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*<sup>-/-</sup> 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*<sup>-/-</sup> 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*<sup>-/-</sup> 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*<sup>-/-</sup> mice are viable and have been used to model

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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 Endoglin 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 precursor 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.
Endoglin expression levels drop in most vessels at peri-circulation stages (Fig. 1F,H). In the endocardium, expression is prominent at HH12 (Fig. 3F,G), but decreases significantly, although still detectable, at HH13 (Fig. 3L). A similar drop was seen in lateral plate vessels (Fig. 3H,I,M). Endoglin expression in small cell clusters in the somatopleure is still strong at HH12 (Fig. 1G; arrows in Fig. 3H,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′-AGAACCTCCTCATCACACT-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 parafilin-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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References


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