Manipulation of Growth Factors and ECM to Optimize Patency and Endothelialization of iPSCs

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ABSTRACT

Coronary artery bypass graft surgeries are among the most common heart surgeries. While synthetic vascular grafts are used, autologous vascular grafts are favored due to the lack of immune rejection from the patient. However, current autologous vascular grafts primarily use direct tissue extraction placed onto arteries, which may lead to adverse long-term effects on the patient. Thus, in-vitro-derived vascular grafts are needed. One of the methods used to produce a vascular graft is iPSC-derived endothelial cells. However, although it is known that iPSCs may induce differentiation into endothelial cells using growth factors, the optimal conditions for doing so have yet to be understood. This proposal aims to identify the optimal combination as well as the sequence of growth factors and extracellular matrix signals to yield the greatest patency and differentiation of iPSCs into endothelial cells inside extracellular matrices.

1. Introduction

1.1: Background

Annually, up to 400,000 patients in the United States receive Coronary Artery Bypass Graft (CABG) surgeries (Baystate Health, 2024). Regenerative medicine of the vascular system has focused on restoring or reversing vascular aging, promoting new blood vessel growth, and restoring the proper structure and function of arteries. These efforts have yielded advancements in synthesizing grafts, providing patients with the option to use synthetic or cultured grafts for

patients. Synthetic grafts are often made of materials combining ceramics, composites, bioglasses, polymers, and metals (Himed, 2024). Of these, polyethylene terephthalate (Dacron) and Expanded Polytetrafluoroethylene (ePTFE) are significant components of vascular graft synthesis (Ravi, Chaikof, 2010). *Dacron*is is a polymer produced from polymerizing ethylene glycol and terephthalic acid (Qiu et al., 2024). ePTFE comprises water, chloroform, fluorspar, and hydrofluoric acid (Mahmoud, Sherif, and Widrich, 2023). While Dacron and ePTFE are considered non-toxic to the human body, allowing new blood vessel linings to grow over time, they pose a significant limitation. Due to the synthetic nature of these materials, they may cause immune rejection, infection, and stenosis of the vascular wall (Shakeel et al., 2023). Thus, autologous vascular grafts are favored over synthetic ones.

Most commonly, autologous grafts used for CABG procedures are extracted from a different part of the body, often from the leg, arm, or chest, which are then attached to the blockage area of the artery (Mahmoud et al., 2023). While this method is common, side effects of vein harvesting for CABG may result in damage to nearby nerves and blood vessels, potentially resulting in blood clots in the area of harvest (Dick et al., 2010). Thus, research has moved away from vein harvesting into stem cell vascular grafts as a less invasive method of graft production.

Stem cells derived from the patient are used to create autologous vascular grafts. These stem cells can be harvested from the bone marrow or blood, which are then cultured in vitro to increase their quantity of differentiation into endothelial cells (Lucie et al., 2018). Endothelial cells form the inner lining of all blood vessels. They function to regulate nutrient and signal exchange to the surrounding tissue, regulate homeostasis, maintain blood fluidity, and help muscles in arteries contract or relax. Autologous vascular grafts created with stem cells derived from bone marrow and blood are advantageous as they prevent immune rejection and mimic blood vessel conditions. However, despite current research, quantification of hematopoietic stem cells (HSCs) and bone marrow-derived stem cells for research remains challenging (Stanford Medicine, 2023).

Another method of deriving stem cells is through embryonic stem cells (ESCs). ESCs are pluripotent, which allows them to differentiate into various cells for research. In addition, ESCs

can be well-maintained in cultures for expansion, which makes ESCs ideal for stem cell research. However, ESCs are largely unused for graft production due to the inefficiency of acquisition, primarily through surgical removal of embryonic cells. In addition, there may be ethical concerns regarding the usage of embryonic stem cells due to debates about at what stage an embryo can be considered a human.

1.2: Induced Pluripotent Stem Cells:

A recent method of obtaining endothelial cells for autologous grafts is induced Pluripotent Stem Cells (iPSCs). iPSCs are derived from the dedifferentiation of the skin or blood cells of the patient back into stem cell form, which is then differentiated into endothelial cells for vascular graft synthesis. iPSCs can differentiate into all three layers of the germ layer, endoderm, ectoderm, and mesoderm, and subsequently can be differentiated into endothelial cells. The advantage of iPSCs is that post-treatment immunosuppressants are minimal or unessential. Using immunosuppressants has many adverse effects on the patient. Physical implications include an increased potential for infections, cardiovascular hypertension, impaired wound healing, etc. Physiologically, mood swings, insomnia, and depression may occur (Ruiz R, Kirk AD, 2015). In addition, immunosuppressants must be taken for years or up to the entire life span of a patient. While the price of immunosuppressants may increase or decrease depending on the type of immunosuppressants used, the costs range between $5,000-$10,000 per year (Kadatz et al, 2019). Thus, interventions with iPSCs are favored. When producing iPSCs, Yamanaka Factors (Transcription Factors) are used to reprogram cells back into iPSCs. The octamer-binding transcription factor-3/4 (Oct 3/4), SRY-related high-mobility group (HMG)-box protein-2 (Sox2), Klf4, and C-myc are often used.

Oct ¾ induces differentiation of iPSCs into endothelial cells. Oct ¾ first binds to motif site OCT, which comprises nucleotides ATTTGCAT. Then, the recruitment of co-activators leads to increased gene expression, thus upregulating hypoxia-inducible factor 1α (HIF), which aids in maintaining the pluripotency of iPSCs (Takahashi et al., 2014). Oct ¾ is a crucial GF for maintaining the pluripotency of iPSCs. Sox2 is a key transcription factor that functions similarly

to Oct ¾. The function of Sox2 is to regulate pluripotent genes involved in iPSCs by either activating or suppressing gene expression (Smith et al., 2009). By doing so, Sox2 can maintain the pluripotency of iPSCs. Sox2 cooperates with Oct ¾ by forming a binary complex that attracts nearby nuclear factors such as Nuclear Factor 1 (NFI), which prevent iPSCs from differentiating. Unlike Sox 2 and Oct ¾, C-myc enhances the efficiency of reprogramming bone marrow or blood cells into iPSCs. In addition, C-myc regulates CDKs (Cyclin-Dependent Kinases), which are used in the cell cycle checkpoints to regulate cell cycle progression. Overall, C-myc is used to reprogram somatic cells (Akifuji et al., 2021). Klf4 is another crucial transcription factor. It is used to maintain the pluripotency of iPSCs by regulating genes that promote pluripotency while deactivating genes used to differentiate iPSCs (Nishimura et al., 2014). Also, Klf4 can be used for reprogramming somatic cells into iPSCs. In addition to transcription factors, some molecules can enhance chromatin remodeling, enhancing the efficiency of iPSC reprogramming. Valproic acid, a histone deacetylase inhibitor, works with Oct ¾ and Sox2 to prevent iPSCs' differentiation into specialized cell types (Zhai et al., 2015).

1.3: Growth Factors

Currently, research has identified four primary GFs used for endothelialization. These GFs have the properties to enhance endothelial cell proliferation, migration, or differentiation of iPSCs into endothelial cells. These GFs are Basic Fibronectin Growth Factors (bFGF), Transforming Growth Factor-β (TGF-β), Hepatocyte Growth Factor (HGF), Insulin-Like Growth Factor (IGF), Notch1, and Vascular Endothelial Growth Factor (VEGF).

The first GF discussed, which aids in iPSC proliferation, migration, and differentiation (Duan et al., 2020), is bFGF. Using bFGF, endothelial cells produced with iPSCs can proliferate and integrate into the extracellular matrix, promoting angiogenesis. bFGF binds to the surface of endothelial cells and activates signaling pathways FGF-R1 and FGF-R4 (Ramos et al., 2015). This indirectly influences gene expression in endothelial cells, thus promoting cell proliferation.

The next growth factor is Transforming Growth Factor-β (TGF-β). It binds to receptors on endothelial cells, activating signaling pathways such as MAPK. Once the signaling pathways

have been activated, it encourages endothelial cells to migrate and proliferate, thereby promoting angiogenesis inside the extracellular matrices (Zachman et al., 2016). TGF-βs can also interact with VEGF, promoting the growth and migration of endothelial cells inside the ECM. Specifically, TGF-βs stimulate VEGF production, thus promoting the migration of endothelial cells inside the extracellular matrices (Goumans et al., 2008).

Hepatocyte Growth Factors (HGF) are normally used to differentiate stem cells into liver cells (Park et al., 2020). However, recent studies have revealed that HGF can also be used to increase the proliferation, migration, and survival rates of endothelial cells. Specifically, when under high dosages of HGF (100-250 ng/ml), HGF activates pathways such as STAT, MEK, and Akt. Thus, HGF can be used after iPSCs have been differentiated into endothelial cells to improve the overall outcome of cell survival (Frankl et al., 2024).

Insulin-like Growth Factor (IGF) promotes angiogenesis by increasing migration and proliferation of endothelial cells inside of an extracellular matrix. Endothelial cells migrate inside the matrix, forming tube-like structures that create a network appearance. IGF also upregulates VEGF, which expands the size of the tube-like networks, thus stimulating angiogenesis (Nwachukwu et al., 2023). This can be utilized inside of an extracellular matrix as it allows for angiogenesis, which enables blood circulation and maintains the cells inside of the matrix.

Notch 1 contributes to the cell fate determination of iPSCs and plays a role in cell proliferation and apoptosis. Notch1 can act as a critical regulator for endothelialization to differentiate iPSCs into endothelial cells. It achieves this by first promoting tip and stalk cell differentiation. Afterward, Notch1 promotes angiogenesis by allowing nascent sprouting when the tip and stalk cells grow toward Notch1 signals and create a vascular branch (Jiao Tian et al., 2018). By controlling various target genes that determine cell physiology and acting as a GF for angiogenesis, Notch1 allows vascular stabilization and the differentiation of iPSCs.

VEGF, a key player in promoting angiogenesis and regulating vascular permeability, holds significant promise in the field of regenerative medicine. VEGF is known to have properties that promote angiogenesis and induce tissue growth (Ahmad et al., 2022) by

activating MAP kinase pathways and PKB/Akt pathways and releasing endothelial nitric oxide, which elevates the rate of endothelialization of iPSCs.

Despite the knowledge that iPSCs may induce differentiation into endothelial cells using growth factors (GFs), the exact conditions for optimizing GF composition and concentration on iPSC differentiation are yet be understood. To be precise, while individual GF functionalities are known, some GFs have similar functions, while others have completely different uses. By identifying the GFs that most efficiently help differentiate iPSCS into endothelial cells, current vascular graft interventions could see a decrease in infection, immune-rejection, and thrombosis (blood clot) rates in addition to an increased patient life expectancy.

1.4: Extracellular Matrices

GFs alone cannot enhance iPSC reprogramming into functional vascular grafts; extracellular matrices (ECMs) are needed. ECMs comprise proteins, signaling molecules, carbohydrates, and enzymes (Shi et al., 2023). These materials surround cells to provide structural stability and GFs to promote cell growth and homeostasis (Kim et al., 2016).

The primary structural proteins composing ECMs include collagen (the common types of collagens used in vascular graft ECMs are Collagen Type I, Type III, and Type IV), fibronectin, and laminin (Kular et al., 2014). These proteins facilitate the adhesion of cells to the ECM and offer mechanical support for attached cells in the ECM. *Collagens* are triple-helical, structural proteins that give tensile strength properties, such as cell migration, adhesion, and proliferation, to the ECM. They bind to integrins to facilitate cell signaling (Fidler et al., 2018). This is crucial for the iPSC differentiation into endothelial cells, as iPSCs are provided with a microenvironment that mimics blood vessel structural properties, which is suitable for cell adhesion and proliferation. *Fibronectin is* a dimeric glycoprotein that binds to multiple cell surface receptors of iPSCs and ECM components. It enhances cell migration, adhesion, and proliferation of iPSCs inside ECM (Hsiao et al., 2017). Fibronectin stimulates the expression of endothelial cell-specific genes in iPSCs, allowing for differentiation into endothelial cells. *Laminin* is a tri-disulfide polypeptide that promotes cell adhesion, differentiation, and migration. It is a crucial component

in the ECM because it creates the basement membrane, which is a sheet-like tissue that separates various types of cells. This provides a microenvironment suitable for signaling and homeostasis of endothelial cells (Amato, 2014).

The carbohydrates in ECMs are most commonly glycosaminoglycans (GAGs), heparan sulfate, and hyaluronic acid. These carbohydrates aid in maintaining ECM homeostasis, including maintaining hydration for cells (Kim et al., 2016). Structurally, GAGs bond with collagen and other proteins and increase the tensile strength of the ECM. GAGs also interact with integrin to facilitate cell signaling alongside collagen. GAGs can bind to GFs like VEGF to facilitate their activity in the ECM. Overall, glycosaminoglycans play a significant role in cell migration, differentiation, and signaling. Heparan sulfate is a GAG composed of disaccharides and uronic acid. Heparan sulfate can bind to integrins to promote cell adhesion and migration of iPSCs (Silva, et al, 2019). It does so through a co-receptor that prevents degradation and releases signaling molecules to allow iPSC differentiation. In addition, it regulates cell signaling by binding to GFs. Hyaluronic acids are composed of disaccharides and glucuronic acid. They help maintain an ECM microenvironment suitable for iPSCs due to their ability to attract water molecules (Garantziotis et al.; Savani, 2019).

Major enzymes seen in ECMs are matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs). These enzymes regulate ECM remodeling, which is crucial for tissue development and repair when developing the ECM into a vascular graft. MMPs act as a degrading enzyme that breaks down structural proteins like collagen. Doing so is crucial for tissue development and angiogenesis since during these processes the ECM needs to be broken down and replaced by endothelial cells over time. This thus creates space for endothelial cells inside the ECM. TIMPs function as an inhibitor for MMPs, preventing MMPs from excessively degrading the ECM. This would otherwise cause damage to iPSCs and thus prevent differentiation. Thus, TIMPs can ensure a stable micro-environment for the growth of endothelial cells (Cabral-Pacheco et al. et al., 2020).

1.5: Differentiation Work in iPSCs Has Been a Limiting Factor

Research on iPSC differentiation into endothelial cells has had significant success. However, the differentiation process has three major limiting factors, the first being efficiency. The differentiation process produces low amounts of endothelial cells needed for vascular grafts. This means the cell culturing process may take longer than the ideal rate to produce functional vascular grafts for the market (Zhong et al., 2022). Due to this, further development is needed to optimize the combination and sequence of ECM signals and GFs to create an ideal microenvironment that maximizes healthy iPSC differentiation and endothelial cell growth. The second limiting factor is tumorigenicity. Factors such as incomplete reprogramming and improper differentiation of iPSCs can create cancer cells rather than endothelial cells, forming tumors (Sarker et al., 2024). Further research in optimizing the growth conditions of iPSCs in ECMs may increase the patency rate and prevent tumor formation. This includes the GFs and ECM signals iPSCs are exposed to. An optimized ECM condition may decrease tumorigenesis. The third limiting factor is functionality. Endothelial cells differentiated from iPSCs may lack endothelial cell-specific surface markers and have reduced capabilities of angiogenesis due to incomplete differentiation (Williams and Wu, 2019). Incomplete differentiation results from lack of nutrition, growth factors, or when differentiation sequence errors occur for iPSCs. Research to optimize ECM signals and GFs may decrease the concerns about iPSC-differentiated endothelial cells. This raises the question: How can growth factors and ECM signals be manipulated to optimize the patency and endothelialization of iPSCs?

This proposal describes a series of experiments that will address this question using different combinations and sequences of GF and ECM signals to find the optimized environment for endothelial cell growth within the ECM. Upon optimization, the ECM can be used to synthesize autologous vascular grafts for clinical applications.

2. Experimental Design

The investigation will be split into two sections. The first set of experiments will prioritize optimizing the ECM signals and GFs crucial for iPSC differentiation into endothelial cells. This will be measured based on a comparison of both the quality and quantity of in-vitro

endothelial cell characteristics and native (host) endothelial cell characteristics. This approach will allow us to understand which ECM signals and GFs are most needed to optimize the ECM's efficacy for vascular graft production. The iPSCs will be introduced into the porcine dECM (a type of ECM), where ECM signals and GFs will interact with iPSCs to develop into endothelial cells. Under the dECM conditions, iPSCs are expected to differentiate into endothelial cells and thus exhibit endothelial cell surface markers such as CD34, CD151, CD143, and Von Willebrand Factors A1 and A2. Also, it is expected to form tube-like structures that resemble native capillary vessels. To identify if the endothelial cells are optimized, immunostaining will be conducted to detect the presence of endothelial cell surface markers. Functional Assays will be conducted to verify the presence of tube-like structures. An automatic cell counter will be used to analyze the cell density of endothelial cells inside the dECM to conclude the proliferation rate of endothelial cells inside the dECM. The second set of experiments will optimize the sequence of ECM signals and GFs added, thereby creating an effective method of utilizing GFs and ECM signals for different stages of iPSC differentiation inside the ECM. Each sequence will have the same set of experiments. The methods for determining optimization will be identical to the first set of experiments.

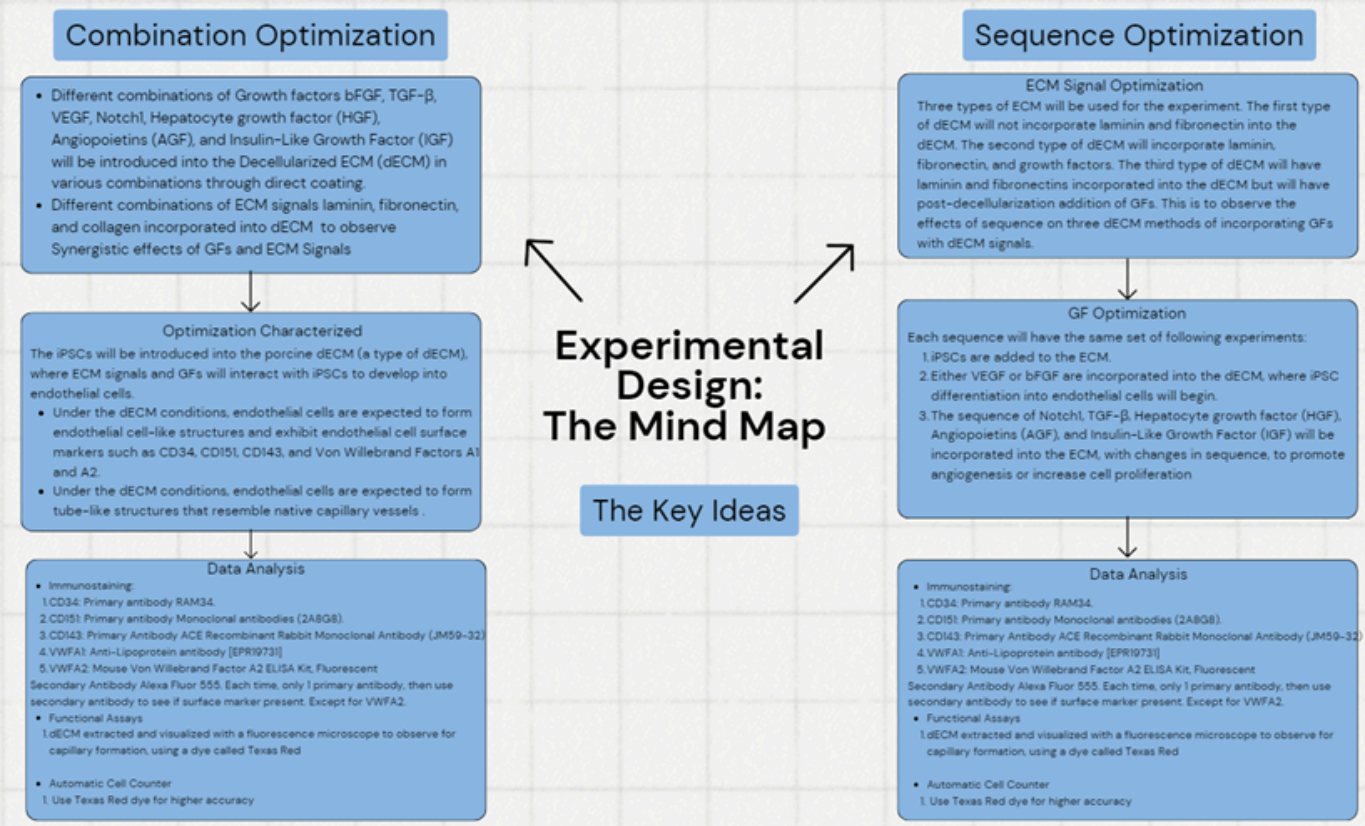

Figure 1: Experimental design mind map to demonstrate combination and sequence experiment layouts. Each experiment is separated into three sections: Experiment overview, optimization method, and data analysis

3. Methodology

3.1: ECM Signal and Growth Factor Optimization

3.1.1: iPSC Culture

Cell cultures of iPSC cell lines will be purchased from ALSTEM Cell Advances. Cells will be grown in the iPSC Essential 8™ Medium, developed by Chen et al., 2011 and sold by ThermoFisher, for cell culture expansion. In order to maintain cell pluripotency, Basic Fibronectin Growth Factors (bFGF) will be added. To maintain cell adhesion, Matrigel (Corning), invented by

Hynda Kleinman, will be put into the growth medium. The incubation process will be conducted in a humid incubator with a temperature of 37°C and a CO_2 concentration of 5%.

3.1.2: Extracellular Matrices Preparations

Xenogenetic decellularized extracellular matrices (dECM) will be prepared for iPSC experimentation. The dECM used is the porcine dECM. Porcine menisci will be derived from a local slaughterhouse, frozen (-20° C or lower), and stored. Following the protocol by Porzucek et al. (2024), the porcine dECM will be made by first washing heart valves obtained from slaughterhouses. Then the cell membranes will be removed with a detergent, and DNA/RNA are to be degraded with pepsin enzymes. Finally, after removing the detergent and enzymes from the ECM, the ECM is dehydrated to become a porcine dECM. However, while dECM contains collagen, it lacks laminin and fibronectin in its composition. Thus, laminin and fibronectin will be reintroduced into the dECM for experimentation through direct coating.

3.1.3: iPSC Differentiation and Characterization

To determine optimization, three criteria of the dECM need to be fulfilled. First, by using an automatic cell counter, endothelial cells inside the dECM can be counted to calculate the density and condition of endothelial cell growth and proliferation (endothelial cells will be dyed prior to cell counting for higher accuracy). If optimized, the dECM should contain a cell density of 2468–2892 cells/mm2 (Lass, et al., 2010). Secondly, using immunostaining, accurate identification of endothelial cell surface markers inside the dECM could be done to measure whether the iPSC-derived endothelial cells exhibit native endothelial cell properties. If endothelial cells exhibit all cell surface markers, the process is optimized. Lastly, using functional assays, the formation of capillaries can be identified. Cells will be dyed and visualized under a fluorescence microscope. If the process is optimized, angiogenesis (formation of blood vessels or capillaries) should occur.

To establish and optimize iPSC differentiation, growth factors TGF-β, VEGF, HGF, IGF, Notch1, and bFGF will be introduced into the ECM in various combinations. The iPSCs will be introduced into the porcine dECM, where ECM signals (laminin, fibronectin, and collagen) and GFs will interact with iPSCs to develop endothelial cells. Under the ECM conditions, endothelial cells are expected to form endothelial cell-like structures and exhibit endothelial cell surface markers such as CD34, CD151, CD143, and Von Willebrand Factors A1 and A2. In addition, under the dECM conditions, endothelial cells are expected to form tube-like structures that resemble native capillary vessels.

3.1.4: Data Analysis

Quantitative and qualitative data analysis will be conducted. Quantitative analysis will observe endothelial cell density inside the dECM to track iPSC proliferation rates via cell counting. BioProfile FAST CDV (NOVA Biomedical) is a reliable and efficient tool for counting iPSC cell differentiation into endothelial cells. It can analyze 45 samples/hour, each containing 100μL of samples. The samples are prepared by coating the Texas Red dye onto a slice of the dECM. The Texas Red dye binds to primary antibodies of the CD34 endothelial cell surface marker. This allows the automatic cell counter to excite the Texas Red with a laser, thereby counting the quantity of iPSC-derived endothelial cells. Qualitative analysis is conducted using functional assays and immunostaining. Functional assays evaluate the level of capillary formation in the dECM, which allows inference of the quality of the dECM in creating a functioning vascular graft. The dECM will be extracted and visualized with a fluorescence microscope to observe capillary formation, using Texas Red dye to visualize the sample. Immunostaining is used to evaluate and identify endothelial cell surface markers inside the dECM to identify the quality of endothelialization. A primary antibody from rabbits and a secondary antibody derived from goats will be used for immunostaining. The secondary antibody will be labeled to give it fluorescence. For endothelial cell surface marker CD34, primary antibody RAM34 will be used. For endothelial cell surface marker CD151, primary antibody monoclonal antibodies (2A8G8) will be used. For CD143, primary antibody ACE Recombinant Rabbit Monoclonal Antibody (JM59-32) will be used. For VWFA1, Anti-Lipoprotein

antibody [EPR19731] will be used. For VWFA2, Mouse Von Willebrand Factor A2 ELISA Kit will be used. The secondary antibody will be Goat anti-rabbit IgG (H+L), labeled with Alexa Fluor 555 for all cell surface markers, except for VWFA2. To conduct immunostaining, one primary antibody will be used per experiment to prevent multiple staining of cell surface markers.

3.1.5: In-Vivo Evaluation of dECM-Derived Vascular Graft

If data analysis of the dECM is promising, in-vivo testing of the efficacy of the vascular graft will be conducted on nude immunodeficient mice. Immunodeficient mice are crucial for minimization of immune rejection of the vascular graft (Radaelli et al., 2018). The mice will be anesthetized, and the vascular graft will be implanted into the aorta through an incision into the groin area of the mice (Boston University, 2024). Post surgery, the mice will be monitored daily for signs of thrombosis. If the mice show no signs of infection and the wound becomes fully healed, the next steps of experiments can continue. The next set of experiments will be oriented towards subjecting the mice to physical stress tests. Specifically, since exercise causes increased pressure in the blood vessels, the blood flow dynamic of the graft could be implicitly tested by making mice run on a mice wheel. It is expected that the graft could withstand the changes in blood pressure, demonstrating its compatibility with the host.

3.2: Growth Factor Sequence Optimization

3.2.1: Extracellular Matrices Preparations

Instead of reintroducing laminin and fibronectin into the dECM simultaneously like **Step 3.1.2,** two sets of dECM will be prepared. The first set will introduce laminin, and the latter will use fibronectin as the first. This is to observe how ECM signal sequence impacts iPSC differentiation and endothelial cell proliferation.

3.2.2: Sequencing Experimentation

Three types of ECM will be used for the experiment. The first type of dECM will not incorporate laminin and fibronectin into the dECM. The second type of dECM will incorporate laminin, fibronectin, and growth factors. The third type of dECM will have laminin and fibronectins incorporated into the dECM but will have post-decellularization addition of GFs. This is to observe the effects of sequence on three dECM methods of incorporating GFs with dECM signals. Each sequence will have the same set of following experiments:

1. iPSCs are added to the ECM.
2. Either VEGF or bFGF are incorporated into the dECM, where iPSC differentiation into endothelial cells will begin.
3. The sequence of Notch1, TGF-β, will be incorporated into the ECM, with changes in sequence, to promote angiogenesis.

3.2.3: Optimization Characterization

Immunostaining will be conducted to optimize iPSC differentiation into endothelial cells and identify endothelial cell surface markers. Significant endothelial cell surface markers are CD34, CD151, CD143, vWFA1, and vWFA2. Identical protocols from **Step 3.1.4.** will be used here. Functional assays (migration assays) will be conducted to identify capillary formation. Specifically, by time-lapsing the migration of endothelial cells inside the dECM over the course of 7 days, the presence of tube-like structures can be identified. Identical protocols from **Step 3.1.4.** will also be used here.

3.2.4: Data Analysis

Data analysis will follow the identical protocols as **Step 3.1.4.**

3.2.5: In-Vivo Evaluation of dECM-Derived Vascular Graft

If data analysis yields promising results, **Step 3.1.5** will be conducted, but with the vascular graft derived from the ECM post-sequence optimization.

3.3: Biohazard Safety

3.3.1: BioSafety Lab

To ensure safe handling of biomaterials, the laboratory is classified as Biosafety Level 2 (BSL-2) and Animal Biosafety Level 1 (ABSL-1). This means that access to the laboratory is restricted and regulated under constant video surveillance. Research personnel are required to wear appropriate protective equipment. PPE items, which include lab coats, latex gloves, surgical masks, and goggles, are to be worn while conducting research to prevent contamination of samples and harm to researchers. In addition, respirators are required when operating with dangerous agents.

3.3.2: Lab Protocols

The National Institute of Health (NIH) provides a lab safety and regulation protocol that includes guidelines for handling iPSCs and biohazardous materials and maintaining a clean laboratory workspace.

3.3.3: Storage Protocols

Storage protocols follow the American Type Culture Collection Guidelines, which provide procedures for safely handling materials and samples.

3.3.4: Waste Disposal

The cleaning protocol for tables and cabinets may use 70% Ethanol or Sodium Hypochlorite, which disinfects surfaces from spills, stains, and other materials. Waste disposal is strictly regulated based on the EPA Waste Disposal Guidelines. The guidelines include protocols for proper biohazardous waste disposal, organic and inorganic disposal, and medical waste disposal.

3.4: Acquisition and Culture of iPSCs

Episomal iPSC cell lines will be acquired from ALSTEM Cell Advances. The cells will be cultured in iPSC Essential 8™ Medium (ThermoFisher). Growth factors TGF-β, bFGF, Notch1, and VEGF are all acquired from Bio-Techne R&D Systems.

3.5: Acquisition of ECMs

The Xenogenetic Decellularized Extracellular Matrices (ECM) will be made following the protocol of Porzucek et al. (2024). One KG of porcine menisci will undergo a first round of water extraction under room temperature and constant mechanical stirring overnight. Next, the second extraction phase will be conducted using lactic acid under the same conditions as the first extraction to further extract porcine menisci. Next, the porcine menisci will undergo filtration bag extraction and hydrolysis three times. Following this, a lyophilization process, where the filtrated porcine menisci will be rapidly frozen and then vacuumed, will occur to turn the filtered sample into powder form. Subsequently, the powder will be processed through a cryo-miller three times. Lastly, the sample will be strained, undergo three cycles of centrifugation, supplied with anhydrous ethanol, and decompressed and sealed in an extraction vessel.

3.6: Acquisition of Vascular Graft on Mice

Nude immunodeficient athymic mice (Charles River) are optimal for evaluating vascular graft efficacy because they significantly decrease the immune rejection rate, allowing for direct testing of the efficacy of the vascular graft of mice's aortas.

4. Discussion

In the domain of tissue engineering technologies, it is crucial to acknowledge that further research in endothelial cell properties, such as discoveries of new growth factors or alternatives to current dECM models, could further optimize iPSC differentiation into endothelial cells. Since the effects of optimizing combinations and sequences of ECM signals and GFs remain unknown, further research is required to uncover the synergetic effects of GFs and ECM signals to solidify an optimized dECM vascular graft. Experimentation to optimize the GF and ECM signal combination and sequence is expected to yield high patency and efficient iPSC-derived dECM vascular graft. This is due to the optimized growing conditions of iPSC-derived endothelial cells, which leads to proper cell density and the presence of blood vessels inside the dECM. Both are crucial for maintaining graft health and conditions once grafted onto an individual. While there are some options to use vascular grafts for disease treatment, an optimized dECM-derived vascular graft would offer new treatments for coronary heart disease, ischemic heart disease, and more. The dECM-based vascular graft could make CABG surgeries more effective for treating cardiovascular diseases, such as ischemic heart disease (which causes a narrowing of heart arteries), since it more closely resembles healthy native tissue. Thus, the optimized dECM would yield new optimal interventions for CABG compared to traditional methods, such as synthetic grafts.

If optimizing the combination and sequence of ECM signals and GFs succeeds, the intervention may pave the way for further research in cardiovascular tissue engineering. Precisely, success in this area would foster research towards graft synthesis of other tissues and organs to treat diseases. For example, the liver contains a complex network of blood vessels, which makes it difficult for current liver graft interventions to replicate. If angiogenesis is optimized inside of the dECM for the vascular graft, this process may be imitated to create

functional grafts for the liver, and perhaps organs, thus treating various diseases. Compared to other current interventions of ECM-derived grafts, the dECM should have decreased infection, immune-rejection, and thrombosis rates. This would lead to increased patient life expectancy. By creating an optimized dECM-derived vascular graft, the long-term outcomes of CABG surgery patients may be improved, and new fields of research towards optimizing other grafts for disease treatment can be within reach.

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