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Dynamic expression of *Endoglin,* a TGF-β co-receptor, during pre-circulation vascular development in chick

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ABSTRACT Mutations in the human *Endoglin* gene, encoding a dimeric TGF-β co-receptor, lead to type 1 hereditary hemorrhagic telangiectasia. Studies in mice have revealed important roles of *Endoglin* in endothelial cell proliferation, differentiation and integrity. *Endoglin*$^{-/-}$ mouse embryos die at mid-gestation due to cardiac defects and vessel rupture. Its role during early vasculogenesis is unclear, as the initial phase of vascular endothelial cell formation appears unaffected in *Endoglin*$^{-/-}$ embryos. In order to understand possible roles of *Endoglin* in early vascular development, we used the chick model and analyzed the temporal and spatial expression pattern of *Endoglin* during vasculogenesis in pre-circulation stage chick embryos. Weak *Endoglin* expression was detected at HH4 in the node and in the extraembryonic mesoderm. The node-specific expression is transitory and disappears after HH5. Strong up-regulation of *Endoglin* expression is seen at HH8 in all endothelial progenitors undergoing morphological changes to become endothelial cells. Most extraembryonic splanchnopleural vascular endothelial cells down-regulate *Endoglin* after their morphological differentiation, whereas lateral plate and cardiac endothelial cells remain positive until HH12, followed by a clear drop after circulation starts at HH13. Progenitors for the pronephric duct are positive from HH10 to HH12, but down-regulate *Endoglin* after epithelialization of duct cells. Overall, these data reveal a dynamic expression pattern of *Endoglin* in pre-circulation chick development and indicate that *Endoglin* may play an important role in the transition from endothelial progenitors to functional endothelial cells during early vascular development.

KEY WORDS: *CD105, vasculogenesis, vasculature, endothelial cell, hematopoiesis, progenitor, chicken*

Introduction

Endoglin (also known as CD105) is a co-receptor for the transforming growth factor-beta (TGF-β) superfamily (Goumans and Mummery, 2000; Lebrin and Mummery, 2008; ten Dijke *et al.*, 2008). In mammals, it has been reported to be expressed in active endothelial cells and during early extraembryonic mesoderm differentiation (Ema *et al.*, 2006; Jonker and Arthur, 2002). In chick, embryonic circulation starts at stage Hamburger and Hamilton (HH) 13 (Eichmann *et al.*, 2005; Hamburger and Hamilton, 1992). The expression pattern of *Endoglin* had been studied in chick with respect to cardiac differentiation (Vincent *et al.*, 1998) and in adult lung and post-circulation yolk sac vasculature (Raab *et al.*, 1999). During post-circulation chick development, *Endoglin* was also found to be expressed intraembryonically in cardiac endothelium and in developing vessels, and was shown to be involved in the process of epithelial-to-mesenchymal transformation (EMT) during cardiac valve formation (Mercado-Pimentel *et al.*, 2007). The *Endoglin*$^{-/-}$ mouse is embryonic lethal with angiogenic defects in addition to vessel rupturing and lack of cardiac EMT by mid-gestation, but with the vasculature appearing normal before E8.5 (Arthur *et al.*, 2000; Bourdeau *et al.*, 1999; Li *et al.*, 1999). *Endoglin*$^{+/-}$ mice are viable and have been used to model

Abbreviations used in this paper: EMT, epithelial-mesenchymal transformation; TGF-β, transforming growth factor beta.

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hereditary hemorrhagic telangiectasia type 1 (HHT1; MIM#187300) (ten Dijke *et al.*, 2008), a condition observed in humans with mutations in the *Endoglin* gene resulting in haploinsufficiency (Abdalla and Letarte, 2006). Although not normally lethal, patients with HHT1 suffer from chronic nosebleeds (epistaxis), telangiectases, have high incidence of pulmonary and cerebral arteriovenous malformations and appear to be predisposed to developing pulmonary arterial hypertension (Lenato and Guanti, 2006), all of which underscore the important underlying function of *Endoglin* in the establishment and maintenance of the circulatory system.

Furthermore, it has been suggested that *Endoglin* is involved in early hematopoietic development, with *Endoglin* being expressed in mouse embryonic stem cell (mESC)-derived mesodermal FLK1^{+} precursors (Cho *et al.*, 2001) and blast colony-forming cells (BL-CFCs) (Perlingeiro, 2007). *In vitro* differentiation assays showed that *Endoglin*$^{-/-}$ mESCs have reduced myelopoiesis and erythropoiesis, whereas the formation of lymphoid and vascular precursors appeared unaffected (Cho *et al.*, 2001). Anemia observed in patients with HHT1 and in *Endoglin*$^{-/-}$ mice is another indicator for a possible role of *Endoglin* in erythropoiesis. In the adult setting, in addition to its involvement in angiogenesis and vascular repair (Hayrabedyan *et al.*, 2005; van Laake *et al.*, 2006), *Endoglin* is expressed in long-term repopulating hematopoietic stem cells from bone marrow (Chen *et al.*, 2002) and was reported to play a role in erythroid lineage differentiation (Moody *et al.*, 2007). Increased levels of *Endoglin* expression were also found in tumors (Fonsatti *et al.*, 2001), atherosclerotic plaques (Conley *et al.*, 2000) and during inflammation and wound healing (Torsney *et al.*, 2002). *Endoglin* expression is thus closely correlated with neo-vascular formation and may play a role in the pathogenesis of vascular diseases. However, *in vitro* angioblast differentiation from *Endoglin*$^{-/-}$ mESCs was reported to be unaffected (Perlingeiro, 2007) and the primary endothelial network in *Endoglin*$^{-/-}$ mouse embryos appears normal (Li *et al.*, 1999), indicating that *Endoglin* may be dispensable for early vascular formation.

Results and Discussion

The chick *Endoglin* gene (AY702002) encodes a 644 amino acid polypeptide, with an overall 34% identity to the human L-Endoglin protein. Searches of EST databases did not indicate the existence of the alternatively spliced S-Endoglin in chick, an isoform up-regulated in senescent endothelial cells in mammals (Bellon *et al.*, 1993; Blanco *et al.*, 2008; Perez-Gomez *et al.*, 2005; Velasco *et al.*, 2008). A high degree of identity between chick and mammalian Endoglins was found in the transmembrane and short cytoplasmic tail domains, with 100% conservation in the last 24 amino acid residues containing the sites that are known to be phosphorylated by activated TβRII, Alk1 and Alk5 and a PDZ motif (Koleva *et al.*, 2006). Its expression pattern in the extraembryonic regions and in pre-circulation development has not been examined. We generated an anti-sense probe corresponding to amino acid residues 111-304 of the full length chick Endoglin (see Materials and Methods), and performed whole-mount *in situ* hybridization on embryos from stage HH4, when blood/endothelial progenitors start to be generated from the posterior primitive streak (Jaffredo *et al.*, 2005; Nakazawa *et al.*, 2006; Shin *et al.*, 2009), to HH13, when circulation is initiated.

At HH4, the earliest time point examined, weak *Endoglin* expression was observed in the Hensen's node and the head process (Fig.1A; arrowhead). This is transitory, as no *Endoglin*

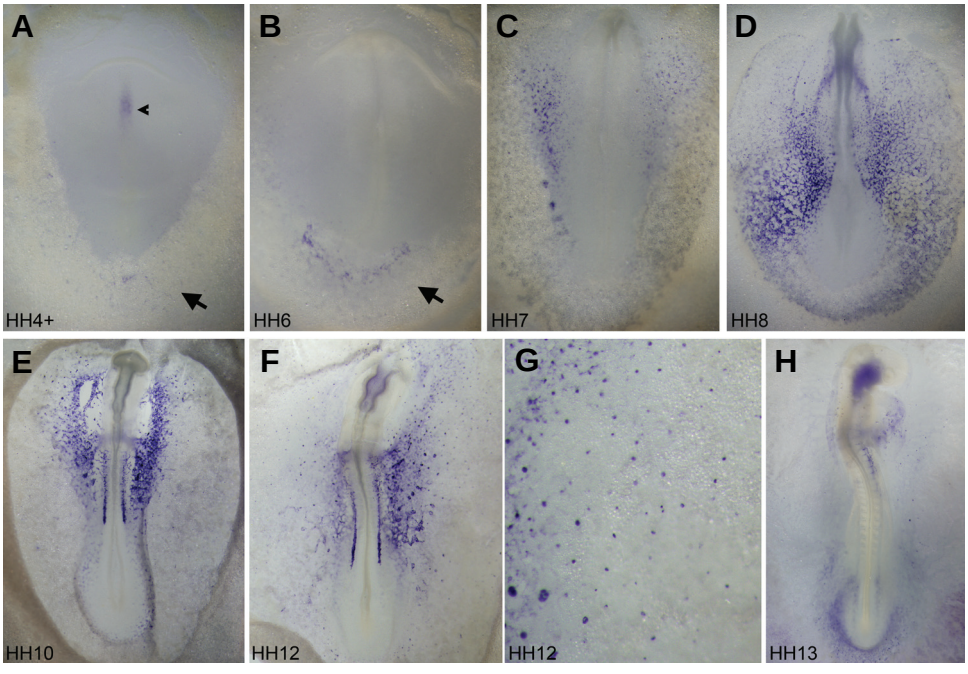

Fig. 1. Whole-mount *in situ* hybridization analysis of *Endoglin* from stage HH4 to stage HH13. (A) *At HH4+,* Endoglin *is expressed very weakly in the Hensen's node and head process (arrowhead) and in the newly formed extraembryonic mesoderm (arrow).* **(B)** *At HH6, expression in the node and node-derived tissues is not detected. Broader, yet still very weak,* Endoglin *expression is observed in the extraembryonic mesoderm (arrow).* **(C)** *By HH7, expression in the area vasculosa becomes stronger, especially in the future medial extraembryonic and lateral plate regions.* **(D)** *At HH8, robust expression is observed throughout extraembryonic and intraembryonic vasculature.* **(E)** *By HH10, extraembryonic vascular expression is reduced significantly, with intraembryonic vasculature (including cardiac and lateral plate vessels) strongly positive for* Endoglin. *Progenitors for the pronephric duct are also strongly positive.* **(F)** *At HH12, vascular expression of* Endoglin *is further reduced, with strong positive cells confined to mid-level medial splanchnopleural vasculature. Pronephric duct progenitors remain strongly positive. Somatopleural vessels are undergoing active vasculogenesis, marked by strong expression of* Endoglin *in numerous small clusters in both extra- and intra-embryonic regions.* **(G)** *Magnified view of the extraembryonic somatopleural* Endoglin *positive clusters at HH12.* **(H)** *By HH13,* Endoglin *expression is much reduced throughout the entire developing embryo. Weak expression is still detected in the heart and the posterior part of the embryo, where new vessels continue to be formed.*

expression was detected in the node or node-derived tissues at later stages. Weak expression could also be observed at this stage in the extraembryonic-fated mesoderm territory (Figs. 1A, 2A; arrows). The extraembryonic mesoderm at this stage strongly expresses hemangioblast, hematopoietic and endothelial precursor markers such as Gata2, Scl, Lmo2, Ets1 and Vegfr2 (Bollerot *et al.*, 2005; Minko *et al.*, 2003; Nakazawa *et al.*, 2006; Shin *et al.*, 2009), indicating that *Endoglin* expression does correlate with the initial steps of endothelial precursor specification.

By HH5 and HH6, *Endoglin* expression in the extraembryonic mesoderm covers a slightly wider area, but remains weak (Fig. 1B; arrow). Stronger and wider expression was observed at HH7 (Fig. 1C). The extraembryonic mesoderm at this stage starts to be segregated morphologically into three cell lineages: the blood, endothelial and smooth muscle cells (Shin *et al.*, 2009). The first two cell lineages come from blood island aggregates and it is at HH7 when hemoglobin gene expression, a marker for terminal differentiation of blood cells, starts to be seen in scattered cells within the blood island population (Alev *et al.*, 2008; Nagai and Sheng, 2007; Nakazawa *et al.*, 2009; Nakazawa *et al.*, 2006; Weng *et al.*, 2007). *Endoglin* up-regulation at HH7 thus seems to mark the initiation of the morphological differentiation of endothelial fated cells. In blood islands located more medially in the embryo, *Endoglin* expression was seen in most cells (Fig. 2B; right arrow), correlating with the fact that most of medially located blood islands contribute only to endothelial cells (Nakazawa *et al.*, 2006). More laterally located blood islands have less *Endoglin* positive cells (Fig. 2B; middle and left arrows; Fig. 2 C,D) and in most cases these positive cells are located at the periphery and on one side of a blood island cluster (Fig. 2D).

At HH8, *Endoglin* expression becomes very robust (Fig. 1D). Strong expression was detected in all endothelial precursors in the process of undergoing morphological changes to become endothelial cells, including endocardial progenitors (Fig. 2E), dorsal aorta progenitors (Fig. 2F) and all other splanchnopleural-associated vessel progenitors in both non-hemogenic (Fig. 2F) and hemogenic (Fig. 2 G,H) regions. Newly formed vascular endothelial cells are also positive for *Endoglin* (Fig. 2I). Morphological differentiation of endothelial progenitor cells and formation of the vascular plexus take place actively at HH8 and HH9. Most vascular endothelial cells down-regulate *Endoglin* expression soon after their morphological differentiation, and by HH10 (Fig. 1E), strong *Endoglin* expression was only seen in more medially located fusing endocardia (Fig.3 A,B) and lateral plate vessel cells (Fig. 3C), whereas extraembryonic vessels do not express *Endoglin* at all by this stage (Fig. 3E). Small cell clusters located in the somatopleure express *Endoglin* strongly (Fig. 3D). These cells, similar to blood island aggregates in the splanchnopleure (our unpublished data), have a slightly delayed differentiation program and *Endoglin* positive cells there will differentiate into small somatopleural vessels (Fig. 3K).

Circulation in chick embryos starts at about HH12 to HH13.

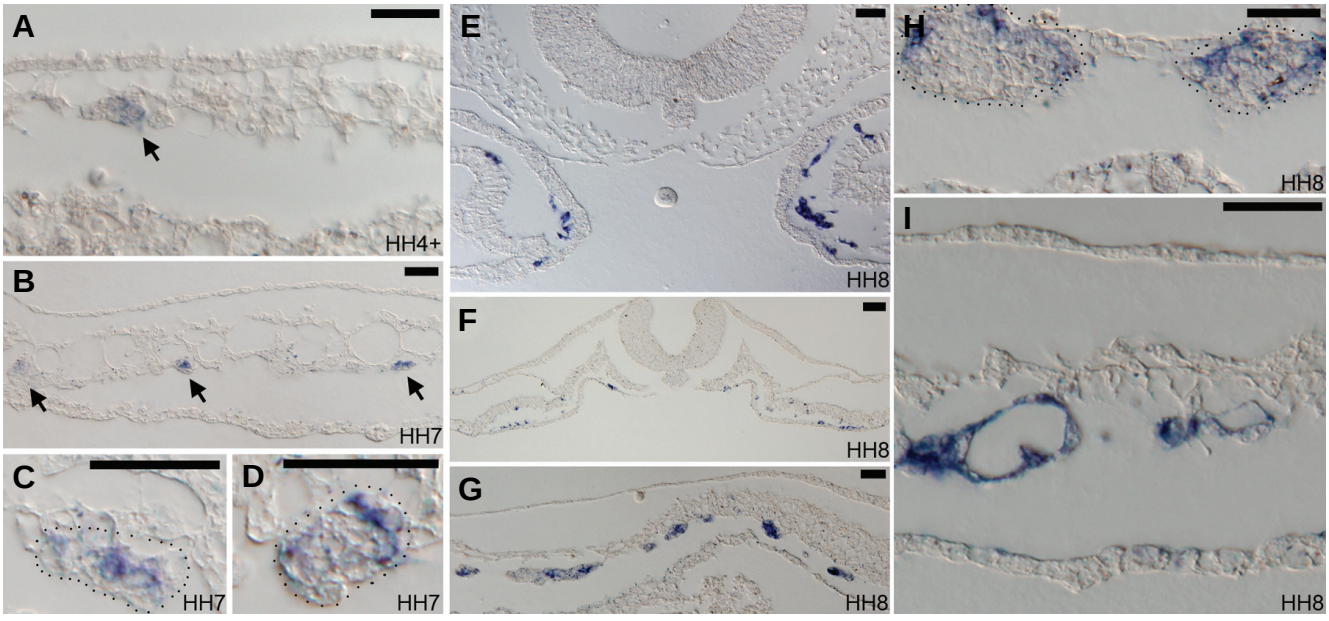

Fig. 2. Paraffin sections of *Endoglin* expressing embryos from HH4 to HH8. (A) *Section through posterior region of the HH4+ embryo shown in Fig.1A. Arrow indicates very weak expression of* Endoglin *in a small number of extraembryonic mesoderm cells.* **(B-D)** *Sections through the posterior part of the HH7 embryo in Fig.1C. Arrows in (B) indicate that some cells within forming blood island clusters are positive for* Endoglin. **(C,D)** *Magnified views of lateral blood island cluster (marked by dotted lines), with some cells in the cluster positive for* Endoglin. *Blood island clusters in this region normally give rise to both blood cells and endothelial cells, whereas blood islands in more medial regions give rise only to endothelial cells. In these lateral blood island clusters, most* Endoglin *positive cells are located in the periphery of the cluster as shown in (D). Some are located within the cluster.* **(E-F)** *Sections of the HH8 embryo in Fig.1D.* **(E)** *A section through the cardiogenic region, with endocardial precursors strongly positive for* Endoglin. **(F)** *A section through mid-level of the embryo, with all embryonic endothelial progenitors positive for* Endoglin. **(G)** *A section through the extraembryonic region, with medially located blood islands having most of their constituent cells positive for* Endoglin *and laterally located blood islands having a few positive cells.* **(H)** *A magnified view of lateral extraembryonic blood islands, showing peripheral locations of* Endoglin *positive cells within blood islands (dotted lines).* **(I)** *In many lateral plate vessels, strong* Endoglin *positive signals remain in morphologically differentiated endothelial cells. Scale bar in all panels: 50 μm.*

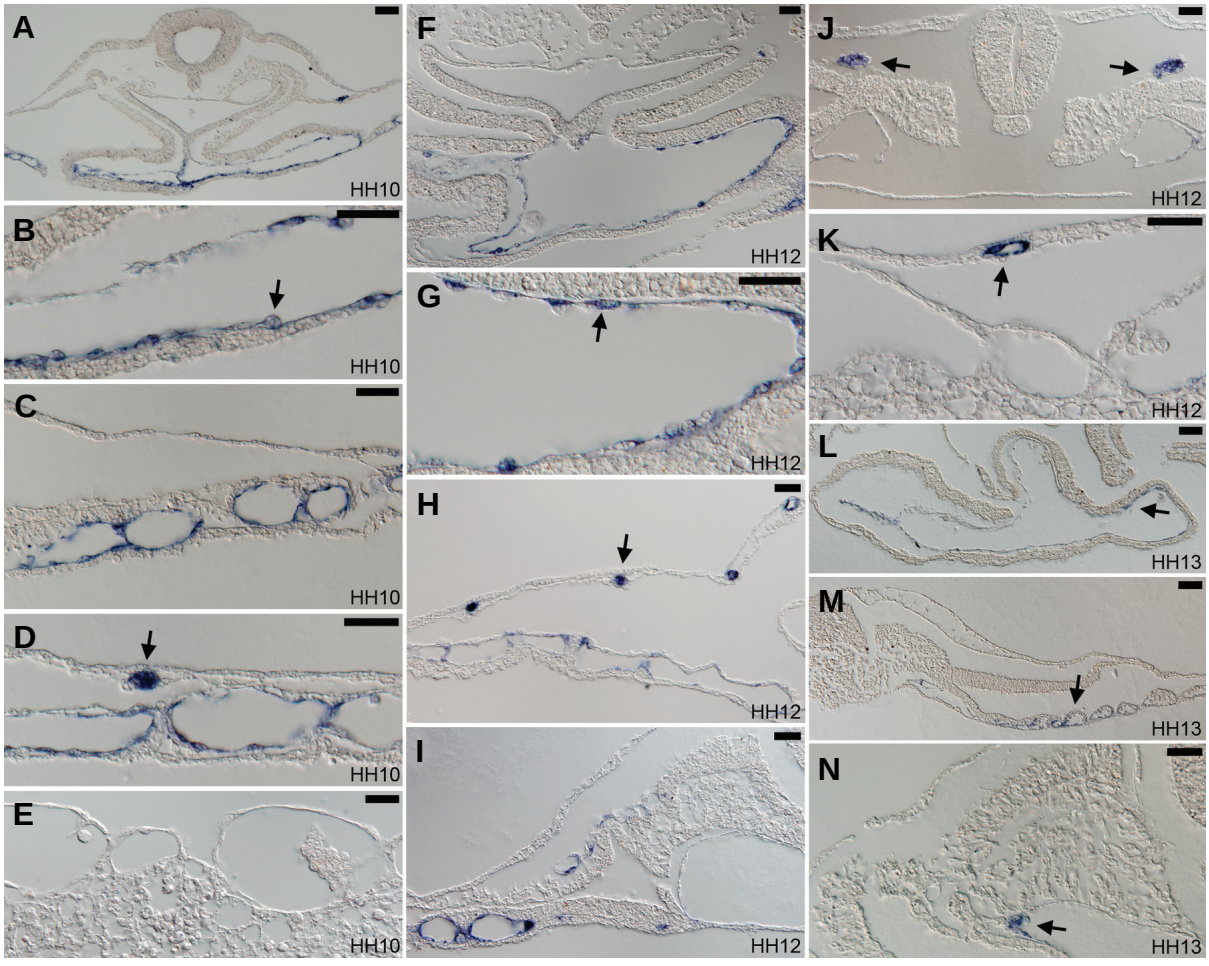

Fig. 3. Paraffin sections of *Endoglin* expressing embryos from HH10 to HH13. (A-E) *Sections of the HH10 embryo shown in Fig.1E.* **(A)** *Fusing endocardia are strongly positive for* Endoglin *(magnified in (B), with arrow indicating a positive endocardial cell).* **(C)** *Lateral plate splanchnopleural vessels remain positive for* Endoglin. **(D)** *In lateral plate and extraembryonic regions, somatopleural vessel precursors are strongly positive for* Endoglin *(arrow).* **(E)** *Lateral extraembryonic vessels are negative for* Endoglin. *Blood cells and blood precursors are always negative for* Endoglin. **(F-K)** *Sections of the HH12 embryo shown in Fig.1F.* **(F)** *Fused endocardium remains positive for* Endoglin *(magnified view in (G), with arrow indicating a positive endocardial cell).* **(H)** *Somatopleural vessel precursors are strongly positive for* Endoglin *(arrow), whereas splanchnopleural vessels have much reduced expression.* **(I)** *Only in the mid-level of the embryo* Endoglin *in splanchnopleural vessels persists.* **(J)** *Developing pronephric duct progenitors (arrows) are strongly positive for* Endoglin. **(K)** *Somatopleural vessels (arrow) remain positive for a short period of time after their formation.* **(L-N)** *Sections of the HH13 embryo shown in Fig.1H.* **(L)** *The endocardium remains weakly positive for* Endoglin *in some cells (arrow).* **(M)** *Newly developed vessels in the posterior part of the embryo are weakly positive for* Endoglin. **(N)** *A few cells in the aortic branch in contact with the forming pronephron express* Endoglin. *Scale bar in all panels: 50 μm.*

Endoglin expression levels drop in most vessels at peri-circulation stages (Fig.1 F,H). In the endocardium, expression is prominent at HH12 (Fig.3 F,G), but decreases significantly, although still detectable, at HH13 (Fig. 3L). A similar drop was seen in lateral plate vessels (Fig.3 H,I,M). *Endoglin* expression in small cell clusters in the somatopleure is still strong at HH12 (Fig.1G; arrows in Fig.3 H,K), but disappears at HH13 (Fig.1H). At HH13, weak *Endoglin* signals were detected in the contact region between the dorsal aorta and the developing pronephric duct and tubule (Fig. 3N), possibly representing the aortic branch of the developing external glomerulus (Hiruma and Nakamura, 2003). From HH10 to HH12, the precursors for the pronephric duct are strongly positive for *Endoglin* (Figs.1E,F, 3J). This and the node cells at HH4 are the only two non-endothelial cell types found to express *Endoglin* in our study.

In summary, we report here that the expression of *Endoglin* during embryonic pre-circulation stages in the chick is mainly confined to areas of vasculogenesis, being strongest in endothelial cells undergoing active vascularization and lower or undetectable in differentiated vessels. At all stages examined here, vascular smooth muscle cells and their progenitors do not express *Endoglin*. Our observations support the idea, as proposed in several mouse studies (Carvalho *et al.*, 2004; Ema *et al.*, 2006; Jonker and Arthur, 2002), that Endoglin plays an important role during vasculogenesis. In chick, however, Endoglin does not appear to be involved in early hemangioblast (common blood and endothelial progenitor) specification or hematopoietic development. During the differentiation of endothelial progenitors, *Endoglin* expression seems to correlate best with the phase of morphological changes required for the formation of functional vessel cells,

and less well with the specification of endothelial progenitor cells or the maintenance of vascular morphology or integrity after their formation. In formed vascular structures undergoing active remodeling, such as in the endocardium and lateral plate splanchnopleural vessels, *Endoglin* expression is maintained for a short while after initial morphological differentiation. It is therefore possible that the Endoglin mediated TGF-β signaling cascade regulates the molecular cues involved in the terminal fate choice of endothelial progenitors to adopt a differentiated endothelial morphology via the initiation of synthesis of intracellular and extracellular molecular components unique for functional endothelial cells (e.g., endothelial specific cell-cell junctional and extracellular matrix proteins). During chick early development, *Endoglin* is in our opinion the best molecular marker yet described for the initiation of terminal differentiation of endothelial progenitor cells.

Materials and Methods

Fertilized *Gallus gallus domesticus* eggs were purchased from Shiroyama Farm (Kanagawa, Japan) and incubated to desired stages at 38.5°C. The DNA fragment for generating *Endoglin in situ* probe was amplified by PCR from stage HH12 cDNA preparation with the following two primers: 5'-AGAACCTCCTCATCCACACT-3' and 5'-GCGATGATGCTGTAGTTCTT-3'. The amplified fragment was confirmed by sequencing and corresponds to nucleotides 332-911 of NCBI #NM_001080887. Whole-mount *in situ* analysis was carried out as previously described (Nakazawa *et al.*, 2006). All *in situ* hybridization experiments were performed at least in triplicate for each stage described. Stained embryos were photographed with an Olympus SZX12 microscope using a DP70 camera, followed by paraffin-embedded sectioning (10 μm). Sections were photographed with an Olympus BX51 microscope. All panels in Figure 1 have stained embryos oriented with the rostral side up and viewed from the ectoderm side. All section panels in Figures 2 and 3 are oriented with the ectoderm side up.

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