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LACK OF EMBRYONIC HOMOZYGOUS OR ADULT HETEROZYGOUS LYMPHATIC PHENOTYPES FOR A *Sos1* MUTATION AND LACK OF LYMPHATIC EMBRYONIC PHENOTYPES FOR A HOMOZYGOUS *Cx47* MUTATION IN MICE

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Abstract

We have studied the lymphatic phenotypes of 2 mutations, known to cause abnormalities of lymphatics in humans, in mice. The Cx47 R260C mutation (variably penetrant in humans heterozygous for it and causing limb lymphedema) had an adult mouse phenotype of hyperplasia and increased lymph nodes only in homozygous condition but we did not find any anatomical phenotype in day 16.5 homozygous embryos. Mice harboring the Sos1 mutation E846K (causing Noonan's 4 in man which occasionally shows lymphatic dysplasia) had no adult heterozygous phenotype in lymphatic vessel appearance and drainage (homozygotes are early embryonic lethals) while day 16.5 heterozygous embryos also had no detectable anatomical phenotype.

Keywords

SOS1 ; *Connexin 47* ; mutations; embryonic/adult phenotypes; lymphatic mice; Noonan syndrome; primary Lymphedema

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Although finding a pathological effect of a "possibly deleterious" human SNP when placed in the corresponding mouse gene is considered a "gold standard" to confirm pathogenicity, mice frequently do not confirm human pathogenicity because a mouse isn't always "like a man" (1). This is frequently true of the lymphatic system. We present two examples of this: *SOS1* and *Cx47*. The former, for which heterozygous mutations in humans cause Noonan's

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CONFLICT OF INTEREST AND DISCLOSURE

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syndrome 4 with characteristically webbed neck, a sign of in utero lymphatic defects (2), has neither an adult heterozygous nor embryonic homozygous lymphatic ab-normality in mice. On the other hand, patients with the induced mouse mutation E846K have only had cardiac defects described (3). The latter, for which heterozygous gain of function mutations cause limb lymphedema in adult humans, when introduced in mice, only homozygous adults have lymphatic defects (4) but show no embryonic defects as precursors of this adult pathology.

MATERIALS AND METHODS

Animals

Mice were housed in clean cages and maintained on a 12-h light/dark schedule with free access to food (NIH-31 7913, Envigo Teklad, Indianapolis, IN) and distilled water. All animal procedures, including generation of transgenic strains, husbandry and experimental protocols, were approved by the University of Arizona's Institutional Animal Care and Use Committee and conformed with the Guide for the Care and Use of Laboratory Animals, 8th edition, from the National Research Council.

Adult Lymphatic Phenotype

Mice were weighed, anesthetized, placed on a warming pad, and examined grossly under a dissecting microscope (Weck, Evergreen, CO, USA) for external physical characteristics indicative of lymphatic abnormalities, i.e., distal edema, distichiasis, ocular abnormalities, and serous effusions, followed by Evans blue dye (EBD) injection. In some cases, mice were first sacrificed since lymphatics continue to drain after death. EBD (<50 μl of 0.5 g/dl) was sequentially injected intradermally into the ear, hind paws, fore paws and snout. Sequential dissection of the peritoneal, thoracic, axillary, jugular, popliteal, and sacral regions revealed EBD-stained lymphatic vessels, regional lymph nodes, cisternal chyli, and, after opening the thoracic cavity, thoracic duct. The latter was revealed traveling alongside the azygous vein until its final entry into the left subclavian vein.

Embryonic Phenotypes

Timed pregnancies were set up and pregnant females sacrificed on day 16.5. The embryos were removed, a small incision made in the anterior abdominal wall for better penetration of fixative and immersed in 2.5% cold paraformaldehyde diluted into PBS.

The placenta were frozen for later DNA extraction and genotyping. Embryos were left overnight in the fixative and then washed in and stored in PBS.

Immunohistochemistry

Mouse embryonic dermal skin was peeled and immunolabeled as described by Geng and Srinivasan (5). Primary antibodies for immunohistochemistry included rabbit anti-PROX1 (catalog 11-002, Angiobio), rat anti-mouse CD31 (catalog 553370, BD Pharmingen), and goat anti-mouse VEGRF3 (catalog AF743) from R&D Systems. Secondary antibodies for IHC included Cy3-conjugated donkey anti-rabbit, Cy5-conjugated donkey anti-rat, and Alexa 488-conjugated donkey anti-goat, purchased from Jackson Immuno-Research Laboratories (catalogs 711-165-152, 712-175-150 and 705-545-147 respectively).

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Genotyping

Cx47—Genomic DNA was extracted and the region surrounding the *Cx47* mutation, and nearby BaeGI restriction site was amplified (KAPA HS Mouse Genotyping kit, Kappa Biosystems, Wilmington, MA) using the following primers: forward: 5'-GCAAGACGG TGGTCACTCC and reverse: 5'-GCCATCT CACAGAGGTTGAGC. PCR amplification of the wildtype gene generates a single 304 bp product that is unaffected by BaeG1 digestion (per manufacturer, NEB, Ipswich, MA), while amplification of the Cx47R259C mutant gene, followed by BaeG1 digestion, produces 2 bands (222 bp and 82 bp). Finally, amplification of DNA isolated from heterozygous mice, followed by BaeG1 digestion, produces 3 bands (304 bp, 222 bp, and 82 bp). Following digestion, PCR products were run on a 1.5% agarose/TBE gel containing GelRed for detection (1 μl GelRed/10 ml agarose gel (VWR)). The 82 bp band was not routinely detected, thus genotypes were distinguished as follows: Wildtype (Wt): single 304 bp band, Heterozygous (Het): 304 bp and 222 bp bands, and Mutant (Mut): single 222 bp band.

Sos1—The primers (listed 5' - 3'): *Sos1* forward: AACCCTTCAGTGTGCATATGTCT; *Sos1* reverse: TGACAACTTCCAGGACACCA were used to amplify a 191 bp segment which was sequenced by Sanger sequencing to identify the point G>A change representing the mutation.

RESULTS

Adult (>8 weeks) Sos1 Heterozygous Mice

One male and two female had the 7 site injections of Evan's blue dye while 3 more females only had the rear paws injected. Examination of the number of lymph nodes, lobes per node, filled vs. unfilled nodes, presence of extra afferent and/or efferent lymphatic vessels and the presence of branched vessels were noted and were completely normal. EBD was rapidly carried anterograde to the patent thoracic duct.

Day 16.5 Embryonic Heterozygous Sos1 Mice

LVV—Lymph returns to blood circulation through 2 pairs of LVVs located bilaterally at the junction of jugular and subclavian veins. We prepared cryosections of this location and analyzed the LVVs of E16.5 control and $Sos1^{+/-}$ littermates as described previously (6). No obvious edema was observed in the mutant embryos and LVVs appeared morphologically indistinguishable from controls (Fig. 1).

Dermal lymphatics were also normal in these embryos (Fig. 2).

Cx47 Homozygous Mutants—LVVs and dermal skin of E17.5 $Cx47^{R260C/R260C}$ homozygous embryos were analyzed as described above and were found to be normal (Figs. 3,4).

DISCUSSION

Mouse models of the most common human genetic lymphatic disorders, Milroy's and lymphedema-distichiasis, have been very useful for understanding abnormal

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lymphangiogenesis. For the former, both mutations in the agonist (VEGF-C) and the receptor (VEGFR-3, FLT-4) cause a hypoplastic phenotype in both mice (7,8) and man (7,9,10).

For the latter, both haplodeficiency and duplication cause a hyperplastic phenotype, both in mice (11) and man (12–14). However, only rarely do the mouse models show limb lymphedema – the *Vegfr-3* mutant does (7) while the *Foxc2* mutant does not. However, in the latter case, the EBD studies and histology show the hyperplastic phenotype (11).

Noonan syndrome (NS) is an autosomal dominant disorder with a resemblance to Turner's syndrome. It is characterized by facial dysmorphism, short stature, slow development, cardiac and, frequently, lymphatic defects (well summarized by Tartaglia et al, 15). Facial features include broad forehead, downslanting palpebral fissures, hypertelorism, high palate, and low-set, posteriorly rotated ears. Hypertrophic cardiomyopathy and pulmonic stenosis are frequent as are skeletal defects and webbed neck, the most common lymphatic defect in Noonan's but there are many more (16). Mutations in 11 genes, all coding for proteins in the RAS pathway and constitute the RASopathies. The RAS pathway is highly involved in lymphatic development (17).

Heterozygous mutations in *SOS1* cause Noonan syndrome 4 and which has a phenotype with more ectodermal features than other types (2). The patients' lymphatic findings are mostly limited to webbed neck. Our extensive study of the E846K *Sos1* mutation in adult heterozygotes demonstrated a normal anatomy and normal drainage of Evans blue dye. Day 16.5 embryonic heterozygous mutants did not disclose any anatomic abnormalities of lymphatic valves or dermal lymphatics. Thus, this mutant does not provide a mouse model of Noonan's syndrome 4.

Missense *CX47* mutations co-segregating with primary lymphedema have been reported in humans (18–20). These were thought to be gain of function mutations as homozygous recessive mutations in the gene cause a severe childhood neurological disease (20). We used CRISPR technology to create a mouse carrying a *Cx47* missense mutation (Cx47R259C) equivalent to the human CX47R260C missense mutation associated with human primary lymphedema (4). Intradermal Evans blue dye injection identified a 2-fold increase in regional lymph nodes in homozygous Cx47R259C mice compared to wildtype, particularly in the jugular region (4.8 ± 0.4 and 2.0 ± 0.0, respectively, $p < 0.01$. Lymphatic channels were increased and mesenteric reflux occurred (vs. none in wild type). Contractility of superficial cervical lymphatics, assessed by pressure myography, was also reduced. We sought to discover the origin of these alterations by studying da 16.5 embryos, hoping to see signs of increase branching of lymphatics and excess nodal development. However, as described above, no structural abnormalities were found.

REFERENCES

1. Erickson RP: Why isn't a mouse more like a man? Trends Genet. 5 (1989), 1–3, 1989. [PubMed: 2711421]
2. van Trier DC, Rinne T, Noordam K, et al. : Variable phenotypic expression in a large Noonan syndrome family segregating a novel SOS1 mutation. Am. J. Med. Genet 173A (2017), 2968–2972.

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3. Chen P-C, Wakimoto H, Conner D, et al. : Activation of multiple signaling pathways causes developmental defects in mice with a Noonan syndrome-associated Sos1 mutation. J. Clin. Invest 20 (2010), 4353–4365.
4. Mustacich DJ, Kylat RI, Bernas MJ, et al. : Abnormal lymphatic phenotype in a crispr mouse model of the human lymphedema-causing connexin47 R260c point mutation. Lymphology 54 (2021), 78–91. [PubMed: 34735753]
5. Geng X, Srinivasan RS: Lymphatic specification and development, EMT-MET, and cancer spread. In: Leong SP, Nathanson SD, Zager JS (Eds.) Cancer Metastasis through the Lymphovascular System. Springer, Cham, 2022. 10.1007/978-3-030-93084-4_20
6. Geng X, Cha B, Mahamud MR, et al. : Multiple mouse models of primary lymphedema exhibit distinct defects in lymphovenous valve development. Dev Biol. 409 (2016), 218–233. doi: 10.1016/j.ydbio.2015.10.022. [PubMed: 26542011]
7. Karkkainen MJ, Saaristo A, Jussila L, et al. : A model for gene therapy of human hereditary lymphedema. Proc. Nat. Acad. Sci 98 (2001), 12677–12682. [PubMed: 11592985]
8. Dellinger M Hunter R, Bernas M, Witte M, Erickson RP: Chy-3 mice are Vegfc haploinsufficient and exhibit defective dermal superficial too deep lymphatic transition and dermal lymphatic hypoplasia. Develop. Dynamics 236 (2007), 2346–2355.
9. Karkkainen MJ, Ferrell RE, Lawrence EC, et al. : Missense mutations interfere with VEGFR-3 signalling in primary lymphoedema. Nature Genet. 25 (2000), 153–159. [PubMed: 10835628]
10. Gordon K, Schulte D, Brice G, et al. : Mutation in vascular endothelial growth Factor-C, a ligand for vascular endothelial growth factor receptor-3, is associated with autosomal dominant Milroy-like primary lymphedema. Circ. Res 112 (2013), 956–960. [PubMed: 23410910]
11. Kriederman BM, Myloyde TL, Witte MH, et al. : FOXC2 haploinsufficient mice are a model for human autosomal dominant lymphedema-distichiasis syndrome. Hum. Mol. Genet 12 (2003), 1179–1185. [PubMed: 12719382]
12. Fang JM, Dagenais SL, Erickson RP, et al. : Mutations in FOXC2 (MFH-1), a forkhead family transcription factor, are responsible for the hereditary lymphedema-distichiasis syndrome. Am. J. Hum. Genet 67 (2000), 1382–1388. [PubMed: 11078474]
13. Vreeburg M, Heitink MV, Damstra RJ, et al. : Lymphedema-distichiasis syndrome: A distinct type of primary lymphedema caused by mutations in the FOXC2 gene. Int. J. Dermatol 447 (2008), 52–55. 10.1111/j.1365-4632.2008.03962.x
14. Witte MH, Erickson RP, Khalil M, et al. : Lymphedema-distichiasis syndrome without FOXC2 mutation: Evidence for chromosome 16 duplication upstream of FOXC2. Lymphology 42 (2009), 152–160. [PubMed: 20218083]
15. Tartaglia M, Zampino G, Gelb B: Noonan syndrome: Clinical aspects and molecular pathogenesis. Molec. Syndrom 1 (2010), 2–26.
16. Cox T, Vance C, Daley S, et al. : Imaging of lymphatic dysplasia in Noonan syndrome: Case studies and historical atlas. Lymphology 54 (2021), 23–40. [PubMed: 34506085]
17. Brouillard P, Witte MH, Erickson RP, et al. : Primary lymphoedema. Nature Reviews Primer 77 (2021), 1–23.
18. Ferrell RE, Baty CJ, Kimak MA, et al. : GJC2 missense mutations cause human lymphedema. Am. J. Hum. Genet 86 (2010), 943–948. [PubMed: 20537300]
19. Ostergaard P, Simpson MA, Brice G, et al. : Rapid identification of mutations in GJC2 in primary lymphoedema using whole exome sequencing combined with linkage analysis with delineation of the phenotype. J. Med. Genet 48 (2011), 251–255. [PubMed: 21266381]
20. Uhlenberg B, Schuelke M, Ruschendorf F, et al. : Mutations in the gene encoding gap junction protein alpha 12 (connexin 46.6) cause Pelizaeus-Merzbacher-like disease. Am. J. Hum. Genet 75 (2004), 251–260. [PubMed: 15192806]

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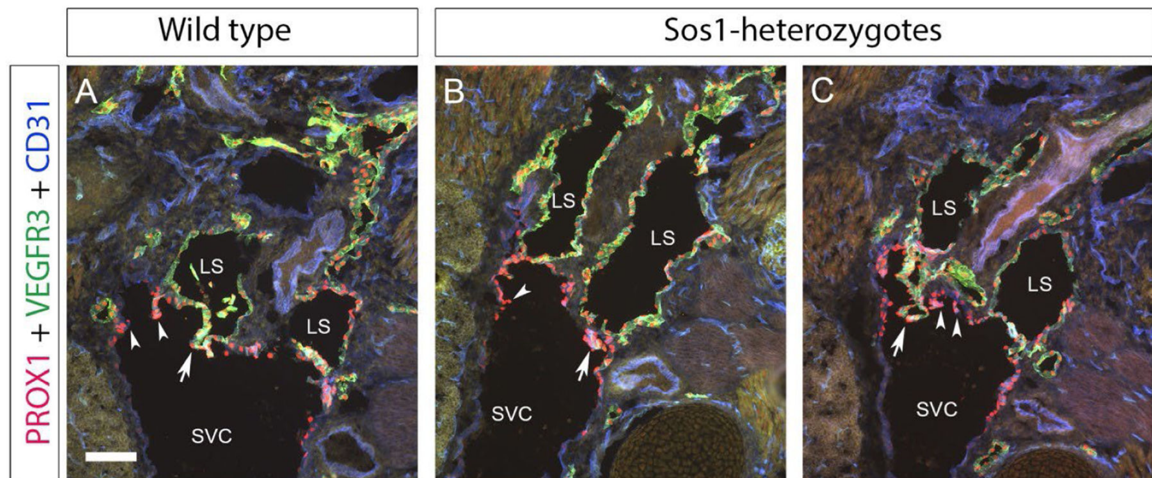

Fig 1:

Venous and lymphovenous valves are normal in Sos1$^{+/-}$ embryos. E16.5 Sos1$^{+/-}$ embryos were frontally sectioned and analyzed using the indicated antibodies. LVVs (arrow) and venous valves (arrowheads) appeared normal in the mutant samples. Abbreviations: SVC, superior vena cava; LS, lymph sacs. Measuring bar: 100 μm.

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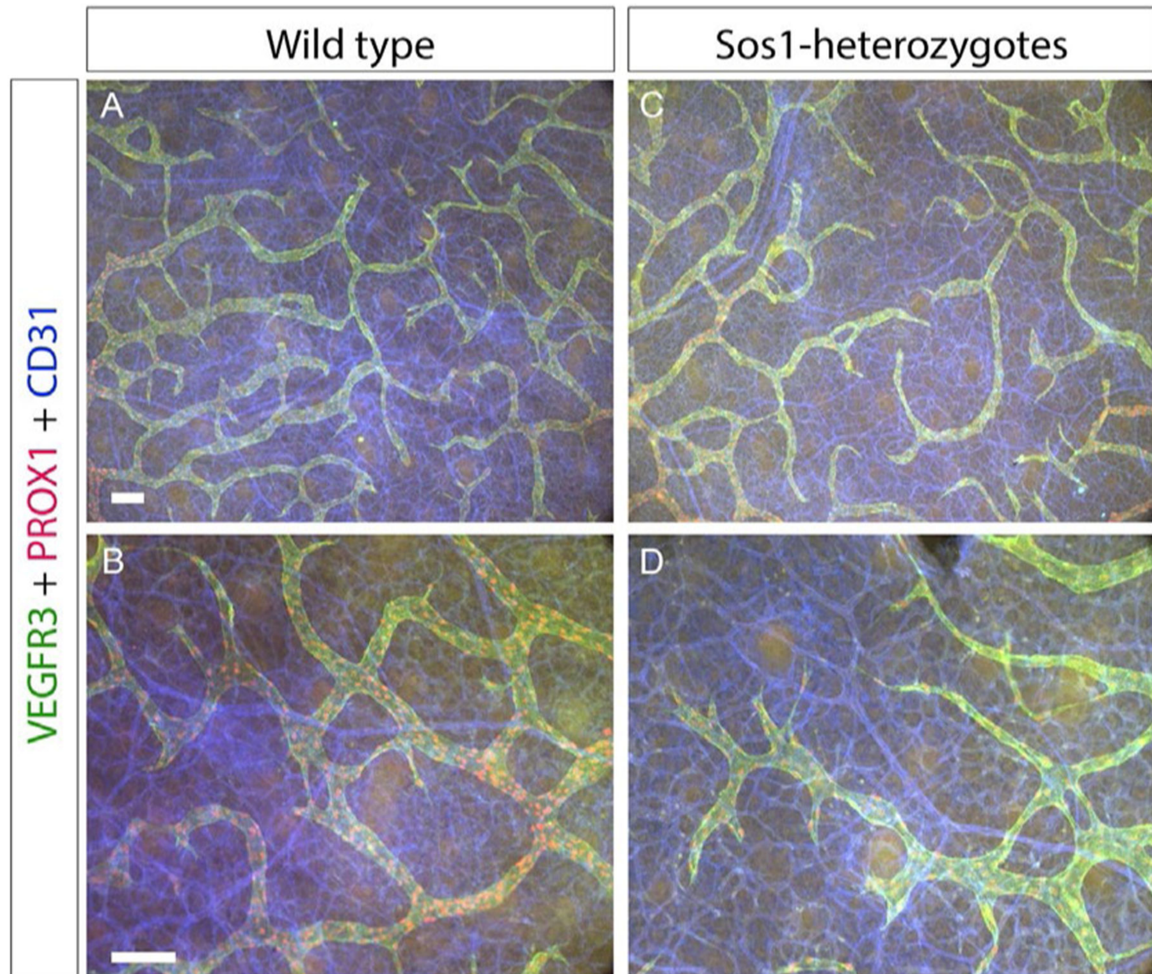

Fig 2:
Dermal lymphatic vessels of $Sos1^{+/-}$ embryos are normal. The dorsal skin of E16.5 wild type (A, B) and $Sos1^{+/-}$ (C, D) embryos was analyzed using the indicated antibodies. No striking defects were observed in the lymphatic vessels of mutant embryos. B and D are higher magnification pictures of the migration front from panels A and C respectively. Measuring bar: 100 μm.

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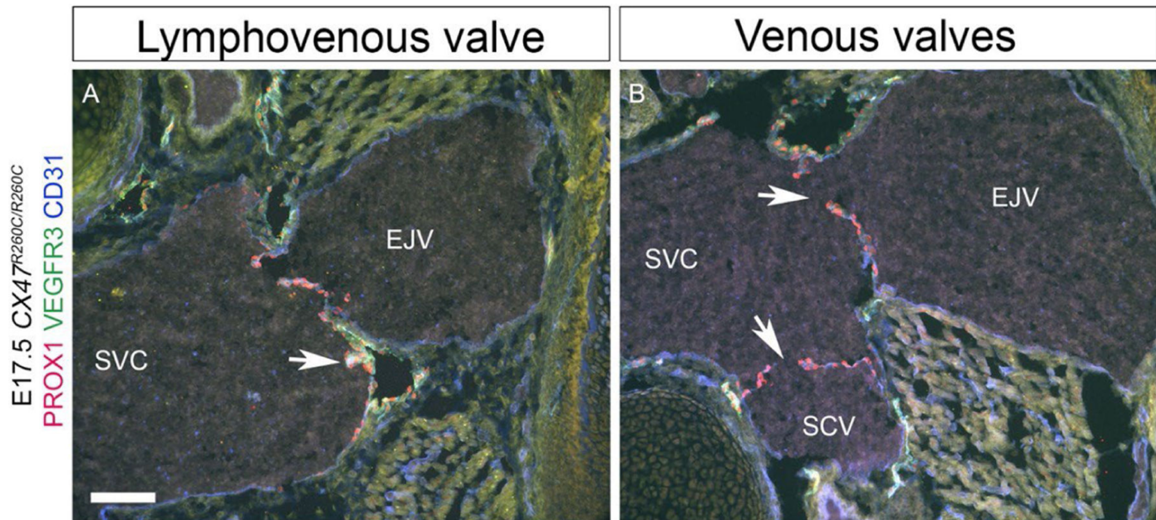

Fig 3:

Venous and lymphovenous valves are normal in Cx47$^{R206C/R206C}$ embryos. E17.5 Cx47$^{R206C/R206C}$ embryos were frontally sectioned and analyzed using the indicated antibodies. LVVs (A, arrow) and venous valves (B, arrows) appeared normal in the mutant samples. Abbreviations: SVC, superior vena cava; EJV, external jugular vein; SCV, subclavian vein. Measuring bar: 100 μm.

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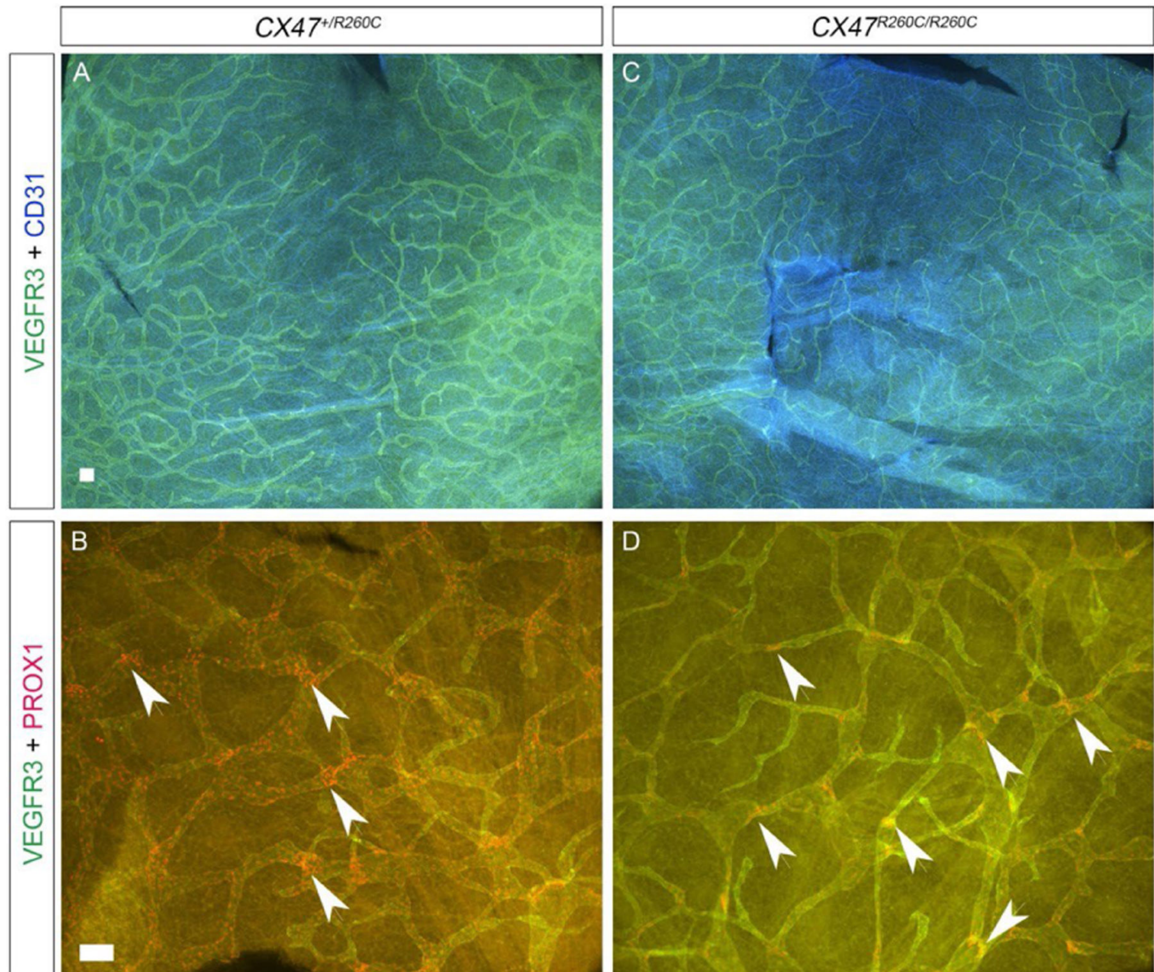

Fig 4:

Dermal lymphatic vessels and lymphatic valves are normal in $Cx47^{R206C/R206C}$ embryos. The dorsal skin of E17.5 $Cx47^{+/R206C}$ and $Cx47^{R206C/R206C}$ embryos were harvested and analyzed using the indicated antibodies. No striking defects were observed in the lymphatic vessels of embryos (A, C). The dermal lymphatic valves also appeared to be developing normally (B, D, arrows). Measuring bar: 100 μm.