Tumor and Stem Cell Biology

Haploinsufficiency in the Prometastasis Kiss1 Receptor Gpr54 Delays Breast Tumor Initiation, Progression, and Lung 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 is yet to be conducted. In this study, we used mouse models of Kiss1r gene knockout and mouse mammary tumor virus–polyoma virus middle T antigen (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 by 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. Cancer Res; 71(20); 6535–46. © 2011 AACR.

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 KISS1R/GPR54 (8–10). In pubertal development, mouse Kiss1 was revealed as a phenocopy of Kiss1r, as 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). These 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 whereas it has been shown that the expression of KISS1 and KISS1R was higher in nonaggressive cancer than in normal and/or metastatic cancer (9, 13, 22–24).

Mouse mammary tumor virus–polyoma virus middle T antigen (MMTV-PyMT) mouse model is widely used to investigate the relationship between human and mouse breast cancer development and metastasis (25, 26). The MMTV-PyMT mouse model is time saving for investigating tumor progression, as 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 the heterozygous condition of Kiss1r 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. Kiss1 or Kiss1r silencing in pubertal breast epithelia confirmed that Kiss1/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 tumorigenecity. In summary, our study suggests that KISS1/KISS1R signaling regulates breast tumor initiation, progression, and metastasis by activating Gαq-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 Drs. Jeffrey M. Rosen and Jianming Xu at Baylor College of Medicine (Houston, TX). All matings were carried out with male mice from Open Biosystems. At 2 weeks of age, fourth mammary glands were injected with lentiviruses through the nipple. At 5 weeks, mice were sacrificed and mammary glands were stained and counted for hyperplastic nodules or lysed for Western blotting. Primary tumors were isolated from breast tumors in 11-week-old PyMT/Kiss1r+/- and PyMT/Kiss1r+/− female mice. For the orthotopic injection of primary tumor cells, 1 × 10^6 cells were mixed with 0.1% Matrigel and then injected into the left fourth 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/Ham’s F12 medium supplemented with 5% horse serum, 1% streptomycin/penicillin, 20 ng/mL epidermal growth factor (EGF), 0.5 mg/mL hydrocortisone, 100 ng/mL cholera toxin, 10 μg/mL insulin at first day and then in DMEM medium. To examine cell proliferation, cells were manually counted every day until 12 days. 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 and cultured in DMEM/Ham’s F12 medium supplemented with 5% horse serum, 1% streptomycin/penicillin, 20 ng/mL EGF, 0.5 mg/mL hydrocortisone, 100 ng/mL cholera toxin, and 10 μg/mL insulin. For RNA quantification, real-time PCR analyses were conducted using SYBR green reagents in Prism 7300 real-time PCR machine (Applied Biosystems), and ΔΔCt values were calculated. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as a reference was used for normalization. Anti-KISS1, KISS1R, RhoA, Cdc42, Rac1, Ras, and actin antibodies were used for Western blotting (Santa Cruz Biotechnology). For the Rho GTPases activity, cell lysates were used in 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). Cells were transfected with pSRE-luc or pSRE-luc for 6 to 24 hours and then subjected to the luciferase activity.

Statistics

Statistics were done using MedCalc (Medcalc Software). All statistics are 2-sided. One-way ANOVA and Student’s t test were appropriately done. Tumor-free survival curves were
carried out by the Kaplan–Meier method and compared with the log-rank test. All data were expressed as means ± 95% CIs. A $P < 0.05$ value was considered as statistically significant.

Results

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

To investigate the roles of Kiss1r in breast tumor development and metastasis in vivo, we generated the MMTV-PyMT/Kiss1r mouse models. As described in Materials and Methods, 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 with 7 weeks in PyMT/Kiss1r$^{+/+}$ mice ($P < 0.0001$), 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.

Figure 1. Kiss1r heterozygosity affects mammary tumor latency, multiplicity, growth, and lung metastasis. A, the Kaplan–Meier plot for palpable tumors until 16 weeks. B, breast tumor numbers in different mammary fat pads between PyMT/Kiss1r$^{+/+}$ and PyMT/Kiss1r$^{+/−}$ mice. 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 PyMT/Kiss1r$^{+/+}$ and PyMT/Kiss1r$^{+/−}$ mice.
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 < 0.00001). 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+/- was smaller than that in PyMT/Kiss1r−/− mice (Fig. 1C; n = 10 per group, P = 0.029). 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 7 to 9 weeks, tumor sizes were smaller in PyMT/Kiss1r−/− mice than those in PyMT/Kiss1r+/- mice (Fig. 2A; arrows in third and fourth panels). Therefore, our data indicated that Kiss1r heterozygosity altered tumor incidence and tumor formation.

To investigate whether Kiss1r heterozygosity 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 (terminal ductal lobular unit) was not found in PyMT/Kiss1r−/− mice, but approximately 22% of normal mammary gland was detected in PyMT/Kiss1r+/− mice (Fig. 2C; P < 0.0001). There was no significant difference in distribution of papilloma and atypical ductal hyperplasia between PyMT/Kiss1r−/− tumor cohort and PyMT/Kiss1r+/− tumor cohort. However, Kiss1r heterozygosity markedly delayed mammary tumor progression with a decrease of ductal carcinoma in situ by approximately 20% (Fig. 2C; P = 0.0068). Moreover, the amount of invasive ductal carcinoma 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 with an approximate 37% in 11-week-old PyMT/Kiss1r+/- mice (Fig. 2C; P = 0.0213). Thus, our data indicated that Kiss1r heterozygosity delayed tumor malignancy and progression.

We next examined an expression of Kiss1r in tumor tissues using the immunohistochemistry. PyMT/Kiss1r+/− tumor expressed higher Kiss1r than PyMT/Kiss1r−/− tumor (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.

**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 as whole-mounting preparations were preferred to address premalignant lesions of mammary glands, termed mammary intraepithelial neoplasia or hyperplastic atypia that results in mammary tumors. When hyperplastic nodules in the fourth 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 fourth inguinal mammary fat pad of PyMT/Kiss1r−/− mice was much less than that in the PyMT/Kiss1r−/− mice (Fig. 2A and B; P < 0.0001). In addition, PyMT/Kiss1r−/− did not develop hyperplastic mammary gland as a result of the defective postnatal mammary gland development (Fig. 2B; P < 0.0001). 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 those in PyMT/Kiss1r+/− mice (Fig. 2A; arrows in third and fourth panels). Therefore, our data indicated that Kiss1r heterozygosity altered tumor incidence and tumor formation.

**Autocrine Kiss1/Kiss1r signaling is sufficient for mouse breast tumor initiation**

To investigate a local function of Kiss1/Kiss1r signaling for hyperplasia, lentiviruses for short hairpin RNAs (shRNA) specific for Kiss1 and Kiss1r were injected into fourth nipple at 2 weeks of age before 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 with 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 C; 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 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<sup>+/+</sup> and PyMT/Kiss1r<sup>+/−</sup> mice and examined the expression of Kiss1 and Kiss1r, respectively. PyMT/Kiss1r<sup>+/−</sup> primary tumor cells showed a significant reduction of Kiss1r expression compared with that in PyMT/Kiss1r<sup>+/+</sup> 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<sup>+/+</sup> and PyMT/Kiss1r<sup>+/−</sup> 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<sup>+/+</sup> and PyMT/Kiss1r<sup>+/−</sup> primary tumor cells was 29.33 and 31.76 hours, respectively (Fig. 4A; n = 3 per group, P = 0.0007), suggesting that Kiss1r heterozygosity negatively regulates mammary tumor cell proliferation.

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

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

To further test that Kiss1r heterozygosity in breast epithelial tumor cells affects tumor growth in vivo, we conducted orthotopic injection assays in NOD.SCID/NCr mice using primary cultured tumor cell lines isolated from PyMT/Kiss1r<sup>+/−</sup> and PyMT/Kiss1r<sup>−/−</sup> mice, respectively. Tumor volume obtained from PyMT/Kiss1r<sup>−/−</sup> tumor cells in NOD.SCID/NCr mice was significantly smaller than those obtained from PyMT/Kiss1r<sup>+/−</sup> 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 tumorigenecity via RhoA**

To understand the molecular mechanisms of Kiss1r signaling in the regulation of tumorigenecity, we next examined the activation of Rho GTPases in breast tumor cells, as Rho GTPases are reported to regulate tumor progression and metastasis (27, 28). In PyMT/Kiss1r<sup>+/−</sup> 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<sup>−/−</sup> tumor cells was also decreased when compared with that in PyMT/Kiss1r<sup>+/−</sup> tumor cells (Fig. 5A), suggesting that Kiss1r signaling in breast tumor cells regulates the activation of RhoA GTPase. To link the finding to our previous observation that autocrine Kiss1/Kiss1r signaling affects tumorigenecity, we next conducted the anchorage-independent growth assays. Dominant-negative RhoA (RhoA DN; RhoA<sup>DN</sup>) decreased the anchorage-independent growth of PyMT/Kiss1r<sup>−/−</sup> cells by approximately 47% (RhoA DN vs. empty; n = 6, P = 0.0001), but wild-type and constitutively dominant active form of RhoA (RhoA<sup>WT</sup>; RhoA<sup>DA</sup>; RhoA<sup>DN</sup>) increased the anchorage-independent colony growth by approximately 29% and 35%, respectively [n = 6; RhoA wild-type (RhoA<sup>WT</sup>) vs. empty, P = 0.0146; RhoA<sup>DA</sup> vs. empty, P = 0.0045]. Similar data were observed in PyMT/Kiss1r<sup>+/−</sup> tumor cells, although the colony number was reduced compared with wild-type tumor cells (Fig. 5B; n = 6; empty/PyMT/Kiss1r<sup>+/−</sup> vs. empty/PyMT/Kiss1r<sup>−/−</sup>, P = 0.0004; RhoA DN/PyMT/Kiss1r<sup>+/−</sup> vs. empty/PyMT/Kiss1r<sup>−/−</sup>, P = 0.0003; RhoA WT/PyMT/Kiss1r<sup>+/−</sup> vs. empty/PyMT/Kiss1r<sup>−/−</sup>, P = 0.0003; and RhoA DA/PyMT/Kiss1r<sup>+/−</sup> vs. empty/PyMT/Kiss1r<sup>−/−</sup>, P = 0.0001). 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 tumorigenecity, as PyMT-induced tumorigenecity was known to require oncogenic activation of Ras (29, 30). As shown in...
Kiss1r (Grp54) Heterozygosity Delays Breast Cancer Progression

Figure 4. Kiss1r signaling regulates tumorigenesis. Kiss1r heterozygosity suppressed primary tumor cell growth (A), cell migration and invasion (B), and anchorage-independent colony formation (C). A, inset, Kiss1r and Kiss1 mRNA level in primary tumor cells isolated from the PyMT/Kiss1r−/− and PyMT/Kiss1r+/− mice. GAPDH used as the internal control for 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+/− mice, respectively.

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. scs shRNA, P = 0.0002), 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 < 0.0001). In addition, RhoA inhibitor (Y-27632) 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 = 0.0001). On the other hand, RhoA DA recovered the inhibitory effect of KISS1R deficiency (knockdown) in Ras-induced tumorigenesis (n = 7, RhoA DA + KISS1R shRNA vs. KISS1R shRNA, P = 0.0097), 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-Gq-q-p63RhoGEF

To delineate KISS1/KISS1R signaling to RhoA, we conducted RhoA activity assays when cells were serum-starved for 24 hours and then incubated for 6 hours in the absence or presence of Kisspeptin-10 (Kp-10; 100 nmol/L). Kp-10 (100 nmol/L) increased GTP-bound RhoA in HEK293 cells overexpressing KISS1R (Fig. 6A). Because serum response factor (SRF) transcriptional activity is widely used for the readout of RhoA GTPase activity (31–33), we conducted the luciferase assays for SRF-dependent promoter activity as the readout of RhoA activation. Kp-10 increased SRF-luciferase activity in cells transfected with KISS1R (Kp-10 in KISS1R cells vs. none in KISS1R cells, P = 0.0309) but not with empty vector (Fig. 6B). Consistently, Kp-10 also activated serum response element–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. RhoA DA highly increased SRF transcriptional activity in HEK293 cells stably expressing KISS1R (RhoA DA vs. empty, P = 0.0027). RhoA WT has little effect on the basal SRF transcriptional activity (RhoA WT + Kp-10 vs. RhoA WT, P = 0.0002; Fig. 6C; 13th, 14th, and 17th bars). RhoA DN suppressed Kp-10 activation of SRF transcriptional activity (Fig. 6C; 13th and 16th bars). Thus, our data indicate that KISS1/KISS1R-mediated SRF activation requires RhoA activation.

To understand how KISS1R activates RhoA, we examined whether the activation of RhoA was mediated by Gq-q, as it was revealed that KISS1R selectively coupled to Gq-q upon Kisspeptin stimulation (1, 4, 8, 34). In HEK293 cells stably transfected with KISS1R, Kp-10 stimulation or constitutively active mutant of Gq-q (Gq-q CA) induced SRF transcriptional activity (Gq-q CA vs. empty, P = 0.0015; Gq-q WT + Kp-10 vs. Gq-q WT, P = 0.0003) whereas Gq-q wild-type (Gq-q WT) alone did not affect SRF transcriptional activity (Fig. 6C; 3rd, 4th, and 7th bars). A dominant-negative mutant of Gq-q (Gq-q DN) attenuated Kp-10–induced SRF transcriptional activation (Fig. 6C; 5th and 6th bars). Thus, our data suggest that the activation of RhoA by Kp-10 is mediated by KISS1R-Gq-q signaling pathway.

As Gq-q activates RhoA via p63RhoGEF (also called GEFT; refs. 35–37), we further examined the Gq-q coupling to p63RhoGEF. In KISS1R-overexpressing cells, p63RhoGEF wild-type (p63RhoGEF WT) did not affect basal or Kp-10–induced SRF transcriptional activity (p63RhoGEF WT + Kp-10 vs. p63RhoGEF WT, P = 0.0003), but catalytic domain-deleted mutant of p63RhoGEF (p63RhoGEF ΔN) inhibited Kp-10

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activation of SRF transcriptional activity (Fig. 6C; 8th–11th bars). Furthermore, p63RhoGEF ΔN suppressed SRF transcriptional activation by Gαq CA (Fig. 6C, 12th bar; Gαq CA vs. p63RhoGEF ΔN + Gαq CA, P = 0.0001), indicating that p63RhoGEF is downstream of Gαq 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 + Gαq CA, P = 0.0001), 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 with that in PyMT/Kiss1r+/+ tumor were 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 showed 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<sup>+/−</sup>) 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 show that KISS1/KISS1R signaling regulates early steps of tumor development through RhoA activation by coupling to G<sub>α</sub>q-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, as 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). Although 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, Kiss1r/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 GqG–p63RhoGEF signaling pathway. Somatic mutation of GqG (GNQ) at codon 209 was frequently found in uveal melanoma and blue naevi (39, 40). Therefore, constitutively active mutation of GqG seems to be involved in melanoma progression. Recent studies delineated that p63RhoGEF linked the activation of Goq and RhoA (36, 37). Our laboratory 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 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, although RhoA promotes pulmonary metastasis of MDA-MB-231 metastatic human breast cancer cell line where endogenous KISS1/KISS1R signaling is absent (43), Kp-10 treatment inhibits TNFα-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, as Kp-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+/− and PyMT/Kiss1r−/− tumors, Kiss1r heterozygosity affected the expression levels of various genes, such as sca-1, for tumor initiation and development (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) showed that Kiss1r/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, as 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 earlier.

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.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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