Pericyte dysfunction due to Shb gene deficiency increases B16F10 melanoma lung metastasis

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Abstract

Intravasation, vascular dissemination and metastasis of malignant tumor cells require their passage through the vascular wall which is commonly composed of pericytes and endothelial cells. We currently decided to investigate the relative contribution of these cell types to B16F10 melanoma metastasis in mice using an experimental model of host Shb gene (Src homology 2 domain-containing protein B) inactivation. Conditional inactivation of Shb in endothelial cells using Cdh5-CreERt2 resulted in decreased tumor growth, reduced vascular leakage, increased hypoxia and no effect on pericyte coverage and lung metastasis. RNAseq of tumor endothelial cells from these mice revealed changes in cellular components such as adherens junctions and focal adhesions by gene ontology analysis that were in line with the observed effects on leakage and junction morphology. Conditional inactivation of Shb in pericytes using Pdgfrb-CreERt2 resulted in decreased pericyte coverage of small tumor vessels with lumen, increased leakage, aberrant platelet-derived growth factor receptor B (PDGFRB) signaling and a higher frequency of lung metastasis without concomitant effects on tumor growth or oxygenation. Flow cytometry failed to reveal immune cell alterations that could explain the metastatic phenotype in this genetic model of Shb deficiency. It is concluded that proper pericyte function plays a significant role in suppressing B16F10 lung metastasis.

KEYWORDS
endothelial cells, melanoma, metastasis, PDGFRB, pericytes, SHB

INTRODUCTION

Metastasis is the major cause of death in malignant cancer disease. For tumor cells to reach the vascular circulation and metastasize, they must become invasive, transmigrate through the layer of pericytes/mural cells surrounding endothelial cells (EC) and penetrate the endothelial barrier. After dissemination, the tumor cells must at sites of metastasis again cross the vasculature before they can seed...
at permissive sites. Although endothelial leakage has received considerable attention as a factor contributing to metastasis, pericyte function has also been suggested to play a role in this context. Problems in assessing the relative contributions of EC and pericytes lie in the fact that these cell types are reciprocally interdependent and that platelet-derived growth factor receptor-β (PDGFRB) expressing tumor stromal cells affect the properties of the tumor cells themselves.

Melanomas are highly invasive and metastatic. Previously, their prognosis was poor but has recently improved markedly due to advances in immunotherapy. Despite tremendous therapeutic success, a number of cases remain refractory to treatment and thus improved understanding of the mechanisms of melanoma metastasis is warranted.

The Shb gene codes for Src homology-2 domain-containing protein B (SHB) which is an adapter protein downstream of several tyrosine kinase receptors such as vascular endothelial growth factor receptor-2 (VEGFR2) and PDGFRB. SHB has been found to play a role in various aspects of tumor biology. These include tumor angiogenesis and immune cell responses. Absence of SHB also influences the characteristics of myeloid and lymphoid leukemia. Of relevance to tumor angiogenesis, SHB is required for VEGF-induced vascular leakage and immune cell infiltration.

B16F10 melanomas grown in Shb+/− mice exhibit certain intricate and contradictory features. That study demonstrated reduced pericyte coverage of small patent venule-like vessels without detectable effects on tumor angiogenesis. Despite increased leakage, CD8α+ cell infiltration was reduced, an effect probably dependent on cell autonomous effects of Shb deficiency in immune cells. Although bone marrow transplantation experiments suggested that the main contribution to the increase in metastasis related to the host genotype (ie, primarily endothelial cells or pericytes), there could have been an immune cell contribution to the increase in metastasis as well, although that would have been of lesser importance.

We currently set out to elucidate the relative importance of Shb deficiency in endothelial cells (EC) and pericytes by usage of conditionally inactivated Shblox/lox in EC (Cdh5-CreERT2) and pericytes (Pdgfrb-CreERT2). Our findings suggest that pericyte dysfunction plays a major role in melanoma metastasis.

## What's new?

Intravasation, vascular dissemination, and metastasis of malignant tumor cells requires their passage through the vascular wall. The relative contributions of vascular wall endothelial cells and pericytes have been difficult to assess, however, due to the interdependence of these cell types.

Here, the authors demonstrate, by using a genetic model of Shb-gene inactivation in endothelial cells and pericytes, that pericyte dysfunction increases melanoma metastasis to the lung. Aberrant platelet-derived growth factor receptor B signaling in pericytes plays a key role in increasing metastasis. The findings highlight ameliorating pericyte dysfunction in malignant tumors as a strategy to prevent metastasis in malignant disease.

All animal experiments were approved by the local animal ethics committee at the Uppsala County Court (C103115/15).

## 2.2 | Tumor studies

All experiments were performed with mycoplasma-free cells. B16F10 (RRID:CVCL_0159 kindly provided by Dr L. Claesson-Welsh at Uppsala University) melanoma cells (2 × 10⁴) were injected subcutaneously in the subscapular region 7 days after the last tamoxifen injection. Mouse weight and tumor size (one tumor per mouse) were monitored and primary tumors were resected under anesthesia when the tumor size reached 0.5 to 1 cm³ at 14 or 16 to 19 days after tumor cell injection. Anesthesia was induced by 4% to 5% isofluorane and maintained at 2% during a 30-minute procedure. The mice were maintained for an additional 21 days before sacrifice (commonly, but not always, there was a tumor relapse deciding the end-point of the experiment). Macroscopically visible (near surface) lung metastases were then counted after separation of the lung into its lobes without tissue clearing.

## 2.3 | Cell isolation

Tumors and lungs were excised and digested with collagenase, and a single cell suspension was prepared as described previously. Alternatively, cell suspensions were prepared from spleens, local lymph nodes and blood as previously described. After lysis of red blood cells, the cell suspension was incubated with CD11b+ microbeads (Miltenyi Biotec, see Table S2 for all immunological reagents) for 20 minutes on ice. CD11b+ cells were eluted using MACS separation columns. The original flow-through suspension upon column application was collected, incubated with CD31-coated Dynabeads (Invitrogen, Waltham, Massachusetts) or...
microbeads, and further column purified as above for CD31+ cells. To enrich for pericytes, the cell suspension from lungs was incubated with biotin-anti-CD45 followed by anti-biotin microbeads. The flow-through, devoid of CD45+ cells purified on the magnetic separation columns, was collected and incubated with biotin-anti-CD80 plus anti-biotin microbeads to purify CD80+ cells predepleted of CD45+ cells by a second round of selection on a magnetic separation column.

2.4 | Immunofluorescence

Resected primary tumors were frozen on dry ice for immunofluorescence staining. Tumor sections (10 μm) were fixed in cold 4% PFA for 10 minutes, permeabilized in 0.2% Triton-X, blocked with 3% BSA and then stained with the following: rat anti-CD31, goat anti-VE-cadherin (vascular endothelial-cadherin, see Table S2 for specifications of reagents), rabbit anti-desmin and goat anti-fibrinogen. Donkey secondary antibodies were used for detection.

Confocal fluorescence images were acquired with a Zeiss LSM780 laser-scanning confocal microscope (Carl Zeiss Microscopy, LLC, Thornwood, New York). The percent area density of blood vessels, the fibrin spread area, hypoxic area relative values by HIF1α staining intensity and pericyte coverage were calculated with Image J by marking relevant areas (whole image field for CD31+ density and HIF1α intensity or for fibrinogen leakage CD31+ enclosed area or area containing surrounding fibrinogen positive staining) after which staining intensity was measured by integrated density.

2.5 | Semiquantitative real-time RT-PCR and RNAseq

Total RNA from tumors or magnetic cell sorting (MACS)-isolated cells was isolated using the RNeasy mini-kit (Qiagen, Hilden, Germany). EC (CD31+ cells after predepletion of CD11+ cells) RNA concentration and quality were assessed using the Agilent 2100 Bioanalyzer, and One-step quantitative real-time RT-PCR was performed with QuantitectTM SYBRGreen RT-PCR-kit (204243, Qiagen, Hilden, Germany) on a LightcyclerTM real-time PCR machine (Lightcycler 2.0; Roche, Mannheim, Germany). Primer sequences are listed in Table S3.

For RNAseq, paired-end sequences were generated using HiSeq2500 high output mode or with Novaseq S1 on libraries made by TruSeq stranded total RNA kit with RiboZero depletion. RNA sequencing was performed at the SNP&SEQ platform, Uppsala University, Sweden. The sequence reads were aligned to the Ensembl mouse gene assembly (GRCm38) using Tophat2 software (version 2.1.1), and FPKM quantification was performed using the Cufflinks tool (version 2.2.1).

2.6 | Flow cytometry

Flow cytometry by fluorescence-activated cell sorting (FACS) was performed on spleen, local lymph nodes, blood and tumors as described. In addition, CD4+/FoxP3+/PD-1+ cells were identified by including a PD-1 pacific blue antibody together with the CD4 and FoxP3 antibodies for FACS analysis. Neutrophilic or monocytic/macrophagic myeloid-derived suppressor cells (nMDSC, mMDSC) were identified as CD11b+/Ly6G+/CD15+ or CD11+/Ly6C+/CD14+ cells, respectively. A list of antibodies currently used is supplied in Table S2.

2.7 | Statistics

Statistical analyses were performed using GraphPad Prism 7.0 Software (GraphPad Software, Inc., La Jolla, California). Tests for comparisons of chance differences between the experimental groups are given in the figure legends. Each observation is based on one tumor-bearing mouse.

3 | RESULTS

3.1 | Tumor growth and metastasis

B16F10 melanoma cells express Shb and tumor cell Shb mRNA was not altered in the current study. Instead, the Shb gene was conditionally deleted in host EC and pericytes. Shb gene inactivation in EC (tamoxifen-treated Shbflx/flox/Cdh5-CreERT2 mice) resulted in reduced B16F10 melanoma growth (Figure S1A). When primary tumor resection was performed on day 14 after cell injection, metastases were infrequent (one wild-type mouse out of nine had two metastases and the other mice had none whereas among five Shbflx/flox/Cdh5-CreERT2 mice none had metastases). For this reason, the time period to primary tumor resection was extended to 16 to 19 days after cell injection depending on when the primary tumor reached the size limit (1 cm³) of the study protocol (Figure S1B). Shb EC-deficient mice exhibited a similar number of metastases as that of wild-type controls although a slight trend (P = .09) toward decreased metastasis was noted (Figure S1B,C). When the Shb gene was inactivated in pericytes (tamoxifen-treated Shbflx/flox/Pdgfrb-CreERT2 mice), no effects on tumor growth were observed whereas lung metastasis was increased when primary tumor resection was performed at 14 days after cell injection (Figure 1A-C).

The data suggest that host Pdgfrb expressing cells play a significant role in melanoma metastasis.

3.2 | Tumor vasculature

Neither the EC-specific, nor the pericyte-specific Shb inactivation, exerted an effect on melanoma vascular density (Figure S2). However, desmin coverage of small patent vessels with a venule-like appearance was significantly reduced (Figure 2) in tumors grown on mice with Shb-deficient pericytes (tamoxifen-treated Shbflx/flox/Pdgfrb-CreERT2 mice). Vascular leakage, defined as the ratio extravascular over
intravascular fibrinogen, was reduced in tumors grown on mice with EC-deficient Shb (tamoxifen-treated Shb\textsuperscript{floxflox}/Cdh5-CreERT2 mice) whereas tumor vascular leakage in mice with Shb-deficient pericytes was increased (Figure 3). To obtain a molecular mechanistic explanation for the reduced pericycle coverage, we isolated lung CD80+/CD45\textsuperscript{−} cells. In lung cells, CD80 is expressed in pericytes, CD31\textsuperscript{+} cells and CD45\textsuperscript{+} cells. CD80+/CD45\textsuperscript{−} cells contain a high percentage desmin positive cells (Figure 4A). Flow cytometry of CD80+/CD45\textsuperscript{−} cells exhibited that more than 50% of this cell population was CD31 negative and thus we considered it to be pericycle enriched (Figure 4B). The total yield of this cell population was 7000 to 10,000 cells per mouse and slightly lower in the tamoxifen-treated Shb\textsuperscript{floxflox}/Pdgfrb-CreERT2 mice (Figure 4C). We have previously shown that SHB operates downstream of PDGFRB\textsuperscript{35} and thus we decided to investigate the importance of PDGFRB signaling for the currently observed reduced desmin coverage by assessing gene expression immediately after isolation to reduce the risk of phenotypic alterations in this cell population during ex vivo maintenance. Tiparp is a poly ADP-ribose polymerase, Sgpl1 a sphingosine-1-phosphate lyase and Arid5b an AT-rich interaction domain DNA binding protein according to GeneCards (https://www.genecards.org). The very low yield of pericycle enriched CD45\textsuperscript{−}/CD80\textsuperscript{+} cells precluded more extensive analysis (RNAseq) of these cells.

The data suggest that increased B16F10 tumor metastasis indeed depends on an inadequate pericycle function and that Shb gene inactivation in pericytes confers a prometastatic phenotype by interfering with PDGFRB signaling.

### 3.3 Immune cell alterations

We previously reported immune cell alterations in B16F10 melanomas as a consequence of host Shb gene deletion (Shb \(+/−\)) that
potentially could explain the metastatic Shb deficient phenotype.\textsuperscript{20} Using the $\text{Shb}^{\text{flox/flox}/\text{Cdh5-CreERt2}}$ transgenic mouse, we detected certain immune cell alterations that resulted from conditional deletion of Shb in hematopoietic cells using this Cre transgene.\textsuperscript{27} Shb deficiency in host EC (tamoxifen-treated $\text{Shb}^{\text{flox/flox}/\text{Cdh5-CreERt2}}$ mouse) presently did not confer increased melanoma metastasis and thus, the reported immune cell alterations\textsuperscript{20,27} were considered irrelevant for the metastatic phenotype. However, increased metastasis due to Shb deficient pericytes could potentially be related to immune cell

**FIGURE 2** Desmin coverage in B16F10 melanomas. Tumor sections from the two wild-type control groups of mice, or mice with Shb deficiency in EC or pericytes were stained for CD31 and desmin and the measured length of desmin staining was divided by the total vascular circumference. Values are percentages as means ± SEM for 6 to 7 mice (tumors) each genotype. Representative images are shown. **\textsuperscript{26}P < .01 when compared to a Student’s t test against corresponding wild-type [Color figure can be viewed at wileyonlinelibrary.com]**

**FIGURE 3** Vascular leakage in B16F10 melanomas. Tumor sections from the two wild-type control groups of mice, or mice with Shb deficiency in EC or pericytes were stained for CD31 and fibrinogen to determine leakage as the ratio extravasated/intravascular fibrinogen. Means ± SEM for 5 to 7 mice (tumors) are shown. *\textsuperscript{P < .05 when compared against corresponding control with a Student’s t test [Color figure can be viewed at wileyonlinelibrary.com]**
alterations because of either an unexpected conditional Shb deletion in certain immune cells by Pdgfrb-CreERt2 or by immune cell effects secondary to altered pericyte function. Thus, immune cell profiling of spleen, local lymph nodes and tumors was performed, determining CD4+, CD8a+, CD4/FoxP3+/-, CD4/FoxP3/IL-35+, CD4+/FoxP3−/PD-1+, CD11b+, CD11b/CD8a+, CD11c+, CD11c/CD8a+, nMDSC and mMDSC cells. None of these cell populations was altered (Figure S3) suggesting that the metastatic phenotype was unlikely to be related to immune cell alterations as a consequence of Shb gene inactivation in pericytes.

### 3.4 RNAseq of tumor CD31+ RNA

To investigate further the observed decreased vascular leakage in melanomas grown on mice with EC-specific Shb deficiency, EC from B16F10 melanomas grown on wild-type mice or mice with Shb conditionally inactivated in EC (tamoxifen-treated Shbflox/flox/Cdh5-CreERt2) were isolated and subjected to RNAseq (Table S1). Changes in gene expression that were significant by a t-test without correction for multiple comparisons were analyzed by gene ontology analysis (GO) at the Toppgene website (https://toppgene.cchmc.org/enrichment.jsp).
Biological processes that were significantly enriched were, among others, related to the vasculature, circulation, vascular function and vascular development (Table S1). Enriched cellular components were junctions (adherens junctions) and focal adhesions (Table 1) by GO analysis. These are both key components regulating vascular leakage and thus provide a structural basis for the reduction of tumor leakage shown under these conditions (Figure 3). Consequently, we investigated the morphology of adherens junctions by staining tumors for VE-cadherin.

**FIGURE 5** Adherens junctions in B16F10 melanomas. Tumors grown on wild-type (wild-type/Cdh5-CreERT2 or wild-type/Pdgfrb-CreERT2) or mice with Shb deficient EC (Shb<sup>lox/lox</sup>/Cdh5-CreERT2) or pericytes (Shb<sup>lox/lox</sup>/Pdgfrb-CreERT2) mice were stained for VE-cadherin. The lengths of adherens junctions in vascular structure were measured and normalized to the length of that vascular structure. Values are ratios as means ± SEM for 3 to 4 mice (tumors) in each genotype and * indicates $P < .05$ when compared to wild-type using a Student’s t test. Representative images are shown. The corresponding images with merged CD31 signal can be seen in Figure S5. For the two Cdh5-CreERT2 groups, 16 to 20 images were analyzed each and for the Pdgfrb-CreERT2 groups, five images were analyzed each [Color figure can be viewed at wileyonlinelibrary.com]

| Wild-type/Cdh5-CreERT2 | Shb<sup>lox/lox</sup>/Cdh5-CreERT2 |
|------------------------|-----------------------------------|
| ![Image](image1.png)   | ![Image](image2.png)             |

| Wild-type/Pdgfrb-CreERT2 | Shb<sup>lox/lox</sup>/Pdgfrb-CreERT2 |
|--------------------------|-------------------------------------|
| ![Image](image3.png)     | ![Image](image4.png)               |

Relative length of adherens junctions in vascular structures in B16F10 melanomas

![Graph](graph.png)

| Wild-type/Cdh5-CreERT2 | Shb<sup>lox/lox</sup>/Cdh5-CreERT2 |
|------------------------|-----------------------------------|
| ![Image](image1.png)   | ![Image](image2.png)             |

| Wild-type/Pdgfrb-CreERT2 | Shb<sup>lox/lox</sup>/Pdgfrb-CreERT2 |
|--------------------------|-------------------------------------|
| ![Image](image3.png)     | ![Image](image4.png)               |

**50 μm**

VE-cadherin
B16F10 tumor hypoxia. Tumors grown on wild-type mice (wild-type/\textit{Cdh5-CreERt2} or wild-type/\textit{Pdgfrb-CreERt2}) or mice with \textit{Shb} deficient EC (\textit{Shbflox/flox/Cdh5-CreERt2}) or pericytes (\textit{Shbflox/flox/Pdgfrb-CreERt2}) mice were stained for the hypoxia marker HIF1a. Staining intensity was quantified by ImageJ and presented as arbitrary units. Means ± SEM for five mice (tumors) each genotype is given. * indicates \( P < .05 \) when compared against corresponding control using a Student’s \( t \) test.

[Color figure can be viewed at wileyonlinelibrary.com]
and observed an increased ratio VE-cadherin positive length per unit vessel length in Shb deficient EC (Figure 5) which is in line with the functional (leakage) and structural data (RNAseq). Four genes belonging to the GO component “junctions” of which three also belong to “focal adhesions” were confirmed to be increased by qPCR in CD31+ RNA from EC with Shb conditionally deleted (Figure S4). The increase in Cdh5 mRNA in Shb deficient tumor EC assessed by qPCR was too small to achieve statistical significance (125% of wild-type, \( P = .38 \)).

RNAseq data analysis supports a role of SHB in EC junction and focal adhesion function/morphology correlating with decreased leakage.

3.5 | Tumor oxygenation

Due to dysfunctional pericytes as a consequence of Shb deficiency, tumor oxygenation could decrease, thus causing a metastatic phenotype.6 Consequently, we stained tumor sections of the genotypes (Figure 6) for hypoxia-inducible factor 1 alpha (HIF1a). HIF1a staining was elevated in tumors grown on mice with Shb deficient EC whereas it was comparable in the other three groups. The poor oxygenation in Shb-deficient EC is a likely consequence of a vascular phenotype that cannot be detected by measuring vascular density but could relate to junction morphology. However, it does not correlate with metastasis, excluding this as a factor of relevance in the present context.

4 | DISCUSSION

Our present findings support the notion that proper pericyte function is highly relevant for reducing melanoma metastasis. Previous studies have implicated vascular leakage over the endothelial barrier as the main component responsible for tumor cell dissemination into the vasculature.5 This is probably a consequence of tumor-induced release of factors causing disassembly of endothelial junctions.2,4,37 Our findings demonstrate that decreased pericyte coverage correlates with metastasis in the current experimental models of conditional Shb gene inactivation in EC or pericytes, strongly implicating a role of pericytes in this process.

The role of the immune system in this process appears to be of minor importance, despite our previous finding of reduced CD8a+ cell infiltration in melanomas grown on Shb deficient mice.20 By flow cytometry, we determined T helper (CD4+) and killer cells (CD8a+), T regulatory cells (CD4+/FoxP3+ and CD4+/FoxP3+/IL-35+), nonconventional inhibitory CD4+/FoxP3+/PD-1+ cells, CD11b+ macrophages, CD11c+ dendritic cells, CD11b+/CD8a+ and CD11c/CD8a+ phagocytic myeloid cells21 and MDSC.38 Infiltration of the latter into tumors has been shown to be regulated by pericytes.32 The lack of detectable changes suggests that these cell populations are of little relevance to the metastatic phenotype in the current experimental models. In addition, we obtained no evidence suggesting that impaired tumor oxygenation due to dysfunctional pericytes could explain the increased rate of lung metastasis. On the contrary, increased hypoxia, determined as intensity of HIF1a staining, was observed in tumors grown on Shb-deficient EC. These showed no increase in metastasis and exhibited signs of vessel normalization as indicated by decreased vascular leakage.39 Angiogenesis inhibition may similarly cause decreased vascular leakage.39

In our previous study using Shb +/- mice,20 all host cells would have exhibited Shb deficiency, making it difficult to establish an exact cause of the leakage/metastasis phenotype. Currently, by conditionally deleting Shb in EC and pericytes separately, we clearly demonstrate a significant role of pericytes in this process. However, due to the interdependence of EC and pericytes, we cannot exclude the possibility that some of the effects presently observed are consequences of pericyte-derived cues affecting EC function.

We have previously shown that SHB operates downstream of PDGFRB35 and the observed reduction in pericyte Sgl1, Arid5b and Tiparp gene expression as a consequence of Shb deficiency supports the notion that aberrant PDGFRB signaling in the absence of Shb confers a phenotype that increases leakage and metastasis. Indeed, in a model of α6-integrin deficiency in pericytes, pericyte coverage of tumor vessels was reduced and leakage increased, effects attributed to decreased PDGFRB signaling.40 Metastasis remained unaffected in that study but in the wild-type situation, metastasis was higher than presently observed, suggesting that variations in the experimental conditions might explain the discrepancy. The combined data suggest that aberrant PDGFRB signaling confers poor pericyte function and that this may have an impact on the ability of pericytes to suppress metastasis.

Shb deficiency in EC reduced leakage and primary tumor growth. This finding is in agreement with previously observed effects in the Shb knockout mouse18,19 and relates to decreased SHB-dependent VEGFR2 signaling19,41,42 causing reduced leakage.19,25,26 Shb deficient EC display elevated basal extracellular signal-regulated kinase (ERK), focal adhesion kinase (FAK) and Rac1 activities with reduced responsiveness to VEGFA19,41 leading to increased VEGFR2/VE-cadherin association in response to VEGFA relative that of the wild-type cells.51

EC and pericytes are interdependent8-10 and tumors may disrupt normal EC-pericyte homeostasis by releasing factors that serve an advantageous role for tumor expansion and metastasis. VEGFA is a major player in this context2 and tumor overproduction of this factor may cause leaky vessels with poor pericyte coverage.39 The present study demonstrates that poor pericyte coverage due to cell autonomous effects in this cell type by itself causes increased leakiness and metastasis. This implicates ameliorating pericyte dysfunction in malignant tumors as a strategy to prevent metastasis in malignant disease under certain conditions.

ACKNOWLEDGEMENTS

We gratefully acknowledge Mihaela M. Martis at the National Bioinformatics Infrastructure Sweden (NBIS) who contributed to the bioinformatical analysis of three samples. We also acknowledge Carlos Villacorta Martin at the Center for Regenerative Medicine at Boston University for helpful advice. The project was supported by grants...
from the Swedish Research Council (2016-08015 and support for SNP&SEQ project 2017040), the Swedish Cancer Foundation (150880), the family Ermfors Fund, Exodiab and the Knut och Alice Wallenbergs Stiftelse (SNP&SEQ). The SNP&SEQ platform, Uppsala Universitet, Sweden is a part of the National Genomics Infrastructure, Sweden, supported by the Swedish Research Council and the Knut och Alice Wallenbergs Stiftelse. The computations were performed on resources provided by SNIC through Uppsala Multidisciplinary Center for Advanced Computational Science (UPPMAX) under Projects SNIC 2017/7-240 and sllstore2017069.

CONFLICT OF INTEREST
The authors have no competing interests to declare.

DATA ACCESSIBILITY
The raw and processed single-cell RNA sequencing data were submitted to the NCBI GEO database, under the series accession number GSE145201 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE145201). Additional data can be obtained from the authors on request.

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SUPPORTING INFORMATION
Additional supporting information may be found online in the Supporting Information section at the end of this article.

How to cite this article: He Q, Li X, He L, Li Y, Betsholtz C, Welsh M. Pericyte dysfunction due to Shb gene deficiency increases B16F10 melanoma lung metastasis. Int. J. Cancer. 2020;147:2634–2644. https://doi.org/10.1002/ijc.33110