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Semaphorin 5A promotes angiogenesis by increasing endothelial cell proliferation, migration, and decreasing apoptosis

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Abstract

Semaphorin 5A (mouse, Sema5A; human, SEMA5A), is an axon regulator molecule and plays major roles during neuronal and vascular development. The importance of Sema5A during vasculogenesis, however, is unclear. The fact that Sema5A deficient mice display a defective branching of cranial vasculature supports its participation in blood vessel formation. In this study, we tested our hypothesis that Sema5A regulates angiogenesis by modulating various steps during angiogenesis. Accordingly, we demonstrated that the treatment of immortalized endothelial cells with recombinant extracellular domain of mouse Sema5A significantly increased endothelial cell proliferation and migration and decreased apoptosis. We also observed a relative increase of endothelial expression of anti-apoptotic genes relative to pro-apoptotic genes in Sema5A–treated endothelial cells suggesting its role in inhibition of apoptosis. In addition, our data suggest that Sema5A decreases apoptosis through activation of Akt, increases migration through activating Met tyrosine kinases and extracellular matrix degradation through matrix metalloproteinase 9. Moreover, *in vivo* Matrigel plug assays demonstrated that Sema5A induces endothelial cell migration from pre-existing vessels. In conclusion, the present work shows the pro-angiogenic role of Sema5A and provides clues on the signaling pathways that underlie them.

Keywords

Angiogenesis; Endothelial cells; Semaphorin; Sema5A; Matrix metalloproteinase; Migration; Akt phosphorylation; Proliferation; Met receptor

Introduction

Angiogenesis plays a key role in normal development as well as several pathological conditions including cancer. The process of angiogenesis is a complex multistep process, and one of the mechanisms by which angiogenesis occurs is through sprouting and

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remodeling of new blood vessels from existing blood vessels into a complex network. The process of angiogenesis is initiated by destabilization of existing matured vessels through vascular permeability, relaxation of intraendothelial cell contacts and alleviation of periendothelial support. Endothelial cells from destabilized matured vessels undergo proliferation followed by lumen formation, migration to an area of new blood vessel formation, attenuation of cells and fusion to pre-existing vessels. Increased survival and differentiation allow further development depending on the tissue origin. Finally, endothelial cells remodel and reorganize into mature blood vessels by formation of three dimensional networks (Carmeliet, 2000; Folkman, 2006). Multiple key molecular regulators direct single or multiple steps of this process (Carmeliet, 2000). Recent reports suggest that members of semaphorin family of proteins mediate several steps during angiogenesis (Eichmann et al., 2005; Serini et al., 2003; Weinstein, 2005).

Semaphorins are members of a large family of highly conserved, secreted glycosylphospatidylinositol (GPI)-anchored and transmem-brane signaling proteins that share a common sema domain of ~500 amino acids at their amino-terminal region. They are classified into eight sub-families based on their structural similarity, species of origin, and presence of class-specific carboxy-terminal domains (Goodman et al., 1999; Tamagnone et al., 1999; Tamagnone and Comoglio, 2000). The functions of semaphorins are exerted by binding to a family of transmembrane proteins called plexins that share the sema domain with semaphorins as well as with the neuropilins (Ellis, 2006) and receptor tyrosine kinases, Met and Ron (Kruger et al., 2005; Tamagnone et al., 1999). Recent reports demonstrate the non-neuronal role of semaphorins in the immune response (Walzer et al., 2005; Yamada et al., 1999), cardiac (Fiore et al., 2005; Gu et al., 2005; Torres-Vazquez et al., 2004) and skeletal development (Behar et al., 1996) and tumor formation (Sekido et al., 1996; Xiang et al., 2002). Although the most thoroughly studied sub-families of semaphorins in angiogenesis are sub-families III and IV, the role of the other subfamilies of semaphorins remains largely unknown. It has already been shown that Sema3A and Sema3F have been shown to inhibit the motility of endothelial cells as well as angiogenesis (Bielenberg et al., 2004; Favier et al., 2006; Kessler et al., 2004; Miao et al., 1999). In contrast, other members of the Semaphorin family such as Sema4D has been found to increase angiogenesis (Banu et al., 2006; Basile et al., 2004; Conrotto et al., 2005). However, the roles of the Semaphorin V sub-family of proteins in angiogenesis remain unclear.

Sema5A belongs to sub-family V of semaphorins, and is an integral membrane protein with characteristic seven thrombospondin specific repeats (TSP-1) (He et al., 2002; Kruger et al., 2005). Neuroepithelial cells ensheathing retinal axons express SEMA5A, which functions as an inhibitory cue for neurite outgrowth as well as a stimulatory cue for growth cone collapse, thus leading to the proper guidance of retinal projections to their targets (Goldberg et al., 2004; Oster et al., 2003). Sema5A was reported to be a bi-functional molecule, exerting its repelling or attracting signals depending on the type of receptor it binds (Kantor et al., 2004). A recent report (Artigiani et al., 2004) and our previous study (Sadanandam et al., 2008) demonstrated that Sema5A interacts with Plexin B3.

In spite of the promiscuous function of Sema5A inactivation leads to embryonic lethality, which can be attributed to defects in the regional patterning of the cranial vasculature (Fiore

et al., 2005). In addition, a recent report demonstrates that the migration of cells (including human umbilical vein endothelial cells) depends on the signaling of Sema5A through the hepatocyte growth factor receptor (Met) (Artigiani et al., 2004). Together, these studies suggest a role for Sema5A in physiological angiogenesis during embryonic development. However, the mechanism(s) by which SEMA5A mediates angiogenesis is not known.

In this study, we hypothesized that Sema5A regulates multiple events during angiogenesis by modulating endothelial cell proliferation, apoptosis and migration. Our data suggest that Sema5A decreases apoptosis through activation of Akt, increases migration through activation of Met tyrosine kinases and extracellular matrix degradation through matrix metalloproteinase 9 (MMP9). The present work shows the pro-angiogenic role of Sema5A and provides clues on the underlying signaling pathways.

Materials and methods

Cell lines and reagents

Immortalized human dermal microvascular endothelial cells (HMEC-1) were obtained from the Center for Disease Control and Prevention (Atlanta, GA) (Ades et al., 1992). The cells were maintained in culture as an adherent monolayer in RPMI-1640 at 37 °C temperature and 5% CO_2 supplemented with 5% fetal calf serum (FCS), 1X nonessential amino acids, 2 mM L-glutamine, 1X vitamin solution (Catalogue #MT-25-020-CI) and 40 μg/ml gentamycin (Mediatech, Herndon, VA). The cultures were free of mycoplasma and pathogenic murine viruses, and were maintained for no longer than eight weeks after recovery from frozen stock.

Recombinant Sema5A protein was purified from conditioned media of Panc-1 cells (transfected with the cDNA construct of the extracellular domain of mouse Sema5A conjugated to human IgG-Fcγ; a generous gift from Dr. David Stretavan, Department of Ophthalmology and Physiology, University of California San Francisco) (Oster et al., 2003) using Protein-A affinity chromatography. Protein purity was verified using silver staining (Fischer Chemical, Fairlawn, NJ). Recombinant VEGF-A was purchased from R and D systems (Minneapolis, MN). The following antibodies were used: anti-phospho-Akt antibody (Cell Signaling Technology, Danvers, MA), anti-Akt antibody (BD Biosciences, Franklin Lakes, NJ), monoclonal antibody against the C-terminal region of β-catenin (a generous gift from Dr. Keith R. Johnson, University of Nebraska Medical Center, Omaha NE), Texas red phalloidin (Molecular Probes, Eugene, OR), anti-human HGF receptor (Met) antibody (R and D Systems, Minneapolis MN), HRP-conjugated secondary anti-rabbit antibody (Vector Laboratories, Burlingame, CA) and cy3-conjugated anti-mouse antibody (Jackson Immunoresearch, West Grove, PA). In addition, we used the Akt inhibitor, 2-(4-Morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (IC_{50} = 1.4 μM) purchased from EMD Biosciences (Gibbstown, NJ).

In vitro endothelial cell proliferation and apoptotic assays

HMEC-1 cells were seeded into 96-well (5000 cells/well) flat-bottom plates in triplicate with medium alone (control) or medium containing different concentrations of recombinant

Sema5A. Following 72 h of incubation, cell proliferation was determined using 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, EMD Biosciences), a tetrazole assay as described earlier (Li et al., 2003). The absorbance was read at 570 nm using a microplate reader (BioTek Instruments, Inc., Vermont, VA). Recombinant VEGF-A (10 ng/ml) was used as a positive control. To examine the effect of inhibition of Akt phosphorylation on proliferation, HMEC-1 were incubated with media containing Sema5A (10 or 100 ng/ml) alone or in combination with an Akt inhibitor (10 μM) and cell proliferation was examined by MTT assay. In order to examine the role of Sema5A on endothelial cell apoptosis (Li et al., 2003), HMEC-1 cells (1×10^5 cells) were incubated with medium alone or medium containing 10 ng/ml of Sema5A for 24 h and stained with CaspACE FITC-VAD-FMK *in situ* marker kit (Promega Corporation, Madison, WI). The number of cells undergoing apoptosis was quantitated by counting the number of immunostained cells in five independent fields (200×) using a Nikon fluorescent microscope. The number of positively stained cells in experimental and control samples were plotted using bar graph. Two independent observers (AS and RKS) examined each slide; their observations were positively correlated with each other ($p<0.05$). If the two observers differed in their scoring a third observer examined the slide.

Endothelial cell migration assay

Endothelial cell migration in response to Sema5A was determined as described earlier (Li et al., 2003). HMEC-1 cells (1×10^5) were plated in duplicate onto the upper transwell chamber (6.5 mm, Corning Costar, Cambridge, MA). The bottom chamber contained 1.0 ml of medium (serum free) alone or medium containing different concentrations of Sema5A. The cells were incubated for 24 h at 37 °C and cells that did not pass through the membrane pores were removed. Migrated cells were stained using Hema 3 kit (Fisher Scientific Company L.L.C., Kalamazoo, MI) as per the manufacturer's instructions and counted in ten random fields (200×). Recombinant VEGF-A (10 ng/ml) was used as a positive control. To examine the effect of neutralization of Met receptor on migration, endothelial cells were incubated with medium containing Sema5A (10 or 100 ng/ml) alone in the lower chamber or in a combination with Met neutralizing antibody (2 μg/ml) in the upper chamber, followed by cell counting.

Zymogram for MMP activity

Culture supernatants from 5×10^5 HMEC-1 cells treated with media alone or media containing Sema5A for 24 h were used for gelatin zymography as described previously (Li et al., 2003). Briefly, the gelatin-degrading activity in the culture supernatants was analyzed by electrophoresis on an 8% SDS-PAGE-containing gelatin (1.5 mg/ml) without prior heating or reduction. Following electro-phoresis, the gel was processed for gelatinolytic activity and stained with 0.1% Coomassie brilliant blue.

Immunohistochemical analysis

HMEC-1 cells (1×10^5) were grown for 48 h on gelatin-coated coverslips, washed twice with PBS and fixed with 4% glutaraldehyde. Later, the cells were permeabilized for 5 min in 0.1% Triton X-100. Following nonspecific blocking, sections were incubated with Texas red

phalloidin (1:200) for 30 min or β-catenin (1:500) for 1 h at room temperature. For β-catenin the sections were then washed and subsequently incubated with cy3-conjugated secondary antibody (1:1000 in PBS) for 30 min . Cells were washed thrice with PBS-T (PBS containing 0.1% Tween20) and mounted with antifade Vectashield mounting medium (Vector laboratories). Immunostained cells were observed using Nikon fluorescent microscope.

For CD31 immunostaining, briefly, 6-μm thick tumor sections were deparaffinized by EZ-Dewax (Biogenex, SanRoman, CA) and blocked for 30 min . Tumor sections were incubated overnight with the anti-CD31 antibody (1:100; Novacastra). The slides were rinsed and incubated in biotinylated secondary antibody (1:500). Immunoreactivity was detected using the ABC Elite kit and DAB substrate (Vector Laboratories, Burlingame, CA) per the manufacturer's instructions.

In vivo Matrigel plug assay

A Matrigel plug assay in BALB/c mice was performed as described previously (Conrotto et al., 2005). BALB/c mice were maintained under specific pathogen-free conditions in facilities approved by the University of Nebraska Medical Center (UNMC) Institution Animal Care and Use Committee (IACUC) guidelines. Mice were injected with 500 μl of Matrigel (BD Biosciences) containing either 100 ng/ml of recombinant Sema5A or PBS alone (negative control). Additional mice were injected with Matrigel containing recombinant VEGF-A (100 ng/ml; positive control). The mice were sacrificed after seven days and the Matrigel plugs were embedded in paraffin. Each sample was processed, sectioned and stained by hematoxylin–eosin and viewed, immunostained for CD31 and quantified.

mRNA analysis

Total cellular RNA was isolated from HMEC-1 cells (1×10^6 cells) using TRIZOL reagent (Invitrogen, Carlsbad, CA). RT-PCR was performed as described earlier (Sadanandam et al., 2007) using PCR primer sets (Table 1). Relative intensity of specific gene expression was determined using Image Quant (GE Healthcare, Piscataway, NJ). mRNA expression is presented as an expression index, the ratio of each gene-specific signal to the signal from the housekeeping gene, β-actin. The expression index for cells cultured with media alone was arbitrarily set to 1 to represent the difference in the expression level among endothelial cells treated with different concentrations of Sema5A.

Western blot analysis

Cell lysates from Sema5A–treated and -untreated HMEC-1 were prepared using lysis buffer containing 20 mM Tris–HCl pH 7.5, 150 mM NaCl, 1% Triton X-100 and complete protease inhibitor cocktail (Roche, Indianapolis, IN). Western blot was performed as described earlier (Li et al., 2003). The membranes were blocked with 3% BSA and probed with anti-Akt antibody, followed by HRP-conjugated anti-rabbit antibody. For phosphorylation experiments, cell lysates from HMEC-1 cells treated for different durations (15 min , 1, 4, 8 or 24 h) with Sema5A recombinant protein were examined using anti-phospho-Akt antibodies. Protein bands were visualized by an enhanced chemiluminescent

detection system (GE Healthcare) according to the manufacturer's protocol, and analyzed using a Typhoon and ImageQuant software (GE Healthcare).

Plexin B3 knock-down using small interfering RNA

In order to determine the role of Plexin B3 in Sema5A–induced endothelial cell proliferation, we transiently knock-down Plexin B3 expression, using short hairpin RNA (shRNA) technology. Two shRNAs targeting Plexin B3 (sh4–5′-AGC AGA TGG TGG AGA GGT A-3′; and sh5, 5′-GGA AGA GAC TCA ACA CCT T-3′) and scrambled (control, 5′-GGC TAC GTC CAG GAG CGC A-3′) were generated with BglII and HindIII overhangs to allow for cloning into the pSuper.neo vector (Oligoengine, Seattle, WA). HMEC-1 cells were transiently transfected with pSuper.neo/scrambled (control vector), pSuper.neo/sh4 Plexin B3 (Plexin B3-sh4), pSuper.neo/sh5 Plexin B3 (Plexin B3-sh5) or plasmid using Lipofectamine reagent (Invitrogen) following the manufacturer's protocol. Plexin B3 knock-down was confirmed by mRNA analysis and its effect on Sema5A–induced cell proliferation was analyzed using the MTT assay as described previously.

Statistical analysis

The significance of the data was determined by Student's *t*-test (two-tailed) using SPSS software (SPSS, Chicago, IL). A value of $p<0.05$ was deemed significant.

Results

Sema5A enhances proliferation and inhibits apoptosis of endothelial cells

We examined whether recombinant Sema5A (extracellular domain) modulates endothelial cell proliferation, an important step during the angiogenic process. HMEC-1 cells were incubated with media alone or media containing different concentrations of recombinant Sema5A or VEGF-A (positive control) and cell proliferation was determined. We observed an increase in endothelial cell proliferation at different concentrations following treatment with Sema5A compared to media alone (Fig. 1A), indicating that Sema5A stimulates endothelial cell proliferation.

Next, we examined whether Sema5A also regulated apoptosis in endothelial cells. HMEC-1 cells (1×10^5 cells/well) cultured on gelatin-coated coverslips in 6-well plates were treated with media alone or media containing Sema5A (10 ng/ml) for 24 h. Cell apoptosis was examined by immunostaining with CaspACE-FITC. We observed a significantly lower frequency of apoptotic cells when HMEC-1 cells were incubated with Sema5A as compared to cells incubated with media alone (Fig. 1B).

Akt phosphorylation mediates Sema5A–induced proliferation

To understand the downstream signaling mechanism by which Sema5A enhanced proliferation and apoptosis in endothelial cells, relative Akt phosphorylation (p-Akt) was examined. Serum-starved HMEC-1 cells were incubated with media alone or media containing Sema5A (10 ng/ml) for different durations (15 min , 1, 4, 8 or 24 h). An increase in p-Akt levels was observed in endothelial cells treated with Sema5A up to 8 h compared to untreated cells (Fig. 2A). In order to understand the direct role of Akt in the proliferation of

endothelial cells, we treated HMEC-1 cells with 10 ng/ml Sema5A in the presence or absence of Akt inhibitor. Interestingly, the presence of Akt inhibitor significantly inhibited Sema5A–induced endothelial cell proliferation compared to the absence of Akt inhibitor (Fig. 2B). Together, these data suggest that Akt phosphorylation might be involved in Sema5A–induced endothelial cell proliferation.

Sema5A modulates anti-apoptotic and pro-apoptotic gene expression in endothelial cells

Considering that Sema5A decreased the apoptotic index of HMEC-1 cells (Fig. 1B), the effect of recombinant Sema5A on the expression of anti-apoptotic (BCL-2, survivin and BCL-x_L) and pro-apoptotic (BAX and BCL-x_S) genes was assessed by semi-quantitative RT-PCR analysis in Sema5A treated and untreated HMEC-1 cells. Sema5A protein had no effect on survivin mRNA expression in HMEC-1 cells (Fig. 2C). In contrast, Sema5A treatment increased the expression of BCL-x_L and decreased the expression of BCL-x_S (Fig. 2C) to significantly increased BCL-x_L to BCL-x_S mRNA expression ratio (Fig. 2D). There was also an increase in the mRNA expression of BCL-2 and BAX, but no change in BCL-2 to BAX mRNA expression ratio in cells treated with Sema5A (data not shown). These results demonstrate that Sema 5A may increase survival by increasing expression of pro-survival and decreasing expression of pro-apoptotic molecules.

Sema5A induces migration of endothelial cells through Met kinase activity

To further elucidate the role of Sema5A in endothelial cell function, we examined the directed migratory activity of Sema5A on immortalized endothelial cells using a modified migration assay using transwell chambers. The migration of HMEC-1cells from the upper chamber of the transwell chamber towards the lower chamber containing the chemo-attractant (Sema5A or VEGF-A) was examined. The assay was performed with different concentrations of Sema5A recombinant protein. Sema5A significantly increased HMEC-1 migration similar to the positive control, VEGF-A (Fig. 3A and B). A recent report demonstrated that Sema5A–mediated cellular migration involves phosphorylation of Met receptor via Plexin B3 (Artigiani et al., 2004). Therefore, we examined whether Sema5A–induced endothelial cell migration is mediated through a Met-dependent signaling pathway. Sema5A–dependent endothelial cell migration was inhibited using a neutralizing antibody against Met activity (Fig. 3C) suggesting that Sema5A may induce migration through the Met receptor in endothelial cells.

Sema5A modulates actin rearrangement, β-catenin localization and active MMP-9 production in endothelial cells

Endothelial cell migration requires cytoskeletal rearrangements by F-actin depolymerization and relocation of β-catenin to the nucleus or the cytoplasm from the cell membrane (Wright et al., 2002). The effect of Sema5A recombinant protein on F-actin organization in endothelial cells was assessed by treating the HMEC-1 cells with Sema5A for increasing periods of time (5, 10, 20 and 60 min),followed by fixing and immunostaining using phalloidin. The control treated HMEC-1 cells showed several stress fibers that were aligned along the major cell axis (Fig. 4Aa). In contrast, Sema5A protein stimulated the progressive dismantling of stress fibers and reorganization of the actin network in HMEC-1 cells as early as 5 min (Fig. 4Ab). In subsequent experiments, the relocalization of β-catenin in

endothelial cells treated with Sema5A recombinant protein was examined by immunostaining using anti-β-catenin monoclonal antibody. The staining pattern showed increased localization of β-catenin to the cytoplasm and nucleus in HMEC-1 cells treated with Sema5A whereas β-catenin was localized in the cell membrane in untreated cells (Fig. 4B). Together, these results demonstrate that Sema5A–induced F-actin depolymerization and β-catenin relocalization to nucleus lead to enhanced directed migratory potential in endothelial cells.

Increased expression and activity of MMP-2 and MMP-9 leading to the degradation of extracellular matrix (ECM) is a critical step for endothelial cell migration. Therefore, we examined MMP-2 and MMP-9 activity using zymography in endothelial cells cultured in media alone or with different concentrations of Sema5A recombinant protein. The levels of pro- and active MMP-9 were increased in HMEC-1 treated with Sema5A compared to untreated cells. However, there was no difference in MMP-2 levels (Fig. 4C). This suggests that Sema5A may mediate extracellular matrix degradation through MMP-9 activation.

Knock-down of Plexin B3 inhibits Sema5A–induced cell proliferation

In the next set of experiments, we analyzed whether HMEC-1 cells express Plexin B3, a putative receptor for Sema5A. Our data demonstrate that HUVEC and HMEC-1 cells express Plexin B3 mRNA (Fig. 5A). Using shRNA technique, we transiently knock-down Plexin B3 mRNA expression (Fig. 5B). Further more, we examined, whether knock-down of Plexin B3 modulated Sema5A–dependent endothelial cell proliferation. Results shown in Fig. 5B demonstrate that knock-down of Plexin B3 expression inhibited Sema5A–stimulated HMEC-1 cell proliferation (Fig. 5C).

Sema5A enhances sprouting of blood vessels

In order to determine whether Sema5A modulates sprouting of blood vessels from pre-existing vessels, we performed a Matrigel plug assay in mice. Matrigel containing Sema5A, PBS or VEGF-A was injected subcutaneously into BALB/c mice and blood vessel sprouting was observed for 7 days. A significantly higher level of blood vessels sprouting from already existing vessels was observed in mice injected with Matrigel containing Sema5A compared to those with PBS control (Fig. 6A). Similarly, VEGF-A (positive control) induced higher vascularization of Matrigel plugs. The average number of microvessels per square area in Matrigel plugs containing Sema5A or VEGF-A was counted and was significantly higher compared to PBS control (Fig. 6B and C).

Discussion

Although angiogenesis has been widely studied, the understanding of the molecular events involved in this complex process is still in its infancy. Recent reports suggest that in addition to known first generation angiogenic growth factors (VEGF, fibroblast growth factor (FGF)-2), other molecules implicated in axonal guidance may also play critical roles in blood vessel guidance, endothelial cell proliferation, migration, cell collapse and endothelial cell progenitor homing during pathological and physiological angiogeneses (Autiero et al., 2005; Carmeliet, 2000; Chedotal et al., 2005). In this study, we demonstrate that SEMA5A

induces endothelial cell proliferation and migration, and inhibits apoptosis, important requirements for angiogenesis. Moreover, our data suggest that Sema5A–induced proliferation and migration of endothelial cells are achieved by activation of Akt and Met tyrosine kinases, respectively. The anti-apoptotic activity of Sema5A is mediated through increased expression of anti-apoptotic proteins, BCL-2 and BCL-x_L in endothelial cells. In addition, our data suggest that extracellular matrix degradation by endothelial cells, needed for migration, accomplished by increased activation of MMP-9 in endothelial cells. Since, the study was performed using transformed endothelial cells and the specific effects of Sema5A in endothelial cells from various tissues still need to be examined.

The Akt/PKB family of kinases has been shown to play critical role in growth, proliferation and apoptosis by phosphorylation-mediated regulation of multiple downstream substrates [for review see Brazil et al. (2004)]. Our data reveal that Sema5A triggers phosphorylation of Akt leading to increased proliferation in endothelial cells, and this endothelial cell proliferation is abrogated by Akt inhibition. Together these data suggest the involvement of Akt phosphorylation as a downstream signaling event in Sema5A–mediated endothelial cell proliferation. In addition to cell proliferation, endothelial cell survival is also important for angiogenesis (Nor et al., 1999). A cell cycle-regulated apoptosis inhibitor, survivin (Ambrosini et al., 1998), and the cell death-related gene family, Bcl-2 (Antonsson and Martinou, 2000), are associated with VEGF-induced angiogenesis (Tran et al., 1999). Our data demonstrated inhibition of apoptosis and differential expression of BCL-2 family members in endothelial cells treated with Sema5A. The relative expression of anti-apoptotic genes as compared to pro-apoptotic genes was higher in Sema5A–treated endothelial cells leading to inhibition of apoptosis.

Another critical step in angiogenesis involves motility of endothelial cells (Carmeliet, 2000) and is a characteristic feature of angiogenic endothelium (Wright et al., 2002). We demonstrate that Sema5A induced the concentration-dependent migration of endothelial cells, and this activity was associated with rearrangement of cytoskeleton (based on F-actin reorganization) and relocalization of β-catenin. An earlier report suggests that Sema5A can trigger the intracellular signaling of the Met receptor independent of HGF leading to attracting cues during cellular migration (Artigiani et al., 2004). Similar results have been demonstrated for another member of the semaphorin family, Sema4D (Basile et al., 2006; Conrotto et al., 2005). In this study, attenuation of Met activity using neutralizing antibody to Met, inhibited Sema5A–induced endothelial cell migration. These data suggest that like most of the common angiogenic molecules, Sema5A can stimulate migration of endothelial cells, a process that might be mediated through Met-phosphorylation. Even though a recent report (Artigiani et al., 2004) and our previous study (Sadanandam et al., 2007), demonstrate that Sema5A interacts with Plexin B3, our *in vitro* knock-down experiments partially abrogated Sema5A–induced cell proliferation. However, there may be additional Sema5A binding receptor in HMEC-1 cells, which is not known. Subsequent studies will be required to identify any Sema5A binding partner(s) and the role of Sema5A–Plexin B3 interaction in HMEC-1 cells to determine the precise molecular mechanism(s) of Sema5A–mediated responses in endothelial cells caused by binding to its receptor.

The degradation of ECM by MMPs secreted by pre-existing endothelial cells guides them to migrate properly towards the area of angiogenesis to form organized endothelial tubes leading to angiogenesis (Carmeliet, 2000). In the current study, we have reported an increase in active MMP-9 in endothelial cells by Sema5A. These data suggest that Sema5A may induce the degradation of ECM through MMP-9, leading to endothelial cell migration, invasion, and capillary tube organization. Our *in vitro* results were supported by an *in vivo* Matrigel angiogenesis assay, where Sema5A enhanced blood vessel sprouting from pre-existing vessels in mice and the levels of sprouting were similar to that observed with a known angiogenic factor, VEGF-A.

In conclusion, our data reveal that Sema5A is a novel pro-angiogenic molecule. Sema5A potently induces endothelial cell proliferation, migration and inhibits apoptosis. The studies presented in this report suggest that these observed activities are similar to VEGF-A stimulation. However, it might be possible that Sema5A and other known angiogenic factors might work in concert at different stages in the process of blood vessel generation, remodeling and renewal. The development of a properly patterned vascular tree depends on the modulation of a complex process controlled by angiogenic growth factors, semaphorins and integrins (Serini et al., 2009). In the future, we will analyze the collaboration, if any, between Sema5A and VEGF-A in this process. Identification of a novel angiogenic molecule such as Sema5A and its downstream signaling events provide clues to understanding its role in physiological as well as pathological angiogenesis and additional opportunities to develop therapeutic strategies using Sema5A to block/induce abnormal angiogenesis occurring during pathological conditions.

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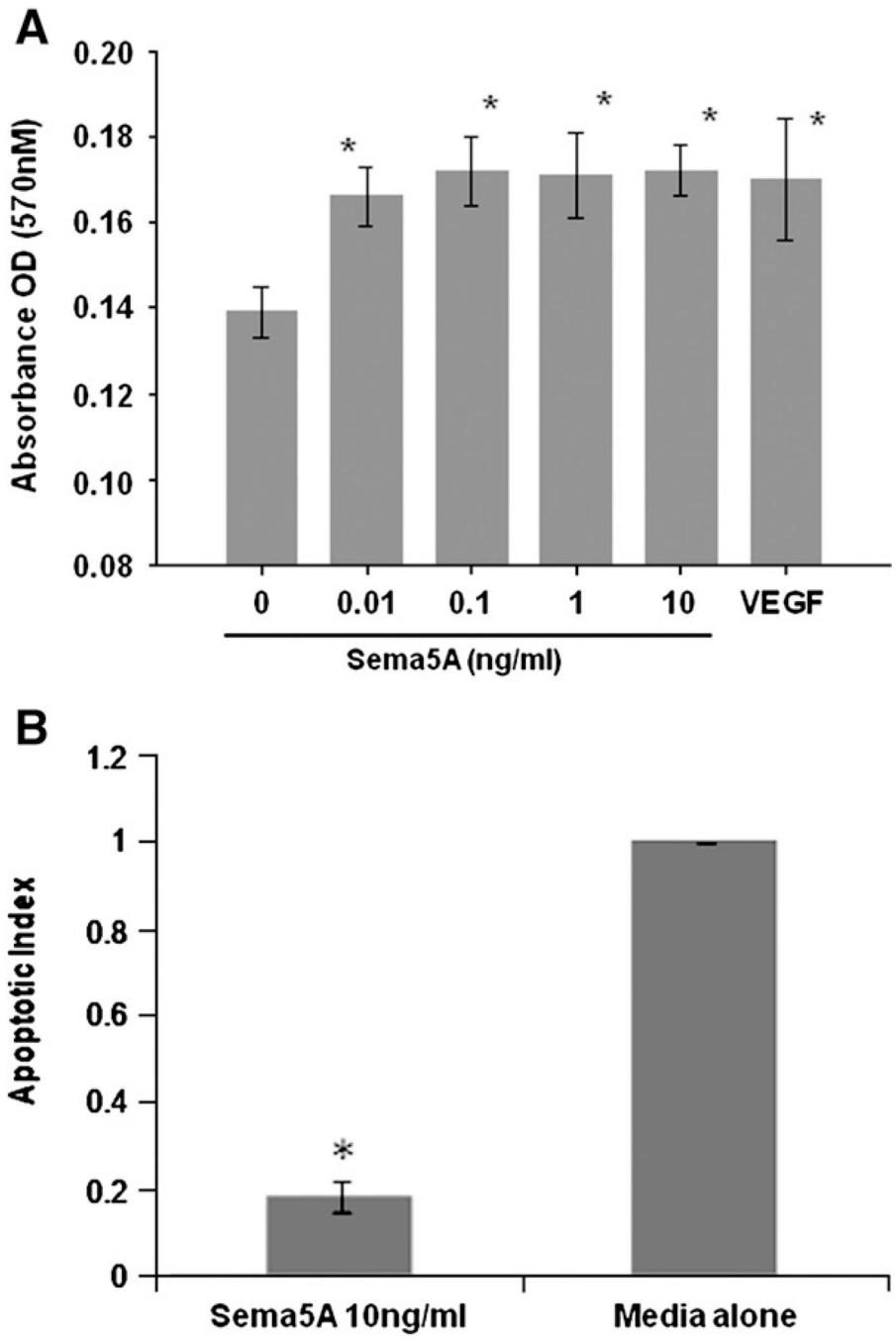

Fig. 1.

Sema5A enhances the proliferation and apoptosis of endothelial cells. (A) Increase in proliferation of endothelial cells in response to Sema5A. HMEC-1 were assessed for proliferation by MTT assay after a 72 h incubation with media alone or media with different concentrations of recombinant Sema5A. The values are mean OD_{570}±SEM (bars) of triplicate culture. This is a representative of five independent experiments performed in triplicate. VEGF-A was used as a positive control. (B) Sema5A inhibited endothelial cell apoptosis. HMEC-1 cells incubated with media alone or media containing Sema5A for 24 h

were examined for apoptosis using the CaspACE FITC-VAD-FMK assay (see Materials and methods for description). The numbers of apoptotic cells were counted and the apoptotic index was calculated by comparing the percentage of apoptotic cells. This is a representative of three independent experiments performed in duplicate. *Significantly different from HMEC-1 cells cultured in media alone ($p<0.05$).

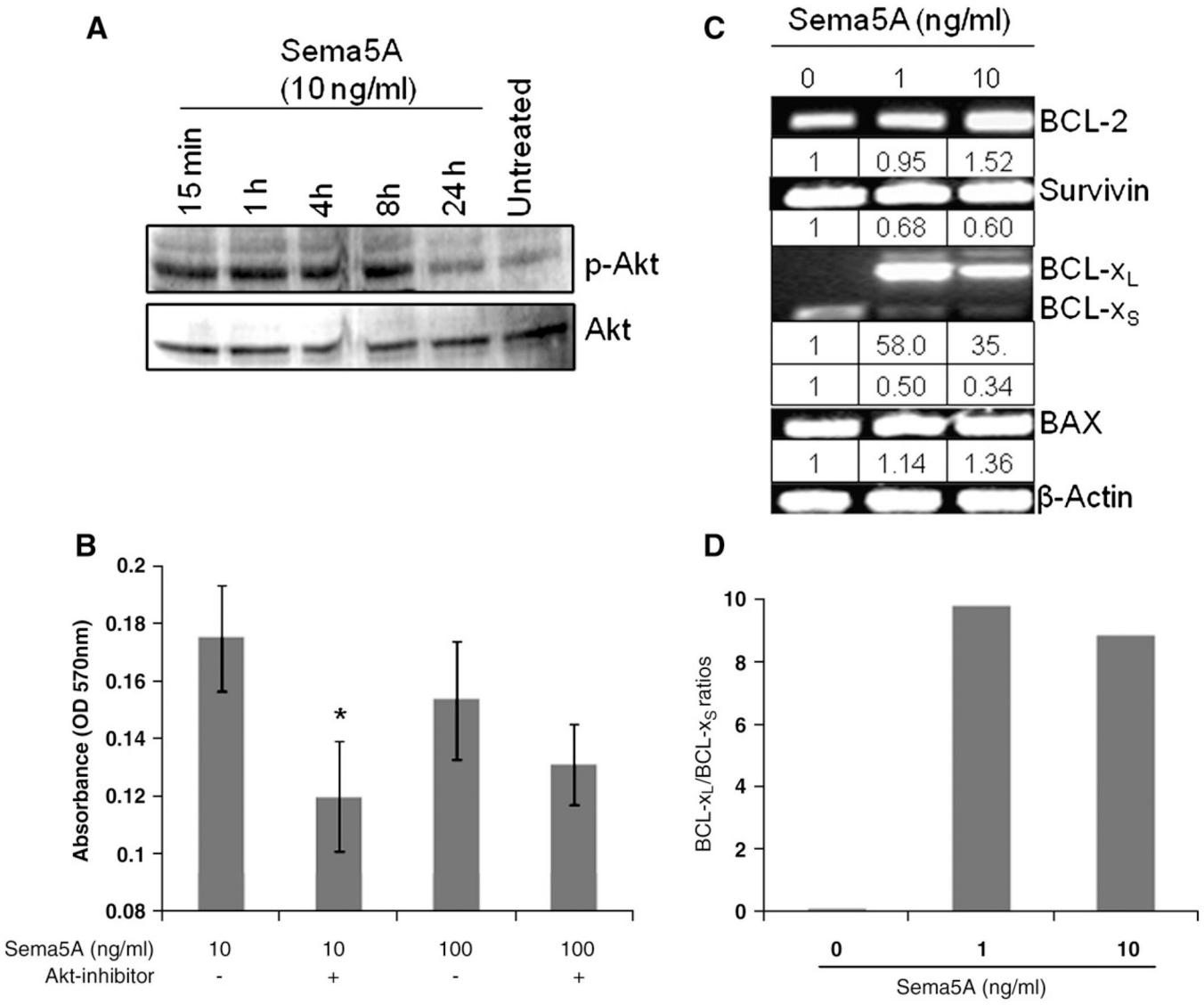

Fig. 2.

Sema5A–induced proliferation and apoptosis is mediated by the activation of Akt phosphorylation and anti-apoptotic gene expression. (A) Phosphorylation of Akt (p-Akt) and the expression of total-Akt in HMEC-1 cells at different time points after Sema5A treatment were examined using Western blot analysis. There was an increase in p-Akt from 15 min to 8 h in HMEC-1 treated with Sema5A compared to untreated cells. (B) Akt inhibitor decreased the proliferation of HMEC-1 cells treated with Sema5A recombinant protein. Proliferation of HMEC-1 cells treated with different concentrations of Sema5A alone or in combination with Akt inhibitor was assessed by MTT assay after 72 h of incubation. The values are mean OD_{570}±SEM (bars) of triplicate culture. *Significantly different from HMEC-1 cells cultured in media alone($p<0.05$). (C) Increased mRNA expression of anti-apoptotic genes compared to pro-apoptotic genes inendothelial cells treated with Sema5A. The expression of anti-apoptotic genes, BCL-2, Survivin and BCL-x_L and pro-apoptotic genes BAX and BCL-x_L in HMEC-1 treated with media containing different concentrations

of Sema5A or media alone were evaluated by semi-quantitative RT-PCR analysis. Expression index displayed below the agarose gel photograph was calculated by comparing gene-specific mRNA transcript levels to that of the housekeeping gene, β-actin. (D) Increased expression ratio between anti-apoptotic and pro-apoptotic genes in endothelial cells treated with Sema5A. The expression ratio of$BCL\text{-}x_L$ and $BCL\text{-}x_S$ were calculated from the relative expression indices of HMEC-1 cells treated with Sema5A or media alone.

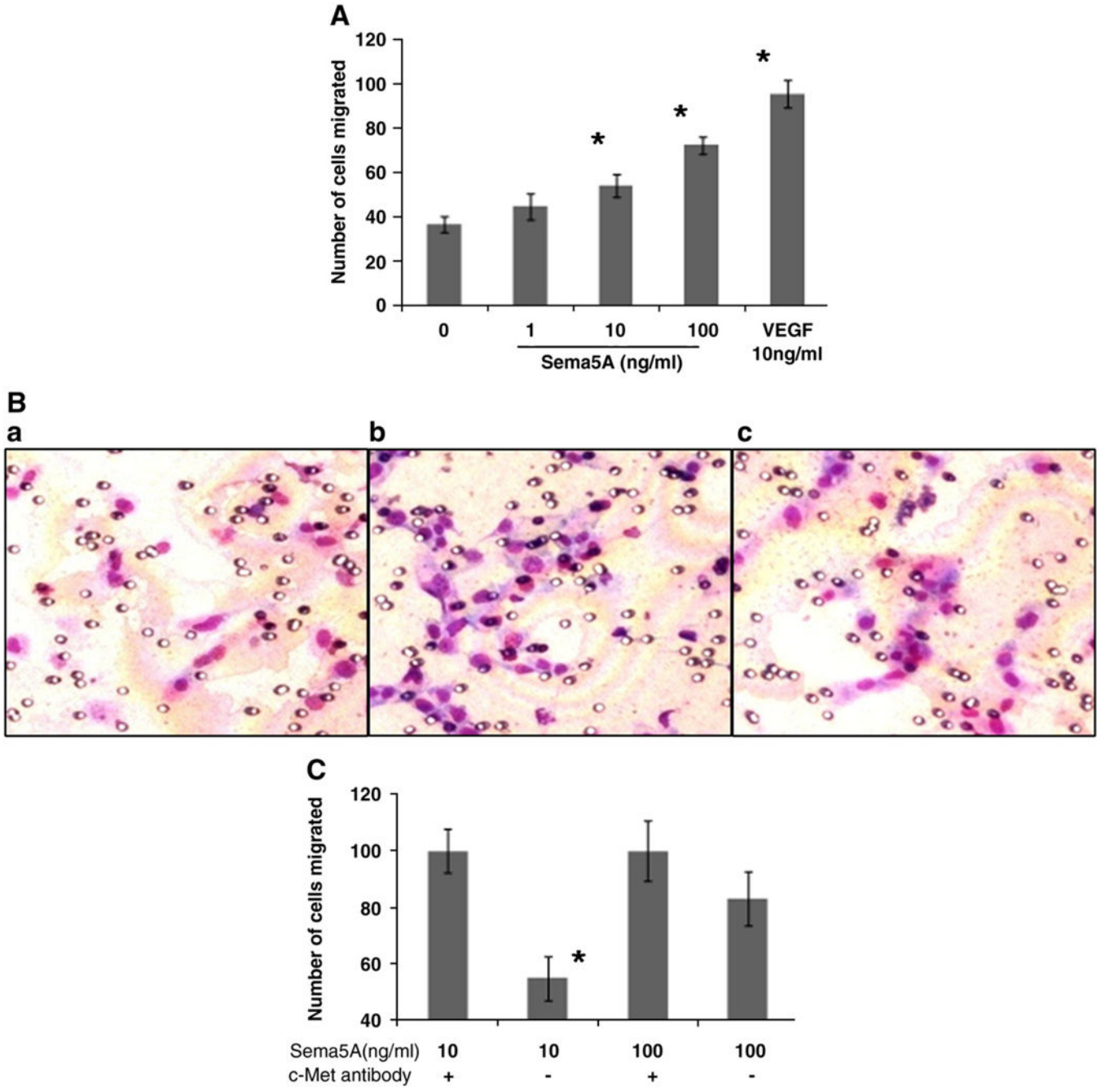

Fig. 3.

Sema5A induces migration of endothelial cells through Met kinase activity. (A) Increased migration of HMEC-1 cells treated with Sema5A. Migration of HMEC-1 cells in response to Sema5A was examined using a transwell chamber assay (see Materials and methods for description). The values represent the average number of cells migrated±SEM. *Significantly different from HMEC-1 cells cultured in media alone (p<0.05). (B) HMEC-1 cells undergoing migration in response to Sema5A were captured using a light microscope at 200× magnification. HMEC-1 treated with a. media alone, b. 10 ng/ml of Sema5A and c. 10 ng/ml of VEGF-A. (C) Sema5A–induced migration of endothelial cells is mediated through

Met receptor. Migration of HMEC-1 cells in response to Sema5A with or without neutralizing antibody for Met receptor was examined using a transwell chamber assay. The values are percent cells migrated in the presence of Sema5A with or without Met antibody ± SEM. *Significantly different from endothelial cells treated with Sema5A alone.

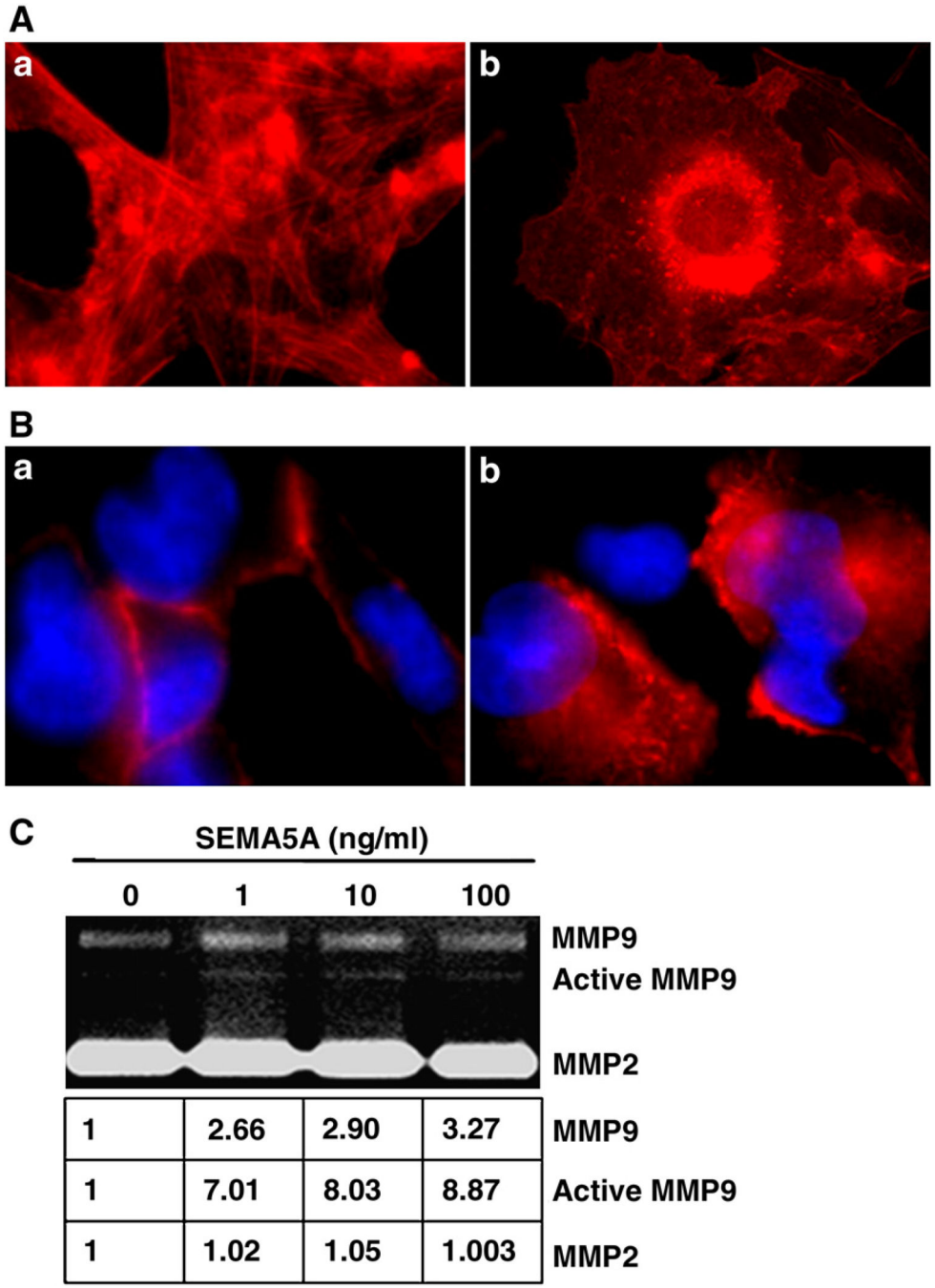

Fig. 4.

Cytoskeletal rearrangement and active MMP-9 production in endothelial cells following treatment with Sema5A. (A) F-actin rearrangement in HMEC-1 cells in response to Sema5A was examined using immunofluorescent analysis by phalloidin staining. a. Stress fibers were found aligned across the cell axis in HMEC-1 treated with media alone. b. Stress fibers disappeared forming membrane ruffles in HMEC-1treated with 10 ng/ml of Sema5A in media for 5 min.(B)Immunocytochemistry to show relocalization of β-catenin in HMEC-1 treated with Sema5A was performed. A.β-catenin was localized on the membrane of

HMEC-1cells treated with media alone whereas b. it was found more in the cytoplasm and nucleus of cells treated with 10 ng/ml of Sema5A. (C) MMP-2 and MMP-9 activity in the culture supernatants of HMEC-1 treated with different concentrations of Sema5A or media alone was determined. The zymogram wasscanned using an Alpha Imager and the in tensity of bands was quantitated using Image Quant software. The values, shown in the lower boxes under the zymogram represent the fold increase in MMP activity as compared with endothelial cells treated with media alone. This is a representative of two independent experiments.

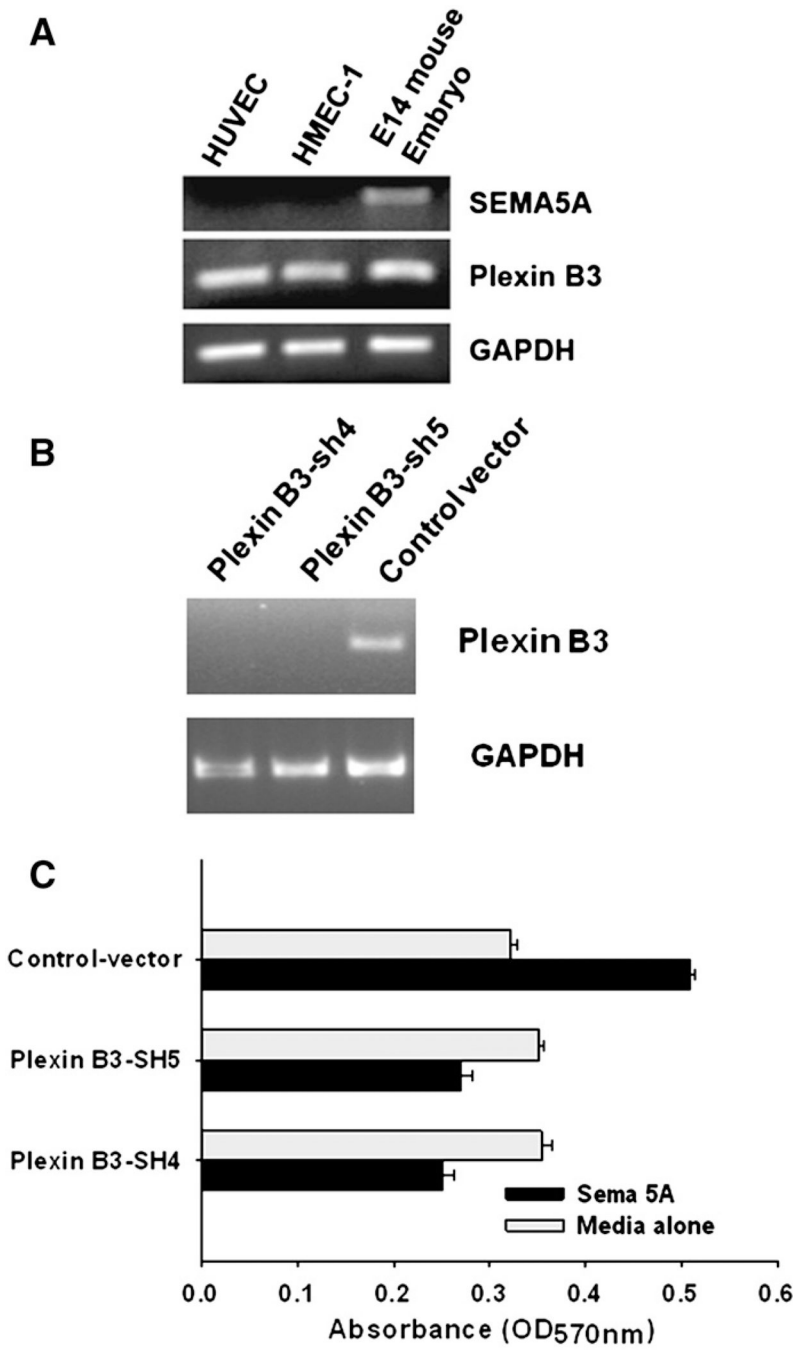

Fig. 5.

Plexin B3 in Sema5A–induced endothelial cell proliferation. (A) Expression of SEMA5A, Plexin B3 and GAPDH (control) mRNA in HUVEC and HMEC-1 cells as examined by RT-PCR analysis. E14 mouse embryo was used as control tissue for SEMA5A expression. (B) Expression of Plexin B3 mRNA in HMEC-1 cells transfected with shRNA vectors containing Plexin B3 shRNAor scrambled shRNA. GAPDH mRNA expression was used as control. (C) Inhibition of Sema5A–induced HMEC-1 cell proliferation following knock-down of Plexin B3 as determined by MTT assay. HMEC-1 cells transfected with shRNA

vector containing Plexin B3 or scrambled shRNA were treated with 1 μg/ml of recombinant Sema5A or media alone. The result is a representative of two independent experiments done in triplicate.

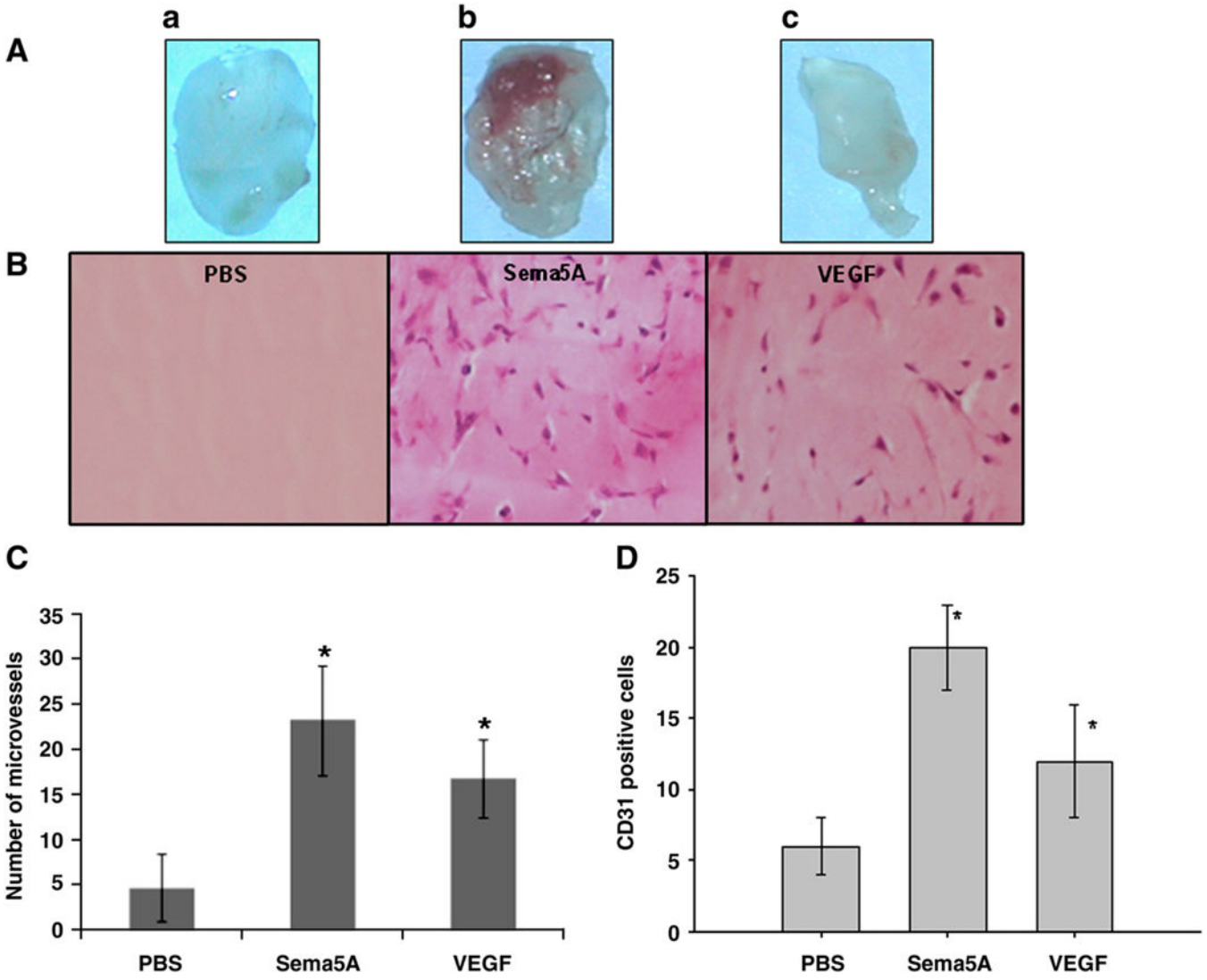

Fig. 6.

Sema5A enhances angiogenesis *in vivo*. Matrigel containing Sema5A (100 ng/ml), PBS (negative control), or VEGF-A (100 ng/ml; positive control) was injected subcutaneously into BALB/C mice and the level of angiogenesis was examined. (A) Representative photomicrographs of Matrigel plugs; Matrigel plug containing a. PBS; b. Sema5A; or c. VEGF-A. (B) Histological analysis of Matrigel plugs to demonstrate angiogenesis. (C) Capillary number quantitation in Matrigel plugs containing PBS, Sema5A or VEGF-A. The values are mean number of microvessels ± SEM. This is a representative of two independent experiments done using three mice per group. *Significantly different from PBS control ($p<0.05$). (C) CD31 positive endothelial cells identified by immunohistochemistry were counted in Matrigel plugs. The values are mean number of CD31 positive cells ± SEM. This is a representation of two independent experiments done using three mice per group. *Significantly different from PBS control ($p<0.05$).

Table 1

Primers used for RT-PCR analysis.

Gene	Orientation	Sequence	Product size
Sema5A	Sense	5′-GAACCGGAAGCGTGTT-3′	755 bp
	Antisense	5′-CAGTGAGATGTGGGTTGAAG-3′	
Plexin B3	Sense	5′-GTGCGGAACCTTCAACATTT-3′	232 bp
	Antisense	5′-AAAGAGCATGGGTGTTGTCC-3′	
Survivin	Sense	5′-GTGTCTGTCAGCCCAACC-3′	208 bp
	Antisense	5′-TGACCTCCAGAGGTTTCCAG-3′	
BCL-x	Sense	5′-GACGAGTTTGAACTGCGGTA-3′	BCL-x_L 378 bp;
	Antisense	5′-CACAGTCATGCCCGTCAG-3′	BCL-x_S 190 bp
BCL-2	Sense	5′-TCCATGTCTTTGGACAACCA-3′	203 bp
	Antisense	5′-CTCCACCAGTGTTCCCATCT-3′	
BAX	Sense	5′-TCTGACGGCAACTTCAACTG-3′	188 bp
	Antisense	5′-TTGAGGAGTCTCACCCAACC-3′	
β-actin	Sense	5′-TGAAGTGTGACGTGGACATC-3′	246 bp
	Antisense	5′-ACTCGTCATACTCCTGCTTG-3′	