Importance of endothelial Hey1 expression for thoracic great vessel development and its distal enhancer for Notch-dependent endothelial transcription

Thoracic great vessels such as the aorta and subclavian arteries are formed through dynamic remodeling of embryonic pharyngeal arch arteries (PAAs). Previous work has shown that loss of a basic helix-loop-helix transcription factor Hey1 in mice causes abnormal fourth PAA development and lethal great vessel anomalies resembling congenital malformations in humans. However, how Hey1 mediates vascular formation remains unclear. In this study, we revealed that Hey1 expression in vascular endothelial cells, but not in smooth muscle cells, played essential roles for PAA development and great vessel morphogenesis in mouse embryos. Tek-Cre–mediated Hey1 deletion in endothelial cells affected endothelial tube formation and smooth muscle differentiation in embryonic fourth PAAs and resulted in interruption of the aortic arch and other great vessel malformations. Cell specificity and signal responsiveness of Hey1 expression were controlled through multiple cis-regulatory regions. We found two distal genomic regions that had enhancer activity in endothelial cells and in the pharyngeal epithelium and somites, respectively. The novel endothelial enhancer was conserved across species and was specific to large-caliber arteries. Its transcriptional activity was regulated by Notch signaling in vitro and in vivo, but not by ALK1 signaling and other transcription factors implicated in endothelial cell specificity. The distal endothelial enhancer was not essential for basal Hey1 expression in mouse embryos but may likely serve for Notch–dependent transcriptional control in endothelial cells together with the proximal regulatory region. These findings help in understanding the significance and regulation of endothelial Hey1 as a mediator of multiple signaling pathways in embryonic vascular formation.

Transcription factors play essential roles in complex arrays of developmental events and are implicated in the etiologies of various human diseases (1, 2). Multiple upstream signals regulate their expression and function in a cell type– and stage–specific manner, which in turn deeply influences cellular differentiation, proliferation, and movement through transcriptional control of downstream target genes. We and others previously identified the Hey family of basic helix-loop-helix transcriptional repressors that were enriched in the embryonic cardiovascular system (3–8). Among three family members, the mice null for Hey2 die soon after birth, showing cardiac malformations and abnormal chamber gene expression (9–12). Combined loss of Hey1 and Hey2 resulted in embryonic lethality due to impaired vascular network formation (11, 13, 14). In addition, we recently reported that the Hey1 deficiency caused lethal anomalies of the thoracic great vessels (15), which were similar to human congenital defects observed as isolated cardiovascular anomalies or as a manifestation of multiorgan syndromes such as 22q11.2 deletion syndrome (16). Hey1 null mice should serve as a new experimental model for human great vessel malformations, which possesses unique as well as overlapping features compared with existing mouse models (17–27). However, the relative importance of Hey1 actions in vascular cell types is not fully elucidated.

Among a variety of cellular signaling pathways involved in embryonic development, it was first demonstrated that Notch signal activation stimulated the expression of Hey family genes through the Rbpj-dependent transcriptional control (28, 29). Hey1 appears sensitive to Notch signaling in vivo, and the deletion of Rbpj or the Notch ligand Dll1 results in down-regulation of Hey1 expression in mouse embryos (5, 30–32). In addition, bone morphogenetic protein (BMP)-ALK receptor signaling activates the Hey1 and Hey2 transcription, which acts synergistically with Notch signaling in endothelial cells (33–35). Members of the Hey genes are under the control of other signaling pathways, such as those mediated by transforming growth factor β, hepatocyte growth factor, fibroblast growth factor, and Wnt (36–39). Despite these lines of evidence, it is not clear how these upstream signaling pathways control cell type specificity.
of the Hey family expression in various embryonic tissues. For example, Hey1 and Hey2 are mutually exclusive in the atrial and ventricular myocardium, whereas they look co-regulated in the atrioventricular canal and endocardium (3, 4). Expression in the pharyngeal epithelium is observed only for Hey1, whereas that in the dorsal ganglion is specific to Hey2. Detailed analyses of transcriptional regulation are necessary to further dissect distinct and complementary roles of Hey family genes in different embryonic tissues.

In this study, we generated Hey1 conditional knockout (cKO) mice and demonstrated that the Hey1 expression in endothelial cells was indispensable for proper pharyngeal arch artery (PAA) development and great vessel morphogenesis. We further performed a bacterial artificial chromosome (BAC)-based enhancer screen and identified distal enhancers that were specific to different cell types with Hey1 expression. The novel endothelial enhancer possesses transcriptional activity recapitulating endogenous endothelial expression in large-caliber arteries downstream of Notch signaling. These data will provide important information to further understand Hey1 function and its regulatory mechanisms in cardiovascular development and disease.

**Results**

**The Hey1 gene in endothelial cells is essential for thoracic great vessel development**

To clarify a cell type that requires Hey1 function for the great vessel formation, we generated a novel Hey1 cKO mouse line (Fig. S1). Hey1 is expressed in both endothelial and smooth muscle cells in developing vasculature (14), so we ablated the Hey1 gene in endothelial and smooth muscle cells using Tek-Cre and Tagln-Cre mice (40, 41), respectively. The Tek-Cre-mediated Hey1 deletion in endothelial cells caused abnormalities of great vessel structure, namely interruption of the aortic arch type B (IAA-B), right-sided aortic arch (RAA), and aberrant origin of the right subclavian artery (ARSA) at embryonic day 18.5 (E18.5) and postnatal day 0.5 (Fig. 1, A and B), which reproduced congenital anomalies observed in Hey1 null mice (15). We did not detect morphological defects of the heart and signs of circulation failure in Tek-Cre–mediated cKO embryos. Micro-CT analysis clearly showed the three-dimensional image of unordinary running and branching of the aorta in cKO embryos with RAA at E18.5 (Fig. 1C and Movies S1 and S2). On the other hand, the Hey1 deletion in vascular smooth muscle cells did not affect great vessel morphogenesis (Fig. 1, A and B). These results indicated that endothelial Hey1 expression was indispensable for the thoracic great vessel formation.

**Loss of endothelial Hey1 expression leads to disrupted tubular structure of fourth PAAAs**

IAA-B, RAA, and ARSA are all attributable to the fourth PAA defects at early developmental stages (42). We then analyzed the structures of pharyngeal arches and PAAAs of endothelial cKO embryos. In control embryos, third, fourth, and sixth PAAAs were well-formed in corresponding pharyngeal arches by E10.5 (Fig. 2A). In contrast, the disruption of endothelial tube structure was observed in fourth PAAAs of endothelial cKO embryos at E10.5 and E11.5 (Fig. 2A), whereas the size and structure of pharyngeal arches were maintained. Pan-endothelial (Pecam1) and arterial endothelial (Nrp1 and Gja5) markers were clearly expressed in the disorganized vasculature (Fig. 2, A and B). Quantitative RT–PCR analysis of Pecam1+ endothelial cells indicated that other endothelial marker genes were also expressed at normal levels in Hey1 cKO as well as null embryos (Fig. S2, A and B), suggesting that general endothelial cell differentiation was not compromised. Migration and distribution of neural crest–derived cells, which were marked with Tfax2a (AP2α) and Crabp1, were not altered by the Hey1 deficiency (Fig. S3, A and B); on the other hand, the expression of a smooth muscle marker Acta2 (α smooth muscle actin) was almost undetectable in the affected fourth PAA (Fig. 2C). These characteristics were identical to that in Hey1 null embryos (15), substantiating the importance of endothelial Hey1 for fourth PAA-derived great vessel formation.

Great vessel anomalies occurred in some endothelial cKO mice but not in others (Fig. 1B), which was unrelated to the extent of Hey1 down-regulation because its mRNA level in Pecam1+ cells did not markedly vary among cKO embryos (Fig. S2A). As is generally accepted for other genetic models showing great vessel malformations (24, 43–45), it is likely that endothelial Hey1 deficiency predisposes cKO mice to fourth PAA defects. cKO embryos retained ~30% of Hey1 transcripts in the Pecam1+ endothelial cell population, whereas null embryos showed only negligible Hey1 mRNA expression (Fig. S2A). As described in its original paper (40), the Tek-Cre–mediated recombination of the Rosa allele was observed in all identifiable endothelial cells by E10.5 (Fig. S4); however, the Hey1 floxed allele should have relatively low efficiency of Cre–mediated recombination. There may be mosaicism of the Hey1 allele recombination, although it is difficult to examine it with single-cell resolution. Nevertheless, it is of note that even a partial reduction of endothelial Hey1 expression was enough to affect fourth PAA development.

**Hey1 expression in embryonic tissues is regulated through multiple cis-regulatory regions**

Considering the importance of endothelial Hey1 expression for proper vascular development, we attempted identification of tissue-specific enhancers for Hey1 transcription to understand how Hey1 expression was regulated during embryonic development. The BAC construct that encompassed ~173 kb surrounding mouse Hey1 gene (~140 to +33kb) was used for F0 transgenic mouse LacZ reporter analysis (Fig. 3A). As shown in Fig. 3 (A and B) and Fig. S5 (A and B), the full-length BAC-LacZ reporter (designated as “a”) showed transcriptional activity that reproduced endogenous Hey1 expression in the cardiovascular system, pharyngeal epithelium, and somites at E9.5. A shorter reporter that lacked the ~140 to ~43 kb region (b) lost the activity in the atri, pharyngeal epithelium, and somites but was positive specifically in large-caliber arteries, including PAA, the dorsal aorta, and intersomitic vessels. On the other hand, the deletion without the ~43 to ~0.3 kb region (c) did not drive the reporter expression in these
arteries, whereas it retained the activity in the pharyngeal epithelium and somites. These results suggested presence of multiple cis-regulatory regions for Hey1 transcription in different embryonic tissues.

The BAC-LacZ reporter analysis located the pharyngeal/somitic enhancer to the region between 2140 and 243 kb. Consistently, a 0.6-kb fragment in the 250 kb region showed robust enhancer activity in the pharyngeal epithelium and somites (Fig. S6A). The activity of full-length BAC-LacZ reporter was reduced by the lack of the 0.6-kb fragment selectively in these tissues, whereas that in the vasculature was maintained (Fig. S6A). In addition, CRISPR/Cas9-mediated excision of the corresponding area significantly decreased endogenous Hey1 expression in a tissue-specific manner (Fig. S6B), clearly indicating its sufficiency and necessity as a new pharyngeal/somitic enhancer.

Figure 1. Loss of endothelial Hey1 leads to thoracic great vessel malformations. A, Hey1 endothelial cKO (Hey1fl/fl;Tek-Cre+) mice showed IAA-B, whereas control (Hey1wt/wt;Tek-Cre+) and smooth muscle cKO (Hey1fl/fl;Tagln-Cre+) mice had normal great vessel structures at E18.5. Ao, aorta; RCCA/LCCA, right/left common carotid artery; RSA/LSA, right/left subclavian artery. Scale bars, 1 mm. B, summary of great vessel phenotypes at E18.5 and postnatal day 0.5 (P0.5). C, front and right/left anterior oblique views of micro-CT images at E18.5. Right-sided aortic arch and unordinary branching were observed in an endothelial cKO (Hey1fl/fl;Tek-Cre+) mouse. Asc, ascending aorta; Desc, descending aorta. Three-dimensional movies are available in the supporting information.
Hey1-proximal region responds to vascular signals but is insufficient for endothelial specific transcription in vivo

BAC-LacZ studies indicated that the endothelial enhancer was present between −43 and −0.3 kb (Fig. 3, A and B), and we further examined how endothelial Hey1 transcription was controlled in mouse embryos. It was previously reported that the HEY1 expression was synergistically activated by Notch and ALK1 signaling in endothelial cells (Fig. S7A) (34). Transcriptional activity driven by the proximal region (−0.5 to +0.2 kb) was up-regulated by the Notch 1 intracellular domain (N1ICD) expression and BMP9 treatment (Fig. 4A) in luciferase reporter assays, which was consistent with the previous finding using BMP6 (33). The proximal region contained three potential Rbpj-binding sites, one of which was required for transcriptional activation (Fig. 4A and Fig. S7B).

Because Notch and ALK1 signaling pathways are heavily involved in endothelial differentiation and vascular formation (34, 35), we first tested the importance of the proximal region for endothelial specific transcription in mouse embryos. Unexpectedly, the proximal region did not show reproducible enhancer activity in the vasculature of LacZ reporter embryos (Fig. 4B and Fig. S7C), strongly suggesting that the proximal region could serve for signal responsiveness but was insufficient to achieve endothelial transcription in vivo.

A distal endothelial enhancer is located 18 kb upstream of the mouse Hey1 gene

With this result, it was the best conceivable that an additional enhancer for endothelial Hey1 expression was present in the region between −43 and −0.3 kb. We then analyzed publicly available ChIP sequencing (ChIP-Seq) (Gene Expression Omnibus, under accession number GSE88789) data sets and found the transcriptional coactivator p300 binding 18 kb upstream of the mouse Hey1 gene in embryonic endothelial cells (Fig. 5A) (46). Indeed, the ablation of a 1.6-kb sequence at the −18 kb region clearly deprived the full-length BAC-LacZ reporter of vascular transcriptional activity at E9.5 (Fig. 5A and B) and Fig. S8A). In addition, the LacZ reporter analysis indicated that the 1.6-kb fragment had highly specific enhancer activity in embryonic vasculature (Fig. 5A and B and Fig. S8A). A detailed analysis using the reporter mouse lines revealed that the transcriptional activity was restricted to endothelial cells of large-
caliber arteries, such as PAAs and the dorsal aorta at E9.5 and E10.5 (Fig. 5C and Fig. S8B). The 1.6-kb sequence was highly conserved among species (Fig. 5A). A comparable 1.9-kb fragment in the human HEY1 16 kb region also showed LacZ reporter expression in vascular endothelial cells/embryos positive in any tissues. B, representative images of E9.5 transgenic embryos are shown with endogenous Hey1 mRNA expression detected in in situ hybridization. β-Gal activity by the full-length BAC-LacZ reporter (a) reproduced endogenous Hey1 mRNA expression in the dorsal aorta (DA), atrium (A), sinus venosus (SV), pharyngeal arch (PA), somites, and floor plate (FP) of neural tube (NT). Deletion of the −140 to −43 kb region resulted in the loss of activity in the atrium, pharyngeal epithelium, and somites (b), whereas that of the −43 to −0.3 kb region diminished the activity in DA, PAA, and intersomitic vessels (ISV) (c). Sections of whole-mount in situ hybridization are displayed to compare mRNA expression and reporter activity in PAAs. Arrowheads, PAAs with significant signals. Magnified views of DA are also shown at the bottom. PAAs are numbered. OV, otic vesicle. Scale bars in whole-mount images, sections, and magnified DA images are 500, 200, and 50 μm, respectively. Results of other F0 embryos are displayed in Fig. S5.

The newly identified distal enhancer drove endothelial transcription that recapitulated endogenous Hey1 expression in large caliber arteries. Contrary to our expectation, however, CRISPR/Cas9-mediated excision of this region did not cause significant decrement of endogenous Hey1 mRNA level in mouse embryos (Fig. S9, A and B). These results suggest that the proximal region or an unidentified enhancer may compensate the lack of the distal endothelial activity, whereas it was technically difficult to test such a hypothesis in mouse embryos by deleting multiple candidate regions around the Hey1 gene.

Hey1 distal endothelial enhancer is controlled by Notch signaling

Last, we examined regulatory mechanisms of the distal enhancer for its endothelial activity. In a screen for potential transcriptional regulators based on the consensus binding
motif analysis, Foxc1, Foxc2, and N1ICD showed highest induction of the luciferase reporter expression driven by the distal enhancer (Fig. 6, A and B). The responsiveness to Foxc proteins was assigned to a 430-bp fragment (Fig. S10 A) and three binding sites (Fig. 6, A and C). Nevertheless, their mutations in the 1.6-kb enhancer did not affect LacZ reporter expression in mouse embryos (Fig. 6 D and Fig. S10 B). Scale bars, 500 μm. Results of other F0 embryos are displayed in Fig. S7.

As previously reported (31), Hey1 mRNA expression in multiple embryonic tissues, including PAAs and the aorta, was markedly decreased in Rbpj null embryos, whereas severe developmental defects made its interpretation difficult (data not shown). We then analyzed whether the distal endothelial enhancer activity was dysregulated in Rbpj null embryos by intercrossing them with EGFP reporter mice. As shown in Fig. 6 F, vascular EGFP expression was suppressed to a virtually negligible level with the Rbpj null background even in E8.5 embryos showing relatively mild developmental defects. These results verified complete Notch dependence of the distal endothelial enhancer and further indicated its usefulness as a surrogate to monitor the Notch signal activity in the embryonic vasculature.

Discussion
In this study, we demonstrate that the conditional deletion of Hey1 in endothelial cells causes abnormalities of thoracic great
vessel morphogenesis. Embryonic arterial defects occur only in fourth PAAs, which is identical to those observed in Hey1 null mice (15). Fourth PAAs are prone to be disorganized during remodeling due to unique characteristics, including a nonmuscular region where the expression of α-smooth muscle actin is reduced or absent (22, 47). Left and right fourth PAAs normally form a part of the aortic arch and right subclavian artery, and their defects cause IAA-B and ARSA, respectively. RAA results from the connection of right fourth PAA to the aorta, which compensates for the abnormal involution of left fourth PAA. It is still unclear how the Hey1 deficiency in endothelial cells acts as a predisposing factor for such abnormalities. Among numerous signaling factors implicated in fourth PAA development (17, 19–21, 23, 24, 27, 48–51), Hey1 may be functionally interconnected with endothelial regulatory genes, such as Pnxn1.
We performed RNA-Seq analysis of embryonic endothelial cells from Hey1 endothelial cKO as well as null mice, but the results did not show the disturbance of a single gene or a few genes that explained vascular abnormalities by the Hey1 deficiency. There was no significant enrichment that could be directly linked to the pathogenesis in gene ontology or KEGG pathway analysis (data not shown). ChIP-grade antibodies against Hey1 are not available, but ChIP-Seq was previously performed using FLAG-tagged HEY1 overexpression in cultured cells (52, 53). These studies suggested possible Hey1 target genes, includingDll4, Kdr, and Foxc1, but the expression of those candidate genes did not change in endothelial cells of Hey1 cKO and null embryos (data not shown). Mosaicism of the Hey1 allele recombination may have made such expression analyses difficult, and a specific population of endothelial cells in a particular region of the embryos might be more sensitive to the Hey1 dosage. Importance in endothelial cells is also evident for complementary functions by Hey1 and Hey2 at earlier vascular formation because their loss in endothelial cells reproduces vascular defects and embryonic lethality observed in double-null mice (14). Advanced technologies such as multomics

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analyses at the single-cell level may give a clue to downstream signaling pathways that are central to Hey-dependent endothelial functions.

Whereas the Hey1 deletion in smooth muscle cells did not cause great vessel anomalies, that does not necessarily negate its supportive functions in smooth muscle cells. Consistently, the incidence of great vessel anomalies appeared lower in endothelial Hey1 cKO mice compared with null mice (15). Although significant retention of Hey1 transcripts in endothelial cells, probably due to low recombination efficiency of the Hey1 floxed allele, can explain low phenotypic penetrance in cKO mice, Hey1 functions in smooth muscle cells or other cell types may also be required for proper fourth PAA development. However, it is noteworthy that Hey1-deficient phenotypes are clearly distinguishable from neural crest–related abnormalities. IAA-B and other great vessel malformations are often observed in patients with 22q11.2 deletion syndrome, and TBX1 is one of the most important genes in the minideletion region (19, 54–56). Tbx1 transcription factor is required for migration, proliferation, and/or survival of neural crest–derived cells, and its mutant mice are defective also in the outflow tract, thymus, and craniofacial structures (19, 44, 55–57). In sharp contrast, Hey1 null (15) as well as endothelial cKO mice (Fig. S3) did not show abnormalities in neural crest–derived cell behavior as well as malformation of the pharyngeal arches, cardiac outflow tract, and other neural crest–related structures. Mouse embryos with inactivation of Notch signaling in neural crest–derived cells showed down-regulation of Hey family expression in the smooth muscle layer of PAA s, but the fourth PAA defects only rarely occurred in these mutant mice (58). Furthermore, the heterozygous Tbx1 deletion in the pharyngeal epithelium leads to great vessel abnormalities (59), indicating that Tbx1 and Hey1 control distinct signaling pathways for PAA development in different cell types.

The present study further elucidates that Hey1 transcription in embryonic tissues is controlled through multiple cis-regulatory regions. In particular, endothelial Hey1 expression essential for vascular development appears maintained through at least two complementary regions. The proximal region adjacent to exon 1 responds to both Notch and ALK1 signaling but is not sufficient to implement endothelial transcription in mouse embryos. The novel enhancer at the distal promoter is differentially expressed in the ventricle (4), and its deletion clearly shows importance as a specific enhancer, but a low level of Hey1 mRNA is still detectable in these tissues. Because the BAC-LacZ reporter activity in these two areas is suppressed by the lack of proximal region (Fig. 4B), the proximal region may serve for Hey1 transcription also in the pharyngeal epithelium and somites. In addition, Hey1 expression restricted to the atrium of the heart is particularly interesting because its family gene Hey2 is differentially expressed in the ventricle (3, 4). We recently reported that ventricular Hey2 expression was controlled by Tbx20 and Gata family proteins through its distal enhancer (71). Although the full-length BAC apparently contains transcriptional activity in the atrium, we have not specified where the Hey1 atrial enhancer is located. Studying the precise mechanisms of Hey1 transcriptional regulation will help understand how tissue-specific gene expression is achieved in the cardiovascular system and other organs during embryonic development.

Increasing evidence supports the importance of the HEY family in human physiology and disease. Their expression is often dysregulated downstream of Notch signaling in various diseases (72–74). In addition, a fusion of HEY1 and NCOA1 causes chondrosarcoma and aberrant HEY1 expression is correlated with the metastasis, therapeutic response, and patient survival in multiple cancer types (38, 75–78). A variant near the HEY2...
locus is associated with clinical characteristics of Brugada syndrome (79), and HEY2 also acts as a key factor for the differentiation of pluripotent stem cells into ventricular myocytes (80). It is tempting to hypothesize that variations in the protein-coding or enhancer regions of the HEY1 gene affect its molecular function or expression level in human patients with isolated great vessel anomalies. HEY1 may also be associated with clinical variability of 22q11.2 deletion syndrome as a modifier gene. It will be of clinical interest to examine possible involvement of HEY1 in such entities of human congenital diseases.

**Experimental procedures**

**Mouse strains**

The founder mice with the Hey1 floxed allele were generated as follows (Fig. S1). The targeting vector was designed to delete exons 2, 3, and 4 of the Hey1 gene by Cre-mediated recombination and was electroporated into HK3i ES cells (81) with two single guide RNAs (Table S1) and the human optimized Cas9 expression plasmid px330 (Addgene plasmid 42230) (82). Recombinant ES cells were injected into eight-cell stage embryos to produce chimera mice. The Pkg-Neo cassette flanked by FRTs was removed by crossing the chimera mice with Flp-expressing female mice (Jackson Laboratory, JAX accession no. CDB1302K). Hey1(loxP) SM22α-Cre (JAX 004746) mice were used for expression and phenotype analyses.

**Micro-CT imaging**

A contrast reagent eXIA 160XL (Summit Pharmaceutical International) was introduced from the right ventricle of E18.5 mouse embryos, and micro-CT images were taken with an 8-μm pixel size using SkyScan 1276 (Bruker).

**Fluorescence and magnetic activated cell sorting (FACS and MACS)**

E10.5 mouse embryos were minced and incubated in 0.1% collagenase type II (Worthington, catalog no. 4176) with mixing and suspension. The dissociated cells were filtered through a BD Falcon Cell Strainer (70 μm, catalog no. 352350) and resuspended in 0.1% BSA and 2 mM EDTA in Ca2+/Mg2+-free PBS. For FACS, the cells were incubated with phycoerythrin- or APC-Cy7-conjugated α-mouse CD31 (Pecam1) antibody MEC13.3 (BioLegend, catalog no. 102507 or 561814) and FITC-conjugated α-mouse CD45 antibody 30-F11 (BioLegend, catalog no. 103107), followed by sorting with FACS Aria (BD Biosciences). For MACS, the cells were incubated with CD31 microbeads (Miltenyi Biotec, catalog no. 130-097-418) and sorted using MS columns (Miltenyi Biotec, catalog no. 130-042-201).

**Expression analysis**

Total RNA was extracted from the FACS- or MACS-sorted cells using Nucleospin RNA XS (Macherey Nagel), and cDNA was synthesized using the PrimeScript RT reagent kit (Takara). Real-time PCR analysis was performed with KAPA SYBR FAST qPCR Master Mix (KAPA Biosystems) and a LightCycler 96 system (Roche Applied Science). 18S rRNA was used as a reference. Primer sequences are listed in Table S1. The following antibodies were used for immunohistochemistry on frozen sections: α-Pecam1 (MEC13.3, BD Pharmingen, catalog no. 550274), phycoerythrin-conjugated α-Pecam1 (MEC13.3, BioLegend, catalog no. 102507), α-Nrp1 (Abcam, catalog no. ab81321), α-Gia5 (Invitrogen, catalog no. 36-4900), Cy3-conjugated α-Acta2 (Sigma, catalog no. 6198), and α-Tap2a (Developmental Studies Hybridoma Bank, catalog no. 3B5). Whole-mount in situ hybridization was performed as we described previously (71). Statistical analysis was performed using Student’s t test.

**LacZ reporter analysis**

A BAC clone encompassing the mouse Hey1 gene, RP23-255P16, was purchased from Advanced GenoTechs Co. The BAC-LacZ reporter construct was generated by inserting the nuclear localization signal (nls)-LacZ-poly(A) fragment at the first ATG site of the Hey1 gene as we reported previously (84). Deletion BAC series were prepared with the kanamycin insertion. Plasmid-based LacZ reporters of the proximal region and distal enhancers were made using the nls-LacZ-poly(A) and hs68 promoter-nls-LacZ-poly(A) vectors, respectively. Rbpj-binding sites in the −18 kb enhancer were mutated as follows: site 1, ACGTGATGGGAAATGGA → ACGTGACCTTAATTGGA; site 2, GGAGCGTGGGAAACCCGG → GGA-GCGCATTAAACCCCG. Foxc-binding sites were mutated as follows: site 1, AGCTTTATTGAGATACA → AGCTTATAAGGA-GATA; site 2, TCACCTAAAATATGTTGA → TCACCT-TAATCTGTGA; site 3, TAGAATATTTTCATTATT → TAGAAaAaTTACTATT. Transgenic mice were generated using standard methods (71). In brief, the circular BAC reporter or linear enhancer reporter fragment (1–3 ng/μl) was injected into the pronuclei of BDF1 fertilized eggs. β-Gal reaction of mouse embryos was performed as we described previously (71).

**Luciferase reporter analysis**

A DNA fragment of the −18 kb enhancer was inserted into the pGL4.10[luc2] plasmid containing the MLP promoter (85). Human umbilical vein endothelial cells were seeded in the EGM2 medium (Lonza) 24 h before the transfection, and the plasmids for the luciferase, CMV-β-gal, and mouse N1ICD expression were introduced using Lipofectamine PLUS and

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LTX reagents (Life Technologies, Inc.). After 1 h of transfection, the cells were preconditioned in the EBM2 medium (Lonza) containing 0.2% BSA and 0.2% fetal bovine serum for 4 h, followed by the treatment with BMP9 (R&D Systems) or vehicle. Transfection of 293T cells was performed according to our previous report (86). Luciferase and β-gal activities were measured using FLUOStar Omega (BMG LABTECH). Triplicated assays were independently performed three times, which gave reproducible results. Statistical analysis was performed using Tukey’s test.

**Data availability**

All data are contained within the article and supporting information.

**Acknowledgments**—We thank T. Higuchi and A. Ohki for instruction on micro-CT imaging; K. Miyazono, N. Mochizuki, and Y. Okada for reporter or expression plasmids; N. Yoshida, N. Nagata, S. Kusumoto, H. Kawakami, and K. Endo for laboratory assistance; and T. Fukayama, T. Tanaka, and N. Liu for experimental support.


**Funding and additional information**—This work was supported in part by the grants from the Japan Society for the Promotion of Science (Grants-in Aid for Scientific Research 15H04883, 15K15407, 16K08605, 18H02787, and 19H03398) (to Y. W. and O. N.), the Novartis Foundation for Cardiovascular Diseases (to O. N.), the Cooperative Research Project Program of Joint Usage/Research Center at the Institute of Development, Aging, and Cancer, Tohoku University (to Y. W.), Japan Agency for Medical Research and Development (AMED-CREST) Grant JP19gm0810001 (to T. O.), and the Intramural Research Fund for Cardiovascular Diseases of National Cerebral and Cardiovascular Center (to Y. W. and O. N.).

**Conflict of interest**—The authors declare that they have no conflicts of interest with the contents of this article.

**Abbreviations**—The abbreviations used are: BMP, bone morphogenetic protein; cKO, conditional knockout; PAA, pharyngeal arch artery; BAC, bacterial artificial chromosome; IAA-B, aortic arch type B; RAA, right-sided aortic arch; ARSA, aberrant origin of the right subclavian artery; E, embryonic day; N1ICD, Notch 1 intracellular domain; MACS, magnetic activated cell sorting; CT, computed tomography; nls, nuclear localization signal.

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doi: 10.1074/jbc.RA120.015003 originally published online October 16, 2020

Access the most updated version of this article at doi: 10.1074/jbc.RA120.015003

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