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ORIGINAL PAPER

VEGFR2 activation mediates the pro-angiogenic activity of BMP4

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Abstract

The Bone Morphogenetic Protein 4 (BMP4) regulates multiple biological processes, including vascular development and angiogenesis. Here, we investigated the role of Vascular Endothelial Growth Factor Receptor 2 (VEGFR2) in mediating the angiogenic activity of BMP4. BMP4 induces a rapid relocation and phosphorylation of VEGFR2 on the endothelial cell membrane. These effects occur in the absence of a direct interaction of BMP4 and/or BMP receptors with VEGFR2. At variance, BMP4, by interacting with the BMPRI-II hetero-complex, induces c-Src phosphorylation which, in turn, activates VEGFR2, leading to an angiogenic response. Accordingly, the BMPR inhibitor dorsomorphin prevents c-Src activation and specific inhibition of c-Src significantly reduces downstream VEGFR2 phosphorylation and the angiogenic activity exerted by BMP4 in a chick embryo chorioallantoic membrane assay. Together, our data indicate that the pro-angiogenic activity exerted by BMP4 in endothelial cells is mediated by a BMPR-mediated intracellular transactivation of VEGFR2 via c-Src.

Keywords Angiogenesis · BMP4 · VEGFR2 · c-Src

Introduction

Angiogenesis, the process of new blood vessel formation from pre-existing vessels, plays a key role in various physiological and pathological conditions, including embryonic development, wound healing, diabetic retinopathy, arteriosclerosis, tumor growth, and metastasis [1]. Several growth factors, including vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF), cytokines, and chemokines orchestrate the angiogenic process by interacting with their receptors on the membrane of endothelial cells (ECs) [2].

VEGF receptor 2 (VEGFR2), a tyrosine kinase receptor mainly expressed on ECs, modulates several physiological responses such as cell proliferation, survival, migration, and differentiation by binding its canonical and non-canonical ligands [3, 4]. In addition, a rapid ligand-independent c-Src-mediated VEGFR2 activation has been described in various cell types, including embryonic [5], endothelial [6, 7], and tumor cells [8, 9]. For instance, the parathyroid hormone-related protein (PTHrP) modulates osteoblastic functions in human osteosarcoma via c-Src-dependent transactivation of VEGFR2 [8, 9]. Moreover, sphingosine-1-phosphate (S1P1) induces mouse embryonic stem cell proliferation through the transactivation of VEGFR2 mediated by a S1P1/3-dependent β-arrestin/c-Src pathway [5]. Together, these data point to a newly identified non-conventional mechanism of activation of VEGFR2, whose contribution to the angiogenic process has not been fully elucidated.

The Bone Morphogenetic Protein (BMP) family is composed by several growth factors which regulate multiple biological processes, including dorso-ventral axis formation [10] and the development of mesoderm-derived cell lineages during embryonic development [11]. Accordingly, mice lacking BMP4, BMP receptor type I receptor (BMPRI,

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also known as ALK3), or type II (BMPRII) fail to initiate gastrulation or show defects in mesoderm differentiation (see [12] and references therein). In addition, BMP signaling affects EC behavior in both pro- and anti-angiogenic ways. Indeed, BMPs regulate the permeability of vascular networks [13–15], promote angiogenesis in in vivo murine and avian models [16, 17], and enhance neovascularization in developing tumors [18]. Also, mutations in BMP signaling molecules are associated with vascular pathological conditions such as type 1 and 2 hemorrhagic hereditary telangiectasia [19, 20] and pulmonary arterial hypertension [21]. On the contrary, BMP9 inhibits angiogenesis by reducing microvascular EC migration and neovascularization both in vitro and in vivo [22].

In this frame, the binding of BMP4 to a hetero-tetrameric receptor complex formed by two BMPRI and two BMPRII serine-threonine kinase receptors plays a central role in vascular development [23, 24]. Nevertheless, the effect of BMP4 on vascular homeostasis and angiogenesis is still controversial. On one hand, BMP4 increases reactive oxygen species (ROS) production leading to EC apoptosis [25] and contributes to EC dysfunctions by promoting protein carbonylation [26]. Also, BMP4 inhibits experimental choroidal neovascularization by modulating VEGF and MMP-9 expression [27]. On the other hand, BMP4 induces EC activation, recruits blood vessels in tumors, and regulates vasculogenic mimicry in malignant melanoma [28]. Furthermore, several evidences demonstrate that BMP4 increases bovine aortic and human microvascular EC proliferation, drives their morphogenesis in tubular-like structures [29], and exerts a pro-angiogenic activity in the chick embryo chorioallantoic membrane (CAM) assay [17]. Relevant to this point, BMP4 induces a significant increase in VEGFR2 expression which mediates, at least in part, the BMP-dependent EC activation [30].

Here, we investigated the role of VEGFR2 in mediating the angiogenic activity of BMP4. Our data demonstrate that BMP4, by interacting with the BMPRI-BMPRII heterocomplex, induces c-Src phosphorylation. In turn, c-Src activation mediates the early phases of the angiogenic response to BMP4, including VEGFR2 phosphorylation, leading to EC activation.

Materials and methods

Reagents

All reagents used were of analytical grade. M199, DMEM, and SFM media and fetal calf serum (FCS) were purchased from GIBCO Life Technologies (Grand Island, NY). Endothelial cell growth factor, porcine gelatin, porcine heparin, methylcellulose, bovine fibrinogen, thrombin, aprotinin, 4′,6-diamidino-2-phenylindole (DAPI), PP2, and Dorsomorphin were purchased from Sigma-Aldrich (St. Louis, MO). PVP-free polycarbonate filters were obtained from Costar (Cambridge, MA). Diff-Quik reagent was obtained from Dade-Behring (Deerfield, IL). Recombinant human VEGF (VEGF-A_{165} isoform) and pcDNA3.1 vector harboring VEGFR2-HA were kindly provided by K. Ballmer-Hofer (PSI, Villigen, Switzerland). Anti-phosphotyrosine (4G10 clone) and anti-HA-tag antibodies were purchased from Upstate Biotechnology (Lake Placid, NY). Anti-phospho-tyrosine (PY99 clone), anti-phospho-c-Src (pTyr 418 clone), anti-Src, anti-BMPR-I, anti-BMPR-II, and anti-GAPDH antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phospho-VEGFR2 (pTyr 951 and pTyr1175 clones), anti-SMAD1, anti-phospho-SMAD1,5,8, and anti-phospho-$ERK_{1/2}$ antibodies were purchased from Cell Signaling Technology (Beverly, MA). Anti-VEGFR1, anti VEGFR2, and anti-VEGFR3 were from Abcam (Cambridge, UK). Alexa Fluor antibodies were obtained from Molecular Probes (Eugene, OR). Recombinant BMP4, BMP7, and anti-BMP4 antibody were purchased from R&D system (Minneapolis, MN). VEGFR2-KI and SU5416 were obtained from Calbiochem (La Jolla, CA). FGF2 was purchased from Tecnogen (Caserta, Italy). Bis(sulfosuccinimidyl)suberate (BS3) cross-linker was obtained from Thermo Fisher Scientific (Waltham, MA).

Surface plasmon resonance (SPR) analysis

A BIAcore X apparatus (BIAcore Inc., Piscataway, NJ) was used to analyze VEGF-A and BMP4 interaction with sVEGFR2 D1-7 (sVEGFR2, Calbiochem Biochemical) immobilized to the sensorchip. To this purpose, recombinant human sVEGFR2 (40 µg/mL in 10 mM sodium acetate, pH 5.3) was allowed to react with a flow cell of a CM5 sensorchip that was previously activated with a mixture of 0.2 M *N*-ethyl-*N*′-(3-dimethylaminopropyl)-carbodiimide hydrochloride and 0.05 M *N*-hydroxysuccinimide (35 µL, flow rate 10 µL/min). These experimental conditions allowed the immobilization of 0.083 pmol/mm^2 of sVEGFR2. After ligand immobilization, matrix neutralization was performed with 1.0 M ethanolamine (pH 8.5) (35 µL, flow rate 10 µL/min). Activated/deactivated sensorchip was used as a negative control and for blank subtraction. To allow the association with immobilized sVEGFR2, VEGF-A or BMP4 were injected in HBS-EP buffer (0.01 M Hepes pH 7.4 plus 0.005% surfactant P20, 0.15 M NaCl, 3.0 mM EDTA) over the sVEGFR2 surface for 4 min (sample volume 40 µL, flow rate 10 µL/min and dissociation time 2 min). Binding of ligand to immobilized sVEGFR2 was monitored as a function of time by tracking the SPR intensity change (response units, RU) upon binding progression.

Cell cultures

Human umbilical vein endothelial cells (HUVECs) were isolated from umbilical cords and used at early (I–IV) passages. Cells were grown on culture plates coated with porcine gelatin in M199 medium supplemented with 20% FCS, endothelial cell growth factor (10 µg/mL), and porcine heparin (100 µg/mL).

Fetal bovine aortic endothelial GM7373 cells stably transfected with pcDNA3.1 vector harboring mouse VEGFR2 or VEGFR2-HA cDNA to generate VEGFR2-overexpressing ECs were grown in DMEM medium supplemented with 10% FCS.

All cells were maintained in a humidified 5% CO_2 incubator at 37 °C, with medium replaced every 2–3 days until cells reached confluency. Cells were tested regularly for *Mycoplasma* negativity.

EC chemotaxis assay

HUVECs were seeded at 1.0×10^6 cells/mL in the upper compartment of a Boyden chamber containing gelatin-coated PVP-free polycarbonate filters (8 µm pore size). 50 ng/mL of BMP4 or BMP7 dissolved in M199 with 1.0% FCS was placed in the lower compartment in the absence or in the presence of 20 nM VEGFR2-KI. After 4 h of incubation at 37 °C, cells migrated to the lower side of the filter were stained with Diff-Quik reagent. Five random fields were counted for each triplicate sample.

Alternatively, chemotaxis filters were fixed in 4.0% paraformaldehyde (PFA) and immunostained with anti-VEGFR2 and anti-BMPRII antibodies. Migrating cells were analyzed using a LSM510 Meta confocal microscope equipped with a Plan-Apochromat 63x/1.4 NA oil objective (Carl Zeiss). 3D-reconstruction images were obtained through AxioVision Inside 4D module (Carl Zeiss).

EC motility assay

Cell motility was assessed by time lapse videomicroscopy. HUVECs were seeded at 150 cells per mm^2 in 24 well-plates for 4 h and then stimulated with 50 mg/mL of BMP4 dissolved in M199 plus 0.5% FCS in the absence or in the presence of 20 nM VEGFR2-KI. Constant temperature (37 °C) and pCO_2 (5%) were maintained throughout the experimental period by means of a heating stage and climate chamber. Cells were observed under an inverted microscope (Zeiss Axiovert 200 M) and phase-contrast snap photographs (one frame every 5 min) were digitally recorded for 6 h. Cell paths (20–30 cells per experimental point) were generated from centroid positions and migration parameters were analyzed with the *Chemotaxis and Migration Tool* of FIJI software (http://rsbweb.nih.gov/ij).

EC sprouting assay

Fibrin gel invasion assay was performed on HUVEC spheroids as described [31]. Briefly, spheroids were prepared in 20% methylcellulose medium, embedded in fibrin gel, and stimulated with 50 ng/mL of BMP4 in the absence or in the presence of 20 nM VEGFR2-KI or 1.0 µm PP2. Formation of radially growing cell sprouts was observed during the next 24 h. Sprouts were counted and photographed using an Axiovert 200 M microscope equipped with a LD A PLAN 20X/0,30PH1 objective (Carl Zeiss).

In vitro angiogenesis assay

Wells of µ-Slide Angiogenesis chamber (Ibidi) were coated with 0.8 mm thick layer of gel matrix by adding 10 µL of Cultrex Reduced Growth Factor Basement Membrane Matrix. After gel polymerization, 5000 HUVECs were seeded in M199 added with 5.0% FCS and treated with 50 ng/mL of BMP4 in the absence or in the presence of 1.0 µM of PP2. After 5 h, samples were photographed using an inverted microscope Axiovert 200 M microscope equipped with a LD A PLAN 20X/0,30PH1 objective (Carl Zeiss).

Artery ring assay

One-millimeter human umbilical artery rings were embedded in fibrin gel and cultured in human EC medium SFM [32] with 50 ng/mL of BMP4 in the absence or in the presence of 20 nM VEGFR2-KI. After 3 days, EC sprouts were counted under an inverted microscope Axiovert 200 M microscope equipped with a LD A PLAN 20X/0,30PH1 objective.

Western blotting and receptor immunoprecipitation

Confluent HUVECs or VEGFR2-HA-overexpressing GM7373 ECs were made quiescent by a 20 h-starvation and stimulated with BMP4. When indicated, BS3 cross-linker was added during the stimulation. After the treatment, cells were lysed in 50 mmol/L Tris–HCl buffer (pH 7.4) containing 1.0% Triton-X100, 0.1% BriJ, 1.0 mM sodium orthovanadate, and protease inhibitor cocktail. Aliquots of each sample containing equal amount of proteins (50–100 µg) were subjected to SDS-PAGE. Western blotting analysis was performed with anti-BMP4, anti-HA-tag, anti-phospho-SMAD1,5,8, anti-phospho-c-Src (pTyr418), anti-GAPDH, anti-phospho-VEGFR2 (pTyr951), anti VEGFR1, anti-VEGFR2, or anti VEGFR3 antibodies. For immunoprecipitation experiments, 1.0 mg of lysates were immunoprecipitated with anti-BMPRI, anti-BMPRII, or anti-phospho-Tyr antibodies, separated by SDS-PAGE and

probed with anti-BMP4, anti-VEGFR2, anti-BMPRI, or anti-BMPRII antibodies.

Immunofluorescence analysis

HUVECs were seeded on gelatin-coated glass coverslips in M199 added with 5.0% FCS. After O/N incubation, cells were treated with 50 ng/mL BMP4 for 15 min at 37 °C, washed, fixed in 4.0% paraformaldehyde (PFA)/2.0% sucrose in PBS, permeabilized with 0.5% Triton-X100, and saturated with goat serum in PBS. Then, cells were incubated with anti-phospho-VEGFR2 (pTyr1175) antibody. Cells were analyzed using a Zeiss Axiovert 200 M epifluorescence microscope.

As for receptor relocation experiments, HUVECs were seeded on fibrinogen- or BMP4-coated glass coverslips. Adherent cells were fixed, permeabilized, and incubated with anti-VEGFR2 or anti-BMPRII antibodies. Cells were analyzed with a LSM510 confocal microscope (Carl Zeiss) equipped with Plan-Apochromat 63x/1.4 NA oil objective. Importantly to collect signal only from the cell ventral membrane in close contact with ligand-enriched matrix, pinhole was closed to obtain 100 nm Z plane.

Ventral plasma membrane (VPM) preparation

Glass coverslips were coated with 2.0 µg/mL BMP4. After 16 h of incubation at 4 °C, the solution was removed and the coverslips were incubated for further 30 min at 37 °C with 1.0 mg/mL BSA in PBS. VEGFR2-overexpressing GM7373 cells (75,000/cm^2 in DMEM containing 1.0% FCS) were allowed to adhere to the coverslips for 6 h in the absence or in the presence of 20 nM VEGFR2-KI or dorsomorphin. VPMs were prepared using a modification of the squirting lysis technique [33]. Briefly, cells were washed twice with ice-cold water; after 1 min, cells were squirted over by using a jet of ice-cold water from a water bottle, and immediately fixed for immunocytochemistry analysis.

ELISA assay

Anti-VEGFR2 antibody was absorbed onto a 96 well plate overnight at 4 °C. After blocking with 1 mg/mL BSA, 100 µL of lysates of BMP4-stimulated HUVECs were added and incubated for 1 h. Bound phospho-VEGFR2 was detected with an anti-phospho-VEGFR2 (pTyr951) antibody.

As for competition experiments, 1.0 µM VEGF-A was overnight added onto the anti-VEGFR2 coated plate in the presence of increasing concentrations of BMP4, Gremlin, or FGF2. Bound VEGF was detected with an anti-VEGF antibody.

Chick embryo chorioallantoic membrane (CAM) assay

CAM assay was performed as previously described [34]. Briefly, alginate beads (4.0 µL) containing vehicle or 100 ng/embryo BMP4 in the absence or in the presence of 3.0 µL of 5.0 µM SU5416, 20 nM VEGFR2-KI, or 1.0 µM PP2 were placed on the CAM of fertilized White Leghorn chicken eggs at day 11 of incubation. After 72 h, newly formed blood vessels converging towards the implant were counted at ×5 magnification using a STEMI SR stereomicroscope equipped with an objective f equal to 100 mm with adapter ring 475070 (Carl Zeiss).

Data representation and statistical analyses

Statistical analysis was performed with GraphPad Prism 7 (San Diego, CA, USA) using one-way ANOVA followed by Tukey multiple comparison post-test or Student's *t* test. The data are expressed as mean ± SEM and p values < 0.05 were considered statistically significant.

Results

VEGFR2 mediates the pro-angiogenic activity of BMP4

In order to evaluate the involvement of VEGFR2 in the pro-angiogenic activity of BMP4, we investigated the effect of the selective VEGFR2 tyrosine kinase inhibitor (VEGFR2-KI) [35] in various BMP4-mediated angiogenesis assays. As shown in Fig. 1a, b, VEGFR2-KI hampers the chemotactic response of human umbilical vein ECs (HUVECs) to BMP4. Accordingly, VEGFR2-KI significantly inhibits the motility of BMP4-treated HUVECs, as assessed by time lapse microscopy (Fig. 1c, d and the related videos in the Online Resources 1, 2). Furthermore, VEGFR2-KI prevents the sprouting of fibrin-embedded HUVEC spheroids, a 3D-assay that recapitulates in vitro the proteolytic, migratory and proliferative events that characterize the angiogenic process (Fig. 1e, f) [31]. In keeping with these observations, VEGFR2-KI inhibits the angiogenic response in an ex vivo angiogenesis assay in which fibrin-embedded human umbilical cord artery rings [32] are grown in the presence of BMP4 (Fig. 1g, h).

Finally, the role of VEGFR2 in mediating the pro-angiogenic activity of BMP4 was assessed in vivo in the CAM assay [34]. To this aim, alginate beads adsorbed with 100 ng/pellet of BMP4 alone or added with the tyrosine kinase VEGFR2-inhibitor SU5416 [36] were implanted on the top of 11-day-old chick embryo CAMs. As anticipated, BMP4 exerts a potent angiogenic response characterized by

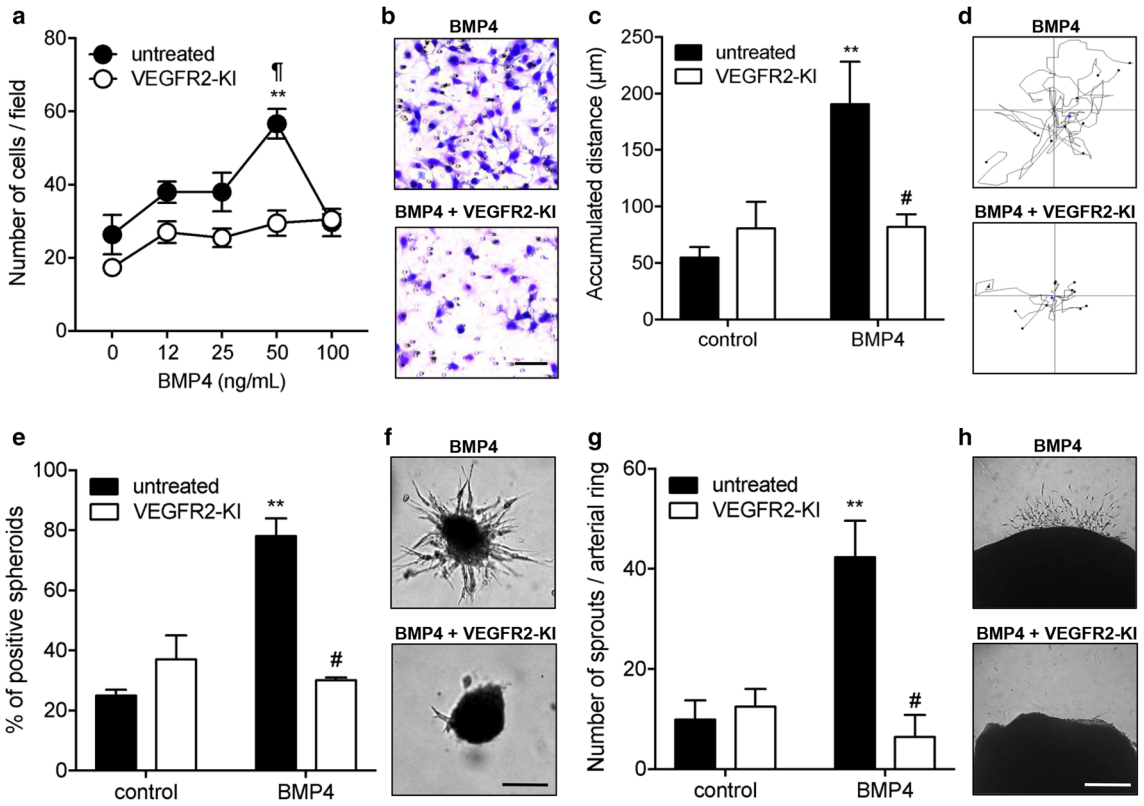

Fig. 1 VEGFR2 mediates the angiogenic activity of BMP4. **a** HUVECs were assessed for their capacity to migrate in response to different doses of BMP4 dissolved in M199 plus 1.0% FCS in the absence or in the presence of 20 nM VEGFR2-KI in a Boyden chamber. After 4 h, cells migrated to the lower side of the filter were counted. Data are the mean ± SEM of 2 independent experiments (**$p < 0.01$ vs. control; $^{¶}p < 0.01$ vs. VEGFR2-KI-treated cells, Student's *t* test). **b** Representative images of cells migrated across the PVPF filter (bar: 200 µm). **c** HUVECs were stimulated with 50 ng/mL BMP4 with or without 20 nM VEGFR2-KI and cell motility was assessed by time lapse videomicroscopy using an inverted photomicroscope (Zeiss Axiovert 200 M). Phase-contrast snap photographs were digitally recorded for 6 h. Cell paths (20–25 cells per experimental point) were generated from centroid positions and accumulated distances were analyzed with "Chemotaxis and Migration Tool" of FIJI Software. Data are the mean ± SEM of 3 independent experiments (**$p < 0.01$ vs. control, $^{#}p < 0.01$ vs. untreated, ANOVA). **d** Representative plots of cell tracks. The intersection of the x- and y-axis is the starting point for each cell track. **e** HUVEC spheroids embedded in fibrin gel were incubated with 50 ng/mL of BMP4 in the absence or in the presence of 20 nM VEGFR2-KI. Formation of radially growing sprouts was evaluated after 24 h of incubation. Data are the mean ± SEM of 3 independent experiments (**$p < 0.01$ vs. control; $^{#}p < 0.01$ vs. untreated, ANOVA). **f** Representative images of BMP4-treated spheroids in the absence (upper panel) or in the presence (lower panel) of VEGFR2-KI (bar: 100 µm). **g** One-millimeter human umbilical artery rings were embedded in fibrin gel and incubated with 50 ng/mL of BMP4 with or without 20 nM VEGFR2-KI. After 3 days, EC sprouts, morphologically distinguishable from scattering fibroblasts/smooth muscle cells, were counted at Axiovert 200 M microscope equipped with a LD A PLAN 20X/0,30PH1 objective. Data are the mean ± SEM (n = 8) (**$p < 0.01$ vs. control; $^{#}p < 0.01$ vs. untreated, ANOVA). **h** Representative images of BMP4-treated umbilical artery ring in the absence (upper panel) or in the presence (lower panel) of VEGFR2-KI (day 6) (bar: 500 µm)

the formation of newly formed vessels converging towards the implant, which was significantly impaired by SU5416 (Fig. 2a, b). Of note, neither VEGFR2-KI nor SU5416 affect cells viability (Online Resources 3). All data confirmed the involvement of VEGFR2 in BMP4-driven angiogenesis.

BMP4 induces VEGFR2 phosphorylation

The above observations prompted us to assess the possibility that BMP4 treatment may induce the activation of VEGFR2 in ECs. As shown in Fig. 3, BMP4 induces the rapid phosphorylation of VEGFR2, in the absence of significant alteration of VEGFR1, VEGFR2 expression (Online Resources 4). Indeed, VEGFR2 is detectable in the cell extract of BMP4-stimulated ECs after immunoprecipitation with an anti-phospho-tyrosine antibody (Fig. 3a). Accordingly, western blot, ELISA and immunofluorescence analyses on serum-starved BMP4-treated HUVECs demonstrate the activation of the specific Y951 and Y1175 VEGFR2 tyrosine residues (Fig. 3b–d). Also, the phosphorylation of VEGFR2 induced by BMP4 was not affected by co-treatment with the anti-VEGF antibody bevacizumab, ruling out the possibility that the observed effect was mediated by the release of endogenous VEGF or by a contamination

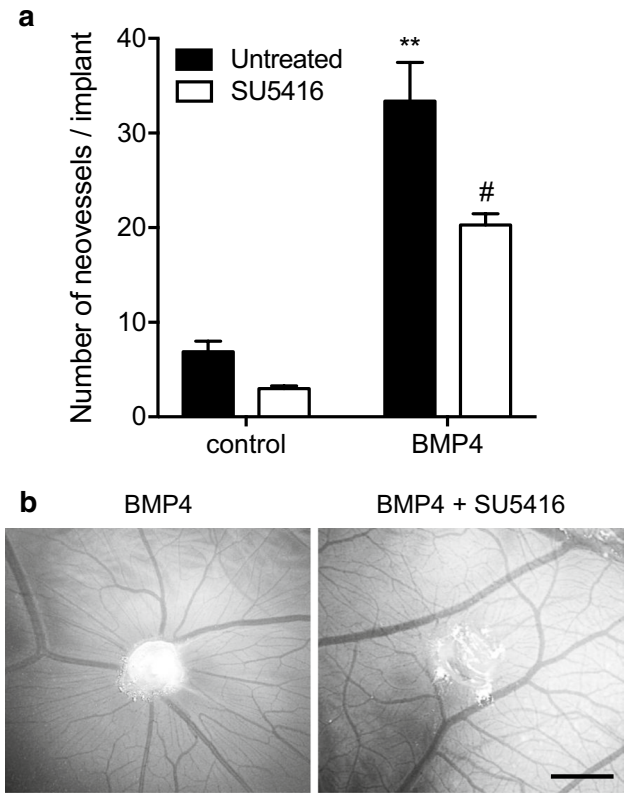

Fig. 2 VEGFR2 inhibitor reduces the BMP4-induced angiogenesis. **a** Alginate beads containing vehicle or 100 ng of BMP4 were implanted on the top of chick embryo CAMs at day 11 of development. When indicated, pellets also contained 3.0 µL of 5.0 µM SU5416. After 72 h, newly formed blood vessels converging toward the implant were counted in ovo at ×5 magnification using a STEMI SR stereomicroscope equipped with an objective f equal to 100 mm with adapter ring 475070 (Carl Zeiss). Data are the mean ± SEM ($n = 6$–8) (**$p < 0.01$ vs. control; $^{\#}p < 0.01$ vs. untreated, ANOVA). **b** Representative images of BMP4-treated CAMs in the absence (left panel) or in the presence (right panel) of SU5416 (bar: 2.0 mm)

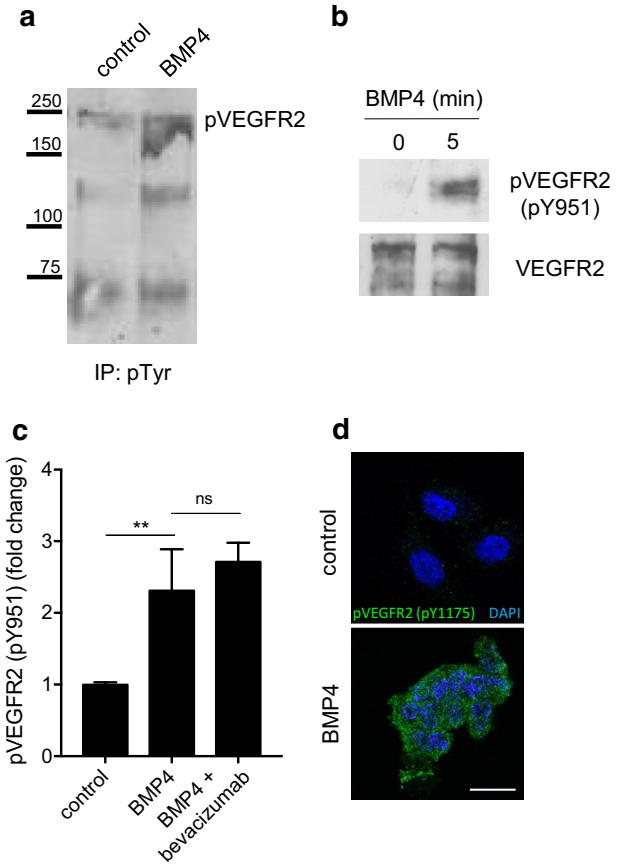

Fig. 3 BMP4 activates VEGFR2 in endothelial cells. **a** Serum-starved ECs were stimulated for 15 min with 50 ng/mL of BMP4. At the end of incubation, 1.0 mg of cell extract was immunoprecipitated with anti-pTyr antibody and probed by Western blotting with anti-VEGFR2 antibody. 250 kDa band corresponds to phosphorylated VEGFR2. **b** Serum-starved HUVECs were stimulated for 5 min with 50 ng/mL of BMP4. Cell extracts were loaded on a 6% SDS-PAGE gel. Membrane was probed with an anti-pVEGFR2 (Y951) antibody. Uniform loading was confirmed with an anti-VEGFR2 antibody. Data are representative of 2 independent experiments that provided similar results. **c** Serum-starved HUVECs were stimulated for 15 min with 50 ng/mL of BMP4 in the absence or in the presence of 10 nM bevacizumab. pVEGFR2 (Y951) levels were then assessed by ELISA. Data are the mean ± SEM of 2 independent experiments (**$p < 0.01$ vs. control, ANOVA). **d** HUVECs were seeded on gelatin-coated glass coverslips and treated for 15 min with 50 ng/mL of BMP4. After fixation, cells were incubated with anti-phospho-VEGFR2 (pTyr1175) antibody and analysis was performed using a Zeiss Axiovert 200 M epifluorescence microscope. Images show control (upper panel) or BMP4-treated (lower panel) cells (scale bar: 20 µm)

in the BMP4 preparation (Fig. 3c). To exclude the possible involvement of other VEGFRs in the modulation of BMP4 dependent angiogenesis, we investigated the phosphorylation of VEGFR1 and VEGFR3 induced by BMP4 stimulation. As shown in Online Resources 4, BMP4 does not affect the phosphorylation nor of VEGFR1 neither of VEGFR3.

In apparent contrast with VEGFR2 activation data, surface plasmon resonance (SPR) analysis indicates that, at variance with the canonical VEGFR2 ligand VEGF-A, BMP4 does not interact with the extracellular domain of VEGFR2 (sVEGFR2) immobilized to a BIAcore sensorchip (Fig. 4a). In addition, BMP4 is not able to prevent the VEGF-A/VEGFR2 interaction in a competitive ELISA assay (Fig. 4b). Finally, cell-surface cross-linking experiments failed to demonstrate the formation of a BMP4/VEGFR2 complex when BMP4 is administered to endothelial GM7373 cells overexpressing ECD-VEGFR2-HA (Fig. 4c). Together, these results suggest that BMP4 may indirectly activate VEGFR2 without binding to the receptor.

BMP4 induces the relocation of VEGFR2 on the EC membrane

Angiogenic growth factors accumulated in the extracellular matrix may cause a rapid relocation of their cognate

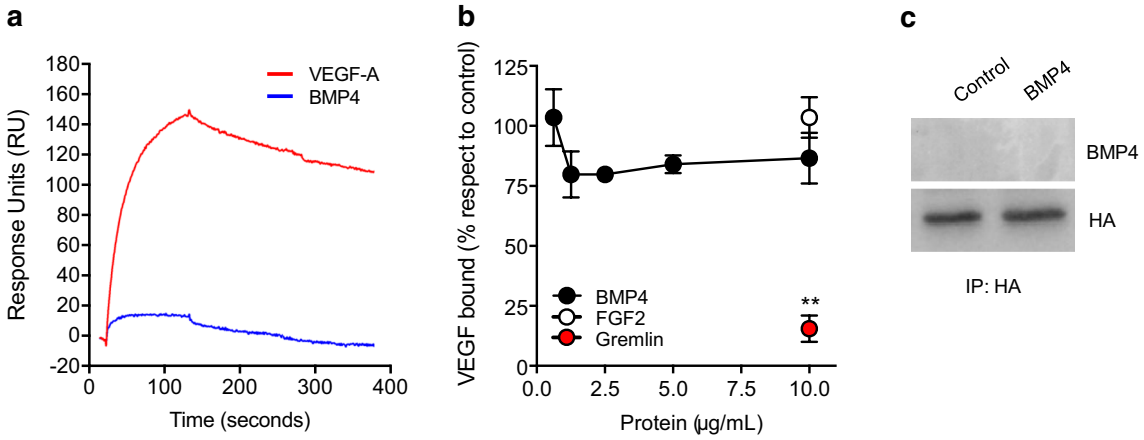

Fig. 4 BMP4 does not directly interact with VEGFR2. **a** BMP4 or VEGF-A were injected in HBS-EP buffer (BIAcore) for 4 min (sample volume: 40 μL; flow rate: 10 μL/min; dissociation time: 2 min) on sVEGFR2 functionalized CM5 sensorchip. The response (in response units RU) was recorded as a function of time. An overlay plot of all sensorgrams is shown after subtraction of their respective control sensorgrams. Data are representative of 3 independent experiments that provided similar results. **b** As for competition experiments, 1.0 μM VEGF-A was overnight added onto an anti-VEGFR2-coated 96-well plate in the presence of increasing concentrations of BMP4. Bound VEGF-A was detected with an anti-VEGF antibody. Gremlin and FGF2 were used as positive and negative controls, respectively. Data are the mean ± SEM of 3 independent experiments (**$p < 0.01$ vs. VEGF-A alone). **c** VEGFR2-HA-overexpressing GM7373 cells were stimulated with 50 ng/mL of BMP4 in the presence of BS3 cross-linker. VEGFR2 complexes, immunoprecipitated by anti-HA antibody, were loaded on a 6% SDS-PAGE gel. Membrane was probed with an anti-BMP4 antibody. Data are representative of 2 independent experiments that provided similar results

receptors on the leading edge of migrating ECs during neovessel formation, leading to the polarization of intracellular signaling molecules [37]. To evaluate VEGFR2 relocation during EC migration in response to the chemotactic stimulus exerted by BMP4, HUVECs were seeded on the upper face filter of a Boyden chamber and allowed to migrate for 2 h in response to BMP4 placed in the lower chamber. After fixation, cells on the upper face of the filter were analyzed by confocal microscopy for the presence of immunoreactive BMPRII and VEGFR2 in the cytoplasmic protrusions extending into the filter pores [37]. 3D reconstructions of the cellular protrusions demonstrate that, in response to BMP4 stimulation, 48% of BMPRII-positive protrusions are also positive for VEGFR2, meaning that BMP4 recruits VEGFR2 at the leading edge of migrating cells (Fig. 5a). In chemotaxis experiments, BMP7 was used as an alternative BMPRII ligand. The analysis of cell protrusions induced by BMP7 demonstrated that only the 3% of BMPRII-positive protrusions are also positive for VEGFR2, further supporting the specificity of BMP4 in the recruitment of VEGFR2 (Fig. 5a). In addition, in order to evaluate the capacity of BMP4 accumulated into the extracellular matrix to recruit VEGFR2 at the basal plasma membrane, confocal analysis of the basal aspect of substratum-adherent HUVECs was performed. As shown in Fig. 5b, adherent HUVECs seeded on immobilized BMP4 show a significant increase of both BMPRII and VEGFR2 recruited at the basal plasma membrane when compared to cells seeded on fibrinogen-coated wells. To further prove that VEGFR2 relocation occurs downstream to BMPRs activation, VEGFR2-overexpressing GM7373 ECs (VEGFR2-GM7373 cells) were seeded on immobilized BMP4 or uncoated coverslips in the absence or in the presence of dorsomorphin, an inhibitor of BMP signaling [38]. Then, ventral plasma membranes (VPMs) were prepared from adherent cells by osmotic shock and decorated for the presence of total VEGFR2 [33]. Absence of DAPI staining and the persistence of actin filaments were used to unequivocally identify the VPM remnants bound to the substratum. As shown in Fig. 5c, VEGFR2 is recruited in VPMs of cells seeded on immobilized BMP4 whereas negligible VEGFR2 immunoreactivity was observed in VPMs from cells seeded on uncoated coverslips. In keeping with a role of BMP signaling in VEGFR2 transactivation, dorsomorphin prevents the relocation of VEGFR2 in VPMs obtained from BMP4-adherent cells.

The ability of BMP4 to induce the relocation of VEGFR2 and BMPRII in specific membrane regions of ECs encouraged us to evaluate the possible formation of VEGFR2/BMPRs complex following stimulation with BMP4. However, co-immunoprecipitation experiments demonstrated the absence of VEGFR2/BMPRI and/or VEGFR2/BMPRII complexes in BMP4-treated cells (Fig. 5d).

BMP4 induces VEGFR2 activation and angiogenesis via c-Src

The absence of a direct interaction of BMPRs and/or BMP4 with VEGFR2 prompted us to evaluate whether

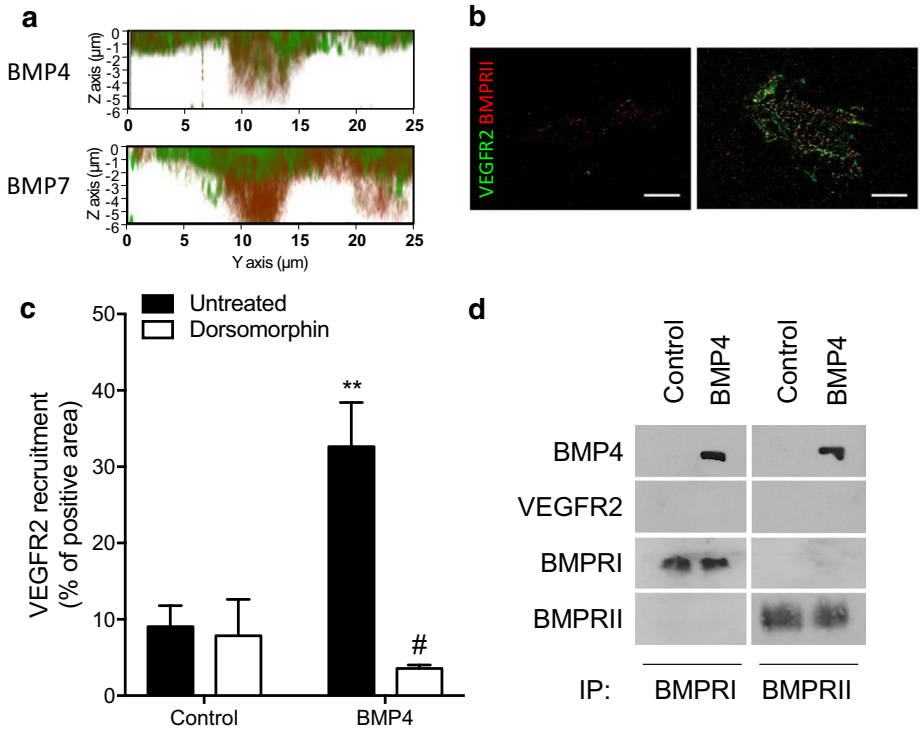

Fig. 5 BMP4 mobilizes VEGFR2 on EC membrane. **a** 8 µm pore chemotaxis filters were inserted in a Boyden chamber and HUVECs were seeded at 1.0×10^6 cells/mL in the upper compartment. After 1 h of migration in response to BMP4 or BMP7, filters were fixed, stained for VEGFR2 (green) and BMPRII (red) and analyzed using a LSM510 Meta confocal microscope equipped with Plan-Apochromat 63x/1.4 NA oil objective. BMPRII-positive cell protrusions extending into pores were counted and their positivity for VEGFR2, at the depth of 5 µm, was quantified (35 protrusions/sample). Representative images of orthogonal higher magnification projection of BMP4- or BMP7-attracted HUVEC in-pore protrusions. **b** HUVECs seeded on substrate-bound fibrinogen or BMP4 were stained for VEGFR2 (green) or BMPRII (red) and analyzed using a LSM510 Meta confocal microscope equipped with Plan-Apochromat 63x/1.4 NA oil objective. Images show the basal portion of fibrinogen-(left panel) or BMP4-(right panel) adherent cells (bar: 20 µm). **c** VEGFR2-overexpressing GM7373 cells were seeded on immobilized BMP4 or uncoated coverslips in the absence or in the presence of 10 µM Dorsomorphin for 6 h and subjected to squirting lysis. Ventral plasma membranes (VPMs) were stained for VEGFR2, actin and nuclei and samples were analyzed with epifluorescence microscope (×630). Total VEGFR2 was quantified in 30 cells/sample using Image-Pro Plus software. Data are expressed as percentage ± SEM of VEGFR2 positive area in respect to the total VPM area, as defined by actin staining (**$p<0.01$ vs. control; $^{\#}p<0.01$ vs. BMP4, ANOVA). **d** HUVECs were stimulated with 50 ng/mL of BMP4 in the presence of BS3 cross-linker. BMPRI and BMPRII receptor complexes were immunoprecipitated by anti-BMPRI and anti-BMPRII antibodies, respectively, and loaded on a 6% SDS-PAGE gel. Membrane was probed with the indicated antibodies. Data are representative of 2 independent experiments that provided similar results

the engagement of BMPRs by BMP4 may transactivate VEGFR2 via an indirect mechanism.

Previous observations had shown that VEGFR2 can be activated in a ligand-independent manner in different cell types [39–41]. In this regard, c-Src plays an intermediary signaling role in ligand-independent VEGFR2 activation and c-Src-mediated VEGFR2 activation plays a non-redundant role in S1P1-dependent embryonic stem cell proliferation, pointing to a relevant function of c-Src in VEGFR2-dependent biological responses [39–41].

To evaluate whether BMP4 modulates the angiogenic process by VEGFR2 activation via c-Src, HUVECs were treated with BMP4 and assessed for c-Src activation as demonstrated by the phosphorylation of its Tyr 418 residue [42]. As shown in Fig. 6a, BMP4 induces a rapid Tyr 418 c-Src phosphorylation that was significantly impaired by dorsomorphin treatment, thus confirming that it depends on BMPR engagement. Next, we tested whether BMP4-activated c-Src induces VEGFR2 phosphorylation. To this aim, cells were treated with BMP4 in the absence or in the presence of PP2, a specific c-Src inhibitor. As shown in Fig. 6b, PP2 prevents BMP4-driven VEGFR2 activation. In addition, PP2 also inhibits VEGFR2 activation driven by VEGF, in keeping with the capacity of c-Src to regulate VEGFR2 phosphorylation [43]. Accordingly, PP2 completely abrogates the BMP4-mediated HUVEC organization in tube-like structures when cells are seeded on Cultrex matrix (Fig. 6c). PP2 also inhibits BMP4 activity in the 3D sprouting angiogenesis assay (Fig. 6d).

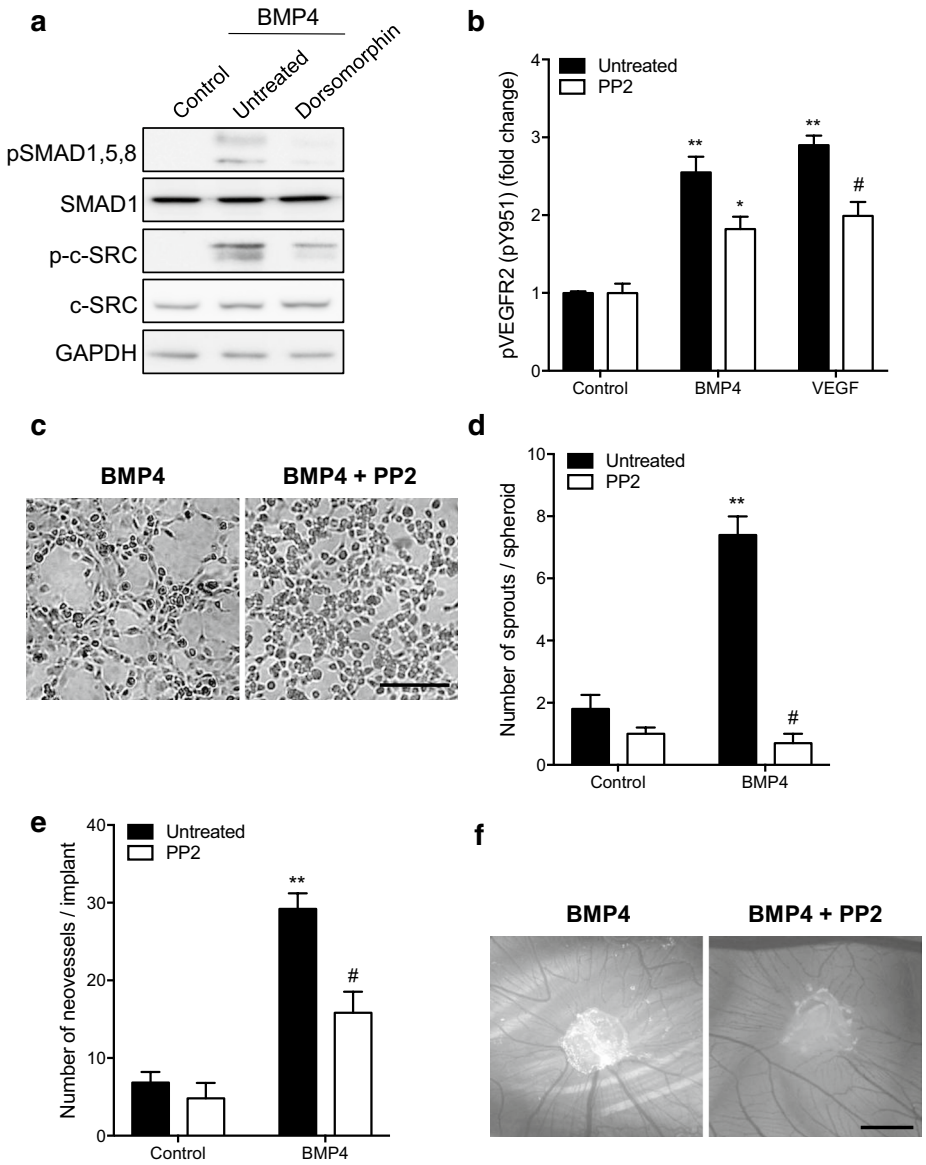

Fig. 6 c-Src controls BMP4-induced angiogenesis. **a** HUVECs were stimulated with 50 ng/mL BMP4 for 30 min in the absence or in the presence of dorsomorphin. 50 µg of total cell lysates were loaded on SDS-PAGE and assessed for total or phosphorylated SMAD1,5,8 and c-Src. Data are representative of 2 independent experiments that provided similar results. **b** Serum-starved HUVECs were stimulated with 50 ng/mL of BMP4 for 15 min in the absence or the presence of 1.0 µM PP2. pVEGFR2 (Y951) levels were then assessed by ELISA. Data are the mean ± SEM of 3 independent experiments (**$p < 0.01$ vs. control; *$p < 0.05$ and $^{\#}p < 0.01$ vs. untreated, ANOVA). **c** HUVECs were seeded on Cultrex matrix in the presence of 50 ng/mL of BMP4 with or without 1.0 µM PP2. Tube-like structure formation was evaluated after 5 h (bar: 200 µm) **d** HUVEC spheroids embedded in fibrin gel were incubated with 50 ng/mL of BMP4 in the absence or in the presence of 1.0 µM PP2. Formation of radially growing sprouts was evaluated after 24 h of incubation. Data are the mean ± SEM of 40 spheroids per experimental point (**$p < 0.01$ vs. control; $^{\#}p < 0.01$ vs. untreated, ANOVA). **e** Alginate beads containing vehicle or 100 ng of BMP4 were implanted on the top of chick embryo CAMs in the absence or in the presence of 1.0 µM PP2 at day 11 of development. After 72 h, newly formed blood vessels converging toward the implant were counted in ovo at ×5 magnification using a STEMI SR stereomicroscope. Data are expressed as mean ± SEM (n = 8) (**$p < 0.01$ vs. control; $^{\#}p < 0.01$ vs. untreated, ANOVA). **f** Representative images of BMP4-treated CAM in the absence (left panel) or in the presence (right panel) of c-Src inhibitor PP2 (bar: 2.0 mm)

Finally, we evaluated the capacity of PP2 to prevent BMP4-dependent angiogenesis in an in vivo assay. As anticipated, PP2 hampers the angiogenic activity exerted by BMP4 in the in vivo CAM assay (Fig. 6e, f). Together, these data demonstrate that BMP4 induces VEGFR2 activation via c-Src activation.

Discussion

BMP4 regulates multiple biological processes during embryogenesis, including dorso-ventral axis formation [10], mesoderm-derived cell lineages [44], and venous specification [23]. In addition, increasing evidence suggests that BMP signaling dynamically regulates vascular homeostasis and permeability, modulating endothelial response to hypoxia and inflammatory stimuli. As an example, BMP4 promotes endothelial proliferation and angiogenesis in response to low oxygen or high ROS levels. In contrast, oxidative damage via ROS can lead to BMP-mediated endothelial apoptosis and vasoconstriction [25]. Therefore, the effect of BMP4 on ECs and its role in the angiogenic process remain controversial and partially uncovered.

BMP4 acts as a growth factor for mouse embryonic stem cell-derived ECs and human microvascular ECs [30]. Also, BMP4 upregulates VEGFR2 expression in ECs [30] and induces neovessel formation when engrafted onto the chick embryo CAM [17].

Here, we examined in depth the role of VEGFRs in BMP4-dependent angiogenesis. We demonstrated that among the VEGFRs, BMP4 triggers only the activation of VEGFR2, although it does not bind the receptor nor in cell-free conditions neither on cell membrane. Even though BMP2 and BMP6 [45] modulate EC migration and angiogenesis in vitro and in vivo by increasing the expression of membrane receptors, including VEGFR2 and Notch, there are no evidences about the ability of BMPs to induce the phosphorylation and activation of VEGFR2 [17]. Also, the analysis of BMP4-activated BMPRI-II receptosomes rules out the possibility of the formation of BMPRI or BMPRII/VEGFR2 complexes. Despite these results, we observed a rapid relocation and phosphorylation of VEGFR2 upon BMP4 stimulation.

BMPs can signal through both canonical and non-canonical pathways. Both pathways are initiated by the formation of a hetero-tetrameric complex comprising two dimers of type I and type II BMPRs. In the canonical signaling, active type I receptor mediates the phosphorylation of the receptor-regulated SMADs, which in turn associate with the co-mediator SMADs, translocate to the nucleus and regulate gene expression [46]. In addition, BMPs can activate several SMAD-independent pathways. In particular, BMP4 activates MAPK, PI3K/Akt, and Rho-GTPase pathways [12, 47]. In keeping with the recently shown capacity of BMP6 to trigger c-Src activation in HUVECs [14], here we provide evidences about c-Src activation induced by BMP4. In contrast with our results, a phosphorylated form of c-Src is constitutively associated with BMPRII in smooth muscle cells, and mutations in BMPRII or BMP2/4/7 stimulation reduce c-Src phosphorylation and its detachment from the receptor, supporting the pathogenesis of pulmonary arterial hypertension [48]. We hypothesize that BMP4-dependent

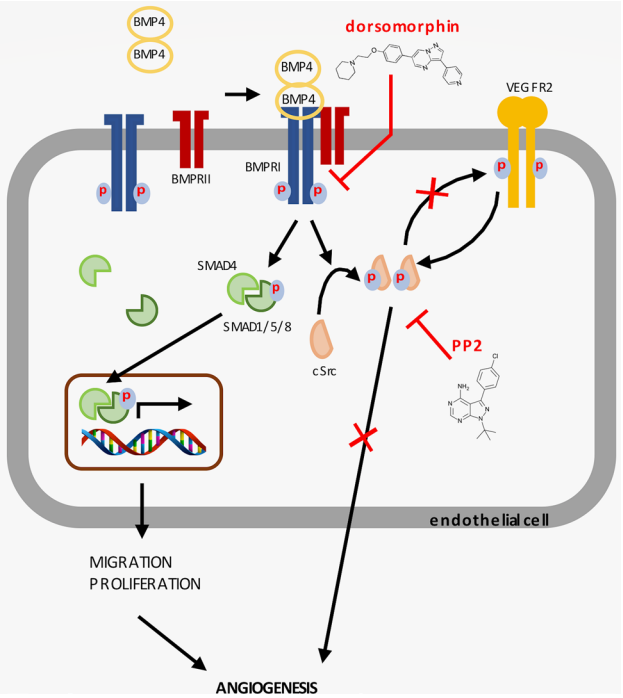

Fig. 7 BMP4 interacting with BMPRI/BMPRII complex mediates c-Src activation, which, in turn, phosphorylates and activates VEGFR2 eventually leading to angiogenesis

effects on phospho-c-Src levels might be context- and/or cell type-dependent.

c-Src is a cytoplasmic tyrosine kinase protein which plays a key role in many cellular responses. Its activity is dependent on a myriad of protein–protein interactions through its SH2 and SH3 domains. c-Src can be phosphorylated and activated by several tyrosine kinase receptors including VEGFR2 [49] and epidermal growth factor receptor [50]. Moreover, c-Src can drive receptor phosphorylation by physically interacting with tyrosine kinase receptors [51]. Here we show that the pro-angiogenic activity of BMP4 is dependent, at least in part, on VEGFR2 transactivation induced by c-Src. These data are supported by the negative regulation exerted by PP2 on both BMP4-dependent VEGFR2 activation and angiogenesis. Of note, in our experimental conditions, EC stimulation by BMP4 does not induce VEGF production (data not shown). In ECs the transactivation of VEGFR2 by a VEGF-independent mechanism has been previously described for several factors, including S1P1, interleukin 8, and PTHrP. All these reports come to the conclusion that VEGFR2 transactivation is driven by the activation of c-Src [6, 52–55].

Overall, we showed that BMP4 activates both the canonical SMAD-dependent pathway and the alternative c-Src pathway by binding to BMPRI/BMPRII receptor complex in ECs. Importantly, c-Src triggers the

ligand-independent transactivation of VEGFR2, leading to EC pro-angiogenic activation. Since the active form of VEGFR2 phosphorylates c-Src [43], it is possible to hypothesize that, following BMP4 stimulation, c-Src phosphorylation is firstly due to BMPR engagement, to be fully activated by the subsequent VEGFR2 activation. Figure 7 schematically illustrates the intracellular events that mediate the pro-angiogenic activity of BMP4. Here we highlighted the previously unrecognized role of c-Src in mediating the pro-angiogenic activity of BMP4, and suggest that both SMAD-dependent and c-Src/VEGFR2 dependent signaling pathways significantly contribute to the final outcome exerted by BMP4 stimulation on ECs.

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