Oncogenes and Tumor Suppressors

CCR2 Chemokine Receptors Enhance Growth and Cell-Cycle Progression of Breast Cancer Cells through SRC and PKC Activation

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Abstract

Basal-like breast cancers are an aggressive breast cancer subtype, which often lack estrogen receptor, progesterone receptor, and Her2 expression, and are resistant to antihormonal and targeted therapy, resulting in few treatment options. Understanding the underlying mechanisms that regulate progression of basal-like breast cancers would lead to new therapeutic targets and improved treatment strategies. Breast cancer progression is characterized by inflammatory responses, regulated in part by chemokines. The CCL2/CCR2 chemokine pathway is best known for regulating breast cancer progression through macrophage-dependent mechanisms. Here, we demonstrated important biological roles for CCL2/CCR2 signaling in breast cancer cells. Using the MCF10CA1d xenograft model of basal-like breast cancer, primary tumor growth was significantly increased with cotransplantation of patient-derived fibroblasts expressing high levels of CCL2, and was inhibited with CRISP/R gene ablation of stromal CCL2. CRISP/R gene ablation of CCR2 in MCF10CA1d breast cancer cells inhibited breast tumor growth and M2 macrophage recruitment and validated through CCR2 shRNA knockdown in the 4T1 model. Reverse phase protein array analysis revealed that cell-cycle protein expression was associated with CCR2 expression in basal-like breast cancer cells. CCL2 treatment of basal-like breast cancer cell lines increased proliferation and cell-cycle progression associated with SRC and PKC activation. Through pharmacologic approaches, we demonstrated that SRC and PKC negatively regulated expression of the cell-cycle inhibitor protein p27KIP1, and are necessary for CCL2-induced breast cancer cell proliferation.

Implications: This report sheds novel light on CCL2/CCR2 chemokine signaling as a mitogenic pathway and cell-cycle regulator in breast cancer cells.

Introduction

Breast cancer is the most common form of cancer diagnosed in women in the United States, with 250,000 cases diagnosed in the United States, and ranks second in the cause of cancer-related deaths (1). Treatment is complicated by the presence of multiple breast cancer subtypes. Luminal A/B breast cancers express estrogen receptor (ER), progesterone receptor (PR), and/or Her2. Others overexpress Her2. Basal-like breast cancers (BLBC) often lack ER, PR, and Her2 (2, 3). BLBC comprise approximately 15% of all of cases diagnosed in North America, but are considered the most aggressive, and are resistant to most treatments other than chemotherapy (4, 5). Understanding the mechanisms that regulate BLBC progression would lead to new therapeutic targets and improved treatment strategies.

Cancer progression is associated with recruitment of myeloid immune cells, increased angiogenesis, and fibroblast accumulation. These stromal cell responses are regulated in part by chemokines, soluble molecules (8 kda), which form molecular gradients to induce cellular chemotaxis during tissue development, inflammation, and cancer, by signaling to seven-transmembrane G coupled receptors (6, 7). The Chemokine C-C Ligand 2 (CCL2) regulates recruitment of macrophages and endothelial cells during acute inflammation by primarily signaling to CCR2 receptors (6, 8). CCL2 overexpression in breast tumors correlates with macrophage levels (9, 10). CCL2 expression in the stroma correlates with poor prognosis for breast cancer patients (10, 11). Antibody neutralization of CCL2 inhibits growth, survival, and invasion of breast tumor xenografts (10, 12, 13), correlating with decreased macrophage recruitment. These studies indicate an important role for CCL2 in regulating macrophage recruitment during breast cancer progression.

Studies on CCL2/CCR2 signaling in cancer have focused on its role in regulating immune cell recruitment. Yet, CCR2 expression is overexpressed in epithelial tissues of invasive ductal carcinomas and luminal and BLBC cell lines (14). shRNA knockdown or antibody neutralization of CCL2 inhibits fibroblast-induced survival and invasion of luminal breast cancer cells (14, 15). Although these studies indicate an important role for epithelial CCR2 signaling in cancer progression, breast tumors exhibit...
variations in CCL2/CCR2 expression and signaling (14) that are poorly understood.

Compared with other molecular subtypes, BLBC show the highest levels of stromal CCL2 expression (11). Using the MCF10CA1d xenograft model of BLBC, we demonstrate that breast tumor growth was significantly increased with cotransplantation of patient-derived fibroblasts, correlating with CCL2 expression. CRISP/R gene ablation of stromal CCL2 or CCR2 in MCF10CA1d breast cancer cells inhibited breast tumor growth and M2 macrophage recruitment. The effects of CCR2 deficiency on tumor growth were validated in the 4T1 model. CCL2 treatment of multiple BLBC cell lines increased proliferation and cell-cycle progression associated with SRC and PKC activation, which were decreased in CCR2 knockout cells. Pharmacologic approaches demonstrate that SRC and PKC negatively regulate CCL2/CCR2 chemokine signaling in breast cancer cells, with important implications on therapeutic targeting.

Materials and Methods

Cell culture

Unless indicated, cell lines were cultured in DMEM/10% FBS/2 mmol/L l-glutamate/1% penicillin–streptomycin. MCF10CA1d cells (16, 17) were kindly provided by Fred Miller (University of Michigan, Ann Arbor, MI). BT-20 and HCC1937 cells were kindly provided by Roy Jensen (University of Kansas Medical Center, Kansas City, KS). 4T1 cells were purchased from the ATCC provided by Roy Jensen (University of Kansas Medical Center, Michigan, Ann Arbor, MI). BT-20 and HCC1937 cells were kindly provided by Roy Jensen (University of Kansas Medical Center, Kansas City, KS). 4T1 cells were purchased from the ATCC provided by Roy Jensen (University of Kansas Medical Center, Michigan, Ann Arbor, MI). 4T1 cells were purchased from the ATCC provided by Roy Jensen (University of Kansas Medical Center, Michigan, Ann Arbor, MI). BT-20 and HCC1937 cells were kindly provided by Roy Jensen (University of Kansas Medical Center, Kansas City, KS). 4T1 cells were purchased from the ATCC provided by Roy Jensen (University of Kansas Medical Center, Michigan, Ann Arbor, MI).

Breast cancer cells and 250,000 fibroblasts were transplanted as described (18). Brieferly, 100,000 cancer cells and 250,000 fibroblasts were seeded into 1% low density. Rat Tail Collagen type I overnight. Mice were anesthetized with % isoflurane. An incision was made into the skin flap to expose the #4–5 mammary glands. One collagen plug was inserted into a pocket that was made underneath the mammary lymph node using spring scissors. Wounds were closed by gut-absorbable suture. Mice were monitored twice a week and measured for tumor size by caliper. Mice bearing MCF10CA1d tumors were sacrificed 6 weeks after transplantation when control tumors grew 1.5 cm in diameter, the largest allowable size allowed by the Institutional Animal Care and Use Committee (IACUC). 4T1 tumor–bearing mice were sacrificed 4 weeks after transplantation. All animal procedures were approved by the IACUC under Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) guidelines.

Histology/immunohistochemistry

Tissues were fixed in 10% neutral formalin buffer (NBF), paraffin embedded, and hematoxylin and eosin (H&E) stained as described (20). For immunostaining, 5-μm sections were dewaxed and heated in 10 mmol/L sodium citrate (pH 6.0) at low pressure for 2 minutes using a pressure cooker. Peroxidases were quenched in PBS/10% methanol/1% H2O2 for 30 minutes. Slides were blocked in PBS/3% FBS, and incubated overnight with antibodies (1:100) to: arginase-1 (Santa Cruz Biotechnology; cat# 20150), Ly6G (BioLegend; cat# 127602), or Von Willebrand Factor 8 (Millipore; cat# 7356). Arginase I and VWF8 were detected with secondary rabbit biotinylated antibodies (1:1,000). Ly6G was detected with secondary rat biotinylated antibodies (1:1,000). Slides were immunostained with anti-human Cytokeratin 5 (Thermo Fisher; cat #M5-12596) or anti-GFP (Santa Cruz Biotechnology; cat# SC9996), using the MOM kit (Vector Laboratories; cat #B1M-2202). Antigens were detected using DAB substrate (Vector Laboratories; cat #SK-4100). Slides were counter-stained with Mayer’s hematoxylin and mounted with Cytoseal (Thermo Fisher; cat #348976). Five images were captured per sample at 10x magnification using the EVOS EL Auto Imaging system (Invitrogen). Expression was quantified by Image J using methods described (11).

CRISP/R gene ablation

Exon 1 of human CCL2 was targeted by CRISPR/Cas9 using the gRNA: 5′-TGTTCTGCGCTGAGCCCGCT-3′. The gRNA was cloned into lentivirus vector pLKO5.sgRNA.EFS.GFP (Addgene; cat #57822). hCAF-1 was transfected with lentivirus containing Cas9 and a GFP reporter. Transduced cells were selected with 4 μg/mL blasticidin. Cells were transduced with lentivirus expressing CCL2 gRNA or control vector, flow sorted for GFP expression, and seeded into 96-well plates. Single-cell colonies were screened by CCL2 ELISA to identify CCL2-deficient clones. CCR2 was targeted by CRISPR/Cas9 with gRNA: 5′CCAGAGCTGCTAAGAGTTGTA-3′. The gRNA was cloned into the pL-CRISPR.EFS.GFP vector [86] (Addgene; cat #57818), a lentivirus vector containing Cas9 and a GFP reporter. Transduced cells were flow sorted for GFP expression and seeded into 6-well plates, 1,000 cells/well. Individual colonies were manually picked and seeded into 96-well plates. Colonies were screened for CCR2 gene alterations by PCR, using primers flanking the targeting site (primer-F: ACATGCTGTGCTGCTATTTATGTCATC-3′ and primer-R: AACAGGCGGAGACTTTCGTC). Wild-type and CCR2-mutant clones were confirmed by DNA sequencing.

shRNA knockdown

The targeting sequences were 5′-TGTTCTGCGCTGAGCCCGCTAAGAGTTGTA-3′ for CCR2KD#1 and 5′-CCAGAGCTGCTAAGAGTTGTA-3′ for CCR2KD#3. The targeting sequence to silence-enhanced GFP as a negative control (CTRL) was 5′-GCTGACAGCTTAGTACAC-3′. The oligonucleotides were phosphorylated by kinase treatment; complementary oligos were annealed and subcloned into Bgl II and HindIII sites of pRETSRO-SUPER vector (21), which was generously provided by Reuven Agami (Netherlands Cancer
CCL2 ELISA
Note that 10,000 fibroblasts/well were seeded in 24-well plates, and incubated in serum-free medium for 24 hours. Conditioned medium was assayed for human CCL2 (ProTecTech, cat# 900-K31) or murine CCL2 (ProTecTech, cat# 900-K126) according to commercial ELISA protocols. Absorbance was read at OD450 nm using a BioTek plate reader. CCL2 levels were normalized to cell density by crystal violet staining as described (22).

Flow cytometry
For antibody staining, 200,000 cells were seeded in 6-cm dishes overnight. Cells were detached from plates with Accutase (EMD Millipore; cat# SCR005) at 37°C for 5 minutes, washed in PBS, and incubated with anti–C–CR2 PE diluted 1:50 (R&D Systems; cat# FAB215P) for 1 hour.

For cell-cycle studies, 200,000 cells were seeded in 6-cm dishes overnight. Cells were treated with or without 2 mmol/L Thymidine (Sigma; cat# T9895-1G) for 16 hours. The cells were washed 3 times with serum-free media (BT-20: EMEM, HCC1937: RPMi, and MCF10CA1d: DMEM). Growth media were added for 2 hours for MCF10CA1d cells and 10 hours for BT-20 and HCC1937 cells. Cells were incubated with 2 mmol/L thymidine for 16 hours, washed with serum-free media, and incubated with growth media, with or without 100 ng/mL CCL2. Cells were detached with Accutase and fixed with 70% ethanol at −20°C. Samples were incubated in 500 µL of PBS/0.1% Triton X-100/2 mg/mL RNase A (VWR; cat# 97064-064) and 200 µg/mL propidium iodide (Invitrogen; cat# P3566) for 15 minutes at 37°C.

For aldehyde dehydrogenase (ALDH) activity assay, cells were seeded in 6-well plates (200,000/well), serum starved for 24 hours, and incubated in serum-free medium with or without 100 ng/mL CCL2 for 24 hours. Cells were subject to AldeRed Assay (EMD Millipore; cat# SCR150) according to commercial protocol. All assays were analyzed using a BD LSRII Flow Cytometer.

3D Matrigel:Collagen cultures
One million cells were cultured in 10-cm dishes in 5 mL DMEM/10% FBS for 24 hours. Medium was collected, centrifuged, and filtered through 0.45 µm pore cellulose acetate membranes. Note that 36-well plates were coated with 40 µL of matrix containing 1:1 ratio of collagen (Corning; cat# 354236) and Growth Factor Reduced Matrigel (Corning; cat# 354230). MCF10CA1d cells were seeded 3,000 cells/well in 100 µL DMEM/10%FBS containing 2.5% Matrigel, with 100 µL conditioned medium, or 200 µL of DMEM/10% FBS/2% Matrigel with or without CCL2. The media were replaced every 2 days for up to 8 days. Bright field images were captured every 2 days using an EVOS FL-Auto Imaging system at 10X magnification, 4 fields/well. Sphere size was quantified using Image J.

Mammosphere assay
Three thousand cells/well were seeded in low-attachment 24-well plates (Corning) in 500 µL of DMEM/10% FBS with or without 100 ng/mL CCL2, and incubated for 5 days. Mammoospheres were pelleted and disassociated with 20 mmol/L Trypsin/2 mmol/L EDTA for 7 minutes at 37°C. Cells were quenched in DMEM/10% FBS, pelleted, and replated. Images were captured at 4X magnification using the EVOS FL-Auto prior to passaging. Mammospheres were counted using Image J, with minimum size of 160 µm².

Reverse phase protein array
Reverse phase protein array (RPRA) assays were adapted from previous studies (1–4). Protein lysates were prepared from quadruplicate samples of CCR2 knockout (CCR2KO-G10) or wild-type CCR2 control (WT-A1) using Pierce Tissue Protein Extraction Reagent (VWR; cat# 78510) with protease and phosphatase inhibitors. Note that 0.5 mg/mL of protein lysis were denatured in SDS sample buffer. The Aushon 2470 Arrayer (Aushon BioSystems) with a 40 pin (185 µm) configuration was used to spot samples and control lysates onto nitrocellulose-coated slides (Grace Bio-Labs) using an array format of 960 lysates/sample (2,880 spots/slide). Each sample was probed on triplicate slides. The slides were probed with 345 antibodies against total and phosphoprotein proteins using an automated slide stainer Auto- link 48 (Dako). A negative control slide was incubated with antibody diluent only. Primary antibody binding was detected using a biotinylated secondary antibody followed by streptavidin-conjugated IRDye680 fluorophore (LI-COR Biosciences; cat# 926-68079). Total protein content/sample was assessed by Sypro Ruby Protein Blot staining (Invitrogen; cat# S11791).

Fluorescence-labeled and negative control slides were scanned on a GenePix 4400 AL scanner at an appropriate PMT. The images were analyzed with GenePix Pro 7.0 (Molecular Devices). Total fluorescence signal intensities/sample were obtained after subtraction of the background signal for each slide and were normalized for variation in total protein, background, and nonspecific labeling using a group-based normalization method as described (23). For each spot, the background-subtracted foreground signal intensity was subtracted by the corresponding signal intensity of the negative control slide (omission of primary antibody) and normalized to the corresponding signal intensity of total protein for that spot. The median of normalized signal intensities/sample was used for statistical analysis.

Immunoblot analysis
Cancer cells were seeded in 6-well plates (200,000 cells/well) in DMEM/10% FBS, serum deprived overnight, and incubated with serum-free media with or without recombinant CCL2, DMSO, PP2, or Gö 6983 for 15 minutes. Cells were lysed with RIPA buffer containing protease inhibitors and phosphatase inhibitors. Twenty-five microgram proteins were resolved on SDS polyacrylamide gels and transferred to nitrocellulose membranes. Membranes were blocked in PBS/0.05% Tween-20/5% milk and incubated with primary antibodies (1:1,000) to: -PKC (pan, BI Ser660, CST; cat# 9371), p-SRC (Tyr416, CST; cat# 6943), SRC (CST; cat# 2123), p27KIP1 (Fisher Scientific; cat# BDB610241), p21 (CST; cat# 2947), p-ERK (Thr202/Tyr204, CST; cat# 4370), ERK1/2 (CST; cat# 9107), p-SMAD3 (Ser423/425, CST; cat# 9520), SMAD3 (CST; cat# 9523), and ACTIN (Sigma; cat# A5441). Proteins were detected with corresponding secondary antibodies conjugated to horseradish peroxidase (1:1,000), developed with West Femto chemiluminescence substrate (Thermo Fisher; cat #34094), and imaged using a Biospectrum Imaging System.
Immunocytochemistry/immunofluorescence
Five thousand cells/well were seeded in 96-well plates. Cells were fixed in 10% NBF, permeabilized with methanol at −20°C for 10 minutes, and blocked in PBS/3% FBS for 1 hour. For immunocytochemistry studies, cells were incubated for 24 hours with antibodies (1:100) to alpha smooth muscle actin (α-SMA, Abcam; cat# 7187), Fibroblast specific protein 1 (FSP1, Abcam; cat# 5550), N cadherin (Santa Cruz Biotechnology; cat #sc7939), E-cadherin (BD Biosciences; cat # 610181), or Pan-cytokeratin (Santa Cruz Biotechnology; cat #8018). α-SMA, Pan-cytokeratin, and N-cadherin were detected using anti–mouse-hp (1:500). FSP1 and N-cadherin were detected using anti–rabbit-hp (1:500). Antigens were detected using DAB. For immunofluorescence studies, cells were incubated with antibodies (1:300) to proliferating cell nuclear antigen (PCNA; BioLegend; cat #307901) or p27KIP1 (Fisher Scientific; cat #BD810241) for 24 hours. Cells were incubated with secondary mouse antibodies conjugated to Alexa-Fluor-647 (1:500). Antigens were detected using DAB. Statistical analysis was performed using GraphPad software. The two-tailed Student t test was used for two groups. One-way ANOVA with Bonferroni post hoc comparison was used for more than two groups. Associations between continuous variables in The Cancer Genome Atlas datasets were analyzed by Spearman correlation analysis. RPPA data were analyzed using two-tailed Student t test (23), using 1.2-fold change as a cutoff. Heat mapping was performed using https://software.broadinstitute.org/morpheus/. Statistical significance was determined by P < 0.05, ‘’, P < 0.01; ‘‘’, P < 0.001; and n.s., not significant. For cell culture experiments, samples were plated in triplicates/group; experiments were repeated 3 times.

Results
BLBC growth is associated with stromal CCL2 expression
To characterize the significance of CCL2 derived from fibroblasts to progression of BLBC, we utilized the MCF10CA1d model, a breast cancer cell line derived from Ras-transformed MCF10A cells (26, 27). Fibroblasts were isolated from invasive ductal carcinoma tissues (hCAF) or normal adjacent tissues (hNAF), and characterized for expression of mesenchymal markers and absence of epithelial markers (Supplementary Fig. S1). By ELISA, CCL2 expression in fibroblasts was significantly higher than in MCF10CA1d breast cancer cells (Fig. 1A). To determine associations between stromal CCL2 expression and breast cancer progression, MCF10CA1d breast cancer cells were orthotopically grafted alone, or cocultured with fibroblasts for 6 weeks. Compared
Figure 2.
Knockout of stromal CCL2 inhibits growth of primary MCF10CA1d breast tumor xenografts. A, CCL2 ELISA of conditioned medium from Parental (Par), control WT, or CCL2 knockout (CCL2KO#7 and CCL2KO#22) hCAF-1 cell lines. B and C, MCF10CA1d breast cancer cells were cografted with WT or CCL2KO fibroblasts for up to 6 weeks and measured for changes in tumor volume over time (B) or endpoint tumor mass (C). D–G, Breast tumor xenografts were immunostained for GFP (D), arginase I (E), Ly6G (F), or Von Willebrand Factor 8 (VWF; G). -GFP control is MCF10CA1d cografted with hCAF-1 parental fibroblasts. Expression was quantified by Image J. Statistical analysis was performed using one-way ANOVA with Bonferroni post hoc comparison. Statistical significance was determined by P < 0.05, ***, P < 0.005, ****, P < 0.0001; and n.s., not significant. Mean ± SEM are shown.
with MC10CA1d cells grafted alone, cotransplantation with hCAF-1 and hNAF-3 significantly enhanced primary tumor mass (Fig. 1B). MCF10CA1d breast tumors in all groups were characterized as invasive carcinomas with microvasculature (Fig. 1C).

Fibroblasts did not significantly affect lung metastasis as determined by analysis of H&E staining (Supplementary Fig. S2A and S2B). These data indicate that MCF10CA1d breast tumor growth is associated with fibroblast expression of CCL2.

Figure 3.
Knockout of CCR2 inhibits growth of primary MCF10CA1d breast tumor xenografts. A, Spearman correlation analysis of CCL2 and CCR2 expression in the METABRIC datasets (n = 2051). B, Flow cytometry analysis of CCR2 expression in Parental (Par), control wild-type (WT-A1), or CCR2 knockout (CCR2KO-F1 and CCR2KO-G10) MCF10CA1d breast cancer cells. C, WT or CCR2KO breast cancer cells were cografted with hCAF-1 fibroblasts for up to 6 weeks and analyzed for changes in tumor mass. D, Immunostaining for arginase I expression. Expression was quantified by Image J. Expression was normalized to hematoxylin and expressed as percentage per field. Statistical analysis was performed using one-way ANOVA with Bonferroni post hoc comparison. Statistical significance was determined by P < 0.05. **, P < 0.01; *** P < 0.001; and n.s., not significant. Mean ± SEM are shown.
Gene ablation of stromal CCL2 or epithelial CCR2 inhibits breast tumor growth

To determine the contribution of stromal CCL2 derived to MCF10CA1d breast tumor growth, exon 1 of CCL2 was targeted by CRISPR/R in hCA1-F. Two clones showed significantly decreased CCL2 expression (CCL2KO#7 and CCL2KO#22), compared with wild-type control cells (WT; Fig. 2A). MCF10CA1d cells were cotransfected with WT or CCL2-deficient fibroblast lines, and analyzed for tumor growth over time for up to 42 days (Fig. 2B). Up until day 35, CCL2 KO#7 tumors showed decreased tumor volume over time compared with WT tumors, indicating decreased tumor growth rate contributed to early growth delay. From days 35 to 42, CCL2KO#7 appeared to reach a similar growth rate to WT tumors. Throughout the study, the CCL2KO#22 tumor growth rate appeared distinctly lower than WT tumors. At 42 days, CCL2KO#7 and CCL2KO#22 tumors showed significantly decreased mass compared with WT tumors (Fig. 2C). CRISPR/R resulted in insertion of a GFP reporter, whose expression was detected in stromal tissues, indicating retention of transplanted fibroblasts (Fig. 2D). Furthermore, MCF10CA1d breast tumor xenografts with CCL2-deficient fibroblasts showed decreased expression of arginase I, an M2 macrophage marker (28), but not Ly6G, a neutrophil marker (29) or VWF8, an angiogenesis marker (ref. 30; Fig. 2E-G). Overall, these studies indicate that CCL2 derived from fibroblasts regulates MCF10CA1d tumor growth and M2 macrophage levels.

The METABRIC mRNA dataset (24, 25) revealed significant correlations between CCL2 and CCR2 expressions in invasive breast cancers (Fig. 3A). To determine the significance of epithelial CCR2 expression to fibroblast-mediated breast tumor growth, the exon encoding the third transmembrane domain of CCR2 was targeted by CRISPR/R. Frameshifts in the coding region generated a premature stop codon, resulting in mutants lacking a C-terminal region. PCR screening identified two mutant clones, CCR2KO-F1 and CCR2KO-G10. DNA sequencing revealed that the CCR2KO-F1 possessed an 81 bp deletion with 7 bp insertion in the coding region. CCR2KO-G10 appeared to show trans-heterozygous alleles, with one allele containing an 11 bp deletion, and the other allele containing a 263 bp deletion. To determine the overall levels of CCR2 expression in WT and knockout cells, which expressed the C-terminal–truncated mutants, we used an antibody that recognized the N-terminus of CCR2. CCR2KO-F1 cells showed a 10% reduction in CCR2 expression, and CCR2KO-G10 cells showed a 75% reduction in CCR2 expression compared with wild-type (WT-A1) control cells (Fig. 3B). Despite residual CCR2 expression, we hypothesized that CCR2 truncation would inhibit fibroblast-mediated tumor growth. CCR2KO-G10 and CCR2KO-F10 grafted alone showed decreased tumor growth compared with WT-A1 control cells; only the decreased growth of CCR2KO-G10 tumors was statistically significant. CCR2KO-F1 and CCR2KO-G10 cells cotransfected with fibroblasts showed a significant decrease in tumor growth, compared with WT-A1 cells cotransfected with fibroblasts (Fig. 3C). CCR2-deficient tumors showed decreased arginase I expression, indicating decreased M2 macrophage levels (Fig. 3D). In summary, CCR2 knockout in MCF10CA1d breast cancer cells inhibits fibroblast-mediated tumor growth.

We further examined the effects of CCR2 knockout on fibroblast-mediated progression of BLBC using the 4T1 model, which overexpress CCR2 (14, 31). Previous work has suggested a tumor-promoting role for mammary fibroblasts isolated from MMTV-PyVmyt transgenic mice (C57Bl6xFVB; refs. 15, 18). Compared with murine normal fibroblasts (mNAP) or 4T1 cells, carcinoma-associated fibroblasts (mCAF) expressed the highest levels of CCL2 (Supplementary Fig. S3A). Stable CCR2 shRNA in 4T1 cells resulted in approximately 30% knockdown in CCR2KD#1 cells and 20% knockdown in CCR2KD#3 cells, compared with control shRNA expressing cells (Supplementary Fig. S3B). CCR2 knockdown did not affect CCL2 expression in 4T1 cells (Supplementary Fig. S3C). CCR2KD#1 but not CCR2KD#3 cells cotransfected with mCAFs showed decreased tumor growth and arginase I expression, compared with co-culturing mCAFs with control 4T1 cells (Supplementary Fig. S3D–S3F). CCR2 knockdown did not significantly affect lung metastasis (Supplementary Fig. S3G). These data indicate that a 30% CCR2 knockdown but not at 20% is sufficient to inhibit 4T1 tumor growth. Overall, these data support a role for CCR2 expression in regulating growth of BLBC.

Effects of CCL2 and CCR2 expression on MCF10CA1d cell growth were analyzed in 3D Matrigel/Collagen cultures (Fig. 4A). CCR2KO-G10 breast cancer cells treated with huCAF-1 conditioned medium showed increased spheroid growth compared with conditioned medium from MCF10CA1d cells (Fig. 4B). CCL2 treatment enhanced spheroid growth (Fig. 4C). Conditioned medium from CCL2-deficient fibroblasts inhibited spheroid growth, indicating a CCL2-deficient spheroid growth.
MCF10CA1d spheroid growth, which was rescued with CCL2 treatment (Fig. 4D). CCR2 deficiency in MCF10CA1d cells inhibited fibroblast-mediated spheroid growth (Fig. 4E). These data indicate that CCL2/CCR2 signaling enhances growth of MCF10CA1d cells.

As stemness is an important factor in tumor growth (32, 33), we examined for mammosphere formation and activity of ALDH, a breast cancer stem cell marker (ref. 34, 2007 #921). CCL2 treatment did not affect mammosphere formation. CCR2 knockout significantly inhibited mammosphere formation and ALDH activity (Fig. 4E and F). These data indicate that CCR2 expression regulates stemness of MCF10CA1d cells.

CCL2/CCR2 signaling mediates BLBC cell growth through PKC and SRC pathways

To identify the downstream molecular mechanisms associated with CCL2/CCR2-mediated cell proliferation, we first examined expression of phosphorylated AKT, SMAD3, and ERK1/2 and PKC. These pathways were important in CCL2 signaling in normal cells and mediated survival and motility of prostate and mammary carcinoma cells (14, 35, 36). By immunoblot analysis, CCL2 treatment of MCF10CA1d cells increased phospho-PKC expression (Fig. 5A), but did not significantly affect SMAD3, ERK1/2, or AKT phosphorylation (Supplementary Fig. S4). For a broader, unbiased analysis of the molecular changes associated with CCR2 expression, CCR2 knockout (CCR2-G10), and wild-type control (WT-A1) cells was subject to RPPA analysis. CCR2 knockout cells showed significant differences in expression of cell-cycle and proliferation-related proteins, compared with control cells (Supplementary Fig. S5; Supplementary Table S1). Notably, CCR2-deficient cells showed decreased phospho-SRC expression and increased expression of the CDK inhibitor p27KIP1. CCL2 treatment of MCF10CA1d cells increased expression of phospho-SRC, increased expression of PCNA, and decreased expression of total p27KIP1. p21 CDK inhibitor expression was weak in BT-20 cells, and not affected with CCL2 treatment in the other cell lines (Fig. 5A). PCNA and p27KIP1 expressions in the cytoplasm and nucleus reflect different activity states.
Therefore, changes in expression and cellular localization of PCNA and p27KIP1 were analyzed by immunofluorescence staining. CCL2 significantly decreased nuclear expression of PCNA and p27KIP1 (Fig. 5B and C). These data support a mitogenic role for CCL2/CCR2 signaling in MCF10CA1d breast cancer cells. Consistent with RPPA data (Supplementary Table S1), CCL2 treatment of MCF10CA1d cells did not affect cell apoptosis or autophagy, as indicated by immunostaining for cleaved caspase-3 and LC3B (Supplementary Fig. S6).

The effects of CCL2 on BLBC cell growth were examined in BT-20 and HCC1937 cells, which expressed comparable levels of CCR2 (Supplementary Fig. S7). Like MCF10CA1d cells, CCL2 treatment of BT-20 and HCC1937 cells increased expression of phospho-SRC, phospho-PKC, and PCNA (Fig. 5A and B), and decreased p27KIP1 expression (Fig. 5B and C). To examine the effects of CCL2 on cell-cycle progression, breast cancer cells were synchronized through thymidine blocking, treated with CCL2, and analyzed by flow cytometry for propidium iodide staining. CCL2 enhanced the percentage of cells in G2–M phase after 2 hours for MCF10CA1d cells and 10 hours for BT-20 and HCC1937 cells (Fig. 6A–D). CCR2-deficient MCF10CA1d cells showed a higher percentage of cells in G1–S phase and decreased percentage of cells in G2–M compared with the wild-type CCR2 control (Fig. 6E). These data indicate that CCL2/CCR2 signaling promotes proliferation and cell-cycle progression in BLBC cells.

To determine the relevance of SRC and PKC to CCL2-induced cell growth, we treated breast cancer cells with PP2, a small-molecule inhibitor that targets SRC kinases (39), or with the pan-PKC inhibitor G0 6983 (40). MCF10CA1d cells were tested for responsiveness to PP2 at 10 and 20 μmol/L, and G0 6983 was tested at 5 and 10 μmol/L, based on previous studies showing 40% to 50% inhibition of biological activity at these concentrations.
concentrations (41–44). PP2 inhibited CCL2-induced phospho-SRC similarly at both concentrations (Fig. 7A). Gö 6983 increased phospho-PKC expression in untreated and CCL2-treated cells similarly at both concentrations (Fig. 7A). The increased phospho-PKC expression is consistent with studies showing that Gö 6983 conformational binding to PKC inhibits dephosphorylation, preventing degradation (45). PP2 or Gö 6983 treatment at lower concentrations inhibited CCL2-mediated expression of PCNA, enhanced expression p27KIP1 (Fig. 7B and C). These data indicate the CCL2-mediated breast cancer cell growth through PKC- and SRC-dependent mechanisms.

**Discussion**

We report that stromal-derived fibroblasts constitute a major source of CCL2 and regulate growth of BLBC by signaling directly to CCR2-expressing cancer cells. CCL2/CCR2-mediated tumor growth is regulated by PKC and SRC signaling pathways. As CCL2 and CCR2 are most highly expressed in BLBC, the proposed pathways may represent viable therapeutic targets for this subtype.

Breast fibroblasts increased MCF10CA1d breast cancer cell growth through CCL2-dependent mechanisms. CCL2 is not
Mediated through immune cells. Although the use of immunocompromised mice prevented analysis of T cells in breast tumors, aggressive breast cancers are associated with elevated expression of CCL2, which is important for tumor growth and metastasis. Studies from our laboratory support a relationship between CCR2-mediated tumor growth and immune cell activity. Targeting CCR2 expression in MMTV-PyVMT mice (FVB) inhibited tumor growth associated with decreased M2 macrophage polarization and increased cytotoxic CD8+ T-cell recruitment and activity (unpublished data). Because epithelial CCR2 expression was associated with M2 macrophages in the MCF10CA1d and 4T1 models, future studies could determine the contribution of tumoral CCR2 expression in T-cell recruitment activity using syngeneic transgenic or transplantation models of BLBC.

We reveal an important in vivo contribution for CCL2/CCR2 signaling to breast cancer cells. The CCL2/CCR2 pathway is a therapeutic target of interest due to its significant effects on tumor growth and metastasis, but translation to therapy may be complex. Ceasing treatment with CCL2 inhibitors resulted in regrowth of breast tumor xenografts in animal models (66). However, this rebound may be due to neutralizing antibodies themselves, rather than CCL2 as a target (20). These present studies indicate that targeting CCL2 or CCR2 expression inhibits tumor growth and could provide a therapeutic benefit when combined with other therapies.

Disclosure of Potential Conflicts of Interest
N. Alvarez is president and has an ownership interest (including stock, patents, etc.) in De Novo Genomics. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions
Conception and design: M. Yao, N. Cheng
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