Tmem100, an ALK1 receptor signaling-dependent gene essential for arterial endothelium differentiation and vascular morphogenesis

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Members of the transforming growth factor-β superfamily play essential roles in various aspects of embryonic development and physiological organ function. Among them, bone morphogenetic protein (BMP) 9 and BMP10 regulate embryonic vascular development by activating their endothelial receptor ALK1 (activin receptor-like kinase 1, also called Acvrl1). ALK1-mediated intracellular signaling is implicated in the etiologies of human diseases, but their downstream functional proteins are largely unknown. In this study, we identified *Tmem100*, a gene encoding a previously uncharacterized intracellular transmembrane protein, to be an embryonic endothelium-enriched gene activated by BMP9 and BMP10 through the ALK1 receptor. *Tmem100* null mice showed embryonic lethality due to impaired differentiation of arterial endothelium and defects of vascular morphogenesis, which phenocopied most of the vascular abnormalities observed with the *Acvrl1/Alk1* deficiency. The activity of Notch- and Akt-mediated signaling, which is essential for vascular development, was down-regulated in *Tmem100* null mice. *Cre*-mediated deletion of *Tmem100* in endothelial cells was sufficient to recapitulate the null phenotypes. These data indicated that TMEM100 may play indispensable roles downstream of BMP9/BMP10-ALK1 signaling during endothelial differentiation and vascular morphogenesis.

Formation of the cardiovascular network is essential for proper embryonic development, and neovascularization is associated with various adult diseases, such as ischemic heart diseases, retinopathy, and cancer, acting either protectively or deteriorately in those pathological states (1, 2). At early embryonic stages, vasculogenesis occurs as a de novo organization of endothelial cell plexus. A series of processes for vascular remodeling and maturation follows, including an initial phase of endothelial cell migration, proliferation, and tubular reorganization, collectively called angiogenesis, and a maturation phase when the structure of blood vessels is established by the stabilization of endothelial cells, acquirement of arterial or venous identity, and recruitment of mural cells to vascular walls (1, 2).

A variety of cellular signaling pathways controlling vascular development involve cytokines and growth factors, such as vascular endothelial growth factors (VEGFs), angiopoietins, transforming growth factor β (TGF-β), and bone morphogenetic proteins (BMPs) (3, 4). Among them, BMP9 and BMP10 act mainly through an endothelium-specific ALK1 (activin receptor-like kinase 1) receptor and promote arterial endothelial maturation and quiescence (5–7). Targeting disruption of the genes for the ALK1 receptor, a type III coreceptor endoglin, and downstream signaling components such as Tak1/Map3k7 caused embryonic lethality with remarkably similar defects of arterial endothelium differentiation and vascular morphogenesis (8–11). Despite clear demonstration of its critical roles in embryonic development, endothelial functional proteins downstream of the BMP9/BMP10-ALK1 signaling pathway remained unclear.

In a microarray screen to search for endothelial genes downstream of BMP9/BMP10-ALK1 signaling, we found that the expression of *Tmem100*, a gene encoding an intracellular transmembrane protein of unknown functions, was markedly augmented by BMP9 and BMP10. It was recently reported that *Tmem100* mRNA expression significantly decreased in the lung of *Acvrl1/Alk1* conditional knockout mice and that *Tmem100* drove endothelial-enriched expression of lacZ reporter in mouse embryos (12). In this paper, we demonstrated that *Tmem100* null mice and endothelial-specific *Tmem100* knockout mice showed fatal defects of arterial endothelium differentiation and vascular morphogenesis, which are virtually identical to the abnormalities due to the *Acvrl1/Alk1* deficiency. These results suggest that TMEM100 may play essential roles as a downstream target protein of BMP9/BMP10-ALK1 signaling in embryonic vascular development.

Results

***Tmem100*: An Arterial Endothelium-Enriched Gene Downstream of BMP9/BMP10-ALK1 Signaling.** In an attempt to identify endothelial genes downstream of BMP9/BMP10-ALK1 signaling, we first analyzed whether human umbilical artery endothelial cells (HUAEC) responded to the treatment with various TGF-β–related factors. Among several ligands tested, BMP9 potently stimulated the phosphorylation of Smad1/5/8 (Fig. S1 *A* and *B*). We then compared gene expression profiles of BMP9-treated HUAEC and control cells by an RNA microarray analysis (Fig. S1*C*). In addition to known BMP9-target genes such as *SMAD6, SMAD7*, *ID1*, and *ID2*, we found that *TMEM100* showed a marked activation of mRNA expression by the BMP9 treatment (Fig. 1*A*). Quantitative RT-PCR and Western blot analysis confirmed a dose-dependent induction of the *TMEM100* expression by BMP9, up to more than 100-fold (Fig. 1*A*). Similar levels of activation were observed with BMP10 (Fig. S1*D*), and those effects were significantly inhibited by knockdown of *ACVRL1/ALK1*, *BMPR2*, and *SMAD4* (Fig. 1*B* and Fig. S1*E*). These results indicated that BMP9/BMP10-induced *TMEM100* expression occurred through the ALK1/BMPR2 receptor complex and Smad-mediated transcriptional regulation.

TMEM100 is a gene encoding a protein with two putative transmembrane domains, but its physiological significance has not been described. The structure of TMEM100 protein is highly

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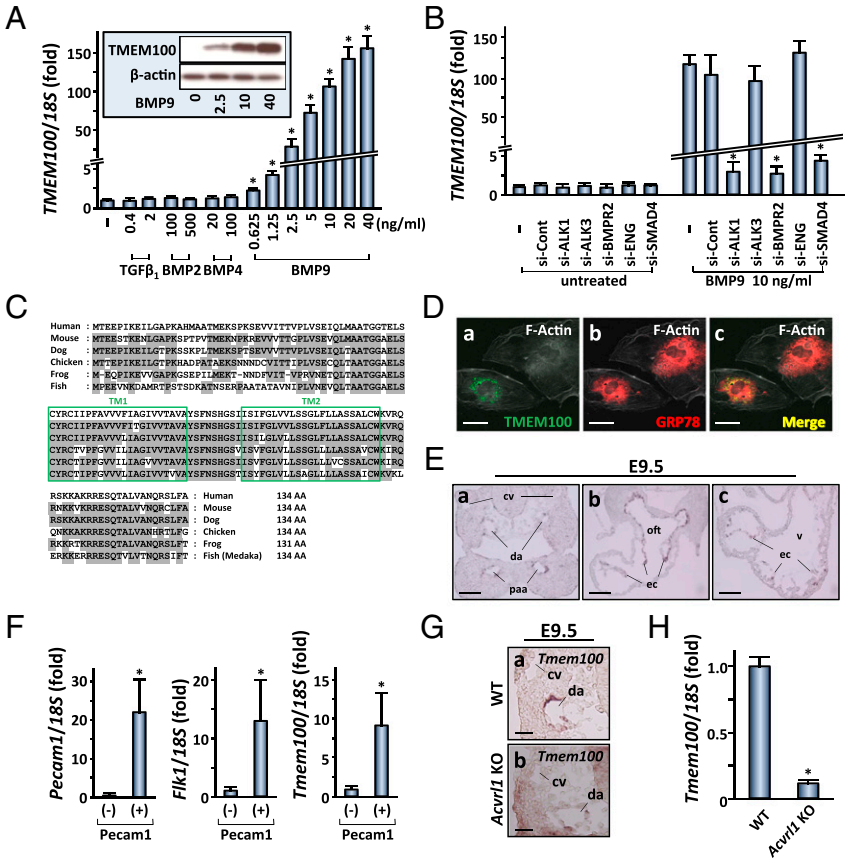

Fig. 1. *Tmem100*: an arterial endothelium-enriched gene downstream of BMP9/BMP10-ALK1 signaling. (*A*) BMP9 markedly increases *TMEM100* mRNA expression at 24 h in a dose-dependent manner. Quantitative RT-PCR analysis is shown. Fold increase relative to the untreated cells is shown with SD. (*Inset*) Western blot result shows a dose-dependent increase in TMEM100 protein expression. –, untreated. (*B*) BMP9-induced *TMEM100* mRNA expression is inhibited by pretreatment with ACVRL1/ALK1 siRNA (si-ALK1), BMPR2 siRNA (si-BMPR2), or SMAD4 siRNA (si-SMAD4), but not with BMPR1A/ALK3 siRNA (si-ALK3), Endoglin siRNA (si-ENG), or control siRNA (si-Cont). Quantitative RT-PCR is shown. –, untreated. (*C*) Amino acid sequences of TMEM100 from various species. Boxes represent two putative transmembrane (TM) domains. Residues conserved with the human sequence are shaded in gray. (*D*) TMEM100 protein predominantly resides in the perinuclear region overlapping endoplasmic reticulum marked by anti-GRP78/HSPA5 antibody. Immunocytochemistry of HUAEC transfected with a TMEM100-FLAG expression plasmid is shown. (*E*) *Tmem100* mRNA is expressed specifically in arterial endothelial cells of pharyngeal arch artery (paa) and dorsal aorta (da) as well as in endocardium (ec), but not in cardinal vein (cv), at E9.5. In situ hybridization is shown. oft, outflow tract; v, ventricle. (*F*) *Tmem100* expression is enriched in endothelial cells also at earlier developmental stages. Shown is FACS sorting of Pecam1-positive cells from E8.5 embryos followed by quantitative RT-PCR. *Pecam1* and *Kdr/Flk1* expression was examined to confirm proper selection of the endothelial population. (*G*) *Tmem100* expression in dorsal aorta is reduced in *Acvrl1/Alk1* null embryos at E9.5. In situ hybridization is shown. (*H*) *Tmem100* expression significantly decreases in the yolk sac of *Acvrl1/Alk1* null mice at E9.5. Quantitative RT-PCR is shown. In *A*, *B*, *F*, and *H*, asterisks indicate data with statistical significance (*$P < 0.05$). (Scale bars: *D*, 10 μm; *E*, 50 μm; and *G*, 20 μm.)

conserved from fish to humans, especially in its putative transmembrane domains (Fig. 1*C*); however, no structurally related family of proteins was found in any species, indicating that TMEM100 represents a previously uncharacterized entity of functional proteins. Levels of endogenous TMEM100 expression in HUAEC were below detection limits in immunocytochemical analyses, whereas TMEM100 protein expressed using a mammalian expression plasmid resided in the perinuclear region marked by endoplasmic reticulum (ER) proteins such as GRP78/HSPA5 (Fig. 1*D* and Fig. S2*A*). Endogenous TMEM100 protein in BMP9-treated HUAEC was recovered to the subcellular membrane fraction and was enriched in the ER microsome (Fig. S2 *B* and *C*). In adult mice, *Tmem100* was most abundantly expressed in the lung with a lower level of expression in the brain, heart, and muscle (Fig. S2*D*). In mouse embryos, *Tmem100* mRNA was detected from embryonic day (E) 8.5 (Fig. S2*E*) and was enriched in arterial endothelium and endocardium (Fig. 1*E* and see Fig. S4*A*). Quantitative RT-PCR confirmed the enrichment of *Tmem100* mRNA in the platelet/endothelial cell adhesion molecule 1 (Pecam1)-positive endothelial cell population sorted from E8.5 embryos (Fig. 1*F*). Consistent with ALK1-mediated *Tmem100* expression in HUAEC (Fig. 1 *A* and *B*) and the previous report (12), *Tmem100* mRNA expression in arterial endothelium was significantly reduced in *Acvrl1/Alk1* null embryos (Fig. 1 *G* and *H*).

These results prompted us to examine functional significance of TMEM100 in vascular differentiation and morphogenesis during development.

Embryonic Lethality by Targeted Disruption of *Tmem100*. We generated *Tmem100*-deficient mice in which exon 3 encoding the entire coding region was replaced with a lacZ-neo cassette (Fig. S3 *A*–*C*). Mice heterozygous for the *Tmem100* mutation survived to adulthood and were fertile. LacZ reporter driven by the *Tmem100* locus (*Tmem100-lacZ*) was predominantly expressed in developing arteries, including dorsal aorta and pharyngeal arch; intersomitic, umbilical, and vitelline arteries; and endocardium (Fig. 2 *A* and *B* and Fig. S4 *B*–*D*). Vascular expression of *Tmem100-lacZ* overlapped with that of lacZ reporter knocked into the *Acvrl1/Alk1* locus (Fig. 2 *A* and *B*) and was observed in

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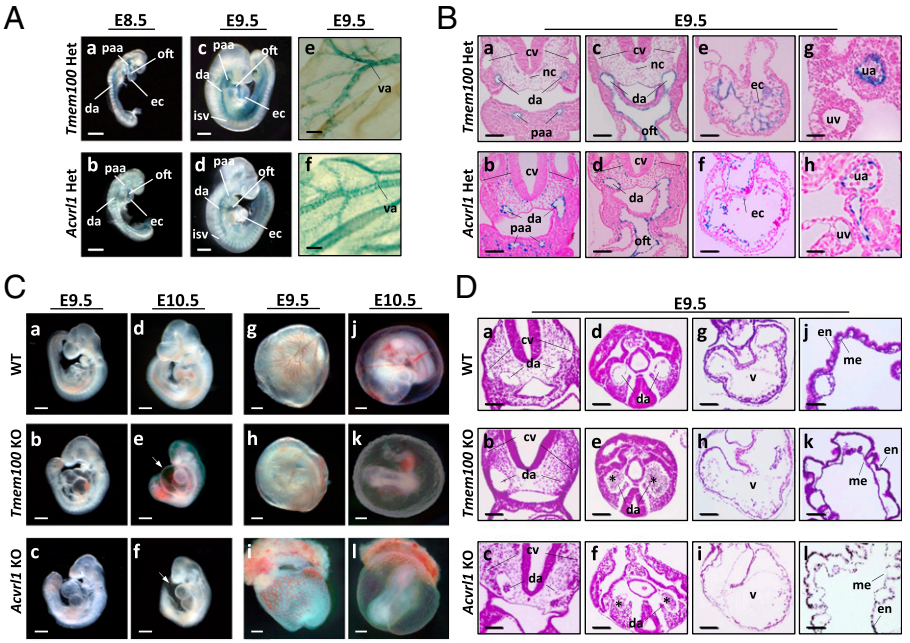

Fig. 2. *Tmem100* and *Acvrl1/Alk1*: arterial expression of knock-in lacZ reporter and angiogenesis defects in null embryos. (*A* and *B*) Knock-in lacZ reporter activity is detected in major arteries and endocardium in both *Tmem100* and *Acvrl1/Alk1* heterozygous mice. Sections counterstained with nuclear fast red are shown in *B*. (*C*) *Tmem100* null embryos die in utero, showing cardiac dysmorphogenesis and enlargement at E9.5, massive pericardial effusion (arrow) and severe growth retardation at E10.5, and absence of large vitelline vessels in the yolk sac at E10.5. These phenotypes are also observed in *Acvrl1/Alk1* null embryos. (*D*) *Tmem100* null and *Acvrl1/Alk1* null embryos have remarkably similar defects of cardiovascular morphogenesis. Paired dorsal aortas show marked dilatation and narrowing, and clumps of blood cells are frequently seen in the caudal region (asterisk). Mural layers surrounding the aorta and myocardial wall are thinner and ventricular trabeculation is not formed well. The yolk sac shows detachment of endodermal (en) and mesodermal (me) layers and abnormal vessel dilatation. H&E staining at E9.5 is shown. cv, cardinal vein; da, dorsal aorta; isv, intersomitic vessel; nc, notochord; oft, outflow tract; paa, pharyngeal arch artery; ua, umbilical artery; uv, umbilical vein; v, ventricle; va, vitelline artery. (Scale bars: *A*, *a–d*, and *C*, 200 μm; *A*, *e* and *f*, *B*, a–f, and *D*, *a–i*, 50 μm; and *B*, *g* and *h*, and *D*, *j–l*, 20 μm.)

the endothelial layer of dorsal aorta but not in smooth muscle cells (Fig. S4 *C* and *D*). At later embryonic stages, *Tmem100-lacZ* expression was also detected in the limb buds, somites, and other tissues (Fig. S4*B*). In the adult lung, *lacZ* reporter was expressed in vascular endothelial cells as well as in alveolar cells (Fig. S4*E*).

Breeding of heterozygous mice revealed a significant deviation from expected inheritance, and no surviving embryos were recovered at and after E11.0 (Fig. S3*D*). *Tmem100* null embryos at E10.5 were strongly affected, showing abnormal cardiac morphology, massive pericardial effusion, and growth retardation (Fig. 2*C*), suggesting that cardiovascular failure caused embryonic lethality of *Tmem100* null mice.

Vascular Defects in *Tmem100* Null Mice. *Tmem100* null embryos showed no detectable phenotypes until E8.5, and the formation of primitive vasculature appeared normal (Fig. S5*A*). Earliest signs of deficiency were observed in the vasculature at E9.0–9.5. The yolk sac of *Tmem100* null embryos showed reduction of vitelline circulation (Fig. 2*C*). Consistently, *Tmem100* null embryos displayed a variety of severe abnormalities in vascular morphology, as shown in Fig. 2*D*. One of the paired dorsal aortae frequently showed a marked dilatation with a narrowing or closure on the opposite side, and clumps of blood cells were often observed in the caudal region, which is likely to be due to vascular obstruction. Cardiac myocardium was thinner, and the yolk sacs showed detachment of endodermal and mesodermal layers and abnormal vessel dilatation (Fig. 2*D*).

Whole-mount Pecam1 staining revealed abnormally coarse vascular patterns, indicative of impaired vascular remodeling (Fig. 3*A*), and India ink injection identified arteriovenous malformation from dorsal aorta toward sinus venosus in *Tmem100* null embryos (Fig. 3*B* and Fig. S5*B*). These phenotypes of *Tmem100* null embryos were mostly identical to those observed in *Acvrl1/Alk1* null embryos (Fig. 2 *C* and *D* and Fig. S6*A*) and reported in previous studies (8, 9).

Defects of Arterial Differentiation in *Tmem100* Null Embryos. ALK1-mediated signaling is implicated in establishing arterial identity of vascular endothelial cells (8, 9). Indeed, the expression of arterial marker genes *Efnb2* and *Gja5/Cx40* was significantly decreased in dorsal aorta of *Tmem100* null embryos as early as E8.5 (Fig. 3 *C* and *D*). In contrast, expression of *Cdh5* (encoding VE-cadherin), *Kdr/Flk1*, and *Pecam1* was maintained (Fig. 3 *C–E*), suggesting that arterial specification is compromised by *Tmem100* deficiency. Upon normal arterial maturation, mural smooth muscle precursors marked by Sm22 are recruited (1, 2); however, the *Sm22/Tagln* expression around the dorsal aorta was significantly reduced in *Tmem100* null embryos (Fig. 3*F*). These defects of arterial differentiation and maturation were also observed in *Acvrl1/Alk1* null mice (Fig. S6 *B–E*).

Down-Regulation of Endothelial Signaling Pathways in *Tmem100* Null Embryos. To begin to understand mechanisms of vascular abnormalities caused by the *Tmem100* deficiency, we performed a microarray analysis using the yolk sac, which is a vascular-rich tissue with obvious null phenotypes (Fig. S7*A*). We observed a significant decrease in the expression of arterial markers, such as *Efnb2*, *Gja4/Cx37*, and *Gja5/Cx40*, and an increase in vein-specific *Ephb4* expression in the *Tmem100* null yolk sac (Fig. 4*A* and Fig. S7*B*), which is consistent with our notion that *Tmem100* is essential for arterial endothelium differentiation.

We also found that the expression of Notch downstream genes *Hrt1/Hey1*, *Hrt2/Hey2*, *Hrt3/Heyl*, and *Hes5* (13, 14) was signifi-

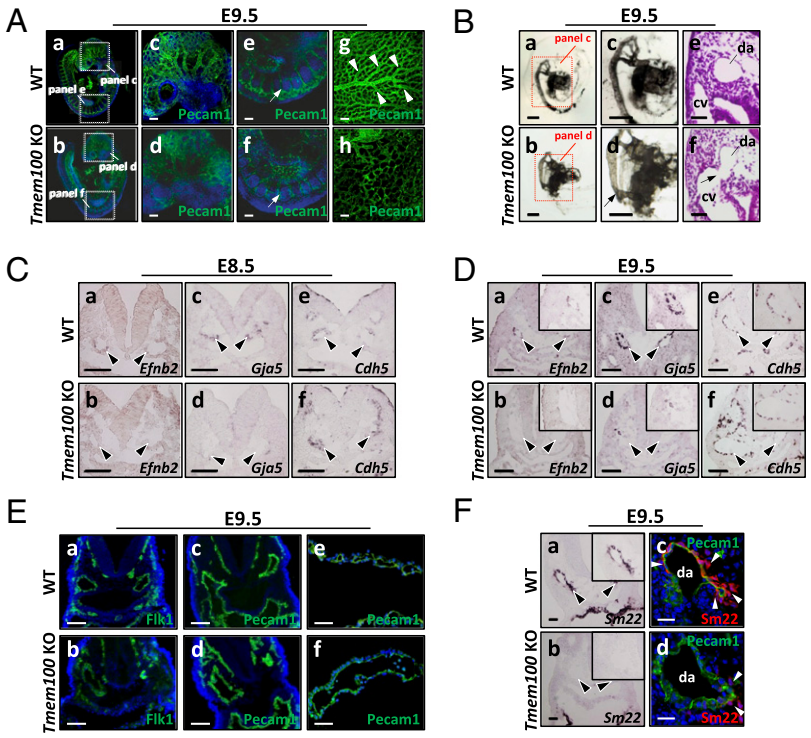

Fig. 3. Impairment of vascular remodeling and arterial endothelium differentiation in *Tmem100* null embryos. (*A*) Impaired vascular remodeling is visualized by whole-mount Pecam1 immunostaining in the *Tmem100* null embryos (*b*, *d*, and *f*) and yolk sac (*h*). Arrows indicate intersomitic vessels. Arrowheads indicate highly organized vascular branches in the wild-type yolk sac. *a*–*f* are shown with nuclear DAPI stain. (*B*) Abnormal vascular connections (arrow) between dorsal aorta and cardinal veins are observed in null embryos. Shown is microangiography by India ink injection and H&E staining. (*C* and *D*) Expression of arterial endothelium marker genes *Efnb2* and *Gja5/Cx40* diminishes in dorsal aorta (arrowheads) of *Tmem100* null embryos at E8.5 (*C*) and E9.5 (*D*), whereas maintenance of *Cdh5* expression indicates existence of endothelial layers. In situ hybridization is shown. (*E*) Flk1 and Pecam1 expression is preserved in dorsal aorta of *Tmem100* null embryo (*b* and *d*) and yolk sac vasculature (*f*). Immunohistochemistry with nuclear DAPI stain is shown. (*F*) Expression of *Sm22/Tagln* mRNA and SM22 protein in the mural layers of dorsal aorta is significantly reduced in *Tmem100* null embryos (arrowheads). In situ hybridization (*a* and *b*) and immunohistochemistry with nuclear DAPI stain (*c* and *d*) are shown. *Insets* in *D* and *F* show magnified views of dorsal aorta. (Scale bars: *A*, *C*, *D*, and *E*, *a*–*d*, 50 μm; *B*, *a*–*d*, 200 μm; and *B*, *e* and *f*, *E*, *e* and *f*, and *F*, 20 μm.)

cantly decreased in *Tmem100* null yolk sac (Fig. 4*A* and Fig. S7*C*). Because Notch signaling was known to be essential for arterial differentiation (1, 2, 15), we further examined whether the activity of Notch signaling is down-regulated in *Tmem100* null embryos. Upon activation by the ligands such as Delta and Jagged, the intracellular domain of Notch receptors, NICD, is enzymatically cleaved and translocated to the nucleus, where it forms a transcriptional activator complex with RBP-J and other cofactors (14). Western blot analysis demonstrated a significant decrement of NICD expression in *Tmem100* null embryos and yolk sac (Fig. 4*B*). Immunohistochemical staining using an anti–NICD-specific antibody indicated that down-regulation of NICD expression was specific to the arterial endothelium and endocardium and was not observed in other Notch-regulated tissues such as somite and neural tube (Fig. 4*C*). The total amount of Notch receptors, which was examined using an antibody recognizing both full-length Notch receptors and NICD, appeared unchanged (Fig. 4*D*). Furthermore, the expression of Notch-target transcription factor Hrt2/Hey2 was significantly decreased in the vasculature of *Tmem100* null embryos, whereas its expression in the cardiac muscle was unaltered (Fig. 4*E*).

In addition, we observed a decrease in Akt phosphorylation in *Tmem100* null embryos and yolk sac (Fig. 4*B*). Consistently, the expression of *Klf2* and *eNOS/Nos3* (Fig. 4*A*) and the cleavage of Presenilin-1 (Ps1) (Fig. 4*F*), which can be activated by Akt signaling (16–18), were suppressed in null embryos and yolk sac, suggesting down-regulation of Akt-mediated signaling important for endothelial survival, migration, and homeostasis. Importantly, dysregulation of Notch and Akt signaling was also observed in *Acvrl1/Alk1* null mice (Fig. S6 *F*–*K*).

In contrast, other signaling pathways implicated in embryonic vascular development did not appear compromised (1, 2, 19–22). The levels of ERK and Smad1/5/8 phosphorylation did not alter in *Tmem100* null embryos and yolk sac (Fig. S7 *D*–*F*). Components of Angiopoietin, Hedgehog, Semaphorin, and fibroblast growth factor signaling pathways and their downstream target genes did not show aberrant expression in *Tmem100* null yolk sac (Fig. S7*C*). These data suggested that the *Tmem100* deficiency caused impairment of arterial differentiation and morphogenesis through, at least in part, down-regulation of Notch- and Akt-mediated signaling.

Embryonic Vascular Defects by Cre-Mediated Deletion of *Tmem100* in Endothelial Cells. We further examined phenotypes of the mice with *Tek-Cre*–mediated endothelial cell-specific deletion of the *Tmem100* gene (Fig. S8 *A*–*D*). Endothelial-specific deletion of *Tmem100* caused vascular defects remarkably similar to those observed in global *Tmem100* null mice and embryonic lethality around E11.0 (Fig. S8 *E* and *F*). Expression of arterial endothelium-specific genes and a vascular smooth muscle marker SM22 apparently decreased, and Notch- and Akt-mediated signaling was down-regulated also by the endothelial-specific *Tmem100* deletion (Fig. S9 *A*–*D*). These results indicated that the endothelial expression of *Tmem100* is essential for arterial differentiation and embryonic development.

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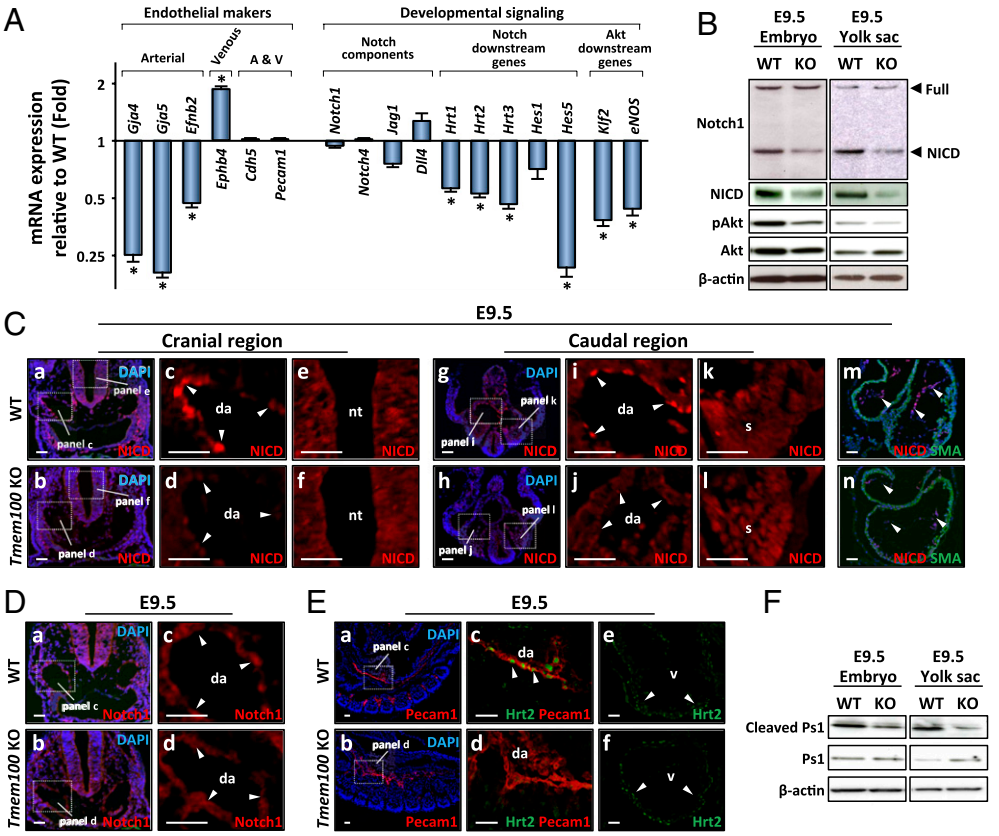

Fig. 4. Notch- and Akt-mediated signaling pathways are down-regulated in *Tmem100* null embryos and yolk sac. (*A*) Expression of arterial endothelium markers, Notch downstream genes, and Akt downstream genes markedly decreases in *Tmem100* null yolk sac at E9.5. Expression of a venous endothelium marker, *Ephb4*, is significantly up-regulated. Quantitative RT-PCR is shown. (*B*) The amount of the intracellular domain of Notch receptor (NICD) and phosphorylated Akt significantly decreases in *Tmem100* null embryos and yolk sac at E9.5, whereas that of full-length Notch1 receptor and total Akt remains unchanged. Western blot analysis is shown. (*C*) NICD expression markedly decreases in dorsal aorta (da) and endocardium (arrowheads in *d*, *j*, and *n*), but not in the neural tube (nt) and somites (s) (*f* and *l*), of E9.5 null embryos. Immunohistochemistry using a NICD-specific antibody is shown. *a*, *b*, *g*, *h*, *m*, and *n* are shown with nuclear DAPI stain. (*D*) Total amount of Notch receptors does not decrease in vascular endothelium of null embryos. Immunohistochemistry using an antibody recognizing full-length Notch receptors and NICD is shown. *a* and *b* are shown with nuclear DAPI stain. (*E*) Expression of Hrt2, a Notch downstream transcription factor, significantly decreases in the vasculature of *Tmem100* null embryos (*d*), whereas its expression in cardiac muscle was unchanged (*f*). Immunohistochemistry is shown. *a* and *b* are shown with nuclear DAPI stain. (*F*) Cleaved Presenilin-1 (Ps1) decreases in null embryos and yolk sac at E9.5. Western blot analysis is shown. (Scale bars in *C*–*E*, 20 μm.)

Discussion

In searching for genes involved in the regulation of endothelial differentiation and vascular development, we identified *Tmem100* to be a gene activated downstream of BMP9/BMP10-ALK1 signaling. *Tmem100* and *Acvrl1*/*Alk1* were expressed in arterial endothelial cells during vascular development in mouse embryos, and their targeting disruption caused embryonic lethality with remarkably similar abnormalities of arterial endothelium differentiation and vascular morphogenesis. *Tmem100* expression significantly decreased in *Acvrl1*/*Alk1* null embryos, which is consistent with down-regulation of *Tmem100* expression in the adult lung of *Acvrl1*/*Alk1* conditional knockout mice (12). It has not been studied whether BMP9 and BMP10 are major regulators of *Tmem100* expression in vivo. *Bmp10* null mice show impaired cardiac growth but not defects of angiogenesis (23), whereas the phenotypes of *Gdf2* (encoding BMP9) knockout mice have not been reported. Examining whether the mice null for both *Gdf2* and *Bmp10* show embryonic vascular defects will provide further insights into in vivo regulatory mechanisms of *Tmem100* expression.

Notch-mediated signaling was suppressed in arterial endothelium of *Tmem100* null embryos as well as in endothelial-specific *Tmem100* knockout embryos. We also found that Notch signaling was down-regulated in *Acvrl1*/*Alk1* null embryos. Notch receptors and ligands are enriched in arterial but not venous endothelium of mouse embryos, and target disruption of Notch signaling components such as *Notch1*, *Notch4*, *Dll4*, *Rbpj*, *Hrt1*/*Hey1*, and *Hrt2*/*Hey2* revealed that Notch activity is crucial for promoting arterial cell fate (1, 2, 15). Impairment of endothelial Notch signaling affects vascular smooth muscle cell recruitment or differentiation during arterial maturation (24–26), which was observed in *Tmem100* and *Acvrl1*/*Alk1* knockout mice in the present study. In addition to Notch signaling, the activities of Akt kinase and Ps1 protease appeared repressed in *Tmem100* and *Acvrl1*/*Alk1* knockout embryos. It was reported that Akt enhances Ps1-mediated Notch cleavage (16, 27) and that Ps1 provokes Akt activation in turn (28). The signaling relay or mutual interaction involving Akt, Ps1, and Notch might play a role in the pathogenesis of vascular defects by the *Tmem100* and *Acvrl1*/*Alk1* deficiency.

A number of biological stimuli including fluid shear stress could influence Akt- and Notch-mediated signaling as a result of insufficient circulation (1, 2, 15). Markers of arterial endothelium differentiation, however, became down-regulated before apparent structural abnormalities in *Tmem100* null embryos, suggesting a possibility that TMEM100 is directly involved in acquiring arterial cell fate. Previous studies reported *Tmem100*

expression in embryonic vasculature and the adult lung, prostate, and kidney in humans and mice (12, 29, 30), but cellular functions of TMEM100 have not been investigated. We found that TMEM100 protein was mainly localized in ER, but not in plasma membrane, of cultured endothelial cells. It is tempting to speculate that TMEM100 works for posttranslational protein modification or intracellular sorting in the membrane of ER and surrounding structures. Elucidating molecular functions of TMEM100 is essential to clarify how TMEM100 conveys the BMP9/BMP10-ALK1 signals toward the downstream signaling cascade for proper endothelial differentiation.

Mutations in *ACVRL1/ALK1*, *BMPR2*, *ENG*, or *SMAD4* cause hereditary hemorrhagic telangiectasia as well as pulmonary arterial hypertension in humans (31, 32). *TMEM100* might be involved in the mechanisms of these diseases as an additional causative gene or a modifier. At late embryonic and adult stages, *Tmem100* expression appears to be not restricted to vascular endothelial cells. Studying the significance of TMEM100 may lead to a better understanding of human diseases in the cardiovascular system and other organs.

Materials and Methods

Cell Culture and Microarray Analysis. HUAEC (Takara Bio) were precultured in Endothelial Basal Medium 2 medium with 0.2% FBS for 3 h and treated with TGF-β superfamily ligands (R&D Systems) for 24 h. siRNA was electroporated to HUAEC 36 h before the BMP9 or BMP10 treatment. Other cell culture experiments were performed using standard procedures.

Two-color microarray analyses using the human and mouse whole-genome 4x44K v2 oligo microarray systems (Agilent Technologies) were performed to examine mRNA expression profiles of BMP9-treated HUAEC and the yolk sac of *Tmem100* null mice, respectively.

Generation of *Tmem100* Null Mice. The *Tmem100*-lacZ targeting vector was constructed using a BAC clone containing the *Tmem100* locus genomic DNA (BACPAC Resources) and was electroporated into C57BL/6 embryonic stem cells for homologous recombination (Fig. S3*A*). A correctly targeted clone, as identified by Southern blot analysis, was injected into BALB/c blastocysts. Chimeras were bred to obtain heterozygous mice that carry the targeted *Tmem100* locus in their germ line. The floxed PGK-neo cassette was removed by breeding with a *CAG-Cre* transgenic line. All breeding was done with mice under the C57BL/6 genetic background. Procedures to generate mice for conditional *Tmem100* deletion are described in *SI Materials and Methods*. All animal experiments were approved by the institutional committee.

Biochemical, Histological, and Anatomical Analyses. Quantitative RT-PCR, Northern blot analysis, Western blot analysis, in situ hybridization, immunohistochemistry, and lacZ staining were performed using standard procedures. A rabbit polyclonal antibody generated against a carboxyl-terminal 27-aa fragment of mouse TMEM100 protein was used for Western blot analysis of HUAEC. Details of PCR primers, Northern probe, and primary antibodies are described in *SI Materials and Methods*. Microangiography was performed by India ink injection to the outflow tract of mouse embryos.

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