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XRASGRP2 is essential for blood vessel formation during *Xenopus* development

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ABSTRACT Ras guanyl nucleotide-releasing protein 2 (RASGRP2), one of the Ras guanine exchange factors, is implicated as a critical regulator of inside-out integrin activation in human lymphocytes, neutrophils and platelets. However, the activities of this protein in endothelial cells remain unclear. In the current study, we identify a physiological function in blood vessel formation for *XRASGRP2*, which is the *Xenopus* ortholog of mammalian *RASGRP2*. *XRASGRP2* over-expression induced ectopic vascular formation, and *XRASGRP2*-knockdown embryos showed delayed vascular development. We also investigated the upstream signaling of *XRASGRP2* in endothelium formation. *XRASGRP2* expression was up-regulated in the presence of VEGF-A and down-regulated following VEGF-A depletion. *XRASGRP2* knockdown abolished the ectopic induction of endothelial cells by VEGF-A in the posterior ventral blood island. These results suggest that XRASGRP2 is essential for vascular formation during *Xenopus* development.

KEY WORDS: *XRASGRP2, Xenopus laevis, VEGF-A, RASGRP, vasculogenesis*

Introduction

Vascular and hematopoietic cells are thought to arise from a common progenitor, the hemangioblast. In *Xenopus*, primitive red blood cells are produced exclusively in the ventral blood island (VBI), which is functionally equivalent to the extra-embryonic yolk sac blood island in mammals. The embryonic endothelial cells arise synchronously with the primitive red blood cells in the VBI. The close spatial and temporal relationships between the blood cells and endothelial cells support the hypothesis that they have a bipotential precursor, the hemangioblast (Sabin, 1920; Murray, 1932). However, the developmental mechanism underlying the differentiation of endothelial and primitive blood cells from the hemangioblast remains unclear.

Vascular endothelial growth factors (VEGFs) are key regulators in vasculogenesis and angiogenesis (Ferrara *et al.*, 2003). In particular, VEGF-A is involved in the regulation of processes required for angiogenesis, i.e., endothelial cell activation, proliferation, migration, and tubule formation (Ferrara *et al.*, 2003). In *Xenopus*, the *VEGF-A* gene is alternatively spliced to produce the VEGF122, VEGF170, and VEGF190 isoforms, which are equivalent to murine VEGF120, VEGF164, and VEGF188, respectively (Cleaver *et al.*, 1997). Ectopic expression of *VEGF122* changes the architecture of the developing vascular network (Cleaver *et al.*, 1997). Over-expression of VEGF170 induces the inhibition of expression of the hematopoietic genes α-*globin* and *GATA-1* in the posterior blood island, as well as the excessive production of endothelial cells (Koibuchi *et al.*, 2006).

Signaling through Ras is one of the intracellular pathways downstream of VEGF stimulation (Doanes *et al.*, 1999; Hood *et al.*, 2003; Meadows *et al.*, 2001). Genetic ablation of either *SOS*, which encodes a Ras guanine nucleotide exchange factor, or *NF1*, which encodes a Ras GTPase-activating protein, results in cardiovascular defects (Brannan *et al.*, 1994; Henkemeyer *et al.*, 1995; Wang *et al.*, 1997). *K-ras*-deficient mice die having multiple defects, including defects of the hematopoietic and cardiovascular systems (Johnson *et al.*, 1997; Koera *et al.*, 1997). Targeted deletion of *NF1* in

Abbreviations used in this paper: RASGRP, Ras guanyl nucleotide-releasing protein; VBI, ventral blood island; VEGF, vascular endothelial growth factor; Xmsr, Xenopus mesenchyme associated serpentine receptor.

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endothelial cells leads to multiple cardiovascular defects (Gitler *et al.*, 2003). The small GTPase Rap1b is required for normal angiogenesis and plays a role in the regulation of pro-angiogenic signaling in endothelial cells (Chrzanowska-Wodnicka *et al.*, 2007).

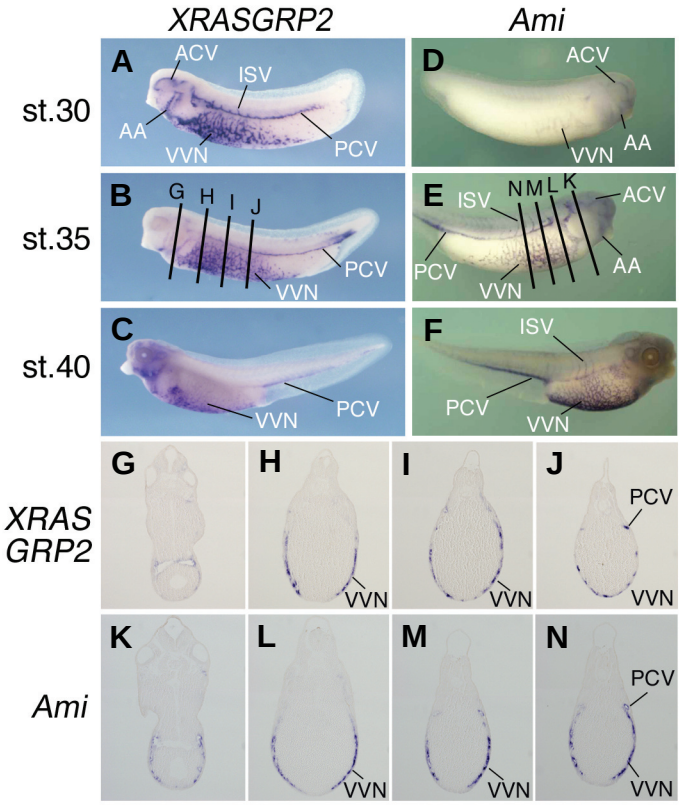
RasGRP2/CalDAG-GEFI is a member of the CalDAG-GEF/RasGRP family of intracellular signaling molecules involved in the activation of the Ras superfamily (Kawasaki *et al.*, 1998; Springett *et al.*, 2004). RasGRP2 contains binding sites for Ca^{2+} and DAG, and a GEF domain that predominantly activates Rap1 (Kawasaki *et al.*, 1998). Recently, RasGRP2 was identified as a critical regulator of inside-out integrin activation in human T lymphocytes, neutrophils, and platelets (Pasvolsky *et al.*, 2007). *RasGRP3* is expressed in embryonic blood vessels and newly formed vessels during pregnancy and tumorigenesis in adults. *RasGRP3* expression is up-regulated by VEGF stimulation of endothelial cells (Roberts *et al.*, 2004). In *Xenopus*, *XRASGRP2* is expressed in the vascular region of the embryo (Nagamine *et al.*, 2008).

In the present study, we reveal the role of XRASGRP2 in *Xenopus* vascular development by showing that: 1) over-expression of *XRASGRP2* induces ectopic endothelial cell differentiation; 2) *XRASGRP2*-knockdown embryos show reduction or delay of endothelial cell differentiation; and 3) *XRASGRP2* expression is induced by VEGF-A signaling. Our findings indicate that XRASGRP2 is essential for vascular development in downstream of VEGF-A signaling in *Xenopus* embryos.

Results

XRASGRP2 *expression is restricted in* Xenopus *developing vessels*

Previously, it was shown that *Xenopus laevis RASGRP2* (*XRASGRP2*) is expressed in the vascular regions of stage 35 embryos (Nagamine *et al.*, 2008). We examined in detail *XRASGRP2* expression during vascular development. *XRASGRP2* mRNA was found to be expressed in vascular regions, such as the anterior cardinal vein (ACV), aortic arch (AA), intersomitic vein (ISV), posterior cardinal vein (PCV), and vascular vitelline network (VVN) at stage 30 (Fig. 1A). The expression levels of *XRASGRP2* mRNA in the ACV, AA, and ISV were reduced at stage 35 (Fig. 1B). At stage 40, *XRASGRP2* expression was restricted to the PCV and VVN (Fig. 1C). In contrast, the expression of *Ami*, which is a vascular-specific gene, was detected in the ACV, AA, and VVN, but not in the ISV and PCV, at stage 30 (Fig. 1D). At stage 35, expression of *Ami* was detected in the ACV, AA, ISV, PCV, and VVN (Fig. 1E). These results



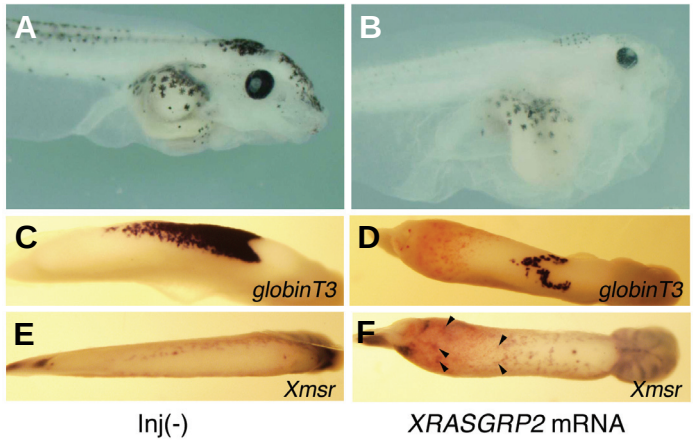


Fig. 1 (Left). *XRASGRP* expression precedes *Ami* expression. (A) XRASGRP2 *transcripts localized in the anterior cardinal vein (ACV), aortic arch (AA), intersomitic veins (ISV), posterior cardinal veins (PCV), and vascular vitelline network (VVN) at stage 30.* **(B)** XRASGRP2 *expression is detected in the PCV and VVN at stage 35. Lines indicate the positions of the sections shown in (G-J).* **(C)** *At stage 40,* XRASGRP2 *expression is restricted to the PCV and VVN.* **(D)** *The expression of* Ami *is weakly detected in the ACV, AA, and VVN at stage 30.* **(E)** Ami *expression is evident in the ACV, AA, ISV, PCV, and VVN at stage 35. Lines indicate the positions of the sections shown in (K-N).* **(F)** Ami *expression is detected continuously in the PCV, ISV, and VVN until stage 40.* **(G-J)** *Histologic section of the embryo shown in (B).* **(K-N)** *Histologic section of the embryo shown in (E). Both* XRASGRP2 *and* Ami *are expressed in the endothelial cells (PCV and VVN).*

Fig. 2 (Right). Ectopic expression of *XRASGRP2* affects vascular formation and induces edema. (A) *An uninjected control embryo at stage 43.* **(B)** *An embryo in which 1 ng of* XRASGRP2 *mRNA was injected into the dorsal vegetal blastomeres (DV) at the 8-cell stage. The embryo shows edema.* **(C-F)** *Whole-mount* in situ *hybridization for a hematopoietic marker,* globin T3, *and an endothelial marker,* Xmsr, *at stage 31.* **(C)** *The expression of* globin T3 *in an uninjected control embryo (ventral view).* **(D)** *Expression of* globin T3 *in an embryo that was co-injected with* XRASGRP2 *and* β-galactosidase *(*β-gal*) into the ventral vegetal blastomeres (VV) at the 8-cell stage. The expression of* globin T3 *is abolished at the injection site in the VBI.* **(E)** *The expression of* Xmsr *in an uninjected control embryo (ventral view).* **(F)** *The expression of* Xmsr *in an embryo that was co-injected with* XRASGRP2 *and* β-gal *into the VV. Ectopic expression of* Xmsr *is evident at the injection site in the VBI. Arrowheads indicate* Xmsr*-positive cells.*

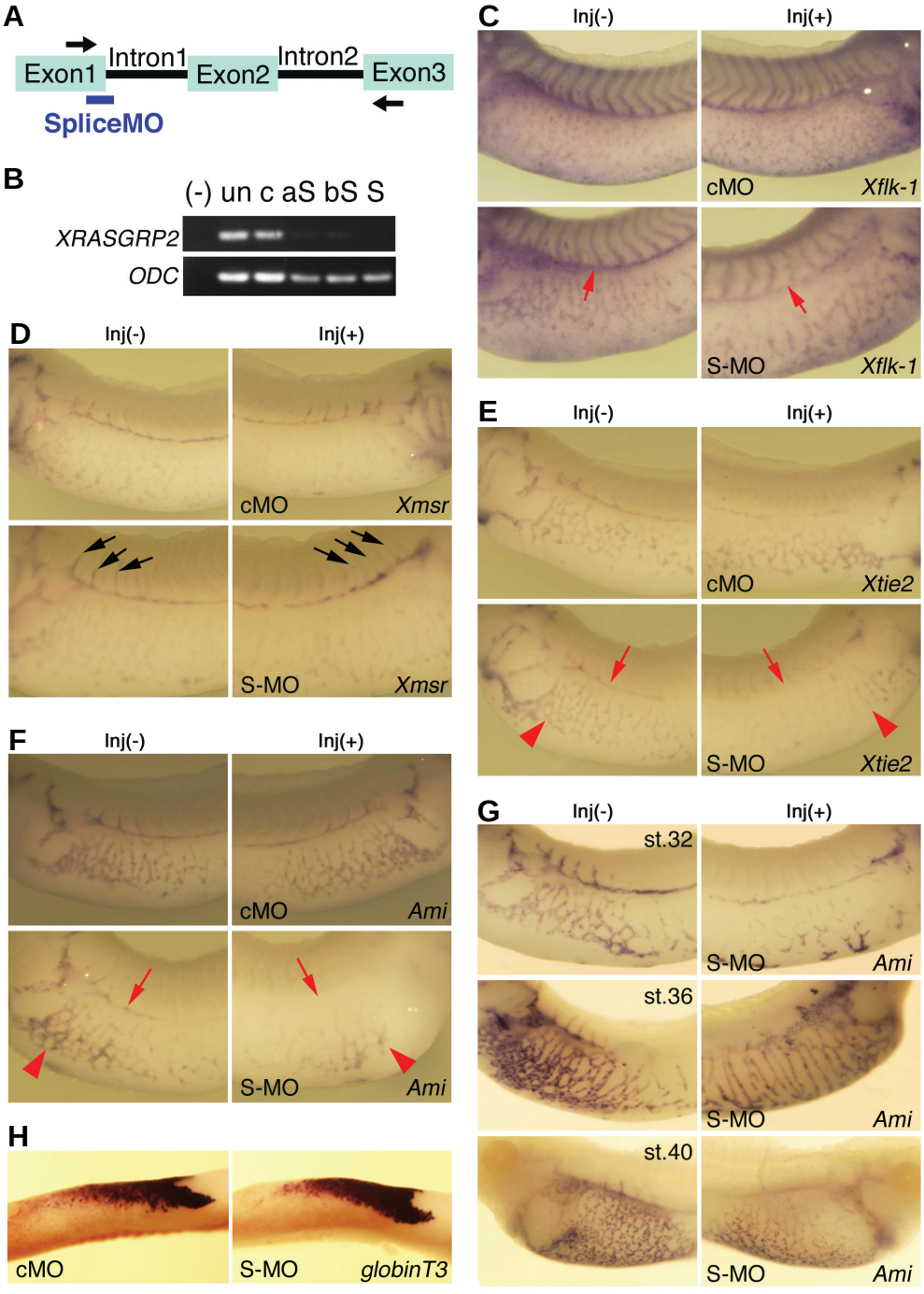


Fig. 3. XRASGRP2 depletion results in aberrant development of blood vessels. (A) *Schematic model for the splice inhibition antisense morpholino oligonucleotides (S-MOs). The binding site of MO is represented by a bolded blue line. Arrows indicate the primers used in the RT-PCR to examine the efficacies of the S-MOs.* **(B)** *The control MO (c, 40 ng), aS-MO (aS, 40 ng), bS-MO (bS, 40 ng), and S-MO (S, 40 ng, comprising 20 ng aS-MO plus 20 ng bS-MO) were injected into 2-cell-stage embryos, and the embryos were analyzed by RT-PCR at stage 30. The presence of the 312-bp band indicates amplification of the normally spliced mRNA. The intensity of this band is reduced in both the aS-MO-injected and bS-MO-injected embryos, as compared to the uninjected embryos and control MO-injected embryos, and this band is not detected for the S-MO-injected embryos. This indicates that the S-MO-injected embryos do not produce a functional XRASGRP2 protein. '-', Sample without reverse transcriptase; 'un', uninjected embryos.* **(C-F)** *Expression patterns of blood vessel marker genes. The 2-cell-stage embryos were injected with the control MO (40 ng) or S-MO (40 ng) into one blastomere (corresponding to the future right-hand side), and harvested at stage 31. The injected sides are indicated as [Inj(+)] and the uninjected sides are indicated as [Inj(-)]. The expression levels of* Xflk-1 *in the PCV (C, red arrows) and of* Xmsr *in the ISV (D, black arrows) are diminished in the S-MO-injected side. The expression levels of Xtie2 (E) and Ami (F) in the PCV (red arrows) and VVN (red arrowheads) are diminished in the S-MO-injected side. The expression level of* Ami *(F) is greatly reduced in the S-MO-injected side. No differences are seen in the control MO-injected embryos.* **(G)** *The expression of* Ami *in VVN is gradually mitigated in the S-MO-injected side.* **(H)** *Expression of* globin T3 *in the control MO-injected embryos. S-MO injection does not affect the level of* globin T3 *expression.*

suggest that the expression of *XRASGRP2* is transient and occurs earlier than the expression of *Ami* in developing vascular regions. Examination of the sections of the stage 35 embryos showed that both *XRASGRP2* and *Ami* were strongly expressed in the VVN and PCV (Fig. 1 G-N). The expression of *Ami* was reduced in the ACV and AA at stage 40 (Fig. 1F).

Over-expression of XRASGRP2 *induces ectopic expression of* Xmsr

We examined the function of *XRASGRP2* in vascular development. *XRASGRP2* mRNA and *β-galactosidase* (*β-gal*) mRNA were injected into dorsal-vegetal (DV) or ventral-vegetal (VV) blastomeres at the 8-cell stage. DV and VV blastomeres contain components of future VBI cells. The injected embryos showed the edema phenotype at the tail-bud stage, and this phenotype was more severe at stage 43 (75%, n=56), as compared to uninjected control embryos (6%, n=72) (Fig. 2 A,B). It was assumed that over-expression of *XRASGRP2* influences cardiovascular development. Whole-mount *in situ* hybridization revealed that the expression of *globin T3* was suppressed in the VBI (Fig. 2D), and that ectopic expression of *Xmsr* was induced (Fig. 2F) in the *XRASGRP2*-injected embryos (Fig. 2 C,E). Xmsr, which is the

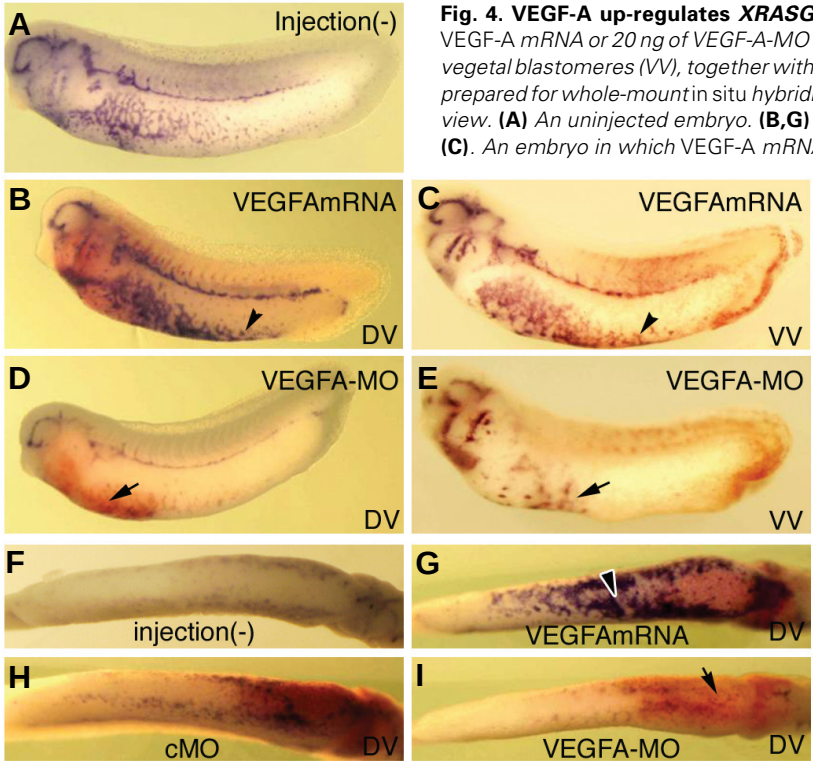


Fig. 4. VEGF-A up-regulates *XRASGRP2* expression. *Embryos were injected with either 1 ng of* VEGF-A *mRNA or 20 ng of VEGF-A-MO into the two dorsal-ventral blastomeres (DV) or the two ventral-vegetal blastomeres (VV), together with 200 pg of* β-gal *mRNA, at the 8-cell-stage. These embryos were prepared for whole-mount* in situ *hybridization of* XRASGRP2 *at stage 32. (A-E) Lateral view. (F-I) Ventral view.* **(A)** *An uninjected embryo.* **(B,G)** *An embryo in which* VEGF-A *mRNA was injected into the DV.* **(C)**. *An embryo in which* VEGF-A *mRNA was injected into the VV.* **(D,I)** *An embryo in which VEGF-A-MO was injected into the DV.* **(E)** *An embryo in which VEGF-A-MO was injected into the VV.* **(F)** *An uninjected embryo.* **(H)** *An embryo in which the control MO (20 ng) was injected into the DV. Black arrows indicate inhibition of* XRASGRP2 *expression in the VVN (D,E) and VBI (I). Black arrowheads indicate ectopic expression of* XRASGRP2 *in the VVN (B,C) and VBI (G).*

Xenopus homolog of the G-protein-coupled receptor APJ, functions as the apelin receptor. *Xmsr* is expressed by endothelial cells (Devic *et al.*, 1996; Inui *et al.*, 2006). These results indicate that the over-expression of *XRASGRP2* alters vascular and hematopoietic cell fates, leading to the replacement of blood cells with endothelial cells.

Knockdown of XRASGRP2 *disrupts vascular development*

The antisense XRASGRP2-MOs (aS-MO and bS-MO) were designed to block splicing at the first exon/intron boundaries (Fig. 3A, see *Materials and Methods*). These MOs inhibit the normal splicing of *XRASGRP2* pre-mRNA, resulting in the production of a truncated protein that lacks the functional domain. RT-PCR analysis revealed that the level of the normally spliced transcript (312-bp band) was reduced in aS-MO- or bS-MO-injected embryos (Fig. 3B, lanes aS and bS). Normally spliced transcripts were not detected in the aS-MO and bS-MO co-injected embryos (Fig. 3B, lane S). These results indicate that aS-MO and bS-MO effectively inhibit *XRASGRP2* gene splicing and production of the intact protein. To investigate the role of *XRASGRP2* in normal vascular development, a mixture of XRASGRP2-MOs (S-MO) was injected into one side of the 2-cell-stage embryos. The embryos were fixed at stage 31, to analyze the expression levels of the vascular-specific marker genes *Xflk-1*, *Xmsr*, *Xtie2*, and *Ami* (Fig. 3 C-F). The expression levels of *Xflk-1* and *Xmsr* were reduced in the PCV and ISV, respectively (Fig. 3 C,D, arrows), and the expression levels of *Xtie2* and *Ami* were suppressed in the PCV and VVN (Fig. 3 E,F, arrows and arrowheads) of the S-MO-injected sides. The suppression of *Ami* expression at the S-MO-injected side was gradually mitigated as the embryos developed (Fig. 3G). The S-MO-injected embryo showed no significant changes in *globin T3* expression under the conditions used in the present study (Fig. 3H).

VEGF-A signaling regulates the expression of XRASGRP2 *and acts through XRASGRP2 in vascular development*

VEGF-A is a key factor in vasculogenesis and induces endothelial gene expression. A functional analysis of VEGF in *Xenopus* embryos was performed by injecting *VEGF-A* mRNA or VEGF-A-MO into dorsal-vegetal blastomeres or ventral-vegetal blastomeres at the 8-cell stage (Koibuchi *et al.*, 2006). We then examined the impact of signaling upstream of *XRASGRP2* on vascular development. *VEGF-A* mRNA or VEGF-A-MO was injected into dorsal-vegetal blastomeres or ventral-vegetal blastomeres, together with *β-gal* mRNA, at the 8-cell

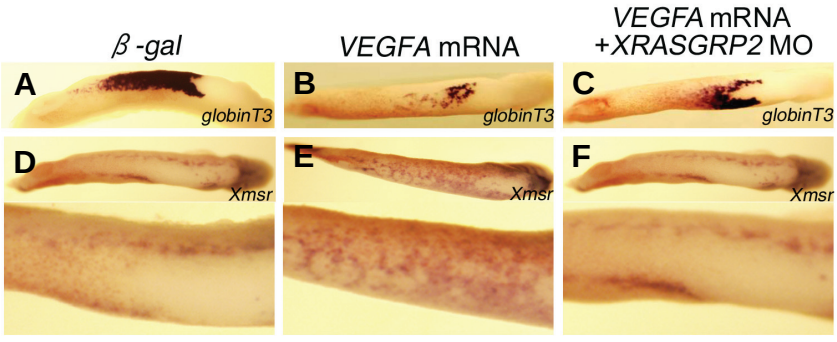


Fig. 5. RasGRP2 mediates VEGF-A signaling. *Embryos were injected with 200 pg of* β-gal *mRNA (A,D), 1 ng of* VEGF-A *mRNA (B,E) or 1 ng of* VEGF-A *mRNA plus 40 ng of XRASGRP2 S-MO (C,F) into two ventral-vegetal blastomeres at the 8-cell stage. The embryos were cultured until stage 31, for whole-mount* in situ *hybridization analysis.* **(A-C)** *Expression patterns of* globin T3. **(D-F)** *Expression patterns of* Xmsr. VEGF-A *inhibits* globin T3 *expression in the VBI. Higher-magnification images showing the* Xmsr *expression patterns in the VBI region are shown (lower panels). (B).* VEGF-A*-mediated suppression of* globin T3 *expression is partially rescued by co-injection of the* XRASGRP2 *S-MO (C).* VEGF-A *induces ectopic* Xmsr *expression in the VBI (E).* VEGF-A*-induced ectopic expression of* Xmsr *is partially rescued by co-injection of the* XRASGRP2 *S-MO (F).*

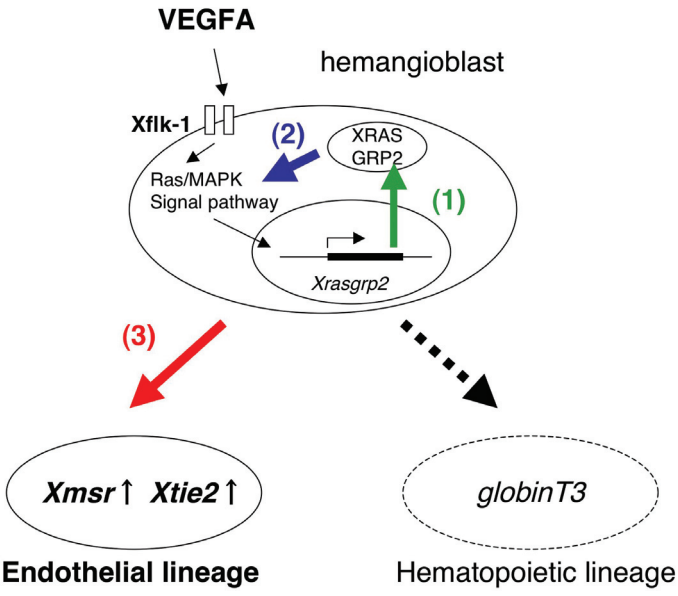


Fig. 6. A model for *XRASGRP2* function in hemangioblast cells. (1) *The expression of* XRASGRP2 *is induced by VEGF-A in hemangioblast cells.* **(2)** *XRASGRP2 reinforces the VEGF-A/Ras signal pathway.* **(3)** *The stimulated hemangioblast cells differentiate to endothelial cells that express* Xmsr *and* Xtie2. *In this case, differentiation to the hematopoietic lineage is suppressed.*

stage. The number of *XRASGRP2*-positive cells increased in the area that was injected with *VEGF-A* mRNA (Fig. 4 B,C,G). In contrast, *XRASGRP2* expression was reduced in the VVN at stage 32 in the VEGF-A-MO-injected embryos (Fig. 4 D,E,I, arrow). These results indicate that VEGF-A induces *XRASGRP2* expression, and that VEGF-A is required for *XRASGRP2* expression. In the *VEGF-A* mRNA-injected embryos, ectopic induction of endothelial cells that expressed *Xmsr* and inhibition of *globin T3* expression were observed (Fig. 5 B,E). When *VEGF-A* mRNA and the *XRASGRP2* S-MO were co-injected, the ectopic expression of *Xmsr* was decreased (Fig. 5 E,F) and the expression of *globin T3* was partially rescued (Fig. 5 B,C). These results suggest that VEGF signaling acts through XRASGRP2 in vascular development.

Discussion

In humans, RasGRP2 has been identified as a critical regulator of inside-out integrin activation in T lymphocytes, neutrophils, and platelets (Pasvolsky *et al.*, 2007). In adult rodents, *RasGRP2* is expressed in platelets, megakaryocytes, and neutrophils within the hematopoietic system, as well as in neurons, especially in the striatum of the basal ganglia (Crittenden *et al.*, 2004; Kawasaki *et al.*, 1998). The expression of *XRASGRP2* in the developing vascular system was reported in *Xenopus* (Nagamine *et al.*, 2008). This *XRASGRP2* expression coincided with that of *Xflk-1* and *Xmsr*, and occurred earlier than the expression of *Ami*. *XRASGRP2* expression was found to be transient in the developing vascular regions (Fig. 1) (Cleaver *et al.*, 1997; Devic *et al.*, 1996; Inui and Asashima, 2006). These results indicate that *XRASGRP2* plays a role in the early phase of vasculogenesis.

In the present study, the edema phenotype was observed for both the up-regulation and down-regulation of *XRASGRP2*. It was reported that *VEGF-A* overexpression induced edema (Koibuchi *et al.*, 2006). *VEGF-A* overexpression led to the formation of ectopic blood vessels and reduced blood circulation. In contrast, *c-myc*-knockdown embryos showed decreased vessel formation and had the edema phenotype (Rodrigues *et al.*, 2008). These results indicate that an appropriate level and precise timing of blood vessel formation are required for normal development.

The *XRASGRP2*-knockdown embryos showed the edema phenotype, which may be due to circulation problems (data not shown) and inhibition of endothelial gene expression (Fig. 3 C-F). This outcome indicates that *XRASGRP2* is necessary for endothelial differentiation. However, the delayed vessel formation observed on the *XRASGRP2*-depleted side was gradually mitigated (Fig. 3G). This indicates that some other molecules partly compensate for the lack of XRASGRP2 function. It has been reported that *RasGRP3* is expressed in endothelial cells in the developing mouse embryo, although a loss-of-function mutation in *RasGRP3* did not affect mouse embryo viability (Roberts *et al.*, 2004). Genetic ablation of *Sos* in the mouse resulted in death at mid-gestation, with evidence of cardiovascular and yolk sac defects (Qian *et al.*, 2000; Wang *et al.*, 1997). These orthologs are candidates for the factors that compensate for the loss of XRASGRP2 function in *Xenopus* vasculogenesis.

VEGF plays a central role in vascular development (Ferrara *et al.*, 2003). VEGF-A signaling is necessary and sufficient for promoting early endothelial differentiation in *Xenopus*. Overexpression of *VEGF-A* caused ectopic expression of *XRASGRP2* (Fig. 4 B,C,G), similar to that of *Xmsr* and *Xtie2* (Fig. 5, Koibuchi *et al.*, 2006). In addition, XRASGRP2 expression was suppressed in VEGF-A-disrupted embryos (Fig. 4). The over-expression of *XRASGRP2* resulted in ectopic expression of *Xmsr*, similar to the over-expression of *VEGF-A* (Figs. 2 and 4). These results indicate that VEGF-A is the endogenous upstream factor of *XRASGRP2* in *Xenopus* endothelial cell differentiation.

The XRASGRP2 S-MO inhibited the ectopic expression of the genes induced by VEGF-A over-expression (Fig. 5), which suggests that XRASGRP2 is necessary for VEGF-A to induce endothelial cell differentiation. Therefore, the expression of *XRASGRP2* is regulated by VEGF-A signaling, and the induced XRASGRP2 facilitates or maintains VEGF-A signaling for endothelial cell differentiation. We propose a model for the function of RASGRP2 in vasculogenesis (Fig. 6). In hemangioblasts, the VEGF signal induces *XRASGRP2* expression. XRASGRP2 directs the cell fate towards the endothelial lineage. Since RasGRP is an activator of members of the small GTPase family, such as Ras and Rap1, these molecules are candidate targets of RASGRP2 in endothelial differentiation. Further studies are required to elucidate the role of XRASGRP2 in VEGF signal transduction and to identify the target molecule of XRASGRP2 in vasculogenesis.

Materials and Methods

Plasmid constructs

The following constructs were generated for *in vitro* RNA synthesis: pCS2P-*XRASGRP2*, which contains the ORF of *Xenopus RASGRP2*; and pCS2-*VEGF-A*, which contains the ORF of *Xenopus VEGF-Ab* (isoform 4, VEGF168, DQ481238). The plasmids were generated by PCR amplification using the Phusion High Fidelity PCR Kit (Finnzymes, Fin-

land) followed by subcloning into the pCS2+ vector (Turner and Weintraub, 1994).

Morpholino oligonucleotide design and validation

We obtained the sequences of the *XRASGRP2a* and *XRASGRP2b* genes of *X. laevis* (corresponding to the pseudo-tetraploid genome) from the database. Through prediction using the *Xenopus tropicalis* genomic sequence, the following common primers for exons 1 and 3 of *XRASGRP2a* and *XRASGRP2b* were generated:
forward, 5'-CTGATCTTGATAAGGGTCTCACCA-3';
reverse, 5'-CTGTTTCCTTTGTTCTCCAG-3'. DNA fragments that encompassed intron 1, exon 2, and intron 2 were amplified from *X. laevis* genomic DNA, and then sequenced. The following XRASGRP2 antisense morpholino oligonucleotides (MOs) were designed based on the boundary between exon 1 and intron 1: XRASGRP2a splice inhibition MO (aS-MO), 5'-CAGAACTTTAGAAGCCTTACCAAAG-3'; and XRASGRP2b splice inhibition MO (bS-MO), 5'-AGAAATTTAGAACCCATACCGAAGC-3'. The MOs were obtained from Gene Tools LLC. The effects of the MOs were confirmed by RT-PCR using the above-mentioned *XRASGRP2* common primers. VEGF-A-MO has been described previously (Kalin *et al.*, 2007).

Embryos and microinjection

Embryonic stage was determined according to the scheme of Nieuwkoop and Faber (Nieuwkoop and Faber, 1994). The jelly coat was removed with Steinberg's solution that contained 4% cysteine hydrochloride (pH 8.0). Microinjection was carried out according to the previously described method (Chan *et al.*, 2000). The *β-galactosidase (β-gal)* mRNA was used as a lineage tracer. The *β-gal*-injected embryos were processed for Red-Gal staining (Research Organics), to reveal β-galactosidase activity.

Whole-mount in situ *hybridization*

Whole-mount *in situ* hybridization was performed as previously described (Harland, 1991; Abe *et al.*, 2004). Digoxigenin-labeled probes were synthesized from linearized plasmids that encode *Xflk-1* (Cleaver *et al.*, 1997), *Xmsr* (Devic *et al.*, 1996), *Xtie2* (Iraha *et al.*, 2002), *Ami* (Inui and Asashima., 2006), *XRASGRP2*, and *globin T3* (Banville and Williams, 1985).

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