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Loss of Transforming Growth Factor-β Signaling in Mammary Fibroblasts Enhances CCL2 Secretion to Promote Mammary Tumor Progression through Macrophage-Dependent and -Independent Mechanisms[1]

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Abstract

Whereas the accumulation of fibroblasts and macrophages in breast cancer is a well-documented phenomenon and correlates with metastatic disease, the functional contributions of these stromal cells on breast cancer progression still remain largely unclear. Previous studies have uncovered a potentially important role for CCL2 inflammatory chemokine signaling in regulating metastatic disease through a macrophage-dependent mechanism. In these studies, we demonstrate a significant regulatory mechanism for CCL2 expression in fibroblasts in mediating mammary tumor progression and characterize multiple functions for CCL2 in regulating stromal-epithelial interactions. Targeted ablation of the transforming growth factor-β (TGF-β) type 2 receptor in fibroblasts ($Tgfbr2^{FspKO}$) results in a high level of secretion of CCL2, and cografts of $Tgfbr2^{FspKO}$ fibroblasts with 4T1 mammary carcinoma cells enhanced tumor progression associated with recruitment of tumor-associated macrophages (TAMs). Antibody neutralization of CCL2 in tumor-bearing mice inhibits primary tumor growth and liver metastases as evidenced by reduced cell proliferation, survival, and TAM recruitment. Both high and low stable expressions of small interfering RNA to CCL2 in $Tgfbr2^{FspKO}$ fibroblasts significantly reduce liver metastases without significantly affecting primary tumor growth, cell proliferation, or TAM recruitment. High but not low knockdown of CCL2 enhances tumor cell apoptosis. These data indicate that CCL2 enhances primary tumor growth, survival, and metastases in a dose-dependent manner, through TAM-dependent and -independent mechanisms, with important implications on the potential effects of targeting CCL2 chemokine signaling in the metastatic disease.

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Introduction

The induction of host inflammatory responses toward breast cancer is characterized by the recruitment of stromal cells to the primary tumor, including macrophages and fibroblasts [1,2]. Accumulation of these stromal cells is associated with an increased expression of soluble factors, which alters the tumor microenvironment including extracellular matrix proteins, growth regulators, cytokines, and angiogenic factors, correlating with invasive breast cancer and poor patient prognosis [3,4]. Whereas the accumulation of fibroblasts and macrophages in breast cancer are a well-documented phenomenon, the signals that regulate the interplay between stromal and epithelial cancer cells remain complex and largely unclear.

Abbreviations: siRNA, small interfering RNA; TAM, tumor-associated macrophage; *Tgfbr2*, type 2 TGF-β receptor gene; $Tgfbr2^{Flox/Flox}$, *Tgfbr2* with loxP sites flanking exon 2; $Tgfbr2^{FspKO}$, conditional knockout of *Tgfbr2* by Fsp-1 Cre

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A number of studies have indicated an important role for transforming growth factor-β (TGF-β) signaling in regulating fibroblast activation. TGF-β signaling activates mammary fibroblasts by inhibiting cell proliferation and inducing production of growth factors, angiogenic factors, extracellular matrix proteins, and proteases [5,6]. Whereas the role of autocrine TGF-β signaling in regulating the mesenchymal phenotype has been well documented [6,7], recent reports have indicated a significant role for TGF-β signaling in regulating interactions between stromal and epithelial cells. In recent studies, the role of TGF-β signaling in fibroblasts was analyzed by disrupting the expression of the TGF-β type 2 receptor (*Tgfbr2*) in a subset of fibroblasts by Cre-Lox ($Tgfbr2^{FspKO}$). Cotransplanting mammary $Tgfbr2^{FspKO}$ fibroblasts with PyVmT or 4T1 mammary carcinoma cells significantly enhanced primary tumor growth and progression [8,9]. Whereas the protumorigenic phenotypes observed were associated with increased receptor tyrosine kinase signaling, including the hepatocyte growth factor/c-Met signaling pathway, abrogation of receptor tyrosine kinase signaling pathways did not completely inhibit tumor progression enhanced by the TGF-β signaling-deficient fibroblasts [8,9], indicating that TGF-β signaling regulates stromal-epithelial interactions through mechanisms independent of receptor tyrosine kinases.

The CCL2/CCR2 inflammatory chemokine signaling pathway has been shown to be important in regulating macrophage recruitment during inflammation and wound healing events [10,11]. Recent studies have demonstrated a potentially important role for CCL2 in regulating breast cancer progression in part through the recruitment of tumor-associated macrophages (TAMs). A high level of CCL2 expression in primary breast tumor has been shown to correlate with breast cancer invasiveness and decreased patient survival [12,13]. In mouse studies, a high level of expression of CCL2 in primary mammary tumors has also been associated with high levels of TAMs, cells that lack immunostimulatory function and secrete growth, survival, and angiogenic factors [14,15]. Tumor cells overexpressing recombinant CCL2 have been shown to recruit TAMs that enhance tumor growth and metastatic spread in mouse xenograft models of cancer [16,17], further implicating CCL2 in a protumorigenic role in breast cancer through a TAM-dependent mechanism.

This report shows that CCL2 and TGF-β signaling interacts to regulate stromal-epithelial interactions during mammary tumor progression. In these studies, $Tgfbr2^{FspKO}$ fibroblasts cografted with mammary carcinoma cells enhanced primary tumor growth and liver metastases statistically correlating with the accumulation of TAMs and increased expression of CCL2 in $Tgfbr2^{FspKO}$ fibroblasts. Antibody neutralization of CCL2 in tumor-bearing mice inhibited tumor growth and metastatic spread to the liver associated with reduced numbers of TAMs. Knockdown of CCL2 by stable small interfering RNA (siRNA) expression in $Tgfbr2^{FspKO}$ fibroblasts did not significantly affect primary tumor growth but did significantly reduce liver metastases and moderately reduce TAM recruitment. These data indicate that, whereas CCL2 in the breast cancer microenvironment functions to enhance primary tumor growth, survival, and metastases in part through TAM recruitment, CCL2 derived from mammary fibroblasts specifically functions to stimulate mammary carcinoma cells to promote cell survival and metastatic spread. In summary, these data are the first to demonstrate that TGF-β signaling in fibroblasts regulates metastatic disease through the CCL2 chemokine signaling pathway. Furthermore, these studies demonstrate a fibroblast-specific contribution of CCL2 to mammary tumor progression and are the first to demonstrate dose-dependent effects of CCL2 on primary tumor progression and TAM recruitment, with important implications on the potential effects of targeting CCL2 inflammatory chemokines signaling in metastatic disease.

Materials and Methods

Culture of Cell Lines

Floxed TGF-β type 2 receptor (*Tgfbr2*) control fibroblasts and $Tgfbr2^{fspKO}$ fibroblasts were immortalized and characterized in previous studies [8]. Fibroblasts, including stably expressing CCL2 siRNA cell lines, were cultured in Dulbecco's modified Eagle medium (DMEM)/F12/10% fetal bovine serum (FBS). 4T1 mammary carcinoma cells (American Type Culture Collection, Manassas, VA) were cultured in DMEM/10 % FBS. Phoenix cells were kindly provided by Jin Chen, MD, PhD, (Vanderbilt University, Nashville, TN) and were cultured in DMEM/10% FBS.

siRNA Silencing of CCL2 Expression in $Tgfbr2^{FspKO}$ Fibroblasts

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 previously mentioned constructs with *Hin*dIII and *Eco*RI and were cloned into the same sites of an siRNA expression vector (pSUPER) or an 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 Brummelkamp et al. [18]. The two targeting sequences used for siRNA-mediated knockdown of CCL2 are 5′-CAGAACCTACAACTTTATT-3′ for 1CCL2– and 5′-TAAATCTGAAGCTAATGCA-3′ for 3CCL2–. The targeting sequence to silence enhanced GFP (eGFP) as a negative control is 5′-GCTGACCCTGAAGTTCATC-3′. 1CCL2–, 3CCL2–, and eGFP targeting oligonucleotides were designed as previously described [18]. The oligonucleotides were phosphorylated by kinase treatment; complementary oligos were then annealed and subcloned into the *Bgl*II and *Hin*dIII sites of pRETRO-SUPER. Plasmids were transfected into Phoenix cells by Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Forty-eight hours after transfection, $Tgfbr2^{FspKO}$ fibroblasts were transduced with virus-conditioned medium and selected with 1.5 μg/ml puromycin.

Mouse Strains and Maintenance

Female nude (*nu/nu*) mice (6-8 weeks of age) were obtained from Harlan Laboratories (Denver, CO). The experimental research on rodents reported here has been performed with the approval of the appropriate ethics committees, including the Association for Assessment and Accreditation of Laboratory Animal Care and University of Kansas Institutional Animal Care and Use Committee.

Subrenal Capsule Grafting

Grafting of collagen-embedded cells was performed according to the methods of Hayward et al. [19]. Briefly, 1×10^5 4T1 cells were resuspended together with 2.5×10^5 $Tgfbr2^{FspKO}$ or $Tgfbr2^{Flox/Flox}$ fibroblasts in 50 μl of collagen per graft. The collagen-embedded cells were cultured in DMEM/F12 10% FBS for 24 hours and then implanted under the renal capsule layer of the kidneys in female nude mice, 6 to 8 weeks of age. Neutralizing antibodies that recognize murine-specific CCL2 (R&D Systems, Minneapolis, MN) or control immunoglobulin G (IgG; Sigma, St Louis, MO) were solubilized in 0.9% saline and injected into the intraperitoneum into mice 7 days after grafting at 5 mg/kg, every 2 days for 14 days. Tumor tissues were collected 21 days after implantation and weighed. Liver metastases were counted by examination of

gross tissues. Metastatic lesions on the liver were confirmed by hematoxylin and eosin stain as previously shown [9].

Histology and Immunohistochemistry

Tumor tissues were fixed in 10% neutral formalin buffer, subjected to dehydration in 50%, 70%, 80%, 95%, and 100% ethanols and xylene and then paraffin-embedded. Five-micrometer sections were prepared for immunohistochemistry. The sections were stained with hematoxylin and eosin. Immunofluorescence staining of F4/80 (rat monoclonal; Abcam, Cambridge, MA) was performed by 10 nM sodium citrate antigen retrieval for 10 minutes at 105°C and by incubating primary antibodies at a 1:50 dilution overnight at 4°C. Sections were incubated with rat biotinylated secondary antibodies at a 1:500 dilution, conjugated to streptavidin–Alexa 568 (1:500; Molecular Probes, Invitrogen, Carlsbad, CA) and counterstained with 4′6′-diamidino-2-phenylindole. Sections were mounted with Prolong Antifade (Molecular Probes). Immunoperoxidase staining for cleaved caspase-3 (rabbit polyclonal; Cell Signaling Technologies, Boston, MA) and Ki67 (rabbit polyclonal; Dako, Carpinteria, CA) was performed by citrate antigen retrieval and by incubating primary antibodies at a 1:100 dilution overnight at 4°C. Sections were incubated with appropriate secondary biotinylated antibodies at a 1:500 dilution and conjugated to streptavidin peroxidase (Vectastain Elite Kit; Vector Laboratories, Burlingame, CA) according to a commercial protocol. Sections were visualized by peroxidase staining (Vectastain Elite Kit; Vector Laboratories) and counterstained with hematoxylin. Proliferative and apoptotic indices were calculated by determining the relative area of positively stained cells to the total number of cells in at least five high-powered fields using Scion Image software (Frederick, MA).

Flow Cytometry Analysis

Primary tumor tissues were digested into single-cell suspensions according to previously described studies [20]. Briefly, tumor tissues were digested in PBS buffer containing 0.4 mg/ml collagenase, 2 mg/ml

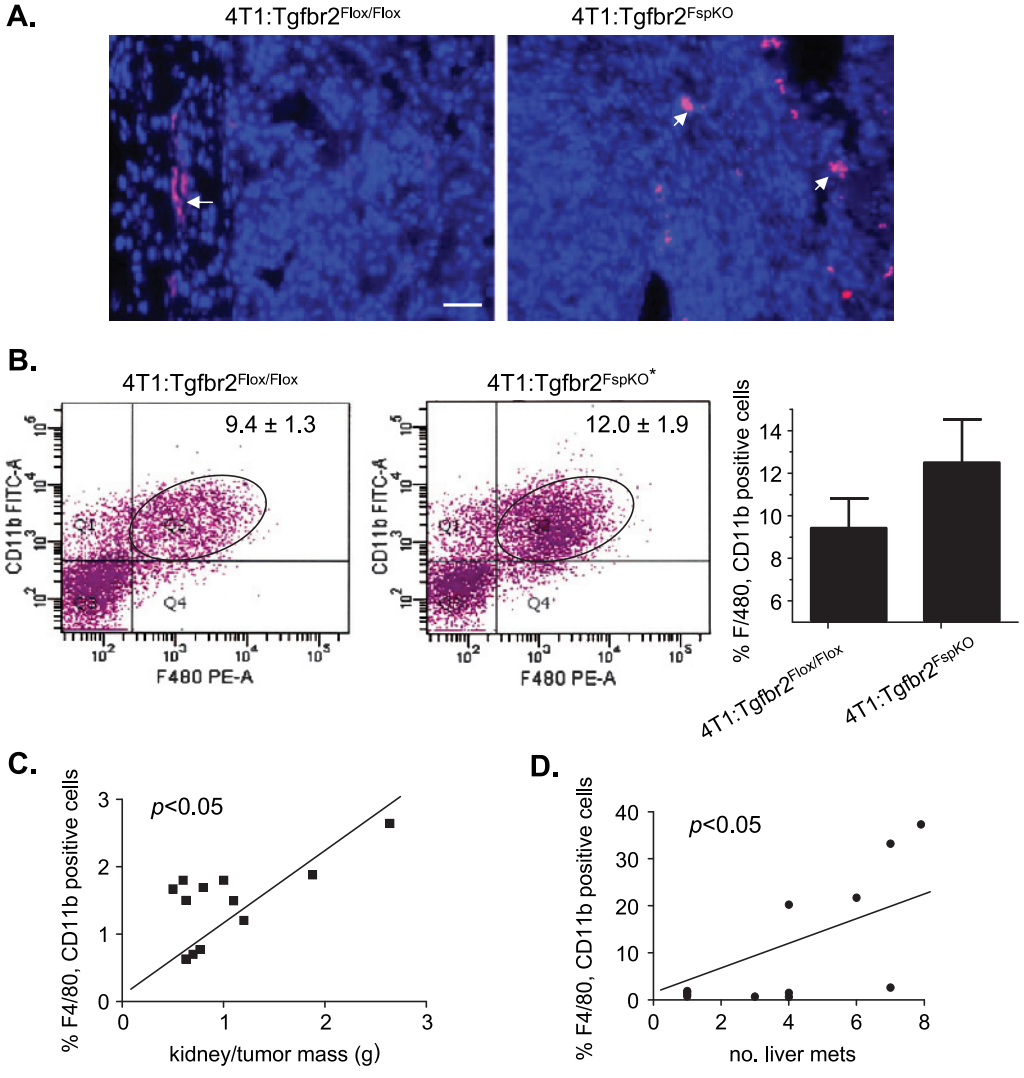

Figure 1. Tgfbr2^{FspKO} fibroblasts enhance recruitment of TAMs. (A) Primary tumor grafts were stained by immunofluorescence for F4/80 expression to detect TAMs. Sections were counterstained with DAPI. Arrows point to F4/80-positive cells. Scale bar, 40 μm. (B) Flow cytometry analysis of primary tumor digests for F4/80, Cd11b-positive cells from 4T1 cells cografted with control Tgfbr2$^{Flox/Flox}$ (n = 10) or Tgfbr2^{FspKO} fibroblasts (n = 10). Values are depicted as mean ± SEM. *Statistical significance was determined by two-tailed Student's t test, $P < .05$ in comparison to 4T1:Tgfbr2$^{Flox/Flox}$ controls. Statistical significance was determined by $P < .05$. Spearman rank correlation analysis of primary tumor masses (C) and number of liver metastases (D) with F4/80, Cd11b-positive cell recruitment to the primary tumor of 4T1 cells grafted with Tgfbr2^{FspKO} fibroblasts. Statistical significance was determined by $P < .05$.

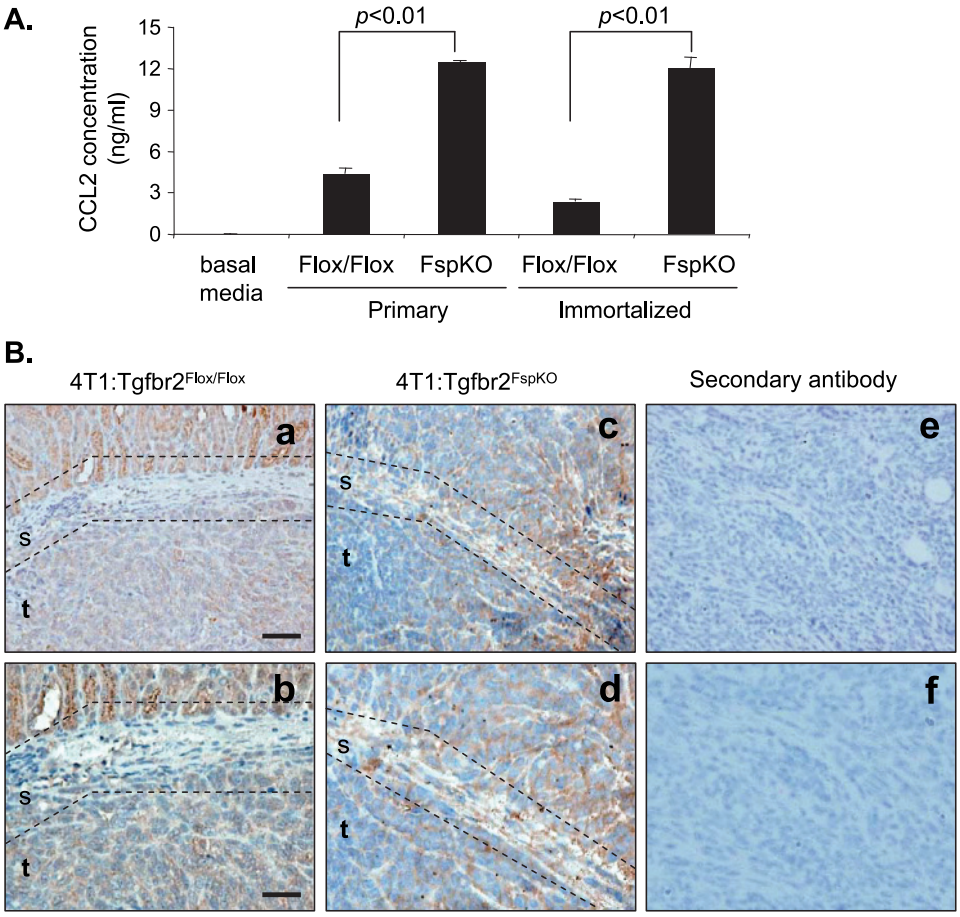

Figure 2. Increased expression of CCL2 in Tgfbr2^{FspKO} stromal cells. (A) Secretion of CCL2 from fibroblast conditioned medium from primary and immortalized Tgfbr2$^{Flox/Flox}$ and Tgfbr2^{FspKO} fibroblasts was quantified by ELISA. Statistical significance was determined by two-tailed Student's *t* test. Statistical significance was determined by $P < .05$, in comparison to Flox/Flox controls. (B) Immunohistochemistry staining of CCL2 in the stroma of primary tumor subrenal grafts. *s* indicates stroma; *t*, tumor. Scale bar, 40 μm (a, c, e). Scale bar, 20 μm (b, d, f).

hyaluronidase, and 2 mg/ml trypsin and separated into a single-cell suspension using a manual tissue homogenizer. Debris were removed by passing through a mesh filter, and red blood cells were lysed using a buffer containing 10 mM $KHCO_3$, 150 mM NH_4Cl, and 0.1 mM EDTA. Samples were incubated with anti–F4/80-PE (AbdSerotec, Oxford, UK), anti–Cd11b-FITC, and anti–Gr1-APC (BD Pharmingen, San Jose, CA), 1:100 on ice, washed with PBS, and then analyzed by flow cytometry using a BD FACS analyzer (BD Biosciences, San Jose, CA).

Determination of CCL2 Levels in Fibroblasts

To determine the levels of CCL2 secreted from fibroblasts in culture by ELISA, immortalized and primary cultures of fibroblasts from Tgfbr2$^{Flox/Flox}$ and Tgfbr2^{fspKO} mammary glands were grown in 10-cm plates in DMEM/F12 medium containing 10% FBS and antibiotics. At 80% confluence, the cells were starved in DMEM F12 medium containing 0.1% FBS for 24 hours and were then incubated in 4 ml of complete medium for another 24 hours. Levels of secreted CCL2 protein were determined by ELISA (R&D Systems) using 100 µl of conditioned medium. Statistical significance was determined by two-tailed Student's *t* test.

Determination of Anti-CCL2 and IgG Levels in Tumor-Bearing Mice

To determine the levels of anti-CCL2 and control IgG in tumor-bearing mice by ELISA, blood sera were collected from the tail vein of grafted mice 4 and 24 hours after the last treatment with anti-CCL2 or IgG. The samples were then diluted in buffer containing 50 mM $NaHCO_3$ and 50 mM Na_2CO_3, pH 9.6, and coated onto 96-well plates overnight at room temperature. The samples were incubated with anti-rat biotinylated antibodies (1:500) for 1 hour and then with streptavidin peroxidase (Vectastain Elite Kit; Vector Laboratories) for an additional 30 minutes. The samples were visualized by incubating with tetramethylbenzidine substrate (R&D Systems) according to the manufacturer's instructions. The reaction was stopped with 1 M HCl, and the absorbance was read at OD 450 nm.

Statistical Analyses

Data were analyzed for statistical significance by two-tailed Student's *t* test, ANOVA with Bonferroni's multiple comparison tests of all groups, or Spearman rank correlation test as indicated, using GraphPad Prism software (GraphPad Software, La Jolla, CA). Statistical significance was determined by $P < 0.05$.

Results

Tgfbr2^{FspKO} Fibroblasts Enhance Recruitment of TAMs to the Primary Tumor Associated with Increased Expression of CCL2

In previous studies, *Tgfbr2*-deficient fibroblasts cografted with mammary carcinoma cells [8,9] resulted in enhanced primary tumor growth

and invasiveness and metastatic spread. Studies have shown that accumulation of immune cells, in particular, TAMs, correlates with the invasive phenotype of breast cancers; therefore, it was important to determine whether *Tgfbr2*-deficient fibroblasts significantly affected recruitment of macrophages to the primary tumor. Tgfbr2^{FspKO} fibroblasts were cografted with 4T1 mammary carcinoma cells in the subrenal capsule of nude mice for 21 days and subjected to flow cytometry analysis for a number of cells coexpressing F4/80 and Cd11b, markers associated with TAMs [14]. Tgfbr2^{FspKO} fibroblasts cografted with 4T1 mammary carcinoma cells resulted in significantly higher levels of F4/80, Cd11b double-positive macrophages compared with mammary carcinoma cells cografted with control Tgfbr2$^{Flox/Flox}$ fibroblasts (Figure 1, *A* and *B*). Furthermore, correlation analyses revealed a significant association between the increased numbers of F4/80, Cd11b-positive cells recruited to the primary tumor, with the increased primary tumor growth and liver metastases of mammary carcinoma cells cografted with Tgfbr2^{FspKO} fibroblasts (Figure 1, *C* and *D*).

Previous studies have shown that macrophage recruitment in part is regulated by the expression of chemokines in the tissue microenvironment [16,21]. In particular, CCL2 was previously shown a significant regulator of macrophage recruitment during inflammation and tumorigenesis [1,22]. By Affymetrix complementary DNA microarray analysis, CCL2 was observed to be the most significantly upregulated of all chemokines in Tgfbr2^{FspKO} fibroblasts, in comparison with control fibroblasts (data not shown). This upregulated expression was confirmed by ELISA of supernatants isolated from cultured primary and immortalized Tgfbr2^{FspKO} and control Tgfbr2$^{Flox/Flox}$ fibroblasts (Figure 2*A*). Furthermore, primary tumors cografted with Tgfbr2^{FspKO} fibroblasts also showed high levels of CCL2 expression localized to the stromal fibroblast compartment, which was absent in the stromal tissues of primary tumors cografted with control fibroblasts (Figure 2*B*). These data indicated an association between enhanced CCL2 expression in Tgfbr2^{FspKO} fibroblasts and recruitment of TAMs, leading to the hypothesis that CCL2 derived from TGF-β signaling–deficient fibroblasts enhances mammary tumor progression through TAM recruitment.

Antibody Neutralization of CCL2 Inhibits Mammary Tumor Progression and TAM Recruitment

To determine the functional contribution of CCL2 derived from Tgfbr2^{FspKO} fibroblasts in mammary tumorigenesis, two approaches were used to inhibit CCL2 activity. First, tumor-bearing mice were

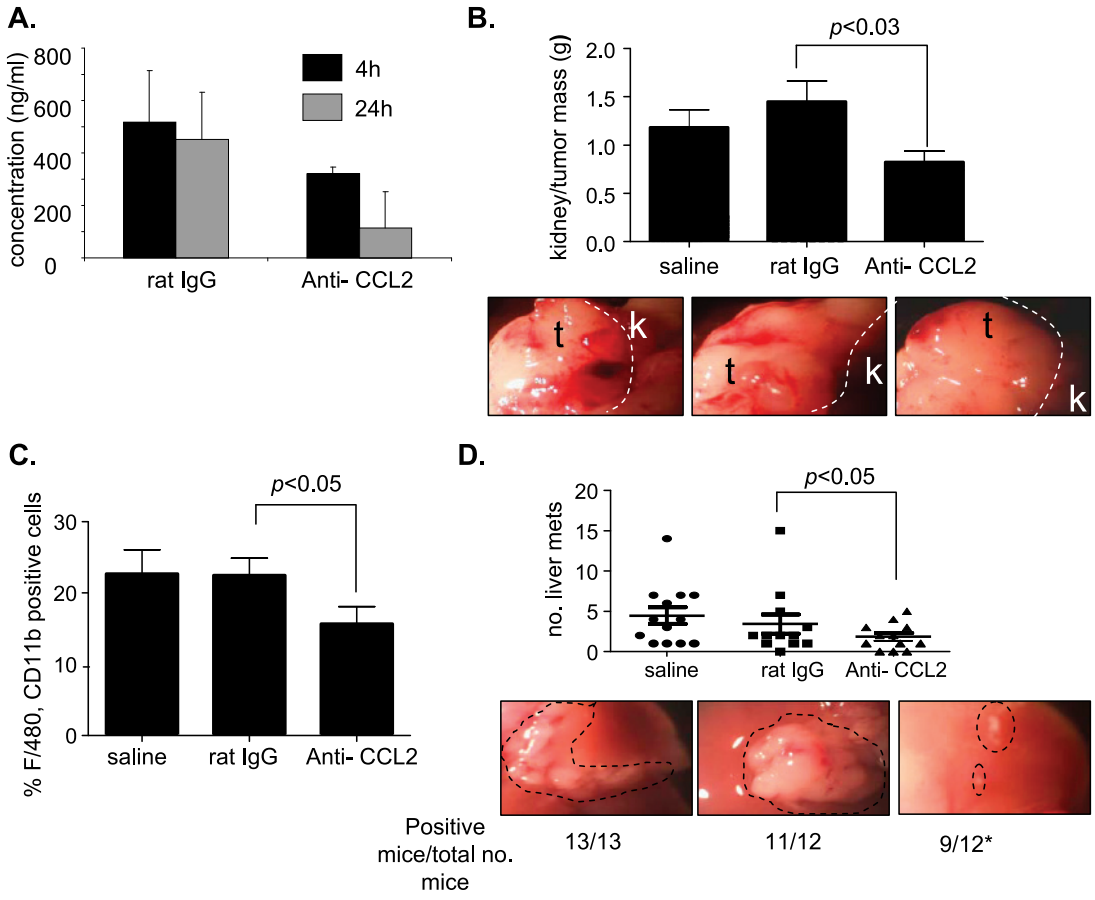

Figure 3. Effect of CCL2 neutralization on primary tumor and recruitment of TAMs. (A) Peripheral blood samples were harvested from tumor-bearing mice 4 and 24 hours after injection and analyzed for the presence of anti-CCL2 or control IgG by ELISA. (B) Primary tumor mass of 4T1:Tgfbr2^{FspKO} graft recombinants treated with saline vehicle control, rat IgG control, or anti-CCL2. Tumor (t) and kidney (k) delineated by dotted line. (C) Primary tumor cell digests from each treatment group were analyzed for the presence of F4/80, Cd11b-positive cells by flow cytometry. (D) Liver metastases were detected by stereomicroscopy of gross tissues and counted. Representative lesions on liver tissues are outlined. Liver incidence is noted below micrographs. Graphs are represented by mean ± SEM. Statistical analysis was performed by ANOVA with Bonferroni's multiple comparison tests of all groups. Statistical significance was determined by $P < .05$ in comparison to rat IgG.

treated with neutralizing antibodies to CCL2, which has been previously shown to inhibit CCL2-induced inflammation in mice [23,24]. Tgfbr2^{FspKO} fibroblasts were cografted with 4T1 mammary carcinoma cells in the subrenal capsule of nude mice. Seven days after treatment, grafted mice were injected in the intraperitoneum with 5 mg/kg of anti-CCL2, control IgG, or 0.9% saline vehicle control for an additional 14 days. To determine the stability of anti-CCL2 injected in mice, samples of blood serum were collected from mice by tail vein and were analyzed for levels of anti-CCL2 4 and 24 hours before the last injection. ELISA of blood serum indicated significant levels of anti-CCL2 24 hours after injection, although the levels were lower compared with the levels of control rat IgG (Figure 3*A*). Compared with IgG controls, anti-CCL2 treatment resulted in a significant reduction in primary mammary tumor mass (Figure 3*B*) associated with decreased TAM recruitment to the primary tumor (Figure 3*C*). In addition, anti-CCL2 treatment also significantly decreased the number but not the incidence of liver metastases compared with rat IgG- or saline-treated mice (Figure 3*D*). These data indicate that systemic inhibition of CCL2 significantly inhibits tumor growth and metastatic spread associated with TAM recruitment.

Immunohistochemistry analyses were performed on primary tumor sections to determine the effects of anti-CCL2 treatment on tumor cell proliferation and survival. Anti-CCL2–treated tumors exhibited significant decreases in cellular proliferation as indicated by K67 staining (Figure 4*A*) compared with IgG- and saline-treated mice. Whereas anti-CCL2 treatment resulted in a significantly increased cleaved caspase-3 staining compared with saline-treated groups, there were no significant differences in cleaved caspase-3 expression between IgG- and anti-CCL2–treated groups because IgG-treated groups also exhibited increased apoptosis compared with saline (Figure 4*B*). In summary, these data indicate that the reduction in cellular proliferation and cell survival in anti-CCL2–treated mice was associated with an overall reduction in primary tumor growth and liver metastases.

siRNA Silencing of CCL2 in Tgfbr2^{FspKO} Fibroblasts Inhibits Liver Metastases but Does Not Significantly Reduce TAM Recruitment

Studies have shown that other cell types in the tumor microenvironment including macrophages, endothelial cells, and mammary epithelial cells expressed CCL2 [25,26]. To determine the functional contribution of CCL2 derived from *Tgfbr2* deficient fibroblasts, an siRNA approach to target CCL2 expression was used. siRNA targeting two different regions of the *Ccl2* transcript were stably expressed in Tgfbr2^{FspKO} fibroblasts, resulting in the generation of two cell lines, one exhibiting 35% knockdown (3CCL2–) and the other exhibiting 82% knockdown (1CCL2–) of CCL2 expression compared with control Tgfbr2^{FspKO} fibroblasts expressing siRNA to GFP (GFP–) (Figure 5*A*). Cografting 4T1 mammary carcinoma cells with fibroblast cell lines expressing varying levels of CCL2 allowed the determination of dose-dependent effects of CCL2 derived from mammary fibroblasts on mammary tumor progression. Compared with cografting 4T1 cells with GFP– control fibroblasts, cografting of 3CCL2– fibroblasts did not result in significant changes to tumor mass (Figure 5*B*), TAM recruitment (Figure 5*C*), or liver metastases (Figure 5*D*). These data indicate that a 35% knockdown in CCL2 secretion in Tgfbr2^{FspKO} fibroblasts was not sufficient to inhibit mammary tumor growth, TAM recruitment, or metastatic spread. Cografting with 1CCL2– fibroblasts did not result in significant changes to primary tumor growth (Figure 5*B*). We observed a small decrease in TAM recruitment, which was not statistically significant (Figure 5*C*). In addition, cografting with 1CCL2– fibroblasts resulted in a significant decrease in the number but not in the incidence of liver metastases (Figure 5*D*). These data indicate that CCL2 derived from Tgfbr2^{FspKO} fibroblasts does not significantly regulate primary tumor growth or TAM recruitment but does, in part, regulate liver metastases of 4T1 mammary carcinoma cells, possibly in a concentration-dependent manner.

Immunohistochemistry analyses were performed to determine the effects of inhibiting CCL2 by knockdown in fibroblasts on primary tumor cell proliferation and survival. Transplantation of 4T1 mammary carcinoma cells with 3CCL2– fibroblasts did not result in significant changes in Ki67 staining or cleaved caspase-3 staining (Figure 6, *A* and *B*). Cografting of 4T1 carcinoma cells with 1CCL2– fibroblasts also did not result in changes to Ki67 staining but did result in significantly increased cleaved caspase-3 staining. These data indicate that CCL2 derived from Tgfbr2^{FspKO} fibroblasts does not regulate tumor cell proliferation but does regulate tumor cell survival, possibly in a concentration-dependent manner.

Discussion

Previous studies have shown that TGF-β signaling in fibroblasts suppresses mammary carcinoma cell growth, invasion, and metastatic spread

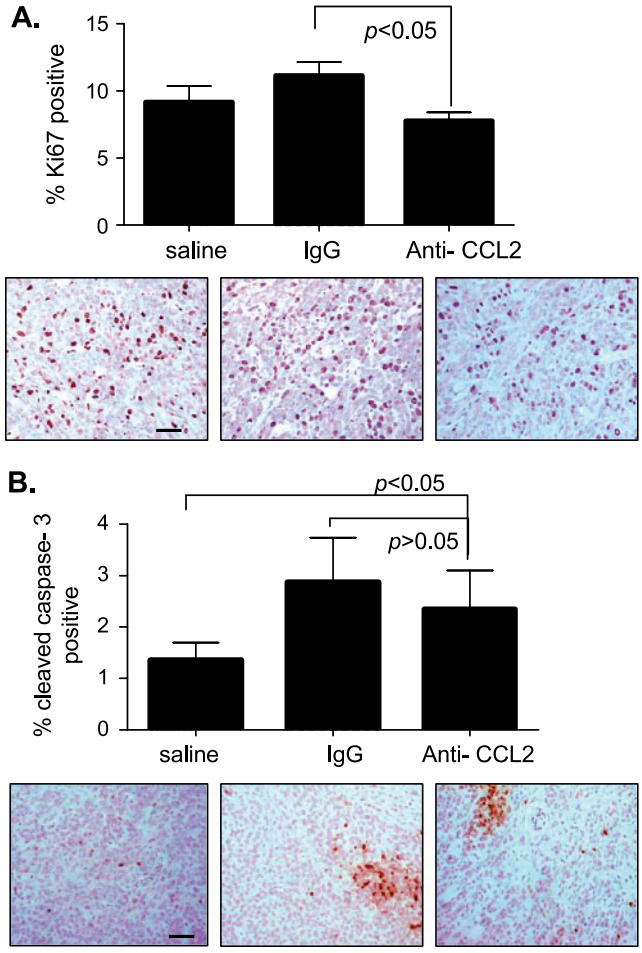

Figure 4. Effect of anti-CCL2 treatment on tumor cell proliferation and apoptosis. Primary tumor graft recombinants were paraffin-embedded, sectioned, and immunostained for the expression of (A) Ki67 as a marker for cell proliferation and (B) cleaved caspase-3 as a marker for apoptosis. Statistical analysis was performed by ANOVA with Bonferroni's multiple comparison tests of all groups. Statistical significance was determined by $P < .05$. Scale bar, 40 μm.

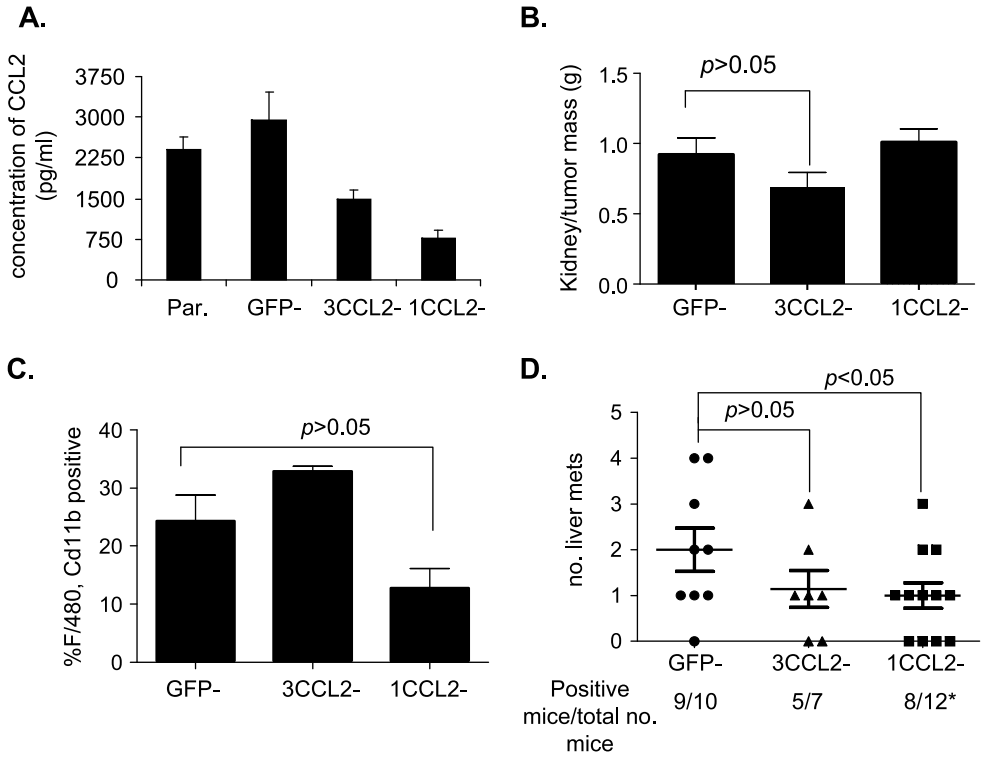

Figure 5. Effect of siRNA knockdown in Tgfbr2^{FspKO} fibroblasts on 4T1 tumor growth and recruitment of TAMs. (A) Conditioned medium from parental fibroblasts (Par), fibroblasts stably expressing control siRNA (GFP−), or siRNA targeting CCL2 (3CCL2− and 1CCL2−) were analyzed for the secretion of CCL2 by ELISA. (B) Primary tumors from mice grafted with 4T1 cells with Tgfbr2^{FspKO} fibroblasts expressing siRNA to control GFP or CCL2 were weighed 21 days after grafting. (C) Primary tumor cell digests from each experimental group were analyzed for the presence of F4/80, Cd11b-positive cells by flow cytometry. (D) Metastatic surface lesions on the liver were detected by stereomicroscopy and counted. The counts are represented as the number of lesions per mouse. The incidence of liver metastases is noted below graph and is represented as the number of mice exhibiting lesions/total number of mice examined. Statistical analysis was performed by ANOVA with Bonferroni's multiple comparison tests of all groups. Statistical significance was determined by $P < .05$.

by regulating expression of soluble factors that mediate tyrosine receptor kinase signaling in mammary carcinoma cells [8]. In these present studies, the data collectively support that TGF-β signaling in mammary fibroblasts inhibits CCL2 chemokine expression to negatively regulate TAM recruitment and tumor metastases. These studies reveal important insight into the relationship between TGF-β and CCL2 signaling pathways during breast cancer progression and reveal important roles for CCL2 in regulating fibroblast interactions with mammary carcinoma cells and other stromal cells in the mammary tumor microenvironment.

Whereas previous studies have demonstrated that CCL2 regulates breast cancer progression through TAM recruitment, the effects of siRNA knockdown in CCL2 in mammary fibroblasts presented here indicate that CCL2 regulates breast cancer progression in part by mediating fibroblast-carcinoma cell interactions. We observed that a 35% knockdown in CCL2 expression in Tgfbr2^{FspKO} fibroblasts was not sufficient to inhibit macrophage recruitment, although a slight reduction in the number of liver metastases was noted. An 82% knockdown in CCL2 expression resulted in a small but not statistically significant reduction in macrophage recruitment but did significantly reduce liver metastases and cell survival. The different effects of CCL2 knockdown in fibroblasts on macrophage recruitment and 4T1 metastases indicate that CCL2 secreted from Tgfbr2^{FspKO} fibroblasts acts directly on mammary carcinoma cells to regulate cell survival and metastatic spread (Figure 7*A*). Interestingly, a recent study demonstrated a different mechanism through which CCL2 may regulate the metastatic spread. This particular study demonstrated that overexpression of CCL2 in prostate cancer cells resulted in increased bone metastases in mice, associated with increased osteoclast formation, indicating that CCL2 may act directly or indirectly on stromal cells of distal sites to regulate metastatic spread [16]. In summary, these studies indicate that CCL2 may regulate late-stage carcinoma progression at the primary tumor site and in distal sites of metastasis, through complex, multiple mechanisms.

Currently, the functional contribution of CCL2/CCR2 signaling in cancer cells remains largely unclear. The polymorphism CCR264-I has been associated with the development sporadic breast cancer [27], and CCR2 overexpression significantly correlates with the development of glioblastoma [28]. Studies of CCL2 signaling in the human PC-3 prostate cancer cell line demonstrate that CCL2 promotes cell survival through an AKT-independent mechanism, by negatively regulating AMP-activated protein kinase resulting in mammalian target of rapamycin complex activation and sustained expression of survivin [29]. In addition, current studies in our laboratory indicate that CCL2/CCR2 signaling regulates mammary carcinoma cell survival and invasion through a mitogen-activated protein kinase–dependent mechanism (unpublished observations). These studies indicate that CCL2 promotes cell survival in different cancer cell types, potentially through multiple mechanisms. Further studies are underway in our laboratory to further understand the molecular mechanisms through which CCL2 mediates stromal–breast cancer cell interactions.

An important association between TAM recruitment and CCL2 in mammary tumor progression was also observed in these current studies. Given that anti-CCL2 antibody inhibited mammary tumor progression

more significantly than siRNA knockdown of CCL2 in mammary fibroblasts, it is possible that CCL2 derived from TAMs also promotes primary tumor growth and TAM recruitment (Figure 7*B*). Anti-CCL2 treatment but not siRNA knockdown of CCL2 in fibroblasts inhibited primary tumor growth, indicating that systemic inhibition of CCL2 expressed in other cell types may also contribute to mammary tumor growth. Because recombinant CCL2 does not significantly affect cellular proliferation *in vitro* (data not shown), CCL2 may enhance primary tumor growth, indirectly through enhancement of TAMs that have been shown to express a number of growth factors [1,30]. This possibility is also supported in previous studies, in which anti-CCL2 treatment of tumor-bearing mice inhibited growth and metastases of MCF7 and MDA-231 cells orthotopically grafted in severe combined immunodeficient mice along with a decrease in macrophage recruitment [17,31]. These studies further suggested that CCL2 expression was derived from TAMs that were recruited to the primary tumor through a CCL2-dependent positive feedback mechanism [31]. CCL2-dependent recruitment of TAMs to the primary tumor has also been demonstrated in a transplantation model of prostate cancer [16]. Anti-CCL2 treatment of prostate tumor-bearing mice inhibited tumor growth associated with decreased macrophage recruitment, indicating that CCL2 may regulate tumor progression through a macrophage-dependent mechanism in multiple tumor types. Because $Tgfbr2^{FspKO}$ fibroblasts cografted with mammary carcinoma cells resulted in enhanced recruitment of TAMs, it is possible that the TAM recruitment was initiated by $Tgfbr2^{FspKO}$ fibroblasts, subsequently resulting in a CCL2-dependent positive feedback mechanism to recruit additional TAMs over time. The data presented here support an important association between CCL2 and TAM recruitment in metastatic disease. Collectively, these studies demonstrate multifunctional roles for CCL2 signaling in breast cancer and indicate that targeting CCL2 in breast cancer would inhibit both epithelial and mesenchymal cell types.

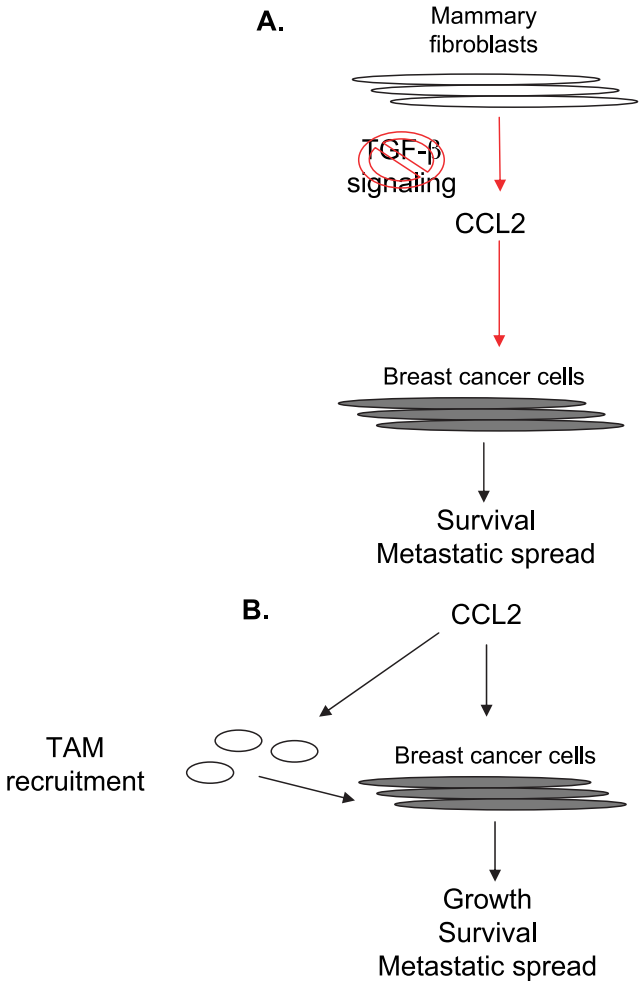

Figure 7. Model for the role of CCL2 signaling in the breast tumor microenvironment. (A) Mammary fibroblasts increase CCL2 expression when TGF-β signaling is downregulated. The increased expression of CCL2 derived from mammary fibroblasts acts directly on mammary carcinoma cells to enhance carcinoma survival and invasion. (B) The expression of CCL2 in the breast cancer microenvironment enhances mammary tumor growth, survival, and invasion through a dual mechanism: directly stimulating mammary carcinoma cells and recruitment of TAMs.

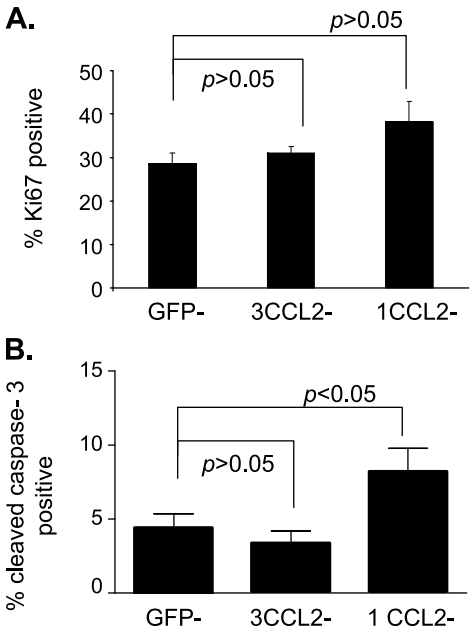

Figure 6. Effect of CCL2 knockdown in fibroblasts on tumor cell proliferation and survival. Immunostaining of primary sections of control, 3CCL2−, and 1CCL2− cocultures for the expression of Ki67 as a marker for cell proliferation (A) and of cleaved caspase-3 as a marker for apoptosis (B). Statistical analysis was performed by ANOVA with Bonferroni's multiple comparison tests. Statistical significance was determined by $P < .05$.

Studies presented here indicate that differing levels of CCL2 in the tumor microenvironment significantly affect the activity of stromal cells and mammary carcinoma cells. Cografting 4T1 cells with 1CCL2– fibroblasts but not 3CCL2– fibroblasts significantly inhibited liver metastases. In addition, cografting experiments with 1CCL2– fibroblasts but not with 3CCL2– fibroblasts resulted in a small decrease in macrophage recruitment, indicating that local concentrations of CCL2 may stimulate stromal cells at levels different from mammary carcinoma cells. This notion is supported in previous studies that show that high concentrations of CCL2 in mammary tumors desensitize and downregulate chemokine receptor signaling of T cells while supporting tumor growth [32]. Although CCL2 has been found to bind and activate CCR2 in mouse and human macrophages with k_d values of 22.5 and 25.7 nM, respectively [33], this study, as well as previous studies [32], indicates that CCL2/CCR2 binding kinetics differ among mammary

epithelial cells or other mesenchymal cell types. Given that chemokine receptor desensitization and down-regulation is a common mechanism in regulating G-coupled receptor signaling in multiple cell types [32,34,35], it would be important in the future to clarify and understand the signaling kinetics of CCL2 chemokine signaling among stromal cells and carcinoma cells to predict its biological effects on tumor progression.

This study of siRNA knockdown in CCL2 in fibroblasts demonstrates a fibroblast-specific contribution of CCL2 signaling in regulating mammary tumor progression. In addition, data presented here support the potential effectiveness for targeting the CCL2/CCR2 network in metastatic disease. However, given that CCL2 acts on multiple cell types in the tumor microenvironment and that chemokine signaling is dependent on ligand concentrations, it is important to clarify the molecular and cellular mechanisms of inflammatory chemokine signaling in breast cancer to design an effective therapeutic regimen for the treatment of metastatic disease.

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