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Transcription regulation of the *vegf* gene by the BMP/Smad pathway in the angioblast of zebrafish embryos ☆

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Abstract

Vascular endothelial growth factor (VEGF) is a mitogen that is critically involved in vasculogenesis, angiogenesis, and hematopoiesis. However, what and how transcription factors participate in the regulation of *vegf* gene expression are not fully understood. Here we report the cloning and sequencing of the zebrafish *vegf* promoter which revealed that the promoter contains a number of bone morphogenetic protein (BMP)-activated Smad binding elements (SBE), implicating Smad1 and Smad5 in the regulation of BMP-induced expression of *vegf*. Electrophoretic mobility shift assays of adding recombinant Smad proteins to the SBE-containing DNA oligonucleotides that represent portions of zebrafish *vegf* promoter resulted in mobility shift of the oligonucleotides. These changes demonstrate potential interactions between Smad1/5 and the *vegf* promoter. Reporter activity assays using the wild-type or SBE-deleted *vegf* promoters to drive the luciferase reporter gene expression revealed that Smad1 stimulated while Smad5 repressed the *vegf* promoter activity in zebrafish embryos. These data indicate that the BMP/Smad signaling pathway is involved in the regulation of zebrafish *vegf* transcription. In addition, we demonstrate that transgenic expression of human BMP4 in zebrafish embryos induced an expansion of the posterior intermediate cell mass (ICM, also commonly called blood island), a population of cells containing endothelial and hematopoietic precursors. In the expanded ICM, *vegf* and VEGF receptor 2 (*flk-1*) were ectopically co-expressed, suggesting that an autocrine/paracrine regulation of *vegf* expression may exist and contribute to the BMP-induced hemangiogenic cell proliferation.

Keywords: VEGF; BMP; Smad; Transcription; Zebrafish; Signal transduction; Gene regulation; Posterior intermediate cell mass; Vasculogenesis; Hemangiogenesis; Embryonic development

Vascular endothelial growth factor (VEGF) or VEGF-A is one of the most potent growth factors directing the development of endothelial and hematopoietic cells [1,2]. VEGF plays important roles in specification of blood islands [3,4], regulation of proliferation, survival, and migration of endothelial as well as hematopoietic cells [5–7]. Multiple isoforms of VEGFs, which resulted from alternative splicing of VEGF mRNA, exert the hemangiogenic activities through membrane receptor tyrosine-kinases, VEGFR-1 (Flt-1) and VEGFR-2 (KDR or Flk-1), and their co-receptors, Neuropilin-1 (NrP-1) and Neuropilin-2 (NrP-2) [2]. Loss of a single *vegf* allele or VEGF tyrosine-kinase-receptors results in growth arrest and

☆ *Abbreviations:* BMP, bone morphogenetic protein; EMSA, electrophoretic mobility shift assays; hpf, hours post-fertilization; ICM, intermediate cell mass; ORF, open reading frame; RACE, rapid amplification of cDNA ends; SBE, Smad binding elements; VEGF, Vascular endothelial growth factor; WISH, whole-mount in situ hybridizations.

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embryonic lethality [8]. Although the physiological functions of VEGF have been well documented [9], the signaling pathway(s) regulating *vegf* transcription during developmental vasculogenesis and hematopoiesis are not completely understood.

BMP signaling is known to induce embryonic mesodermal patterning and programs the development of hemangioblasts [10,11], and therefore serves as an attractive candidate for an upstream regulator of *vegf* expression. In the BMP signaling pathway, the BMP receptor-regulated Smads (R-Smads including Smad1, 5, and 8) are phosphorylated by the BMP type I receptor (Alk3). The phosphorylated R-Smads form a heteromeric complex with Co-Smads (Smad4) and translocate into the nucleus, where the activated Smad complex binds to the SBE in the promoter and regulates transcription [12]. In mouse embryos, Smad1 is uniquely expressed in visceral endoderm where VEGF is expressed [13,4]. In addition, Smad5 mutant mouse embryos demonstrate ectopic vasculogenesis and hematopoiesis in the amnion [14]. Therefore, we hypothesized that the signaling via Smad1/5 plays a role in the regulation of *vegf* transcription.

In this study, we cloned the zebrafish *vegf* promoter and identified SBEs in the promoter sequence. We further investigated the binding of Smad proteins to these elements in vitro and provided evidence that these elements were also functional in vivo. Finally, coupling specific DNA deletions with a gene reporter assay, we examined how Smads regulate *vegf* gene expression.

Materials and methods

Zebrafish maintenance and plasmid microinjection. Zebrafish were maintained as described [15]. The embryos were staged as hours post-fertilization (hpf) [16]. Microinjections of plasmids were performed as described [17].

Rapid amplification of cDNA ends (RACE). In order to identify the 5' end of zebrafish *vegf* mRNA, total RNA was extracted from 24 hpf zebrafish embryos using RNA-Bee (TEL-TEST, Friendswood, TX). DNA contaminants were removed using the DNA-Free kit (Ambion). 5′-RACE *vegf* mRNA was performed following the instructions of the FirstChoice RLM-RACE Kit (Ambion) with some minor modifications. The reverse transcription was performed at 60 °C for 1 h using thermostable *C. therm.* polymerase (Roche). The following PCR was performed using PfuUltra high-fidelity DNA polymerase (Stratagene). The *vegf* mRNA specific primers used for the first round ligation-mediated PCR and the second round nested PCR were 5′-GAT TAT CAT CAA GAG CAA AGG CTT GCT G-3′, and 5′-CAA AGG CTT GCT GTA AAG AGA TCC AGT-3′, respectively. The cycling program was set as follows: 95 °C, 1 min (denaturing), 1 cycle only; 95 °C, 30 s (denaturing); 60 °C, 30 s (annealing); 72 °C, 2 min (elongating), 25 cycles; and 72 °C, 10 min, 1 cycle only. The amplified DNA fragment was cloned into pCR-Blunt (Invitrogen) for subsequent sequencing.

Isolation and characterization of the zebrafish vegf promoter. The *vegf* promoter was isolated using the Universal Genome Walker Kit (Clontech). The genomic walk started from 54 bp downstream of the *vegf* translation start codon and proceeded in the 5′ direction. The promoter region of *vegf* gene was isolated from zebrafish genomic DNA by PCR, using a 5′ primer of 5′-GGG TAA TTA GGC AAT TTA TTG TAT ATC GAT GG-3′ and a 3′ primer of 5′-GCT GGT TCG TAC TGC CGT GGA TG-3′, and/or a pair of nested primers, 5′-CAA TTT ATT GTA TAT CGA TGG TTT GTT TTG TAA GC-3′ as the 5′ primer and 5′-CTG CCG TGG ATG TTA GCA CGC G-3′ as the 3′ primer. The 1.2 kb of zebrafish *vegf* promoter including 85 bp of 5′ untranslated region (5′-UTR) was amplified and subsequently cloned into the pCRII-TOPO vector (Invitrogen).

Site-directed mutagenesis. To determine if the putative SBEs in zebrafish *vegf* promoter were functional, the Smad1 SBEs (GCAT) and a Smad5 SBE (TGTCTGAGAC) were removed from the zebrafish *vegf* promoter using a PCR mutagenesis protocol as previously described [18].

Protein expression and affinity purification. To produce recombinant Smad1 DNA binding domain, MH-1, and Smad5 protein, cDNA encoding the MH-1 domain (residues 1–146) of Smad1 was isolated from zebrafish mRNA of 24 hpf zebrafish embryos by reverse transcription polymerase chain reaction (RT-PCR). The 5′ primer (5′-ACCGCGGATGAATTCACCTCACGCTTTTC-3′) harbors a *Sac*II site 'CCGCGG' and the 3′ primer (5′-TCA GAG TTT AGC GTT GAA CTC GCT GTT TCG-3′) contains a stop codon engineered in it. The PCR product was cloned into pCRII-TOPO (invitrogen). To generate the expression constructs of Smad1 MH-1 domain, the PCR product was cleaved from pCRII-TOPO by *Eco*RI and inserted in-frame into the *Eco*RI site in the pET14b (Novagen) vector that directs the expression of an amino-terminal His-tag. The orientation of the insertions was determined by restriction digestion with *Sac*II. The construct was transformed into the BL21 (DE3) strain of *E. coli* (Stratagene) for MH-1 domain protein production. The Smad5 open reading frame (ORF) was amplified from pSP64TS-Smad5wt, a plasmid containing the full-length cDNA (cloned by Marc Hild, the Hammerschmidt laboratory at Spemann Labs, Max-Planck-Institut für Immunbiologie, Freiburg, Germany, as a generous gift). The smad 5 ORF fragment was amplified by PCR with a 5′ primer (5′-GGA TCC ATG ACC TCC ATG TCT AGT CTG TTT TCC TTC-3′) encompassing a *Bam*HI site and a 3′ primer (5′-GGA TCC TCA CTA TTA CGA GAC AGA AGA GAT GGG GTT CAG AG-3′) containing a *Bam*HI site and three contiguous stop codons. The resulting Smad5 ORF was sub-cloned into pET14b (Novagen) for the expression of the N-terminal His-tagged protein in *E. coli* strain BL21 (DE3). The recombinant proteins were purified under native condition using His•Bind Purification Kits (Novagen). The recombinant proteins were quantified by the Bradford method (Bio-Rad).

Electrophoretic mobility shift assay (EMSA). The PAGE-purified synthetic single-strand oligonucleotides which contain SBEs (Table 1) were annealed between complementary strands to form double-stranded oligonucleotides that were labeled using terminal transferase and Digoxigenin-11-ddUTP (DIG-ddUTP) following the protocol of DIG Gel Shift Kit (Roche). The binding reactions were carried out in a total volume of 20 μl containing 310 fmol of labeled oligo DNA, 500 ng His-tagged Smad protein, 1 μg poly(dI–dC), 0.1 μg poly L-lysine, 10% glycerol, and 5 mM $MgCl_2$. To generate supershift, 4.2–8.4 μg of mouse anti-His monoclonal antibody (Amersham) was added to the binding reaction, while an equal amount of non-immune mouse IgG was added as control. For the competition experiments, a 280-fold excess of the un-labeled DNA fragments was added to the reaction. The binding reaction mixture was kept at 4 °C for 8 h. The assay mixture was separated on a 4–20% gradient TBE native PAGE-gel (Bio-Rad) running at 210 V in 0.5× TBE buffer. The gel was then electro-blotted to a positively charged nylon membrane (Roche) by electrophoresing at 400 mA for 30 min with 0.5× TBE buffer in a Mini Trans-Blot Transfer Cell (Bio-Rad). The DIG-labeled DNA–protein complex was detected with an alkaline phosphatase-conjugated anti-DIG antibody followed by chemiluminescence and X-ray film exposure.

Luciferase assay. The wild-type or mutant zebrafish *vegf* promoter was cloned into the *Eco*RI site of phRL-null vector (Promega). The orientation of the cloned zebrafish *vegf* promoter was verified by a *Sac*I restriction digestion. The phRL *Renilla* luciferase constructs with wild-

Table 1
The sequence of the zebrafish *vegf* promoter fragments used in EMSA assays

	Position in zebrafish *vegf* promoter	Sequence
Oligonucleotides with the Smad1 SBE		
S1BE1	−97 to −58	5′-AAACCCTTCACAGAGCATCATCATCTACGTCACCTCCCAC-3′
S1BE2	−295 to −255	5′-AATAAGATCAAGAGCATGATCTTTACCCGAGAAATCAGAA-3′
S1BE3	−545 to −506	5′-ATAATAACATCACTGCATTGAAGAAAAAATACAATAACAA-3′
S1BE4	−794 to −755	5′-ATTTAGAGGCTTTGGCATCTGAGCTCTTCATGTTTAATAT-3′
S1BE5	−812 to −773	5′-GAAGAAAATTCAGATGGCATTTAGAGGCTTTGGCATCTGA-3′
S1BE6	−1002 to −963	5′-AGCCGAAATAAAATGCATTAAAATGTATTCTAGCCAAAAT-3′
Oligonucleotides with the Smad5 SBE		
S5BE	−727 to −683	5′-AATTGGAAAAAGATGCAGACTGTCTGAGACATCCTGAGGATTTCA-3′

The underlines identify the Smad binding elements.

type zebrafish *vegf* promoter, the Smad1-SBE-deleted *vegf* promoter, or the Smad5-SBE-deleted *vegf* promoter were designated as VptrRluc, VptrΔ S1BERluc, and VptrΔ S5BERluc, respectively. The construct for the expression of human BMP4, which was cloned into pCI vector (Promega) between *Nhe*I and *Kpn*I, was co-injected with either the empty phRL *Renilla* luciferase vector (control) or the phRL vector inserted with zebrafish *vegf* promoters into one-cell stage zebrafish embryos. At 24 hpf, embryos with the expanded ICM were selected for luciferase assay. Luciferase assays were carried out using a *Renilla* luciferase Assay System (Promega). The luciferase activities were measured by Lumat LB 9501 (EG & G Berthold) and normalized to the number of embryos used in each sample. The data were analyzed with ANOVA and the Student–Newman–Keuls tests.

Microscopic imaging. For in vivo red fluorescence protein (DsRed2) reporter assays, the *vegf* promoter was inserted at the 5′ end of DsRed2 gene in the pDsRed2-1 vector (Clontech). The injected embryos were imaged alive or fixed for 12 h in 4% paraformaldehyde at 4 °C, pH 7.4. The epifluorescence images were taken with a Nikon Eclipse E600, using TRITC filter sets while the laser scan image was taken with a Zeiss LSM 510 confocal laser scanning microscope, using visual HeNe1 laser (543 nm) and DsRed filter.

Whole-mount in situ hybridization. The DNA templates of probes were synthesized in segments as overlapping single-strand DNA oligonucleotides and annealed as double-strand DNA fragments before cloning into pGEM3f(+) vectors. The *vegf* probe, which was cloned into pGEM3f(+) (Promega) between *Bam*HI at 5′ end and *Hin*cII at 3′ end, was 111 bp in length, representing the sequence from 753 to 864 bp in the *vegf* cDNA (GenBank Accession No. AF016244) [19]. The *flk-1* probe, which was cloned into pGEM3f(+) between *Bam*HI at the 5′ end and *Sph*I at the 3′ end, was 152 bp in length, representing the sequence from 3751 to 3903 bp in the *flk-1* cDNA (GenBank Accession No. AF487829) [20]. The *smad1* probe, which was cloned into pGEM3f(+) between *Bam*HI at the 5′ end and *Sph*I at the 3′ end, was 150 bp in length, representing the sequence from 541 to 690 bp in the zebrafish *smad1* cDNA (GenBank Accession No. AF174434) [21]. The constructs containing the DNA templates of the whole-mount in situ hybridization (WISH) probes were linearized at *Bam*HI before in vitro transcription. The riboprobes used in the in situ hybridization were synthesized from the corresponding DNA templates using the DIG RNA labeling kit (Roche). WISH assays were performed following the protocol as described [22].

Results and discussion

Isolation and characterization of the zebrafish vegf promoter

In order to investigate the transcription-regulation of zebrafish *vegf* gene, we first identified the transcription start site (TSS) of zebrafish *vegf* mRNA by 5′-RACE (Fig. 1). Using the sequence information of TSS, we isolated and cloned the putative *vegf* promoter, a genomic DNA fragment that covers 1.2 kb upstream of the 5′-untranslated region of the zebrafish *vegf* gene. To determine if the 1.2 kb fragment was sufficient to direct the correct pattern of *vegf* gene expression, we inserted this putative promoter in front of a DsRed2 reporter (red fluorescence protein) and monitored the pattern of expression using laser-scan and epi-fluorescence microscopy (Fig. 2A). By 33 hpf, the DsRed reporter expressed exclusively in the somites of embryos along the trunk region (Figs. 2B and C). The expression pattern of DsRed2 reporter was identical to that of endogenous

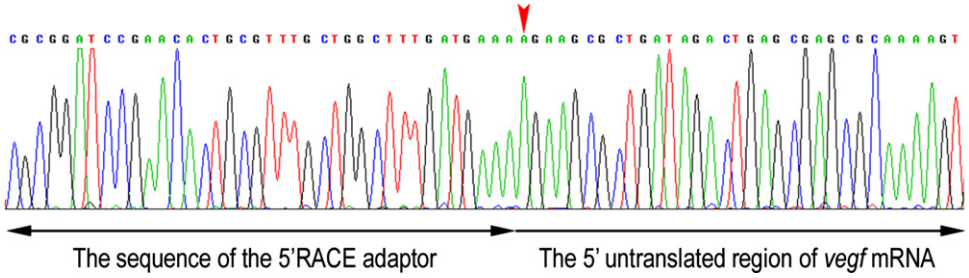

Fig. 1. The 5′ end of the zebrafish *vegf* mRNA. In the sequencing result of the 5′-RACE, the transcription start site is designated +1 and indicated with an arrow.

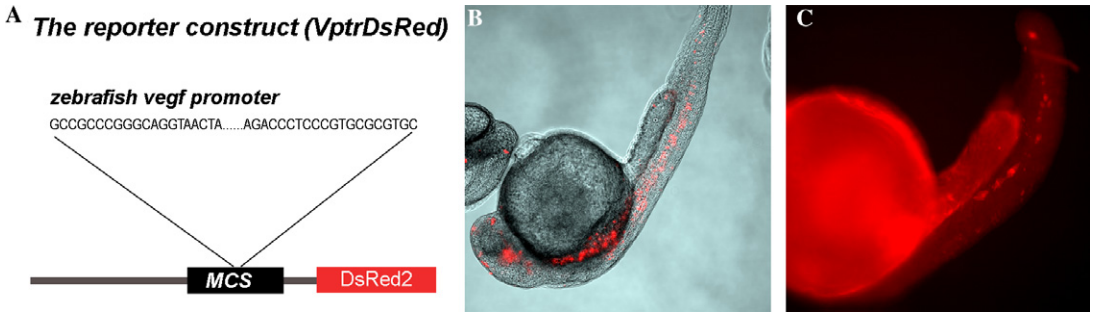

Fig. 2. Expression pattern of the zebrafish *vegf* promoter-directed DsRed2 reporter in zebrafish embryos. The zebrafish *vegf* promoter-directed expression of DsRed2 reporter revealed an expression pattern that is the same as the endogenous *vegf* along the somites as previously reported [23,24]. (A) The genomic DNA representing zebrafish *vegf* promoter was inserted into pDsRed2-1 vector, designated as VptrDsRed. (B) A laser-scan image of a wild-type embryo expressing the circular VptrDsRed plasmid at 36 hpf. (C) An epifluorescence image of a wild-type embryo expressing the linearized VptrDsRed plasmid at 33 hpf.

vegf in wild type embryos at the same developmental stage [23,24], indicating that the 1.2 kb fragment was able to serve as a functional *vegf* promoter.

We further searched the promoter sequence for putative transcriptional factor-binding sites (Fig. 3). A total of 12 DNA elements (−83 to −80, −133 to −130, −282 to −279, −531 to −528, −603 to −600, −617 to −614, −715 to −712, −780 to −777, −796 to −793, −824 to −821, −988 to −985, and −990 to −987) were identical to the Smad1 SBEs (GCAT) previously characterized in the *Xvent-2B* [25] and *Xretpos* [26] promoters in *Xenopus*. In addition, we found a DNA motif (TGTCTGAGAC) similar to that of the Smad5 SBE (TGTCTAGAC) identified in the mouse *smad7* promoter [27]. The presence of this sequence led us to speculate that this motif was a putative Smad5 SBE in the zebrafish *vegf* promoter. Since both Smad1 and Smad5 are downstream BMP signaling mediators [28], the existence of putative SBEs in the *vegf* promoter fosters the hypothesis that BMP/Smad

Fig. 3. The genomic DNA sequence of the zebrafish *vegf* promoter (GenBank Accession No. AY278863). The putative consensus binding sites of transcriptional factors are marked directly on the promoter sequence. The arrows indicate the putative transcriptional factor binding sequences on the complementary strand.

signaling may be involved in transcriptional regulation of zebrafish *vegf* gene.

Smad1 and Smad5 proteins bind to the SBEs in the zebrafish vegf promoter in vitro

In order to investigate whether Smad1 and 5 interact with the putative SBEs on zebrafish *vegf* promoter, we synthesized DNA oligonucleotides representing the SBE-containing promoter fragments, and used them as probes (Table 1) in EMSA for molecular interactions with either the recombinant His-tagged zebrafish Smad1 DNA binding domain (MH-1 domain) or His-tagged zebrafish Smad5 proteins. The individual addition of the Smads to the SBE-containing fragments resulted in band shifts of all six DIG-labeled promoter fragments (Fig. 4). The shifts were greatly reduced by addition of 280-fold excess unlabeled DNA fragments to the binding reaction (Fig. 4, ''specific competitor''), indicating the specific binding between Smad proteins and oligonucleotides. Furthermore, the inclusion of an anti-His-tag monoclonal antibody, but not non-immune IgG (data not shown), generated supershifts of the DNA–Smad complex (Fig. 4, ''His-tag antibody''),. This observation indicates that Smad proteins (Smad1 MH-1 domain or Smad5) physically associate with the promoter fragments that contain the corresponding putative SBEs. This result strongly suggests the presence and functionality of both Smad1 and Smad5 binding elements in the zebrafish *vegf* promoter.

BMP/Smad signaling regulates the activity of zebrafish vegf promoter

To evaluate the biological function of the SBEs located in the zebrafish *vegf* promoter in vivo, we performed luciferase reporter assays in zebrafish embryos under the challenge of an ectopic BMP-signaling. The wild-type or mutant *vegf* promoters were fused to a luciferase reporter and co-injected with a human BMP4 expression construct (pCI-hBmp4) into one-cell stage embryos. Ectopic expression of *bmp* genes led to

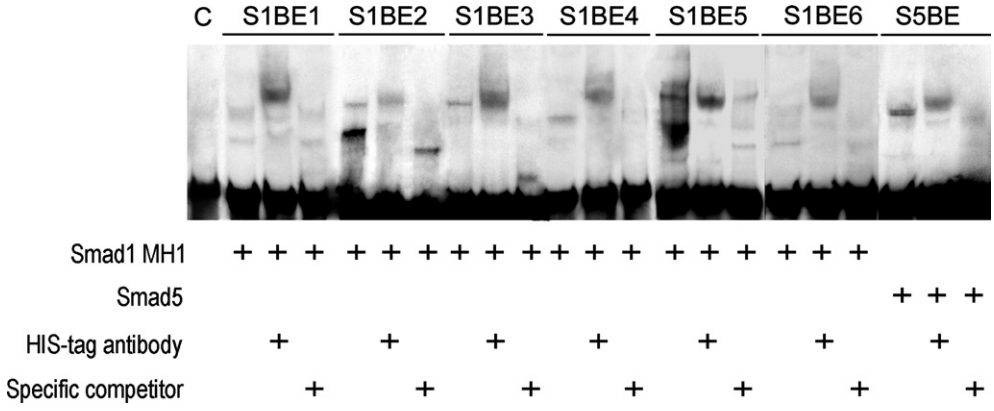

Fig. 4. Electrophoretic mobility assays (EMSAs) on the binding of recombinant Smad1 MH1 domain or Smad5 to zebrafish SBE-containing *vegf* promoter fragments. Samples S1BE1 to S1BE6 demonstrate the binding reactions that contain the His-tagged MH-1 domain of Smad1 protein and the Smad1 SBE-containing promoter fragments (GCAT motif). S5BE samples demonstrate the binding reactions that contain the His-tagged Smad5 protein and the Smad5 SBE-containing promoter fragment.

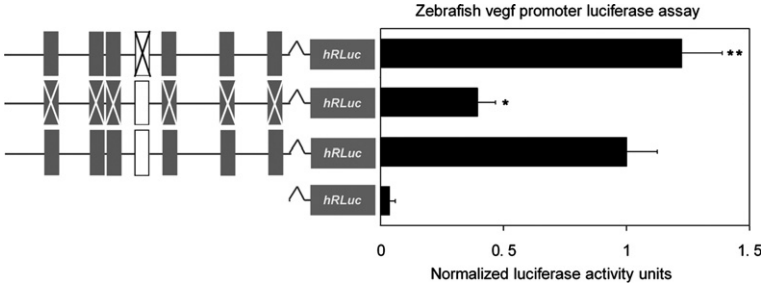

Fig. 5. Luciferase activity assays of zebrafish *vegf* promoter in zebrafish embryos. Zebrafish embryos were co-injected with the CMV promoter driven human *bmp* 4 expression constructs and the luciferase reporter constructs driven by either wild-type zebrafish *vegf* promoter (Smad1 SBEs are indicated by gray boxes while the Smad5 SBE is indicated by a white box), or mutant zebrafish *vegf* promoters (Deletion of Smad1 SBE is indicated by a white cross over the gray box, while deletion of Smad5 SBE is indicated by a black cross over the white box) (0.1 ng/embryo/construct). Background expression of the *Renilla* luciferase vectors was used as the internal reference control, and the luciferase activity derived from the transcription driven by the wild-type zebrafish *vegf* promoter was set as 100%. $*P < 0.05$ and $**P < 0.001$, respectively. The reported luciferase activity results are means of duplicate assays and are representative of four independent experiments with standard deviations $\leqslant 15\%$.

expansion of the posterior ICM compartment of zebrafish embryos [29], which is a typical phenotype associated with elevated levels of BMP signaling in zebrafish embryos described previously [30]. In this study, 44% of the injected embryos at 25 hpf ($n = 360$) displayed the expanded posterior ICM. Luciferase activities were examined only in the zebrafish embryos with enlarged posterior ICM compartments. As shown in Fig. 5, deletion of the six Smad1 SBEs from the *vegf* promoter reduced the transcription activity by about 50% in comparison with the wild-type *vegf* promoter (40 embryos were examined in 8 groups for the wild-type promoter; 45 embryos were examined in 9 groups for the Smad1-SBE-deleted mutant promoter, $P < 0.001$). This result suggests that Smad1 contributes to the activation of the *vegf* promoter. In contrast, deletion of the Smad5 SBE enhanced the reporter activity by about 30% (50 embryos examined in 10 groups, $P < 0.05$), implying that Smad5 may negatively affect *vegf* transcription. These luciferase reporter activity assays suggest that Smad1 and Smad5 possibly play opposite roles in regulation of *vegf* promoter activity.

Since Smad proteins always need transcriptional co-factors to modulate transcriptional activity of target genes [12], transcriptional co-activators or co-repressors may interact with Smad1 or Smad5 to generate opposite effects of Smad1 and Smad5 in regulating *vegf* promoter activity. In the zebrafish *vegf* promoter, four putative E-box elements [30] are present in proximity to Smad1/5 SBEs (Fig. 3). The zinc finger E-box binding protein (ZEB) is known to interact with R-Smads. When transcription co-activators p300 and P/CAF are associated with ZEB-1/δEF-1, the Smad/ZEB complex activates transcription. However, upon recruitment of the transcription co-repressor CtBP to ZEB-2/SIP-1, the Smad/ZEB complex represses transcription [31]. It will be of interest to see whether Smad1 and Smad5 interact with ZEB/δEF1 or ZEB-2/SIP1 in regulation of the *vegf* expression.

A co-expression of vegf, flk-1, and smad1 in the angioblasts of zebrafish embryos expressing human BMP4

Based on the observations that ectopic expression of *bmp* genes led to expansion of the posterior ICM compartment of zebrafish embryos (Fig. 6A) [28] and the posterior ICM cells in wild-type zebrafish embryos represent the angioblast [32], we characterized the cells of the expanded posterior ICM using the probes that identify *vegf* and the angioblast marker, *flk*-1 [33], in a series of WISH. Injection of human *bmp4* construct (pCI-hBmp4) into zebrafish embryos led to an ectopic expression of *vegf* by 24 hpf in the expanded posterior ICM (Figs. 6B(a) and (b)) and an ectopic, elevated expression of VEGF receptor-2 (*flk-1*) in the posterior ICM (Figs. 6B(c) and (d)). In wild-type zebrafish embryos, only weak expression of *vegf* was previously reported in the posterior ICM at 48 hpf [24]. The co-localization of *vegf* and *flk-1* mRNAs in the posterior ICM suggests that ectopic expression of BMP proteins possibly induces a VEGF autocrine- or paracrine-signaling that leads to simultaneous up-regulation of *vegf* and *flk-1* genes, and to proliferation of the hematopoietic and endothelial precursors in the expanded ICM. In support of this hypothesis, it was previously reported that both *vegf* and *flk-1* were detected by 48 hpf in the posterior ICM region of wild-type zebrafish [24] and microinjection of *vegf* mRNA in zebrafish embryos induced an elevated expression of *flk-1* in ICM [24].

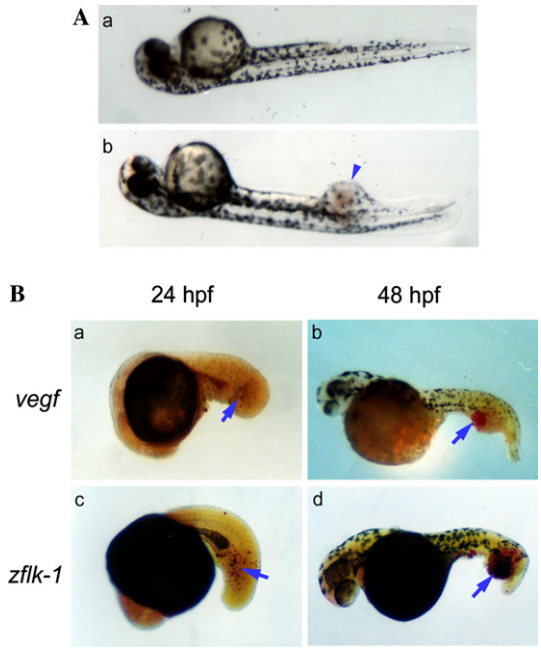

Fig. 6. Expansion of the posterior ICM and ectopic expression of *vegf* and *flk-1 induced by* human *bmp4* in zebrafish embryos. Wild-type zebrafish embryos at one cell stage were injected with CMV promoter directed human *bmp4* constructs or the expression vectors alone (0.1 ng/embryo/construct) as negative controls. DIG-labeled riboprobes for genes of *vegf* and *flk-1* were used in the WISH. (A) The expanded posterior ICM induced by human BMP4. (a) The control embryo developed normally at 48 hpf; (b) the transgenic embryo expressing human *bmp4* at 48 hpf. The arrowhead shows the enlarged posterior ICM. (B) The results of WISH in embryos injected with human *bmp4*. (a,b) Ectopic expression of the zebrafish *vegf* in the posterior ICM compartment at 24 and 48 hpf, and (c,d) elevated expression of the zebrafish *flk-1* in the same region at 24 and 48 hpf. The arrows indicate the hybridized area in the embryos.

In summary, we cloned the zebrafish vegf promoter and identified the Smad1/Smad5 binding elements located in the promoter. Using in vitro EMSA and in vivo reporter assay, we provided experimental evidence that Smad1 and Smad5 interacted with these elements to differentially regulate the expression of the *vegf* gene. We further showed that the ectopic BMP signaling induced co-expression of *flk-1* and *vegf* in zebrafish embryonic

endothelial and blood precursor cells. Our findings provide evidence that BMP/Smad pathway is one of the players regulating *vegf* expression in vasculogenesis and hemangiogenesis in zebrafsih.

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