Mesenchymal CD44 Expression Contributes to the Acquisition of an Activated Fibroblast Phenotype via TWIST Activation in the Tumor Microenvironment

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Abstract
Tumor–stroma interactions play a crucial role in cancer progression by eliciting factors that promote proliferative, angiogenic, and invasive supports to the tumor microenvironment. Mesenchymal stromal/stem cells (MSC) contribute to stroma in part as cancer-associated fibroblasts (CAF), but a complete understanding of how MSC contribute to the tumor stroma is lacking. In this study, we show how CAF phenotypes rely upon MSC expression of the multifunctional cell surface glycoprotein CD44, a putative stem cell marker. Through bone marrow transplantation experiments in a transgenic mouse model of cancer, we determined that CD44 deficiency leads to a relative reduction in the contribution of bone marrow–derived cells to tumor stroma. CD44 attenuation in MSC limited their expression of CAF markers induced by tumor conditioning, and these MSC migrated poorly and provided weak angiogenic support compared with wild-type MSC. These defects were linked to deficiencies in the ability of CD44-attenuated MSC to transcriptionally upregulate Twist expression. Together, our results establish that CD44 expression contributes to critical functions in the tumor stroma. Cancer Res; 73(17); 5347–59. ©2013 AACR.

Introduction
Mesenchymal stem/stromal cells (MSC) are a population of multipotent stem cells that exist in most tissues of the body. Under circumstances of growth or repair, these resident progenitor cells repopulate the tissue and provide structural support and paracrine stimulation. They are known to migrate toward inflamed/injured environments, including tumors (1–3) and can be induced to express markers associated with tumor/cancer-associated fibroblasts (TAF/CAF) and myofibroblasts (4–6).

Stromal cells of mesenchymal phenotype fill the interstitial space of tumors and provide growth factors, matrix remodeling factors, and other tumoriganic supportive factors (7, 8). They are often referred to as activated (myo)fibroblasts or TAF and have been shown to originate from multiple sources including local tissue–derived fibroblasts, local tissue–derived MSC (9), bone marrow–derived MSC (4), and cancer cells through the epithelial-to-mesenchymal transition (EMT; refs. 10, 11). Regardless of their origin, we and others have shown the protumorigenic (4, 12, 13) and prometastatic (14) effects of these stromal cells within the tumor microenvironment. Furthermore, the presence of stromal cells is not restricted to a specific tumor type as TAF, myofibroblasts, MSC, and fibroblasts have been isolated from several different tumor types, including breast (15), pancreatic (16), gastric (17), and lung (18).

The TAF population is a heterogeneous population of cells intertwined throughout the tumor parenchyma. We now divide this population into at least two subpopulations: one that is defined by a pericytic role, termed an activated myofibroblast, and defined by the expression of α-smooth muscle actin (α-SMA) and the chondroitin sulfate neural glial proteoglycan (NG2); the other that is defined by a fibroblastic role, termed an activated fibroblast, and defined by the expression of the seprase, fibroblast activation protein (FAP), and the calcium-binding protein, fibroblast-specific protein (FSP; ref. 5).

Although our TAF population is loosely defined by a selection of markers, all the stromal cells express CD44. This multifunctional glycoprotein is expressed on many cell types from progenitor populations to lineage-specific cells and commonly regulates cell–cell adhesion and migration. Its most noted ligands include hyaluronan and osteopontin (19–21). Both ligands have been implicated as prognostic markers of cancer progression and metastases in breast, prostate, lung, and ovarian cancers (22–26).

In this study, we examined the deficiencies of tumor stromal mesenchyme lacking CD44 using murine tumor models. We...
found that CD44<sup>−/−</sup> MSC form stroma-deficient tumor microenvironments: first, because of their inability to fully differentiate into a TAF subpopulation, the activated fibroblast, as identified by the expression of FAP and FSP; second, because of impaired migratory capacity; and third, because of their diminished proangiogenic supportive capabilities.

**Materials and Methods**

**Cell culture**

Primary human bone marrow MSC, wild-type (WT) murine MSC, and transgenic CD44<sup>−/−</sup> murine MSC (The Jackson Laboratory) were isolated as previously described (3) and grown in α-minimum essential medium (α-MEM) with 20% FBS and 10% penicillin/streptomycin/gentamicin (Gibco/Invitrogen). All primary MSC were used between passages 1 and 6. Expression of cell surface markers CD105, CD90, CD44, CD73, CD45, and CD34 were validated in human MSC by flow cytometry. MSC were also subjected to colony formation assays and differentiation assays (Supplementary Figs. S3 and S4), including assays of adipocyte, osteoblast, and chondrocyte differentiation as previously described (3). Tumor cell lines were purchased from American Type Culture Collection (ATCC) and maintained in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% FBS and 10% penicillin/streptomycin/gentamicin.

**Antibodies**

Antibodies to CD31, α-SMA, FAP, CD44, platelet-derived growth factor receptor-β PDGFR-β, vimentin, GFP (Abcam Inc.), β-gal (Novus Biologicals), red fluorescent protein (RFP; Rockland), FSP (Dako), NG2 (Millipore), osteopontin (R&D Systems), and secondary Alexa Fluor antibodies 464, 594, and 488 (Invitrogen) were purchased and used according to the manufacturer’s instructions. Chromatin immunoprecipitation

**Immunohistochemistry/immunofluorescence**

Tumor sections were deparaffinized in a series of xylene and ethanol gradient incubations before being subjected to boiling sodium citrate buffer. Sections were blocked in 3% bovine serum albumin, 2% FBS for 2 hours before being stained with primary antibody (consecutively for multiple primaries) for 2 hours at room temperature or overnight at 4°C and secondary antibody for 2 hours at room temperature. Stained slides were analyzed using a Nucen camera attachment and InForm (PerkinElmer). All images were taken and analyzed with a single acquisition algorithm using the Nuance camera attachment and InForm software (PerkinElmer). Fluorescent intensity was calculated per cell based on nuclear DAPI stain.

**Fluorescence-activated cell sorting**

Cells were subjected to trypsinization, washed, filtered, and resuspended in ice-cold PBS supplemented with 2% FBS. Cells were then sorted into GFP<sup>+</sup> and GFP<sup>−</sup> subpopulations (BD FACSAria).

**Western blotting**

Cells were lysed in protein lysis buffer containing 50 mmol/L HEPES (238.3 g/mol), 300 mmol/L NaCl (58.4 g/mol), 2 mmol/L EDTA (372.24 g/mol), 50 mmol/L NaF (41.99 g/mol), 2 mmol/L sodium orthovanadate (183.91 g/mol), 10% (v/v) glycerol, 2% (v/v) NP-40, 1% (v/v) Triton X-100, and a cocktail of protease and phosphatase inhibitors (Sigma). After 1 to 2 hours on ice, lysates were spun down for 30 minutes at 13,000 rpm, lysate supernatant was quantified by Bradford assay (Bio-Rad), and then lysate was boiled with loading buffer and loaded onto a 12% SDS-PAGE gel (Bio-Rad). After electrophoresis, proteins were transferred onto Hybond-P membranes (Amersham Pharmacia Biotech), or nitrocellulose odyssey membranes (LI-COR Biosciences), which were subjected to immunoblotting. Signals were detected using ECL-Plus (Bio-Rad). Alternatively, if a fluorophore-conjugated secondary antibody was used, the immunoblot signals were detected on the Odyssey Infrared Imaging System (LI-COR Biosciences).

**Chromatin immunoprecipitation**

CD44 (Abcam), Stat3, and p300 (Cell Signaling Technology) antibodies were used in the chromatin immunoprecipitation (ChIP) assay conducted using the EZ-ChIP Kit (Millipore) according to the manufacturer’s instructions. Briefly, MSC were first cross-linked with 1% formaldehyde for 10 minutes. The reaction was stopped by adding 2.5 mol/L glycine at a 1:20 volume. Cell lysis and sonication were followed by overnight antibody incubations at 4°C. Finally, cross-linking was reversed by a 3-hour incubation at 65°C and purified DNA analyzed by quantitative PCR. Twist1 proximal promoter forward: 5′-CGGGGGAGGAGGACTGGAACGC-3′; Twist1 reverse: 5′-AGGCCCTCTGGAAACGTCGG-3′; Twist1 distal promoter forward: 5′-TACCCAGGCTGGTCACAAACACT-3′; Twist1 distal promoter reverse: 5′-AACGAAAGCCCCAAAGGTTG-3′ (27).

**Viral transduction**

**Adenoviral vectors.** Soluble CD44 (s44) adenoviral vector was (28) amplified in 293T cells and concentrated on a cesium chloride gradient.

**Lentiviral vectors.** Five GIPZ short hairpin RNA (shRNA) lentiviral constructs against CD44 (clone ID: 1-V2LHS_111680; 2-V2LHS_111682; 3-V2LHS_111684; 4-V3LHS_334833; 5-V3LHS_334830) and one negative control shRNA (RHS4346; Open Biosystems) were transfected into HEK-293T cells with pPax and pMD2 plasmids using JetPrime Transfection reagent or 50 mmol/L BES solution. Supernatants were collected and filtered after 72 hours for a total of 40 mL. Virus was concentrated in 8.5% polyethylene glycol (8.4 mL PEG from 50% stock solution) and 0.3 mol/L sodium chloride (3.75 mL NaCl from 4 mol/L stock solution) and balanced with PBS to equal volume. The solution was placed on a shaker at 4°C for 90 minutes and then centrifuged at 4,000 rpm at 4°C for 45 minutes. The resulting pellet was resuspended in 300 μL PBS,
snap-frozen, and stored at −80°C. For transduction, 20 μL of each concentrate was combined with 5 μL of 5 mg/mL poly-b- ene stock in 10 μL of fresh 20% serum medium. The medium was changed 24 hours after transduction. Cells were sorted by FACS for GFP positivity. fireRed/cGFP coexpressing vector was made by subcloning the fireRed from pGL4.51[fireRed/CMV/Neo] Vector (Promega) into pCDH-CMV-MCESEF1-copGFP_CDS11B-1 (System Biosciences).

Migration assay
MSC migration was assayed by using 8-μm pore, 6.5-mm Transwell inserts in a 24-well plate. MSC serum-starved for 18 hours were plated at 4 × 10^4 cells per well, with tumor-conditioned medium (TCM), serum medium, or serum-free medium for 24 hours. Transwell inserts were analyzed by manual cell counts from captured images (Olympus BX41 microscope with an Olympus DP70 camera attachment) and by quantitative counts obtained by solubilizing stained Transwell membranes in 300 μL 2% deoxycholic acid for 4 hours, then measuring optical density at a fixed wavelength of 595 nm for each sample (Beckman Du Maxima Instruments). The following migratory inhibitors used were: (i) matrix metalloproteinase (MMP) inhibitor V (Calbiochem; #444285), this MMP inhibitor is selective for MMP2 at <20 nmol/L, MMP9 at <200 nmol/L, and MMP14 at <1 μmol/L. We used multiple concentrations because our target inhibition was of MMP14, however, migration is also inhibited when MMP2 and MMP9 are inhibited at concentrations lower than 1 μmol/L; (ii) hyaluronan oligomer (oHA; ref. 29); (iii) s44, an adenoviral vector expressing s44, was transfigured into MSC or into Skov-3 tumor cells. Secreted s44 was present in the medium 48 to 72 hours posttransfection and was used for subsequent experiments.

Tube formation assay
Human umbilical vein endothelial cells (HUVEC; ATCC) were cultured in EGM2 (Clonetics) and plated (3 × 10^5/well) into a 24-well plate in triplicate. Medium was replaced 12 hours later with MSC-conditioned medium and the cells were imaged every 12 hours for 12 days. Wells were imaged and analyzed with an Olympus 1×81 microscope with a DSU confocal attachment and SlideBook software. Four images were taken per well. Tubes were manually counted and averaged among groups.

PCR
Maxima Hot Start 10× buffer, 25 mmol/L MgCl_2, 25 mmol/L deoxynucleotide triphosphate (dNTP), and 5 U Taq, was purchased from Fermentas Life Sciences. Primers purchased from Integrated DNA Technologies were prepared as 10 μmol/L stock solutions and are listed in Supplementary Table S1. Real-time PCR. RNA (1 μg) was reverse transcribed using a kit from Signalway Biotechnology. cDNA (20 μL) was diluted with 100 μL of distilled RNase/DNase-free water. SYBR-Green Master Mix was purchased from Applied Biosystems. 1.0 μL cDNA was used per reaction. Of note, 0.5 μL forward and reverse primer stocks, 10 μL distilled water, and 12 μL SYBR-Green Master Mix was added per well for a total volume of 25 μL. All reactions were carried out in triplicate and carried out in a 96-well fast plate in an ABI7900HT Thermocycler (Applied Biosystems). Relative expression was calculated by normalizing samples to glyceraldehyde-3-phosphate dehydrogenase (GAPDH; human) or 18s (murine) and then compared between control and sample sets using the ΔΔCt method. All data are presented as fold change over control (2−ΔΔCt).

In vivo murine tumor models
All transgenic mice used were female between the ages of 5 to 12 weeks. All mice weighed an average of 25 g. Human xenograft model. Nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice (The Jackson Laboratory strain NOD.CB17-Prkdcscid/J; stock #001303) were housed and treated according to protocols approved by MD Anderson Cancer Center (Houston, TX) Institutional Animal Care and Use Committee (IACUC). Mice (n = 20) were anesthetized and were injected subcutaneously with 5 × 10^5 Renilla luciferase (rLuc)–labeled Skov-3 cells suspended in 100 μL of PBS into both hind limbs. Fifty-six days after injection, bioluminescent imaging (BLI; IVIS-Xenogen 100 system; Caliper Lifesciences) confirmed tumor engraftment. Then, each mouse received fireRed-labeled MSC (1 × 10^6) in 100 μL PBS by tail vein injection. The mice were divided into four equal groups of 5 mice each: two groups received an intratumoral injection of the s44-expressing adenovirus (Ad-s44) in the right hind limb 48 hours before intravenous administration of MSC. BLI was conducted daily for 5 consecutive days after MSC administration. The fireRed substrate, n-luciferin (100 μL of 4 mg/mL in PBS; Biosynth International Inc.) or rLuc substrate coelenterazine (100 μL of 200 ng/mL in PBS; Biotium, Inc.) was injected intraperitoneally 5 minutes before imaging. Images were analyzed with the Living Image Software (IVIS-Xenogen).

Murine models. WT and transgenic CD44-knockout murine MSC were admixed with 1 × 10^6 4T1 tumor cells and injected into the mammary fat pad of NOD/SCID mice (n = 20). Three weeks following injection, mice were sacrificed and tumors and organs removed for immunohistochemical analysis. These tumors were weighed to compare the growth differences between the tumors in each group, however, upon analysis, the weights were not taken into account because we acquired, at random, the same number of images per tumor, which were then used for comparative, quantitative analysis.

EO771 murine mammary carcinoma cells (2 × 10^5) were injected into the mammary fat pad of C57/B6 CD44 transgenic mice (The Jackson Laboratory strain B6.129(Cg)-Cd44tm1Hbg/J; stock #005085) and control WT C57/B6 mice (The Jackson Laboratory strain C57BL/6j; stock #006664; n = 20). Tumor growth was followed for 4 weeks with daily caliper measurements and mice were sacrificed just before reaching excessive tumor burden, in compliance with IACUC guidelines. Excised tumors were weighed and suspended in formalin in preparation for immunohistochemical analysis.
It is important to note that we were unable to observe lung metastases in any of the tumor model groups. 

**Bone marrow transplant model.** The tumors from the RFP\(^+\) bone marrow transplant (BMT) mice (n = 15) were analyzed for coexpression of RFP (to label the BMT cells) or GFP (to label the host cells) and the TAF markers FAP, FSP, and \(\alpha\)-SMA, and the endothelial marker CD31. Likewise, tumors from the CD44-knockout BMT mice (n = 15) were analyzed for coexpression of LacZ (to label the BMT cells) or GFP (to label the host cells) and FAP, FSP, \(\alpha\)-SMA, and CD31. Tumor size, by weight was not significantly different between analyzed samples. Coexpression was characterized by InForm software to identify immunostaining colocalization. Briefly, each cell was identified on the basis of nuclear DAPI stain (average of 2,000 cells/image). The fluorescence intensity of each fluorescent label per cell was then enumerated. The fluorescence intensities were plotted (488 label on the Y-axis and 594 on the X-axis), and costained cells were quantified.

Percentages displayed in Fig. 6A–C of GFP\(^+\) or RFP\(^+\) or \(\beta\)-gal costained cells in tumors from BMT mice were calculated averages of costained (double-positive staining) cells per total number of cells per tumor image among 5 mice (10 images/tumor). Statistical significance was determined by the one-tailed Student \(t\) test with 90% confidence interval (CI).

**Data analysis**

Analysis of the BLI was conducted by creating standard regions of interest around the right and left hind legs to calculate the flux (photons/s) of the target signal in the given area. Relative quantities of MSC and tumor sizes were then calculated by normalizing the MSC flux against the tumor flux to achieve comparable numbers of migrated MSC toward each tumor individually. Statistical significance was determined by the Student \(t\) test.

**Results**

**CD44\(^{-/-}\) mice show delay in tumor progression**

We engrafted EO771 murine breast carcinoma cells (2 \(\times\) 10\(^5\)) into the mammary fat pads of transgenic CD44-knockout (CD44\(^{-/-}\)), LacZ-expressing mice, and WT, RFP-expressing mice. Despite 100% tumor engraftment in both groups, the CD44\(^{-/-}\) mice survived significantly longer (\(P < 0.05\); log-rank test) than the WT mice (Fig. 1A). Because mice were sacrificed according to institutional/IACUC standards, mice were euthanized when tumor burden became too great, therefore our survival curve suggests that CD44\(^{-/-}\) mice bearing tumors develop slowly than tumors in WT mice. Immunofluorescent staining for tumor stromal markers FAP, FSP, and \(\alpha\)-SMA revealed that tumors from WT mice had significantly more accumulative stroma than tumors from CD44\(^{-/-}\) mice (Fig. 1B). Eosinophilic structures including large, elongated stromal cytoplasm and collagen fibers were more readily visualized by hematoxylin and eosin (H&E) staining in the WT group than in the CD44\(^{-/-}\) group revealing general differences in stromal architecture between the two groups (Fig. 1C).
Endogenous tumor incorporation of bone marrow–derived stromal components

Having observed diminished tumor stroma incorporation within CD44−/− mice compared with WT mice, we questioned whether the reduction in tumor-associated stromal cells was niche-specific. There are two major sources of tumor stroma: bone marrow and local neighboring tissue. We conducted BMT studies to examine the contribution of CD44−/− bone marrow compared with WT bone marrow in WT recipient mice. Briefly, irradiated WT GFP− mice underwent bone marrow transplantation with 2 × 10^6 whole bone marrow cells from either CD44−/− donor mice (henceforth defined as CD44−/− BMTWT) or WT RFP+ donor mice (WT BMTWT; Supplementary Fig. S1A and S1B). Four weeks after BMT, following confirmation of bone marrow engraftment by flow cytometry (Supplementary Fig. S1C), mice received an orthotopic injection of EO771 tumor cells (5 × 10^4). We compared the nonhematopoetic populations of bone marrow in WT and CD44−/− mice (n = 3 each group) to confirm that population percentage of potential stromal progenitors were statistically insignificant based on CD45−/−Ter119−/− bone marrow–derived cells (Supplementary Fig. S1D).

Tumors were excised 5 weeks after engraftment and subjected to multiplex immunofluorescent imaging to infer the origin of the tumor stromal participants: donor bone marrow (RFP+ WT or β-gal− CD44−/−) or recipient host (GFP− WT). The activated fibroblasts in this model were defined by FAP and FSP; whereas the activated myofibroblasts were defined by α-SMA and NG2. Bone marrow–derived incorporation of activated fibroblasts into tumors showed significant discrepancy between transplant groups, whereas there was no significant difference in the activated myofibroblast derived from the bone marrow or host tissue (Supplementary Fig. S2A–S2C). A small percentage (0.05% ± 0.018%) of the activated fibroblasts were CD44−/− bone marrow–derived, whereas 0.27% ± 0.044% of the activated fibroblasts were WT (RFP+−) bone marrow–derived (P < 0.001; Fig. 2A). Furthermore, the lack of CD44−/− bone marrow–derived incorporation of activated fibroblasts was compensated by a significant increase (P < 0.05) in WT host-derived (GFP+) activated fibroblasts; 2.5% ± 0.47% in the CD44−/− BMT group versus 1.4% ± 0.25% in the WT BMT group (Fig. 2B and C).

MSC Participation as Activated Fibroblasts Requires CD44

Endogenous tumor incorporation of local tissue–derived stromal components

This model was recapitulated in an inverse transplant series to assess the derivation of tumor stroma in a CD44−/− recipient/host background with WT BMT (WT BMTCD44+/−) compared with WT recipient/host with CD44−/− BMT (CD44−/− BMTWT). Using the orthotopic C57BL/6 ID8 ovarian tumor model (30) that mimics human progression of ovarian cancer and recruits more stroma than the C57BL/6 EO771 breast cancer tumor used previously, we compared the incorporation of the activated fibroblast and myofibroblast populations derived from host tissue or donor bone marrow. The bone marrow–derived activated fibroblasts from WT BMTCD44+/− mice were more abundant in engrafted tumors compared with those from CD44−/− BMTWT mice, which followed the trend from the first transplant series without statistical significance (Fig. 2D). But, we show that the host-derived activated fibroblast was significantly higher (P < 0.01) in the tumors engrafted in the CD44−/− BMTWT mice (4.63% ± 1.13%) compared with those in the WT BMTCD44+/− mice (0.69% ± 0.21%; Fig. 2E and F), suggesting a compensation of the decreased activated fibroblast population derived from the CD44−/− bone marrow. In this model, we also observed a significant increase in the bone marrow–derived myofibroblast populations from the CD44−/− BMTWT versus the WT BMTCD44+/− (0.34% ± 0.15% vs. 6.3% ± 1.05%; Supplementary Fig. S2D–S2F).

CD44−/− murine MSC produce fewer TAF in vivo

The BMT studies showed limited tumor stroma participation among the activated fibroblast populations derived from CD44−/− compared with WT. The MSC is a stromal precursor; therefore, we next wanted to investigate whether the loss of CD44 expression on MSC could hinder the capacity to form FAP+/FSP−–activated fibroblasts. To address this question, we increased the physiologic ratio of stromal cells to tumor cells to emphasize and clearly visualize the stromal contribution within the tumor microenvironment. MSC derived from the CD44−/− mice were phenotypically similar to WT controls and could differentiate into chondrocyte, osteoblast (Supplementary Fig. S3B–S3D), and adipocyte lineages (not shown). To generate tumors, we used the 4T1 murine breast carcinoma line, which produces a solid orthotopic tumor mass as opposed to the hemolytic EO771 breast cancer tumor model and is more easily accessible in the mammary fat pad as opposed to the intraperitoneal cavity. Briefly, 4T1 tumor cells were engrafted into one mammary fat pad of SCID mice, and a 1:1 admixture of 4T1 cells with WT MSC or CD44−/− MSC was injected into the contralateral mammary fat pad of these mice.

We measured the FAP and FSP expression in the admixed tumors to confirm the presence of the activated fibroblasts as in the previous BMT models. We also looked at CD31 incorporation within the tumors to assess the potential to promote angiogenesis. FAP (P < 0.001), FSP (P < 0.001), and CD31 (P < 0.005) were expressed at significantly higher levels in the WT MSC admixed tumors compared with the 4T1-only tumors. FAP (P < 0.001; Fig. 3A), FSP (P < 0.001; Fig. 3B), and CD31 (P < 0.05; Fig. 3C) were expressed significantly lower in CD44−/− MSC admixed tumors than in WT MSC admixed tumors. These data confirm that CD44−/− MSC are defective in producing FAP+/FSP−–activated fibroblasts, and the lack of stromal support is associated with a decrease in CD31+ cells within the tumor.

Tumor-conditioned medium activates differentiation and signaling cascades in MSC through CD44

Our preliminary studies suggest that binding of CD44 to its ligands (i.e., hyaluronan and osteopontin) can trigger signaling cascades in MSC that can lead to TAF differentiation and increased migration potential. To analyze the effects of long-term tumor stimulation on MSC in vitro, we first needed
to identify a model tumor cell line that secreted a known CD44 ligand. We screened several pancreatic, breast, and ovarian human cancer cell lines for expression of CD44 ligands. More specifically, we looked at the potential for the tumor cell lines to produce osteopontin, as measured by ELISA, and hyaluronan by assessing the hyaluronan synthetase 2 (HAS2) expression levels. Most of the analyzed tumor cell lines expressed low/negligible levels of HAS2 and osteopontin, however, the ovarian carcinoma cell line Skov-3 expressed and secreted high quantities of osteopontin (Supplementary Fig. S4A and S4B). More importantly, we found that MSC cultured with Skov-3 TCM expressed high levels of HAS2, suggesting that MSC use CD44 for autocrine and paracrine signals (Supplementary Fig. S4A). Skov-3 was thus chosen as our human tumor model for all subsequent experiments.

**Human CD44 knockdown MSC are TAF-deficient in vitro**

Next, we wanted to confirm the role of CD44 deficiency in primary human MSC. We constructed a stable knockdown of CD44 in MSC using four shRNA hairpins (MSCsh44). By flow cytometry, MSCsh44 expressed surface markers common to the nonspecific shRNA knockdown MSC (MSCshNS) including positive expression of CD105, CD90, CD73, CD146, CD166, and CD140b, and negative expression of CD45, CD31, and CD34 but the MSCsh44 were slightly hindered in

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their adipocyte differentiation capacity (Supplementary Fig. S3G) compared with the MSCshNS.

We then conditioned MSC sh44 and MSCshNS with TCM for 21 days in vitro to induce TAF marker expression. After long-term tumor conditioning, the MSCshNS expressed FAP, FSP, and α-SMA—three markers that we use as hallmarks of TAF. Importantly, the MSC sh44 expressed nearly negligible levels of FAP and FSP, similar to the results observed by immuno-fluorescence in our mouse models. The MSC sh44 also expressed less α-SMA, vimentin, and PDGFR-β than WT MSC, and expressed negligible levels of TSP1 and Twist1 (Fig. 4A).

After we confirmed a deficiency in TAF marker expression in MSC sh44 compared with MSCshNS by Western blot analysis, we asked whether the knockdown of CD44 contributed to a migratory deficient phenotype in the MSC sh44 compared with MSCshNS. A Transwell migration assay confirmed the defective migration of the MSC sh44 compared with MSCshNS toward normal serum media (Fig. 4B). We then used a small oHA (29) to competitively bind to CD44 and block activity to show an alternative means of migratory inhibition through CD44 (Fig. 4B). We next conducted an in vivo migration study using a s44, which acts as a CD44 antagonist by competing for the available ligand (31).

Figure 3. TAF marker expression is significantly decreased in tumors admixed with CD44−/−-derived murine MSC. A, expression of FAP was significantly lower (P < 0.001) in CD44−/− MSC admixed 4T1 tumors than in WT MSC admixed 4T1 tumors (n = 20 total). B, similarly, FSP expression was significantly reduced (P < 0.001) in CD44−/− MSC admixed 4T1 tumors than in WT MSC admixed 4T1 tumors. C, CD31 expression, used as evidence of vascularization, was also significantly reduced (P < 0.05) in CD44−/− MSC admixed 4T1 tumors compared with WT MSC admixed 4T1 tumors. Mock brightfield (BF) images were digitally generated from the fluorescent images and are shown in this figure to emphasize the staining and the tissue architecture on a white background. IF, immunofluorescence.
an intratumoral injection of PBS. Forty-eight hours after fLuc-MSC injection, we assessed the capacity of these MSC to migrate to the tumor site by bioluminescent activity measured within the tumors. Bioluminescent signal was observed at significant lower levels ($P < 0.05$) in the tumor injected with s44 (Supplementary Fig. S5A).

**TCM induces CD44 activation on MSC**

By flow cytometry, the surface expression of CD44 following 30-minute exposure to TCM was slightly lower than that of serum-cultured MSC and of MSC exposed to 24-hour TCM (Supplementary Fig. S5B). Because activation and signaling via CD44 is known to be induced by a series of extracellular and intracellular cleavages (32), we sought to test whether TCM was activating CD44 by cleaving it. Because MMP14 has been reported to cleave the extracellular and intracellular portion of CD44 (33), we treated MSC with an MMP14 inhibitor and showed a decrease in MSC Transwell migration similar to the migration inhibition observed when cells were treated with s44 or oHA antagonist (Supplementary Fig. S5C). Furthermore, the migration inhibition at 1 μmol/L MMP inhibitor coincided with the abrogation of CD44 cleavage products seen by Western blot analysis (Supplementary Fig. S5D).

**CD44 activation by TCM stimulates Twist expression in MSC**

One of the consequences of intracellular cleavage of CD44 is nuclear localization and transcriptional activation of target genes through association with Stat3 (34). By screening a number of Stat3-activated genes following TCM by PCR (data not shown), we only observed one gene, Twist1 that had a correlated increase in protein expression by immunofluorescence (Fig. 5A). This observation was confirmed by Western blot analysis in the MSCshNS cells following TCM. Furthermore, the MSCsh44 did not show increases in Twist1 expression comparable with MSCshNS by Western blot analysis (Fig. 5B). We then confirmed by real-time PCR (RT-PCR) that Twist1 RNA expression was lower in the MSCsh44 than in naïve MSC or MSCshNS; this decrease was similar to the decrease in expression observed by s44 and MMP inhibition (Fig. 5C), suggesting that the regulation of Twist1 by CD44 is transcriptional.

To confirm the direct transcriptional regulation of Twist1 by CD44 in tumor-conditioned MSC, we conducted quantitative ChIP analysis. Results revealed that after 30-minute exposure to TCM, CD44 association at the Twist1 promoter increased by 3.5-fold (Fig. 5D). Furthermore, association of acetylated Stat3, Stat3, and p300 with the Twist1 promoter in MSC following TCM exposure increased by 2-, 6.5-, and 5-fold, respectively (Fig. 5D).

**Modulation of tumor-conditioned MSCsh44 with Twist reexpression**

Next, we exogenously expressed Twist1 in MSCsh44 (Fig. 6A and B) to see if we could regain function of MSC under TCM exposure. Expression of Twist in MSCsh44 increased the migration capacity under TCM by 25%, similar to the increase in migration capacity of MSCshNS (Fig. 6C). We also examined expression of proangiogenic genes with and without exogenous Twist1 expression. After 24-hour exposure to TCM, MSCsh44 ectopically expressing Twist1 had increases in Il8, VegfC, and EGF expression (Fig. 6D). Although protein secretion was not measured, we did analyze the functional effects of the proangiogenic factors produced by MSCsh44 ectopically expressing Twist1 compared with MSCshNS and MSCshNS. Ectopic expression of Twist1 partially restored the ability of MSCsh44-conditioned medium to promote HUVEC tube formation (Fig. 6E), suggesting the reestablishment of proangiogenic function of the MSCsh44.
Discussion

It is well established that MSC contribute to the tumor microenvironment in support of the tumor parenchyma. The interactions between tumor cells and stromal cells dictate tumorigenesis; stroma-rich breast tumors have a higher risk of relapse and confer shorter overall survival (35). In this current study, we showed that CD44 expression on tumor stromal precursors is necessary for their migration, incorporation, and functionality within the tumor microenvironment as TAF—or more specifically as FAP/FSP—expressing activated fibroblasts.

Previous studies have shown the significance of CD44 in migration of MSC (20). Okamoto and colleagues proposed that CD44 transcription can be self-regulated and mediated by CBP/p300 in a glioma cell model (36). CD44, when appropriately stimulated, undergoes an extracellular cleavage that is followed by an intracellular cleavage leading to the translocation of the intracellular domain of CD44 into the nucleus where it has been shown to associate with Stat3 to promote transcription in a lung carcinoma model (34). We sought to identify the genes transcriptionally regulated by CD44 in MSC under exposure to TCM. Assessment of Stat3-regulated genes lead us to Twist, which was upregulated in MSC under tumor conditioning, but significantly downregulated under CD44 inhibition with s44. The mesoderm regulator Twist has been associated with migration, metastases, drug resistance, angiogenesis, and EMT (37–39). Decreased survival has been correlated to stromal fibroblast Twist expression in patients with gastric cancer (40). Our data support these findings and provide evidence that MSC whose CD44 expression is blocked results disruption of mesenchyme markers including Twist, FAP, or FSP expression thereby producing dysfunctional activated fibroblasts.

TAF are known to have an inflammatory gene expression profile compared with normal fibroblasts (41), particularly in the breast tumor microenvironment (42). Proinflammatory gene expression leads to recruitment of macrophages, myofibroblasts, MSC, pericytes, and endothelial precursors that aid in tumor progression and angiogenesis. We showed a global decrease in the expression of proangiogenic factors from MSC shCD44 under TCM in vitro. We suggest that the observed decrease in endothelial CD31 presence within the CD44+/− MSC admixed 4T1 tumors in vivo was due to lack of proangiogenic expression potential. All tumor models used in these studies were highly aggressive and therefore did not show a strong dependence on stromal support. This suggests that the differences observed between the CD44+/− and CD44−/− groups as well as the overall tumor aggressiveness as measured by the Kaplan–Meier survival curves in Figure 4B are due to a lack of proangiogenic support.
and WT stromal microenvironments may be more pronounced in a less aggressive, or earlier stage tumor model.

Differences in tumor stroma recruitment have previously been described in the context of circulating bone marrow–derived cells and the "potency" of the tumor (43). The complex interface between the tumor parenchyma and the stroma collectively influences tumor progression. Our model shows a division of stroma participants based on marker specificity: the activated myofibroblast TAF and the activated fibroblast TAF. Individual expression of activated fibroblast markers FAP and FSP have been associated with disease and metastases (44–46). Our studies suggest that decrease in CD44 expression leads to decreases in both FAP and FSP expression, but further studies are needed to determine the complete mechanism of depletion of TAF gene expression as a result of CD44 knockdown. We did not observe a significant difference in size between tumors engrafted in the transgenic BMT models, nor did we observe metastatic lesions at the time the mice were sacrificed.

Although the most profound difference in the transgenic BMT models was the activated fibroblast populations, we did see a significantly higher population of bone marrow–derived activated myofibroblast (P < 0.001) in the tumors compared with those in the WT BMTCD44+/C0/C0 mice (6.32% ± 1.05%) when conditioned with medium from shCD44MSC compared with medium from naïve MSC or shNS MSC but ectopic Twist expression restored proangiogenic potential in shCD44MSC. Graph represents the number of branch points counted per image.

Figure 6. Ectopic expression of Twist in CD44 knockdown MSC partially restores phenotype under TCM in vitro. A and B, validation of hemagglutinin-tagged, ectopic Twist expression by RT-PCR (A) and Western blot analysis (B). C, Transwell migration assay showed that ectopic expression of Twist in shCD44MSC increased the migration capacity 2-fold toward TCM and that this effect was not inhibited by oHA. D, ectopic Twist expression in shCD44MSC showed an increase in angiogenesis-associated growth factor expression by RT-PCR compared with shCD44MSC. Fold changes relative to shNSMSC expression levels. E, HUVEC tube formation potential was significantly decreased (P < 0.05) when conditioned with medium from shCD44MSC compared with medium from naïve MSC or shNS MSC but ectopic Twist expression restored proangiogenic potential in shCD44MSC. Graph represents the number of branch points counted per image.
significant difference in size between the 4T1 tumors contralateral to the CD44+/− admixed tumors compared with the 4T1 tumors contralateral to the WT admixed tumors, suggesting that the CD44−/− MSC were not able to generate the factors necessary to elicit a proinflammatory signature that recruits additional tumor-supportive stromal participants including macrophages and endothelial cells (data not shown).

Anti-CD44–targeting methods have shown promising antitumor efficacy in animal model systems including the use of a s44 antagonist (28, 31). We used an intratumoral injection of adeno-viral-expressed s44 to reduce MSC incorporation into the tumor microenvironment, indicating that therapeutic avenues to suppress stroma incorporation within the tumor are possible. However, further studies are required to assess the global effects of CD44 inhibition on the normal cells and not just the tumor-associated cell populations. One potential therapeutic avenue is to distinguish between CD44 variant forms among normal cells and tumor-associated cells.

Our findings about the formation of activated fibroblasts evoke broader implications on tumor biology as CD44 expression has been used as one of the definitive markers of cancer stem cell populations, particularly in breast cancer (47). We provide evidence in MSC that CD44 can be activated by tumor paracrine factors to induce and directly regulate Twist expression. This suggests that CD44 expression in mesenchymal transitioned cancer cells may also have the ability to propagate their mesenchymal phenotype (10) and thus their own microenvironment through CD44 regulation of Twist. In fact, CD44 stimulation in breast cancer cells has been shown to activate Twist expression through lysyl oxidase activation to regulate an EMT phenotype (48). Recently, Shangguan and colleagues reported that inhibition of TGF-β signaling by transducing MSC with bone morphogenetic protein and Activin Membrane Bound Inhibitor blocked expression of FAP, FSP, and α-SMA in tumor-conditioned MSC (49). Furthermore, the TGF-β receptor contains a CD44 binding site through which CD44 and/or Sema in tumor-conditioned MSC (49). In addition, TGF-β stimulation has previously been shown to activate downstream TGF-β signaling (50). Our findings suggesting the importance of CD44 signaling in MSC role within the tumor microenvironment support the potential for CD44 activation to stimulate multiple downstream pathways including TGF-β signaling.

In summary, we show that CD44-deficient stromal mesenchyme is incapable of participating in the tumor microenvironment as fully functional TAF. In particular, they are unable to form activated fibroblasts, but retain the ability to express activated myofibroblast markers. They display deficiencies in (i) tumor-tropic migratory capacity, (ii) ability to induce angiogenesis, and (iii) expression of activated fibroblast markers FAP and FSP. We show that tumor paracrine stimulation on the MSC activates CD44, which is able to directly regulate expression of Twist through interaction with Stat3. These results implicate CD44 on the tumor stromal population as a potential therapeutic target as tumor progression is associated with an increased presence of stromal cells. Furthermore, because of the breadth of potential cells from which stromal cells can be derived including cancer cells themselves, CD44 provides a potential antitumor target on the supportive stromal population as well as cancer cell population. Ongoing studies using a pharmacologic anti-CD44 antibody to inhibit tumor stroma formation are currently underway.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: E.L. Spaeth, A.M. Labaff, M. Andreeff, F.C. Marini
Development of methodology: E.L. Spaeth, A.M. Labaff
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): E.L. Spaeth, A.M. Labaff
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): E.L. Spaeth, A.M. Labaff, B.P. Toole, A. Kloppe, M. Andreeff, F.C. Marini
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References


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