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LYMPHANGIOGENESIS REVIEWS

THE EMERGENCE OF MOLECULAR AND TRANSGENIC LYMPHOLOGY: WHAT DO WE (REALLY) KNOW SO FAR?

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ABSTRACT

Since its first sequential visualization in 1902 by Florence Sabin, the development and maintenance of the lymphatic system has intrigued scientists and clinicians worldwide. Its close ties to the vascular network, relevance in the spread and control of parasitic and cancerous diseases, and involvement in the development of other disease states manifested by lymphedema are well known. What is still not clear is how the system develops in the first place, and this limits its effective manipulation for the management of disease states. The aim of the current article is to summarize advances that have been made via genetic approaches using transgenic and knockout mice. It should be noted that studies of lymphatic vessel growth utilizing protein reagents or transgenic technology alone, in a tissue/cell culture environment, during tumor metastasis, in a lymphatic disease paradigm, or during tissue repair, have shown that various growth factors, such as platelet-derived growth factor BB (1), hepatocyte growth factor (2), fibroblast growth factor (3), and VEGF-A (4) appear to play a role. This article is a commentary on the usefulness of specific genetic engineering tools in understanding the development of the lymphatic system — with a focus on the questions that have been addressed using these tools, the extent to which the questions have actually been answered, and the questions that have subsequently been raised. It is not meant to be a discussion of protein reagents or of specific biological situations that exhibit lymphangiogenesis (see reviews 5-7).

Keywords: molecular lymphangiogenesis, transgenic technology, lymphatic development, lymphedema, VEGF, angiopoietins, Prox-1, Foxc2

Genetic manipulations afforded by the techniques of molecular biology have been crucial in providing early clues about the development and maintenance of the lymphatic system *in vivo* (see technology review in *Fig. 1*). We now know some of the genes, and hence the proteins, that are essential at different stages of development. So far, nearly all results have been based on straightforward genetic manipulations, such as those involving the over-expression or removal of a single gene. In each case, the gene under study was chosen either because of its already established role in angiogenesis (e.g., *Vegfr-3*), or because of its relationship to or interaction with an angiogenic factor

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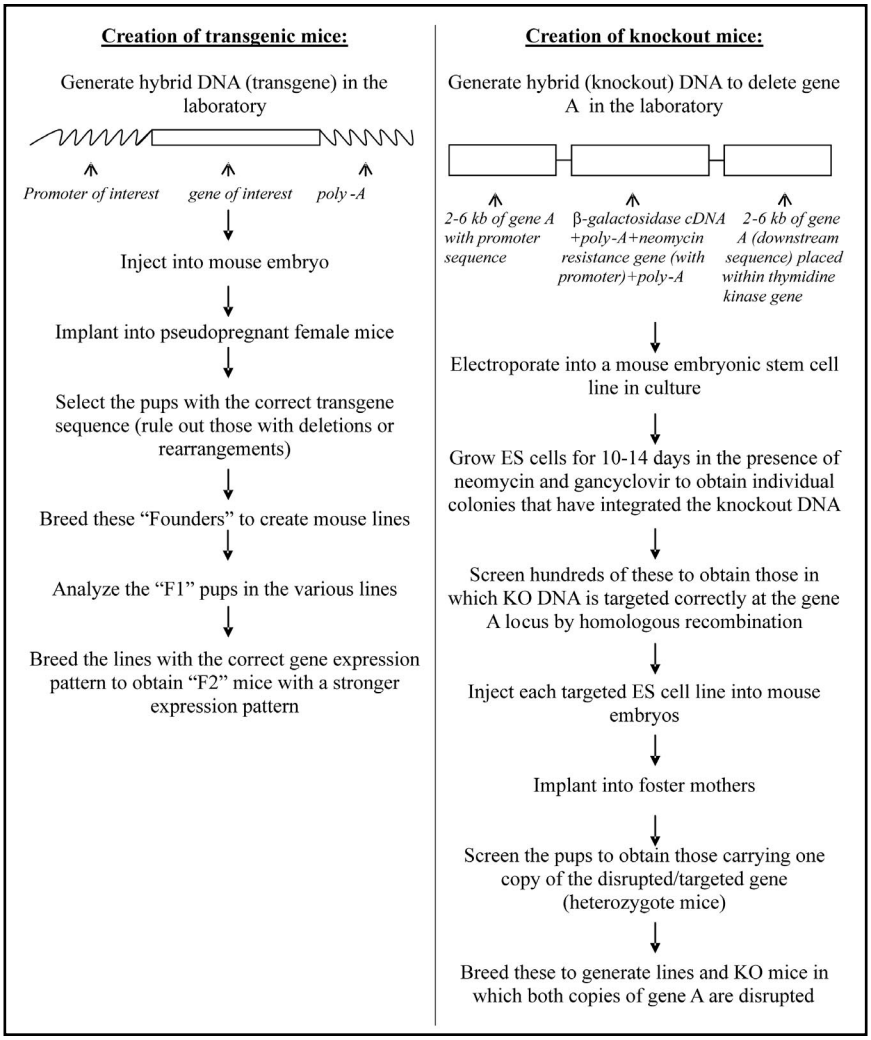

Fig. 1. Molecular techniques for the creation of transgenic and knockout mouse models.

(e.g., *Vegf-C, Vegf-D, Ang2, Nrp-2*). *PROX-1*, a homeobox gene cloned by homology to a *Drosophila melanogaster* (fruit fly) gene, appears to have been a serendipitous finding, which then led to the discovery of a *Prox-1* induced gene, namely *T1α/podoplanin*. Another gene, *FOXC-2*, was the result of genetic mapping studies relating to a specific human lymphatic disorder – lymphedema-distichiasis (double row of eyelashes).

In thinking about lymphatic system development, what do we know with reasonable certainty? It might be helpful to address this question in parts, starting from the very beginning:

Emergence of the First Lymphatic Cells

The controversy over whether lymphatic cells develop as centrally located sacs from pre-existing blood vessels (8) or are direct derivatives of a peripherally-located precursor cell (a lymph-hemangioblast) (9) is far from settled. In mammals, there is strong evidence for Sabin's theory (though it may be that both processes are utilized within the

developing embryo at different locations) (9). In mice lacking the *Prox-1* gene, lymphatic cells can be visualized budding from the cardinal vein but they fail to form lymph sacs centrally and completely lack a lymphatic vasculature peripherally (10). These lymphatic cells are arrested at E (embryonic day) 11.5-E12.0, and they show weak expression of Vegfr-3 and no expression of Lyve-1 or Slc, which are hallmarks of lymphatic cells in a normal, wild-type mouse (11). In these nullizygous embryos, the *Prox-1* gene had been inactivated but its upstream and downstream control elements were kept intact and used to express β-gal, a marker gene. With this genetic manipulation, Wigle et al (10) could clearly show that in the nullizygous embryos, the early lymphatic cells were indeed born (as determined by the presence of β-gal-positive cells), and they did not just die out as might have been expected if they were not going to progress any further. Surprisingly, by E11.5, these cells had switched their protein expression to resemble that of blood vascular endothelial cells (as determined by the presence of β-gal-positive cells co-expressing laminin and CD34, both of which identify blood vessels). These observations demonstrate that early lymphatic cells are born from the cardinal vein and need a specific signal (Prox-1) to maintain their lymphatic lineage. In its absence, they will revert to a blood vascular phenotype!

The actual signals that lead to the emergence of Prox-1-positive cells from the cardinal vein are still not known. The localized expression of Prox-1 suggests the presence of a localized factor which is probably matrix bound and synthesized by the surrounding mesenchyme. The endothelial cell-matrix/smooth muscle cell interaction that has been shown to be a critical factor in hemangiogenesis (12) should perhaps be looked at in greater detail during lymphangiogenesis as well.

Continued Embryonic Development of the Lymphatic Vasculature

The latest genetic manipulations have shed further light on the presumed roles of the *Vegf* family members. One of the receptors, Vegfr-3, has been strongly implicated in lymphatic system development due to its expression pattern within the embryo (13) and because inactivating mutations have been found in human hereditary lymphedema (14). However, mice lacking *Vegfr-3* cannot be studied for the gene's effect on the lymphatics since they die at E9.5 from vascular abnormalities prior to the emergence of the lymphatic system (15). The only two known ligands of Vegfr-3, Vegf-C and Vegf-D, were both presumed to play a role in early development mainly because of results with adult transgenic mice (16). When either of these proteins was expressed at high levels in the skin, the growth of the lymphatic vessels was visibly enhanced (17). However, now that mice lacking one or the other of these two proteins have been created, the early theories have had to be modified! Whereas mice nullizygous for *Vegf-C* fail to develop lymphatic vessels (18), those lacking *Vegf-D* are completely normal (19). These results underscore the importance of using different genetic approaches to delineate the role of any one gene.

In the embryos that lacked *Vegf-C*, there was no sprouting of lymphatic endothelial cells from the cardinal vein and neither jugular lymph sacs nor any of the subsequent structures were formed (18). It is quite clear now that during embryogenesis, *Vegf-C*, not any of its other relatives, is essential for the continued development of the lymphatic vasculature in the presence of Prox-1. These experiments also show that Vegf-C is not required for the expression of Prox-1. However, it should be noted that since these cells are presumably normal in their expression of Vegfr-3, it is still not clear if the early developmental expression of Vegfr-3 is needed for the initial expression of *Prox-1* in the cardinal vein. The diversity of ligand-receptor interactions is further highlighted by other experiments which suggest that Vegf-C

probably exerts its effect not just via Vegfr-3 but also via neuropilin-2 (Nrp-2). Vegf-C has been shown to bind to the latter receptor which is also co-expressed with Vegfr-3 on lymphatic endothelium (20). Targeted disruption of *Nrp-2* shows severe loss or absence of lymphatic capillaries beginning at E13 in a variety of developing organs (21). There appears to be a redundancy in the system at later stages because from P7 (post-natal day 7) onwards the lymphatic vessels in these mutant mice can be detected again, albeit arranged in a slightly different pattern. Interestingly, the developing lymphatic tree at this point is not just being modified and maintained by Vegf-C! There are other factors, discussed below, whose roles in the developing lymphatic system have only become apparent as a result of genetic manipulations.

Postnatal Remodeling of the Lymphatic Vessels

It appears that once the lymphatics are formed in the embryo, another class of genes, the Angiopoietin family, comes into play to help maintain the integrity of the system. By genetic engineering, it has recently been shown that two of the most studied ligands of this family, Ang1 and Ang2, are both capable of adopting this role (22). *Ang1* nullizygous mice show major blood vascular defects at E9.5 and die soon after, establishing the vital role of this gene in hemangiogenesis (12). *Ang2*, a close relative of *Ang1*, when disrupted in mice, causes severe abnormalities in both the large and small lymphatics that coincide with the presence of chylous ascites and lymphedema (22). However, if *Ang1* is expressed at all sites and at all times when *Ang2* is normally expressed, the lymphatics appear completely normal. This observation shows that while Ang1 normally acts on the blood vascular endothelium (via the Tie-2 receptor), it can act on the lymphatic endothelium as well via the same receptor. Clearly, the blood and the lymphatic vascular systems share genes whose effects are controlled at both spatial and temporal levels. These experiments raise new questions – why is it that *Ang2* nullizygous mice only show a phenotype late in development (whereas the Ang2 receptor, Tie2r, is clearly there much earlier)? Is it that *Ang1* functions early on and is then switched off just as *Ang2* is turning on? In other words, if *Tie2r* were ablated just in the lymphatic endothelium, would its effect be any different from when the *Ang2* is removed? Also, what is the role of the *Vegf* family members at this late developmental stage?

Ang2 is not the only gene that when deleted affects lymphatic vessel patterning and function at this stage. There are two others – *T1α/podoplanin* and *Foxc2*. The gene for *T1α/podoplanin* is induced by *Prox-1* (23), co-expressed with *Prox-1* by E11, and highly expressed in the lymphatic endothelium after birth (24). Mice that carry this disrupted gene exhibit enlarged, abnormally patterned and functionally impaired lymphatics at P0 (24). Unfortunately, the lymphatic system development cannot be pursued further in these mice since, due to the absence of the normally widespread expression of this gene, the pups die at birth from other complications. Regardless, these mice still demonstrate that while *T1α/podoplanin* is expressed early during development, its effect is seen much later, and is clearly downstream of the early onset genes such as *Lyve-1* (the expression pattern of which is unchanged in these mutant mice). In contrast to these genes, which when removed, lead to a generally hypoplastic lymphatic system, mice lacking just one copy of *Foxc2* exhibit a uniquely hyperplastic refluxing lymphatic system. Incidentally, this phenotype, along with the occasional lymphedema and a double row of eyelashes (25), closely mimics the human FOXC2-deficient autosomal dominant Lymphedema-Distichiasis syndrome (26). One of the questions that arises is whether *Ang2*, *T1α/podoplanin*, and *Foxc2* act along separate pathways or if any one of these might be turned on by the other(s).

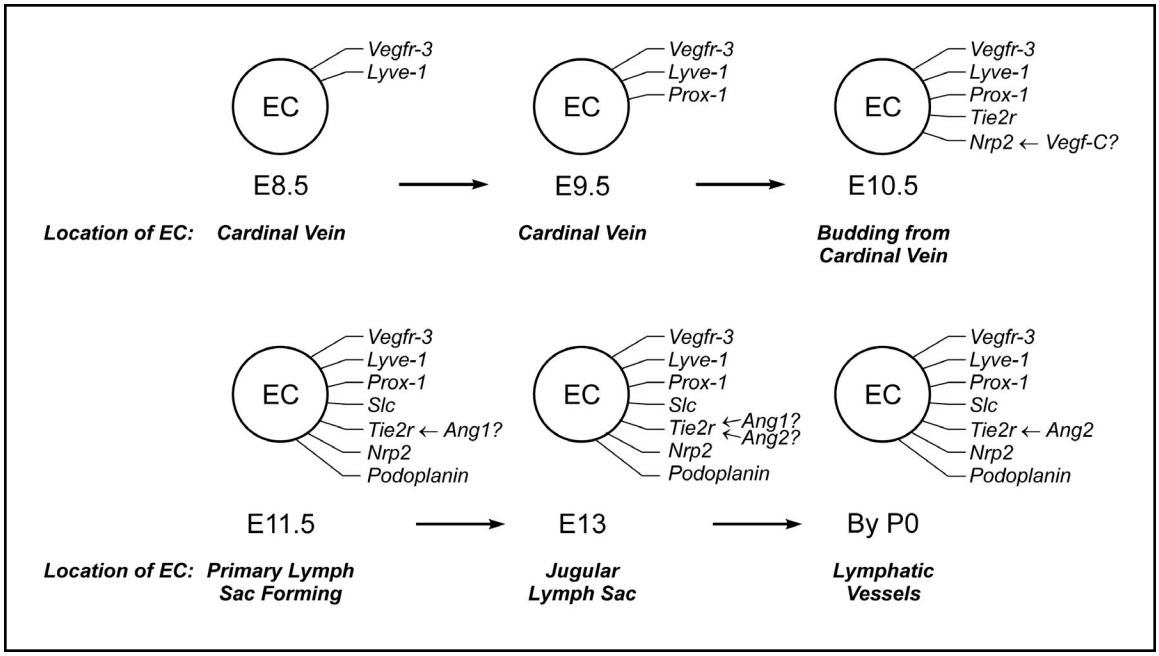

Fig. 2. Timeline of endothelial cell (EC) gene expression in the development of the murine lymphatic system.

Cumulatively, these pivotal experiments within the last six years have generated enough data to create a preliminary developmental timeline for the expression of some genes involved in the development of the murine lymphatic system (*Fig. 2*). Without any doubt, the techniques of molecular biology have been utilized quite successfully to uncover some of the critical events in the emergence and maintenance of the lymphatic system. However, as with any technology, one needs to be aware of its limits. Transgenic mice, for instance, in which particular genes are expressed under tissue-specific promoters, are often just models of ectopic gene expression. As such, they are good at identifying the manipulative power of the gene of interest. A transgenic effect implies that the system is susceptible to high levels of a particular gene at a particular point in time. Under normal circumstances, that gene may not even be expressed at that particular developmental stage. The advantages of transgenics are that the genetic engineering required is often minimal, the mice are generated very easily, often the results can be seen in the first generation, and as long as a reasonable promoter is used, a positive result is a good indication that the gene is relevant to the system under study (*Fig. 1*). The hazards of over-interpretation are well illustrated by the series of experiments with *Vegf-C* and *Vegf-D*. A knockout experiment, on the other hand, is more powerful because it removes just one gene from the genetic make-up of the mouse and thus serves to establish a clear role for the gene during development. A transgenic experiment is especially reliable when supplemented with a knockout experiment. The problem is that genes often have multiple roles and they may be required for the development of more than one system, causing embryos to die from defects unrelated to the system of interest and leading to an inconclusive experiment. A straightforward knockout experiment also has its limitations – it cannot be used to delineate a gene's

ongoing role during different developmental stages. Such problems can only be alleviated by genetic manipulations that would allow gene expression to be ablated at specified times and in specific locations, not just when the gene is first switched on. Such second-stage conditional knockouts, along with sophisticated manipulations that allow gene expression to be switched during development (refer to the *Ang1*/*Ang2* study), will be essential in addressing the next level of questions: what role do any of these genes (*Prox-1, Vegfr-3, Vegf-C, Ang1, Ang2*) play during lymphangiogenesis in the adult, as in wound healing, or cancer; what is their role in the development and maintenance of adult-onset lymphedema; and what is the functional overlap between the different gene groups at different stages of development? As mouse models become available, their inter-crosses can give hints of whether any two genes lie within the same pathway and, if so, which one of them may be upstream. As lymphatic cell lines become established, they can be used for multiple gene targeting events to shed light on lymphangiogenesis under controlled conditions.

The dawn of molecular lymphology is indeed here – age-old questions are beginning to be answered. The technology, with its limitations, is powerful, and there are strong indications that with each technological advance, the biology of lymphatic system development will be that much better understood.

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