

Znf45l affects primitive hematopoiesis by regulating transforming growth factor- β signaling

Huijuan Chen^{1,#}, Huaqin Sun^{2,#}, Dachang Tao^{1,#}, Ping Yang¹, Shasha Bian¹, Yunqiang Liu¹, Sizhong Zhang¹ & Yongxin Ma^{1,*}

¹Department of Medical Genetics, State Key Laboratory of Biotherapy, West China Hospital, Sichuan University, ²Sichuan University-The Chinese University of Hong Kong Joint Laboratory for Reproductive Medicine, West China Institute of Women and Children's Health, West China Second University Hospital, Sichuan University, Chengdu, China

Znf45l, containing classical C₂H₂ domains, is a novel member of Zinc finger proteins in zebrafish. In vertebrates, TGF- β signaling plays a critical role in hematopoiesis. Here, we showed that Znf45l is expressed both maternally and zygotically throughout early development. Znf45l-depleted Zebrafish embryos display shorter tails and necrosis with reduced expression of hematopoietic maker genes. Furthermore, we revealed that znf45l locates downstream of TGF- β ligands and maintains normal level of TGF- β receptor type II phosphorylation. In brief, our results indicate that znf45l affects initial hematopoietic development through regulation of TGF- β signaling. [BMB Reports 2014; 47(1): 21-26]

INTRODUCTION

Zinc finger protein 45-like (znf45l), a novel member of zinc finger protein with seven Cys²His² zinc-finger domains in zebrafish, was discovered by Strausberg *et al.* in 2002 (1). So far, little about the underlying mechanism of znf45l in bioprocesses has been reported.

The establishment of the hematopoietic system involves multiple developmental steps ranging from induction and patterning of mesoderm to specification of the earliest blood cell progenitors (2). In zebrafish, HSC generation is underway from 5 hours post-fertilization (hpf), when gastrulation takes place. During the gastrula period, embryos develop three germ layers - ectoderm, mesoderm and endoderm. Among these three germ layers, mesoderm is specified to the hematopoietic lineages. Hematopoiesis is a dynamic process involving the interplay between lineage-specific transcription factors and epigenetic regulators (3, 4). Once mesoderm is defined by transforming growth factor β

(TGF- β) signaling, mesoderm is further specified into either dorsal fate (notochord, somites) or ventral fate (blood, vasculature and pronephros) (2, 5, 6). The roles of TGF- β , BMP, and canonical Wnt signaling pathways were defined using a variety of model systems, including embryonic stem cell differentiation system, at three distinct developmental stages during hematopoietic ontogeny: induction of a primitive streak (PS)-like population, formation of Flk1 + mesoderm, and induction of hematopoietic progenitors (2).

Two sets of identified membrane receptors are involved in TGF- β subfamily ligands signal. In detailed, Type II receptor phosphorylates and activates type I receptor in response to ligands binding (7). Type I Phosphorylated receptors predominantly involved in the activation of downstream transducers, such as smad2/3. Then, phosphorylated smad2/3 form complex with Smad4 and are transported into the nucleus, where Smads cooperate with specific DNA-binding transcription factors to regulate gene transcription in a context-dependent manner (7-10). Here, we present that znf45l affect initial hematopoietic development in the early embryo by regulating transforming growth factor β signaling.

RESULTS

Znf45l is expressed on general body and then focus on branchial and pharyngeal arches

The full-length mRNA sequence of znf45l (GenBankBC090439) is 2,269 bp, the reading frame from 307 to 1,530 is 1,223 bp encoding a polypeptide of 407 amino acid residues. Similar to other member of Zinc finger protein, znf45l has conserved domains of Zinc finger protein, suggesting functional similarity and conservation (Supplemental Fig. S1).

We analyze the spatiotemporal expression pattern of znf45l during zebrafish embryogenesis by whole-mount in situ hybridization from two-cell stage to 3 days post-fertilization (dpf) using Digoxigenin-labeled antisense RNA probe. Znf45l transcript first appears in two-cell stage and is ubiquitously expressed in gastrula stage, thus pointing to a maternal origin of the transcript and suggesting multiple functions. When segmentation period starts, znf45l is expressed in axis ubiquitously, and then branchial and pharyngeal arches gradually gain stronger expression

*Corresponding author. Tel: +86-28-85164010; Fax: +86-28-85164009; E-mail: mayongxin@gmail.com

#These authors contributed equally to this work.

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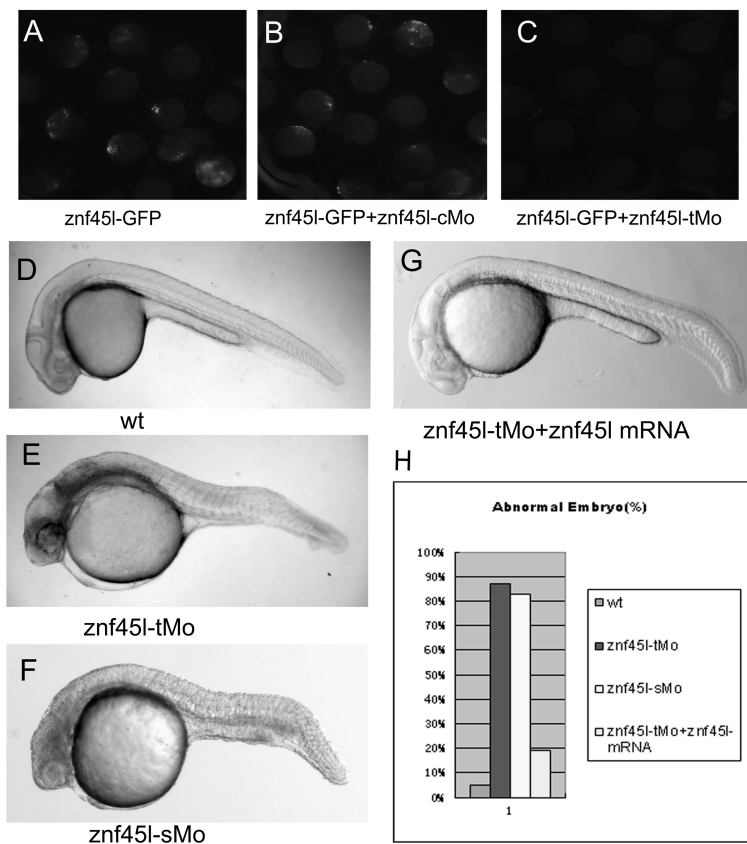


Fig. 1. Effects of Znf45l knockdown and overexpression in zebrafish embryos. (A-C) Live embryos at the 70%-epiboly stage. (D-G) Lateral views of live embryos at 24 hpf are shown. (A) Embryos injected with 120 pg znf45l-GFP DNA produce green fluorescent fusion protein. (B) Expression of the fusion protein is not inhibited by coinjecting with 4 ng control morpholino (znf45l-cMo). (C) Expression of the fusion protein is inhibited by coinjecting with 4 ng znf45l-tMo. (D) Wild-type. (E) Injection with 4 ng znf45l-tMo leads to shortened body length, moderate necrosis in the anterior region and ventrally curved tail. (F) Injection with 8 ng znf45l-sMo. (G) Injection with 4 ng of znf45l-tMo and 120 pg znf45l-mRNA, the abnormal phenocopy is rescued. (H) Statistical data for (D, E, F, G).

(Supplemental Fig. S2).

Znf45l is necessary for zebrafish early embryo development

To study the function of znf45l, endogenous expression of znf45l is knocked down by injecting a translation blocker antisense morpholino (znf45l-tMo), which is able to block production of the Znf45l-GFP fusion protein from a znf45l-GFP fusion expression plasmid (Fig. 1A-C). Embryos injected with 4 ng of znf45l-tMo show shortened body length, moderate necrosis in the anterior region and ventrally curved tail at 24 hpf. To test the specification of znf45l-tMo, znf45l mRNA is synthesized for rescuing the phenocopy mediated with znf45l-tMo. The results show that the developmental defects of znf45l morphants could be rescued by coinjection of znf45l mRNA in 78.2% (n = 108) embryos, suggesting that znf45l-tMo could specially block translation of znf45l mRNA (Fig. 1D-G). A splice inhibitor (znf45l-sMo) is designed and synthesized against znf45l to identify the specific effects of znf45l-tMo once again (Supplemental Fig. S3). Consistent with znf45l-tMo, sMo injections produce the same abnormalities in appearance. These results suggest that znf45l is necessary for zebrafish early embryogenesis.

Znf45l affects primitive hematopoiesis in early embryo

The phenocopy caused by znf45l knockdown is similar to the

embryos depleted of β -arrestin1 and mta3 (metastasis-associated protein number3), which were described by Yue *et al.* and Li *et al.* as critical factors for hematopoiesis (4, 11). Reasonably, we ask that whether znf45l functions to initial primitive hematopoiesis. So, we inject zebrafish embryo with 120 pg znf45l mRNA or 4 ng znf45l-tMo at one-cell stage, then detect expression level of hematopoietic marker gene *scl*, *lmo2*, *gata2* and *fli1a* at 8-somite stage. *Scl* (stem cell leukemia protein) is a critical transcription factor, indicating initiation of HSC and angioblast (vascular precursor) formation. Other hematopoietic transcription factors (such as *lmo2* and *gata2*) and vascular transcription factors (*fli1a* and *flk1* for example) are also co-expressed with *scl* (5, 12-14). The expression levels of these hematopoietic marker genes are enhanced in embryos injected with znf45l mRNA while reduced in embryos injected with znf45l-tMo (Fig. 2). And then, O-dianisidine staining of embryos at 36hpf reveals reduction of red blood cells in znf45l morphants (Fig. 2). The two results indicate that znf45l affects in primitive hematopoiesis in early zebrafish embryo.

The embryos injected with znf45l-tMo show moderate necrosis in the anterior region, and Robu *et al.* report that off-target effects of morpholino injections in fish embryos, manifested mainly as extensive cell death, are induced through activation of p53 and can be ameliorated by co-knockdown of p53 gene (15).

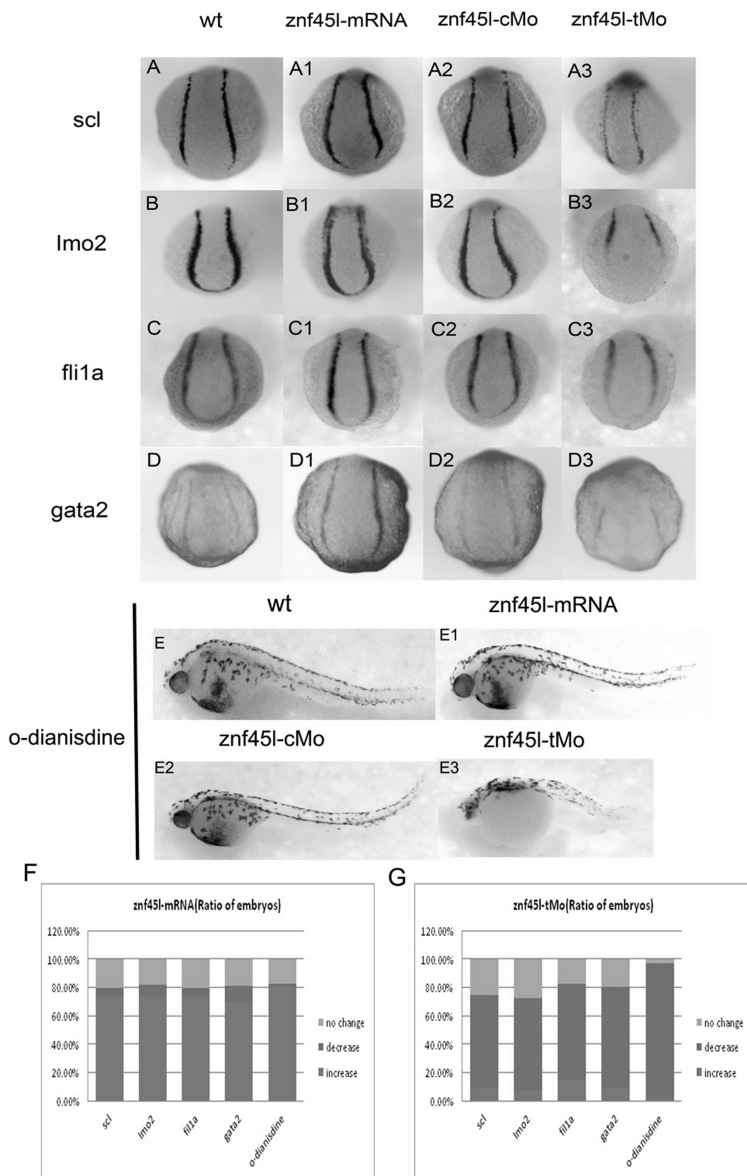


Fig. 2. Effects of Znf45l overexpression and knock-down on hematopoietic marker genes and hemocyte. (A-D) Wide type; (A1-D1) Expression patterns of marker genes in embryos injected with 120 pg znf45l-mRNA; (A2-D2) injected with 4 ng znf45l-cMo; (A3-D3) injected with 4 ng znf45l-tMo. All embryos are indicated by ISH at the 8-somite stage and dorsal views with anterior to the top. (E-E3) Hemoglobin staining by O-dianisidine. Embryos stained by O-dianisidine at 36hpf. (F, G) Statistical data respectively for (A1-E1) and (A3-E3).

We ask whether the loss of hematopoietic cells in znf45l morphants could be attributed to the mechanism of extensive apoptosis of hematopoietic cells. And then we find that, similar to injection with 4 ng of znf45l-tMo alone, coinjection of 4 ng of znf45l-tMo and 5 ng of p53-Mo still cause the loss of scl expression (Supplemental Fig. S4), which excludes the possibility that inhibition of scl expression by znf45l-tMo injected is a p53-dependent off-target effect. Taken together, our results indicate that znf45l functions in the specification of primitive hematopoietic precursors and affects primitive hematopoiesis.

Znf45l locates downstream of TGF- β ligand to transduce the signal and promote hematopoiesis

Hematopoiesis is a highly complex differentiation process that ultimately originates from a rare population of primitive hematopoietic stem cells (HSCs) (16). Although the molecular mechanisms behind hematopoietic regulation are not fully understood, it is recognized that HSCs are governed by an intricate network of regulatory factors. Recent studies give credence to certify that TGF- β superfamily of growth factors and the downstream Smad signaling pathway plays an important role in this network (16, 17). Here, we ask whether znf45l is involved in TGF- β signals and mediated their activities to promote hematopoiesis. We test

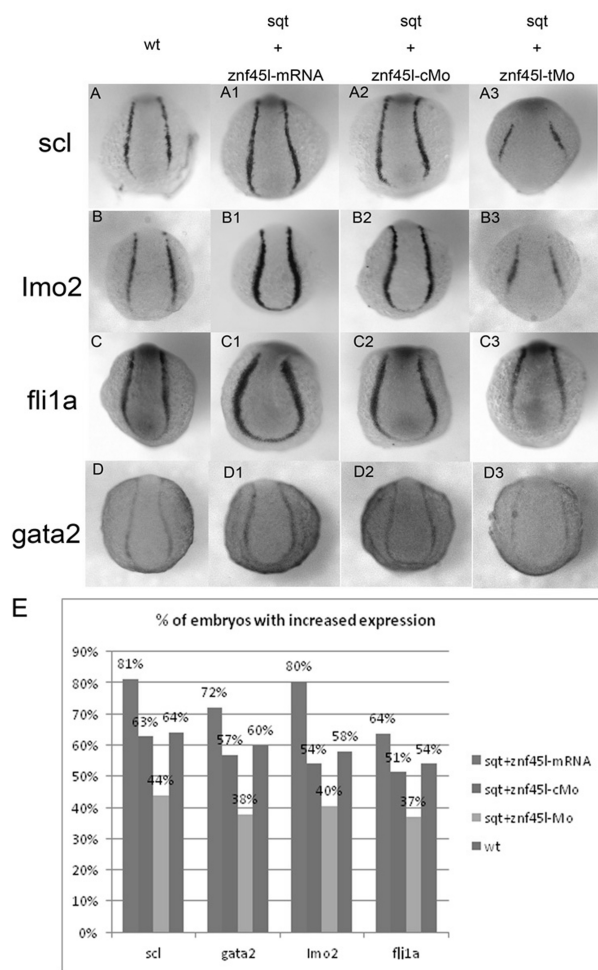


Fig. 3. Genetic interactions between Znf45l and nodal signals. (A-D3) Expression of the marker genes indicated on the left. (A-D) Wild type. (A1-D1) Embryos injected with 0.24 pg sqt mRNA plus 120 pg znf45l mRNA; (A2-D2) 0.24 pg sqt mRNA plus 4 ng znf45l-cMo; (A3-D3) 0.24 pg sqt mRNA plus 4 ng znf45l-tMo; Dorsal views with the animal pole oriented toward the top for scl, lmo2, fli1a and gata2 at 8-somite stage are shown. (E) Statistical data for (A-D3).

the genetic interaction between znf45l and sqt that is essential for primitive hematopoiesis. As shown in Fig. 3, injection with 0.24 pg of zebrafish sqt mRNA lead to expanded expression of scl in 62.7% (n = 43) embryos at 8-somite stage (sqt, also known as ndr1, Nodal-related ligands in frogs and zebrafish). The percentage of expanded scl increases to 81.3% (n = 48) when the same amount of sqt mRNA and znf45l mRNA are coinjected. In contrast, coinjection with sqt mRNA and znf45l-tMo cause the percentage of embryos with expanded scl expression decreasing to 44% (n = 45), and a similar case occurs when we examine the other hematopoietic development marker genes lmo2, gata2 and fli1a expression, suggesting that Znf45l locates downstream of

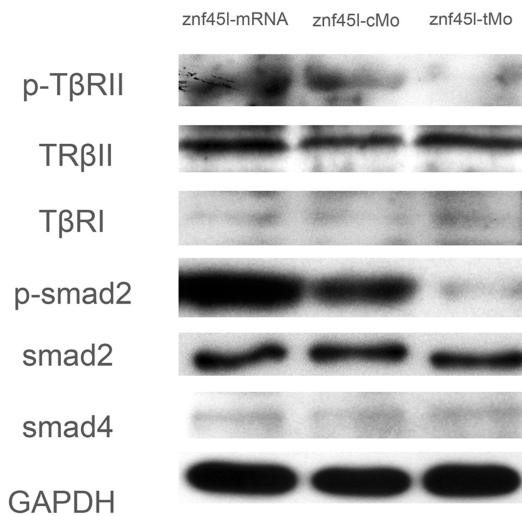


Fig. 4. Effects of Znf45l on TGF- β signaling at the level of TGF- β receptor II and Smad2 phosphorylation. Embryos injected with 120 pg znf45l-mRNA (lane1) or 4 ng znf45l-cMo (lane2) or 4 ng znf45l-tMo (lane3) was harvested at 50%-epiboly stage. Embryos were used for Western blot analysis with indicated antibody. p-T β R II, phospho-TGF- β receptor II; T β R II, TGF- β receptor II; T β R I, TGF- β receptor I; P-smad2, phospho-Smad2; GAPDH as loading control.

TGF- β ligand to transduce the signal and promote hematopoiesis.

Znf45l enhances TGF- β receptor II phosphorylation to promote hematopoiesis

TGF- β signaling has been triggered by TGF- β subfamily ligands binding to trans-membrane serine-threonine kinase receptors. Type II receptors phosphorylate and activate type I receptors in response to ligands binding. Activated type I receptors induce Smad2/3 phosphorylation to regulate a cascade of downstream events (7, 18-20). To clarify a role of znf45l in TGF- β signaling, we examine whether TGF- β signaling related proteins can be regulated by Znf45l. Initially, we injected zebrafish embryo with 120 pg znf45l mRNA, 4 ng znf45l-cMo or 4 ng znf45l-tMo at one-cell stage. Then, we detect expression levels of smad2, p-smad2, smad4, TGF- β receptor I, TGF- β receptor II and phosphorylated TGF- β receptor II. As shown in Fig. 4, the expression levels of phosphorylated TGF- β receptor II and p-smad2 are increased in zebrafish embryo injected with 120 pg znf45l mRNA. Conversely, the expression levels of these factors are decreased in zebrafish embryo injected with 4 ng znf45l tMo. However, the expression levels of other TGF- β signaling related proteins do not appear significant change. These results indicate that znf45l regulates TGF- β signaling by enhancing TGF- β receptor II phosphorylation and then increasing phosphorylation level of smad2.

DISCUSSION

One of the most important processes in the generation of verte-

brate embryo is the formation of the mesoderm, from which blood and angioblasts (endothelial progenitors) originate. In early hematopoietic development and specification, TGF- β and other pathways play critical roles in the proper formation of the (PS) (2, 21, 22). In this study, we provide several lines of evidence showing that znf45l affects hematopoiesis through regulating TGF- β signaling.

To uncover the function of znf45l gene in zebrafish, we detect its expression patterns during embryogenesis. In zebrafish, znf45l is expressed both maternally and zygotically, which raises the possibility that znf45l may regulate primitive hematopoiesis indirectly by controlling ventral mesoderm development. Now, the presupposition is supported by two lines of evidence in our study. First, knockdown of znf45l shows shortened body length and moderate necrosis in the anterior region and ventrally curved tail. Second, knockdown of znf45l inhibits the expression of the hemangioblast/hematopoietic precursor markers *scl*, *gata2*, *lmo2* and *fli1a* at early segmentation period. Meanwhile, coinjection with znf45l-tMo and P53-Mo still caused loss of *scl* expression, which certifies znf45l functions in specification of hematopoiesis. It is well-known that TGF- β signaling induces mesoderm formation and is required to primitive hematopoiesis. We checked genetic interaction between znf45l and TGF- β signaling and indicated that znf45l located downstream of TGF- β ligand. The most importantly, we examined TGF- β signaling related receptors and smads proteins to illustrate how znf45l affected TGF- β signaling. The result reveals that znf45l enhances TGF- β signaling through increasing TGF- β receptor II phosphorylation and furthermore promotes smad2 phosphorylation. Notably, our study mainly focuses on znf45l function regulating hematopoiesis through TGF- β signaling, without excluding the possibilities that znf45l may play roles in other pathways.

Our work shows that znf45l functions to early embryogenesis as a novel positive regulator of TGF- β signaling to promote hematopoiesis. Considering the significance of TGF- β signaling in embryonic development, our study might offer a new perspective to novel molecular mechanism of TGF- β signaling and hematopoiesis.

MATERIALS AND METHODS

Animals

The zebrafish (*Danio rerio*) AB strain was maintained and raised at 28.5°C. Fertilized eggs were obtained by natural mating and cultured in embryo medium. Staging of the embryos was carried out according to Kimmel *et al.* (23).

In vitro RNA extraction, RT-PCR, cloning

Total RNA was extracted from embryo at 50% epiboly stage by using TRIzol (Invitrogen, USA). cDNA was reverse-transcribed from total RNAs templates by using SuperScript™ Reverse Transcriptase (Fermentas). The coding sequence of znf45l was cloned into the vector pcDNA3.0 (Invitrogen, USA) for capped

mRNA synthesis and antisense RNA probe synthesis.

Synthesis of mRNA and probe, morpholino oligonucleotides

The mRNA of znf45l is synthesized by using mMESSAGE-mMACHINE Kit (Ambion, Austin, Texas, USA). The probe labeled with Digoxigenin-labeled UTP is synthesized by using DIG RNA Labeling Kit (SP6/T7) (Roche, German). The full-length coding region of znf45l was also cloned into pEGFP-N1 for expression of znf45l-GFP fusion protein to generate plasmid that was used for testing the effectiveness of znf45l-tMo. Znf45l translation blocker morpholino (znf45l-tMo, 5'-GGTTCTGGATCACTCATCTTCTCT C-3'), the splicing blocker morpholino (znf45l-sMo, 5'-CAGGAA TATACACCAACCTATTTGT-3'), the P53 morpholino (5'-CGGCC ATTGCTTTGCAAGAAT TG-3') and 5-mis-pair control morpholino (znf45l-cMo, 5'-GGTTGTCGATCAGTCATGTTGTCTC-3') were used in this study.

Zebrafish whole mount in situ hybridization (ISH) and microinjection

The morpholino, linearized plasmid or mRNA was microinjected into one-cell stage embryos. In situ hybridization protocols were performed as described in Sun *et al.* (20). Fragments of marker genes *scl*, *gata2*, *lmo2*, *fli1a* were PCR-amplified and cloned into the PGM-T (Tiagen, China) vector respectively. After lineage by appropriate restriction enzymes, the probes were synthesized using DIG RNA Labeling Kit (SP6/T7) (Roche, German).

Western blot analysis

Composite of capped mRNAs or morpholinos were injected into single-cell embryos, and 100 embryos at the 50% epiboly stage were used for western blot analysis. The Universal protein extraction buffers were purchased from Biotek Corporation (Beijing, China). Immunoblotting were performed as described in the product information. Antibodies against p-Smad2 (3101s) from Cell Signaling Technology, smad2 (1736-1) from Epitomics, TGF- β receptor I (ab31013) from Abcam, TGF- β receptorII (sc-17792), smad4 (sc-1909) from Santa Cruz Biotechnology; TGF- β receptorII (phosphor) (sy-165843) from Orbigen are used in this study.

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