An important role of endothelial Hairy-related transcription factors in mouse vascular development

Takashi Morioka,[1,2,*] Masahide Sakabe,[1,*,§] Tomoko Ioka,[1] Tomoko Iguchi,[1] Ken Mizuta,[1] Miwa Hattammaru,[1,3] Chihiro Sakai,[1] Munehiro Itoh,[1] Genki E. Sato,[1] Aya Hashimoto[1], Masahide Fujita[1], Kazuki Okumura[1], Mutsumi Araki[1], Mei Xin,[4,&] Roger A. Pedersen,[5,#] Manuel F. Utset,[6] Hiroshi Kimura,[2] Osamu Nakagawa[1,§] (* These authors equally contributed to this study.)

[1]Laboratory for Cardiovascular System Research, Nara Medical University Advanced Medical Research Center, Kashihara, Nara, Japan; [2]The Second Department of Internal Medicine, Nara Medical University, Kashihara, Nara, Japan; [3]Department of Internal Medicine, Tokyo Women's Medical University Medical Center East, Tokyo, Japan; [4]Department of Molecular Biology, The University of Texas Southwestern Medical Center at Dallas, Dallas, TX, U.S.A.; [5]Reproductive Genetics Division, Department of Obstetrics and Gynecology, The University of California San Francisco, San Francisco, CA, U.S.A.; [6]Department of Pathology, The University of Illinois at Chicago, Chicago, IL, U.S.A.

[&] Present address: The Division of Experimental Hematology and Cancer Biology, Cincinnati Children's Hospital Medical Center and Department of Pediatrics, The University of Cincinnati, Cincinnati, Ohio.

[#] Present address: The Anne McLaren Laboratory for Regenerative Medicine, Stem Cell Institute, University of Cambridge, Cambridge, U.K.

[§] Corresponding authors: Osamu Nakagawa, M.D., Ph.D. (Email: nakagawa-nmu@umin.org) and Masahide Sakabe, Ph.D. (Email: sakabe@naramed-u.ac.jp), Laboratory for Cardiovascular System Research, Nara Medical University Advanced Medical Research Center, 840 Shijo-cho, Kashihara, Nara, 634-8521, Japan, Phone 81-744-29-8950.

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ABSTRACT

The Hairy-related transcription factor family of Notch- and ALK1-downstream transcriptional repressors, called Hrt/Hey/Hesr/Chf/Herp/Gridlock, has complementary and indispensable functions for vascular development. While mouse embryos null for either *Hrt1/Hey1* or *Hrt2/Hey2* did not show early vascular phenotypes, *Hrt1/Hey1; Hrt2/Hey2* double null mice ($H1^{ko}/H2^{ko}$) showed embryonic lethality with severe impairment of vascular morphogenesis. It remained unclear, however, whether Hrt/Hey functions are required in endothelial cells or vascular smooth muscle cells. In this study, we demonstrate that mice with endothelial-specific deletion of *Hrt2/Hey2* combined with global *Hrt1/Hey1* deletion ($H1^{ko}/H2^{eko}$) show abnormal vascular morphogenesis and embryonic lethality. Their defects were characterized by the failure of vascular network formation in the yolk sac, abnormalities of embryonic vascular structures and impaired smooth muscle cell recruitment, and were virtually identical to the $H1^{ko}/H2^{ko}$ phenotypes. Among signaling molecules implicated in vascular development, *Robo4* expression was significantly increased and activation of Src family kinases was suppressed in endothelial cells of $H1^{ko}/H2^{eko}$ embryos. The present study indicates an important role of Hrt1/Hey1 and Hrt2/Hey2 in endothelial cells during early vascular development, and further suggests involvement of Robo4 and Src family kinases in the mechanisms of embryonic vascular defects caused by the *Hrt/Hey* deficiency.

KEYWORDS

mouse embryo, vascular morphogenesis, *Hrt/Hey*, *Robo4*, Src family kinases

INTRODUCTION

Among a variety of cellular signaling pathways implicated in embryonic vascular development, Notch signaling regulates arterial endothelium differentiation and vascular morphogenesis (Herbert and Stainier, 2011; Potente et al., 2011). Mice null for Notch receptors or other signaling components die *in utero* due to abnormalities of vascular formation (Krebs et al., 2000; Duarte et al., 2004; Benedito et al., 2008).

We and others previously identified the Hairy-related transcription factor family, independently named Hrt, Hey, Hesr, Chf, Herp or Gridlock, as downstream mediators of Notch signaling in the developing vasculature (Kokubo et al., 1999; Leimeister et al., 1999; Nakagawa et al., 1999; Chin et al., 2000; Zhong et al., 2000; Iso et al., 2001). Endothelial *Hrt/Hey* expression is also controlled by bone morphogenetic protein 9 (BMP9)/BMP10-dependent ALK1 receptor signaling, which is indispensable for embryonic vascular development (Larrivée et al, 2012; Somekawa et al., 2012). Among the three gene family members (*Hrt1/Hey1*, *Hrt2/Hey2 and Hrt3/HeyL*), mice null for *Hrt2/Hey2* show perinatal lethality due to ventricular septal defects and mitral valve insufficiency, while no cardiovascular phenotypes have been reported as yet for *Hrt1/Hey1* null or *Hrt3/HeyL* null mice (Donovan et al., 2002; Fischer et al., 2004; Kokubo et al., 2005; Fischer et al., 2007; Xin et al., 2007). Of note, combined loss of *Hrt1/Hey1* and *Hrt2/Hey2* resulted in embryonic lethality due to vascular demise similar to that observed in Notch or ALK1 signaling deficient embryos (Fischer et al., 2004; Kokubo et al., 2005). Despite their clear significance in vascular development, it remained unclear whether the complementary functions of Hrt1/Hey1 and Hrt2/Hey2 were required in endothelial cells (EC) or vascular smooth muscle cells (VSMC).

In this study, we demonstrate an important role of endothelial Hrt/Hey functions during early vascular development of mouse embryos, and further suggest involvement of Robo4, an EC-specific Roundabout family membrane protein (Park et al., 2003), and Src family kinases (SFKs) (Eliceiri et al., 1999) in the mechanisms of vascular abnormalities due to the *Hrt/Hey* deficiency.

RESULTS AND DISCUSSION

As shown in Fig. 1a-b, *in situ* hybridization demonstrated that *Hrt1/Hey1* and *Hrt2/Hey2* were expressed in vascular walls of embryonic arteries such as the dorsal aorta. Hrt2/Hey2 protein was co-localized with platelet endothelial cell adhesion molecule-1 (PECAM1) and smooth muscle alpha actin (αSMA), established EC and VSMC markers, respectively (Fig. 1c-d). While Hrt1/Hey1 antibodies were not available for immunohistochemistry, broad expression of Hrt1/Hey1 mRNA in vascular walls suggested that Hrt1/Hey1 protein was also expressed in EC and VSMC (Fig. 1a-b).

The present study attempted to analyze if *Hrt1/Hey1* and *Hrt2/Hey2* functions are necessary in EC during vascular development, using a new *Hrt1/Hey1* null mouse line (supplemental Fig. 1), the *Hrt2/Hey2* null and floxed lines that we previously described (Xin et al., 2007) and *Tek/Tie2*-Cre transgenic mice (Kisanuki et al., 2001). As reported (Donovan et al., 2002; Fischer et al., 2007), mice null for either *Hrt1/Hey1* or *Hrt2/Hey2* did not show early vascular phenotypes. At embryonic day (E) 10.5, *Hrt1/Hey1* null ($H1^{ko}$) embryos as well as *Hrt2/Hey2* null ($H2^{ko}$) embryos were indistinguishable from wild-type littermates (Fig. 2a-c). Consistent with previous studies (Fischer et al., 2004; Kokubo et al., 2005), however, the embryos null for both *Hrt1/Hey1* and *Hrt2/Hey2* (*Hrt1/Hey1*$^{ko/ko}$; *Hrt2/Hey2*$^{ko/ko}$, hereafter called $H1^{ko}/H2^{ko}$) showed characteristic defects of cardiovascular morphogenesis (Fig. 2d, f, h, and described below), and all died *in utero*.

Of note, the embryos in which the *Hrt2/Hey2* gene was deleted specifically in EC with the global *Hrt1/Hey1* null background (*Hrt1/Hey1*$^{ko/ko}$; *Hrt2/Hey2*$^{fl/ko}$; *Tek/Tie2-Cre*$^{+}$, hereafter called $H1^{ko}/H2^{eko}$) showed apparent vascular abnormalities and died between E10.5-12.5 with complete penetrance (supplemental Table 1, supplemental Fig. 2). The embryos without the endothelial loss of *Hrt2/Hey2* (*Hrt1/Hey1*$^{ko/ko}$; *Hrt2/Hey2*$^{fl/ko}$) showed normal vascular morphogenesis despite the deletion of three out of four *Hrt1/Hey1; Hrt2/Hey2* alleles. *Hrt2/Hey2*$^{fl/ko}$; *Tek/Tie2-Cre*$^{+}$ ($H2^{eko}$) mice showed no defects in vascular formation in the absence of homozygous *Hrt1/Hey1*

deficiency (supplemental Table 1). These analyses indicated that *Hrt1/Hey1* and *Hrt2/Hey2* played complementary roles in vascular development, and that the complete loss of *Hrt1/Hey1* and *Hrt2/Hey2* in EC was sufficient to cause severe defects of early vascular formation.

$H1^{ko}/H2^{eko}$ embryos started to display impairment of yolk sac angiogenesis at E9.5 (Fig. 3c), and did not form a proper vascular network in the yolk sac even at E10.5 (Fig. 3g). Embryo growth was apparently retarded and pericardial effusion was observed in $H1^{ko}/H2^{eko}$ embryos (Fig. 3d, h). Histological studies detected frequent narrowing of the dorsal aorta in $H1^{ko}/H2^{eko}$ embryos, while the structure of the anterior cardinal veins appeared intact at E9.5-10.5 (Fig. 4b, d). Recruitment of VSMC to the dorsal aorta was suppressed in $H1^{ko}/H2^{eko}$ embryos, as demonstrated by αSMA staining (Fig. 4f). Ventricular wall thickness and trabeculation were not significantly altered in $H1^{ko}/H2^{eko}$ embryos (Fig. 4h). The yolk sac showed detachment of endodermal and mesodermal layers and abnormal vessel dilatation in $H1^{ko}/H2^{eko}$ embryos (Fig. 4j). These cardiovascular abnormalities were virtually identical to those in $H1^{ko}/H2^{ko}$ embryos (Fig. 2d, f, h).

Whole mount PECAM1 immunostaining indicated a defective endothelial network in $H1^{ko}/H2^{eko}$ embryos (Fig. 5a-b). Although a highly organized vascular tree with large, intermediate and small branches was observed in the head region of control embryos (Fig. 5a'), $H1^{ko}/H2^{eko}$ embryos exhibited a primitive vasculature with uniformly sized and poorly organized vessels (Fig. 5b'). Intersomitic vessels in the trunk regions of $H1^{ko}/H2^{eko}$ embryos were shorter and thicker than those of control embryos (arrows in Fig. 5a"-b", c-d and supplemental Fig. 3), suggesting a failure of their correct elongation. While the perineural vascular plexus showed normal arborization in the dorsal region of control embryos (surrounded with dashed lines in Fig. 5a"), it was poorly organized and less branched in $H1^{ko}/H2^{eko}$ embryos (Fig. 5b", e, and supplemental Fig. 3).

To begin to understand how abnormal vascular morphogenesis occurred in $H1^{ko}/H2^{eko}$ embryos, we analyzed the expression of signaling molecules implicated in embryonic vascular

development (Herbert and Stainier, 2011; Potente et al., 2011). Among those tested, mRNA expression of *Robo4* and *Kdr/Vegfr2* was significantly increased in $H1^{ko}/H2^{eko}$ embryos at E9.5 (Fig. 6a). Their mRNA expression was not altered in a stage-dependent manner among E9.0, E9.5 and E10.0 wild-type embryos, suggesting that the increase was not due to the growth delay of $H1^{ko}/H2^{eko}$ embryos (data not shown). Since increased expression of Robo4 inhibits vascular endothelial growth factor (VEGF)-dependent SFK activation in EC (Jones et al., 2008; Koch et al., 2011), we assessed the expression of *Robo4* in EC of $H1^{ko}/H2^{eko}$ embryos. *Robo4* expression was highly restricted to the EC population (Fig. 6b) and was significantly up regulated in EC isolated from $H1^{ko}/H2^{eko}$ embryos (Fig. 6c). We then examined the expression patterns of activated SFKs containing a phosphotyrosine residue in the kinase domain in control and $H1^{ko}/H2^{eko}$ embryos. Localization of activated SFKs closely overlapped with that of PECAM1 in control embryos, indicating that SFKs were preferentially activated in developing EC (Fig. 6d'-f'). The levels of SFK activation was reduced in EC of $H1^{ko}/H2^{eko}$ embryos (Fig. 6g') compared to control embryos (Fig. 6d'). A significant decrement of activated SFKs in $H1^{ko}/H2^{eko}$ embryos was confirmed by Western blot analysis, while the total protein levels of SFKs were unchanged between control and $H1^{ko}/H2^{eko}$ embryos (Fig. 6j).

In the present study, we demonstrate that the loss of *Hrt2/Hey2* in EC in mouse embryos with the *Hrt1/Hey1* null background causes severe vascular defects and embryonic lethality, which are virtually identical to *Hrt1/Hey1; Hrt2/Hey2* double null phenotypes. While we have not examined the phenotypes caused by the endothelial-specific deletion of *Hrt1/Hey1* and *Hrt2/Hey2*, the results strongly suggest that Hrt/Hey proteins in EC play crucial roles during early vascular development. Hrt1/Hey1 and Hrt2/Hey2 control target gene expression as transcriptional repressors by binding to consensus DNA elements or by associating with other DNA-binding transcription factors (Kathiriya et al., 2004; Heisig et al., 2012), yet very little is known about their target genes directly involved in cardiovascular abnormalities observed in *Hrt/Hey* mutant mice. Up regulation of *Robo4* expression in $H1^{ko}/H2^{eko}$ embryos suggests a possible link between

Hrt/Hey function and *Robo4* expression. Interestingly, a significant increase in *Robo4* expression is observed concurrently with reduction of *Hrt/Hey* expression in *Dll4* null and *Alk1/Acvrl1* null embryos (Park et al., 2003; Benedito et al., 2008; Somekawa et al., 2012). Since shear stress can suppress *Robo4* expression in cultured EC (Mura et al., 2012), increased *Robo4* expression in $H1^{ko}/H2^{eko}$ embryos and other mutant embryos might, at least in part, be due to vascular dysmorphogenesis and circulation failure. Nevertheless, it was also reported that *Robo4* expression was decreased concurrently with increased *Hrt/Hey* expression in mouse embryos with transgenic *Dll4* overexpression, although these embryos showed significant vascular defects (Trindade et al., 2007). Endothelial-specific expression of human *ROBO4* gene is regulated by the transcription factors such as Sp1, GABA and Sox18 as well as through the promoter DNA methylation (Samant et al., 2011; Okada et al., 2014), but little is known about the regulatory mechanisms of mouse *Robo4* expression. It will be important to study how endothelial *Robo4* expression is regulated in mouse embryos and clarify if Hrt/Hey proteins are directly involved in transcriptional repression of *Robo4* expression.

Amidst other components of endothelial signaling pathways, Hrt/Hey proteins repress the mRNA expression of *Kdr/Vegfr2* in cultured EC (Holderfield et al., 2006; Larrivée et al., 2012), and *Kdr/Vegfr2* expression was indeed increased in $H1^{ko}/H2^{eko}$ embryos (Fig. 6a). However, the activity of VEGF downstream signaling molecules showed different changes in $H1^{ko}/H2^{eko}$ embryos. As described above, phosphorylation of SFKs was significantly down regulated, while that of p38 mitogen activated protein kinases and AKT was rather increased and variable, respectively (Fig. 6j). It is likely that the activity of VEGF downstream factors is differentially modulated by various other signaling pathways in $H1^{ko}/H2^{eko}$ embryos.

The activation of SFKs by VEGF signaling is fundamental to vascular development (Eliceiri et al., 1999; Koch and Claesson-Welsh, 2012). While increased Robo4 expression represses VEGF-dependent SFK activation, its mechanisms appear compound or context-dependent. It was first proposed that Slit2-dependent activation of Robo4 receptor signaling suppressed SFK

activity (Jones et al., 2008), whereas Eichmann and colleagues reported that Robo4 mainly acted as a ligand for Unc5B, redistributed SFKs associated with VEGFR2 to Unc5B, and suppressed VEGF-dependent SFK activation (Koch et al., 2011). Similar to Robo4, Ang1-Tek/Tie2 signaling induces the association of the RhoA effector mDia with SFKs, depriving VEGFR2 of SFKs and suppressing VEGF-dependent SFK activation (Gavard et al., 2008). However, *Ang1* mRNA expression was not significantly increased in $H1^{ko}/H2^{eko}$ embryos (Fig. 6a). Other regulatory molecules such as C-terminal Src kinases and receptor-type protein tyrosine phosphatases might be involved (Kaimachnikov et al., 2009), and it is still unclear if Robo4 is a major effector that suppresses SFK activity in $H1^{ko}/H2^{eko}$ embryos. It awaits further studies to clarify how the *Hrt1/Hey1; Hrt2/Hey2* deficiency causes down regulation of SFK activity in embryonic EC.

In addition to Notch signaling, ALK1 signaling markedly stimulates endothelial expression of *Hrt1/Hey1* and *Hrt2/Hey2* (Larrivée et al, 2012), and *Alk1/Acvrl1* null embryos show a significant decrease in their expression (Somekawa et al., 2012). As a nexus of these important signaling pathways, Hrt1/Hey1 and Hrt2/Hey2 are likely to be involved in various phenomena for EC differentiation and maturation. Furthermore, *Hrt1/Hey1* and *Hrt2/Hey2* may have additional roles in VSMC, especially at later stages of vascular development. Future studies including VSMC-specific deletion of *Hrt1/Hey1* and *Hrt2/Hey2* in mice will clarify how the Hrt/Hey genes work in EC, VSMC and their interrelationship during vascular morphogenesis.

METHODS

Mice

Animal experiments were performed with the approval of the institutional animal care and use committee of Nara Medical University. The *Hrt1/Hey1* null allele was created by replacing exons 2-4 with a PGK-neo cassette (supplemental Fig. 1). The *Hrt1/Hey1* null mouse line will be made available to the research community. Mouse lines harboring the *Hrt2/Hey2* null and floxed alleles and *Tek/Tie2*-Cre transgenic mice were described previously (Kisanuki et al., 2001; Xin et

al., 2007). Sequences of genotyping PCR primers were listed in supplemental Table 2. All breeding was done using the mice with mixed genetic background (129/SvEv and C57BL/6). Specific loss of *Hrt2/Hey2* expression in EC, but not in other cell types, in $H1^{ko}/H2^{eko}$ embryos was confirmed, as shown in supplemental Fig. 2.

Immunohistochemistry, section *in situ* hybridization and Western blot analysis

For immunohistochemistry, embryos were fixed in 4% paraformaldehyde, embedded in OCT compound, and sectioned at 10 μm. Sections were blocked with 1% bovine serum albumin (BSA) in phosphate buffered saline (PBS) and incubated with primary antibodies against PECAM1 (BD Biosciences, #550274), Hrt2/Hey2 (ProteinTech, #10597-1-AP), αSMA (Sigma-Aldrich, A2547), phosphorylated SFKs (tyrosine^{416} in human Src, Cell Signaling, #6943). Sections were further incubated with Alexa Fluor-conjugated secondary antibodies against mouse, rabbit or rat IgG. Hematoxylin and eosin (H&E) staining was performed using the standard protocol.

For whole mount immunohistochemistry using anti-PECAM1 antibody (BD Biosciences, #550274), embryos fixed in 4% paraformaldehyde were dehydrated in methanol and stored at -20°C until use. After the rehydration in PBS, samples were blocked in PBS containing 10% fetal bovine serum and 1% Tween20, and incubated at 4°C with anti-PECAM1 antibody overnight. Samples were washed in PBS containing 1% Tween20, and further incubated at 4°C with Alexa Fluor 488-labeled secondary antibody. Following clearing, Z-stack images were captured using FLUOVIEW1000 laser-scanning confocal microscope (OLYMPUS).

To detect the expression of *Hrt1/Hey1* or *Hrt2/Hey2* mRNA, frozen sections were hybridized with a digoxigenin-labeled antisense RNA probe, followed by detection using the alkaline phosphatase-conjugated antibody (Roche) and counterstaining with eosin.

Western blot analysis was performed using standard procedures. Manufacturers of primary antibodies were as follows: SFKs (Cell Signaling, #2109), phosphorylated SFKs (Cell Signaling, #6943), p38 (Cell Signaling, #8690), phosphorylated p38 (Cell Signaling, #4511), AKT (Cell

Signaling, #4691), phosphorylated AKT (Cell Signaling, #4060), β-actin (Sigma-Aldrich, A5441). Secondary antibodies were purchased from Santa Cruz Biotechnology.

Embryonic EC isolation

Embryos harvested at E9.5 were individually digested in 0.1% collagenase II (Worthington) for 10 min at 37°C. After washing digested cells with Dulbecco's modified Eagle medium containing 10% fetal bovine serum and with PBS containing 0.1% BSA, each cell suspension was incubated with 1 μg of anti-PECAM1 antibody (BD Biosciences, #550274) for 20 min at 4°C. After several washes with PBS containing 0.1% BSA, PECAM1-positive and negative cell populations, referred to as EC and non-EC populations, respectively, were separated by the magnet bead-based system using Dynabeads (Life Technologies) coupled with anti-rat IgG antibody (supplemental Fig. 2).

Reverse transcription (RT) PCR analysis

Total RNA from whole embryos or EC/non-EC populations was extracted using TRIzol reagent (Life Technologies). cDNA was synthesized using 500ng of total RNA using PrimeScript RT Master Mix (TaKaRa), and real-time PCR was carried out using the KAPA SYBR FAST qPCR kit (KAPA). Conventional PCR was performed using KAPA Taq Ready Mix (KAPA). Sequences of PCR primers were listed in supplemental Table 2.

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FIGURE LEGENDS

Fig. 1. *Hrt1/Hey1* and *Hrt2/Hey2* are expressed in embryonic vasculature.

(a, b) *in situ* hybridization of *Hrt1/Hey1* and *Hrt2/Hey2* mRNA showed that they were expressed in the dorsal aorta (DA), but not in the anterior cardinal veins (CV), in E10.5 mouse embryos. Scale bars represent 100 μm (a-b) or 50 μm (a'-b').

(c, d) Immunohistochemistry co-stained with PECAM1 or αSMA indicated that Hrt2/Hey2 protein

was expressed in both EC (arrows) and VSMC (arrowheads) in the dorsal aorta (E10.5). Scale bars represent 20 μm.

Fig. 2. *Hrt1/Hey1* and *Hrt2/Hey2* are complementary and essential for embryonic vascular development.

(a-f) Mice null for either *Hrt1/Hey1* ($H1^{ko}$, panel b) or *Hrt2/Hey2* ($H2^{ko}$, panel c) were indistinguishable from wild-type controls (a) at E10.5. $H1^{ko}/H2^{ko}$ embryos lacking both *Hrt1/Hey1* and *Hrt2/Hey2* displayed severe abnormalities such as growth retardation (d), pericardial effusion (d') and defective yolk sac angiogenesis (f'). Scale bars represent 500 μm (a-d, d', e-f) or 250 μm (e'-f').

(g-h) H&E staining of the transverse sections of E10.5 embryos showed that paired dorsal aorta (DA) were markedly reduced in size in $H1^{ko}/H2^{ko}$ embryos (arrows in g'-h'). CV, cardinal vein. Mice with the *Hrt1/Hey1*$^{wt/ko}$; *Hrt2/Hey2*$^{wt/ko}$ genotype were used as controls in panels e and g. Scale bars represent 200 μm (g-h) or 100 μm (g'-h').

Fig. 3. Endothelial functions of *Hrt1/Hey1* and *Hrt2/Hey2* are indispensable for vascular development.

Gross morphology of $H1^{ko}/H2^{eko}$ (*Hrt1/Hey1*$^{ko/ko}$; *Hrt2/Hey2*$^{fl/ko}$; *Tek/Tie2-Cre*$^{+}$) embryos is shown. $H1^{ko}/H2^{eko}$ embryos displayed apparent growth retardation (d, h), pericardial effusion (d', h') and impairment of yolk sac angiogenesis (c', g') at E9.5-10.5. Mice with the *Hrt1/Hey1*$^{wt/ko}$; *Hrt2/Hey2*$^{wt/fl}$; *Tek/Tie2-Cre*$^{+}$ genotype did not display detectable vascular defects, and were used as littermate controls (a, b, e, f). Scale bars represent 500 μm.

Fig. 4. Vascular morphogenesis is impaired by the lack of *Hrt1/Hey1* and *Hrt2/Hey2* in EC.

(a-d) H&E staining of transverse sections of E9.5-10.5 embryos showed that the lumen size of

dorsal aorta (arrows) was markedly reduced in $H1^{ko}/H2^{eko}$ embryos. Scale bars represent 200 μm (a-d), 100 μm (a'-d').

(e-f) Double-staining of PECAM1 and αSMA indicated that few VSMC were observed around EC of the dorsal aorta (arrows) in $H1^{ko}/H2^{eko}$ embryos. Scale bars represent 100 μm.

(g-h) H&E staining showed that ventricular wall thickness and trabeculation were not significantly altered in $H1^{ko}/H2^{eko}$ embryos. Scale bars represent 100 μm.

(i-j) H&E staining showed detachment of endodermal and mesodermal layers and abnormal vessel dilatation in the yolk sac of $H1^{ko}/H2^{eko}$ embryos. Scale bars represent 100 μm.

Fig. 5. The *Hrt1/Hey1* and *Hrt2/Hey2* deficiency in EC causes failure of the formation of higher vascular structure.

(a-b) Whole-mount PECAM1 immunostaining demonstrated that $H1^{ko}/H2^{eko}$ embryos had defective formation of vascular tree compared to control embryos. Enlarged views of the head region (a'-b') and the dorsal trunk region (a"-b") are shown. Scale bars represent 500 μm (a-b) or 250 μm (a', a", b', b").

(c-e) The length and diameter of intersomitic vessels (ISV) and branching frequency of perineural vascular plexus in the dorsal trunk region were quantitated as described in supplemental Fig. 3. Cont., control. P values were calculated by Student's t test.

Fig. 6. The *Hrt1/Hey1* and *Hrt2/Hey2* deficiency in EC alters the expression and activity of endothelial signaling molecules.

(a) Expression of endothelial genes was examined by real-time PCR using whole embryo lysate. Relative mRNA expression levels in $H1^{ko}/H2^{eko}$ compared to control embryos are shown. *Robo4* and *Kdr/Vegfr2* mRNA expression was significantly increased in $H1^{ko}/H2^{eko}$ embryos. P values were calculated by Student's t test. * $P<0.01$, ** $P<0.05$.

(b-c) EC was isolated from whole embryo cell suspension, and RT products were subjected to conventional and real-time PCR. Semi-quantitative RT-PCR indicated efficient separation of EC and non-EC populations and EC-specific expression of *Robo4* (b). Real-time PCR demonstrated that *Robo4* expression was significantly increased in EC of $H1^{ko}/H2^{eko}$ embryos (c). Cont., control.

(d-i) Immunostaining of E10.5 embryos indicated that activated SFKs containing a phosphotyrosine residue in the kinase domain (P-SFK) was enriched in PECAM1-positive EC (d-f). P-SFK signals appeared decreased in $H1^{ko}/H2^{eko}$ embryos (g-i). Scale bars represent 200 μm (d-f) or 100 μm (d'-f').

(j) Western blot analysis confirmed that the levels of activated SFKs (P-SFK) were reduced in $H1^{ko}/H2^{eko}$ embryos, while total protein expression of SFKs (SFK) was unchanged. The levels of activated p38 (P-p38) were increased, while those of activated AKT (P-AKT) were variable in $H1^{ko}/H2^{eko}$ embryos. β-actin expression levels are shown as loading controls.

SUPPLEMENTAL INFORMATION

FIGURE LEGENDS

Supplemental Fig. 1. Generation of *Hrt1/Hey1* null mouse line.

(a) A schematic diagram of the *Hrt1/Hey1* targeting strategy. The targeting construct was generated using 4.2-kb *Hin*dIII/*Sac*I fragment and 1.5-kb *Bgl*II/*Hin*dIII fragment from the *Hrt1/Hey1* locus. A homologous recombination event replaced a *Hrt1/Hey1* genomic region, which includes a part of the first coding exon, the entire second and third coding exons, and a small portion of the fourth coding exon including the splice acceptor, with a PGK-neo expression cassette in the opposite transcriptional orientation to the *Hrt1/Hey1* gene. Sequences of genotyping PCR primers (F1, R1, F2, R2) were listed in supplemental Table 2.

(b) Representative genotyping results of *Hrt1/Hey1* null mouse line. WT, wild-type; Het, heterozygous; KO, null.

Supplemental Fig. 2. Gene expression analysis of mouse embryos with EC-specific deletion of *Hrt2/Hey2* combined with global *Hrt1/Hey1* deletion ($H1^{ko}/H2^{eko}$).

(a) A schematic of the EC isolation protocol from individual embryos is shown. EC isolation was performed using anti-PECAM1 antibody (Ab).

(b) Confirmation of *Hrt1/Hey1* and *Hrt2/Hey2* expression in EC and non-EC populations sorted from control and $H1^{ko}/H2^{eko}$ embryos. *Hrt1/Hey1* and *Hrt2/Hey2* mRNA was expressed in EC as well as non-EC populations in control embryos. *Hrt2/Hey2* mRNA expression was eliminated only in EC population but not in non-EC population, while *Hrt1/Hey1* mRNA expression was not detectable in EC or non-EC populations from $H1^{ko}/H2^{eko}$ embryos.

Supplemental Fig. 3. Quantitation of altered vascular structure in EC-DKO embryos.

The representative confocal image Z-stacks of E10.5 embryos are shown. (a-b) The length and

diameter of intersomitic vessels were measured. (c-d) Branching points (red points) in perineural vascular plexus were counted. Quantitative data are shown in Fig. 5.

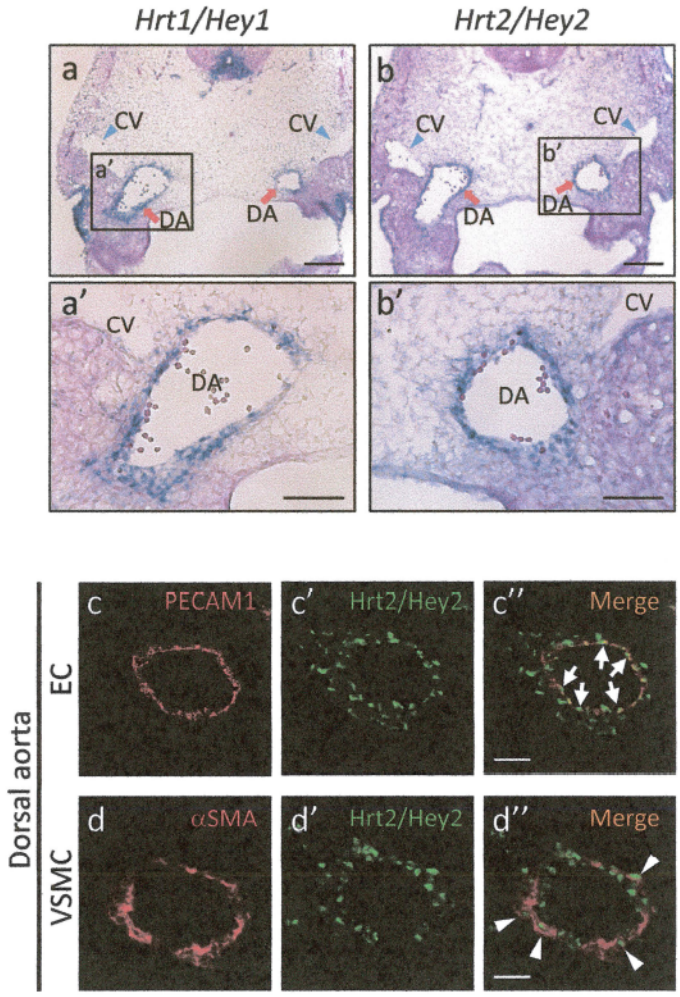

Hrt1/Hey1
Hrt2/Hey2
a
b
CV
CV
CV
CV
a'
b'
DA
DA
DA
DA
a'
b'
CV
CV
DA
DA
Dorsal aorta
EC
VSMC
c
PECAM1
c'
Hrt2/Hey2
c''
Merge
d
αSMA
d'
Hrt2/Hey2
d''
Merge

Fig.1

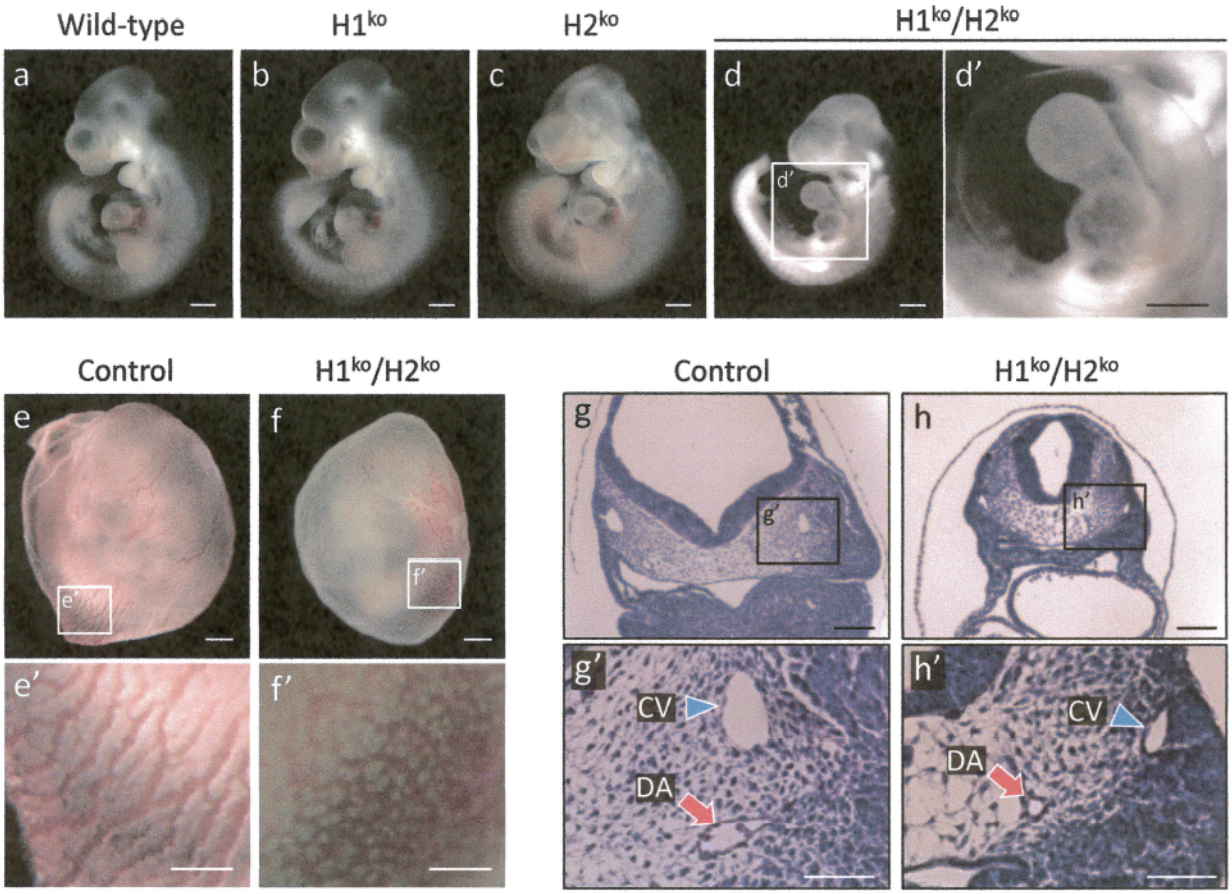

Wild-type
H1ko
H2ko
H1ko/H2ko
a
b
c
d
d'
d'
Control
H1ko/H2ko
e
f
e'
f'
e'
f'
Control
H1ko/H2ko
g
h
g'
h'
g'
h'
CV
DA
CV
DA

Fig.2

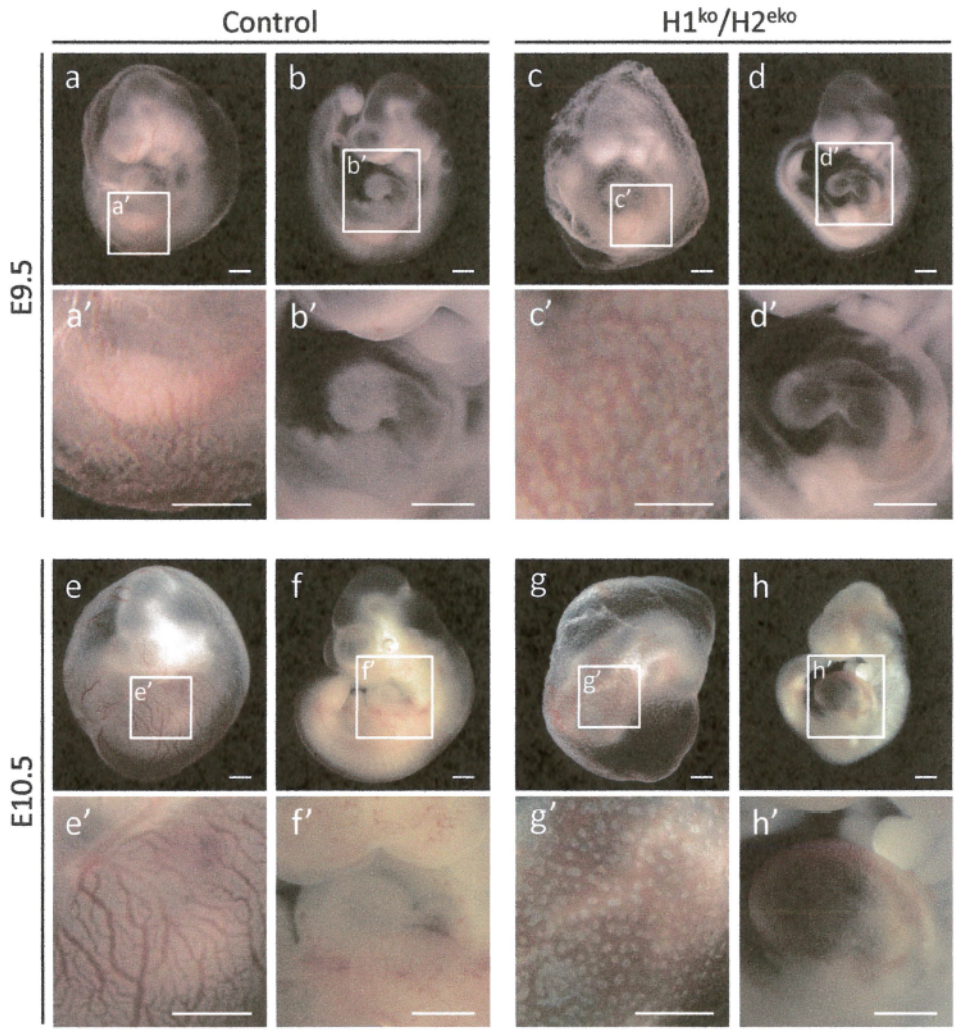


Fig.3

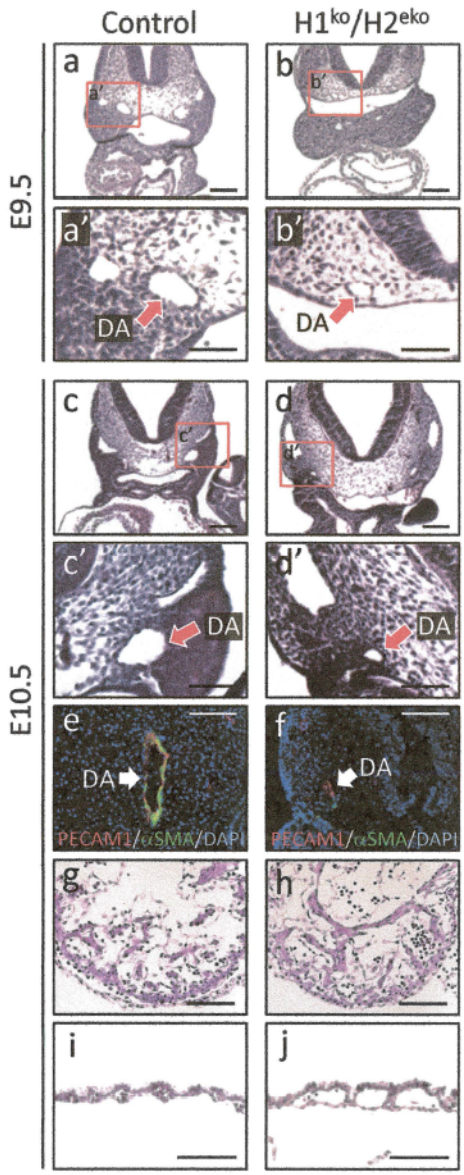

Control
H1ko/H2eko
E9.5
E10.5
a
b
a'
b'
DA
c
d
c'
d'
e
f
PECAM1/αSMA/DAPI
g
h
i
j

Fig.4

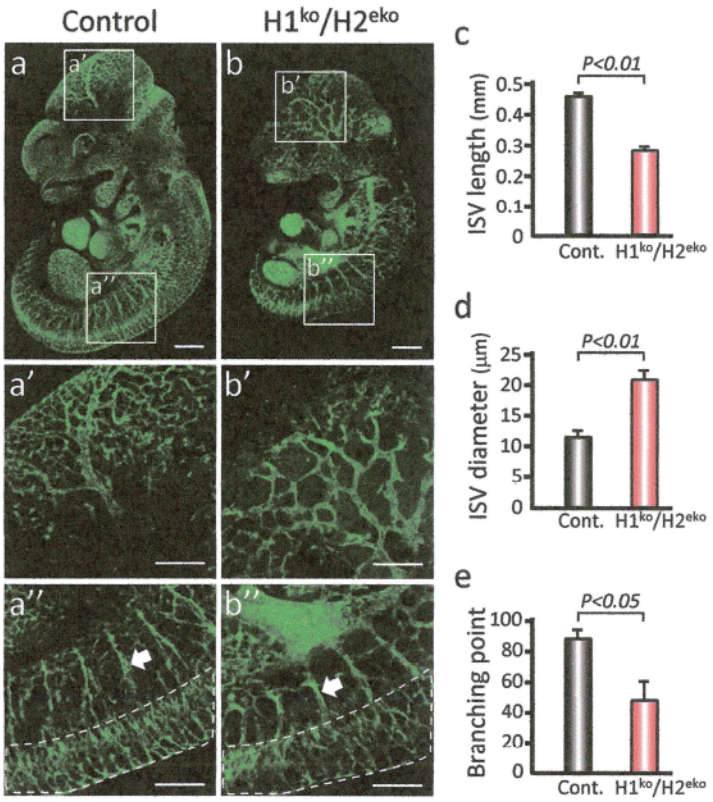

Control
H1ko/H2eko
a
a'
a''
b
b'
b''
c
P<0.01
ISV length (mm)
0.5
0.4
0.3
0.2
0.1
0
Cont.
H1ko/H2eko
d
P<0.01
ISV diameter (μm)
25
20
15
10
5
0
Cont.
H1ko/H2eko
e
P<0.05
Branching point
100
80
60
40
20
0
Cont.
H1ko/H2eko

Fig.5

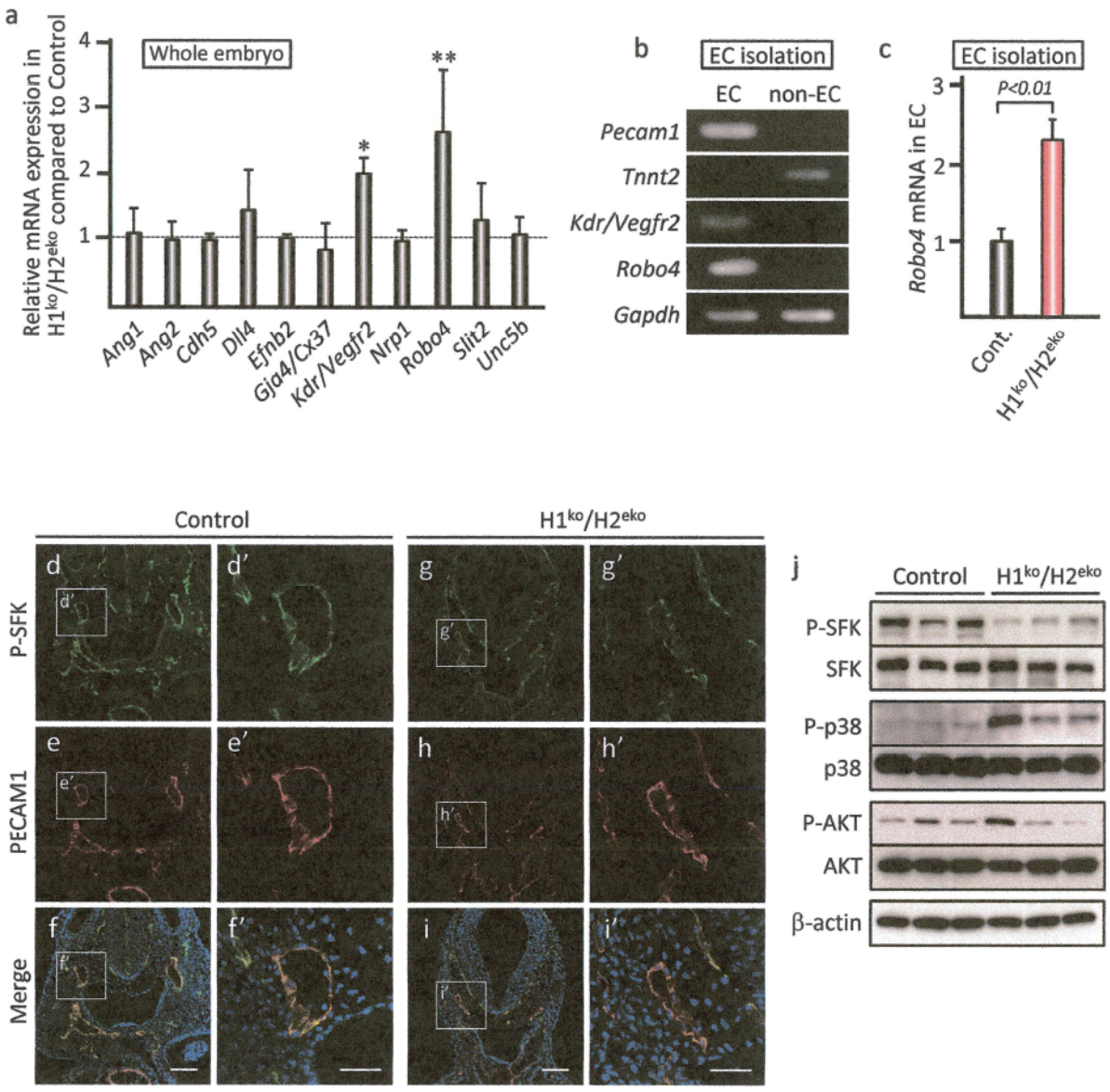
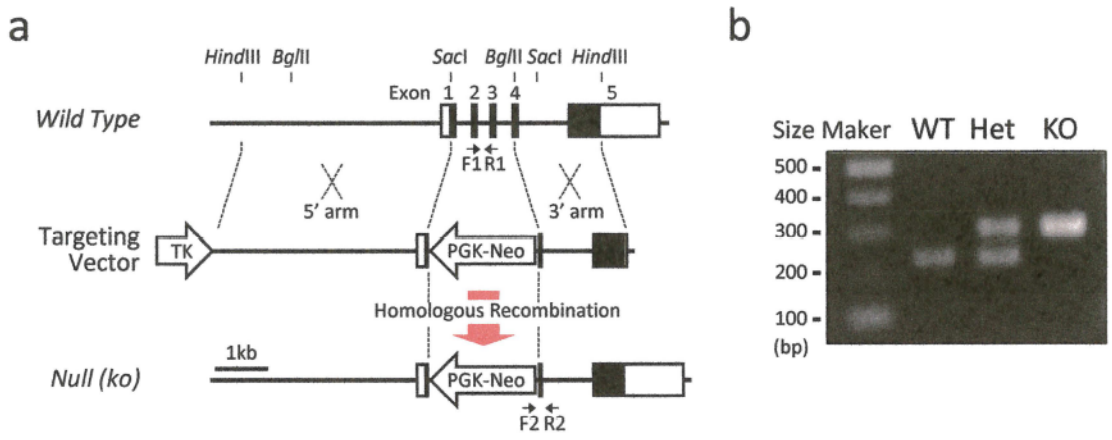
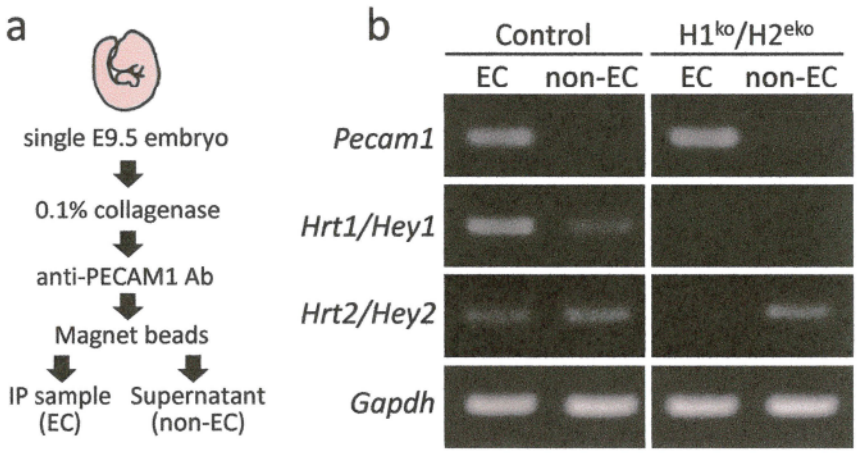

a
Relative mRNA expression in H1ko/H2eko compared to Control
Whole embryo
Ang1
Ang2
Cdh5
Dll4
Efnb2
Gja4/Cx37
Kdr/Vegfr2
Nrp1
Robo4
Slit2
Unc5b
b
EC isolation
EC
non-EC
Pecam1
Tnnt2
Kdr/Vegfr2
Robo4
Gapdh
c
EC isolation
P<0.01
Robo4 mRNA in EC
Cont.
H1ko/H2eko
Control
H1ko/H2eko
P-SFK
PECAM1
Merge
j
Control
H1ko/H2eko
P-SFK
SFK
P-p38
p38
P-AKT
AKT
β-actin

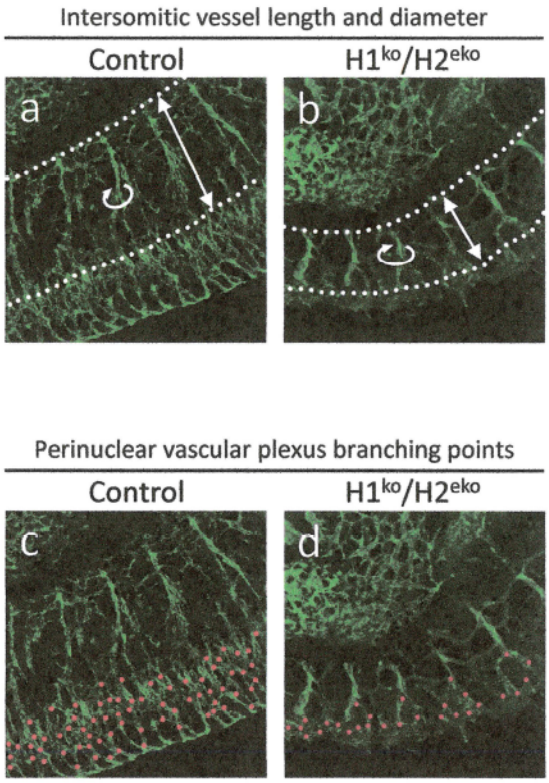
Fig.6



Supplemental Fig. 1



Supplemental Fig. 2



Supplemental Fig. 3