

TRANSCRIPTION FACTOR FOXC2 DEMARCATES THE JUGULAR LYMPHANGIOGENIC REGION IN AVIAN EMBRYOS

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ABSTRACT

In the human, mutations of the forkhead winged-helix transcription factor FOXC2 cause the lymphedema-distichiasis syndrome, which is characterized by a double row of eyelashes and pubertal onset lymphedema of the legs due to hyperplasia and malformation of lymphatic collectors. While a function of FOXC2 for the differentiation of lymphatic collectors is well documented, recent studies have indicated an early function for the sprouting of lymphatics from embryonic veins. We studied the expression of FoxC2 in early avian embryos and compared its expression pattern with that of the homeobox transcription factor Prox1, which is essential for lymphatic endothelial cell (LEC) development. We show that FoxC2 demarcates a segment of the somatopleura in the cervical region on embryonic day (ED) 3, before Prox1 is expressed. On ED 4, its expression domain coincides with that of Prox1 in the jugular region. This region is characterized by the confluence of Tie2-positive anterior and posterior cardinal veins. It has been shown that Prox1 expression in a subpopulation of venous endothelial cells induces transdifferentiation into LECs. Our data suggest that FoxC2, in addition to its late functions during lymph collector differentiation, has an early function during lymphendothelial commitment of venous ECs in the jugular region.

Keywords: FoxC2, lymphangiogenesis, lymphatic endothelial cell, jugular lymph sac, Prox1, Tie2, transdifferentiation, lymphedema-distichiasis

FoxC2/Mfh-1 is a forkhead/winged helix transcription factor, which is expressed in the paraxial and intermediate mesoderm of avian and murine embryos. It is also expressed in various organ rudiments, such as the eyelashes, kidney, heart and blood vessels, and has pleiotropic functions (1-4). In the human, FOXC2 mutations cause lymphedema-distichiasis (LD), which is a syndrome characterized by pubertal onset of lymphedema of the legs and a double row of eyelashes. Other manifestations may include cardiac defects, cleft palate and spinal extradural cysts. Although the number of lymphatic collectors in LD patients is greater than normal (hyperplasia), lymphedema develops at least in part because of the lack of competent valves that promote the centripetal flow of lymph in the collectors (5,6). Failure of FoxC2 functions in the mouse closely mimics the human syndrome (7-9). FoxC2 deficient mice exhibit abnormal eyelashes and other LD-associated phenotypic features. Morphogenesis of lymph vessels is disturbed: lymphatic capillaries are abnormally covered by pericyte-like cells, and lymphatic collectors fail to develop valves (8). The data show that in both mouse and man, FoxC2 is

essential for the development of a functional lymphatic system. It acts in the late stages of lymphangiogenesis, when lymphatic collectors become invested with contractile smooth muscle cells and develop valves, which control the direction of lymph flow.

Early functions of FoxC2 seem to reside in the induction of lymphatic sprouts from embryonic veins, and loss of FoxC2 can be rescued by the paralogous FoxC1 (10). It has been shown that the major part of the lymphovascular tree develops by sprouting from specific segments of the deep venous system (11). In the anterior part of the embryo, lymphatics are derived from the jugular lymph sacs, which, in turn, are derived from the jugular segment of the cardinal veins (11-14). Endothelial cells (ECs) in the jugular segment of the cardinal veins are characterized by the expression of the homeobox transcription factor Prox1, which is essential for the development of lymphatic endothelial cells (LECs) (12). Prox1 is the earliest and most stable marker of LECs during development, however, what drives Prox1 expression in the specific segments of embryonic veins is not yet known. We have studied FoxC2 expression in avian embryos before and during development of jugular lymph sacs. We show that chicken FoxC2 is expressed before Prox1 in a segment of the lateral plate, which corresponds to the lymphangiogenic jugular region. The spatio-temporal expression pattern of cFoxC2 suggests a function for the specification of venous ECs into lymphangioblasts.

MATERIALS AND METHODS

Embryos

Fertilized chick and quail eggs were incubated in a humidified atmosphere at 37.8°C. Staging (HH stages) was performed according to previous work (15). The embryos were removed from the eggs and fixed at various stages of development.

In Situ Hybridization

In situ hybridization (ISH) was performed as described previously (14,16). Briefly, embryos were fixed in 4% paraformaldehyde (PFA), rinsed, dehydrated in 100% methanol, and frozen. They were then rehydrated, treated with proteinase K, rinsed, and post-fixed with 4% PFA. Hybridization was performed at 70°C overnight. Specimens were rinsed and incubated with alkaline phosphatase-conjugated anti-digoxigenin antibody diluted 1:2000 in a blocking agent (Roche, Mannheim, Germany). The antibody was detected with 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium chloride (BCIP/NBT) in alkaline phosphatase buffer (Roche). The reaction was stopped with 1mM EDTA in phosphate buffered saline with Tween 20 (PBT), the specimens cleared in dimethyl formamide and stored in 4% PFA. For the detection of cProx1 mRNA, we used a chick probe that has been described by Tomarev et al (1996). The probe was cloned into pBluescript SK- and corresponded to positions 1442-3322 of the coding region of the *Prox1* gene. Linearization was performed with *EcoRI* (T3) and *SacI* (T7) for the preparation of sense and anti-sense probes, respectively. For the detection of cTie2 mRNA, a chick probe was used as previously described (17). The probe was cloned into pBluescript SK-. Linearization was performed with *XhoI* (T7) and *NotI* (T3) for the preparation of sense and anti-sense probes, respectively. For the detection of cFoxC2 mRNA, a chick probe was used described by Sudo et al (2001). The probe was cloned into pBluescript SK+. Linearization was performed with *NotI* (T3) and *XbaI* (T7) for the preparation of sense and anti-sense probes, respectively. Probe labeling was performed with the digoxigenin RNA labeling kit as recommended (Roche).

Immunofluorescence

For immunofluorescence studies,

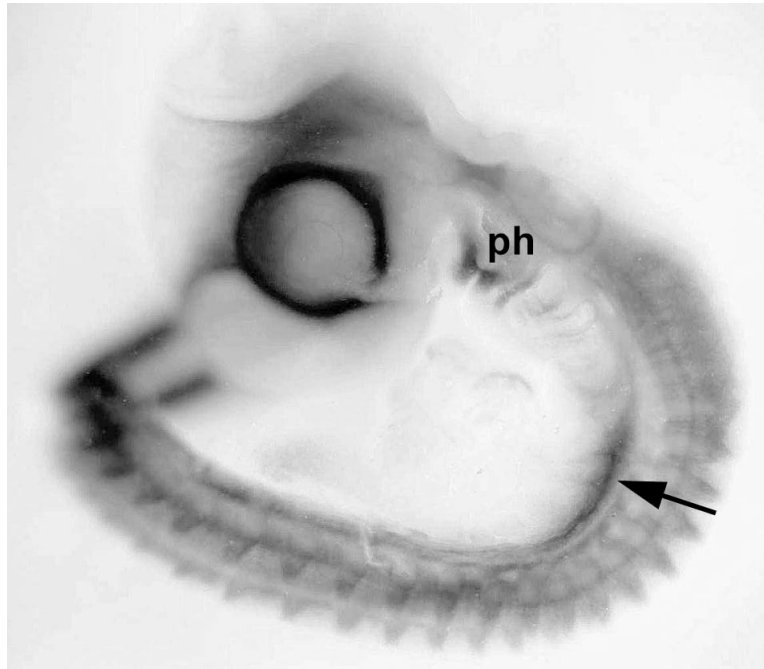


Fig. 1. In situ hybridization showing expression of cFoxC2 in ED 3 (stage 18 HH) chick. Strongest expression is found in developing eyelashes, vertebral arches, pharyngeal arches I and II (ph), and in the cervical somatopleure (arrow) adjacent to the heart.

specimens were embedded in tissue freeze medium (Leica). Cryosections of 16 μm thickness were prepared. Non-specific binding of antibodies was blocked by incubation with 1% bovine serum albumin (BSA) for 10 min. The monoclonal QH1 antibody was diluted 1:100 and incubated with the sections for 1h, as described previously (18,19). After rinsing, the secondary Alexa 594-conjugated goat-anti-mouse IgG (Molecular Probes, Eugene, US) was applied at 1:200 for 1h. Staining with polyclonal Prox1 antibody was performed as described previously (20) at a dilution of 1:500. The secondary Alexa 594-conjugated goat-anti-rabbit IgG (Molecular Probes) was applied at a dilution of 1:200. In controls, primary antibodies were omitted. After rinsing, sections were mounted under cover slips and viewed with an epifluorescence microscope (Zeiss, Göttingen, Germany).

RESULTS AND DISCUSSION

Expression patterns of FoxC2 have been studied in both mouse and chicken. The studies have shown that FoxC2 is strongly expressed in the paraxial mesoderm, and that, together with FoxC1, FoxC2 regulates segmentation of the paraxial mesoderm (somitogenesis) and specification of paraxial versus intermediate mesodermal cell fate (1,21). In the lateral plate, functions of FoxC2 have been studied at inter-limb levels, where it is involved in rib development under the control of Bone Morphogenetic Proteins (BMPs) (3). Otherwise, spatio-temporal expression patterns of FoxC2 in the lateral plate have not been studied in detail. Whereas in early ED 3 embryos (stages 16-17 HH), we could not detect a specific expression pattern in the lateral plate, we observed that chicken FoxC2 is expressed in the lateral plate of ED 3 chick and quail embryos (stage 18 HH) at the level of somites 7-11 (*Fig. 1*). This corresponds to the cervical region, which

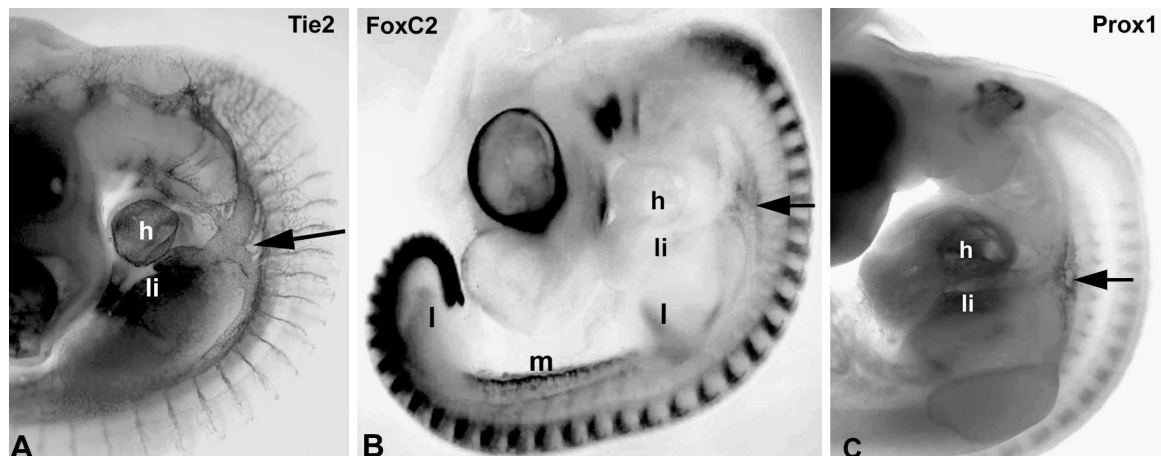


Fig. 2. *In situ* hybridization showing expression of *cTie2* (A) *cFoxC2* (B) and *cProx1* (C) in ED 4 (stage 21 HH) chick. A) *cTie2* is expressed in blood vascular endothelial cells. Note confluence of the anterior and posterior cardinal veins (arrow) into the duct of Cuvier. h, heart; li, liver. B) *cFoxC2* is expressed in the eyelashes, vertebral arches, occipital mesenchyme, pharyngeal arches I and II, limb buds (l), mesonephros (m), and in the cervical somatopleure (arrow) in the angle formed by the anterior and posterior cardinal veins. C) *cProx1* is expressed in the retina, otic placode, liver (li), heart (h), spinal and sympathetic ganglia, and in the angle formed by the anterior and posterior cardinal veins (arrow).

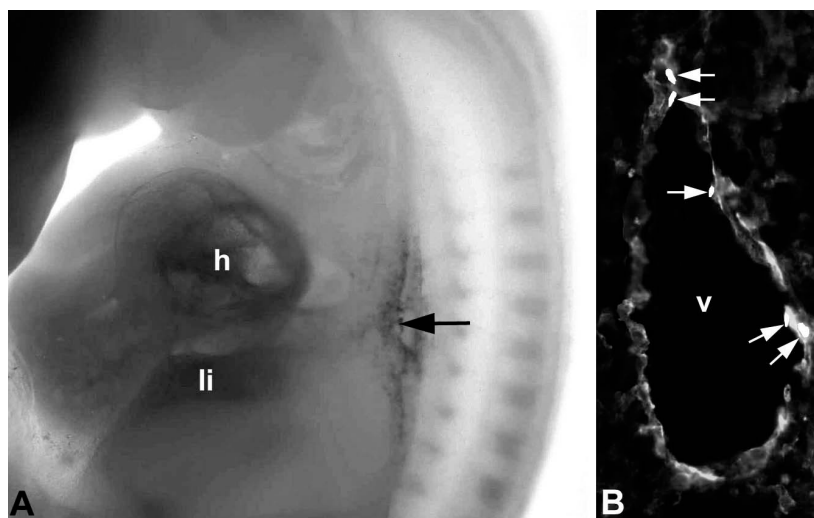


Fig. 3. Expression of *cProx1* in ED 4 (stage 21 HH) chick and quail. A) Higher magnification Fig 2C. *Prox1* is expressed in veins of the jugular region (arrow) but not by other vessels. li, liver; h, heart. B) Quail embryo stained with QH1 antibodies (green in original color photograph) and anti-*Prox1* antibodies (red in original color photograph). Note expression of *Prox1* in the nuclei (arrows) of a subpopulation of ECs in the jugular segment of the cardinal vein (v).

contains the venous inflow to the heart. Expression of *cFoxC2* in this region precedes that of *Prox1* in venous endothelial cells (14,22,23). On ED 4 (stage 21 HH), *cFoxC2*

is strongly expressed in the angle formed by the confluence of the anterior and posterior cardinal veins (Fig. 2A,B). This region corresponds to the jugular region, which, in

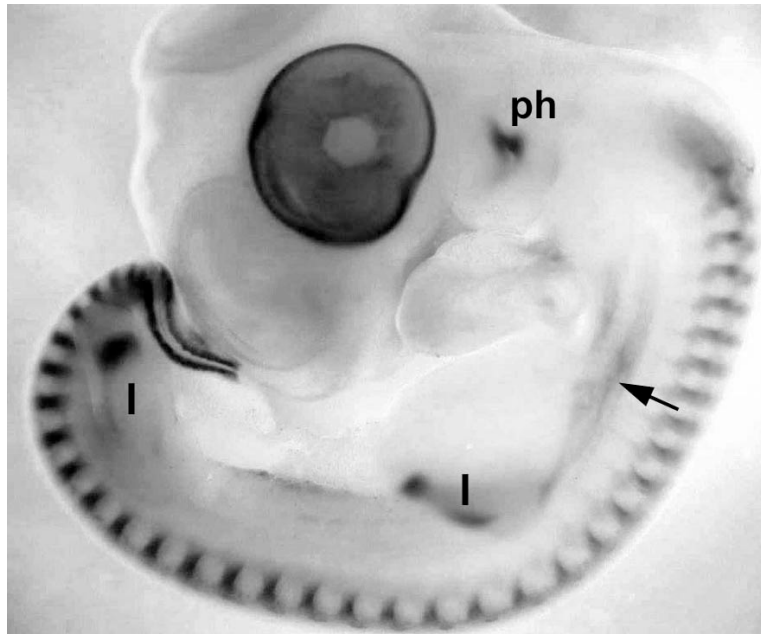


Fig. 4. Expression of *cFoxC2* in ED 5 (stage 25 HH) chick in the eyelashes, vertebral arches, pharyngeal arches I and II (ph), limb buds (l), and in the cervical somatopleure (arrow).

the adult, contains the anastomosis of the thoracic duct (or the right lymphatic duct) with the venous angle, which is formed by the internal jugular and the subclavian veins. In the embryo, the jugular region is the main lymphangiogenic region in the anterior part of the vertebrate body (11,12,14,24). The expression of *cFoxC2* now overlaps with that of *Prox1* in the confluence of the anterior and posterior cardinal veins (Fig 2B,C). The expression domain in the lateral plate is adjacent to somites 8-11/12. Studies on murine embryos have shown that *FoxC2* is expressed in both the veins and the surrounding mesenchymal cells (9,10). *Prox1* is expressed in a subpopulation of venous ECs (Fig. 3), and it has been shown that these cells sprout into the mesenchyme to form the jugular lymph sacs (11,14). In ED 5 (stage 24-25 HH) embryos, *cFoxC2* is still expressed in the jugular region, which has descended slightly and now is adjacent to somites 9-13 (Fig. 4). In chick and quail, jugular lymph sacs can be detected on ED 5-6 (23).

Recent studies have shown that embryonic LECs are derived from specific segments of the deep intra-embryonic venous system (11), although some LECs may also develop from scattered mesenchymal lymphangioblasts (14,19). Expression of *Prox1* in venous ECs is a prerequisite for the commitment of LECs (11,12) but what specifies selected venous segments to become lymphendothelial progenitors is not known.

Our studies suggest that *FoxC2* expression in the jugular segment of the lateral plate is involved in this process. *FoxC2* is expressed before *Prox1* and then overlaps with *Prox1* in the jugular region. Overlapping expression of *FoxC2* with the lymphendothelial markers *Prox1*, Vascular Endothelial Growth Factor Receptor-3 and *Lyve-1* has also been observed in the jugular region of mouse embryos (9). There is clear evidence that *FoxC2* is involved in lymphangiogenesis. It regulates the differentiation of lymphatic collectors and, thereby, seems to prevent lymphatic overgrowth. Nonsense and

frameshift mutations of *FOXC2* cause the lymphedema-distichiasis syndrome (5,6). This is characterized by hyperplasia of lymphatic collectors, which, however, are incompetent because of the lack of functional valves. A similar phenotype has been observed in *FoxC2*-haploinsufficient and null mice (7,8,25). In addition to control of lymph collector development, *FoxC2* seems to have an additional early function during the specification of LECs from venous precursor cells. Our data show that *FoxC2* is expressed in the jugular region of the somatopleure before *Prox1*, which is the major determinant of LEC development (12). *Prox1* is capable of transdifferentiating blood vascular ECs into LECs (26,27). The spatio-temporal expression of *FoxC2* suggests a function during the induction of *Prox1* in the jugular segment of the embryonic veins. The next step is the formation of jugular lymph sacs. This involves paracrine interactions between Vascular Endothelial Growth Factor-C-expressing mesenchymal cells and VEGFR-3-positive LEC precursors (28), and it appears that VEGF-C expression in the mesenchyme is also under the control of *FoxC2* (10). *FoxC2* functions can be taken over by *FoxC1*, which is expressed in overlapping domains (10). Additionally, *FoxC2* directly induces the chemokine receptor, *CXCR4*, in ECs, which directs migration of ECs toward its ligand *CXCL12* (29).

In summary, there is evidence that *FoxC2* has a threefold function in lymphangiogenesis: two early ones for the specification and migration of lymphangioblasts in the jugular region, and a late one for the differentiation of lymphatic collectors.

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