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A Transmembrane Protein EIG121L Is Required for Epidermal Differentiation during Early Embryonic Development*[S]

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Epidermal differentiation in the ventral ectoderm of *Xenopus* embryos is regulated by the bone morphogenetic protein (BMP) pathway. However, it remains unclear how the BMP pathway is activated and induces the epidermal fate in the ventral ectoderm. Here, we identify a novel player in the BMP pathway that is required for epidermal differentiation during *Xenopus* early embryonic development. We show that *Xenopus* EIG121L (xEIG121L) protein, an evolutionarily conserved transmembrane protein, is expressed in the ventral ectoderm at the gastrula and neurula stages. Almost complete knockdown of xEIG121L protein with antisense morpholino oligonucleotides in early *Xenopus* embryos results in severe developmental defects, including the inhibition of epidermal differentiation and the induction of neural genes. Remarkably, our analysis shows that BMP/Smad1 signaling is severely suppressed in the xEIG121L knockdown ectoderm. Moreover, immunoprecipitation and immunostaining experiments suggest that xEIG121L protein physically interacts, and co-localizes, with BMP receptors. Thus, our results identify a novel regulator of the BMP pathway that has a positive role in BMP signaling and plays an essential role in epidermal differentiation during early embryonic development.

The bone morphogenetic protein (BMP)[4] signaling pathway has key roles in embryonic development, adult homeostasis, and diseases (1–6). A secreted dimeric ligand binds to a heterotetrameric cell surface complex of two type II and two type I kinase receptors. The type II receptor phosphorylates the type I receptor and thereby activates it. The activated type I receptor phosphorylates receptor-activated Smad (Smad1, Smad5, and Smad8 in mammals) at C-terminal serines. These phosphorylated Smad proteins form heterotrimeric complexes with the common-mediator Smad (Smad4 in mammals), and these complexes accumulate in the nucleus where they participate in the transcriptional control of target genes with sequence-specific transcription factors, co-activators, and co-repressors (1–6).

In *Xenopus* embryos, the BMP pathway determines cell fates at the gastrula stage (7, 8). In the ectoderm, the ventral region, in which the BMP pathway is activated, differentiates into epidermal tissues, and the dorsal region, in which the BMP pathway is inhibited, differentiates into neural tissues. Previous studies have shown that various factors regulate the BMP pathway during early embryonic development (7, 9–18). Secreted factors such as Chordin, Noggin, and Follistatin bind to BMPs (BMP2, BMP4, BMP7, and anti-dorsalizing morphogenetic protein) in the extracellular space and inactivate BMP signaling at the gastrula stage, thereby inducing neural differentiation in the dorsal ectoderm (9, 10, 12, 19). In the ventral region, secreted factors Sizzled (Szl) and Crossveinless-2 (Cv2) are induced by the BMP pathway and serve as BMP feedback inhibitors (15, 17). These molecules comprise a network of BMP interacting proteins to establish the dorsoventral body axis in early embryos.

In epidermal differentiation, the BMP pathway activates the expression of target genes, including *Xvent2* and *Msx1*, in the ventral ectoderm (20, 21). These transcription factors induce more restricted proepidermal genes such as *Dlx3/5*, *Grhl1*, and *Xap2* (8, 22, 23). These genes in turn regulate epidermal structural genes such as epidermal keratin. Thus, the epidermal fate is determined by the regulatory network in the downstream of the BMP pathway. However, it remains unclear how the BMP pathway is activated in the ventral ectoderm in epidermal differentiation.

We previously reported that *Xenopus* EIG121L (xEIG121L) mRNA has specific expression patterns during early *Xenopus* embryogenesis and is expressed in the ventral ectoderm at the gastrula and the neurula stages (24). *EIG121L* is an evolutionarily conserved gene, but its function has not been elucidated. Its specific expression pattern strongly suggests that xEIG121L has some important role in epidermal differentiation during early embryonic development. In this study, we show that xEIG121L protein is a novel regulator of the BMP pathway and plays an essential role in epidermal differentia-

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[4] The abbreviations used are: BMP, bone morphogenetic protein; dnBMPR, dominant negative BMP receptor; mEIG121L, mouse EIG121L; MO, morpholino oligonucleotide; rhBMP2, recombinant human BMP2; Szl, Sizzled; xEIG121L, *Xenopus* EIG121L; 5misMO, five-bp mismatch MO.

tion during early embryonic development. Our results show that xEIG121L protein is expressed in the ventral ectoderm and localizes in the plasma membrane and the perinuclear foci in early *Xenopus* embryos and that knockdown of xEIG121L causes the inhibition of epidermal differentiation, resulting in severe developmental defects. Remarkably, our analysis demonstrates that xEIG121L protein is required for Smad1 phosphorylation in the ventral ectoderm. In addition, xEIG121L physically interacts and co-localizes with BMP receptors. We have also found that EIG121L overexpression enhances BMP signaling both in mammalian cultured cells and *Xenopus* embryos. These results demonstrate that xEIG121L is a novel player of BMP signaling during epidermal differentiation.

EXPERIMENTAL PROCEDURES

Embryo Manipulations—*Xenopus* embryos were obtained by *in vitro* fertilization and cultured in 0.1 × MBS (1.0 mM HEPES, pH 7.4, 8.8 mM NaCl, 0.1 mM KCl, 0.24 mM $NaHCO_3$, 0.082 mM $MgSO_4$, 0.03 mM $Ca(NO_3)_2$, and 0.041 mM $CaCl_2$). Embryos were staged according to Nieuwkoop and Faber (25). Antisense morpholino oligonucleotides (MOs) or mRNAs were injected into animal poles of four-cell stage embryos in 4% Ficoll in 0.1× MBS. *In vitro* synthesis of capped mRNA was performed using mMESSAGE mMACHINE (Ambion) according to the manufacturer's instructions. Antisense morpholino oligonucleotides were obtained from Gene Tools, Inc. The MO sequences were as follows: xEIG121L MOα, 5′-ATCCACTCCAGCAGAAAACCCCCAT-3′; xEIG121L MOβ, 5′-TCCGCTCCAGCAGAAAAACCCCATC-3′; xEIG121L 5misMOα, 5′-ATGCAGTCCAGCACAAAACGCCGAT-3′; xEIG121L 5misMOβ, 5′-TCGGCTGCACCAGAAAAAGCCAATC-3′; and a standard control oligo (control MO), 5′-CCTCTTACCTCAGTTACAATTTATA-3′. Sequences complementary to the predicted start codon are *underlined*. Ectodermal explants were dissected at stage 8 and cultured in 1 × Steinberg's solution (10 mM HEPES, pH 7.4, 60 mM NaCl, 0.67 mM KCl, 0.83 mM $MgSO_4$, and 0.34 mM $Ca(NO_3)_2$). The explants were harvested at indicated stages for RT-PCR.

Molecular Cloning and Plasmid Construction—We found several sequences in *Xenopus laevis* expressed sequence tags database (GenBank™/EMBL/DDBJ accession nos. BU908560, CA987114, BP729964, DC077406, BJ636768, BJ048271, and BJ081522) that are homologous to *Xenopus tropicalis EIG121L* (GenBank™/EMBL/DDBJ accession no. CT025403). We designed primers based on the sequence of BU908560 and isolated the full length of xEIG121L. For rescue experiments, an xEIG121L MO-resistant construct (xEIG121L(MOres)) was generated by changing nucleotides at the morpholino target site of xEIG121L as follows, indicated in lowercase letters; 5′-GATGGGtGTaTTtTGtTGGtcgGGt-3′. Sequences of the predicted start codon are *underlined*. Expression plasmids were constructed as described (26). Myc tag was added to the C terminus of xEIG121L(MOres), xALK2, xALK3, AcvR2b, xBMPR2, or xFGFR1. FLAG tag was added to the C terminus of xEIG121L and to the N terminus of xSmad1.

Antibody Production and Affinity Purification—Anti-xEIG121L and anti-mouse EIG121L (mEIG121L) polyclonal antibodies were produced in rabbits by immunizing them with a synthetic peptide corresponding to residues 979–995 of xEIG121L (EHNFESVQLKSSRAQNI) and a synthetic peptide corresponding to residues 1005–1021 of human EIG121L (KSLATKEKEDHFESVQL), respectively, plus an additional N-terminal cysteine for conjugation. Rabbit antisera were affinity purified using the synthetic peptide, and then antibodies were dialyzed and concentrated. The peptide synthesis, immunizations, and affinity purification were performed by MBL Co. (Nagoya, Japan).

Cell Culture and Transfection—C2C12 and HEK293 cells were cultured in Dulbecco's modified Eagle's medium containing 15 and 10% fetal calf serum, respectively. Cells were transfected by using Lipofectamine Plus reagent (Invitrogen) or Lipofectamine 2000 (Invitrogen), according to the manufacturer's protocols. Transfected cells were stimulated by recombinant human BMP2 (rhBMP2; R&D Systems, 355-BEC) at the indicated concentrations and were harvested after 3 h.

Co-immunoprecipitation and Immunoblotting—Transfected cells, injected embryos, or ectodermal explants were lysed in a buffer consisting of 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1.5 mM $MgCl_2$, 2 mM EGTA, 25 mM β-glycerophosphate, 10 mM sodium pyrophosphate, 1% Nonidet P-40, 10 mM NaF, 1 mM vanadate, 2 mM DTT, 1 mM PMSF, 0.5% aprotinin, 2 μg/ml leupeptin, and 2 μg/ml pepstatin A. Mouse anti-Myc antibody (9E10; Santa Cruz Biotechnology) and mouse anti-FLAG antibody (M2; Sigma) were used for immunoprecipitation with protein G-Sepharose beads (GE Healthcare). Precipitates were subjected to immunoblotting with the indicated antibodies.

For immunoblotting, the following antibodies were used at the indicated dilutions. The primary antibodies were rabbit anti-Myc (A-14, 1:500; Santa Cruz Biotechnology), rabbit anti-phosopho-Smad1/5/8 (1:1000; Cell Signaling), mouse anti-actin (Ab-5, 1:400; Thermo Scientific), mouse anti-α-tubulin (DM1A, 1:1000; Sigma), mouse anti-FLAG (M2, 1: 3000; Sigma), rabbit anti-FLAG (1:1000; Sigma), rabbit anti-mEIG121L (1:500), and rabbit anti-xEIG121L (1:1000). The secondary antibodies were sheep anti-mouse IgG HRP-conjugated (1:5000; GE Healthcare) and donkey anti-rabbit IgG HRP-conjugated (1:5000; GE Healthcare).

Whole-mount in Situ Hybridization and Immunostaining—Whole-mount *in situ* hybridization and whole-mount immunostaining were performed as described previously (27). For cryosectioning, embryos were devitellinized at stage 20, fixed in MEMFA (100 mM MOPS, pH 7.4, 2 mM EGTA, 1 mM $MgSO_4$, and 3.7% formaldehyde) for 1 h, washed with PBS, and embedded in 7.5% gelatin, 15% sucrose in PBS. The embedded embryos were frozen in liquid N_2 and sectioned at 10 μm with Microm HM500 OM microtome cryostat (Carl Zeiss, Germany). Transverse sections were permeabilized with 0.5% Triton X-100 in PBS for 5 min and then blocked in 2% bovine serum albumin/6% goat serum in PBS. Sections were incubated with primary antibodies at 4 °C overnight and then with the appropriate secondary antibodies at room temperature for 2 h. The following antibodies were used at the

indicated dilutions. The primary antibodies were rabbit anti-xEIG121L antibody (1:100) and mouse anti-Myc antibody (9E10, 1:50; Santa Cruz Biotechnology). The secondary antibodies were Alexa Fluor 488-conjugated anti-rabbit IgG (1:500; Invitrogen) and Alexa Fluor 546-conjugated anti-mouse IgG (1:500; Invitrogen).

Quantification of Forebrain Area—The areas of the forebrain in photos of embryos in the lateral view were determined by the pen tool in Adobe Photoshop CS2 (Adobe Systems) by hand. The number of pixels was obtained in the histogram tool in the image menu in Adobe Photoshop CS2.

RT-PCR—Semi-quantitative RT-PCR and quantitative RT-PCR were performed as described previously (24). The primers for xEIG121L have been described elsewhere (24). The sequences of other primer pairs used were described in Table 1.

RESULTS

A Transmembrane Protein xEIG121L Is Expressed in Ventral Ectoderm—We previously isolated a novel gene *xEIG121L* and examined its expression pattern during early *Xenopus* development and found that xEIG121L mRNA is expressed in the ventral ectoderm at the gastrula and neurula stages (24). A database search revealed that *xEIG121L* has orthologs in other vertebrates (24). Subsequent comparison of the amino acid sequences across the species raised the possibility that the previously deposited sequence of *xEIG121L* in the public database (Genbank™/EMBL/DDBJ accession no. AAH77391) might not be a full-length sequence, as it seemed to lack a putative transmembrane domain, which should be located in the C-terminal region. By searching the public database, we found several *X. laevis* expressed sequence tag clones that contain the C-terminal end of EIG121L orthologs in other vertebrates. We then designed the primers based on the sequences of these expressed sequence tag clones and performed RT-PCR to isolate a full-length cDNA of xEIG121L. The obtained full-length sequence encodes a 995-amino acid protein, which contains additional 176 amino acids at the C terminus of the previously deposited sequence. It shows 72.2% sequence identity with mouse EIG121L at the protein level (Fig. 1*A* and supplemental Fig. S1). PSORTII analysis revealed that the full-length sequence encodes a putative type I one-pass transmembrane protein, like all other orthologs in vertebrates (supplemental Fig. S1). At the C-terminal end, it has a putative intracellular domain, whose amino acid sequence shows high sequence identity (86.1%) to that of mouse EIG121L (supplemental Fig. S1). xEIG121L contains three GCC2_GCC3 domains (Pfam ID PF07699). The GCC2_GCC3 domain is found in a wide variety of extracellular proteins, although its function has been unknown. These results collectively indicate that a full-length xEIG121L cDNA encodes a

TABLE 1
Primer sets for quantitative RT-PCR

Gene	Forward (5′ to 3′)	Reverse (5′ to 3′)
Cv2	GGCTGGGTTCTTGTTCTCTCTTC	TCGCATTTGGCGATCGAT
Dlx3	AGAAGAAGATGGCCGTGTTGA	GGTGGGCGAGTCCTTGGT
Grhl1	ACACAACAGCTGCAGTCTCCAT	AGACCCACCGAGAAGCAATG
Msx1	GCTCCCTCAACCTCACAGAG	CATGGGTTTAGCAGCCATTT
ODC	GCCATTGTGAAGACTCTCTCCATTC	TTCGGGTGATTCCTTGCCAC
Szl	GTCTTCCTGCTCCTCTGC	AACAGGGAGCACAGGAAG
Xlr	CGTGCGATGAACATGCCTTCTT	GCCACCGCACGCAAAG
Zicr1	TCAACCCGAGTAGCCACGAT	CGGAGCCTGCGAAGTGA
Zic2	GCAGGAGAGGGAGCTGAGTCT	GCTTAAATGCGCCCATGTG
Zic3	CAGGCTTCTGGATATGCCAATT	AGGCTGCACCACCGTAAGAT

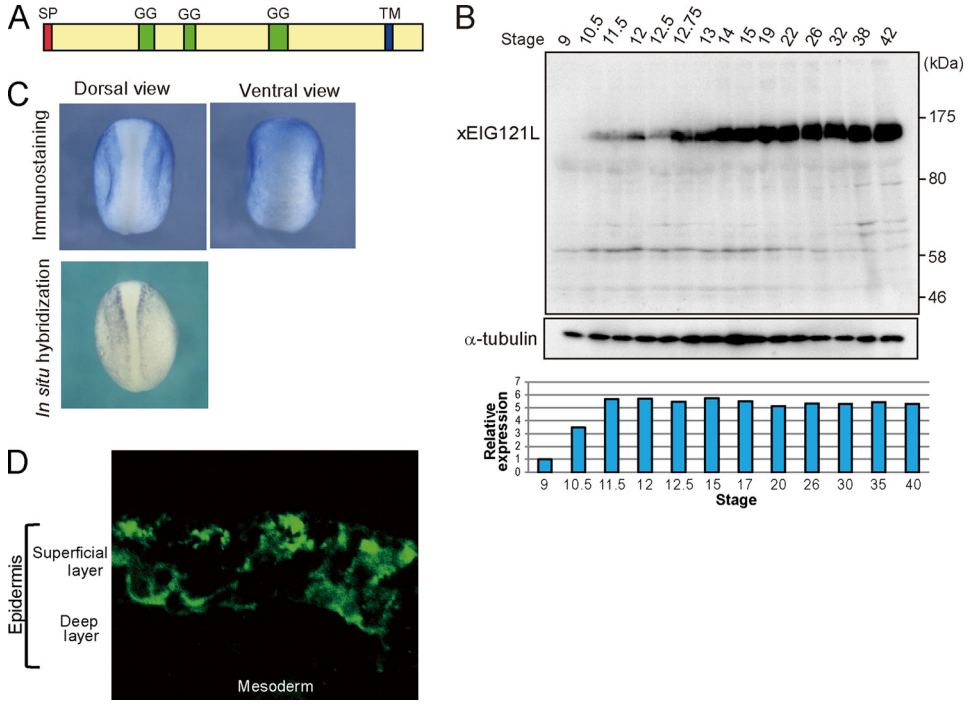

FIGURE 1. **Expression patterns of xEIG121L protein during early *Xenopus* development.** *A*, schematic representation of xEIG121L. *SP*, signal peptide sequence; *GG*, GCC2_GCC3 domain (Pfam ID PF07699); *TM*, transmembrane domain. *B*, temporal expression of xEIG121L at the protein level (*upper panel*) and the mRNA level (*lower panel*). In the immunoblotting analysis, α-tubulin was used as a loading control. In the semiquantitative RT-PCR analysis, the expression level of *xEIG121L* was normalized to that of ornithine decarboxylase (*ODC*). *C*, spatial expression of xEIG121L in neurula embryos analyzed by whole-mount immunostaining (*upper panel*) and whole-mount *in situ* hybridization (*lower panel*). *D*, subcellular localization of xEIG121L. The transverse section of a neurula embryo was stained with an anti-xEIG121L antibody (*green*).

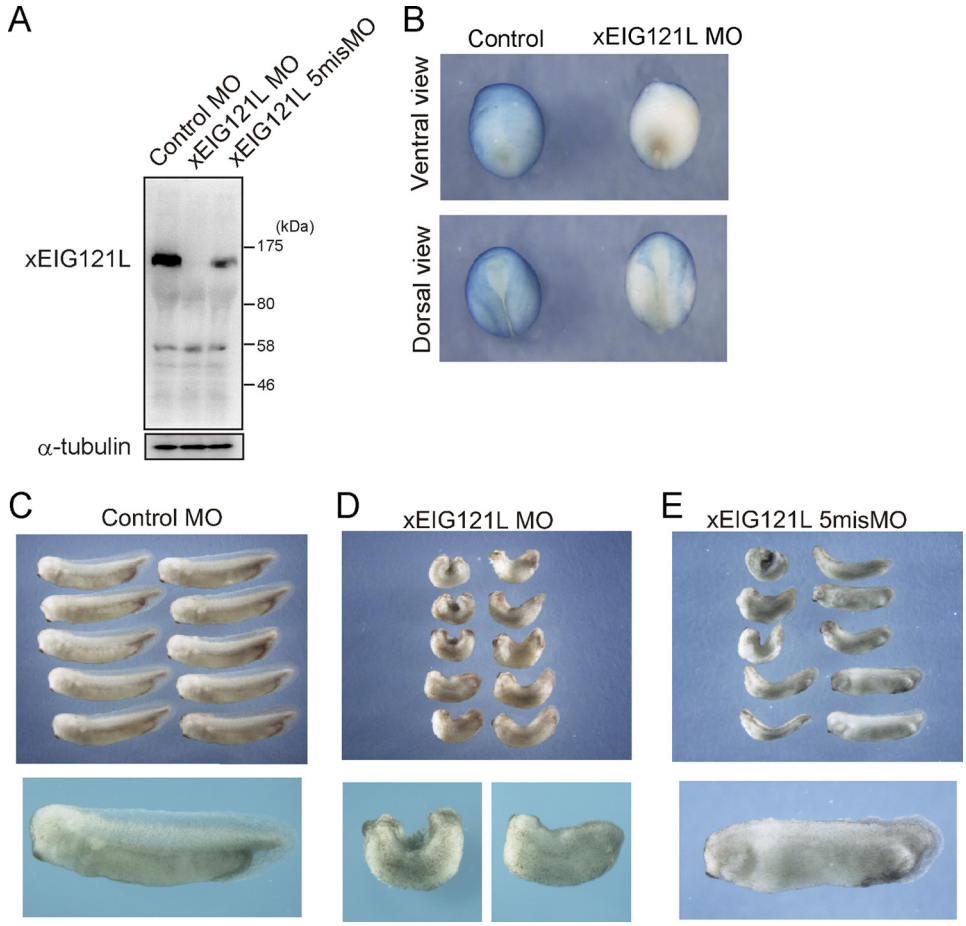

FIGURE 2. **xEIG121L is required for early development in *Xenopus* embryos.** *A* and *B*, evaluation of xEIG121L knockdown by antisense MOs using an anti-xEIG121L antibody. *A*, control MO (20 ng), xEIG121L MO (MOα (10 ng) plus MOβ (10 ng)) or xEIG121L 5misMO (5misMOα (10 ng) plus 5misMOβ (10 ng)) were injected into the animal pole of all blastomeres at the four-cell stage. Embryos were harvested at the tailbud stage. α-Tubulin was used as a loading control. *B*, xEIG121L MO (MOα (5 ng) plus MOβ (5 ng)) was injected into the animal pole of two ventral blastomeres at the four-cell stage. Embryos were fixed at the neurula stage and then subjected to the whole-mount immunostaining analysis with an anti-xEIG121L antibody. Sibling embryos were used as a control. *C–E*, knockdown experiments by MOs. Control MO (20 ng) (*C*), xEIG121L MO (MOα (10 ng) plus MOβ (10 ng)) (*D*), or xEIG121L 5misMO (5misMOα (10 ng) plus 5misMOβ (10 ng)) (*E*) were injected into the animal pole of all blastomeres at the four-cell stage. Embryos injected with xEIG121L MO ($n = 69$) showed dorsal open phenotypes (29%, *lower left panel*) or small head and tail phenotypes (39%, *lower right panel*), whereas only 5% of embryos injected with control MO showed defects ($n = 82$). Embryos injected with xEIG121L 5misMO ($n = 85$) showed dorsal open phenotypes (12%) or small head and tail phenotypes (43%).

one-pass transmembrane protein that is highly conserved across vertebrates.

We made an anti-xEIG121L rabbit polyclonal antibody by immunizing animals with a synthetic peptide corresponding to the C-terminal end of xEIG121L. Immunoblotting analysis showed that the anti-xEIG121L antibody strongly reacted with a protein with an apparent molecular mass of ~150 kDa (Fig. 1*B*, *upper panel*). The protein band was first detected at stage 10.5, an early gastrula stage, and its amount increased gradually until the tadpole stage. This expression pattern of xEIG121L protein correlated very well with the xEIG121L mRNA expression profile (Fig. 1*B*, *lower panel*) (24). We then performed immunostaining experiments using this antibody. Whole-mount immunostaining analysis showed that strong signals were detected in the epidermal ectoderm at the neurula stage (Fig. 1*C*, *upper panel*). This result is consistent with the expression pattern of xEIG121L mRNA (Fig. 1*C*, *lower panel*), which was reported previously (24). We next examined subcellular localization of xEIG121L using the transverse section of the neurula embryo. Our results clearly show that xEIG121L protein was specifically expressed in the superficial layer in the ventral ectoderm. xEIG121L protein was present both in the plasma membrane and the perinuclear foci (Fig. 1*D*; see also Fig. 6*C*). These results indicate that xEIG121L protein is a one-pass transmembrane protein, which is expressed in the ventral ectoderm in early *Xenopus* embryos.

xEIG121L Plays an Essential Role in Early Embryonic Development—To determine the function of EIG121L during early *Xenopus* development, we performed knockdown experiments by antisense MOs. We used a mixture (xEIG121L MO) of two sequences of MOs (xEIG121L MOα and xEIG121L MOβ) against each pseudoallele of xEIG121L. Immunoblotting analysis with the anti-xEIG121L antibody of the whole extracts of the embryos demonstrated that the xEIG121L MO injection resulted in a dramatic reduction in the protein level of xEIG121L, and the xEIG121L protein band was scarcely detected (Fig. 2*A*). Furthermore, the drastic reduction of the xEIG121L protein level by the xEIG121L MO injection was confirmed by immunostaining experiments, in which xEIG121L expression in the ventral side was almost

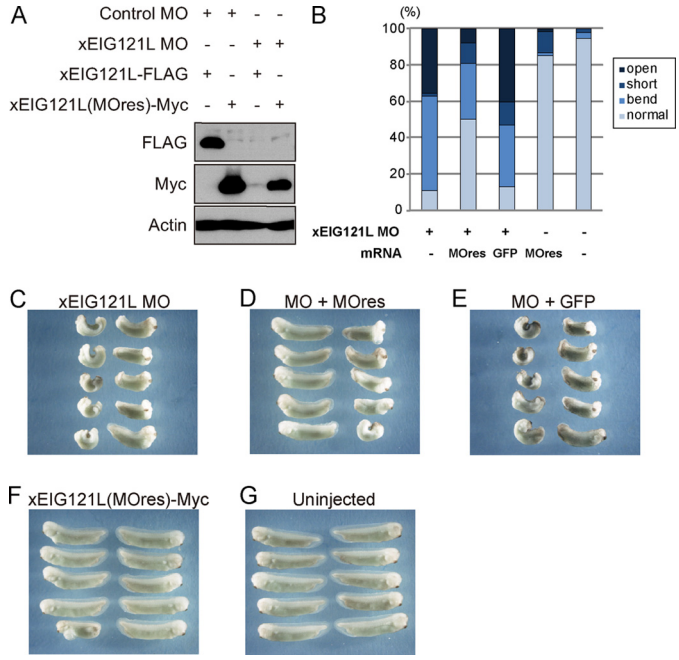

FIGURE 3. **Rescue experiments in xEIG121L knockdown embryos.** *A*, indicated sets of morpholino oligonucleotides (20 ng) and mRNA (1 ng) were injected, and the protein level was examined by immunoblotting with anti-FLAG and anti-Myc antibodies. Actin was used as a loading control. *B–G*, embryos were co-injected with indicated MOs and/or mRNAs into the animal pole of the right side blastomeres at the four-cell stage for the rescue experiment. Embryos injected with xEIG121L MO (MOα (4 ng) plus MOβ (4 ng)) alone showed dorsal open (open, 36%), short axis (short, 1%), or bending phenotypes (bend, 52%) (*C*, $n = 73$). Fifty percent of embryos co-injected with xEIG121L(MOres)-Myc mRNA (MOres) (200 pg) showed no abnormality (*D*, $n = 62$), whereas only 13% of embryos co-injected with GFP mRNA (200 pg) showed no abnormality (*E*, $n = 47$). Embryos injected with xEIG121L(MOres)-Myc mRNA alone (*F*, $n = 68$) and uninjected embryos (*G*, $n = 91$) were almost normal (85 and 95%, respectively).

completely inhibited, whereas faint expression was still detected in the dorsal region around the neural plate (Fig. 2*B*). Embryos injected with xEIG121L MO into the animal pole at the four-cell stage showed severe developmental defects, which include the dorsal open phenotype (Fig. 2*D*, *lower left panel*), and the reductions in the head and tail structures at the tailbud stage (Fig. 2*D*). Embryos injected with the same dose of control MO showed no abnormality (Fig. 2*C*). When xEIG121L 5misMO was injected, the protein level of xEIG121L was modestly but significantly reduced (Fig. 2*A*) and milder, as compared with xEIG121L MO, but significant defects were observed (Fig. 2*E*). To further show the specificity of xEIG121L MO, we performed the rescue experiment using an MO-resistant construct of xEIG121L (xEIG121L(MOres)) (Fig. 3). Immunoblotting analysis showed that the expression of xEIG121L(MOres), which contains sequences that do not match xEIG121L MO, was not suppressed significantly by xEIG121L MO (Fig. 3*A*). Embryos, in which both xEIG121L MO and xEIG121L(MOres) were unilaterally injected, showed a significantly milder phenotype in the body axis bending (Fig. 3, *B* and *D*), whereas embryos in which xEIG121L MO alone was unilaterally injected showed a severe bending phenotype in the body axis (Fig. 3, *B* and *C*). Co-injection of GFP failed to rescue the xEIG121L knockdown phenotypes (Fig. 3, *B* and *E*). Embryos injected with the same dose of xEIG121L(MOres) mRNA alone as well as uninjected embryos showed almost no abnormality (Fig. 3, *B*, *F*, and *G*). These results confirm the specificity of xEIG121L MO and thus suggest that xEIG121L plays an essential role in early embryonic development.

Knockdown of xEIG121L Causes Inhibition of Epidermal Differentiation and Induction of Neural Differentiation—As xEIG121L is first expressed at the gastrula stage in the ventral ectoderm, which is the presumptive epidermal region during early embryonic development (24), we hypothesized that xEIG121L should regulate epidermal differentiation. To test this hypothesis, we examined the expression levels of epidermal and neural marker genes in xEIG121L-depleted ectodermal explants. Our quantitative RT-PCR analyses showed that the expression levels of epidermal genes, *Msx1* (21), *Dlx3* (22), *Grhl1* (23), *Cv2* (17), *Xlr* (Xolloid-related) (11), and *Szl* (15), were dramatically decreased in the xEIG121L-depleted ectodermal explants, compared with those in control MO-injected ectodermal explants (Fig. 4*A*). On the other hand, the expression levels of neural genes (*Zicr1*, *Zic2*, and *Zic3* (28)) were dramatically up-regulated in the xEIG121L-depleted ectodermal explants (Fig. 4*B*). These results strongly suggest that xEIG121L is required for epidermal differentiation, and thus, the knockdown of xEIG121L induces neural differentiation. These changes in gene expression patterns (inhibition of epidermal genes and induction of neural genes) were reversed by co-injection of xEIG121L(MOres) mRNA (Fig. 4, *A* and *B*), indicating again the specificity of xEIG121L MO. We then examined whether these changes in gene expression profiles are observed in whole embryos. xEIG121L MO was injected into two unilateral blastomeres at the four-cell stage, and the embryos were analyzed by whole-mount *in situ* hybridization. Although epidermal keratin, a terminal epidermal marker gene, was expressed normally in the uninjected ventral side, its expression in the xEIG121L MO-injected side was severely suppressed (Fig. 4*C*). On the other hand, the region expressing a dorsal marker gene *Chordin* (29) and a neural marker gene *Sox2* (28) was expanded in the xEIG121L MO-injected right side (Fig. 4, *D* and *E*). These results are consistent with the results of the quantitative RT-PCR analyses. Collectively, these results show that xEIG121L knockdown results in the inhibition of epidermal differentiation and the induction of neural differentiation in the ventral ectoderm.

xEIG121L Is Involved in BMP Signaling in Ventral Ectoderm—As the BMP signaling pathway has been shown to play a key role in determining cell fates in the ectoderm (30, 31), we compared the xEIG121L knockdown phenotype with the phenotype of embryos injected with dominant negative BMP receptor (dnBMPR) (32, 33). The result showed that embryos, in which dnBMPR was injected into the animal pole (the presumptive epidermal ectoderm), resembled xEIG121L knockdown embryos (Fig. 5*A*, also see Fig. 2*D*). At the earlier stages, the reduction of epidermal ectoderm (epidermal keratin) and the expansion of neural ectoderm (Sox2) were also observed in the injected side in dnBMPR mRNA-injected embryos as well as in xEIG121L knockdown embryos (Fig. 5, *B* and *C*, also see Fig. 4, *C* and *E*, respectively). To see this phenotypic similarity in more detail, we examined the expression

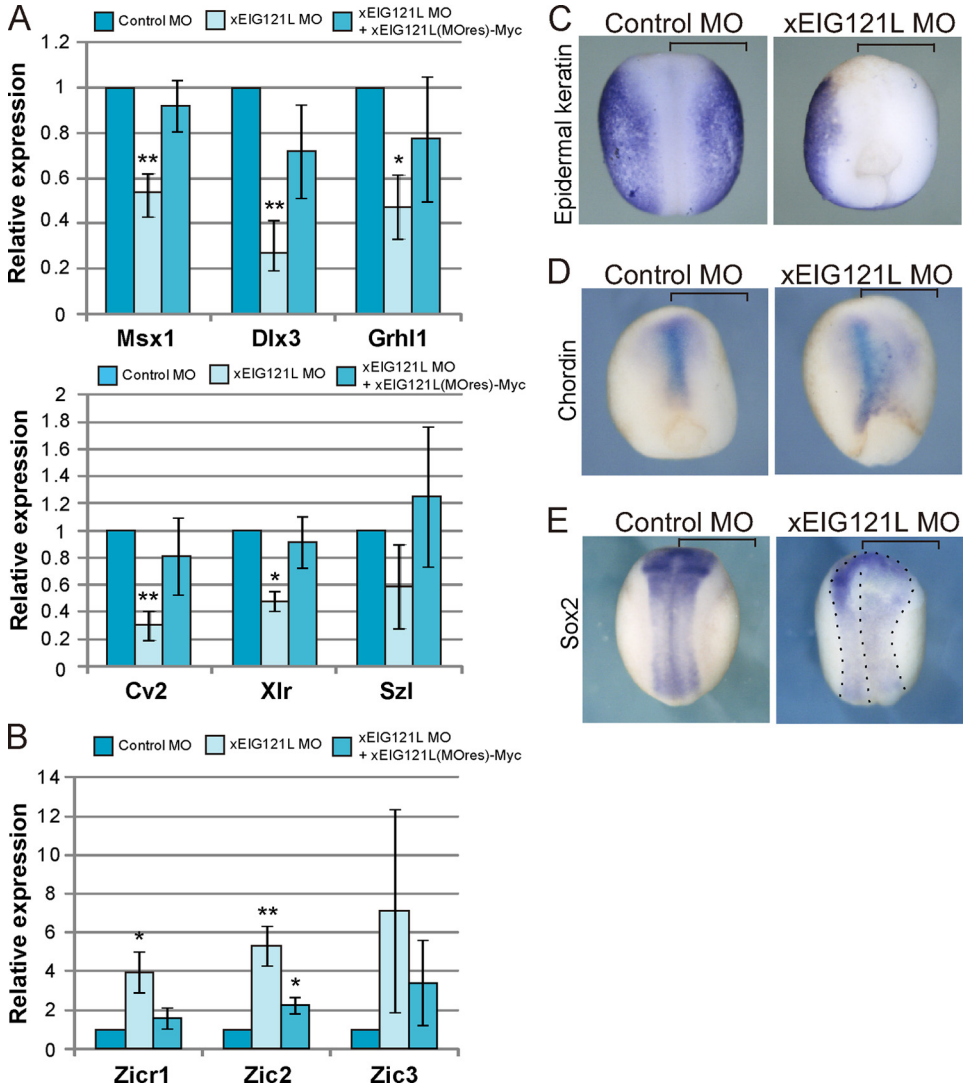

FIGURE 4. **Knockdown of xEIG121L affects the expression of epidermal and neural genes.** *A* and *B*, ectodermal explants injected with control MO (16 ng), xEIG121L MO (MOα (8 ng) plus MOβ (8 ng)), or xEIG121L MO (MOα (8 ng) plus MOβ (8 ng)) plus xEIG121L(MOres)-Myc mRNA (400 pg) were harvested at the late gastrula stage. The relative expression levels of epidermal (*A*) and neural (*B*) genes were determined by quantitative RT-PCR. The expression level of each gene was normalized to that of ornithine decarboxylase. *Error bars*, S.E.; ($n = 3$). **, $p < 0.01$; *, $p < 0.05$, significant difference from control MO. *C* and *D*, control MO (10 ng) or xEIG121L MO (MOα (5 ng) plus MOβ (5 ng)) were injected into the animal pole of right side blastomeres at the four-cell stage. The expression patterns of epidermal keratin at the neurula stage (*C*) and of *Chordin* at the late gastrula stage (*D*) were analyzed by whole-mount *in situ* hybridization. Dorsal views of embryos are shown with the anterior side at the *top*. *Brackets* indicate the injected sides. *E*, control MO (8 ng) or xEIG121L MO (MOα (4 ng) plus MOβ (4 ng)) were injected into the animal pole of right side blastomeres at the four-cell stage. The expression pattern of *Sox2* at the neurula stage was analyzed by whole-mount *in situ* hybridization. Dorsal views of embryos are shown with the anterior side at the *top*. *Brackets* indicate the injected sides.

levels of epidermal and neural marker genes in ectodermal explants. Quantitative RT-PCR analyses showed that the expression levels of epidermal genes were dramatically decreased both in the dnBMPR-overexpressed ectodermal explants and in the xEIG121L knockdown ectodermal explants, compared with those in control MO-injected ectodermal explants (Fig. 5*D*). On the other hand, the expression levels of neural genes were dramatically up-regulated both in the dnBMPR-overexpressed ectodermal explants and in the xEIG121L knockdown ectodermal explants (Fig. 5*E*). These results strongly suggest that knockdown of xEIG121L phenocopies the inhibition of the BMP pathway in the ventral ectoderm in *Xenopus* embryos and that xEIG121L is involved in BMP signaling in the ventral ectoderm.

We then considered the possibility that xEIG121L should be involved in the control of BMP signaling. To test the potential role of xEIG121L in BMP signaling, we examined the effect of xEIG121L knockdown on the Smad1 phosphorylation, the readout of BMP signaling, in the ectoderm. Our result showed a marked reduction in the Smad1 phosphorylation level in the xEIG121L MO-injected ectodermal explants (Fig. 6*A*). This result suggests that xEIG121L plays a positive role in BMP signaling to determine the cell fate into epidermal differentiation in the ventral ectoderm.

As EIG121L is a transmembrane protein, we considered the possibility that xEIG121L might interact with BMP receptors. To test this idea, we performed immunoprecipitation experiments by expressing xEIG121L and BMP receptors in C2C12

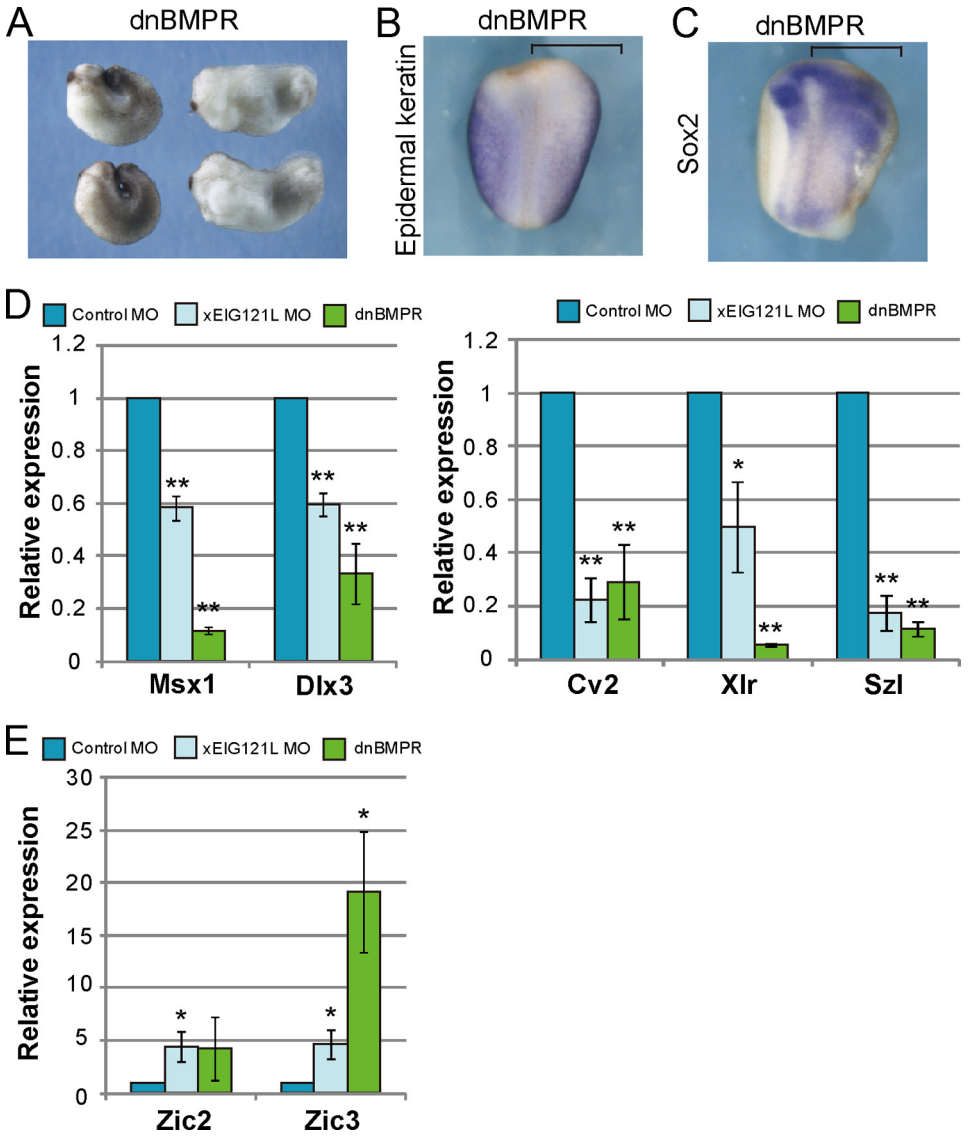

FIGURE 5. **Phenotypes caused by dnBMPR.** *A*, dnBMPR mRNA (200 pg) was injected into the animal pole of all blastomeres at the four-cell stage. *B* and *C*, dnBMPR mRNA (200 pg) was injected into the animal pole of the right side blastomeres at the four-cell stage. The expression patterns of epidermal keratin (*B*) and *Sox2* (*C*) at the neurula stage were analyzed by whole-mount *in situ* hybridization. Dorsal views of embryos are shown with the anterior side at the *top*. *Brackets* indicate the injected sides. *D* and *E*, ectodermal explants injected with control MO (16 ng), xEIG121L MO (MOα (8 ng) plus MOβ (8 ng)), or dnBMPR mRNA (400 pg) were harvested at the late gastrula stage. The relative expression levels of epidermal (*D*) and neural (*E*) genes were determined by quantitative RT-PCR. The expression level of each gene was normalized to that of ornithine decarboxylase. *Error bars*, S.E.; ($n = 3$). **, $p < 0.01$; *, $p < 0.05$, significant difference from control MO.

cells. The obtained result showed clearly that two type I BMP receptors (xALK2 and xALK3) and two type II BMP receptors (xAcvR2b and xBMPR2) co-immunoprecipitated with xEIG121L protein (Fig. 6*B*) and thus suggest that these BMP receptors can physically interact with xEIG121L protein. In our immunoprecipitation assays, xFGFR1 also co-immunoprecipitated with xEIG121L, although faintly. Whether this weak interaction would have a physiological role is not known at present. To examine the interaction of xEIG121L with BMP receptors in *Xenopus* embryos, we next determined the subcellular localization of endogenous xEIG121L and a Myc-tagged type I BMP receptor (xALK2-Myc) in the ventral ectoderm. Immunostaining showed that xALK2-Myc co-localized with xEIG121L in the basolateral membrane in the superficial layer of the ventral ectoderm (Fig. 6*C*). These results support our idea that xEIG121L interacts with BMP receptors and functions as a positive regulator of the BMP pathway at the plasma membrane to promote epidermal differentiation in the ventral ectoderm in *Xenopus* embryos.

Overexpression of EIG121L Can Enhance BMP Signaling in Mammalian Cultured Cells and in Xenopus Embryos—Finally, we examined whether overexpression of EIG121L activates BMP signaling. Our results show that increasing amounts of a mouse ortholog of EIG121L (mEIG121L) in HEK293 cells enhanced the phosphorylation level of Smad1 in a dose-dependent manner, especially when cells were stimulated by lower concentrations (10 or 30 ng/ml) of BMP2. (Fig. 7*A*). This result suggests that EIG121L can activate the BMP pathway directly.

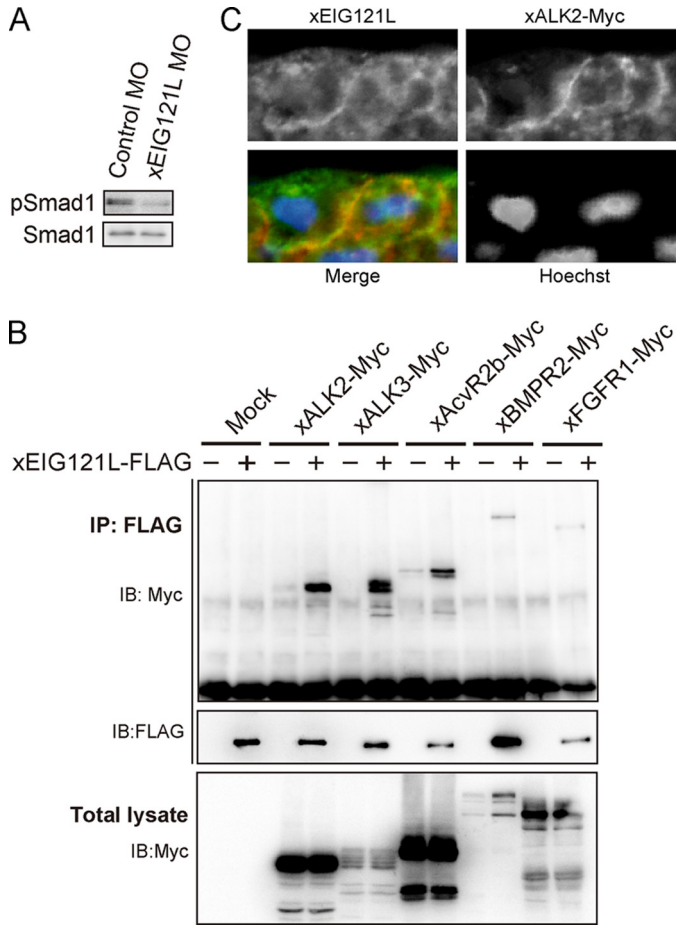

FIGURE 6. **Involvement of xEIG121L in BMP/Smad1 signaling in the ventral ectoderm.** *A*, Smad1 phosphorylation was analyzed by immunoblotting in ectodermal explants co-injected with Myc-xSmad1 mRNA and control MO (10 ng) or xEIG121L MO (MOα (5 ng) plus MOβ (5 ng)). Explants were harvested at the late gastrula stage. Lysates were subjected to immunoprecipitation with an anti-Myc antibody, followed by immunoblotting (*IB*) with an anti-phospho-Smad1 antibody. *B*, C2C12 cells were transfected with the indicated combinations of expression vectors. Physical interactions between each receptor and xEIG121L were detected by immunoprecipitation (*IP*). *C*, subcellular localization of xEIG121L and a type I BMP receptor xALK2. xALK2-Myc mRNA (200 pg) were injected into the animal pole, and embryos were fixed at the neurula stage. The transverse section was stained with an anti-xEIG121L antibody (*green*), an anti-Myc antibody (*red*), and Hoechst (*blue*).

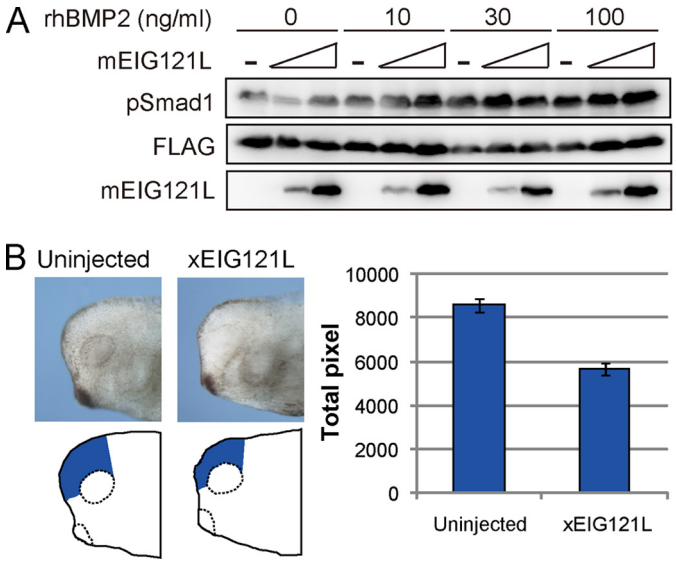

FIGURE 7. **EIG121L enhances BMP signaling.** *A*, HEK293 cells were transfected with FLAG-xSmad1 (3 μg) alone or together with mouse EIG121L (mEIG121L) (0.3 μg or 1 μg). Transfected cells were stimulated by recombinant human BMP2 (rhBMP2) at the indicated concentrations, and were harvested after 3 h. (*B*) xEIG121L mRNA (3 ng) was injected into the animal pole of all blastomeres at the four-cell stage. Each area of the forebrain region in the lateral view, indicated as blue, was estimated in uninjected embryos ($n = 10$) and xEIG121L mRNA-injected embryos ($n = 14$). Error bars, S.E. $p <$ 0.000001, significant difference from uninjected control.

We then tested whether overexpression of xEIG121L inhibits its neural differentiation in *Xenopus* embryos. Embryos injected with xEIG121L mRNA showed reduced brain structures at the tailbud stage (Fig. 7*B*, *left panel*). The area of the forebrain region (Fig. 7*B*, *lower left panel*, *blue*) was significantly decreased in embryos injected with xEIG121L mRNA (Fig. 7*B*, *right panel*). This result may suggest that overexpression of xEIG121L suppressed neural differentiation by activating the BMP pathway.

DISCUSSION

EIG121L is an evolutionarily conserved gene among vertebrates, but its function has been unknown. In *Xenopus* embryos, xEIG121L mRNA has previously been shown to be expressed in the ventral ectoderm at the gastrula and the neurula stages (24). We have here demonstrated that a one-pass transmembrane protein xEIG121L is expressed in the ventral ectoderm and plays an essential role in epidermal differentiation. Our analyses then strongly suggest that xEIG121L protein is involved in the BMP signaling pathway and may have a positive role in BMP signaling during epidermal differentiation in the ventral ectoderm. As the BMP signaling pathway has been shown to be essential for epidermal differentiation (30, 31), we can conclude that xEIG121L plays a role in epidermal differentiation by positively regulating BMP signaling. The RGM (repulsive guidance molecule) family was previously reported as co-receptors in the BMP pathway (34–36). However, recent studies have shown that RGMa is dispensable for epidermal differentiation in *Xenopus* (37, 38). Our results might imply that EIG121L would be another co-receptor of the BMP pathway.

This is the first study to identify the function of EIG121L *in vivo*. *EIG121*, a close homolog of *EIG121L*, has been identified as a biomarker in human endometrial adenocarcinoma (39). *EIG121* and *EIG121L* comprise a putative gene family (EIG121 family) in fish, zebra finch, and mammals (data not shown) (24). However, we could not detect a putative ortholog of *EIG121* in chicken and *Xenopus laevis*. In our BLAST search, an ortholog of the EIG121 family is detected in a variety of species, including amoeba, hydra, nematostella, nematodes, sea urchin, amphioxus, ascidian, and etc. (24 and data not shown). These orthologs are more similar to vertebrate EIG121L than EIG121. This may imply that EIG121L has an evolutionarily conserved role *in vivo*. As we observed that mEIG121L can increase the phosphorylation level of Smad1 in mammalian cultured cells (Fig. 7*A*), EIG121L may have an evolutionarily conserved activity to enhance BMP signaling. Future studies in other species will elucidate this putative conserved function of the EIG121 family.

A Novel Regulator of BMP Signaling

Recently, EIG121 has been shown to regulate autophagy and cell survival in mammalian cultured cells (40). In this work, EIG121 was shown to be localized in the plasma membrane and the perinuclear intracellular dot-like structures (trans-Golgi/late endosome-lysosome compartments) (40). This subcellular localization of EIG121 in mammalian cells is very similar to that of xEIG121L in embryos (Fig. 6*B*). This may imply the potential involvement of EIG121L in lysosomal degradation of membrane proteins. The detailed molecular mechanism by which EIG121L regulates BMP signaling should be elucidated in future studies.

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