Development of Methods to Enhance Stem Cell Derived Islet Survival

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Development of Methods to Enhance Stem Cell Derived Islet Survival

by

Aining Fan

A thesis presented to
the McKelvey School of Engineering
of Washington University in
partial fulfillment of the
requirements for the degree
of Master of Science

May 2023

St. Louis, Missouri
# Table of Contents

List of Figures ........................................................................................................................................ iv

List of Tables ........................................................................................................................................ v

Acknowledgments .................................................................................................................................. vi

Abstract .................................................................................................................................................. vii

Chapter 1: Introduction and Background ............................................................................................................. 1

1.1 The Impact of Hypoxia on Cellular Function ................................................................................................. 1

1.1.1 The Association Between Vascular Formation and Cell Survival ................................................................. 3

1.1.2 Tissue Geometry Impacts Cell Function ........................................................................................................ 4

1.2 Diabetes and Stem Cell Derived β Cell ............................................................................................................ 6

1.2.1 The 6-Stage differentiation of Stem Cell derived Islet .................................................................................. 7

1.2.2 Hypoxia Negatively Impact β Cell Function .................................................................................................. 9

Chapter 2: Endothelial Cell Generation from Pluripotent Stem Cells ........................................................................ 11

2.1 Introduction .................................................................................................................................................. 11

2.2 Material and Methods .................................................................................................................................. 13

2.2.1 CRISPRa induction and sgRNA generation ................................................................................................. 13

2.2.2 Cell Culture for SC-EC differentiation ....................................................................................................... 13

2.3 Results ....................................................................................................................................................... 16

2.3.1 Validation of CRISPRa System ................................................................................................................... 16

2.3.2 ETV2 Promotes Endothelial Differentiation ............................................................................................... 18

2.4 Discussion ................................................................................................................................................ 20

Chapter 3: “Pancake” Islet Development ............................................................................................................. 22

3.1 Introduction ................................................................................................................................................ 22

3.2 Material and Methods .................................................................................................................................. 22

3.2.1 Oxygen Diffusion Modeling ...................................................................................................................... 22

3.2.2 Pancake islets culture ............................................................................................................................... 25

3.2.3 Pancake functional assays ....................................................................................................................... 26

3.3 Results ....................................................................................................................................................... 27

3.3.1 Pancake islets show better GSIS performance .......................................................................................... 27
3.3.2 Gene and protein expression of pancake islets .......................................................... 28
3.4 Discussion ....................................................................................................................... 30
References ............................................................................................................................ 33
List of Figures

Figure 1.1: Schematic diagram of HIF-1α pathway ................................................................. 2
Figure 1.2: Key processes of angiogenesis ................................................................................. 4
Figure 1.3: The protocol of stem cell-derived β cell differentiation ........................................... 9
Figure 2.1: Direct and 2-stage differentiation approaches to generated SC-ECs...................... 14
Figure 2.2: Validation of CRIPSRα system.................................................................................. 17
Figure 2.3: Temporal changes of ETV2 and PECAM1 expression during direct differentiation 19
Figure 2.4: Temporal change ETV2 and PECAM1 expression during 2-stage differentiation... 19
Figure 3.1: Oxygen diffusion modeling of SC-islet aggregate and pancake islet....................... 24
Figure 3.2: Working principle of Nunc UpCell surface from Thermo Fisher Scientific™...........26
Figure 3.3: Static and dynamic GSIS of Pancake and control group........................................... 27
Figure 3.4: Pancake gene expression.......................................................................................... 28
Figure 3.5: Immunostaining stained for C-peptide, glucagon (GCG), NKX6-1, chromogranin A (CHGA) and somatostatin (SST).................................................................................. 29
List of Tables

Table 2.1 Designed gRNAs targeting ETV2, with sequences and tools ........................................13
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Washington University in St. Louis

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ABSTRACT OF THE THESIS

Development of Methods of Enhance Stem Cell Derived Islet Survival

by

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Master of Science in Biomedical Engineering

Washington University in St. Louis, 2023

Professor Jeffrey Millman, Chair

Hypoxia in tissue is a condition that oxygen is lacking. The oxygen consumption of cells is closely associated with their function and fate. The human pluripotent stem cell-derived islets, a promising cell source for diabetes treatment, suffer from hypoxia after transplantation. To alleviate hypoxia and enhance cell functions, we developed two platforms as potential strategies, i) Endothelial generation from pluripotent stem cells through CRISPR activation of ETV2; ii) Stem cell-derived islets geometry alternation into a planar shape. The doxycycline-inducible CRISPR activation system was validated, and the endothelial markers gene was upregulated up to 4000 times fold, compared to that of stem cells. The "pancake" islets also displayed advanced insulin production under low and high glucose stimulation. Further assessments are necessary to validate their effects on hypoxia and organoid functions.
Chapter 1: Introduction and Background

1.1 The Impact of Hypoxia on Cellular Function

Oxygen plays an important role in ensuring tissue functioning and supporting biological activities. After being uptaken by the lungs, oxygen diffuses into the bloodstream and binds to hemoglobin, a protein in red cells. The oxygen-rich blood reaches the tissues through the complicated branching networks of blood vessels. The oxygen delivery is demand-dependent, and the oxygen take-up is controlled by the metabolic rate of cells. Normal healthy human tissues exhibit a wide range of oxygen supply and demands. For example, the oxygen consumption (mL O$_2$/min per 100g) for the brain is 5, much higher compared to 0.2 for the skin (Pittman, 2011).

Hypoxia, a condition characterized by a lack of oxygen supply to tissues, can elicit a cellular and molecular effect on different levels. Hypoxia takes place in many pathological conditions and leads to impaired ability of cells to generate ATP through oxidation phosphorylation. In hypoxia conditions, hypoxia-inducible transcription factors (HIF) upregulate hypoxia-responsive genes and change cellular metabolism and cell fate. The HIF-1α pathway leads to cell adaption in low oxygen concentrations, where HIF-1α transcription plays an essential role in the process, which is summarized in Figure 1.1.

The HIF pathway consists of two subunits, HIF-α and HIF-β, that dimerize to form the active transcription factor (Akhtar et al., 2014). Under normoxic conditions, HIF-1α is hydroxylated by the prolyl hydroxylates (PHD) enzymes and results in proteasomal degradation of HIF through
ubiquitination of HIF-1α. In hypoxic conditions, HIF-α is accumulated through the inhibition of PHD and FIH (factor inhibiting HIF). The HIF-α is then translocated to the nucleus and regulates the expression of genes involved in angiogenesis, glucose metabolism, and cell survival. The HIF pathway is involved in various physiological processes. Although many cell types can endure low oxygen concentration by reducing metabolic rates and adapting to hypoxia conditions, anoxia or extreme hypoxia conditions (O$_2$ <0.1% ) turn on the cellular intrinsic apoptosis pathway (Lenihan & Taylor, 2013). Hypoxia also impacts the expression of other transcriptional factors involved in apoptosis determination, such as p53 and NF-κB. In general, hypoxia is an important aspect in numerous pathological processes, such as tumorigenesis, inflammation, and cardiovascular diseases(Lenihan & Taylor, 2013).
Figure 1.1 Schematic diagram of HIF-1α pathway. The HIF-1α expression has an exponential negative correlation with oxygen concentration. Figure adapted from (Akhtar et al., 2014)

1.1.1 The Association Between Vascular Formation and Cell Survival

The blood delivers oxygen and nutrients to the tissues and removes wastes through the circulatory system. As stated, tissues vary widely in their oxygen sensitivity. The neurological cells survive for a few minutes without a blood supply, while the vascular smooth muscle can survive for 24-72 hours under the same condition (Leach & Treacher, 1998). A primary therapeutic goal is to maintain the blood supply to the organs with high oxygen demands, especially the grafts that experience extreme hypoxia after being transplanted before the process of the surrounding vascularization begins. To achieve this goal, it is essential to perceive the mechanism of angiogenesis and the factors that influence the process.

The five discrete steps involved in angiogenesis are depicted in Figure 1.2. First, angiogenesis factors, mainly vascular endothelial growth factor (VEGF-A), bind to endothelial cell receptors and activate the signal transduction pathways and endothelial cell growth. The tumor microenvironment usually induces abnormal angiogenesis, as a disproportionately large amount of pro-angiogenesis factors is produced from tumor cells and immune cells (Yoo & Kwon, 2013). Hypoxia and inflammation elevate the expression of VEGF-A, and continued tumor growth is promoted in this way. Second, the pre-existing vasculature is destabilized and allows the sprouting of new vessels. The angiopoietin (Ang-1), a ligand secreted by endothelial cells and pericytes, induces signaling of tyrosine kinase receptor Tie-2 and promotes endothelial cell survival via PI3K pathway. Third, the endothelial cells start to form sprouts extended from pre-
existing vessels via the activation of Notch signaling. Notch signaling plays a complicated role in regulating angiogenesis. It elevates the expression of VEGF and enhances the survival of endothelial cells. However, improper enhancement of DLL4-induced Notch signaling also inhibits tip cell formation and angiogenic sprouting (Kofler et al., 2011). Then, a denser vasculature is formed through migration, survival, and proliferation of endothelial cells, contributed by factors such as bFGF, Ang-1, and VEGF-A. Integrin expressed by endothelial cells facilitates their migration for tube formation, leading to the eventual maturation and stabilization of new vessels.

Figure 1.2 Key processes of angiogenesis. All the events arise in both normal vessel growth and tumorous environments. Figure extracted from (Hall & Ran, 2010)

1.1.2 Tissue Geometry Impacts Cell Function

The shape and organization of tissue are one of the leading factors that determine cellular behavior, function, and communication. For example, the villi and microvilli located in the small
intestine increase the surface area for the absorption of nutrients. The finger-like villi protrude from the inner intestinal walls, covered with column-shaped epithelial cells and thousands of tightly packed microvilli. The brush border formed by villi and microvilli increases the efficiency of nutrient diffusion and protects against bacterial infection (McConnell et al., 2009). On the other hand, the epithelium locates at the inner surface of vessels constitutes the thin, small, flat cells from a semipermeable membrane, allowing selective transport of substances. The geometry also impacts cell viability and tissue growth. For example, the spatial organization of the fibrosis and mesenchymal compartment plays a crucial role in liver regeneration after injury (Aloia, 2022). The distinct groups of mesenchymal and epithelial cells are identified in the periportal and pericentral locations in the liver lobule and exhibit different molecular signatures. These subpopulations of the cells activate the epigenetic mechanisms to different extents, which are crucial in regulating cell fate and impacting regeneration efficiency. The spatial location of the hepatic stellate cells affects the amount of fibrosis that is generated prevalently in the lobule, executing either a pro-regenerative effect or a detrimental effect on the injured liver. In terms of oxygen uptake, the tissue, and cellular shape is essential in ensuring oxygen uptake and transportation. The alveolus in the lungs is surrounded by a rich network of capillaries. Their thin, flat walls that are only one cell thick allow short diffusion distance of oxygen. Then, the flattened-disk shape red blood cell rapidly picks up oxygen through its broad surface area and transports the oxygen throughout the body. Together, these findings raise the possibility of alleviating hypoxia conditions by altering cellular or tissue geometry. The design of cellular shape and the spatial location of cell groups may change the oxygen diffusion rates and the regulation profile of HIF.
1.2 Diabetes and Stem Cell-Derived β Cell

Diabetes is a chronic metabolic disorder characterized by abnormally high blood glucose levels. Glucose is generated through the breakdown of food by acids and enzymes in the stomach. The intestine absorbs it and gets into the bloodstream. In a healthy body, the hormone insulin is produced by functional pancreatic β cells and then released from the pancreas islet, removing the glucose from the blood for energy use and storage. In people with diabetes, insulin can not be produced appropriately to reduce glucose to normal levels. The sustaining high level of glucose in the blood may cause long-term damage to the nerve, kidneys, skin, heart, and blood vessels (Dean & McEntyre, 2004).

There are two main types of diabetes. Type 1 diabetes is caused by the autoimmune destruction of pancreatic β cells through a humoral and inflammatory response. Type 1 diabetes often leads to absolute insulin deficiency, and the underlying reason for the autoimmune attack is unknown. Type 1 diabetes usually occurs early, and accounts for 80%-90% of diabetes in children and adolescents, and around 10% of the general population is affected by diabetes (Kharroubi & Darwish, 2015). Type 2 diabetes is a metabolic disorder in which the body becomes resistant to insulin, resulting in β cell dysfunction and a progressive loss of insulin secretion. It accounts for around 90% of the diabetes population and occurs predominantly after 40 years of age. Diabetes has turned into an epidemic with increasing prevalence worldwide. In 2021, 10.5% (536 million) of people between 20-79 years old had diabetes, and the number is estimated to rise to 12.2% (732 million) in 2045 (Sun et al., 2022).
Current treatments for type 1 diabetes include exogenous insulin injections and cadaveric donor islet transplantation. The peripheral administration of insulin can induce iatrogenic hyperinsulinemia that causes short- and long-term unfavorable effects (Schwartz et al., 2017). The off-target effect of insulin causes acute asymptomatic hypoglycemia, which occurs in nearly 50% of patients. Other consequences of hyperinsulinemia include endothelial dysfunction, chronic inflammation, and even cancer. Subcutaneous insulin delivery, as the most common route of insulin administration, is associated with injection pain, lipodystrophy, and peripheral hyperinsulinemia (Shah et al., 2016).

On the other hand, the donor islet transplant suffers from the limited supply of cadaveric donors and the implications of immunosuppression therapy. The immunosuppression-related events include adverse drug reactions, cytopenia, abdominal pain, and renal dysfunction. In a group of 65 patients who received an islet transplant, only 10% maintained insulin independence with a median duration of 15 months (Edmond et al., 2005). Although C-peptide secretion was maintained for up to 5 years, most patients resumed insulin therapy. The islet transplantation improves insulin stability and relieves hypoglycemia but is compromised with toxic side effects and limited preservation of function. Considering the unfavorable conditions developed in conventional treatments, it is essential to develop novel treatments that reduce toxic immunosuppression, improve patient compliance, and protection of long-term insulin provision.

1.2.1 The 6-Stage Differentiation of Stem Cell-derived Islet

Stem cells are unspecialized cells and can differentiate into any cell of an organism in the human body. They work as a renewal and repair system for damaged cells or injured organisms. Stem
cells can be divided between embryonic stem cells and adult stem cells. Adult stem cells are
tissue-specific stem cells, whereas embryonic stem cells can give rise to all types of cells in the
body. The pluripotent embryonic stem cells are derived from the inner cell mass of a blastocyst
and can form cells of all germ layers: ectoderm, mesoderm, and endoderm. The endoderm forms
linings of respiratory and gastrointestinal tracts, including pancreatic β cells (Zakrzewski et al.,
2019). In the past decade, the idea of inducing differentiation of human pluripotent stem cell
(hPSC) into pancreatic β cell have been implemented and brought up promising outcomes.
A breakthrough occurred in 2014 when stem cell (SC) - derived β cells were developed *in vitro*
and demonstrated the ability to secrete insulin in response to glucose change after mice
transplantation (Pagliuca et al., 2014). The protocol was further revised for more efficient
differentiation of highly functional β cells. The protocol is displayed in Figure 1.3. The process
includes six stages: definitive endoderm, primitive gut tube, pancreatic progenitors, endocrine
and SC-β cells. The cell fate is stepwise narrowed and eventually targeted to final β cell
differentiation. The process is navigated by the application of growth factors and small
molecules.

The generated SC-β cells are capable of reversing severe diabetes in mice within two weeks,
similar to the rate of primary human islets (Hogrebe et al., 2021). However, the SC islets are less
mature compared to adult islets in terms of both insulin secretion function and the expression of
important maturation genes. With more matured techniques and scaled-up manufacture, the SC-
islets can generate from iPSC reprogrammed from patient tissue and provide an autologous
replacement for patients with diabetes in the future (Maxwell & Millman, 2021). It might
potentially provide unlimited cell sources, as well as avoid the adverse effects of
immunosuppressive drugs and exogenous insulin take-up.
1.2.2 Hypoxia Negatively Impacts β Cell Function

The transplanted SC-β cells have exhibited assembling to the primary human islet, as well as a notable increase in expression of maturation genes, such as MAFA and G6PC2, compared to stage 6 SC-β cells (Augsornworawat et al., 2020). However, the transplanted clusters suffer from cell loss and functional impairment resulting from hypoxia due to the inevitable delay of
vascularization on site after transplantation. The depletion of oxygen is even more intensified because of the sphere shape of islets, making it difficult for oxygen diffuse toward the core. The pancreatic β cells are highly metabolically active and have an elevated oxygen consumption rate compared to other cell types. It is reported that over half of transplanted islets do not successfully engraft after transplantation (Komatsu et al., 2017). Therefore, it is essential to develop methods that alleviate hypoxia and helps to preserve SC-β cell viability and function. The goal of this study is to develop innovative technological approaches and platforms that can increase the oxygen exposure of SC-islets, with the potential to mitigate hypoxia and enhance the survival of islets in vivo. Our investigation will explore pathways for promoting angiogenesis and altering organoid geometry as potential strategies for achieving this goal.
Chapter 2: Endothelial Cell Generation from Pluripotent Stem Cells

2.1 Introduction

In 2013, the RNA-guided CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) – Cas nuclease system was developed to facilitate efficient genome engineering. The CRISPR system consists of two components: the CRISPR-associated (Cas) nuclease protein and RNA molecules that guide the Cas protein to a specific site in the DNA. Cas9 induces DNA double-strand break directly upstream of PAM, which is about 2-6 nucleotides downstream of the targeted DNA sequence. An exogenous repair template can be introduced to allow for high fidelity and precise editing. CRISPR activation (CRISPRa) system uses a modified Cas protein fused with a transcriptional activator to activate gene expression by binding to the DNA and initiating the process of transcription instead of cutting DNA. The function diagram is displayed in Figure 2.1 (B). Doxycycline (DOX) is an antibiotic that can facilitate gene upregulation by binding to Ted-Off protein and allowing binding released transcriptional activator to bind with the target gene.

As previously mentioned, SC-islets are a promising unlimited, and cost-effective source of insulin-producing cells for cell transplantation therapy. However, SC-islets completely lack intra-islet vasculature, which is essential for proper nutrient supply and function. Further, simultaneously differentiating hPSCs into SC-islets and endothelial cells is challenging due to mutually exclusive culture conditions. The major vasculature component is endothelial cells, which form a single layer that lines the blood vessel and plays an important role in regulating
surrounding tissue growth (Albert B et al., 2002). The ETV2 gene is an essential transcription factor for the development of endothelial lineage. ETV2 was found to be a pioneer factor in regulating endothelial cell reprogramming (Gong et al., 2022). ETV2 binds the nucleosomal DNA and targets the close chromatin domains independent of BRG1, an ATP-dependent chromatin factor regulating the chromatin state and DNA accessibility to transcription factors. BRG1 is then recruited to relax chromatin and recruit other co-factors in endothelial lineage specification. Wang et al. developed a protocol for hPSC-derived Endothelial cells, in which the generated endothelial cell to hPSC ratio was around 70-fold after three weeks of culture, with the use of chemically modified mRNA (Kai Wang et al.,2020). The protocol consisted of stage 1 (mesodermal induction) and stage 2 (endothelial induction), each lasting for 48 days. The early ETV2 activation in stage 1 was found critical in vascular function, including cell migration, angiogenesis, and muscle proliferation.

The modRNA gene editing technique used in the above study alters gene expression by delivering modified mRNA into nucleotides sequence and promoting the translation of the mRNA molecule into protein (Roundtree et al., 2017). The CRISPR system, on the other hand, directly introduces specific changes into the DNA sequence instead of chemically modifying the mRNA molecule after transcription. CRISPR permanently edits the DNA and might potentially generate a more stable gene expression. Because of the advanced performance of the CRISPR technique in accuracy, efficiency, quickness, and ease of implementation, we wondered whether higher Endothelium differentiation efficiency would be obtained by the CRISPR activation system. Here, we utilized a DOX-inducible CRISPRa system to generate hPSC-derived endothelial cells (SC-ECs) via activation of ETV2. Ultimately, this work aims to provide an approach for developing vascularized SC-islets for Type 1 Diabetes cell replacement therapy.
2.2 Material and Methods

2.2.1 CRISPRa induction and sgRNA generation

Stable CRISPRa H1 hPSCs, expressing dead Cas9 (dCAS9) fused to the VPR(VP65-p65-Rta) activation domain and an EGFP reporter at the AAVS1 safe-harbor locus, were cultured on Matrigel-coated flasks. ETV2 guide RNAs were designed and introduced via lentiviral transduction. The sources of single-guide RNA are summarized in Table 2.1.

Table 2.1 Designed gRNAs were targeting ETV2 with sequences and tools.

<table>
<thead>
<tr>
<th>sgRNA</th>
<th>Sequence</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>ETV2.1</td>
<td>GAGCCTACAAGTGCTTCTAC</td>
<td>Kai Wang et al</td>
</tr>
<tr>
<td>ETV2.2</td>
<td>ATTGTGACGTACGCTGACGCTGG</td>
<td>CHOPCHOP Sequence Rank 1 <a href="http://chopchop.cbu.uib.no/">http://chopchop.cbu.uib.no/</a></td>
</tr>
<tr>
<td>ETV2.3</td>
<td>GAAATGGTACAGTCCGTGCG</td>
<td>BROAD Sequence Rank 1 <a href="https://portals.broadinstitute.org/gppx/crispick/public">https://portals.broadinstitute.org/gppx/crispick/public</a></td>
</tr>
<tr>
<td>ETV2.4</td>
<td>CAATCGGCAGTACACTCGGGGG</td>
<td>CHOPCHOP Sequence Rank 2</td>
</tr>
<tr>
<td>ETV2.5</td>
<td>CCCGACGGCGATACCTACTG</td>
<td>BROAD Sequence Rank 2</td>
</tr>
</tbody>
</table>

2.2.2 Cell Culture for SC-EC Differentiation

To generate SC-ECs, we tested two approaches: direct differentiation and 2-Stage differentiation (Figure 2.1 A). For direct differentiation, hPSCs were treated with doxycycline (DOX), vascular
endothelial growth factor (VEGF), and ALK4 inhibitor (SB431542) for six days. The 2-Stage approach consisted of 48-hour DOX treatment (starting from 24-hour and 48-hour) and 48-hour exposure to CHIR99021 to specify the mesoderm germ layer, followed by 48-hour or 96-hour treatment with VEGF and SB431542 to induce endothelial differentiation. Differentiation efficiency was quantified by quantitative reverse transcription PCR (qRT-PCR). Statistical significance was determined by one-way ANOVA with multiple comparisons in PRISM8. All works were done in accordance with the policy declared by Embryonic Stem Cell Research Oversight (ESCRO) committee.
Figure 2.1 (A) Direct and 2-stage differentiation approaches to generated SC-ECs (B) CRISPRa function diagram. The system includes dCas9 fused to activator proteins (VPR), and a gRNA targets the region immediately upstream of transcription factors. In this study, Green Fluorescent Protein (GFP) is fused to dCas9. Figure adapted from (Crisprmod crispra synthetic crrna) (C) Structure of GFP. GFP contains chromophores comprising three amino acids: serine, tyrosin, and glycine. Figure extracted from (Piston et al.) (D) GFP allows visualization of the genomic locus of CRISPR complex in living cells. The expression of GFP is led by the activation of the promoter upstream of the GFP gene and emits green fluorescence when excited by a specific wavelength (488 nm). Figure extracted from (Chen et al., 2013)
2.3 Results

2.3.1 Validation of CRISPRa System

dCas9-VPR H1 hPSCs transduced with gRNA were treated with or without 5µg/mL DOX. The DOX and control samples were both imaged under fluorescent microscopy after 24-hour and 48-hour treatment. The DOX group shows strong fluorescent signals, compared to the dark appearance of the control group on the image. The flow cytometry analysis is coherent with fluorescent observation. The 48-hour DOX-treated H1 hPSCs exhibit 99% positive signals and have <2% signal overlap with the control group. The results suggest a successful induction of the DOX-inducible CRISPRa system. The gRNA is validated by RT-qPCR analysis. H1 hPSCs transduced with ETV2, and ETV4 had ETV2 gene upregulation to over 20 expressions fold change, indicating a comparably precise DNA site specification of ETV2 targeting. Therefore, H1 hPSCs transduced with ETV2, ETV4, and ETV5 gRNA were propagated for further EC differentiation.
Figure 2.2 (A) Fluorescent microscopy images demonstrating expression of GFP in dCas9-VPR H1 hPSCs transduced with gRNA ETV2.3 and imaged 24hr and 48hr treatment with 5µg/mL.
DOX. (B) Flow cytometry analysis of GFP fluorescence in dCas9-VPR H1 hPSCs treated with and without 5µg/mL DOX for 48hrs. (C) gRNA validation manifested by ETV2 gene up-regulation. Cell collected 24hr after DOX treatment.

2.3.2 ETV2 Promotes Endothelial Differentiation

During the 6-day-direct differentiation, the ETV2.5 transduced cells were collected every 24 hours and analyzed for gene expression of ETV2 and the endothelial cell marker PECAM1 (or CD31). As shown in Figure 2.3 A, both ETV2 and PECAM1 genes are upregulated at around 40-fold expression change at day 6, compared with H1 hPSC at day 0. It demonstrated a gene-upregulation-promoting effect of DOX treatment in EC differentiation in combination with VEGF and SB431542.

The RT-qPCR analysis of 2-stage differentiation in Figure 2.3 B shows a notably promoted ETV2 and PECAM1 expression compared to that of direct differentiation. The robust expression of MIXL at stage 1 suggests a certain mesoderm specification. At the end of stage 2, the ETV2 and PECAM2 are shown to reach 250–450-hundred-fold upregulation. Interestingly, DOX treated within stage 2 shows a higher capability in promoting EC differentiation efficiency.

Lastly, we sought to determine if differentiation could be further improved if stage 2 is prolonged to 4 days with DOX treatment. As displayed in Figure 2.4, the PECAM1 was shown to be upregulated to 3922-fold higher than that of H1 hPSCs, indicating a high efficiency of EC derivation.
Figure 2.3 (A) Temporal changes of ETV2 and PECAM1 expression during direct differentiation. (B) Comparison between control (no DOX) and DOX-treated groups. RNA was collected from cells for qPCR at the end of the Mesoderm Induction Stage (Day 2) and Endothelial Specification Stage (Day 4). Decreased expression of MIXL, a mesoderm marker, and increased expression of ETV2 and PECAM1 indicate differentiation towards an endothelial lineage. The presence of DOX is shown to promote differentiation dramatically.
Figure 2.4 Temporal change ETV2 and PECAM1 expression during 2-stage differentiation. A One-Way ANOVA with Dunnett’s multiple comparison test was used for the statistical analysis. ns: P>0.05; **:P<0.01; ***: P<0.001.

2.4 Discussion

In this study, we have successfully validated a DOX-inducible CRIPSRa system targeting ETV2 and demonstrated the ability to generate SC-ECs from hPSCs. We also found DOX treatment in 2\textsuperscript{nd} stage of 2-stage differentiation generates the best performance. However, we still need further assessments to validate the efficiency of ECs differentiation. Firstly, we will measure multiple endothelial markers other than PECAM1, including endothelial-specific VE-cadherin, VEGFR2, and Tie2. Although PECAM1 is primarily expressed in endothelial cells, it can also be expressed in some non-endothelial and hematopoietic progenitor cells. Secondly, protein generation should be further validated to safely conclude that ECs are generated abundantly because the qPCR only detects and quantifies the gene expression. Additionally, errors of qCPR might generate from inaccuracy in pipetting and input DNA quantity. Techniques including flow cytometry, enzyme-linked immunosorbent assay, or fluorescent-based assays can be utilized to quantify protein generation. Given this idea, we sought to measure PECAM1 by flow, obtaining a result not in accordance with that of qPCR. It was estimated that 7.21% of cells expressing PECAM1 were generated in the 6-day control group without DOX treatment, whereas the number was 0.89% for DOX treated two-stage differentiation group. A potential underlying reason is protein degradation. PECAM1 degradation rate could be affected by prolonged exposure to fixed PBS buffer and the light during flow preparation. To revise the flow process, we would proceed quickly to minimize the exposure, with protease inhibitor metalloproteinases (MMPs) added in cell storage to prevent protein degradation. If it succeeds, several ECs-specific
proteins stated above will also be measured to confirm the generation of ECs, rather than other types of cells expressing PECAM1.

The endothelial tube formation assays will be conducted to study angiogenesis formation \textit{in vitro} (DeCicco-Skinner et al., 2014). The endothelial cells will be cultured on an extracellular matrix that mimics the basement membrane of blood vessels. The tubes forming from endothelial cells can be observed under a microscope, and tube parameters determine the number of tubes. The time for tube formation depends on various factors that can be further studied on optimized angiogenesis stimulus, including extracellular matrix proteins, growth factors, and cytokines.

In summary, we validated a differentiation model that can potentially be utilized for ECs generation. The result suggests a better efficiency of differentiation following two stages: mesodermal induction and endothelial cell specification. If further assays confirm a steady and adequate generation of ECs, we aim to differentiate ECs orthogonally with SC-islets and test whether their combination promotes angiogenesis and alleviates hypoxia \textit{in vivo}. 
Chapter 3: "Pancake" Islet Development

3.1 Introduction

Because a SC-islet cluster may contain several hundred to several thousand cells, the oxygen and nutrient requirements of these cells in culture or at the transplantation site are different from those of individual cells. While necrosis is a rare occurrence in single-cell culture, central necrosis of islet cluster is commonly observed during culture due to its sphere geometry.

The temperature-responding culture dishes are designed to allow for the non-enzymatic detachment of adherent cells. The dishes are coated with a polymer with a critical solution temperature (LCST) of around 32 degrees Celsius. Above the LCST, the polymer coating is hydrophobic, allowing cells to adhere and spread on the surface through the hydrophobic interactions between the cell membrane and surface. Below the LCST, the cells detach from the polymer as water molecules are absorbed into the hydrophilic polymer layer. Here, we sought to alter the geometry of the cluster to a planar shape to allow for a higher amount of oxygen diffusion into the center of the cluster. The stage 6 SC-β cells were transferred to a temperature-responsive culture dish instead of forming aggregate on the shaker. The cell sheets, namely "pancake" SC-islets, were collected for function assays after detachment from the dish.

3.2 Material and Methods

3.2.1 Oxygen Diffusion Modeling

The oxygen diffusion simulation is produced with COMSOL 5.0 (COMSOL, Los Angeles, CA, USA). The diffusive mass transports of oxygen are governed by the generic stationary-state
reaction-diffusion equation in the chemical species transport module of COMSOL. The aggregate is modeled as a circle with a diameter of 500 μm, and the pancake islet is modeled as an ellipse with 220 μm in major diameter, according to their average size captured under the microscope. As the thickness of the pancake clusters is not determined, three different minor diameters were set in pancake islet modeling: 20 μm, 60 μm, and 100 μm. The model simulates the islet condition \textit{in vivo}, where the oxygen consumption rate (OCR) of the islet is $-0.034 \frac{mol}{m^3*sec}$, the oxygen diffusion rate is $1.52\times10^{-9} \frac{m^2}{s}$, and the oxygen concentration at the cluster boundary is $0.0452 \frac{mol}{m^3}$ (36mmHg). The simulation is shown in Figure 3.1. In SC-islet aggregate, the oxygen concentration progressively decreases in square proportion to the radial distance inward. The simulation demonstrates a more homogeneous oxygen diffusion within the planar ellipse shape cell cluster by theory. To display visually, the color scale bar for both pancake and the aggregate cluster are identical.
Figure 3.1 Oxygen diffusion modeling of SC-islet aggregate and pancake islet. (A) Visualization of oxygen concentration within the aggregate clusters represented by the color scale bar. With a diameter of 200 μm, the minimum concentration to maximum concentration ratio is 17.49%. (B)
The ratio of minimum oxygen concentration of pancake to boundary oxygen concentration is 0.973, 0.793, and 0.48 for pancake clusters with minor diameters of 20 μm, 60 μm, and 100 μm, respectively. The value for the aggregate cluster is -0.067.

### 3.2.2 Pancake islets culture

The SC-β cells were generated through the 6-stage differentiation protocol discussed in Chapter 1, using the genetic background of hPSC origin. The HUES8 cell line was provided by Harvard University. The SC-β cells were transferred to the Nunc UpCell thermos responding dish at stage 6, day 7, where they were cultured for another 7 days at 37 °C. When collecting the pancake islets, the dish was laid on the ice for temperature drop and cell detachment. The cells were then distributed for multiple assays. The Nunc UpCell thermos-responding dish surface was coated with a nanometer-scale layer of thermos-sensitive hydrogel Poly(N-isopropyl acrylamide), abbreviated PIPAAm. PIPAAm remains hydrophobic at 37 °C while becoming hydrophilic when the solution is below its critical temperature, allowing cell detachment.
3.2.3 Pancake functional assays

To investigate the function and gene expression of the Pancake islets, the following *in vitro* assays were conducted: the single cell RNA-sequencing, RT-qPCR, flow cytometry, immunohistochemistry (IHC) staining, static and dynamic glucose-stimulated insulin secretion (GSIS). The investigation focused on the insulin secretion-functional performance, cell type identifications, and β cell-associated gene expression compared with aggregate control. In addition, the Pancake clusters were subcutaneously transplanted into the mice, and the blood samples were collected afterward for c-peptide measurement using ELISA.
3.3 Results

3.3.1 Pancake islets show better GSIS performance.

Figure 3.2 presents the diagram of GSIS values of both pancake and control aggregate in terms of glucose concentration and time change. In both static and dynamic GSIS, the pancake secretes around or over double the times of insulin amount compared to the control aggregates, demonstrating a higher sensitivity to glucose stimulation.

Figure 3.3 Static and dynamic GSIS of Pancake and control group, in which the average cell count is 0.1 million, and the dilution for static GSIS is 10X. In both static and dynamic GSIS, the glucose concentration ranges from 2 mM (low concentration) to 20 mM (high concentration). An unpaired two-tailed t test was used for statistical analysis. **: P<0.01; ***: P<0.001.
3.3.2 Gene and protein expression of pancake islets

Figure 3.3. shows the RT-qPCR results on gene expression of pancake and stage 6 clusters. The pancake has a notably higher expression of SLC2A1 and a comparably higher expression of ALDOA and UCN3. The SLC2A1 regulates the glucose transporter protein 1 (GLUT1), which plays an important role in glucose transport. The rise in SLC2A1 can either suggest an elevated glucose sensitivity or an increased amount of enterochromaffin cells, which also encode the GLUT1. The ALDOA encodes the enzyme aldolase A and is upregulated in response to hypoxia to participate in the cells' metabolic reprogramming. The UCN3 is expressed in β cells and regulates glucose homeostasis and insulin secretion. The VEGFA is expressed in endothelial cells and is involved in the regulation of angiogenesis. In combination, the pancake might show an inclination for better glucose regulation and angiogenesis performance. However, no evidence in RT-qPCR results shows pancakes are subjected to less hypoxia.

![Pancake Normalized to Stage6 clusters](image)

Figure 3.4 Pancake gene expression
Figure 3.5 Immunostaining stained for C-peptide, glucagon (GCG), NKX6-1, chromogranin A (CHGA), and somatostatin (SST). (A) Pancake clusters (B) stage 6 aggregates, figure extracted from (Augsornworawat et al., 2020) and (Velazco-Cruz et al., 2019)
Figure 3.4 displays the protein markers visualized with a fluorescent microscope. There is an abundant generation of SST and much less C-peptide compared to the amount in control aggregates. Neither NKX6-1, GCG, nor CHGA was observed. The stained figure suggests the existence of δ cells, as SST is mainly produced by δ cells. However, the dim in C-peptide suggests a lack of, or a very limited number of β cells, compared to the strong green fluorescent signals in stage 6 control aggregates. Because of the absence of NKX6-1, GCG, and CHGA, the immunostaining of pancake islets does not provide solid proof of the existence of β cells or α cells.

3.4 Discussion

Pancake islets were designed with the intention of reducing β cell mortality and enhancing β cell function. Because of their planar geometry and smaller size compared to aggregate clusters, the cells are assumed to have more exposure to oxygen and are less likely to undergo anoxia-induced intrinsic apoptosis pathways. As we expected, we have obtained promising data showing the advanced insulin production and higher sensitivity to glucose of pancake islets in GSIS assays. While no evidence supports the hypothesis that pancake undergoes less hypoxia, the higher expression of SLC2A1, UCN3, and VEGFA indicates a potential for improved glucose regulation and angiogenesis performance. However, the results from other assays do not show the advantage of pancakes or even fail to give out useful data. Here, we will discuss the potential underlying causes and the alternative methods for future study on pancake islet function.

In immunohistochemistry staining, a high background staining was observed in C-peptide. This suggests that a certain amount of non-specific staining has taken place. In addition, several cell samples could no longer be identified after antigen retrieval. Although exact causes are not
determined, an optimized retrieval process and tissue fixation might help to produce more accurate staining. To measure the geometry of the pancake islets accurately and revising a more delicate model, a confocal microscope can be used in the future for capturing images at different depths and measuring the thickness.

In flow cytometry analysis of C-peptide and NKX6-1, neither the pancake group nor the control aggregate showed positive expression. It is speculated that single-cell dispersing was not successful, leading to inaccurate identification of cell population or false negatives. We are developing a revised protocol to tackle this problem, potentially altering the amount of trypsin while breaking down the cluster. In addition, little C-peptide was found in blood samples of mice receiving pancake islets and aggregate islets injection. This might have resulted from two factors: i) cell loss because of the unsteady flow within hypodermic tissue; ii) inadequate cell population to stimulate noticeable insulin production. To tackle these problems, we would transplant the clusters into kidneys or encapsulate them in biocompatible hydrogel devices, which will be retrieved later for in vitro functional assessments. In future transplantation, we will start with transplanting 100 million cells per mouse, then measuring C-peptide production every other week. The test time would range from minutes to hours, depending on the observation and size measured under the microscope. Lastly, we will investigate whether and how long pancake islets maintain their geometry after detaching from the temperature-sensitive plate.

In summary, the pancake islets are perceived to be promising but still need validation in their function through multiple assays. The two studies presented in this paper serve as an insight or technological platform for the further development of strategies for alleviating hypoxia and promoting function for stem cell-derived islets within a physiological environment. There is much more that needs to be done, as the transplanted SC-islets suffer from both hypoxia and
immune inflammation in the current stage, leading to great mortality in β cells. It is essential to overcome these challenges and take concrete steps for treating Type 1 diabetes.
References


