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Hippo signaling components, Mst1 and Mst2, act as a switch between self-renewal and differentiation in *Xenopus* hematopoietic and endothelial progenitors

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ABSTRACT Hippo signaling is a conserved pathway that regulates cell proliferation and organ size control. *Mst1* and *Mst2* were identified as homologs of *hippo* and as core kinases of the Hippo pathway in mammals. Here, we have characterized the role of *Mst1* and *Mst2* during *Xenopus* primitive hematopoiesis. We showed that *Mst1* and *Mst2* were strongly expressed in the *Xenopus* ventral blood island, where primitive hematopoiesis is initiated. Loss-of-function analysis of Mst1/2 revealed morphogenetic defects, including short axis, smaller eyes and abnormal epidermis, and decreased phosphorylation of Yap. Mst1/2 morphants did not exhibit any change in the expression of hematopoietic and endothelial progenitor markers in early hematopoietic development. In addition, we have shown that such progenitor markers were continuously expressed through to the late hematopoietic development stage. As a result, the expression of erythroid, myeloid and endothelial differentiation markers were decreased in Mst1/2 morphants. Our results indicate that Mst1/2 act as a differentiation switch in *Xenopus* hematopoietc and endothelial progenitors.

KEY WORDS: *Hippo signaling, Mst, Yap, primitive hematopoiesis, Xenopus tropicalis*

Introduction

The Hippo signaling pathway plays a key role in regulating cell growth and organ size, and the core components of Hippo signaling are highly conserved from vertebrates to fly (Zhao *et al*., 2011). In vertebrates, mammalian sterile 20-like kinase 1 and 2 (Mst1/2; homolog of Hippo in fly) are serine/threonine kinases possessing the SARAH (Sav/Rassf/Hippo) domain. Mst1/2 form hetero- and homo-dimers, interacting with Sav1 and Rassf family members, which activate Mst1/2 (Scheel and Hofmann, 2003; Callus *et al*., 2006; Guo *et al*., 2007). Mst1/2 are also activated by Tao1 (Poon *et al*., 2011), a putative upstream regulator, as well as by caspase-dependent proteolytic cleavage (Lee *et al*., 2001), and/or autophosphorylation (Glantschnig *et al*., 2002). Activated Mst1/2 directly phosphorylate and activate large tumor suppresser homolog 1 and 2 (Lats1/2; homolog of Warts in fly), which are other core kinases in the Hippo signaling pathway (Dong *et al*., 2007). Phosphorylated Lats1/2 phosphorylate and inhibit Yes-associated protein (Yap; homolog of Yki in fly) and the transcriptional coactivator with PDZ domain (TAZ; ortholog of Yap). The phosphorylation of Yap/TAZ corresponding to serine 127 in human Yap induces binding to 14-3-3 and consequently, leads to cytoplasmic retention of Yap/TAZ (Zhao *et al*., 2007; Hao *et al*., 2008; Lei *et al*., 2008). In addition, phosphorylation of Yap/TAZ corresponding to serine 381 in human Yap triggers ubiquitination and subsequent degradation (Zhao *et al*., 2010; Liu *et al*., 2010). Intact Yap/TAZ proteins are primarily localized in the nucleus, where they interact with transcriptional factors (TFs), including TEAD1-4 (via the TEAD-binding domain of Yap/TAZ), Runx1/2 (via the WW domain), and regulate gene expressions related to growth and proliferation (Zhao *et al*., 2008;

Abbreviations used in this paper: VBI, ventral blood island.

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Zhang *et al*., 2009; Yagi *et al*., 1999; Cui *et al*., 2003).

In mice, gene ablation of *Mst1/2* results in death at approximately embryonic day 8.5 with the following phenotypes: severe growth retardation, failed placental development, impaired yolk sac/embryo vascular patterning and primitive hematopoiesis, increased apoptosis in placenta and embryo, and disorganized proliferating cells (Oh *et al*., 2009). In addition, *Yap*-depletion in mouse is embryonically lethal due to defective embryonic axis elongation and yolk sac vasculogenesis (Morin-Kensicki *et al*., 2006). These results implicated Hippo signaling in primitive hematopoiesis.

The ontogeny of embryonic hematopoiesis and vasculogenesis, known as primitive hematopoiesis, is conserved in vertebrates (Galloway and Zon, 2003; Medvinsky *et al*., 2011), in which it usually take place in the blood island. The mammalian and avian blood island is formed in the early yolk sac. In *Xenopus* embryo, primitive hematopoiesis initiates in the ventral blood island (VBI), which constitutes the anterior VBI (aVBI) and the posterior VBI (pVBI) domains. Previous attempts to characterize fate-mapping blastomeres of the 32-cell embryo indicated that aVBI derives from the dorsal vegetal blastomeres and that pVBI derives from the ventral vegetal blastomere (Ciau-Uitz *et al*., 2000; Lane and Sheets, 2002). The primary site for primitive myeloid cell differentiation is the aVBI, which initially expresses hematopoietic and endothelial progenitor markers, including *Scl*, *Runx1*, *Lmo2*, and *Fli1*. Subsequently, pVBI expresses several hematopoietic and endothelial markers and gives rise to embryonic erythrocyte, myeloblast, and vascular endothelial cells (Ciau-Uitz *et al*., 2010; Liu *et al*., 2008). It is also known that these hematopoietic lineage and endothelial cells are derived from the common multipotent precursor cells of the hemangioblast (Sabin 1920; Murray, 1932).

Recently, Yap/TAZ was shown to regulate proliferation, self-renewal, and pluripotency in the following undifferentiated cells: embryonic stem cell (ESC) and induced pluripotent stem cell (iPSC) populations (Lian *et al*., 2010), intestinal stem cell (ISC) progenitors (Camargo *et al*., 2007), epidermal progenitors (Zhang *et al*., 2011), liver progenitors (Camargo *et al*., 2007; Dong *et al*., 2007), neural progenitors (Cao *et al*., 2008), and other stem cells (Hiemer and Varelas, 2012). Hippo signaling plays an important role in the stemness of undifferentiated/progenitor cells as well as organ size control. However, little is known about the role of Hippo signaling in hemangioblast and hematopoietic and endothelial progenitor.

Here, we report that *Mst1/2* are expressed in VBI, where they act as a switch between self-renewal and differentiation in *Xenopus* hematopoietic and endothelial progenitor.

Results

Mst1 *and* Mst2 *expression is localized in the ventral blood island in* Xenopus tropicalis *embryos*

To determine the spatiotemporal expressions of *Mst1/2*, we performed semi-quantitative RT-PCR and WISH analyses as the developmental stages progressed. *Mst1/2* were continuously expressed throughout the early developmental stages, although *Mst1* expression was temporally decreased at the early gastrula stage (Fig. 1A). Whole-mount *in situ* hybridization (WISH) analyses confirmed that these genes were ubiquitously expressed before the neurula stage (data not shown). The localized expression of *Mst1* was initially detected in the aVBI at stage 15 (Fig. 1B,B'). Subsequently, *Mst1* was expressed in the aVBI and in scattered cells corresponding to migrating myeloblasts, with weak expression also detected in the pVBI (Fig. 1C-F,C'-F'). At the tadpole stages, *Mst1* was detected in the branchial arches, intestine, and duodenum (Fig. 1 G,H). Hemi-sectioning of a stage-18 embryo revealed detailed

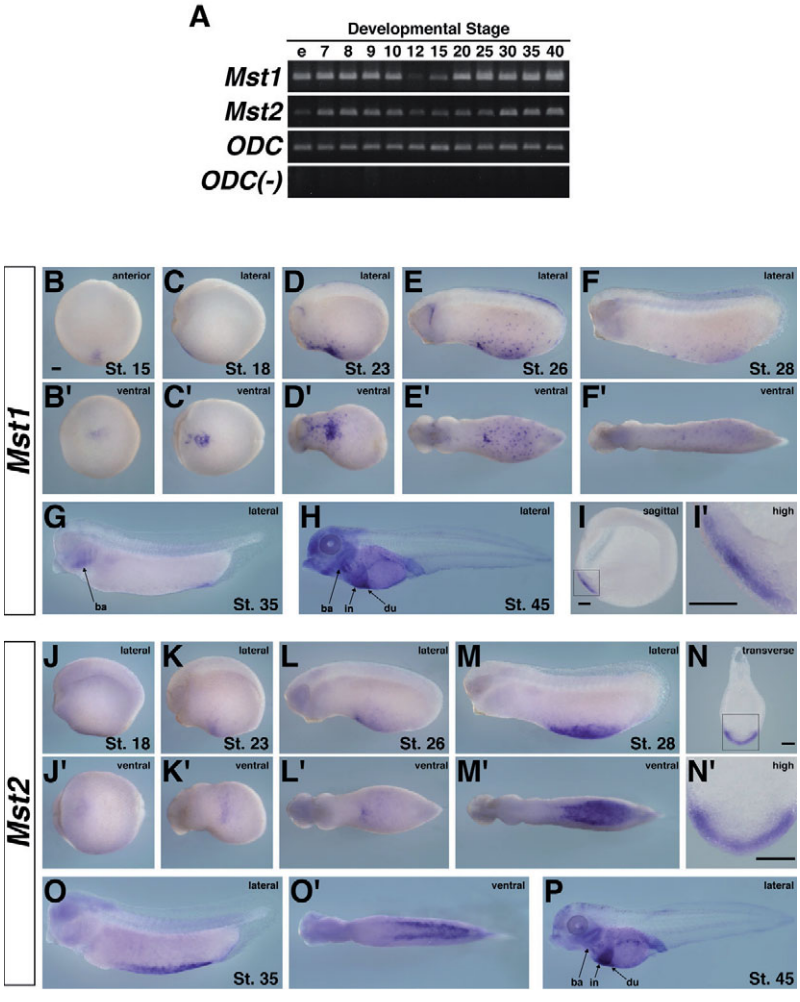

Fig. 1. Expression pattern of *Mst1* and *Mst2* in *Xenopus tropicalis* development. (A) *RT-PCR analysis revealed the temporal expression patterns of Mst1 and Mst2. RNA was extracted from* Xenopus tropicalis *embryos at the stages indicated above each lane.* ODC *were used as internal controls.* ODC(-), *RT-PCR without reverse transcriptase in* ODC *reaction. e, unfertilized egg.* **(B-P)** *Whole-mount* in situ *hybridization analysis of* Mst1 *and* Mst2. **(B-H)** *Localized expression pattern of* Mst1. **(B')** *The anterior view corresponding to (B).* **(C'-F')** *The ventral view corresponding to C-F.* **(I)** *Hemi-sectioning of a stage-18 embryo revealed detailed localization of* Mst1 *in the aVBI.* **(I')** *High magnification view of the boxed region in (I).* **(J-M,O,P)** *Localized expression pattern of* Mst2. **(J'-M',O')** *The ventral view corresponding (J-M, P,O).* **(N)** *Transverse sectioning of a stage-28 embryo revealed detailed localization of* Mst2 *in the pVBI.* **(N')** *High magnification view of the boxed region in (N). Scale bars, 100 μm. View direction is indicated in the upper right of each panel. Developmental stage is indicated in the lower right. Abbreviations: ba, branchial arch; du, duodenum; in, intestine.*

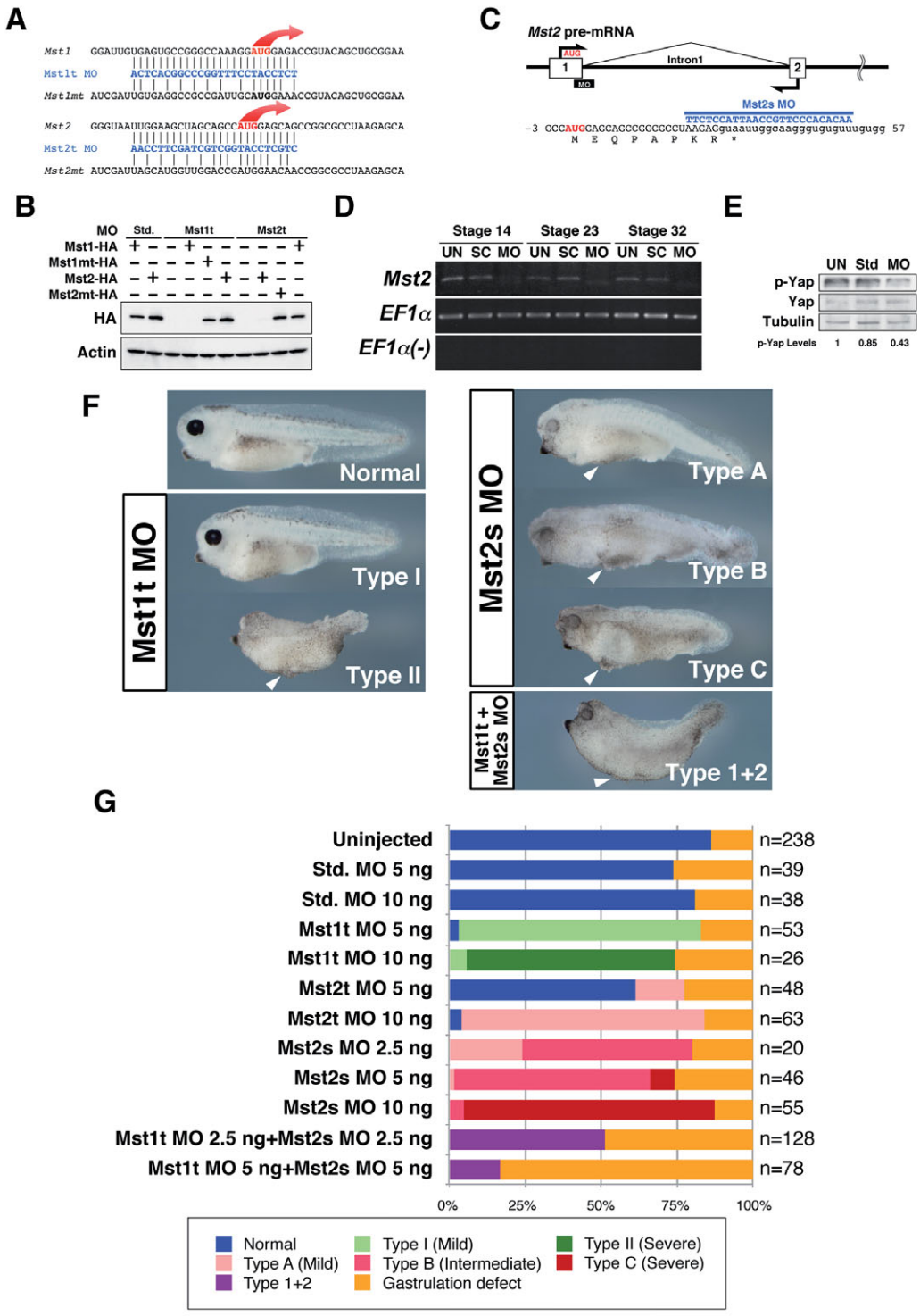

Fig. 2. Phenotype of Mst1 and Mst2 morphants. (A) *Mst1 and Mst2 translational blocking MOs (Mst1t MO and Mst2t MO) targeting* Mst1 *and* Mst2 *genes. The target regions of* Mst1 *and* Mst2 *are indicated in black, the sequences of the Mst1t MO and Mst2t MO are shown in blue, and the start codons are shown in red.* **(B)** *Western blotting analysis showing the translation level of HA-tagged Mst1 or Mst2 protein in* Xenopus laevis *embryo injected with standard control MO (20 ng), Mst1t MO (20 ng), or Mst2t MO (20 ng). Mst1t and Mst2t MO completely blocked the translations of Mst1-HA and Mst2-HA mRNA (500 pg), but did not block the translations of Mst1mt-HA and Mst2mt-HA mRNA (500 pg), which were mutated in the MO complementary sequences, respectively. Actin was used as an internal control.* **(C)** *Genomic structure including exon 1 (1) and exon 2 (2) of* Mst2*. The primer pair designed to amplify in the case of the proper splicing of* Mst2 *mRNA. The pre-mRNAs of* Mst2 *generated by splice blocking have a termination codon immediately following exon 1. The primer pair is indicated by arrows. The target region of* Mst2 *pre-mRNA is indicated in black. The sequence of the Mst2 splicing blocking MO (Mst2s MO) is indicated in blue. The start codon is indicated in red. Exon is shown in upper case, and the intron in lower case. Amino acid sequences are also indicated. Asterisk corresponds to stop codon.* **(D)** *RT-PCR analysis of* Mst2 *mRNA in MO-injected embryos. Embryos were injected with* Mst2 *MO (MO, 5 ng) or standard control MO (SC, 5 ng), in the animal pole of both blastomeres at the 2-cell stage, and cultured for RT-PCR until various stages as indicated above each lane. In Mst2 MO-injected embryos, the mature mRNA was not detected from stage 14. Splicing of* Mst2 *pre-mRNA was not affected by standard control MO injection. UN, uninjected embryo.* EF1α *was used as an internal control.* EF1α(-)*, RT-PCR without reverse transcriptase in* EF1α *reaction.* **(E)** *Western blotting analysis of the Mst1/2 knockdown embryos. Embryos were injected with Mst1t MO and Mst2s MO (MO, Mst1t+2s MO 2.5 ng/2.5 ng) or standard control MO (SC, 5 ng), in the animal pole of both blastomeres at the 2-cell stage, and cultured until stage 30. Phospho-Yap levels were described in the bottom of the panel. UN, uninjected embryo.* **(F)** *Phenotypes of Mst morphant. Mst1 or Mst2 MO injection induced a morphological abnormality in a concentration-dependent manner. Discriminations of phenotypes were indicated using the following abbreviations. Mst1 morphant; Type I, short axis. Type II, short axis and abnormal epidermis. Mst2 morphant, Type A; smaller eyes and bent axis. Type B; smaller eyes, bent axis and abnormal epidermis. Type C, smaller eyes abnormal episermis and short axis. Mst1/2 morphant; Type 1+2, short axis and smaller eyes. Arrowheads indicate overgrowth of epidermis.* **(G)** *Frequency of observed Mst morphant. Injected MOs are described at the left side of the panel. Numbers of injected embryos are indicated on the right side. Distinctions of phenotypes were indicated using the following colors. blue; Normal, light-green; Type I, green; Type II, light-pink; Type A, pink; Type B, red, Type C, purple; Type 1+2, yellow, Gastrulation defect.*

localization of *Mst1* in the aVBI (Fig. 1 I,I'). The *Mst2* transcripts were weakly expressed in aVBI with a pattern identical to that of *Mst1* (Fig. 1 J,J'). *Mst2* was weakly expressed in pVBI until the early tailbud stage, after which it was strongly expressed in pVBI from stage 28 until stage 35. (Fig. 1 K-M,K'-M',O,O'). Hemisectioning of a stage-28 embryo revealed detailed localization of *Mst2* in the pVBI (Fig. 1 N,N'), and at stage 45, it was detected in the branchial arches, intestine, and duodenum as for *Mst1* (Fig. 1P). Thus, *Mst1/2* were transiently and specifically expressed in VBI, suggesting that Mst1/2 may function in hematopoiesis and vascular formation.

Mst1 and Mst2 depletion synergistically induced morphogenic defects

We generated two kinds of Mst1/2 MOs, the translation-blocking MO, Mst1t MO and Mst2t MO, and splice-blocking MO, Mst1s MO and Mst2s MO (Fig. 2 A,C). We first tested whether the translation-blocking MOs could inhibit translation by coinjecting HA-tagged Mst1/2 mRNA including sequences of the AUG initiation codon and 5'UTR to which MO bind (Mst1-HA and Mst2-HA) or mutated sequences of them (Mst1mt-HA and Mst2mt-HA) together with Mst1t MO and Mst2t MO into 2-cell-stage *Xenopus laevis* embryos. Western blotting analysis performed at stage 10.5 showed that Mst1t MO and Mst2t MO specifically and completely inhibited the translation of Mst1-HA and Mst2-HA respectively, but not of Mst1mt-HA and Mst2mt-HA (Fig. 2B). To address the splice-blocking effects of Mst1s MO and Mst2s MO, we performed RT-PCR analysis of MO-injected *Xenopus tropicalis* embryos. Mst2s MO (5 ng) efficiently blocked the proper splicing (Fig. 2D), whereas Mst1s MO (10 ng) had no effect on splice blocking (data not shown).

It has reported that phosphorylation level of Yap was significantly reduced by depletion of Mst1 and Mst2 in the mouse liver (Zhou *et al*., 2009; Song *et al*., 2010; Lu *et al*., 2010). We tested whether Yap phosphorylation was decreased in the Mst1/2 double-knockdown embryos with western blots. Compared to standard control MO-injected embryos phospho-Yap level was clearly decreased in the Mst1/2 double-knockdown embryos (Fig. 2E). These results suggested that Mst1 and Mst2 MO effectively inhibited production of Mst1 and Mst2, resulting in reduction of Yap phosphorylation.

We then performed loss-of-function analysis of Mst1/2 using Mst1t MO, Mst2t MO, and Mst2s MO. Mst1-depleted embryos exhibited a short axis and impaired normal morphogenesis including malformation of the ventral epidermis at higher MO concentrations. (Fig. 2 F,G). Mst2 morphants were severe phenotypes in a concentration-dependent manner, including epidermodysplasia (Fig. 2 F,G), and the embryo phenotypes were equivalent for Mst2t MO-injected and Mst2s MO-injected embryos. In addition, we found that Mst2s MO-injected embryos showed more severe phenotypes than Mst2t MO-injected embryos (Fig. 2G). Based on these results, we used Mst1t MO and Mst2s MO for the subsequent morphant analyses. Mst1/2 double-knockdown embryos showed a shortened body axis phenotype and gastrulation defect in a concentration-dependent manner (Fig. 2 F,G).

Mst1/2 is required for normal formation of embryonic erythrocytes, myeloblasts and vascular endothelium

As *Mst1/2* were specifically expressed in VBI, we next examined the effects of Mst1/2 depletion on embryonic erythrocytes, using *globin* as a marker of erythrocyte differentiation. Compared to standard control MO (5 ng)-injected embryos, the expression pattern of α-*globin* did not alter in the Mst1 MO (5 ng)-injected embryos (Fig. 3 A,B,A',B'). However, Mst2 and Mst1/2-depleted embryos exhibited decreased α-*globin* expression (Fig. 3 C,D,C',D'). At stage 40, circulating erythrocytes were observed in the veins of standard control MO-injected embryos, whereas knockdown of Mst1, Mst2 and Mst1/2 inhibited the formation and circulation of erythrocytes (Fig. 3 E-H). In addition, *o*-dianisidine staining to visualize circulating erythrocyte (O'Brien, 1961) showed hardly any staining in the Mst2 and Mst1/2 morphants but not Mst1 morphant, compared to standard control MO-injected embryos (Fig. 3 I-L). Moreover, RT-qPCR analysis of β-*globin* revealed

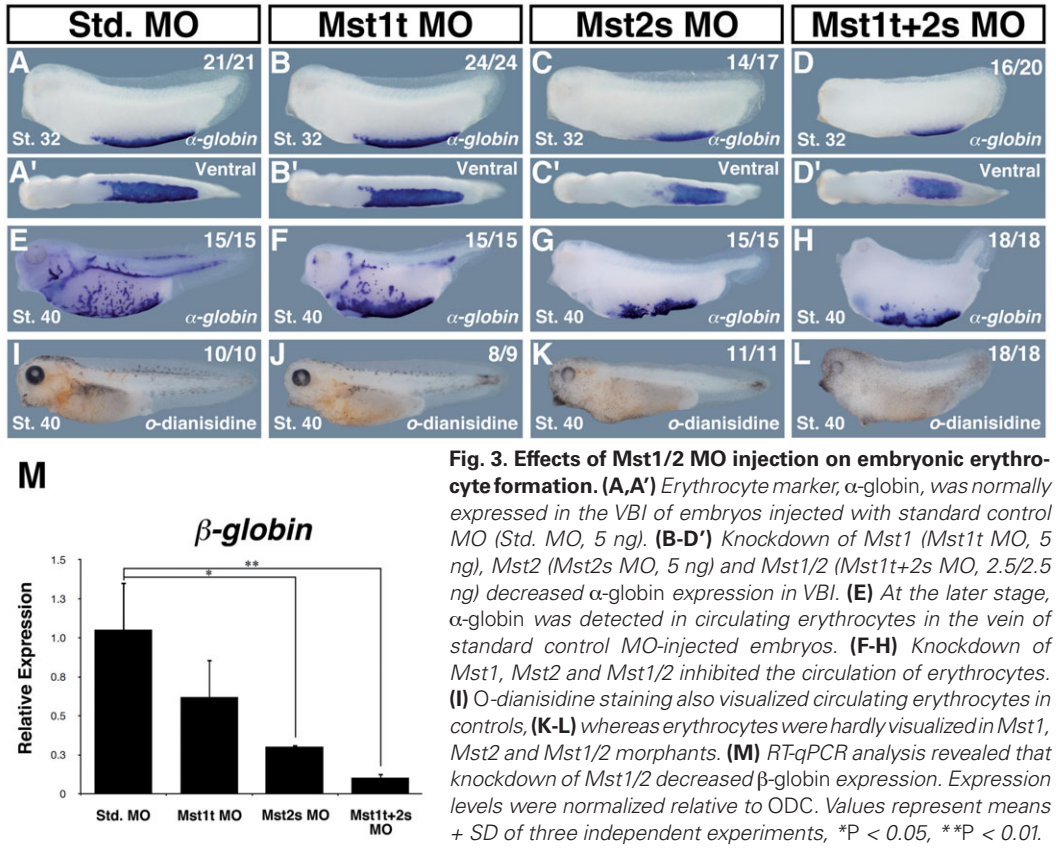

Fig. 3. Effects of Mst1/2 MO injection on embryonic erythrocyte formation. (A,A') *Erythrocyte marker,* α-globin, *was normally expressed in the VBI of embryos injected with standard control MO (Std. MO, 5 ng).* **(B-D')** *Knockdown of Mst1 (Mst1t MO, 5 ng), Mst2 (Mst2s MO, 5 ng) and Mst1/2 (Mst1t+2s MO, 2.5/2.5 ng) decreased* α-globin *expression in VBI.* **(E)** *At the later stage,* α-globin *was detected in circulating erythrocytes in the vein of standard control MO-injected embryos.* **(F-H)** *Knockdown of Mst1, Mst2 and Mst1/2 inhibited the circulation of erythrocytes.* **(I)** *O-dianisidine staining also visualized circulating erythrocytes in controls,* **(K-L)** *whereas erythrocytes were hardly visualized in Mst1, Mst2 and Mst1/2 morphants.* **(M)** *RT-qPCR analysis revealed that knockdown of Mst1/2 decreased* β-globin *expression. Expression levels were normalized relative to* ODC. *Values represent means + SD of three independent experiments,* *P < 0.05, **P < 0.01.

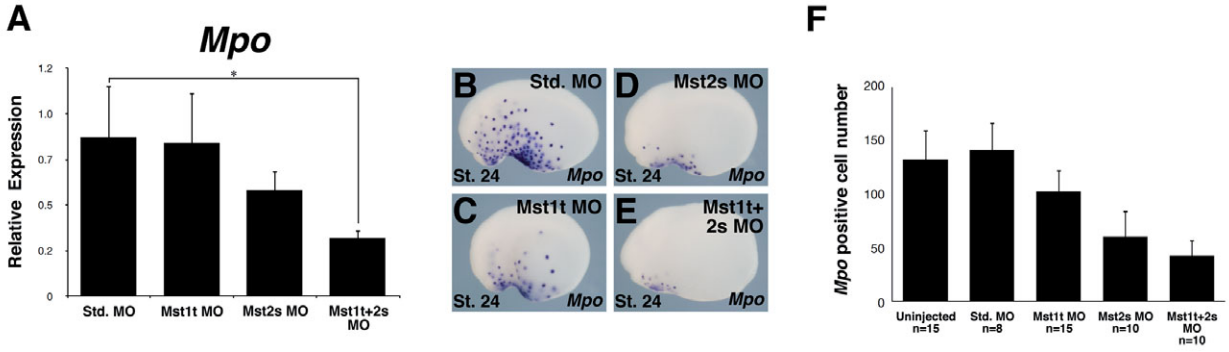

Fig. 4. Effects of Mst1/2 MO injection on embryonic myeloblast formation. (A) Mpo *is detected in myeloblast in standard control MO-injected embryo.* **(B-D)** *Knockdown of Mst1, Mst2 and Mst1/2 decreased myeloblast numbers, and* **(E)** Mpo*-positive cell number was synergistically decreased by Mst1/2 MO injection.* **(F)** *RT-qPCR analysis revealed that the knockdown of Mst1/2 decreased expression of* Mpo*, a myeloblast marker. Values represent means + SD of three independent experiments, *P < 0.05, **P < 0.01.*

synergistically decreased expression of *β-globin* in the Mst morphants (Fig. 3M). We also assessed primitive myelopoiesis following Mst depletion. Expression of myeloblast marker, *Mpo,* was decreased by Mst depletion (Fig. 4A). WISH analysis of *Mpo* was also revealed that *Mpo*-positive cell numbers were decreased in Mst1, Mst2 and Mst1/2 MO-injected embryos (Fig. 4 B-F). Furthermore, we found that depletion of Mst impaired appropriate formation of the embryonic vasculature based on a decrease in vascular endothelium markers, *Ami*, *Tie2*, and *Msr,* and abnormal vascular patterning in the Mst morphants (Fig. 5).

To address the specificity of the Mst1/2 knockdown, we assessed whether *Mst1* or *Mst2* construct could rescue the phenotype and knockdown effects of Mst1/2 morphant. The injection of *Mst1* or *Mst2* expression constructs, which were driven by EF1α promoter, rescued morphogenetic abnormality of Mst1/2 knockdown embryo (Supplemental Fig. 2A-E). Furthermore, markedly decreased expression of β-*globin* in the Mst1/2 knockdown embryo was partially rescued by injection of *Mst1* or *Mst2 DNA* (Supplemental Fig. 2F). The disruption of embryonic vascular patterning in the Mst1/2 morphant was also rescued with injection of *Mst1* or *Mst2 DNA* but not *LacZ DNA* (Supplemental Fig. 2 G-J). These results suggested that Mst1/2 are required for erythropoiesis, myelopoiesis, and vascular formation.

Mst1/2 depletion promotes maintenance of the hematopoietic and endothelial progenitor

The hemangioblast is a common precursor of hematopoietic and endothelial lineages (Orkin and Zon, 2008), and the Mst1/2-depleted embryos studied here exhibited defects in both hematopoietic cells and endothelial cells. Therefore, we examined whether hematopoietic and endothelial progenitor, and/or early differentiation markers, *Scl*, *Runx1*, *Lmo2*, *Gata2*, *C/EBPα*, *Spib*, *Gata1*, *Fli1*, and *Etv2* were affected by depletion of Mst1/2. In the early tailbud stage, the early hematopoietic and endothelial progenitor marker expressions were not affected (Fig. 6 A-H). In aVBI of same stage and neurula stage, WISH analyses of *C/EBPα* and *Spib*, which were myeloid progenitor markers (Chen *et al*., 2009), revealed that expression of these markers were maintained, but migrating myeloid cells were obviously decreased in the Mst1/2 knockdown embryo (Fig. 6 I-N, Fig. 4). At the early tadpole stage, these markers were gradually downregulated in standard control MO-injected embryos as cells differentiated into hemocytes and endothelia, whereas Mst1/2-knockdown embryos maintained the earlier expression levels of these markers (Fig. 6 O-V). In this stage, *Gata1* is one of the most important transcriptional factors for erythroid differentiation (Pevny *et al*., 1991), under the regulation of *Gata2* (Bresnick *et al*., 2010). Depletion of Mst1/2 decreased expression of *Gata1* in VBI (Fig. 6

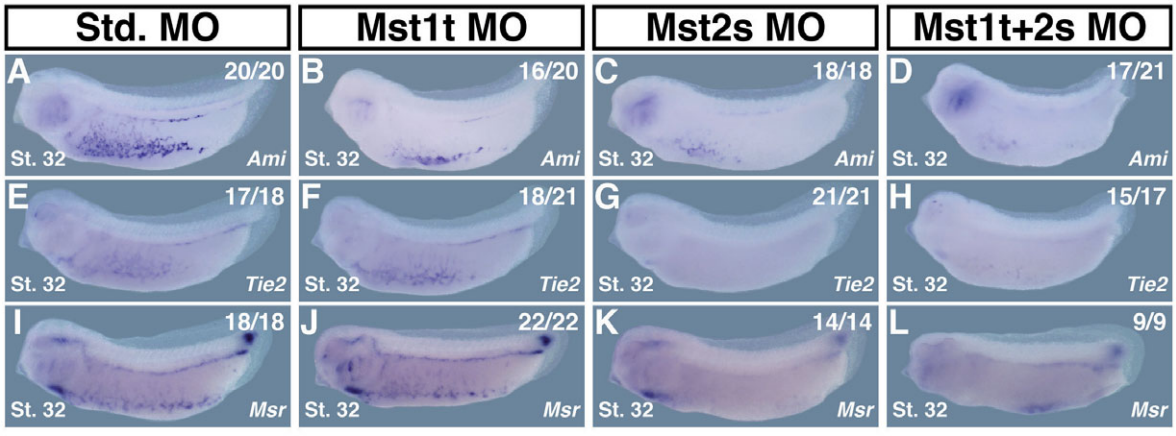

Fig. 5. Effects of Mst1/2 MO injection on embryonic vascular plexus formation. *WISH analysis of embryonic vascular markers,* Ami, Tie2, *and* Msr. **(A,E,I)** *The embryonic vascular network had started forming at stage 32 in standard control MO-injected embryo,* **(B-D,F-H,J-L)** *and this was disrupted by Mst1, Mst2, and Mst1/2 knockdown. Sample numbers are indicated in the upper right of each panel. Developmental stage is indicated in the lower left. Assessed marker is indicated in the lower right all panels.*

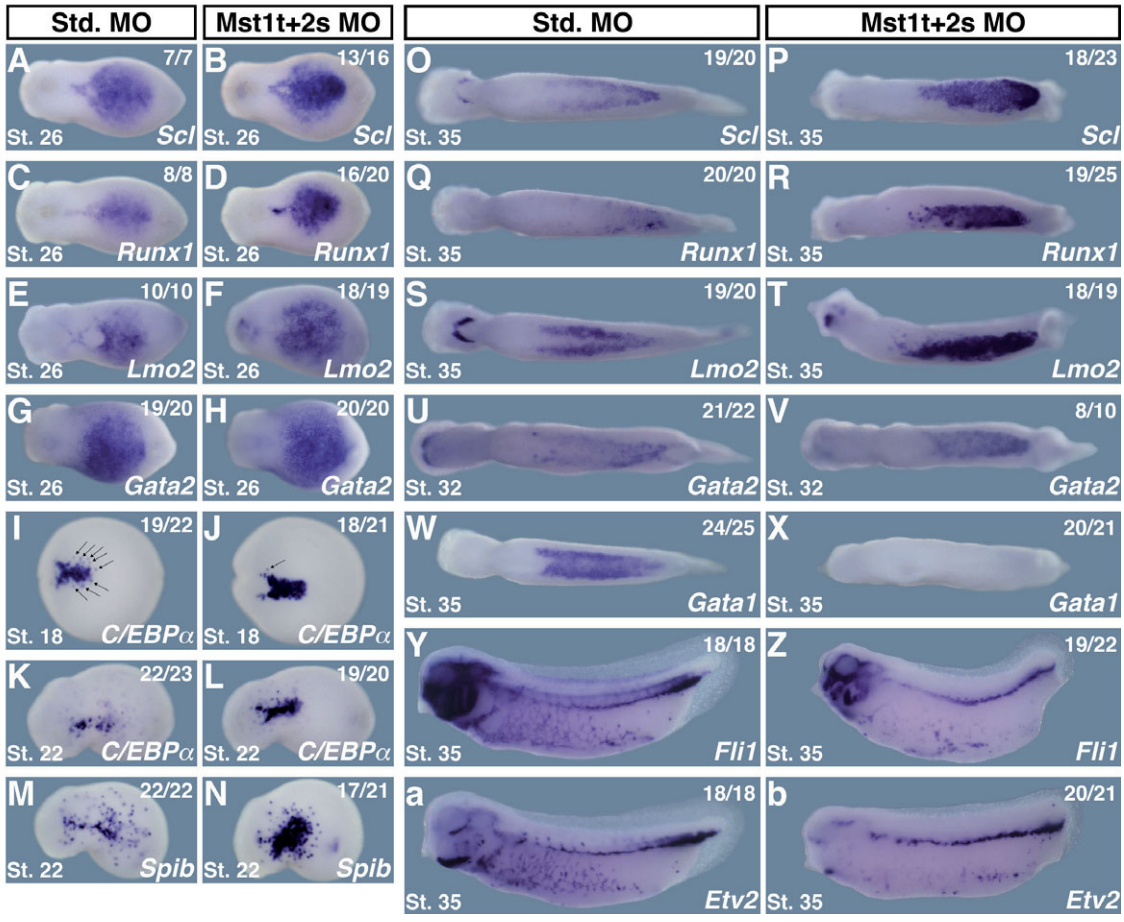

Fig. 6. Effects of Mst1/2 MO injection on hematopoietic and endothelial progenitors. (A-H) *At the early stage, no differences in the expression of hematopoietic and endothelial progenitor markers,* Scl, Runx1, Lmo2, *and* Gata2, *were observed between standard control MO (Std. MO, 5 ng)- and Mst1/2 MO (Mst1t+2s MO, 2.5/2.5 ng)-injected embryos.* **(I-N)** *In the Mst1/2 morphant, expressions of early myeloid progenitor marker,* C/EBPα *and* Spib *were maintained in aVBI, but reduction of migrating differentiated myeloid cells was observed. Arrow indicate migrating myeloid cells from aVBI.* **(O-V)** *At the later stage, following the differentiation of hematopoietic cells,* Scl, Runx1, Lmo2, *and* Gata2 *expression gradually decreased in the VBI of standard control MO-injected embryos, whereas these markers were continuously expressed at similar levels in the Mst1/2 knockdown embryo.* **(W,X)** *The pre-erythroblast marker,* Gata1, *was markedly decreased by Mst1/2 knockdown.* **(Y-b)** *The expression patterns of the endothelial progenitor marker,* Fli1 *and* Etv2, *were disrupted by depletion of Mst1/2. Sample numbers are indicated in the upper right of each panel, and developmental stage is indicated in the lower left. Assessed marker is indicated in the lower right of each panel.*

W,X), as well as the early endothelial markers, *Fli1* and *Etv2* (Fig. 6 Y-b). Thus, Mst1/2 play an important role in cell differentiation from hematopoietic and endothelial progenitors.

Discussion

Mst1/2 expression level regulates Hippo signaling

Our experiments revealed that Mst1/2 are transiently and locally expressed in VBI (Fig. 1). Loss-of-function experiment showed that decrease of phosphorylation of Yap protein (Fig. 2E). This is the first report that expression levels of Hippo signal transducers regulate the lineage-specific intensity of signal transduction. Previous studies have shown that TFs expressed in hemangioblast and HSC/early hematopoietic and endothelial lineages, such as *Scl*, *Runx1*, *Lmo2*, and *Gata2*, form a robust transcriptional regulatory network (Wilson *et al*., 2010). Additionally, stage-specific transcriptional programs control hematopoietic gene expression and the subsequent differentiation into multiple mature hematopoietic lineages (Pal *et al*., 2004; Orkin and Zon, 2008; Monteiro *et al*., 2011). The difference in expression patterns of *Mst1* and *Mst2* suggest individual regulation via distinct hematopoietic lineage-specific TFs. In fact, the binding sites for various hematopoietic TFs that are highly expressed in pVBI, including Gata, Ets, Runx, and Scl, are conserved in the *Mst2* gene promoter region, but not in the equivalent regions of the *Mst1* gene, which is not highly expressed in pVBI (data not shown). Such programmed and localized expression of *Mst1/2* resulted in enhanced Hippo signaling in VBI. Although Tao1 is known to directly phosphorylate Mst and promote Hippo pathway activation in mammalian cells (Poon *et al*., 2011), it remains less clear how the pathway is regulated upstream of Mst.

Hippo signaling acts as a switch between self-renewal and differentiation in hematopoietic and endothelial progenitors

In this study, we found *Mst1/2* to be transiently and locally expressed in VBI in a hematopoietic differentiation stage-progressive manner (Fig. 1), and revealed that Mst1/2 double knockdown strikingly disrupted erythropoiesis, myelopoiesis, and vasculogeneis and maintained stem cell or progenitor population (Fig. 3-6). Due to the disruption of hippo signaling in the Mst1/2 knockdown embryo, activated Yap/TAZ may inhibit hematopoietic and endothelial differentiation. Consistent with this finding, previous studies demonstrated that Yap/TAZ regulates proliferation, self-renewal, and pluripotency in several stem/progenitor cells (Hiemer and Varelas, 2012). In mouse intestine, *Yap* expression was restricted in undifferentiated/progenitor cells and Yap overexpression induced loss of differentiation and expansion of progenitor cells (Camargo *et al*., 2007). In addition, Mst1/2 restrained intestinal stem cell proliferation by inhibiting the nuclear localization of Yap (Zhou

et al., 2011). It seems quite probable that the same mechanism of Yap regulation could act to control stemness in hematopoietic and endothelial progenitors. Our preliminary data showed that besides decreased expression of embryonic erythrocyte and vascular marker and network-dissipated abnormal vasculature, decreased stem cell marker were observed in the Yap morphant (data not shown). These observations suggested that Yap was also required progenitor to proliferate in the early stage. Furthermore, our previous study showed that *Yap* expression is transiently suppressed in VBI at stage 30, the most prominent blood-producing stage (Nejigane *et al*., 2011), suggesting that Yap is also regulated at the transcriptional level in VBI. Taken together, the evidence indicates that Yap is required but transient elimination of both Yap protein and transcript in hematopoietic and endothelial progenitors is necessary during hematopoietic and endothelial differentiation.

Our study demonstrated that Hippo signaling components participated in hematopoietic and endothelial progenitors regulation in *Xenopus* embryogenesis. These finding is important for identifying potential therapeutic targets of hematopoietic and vascular diseases.

Materials and Methods

Plasmid construction

Using JGI *Xenopus tropicalis* v4.1, we searched and identified the genomic clones corresponding to *Xenopus tropicalis Mst1* and *Mst2*. The full-length ORFs of *Mst1* and *Mst2* were then amplified by PCR from the cDNA of *Xenopus tropicalis* gastrula-stage embryos. Primer sequences are shown in the supplementary table. Each clone was subcloned into the pCS2p (+) vector, which was digested by *Stu*I. pCS2p-*Mst1* and pCS2p-*Mst2* (Genbank Accession No. AB744231 and AB744232, respectively) were used for synthesis of the *in situ* hybridization probes and capped mRNAs. MacVector software (MacVector Inc) was used for multiple protein sequence alignment and phylogenetic tree analysis of the *Mst* genes (see Supplemental Fig. 1). HA-tagged Mst1/2 including either the 5'-untranslated region (UTR) to which morpholino antisense oligos (MO) could bind or a mutated 5'UTR to which MO could not bind were amplified by PCR using pCS2p-*Mst1* and pCS2p-*Mst2*, respectively, which were digested by *Cla*I and subcloned into the equivalent sites of the pCS2-HA vector (Nitta *et al*., 2004). The rescue analyses were performed using pENL, pE-*Mst1*, and pE-*Mst2*, which were *LacZ*, *Mst1*, and *Mst2* driven by Chick *EF1*α promoter, respectively.

Xenopus *embryo manipulation, mRNA synthesis for microinjection, and morpholino antisense oligos*

Xenopus embryos were obtained by artificial fertilization according to the method of Ariizumi *et al.,* (Ariizumi *et al*., 2009). Embryos were cultured in 10% Steinberg's solution and staged according to the scheme of Nieuwkoop and Faber (Nieuwkoop and Faber, 1994). Capped mRNAs were synthesized using an mMESSAGE mMACHINE SP6 Kit (Life Technologies) according to the manufacturer's instructions. Mst1 and Mst2 translational blocking MOs (Mst1t MO, 5'-TCTCCATCCTTTGGCCCG-GCACTCA-3'; Mst2t MO, 5'-CTGCTCCATGGCTGCTAGCTTCCAA-3'; Gene Tools, LLC) were targeted to the 5'UTR including the start codons of *Mst1* and *Mst2* transcripts, respectively. The splice site-targeted Mst2 MO (Mst2s MO, 5'-AACACACCCTTGCCAATTACCTCTT-3') was designed against the first exon-intron boundary of *Mst2* pre-mRNA (the underlining indicates the exonic region). The standard control MO (Gene Tools, LLC) was used as a control.

Whole-mount in situ *hybridization and* o-*dianisidine staining*

Digoxigenin (DIG)-labeled anti-sense RNA probes were synthesized by T7 or SP6 RNA polymerase (Promega) according to the manufacturer's instructions. WISH analysis was performed as described previously (Harland, 1991). After WISH, embryos were embedded in 3% low melting point agarose/3% sucrose and sectioned. To synthesize RNA probes, *α–globin, Mpo, Ami, Tie2, Msr, C/EBPα, Spib, Scl, Runx1, Lmo2, Gata2, C/EBPα, Spib,Gata1, Fli1, and Etv2* were amplified by PCR from the cDNA of the tailbud-stage embryos and subcloned into vectors. Primer sequences and vectors are shown in the supplementary table (see supplementary table). These sequences were verified by ABI PRISM® Genetic Analyzer 310 (Applied Biosystems). *O*-dianisidine staining was performed as described previously (O'Brien, 1961).

RT-PCR, RT-qPCR

Total RNA samples were isolated using RNAiso Plus (Takara Bio Inc), and 1 μg of total RNA was used as the template for first-strand cDNA synthesis using SuperScript II Reverse Transcriptase according to the manufacturer's instructions (Invitrogen). The cDNA (1 μl) was used as a template for PCR, and quantitative RT-PCR (RT-qPCR) was performed using a StepOnePlus™ Real-time PCR system (Applied Biosystems) and the Fast SYBR Green Master Mix (Applied Biosystems). Primer sequences are shown in the supplementary table (see Supplemental table). *Ornithine decarboxylase* (*ODC*) and *elongation factor 1 α* (*EF1α*) were used as internal controls.

Western blotting

Western blotting was performed by a standard protocol. Briefly, embryos were harvested at stage 11 or stage 30 and lysed in RIPA buffer containing a Complete Protease Inhibitor Cocktail (Roche). The equivalent of one embryo was loaded and run on a sodium dodecyl sulfate–polyacrylamide gel electrophoresis gel. After electrophoresis, proteins were transferred to Amersham Hybond-P PDVF Membrane (GE Healthcare), and immunostained by anti-HA (F-7; Santa Cruz), anti-Yap (H-9; Santa Cruz) anti-pYap (4912; Cell Signaling Technology), anti-actin (AC-40; SIGMA) and anti-Tubulin (T3526; SIGMA) as primary antibodies. Phospho-Yap levels were calculated by gel densitometry using ImageJ software (http://rsbweb.nih.gov/ij/), normalizing against Yap levels.

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