Published in final edited form as:

Clin Exp Metastasis. 2011 April ; 28(4): 351–366. doi:10.1007/s10585-011-9373-0.

Loss of one *Tgfb β 2* allele in fibroblasts promotes metastasis in MMTV: polyoma middle T transgenic and transplant mouse models of mammary tumor progression

Wei Bin Fang,

Department of Pathology and Laboratory Medicine, University of Kansas Cancer Center, University of Kansas Medical Center, Lied 3001, 3901 Rainbow Blvd, Kansas City, KS 66160, USA

Iman Jokar,

Department of Pathology and Laboratory Medicine, University of Kansas Cancer Center, University of Kansas Medical Center, Lied 3001, 3901 Rainbow Blvd, Kansas City, KS 66160, USA

Anna Chytil,

Department of Cancer Biology, Vanderbilt-Ingram Cancer Center, Nashville, TN 33232, USA

Harold L. Moses,

Department of Cancer Biology, Vanderbilt-Ingram Cancer Center, Nashville, TN 33232, USA

Ty Abel, and

Department of Pathology, Vanderbilt University Medical Center, Nashville, TN 33232, USA

Nikki Cheng

Department of Pathology and Laboratory Medicine, University of Kansas Cancer Center, University of Kansas Medical Center, Lied 3001, 3901 Rainbow Blvd, Kansas City, KS 66160, USA

Abstract

Accumulation of fibroblasts is a phenomenon that significantly correlates with formation of aggressive cancers. While studies have shown that the TGF- β signaling pathway is an important regulator of fibroblast activation, the functional contribution of TGF- β signaling in fibroblasts during multi-step tumor progression remains largely unclear. In previous studies, we used a sub-renal capsule transplantation model to demonstrate that homozygous knockout of the *Tgfb β 2* gene (*Tgfb β 2*^{FspKO}) enhanced mammary tumor growth and metastasis. Here, we show for the first time a significant role for loss of one *Tgfb β 2* allele during multi-step mammary tumor progression. Heterozygous deletion of *Tgfb β 2* in stromal cells in MMTV–PyVmT transgenic mice (PyVmT/*Tgfb β 2*^{hetFspKO} mice) resulted in earlier tumor formation and increased stromal cell accumulation. In contrast to previous studies of *Tgfb β 2*^{FspKO} fibroblasts, *Tgfb β 2*^{hetFspKO} fibroblasts did not significantly increase tumor growth, but enhanced lung metastasis in PyVmT transgenic mice and in co-transplantation studies with PyVmT mammary carcinoma cells. Furthermore, *Tgfb β 2*^{hetFspKO} fibroblasts enhanced mammary carcinoma cell invasiveness associated with expression of inflammatory cytokines including CXCL12 and CCL2. Analyses of *Tgfb β 2*^{FspKO} and

© Springer Science+Business Media B.V. 2011

ncheng@kumc.edu.

Conflict of interest None.

Electronic supplementary material The online version of this article (doi:10.1007/s10585-011-9373-0) contains supplementary material, which is available to authorized users.

Tgfb²^{hetFspKO} fibroblasts revealed differences in the expression of factors associated with metastatic spread, indicating potential differences in the mechanism of action between homozygous and heterozygous deletion of *Tgfb²* in stromal cells. In summary, these studies demonstrate for the first time that loss of one *Tgfb²* allele in fibroblasts enhances mammary metastases in a multi-step model of tumor progression, and demonstrate the importance of clarifying the functional contribution of genetic alterations in stromal cells in breast cancer progression.

Keywords

Mammary carcinoma; Metastasis; Polyoma middle virus T transgenic mice; Stroma; *Tgfb²*; Chemokine

Introduction

The accumulation of reactive stromal cells, particularly fibroblasts in primary tumors, strongly correlates with metastatic disease and poor patient prognosis in various cancers [1-3]. The functional contribution of stromal fibroblasts in regulating the growth and progression of numerous types of cancer has been well established in transplantation studies in mice [4-6]. Recent studies have focused on identifying the molecular mechanisms that regulate the activity of reactive stromal cells in the tumor microenvironment. Reported mutations in the TP53 and PTEN genes in stromal cells correlate with malignancy of breast cancers [7] indicating that heritable changes in fibroblasts alter the signaling interactions with epithelial tumor cells to promote tumor progression.

A number of studies have indicated that deregulation of TGF- β signaling in the stroma may have profound effects on tumor progression. In mouse xenograft models of prostate cancer, treatment of primary tumors with TGF- β promotes the accumulation of reactive stromal cells, including fibroblasts, enhancing the growth and progression of these tumors [8-10]. These studies indicate a tumor promoting role for TGF- β signaling in the stroma. In contrast, overexpression of a kinase deficient TGF- β type II receptor in the mammary stroma of transgenic mice results in mammary epithelial hyperplasia and increased hepatocyte growth factor expression [11], indicating a tumor suppressive role for TGF- β signaling in the stroma. Furthermore, studies indicate that senescent stromal fibroblasts, show increased replicative errors in transcription of the *Tgfb²* gene [12], correlating with increased expression of secretory growth factors and enhanced tumor progression [13, 14]. Mutations in the *Tgfb¹* genes have been reported in the stroma of head and neck cancers and colon cancers leading to decreased TGF- β signaling in the stroma [15], indicating a clinically relevant role for TGF- β signaling deficiency in the tumor stroma. The different outcomes caused by up-regulation or down-regulation of TGF- β signaling in the stroma may in part depend on several factors including: type of tissues involved, state of oncogenic transformation and age. These factors have been shown to affect expression of TGF- β receptors and activity of TGF- β signaling pathways regulating the anti-proliferative response and epithelial mesenchymal transition [16-18]. In summary, studies indicate complex multiple roles for stromal TGF- β signaling during tumor progression. In previous studies, we studied the effects of stromal TGF- β signaling on tumorigenesis by inactivating the *Tgfb²* gene in a subset of fibroblasts using Cre-Lox. Mice expressing Cre under the control of the Fibroblast Specific Protein 1 promoter (Fsp1-Cre) were bred with mice carrying Lox P sites flanking exon 2 of *Tgfb²* (*Tgfb²*^{Flox/Flox}). Subsequent progeny which exhibited homozygous knockout *Tgfb²* (*Tgfb²*^{FspKO}) developed carcinoma of the fore stomach and prostatic intraepithelial neoplasias [19]. Interestingly, while mammary development was severely inhibited in these *Tgfb²*^{FspKO} mice, mammary *Tgfb²*^{FspKO}

fibroblasts grafted with PyVmT or 4T1 mammary carcinoma cells in the sub-renal capsules of nude mice resulted in enhanced primary tumor growth and metastasis correlating with enhanced secretion of oncogenic signaling factors from *Tgfr2*^{FspKO} fibroblasts including HGF [20].

Based on the transplantation studies involving *Tgfr2*^{FspKO} fibroblasts, we hypothesized that loss of TGF- β signaling in the stroma enhanced the growth and progression of mammary carcinomas. To address this hypothesis, we used the MMTV–PyVmT mouse model in order to investigate the effect of *Tgfr2* deficiency during different stages of mammary tumor development and progression. Homozygous deletion of *Tgfr2* knockout mice results in severe phenotypic defects, including infertility and early death [19]. Heterozygous deletion of *Tgfr2* resulted in mice that were viable and fertile without visible defects in mammary gland development [19, 20], indicating very different developmental outcomes from homozygous *Tgfr2* deletion in stromal cells. These different developmental phenotypes and the conflicting nature of TGF- β signaling in tumor progression raised the possibility that differences in response to stromal TGF- β signaling in tumors could be due to differences in levels of *Tgfr2* and downstream signaling pathways. Therefore, in these present studies, we conditionally targeted one *Tgfr2* allele in stromal cells, and examined the effects of heterozygous knockout of stromal specific *Tgfr2* on mammary tumor progression by crossing MMTV–PyVmT mice with mice exhibiting loss of one *Tgfr2* allele in stromal cells (*Tgfr2*^{hetFspKO}).

Subsequent PyVmT positive progeny showing loss of one *Tgfr2* allele in fibroblasts (PyVmT/*Tgfr2*^{hetFspKO}) exhibited significantly shorter tumor latency and increased numbers of lung metastases associated with reduced tumor cell survival and the accumulation of reactive stromal cells. The enhanced metastasis was further observed in cotransplantation studies of heterozygous knockout *Tgfr2* fibroblasts with PyVmT carcinoma cells in the sub-renal capsule of nude mice. Furthermore, *Tgfr2*^{hetFspKO} fibroblasts enhanced mammary carcinoma cell invasiveness associated with expression of inflammatory cytokines including CXCL12 and CCL2. Analyses of *Tgfr2*^{FspKO} and *Tgfr2*^{hetFspKO} fibroblasts revealed certain differences in the expression of factors associated with metastatic spread including CXCL12, indicating potential differences in the mechanism of action between homozygous and heterozygous deletion of *Tgfr2* in stromal cells. In summary, these studies demonstrate for the first time that loss of one *Tgfr2* allele in fibroblasts enhances mammary metastases in a multi-step model of tumor progression, and demonstrate the importance of clarifying the functional contribution of genetic alterations in stromal cells in breast cancer progression.

Results

Targeted deletion of one *Tgfr2* allele in mammary fibroblasts enhances tumor progression in PyVmT transgenic mice

In order to determine the effects of *Tgfr2* deficiency in fibroblasts on stage dependent mammary tumor progression, we utilized the MMTV–PyVmT mice which express the Polyoma Virus Middle T antigen under the control of the mouse mammary tumor virus promoter (MMTV), an active promoter in mammary epithelium. Because MMTV–PyVmT mice form mammary tumors analogous to stage IV breast cancers and tumor progression can be examined at specific stages [21, 22], the MMTV–PyVmT transgenic mouse model represents a useful and relevant model for mammary tumor progression. Homozygous deletion of the *Tgfr2* allele in fibroblasts results in severe defects in mammary gland development, and tumorigenic lesions in the prostate and fore-stomach. Therefore neither male nor female *Tgfr2*^{FspKO} mice were viable for breeding [19]. Mice expressing Fsp1-Cre and one Floxed *Tgfr2* allele resulted in loss of one *Tgfr2* allele in fibroblasts

(*Tgfr2*^{hetFspKO} mice). *Tgfr2*^{hetFspKO} mice were observed to be viable and fertile [19, 20]. In addition, female *Tgfr2*^{hetFspKO} mice did not exhibit visible differences in ductal outgrowth and morphogenesis compared to wildtype mice, as determined by whole mount staining and H&E stain of inguinal and thoracic mammary gland tissues. These data demonstrated that loss of one *Tgfr2* allele in stroma did not significantly affect mammary gland development in *Tgfr2*^{hetFspKO} mice.

We crossed *Tgfr2*^{hetFspKO} mice with MMTV-PyVMT mice in order to achieve targeted deletion of *Tgfr2* in mammary fibroblasts in subsequent PyVMT positive progeny. Female PyVMT mice exhibiting loss of one *Tgfr2* allele (PyVMT/*Tgfr2*^{hetFspKO}) and control mice (PyVMT with intact wildtype or floxed *Tgfr2* alleles, also referred to as PyVMT controls) were palpated for tumor formation twice weekly. The tumors in PyVMT/*Tgfr2*^{hetFspKO} mice became physically palpable between the ages of 5.4 and 10.4 weeks with a median age of 7.6 weeks for tumor development. In control PyVMT mice, tumor nodules became physically palpable between 6.5 and 14.5 weeks with a median age of 9.0 weeks for tumor development (Fig. 1a). Control mice and PyVMT/*Tgfr2*^{hetFspKO} mice were sacrificed and organs were harvested at 13.4 weeks of age, when the earliest time when tumor burden exceeded 10% of initial body weight. Multifocal tumors formed at the thoracic and inguinal mammary glands in control and PyVMT/*Tgfr2*^{hetFspKO} mice. As determined at 13.4 weeks, PyVMT/*Tgfr2*^{hetFspKO} mice showed a small but not statistically significant increase in tumor mass compared to control mice (Fig. 1b). The most striking features of PyVMT/*Tgfr2*^{hetFspKO} mice were the presence of large areas of necrosis, and solid sheets of cells, without discernible ductal structures (Fig. 1c), indicating that the presence of poorly differentiated tumors, in particular high grade adenoductal carcinoma. Control PyVMT tumors exhibited less areas of necrosis and the presence of tubule structures with atypical hyperplasia indicating the presence of moderately differentiated tumors. These data indicate that loss of one *Tgfr2* allele in stromal cells promotes earlier tumor onset, resulting in poorly differentiated, more invasive tumors.

In previous studies, we showed that *Tgfr2*^{FspKO} fibroblasts co-grafted with 4T1 mammary carcinoma cells resulted in increased numbers of lung metastases [20]. To determine if loss of one *Tgfr2* allele in mammary fibroblasts significantly affected the metastatic spread of PyVMT mammary carcinomas, we counted the number of metastatic nodules visualized by hematoxylin whole-mount staining of lung tissues from PyVMT/*Tgfr2*^{hetFspKO} and PyVMT control mice. The presence of these metastatic nodules was confirmed microscopically by H&E stain (Fig. 1d), and indicated increased numbers of lung metastases in PyVMT/*Tgfr2*^{hetFspKO} mice compared to control mice. These data indicate that loss of one *Tgfr2* allele in fibroblasts results in increased lung metastases in PyVMT transgenic mice. In summary, these data indicate that earlier tumor formation resulted in further carcinoma progression of PyVMT/*Tgfr2*^{hetFspKO} mice.

Primary tumors in PyVMT/*Tgfr2*^{hetFspKO} mice are associated with enhanced tumor cell survival and growth associated with increased accumulation of stroma

To further determine the effects of stromal *Tgfr2* deficiency on mammary tumor growth and survival, we performed immunostaining for Ki67, a proliferation marker, and TUNEL, an apoptotic marker, on primary tumor sections of PyVMT/*Tgfr2*^{hetFspKO} and PyVMT control mice. Tumors from PyVMT/*Tgfr2*^{hetFspKO} mice exhibited a 20% increase in levels of Ki67 staining that was statistically significant, indicating increased tumor cell proliferation. In addition, PyVMT/*Tgfr2*^{hetFspKO} tumors exhibited a significant 80% decrease in TUNEL-positive cells, indicating increased tumor cell survival, respectively (Fig. 2a-b).

To determine the effects of *Tgfb2* deletion in fibroblasts on formation of reactive stroma, we assayed for levels of fibroblasts, macrophages and endothelial cells by immunohistochemistry staining of mammary tumors. Previous studies have shown that fibroblasts represent a heterogeneous population of cells, with overlapping expression of alpha-smooth muscle actin (α -sma), vimentin and Fsp1 [23]. Therefore, immunostaining was performed to examine the presence of the fibroblast population using these biomarkers. Immunostaining was also performed to analyze macrophage recruitment by F4/80 staining and blood vessel density by Von Willebrand Factor 8 (VWF8) staining. PyVmT/*Tgfb2*^{hetFspKO} mice exhibited increased staining for α -sma, Fsp1, vimentin and F4/80 (Fig. 3a-d), but did not show any significant changes in VWF8 staining (Fig. 3e). These studies indicate that targeting of one *Tgfb2* allele in stromal cells in PyVmT transgenic mice results in increased accumulation of fibroblasts and macrophages, but does not significantly change vascular density in primary mammary carcinomas.

Effect of Fsp1-Cre mediated deletion of one *Tgfb2* allele in mammary stromal cells

As targeted deletion of one *Tgfb2* allele in mammary fibroblasts resulted in enhanced mammary tumor progression of PyVmT mice, we further investigated the effects of Cre-mediated deletion of one *Tgfb2* allele in mammary fibroblasts. In previous studies, the Fsp1 promoter was observed to be active in a subset of fibroblasts in reporter mice expressing Green Fluorescence Protein under the control of the Fsp1 promoter. In addition, similar specific patterns of Fsp1 promoter activity were observed in Rosa26 reporter mice, which expressed β -galactosidase upon Fsp1-Cre mediated deletion of the stop codon flanking the β -galactosidase gene [20]. In these studies, spatial analysis of Cre expression was determined by Cre immunohistochemistry in the mammary tissues of PyVmT/*Tgfb2*^{hetFspKO} mice sacrificed at 13.4 weeks. Cre was expressed primarily in the stroma of primary mammary tumors, including cells exhibiting a fibroblastic morphology (Fig. 4A). To further quantitate the number of fibroblasts exhibiting loss of one *Tgfb2* allele, Southern blot analyses were performed on mammary fibroblasts isolated from PyVmT/*Tgfb2*^{WT/flox} control and PyVmT/*Tgfb2*^{hetFspKO} mice. By phosphoimager analysis, we observed that approximately 30% of the fibroblasts isolated from PyVmT/*Tgfb2*^{hetFspKO} mice showed Cre-mediated deletion of exon 2 of one *Tgfb2* allele (Fig. 4B). These data indicate that mammary fibroblasts represent a heterogeneous population of cells and that a subset of mammary fibroblasts from this heterogeneous population exhibit loss of one *Tgfb2* allele.

We next determined if loss of one *Tgfb2* allele affected TGF- β responsiveness in cultured mammary fibroblasts. To circumvent the possibility that the presence of wildtype (WT) fibroblasts would obscure the TGF- β responsiveness of heterozygous *Tgfb2* knockout fibroblasts in a heterogeneous population of fibroblasts, the subset of fibroblasts exhibiting heterozygous knockout of *Tgfb2* were sorted from a heterogeneous population of *Tgfb2*^{hetFspKO} cells by flow cytometry as previously described [20]. Briefly, *Tgfb2*^{hetFspKO} fibroblasts were initially flow sorted into single cells in 96 well plates. Single cells were expanded into clonal populations of cells and analyzed for the presence of WT and Flox *Tgfb2* alleles and for Cre by PCR analyses [20]. The control fibroblasts and heterozygous *Tgfb2* knockout clonal cell populations derived from the heterogeneous population of *Tgfb2*^{hetFspKO} cells were used in subsequent experiments.

To determine the effect of heterozygous loss of *Tgfb2* on cell proliferation, clonal and heterogeneous fibroblasts were treated with TGF- β and analyzed for changes in ³[H] thymidine incorporation. Statistical analyses were performed to determine possible statistical significances between cells treated with or without TGF- β , between control and heterozygous *Tgfb2* knockout fibroblasts, and between cell lines in each experimental group. As shown in Fig. 5a and b, TGF- β treatment significantly decreased ³[H] thymidine incorporation in *Tgfb2*^{hetFspKO} fibroblasts and control fibroblasts. These data indicate that

loss of one *Tgfb2* allele did not significantly affect responsiveness of heterogeneous or clonal mammary fibroblasts to exogenous TGF- β stimulation. Also, there were no significant differences between cell lines in either the control or *Tgfb2*^{hetFspKO} group. However, each of heterogeneous and clonal *Tgfb2*^{hetFspKO} fibroblast cell lines showed significant increases in overall cell proliferation compared to each of the control fibroblasts (Fig. 5a, b) and correlated with the accumulation of stroma observed in PyVmT/*Tgfb2*^{hetFspKO} mice. These data indicate that Fsp1-Cre mediated deletion of one *Tgfb2* allele does not significantly affect the ability of exogenous TGF- β to inhibit mammary fibroblasts, but does enhance overall cell proliferation.

***Tgfb2*^{hetFspKO} fibroblasts significantly enhance invasiveness of PyVmT mammary carcinoma cells associated with inflammatory cytokine expression**

As we observed a pro-tumorigenic phenotype in PyVmT/*Tgfb2*^{hetFspKO} mice, we further determined the effects of *Tgfb2* deficient mammary fibroblasts on mammary tumor progression using a sub-renal transplant model. PyVmT mammary carcinoma cells were grafted in the presence of clonal *Tgfb2*^{WT/Flox} or clonal *Tgfb2*^{hetFspKO} fibroblasts in the sub-renal capsule of nude mice, a site devoid of host fibroblasts and ideal for examining stromal: epithelial interactions [24]. 45 days post-transplantation, the mice were then analyzed for changes in primary tumor growth and metastatic spread. As shown in Table 1, PyVmT mammary carcinoma cells grafted with clonal *Tgfb2*^{hetFspKO} fibroblasts exhibited decreased tumor growth and increased lung metastases compared to co-grafting with control fibroblasts. These data indicate that loss of one *Tgfb2* allele in mammary fibroblasts specifically contributes to mammary tumor progression by enhancing metastatic spread.

To determine the mechanisms through which *Tgfb2*^{hetFspKO} fibroblasts enhanced metastatic spread, we hypothesized that *Tgfb2* deficiency de-regulated paracrine signaling interactions between fibroblasts and carcinoma cells to promote cellular invasiveness. First, carcinoma cell invasiveness was determined using a 3D collagen assay in which PyVmT carcinoma cells labeled with CMFDA CellTracker Fluorochrome were embedded in collagen, and monitored for invasion through the collagen over time (Fig. 6). This in vitro invasion assay enabled us to mimic the in vivo process through which carcinoma cells invade through extracellular matrix in primary tumors. PyVmT cells co-cultured with *Tgfb2*^{hetFspKO} fibroblasts resulted in a significant increase in carcinoma cell invasion compared to PyVmT cells cultured alone or co-cultured with control fibroblasts (Fig. 6). To identify the specific factors secreted by *Tgfb2*^{hetFspKO} fibroblasts that contributed to enhanced mammary carcinoma cell invasion, we profiled conditioned medium from control or *Tgfb2*^{hetFspKO} fibroblasts using Raybiotech antibody cytokine arrays (Supplemental Fig. 1). *Tgfb2*^{hetFspKO} fibroblasts showed significantly increased expression of cytokines previously associated with carcinoma cell invasiveness and breast cancer metastases including CXCL7 and CXCL12 (Table 2) [25-27]. Other cytokines found to be significantly increased in *Tgfb2*^{hetFspKO} fibroblasts were previously shown to modulate bone marrow derived immune cell recruitment during inflammation and mammary tumor progression including GCSF, CCL2 and CCL12 [28-30]. Other inflammatory cytokines and secreted factors on the array, which did not show significant levels in expression in either experiment group included members of the IL-1 family (IL-1b, IL-1 through 4), members of tumor necrosis factor family (CD30L, CD40L) and IFN- γ . Other various secreted factors which were expressed in both experimental groups, but did not show significant changes in expression included: CCL19, CCL21, CCL9, CXCL2, TIMP-2, osteopontin, MMP-2, TNF- α , bFGF and VEGF-D. These data indicate that increased metastases in PyVmT/*Tgfb2*^{hetFspKO} mice is associated with expression of specific cytokines involved in mediating breast cancer metastases and recruitment of bone marrow derived immune cells.

To further determine the functional significance of the factors secreted by *Tgfr2*^{hetFspKO} fibroblasts in carcinoma cell invasion, we compared the expression patterns of secreted proteins from heterozygous *Tgfr2* knockout fibroblasts with factors from homozygous *Tgfr2* knockout fibroblasts which were identified through proteomics profiling and candidate screening [31]. We observed a number of similarities and differences in the expression of particular proteins. Of note, CXCL12, an important regulator of metastatic spread [32, 33], was shown to be decreased in homozygous *Tgfr2* knockout fibroblasts and increased in heterozygous *Tgfr2* knockout fibroblasts (Table 3). HGF was previously shown to be upregulated in homozygous *Tgfr2* knockout fibroblasts by ELISA and Northern blot analysis [20, 34], and is a factor whose expression in stromal cells was shown to promote mammary tumor growth and metastases [31, 34]. In these studies, ELISA analysis of HGF levels showed a small but not significant decreased in expression in heterozygous *Tgfr2* knockout fibroblasts, compared to control fibroblasts (Fig. 7a). Other proteins associated with regulating mammary carcinoma cell proliferation and migration including: CXCL10, IGF-I and -II showed significant changes in homozygous *Tgfr2* knockout fibroblasts [31], but did not show changes in expression in heterozygous *Tgfr2* knockout fibroblasts compared to control fibroblasts, as summarized in Table 3. CCL2 was shown to be increased in both homozygous [35] and heterozygous *Tgfr2* knockout fibroblasts (Fig. 7b, c). CCL2 was of particular interest due to previous studies in our laboratory showing that targeting CCL2 in *Tgfr2*^{FspKO} fibroblasts decreased metastases without significantly affecting macrophage recruitment, indicating a possible direct effect on mammary carcinoma cells [35]. To determine the functional significance of CCL2 derived from *Tgfr2*^{hetFspKO} fibroblasts on mammary carcinoma invasion, PyVmT cells were treated with neutralizing antibodies to CCL2 in the presence of *Tgfr2*^{hetFspKO} fibroblasts. Compared to IgG control treatment, anti-CCL2 significantly inhibited invasion of PyVmT mammary carcinoma cells induced by *Tgfr2*^{hetFspKO} fibroblasts (Fig. 7d) indicating an important role for CCL2 in regulating carcinoma cell invasiveness. In summary, these data indicate that loss of one *Tgfr2* knockout fibroblasts results in similarities and also significant differences in secretion of cytokines associated with mammary tumor progression, compared to loss of both *Tgfr2* alleles.

Discussion

In these studies, we show for the first time that targeted deletion of one *Tgfr2* allele in mammary fibroblasts enhances stromal cell accumulation in primary tumors and promotes metastasis of mammary carcinomas in the MMTV–PyVmT transgenic mouse model. A number of factors affect TGF- β signaling in stromal cells contributing to the differences in tumor phenotypes in these studies compared to previous studies [19, 20], including the background pathology, type of mouse model used, and allelic expression. While a subset of fibroblasts showed heterozygous deletion of *Tgfr2*, our studies indicate significant functional consequences on the progression of mammary carcinomas that are potentially regulated through multiple, complex mechanisms.

In previous studies, *Tgfr2*^{hetFspKO} mice did not exhibit significant changes to mammary gland development [19]. In these studies, heterozygous *Tgfr2* deletion significantly enhanced progression of mammary carcinomas in MMTV–PyVmT transgenic mice. The mechanisms responsible for the differences in phenotype are most likely related to the background pathology, in which oncogenic expression of PyVmT was necessary for tumor initiation because heterozygous *Tgfr2* deletion in stromal cells was not sufficient to induce tumors in normal mice. In the normal mammary gland, expression of oncogenes and tumor suppressors including HGF and TGF- β are tightly regulated at a temporal and spatial level to mediate cell proliferation, survival and migration during ductal outgrowth and morphogenesis [36, 37]. Cellular stress such as sustained signaling in normal mammary

epithelial cells leads to cell cycle arrest and apoptosis [38], providing additional protective mechanisms against tumor growth. The lack of a discernible phenotype in *Tgfr2*^{hetFspKO} mice indicate that the presence of these strict regulatory mechanisms in normal mammary epithelial cells compensate for the de-regulated growth factor expression in *Tgfr2*^{hetFspKO} fibroblasts. As a potent viral oncogene, constitutive overexpression of PyVmT in mammary epithelial cells deregulates multiple signaling pathways and overrides cell cycle checkpoints [22, 39], leading to consistent tumor formation. These studies indicate that heterozygous *Tgfr2* deletion in stromal cells enhances progression of mammary carcinomas initiated by oncogenes such as PyVmT.

In this report, we observed that heterozygous deletion of *Tgfr2* in stromal cells resulted in similar patterns of metastatic spread but differences in tumor growth between PyVmT transgenic mice and in grafted mice. In both studies, heterozygous deletion of *Tgfr2* in stromal cells enhanced metastatic spread of PyVmT mammary carcinoma cells, indicating that TGF- β signaling in stromal cells acts as a metastasis suppressor. In contrast, PyVmT mice/*Tgfr2*^{hetFspKO} mice exhibited a small but not significant increase in tumor mass, while co-grafting of PyVmT cells with *Tgfr2*^{hetFspKO} fibroblasts resulted in significantly decreased tumor mass. Despite the differences in tumor growth, the enhanced metastatic phenotype in both studies indicates that tumor growth is not a reliable indicator for mammary tumor malignancy. The observations here are consistent with previous studies showing that some small breast tumors harbor highly metastatic cells [40]. It is possible that increased metastatic dissemination contributed to the decrease in tumor size of subrenal grafts containing *Tgfr2*^{hetFspKO} fibroblasts and also in the size of PyVmT/*Tgfr2*^{hetFspKO} tumors despite the increase in cell proliferation observed in these tumors. This hypothesis is supported by previous gene profiling studies identifying metastasis specific genes such as SPARC and MMP2 [41], and data from these present studies showing increased expression of pro-metastatic factors and level expression of growth factors from *Tgfr2*^{hetFspKO} fibroblasts.

The differences in mouse models would have also influenced the effects of *Tgfr2*^{hetFspKO} stromal cells on mammary tumor growth or invasion. As the effect of kidney cells on mammary stromal: epithelial interactions remain unclear, transplantation in the subrenal capsule could affect activity of *Tgfr2*^{hetFspKO} fibroblasts and mammary carcinoma cells resulting in decreased tumor growth compared to tumors formed in the mammary gland. Another factor affecting tumor growth would be the differences in T cell recruitment in each tumor model. T cells play critical roles in mediating anti-tumor immunity through recognition of tumor specific antigens and subsequent targeting of tumor cells, inhibiting metastatic spread [42]. It is possible that the lack of cytotoxic T cells in nude grafted mice would enhance metastatic spread of mammary tumor cells in tumor bearing mice, resulting in smaller tumors. In PyVmT/*Tgfr2*^{hetFspKO} tumors, increased macrophage recruitment was observed. As macrophages in breast tumors exhibit increased expression of immunosuppressive factors [43, 44], it is possible that the increased macrophage recruitment would lead to changes in T cell activity or recruitment, affecting tumor growth and metastasis. A third possible factor affecting tumor growth is the malignancy of PyVmT cells in each tumor model. As metastatic carcinoma cells are more responsive to pro-invasive factors compared to benign cells [45], it is possible that PyVmT cells in the subrenal graft model would exhibit increased invasion and decreased growth in response to the secretory factors from *Tgfr2*^{hetFspKO} fibroblasts. In contrast, in PyVmT transgenic mice, benign mammary epithelial cells would first be exposed to *Tgfr2*^{hetFspKO} fibroblasts due to early embryonic deletion of *Tgfr2* [19, 46]. Studies indicate that signaling interactions between stromal cells and carcinoma cells evolve over the progression of carcinomas [47, 48]. As such, exposure of early stage PyVmT carcinoma cells to *Tgfr2*^{hetFspKO} fibroblasts would change the pattern of signaling interactions between stromal cells and epithelial cells over

time, possibly leading to the tumor phenotypes observed in PyVmT/*Tgfr2*^{hetFspKO} mice. Understanding how the differences in tumor models affect mammary tumor progression would contribute to identification of relevant signaling pathways that regulate stromal: epithelial interactions during mammary tumor progression. Despite these differences in tumor models, the data indicate that TGF- β signaling in stromal cells suppresses tumor growth and metastasis, and specific effects are dependent on allelic expression of *Tgfr2*.

In previous studies, homozygous *Tgfr2* knockout fibroblasts showed significant impairment in TGF- β responsiveness [20]. In contrast, heterozygous *Tgfr2* knockout fibroblasts did not exhibit any differences in TGF- β responsiveness compared to control fibroblasts but did show significant increases in overall cell proliferation. These data indicate that loss of one *Tgfr2* allele is not sufficient to inhibit paracrine TGF- β signaling in mammary fibroblasts but inhibits autocrine TGF- β signaling. The increased fibroblast growth in vitro is consistent with increased stromal cell growth observed in PyVmT/*Tgfr2*^{hetFspKO} mice (Fig. 3). Other changes to the stroma of PyVmT/*Tgfr2*^{hetFspKO} mice include increased macrophage recruitment, which may be due in part to increased expression of chemokines from *Tgfr2*^{hetFspKO} fibroblasts, including CCL2 [49, 50]. Given that the presence of reactive stroma significantly correlates with breast cancer malignancy and poor patient prognosis [51-53], our data suggest that subtle genetic changes to *Tgfr2* in stromal cells would affect the overall composition of the tumor microenvironment through expression of paracrine factors, thereby also affecting mammary tumor progression indirectly.

Analysis of the protein expression profiles between *Tgfr2*^{FspKO} and *Tgfr2*^{hetFspKO} fibroblasts indicate that the differences in expression of soluble factors may contribute to the differences in tumor phenotype. In previous studies, homozygous deletion of *Tgfr2* in fibroblasts resulted in increased expression of growth factors, including IGF-I, IGF-II, CXCL10 and HGF. These stromal derived soluble factors have been shown to promote mammary tumor cell proliferation in vitro [54-56]. Inhibition of these factors through siRNA targeting or pharmacologic inhibition in mammary tumor bearing mice significantly reduces tumor growth, indicating pro-tumorigenic roles for these soluble factors [34, 57-59]. The role of these growth factors is consistent with the increased mammary tumor growth observed in co-grafting of *Tgfr2*^{FspKO} fibroblasts with mammary carcinoma cells [20]. In these studies, *Tgfr2*^{hetFspKO} fibroblasts did not exhibit significant differences in expression of these growth factors, correlating with the lack of tumor growth in these studies. While the molecular mechanisms regulating expression of IGF-I, IGF-II and CXCL10 in mammary fibroblasts remain unclear, the mechanisms may be similar to HGF, in which TGF- β mediates transcriptional suppression through Smad dependent mechanisms. Previous studies have shown that the HGF promoter region contains Smad binding inhibitor elements, and that HGF expression is suppressed by Smad2/3 and SP-1 transcription factors mediated by TGF- β [60]. Alternatively, TGF- β may suppress transcriptional activators of IGF-I, IGF-II or CXCL10, for example inhibiting Stat5 transcription of IGF-I, [61, 62]. Homozygous [20], but not heterozygous deletion of *Tgfr2* significantly impaired TGF- β responsiveness indicating that gene expression of these specific growth factors is related to stoichiometric expression of *Tgfr2*. Loss of one *Tgfr2* allele was not sufficient to affect expression of factors affecting tumor growth; instead, *Tgfr2*^{hetFspKO} stromal cells showed enhanced expression of factors promoting metastasis.

Tgfr2^{hetFspKO} fibroblasts also exhibited changes in expression of particular factors associated with cellular invasion and metastatic spread, in particular CXCL12. Previous studies have shown that breast cancer associated fibroblasts express high levels of CXCL12 which functions to regulate metastatic homing [26, 32]. Here, we observed that loss of both *Tgfr2* alleles decreases CXCL12 expression [31], while loss of one *Tgfr2* allele increases

CXCL12 expression in mammary fibroblasts. The molecular mechanisms responsible for the differences in CXCL12 expression remain unclear, but may involve TGF- β type II receptor interactions with other cell surface proteins to regulate CXCL12 expression. TGF- β receptors have been shown to interact with erbB2 receptors to potentiate erbB2 signaling pathways [63]. In addition, enhanced erbB2 signaling is associated with increased transcription of CXCL12 in mammary tumors [64]. Changes in *Tgfb2* expression may alter interactions between the TGF- β receptors and erbB2 leading to changes in signaling pathways that regulate CXCL12 so that heterozygous deletion of *Tgfb2* enhances CXCL12 expression while homozygous deletion of *Tgfb2* inhibits CXCL12 expression. Possible changes in downstream mechanisms include alterations in CpG methylation of the CXCL12 promoter [65], and HIF-1 α transcriptional activity [66]. Given the importance of CXCL12 in regulating mammary metastasis, it would be of interest to further investigate the interactions between stromal CXCL12 and TGF- β signaling in mammary tumor progression. Studies are currently being performed to dissect the mechanisms of TGF- β dependent CXCL12 expression in mammary fibroblasts and the contribution of CXCL12 in mammary tumor progression enhanced by *Tgfb2*^{hetFspKO} stromal cells.

In these studies, we also characterize a novel function for CCL2 derived from *Tgfb2*^{hetFspKO} fibroblasts in regulating mammary tumor progression. In previous studies, CCL2 has been shown to primarily regulate mammary tumor metastasis through recruitment of macrophages [43, 67]. In our laboratory, we had shown that *Tgfb2*^{FspKO} fibroblasts expressed higher levels of CCL2 compared to control fibroblasts. Targeting CCL2 specifically in *Tgfb2*^{FspKO} fibroblasts reduced metastasis through a macrophage independent mechanism [35], indicating that CCL2 signaled directly to mammary carcinoma cells during mammary tumor progression. These current studies show that CCL2 derived from *Tgfb2*^{hetFspKO} significantly contributes breast cancer cell invasion, indicating that a novel role for CCL2 in regulating mammary carcinoma cell invasion. In addition, these studies indicate that enhanced expression associated with loss of *Tgfb2* directly signals to mammary carcinoma cells to regulate cellular invasiveness and may contribute to the enhanced metastatic phenotype in PyVmT/*Tgfb2*^{hetFspKO} mice. Given that inflammatory cytokines are being considered as therapeutic targets in cancer [68, 69], it is important to fully understand the molecular mechanisms responsible for chemokine expression during mammary tumor progression. Studies are currently underway to further determine the functional connection between CCL2 mediated breast cancer invasion and metastatic spread.

In summary, our studies have identified several important mechanisms through which loss of one *Tgfb2* allele in stromal cells promotes mammary tumor progression. These mechanisms include alterations in gene expression of soluble factors to affect autocrine TGF- β signaling and paracrine signaling interactions between stromal and epithelial cells in the tumor microenvironment. In addition, we demonstrate a functional cooperativity between inactivation of one *Tgfb2* allele in fibroblastic cells and expression of the PyVmT oncogene in epithelial cells. While hypomorphic mutations in the TGF- β signaling pathway have been reported in a number of cancers [15, 70], current studies are underway to determine the relevance of genetic mutations of TGF- β signaling components in the breast cancer stroma. Further understanding of the complex, multiple effects of TGF- β signaling in both the stroma and tumor cells will facilitate the development and appropriate use of cancer therapies targeting the TGF- β signaling pathways.

Methods

Mouse strains and maintenance

PyVmT positive animals (FVB) were maintained by crossing PyVmT positive males with Wildtype (WT) females. *Tgfb2*^{hetFspKO} mice were generated by crossing FSP-Cre positive

mice (C57BL/6) with $Tgfr2^{flox/flox}$ mice (C57BL/6). PyVmT males were crossed with $Tgfr2^{flox/flox}$ females to generate PyVmT/ $Tgfr2^{flox/flox}$ progeny. PyVmT/ $Tgfr2^{flox/flox}$ males were then crossed with $Tgfr2^{hetFspKO}$ females to generate PyVmT/ $Tgfr2^{hetFspKO}$ mice. Animals were genotyped for the *PyVmT* gene by PCR analysis of genomic DNA as previously described [71]. The presence of the *Cre* and Floxed *Tgfr2* genes were identified by PCR analysis of genomic DNA from tail biopsy as previously described [19, 72]. For transplantation studies, female nude (nu/nu) mice (6–8 weeks of age) were obtained from Harlan Laboratories (North Carolina). These animals were maintained in accordance with AAALAC and University of Kansas Medical Center guidelines.

Assessment of tumor formation and tumor burden in transgenic mice

PyVmT/ $Tgfr2^{WT/flox}$ and PyVmT/ $Tgfr2^{hetFspKO}$ mice were palpated twice weekly for tumor growth. Mice were sacrificed at 13.4 weeks, when tumor burden exceeded 10% of initial bodyweight, according to AAALAC and University of Kansas Medical Center guidelines. Tumor burden was assessed as a measurement of whole body weight at the time of sacrifice.

Histology/immunohistochemistry

Tumor tissues were fixed in 10% neutral formalin buffer and embedded in paraffin using the Vanderbilt University Immunohistochemistry Core and the University of Kansas Medical Center Histology Core facilities. Paraffin embedded sections were subject to H&E staining.

Paraffin embedded sections were subject to immunostaining using antibodies to: TUNEL staining (Cell Signaling Technologies, Danvers, MA), Ki67 (BD-Pharmingen, San Diego, CA), α -sma (Abcam, Cambridge, UK), Fsp-1 (Abcam), vimentin (Abcam), F4/80 (Abcam), Cre (Abcam), VWF8 (BD-Pharmingen), and visualized by peroxidase staining (Vectastain Elite kit, Vector Laboratories, Burlingame, CA). Sections were counterstained with hematoxylin. The density of α -sma, Fsp1, F4/80 and VWF8 positive staining were measured in at least 5 fields at 109 magnification by Image J software. Proliferative (Ki67 positive) and apoptotic indices (TUNEL) were calculated by determining the relative area of positive stained cells to total number of cells in at least five fields at 109 magnification using Image J software.

Lung tissues from control PyVmT mice and PyVmT/ $Tgfr2^{hetFspKO}$ mice were subjected to whole mount staining according to methods by Medina et al. [73]. Briefly, lung tissues were fixed in 4% paraformaldehyde at 4°C overnight and dehydrated in 70, 95 and 100% ethanols for 1 h each at 4°C. The tissue was then subjected to treatment in acetone, 100 and 95% ethanols, stained with Mayer's hematoxylin for 3 min dehydrated in 70, 90 100% ethanols and 100% xylene. The metastatic nodules in the lung were visualized under the stereomicroscope and counted. The lung tissues were then fixed, embedded in paraffin, sectioned and stained by hematoxylin and eosin to confirm the presence of metastatic lesions, which were identified by a clinical pathologist for the presence of acini with atypical nuclei, with cell morphology resembling that of cells in the primary mammary tumors.

Fibroblast and mammary carcinoma cell culture

Primary fibroblasts were isolated from the thoracic and inguinal mammary glands from 13.4 week old PyVmT/ $Tgfr2^{WT/flox}$ and PyVmT/ $Tgfr2^{hetFspKO}$ or $Tgfr2^{hetFspKO}$ mice using a modified protocol as described [74]. The identity of fibroblasts was confirmed by positive immunostaining for Fsp1, vimentin, α -sma and desmin. Fibroblasts were cultured onto 10 mm dishes coated with rat tail collagen type I (BD Biosciences, Palo Alto, CA) in DMEM/F12 medium containing 5% ABS, for a minimum of three passages prior to in vitro and in vivo studies. PyVmT cells were isolated from invasive carcinomas of PyVmT transgenic

mice on an FVB genetic background as described [73] and cultured on plastic in DMEM media containing 10% FBS with antibiotics.

Southern blot analysis

Recombination of the *Tgfr2* allele was assessed by Southern blot analysis in cultured mammary fibroblasts isolated from PyVmT/*Tgfr2*^{WT/flox} and PyVmT/*Tgfr2*^{hetfspKO} mice according to the methods described by Chytil et al. [72].

[³H] Thymidine incorporation

Fibroblast cell proliferation was measured by [³H] Thymidine incorporation and by manual cell counting. To assay [³H] Thymidine incorporation, fibroblasts (20,000) were plated per well in triplicate in 24 well plates (Nalge NUNC International, Rochester, NY), starved for 24 h in growth factor deprived media, then incubated in DMEM/ F12/5% ABS in the presence or absence of 5 ng/ml TGF- β 1 (R&D systems, Minneapolis, MN), treated with 1 μ Ci [³H]dTh (MP Radiochemicals, Solon, OH) for 24 h, and then assayed for [³H] Thymidine incorporation using a beta scintillation counter.

Sub-renal capsule assay

Grafting of collagen embedded cells was performed according to the methods of Hayward et al. [24]. Briefly, 1×10^5 PyVmT cells were re-suspended together with 2.5×10^5 clonal *Tgfr2*^{hetfspKO} or *Tgfr2*^{WT/flox} fibroblasts in 50 μ l of rat tail collagen type I (BD Biosciences, San Jose, CA) for one graft. The collagen embedded cells were cultured in DMEM/F12 5% ABS for 24 h and then implanted under the renal capsule layer of the kidneys in female nude mice, 6-8 weeks of age. Tumor tissues were collected 45 days post-implantation, and weighed.

ELISA

Control and *Tgfr2*^{hetfspKO} fibroblasts (250,000) were grown in 6 cm plates in DMEM media containing 10% FBS and antibiotics. The cells were incubated in DMEM media in the absence of serum for 24 h. Levels of secreted HGF and CCL2 proteins were determined by ELISA (R&D systems), using 100 μ l of conditioned media. Statistical significance was determined by two tailed Student's *t*-test.

Collagen invasion assay

PyVmT mammary carcinoma cells (62,000) were labeled with 3 μ M CMFDA CellTracker dye (Invitrogen, Carlsbad, CA) and embedded in 25 μ l rat tail collagen type I in the presence or absence of fibroblasts (125,000) in 24 well dishes using procedures previously described [15]. Co-cultures were treated with 3 μ g/ml control rat IgG (Sigma, St. Louis, MO) or anti-CCL2 (R&D systems, Minneapolis, MN) for up to 72 h. Images of collagen embedded cells (three fields per sample, samples plated in duplicate) were taken by fluorescence microscopy every 24 h at 109 magnification and quantified by Image J analysis.

Cytokine array profiling

(500,000) PyVmT/*Tgfr2*^{hetFspKO} or PyVmT/*Tgfr2*^{WT/Flox} fibroblasts were cultured in 10 cm dishes for 24 h in 5 ml DMEM/F12/5% FBS. After 24 h incubation, basal medium or fibroblast conditioned medium were harvested and subject to cytokine array profiling using the Mouse Cytokine Antibody Arrays 3 and 4 (Raybiotech) according to manufacturer protocol.

Statistical analysis

Statistical significance between groups was determined by Two-Way ANOVA, Bonferroni–Dunn test, two-tailed Student's *t*-test or Poisson Regression where appropriate. Statistical analyses were performed in consultation with the University of Kansas Biostatistics Core, using Graphpad Prism software (Graphpad Software, San Diego, CA).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Stacey Hembruff for technical assistance, Fang Fan, M.D (University of Kansas Medical Center), Osama Tawfik, M.D (University of Kansas Medical Center), Roy Jensen, M.D (University of Kansas Medical Center), and Rebecca Cook, Ph.D (Vanderbilt University, Nashville, TN) for scientific discussion. This work was supported by the following grants: CA102162 and CA85492 (National Cancer Institute DHHS), and the TJ Martell Foundation to HL Moses, CA127357-01A2 (National Cancer Institute DHHS) and University of Kansas Endowment to N Cheng.

Abbreviations

CCL2	Chemokine (C–C motif) ligand 2
CXCL12	Chemokine (C–X–C motif) ligand 12
Fsp1	Fibroblast specific protein 1
HGF	Hepatocyte growth factor
MMTV	Mouse mammary tumor virus promoter
PyVmT	Polyoma virus middle T
α-sma	Alpha smooth muscle actin
TGF-β	Transforming growth factor beta
Tgfb2	TGF-β type II receptor gene
Tgfb2^{FspKO}	Homozygous deletion of <i>Tgfb2</i> in stromal cells
Tgfb2^{hetFspKO}	Heterozygous deletion of <i>Tgfb2</i> in stromal cells
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
VWF8	Von Willebrand factor 8
WT	Wildtype

References

1. Barrett-Lee P, Travers M, Luqmani Y, et al. Transcripts for transforming growth factors in human breast cancer: clinical correlates. *Br J Cancer*. 1990; 61(4):612–617. [PubMed: 2331446]
2. Desmouliere A, Guyot C, Gabbiani G. The stroma reaction myofibroblast: a key player in the control of tumor cell behavior. *Int J Dev Biol*. 2004; 48(5-6):509–517. [PubMed: 15349825]
3. Kalluri R, Zeisberg M. Fibroblasts in cancer. *Nat Rev Cancer*. 2006; 6(5):392–401. [PubMed: 16572188]
4. Camps J, Chang S, Hsu T, et al. Fibroblast-mediated acceleration of human epithelial tumor growth in vivo. *Proc Natl Acad Sci USA*. 1990; 87:75–79. [PubMed: 2296606]
5. Sakakura T, Sakagami Y, Nishizuka Y. Accelerated mammary cancer development by fetal salivary mesenchyma isografted to adult mouse mammary epithelium. *J Natl Cancer Inst*. 1981; 66(5):953–959. [PubMed: 6262561]

6. Shekhar MP, Werdell J, Santner SJ, et al. Breast stroma plays a dominant regulatory role in breast epithelial growth and differentiation: implications for tumor development and progression. *Cancer Res.* 2001; 61:1320–1326. [PubMed: 11245428]
7. Kurose K, Gilley K, Matsumoto S, et al. Frequent somatic mutations in PTEN and TP53 are mutually exclusive in the stroma of breast carcinomas. *Nat Genet.* 2002; 32(3):355–357. [PubMed: 12379854]
8. Hagedorn H, Bachmeir B, Nerlich A. Synthesis and degradation of basement membranes and extracellular matrix and their regulation by TGF-beta in invasive carcinomas (review). *Int J Oncol.* 2001; 18(4):669–681. [PubMed: 11251160]
9. Lei X, Bandyopadhyay A, Le T, Sun L. Autocrine TGFbeta supports growth and survival of human breast cancer MDA-MB-231 cells. *Oncogene.* 2002; 21:7514–7523. [PubMed: 12386814]
10. Tuxhorn J, McAlhany S, Yang F, et al. Inhibition of transforming growth factor B activity decreases angiogenesis in human prostate cancer-reactive stroma-xenograft model. *Cancer Res.* 2002; 62:6021–6025. [PubMed: 12414622]
11. Joseph H, Gorska AE, Sohn P, et al. Overexpression of a kinase-deficient transforming growth factor-B type II receptor in mouse mammary stroma results in increased epithelial branching. *Mol Biol Cell.* 1999; 10:1221–1234. [PubMed: 10198068]
12. Paoloni-Giacobino A, Rossier C, Papasavvas MP, et al. Frequency of replication/transcription errors in (A)/(T) runs of human genes. *Hum Genet.* 2001; 109(1):40–47. [PubMed: 11479734]
13. Krtolica A, Parrinello S, Lockett S, et al. Senescent fibroblasts promote epithelial cell growth and tumorigenesis: a link between cancer and aging. *Proc Natl Acad Sci USA.* 2001; 98(21):12072–12077. [PubMed: 11593017]
14. Lawrenson K, Grun B, Benjamin E, et al. Senescent fibroblasts promote neoplastic transformation of partially transformed ovarian epithelial cells in a three-dimensional model of early stage ovarian cancer. *Neoplasia.* 2010; 12(4):317–325. [PubMed: 20360942]
15. Bian Y, Knobloch TJ, Sadim M, et al. Somatic acquisition of TGFBR1*6A by epithelial and stromal cells during head and neck and colon cancer development. *Hum Mol Genet.* 2007; 16(24):3128–3135. [PubMed: 17890272]
16. ten Dijke P, Hill CS. New insights into TGF-beta-Smad signalling. *Trends Biochem Sci.* 2004; 29(5):265–273. [PubMed: 15130563]
17. McCaffrey TA, Falcone DJ. Evidence for an age-related dysfunction in the antiproliferative response to transforming growth factor-beta in vascular smooth muscle cells. *Mol Biol Cell.* 1993; 4(3):315–322. [PubMed: 8387357]
18. Angeli F, Koumakis G, Chen MC, et al. Role of stromal fibroblasts in cancer: promoting or impeding? *Tumour Biol.* 2009; 30(3):109–120. [PubMed: 19440007]
19. Bhowmick N, Chytil A, Plieth D, et al. TGF- β signaling in fibroblasts modulates the oncogenic potential of adjacent epithelia. *Science.* 2004; 303:847–851.
20. Cheng N, Bhowmick NA, Chytil A, et al. Loss of TGF-beta type II receptor in fibroblasts promotes mammary carcinoma growth and invasion through upregulation of TGF-alpha-, MSP- and HGF-mediated signaling networks. *Oncogene.* 2005; 24(32):5053–5068. [PubMed: 15856015]
21. Guy C, Cardiff R, Muller W. Induction of mammary tumors by expression a polyomavirus middle T oncogene: a transgenic mouse model for metastatic disease. *Mol Cell Biol.* 1992; 12:954–961. [PubMed: 1312220]
22. Lin E, Jones J, Zhu L, et al. Progression to malignancy in the polyoma middle T oncoprotein mouse breast cancer model provides a reliable model for human diseases. *Am J Path.* 2003; 163(5):2113–2126. [PubMed: 14578209]
23. Sugimoto H, Mundel TM, Kieran MW, et al. Identification of fibroblast heterogeneity in the tumor microenvironment. *Cancer Biol Ther.* 2006; 5(12):1640–1646. [PubMed: 17106243]
24. Hayward S, Haughney PC, Rosen MA, Greulich KM, Weier HU, Dahiya R, Cunha GR. Interactions between adult human prostatic epithelium and rat urogenital sinus mesenchyme in a tissue recombination model. *Differentiation.* 1998; 63(3):131–140. [PubMed: 9697307]
25. Tang Z, Yu M, Miller F, et al. Increased invasion through basement membrane by CXCL7-transfected breast cells. *Am J Surg.* 2008; 196(5):690–696. [PubMed: 18954601]

26. Allinen M, Beroukhi R, Cai L, et al. Molecular characterization of the tumor microenvironment in breast cancer. *Cancer Cell*. 2004; 6(1):17–32. [PubMed: 15261139]
27. Orimo A, Gupta PB, Sgroi DC, et al. Stromal fibroblasts present in invasive human breast carcinomas promote tumor growth and angiogenesis through elevated SDF-1/CXCL12 secretion. *Cell*. 2005; 121(3):335–348. [PubMed: 15882617]
28. Yamashiro S, Takeya M, Nishi T, et al. Tumor-derived monocyte chemoattractant protein-1 induces intratumoral infiltration of monocyte-derived macrophage subpopulation in transplanted rat tumors. *Am J Pathol*. 1994; 145(4):856–867. [PubMed: 7943176]
29. Jia GQ, Gonzalo JA, Lloyd C, et al. Distinct expression and function of the novel mouse chemokine monocyte chemotactic protein-5 in lung allergic inflammation. *J Exp Med*. 1996; 184(5):1939–1951. [PubMed: 8920881]
30. Abramson N, Castro S, Goldstein JD. Lipid-laden macrophage infiltration of human adenocarcinoma in vivo associated with taxol and GCSF treatment. *Cancer Invest*. 1997; 15(1):18–22. [PubMed: 9028386]
31. Xu BJ, Yan W, Jovanovic B, et al. Quantitative analysis of the secretome of TGF-beta signaling-deficient mammary fibroblasts. *Proteomics*. 2010; 10:2458. [PubMed: 20405477]
32. Muller A, Homey B, Soto H, et al. Involvement of chemokine receptors in breast cancer metastasis. *Nature*. 2001; 410(6824):50–56. [PubMed: 11242036]
33. Kim SY, Lee CH, Midura BV, et al. Inhibition of the CXCR4/CXCL12 chemokine pathway reduces the development of murine pulmonary metastases. *Clin Exp Metastasis*. 2008; 25(3):201–211. [PubMed: 18071913]
34. Cheng N, Chytil A, Shyr Y, et al. Enhanced hepatocyte growth factor signaling by type II transforming growth factor- β receptor knockout fibroblasts promotes mammary tumorigenesis. *Cancer Res*. 2007; 67(10):4869–4877. [PubMed: 17495323]
35. Hembruff SL, Jorak I, Yang L, et al. Loss of transforming growth factor-beta signaling in mammary fibroblasts enhances CCL2 secretion to promote mammary tumor progression through macrophage-dependent and -independent mechanisms. *Neoplasia*. 2010; 12(5):425–433. [PubMed: 20454514]
36. Robinson S, Silberstein G, Roberts A, et al. Regulated expression and growth inhibitory effects of transforming growth factor-beta isoforms in mouse mammary gland development. *Development*. 1991; 113:867–878. [PubMed: 1821856]
37. Cunha G, Hom Y. Role of mesenchymal-epithelial interactions in mammary gland development. *J Mamm Gland Biol*. 1996; 1(1):21–34.
38. Gal A, Sjoblom T, Fedorova L, et al. Sustained TGF beta exposure suppresses Smad and non-Smad signalling in mammary epithelial cells, leading to EMT and inhibition of growth arrest and apoptosis. *Oncogene*. 2008; 27(9):1218–1230. [PubMed: 17724470]
39. Webster M, Hutchinson JN, Rauh MJ, Muthuswamy SK, Anton A, Tortorice CG, Cardiff RD, Graham FL, Hassell JA, Muller WJ. Requirement of both Shc and Phosphatidylinositol 3' kinasesignaling pathways in polyomavirus middle T-mediated mammary tumorigenesis. *Mol Cell Biol*. 1998; 18(4):2344–2359. [PubMed: 9528804]
40. Foulkes WD, Reis-Filho JS, Narod SA. Tumor size and survival in breast cancer—a reappraisal. *Nat Rev Clin Oncol*. 2010; 7(6):348–353. [PubMed: 20309006]
41. Minn AJ, Gupta GP, Siegel PM, et al. Genes that mediate breast cancer metastasis to lung. *Nature*. 2005; 436(7050):518–524. [PubMed: 16049480]
42. DeNardo DG, Johansson M, Coussens LM. Immune cells as mediators of solid tumor metastasis. *Cancer Metastasis Rev*. 2008; 27(1):11–18. [PubMed: 18066650]
43. Allavena P, Sica A, Solinas G, et al. The inflammatory micro-environment in tumor progression: the role of tumor-associated macrophages. *Crit Rev Oncol Hematol*. 2008; 66(1):1–9. [PubMed: 17913510]
44. Gordon S. Alternative activation of macrophages. *Nat Rev Immunol*. 2003; 3(1):23–35. [PubMed: 12511873]
45. Anders CK, Carey LA. Biology, metastatic patterns, and treatment of patients with triple-negative breast cancer. *Clin Breast Cancer*. 2009; 9(Suppl 2):S73–S81. [PubMed: 19596646]

46. Strutz F, Okada H, Lo CW, Danoff T, Carone RL, Tomaszewski JE, Neilson EG. Identification and characterization of a fibroblast marker: FSP1. *J Cell Biol.* 1995; 130(2):393–405. [PubMed: 7615639]
47. Joyce JA, Pollard JW. Microenvironmental regulation of metastasis. *Nat Rev Cancer.* 2009; 9(4): 239–252. [PubMed: 19279573]
48. Brier B, Moses HL. Under pressure: stromal fibroblasts change their ways. *Cell.* 2005; 123(6): 985–987. [PubMed: 16360028]
49. Fujimoto H, Sangai T, Ishii G, et al. Stromal MCP-1 in mammary tumors induces tumor-associated macrophage infiltration and contributes to tumor progression. *Int J Cancer.* 2009; 125(6):1276–1284. [PubMed: 19479998]
50. Lu X, Kang Y. Chemokine (C-C motif) ligand 2 engages CCR2? stromal cells of monocytic origin to promote breast cancer metastasis to lung and bone. *J Biol Chem.* 2009; 284(42):29087–29096. [PubMed: 19720836]
51. Anastassiades OT, Pryce DM. Fibrosis as an indication of time in infiltrating breast cancer and its importance in prognosis. *Br J Cancer.* 1974; 29(3):232–239. [PubMed: 4364382]
52. Hasebe T, Tsuda H, Tsubono Y, et al. Fibrotic focus in invasive ductal carcinoma of the breast: a histopathological prognostic parameter for tumor recurrence and tumor death within three years after the initial operation. *Jpn J Cancer Res.* 1997; 88(6):590–599. [PubMed: 9263537]
53. Tamiolakis D, Papadopoulos N, Cheva A, et al. Immuno-histochemical expression of alpha-smooth muscle actin in infiltrating ductal carcinoma of the breast with productive fibrosis. *Eur J Gynaecol Oncol.* 2002; 23(5):469–471. [PubMed: 12440829]
54. Karas M, Amir H, Fishman D, et al. Lycopene interferes with cell cycle progression and insulin-like growth factor I signaling in mammary cancer cells. *Nutr Cancer.* 2000; 36(1):101–111. [PubMed: 10798222]
55. Sciacca L, Costantino A, Pandini G, et al. Insulin receptor activation by IGF-II in breast cancers: evidence for a new autocrine/paracrine mechanism. *Oncogene.* 1999; 18(15):2471–2479. [PubMed: 10229198]
56. Rahimi N, Saulnier R, Nakamura T, et al. Role of hepatocyte growth factor in breast cancer: a novel mitogenic factor secreted by adipocytes. *DNA Cell Biol.* 1994; 13(12):1189–1197. [PubMed: 7811385]
57. Ma X, Norsworthy K, Kundu N, et al. CXCR3 expression is associated with poor survival in breast cancer and promotes metastasis in a murine model. *Mol Cancer Ther.* 2009; 8(3):490–498. [PubMed: 19276169]
58. Dunn SE, Ehrlich M, Sharp NJ, et al. A dominant negative mutant of the insulin-like growth factor-I receptor inhibits the adhesion, invasion, and metastasis of breast cancer. *Cancer Res.* 1998; 58(15):3353–3361. [PubMed: 9699666]
59. Sachdev D, Li SL, Hartell JS, et al. A chimeric humanized single-chain antibody against the type I insulin-like growth factor (IGF) receptor renders breast cancer cells refractory to the mitogenic effects of IGF-I. *Cancer Res.* 2003; 63(3):627–635. [PubMed: 12566306]
60. Zhang X, Yang J, Li Y, et al. Both Sp1 and Smad participate in mediating TGF-beta1-induced HGF receptor expression in renal epithelial cells. *Am J Physiol Renal Physiol.* 2005; 288(1):F16–F26. [PubMed: 15339794]
61. Chia DJ, Ono M, Woelfle J, et al. Characterization of distinct Stat5b binding sites that mediate growth hormone-stimulated IGF-I gene transcription. *J Biol Chem.* 2006; 281(6):3190–3197. [PubMed: 16339156]
62. Cocolakis E, Dai M, Drevet L, et al. Smad signaling antagonizes STAT5-mediated gene transcription and mammary epithelial cell differentiation. *J Biol Chem.* 2008; 283(3):1293–1307. [PubMed: 18024957]
63. Muraoka RS, Koh Y, Roebuck LR, et al. Increased malignancy of Neu-induced mammary tumors overexpressing active transforming growth factor beta1. *Mol Cell Biol.* 2003; 23(23):8691–8703. [PubMed: 14612410]
64. Schade B, Lam SH, Cernea D, et al. Distinct ErbB-2 coupled signaling pathways promote mammary tumors with unique pathologic and transcriptional profiles. *Cancer Res.* 2007; 67(16): 7579–7588. [PubMed: 17699761]

65. Wendt MK, Johannesen PA, Kang-Decker N, et al. Silencing of epithelial CXCL12 expression by DNA hypermethylation promotes colonic carcinoma metastasis. *Oncogene*. 2006; 25(36):4986–4997. [PubMed: 16568088]
66. Plerixafor. AMD 3100, AMD3100, JM 3100, SDZ SID 791 Drugs R D. 2007; 8(2):113–119.
67. De Paepe B, Creus KK, De Bleecker JL. Chemokines in idiopathic inflammatory myopathies. *Front Biosci*. 2008; 13:2548–2577. [PubMed: 17981734]
68. Bieche I, Chavey C, Andrieu C, et al. CXC chemokines located in the 4q21 region are up-regulated in breast cancer. *Endocr Relat Cancer*. 2007; 14(4):1039–1052. [PubMed: 18045955]
69. Hembruff S, Cheng N. Chemokine signaling in cancer: implications on the tumor microenvironment and therapeutic targeting. *Cancer Therapy*. 2009; 7:254–267. [PubMed: 20651940]
70. Bacman D, Merkel S, Croner R, et al. TGF-beta receptor 2 downregulation in tumour-associated stroma worsens prognosis and high-grade tumours show more tumour-associated macrophages and lower TGF-beta1 expression in colon carcinoma: a retrospective study. *BMC Cancer*. 2007; 7:156. [PubMed: 17692120]
71. Bugge TH, Lund LR, Kombrinck KK, et al. Reduced metastasis of Polyoma virus middle T antigen-induced mammary cancer in plasminogen-deficient mice. *Oncogene*. 1998; 16(24):3097–3104. [PubMed: 9671388]
72. Chytil A, Magnuson MA, Wright CVE, et al. Conditional inactivation of the TGF-B type II receptor using Cre-Lox. *Genesis*. 2002; 32:73–75. [PubMed: 11857781]
73. Medina, D.; Kittrell, F. Establishment of mouse mammary cell lines. In: Ip, MM.; Asch, BB., editors. *Methods in mammary gland biology and breast cancer research*. Kluwer Academic/Plenum Publishers; New York: 2000. p. 137-147.
74. Bitzer M, von Gersdorff G, Liang D, Dominguez-Rosales A, Beg AA, Rojkind M, Bottinger EP. A mechanism of suppression of TGF-beta/SMAD signaling by NF-kappa B/RelA. *Genes Dev*. 2000; 2(14):187–197. [PubMed: 10652273]

Fig. 1.

Loss of one *Tgfr2* allele enhances mammary tumor progression in PyVmT transgenic mice. a PyVmT controls (n = 38) and PyVmT/*Tgfr2*^{hetFspKO} (n = 24) mice were palpated for tumors and scored for onset of primary tumor growth. b Tumor burden was assessed by a measurement of total tumor volume at 13.4 weeks of age for control mice and PyVmT/*Tgfr2*^{hetFspKO} mice. c H&E stain of primary mammary tumors. The areas outlined with dotted lines indicate necrosis. d Lung metastases were visualized by whole mount hematoxylin staining of lung tissue extracted from PyVmT controls or PyVmT/*Tgfr2*^{hetFspKO} mice and scored. Each point in the graph represents the number of lung metastases in one mouse. Bars represent the mean ± standard error of the mean (SEM). The presence of nodules was confirmed by H&E stain of 5 micron sections. Representative nodule in PyVmT control is outlined by dotted line. Statistical analysis of PyVmT/*Tgfr2*^{hetFspKO} compared to PyVmT controls was performed using two tailed Student's *t*-test. Statistical significance determined by P value less than 0.05. **p* < 0.01, ***p* > 0.05, ****p* < 0.05. Scale bar = 80 μm

Fig. 2.

Primary tumors in PyVmT/Tgfr2^{hetFspKO} mice show increased tumor cell proliferation and survival. a Ki67 immunostaining as a measure of tumor cell proliferation. b TUNEL immunostaining as a measure of cellular apoptosis. Five random sections were photographed and quantitated for positive staining. PyVmT/Tgfr2^{hetFspKO} measurements were compared to PyVmT controls using two tailed Student's *t*-test. Statistical significance determined by P-value less than 0.05. **p* < 0.05, ***p* < 0.02. Scale bar of low magnified image = 150 μ m. Smaller boxed areas within each image refer to magnified inset. Scale bar of inset = 15 μ m. Images are representative of the entire area of each the five random sections counted

Fig. 3. PyVmT/Tgfr2^{hetFspKO} primary tumors exhibit increased stromal cell accumulation. Tumor sections of PyVmT controls and PyVmT/Tgfr2^{hetFspKO} were stained with antibodies. a Alpha smooth muscle actin (α-sma), b Fsp1, c vimentin, d F4/80, e Von Willibrand Factor 8. Five random sections were photographed and quantitated for positive staining. Positive DAB staining is emphasized by arrowhead. Statistical analysis of PyVmT/Tgfr2^{hetFspKO} mice compared to PyVmT controls was performed using Poisson Regression. Statistical significance determined by P-value less than 0.05. * $p < 0.04$, ** $p < 0.01$, *** $P = 0.05$. Scale bar of low magnified image = 150 μ m. Small boxed areas within each image refer to magnified inset. Scale bar of inset = 15 μ m. Images are representative of the entire area of each the five random sections counted

Fig. 4.

Loss of one *Tgfr2* allele occurs in a subset of mammary fibroblasts from PyVmT/*Tgfr2*^{hetFspKO} mice. A Cre immunostaining of primary tumor sections at low (a, b) and high magnification (c, d). Arrows indicate Cre staining in mammary stromal cells. (a, b; bar = 40 μm. c, d; bar = 10 μm). B Southern blot analysis of mammary fibroblasts isolated from control PyVmT or PyVmT/*Tgfr2*^{hetFspKO} tumors. Controls include: WT Wildtype fibroblasts, *Flox/Flox* Fibroblasts carrying homozygous Floxed *Tgfr2*, *FspKO* Homozygous *Tgfr2* knockout fibroblasts. Percent recombination (% recom) was determined by Phosphorimager analysis. *Flox* Floxed *Tgfr2*, *KO* Knockout of the *Tgfr2* allele by excision of exon 2, *WT* Wildtype *Tgfr2*

Fig. 5.

Loss of one *Tgfr2* allele in mammary fibroblasts enhances overall fibroblast proliferation but does not significantly inhibit cell proliferation in response to TGF- β treatment. a Heterogeneous or b clonal fibroblasts were treated with 5 ng/ml TGF- β and analyzed for changes in cell proliferation by $^3\text{[H]}$ thymidine incorporation. Statistical analysis was performed using Two-Way ANOVA, accompanied by post-hoc pair-wise analysis using Bonferroni–Dunn test. Statistical significance determined by P-value less than 0.05. *(-) TGF- β versus (+)TGF- β treatment in a given sample; $P < 0.05$. **WT/Flox #1 versus WT/Flox #2; $P > 0.05$. ***hetFspKO #1 versus hetFspKO #2; $P > 0.05$. ****hetFspKO #1 or #2 versus WT/Flox #1 or #2; $P < 0.01$

Fig. 6.

Effect of $Tgfr2^{hetFspKO}$ fibroblasts on PyVmT carcinoma cell invasion. PyVmT carcinoma cells labeled with CMFDA CellTracker dye were embedded in collagen with clonal $Tgfr2^{WT/Flox}$ or $Tgfr2^{hetFspKO}$ fibroblasts and analyzed for invasion through collagen for 72 h. Representative images of PyVmT cells embedded in collagen alone for 48 h are shown. The border of the collagen through which tumor cells invade is indicated by arrow in the magnified images on the right. Experiments were performed in triplicate, $n = 3$ per experimental group. Statistical analysis was performed using two-tailed Student's t -test. Statistical significance determined by P-value less than 0.05. * $p < 0.05$ versus WT/Flox

Fig. 7. $Tgfr2^{hetFspKO}$ fibroblasts enhanced mammary carcinoma invasion through CCL2 but not HGF dependent mechanisms. Conditioned medium was analyzed by ELISA for levels of a HGF, b CCL2 from heterogeneous fibroblasts and c CCL2 from clonal fibroblasts. d PyVmT cells were embedded in collagen with $Tgfr2^{hetFspKO}$ fibroblasts in the presence or absence of control IgG, anti-CCL2 for 72 h and analyzed for degree of invasion through the collagen. Experiments were performed in triplicate, $n = 3$ per experimental group. Statistical analysis of was performed using two-tailed Student's t -test. Statistical significance determined by P-value less than 0.05. * $p > 0.05$ versus WT/Flox, ** $p < 0.05$ versus WT/Flox, *** $p < 0.01$ versus WT/Flox, **** $p < 0.05$ versus Rat IgG

Table 1
Effect of clonal Tgfr2^{hetFspKO} fibroblasts on PyVmT mammary tumor growth and metastases

Graft components	Primary tumor/mass (g) Mean ± SEM	P -value	No. lung metastases/mouse Mean ± SEM	P- value	n
PyVmT: clonal WT/Flox	0.86 ± 0.12	0.01	1.6 ± 0.308	0.02	7
PyVmT: clonal hetFspKO	0.45 ± 0.02		6.8 ± 1.8		6

Clonal control or Tgfr2^{hetFspKO} fibroblasts were co-transplanted with PyVmT mammary carcinoma cells in the subrenal capsule of nude mice. 45 days post-transplantation, the organs were harvested and analyzed for changes in primary tumor mass and lung metastases. Statistical analysis was determined by two-tailed Student's *t*-test. Statistical significance was determined by *P*-value less than 0.05

Table 2Summary of proteins secreted by *Tgfb β 2^{hetFspKO}* fibroblasts

Protein	Fold change $\pm$ SEM	P-value	Reported function in cancer	Reference
CXCL7	8.5 $\pm$ 2.5	0.004	Enhances breast cancer cell invasiveness	[19]
GCSF	4.5 $\pm$ 1.9	0.01	Macrophage infiltration	[24]
CXCL12	3.7 $\pm$ 1.35	0.01	Enhances breast cancer cell invasiveness, promotes metastatic spread, tumor angiogenesis	[20, 21]
CCL2	3.5 $\pm$ 1.62	0.008	Promotes macrophage recruitment, associated with increased metastases during mammary tumor progression	[32, 33]
CCL12	2.6 $\pm$ 0.45	0.008	Regulates macrophage recruitment during inflammation and host response to pathogens	[23]

Secreted proteins were identified by cytokine array profiling of conditioned medium from control fibroblasts and *Tgfb β 2^{hetFspKO}* heterogeneous fibroblasts ($n = 3$ per group) or basal medium (DMEM/F12/5% FBS). Expression of proteins were quantitated by densitometry analysis using Image J software. Statistical significance determined by *P*-value less than 0.05; * $P < 0.05$ vs. WT/Flox. Only the proteins showing a statistically significant change in expression compared to control fibroblasts are listed here

Table 3Comparison of secreted proteins between Tgfr2^{hetFspKO} and Tgfr2^{FspKO} fibroblasts

Protein	Tgfr2 ^{hetFspKO}	Tgfr2 ^{FspKO}
CCL2	↑	↑
CXCL10	=	↑
CXCL12	↑	↓
HGF	=	↑
IGF-I	=	↑
IGF-II	=	↓
IGFBP2	=	↑

The expression patterns of secreted proteins from Tgfr2^{hetFspKO} fibroblasts from cytokine array profiling were compared to the expression patterns of secreted proteins from Tgfr2^{FspKO} fibroblasts identified in previous studies [25, 29, 31]. The symbols are defined as statistically significant changes in expression compared to control fibroblasts: Increased expression: ↑; decreased expression: ↓; no change in expression: =. HGF is not included in the cytokine array profiling and its expression in Tgfr2^{hetFspKO} fibroblasts was analyzed independently as a candidate protein