Research Article

EphB4 Overexpression in B16 Melanoma Cells Affects Arterial-Venous Patterning in Tumor Angiogenesis

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Abstract

EphB4 receptor and its ligand ephrinB2 play an important role in vascular development during embryogenesis. In blood vessels, ephrinB2 is expressed in arterial endothelial cells (EC) and mesenchymal supporting cells, whereas EphB4 is only expressed in venous ECs. Previously, we reported that OP9 stromal cells, which support the development of both arterial and venous ECs, in which EphB4 was overexpressed, could inhibit ephrinB2-positive (ephrinB2$^+$) EC development in an embryonic tissue organ culture system. Although the EphB4 receptor is expressed in a variety of tumor cells, its exact function in regulating tumor progression has not been clearly shown. Here we found that overexpression of EphB4 in B16 melanoma cells suppressed tumor growth in a s.c. transplantation tumor model. Histologic examination of these tumors revealed that EphB4 overexpression in B16 cells selectively suppressed arterial ephrinB2$^+$ EC development. By coculturing ephrinB2-expressing SV40-transformed mouse ECs (SVEC) with EphB4-overexpressing B16 cells, we found that EphB4 induced the apoptosis of SVECs. However, ephrinB2 did not induce the apoptosis of EphB4-overexpressing B16 cells. Based on results from these experiments, we concluded that EphB4 overexpression in B16 tumor cells suppresses the survival of arterial ECs in tumors by a reverse signaling via ephrinB2. [Cancer Res 2007;67(20):9800–8]

Introduction

The growth of solid tumors is closely associated with the ability to recruit blood vessels, which can supply tumors with the growth factors, oxygen, and nutrients necessary for their survival and growth and for the maintenance of the malignant state. In embryos, blood vessels are initially formed by a process called vasculogenesis but become remodeled and mature through a second process called angiogenesis, which results in the development of a highly hierarchical architecture of blood vessels ranging from small to large (1). During these processes, a distinction develops between arterial and venous endothelial cells (EC); eventually, the arterial ECs selectively express ephrinB2 and the venous ECs preferentially express EphB4, which is a cognate receptor tyrosine kinase for ephrinB2 (2–4).

Eph receptors and ephrins are frequently expressed in reciprocal patterns that correlate with cellular boundaries during embryonic development (5). Consistent with this expression pattern, Eph-ephrin signaling regulates the boundary of distinct cells in culture (6) and is required for vascular modeling (2, 7), axon guidance (8, 9), and epithelial-mesenchymal transitions (10). Reciprocal expression of ephrinB2 and EphB4 in arterial and venous ECs, respectively, suggests that ephrinB2 and EphB4 might interact at the arterial-venous interface and regulate angiogenesis (2–4). Targeted disruption of either ephrinB2 or EphB4 in mice has been shown to lead to early embryonic lethality through the disruption of blood vessel formation in angiogenesis but not in vasculogenesis (2–4). EphrinB2 contains transmembrane and cytoplasmic domains; therefore, it has been suggested that the functioning of this receptor/ligand system is dependent on cell-to-cell contact (5). EphB4 is a member of the receptor tyrosine kinase family and initiates signal transduction through autophosphorylation after ligand binding (forward signaling); however, in contrast to other soluble ligands for receptor system, ephrinB2 also has the ability to initiate receptor-like active signaling (reverse signaling; refs. 5, 11, 12). Indeed, a loss-of-function experiment, in which the cytoplasmic domain of ephrinB2 was deleted, showed that bidirectional EphB4/ephrinB2 signaling was required for proper arterial and venous development (13).

It was originally assumed that the blood vessel system in tumors was composed of homogeneous capillaries, based on their uniformly small size and the sparse adhesion of mural cells to ECs. However, it has been reported that the vessels in tumors can be divided into ephrinB2-positive (ephrinB2$^+$) arterial and ephrinB2-negative (ephrinB2$^-$), and therefore presumably, venous ones (7). This suggests that the EphB4/ephrinB2 system is involved in tumor angiogenesis and that it may have an affect on the specification of arteries and veins in the tumor environment. Moreover, EphB4 expression has been reported in numerous tumors such as breast, liver, gastrointestinal, prostate, bladder, lung, and ovarian cancers, as well as leukemia, mesothelioma, and melanoma (14–19). Recent research reported that reduction of EphB4 activity accelerated tumorigenesis in the colon and rectum and that loss of EphB4 expression represented a critical step in colorectal cancer progression (15). Furthermore, a highly significant correlation was reported between EphB4 positivity and low histologic grading of tumor cells in breast cancer (20). Other reports also showed that overexpression of EphB4 is inversely related to a poor prognosis in head and neck squamous cell carcinoma and endometrial carcinoma (21, 22). These results indicated that EphB4 expression is not compatible with tumor progression. However, in mesothelioma, up-regulation of EphB4 was shown to provide a survival advantage in tumor tissue (17)

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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and resulted in growth of the tumor. At present, little is known about this apparent discrepancy in terms of the function of EphB4 in tumor progression, but it is possible that coexpression of another member of the EphB family, such as EphB1, EphB2, EphB3, and EphB6, or coexpression of EphB4 ligands such as ephrinB2 in tumor cells may affect tumor cell viability and proliferation. Indeed, it was reported that, in mesothelioma, ephrinB2 and EphB4 were almost exclusively coexpressed in cells from tumor cell lines and primary cancers, and that knockdown of either ephrinB2 or EphB4 by small interfering RNA inhibited the viability of tumor cells coexpressing EphB4/ephrinB2 (17). This suggested that the expression of ephrinB2 in tumor cells has a reciprocal affect on EphB4 expression in an autocrine or paracrine manner. Similar coexpression of this receptor-ligand pair has been reported in other tumor types, including endometrial, lung, and colon carcinoma, both in the corresponding tumor cell lines and primary cancers (16, 23, 24).

Although the expression of EphB4 in various tumors and its effect on tumor progression have been reported, its other role in the development of arteries and veins in tumors has not been clarified. To investigate this, we searched for tumor cells that expressed EphB4 on their surface, but not ephrinB2, to exclude any autocrine or paracrine effects of EphB4 on tumor cells. Furthermore, we looked for murine tumor cell lines because of the availability of an *in vivo* tumor angiogenesis model, in which murine EphB4 affects murine ephrinB2 of ECs. Among tumor cell lines tested, such as melanoma, colon cancer, lung cancer, and mammary gland tumor, we found a B16 mouse melanoma cell line that matched these criteria. Using this cell line, we overexpressed or knocked down EphB4 and observed the effect of EphB4 on blood vessel formation and tumor growth. Moreover, to test the viability of ephrinB2$^+$ ECs on stimulation with EphB4$^+$ tumor cells, we cocultured an ephrinB2-induced EC line, a SV40-transformed mouse EC line (SVEC; ref. 25), with B16 melanoma cells *in vitro*, and observed dose dependency of cell-surface EphB4 and apoptosis of ephrinB2$^+$ ECs.

Materials and Methods

Animals. C57BL/6 mice and Wistar rats were purchased from Japan SLC at 8 weeks of age and used between 8 and 12 weeks of age. EphB4$^{LacZ/+}$ and ephrinB2$^{LacZ/+}$ mice (2, 4) were provided by Dr. D.J. Anderson (California Institute of Technology, Pasadena, CA). Animal care in our laboratory was in accordance with the guidelines of Kanazawa and Osaka University for animal and recombinant DNA experiments.

Cell lines and transfection. B16 (mouse melanoma) cells were cultured in DMEM supplemented with 10% FCS. BaF3 pre-B hematopoietic cells were cultured in RPMI 1640 supplemented with 10% FCS and 1 ng/mL interleukin-3.

Transfection of mouse full-length *EphB4* or *ephrinB2* cDNA (26) was done using Lipofectamine Plus reagent (Invitrogen). Stable transfection was obtained by antibiotic resistance selection using G418 (Life Technologies, Inc.). Stable knockdown of *EphB4* gene in B16 cells was done using the short hairpin RNA (shRNA) method. shRNA coding for *EphB4* was introduced into the pSINsi-hU6 DNA vector (Takara) at the *Bam*H1 and *Cla*1 ligation sites according to the manufacturer's instruction. shRNA oligonucleotides were synthesized corresponding to the published sequence of *EphB4* mRNA (NM_010144). The insert for *EphB4* shRNA (forward strand, 5′-GCATCA-CAGTCAGACTCAACT-3′; reverse strand, 5′-AGTTGAGTCTGACTGTG-ATGC-3′) and a nonspecific oligonucleotide insert for negative control (forward strand, 5′-gatccGATCGTTGGTGTGGTGGGTCGttcaagagaACTAC-CATGCTCCCATGAACAtttttttat-3′; reverse strand, 5′-cgataaaaaaTGTTCAT-GGGAGCATGGTAGTtctcttgaaCGACCCACCACACCAACGATCg-3′) were used. Plasmids were transfected into B16 by electroporation (Amaxa Biosystems); 48 h after electroporation, the cells were harvested by trypsin-EDTA (Life Technologies) and reseeded at a density of 2×10^4 cells in 10-cm culture dishes. The following day, geneticine (Life Technologies) was added to a final concentration of 1,000 μg/mL. Eight days after addition of geneticine, colonies were picked with a cloning ring (Asahi Techno Glass) and reseeded in the culture plate. For the evaluation of *EphB4* knockdown, *EphB4* expression was determined by real-time reverse transcription and reverse transcription-PCR (RT-PCR).

RT-PCR analysis. Total RNA was extracted from cells using the RNeasy plus mini kit (Qiagen) according to the manufacturer's protocol. Total RNA was reverse transcribed into cDNA using Exscript RT Reagent Kit (Takara). Primer pairs for studying the expression of mouse *EphB4*, mouse *ephrinB2*, human *EphB4*, human *ephrinB2*, mouse *β-actin,* or human *glyseraldehyde-3-phosphate dehydrogenase* (*GAPDH*) were, for real-time PCR, *EphB4* (sense, 5′-AATGTCACCACTGACCGTGA-3′; antisense, 5′-TCAGGAAACGAA-CACTGCTG-3′) and *GAPDH* (sense, 5′-TGGCAAAGTGGAGATTGTTGCC-3′; antisense, 5′-AAGATGGTGATGGGCTTCCCG-3′), and for RT-PCR primers, mouse *EphB4* (sense, 5′-CGTCCTGATGTCACCTATACCTTTGAGG-3′; antisense, 5′-GAGTACTCAACTTCCCTCCCATTGCTCT-3′), mouse *ephrinB2* (sense, 5′-AGGAATCACGGTCCAACAAG-3′; antisense, 5′-GTCTCCTGCGG-TACTTGAGC-3′), human *EphB4* (sense, 5′-GGCTGCTCGCAACATCCTAGT-3′; antisense, 5′-CCACATCACAATCCCGTAAC-3′), human *ephrinB2* (sense, 5′-CTGCTGGATCAACCAGGAAT-3′; antisense, 5′-GATGTTGTTCCCCG-AATGTC-3′), mouse *β-actin* (sense, 5′-CCTAAGGCCAACCGTGAAAAG-3′; antisense, 5′-TCTTCATGGTGCTAGGAGCCA-3′), and human *GAPDH* (sense, 5′-GAAGGTGAAGGTCGGAGTC-3′; antisense, 5′-GAAGATGGTGATGG-GATTTC-3′). Real-time PCR analysis was done with Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen). The levels of PCR products were monitored with an ABI PRISM 7900HT sequence detection system and analyzed with ABI PRISM 7900 SDS software (Applied Biosystems). The adjustment of baseline and threshold was done according to the manufacturer's instructions. The relative abundance of transcripts was normalized to the constitutive expression level of *GAPDH* RNA.

Generation of EphB4 monoclonal antibody. The extracellular ligand binding domain of mouse *EphB4* (amino acids 39–165) was cloned into pGEX-2T to generate glutathione *S*-transferase (GST)–fused protein. EphB4 ligand binding domain expressed as a GST fusion protein in DH5α *E. coli* was purified by affinity chromatography and used as an immunogen for Wistar rats. The methods for generation of hybridoma cells using X63Ag8 mouse myeloma cells and the purification of monoclonal antibody (mAb) were the same as previously described (27). The sensitivity of the mAb produced from the hybridoma clone (VEB4-7E4) was confirmed by Western blotting and fluorescence-activated cell sorting (FACS) analysis with BaF3 cells stably transfected with EphB4 (BaF3/EphB4). Furthermore, by immunohistochemistry, the specificity of the mAb was examined by reciprocal expression of ephrinB2 and EphB4 or colocalization of ECs stained with anti–β-galactosidase and anti-EphB4 antibody on the hind limb section of ephrinB2$^{Lac/+}$ or EphB4$^{Lac/+}$ mice, respectively.

Immunoprecipitation and Western blotting. Cells were lysed on ice with lysis buffer [50 mmol/L HEPES (pH 7.4), 1% Triton X-100, 10% glycerol, 10 mmol/L sodium pyrophosphate, 100 mmol/L sodium fluoride, 4 mmol/L EDTA, 2 mmol/L sodium orthovanadate, 50 μg/mL aprotinin, 1 mmol/L phenylmethylsulfonyl fluoride, 100 mmol/L leupeptin, 25 μmol/L pepstatin A]. Cell lysates were cleared by centrifugation for 15 min at $15{,}000 \times g$ at 4°C, and then proteins were subjected to SDS-PAGE and transferred onto polyvinylidene difluoride membranes (Nihon Millipore). Membranes were blocked with 5% normal goat serum, 1% bovine serum albumin, 5% skimmed milk in TBST [20 mmol/L Tris (pH 7.4), 150 mmol/L sodium chloride, 0.1% Tween 20] for 60 min at room temperature. Membranes were washed and then incubated for 60 min at room temperature with anti-EphB4 mAb or anti-ephrinB2 polyclonal antibody (Santa Cruz Biotechnology), followed by 60 min at room temperature with horseradish peroxidase–conjugated goat anti-mouse immunoglobulins (Amersham Pharmacia Biotech) or goat anti-rabbit immunoglobulins antibody (Biosourse). Proteins were visualized by enhanced chemiluminescence detection system (LAS-3000 mini, Fuji).

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FACS analysis. Flow cytometric analysis was done as previously described (28). The antibodies used in this experiment were anti-EphB4 mAb (VEB4-7E4) and Alexa Fluor 488 goat anti-rat immunoglobulin G (IgG) (H+L) antibody (Molecular Probes). For the analysis of specific binding of EphB4 mAb to EphB4 on BaF3/EphB4 cells, cells were preincubated with soluble EphB4 receptor (sEphB4; extracellular domain of EphB4 fused with human Fc of IgG; ref. 26) for 30 min on ice. The stained cells were analyzed by FACSCalibur (Becton Dickinson).

Immunohistochemistry. Tissue fixation, preparation of tissue sections, and staining of sections with antibodies were done as previously described (29). Antibodies used in immunohistochemical staining were nonlabeled or FITC-conjugated anti–platelet/endothelial cell adhesion molecule 1 (PECAM-1) mAb (PharMingen), anti–β-galactosidase antibody (Chemicon), and anti-EphB4 mAb (VEB4-7E4). Secondary antibodies used were horseradish peroxidase– or Alexa Fluor 488–conjugated goat anti-rat IgG(H+L) antibody for anti-EphB4 mAb (Biosource) and nonlabeled anti–PECAM-1 mAb or HRP-, alkaline phosphatase–, or Alexa Fluor 546–conjugated goat anti-rabbit IgG antibody (Molecular Probes) for anti–β-galactosidase antibody. Anti–PECAM-1 antibody was developed with HRP-conjugated anti-rat IgG antibody (Biosource). For color reaction of HRP, samples were soaked in PBS containing 250 μg/mL diaminobenzidine (Dojin Chem.) in the presence of 0.05% $NiCl_2$ for 10 min, and 0.01% hydrogen peroxidase was added for the enzymatic reaction. For the color reaction of alkaline phosphatase, new Fuchsin substrate kit (DAKO) was used. Nuclear staining was done with Hoechst (Sigma) or 4′,6-diamidino-2-phenylindole (DAPI; Invitrogen) to obtain fluorescent images. Finally, the sections were observed and photographed under a microscope (IX-70, Olympus) with UV lamp.

***In vitro* proliferation assay.** Cells (10^3 per well) were seeded into 96-well plates and cultured in DMEM supplemented with 10% FCS under 37°C, CO_2 0.5%. The cell number was counted daily.

Mouse xenograft assay. Tumor cells (5×10^6 per mouse in 0.1-mL PBS) were injected s.c. into 8-week-old female wild-type C57BL/6, ephrinB2$^{LacZ/+}$, or EphB4$^{LacZ/+}$ mice, as previously reported (30). Tumor volumes were measured with calipers every 3 days and calculated as width × width × length × 0.52. Tumor tissues were removed from mice on day 18 postinjection.

Coculture system and apoptosis assay. B16/mock (B16 cells induced with mock vector) or B16/EphB4 (B16 cells induced with mouse EphB4 expression vector) tumor cells labeled with PKH26 Red Fluorescent Cell Linker Mini Kit (Sigma) were seeded into 12-well plates in culture medium (DMEM supplemented with 10% FCS). When cells had reached 80% confluency, they were serum starved for 12 h in DMEM. SVECs transduced with mouse ephrinB2 (SVEC/ephrinB2 cells) were prelabeled with PKH67 Green Fluorescent Cell Linker (Sigma) and serum starved for 12 h in DMEM, and then added in medium (DMEM, 2% FCS) either into B16/mock or B16/EphB4 tumor cells or trans-well plates with 0.4-μm pores (Corning, Inc.). For binding inhibition assays of EphB4 in B16/EphB4 cells and ephrinB2 in SVEC/ephrinB2 cells, soluble EphB4-Fc (sEphB4; 5 μg/mL) was added. After 24 h of coculture, cells were harvested from the culture plate using trypsin-EDTA and stained with Annexin V-Cy5 Apoptosis Detection Kit (BioVision) and DAPI (Invitrogen). Stained cells were analyzed by flow cytometry with UV laser (JSAN, Bay bioscience).

Statistical analysis. All date are presented as mean ± SE. For statistical analysis, Microsoft Excel software was used for two-sided Student's *t* test.

Results

Generation of mAb against mouse EphB4. To investigate EphB4 expression at the protein level in tumor cells, we generated antimouse EphB4 mAb (clone VEB4-7E4) and first observed EphB4 expression in the BaF3 hematopoietic cell line induced with ectopic EphB4 (BaF3/EphB4, Fig. 1*A*) by flow cytometric analysis (Fig. 1*B*) and Western blotting (Fig. 1*C*). As observed in Fig. 1*B*, this mAb recognized EphB4 on BaF3 cells and this reaction to EphB4 by the mAb was almost completely suppressed by soluble EphB4 protein, suggesting that this reaction was specific to EphB4. Moreover, this mAb could recognize a 120-kDa protein from BaF3/EphB4 cell lysates but not from BaF3/mock cells (Fig. 1*C*). In immunohistochemical staining of hind limb muscle using LacZ reporter strain to detect expression of ephrinB2 (arteries; ref. 4), together with antibody to CD31/PECAM-1, a pan-endothelial marker, this mAb against EphB4 (VEB4-7E4) did not recognize CD31$^+$ephrinB2$^+$ arterial ECs but was able to mark CD31$^+$ephrinB2$^-$ ECs (Fig. 1*D*). These reciprocal expression profiles indicated that this mAb could

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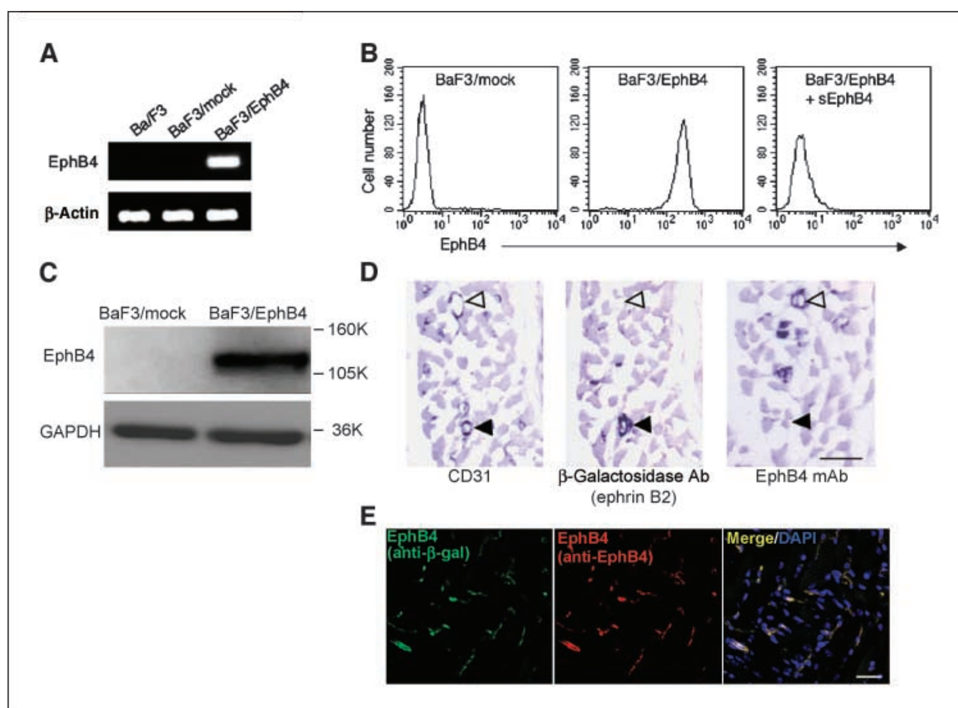

Figure 1. Specificity of generated EphB4 mAb. *A,* RT-PCR analysis of *EphB4* mRNA in BaF3 cells, BaF3 cells transfected with mock (BaF3/mock), or *EphB4* (BaF3/EphB4) plasmid. *B,* FACS analysis of EphB4 expression in BaF3/mock and BaF3/EphB4 cells. Soluble EphB4-FC chimeric protein (sEphB4) was used for analyzing specific binding of EphB4 mAb to EphB4. *C,* Western blotting analysis for detecting EphB4 protein from cell lysates of BaF3/mock or BaF3/EphB4 with EphB4 mAb and anti-GAPDH antibody. *D,* serial sections from mouse hind limb muscle of 8-week-old ephrinB2$^{LacZ/+}$ mice were stained with anti-CD31, anti–β-galactosidase, or anti-EphB4 antibodies. *Open* and *closed arrows,* CD31$^+$ephrinB2$^-$EphB4$^+$ and CD31$^+$ephrinB2$^+$EphB4$^-$ blood vessels, respectively. *Bar,* 40 μm. *E,* sections from mouse hind limb muscle of 8-week-old EphB4$^{LacZ/+}$ mice were stained with anti–β-galactosidase and anti-EphB4 antibodies. Nuclei were stained with DAPI. *Bar,* 40 μm.

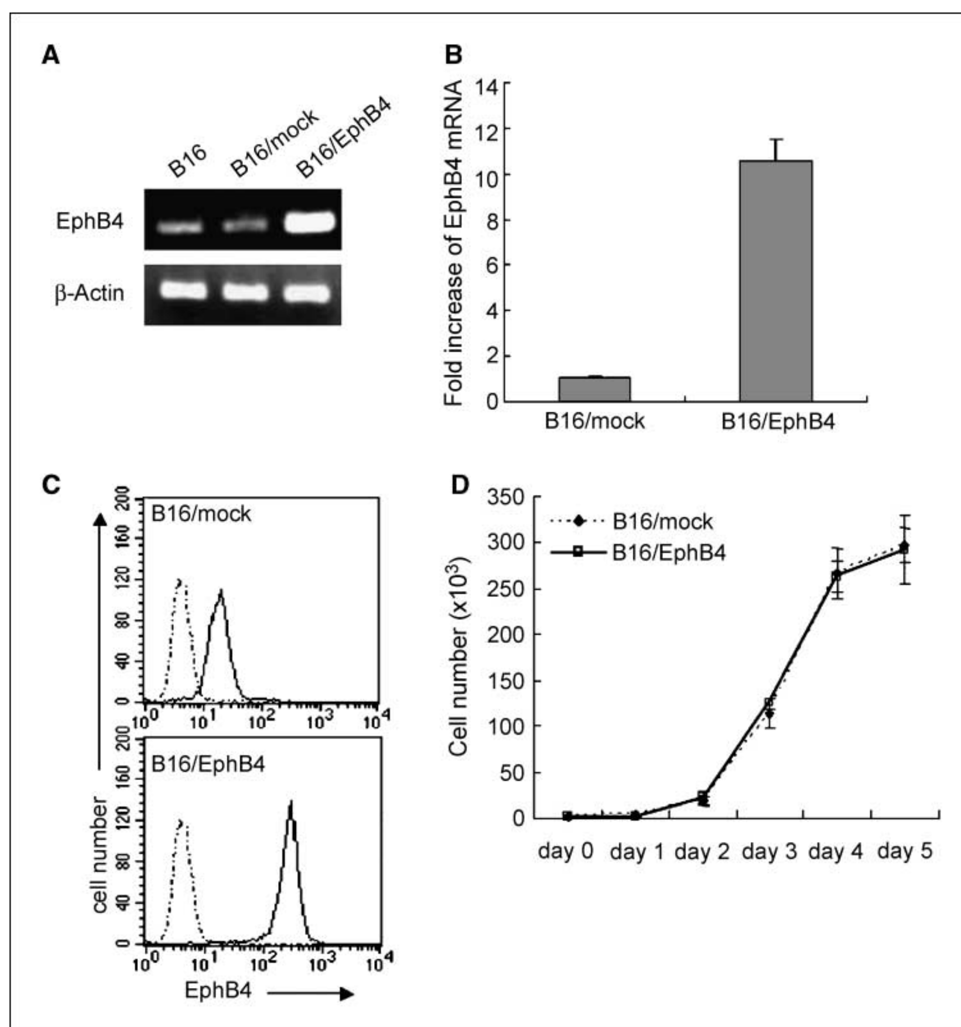

Figure 2. Effect of EphB4 overexpression in B16 cells on *in vitro* proliferation. B16 cells were transfected with mock (B16/mock) or *EphB4* (B16/EphB4) expression vectors. *A,* RT-PCR analysis of *EphB4* mRNA expression in B16, B16/mock, or B16/EphB4 cells. *B,* real-time PCR analysis of *EphB4* mRNA in B16/mock or B16/EphB4 cells. *C,* FACS analysis of *EphB4* protein expression by EphB4 mAb in B16/mock or B16/EphB4 cells. *Dashed line,* intensity of negative control. *D, in vitro* proliferation analysis.

react with EphB4. Moreover, to detect the expression of EphB4 in hind limb muscle from LacZ reporter mice (2), β-galactosidase–positive ECs were exclusively stained with anti-EphB4 antibody (Fig. 1*E*). Taken together, we concluded that this mAb (VEB4-7E4) reacts specifically with EphB4.

EphB4 overexpression did not alter *in vitro* proliferation of B16 melanoma cells. We looked for a tumor cell line that endogenously expresses EphB4 but not ephrinB2. Among several mouse and human tumor cell lines tested, we found that B16 mouse melanoma cells matched these criteria (Supplementary Table S1). Therefore, using B16 melanoma cells, we first observed whether overexpression of EphB4 in B16 cells affects cell growth. Overexpression of EphB4 stably transduced in B16 cells (B16/EphB4) was compared with that in control B16 cells transduced with mock vector (B16/mock) by RT-PCR (Fig. 2*A*) and real-time PCR (Fig. 2*B*) analyses at the mRNA level. Overexpression of EphB4 in B16/EphB4 cells was also confirmed by FACS analysis at the protein level (Fig. 2*C*). An *in vitro* proliferation of B16/EphB4 and B16/mock cells was observed. Results indicated that B16 overexpression did not alter the growth of B16 cells (Fig. 2*D*). We cloned several EphB4-overexpressing B16 cells and there was no difference from the results obtained with the subclones (data not shown).

EphB4 overexpression suppressed tumor growth in *in vivo* xenograft assay. To investigate the function of EphB4 in B16 cells *in vivo*, B16/EphB4 and B16/mock cells were injected s.c. into C57BL/6 mice. To observe tumor growth, the tumor size was measured every 3 days. Eighteen days after inoculation of tumor cells, the tumors were removed from the mice and the tumor mass was weighed. Although EphB4 overexpression did not affect the *in vitro* growth of B16 cells (see above), the *in vivo* growth of B16/EphB4 cells was clearly inhibited in comparison with that of B16/mock cells (Fig. 3*A*).

Although EphB4 protein expression level in parental B16 cells was low (Fig. 2*C*), to confirm dose dependency of EphB4 in tumor growth, we knocked down the *EphB4* gene in B16 cells and then studied *in vitro* and *in vivo* growth. As shown in Fig. 3*B*, on transfection with plasmids containing shRNA oligonucleotides that specifically recognized *EphB4* sequences, *EphB4* expression of B16 cells (B16/shRNA) was reduced compared with the level of expression when the cells were transfected with a plasmid containing nonspecific shRNA (B16/scr). *In vitro* cell proliferation was not affected by the depletion of EphB4 from B16 cells (Fig. 3*B, middle*); however, we confirmed that, *in vivo*, EphB4 depletion in B16 cells enhanced tumor growth (Fig. 3*B, right*). These results clearly indicated that reduction of EphB4 expression accelerates tumor growth in B16 cells.

EphB4 overexpression in B16 cells inhibits formation of blood vessels with an arterial phenotype in tumors. To clarify the

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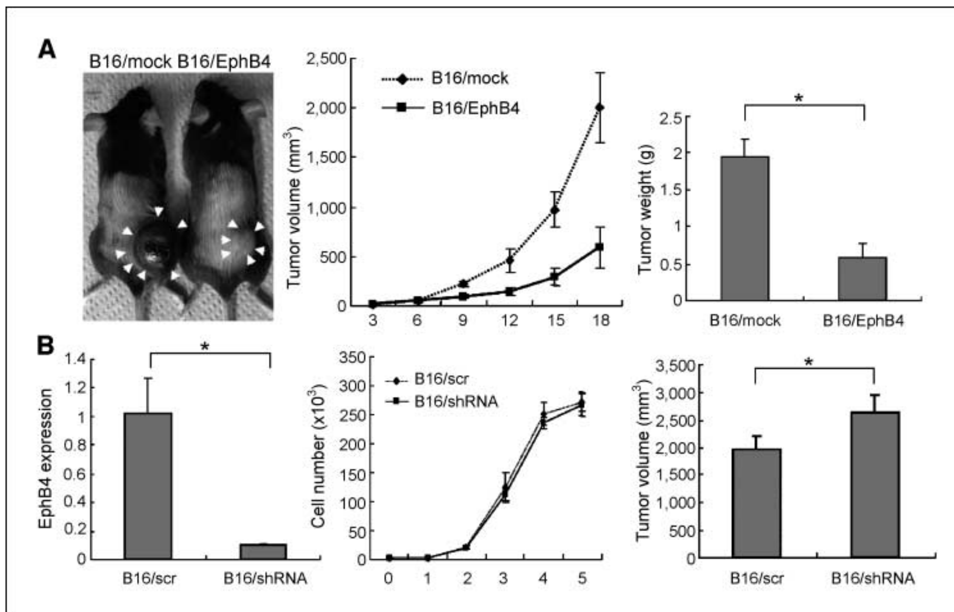

Figure 3. Tumor growth in inverse proportion to the expression levels of EphB4. *A, left,* gross appearance of tumors derived from B16/mock or B16/EphB4 cells on day 18 after tumor cell inoculation. *Arrows,* tumor area. *Middle,* tumor volume measured every 3 d after tumor cell inoculation. *Right,* tumor weight on day 18 after tumor cell inoculation. *, $P < 0.05$ ($n = 5$). *B, left,* real-time RT-PCR analysis of *EphB4* mRNA expression in B16 cells transduced by an *EphB4*-specific shRNA (B16/shRNA) or a nonspecific insert (B16/scr). *, $P < 0.05$ ($n = 3$). *Middle, in vitro* proliferation analysis. *Right,* tumor volume was determined on day 18 after s.c. inoculation of B16/shRNA or B16/scr cells into C57BL/6 mice. *, $P < 0.05$ ($n = 5$).

suppressive effect on tumor growth of EphB4 overexpression in B16 cells, we stained tumor tissues with anti-CD31 antibody and found that the number of blood vessels in tumors from B16/EphB4 cells was almost half that found in tumors from B16/mock cells (Fig. 4*A* and *B*). On the other hand, the difference in vessel density between B16/mock and B16/shEphB4 was not large compared with that between B16/mock and B16/EphB4; however, vessel density was slightly, but statistically significantly, higher in the B16/shEphB4 tumor than in the control B16/mock tumor (Fig. 4*A* and *B*).

These results suggested that EphB4 overexpression in B16 cells suppressed tumor angiogenesis.

Nevertheless, the method by which EphB4 affects blood vessel development was not clear. To investigate the properties of blood vessels in tumors, we inoculated B16/EphB4 and B16/mock cells s.c. into LacZ reporter mice to detect the expression of ephrinB2 (4) or EphB4 (2), together with anti-CD31 antibody, in tumor sections. As shown in Fig. 5, in tumors derived from B16/mock cells, nearly half of the ECs were ephrinB2$^+$ and had an arterial phenotype. On

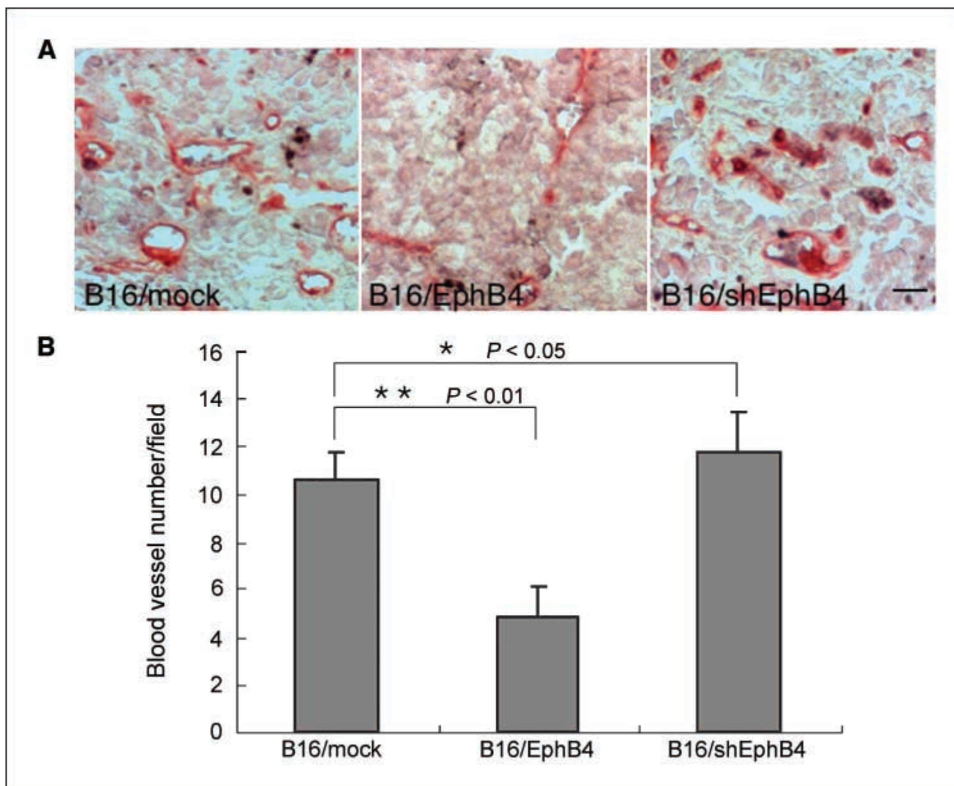

Figure 4. Effect of EphB4 overexpression in B16 cells on tumor angiogenesis. *A,* blood vessel formation in tumors derived from B16/mock (*left*), B16/EphB4 (*middle*), or B16/shEphB4 (*right*) cells. Sections were obtained from the tumors on day 18 after inoculation with the respective tumor cells and stained with anti-CD31 mAb (*red*). *Bar,* 40 μm. *B,* quantitative evaluation of blood vessels from 30 random fields of three independent tumors, as indicated. *, $P < 0.05$; **, $P < 0.01$.

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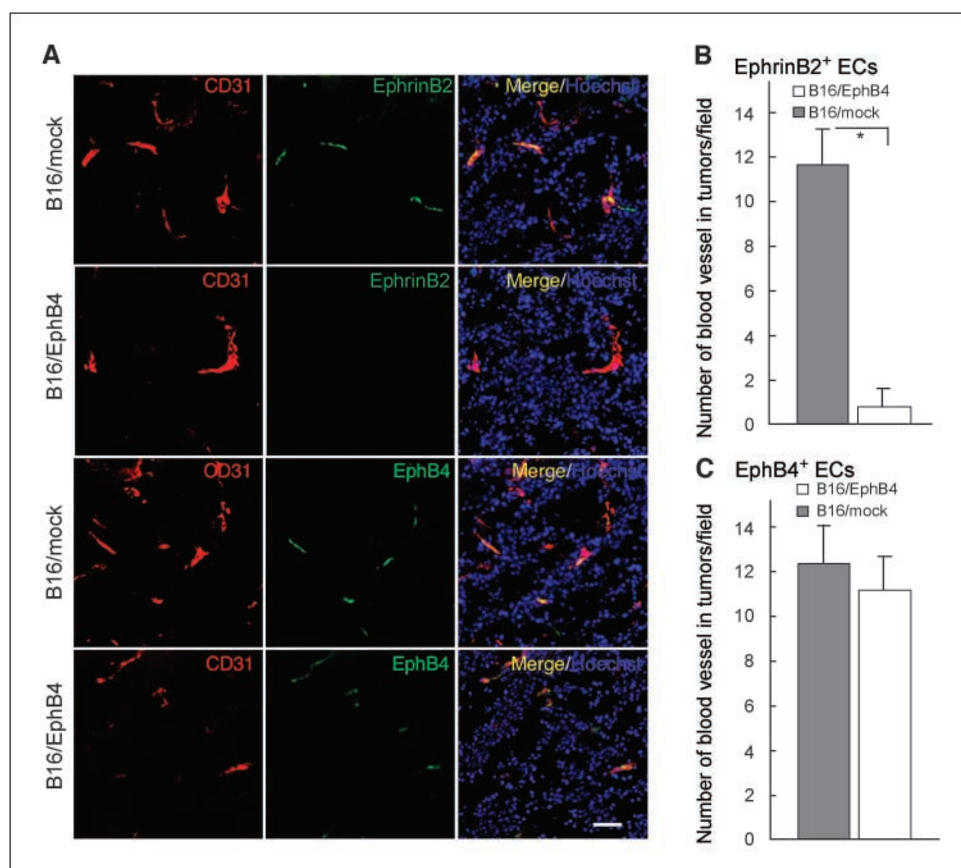

Figure 5. Effect of EphB4 overexpression in B16 cells on the arterial-venous patterning in tumors. *A,* sections derived from tumors generated by s.c. inoculation of B16/mock (*top lane*) or B16/EphB4 (*second lane*) into ephrinB2$^{LacZ/+}$ mice for 18 d were doubly stained with anti-CD31 (*red*) and anti–β-galactosidase (*green*) antibodies. Merged image with CD31 and β-galactosidase staining was shown as indicated. Nuclei were counterstained with Hoechst. Sections derived from tumors generated by s.c. inoculation of B16/mock (*third lane*) or B16/EphB4 (*bottom lane*) into EphB4$^{LacZ/+}$ mice after 18 d were doubly stained with anti-CD31 (*red*) and anti–β-galactosidase (*green*) antibodies. Merged image with CD31 and β-galactosidase staining was shown as indicated. Nuclei were counterstained with Hoechst. *Bar,* 40 μm. *B* and *C,* quantitative evaluation of ephrinB2$^+$ arteries (*B*) or EphB4$^+$ veins (*C*) from 30 random fields of three independent tumors, as indicated. *, $P < 0.05$.

the other hand, in B16/EphB4 tumors, ephrinB2$^+$ ECs were difficult to detect; only ~6% of ECs in these tumors expressed ephrinB2. By contrast, EphB4$^+$ ECs having a venous phenotype were observed in equal numbers in tumors derived from both B16/mock and B16/EphB4 cells. Therefore, we concluded that EphB4 in B16 cells selectively suppressed the formation of blood vessels with an arterial phenotype.

EphB4 in B16 cells induces apoptosis of ephrinB2$^+$ ECs. Because EphB4 suppressed the appearance of ephrinB2$^+$ ECs in tumors, the final aspect we investigated was how EphB4 overexpression in B16 cells might be affecting ephrinB2$^+$ cells *in vitro*. To achieve this, we induced ephrinB2 into the murine SVEC EC line and generated stably and strongly ephrinB2-expressing ECs (SVEC/ephrinB2), because endogenous ephrinB2 expression was weak in the original SVECs (Supplementary Fig. S1), and cocultured these cells with B16/mock or B16/EphB4 cells (Fig. 6). After coculturing for 24 h, apoptosis of SVEC/ephrinB2 in B16/mock cells or B16/EphB4 cells, B16/mock cells cocultured with SVEC/ephrinB2, and B16/EphB4 cells cocultured with SVEC/ephrinB2 cells was observed by staining with Annexin V and nuclear dye DAPI (Fig. 6*A* and *B*). Results showed that B16/EphB4 cells enhanced apoptosis (Annexin V$^+$DAPI$^-$) and cell death (Annexin V$^+$DAPI$^+$) in SVEC/ephrinB2 cells compared with B16/mock cells (Fig. 6*A* and *B*). When compared with cell death of SVEC/ephrinB2 in B16/mock cells, similar apoptosis and cell death of SVEC/ephrinB2 cells in normal culture conditions (i.e., SVEC/ephrinB2 cells not cocultured with B16 cells) was observed (data not shown). The apoptotic effect of B16/EphB4 cells on SVEC/ephrinB2 cells in this coculturing system was suppressed by the separation of the two cell types by means of a 0.4-μm-pore filter (Fig. 6*A* and *B*). Moreover, blockade of interactions between SVEC/ephrinB2 and B16/EphB4 cells with soluble EphB4 proteins abolished the B16/EphB4-mediated increase in cell death of SVEC/ephrinB2 (Fig. 6*A* and *B*). Therefore, we concluded that apoptosis of SVEC/ephrinB2 cells was accelerated by cell-to-cell contact with B16/EphB4 cells.

On the other hand, in the case of B16 cells, the percentage (Fig. 6*A* and *B*) and absolute cell number (data not shown) of apoptotic and dead cells from B16/EphB4 cells cocultured with SVEC/ephrinB2 cells were not statistically significantly different compared with those from B16/mock cells cocultured with SVEC/ephrinB2 cells. Moreover, such apoptosis and cell death was also observed in B16/mock and B16/EphB4 under normal culture conditions without coculturing with SVEC/ephrinB2 cells (data not shown). This suggested that ephrinB2 does not affect EphB4 in B16 cells, in terms of cell viability, as a forward signaling.

Taken together, we concluded that EphB4 overexpression in B16 melanoma cells induces cell apoptosis of ephrinB2$^+$ ECs. Therefore, when inoculated into mice, B16/EphB4 cells might suppress the survival of ephrinB2$^+$ ECs, resulting in insufficient arterial-venous blood vessel distribution and the inhibition of tumor growth.

Discussion

Although the EphB4 receptor has been reported to be expressed in various tumor cells (14–19), its exact function in arterio-venous specification has not yet been elucidated. In this study, we showed

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that EphB4 overexpression in B16 melanoma cells suppressed tumor angiogenesis, especially the development of ephrinB2$^+$ ECs with an arterial phenotype in tumors. By coculturing ephrinB2-expressing ECs (SVEC induced with ephrinB2) with EphB4-overexpressing B16 cells, we found that EphB4 reverse signaling via ephrinB2 was involved in the apoptosis of ephrinB2$^+$ ECs. Ideally, it was better to coculture ephrinB2$^+$ ECs in tumors derived from B16 cells with B16/EphB4 cells to examine the nature of the arterial ECs affected by EphB4 in tumor cells. However, due to technical problems, we experienced difficulties in culturing primary ECs from tumors *in vitro*. Moreover, there was no antibody available to isolate ephrinB2$^+$ ECs from tumors using a cell sorter. Therefore, we used SVEC, an EC line, stably and strongly induced with ephrinB2.

Thus far, a variety of functions of Eph receptors and their ephrin ligands have been reported in the areas of cell migration, repulsion, and adhesion (5, 6, 8, 31, 32). Although it has been proposed that this receptor/ligand system is not involved in cell proliferation, nevertheless, it has also been reported that ephrin-A/EphA receptor signaling plays a key role in controlling the size of the mouse cerebral cortex by regulating cortical progenitor cell apoptosis (33). Moreover, we previously reported that EphB4 reverse signaling via ephrinB2 inhibited the mitotic activity of ephrinB2$^+$ ECs (25). Because of bidirectional signals termed forward signaling via EphB4 and reverse signaling via ephrinB2, we suggested that EphB4/ephrinB2 works on the arterial-venous interface through this bidirectional repulsion to form the arterial-venous boundary. However, in this report, we have clearly shown that reverse signaling via ephrinB2 led to the apoptosis of ephrinB2$^+$ cells (SVEC/ephrinB2), whereas forward signaling via EphB4 in tumor cells did not induce the apoptosis of EphB4$^+$ tumor cells (B16/EphB4). Taken together, this suggested that reverse signaling via ephrinB2 in ECs might terminate arteriogenesis during angiogenesis or induce regression of newly developed arterial blood vessels.

The function of EphB4/ephrinB2 in the development of blood vessel formation during embryogenesis has been the focus of research (2–4). In addition, its role in tumor angiogenesis has also been examined by a variety of methods, such as systemic or local administration of soluble EphB4 receptors (34, 35), site-specific expression of EphB4 or kinase-dead EphB4 in ECs retrovirally (36), and a xenograft model using breast cancer cells expressing kinase-dead EphB4 receptor (37). In the case of soluble EphB4 administration, tumor angiogenesis was perturbed (34, 35). This effect might result from the suppression of forward and reverse signaling via EphB4 and ephrinB2, respectively. Recently, the enhancement of EphB4 reverse signaling via ephrinB2 in ECs, in which EphB4 was ectopically induced in the ECs of tumors, has been reported to increase vascular density and permeability of blood vessels in tumors (36). These results indicated that EphB4/ephrinB2 signaling is active in tumor angiogenesis. However, the function of EphB4 in tumor cells for blood vessel formation has not been well clarified. One piece of research has shown that kinase-dead EphB4 expressed in tumor

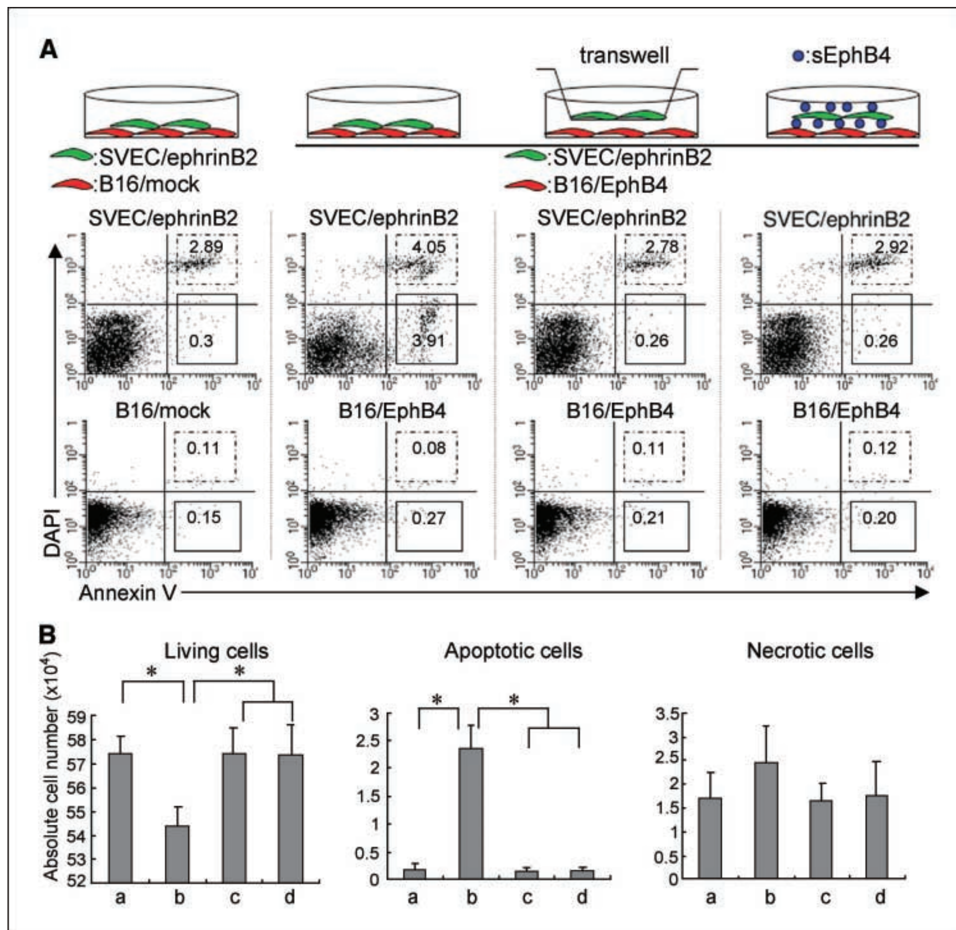

Figure 6. Effect of EphB4 overexpression in B16 cells on ephrinB2$^+$ SVECs as a reverse signaling via ephrinB2. *A, first* and *second columns,* direct coculture of SVEC/ephrinB2 cells with B16/mock or B16/EphB4 cells, respectively. *Third column,* indirect coculture of SVEC/ephrinB2 cells with B16/EphB4 cells separated with a transmembrane having 0.4-μm pores. *Fourth column,* direct coculture of SVEC/ephrinB2 cells with B16/EphB4 cells in the presence of soluble EphB4 (sEphB4). Schematic presentation of coculturing is presented on the top. B16 tumor cells or SVECs were prelabeled with PKH26 red fluorescence or PKH67 green fluorescence, respectively. After 24 h of coculture, cells gated by PKH67 or PKH26 discrimination (Supplementary Fig. S2) were analyzed by staining with anti-Annexin V-Cy5 and DAPI by FACS. Numbers in each quadrant indicate the percentage of Annexin V$^+$DAPI$^-$ apoptotic cells (*solid squares*) or Annexin V$^+$DAPI$^+$ dead cells (*dashed squares*) among the total cells. *B,* absolute number of living, apoptotic, or necrotic SVEC/ephrinB2 cells among the number of harvested whole cells in each culture condition as in *A*. *a* to *d,* culture conditions described in *A*: *a,* left; *b,* second; *c,* third; *d,* right. *, $P < 0.05$ ($n = 3$).

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cells, in which only reverse signaling via ephrinB2 was enhanced, increased tumor angiogenesis and tumor growth (37). At first glance, there seems to be a discrepancy between these data and those presented here. However, in our experiment, wild-type EphB4 overexpression in B16 tumor cells, in which both forward signaling via EphB4 for tumor cells and reverse signaling via ephrinB2 for ECs cells were enhanced, suppressed the development of ephrinB2$^+$ blood vessels and resulted in the retardation of tumor growth. Therefore, this suggests that secondary inside-out signaling molecules from EphB4$^+$ tumor cells, through forward signaling via EphB4, may induce apoptosis of ephrinB2$^+$ cells with reverse signaling. Recently, it was reported in humans that EphB4 was expressed in human colonic crypts and in early colorectal cancer lesions and that EphB4 expression was lost in advanced colorectal tumors (15). This examination correlates with our data and suggests that, within tumors, the expression of EphB4 inhibits arteriogenesis whereas the loss of EphB4 permits the supply of oxygen and nutrients by establishing arterial-venous circulation.

Recently, it was reported that the expression level of EphB4 in human ovarian cancers is correlated with poor response to chemotherapy (19). Our results can offer two possible, complementary, explanations for this finding. From our study, it seems that EphB4 overexpression in tumor cells inhibits the organization of arterio-venous patterning in tumors, which could increase the difficulty anticancer drugs have in penetrating deep into tumor tissue, whereas, additionally, this overexpression has an inhibitory effect on rapid tumor growth. It is well known that blood vessels in tumors are disorganized compared with those observed in normal tissue, and permeability is relatively suppressed in the tumor environment. Recently, a new therapeutic concept has emerged whereby the artificially induced normalization of blood vessels in tumors could allow the penetration of anticancer drugs deep into the site of the tumors (38); normalization of arterial-venous patterning might be one such strategy for cancer therapy. In this study, we used a B16 mouse melanoma cell line to observe the function of EphB4 in ephrinB2$^+$ EC development precisely because of the lack of ephrinB2 expression in B16 cells. In humans, there are no published reports showing EphB4 expression in primary melanomas but several articles have indicated that most human melanoma cell lines express EphB4 (14, 39). However, although coexpression of EphB4/ephrinB2 in human melanoma tissue samples or melanoma cell lines has not been reported, in both settings the expression level of ephrinB2 has been reported to vary, and it has been proposed that expression level correlates with malignancy (14, 40). The functions of ephrinB2 and EphB4 in melanomas seem to be redundant for tumorigenesis in which ephrinB2 and EphB4 are associated with both tumor cell viability and generation of microenvironment. To further clarify the function of the EphB4/ephrinB2 system, it will be necessary to examine how EphB4 and ephrinB2 expression in tumor cells is regulated during the ontogeny of tumors.

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