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SRG3, a core component of mouse SWI/SNF complex, is essential for extra-embryonic vascular development

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Abstract

The SWI/SNF chromatin-remodeling complex functions as a transcriptional regulator and plays a significant role in cell proliferation, differentiation and embryonic development. SRG3, a homologue of human BAF155, is a core component of the mouse SWI/SNF chromatin remodeling complex. Mutant mice deficient in *Srg3* expression are peri-implantation lethal. To investigate the role of SRG3 in the post-implantation stage, we generated SRG3 transgene-rescued (*Srg3*−/−Tg+) embryos by inducing exogenous gene expression. These *Srg3*−/−Tg+ embryos overcame early embryonic lethality and extended the life span until mid-gestation. However, the embryos displayed significant defects in blood vessel formation and fetal circulation within the yolk sac around embryonic day 10.5, which led to developmental retardation and death. We found that SRG3 expression was absent in the visceral endoderm of *Srg3*−/−Tg+ yolk sacs, while SRG3 was normally expressed in wild-type embryos. In addition, expression of angiogenesis-related genes, including *Angiopoietin1*, *Tie2*, *EphrinB2*, *Ihh* and *Notch1*, was markedly reduced in *Srg3*−/−Tg+ yolk sacs. During normal angiogenesis, maturation of the visceral endoderm development is observed in the yolk sac. However, in *Srg3*−/−Tg+ yolk sacs, the visceral endoderm did not develop normally. Our results indicate that SRG3 is required for angiogenesis and visceral endoderm development in the yolk sac.
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Keywords: SRG3; SWI/SNF complex; Transgene-rescue; Angiogenesis; Yolk sac; Blood vessel; Visceral endoderm

Introduction

The SWI/SNF chromatin-remodeling complex is involved in gene activation and repression by regulating the accessibility of transcriptional machineries to DNA (Kingston and Narlikar, 1999). Since the complex functions as a transcriptional regulator, it is presumed to have a significant role in cell proliferation, differentiation and embryonic development (Bultman et al., 2000; Kim et al., 2001; Muchardt and Yaniv, 2001). The SWI/SNF complex was originally identified in the yeast. Its core subunits, the main components of the minimum-catalytic complex that possess chromatin-remodeling activity, are well conserved from yeast to mammals (Imbalzano, 1998; Sudarsanam and Winston, 2000). BRG1 (brama-related gene 1) or hBRM, SRG3/BAF155 and SNF5/INI1 (intergrase interactor 1) are the core members of the complex (Phelan et al., 1999). SRG3 serves as a scaffold, controlling the stability of the other major components of the complex (Sohn et al., 2007).

Previous studies have shown that SRG3 is essential for embryonic viability. Null mutant mice for SRG3 die during the time of embryonic implantation. Some heterozygous mutant embryos show defects in brain development (Kim et al., 2001). These characteristics are similar to the phenotype of embryos lacking *Brg1* (Bultman et al., 2000). Recent studies using targeted mutation of core subunits in the mouse have extended the understanding of the roles of the mammalian SWI/SNF complex *in vivo* (Bultman et al., 2000; Guidi et al., 2001; Kim

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et al., 2001; Klochendler-Yeivin et al., 2000). Mice with null mutations of the core subunits were peri-implantation lethal and failed to form the inner cell mass from blastocysts *in vitro* (Bultman et al., 2000; Kim et al., 2001; Klochendler-Yeivin et al., 2000).

To overcome the barrier of early lethality, various hypomorphic methods have been used, including conditional knockout of the cre-loxP system (Klochendler-Yeivin et al., 2006) or partial loss-of-function, such as an *N*-ethyl-*N*-nitrosourea (ENU)-induced mutation (Bultman et al., 2005). These methods demonstrated that the SWI/SNF complex is involved in various events during embryonic development (Bultman et al., 2005; Klochendler-Yeivin et al., 2006). However, the function of the SWI/SNF complex in yolk sac development and in blood vessel formation, which occurs in the yolk sac during embryonic development, has not been elucidated.

Embryonic blood vessel formation has two main steps: vasculogenesis and angiogenesis. Embryonic vessel development begins as angioblast formation in the yolk sac at 7.0–7.5 days post conception (dpc) (Conway et al., 2001; Neufeld et al., 1999; Patan, 2000). This leads to the formation of the vascular plexus, via vasculogenesis. In vasculogenesis, *vascular endothelial growth factor* (*VEGF*) promotes the differentiation of angioblasts into endothelial cells in mesoderm adjacent to visceral endoderm. VEGF binds to two tyrosine kinase receptors, KDR and Flt1. KDR is a mouse homolog of Flk1 and is related to vasculogenesis and blood island formation (Shalaby et al., 1995, 1997). Flt1 is also important to blood island formation, but is not involved in endothelial cell formation (Fong et al., 1995). Null mutant mice of these receptors die in an early embryonic development stage. However, mutant mice lacking VEGF in a single allele are lethal at 9 dpc and show severe defects in vasculogenesis. This indicates that the concentration of VEGF is important to blood vessel formation (Ferrara et al., 1996).

Angiogenesis involves the maturation and expansion of the primitive vascular network by remodeling and sprouting branches from pre-existing vessels. Angiopoietin1 plays a key role in this step. This soluble ligand phosphorylates the endothelial tyrosine kinase receptor Tie2. Mutant mice lacking these signaling partners show defects in vascular remodeling at mid-gestation (Gale and Yancopoulos, 1999; Suri et al., 1996).

Visceral endoderm plays an important role in nutrient uptake and delivery. The visceral endoderm affects mouse development by the production and secretion of various signaling molecules throughout development (Bielinska et al., 1999). The yolk sac visceral endoderm, a terminal station of visceral endoderm differentiation, is formed as the proximal visceral endoderm migrates onto the extra-embryonic mesoderm cell layer. Yolk sac visceral endoderm takes charge in the process of nutrient and waste exchange until 10 dpc (Cross et al., 1994). The most important role of the yolk sac visceral endoderm is to enhance the formation of blood islands and the yolk sac blood vessel network (Bielinska et al., 1996; Boucher and Pedersen, 1996; Palis et al., 1995). As vasculogenesis commences in the yolk sac and embryonic circulation begins, the function of exchanging nutrients and gases is transferred from the visceral endoderm to the vasculature. Studies of the visceral endoderm in chicken and mouse embryos demonstrate that visceral endoderm plays an important role in induction of blood and vessel precursors (Belaoussoff et al., 1998; Miura and Wilt, 1969).

We investigated the role of a SWI/SNF core component, SRG3, during vascular development and visceral endoderm maturation in the yolk sac. We focused our study of vascular development on the yolk sac because it is the organ in which the first blood vessels form and is the final destination of visceral endoderm development. We have generated a SRG3 transgene-rescued (*Srg3*−/−Tg+) mouse by inducing an exogenous transgene into a null mutant embryo. We examined the role of SRG3 in vascular development and visceral endoderm maturation during yolk sac development. In this study, we found that SRG3 is absent in the *Srg3*−/−Tg+ yolk sac visceral endoderm, while SRG3 is present in the wild-type yolk sac. Our data reveal for the first time that SRG3 is required for maturation of the primitive endothelial plexus to rebuild into a functional vascular system. However, SRG3 is not required for the process of vasculogenesis. In addition, our results suggest that SRG3 also plays an essential role in development of the yolk sac visceral endoderm.

Materials and methods

Generation of SRG3 transgene-rescued (Srg3−/−Tg+) embryos

Generation of SRG3 transgenic mice was described previously (Lee et al., 2007). SRG3 transgene-rescued (*Srg3*−/−Tg+) embryos were generated using the following steps. First, double transgenic (*Srg3*+/−Tg+) mice were produced by crossing SRG3 transgenic mice (Tg+) with SRG3 heterozygous mutant mice (*Srg3*+/−). Then, these *Srg3*+/−Tg+ mice were intercrossed to generate SRG3 transgene-rescued (*Srg3*−/−Tg+) embryos. All experiments were carried out in a specific pathogen-free facility in compliance with animal policies of Seoul National University.

Genotyping of mouse embryos

Offspring were genotyped using the five-primer PCR method for selecting double transgenic (*Srg3*+/−Tg+) mice, using genomic DNA extracted from mice tails. For embryos of mid-gestational stage, genomic DNA was prepared from a small piece of yolk sac. The following transgene-specific primers were used: P1, 5′-GAC TAG ACC AAA CAT CTA CCT C-3′; and P2, 5′-GTC AAC TGA GCG ACT TGG ATC-3′. For screening of the SRG3 allele, a common 5′ primer was used (P3, 5′-ACA ACG AAA TCT GTG GAG TAG C-3′) in combination with a SRG3-specific 3′ primer (P4, 5′-GGG ATG GGT TCT GAA GAT CA-3′) and a Neo-specific 3′ primer (P5, 5′-CTA AAG CGC ATG CTC CAG AC-3′).

Blastocyst outgrowth analyses

Double transgenic (*Srg3*+/−Tg+) mice, possessing an SRG3 mutant allele and transgene constructs, were intercrossed. At 3.5 dpc, blastocysts were flushed from the uterine tract. Blastocysts were placed on a cell layer of mouse embryonic fibroblasts in embryonic stem cell medium (DMEM, 15% FBS) in a 4-well dish (Nunc, Denmark). Blastocysts were cultured at 37°C in a humid incubator infused with 5% CO_2. The fibroblast cells were treated with Mitomycin C (10 μg/ml for 2.5 h, M4287, Sigma-Aldrich, MO) for proliferation inhibition and washed for 2 h before analysis. After 5 days, explants were scored for degree of outgrowth, photographed and genotyped by PCR.

RT–PCR analysis

Total RNA was extracted from the yolk sacs and fetuses of wild-type and SRG3 transgene-rescued embryos using Trizol reagent (Invitrogen, Carlsbad, CA). RNA extracts were treated with RNAse-free DNaseI prior to reverse

transcription. Using oligo-dT and Superscript Reverse Transcriptase (Invitrogen, Carlsbad, CA) according to the manufacturer's directions, the first strand was synthesized. For real-time PCR analysis, Cybergreen (Bio-Rad, CA) was added into the reactions. The MyiQ real-time PCR detection system (Bio-Rad, CA) was used for PCR amplification. Each analysis was performed using three independent PCR amplifications for each sample. Reverse transcripts were synthesized three times from different embryos. Gapdh was used as an internal control. PCR was performed in 50 mM Tris (pH 9.0), 16 mM $(NH_4)_2SO_4$, 1.75 mM $MgCl_2$, 1 mM dNTPs and 1 U *Taq* polymerase. The cycling parameters were 94 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s for 30 cycles. Primer sequences are available upon request.

Whole-mount PECAM staining

The yolk sacs were fixed in a solution of methanol and DMSO (4:1) for 3 h at 4 °C. Then, yolk sacs were blocked in a solution containing PBS, 2% nonfat skim milk and 0.5% Triton X-100 (blocking solution) for 1 h at room temperature. The yolk sacs were incubated with PECAM1 antibody (1:200 in blocking solution, clone MEC 13.3, catalog no. 5550274; Pharmingen, CA) overnight at 4 °C, washed three times with blocking solution for 3 h, and incubated in anti-rat biotinylated antibody (1:500 in blocking solution) overnight at 4 °C. Finally, yolk sacs were incubated in peroxidase-conjugated streptavidin (ABC kit, Vector Laboratories, Burlingame, CA) for 1 h at room temperature. Peroxidase activity was detected using the DAB method.

Histology and immunohistochemistry

All specimens (whole embryo and yolk sac) were fixed in 4% paraformaldehyde and 0.05% glutaraldehyde in phosphate-buffered saline (PBS) for 2 h at 4 °C, embedded in paraffin, and serially sectioned (5 μm). Some sections were stained with routine hematoxylin–eosin, while others were prepared for immunolocalization of several specific molecules. Briefly, after three washes in PBS, deparaffinized slides were incubated in 3% H_2O_2 in methanol to quench endogenous peroxidase activity. Nonspecific binding sites were blocked with serum for 1 h, followed by overnight incubation at 4 °C with primary antibodies SRG3/BAF155 (1:100, sc-9747), BRG1 (1:80, sc-8749), VEGF (1:50, sc-507) or ANG1 (1:50, sc-6319), each purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Slides were rinsed with PBS and incubated for 30 min at room temperature in biotinylated antibody (1:200 in PBS, 1.5% serum). Following incubation with the antibody, slides were incubated for 1 h in preoxidase-conjugated streptavidin (ABC kit; Vector Laboratories, Burlingame, CA). Peroxidase activity was detected using the DAB method. Counterstaining was performed using methyl green (Sigma-Aldrich, MO). For negative controls, the primary antibody was omitted from the procedure.

Transmission electron microscopy

The yolk sacs of wild-type and transgene-rescued embryos were collected at 9.5 and 10.5 dpc, and fixed in cold fixative of 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4. After overnight fixation, the yolk sacs were postfixed in 1% OsO_4, and dehydrated in graded alcohol. Decreasing concentrations of propylene oxide and increasing EmBed-812 (Electron Microscopy Services, Fort Washington, PA) were employed. After pure fresh resin embedding and polymerization, 1-μm-thick sections were initially cut and stained with Richardson's blue. Once the yolk sac visceral endoderm layer was observed in the same section, ultra-thin sections, 60–80 nm in thickness, were made, stained with uranyl acetate and Reynold's lead citrate, and observed with transmission electron microscopy (TEM) at 80 kV using a Hitachi H7600 transmission electron microscope (Hitachi, Japan).

Results

SRG3 is essential for embryonic viability

To examine the role of SRG3 during mouse embryonic development, we designed a transgene rescue system. SRG3 transgenic mice were generated using a DNA construct expressing SRG3 cDNA, driven by the cytomegalovirus (CMV) enhancer/chicken β-actin promoter (Fig. 1A). SRG3 transgenic mice were fertile and showed a normal phenotype, except for higher levels of SRG3 expression in the region of heart (Fig. 1B). Transgenic mice were crossed with SRG3 heterozygous mutant (*Srg3*+/−) mice to obtain the SRG3 double transgenic (*Srg3*+/−Tg+) mice, which carried both the transgenic construct and SRG3 knockout allele. The *Srg3*+/−Tg+ mice were intercrossed to produce SRG3 transgene-rescued (*Srg3*−/−Tg+) mice.

In vitro implantation analysis revealed that *Srg3*−/−Tg+ blastocysts form both an inner cell mass and trophoblast. Blastocysts at 3.5 dpc were cultured on the feeder layer of Mitomycin C-treated mouse embryonic fibroblasts. Wild-type (*Srg3*+/+), transgenic (*Srg3*+/+Tg+) and SRG3 heterozygous knockout mice with (*Srg3*+/−Tg+) or without (*Srg3*+/−) the SRG3 transgene hatched normally and produced both an inner cell mass and trophoblast giant cells (Fig. 1C). All blastocysts from SRG3 homozygous knockout mice lacking the SRG3 transgene (*Srg3*−/−) failed to induce *in vitro* implantation (Fig. 1C). However, The SRG3 homozygous knockout blastocysts expressing the transgene (*Srg3*−/−Tg+) showed normal implantation and inner cell mass formation (Fig. 1C). Thus, transgene-rescued (*Srg3*−/−Tg+) mice developed normally until the formation of blastocyst and the peri-implantation stage.

Transgene-rescued (*Srg3*−/−Tg+) mouse embryos developed normally up to 8.5 dpc, after which time development became severely retarded, with embryonic developmental defects more severe. However, a large number of embryos survived up to 10.5 dpc, although they did not develop normally. These surviving *Srg3*−/−Tg+ embryos generally appeared grossly abnormal by 10.5 dpc and died by 11.5 dpc. Intriguingly, at 10.5 dpc, neither blood vessels nor the circulation of fetal blood cells were observed the yolk sacs of *Srg3*−/−Tg+ embryos (Fig. 2B).

Many other developmental defects were evident in *Srg3*−/−Tg+ embryos. These embryos were smaller, stickier and showed developmental retardation, compared with littermate controls (Fig. 2D). A heartbeat was observed in *Srg3*−/−Tg+ embryos, but its pulse rate was slow and weak (data not shown). Incoming and outgoing embryonic blood cells were not identified in the heart. Additionally, limb bud formation did not occur (Fig. 2D). Development of the head did not occur normally in *Srg3*−/−Tg+ embryos, as the neural fold did not close (Figs. 2F, H), as it did in wild-type embryos (Figs. 2E, G).

Expression of *Srg3* in the *Srg3*−/−Tg+ conceptuses was significantly lower than in the wild-type embryos (Fig. 2I). Interestingly, *Brg1* transcription levels were also significantly down-regulated in *Srg3*−/−Tg+ embryos (Fig. 2I). The reduced levels of *Srg3* and *Brg1* and the unclosed neural fold in the head of *Srg3*−/−Tg+ embryos recapitulate the defects in brain development of SRG3 and BRG1 heterozygous mutant mouse embryos (Bultman et al., 2000; Kim et al., 2001).

Vascular abnormalities in the Srg3−/−Tg+ yolk sac

The yolk sacs of *Srg3*−/−Tg+ mouse embryos showed vascular development defects. By 10.5 dpc, wild-type yolk

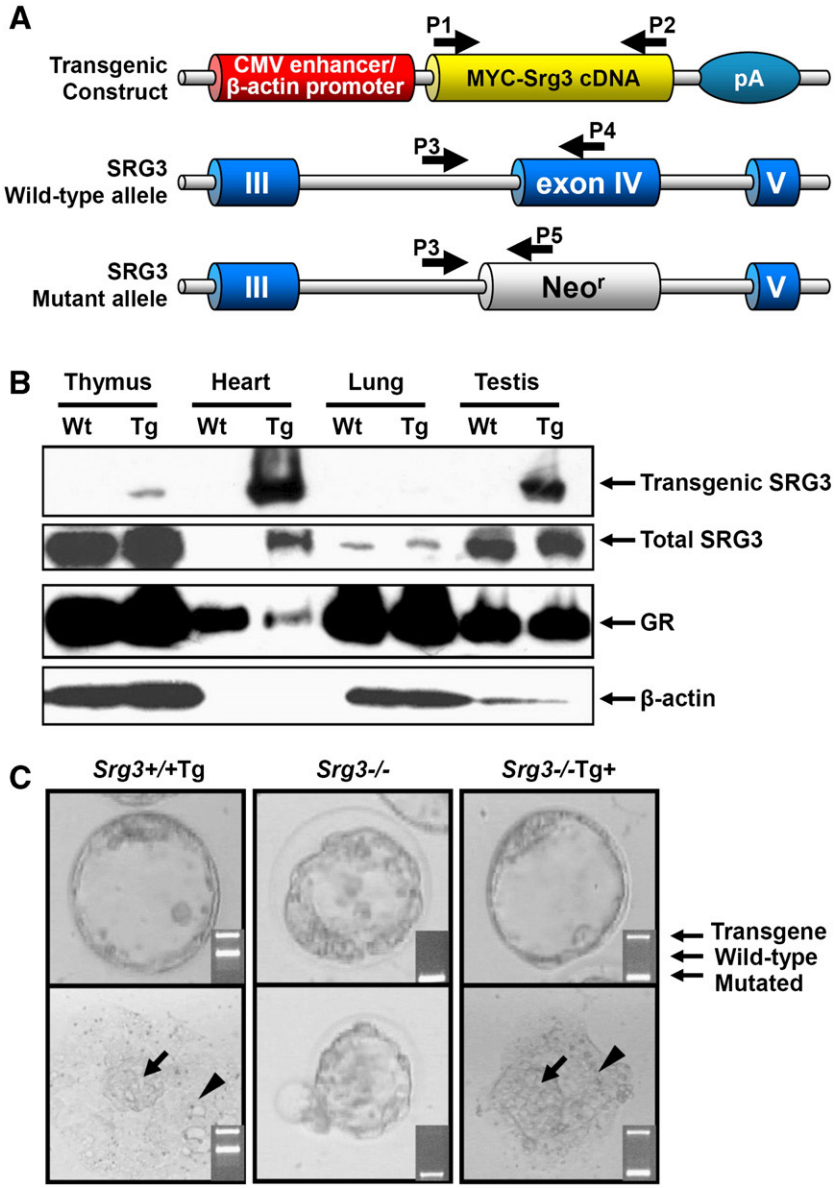

Fig. 1. Preparation of SRG3 transgenic mice. (A) Transgenic construct and schematic diagram of the *Srg3* locus and targeted allele. The blue columns represent *Srg3* exons (III–V) and the white column depicts a *neomycin resistance cassette* (*Neo*^{r}). Arrows P1–P5 indicates the position and orientation of oligomers for genotyping in (C). pA, polyadenylation sequence. Oligomer P3 is common to both wild-type and mutant alleles. (B) Western blot analysis of SRG3 expression in adult organs of both transgenic mice and littermate controls (8 weeks old). Glucocorticoid receptor (GR) and actin were used as internal controls. (C) Phase contrast view of E3.5 blastocysts from *Srg3*+/−Tg+ mice intercrosses. Genotyping results are shown on the bottom right side of each panel. The transgenic fragment (800 bp) was amplified by the P1–P2 primer pair, while the wild-type fragment (450 bp) was amplified by the P3–P4 primer pair and the mutant fragment (250 bp) was amplified by the P3–P5 primer pair. Arrow, inner cell mass; arrowhead, a single layer of trophoblast giant cells.

sacs showed an extensive and highly organized vasculature filled with blood (Figs. 2A and 3A), while the vasculature within *Srg3*−/−Tg+ yolk sacs was poorly developed (Figs. 2B and 3B). In the *Srg3*−/−Tg+ embryos, mature vasculature did not develop, *although* blood island formation was observed (Fig. 3B). These defects in vascular development led to extensive leakage of fetal blood cells or hematopoietic cells within embryos (Fig. 2D).

Histological analysis indicated that mature blood vessels were absent in the *Srg3*−/−Tg+ yolk sacs (Fig. 3B). On the other hand, blood vessels in wild-type yolk sacs were well-formed and contained hematopoietic cells (Fig. 3A). Immunohistochemical analyses showed that SRG3 expression was dramatically reduced in *Srg3*−/−Tg+ yolk sacs compared to wild-type (Figs. 3C, D). Note that SRG3 expression in the region of yolk sac visceral endoderm was totally absent (Fig. 3D, asterisk). BRG1 was detected in *Srg3*−/−Tg+ yolk sacs at significantly down-regulated levels (Fig. 3F). Semi-quantitative RT–PCR analysis confirmed that *Srg3* and *Brg1* expression in *Srg3*−/−Tg+ yolk sacs were significantly reduced as compared to wild-type yolk sacs (Fig. 3G). The lack of SRG3 in *Srg3*−/−Tg+ yolk sacs suggests a role of SRG3 in the vascular development of the yolk sac.

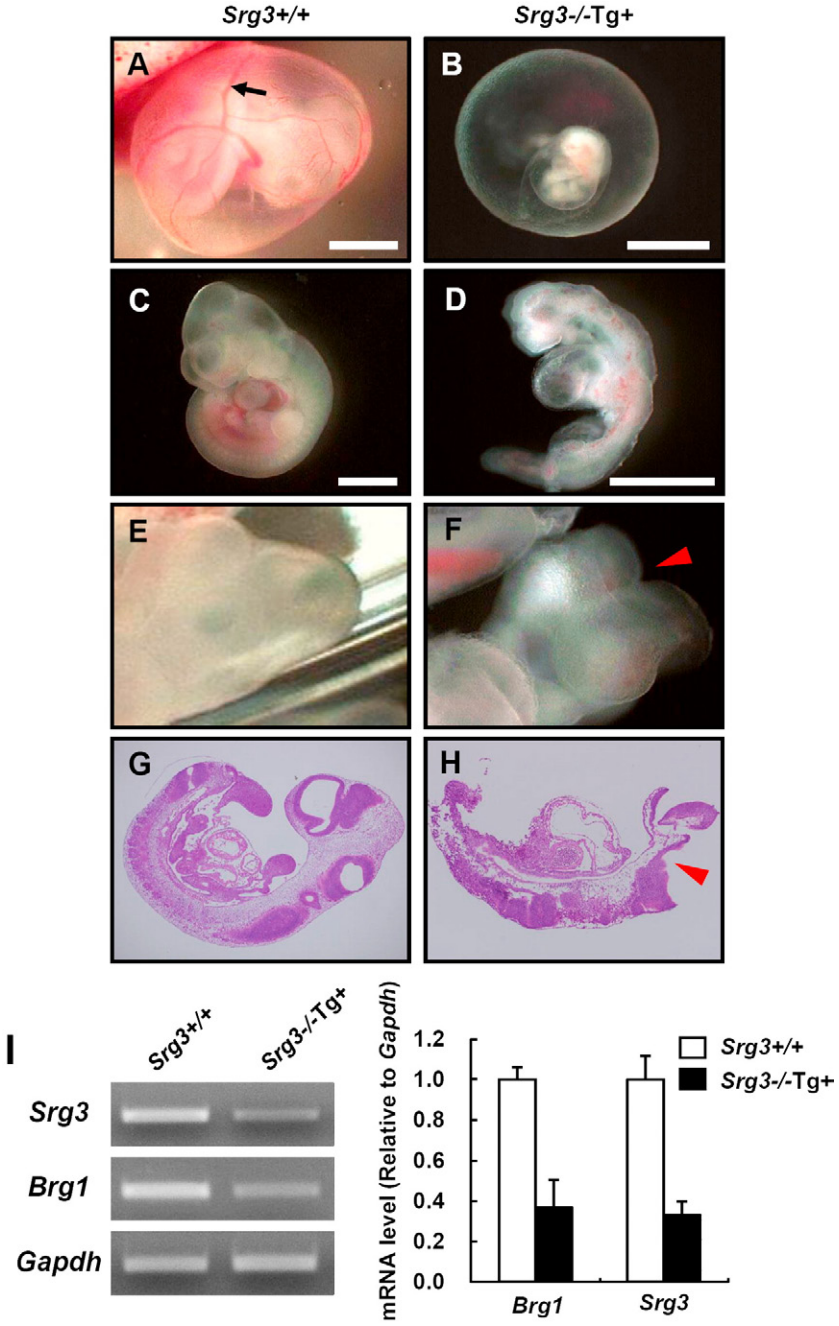

Fig. 2. Phenotype of SRG3 transgene-rescued (*Srg3*−/−Tg+) embryos. (A–H) Conceptuses of 10.5 dpc wild-type (A, C, E, G) and *Srg3*−/−Tg+ embryos (B, D, F, H). Note the presence of large collection vessels and circulation (arrow). (E) Normal developed head region of 10.5 dpc wild-type embryo. (F) A head region of 10.5 dpc *Srg3*−/−Tg+ embryo. Note that the neural fold remains unclosed (arrowhead). (G, H) Sagittal sections of 10.5 dpc wild-type and *Srg3*−/−Tg+ embryos stained by hematoxylin–eosin. The arrowhead indicates the unclosed neural fold in the head region of an *Srg3*−/−Tg+ embryo. (I) The expression level of *Srg3* and *Brg1* transcripts in the conceptuses of 10 dpc wild-type and *Srg3*−/−Tg+ embryos. *Srg3*+/+ is the littermate control for the 10.5 dpc *Srg3*−/−Tg+ embryo. Semi-quantitative RT–PCR and real-time PCR were performed with cDNA from each embryo. Error bars represent SD ($n=3$). Scale bars: 1 mm.

Lack of blood vessel remodeling in the Srg3−/−Tg+ yolk sac

During mouse embryonic development, the yolk sac is the first place where blood vessel formation occurs. Blood vessel formation occurs in two main steps: vasculogenesis and angiogenesis. Vasculogenesis leads to the beginning of vessel formation. In the yolk sac, the differentiation and aggregation of angioblasts occurs, along with the formation of a primary capillary plexus of simple endothelial cells. To observe whether primitive networks exist in the yolk sac of *Srg3*−/−Tg+ embryos, we examined whole-mount staining using PECAM1, an endothelial cell marker (Fig. 4). The presence of a primary plexus suggests that the early step of vasculogenesis commences in *Srg3*−/−Tg+ embryos. Additionally, the formation and aggregation of endothelial cells is not dependent on SRG3 expression.

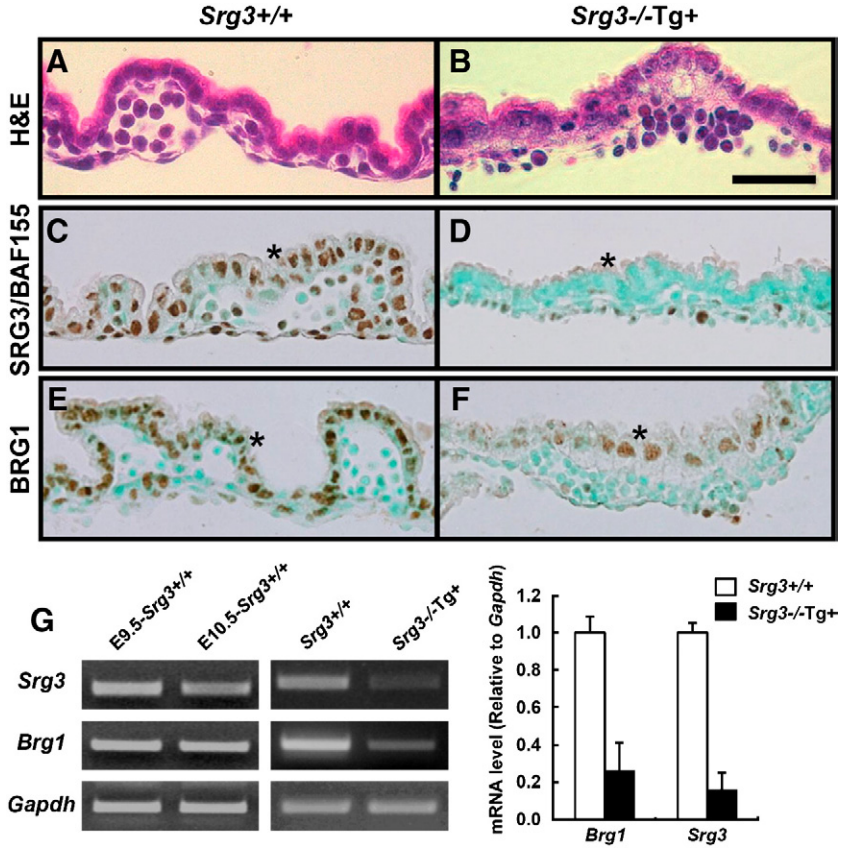

Fig. 3. Yolk sac defects in *Srg3*−/−Tg+ embryos. Histological and immunohistochemical analyses were performed in 10.5 dpc wild-type and SRG3 transgene-rescued (*Srg3*−/−Tg+) yolk sacs. (A, B) Hematoxylin–eosin staining of yolk sacs from 10.5 dpc wild-type and *Srg3*−/−Tg+ embryos. (C) Anti-SRG3/BAF155 antibody was used to stain the nuclei of yolk sacs (asterisk) in wild-type mouse embryos. In contrast, SRG3 is not detected in the yolk sacs of the *Srg3*−/−Tg+ embryos (D, asterisk). (E, F) Anti-BRG1 antibody stained the nuclei of yolk sacs (asterisk). (G) The expression level of *Srg3* and *Brg1* transcripts in yolk sacs of 9.5 dpc and 10.5 dpc wild-type and 10.5 dpc *Srg3*−/−Tg+ embryos. *Srg3*+/+ is littermate control of 10.5 dpc *Srg3*−/−Tg+ embryo. Semi-quantitative RT–PCR and real-time PCR were performed with cDNA from each yolk sac. Error bars represent SD ($n=3$). Scale bar: 50 μm.

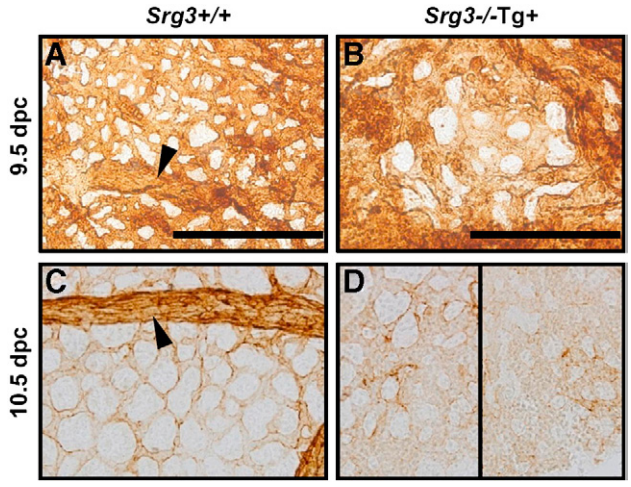

Fig. 4. Blood vessel formation in *Srg3*−/−Tg+ yolk sacs. Visualization of endothelial cells expressing PECAM1 in yolk sacs. Whole-mount immunostaining of 9.5 dpc and 10.5 dpc wild-type yolk sacs (left) and *Srg3*−/−Tg+ yolk sacs (right). Wild-type yolk sacs show large maturated vessels at both 9.5 dpc and 10.5 dpc (arrowheads). *Srg3*−/−Tg+ yolk sacs lack mature vasculature. Scale bars: 200 μm.

After the formation of the primary plexus in vasculogenesis, the process of vascular remodeling and refining occurs during angiogenensis. During angiogenesis, pre-existing vessels develop into distinguishable large and small vessels. Endothelial cells of *Srg3*−/−Tg+ yolk sacs were not developed into mature blood vessel network. Rather, they remained a primitive network showing a honeycomb-like structure (Figs. 4B, D), a feature of an immature vascular plexus. However, wild-type embryos showed extensive organized and mature vasculature, consisting of both large vessels and capillary beds (Figs. 4A, C). Deficiency in blood vessel refinement in *Srg3*−/−Tg+ yolk sacs suggests that vascular development in the *Srg3*−/−Tg+ yolk sac is arrested before angiogenesis commences.

SRG3 regulates the expression of angiogenic genes

Numerous signaling pathways are involved in regulating vasculature formation during embryonic development (Rossant and Howard, 2002). To examine how SRG3 plays a role in blood vessel development in yolk sacs, we analyzed the expression of vasculature-related genes using semi-quantitative RT–PCR (Fig. 5).

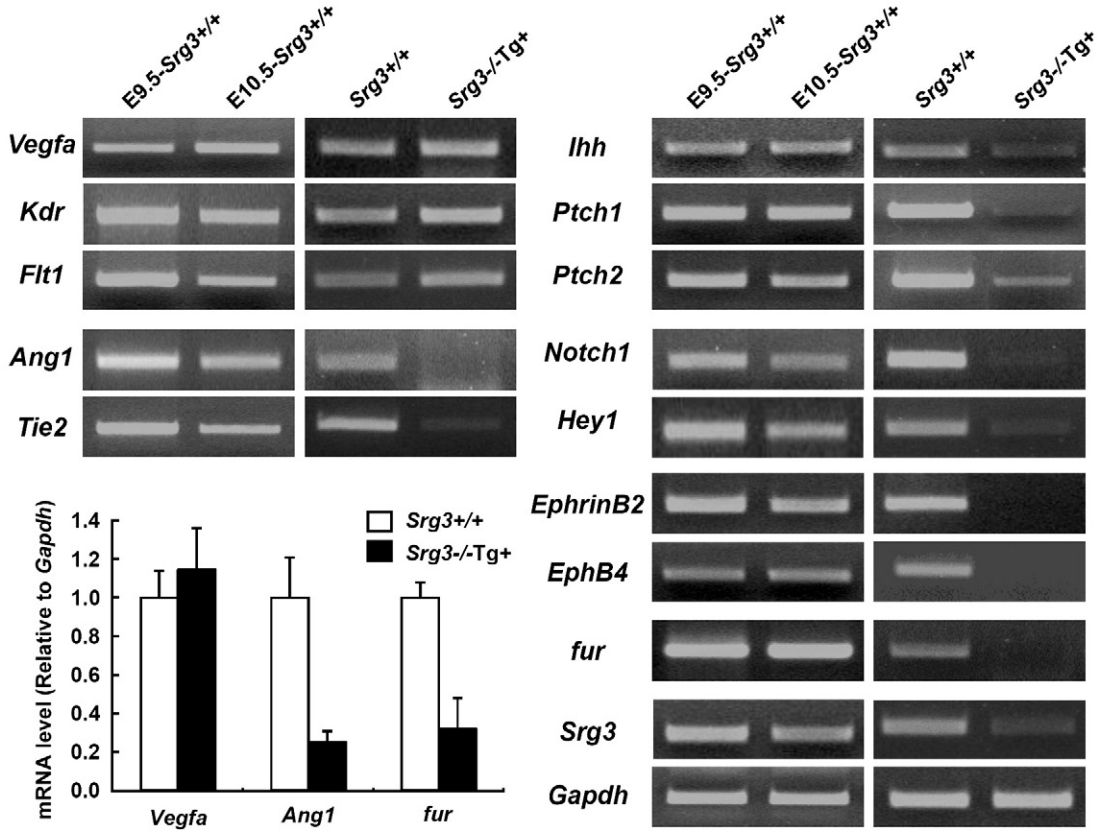

Fig. 5. RT–PCR analysis of angiogenic genes in SRG3 transgene-rescued (*Srg3*−/−Tg+) yolk sacs. cDNA was synthesized from RNA extracted from the individual yolk sacs of 10.5 dpc *Srg3*−/−Tg+ and 10.5 dpc *Srg3*+/+ littermates, along with 9.5 dpc *Srg3*+/+ embryos. Semi-quantitative RT–PCR and real-time PCR were performed with synthesized cDNA. *Vegfa* and *Ang1* and their related genes are shown on the left. Expression of *Srg3* is shown on the bottom right side. *Ang1*, *EphrinB2*, *Ihh*, and their receptors and *Notch1* and *Hey1* were down-regulated in *Srg3*−/−Tg+ yolk sacs. *Gapdh* was used to normalize each sample. Error bars represent SD ($n=3$).

We first focused on the main vasculature-related growth factor genes, vascular endothelial growth factor (VEGF) and Angiopietin1 (ANG1). While VEGF plays an essential role in the formation of new vessels, ANG1 is involved in the maturation and remodeling of the primary plexus. The expression of *Vegf* did not notably change in *Srg3*−/−Tg+ yolk sacs, while *Ang1* expression was considerably down-regulated (Fig. 5, left).

Hypoxia is also known to affect the regulation of these factors by inducing hypoxia-inducible factor 1α (Hif1α) (Iyer et al., 1998). Thus, we inspected whether the hypoxia condition is changed in *Srg3*−/−Tg+ embryos by checking the Hif1α expression level. Consequently, there was no significant change in Hif1α expression compared to the wild-type (data not shown), indicating that hypoxia condition did not affect *Srg3*−/−Tg+ embryos.

Vascular development is regulated through interactions between soluble vasculature-related factors and their receptors. Therefore, we examined the expression of the VEGF receptors KDR and Flt1. KDR is a mouse homologue of Flk1, while Flt1 is the earliest marker of the developing endothelial lineage. We also examined the expression of the ANG1 receptor Tie2, which is expressed in endothelial cells. The expression of *Kdr* and *Flt1* in the *Srg3*−/−Tg+ yolk sacs was not significantly different from controls. However, the expression of *Tie2* was down-regulated in *Srg3*−/−Tg+ yolk sacs (Fig. 5, left). These data implicate a role of SRG3 in angiogenesis, but not vasculogenesis, during blood vessel development in the yolk sacs.

Next, we examined other vasculature-related signaling pathways essential for vascular remodeling in the yolk sac, including Hedgehog, Eph/ephrin and Notch signaling. Deficiency in the members of these pathways leads to severe defects in blood vessel remodeling in yolk sacs during mouse embryonic development (Adams, 2002; Byrd et al., 2002; Krebs et al., 2000). Such deficiencies are morphologically very similar to *Srg3*−/−Tg+ embryos. In *Srg3*−/−Tg+ yolk sacs, transcript levels of both Ihh (Indian hedgehog) and its receptor *Ptch1/2* were down-regulated (Fig. 5, right). *Notch1* and its target molecule, *Hey1*, were reduced in *Srg3*−/−Tg+ yolk sacs (Fig. 5, right). Moreover, both *ephrinB2* and *EphB4*, a key ligand and receptor of Eph/ephrin pathway, were also down-regulated in the *Srg3*−/−Tg+ yolk sac (Fig. 5, right).

Another important molecule that plays an essential role in yolk sac vascular development is Furin. Deficiency of the Furin gene (*fur*) leads to early embryo lethality and shows disorganized vasculature in the yolk sac (Roebroek et al., 1998). Semi-quantitative RT–PCR analysis showed that *fur* was significantly down-regulated in the *Srg3*−/−Tg+ yolk sac compared with wild-type yolk sacs (Fig. 5, right). Our results indicate that the expression of most angiogenesis-related genes, but not vasculature-related genes, are dependent on the expression of

SRG3, strongly supporting the hypothesis that SRG3 is required for angiogenesis during blood vessel development in yolk sacs.

Immunohistochemical analyses of yolk sacs in 10.5 dpc wild-type and *Srg3*−/−Tg+ embryos were performed using anti-ANG1 and anti-VEGF antibodies. We confirmed that ANG1 protein levels were down-regulated in *Srg3*−/−Tg+ embryos. In *Srg3*−/−Tg+ yolk sacs, the expression level of ANG1 significantly decreased, while the VEGF expression did not change (Figs. 6B, D).

Aberrant visceral endoderm development in the Srg3−/−Tg+ yolk sac

The visceral endoderm has essential roles during mouse embryonic development, such as nutrient uptake, nutrient transport and guiding the early development of the embryo. The yolk sac is the final destination in visceral endoderm development; the yolk sac visceral endoderm affects embryonic development until birth (Bielinska et al., 1999; Cross et al., 1994). A number of signaling molecules released from the visceral endoderm of the yolk sac regulate the formation of blood islands and the differentiation into hematopoietic and endothelial cells. Electron microscopy analysis showed that distorted microvilli and excessive amount of vesicles and vacuoles were present in the visceral endoderm of *Srg3*−/−Tg+ yolk sacs (Figs. 7A–C). The absence of SRG3 expression in the yolk sac visceral endoderm region of *Srg3*−/−Tg+ embryos suggests that SRG3 plays a role in yolk sac visceral endoderm development.

GATA-4 and GATA-6, two zinc finger transcription factors, induce various genes required for early embryonic development. Failure of induction of these genes leads to severe defects in visceral endoderm formation (Bielinska and Wilson, 1997; Soudais et al., 1995). In the *Srg3*−/−Tg+ yolk sac, the expression level of GATA-4 was reduced by a small amount, while there was no significant change in GATA-6 expression (Fig. 7D). Interestingly, reduction in the expression of Furin, an important molecule for visceral endoderm development (Bielinska et al., 1999), indicates that SRG3 affects the maturation of yolk sac visceral endoderm development (Fig. 7D).

Discussion

Since SRG3 null mutant mice die at the implantation stage, the role of SRG3 during development has not been determined. In this study, we investigated the role of SRG3 using a transgene-rescue strategy. The peri-implantation lethality of the SRG3 null mutant (*Srg3*−/−) embryos was overcome by exogenous expression of the SRG3 transgene driven by the chicken β-actin promoter. The transgene recovered the loss of SRG3 expression partially. Therefore, the transgene-rescued (*Srg3*−/−Tg+) embryo showed defects in both extra-embryonic tissues including the yolk sac and retardation in the developing embryo. The arrest of growth and development in the embryo suggests that SRG3 is essential for developmental events.

We determined that the yolk sac is a region where SRG3 is normally expressed. SRG3 is expressed in the yolk sac visceral endoderm throughout development. This region is severely affected by the absence of SRG3. The Srg3−/−Tg+ embryo showed defects in the visceral endoderm development of the yolk sac, indicating that SRG3 is required for formation and maturation of the yolk sac visceral endoderm. A major defect in *Srg3*−/−Tg+ yolk sacs is an arrest in vascular development, which results in a failure of blood to circulate. The yolk sac is the first organ in which hematopoiesis and angiogenesis occur during embryonic development. Severe defects in the yolk sacs led to developmental failure in *Srg3*−/−Tg+ embryos.

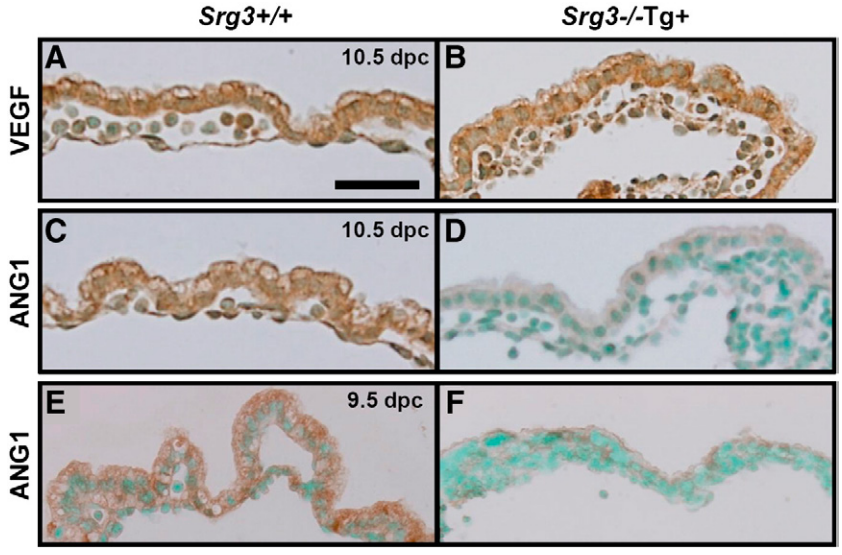

Fig. 6. VEGF and Angiopoietin1 (ANG1) expression in yolk sacs. Sections of yolk sacs from 9.5 dpc and 10.5 dpc wild-type and SRG3 transgene-rescued (*Srg3*−/−Tg+) embryos were stained with each antibody. (A, B) VEGF expression did not significantly change in 10.5 dpc *Srg3*−/−Tg+ yolk sacs. (C, D) ANG1 expression was remarkably reduced in 10.5 dpc *Srg3*−/−Tg+ yolk sacs. (E, F) ANG1 expression was also reduced in 9.5 dpc *Srg3*−/−Tg+ yolk sacs. Scale bar: 50 μm.

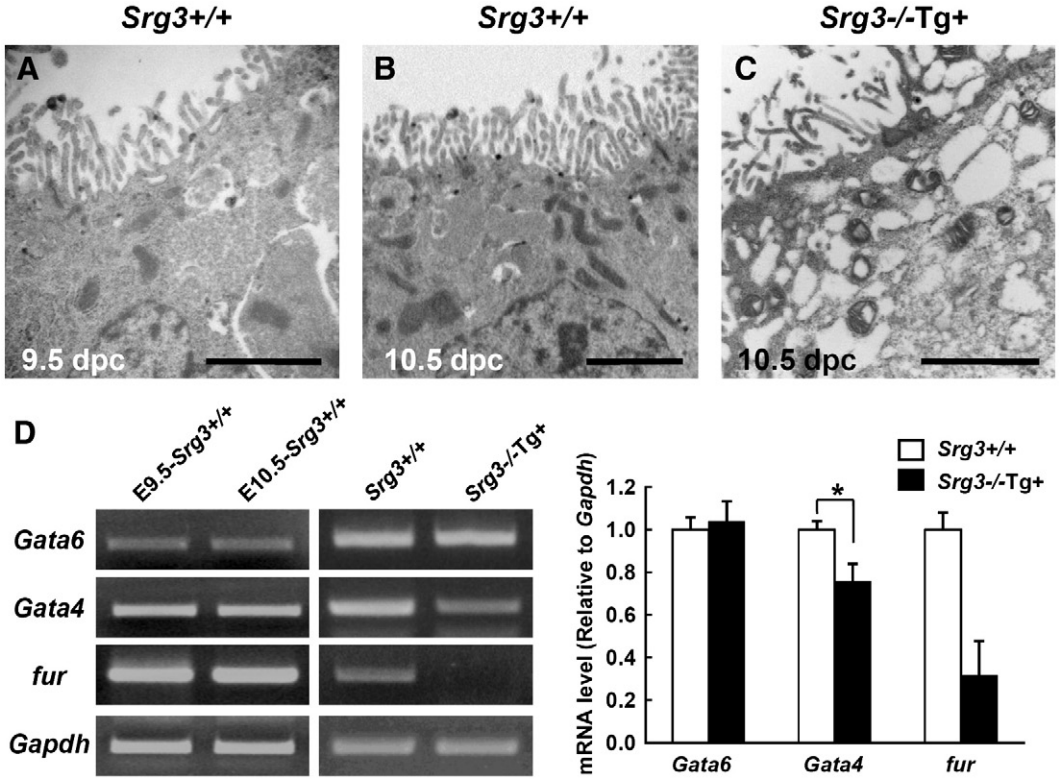

Fig. 7. Impairment in visceral endoderm development of the *Srg3*−/−Tg+ yolk sac. (A–C) Electron microscopy of the visceral endoderm region in 9.5 dpc (A) and 10.5 dpc wild-type yolk sacs (B), and 10.5 dpc *Srg3*−/−Tg+ yolk sacs (C). Note the normal arrangement of microvilli and mature visceral endoderm cells in both 9.5 dpc and 10.5 dpc wild-type yolk sacs (A, B). However, distortion of microvilli and numerous vesicles and vacuoles were present in the visceral endoderm cells of *Srg3*−/−Tg+ yolk sacs (C). (D) The expression level of visceral endoderm development-related genes. Semi-quantitative RT–PCR and real-time PCR were performed with cDNA from each yolk sac of 9.5 dpc and 10.5 dpc wild-type and 10.5 dpc *Srg3*−/−Tg+ embryos. *Srg3*+/+ served as a littermate control for 10.5 dpc *Srg3*−/−Tg+ embryos. In the yolk sac of *Srg3*−/−Tg+ embryos, *Gata4* was slightly down-regulated, but *Gata6* was not changed significantly. Error bars represent SD (n=3). *P<0.05 (Student's t test). Scale bar: 2 μm.

SRG3 expression in the murine yolk sac

Several studies have demonstrated the importance of the SWI/SNF chromatin remodeling complex during mouse embryonic development. However, the expression patterns of its components, including SRG3 and BRG1, during development have not been determined. Although expression patterns and transcription profiling of *Srg3* and *Brg1* in whole embryos have been examined (Bultman et al., 2000; Kim et al., 2001), their expression patterns in extra-embryonic tissues have not been reported.

In this study, we found that SRG3 is expressed in the yolk sac during embryonic development. The expression of *SRG3* is observed in the yolk sac at 7.5 dpc and persists after 10.5 dpc (data not shown). In the yolk sac of *Srg3*−/−Tg+ embryos, SRG3 was barely detected, while severe defects in yolk sac development were found. Intriguingly, decreased expression of BRG1 and its reduced transcript level was found in the same regions. SRG3 interacts with and stabilizes major components of the SWI/SNF complex, including BRG1, SNF5, and BAF60a, indicating a scaffold function of SRG3 (Sohn et al., 2007). It is interesting to note that the BRG1 transcript was also down-regulated in the *Srg3*−/−Tg+ yolk sac, suggesting that SRG3 may regulate the SWI/SNF complex activity at a transcriptional as well as a protein level.

Regulation of angiogenesis by SRG3

Normal differentiation of endothelial cells occurred in the yolk sac of *Srg3*−/−Tg+ embryo. However, the remodeling of the primary vascular plexus was abnormal. This feature of endothelial cell development in *Srg3*−/−Tg+ embryos is similar to the phenotype of mouse embryos carrying a mutation for Angiopoeitin1 (ANG1) and its receptor, Tie1/2 (Sato et al., 1995). Thus, in the yolk sacs of *Srg3*−/−Tg+ embryos, vasculogenesis occurs, while angiogenesis is arrested at an early stage.

Various signaling pathways involving soluble growth factors and their receptors direct blood vessel formation in embryonic development. In the *Srg3*−/−Tg+ yolk sac, vasculogenesis-related genes such as *VEGF*, *KDR*, and *Flt1* were expressed normally. Furthermore, normal expression of KDR indicates that endothelial cells are developed and well-aggregated (Millauer et al., 1993). Expression of these receptors is enhanced by VEGF. Their expression was detected in the yolk sac, where SRG3 expression is significantly reduced. Therefore, SRG3 does not affect the expression of VEGF and its receptors or endothelial cell differentiation in the yolk sac.

Several signaling molecules essential for angiogenesis have been reported, such as Ihh, Notch and Eph/ephrin signaling pathway. We tested the expression of these genes in *Srg3*−/−Tg+ yolk sacs. Consistent defects in the expression of these genes

were found. Ihh is normally expressed in the yolk sac visceral endoderm after gastrulation; its expression is maintained until after 12.5 dpc (Byrd et al., 2002). The loss of Ihh (Indian hedgehog) results in severe vascular defects during blood vessel maturation (Byrd et al., 2002). In the yolk sac, Ihh acts through binding to its receptor patched (*Ptch*). We determined that *Ihh* and its receptors, *Ptch1*/2, were down-regulated in the yolk sacs of *Srg3*−/−Tg+ embryos.

The Notch signaling pathway has been shown to be a determining factor of cell fate specification (Kojika and Griffin, 2001). In vascular development, Notch signaling is hypothesized to influence the commitment of the angioblast to an artery versus vein fate (Rossant and Howard, 2002). Mice with mutations in the Notch signaling pathway show vascular defects similar to those observed in Srg3−/−Tg+ yolk sacs (Krebs et al., 2000). As expected, *Hey1*, a gene downstream of Notch1, was also down-regulated in Srg3−/−Tg+ yolk sacs.

The Eph/ephrin pathway is also required for blood vessel maturation during embryonic development (Adams, 2002). Both ephrinB2 ligand and EphB4 receptor are normally expressed in yolk sac blood vessels. While ephrinB2 is expressed in the endothelial cells that differentiate into arteries, EphB4 is expressed in venous endothelial cells from an early stage of vascular development (Wang et al., 1998). However, when ephrinB2 or EphB4 are mutated, a normal primary plexus forms, but defects in vascular remodeling in the yolk sacs are evident (Gerety et al., 1999; Wang et al., 1998). This is morphologically similar to the Srg3−/−Tg+ embryos. Consistent with these results, we found that EphrinB2 and EphB4 were also down-regulated in Srg3−/−Tg+ yolk sacs.

From 7.5 dpc, Furin is produced in the extra-embryonic endoderm, which becomes the visceral endoderm. Mice lacking Furin die at 10.5–11.5 dpc and show developmentally severe defects (Roebroek et al., 1998). The yolk sacs of Furin knockout mouse embryos fail to develop large blood vessels. However, endothelial precursors expressing KDR, an endothelial cell marker, are present (Roebroek et al., 1998). The expression level of Furin was significantly down-regulated in the *Srg3*−/−Tg+ yolk sac.

SRG3 is required for yolk sac visceral endoderm development

Visceral endoderm originates from the hypoblast at the blastocyst stage (Gardner, 1983; Hogan et al., 1994). In the early stage, the visceral endoderm plays important roles in the absorption and digestion of maternal nutrient and in the exchange of gases between mother and embryo (Bielinska et al., 1999). Therefore, the visceral endoderm contains a number of phagocytic and pinocytic vesicles. Visceral endoderm synthesizes and releases many molecules related to nutrient transport and embryonic development, including the differentiation and formation of blood islands and blood vessels (Boucher and Pedersen, 1996; Cross et al., 1994; Freeman, 1990). Hence, severe defects in visceral endoderm development affect blood cell and blood vessel formation during vertebrate development (Winnier et al., 1995). As vasculogenesis commences in the yolk sac and embryonic circulation begins, the function of exchanging nutrients and gases is transferred from the visceral endoderm to the vasculature (Bielinska et al., 1999). In addition, phagocytic and pinocytic vesicles disappear after fetal blood cell begins to flow. *Srg3*−/−Tg+ yolk sac contained several vesicles and distorted microvilli in the yolk sac visceral endoderm, indicating that the visceral endoderm of the *Srg3*−/−Tg+ yolk sac did not develop normally.

GATA-4 and GATA-6 are important factors for visceral endoderm development. There are zinc finger transcription factors that induce the expression of numerous genes in cardiomyocytes and endoderm derivatives, including the visceral endoderm (Bossard and Zaret, 1998; Heikinheimo et al., 1997). The examination of embryoid bodies differentiated from embryonic stem cell shows that deficiency of GATA-4 and GATA-6 leads to defects in visceral endoderm formation and reduction of many visceral endoderm markers (Bielinska and Wilson, 1997; Soudais et al., 1995). However, mice lacking GATA-6 die 2–4 days earlier than GATA-4 knockout mice, indicating that GATA-6 is an early factor required for proper differentiation of the visceral endoderm (Bielinska et al., 1999; Koutsourakis et al., 1999; Molkentin et al., 1997). In the yolk sacs of *Srg3*−/−Tg+ embryos, GATA-4 expression was slightly reduced, but GATA-6 expression remained normal. GATA-4 heterozygous mutant embryos shows normal developmental features (Molkentin et al., 1997), suggesting that a slight reduction of GATA-4 in the *Srg3*−/−Tg+ yolk sac likely do not significantly contribute to the defects in yolk sac visceral endoderm development.

Furin is another important factor in visceral endoderm development. Furin activates precursors of signaling molecules related to development, such as insulin-like growth factor-I and members of the transforming growth factor β (TGF-β) family (Dubois et al., 1995; Duguay et al., 1997). Furin is made in the visceral endoderm from 7.5 dpc, which is later in development than GATA-4/6 expression (Roebroek et al., 1998). Therefore, reduced expression of Furin in the *Srg3*−/−Tg+ yolk sac suggests that SRG3 is involved in the late stage of yolk sac visceral endoderm development by regulating Furin expression. However, it is unclear whether an insufficient vasculature system leads to abnormal visceral endoderm development, or vice versa.

Down-regulation of angiogenesis-related genes in the yolk sacs of *Srg3*−/−Tg+ embryos accounts for severe defects observed in yolk sac vasculature maturation. In *Srg3*−/−Tg+ yolk sacs, several genes involved in angiogenesis and visceral endoderm development were down-regulated. In addition, reduced *Brg1* expression in *Srg3*−/−Tg+ embryos supports the hypothesis that SRG3 is a key regulator of activity of SWI/SNF complex. It is unclear whether these genes are regulated directly by the SWI/SNF complex or indirectly through the control of comodulators of their upstream targets. Nevertheless, it is clear that the SWI/SNF chromatin remodeling complex plays a critical role in the regulation of angiogenesis-related genes during embryonic development.

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