OPTOGENETIC DIFFERENTIATION OF CARDIOVASCULAR CELLS FROM PLURIPOTENT STEM CELLS

by

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A Thesis

Submitted to the Faculty of Purdue University

In Partial Fulfillment of the Requirements for the degree of

Master of Science in Chemical Engineering

Davidson School of Chemical Engineering

West Lafayette, Indiana

May 2021

THE PURDUE UNIVERSITY GRADUATE SCHOOL
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To my wife, Riley Jane, who is a source of good in the world. She inspires me to do my best.
And to my dog, Archie, who doesn't understand what a thesis is.

ACKNOWLEDGMENTS

For any success of mine, I thank my faculty advisor and mentor, Dr. Xiaoping Bao. He has taught me how to perform research, investigate the world around me, and think critically. He has demonstrated the greatest levels of understanding and patience with me. He has taught me much in and out of the lab, for which I am very grateful.

Thank you to the members of the Bao Lab: Dr. Yun Chang, Ju Jung, Ari Das, and Po-Yu Liang. This community has been integral to my success and has helped with both personal and academic growth. I look forward to seeing the future successes of the Bao Lab.

Thank you to Dr. Nicole Repina and Dr. Xiaojun Lian, who have been of great assistance behind the scenes. Dr. Repina designed the LAVA device, which illuminates our cells. Dr. Lian designed a plasmid vector that is essential for the gene editing that we perform in the lab. Without their contributions, this project would have not been possible.

Thank you to my friends and family who have supported me on my journey. Through the good and bad times, I relied on you. I truly appreciate you.

Finally, thank you to the Davidson School of Chemical Engineering. Through both my Bachelor and Master of Science degrees, this school has been a home to me. Within the walls of Forney, I have made lasting friendships, developed skills, and learned as much about life as I have engineering. I am extremely grateful for the opportunities made available to me by this school, and I am indebted to Purdue ChE for all future successes.

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ABBREVIATIONS

hPSCs – human Pluripotent Stem Cells
hESCs – human Embryonic Stem Cells
iPSCs – induced Pluripotent Stem Cells
EPCs – Endothelial Progenitor Cells
CMs – Cardiomyocytes
LAVA – Light Activation at Variable Amplitudes device
AAVS1 – Adeno Associated Virus Site 1
CRISPR – Clustered Regularly Interspaced Short Palindromic Repeats
HDR – Homology Directed Repair
SOX2 – SRY Box Transcription Factor 2
OCT4 – Octamer Binding Protein 4
CD31 – Cluster of Differentiation 31
    Also PECAM-1 – Platelet and Endothelial Cell Adhesion Molecule 1
CD34 – Cluster of Differentiation 34, Hematopoietic Progenitor Cell Antigen
SSEA4 – Stage-Specific Embryonic Antigen 4
VECAD – Vascular Endothelial Cadherin
BRA – Brachyury
    Also T – T-Box Transcription Factor
cTnT- cardiac Troponin T
MF20 – sarcomere Myosin heavy chain
vWF – von Willebrand Factor
LDL – Low Density Lipoprotein
GSK3 – Glycogen Synthase Kinase 3
Fz – Frizzled
PBS – Phosphate Buffer Solution
BSA – Bovine Serum Albumin
DMEM – Dulbecco's Modified Eagle Medium
DMSO – Dimethyl Sulfoxide
CHIR – CHIR99021, Wnt Activator
VEGF – Vascular Endothelial Growth Factor
EDTA – Ethylenediaminetetraacetic acid
DAPI – 4',6-Diamidino-2-Phenylindole
BF – Brightfield
Wnt – from "Wingless-related integration site"
hv – Light Activation (at 470 nm)
PuroR – Puromycin resistance

ABSTRACT

Stem cell technologies hold great promise in solving problems within fields such as drug development, regenerative medicine, and disease modeling. Stem cell engineering provides a mechanism that will help stem cells achieve this promise. Currently, many applications within tissue engineering are limited by a lack of ability to create accurate micro-physiological structures that recapitulate multicellular tissue patterns *in vivo*. Precise control of spatial and temporal signaling is desired to perform concurrent differentiation to multiple cell types intentionally. The OptoWnt construct, a novel optogenetic system activating the Wnt signaling pathway, achieves precise spatiotemporal regulation, in pursuit of greater control in stem cell differentiation. We utilize OptoWnt, to differentiate stem cells into cardiovascular cells: endothelial progenitor cells and cardiomyocytes, valuable cell types for designing microtissues. Endothelial cells comprise the luminal lining of blood and lymphatic vessels, providing the integral structure for distribution within the body, separating mobile and stationary tissues. Cardiomyocytes provide the force required to pump blood throughout the human body and are a highly desired cell type in regenerative medicine.

In this project, we have applied an optogenetic induced signaling pathway, OptoWnt, to differentiate human pluripotent stem cells (hPSCs) into cardiovascular cells via light-induced activation of Wnt signaling pathway. In the analysis of these cells and comparison to previous small molecule approaches to cardiovascular cell differentiation, we demonstrate the robustness of the optogenetic approach and similar efficiency that it has with the small molecule approach. In short, we have further demonstrated the utility and potential of optogenetic induction of developmental pathways, via the OptoWnt construct.

1. INTRODUCTION

1.1 Motivation

Every single cell of any human being once originated from a stem cell. Stem cells have a property called pluripotency, which refers to their ability to differentiate, or sequentially refine through defined developmental pathways, into a more mature phenotype, sequentially becoming less pluripotent, but more nuanced[1]. This is how we all came to be.

Stem cells hold immense promise in fields such as regenerative medicine, drug development, and disease modeling. Stem cells, as tools for developmental biology, help us to identify where exactly certain mechanisms and changes occur in embryogenesis and what occasionally goes wrong to create diseases. Stem cells have the capacity to differentiate into any tissue within the human body. For instance, with proper signaling pathway control, human pluripotent stem cells (hPSCs) may be differentiated to form different cardiovascular cells (**Figure 1.1**). One of the attractive characteristics of stem cells is a seemingly unlimited renewal capacity[2], which allows for a great source of cells for tissue generation. With induced pluripotent stem cells[3] and genome editing[4], we now have the tools to perform greater investigations of genetic-related diseases, permitting more accurate genetic and cellular models of diseased phenotypes.

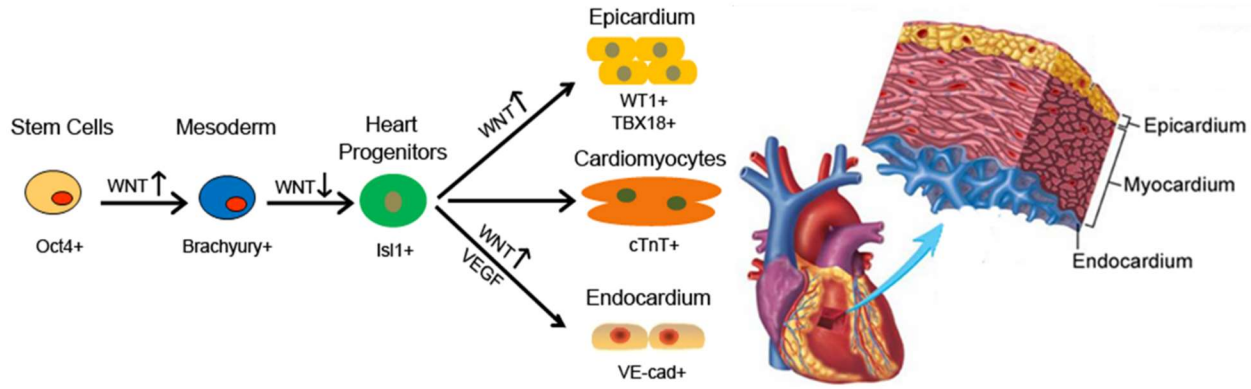

Figure 1.1. Wnt signaling modulation to develop different cell types within heart tissue. With the activation and inhibition of the Wnt pathway, we are able to sequentially differentiate stem cells into more specified cell types, ultimately deriving the cells that compose the three major layers of the heart: endocardium, myocardium, and epicardium.

During the conception of stem cell technology, researchers and healers alike dreamed of a future with modular medicine. That would be the pinnacle of regenerative medicine: where an injured tissue may simply be replaced with a stem cell-derived substitute. Priority stem cell transplant treatments primarily involve cells that do not heal or replicate well, such as neurons and cardiomyocytes. Slightly over 20 years have passed since the discovery that allows for the maintenance of pluripotency in stem cells[5,6], functionalizing these cells for studies in the lab; however, stem cell treatment has not yet become standard care. While no stem cell therapies have arrived in phase 3 clinical trials[7], there have been several promising investigations in phases 1 and 2, looking to repair cardiomyopathy[8] and retinal macular degeneration[9]. Clearly, there is much more to investigate and discover for stem cell applications.

Current progress is limited by the inability to create physiologically accurate tissues. *In vivo,* animal tissues contain a heterogenous mixture of cells with defined and purposeful relations to each other. Pursuits in physiologically relevance include creating integrated heterogeneous composites of multiple cell types, achieving mature phenotypes comparable to tissues *in vivo*, and designing tissues that are sufficiently complex to mimic tissue. Pursuing these concepts within stem cell cultures will help us to create synergistic interactions between cells within these tissues and create tissues that model those required to meet the complicated demands of multicellular life.

Historically, most differentiation and cell culture protocols focus on creating a monoculture of a specific cell phenotype. However, in many of the body's microstructures, there is heterogeneity in cell types. Current approaches in designing intentional multicellular tissues rely on these different approaches: matrix bound substrates[10], 3D printing[11,12], or signaling gradients via microfluidics[13]. Matrix bound substrates refer to tagging the matrices that cells grow on with signaling molecules or substrates, such that only the cells in the very direct vicinity of said tag are signaled and differentiate accordingly. 3D printing involves suspending cells of interest into different 3D printing inks to be layered spatially. A third approach requires utilizing microfluidics to create signaling molecule gradients. Difficulties with the aforementioned approaches to multicellularity in differentiation involve a lack of spatial control after the initial design, lack of cell-to-cell interactions, and lack of tunability, all of which are imperative to creating a physiologically relevant model. OptoWnt, an optogenetic construct which activates the Wnt pathway, meets these needs in supplying a robust system that provides precise spatially controlled signaling[14].

The canonical Wnt pathway is a well-studied, highly conserved developmental pathway common to fruit flies, humans, and all animals in-between. This pathway occurs in early embryogenesis and helps to establish fate determination among different subpopulations of stem cells[15,16]. Upon activation, the extracellular Wnt protein binds to the Wnt receptors, Frizzled and LRP6 (**Figure 1.2**). As Frizzled and LRP6 bind to the extracellular Wnt protein, the intracellular components of the receptors create a condition which attracts and inhibits GSK3[17]. While GSK3 is inhibited, β-catenin accumulates and resultingly migrates into the nucleus of the cell. Once in the nucleus, β-catenin binds to other transcription factors and activates the Wnt pathway associated genes.

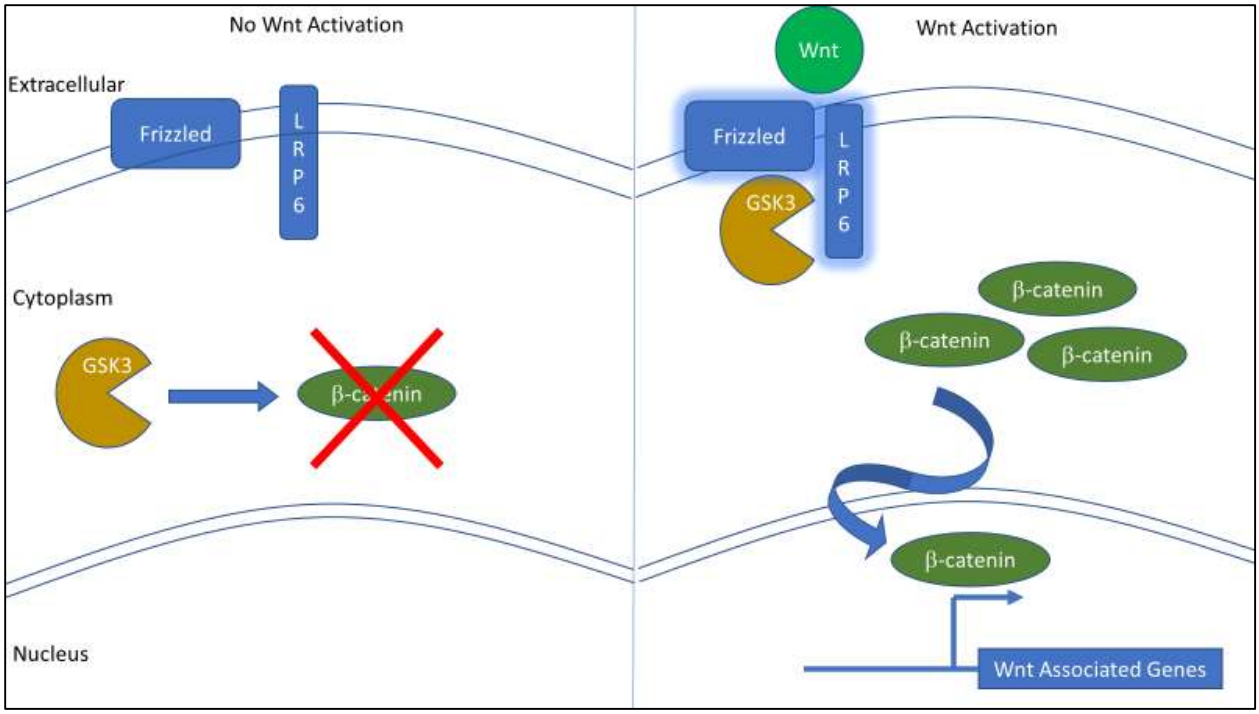

Figure 1.2. Simplified schematic of the canonical Wnt pathway. The activation of the Wnt pathway relies on the inhibition of a protein complex centered on GSK3. Uninhibited, GSK3 marks β-catenin for ubiquitination, or degradation. In vivo the canonical Wnt pathway is activated via an extracellular Wnt signaling protein, such as Wnt-8. This protein binds to the receptors Frizzled and LRP6. Once activated by the extracellular Wnt ligand, LRP6 becomes phosphorylated. Phosphorylated LRP6 inhibits GSK3, allowing β-catenin to accumulate in the cytosol, diffuse into the nucleus, and resultingly, up-regulate Wnt targeted genes.

1.2 **Optogenetics**

Optogenetics refers to the process of utilizing light as a medium to alter genetic expression within the cell. This can refer to utilizing light to induce the production of a specific protein[18], or, as in our case, utilizing light to activate a fundamental developmental pathway[14,19,20]. We will be

referring to the latter. The primary purpose of the OptoWnt system is to provide precise spatiotemporal control over the activation of Wnt signaling[14]. In application, modulation of signaling permits researchers to have greater spatiotemporal control in differentiating stem cells intentionally and concurrently into cells of interest within the same culture dish (**Figure 1.3**).

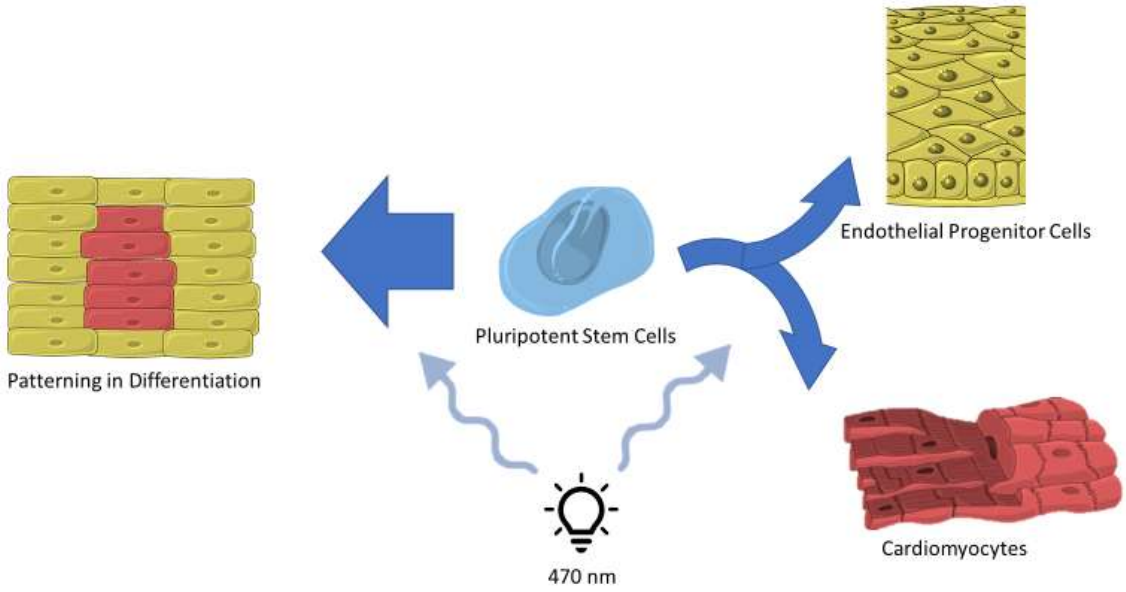

Figure 1.3. Graphic demonstrating OptoWnt's ability to selectively differentiate into multilineage patterns. Through this research, we demonstrate that optogenetics may be utilized to modulate the Wnt pathway and induce differentiation into different cell types, such as cardiomyocytes and endothelial progenitor cells. In taking the research a step further, we use spatial control of light exposure to create patterning in cell differentiation, leading to intentional and controlled patterning in stem cell differentiation.

As compared to other approaches for intentionally heterogeneous differentiation, optogenetics imparts several comparative benefits: orthogonality in signaling, precise control over expression, low impact on non-optogenetic cells, and concurrent differentiation. Orthogonality in signaling refers to being able to supply other means by which impact the differentiation of the stem cells, aiding in the adaptability of this approach. Other methods of co-differentiation are unable to achieve the spatial precision that optogenetics achieves. With optogenetics, boundaries between different cell types are defined by the exposure to the activating light[14,21]. It is difficult to achieve this discrete of a boundary with other mechanisms of signaling control. It may be noted that the exposure to light has a negligible effect on non-engineered stem cells. Finally, the greatest benefit and perhaps most influential achievement of OptoWnt and the Light Activation at Variable

Amplitude (LAVA) device is the ability to create distinct populations of desired cell types via Wnt pathway modulation from a source of light alone[22]. Specifically, this may be noted in the example of differentiation hPSCs into cardiomyocytes and epicardial cells, where distinct subpopulations of each cells are shown. This precise spatiotemporal control is paramount to designing more physiologically relevant tissues[23,24].

OptoWnt was designed by inserting a conjugated protein Cry2-LRP6c (Cry2 connected to the cytoplasmic domain of LRP6) into the H9 hPSCs' *AAVS1* safe harbor locus[14]. Cry2-LRP6c is expressed constitutively within the cell, permitting an abundance of this protein to be available for the activation of the Wnt pathway. This creates an alternative entry into the standard canonical Wnt pathway. Instead of relying on the exogenous exposure to the Wnt protein or a substitute, cells may be exposed to light to induce the Wnt pathway, a much more spatially controllable option. When the OptoWnt cells are exposed to 470 nm light, the Cry2 domains of the Cry2-LRP6c protein clump together, forming aggregate bodies[25]. These aggregate bodies recapitulate the Wnt receptor protein interactions on the cell surface, leading to a similar activation. The clustering of the LRP6c domains inhibits GSK3, leading to the accumulation of β-catenin. Once again, β-catenin is able to migrate to the nucleus and interact with transcription factors activating Wnt-targeted genes.

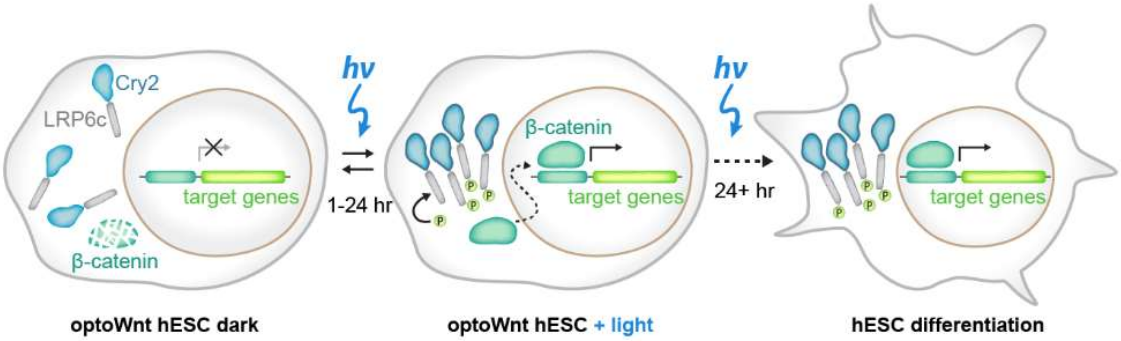

Figure 1.4. Mechanism of light activated Wnt pathway in OptoWnt cells[14]. To create an optogenetic approach to activation of the Wnt pathway, the cytoplasmic domain of LRP6 was conjugated to the Cry2 protein. When exposed to light at 470 nm, Cry2 aggregates similarly to the endogenous triggering of the Wnt receptors. LRP6c becomes phosphorylated due to aggregation and inhibits GSK3. As GSK3 is inhibited, β-catenin accumulates in the cytosol, diffuses into the nucleus, and activates Wnt target genes. Reproduced without edit under the Creative Commons license 4.0.[14]

1.3 **Cardiovascular Cells**

Endothelial progenitor cells are the precursor to endothelial cells. These progenitors demonstrate similarities and the potential to become endothelial cells, but they do not exhibit the expected qualities and expression levels characteristic of mature endothelial cells, such as vWF

expression or acetylated LDL uptake[26]. Endothelial cells originate from the mesoderm germ layer and compose the luminal lining of blood and lymphatic vessels, present all over the body. These endothelial cells act as a barrier between mobile and stationary tissues. Endothelial cells have distinct and vital functions such as structural integrity, nutrient and waste transport, protection, tissue separation, specialized cellular transport, promoting proper hemostasis, and chemical production[27–30].

As endothelial cells define the luminal surface of blood and lymph vessels, the relative spatial properties of these cells are very important in practice[31]. Without the annular geometry of blood vessels, the body would not be able to distribute oxygen and nutrients as efficiently. Therefore, the spatial patterning of tissues is especially essential in regards to endothelial cell placement. OptoWnt provides a way to meet these spatial demands.

For any significant orthotopic tissue repair via stem cell derived tissues, endothelial cells are necessary to ensure proper vascular supply to the donor tissue[30]. Similarly, in drug development, endothelial tissues provide a system to characterize pharmacokinetics[32], investigating how a drug is able to permeate the walls of the blood vessels. Endothelial cells have been shown to increase the maturity of stem cell-derived tissues, leading to a more phenotypically relevant tissue[33]. With greater endothelial cell models, we would be able to investigate findings of angiogenesis, leading to better methods of repairing vascular injuries[34] and better methods to prevent the growth and spread of tumors[35].

Cardiomyocytes comprise the muscle tissue that enables the heart to beat, pumping blood throughout the body. These cells rhythmically contract from birth to death without interruption. Cardiomyocytes are another prime cell fate to organize spatially, due to a necessity for proper vasculature and cooperation among the cells. In muscle fibers, cells are all oriented in the same direction, allowing for the cooperation of each muscle cell's contraction. Without spatial control in the differentiation of cardiomyocytes, the orientation and direct vicinity of each cell is random[36], which is difficult to implement downstream or clinically. Cardiovascular and heart disease are the leading causes of mortality in the world[37]. These diseases result from blockages in the coronary arteries that provide nutrients and oxygen to the cardiomyocytes. With a blockage, these cells die, commonly referred to as a myocardial infarction, or heart attack. Cardiomyocytes are not a highly proliferative cell; therefore, the heart is not typically repaired. Cardiomyocytes prove to be a prime target for the regenerative medicine potential of stem cells.

Previous methods for cardiovascular cell differentiation call for co-culturing with stromal cells[38], embryoid body formation, or 2-dimensional (2D) monolayer[39]. These techniques lead to heterogeneity in cell population, with the 2D monolayer having the greatest efficiency of the three. Stromal cells, also called feeder cells, provide hPSCs with the proper environment to proliferate and differentiate into endothelial cells. Embryoid body formation relies on spontaneous differentiation in the stem cells to produce a subpopulation of endothelial progenitors. The resulting cell mixtures have a high variability in composition and are not ideal for tissue modeling. 2D monolayers demonstrate good efficiencies in differentiating, but do not grow integrated with other cell types. While modulating the OptoWnt spatial signaling does pursue heterogeneity in culture, it is controlled heterogeneity, as opposed to what stromal cell and embryoid body techniques allow. This control allows for the production of complex stem cell-derived multicellular tissues, driving the field of stem cell technology forward.

1.4 Project Objectives

This research is an exploration of an optogenetic-induced Wnt pathway, OptoWnt. Primarily, we introduce this construct as an alternate mode of signal induction and, resultingly, differentiation. We investigate the usage of OptoWnt and LAVA device to provide **precise spatiotemporal signaling control**. Through our investigation, we first demonstrate the similar efficiency in endothelial progenitor differentiation obtained via optogenetics compared to the established small-molecule approach. Next, we demonstrate the ability of this technology to establish patterns in differentiation via modulation of light exposure spatially. In achieving these objectives, we provide a proof of concept for the potential and efficacy of optogenetics in stem cell populations.

2. RESULTS

2.1 Genomic Insertion of OptoWnt Construct

In order to create the stable OptoWnt cell line, we prepared a vector to insert into the *AAVS1* safe harbor site, a commonly utilized locus for gene editing. As shown in **Figure 2.1** below, the donor plasmid has a puromycin resistance (PuroR) cassette for drug selection and a constitutive CAG promoter producing Cry2-LRP6c and mCherry. Homologous 5'arm and 3'arm were also included in this construct to achieve targeted knockin via homology directed repair (HDR), which permits any transgene of interest to insert into and repair the genomic DNA after a double stranded break (DSB) induced by a nuclease such as Cas9.

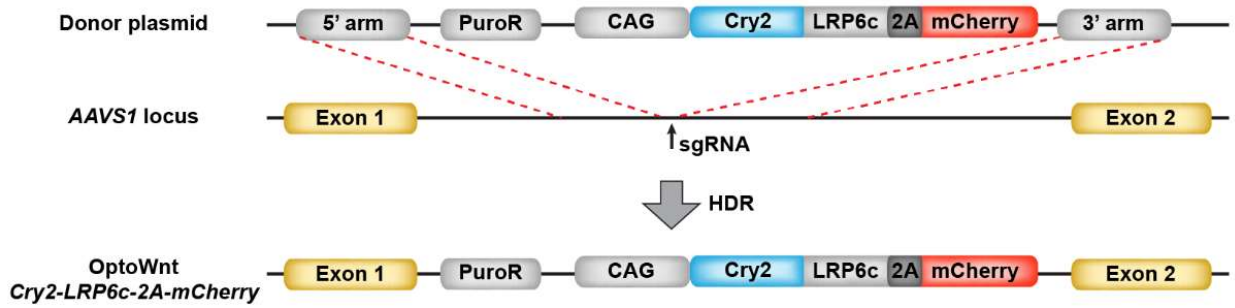

Figure 2.1. Insertion of OptoWnt construct into *AAVS1* safe harbor locus via homology directed repair[14]. Cells were transfected with plasmids coding for Cas9, sgRNA for the *AAVS1* locus, and the OptoWnt donor construct. The Cas9-sgRNA complex creates a double stranded break at the *AAVS1* locus. Using homology directed repair, the OptoWnt donor construct inserts into the *AAVS1* site.

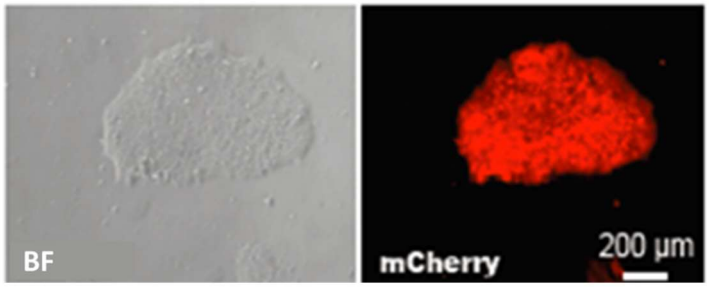

Figure 2.2. Identification of a positive insertion clone with mCherry. After a successful transfection, mCherry positive colonies are identified. As mCherry and Cry2-LRP6c are transcribed under the same promoter, CAG, mCherry presence is indicative of Cry2-LRP6c.

We transfected the hPSCs via electroporation. Initially, we investigate if the stabilized colonies, post-transfection and selection, expressed mCherry (**Figure 2.2**). After we have

identified drug-resistant individual mCherry+ hPSC clones from the transfection, we utilized PCR genotyping to determine their homozygosity (**Figure 2.3**). We prepare primers that identify the unedited *AAVS1* site and then perform PCR to determine which clones still retain the unedited *AAVS1* locus. If the clones display a band in the homozygosity assay below, then they do not have a homozygous insertion.

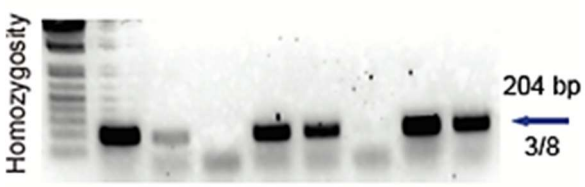

Figure 2.3. Homozygosity PCR assay to determine if the OptoWnt construct is inserted into both alleles of the *AAVS1* site[14]. The clones with 204 bp results only have a single OptoWnt insertion. The negative clones are identified as having a homozygous insertion of OptoWnt into both alleles of the *AAVS1* site.

2.2 OptoWnt Retains Pluripotency

Prior to the usage and differentiation of the stable OptoWnt cell line, it is important to ensure that the engineered cell line itself retains pluripotency, despite having an exogenous gene expressed. Without pluripotency under maintenance stage, there would be no subsequent directed differentiation into desired cell types. We cultured OptoWnt cells in pluripotency media for the duration of 3 passages, or roughly 10 days, and analyzed the expression of two pluripotency markers, SOX2 and OCT4, via immunostaining and flow cytometry. As can be seen in **Figure 2.4** by the 98.3% double positive cells in the flow cytometry plot, the OptoWnt cells retain pluripotency.

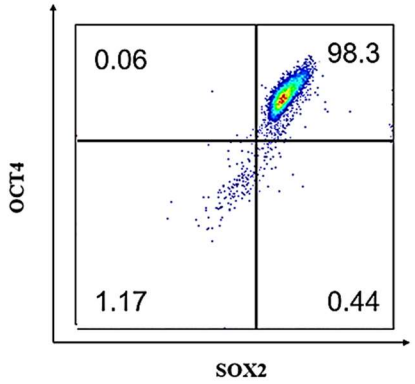

Figure 2.4. Flow cytometry analysis of OptoWnt for pluripotency markers, OCT4 and SOX2. Cells were treated with antibodies binding to the pluripotency markers OCT4 and SOX2. Each axis demonstrates a logarithmic scale of fluorescence. As 98.3% of these cells demonstrate presence of the pluripotency markers, we can properly state that the engineered OptoWnt cells retain pluripotency.

Furthermore, we performed immunostaining analysis to demonstrate the typical stem cell phenotype. The strong expression of OCT-4 and SSEA-4 in OptoWnt cells confirmed their pluripotency (**Figure 2.5**). The mCherry expression suggests the expression of OptoWnt transgene, as these two transgenes are driven by the same constitutive CAG promoter.

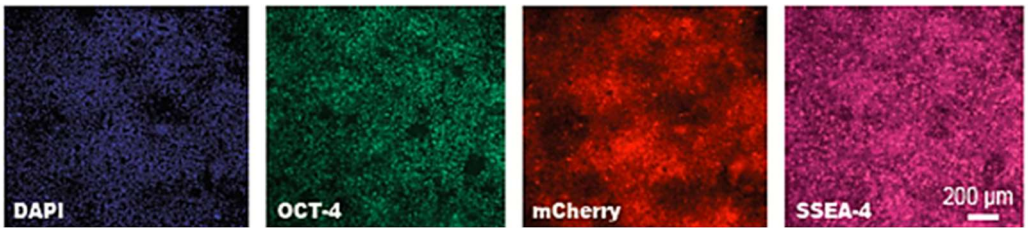

Figure 2.5. Immunostaining analysis for pluripotency markers OCT-4 and SSEA-4, along with mCherry[14]. OCT4 and SSEA4 presence further demonstrates the pluripotent state of the OptoWnt engineered hPSCs.

2.3 Differentiation of Endothelial Progenitor Cells

In order to differentiate OptoWnt cells into endothelial progenitor cells, we modified an established protocol (**Figure 2.6**), which utilizes small molecule CHIR99021 (CHIR) to activate the canonical Wnt signaling pathway. The modification of the Wnt activation replaces the small molecule CHIR with light activation in the OptoWnt cells (**Figure 2.7**).

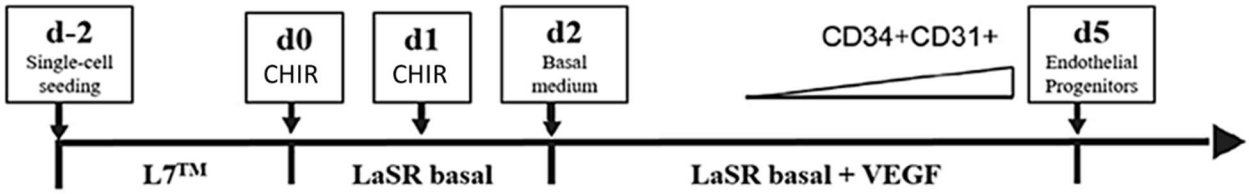

Figure 2.6. Small-molecule approach for endothelial progenitor differentiation using Wnt pathway activator CHIR99021 (CHIR)[40]. The small-molecule CHIR activates the Wnt pathway via inhibition of GSK3 during days 0 and 1 of endothelial progenitor differentiation. On days 2 through 5, the cells are cultured in a basal media with 100 ng/mL of VEGF.

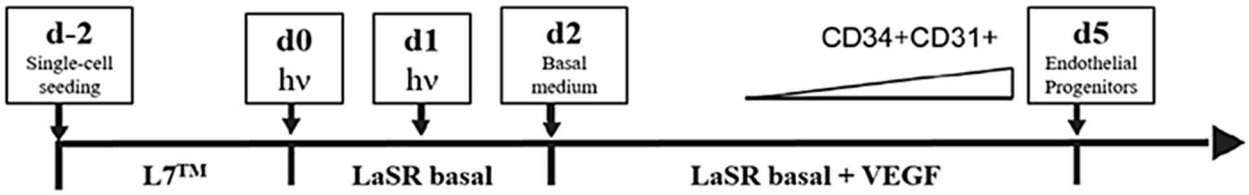

Figure 2.7. Modified light-activated approach to endothelial progenitor differentiation for OptoWnt cells. The standard small-molecule differentiation protocol is altered by utilizing the light activation of OptoWnt in replacement of the CHIR activation. Wnt activation occurs on days 0 and 1. On days 2 through 5, the cells are cultured in a basal media with 100 ng/mL of VEGF.

Through endothelial progenitor cell differentiation via OptoWnt activation with the LAVA device, we achieved a 19.0% CD34+CD31+ endothelial progenitor cells (**Figure 2.8**). In comparison to other tested cell lines, this demonstrates great promise in the potential for differentiation via optogenetics.

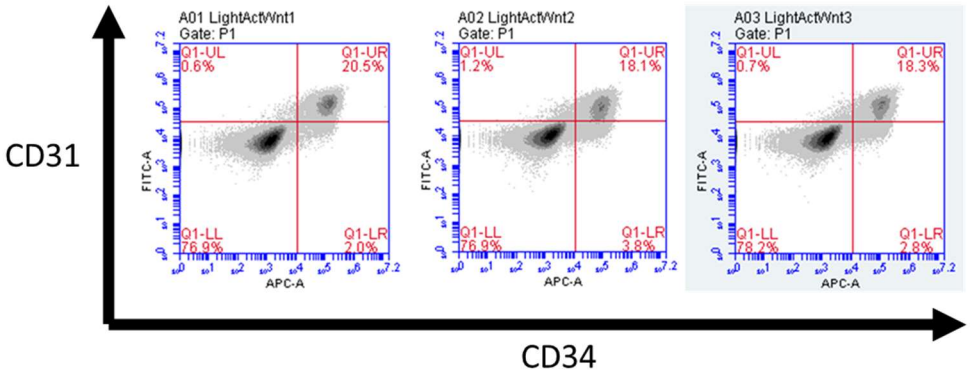

Figure 2.8. Flow cytometry analysis of OptoWnt endothelial progenitor cells via light activation. OptoWnt endothelial progenitor cells are stained for established surface markers CD31 and CD34 on day 5 of differentiation. The light-activation of OptoWnt demonstrates a CD31+CD34+ rate of 19.0% with a standard deviation of 1.3%.

21.8% of these light-activated endothelial progenitor cells are CD34+. This is slightly higher than the small-molecule approach of 20.4% CD34+, but not significantly (**Figure 2.9**). Furthermore, the light-activated endothelial progenitor differentiation approach shows a comparable efficiency to the other cell lines achieved via the small-molecule approach. The greatest efficiency of the cell lines was by H13 at 32.0% and the smallest efficiency by H9 at 20.4% CD34+. The H9 optogenetic differentiation results are statistically similar to the small-molecule approaches of cell lines 19-9-7, 6-9-9, H9, and H14. It may be noted that this range in differentiation efficiencies is typical in stem cell technologies. Different cell lines may be predisposed to a specific lineage compared to other cell lines[41]. To further explain potential variations, certain induced pluripotent stem cell (iPSC) lines may be predisposed to differentiate to similar tissues from which the iPSC was originally derived. Additionally, these biological science experiments have a tendency towards larger variations. This exciting result demonstrates the potential that the OptoWnt cell line has to offer in terms of differentiation induction.

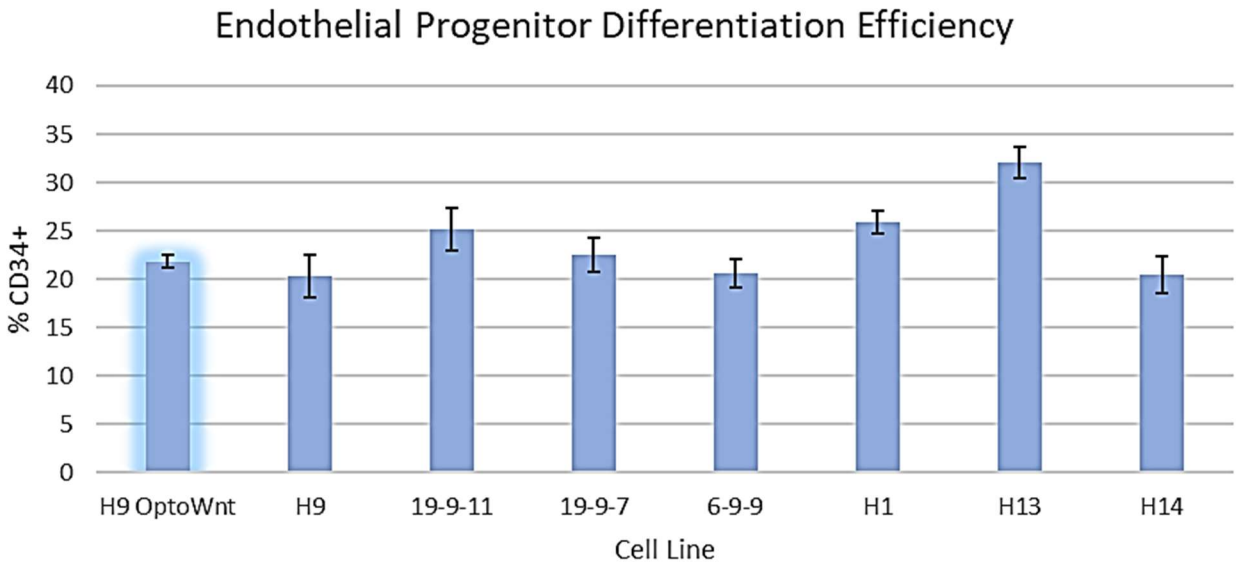

Figure 2.9. Comparison of light activated H9 OptoWnt CD34+% with other cell lines using small molecule differentiation via CHIR[40]. In comparing the H9 cells differentiated into endothelial progenitor cells via the light-activation approach and the H9 differentiated via the CHIR, we can see comparable results. Light-activation achieved an average of 21.8% CD34+ cells while small molecule approach averaged at 20.4%, with standard deviations of 0.7 and 2.2%, respectively. Data received from Bao et al. *Stem Cell Research* 2015.

To further characterize the cells derived from light activated OptoWnt-derived endothelial progenitor cells, we performed immunostaining analysis. Cells were stained for surface markers CD31, CD34, and VECAD (**Figure 2.10**). Brightfield images presented a cobblestone-like phenotype in cells, along with some apparent 3-dimensional structure. This cobblestone-like characteristic packing of endothelial cells is what allows them to create the layers that seal the blood vessels circulating throughout the body.

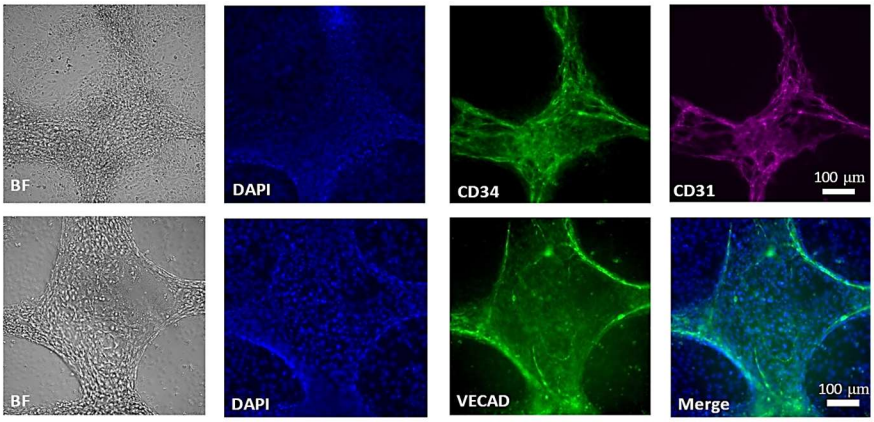

Figure 2.10. Immunostaining analysis of CD31, CD34, and VECAD in light-induced endothelial progenitor cells. CD31, CD34, and VECAD are representative of endothelial progenitor cells, demonstrating the effectiveness of the optogenetic protocol.

2.4 Differentiation of Cardiomyocytes

Similar to the protocol for differentiation of endothelial progenitor cells, we replace the small-molecule Wnt-activation with light-activated Wnt-activation via OptoWnt to differentiate cardiomyocytes (**Figure 2.11**). Following Wnt-activation on day 1, Wnt-inhibition is induced by Wnt-C59 at day 3. Following this, the cells are retained in basal media until day 15, when they are characterized via flow cytometry analysis or immunostaining. The differentiated cardiomyocytes may be noticed contracting after day 7, a well-known example of cardiomyocytes' ability to beat spontaneously.

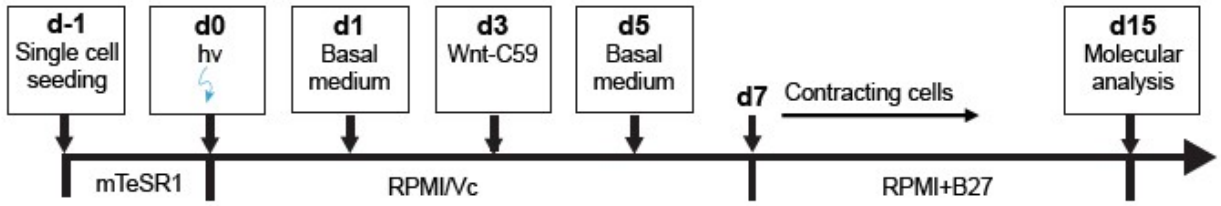

Figure 2.11. Light-activated approach to cardiomyocyte differentiation from OptoWnt cells. On day 0, the Wnt pathway is activated via light exposure. On day 3, Wnt-C59 inhibits Wnt signaling. These cells are maintained on basal medium until characterization via immunostaining and flow cytometry on day 15.

In further analysis, optogenetic differentiated cardiomyocytes were stained for cTnT and MF20 (**Figure 2.12**). These cells demonstrate a similar phenotype to the small-molecule approach for cardiomyocytes. Furthermore, they demonstrate a high efficiency of differentiation, judged by the presence of cTnT. It may be noted that mCherry still remains present from the OptoWnt transgene.

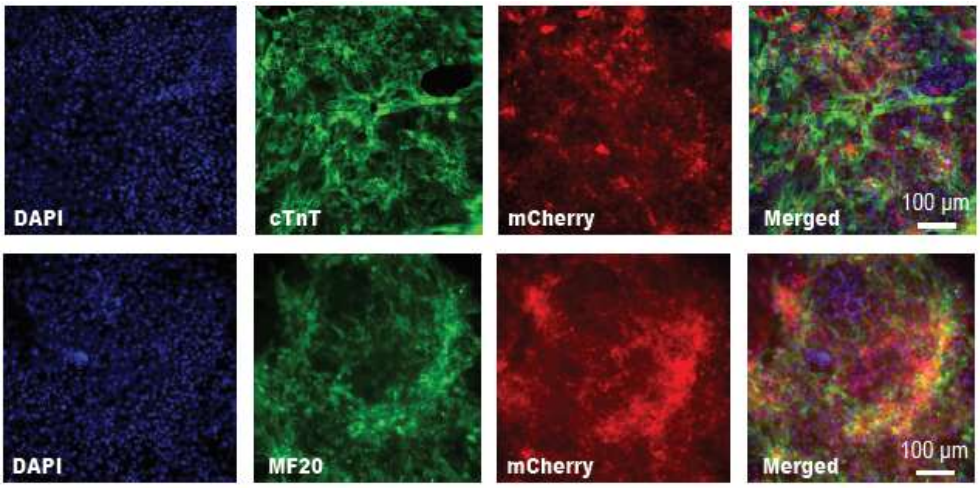

Figure 2.12. Immunostaining of optogenetic-differentiated cardiomyocytes. Cells are stained with cardiomyocyte specific marker cTnT and muscle cell specific marker MF20. mCherry is produced from the OptoWnt gene construct.

To investigate the efficiency of cardiomyocyte differentiation from hPSCs, flow cytometry analysis was performed, quantifying cTnT in the differentiated cultures (**Figure 2.13**). The isotype utilized compared to was non-differentiated OptoWnt. When compared to a small molecule protocol for cardiomyocyte differentiation, the optogenetic efficiency of 81.8% cTnT+ approaches the small molecule efficiency of 96% cTnT+[42].

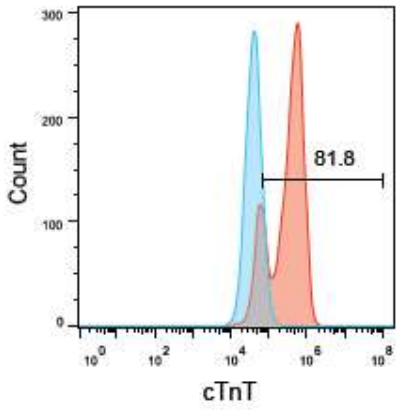

Figure 2.13. Histogram defining difference in cTnT expression between optogenetic-derived OptoWnt cardiomyocytes and pluripotent OptoWnt cells. The differentiated cardiomyocytes, red curve, are found to be 81.8% cTnT positive when using pluripotent OptoWnt cells as an isotype, blue curve.

2.5 Spatial Modulation of the Wnt Pathway

To demonstrate the spatial modulation that the OptoWnt cell line and the LAVA device may serve, we set up two scenarios of light exposure: a ring and a vertical bar (**Figure 2.14**). The ring exposes all cells to light except for those under the ring, while the vertical bar only exposes those within the bar to light. The 470 nm light activates the Wnt pathway and induces illuminated cells to progress into the mesoderm germ layer. The brachyury (or T) is a mesendoderm specific marker, and can be witnessed in the illuminated cells below. We can see that only the cells that have been exposed to the light have progressed into the mesendoderm lineage, demonstrating spatial modulation as a proof-of-concept. In the bright field panels of each image, we may see a black ring and light vertical bar. These demonstrate the light exposure geometries that these cells were cultured in. Therefore, the black region in the brightfield images indicates a region that was not illuminated and did not have induce the Wnt pathway.

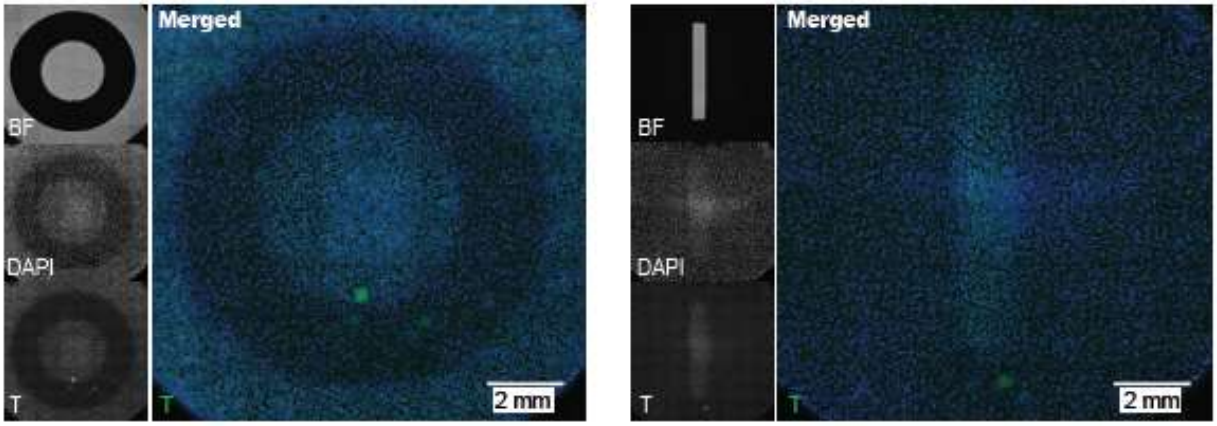

Figure 2.14. Spatial modulation of Wnt signaling creates patterning of mesoderm lineage cells[14]. Transparent films were utilized to control the spatial differentiation and were placed between the culture plate and the LAVA light source. Two conditions, a ring and a vertical bar, were utilized to test the capabilities of this technology. The ring demonstrates a majority of the cell culture exposed to light, while the vertical bar exposes less than half of the cells, with two different geometries.

After the proof-of-concept in delivering spatial patterning within the mesendoderm, we induced differentiation into two different cell types, cardiomyocytes and epicardial cells (**Figure 2.15**). All cells were initially illuminated on day 0, in order to activate the Wnt pathway. On day 3, Wnt-C59 was added to the media to inhibit the Wnt pathway for all cells. At this point, the cells may be classified as cardiac progenitors. A transparent film with an opaque ring was placed between the LAVA device and the cell culture, inducing spatial patterning. The cells exposed to the light once again had the Wnt pathway activated and consequentially differentiated into epicardial cells. The cells shaded by the mask further developed into cardiomyocytes.

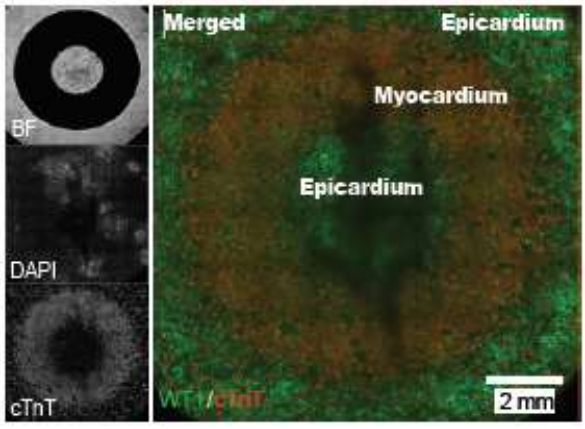

Figure 2.15. Spatial modulation of Wnt signaling creates patterning of cardiomyocytes and epicardial cells. After Wnt-inhibition via Wnt-C59, a ring mask blocked exposure during a second light-activated Wnt-activation. The secondary Wnt-activation induced the development of epicardial cells around a ring of cardiomyocytes.

3. MATERIALS AND METHODS

3.1 Stem Cell Culture and Passaging

Original H9 wildtype hPSCs were obtained from WiCell. hPSCs were cultured on Matrigel coated 6-well plates in stem cell culture media: mTeSR 1™, mTeSR Plus™, E8 or LaSR. Cells were cultured in an incubator at 5% CO_2, 37°C. When cell confluency reached approximately 90%, cells were passaged using 0.5 mM EDTA solution to a new well with 5 μM Y-27632, a ROCK inhibitor.

3.2 Genomic Editing

CRISPR/Cas9-mediated homology-directed repair (HDR) was used to knock the OptoWnt construct into H9 hPSCs. To construct the donor plasmid, Cry2-LRP6c-P2A-mCherry was synthesized and cloned into *AAVS1*-Puro CAG FUCCI (Addgene #136934) via Gibson Assembly. The resulting donor plasmid was electroporated into wildtype H9 hPSCs along with the SpCas9 *AAVS1* gRNA T2 (Addgene; #79888). The resulting cells were seeded onto the Matrigel-coated 6-well plate and subjected for drug selection (1 μg/ml puromycin) once reaching confluency. Successfully-targeted mCherry+ clones were then picked and subjected for homozygosity assay using PCR genotyping.

3.3 Light Activated Wnt Activation

In order to induce the light activated Wnt pathway in OptoWnt cells, we utilized the LAVA board. The LAVA device was set to expose the cells to 1 μW/mm^2 of 470 nm light, the equivalent of Wnt activation by 6 μM of CHIR99021. The 24-well plate with the differentiating OptoWnt cells was placed on top of the LAVA device (**Figure A1**) and cultured for 24 or 48 hours. After 24 hours, the cells were inspected for any apoptosis. For desired differentiation patterning, films were created with transparent and opaque regions to either expose or block cell culture from the light, resultingly modulating the Wnt activation across a single cell culture well.

3.4 Flow Cytometry Analysis

Flow cytometry allows us to quantify subpopulations of cells based on fluorescent molecules. Antibodies may be tagged with fluorophores to identify cells with specific markers. For surface markers, live cell flow cytometry analysis was performed. Cells were treated with Accutase™ or 0.5 mM EDTA and filtered through a strainer to obtain single cell suspension. Singularized cells were centrifuged and washed once with FlowBuffer-1 (0.5% BSA in PBS). The cell pellet was then suspended in 50 μL of FlowBuffer-1 with appropriate antibodies and incubated for 30 minutes at room temperature. Cells were then washed and analyzed in a BD Accuri C6 plus flow cytometry machine.

3.5 Immunostaining

Cells were washed with PBS to remove any debris and fixed in 4% paraformaldehyde/PBS for 15 minutes. Cells were washed twice with PBS and stained with desired antibodies in 5% nonfat milk powder and 0.4% Triton X-100 solution overnight at 4°C on a plate rocker. After PBS washing, cells were incubated with appropriate secondary antibodies in the 5% milk blocking solution for 30 mins at room temperature or overnight at 4°C. Nuclei were stained with DAPI solution for 5 mins. After PBS washing, the stained cells were imaged and processed with a Leica DMi-8 fluorescent microscope and ImageJ, respectively.

4. CONCLUSIONS AND FUTURE DIRECTIONS

4.1 Summary of Current Progress

The OptoWnt gene construct has shown its ability to activate the Wnt pathway, differentiating hPSCs into endothelial progenitor cells, cardiomyocytes[43], and epicardial cells[21]. In creating results capable of direct comparison with the small-molecule approach of differentiation, we have demonstrated the capacity of the OptoWnt construct to replace the standard Wnt activator, CHIR, while permitting precise spatial control at the same time. Comparable percentages of CD34+ endothelial progenitor cells were achieved between the OptoWnt optogenetic differentiation and previous small molecule approach. Future work may investigate the ability of OptoWnt cells to differentiate into other cell types achieved via Wnt pathway modulation.

Further investigations should look at the gene expression levels of OptoWnt cells. It should be investigated if the cells display any unusual markers that may be attributed to the constitutive expression of Cry2-LRP6c. The cell line being able to maintain a pluripotent state is a good start to this investigation. If the cells were under stress from the constant expression of this transgene, we would anticipate that the cells would spontaneously differentiate and would not remain proliferative in culture[44]. Any findings of potential stress indicators as a result of the constitutive expression of Cry2-LRP6c would provide valuable insight into micro-physiological effects that this approach may have on other cells.

4.2 Applications of OptoWnt Endothelial Progenitor Cells for Future Work

In order for a tissue to be effective in orthotopic transplantation, it must have a proper supply of blood. Similarly, for the scaling-up of lab grown tissues for transplantation experiments, vascular supply is necessary. OptoWnt-derived endothelial cells provide as source of vascularization for creating physiologically relevant tissues. With the spatial patterning capability that the LAVA device allows, more physiologically relevant tissues may be achieved. A next step in this investigation may examine the potential of OptoWnt-derived endothelial tissue to benefit in vascular repair[45].

Future analysis to be done with OptoWnt-derived endothelial cells includes performing functional assays to demonstrate the phenotype of these cells. Function assays include acetylated-

LDL uptake[26] and vascular tube formation[46], which may further demonstrate the mature phenotype achieved by cells derived from the optogenetic differentiation of hPSCs.

4.3 Other Applications of Optogenetics in hPSCs

The approach discussed in this study is flexible. Additional cell lines may be engineered for optogenetic control of other developmental pathways. However, a requirement is that the receptor protein must have a similar mechanism to LRP6c, which may be recapitulated by the light-activated aggregation of Cry2. For example, Humphreys *et al*. has created a system in which the BMP signaling pathway was activated via optogenetics[19]. With the increasing availability of stem cell lines with different optogenetic pathways, tissue development will have access to more control in permitting the sequential addition and differentiation of different stem cell subpopulations.

APPENDIX A

Table A.1. Values for CD34+% in comparison of endothelial progenitor differentiation from stem cell lines[43]. Data from Bao et al. *Stem Cell Research* 2015.

Cell Line	Wnt Activation	CD34+ Endothelial Progenitors %	Standard Dev.%
H9 OptoWnt	Light	21.8	±0.7
H9 ESCs	CHIR	20.4	±2.2
19-9-11 iPSCs	CHIR	25.2	±2.2
19-9-7 iPSCs	CHIR	22.5	±1.8
6-9-9 iPSCs	CHIR	20.6	±1.4
H1 ESCs	CHIR	25.9	±1.2
dH13 ESCs	CHIR	32.0	±1.6
H14 ESCs	CHIR	20.5	±1.9

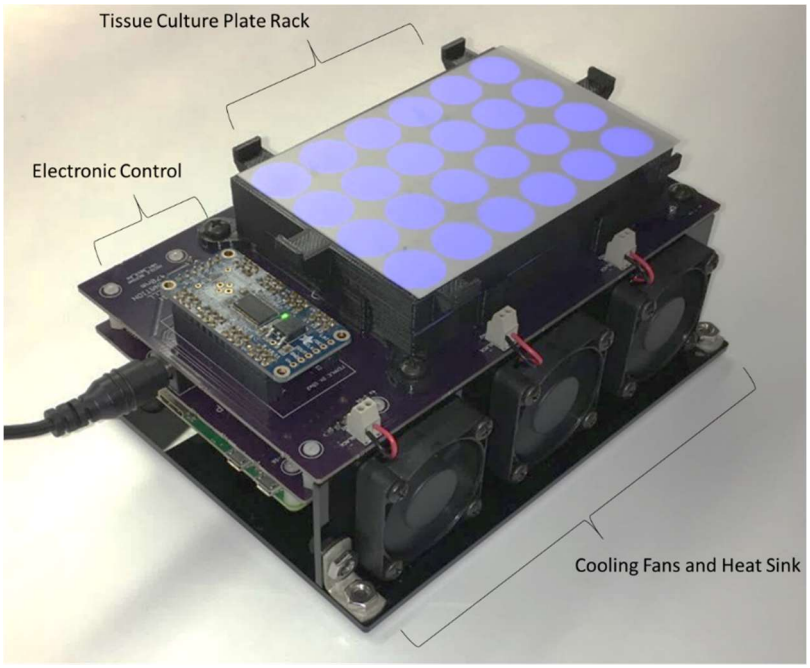

Figure A.1. Descriptive Diagram of Light Activation at Variable Amplitude (LAVA) device. Cells are placed on top of the tissue culture plate rack in a 24-well tissue culture plate. If spatial patterning is desired, a regionally-opaque and regionally-transparent film is placed underneath the culture plate, leading to spatially-controlled activation of the Wnt pathway.

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