TR&D 1: Rapid, Efficient and Cost-effective Cell Programming Platform

Goal: Current hiPSC differentiation protocols rely on an outside-in approach, where periodic application of external growth factors triggers intracellular pathways to control cellular differentiation. Growth factors are expensive. In addition, they lose bioactivity due to their fragile structures during cell culture. As cell differentiation is highly sensitive to the growth factor composition and concentration, the inevitable variation of growth factors leads to the production of mixed cell subtypes. It reduces the yield of producing desired cell types and increases the difficulty of cell purification. This project pioneers an intrinsic-in approach for rapid, cost-effective hiPSC programming by developing inducible transcription factor (TF) expression systems. This intrinsic-in approach leverages forward programming and forced TF expression to determine cell fate with high purity and speed. Additionally, this project will leverage state-of-the-art molecular engineering toolsets to target safe genomic sites with inducible expression to overcome bottlenecks in traditional protocols. Resultantly, an innovative approach will be developed to revolutionize large-scale hiPSC-derived beta cell production, paving the way for transformative therapeutic applications in diabetes treatment and beyond.

Published and Preliminary Data:

(1) Identification of SOXF factors enriched in hPSC-EPs.

To identify TFs enriched in the EP population differentiated from hPSCs, we performed single cell RNA-seq (scRNA-seq) analysis of day 5 differentiated cells (**Fig. 1A**). Dimensional reduction and supervised clustering of the 2000 cells showed five distinct clusters of cells (**Fig. 1B-C**). Among all the clusters, **cluster 1** separated further away from the other four clusters in the UMAP projection and showed increased expression of *PECAM1* (*CD31*), *CD34*, and *CDH5* (*VEC*), indicating their EP identity (**Fig 1D**). Inspection of differentially expressed TFs uncovered high expression of all three SOXF factors in the EPs (**Fig. 1E**). No other SOX factors were differentially expressed in cluster 1. To confirm SOXF expression in EPs, we used SOX17-

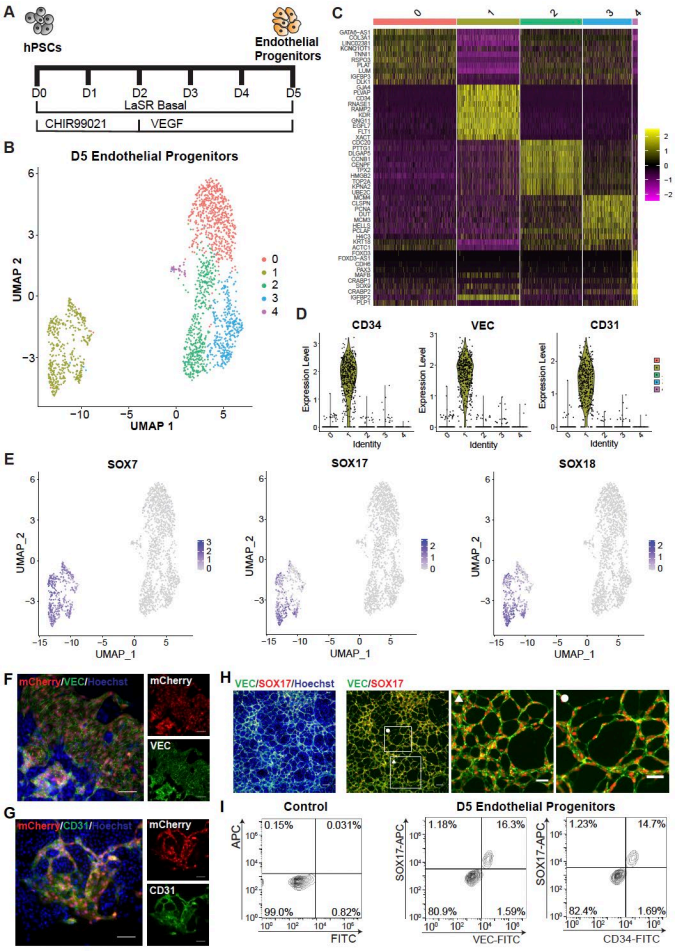

Fig 1. **SOXF factors expressed in hPSC-EP population. A)** Schematic of EP differentiation. **B)** UMAP dimensional reduction projection showing clustering of cells produced on day 5 (D5). **C)** Heat map indicating the top 10 variably expressed genes for each cluster. **D)** Violin plots identifying CD34, VEC, and CD31 expression in cluster 1. **E)** SOXF family member expression in D5 population. **F-G)** Immunofluorescence images of D5 EPs derived from SOX17-mCherry reporter cells. mCherry expression is seen in cells that express VEC **(F)** and CD31 **(G)**. Scale bars are 100 μm. **H)** Immunofluorescent images showing VEC and SOX17 co-expression in D5 cells differentiated from 6-9-9 cells. Left two scale bars are 100 μm and right two scale bars are 50 μm. **I)** Flow cytometry showing co-expression of SOX17 and VEC, SOX17 and CD34 in D5 cells.

mCherry knockin reporter H9 cells[58] and differentiated the knockin cells to EPs. We found that mCherry expression only occurred in cells also expressing CD31 and VEC (**Fig. 1F-G**). The co-expression of SOX17 with EP markers VEC and CD34 was also observed in EP differentiation of hiPSC 6-9-9 cells by immunostaining and flow cytometry (**Fig. 1H-I**). Furthermore, SOX17 in particular has been identified as a marker of arterial EPs in both mouse and human models[59–61]. These data provide strong evidence that EPs differentiated from hPSCs via WNT activation exhibited upregulated expression of SOXF factors, including SOX17.

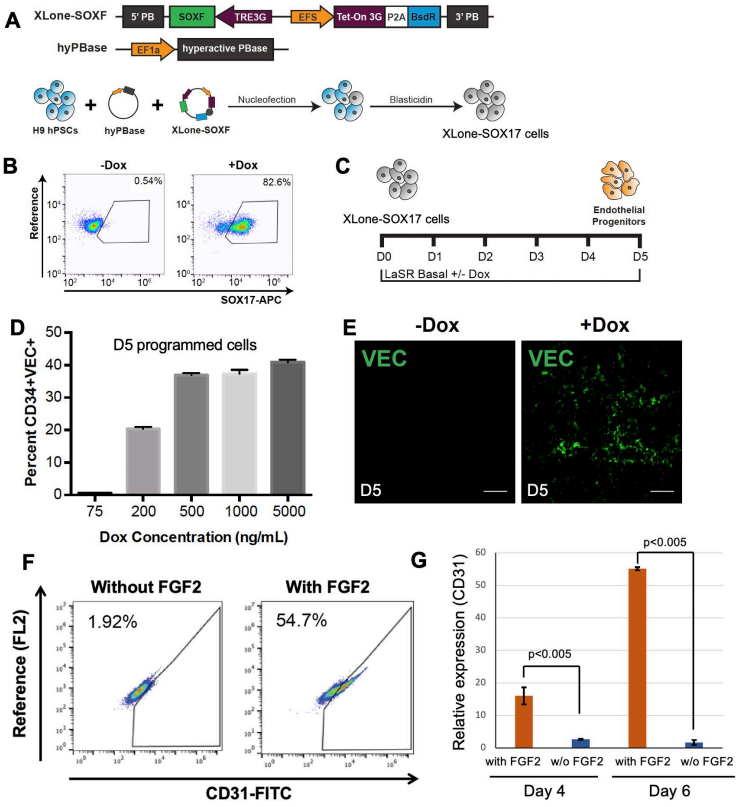

Fig 2. SOX17 is sufficient to program hPSCs into EPs in the presence of FGF2. A) Schematic of establishing XLone cell lines. **B)** XLone-SOX17 cells efficiently express SOX17 after Dox treatment. **C)** Schematic of SOX17 programming protocol. **D)** $CD34^{+}VEC^{+}$ cells evaluated on D5 after SOX17 programming. **E)** SOX17 programmed cells expressed VEC. **(F-G)** FGF2 enabled expression of CD31 in SOX17 programmed cells.

(2) SOX17 and FGF2 are sufficient for direct EP programming of hPSCs

Upon discovering all 3 SOXF factors are expressed in EPs, we sought to understand which, if any of these factors, can directly program hPSCs into EPs. To address this question, we generated cell lines with inducible overexpression of *SOX7*, *SOX17*, and *SOX18* by cloning each TF cDNA into our doxycycline (Dox) inducible, PiggyBac-based XLone construct[62] (**Fig. 2A**). This construct was then introduced into hPSCs, and cells successfully incorporating the construct were purified by drug selection (**Fig. 2A**). To ensure the desired function of resulting cells, XLone-SOX17 cells were treated with or without Dox for 24 hours, which revealed robust SOX17 expression in 82.6% of cells, with minimal leakage in cells without Dox treatment (**Fig. 2B**). This demonstrated that we could express gene of interest in hPSCs efficiently with XLone. Next, we treated XLone-SOX17 hPSCs with Dox to induce SOX17 expression to evaluate its ability to program hPSCs into EPs (**Fig. 2C**). We found that SOX17 can efficiently program hPSCs into $CD34^{+}VEC^{+}$ cells (**Fig. 2D,E**) and this programming is strictly dependent on Dox concentration. Furthermore, the differentiated $CD34^{+}VEC^{+}$ cells did not express CD31, unless cells were also treated with FGF2 during SOX17-programming (**Fig. 3F-G**).

(3) Dynamic expression of SOX17 during hPSC differentiation into EPs via Wnt activation.

To better understand the role of SOX17 during directed differentiation, we characterized SOX17 expression kinetics. We collected differentiated cells daily until day 3 and every six hours after day 3 until day 5. Western blot analysis showed SOX17 and VEC are first detected on day 3.75 (**Fig. 3A-B**). Immunofluorescent analysis revealed there are more cells expressing SOX17 alone on day 3.75, and this SOX17 single positive cell

number gradually decreases as cells become double positive for SOX17 and VEC over time (**Fig. 3C-D**). This data indicates expression of SOX17 occurred before the appearance of VEC^{+} cells. To further study **the role of SOX17 in EP differentiation**, we sought to perform a loss-of-function analysis using a Cas13d-meditated knockdown approach[67,68]. Cas13d can knock down RNA transcripts efficiently[69]. We cloned Cas13d into our XLone plasmid construct under the control of the inducible TRE3G promoter (**Fig. 3E**)[62]. We also cloned a U6 promoter expressing SOX17 gRNA targeting SOX17 cDNA region into the same plasmid to establish a single transposon system for SOX17 knockdown. We transfected this plasmid into H9 cells and used puromycin drug selection to purify the cells that integrated XLone-Cas13d system (**Fig. 3E**). EP differentiation of these SOX17 inducible knockdown cells with and without Dox treatment revealed reduced CD31, CD34 and VEC expression upon SOX17 knockdown (**Fig. 3F-G**). These results demonstrate that SOX17 is required for the EP differentiation from hPSCs.

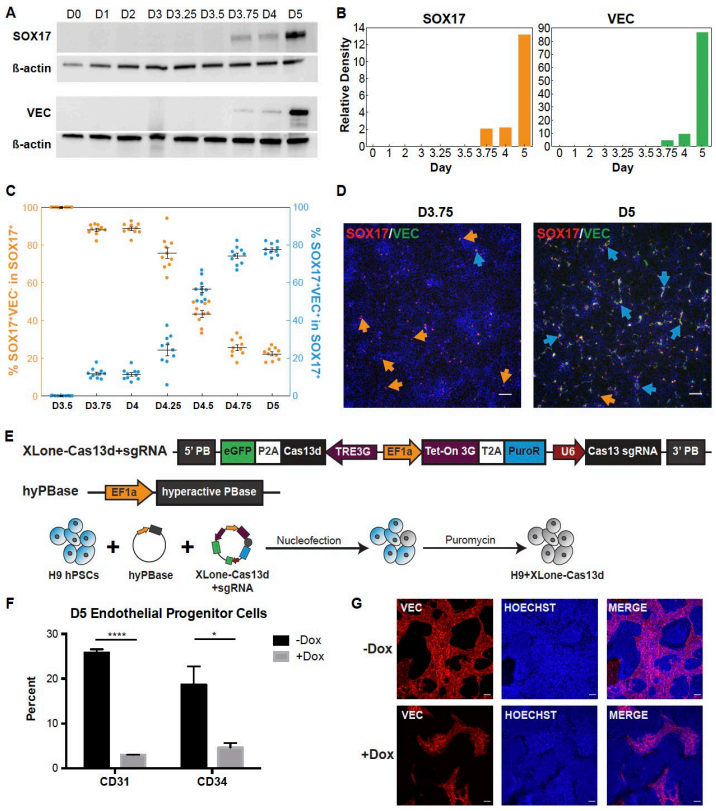

Fig 3. SOX17 is required for EP differentiation. **A**) Western blots showing SOX17 and VEC protein levels over the course of differentiation. **B**) Quantification of blots shown in (A) normalized to ß-actin. **C**) Quantification of SOX17+VEC- cells (left axis, orange) and SOX17+VEC+ cells (right axis, blue) in the SOX17+ population. **D**) Representative immunofluorescence images used for quantification in (C) for D3.75 and D5. Orange arrows highlight SOX17+VEC- cells and blue arrows highlight SOX17+VEC+ cells. Scale bars are 100 µm. E) Schematic illustrating the generation of a cell line with Cas13d-based inducible SOX17 knockdown. F) Quantification of flow cytometry experiments analyzing the change in size of the D5 population expressing CD31 or CD34 for cells treated with and without Dox (n=3). * $p < 0.05$ and **** $p < 0.0001$. G) Immunofluorescence images of D5 cells differentiated with or without Dox stained with VEC. Scale bars are 100 µm.