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Intracellular Notch1 Signaling in Cancer-Associated Fibroblasts Dictates the Plasticity and Stemness of Melanoma Stem/Initiating Cells

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ABSTRACT

Cancer stem cells (CSCs) play critical roles in cancer initiation, metastasis, recurrence, and drug resistance. Recent studies have revealed involvement of cancer-associated fibroblasts (CAFs) in regulating CSCs. However, the intracellular molecular mechanisms that determine the regulatory role of CAFs in modulating the plasticity of CSCs remain unknown. Here, we uncovered that intracellular Notch1 signaling in CAFs serves as a molecular switch, which modulates tumor heterogeneity and aggressiveness by inversely controlling stromal regulation of the plasticity and stemness of CSCs. Using mesenchymal stem cell-derived fibroblasts (MSC-DF) harboring reciprocal loss-of-function and gain-of-function Notch1 signaling, we found that MSC-DF$^{Notch1-/-}$ prompted cocultured melanoma cells to form more spheroids and acquire the phenotype (CD271$^+$ and Nestin$^+$) of melanoma stem/initiating cells (MICs), whereas MSC-DF$^{N1IC+/+}$ suppressed melanoma cell sphere formation and mitigated properties of MICs. MSC-DF$^{Notch1-/-}$ increased stemness of CD271$^+$ MIC, which resultantly exhibited stronger aggressiveness in vitro and in vivo, by upregulating Sox2/Oct4/Nanog expression. Consistently, when cografted with melanoma cells into NOD scid gamma (NSG) mice, MSC-DF$^{Notch1-/-}$ increased, but MSC-DF$^{N1IC+/+}$ decreased, the amounts of CD271$^+$ MIC in melanoma tissue. The amounts of CD271$^+$ MIC regulated by MSC-DF carrying high or low Notch1 pathway activity is well correlated with capability of melanoma metastasis, supporting that melanoma metastasis is MIC-mediated. Our data demonstrate that intracellular Notch1 signaling in CAFs is a molecular switch dictating the plasticity and stemness of MICs, thereby regulating melanoma aggressiveness, and therefore that targeting the intracellular Notch1 signaling pathway in CAFs may present a new therapeutic strategy for melanoma. STEM CELLS *2019;37:865–875*

SIGNIFICANCE STATEMENT

Tumor–stroma interactions play an important role in cancer progression. However, the intracellular molecular mechanisms that determine the regulatory role of cancer-associated fibroblasts (CAFs) in modulating the plasticity and stemness of cancer stem cells (CSCs) remain unknown. This study uncovers that intracellular Notch1 signaling in CAFs is a molecular switch, inversely controlling stromal regulation of the plasticity and stemness of melanoma stem/initiating cells (MICs) and thereby modulating melanoma heterogeneity and aggressiveness. This study also indicates that therapeutic activation of the Notch1 pathway in CAFs can control MIC plasticity and inhibit MIC-mediated melanoma aggressiveness/metastasis. Hence, targeting the intracellular Notch1 signaling pathway in CAFs may present a new therapeutic strategy for melanoma.

INTRODUCTION

Accumulating evidence suggests that diverse tumors are hierarchically organized and a subset of cancer cells possessing stem cell-like properties, referred to as cancer stem cells (CSCs) or tumor-initiating cells (TICs), primarily accounts for tumor initiation, metastasis, drug-resistance, and cancer recurrence [1, 2]. Targeting CSCs has, thus, been proposed as a new paradigm in anticancer therapy. Melanoma is the deadliest form of skin cancer; it is notorious for its tendency to metastasize and for its resistance to most therapies, including drugs targeting oncogenic BRAF^{V600E} [3]. Melanoma stem cells or melanoma-initiating cells (MICs) may be the driving force behind melanoma metastasis/aggressiveness and may contribute to drug-resistance [4–6]. MICs have been reported to express a variety of markers, including CD271 (nerve growth factor receptor), Nestin, ABCB5,

CD133, ALDH1, and JARID1 [7–13], although none of them are consensual markers and expressed simultaneously in a given subpopulation of MICs. Melanoma cells are heterogeneous populations and show a high degree of plasticity. Both CSCs and non-CSCs are plastic and capable of undergoing phenotypic transitions in response to appropriate stimuli. Tumor heterogeneity and tumor cell plasticity are determined not only by the intrinsic properties of tumor cells, but also largely by external influences from the tumor stroma/microenvironment [14–16]. Cell–cell interactions between tumor cells and their stroma or niche have a profound influence on the plasticity of CSCs, including MICs. Stromal fibroblasts, or cancer-associated fibroblasts (CAFs), are major components of stroma in solid tumors and are critically involved in regulating tumor growth, metastasis, recurrence, and drug resistance through secretion of soluble factors, synthesis of extracellular matrix (ECM), release of exosomes, and direct cell–cell interaction [17–22]. Recent studies have revealed that CAFs play pivotal roles in regulating CSCs. For instance, CAFs may stimulate epithelial–mesenchymal transition (EMT)-driven cancer stemness in prostate cancer [23, 24]. In addition, CAFs induce phenotype and self-renewal of CSCs in breast cancer cells by releasing chemokine (C—C motif) ligand 2 (CCL2) [25]. Moreover, CAFs constitute a supporting niche and secrete insulin-like growth factor (IGF)-II to regulate plasticity of lung cancer stemness via the IGF-II/IGF1R signaling pathway [26]. However, the molecular mechanisms that control the regulatory function of CAFs in modulating the plasticity and stemness of CSCs remain largely uncharacterized.

CAFs consist of a heterogeneous population of cells and can be derived from multiple origins, including infiltrated local tissue fibroblasts, recruited bone marrow-derived mesenchymal stem cells (MSCs), and perhaps trans-differentiated epithelial and endothelial cells [27–29]. MSCs are one of the critical and major sources of CAFs [28, 30, 31]. Approximately 40% of the total CAF population within engrafted pancreatic cancers [28] and 60% of CAFs in engrafted ovarian and breast cancers originate from MSC [32]. In addition, thanks to the accessibility of bone marrow MSC, availability of ex vivo/in vitro MSC engineering, and intrinsic tumor-homing capability of MSC; MSC-derived fibroblasts (MSC-DF) may be used as a therapeutic tool or target for cancer therapeutic interventions on the tumor microenvironment (TME). In this study, we used MSC-DF as a type of CAF and explored the intracellular signaling pathway that determines the regulatory function of MSC-DF in modulating the plasticity of MICs and melanoma heterogeneity. We observed a fibroblast-dependent stem-cell-like sphere-forming phenotype in melanoma cells cocultured with MSC-DF. Most importantly, MSC-DF regulation of melanoma cell sphere-formation and induction of MIC markers, $CD271^+$ and $Nestin^+$, is determined inversely by intracellular Notch1 pathway activity. In vitro, loss-of-function (LOF) Notch1 signaling in MSC-DF ($MSC\text{-}DF^{Notch1-/-}$) prompted cocultured melanoma cells to form more spheroids and induced melanoma cells to express MIC markers $CD271^+$ and $Nestin^+$. In contrast, gain-of-function (GOF) Notch1 signaling in MSC-DF ($MSC\text{-}DF^{N1IC+/+}$) inhibited melanoma cell sphere-formation and mitigated expression of MIC markers. Consistently, when cografted with melanoma cells into NOD scid gamma (NSG) mice, $MSC\text{-}DF^{Notch1-/-}$ increased, whereas $MSC\text{-}DF^{N1IC+/+}$ decreased, the amounts of $CD271^+$ MICs in melanoma tissue. Moreover, $MSC\text{-}DF^{Notch1-/-}$ increased stemness of $CD271^+$ MICs by upregulating Sox2/Oct4/Nanog expression and resulted in more aggressive behavior of melanoma cells in vitro and in vivo. These findings uncover intracellular Notch1 signaling in fibroblasts as a molecular switch dictating the plasticity and stemness of MICs, thereby regulating melanoma heterogeneity and aggressiveness, and further justify this pathway as a new target for therapeutic interventions on the TME.

Materials and Methods

Cells and Cell Culture

MSC-DF were generated and characterized as described [33]. Briefly, murine MSCs were enriched by culturing BM-mononuclear cells in MesenCult medium supplemented with *MSC Stimulatory Supplements* (#05502; StemCell Technologies, Vancouver, Canada) for 10 days with periodic medium changes. These MSCs were characterized as $CD73^+/CD105^+/Lin^-$. MSCs were subsequently cultured with complete Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA) for an additional 2 weeks to differentiate into fibroblasts. Derived MSC-DF were transduced with Cre-*ires*-green fluorescent protein (GFP)/lentiviral or GFP/lentiviral vector and sorted by fluorescence-activated cell sorting (FACS), respectively. Human metastatic melanoma cells (C8161 and 1205Lu) were cultured in W489 medium as described [34]. MeWo (ATCC HTB-65) cells were cultured in DMEM with 10% fetal bovine serum (FBS) except for coculture.

Cell Coculture Sphere-Formation and Flow Cytometry

Cell mixtures of $DsRed^+$-melanoma cells ($DsRed^+$/C8161 or $DsRed^+$/1205Lu or $DsRed^+$/MeWo) and various GFP^+-Cre^+/MSC-DF or control GFP^+/MSC-DF were seeded into 6-well plates and cultured in serum-free W489 and DMEM mixture (1:1) or serum-free DMEM (for $DsRed^+$/MeWo coculture with MSC-DF). The optimal cell density per well for the formation of spheroid by different melanoma cells varied slightly: (a) 3×10^4 C8161 + 6×10^4 MSC-DF; (b) 2×10^4 1205Lu + 2×10^4 MSC-DF; and (c) 1×10^4 MeWo + 1×10^4 MSC-DF. $DsRed^+$/melanoma cells started to form three-dimensional (3D) multicellular tumor spheroids in approximately 3 days, peaked at 4–5 days, and sustained the spheroids until 12–14 days. For subsequent analyses, spheroids at day 4 were dispersed into single cell suspension by gentle pipetting in 0.5 mM EDTA solution. Expression of CD271 (using antibody: 5170823097, Miltenyi Biotec, Auburn, CA, USA) or Nestin (using antibody: 560393, BD Biosciences, Rockville, MD) in gated $DsRed^+$/melanoma cells was subsequently analyzed by flow cytometry (FACSAria II; BD Biosciences). To determine melanoma cell proliferation in coculture, spheroids in portions (individual wells) of cocultures at day 4 were dispersed into single cell suspension by gentle pipetting and subject to flow cytometry analysis to count the numbers of melanoma cells ($DsRed^+$).

Cell Proliferation and Migration Assays

Cell growth was tested using the water-soluble tetrazolium (WST) cell proliferation kit (K302-500, BioVision, Mountain Views, CA) according to the manufacturer's instruction. 5×10^3 cells were cultured in 96-well plates overnight with serum-free W489 before the WST assay. Cell migration was tested using BD Falcon FluoroBlok Systems with 8 μm porous membrane insert (BD Biosciences). 5×10^3 cells were suspended in 0.5 ml serum-free W489 and seeded in inserts to migrate toward the low chamber filled with 0.7 ml of W489 containing 10% FBS. After 16 hours, migrated cells ($DsRed^+$) were counted under a fluorescence microscope. Both cell growth

and migration assays were tested in triplicates and assays were repeated three times.

Lentivirus and Cell Transduction

GFP/lentiviral, DsRed/lentiviral, Cre/lentiviral, and Luc2$^+$/lentiviral vectors were constructed as described ([33]. Production of pseudotyped lentivirus and transduction of cells were performed as described [34]. Transduced cells were cultured with a regular complete medium for 3 days, sorted by FACS, and then tested in subsequent analyses.

Mice, Skin Cograft, and Melanoma Skin Xenograft Models

Notch1$^{F/F}$ mice were described [35]. *ROSA*$^{LSL\text{-}N1IC}$ (#006850) mice, which carry *STOP* codon floxed Notch1 intracellular domain (LSL-N1^{IC}) allele knocked-in in *ROSA* mice, were purchased from The Jackson Lab (Bar Harbor, ME). *SCID* mice were purchased from Charles River (Wilmington, MA). *NSG* mice were purchased from the Jackson Lab. Mice were maintained at the DVR animal facility under standard conditions. All animal studies were approved by the University of Miami Institutional Animal Care and Use Committee. To perform cografting experiments, 2×10^6 cell mixtures of melanoma cells (Luc$^+$ and DeRed$^+$/C8161) and MSC-DFs (at a ratio of 1:1) suspended in 0.1 ml of saline were injected (*intradermally*) into the dorsal skin of 8-week-old to 10-week-old male *SCID* mice. Melanoma skin xenograft experiments were conducted by injecting 4×10^4 melanoma cells (Luc$^+$ and DeRed$^+$) suspended in 0.1 ml of saline into the dorsal skin (intradermally) of 8- to 10-week-old male *NSG* mice.

Bioluminescence Imaging of In Vivo Imaging System

D-Luciferin was injected intraperitoneally 10 minutes prior to imaging (150 mg/kg). Mice were anesthetized with isoflurane and the whole body was scanned using in vivo imaging system (IVIS) 200B (PerkinElmer, Waltham, MA) with a 3-minute capture and medium binning. Following the whole-body scan, major organs were harvested and rescanned with a 1 minute capture. Scans were completed within 30 minutes of D-luciferin injection. Bioluminescence signals were quantified using the Living Image software and reported as total light emission within the region of interest (photon/second). A signal was defined as positive when it was greater than the sum of the mean background signal plus 2 SD of the background signal.

Histology, Immunofluorescence, and Immunoblot

H&E and immunofluorescence (IF) were performed as described [36]. Tumor local invasion was evaluated by histological assessment (H&E staining) of 120 tissue sections per group (20 sections/tumor × 6 tumors/group = 120 sections/group) by means of "yes" (+) or "no" (−). Tumor invasion rate was represented in percentage. For IF, following a phosphate-buffered saline wash, sections were blocked with Protein Block (Dako, Carpinteria, CA), then incubated with antibodies (Abs) against CD271 or Luc (ab3125 or ab181640, Abcam, Cambridge, MA, USA), and then with Alexa Fluor 594-anti-mouse IgG (A21203) or Alexa Fluor 594-anti-goat IgG (A11055, Thermo Fisher Scientific, Waltham, MA, USA). Nuclei were stained with DAPI (Sigma–Aldrich, St. Louis, MO). Isotype-matched nonspecific Abs was used as control. Immunoblot was performed as described [37]. Membranes were probed with Abs against Oct-4, Sox-2, and Nanog (#2750, #4900, #4893, Cell Signaling Technology, Danvers, MA, USA) or GAPDH (sc-25,778, Santa Cruz, Santa Cruz, CA, USA) accordingly. Auto photographs of blots were scanned by densitometer (Molecular Dynamics, Caesarea, Israel) to quantify the bands. Relative levels of protein expression (fold) are presented by setting that expressed in CD271$^-$ tumor cells as "1."

Statistical Analysis

Data were statistically analyzed using two-tailed Student's *t* test and is expressed as mean ± SD. The values are considered statistically significant when $p < .05$.

Results

The Intracellular Notch1 Signaling Determines Capability of MSC-DF in Regulating Melanoma Cell Sphere-Formation

As recent studies demonstrated that CAFs play pivotal roles in regulating CSCs [25, 26, 38, 39], we explored the role of MSC-DF in regulating properties of MICs using melanoma cell sphere-formation assay, a commonly used assay to evaluate CSC-like activity in vitro. MSC-DF generated from bone marrow cells of Notch1$^{F/F}$ and ROSA$^{LSL\text{-}N1IC}$ mice exhibited typical spindle-shaped fibroblast appearance and were characterized as α-SMA$^+$, vimentin$^+$, and FSP-1$^+$ cells by immunostaining [33]. MSC-DF were then labeled with GFP by lentiviral vector and GFP$^+$/MSC-DF were sorted by FACS. Because stem cell-like markers for mouse melanoma cells, such as B16, are not well characterized, we investigated three human metastatic melanoma cells—C8161 [40], 1205Lu [34], and MeWo (ATCC HTB-65)—which have different mutation backgrounds. 1205Lu carries the BRAF^{V600E} mutation. C8161 and MeWo cells do not have the BRAF mutation, yet C8161 cells express high levels of CDK4/Kit. Many mouse cells and human cells can communicate with each other, because numerous molecules between two species share high homology. Melanoma cells were prelabeled with DsRed by lentiviral vector and DsRed$^+$/C8161, DsRed$^+$/1205Lu, and DsRed$^+$/MeWo cells were sorted by FACS, respectively. When cultured solely in serum-free tumor medium, three human melanoma cells did not form clusters until day 7 (Fig. 1). When tumor cells were cocultured with MSC-DF in serum-free medium, they formed typical spheroids at approximately day 3, revealing a critical role of MSC-DF in inducing MIC phenotype.

Next, this study addressed the potential role of Notch1 signaling in determining the function of MSC-DF in regulating MIC phenotype, because our previous studies demonstrated that the tumor-regulating function of CAFs could be modulated by the Notch1 signaling pathway [33, 36, 41]. To this end, we generated MSC-DF harboring reciprocal LOF and GOF Notch1 signaling. MSC-DF carrying null Notch1 were created from *Notch1*$^{LoxP/LoxP}$ mice, whereas MSC-DF expressing N1^{IC} (Notch1 intracellular domain, an active Notch1 mutant) were generated from *ROSA*$^{LSL\text{-}N1IC}$ mice. MSC-DF were transduced with GFP/Lentivirus. GFP$^+$ cells were sorted by FACS and subsequently transduced with Cre/Lentivirus to either delete the *Notch1* gene or induce expression of N1^{IC}, resulting in MSC-DF$^{Notch1-/-}$ and MSC-DF$^{N1IC+/+}$, respectively. Corresponding MSC-DF transduced with GFP/Lentivirus as described above, namely MSC-DF$^{Notch1F/F}$ and MSC-DF$^{LSL\text{-}N1IC}$, were used as controls. When melanoma cells were cocultured with MSC-DF$^{Notch1-/-}$, sphere-formation was robustly enhanced in terms of both number and size compared with those cocultured with MSC-DF$^{Notch1F/F}$. In contrast, MSC-DF$^{N1IC+/+}$ significantly suppressed melanoma cells to form

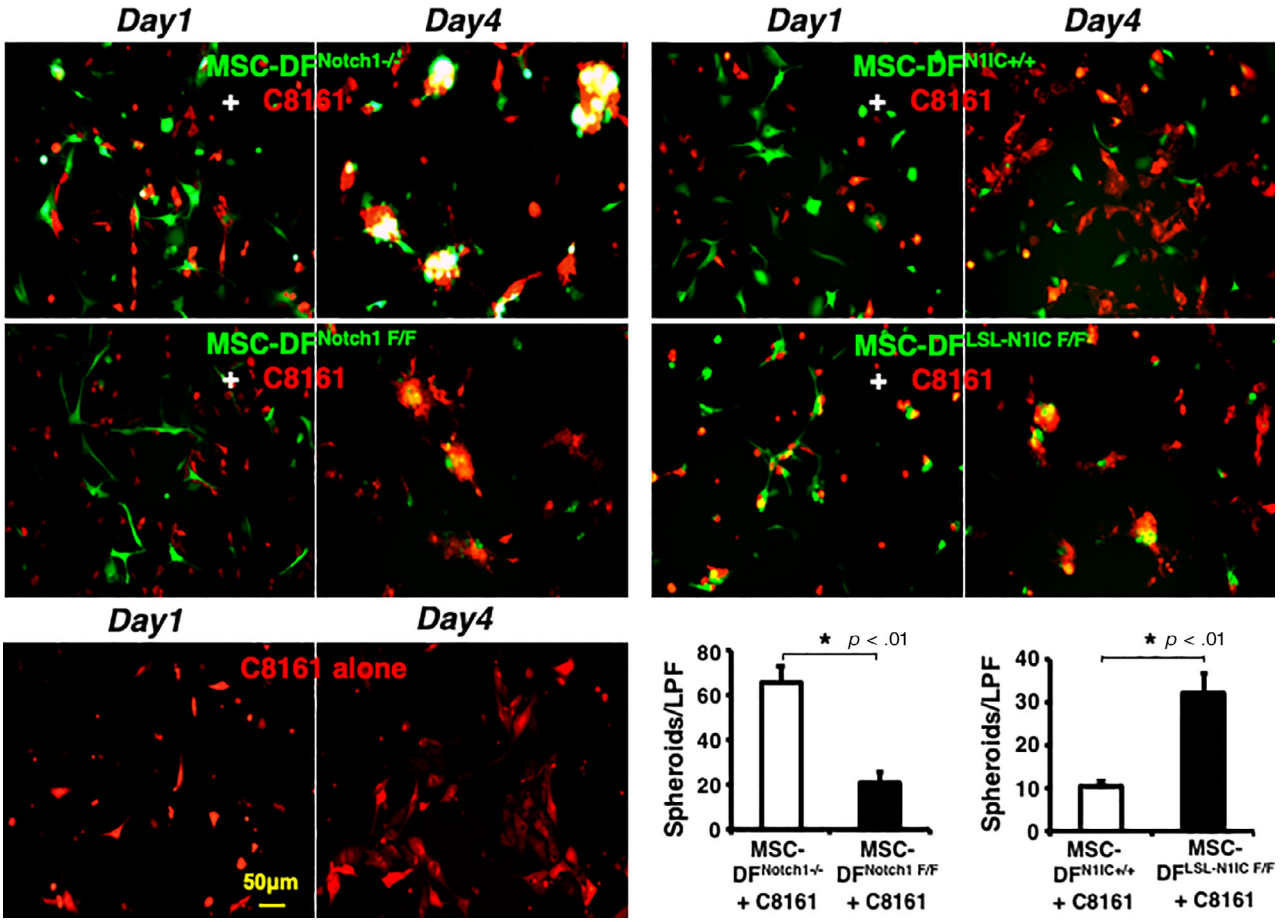

Figure 1. Mesenchymal stem cell-derived fibroblasts (MSC-DFs)—melanoma cell coculture experiments. MSC-DF$^{Notch1-/-}$ robustly promotes sphere-formation of C8161 melanoma cells compared with MSC-DF$^{Notch1F/F}$, whereas MSC-DF$^{N1IC+/+}$ mitigate sphere-formation of C8161 cells compared with MSC-DF$^{LSL-N1IC}$ in cocultures. Melanoma cells (C8161) are DsRed^{+} and MSC-DF are GFP^{+}. C8161 melanoma cells alone do not form spheroids. Quantification of melanoma spheroids is shown. Data were analyzed using the Student's *t* test and are presented as mean ± SD based on three independent experiments.

spheroids compared with MSC-DF$^{LSL-N1IC}$. Results of sphere-formation by cocultured melanoma cell C8161 are shown in Figure 1. See Supporting Information Figures S1 and S2 for the similar results of 1205Lu and MeWo. Results of size of spheroids formed by cocultured melanoma cell C8161 are shown in Supporting Information Figure S3A. At day 4 of cell coculture, spheroids in portions (individual wells) of cocultures were dispersed into single cell suspension by gentle pipetting and subject to flow cytometry analysis to count the numbers of melanoma cells (DsRed^{+}). Consistent with the number of spheroids formed in each coculture condition, MSC-DF$^{Notch1-/-}$ promoted melanoma cell proliferation, whereas MSC-DF$^{N1IC+/+}$ significantly suppressed melanoma cell proliferation (Supporting Information Fig. S3B). These results demonstrated that LOF Notch1 signaling results in MSC-DF promoting melanoma cell sphere-formation, whereas GOF Notch1 signaling causes MSC-DF to suppress MIC phenotype. Therefore, our data indicate that Notch1 signaling serves as a molecular switch dictating the MIC-regulating function of MSC-DF.

The Intracellular Notch1 Signaling Determines Capability of MSC-DF in Regulating Phenotype of MICs

To further study the role of intracellular Notch1 signaling in determining the function of MSC-DF in regulating MIC phenotype, we examined the effect of intracellular Notch1 pathway activity in MSC-DF on regulating expression of MIC markers in melanoma cells. MICs have been reported to express a variety of markers (e.g., CD271, Nestin, ABCB5, CD133, ALDH1, and JARID1 [7–13]), none of which, however, are consensual markers. As mounting evidence suggests that MICs exhibit an undifferentiated and immature phenotype, we examined two markers that are tightly correlated with undifferentiated and immature state—CD271, which is the most commonly used marker for MICs, and Nestin, in melanoma cells. By analysis of CD271 and Nestin expression in gated DsRed^{+}/C8161 melanoma cells dispersed from tumor spheroids at day 4 using FACS, we found that MSC-DF$^{Notch1-/-}$ increased, but MSC-DF$^{N1IC+/+}$ decreased the size of subpopulations of CD271^{+} and Nestin^{+} melanoma cells in tumor spheroids. Melanoma cell C8161 expressed very low levels of CD271 when cultured solely as shown in Figure 2A. However, cocultured MSC-DF could induce a fraction of melanoma cells to express CD271. Importantly, MSC-DF$^{Notch1-/-}$ induced ∼45% melanoma cells to express CD271, significantly higher than ∼26% tumor cells induced by the control MSC-DF$^{Notch1F/F}$. In contrast, MSC-DF$^{N1IC+/+}$ decreased CD271^{+} melanoma cells to ∼14% from ∼22% induced by the control MSC-DF$^{LSL-N1IC}$ (Fig. 2A). Nestin expression in melanoma cells regulated by various MSC-DF exhibited a similar, yet slightly different pattern from CD271 (Fig. 2B). MSC-DF$^{Notch1-/-}$ increased Nestin expression in all melanoma cells (∼100%)

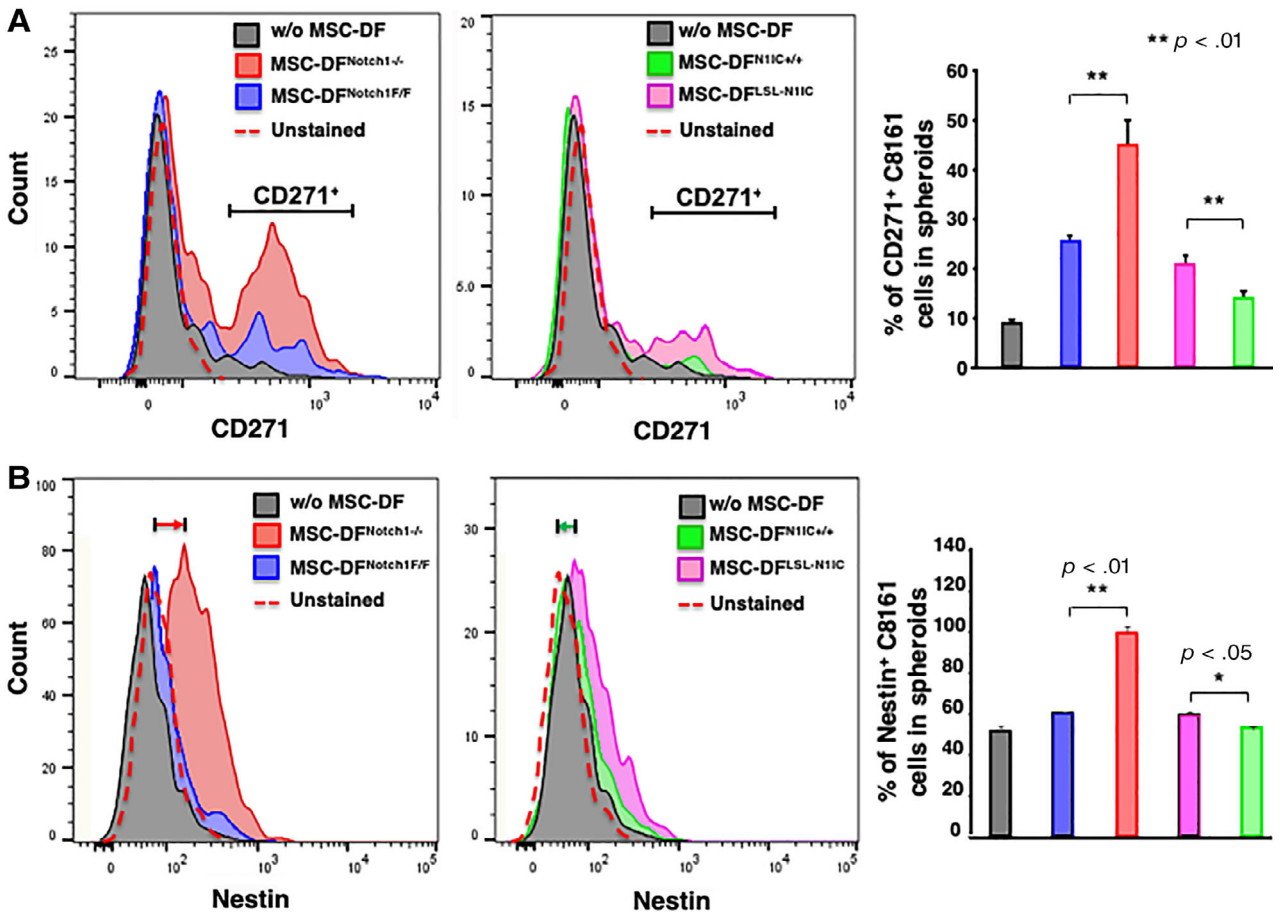

Figure 2. Flow cytometry analysis of melanoma stem/initiating cells phenotypes induced by cocultured mesenchymal stem cell-derived fibroblasts (MSC-DFs) with loss-of-function or gain-of-function Notch1 signaling. MSC-DF$^{Notch1-/-}$ increased, but MSC-DF$^{N1IC+/+}$ decreased, subpopulation of CD271$^+$ and nestin$^+$ C8161 melanoma cells in tumor spheroids. **(A):** Cocultured MSC-DF$^{Notch1-/-}$ could increase, whereas MSC-DF$^{N1IC+/+}$ could decrease, the fraction of melanoma cells that express CD271 compared with solo-cultured C8161 cells and C8161 cocultured with the counterpart control MSC-DF. **(B):** MSC-DF$^{Notch1-/-}$ induced all melanoma cells (100%) to express more nestin compared with the control MSC-DF$^{Notch1F/F}$, whereas MSC-DF$^{N1IC+/+}$ downregulated melanoma cell expression of nestin in comparison to the control MSC-DF$^{LSL-N1IC}$. Data are presented as mean ± SD based on three independent experiments.

compared with control MSC-DF$^{Notch1F/F}$. Conversely, MSC-DF$^{N1IC+/+}$ downregulated melanoma cell expression of Nestin compared with that of the control MSC-DF$^{LSL-N1IC}$. Overall, the capability of MSC-DF harboring reciprocal LOF and GOF Notch1 signaling in inducing tumor cell MIC marker expression is consistent with melanoma cell sphere-formation as described above. Taken together, our results reveal that turning "OFF" Notch1 signaling results in MSC-DF modulating melanoma cells to acquire a stem-cell-like phenotype, whereas turning "ON" Notch1 signaling confers an inhibitory function on MSC-DF in regulation of MIC phenotype. Hence, our data indicate that intracellular Notch signaling in MSC-DF dictates the plasticity of MICs.

The Intracellular Notch1 Pathway Activity in MSC-DF Determines Competency of MSC-DF in Modulating MIC Phenotype, Melanoma Heterogeneity, and Melanoma Metastasis In Vivo

Next, we investigated the effect of intracellular Notch1 pathway activity in MSC-DF on regulating MICs and tumor heterogeneity in mouse melanoma models. Because CD271 and Nestin are not always simultaneously expressed in a given subpopulation of MICs, we tested the CD271$^+$ subpopulation of MICs, as CD271 is the most commonly used marker for MICs. We carried out melanoma cell and MSC-DF cograft experiments. Melanoma cell C8161 was prelabeled with luciferase by transduction with Luc2/Lentivirus. Four groups of cell mixtures of MSC-DF$^{Notch1-/-}$ + Luc2$^+$/C8161 versus MSC-DF$^{Notch1F/F}$ + Luc2$^+$/C8161 and MSC-DF$^{N1IC+/+}$ + Luc2$^+$/C8161 versus MSC-DF$^{LSL-N1IC}$ + Luc2$^+$/C8161 (totally 2×10^6 cells at a ratio of 1:1 per group) suspended in 0.1 ml of saline were injected intradermally into the dorsal skin of 8- to 10-week-old male NSG mice ($n = 6$ per group). Mice were sacrificed 6 weeks after cografting. Primary skin tumors were resected and weighted to evaluate tumor growth. The lungs, hearts, livers, spleens, brains, and kidneys were harvested and scanned instantly by IVIS to detect distant metastasis of melanoma. Although melanoma growth on skin was comparable between MSC-DF$^{Notch1-/-}$ and MSC-DF$^{Notch1F/F}$ groups as well as between MSC-DF$^{N1IC+/+}$ + Luc2$^+$/C8161 and MSC-DF$^{LSL-N1IC}$ + Luc2$^+$/C8161, MSC-DF$^{Notch1-/-}$ increased melanoma local invasion whereas MSC-DF$^{N1IC+/+}$ decreased melanoma local invasion (Fig. 3A). Consistently, MSC-DF$^{Notch1-/-}$ robustly increased lung metastasis [33]. These results indicate that turning "OFF" Notch1 signaling in MSC-DF promotes melanoma invasion and metastasis whereas turning "ON" Notch1 signaling in MSC-DF suppresses melanoma invasion and metastasis.

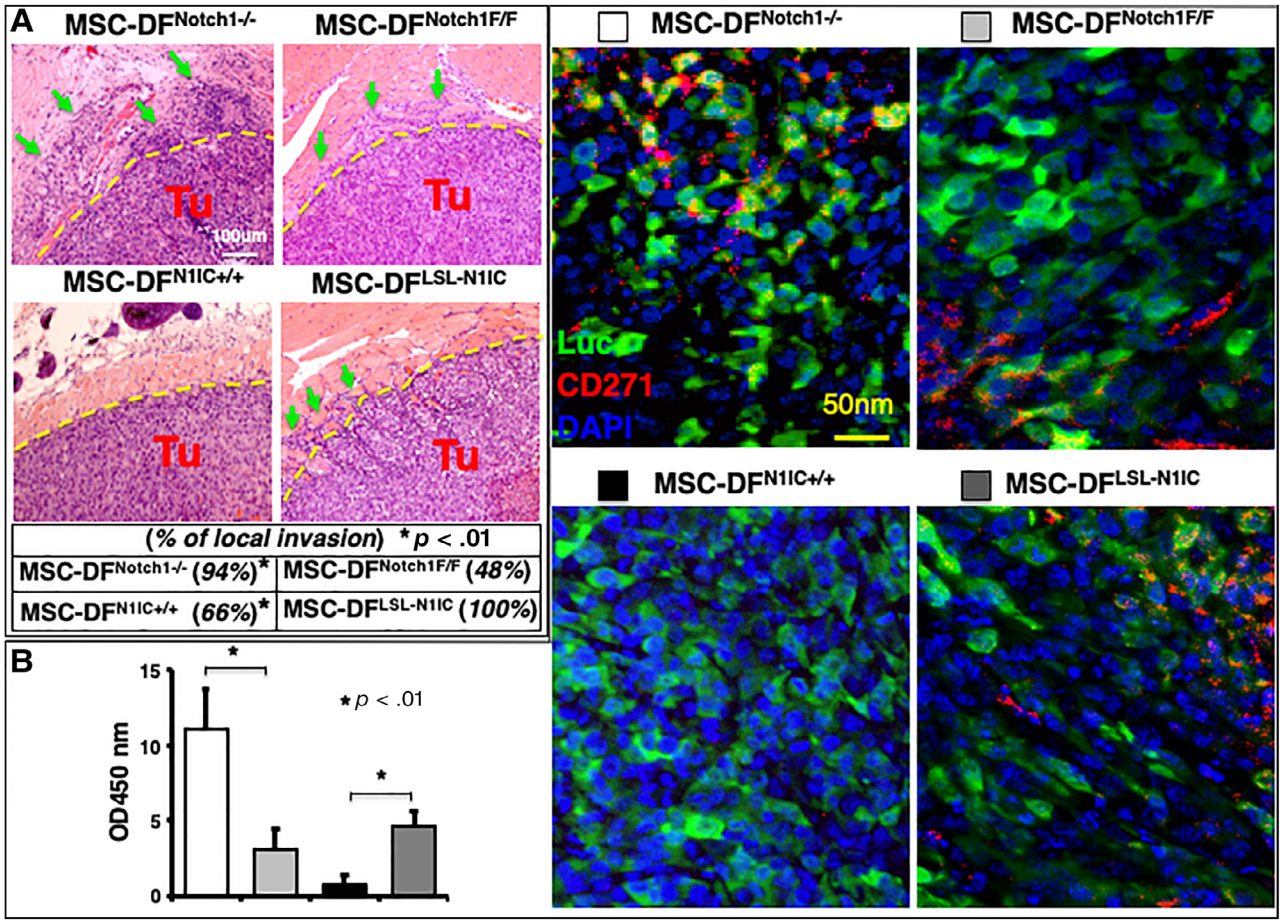

Figure 3. Intracellular Notch1 pathway activity determines competency of mesenchymal stem cell-derived fibroblast (MSC-DF) in modulating melanoma aggressiveness, melanoma stem/initiating cells (MICs) phenotype, and melanoma heterogeneity in vivo. **(A):** Representative H&E images of sections from resected skin melanoma show that MSC-DF$^{Notch1-/-}$ promotes, whereas MSC-DF$^{N1IC+/+}$ suppresses, melanoma local invasion compared with their counterpart controls. Green arrows point to invasive edges of xenografted skin melanoma samples. Percentage of local invasion is summarized (n = 6 tumors per group; *, $p < .01$ between MSC-DF$^{Notch1-/-}$ and MSC-DF$^{N1IC+/+}$ versus their counterpart controls). **(B):** MSC-DF$^{Notch1-/-}$ robustly increased, but MSC-DF$^{N1IC+/+}$ significantly decreased the amount of CD271$^+$ subpopulation of MICs in tumor tissues compared with the control MSC-DF$^{Notch1F/F}$ and MSC-DF$^{LSL-N1IC}$, respectively. Images of immunofluorescence with the combination of three colors (CD271, Luc, and DAPI) are shown.

We examined the amount of CD271$^+$ subpopulations of MICs in the primary lesions of four groups of cografted melanomas. CD271$^+$ subpopulations of MICs were detected by double staining of melanoma sections with anti-Luc and anti-CD271 Abs. We observed that MSC-DF$^{Notch1-/-}$ robustly increased, but MSC-DF$^{N1IC+/+}$ significantly decreased CD271$^+$ subpopulation size of MICs in tumor tissues compared with the controls MSC-DF$^{Notch1F/F}$ and MSC-DF$^{LSL-N1IC}$, respectively. Images of IF with the combination of three colors (CD271, Luc, and DAPI) are shown in Figure 3B, whereas images with individual color are shown in Supporting Information Figures S3–S5. Interestingly, the frequency of CD271$^+$ subpopulations of MICs in the primary lesions of melanomas is well correlated with the capability of melanoma invasion and metastasis, suggesting that CD271$^+$ subpopulation of MICs may be responsible for melanoma metastasis. Hence, our in vivo data is consistent with in vitro data. Overall, the data show that Notch1 activation incites MSC-DFs to promote MIC phenotype, whereas Notch1 inactivation causes MSC-DF to suppress MIC phenotype, demonstrating that intracellular Notch signaling in MSC-DF serves as a molecular switch that inversely dictates the plasticity of MICs.

The Intracellular Notch1 Signaling in MSC-DF Determines Stemness of MICs

MICs possess stemness properties that are regulated by the triad of master regulators of pluripotency Sox2, Oct4, and Nanog [42]. Therefore, we examined levels of the stemness factors Sox2, Oct4, and Nanog induced by MSC-DF$^{Notch1-/-}$ in CD271$^+$ subpopulations of MICs versus CD271$^-$ subpopulations of melanoma cells. CD271$^+$ and CD271$^-$ melanoma cells were sorted by FACS from single cell suspensions dispersed from spheroids formed in MSC-DF$^{Notch1-/-}$ and DsRed$^+$-C8161 cell cocultures at day 4. By immunoblotting analysis, we observed that expression of Sox2, Oct4, and Nanog is drastically greater in CD271$^+$ subpopulations of MICs compared with that in the CD271$^-$ fraction of melanoma cells (Fig. 4). Our data demonstrate that MSC-DF$^{Notch1-/-}$induced CD271$^+$ subpopulations of MICs express high levels of stemness factors, indicating that these cells possess stemness properties.

MSC-DF-Induced CD271$^+$ Subpopulations of MICs Exhibit Aggressive Behaviors In Vitro

MICs are known to possess dynamic proliferative potential and exhibit more aggressive behavior once excited from dormancy.

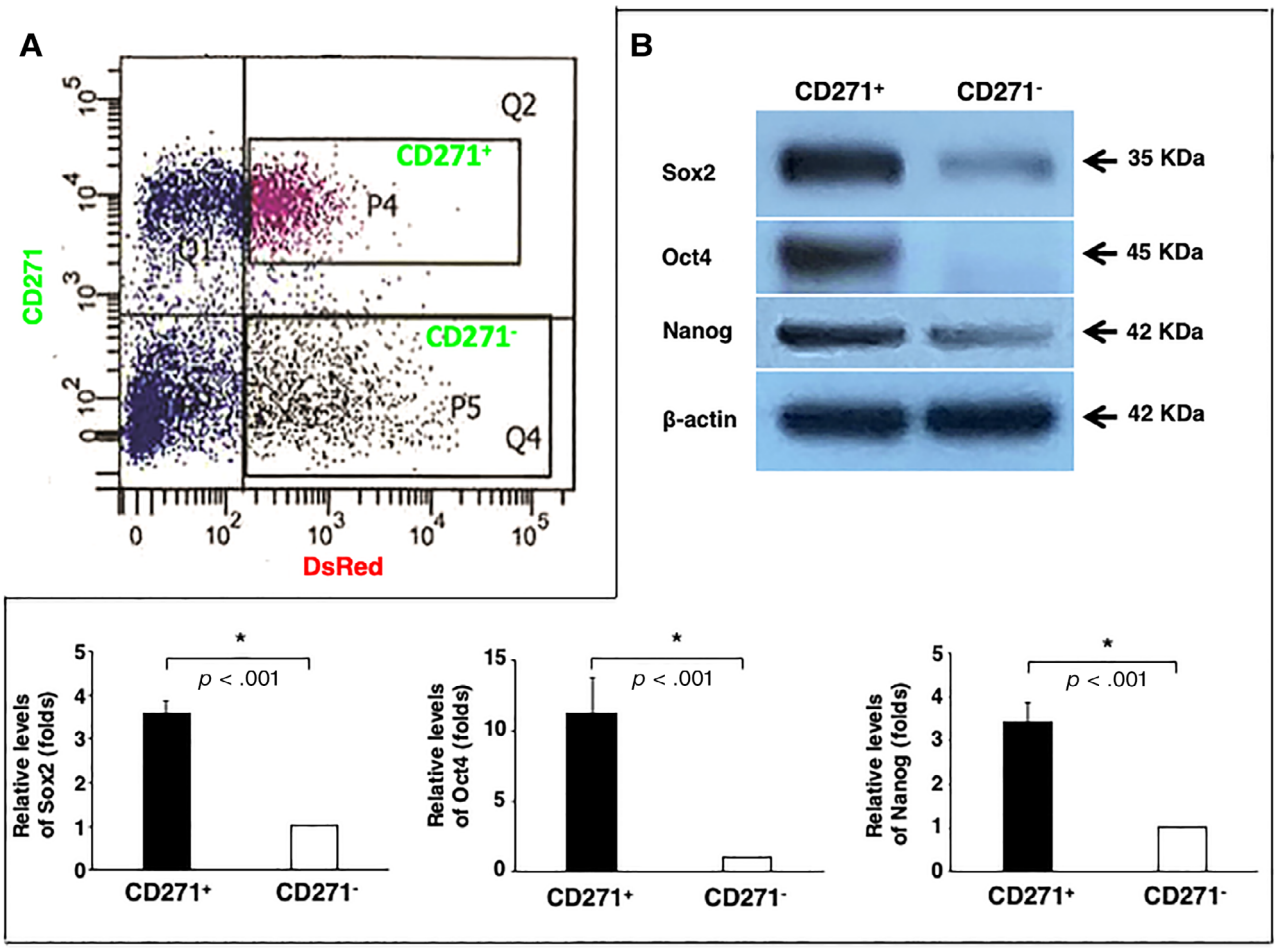

Figure 4. Immunoblotting analysis of expression of Sox2, Oct4, and Nanog in the CD271$^+$ subpopulation of melanoma stem/initiating cells (MICs) compared with the CD271$^-$ fraction of melanoma cells. **(A):** Isolation of the CD271$^+$ subpopulation of MICs and the CD271$^-$ fraction of melanoma cells by FACS. Image shows gated CD271$^+$ and CD271$^-$ fractions of DeRed$^+$/C8161 melanoma cells. **(B):** *Left*: Expression of Sox2, Oct4, and Nanog is drastically greater in the CD271$^+$ subpopulation of melanoma stem/initiating cells compared with the CD271$^-$ fraction of melanoma cells. GAPDG was used as loading control. Molecular weights indicated are the expected sizes of the respective molecules. *Right*: Relative levels of SOX2, Oct4, and Nanog expression. Data are from three different Western blots.

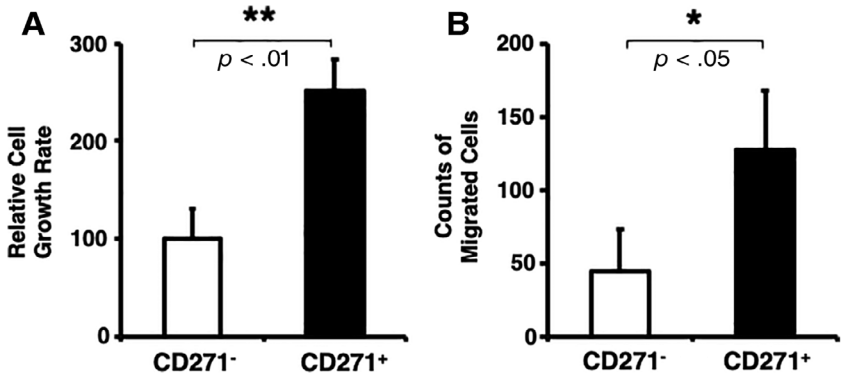

Figure 5. Cell proliferation and migration assays of CD271$^+$ versus CD271$^-$ subpopulations. **(A):** WST cell proliferation assay shows that the CD271$^+$ subpopulation of melanoma stem/initiating cells (MICs) exhibits faster growth rate than the CD271$^-$ subpopulation of melanoma cells. **(B):** The CD271$^+$ subpopulation of MICs migrates faster than the CD271$^-$ subpopulation of melanoma cells in transwell assay. Both assays were tested in triplicates and were repeated three times.

We tested proliferative and migratory properties of CD271$^+$ subpopulations of MICs induced by MSC-DF$^{Notch1-/-}$ and compared these with the properties of the CD271$^-$ fraction of melanoma cells in vitro. We sorted out CD271$^+$ and CD271$^-$ subpopulations by FACS from single cell suspensions dispersed from spheroids formed in MSC-DF$^{Notch1-/-}$ and DsRed$^+$-C8161 cell cocultures at day 4. Cell proliferation was tested by WST cell proliferation assay (BioVision, Mountain Views, CA) and migration was examined by

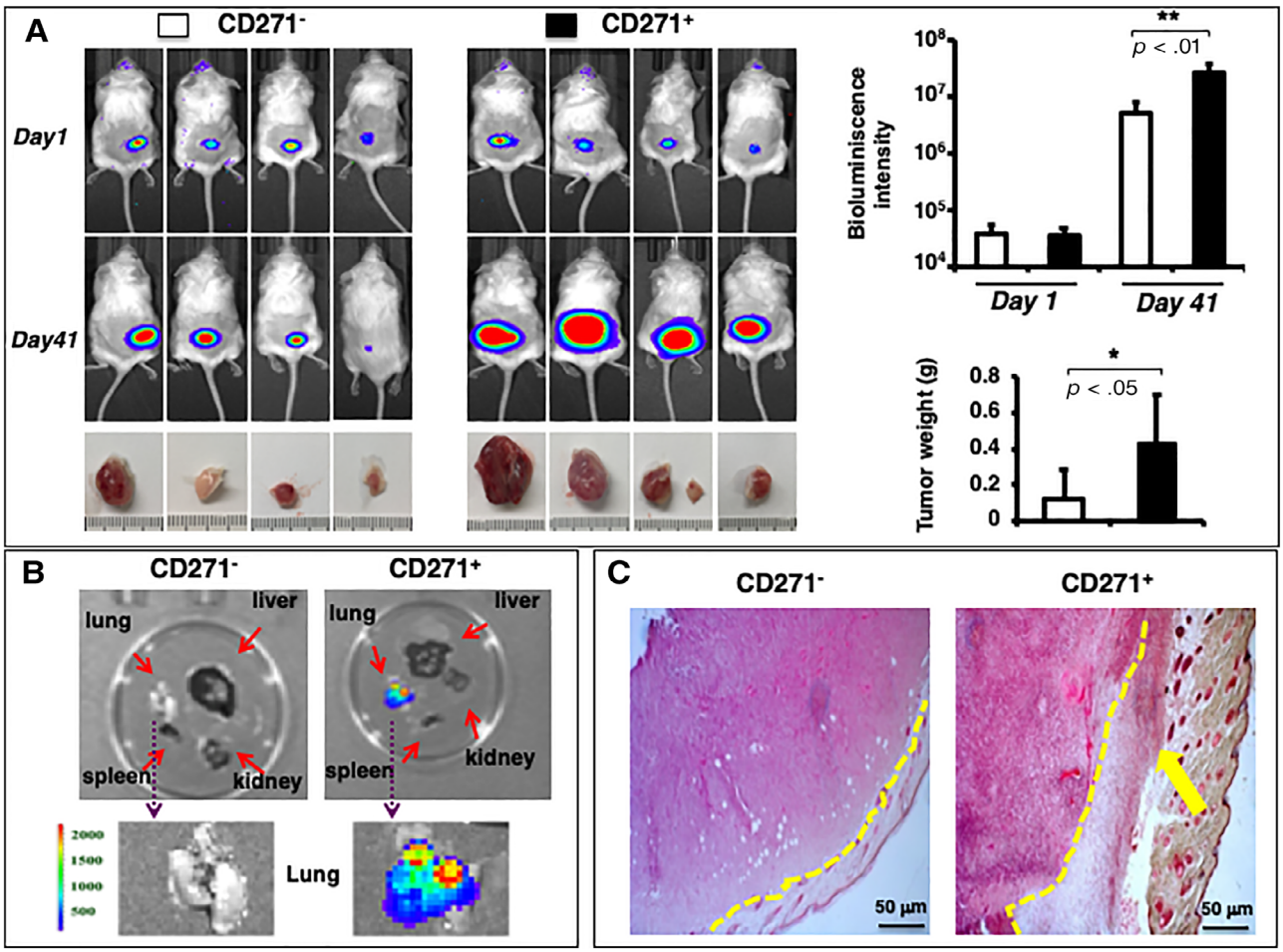

Figure 6. Melanoma skin growth, invasion, and distal metastasis of xenografted CD271$^+$ versus CD271$^-$ subpopulations of Luc$^+$/C8161 melanoma cells. **(A):** Representative images of whole-body IVIS scanning and resected skin tumors from mice xenografted with CD271$^+$ versus CD271$^-$ subpopulations of melanoma cells. Quantitative bioluminescent signals measured in day 1 and day 41 and weights of resected tumors at day 41 are shown (n = 5 mice per group). **(B):** Representative images of IVIS scanning of harvested major organs from mice xenografted with CD271$^+$ versus CD271$^-$ subpopulations of melanoma cells are shown. The CD271$^+$ subpopulation of melanoma stem/initiating cells (MICs) exhibits lung metastasis. **(C):** Representative images of H&E staining of tumor sections show that the CD271$^+$ subpopulation of MICs invades into local skin tissue.

transwell assay using BD Falcon FluoroBlok Systems with 8 µm porous membrane insert (BD Biosciences). We found that the CD271$^+$ subpopulation of MICs exhibited faster growth and migration rates as compared with the CD271$^-$ subpopulation of melanoma cells (Fig. 5A, 5B). These results reveal that MSC-DF$^{Notch1-/-}$-induced CD271$^+$ subpopulation of MICs exhibits more aggressive behaviors than CD271$^-$ melanoma cells in vitro.

The CD271$^+$ Subpopulation of MICs Exhibits More Aggressive Behaviors In Vivo

We further tested tumor growth, invasion, and metastasis of the CD271$^+$ versus CD271$^-$ subpopulations of MICs in vivo. 4×10^4 of Luc2$^+$/DsRed$^+$-CD271$^+$ and Luc2$^+$/DsRed$^+$-CD271$^-$ subpopulations of MICs sorted by FACS as described above were inoculated intradermally onto dorsal skin of NSF mice (n = 5 per group), respectively. Skin growth of melanoma xenografts was measured by endpoint whole-body IVIS scanning at day 47 after tumor inoculations and endpoint tumor weight. The CD271$^+$ subpopulation of MICs grew faster than the CD271$^-$ subpopulation of MICs (Fig. 6A). Lungs, livers, spleens, and kidneys were harvested immediately after whole-body IVIS scanning and rescanned by IVIS to detect distant metastasis of skin melanoma. Lung metastasis was found in mice xenografted with the CD271$^+$ subpopulation of MICs, but not in those xenografted with the CD271$^-$ subpopulation of MICs (Fig. 6B). Melanoma local invasion was evaluated by histological assessment of tissue sections of resected skin melanoma. H&E staining of resected melanoma tissues illustrated that 5/5 (100%) of mice grafted with the CD271$^+$ subpopulation of MICs had local invasion into adjacent skin tissues, compared with 0/5 (0%) of mice grafted with the CD271$^-$ subpopulation of MICs (Fig. 6C). Overall, the data show that the CD271$^+$ subpopulation of MICs exhibits more aggressive behaviors than CD271$^-$ melanoma cells in vivo. Our in vivo data is consistent with in vitro data.

Discussion

A correlation between the status of Notch signaling and activity of fibroblasts was previously reported. Loss of *Notch1* in mouse embryonic fibroblasts (MEFs) conferred faster cell growth and motility rate, whereas constitutive activation of the Notch1

pathway slowed cell growth and motility of human fibroblasts [43]. Consistently, Notch activation resulted in cell-cycle arrest and apoptosis in MEFs [44]. These studies indicated that the Notch signaling pathway serves as a negative regulator or "brake" on fibroblast cell growth. Activation of Notch pathway can down-regulate cellular activity of fibroblasts. In addition, analysis of Notch pathway gene expression profiling exhibited striking differential patterns between proliferating and quiescent human dermal fibroblasts. Proliferating fibroblasts expressed either undetectable or low levels, whereas quiescent fibroblasts manifested increased levels, of genes of Notch pathway components [43]. These findings suggested that Notch signaling is maintained in an inactivated status or at a low level of activity in proliferating fibroblasts, whereas Notch signaling is activated or has a high level of activity in quiescent fibroblasts. However, it is unclear how the Notch1 pathway is turned "OFF" in CAFs. Likely, Notch1 inactivation in CAFs is a genetic or epigenetic consequence resulting from the influence of the tumor cells and/or the TME. Notch1 signaling in melanoma CAFs may be turned "OFF" by a yet unidentified inhibitory signaling cascade(s) initiated by cytokine(s) and ECM in tumor tissue or by cell–cell interaction between CAFs and melanoma cells or other tumor stromal cells. Invasion of melanoma cells and infiltration/recruitment of other tumor stromal cells result in a switch of the partner cells with which normal fibroblasts interact in the physiological condition. Switching of partner cells may interrupt existing communication or initiate new communication that causes diminished Notch1 signaling in melanoma CAFs.

Our current work focused on exploring the role of intracellular Notch1 signaling in stromal fibroblasts in regulating MIC plasticity and tumor heterogeneity. It should be noted that the roles of intracellular Notch signaling in stromal fibroblasts and the roles of Notch/ligand in mediating intercellular communication are two different topics. It is unclear whether Notch/ligands participate in cell–cell communication between fibroblasts and melanoma cells and whether they are involved in regulating MIC phenotype and tumor heterogeneity. For example, it was found that CAF-released CCL2 inducing the self-renewal of CSCs is mediated by inducing elevated Notch1 expression and Notch pathway activation in breast cancer cells [25]. However, it is unknown whether Notch pathway activation in breast cancer cells is triggered by Notch ligands expressed in CAFs or in neighboring cancer cells.

We previously reported that cografted fibroblasts, pre-engineered to carry high Notch1 activity, inhibited melanoma growth and angiogenesis in a melanoma xenograft model [36], revealing that Notch1 activation antagonizes the tumor-promoting effect of stromal fibroblasts. Consistently, we showed that CAFs carrying elevated Notch1 activity significantly inhibited melanoma growth and invasion, whereas those with a null Notch1 promoted melanoma invasion [41]. Our current work is not only consistent with the general role of Notch1 signaling in governing the tumor-regulating function of CAFs, but also expands the repertoire of Notch1 signaling as a molecular switch in regulating the plasticity of CSCs and tumor heterogeneity and further indicates a mechanism underlying opposite tumor regulatory effects of CAFs carrying high or low Notch1 pathway activity in regulating tumorigenicity. Although most of the data demonstrating a critical role of intracellular Notch1 signaling in CAFs in determining the plasticity and stemness of MICs were derived from a single melanoma cell line (C8161), which are the limitations of our current study, the fact that Notch1 signaling activity in CAFs uniformly determines the ability of three different melanoma cell lines (1205Lu, C8161, and MeWo) to form spheres, which is a hallmark of CSCs, strongly suggest that intracellular Notch 1 signaling in CAFs plays a general role in determining MIC phenotype. Our study indicates that activation of the Notch1 pathway in CAFs can potentially be a therapeutic intervention approach to decrease the frequency of MICs in melanoma and inhibit MIC-mediated melanoma aggressiveness/metastasis. This novel approach may lead to the development of novel adjuvant/neoadjuvant melanoma therapies that can ultimately be part of integrative cancer care targeting cancer cells and TME simultaneously. An additional advantage of this approach is that activation of Notch1 signaling in CAFs uniformly suppresses three different melanoma cell lines (1205Lu, C8161, and MeWo), which harbor different mutations. 1205lu carries $BRAF^{V600E}$ mutation while the C8161 and MeWo cells do not have the BRAF mutation. It suggests that targeting CAFs can potentially be an ideal approach which does not depend upon types of mutations the melanoma cells carry. Our findings open a new avenue to target the TME by reprograming and converting CAFs from "MIC promoters" to "MIC suppressors" through therapeutic activation of the Notch1 pathway. There are several options for activation of the Notch pathway in CAFs, such as using a gene therapy approach or the novel genome editing method, CRISPR/Cas9, to introduce $N1^{IC}$ or applying a Notch pathway-activating compound, which can be identified through a similar high-throughput screening method [45] and activating Notch signaling specifically in CAFs. However, as the function of Notch signaling is cell context-dependent [46] and high Notch activity is oncogenic to melanoma [34, 47, 48], the development and clinical application of Notch-targeting agents that can selectively increase Notch pathway activity in CAFs without simultaneously increasing Notch pathway activity in melanoma cells will be the key for precision medicine. An alternative is to identify and use Notch pathway downstream targets, which are responsible for mediating the Notch-induced tumor-suppressing phenotype of CAFs [33, 36, 43]. Our previous finding of WNT1-inducible-signaling pathway protein-1 (WISP-1) as a functional mediator of Notch1 signaling provides a practicable agent to control melanoma progression, since WISP-1 is a soluble molecule and is thus easy to be directly administered. In addition, because melanoma cells barely express WISP-1 [36], WISP-1 can be an ideal and manageable therapeutic candidate to treat melanoma. Future study is warranted to investigate whether WISP-1 is responsible for mediating the effect of Notch1 signaling in CAFs on regulating MIC plasticity and melanoma heterogeneity. Another alternative strategy is to develop cell-based therapy through targeted delivery of autologous MSC-DF, pre-engineered "ex vivo" to either overexpress WISP-1 or carry high Notch1 activity using methods mentioned above into tumor tissue. Such engineered fibroblasts expressing high Notch activity tend to undergo cell cycle arrest [43, 44]. This characteristic makes MSC-DF carrying high Notch activity especially appealing as therapeutic cells because they will not expand uncontrollably after residing in the tumor tissue and are eventually cleared by immune cells. Therefore, they can be repeatedly administered to patients to enhance therapeutic efficacy.

The molecular mechanisms underpinning the role of intracellular Notch1 signaling in CAFs as a molecular switch in controlling stromal regulation of the plasticity and stemness of MICs and melanoma heterogeneity remain unclear. The potential role of WISP-1 in mediating Notch1's effect on regulating the plasticity and stemness of MICs will be explored in future

studies. In addition, other effector(s) that mediate effect of intracellular Notch1 signaling in CAFs on regulation of the plasticity and stemness of MICs and melanoma heterogeneity will be identified and validated from the list of 689 candidates generated by microarray analysis using the Illumina mouse whole-genome-6 v2.0 to discover differentially expressed gene profiles of MSC-DF$^{Notch1-/-}$ versus MSC-DF$^{Notch1F/F}$ [33].

Conclusion

We uncovered that intracellular Notch1 signaling in CAFs is a molecular switch, inversely controlling stromal regulation of the plasticity and stemness of MICs and thereby modulating melanoma heterogeneity and aggressiveness. Our study indicates that therapeutic activation of the Notch1 pathway in CAFs can control MIC plasticity and inhibit MIC-mediated melanoma aggressiveness/metastasis, and further may be able to mitigate drug resistance and melanoma recurrence.

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Author Contributions

Y.D., H.S.: collection and assembly of data, data analysis and interpretation; M.M.: provision of study material or patients, data analysis and interpretation; R.P.: collection and assembly of data, data analysis and interpretation, proofreading of manuscript; Y.T.T.: collection and assembly of data; Z.-J.L.: conception and design, financial support, manuscript writing, final approval of manuscript.

Disclosure of Potential Conflicts of Interest

The authors indicated no potential conflicts of interest.

Data Availability Statement

All data generated or analyzed during this study are included in this published article (and its Supporting Information files).

References

1 Visvader JE, Lindeman GJ. Cancer stem cells in solid tumours: Accumulating evidence and unresolved questions. Nat Rev Cancer 2008;8:755–768.

2 Ailles LE, Weissman IL. Cancer stem cells in solid tumors. Curr Opin Biotechnol 2007; 18:460–466.

3 Schadendorf D, Fisher DE, Garbe C et al. Melanoma. Nat Rev Dis Primers 2015;1:15003.

4 Refaeli Y, Bhoumik A, Roop DR et al. Melanoma-initiating cells: A compass needed. EMBO Rep 2009;10:965–972.

5 Shakhova O, Sommer L. Testing the cancer stem cell hypothesis in melanoma: The clinics will tell. Cancer Lett 2013;338:74–81.

6 Parmiani G. Melanoma cancer stem cells: Markers and functions. Cancers 2016;8:E34.

7 Boiko AD, Razorenova OV, van de Rijn M et al. Human melanoma-initiating cells express neural crest nerve growth factor receptor CD271. Nature 2010;466:133–137.

8 Civenni G, Walter A, Kobert N et al. Human CD271-positive melanoma stem cells associated with metastasis establish tumor heterogeneity and long-term growth. Cancer Res 2011;71:3098–3109.

9 Piras F, Perra MT, Murtas D et al. The stem cell marker nestin predicts poor prognosis in human melanoma. Oncol Rep 2010;23:17–24.

10 Ma J, Lin JY, Alloo A et al. Isolation of tumorigenic circulating melanoma cells. Biochem Biophys Res Commun 2010;402:711–717.

11 Fusi A, Ochsenreither S, Busse A et al. Expression of the stem cell marker nestin in peripheral blood of patients with melanoma. Br J Dermatol 2010;163:107–114.

12 Schatton T, Murphy GF, Frank NY et al. Identification of cells initiating human melanomas. Nature 2008;451:345–349.

13 Monzani E, Facchetti F, Galmozzi E et al. Melanoma contains CD133 and ABCG2 positive cells with enhanced tumourigenic potential. Eur J Cancer 2007;43:935–946.

14 Kalluri R, Zeisberg M. Fibroblasts in cancer. Nat Rev Cancer 2006;6:392–401.

15 Chiang AC, Massague J. Molecular basis of metastasis. N Engl J Med 2008;359:2814–2823.

16 Lee JT, Herlyn M. Microenvironmental influences in melanoma progression. J Cell Biochem 2007;101:862–872.

17 Olumi AF, Grossfeld GD, Hayward SW et al. Carcinoma-associated fibroblasts direct tumor progression of initiated human prostatic epithelium. Cancer Res 1999;59:5002–5011.

18 Allinen M, Beroukhim R, Cai L et al. Molecular characterization of the tumor microenvironment in breast cancer. Cancer Cell 2004;6:17–32.

19 Midwood KS, Williams LV, Schwarzbauer JE. Tissue repair and the dynamics of the extracellular matrix. Int J Biochem Cell Biol 2004;36: 1031–1037.

20 Lynch CC, Matrisian LM. Matrix metalloproteinases in tumor-host cell communication. Differentiation 2002;70:561–573.

21 Bhowmick NA, Neilson EG, Moses HL. Stromal fibroblasts in cancer initiation and progression. Nature 2004;432:332–337.

22 Luga V, Zhang L, Viloria-Petit AM et al. Exosomes mediate stromal mobilization of autocrine Wnt-PCP signaling in breast cancer cell migration. Cell 2012;151:1542–1556.

23 Giannoni E, Bianchini F, Masieri L et al. Reciprocal activation of prostate cancer cells and cancer-associated fibroblasts stimulates epithelial-mesenchymal transition and cancer stemness. Cancer Res 2010;70:6945–6956.

24 Tirino V, Desiderio V, Paino F et al. Cancer stem cells in solid tumors: An overview and new approaches for their isolation and characterization. FASEB J 2013;27:13–24.

25 Tsuyada A, Chow A, Wu J et al. CCL2 mediates cross-talk between cancer cells and stromal fibroblasts that regulates breast cancer stem cells. Cancer Res 2012;72:2768–2779.

26 Chen WJ, Ho CC, Chang YL et al. Cancer-associated fibroblasts regulate the plasticity of lung cancer stemness via paracrine signalling. Nat Commun 2014;5:3472.

27 Orimo A, Weinberg RA. Heterogeneity of stromal fibroblasts in tumors. Cancer Biol Ther 2007;6:618–619.

28 Ishii G, Sangai T, Oda T et al. Bone-marrow-derived myofibroblasts contribute to the cancer-induced stromal reaction. Biochem Biophys Res Commun 2003;309:232–240.

29 Kalluri R, Neilson EG. Epithelial-mesenchymal transition and its implications for fibrosis. J Clin Invest 2003;112:1776–1784.

30 Price JE. Xenograft models in immunodeficient animals: I. Nude mice: Spontaneous and experimental metastasis models. Methods Mol Med 2001;58:205–213.

31 Spaeth EL, Dembinski JL, Sasser AK et al. Mesenchymal stem cell transition to tumor-associated fibroblasts contributes to fibrovascular network expansion and tumor progression. PLoS One 2009;4:e4992.

32 Kidd S, Spaeth E, Dembinski JL et al. Direct evidence of mesenchymal stem cell tropism for tumor and wounding microenvironments using in vivo bioluminescent imaging. Stem Cells 2009;27:2614–2623.

33 Shao H, Cai L, Moller M et al. Notch1-WISP-1 axis determines the regulatory role of mesenchymal stem cell-derived stromal fibroblasts in

melanoma metastasis. Oncotarget 2016;7:79262–79273.

34 Balint K, Xiao M, Pinnix CC et al. Activation of Notch1 signaling is required for beta-catenin-mediated human primary melanoma progression. J Clin Invest 2005;115:3166–3176.

35 Radtke F, Wilson A, Stark G et al. Deficient T cell fate specification in mice with an induced inactivation of Notch1. Immunity 1999;10:547–558.

36 Shao H, Cai L, Grichnik JM et al. Activation of Notch1 signaling in stromal fibroblasts inhibits melanoma growth by upregulating WISP-1. Oncogene 2011;30:4316–4326.

37 Liu ZJ, Tian R, An W et al. Identification of E-selectin as a novel target for the regulation of postnatal neovascularization: Implications for diabetic wound healing. Ann Surg 2010;252:625–634.

38 Albini A, Bruno A, Gallo C et al. Cancer stem cells and the tumor microenvironment: Interplay in tumor heterogeneity. Connect Tissue Res 2015;56:414–425.

39 Boesch M, Sopper S, Zeimet AG et al. Heterogeneity of cancer stem cells: Rationale for targeting the stem cell niche. Biochim Biophys Acta 2016;1866:276–289.

40 Welch DR, Bisi JE, Miller BE et al. Characterization of a highly invasive and spontaneously metastatic human malignant melanoma cell line. Int J Cancer 1991;47:227–237.

41 Shao H, Kong R, Ferrari ML et al. Notch1 pathway activity determines the regulatory role of cancer-associated fibroblasts in melanoma growth and invasion. PLoS One 2015;10:e0142815.

42 Boyer LA, Lee TI, Cole MF et al. Core transcriptional regulatory circuitry in human embryonic stem cells. Cell 2005;122:947–956.

43 Liu ZJ, Li Y, Tan Y et al. Inhibition of fibroblast growth by Notch1 signaling is mediated by induction of Wnt11-dependent WISP-1. PLoS One 2012;7:e38811.

44 Ishikawa Y, Onoyama I, Nakayama KI et al. Notch-dependent cell cycle arrest and apoptosis in mouse embryonic fibroblasts lacking Fbxw7. Oncogene 2008;27:6164–6174.

45 Pinchot SN, Jaskula-Sztul R, Ning L et al. Identification and validation of Notch pathway activating compounds through a novel high-throughput screening method. Cancer 2011;117:1386–1398.

46 Radtke F, Raj K. The role of Notch in tumorigenesis: Oncogene or tumour suppressor? Nat Rev Cancer 2003;3:756–767.

47 Liu ZJ, Xiao M, Balint K et al. Notch1 signaling promotes primary melanoma progression by activating mitogen-activated protein kinase/phosphatidylinositol 3-kinase-Akt pathways and up-regulating N-cadherin expression. Cancer Res 2006;66:4182–4190.

48 Pinnix CC, Lee JT, Liu ZJ et al. Active Notch1 confers a transformed phenotype to primary human melanocytes. Cancer Res 2009;69:5312–5320.

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