Twist1 Induces CCL2 and Recruits Macrophages to Promote Angiogenesis

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Abstract
The transcription factor Twist1 induces epithelial–mesenchymal transition and extracellular matrix degradation to promote tumor metastasis. Although Twist1 also plays a role in embryonic vascular development and tumor angiogenesis, the molecular mechanisms that underlie these processes are not as well understood. Here, we report a novel function for Twist1 in modifying the tumor microenvironment to promote progression. We found that expression of Twist1 in human mammary epithelial cells potently promoted angiogenesis. Surprisingly, Twist1 expression did not increase the secretion of the common proangiogenic factors VEGF and basic fibroblast growth factor but rather induced expression of the macrophage chemoattractant CCL2. Attenuation of endogenous Twist1 in vivo blocked macrophage recruitment and angiogenesis, whereas exogenous CCL2 rescued the ability of tumor cells lacking Twist1 to attract macrophages and promote angiogenesis. Macrophage recruitment also was essential for the ability of Twist1-expressing cells to elicit a strong angiogenic response. Together, our findings show that how Twist1 recruits stromal macrophages through CCL2 induction to promote angiogenesis and tumor progression. As Twist1 expression has been associated with poor survival in many human cancers, this finding suggests that anti-CCL2 therapy may offer a rational strategy to treat Twist1-positive metastatic cancers. Cancer Res; 73(2): 662–71. ©2012 AACR.

Introduction
Increased expression of the Twist1 transcription factor has been correlated with metastasis and poor survival in many human cancers including breast cancer (1–3). To date, the prominent role of Twist1 in tumor progression has been to induce epithelial–mesenchymal transition (EMT) and extracellular matrix degradation (4–6). During EMT, Twist1 promotes stationary epithelial cells to lose cell–cell junctions and gain migratory and invasive capacities. Interestingly, Twist1 is also indicated in vascularization both during development and in tumor models. In Xenopus, Twist1 is required for proper embryonic vascular development (7). During human embryoid body formation, Twist1 is co-upregulated with several genes regulating vascular development (8). Under hypoxia, Twist1 expression in tumor cells is directly induced by hypoxia-inducible factor (HIF)-1α and -2α transcription factors, molecules known to regulate proangiogenic genes such as VEGF (9, 10). Finally, Twist1 expression promotes vascularization in tumor xenograft models (11, 12). Together, these observations suggest that Twist1 is involved in tumor angiogenesis.

The ability of tumor cells to promote angiogenesis, or blood vessel growth, is one of the critical contributing factors to metastatic spread. Tumor-associated neovascularization exhibits increased permeability due to poorly aligned endothelial cells with wide fenestrations and sparse pericyte coverage (13). Besides supporting tumor growth by increasing oxygen and nutrient influx, angiogenesis gives malignant tumor cells access into the systemic circulation for metastasis. Tumor angiogenesis may be modulated by the presence of myeloid cells such as macrophages and neutrophils, important agents within the tumor microenvironment (14–18). In particular, several lines of evidence support the role of macrophages in tumor angiogenesis and metastasis. In invasive human breast cancers, the presence of macrophages correlates with microvessel density, VEGF content, tumor grade, lymph node metastasis, and poor overall survival (19, 20). In the PyMT mammary tumor model, macrophage depletion delays the onset of angiogenesis, tumor formation, and metastasis, whereas addition of exogenous macrophages shortens tumor latency (18). In various tumor xenograft models, co-injection with macrophages increases VEGF abundance and neovascularization (21). Importantly, macrophages have been shown to contribute to later steps of the metastatic cascade such as extravasation and metastatic seeding (22–24). Together, these studies show the importance of tumor-associated macrophages in regulating tumor angiogenesis and promoting metastasis.
Currently, it is unknown whether Twist1 can modulate the tumor microenvironment to promote angiogenesis. In this study, we have used various direct and quantitative assays to uncover a novel function of Twist1 in recruiting macrophages to facilitate angiogenesis.

Materials and Methods

Cell lines and cell culture

Human mammary epithelial cells (HMLE), human breast cancer cell line SUM1315, and mouse mammary tumor cell lines 168FARN and 4T1 were obtained from Dr. Robert Weinberg and cultured as described (4). The MDA-MB-468 line was obtained from the American Type Culture Collection. All cell lines were reauthenticated by microsatellite profiling immediately before manuscript submission. Primary bone marrow-derived macrophages (BMDM) were harvested and differentiated as described (25).

DNA constructs

The Twist1, Twist1-ER, and shTwist1 vectors were previously described (4, 26). Control short hairpin RNA (shRNA) pl.KO.1 was obtained from the Sahatani lab (Addgene) and 2 Mission shRNAs against CCL2 in pl.KO.1 (Sigma) contain the following targeting sequences:

shCCL2.1: GCTCGGCAAGCTATAGAAGAAT
shCCL2.4: GATGTGAAACATTATGCCTTA

Chick chorioallantoic membrane angiogenesis assay

The shell-less chick chorioallantoic membrane (CAM) angiogenesis assay was conducted as previously described (27). Test cells were suspended in neutralized collagen and pipetted onto 2 layers of nylon mesh (Sefar America) at a final density of 75,000 cells per 30 mL. "onplant." Angiogenesis was scored in the onplants of the live embryos 72 to 96 hours later using a dissecting microscope.

ELISA and cytokine array

ELISAs for VEGF, basic fibroblast growth factor (bFGF; Peprotech), and CCL2 and the human cytokine arrays (Ray
tech) were conducted according to the manufacturers' instructions. All assay reagents were neutralized by microsatellite profiling immediately before manuscript submission. Primary bone marrow-derived macrophages (BMDMs) were harvested and differentiated as described (25).

Quantitative PCR

Total RNA was purified using the RNeasy Mini Kits with DNase treatment (Qiagen). About 2 μg of RNA was reverse-transcribed using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Resulting cDNAs were analyzed in triplicate using SYBR-Green Master PCR mix (Applied Biosystems). Relative mRNA levels were determined by 2^ -ΔΔCt, where Ct is the mean threshold cycle differences after normalizing to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) values. Primers used for quantitative PCR (qPCR) are as follows:

shCCL2: CAGCCAGATGCAATCAATGCC, GATGGTACATGACAAGGTGC
hCCL2: GAGAGACCCTCACTGCTG, TGGAAATCCTGAAACCACCTCT
hGAPDH: GAGAGACCCCTCAGTGCTG, GATGGTACATGACAAGGTGC
mCcl2: TTTAACACCTGATCGAACCMAA, GCATTAGCTTCAGTTACGGG
mGapdh: GACCCCTTCATGGACCTCAAC, CTCCATGGTGTGGTAAGA

Macrophage migration assay

On day 10 of differentiation, BMDMs were scraped and resuspended in RPMI-1640, 0.5% heat-inactivated FBS, and 20 ng/mL macrophage colony-stimulating factor (M-CSF; She
nandoah Biotechnology). Cell suspensions (200,000 cells/100 μL) were added to the upper chamber of 8-μm pore Transwell inserts (Corning) for 2 hours at 37°C to attach to the membrane. Transwells were then moved to 24-well plates containing 0.6-μL cell-conditioned media and incubated at 37°C for 2 to 4 hours. Cells were fixed in 4% paraformaldehyde and stained with 0.1% crystal violet. Cells in the upper chamber were removed with a cotton swab, and migrated cells in the lower chamber were quantified using 12 to 15 random fields. Two to three independent experiments were carried out for each assay.

Matrigel plug injection

All animal care and experiments were approved by the Institutional Animal Care and Use Committee of University of California, San Diego (San Diego, CA). Adult BALB/c mice were given 2 to 3 s.c. injections of 200 ng/mL of a 3:1 mixture of growth factor–reduced Matrigel (BD Biosciences) and 2 × 10^6 cells in Dulbecco’s Modified Eagle’s Media (DMEM). After 5 days, plugs were harvested, imaged, and fixed in 10% zinc formalin followed by paraffin sectioning. For macrophage depletion, 200 μL of liposome suspension was injected via tail vein 2 days before plug injections or 50 μL of liposome suspension was co-injected with the plug. C2MDP (or clodro
nate) was a gift of Roche Diagnostics GmbH. Clodronate liposomes and control liposomes containing PBS were prepared as described previously (29). Where indicated, Matrigel plugs were supplemented with recombinant hCCL2 (10 ng/mL, R&D).

Immunohistochemistry

Following antigen retrieval in citrate buffer and peroxidase blocking in H_2O_2, tissues were blocked with 2% goat serum and 2% bovine serum albumin in PBS. Serial sections were stained using rabbit anti-CD31 (1:50, Abcam) or rat anti-F4/80 (1:50, #CLEA13-1 Santa Cruz Biotechnology) antibodies, species-specific biotinylated secondary antibodies, followed by ABC reagent, developed with 3,3’-diaminobenzidine (DAB) substrate and counterstained using Hematoxylin QS (Vector Labs).

For blood vessel quantification, CD31 vessels were counted in 5 random fields (×20) in vascular hot spots and averaged for each section. A vessel was defined as a positively stained capillary, a branch from a multibranched structure, or a solitary branch. For macrophage quantification, 4 random fields (×20)
in F4/80-positive hot spots were scored on a scale of 0 to 6 for staining intensity and distribution within a field: 0, undetectable; 1, faint, discrete patches; 2 faint, all over; 3 medium, discrete patches; 4 medium, all over; 5 intense, discrete patches; 6 intense, all over.

Statistics
Statistical analysis was conducted using GraphPad Prism.

Results
Twist1 promotes angiogenesis in a CAM angiogenesis assay
To directly and quantitatively test whether Twist1 can promote angiogenesis, we stably expressed Twist1 in immortalized HMLEs (Fig. 1A; Supplementary Fig. S1) and examined their angiogenic potential using a quantitative CAM angiogenesis assay (27). For each cell type tested, cells were suspended in collagen that solidified over 2 nylon meshes to form an "onplant." Thirty-six onplants were evenly distributed onto the CAMs of approximately 6 chicken embryos at 10 days after fertilization, a time when the developing vasculature is able to sprout in response to additional angiogenic stimuli. Between 72 and 96 hours after grafting, nascent vessels had grown vertically into the onplants and were visible within the grids of the upper mesh (Fig. 1B). Frequently, nucleated red blood cells could be seen moving within the vessels, indicating that these vessels were functional. As shown in Figure 1C of one representative experiment, we quantified angiogenic index, defined as the percentage of grids that contained nascent vessels, in at least 25 onplants per condition and found that onplants containing HMLE-Twist1 cells presented a significantly higher angiogenic index than onplants with control HMLE cells where new vessels were nearly absent (0.43 vs. 0.10). Consistently, results from 11 independent experiments showed that HMLE-Twist1 cells exhibited a robust and repeatable average 2.9-fold increase in angiogenesis (Fig. 1D). These results show that expression of Twist1 in human mammary epithelial cells is sufficient to promote angiogenesis.

Twist1 is necessary and sufficient to induce CCL2 expression
To investigate the mechanism underlying Twist1-induced angiogenesis, we examined factors secreted from Twist1-expressing cells that could attract or stimulate proliferation of endothelial cells. Previous studies suggested that Twist1 could regulate VEGF mRNA or protein expression (11, 12). To explore how Twist1 promotes angiogenesis in our system, we compared the levels of 174 cytokines in conditioned media from HMLE cells using a cytokine array. Interestingly, HMLE-Twist1 cells did not significantly increase the expression of 2 major proangiogenic molecules bFGF and VEGF (Supplementary Fig. S2A). This result was further validated by quantitative measurement of secreted bFGF and VEGF in conditioned media by ELISA (Supplementary Fig. S2B and S2C).

Interestingly, the cytokine array detected increased levels of CCL2 (monocyte chemotactic protein-1 or MCP-1) in conditioned media from HMLE-Twist1 cells (Fig. 2A). CCL2 is a major chemoattractant for macrophages, cells that have been shown to have proangiogenic functions in physiologic and disease states (30, 31). On the basis of this knowledge, we focused our studies on examining the role of CCL2 in Twist1-induced angiogenesis. ELISA analysis confirmed that Twist1 expression increased secreted CCL2 from HMLE cells by 15.0-fold (Fig. 2B). Furthermore, qPCR analysis revealed that CCL2 mRNA in HMLE-Twist1 cells was 10.2-fold higher than in control cells (Fig. 2C). Using HMLE cells that express an inducible Twist1-ER construct, we found that CCL2 mRNA increased by 5.2-fold within 1.5 hours of Twist1 activation and continued to increase up to 4 days later (Fig. 2D), which is consistent with a previous study showing that Twist1 directly regulates CCL2 in white adipose tissue (32). It is worth noting...
that the immediate induction of \( \text{CCL2} \) in response to Twist1-ER activation is higher than the CCL2 level in HMLE cells stably expressing Twist1. We found that this difference is largely due to reduced Twist1 transgene expression upon long-term culture of HMLE-Twist1 cells (data not shown). Importantly, direct induction of \( \text{CCL2} \) transcription was specific to Twist1, as another EMT-inducing transcription factor Snail only induced CCL2 by 1.6-fold 4 days after activation (Supplementary Fig. S2G). To extend our studies to breast tumor cells, we expressed Twist1 in MDA-468 breast cancer cells (Supplementary Fig. S2D). Consistent with results from HMLE cells, Twist1 expression increased secreted CCL2 protein by 5.1-fold and \( \text{CCL2} \) mRNA by 2.9-fold in MDA-468 cells (Supplementary Fig. S2E and S2F). Collectively, these results suggest that Twist1 expression is sufficient to induce CCL2 in normal and cancer mammary epithelial cells.

To determine whether Twist1 is important for CCL2 expression in breast tumor cells, we knocked down the high level of endogenous Twist1 in multiple cell lines. In 168FARN mouse mammary tumor cells, we used 2 independent shRNA constructs against Twist1 (Fig. 2E; Supplementary Fig. S2D; ref. 4) and found that knocking down Twist1 reduced the level of secreted CCL2 protein by approximately 65% as measured by ELISA (Fig. 2F). Similarly, using qPCR, we found that \( \text{Ccl2} \) mRNA was reduced by approximately 80% upon knocking down Twist1 in 168FARN cells (Fig. 2G), further indicating an essential role of Twist1 in CCL2 induction. Using the more aggressive 4T1 mouse mammary tumor cell line and the SUM1315 human mammary cancer cell line, which both also express high levels of endogenous Twist1 (4, 5), knockdown of Twist1 also reduced \( \text{CCL2} \) mRNA (Supplemental Fig. S2H and S2I). Together, results from these complementary sets of...
overexpression and knockdown experiments show that Twist1 is both necessary and sufficient to induce CCL2 expression in mammary epithelial cells.

CCL2 mediates Twist1-induced angiogenesis in the CAM assay

To test whether induction of CCL2 is important to mediate Twist1-induced angiogenesis, we stably knocked down CCL2 in HMLE-Twist1 cells and tested the effect on angiogenesis using the CAM assay. Two independent shRNA constructs efficiently repressed CCL2 mRNA and protein levels in HMLE-Twist1 cells, close to the baseline levels of control HMLE cells (Fig. 3A and B). Knocking down CCL2 did not affect Twist1 expression (Fig. 3C). Likewise, knockdown of CCL2 did not alter the EMT phenotype induced by Twist1, as expression of the epithelial marker E-cadherin and the mesenchymal marker fibronectin were unchanged and the cells remained elongated and scattered, similar to parental HMLE-Twist1 cells and HMLE-Twist1 cells expressing a control shRNA (Fig. 3C, Supplementary Fig. S3A and S3B). Importantly, in the CAM assay, we found that knocking down CCL2 in HMLE-Twist1 cells reduced the angiogenic index by approximately 50% from 0.35 to 0.16 and 0.18, respectively (Fig. 3D). These results indicate that Twist1 induces CCL2 to promote angiogenesis, and this function of Twist1 in angiogenesis is independent of its ability to induce EMT.

Twist1 expression in mammary epithelial cells promotes macrophage attraction in a CCL2-dependent manner

We next set out to determine how CCL2 mediates Twist1-induced angiogenesis. Because CCL2 is a chemoattractant for macrophages (30) and macrophages have been shown to promote angiogenesis (31), we asked whether induction of CCL2 by Twist1 promotes macrophage attraction in culture. As the literature about the function of avian cytokines and macrophage regulation is limited (33, 34), we used mouse models to address this question. We isolated bone marrow from mice and differentiated the monocytes into macrophages in vitro. Flow cytometric analysis revealed strong surface expression of the CCL2 receptor CCR2 in these BMDMs (Fig. 4A). We seeded isolated BMDMs on the top of a Boyden chamber to determine their ability to migrate toward various conditioned media. As shown in Fig. 4B, significantly more macrophages were attracted to the conditioned media from HMLE-Twist1 cells than HMLE control cells. Alternatively, when we knocked down Twist1 in 168FARN cells, BMDM migration was significantly reduced compared with 168FARN control knockdown conditioned media (Fig. 4C). Importantly, we were able to rescue this migration defect by supplementing conditioned media from 168FARN-shTwist1 cells with recombinant CCL2 (rCCL2; Fig. 4D). Finally, macrophage migration was significantly reduced toward conditioned media from HMLE-Twist1 cells expressing CCL2 shRNAs.
when compared with HMLE-Twist1 cells expressing a control shRNA (Fig. 4E). Collectively, these results show that induction of CCL2 is important for the ability of Twist1 to promote macrophage attraction.

**Figure 4.** Twist1 expression in mammary epithelial cells promotes macrophage attraction in a CCL2-dependent manner. A, cell surface expression of CCR2 on isolated mouse BMDMs as measured by flow cytometric analysis using anti-CCR2 (open) versus control IgG (shaded) antibody staining. B, BMDMs were subjected to Boyden chamber migration assays toward conditioned media from HMLE-Ctrl or HMLE-Twist1 cells. Representative images of migrated macrophages stained with crystal violet (top) and quantification of relative migration (bottom) for one representative experiment. *, *P < 0.05 by Student t test. C, macrophage migration toward conditioned media from 168FARN cells with indicated shRNAs for one representative experiment. *, *P < 0.05 versus shCtrl by ANOVA followed by Tukey Test. D, macrophage migration toward conditioned media from 168FARN cells with shRNAs against Twist1, co-incubated with either PBS or rCCL2 (10 ng/mL) for one representative experiment. *, *P < 0.05 by Student t test. E, macrophage migration toward conditioned media from HMLE-Twist1 cells expressing indicated shRNAs for one representative experiment. *, *P < 0.05 versus shCtrl by ANOVA followed by Tukey Test.  

**Twist1 expression promotes angiogenesis and macrophage infiltration in the Matrigel plug assay**

We next aimed to determine whether Twist1 expression in mammary tumor cells leads to macrophage recruitment...
in vivo. To do this, we conducted Matrigel plug assays in mice using 168FARN cells in which CCL2 expression depends on endogenous Twist1 (Fig. 2E–G). We harvested the plugs 5 days after implantation for analysis. Macroscopic examination revealed that Matrigel plugs containing 168FARN cells expressing a control shRNA appeared to contain more blood than plugs with 168FARN cells expressing Twist1 shRNAs (Fig. 5A, top row). Using CD31 as a marker for endothelial cells, we found that knocking down Twist1 significantly reduced the number of blood vessels in Matrigel plug tissue sections by approximately 50%, further supporting our conclusion that Twist1 promotes angiogenesis in vivo (Fig. 5A, middle row, Fig. 5B). Importantly, we measured macrophage content in serial sections by staining for the mature macrophage marker F4/80 and found that Twist1 knockdown also significantly decreased macrophage content by approximately 50% (Fig. 5A, bottom row, Fig. 5C). We conclude that Twist1 expression in tumor cells is required for macrophage recruitment and angiogenesis in vivo.

CCL2 and macrophage recruitment are essential for Twist1-induced angiogenesis

We next set out to understand whether macrophages and CCL2 are important for Twist1 to promote angiogenesis. First, we depleted macrophages using clodronate liposomes in the Matrigel plug assay. In vivo, macrophages recognize liposomes as foreign bodies. Upon phagocytosis and release of the encapsulated clodronate, macrophages undergo apoptosis and are not replenished during the experimental period (29). Indeed, clodronate liposome injection compared with control liposome injection, effectively reduced the presence of F4/80-positive macrophages to nearly undetectable levels in Matrigel plugs containing 168FARN cells (Fig. 6A, bottom row, and C). As expected, control liposome injection did not affect the strong angiogenic response induced by 168FARN cells as observed both macroscopically and as measured by CD31 staining (Fig. 6A, top and middle rows, Fig. 6B). In contrast, macrophage depletion by clodronate liposome treatment significantly reduced Twist1-induced angiogenesis by 74.4% (Fig. 6A, top and middle rows, Fig. 6B). These data indicate that macrophage recruitment is essential for the ability of Twist1 to promote angiogenesis.

Second, we asked whether addition of exogenous CCL2 could rescue the ability of 168FARN tumor cells with Twist1 knockdown to attract macrophages and promote angiogenesis. To test this, we added rCCL2 to Matrigel plugs with 168FARN cells expressing 2 independent Twist1 shRNA constructs. Significantly, addition of rCCL2 increased macrophage influx by approximately 1.9- and 2.3-fold compared with PBS-added Matrigel plugs (Fig. 6D, bottom row, and F). Importantly, rCCL2 addition increased the number of CD31-positive vessels by 1.9-fold in both groups compared with Matrigel plugs supplemented with PBS (Fig. 6D, top and middle rows, and E). Together, results from macrophage depletion and CCL2 rescue experiments indicate that CCL2-dependent macrophage recruitment is essential for the ability of Twist1 to promote angiogenesis in vivo.

Discussion

This study supports and expands upon the observations that Twist1 facilitates multiple important steps in metastasis including angiogenesis. Importantly, we have shown the ability of Twist1-expressing cells to promote angiogenesis via a non–cell-autonomous mechanism involving
We show that Twist1 induces robust CCL2 expression of CCL2 and knockdown of CCL2 leads to a decrease in Twist1-induced angiogenesis in a quantitative CAM angiogenesis assay. We show that isolated macrophages, which express high levels of CCR2, can migrate toward conditioned medium from Twist1-expressing cells in a CCL2-dependent manner. Using mouse Matrigel plug assays, we show that expression of Twist1 promotes both angiogenesis and macrophage infiltration, and both CCL2 and macrophage influx are important for Twist1-induced neovascularization. On the basis of these results, we propose a model in which upregulation of Twist1 in carcinoma cells activates transcription of CCL2 mRNA; secretion of CCL2 protein from Twist1-expressing cells creates a chemotactic gradient to promote macrophage infiltration and subsequent angiogenic stimulation (Fig. 7). To date, the predominant role of Twist1 in tumor progression is thought to be cell autonomous through the induction of EMT (4). Our current study uncovers a novel function of Twist1 to generate tumor–stromal cell communication by recruiting macrophages, a cell type previously shown to facilitate angiogenesis and metastasis in a mammary tumor model (18). Interestingly, ovarian tumor cells that have undergone EMT are shown to promote monocyte differentiation to a proangiogenic phenotype (35). Importantly, we show that the ability of Twist1 to promote angiogenesis is independent of its role

![Diagram](image.png)

Figure 6. CCL2 and macrophage recruitment are essential for Twist1-induced angiogenesis. A, a mixture of Matrigel and 168FARN cells was subcutaneously injected in mice either previously depleted of macrophages by clodronate-encapsulated liposomes (9 mice) or treated with control liposomes (6 mice). Resulting plugs were harvested 5 days later and processed for immunohistochemical staining. Top row, macroscopic images of plugs; middle row, images of tissue sections stained for blood vessel marker CD31(brown); bottom row, images of tissue sections stained for mature macrophage marker F4/80 (brown). Nuclei were stained with hematoxylin (blue). Scale bar, 2 mm. B and C, quantification of stains in A; 5 random high-powered fields per section were counted for number of CD31-stained vessels or were scored for F4/80 staining intensity and distribution; 10 to 15 plugs/group and 1 to 2 sections/plug were analyzed. Scale bar, 50 μm. Error bars are SEM. *P < 0.05 by Student t test. D, Matrigel plug assays were conducted using 168FARN mouse mammary tumor cells and supplemented with recombinant CCL2 (10 ng/mL final concentration) or PBS using at least 3 mice/group. Scale bar, 2 mm. E and F, quantification of stains in D, with 6 to 8 plugs/group and 1 to 2 sections/plug analyzed. Scale bar, 50 μm. Error bars are SEM. *P < 0.05 by Student t test.
in EMT, as CCL2 suppression specifically hindered the angiogenic response without affecting the Twist1-induced EMT phenotype.

We show that knocking down Twist1 in 168FARN tumor cells caused 70% reduction of CCL2. Such CCL2 reduction resulted in more than 50% suppression of macrophage infiltration in vivo but less than 30% reduction in macrophage migration in vitro. One explanation for this difference is that mouse macrophages are highly sensitive to mouse CCL2 (36), and the conditioned media used for the in vitro macrophage migration assay contain CCL2 at concentrations well above the level required for macrophage sensitivity. Another possibility is that Twist1 regulates other molecules to attract additional stromal cell types to indirectly recruit macrophages, which could also explain why addition of CCL2 alone did not completely rescue Twist1-dependent angiogenesis and macrophage recruitment.

This study focuses mainly on the role of Twist1-induced CCL2 in angiogenesis. Angiogenesis gives invading tumor cells access to blood circulation for systemic dissemination; therefore, this function of Twist1 likely aids its role in metastasis. Because macrophages have been shown to contribute to many steps of tumor progression, Twist1-induced CCL2 production and macrophage recruitment likely play critical roles in additional steps of tumor development, which merits future studies. First, macrophages are known to secrete proteases such as matrix metalloproteinase, which can degrade extracellular matrix and release matrix-bound growth factors (37). Functionally, macrophage-mediated matrix degradation could allow endothelial cells to invade tumor tissues during angiogenesis (38) and also could facilitate tumor cell invasion. Of note, a previous study showed that co-culture of tumor cells and macrophages promoted collagen degradation ability, highlighting a symbiotic relationship between them (39). Twist1, in addition to inducing EMT and invadopodia formation (4–6), may engage macrophages to promote tumor invasion.

Second, several new studies emphasize important roles that CCL2 and macrophages play not only at the primary tumor site but also at the site of metastasis. The Pollard group found that the onset of metastasis was delayed when mice lacking macrophages were crossed to the PyMT mammary tumor model (40). Interestingly, they found that metastatic tumor cells secrete CCL2 to engage macrophages, thus increasing endothelial permeability and facilitating extravasation and metastatic seeding (22, 23). In parallel, another study found that tumor cell-derived CCL2 engaged 2 CCR2-positive cell types derived from the monocyte lineage to facilitate metastasis: macrophages for lung metastasis and osteoclasts for osteolytic bone metastasis (24). Our study identified Twist1 as a potent inducer of CCL2 in metastatic tumor cells. Potentially, induction of CCL2 by Twist1 could likewise function to attract macrophages to promote extravasation and metastatic seeding in distant organs.

In summary, we show that Twist1 has important non-cell-autonomous functions to aid in metastasis. Twist1 expression in tumor cells allows interactions with macrophages through upregulation of CCL2. As macrophages have important roles in many steps of tumor progression, our study has extended the depth at which Twist1 may impact the metastatic cascade and suggests the rationale for anti-CCL2 therapy in treating Twist1-positive metastatic tumors.

Disclosure of Potential Conflict of Interest
No potential conflicts of interest were disclosed.

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