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# FOXC2 TRANSCRIPTION FACTOR: A NOVEL REGULATOR OF LYMPHANGIOGENESIS

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*ABSTRACT*

*Lymphangiogenesis is the critical process of forming new lymphatic vessels under physiological and pathological conditions and involves both molecular and morphological changes. Despite evidence that lymphangiogenic factors, including vascular endothelial growth factors (VEGFs) and Prox1, regulate lymphangiogenesis, the molecular mechanisms underlying gene regulation in lymphatic vessel remodeling and maturation are not fully understood. Importantly, recent studies demonstrate that Forkhead transcription factor FOXC2 controls later steps of lymphatic vascular development and is responsible for establishing a collecting lymphatic vessel identity by regulating expression of downstream genes involved in lymphangiogenesis, including PDGF-ß, Delta-like 4 (Dll4) and angiopoietin (Ang)-2. Thus, FOXC2 is now recognized as a novel regulator of lymphatic vascular formation and remodeling. This review summarizes current knowledge about the function of FOXC2 in lymphangiogenesis and discusses prospects for future research in FOXC2-mediated pathological lymphangiogenesis in lymphatic-related disease.*

**Keywords:** FOXC2, remodeling and maturation, lymphangiogenesis.

Lymphatic network formation is vital for embryonic development as well as postnatal life. Although embryonic vein endothelial cells sprout and incorporate to form primary lymph sacs and primary lymphatic plexus (1), subsequent processes of vascular remodeling and maturation gives rise to a functional network of lymphatic vessels, including lymphatic capillaries responsible for absorption of interstitial fluid and collecting lymph vessels that transport the lymph back to the blood circulation. While lymphangiogenesis occurs normally in almost all tissues under physiological conditions (with the notable exceptions of the central nervous system, bone marrow, cartilage, cornea and epidermis), elucidation of the mechanisms involved in pathological lymphangiogenesis such as solid tumor metastasis, inflammation and lymphedema is clearly of great importance. Recent work has discovered several regulators including the transcription factors Prox1 (2), VEGFR-3/ VEGF-C (3), Tie/Ang2 (4), and EphrinB2 (5), which contribute to early lymphangiogenesis. However, the later steps of lymphatic vascular development including the maturation of the primary lymphatic plexus into functional collecting vessels and capillaries, remain largely unknown and need further investigation. Here we discuss the rapidly accumulating evidence that transcription factor FOXC2 is a key regulator during lymphatic remodeling and maturation.

*Physiological Roles of FOXC2 during*

*Development and in the Adult*

*FOXC2* is a member of the forkhead/ winged-helix family of transcription factor genes that is located on the long (q) arm of chromosome 16 at position 24.1 (6). The gene, with a single exon which is highly GC rich, encodes for a 2.2 Kb protein (7). Forkhead box proteins are characterized by the forkhead box, a DNA binding Motif with a sequence of 80 to 100 amino acids (8). In humans, the FOX family of transcription factors is present in various embryonic organs and tissues during development and plays crucial roles in a variety of processes such as proliferation, differentiation and survival (9). FOXC2 protein, also known as forkhead-related protein FKHL14 (FKHL14), transcription factor FKH-14, or mesenchyme forkhead protein (MFH1), belongs to the "C" subfamily and is required for cardiovascular development (10) as well as the development of the lungs (11), eyes (12), kidneys (13) and urinary tract (14). Recently, FOXC2 has been shown to be involved in cancer angiogenesis and metastasis (15) and is particularly implicated in cancer progression through its induction of epithelial-to-mesenchymal transition (16). Moreover, suppression of FOXC2 expression using shRNA in a highly metastatic breast cancer model blocked metastatic ability (17), indicating FOXC2 might be another therapeutic target for cancer therapy.

*Role of Foxc2 in Lymphatic Vascular Development*

Recently, Foxc2 has been shown to be implicated in lymphatic vascular development and disease (18,19). The fact that Foxc2 is highly expressed in the developing lymphatic vessels as well as lymphatic valves in adult mice raises the possibility that it serves a function in the development and maintenance of the lymphatic vasculature (19-21). Moreover, loss of Foxc2 leads to abnormal lymphatic patterning, failure to form lymphatic valves and increased mural cell investment of lymphatic capillaries in a mouse model, indicating Foxc2's role in remodeling and maturation of lymphatic vessels (19). Further investigations using models of animal development showed that change in Foxc2 expression is the critical first event of the maturation process of lymphatic vessels (18), configuring Foxc2 as an important transcriptional regulator to control expression of multiple genes in the process of lymphatic maturation. This conclusion is supported by the observation that Foxc2 inhibited SMC coverage of initial lymphatic vessels through suppressing the expression of PDGF-ß, a potent chemoattractant for vSMCs, in lymphatic vessels (19,22). Notably, Foxc2 regulated the transcriptional network in this process in cooperation with nuclear factor of activated T cells (NFATc)-1 that has been found to control the morphogenesis of cardiac valves (18,23).

Interestingly, patients with Lymphedema-Distichiasis syndrome (LD) [Online Mendelian Inheritance in Man (OMIM)], a disease caused by mutations in *FOXC2*, have a similar phenotype to *Foxc2* null (19) and haploinsufficient mice (20). So far, mutations in *FOXC2* have been found to be the only known cause of LD. Moreover, frameshift mutations that involve a probable loss-of-function mechanism (24-26) were the predominant mutations, whereas nonsense and missense mutations were rare (7,26,27). While genotype/phenotype correlation has not been clearly delineated, all mutations including frameshift and nonsense mutations that are responsible for LD truncate the protein, leading to *FOXC2* haploinsufficiency (24,26,28,29). Clinically, affected patients commonly have bilateral lymphedema of the lower limbs that usually develops around puberty and abnormal eyelashes (a double row of eyelashes) (7). In addition to these specific signs of LD, other associated complications may include ptosis (31%), varicose veins (25%), congenital heart diseases (6.8-10%), and cleft palate (4-10%). Scoliosis,

renal anomalies, hydrocele, strabismus have also been reported but are less common (26), suggesting the phenotypic spectrum of LD could be variable.

Further investigation of the lymphedema-distichiasis phenotype through lymphscintigraphy demonstrates lymph reflux in the large lymphatic vessels of the leg, suggesting agenesis or absence of valves in the collecting lymphatic vessels (7). In addition, in contrast to the lymphatic vessel hypoplasia or aplasia seen in other forms of primary lymphedema, FOXC2 haploinsufficient humans and mice exhibit a hyperplastic lymphatic system (7,18,20,22,30). Indeed, failure of downregulation of VEGFR3 and active VEGFR3 signaling have been observed in FOXC2-deficient lymphatic vessels (18).This finding indicates that reduced FOXC2 expression disrupts the normal balance between lymphatic vessel growth promoting and inhibiting genes. Interestingly, histological study revealed that the major proportion of skin lymphatic vessels was abnormally invested by smooth muscle cells in patients with *FOXC2* mutations (19), which is in agreement with the findings in *Foxc2*-/- mice and indicates that FOXC2 is essential for the morphogenesis of lymphatic valves and the establishment of a pericyte-free initial lymphatic network (19).

In addition, varicose veins are another typical sign of LD. Recently *FOXC2* mutations have been shown to be implicated in primary valve failure in veins (31), suggesting a possible developmental role for FOXC2 in both venous and lymphatic systems.

Recently, Seo et al have reported that compound null mouse mutant embryos for *Foxc2* and related *Foxc1*, have reduced sprouting of Prox1 positive cells from the cardinal vein by reducing mesenchymal Vegf-C expression (32), indicating Forkhead transcription factors might act upstream of Vegf-C in the formation of primary lymph sacs. On the other hand, while Foxc1 and Foxc2 have very similar expression patterns in various embryonic tissues and overlapping roles in development of some organs (33-36), Foxc1 has been shown to be absent in lymphatics (21). These observations suggest that Foxc2 regulates the maturation of lymphatic vessels, whereas early lymphatic specification depends to some extent on proper Foxc1 activity.

Although deficiency of forkhead transcription factor Foxc2 results in defects in lymphatic remodeling and failure to form lymphatic valves (19), the precise function of Foxc2 in this process has yet to be defined. Recently, it has been reported that integrin-$\alpha$9 mutant mice have a similar phenotype to *Foxc2*-null mice, displaying abnormal lymphatic valves and retrograde lymph flow and impaired fluid transport. Integrin-$\alpha$9 has been shown to be implicated in controlling the formation of lymphatic valve leaflets (37). Further analysis shows that upregulation of *Foxc2* transcription factor initiated the formation of the lymphatic valve followed by the development of the valve leaflet, which was initiated by upregulation of integrin-$\alpha$9 expression during embryogenesis (18,37). On the other hand, Foxc2 like integrin-$\alpha$9 is present in mature and developing lymphatic valves (37). In this light, it is tempting to speculate that Foxc2 acts upstream of integrin-$\alpha$9 in the formation of lymphatic valves. But this possibility needs to be further investigated.

### *Foxc2 Regulates Notch Signaling in Lymphangiogenesis?*

Notch signaling is an evolutionarily conserved pathway that modulates arterial cell-fate decisions (38,39). Interestingly, Notch, in addition to its role in blood vessel morphogenesis and arterial development (40,41), also regulates lymphatic development. Notch1 and Notch4 have been found to be present in some murine lymphatics as well as tumoral lymphatics of human mammary carcinomas (42). Moreover, some *in vitro* studies have displayed that notch signaling participates in and regulates

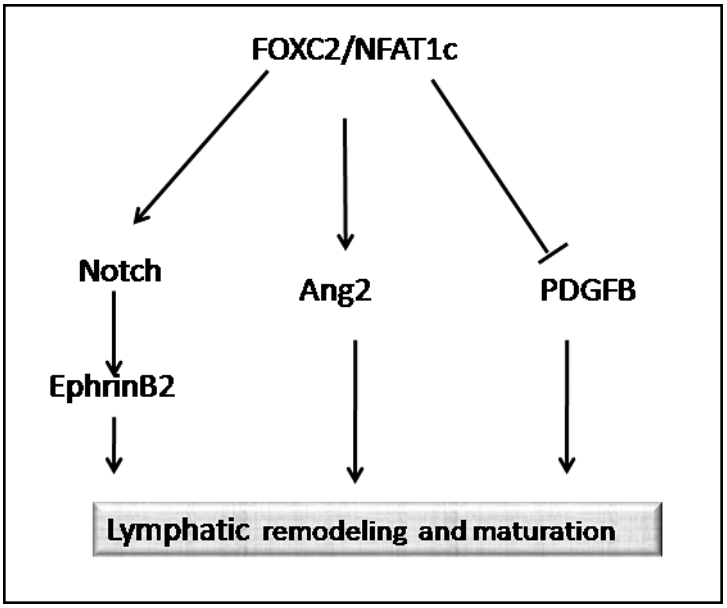


*Fig. 1. Proposed upstream location of FOXC2 and NFAT1c in lymphatic remodeling and maturation.*

lymphangiogenesis (43), and a recent *in vivo* study using gene silencing methods in zebrafish demonstrated a role for Notch signaling in lymphangiogenesis (44). Knockdown of Dll4 or its receptors (Notch-1b or Notch-6) in zebrafish resulted in impaired lymphangiogenesis at multiple steps during early lymphatic vascular development, indicating critical roles for Notch signaling in the formation and wiring of the lymphatic network (45).

Recently, it has been found that Foxc1 and Foxc2 upregulate the expression of Notch1, Notch4, and Dll4, as well as the arterial specific markers, ephrinB2 in mouse embryonic endothelial cells (MEECs) (32). Furthermore, recent studies showed that Foxc2 directly induces the transcription of delta-like4 (a novel ligand for notch receptors) by activating its promoters, suggesting that Foxc2 transcription factors act upstream of Notch signal to mediate arterial specification. Thus, these findings lead to speculation of a similar relationship between Foxc2 and Notch signaling in lymphatic endothelial cells. On the other hand, Notch promotes arterial endothelial differentiation in part through regulation of ephrinB2 expression in arterial endothelium (46). Equally, Dll4/Notch increased expression levels of ephrinB2 in the lymphatic endothelium *in vitro*. Moreover, ephrinB2 has recently been shown to contribute to remodeling of the lymphatic plexus into lymphatic capillaries and collecting vessels (5). It is thus possible that Foxc2 might mediate later steps of lymphatic vascular remodeling and maturation via Notch- ephrinB2 pathway (*Fig. 1*).

### *Foxc2-Dependent Autocrine Effects on Lymphangiogenesis*

Recent studies have demonstrated that Foxc2 indirectly controls angiogenesis as well as remodeling and maturation of the vasculature by inducing Ang2 expression in adipocytes in a paracrine manner (47). Ang2 is a ligand for the tyrosine kinase receptor Tie2 on endothelial cells and plays an important role in remodeling and maturation of blood vessels as well as lymphatic vessels (4,48-50). Ang2 is produced by LECs *in vivo*

(18,29). Moreover, the activation of *FOXC2* results in release of Ang-2 from Weibel-Palade bodies in endothelial cells (51,52). These findings suggest that FOXC2 promotes lymphangiogenesis in an autocrine manner in lymphatic endothelial cells. On the other hand, *Ang2* and *Foxc2* mutant mice exhibit some similarities in lymphatic phenotype, e.g., improper recruitment of smooth muscle cells into lymphatic capillaries (4,19,53). Could the lymphatic defects in *Foxc2* mutant mice be in part due to decreased Ang2 expression as well?

## *CONCLUSION*

Accumulated studies on FOXC2's role in lymphangiogenesis, using animal models development as well as clinical investigations, have revealed complicated functions of FOXC2 in lymphangiogenesis. It is well recognized that FOXC2 regulates later steps of lymphatic vascular development, the maturation of the primary lymphatic plexus into functional collecting vessels and capillaries in cooperation with NFATc1 by controlling expression of multiple genes in critical aspects of lymphangiogenesis. In addition to PDGF-ß, FOXC2 might also contribute to lymphatic vascular remodeling and maturation via Notch- ephrinB2 pathway and Ang2. Elevated FOXC2 expression in the developing lymphatic vessels as well as in lymphatic valves in adults have established FOXC2 as an attractive therapeutic target for lymphatic-related diseases.

Future studies should focus on identifying the direct FOXC2 target genes and enhancing understanding of the interplay of FOXC2 with other prolymphangiogenic molecules such as VEGFR3, which is linked to a subset of primary congenital lymphedema syndromes (Milroy disease; OMIM 153100) (54,55). Although it has been reported that Foxc2 may act downstream of Vegfr3 in blocking SMC coverage to lymphatic capillaries (19), many key questions remain. What are the cellular and molecular processes involved in the interactions between FOXC2 and VEGFR3? How does such an interaction affect gene expression in lymphatic endothelial cells?

Taken together, further analysis of the role of FOXC2 in lymphangiogenesis may promote a new treatment approach for LD and perhaps secondary lymphedemas.

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