Lymphology 44 (2011) 95-102

LYMPHATIC COMMUNICATION: CONNEXIN JUNCTION, WHAT'S YOUR FUNCTION?

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ABSTRACT

This article reviews recent findings on expression and function of connexin proteins – the structural subunits of gap junction intercellular channels in the lymphatic vasculature – both during development and in the mature lymphatic vessel. Highlighted in particular are recent mouse connexin knockout studies which show that connexins are crucial for normal lymphatic development. We discuss, in general terms, both channel-dependent as well as channel-independent functions of connexins and raise some of the many unanswered questions about the mechanism(s) of action and physiological roles of connexins in the lymphatic vasculature.

Keywords: connexin, gap junction, lymphatic development, lymphatic vasculature, lymphatic valve

"Man is by nature a social animal," Aristotle wrote in the 4th century BC, but perhaps he did not appreciate at the time how relevant his statement would be for the microscale of our very existence. Humans are indeed social creatures. Communication is a critical part of our daily lives and occurs at various levels, from interactions with our family, friends, and colleagues to larger scale community interactions (such as those within our respective fields or across fields). Additionally, the different modes in which we communicate each carry their own speeds, advantages, disadvantages, and particular functions. Much in the same way, cells of our body communicate with each other at different levels and have diverse tools with which they carry out that communication. One of the most robust tools cells use in their interactions comes in the form of a protein family known as connexins.

Connexins are well recognized for their ability to facilitate direct cell-to-cell communication. The connexin protein family in humans comprises 21 isoforms, each with distinct functional properties, which are differentially expressed and dynamically regulated in a multitude of tissues and organs throughout the body (1,2). Six connexin proteins assemble together to form membrane channels termed hemichannels. On their own, hemichannels can act as portals that allow signaling molecules such as ATP (3), glutamate (4), and prostaglandins (5) to pass into the extracellular space (6). When docked with a hemichannel from another cell, they can directly link the cytoplasm of neighboring cells through the formation of intercellular channels, which often organize into tightly packed clusters called gap junctions. The "gap" here refers to the very close apposition of membranes at these junctions, visualized via electron microscopy as a 3 nm separation between cells. Gap junctions allow for the exchange, sometimes selectively, of a variety of substances typically less than ~1 kDa in size such as ions, metabolites, signaling molecules (e.g., Ca^{2+}, cAMP, cGMP, IP_3),

short peptides (7), and even short nucleotide sequences in the form of miRNA (8) and siRNA (9) between cells (1). One of the most striking consequences of this cell-cell link is exemplified by the heart, where the coordinated contraction of the organ depends on gap junctions to allow the spread of ions and thus the electrical impulses that drive the muscle. Gap junctions not only mediate direct links between the same cell type (cardiomyocyte to cardiomyocyte, for instance), but also between different cell types (such as endothelial cells and vascular smooth muscle cells) (10). In addition to their importance in mediating direct cell-to-cell communication, connexins have increasingly been recognized to have channel-independent functions through their interaction with other cellular proteins (11). Connexins, through their channel-dependent and channel-independent functions, have been found to be involved in a diverse array of cellular processes including growth (12), migration (13) , and differentiation (14,15) among others. These features of connexin function have profound consequences for the development of organisms, coordination of normal function of tissues and organs, and responses of cells and systems to pathophysiological situations.

While connexins have been studied extensively in the blood vasculature, the exploration of their expression and function in the lymphatic vasculature has been quite limited until recently. About three decades ago, Rhodin and Sue suggested that gap junctions might exist between lymphatic endothelial cells, but the electron micrographs were inconclusive (16). Later, McHale and Meharg cited electron microscopy data reportedly displaying gap junctions between lymphatic smooth muscle cells (17). Gap junctions between sinus-lining endothelial cells of the rabbit lymph node were clearly visualized by electron microscopy (18) and later immunohistochemistry revealed that a specific connexin isoform, connexin43 (Cx43), was expressed by the sinus-lining endothelial cells of human lymph nodes (19). Moreover, studies of bovine and rat mesenteric lymphatic vessels provided evidence that synchronized and propagated contractions of lymphatic vessels could be impaired through the use of non-specific gap junction inhibitors (17,20). More recently, genetic linkage and DNA sequencing studies identified missense mutations in Cx47 (*GJC2*) that underlie the development of some forms of human lymphedema (21,22). Despite these important discoveries, a great deal of mystery continues to shroud connexins and their relationship with the lymphatic vasculature.

Recently, our group characterized the *in vivo* expression of three connexin isoforms – Cx37 (*Gja4*), Cx43 (*Gja1*), and Cx47 (*Gjc2*) – at various levels of the mouse lymphatic vasculature and showed that they are differentially expressed throughout embryonic and post-natal lymphatic development (23). Cx37 and Cx43 are both found during early lymphatic development in the lymphatic endothelial cells (LECs) of the jugular lymph sacs. Interestingly, their expression in the jugular lymph sac often occurs in distinct domains, with regions of high Cx37/low Cx43 expression next to regions of high Cx43/low Cx37 expression. Within developing and mature lymphatic collecting vessels, Cx37 and Cx43 colocalize in the general (non-valve) endothelium. However, during development these connexins become progressively enriched at lymphatic valves and, in mature valves, they are exquisitely differentially expressed in the upstream versus downstream sides of the valve leaflets (*Fig. 1*). Cx43 is found exclusively in the LECs of the upstream valve leaflet, whereas Cx37 is expressed exclusively in the LECs of the downstream leaflet. The significance of this segregated expression is not yet clear, but one idea is that it may represent a physiological response to unequal mechanical stress experienced by the two sides of the valve leaflet. Cx47 is also highly enriched in the endothelium of lymphatic valves and, curiously, its expression is

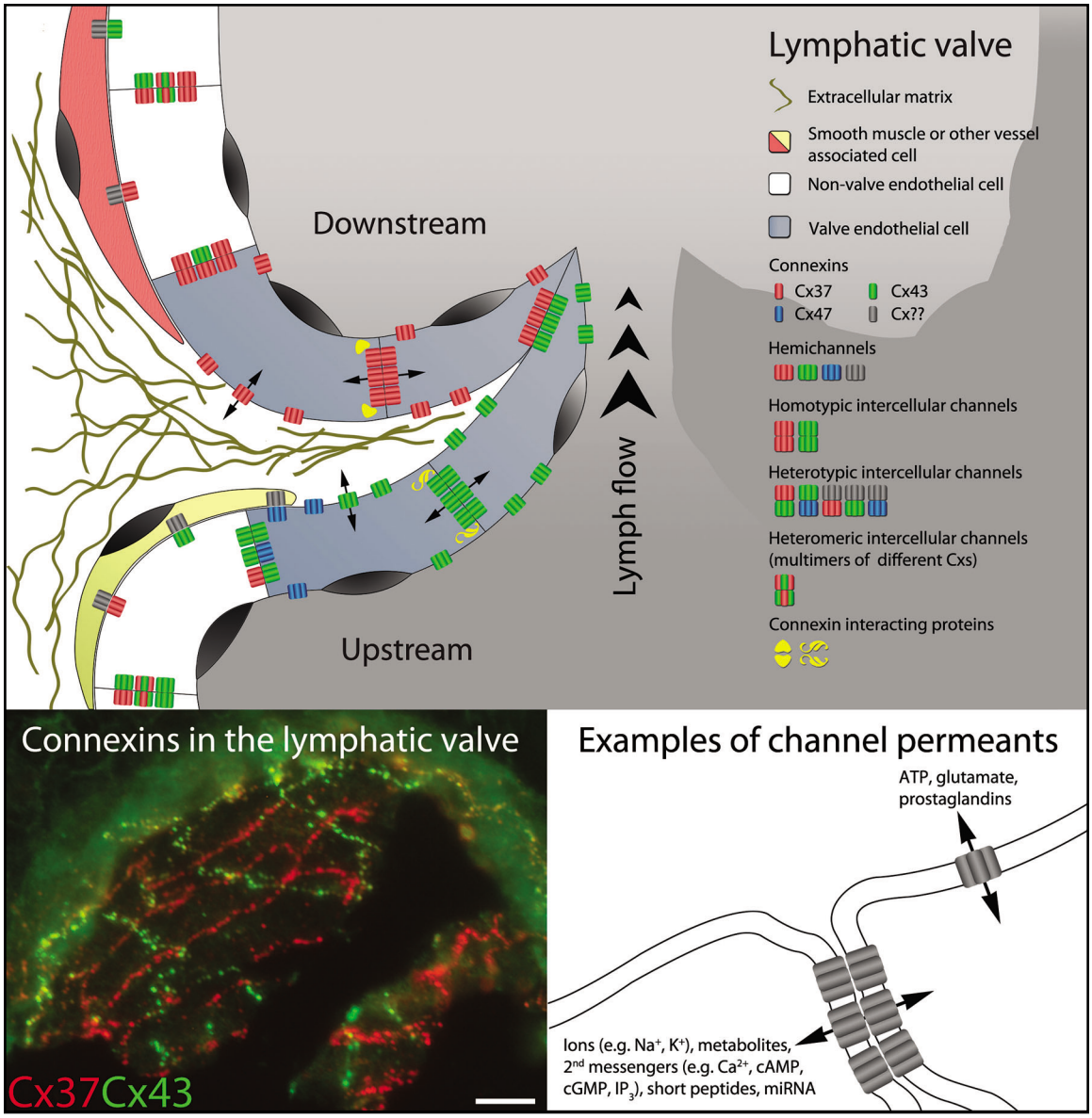

Fig. 1: **Top.** ***Schematic representation of a longitudinal section through a lymphatic collecting vessel with emphasis on a valve. Depicted are potential connexin-containing channel configurations that may exist on or between cells of the vessel wall. Channel types: hemichannel (six connexin subunits assembled into a channel in one membrane), homotypic (composed of matching hemichannels), heterotypic (composed of non-matching hemichannels), and heteromeric (composed of hemichannels containing more than one connexin type). Proposed channel combinations are not exhaustive.*** **Bottom left.** ***Cx37 (red) and Cx43 (green) immunofluorescent staining of a transverse section through a mesenteric lymph valve (mouse, 4 weeks old). The valve leaflet shown appears*** **en face** ***in the section and extends into the lumen of the vessel. Cx37 and Cx43 are highly enriched in the valve and are differentially expressed on the two sides of the valve leaflet. Cx43 is present on the upstream side of the valve leaflet and Cx37 is present on the downstream side. Colors correspond with connexin colors in the accompanying schematic. Scale = 10 µm. This image is reprinted from Kanady et al. (23) with permission from Elsevier.*** **Bottom right.** ***Examples of hemichannel and gap junction intercellular channel permeants as documented in other cell types.***

restricted to a small subset of valve LECs, where it colocalizes with Cx43. In contrast to the abundant presence of these connexins within lymph collecting vessels, their expression in the lymphatic capillary network is very low or undetectable.

Both Cx37 and Cx43 have critical roles in lymphatic vascular development and function as evidenced by the consequences of their loss in knockout mice (23). In the early stages of lymphatic development, profound enlargement of the jugular lymph sac occurs with the loss of Cx37 alone and this effect is exacerbated when combined with the loss of Cx43. Embryos lacking both Cx37 and Cx43 also exhibit widely dilated superficial lymphatics in the skin and severe lymphedema. Amazingly, ablation of Cx43 alone results in the complete loss of valve formation in the collecting lymphatics of the mesentery. In addition, knockout of Cx43 results in an abnormally patterned thoracic duct characterized by erratic caliber, blind-ended outcroppings, and bifurcated segments. Loss of Cx37, on the other hand, results in a partial reduction in valve number in mesenteric lymphatics and adult mice that display lymphatic reflux. Combining the Cx37 knockout with the loss of a single copy of Cx43 leads to an interesting mouse model in which adult mice exhibit severe lymphatic reflux and often develop sudden lethal chylothorax – defects stemming from the deficit of lymphatic valves. In the case of chylothorax, the likely scenario is that the thoracic duct valve deficits observed in these mice encumber the capacity of the lymphatic system to the point where sudden rupture of vessels in the thoracic cavity occurs. These findings illustrate crucial functions for Cx37 and Cx43 in collecting vessel and lymphatic trunk development and function.

Given their lack of expression in lymphatic capillaries, it is perhaps not surprising that the lymphatic capillary network per se is seemingly unaffected by the loss of Cx37 or Cx43 (23). However, that is not to say that connexins are unimportant for lymphatic capillaries. Dicke et al have recently shown that ectodermal expression of a different connexin, Cx26 (*Gjb2*), was essential for normal peripheral lymphangiogenesis in mice, as either the ablation of Cx26 or its substitution with Cx32 (*Gjb1*) led to abnormal lymphatic capillary network development and lymphedema (24). The authors propose that Cx26 may regulate lymphangiogenic signals that arise from developing epidermis, and thus influence dermal lymphatic development. In this light, it is intriguing to consider the implications of prior results documenting Cx26 expression in human breast tumors and its association with lymphatic vessel invasion (25) as well as the relationship between Cx26 expression and the likelihood of metastasis or poor prognosis in other types of tumors (26,27). From these recent studies, it is becoming more and more apparent that connexins play influential roles in the development and function of the lymphatic vasculature. Looking ahead, however, there remain many unanswered questions and a multitude of investigative avenues to explore.

Basic questions about connexins and lymph vessels remain unanswered – namely are there *bona fide* gap junction structures in the lymphatic wall and, if so, where are they? Connexins have been studied extensively in the cardiovascular system and are known to couple blood vascular endothelial cells, vascular smooth muscle cells, as well as coupling blood vascular endothelial cells with vascular smooth muscle cells (myoendothelial coupling) (28). Whereas morphologically identifiable gap junctions have been demonstrated between the sinus-lining endothelial cells of the lymph node, gap junctions between endothelial cells of lymphatic vessels have not been documented. Importantly as well, functional coupling between lymphatic endothelial cells via gap junctions remains to be demonstrated. While studies employing the use of non-specific gap junction inhibitors have shed some light on a possible functional role for gap junctions in

lymphatic vessel contraction (17,20), the non-specific inhibitors used in those studies could have had effects on other membrane proteins. Thus, studies that explore the functional coupling of cells (dye transfer or electrical coupling experiments) within lymphatic vessels are needed. It also remains unclear whether there are myoendothelial junctions between lymphatic endothelial cells and lymphatic smooth muscle. These issues are particularly pertinent in light of experiments that, through the targeted destruction of the endothelial cell layer versus the vascular smooth muscle layer of arterioles, showed that dilatory and vasoconstrictive signals could be differentially blocked by the destruction of one cell layer versus the other (29). Clearly, elucidating the communicating partners within and between layers of the lymphatic vessel wall will be critical to enhancing our understanding of the cellular interplay that coordinates lymphatic vessel contraction, relaxation, and propagated vascular responses.

The previous questions inevitably lead to the following – what are connexins and/or gap junctions actually doing in the lymphatic system? In considering this, insight may be gleaned from their functions in other cells and systems. Their possible role in mediating lymphatic vessel contraction has been alluded to previously and likely involves their ability to facilitate the spread of depolarizing or hyperpolarizing currents through the vessel wall. However, gap junctions in lymphatics may also mediate the transfer of other permeants such as second messengers (e.g., Ca^{2+}, cAMP, cGMP, IP_3) or something akin to endothelium-derived hyperpolarizing factor, as has been hypothesized in the blood vasculature (30). But, this assumes gap junctions are indeed coupling cells within lymphatic vessels. In addition (or perhaps, alternatively), connexins could be forming hemichannels, and their ability to allow substances to be released into the surrounding tissues or lumen of the vessel, perhaps in response to stretching of the vessel wall, could theoretically affect lymphatic vessel dynamics through paracrine signaling (31).

In terms of development, the potential influences of connexins on the lymphatic vasculature are numerous. Cx37 and Cx43 have both been shown to mediate growth control effects in *in vitro* settings (32,33). Hence, these connexins may be exerting sway over lymphangiogenesis through modulation of proliferation. Connexins have also been implicated in developmental patterning (34-36) and in the control of cell migration (37). Studies in the blood vasculature suggest that the precise level of connexin expression can affect vascular network formation, as the loss of a single copy of Cx43 in mice resulted in variable branching and abnormal patterning of coronary vessels (38). We have demonstrated the profound impact on collecting vessel formation that occurs with the loss of Cx37 and Cx43, particularly for valve development, but these connexins may also be involved in the patterning of the lymphatic network, as suggested by the abnormal patterning of the thoracic duct and intercostal lymphatic trunks in Cx43 knockout mice (23). However, connexin involvement in the development of vascular beds (blood or lymph) remains poorly understood. In both these regards (growth and development), connexins represent very attractive targets of study for their possible involvement in directing and modulating physiological and pathophysiological lymphangiogenesis.

An important challenge ahead will be to understand how connexins are integrated with other signaling pathways and genetic programs during lymphatic development, particularly because it is still uncertain whether their effects are mediated through their channel functions or through their ability to potentially interact with a host of intracellular proteins such as scaffolding proteins, cytoskeletal elements, kinases, and, in the case of Cx37, endothelial nitric oxide synthase (39). It is beyond the scope of this communication to review in depth the substantial progress that has been made in

recent years in identifying crucial genes and proteins involved in lymphvasculogenesis and lymphangiogenesis. A number of approaches have established the importance of key transcription factors, signaling proteins, receptors, and cell-matrix interactions in the developmental sequence (40). One of the transcription factors found to be important for lymphatic collecting vessel development is Foxc2, a forkhead family transcription factor that is mutated in human lymphedema-distichiasis syndrome and is required for lymphatic valvulogenesis (41-43). Interestingly, Cx37 expression is drastically reduced in the jugular lymph sac and mesenteric collecting vessels of mice that lack Foxc2, suggesting that Cx37 may be a target of regulation by Foxc2 (23). Besides Foxc2, a number of other proteins have been shown to be critical for lymphatic collecting vessel development and valve formation, including Integrin-α9 (44), Ephrin-2 (45), and NFATc1 (46), and it will be interesting to see if connexins in some way contribute to these signaling pathways in lymphatic development.

The exploration of connexins in the lymphatic system represents fertile scientific territory. Some of the groundwork has been laid in the last 30 years, but there are still many unanswered questions. The importance of connexins in facilitating the vascular tone of blood vessels is well recognized, yet knowledge of their function in the contractile activity of lymph vessels is minimal. The developmental roles of connexins are diverse, but we are still only scratching the surface in terms of figuring out their precise functions and mechanisms of action both in non-lymphatic and lymphatic systems. If we are to understand the lymphatic vasculature more completely, we will need to appreciate clearly how the cells of the system communicate with each other. And, while cells may not communicate with words, the better our understanding of their language and the ways in which that communication affects their behavior, the shorter our stay will be in the shadow of ignorance.

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