TECHNOLOGY REPORT

An inducible Cldn11-CreERT2 mouse line for selective targeting of lymphatic valves

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Abstract
Luminal valves of collecting lymphatic vessels are critical for maintaining unidirectional flow of lymph and their dysfunction underlies several forms of primary lymphedema. Here, we report on the generation of a transgenic mouse expressing the tamoxifen inducible CreERT2 under the control of Cldn11 promoter that allows, for the first time, selective and temporally controlled targeting of lymphatic valve endothelial cells. We show that within the vasculature CLDN11 is specifically expressed in lymphatic valves but is not required for their development as mice with a global loss of Cldn11 display normal valves in the mesentery. Tamoxifen treated Cldn11-CreERT2 mice also carrying a fluorescent Cre-reporter displayed reporter protein expression selectively in lymphatic valves and, to a lower degree, in venous valves. Analysis of developing vasculature further showed that Cldn11-CreERT2-mediated recombination is induced during valve leaflet formation, and efficient labeling of valve endothelial cells was observed in mature valves. The Cldn11-CreERT2 mouse thus provides a valuable tool for functional studies of valves.

KEYWORDS
Cre recombinase, lymphatic vessel, valve

1 | INTRODUCTION

A major function of the lymphatic vasculature is to return excess interstitial fluid and macromolecules to the blood circulation. Uptake into lymphatic vessels occurs in blind-ended lymphatic capillaries that are characterized by endothelial cells (ECs) linked together via discontinuous button-like cell–cell junctions. Absorbed fluid is drained from the capillaries via so called precollecting vessels to a network of collecting vessels and large collectors that ultimately drain into the venous blood. Collecting lymphatic vessels, in turn, are composed of ECs that are interconnected by continuous zipper-like junctions. Unlike lymphatic capillaries, collecting vessels also have smooth muscle cell coverage and intraluminal valves that prevent backflow (reflux) of fluid (Geng, Cha, Mahamud, & Srinivasan, 2017; Potente & Mäkinen, 2017).

The critical importance of intraluminal valves for normal lymphatic vessel function is highlighted by the fact that their dysfunction underlies primary lymphoedema—a progressive and lifelong condition characterized by gross swelling of the affected tissue. Indeed, mutations in genes regulating lymphatic valve morphogenesis including FOXC2 (Petrova et al., 2004), GATA2 (Kazenwadel et al., 2012; Ostergaard et al., 2011), EPHB4 (Martin-Almedina et al., 2016; Zhang et al., 2015), and CELSR1 (Gonzalez-Garay et al., 2016; Tatin et al., 2013) were identified as causative of primary lymphoedema in humans. Detailed analyses of genetic mouse models for these and an increasing number of other involved genes have greatly increased our
understanding of the mechanisms of valve morphogenesis during the past decade (reviewed in Geng et al., 2017). In addition, lymph flow-derived mechanical forces play an important role in the development and maintenance of lymphatic valves (Sabine et al., 2012, 2015; Sweet et al., 2015). Notably, shear stress is also important for collecting vessel remodeling, while valve disruption may lead to alterations in the lymph flow patterns thereby affecting collecting vessels globally. This, together with the fact that the expression of most if not all regulators of valve formation identified so far are not restricted to valve lymphatic endothelial cells (LECs), makes mechanistic interpretation of phenotypes resulting from pan-LEC-specific gene deletion difficult. Understanding cell-autonomous functions of genes in valve LECs will thus benefit from tools that allow specific targeting of this cell population.

Here, we report on the generation of a transgenic mouse expressing the tamoxifen inducible CreERT2 under the control of Cldn11 promoter that allows, for the first time, selective and temporally controlled targeting of lymphatic valve ECs and thus provides a valuable tool for functional studies of valves.

2 | RESULTS

2.1 | CLDN11 is specifically expressed in lymphatic valves but not required for their development

To determine the unique molecular identity of LECs of collecting vessels and valves, we utilized data from global transcriptome analysis of FACS purified dermal LECs based on LYVE1 protein levels (Hernández Vásquez et al., 2021). Capillary LECs are characterized by high LYVE1 expression whereas LECs of collecting vessels, including those of luminal valves, display low or absent LYVE1 expression (Mäkinen et al., 2005). Affymetrix GeneChip analysis of the sorted cells identified Cldn11 as a LYVE1low (i.e., collecting vessel) LEC-enriched gene (Hernández Vásquez et al., 2021). Consistent with this finding, single-cell RNA sequencing of LECs of human lymph nodes revealed specific expression of Cldn11 in lymphatic valves (Takeda et al., 2019). We confirmed valve-specific expression of CLDN11 by immunofluorescence analysis of neonatal murine mesenteric lymphatic vessels (Figure 1a).

To evaluate the potential function of CLDN11 in the development of lymphatic valves, we analyzed a global Cldn11 knock-out mouse model that exhibits defective tight junctions in central nervous system myelin and between Sertoli cells but is viable to adulthood (Gow et al., 1999). Whole-mount immunofluorescence of neonatal mesenteric vasculature showed normal gross morphology of lymphatic vessels and valves in Cldn11-/- mice in comparison to littermate controls (Figure 1b). These results demonstrate that, although a specific marker of lymphatic valves, CLDN11 is not required for their morphogenesis.

2.2 | Cldn11-CreER<sup>T2</sup> line allows selective targeting of lymphatic valves

Next, we aimed to utilize the LEC subtype-specific expression of Cldn11 for valve-specific targeting in vivo. To this aim, we generated a

(a) (b)

FIGURE 1 The lymphatic valve-specific tight junction protein CLDN11 is not required for valve morphogenesis. (a) Whole mount immunofluorescence of a mesenteric lymphatic vessel of P3 wild type mouse showing valve-specific expression of Cldn11. (b) Whole mount immunofluorescence of P2 Cldn11 knock-out mouse mesenteries labeled for the indicated antibodies, showing normal vessel morphology and organization of PROX1 valve LECs in Cldn11-/- compared to Cldn11-/- littermate mouse. Scale bars: 100 µm
FIGURE 2  Legend on next page.
tamoxifen-inducible Cldn11-CreERT2 mouse line by introducing CreERT2 encoding sequence followed by Cldn11 3’UTR and human growth hormone (hGH) polyadenylation signal into the start codon of Cldn11 in a bacterial artificial chromosome (BAC) (Figure 2a). Five transgenic founder lines were generated by pronuclear injection of the BAC into fertilized oocytes and tested for efficiency and specificity of Cre-mediated recombination. Mice were crossed with the double fluorescent R26-mTmG reporter mouse (Muzumdar et al., 2007) and the progeny was screened for green fluorescent protein (GFP) expression after tamoxifen administration. Whole-mount immunofluorescence of the ear skin of Cldn11-CreERT2;R26-mTmG mice treated at 3 weeks of age with three daily consecutive administrations of tamoxifen revealed lymphatic valve-specific expression of GFP in all but one founder line (Figure S1, Figure 2b). Although within the vasculature GFP expression was restricted to lymphatic valves, additional GFP+ nonvascular cells, including cells of the hair follicles, were observed in the ear skin of Cldn11-CreERT2;R26-mTmG mice (Figure S2a). In addition, the extended cell membranes of OLIG2+ oligodendrocytes in the brain that are known to express CLDN11 (Gow et al., 1999) were visualized by GFP staining (Figure S2b). Founder #151 showed the highest Cre recombination efficiency in lymphatic valves (Figure S1, Figure 2b), and was selected for further studies. To further assess the specificity and efficiency of the Cldn11-CreERT2 line, we quantified valves containing reporter expressing LECs using two reporter alleles of different sensitivity (Álvarez-Aznar et al., 2020). Efficient targeting of lymphatic valves was observed in 5-week old mice treated 2 weeks earlier with three daily consecutive administrations of tamoxifen independent of the reporter used: 95 ± 6.1% (n = 60 valves from six mice) in the R26-mTmG mice and 98 ± 5.6% (n = 26 valves from five mice) in the more sensitive R26-tDTom mice. Recombination in the dermal vasculature was highly specific to valves as in all images analyzed we could identify only one reporter expressing dermal LEC (in the R26-mTmG line) that was not confined to the valve, and no reporter positive BECs. Flow cytometry analysis of dermal ECs from Cldn11-CreERT2;R26-tDTom mice confirmed lack of recombination in BECs, while 7 ± 3.2% (n = 4 mice) of LECs were targeted (Figure 2c).

### 2.3 Cldn11-CreERT2 targets venous valves with low efficiency

Given that the molecular mechanisms controlling the development of lymphatic and venous valves are shared (Bazigou et al., 2011), we next asked whether Cldn11-CreERT2 can also target valves in the veins. Whole-mount immunofluorescence of lower iliac veins of 8 days old (P8) Cldn11-CreERT2;R26-mTmG mice administered with 4-OHT at P5 and P6 revealed scattered GFP+ cells within the PROX1+ valve leaflets in all mice analyzed (n = 5, Figure 2d). Quantification revealed on average 33 GFP+ cells per valve, which corresponds to 22 ± 6.2% (n = 6 valves from four mice) of all PROX1high venous valve ECs. Analysis of mesenteries of the same animals showed markedly higher recombination efficiency in most ECs forming the lymphatic valve leaflets (Figure 2d). These results demonstrate specific targeting of venous valves, albeit with low efficiency, in neonatal Cldn11-CreERT2 mice.

Specialized lymphovenous valves (LVVs) forming at the connection sites between the primordial thoracic duct (a.k.a. lymph sac) and the cardinal vein also share molecular mechanisms with lymphatic valves (Geng et al., 2017). To analyze the potential Cldn11-CreERT2-mediated targeting of LECs of LVVs, pregnant females were administered with two consecutive intraperitoneal injections of 1 mg of 4-OHT at embryonic day (E)11 and (E)12, when LVV formation is initiated (Geng et al., 2017), and embryos were collected at E13 (Figure 2e). Analysis of immunostained coronal vibratome sections of Cldn11-CreERT2; R26-mTmG embryos revealed rare scattered GFP+ cells within the LVVs and the lymph sac, but also outside of lymphatic vessels (Figure 2e). The Cldn11-CreERT2 line is thus not suitable for targeting of LECs of the developing LVVs.

### 2.4 Cldn11-CreERT2 is induced during valve leaflet formation and allows targeting of mature valves

To characterize Cldn11-CreERT2 mediated recombination in the developing lymphatic valves, we next analyzed Cre reporter expression in mesenteries of 4-OHT-treated Cldn11-CreERT2 embryos and neonates carrying the R26-mTmG (Muzumdar et al., 2007) or R26-tDTom
(Madisen et al., 2010) transgene. We administered 4-OHT to pregnant females at E15, when valve formation is initiated (Bazigou et al., 2009; Normén et al., 2009; Sabine et al., 2012). Unexpectedly, this resulted in labeling of scattered ECs in the mesenteric vein and only rare LECs at E18 (Figure 3a). Consistent targeting of valve LECs was first observed in embryos treated with 4-OHT at E17 (Figure 3a), coinciding with the initiation of valve leaflet formation (Bazigou et al., 2009; Normén et al., 2009; Sabine et al., 2012). Efficient labeling of valve ECs was observed in mice that were treated with 4-OHT neonatally at P5 (Figure 3a) or at weaning age (Figure 3b). A similar valve-specific pattern of recombination was observed in neonatal Cldn11-CreERT2; R26-ttdTom mice (Figure 3a). Quantification of neonatal Cldn11-CreERT2; R26-ttdTom further revealed that 84 ± 11.4% PROX1^{high} valves (n = 26 valves from n = 4 mice), contained tomato^+ cells. Rare individual ECs observed in the veins (data not shown) suggested low level of expression of Cldn11 in developing venous endothelia that can be detected using a sensitive Cre reporter. Similar quantification of adult Cldn11-CreERT2; R26-mTmG mesenteric lymphatic vessels revealed that 83 ± 12.2% (n = 18 valves from two mice) of valves had reporter^+ LECs within the leaflets, with approximately 25% (4 out of 18) of them also contained individual LECs outside of the leaflet but in close proximity to the valve (arrowhead in Figure 3b).

Taken together, our data, based on two different Cre reporters, demonstrate efficient and selective targeting of lymphatic valves in

![FIGURE 3](image-url)

_Cldn11-CreERT2^2 targets lymphatic valves during postnatal development and in postweaning mice. Whole-mount immunofluorescence of mesenteries of the indicated developmental stages (a) or in a juvenile mouse (b). The time points of 4-OHT (developmental stages) or tamoxifen (3-week old) treatment and analysis are indicated. Note efficient Cre-mediated recombination (reporter expression) in valve LECs (arrows) upon postnatal but not embryonic Cre induction. Asterisks indicate valves composed of PROX1^{high} cells, arrowheads indicate recombined nonvalve LECs, open arrowheads indicate recombined venous ECs. Scale bars: 50 μm.
neonatal and adult mice. Targeting of scattered nonvalve LECs and venous ECs at developmental stages is likely a consequence of dynamic cell movements during vessel morphogenesis and low Cldn11 expression, respectively.

3 | DISCUSSION

Temporally controlled and cell type-specific Cre lines are important tools for studying gene functions and cell lineages. Over the past decade, several valuable inducible Cre lines targeting different EC types have been generated. With the realization that ECs in different organs, and even within the vessels have distinct identities and functions (Petrova & Koh, 2018; Potente & Mäkinen, 2017), there is a need for more specific tools that allow targeting of EC subpopulations (Han et al., 2021; Pu et al., 2018). In this study, we describe a transgenic tamoxifen-inducible Cldn11-CreERT2 mouse that allows, for the first time, selective targeting of ECs of lymphatic valves and, with lower efficiency, venous valves.

Expression of Cldn11 was previously reported in oligodendrocytes and Sertoli cells, where it forms an important component of the intercellular tight junctions (Gow et al., 1999). Recently, single cell RNA sequencing of human lymph node LECs additionally identified Cldn11 as a lymphatic valve-specific gene (Takeda et al., 2019). Our expression analysis of Cldn11 protein and a genetic reporter driven by the Cldn11-CreERT2 BAC transgene confirmed valve-specific expression within murine lymphatic vessels. In addition, venous valves were targeted, albeit with lower efficiency, providing further support to the notion of a common gene regulation in venous and lymphatic valves is shared (Bazigou et al., 2011; Geng et al., 2017). Analysis of five different Cldn11-CreERT2 founder lines showed that within the vasculature, the reporter expression was indeed consistently restricted to valves. However, additional nonvascular cells including oligodendrocytes were also positive, but it should be noted that only a limited number of tissues and developmental stages were studied. Scattered Cre-targeted venous ECs detected using the sensitive R26-tdTom reporter suggests low level of Cldn11 expression also in the developing veins. The Cldn11-CreERT2 line should thus allow studying temporal pattern of Cldn11 expression also beyond the lymphatic vasculature, but faithful recapitulation of endogenous Cldn11 expression should be confirmed in each situation.

Specific localization to lymphatic valves suggested a potential function for Cldn11 in valve morphogenesis. Analysis of neonatal mesenteric lymphatic vessels in Cldn11 deficient mice, however, revealed normal valves. This demonstrates that Cldn11 is dispensable for valve morphogenesis, but does not exclude a function of Cldn11 in long-term maintenance of lymphatic valves that should be addressed in future research.

Analysis of Cldn11-CreERT2-driven Cre recombination during development of mesenteric lymphatic vessels showed that valve LECs were first targeted at E17, at the onset of valve leaflet formation (Bazigou et al., 2009; Sabine et al., 2012). Efficient targeting was, however, only observed in postnatal stages, when Cre was induced neonatally at P5 or later. Analysis of two Cre reporters of different sensitivity (Álvarez-Aznar et al., 2020), demonstrated similar recombination efficiency and selectivity. Our recent report on valve defects observed upon neonatal Cldn11-CreERT2-mediated deletion of the key valve regulator Efnb2 further demonstrates the value of the line for functional studies of lymphatic valves (Frye et al., 2020). Cldn11-CreERT2 thus represents a novel tool for studying gene functions in lymphatic valves during postnatal development, maintenance and remodeling.

4 | METHODS

4.1 | Mice

R26-mTmG (Muzumdar et al., 2007) and R26-tdTom (Madisen et al., 2010) lines were obtained from the Jackson Laboratory. Generation of the Cldn11 global knock-out mouse was described previously (Gow et al., 1999). Experimental procedures involving the use of animals were in conformity with Swedish laws for the care and use of laboratory animals and approved by the Uppsala Animal Experiment Ethics Board (permit numbers C130/15 and 5.8.18-06383/2020).

4.2 | Generation of Cldn11-CreERT2 mice

To generate transgenic mice expressing the tamoxifen inducible CreERT2 under the control of the Cldn11 promoter, a BAC (RP23-128D19) carrying the mouse Cldn11 gene (RefSeq NM_008770) was obtained from the mouse C57BL/6J RPCI-23 library. The BAC vector was engineered to replace the coding sequence of exon 1 with a cassette containing the open reading frame for a CreERT2 element and the Cldn11 3’ untranslated region containing a stop codon, such that the endogenous translation initiation codon from the Cldn11 gene is used for the expression of the CreERT2 protein (Figure 2a). The engineered BAC vector was used for pronuclear injection into fertilized C57BL/6NTac oocytes. Mice were generated by Taconic Biosciences.

To detect mice positive for the transgene we performed PCR with primers designed to amplify a region at the junction of mouse genomic regions and the CreERT2 open reading frame in the transgene (Figure 2a). Primers specific for the Cre transgene (sense: 5’-AACAT CCGTGTGAGTGGACGC-3’; anti-sense: 5’-AGCTGGCCAAATGTTGC-3’) were used to amplify a 328 bp fragment. Additional primers (sense 5’- GAGACTCTGGCTACTCATCC-3’ and anti-sense 5’- CCTTCAGC AAGAGCTGGGGAC-3’) were included in the reaction to amplify a 585 bp control fragment. Standard amplification reactions (25 μl) were prepared using 0.4 μM of each primer and 0.2 mM dNTPs. After initial denaturation at 95 °C for 5 min, reactions were subjected to 35 cycles of 95 °C (30 s), 61 °C (30 s), and 72 °C (60 s). Reactions were incubated for a final elongation at 72 °C for 10 min. PCR products were separated on a 2% agarose gel containing Sybr Safe. The line will be available to the research community.
4.3 | Cre induction

To induce Cre activity in Cldn11-CreERT2 mice, tamoxifen (Sigma T5648, 1 mg dissolved in peanut oil) was administrated via oral gavage once per day for three consecutive days right after the time of weaning. Alternatively, 4-hydroxytamoxifen (4-OHT, Sigma, H7904) was administrated by intraperitoneal injection(s) to pregnant females (1 mg dissolved in peanut oil) or neonatal pups (50 µg dissolved in ethanol). All analyzed mice were heterozygous for the Cldn11-CreERT2 transgene. Potential phenotype(s) associated with homozygosity for the transgene insertion were not tested.

4.4 | Immunofluorescence

For whole-mount immunostaining ear skin, mesentery, or iliac and femoral veins (as described in [Lyons et al., 2017]) were fixed in 4% paraformaldehyde (PFA) for 2 hr (all samples except for Cldn11 knock-out) or overnight (Cldn11 knock-out) at room temperature, permeabilised in 0.3% Triton-X100 in PBS (PBSTx) and blocked in PBSTx plus 3% milk or 3% BSA (blocking buffer) for 2 hr. Primary antibodies were incubated at 4°C overnight in blocking buffer. After washing in PBSTx, the samples were incubated with fluorochrome-conjugated secondary antibodies in blocking buffer, before further washing and mounting in Mowiol Mounting Medium. For preparation of brain sections, mice under full anesthesia were euthanized by transcardial perfusion with PBS followed by 4% PFA. Brains were removed and postfixed in 4% PFA over night at 4°C followed by equilibration in 50% sucrose. Sagittal cryosections (20 µm) were cut, and stained and mounted as described above. For analysis of LVVs (n = 3 valves from two embryos), 100-µm coronal vibratome sections of E13 embryos were cut and stained as described above. The following antibodies were used: rabbit anti-mouse LYVE1 (RELIATech 103-PAS04AG, 1:500), rat anti-mouse PECAM1 (BD 553370, 1:200), rabbit anti-PROX1 (generated against human PROX1 C-terminus (567-737aa) (Stanczuk et al., 2015), 1:200), chicken anti-GFP (Abcam ab13970, 1:100), goat anti-mouse CLDN11 (Abcam ab13970, 1:100), hamster anti-mouse PDPN (clone 8.1.1 Developmental Studies Hybridoma Bank, 1:200), mouse anti-α-smooth muscle actin-Cy3 (Sigma C6198, 1:400), rabbit anti-mouse Collagen IV (Bio-Rad 2150-1470, 1:1000), rabbit anti-mouse CLDN11 (Abcam ab53041, 1:50), goat anti-mouse integrin-α9 (R&D Systems AF3827, 1:200) or OLIG2 (Millipore AB9610, 1:500). Secondary antibodies conjugated to AF488, AF594 or AF647 were obtained from Jackson ImmunoResearch (all 1:200). AF488, AF594 or AF647 were obtained from Jackson ImmunoResearch (Millipore AB9610, 1:50), goat anti-mouse integrin-α9 (R&D Systems AF3827, 1:200) or OLIG2 (Millipore AB9610, 1:500). Secondary antibodies conjugated to AF488, AF594 or AF647 were obtained from Jackson ImmunoResearch (all 1:200).

4.5 | Image acquisition and quantification

Confocal images of tissues represent maximum intensity projections of Z-stacks that were acquired using Leica SP8 inverted microscope with HCX PL APO CS 10×/0.40 DRY, Fluotar VISIR 25×/0.95 water or HC PL APO CS2 63×/1.30 GLYC objectives and LAS X version 3.5.5.19976, except for Figure 1a that was acquired using Zeiss 710 LSM with Plan-Apochromat 20×/0.8 objective. Quantification of Cre-recombined venous ECs was performed by manually counting GFP+PROX1+ of the total PROX1+ venous EC population (n = 7 VVs from four mice).

4.6 | Flow cytometry

For FACS analysis of tomato+ BECs and LECs, single cell suspensions of the ear skin were prepared as previously described (Martinez-Corral et al., 2020). For immunostaining, the cells were first incubated with rat anti-mouse CD16/CD32, (eBioscience, cat 14–0161-85) and then stained with antibodies targeting PDPN (clone 8.1.1, PE, eBioscience cat 12-5381-82), CD31/PECAM1 (390, PE-Cyanine7, eBioscience, cat 25-0311-82), Lyve1 (ALY7, eFluor660, eBioscience, cat 50-0443-82) and CD45 (30-F11, eFluor 450, eBioscience cat 48-0451-82), CD11b (M1/70, eFluor 450, eBioscience, cat 48-0112-82) and Ter119 (TER-119, eFluor450, eBioscience, cat 48-5921-82). The cell death dye Sytox blue (Life technologies) was added and cells were analyzed on a BD LSR Fortessa cell analyzer equipped with violet (405), blue (488), yellow/green (561), and red (633) lasers. Compensation was performed using the anti-rat/hamster compensation bead kit (Life technologies). Single viable cells were gated from FSC-A/SSC-A, FSC-H/SSC-W, and SSC-H/SSC-W plots followed by exclusion of immune cell and dead cells in a common violet laser dump channel. CD31/PECAM1+PDPN+ cells were considered as BECs while CD31/PECAM1+PDPN+ cells were LECs. The fraction of tomato+ cells was measured in both populations.

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AUTHOR CONTRIBUTIONS

Henrik Ortsäter, Magda N. Hernández-Vásquez, and Maria H. Ulvmar performed experiments and analyzed data. Henrik Ortsäter characterized transgenic founders and recombination in the lymphatic vasculature, Magda N. Hernández-Vásquez characterized venous valves, Maria H. Ulvmar characterized CLDN11 expression. Alexander Gow provided Cldn11 knock-out mouse tissues. Taija Mäkinen conceived the study and wrote the paper with HO, with input from other authors.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.
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SUPPORTING INFORMATION
Additional supporting information may be found online in the Supporting Information section at the end of this article.

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