ASPSCR1-TFE3 orchestrates the angiogenic program of alveolar soft part sarcoma

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Abstract

Alveolar soft part sarcoma (ASPS) is a soft part malignancy affecting adolescents and young adults. ASPS is characterized by a highly integrated vascular network, and its high metastatic potential indicates the importance of ASPS's prominent angiogenic activity. Here, we found that the expression of ASPSCR1-TFE3, the fusion transcription factor causatively associated with ASPS, is dispensable for in vitro tumor maintenance; however, it is required for in vivo tumor development via angiogenesis. ASPSCR1-TFE3 is frequently associated with super-enhancers (SEs) upon its DNA binding, and the loss of its expression induces SE-distribution dynamic modification related to genes belonging to the angiogenesis pathway. Using epigenomic CRISPR/dCas9 screening, we identified Pdgfb, Rab27a, Sytl2, and Vwf as critical targets associated with reduced enhancer activities due to the ASPSCR1-TFE3 loss. Upregulation of Rab27a and Sytl2 promotes angiogenic factor-trafficking to facilitate ASPS vascular network construction. ASPSCR1-TFE3 thus orchestrates higher ordered angiogenesis via modulating the SE activity.

Introduction

Tumor angiogenesis is one of the most important processes in malignant progression and distant metastasis of cancer[1]. Migration, extension, and sprouting of endothelial cells are key mechanisms that initiate angiogenesis to construct vascular tubes that are required for oxygen and nutrition transport to cancer cells as well as cancer-cell intravasation[2]. However, this cancer-induced neovascularization frequently induces the construction of fragile blood vessels that exhibit abnormal distributions and reduced pericyte numbers, resulting in frequent vessel destruction and obstruction and insufficient blood supply in common cancers[3,4]. In contrast, there is a group of cancers that induce highly integrated blood vessels with abundant pericyte wrapping, and the presence of the pericytes and their derivatives affects the malignant potential of cancer. The group includes cancers originating from blood vessel-rich organs, such as the kidney, liver, and endocrine organs[5-7]. Platelet-derived growth factor b (PDGFB), which is provided by both cancer cells and non-cancerous stromal cells, is important for pericyte recruitment, and the interaction between PDGFB and PDGF receptor b (PDGFRB) on pericytes is responsible for mature-blood-vessel stabilization[8,9].

Alveolar soft part sarcoma (ASPS) is a slow-growing malignant neoplasm affecting adolescents and young adults[10,11]. Despite its less aggressive nature in the primary site, ASPS is highly metastatic, and patients exhibit poor outcomes due to the limitations of the current therapies[12-14]. ASPS is characterized by a blood vessel-rich alveolar structure with a highly integrated vascular network, which is responsible for frequent metastasis[11,15]. Blood-vessel abundance in ASPS suggests that the vascular network is an ideal target for effective therapies; however, vascular endothelial growth factor receptor (VEGFR)-inhibitor administration has not provided a cure for ASPS[12,14,16]. These findings indicate that there is an unknown ASPS angiogenic mechanism that should be clarified to facilitate enhanced therapy.

We established a mouse model for human ASPS[15] that effectively recapitulates ASPS phenotypes, such as alveolar structure with fine capillary network, frequent intra- and extravasation of tumor nests, and distant metastasis. Importantly, tumor-blood-vessel analysis in the mouse model and human ASPS clarified that these vessels are well encapsulated with PDGFRB-positive pericytes.

ASPS is invariably associated with *ASPSCR1-TFE3* gene fusion that encodes an oncogenic transcription factor using TFE3's basic helix-loop-helix (bHLH) domain as a DNA-binding domain[17-19]. Like other sarcoma-associated fusion genes, *ASPSCR1-TFE3* is essential for transforming target cells to induce ASPS *in vivo*[15,20]. ASPSCR1-TFE3 upregulates genes important for angiogenesis and tumor metastasis, such as *PDGFB, ANGPTL2*, and *GPNMB*[15,21,22], suggesting that ASPSCR1-TFE3 plays a central role in the angiogenesis characteristic of ASPS. In addition, its function in rather restricted cells-of-origin, such as embryonic mesenchymal cells, suggests that a proper epigenetic environment is important for the oncogenic and angiogenic function of ASPSCR1-TFE3. Despite the strong circumstantial evidence for the association between ASPSCR1-TFE3 and angiogenesis, the mechanism by which ASPSCR1-TFE3 orchestrates angiogenesis in association with the proper epigenetic conditions remains unclear.

Results

ASPSCR1-TFE3 is dispensable for cell growth and survival of ASPS cells *in vitro* but required for *in vivo* tumorigenesis

In our previous study, *ASPSCR1-TFE3* expression in murine embryonic mesenchymal cells could effectively induce highly metastatic tumors with human ASPS phenotypes when they were transplanted into nude and Balb/c mice[15]. These sarcoma cells proliferate well *in vitro* and are serially transplantable. However, we observed frequent *ASPSCR1-TFE3*-expression loss or decrease during *in vitro* passage (Fig. 1a-c). There were no significant differences in cell proliferation or morphology (Fig. 1d and Extended Data Fig. 1a). When *ASPSCR1-TFE3* was knocked down in the human ASPS-KY cell line, the *in vitro* growth potential was not significantly affected (Fig. 1d). Although the cause of the frequent *ASPSCR1-TFE3*-expression loss has not been fully clarified, we detected a loss of the 5' long terminal repeat promoter/enhancer of the retroviral vector used for *ASPSCR1-TFE3* transduction in at least one pair of murine ASPS cell lines, ASPS17 and ASPS null (Extended Data Fig. 1b). These results indicate that *ASPSCR1-TFE3* expression is dispensable for *in vitro* ASPS-cell growth once they are transformed. In contrast, no tumor growth was observed when ASPS-null cells were transplanted into nude mice (Fig. 1e). Histological examination of early tumorigenic lesions 4 days after transplantation revealed that FLAG-positive ASPS-cell foci were accompanied by CD31-positive endothelial cells and PDGFB-positive pericytes in the ASPS17 transplanted areas, whereas no FLAG-positive cells and very few CD31-positive and PDGFRB-positive blood vessel components were observed in the subcutaneous part of ASPS-null transplanted recipients (Fig. 1f). Gene expression profiling identified 3,123 upregulated genes and 2,400 downregulated genes (1.5-fold threshold) in ASPS-null cells compared with those in ASPS17 cells as well as 450 upregulated and 376 downregulated genes in ASPS-KY cells by knockdown of *ASPSCR1-TFE3*

(Supplementary Table 1-4). Gene set enrichment analysis (GSEA) revealed enrichment of angiogenesis-related pathways, such as the VEGF, PDGF, and intracellular vesicle/granule-related pathways (Fig. 1g and Extended Data Fig. 1c). Similar enrichment results were obtained in the *ASPSCR1-TFE3* knockdown experiment using the human ASPS-KY cell line (Fig. 1h and Extended Data Fig. 1d). Downregulated expression of individual genes in these pathways such as *Rab27a, Sytl2, Pdgfb*, *Vwf,* and *Gpnmb* was confirmed by quantitative reverse transcription polymerase chain reaction (qRT-PCR) (Fig. 1g,h and Extended Data Fig. 1c,d). Collectively, these results suggest that ASPSCR1-TFE3 regulates downstream target genes involved in angiogenesis to induce ASPS's blood-vessel-rich alveolar structure and *in vivo* tumorigenesis.

ASPSCR1-TFE3 affects chromatin remodeling and activates its downstream target genes

Drastic modulation of gene expression profiles in the absence of ASPSCR1-TFE3 suggests that chromatin activity may be affected. ChIP-seq analysis demonstrated that ASPSCR1-TFE3 binding peaks were predominantly associated with distal regions (69–71%) and 21–22% of the peaks were located at gene promoters both in mouse and human ASPS (Fig. 2a). The Genomic Regions Enrichment of Annotations Tool (GREAT) pathway analysis for these ASPSCR1-TFE3 binding peaks identified genes involved in lysosomes, cytoplasmic vesicles, or vacuolar membranes, whose expression was upregulated in ASPS (Extended Data Fig. 2a). The consensus binding motif for the MiT/TFE family transcription factors, GTCACGTG/CACGTGAC, was most highly enriched in mouse and human ASPS (Fig. 2b). A frequent association between ASPSCR1-TFE3 and histone H3K27ac binding was observed (86% and 65% of ASPSCR1-TFE3 binding in mice and humans, respectively) (Fig. 2c,d). H3K27ac-binding signals around the ASPSCR1-TFE3 binding regions were significantly reduced by loss of or diminished *ASPSCR1-TFE3* expression (Fig. 2d and Extended Data Fig. 2b), suggesting that ASPSCR1-TFE3 potentially modulates global histone modifications and chromatin activation. Thereafter, we compared SE profiles between ASPS17 and ASPS-null cells and found that 49% of SEs were lost by loss of *ASPSCR1-TFE3*-expression (Fig. 2e,f and Supplementary Table 5–7). Pathway analysis of 258 SEs that disappeared after *ASPSCR1-TFE3* loss revealed blood vessel-related pathway enrichment, whereas angiogenesis-related pathways were not enriched in 269 common SEs for ASPS17 and null cells and 187 null cell-specific SEs (Fig. 2f,g and Extended Data Fig. 2c). Significant differences in SE distribution were also evident in human ASPS cells after *TFE3* knockdown (Extended Data Fig. 2d-f). These results strongly suggest that ASPSCR1-TFE3 partially regulates angiogenesis-associated target genes by modulating SE.

The BET protein inhibitor JQ1 suppresses angiogenesis-associated SEs and inhibits ASPS *in vivo* growth

BET domain proteins play an important role in the SE functions, and the inhibition of their activities renders effective targets for the SE-associated malignant potential of cancer[23,24]. *In vivo* treatment of murine ASPS cells transplanted into nude mice with JQ1, a BET-domain inhibitor, significantly suppressed tumor growth (Fig. 3a). This growth suppression was accompanied by remarkable angiogenic inhibition, exhibiting a reduction in CD31-positive endothelial cells and PDGFRB-positive pericytes (Fig. 3b). Gene expression analysis using GSEA and Ingenuity Pathway Analysis (IPA) demonstrated PDGF-associated

and angiogenesis-related pathway enrichment (Fig. 3c,d and Extended Data Fig. 3a). Brd4 is a major JQ1 target, and ChIP-seq analysis revealed a significant reduction in Brd4 binding around the ASPSCR1-TFE3 binding peaks (Fig. 3e). A total of 23,924 out of 34,800 Brd4-binding peaks disappeared after JQ1 treatment, and the GREAT analysis of these peaks revealed angiogenesis-related pathway enrichment (Fig. 3f). Moreover, the angiogenesis pathway was affected in an ASPSCR1-TFE3-dependent manner (Extended Data Fig. 3b). In addition, Brd4-binding signals within SEs were significantly decreased by JQ1 treatment (Extended Data Fig. 3c). ChIP-seq analysis also demonstrated that Brd4-binding signals were reduced in SEs and active enhancers associated with ASPSCR1-TFE3 target genes, namely, *Rab27a, Sytl2, Pdgfb,* and *Vwf*, whose expression was downregulated by *ASPSCR1-TFE3* loss (Fig. 3g,h). In contrast, there was also a subset of genes, including *Myh9*, *Neat1,* and *Atf4,* whose enhancers were not affected by the JQ1 treatment. The CDK7/8 inhibitor, THZ1, which also targets SEs in certain cancers[25], did not exhibit growth suppressive effects on ASPS or angiogenesis-related gene downregulation (Extended Data Fig. 3d,e), suggesting that chromatin modifications on angiogenesis-associated SEs by ASPSCR1-TFE3 may be effective in a Brd4-specific manner.

Epigenomic CRISPR screening identifies ASPSCR1-TFE3 target genes involved in angiogenesis and *in vivo* tumor development

SE modification by ASPSCR1-TFE3 and JQ1 treatment strongly suggests that downstream target genes regulated by these SEs play a critical role in ASPS-related angiogenesis, and that the identification of these targets will provide important information for novel ASPS therapies. Therefore, epigenetic CRISPR screening with dCas9-KRAB was performed targeting 494 SEs and active enhancers in which H3K27ac signals were reduced by > 40% due to *ASPSCR1-TFE3* loss were selected, and 7,716 gRNAs per enhancer (average 12.8) were designed (Fig. 4a and Supplementary Table 8). To suppress target-enhancer activities, ASPS17 cells were introduced with lentiviral vectors bearing gRNAs and dCas9-KRAB, and pools of lentivirus-transduced cells were transplanted into nude mice (Extended Data Fig. 4a). Locus-specific repression effects were confirmed by introducing individual lentiviruses (Extended Data Fig. 4b), and 63 loci with over five gRNAs reduced two-fold reduced in tumors were considered positive candidates (Fig. 4b, Extended Data Fig. 4c, and Supplementary Table 9). Topologically associated domains (TADs) were defined by Hi-C analysis (Fig. 4c), and 345 candidate target genes included in the same TAD as each SE were selected. Fifty-six candidate genes were further selected by downregulated expression in ASPS-null cells and functional annotations (Fig. 4d and Supplementary Table 10). Six candidate genes, namely, *Ccbe1, Pdgfb, Rab27a, Syngr1, Sytl2*, and *Vwf*, were subjected to an *in vivo* validation assay (Extended Data Fig. 4d). After effective knockout of each gene was validated (Extended Data Fig. 4e), the tumor clones were transplanted into nude mice. Despite comparable proliferation rates *in vitro* (Extended Data Fig. 4f), significant suppression of *in vivo* tumor development was observed by *Pdgfb, Rab27a, Sytl2*, and *Vwf* knockout (Fig. 4e). As shown by *ASPSCR1-TFE3*-expression loss and JQ1 treatment, tumor-growth suppression by each knockout cell was accompanied by significant angiogenic inhibition (Extended Data Fig. 4g). The present epigenetic screening identified Sytl2 and Rab27a, both of which are involved in intracellular trafficking of cytoplasmic vesicles[26], as well as angiogenic factors Pdgfb and Vwf, indicating

that the ASPSCR1-TFE3-modulated chromatin activity orchestrates the core angiogenesis program of ASPS.

Expression of ASPSCR1-TFE3 target genes identified in the present study was examined using clinical sarcoma samples and human sarcoma cell lines. Increased *RAB27A*, *SYTL2*, and *VWF* expression was observed in human ASPS among the six sarcoma types (Fig. 4f). *PDGFB* expression in ASPS was the second highest following dermatofibrosarcoma protuberance, in which *PDGFB* fuses with *COL1A1* and is highly upregulated[27]. Upregulated *RAB27A, SYTL2,* and *VWF* expression was confirmed in two human ASPS cell lines, ASPS-KY and ASPS1, in comparison with Ewing, synovial, and osteosarcoma cell lines (Extended Data Fig. 4h). Immunohistological examination revealed diffuse cytoplasmic RAB27A and SYTL2 staining, in accordance with their upregulated expression (Fig. 4g). VWF was also positive with focally strong staining. The mechanism of uneven VWF accumulation remains unclear, and the clarification of its relationship with endothelial sprouting is rather intriguing.

Intracellular trafficking of angiogenic factors is promoted by the Rab27a/Sytl2 axis

To clarify the role of the Rab27a/Sytl2 axis in ASPS, Pdgfb and Gpnmb secretion, the latter of which is highly expressed in ASPS and is required for extravasation of tumor cells, was examined. *Rab27a* or *Sytl2* knockouts significantly suppressed Pdgfb and Gpnmb secretion (Fig. 5a). A co-immunoprecipitation experiment showed interaction between Rab27a and Sytl2, and the Rab27a W73G mutant, in which the binding activity for Sytl2 was lost[28], failed to exhibit increased secretion activity (Fig. 5a and Extended Data Fig. 5a,b). *Rab27a, Sytl2*, or *Pdgfb* knockout and *ASPSCR1-TFE3* loss resulted in decreased pericyte growth induction when these cells' conditioned media were used (Fig. 5b). Fluorescence recovery after photobleaching (FRAP) demonstrated significant delay of Pdgfb, Gpnmb, Angptl2 trafficking in *Rab27a* knockout, *Sytl2* knockout, and ASPS-null cell membrane trafficking (Fig. 5c and Extended Data Fig. 5c). *Rab27a* re-expression rescued Pdgfb and Gpnmb trafficking, whereas the W73G mutant did not (Extended Data Fig. 5d). Likewise, von Willebrand factor (VWF) secretion was also significantly reduced by *Rab27a* and *Sytl2* knockout and by *ASPSCR1-TFE3*-expression loss (Fig. 5d). Although its large molecular weight precluded the FRAP assay, the intra-cytoplasmic distribution of VWF indicated that VWF is predominantly localized in the perinuclear region in ASPSCR1-TFE3-, Rab27a-, or Sytl2-reduced conditions (Fig. 5e and Extended Data Fig. 5e). Nexinhib 20, a small-molecule inhibitor for the interaction between Rab27a and Sytl1 (Extended Data Fig. 5f), a close Sytl2 homolog[29], suppressed Pdgfb, Gpnmb, and VWF secretion and Pdgfb, Gpnmb, and Angptl2 in membrane trafficking in ASPS 17 cells (Fig. 5f,g and Extended Data Fig. 5g). Overall, these results indicate that intracytoplasmic trafficking of angiogenic factors is promoted in ASPS cells and regulated by ASPSCR1-TFE3 via the Rab27a/Sytl2 axis.

Rab27a or *Sytl2* loss affects vascular sprouting in the microfluidic device

To confirm the significant role of the Rab27a/Sytl2 axis in angiogenesis, three-dimensional (3D) tissue culture/microfluidic devices were used to mimic *in vivo* angiogenesis. The microfluidic device included a tumor spheroid comprising murine ASPS cells covered with pericytes to constitute "core shell" structures

(Fig. 6a,b and Extended Data Fig. 6a). Spheroid growth varied, despite the equal growth property of each ASPS clone (Extended Data Fig. 6a). The tumor spheroids were subsequently co-cultured with human umbilical vein endothelial cells (HUVEC) to evaluate the on-chip vascular extension. During 5 days of co-culturing, HUVEC sprouting toward the tumor spheroids of ASPS cells occurred; however, the sprouting effect was significantly suppressed by *Rab27a* or Sytl2 knockout and *ASPSCR1-TFE3*-expression loss (Fig. 6c,d and Extended Data Fig. 6b,c). Decreases in vascular components of Rab27a or Sytl2 knockout cells and ASPS-null cells were also confirmed by immunohistochemical spheroid analysis (Fig. 6e). These results suggest that ASPSCR1-TFE3 induces endothelial sprouting via the Rab27a and Sytl2 axis in ASPS cells in collaboration with pericytes.

Discussion

A prominent vascular network is a hallmark of ASPS, both in morphology and biological behavior. Abundant capillary blood vessels in ASPS are associated with its slow growth and resistance to conventional chemotherapy. Our study demonstrated that angiogenic promotion occurs at the early stage of tumor growth *in vivo* (Fig. 1f), indicating that this mechanism is essential for the ASPS development.

Previous studies identified numerous ASPSCR1-TFE3 targets that are involved in growth signaling, lysosomal functions, autophagy, and angiogenesis in both ASPS and fusion-positive renal cell carcinoma, with the latter exhibiting a vascular structure similar to that of ASPS[19-22,30]. However, the reported targets are rather diverse, probably due to differences in model systems and the lack of appropriate biological evaluation. Furthermore, *in vitro* studies using cell lines are considerably limited, and the clarification of angiogenic mechanisms requires both *in vivo* studies and advanced co-culture techniques, such as the organ-on-a-chip system used in this study. The similar tumor suppression and angiogenesis phenotypes and gene expression profiles between *ASPSCR1-TFE3* loss and the JQ1 treatment suggests the critical role of SEs modulated by ASPSCR1-TFE3, although the mechanism by which Brd4-inhibition targets certain SEs awaits clarification. A previous study indicated that key SEs define cell lineage, stemness, plasticity, and differentiation, which are remodeled by pioneer transcription factors such as SOX9, in hair follicle stem cells[31]. ASPSCR1-TFE3, as a pioneer factor, may also play a key role in SE modulation during ASPS development and angiogenesis.

RAB27 and SYTL/SLP proteins collaborate to facilitate cytoplasmic trafficking of vesicles, secretory granules, and target proteins, such as membrane-bound receptors[32,33]. These molecules are important in non-neoplastic pathological conditions, such as degranulation of neutrophils, cytotoxic T-cells, and platelets[33,34]. Although *RAB27A* and *SYTL2* expression is upregulated in multiple cancer types[35], the biological significance of this upregulation is not well understood. We previously identified *Sytl1*, a close homolog of *Sytl2*, as a direct target gene of Meis1 in AML[36]. Sytl1, a Rab27b partner in hematopoietic cells, facilitates CXCR4 membrane trafficking and promotes occupancy of the bone marrow niche by AML, indicating the importance of the Rab27/Sytl axis in the interaction between tumor cells and the

microenvironment. Rab27a and Sytl2 upregulation indicates the importance of targeting this pathway for effective ASPS therapy using specific inhibitors.

Multiple factors that promote angiogenesis were found to be upregulated in ASPS[15,16,19,37]. Our present study revealed important angiogenic factors, such as PDGFB, GPNMB, and ANGPTL2, and they are included in the cargo transported from the cytoplasm to the membrane by RAB27A and SYTL2. However, we did not identify all the components sufficient for angiogenesis in ASPS. Further studies are required to clarify the complete angiogenic process of ASPS. Nevertheless, this study revealed that PDGFB is important for pericyte migration and proliferation[8,9,38], a key vascular-network component in ASPS. Moreover, GPNMB is essential for tumor-cell intravasation and extravasation in ASPS, given that *Gpnmb* knockdown significantly reduces the interaction between ASPS and endothelial cells[15]. GPNMB also induces endothelial-cell migration[39], indicating its importance in tumor vascular network formation. ANGPTL2 binds to endothelial cells and promotes vascular sprouting in collaboration with ANGPTL1[40,41]. VWF also plays an important role in angiogenesis and it functions as a reservoir of angiogenic growth factors[42]. Angiogenic factors, such as PDGFB and VEGF, possibly bind to VWF to facilitate interactions between pericytes and endothelial cells.

The organ-on-a-chip system, using microfluidic devices and tumor spheroids, greatly improves monolayer-culture deficit[43]. In this study, we created a core shell structure containing ASPS tumor cells covered with pericytes, reflecting the alveolar structure of ASPS *in vivo*. Proper and efficient interaction between ASPS and pericytes induces endothelial-cell sprouting. Significant ASPSCR1-TFE3, Rab27a, and Sytl2 contributions were highlighted in the induction of vascular sprouting. Inhibitory drugs for angiogenesis in ASPS will be evaluated using this system, providing novel and effective ASPS therapies.

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Methods

Cell lines and culture. The mouse ASPS cell line ASPS17 was established from the tumors induced in embryonic mesenchymal cells expressing ASPSCR1-TFE3 as previously described[17]. The human ASPS cell lines, ASPS-KY and ASPS-1, are described previously[44,45]. Mouse pericytes were purified from the dpc 17 mouse embryo mesenchyme by sorting the PDGFRB+, CD105+, and CD31- fraction, and the cells were immortalized by introduction of the SV40 large T antigen. All cell lines were grown in DMEM supplemented with 10% FBS.

Allograft transplantation studies. Tumor cell transplantation experiment was performed by injecting 5 x 10^6 ASPS cells mixed in Matrigel (Corning) into the subcutaneous regions of 10- to 12-weeks old Balb/c nude mice as described previously[17]. JQ1 and THZ1 were intraperitoneally administrated 50 mg/kg daily for two weeks. Mice were carefully observed daily, and were euthanized 17 days after transplantation (n = 10 per each group). All animal experiments described in this study were performed in strict accordance with standard ethical guidelines and were approved by the animal care committee at the Japanese Foundation for Cancer Research under licenses 10-05-9 and 0604-3-13.

Immunoblotting. Cells were lysed in RIPA buffer and precleaned by centrifugation at 10,000g for 10 min at 4°C. Protein concentrations were measured by the DC protein assay (Bio-Rad). Equal amout of protein lysates were boiled for 5 min in sample buffer (0.5 M dithiothreitol, 25% glycerol, 2% SDS, 60 mM Tris-HCl pH 6.8 and bromophenol blue. The samples were separated by SDS-PAGE on SuperSep gels (Fuji Film) and transferred onto nitrocellulose membrane (Amersham). Immunoblots were probed with primary antibodies in 5% skim milk overnight at 4°C and respective secondary antibodies for 1 h at room temperature. Primary antibodies used were ASPSCR1 (Sigma-Aldrich, HPA026749), Cas9 (Novus Biologicals, NBP2-36440), FLAG-tag (Sigma-Aldrich, F3165), Myc-tag (Santa Cruz Biotechnology, sc-40), GFP (Merck Millipore, MAB3580), and mCherry (Cell Signaling, 43590).

Immunofluorescence. Mouse ASPS cells were fixed with 4% paraformaldehyde and were subjected to immunofluorescence using the specific antibodies and the respective fluorochrome-labeled secondary antibodies. Immunofluorescent images were photographed with a Zeiss LSM 770 laser scanning microscope with a 60x objective (Zeiss) and LSM Software ZEN 2009 (Zeiss). Captured images were

analyzed using ImageJ. The mean distance of Vwf from the nucleus was calculated in 10 different cells for each condition per experiment by tracing lines (6 in each cell) from the nuclear membrane to the most distal fluorescent foci. For evaluation of 3D-culture in the microfluidic device, fluorescence and immunofluorescence images were captured using Olympus IX71 and Olympus FV3000, and recorded every other day and quantified as described in the figure legends. Antibodies used were VWF (DAKO, N1505), FLAG-tag (Sigma-Aldrich, F7425), and goat anti-rabbit IgG conjugated with RRX (Jackson Immunoresearch, 111-295-144).

Quantitative RT-PCR (qRT-PCR). Total RNA extraction, reverse transcription and RNA quantification were performed by standard methods. Conventional RT-PCR and real-time quantitative RT-PCR (Q-RT-PCR) were performed with a Gene Amp 9700 thermal cycler (Applied Biosystems) and a 7500 Fast Real-Time PCR System (Applied Biosystems), respectively. The sequences of the oligonucleotide primers are shown in Supplementary Table 11.

Histopathology and immunohistochemistry. For light microscopic analysis, tumor tissues were fixed with 3% formaldehyde, paraffin embedded, and stained with hematoxylin and eosin (H&E) using standard techniques. Antibodies used were mouse CD31 (Cell Signaling, 77699), PDGFRB (R & D Systems, BAF1042), RAB27A (Cell Signaling, 69295), SYTL2 (Santa Cruz Biotechnology, sc393847), VWF (Santa Cruz Biotechnology, sc365712), human CD31 (Abcam, ab28346), and FLAG-tag (Sigma-Aldrich, F3165). Heat-mediated antigen retrieval was performed in Tris-EDTA buffer at pH 6.0. Immunohistochemical staining was performed using the Simple Stain MAX-PO kit (Nichirei Bioscience), the Histofine SAB-PO (R) kit (Nichirei Bioscience).

Gene expression profiling. ASPS cell pellets were processed to extract total RNeasy Mini Kit (Qiagen). RNA quality was assessed using the Bioanalyzer 2100 (Agilent). The murine Genome HT MG-430 PM Array and the human HG-U133 + PM Array (Affymetrix, Santa Clara, CA, USA) were hybridized with aRNA probes generated from the total RNA samples. After staining with streptavidin-phycoerythrin conjugates, arrays were scanned using an Affymetrix GeneAtlas Scanner.

Microarray data processing and analysis. The data were analyzed with Microarray Suite version 5.0 (MAS 5.0) using Affymetrix default analysis settings and global scaling as normalization method. The trimmed mean target intensity of each array was arbitrarily set to 100. Microarray data were analyzed using GeneSpring GX 12.6 (Agilent). Gene pathway analysis was performed using Gene Set Enrichment Analysis (GSEA) was performed using GSEA-P 2.0 software[46], and Ingenuity Pathway Analysis (Qiagen).

Chromatin immunoprecipitation (ChIP)-Sequencing. ChIP-Seq was performed using the method previous described with modifications[47]. A total of 5 x 10^6 ASPS cells per immunoprecipitation were cross-linked with 1% formaldehyde for ten minutes at room temperature. Chromatin was sheared in sodium dodecyl sulfate (SDS) lysis buffer containing 1% SDS, 10 mM EDTA, and 50 mM Tris pH 8.0 to an average size of 400 to 500 bp using a Covaris S220 sonicator for 15 min. ChIP was performed with 5 µg anti-histone H3K27ac (Active Motif, 39133), anti-H3K4me3 (Abcam, ab8580), anti-H3K27me3 (Millipore, 07-449), anti-

FLAG (Sigma-Aldrich, F7425), anti-ASPSCR1 (Sigma-Aldrich, A026749), anti-BRD4 (Bethyl Laboratories, A301-985A100) antibodies. The antibody-bound protein/DNA complexes were immunoprecipitated using protein G magnetic beads. Immunoprecipitated DNA was then purified and subjected to secondary sonication to an average size 150 to 350 bp. Libraries were prepared according to instructions accompanying the ThruPLEX DNA-Seq kit (Rubicon Genomics). The ChIP DNA was end modified and adapters were ligated. DNA was PCR amplified with Illumina primers and Illumina-compatible indexes were added. The library fragments of approximately 300-500 bp were band-isolated from an agarose gel. The purified DNA was sequenced on an Illumina MiSeq next-generation sequencer following the manufacturer protocols.

ChIP-seq data analysis. Base calls were performed using Bowtie 2 (http://bowtie-bio.sourceforge.net/bowtie2/index.shtml). ChIP-Seq reads were aligned to the mm9 (https://www.ncbi.nlm.nih.gov/assembly/GCF_000001635.18) or hg19 (https://www.ncbi.nlm.nih.gov/assembly/GCF_000001405.13/) genome assembly using samtools 1.2 (http://www.htslib.org). Peak calling was performed using MACS1.4 (http://liulab.dfci.harvard.edu/MACS). Peak distribution was calculated by Cistrome (http://cistrome.org/ap/root). Neighbor genes on enriched genomic regions were determined using by Nucleus (https://rias.rhelixa.com). The genomic distributions of DNA-binding peaks were visualized by NGSplot (https://anaconda.org/bioconda/r-ngsplot). DNA-binding of each ChIP-seq data was visualized using IGV_2.3.80 (http://software.broadinstitute.org/software/igv). The de novo motif enrichment was performed using HOMER v 4.11.1 (http://homer.ucsd.edu/homer/motif). Super-enhancers were identified using the method previously described with the ROSE program (http://younglab.wi.mit.edu/super_enhancer_code.html). Gene ontology analysis for nearby genes on super-enhancers were performed by GREAT version 4.0.4 (http://great.stanford.edu/public/html/).

Epigenomic CRISPR/dCas9 screening. CRISPR/Cas9-based epigenomic screening was performed according to the method described with modifications[48,49]. Total of 494 SEs and active enhancers in which H3K27ac signals were reduced more than 40% by *ASPSCR1-TFE3* loss were selected, and average 12.8 gRNAs per each enhancer were designed to target these enhancers using CRISPR direct (https://crispr.dbcls.jp) and GuideScan (http://www.guidescan.com) (Supplementary Table S8). 397 gRNAs for enhancers without ASPSCR1-TFE3 binding and 1,000 non-target gRNAs (https://www.addgene.org/pooled-library/zhang-mouse-gecko-v2/) were designed as negative controls. Gibson overhangs were fused to sense and antisense nucleotides corresponding to each gRNA. The gRNA library was synthesized by CustomeArray and inserted into pLV-U6-gRNA-UbC-DsRed-P2A-Bsr (Addgene) using Gibson Assembly (New England BioLabs). A high titer gRNA library was constructed in Endura ElectroCompetent cells (Lucigen), and 293FT cells were transfected with the purified library DNA, psPAX2 and pCMV-VSV-G. ASPS17/dCas9-KRAB cells were generated by infecting Lenti-Ef1a-dCas9-KRAB-Puro lentivirus (Addgene) and the cells were transduced with lentivirus bearing the gRNA library at 100x fold coverage. DsRed-positive cells were sorted by FacsAria II and selected using 2 μg/ml of Blasticidin four days after infection. Then, $1x10^6$ infected cells were transplanted subcutaneously to nude

mice 10 days after infection. Subcutaneous tumors were removed a month after transplantation and genomic DNA was extracted, PCR amplified, and subjected to target sequencing using screening primers listed in Supplementary Table 12. Sequencing reads were compared between pre-tranplanted and post-transplanted ASPS 17 cells using DESeq2, and enhancer loci that contain more than 50% of gRNAs reduced <0.5 with and adjusted p-value <0.05 were defined as enhancers required for *in vivo* ASPS growth.

Hi-C. The Hi-C libraries were constructed using an Arima-HiC Kit (Arima Genomics) according to the manufacturer's instructions for Mammalian Cell Lines (A160134 v00) and Library Preparation (A160137 v00). **In brief,** one million cells were collected and crosslinked with 37% formaldehyde solution. DNA isolated from the crosslinked cells was digested with two restriction enzymes (^GATC and G^ANTC). After the incorporation of biotinylated nucleotides at the digested DNA ends, both ends were ligated with the spatially proximal ends. The ligated DNA was sheared into 200-600 bp fragments using the Focused-ultrasonicator M220 (Covaris) and the ligation junctions were enriched with streptavidin magnetic beads. The sequencing libraries from enriched DNA fragments were prepared with a TruSeq DNA PCR-Free Library Prep Kit (Illumina). The resulting libraries were amplified with 10 PCR cycles and purified with SPRI beads. The quality and concentration of the paired-end libraries were evaluated using the Qubit 4 Fluorometer (Thermo Fisher Scientific), the 2100 Bioanalyzer system (*Agilent* Technologies), and the 7900HT Fast Real-Time PCR System (Thermo Fisher Scientific). The final libraries were sequenced on the Illumina NovaSeq 6000 sequencer with a read length of 150 bp. Reads were processed with Juicer pipeline, using reference of hg38 for human samples and mm10 for mouse samples, yielding *.hic* files[50]. These files were further used for Juicebox visualization and downstream analysis, including finding loops and contact domains using Juicer tools. Loops were called by HICCUPS and those located in Chr1-22 (human), 1-19 (mouse), and X.

CRISPR/Cas9-mediated gene editing. Lentivirus plasmids containing short guided RNA (sgRNA) for *Ccbe1, Pdgfb, Rab27a, Syngr1, Sytl2,* and *Vwf* were introduced into the lentiCRISPRv2-puro vector (Addgene). The sgRNA sequences are listed in Supplementary Table 12. Knockdown efficiencies were confirmed by western blotting and/or RT-PCR.

ELISA. For the ELISA, 5 x 10^6 mouse ASPS cells were seeded and cultured overnight. The culture media were collected and immediately analyzed using the mouse Gpnmb (Abcam), Vwf (Abcam), and Pdgfb (Proteintech) ELISA kits according to manufacturers' instructions.

Fluorescence recovery after photobleaching. Trafficking of Pdgfb, Gpnmb, and Angptl2 was evaluated by FRAP following previously described procedures[51]. ASPS17 and null cells were transfected with with plasmids containing, DsRed-tagged Pdgfb, Gpnmb, or Angptl2. RFP-tagged actin (Invitrogen) was used as a negative control of trafficking. FRAP images were acquired with an LSM880 confocal microscope equipped with a live cell chamber (set at 37°C) and ZEN software (Zeiss) with a 40X objective. Cells were excited with a 561 nm laser and the emission between 566 and 689 nm recorded. Images were acquired with 12 bits image depth and 512 × 512 resolution using a pixel dwell of ~1.52 µs. At least three (n ≥ 3)

pre-bleaching images were collected and then the region of interest was bleached with 100% of laser power. The recovery of fluorescence was traced at every 5 min for 1 hr. For drug inhibition experiments, 10 μM of Nexinhib 20 (Tocris) was added into culture media 3 hr before bleaching. Fluorescence recovery was calculated as previously described[51].

Fabrication of microfluidic device. The micropatterned master with 100 μm in height features was fabricated based on standard photolithography. SU-8 3050 (MicroChem) was coated and patterned on a 4 inch silicon wafer by exposing ultraviolet (UV) light to the top of a photomask on which the desired 3-channel patterns are printed. A mixture of PDMS (Sylgard 184, Dow-Corning) and its curing agent was mixed to a 10:1 weight ratio, poured onto the patterned master to a 5-mm thickness, degassed to remove air bubbles for 1-2 h, and cured in an oven at 80 °C for 3-4 h. The cured PDMS was removed from the wafer. The inlets (diameter: 2 mm), outlets (diameter: 6 mm), and center hole (diameter: 1 mm) for media filling and spheroid injection were punched using biopsy punches unless otherwise noted. After cleaning with adhesive tape, the PDMS device and glass coverslip were permanently bonded using oxygen plasma treatment (40 s, 50 sccm, 40 mW). Furthermore, the microfluidic devices were then stored in a 35mm dish and cured in an oven at 80 °C for more than 2 h. After sterilization under UV for 1-2 h, the microfluidic devices were ready for cell seeding.

ASPS spheroid formation. The prewarmed DMEM was added to the dish, and the ASPS cell suspensions were transferred into 15 mL tubes and centrifuged at 1000 rpm for 3 min. The cells were resuspended at desired density. Cell suspension was then added in a prime surface 96-well plate with U-shaped bottom well (Sumitomo Bakelite), which significantly caused self-aggregation of cells, namely a spheroid. Monoculture core spheroids were prepared with a density of 5.0×10^4 cells/mL in the IMDM medium for two days. Co-culture core-shell spheroids were initiated by core spheroid formation. After two days, the suspension culture of core spheroids was then replaced by pericytes suspension at a density of 7.5×10^4 cells/mL in the IMDM medium for shell formation for another day. Finally, the spheroids were introduced into a microfluidic device.

In vitro angiogenesis. A spheroid was transferred into fibrin-collagen gel (2.5 mg/mL fibrinogen) (Sigma-Aldrich), 0.15 U/mL aprotinin (Sigma-Aldrich), 0.2 mg/mL collagen type I (Corning), and thrombin 0.5 U/mL (Sigma-Aldrich) in D-PBS. The spheroid suspended in gel solution was then injected through the center hole into channel 2 without leakage into channels 1 and 3 and incubate at a 37 °C CO_2 incubator for 15 min for gelation. Channels 1 and 3 were filled with EGM-2 and incubated overnight at the incubator to eliminate the bubbles at the boundary of gel and medium. For HUVEC adhesion at the gel interface, the HUVECs (5.0×10^6 cells/mL in the EGM-2) were injected into channel 1. With a 90° tilt device at a 37 °C CO_2 incubator for 15 min, HUVECs adhered to the gel surface in the microfluidic device. This process was repeated for channel 3. After spheroid injection and HUVEC adhesion, the inlets and outlets of channels 1 and 3 were filled with EGM-2 and kept at 37 °C and 5% CO_2 in an incubator. For culturing cells on the device, EGM-2 was replaced daily unless otherwise noted.

Human sarcoma specimens. Alveolar soft part sarcoma, synovial sarcoma, Ewing sarcoma, myxoid liposarcoma, dermatofibrosarcoma protuberans and solitary fibrous tumor surgical specimens were obtained from The Cancer Institute Hospital. Informed consent was obtained from donors, and the study was approved by Institutional Review Board at the Japanese Foundation for Cancer Research under license 2013-1155.

Statistical analysis. All data are representative results from at least three independent experiments unless otherwise specified in the figure legends. The mean ± SD of individual experiments is shown. Student *t*-test and one-way ANOVA statistical method were used.

Material availability. Plasmids generated in this study are available upon request (T.N.)

Data availability

Microarray and ChIP-seq data are accessible through the NCBI Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo), with the accession number GSE186789 and GSE189163, respectively. Hi-C data are available in the DDBJ Sequenced Read Archive under the accession numbers DRR330423 and DRR330424.

Code availability

We did not use any custom code or mathematical algorithm that is deemed central to the conclusions. All software and packages used are listed in the Reporting Summary and are publicly available.

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Declarations

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Authors' Contributions

M. Tanaka: Investigation, formal analysis, data curation, visualization, validation, funding acquisition and writing-original draft. **S. Chuaychob:** Data curation, investigation, methodology and visualization. **M. Homme:** Investigation and validation. **Y. Yamazaki:** Investigation. **R. Lyu:** Investigation. **K. Yamashita:** Resources. **K. Ae:** Resources. **S. Matsumoto:** Resources. **R. Maruyama:** Data curation and formal analysis. **W. Qu:** Formal analysis and data curation. **Y. Miyagi:** Resources. **R. Yokokawa:** Methodology, conceptualization, funding acquisition and supervision. **T. Nakamura:** Conceptualization, investigation, project administration, supervision, funding acquisition, writing-original draft and editing.

Competing interests

The authors declare no potential conflict of interests.

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Figures

Figure 1

***ASPSCR1-TFE3* expression is dispensable for cell maintenance in vitro but required for in vivo tumor development in ASPS. a,** Schematic illustration of functional analysis in ASPS cells with or without *ASPSCR1-TFE3* expression. **b,** ASPSCR1-TFE3 expression in ASPS17 and ASPS null cells at the transcriptional level (top) and protein levels (bottom). *** $P < 0.001$. **c,** Loss of the ASPSCR1-TFE3 protein in ASPS null cells exhibited by immunofluorescence using the anti-FLAG antibody. Scale bar: 20 μm. **d,** Cell proliferation of mouse ASPS17 and ASPS null cells (left, top), and human ASPS-KY cells with siRNA treatment (left, bottom). No significant growth changes were observed in the presence or absence of ASPSCR1-TFE3. ns: no significance. The efficiency of *ASPSCR1-TFE3* knockdown is shown at transcriptional and protein levels (right). * $P < 0.05$. **e,** Suppression of tumor development by loss of *ASPSCR1-TFE3* expression. Average tumor volumes with SD are shown in the recipient transplanted with ASPS17 and ASPS null cells. *** $P < 0.001$. **f,** Histology of transplanted area with ASPS17 and ASPS null cells 4 days after transplantation. Hematoxylin and eosin (HE) staining and immunohistochemistry with indicated antibodies. Significant reduction of FLAG-positive tumor cells, CD31-positive endothelial cells, and PDGFRB-positive pericytes in recipients with ASPS null cells. Scale bar: 50 μm. **g,** Gene set enrichment analysis (GSEA) showing enrichment of VEGF and exocytic vesicle pathways between ASPS17 and ASPS null cells. Normalized enrichment scores (NES), nominal *p*-values, and FDR *q*-values are indicated (left). Quantitative RT-PCR (qRT-PCR) showing downregulation of *Rab27a, Sytl2, Pdgfb*, and *Vwf* in ASPS null cells while *Myh9* expression was increased. *** $P < 0.001$. **h,** GSEA showing enrichment of VEGFA and pigment granule pathways by comparing human ASPS-KY cells with and without knockdown of ASPSCR1-TFE3 (left). Downregulation of *RAB27A, SYTL2, PDGFB*, and *VWF* is shown. * $P < 0.05$.

Figure 2

The loss of *ASPSCR1-TFE3* expression modulates distribution of super-enhancers (SEs). a, The genomic distribution of ASPSCR1-TFE3 2,884 and 5,146 bound sites in mouse and human ASPS, respectively, showing predominant binding in distal regions. **b,** De novo motif enrichment analysis of ASPSCR1-TFE3 binding regions in mouse (left) and human (right) ASPS. The top three motifs identified are shown. **c,** Venn diagrams showing the number of common binding regions between ASPSCR1-TFE3 and H3K27ac in mouse and human ASPS. **d,** Composite plots showing a significant reduction in H3K27ac signals in the absence of ASPSCR1-TFE3 in both mouse and human ASPS (left). Heat maps showing ASPSCR1-TFE3, H3K27ac, H3K4me3, and H3K27me3 signals in murine ASPS17 and human ASPS-KY cells. Reduction in H3K27ac signals is observed in both cells (right). **e,** Enhancers are ranked by increasing H3K27ac signals in ASPS17 and ASPS-null cells. Using the ROSE algorithm, 527 and 456 enhancers were defined as SEs in ASPS17 and ASPS-null cells, respectively. **f,** Venn diagram showing overlapping and distinct SEs (left). Enrichment of Gene Ontology biological process for 258 ASPS17-specific SEs, showing inclusion of angiogenesis pathways in red (right). **g,** ChIP-seq track at *Pdgfb, Vwf, Rab27a, Sytl2,* and *Myh9* genomic loci, showing the association between ASPSCR1-TFE3 and H3K27ac binding in ASPS17 cells. Significant

loss of H3K27ac signals and/or SEs in ASPS-null cells are exhibited. The *Myh9* genomic locus is shown as an example of SEs unaffected by *ASPSCR1-TFE3* loss.

Figure 3

JQ1 suppresses angiogenesis-associated SEs and inhibits ASPS *in vivo* growth. a, Suppression of *in vivo* tumor growth by JQ1 treatment. Growth curves of the transplanted tumors and treated with JQ1 or vehicle and the experimental schedule are shown (left). Gross appearances of tumors resected on day 18 (right). * $P < 0.05$. **b,** Immunohistological examination of the tumor samples treated with JQ1 or vehicle. Anti-CD31 (endothel), anti-PDGFRB (pericyte) and FLAG (tumor cell) were used. CD31- and PDGFRB-positive areas were measured and quantitated using the image J software. Positive areas were normalized by the number of FLAG-positive cells (right). scale bar: 50 μm. * $P < 0.05$, ** $P < 0.01$. **c,** GSEA showing PDGF-pathway enrichment by comparing vehicle-treated ASPS treated with JQ1 gene expression signatures. **d,** Ingenuity pathway analysis (IPA) exhibiting multiple genetic pathways including angiogenesis/vasculogenesis (described in red) by JQ1 treatment. **e,** Composite plots showing a significant reduction in Brd4-binding signals around ASPSCR1-TFE3-binding peaks by JQ1 treatment (left). Heat maps showing ASPSCR1-TFE3 and Brd4 with or without JQ1 treatment, in murine ASPS17 cells (right). **f,** Venn diagram showing overlapping and distinct Brd4-binding peaks in ASPS17 cells treated with JQ1 and vehicle (top). IPA using 23,924 genetic loci specific for vehicle-treated ASPS17 showing the enrichment of angiogenesis-related pathways (bottom). **g,** ChIP-seq track at *Rab27a, Sytl2, Pdgfb, Vwf, Myh9, Neat1,* and *Atf4* genomic loci with ASPSCR1-TFE3-binding peaks in ASPS17 cells. Significant reduction of Brd4 signals at *Rab27a, Sytl2, Pdgfb,* and *Vwf* loci, with Brd4 signlas at *Myh9, Neat1,* and *Atf4* loci remaining unchanged. **h,** qRT-PCR showing downregulation of *Rab27a, Sytl2, Pdgfb*, and *Vwf* in ASPS17 treated with JQ1, with the expression of *Myh9, Neat1*, and *Atf4* remaining unchanged. * $P < 0.05$.

Figure 4

Identification of ASPSCR1-TFE3 targets by epigenomic CRISPR/dCas9 screening. a, Schematic illustration of the screening. ASPS17-specific super-enhancers and active enhancers positive for ASPSCR1-TFE3-binding peaks were selected for the construction of the gRNA library. The library consisting of 7716 gRNAs was inserted into the pLV-U6-gRNA-UbC-DsRed-P2A-Bsr lentivirus vector (library lentivirus). ASPS17 cells were serially transduced with lenti-Ef1a-dCas9-KRAB-Puro and library lentivirus. After selection by puromycin and blasticidin the cells were transplanted into the subcutaneous parts of nude mice. Tumors were obtained 4 weeks after transplantation, and tumor DNA samples were subjected to deep sequencing by MiSeq. Enhancers containing significantly decreased gRNA were identified and candidate target genes located within the same topologically associated domains (TADs) as enhancers were selected. After functional annotation of 56 genes, six genes were selected for *in vivo*

validation. **b,** A detailed view of the region around *Rab27a* for the dCas9-KRAB screen. Log_2 fold changes in gRNA abundance are shown. **c,** Hi-C showing topologically associated domains (TADs) at *Rab27a, Sytl2, Pdgfb*, and *Vwf* loci. The genetic lengths are labeled in each diagram. **d,** Volcano plot comparing the expression of 345 candidate genes between ASPS17 and ASPS-null cells. Dots labeled in red were determined as having a log_2 (fold change) of ≤ -1 in null cells and an adjusted *P* value ≤ 0.01 (Bonferoni correction). **e,** Growth curves of the transplanted tumors with ASPS17 cells indicating deleted genes. * $P < 0.05$. **f,** qRT-PCR of *RAB27A, SYTL2, PDGFB*, and *VWF* in human sarcoma patients. ASPS: alveolar soft part sarcoma (n = 7), SS: synovial sarcoma (n = 7), ES: Ewing sarcoma (n = 5), MLS: myxoid liposarcoma (n = 6), DFSP: dermatofibrosarcoma protuberance (n = 5), SFT: solitary fibrous tumor (n = 7). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. **g,** Immunostaining of RAB27A, SYTL2, and VWF in human ASPS. Scale bar, 50μm.

Figure 5

Intracellular trafficking of angiogenic factors is promoted by the Rab27a/Sytl2 axis. a, Secretion of Pdgfb and Gpnmb in conditional media of ASPS17 cells measured by ELISA. Marked suppression in secretion of both proteins by knockout of *Rab27a, Sytl2*, or *Pdgfb* is shown. sgNTC and ASPS null (Null) are used as positive and negative controls, respectively. Introduction of wild type Rab27a cDNA, with this effect being diminished by introducing the W73G mutant. * $P < 0.05$, ** $P < 0.01$. **b,** Induction of pericyte growth promotion by an ASPS17-conditioned medium was inhibited by *Rab27a, Sytl2,* or *Pdgfb* knockout and *ASPSCR1-TFE3* loss. Culture medium containing 0.5% FBS was used as negative control, and the addition of 5 ng/mL recombinant PDGFB promoted growth. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. **c,** Fluorescence recovery after photobleaching (FRAP) showing a marked delay in the trafficking of DsRed-labeled Pdgfb or Gpnmb by *Rab27a* knockout, *Sytl2* knockout, and ASPS-null cells. Representative FRAP images of ASPS17 cells are shown in the upper panels, and representative normalized traces of FRAP for each experiment are shown in the lower panels. Each point represents one image acquired every 5 min. RFP-tagged actin was used as a negative control. Recovery rates were corrected for internal photobleaching and background, and they were normalized to pre-photobleaching intensities. **d,** Vwf secretion in conditional media of ASPS17 cells measured by ELISA. Marked suppression of secretion of both proteins by *Vwf, Rab27a,* or *Sytl2* knockout is shown. sgNTC and ASPS null (Null) are used as positive and negative controls, respectively. Introduction of wild type human *RAB27A* cDNA, with this effect being diminished by introducing the W73G mutant. * $P < 0.05$, ** $P < 0.01$. **e,** Immunofluorescence showing intracellular localization of Vwf. Localization in peripheral areas of ASPS17 cells treated with negative-control sgRNA was observed, whereas perinuclear Vwf localization was indicated by *Rab27a* or *Sytl2* knockout, and ASPS-null cells (left). Intracellular Vwf distribution in each cell type was plotted as mean intensities of fluorescence at indicated distances from the nucleus (right). Scale bar, 10 μm. * $P < 0.05$. **f,** Suppressed secretion of Pdgfb, Gpnmb and Vwf by Nexinhib 20 measured using ELISA. * $P < 0.05$. **g,** FRAP showing significant delay in Pdgfb and Gpnmb trafficking by Nexinhib 20 treatment.

Figure 6

Induction of vascular network by co-culture of tumor spheroids, pericytes, and HUVECs in the microfluidic device. a, Schematic representation of the spheroid formation on the three-channel microfluidic device. **b,** Core shell spheroids composed of ASPS cells (red) and pericytes (green) and HUVECs (green) in the microfluidic device. The time-course fluorescent images of the *in vitro* angiogenesis using the core-shell ASPS spheroids (sgNTC, sgRab27a, sgSytl2, and null) on days 0, 1, 3, and 5. **c,** Quantification for surface coverage of angiogenic sprouts on day 5. The data are represented as the mean ± SD (n = 3; one-way ANOVA, $P < 0.05$). **d,** Representative images at day 5 clearly show the presence of angiogenic sprouts by CD31 staining. Scale bar, 200 μm. **e,** Immunohistochemical analysis of spheroids showing the expression of ASPSCR1-TFE3 (FLAG), PDGFRB, and CD31, for ASPS cells, pericytes, and HUVECs, respectively. Increase in PDGFRB-positive pericytes and CD31-positive HUVECs in sgNTC spheroids. Scale bar, 100 μm.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- ExtendedDataFigures.v1.docx
- SupplementaryTable1.xlsx
- SupplementaryTable2.xlsx
- SupplementaryTable3.xlsx
- SupplementaryTable4.xlsx
- SupplementaryTable5.xlsx
- SupplementaryTable6.xlsx
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