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Endothelial cells differentiated from patient dermal fibroblast-derived induced pluripotent stem cells resemble vascular malformations of Port Wine Birthmark

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Conflicts of interest: None to declare.

Data availability: The processed bam files of RNA-seq are deposited into NIH SRA (Sequence Read Archive) (BioProject ID: PRJNA997591). The transcriptome data (raw counts and pseudo counts for each sample) are to be deposited into the NCBI GEO (Gene Expression Omnibus) (Accession number: GSE240770). Additional data can be found in the supporting material version of this study at bioRxiv (BIORXIV/2023/547408)

Ethics statement: The study (#1853132) was approved by the Institutional Review Board at the Prisma Health Midlands.

Dear Editor, Port-wine birthmark (PWB) is a congenital vascular malformation with an estimated prevalence of 0.1–0.3% per live births.[1] PWB lesions typically show the proliferation of endothelial cells (ECs) and smooth muscle cells, replication of basement membranes, disruption of vascular barriers, and the progressive dilatation of vasculature.[2-4] Pathologically, PWB ECs are differentiation-defective ECs.[2, 5] In this study, we generated PWB patient-derived-induced pluripotent stem cells (iPSCs) and differentiated them into ECs, followed by characterization of their vascular phenotypes.

The study (#1853132) was approved by the Institutional Review Board at the Prisma Health Midlands. One surgically excised nodular PWB lesion from one patient (Caucasian male, 46 years old) and one age and gender-matched de-identified surgically discarded normal skin tissue were collected for this study. The donor had very large lesions with nodular and hypertrophic PWB on back, chest, arm, and hand and received multiple rounds of PDL treatments prior to surgical procedures. The skin biopsies were collected for outgrowth of human dermal fibroblasts. The generation of iPSCs was performed using a CytoTune-iPS Sendai Reprogramming Kit (ThermoFisher, Waltham, MA). The iPSC colonies were selected, isolated, and propagated. The differentiation of iPSCs into ECs followed a previous report.[6] RNA-sequencing (RNA-seq) was performed on iPSCs, mesenchymal cells (MSCs), and ECs. The STAR, FeatureCounts, and edgeR were used for data analysis. For functional characterization of the intersecting gene sets, we used the ToppCluster

(https://pubmed.ncbi.nlm.nih.gov/20484371/), an extended version of ToppFun application of the ToppGene Suite.[7]

For *in vitro* capillary-like structure (CLS) formation assay, ECs (n=4 for each cell model, $4.5x10^4$ in 200 µl) was added into Geltrex-precoated wells. After CLS formation, the cells were fixed, and Images were acquired. The experimental procedures for *in vivo* xenograft assay in severe combined immune deficient (SCID) mice was approved by the University of South Carolina institutional animal care and use committee. We implanted iPSC-derived control and PWB ECs and MSCs (2×10^6 cells with a ratio of EC:MSC as 2:3) with Geltrex into the subcutaneous layer of the skin of SCID mice using a protocol previously reported.[8] Male SCID mice (Nude NIH-III) from Charles River were used. The animals were sanctified on day 10 post-injection. The plug-ins were removed, fixed, embedded, and sectioned.

About 12 - 14 days after the delivery of Yamanaka factors into hDFs, the formation of iPSC colonization was observed (Fig 1A). The iPSC colonies were confirmed using alkaline phosphate live staining (Fig 1B). Two iPSC lines from a human PWB lesion (#4221_3 and #4221_6) and three lines from normal skin (#52521_4, #52521_8 and #52521_9) were successfully expanded and maintained for more than 50 passages, respectively. These cells were further verified to express the iPSC biomarkers, Tra1-60, Nanog, Oct4, and Sox2 (Figs 1C-D). We next differentiated these iPSCs into ECs. Fully differentiated monolayer ECs from PWB iPSCs were verified using IF staining with antibodies recognizing specific biomarkers CD31 and CD144 (Figs 1E-F).

Next, we performed CLS formation on Geltrex *in vitro*. The CLS formed by PWB EC_4221 in Geltrex *in vitro* had larger perimeters and greater branches thickness than those formed by normal EC_52521 (Figs 1G-I). In xenograft assay, perfused PWB or normal vasculatures were formed in dermal implants ten days after xenografting, which comprised human ECs recognized by anti-human UEA1 antibody (Figs 1J-K). The vasculature formed by PWB ECs had larger perimeters and higher density than those formed by normal ECs; size distribution showed that the total percentage of perfused large vessels (perimeters > 100 µm) formed by PWB iPSC-derived ECs was significantly higher as compared to those formed by the control iPSC-derived ECs (41.2% vs 16.5%) (Figs 1L). This was consistent with data from patients' lesions in our previous report[2] and a xenografted animal model.[8] Last, we determined the differentially expressed genes (DEGs) in PWB iPSCs, MSCs, and ECs as compared with their control counterparts. Functional enrichment networks showed that differentially expressed genes (DEGs) in Hippo and Wnt pathways confer significant functions in vasculature development and

EC differentiation (Fig 1M). Other top significantly dysregulated pathways in PWB iPSCs and ECs included regulating pluripotency of stem cells, calcium signaling, gap junction, fatty acid biosynthesis, and vascular smooth muscle cell contraction, TNF, NF-kappa B, MAPK, cholesterol metabolism, TGF-beta, axon guidance, and focal adhesion (Figs 1N-O).

In conclusion, lesional iPSCs-derived ECs can resemble pathological vascular phenotypes of PWB. Our data demonstrate multiple pathways, such as Hippo and Wnt, NF-kappa B, TNF, MAPK, and cholesterol metabolism, are dysregulated. This data suggests new therapeutics to be developed for targeting such dysregulated pathways for treatment of PWB.

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Figure legend

Figure 1: Generation and functional characterization of PWB-derived iPSCs and their differentiated ECs. (A) Typical morphology of a control iPSC colony, ctl_iPSC_52521_8. (B) AP staining on the PWB iPSC 4221_3 colony. (C) PWB_iPSC_4221_6 colony expressing stem cell biomarkers Nanog and Tra1-60 (overlay in the image). (D) Stem cell biomarkers Sox2 and Oct4 (overlay in the image) were used to stain the control iPSCs. (E) fully differentiated monolayer ECs from PWB IPSCs were observed on day 8 during differential induction. (F) PWB iPSC-derived ECs were expressed EC membrane biomarkers CD31 (red) and CD144 (green) (overlay in the image). Nuclei were stained by DAPI. Yellow scale bar: 100 μm. (G) Control EC_52521_9 and (H) PWB EC_4221_3 formed CLS on Geltrex. (I) PWB EC_4221_3 showed impaired CLS *in vitro* with larger perimeters (p=5.09x10^{-32}) as compared to the control EC_52521_9. Whiskers: mean ± S.D.; Diamond box: interquartile range (IQR); Dotted curve: data distribution. A Mann-Whitney U test was used. (J-K) Formation of perfused human vasculature 10 days after intradermal xenograft of the control (J) and PWB (K) ECs with corresponding MSCs into SCID mice. Arrows: perfused blood vessels in xenografts comprising human ECs were confirmed using immunohistochemistry (IHC) by an anti-human UEA1 antibody. (L) Perimeter distribution of xenografted vasculature formed by PWB ECs compared to control vasculature. Pink dashed rectangle: the total percentage (41.2%) of perfused vessels formed by PWB iPSC-derived ECs as compared with 16.5% of perfused vessels formed by control iPSC-derived ECs with perimeters over 100 μm. (M) Functional interactive network of Hippo- and Wnt-related DEGs in PWB vasculature showing significant enrichment (FDR<0.05) for tube morphogenesis, endothelium and vasculature development, and EC differentiation. (N) and (O) KEGG enrichment analysis showing top significantly upregulated (N) and downregulated (O) pathways related to vascular differentiation and development in PWB iPSCs and ECs.

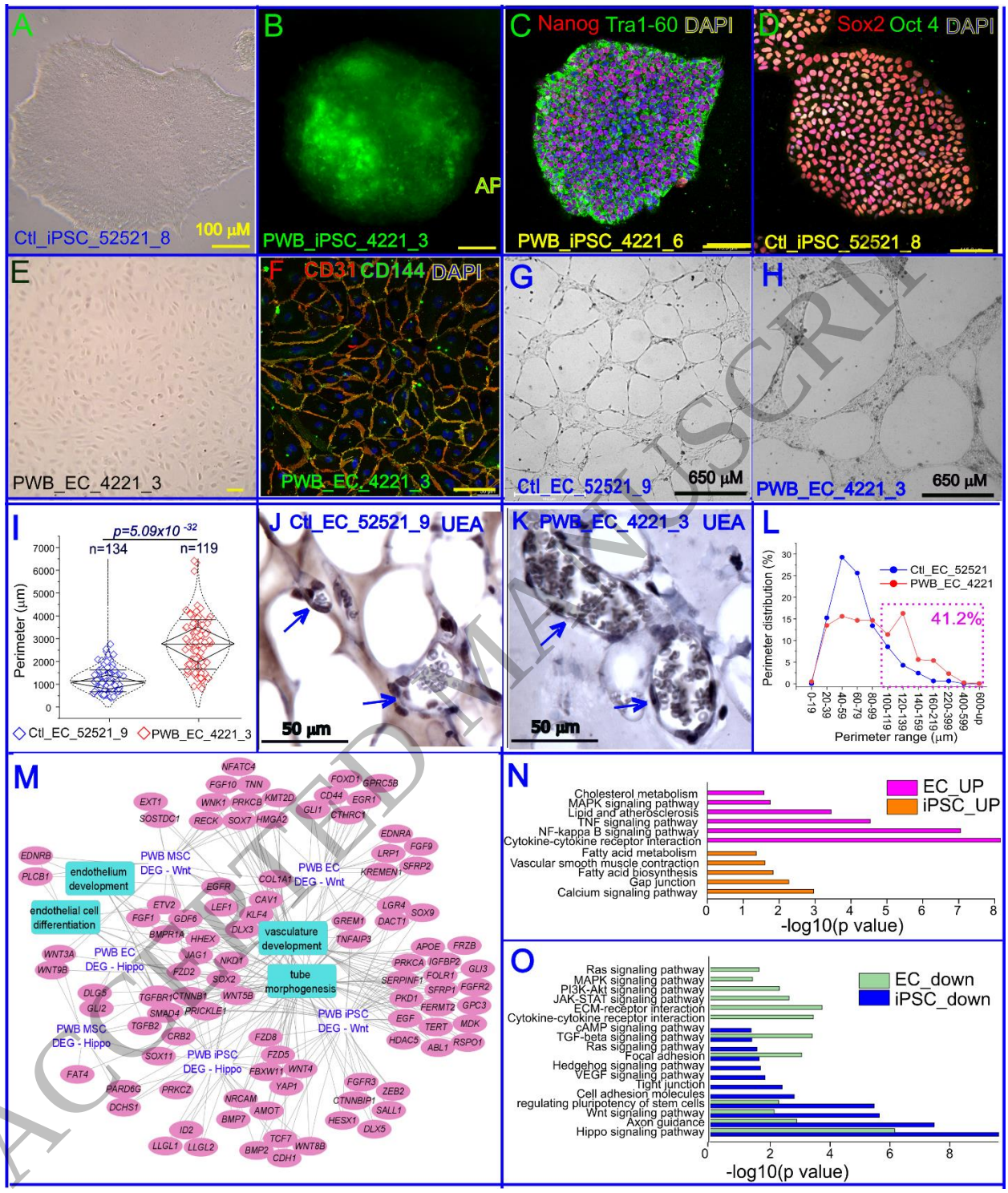

Figure 1
472x559 mm (x DPI)