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Infantile hemangioma-derived stem cells and endothelial cells are inhibited by class 3 semaphorins

Hironao Nakayama [a, b, d, 1], Lan Huang [a, b, 1], Ryan P. Kelly [a], Clara R.L. Oudenaarden [a], Adelle Dagher [a, b], Nicole A. Hofmann [a, b], Marsha A. Moses [a, b], Joyce Bischoff [a, b, **], Michael Klagsbrun [a, b, c, *]

[a] Vascular Biology Program, Boston Children's Hospital, Harvard Medical School, Boston, MA 02115, USA
[b] Department of Surgery, Boston Children's Hospital, Harvard Medical School, Boston, MA 02115, USA
[c] Department of Pathology, Boston Children's Hospital, Harvard Medical School, Boston, MA 02115, USA
[d] Division of Cell Growth and Tumor Regulation, Proteo-Science Center, Ehime University, Toon, Ehime 791-0295, Japan

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ABSTRACT

Class 3 semaphorins were discovered as a family of axon guidance molecules, but are now known to be involved in diverse biologic processes. In this study, we investigated the anti-angiogenic potential of SEMA3E and SEMA3F (SEMA3E&F) in infantile hemangioma (IH). IH is a common vascular tumor that involves both vasculogenesis and angiogenesis. Our lab has identified and isolated hemangioma stem cells (HemSC), glucose transporter 1 positive (GLUT1^{+}) endothelial cells (designated as GLUT1^{sel} cells) based on anti-GLUT1 magnetic beads selection and GLUT1-negative endothelial cells (named HemEC). We have shown that these types of cells play important roles in hemangiogenesis. We report here that SEMA3E inhibited HemEC migration and proliferation while SEMA3F was able to suppress the migration and proliferation in all three types of cells. Confocal microscopy showed that stress fibers in HemEC were reduced by SEMA3E&F and that stress fibers in HemSC were decreased by SEMA3F, which led to cytoskeletal collapse and loss of cell motility in both cell types. Additionally, SEMA3E&F were able to inhibit vascular endothelial growth factor (VEGF)-induced sprouts in all three types of cells. Further, SEMA3E&F reduced the level of p-VEGFR2 and its downstream p-ERK in HemEC. These results demonstrate that SEMA3E&F inhibit IH cell proliferation and suppress the angiogenic activities of migration and sprout formation. SEMA3E&F may have therapeutic potential to treat or prevent growth of highly proliferative IH.

1. Introduction

Infantile hemangioma (IH) is the most common tumor of infancy [1,2]. It displays a unique life cycle that can be divided into three major phases: proliferating, involuting and involuted [3]. We have identified CD133-positive cells in the proliferating IH as hemangioma stem cells (HemSC) [4]. They are able to differentiate into endothelial cells (EC), pericytes and adipocytes *in vitro* and *in vivo*. When mixed in Matrigel and injected subcutaneously into nude mice, HemSCs form hemangioma-like blood vessels and recapitulate the life cycle of IH [3,4].

Glucose transporter 1 (GLUT1)-positive ECs are a hallmark of IH, and our recent data shows that these cells diminish as the tumor involutes. GLUT1-positive ECs in IH behave as facultative stem cells. They function as bona fide EC *in vivo* but when removed from the tumor and cultured *in vitro* as a purified cell population (called GLUT1^{sel} cells), they display stem cell-like properties. The GLUT1^{sel} cells are clonogenic and can undergo multi-lineage differentiation [5]. GLUT1-negative ECs, unlike from the GLUT1-positive ECs, consistently exhibit endothelial phenotype *in vitro* [5]. They were

Abbreviations: SEMA3, class 3 semaphorin; SEMA3E&F, semaphorin 3E and 3F; IH, infantile hemangioma; HemSC, hemangioma stem cells; GLUT1, glucose transporter 1; EC, endothelial cell; HemEC, hemangioma endothelial cell; VEGF, vascular endothelial growth factor; VEGFR2, vascular endothelial growth factor receptor 2; NRP2, neuropilin 2.

* Corresponding author. Boston Children's Hospital, Harvard Medical School, Karp Family Research Laboratories 12.210, 1 Blackfan Circle, Boston, MA 02115, USA.
** Corresponding author. Boston Children's Hospital, Harvard Medical School, Karp Family Research Laboratories 12.212, 1 Blackfan Circle, Boston, MA 02115, USA.
E-mail addresses: joyce.bischoff@childrens.harvard.edu (J. Bischoff), michael.klagsbrun@childrens.harvard.edu (M. Klagsbrun).

[1] These authors contributed equally to this work.

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previously designated as hemangioma endothelial cells (HemEC) [6]. Noticeably, HemECs constitutively express phosphorylated vascular endothelial growth factor receptor 2 (VEGFR2) and low levels of VEGFR1 in comparison with normal EC from newborn foreskin [7].

Although benign, IH ranges in severity from cutaneous discolorations to massive, life-threatening lesions. Despite advances in treatments for children with IH, drugs such as corticosteroid and propranolol are accompanied by side effects [8,9]; for example, the hemangioma starts to regrow in 10–20% of cases when propranolol is stopped and not all patients respond well [10,11]. There is still a pressing need for improved therapies that will shorten the treatment duration or, ultimately, prevent problematic IH from forming.

Class 3 semaphorins (SEMA3s), a family of seven members of axon guidance molecules (SEMA3A-G), have been well studied in our lab [12,13]. They first were discovered as mediators of neuronal guidance during neuronal development. More recently, their critical roles in the vascular system and in tumor biology have been recognized [12,13]. To elicit the regulatory signaling in the cells, SEMA3s are required to bind to neuropilin receptors and to form a complex with Plexin family receptors. Semaphorin 3F (SEMA3F) negatively regulates tumor cell and EC migration *in vitro* and tumor angiogenesis and metastasis *in vivo* [14,15]. It binds to neuropilin 2 (NRP2) and Plexin A1 receptors and induces signaling that causes cytoskeletal collapse in tumor cells and ECs [15]. Semaphorin 3E (SEMA3E) can directly bind to Plexin D1 receptor without NRPs [16] and can also cause cytoskeletal collapse, thus inhibiting EC sprouting and adhesion [17,18].

In our previous studies, NRP2 and Plexin D1 were significantly upregulated during HemSC/GLUT1^{sel}-to-EC differentiation [5,19], suggesting SEMA3s may gain the ability to influence IH growth as endothelial differentiation occurs. Therefore, we set out to understand, for the first time, whether and how SEMA3s, particularly SEMA3E and SEMA3F (SEMA3E&F), affect HemSC, GLUT1^{sel} cell and HemEC behavior and thus modulate IH growth. We demonstrated that SEMA3E&F inhibit the angiogenic activity of HemSC, GLUT1^{sel} cells and HemEC, suppressing cell migration and proliferation as well as VEGF-A-induced sprouting. In addition, SEMA3E&F reduced actin stress fibers in IH cells, leading to cytoskeletal collapse and loss of cell motility. In particular, in HemEC, SEMA3E&F noticeably inhibited p-VEGFR2 and p-ERK. In summary, our data show that SEMA3E&F can suppress the angiogenic ability of IH-derived cells and thus may potentially inhibit IH growth, which suggests a therapeutic application of SEMA3E&F in the treatment and prevention of highly proliferative IH.

2. Materials and methods

2.1. Antibodies & ELISA

The following antibodies were purchased from Cell Signaling Technology: rabbit polyclonal anti-phospho-ERK1/2 antibody (#9101); mouse monoclonal anti-ERK1/2 antibody (#4696); rabbit polyclonal anti-Plexin A1 antibody (#3813); rabbit monoclonal anti-phospho-VEGFR2 antibody (#2478); rabbit monoclonal anti-VEGFR2 antibody (#2479). The goat polyclonal anti-Plexin D1 antibody (AF4160) was purchased from R&D Systems. The mouse monoclonal anti-NRP2 antibody (sc-13117) was purchased from Santa Cruz Biotechnology. The rabbit anti-VE-cadherin antibody (HPA030562) and mouse monoclonal anti-β-actin antibody (AC-15) were from Sigma–Aldrich. Human VEGF Quantikine ELISA Kit (DVE00) was obtained from R&D Systems.

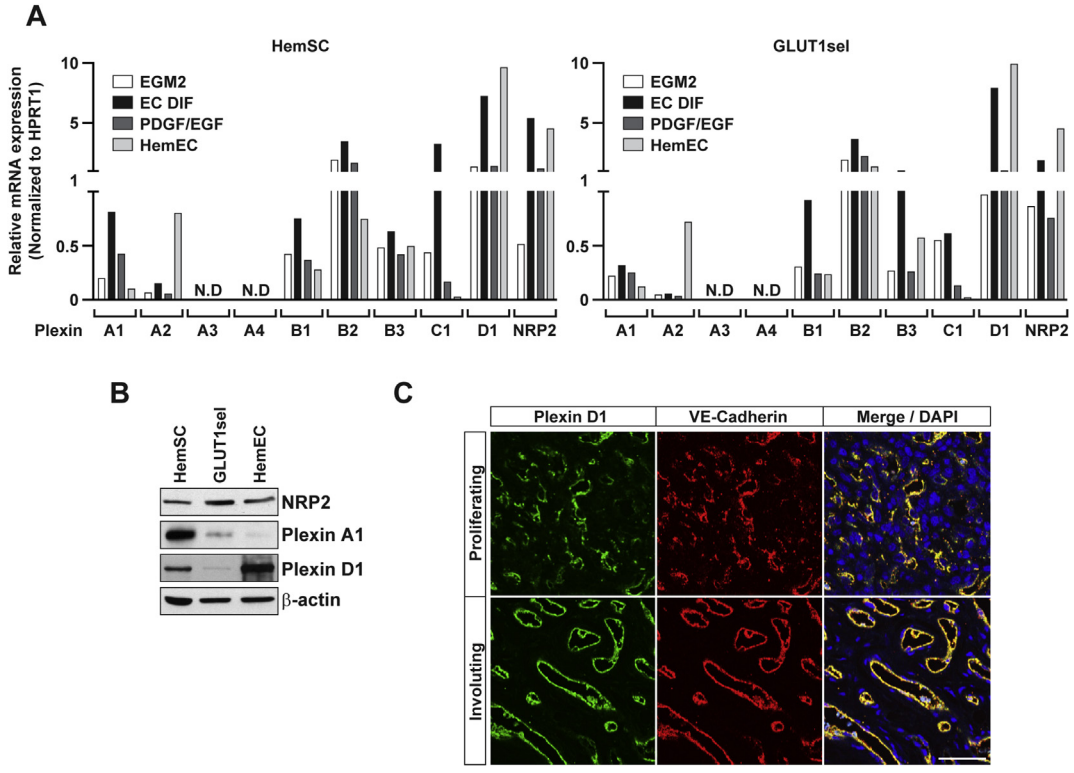

Fig. 1. Class 3 semaphorin receptors expressed on IH cells. A, HemSC and GLUT1^{sel} cells were cultured for 5 days in EGM-2, EC differentiation media or PDGF/EGF (negative control) media. Nine Plexins and NRP2 mRNA expression were analyzed by qPCR. mRNA levels were normalized to HPRT1 mRNA. N.D, not detected. B, Western blot for NRP2, Plexin A1 and Plexin D1 protein expression in HemSC, GLUT1^{sel} and HemEC. β-actin served as loading control. C, Expression of Plexin D1 in IH tissue. Immunofluorescence staining for Plexin D1 (green) and VE-cadherin (red) in proliferating (6 months) and involuting (31 months) IH tumor sections. Nuclei were counterstained with DAPI (blue). The scale bar indicates 50 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

2.2. Cell isolation and culture

HemSC (IH specimen #124, 125, 135), GLUT1^{sel} cells (IH specimen #159, 163, 164) and HemEC (IH specimen #159, 163, 17b) were isolated and maintained in our lab as previously described [4,5,7]. They were cultured on 1%-gelatin-coated plates in EBM-2 medium supplemented with EGM-2 SingleQuot (Lonza) and 10% fetal bovine serum in 5% CO_2 at 37 °C.

2.3. EC differentiation

To induce endothelial differentiation, HemSC and GLUT1^{sel} cells were cultured for 5 days in serum-free EBM-2 medium containing 1x insulin-transferrin-selenium, 1x linoleic acid-albumin, 100 μM ascorbic acid 2-phosphate, 1 μM dexamethasone. The same media with 10 ng/ml epidermal growth factor (EGF) and 10 ng/ml platelet derived growth factor (PDGF)-BB served as a control,

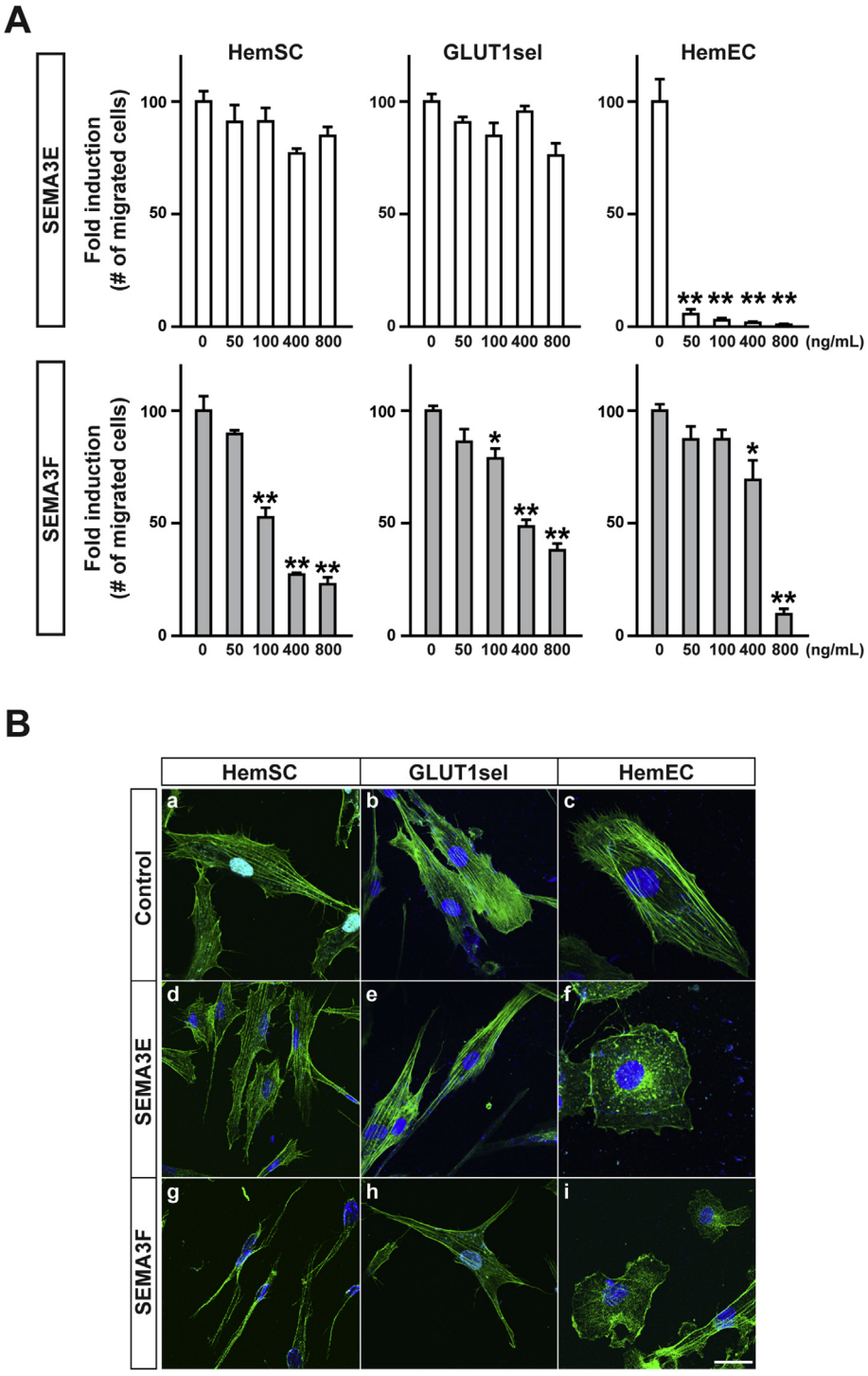

Fig. 2. Inhibitory effect of SEMA3E and SEMA3F on IH cell migration and cytoskeletal stress fibers. A, Cells were treated with SEMA3E (top) or SEMA3F (bottom) at the indicated dose and assessed for migration through gelatin-coated transwells. Data represent the mean ± SD, $^{*}p < 0.05$, $^{**}p < 0.01$ vs. untreated group. B, Cells were treated with 800 ng/ml of SEMA3E or SEMA3F. After 45 min, cells were fixed stained with Alexa Fluor 488-conjugated Phalloidin and Hoechst and observed by confocal microscopy. The scale bar indicates 10 μm.

maintaining cells without differentiation, as shown previously [4,5].

2.4. Immunohistochemistry

Formalin-fixed, paraffin-embedded tissue sections (5 μm) of Hem 137 and Hem I-84 were deparaffinized and either directly stained with hematoxylin and eosin (H&E) or immersed in a retrieval solution (Citrate-EDTA buffer: 10 mM Citric Acid, 2 mM EDTA, 0.05% Tween-20, pH 6.2) for 20 min at 95 °C–99 °C. Sections were blocked for 30 min in 5% serum and stained with anti-human VE-cadherin and anti-human Plexin D1. For immunofluorescence staining, sections were incubated with appropriate FITC or Texas Red conjugated secondary antibody (Vector Lab). All slides were mounted using DAPI (Molecular Probe, Eugene) as a nuclear marker. Images were acquired using a Leica TCS sp2 Acousto-Optical Beam Splitter confocal system equipped with a DMIRE2 inverted microscope camera (Leica Microsystems).

2.5. Migration

Migration assays were performed in Transwells (Corning Glass) with an 8.0-μm pore size coated with 1% gelatin. Cells (2.5×10^4) were seeded on the semipermeable membrane of the upper Transwell compartment. EMB-2 containing 1% fetal bovine serum (FBS, Denville Scientific, Inc.), SEMA3A and SEMA3F were added to the lower wells. Cells then were allowed to migrate for 18–20 h at 37 °C. Migrated cells were fixed and stained with Diff-Quick cell staining kit (Dade Behring, Inc.), and four fields were counted by phase microscopy.

2.6. Proliferation

Cells (1.0×10^4) were seeded in gelatin-coated 48-well plates with EGM-2. The next day, cell numbers were counted as a baseline (at 0 h). SEMA3E and SEMA3F were added twice a day in a concentration of 800 ng/ml each time. MEK-inhibitor U0126 (10 μM, EMD Millipore) was added once at time point 0 h. Cell number was counted at the indicated time points.

2.7. Collapse

Cells were cultured in 0.5% FBS/EBM-2 medium and were treated with 800 ng/ml of SEMA3E or SEMA3F for 45 min. Cells were fixed with 4% paraformaldehyde (PFA), followed by permeabilization with 0.05% Triton X-100 in phosphate buffered saline (PBS). F-actin stress fibers were stained with Alexa Fluor 488 Phalloidin and nuclei were stained with Hoechst.

2.8. Spheroids

Cells were suspended and aggregated overnight to form cellular spheroids (500 cells/spheroid) in a hanging drop of EGM-2 supplemented with methylcellulose. The next day, spheroids were collected and embedded into collagen gels treated with VEGF-A (15 ng/ml) and with or without SEMA3E or SEMA3F (800 ng/ml). The cumulative length of sprouts that had grown out of each spheroid was measured using NIH ImageJ software.

2.9. Quantitative real-time polymerase chain reaction (qPCR)

Total cellular RNA was isolated with the RNeasy Micro extraction kit (Qiagen). Reverse transcription of RNA was performed with SuperScript II Transcriptase (Invitrogen), according to the manufacturer's protocol. Quantitative Real-time Polymerase Chain Reaction (qPCR) was performed using FastStart Universal SYBR Green Master (Roche). Amplification was carried out in an ABI 7500 (Applied Biosystems). A relative standard curve of each gene amplification was generated to determine the amplification efficiency. Hypoxanthine phosphoribosyltransferase 1 (HPRT1) was used as a housekeeping gene expression reference. The qPCR primers used in the present study are listed in Supplementary Table 1.

2.10. Statistical analysis

All assays were independently performed three times. The results are represented as mean ± SD. Analysis of variance (ANOVA) with Bonferroni post-hoc test was used for multiple comparisons. $p < 0.05$ was considered statistically significant.

3. Results and discussion

Several studies have demonstrated that the association of SEMA3s with their receptors can inhibit tumor growth and angiogenesis [12,13]. Previously, NRP2 was shown to be expressed in IH [19,20]; however, the other SEMA3 receptors, including Plexin family receptors, have not been studied. By qPCR analysis, all receptors tested were detectable at the mRNA level in HemSC and GLUT1^{sel} cells, except for Plexin A3 and A4 (Fig. 1A). The expression of these Plexin receptors was increased to varying levels in HemSC and GLUT1^{sel} cells subjected to an EC differentiation protocol (Fig. 1A). NRP2 (SEMA3F receptor) and Plexin D1 (SEMA3E receptor) were noticeably increased in HemSC/GLUT1^{sel} undergoing endothelial differentiation, to levels similar to those in HemEC. The expression of Plexin A1, A2 and of the Plexin B family were changed during endothelial differentiation, but these alterations were not consistent in HemSC versus GLUT1^{sel} cells. Plexin C1 was induced during HemSC/GLUT1^{sel}-to-EC differentiation, but its expression in HemEC was negligible. Thus, we focused on NRP2 and Plexin D1 and examined protein levels in HemEC compared to HemSC and GLUT1^{sel} cells in normal growth conditions. NRP2 was expressed in all three types of cells (Fig. 1B). Plexin D1 was dominantly expressed in HemEC, which is consistent with immunohistochemistry: Plexin D1 was detected along the endothelium in IH (Fig. 1C). In contrast, Plexin A1 (also a receptor for SEMA3F) was detected in HemSC, but minimally expressed in GLUT1^{sel} cells and HemEC. These data showed the expression of SEMA3E and SEMA3F receptors in IH-derived cells, indicating that SEMA3s may affect IH cellular properties.

SEMA3E&F are potent migration inhibitors of tumor cells and EC [15,17,21]. For example, SEMA3E-induced EC collapse results in the loss of focal adhesions and disarrangement of the actin cytoskeleton [15,22]. First, we examined the dose-dependent effects of SEMA3E&F on the migration of IH-derived cells (Fig. 2A). Migration of HemECs was 90% inhibited by SEMA3E at concentrations as low as 50 ng/ml. In contrast, SEMA3E had no effect on the migration of HemSC and GLUT1^{sel} cells under the same experimental conditions (Fig. 2A, upper panel). SEMA3F was able to suppress HemEC migration only at high concentrations (>400 ng/ml) and to inhibit HemSC and GLUT1^{sel} cell migration at low concentrations (>100 ng/ml) (Fig. 2A, lower panel). Thus, SEMA3E inhibited the migration of only HemEC, while SEMA3F inhibited the migration of HemSC, GLUT1^{sel} cells and HemEC.

We showed SEMA3F induces glioblastoma cell and HUVEC cytoskeletal collapse, leading to loss of cell motility [15,22]. SEMA3E also strongly reduces EC adhesion and stress fibers [17,18,23]. Phalloidin staining showed abundant stress fibers in IH cells (Fig. 2Ba–c). SEMA3E strongly inhibited stress fibers in HemEC (Fig. 2Bf) compared to non-treated cells, thereby collapsing the F-

actin cytoskeleton. However, SEMA3E did not alter HemSC and GLUT1^{sel} cell morphology (Fig. 2Bd and e), perhaps due to the lower level of expression of its receptor, Plexin D1, in these cells (Fig. 1B). SEMA3F modulated cell morphology at different levels; there was a strong inhibition of stress fibers in HemSC, a partial inhibition in HemEC (Fig. 2Bg and i), and a slight inhibition in the GLUT1^{sel} cell (Fig. 2Bh). Thus, SEMA3F mostly affected cells in which its receptor Plexin A1 was highly expressed (Fig. 1B). These results demonstrate that, consistent with the migration assay (Fig. 2A), SEMA3E&F inhibit stress fibers in IH cells, resulting in cytoskeletal collapse and loss of cell motility.

We next evaluated how SEMA3E&F affect the sprout formation in the presence of pro-angiogenic factor VEGF-A [24]. As the baseline, there were some sprouts in HemSC (3.6 ± 2.1 mm) and GLUT1^{sel} cells (2.0 ± 1.0 mm), but none from HemEC spheroids (0.4 ± 0.1 mm) (Fig. 3A). VEGF-A (15 ng/ml) increased total sprout length compared to non-treated: HemSC (1.5 ± 0.3-fold), GLUT1^{sel} cells (1.8 ± 0.7-fold) and HemEC (6.0 ± 0.4-fold). SEMA3E (800 ng/ml) reduced VEGF-induced sprouting in all cell types tested (Fig. 3B). Sprouts were decreased in HemSC (by 47% ± 18.1), in GLUT1^{sel} cells (by 23% ± 13.0) and in HemEC (by 69% ± 16.1) (Fig. 3B). Similarly, SEMA3F reduced the VEGF-A-induced sprouting in HemSC (by 59% ± 1.8), GLUT1^{sel} cells (by 53% ± 18.3) and HemEC (by 51% ± 12.1) (Fig. 3C). The inhibitory effect of SEMA3E on the HemSC and GLUT1^{sel} cells was not significant as that observed in HemEC. This may be due to the level of Plexin D1, as its expression was much higher in HemEC than in non-EC (Fig. 1B). In contrast, SEMA3F has more universal inhibitory effects on the pro-angiogenic properties of IH-derived stem cells and EC, including cell migration, collapse and sprouting.

We further assessed the effects of SEMA3E&F on IH cell proliferation at 800 ng/ml, which is sufficient to inhibit the migratory activity of IH cells (Fig. 2A). SEMA3E had no effect on the proliferation of HemSC and GLUT1^{sel} cells, but it inhibited HemEC proliferation at 48 h (Fig. 4A & Fig. S1A). SEMA3F produced a modest inhibitory effect on HemSC and GLUT1^{sel} cells at 72 h and showed an inhibitory effect similar to SEMA3E on HemEC at 72 h (Fig. 4A). In HemSC, VEGF-A or VEGF-B, via VEGFR1, phosphorylates ERK and thus promotes HemSC-to-EC differentiation [19], suggesting that the inhibition of MAPK could suppress the angiogenic properties of

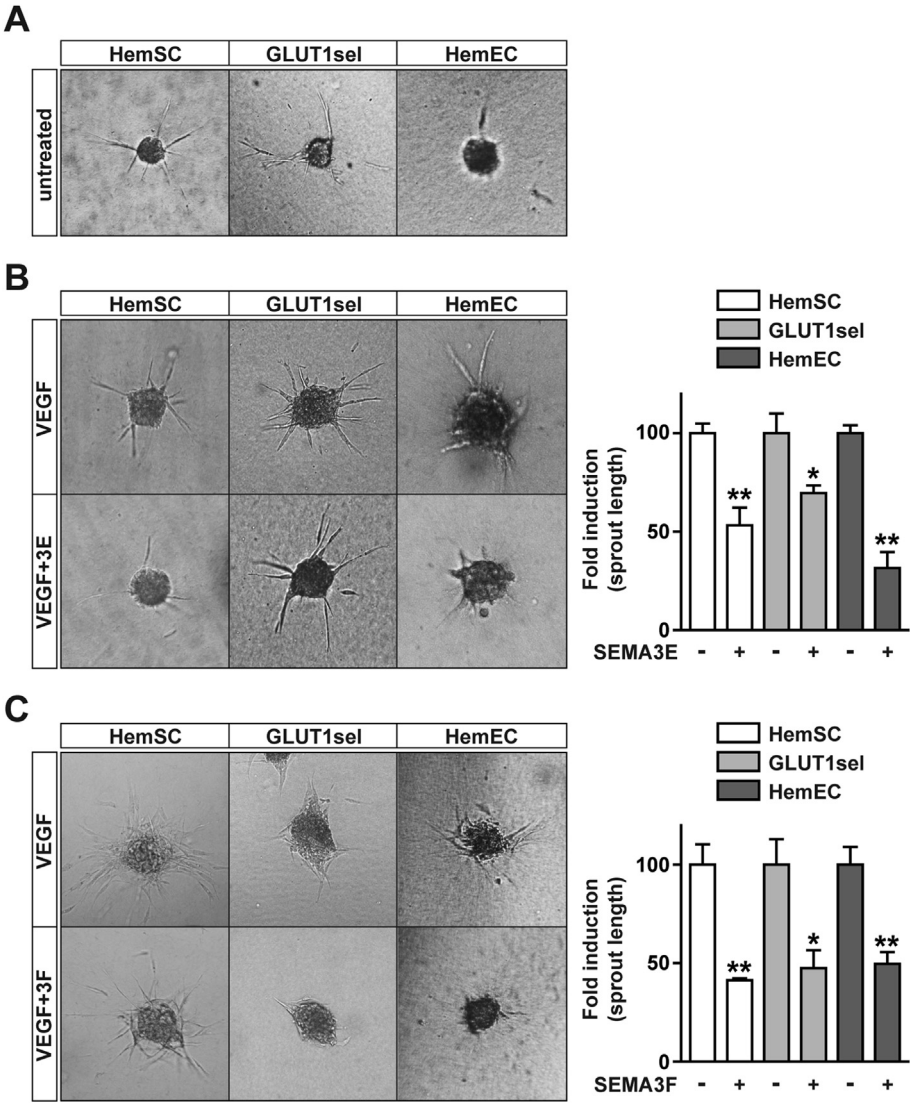

Fig. 3. SEMA3E and SEMA3F inhibit VEGF-induced sprouts in IH cells. A, IH cell spheroids were assessed for total sprout length in untreated group. B,C, Spheroids were treated with SEMA3E (top) or SEMA3F (bottom) at 800 ng/ml in the presence of VEGF-A (15 ng/ml) and assessed for total sprout length. Data represent the mean ± SD, *p < 0.05, **p < 0.01, vs. untreated group.

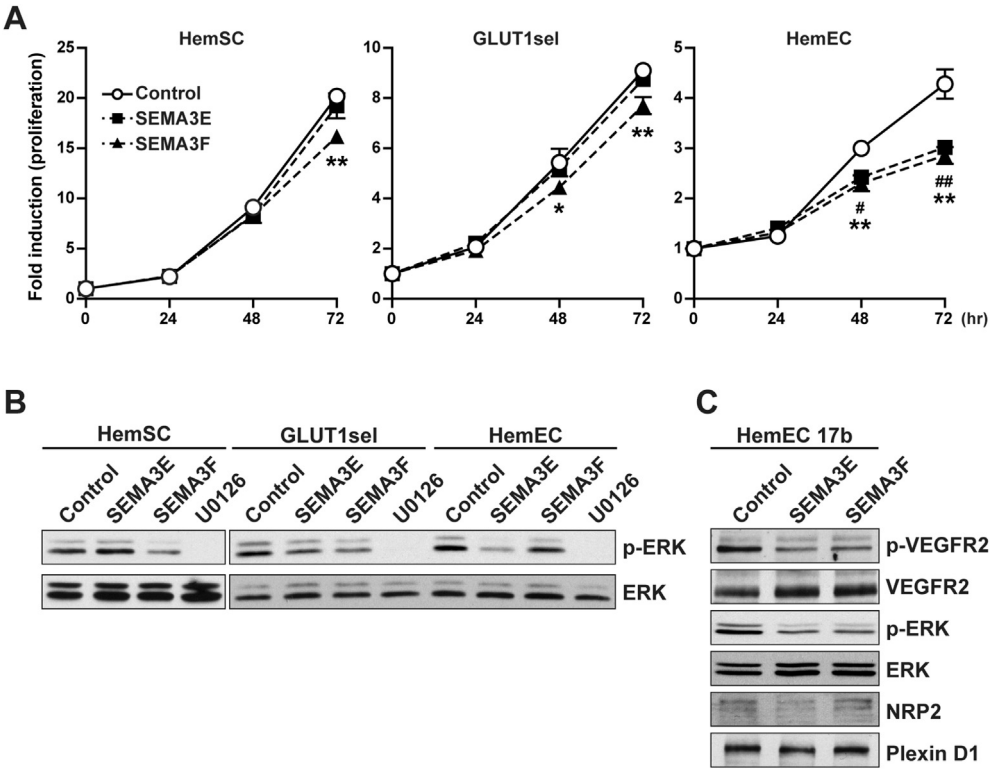

Fig. 4. SEMA3E and SEMA3F inhibit IH cell proliferation in a MAPK dependent pathway. A, Cells (1.0×10^4) were cultured in EGM-2 in the absence or presence of SEMA3E or SEMA3F (800 ng/ml). Cell number was counted at 24, 48 and 72 h. Data represent the mean ± SD, *p < 0.05, **p < 0.01 (SEMA3E-treated vs. untreated group), #p < 0.05, ##p < 0.01 (SEMA3F-treated vs. untreated group). B, SEMA3E and SEMA3F inhibit the MAPK pathway in IH cells. Cells were treated with SEMA3E or SEMA3F (800 ng/ml). After 30 min, cell lysates were collected and analyzed by western blot. The MEK inhibitor U0126 (10 μM) was used as a positive control. C, Cells were treated with SEMA3E or SEMA3F (800 ng/ml). After 30 min, cell lysates were collected and analyzed by western blot.

IH cells. Therefore, we investigated if SEMA3E&F could affect MAPK signaling in IH derived cells and thus potentially inhibit IH growth. SEMA3E inhibited p-ERK expression in GLUT1^{sel} cells (by 55%) and HemEC (by 76%); however, there was no change in HemSC (Fig. 4B). SEMA3F inhibited ERK phosphorylation in HemSC (by 52%), GLUT1^{sel} cells (by 60%) and HemEC (by 34%) (Fig. 4B). Collectively, SEMA3E&F can decrease p-ERK levels in IH cells and slow proliferation, similar to the pharmacological MEK inhibitor U0126 that was able to inhibit the proliferation of GLUT1^{sel} cells and HemEC (Fig. S1B).

IH is a highly proliferative vascular tumor, and VEGF signaling has been implicated in the pathogenesis of hemangiogenesis. In particular, HemEC express increased phosphorylated VEGFR2 but low levels of VEGFR1 [7], suggesting that low levels of VEGFR1 may facilitate constitutive signaling through VEGFR2 by not trapping VEGF-A. The HemEC from IH specimen 17b tested here show constitutively high levels of p-VEGFR2 and p-ERK [7]; thus, we next examined whether SEMA3E or SEMA3F treatment will reduce VEGF signaling in HemEC. Significantly, the administration of SEMA3E inhibited the phosphorylation of VEGFR2 and ERK but did not affect VEGFR1 expression (data not shown). This is also true for SEMA3F, suggesting that SEMA3E&F reduce VEGFR2 signaling through a non-VEGFR1-dependent mechanism. Plexin D1 signaling can induce soluble VEGFR1 (sFlt1) expression and thus inhibit VEGF signaling in zebrafish [25]. Herein, we could speculate that SEMA3E treatment induces the sFlt1 level, but does not affect the total VEGFR1 level in HemEC, resulting in a decrease of p-VEGFR2. On the other hand, SEMA3F competitively inhibits VEGF165 binding to EC [26], as SEMA3F and VEGF165 share the binding domain on NRPs as a co-receptor to enhance VEGFR2 activity [27,28]. When adding SEMA3F to HemEC, SEMA3F competed the association of VEGF with NRPs, thus reducing the level of p-VEGFR2. Thus, both SEMA3E&F inhibit the activity of VEGFR2 and its downstream ERK in HemEC, possibly through distinct molecular mechanisms.

VEGF-VEGFR2 signaling is a therapeutic target of IH. For example, corticosteroids, a mainstay therapy for IH, suppress VEGF-A expression in HemSC and blood vessel formation in HemSC/Matrigel suspensions injected into mice [8]. SEMA3E&F significantly reduced the level of p-VEGFR2 in HemEC but had no significant influence on VEGF production (by qPCR, Fig. S2A and B) or secretion into the conditioned media (by ELISA, Fig. S2C and D) in either HemSC or GLUT1^{sel} cells. This indicates a distinct mechanism of action from corticosteroids.

In summary, our data first demonstrate that axon guidance molecules SEMA3E&F inhibit the angiogenic properties of HemSCs, GLUT1^{sel} cells and HemECs. The inhibitory effects are mainly mediated by decreasing MAPK signaling. Particularly, in HemEC, SEMA3E&F can interrupt the VEGF-A-VEGFR2-ERK signaling pathway and thus neutralize VEGF-induced angiogenesis. This information provides a novel insight into the role of SEMA3E&F in IH growth and as well as its potential to target tumor angiogenic properties.

Conflict of interest

The authors declare that there are no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.06.087.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.06.087.

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