Enhanced Hepatocyte Growth Factor Signaling by Type II Transforming Growth Factor-β Receptor Knockout Fibroblasts Promotes Mammary Tumorigenesis

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Abstract
Transforming growth factor-β (TGF-β) plays complex dual roles as an inhibitor and promoter of tumor progression. Although the influence of the stromal microenvironment on tumor progression is well recognized, little is known about the functions of TGF-β signaling in the stroma during tumor progression. Using cre-lox technology, expression of the type II TGF-β receptor was selectively knocked out in fibroblasts (Tgfbr2FspKO). In a co-xenograft model, we show that Tgfbr2FspKO fibroblasts enhance mammary carcinoma growth and metastasis in mice while increasing hepatocyte growth factor (HGF) expression and c-Met signaling downstream pathways including signal transducers and activators of transcription 3 (Stat3) and p42/44 mitogen-activated protein kinase (MAPK). Treatment of tumor-bearing mice with a pharmacologic inhibitor (EXEL-7592) of c-Met blocks tumor progression. Using cre-lox technology, expression of Tgfbr2FspKO fibroblasts alter the signaling interactions with epithelial tumor cells to promote tumor progression.

Introduction
The transformation of normal epithelial tissue to invasive cancer is a multistep process that depends heavily on interactions between epithelial cells and their surrounding stromal microenvironment. Fibroblasts are a major cellular component of the stroma and influence tumor cell behavior directly and indirectly through secretion of growth regulators and angiogenic factors, extracellular matrix proteins, and proteases (1, 2). Activation and accumulative growth of fibroblasts characterize desmoplasia, a common occurrence in many human cancers including breast, prostate, and melanoma (3, 4), which correlates with poor prognosis. Whereas the molecular mechanisms of desmoplasia remain unclear, studies suggest that changes in the stroma may precede neoplastic transformation. Genetic alterations including chromosomal rearrangements and mutations in the TP53 and PTEN genes have been detected in the stroma, correlating with formation of malignant breast cancers (5). Coimplantation of prostate, ovarian, or mammary epithelial cells with carcinoma-associated fetal or irradiated fibroblasts results in enhanced tumor growth and invasion (4, 6). These studies indicate that heritable changes in fibroblasts alter the signaling interactions with epithelial tumor cells to promote tumor progression.

As a growth factor expressed primarily by fibroblasts, hepatocyte growth factor (HGF) acts on epithelial cells expressing c-Met receptor tyrosine kinases to promote cell proliferation, motility, and regulating differentiation. All of these cellular functions are achieved by binding of the TGF-β ligand to its cell-surface type II receptor, which leads to recruitment and activation of its type I receptor and subsequent downstream signaling in the cytoplasm of multiple pathways including Smad, mitogen-activated protein kinase (MAPK), and Rho pathways (7). In vitro, TGF-β regulates desmosplastic responses by activating fibroblasts to acquire a myofibroblast state inducing production of growth factors, angiogenic factors, extracellular matrix proteins, and proteases (8, 9). Conditional knockout of the TGF-β type II receptor in fibroblasts (Tgfbr2FspKO) has been shown to inhibit normal mammary gland development in Tgfbr2FspKO mice but enhance growth of tumor cells in a xenograft model (10). Studies in our laboratory suggest that alterations in paracrine signaling between Tgfbr2FspKO fibroblasts and tumor cells may, in part, contribute to the enhanced tumor growth.

Whereas studies in our laboratory (10, 15) show an inverse correlation between HGF/c-Met signaling in tumor growth and TGF-β signaling in the stroma, the molecular mechanisms and functions for HGF/c-Met signaling in mammary tumor progression mediated by stromal TGF-β signaling have remained unclear. In the present study, we define a functional contribution for HGF/c-Met signaling in mammary tumor progression by showing that coimplantation of 4T1 mammary carcinoma cells with Tgfbr2FspKO fibroblasts in the subrenal capsule of nude mice resulted in enhanced tumor growth with increased formation of distant metastases to the spleen, liver, and lungs. These phenotypes were directly associated with increased phosphorylation of c-Met.
receptor and downstream signaling proteins including Stat3 and p42/44 MAPK. Treatment of tumor-bearing mice with a small-molecule c-Met inhibitor, EXEL-7592, significantly inhibited primary tumor growth and metastatic dissemination and also inhibited HGF/c-Met signaling. Similarly, small interfering RNA (siRNA) knockdown of c-Met expression in mammary tumor cells reduced metastatic spread and c-Met signaling caused by Tgfbr2FspKO fibroblasts. These studies show that TGF-β signaling in fibroblasts suppresses HGF/c-Met signaling in epithelial cells to negatively regulate epithelial tumor cell metastasis.

Materials and Methods

Mouse strains and maintenance. Female nude (nu/nu) mice were obtained from Harlan Mutant Mouse Research Center (North Carolina). FSP.Cre mice (CF57Bl/6) were crossed with the Tgfbr2Flox/Flox mice (C57Bl/6) to generate Tgfbr2Flox/Flox mice positive for Cre expression (Tgfbr2FspKO). Animals positive for the Cre gene and homozygous for the Tgfbr2Flox/Flox gene were identified by PCR analysis of genomic DNA from tail biopsy (16). These animals were maintained in accordance with Association for Assessment and Accreditation of Laboratory Animal Care and Vanderbilt University guidelines.

Cell lines. Primary fibroblasts were isolated from the thoracic and inguinal mammary glands from Tgfbr2FspKO and Tgfbr2Flox/Flox mice at –6 weeks of age according to Medina and Kittrell (17). Fibroblasts were cultured for a minimum of three passages before in vivo studies. Spontaneously immortalized Tgfbr2FspKO fibroblast cell lines (10) used for in vitro studies were cultured on plastic in DMEM/F12/5% adult bovine serum (ABS). The 4T1 parental mammary carcinoma cell line was generously provided by the laboratory of Fred Miller (University of Michigan, Ann Arbor, MI). c-Met receptor expression was targeted in 4T1 parental cell lines by siRNA silencing (4T1.2Met, 4T1.3Met) as described below. The siRNA control cell line 4T1.LGFp was generated as described above. 4T1 parental line and the sublines 4T1.LGFp, 4T1.2Met, and 4T1.3Met were cultured on plastic in DMEM containing 10% fetal bovine serum with antibiotics.

For HGF treatment, 4T1 tumor cells were cultured to 80% confluency, starved in serum-free medium for 24 h, and then treated with 60 ng/ml HGF (R&D Systems) in serum-free medium for 30 min.

Chemicals. EXEL-7592 and EXEL-1075 were synthesized and purified in the Medicinal Chemistry Department at Exelixis, Inc. (18). A 10 mM stock solution was prepared in DMSO and diluted in optimal assay buffers or culture medium.

Assessment of tumor progression. Granting of collagen-embedded cells was done according to the methods of Hayward et al. (19). Briefly, 1 × 10^5 4T1 cells were resuspended together with 2.5 × 10^5 Tgfbr2FspKO or Tgfbr2Flox/Flox fibroblasts in 50 μl of collagen per graft. The collagen-embedded cells were cultured in DMEM/F12/5% ABS for 24 h. The implanted under the renal capsule layer of the kidneys in female nude mice, 8 to 10 weeks of age. Grafted kidneys were collected 21 days post-implantation and weighed. Primary tumor mass was determined by subtracting the contralateral kidney mass from the grafted kidney.

In vivo treatment with EXEL-7592 and EXEL-1075 inhibitors. Tgfbr2FspKO fibroblasts were grafted with 4T1 cells in the subrenal capsule of nude mice as described above (one graft per mouse). Seven days postgrafting, mice were injected with 30 mg/kg of EXEL-7592, EXEL-1075 in 0.9% NaCl solution, or saline vehicle i.p. for 14 days before sacrifice for tumor analysis. Levels of EXEL-7592 and EXEL-1075 in plasma were analyzed by liquid chromatography-mass spectrometry (Exelixis) of blood extracted 4 and 24 h after the 14th day of treatment.

SiRNA silencing of c-Met receptor expression in 4T1 cells. The siRNA construct to target c-Met was obtained from Dr. Martin Schwartz (University of Virginia, Charlottesville, VA). The H1 promoter and targeting sequences were digested from the above constructs with HindIII and EcoRI and cloned into the same sites of a siRNA expression vector (pSUPER) or a siRNA retroviral vector (pRETRO-SUPER). The pRETRO-SUPER vector was generously provided by Dr. Reuven Agami (Division of Tumor Biology, The Netherlands Cancer Institute, Amsterdam, the Netherlands) and described in ref. 20. The two targeting sequences used for siRNA-mediated knockdown of c-Met were 5′-TCAGGTCTCTGGTCTGACT-3′ and 5′-GGAGGACCGTGTTGACCAT-3′. Oligonucleotides for enhanced GFP (eGFP) were also designed and used as a negative control for RNA interference (5′-GCTGACCTGAAATCTC-3′, 2MET–, 3MET–, and eGFP targeting oligonucleotides were designed as previously described (20). Oligonucleotides for eGFP were phosphorylated by kinase treatment and complementary oligos were annealed and subcloned into the BgII and HindIII sites of pSUPER or pRETO-SUPER.

Immunohistochemistry and histology. Primary tumor, spleen, lung, and liver tissues were fixed in 4% paraformaldehyde/PBS overnight at 4°C; subjected to dehydration in 50%, 70%, 80%, 95%, and 100% ethanol and xylene; and then paraffin embedded. Five-micrometer sections were prepared for immunohistochemistry. The sections were stained with H&E. Immunoperoxidase staining for Ki67 (PharMingen) and von Willebrand factor 8 (vWF8) expression (PharMingen) was done by the Vanderbilt Histology Core Facility. APOPTOSIS in tumor sections was analyzed by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL; Intergen) and visualized by peroxidase staining (Vectastain Elite kit, Vector Laboratories). Sections were counterstained with hematoxylin Blood vessel density was calculated by determining the relative area of vWF8 positive staining in at least five high-power fields whereas proliferative and apoptotic indices were calculated by determining the relative area of positively stained cells to total number of cells in at least five high-power fields using Scion Image software. Lung tissues from grafted mice were analyzed by whole mount staining according to methods by Maglione et al. (21).

Western blot analyses of tumor cell lysates. Primary tumors or cultured cell lysates were lysed in radioimmunoprecipitation assay buffer containing 1% Igepal CA-630 (Sigma), 0.5% sodium deoxycholate, 0.1% SDS in PBS, supplemented with a protease inhibitor cocktail (Sigma) containing aprotinin and leupeptin and 10 mM/L sodium orthovanadate (Sigma). Fifty micrograms of protein were fractionated on 8% to 12% SDS-PAGE gels. The proteins were transferred to nitrocellulose membranes and then probed with antibodies (1:1,000) to the phosphorylated and unphosphorylated forms of c-Met, p42/44 MAPK, and Stat3 (Cell Signaling Technologies). Specific immunoreaction was detected using horseradish peroxidase–conjugated immunoglobulin G antibodies and ECL Plus chemiluminescence (Amersham Pharmacia Biotechnology). The blots were reprobed with α-actin antibodies (Santa Cruz Biotechnology; 1:500) to confirm uniform loading. Data are a representation of three independent samples per cell line.

Determination of HGF levels. HGF secretion in 4T1 tumor cells and fibroblasts was determined by ELISA (R&D Systems) using 100 mM of conditioned medium as described (10).

Transwell migration assay. 4T1 tumor cells (50,000) were seeded on an 8-μm pore transwell filter coated with fibronectin (50 μg/mL; Sigma), 0.1% gelatin in 5 h at 37°C in the presence or absence of HGF (60 ng/mL) or fibroblast-conditioned medium from Tgfbr2FspKO fibroblasts. The cells were fixed in neutral formalin buffer (vWR) for 10 min. Cells on the top side of the filter were removed by cotton swab and the migrated cells were visualized by staining with 0.1% crystal violet and quantitated.

To generate fibroblast-conditioned medium, 500,000 Tgfbr2FspKO fibroblasts were plated in 100-mm dishes and incubated in DMEM/F12/0.1% ABS for 24 h.

Statistical analysis. Analyses of study results focused on estimating the association between the study groups (e.g., Tgfbr2FspKO fibroblasts versus Tgfbr2Flox/Flox fibroblasts) and study end points (e.g., tumor mass). The tests of hypotheses about the single measurement of outcome data (e.g., tumor mass) were carried out using the nonparametric Kruskal-Wallis test, the general linear model, or one-way ANOVA. Tests of hypotheses about the multiple measurements of the outcome data (e.g., Ki67) were made using the restricted/residual maximum likelihood–based mixed-effect model to adjust the intracorrelation effect for the animals that had multiple measurements. The model reported in the article was selected based on Akaike’s Information Criterion and Schwarz’s Bayesian Criterion with
different covariance structures (e.g., Toeplitz, first-order autoregressive, and compound symmetry, etc.). All tests of significance were two sided, and differences were considered statistically significant when \( P < 0.05 \). All data were expressed as mean ± SD. SAS version 9 was used for all analyses.

Results

Tgfb2FspKO fibroblasts enhance 4T1 tumor growth and metastasis with increased c-MET activation. To address the role of TGF-\( \beta \) signaling by stromal cells on the metastatic process.

Figure 1. Tgfb2FspKO fibroblasts grafted with 4T1 mammary carcinoma cells lead to enhanced growth and invasion. A, primary tumor masses from nude mice grafted with 4T1 alone, with Tgfbr2Flox/Flox, or Tgfbr2FspKO fibroblasts. Columns, mean weight (n = 9 per experimental group); bars, SE. Statistical comparison among 4T1 alone, 4T1:Tgfbr2Flox/Flox, and 4T1:Tgfbr2FspKO tumors was done using the Kruskal-Wallis test. B, primary tumor tissue sections were immunostained for the presence of Ki67 (cell proliferation), TUNEL-positive nuclei (apoptosis), and vWF8 (tumor angiogenesis). Bar, 40 µm. Positive staining was quantified with the Scion image software. Statistical comparison among experimental groups was done using the Mixed Procedure test. C, the concentration of HGF secreted into the conditioned medium of cultured Tgfbr2Flox/Flox and Tgfbr2FspKO fibroblasts and 4T1 tumor cells was analyzed by ELISA. SF, serum-free; Basal medium, DMEM/F12/0.1% ABS. Statistical comparison among experimental groups was done using one-way ANOVA. D, primary tumor cell lysates were analyzed by Western blot and densitometry for the phosphorylated status of the indicated proteins. Representative of triplicate samples analyzed.
during mammary tumor progression, we used 4T1 mammary carcinoma cells, which were derived from a spontaneous tumor in BALB/c mice (22). When implanted in mice, 4T1 tumor formation and metastatic spread are rapid and histologically analogous to human breast cancers, making it a relevant model in which to study mammary tumor progression (22). To prevent the potential interference of endogenous host fibroblasts, 4T1 tumor cells were implanted alone or with either Tgfbr2 FspKO or Tgfbr2 Flox/Flox fibroblasts under the renal kidney capsules of nude mice, a site shown to be largely devoid of fibroblasts (23). Grafting of 4T1 tumor cells with Tgfbr2FspKO fibroblasts resulted in an f1.7-fold increase in primary tumor mass compared with grafting with Tgfbr2Flox/Flox fibroblasts. Increased vascularity in the primary tumor was observed in the gross morphology whereas increased invasion was observed in the kidney parenchyma, as determined by examination of H&E-stained sections (Fig. 1A). Immunostaining of tumor tissue with Ki67 indicated a significant 1.8-fold increase in cell proliferation of 4T1 cells grafted with Tgfbr2FspKO fibroblasts whereas immunostaining for TUNEL-positive nuclei revealed a 1.5-fold decrease in apoptosis, although not statistically significant. By immunostaining for the endothelial cell marker vWF8, we measured a significant 1.8-fold increase in blood vessel density in 4T1 cells grafted with Tgfbr2FspKO fibroblasts (Fig. 1B). The data indicate increased 4T1 tumor cell proliferation, survival, and angiogenesis in the presence of Tgfbr2FspKO fibroblasts. Taken together, the data show that Tgfbr2-deficient stromal fibroblasts interact with 4T1 mammary carcinoma cells to enhance growth and invasion.

As Tgfbr2FspKO fibroblasts secreted high levels of HGF compared with control fibroblasts and 4T1 tumor cells (Fig. 1C), we did Western blot analyses on 4T1 primary tumor xenografts to determine possible changes in HGF/c-Met signaling. 4T1 tumor...
cells grafted with Tgfb2FspKO fibroblasts exhibit increased phosphorylation levels of c-Met, Stat3, and p42/44 MAPK proteins relative to those grafted with Tgfb2Flox/Flox fibroblasts or to 4T1 tumor cells grafted alone (Fig. 1D). Taken together, the data indicate that Tgfb2FspKO fibroblasts enhance 4T1 mammary carcinoma growth correlating with enhanced HGF/c-Met signaling.

The effect of Tgfb2FspKO fibroblasts on metastatic spread was assessed in lung, liver, and spleen tissues, which were dissected from tumor-bearing mice 21 days post grafting. Metastases to the lungs, spleen, and liver tissues were confirmed by H&E staining. By observing gross morphology, we detected a significant 3-fold increase in the number of nodules on the spleen and liver, whereas whole mount staining revealed a 5-fold increase in the number of lung metastases in 4T1:Tgfb2FspKO xenografts (Figs. 2A–C). These data indicate that Tgfb2FspKO fibroblasts enhance the metastatic process of mammmary carcinoma cells.

EXEL-7592 significantly inhibits 4T1 tumor growth and metastasis. To investigate a possible function for HGF/c-Met signaling during tumor progression mediated by Tgfb2FspKO fibroblasts, we used the pharmacologic inhibitor EXEL-7592, which competitively binds the ATP binding pocket of the c-Met receptor as well as the vascular endothelial growth factor (VEGF) ligand binding receptor KDR (Exelixis, U.S. patent WO2005030140). To control for the effects of KDR inhibition in our studies, we included a KDR-specific inhibitor, EXEL-1075. By in vitro kinase assay, EXEL-7592 was found to potently inhibit c-Met (IC50 0.4 nmol/L) and KDR (IC50 0.15 nmol/L). EXEL-1075 did not significantly affect c-Met phosphorylation (IC50 4,000 nmol/L) but did potently inhibit KDR phosphorylation (IC50 7 nmol/L; Supplementary Table I). EXEL-7592 also inhibited HGF- and VEGF-induced migration of multiple cell types at concentrations <1 μmol/L whereas EXEL-1075 specifically inhibited VEGF-induced migration at concentrations <1 μmol/L (Supplementary Table II). To determine the effect of EXEL-7592 on 4T1 cells, cultured tumor cells were treated with HGF in the presence or absence of EXEL-7592 or EXEL-1075. By Western blot analyses, c-Met phosphorylation was blocked by EXEL-7592 treatment and was unaffected by EXEL-1075 (Fig. 3A). These data indicate that low concentrations of EXEL-7592 but not EXEL-1075 are sufficient to inhibit HGF-induced phosphorylation and activity of c-Met.

We next determined the contribution of c-Met phosphorylation enhanced by Tgfb2FspKO fibroblasts in mammary tumor progression. 4T1:Tgfb2FspKO xenografts were established in mice and grown for 7 days, at which time tumor-bearing mice were injected i.p. with 30 mg/kg of EXEL-7592, EXEL-1075, or saline vehicle daily for the following 14 days. High-performance liquid chromatography analyses of plasma samples 24 h after the final treatment indicated that EXEL-1075 was present at ~2,500 ng/mL whereas EXEL-7592 was present at 250 ng/mL (Fig. 3B), indicating a higher rate of plasma clearance for EXEL-7592.

Treatment with EXEL-7592 resulted in a significant 3.3-fold decrease in primary tumor growth compared with saline vehicle treatment and a 2-fold decrease in growth compared with EXEL-1075 treatment (Fig. 4A). By immunostaining for Ki67, EXEL-7592 inhibited primary tumor cell proliferation by 2-fold compared with saline or EXEL-1075 treatment. TUNEL analysis revealed that EXEL-1075 and EXEL-7592 treatment increased cellular apoptosis by 2-fold compared with saline-treated mice. Immunostaining for vWF8 indicated that EXEL-1075 treatment decreased blood vessel density by 2-fold whereas EXEL-7592 reduced blood vessel density by 3-fold, compared with saline treatment (Fig. 4B). These data indicate that whereas EXEL-7592 was present at lower levels in vivo than EXEL-1075, EXEL-7592 exerted a more potent effect on tumor growth.

By Western blot analyses of primary tumor cell extracts, EXEL-7592 treatment significantly decreased levels of phosphorylated c-Met, Stat3, and p42/44 MAPK proteins, which were unaffected by EXEL-1075 (Fig. 4C). These results indicate that EXEL-7592 effectively blocked HGF/c-Met signaling in primary tumors. Whereas the whole tumor extracts contain proteins from both carcinoma and stromal cells, the majority of the tumor is composed of carcinoma cells (10); thus, the changes observed likely represent those occurring in carcinoma cells in response to the fibroblasts. Taken together, these data suggest that EXEL-7592 decreases tumor growth through inhibition of c-Met activity.

Spleen, liver, and lung tissues of mice treated with EXEL-7592 displayed a reduction in the total number of metastatic lesions as compared with tissues from saline- or EXEL-1075–treated mice (Fig. 4D). EXEL-1075 did not significantly affect metastatic spread to liver and spleen (Fig. 4D) and slightly decreased metastasis to the lungs (Fig. 4A). These data indicate that systemic blockade of HGF/c-Met signaling by EXEL-7592 significantly inhibits the metastatic process during mammary tumor progression.

SilRNA silencing of c-Met receptor expression in 4T1 cells significantly reduces tumor metastatic formation. Because systemic delivery of EXEL-7592 would inhibit HGF/c-Met and KDR signaling of multiple cell types, we sought to clarify a specific role for HGF/c-Met signaling in stromal-epithelial interactions by targeting expression of c-Met on tumor cells by siRNA silencing. 4T1 cells were generated to stably express two different siRNAs against the c-Met receptor gene and resulted in two different cell
lines silenced for c-Met expression: 4T1.2Met− and 4T1.3Met−. To control for target specificity of siRNA silencing, 4T1 parental cells were transduced with retrovirus-expressing siRNAs designed to target GFP (4T1.GFP−). Western blot analyses of the targeted 4T1 cell lines indicated a 47% and 87% reduction in c-Met receptor expression in the 4T1.2Met− and 4T1.3Met− sublines, respectively (Fig. 5A). 4T1.GFP− did not show a significant difference in c-Met receptor expression compared with the 4T1 parental line. 4T1.2Met− (47% c-Met reduction) showed an intermediate decrease in phosphorylated c-Met, p42/44 MAPK, and Stat3 proteins whereas 4T1.3Met− (87% c-Met reduction) exhibited the most significant decrease in phosphorylated proteins (Fig. 5B). Both 4T1.2Met− and 4T1.3Met− tumor cells showed significantly decreased transwell migration in response to HGF or conditioned medium from Tgfbr2FspKO fibroblasts, compared with control tumor cells (Fig. 5C). These data indicate that HGF responsiveness in the 4T1 tumor cells is dependent on c-Met expression levels.

To determine the effects of c-Met deficiency on tumor progression, the 4T1 cell lines 4T1.GFP−, 4T1.2Met−, and 4T1.3Met− xenografts as determined by Ki67 or TUNEL immunostaining. However, 4T1.3Met− xenografts displayed a significant reduction in vWF8 blood vessel density as compared with 4T1.GFP− xenografts, whereas 4T1.2Met− xenografts did not (Fig. 6B), suggesting that HGF/c-Met signaling in epithelial cells plays a role in tumor vascularization. By Western blot analyses of tumor cell extracts, 4T1.2Met− and 4T1.3Met− xenografts showed a significant reduction in levels of phosphorylated c-Met, p42/44 MAPK, and Stat3 proteins, compared with 4T1.GFP− controls (Fig. 6C). These data indicate that silencing of c-Met impaired HGF/c-Met signaling in both cell lines.

We next examined the effect of c-Met silencing on tumor metastasis. We observed that 4T1.2Met− and 4T1.3Met− xenografts produced fewer metastatic lesions of the liver, spleen, and lungs (Fig. 6D). These data indicate that silencing of c-Met in tumor cells significantly blocks tumor metastases enhanced by Tgfbr2FspKO fibroblasts.

Discussion

We have previously reported that fibroblasts deficient in TGF-β signaling enhance growth and invasiveness of PyVmT mammary...
carcinomas, correlating with enhanced c-Met signaling in primary tumors (10). As metastatic spread was not observed at the time of primary tumor examination, we were unable to evaluate the effect of Tgfbr2-deficient fibroblasts on mammary tumor metastases and thus prove the involvement of the HGF/c-Met in these processes.

In these current xenograft studies, we show that Tgfbr2FspKO fibroblasts enhanced 4T1 primary tumor growth at 21 days and metastatic spread to the lungs, liver, and spleen compared with control xenografts. Similar to the PyVmT:Tgfbr2FspKO xenografts (10), 4T1:Tgfbr2FspKO xenografts showed significantly increased levels of phosphorylated c-Met, p42/44 MAPK, and Stat3 proteins, which correlates with increased expression of HGF by Tgfbr2FspKO fibroblasts observed in vitro. Taken together, these studies indicate that the tumor-promoting effects of Tgfbr2FspKO fibroblasts are not restricted to a particular cell line but may represent a more common mechanism of tumor growth. Moreover, these data show a correlation between enhanced primary tumor progression caused by Tgfbr2-deficient fibroblasts and enhanced HGF/c-Met signaling.

To determine the significance of HGF derived from Tgfbr2FspKO fibroblasts in mammary tumor progression, we inhibited c-Met in vivo using pharmacologic inhibition and siRNA silencing of c-Met of 4T1 tumor cells. Both methods reduced 4T1 metastatic spread caused by Tgfbr2FspKO fibroblasts, indicating that intact c-Met signaling was necessary for 4T1 metastasis and Tgfbr2FspKO fibroblasts were a significant source of HGF. Consistently, we observed that HGF and Tgfbr2FspKO fibroblast-conditioned medium potently stimulated migration in 4T1 cells; these effects were abrogated by silencing of c-Met expression. These observations are consistent with previous studies done in PyVmT tumor cells, in which neutralizing antibodies to HGF inhibited PyVmT tumor cell migration induced by Tgfbr2FspKO fibroblasts (10). Interestingly, unlike PyVmT tumor cells, 4T1 cell proliferation was not affected by HGF treatment. Consistently, silencing of c-Met expression in the 4T1 cells did not significantly affect primary tumor growth in vivo. In comparing the PyVmT xenograft model with the 4T1 model, we suggest that PyVmT tumor cells rely on c-Met signaling for cell growth and motility, whereas 4T1 cells rely on c-Met signaling for cell motility but not cell growth. Taken together, these data suggest that enhanced HGF secretion by Tgfbr2-deficient fibroblasts promotes tumor cell motility to enhance 4T1 metastatic spread. We do recognize that other mechanisms may also contribute to tumor progression enhanced by Tgfbr2FspKO fibroblasts, including the possibility that the increased HGF expression by Tgfbr2FspKO fibroblasts may also act on other cell types, including endothelial cells, to promote tumor angiogenesis. It would be of interest in the future to study the role of fibroblast-endothelial interactions in tumor progression.

Therapeutic targeting of HGF signaling has been shown to block primary tumor growth and metastatic spread of various cancers in mice including those of the gall bladder, pancreas, breast, and lung (14). Consistent with these studies, we show that treatment of tumor-bearing mice with EXEL-7592, a KDR/c-Met inhibitor, resulted in a significant reduction in primary tumor growth and metastasis compared with saline vehicle treatment and KDR inhibition by EXEL-1075 treatment. Whereas previous studies have shown that targeting of the VEGF/KDR pathway has been effective in some breast cancers (24), KDR inhibition in our xenograft model did not significantly inhibit primary tumor growth compared with vehicle saline treatment, nor did it significantly affect liver or spleen metastasis. We did observe that EXEL-1075 treatment reduced the number of lung metastases. Whereas the cause of this reduction is unclear, one possibility is that lung tissues in the tumor-bearing mice were exposed to EXEL-1075 due to systemic delivery of the drug, subsequently suppressing vascular function and inhibiting the ability of tumor cells to colonize to these tissues. Despite higher levels of EXEL-1075 detected in peripheral blood compared with EXEL-7592, a significantly greater therapeutic effect was observed with EXEL-7592 treatment. The potent effects of EXEL-7592 may be due its high binding affinity to c-Met with an IC50 10,000-fold higher than EXEL-1075. In addition, EXEL-7592...
was found to bind KDR receptor with a 7-fold greater affinity than EXEL-1075, indicating that low levels of EXEL-7592 would be sufficient to effectively block tumor progression. Thus, synergistic inhibition of c-Met and KDR by EXEL-7592 would have also resulted in more potent inhibition of tumor progression.

To further examine the functional role of HGF derived from Tgfb2
fibroblasts in mammary tumor progression, we specifically targeted c-Met expression in tumor cells. Whereas both the 4T1.2Met– and 4T1.3Met– cell lines exhibited deficiencies in c-Met signaling in response to HGF in vitro, neither cell lines showed significant changes in primary tumor growth, cell proliferation, or apoptosis compared with 4T1 cells with intact c-Met in vivo. These observations are consistent with in vitro observations in which 4T1 cell proliferation was not affected by HGF. It is likely that the reduction in primary tumor growth observed in EXEL-7592 treatment was caused by inhibition of all KDR- and c-Met–expressing cell types, including endothelial cells and immune cells (25, 26). 4T1.3Met– primary tumors, but not 4T1.2Met– primary tumors, showed decreased blood vessel density. As HGF has been shown to regulate the expression of angiogenic factors secreted by tumor cells to decrease blood vessel density. Both c-Met–deficient tumor cell lines did exhibit decreased metastasis, suggesting that intact c-Met expression is required for 4T1 metastatic spread but not primary tumor growth.

Whereas our data show that HGF/c-Met is important for 4T1 tumor progression, previous studies have shown that c-Met also interacts with other molecules to mediate tumor progression. c-Met has been shown to interact with Ron, a c-Met–related tyrosine kinase, to cooperate in ovarian tumor cell invasiveness (28). In addition, c-Met has been shown to interact with semaphorins (29), integrins (30), and heparin sulfate proteoglycans (31) to mediate tumor cell migration and invasion in various tumor cell types, including colon, gastric, and breast tumors. Therefore, it is likely that multiple mechanisms involving c-Met contribute to 4T1 tumor growth and metastasis. It would be of interest in the future to investigate more fully the effects of c-Met knockdown in mammary tumor cells on tumor cell growth and metastasis.

In summary, the present study shows that loss of TGF-β signaling in stromal fibroblasts can dramatically enhance metastasis of mammary carcinomas and provide overwhelming evidence that HGF/c-Met signaling is involved in this process. In particular, we conclude that HGF derived from Tgfb2
fibroblasts...
fibroblasts enhances c-Met signaling to promote mammary tumor progression. This tumor xenograft mouse model represents a unique context in which to study stromal TGF-β and HGF/c-Met signaling in mammary tumorigenesis. As metastasis to vital organs remains a leading cause of death among cancer patients, clarifying the mechanisms regulating this process, such as TGF-β and HGF signaling, will aid the design of more efficient therapies for cancer.

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