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The forkhead transcription factors, Foxc1 and Foxc2, are required for arterial specification and lymphatic sprouting during vascular development

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Abstract

Accumulating evidence suggests that in the vertebrate embryo, acquisition of arterial and venous identity is established early by genetic mechanisms, including those regulated by vascular endothelial growth factor (VEGF) and Notch signaling. However, although the COUP-TFII nuclear receptor has recently been shown to regulate vein identity, very little is known about the molecular mechanisms of transcriptional regulation in arterial specification. Here, we show that mouse embryos compound mutant for *Foxc1* and *Foxc2*, two closely related Fox transcription factors, exhibit arteriovenous malformations and lack of induction of arterial markers whereas venous markers such as COUP-TFII are normally expressed, suggesting that mutant endothelial cells fail to acquire an arterial fate. Notably, consistent with this observation, overexpression of *Foxc* genes in vitro induces expression of arterial markers such as *Notch1* and its ligand *Delta-like 4* (*Dll4*), and Foxc1 and Foxc2 directly activate the *Dll4* promoter via a Foxc-binding site. Moreover, compound *Foxc* mutants show a defect in sprouting of lymphatic endothelial cells from veins in early lymphatic development, due to reduced expression of *VEGF-C*. Taken together, our results demonstrate that Foxc transcription factors are novel regulators of arterial cell specification upstream of Notch signaling and lymphatic sprouting during embryonic development.

Keywords: Vascular development; Arterial-venous specification; Lymphatic development; Notch; Forkhead; VEGF

Introduction

Blood vessels in the embryo and extraembryonic membranes first form as a primitive vascular network by differentiation and proliferation of endothelial cell precursors, angioblasts (vasculogenesis). The vessels subsequently remodel by the process of angiogenesis to form the small and large vessels of the mature vasculature. These complex processes depend on the precisely coordinated and sequential actions of several angiogenic growth factors such as the vascular endothelial growth factor (VEGF) (Gale and Yancopoulos, 1999; Rossant and Howard, 2002). Whereas anatomical differences between arteries and veins have long been understood, recent evidence demonstrates that in the vertebrate embryo, angioblasts become committed to arterial or venous lineages before circulation begins (Lawson et al., 2001, 2002, 2003; Mukouyama et al., 2002; Zhong et al., 2001). The Notch pathway induces arterial specification, and the VEGF pathway functions upstream of the Notch pathway in this process (Alva and Iruela-Arispe, 2004; Lawson et al., 2002, 2003; Mukouyama et al., 2002; Shawber et al., 2003). Consistent with this idea, vascular expression of Notch pathway receptors and ligands has been shown to be restricted to arterial endothelial cells in the early mouse and zebrafish embryo (Lawson and Weinstein, 2002; Shutter et al., 2000; Uyttendaele et al., 2001; Villa et al., 2001). On the other hand, a recent study demonstrates that the COUP-TFII transcription factor expressed in venous endothelium regulates a venous cell fate by repressing Notch signaling (You et al., 2005). Although ephrinB2 and EphB4 are expressed in arteries and veins, respectively, genetic studies suggest that the ephrin/Eph pathway is not required for the initial specification or determination of arterial-venous cell

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fate (Adams et al., 1999; Wang et al., 1998). Consequently, mouse and zebrafish embryos mutant for Notch signaling genes, including the *Delta-like 4* (*Dll4*) ligand and the downstream targets *Hey1/Hey2*, fail to express arterial markers such as ephrinB2 in endothelial cells and these mouse mutants show severe vascular remodeling defects (Duarte et al., 2004; Fischer et al., 2004; Kokubo et al., 2005; Krebs et al., 2004; Lawson et al., 2001). Moreover, mutant embryos for Notch signaling genes such as *Dll4* result in the formation of arteriovenous malformations (AVMs), the fusion of arteries and veins without an intervening capillary bed (Duarte et al., 2004; Kokubo et al., 2005; Krebs et al., 2004; Lawson et al., 2001, 2002; Zhong et al., 2001). After the differentiation of endothelial cells into veins, they subsequently sprout to form lymphatic vasculature in the embryo (Hong et al., 2004; Oliver, 2004). However, the cellular and molecular mechanisms governing these complex processes still remain to be elucidated, and much more needs to be discovered about how transcription factors respond to extracellular signals and regulate gene expression at the onset of arterial-venous specification and differentiation.

Foxc1 and *Foxc2* (formerly *Mf1* and *Mfh1*, respectively) encode forkhead/Fox transcription factors (Carlsson and Mahlapuu, 2002; Kaestner et al., 2000) with virtually identical DNA-binding domains and very similar expression patterns in various embryonic tissues. The two genes are first transcribed in a dorsoventral gradient in the non-axial mesoderm with the highest levels in the region closest to the neural tube (paraxial) and the lowest levels ventrally (lateral) during gastrulation and in numerous embryonic tissues, including the endothelial and mesenchymal cells of blood vessels (Hiemisch et al., 1998; Iida et al., 1997; Kume et al., 1998, 2000b, 2001; Sasaki and Hogan, 1993; Winnier et al., 1999). Since NMR structural analysis suggests that the Foxc proteins have the same binding specificity to a target sequence (van Dongen et al., 2000), they are likely to regulate the same downstream target(s) where they are coexpressed in the same cells. Although the consensus DNA-binding sequence for Foxc1 has already been reported using PCR-mediated DNA-binding site selection (Pierrou et al., 1994), direct targets of Foxc in vivo remain largely unknown. It has recently been shown that Foxc1 and Foxc2 can bind to the enhancer of the T-box transcription factor *Tbx1*, regulating its expression in the head mesenchyme (Yamagishi et al., 2003). Although *Foxc1* and *Foxc2* are expressed in a variety of embryonic tissues, this is the only known direct target of Foxc during development.

Mice that are homozygous for either a spontaneous mutation in *Foxc1* (*congenital hydrocephalus*, $Foxc1^{ch}$) or an engineered null mutation ($Foxc1^{lacZ}$) die both prenatally and perinatally with identical phenotypes (Kume et al., 1998). These include hemorrhagic hydrocephalus and multiple skeletal, ocular, genitourinary, and cardiovascular defects, including the interruption or coarctation of the aortic arch, ventricular septal defects (VSD), and aortic and pulmonary valve dysplasia (Kidson et al., 1999; Kume et al., 1998, 2000b, 2001; Smith et al., 2000; Winnier et al., 1999). *Foxc2* null mutants die pre- and perinatally with skeletal, genitourinary, and cardiovascular defects similar to those seen in *Foxc1* homozygous mutants (Iida et al., 1997; Kume et al., 2000b, 2001; Winnier et al., 1997), suggesting the functional interaction between the two genes. Surprisingly, the majority of compound heterozygotes die soon after birth with a similar spectrum of cardiovascular, genitourinary, and eye abnormalities as those seen in each single homozygous null mutant (Kume et al., 2000b, 2001; Smith et al., 2000; Winnier et al., 1999). These data suggest that they functionally overlap in vivo. Moreover, compound homozygous and hetero/homozygous embryos die in utero with a phenotype that is much more severe than that of a single homozygote and a compound heterozygote in a dose-dependent manner (Kume et al., 2001). For example, most blood vessels including the yolk sac vasculature in compound homozygous mutants are disorganized plexi that have undergone very little remodeling, compared to the wild type. Compound *Foxc1*+/−; *Foxc2*−/− mutant embryos also have less severe but extensive defects in the morphology and remodeling of the blood vessels in the head and branchial arches. However, the molecular mechanisms underlying the overlapping role of Foxc in vascular development is still poorly understood.

A role for Foxc2 in lymphatic development is suggested by the fact that humans and mice heterozygous for *FOXC2/Foxc2* have lymphedema (Fang et al., 2000; Kriederman et al., 2003). Recent studies have demonstrated that *Foxc2* is expressed lymphatic vessels in the developing embryos as well as in the adult (Dagenais et al., 2004; Petrova et al., 2004), and that *Foxc2* homozygous mice show agenesis of lymphatic valves and increased pericyte investment of lymphatic vessels (Petrova et al., 2004). We show here that compound *Foxc1*+/−; *Foxc2*−/− mutants exhibit defects in the early sprouting and formation of lymphatic vessels. Most importantly, compound *Foxc1*; *Foxc2* homozygotes have AVMs and their endothelial cells fail to express arterial markers including *Dll4*, consistent with our finding that Foxc can directly transactivate the *Dll4* promoter in vitro. Taken together, we propose that the *Foxc* genes are important new components of the genetic cascade regulating arterial specification upstream of Notch signaling and lymphatic sprouting during vascular development.

Materials and methods

Breeding mutant mice and genotyping

Mice heterozygous for the null mutations, *Foxc1* and *Foxc2*, were maintained by interbreeding and genotyped, and compound mutant embryos were obtained by interbreeding compound *Foxc1*; *Foxc2* heterozygotes as described (Kume et al., 2001). At least two embryos of each genotype were analyzed to determine expression patterns of marker genes. The number of the somites was counted for staging wild-type and compound mutant embryos to confirm no developmental delay in compound mutants. Since compound homozygotes do not have the somites (Kume et al., 2001), their embryonic stages were determined by comparing the body length of wild-type and compound homozygous embryos.

Histological analysis

Histological analysis including H/E staining and section in situ hybridization was performed as described (Kume et al., 1998, 2001, 2000a,b). For immunohistochemical analysis, embryos were fixed with 4% paraformaldehye in PBS, dehydrated, embedded in paraffin with standard procedures, and then

sectioned at 8 μm. The antigen retrieval of paraformaldehyde-fixed tissue sections was performed by heat treatment for 30 min in antigen retrieval reagent Retrievit 6 (InnoGenex) for Prox1 antibody or by incubation with 0.025% trypsin for 8 min at 37°C for PECAM-1, COUP-TFII, and EphB4 antibodies. The sections were blocked for 20 min in PowerBlock (BioGenex) followed by incubation with primary antibodies. The primary antibodies used were rat anti-mouse CD31 (PECAM-1) (Pharmingen), goat anti-mouse EphB4 (R&D System), mouse anti-human COUP-TFII (R&D System), rabbit anti-mouse Lyve1 (Abcam), and rabbit anti-mouse Prox1 (Covance). After washing 3 times for 10 min in PBS/0.05% Tween 20 (PBST), the sections were incubated with secondary antibodies. The secondary antibodies used were Alexa 488 donkey anti-goat IgG (Molecular Probes) for EphB4, Alexa 488 goat anti-mouse IgG (Molecular Probes) for COUP-TFII, Alexa 488 donkey anti-rabbit IgG (Molecular Probes) for Lyve1, and Cy-3 donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories) for Prox1. For double immunofluorescence to define endothelial cells, PECAM-1 was detected using either Alexa 568 goat anti-rat IgG (Molecular Probes) for PECAM-1/COUP-TFII or rabbit anti-rat IgG (Vector Laboratories) and Cy-3 donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories) for PECAM-1/EphB4. Following extensive washes for 3 × 10 min in PBST, the sections were mounted with anti-fading agent Gel/Mount (Biomeda).

Cell lines

COS-7 and 10T1/2 cells were purchased from American Type Culture Collection (ATCC) and cultured according to the manufacturer's protocol. Mouse embryonic endothelial cells (MEECs) were cultured as previously described (Goumans et al., 2002).

RNA extraction and semi-quantitative RT-PCR analysis

Total RNA was isolated from MEECs or 10T1/2 cells using Trizol (BD Biosciences) after 24 h of transfection with expression vectors and subjected to semi-quantitative RT-PCR using AmpliTaq Gold (Applied Biosystems). The cycle number for each primer set was determined within the linear range of amplification of target cDNA (25 to 32 cycles). Primers used for RT-PCR were as follows: Gapdh, 5′-ACATCAAGAAGGTAGTGAAGCTGGC-3′ and 5′-TTCAAGAGATTAGGGCTCCCTAGG-3′; *Flk1*, 5′-CCTACCCCACACATTACATGG-3′ and 5′-TTTTCCTGGGCACCTTCTATT-3′; *Notch1*, 5′-CCAGCATGGCCAGCTCTGG-3′ and 5′-CATCCAGATCTGTGGCCCTGTT-3′; *Notch4*, 5′-ACCCTGCTCCAATGGAGGAT-3′ and 5′-AGAACCTCCGATTCACACTCC-3′; *Dll4*, 5′-CGAGAGCAGGGAAGCCATGA-3′ and 5′-CCTGCCTTATACCTCTGTGG-3′; *Hey2*, 5′-GTGGGGAGCGAGAACAATTACCCTGG-3′ and 5′-TGCTGAGATGAGAGACAAGGCGCACG-3′; *ephrinB2*, 5′-CTGTGCCAGACCAGACCAAGA-3′ and 5′-CAGCAGAACTTGCATCTTGTC-3′; *EphB4*, 5′-CAGGTGGTCAGCGCTCTGGAC-3′ and 5′-ATCTGCCACGGTGGTGAGTCC-3′; *PECAM-1*, 5′-GACCCAGCAACATTCACAGAT-3′ and 5′-TCTTTCACAGAGCACCGAAGT-3′; *Tie2*, 5′-CGGCCAGGTACATAGGAGGAA-3, and 5-CCCCCACTTCTGAGCTTCAC-3′, *Flt4*, 5′-CGAGACTGGAAGGAGGTGAC-3′ and 5′-GTACGTGTAGTTGTCCGCCC-3′; *COUP-TFII*, 5′-AAGCTGTACAGAGAGGCAGGA-3′ and 5′-AGAGCTTTCCGAACCGTGTT-3′; *Foxc1*, 5′-GCGGAAATTG TAGGAGTTCCCTAG-3′ and 5′-TTTGGCATCTGGCTCACAGG-3′; *Foxc2*, 5′-ACGAGTGCGGATTTGTAACCAG-3′ and 5′-GTGTTTTTGGAATACCCCAGATGG-3′.

Electrophoretic mobility shift assay (EMSA)

Foxc1 and Foxc2 proteins were synthesized using TNT-coupled reticulocyte lysate system (Promega). Oligonucleotide probes were generated by annealing complementary deoxyoligonucleotides at 85°C, end-labeled with [γ-^{32}P] ATP using T4 polynucleotide kinase, and were purified using Sephadex G-50 columns (Roche). For competition studies, the excess cold DNA fragments were added and incubated for 15 min at RT before adding the DNA probe. Binding reaction was performed at 25°C for 20 min in 20 μl of the slurry containing 2 μl of in vitro translation product, 1 μg of poly (dI–dC), and typically 0.1 ng probe (10,000 cpm, Cerenkov) in 20 mM HEPES, pH 7.9; 50 mM KCl; 2 mM $MgCl_2$; 0.5 mM EDTA; 10% glycerol; 0.1 mg/ml BSA; 2 mM DTT; 0.5 mM PMSF. The samples were run on 6% polyacrylamide gel containing 1% Ficoll 400 and 5% glycerol in Tris–glycine buffer (25 mM Tris, 190 mM glycine, 1 mM EDTA) at 10 V/cm at 4°C for 110 min. Gels were vacuum dried and subjected to autoradiography. Deoxyoligonucleotides used for the FBE in the *Dll4* promoter were 5′-TTGTAAAATCGATTTATTGACCGGCAGGTGCGAGC-3′ and 5′-GCTCGCACCTGCCGGTCAATAAATCGATTTTACAA-3′.

Plasmid constructs

A 3.7-kb fragment of the mouse *Dll4* promoter (−3631/+76; XhoI/XhoI fragment) isolated from a mouse 129SV genomic BAC library (BACPAC Resources) was subcloned into the XhoI site of pGL3Basic (Promega) (pDll4-3.7k-Luc). pDll4-3.7kmut-Luc was generated to mutate the FBE (ATTTATTGAC) to AAGCTTGAC using QuikChange site-directed Mutagenesis Kit (Staratagene). A 2.1-kb region (−2028/+76; EcoRV/XhoI fragment) was subcloned into SmaI/XhoI sites of pGL3Basic (pDll4-2.1k-Luc).

Luciferase reporter assay

COS-7 cells were transfected with 400 ng of expression vectors along with 100 ng of reporter constructs and 1 ng of pRL-SV40 Renilla using Lipofectamine 2000 (Invitrogen). The total amount of expression plasmid transfected per well was kept constant by addition of varying amounts of pcDNA3.0 (Invitrogen). Luciferase assays were performed using Dual-Luciferase Reporter Assay System (Promega) at 48 h after transfection as described (Fujita et al., 2006). Each transfection was performed in triplicate and replicated three times.

Statistics

Student's *t* test was performed to compare the average of two groups. A value of $P < 0.05$ was considered statistically significant.

Results

Compound Foxc1; Foxc2 mutant embryos have arteriovenous malformations resulting from vascular fusions

It has previously shown that *Foxc1* and *Foxc2* are transcribed in blood vessels of the embryo, and that compound *Foxc1*; *Foxc2* mutant embryos have abnormalities in cardiovascular development (Kume et al., 2001; Winnier et al., 1999). In particular, compound homozygous and hetero/homozygous mutant embryos exhibit severe vascular defects in branching morphogenesis and vessel remodeling in the yolk sac and embryo proper (Kume et al., 2001). Whereas either the majority of compound heterozygotes or all of each single homozygous mutants die prenatally (from around embryonic day E14.5) or soon after birth with a similar spectrum of cardiovascular abnormalities such as the interruption of the aortic arch, a small population of compound heterozygotes survive until adulthood and are fertile. Compound *Foxc1*+/−; *Foxc2*−/−; and *Foxc1*−/−; *Foxc2*+/− mutants can survive until E12.0–12.5, whereas compound homozygous mutants die around E9.5. Although these results suggest that Foxc1 and Foxc2 functionally overlap and are required for embryonic development in a dose-dependent manner, the precise mechanisms of the cooperative role of Foxc1 and Foxc2 in vascular development, including the identification of their direct targets, have not yet been studied. Here, we found that at E8.25, when both circulation

and cardiac function begin (McGrath et al., 2003), the dorsal aorta and cardinal vein in compound homozygotes were fused together (Fig. 1C). In addition, significantly enlarged blood vessels possibly due to vascular fusions were observed in compound homozygotes at E9.0 (Figs. 2B, D, F, H, J, L, N, P, R, T, V, and X). Moreover, some compound *Foxc1*+/−; *Foxc2*−/− mutants (2/8) had similar but less severe vascular shunts at E9.0 (Fig. 1F) indicating incomplete penetrance, whereas compound *Foxc1*−/−; *Foxc2*+/− mutants (0/8), as well as single mutants (0/10) and compound heterozygotes (0/9), showed no vascular fusions (data not shown). These AVMs were frequently seen between the dorsal aorta and the anterior cardinal vein at the level of the aortic sac. These data suggest that the effects of the two *Foxc* genes on vascular development are dose-dependent. It is suggested that AVMs result from the defective separation of uncommitted angioblasts as arteries and veins during migration and/or formation of blood vessels (Lawson and Weinstein, 2002). Importantly, in addition to the failure of blood vessels to remodel during development, similar AVMs have been observed in mouse and zebrafish embryos mutant for Notch signaling genes such as *Dll4* (Duarte et al., 2004; Krebs et al., 2004; Lawson et al., 2001), indicating that impaired arterial specification/differentiation commonly leads to AVMs.

Compound Foxc1; Foxc2 homozygous null mutants exhibit lack of induction of arterial markers

We next examined the expression patterns of vascular markers, including Notch signaling genes in compound homozygotes (Fig. 2). A pan-endothelial marker, *Flk1* (also known as *VEGFR-2*), was expressed in endothelial cells of compound homozygotes (Fig. 2B), indicating that mesodermal cells were normally specified to endothelial cells in compound homozygotes. Intriguingly, transcripts for the arterial markers, *Notch1*, *Notch4*, *Dll4*, *Jagged1*, *Hey2*, and *ephrinB2*, were all lost or reduced in endothelial cells of compound homozygotes (Figs. 2D, F, H, J, L, and N). These data suggest that arterial specification is impaired in compound homozygotes.

The VEGF pathway plays a crucial role in inducing arterial specification upstream of Notch signaling (Lawson et al., 2002; Mukouyama et al., 2002; Visconti et al., 2002). *Neuropilin-1* is a coreceptor for VEGF receptors and is expressed in arterial endothelial cells (Moyon et al., 2001). In compound homozygotes, *Neuropilin-1* expression was significantly reduced (Fig. 2P). By contrast, whereas *VEGF* is normally expressed throughout the mouse embryo with highest levels in the gut endoderm at E8.25 (Dumont et al., 1995), in compound homozygotes *VEGF* expression was upregulated in the paraxial mesoderm, neural tube, and endoderm as early as E8.25 (Fig. 2R and data not shown). Similar upregulation of *VEGF*, possibly because of hypoxia caused by cardiovascular defects, is observed in *Mef2c* mutant embryos at E9.5, but not at E8.25 when circulation is under way (Bi et al., 1999). This suggests that the upregulation of *VEGF* in compound *Foxc* mutants is unlikely to result from hypoxia. Because both *Foxc1* and *Foxc2* are normally expressed in the dorsal aorta and cardinal vein (Supplementary Fig. 1), we also examined the expression of venous markers in compound homozygotes. Interestingly, compared to the wild type (Figs. 2S and U), COUP-TFII and EphB4 proteins were similarly detected in endothelial cells of compound homozygotes (Figs. 2T and V), suggesting proper processes of venous specification/differentiation. This is consistent with recent evidence that endothelial cells of *Dll4* mutant embryos express EphB4 (Duarte et al., 2004), and it is possible that COUP-TFII counteracts Foxc to repress Notch signaling in veins (You et al., 2005). The expression of the lymphatic vessel endothelial hyaluronan receptor 1 (Lyve1) (Banerji et al., 1999) in the anterior cardinal vein at E9.0–9.5 is thought to indicate that venous endothelial cells are already competent to respond to a lymphatic-inducing signal (Oliver, 2004). The observation that Lyve1 protein was detected in

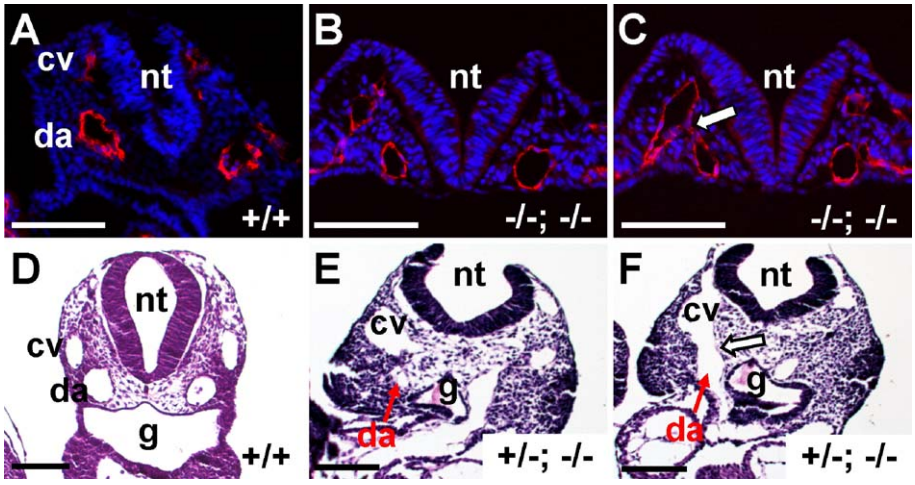

Fig. 1. Arteriovenous malformations (AVMs) in compound *Foxc1*; *Foxc2* mutant embryos. (A–C) PECAM-1 immunostaining of wild-type and compound homozygous embryos at the level of the heart tube at E8.25. Serial sections (B, C) reveal that the compound homozygote has an AVM caused by the fusion (white arrow) of blood vessels (C) compared with the dorsal aorta (da) and cardinal vein (cv) in the wild type (A). (D–F) Transverse sections of wild-type and compound *Foxc1*+/−; *Foxc2*−/− mutants at the level of the heart at E9.0. Serial sections (E, F) reveal that the compound *Foxc1*+/−; *Foxc2*−/− mutant embryo shows a vascular shunt (white arrow) between the dorsal aorta and anterior cardinal vein (F). g, gut; nt, neural tube. Scale bars, 100 μm.

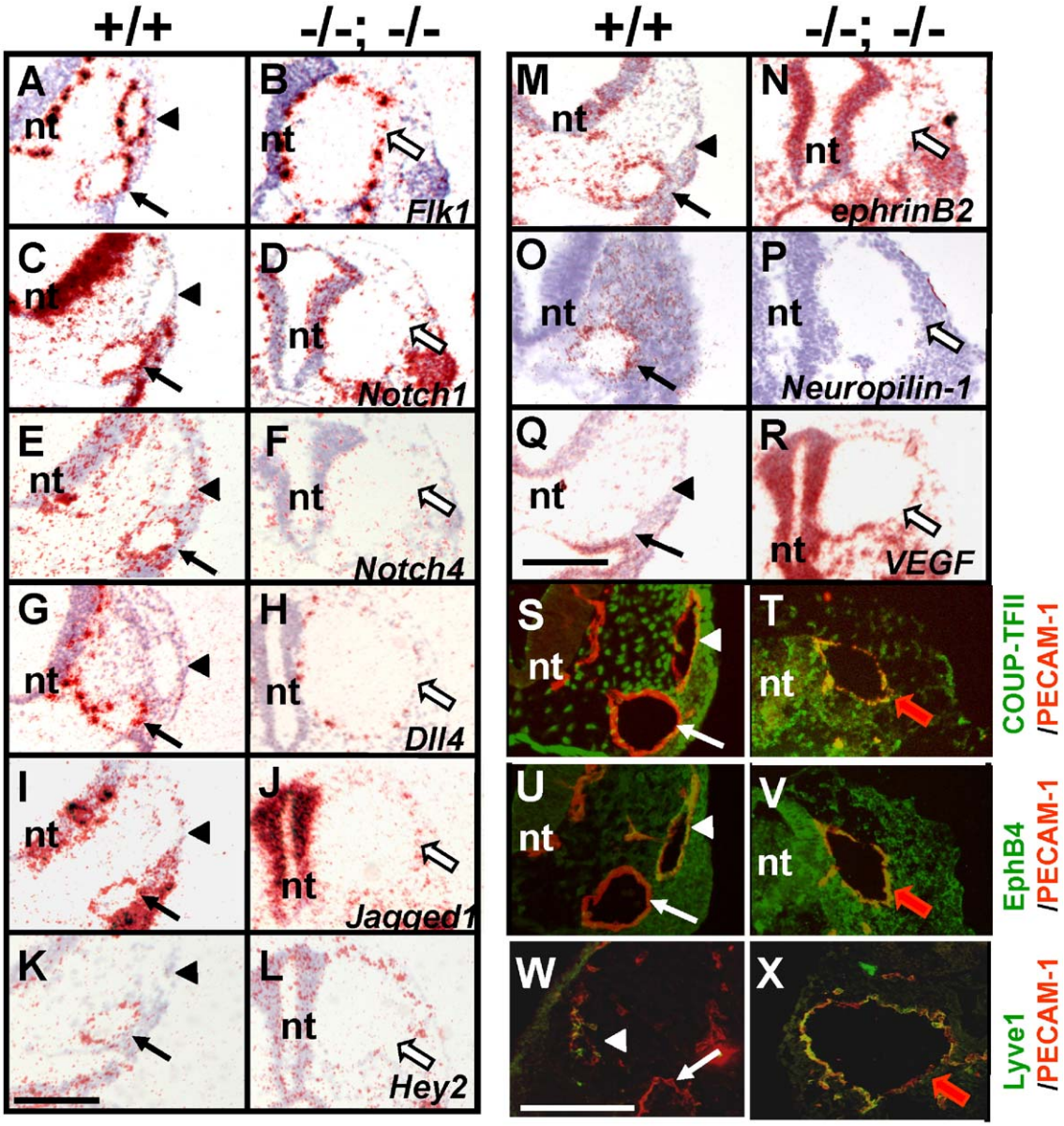

Fig. 2. Failure of induction of arterial markers in compound *Foxc1*; *Foxc2* homozygotes. (A–R) Section in situ hybridization at E9.0 at the level of the heart. Sections are counterstained with hematoxylin, and positive signals are shown in brown/red. In the wild type, *Notch1*, *Notch4*, *Dll4*, *Jagged1*, *Hey2*, *ephrinB2*, and *Neuropilin-1* are expressed in arteries (black arrows) but not in veins (black arrowheads) (C, E, G, I, K, M, and O). In compound homozygous mutant embryos, none of these arterial markers are induced in endothelial cells (white arrows) (D, F, H, J, L, N, and P). Expression of *VEGF* is upregulated in the compound homozygote (R) compared with the wild type (Q). (S–V) Immunohistochemical analysis at E9.0 to detect expression of venous markers. In the wild type, COUP-TFII and EphB4 are expressed in veins (white arrowheads) but not in arteries (white arrows) (S and U). Similarly, PECAM-1+ endothelial cells (red arrows) of compound homozygotes express both COUP-TFII (T) and EphB4 (V). (W, X) Immunohistochemical analysis at E9.0 to detect Lyve1 in the cardinal vein. Similar to the wild type (W), Lyve1 protein is localized in endothelial cells of the compound homozygote (red arrow) (X). nt, neural tube. Scale bar, 100 μm.

endothelial cells of compound homozygotes (Fig. 2X) further reinforces their acquirement of a venous fate.

Taken together, these results indicate that endothelial cells of compound *Foxc* mutants exhibit a failure of arterial specification leading to AVMs as well as vascular remodeling defects and that this phenotype is not due to cardiac defects (Kume et al., 2001), but primary to vascular abnormalities, consistent with recent evidence that the initiation of arterial marker expression is genetically predetermined and independent of blood flow (Le Noble et al., 2004; Moyon et al., 2001).

Compound Foxc1+/−; Foxc2−/− mutants exhibit defective formation of lymphatic vessels

Recent studies have shown that single *Foxc2* homozygous mutant embryos have normal development of the initial budding of lymphatic primordia and subsequent formation of lymphatic sacs at E12.5 (Dagenais et al., 2004; Petrova et al., 2004), although these mutants exhibit defective lymphatic valves and abnormal pericyte investment of lymphatic vessels in late development of the lymphatic vasculature (Petrova et al.,

2004). In agreement with the previous report (Dagenais et al., 2004), we observed that *Foxc2* expression is detected in the mesenchymal cells surrounding the cardinal vein where the specification and sprouting of lymphatic endothelial cells occur, and, interestingly, its expression is overlapped with that of *Foxc1* (Figs. 3F and G). Furthermore, their expression domains are localized in the mesenchyme expressing *VEGF-C* (Fig. 3H), which is required for the initial sprouting of lymphatic vessels from the veins (Karkkainen et al., 2004). Therefore, we tested whether compound *Foxc* mutants have normal development of lymphatic vessels. Because compound homozygotes cannot survive during lymphangiogenesis (starting from E10.5), we examined the formation of lymphatic vessels in compound *Foxc1*+/−; *Foxc2*−/− mutant embryos whose embryonic stages were matched with the wild-type embryos at E10.5 and E11.5 (Fig. 3). At E10.5, Prox1-positive cells have started sprouting from the cardinal vein in the wild type (Fig. 3A). Interestingly, although compound *Foxc1*+/−; *Foxc2*−/− embryos showed the budding of lymphatic endothelial cells from the cardinal vein (Fig. 3B), our quantitative analysis revealed that the number of Prox1-positive cells was significantly reduced compared to the wild type (Fig. 3E). This observation was more evident at E11.5 (Figs. 3D and E). Moreover, in contrast with wild-type embryos, the abnormal formation of the lymph sacs was seen in compound *Foxc1*+/−; *Foxc2*−/− mutants (Fig. 3D).

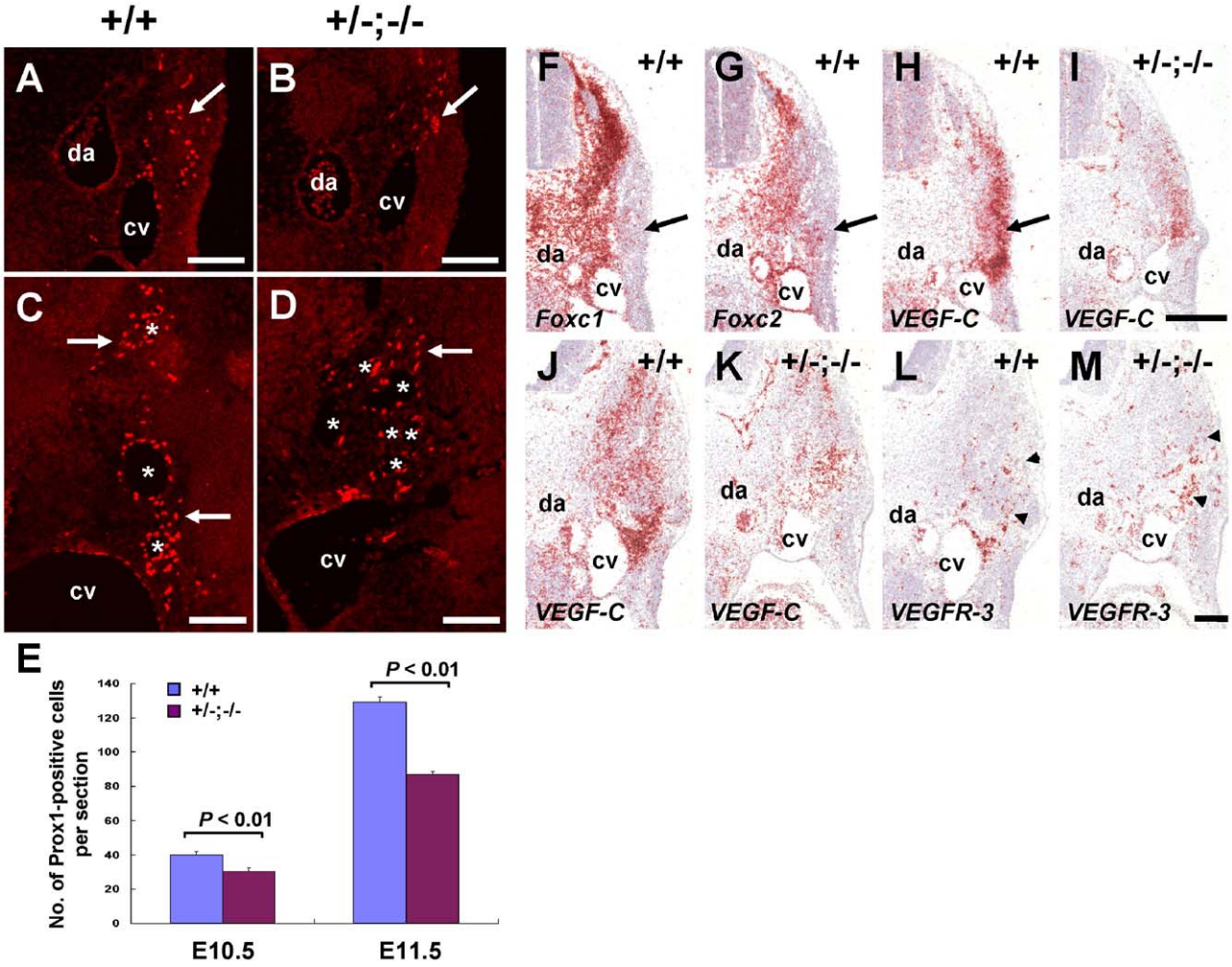

Fig. 3. Defective lymphatic vessel development in compound *Foxc1*+/−; *Foxc2*−/− mutants. (A–D) Immunohistochemical analysis to detect a lymphatic endothelial cell marker, Prox1, using transverse sections at the level of the heart at E10.5 (A, B) and E11.5 (C, D). (A, B) Although compound *Foxc1*+/−; *Foxc2*−/− mutant embryos (B) show the budding of lymphatic endothelial cells (arrows) from the cardinal vein, the number of Prox1-positive cells appears to be reduced compared to the wild type (A). (C, D) At E11.5, wild-type embryos (C) have well-formed lymph sacs (asterisks) and the sprouting of lymphatic endothelial cells (arrows). By contrast, abnormal formation of the lymph sacs and the reduced sprouting of lymphatic endothelial cells are observed in compound *Foxc1*+/−; *Foxc2*−/− mutants (D). (E) Quantitative analysis of Prox1-positive cells in wild-type and compound *Foxc1*+/−; *Foxc2*−/− mutant embryos at E10.5 and E11.5. The number of Prox1-positive cells is significantly reduced in compound *Foxc1*+/−; *Foxc2*−/− mutants at both E10.5 ($P < 0.01$) and E11.5 ($P < 0.01$). Data are collected from three embryos per genotype. (F–M) Section in situ hybridization at E10.5 (F–I) and E11.5 (J–M) at the level of the heart. In adjacent sections of wild-type embryo at E10.5 (F–H), expression domains of *Foxc1* (F) and *Foxc2* (G) in the developing lymphatic region are partially overlapped with those of *VEGF-C* (H) as indicated by arrows. *VEGF-C* expression is significantly reduced in compound *Foxc1*+/−; *Foxc2*−/− mutant embryos at E10.5 (I) and E11.5 (K), compared to wild-type embryos (H, J). By contrast, expression levels of *VEGFR-3* in lymphatic endothelial cells (arrowheads) are normal in compound *Foxc1*+/−; *Foxc2*−/− mutant (M), compared to wild-type embryo (L). cv, cardinal vein; da, dorsal aorta. Scale bars, 50 μm (A–D) and 100 μm (I and M).

Because the signaling pathway of VEGFR-3 and its ligand, VEGF-C, plays an important role in sprouting lymphatic vessels to form the initial lymph sacs (Karkkainen et al., 2004), we next examined their expression levels in compound *Foxc1*+/−; *Foxc2*−/− mutants. Whereas *VEGFR-3* expression was normally detected in lymphatic endothelial cells (Fig. 3M), levels of *VEGF-C* transcripts were significantly reduced in compound mutants (Figs. 3I and K), compared with the wild type (Figs. 3H and J) at E10.5 and E11.5. In addition, in compound mutants, no obvious reduction in cell proliferation was observed in Prox1-positive lymphatic endothelial cells and surrounding mesenchymal cells, as well as other tissues at E10.5 and E11.5 (Supplementary Fig. 2). Taken together, these data suggest that compound *Foxc* mutants have defects in the early processes of lymphatic development.

Foxc1 and Foxc2 transcription factors induce expression of arterial markers in vitro

To confirm defects in arterial specification/differentiation observed in compound homozygotes (Fig. 2), we examined whether Foxc1 and Foxc2 directly regulate transcripts of arterial markers in vitro. As shown in Fig. 4, we found that transient overexpression of either *Foxc1* or *Foxc2* significantly upregulates endogenous expression of *Dll4*, *Notch1*, *Notch4*, *Hey2*, and *ephrinB2* in mouse embryonic endothelial cells (MEECs) (Goumans et al., 2002) as well as multipotent mesenchymal 10T1/2 cells, whereas expression of pan-endothelial markers such as *Flk1* was not changed. Moreover, no change in expression of the venous markers, *COUP-TFII* and *EphB4*, was observed by overexpression of *Foxc* genes in these cell lines. Together, these data suggest that Foxc1 and Foxc2 are pivotal transcriptional regulators acting upstream of Notch signaling in arterial specification.

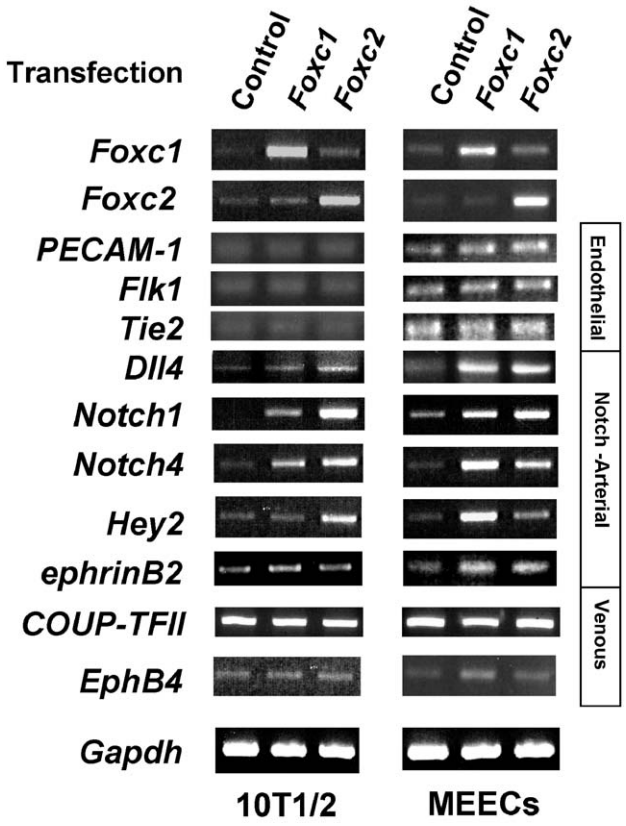

Fig. 4. Induction of arterial markers by overexpressing *Foxc* genes in vitro. *Foxc1* and *Foxc2* significantly upregulate transcripts of arterial markers in MEECs and 10T1/2 cells at 24 h after transfection. By contrast, transcripts of pan-endothelial and venous markers are not changed by overexpressing *Foxc*. Control indicates transfection of a mock expression vector. A representative experiment is depicted ($n = 4$).

Foxc proteins bind and activate the Dll4 promoter through a forkhead-binding element

It has recently been shown that *Dll4* mutant embryos have similar vascular abnormalities including AVMs (Duarte et al., 2004; Gale et al., 2004; Krebs et al., 2004). Given that expression of *Dll4* is absent in endothelial cells of compound *Foxc* homozygotes (Fig. 2H) and is upregulated by overexpression of *Foxc* in vitro (Fig. 4), we sought to analyze the effects of Foxc on the promoter activity of *Dll4*. In upstream regions in the mouse and human *Dll4* loci, there are two highly conserved regions (HHRs), HHR1 and HHR2. Significantly, we found that the HHR2 contains a putative Forkhead-binding element (FBE), which fits the core consensus sequence (Carlsson and Mahlapuu, 2002) (Fig. 5A). Using electrophoretic mobility shift assay (EMSA), we found that Foxc1 and Foxc2 proteins can directly bind to the FBE, whereas no DNA–protein complex was detected with the mock expression vector (Fig. 5B). It should be noted that the binding of Foxc proteins to the FBE probe was strongly competed by the excess cold probe. Next, in order to test whether Foxc can transactivate the mouse *Dll4* promoter by luciferase assay, we generated three reporter plasmids, which contain the −3.7 kb promoter, −3.7 kb promoter with a mutated FBE, and −2.1 kb promoter lacking the HHR2 (Fig. 5C). We found that both Foxc1 and Foxc2 significantly activated the −3.7 kb promoter in a dose-dependent manner (Fig. 5D). In addition, the induction of luciferase activity by Foxc1 and Foxc2 using either the −2.1 kb promoter or the −3.7 kb promoter with the mutated FBE was greatly reduced in COS-7 cells and MEECs (Fig. 5D and data not shown). These data strongly demonstrate that Foxc1 and Foxc2 directly activate the *Dll4* promoter via the FBE. Consistent with the phenotype of compound *Foxc1*; *Foxc2* mutants, these results indicate that the two *Foxc* genes act upstream of Notch signaling regulating arterial cell identity.

Discussion

Our data demonstrate that Foxc1 and Foxc2 have dose-dependent roles in arterial cell fate determination and that complete absence of *Foxc* genes results in a failure in arterial specification and malformations between arteries and veins. Most significantly, we show in this study that the Notch ligand, *Dll4*, is a downstream target of Foxc transcription factors. Therefore, we propose that Foxc transcription factors directly

act upstream of Notch signaling in this process, an idea consistent with recent studies that several mutant mice for Notch signaling genes such as *Dll4*, *RBP-J*, and *Hey1/Hey2* also do not express arterial markers such as *ephrinB2* (Duarte et al., 2004; Fischer et al., 2004; Kokubo et al., 2004; Krebs et al., 2004). Another intriguing finding is that compound *Foxc* mutants have reduced expression of *VEGF-C* in the mesenchyme surrounding the cardinal vein, leading to impaired sprouting of lymphatic vessels from the vein.

Foxc acts upstream of Notch signaling in arterial specification

Initial differentiation of endothelial cell lineage begins as a subset of mesodermal cells. They are thought to differentiate into bipotential progenitor cells, hemangioblasts (Huber et al., 2004). These progenitor cells subsequently differentiate into either endothelial or hematopoietic lineages. Endothelial progenitors (angioblasts) become either arterial or venous endothelial cells. Although it remains to be determined whether endothelial cells in veins and arteries have distinct precursors, molecular mechanisms underlying the development of arteries and veins have recently been discovered. As described in the Introduction, the VEGF signaling pathway activates Notch signaling in endothelial cells, regulating arterial specification/differentiation through the induction of artery-specific genes (Lawson et al., 2002; Mukouyama et al., 2002; Stalmans et al., 2002; Visconti et al., 2002). Because transcripts of *Foxc1* and *Foxc2* are first detected in mesoderm during gastrulation, they are likely to be expressed in arterial and venous precursors before and at the time of cell fate specification. Our results demonstrate that endothelial cells of compound homozygotes fail to acquire an arterial cell fate although they appear to differentiate from mesodermal cells and normally express pan-endothelial markers such as Flk1 and PECAM-1 (Fig. 2B and Kume et al., 2001). We cannot exclude the possibility that other cell types of the surrounding tissues affect arterial specification of endothelial cells in compound *Foxc* mutants since the *Foxc* genes are broadly expressed in the mesoderm of the early embryo. For example, although vascular smooth muscle differentiation appears to be normal in compound homozygotes (Kume et al., 2001), the problem of an earlier function for *Foxc* genes in mesodermal patterning may be partly associated with their vascular phenotype. Thus, in order to elucidate the precise role of *Foxc* in vascular development, conditional ablation of *Foxc* genes in an endothelial-specific manner needs to be performed in the future.

The upregulation of *VEGF* in compound homozygotes (Fig. 1R) is quite intriguing although the cause of this phenotype remains unclear. It has been shown that the VEGF concentration gradient is spatially restricted (Ruhrberg et al., 2002), and that *VEGF* expression in the somites of zebrafish embryos is vital for the induction of arterial specification (Lawson et al., 2002). The two *Foxc* genes are expressed in a dorsoventral gradient in the non-axial mesoderm with the highest levels in paraxial mesoderm (Kume et al., 2000b; Sasaki and Hogan, 1993), and Foxc1 and Foxc2 regulate the specification of mesoderm to paraxial versus intermediate fates (Wilm et al., 2004). Therefore, the two genes may normally repress expression of *VEGF* in the paraxial mesoderm and somite as a feedback loop, controlling the arterial identity of angioblasts derived from the lateral mesoderm as proposed (Lawson and Weinstein, 2002). Wild-type and compound homozygous embryos showed no significant differences in the number and distribution of either proliferating or apoptotic cells in endothelial cells as well as other tissues in both the embryo proper and yolk sac (data not shown). Given that endothelial cell proliferation in compound homozygotes appeared normal despite the upregulation of *VEGF* expression, one possibility is that the primary defect is a failure of *Foxc1/c2*-null endothelial cells to respond to the VEGF signal. As described below, the downregulation of *Neuropilin-1* expression also supports this idea. Another non-mutually exclusive possibility is that the dysregulation of *VEGF* expression in compound homozygotes may affect the precise control of arterial specification of endothelial cells because the definitive endoderm in mice is a potent source of VEGF (Miquerol et al., 1999).

Recent studies indicate that VEGF-induced specific signal to induce arterial specification is transmitted via the phospholipase Cγ-1 (Lawson et al., 2003) or phosphatidylinositol 3-kinase/Akt pathway (Liu et al., 2003), and that VEGF induces expression of *Notch1* and *Dll4* in arterial endothelial cells (Liu et al., 2003). However, transcription factors that act downstream of the VEGF-induced arterial signal to regulate gene expression have not been identified. Although the precise functional interactions between Foxc and VEGF remain to be elucidated, Foxc1 and Foxc2 may act as intracellular regulators downstream of VEGF signaling in arterial specification. Coupled with evidence that Foxc1 and Foxc2 are phosphoproteins in which a number of residues predicted to be phosphorylated (Berry et al., 2002; H.F. and T.K., unpublished results), it is plausible that precise transcriptional activity of Foxc proteins is regulated by VEGF-mediated phosphorylation/dephosphorylation. These possibilities are now being investigated.

It is still not clear how VEGF can discriminate one type of endothelial cell from another (e.g., a preference for arterial or venous endothelial precursors). Because *Neuropilin-1*, a coreceptor for VEGF receptors, is preferentially expressed in arterial endothelial cells (Moyon et al., 2001), it is thought that this coreceptor is a determinant for VEGF-mediated arterial signal or possibly induced to enhance/maintain the expression of artery-specific markers. This is consistent with our observation that in compound homozygotes *Neuropilin-1* expression was significantly reduced as early as E8.25 (Fig. 1P and data not shown). A recent study provides evidence that COUP-TFII determines the venous cell fate by suppressing expression of *Neuropilin-1* and Notch signaling genes, whereas *COUP-TFII*-mutant veins can acquire arterial characteristics (You et al., 2005). By contrast, *Dll4* mutant embryos exhibit the ectopic expression of the venous marker *EphB4* in the artery (Duarte et al., 2004), consistent with those seen in compound *Foxc1*; *Foxc2* mutants (Fig. 2V). Together, these data suggest that the precise control of the establishment and maintenance of arterial-venous identity is a complex process that may be regulated by counteraction between Foxc and COUP-TFII.

Defective lymphangiogenesis in compound Foxc1; Foxc2 mutants

Mutations of the human *FOXC2* gene are associated with dominantly inherited lymphedema (Fang et al., 2000). *Foxc2* homozygous mutants have agenesis of lymphatic valves and aberrant pericyte recruitment of lymphatic vessels (Petrova et al., 2004), although they have been reported to be normal in early lymphatic development. We show in this paper that compound *Foxc1*+/−; *Foxc2*−/− mutant embryos exhibit a reduced number of lymphatic endothelial cells sprouting from the vein in the early stage of lymphatic development. Although *Foxc1* expression has been shown to be negative in lymphatic vessels (Dagenais et al., 2004), *Foxc1* and *Foxc2* are coexpressed in mesenchymal cells surrounding the cardinal veins (Figs. 3F and G). Because these mesencymal cells also produce VEGF-C, which is required for initial sprouting of lymphatic endothelial cells (Karkkainen et al., 2004), the lymphatic phenotype observed in compound *Foxc* mutants is most likely attributed to the reduction of *VEGF-C* expression. It is currently unclear whether Foxc proteins directly regulate *VEGF-C* gene expression, and this possibility needs to be investigated further. Thus, coupled with the previous studies (Dagenais et al., 2004; Fang et al., 2000; Kriederman et al., 2003; Petrova et al., 2004), our results indicate that the dosage effects of *Foxc1* and *Foxc2* are involved in multiple processes of lymphatic vascular development.

Foxc directly regulates Dll4 gene expression

One of the most important findings is that Foxc1 and Foxc2 directly activate *Dll4* transcription via a Foxc-binding element

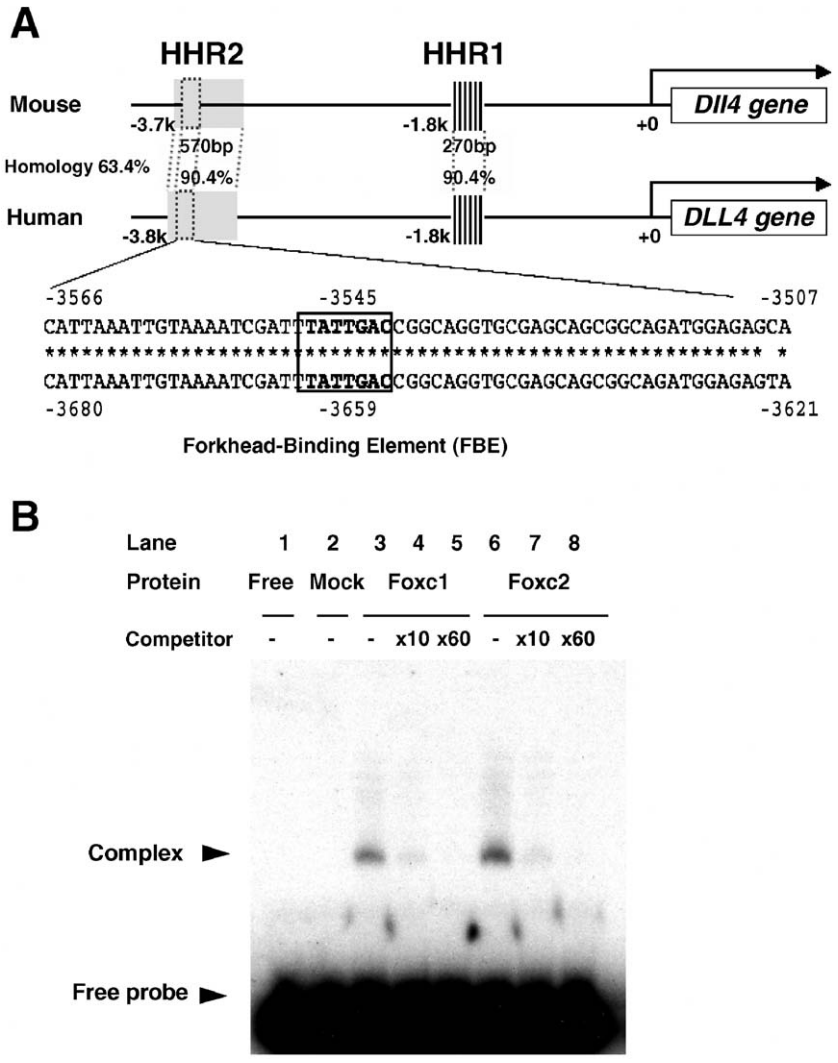

Fig. 5. *Dll4* is a direct downstream target of Foxc proteins. (A) Promoter regions of murine *Dll4* and human *DLL4*. They share two highly homologous regions (HHRs) with more than 90% identity. The HHR2 contains a consensus forkhead-binding element (FBE) shown in bold. (B) Electrophoretic gel mobility shift assays (EMSA). Both in vitro translated Foxc1 and Foxc2 proteins form DNA–protein complexes with the FBE in the *Dll4* promoter (lanes 3 and 6). The probes are competed by unlabeled oligonucleotides in a dose-dependent manner (lanes 4, 5, 7, and 8). No DNA–protein complex (lane 2) is observed with the blank expression vector pcDNA3.0 used as a control (Mock). (C) Luciferase reporter constructs. Note that the FBE in p*Dll4*-3.7kmut-Luc is disrupted. (D) Luciferase reporter assays. Both Foxc1 and Foxc2 transactivate p*Dll4*-3.7k-Luc in COS-7 cells in a dose-dependent manner, while luciferase activity of p*Dll4*-3.7kmut-Luc and p*Dll4*-2.1k-Luc by Foxc is significantly reduced. *Significantly different from control (pcDNA3.0), $P < 0.05$.

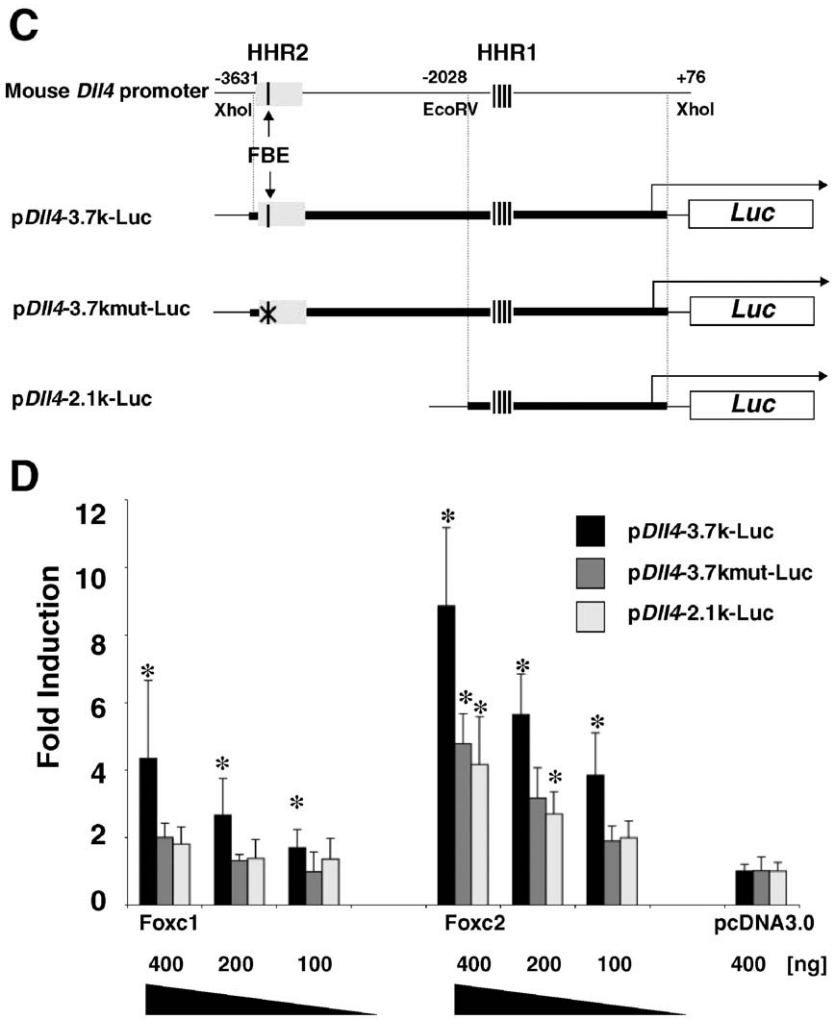

Fig. 5 (*continued*).

(FBE) (Fig. 5). To our knowledge, Foxc proteins are the first transcription factors identified to directly regulate *Dll4* expression during vascular development. Although a single Foxc-binding element is significantly important for the induction of *Dll4* expression, it is interesting that the mutation of FBE is not completely able to attenuate *Dll4* induction by Foxc2. Given that combinatorial regulation of gene expression by different classes of transcription factors is critical for vascular development (Kappel et al., 2000; Minami et al., 2003; Oettgen, 2001), other transcription factor(s), which binds to the HHR1 and interacts with Foxc2, may be responsible for the total regulation of *Dll4* transcription. Interestingly, we found that the HHR1 includes one putative biding site for the basic helix–loop–helix transcription factor SCL/Tal-1, whereas there are two SCL/Tal-1 binding sites and one Ets-binding site in the HHR2 (data not shown).

It should be noted that inactivation of all four alleles for *Foxc* result in more severe vascular defects than only two or three alleles are absent in vivo. Because there are putative Foxc-binding sites in conserved regions upstream of the 3.7 kb promoter (data not shown), multiple binding elements for Foxc proteins may be essential for the full induction of *Dll4* expression. In any case, it will be important to test the significance of the newly-identified FBE in the *Dll4* promoter in vivo using transgenic approaches, as in the case of a recently identified target of Foxc, *Tbx1*, in the development of the pharyngeal arches (Yamagishi et al., 2003). Such experiments are underway to validate the direct regulation of *Dll4* gene expression by Foxc.

We found that Foxc2 can more strongly transactivate the *Dll4* promoter than Foxc1. Importantly, although some compound *Foxc1*+/−; *Foxc2*−/− mutants exhibited AVMs as those seen in compound homozygotes, compound *Foxc1*−/−; *Foxc2*+/− mutants did form normal dorsal aortae and cardinal veins (Fig. 1 and data not shown), suggesting that there are some functional differences between the two genes. Although a duplication of the ancestral *Foxc* gene is quite likely to occur in the course of evolution (Carlsson and Mahlapuu, 2002), they may develop independent roles during evolution. For example, each single mutant dies with some distinct phenotypes (e.g., hydrocephalus in *Foxc1*−/−) (Kume et al., 1998). Thus, as described above, we predict that Foxc1 and Foxc2 have mostly

overlapping, but some distinct functions during vascular development.

In addition to *Dll4*, transcripts of other arterial markers such as *Notch1/4* were reduced in endothelial cells of compound homozygotes (Fig. 2), and expression of these genes was significantly induced by Foxc in vitro (Fig. 4). It has been shown that *C.elegans* Pha-4, a Foxa homologue, controls the expression of many regulatory genes throughout pharynx development (Gaudet and Mango, 2002), and that Foxo1 regulates many genes associated with vascular destabilization/remodeling and endothelial cell apoptosis (Daly et al., 2004). A recent study has demonstrated that the AP-1 transcription factor controls the transcription of the human *NOTCH4* gene in vascular endothelial cells via an AP-1 binding site in its promoter (Wu et al., 2005). Interestingly, we found that this AP-1 binding site is adjacent to a putative Forkhead binding site (data not shown). It is, therefore, tantalizing to speculate that Foxc1 and Foxc2 also regulate expression of *Notch4* in the process of arterial specification.

In conclusion, our results suggest that the two *Foxc* genes function together at an earlier stage to critically control the proper process of arterial specification by regulating expression of Notch signaling genes such as *Dll4*. In addition, while Foxc2 alone has been shown to play a role in late lymphatic developmental processes such as the formation and maintenance of the valves (Petrova et al., 2004), Foxc1 and Foxc2 cooperatively regulate sprouting of lymphatic endothelial cells. Therefore, the phenotype of compound *Foxc1*; *Foxc2* mutants reported here provides new insights into the molecular mechanisms underlying arterial specification and lymphatic sprouting during early embryonic development.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2006.03.035.

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