TUMORIGENESIS AND NEOPLASTIC PROGRESSION

Insights into Fibroblast Plasticity

Cellular Communication Network 2 Is Required for Activation of Cancer-Associated Fibroblasts in a Murine Model of Melanoma

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Tumor stroma resembles a fibrotic microenvironment, being characterized by the presence of myofibroblast-like cancer-associated fibroblasts (CAFs). In wild-type mice injected with melanoma cells, we show that the stem cell transcription factor Sox2 is expressed by tumor cells and induced in CAFs derived from synthetic fibroblasts. These fibroblasts were labeled postnatally with green fluorescent protein using mice expressing a tamoxifen-dependent Cre recombinase under the control of a fibroblast-specific promoter/enhancer. Conversely, fibroblast activation was impaired in mice with a fibroblast-specific deletion of cellular communication network 2 (Ccn2), associated with reduced expression of α -smooth muscle actin and Sox2. Multipotent Sox2-expressing skin-derived precursor (SKP) spheroids were cultured from murine back skin. Using lineage tracing and flow cytometry, approximately 40% of SKPs were found to be derived from type I collagen-lineage cells and acquired multipotency in culture. Inhibition of mechanotransduction pathways prevented myofibroblast differentiation of SKPs and expression of Ccn2. In SKPs deleted for Ccn2, differentiation into a myofibroblast, but not an adipocyte or neuronal phenotype, was also impaired. In human melanoma, CCN2 expression was associated with a profibrotic integrin alpha (ITGA) 11-expressing subset of CAFs that negatively associated with survival. These results suggest that synthetic dermal fibroblasts are plastic, and that CCN2 is required for the differentiation of dermal progenitor cells into a myofibroblast/CAF phenotype and is, therefore, a therapeutic target in melanoma. (*Am J Pathol* 2020, 190: 206–221; <https://doi.org/10.1016/j.ajpath.2019.09.006>)

Melanoma is highly metastatic and the most fatal skin cancer.¹ Although drugs, including those targeting specific mutations (eg, in *BRAF*) and checkpoint inhibitors, have been discovered that can retard melanoma progression in some patients, ultimately patients develop resistance to these regimens.^{2,3} Although much work is currently being expended on how tumors evade immunosurveillance, alternative strategies to block metastasis are needed, yet such novel approaches are relatively underresearched.

Fibroblasts, the cells responsible for generating and maintaining extracellular matrix (ECM), are fundamental contributors to diverse physiological and pathologic

processes, including cancer. Within the tumor stroma, fibroblasts, intimately associated with cancer cells at all stages of cancer progression, actively contribute to both

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tumor growth and metastasis.⁴ More specifically, in melanoma, the stiffness of the surrounding ECM has been linked to tumor aggressiveness and the acquisition of drug resistance.^{4–6} This phenomenon can be conceptually described as follows. When located within the tumor, cancer cells attach to each other via cell/cell contacts. In this environment, mutations (eg, those activating BRAF) that activate prosurvival and proliferative signals (eg, extracellular signal-regulated kinase and Akt) can be directly targeted by inhibitors targeting these mutations (eg, BRAF inhibitors). Conversely, when cells leave this niche and migrate on a stiff ECM, cancer cells now attach to the surrounding ECM via focal adhesion kinase/integrin β 1; this engagement now results in the activation of downstream prosurvival signals (eg, extracellular signal-regulated kinase and Akt) in an adhesion/stiffness-dependent yet BRAF-independent manner, resulting in resistance to anti-BRAF inhibitor chemotherapy.^{7,8} Thus, a stiff ECM may be sufficient for the activation and metastasis of melanoma cells. This stiff ECM is generated by so-called cancer-associated fibroblasts (CAFs) that are similar to the myofibroblasts seen in fibrotic conditions, as these CAFs express the highly contractile protein α -smooth muscle actin (α -SMA).⁹ Proteins secreted by CAFs into the tumor microenvironment are essential mediators of cellular signaling responses that contribute to cancer cell proliferation and metastasis.¹⁰ Therefore, understanding how CAFs originate and promote melanoma progression is of major importance.

Matricellular proteins, nonstructural ECM components that are secreted by fibroblasts into the microenvironment during conditions of pathologic tissue remodeling, are emerging therapeutic targets in fibrotic disease and cancers, including in melanoma.^{11,12} Specifically, the central communication network (CCN) family of matricellular proteins is of current therapeutic interest.¹³ Cellular communication network 2 (CCN2), a CCN family member formerly referred to as connective tissue growth factor,¹⁴ is not normally expressed by dermal fibroblasts, but is potently induced in normal tissue repair and pathologies, including fibrosis and cancers.¹⁵ In human melanoma patients, CCN2 expression does not correlate with *BRAF* mutational status, but instead correlates with expression of stroma and angiogenic gene subsets, and negatively correlates with survival.^{16,17} In mice, conditional knockout strategies, using a tamoxifen-dependent Cre recombinase expressed under the control of fibroblast-specific *Col1a2* promoter/enhancer,¹⁸ have shown that loss of CCN2 expression by fibroblasts results in resistance to melanoma metastasis and tumor-induced neovascularization, concomitant with a reduced production of the proangiogenic mediators periostin and vascular endothelial growth factor.^{16,17} Accordingly, CCN2 represents a novel therapeutic target for BRAF inhibitor-resistant melanoma.

Although much work has been done to investigate the plasticity of tumor cells and their acquisition of stem-like characteristics in cancer progression, relatively little is

known about the specific mechanisms through which resident fibroblasts become recruited and activated within the tumor stroma to become CAFs. In a model of bleomycin-induced skin fibrosis, it has previously been shown that dermal cells expressing the progenitor cell marker Sox2 represent an important source of α -SMA-expressing myofibroblasts and differentiate in a CCN2-dependent manner to result in pathologic skin thickening.¹⁹ Interestingly, in human melanoma, expression of Sox2 correlates with tumor thickness and is markedly induced in spindle-like cells occurring at the tumor/stroma interface (ie, in cells resembling fibroblasts),²⁰ suggesting the intriguing possibility that Sox2 may be expressed in CAFs and, as in during skin fibrosis, may be involved with the acquisition of a myofibroblast phenotype.

In this report, conditional knockout and lineage tracing strategies using a tamoxifen-dependent Cre recombinase expressed under the control of a fibroblast-specific promoter/enhancer derived from the *Col1a2* gene¹⁸ were used to investigate how dermal fibroblasts contribute, in a CCN2-dependent manner, to the appearance of progenitor-like cells and CAFs in tumor stroma. Skin-derived precursor (SKP) spheroids were further used as an *in vitro* model of fibroblast plasticity to demonstrate the mechanistic role of CCN2 in myofibroblast differentiation of progenitor cells. These data provide new and valuable insights into the potential role of fibroblast plasticity in melanoma progression.

Materials and Methods

Generation of Transgenic Mice

For lineage tracing, mice hemizygous for a tamoxifen-dependent Cre recombinase under the control of a *Col1a2*¹⁸ or Sox2 (Institut Clinique de la Souris, Alsace, France) promoter [*Col1a2*-Cre(ER)-T; *Sox2*-Cre(ER)-T] were bred with mice homozygous for a double-fluorescent reporter transgene (mTmG) integrated into the Gt(ROSA) 26Sor locus (Jackson Laboratories, Bar Harbor, ME), thereby generating *Col1a2*-Cre(ER)T; *Rosa26mTmG* or *Sox2*-Cre(ER)T; *Rosa26mTmG* mice. Please note *Col1a2*-Cre(ER)-T mice contain a fibroblast-specific far upstream enhancer, initially identified in the laboratory of Benoit de Crombrughe, subcloned upstream of the *Col1a2* minimal promoter; previous publications have extensively shown that this construct permits transgene expression only in fibroblasts and not in other collagen-expressing cells, such as bone and cartilage.^{18,21–23} In these mice, when Cre recombinase is induced via tamoxifen, the mTmG transgene results in permanent green fluorescent protein (GFP) expression in cells expressing the promoter construct (and all of their progeny), whereas all other cells express tdTomato. To generate *Ccn2* conditional knockout mice, *Col1a2*-Cre(ER)T; *Rosa26mTmG* mice were bred for two generations with mice homozygous for a floxed *Ccn2* allele^{16–19} to produce *Col1a2*-Cre(ER)T; *Rosa26mTmG*; *CCN2*^{fl/fl} mice. In these mice, induction of Cre

via tamoxifen results in a Col1a2-specific *Ccn2* knockout (*Ccn2*^{-/-}) in addition to activating the mTmG reporter described above. To induce Cre recombinase, a stock solution of 4-hydroxy-tamoxifen in ethanol (Sigma, St. Louis, MO; 100 mg/mL) was diluted in corn oil (Sigma) to 10 mg/mL. Three-week-old littermate mice were given 0.1 mL i.p. injections of the tamoxifen solution (for lineage tracing and *Ccn2*^{-/-}) or corn oil (for *Ccn2*^{fl/fl}) for 5 consecutive days. Two weeks after tamoxifen injection, loss of *Ccn2* was confirmed by PCR genotyping, and mice were sacrificed for experimental use or injected with melanoma cells.

Melanoma Mouse Model

B16F10 murine melanoma cells (ATCC, Manassas, VA) were injected subcutaneously (330,000 cells) into the right flank of transgenic mice described above. Tumors grew for 14 days after the appearance of a palpable tumor, and skin samples containing tumor and tumor stroma were collected. Samples were put in OCT compound (Sakura Finetek, Torrance, CA), frozen at -80°C, and stained as described below.

Cell Extraction and Culture

Full-thickness dorsal skin was dissected from sacrificed mice, washed in phosphate-buffered saline (PBS), and then placed into high-glucose Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Burlington, ON, Canada) containing 2 mg/mL collagenase type II and incubated for 3 hours at 37°C. Dermal tissue was scraped off into DMEM and mechanically dissociated by pipetting. Samples were centrifuged for 1 minute at 55 × g, and supernatant containing cells was collected and spun down again for 5 minutes at 250 × g. Cell pellets were collected and resuspended in media, filtered with a 40-µm cell strainer, and seeded in a T75 cell culture flask. Fibroblasts were cultured in high-glucose DMEM (Invitrogen) supplemented with 10% fetal bovine serum (FBS; Invitrogen) and 1% antibiotic-antimycotic (Invitrogen). SKP spheroids were cultured in SKP base media: three parts DMEM glutaMAX and one part Hams F12 (Invitrogen); supplemented with 1% antibiotic-antimycotic, 2% B27 (Invitrogen; A35828-01), 1% N2 (Invitrogen; 17502048), epidermal growth factor (20 ng/mL; Invitrogen; PHG0311), and basic fibroblast growth factor (20 ng/mL; Invitrogen; PHG0026).^{18,19} Alternatively, to generate larger quantities of SKPs, similar to a previously described report,²⁰ confluent 10-cm plates of fibroblasts were removed with 0.25% trypsin (Invitrogen) and resuspended in SKP base media, as described above. Cells were then seeded into 3 wells of a 6-well plate per 10-cm plate of fibroblasts. Basic fibroblast growth factor (20 ng/mL) supplement was added Monday, Wednesday, and Friday for 2 weeks. Melanoma B16(F10) (ATCC) cells were cultured in high-glucose DMEM supplemented with 10% FBS and 1% antibiotic-antimycotic.

SKP Differentiation

SKP differentiation was conducted exactly as previously described.²⁴ Briefly, plates were coated with 0.001% poly-L-lysine (Sigma) and laminin (2.5 mg/mL; Sigma) in PBS (Invitrogen) overnight. SKPs were collected and plated on coated plates in various media.

SKP to Myofibroblasts

SKPs were transferred to DMEM supplemented with 0.5% FBS for 24 hours, at which point images were taken and samples were collected for further analysis. For inhibitor experiments, inhibitors were added 30 minutes before FBS exposure: PP2 (Sigma; 10 µm), PF228 (Tocris, Oakville, ON, Canada; 10 µm), and CCG1423 (Tocris; 50 µm).

SKP to Neurons

SKPs were differentiated in medium containing 5% FBS for 5 days, then medium was replaced and supplemented with NGF (40 ng/mL; Invitrogen; 13257-019) for 5 additional days.

SKP to Adipocytes

SKPs were differentiated in medium containing 5% FBS for 24 hours, then medium was replaced with adipocyte differentiation medium (A1007001; ThermoFisher, St. Laurent, QC, Canada) every approximately 5 days and stained with oil red O after a total of 14 days (Sigma).

Histology

Cryosections of mouse dorsal skin were acquired by freezing tissue in OCT compound at -80°C and dividing into sections (20 µm thick) at -22°C using a Leica CM1900 UV microtome cryostat (Leica, Concord, ON, Canada). To detect endogenous GFP and tdTomato expression in mTmG reporter mice, slides were washed in PBS, mounted with mounting media containing DAPI (Vector Laboratories, Burlington, ON, Canada), and imaged using a Zeiss Imager M1 fluorescence microscope and Northern Eclipse software version 8 (Empix, Mississauga, ON, Canada).

Immunofluorescence

Cultured cells or skin tissue cryosections were fixed with 4% paraformaldehyde for 10 minutes, followed by permeabilization with 0.5% PBS/0.05% Tween 20 (Sigma) for 10 minutes. Samples were incubated in blocking solution (0.5% PBS/0.05% Tween 20 with 10% donkey serum; Jackson ImmunoResearch, West Grove, PA) for 30 minutes, followed by incubating with primary antibody in the blocking solution overnight. The following primary antibodies were used: anti-α-SMA (1:500; Sigma; A5228), anti-CCN2 (1:100; Santa Cruz Biotechnology, Dallas, TX;

Figure 1 Col1a2-specific *Ccn2* knockout decreases expression of Sox2 and α -smooth muscle actin (α -SMA) in Col1a2-derived melanoma tumor stroma cells. *Col1a2-CreER(T); Rosa26mTmG; Ccn2^{fl/fl}* and *Ccn2^{-/-}* mice were implanted with B16F10 melanoma cells. In this model, host-derived cells express green fluorescent protein (GFP) or tdTomato, whereas tumor cells express neither GFP nor tdTomato. GFP⁺ refers to cells expressing GFP and are, therefore, host cells that were expressing the fibroblast-specific Col1a2 promoter/enhancer at the time of tamoxifen injection, before the injection of tumor cells. **A** and **B**: *Ccn2^{fl/fl}* (**A**) and *Ccn2^{-/-}* (**B**) tissue sections were stained for GFP (green), α -SMA (red), and Sox2 (gray) with representative low-power (**left panel**) and high-power (**right panel**) magnification images shown. **C**: Col1a2-derived GFP⁺ cells, expressed as percentage of total tumor stroma cells in *Ccn2^{fl/fl}* and *Ccn2^{-/-}* sections. **D** and **E**: Percentage of GFP⁺ cells coexpressing either α -SMA or Sox2 in *Ccn2^{fl/fl}* and *Ccn2^{-/-}* sections. **F** and **G**: Percentage of GFP⁺ cells either coexpressing both α -SMA and Sox2 (**arrow** in **A**) or expressing Sox2 but negative for α -SMA, in *Ccn2^{fl/fl}* and *Ccn2^{-/-}* sections. Data are expressed as means $\pm$ SEM (**C–G**). *N* = 3 mice with tumors (**A–G**). **P* < 0.05, ****P* < 0.001 versus *Ccn2^{fl/fl}* (*t*-test). Scale bars: 100 μ m (**A** and **B**, **left two columns**); 25 μ m (**A** and **B**, **right two columns**).

Figure 2 Col1a2-expressing dermal fibroblasts express Sox2 when cultured as skin-derived precursor (SKP) spheroids. **A**: Expression of neural crest stem cell markers Sox2 and nestin by SKP spheroids; representative images shown. Lineage tracing was performed using Col1a2-CreER(T); Rosa26mTmG and Sox2-CreER(T); Rosa26mTmG reporter mice that were injected with tamoxifen 3 weeks after birth. **A–G**: Expression of green fluorescent protein (GFP) and tdTomato was assessed in skin histologic sections via fluorescence microscopy (**A**, **B**, and **E**) and in cultured SKPs via fluorescence microscopy (**C** and **F**) and flow cytometry (**D** and **G**). Representative images are shown. **H**: Percentage of GFP⁺ cells in SKPs cultured from Sox2 and Col1a2 lineage reporter mice via quantification of flow cytometry. **I**: Flow cytometry performed on Col1a2-GFP SKPs detecting expression of Sox2 with (left panel) representative flow cytometry quadrants (right panel) graph representing percentage of Sox2⁺ cells in the GFP⁺ and GFP[−] populations. Data are expressed as means $\pm$ SEM (**H** and **I**). $N = 4$ mice (**H** and **I**). $^{**}P < 0.01$ versus Col1a2 (t -test). Scale bars: 100 μ m (**A**, **B**, and **E**); 300 μ m (**C** and **F**). Q, quartile.

Figure 3 Fibroblast-derived skin-derived precursors (SKPs) possess multipotent differentiation potential. SKPs cultured from the dorsal skin of Col1a2-CreER(T); Rosa26mTmG reporter mice were grown in media containing 0.5% fetal bovine serum (FBS), 40 ng/mL NGF, or adipogenesis supplemented, allowing for differentiation into myofibroblast, neuronal, or adipocyte (Adipo)-like cells, respectively. Indirect immunofluorescence analysis was performed to assess for colocalization (arrow) of green fluorescent protein (GFP) and markers of myofibroblast [α -smooth muscle actin (α -SMA); **A**], neuronal (β III-tubulin; **B**), and adipocyte differentiation (oil red O; **C**) to indicate a Col1a2 origin. Representative images are shown. Arrows denote Col1a2-lineage cells that have differentiated into myofibroblast- (**A**), neuronal- (**B**) or adipocyte-like (**C**) cells. $N = 3$ independent experiments (**A–C**). Scale bars = 100 μ m (**A–C**).

sc-14939) anti- β III-tubulin (1:1000; PhosphoSolutions, Aurora, CO), anti-GFP (1:100; Santa Cruz Biotechnology; sc-9996), antinestin (1:100; Santa Cruz Biotechnology; sc-21248), and anti-Sox2 (1:100; Santa Cruz Biotechnology; sc-17320). Appropriate IgG controls were from Jackson ImmunoResearch. Appropriate fluorophore-conjugated secondary antibodies (raised in donkey; 1:5000; Jackson ImmunoResearch) diluted in blocking solution were added (1 hour) and washed, and cells were mounted using DAPI-containing mounting media (Vector Laboratories). Imaging was performed using a Leica Microsystems DMI6000B inverted microscope. Images were analyzed using ImageJ software version 1.49 (NIH, Bethesda, MD; [https://imagej.](https://imagej.nih.gov/ij)

[nih.gov/ij](https://imagej.nih.gov/ij)), and cells within the tumor stroma staining positively for GFP were manually counted. Of those cells, those that were coexpressing α -SMA, SOX2, both α -SMA and SOX2, or SOX2 without α -SMA were manually counted.

RNA Analysis

RNA was extracted from cell cultures using a TRIzol-Chloroform (Invitrogen) method and analyzed as described previously.^{21,22} Microarray analysis was performed using Affymetrix GeneChip Mouse Gene 2.0 ST arrays (Affymetrix, Santa Clara, CA), as described previously^{15,21,22,25–29} by

Figure 4 Inhibition of Src, focal adhesion kinase (FAK), and serum response factor/myocardin-related transcription factor (SRF/MRTFA) prevents myofibroblastic differentiation of skin-derived precursors (SKPs) and suppresses the activation of CCN2. SKP spheroids were pretreated with dimethyl sulfoxide (DMSO), PP2 (Src-family kinase inhibitor; 10 μ mol/L), PF228 (FAK inhibitor; 10 μ mol/L), or CCG-1432 (SRF/MRTF inhibitor; 50 μ mol/L) for 30 minutes before 24 hours of treatment with or without (negative control) 0.5% fetal bovine serum (FBS). Representative phase contrast and immunofluorescence images for α -smooth muscle actin (α -SMA) and CCN2 are shown. RNA was harvested and subjected to real-time quantitative PCR analysis for α -SMA and CCN2 expression; fold change is shown. Data are expressed as means $\pm$ SEM. $N = 3$. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ (one-way analysis of variance, Tukey post-hoc test). Scale bars = 300 μ m.

the London Regional Genomics Center; cluster analysis was performed using DAVID Functional Annotation Software version 6.7 (<https://david.ncifcrf.gov>).³⁰ Experiments were performed twice; the complete data set after average values was calculated, is shown in [Supplemental Table S1](#), and as such represents an initial approach to identifying genes whose expression is potentially CCN2 dependent (fold change $> |1.4|$; $N = 2$). Data were analyzed as previously described.^{17,25–29} Real-time PCR using 40-ng RNA samples was run in triplicate on an ABI Prism 7900 HT PCR machine (ThermoFisher). 18S was used as internal control, and fold change was calculated using the $\Delta\Delta C_t$ method compared with one RNA sample derived from wild-type cells treated with serum.

Western Blot Analysis

Protein was harvested by cell lysis by incubating cultured cells in radioimmunoprecipitation assay buffer supplemented with complete protease inhibitor cocktail (Sigma). Protein samples (50 μ g) were run on 10% SDS-acrylamide gels. Protein was transferred to a nitrocellulose membrane and blocked in 5% milk in 0.01% Tris-buffered saline/Tween 20 for 1 hour. Primary antibodies were incubated in blocking milk overnight at 4°C: anti- α -SMA (1:2500), anti-CCN2 (1:100), anti- β III-tubulin (1:10,000), or anti- β -actin (1:5000; Sigma). Horseradish peroxidase-conjugated secondary antibodies were incubated for 1 hour at room temperature after washes in 0.01% Tris-buffered saline/Tween 20, and membranes were treated with SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, St. Laurent, QC, Canada) and visualized

using X-ray film. Densitometry quantification was performed using ImageJ software.

Flow Cytometry

Cells were brought to single-cell suspension by incubation with 1 U/ μ L collagenase type IV (Invitrogen; 17104-019) for 1 hour at 37°C in Hanks' balanced salt solution, followed by mechanical dissociation. Cells were then filtered through a 40- μ m cell strainer and incubated in Zombie Aqua dye (423101; Biolegend, San Diego, CA) and PBS for 20 minutes in the dark. Cells were fixed and permeabilized using nuclear fixation kit (Biolegend), followed by staining with 647-fluorophore-conjugated Sox2 antibody (1:50) in cell staining buffer (Biolegend). Cells were washed three times in cell staining buffer, and 50,000 cells were run through the FACSCanto flow cytometer (BD Biosciences, San Jose, CA) to determine the number of tdTomato-, GFP-, and Sox2-positive cells using FACSDiva software version 8.01 (BD Biosciences). Analysis was performed on FlowJo software version 10.6.1 (Tree Star Inc., Ashland, OR).

Data Sets and Survival Analysis

Single-cell RNA-sequencing data, derived from 6879 melanoma tumor cells, were obtained from Gene Expression Omnibus GSE115978 in July 2019 (<https://www.ncbi.nlm.nih.gov/geo>; accession number GSE115978). Please note that the data set analyzed is distinct from that analyzed previously.¹⁷ For single-cell analysis, transcript-per-million (TPM) values were divided by 10, given that the complexity of single-cell libraries is estimated to be in the order of 100,000 transcripts. Level 3 The Cancer Genome

Figure 5 Col1a2-specific CCN2 knockdown impairs myofibroblast skin-derived precursor (SKP) differentiation. SKPs cultured from Col1a2-CreER(T); Rosa26mTmG; *Ccn2*^{fl/fl} or Col1a2-CreER(T); Rosa26mTmG; *Ccn2*^{-/-} mice were grown in the presence [+ fetal bovine serum (FBS)] or absence (- FBS) of myofibroblast differentiation media. **A:** Representative immunofluorescence images for α -smooth muscle actin (α -SMA), CCN2, and Ki-67 expression in response to myofibroblast differentiation media are shown. Immunostaining for Ki-67 is expressed as percentage of proliferating cells. **B and C:** Real-time quantitative PCR (**B**) and Western blot analysis (**C**) were performed to assess α -SMA and CCN2 expression. Data are expressed as means $\pm$ SEM (**B and C**). N = average of 10 fields (**A**); N = 3 independent experiments (**B and C**). * P < 0.05, ** P < 0.01, and *** P < 0.001 (one-way analysis of variance, Tukey post-hoc test). Scale bars = 300 μ m (**A**).

Atlas RNAseqV2 gene expression was obtained from The Cancer Genome Atlas Data Portal in January 2016. RNA-Seq by Expectation Maximization (RSEM) expression data were used in downstream analyses.³¹ Patient survival information was downloaded in December 2017. Z-score unsupervised hierarchical clustering was conducted using 1-c (where c is the Pearson correlation coefficient) as the distance and the Ward agglomeration method (ward.D2).

CAF-specific *CCN2* scores were calculated for tumors by summing Z-scores for genes that were i) strongly correlated with *CCN2* (Pearson r > 0.5), ii) had 10-fold higher mean expression in CAFs than any other cell type, and iii) had mean expression <0.1 TPM/10 in malignant cells. This resulted in a nine-gene score composed of the following genes: *ZFHX4*, *DCN*, *ITGA11*, *COL6A3*, *COL1A1*, *ITGBL1*, *COL8A1*, *INHBA*, and, *MEG3*. CAF-specific *CCN2* scores were dichotomized with receiver operating characteristic curves to

determine the optimal cutoff for the end point of overall survival censorship. Survival curves were constructed using the Kaplan-Meier method on primary tumor samples that had survival information available from The Cancer Genome Atlas. Significance was determined by log-rank test.

All analyses and visualizations were conducted in the RStudio programming environment version 1.1.463 (RStudio, Inc., Boston, MA). R/Bioconductor packages ggplot2, plyr, pROC, gplots, and survival were used, where appropriate.

Statistical Analysis

Statistical analysis using either unpaired t -test or one-way analysis of variance with Tukey multiple comparison post-hoc testing, as appropriate, was performed using GraphPad Prism version 7 (GraphPad Software, La Jolla, CA).

Figure 6 Col1a2-specific *Ccn2* knockdown does not impair neuronal or adipocytic skin-derived precursor (SKP) differentiation. SKPs cultured from Col1a2-CreER(T); Rosa26mTmG; *Ccn2^{fl/fl}* or Col1a2-CreER(T); Rosa26mTmG; *Ccn2^{-/-}* mice were grown in neuronal (NGF) and adipocytic (Adipo) differentiation media. **A:** Neuronal differentiation in the presence or absence of *Ccn2* was assessed through βIII-tubulin and *Ccn2* expression via immunofluorescence and Western blot analysis. **B:** Adipocytic differentiation was assessed via quantification of oil red O staining in green fluorescent protein–positive (GFP⁺; cells with activated Cre) in *Ccn2^{fl/fl}* and *Ccn2^{-/-}* SKPs. Representative images are shown. *N* = 3 independent experiments (**A**); *N* = average of 10 fields (**B**). **P* < 0.05 versus *Ccn2^{fl/fl}* + NGF. Scale bars = 300 μm (**A** and **B**).

Results

In a Murine Melanoma Model, Dermal Fibroblasts Acquire Expression of Sox2 and α-SMA in a CCN2-Dependent Manner

To address the potential role of Sox2-expressing progenitor cells in the *Ccn2*-mediated activation of CAFs, a syngeneic mouse model was used in which Col1a2-CreER(T); Rosa26mTmG C57/BL6 mice deleted (*Ccn2^{-/-}* or knockout) or not (*Ccn2^{fl/fl}* or wild type) for *Ccn2* in Col1a2-expressing fibroblasts were injected with B16F10 melanoma cells 2 weeks after induction of Cre recombinase. In this model, host cells are permanently labeled with either GFP or tdTomato, whereas tumor-derived cells express neither GFP nor tdTomato. To test the hypothesis that the induction of α-SMA–expressing CAFs might be occurring via a Sox2-expressing intermediate in the host tumor stroma, it was examined whether, in principle, there

existed any cells in our tumor model that labeled with GFP that were also expressing both Sox2 and α-SMA. Tumors were allowed to grow for 14 days after detection of a palpable tumor. Mice were sacrificed, and tissue sections were stained for GFP (to detect cells derived from Col1a2-expressing fibroblasts at the time of tamoxifen injection and before the s.c. injection of tumor cells), Sox2 (to detect progenitor-like cells), and α-SMA (to detect CAFs; ie, myofibroblasts) (Figure 1, A and B).

GFP was readily detected in the tumor stroma of both wild-type and knockout mice, indicating that these cells were host derived and contained an active Col1a2 promoter at the time of tamoxifen injection (Figure 1C). In wild-type mice, most GFP⁺ cells also expressed either Sox2 (Figure 1D) or α-SMA (Figure 1E). Notably, a subset of GFP⁺ cells coexpressed both Sox2 and α-SMA (Figure 1F). In contrast, in the tumor stroma of *Ccn2*-deficient mice, cells coexpressing GFP and α-SMA were undetected, resulting in an absence of cells triple positive for GFP, Sox2, and

Table 1 Microarray Was Performed on SKPs Cultured from *Ccn2^{fl/fl}* and *Ccn2^{-/-}* Mice before and after 24 Hours of Treatment with 0.5% FBS to Induce Myofibroblast Differentiation

Gene name	Gene symbol	Fold change
Extracellular matrix		
Matrix metalloproteinase 9	<i>Mmp9</i>	1.83033
Matrix metalloproteinase 1a	<i>Mmp1a</i>	1.77374
Connective tissue growth factor	<i>Ctgf</i>	-3.03684
Tissue inhibitor of metalloproteinase 3	<i>Timp3</i>	-1.77853
ADAMTS-like 1	<i>Adamts1</i>	-1.69146
Collagen, type XII, α 1	<i>Col12a1</i>	-1.42349
Aggrecan	<i>Acan</i>	-1.41547
Cytoskeleton		
Myosin, light chain 9	<i>My19</i>	-2.38173
Actin, α 2, smooth muscle	<i>Acta2</i>	-1.64337
Transgelin	<i>Tagln</i>	-1.52242
Actin, α 1, skeletal muscle	<i>Acta1</i>	-1.52047
A kinase anchor protein 12	<i>Akap12</i>	-1.51423
Nexilin	<i>Nexn</i>	-1.41814
Cell adhesion		
Connective tissue growth factor	<i>Ctgf</i>	-3.03684
Sorbin and SH3 domain containing 1	<i>Sorbs1</i>	-1.96531
LIM and senescent cell antigen—like domains 2	<i>Lims2</i>	-1.68726
Multiple EGF-like domains 10	<i>Megf10</i>	-1.59757
Thymus cell antigen 1, θ	<i>Thy1</i>	-1.58788
Contactin 1	<i>Cntn1</i>	-1.50956
Dermatopontin	<i>Dpt</i>	-1.45043
Integrin α 11	<i>Itga11</i>	-1.41949
Cell migration		
Connective tissue growth factor	<i>Ctgf</i>	-3.03684
Nerve growth factor receptor	<i>Ngfr</i>	-1.50912
Epidermal growth factor receptor	<i>Egfr</i>	-1.42589
Integrin α 11	<i>Itga11</i>	-1.41949

Cluster analysis using DAVID Functional Annotation Software version 6.7 showed alterations in cytoskeleton, extracellular matrix, cell adhesion, and cell migration gene clusters in *Ccn2^{-/-}* versus *Ccn2^{fl/fl}* SKPs in response to FBS. A negative fold change value indicates lower expression in *Ccn2^{-/-}*, whereas a positive value indicates increased expression in *Ccn2^{-/-}*.

ADAMTS, a disintegrin and metalloproteinase with thrombospondin motifs; EGF, epidermal growth factor; FBS, fetal bovine serum; SKP, skin-derived precursor.

α -SMA (Supplemental Figure S1). *Ccn2*-deficient mice also displayed a marked reduction in the overall proportion of Sox2-expressing GFP⁺ cells ($P < 0.05$). When measuring the proportion of GFP⁺ cells that were α -SMA⁺ and Sox2⁺, there was no significant difference between wild-type and knockout mice ($P = 0.97$) (Figure 1G). These data suggested that, in a murine model of melanoma, host stromal cells derived from Col1a2-expressing cells (ie, synthetic fibroblasts) are plastic and can express the progenitor cell marker Sox2 *in vivo*. Moreover, expression of *Ccn2* appeared necessary for the activation and differentiation of these Sox2-expressing cells into α -SMA-expressing CAFs (myofibroblasts) in the stroma.

A Subset of Multipotent Dermal Skin Progenitor Cells (SKPs) *in Vitro* Is Derived from Col1a2-Expressing Fibroblasts

On the basis of these observations, it was tested if GFP-expressing (ie, synthetic) dermal fibroblasts could acquire progenitor cell—like features *in vitro*, and if their ability to differentiate into myofibroblasts was CCN2 dependent. To test this hypothesis, mouse dermal cells were cultured in stem cell media containing basic fibroblast growth factor, which results in the formation of free-floating spheroid aggregates that express Sox2 and nestin (Figure 2A). These spheroids, which can be readily isolated directly from the dermis or from established fibroblast monolayer cultures, are well described in the literature, possess multipotent differentiation potential, and are often referred to as SKPs.^{18–20}

To test if cells derived from synthetic fibroblasts could display progenitor cell—like features, it was tested if cells labeled postnatally with GFP using a fibroblast-specific Col1a2-derived promoter/enhancer (ie, cells derived from synthetic fibroblasts) could be incorporated into SKP spheroids and differentiate into other cell types *in vitro*. Alternatively, it was tested if SKPs were derived from dermal cells that had previously expressed Sox2 *in vivo*. Accordingly, SKP spheroids were cultured from the dermis of Col1a2-CreER(T); Rosa26mTmG or Sox2-CreER(T); Rosa26mTmG promoter mice, where Col1a2- or Sox2-expressing cells were permanently labeled with GFP 3 weeks after birth, respectively. All other cells were labeled with tdTomato. Visualization of promoter activity in skin demonstrated Col1a2 lineage labeling in most dermal fibroblasts (Figure 2B); when fibroblasts were cultured *in vitro* from dermal and s.c. tissue, approximately 25% of monolayer cells expressed GFP (Supplemental Figure S2). Conversely, Sox2 lineage labeling was largely seen in the hair follicle dermal papilla and dermal sheath, and in the rare cell at the dermal-epidermal junction adjacent to hair, likely representing Merkel cells (Figure 2E), consistent with the known expression pattern of Sox2 in skin.³² Thus, the Col1a2 and Sox2 promoters were faithfully expressed. Interestingly, it was found via fluorescence microscopy and quantified via flow cytometry (Figure 2, C, D, and F–H) that a large portion of SKP cells originated from Col1a2-expressing fibroblasts, whereas few were derived from cells that expressed Sox2. Indeed, flow cytometry further confirmed that Sox2 was readily induced in cells of Col1a2 origin in response to culturing in stem cell medium (Figure 2I).

Standard phenotypic assays have been established to assess the capability of SKPs to differentiate into neural and mesodermal lineages.^{24,33,34} Neuronal and adipogenic differentiation can be detected by the appearance of β III-tubulin protein expression and the presence of oil red O-staining lipid droplets, respectively, whereas

Figure 7 Loss of Ccn2 alters cytoskeleton, extracellular matrix, cell adhesion, and cell migration gene expression to impair myofibroblastic differentiation. Microarray was performed on skin-derived precursors (SKPs) cultured from *Ccn2^{fl/fl}* and *Ccn2^{-/-}* mice before and after 24 hours treatment with 0.5% fetal bovine serum (FBS) to induce myofibroblast differentiation. Real-time quantitative PCR was performed to verify the differential expression of *Tagln*, *Itga11*, *Thy1*, *Egfr*, *Mmp9*, and *Timp3*. Graphs represent RNA fold change. Data were calculated relative to sample 1 of *Ccn2^{fl/fl}* SKPs treated with FBS. Data are expressed as means $\pm$ SEM. $N = 3$ independent experiments. * $P < 0.05$, ** $P < 0.01$ versus *Ccn2^{fl/fl}* + FBS (*t*-test).

myofibroblast differentiation is measured by the appearance of α -SMA protein.^{24,33,35,36} To test whether the subset of fibroblast-derived SKPs possessed multipotent differentiation potential, SKPs cultured from *Colla2*-CreER(T); *Rosa26mTmG* promoter mice were exposed to various differentiation media; and it was found that differentiated cells expressing myofibroblast, neuronal, and adipocyte markers could all be traced back to a *Colla2* origin via GFP expression (Figure 3). These data collectively support the notion that fibroblasts are highly plastic and can acquire aspects of a progenitor cell phenotype, and they further suggest that SKP spheroids can be used to model fibroblast plasticity similar to what is seen in melanoma.

The Ability of SKPs to Differentiate into Myofibroblasts in Response to Serum Is Impeded by Focal Adhesion Kinase/Src or Myocardin-Related Transcription Factor/Serum Response Factor Inhibition and Is Dependent on CCN2

Mechanotransduction, mediated through focal adhesions, has been shown, in other systems, to mediate myofibroblast differentiation and to be important for the initiation and maintenance of fibrogenic responses *in vitro* and *in vivo* as well as in the acquisition of drug resistance in melanoma.^{7,8,37} In response to serum, SKP spheroids attach to and spread on the surrounding matrix, forming a monolayer.

To assess for pathways essential for SKP differentiation into myofibroblasts, SKPs were treated with FBS to induce myofibroblast differentiation in the presence or absence of the Src-family kinase inhibitor PP2, the focal adhesion kinase inhibitor PF228, or the myocardin-related transcription factor/serum response factor inhibitor CCG-1423.^{35–37} These inhibitors were chosen as focal adhesion kinase/Src mediates adhesive signaling, resulting from integrin-mediated attachment to ECM; and the myocardin-related transcription factor/serum response factor pathway mediates responses to mechanotransduction.^{37–39} All three inhibitors blocked spreading of spheres into monolayers and prevented myofibroblast differentiation, as revealed by immunofluorescence and real-time PCR analysis examining expression of α -SMA and CCN2 (Figure 4), suggesting that cell attachment to ECM mediates progenitor cell myofibroblast differentiation.

CCN2 Is Required for the Ability of SKPs to Differentiate into Myofibroblasts

The role of CCN2 in SKP differentiation was further elucidated. Although SKPs could readily be isolated from both wild-type CCN2-deficient skin, CCN2 deficiency impaired the ability of SKPs to differentiate into α -SMA-expressing cells (ie, myofibroblasts), as shown previously,¹⁹ as visualized by immunofluorescence analysis (Figure 5A). This effect was independent of cell

Figure 8 *CCN2* expression in human melanoma tumor single cells. **A:** Heat map representation of expression of select genes in 106 human cancer-associated fibroblast (CAF) single cells from melanoma tumors depicts *CCN2* expression in a particular subset of CAFs (<https://www.ncbi.nlm.nih.gov/geo/>; accession number GSE115978). Columns represent single CAF cells, and rows represent Z-score mRNA expression (TPM/10). Horizontal bar denotes three sample clusters. **B:** Boxplot shows *CCN2* gene expression values (TPM/10) in melanoma tumor single cells by cell type [B cells, CAFs, endothelial cells, macrophages, malignant cells, natural killer (NK) cells, CD4⁺ T cells, CD8⁺ T cells, and other T cells]. Boxes represent interquartile ranges, and points represent individual sample values (triangles depict outliers). Please note that the data set analyzed is distinct from that previously analyzed.¹⁷ **C:** Kaplan-Meier curves of overall survival (OS) and disease-free survival (DFS) for patients with high CAF-specific *CCN2* scores and those with low CAF-specific *CCN2* scores. A nine-gene set of CAF-specific genes correlating with *CCN2* (*ZFHX4*, *DCN*, *ITGA11*, *COL6A3*, *COL1A1*, *ITGBL1*, *COL8A1*, *INHBA*, and *MEG3*), as described in *Materials and Methods*, was analyzed. *n* = 818 B cells (**B**); *n* = 106 CAFs (**B**); *n* = 104 endothelial cells (**B**); *n* = 420 macrophages (**B**); *n* = 2018 malignant cells (**B**); *n* = 92 NK cells (**B**); *n* = 856 CD4⁺ T cells (**B**); *n* = 1759 CD8⁺ T cells (**B**); *n* = 706 other T cells (**B**); *n* = 59 OS for patients with high CAF-specific *CCN2* scores (**C**); *n* = 51 DFS for patients with high CAF-specific *CCN2* scores (**C**); *n* = 21 OS for patients with low CAF-specific *CCN2* scores (**C**); *n* = 16 DFS for patients with low CAF-specific *CCN2* scores (**C**). ****P* < 0.001 versus high CAF-specific *CCN2* score (OS) (log-rank test); ††*P* < 0.01 versus high CAF-specific *CCN2* score (DFS). TPM, transcript-per-million.

proliferation, demonstrated by quantification of Ki-67 staining. Results were further confirmed by Western blot and real-time quantitative PCR analysis examining α -SMA and *CCN2* expression, demonstrating significant decreases in both (Figure 5, B and C). SKPs exhibit baseline expression of α -SMA before differentiation in serum, as the protein is expressed by cells on the outer surface of the spheroids. The standard procedure in the literature for measuring neural differentiation and adipogenic differentiation is the appearance of β III-tubulin protein expression and the presence of lipid droplets, respectively; and myofibroblast differentiation is measured by the appearance of α -SMA protein,^{18–20,33} as these are measures of the overall phenotype of the cells. Differentiation into neuronal or adipocyte-like cells was unaffected by loss of *CCN2*, as demonstrated by β III-tubulin expression via immunofluorescence and Western blot analysis (Figure 6A) and quantification of oil red O staining of lipid droplets (Figure 6B), respectively.

Microarray analysis of *Ccn2*^{-/-} versus *Ccn2*^{fl/fl} SKPs treated with FBS revealed 145 differentially expressed

genes (Supplemental Table S1). Cluster analysis was performed using the DAVID Functional Annotation Tool, which demonstrated alterations in gene clusters related to the extracellular matrix, cytoskeleton, cell adhesion, and cell migration (Table 1). Notably, five genes encoding contractile proteins were down-regulated (α -skeletal muscle actin, α -smooth muscle actin, myosin light chain 9, myosin light chain 10, and transgelin). Real-time quantitative PCR was performed to verify the differential expression of select genes associated with each altered gene cluster (Figure 7). In *Ccn2*-deficient SKPs, significant decreases in expression in response to serum were confirmed for transgelin (*TAGLN*), integrin α -11 (*ITGA11*), Thy1 cell surface antigen (*THY1*), epidermal growth factor receptor (*EGFR*), and metalloproteinase inhibitor 3 (*TIMP3*), whereas significantly increased expression was demonstrated for matrix metalloproteinase 9 (*MMP9*). Briefly, *TAGLN* encodes an actin-binding protein that is an early marker of smooth muscle differentiation.⁴⁰ *ITGA11* is a profibrotic gene involved in mediating ECM stiffness and metastasis in cancer

models.⁴¹ *THY1* mediates cell adhesion and is also a mesenchymal stem cell marker whose expression in cancer is associated with disease progression.⁴² *EGFR* plays a critical role in transforming growth factor- β -dependent fibroblast to myofibroblast differentiation and also drives cancer cell proliferation when overexpressed.⁴³ *MMP9* and its inhibitor *TIMP3* are known to play pivotal roles in regulating extracellular matrix degradation, thereby mediating physiological processes of wound healing and angiogenesis as well as driving pathologic processes, including cancer.⁴⁴

Collectively, these results emphasize the selectivity of *CCN2* action and that *CCN2* may represent a specific profibrotic mediator through its requirement for progenitor cells to differentiate CAFs into activated cells through regulation of extracellular matrix, cytoskeleton, cell adhesion, and cell migration genes.

In Melanoma Patients, *CCN2* Expression in CAFs Correlates with Expression of a Fibrotic Signature

In cancers, including in melanoma, α -SMA-expressing CAFs are a heterogeneous population, composed of subsets differing on the basis of their expression profile. To provide a clinical context for these studies, a single-cell RNA-sequencing data set was used to determine which genes correlated with *CCN2* expression in CAF single cells from human melanoma tumors. *CCN2* expression correlated with expression of *FAP* expression and several profibrotic genes, including *ITGA11*, *COL1A1*, *P4HA2*, and *LOX* (Supplemental Table S2 and Figure 8A). These genes are known to contribute to the generation of a stiff, fibrotic ECM, and, hence, to drug resistance.^{8,45,46} As expected, *ACTA2* (the gene encoding α -SMA) expression correlated with a broader set of CAFs (Figure 8A).

When the expression of *CCN2* was examined by cell type in melanoma tumors, median *CCN2* expression was highest in CAFs, similar to what was found previously using a smaller data set (Figure 8B).¹⁷ Biologically, *CCN2* produced by CAFs may have a different functional impact than *CCN2* expressed by other cell types in the tumor microenvironment. However, as *CCN2* is expressed in additional cell types in the melanoma tumor environment, it is difficult to associate CAF-specific *CCN2* expression with patient outcomes using bulk tumor RNA sequencing. Therefore, a surrogate score was generated for CAF-specific *CCN2* expression. Using single-cell RNA sequencing in CAF single cells (<https://www.ncbi.nlm.nih.gov/geo>; accession number GSE115978),⁴⁷ a cohort of nine genes (*ZFHX4*, *DCN*, *ITGA11*, *COL6A3*, *COL1A1*, *ITGBL1*, *COL8A1*, *INHBA*, and *MEG3*) was identified that strongly correlated with *CCN2* expression in CAF cells, and was minimally expressed in other cell types within the tumor microenvironment. These nine genes were used as a surrogate score for CAF-specific *CCN2* expression in bulk tumor RNA sequencing. CAF-specific *CCN2* scores were

dichotomized with receiver operating characteristic curves to ascertain the optimal cutoff for the end point of overall survival censorship. In this data set, a high CAF-specific *CCN2* score was associated with poor overall and disease-free survival ($P < 0.01$) (Figure 8C). These data are consistent with the notions that *CCN2* is expressed in a profibrotic subset of CAFs and, hence, may mediate a profibrotic and prometastatic phenotype in melanoma patients and targeting *CCN2* may be a novel therapeutic strategy.

Discussion

Cancer biologists have proposed a cancer stem cell theory of cancer, which suggests that a subpopulation of self-renewing tumor cells is responsible for tumorigenesis.⁴⁸ In human melanoma, Sox2, an embryonic neural crest stem-cell transcription factor, is abundantly expressed,²⁰ with the 3-year median survival for patients with Sox2-expressing metastatic tumors being 145 days less than those with metastatic tumors that do not express Sox2.⁴⁹ CAFs, one of the most prominent cell types present in the tumor stroma, and of likely heterogeneous origin, are now recognized as playing an active role in cancer progression and metastasis, and have been implicated in providing a survival niche for cancer stem cells.^{50–53} CAFs have also been proposed to possess inherent stemness characteristics similar to mesenchymal stem cells, and Sox2 has been noted to be expressed in tumor stroma cells of human melanoma²⁰ and colorectal cancer.^{50–53}

In this study, using lineage tracing in a murine model of melanoma, it was shown that resident Col1a2-expressing (ie, synthetic) dermal fibroblasts recruited adjacent to tumor can become induced to express the stem cell marker Sox2 (also expressed by tumor cells) and that these cells are activated into an α -SMA-expressing CAF phenotype, all in a *CCN2*-dependent manner. It should be emphasized that by using GFP to specifically label host fibroblasts, this process could be distinguished from tumor cells potentially undergoing epithelial-mesenchymal transition⁵⁴ or host endothelial cells undergoing endothelial-mesenchymal transition,⁵⁵ which in other models have also been shown to express α -SMA and have been proposed to contribute to CAF populations.

Col1a2-expressing dermal fibroblasts were shown to be capable of being induced to express Sox2 when cultured as multipotent SKP spheroids. Lineage tracing experiments demonstrated that these cells could differentiate into neuronal, adipocytic, and myofibroblastic phenotypes. In this model, *CCN2* was required for the differentiation of progenitor cells into contractile myofibroblasts through regulation of extracellular matrix, cytoskeleton, cell adhesion, and cell migration genes. These data support the notion that host fibroblast-derived CAFs are capable of exhibiting

stem cell–like characteristics and emphasize an underappreciated phenotypic plasticity of dermal fibroblasts. Specifically, when first described, SKPs were thought to originate from endogenous Sox2-expressing hair follicle dermal papilla and dermal sheath.³⁶ Alternatively, it has been suggested that a subpopulation of progenitor cells exists in the dermis, and this subpopulation is being selected for in stem cell media.^{24,33,35,56} However, the data showing that, in tumor stroma, cells derived from Col1a2-expressing fibroblasts can acquire the expression of Sox2 suggest that cultured SKPs can partly originate from Col1a2-expressing fibroblasts. These data are consistent with a prior experiment suggesting that SKPs can be isolated from primary cultures of dermal fibroblasts; however, in that study, it was unclear if synthetic (ie, collagen-producing) fibroblasts could actively behave like progenitor cells or if an already existing progenitor cell population was being selected.^{57,58} In this study, the ability of fibroblasts to behave like progenitor cells *in vivo* was triggered by the presence of the tumor microenvironment. It remains unclear if this property is unique to dermal fibroblasts or if it might be also observed in other tissues, although the latter seems likely.

CCN2 is under clinical development as a therapeutic target in idiopathic pulmonary fibrosis and pancreatic cancer.^{13–15,59} Recent evidence has emerged that targeting CCN2 may also be of value in melanoma.^{16,17,60} As a member of the CCN family of matricellular proteins, CCN2 has limited *in vitro* activity as a proadhesive molecule; however, because of its ability to interact with a wide variety of proteins and receptors, CCN2 has profound effects in development and pathologic processes *in vivo*.⁶¹ Uncovering these *in vivo* roles has involved the use of genetic (whole-body and conditional knockout) approaches.^{62,63} Previously, in a mouse model of bleomycin-induced skin fibrosis,¹⁹ and now in a model of cutaneous melanoma, we have shown that CCN2 is likely necessary for the appearance of myofibroblasts involving progenitor cell–like intermediates. Conversely, this mechanism may not play an appreciable role in cutaneous wound healing,^{64–66} suggesting that CCN2 represents a specific target for pathologic fibrosis through differentiation of various types of precursor cells into myofibroblasts. Consistent with this notion, in CAFs of melanoma patients, CCN2 expression correlated with a subset of CAFs, expressing specific fibrogenic genes, including *ITGA11*, which negatively correlates with survival. Conversely, the related protein CCN1 appears to mediate collagen fiber alignment and stability in fibrotic conditions.⁶⁷

It is interesting to note that in a murine model of melanoma, there were fewer fibroblasts expressing Sox2 in CCN2-deficient stroma. Conversely, fibroblasts *ex vivo* were able to form multipotent SKP spheroids in the absence of CCN2. These results suggest that the ability of fibroblasts to express Sox2 does not absolutely require CCN2; rather, CCN2 is required for the differentiation of fibroblasts to α -SMA–expressing CAFs via a Sox2

(progenitor cell–like) intermediate. Alternatively, as there were fewer Sox2-expressing cells in the absence to CCN2, these results also suggest that the mechanism of how fibroblasts acquire a Sox2⁺ phenotype may differ *in vitro* versus *in vivo*. Several different methods have been developed that show dermal fibroblasts transition into progenitor cells/neuropheres.^{20,53,54} *In vivo*, it is likely that activation of fibroblasts to a Sox2-expressing phenotype involves cross talk from tumor (melanoma) cells. CCN2 might represent a competency factor, allowing fibroblasts to maximally respond to these yet unidentified signals.

Overall, the current data show that cells derived from Col1a2-expressing cells are major contributors to SKP spheres and progenitor-like tumor stromal cells that differentiate into CAFs. Collectively, these data point to the fact that at least a subset of fibroblasts, notably those derived from type I collagen-expressing cells, are plastic, can readily acquire a progenitor cell phenotype, and may in the future represent a valuable source of progenitor cells for therapeutic applications. Moreover, the ability of collagen-expressing fibroblasts *in vivo* to acquire a progenitor cell–like phenotype (eg, the acquisition of Sox2 expression) may indicate a pathologic phenotype and an intermediate in the differentiation of fibroblasts to myofibroblasts. That Sox2-expressing cells represent a transient intermediate cell type in CAF differentiation is consistent with prior studies performed in CAFs derived from human colorectal tumors, which have demonstrated expression of Sox2 and other stem cell markers, in association with promigratory effects,⁵² high proliferation potential, and poor prognosis.⁵¹ Furthermore, Sox2 is a recognized marker of both cancer and mesenchymal stem cells; thus, these data are consistent with the notion that mesenchymal stem cells or mesenchymal stem cell–like cells play roles in fibroproliferative responses, including in cancers.^{68–72} Finally, these data suggest that cultured SKPs have a predominantly fibroblast origin and that the use of SKPs appears to be a useful model system for studying fibroblast plasticity in melanoma. Intriguingly, these data extend recent lineage tracing analysis showing mesenchymal precursors (Twist2-expressing cells) contribute to SKPs and can generate a peripheral neural cell type believed to derive directly only from the neural crest.⁵⁸ Finally, these data suggest the intriguing possibility that at least a subset of so-called fibroadipogenic precursors, which are proposed to play a key role in fibroproliferative disorders,⁵⁶ may be synthetic fibroblasts.

In summary, these data collectively suggest that fibroblasts are plastic *in vitro* and *in vivo* and can contribute to the appearance of myofibroblasts, possibly through a progenitor cell intermediate and CCN2. These observations may be of particular relevance in pathologic fibroproliferative conditions involving myofibroblast differentiation, and are consistent with the notion that targeting CCN2 represents a novel therapeutic target.

Supplemental Data

Supplemental material for this article can be found at <https://doi.org/10.1016/j.ajpath.2019.09.006>.

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