KLF4-dependent perivascular cell plasticity mediates pre-metastatic niche formation and metastasis

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A deeper understanding of the metastatic process is required for the development of new therapies that improve patient survival. Metastatic tumor cell growth and survival in distant organs is facilitated by the formation of a pre-metastatic niche that is composed of hematopoietic cells, stromal cells and extracellular matrix (ECM). Perivascular cells, including vascular smooth muscle cells (vSMCs) and pericytes, are involved in new vessel formation and in promoting stem cell maintenance and proliferation. Given the well-described plasticity of perivascular cells, we hypothesized that perivascular cells similarly regulate tumor cell fate at metastatic sites. We used perivascular-cell-specific and pericyte-specific lineage-tracing models to trace the fate of perivascular cells in the pre-metastatic and metastatic microenvironments. We show that perivascular cells lose the expression of traditional vSMC and pericyte markers in response to tumor-secreted factors and exhibit increased proliferation, migration and ECM synthesis. Increased expression of the pluripotency gene Klf4 in these phenotypically switched perivascular cells promoted a less differentiated state, characterized by enhanced ECM production, that established a pro-metastatic fibronectin-rich environment. Genetic inactivation of Klf4 in perivascular cells decreased formation of a pre-metastatic niche and metastasis. Our data revealed a previously unidentified role for perivascular cells in pre-metastatic niche formation and uncovered novel strategies for limiting metastasis.

Microenvironmental signals that arise early in pre-metastatic sites are among the key determinants of successful metastatic colonization. Previously, we defined activated stromal cells, altered ECM and recruited bone-marrow-derived cells (BMDCs) as components of a tumor-conducive microenvironment at distant sites that arises in response to factors released by the primary tumor, which we termed the pre-metastatic niche. Expansion of PDGFR-α* stromal cells and an associated localized increase in fibronectin supports the recruitment of hematopoietic cells to the pre-metastatic niche. These recruited hematopoietic cells develop into myeloid cells at pre-metastatic sites and exhibit immunosuppressive features that support metastatic tumor cell colonization and proliferation. Although there is an increased understanding of the role of myeloid cells in the pre-metastatic environment and in tumor metastases, less is known about the contribution of stromal cells to pre-metastatic niche formation and their functional role in metastatic outgrowth.

Perivascular cells, including vSMCs and pericytes, support vascular stability through close contact and signaling cross-talk with the endothelium and through their contractile role in regulating blood vessel tone, diameter, and permeability. There is increasing evidence to suggest that perivascular cells are also the key stromal component of stem cell niches, in which they regulate stem cell maintenance and proliferation, and as such are critical to tissue regeneration and organ homeostasis. Perivascular cells are traditionally identified by the expression of a combination of genes involved in blood vessel contractility, such as Myh11, Acta2 and Tagln (vSMCs), and of cell surface marker proteins, such as CSPG4 (also known as NG2), PDGFR-β and RGS5 (pericytes), with extensive overlap in marker expression being observed in vSMC and pericyte populations. Perivascular cells also show remarkable plasticity in the settings of inflammation and vascular disease, where they lose expression of the genes involved in contractility (such as Myh11, Acta2 and Tagln) and acquire a proliferative, migratory and ECM synthesizing or modulating phenotype. This process has been termed phenotypic switching. Recent studies have demonstrated that perivascular cells can acquire macrophage and mesenchymal stem cell (MSC) phenotypes in individuals with atherosclerosis and that this is dependent on the expression of Krüppel-like factor 4 (Klf4) and POU domain, class 5, transcription factor 1 (Pou5f1, also known as Oct4), which play critical roles in pathogenesis. As a result of phenotypic switching, perivascular cells can be overlooked or misidentified owing to a lack of expression of their classic markers. Although there is evidence for

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perivascular phenotypic switching in inflammation and vascular disease, the role of perivascular cells in pre-metastatic niche formation and metastatic progression remains underappreciated.

Here we describe perivascular cell phenotypic switching that coincides with Klf4 and PDGFR-α expression in pre-metastatic lung in response to a growing, localized primary tumor or to tumor-secreted factors. Tumor-induced perivascular cell phenotypic switching initiated pre-metastatic niche formation, at least in part by contributing to fibronectin protein production that promoted tumor cell metastatic behavior. Disruption of perivascular-cell-specific Klf4 expression inhibited perivascular phenotypic switching and decreased metastasis. Our results reveal a previously unknown role for perivascular cells in pre-metastatic niche formation and identify Klf4 as a critical inducer of perivascular cell phenotypic switching. By identifying perivascular cell plasticity in the pre-metastatic niche, we uncover a new opportunity to redirect stromal involvement in this setting and limit metastatic progression.

RESULTS

Lineage-traced perivascular cells demonstrate that phenotypic switching occurs in pre-metastatic sites

Perivascular cell phenotypic switching is characterized by loss of marker gene expression (such as Myh11 and Acta2) and an increase in proliferation, migration and ECM production. Due to this reduced perivascular marker gene expression and the potential misidentification of these cells in their activated state, in vivo studies that carefully trace and investigate the function of phenotypically switched perivascular cells are required. To determine whether perivascular cells undergo phenotypic switching in pre-metastatic tissue, we used the recently described Myh11-ERT-crt2 ROSA-STOP-flox-eYFP lineage-tracing mice (hereafter referred to as ‘Myh11 lineage-tracing mice’), in which the promoter of perivascular-cell-specific gene Myh11 drives expression of a tamoxifen-inducible Cre recombinase (Supplementary Fig. 1a). In adult Myh11 lineage-tracing mice, tamoxifen treatment induces stable expression of enhanced yellow fluorescent protein (eYFP) in SMCs and pericytes, which enables the detection of cells that express the Myh11 gene only after tamoxifen administration, including in pre-existing vSMCs and pericytes, as well as their progeny, even when expression of this perivascular marker is subsequently lost. Notably, we found that nearly all MYH11+ cells in the lungs of healthy Myh11 lineage-tracing mice treated with tamoxifen were eYFP+ and co-expressed MYH11 and ACTA2 (Supplementary Fig. 1b). eYFP+ cells also expressed ACTA2, a known marker of perivascular cells and myofibroblasts (Supplementary Fig. 1c).

To interrogate the role of perivascular cells during metastatic development, we orthotopically injected metastatic melanoma B16-F10 or metastatic rhabdomyosarcoma M3-9M tumor cells into syngeneic Myh11 lineage-tracing mice and analyzed the pre-metastatic lung at multiple time points for evidence of perivascular phenotypic switching in eYFP-expressing cells that had lost expression of perivascular cell markers MYH11 and ACTA2 (Supplementary Fig. 1d,e). We found that there was an increase in the total number of eYFP+ cells in the lungs of mice with the B16-F10 or M3-9M tumors from day 10 to day 18 after tumor cell injection (Fig. 1 and Supplementary Fig. 2a). By careful confocal imaging analysis of the pre-metastatic lung at multiple time points, we demonstrated the loss of the perivascular cell markers MYH11 and ACTA2, the displacement of perivascular cells away from blood vessels by day 3, the appearance of eYFP+ cells away from blood vessels by day 5, and an increase in the number of eYFP+MYH11+ACTA2− cells found in clusters within the parenchyma that was separated from the vasculature of the pre-metastatic lung by day 10 after tumor cell injection (Fig. 1e and Supplementary Fig. 2a). The total number of eYFP+ cells and the proportion of phenotypically modulated eYFP+ cells that were MYH11+ACTA2− were increased in the pre-metastatic lung (as compared to those in the
normal lungs of control mice (Fig. 1c,f,j,k). This expanded population of eYFP+ cells may be due to vSMC recruitment to these pre-metastatic sites, proliferation in situ or a combination of both. Consistent with the increase in the number of eYFP+ cells identified in stained tissue sections, we determined that many eYFP+MYH11−ACTA2− cells expressed the proliferation marker Ki67 (Supplementary Fig. 2b). More eYFP+ cells were found at distances further away from blood vessels in the lungs of mice with the B16-F10 (Fig. 1d) or M3-9M (Fig. 1l) tumors than in the lungs of control mice. These findings suggested that perivascular cells in the pre-metastatic...
lung showed enhanced proliferation and migration, either from the existing lung vasculature or from other unappreciated sites, which are hallmarks of perivascular phenotypic switching. This increase in the number of phenotypically modulated perivascular cells was specific to pre-metastatic lung tissue, in that a similar expansion of perivascular cells was not seen in other tissues of tumor-bearing mice such as the liver (Supplementary Fig. 3), to which the B16-F10 and M3-9M cells rarely metastasize to, implying that perivascular phenotypic switching is associated with the site specificity of metastasis.

Using flow cytometric analysis, we confirmed and quantified the expansion of eYFP+ cells in the pre-metastatic lungs of B16-F10–tumor-bearing mice over a range of time points (Fig. 1g and Supplementary Fig. 4a,b). We used pathology review, an in vivo imaging system (IVIS) and flow cytometry to assay for tumor cells that expressed a firefly luciferase and mCherry fusion protein and determined that metastasis was below the level of detection between days 5 and 16 after tumor cell injection (Supplementary Fig. 1g). A significant 4.39-fold increase in the total number of eYFP+ cells was observed in the pre-metastatic lungs of B16-F10–tumor-bearing Myh11 lineage-tracing mice at day 10 after tumor cell injection, which persisted to later time points, as compared to that in HBSS-injected non-tumor-bearing Myh11 lineage-tracing littermate control mice (Fig. 1g). A concurrent increase in the total number of eYFP+Ki67+ cells was observed at all time points in the pre-metastatic lung, including at day 3 after tumor cell injection, before the observed increase in eYFP+ cells at day 10 after tumor cell injection. Ki67 marks cells in all stages of the cell cycle that lead to cell division; thus, the day 3 time point may represent the early transition of quiescent eYFP+ cells to a cycling eYFP+ cell population70. Taken together, these data indicated that eYFP+ cells proliferate in the pre-metastatic lung (Fig. 1g and Supplementary Fig. 4d). Similarly, there were 3.2-fold more eYFP+ cells in the pre-metastatic lungs of Myh11 lineage-tracing mice with M3-9M tumors than in the lungs of HBSS-injected non-tumor-bearing Myh11 lineage-tracing littermate control mice (Supplementary Fig. 4c). A decrease in the total number of eYFP+ACTA2+ cells over time was observed in the pre-metastatic lungs of Myh11 lineage-tracing mice with B16-F10 tumors (Fig. 1g). These data were consistent with our immunofluorescence analysis of stained tissue sections of the similar, but more specific, eYFP+ACTA2+MYH11+ cell population, highlighting the complementary nature of analysis by confocal imaging and flow cytometry to examine perivascular cells in the pre-metastatic lung (Fig. 1c,f,j,k). Taken together, these data are indicative of marker downregulation that is characteristic of perivascular phenotypic switching.

To further characterize perivascular eYFP+ cells in our lineage-tracing mouse models of pre-metastasis, we evaluated pericyte markers in eYFP+ cells in the Myh11 lineage-tracing model. We discovered many eYFP+ pericytes in the microcirculation of the heart, skeletal muscle and lung (Supplementary Fig. 5). A subset of eYFP+ cells within the microvasculature of non-tumor-bearing lungs of Myh11 lineage-tracing mice expressed the pericyte markers PDGFR-β and NG2 (Supplementary Fig. 5c). We identified eYFP+ cells that were dissociated from the microvasculature within the pre-metastatic lungs of Myh11 lineage-tracing mice with B16-F10 tumors (Supplementary Fig. 5c). Of note, eYFP+ cells in the pre-metastatic lungs of the Myh11 lineage-tracing mice with B16-F10 tumors did not express the macrophage markers F4/80 or LGALS3, as reported for atheromas17 (Supplementary Fig. 4e). Although PDGFR-α+ cells are found in normal healthy lungs, this population was increased at only later time points (day 18) in the pre-metastatic lung (Supplementary Fig. 6a-c).

By contrast, we observed that the number of eYFP+PDGFR-α+ cells was dramatically increased as early as day 10 after tumor cell injection (Supplementary Fig. 6a), and we found that the number of eYFP+PDGFR-α+CD51+ cells increased throughout the time course in pre-metastatic lungs (Fig. 1g). This was consistent with previous findings that identified the expansion of a PDGFR-α+ fibroblast-like population in the pre-metastatic niche71. Taken together, these data demonstrate that pre-metastatic lungs contain an expanded population of eYFP+ perivascular cells that are not in close association with blood vessels and that may contribute to a PDGFR-α+ activated stromal cell population previously described in the pre-metastatic niche.

NG2+ pericytes contribute to the activated perivascular population in the pre-metastatic niche

In addition to the stable eYFP labeling of vSMCs in the Myh11 lineage-tracing model, eYFP+ pericytes were observed in many tissues, including the microvasculature of the heart and spinotrapezius muscle (Supplementary Fig. 5a,b). Furthermore, eYFP+ cells in the lungs of non-tumor-bearing Myh11 lineage-tracing mice expressed the pericyte markers NG2 and PDGFR-β (Fig. 2a and Supplementary Fig. 5c), and the total number of eYFP+NG2+ cells were found to increase in pre-metastatic lungs over time (Fig. 2b). Taken together, these data suggested that pericytes could substantially contribute to the expansion of eYFP+ cells in the pre-metastatic lungs of Myh11 lineage-tracing mice. Given that NG2+ perivascular cells are a known key component of stem cell niches, particularly in the bone marrow where they support hematopoietic stem cell maintenance72 and in the fetal liver where they induce hematopoietic stem cell proliferation19, we sought to determine the contribution of the NG2-expressing pericyte population to the perivascular eYFP+ population in pre-metastatic tissue of Myh11 lineage-tracing mice.

To elucidate whether pericytes, in particular, participate in pre-metastatic niche formation, we generated conditional NG2-ERT-creER2 ROSA-STOP-flox-eYFP lineage-tracing mice (hereafter referred to as ‘NG2 lineage-tracing mice’) (Supplementary Fig. 7a)21, in which NG2-expressing pericytes were stably labeled by eYFP expression in many tissues after injection of tamoxifen in the adult mice (Supplementary Fig. 7b,c). Using this NG2 lineage-tracing model, we observed an expansion of eYFP+ cells in the lungs of mice bearing B16-F10, M3-9M or E0771 tumors that was comparable to the expanded perivascular population that we identified in the tumor-bearing Myh11 lineage-tracing mice, suggesting that NG2+ pericytes represent an expanded perivascular population in the pre-metastatic setting (Fig. 2c-j and Supplementary Fig. 7b). We confirmed and quantified this expansion by immunofluorescence image analysis and flow cytometric analysis (Fig. 2j,k and Supplementary Fig. 7d). eYFP+ cells were only rarely identified in the lungs of NG2 lineage-tracing mice that received non-metastatic melanoma B16-F0 tumor cells, suggesting that the increased number of eYFP+ cells in the NG2 lineage-tracing mice correlated with metastatic potential (Fig. 2d). Taken together, the data reveal that perivascular cells, including NG2-expressing pericytes, show expansion in the parenchyma of pre-metastatic lungs in a number of mouse models of metastasis.

Tumor-derived factors induce KLF4 expression in perivascular cells of the pre-metastatic niche

Perivascular cell phenotypic switching is dependent on the expression of KLF4 in several disease models7,8,14,16,17,22 (Fig. 3a). Direct KLF4 binding to the promoters of vSMC marker genes results in downregulation of marker gene expression and a transition from a contractile SMC phenotype to a plastic, reactive state17,22,23. We observed an
NG2-expressing pericytes are activated in the pre-metastatic lung. (a) Representative low-magnification image (left) of lungs from Myh11 lineage-tracing mice showing eYFP+ cells (orange), as well as cells expressing the pericyte marker NG2 (red) around blood vessels (V) and the perivascular markers ACTA2 (green) and MYH11 (magenta) (n = 8 mice per group; n = 5 images per mouse). DAPI-stained nuclei are shown in blue. Higher-magnification (20×) confocal images with single-channel fluorescence images are shown to the right. The arrows depict an eYFP+NG2+MYH11+ACTA2+ cell. Scale bars, 20 μm.

(b) Quantification of CD31−CD45−TER119−eYFP+NG2+ cells, using flow cytometry, in lungs from Myh11 lineage-tracing mice harvested at 3, 5 and 10 d after injection with B16-F10 tumor cells or at day 0 after injection with HBSS (HBSS day 0, n = 6; B16-F10 day 3, n = 6; B16-F10 day 5, n = 5; B16-F10 day 10, n = 5). Center lines indicate median values, and top and bottom lines indicate the 25th and 75th percentiles, respectively. ** P < 0.001, ANOVA.

(c–g) Representative images of lungs from NG2 lineage-tracing mice that were harvested at day 10 after injection with HBSS (c), nonmetastatic B16-F0 melanoma cells (d), metastatic B16-F10 melanoma cells (e), E0771 tumors cells (f) or M3-9M tumor cells (g) (n = 3 mice per group; n = 5 images per mouse). Blood vessels (V) were stained for PECAM1 (red), and perivascular cells were stained for ACTA2 (green). Nuclei were stained with DAPI (blue). Scale bars, 20 μm.

(h, i) Representative confocal images of lungs harvested from NG2 lineage-tracing mice 10 d after injection with HBSS (h) or B16-F10 tumor cells (i) showing NG2+ (red), PDGFR-β (green) and/or MYH11+ (magenta) eYFP+ perivascular cells (orange) (n = 5 mice per group; n = 5 images per mouse). Nuclei were stained with DAPI (blue). Scale bars, 10 μm.

(j) Quantification of DAPI+eYFP+ particles in immunofluorescence images of lungs from three mouse models of pre-metastasis—B16-F10 melanoma cells (n = 8 mice per treatment group) (left), E0771 breast carcinoma cells (HBSS, n = 9; E0771, n = 8) and M3-9M metastatic rhabdomyosarcoma cells (HBSS, n = 6; M3-9M, n = 7)—at day 10 after HBSS or tumor cell injection in NG2 lineage-tracing mice. Center lines indicate median values, and top and bottom lines indicate the 25th and 75th percentiles, respectively. **** P < 0.0001 by Student’s t-test.

(k) Flow cytometric analysis of CD31−CD45−TER119−eYFP+ cells in the lungs from NG2 lineage-tracing mice at days 3, 5 and 10 after injection with B16-F10 tumor cells or at day 0 after injection with HBSS (HBSS day 0, n = 6; B16-F10 day 3, n = 5; B16-F10 day 5, n = 5; B16-F10 day 10, n = 6). Center lines indicate median values, and top and bottom lines indicate the 25th and 75th percentiles, respectively. *** P < 0.0001 and **** P < 0.00001 by ANOVA.
increase in the percentage of eYFP⁺ cells that were KLF4⁺ in the pre-metastatic lungs of Myh11 lineage-tracing mice injected with B16-F10 tumor cells at day 10 after tumor cell injection, as compared to that in HBSS-injected Myh11 lineage-tracing littermate control mice (Fig. 3b–c). As such, we sought to determine whether tumor-derived factors might induce KLF4 expression in perivascular cells that, in turn, could mediate perivascular cell plasticity and phenotypic switching.

Previous investigations have demonstrated that in vivo administration of tumor-derived factors, such as those in tumor-conditioned medium (TCM) or in tumor-derived exosomes, potentiates pre-metastatic niche formation. To determine whether perivascular cell activation in the pre-metastatic niche is initiated by tumor-derived factors, we intraperitoneally injected non-tumor-bearing Myh11-lineage-tracing mice with TCM or tumor-derived exosomes and assessed them for phenotypic switching by assaying for eYFP⁺ cells in the Myh11 lineage-tracing mice that no longer expressed MYH11. There were more eYFP⁺ MYH11−ACTA2− cells that were neither in close association with blood vessels nor could be identified by perivascular marker expression, in the lungs of Myh11 lineage-tracing mice that had been exposed to tumor-derived factors, as compared to that in the lungs of Myh11 lineage-tracing mice that had been exposed to non-tumor melanocyte factors, which was indicative of their activated state and was similar to our observations in the pre-metastatic lungs at various time points of tumor-bearing mice (Fig. 3f–j). Preconditioning of mice with exosomes from B16-F10 tumor cells in vitro or TCM followed by tail vein injection of B16-F10 melanoma cells resulted in increased metastasis, as compared to that in mice that were treated with exosomes or TCM from non-tumor melanocyte cultures, indicating that pretreatment with tumor-derived factors is sufficient to induce perivascular cell plasticity and establish a pre-metastatic niche that enhances tumor cell colonization and/or proliferation (Supplementary Fig. 8a). These studies indicate that tumor-derived factors contribute to perivascular cell phenotypic switching and pre-metastatic niche formation.

The activated state of perivascular cells was characterized by a decrease in marker gene expression and an increase in proliferation, migration and matrix production (Fig. 3a). To test whether tumor-derived factors induced KLF4-dependent perivascular
phenotypic switching, we sought to establish an in vitro model by culturing vSMCs with either TCM from B16-F10 metastatic melanoma cultures or exosomes purified from B16-F10 TCM. qRT–PCR profiling of vSMCs that were exposed to TCM over the course of 72 h revealed an increase in Klf4 gene expression as early as 2 h after TCM treatment to levels similar to those seen in vSMCs that were treated with the cytokine PDGF-BB (Supplementary Fig. 8b), a known inducer of KLF4-dependent phenotypic switching23,27. We observed...
a subsequent decrease in the expression of vSMC marker genes, including Acta2, Myh11 and Tagln (Supplementary Fig. 8b). Notably, TCM derived from culturing the nonmetastatic melanoma cell line B16-F0 did not induce these gene expression changes (Supplementary Fig. 8b), indicating that tumor-factor-induced vSMC phenotypic switching may be specific to tumor metastatic potential.

We extended these gene expression data by performing RNA sequencing (RNA-seq) analysis of cultured vSMCs that were exposed to TCM over the course of 72 h. We confirmed that expression of Klf4 was elevated, and the expression of the vSMC marker genes Acta2 and Tagln was decreased, in vSMCs treated with B16-F0 TCM, as compared to that in vSMCs treated with serum-free medium (SFM) (Fig. 3k). Pathway analysis demonstrated that genes set related to proliferation, migration and invasion—all hallmarks of perivascular phenotypic switching—were activated over the course of 72 h (Fig. 3l). We confirmed that there was an increase in proliferation by vSMCs cultured in B16-F0 TCM (Supplementary Fig. 8d), which was indicative of their activated, phenotypically modulated state.

Consistent with our finding that the number of eYFP+PDGFR-α+ cells was increased in the pre-metastatic lungs of mice with B16-F10 tumors (Fig. 1g and Supplementary Fig. 6b,c), vSMCs that were cultured in vitro with pre-differentiated PDGFR-α, an activated stromal marker (Fig. 3k and Supplementary Fig. 8b,c), suggesting that phenotypically modulated vSMCs may represent a PDGFR-α+ cell population previously reported in pre-metastatic tissue in response to tumor-derived factors.

Treatment with TCM from the metastatic B16-F10 cell line but not from the nonmetastatic B16-F0 cell line was sufficient to induce vSMC phenotypic switching in vitro (Supplementary Fig. 8b,c), indicating that factors secreted specifically by tumor cells with metastatic potential contributed to the activated vSMC phenotype. As such, we sought to determine which cytokines were increased in the conditioned medium from the metastatic B16-F10 cell line, as compared to that in the TCM from the nonmetastatic B16-F0 cell line, by using a cytokine array (Supplementary Table 1). Of the eight cytokines found to be elevated in this assay, WISP1, ANG2 and interleukin (IL)-1B all have known roles in vascular biology and inflammation and can, in some reports, induce KLF4 expression.29–33 We used a Klf4 reporter assay, which results in the secretion of luciferase in response to Klf4 promoter activity, in combination with qRT–PCR analysis to determine that all three cytokines were capable of inducing Klf4 expression in vSMCs to levels comparable to those induced by treatment with B16-F10 TCM, and knockdown of KLF4 expression with a short interfering RNA (siRNA) specific for Klf4 (siKlf4) significantly decreased Klf4 promoter activity, in combination with qRT–PCR analysis to determine that all three cytokines were capable of inducing Klf4 expression in vSMCs to levels comparable to those induced by treatment with B16-F10 TCM, and knockdown of KLF4 expression with a short interfering RNA (siRNA) specific for Klf4 (siKlf4) significantly decreased Klf4 promoter activity.
inhibited this activation (Fig. 3m). Treatment of vSMCs cultured in vitro with WISP1 or IL-1β, but not ANG2, induced KLF4-dependent suppression of vSMC marker gene expression (Fig. 3n). However, pretreatment of B16-F10 TCM with a neutralizing antibody specific for ANG2 before applying the B16-F10 TCM onto vSMCs in vitro blocked TCM-induced vSMC proliferation (Supplementary Fig. 8e), indicating that ANG2-induced effects on vSMC activation may work coordinately through KLF4 and other effector proteins.

In addition to B16-F10 TCM, we found that treatment with exosomes derived from B16-F10 tumor cells was also capable of inducing vSMCs/pericytes to adopt a phenotype associated with tumor cell-secreted factors. This was evident through the expression of KLF4 and other markers associated with activated vSMCs/pericytes (Fig. 3a-c). The use of lentiviral vectors expressing eYFP and FN1 allowed for the identification of eYFP+ cells among the vSMCs/pericytes, providing a clear visualization of the phenotypic changes (Fig. 3d)

Furthermore, the use of eYFP labeling and inactivation of the Klf4 gene in perivascular cells (Fig. 3e) enabled the tracking of these changes over time. The expression of ACTA2, eYFP, FN1, and DAPI was observed in these cells, providing a comprehensive view of the cellular dynamics (Fig. 3f).

The stable eYFP+ cells/lung × 10⁴ (Fig. 3g) and eYFP+ cells/lung × 10³ (Fig. 3h) confirmed the effectiveness of the eYFP labeling and inactivation of the Klf4 gene in perivascular cells. The use of Tamoxifen-induced Cre recombination (Fig. 3i) further validated the approach, providing a stable marker for tracking the phenotype changes.

Overall, these findings highlight the complex interplay between tumor cell-secreted factors and vSMCs/pericytes, emphasizing the role of KLF4 in mediating these responses.
perivascular phenotypic switching in vivo (Fig. 3h–j) and vSMEC phenotypic switching in vitro (Supplementary Fig. 8c). Isolated melanocyte-,-B16-F0- and B16-F10-derived exosomes were found to have a similar size distribution—with peak particle sizes of 133 nm, 85 nm and 99 nm, respectively (Supplementary Fig. 8g)—and to contain exosomal and extracellular-vesicle markers (Supplementary Fig. 8h). We also found that fluorescently labeled B16-F0 and B16-F10 exosomes were capable of entering vSMECs (Supplementary Fig. 8i). We determined whether any of the three candidate cytokines were contained in metastatic-tumor-derived exosomes. We found that only WISP1, and not IL-1β or ANG2, was found in metastatic-tumor-cell-derived exosomes, including those from the B16-F6 melanoma, MNNG-HOS and HO8 osteosarcoma, and Panc02 pancreatic tumor cell lines. WISP1 was not found in exosomes derived from the nonmetastatic B16-F0 cell line or a non-tumor melanocyte cell line, suggesting that the presence of exosomal WISP1 production may be specific to metastatic tumor cell lines, which in combination with non-exosomal cytokines (such as IL-1β and ANG2) may enhance tumor metastatic behavior (Fig. 3o and Supplementary Figs. 8f and 9a,d). Circulating levels of WISP1 were also found to be increased at various time points in the pre-metastatic stages in B16-F10 tumor-bearing mice (Fig. 3p) to concentrations equivalent to those found in B16-F10 exosomes.
generated in vitro (Supplementary Fig. 8f) that promoted perivascular phenotypic switching after administration in vivo (Fig. 3h–j), supporting the idea that this cytokine may contribute to pre-metastatic niche formation (Supplementary Fig. 8a). Neutralizing antibodies specific for β1-integrin that are known to interfere with WISP1 activity inhibited both B16-F10-exosome-induced and recombinant WISP1 (rWISP1)-induced vSMC proliferation in vitro (Fig. 3g). Taken together, the data suggest that multiple factors secreted by the B16-F10 metastatic melanoma cell line, including but not limited to WISP1-containing tumor-derived exosomes, may work in concert through the induction of KLF4 expression to contribute to vSMC phenotypic switching characterized by downregulation of vSMC marker gene expression and increased vSMC proliferation.

Tumor-derived factors induce perivascular phenotypic switching and enhance ECM deposition that supports tumor metastatic behavior

Phenotypically modulated perivascular cells increase production of ECM proteins, such as fibronectin and various collagens, and ECM-remodeling enzymes, such as matrix metalloproteinases, in many disease settings14,3,4. ECM modulation can dictate tumor cell fate and metastasis in a number of models35–38. As seen previously1, we observed more fibronectin staining in the lungs of B16-F10-tumor-bearing Myh11 lineage-tracing mice at day 10 after tumor cell injection than that in the lungs of HBSS-treated littermate control mice (Fig. 4a.b). Increased amounts of fibronectin deposition in the pre-metastatic lungs were found to be more closely associated with eYFP+ cells than with eYFP− cells within the same stained tissue sections (Fig. 4c). eYFP+ aortic vSMCs from Myh11 lineage-tracing mice that were isolated and cultured ex vivo with either B16-F10 TCM or tumor-derived exosomes also showed an increase in fibronectin production as compared to those cultured in defined SFM (Fig. 4d.e). We next used an in vitro model to test whether the matrix produced by phenotypically modulated vSMCs provided a pro-metastatic environment and enhanced metastatic behavior in tumor cells. vSMC phenotypic switching was induced in vitro by treatment with either B16-F10 TCM or tumor-derived exosomes for 72 h, after which the vSMCs were lysed from the culture plates to leave behind the vSMC-derived ECM (Fig. 4f,g). B16-F10 tumor cells that were seeded to plates containing ECM produced by the phenotypically modulated vSMCs with enhanced fibronectin production (Fig. 4g) showed more B16-F10 tumor cell adhesion and proliferation than the B16-F10 tumor cells that were seeded to plates containing ECM produced by vSMCs that did not undergo phenotypic switching (Fig. 4h,i). B16-F10 tumor cells showed increased migration across a Transwell membrane toward ECM components derived from phenotypically switched vSMCs that had been exposed to B16-F10 exosomes, B16-F10 TCM or rWISP1, as compared to the migration seen for tumor cells that had been subjected to ECM components derived from vSMCs conditioned with B16-F0 exosomes or melanocyte exosomes (Fig. 4j). Fibronectin production by vSMCs that had been cultured in B16-F10 TCM was dependent on KLF4, as siRNA-mediated knockdown of Klf4 expression resulted in a reduction of B16-F10-TCM-induced fibronectin production (Fig. 4k and Supplementary Fig. 9b), suggesting that activated vSMCs contribute to a pro-metastatic environment, at least in part, through KLF4-dependent fibronectin production. Indeed, B16-F10 tumor cells that were cultured on the ECM derived from phenotypically switched vSMCs demonstrated an enhanced ability to form tumorspheres (Supplementary Fig. 8j), suggesting that ECM production from phenotypically modulated vSMCs in response to tumor-derived factors promoted a stem-cell-like environment that had features of metastasis-initiating tumor cells39,40.

Knockout of Klf4 in perivascular cells limits phenotypic switching and pre-metastatic niche formation

To evaluate whether KLF4-dependent perivascular phenotypic switching is essential for pre-metastatic niche formation and metastasis, we crossed the perivascular-cell-specific Myh11 lineage-tracing mice to mice withloxP-flanked (floxed) Klf4 (referred to as Klf4-flox mice), which effectively resulted in knocking out Klf4 in all of the perivascular cells in the progeny (Klf4-flox;Myh11 lineage-tracing) mice as previously described17. This Klf4-flox;Myh11 lineage-tracing model enables simultaneous tamoxifen-inducible perivascular-cell-specific Klf4 deletion (Klf4Δ) and eYFP labeling before orthotopic tumor cell injection (Fig. 4l). Despite the potential effect that perivascular phenotypic switching may have on primary tumor angiogenesis and tumor growth, we did not observe a significant difference in the diameters of B16-F10 primary tumors between Klf4Δ and Klf4-expressing (Klf4WT) littermate control mice (Supplementary Fig. 10a) nor did we observe involvement of eYFP+ perivascular cells in primary tumor architecture, as eYFP+ cells were located in normal adjacent tissue in full association with well-formed blood vessels (Supplementary Fig. 10c). However, perivascular-cell-specific Klf4 deletion resulted in a marked decrease in the number of phenotypically modulated eYFP+ACTA2+ cells in pre-metastatic lungs of B16-F10-tumor-bearing mice at day 10 after tumor cell injection, relative to those in the lungs of tumor-bearing Klf4WT littermate control mice, as determined by immunofluorescence analysis of lung parenchyma and flow cytometric analysis of whole lung (Fig. 4m.n). Consistent with the decrease observed in perivascular phenotypic switching with a perivascular-cell-specific Klf4 deletion, we saw decreased amounts of fibronectin in the lungs of B16-F10-tumor-bearing Klf4Δ mice at day 10 after tumor cell injection, as compared to that in tumor-bearing Klf4WT littermate control mice (Fig. 4o,p), indicating that KLF4 expression in perivascular cells regulates fibronectin production during the process of pre-metastatic niche formation.

Perivascular-cell-specific Klf4 deletion decreases early tumor cell colonization and metastatic burden

To determine the mechanistic role of KLF4-dependent perivascular-cell-mediated metastasis in early tumor cell seeding, we performed histological analysis of lung sections from Klf4WT and Klf4Δ mice at early metastatic time points and found decreased metastatic colonization (disseminated tumor cells and micrometastasis) in the lungs from the Klf4Δ mice relative to that in the lungs from the Klf4WT mice (Supplementary Fig. 11a,b). To assess whether these changes in early tumor cell seeding affect metastatic burden, we stably transduced our B16-F10 metastatic melanoma, M3-9M metastatic rhabdomyosarcoma and E0771 metastatic breast carcinoma cell lines with a construct expressing an mCherry–Firefly luciferase (LUC) fusion protein to detect the presence of tumors by an immunofluorescence or bioluminescent signal. Analysis of late-stage metastatic lungs revealed a dramatic decrease in metastasis to the lungs of Myh11 lineage-cell-specific Klf4Δ mice in both the model of B16-F10 tumor metastasis at day 23 after tumor cell injection and the model of M3-9M rhabdomyosarcoma metastasis at day 26 after tumor cell injection, as compared to those in the Klf4WT tumor-bearing mice (Fig. 5a–d and Supplementary Figs. 11a and 12a,b). No difference was seen in primary tumor growth in the
B16-F10 or M3-9M models between the \(Klf4^{WT}\) and \(Klf4^{Δ}\) mice, as determined by tumor volume measurements (Supplementary Fig. 10a). Although phenotypically modulated eYFP+ACTA2+/MYH11+ cells were found in the late-stage metastatic lungs of both \(Klf4^{WT}\) and \(Klf4^{Δ}\) mice, relatively few KLF4+eYFP+ cells were detected in lung sections from \(Klf4^{Δ}\) mice, whereas many more KLF4+eYFP− cells were found in the lung sections from \(Klf4^{Δ}\) mice, which confirmed the perivascular-cell-specific deletion of \(Klf4\) in \(Klf4^{Δ}\) mice (Fig. 5e). Furthermore, there was a significant decrease in the average frequency of eYFP+ cells found at long distances from the closest platelet-endothelial cell adhesion molecule 1 (PECAM1)-positive blood vessels in the lungs of \(Klf4^{Δ}\) mice (Fig. 5f).

Consistent with our findings in Myh11 lineage-cell-specific \(Klf4^{Δ}\) mice, we found no significant difference in primary tumor growth (Supplementary Fig. 10a) but did observe a dramatic decrease in metastasis to the lungs of NG2 lineage-cell-specific \(Klf4^{Δ}\) mice in the models for M3-9M rhabdomyosarcoma metastasis at day 29 (Fig. 5g.h and Supplementary Fig. 12c) and E0771 breast carcinoma metastasis at day 25 (Fig. 5i and Supplementary Fig. 12d). Taken together, these data indicate that KLF4-dependent perivascular phenotypic switching is critical for metastatic progression in two genetic mouse models of perivascular lineage-tracing and in three different orthotopic models of spontaneous tumor metastasis.

Inhibition of tumor cell binding to fibronectin recapitulates perivascular-specific KLF4-dependent metastasis

To assess the specific role of phenotypically switched perivascular cell fibronectin deposition in promoting tumor metastasis, we used siRNA-mediated knockdown of either \(Klf4\) or fibronectin 1 (Fn1) or of both genes simultaneously in vSMCs that were cultured with B16-F10 TCM. Following vSMC lysis, we seeded B16-F10 tumor cells on the resultant vSMC-deposited ECM (Fig. 4d) and performed RNA-seq analysis to determine vSMC-specific KLF4- and fibronectin-dependent changes in tumor cell metastatic behavior. Pathways related to tumor cell proliferation, viability and survival were most activated in B16-F10 cells that were cultured on the ECM of vSMCs treated with TCM as compared to those treated with SFM (Fig. 6a). These pathways were not activated when either \(Klf4\) or \(Fn1\) expression was knocked down (Fig. 6a). Hierarchical clustering analysis related to tumor cell proliferation, viability and survival demonstrated that B16-F10 cells cultured on the ECM of siKlf4-, siFn1-, or both siKlf4- and siFn1-treated vSMCs had gene expression profiles that clustered together and that clustered with B16-F10 cells cultured on the ECM of vSMCs treated with nontargeting siRNAs (siNTs) and SFM (Fig. 6b). There was an overlap of 83 genes between the B16-F10 cells that were cultured on the ECM from siKlf4- and TCM (siKlf4-TMC)-treated vSMCs and those cultured with the ECM from siFn1-TCM-treated or siKlf4-siFn1-TCM-treated vSMCs, and only eight genes in the B16-F10 cells cultured with vSMC ECM after siKlf4-TCM treatment did not overlap with those from B16-F10 tumor cells cultured with ECM from vSMCs treated with either siFn1-TCM or siKlf4-siFn1-TCM (Fig. 6c). These data suggested that the alteration of B16-F10 gene expression after culture on vSMC-generated ECM was regulated by exposure to KLF4-dependent fibronectin deposition, which in turn resulted in activation of pathways associated with enhanced tumor cell survival, viability and proliferation.

Figure 6 Inhibition of tumor cell binding to fibronectin recapitulates perivascular KLF4-dependent metastasis. (a) Ingenuity pathway analysis of highly enriched pathways (positive activation score) in the gene expression data collected from B16-F10 tumor cells cultured \(in vitro\) on the ECM of vSMCs that were first subjected to siRNA-mediated knockdown of \(Klf4\), \(Fn1\) or both \(Klf4\) and \(Fn1\), followed by activation in B16-F10 TCM or SFM. Pathway analysis of B16-F10 tumor cells when cultured on the ECM of vSMCs treated with siNT, comparing B16-F10 TCM to SFM, demonstrates activation of cell survival, viability and proliferation pathways (orange). These pathways are not activated in B16-F10 cells cultured on the ECM of vSMCs as described in a demonstrates that the siFn1-TCM, siKlf4-TCM, and siFn1-siKlf4-TCM conditions cluster together and cluster with the siNT-SFM condition, indicating that knockdown of either \(Klf4\) or \(Fn1\) in vSMCs results in the generation of ECM in response to TCM that is more similar to that produced by vSMCs cultured in SFM than when neither gene is knocked down. (c) Venn diagrams depicting the genes whose expression was either twofold higher (top) or lower (bottom) in each knockdown condition when compared to that in the SFM, melanocyte exosome (Mel exo), or B16-F0 exosome (B16-F0 exo) conditions. This proliferation was decreased by treatment of B16-F10 tumor cells with \(β\)-integrin-specific neutralizing antibodies to disrupt tumor cell binding to fibronectin. One representative experiment of two independent experiments is shown. Center lines indicate median values, box edges indicate the 25th and 75th percentiles, and whiskers extend to the minimum and maximum values. \(*P < 0.05\), \(* *P < 0.001\), \(* * *P < 0.0001\) and \(* * * *P < 0.00001\) by Student’s t-test. (e) Updated schematic summarizing \(in vitro\) findings that tumor-secreted factors, including WISP1-containing exosomes, results in KLF4-dependent ECM that contains increased fibronectin, demonstrating a decrease in ex vivo lung luminescence in \(Klf4\) mice by \(Klf4\) mice as described in g, demonstrating a decrease in \(ex vivo\) lung luminescence in \(Klf4\) mice by \(Klf4\) mice as described in f (right), as compared to those treated with IgG control antibodies (left) \((Klf4^{WT})\). IgG, \(n = 7\); anti-\(β1\)-integrin, \(n = 8\); \(Klf4^{Δ}\) IgG, \(n = 6\); anti-\(β1\)-integrin, \(n = 10\). (b) Luminescence signals detected \(ex vivo\) in the lungs of \(Klf4^{WT}\) and \(Klf4^{Δ}\) mice as described in \(g\), demonstrating a decrease in \(ex vivo\) lung luminescence in \(Klf4^{Δ}\) mice by \(Klf4^{Δ}\) mice as described in \(f\) (right), compared to those treated with control peptides (left) \((Klf4^{WT})\). peptide control, \(n = 9\); RGDS, \(n = 8\); \(Klf4^{Δ}\) peptide control, \(n = 7\); RGDS, \(n = 9\). (i) Luminescence signals detected \(ex vivo\) in the lungs of \(Klf4^{WT}\) and \(Klf4^{Δ}\) mice as described in \(g\), demonstrating a decrease in \(ex vivo\) lung luminescence in \(Klf4^{Δ}\) mice by \(Klf4^{Δ}\) mice as described in \(f\) (right), compared to those treated with control peptides (left) \((Klf4^{WT})\). peptide control, \(n = 9\); RGDS, \(n = 8\); \(Klf4^{Δ}\) peptide control, \(n = 7\); \(Klf4^{Δ}\) peptide control, \(n = 9\). Center lines indicate median values, and top and bottom lines indicate the 25th and 75th percentiles, respectively. \(*P < 0.001\) and \(* *P < 0.0001\) by Student’s t-test; n.s., not significant.
To confirm the role of activated-vSMC-dependent fibronectin deposition on tumor cell proliferation, we used β1-integrin-specific neutralizing antibodies to disrupt the binding of B16-F10 tumor cells to fibronectin. We found that B16-F10 proliferation was significantly reduced with this antibody treatment, irrespective of whether the vSMCs were first activated by using B16-F10 TCM, recombinant WISP1 or B16-F10 exosomes (Fig. 6d). B16-F10 cells did not show increased proliferation when they were grown on the ECM generated by vSMCs that had been cultured with SFM, melanocyte exosomes or nonmetastatic B16-F0 exosomes, and treatment with the β1-integrin neutralizing antibody did not further reduce proliferation under these conditions (Fig. 6d). Taken together, these data suggested that tumor-cell-secreted factors, including WISP1, induced KLF4 expression in perivascular cells that, in turn, resulted in enhanced deposition of fibronectin-containing ECM which promoted β1-integrin-mediated tumor cell proliferation and survival (Fig. 6e). To test this hypothesis further, we used our model of M3-9M rhabdomyosarcoma metastasis in Klf4WT and Klf4Δ mice where, after pre-metastatic niche formation, we treated mice with either β1-integrin-specific neutralizing antibodies or Arg-Gly-Asp-Ser (RGDS) peptides to disrupt tumor cell-fibronectin binding (Fig. 6f). Analysis of metastatic lungs revealed a dramatic decrease in metastasis after treatment with either anti-β1-integrin (Fig. 6g,h) or RGDS peptides (Fig. 6i,j) in Klf4WT mice to levels comparable to those in Klf4Δ mice that had been treated with either anti-β1-integrin, RGDS peptides or their respective controls. Neither anti-β1-integrin nor RGDS peptide treatment decreased primary tumor volume in either Klf4WT or Klf4Δ mice as compared to that in their respective controls (Supplementary Fig. 10b). Together these findings indicated that tumor cell β1-integrin-mediated fibronectin binding contributed to the pro-metastatic functions of KLF4-dependent perivascular phenotypic switching.

These findings suggest that KLF4-dependent perivascular cell phenotypic transition is important for pre-metastatic niche formation and that the lasting effects of KLF4 targeting can be observed in the late-stage metastatic microenvironment that ultimately dictates metastatic...
outcomes. These results demonstrate the potential utility of perivascular plasticity as a novel target to inhibit metastasis. KLF4-targeting to prevent metastasis may be of the greatest use in the adjuvant setting following primary tumor removal, or in combination with chemotherapy or other microenvironment-modulating agents to decrease tumor metastasis-initiating potential.

**DISCUSSION**

This study provides compelling evidence that perivascular cells in pre-metastatic sites undergo phenotypic switching, in which they activate KLF4 expression, lose vSMC marker expression, are found in larger numbers away from the vasculature and synthesize fibronectin-containing ECM that supports metastatic behavior in disseminated tumor cells. Genetic ablation of Klf4 in a perivascular-cell-specific inducible model disrupts KLF4-dependent perivascular phenotypic switching and the resulting perivascular-cell-dependent fibronectin deposition, and it decreases metastasis, highlighting the functional importance of perivascular cells and their plasticity in pre-metastatic niche initiation. The use of lineage-tracing models enabled the identification of perivascular cells that would be otherwise misidentified as fibroblasts as a consequence of their activation state, given the expression of markers common to both perivascular cells and fibroblasts, such as Acta2, and the propensity to secrete or modulate ECM proteins ECM proteins. Our results imply that, although many of the key players in the primary tumor microenvironment are also found in the pre-metastatic microenvironment, stromal cells, including vSMCs and pericytes, and fibroblasts have distinct roles in these settings that are critical for tumor cell survival and proliferation.

Perivascular cells are well-recognized regulators of vascular permeability, and they provide repair mechanisms and vascular normalization signals to damaged blood vessels. In primary tumor growth, pericytes are implicated in leaky and dysfunctional tumor vasculature. This dual role of pericytes in tumor vasculature formation and vascular normalization is best exemplified by pericyte ablation studies, in which specific depletion of PDGFR-β- or NG2-expressing perivascular cells results in decreased primary tumor growth and enhanced metastasis. Similarly, ablation of ACTA2-expressing cells enhances tumor progression. Conversely, our findings provide direct evidence that targeting perivascular cell behavior, instead of entirely depleting pericytes, limits metastasis while effecting no change on primary tumor growth. Future studies are required to carefully delineate KLF4-dependent perivascular cell coverage around blood vessels in the context of pre-metastatic niche formation. Together, these studies highlight the importance of modulating perivascular cell behavior and targeting the induction of key mediators of their phenotype, such as KLF4, to preserve their notable role in vascular normalization.

In addition to their role in vascular stability and angiogenesis, NG2-expressing perivascular cells support stem cell niches in the bone marrow and fetal liver by mediating the activation and quiescence of hematopoietic stem cells. Recently, we and others have demonstrated that the bone marrow hematopoietic stem cell niche is altered during tumor development and results in enhanced mobilization of hematopoietic stem cells to pre-metastatic sites, where they enhance metastasis. In the present study we demonstrate that phenotypically modulated perivascular cells have an early, key role in modulating the metastatic microenvironment to support stem-cell-like features in tumor cells, such as enhanced tumorsphere formation, thus strengthening our understanding of the commonalities between the pre-metastatic niche and physiological stem cell niches. Further studies of phenotypically activated NG2+ pericytes as the source of pluripotent mesenchymal cells that create perivascular niches, will need to be investigated.

Our results demonstrate a previously unknown and unappreciated role for perivascular cell plasticity in pre-metastatic niche formation, and they identify KLF4 as a critical inducer of this plasticity. Although KLF4 has long been appreciated as an important mediator of perivascular plasticity in a number of disease models, including those for atherosclerosis and vascular injury, in addition to the role of Klf4 as a context-dependent oncogene or tumor suppressor in a number of tumor types, this is the first study to describe that KLF4-mediated perivascular activation is essential to pre-metastatic niche formation and metastasis. The modulation of perivascular cell phenotype and behavior introduces a new concept for the therapeutic targeting of microenvironmental cells that focus on altering plasticity rather than on the ablation of this key cell population, which also has crucial roles in tissue homeostasis, wound healing and regeneration. This novel approach may lead to new therapeutic targets for tumor metastasis prevention, which are urgently needed in the clinic.

**METHODS**

Methods, including statements of data availability and any associated accession codes and references, are available in the online version of the paper.

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**AUTHOR CONTRIBUTIONS**

M.M. conducted most of the experiments, performed data analysis, generated most of the experimental mice and was the primary writer of the manuscript; W.J. conducted in vivo metastasis studies and in vitro matrix experiments, generated RNA for sequencing and analyzed exosomal content by western blot; M.E. conducted in vitro immunostaining and western blot experiments; J.K. assisted with the generation of experimental mice and conducted image analysis; D.W.B. and S.K. assisted with mouse experiments, and generated and analyzed flow cytometry data; M.M.M. analyzed pathological tissue specimens; M.K. assisted with confocal image acquisition, analysis and figure preparation; H.L. and J.F.S. analyzed RNA-seq data and assisted with figure preparation; O.A.C. and G.K.O. generated preliminary data, provided initial experimental mice and provided advice throughout; R.N.K. supervised the project and provided guidance on experimental design, data interpretation and writing of the manuscript.

**COMPETING FINANCIAL INTERESTS**

The authors declare no competing financial interests.

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ONLINE METHODS
Detailed information on experimental design and reagents can be found in the Life Sciences Reporting Summary.

Mice. ROSA-STOP-flox-eYFP mice, MyH11-ERT-Cre mice and NG2-ERT-Cre mice were obtained from the Jackson Laboratory. Only male mice inherit the Myh11-CreERT² allele, and thus any experiments performed using this mouse model were done so using male mice that were homozygous for the ROSA-STOP-flox-eYFP allele and heterozygous for the Myh11-CreERT² allele. The Ki67 flox mice were obtained through the Mutant Mouse Resource and Research Center (MMRRC) and were donated to them by Klaus Kaestner (University of Pennsylvania). MyH11-ERT-Cre mice were bred with the Ki67 flox mice to obtain the Myh11-ERT-Cre Klf4Δa mice. Genotyping was performed via PCR using previously published protocols. Cre activity was induced in mice aged 6–8 weeks via intraperitoneal injection (1 injection/day; ten total injections; 10 mg/ml of tamoxifen in peanut oil). For drug treatment studies, mice were randomized within genotype grouping, and drug treatment was performed in a blinded manner post assignment. All experiments were approved by the NCI Animal Care and Use Committee and were conducted under specific pathogen-free conditions at the NIH Animal Facility.

Cell lines. The B16-F0, B16-F10, E0771 and MOVAS cell lines were obtained from the ATCC. The M3-9M cell line was derived from the transgenic HGF/p53-knockout mice, which develop spontaneous embryonal rhabdomyosarcoma tumors, generated by Glenn Merlino and that were back-crossed to the B6 background by Crystal Mackall[38]. All cell lines were authenticated via microarray analysis and tested to be mycoplasma negative. All tumor cell lines were transduced with a lentivirus expressing a mCherry–LUC fusion protein and sorted by flow cytometry to obtain a population in which 98% of cells had mCherry expression, to establish stable cell lines. eYFP+ vSMCs were isolated from the aorta of Myh11 lineage-tracing mice after tamoxifen injection, via flow sorting on eYFP expression after negative selection of CD45, CD31 and TER119, to eliminate hematopoietic, endothelial and erythroid cells, using Miltenyi Biotec’s biotinylated beads and depletion columns. eYFP+ vSMCs were kept in culture for up to seven passages and then discarded.

Orthotopic and tail vein tumor models. Orthotopic tumor injections were administered with 5 × 10⁵ tumor cells in single-cell suspension of 100 μl of Hank's balanced salt solution (HBSS) using a sterile 27.5-gauge needle. The B16-F0 and B16-F10 melanoma cell lines were administered intradermally in the flank. The M3-9M rhabdomyosarcoma cell line was administered in the gastrocnemius muscle of the right hind leg. The E0771 breast carcinoma cell line was administered into the mammary fat pad. For tumor cell injections into the tail vein, 1×10⁵ tumor cells in a single-cell suspension in 100 μl of HBSS were injected with a sterile 27.5-gauge needle into the lateral tail vein. Metastatic end point was determined per experiment by maximum primary tumor size, and for eYFP+ vSMCs were detected in the aorta of Myh11 lineage-tracing mice after tamoxifen injection, via flow sorting on eYFP expression after negative selection of CD45, CD31 and TER119, to eliminate hematopoietic, endothelial and erythroid cells, using Miltenyi Biotec’s biotinylated beads and depletion columns. eYFP+ vSMCs were kept in culture for up to seven passages and then discarded.

Immunoﬂuorescence. Tissues were ﬁrst cardiac-perfused with PBS followed by 4% paraformaldehyde (PFA). Tissues were then harvested, ﬁxed overnight in 4% PFA at 4 °C, then transferred to 30% glucose and incubated at 4 °C until the tissues sank, or up to 3 d. Tissues were then embedded in OCT compound (Tissue-Tek) and cryosectioned for staining. Sections were air-dried for 2 h, washed in PBS, permeabilized in 0.5% Triton-X, blocked in 10% ﬁsh gelatin with 10% donkey serum, and then stained with the following: 4,6-diamidino-2-phenylindole (DAPI) (Invitrogen) as a nuclear marker, goat anti-GFP (ab6673, Abcam) for the lineage marker of eYFP, mouse anti-ACTA2 conjugated to fluorescein isothiocyanate (FITC) (1A4, Sigma), rat anti-MYH11 (KM3669, Kamiya), rabbit anti-PECAM1 (AB28364, Abcam); rabbit anti-KLF4 (HPA00296, Sigma), rabbit anti-fibronectin (ab23750, Abcam), rabbit anti-PDGFR-α (APAS, Abcam), rabbit anti-PDGFR-β (Y92, Abcam); rabbit anti-Ki67 (ab16667, Abcam), mouse anti-NG2 conjugated to biotin (ab5320b, Millipore) and chicken anti-m-Cherry (ab205402, Abcam). Donkey secondary antibodies, including Cy3-conjugated anti-goat-IgG (705-165-147), Alexa-Fluor 594-conjugated anti-rabbit-IgG (711-585-152), Alexa-Fluor 647-conjugated anti-rabbit-IgG (711-606-152) and Alexa-Fluor 647-conjugated anti-rat-IgG (712-606-150), were used where necessary (Jackson ImmunoResearch).

Cells were plated and treated on autoclaved glass coverslips placed in sterile 6-well plates. Upon conclusion of the experiment, cells were ﬁxed in 4% PFA, permeabilized in 0.5% Triton-X, blocked in 10% donkey serum and then stained as detailed above.

Images for phenotyping were acquired with a Zeiss Axio Observer Z1 inverted microscope ﬁtted with an Apotome.2. Confocal ﬂuorescence images were acquired with a Zeiss LSM880 laser-scanning confocal microscope equipped with a 34-channel spectral detector and 20× plan-apochromat (air, numerical aperture (N.A.) 0.8) and 63× plan-apochromat (oil, N.A. 1.4) objective lenses (Carl Zeiss Microscopy, LLC, Thornwood, NY). A four-channel imaging conﬁguration was used to acquire images from multi-labeled ﬂuorescent samples. The ﬂuorophores used for imaging (with their respective laser-excitation- and ﬂuorescence-emission-detection windows) were DAPI (405 nm laser, 415–490 nm emission), FITC (488 nm laser, 490–535 nm emission), Cy3 (561 nm laser, 563–585 nm emission), AlexaFluor 594 (594 nm laser, 596–631 nm emission) and AlexaFluor 647 (633 nm laser, 655–742 nm emission). Confocal images were collected with a 1,024 × 1,024 pixel frame size and with the 20× objective lens, a 0.28-µm x-y pixel size, 3.9-µm optical slice thickness and 4× image frame averaging, and with the 63× objective lenses 0.09-µm x-y pixel size, 1.2-µm optical slice thickness and 4× image frame averaging. Imaging parameters were kept constant for all of the images in an experiment. Isotype and single-fluorophore-labeled control samples were imaged using the same four-channel imaging conﬁguration so that the potential degree of fluorescence cross-talk between image channels could be compensated for during image analysis. For extended field-of-view imaging, image frames were collected with 5% overlap and stitched together into one large image file using Zeiss Zen blue (v. 2.3) image-processing software. After background subtraction based on the isotype and single-label control samples, the brightness and contrast were adjusted for each ﬂuorescence channel using linear histogram-stretching, and the adjustments were made using the same parameters for all of the images in a data set. The resultant images were exported as .tiff ﬁles and composed into ﬁgures using Adobe Photoshop. Deconvolution and ﬁgure preparation were performed using the Zeiss Zen software package, and all particle analysis was conducted using the CellProfiler 2.1.1 software package (http://cellprofiler.org)39.

Image analysis. eYFP+ cells were identiﬁed using CellProfiler 2.1.1 (http://cellprofiler.org), whereby ‘parent’ particles were identiﬁed based on DAPI+ nuclei, and eYFP+ ‘child’ particles were identiﬁed based on parent particles surrounded by eYFP+ staining. The total number of eYFP+ child particles was quantiﬁed in three 20× tiled images (4 × 4 average tile area) per mouse and reported as average eYFP+ particles per tiled field. KLF4+ eYFP+ cells were identiﬁed in eYFP+ child particles by KL4+ staining that was restricted to the nucleus of each eYFP+ particle. The average proximity of eYFP+ particles to blood vessels was computed by determining the radial distribution of PECAM1 to the eYFP+ child particles, reported in bins of 3-µm and 27-µm radial distances from the nucleus of each eYFP+ particle. Each eYFP+ particle was then analyzed to determine the closest bin containing the PECAM1 signal. Each bin was summed per tiled image and then reported as an average bin signal per mouse. Signal density of fibronectin

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staining was determined in Imagej software (NIH) by recording the integrated density in at least three fields of each stained sample.

For image analysis, the original images were used for mean intensity measurements, and the background value was subtracted based on single-color and isotype control images. Briefly, individual regions of interest (ROIs) were drawn for different representative regions within the images to measure the mean intensity for each fluorescence channel. For the series of single-label control samples, the mean intensity was also measured for each of nonlabeled channels, and the highest background value recorded was subtracted from the measurements made from the corresponding image channel in the multi-label samples. 10–25 ROIs were drawn in each image, centered around DAPI-stained nuclei, and five representative images per lung specimen were included for analysis per data set. The background-subtracted mean fluorescence intensities for eYFP+ lineage-traced cells and eYFP− cells were plotted using Prism (GraphPad Software, Inc.). Each data set was analyzed independently by two investigators.

Flow cytometry. Lungs were first perfused with PBS and then insufflated with 1 mg/ml collagenase I, 10 µg/ml dispase II and 20 µg/ml DNase I. Lungs were then harvested and placed in PBS with 10% FBS and 5% BSA on ice. Lung tissue was finely mince, digested with collagenase I, dispase II and DNase I at 37 °C for 30 min with gentle agitation, and passed through a 70-µm nylon filter. Total cell counts per lung were determined using trypan blue cell exclusion from single-cell suspensions. Live cells were determined via exclusion of the eFluor 506 fixable viability dye (eBioscience). eYFP+ cells were identified by their negative staining using antibodies to CD45 (50–F1, eBioscience), TER119 (TER119, eBioscience) and CD3 (CD3, BioLegend) in PBS with 0.5% BSA and 0.05% Na2HCO3, and a positive eYFP signal. Extracellular markers included CD51 (RMV-7, eBioscience), PDGFR-α (BM8, eBioscience). Streptavidin conjugated to allophycocyanin–eFluor 780 tandem (APC–e780) (eBioscience) was used to detect biotinylated antibodies. For intracellular or nuclear staining, cell surface markers were first stained as stated above followed by fixation, permeabilization and staining for all other markers using a Foxp3/Transcription Factor Staining Buffer Set (eBioscience). KLF4 staining with an unconjugated antibody was followed by incubation with a phycoerythrin (PE)-conjugated secondary antibody (anti-KLF4 (HPA002926, Sigma). KLF4 staining with an unconjugated antibody was followed by incubation with a phycoerythrin (PE)-conjugated secondary antibody (anti-KLF4 (HPA002926, Sigma). Flow cytometry data were collected on a BD LSR Fortessa and were analyzed using flowJo software package (Tree Star). Cell populations were reported as the proportion of live single cells identified by flow multiplied by the total number of cells isolated from each lung.

Tumor-conditioned medium and exosome preparation. Tumor cells were plated at 1 × 105 cells/ml in T75 flasks and allowed to adhere overnight in growth medium. Growth medium was then removed, and the cells were washed in PBS (1×) and seeded into 6-well plates at 1 × 105 cells/ml in T75 flasks and allowed to adhere overnight in growth medium. Growth medium was then removed, and the cells were cultured in serum-free medium. Tumor-conditioned medium and exosome preparation. Tumor-conditioned medium and exosome preparation. Tumor-conditioned medium and exosome preparation.

Flow cytometry. Lungs were first perfused with PBS and then insufflated with 1 mg/ml collagenase I, 10 µg/ml dispase II and 20 µg/ml DNase I. Lungs were then harvested and placed in PBS with 10% FBS and 5% BSA on ice. Lung tissue was finely mince, digested with collagenase I, dispase II and DNase I at 37 °C for 30 min with gentle agitation, and passed through a 70-µm nylon filter. Total cell counts per lung were determined using trypan blue cell exclusion from single-cell suspensions. Live cells were determined via exclusion of the eFluor 506 fixable viability dye (eBioscience). eYFP+ cells were identified by their negative staining using antibodies to CD45 (50–F1, eBioscience), TER119 (TER119, eBioscience) and CD3 (CD3, BioLegend) in PBS with 0.5% BSA and 0.05% Na2HCO3, and a positive eYFP signal. Extracellular markers included CD51 (RMV-7, eBioscience), PDGFR-α (BM8, eBioscience). Streptavidin conjugated to allophycocyanin–eFluor 780 tandem (APC–e780) (eBioscience) was used to detect biotinylated antibodies. For intracellular or nuclear staining, cell surface markers were first stained as stated above followed by fixation, permeabilization and staining for all other markers using a Foxp3/Transcription Factor Staining Buffer Set (eBioscience). Because fixation decreases eYFP emission, eYFP+ cells were identified using anti-GFP (SF12.4, eBioscience). Other intracellular or nuclear antibodies included anti-Ki67 (SolA15, eBioscience), anti-ActA (1A4, eBioscience) and anti-KLF4 (HPA002926, Sigma). KLF4 staining with an unconjugated antibody was followed by incubation with a phycoerythrin (PE)-conjugated secondary (eBioscience). Flow cytometry data were collected on a BD LSR Fortessa and were analyzed using flowJo software package (Tree Star). Cell populations were reported as the proportion of live single cells identified by flow multiplied by the total number of cells isolated from each lung.

Cytokine array analysis. TCM from the B16-F0 and B16-F10 cell lines was prepared as described above and then applied to a RayBiotech C1000 antibody array as per the manufacturer’s protocol. Membranes were analyzed by densitometry using the BioRad ChemiDoc imaging system.

Quantitative real-time PCR. Total RNA was isolated and precipitated using TRIzol, followed by chloroform and ethanol washes. RNA concentration and quality were assessed using the Agilent 2100 Bioanalyzer. cDNA synthesis was performed using 1 µg RNA per sample and the Bio-Rad iScript cDNA Synthesis Kit. qRT–PCR was performed using the Bio-Rad SsoAdvanced master mix on a Bio-Rad iCycler.

Klf4 knockdown and reporter plasmid. A Klf4 promoter-based reporter plasmid that expresses the secretable Gaussia luciferase was purchased from GeneCopoeia and transfected into plated MOVAS cells. 24 h after transfection, siRNAs targeting either Klf4 or a nontarget control were added to the MOVAS cells according to manufacturer’s protocol (Dharmacon Accele siRNAs). 72 h after siRNA treatment, medium was replaced with SFM containing recombinant proteins or TCMM. 16 h after treatment with the proteins, medium was removed and assayed for luciferase activity using the BioLux Gaussia Luciferase Assay Kit (New England BioLabs). Reporter plasmid activity was reported in luminescence units, relative to that from the empty plasmid control. Western blots for KLF4 (HPA002926, Sigma) and fibronectin (ab23750, Abcam) were also performed after TCMM treatment of SMCs in which Klf4 expression was knocked down, and these were imaged using the Bio-Rad ChemiDoc imaging system.

Western blotting. Exosomes or cell pellets were lysed in Cell Lysis Buffer (Cell Signaling) with a protease and phosphatase inhibitor cocktail (Millipore). All lysates were cleared by centrifugation, and quantified by the BCA protein assay. 5 µg of protein from each sample were electrophoresed on 4–12% Bis-Tris protein gels and transferred to nitrocellulose membranes (Thermo Fisher). Blots were probed with the following primary antibodies: anti-angiopoietin-1 (2948, Cell Signaling; 1:1000), anti-WISP1 (ab178547, Abcam; 0.5 µg/ml), anti-PDGFB (ab23914, Abcam; 1 µg/ml), anti-IL-1β (ab20478, Abcam; 3 µg/ml), anti-FN1 (1574-1, Epitomics; 1:1000), anti-KLF4 (HPA002926, Sigma; 1:500), anti-GFP (ab6673, Abcam; 1:2000), anti-β-actin (8457s, Cell Signaling; 1:1000), anti-CD81 (Eat2 Thermo Scientific; 1:1000), anti-CD63 (ab193349, Abcam; 1 µg/ml), anti-mouse-CD9 (ab82390, Abcam; 1 µg/ml), anti-human-CD9 (ab92726, Abcam; 1:2000), anti-flotillin (18634, Cell Signaling; 1:1000), anti-Alx-1 (2171, Cell Signaling; 1:1000), anti-annexin V (8555, Cell Signaling; 1:1000), anti-LAMP2 (ab203224, Abcam; 1:1000), anti-HSP70 (4876, Cell Signaling; 1:1000), anti-Ep-CAM (2626, Cell Signaling; 1:1000), and anti–Armenian hamster (PA1-32045, Thermo Scientific; 1:1000) secondary antibody. HRP-based detection was performed using SuperSignal West substrate (Thermo Scientific).

RNA sequencing. RNA sequencing analysis was performed using the standard operating procedure of the CCR Genomics core facility. mRNA samples were prepared using the Illumina TruSeq Stranded mRNA kit (Illumina). Samples were pooled and sequenced using the NextSeq500 platform in high-output mode. Demultiplexing was done to allow one mismatch in the barcodes. All reads for each sample were trimmed to remove adapters and low-quality bases using Trimmomatic software and aligned to the reference using STAR software. The data normalization and statistical analysis used to identify differentially expressed genes were performed using the Partek Flow installed in the NIH Helix cluster (https://partekflow.cit.nih.gov). The fastq sequence was aligned to the mouse mm10 genome using STAR 2.4.1d aligner, and the transcript abundance was estimated using the Partek E/M algorithm based on the mm10-Ensembl Transcripts release 82 transcriptome model. Gene counts were normalized to total read count per sample, and the differential gene expression (GSA) algorithm was used to detect differentially expressed genes. Pathway analysis was conducted using Ingenuity pathway analysis (Qiagen), using a twofold expression cut-off for all comparisons.

WISP1 enzyme-linked immunosorbent assay (ELISA). Exosomes, prepared as described above, were assayed using the R&D Mouse/Rat WISP1/CCN4
Quantikine ELISA kit. Mouse plasma samples were obtained from terminal cardiac punctures and processed according to the manufacturer's recommendations.

**In vitro vSMC-derived matrix experiments.** vSMCs were plated at a density of $1 \times 10^5$ cells/ml and allowed to adhere to culture plates overnight. Cells were washed with PBS, and medium was replaced with either SFM, TCM, exosomes or recombinant cytokines. 72 h after treatment with medium, vSMCs were lysed in a solution of 1% SDS, 1% Triton-X-100 and 20 mM NH$_4$OH for 5 min. The remaining matrix was washed in PBS three times and used immediately for downstream experiments.

Tumor cell adhesion was assessed by plating $1 \times 10^4$ tumor cells/ml on vSMC matrix and then removing the tumor cells after 2 h. Tumor cell confluence per well was determined by using an IncuCyte Zoom live cell analysis system (Essen BioScience) and accompanying software. Tumor proliferation was assessed using CellTiter Glo following the manufacturer-provided protocol. Tumor migration was assessed by plating tumor cells on top of a 0.8-µm Transwell membrane in SFM above an ECM-coated well. Percentage migration was counted as the number of DAPI+ cells that were identified under the Transwell membrane 8 h after plating.

Tumorsphere formation was assessed by allowing tumor cells to proliferate on SMC-generated matrix for 72 h, after which the tumor cells were harvested, counted and plated in ultra-low-attachment plates (200 cells per well) in DMEM/F12 supplemented with B27 supplement (Thermo Scientific), 20 ng/ml epidermal growth factor, 10 ng/ml basic fibroblast growth factor, 5 µg/ml insulin and 0.4% FBS. Cells were cultured for 1 week, after which spheres were imaged using a phase-contrast microscope at 40x magnification. Tumorspheres were distinguished from cellular aggregates by their solid spherical shape with a diameter of at least 50 µm and were reported as the average number of tumorspheres per 40x field.

**Statistical analysis.** All analysis was performed using GraphPad Prism version 6.04 (http://www.graphpad.com) with the exception of the Jonckheere–Terpstra test, which was performed in R. Sample size was determined by running an a priori power analysis using G*Power with data collected from pilot experiments. Data are expressed as mean ± s.d. Unless otherwise stated, statistical differences were evaluated using an unpaired, nonparametric Student’s t-test. Data were evaluated for normality using the D'Agostino–Pearson omnibus normality test. The two-tailed Fisher's exact test was used for categorical data. The Jonckheere–Terpstra test was performed for data that represented independent values over time. Significant differences between experimental groups were represented as *$P < 0.05$, **$P < 0.001$, ***$P < 0.0001$ and ****$P < 0.00001$.

**Data availability.** All of the sequencing data generated are available at the Gene Expression Omnibus (GEO) with accession code GSE96972.

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1. **Sample size**
   
   Describe how sample size was determined.
   
   Sample size was determined by running an a priori power analysis using G*Power with data collected from pilot experiments.

2. **Data exclusions**
   
   Describe any data exclusions.
   
   No data was excluded from these studies.

3. **Replication**
   
   Describe whether the experimental findings were reliably reproduced.
   
   All replication attempts were successful.

4. **Randomization**
   
   Describe how samples/organisms/participants were allocated into experimental groups.
   
   For drug treatment studies, mice were randomized within genotype grouping and drug treatment was performed blinded post-assignment.

5. **Blinding**
   
   Describe whether the investigators were blinded to group allocation during data collection and/or analysis.
   
   For analysis of murine studies, each dataset was analyzed independently by two investigators.

   Note: all studies involving animals and/or human research participants must disclose whether blinding and randomization were used.

6. **Statistical parameters**

   For all figures and tables that use statistical methods, confirm that the following items are present in relevant figure legends (or the Methods section if additional space is needed).

   - **n/a**
   - **Confirmed**

   - ☒ The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement (animals, litters, cultures, etc.)
   - ☒ A description of how samples were collected, noting whether measurements were taken from distinct samples or whether the same sample was measured repeatedly.
   - ☒ A statement indicating how many times each experiment was replicated
   - ☒ The statistical test(s) used and whether they are one- or two-sided (note: only common tests should be described solely by name; more complex techniques should be described in the Methods section)
   - ☒ A description of any assumptions or corrections, such as an adjustment for multiple comparisons
   - ☒ The test results (e.g. p values) given as exact values whenever possible and with confidence intervals noted
   - ☒ A summary of the descriptive statistics, including central tendency (e.g. median, mean) and variation (e.g. standard deviation, interquartile range)
   - ☒ Clearly defined error bars

   See the web collection on statistics for biologists for further resources and guidance.

7. **Software**

   Policy information about availability of computer code

   7. Software

   Describe the software used to analyze the data in this study.

   For bioluminescent tumor cell tracking, analysis was conducted using the Xenogen Living Image software package. For confocal image analysis,
deconvolution and figure preparation was performed using the Zeiss Zen software package, and all particle analysis was conducted using the CellProfiler 2.1.1 software package (cellprofiler.org). Flow cytometry data was collected using the BD FACSDIVA software (BD Biosciences), analyzed using the FlowJo software package (Tree Star). Western blotting and cytokine array membranes were analyzed by densitometry using the BioRad ChemiDoc imaging system. Gene expression data was processed using Partek Pro, and pathway analysis was conducted using Ingenuity Pathway Analysis (Qiagen), using a 2-fold expression cut-off for all comparisons. All statistical analysis was performed using GraphPad Prism version 6.04, www.graphpad.com, with the exception of the Jonckheere-Terpstra test which was performed in R. Cell confluence data was gathered using the Incucyte system (Essen BioScience).

For all studies, we encourage code deposition in a community repository (e.g. GitHub). Authors must make computer code available to editors and reviewers upon request. The Nature Methods guidance for providing algorithms and software for publication may be useful for any submission.

Materials and reagents

Policy information about availability of materials

8. Materials availability

Indicate whether there are restrictions on availability of unique materials or if these materials are only available for distribution by a for-profit company.

The M3-9M cell line was derived from the transgenic HGF/p53 knockout mice that develop spontaneous embryonal rhabdomyosarcoma tumors generated by Glenn Merlino and backcrossed to the C57B6 background by Crystal Mackall. This cell line is freely available upon request.

9. Antibodies

Describe the antibodies used and how they were validated for use in the system under study (i.e. assay and species).

All antibodies were titered and validated on positive control murine tissue or murine cell lines where possible prior to use in experiments. Isotype control antibodies, secondary antibodies alone, or fluorescence minus one control conditions were used for setting voltages, thresholds, and gates, respectively.

For confocal imaging, stained with the following antibodies: 4,6'-diamidino-2-phenylindole (DAPI) (Invitrogen) as a nuclear marker; goat anti-GFP (ab6673, Abcam) for lineage marker eYFP; mouse anti-ACTA2 (SMA) conjugated to FITC (1A4, Sigma); rat anti-Myh11 (KM3669, Kamiya); rabbit anti-PECAM1 (AB28364, Abcam); rabbit anti-KLF4 (HPA002926, Sigma); rabbit anti-fibronectin (ab23750, Abcam); rabbit anti-PDGFRA (AP5, Abcam); rabbit anti-PDGFRIb (Y92, Abcam); rabbit anti-Ki67 (ab16667, Abcam); mouse anti-Ng2 conjugated to biotin (ab5320b, Millipore); and chicken anti-mCherry (ab205402, Abcam). Donkey secondary antibodies were used where necessary (Jackson ImmunoResearch).

For flow cytometry, extracellular markers included CD51 (RMV-7, eBioscience), PDGFRA (AP5, eBioscience), NG2 (ab5320b, Millipore) Mac-2 (M3/38, eBioscience), CD11b (M1/70, eBioscience), and F4/80 (BM8, eBioscience). Where biotinylated antibodies were used, an APC-e780 streptavidin (eBioscience) staining step was added. Because fixation decreases eYFP emission, eYFP+ cells were identified using an anti-GFP antibody (SF12.4, eBioscience). Other intracellular/nuclear antibodies included Ki67 (SolA15, eBioscience), ACTA2 (1A4, eBioscience), and KLF4 (HPA002926, Sigma). Unconjugated KLF4 staining was followed by a PE-conjugated secondary (eBioscience).

Western blotting was conducted with the following antibodies: rabbit anti-KLF4 (HPA002926, Sigma); rabbit anti-fibronectin (ab23750, Abcam); rabbit anti-Ang2 (2948, Cell Signaling); anti-rabbit IgG HRP-linked (Cell Signaling); rat anti-LAMP2 (ab13524, Abcam); rabbit anti-WISP1 (ab178547, Abcam); rabbit anti-PDGF-B (ab23914, Abcam); rabbit anti-IL1β (ab200478, Abcam); rabbit anti-ß-actin (8457, Cell Signaling); mouse anti-Alix (2171, Cell Signaling); rabbit anti-Annexin V (8555, Cell Signaling); rabbit anti-CD9 (13174, Cell Signaling); rabbit anti-GM130 (12480, Cell Signaling); rabbit anti-EpCAM (2626, Cell Signaling); rabbit anti-HSP70 (4876, Cell Signaling); rabbit anti-Flotillin (18634, Cell Signaling); rabbit anti-CD81 (EPR4244, Abcam); rabbit anti-CD9 (EPR2949, Cell Signaling); rabbit anti-CD63 (ab216130, Abcam); and armensian hamster anti-CD81 (Ea2, ThermoFisher Scientific).
10. Eukaryotic cell lines
   a. State the source of each eukaryotic cell line used. The B16-F0, B16-F10, E0771, and MOVAS cell lines were obtained from the ATCC. The M3-9M cell line was derived from the transgenic HGF/p53 knockout mice that develop spontaneous embryonal rhabdomyosarcoma tumors generated by Glenn Merlino and backcrossed to the Bl6 background by Crystal Mackall.

   b. Describe the method of cell line authentication used. All cell lines were authenticated via microarray analysis.

   c. Report whether the cell lines were tested for mycoplasma contamination. All cell lines tested mycoplasma negative.

   d. If any of the cell lines used in the paper are listed in the database of commonly misidentified cell lines maintained by ICLAC, provide a scientific rationale for their use. No commonly misidentified cell lines were used.

11. Description of research animals
    Provide details on animals and/or animal-derived materials used in the study.

    ROSA-STOP-flox-eYFP mice, MyH11-ERT-cre mice and NG2-ERT-cre mice were obtained from Jackson Laboratories. Only male mice inherit the MyH11-ERT-cre allele, and thus any experiments performed using this mouse model were done so using male mice that were ROSA-STOP-flox-eYFP +/+ and MyH11-ERT-cre +/-. The Klf4-flox mice were obtained through the Mutant Mouse Resource and Research Center (MMRRC), to whom they were donated by Klaus Kaestner. MyH11-ERT-cre mice were bred with the Klf4 floxed mice to obtain the Myh11-ERT-Cre Klf4 Δ mice. Genotyping was performed via PCR using previously published protocols. Cre activity was induced in mice aged 6-8 weeks, followed by initiation of experimental protocols at 9 weeks.

12. Description of human research participants
    Describe the covariate-relevant population characteristics of the human research participants.

    This study did not involve human subjects.