of PyMT/Kiss1r\(^{+/+}\) (\(n = 20\)), PyMT/Kiss1r\(^{+/−}\) (\(n = 21\)) and PyMT/Kiss1r\(^{−/−}\) (\(n = 11\)) mice at ages from 5 to 9 weeks were counted, respectively, we found that the total number of hyperplastic...
nODULES IN THE 4th INGUINAL MAMMARY FAT PAD OF PyMT/Kiss1r-/- MICE WAS MUCH LESS THAN THAT IN
THE PyMT/Kiss1r+/+ MICE (Fig. 2A AND 2B; P < .0001). IN ADDITION, PyMT/Kiss1r-/- DID NOT
DEVELOP HYPERPLASTIC MAMMARY GLAND AS A RESULT OF THE DEFECTIVE POSTNATAL MAMMARY GLAND
DEVELOPMENT (Fig. 2B; P < .0001). AT 5 WEEKS, MAMMARY DUCTAL HYPERPLASIA IN PyMT/Kiss1r+/+
MICE WAS MUCH LESS SEVERE THAN THAT IN PyMT/Kiss1r+/+ MICE (Fig. 2A, INBOXES IN SECOND PANELS).
AT 7 TO 9 WEEKS, TUMOR SIZES WERE SMALLER IN PyMT/Kiss1r+/+ MICE THAN THAT IN PyMT/Kiss1r+/+ MICE (FIGURE 2A, ARROWS IN THIRD AND FOURTH PANELS). THEREFORE, OUR DATA INDICATED THAT Kiss1r
HETEROZYGOITY AFFECTED TUMOR INCIDENCE AND TUMOR FORMATION.

TO INVESTIGATE WHETHER Kiss1r HETEROZYGOITY FURTHER AFFECTED TUMOR MALIGNANCY, WE
ANALYZED TUMOR COHORTS IN 11 WEEK-OLD PyMT/Kiss1r+/+ AND PyMT/Kiss1r+/+ MICE (N = 7 PER
GROUP). TUMOR COHORTS WERE MORE COMPLEX AND ADVANCED IN THE HISTOLOGY SAMPLES OF
PyMT/Kiss1r+/+ MICE THAN THOSE IN PyMT/Kiss1r+/+ MICE (Fig. 2C). NORMAL MAMMARY GLAND (N;
terminal ductal lobular unit: TDLU) WAS NOT FOUND IN PyMT/Kiss1r+/+ MICE, BUT APPROXIMATELY
22% OF N WAS DETECTED IN PyMT/Kiss1r+/+ MICE (Fig. 2C; P < .0001). THERE WAS NO SIGNIFICANT
DIFFERENCE IN DISTRIBUTION OF PAPILLOMA (P) AND ATYPICAL DUCTAL HYPERPLASIA (ADH) BETWEEN
PyMT/Kiss1r+/+ TUMOR COHORT AND PyMT/Kiss1r+/+ TUMOR COHORT. HOWEVER, Kiss1r HETEROZYGOITY
MARKEDLY DELAYED MAMMARY TUMOR PROGRESSION WITH A DECREASE OF DUCTAL CARCINOMA IN SITU
(DCIS) BY APPROXIMATELY 20% (Fig. 2C; P = 0.0068). MOREOVER, THE AMOUNT OF INVASIVE DUCTAL
CARCINOMA (IDC) IN PyMT/Kiss1r+/+ MICE WAS SIGNIFICANTLY LOWER THAN THOSE IN PyMT/Kiss1r+/+
MICE, AMOUNTING TO AN APPROXIMATE 14% IN 11 WEEK-OLD PyMT/Kiss1r+/+ MICE COMPARED TO AN
APPROXIMATE 37% IN 11 WEEK-OLD PyMT/Kiss1r+/+ MICE (Fig. 2C; P = 0.0213). THEREFORE, OUR DATA
INDICATED THAT Kiss1r HETEROZYGOITY DELAYED TUMOR MALIGNANCY AND PROGRESSION.

WE NEXT EXAMINED AN EXPRESSION OF Kiss1r IN TUMOR TISSUES USING THE IMMUNOHISTOCHEMISTRY.
COMPARED TO PyMT/Kiss1r+/+ TUMOR, PyMT/Kiss1r+/+ TUMOR EXPRESSED HIGHER Kiss1r (Fig. 2D).
ALTHOUGH Kiss1r WAS DETECTED IN FIBROBLAST AND ENDOTHELium, BOTH Kiss1 AND Kiss1r WERE FOUND IN
LUMINAL EPITHELIAL TUMOR CELLS (Fig. 2D), SUGGESTING AUTOCRINE Kiss1/Kiss1r SIGNALING.
**Autocrine Kiss1/Kiss1r signaling is sufficient for mouse breast tumor initiation**

To investigate a local function of Kiss1/Kiss1r signaling for hyperplasia, lentiviruses for shRNAs specific for Kiss1 and Kiss1r were injected into 4th nipples at 2 weeks of age prior to pubertal mammary development. Mice were sacrificed at 5 weeks of age when hyperplasia was developed. Lentiviral knockdown strategies repressed the expression levels of either Kiss1 or Kiss1r in breast epithelia (Fig. 3A and B). Compared to the silencing with control shRNA (scr), both Kiss1 and Kiss1r silencing in breast epithelia caused a significant decrease of hyperplastic nodule number (Fig. 3B and 3C; n = 5 per group, Kiss1 shRNA vs. scr shRNA, $P = 0.0062$; Kiss1r shRNA vs. scr shRNA, $P = 0.0097$), supporting that local Kiss1 and Kiss1 receptor (Kiss1r) are involved in breast tissue hyperplasia and tumor initiation. Data from knockdown of Kiss1 or Kiss1r were consistent with our data from Kiss1r heterozygosity, which indicates that autocrine Kiss1/Kiss1r signaling in breast epithelial cells play a critical role in breast tumor development.

**Kiss1r haploinsufficiency affects tumorigenesis in isolated primary tumor cells and orthotopic mouse tumor models**

To investigate the function of Kiss1/Kiss1r signaling in breast tumor cells, we isolated breast tumor cells from tumor burdens in 11 week-old PyMT/Kiss1r$^{+/+}$ and PyMT/Kiss1r$^{+/−}$ mice, and examined the expression of Kiss1 and Kiss1r, respectively. PyMT/Kiss1r$^{+/−}$ primary tumor cells showed a significant reduction of Kiss1r expression compared with that in PyMT/Kiss1r$^{+/+}$ primary tumor cells (Fig. 4A). On the other hand, no significant alteration of Kiss1 expression was detected (Fig. 4A). To examine cell growth in Kiss1r heterozygosity, primary tumor cells isolated from PyMT/Kiss1r$^{+/+}$ and PyMT/Kiss1r$^{+/−}$ mice were cultured until 12 days and the number of cells was counted every day to calculate the doubling time. Doubling time for PyMT/Kiss1r$^{+/+}$ and PyMT/Kiss1r$^{+/−}$ primary tumor cells was 29.33 hr and 31.76 hr, respectively.
(Fig. 4A; n = 3 per group, \( P = 0.0007 \)), suggesting that Kiss1r heterozygosity negatively regulates mammary tumor cell proliferation.

Since Kiss1/Kiss1r signaling was implicated in cell motility and invasion, we next investigated cell migration of PyMT/Kiss1r\(^{+/+}\) and PyMT/Kiss1r\(^{+/−}\) primary tumor cells. Cells were cultured on Boyden chamber precoated with Matrigel and then the number of cells passed through the Matrigel was counted after 24hr. The invasion capacity of PyMT/Kiss1r\(^{+/+}\) cells was approximately 31\% higher than that of PyMT/Kiss1r\(^{+/−}\) cells (Fig. 4B; n = 4 per group, \( P = 0.0099 \)), indicating that Kiss1r heterozygosity affects tumor cell motility and invasion.

Next, we performed the anchorage-independent colony formation assays to examine whether Kiss1r heterozygosity in mammary tumor cells affects tumorigeneity. Primary tumor cells isolated from PyMT/Kiss1r\(^{+/+}\) and PyMT/Kiss1r\(^{+/−}\) mice were cultured in soft agar for 20 days and then the number of colonies was counted. Compared to PyMT/Kiss1r\(^{+/+}\) cells, the number of colonies formed by PyMT/Kiss1r\(^{+/−}\) cells was reduced by approximately 59\% (Fig. 4C; \( P = 0.00375 \)). Thus, our data suggest that Kiss1r heterozygosity causes haploinsufficiency and negatively regulates breast tumorigeneity in vitro.

To further test that Kiss1r heterozygosity in breast epithelial tumor cells affects tumor growth in vivo, we performed orthotopic injection assays in NOD.SCID/NCr mice using primary cultured tumor cell lines isolated from PyMT/Kiss1r\(^{+/+}\) and PyMT/Kiss1r\(^{+/−}\) mice, respectively. Tumor volume obtained from PyMT/Kiss1r\(^{+/−}\) tumor cells in NOD.SCID/NCr mice was significantly smaller than those obtained from PyMT/Kiss1r\(^{+/+}\) tumor cells (Fig. 4D; n = 13 per group, \( P < 0.0001 \)), indicating that Kiss1r haploinsufficiency in breast cancer cells significantly inhibited tumor growth in vivo.

**Kiss1r sufficiently regulates tumorigeneity via RhoA**

To understand the molecular mechanisms of Kiss1r signaling in the regulation of tumorigeneity, we next examined the activation of Rho GTPases in breast tumor cells since Rho
GTPases are reported to regulate tumor progression and metastasis (27, 28). In PyMT/Kiss1r+/+
primary tumor cells, knockdown of Kiss1r expression significantly decreased RhoA activity in a
dose-dependent manner but did not affect the activity of either Cdc42 or Rac1 (Fig. 5A). RhoA
activity in PyMT/Kiss1r+/− tumor cells was also decreased when compared to that in
PyMT/Kiss1r+/+ tumor cells (Fig. 5A), suggesting that Kiss1r signaling in breast tumor cells
regulate the activation of RhoA GTPase. To link the finding to our previous observation that
autocrine Kiss1/Kiss1r signaling affected tumorigenecity, we next performed the anchorage-
independent growth assays. Dominant negative RhoA (RhoA DN; RhoAN17) decreased the
anchorage-independent growth of PyMT/Kiss1r+/+ cells by approximately 47% (n = 6, RhoA DN
vs. empty, P = .0001), but wild type and constitutively active RhoA (RhoA DA; RhoAV14)
increased the anchorage-independent colony growth by approximately 29% and 35%,
respectively (n= 6; RhoA WT vs. empty, P = .0146; RhoA DA vs. empty: RhoA DA vs. empty, P
= .0045). Similar data were also observed in PyMT/Kiss1r+/− tumor cells although the colony
number was reduced compared to wild type tumor cells (Fig. 5B; n = 6; empty/PyMT/Kiss1r+/− vs.
empty/PyMT/Kiss1r+/+, P = .0004; RhoA DN/PyMT/Kiss1r+/− vs. empty/PyMT/Kiss1r+/+, P
= .0003; RhoA WT/PyMT/Kiss1r+/− vs. empty/PyMT/Kiss1r+/+, P = .0003; RhoA
DA/PyMT/Kiss1r+/− vs. empty/PyMT/Kiss1r+/+, P = .0001). Altogether, our data indicate that
RhoA is a key protein downstream of Kiss1/Kiss1r signaling for breast tumorigenecity and tumor
progression.

To confirm the roles of human KISS1R for tumorigenecity in human breast epithelium, we
transformed MCF10A human normal breast epithelial cells by overexpressing the constitutively
active H-Ras (H-RasV12) to induce tumorigenesis, as PyMT-induced tumorigenesis was known to
require oncogenic activation of Ras (29, 30). As shown in figure 5C, overexpression of the active
H-RasV12 transformed the MCF10A breast epithelial cells and induced anchorage-independent
colony growth on soft agar. Knockdown of KISS1R using specific shRNA for human KISS1R
reduced Ras-induced anchorage-independent colony formation by approximately 37% (Fig. 5C; n
= 7, KISS1R shRNA vs. scr shRNA, \( P = .0002 \), suggesting that human KISS1R plays a key role in Ras-induced MCF10A cell tumorigenesis. We further examined whether KISS1R-mediated RhoA activation was involved in Ras-induced tumorigenesis using the anchorage-independent colony growth assays. Inactive mutant of RhoA (RhoA DN) decreased Ras-induced colony formation of MCF10A (Fig. 5D; \( n = 7 \), RhoA DN vs. control, \( P < .0001 \)). In addition, RhoA inhibitor (Y27632) blocked Ras-induced tumorigenesis in MCF10A cells that express RhoA, indicating that Ras-induced tumorigenesis requires RhoA activation (Fig. 5D; \( n = 7 \), RhoA inhibitor vs. control, \( P = .0001 \)). On the other hand, dominant active form of RhoA (RhoA DA) recovered the inhibitory effect of KISS1R deficiency (knockdown) in Ras-induced tumorigenesis (\( n = 7 \), RhoA DA + KiSS1R shRNA vs. KiSS1R shRNA, \( P = .0097 \)), suggesting that RhoA is the key downstream target of KISS1R in Ras-induced MCF10A cell tumorigenesis. Together, our data indicate that KISS1R-mediated RhoA activation is important for Ras-induced tumorigenesis, similar to our results found in the PyMT/Kiss1r mouse tumor models.

**Kisspeptin activates RhoA-mediated transcription through KISS1R-G\( \alpha \_q \)-p63RhoGEF**

To delineate KISS1/KISS1R signaling to RhoA, we performed RhoA activity assays when cells were serum-starved for 24hr and then incubated for 6hr in the absence or presence of kisspeptin-10 (Kp10, 100nM). Kisspeptin-10 (100nM) increased GTP-bound RhoA in HEK293 cells overexpressing KISS1R (Fig. 6A). Since serum response factor (SRF) transcriptional activity is widely used for the readout of RhoA GTPase activation (31-33), we performed the luciferase assays for SRF-dependent promoter activity as the readout of RhoA activation. Kisspeptin-10 increased SRF-luciferase activity in cells transfected with KISS1R (Kisspeptin-10 in KiSS1R cells vs. none in KiSS1R cells, \( P = 0.0309 \)) but not with empty vector (Fig. 6B). Consistently, kisspeptin-10 also activated serum response element (SRE)-luciferase activity via KISS1R (data not shown). Therefore, our data indicate that KISS1/KISS1R signaling directly activates RhoA.
We next investigated whether KISS1/KISS1R signaling increases SRF transcriptional activity through RhoA activation. Dominant active form of RhoA (RhoA DA) highly increased SRF transcriptional activity in HEK293 cells stably expressing KISS1R (RhoA DA vs. empty, \( P = .0027 \)). RhoA wild type (RhoA WT) has little effect on the basal SRF transcriptional activity (RhoA WT plus kisspeptin-10 vs. RhoA WT, \( P = .0002 \)) (Fig. 6C, 13\textsuperscript{th}, 14\textsuperscript{th}, and 17\textsuperscript{th} bars). A dominant negative mutant of RhoA (RhoA DN) suppressed kisspeptin-10 activation of SRF transcriptional activity (Fig. 6C, 15\textsuperscript{th} and 16\textsuperscript{th} bars). Thus, our data indicate that KISS1/KISS1R-mediated SRF activation requires RhoA activation.

To understand how KISS1 receptor activates RhoA, we examined whether the activation of RhoA was mediated by \( G_{\alpha q} \), as it was revealed that KISS1R selectively coupled to \( G_{\alpha q} \) upon kisspeptin stimulus (1, 4, 8, 34). In HEK293 cells stably transfected with KISS1R, kisspeptin-10 stimulation or constitutively active mutant of \( G_{\alpha q} \) (\( G_{\alpha q} \) CA) induced SRF transcriptional activity (\( G_{\alpha q} \) CA vs. empty, \( P = .0015 \); \( G_{\alpha q} \) WT plus kisspeptin-10 vs. \( G_{\alpha q} \) WT, \( P = .0003 \)), while \( G_{\alpha q} \) wild type (\( G_{\alpha q} \) WT) alone did not affect SRF transcriptional activity (Fig. 6C, 3\textsuperscript{rd}, 4\textsuperscript{th}, and 7\textsuperscript{th} bars). A dominant negative mutant of \( G_{\alpha q} \) (\( G_{\alpha q} \) DN) attenuated kisspeptin-10-induced SRF transcriptional activation (Fig. 6C, 5\textsuperscript{rd} and 6\textsuperscript{th} bars). Thus, our data suggest that the activation of RhoA by kisspeptin-10 is mediated by KiSS1R-\( G_{\alpha q} \) signaling pathway.

As \( G_{\alpha q} \) activates RhoA via p63RhoGEF (also called GEFT) (35-37), we further examined the \( G_{\alpha q} \) coupling to p63RhoGEF. In KISS1R-overexpressing cells, p63RhoGEF wild type (p63RhoGEF WT) did not affect basal or kisspeptin-10-induced SRF transcriptional activity (p63RhoGEF WT plus kisspeptin-10 vs. p63RhoGEF WT, \( P = .0003 \)), but catalytic domain-deleted mutant of p63RhoGEF (p63RhoGEF ΔN) inhibited kisspeptin-10 activation of SRF transcriptional activity (Fig. 6C, 8\textsuperscript{th} to 11\textsuperscript{th} bars). Furthermore, p63RhoGEF ΔN suppressed SRF transcriptional activation by \( G_{\alpha q} \) CA (Fig. 6C, 12\textsuperscript{th} bar; \( G_{\alpha q} \) CA vs. p63RhoGEF ΔN plus \( G_{\alpha q} \) CA, \( P = .0001 \)), indicating that p63RhoGEF is downstream of \( G_{\alpha q} \) in the activation of KISS1R.
We further examined whether KISS1/KISS1R signaling activated RhoA-dependent SRF transcriptional activity through Gαq-p63RhoGEF. RhoA DN inhibited Gαq CA-induced SRF transcriptional activation (Gαq CA vs. RhoA DN plus Gαq CA, \( P = .0001 \)), and p63RhoGEF ΔN did not inhibit RhoA DA-increased SRF transcriptional activity (Fig. 6C, 18th and 19th bars). Therefore, our data indicate that KiSS1/KiSS1R signaling activates Gαq-p63RhoGEF-RhoA signaling pathway.

Finally, we examined whether Kiss1r heterozygosity regulated RhoA-dependent gene expression in tumor progression using real-time PCR assays and MatInspector software (38). Among genes of which promoter region contained SRE, we found that the expression levels of Vegf, ErbB2, c-fos, and Mmp-9 in PyMT/Kiss1r+/− tumor compared to that in PyMT/Kiss1r+/+ tumor was significantly reduced (Fig. 6D), indicating that Kiss1r heterozygosity selectively affected RhoA-dependent expression level of key genes involved in cancer progression.
Discussion

KISS1/KISS1R signaling has been implicated in cancer metastasis, which was first revealed in xenograft tumor growth and experimental metastasis models (4, 6). However, its functional requirement in the early stages of cancer development was unclear. In this study, we demonstrated that mouse Kiss1r heterozygosity delayed breast cancer initiation, progression and metastasis in MMTV-PyMT mouse model system. In our PyMT/Kiss1r mouse models, Kiss1r heterozygosity (PyMT/Kiss1r +/-) diminished hyperplasia, resulting in the delay of tumor formation, progression, and lung metastasis. Furthermore, we confirmed that knockdown of Kiss1 and Kiss1r locally inhibited mammary gland hyperplasia and that the dosage-dependent regulation of breast tumor initiation by Kiss1r was connected to RhoA activity. Accordingly, we demonstrate that KISS1/KISS1R signaling regulates early steps of tumor development through RhoA activation by coupling to Gαq-p63RhoGEF.

In xenograft mouse tumor model systems, KISS1 overexpression blocked cancer metastases without affecting tumorigenesis (4, 6, 7). However, xenograft mouse tumor model has limitations to understand KISS1/KISS1R signaling in early stages of tumor progression since it is not able to recapitulate some aspects of tumorigenesis (25). For example, tumor cells used in xenograft assays already have tumor characteristics, indicating that tumorigenic process is not recapitulated in xenograft assay. Moreover, data from human cancer patients have consistently suggested that the roles of KISS1/KISS1R signaling might not be simple in metastasis because both KISS1 and KISS1R were comparably expressed in normal and/or benign tissues (12). While KISS1/KISS1R signaling inhibited metastasis in xenograft tumor and experimental metastasis mouse model systems, our MMTV-PyMT mouse model system showed that silencing of Kiss1 or Kiss1r as well as Kiss1r heterozygosity in breast epithelial cells attenuated PyMT-induced breast tumor initiation and development. Our ovariectomy study showed that mammary Kiss1r did not affect mammary gland development (data not shown). Thus, in our present study, Kiss1/Kiss1r signaling was selective for PyMT-induced mammary tumor initiation. Meanwhile, our data
showed that KISS1/KISS1R signaling seems to be required for tumor cell growth and motility, as knockdown of KISS1 or KISS1R as well as Kiss1r heterozygosity reduced cell growth and motility. Therefore, KISS1/KISS1R signaling may form a fine-tuned network in the cells and during tumor progression.

Our study further identified that KISS1/KISS1R regulated breast tumorigenesis via the activation of RhoA by Gαq-p63RhoGEF signaling pathway. Somatic mutation of Gαq (GNAQ) at codon 209 was frequently found in uveal melanoma and blue naevi (39, 40). Therefore, constitutively active mutation of Gαq seems to be involved in melanoma progression. Recent studies delineated that p63RhoGEF linked the activation of Gαq and RhoA (36, 37). Our lab also revealed that p63RhoGEF overexpression induced NIH3T3 transformation (35). RhoA upstream of Cdc42 and Rac1 in regulating dynamics of cell growth and motility is known to affect preneoplastic transformation in primary mammary epithelial cells (41). Consistently, RasV12 required ROCK and Cdc42 for apical growth in Madin-Darby canine kidney (MDCK) epithelial cells, indicating that RhoA-ROCK pathway is critical for tumorigenic cell growth (42). Thus, KISS1/KISS1R signaling to RhoA is likely to control the growth of tumorigenic cells. In addition, while RhoA promotes pulmonary metastasis of MDA-MB-231 metastatic human breast cancer cell line where endogenous KISS1/KISS1R signaling is absent (43), kispeptin-10 treatment inhibits TNFalpha-induced RhoA activation in the same cell line (28). Thus, KISS1/KISS1R signaling to RhoA during breast cancer progression is probably stage-specific. However, KiSS1/KiSS1R signaling to RhoA is likely more complex in vivo since kispeptin-10 inhibited cell migration and invasion in Caki-1 and ACHN renal cancer cells or caused apoptosis of Jurkat human leukemic cells by activating RhoA (27, 44). It is possible that the effect of KISS1/KISS1R signaling is also cell type-specific. As tumor microenvironment is complex and changed following cancer progression (45), a different combination of signaling inputs would result in diverse consequences (46).
Genetic alteration in the MMTV-PyMT model may change a predisposition of different breast cell lineages, which can affect particular stages of cancer progression (47-49). In our study, Kiss1/Kiss1r signaling showed dosage-dependent regulation of mouse breast tumor initiation and progression. When we examined the expression patterns of various genes in PyMT/Kiss1r\textsuperscript{+/+} and PyMT/Kiss1r\textsuperscript{+/−} tumors, Kiss1r heterozygosity affected the expression levels of various genes for tumor initiation and development, such as Sca-1 (data not shown). Thus, KISS1/KISS1R signaling is likely to alter the expression pattern of endogenous gene sets for tumor initiating-cell propagation. Meanwhile, gene expression studies for Kiss1 and Kiss1r during mouse cancer progression (data not shown) demonstrated that Kiss1/Kiss1r signaling might be upregulated in benign tumor and then downregulated in malignant tumor. Thus, endogenous KISS1/KISS1R signaling may be necessary for tumor initiation, and be sustained or enhanced during cancer progression, and then disappeared by mechanisms that we previously reported (9, 13). Thus, proper schedules for the usage of exogenous kisspeptin should consider the endogenous KISS1/KISS1R signaling in tumor progression, since recent studies have shown that exogenous KISS1/KISS1R signaling could suppress tumor angiogenesis as well as distant metastasis (7, 50). However, if KISS1/KISS1R signaling exhibits a tonic effect on tumor progression including tumorigenesis and metastasis, exceeding KISS1/KISS1R signaling by administrating kisspeptins may target any steps of cancer progression including metastasis through the net effect as mentioned above.

Altogether, we conclude that Kiss1r heterozygosity attenuates breast cancer initiation, progression and metastasis and that KISS1/KISS1R signaling via RhoA is necessary for breast tumor development and progression.
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Figure legends

Figure 1. Kiss1r heterozygosity affects mammary tumor latency, multiplicity, growth and lung metastasis. (A) Kaplan-Meier plot for palpable tumors until 16 weeks. (B) Breast tumor numbers in different mammary fat pads between PyMT-Kiss1r wild type (Kiss1r+1+) and PyMT-Kiss1r+1/ (Kiss1r+1/). (C) The volume of primary tumors in 11 week-old mice. (D) Metastatic tumor foci number in lung tissues at 11 weeks of age in wild-type (Kiss1r+1+) and heterozygosity mice (Kiss1r+1/).

Figure 2. Kiss1r heterozygosity attenuates the incidence of mammary hyperplasia, solid tumor formation and tumor malignance. (A) Primary tumor development in 4th inguinal mammary fat pad from 5 to 9 weeks of age. Arrows indicate primary tumors. (B) Hyperplastic nodules (the inbox) at 5 weeks of age. Inbox represents the hyperplastic nodule image. (C) Tumor malignancy in PyMT/Kiss1r+/+ (wild type) and PyMT/Kiss1r+1/ (heterozygous mice). (D) Immunohistochemistry for Kiss1r in tumor tissues.

Figure 3. Autocrine Kiss1/Kiss1r signaling is sufficient for breast tissue hyperplasia. (A) Expression of Kiss1 and Kiss1r in mammary glands injected with lentiviral shRNA for scr (control), Kiss1 or Kiss1r, showing down-regulation of Kiss1 and Kiss1r in mammary glands. (B) Immunohistochemistry staining for breast tissues infected with lentiviral shRNA for scr, Kiss1 or Kiss1r. (C) Hyperplastic nodule numbers in mammary glands infected with different shRNAs for control, Kiss1 and Kiss1r, respectively. The representative images were shown in the top of the figure.

Figure 4. Kiss1/Kiss1r signaling regulates tumorigeneity. (A-C) Kiss1r heterozygosity suppressed primary tumor cell growth (A), cell migration and invasion (B), and anchorage-independent colony formation (C). (A- insert) Kiss1r and Kiss1 mRNA level in primary tumor
cells isolated from the PyMT/Kiss1r$^{+/+}$ and PyMT/Kiss1r$^{+/c}$ mice. Gapdh used as the internal control of PCR reaction. (D) Kiss1r heterozygosity significantly inhibits orthotopic tumor growth in xenograft mouse tumor model using primary tumor cells isolated from the PyMT/Kiss1r$^{+/+}$ and PyMT/Kiss1r$^{+/c}$ mouse models, respectively.

**Figure 5. Kiss1/Kiss1r regulates tumorigenesis by activating RhoA.** (A) Activation of Rho family of GTPases by suppressing Kiss1r expression. Kiss1r activates RhoA in a dose-dependent manner while Rac1 and Cdc42 are not affected by the downregulation of Kiss1r. (B) RhoA and its mutants regulate anchorage-independent colony formation in tumor cells isolated from PyMT/Kiss1r$^{+/+}$ and PyMT/Kiss1r$^{+/c}$ mouse models. (C) Knockdown of KISS1R in Ras-transformed MCF10A cell inhibited anchorage-independent cell growth and colony formation. (D) Ras-induced anchorage-independent colony formation of MCF10A cells depends on RhoA signaling pathway using different RhoA plasmids or treated with RhoA inhibitor, Y-27632 (10μmol/L).

**Figure 6. KiSS1/KiSS1R regulates RhoA-dependent gene expression through Gaq-p63RhoGEF-RhoA signaling.** (A) Kisspeptin-10 activation of RhoA is dependent on the expression of KiSS1R. (B) Activation of SRF-dependent transcriptional activity by Kisspeptin-KiSS1R. (C) Activation of SRF-dependent transcriptional activity by Gαq, p63RhoGEF and RhoA signaling pathway as indicated conditions. (D) Regulation of gene expression levels in Kiss1r heterozygosity mouse models.
Cho et al., Fig. 3

A

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shRNA

B

- Kiss1
- Kiss1r

- scr shRNA
- Kiss1 shRNA
- scr shRNA
- Kiss1r shRNA

C

- scr shRNA
- Kiss1 shRNA
- Kiss1r shRNA

Hyperplastic nodule number

- scr
- Kiss1
- Kiss1r

shRNA
Haploinsufficiency in the pro-metastasis Kiss1 receptor Gpr54 delays breast tumor initiation, progression and lung metastasis

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