

SHORT COMMUNICATION

Deficiency of Kruppel-like factor KLF4 in myeloid-derived suppressor cells inhibits tumor pulmonary metastasis in mice accompanied by decreased fibrocytes

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The importance of immunosuppressive myeloid-derived suppressor cells (MDSCs) bearing monocyte markers in tumor metastasis has been well established. Recently, it was reported that these cells possess phenotypic plasticity and differentiate into fibrocytes, very distinct cells that are precursors of tumorigenic myofibroblasts. However, the importance of this transdifferentiation in tumor metastasis has not been explored. Here, we describe the role of MDSC-derived fibrocytes in tumor metastasis that is regulated by Kruppel-like factor 4 (KLF4), a transcription factor that is critical to monocyte differentiation and to promotion of cancer development. Using mouse metastasis models of melanoma and breast cancer, we found that KLF4 knockout was associated with significantly reduced pulmonary metastasis, which was accompanied by decreased populations of MDSCs, fibrocytes and myofibroblasts in the lung. Cause-effect studies by adoptive transfer revealed that KLF4 deficiency in MDSCs led to significantly reduced lung metastasis that was associated with fewer MDSC-derived fibrocytes and myofibroblasts. Mechanistically, KLF4 deficiency significantly compromised the generation of fibrocytes from MDSCs *in vitro*. During this process, KLF4 expression levels were tightly linked with those of fibroblast-specific protein-1 (FSP-1), deficiency of which resulted in no metastasis in mice as has been previously reported. In addition, KLF4 bound directly to the FSP-1 promoter as determined by chromatin immunoprecipitation and overexpression of KLF4 increased the FSP-1 promoter activities. Taken together, our results suggest that MDSCs not only execute their immunosuppressive function to promote metastatic seeding as reported before, but also boost metastatic tumor growth after they adopt a fibrocyte fate. Therefore, KLF4-mediated fibrocyte generation from MDSCs may represent a novel mechanism of MDSCs contributing to tumor metastasis and supports the feasibility of inhibiting KLF4 or FSP-1 to prevent tumor metastasis.

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INTRODUCTION

Myeloid-derived suppressor cells (MDSCs) are a bone marrow-originated heterogeneous population of immature myeloid cells with phenotypic plasticity¹ and involved in multiple inflammatory conditions.^{2,3} They are broadly defined as CD11b⁺Gr-1⁺ or CD11b⁺Ly6G⁺ cells in mice, with a wider range of markers in humans. Increased MDSCs are associated with tumor growth and metastasis in both tumor-bearing mice and cancer patients,⁴ which makes this population a novel therapeutic target.⁵ Recently, MDSCs were found to be differentiated into fibrocytes,⁶ a very distinct cell type that may promote tumorigenesis by incorporating into the tumor stromal and differentiating into myofibroblasts.^{7,8} In addition, expansion of fibrocytes has been observed in the microenvironment of solid tumors⁹ and circulation of subjects with metastatic cancer,¹⁰ which facilitate tumor metastasis.¹¹ However, the importance and the underlying molecular mechanism of MDSCs transdifferentiation into fibrocytes in tumor metastasis have not been clearly elucidated.

Kruppel-like factor 4 (KLF4) is a transcription factor¹² critical to monocyte differentiation,¹³ and has an oncogenic function in

cancer development.^{14,15} Recently, we found that KLF4 deficiency in mammary tumor cells compromised the recruitment and function of MDSCs in primary and metastatic tumors, resulting in attenuated tumor development.¹⁶ However, whether and how KLF4 in MDSCs, a major component of immunosuppressive cells in tumor microenvironment,¹⁷ regulates tumor metastasis has not been reported before.

In this study, we found that KLF4 deficiency in mice led to reduced pulmonary metastasis, which was accompanied by decreased populations of a subset of MDSCs bearing features of CCR2⁺CD11b⁺Ly6G^{int} (CCR2⁺ MDSCs) and reduced numbers of fibrocytes and myofibroblasts in the lung. Cause-effect studies indicated that KLF4 deficiency in CCR2⁺ MDSCs was responsible for compromised lung metastasis. Mechanistically, KLF4 regulated the fibrocyte generation from CCR2⁺ MDSCs *in vitro* and occupied the fibroblast-specific protein-1 (FSP-1) promoter, which may contribute to increased promoter activities. Taken together, our study demonstrates a critical role of KLF4-mediated fibrocyte generation from CCR2⁺MDSCs in tumor metastasis, which may provide a therapeutic strategy to treat metastasis by targeting KLF4 or FSP-1.

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RESULTS AND DISCUSSION

KLF4 deficiency in mouse bone marrow drastically reduced lung metastasis that was accompanied by decreased recruitment of CCR2⁺ MDSCs in the lung

We recently reported that mammary tumor cell-expressing KLF4 facilitated primary tumor growth and pulmonary metastasis.¹⁶ The accumulation of MDSCs in bone marrow, spleen and primary tumors were all decreased in mice that were inoculated with breast cancer cells lacking KLF4 expression. However, KLF4 deficiency is restricted to tumor cells in this study, and the effect of KLF4 in MDSCs on tumor metastasis still remains unknown. Therefore, we generated inducible KLF4-knockout mice (Rosa26CreER/KLF4 (flox^{+/+}) mice on C57BL/6 background) and spontaneous KLF4-knockout mice (FSP-1-Cre/KLF4 (flox^{+/+}) mice on BalB/c background). The efficiency of KLF4 knockout in both models was confirmed by quantitative reverse transcriptase-PCR indicating that KLF4 expression in bone marrow was decreased to < 10% of that in the wild-type mice (data not shown). We then established metastatic mouse models using B16F10-Luc2 melanoma and 4T1-Luc2 breast cancer cells in these mice. Ten days after intravenous tumor cell inoculation, *in vivo* bioluminescent imaging showed that the signals of lung metastasis in KLF4-knockout (KLF4^{-/-}) groups were much lower than those in the control (KLF4^{+/+}) groups in both models (Supplementary Figure S1A). Two weeks after tumor inoculation, mice were sacrificed and a significantly decreased incidence of lung metastasis was found in the KLF4^{-/-} groups (Supplementary Figure S1B). Flow cytometric analysis showed that the percentage of MDSCs in bone marrow of the KLF4^{-/-} group was almost the same as the KLF4^{+/+} group in both of the two metastatic models. In addition, although MDSCs were reduced in spleen and lung after KLF4 was knocked out in these two models, the differences between the KLF4^{-/-} and KLF4^{+/+} groups were not statistically significant (data not shown).

Note that KLF4 deficiency was systemic in the above mentioned mouse models. To exclusively investigate whether the KLF4-knockout effect on tumor metastasis was contributed by bone marrow KLF4, we performed the same experiments in the B16F10-Luc2 melanoma metastatic model using chimeric mice that had received bone marrow cells from B6 Rosa26CreER/KLF4(flox^{+/+})/β-actin-EGFP⁺ donor mice. Similar with the systemic KLF4-knockout mice, average bioluminescence intensity was decreased from 9.31 (±1.92) × 10³ photons/s in the lung of control mice to 2.86 (±1.34) × 10³ photons/second in the lung of mice with bone marrow KLF4 knockout induced by TAM (Figure 1a, *P* < 0.05). Consistently, drastically decreased numbers of pulmonary metastatic nodules were found in the KLF4^{-/-} group when compared with the KLF4^{+/+} group (Figure 1b) and metastatic area was also reduced upon KLF4 deficiency in bone marrow (Figure 1c). Similarly, there were still no significant changes of MDSCs in bone marrow, spleen and lung after KLF4 knockout in the bone marrow (data not shown), even though the percentages of MDSCs in the lung were markedly reduced (Supplementary Figure S2A, *P* = 0.165).

MDSCs are heterogeneous. It was recently reported that a subset of MDSCs characterized by CD11b⁺Gr-1⁺CD115⁺ was preferentially recruited to the lung through the CCL2/CCR2 signaling pathway, resulting in facilitation of pulmonary metastasis of mammary tumor.¹⁸ We therefore postulated that KLF4 may only regulate a specific subpopulation of MDSCs, such as CCR2⁺ MDSCs, leading to compromised lung metastasis in our studies. Indeed, in the B16F10 chimeric metastasis model, the CCR2⁺ MDSC subset decreased from 2.41 (±0.21)% to 0.86 (±0.25)% in lung (Figure 1d, *P* < 0.05), but not in spleen (Supplementary Figure S2B, *P* = 0.057) or bone marrow (data not shown). Consistent with these results in the B16F10 model, CCR2⁺ MDSCs in lung (Supplementary Figure S3A, *P* < 0.001), but not in spleen or bone marrow (data not shown), were also significantly reduced in the 4T1 metastatic model. Collectively, these data suggested

that KLF4 in bone marrow regulated the pulmonary recruitment of CCR2⁺ MDSCs, and decreased CCR2⁺ MDSCs reduced tumor lung metastasis.

KLF4 deficiency in bone marrow was associated with significantly decreased numbers of fibrocytes and myofibroblasts in metastatic lung

MDSCs are well known for their immunosuppressive functions under different pathological conditions including cancer development.¹⁹ As discussed above, KLF4 knockout had limited effects on the recruitment of the whole population of MDSCs, but significantly reduced the numbers of CCR2⁺ MDSC subset in lung. It suggested that the significantly reduced lung metastasis after bone marrow KLF4 knockout was contributed by other factors beyond the immunosuppressive function of CCR2⁺ MDSCs. Recently it was reported that MDSCs had a high potential to differentiate into fibrocytes.⁶ As mesenchymal cells that arise from monocyte precursors, fibrocytes have both the inflammatory features of macrophages and the tissue remodeling properties of fibroblasts.²⁰ Circulating fibrocytes can traffic to lungs and differentiate into fibroblasts,²¹ the precursors of myofibroblast that are crucial tumor stromal component.²² Therefore, there was an intriguing possibility that the CCR2⁺ MDSCs exerted their ability of promoting metastatic tumor growth mostly by adopting a fibrocyte fate after they were recruited to lung. To test this, we first used B16F10 chimeric metastasis model. As the chimeric mice received EGFP-expressing bone marrow cells, we used COL1A1⁺CD45⁺CD11b⁺EGFP⁺ and CD11b⁺α-SMA⁺EGFP⁺ to identify bone marrow-derived fibrocytes and myofibroblasts, respectively. The peripheral blood monocytes and lung single cells of the chimeric mice were examined by flow cytometry. As shown in Figure 2a, the percentage of fibrocytes in peripheral blood monocytes dropped from 2.50 (±0.32) % in the KLF4^{+/+} group to 0.63 (± 0.40) % in the KLF4^{-/-} group (Figure 2a, *P* < 0.05). Although the percentage of fibrocytes and myofibroblasts in the lung was similar between the two groups (KLF4^{+/+} 2.54 (±0.28) % vs KLF4^{-/-} 1.67 (± 0.29) % for fibrocytes, *P* = 0.10, KLF4^{+/+} 17.03 (±1.15) % vs KLF4^{-/-} 16.22 (± 0.52) % for myofibroblasts, *P* = 0.60), the total cell number of COL1A1⁺CD45⁺CD11b⁺EGFP⁺ and CD11b⁺α-SMA⁺EGFP⁺ cells in the KLF4^{-/-} group decreased by 64.30 and 64.34%, respectively (Figure 2b). In parallel with these observations, the immunofluorescent staining of lung sections showed that the numbers of COL1A1⁺EGFP⁺ and α-SMA⁺EGFP⁺ double positive cells were significantly reduced in the KLF4^{-/-} group when compared to those in the KLF4^{+/+} group (Figures 2c and d). Consistently, in the 4T1 metastatic model, decreased populations of fibrocytes (COL1A1⁺CD45⁺CD11b⁺) were also observed in peripheral blood monocytes and lung (Supplementary Figure S3B and S3C, *P* < 0.001 and *P* < 0.01). Together, these findings supported our hypothesis that CCR2⁺ MDSCs are not only preferentially recruited to target organs to prepare for metastatic seeding, but also boost metastatic tumor growth by adopting a fibrocyte fate. In addition, KLF4 regulates both of these two progresses.

KLF4 ablation in CCR2⁺ MDSCs reduced pulmonary metastasis that was associated with decreased numbers of fibrocytes

To exclude the effect of KLF4-mediated development of MDSCs and to test the direct effect of KLF4-regulated fibrocyte generation from CCR2⁺ MDSCs on tumor metastasis, a cause-effect study was performed. We sorted this specific MDSC subset from the lungs of B6 Rosa26CreER/KLF4 (flox^{+/+})/β-actin-EGFP⁺ mice bearing B16F10 melanoma, mixed with B16F10 tumor cells, and then injected wild-type mice with the mixture intravenously, followed by tamoxifen (TAM) injection to induce KLF4 knockout (Figure 3a). In the control group, mice only received B16F10 tumor cells, but still intraperitoneally injected with TAM or sunflower seed oil as the control. At day 7 after tumor cell inoculation, mice were

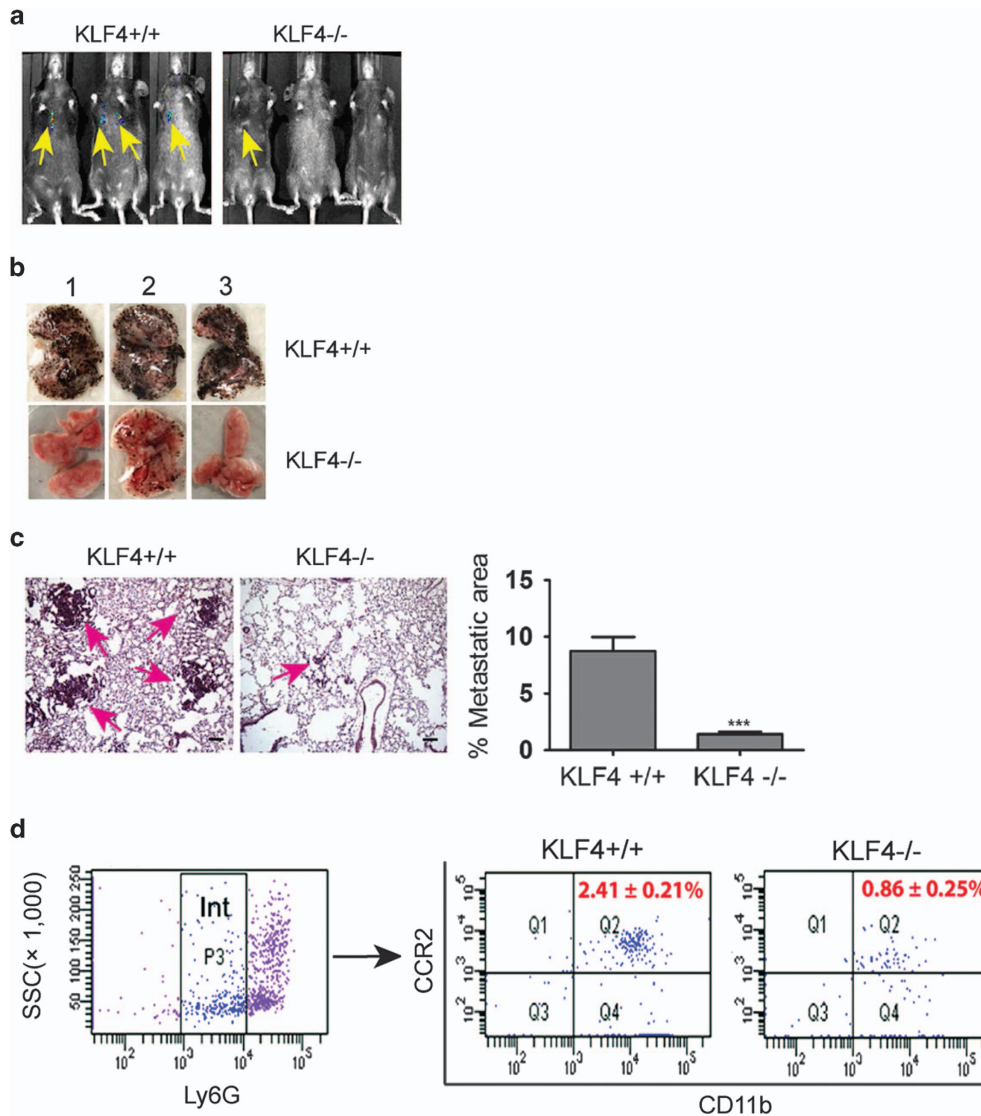


Figure 1. KLF4 deficiency in mouse bone marrow drastically reduced lung metastasis that was accompanied by decreased recruitment of CCR2⁺ MDSCs in the lung. **(a)** Six-week old C57BL/6 (B6) wild-type mice (The Jackson Laboratory, Bar Harbor, ME, USA) were lethally irradiated (900 rad) and then transplanted with bone marrow cells (1.0×10^6 cells per recipient mouse) from B6 Rosa26CreER/KLF4 (floxed^{+/+})/ β -actin-EGFP mice, which were generated by crossing tamoxifen-inducible RosaCreER mice (01XAB, NCI) with KLF4 (floxed) mice (029877-Mu, MMRRC) and β -actin-EGFP mice (The Jackson Laboratory). Four weeks later, KLF4 knockout of chimeric mice was induced by daily intraperitoneal injection of tamoxifen (TAM, Sigma-Aldrich, St Louis, MO, USA; 50 mg/kg) for five consecutive days, followed by intravenous injection of B16F10-Luc2 tumor cells (Caliper Life Sciences, Middlesex, MA, USA). Sunflower seed oil (Sigma-Aldrich) was used as control. These mice were then monitored by IVIS Spectrum Fluorescence and Bioluminescence Imaging System (Caliper Life sciences) to detect the signals of pulmonary metastasis. Results of representative control (KLF4^{+/+}) and TAM-induced (KLF4^{-/-}) mice at day 10 after tumor cell inoculation were shown ($n=8$, $P < 0.05$). **(b)** Representatives of lung macrometastases from the chimeric mice as described in **a** were shown. **(c)** Left, representative HE stained lung sections with red arrows pointing the metastatic lesions were displayed (scale bars = 100 μ m). Right, percentages of metastatic areas in control and TAM-induced chimeric mice were quantified. **(d)** Lungs were minced into small fragments ($< 1 \text{ mm}^3$) and digested in 10 ml of dissociation solution (RPMI 1640 medium supplemented with Collagenase type I (200 U/ml) and DNase I (100 μ g/ml)) for 60 min at 37 °C. Lung-infiltrating immune cells were further purified using Percoll (Sigma-Aldrich) according to the manufacturer's instructions. The cells with intermediate Ly6G (clone RB6-8C5, eBiosciences, San Diego, CA, USA) signals (Ly6G^{int}) were gated (left) followed by further flow cytometry analysis (right) using CD11b (clone M1/70, eBiosciences) and CCR2 (R&D, Minneapolis, MN, USA) antibodies. The flow cytometry were performed and data were analyzed using FACS Aria II (BD Biosciences, San Jose, CA, USA). Representative dot plots in each group were shown ($n=8$). Mean \pm s.e.m., *** $P < 0.001$.

sacrificed. The gross view and hematoxylin and eosin staining of the lung in the control group showed that there was no difference in the incidence of lung metastasis between the mice administered with TAM or sunflower seed oil (data not shown). However, the pulmonary metastatic nodules in the KLF4^{-/-} and control groups were drastically fewer than those in the KLF4^{+/+} group. The percentages of lung metastatic areas in KLF4^{-/-} (2.64 (\pm 0.16)%) and control group (2.87 (\pm 0.21)%) were also significantly lower

than those in the KLF4^{+/+} group (11.71 (\pm 0.88)%; Figure 3b). Note that KLF4 deficiency in CCR2⁺ MDSCs led to similar metastatic areas in KLF4^{-/-} group as in the control group. The results demonstrated that CCR2⁺ MDSCs did facilitate the seeding and growth of pulmonary metastatic nodules in a KLF4-dependent manner. Because the transplanted CCR2⁺ MDSC cells also carried the EGFP marker, we examined their differentiation in the lung by immunofluorescent staining of lung cryosections with COL1A1

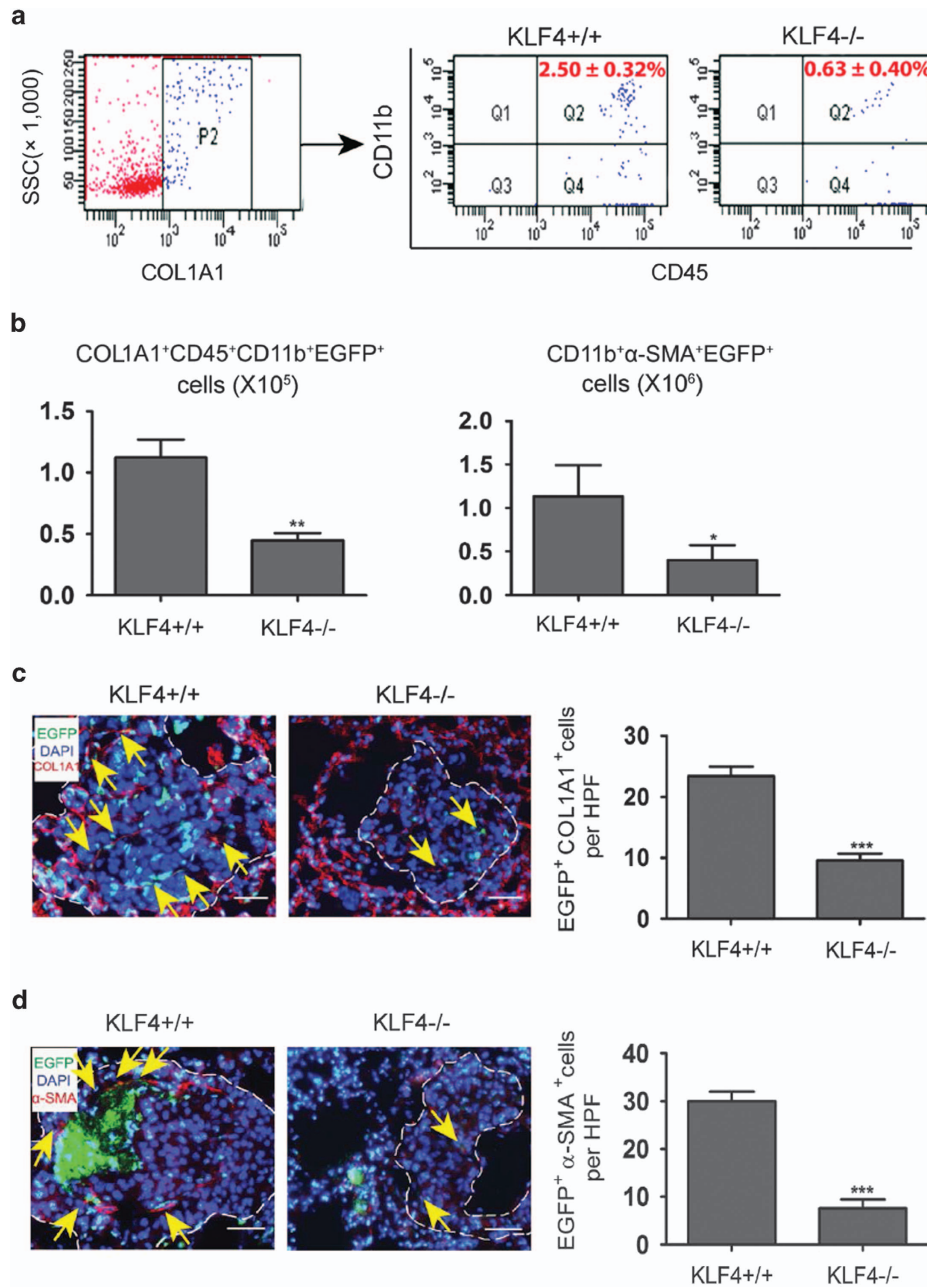


Figure 2. KLF4 deficiency in bone marrow was associated with significantly decreased numbers of fibrocytes and myofibroblasts in metastatic lung. **(a)** The peripheral blood monocytes (PBMCs) from the chimeric mice as described in Figure 1 were isolated using Histopaque (Sigma-Aldrich) according to the manufacturer's instructions. EGFP-expressing PBMCs were stained and gated by biotin-conjugated mouse Collagen Type I alpha1 antibody (COL1A1; Rockland, Gilbertsville, PA, USA; left) and further analyzed using CD45 (clone 30-F11, eBiosciences) and CD11b antibodies (right) by flow cytometry. Representative dot plots were shown ($n = 8$, $P < 0.05$). **(b)** Lung-infiltrating immune cells were stained with biotin-conjugated mouse COL1A1, rabbit anti-mouse α -SMA (Millipore, Billerica, MA, USA), CD11b and CD45 antibodies and then examined using flow cytometry. Quantifications of the total number of COL1A1⁺CD45⁺CD11b⁺EGFP⁺ cells (left) and CD11b⁺ α -SMA⁺EGFP⁺ cells (right) in lung were shown based on cell counting and flow cytometry data. **(c and d)** Representative immunofluorescence staining of lung tissues with rabbit anti-mouse COL1A1 (Rockland; 1:200, scale bars = 100 μ m) and α -SMA (1:200, scale bars = 50 μ m). COL1A1⁺EGFP⁺ and α -SMA⁺EGFP⁺ double positive cells (yellow arrows) within dotted lines encircling metastatic lesions were counted for every five high power fields (HPF) and compared. Mean \pm s.e.m., * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

and α -SMA antibodies. The results showed that although there was no difference in the total number of EGFP⁺ cells between the KLF4^{+/+} and KLF4^{-/-} group (data not shown), COL1A1⁺EGFP⁺ cells decreased from 6.20 (± 0.66)% in the KLF4^{+/+} mice to 1.6 (± 0.51)% in KLF4 deficient mice (Figure 3c, $P < 0.001$). Similarly, α -SMA⁺EGFP⁺ cells also decreased in KLF4^{-/-} mice (KLF4^{+/+} 4.6 (± 0.68)% versus KLF4^{-/-} 1.4 (± 0.40)%, $P < 0.01$, Figure 3c), which further supported

our speculation that KLF4 regulates the differentiation of CCR2⁺ MDSCs after they are recruited to the lung *in vivo*.

KLF4 gene expression was tightly linked with that of FSP-1 in fibrocyte generation from MDSCs

To elucidate the underlying molecular mechanism, we proceeded to examine the role of KLF4 in fibrocyte generation from MDSCs *in vitro*.

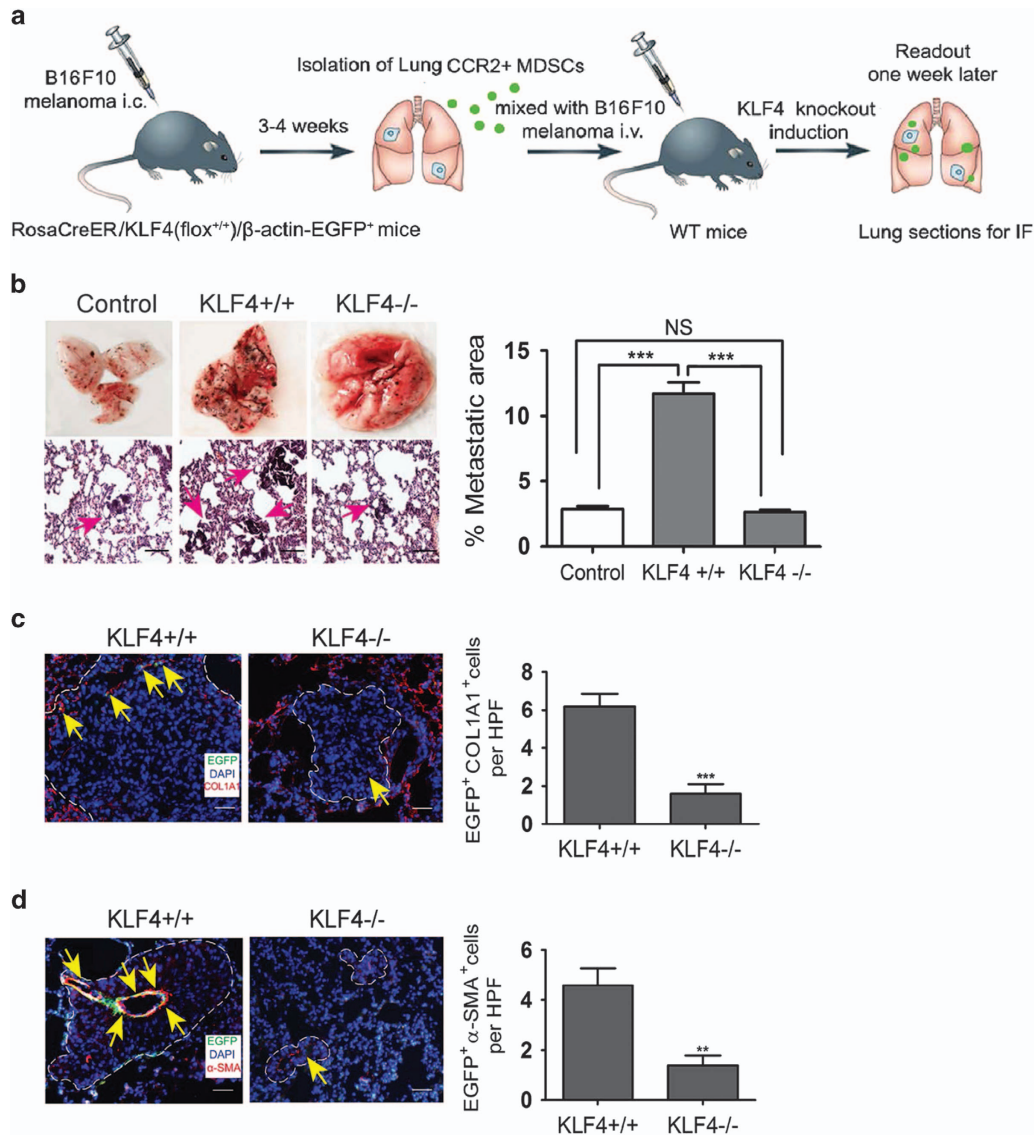


Figure 3. KLF4 ablation in CCR2⁺ MDSCs reduced pulmonary metastasis that was associated with decreased numbers of fibrocytes. **(a)** Rosa26CreER/KLF4 (floxed) mice were inoculated under the dorsal skin with 5×10^5 B16F10-Luc2 cells. Three to four weeks later, CCR2⁺ MDSCs were sorted from the lung of the tumor-bearing mice, mixed with B16F10 melanoma cells and injected into WT mice intravenously, followed by induction of KLF4 knockout as described in Figure 1. The mice in the control group were injected with B16F10 melanoma cells only, but were still treated with tamoxifen or sunflower seed oil. Mice were sacrificed 1 week later for various analyses. **(b)** Left, representative lung macrometastases (top) and hematoxylin and eosin stained sections with red arrows pointing metastatic lesions (bottom) from the mice as described in **a** were shown (scale bars = 100 μ m). Quantification of percentages of metastatic areas in each group was shown on the right. **(c)** and **(d)** Representative IF staining of lung tissues with COL1A1 (1:200) and α -SMA (1:200). COL1A1⁺EGFP⁺ and α -SMA⁺EGFP⁺ double positive cells (yellow arrows) within dotted lines circling metastatic lesions were counted for every five HPF and compared (scale bars = 50 μ m). Mean \pm s.e.m., *** P < 0.01, **** P < 0.001. HPF, high power fields; IF, immunofluorescent; NS, not significant; WT, wild type.

Splenocytes from Rosa26CreER/KLF4 (floxed) mice were purified followed by fibrocyte generation assay with recombinant murine interleukin-13 and macrophage colony-stimulating factor as previously described.²³ 4-OH TAM was used to induce KLF4 knockout. Three days after the cytokine treatment, the cells were stained and the numbers of fibrocytes per 1×10^5 splenocytes in each group were counted. While there were 58 ± 7 fibrocytes per 1×10^5 splenocytes in the control group, the number of fibrocytes decreased to 5 ± 2 fibrocytes per 1×10^5 splenocytes in the presence of 5μ m 4-OH TAM (Figure 4a). In the presence of 10μ m of 4-OH, there were no fibrocytes generated from splenocytes (data not shown). Quantitative reverse transcriptase-PCR analysis revealed that expression levels of KLF4 and FSP-1 were significantly elevated after the application of interleukin-13 and macrophage colony-stimulating

factor, and KLF4 knockout induced by 4-OH TAM correlated with significant downregulation of FSP-1 expression (Figure 4b). Consistently, the expression levels of FSP-1 in bone marrow, spleen and lung of the chimeric metastatic model were all decreased upon KLF4 knockout (Supplementary Figure 4), accompanied with decreased lung metastasis. FSP-1 is a member of S100 superfamily of calcium-binding proteins whose expression level is strongly associated with an aggressive metastatic phenotype and worse prognosis for patients with various malignancies.²⁴ The causative role of FSP-1 in tumor metastasis has been well established in literature.^{24,25} Given the fact that FSP-1 has a specific expression in fibroblasts and is also found in more than 90% of monocytes of the host immune system,²⁶ it is quite possible that there is a lineage link between the two very different cell types. Our *in vivo* data has shown that the populations

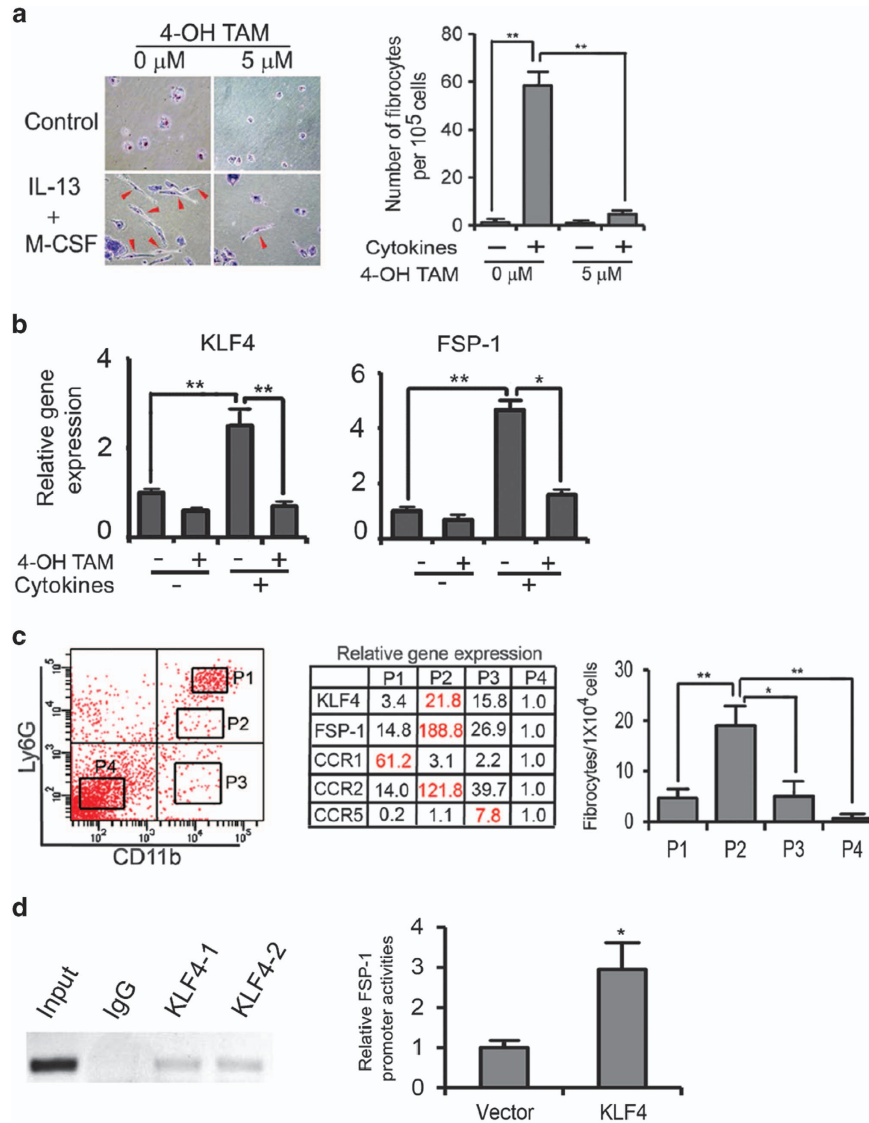


Figure 4. *KLF4* gene expression was tightly linked with that of *FSP-1* in fibrocyte generation from MDSCs. **(a)** Splenocytes from *Rosa26CreER/KLF4* (*flox^{+/+}*) mice were purified and subjected to fibrocyte generation using a recently developed approach with the application of murine interleukin (IL-13 (50 ng/ml) and macrophage colony-stimulating factor (25 ng/ml).²³ 4-OH tamoxifen (4-OH TAM, Sigma-Aldrich) was used to induce *KLF4* knockout *in vitro* and 100% ethanol as a control. Three days later, the cells were stained with Hema 3 staining kit (Fisher Scientific, Waltham, MA, USA) and the numbers of fibrocytes per 1×10^5 splenocytes in each group were counted. Left, representative photographs of fibrocytes generated from splenocytes (red arrows) were shown. Right, the number of fibrocytes per 1×10^5 splenocytes in each group was counted and compared ($n=5$). **(b)** The total RNA of fibrocytes generated as described in **a** were extracted using Trizol Reagent (Invitrogen, Grand Island, NY, USA) according to the manufacturer's instructions. First-strand cDNA synthesis and quantitative reverse transcriptase-PCR (qRT-PCR) were performed as described previously.¹⁵ The relative expression levels of *KLF4* and *FSP-1* were normalized to that of GAPDH. Primer sequences for qRT-PCR were listed in Supplementary Table S1. **(c)** Four different MDSC subsets in mouse splenocytes were gated using CD11b and Ly6G antibodies (left) and then sorted for qRT-PCR (middle) and fibrocyte generation assays (right). **(d)** Left, *KLF4* bound to the promoter region of *FSP-1* by the chromatin immunoprecipitation assay as we previously described²⁸ using NIH 3T3 cells (ATCC, Manassas, VA, USA). Two *KLF4* antibodies (*KLF4-1* antibody from our laboratory²⁹ and *KLF4-2* antibody from Genespin (Milano, Italy)) were used to precipitate DNA-protein complexes followed by PCR to amplify a 135-bp fragment in the *FSP-1* promoter. Normal rabbit immunoglobulin G (IgG, Santa Cruz Biotechnology, Dallas, TX, USA) was used as a negative control. The PCR primers were listed in Supplementary Table 1. Right, *KLF4* overexpression increased the promoter activities of *FSP-1* by transient transfection and dual luciferase assay. A ~2.3 kb mouse *FSP-1* promoter region was amplified (MGI: 1330282, nucleotide position -1104 to 1224) using the primers listed in Supplementary Table 1. It was cloned into pGL2 basic vector (Promega, Sunnyvale, CA, USA) and confirmed by sequencing. *FSP-1* promoter luciferase reporter and *KLF4*-overexpressing construct were co-transfected into NIH 3T3 cells using TurboFect (Thermo Scientific, Waltham, MA, USA) according to the manufacturer's instructions. The empty pGL2 vector was used as a control. Two days after transfection, the cells were lysed and the effect of *KLF4* overexpression on *FSP-1* promoter activity was assessed using the dual luciferase assay system (Promega). Mean \pm s.e.m., * $P < 0.05$, ** $P < 0.01$.

of $CCR2^+$ MDSCs, fibrocytes and myofibroblasts were highly correlated, suggesting that fibrocytes are the key to connect the host immune cells with fibroblasts in the tumor microenvironment by carrying the expression/function of *FSP-1*.

To further test our hypothesis, we sorted four different subsets of MDSCs from murine splenocytes based on CD11b and Ly6G signals (Figure 4c) and performed quantitative PCR analysis and fibrocyte generation assay. In agreement with our speculation, the

highest expression levels of *KLF4*, *FSP-1* and *CCR2* coexisted in CD11b⁺Ly6G^{int} MDSCs (namely CCR2⁺MDSCs, P2 in Figure 4c) among all the MDSC subsets, and this subpopulation showed the most efficient fibrocyte generation as well (Figure 4c). To examine the potential regulation of *FSP-1* transcription by *KLF4*, we first performed chromatin immunoprecipitation assay using two different *KLF4* antibodies to examine the direct binding of *KLF4* with the *FSP-1* promoter. As shown in the left panel of Figure 4d, both *KLF4* antibodies but not control immunoglobulin G pulled down a fragment in the *FSP-1* promoter region as amplified by PCR. We also generated a ~2.3 kb *FSP-1* promoter luciferase reporter construct and performed dual luciferase assays. As shown in the right panel of Figure 4d, overexpression of *KLF4* tripled the *FSP-1* promoter activity when compared with that of the vector control. Together, these data suggest a direct regulation of *FSP-1* expression by *KLF4* at the transcription level in the process of fibrocyte generation from CCR2⁺ MDSCs.

Collectively, our data suggest that *KLF4* in bone marrow promotes tumor metastasis by regulating the recruitment of CCR2⁺ MDSCs, likely in a CCL2/CCR2-dependent manner because *KLF4* regulates *CCR2* expression.²⁷ In addition, once CCR2⁺ MDSCs are recruited to the lung, *KLF4* further regulates their differentiation into fibrocytes resulting in increased metastatic tumor growth probably dependent on *KLF4*-mediated *FSP-1* expression. This finding uncovered, to our knowledge, a novel mechanism whereby *FSP-1* promotes tumor metastasis by fibrocyte generation from MDSCs. One limitation of our study is that we only used *KLF4* deficient mouse models. Future experiments with transgenic mice overexpressing *KLF4* in bone marrow will further confirm our observations. In addition, to confirm our hypothesis that *KLF4* promotes tumor metastasis in a *FSP-1* dependent manner, future experiments will be performed to test whether ectopic expression of *KLF4* can rescue *FSP-1* knockout-induced phenotypes of tumor metastasis. However, in *Fsp-1-Cre/KLF4* (flox^{+/+}) mice, *KLF4* deficiency was correlated with drastic downregulation of *FSP-1* (data not shown). These results not only confirmed regulation of *FSP-1* by *KLF4*, but also supported our overall hypothesis. Finally, further studies are needed to fully characterize the mechanism whereby *KLF4* regulates *FSP-1* transcription.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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