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# *Hes1* and *Hes5* regulate vascular remodeling and arterial specification of endothelial cells in brain vascular development

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ABSTRACT

The vascular system is the first organ to form in the developing mammalian embryo. The Notch signaling pathway is an evolutionarily conserved signaling mechanism essential for proper embryonic development in almost all vertebrate organs. The analysis of targeted mouse mutants has demonstrated essential roles of the Notch signaling pathway in embryonic vascular development. However, Notch signaling-deficient mice have so far not been examined in detail in the head region. The bHLH genes *Hes1* and *Hes5* are essential effectors for Notch signaling, which regulate the maintenance of progenitor cells and the timing of their differentiation in various tissues and organs. Here, we report that endothelial-specific *Hes1* and *Hes5* mutant embryos exhibited defective vascular remodeling in the brain. In addition, arterial identity of endothelial cells was partially lost in the brain of these mutant mice. These data suggest that *Hes1* and *Hes5* regulate vascular remodeling and arterial fate specification of endothelial cells in the development of the brain. *Hes1* and *Hes5* represent critical transducers of Notch signals in brain vascular development.


## 1. Introduction

The vascular system is one of the first organs to form in the developing mammalian embryo. During the early stages of vascular development, endothelial cell (EC) precursors differentiate and coalesce into a primitive network of undifferentiated blood vessels in the process of vasculogenesis. In the later stages of vascular development, this primary vascular plexus is remodeled into a network of large and small vessels by the process of angiogenesis (Carmeliet, 2000; Risau, 1997). This process is controlled by various signaling molecules and their downstream pathways. These pathways include vascular endothelial growth factor (VEGF) and its receptors, transforming growth factor-β (TGF-β) and its receptors, angiopoietin 1 and its receptor Tie2, and ephrin-B ligands and EphB receptors (Carmeliet et al., 1996; Suri et al., 1996; Wang et al., 1998).

The Notch signaling pathway is an evolutionarily conserved signaling mechanism essential for proper embryonic development in almost all vertebrate organs. Mammals express four Notch receptors (Notch1–4) and five ligands [Jagged (Jag) 1 and 2, and Delta-like (Dll) 1, 3 and 4] (Gridley, 2007; Kageyama et al., 2005, 2007). Notch is a transmembrane protein and activated by its ligands, Dll or Jag proteins. Upon

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activation, the intracellular domain (ICD) of Notch is cleaved off and transferred into the nucleus, where the Notch ICD forms a complex with the DNA-binding protein RBP-J. This complex induces expression of *Hes* genes (Kageyama et al., 2008a,b). The analysis of targeted mouse mutants has demonstrated roles of the Notch signaling pathway in embryonic vascular development. In mice, the absence of Notch signaling results in a similar phenotype characterized by defective vascular remodeling in the extraembryonic yolk sac, placenta, and embryo proper (Alva and Iruela-Arispe, 2004; Gridley, 2007). These embryos also exhibited a loss of expression of arterial markers in arterial vessels. Notch signaling regulates the specification of arterial fate in ECs during mouse embryogenesis (Alva and Iruela-Arispe, 2004; Gridley, 2007).

The bHLH genes *Hes1* and *Hes5* are essential effectors for Notch signaling, which regulate the maintenance of progenitor cells and the timing of their differentiation in various tissues and organs (Hatakeyama et al., 2004, 2006; Hojo et al., 2008, 2000; Ohtsuka et al., 1999). These features of *Hes* genes have been reported especially in the development of the nervous system. However, only a few *in vitro* studies have shown a role of *Hes* genes as a downstream effecter of Notch in ECs. *In vitro* studies using human arterial ECs have supported that *Hes1* is a downstream effector of Notch1 in the vasculature similarly as in other tissues (Albig et al., 2008; Liu et al., 2006; Quillard et al., 2008). In humans and mice, the Notch3-Hes5 signaling pathway is crucial for the development of pulmonary arterial hypertension (Li et al., 2009). However, no study using *Hes* mutant mice has so far shown a role of *Hes* genes in vascular development.

The bHLH genes *Hesr* (*Hey*, *HRT*, *gridlock*, or *CHF*) belong to a related but different subclass from *Hes* genes (Fischer and Gessler, 2003). The knockout (KO) of *Hesr2* revealed a critical function during heart development with ventricle septum defect, persistent foramen ovale, tricuspid valve stenosis, and cardiomyopathy as the predominant anomalies. However, *Hesr2* KO mice did not show vascular defects, in contrast to mutants of zebrafish *gridlock*, a homolog of *Hesr2*, which exhibit coarctation of the aorta (Fischer et al., 2004; Kokubo et al., 2005). In addition, *Hesr1* KO mice showed no obvious phenotype. However, mouse embryos lacking both *Hesr1* and *Hesr2* are embryonic lethal due to cardiovascular malformations, and have a single large ventricle and arterial differentiation defect, suggesting that *Hesr1* and *Hesr2* are required in mediating Notch signaling in the developing cardiac and vascular systems (Fischer et al., 2004; Kokubo et al., 2005).

Although mutations in human *Notch3* cause CADASIL, a hereditary vascular dementia, *Notch3* KO mice develop normally (Joutel et al., 1996; Krebs et al., 2003). In adult *Notch3* KO mice, cerebral arteries are enlarged and have thinner smooth muscle cell (SMC) coat (Domenga et al., 2004). Although it is expected that the Notch pathway have an important role in vascular development of the brain, Notch signaling-deficient mice have so far not been examined in detail in the head region. Here, we show that *Hes1* and *Hes5* regulate vascular remodeling of the brain during embryogenesis by analyzing *Hes*-mutant mouse embryos. We also show that *Hes1* and *Hes5* play a role in establishing arterial cell fate and identity of ECs of cerebral blood vessels.

## 2. Results

### 2.1. *Hes*-mutant embryos exhibit abnormal brain vascular development

We first examined *Hes1* expression in the internal carotid artery of mouse embryos by *in situ* hybridization (ISH). *Hes1* was expressed in almost all ECs of the developing internal carotid artery at E10.5 (Fig. 1A), suggesting that *Hes1* plays a role in brain vascular development. To reveal the role of *Hes*

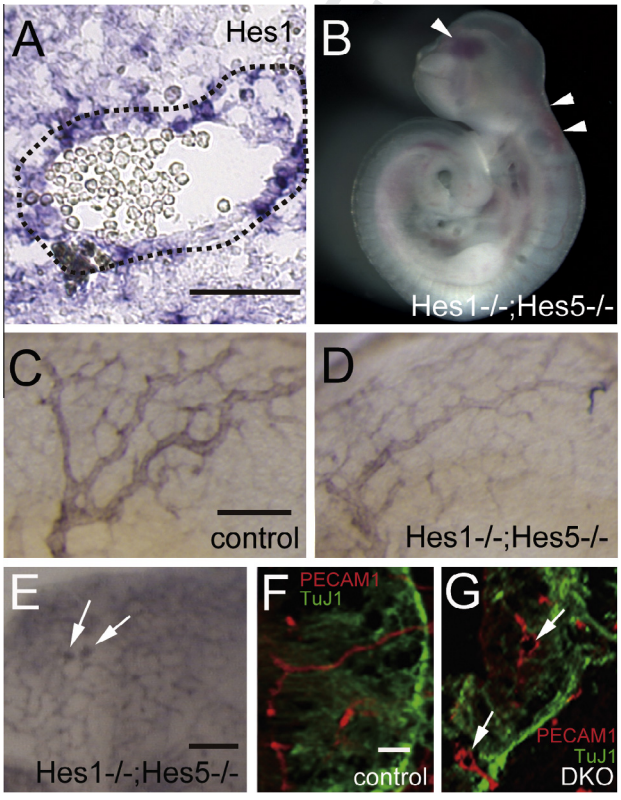


**Fig. 1 – Vascular defects in the brain of *Hes1*; *Hes5* DKO mice. (A) ISH of *Hes1*. *Hes1* was expressed in the internal carotid artery (dashed line) at E10.5. (B) *Hes1*; *Hes5* DKO embryos showed hemorrhage in the brain and the spinal cord at E10.5 (arrowheads). (C–E) Whole-mount PECAM-1-stained control (C) and *Hes1*; *Hes5* DKO (D and E) embryos at E10.5. The surface of the mesencephalon is shown. In *Hes1*; *Hes5* DKO mice, large major branches of the internal carotid artery were decreased in number, and instead, small branches were increased (D and E) compared with the control (C). Moreover, in double-mutant mice, abnormal microvascular networks and vascular malformations (abnormal angiomas, indicated by arrows) were also observed (E). (F and G) Immunostaining for PECAM-1 (red) and Tuj1 (green) on sections through the forebrain of E10.5 control (F) and *Hes1*; *Hes5* DKO (G) embryos. Small fragmented vessels (arrows) were observed in the neural tube of *Hes1*; *Hes5* DKO embryos (G) compared with the control (F). Bars, 50 μm (A, F and G); 200 μm (C and D); 100 μm (E).**



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