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A novel *Podoplanin-GFP*Cre mouse strain for gene deletion in lymphatic endothelial cells

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Abstract

The lymphatic vascular system is a one-direction network of thin-walled capillaries and larger vessels covered by a continuous layer of endothelial cells responsible for maintaining fluid homeostasis. Some of the main functions of the lymphatic vasculature are to drain fluid from the extracellular spaces and return it back to the blood circulation, lipid absorption from the intestinal tract, and transport of immune cells to lymphoid organs. A number of genes controlling the development of the mammalian lymphatic vasculature have been identified in the last few years, and their functional roles started to be characterized using gene inactivation approaches in mice. Unfortunately, only few mouse *Cre* strains relatively specific for lymphatic endothelial cells (LECs) are currently available. In this paper we report the generation of a novel Podoplanin (*Pdpn*) *GFP*Cre transgenic mouse strain using its 5' regulatory region. *Pdpn* encodes a transmembrane mucin-type O-glycoprotein that is expressed on the surface of embryonic and postnatal LECs, in addition to few other cell types. Our detailed characterization of this novel strain indicates that it will be a valuable additional genetic tool for the analysis of gene function in LECs.

Keywords

Podoplanin; Cre recombinase; lymphatic endothelial cells; mouse

INTRODUCTION

Lymphatic vessels run throughout the body and carry lymph, a hydrophobic solution composed of lymphocytes, interstitial fluid and dietary fat. Essential for homeostasis, the lymphatic vasculature regulates tissue fluid levels, immune function, and absorption of lipids from the intestinal tract (Tammela & Alitalo, 2010). In the last few years, lymphatics have been shown to have a variety of additional functional roles in obesity, hypertension, chronic inflammatory conditions, inflammatory bowel disease, cardiovascular disease, and psoriasis (Harvey et al., 2005; Machnik et al., 2009; Martel & Randolph, 2013; Baluk et al., 2005; Dieterich, Seidel, & Detmar, 2014; (Harvey et al., 2005; Machnik et al., 2009; Martel & Randolph, 2013; Baluk et al., 2005; Dieterich, Seidel, & Detmar, 2014; Aspelund, Robciuc, Karaman, Makinen, & Alitalo, 2016), as well as in some neurological disorders and

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glaucoma (Louveau et al., 2015; Thomson et al., 2014). Recently, the identification of a number of genes controlling lymphatic vasculature development helped to better characterize the morphological and molecular processes regulating this process (Escobedo & Oliver, 2016; Ma & Oliver, 2017). Despite this progress, we still lack a detailed understanding of the molecular mechanisms and the stepwise differentiation process converting LEC progenitors into mature LECs.

Relevant to the characterization of the stepwise process leading to the formation of the lymphatic vasculature has been the identification of novel genes and the characterization of their functional roles using gene inactivation approaches in mice. Critical to accomplish those goals is the availability of mouse strains efficiently expressing Cre activity in LECs. Until now, only a few Cre strains driving expression in LECs are available, and most are all also expressed in many other cell types and their efficiency is variable. The most widely used mouse Cre strains for LECs are: *Lyve1GFP-Cre* (lymphatic vessel endothelial hyaluronan receptor 1) (Pham et al., 2010). This transgenic line drives Cre in venous ECs inside the cardinal vein (CV) (including LEC progenitors), as well as in budding LEC progenitors and differentiating LECs in most organs. Although useful, *Lyve1* expression in the lymphatic vasculature is quite heterogeneous, being abundant in smaller capillaries but rarely expressed in larger collecting vessels (Baluk & McDonald, 2008). Moreover, *Lyve1GFP-Cre* is also expressed in the yolk sac endothelium, hematopoietic stem cells and macrophages (Lee et al., 2016); therefore, it leads to early lethality in many instances and the lymphatic phenotype is far from being highly penetrant and widespread in all LECs. *Prox1CreERT2* (Srinivasan et al., 2007) is a knock-in inducible strain that works efficiently in most developing LECs; however, its efficiency depends on the tamoxifen treatment protocol and the stage, as apparently deletion is less efficient at postnatal stages. *Prox1* is also expressed in various other cell types (Oliver et al., 1993). *Tie2Cre* is a transgenic strain used to delete the gene of interest mainly in venous LEC progenitors (Kisanuki et al., 2001); however, it is also expressed in BECs and hematopoietic progenitors (Takakura et al., 1998; Tang, Harrington, Yang, Friesel, & Liaw, 2010), and it is still not clear whether *Tie2* is widely expressed in all LECs. *Vegfr3CreERT2* (Martinez-Corral et al., 2016) is a knock-in mouse strain expressed uniformly in most LECs. However, particularly during development *Vegfr3* is also widely expressed in blood endothelial cells and other cell types (Tammela et al., 2008; Partanen et al., 2000; Schoppmann et al., 2002).

Podoplanin (Pdpn) is a transmembrane mucin-type O-glycoprotein expressed on the LECs surface (Wetterwald et al., 1996). *Pdpn* is a ligand for the C-type lectin-like receptor 2 (CLEC2), which is expressed on platelets and contributes to platelet aggregation (Suzuki-Inoue et al., 2010; Uhrin et al., 2010). *Pdpn* plays also an essential role during lymphatic development. In mice, its expression starts in LECs as soon as they bud off from the CV and it remains in all differentiating and mature LECs (Schacht et al., 2003). Germ line and conditional functional inactivation of *Pdpn* affects pup survival, likely because of abnormal epicardial development (Mahtab et al., 2008) and defects in lymphatic vessels formation (Schacht et al., 2003). In addition to its role in LECs, *Pdpn* main expression is also detected in the developing central nervous system, kidney and aggregating platelets among other cell types (M. C. Williams, Cao, Hinds, Rishi, & Wetterwald, 1996; Mary C. Williams, 2003).

Although a *Podoplanin-Cre* BAC transgenic mouse strain is currently available, this line drives Cre expression very efficiently in stromal cells of secondary lymphoid organs, but has almost no detectable Cre activity in LECs or BECs (Onder et al., 2011). Another *Pdpn-Cre* strain was created by the insertion of a Cre cDNA into exon 1 (*Pdpn^{Cre4}*) to generate a *Pdpn*-null allele (Bertozzi et al., 2010). Therefore, the use of this strain for LEC-specific deletion has the concern that a null allele of *Pdpn* will always be part of the compound genotype to be generated by this cross. Accordingly, we thought that a *PdpnGFPCre* mouse strain will be a valuable asset for those interested in dissecting the molecular steps controlling lymphatic vasculature formation. This strain will provide a genetic tool to uniformly delete the gene of interest in all differentiating and mature LECs independent of their caliber, and without being concerned about deletion in other EC types. Therefore, in this paper we report the generation of a *PdpnGFPCre* transgenic mouse line that specifically expresses Cre recombinase under the control of a 5' upstream regulatory region of the *Pdpn* gene. Characterization of this strain confirmed that it is expressed in all LECs and has a very efficient rate of deletion, providing us with a valuable new tool to better characterize lymphatic-specific gene function.

RESULTS

To generate the *PdpnGFPCre* mouse strain we used a 5' 1.272 bp upstream regulatory region of the mouse *Pdpn* gene including four putative Prox1 binding sites, as previous reports argued that Prox1 is a direct activator of *Pdpn* expression in LECs (Pan, Wang, & Yago, 2014). This 5' regulatory region was fused to an eGFP:Cre cassette containing an ATG start codon and a polyadenylation tail (Fig. 1a). This construct was microinjected into fertilized mouse oocytes and founder mice carrying the desired cassette were further characterized (Fig. 1b). A single male founder was used to expand the line. To start characterizing the generated strain *PdpnGFPCre* mice were crossed with the *Rosa26LacZ* reporter mice to monitor β -galactosidase (*LacZ*) activity. Initial whole mount X-gal staining of E14.5 *PdpnGFPCre;Rosa26LacZ* embryos revealed extensive β -gal expression, particularly in dermal vessels resembling small lymphatic capillaries (Fig. 1c, c'). Similar to *Pdpn* normal endogenous expression (Astarita, Acton, & Turley, 2012), we also detected β -gal expression in parts of the head and other scattered cell types. At E16.5, β -gal expression was evident in dorsal skin small capillaries, as well as in vessels (likely lymphatics) in the mesentery, heart and lungs (Fig. 1d-g)

To confirm that the observed β -gal expression corresponded to lymphatics, we characterized its expression in the skin and mesentery by whole mount immunofluorescence using antibodies against GFP to detect *Pdpn* promoter expression, together with LEC-specific antibodies such as Prox1 and neuropilin 2 (Nrp2). At E15.5 the dermal lymphatic network is normally well developed and GFP co-localized with Prox1 and Nrp2 in those vessels (Fig. 2a-d). At this stage, some lymphatics start to be detected in the mesentery and similar to the skin, GFP expression co-localized with that of Prox1 and Nrp2 in this tissue (Fig. 2e-h). Characterization of GFP expression in those same tissues two days later (E17.5) showed specific co-localization with Prox1 and Nrp2; however, GFP expression levels were reduced in regions where dermal (Fig. 2i-l, arrow) and mesenteric lymphatic valves were forming (Fig. 2m-p, arrow).

To evaluate the expression of *PdpnGFP*Cre in some organs, the same combination of antibodies was used to immunostain P7 diaphragm and meninges by whole mount immunofluorescence. GFP was specifically expressed in LECs in the diaphragm (Fig. 3a-d) and meninges (Fig. 3e-h). These results argue that similar to endogenous *Pdpn*, *PdpnGFP*Cre drives activity in lymphatic vessels during embryonic and postnatal stages.

Next, we evaluated more specifically whether *PdpnGFP*Cre recapitulated the reported *Pdpn* endogenous expression during earlier stages of lymphatic development (Astarita et al., 2012). One of the earliest events during lymphatic development is the specification of LEC progenitors inside the CV. This step requires *Prox1* expression in a subset of venous ECs located in the anterior CV at around E9.75 (J. T. Wigle & Oliver, 1999). That expression will be required for those ECs to acquire LEC fate, bud off from the CV and give rise to most lymphatics (Jeffrey T. Wigle et al., 2002). In contrast to *Prox1*, *Pdpn* expression is not detected in LEC progenitors inside the CV; instead, starts to be expressed once LECs bud off from the CV at around E10.5 (Yang et al., 2012). In agreement with this, GFP (*Pdpn*) expression was strongly detected in *Prox1* expressing LECs outside the CV at E11.5 (Fig. 4a-f). A few spots of weak *Pdpn* promoter activity (GFP expression) might also be present inside the CV at this stage (Fig. 4d-f). These results argued that *PdpnGFP*Cre transgenic mice drive GFP/Cre expression in early budding LECs outside the CV. At E13.5, GFP expression was strongly detected in LECs in the developing cervical lymph sacs (Fig. 4g-h). *Pdpn*/GFP expression was also clearly detected in regions of the brain, spinal cord, retinal pigmented epithelium, lung and heart (data not shown).

To functionally validate the generated *PdpnGFP*Cre mouse strain as a reliable tool to inactivate gene function in LECs, we crossed *PdpnGFP*Cre with a *Prox1* conditional mouse strain (Harvey et al., 2005). Removal of one allele of *Prox1* led to major lymphatics defects and edema in *Prox1*^{+/LacZ} or in *Prox1*^{+/f} mice using *Tie2*Cre (Harvey et al., 2005) or *Lyve1*GFP/Cre (Escobedo et al., 2016). In agreement with those results, E14.5 *PdpnGFP*Cre;*Prox1*^{+/f} embryos also exhibited extensive edema and blood-filled dermal lymphatics (Fig. 5g, h). To confirm these findings, we next compared the phenotypes of conditional *Prox1* heterozygous embryos generated using *PdpnGFP*Cre or *Lyve1*GFP/Cre, with those of standard *Prox1* heterozygous embryos. As seen in Figure 5, *Lyve1*GFP/Cre;*Prox1*^{+/f} and *PdpnGFP*Cre;*Prox1*^{+/f} embryos exhibited extensive edema and blood filled dermal lymphatics (Fig. 5e, g), closely reminiscent of what is observed in *Prox1*^{+/LacZ} embryos at this stage (Fig. 5c). A more detailed characterization of those phenotypes revealed that at least in dermal LECs, *Prox1* deletion using *PdpnGFP*Cre faithfully recapitulates the lymphatic phenotypes of *Prox1*^{+/LacZ} and *Lyve1*GFP/Cre;*Prox1*^{+/f} embryos (less branching and dilated lymphatics, Fig. 5h). As a final validation, we generated conditional *PdpnGFP*Cre;*Prox1*^{f/f} null embryos. Similar to previous reports (J. T. Wigle & Oliver, 1999), no *Prox1*-expressing LEC progenitors were detected inside or outside the CV at E11.5 (Fig. 6e-h); nor were dermal lymphatics seen at E14.5 (Fig. 6l).

These data indicated that the newly generated *PdpnGFP*Cre transgenic strain is a valuable genetic tool for the analysis of gene function in LECs. It is worth mentioning that we have not evaluated whether this strain can be made homozygous for the transgene.

Methods

Generation of the *Pdpn**GFPCre* transgenic mice

A 1.3 kb *Pdpn* upstream regulatory genomic region was inserted between the SalI-EcoRI sites of the pCAG-Cre:GFP vector (Addgene). The Cre:GFP cassette was inserted before the first *Pdpn* exon. To eliminate plasmid sequences, a 4.8 kb *Pdpn*Cre:GFP fragment was isolated by SalI-PstI digestion. This fragment was microinjected into fertilized FVB eggs (Friend Virus B NIH Jackson). The generated transgenic lines were backcrossed into the NMRI background. All of the mouse experiments were approved by the Northwestern University Animal Care and Use Committee. This strain will be available to the research community upon request.

Mice and genotyping

Prox1-tdTomato mice were obtained from the Mutant Mouse Resource & Research Center (STOCK Tg(*Prox1-tdTomato*)TA76Gsat/Mmucd) (Gong et al., 2003). *Lyve1GFPCre* (B6;129P2-*Lyve1*^{tm1.1(EGFP/cre)Cys/J}, Stock No:012601) (Pham et al., 2010) and *Rosa26LacZ* (FVB.129S4(B6)-*Gt(ROSA)26Sor*^{tm1Sor/J}) (Soriano, 1999) mouse lines were obtained from the Jackson Laboratory. *Prox1*^{+/LacZ} and *Prox1*^{+/f} mice were generated and reported previously (J. T. Wigle & Oliver, 1999; Harvey et al., 2005). Embryonic stages were estimated by considering the morning of the vaginal plug as E0.5. Transgenic mice were genotyped by PCR using tail DNA. The presence of the Cre transgene was detected using the following specific primers: *PdpnGFPCre*-F: CATCTTTTCACAACCCACAAAA and *PdpnGFPCre*-R: ATAATCGCGAACATCTTCAGGT, *Prox1-tdTomato*-F: GATGTGCCATAAATCCCAGAGCCTAT and *Prox1-tdTomato*-R: CCTTGGAGCCGTACATGAACTGG, *Lyve1GFPCre*-Wild type F: TGCCACCTGAAGTCTCTCCT, *Lyve1GFPCre*-Mutant F: GAGGATGGGGACTGAACTG, and *Lyve1GFPCre*-R: TGAGCCACAGAAGGGTTAGG, *LacZ*-F: CTACAGGAAGGCCAGACGCGA and *LacZ*-R: ACGTAGTGTGACGCGATCGGC, and *Prox1*^{+/f}-F: CTCTGTGGAAGGACTCCGAATC and *Prox1*^{+/f}-R: AACTAAACAAGCAAATGGAAATGC.

β-galactosidase staining

Embryos were fixed in a solution of 0.2% glutaraldehyde, 1% formaldehyde, 2mM MgCl₂, and 0.02% Nonidet-P40 for 1 hour at 4°C and then washed 3 times in a 0.1 M sodium phosphate buffer solution (PBS, pH 7.5). Embryos were stained with 1 mg/ml of 5-bromo-4-chloro-3-indolyl-beta-D-galactoside (X-gal, Sigma, 10703729001) in a 0.1 M PBS solution of 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide and 2 mM magnesium chloride (MgCl₂) at 30°C. Next day, embryos were washed with PBS and photographed.

Immunohistochemistry—For immunohistochemistry on vibratome sections, embryos were fixed in 4% paraformaldehyde (PFA) overnight at 4°C, embedded in 7% low melting agarose and sectioned in a vibratome (100 μm). Sections were permeabilized with 0.3% PBS Triton X-100 (PBS-T) for 1 hour at 4°C and immunostained. For immunohistochemistry on cryosections, embryos were fixed in 4% PFA diluted in PBS overnight at 4°C, rinsed in PBS, transferred to 30% sucrose and then embedded in OCT/Tissue Tek (Sakura Finetek).

Samples were sectioned in a cryostat (12 μ m) and immunostained. Cryosections were incubated in blocking solution (20% bovine serum albumin, 20% blocking reagent, and 60% maleate buffer) for 1 hour at room temperature. Primary antibodies diluted in blocking solution were incubated at RT overnight. They were washed 3 X in 1% Tris-buffered saline-Tween 20 (TBS-T) and secondary antibodies were added and incubated for 1 hour at RT. After washing 3x in 1% TBS-T, sections were mounted using a mounting medium containing DAPI ProLong Gold antifade medium (Invitrogen, Eugene, OR). The images were acquired from a Carl Zeiss Axio Imager A2 microscope.

The following primary antibodies were used: rabbit anti-*Prox1* (1:400 AngioBio, San Diego, CA); goat anti-*Prox1* (1:200 R&D, Minneapolis, MN); hamster anti-*podoplanin* (1:200 BioLegend, San Diego, CA); rat anti-*CD31* (1: 500 BD Pharmingen, San Jose, CA); goat anti-*lymphatic vessel endothelial hyaluronan receptor 1 (Lyve1)* (1:500 R&D, Minneapolis, MN); and chicken anti-GFP (1:500 Abcam, Cambridge, MA).

The following secondary antibodies were used: Alexa 488-conjugated donkey anti-rabbit (Molecular Probes, Waltham, MA); Alexa 488-conjugated goat anti-hamster (Molecular Probes, Waltham, MA); Alexa 488-conjugated donkey anti-goat (Molecular Probes, Waltham, MA); Cy3-conjugated donkey anti-rabbit (Jackson ImmunoResearch Laboratories, West Grove, PA); Cy3-conjugated donkey anti-goat (Jackson ImmunoResearch Laboratories, West Grove, PA); and Cy5-conjugated donkey anti-rat (Jackson ImmunoResearch Laboratories, West Grove, PA).

Whole-mount immunostaining

To analyze the dermal lymphatics, embryos were fixed in 4% PFA diluted in PBS for 2 hours prior to removing the skin for immunostaining. Tissues were blocked with blocking buffer (5% normal donkey serum, 1% bovine serum albumin, 0.1% Triton X-100, and 0.05% sodium azide (NaN₃) for 1 hour at RT. Primary antibodies diluted in blocking buffer were incubated overnight at 4°C. Next day, tissues were extensively washed with 0.1% PBS-Triton X-100 for 2 hours at RT and the corresponding secondary antibodies conjugated with either Alexa 488, 546, or 647 (1:200, Molecular Probes, Waltham, MA) were incubated for 2 hours at RT. Next, samples were washed with 0.1% PBS-Triton X-100 and mounted using a Vectashield antifade-mounting medium (Vector Laboratories, Burlingame, CA). The images were acquired with a LEICA TCS SPE DM2500 confocal microscope.

The following primary antibodies were used: goat anti-*Prox1* (R&D, Minneapolis, MN); rat anti-*CD31* (BD Pharmingen, San Jose, CA); goat anti-*Nrp2* (1:500 R&D, Minneapolis, MN); goat anti-*lymphatic vessel endothelial hyaluronan receptor 1 (Lyve1)* (1:500 R&D, Minneapolis, MN); chicken anti-GFP (Abcam, Cambridge, MA); and goat-anti-Vegfr3 (1:500. R&D, Minneapolis, MN)

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Fig 1. Generation of *PdpnGFP*Cre transgenic mice and characterization of their Cre activity
 (a) Schematic diagram of the *Pdpn*Cre cassette used to generate the transgenic mice. Arrows indicate the position of the forward and reverse primers. (b) Genotyping of the transgenic mice by PCR using tail DNA. (c, c') Whole-mount X-gal staining of *PdpnGFP*Cre;*Rosa26* *LacZ* embryos showing lymphatic specific X-gal staining in the E14.5 skin. (d-g) X-gal staining of E16.5 *PdpnGFP*Cre;*Rosa26* *LacZ* embryos in the dorsal skin, mesentery, heart and lung.

Fig 2. *PdpnGFP-Cre* is expressed in lymphatic endothelial cells

(a-p) Whole-mount immunofluorescence of dermal and mesenteric lymphatics in E15.5 (a-h) or E17.5 (i-p) *PdpnGFP-Cre* embryos. Arrows indicate lymphatic valves-forming regions. Scale bar=100μM

Fig 3. *Pdpr*GFP^{Cre} is expressed in postnatal LECs

Whole-mount immunofluorescence shows GFP expression in LECs in the diaphragm (a-d) and meninges (e-h) at P7. Scale bar=100 μ M

Fig 4. *PdpnGFP* is expressed in budding LEC progenitors at E11.5
 (a-c) E11.5 endogenous *Pdpn* expression is shown in budding LECs outside the CV on 100 μ m thick vibrotome sections. (d-f) At this stage, GFP is also strongly detected in budding LECs in *PdpnGFP* embryos. (g, h) At E13.5, GFP is seen in LECs in the developing cervical lymph sacs. Scale bar=100 μ m.

Fig 5. *PdpnGFP-Cre;Prox1^{+/f}* embryos recapitulate the lymphatic phenotypes observed in *Prox1^{+/LacZ}* and *Lyve1GFP-Cre;Prox1^{+/f}* embryos

(a, b) Normal lymphatic patterning is seen in the anterior dorsal skin of WT E14.5 embryos. Instead, edema and blood-filled lymphatics are seen at this stage in *Prox1^{+/LacZ}* (c, d) and *Lyve1GFP-Cre;Prox1^{+/f}* (e, f) embryos. (g, h) *PdpnGFP-Cre;Prox1^{+/f}* embryos show the same lymphatic phenotypes (edema, blood filled lymphatics) seen in those embryos. Scale bar=100μm

Fig 6. *PdpnGFP-Cre;Prox1^{fl/fl}* embryos lack LECs

(a-d) *Prox1⁺* lymphatic endothelial cells are detected inside the CV at E11.5 in WT embryos. (e-h) In contrast, no *Prox1⁺* LECs are seen inside or outside the CV of *PdpnGFP-Cre;Prox1^{fl/fl}* embryos. E14.5 *PdpnGFP-Cre;Prox1^{fl/fl}* embryo exhibit edema (k) and are devoid of LECs in the skin (l). Scale bar=100μm