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ADVANCING CARDIAC TISSUE ENGINEERING FROM HUMAN PLURIPOTENT STEM CELLS BY INCORPORATION OF EPICARDIAL AND FIBROBLAST CELL POPULATIONS

By

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Chapter 1: Introduction to hPSC Cardiac Cells and Project Outline

I have written this chapter to explain my research to a broad audience. The end goal of science is to help people by developing new technologies and gaining a better understanding of the world. As such, I think it's important to be able to communicate science to a non-scientific audience. I've formatted this chapter in the form of question and answers so that you can easily find sections that seem interesting to you, and hopefully you will enjoy learning a little bit about what I've been working on. I would like to thank the Wisconsin Initiative for Science Literacy at UW-Madison for supporting the creation of this chapter and helping me develop my communication skills.

What is a thesis?

A thesis is a long document (usually 100+ pages) that serves as a record of everything that a PhD graduate student has accomplished. I was always told that I was a slow learner. Why else would I go to school for another five years after getting an undergraduate degree to write a long boring document?

I like to remind people that a PhD is not like an undergrad degree where you spend most of your time doing homework and taking exams. I usually spent my days doing 4-6 hours of laboratory work, 1-2 hours of reading or hearing about other people's work, and 1-2 hours of analyzing and preparing my data to share with others. In the beginning, I took some courses in chemical and biological engineering and other fields that are important to my work, such as developmental biology. After finishing my courses, I transitioned to focusing on experiments and preparing and presenting my data. My final test, which determines if I can get my PhD, is this long document. A panel of professors reads the document and drills me with questions to see if I learned

anything. If I pass (fingers crossed), I plan to get a “real job” and do biological research for a company.

What is your thesis about?

My thesis focuses on understanding more about the non-muscle cells of the heart using human pluripotent stem cells. Human pluripotent stem cells are a special type of cell which are present during early development and have the potential to become nearly any cell type of the body. I settled on this topic as my professor’s group focuses on developing “recipes” to make heart and brain cells from human pluripotent stem cells. When I joined the lab, we had just developed a protocol to make some of the non-muscle cells in the heart. I optimized this “recipe” and then used these cells to answer interesting questions. In the next few sections, let’s delve a little more into what my project is about.

Why study the heart?

Heart disease covers many conditions affecting the heart’s function and structure and is the leading cause of death in the United States. One symptom of heart disease is a heart attack, although heart disease also includes just high blood pressure. Most heart disease cases can be prevented through not smoking, physical activity, eating a healthy diet, and controlling blood pressure levels (Benjamin Emelia J. et al., 2019). About 31% of adults over the age of 20 suffer from high blood pressure and 9% additionally suffer from stroke, heart failure, or other forms of heart disease (Benjamin Emelia J. et al., 2019). This is where my work comes in; we want to further study early heart development and investigate potential treatment options for heart disease.

Why use human pluripotent stem cells?

Human pluripotent stem cells (hPSCs) provide a proliferative and scalable heart cell source. Typically, hPSCs are defined by two properties 1) capability for self-renewal (able to be

expanded for years in a laboratory) and 2) pluripotent (can make all tissues of the human body). In the past 25 years since isolation and generation of hPSCs, many protocols have been developed to generate nearly every cell type of the body. hPSCs provide a platform for studying early human development, such as what happens as the embryo develops during the first five weeks. Another opportunity is to generate hPSCs from patients to study disease progression, along with differences between patients. Lastly, hPSCs can be used to study whether a drug works and whether that drug causes undesirable effects, such as damaging the heart. What we are most excited about is that hPSCs can provide us with a human specific system to complement animal studies. Additionally, since we don't have the whole animal, we can ask simple, direct questions such as how a drug affects the heart muscle cells (not including the effect on non-muscle heart cells, blood flow rate, the kidney, the brain, etc.).

We can also make engineered tissues derived from hPSCs that can be used clinically to restore damaged tissues. By 2019, 54 hPSC-derived cellular products were in clinical trials with over half of these therapies targeting eye diseases (Kobold et al., 2020), and currently, there are two clinical trials targeting heart diseases (Help Therapeutics, 2021; Toda, 2021). Although there currently aren't any government approved hPSC cell therapies, maybe someday you will be treated with hPSC tissues to replace or repair a failing organ.

How big is a cell?

Well, your human body has approximately 30,000,000,000,000 cells, so that makes them pretty small (Bianconi et al., 2013). Let's look at my hPSCs (Figure 1-1). I typically keep my cells on a plate (approximately 4 in by 6 in) in an incubator, a low-temperature sauna for cells, (approximately 3 ft x 4 ft x 3 ft). On a given plate, there are usually 10,000,000 cells, and a stem cell size is on the scale of microns. This means that I need a microscope to even see my cells!

How do you get your human pluripotent stem cells?

People are often worried when I tell them I'm working with stem cells. Let me tell you a little bit more about them. hPSCs can be taken from the early embryo (known as embryonic stem cells) (Thomson et al., 1998). These samples are typically obtained from discarded artificial insemination treatments. Alternatively, adult patient's skin and blood samples can be manipulated in the lab so that they become embryonic-like in a process called "reprogramming" (Takahashi and Yamanaka, 2006; Yu et al., 2007). Researchers named the resulting cell type "induced pluripotent stem cells" since they were not obtained from an embryo. This is a relatively non-invasive process for the patient and still allows researchers to use hPSCs for their studies. In fact, next time you donate blood, you may also be asked if it would be okay to use some of that blood to generate hPSCs.

As part of my training, I've had to take ethics training and courses where we discuss how to appropriately use hPSCs following the government regulations and how that fits into our own moral viewpoints. Regulations on hPSCs vary significantly between countries and there is no consensus. Current U.S. government regulations do not allow for further generation of embryonic stem cell lines using government funding; we can only use the cell lines approved by the National Institutes of Health (Ludwig et al., 2018). As a result, many labs, including mine, use induced pluripotent stem cells to explore new areas of research. In both cases donor agreement and confidentiality is important; donors are required to sign a consent form which allows researchers to make and/or keep hPSCs from their sample and use them for research studies or clinical trials. Donor samples are typically "de-identified" and the researcher only receives information about the donor sex, age, and if the patient had any genetic diseases. In this way, we try to make sure that

donors understand what they are providing to the scientific community and that the scientists respect what has been donated.

Do you generate your own induced pluripotent stem cells?

Although technically possible, we do not generate our own induced pluripotent stem cells. Generating these hPSCs is typically not very effective; if we are lucky, 0.01-1% of donor cells become hPSCs (Farid and Jenkins, 2018). Also, the reprogramming process takes approximately two weeks and then you need additional quality control to make sure that you still have the correct number of chromosomes, that the genome is stable, and that you have pluripotent stem cells. This validation step takes a long time and can last six months or more. (I don't want to be in school forever!)

We usually purchase our hPSC lines from a cell bank. A cell bank is to researchers is like a seed bank is to farmers. The cell bank is typically a non-profit organization which expands, validates and stores cells in liquid nitrogen (a cold liquid used for deep freezing storage). They sell these lines to academic researchers for approximately $500-$5,000, and we sign a legal agreement with the cell bank saying that we will only use the lines for research purposes. Once we receive the stem cell line from the cell bank, we can expand those cells and have enough for many (10+) years of research. If you think your deep freezer in the basement at home is full of old things, you should check out ours! Some of our samples are over 20 years old!

How do you make heart cells from hPSCs?

Our group and others have developed protocols to generate many cell types of the heart (Bao et al., 2016; Burridge et al., 2014; Floy et al., 2021; Lian et al., 2013). One way to envision differentiation is through Waddington's landscape (Waddington, 1958). Think of this as a skier going down the hill (Figure 1-2). At the top of the hill, the skier has the greatest distance to travel

and could choose to go down the hill in any number of directions and choose any side path. As the skier gets farther down the hill, they can only take paths the lead downward from the one they are currently on. Eventually, the skier reaches the bottom of the hill and must take a lift to go back up the hill. Similarly, hPSCs have the potential to become many different cell types.

Let's follow a path. We start with hPSCs and can go down one of three paths representing an early fate decision to become mesoderm, endoderm, or ectoderm. These are a temporary state which you pass through on the way to making a differentiated cell. Following the mesoderm path, we next reach a decision to become heart cells, and we lose the potential to become brain cells (ectoderm path) or stomach cells (endoderm path). Our next choice may be to become a heart cell and we lose the potential to become kidney cells. Lastly, we can choose to become a heart muscle cell. Since this is at the bottom of the path, the hPSC is said to be fully differentiated. To return to the hPSC state, the cells must be reprogrammed which is much more difficult than going downhill.

So, what does this look like in the lab? Practically, hPSCs are kept on a plate and treated with a red liquid containing salt, sugar, electrolytes, and nutrients. You can think of this like "Powerade" for the cells. We change this "Powerade" daily to keep the cells happy and alive. It usually takes me between 1-4 hours to feed all my cells on any given day (yes, that includes weekends). To differentiate the cells, we follow a "recipe" and add proteins or chemicals to direct the hPSCs toward different paths. A typical differentiation process takes 2-4 weeks and the resulting cells are similar to those found during the first trimester of human development. Honestly, it's impressive that the process of making first trimester-like cells is this simple!

Do you use special equipment to study your heart cells?

Most of the equipment that I use on a routine basis is what you would see in any lab that grows cells (Figure 1-3). We keep the cells on a plate in an incubator. This is like a low-temperature

sauna that mimics the body's conditions. When we want to work with the cells, such as giving them new "Powerade", we put them in a biosafety cabinet. The cabinet provides a clean workbench that keeps the cells away from me and me away from the cells. Sometimes I need to see what the cells look like and how they are behaving. I typically use a microscope that magnifies anywhere between 4 to 100X and where I can see anywhere between 10,000 cells down to 1 cell per field of view. Lastly, when we aren't using our cells, we keep them in long-term deep freezing storage. This storage is basically a tank full of liquid nitrogen (a cold liquid) where we submerge the cells. Have you ever seen a movie such as Star Wars *The Empire Strikes Back*, Marvel's *Captain America,* or the recent movie *Passengers* where the main character's body is frozen and they come back after a long period of time? Well, this is the same idea but for cells instead of a whole body.

What are your specific thesis questions?

There are two major research areas in the field 1) application of hPSC-cardiac cells to answer interesting questions and 2) development of new "recipes" to make specialized hPSC-cardiac cell types. My projects described in this thesis cover both topics and are described below.

Project 1: Study of Interactions between differentiating hPSC-cardiomyocytes and epicardial cells (Chapter 4)

Epicardial cells line the outside of the heart and are important for development. For example, when the epicardial cells are genetically or physically removed, the heart wall does not thicken properly and ruptures during development (Chau et al., 2011; Lie-Venema et al., 2007). Epicardial cells are also interesting as they are a progenitor cell population and can further differentiate, think of this as a skier who is not yet at the bottom of the hill. Thus, we predict that epicardial cells play an important role in communicating with developing muscle cells

(cardiomyocytes). To study this, we combined developing hPSC-cardiomyocytes and hPSC-epicardial cells in a dish and checked back after two weeks to see what changes had occurred (Figure 1-4). Both developing cardiomyocytes and epicardial cells are not fully differentiated; they are like the skier who is almost at the bottom of the hill but not yet there. Interestingly, we found that if the epicardial cells were not allowed to differentiate further, there was an increased number of hPSC-cardiomyocytes and reduced organization of muscle filaments as described in Chapter 4. If we allowed the EpiCs to differentiate (reach the bottom of the hill), the same effect on developing cardiomyocytes was not observed. Our work suggests important communication between these two cell types which can be used to alter cell fate and improve the ability to make engineered heart tissues.

Project 2: Specification of hPSC-cardiac fibroblasts (Chapter 5 and 6)

Cardiac fibroblasts are a type of heart cell which support the cardiac tissue by providing structure and sending important communication signals to other heart cells. As an analogy, they are the builders who construct the frame of a building which everything else is built upon. When I started my PhD, we had two different "recipes" to make cardiac fibroblasts. However, nobody knew if these differences in "recipes" contributed to differences in the resulting cardiac fibroblasts. I started by simply comparing these two cell types. As I was doing these experiments, some new data came out from other labs suggesting that cardiac fibroblasts from different areas of the human heart were not all the same (Asp et al., 2019; Tucker et al., 2020).

I hypothesize that these differences could be caused 1) by nature (their lineage or path down the ski hill) or 2) by nurture (environmental factors). Think of this in terms of two business owners Jane and Billy. Jane comes from a rich family, attended Harvard, and runs a Fortune 500 company. Billy comes from a poorer family, attended a community college, and runs his own small

business in the country. Is Jane richer than Billy because of her prestigious family background or due to her Harvard degree? In terms of cardiac fibroblasts, we can ask the same question. Is the difference in cardiac fibroblasts due to their lineage (path down the ski hill) or due to their environment in the valve versus the heart wall?

Asking a nature versus nurture question in an animal model is impossible; you cannot separate these two events. However, our hPSC model is ideal for isolating these two confounding variables (Figure 1-5). We can make cardiac fibroblasts using two different "recipes" and compare the final cells to look at lineage effects. We can also take one of those differentiated cells and expose them to different environmental factors or signals. Knowledge about the important signals and cues can be used to alter our "recipe" to make new subtypes of cardiac fibroblasts.

Not surprisingly, we found that both lineage (Chapter 5) and regional environment (Chapter 6) affect the structural foundation, called extracellular matrix, produced by the fibroblasts and cardiac fibroblast response to stress. We used this knowledge to suggest which cardiac fibroblast "recipes" to use for a given purpose. For example, fibroblasts derived from epicardial cells would probably be a better choice if you wanted to test the effect of a drug on a stressed heart tissue, because they can more closely mimic cells in the diseased heart wall.

How does your project contribute to the big picture?

My work identifies important communication mechanisms between heart cells and how heart cells become specialized. You can think of my research as one piece of a big puzzle. My work is the fundamental knowledge that can be applied to engineering systems and translated into personal medicine strategies. For example, when we are thinking of engineering heart tissue for transplantation, researchers can use our insights to consider which hPSC-derived cell types to include in their hPSC-tissues. We can also use our understanding of communication between heart

cells to design better models for drug testing and studying heart disease. For example, we can generate hPSCs from a patient suffering from heart disease. Then we can combine hPSC-cardiomyocytes and hPSC-cardiac fibroblasts, stress the cells to mimic heart failure, and see how that specific patient responds to new drugs. This will tell us if the patient should take this new drug or if a different drug or treatment might be a better option for them.

Now that your thesis is done, what happens next?

My work has been published in several biotechnology and heart-specific journals. These are online scientific magazines that other researchers' read to inform their experiments and spread knowledge. The "recipe" I created to make cardiac fibroblasts has been posted on something I'd call the "Pinterest" for scientists. I've also written this long dissertation document which will be available online or on a dusty shelf in the University of Wisconsin's library.

As for my projects, they will be continued in the Palecek lab by a new graduate student. She will make some progress and then hand it on to the next student. However, the end goal for the field is to be able to use hPSC heart cells for fundamental research and applications such as regenerative medicine or personalized medicine approaches (like fixing your heart when you grow old). Since this is such a huge project, it will take the effort of many more researchers across numerous years. Research is slow but has great potential, just like my career.

Figures

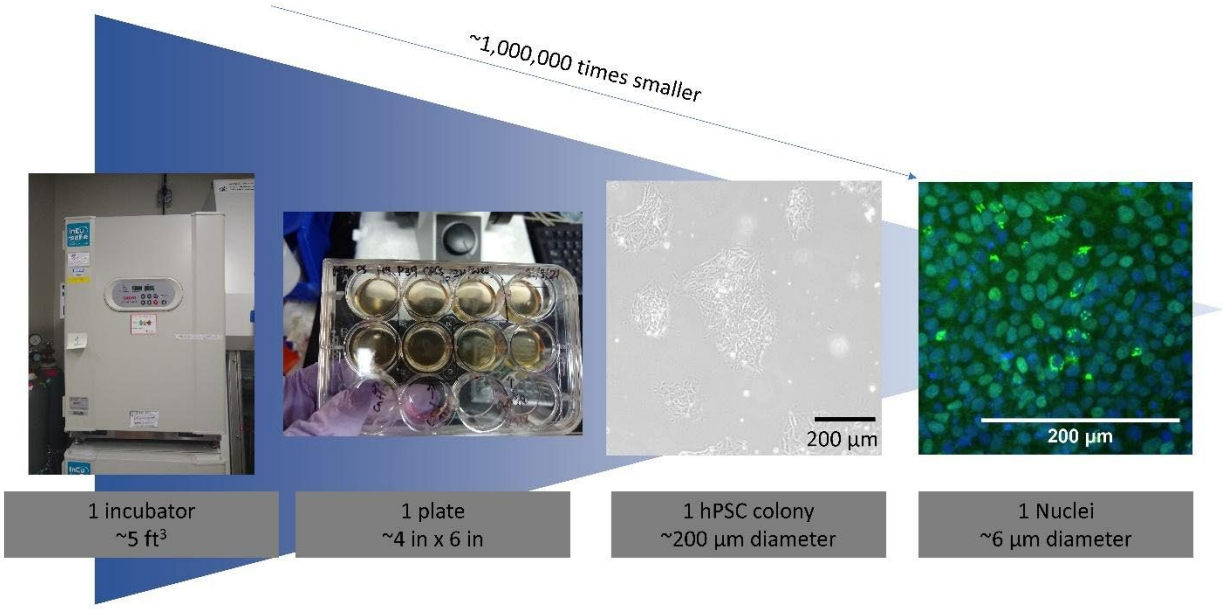

Figure 1 - 1: Relative scale of cells. *Figure shows the relative scale of the cells that I work with. On the left is an incubator, a low temperature sauna, where we keep cells at body-like conditions. Within the incubator, we have 50-100 plates containing approximately 10,000,000 cells/plate. We can then look at an individual well on a plate using a microscope to see a hPSC colony, which are approximately 200 µm in diameter. Zooming in, we can see individual nuclei (stained in blue for nuclei and green for a marker called NANOG demonstrating that they are hPSCs) which are approximately 6 µm in diameter. Cells are approximately 1,000,0000 times smaller than the incubator.*

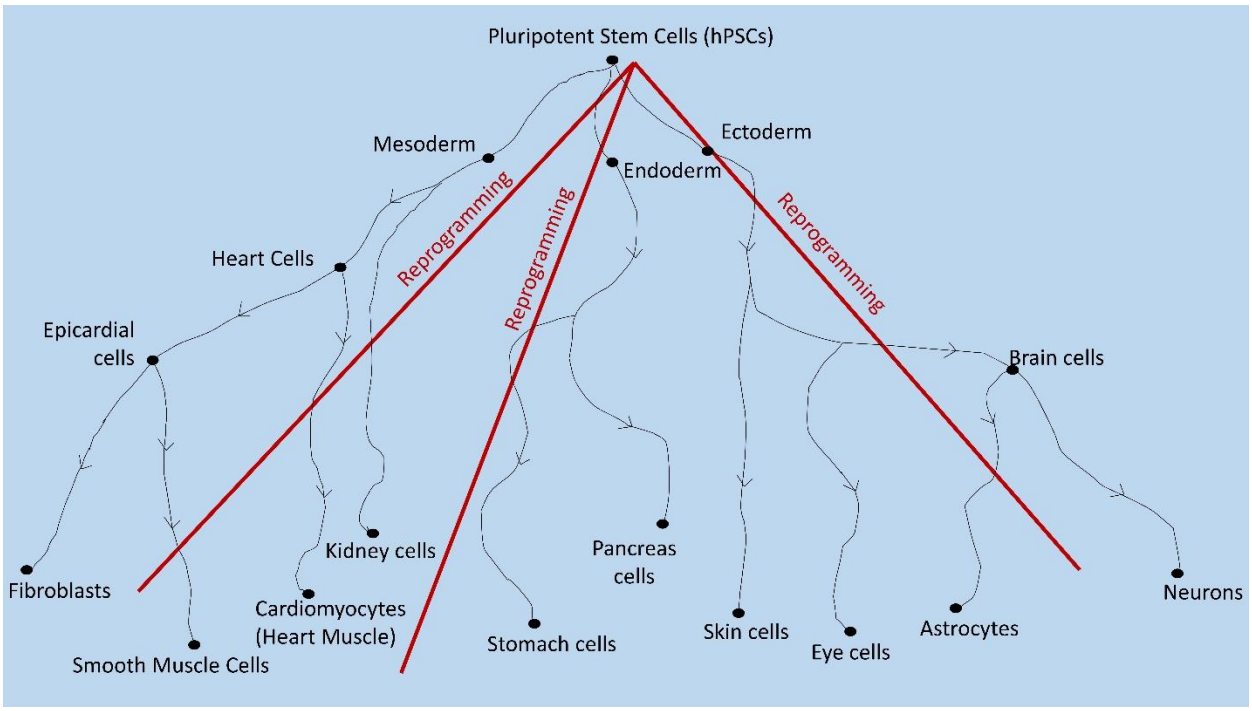

Figure 1 - 2: Schematic interpretation of Waddington's landscape. *This is described by a skier going downhill and selecting routes towards a terminally differentiated cell. Reprogramming is shown as the ski-lift to demonstrate that it is less efficient and more difficult than differentiation.*

Figure 1 - 3: Special equipment for working with cells. *On the top left is an incubator. This is like a low temperature sauna where we store the cells at body-like conditions. On the top right is a biosafety cabinet. This is the workbench where we handle our cells. It is specially designed to keep the cells away from us and us away from the cells. On the bottom right is our microscope. We use the microscope to be able to see a cell since they are only microns wide. On the bottom right is our long-term cell storage. These are tanks where we keep the cells in liquid nitrogen (a cold liquid). This perseveres the cells in a hibernation state until we are ready to use them. We have some samples that are over 20 years old!*

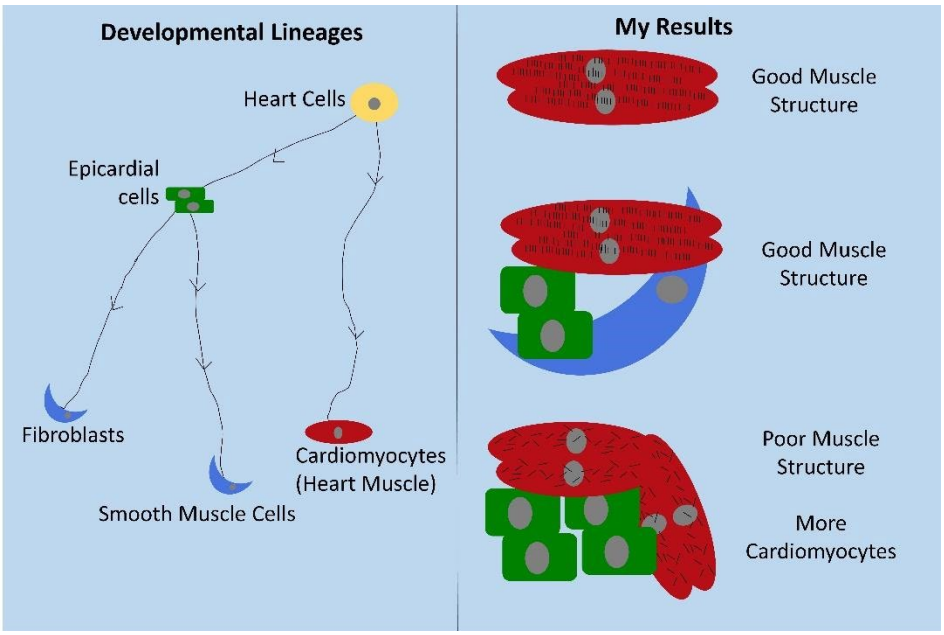

Figure 1 - 4: Study of interactions between developing cardiomyocytes (CPCs) and epicardial cells (EpiCs), Project 1. *Left side shows "ski hill" of heart cells including cardiomyocytes and epicardial cells. Right side shows my experimental conditions and results. We combined cardiomyocytes (red) and epicardial cells (green) in a dish. After two weeks, there were more cardiomyocytes in the conditions with EpiCs and there was reduced muscle structure (shown by black lines in the red cells). If epicardial cells were allowed to differentiate further into fibroblasts or smooth muscle cells (blue cells), there was no change in cardiomyocyte muscle structure.*

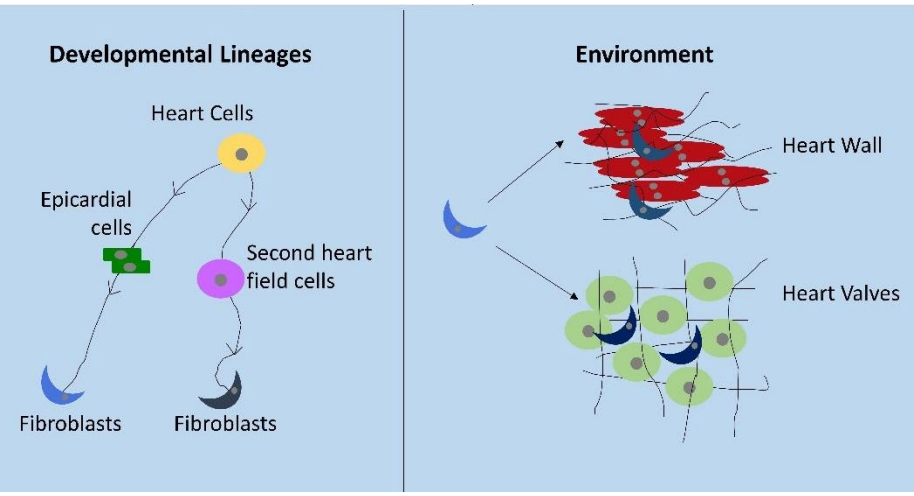

Figure 1 - 5: Theory of cardiac fibroblast lineage specification, Project 2. *Specification is thought to occur either through lineage (path down the ski hill) or regional environmental cues (such as if the cells are in the heart wall versus the valves).*