Zebrafish *scube1* (<u>S</u>ignal Peptide-<u>C</u>UB (Complement Protein C1r/C1s, <u>U</u>egf, and <u>B</u>mp1)-<u>E</u>GF (Epidermal Growth Factor) Domain-containing Protein <u>1</u>) Is Involved in Primitive Hematopoiesis*[S]

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Background: Roles of zebrafish *scube1* in primitive hematopoiesis remain unknown.
Results: *scube1* knockdown by morpholino-oligonucleotides reduced the expression of marker genes associated with primitive hematopoiesis.
Conclusion: *scube1* is critical for and acts at the top of the regulatory hierarchy of primitive hematopoiesis by modulating BMP signaling.
Significance: Scube1 is a key regulator for primitive hematopoiesis in vertebrates.

scube1 (signal peptide-<u>C</u>UB (complement protein C1r/C1s, Uegf, and Bmp1)-<u>E</u>GF domain-containing protein <u>1</u>), the founding member of a novel secreted and cell surface SCUBE protein family, is expressed predominantly in various developing tissues in mice. However, its function in primitive hematopoiesis remains unknown. In this study, we identified and characterized zebrafish *scube1* and analyzed its function by injecting antisense morpholino-oligonucleotide into embryos. Whole-mount *in situ* hybridization revealed that zebrafish *scube1* mRNA is maternally expressed and widely distributed during early embryonic development. Knockdown of *scube1* by morpholino-oligonucleotide down-regulated the expression of marker genes associated with early primitive hematopoietic precursors (*scl*) and erythroid (*gata1* and *hbbe1*), as well as early (*pu.1*) and late (*mpo* and *l-plastin*) myelomonocytic lineages. However, the expression of an early endothelial marker *fli1a* and vascular morphogenesis appeared normal in *scube1* morphants. Overexpression of bone morphogenetic protein (*bmp*) rescued the expression of *scl* in the posterior lateral mesoderm during early primitive hematopoiesis in *scube1* morphants. Biochemical and molecular analysis revealed that Scube1 could be a BMP co-receptor to augment BMP signaling. Our results suggest that *scube1* is critical for and functions at the top of the regulatory hierarchy of primitive hematopoiesis by modulating BMP activity during zebrafish embryogenesis.

SCUBE1 (signal peptide-<u>C</u>UB-<u>E</u>GF (epidermal growth factor) domain-containing protein <u>1</u>) is the founding member of the newly identified, secreted, cell membrane-associated SCUBE family (1–7). This protein family is composed of three distinct members evolutionarily conserved in vertebrates (1, 2, 6–10). The proteins are organized in a modular fashion and share at least five protein domains: (i) an NH_2-terminal signal peptide sequence, (ii) nine copies of EGF-like repeats, (iii) a spacer region, (iv) three cysteine-rich motifs, and (v) one CUB domain at the COOH terminus. In mice and humans, SCUBE genes are all expressed in the endothelium but have unique expression profiles during development. For example, mouse *Scube1* is highly expressed in developing gonads, the central nervous system, somites, limb buds, and surface ectoderm (1). *Scube2* is expressed in embryonic neuroectoderm, developing face, heart, and several sites of the endochondral skeleton (2). *Scube3* transcript was localized to the neural tube, bronchial arches, the fronto-nasal region, somites, limb buds, developing teeth, and hair follicles (11). Thus, *Scube* genes may play important roles during early embryonic development.

However, we lack direct functional studies of these *Scube* genes by gene-targeting approaches to generate the null allele during mouse development, probably because of their large and complex genomic structures. Zebrafish have emerged as a powerful vertebrate model to knock down genes of interest to functionally assess genetic regulatory pathways (12). Indeed, forward genetic screens or knockdown experiments have revealed that the zebrafish *scube* genes are essential for proper Hedgehog signaling in development of slow muscle and ventral spinal cord fates (8–10, 13).

All vertebrates, including zebrafish, have two successive waves of hematopoiesis: primitive and definitive (14, 15). In mammalian and avian species, primitive hematopoiesis occurs in the extraembryonic yolk sac, but in zebrafish, it is at two intraembryonic locations: the anterior lateral meso-

derm (ALM)[2] and the posterior lateral mesoderm (PLM), which later fuses at the midline to form the intermediate cell mass (ICM). Primitive myelopoiesis is in the ALM, whereas primitive erythropoiesis initiates in the PLM/ICM region (14, 15).

The development of hematopoiesis precursor cells is regulated by both intrinsic and extrinsic factors. We have good documentation of the regulatory mechanisms underlying various intrinsic transcription factors (TFs) required for the formation of hematopoietic stems (*e.g. scl*, a basic helix-loop-helix TF) and the differentiation of stem cells along the myeloid (*e.g. pu.1*, an ETS-domain TF) or erythroid lineage (*e.g. gata1*, a zinc finger TF) (16, 17). However, the signaling regulation of extrinsic factors, such as secreted bone morphogenetic proteins (*e.g.* BMP4), upon induction of blood cells is less described (18–21). A recent molecular and genetic study showed that BMP acts upstream via Wnt signaling to activate the *cdx-hox* pathway, which directs the expression of *scl*, a key transcription factor specifying primitive hematopoietic progenitors (19).

In this study, we isolated and characterized the zebrafish *scube1* gene and investigated its role during zebrafish embryonic development by using an antisense morpholino-oligonucleotide (MO) knockdown approach. We found that zebrafish *scube1* plays an upstream role in primitive hematopoiesis by acting as a BMP co-receptor to increase its signal activity during early zebrafish embryonic development.

EXPERIMENTAL PROCEDURES

Ethics Statement—Animal handling protocols used in this study were reviewed and approved by the Institutional Animal Care and Utilization Committee, Academia Sinica (Protocol RMiIBMYR2010063).

Zebrafish Maintenance—Wild-type AB strain, *Tg(kdr:EGFP)* (22), and *Tg(gata1:dsRed)* (23) transgenic zebrafish were maintained in a 14-h light/10-h dark cycle at 28.5 °C. Zebrafish embryos were collected by natural spawning and incubated in 0.3× Danieau's buffer (1× Danieau's buffer: 58 mM NaCl, 0.7 mM KCl, 0.4 mM $MgSO_4$, 0.6 mM $Ca(NO_3)_2$, and 5 mM HEPES (pH 7.6) with double-distilled water) until observation or fixation. The definition of embryo stage was as described (24), and the stages are indicated by hours postfertilization (hpf). An amount of 0.2 mM *N*-phenylthiourea in 0.3× Danieau's buffer was used to suppress melanin formation after bud stage. Dechorionation of embryos was achieved by protease (0.01 g/ml) digestion.

Identification of scube1 Full-length cDNA in Zebrafish—A TBLASTN search of the public database with mammalian SCUBE1 protein sequences identified one expressed sequence tag clone (GenBank[TM] accession number EB778403) containing the 5′-most coding sequences of zebrafish *scube1*. This cDNA clone was then purchased from Open Biosystems (Lafayette, CO). After complete sequencing of this clone, we identi-

fied a full-length cDNA sequence of zebrafish *scube1* (3,842 bp) composed of a 122-bp 5′-UTR, a 3,075-bp protein-coding sequence, and a 645-bp 3′-UTR. This sequence was deposited in the public database with GenBank[TM] accession number JQ740109.

Whole-mount in Situ Hybridization (ISH)—Whole-mount ISH was performed essentially as described (25). A 640-bp cDNA in the 3′-UTR of zebrafish *scube1* was used to synthesize the antisense RNA riboprobe. All other probes were synthesized as described in the following papers: *scl* (26), *gata1* (27), *pu.1* (28), *hbbe1* (29), *l-plastin* (30), *mpo* (31) and *fli1a* (32). Two-color ISH was performed as described (33).

Morpholino-oligonucleotide and mRNA Microinjection—Two *scube1* antisense translation-blocking MOs (tMOs) were designed with the primers tMO1 (5′-AGG CTG TGA GTG AAT GGG TCT CTC C-3′) and tMO2 (5′-CCA GGA GAA GCA GGC AGG ACC CAT G-3′) (Gene Tools, Philomath, OR). The *scube1* tMO1 targets the 5′-UTR, and *scube1* tMO2 blocks the AUG translation start site of zebrafish *scube1*. The *scube1* tMOs diluted in Danieau's buffer (12) were injected at the one- or two-cell stage. Increasing amounts of each tMO were injected to titrate for the proper dose by monitoring the overall embryonic morphology or the expression of a mesoderm marker *myod1* to rule out the nonspecific phenotypic effects (supplemental Fig. 1). As a control for nonspecific effects, each experiment was performed in parallel with a randomized control MO oligonucleotide. For knockdown experiments, we injected 0.625 ng per embryo of *scube1* tMO1 or tMO2. Sense mRNA encoding full-length *scube1* was transcribed *in vitro* from the pCS2+-*scube1* plasmid by use of the mMESSAGE mMACHINE kit (Ambion).

Construction of MO Testing Plasmid and the Expression Plasmids—To construct the *scube1*-GFP testing plasmid, we cloned cDNA containing the region targeted by *scube1* tMOs (−31 to +24) into the EcoRI-XbaI sites of pCS2-EGFP vector. The *scube1* cDNA fragment was obtained by use of the following complementary oligonucleotides: 5′-aat tcG GAG AGA CCC ATT CAC TCA CAG CCT CTA ACC ATG GGT CCT GCC TGC TTC TCC TGG t-3′ and 5′-cta gaC CAG GAG AAG CAG GCA GGA CCC ATG GTT AGA GGC TGT GAG TGA ATG GGT CTC TCC g-3′. The FLAG or Myc epitope-tagged version of zebrafish Scube1 expression plasmid was constructed in the pFLAG-CMV1 (Sigma) or pSecTag2 (Invitrogen) as described (4). The expression plasmids coding for Myc-tagged BMP2 or BMP4 were prepared as described (34).

o-Dianisidine Staining of Erythrocytes—Hemoglobin of erythrocytes was stained as described (35). Zebrafish embryos collected at the proper stage were incubated in fresh prepared *o*-dianisidine solution (40% ethanol, 0.01 M sodium acetate, 0.65% H_2O_2, and 0.6 mg/ml *o*-dianisidine, dissolved in double-distilled water) for 20 min in the dark.

Cell Culture and Transfection—Human embryonic kidney (HEK)-293T cells were maintained as described previously (5) and were transfected by use of Lipofectamine 2000 (Invitrogen).

Immunoprecipitation and Western Blot Analysis—Two days after transfection, cell lysates were clarified by centrifugation at 10,000 × *g* for 20 min at 4 °C. Samples were incubated with 1 μg

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[2] The abbreviations used are: ALM, anterior lateral mesoderm; PLM, posterior lateral mesoderm; ICM, intermediate cell mass; TF, transcription factor; BMP, bone morphogenetic protein; BMPR, BMP receptor; MO, morpholino-oligonucleotide; tMO, translation-blocking MO; cMO, control MO; ISH, *in situ* hybridization; HEK, human embryonic kidney; hpf, hours postfertilization.

of the indicated antibody and 20 μl of 50% (v/v) Protein A-agarose (Pierce) for 2 h with gentle rocking. After three washes with lysis buffer, precipitated complexes were solubilized by boiling in Laemmli sample buffer, fractionated by SDS-PAGE, and transferred onto PVDF membranes. The membranes were blocked with phosphate-buffered saline (pH 7.5) containing 0.1% gelatin and 0.05% Tween 20 and blotted with the indicated antibodies. After two washes, the blots were incubated with horseradish peroxidase-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories) for 1 h. After washing the membranes, the reactive bands were visualized by use of the VisGlow™ chemiluminescent substrate, horseradish peroxidase system (Visual Protein).

Luciferase Activity Assay—Human HepG2 cells (3×10^5 cells/well) were seeded into 24-well plates and transfected with 0.3 μg of the BMP-responsive luciferase reporter I-BRE-Luc, 0.01 μg of the *Renilla* luciferase reporter used as an internal control, and either empty vector or the zebrafish *scube1* expression plasmid. Transfected cells were maintained in low serum medium (0.1% FBS) for 18 h. Luciferase activity was measured after a 24-h treatment with recombinant human BMP2 protein.

RESULTS

Identification and Embryonic Expression Pattern of Zebrafish scube1—A homology search of the public database identified a full-length *scube1* cDNA clone coding for a polypeptide of 1,024 amino acids sharing a conserved protein domain organization (Fig. 1*A* and supplemental Fig. 2) and overall 76 or 77% sequence identity with its mouse or human orthologue, respectively (supplemental Figs. 3 and 4). Furthermore, phylogenetic analysis showed that zebrafish Scube1 clustered closely with mouse and human SCUBE1 and separate from groups of SCUBE2 or SCUBE3 protein sequences (Fig. 1*B*). These results suggest that zebrafish *scube1* is an authentic orthologue of mammalian SCUBE1.

We then examined the spatiotemporal expression pattern of *scube1* during embryonic development by whole-mount ISH. Maternal *scube1* transcripts were distributed evenly in blastomeres (Fig. 1*C*). Likewise, zygotic *scube1* was globally expressed up to the 14-somite stage. From 20 to 31 hpf, strong *scube1* expression was detected in the brain and somitic region (Fig. 1*C*). The maternal and ubiquitous expression patterns of *scube1* during early embryonic stages implied that *scube1* may play critical roles during early embryogenesis.

Zebrafish Scube1 Is a Secreted and Surface-associated Glycoprotein—Because heterologous expression of human SCUBE1 resulted in secreted glycoprotein that could tether on the cell surface (7), we then examined whether overexpressed zebrafish Scube1 protein might also behave in a similar manner. The zebrafish Scube1 protein was heterologously expressed by transient transfection in HEK-293T cells. Overexpressed Scube1 (tagged with FLAG epitope) could be effectively secreted into the conditioned medium, targeted onto the cell surface, and post-translationally modified with *N*-linked oligosaccharides (Fig. 2).

Zebrafish scube1 Is Essential for Primitive Hematopoiesis—To further investigate the role of *scube1* during early embryogenesis in zebrafish, we then designed two non-overlapping
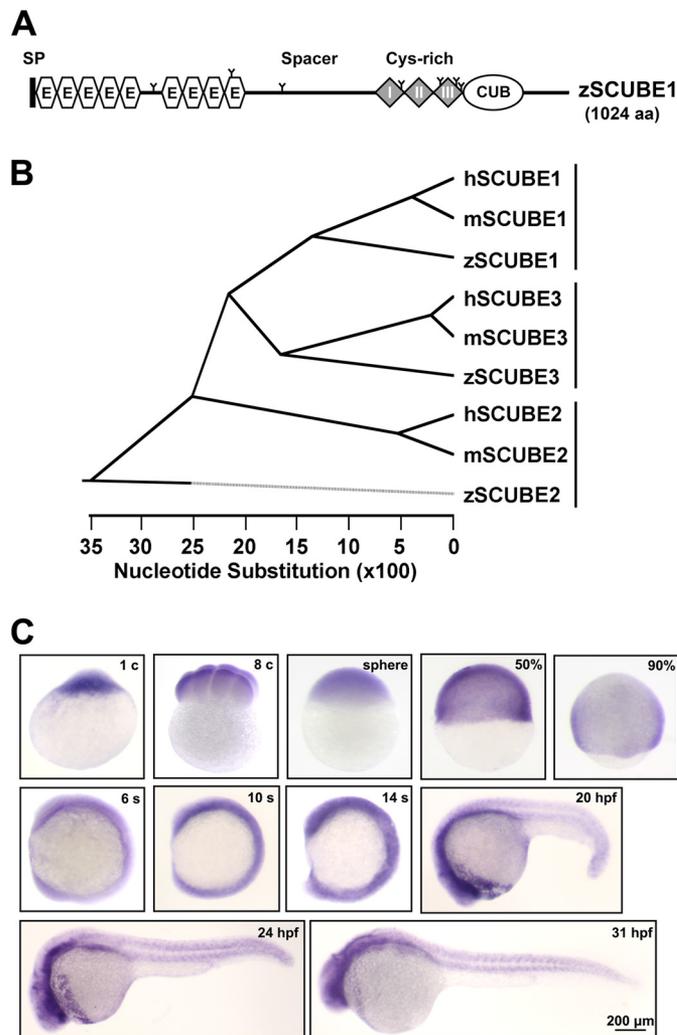

FIGURE 1. **Protein domain organization, phylogenetic analysis, and embryonic expression patterns of zebrafish *scube1*.** *A*, graphic illustration of the domain structure of zebrafish Scube1 protein. *SP*, signal peptide sequence; *E*, EGF-like domain; *Cys-rich*, cysteine-rich repeats. *B*, phylogenetic tree of the SCUBE protein family. Similarity of human (*h*), mouse (*m*), and zebrafish (*z*) SCUBE protein sequences was analyzed by use of the Lasergene MEGALIGN program (ClustalW algorithm). Branch order indicates structural relative, and branch length reflects sequence identity. *C*, whole-mount *in situ* hybridization of zebrafish embryos at the indicated stages with antisense *scube1* probe. Corresponding control sense probes detected no apparent signal (not shown). *c*, cell stage; *s*, somite stage; *hpf*, hours postfertilization.

antisense tMOs targeting the 5′-UTR or the start codon sequence of *scube1* to block translation of *scube1* (named *scube1* tMO1 or tMO2; Fig. 3*A*). A random sequence of antisense MO with minimal effect on embryonic development was used as a control (cMO). Both tMOs could specifically and effectively block translation of *scube1*, whereas cMO had no effect (Fig. 3*D*).

We then injected embryos with *scube1* tMOs (referred as morphants) and examined their effects on embryonic development. Zebrafish embryos injected with a low dose of cMO or *scube1* tMOs (0.625 ng/embryo) produced no gross lethality or morphological defect because the expression of *shh* (the axial mesoderm marker), *pax2.1* (the lateral mesoderm marker), and *myod1* (the somitic mesoderm marker) was not affected (Fig. 3*E*). However, the *scube1* morphants showed a markedly reduced number of circulating blood cells as compared with
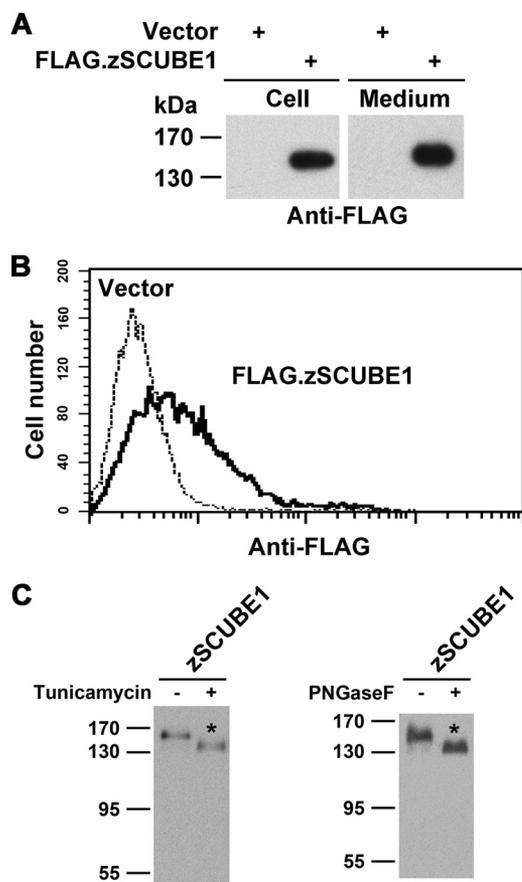
FIGURE 2. **Overexpressed zebrafish Scube1 is a secreted, surface-anchored glycoprotein in HEK-293T cells.** *A*, secretion of zebrafish Scube1 into the conditioned medium. HEK-293T cells were transfected with empty vector (*Vector*) or expression plasmid encoding FLAG-tagged zebrafish Scube1 (*FLAG.zSCUBE1*). Forty-eight hours post-transfection, the serum-free conditioned medium was collected, and cells were detached with phosphate-buffered saline/EDTA. Samples from cell lysate (*Cell*) or the serum-free conditioned culture medium (*Medium*) were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes. Recombinant FLAG.zS-CUBE1 protein was detected by Western blot analysis with anti-FLAG antibody. *B*, cell surface expression of zebrafish Scube1 protein. HEK-293T cells were transfected with empty vector (*Vector*) or expression plasmid encoding FLAG.zSCUBE1 protein. Forty-eight hours post-transfection, cells were detached and probed with anti-FLAG antibody and subjected to flow cytometry analysis. *C*, N-linked glycosylation of zebrafish SCUBE1. HEK-293T cells were transfected with expression vector encoding FLAG.zSCUBE1 protein. Transfected cells were cultured in the absence (−) or presence (+) of tunicamycin (an inhibitor of *N*-glycosylation) for 24 h (*left*). Alternatively, cell lysates were left untreated (−) or treated (+) with peptide *N*-glycosidase F (*PNGaseF*) to remove the *N*-linked oligosaccharide chain (*right*). Cell lysates derived from each treatment were examined by Western blot analysis with anti-FLAG antibody.

cMO-injected embryos at 30 hpf (supplemental Movies 1 and 2).

Because the first wave of blood production, primitive hematopoiesis, predominantly produced erythrocytes and some primitive macrophages in zebrafish (14), we then used *o*-dianisidine staining to detect hemoglobin-containing erythrocytes. The *scube1* morphant embryos showed severe reduction in proportion of differentiated erythrocytes in 48-hpf embryos (Fig. 4). Likewise, the expression of an erythroid-specific gene, β1-embryonic hemoglobin (*hbbe1*), was significantly reduced over the PLM at the 10-somite stage (supplemental Fig. 5) or in the ICM region at 31 hpf (Fig. 5B). Importantly, the loss of

o-dianisidine staining and *hbbe1* expression could be rescued by the co-injection of *scube1* mRNA in *scube1* morphant embryos (Figs. 4 (*E* and *F*) and 5C), which confirmed the specificity of *scube1* tMO targeting. Note that injection of *scube1* mRNA alone produced normal expression of *myod1* or *hbbe1* (supplemental Fig. 6 and Fig. 5D). To investigate whether *scube1* also plays an important role in regulating primitive myelopoiesis, we examined the expression of two myeloid markers, *l-plastin* (an actin-binding protein) and *mpo* (myeloperoxidase), which marks pan-myeloid cells and differentiated granulocytes, respectively (36). As shown in Fig. 5E, *scube1* knockdown significantly decreased the expression of these two myeloid-specific genes.

To further dissect whether *scube1* is critical in these early primitive hematopoietic processes, we then examined the effect of *scube1* tMO1 on gene expression associated with these early hematopoietic lineages. Whole-mount ISH at the 6-somite stage (12 hpf) showed that *scube1* knockdown resulted in reduced expression of marker genes associated with primitive hematopoietic progenitors (*scl*) or primitive myelopoiesis (*pu.1*) and primitive erythropoiesis (*hbbe1* and *gata1*) in the ALM or PLM (Fig. 6A and supplemental Fig. 5). Similar phenotypes were observed in tMO2-injected embryos (data not shown).

Zebrafish scube1 Is Not Involved in the Fate Determination of Vascular Endothelial Progenitors or Blood Vessel Formation—Because primitive hematopoietic and vascular endothelial precursors may be derived from common hemangioblasts in zebrafish (37), we then examined whether *scube1* also plays a role in the fate determination of endothelial precursors and subsequent blood vessel morphogenesis. We examined the expression of a vascular endothelium-specific transcription factor, *fli1a*, in *scube1* morphants and injected *Tg(kdr:EGFP)* (22) transgenic embryos with the *scube1* tMO1. Expression of *fli1a* in the ALM and PLM at early 6-somite stage or in endothelial cells at 31 hpf was virtually unaffected (Fig. 6, *B–E*). In agreement with this finding, the *scube1* tMO-injected *Tg(kdr:EGFP)* embryos showed a morphologically intact vasculature up to 48 hpf (Fig. 6, *F* and *G*). Thus, *scube1* may not be needed for specification of angioblasts at early stages or angiogenesis at late stages of embryogenesis.

Forced Expression of scl Rescues the Primitive Hematopoietic Defects in scube Morphants—To establish the epistatic relationships between *scube1* and genes involved in early primitive hematopoiesis, we further determined whether overexpression of *scl* could rescue the *pu.1* or *gata1* expression defects in *scube1* morphants. Forced expression of *scl* by mRNA microinjection in *scube1* morphants rescued the expression of both *pu.1* and *gata1* (Fig. 7A), which suggests that *scl* acts downstream of *scube1* to specify early primitive myelopoietic and erythropoietic precursor formation.

Overexpression of bmp2b or bmp4 Rescues the Expression of scl in scube1 Morphants—Because human SCUBE1 or SCUBE2 have been implicated in regulating the long range signaling of BMP (3, 34) and because BMP signaling is important for mesoderm patterning and specifying the blood fate (19, 21, 38), we then investigated the genetic relationship between *scube1* and *bmp* genes by co-injection of *bmp2b* or *bmp4* mRNA with
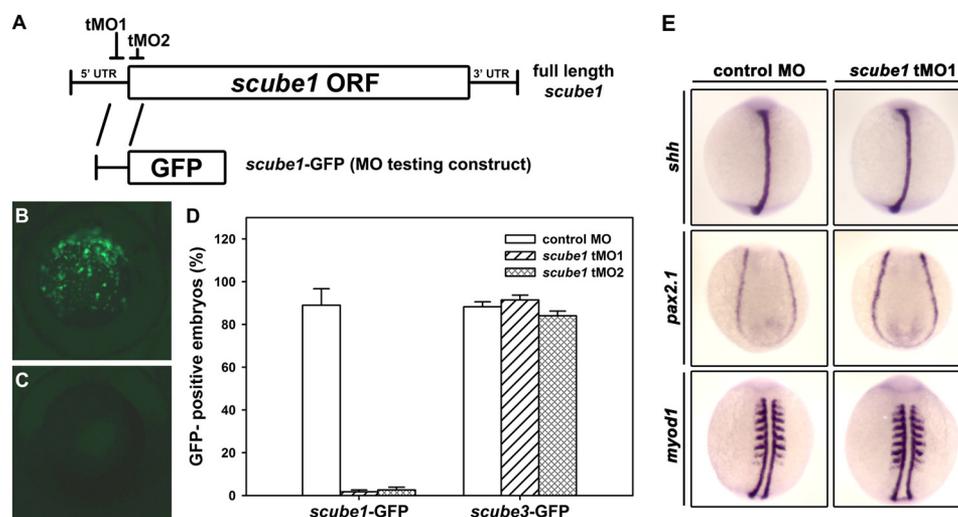
FIGURE 3. **Translational blocking efficiency of two *scube1* tMOs and no effect of *scube1* knockdown on the expression of various mesoderm genes.** *A*, diagram showing the targeting sites of two *scube1* tMOs and the *scube1*-GFP test construct. tMO1 targets the 5′-UTR, and tMO2 blocks at the AUG translation start site. The *scube1*-GFP plasmid contains pCS2-EGFP fused with a fragment of *scube1* gene sequence from −31 to +24. Embryos at the one-cell stage were injected with 250 pg of *scube1*-GFP or *scube3*-GFP testing plasmid without or with 0.625 ng of *scube1* tMO1 or tMO2, respectively. The expression of GFP was examined at 8 hpf and photographed by epifluorescent microscopy. Images are representative of embryos injected with scube1-GFP plasmid (*B*) or *scube1* tMO1 (*C*). *D*, summary of translational blocking efficiency of two *scube1* tMOs. The lack of suppression effect on the *scube3*-GFP testing construct confirms their specificity in blocking the translation of *scube1* protein. *Error bars*, S.E. *E*, whole-mount ISH of *shh* (the axial mesoderm marker), *pax2.1* (the lateral mesoderm marker), and *myod1* (the somitic mesoderm marker) in cMO- or *scube1* tMO1-injected embryos (0.625 ng/embryo) at the 6-somite stage (dorsal view).
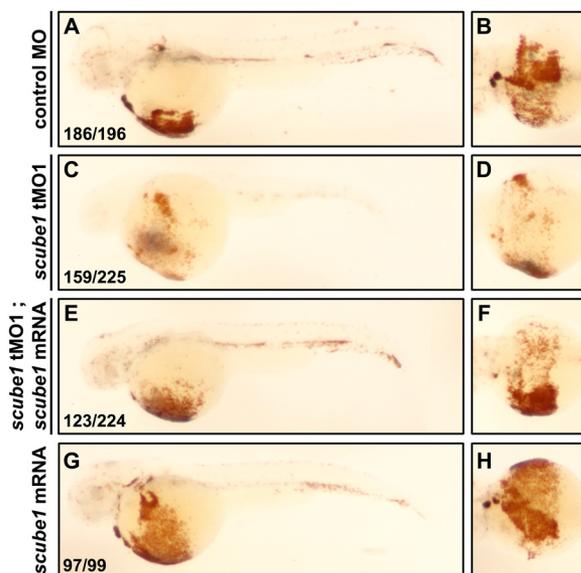
FIGURE 4. **Effect of *scube1* knockdown and *scube1* mRNA rescue on the production of erythrocytes.** *A–D*, detection of hemoglobin in erythrocytes stained with *o*-dianisidine in control MO or tMO1-injected embryos at 48 hpf. *E–H*, forced expression of *scube1* mRNA alone had little phenotypic effect (*G* and *H*) but could partially rescue the anemic defect in the *scube1* tMO1-injected embryos (*E* and *F*). The proportion of erythrocytes was restored by co-injection of 50 pg of *scube1* mRNA in *scube1* morphants at 48 hpf. Embryos are shown in lateral (*A, C, E,* and *G*) and ventral view (*B, D, F,* and *H*). The *numbers* indicated in the *bottom left-hand corners* of photographs are the number of affected embryos with phenotype similar to what is shown in the picture (*left*) and the total number of observed embryos (*right*).
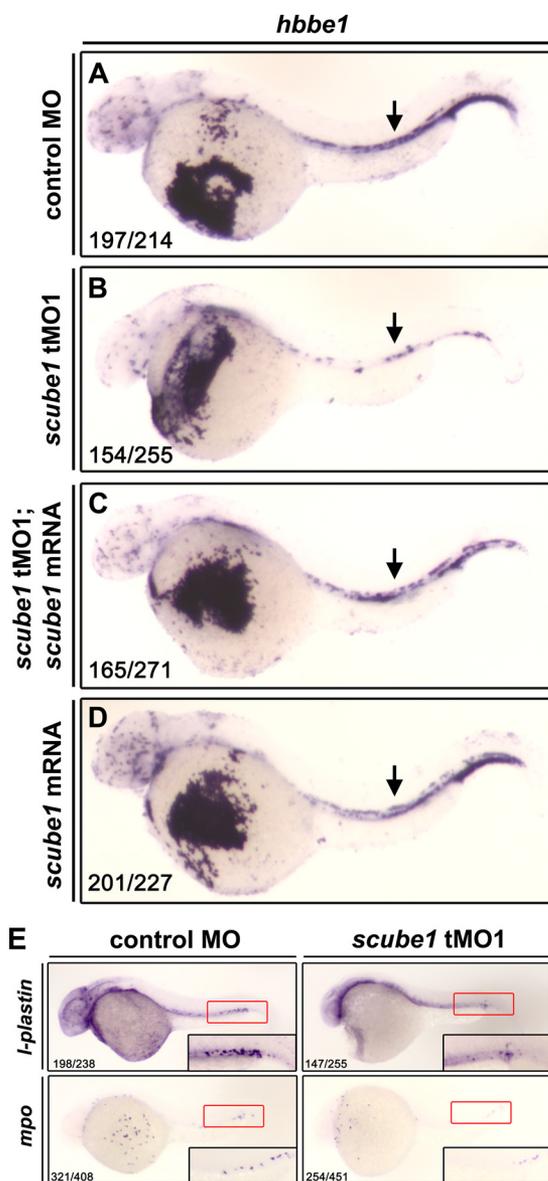
scube1 tMO1 in embryos. Overexpression of *bmp2b* or *bmp4* restored *scl* expression in *scube1* morphants (Fig. 7*B*), which suggests that *bmp* lies downstream or parallel to *scube1* for the initiation of zebrafish primitive hematopoiesis.

Zebrafish Scube1 Can Interact with BMP Ligands or Receptors and Enhance BMP Signaling—Because we observed a genetic interaction between *scube1* and *bmp* genes, we then

examined whether Scube1 could biochemically interact with BMP ligands and their receptors and, if so, whether zebrafish Scube1 could affect BMP signal activity. HEK-293T cells were transfected with the expression plasmid encoding Myc-tagged BMP2 or BMP4 alone or with the FLAG.zSCUBE1 expression construct. Two days after transfection, cell lysates underwent immunoprecipitation with the anti-Myc monoclonal antibody, and precipitates were analyzed by immunoblotting with anti-FLAG monoclonal antibody to determine protein interaction. Immunoprecipitation with anti-Myc antibody resulted in a specific coprecipitation of the Scube1 protein by BMP2/4 but not IL-8 (Fig. 8*A*). Thus, Scube1 may indeed form a complex with BMP2 or BMP4.

BMP signaling is initiated when BMP protein binds to its receptor complex, composed of two type I (BMPR-I) and two type II (BMPR-II) receptors (38, 39). Because Scube1 is also expressed on the cell surface (Fig. 2*B*), we next investigated whether Scube1 could interact with BMPR-I or -II. HEK-293T cells were transfected with BMPR-IA, -IB, or -II expression plasmids alone (tagged with the HA or FLAG epitope for easy detection) or with FLAG-tagged Scube1 (Fig. 8*B*) or Myc-tagged Scube1 (Fig. 8*C*) expression constructs, respectively. At 2 days after transfection, the BMPR-I or -II protein was immunoprecipitated with its corresponding anti-epitope antibody (anti-HA or anti-FLAG), and individual immunoprecipitates were analyzed by immunoblotting for the presence of Scube1 (anti-FLAG or anti-Myc). Scube1 protein co-immunoprecipitated with BMPR-IA, -IB, or -II protein (Fig. 8, *B* and *C*), which suggests that Scube1 can also interact with BMPRs on the plasma membrane. The interaction between Scube1 and BMPRs appears to be selective because Scube1 is incapable of forming a complex with Toll-like receptor 4 (TLR4) (Fig. 8*B*).

Because of the biochemical interactions between Scube1 and BMP ligands or receptors, we then evaluated whether Scube1

FIGURE 5. **Effect of *scube1* knockdown on the expression of erythrocyte and myeloid-specific markers.** *A* and *B,* expression of the erythrocyte marker *hbbe1* in control MO- or tMO1-injected embryos at 31 hpf (lateral view). *C* and *D,* overexpression of *scube1* mRNA produced little phenotypic effect (*D*) but could partially rescue *hbbe1* expression in *scube1* morphants (*C*). The expression of *hbbe1* was restored by co-injection of 50 pg of *scube1* mRNA in *scube1* morphants at 31 hpf. The *arrows* indicate the *hbbe1*-expressing erythrocytes in the ICM region. *E,* expression of the granulocyte marker *l-plastin* and macrophage marker *mpo* in cMO- or tMO1-injected embryos at 31 hpf (lateral view). The ICM regions are *boxed* and shown at higher magnification in the *bottom right-hand corner* of each photograph. The *numbers* indicated in the *bottom left-hand corners* of photographs are the number of affected embryos with phenotype similar to what is shown in the picture (*left*) and the total number of observed embryos (*right*).

could regulate the BMP signal activity. HepG2 cells were transfected with a BMP-responsive luciferase reporter construct, BRE-Luc (40), alone or with plasmid encoding Scube1 (Fig. 8*D*). Transfected cells were then stimulated with or without BMP2 for 24 h before measurement of luciferase activity. In the absence of Scube1, treatment with BMP2 increased the relative luciferase activity as compared with untreated cells (Fig. 8*D*). Co-transfection with Scube1 similarly increased BRE luciferase activity even in the absence of exogenous BMP2 stimulation,

probably because of Scube1-mediated cross-linking of BMPR-I and -II proteins (Fig. 8*D*). Most interestingly, Scube1 markedly enhanced signaling with exogenous BMP2 (Fig. 8*D*). As a control, co-transfection with Scube1 did not increase transforming growth factor (TGF)-β- or NF-κB-responsive luciferase activity above base line (data not shown). Furthermore, knockdown of *scube1*, not *scube2*, significantly decreased the BMP-responsive Smad activation (phosphorylated Smad1/5/8 protein level) (supplemental Fig. 7), which demonstrates the specific role of Scube1 in modulating BMP signaling. Consistently, whole-mount confocal immunofluorescent analysis also showed a reduction of p-Smad1/5/8 level in hematopoietic progenitors in the ICM region (supplemental Fig. 8). Thus, Scube1 may act as a BMP co-receptor and augment BMP-mediated signal activity.

DISCUSSION

In this study, we isolated the zebrafish *scube1* gene and characterized its expression and function in primitive hematopoiesis. Although *Scube1* is highly expressed in developing gonads, nervous system, somites, surface ectoderm, and limb buds in mice (1, 7), its involvement in primitive hematopoiesis in vertebrates has not yet been described. Our results demonstrate that *scube1* is critical for primitive hematopoiesis in zebrafish embryos by positively regulating the expression of *scl*, a master gene in hematopoiesis. Epistasis experiments revealed an ordered *scube1-bmp-scl* pathway. Consistent with this finding, our biochemical and molecular analyses suggested that Scube1 may modulate BMP signal transduction during receipt of the BMP signal at the plasma membrane. This modulation may occur through the action of Scube1 as a BMP co-receptor in enhancing cellular responses to BMP for specifying the earliest hematopoietic precursor cells at the onset of primitive hematopoiesis. Thus, our findings position *scube1* at the top of the regulatory hierarchy of primitive hematopoiesis in zebrafish.

Several lines of evidence indicate that *scube1* is critical for the initiation of primitive hematopoiesis. First, knockdown of *scube1* reduced the expression of the hematopoietic precursor marker *scl* during the early segmentation stage (Fig. 6*A*), and as a result, the expression of the erythroid marker *gata1*, hemoglobin gene *hbbe1*, and myeloid markers *pu.1*, *mpo*, and *l-plastin* was blocked at later stages (Figs. 5*E* and 6*A*). Second, the effect of *scube1* knockdown could be rescued by coinjection of *scube1* mRNA (Figs. 4 and 5). Third, forced expression of *bmp2b* or *bmp4* could overcome the suppressive effect of *scube1* knockdown on primitive hematopoiesis (Fig. 7*B*). Finally, we also validated hematopoietic phenotypes of *scube1* knockdown by two independent splice-blocking MOs that target the exon2-intron2 or intron3-exon4 boundary sequences (data not shown).

BMPs belong to the TGF-β superfamily, initially essential for the patterning of ventral mesoderm from where the primitive hematopoietic stem cells arise. However, recent gain- or loss-of-function manipulation of the BMP signal pathway during middle or late gastrulation or even post-gastrulation suggested that BMP signaling also functions in blood fate specification (19, 21, 41–45). Overall, our genetic data suggest that *scube1* may regulate the BMP signaling for its later role during somitogenesis in the specification of blood progenitors. Further-
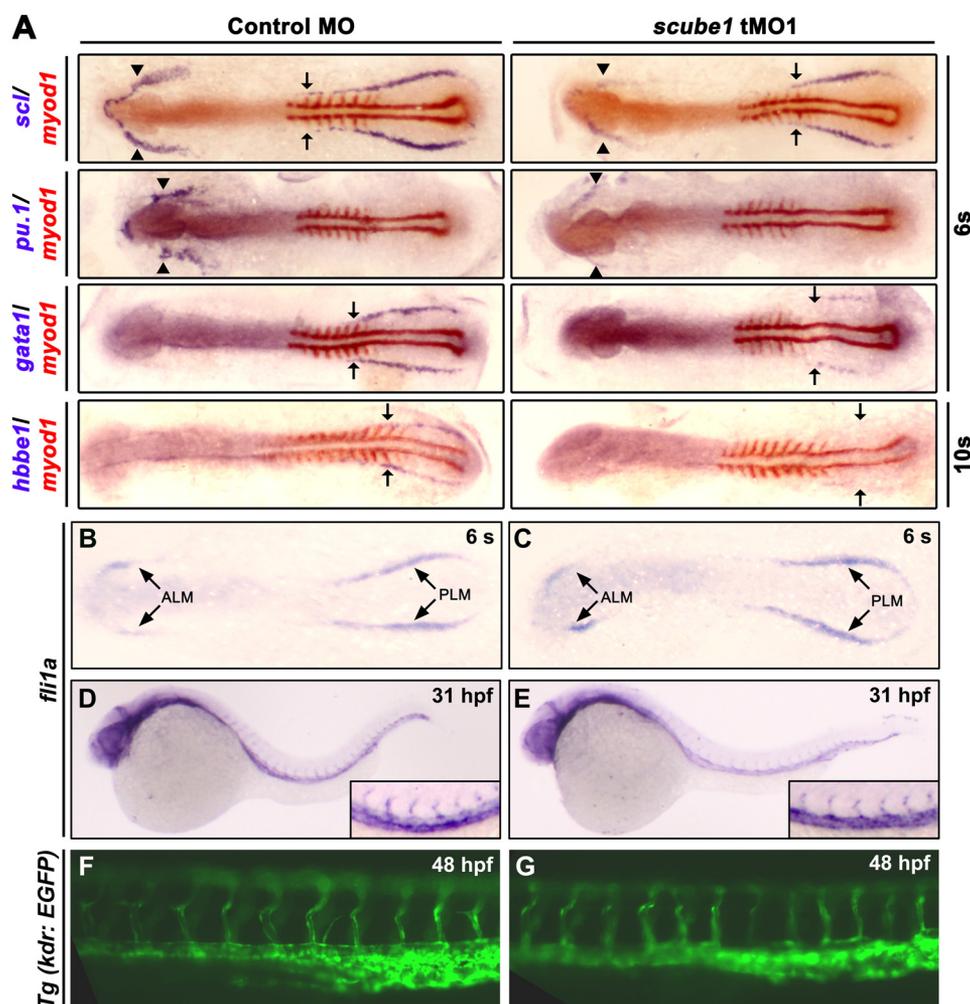
FIGURE 6. **Effect of *scube1* knockdown on the expression of early hematopoiesis precursor markers, a vascular endothelial marker, and blood vessel morphogenesis.** *A*, one-celled embryos were injected with cMO or *scube1* tMO1 (1.25 ng/embryo) and examined for expression of the indicated markers at the 6- or 10-somite stage by two-color RNA ISH. Embryos are shown in dorsal view with anterior to the *left*. Although the expression of *myod1* (*red*) remained relatively unaltered, the expression level of *scl* or *pu.1* at the ALM (*arrowheads*) and the expression margins of *scl*, *gata1*, and *hbbe1* at the PLM (*arrows*) were reduced in the *scube1* morphants as compared with the control embryos. *s*, somite. *B–G*, expression of a vascular endothelial marker, *fli1a*, in cMO- or *scube1* tMO1-injected embryos at the 6-somite stage (*B* and *C*, 6 s, dorsal view with anterior to the *left*) and 31 hpf (*D* and *E*, lateral view with anterior to the *left*). Shown are fluorescent images of *Tg(kdr:EGFP)* embryos at the trunk region injected with cMO (*F*) or *scube1* tMO1 (*G*) at 48 hpf.

more, biochemical and molecular experiments revealed that Scube1 expressed on the cell surface may act as a BMP co-receptor through its interactions with BMP ligands and receptors to facilitate the presentation of BMP ligands to its receptor complexes, thus promoting BMP signal transduction in the responding cells (Fig. 8). Our data suggest an interaction between SCUBE1 and BMP receptor protein when overexpressed in HEK-293T cells; however, it remains to be confirmed whether or not such an interaction indeed occurs *in vivo*.

In addition, we cannot exclude the possibility that a secreted extracellular form of Scube1 may be involved in transporting and/or protecting the BMP ligands against degradation in the extracellular environment. Alternatively, the secreted Scube1 protein may deposit in the extracellular milieu, where it cooperates with yet unknown regulatory molecules to modulate other cell growth signalings and establish a competent stem cell niche or microenvironment suitable for hematopoietic differentiation. We further performed RT-PCR analysis to examine the expression of *scube1* in isolated hematopoietic progenitor cells from transgenic *Tg(gata1:dsRed)* embryos (23). Our

results showed that *scube1* is also expressed in hematopoietic progenitors, suggesting that Scube1 may act cell-autonomously in primitive hematopoiesis (supplemental Fig. 9). However, further cell transplantation experiments are needed to verify the cell-autonomous or non-cell-autonomous role of *scube1*.

During development, blood and endothelial cells differentiate in close proximity, which suggests that these cells may derive from common bipotential precursor cells, termed the hemangioblasts. A recent fate-mapping study provided clear evidence of the existence of hemangioblasts within zebrafish embryos (37). However, the number of such bipotential progenitors is limited *in vivo*, which suggests that not all endothelial and blood cells arise from a common progenitor. Instead, juxtaposed but distinct populations of angioblasts and hematopoietic precursors may develop independently from the posterior mesoderm and further differentiate into vascular endothelial cells or blood cells (46, 47). Consistent with this notion, our data show that *scube1* is solely involved in the BMP regulatory pathway required for specifying hematopoietic fate and completely separate from the vascular endothelial lineage. Likewise,
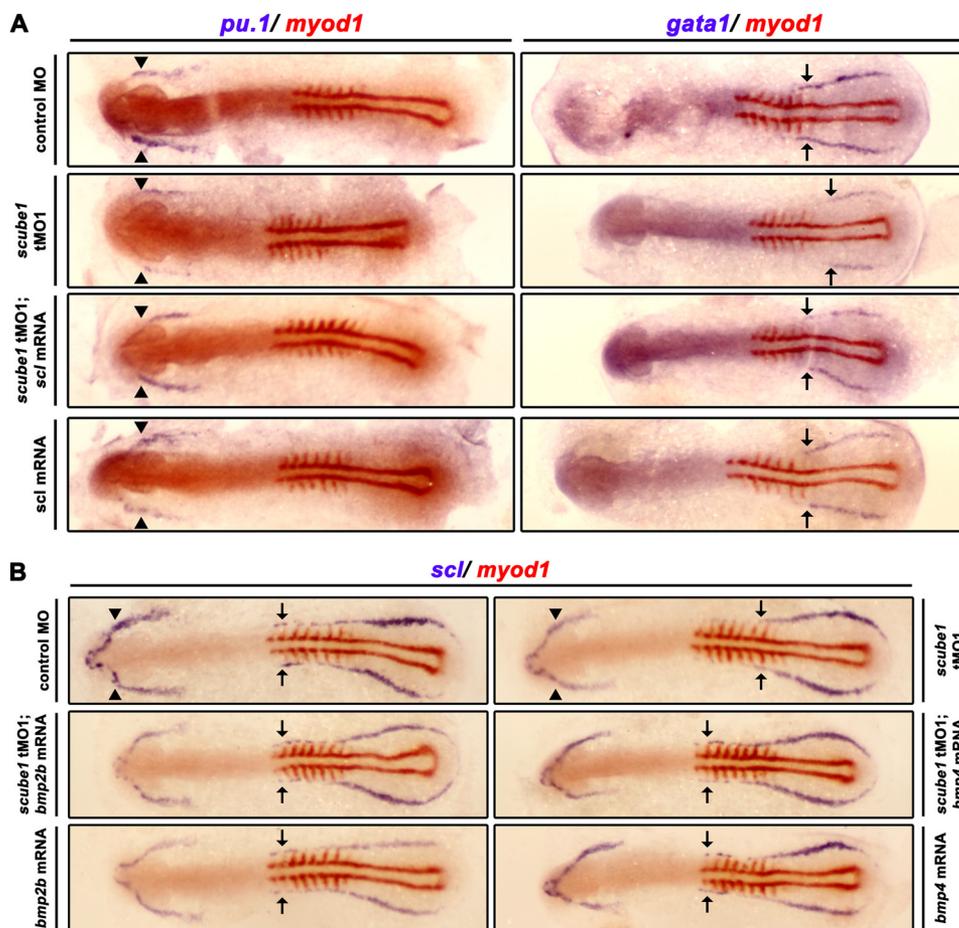
FIGURE 7. ***scube1* acts upstream of *scl* during primitive hematopoiesis, and overexpression of *bmp* rescues *scl* expression in *scube1* morphants.** *A*, injection of 50 pg of *scl* mRNA rescued *pu.1* and *gata1* expression in *scube1* morphants at the 6-somite stage. Embryos are shown in dorsal view with anterior to the *left*. Expression of *pu.1* or *gata1* in ALM or PLM was marked by *arrowheads* or *arrows*, respectively. Note that the expression level of *pu.1* in ALM or the expression margins of *gata1* in PLM were reduced in the *scube1* morphants that can be partially rescued by *scl* overexpression. *B*, injection of 50 pg of *bmp2b* and *bmp4* mRNA restored *scl* expression in *scube1* tMO1-injected embryos at the 6-somite stage. Embryos are shown in dorsal view with anterior to the *left*. Note that the margins of *scl* expression in the anterior region of PLM (*arrows*) were reduced in the *scube1* morphants, which can be restored by *bmp* overexpression.

in *Xenopus* embryos, expression of the blood gene SCL but not endothelial gene Xfli1 depends on BMP signaling (48). However, additional studies are needed to understand the molecular mechanism underlying the context-dependent BMP regulatory function of *scube1* in specifying hematopoietic progenitor cells.

Although the Scube protein family contains three members with similar domain structure and high protein sequence homology, knockdown of two other *scube* genes (*scube2* and *scube3*) produced no apparent defects in primitive hematopoiesis.[3] This observation can be simply due to a distinctive, nonoverlapping spatiotemporal expression pattern and thus nonredundant functions for each *scube* gene during early zebrafish embryogenesis. For example, zebrafish *scube2* has been implicated in the development of slow muscle fibers and the ventral neural tube by modulating the hedgehog signal transduction pathway (8–10). Furthermore, because BMP signaling is also involved in definitive hematopoiesis (42, 43, 49), we are interested in determining whether *scube1* or other *scube* genes are also involved in definitive hematopoiesis.

Because the genetic program that drives hematopoiesis is evolutionarily conserved among vertebrates, whether SCUBE1 is also involved in primitive hematopoiesis in mammals is of interest. Because of the large and complex genomic structure of the mouse *Scube1* gene, designing a targeting strategy for generating a null allele is difficult. Indeed, our previous targeting study disrupted only the COOH-terminal CUB domain of mouse *Scube1* (mutant *scube1*[Δcub] allele), which resulted in brain malformation and perinatal lethality (34). Although this gene-targeting study clearly demonstrated the role of *Scube1* during early stages of central nervous system development, its function in primitive hematopoiesis was not obvious and not thoroughly examined (34). Given the essential role of *Scube1* in primitive hematopoiesis in zebrafish, we are interested in producing a conditional null allele to further explore the biological functions of *Scube1* in mammalian hematopoiesis or other developmental processes.

In summary, our data demonstrate that zebrafish *scube1* plays a critical role in the specification of blood cells by regulating the upstream, extrinsic Bmp signal activity during early somitogenesis. Given the wide spectrum expression of *Scube1*

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[3] K.-C. Tsao, C.-F. Tu, S.-J. Lee, and R.-B. Yang, unpublished data.

A

B

C

D
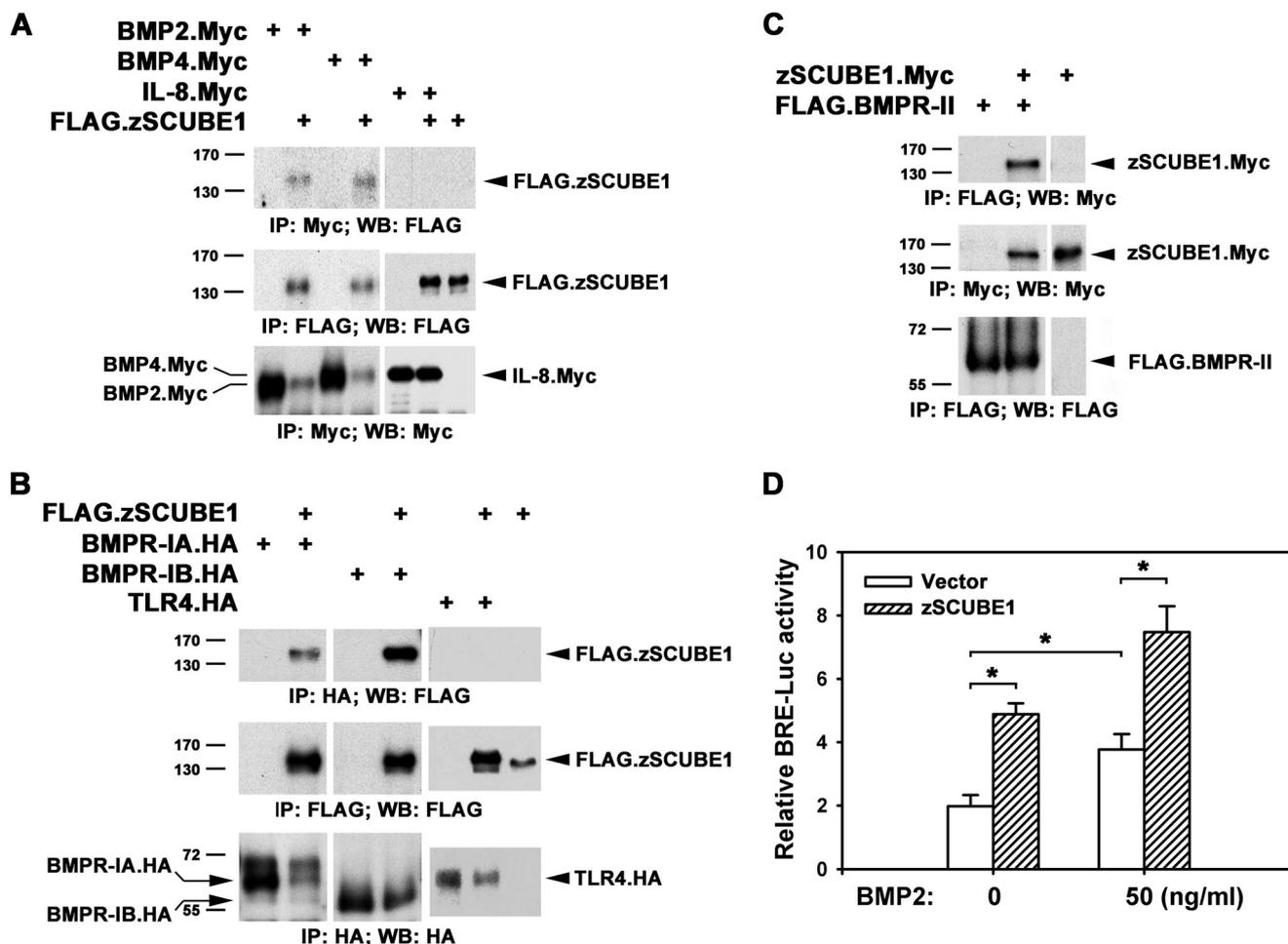
FIGURE 8. **Scube1 forms a complex with BMP ligands and receptors and augments BMP signal activity.** The expression plasmid encoding the Myc-tagged BMP2/BMP4 or IL-8 (*A*), HA-tagged BMPR-IA/IB or TLR4 (*B*), or FLAG-tagged BMPR-II (*C*) was transfected alone or with the FLAG-tagged SCUBE1 or Myc-tagged SCUBE1 in HEK-293T cells. After 2 days, cell lysates underwent immunoprecipitation (*IP*) and then Western blot analysis (*WB*) of expression of the indicated proteins to determine protein-protein interactions. *D*, SCUBE1 enhances BMP signaling. HepG2 cells were transfected with BRE-Luc and pRL-TK alone or with the expression plasmid encoding SCUBE1. Transfected cells were then incubated for 24 h with or without 50 ng/ml BMP2, and luciferase activity was measured. Relative luciferase activity represents firefly luciferase values normalized to *Renilla* activity. Data are means ± S.E. (*error bars*). *, $p < 0.05$.

in various developing tissues (1, 50), further investigation is needed to fully elucidate its stage- and tissue-specific functions during embryonic development.

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