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Title

Tissue Engineered Lymphatic Graft for the Treatment of Lymphedema

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Brief running title

Tissue engineering of lymphatic graft

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ABSTRACT

Background:

Lymphedema is a chronic debilitating condition and the curative treatment is yet to be found. Tissue engineering approach which combines cellular components, scaffold and molecular signals hold great potential in the treatment of secondary lymphedema with the advent of lymphatic graft to reconstruct damaged collecting lymphatic vessel. This review highlights the ideal characteristics of lymphatic graft, the limitation and challenges faced as well as the approaches in developing tissue engineered lymphatic graft.

Methods:

Literatures on tissue engineering of lymphatic system and lymphatic tissue biology were reviewed.

Results:

The prime challenge in the design and manufacturing of this graft is producing endothelialized conduit with intraluminal valves. Suitable scaffold material is needed to ensure stability and functionality of the construct. Endothelialization of the construct can be enhanced via biofunctionalization and nanotopography which mimics extracellular matrix. Nanocomposite polymers with improved performance over existing biomaterials are likely to benefit the development of lymphatic graft.

Conclusions:

In depth understanding of tissue engineering, nanotechnology and improved knowledge on the biology of lymphatic regeneration, the aspiration to develop successful lymphatic graft is well achievable.

1. INTRODUCTION

Lymphedema is a debilitating condition resulting from dysfunction of lymphatic system characterised by progressive soft tissue swelling which commonly involves the limbs and genitalia. Lymphedema is categorised into primary and secondary lymphedema. Primary lymphedema is rare and caused by genetic defects. Secondary lymphedema is common and caused by acquired damage secondary to surgical resection (lymph node clearance), tumour infiltration, parasitic infection and radiation induced fibrosis.

In the United Kingdom alone, there are estimated 240,000 people living with lymphedema (1). Despite the wide practise of breast conserving surgery and minimal lymphatic intervention, the world wide incidence of secondary lymphedema remains high with an estimate of 295,320 patients developing upper limb lymphedema yearly after breast cancer surgery (2). This huge number highlights the critical clinical need to treat surgery induced secondary lymphedema.

The current armamentarium of therapeutic intervention involves non-surgical and surgical treatment. Non-surgical treatment includes bandaging and physiotherapy which does not prevent progression of disease and results in poor quality of life. The surgical treatment of lymphedema has evolved tremendously over the years. In 1990, Baumeister reported the use of lymphatic (lymphatico-lymphatic) bypass graft to bridge damaged lymphatic vessels utilising autologous lymphatic graft harvested from ventromedial part of the thigh (3). Although reporting a volume reduction of 80% compared to pre-operative conditions, this technique did not gain popularity due to the high risk of donor site morbidity. Following the same principle, Campisi used vein graft (lymphatic-venous-lymphatic shunt) and reported volume reduction of 75% in almost half of his patients(4). Several other form of surgical treatment such as liposuction (suction assisted lipectomy), subcutaneous excision and ablative surgery with skin grafting were practiced but with poor outcomes. In the recent years, surgical treatment of lymphedema has evolved into super-microsurgical approach. The techniques undertaken are lymphatic bypass surgery (lymphaticovenular anastomosis) and vascularised lymph node transfer. Most authors reported a modest improvement in limb volumes (decrease of 30%–50%) although some patients experienced more significant improvement (5). However, none of these techniques are curative and they are exclusively for patients in early stages of lymphedema.

With these challenges in mind, tissue engineered lymphatic graft is an ideal alternative that should be explored for the treatment of secondary lymphedema as it is likely to offer greater versatility and therapeutic power. The engineered lymphatic graft can be used to bridge defects involving collecting lymphatic vessels due to surgical resection or as a bypass for congenital cause of blockade to lymphatic circulation. Furthermore, it could also benefit patients with venous impairment in the same lymphedema limb that are not suitable candidate for lymphovenous shunt. To match the properties of native lymphatic vessel, an ideal lymphatic graft should be durable, able to maintain patency, easy to sterilize, non-toxic, non-allergenic and non-carcinogenic. In terms of handling, it should be kink-resistant, easy to suture and have adequate fatigue strength. The goal of tissue engineered lymphatic graft is to reproduce the structure, function and cellular organisation of a native collecting lymphatic vessel.

This article aims to discuss the tissue engineering approaches in developing lymphatic graft and the unique limitations and challenges involved. This review also features the advancement in the knowledge of lymphatic biology, with emphasis on lymphatic regeneration and the molecular signals involved.

2.0 TISSUE ENGINEERING OF LYMPHATIC GRAFT

The lymphatic system can be partitioned into five sections: lymphatic capillary, collecting lymphatic, lymph nodes, lymphatic trunk and lymphatic duct (Figure 1). The collecting lymphatic vessels contain intraluminal (secondary) valves and endothelial cells that are spindle shaped with continuous basement membrane, pericytes and associated smooth muscle cell (SMC) cover enabling intrinsic contractility of the vessels (6). Collecting lymphatic vessels are made up of functional subunits known as lymphangion which propels lymph in a peristalsis manner. Lymph flow is driven by local forces and affected by neighbouring musculoskeletal movement and vasomotion. The collecting lymphatics have similar wall as of blood vessels and its smooth muscle tissue contains specialised pacemaker cells that regulate spontaneous electrical activity (Table 1) (7).

Lymphatic system regulates extracellular fluid homeostasis by preserving tissue fluid balance, involved in immune response and absorption of dietary fat. The amount of lymph

formation and flow rate is dependent on the characteristics of extracellular matrix (ECM), type of tissue and the degree of swelling (8).

The lymphatic network is lined by specialised endothelial cells known as lymphatic endothelial cells (LECs). The LECs embryologically originates from out pouching of blood endothelial cells (BECs) from cardinal vein and they both share close structural similarities. About 300 genes are expressed differentially between BECs and LECs which includes LEC-specific genes (LYVE-1, VEGFR-3, prox1, podoplanin, and β-chemokine receptor D6), integrins, cadherins, pro-inflammatory cytokines and chemokines (9).

Three key aspects that tissue engineering could benefit lymphatic system are development of artificial lymphatic graft (Figure 2), regeneration of lymphatic network via lymphangiogenesis and engineering artificial lymph node.

Developing tissue engineered lymphatic graft involves proper material selection and fabrication of scaffold to create an environment conducive for LEC growth as well as for lymphatic regeneration. Although artificial lymphatic graft has yet to be successful, it holds great potential as there is possibility of translating technology and knowledge from tissue engineered vascular graft. Regenerating lymphatic network is a long-standing challenge for tissue engineers as it required self-organisation of endothelial cells into a network of conduits in-vivo. A possible solution is with the use of stem cell technology and growth factors to form an extensive network of lymphatic capillary which drains into existing or neighbouring collecting lymphatic. Artificial lymph node that is immunologically functional was successfully engineered by Watanabe et al (10). Although this artificial organ does not enhance lymphatic circulatory function, nevertheless this is a step forward in achieving alternative treatment for lymphedema in future.

The challenges involved in developing lymphatic graft differ from vascular graft. The major differences are the mechanical property and hydrostatic pressure. Lymphatic system is a low pressure and pulseless system as compared to vascular system. This allows lymphatic graft to be built with lower strength and elasticity however intraluminal valve is an essential feature to be included to ensure its functionality. The following section discusses on the strategies involved in developing lymphatic graft.

3.0 STRATEGIES IN DEVELOPMENT OF LYMPHATIC GRAFT

3.1 Lymphatic pressure

Two primary mechanical forces that require special attention in tissue engineering of lymphatic graft are the transaxial pressure gradient, which governs fluid shear stress; and average transmural pressure, which governs circumferential stress(16). These two intrinsic pressures work in tandem with extrinsic pressures such as internal tissue pressure, tissue osmotic pressure and venous pressure which determine the flow rate of lymphatic fluid (17). Intrinsic contraction of lymphatic vessel ranges between 12 and 70mmHg in healthy human. The extrinsic pressure in healthy human ranges between 0 and 60mmHg (18). In pathological condition, the intrinsic lymphatic pressure is elevated and the accumulation of fibrotic tissue as well as adipocytes due to lymph stasis drastically alters the surrounding mechanical environment (19, 20). This condition may eventually overwhelm the lymphatic vessels and result in decreased contractile tone and frequency of collecting lymphatics as seen in rat thoracic duct model (negative chronotrophic and inotrophic effect) (21). However, this is not seen in the mesenteric lymphatic vessels which indicated that the amount of reduction varies in different location. The pressure gradient at which flow cessation takes place in human is yet to be known. It is crucial to quantify these values in diseased human lymphatic system as the ability to simulate these pressure ex-vivo is paramount in determining the success of the lymphatic graft.

The advancement of imaging modalities coupled with computational power and image processing algorithms has enabled reliable quantification and correlation between lymph flow rate and intrinsic contractile activity (11, 12). The use of non-invasive imaging modalities, the near-infrared (NIR) fluorescence imaging in combination with the clinically approved Indocyanine Green (ICG) dye has enabled researchers to better understand the draining velocity of initial lymphatics and contractile physiology of collecting lymphatics in human as most previous studies were mostly limited to animal models(13-15). This advancement in imaging has been particularly useful in understanding the changes that takes place in diseased lymphatic system which is a step closer to unravelling ways to manipulate the biological and mechanical changes taking place in lymphedema.

3.2 Mechanical property of Lymphatic Vessel

The elastic modulus, tensile stress and strain at failure and the burst strength of human collecting lymphatic vessel are not as well studied and documented as for arteries and veins (22). Several studies have been conducted on animal models however these values might not precisely reflect that of human due to difference in size and capacity of the vessel (23-25). An ideal tissue engineered lymphatic graft should mimic the mechanical properties of native collecting lymphatic vessel. Features of an ideal tissue engineered lymphatic graft are high strength and flexibility, good hydraulic conductivity, kink resistant and good suture retention. This will ensure that the conduit and anastomosis remains intact especially in regions with great range of motion such as the axilla and groin. In terms of achieving a reliable standard and to ensure patient's safety, the International Standard for cardiovascular implant – tubular vascular prostheses BS ISO 7198:1998 is an excellent guideline to adhere to at the moment.

3.3 lymphatic Valve

The main challenge in the design of artificial lymphatic graft is the inclusion of valves. Lymphatic valves are bileaflet and made up of connective tissue lined by LECs which are anchored to the base by elastin and collagen that are responsible to resist inversion (26). Valves are present at intervals of every few millimetres and usually found in vessel bifurcations and branch points (27, 28). Valves function to prevent backflow and is the anatomical site of fluid shear sensation for production of nitric oxide to regulate lymphatic contractions (29). Valvular dysfunction would exacerbate lymphedema which is witnessed in genetic conditions involving lymphatic valve malformation such as in FOXC2 mutation (30). Valves also play an important role in sectioning the conduit into shorter segments in order to assist capillary action.

Capillary action is described as (31):

$$h = \frac{2\gamma \cos\theta}{pgr} \qquad \text{(a)}$$

Where h is the height of the meniscus, θ is the contact angle, γ is the liquid-air surface tension (force/unit length), g is the gravitational acceleration (length/square of time), p is the density of liquid (mass/volume), and r is the radius of conduit (length).

Equation (a) implies that height of capillary action is inversely proportional to the radius of the conduit. A simplified assumption using properties of water suggests that the height of capillary action is 3.6cm if the r is 0.4mm. However, knowing that the density of lymph is greater than that of water, the height of meniscus is expected to be much lower and hence capillary action alone is insufficient to drive the lymph through the whole length of lymphatic vessels if valves were not present to segment the columns.

3.3 Scaffold

Tissue engineering is aimed at creating a construct which allows harmonious interaction between scaffold, cells and appropriate growth factors (32). An ideal scaffold should bridge the gap between lymphatic vessels and guide regeneration of functional lymphatic tissue. The importance of designing a conduit with intraluminal valves exclude the possibility of using non-scaffold based tissue engineering approach such as ‘sheet-based’ method (22). Scaffold for lymphatic graft can be broadly divided into synthetic and natural (decellularized).

3.3.1 Biodegradable synthetic scaffold

Synthetic scaffold has the advantage of being custom designed and shaped with well-defined physical and biochemical property at production. Biodegradable synthetic scaffold acts as temporary scaffold prior to being substituted by cellular matrix that is balanced by the polymer degradation rate. Polyglycolic acid (PGA) scaffold was used by Dai and colleagues to engineer lymphatic vessel and reported successful endothelialization by LEC (33). PGA was used due to its well documented biocompatibility as well as the ease in manipulating and design. This is the sole reported work on biodegradable lymphatic conduit and despite being a preliminary work; it showed the ability of LEC to attach onto polymer scaffold.

3.3.2 Non-biodegradable synthetic scaffold

This scaffold has stable mechanical strength and able to maintain its shape. The main issue with most of these materials used in vascular grafts are maintaining patency of the graft and high infection rate especially when the diameter is <5mm (34, 35). This is due to the absence of adaptive tissue that is responsive to local environment (36). These limitations have driven

researches to develop vascular graft with endothelial cell lining as well as hybrid graft which has the combination of mechanical strength and biological cues (37, 38).

A particular nanocomposite polymer, the UCL-Nano^{TM} (POSS-PCU) is a novel nanocomposite developed in our laboratory and has great potential in lymphatic graft engineering (39). This polymer has shown several superior properties over present synthetic polymer contributed by its surface nanotopography as well as its viscoelastic properties (Figure 3). It has been successful in cardiovascular applications such as heart valves and bypass graft due to its ideal mechanical and biochemical properties which include biocompatibility, durability, ability for biofunctionalization and endothelialization as well as anti-thrombogenic property (40-42). These merits are expected to be favourable in the engineering of lymphatic graft. This nanomaterial have been used in the development of synthetic trachea, lacrimal drainage conduit and vascular bypass graft (43). POSS-PCU has also been incorporated with a second polymer polycaprolactone (UCL Nano-Bio^{TM}) to produce a degradable nanocomposite polymer (44).

The superior qualities of nanocomposites are contributed by the interaction at nano-scale level resulting in the unique quantal properties instead of bulk properties of conventional material (45). The general advantages offered by nanocomposites are high surface area to mass ratio, thermodynamic stability, bio-stability, amphiphilicity and enhanced mechanical property compared to conventional materials for graft. These properties can be enhanced by nanofibers and nanotubes as fillers (22). Carbon nanotubes are able to reduce macrophage adhesion and proliferation which could prevent blockage of lymph flow (46). However, the potential toxicity of carbon nanotube limits its translation potential (47).

3.3.3 Decellularized scaffold

Decellularized scaffold has been widely used in tissue engineered vascular graft as it overcomes the challenge of design and fabrication (48, 49). The pre-formed scaffold caries the ECM architecture of native vessel and biochemical cues to support cell viability while preserving the bulk mechanical feature of native tissue (50). Wong et al. showed that decellularized scaffold can provide necessary extracellular signalling molecules for lymphatic tissue regeneration by evaluating lymphatic vessel ingrowth in acellular dermal matrices (51). However, decellularized collecting lymphatic vessel has not been used to engineer lymphatic graft thus far. In view of the successful clinical application of autologous lymphatic graft by

Baumeister, the application of decellularized collecting lymphatic as scaffold should be tested. One of the challenges with decellularized scaffold is the extreme density of the matrix which delays cell infiltration upon seeding resulting in excess culture time (52). In terms of translational potential, decellularized scaffold are often more expensive, limited in resource and difficult for surgical manipulation.

3.4 Endothelialization of lymphatic graft

Lymphatic endothelium is essential in modulating the fluid flow and dendritic cell transport function (53). As compared to vascular graft, lymphatic graft does not hold risk of thrombosis however in view of the low flow rate and the content of lymphatic fluid, risk of coagulation in the lymphatic graft may be present. The main concern in producing an endothelialised construct is the attachment of seeded cells onto scaffold material (33).

In order to ensure a higher rate of endothelialization and to increase its potential, scaffolds could be bio-functionalised with appropriate ligands. Peptide arginine-glycine-aspartic acid (RGD) which is the minimal binding domain of fibronectin has been studied comprehensively to increase cell adhesion on vascular grafts (42). Peptide RGD forms receptor-ligand interactions with integrins on BEC. In order to identify the best form of RGD for integrins on LEC, polymer scaffold could be modified with different forms of RGD. This bio-functionalised scaffold would provide environment which mimics ECM to help anchor LECs to the polymer scaffold.

Surface topography that is favourable for LEC has yet to be outlined but this feature has been extensively explored with regards to vascular graft engineering. Nanostructuralisation increases total surface area which in return facilitates protein interaction and promotes cell adhesion (54).

3.5 Interstitial flow

Swartz and colleagues have made great contribution in characterising the importance of interstitial fluid flow in regulation of LEC proliferation, function and migration (55). They found that BEC and LEC have differential responses to interstitial flow leading to variation in cellular morphology and branching patterns indicating that LEC differentiation is influenced

by specialised responses from environmental stimuli (56). Similar concept was demonstrated in a mouse model showing that interstitial fluid flow guides LEC organisation along the direction of flow (57, 58). These suggest that scaffold which encourages interstitial flow is vital in engineering lymphatic graft.

3.6 Growth factor

Regenerative medicine research is likely to produce more effective and better tolerated treatment for lymphedema in the long term and proper understanding of lymphangiogenesis is the essence of it. Lymphangiogenesis occurs physiologically during wound healing as well as pathologically in inflammation, tumours metastasis and transplant rejection (59, 60).

Table 2 and 3 provides a comprehensive summary of the main molecular factors found to be important in normal lymphatic development, function and maintenance.

In pathological condition such as lymphedema, the vascular endothelial growth factor-C (VEGF-C) expression or even over-expression is insufficient to promote lymphatic regeneration (61, 62). In such cases, lymphatic function and regeneration could be improved by inhibition of transforming growth factor beta-1 (TGF- β1) (63). The TGF- β1 has potent anti-lymphangiogenic activity leading to decreased LEC proliferation and in vivo LEC migration, impaired lymphatic tubule formation and down regulation of lymphatic specific gene expression (63). Furthermore, the uses of anti-lymphangiogenic factors are also clinically relevant in cases where VEGF-C is contraindicated such as in immediate post cancer resection since VEGF-C expression could contribute to tumour growth and metastasis (64). Hence tissue engineering approach could augment lymphangiogenesis by regulating the equilibrium between lymphangiogenic and anti-lymphangiogenic forces.

3.7 Gene therapy

Genetic and recombinant protein therapeutic strategies are likely to enhance lymphangiogenesis and treat both primary and secondary lymphedema. Hepatocyte growth factor (HGF) gene therapy has successfully stimulated the growth of lymphatic vascular system in mouse model (65). HGF plasmid DNA was used in vascular system, heart and lung and also reported to be safe in patients with critical limb ischaemia (66, 67). Besides that, VEGF-C activation using viral vectors also reported to have potential in lymphedema treatment (68). However, VEGF-C therapy was reported to exacerbate oedema by causing

poor functioning and hyperplastic lymphatic vessels(62). Nevertheless, gene therapy and growth factors have the potential to be an adjunct to engineered biomaterials which can be administered locally or systemically to enhance collateral lymphatic vessel formation and also to provide greater biocompatibility for the biomaterial.

3.8 Cell source

Common source of LEC isolation is the skin dermis and its isolation from human dermis has been well established (69-71). An alternative source of LEC is from pluripotent stem cells such as embryonic stem cells that have been reported to differentiate into LECs in vitro in the presence of VEGF-C and Ang1 (72). Besides that, mesenchymal stem cells (MSCs) derived from bone marrow or adipose tissue also show good potential to differentiate into LECs in the company of recombinant VEGF-C (73). Adipose-derived MSCs have been reported to improve lymphatic regeneration and restore function upon direct injection into rat hind limb and mouse tail models (74). The huge potential of adipose derived stem cells in the field of lymphedema should be acknowledged and explored further in view of its abundant resource and easy availability (32).

3.9 Animal model

The first step in developing an animal model to evaluate the effectiveness of the tissue engineered lymphatic graft is producing a sustainable model of secondary lymphedema. The animal model should permit quantification of lymphatic function over time and allow assessment of practical effectiveness of the tissue engineered lymphatic graft. Animal models that have been used include dog hind limb models, rabbit ear models and rodent tail and hind limb models (75-78). The main limitation of these models are difficulty in quantifying the lymphatic function, size that is much smaller than that of human and unsustainable effect of lymphedema. Upon lymph node resection, some degree of lymphangiogenesis may take place however transport properties exhibited by the newly formed lymphatics are insufficient to restore flow parameters to its original state(79). Combination of radiotherapy with surgical ablation has shown to induce sustained lympheodema after surgery (80).

Tobbia et al outlined the advantage of using sheep as lymphedema model due to the anatomical dimension of the lymphatic vessel which allows quantification of lymphatic function and therapeutic outcome (81). In their study, the popliteal lymph node was excised due to the ease in identification and sufficient size for cannulation. Furthermore, there is just one post-nodal duct despite having about 6-12 pre-nodal ducts which is ideal for the application of the tissue engineered lymphatic graft. Multiple pre-nodal ducts can be anastomosed to the lymphatic graft distally as performed by Campisi with the use of venous graft (4). Objective quantification of lymphatic function before and after administration of the tissue engineered lymphatic graft can be performed with the usage of radiolabelled human serum albumin (81).

A robust pre-clinical study design is crucial in determining the right time to administer the tissue engineered lymphatic graft. Comparison should be made on the benefit of early application of the lymphatic graft upon surgical resection of lymph nodes over application upon onset of clinical symptoms. Such robust study carries a huge potential to revolutionise the current clinical practise in the management of secondary lymphedema.

4.0 CONCLUSION AND FUTURE PROSPECTIVE

Treatment of lymphedema is evolving positively of late due to the growing knowledge in lymphatic biology which has enhanced our understanding on lymphatic growth and repair. In conjunction with this, more studies need to be carried out to further characterise the human lymphatic system in order to bridge the current limitations that exit alongside the promise that new technological advancement holds. Tissue engineered lymphatic graft is a tool that could benefit many around the world by reducing the morbidity related to lymphedema. Furthermore, tissue engineered lymphatic tissue model would be valuable for lymphatic biology and cancer research. This could reduce the usage of animal models for lymphatic and cancer research. With the in depth understanding of tissue engineering, nanotechnology and improved knowledge on biology of lymphatic regeneration, the aspiration to develop successful lymphatic graft is highly achievable. A successful lymphatic graft requires design which is functional as well as scaffold which encourages and maintains endothelialization and mechanical strength. The next step upon creation of successful lymphatic graft is regenerating lymphatic network in order to channel lymphatic fluid from the distal part of the body to functional host lymphatic vessels.

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6.0 APPENDIX

6.1 TABLES

Table 1. Characteristics of lymphatic vessels and blood vessels

Table 2. List of molecular factors important in lymphatic system function described according to the embryological developmental stage in which discovered.

Table 3. Other factors important in lymphatic function not clearly related to embryological development.

6.2 ILLUSTRATIONS

Figure 1. Organisation of lymphatic system

Figure 2. Illustration of tissue engineered lymphatic graft bridging damaged lymphatic vessel.

Figure 3. SEM image of porous POSS-PCU polymer scaffold extruded with sodium bicarbonate (100 µm particle size) porogen. Images are of a longitudinal section. Note the highly porous structure similar to structure of extra cellular matrix indicative of possible better tissue engineering properties (highlighted by the arrow); 10 x magnification (left), 80x magnification (right).

Table 1.

Characteristic	Lymphatic capillary	Lymphatic collector	Blood vessel
Diameter	10-60um	50-400um	Wide range
Inner lining (intima)	LEC	LEC	BEC
Basement membrane	Patchy or absent. Lacks pericytes.	Continuous, multi-layered with pericytes	Continuous, multi-layered with pericytes
Smooth muscle wall (media)	None	Present	Present
Valves	Primary valve	Secondary valve	Present in vein
Subunit	None	Lymphangion	None

Table 2.

(Key:*gene loss lethal, ^gene mutation related to known cases of human primary lymphedema)

Developmental step	Molecular factor	Function	References
Competence	***LYVE-1*** (Lymphatic vessel hyaluronan receptor-1)	The first proteins expressed by endothelial cells that differentiate into LEC. Likely function is to aid leukocyte migration via hyaluronan receptor. Is a useful LEC marker.	(82)
Bias and Specification	***Prox-1**** (homeobox transcription factor)	Master control gene, required to maintain LEC phenotype. Expression can induce LEC like phenotype in mature blood endothelial cells.	(83)
	***SOX-18**^ (sex determining region Y box 18)	A nuclear transcription factor binds to a proximal promoter of the Prox-1 gene. Not required for sustaining LEC phenotype.	(84)
	***COUP-TFII**(Chicken ovalbumin upstream promoter transcription	An orphan nuclear receptor that has a vital role in organogenesis generally. Acts as a regulator of transcriptional activity of Prox-1, notably by down regulating VEGFR-3 (effects cell migration) and cyclin E1 (cell	(85)

	factor II)	proliferation gene) transcription.	
	VEGFR-3*^(vascular endothelial growth factor receptor 3)	Vascular endothelial growth factor receptor essential for lymphatic as well as blood vessel embryological development. Becomes specifically expressed on LEC's by birth. Not required for LEC phenotype maintenance, but is required or lymphangiogenesis. Useful LEC marker in adults.	(86, 87)
	VEGF-C* and D	Vascular endothelial growth factors - ligands for VEGFR-3. The major lymphoangiogenic factor. VEGF-D is redundant, but can rescue VEGF-C deficiency. Not required to maintain LEC phenotype, but is required for lymphangiogenesis. Both also bind to VEGFR-2 present on blood and lymphatic vessels. Therapeutic use in lymphedema and cancer under investigation.	(88, 89)
	NRP-2(Neuropilin 2)	A glycoprotein trans-membrane protein binds to VEGFR-2 and 3 and has a modulating effect important for normal lymphatic development.	(90)
Maturation	***Syk& SLP-76**** (tyrosine kinase and adaptor protein)	Haemopoeitic signalling proteins essential in maintaining blood and lymphatic systems separation, preventing haemorrhage and organ failure.	(91, 92)
	Spred -1 /2*, fasting-induced adipose factor	Spred-1 and 2 are negative regulators for growth factors including the VEGF-C/VEGFR-3 signalling cascade. Have	(93)

(Fiaf), or angiopoietin-like protein 4 (Angptl4)	similar role to Syk or SLp-76.	
Phospholipase Cγ2 (PLCγ2).	PLCy2 like Syk/SLP-76 and Spreds has a role in the separation of the lymphatic from blood system.	(94)
Foxc2^ (previously known as mesenchyme forkhead 1 (MFH1)	A transcription factor expressed in mesenchymal tissue including the heart and endothelial cells. Foxc2 deficiency leads to severe modelling defects resulting in abnormal lymphatic vessels lacking valves.	(95)
NAFTc1 (Nuclear factor of activated T cells 1)	A transcription factor that interacts with Foxc2 in remodelling the lymphatic vasculature, in particular maturation of collecting vessels.	(96)
Integrins (α9)*	Integrin α9β1 is involved in LEC migration towards VEGF-C. Has a crucial role in organising fibronectin matrix for normal valve development.	(97)
Ang2/Tie2 (Angiopoeitin growth factor binds to Tie receptors)	Essential in recruiting smooth muscle cells to collecting lymphatic vessels and patterning of the lymphatic capillaries. Ang1 can rescue loss of Ang2, Tie 1 may have a modulatory role.	(98)
EphrinB2	Ephrin B2 acts through Eph B4 receptor to regulate maturing lymphatic vessels. Mutation of the Ephrin B2 PDZ interaction site causes failure of hierarchal organisation, failure of valve formation and abnormal smooth muscle recruitment on lymphatic capillaries.	(99)

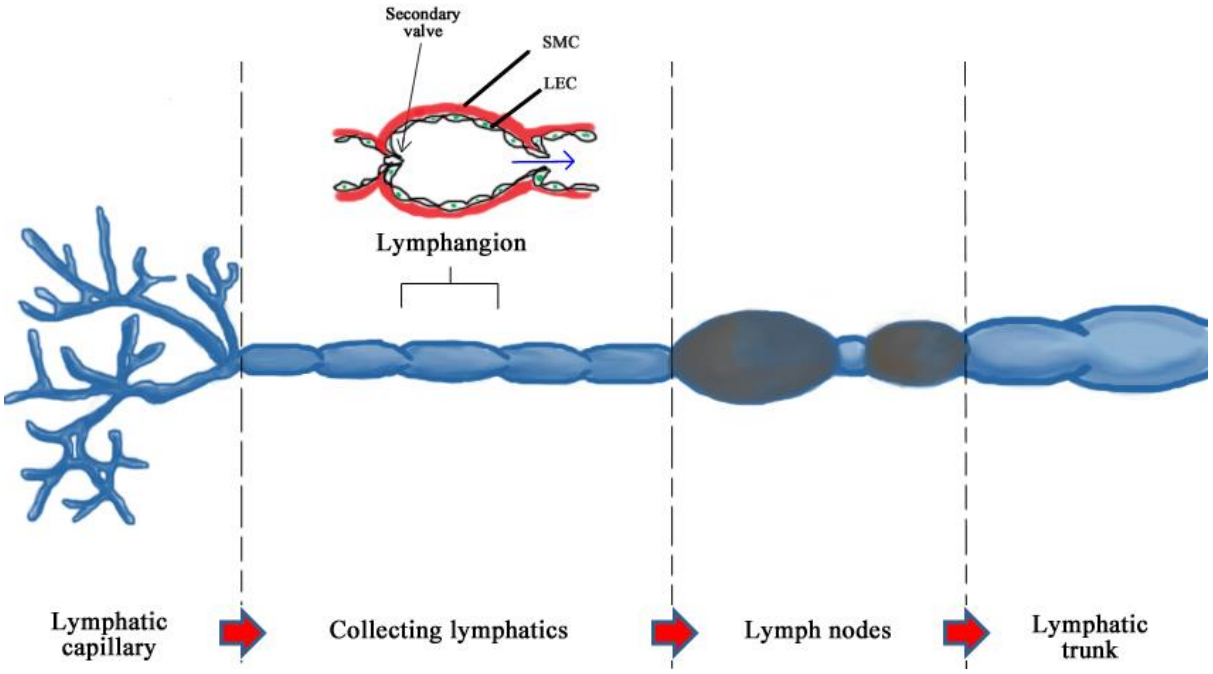
	Podoplanin T1α	A Mucin-type transmembrane glycoprotein. Probable function in cell adhesion, migration and tube formation. Deficiency results in lymphedema secondary to abnormal patterning.	(100)
	Adrenomedullin* (AM)	Multifunction peptide hormone acts via its receptor calcitonin receptor-like receptor (calcrl,) and receptor activity-modifying protein (RAMP2). It is a potent inducer of endothelial cell proliferation, migration and tube formation essential in blood and lymphatic development. Under investigation for treatment of lymphedema.	(101, 102)

Table 3.

(^gene mutation related to known cases of human primary lymphedema)

Molecular factor	**Function**	**References**
Emilin-1 (elastic extra-cellular matrix microfibril associated glycoprotein)	patterning in relation to anchoring filament attachment to the ECM	(103)
Ccbe1^ (Collagen and calcium-binding EGF domain-1)	Essential in lymphatic development in a zebra fish, recent link to human primary lymphedema. Has not been found to be expressed in lymphatic endothelium and is more likely to be found within the ECM.	(104)
HGF (hepatocyte growth factor)	Lymphangiogenic growth factor, investigated for treatment of lymphedema.	(105)
FGF-2 (fibroblast growth factor-2)	Lymphangiogenic growth factor	(106)
PDGF-BB (platelet derived growth factor-BB)	Lymphangiogenic growth factor	(106)
IGF-1/2 (insulin-like growth factors)	Lymphangiogenic growth factor	(106)

Figure 1.



SMC: Smooth muscle cell

LEC: Lymphatic endothelial cell

Figure 2.

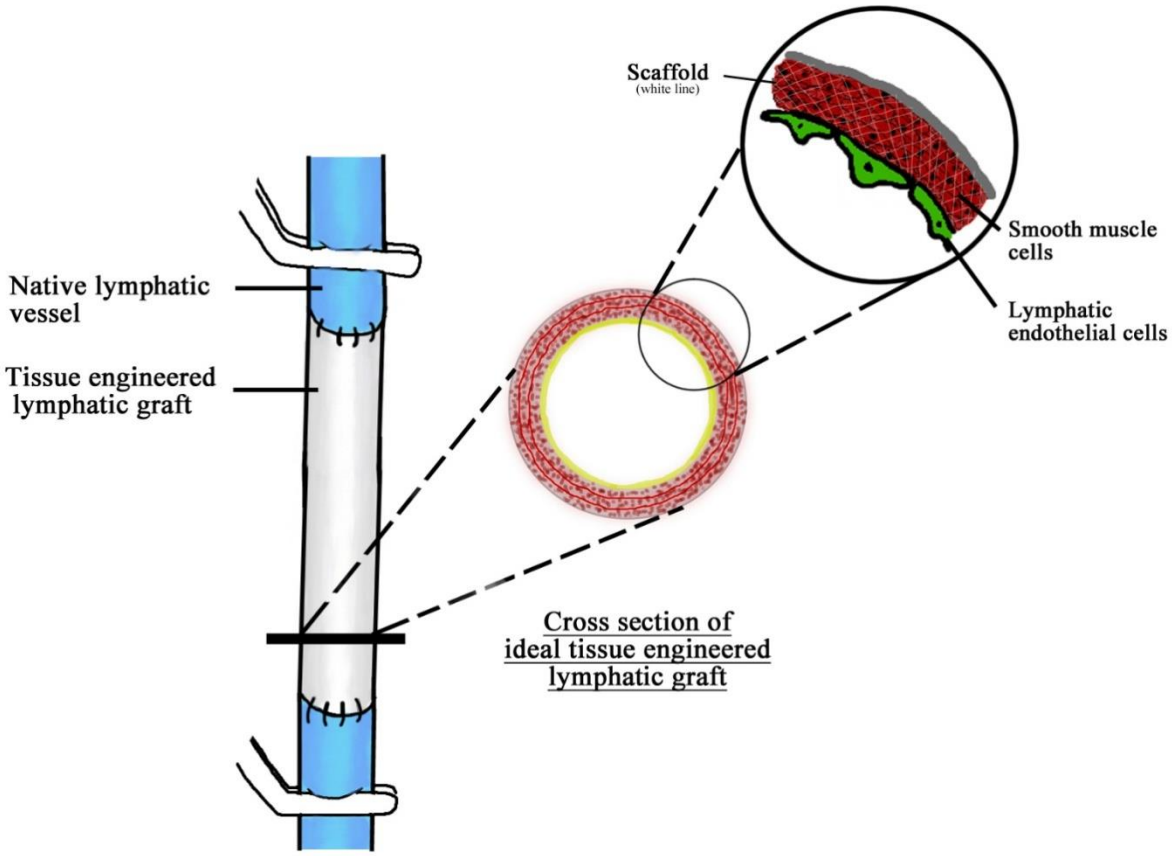

Scaffold
(white line)
Smooth muscle cells
Lymphatic endothelial cells
Native lymphatic vessel
Tissue engineered lymphatic graft
Cross section of ideal tissue engineered lymphatic graft

Figure 3.