Dissecting the biology of lymphedema-distichiasis

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Functions of lymphatic vasculature

**Interstitial fluid balance**
Returning excess interstitial fluid to the blood circulation

**Lipid transport**
Absorption of fat and fat-soluble vitamins from the digestive tract

**Immune surveillance**
Carrying antigen and antigen-presenting cells

Lymphatic vasculature and human diseases

- Primary and secondary lymphoedema
- 140 million cases worldwide
- Highly underestimated cause of disability comparable to Crohn’s disease
Rare disease of lymphatic vessels
PRIMARY LYMPHEDEMA

- Chronic excessive tissue swelling
- Rare disease 1:6’000
- Autosomal dominant
- Affects up to 300’000 patients in Europe
- High morbidity

- Prone to infections
- Fibrosis
- Fat accumulation
- Sometimes lethal (lymphangiosarcoma, pulmonary edema or protein-loosing enteropathy)
Rare disease of lymphatic vessels

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**SECONDARY LYMPHEDEMA**

- Lymph node resection is the main cause
- Up to 30% of breast-cancer patients (350’000 individuals in Europe)
- Other cancers…

- May take several years to develop

Genetic component?
Rare disease of lymphatic vessels

**PRIMARY LYMPHEDEMA**

- Rare disease
- Autosomal dominant
- Affects 1 in 6,000 patients
- Chronic excessive tissue swelling
- High morbidity
- No curative treatment at the moment...
  - Only compression wraps and regular manual lymphatic drainage!

**SECONDARY LYMPHEDEMA**

- Lymph node resection is the main cause
- Up to 30% of breast cancer patients (350,000 individuals in Europe)
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- Lymphedema prone to infections
- Fibrosis
- Fat accumulation
- Sometimes lethal (lymphangiosarcoma, pulmonary edema or protein-losing enteropathy)

Genetic component?

- Rare disease 1:6,000
- Autosomal dominant
- Affects up to 300,000 patients in Europe
Different subtypes of hereditary lymphedema

- 20 causative genes are identified so far

- **Example: Milroy’s disease**
  - mutations in VEGFC and VEGFR3
  - congenital lymphedema
  - *Chy* mouse model with mutations in Vegfr3
  - hypoplastic cutaneous lymphatic vessels
  - Adeno-VEGFC therapy re-grows lymphatic vessels and restores lymphatic flow

- Lymfactin (AdVEGFC) is in clinical trial to treat secondary lymphedema (Herantis Pharma)
Lymphedema-distichiasis

- Autosomal dominant disease with reduced penetrance
- Late onset lymphedema (av. 13 years old)
- Distichiasis - double row of eyelashes

- anomalous pulmonary venous connections
- patent ductus arteriosus

Lymphatic vessels in LD

- Normal (Brice et al., 2002) or hyperplastic (Johnson et al., 1999)
- Lymphoscintigram shows reflux of tracer (Brice et al., 2002)
- Lymph node hyperplasia
Forkhead transcription factor FOXC2

- Highly conserved 100 aa fork head DNA binding domain
- Expressed in lymphatic EC, especially in lymphatic valves
- Multiple mutations cause lymphedema-distichiasis

Fang et al., 2000
Bell et al., 2000
Finegold et al., 2001
Main questions

• What is the role of FOXC2 in lymphatic vasculature?

• What are the mechanisms underlying the lymphatic vessel dysfunction in LD?

• Can such mechanisms be targeted to improve the lymphatic transport in LD patients?
FOXC2 mutants are transcriptionally inactive

Reporter: 6xFOXC2-luci, 2.5 and 0.5 ug of expression constructs

Fold increase

WT, 2.5  WT, 0.5  A, 2.5  A, 0.5  B, 2.5  B, 0.5  C, 2.5  C, 0.5  F, 2.5  F, 0.5  K, 2.5  K, 0.5  WT, 0.5+F, 2.5  WT, 0.5+F, 0.5  WT, 0.5+K, 2.5  control
Mouse mesenteric lymphatic vessels as a model

- Thin 2D-like structure
- Stereotypic location of blood and lymphatic vessels
- Both collecting lymphatic vessel and capillaries
Lymphatic vasculature contains two types of vessels.

- **Lymphatic capillaries** = fluid/proteins/cells uptake
- **Collecting vessels** = lymph transport
Lymphatic capillary = fluid/proteins/cells uptake

+ Collecting vessels = lymph transport

Efficient lymph transport and immune response
What is the role of Foxc2 in the embryonic, postnatal and adult lymphatic vasculature?

Embryo: Foxc2^{fl/fl}; Prox1-Cre^{ERT2} mouse models

Postnatal: Foxc2^{fl/fl}; Prox1-Cre^{ERT2} mouse model

Adult: Petrova et al., Nat Med 2004
Norrmens et al., J Cell Biol 2009
Sabine et al., Dev Cell., 2012
Sabine et al, JCI, 2015
Foxc2 controls formation of collecting lymphatic vessels during embryogenesis.
Agenesis of lymphatic valves in Foxc2 germline knockout mice

VEGFR-3

E17.5 mesenteric vessels

Petrova et al., Nat Med, 2004
Lymphatic valves prevent lymph backflow

Unidirectional movement of lymph
Agenesis of lymphatic valves leads to inefficient lymph transport.
Is Foxc2 important for collecting lymphatic vessel maintenance?

Deletion:
- tissue-specific (lymphatic vasculature)
- time-dependent (tamoxifen injections)
- expression of an orange lymphatic marker

**Prox1-mOrange2**: Friedemann Kiefer

**Prox1-CreERT2**: Tajia Makinen
Degeneration of lymphatic valves in Foxc2lecko mice
Foxc2 postnatal deletion induces leaky collecting vessels
Formation and maintenance of collecting lymphatic vessels and valves

Directional transport of lymph and impermeability of collecting vessels
Analysis of FOXC2 transcriptional network in lymphatic endothelial cells

Primary lymphatic endothelial cells

+ siRNA knockdown
+ FOXC2 overexpression

Transcriptome profiling

- Chromatin immunoprecipitation
- High throughput mapping of FOXC2-DNA interactions

Bioinformatics analysis

- FOXC2 target genes

Novel regulators of lymphatic vascular development
FOXC2 bound enhancers are enriched in NFAT sites
FOXC2 and NFAT cooperate on composite enhancers

Calcineurin/NFAT pathway

- Important in lymphocytes (IL-2), cardiac, skeletal and smooth muscle cells
- Control of pro-inflammatory genes in blood endothelial cells downstream of VEGF/VEGFR-2
- Role in lymphatic vasculature?
NFATc1 in lymphatic vessels: nuclear localization and high expression in valves

LV=lymphatic vessel
Genetic analysis of calcineurin/NFAT signaling in lymphatic vascular development

Tamoxifen-inducible inactivation of CnB1:
- PDGFβ-iCre\textsuperscript{ERT2} $\rightarrow$ blood endothelium
- VEC-Cre\textsuperscript{ERT2} $\rightarrow$ blood and lymphatic endothelium
- Prox1-Cre\textsuperscript{ERT2} $\rightarrow$ lymphatic endothelium
Pan-endothelial or lymphatic endothelial deletion of calcineurin prevents lymphatic valve formation.

E18.5 mesenteric vasculature

CnB1\textsuperscript{ecWT}  

CnB1\textsuperscript{ecKO}

Pecam-1  Prox1
Formation and maintenance of collecting lymphatic vessels and valves

Foxc2

Calcineurin/NFAT

?
Gene expression analysis after FOXC2 knockdown \textit{in vitro}
**Cx37**<sup>-/-</sup> adult mice do not have lymphatic valves

**Cx37<sup>WT</sup>**

**Cx37<sup>KO</sup>**

Adult diaphragm vasculature

VE-cadherin  Laminin α5  Foxc2
Foxc2 → Calcineurin/NFAT → Connexin37 (+other targets) → Formation and maintenance of collecting lymphatic vessels and valves
Formation of collecting lymphatic vessels and valves

Foxc2

Calcineurin/NF AT

Connexin37
Lymphatic valves are frequently located at branching points/bifurcations: role of disturbed flow?

Yan Agalarov
Parallels with atherosclerosis: *role of disturbed flow?*

Predilection sites are: curved vessels, bifurcations at side branches
Low laminar or oscillatory flow induce atherosclerosis

Steinman DA. J. Biomechanics 2000
## Analysis of LEC mechanosensory responses *in vitro*

**Parallel plate flow system**

**Primary human lymphatic endothelial cells**

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Output parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td>Static</td>
<td>Imaging</td>
</tr>
<tr>
<td>Oscillatory shear stress</td>
<td>Western</td>
</tr>
<tr>
<td>Laminar shear stress</td>
<td>Expression profiles</td>
</tr>
</tbody>
</table>

![Parallel plate flow system](image-url)
Reversing flow induces expression of Foxc2

*In vitro*

<table>
<thead>
<tr>
<th>Static</th>
<th>OSS</th>
<th>LSS</th>
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</thead>
<tbody>
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</table>

*FOX2C*

*GAPDH*

*In vivo*

![Image](image4.png)
Formation and maintenance of collecting lymphatic vessels and valves

Shear stress → Foxc2 → Connexin37+other targets

Calcineurin/NFAT
Formation and maintenance of collecting lymphatic vessels and valves

Shear stress → Foxc2 → Calcineurin/NF

Can we exploit this fundamental knowledge to propose treatment approaches?
Foxc2 inactivation disrupts cell-cell junctions in vitro

Oscillatory shear stress (48h - 4 dyn/cm² - 4sec)
Foxc2 inactivation disrupts cell-cell junctions in vivo.

Wildtype vs. Foxc2 lecKO:
- **Pecam 1**
- **VE-cadherin**

Images show a comparison between wildtype and Foxc2 lecKO conditions, highlighting disruptions in cell junctions.
Targeting vascular leakage in the animal LD model?
TheraLymph

Adenoviral therapy
Animal model of Hennekam syndrome

Analysis of lymphatic vessel function
Imaging

TP

Tatiana Petrova

AB

Andrea Brendolan

Stefan Schulte-Merker
Pharmacological therapy for treatment of lymphedema-distichiasis

What is the potential of repurposing approved drugs to treat lymphedema?

<table>
<thead>
<tr>
<th>Therapeutic approach</th>
<th>In vitro</th>
<th>In vivo</th>
</tr>
</thead>
<tbody>
<tr>
<td>ROCK inhibitors</td>
<td>✔️</td>
<td>✗️</td>
</tr>
<tr>
<td>VE-PTP inhibition</td>
<td>✗️</td>
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</tr>
<tr>
<td>Macrophage recruitment</td>
<td></td>
<td>✗️</td>
</tr>
<tr>
<td>VEGFA driven vascular permeability</td>
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<td>✗️</td>
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<tr>
<td>VEGFC/VEGFR-3 blockade</td>
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<td>✔️</td>
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Formation and maintenance of collecting lymphatic vessels and valves

Pathological states: Lymphedema, chylous effusions and inflammation
Lab:
Esther Bovay
Amelie Sabine
Cansaran Saygili

Collaborators:
Brenda Kwak
Friedemann Kiefer

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Mouse model

FOXC2