Minireview

**Lymphatic endothelial differentiation: start out with Sox - carry on with Prox**

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**Abstract**

The transcription factor Prox1 is the master regulator of lymphatic endothelial cell differentiation and its expression initiates the morphogenesis of the lymphatic vasculature in the early embryo. Two new studies now answer some fundamental questions concerning Prox1 biology.

The bodies of higher vertebrates contain two highly branched hierarchical networks of endothelial tubules. One comprises the blood vessels, which provide the conduits for the systemic circulation and transport cells, gases, nutrients and waste products to their appropriate targets. The second endothelial network, the lymphatic vasculature, carries the lymph - draining tissues of plasma, proteins, particles and cells that have actively or passively gained access to the extracellular space [1,2]. Although blood vessels and lymphatic vessels are often found in close proximity, direct contact is avoided, thereby preventing illegitimate shortcuts between the two networks. Two defined connections do exist. These enable lymphatic vessels to return their cargo into the venous circulation after having delivered potential antigens to the adaptive immune system en route, as the lymph percolates through the lymph nodes [3].

Despite being first described in the 17th century by Aselli [4], the lymphatic system until very recently remained the Cinderella of the vascular family. However, the discovery of positively identifying marker proteins for lymphatic endothelial cells (LECs), such as the homeobox transcription factor Prox1, and the generation of targeted gene deletions causing lymphatic defects in the mouse have led to unprecedented progress in our understanding of the biology of lymph vessel formation.

**Initiation and maintenance of lymphatic differentiation**

Endothelial expression of Prox1 is first detected in mice at embryonic day (E)10.5 in a dorsal subset of endothelial cells of the cardinal veins. Prox1-positive cells adopt a lymphatic identity and under the influence of vascular endothelial growth factor-C (VEGF-C) bud from the veins, migrate away and reorganize themselves in the primary lymph sacs of the jugular and mesonephric region [5]. Prox1-deficient embryos do not accomplish specification of the emerging lymphatic subpopulation and lack the subsequent upregulation of lymphatic markers. Endothelial cells bud from the cardinal veins but keep on expressing blood vascular markers and fail to organize into lymph sacs. The result is a complete arrest of lymphatic development [6,7]. The knockout therefore indicates that Prox1 serves as a master gene for lymphatic identity, a notion further bolstered by reports demonstrating that forced expression of Prox1 in blood endothelial cells (BECs) led to the acquisition of many lymphatic markers [8,9].

But despite the overwhelming evidence for the role of Prox1 as a lymphatic master regulator, it is still entirely unclear which molecular mechanism triggers the transcription of Prox1 during the differentiation of the first LECs and, equally important, how lymphatic expression of the transcription factor is maintained throughout life. With
respect to the first part of this question at least, a study by Peter Koopman and co-workers (François et al. [10]) published recently in *Nature* adds an important piece to the puzzle, by elucidating the role of the transcription factor Sox18 in the regulation of Prox1.

Mutations in the gene for Sox18 are known to be responsible for the naturally occurring mouse mutants of the ragged allelic series [11]. Ragged mutations affect the coat hair and also cause vascular malfunctions that result in chylous ascites and edema. In humans, dysfunction of Sox18 is likely to contribute to the development of the hypotrichosis-lymphedema-telangiectasia syndrome [12].

Somewhat unexpectedly, targeted inactivation of Sox18 in the mouse failed to cause vessel defects, which has been attributed to genetic compensation by the related Sox family members Sox7 and Sox17 [13-15]. Whereas this knockout had been generated in a mixed 129/CD1 background, François et al. [10] now report that homozygous Sox8-deficient mice on a pure-bred C57/B6 background develop lethal fetal edema. Heterozygotes already display patterning and remodeling defects of the dermal lymphatic master switch, suggesting an important function for Sox18 during lymphatic development. The absence of polarized Prox1 expression in the cardinal veins of Sox18-deficient embryos indicates that Sox18 is necessary for Prox1 induction during the first steps of lymphatic specification. In the cardinal vein, Sox18 expression precedes the onset of Prox1 expression by a whole day, also displaying the characteristic polarized expression pattern in a subset of endothelial cells within the vessel wall (Figure 1a–c). Furthermore, forced expression of Sox18 in differentiating endothelial cells results in the upregulation of lymphatic signature genes, most notably Prox1. Indeed, a proximal 4.1-kb Prox1 promoter fragment contains two Sox18-binding sites, which are both necessary for Prox1 expression *in vitro* and *in vivo*.

**Sox18: just one day of fame?**

The study by the Koopman lab raises the question of whether Sox18 is the ultimate lymphatic master switch. Clearly, Sox18 is part of an essential decision process upstream of Prox1. However, in contrast to Prox1, Sox18 is neither indispensable nor likely to act single-handedly during lymphatic differentiation, as is indicated by the normal lymphatic development in Sox18-knockout mice on an outbred background. Here, the related transcription factors Sox7 or Sox17 might compensate for the loss of Sox18, and it could prove revealing to test the C57/B6 mouse for defects in one of these genes. Furthermore, François et al. [10] demonstrate abundant Sox18 expression in embryonic blood vessels and blood vessels of the newborn mesentery and skin. This pattern of expression indicates that Sox18 alone cannot be sufficient for the specification of lymphatic vessels and points to the existence of an indispensable, but as yet unidentified, ally of Sox18 during lymphatic specification.

Intriguingly, and in contrast to Prox1, persistent expression of Sox18 is not necessary for the maintenance of lymphatic identity. Obviously, both genes define two different classes of master switch during tissue specification. Sox18 appears to act as an inducer of the lymphatic program in the early embryo and apparently becomes dispensable thereafter. Prox1 rather exerts a sustaining function and its constant presence is necessary for the maintenance of the lymphatic program. The nature of the signals required for this later phase of Prox1 expression are unclear, but one possibility is that Prox1 might stimulate its own promoter either directly or via intermediate transcriptional targets.

**Lymphatic endothelial cell plasticity**

More recently, unexpected plasticity of lymphatic endothelial cells has been reported. Johnson et al. [16] used tamoxifen-inducible Cre-mediated, and therefore temporally controlled, inactivation of the Prox1 gene in mice to study the role of Prox1 in lymphatic vessels at various developmental stages. Loss of Prox1 from venous lymphatic precursors resulted in prominent edema and scattered blood-filled vessels at midgestation, reminiscent of constitutive Prox1-knockout mice...
[6] (Figure 1d). Similarly, targeting of the Prox1 gene during later steps of lymphatic development in the embryo led to the presence of blood in the superficial lymphatics of the developing skin and in the mesenteric lymphatics.

In keeping with the proposed function of Prox1 as a master regulator of lymphatic differentiation, loss of Prox1 expression was accompanied by the loss or downregulation of other lymphatic markers such as podoplanin, CCL21 (SLC) and Lyve1. Concomitantly, markers characteristic for the endothelium of blood vessels, such as endoglin or CD34, were upregulated, and perivascular cells positive for smooth muscle a-actin, a characteristic feature of blood vessels but not of lymphatic capillaries, covered the mutant lymphatic vasculature [3]. Previous work has shown that the endothelial cells of lymphatic capillaries (also termed initial lymphatics) are connected by discontinuous, button-like junctions, which presumably facilitate the uptake of cargo from the extracellular space [17]. Loss of Prox1 compromised the lymph-specific distribution of the junctional adhesion molecule VE-cadherin and consequently impaired the formation of button-like junctions. However, the continuous and zipper-like junctional pattern seen in the endothelium of blood vessels was not reacquired in Prox1 mutants, suggesting that LEC dedifferentiation in these mutants is incomplete or deregulated. Nevertheless, the sum of the findings argues for a change in vessel identity and the partial adoption of a blood-vessel-like phenotype by the dedifferentiated lymphatics.

Lymphatic endothelial cell plasticity in health and disease

The study by Johnson et al. [16] adds to the view that differentiated tissues may retain a surprising degree of plasticity. Continued expression of Prox1 is required for maintaining LEC differentiation even in the adult. Conversely, loss expression or dysfunction of Prox1 might be potentially relevant for certain human diseases such as hereditary lymphedema syndromes, in which malformed lymphatic vessels are seen [18-20]. Moreover, neoplastic endothelial cells in angiosarcoma and Karpoo’s sarcoma express both BEC and LEC markers and lack a clear identity [21-23]. Future work will have to address whether Prox1 plays any part in these or other disease conditions.

The physiological reasons for the remarkable plasticity of the lymphatic endothelium also remain unclear. Are there any circumstances during development, growth or tissue regeneration that might trigger the reprograming of LECs and their incorporation into blood vessels? Equally enigmatic is the question of how the dedifferentiation of LECs leads to the presence of blood cells within the mutant lymphatic vessels. This defect is observed in a number of mouse mutations affecting lymphatic differentiation [20,24-26], and indicates the presence of aberrant connections between blood vessels and lymphatic vessels. An important issue to be resolved is whether such connections are formed by cell-cell interactions between now identically specified Prox1-deficient endothelial cells at sites of closest proximity. Alternatively, dedifferentiated LECs might respond to the same tissue-derived guidance signals as BECs, so that sprouts and growing vessels will make contact.

Owing to the availability of targeted mouse mutations and increasingly refined genetic tools that allow the timed and tissue-directed precise deletion of genes, the regulation of blood vessel specification and differentiation is slowly unfolding. Interestingly, endothelial differentiation appears to entail an unexpected degree of reversibility, which may be encouraging news for future attempts towards therapeutic intervention and regeneration.

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