Generating fluorescent reporter constructs to study cardiovascular development and disease

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Contents

1. Abstract

The cardiovascular system consists of the heart and its blood vessels, and a wide array of problems can arise within the cardiovascular system. The heart consists of three layers: the epicardium (outer layer), the myocardium (middle layer) which forms the muscular walls of the heart and the endothelial layer (inner layer) which comprises the endocardium. Endocardial cells are specialised endothelial cells which form a lining on the inside of the heart. They are an essential source for several lineages of the cardiovascular system including the coronary endothelium, cardiomyocytes, fibroblasts, endocardial cushion mesenchyme, and can also undergo endothelial to mesenchymal transition, to give rise to cells that can form mature valves. The endocardium has also been seen to contribute to trabeculation and revascularisation post myocardial infarction (MI), therefore by understanding the biology of endocardial cells better, we might be able to manipulate and harness its potential regenerative capacity. However, studying endocardial cell biology has been made difficult due to a lack of adult endocardial cell lines. In previous work, our lab, defined induced pluripotent stem cell (iPSC) differentiation protocols to generate endocardial- and coronary endothelial-like cells. To further utilise these cells better, in this project we aimed to generate reporter gene constructs that could be expressed in endocardial and coronary endothelial cells (cEC) to help mark specific stages of differentiation and thus allow us to optimise our current endocardial differentiation and cEC transdifferentiation protocols, along with, helping us model how endocardial cells and their derivatives can contribute to heart regeneration.

Studies have shown genetic factors that control the development of endothelial cells residing in the endocardium are highly related but still distinct from the endothelial cells located elsewhere in the embryo. For example, CDH5 is a pan-endothelial cell marker, the expression of NPR3 is restricted to endocardial cells, and APLN is expressed in cEC. The aim of my study is to focus on NPR3, APLN, and CDH5, and generate fluorescent reporter constructs for them. Our reporter constructs were made by isolating the promoter regulatory regions of NPR3, APLN, and CDH5, and cloning these into a vector, into which I also cloned either a green fluorescent protein or mCherry (red fluorescent protein). Due to time constraints, I only managed to test the CDH5:GFP reporter in adult endothelial progenitor cells, and the cells carrying the reporter emitted fluorescence when imaged. In future work, the remaining reporters will be tested in adult cells and later be used to produce an iPSC reporter line that will stably express NPR3 in iPSC-endocardial cells (iPSC-Endocs), and APLN and CDH5 in iPSC-coronary endothelial cells, which will then be used in studies as outlined above.

2. Introduction

2.1 Cardiovascular diseases

Cardiovascular diseases (CVDs) are a group of disorders affecting the heart and blood vessels, and include, coronary artery disease, congenital heart disease, myocardial infarction, valvular heart disease, stoke amongst other diseases, etc. (Farley *et al*., 2012). Together, cardiovascular diseases are the number one cause of mortality globally with an estimated 17.9 million deaths each year, and also represents the greatest economic healthcare burden, with calculated costs of around $237 billion per year and a projected increase to $368 billion by 2035 (Dunbar *et al*., 2018). Almost half of cardiovascular diseases are due to myocardial infarction (MI); most MIs are due to coronary artery disease, in which the myocardium is deprived of oxygen due to coronary artery occlusion and can lead to myocardial cell death and necrosis (Thygesen *et al*., 2007) (Figure 1). Although many advancements have been made in standard of care for patients with CVD, there remains a need for better and more efficacious treatments, with the aim of, improving patient outcomes, such as reducing symptom severity, readmissions, quality of life, and mortality.

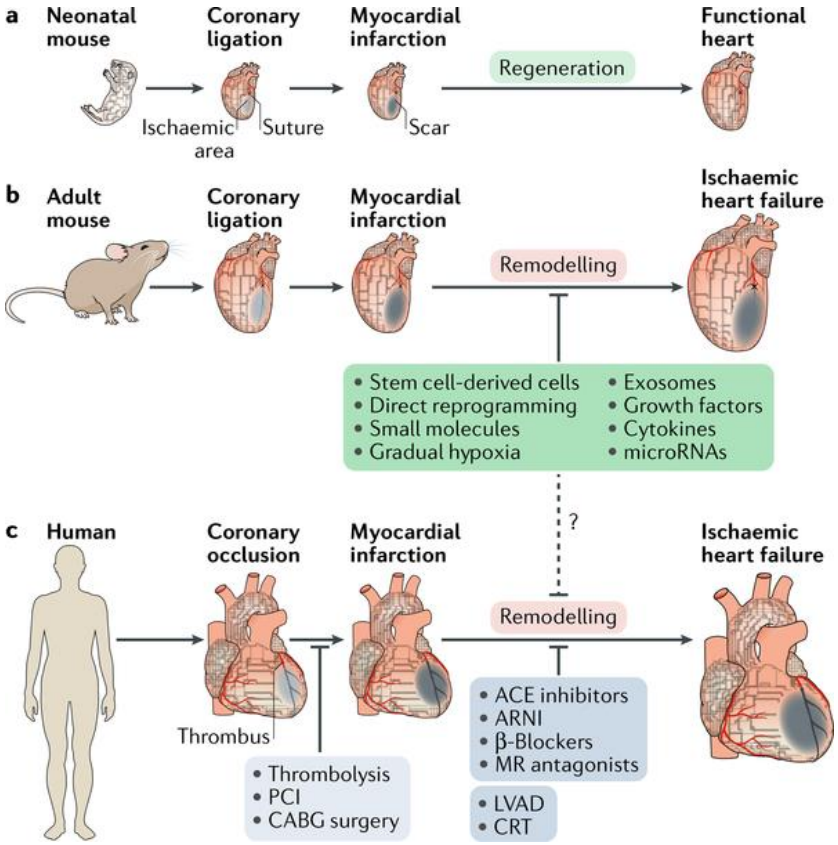


Figure 1. diagram showing the response and therapeutic approaches to myocardial injury. (a) Neonatal mice aged under 1 week are capable of regenerating the heart, with functional recovery after injury. However, this regenerative capacity is lost postnatally after the first week. (b,c) in adult mice and humans, after myocardial infarction fibrosis happens, where the necrotic tissue is replaced with fibrotic scar, causing loss of cardiac contractility. This causes the adult heart to undergo cardiac remodelling and eventually progress to heart failure. Therefore, therapies need to be created to salvage the ischaemic myocardium by early revascularisation or prevent the cardiac remodelling, and stem cells need to be used as a cellular source for cardiac regenerative therapies that improve cardiac function post disease (Hashimoto *et al*., 2018).

2.2 Cardiac regeneration

Cardiomyopathy can lead to myocardial infarction and heart failure which are leading causes of death worldwide. The main hallmark of heart failure is a loss of cardiomyocytes, as a human heart can lose up to 1 billion cardiomyocytes during heart failure, which is followed by scar formation and adverse cardiac remodelling, which is defined as a group of molecular, cellular, and interstitial changes that manifest clinically as changes in size, mass, and function of the heart after injury (Townsend *et al*., 2015; Hashimoto *et al*., 2018; Azevedo *et al*., 2016). Although heart regeneration has been reported in the neonatal mouse model (figure 1), the adult human has very minimal regenerative capacity; the cardiomyocytes annual renewal rate is around 1% at the age of 25 and 0.45% at the age of 75 (Murry *et al*., 2006) (figure 2), which is not sufficient for restoring the homeostatic cardiac function of the heart during normal ageing or in disease (Hashimoto *et al*., 2018). Apart from cardiomyocytes, the adult heart regenerative capacity also relies on replenishing vasculature, lymphatics, conduction system cells, and the interstitium (Sadek and Olson, 2020). For these reasons, the human heart cannot replace cardiomyocytes lost after MI and, as a result, patients have a lower quality of life and often die prematurely (Augoustides and Riha., 2009). Therefore, there is an essential clinical need to develop cardiac regenerative therapies to restore cardiac function for patients suffering from CVDs. Cardiac regenerative therapies aim to repair damaged heart tissue and its functionality, using the body's natural regenerative ability, to restore normal function, using the help of cutting-edge science, including stem cell and cell-free therapy. The three main strategies for myocardial regeneration include, the induction of cardiomyocyte proliferation, the use of pluripotent-stem cell derived cardiomyocytes, and direct reprogramming (Sadek and Olson, 2020). Research is also being conducted to explore genes and growth factor pathways, that can be activated/ induced in patients post disease, which will induce the body's natural ability to regenerate and revascularize, and give rise to different cell/ tissue types, e.g., coronary vessels, to restore cardiac function.

Recent studies have shown that in the developing heart, the sinus venosus and endocardium are the major source of the endothelium for coronary vessel growth, which develop in response to vascular endothelial growth factor B (VEGF-B) in the heart. The study also reveals the role of VEGF-B in cardiac regeneration post-myocardial infarction (Räsänen *et al*., 2021). Vascular endothelial growth factor (VEGF) is a major regulator of developmental and adaptive vascular growth in the heart, as it's a potent angiogenic factor, along with being an essential growth factor for vascular endothelial cells (Ferrera *et al*., 2003). This study used mice with a VEGF-B transgene, mice with a VEGF-B deletion, apelin-CreERT, and natriuretic peptide receptor 3–CreERT recombinase-mediated genetic cell lineage tracing, along with viral vector–mediated VEGF-B gene transfer in adult mice. A left coronary

vessel ligation was also performed on mice to study the development and function of the VEGF-B–induced vessels in the heart. The results from the study show that overexpression of VEGF-B during development promotes the growth of ventricle-derived new vessels, that are connected with the coronary vessels in the subendocardial myocardium. Likewise, in adult mice, adeno-associated viral vector mediated VEGF-B delivery induced endothelial proliferation in the subendocardial coronary vessels (Rasanen *et al*., 2021).

It was also shown that myocardial vessels originate from the ventricular endocardium in VEGF-B transgenic hearts of adult rats, whereas in wildtype hearts, such vessels were not present, highlighting that the VEGF-B gene has the capability to promote the development of subendocardial coronary vasculature which arises from the endocardium. Furthermore, genetic lineage tracing of endocardial cells in the adult heart after MI, revealed an increase in the endocardial cells in the subendocardial vessels of the VEGF-B transduced hearts, showing that VEGF-B can enhance the endocardial contribution to neovascularisation after injury. Alongside, VEGF-b gene therapy can also decrease the myocardial scar and improve cardiac output after MI (Rasanen *et al*., 2021).

Although, in the adult heart, the endocardial contribution to the formation of coronary endothelial cells is low, post injury (Zhang *et al*., 2016, and Dube *et al*., 2017), research has shown that the endocardium has the ability to give rise to subendocardial vessels, and the endocardial cells which become trapped in the myocardium during cardiac remodelling, can contribute to vessel growth in the damaged myocardial tissue (Tang *et al*., 2018). VEGF-B can also induce vessel growth along with endocardial cell delamination and migration into the ischemic myocardium, where endocardial cells can transdifferentiate into vascular endothelial cells and cause de novo coronary vessel formation (Rasanen *et al*., 2021). Therefore, this shows the importance of the endocardium in neovascularisation and cardiac remodelling post injury, such as MI, and the discovery of VEGF-B as a factor that can induce both these processes.

Moreover, endocardial cells line the chambers of the heart and produce multiple cells in the heart, including coronary vessel endothelial cells, valve mesenchyme and mural cells. Another study has explored the angiogenic potential and plasticity of the endocardium, and showed that Bmp2 can induce endocardial angiogenesis both during development and in injured adult hearts, with support from VEGF-B (Rasanen *et al*., 2021) or Vegfr2 (Jiang *et al*., 2021), supporting the idea of other studies that adult endocardial cells do have the potential to differentiate into coronary endothelial cells, and contribute to neovascularisation post-injury, when stimulated by different growth factors (Gennaro D'Amato *et al*., 2022).

Lastly, another recent study has found a factor that could enhance endogenous regeneration of the heart. Smart *et al*., 2006 have identified thymosin β4 (Tβ4) as an essential factor to regulate coronary vessel development in mice, along with, demonstrating its role in stimulating outgrowth from quiescent adult epicardial explants, to restore pluripotency and triggering differentiation of fibroblasts, smooth muscle cells, and endothelial cells. Tβ4 is a G-actin monomer binding protein with roles in reorganisation of the actin cytoskeleton and promoting myocardial and endothelial cell migration in the embryonic heart and retains this property in postnatal cardiomyocytes as well, to minimise cardiomyocyte loss post MI (Bock-Marquette *et al*., 2004). To investigate the role of Tβ4 during heart development and its role in adult heart repair, mouse embryos with Tβ4 deficiency were generated. Knockdown of Tβ4 caused epicardium-derived cells (EPDCs) to become trapped in the epicardium, and this failure to migrate to the myocardium, resulted in impaired vasculogenesis, defective vascular regression, thin myocardium, failed ventricular compaction, and a severe reduction in cardiomyocytes. Therefore, this shows that Tβ4 is a crucial factor in heart development, as it is required for coronary vessel development, promoting vascularisation from embryonic epicardium by inducing EPDCs to differentiate into vascular progenitors, inducing adult epicardial cell migration and differentiation into fibroblasts, smooth muscle cells, and endothelial cells, along with Tβ4's role in maintaining cardiomyocyte survival. Overall, this study on mice shows the role of Tβ4 as a potent stimulator of coronary vasculogenesis and angiogenesis, not only during development, but also in adult epicardium, along with being a source of vascular progenitors for renewal of regressed vessels and neovascularisation following cardiac injury. These findings open up a new avenue and suggest Tβ4 as a potential therapeutic in humans (Smart *et al*., 2006).

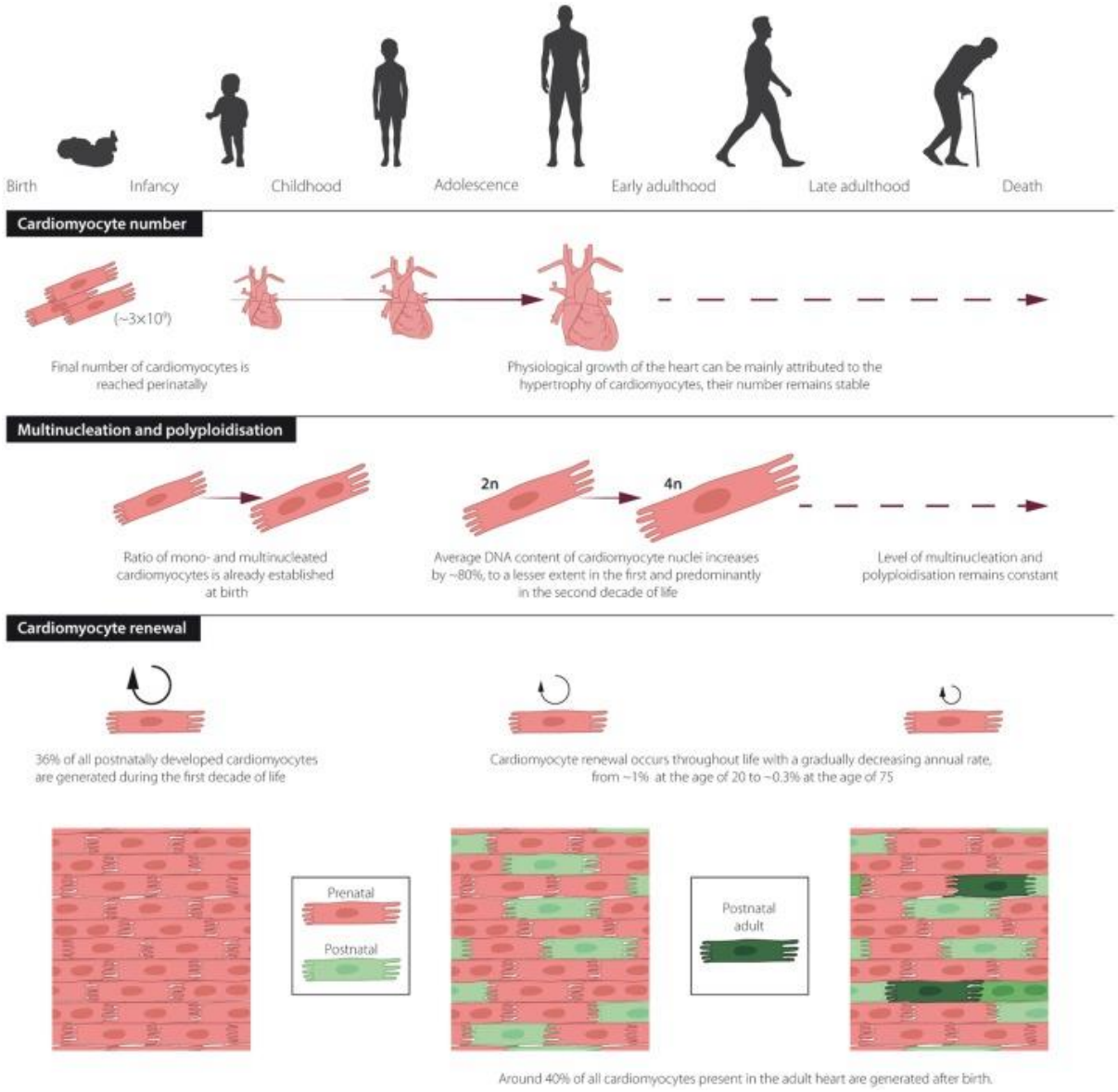


Figure 2. diagram showing the human heart throughout life and how the cardiomyocyte number, multinucleation, polyploidy, and cardiomyocyte renewal reduces over time in the human heart. The renewal capacity decreases significantly over age and hence regenerative therapies need to be created to regenerate the heart post disease (Lazar *et al*., 2017).

2.3 Challenges in cardiac regeneration

Due to the limited regenerative capacity of the heart, scientists have looked at other cellular sources that can be used in cell-based therapies to restore cardiac function post disease. For example, cardiac stem cells (CSCs) are multipotent cells residing in the adult heart, that are capable of self-renewing and generating coronary vessels, endothelial cells (ECs), and cardiomyocytes (Beltrami *et*

al., 2003). However, the density of CSCs was very low in the atrium (1 cell per every 10,000 myocytes). Firstly, although these adult stem cells have been observed, they do not endogenously appear to have the capacity to regenerate the heart on the scale required to restore cardiac function post-myocardial infarction. Secondly, the scarcity of these cells makes them very difficult to isolate (Smith *et al*., 2014). Having said this, when isolated, preclinical studies have shown that CSCs can regenerate the heart of mice post myocardial infarction, through the formation of new myocytes and vasculature, and protecting the pre-existing cardiomyocytes from apoptosis through the secretion of IGF-1 (Kawaguchi *et al*., 2010). CSCs have also been used in phase 1 human trials to treat patients with ischemic cardiomyopathy, and the trial showed that intracoronary administration of CSCs increased left ventricular ejection fraction with decreased infarct size. However, the cell retention in the myocardium was very low despite the high number of infused CSCs (1 million cells) (Chugh *et al*., 2012). Mesenchymal stem cells (MSCs) derived from the bone marrow, have also been used in cardiac regenerative therapies. Several *in vivo* studies showed improvements in myocardial function despite low rates of MSC engraftment and differentiation. However, the differentiated myocytes did not possess the electrical properties of functional cardiomyocytes (Rose *et al*., 2008). In addition, although MSCs showed to reduce scar mass, and improved the early enhancement defect in ischemic cardiomyopathic patients, the MSC- treated groups did not show significant improvements in ejection fraction (Mathiasen *et al*., 2015).

As an alternative to adult stem cells, it has been observed that cardiac fibroblasts play critical roles in maintaining cardiac function and in cardiac remodelling after myocardial infarction, and the field has begun to explore our ability to harness these cells for cardiac regeneration. Cardiac fibroblasts arise from the proepicardium and comprise over 50% of all cells in the heart, along with, providing support structure, secreting signals, and contributing to scar formation after cardiac damage (Ieda *et al*., 2009). Srivastava *et al*., 2010 aimed to directly reprogram these endogenous cardiac fibroblasts into beating cardiomyocytes, as a potential regenerative therapy. This study examined key developmental cardiac regulators and found a specific combination of three transcription factors, GATA4, Mef2c, and Tbx5 (GMT), which were sufficient to generate functional beating cardiomyocytes from postnatal mouse cardiac fibroblasts. These induced-cardiac cardiomyocytes expressed cardiac-specific markers, contracted spontaneously, and had a global gene expression profile similar to cardiomyocytes. However, this reprogramming event appears stable only on epigenetic level, as the global expression of induced cardiomyocytes is similar but not identical to neonatal cardiomyocytes, and it is undetermined whether they're more similar to adult ventricular cardiomyocytes or some other subpopulations, so further research still needs to be conducted. On the other hand, to investigate if these transduced cardiac fibroblasts can be reprogrammed to

express cardiomyocyte-specific genes *in vivo*, cardiac fibroblasts were harvested one day after viral transduction and injected into immunosuppressed mouse hearts. The injected cardiac fibroblasts transduced with GATA4, Mef2c, and Tbx5, expressed α-actin and sarcomeric structures, one day after transplantation, and fully reprogrammed to cardiomyocytes within 2 weeks of transplantation *in vivo* (Ieda *et al*., 2010). Lastly, to investigate whether viral gene transfer of GMT into infarcted mouse hearts induced cardiomyocyte generation or not, coronary artery ligation was used to generate MI in mouse hearts. *In vitro* transduction of GMT retrovirus revealed that cardiac fibroblasts from the infarct region converted into cardiomyocyte-like cells with cardiac-specific gene expression, sarcomeric α-actin structures, and cross striations within the heart (Inagawa *et al*., 2012). This is a big breakthrough, as the ability to reprogram cardiac fibroblasts into cardiomyocytes, has many therapeutic implications, such as, using an individual's own fibroblasts, grown from a cardiac or skin biopsy *in vitro*, for transduction with defined factors and delivery of these cells to damaged hearts. Along with, the potential to introduce these defined factors directly into the patient's heart to reprogram the endogenous fibroblast population, into new cardiomyocytes that can improve heart contractility, post cardiac damage (Ieda *et al*., 2010). However, the reprogramming efficiency needs to be improved, and the safety concerns of using fibroblasts for regenerative therapy should also be addressed, as well as focusing on safe delivery of these defined factors into the heart (Okano *et al*., 2013).

Even though, several cellular sources have been studied for cardiac regenerative therapies, each of them has several disadvantages and have not yet successfully been translated into the clinic. Therefore, other cellular sources need to be investigated to decipher their potential in improving cardiac function post disease, and to be used as cardiac regenerative therapies in the future.

2.4 The endocardium

The cardiovascular system is the first organ system to form and function in the vertebrate embryo. Following gastrulation, the cells of the inner cell mass are specified into three germ layers: endoderm, ectoderm, and mesoderm. Cardiac development is first seen at E6.5 when the cardiac mesoderm forms, along the primitive streak. At E7, the cardiac mesodermal cells migrate to form, the primary heart field, which contributes to the beating primitive heart tube, left ventricle and atria during development, and the secondary heart field contributes to developing the right ventricle, outflow tract and pulmonary trunk. At E9, the transient proepicardial origin which forms the epicardium becomes apparent and cardiac neural crest cells contribute to smooth muscle cells. The heart consists of three layers: the epicardium (outer layer), the myocardium (middle layer) which forms the muscular walls of the heart and the endocardium layer (inner layer) which comprises the

endocardial cells (Abu-Issa and Kirby, 2007 and Spater *et al*., 2014). The major cell types of the heart include, cardiomyocytes, cardiac conduction cells, vascular smooth muscle cells, endothelial cells, cardiac fibroblasts, and endocardial cells. Endocardial cells are specialised endothelial cells which form a lining on the heart chamber and are formed from the vascular endothelial progenitors and the cardiac mesoderm (figure 3). They are an essential source for several lineages of the cardiovascular system including the coronary endothelium, cardiomyocytes, fibroblasts, endocardial cushion mesenchyme, etc. (Figure 4) (Zhang *et al*., 2018). Upon stimulation, during development, endocardial cells within the atrioventricular canal undergo endothelial to mesenchymal transition (EMT) to give rise to precursor cells the form mature valve structures. On the other hand, during development, nonvalvular endocardial cells give rise to endothelial lining of the heart which includes the atria, ventricles, and sinus venosus. Further fate-mapping studies have shown that these endocardial cells also contribute to the formation of coronary vascular endothelial cells and act as novel progenitors for mural cells of the heart, such as cardiomyocytes (Tian *et al*., 2015). Due to the nature of endocardial cells and the diverse cell lineages it gives rise to, the endocardium has several key roles in the body.

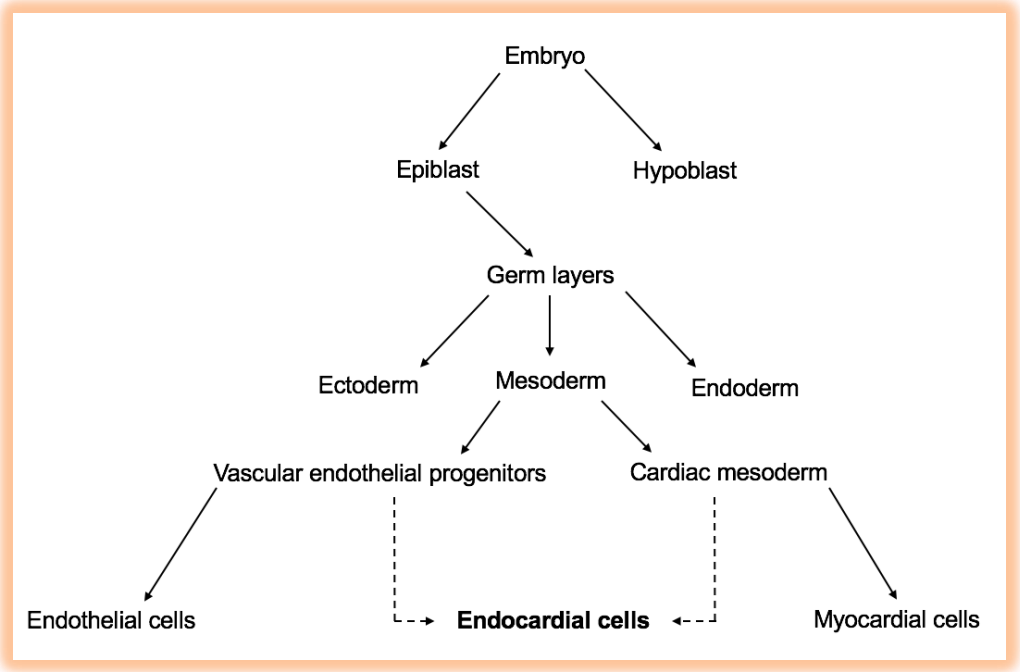


Figure 3. Diagram outlining the origin of endocardial cells (adapted from Puceat, 2013).

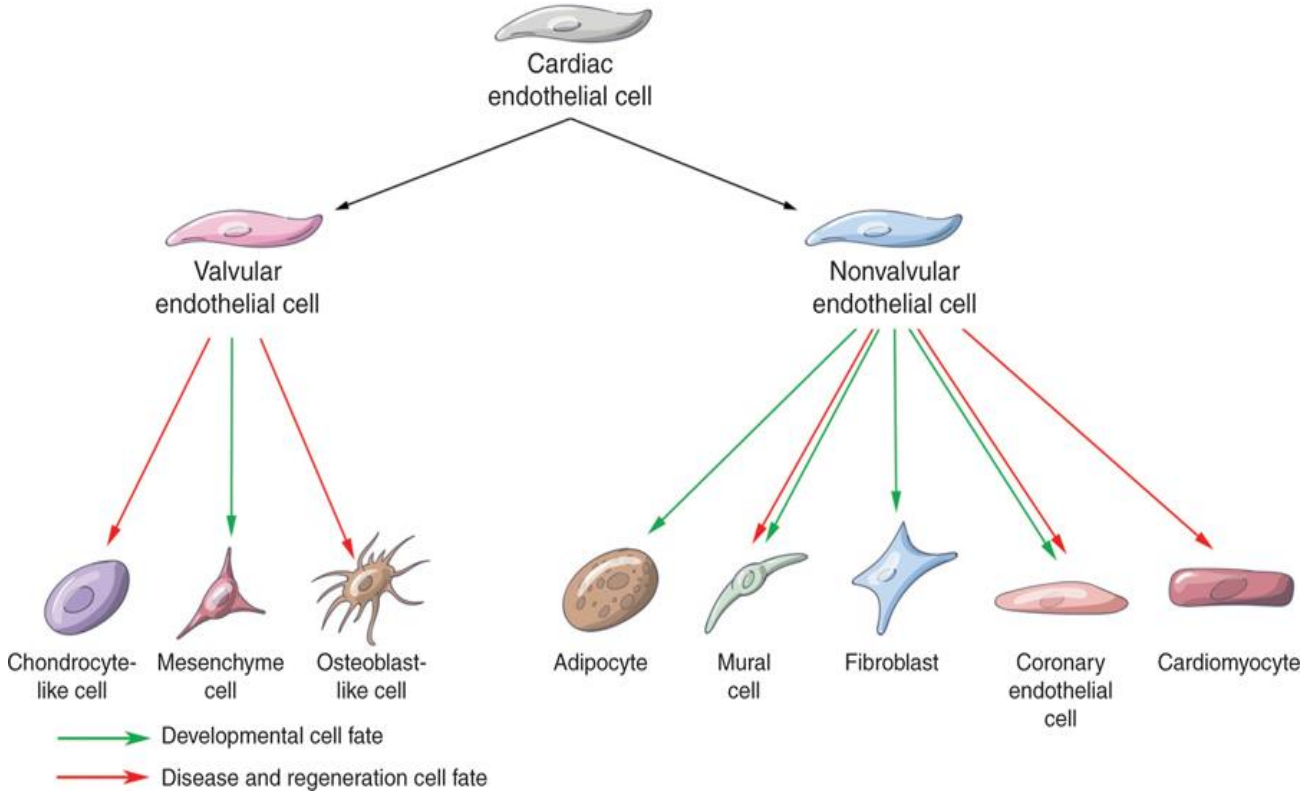


Figure 4. diagram shows the fate of endocardial cells in development, disease, and regeneration. During early cardiogenesis endocardial cells line the nonvalvular sites of the primitive valve structures and myocardium and within these regions endocardial cells give rise to multiple cell fates during development (green arrows) and also contribute to processes involves in adult disease and regeneration (red arrows). Therefore, the cardiac endothelial cells within the endocardium contribute in giving rise to a diverse lineage of cells (Dye and Lincoln, 2020).

2.4.1 Endocardium and its role in forming cushion mesenchymal cells

The heart valve begins developing with the formation of endocardial cushions in the atrioventricular valve (AVC) and outflow tract (OFT) of the heart tube. The endocardial cushion formation is induced by a subset of endocardial cells altering their adherent junctions and migrating into the cardiac jelly where endothelial to mesenchymal (EndoMT) happens in the AVC and OFT (Camenisch *et al*., 2000). Further genetic lineage tracing has shown that majority of the mesenchymal cells that are present in the mature valves are of the endocardial origin, hence demonstrating the importance of endocardial cells in valve formation (de Lange *et al*., 2004). Multiple signalling pathways control the role of endocardium in cushion morphogenesis through regulating EndoMT, such as, BMP, TGF-β, Notch, and Wnt. Other factors such as GATA4, Sox9, and Nfatc1 are also involved in cushion EndoMT and valve maturation processes (Moskowitz *et al*., 2011) (Figure 5). In addition, during valve formation and remodelling, the endocardial derived mesenchymal cells adopt myofibroblast cells fate in the

adult heart, contributing to osteoblast-like cells that play a role in valve calcification (Song *et al*., 2017). Overall, cardiac valve malformations and atrioventricular septal defects account for the most common congenital heart defects, therefore, understanding the mechanisms that govern cardiac valve development during cushion formation, would provide new insights into developing therapies to prevent and repair valve disease (Brown *et al*., 1999).

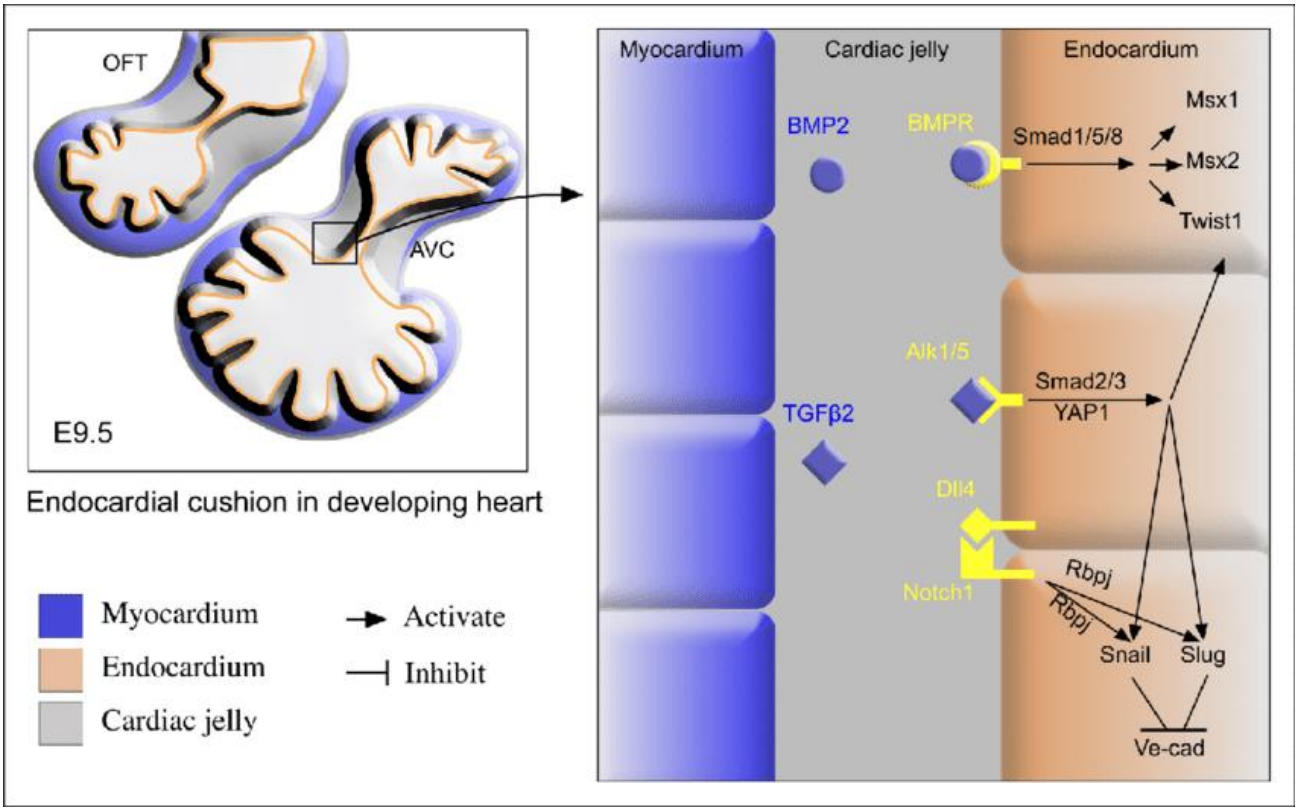


Figure 5. multiple signalling pathways control the endocardial contribution to cushion morphogenesis through regulating endothelial to mesenchymal transition. These signalling pathways include BMP (bone morphogenetic protein), TGF-β (transforming growth factor-β), and Notch (Zhang *et al*., 2018).

2.4.2 Endocardium and its role in trabeculation

Ventricular chamber morphogenesis: a key development process in cardiac ontogeny, happens through trabeculae formation which is crucial for cardiac function and embryonic viability and relies on the cellular interactions between the endocardium and myocardium. The formation of trabeculae is one of the first signs of chamber development and lack of trabeculation causes embryonic lethality in mice, whereas excess trabeculation causes cardiomyopathy and heart failure in humans (Zhang *et al*., 2013). There are several signalling molecules in the myocardium, endocardium, and cardiac extracellular matrix (ECM) which contribute to the process of trabeculation, and the key

communication between the endocardium and myocardium promote cardiomyocyte proliferation and differentiation, which leads to trabeculae formation. Some of these signalling molecules include, BMP10, Nrg1, and EphrinB2 which are all part of the Notch signalling pathway. Another key signalling molecule is Tie (tyrosine kinase immunoglobulin-like and EGF homology) receptors. Tie1 and Tek/Tie2 are the only endothelial cell specific receptor tyrosine kinases (RTKs). A study by Xianghu and Baldwin, 2020, proposed a model of trabeculation which is regulated by endocardial Tie2. In mice, trabeculation happens in three major steps, the first initiation stage happens by endocardial sprout formation and myocardial delamination, which is followed by the next stage, in which endocardial sproutings move laterally under the myocardial lamina to form isolated clusters of myocardial cells in the cardiac ECM, which is covered by the endocardium. In the last stage, the endocardial sprouting enveloped in myocardial lamina assemble to form short trabecular clusters, which is followed by trabecular extension, which finally produces long trabecular structures (Qu and Baldwin, 2020). Overall, this study highlights the key role of the endocardium in the process of ventricular trabeculation, which is dependent on endocardial RTK Tie2 signalling. Studies in mice have shown that the loss of endocardial Tie2 results in impaired endocardial sprouting and embryonic growth, due to decreased endocardial cells proliferation and impaired migration, which can cause lethality of the embryo due to an over-simplified trabecular network and impaired sprouting (Qu *et al*., 2019). Therefore, this shows that Tie2 promotes endocardial proliferation and the development of angiogenic sprouts, which are essential in the initiation process of trabeculation, whereas it can also prevent myocardial hypertrabeculation through the suppression of retinoic acid signalling in the cardiomyocytes (Qu *et al*., 2022).

2.4.3 Endocardium and its role in revascularisation post-MI

Myocardial infarction (MI) is one of the most common causes of heart failure due to the occlusion of the coronary artery, which diminishes the delivery of oxygen and nutrients to the myocardium. The patients also suffer from ischaemic cardiomyopathy which causes left ventricular dysfunction. After MI, the damaged myocardium gets replaced by fibrotic scar tissue as the cardiomyocytes in the adult human heart have a very minimal regenerative capacity. The scar tissue causes loss of pump function in the heart along with circulatory deficiency. This leads to the heart undergoing cardiac remodelling which results in further fibrosis, cardiac dysfunction, loss of myocardium and eventually heart failure, that can be fatal. The irreversible cell loss and scarring is the major cause of morbidity and mortality, post MI (Cohn *et al*., 2000). Revascularisation is the most important factor of regenerative therapies after MI, where neovascularisation needs to happen through the redeployment of coronary vasculature generating mechanisms (Del Monte and Harvey, 2012). Studies have shown that epicardially derived cells and the sinus venosus contribute to the

development of coronary vasculature, and in addition to that, genetic tracing studies using Nfatc1 and APLN sequences have shown the role of the endocardium as a major source of coronary endothelial cells in the early postnatal heart. However, the role of the endocardium in adult heart revascularisation post disease is still unknown (Red-Horse *et al*., 2010, Wu *et al*., 2012). In a recent study Miquerol *et al*., 2015 studied the vascular remodelling of coronary arteries after infarction in mice. In this, permanent left coronary ligation was performed on Connexin40-GFP mice expressing green fluorescent protein in endothelial cells of coronary arteries only. VEGFR2 is a capillary endothelial marker and plays a crucial role in vascular endothelial cell survival and blood vessel formation and is weakly expressed in the endocardium. Endocardial cells expressing VEGFR2 were seen in mice 3 days post MI, which shows that EndoMT may happen in the endocardium. From day 7, endocardial cells form structures named endocardial flowers (figure 6), and connect with vessels, and within these flowers VEGFR2 cells have a tip-cell phenotype and NG2 pericytes are also present within these flowers. Therefore, this suggests that the formation of these endocardial flowers initiates angiogenesis, through the expression of VEGFR2 and active proliferation of adjacent endocardial and smooth muscle cells. Cx40, a marker of endothelial cells of coronary arteries was also expressed in these endocardial flowers. Further immunostaining for proliferation markers such as Ki67 showed that endocardial cells and subendocardial smooth muscle cells (SMCs) linked to endocardial flowers have elevated proliferation levels after MI. Overall, this study reveals that endocardial flowers adopt an arterial phenotype after injury, and that the endocardium can be proposed as a new source of coronary vascular endothelial cells post MI, given that active angiogenesis of endocardial endothelial cells is followed by Cx40 expression and proliferation of subendocardial SMCs (Miquerol *et al*., 2015). Other studies have also shown that endocardial cells have been reported to contribute to coronary endothelial cells (CECs) and are the main source of CECs in the ventricular septum in the postnatal heart (Tian *et al*., 2015). Overall, endothelial cells plasticity has been observed within the infarct zone and researchers can identify the ventricular endocardium as a site of arteriogenesis and a source of endothelial cells to promote revascularisation post MI. However, there is also a completely contradictory viewpoint to this, which claims that the sinus venosus is the major source of coronary vascular ECs, rather than the ventricular endocardium, as proposed in previous studies. A study by Zhang *et al*., 2016, used intersectional lineage tracing studies, and generated *Nfatc1-Cre* and *Nfatc1-Dre* lineage tracers for endocardium labelling in mice. They found that Nfatc1, a transcription factor required for valve formation, labels a significant portion of the sinus venosus (SV) endothelial cells in addition to the endocardium. Along with, using NPR3 genetic lineage tracing to demonstrate that the endocardium minimally contributes to coronary endothelium in the embryonic ventricular free walls. Overall,

there is debate over why two different coronary development pathways coexist, originating from two distinct sources with inverse distribution in the developing heart (Zhang *et al*., 2016). An SV source suggests that cells from venous endothelium become reprogrammed to an arterial fate and invade the ventricle wall in an epicardium-to-endocardium direction to form coronary arteries. Whereas a VE source suggests that ventricular endocardial cells invade ventricle wall in endocardium-to-epicardium direction to form coronary arteries (Red-Horse *et al*., 2010 and Wu *et al*., 2012). In conclusion, there is an urgent need to resolve this controversy, to reveal the developmental origin of the coronary endothelium, along with discovering the developmental mechanisms responsible for coronary vessel formation, to help in creating new therapies for coronary artery diseases and to induce cardiac revascularisation post disease.

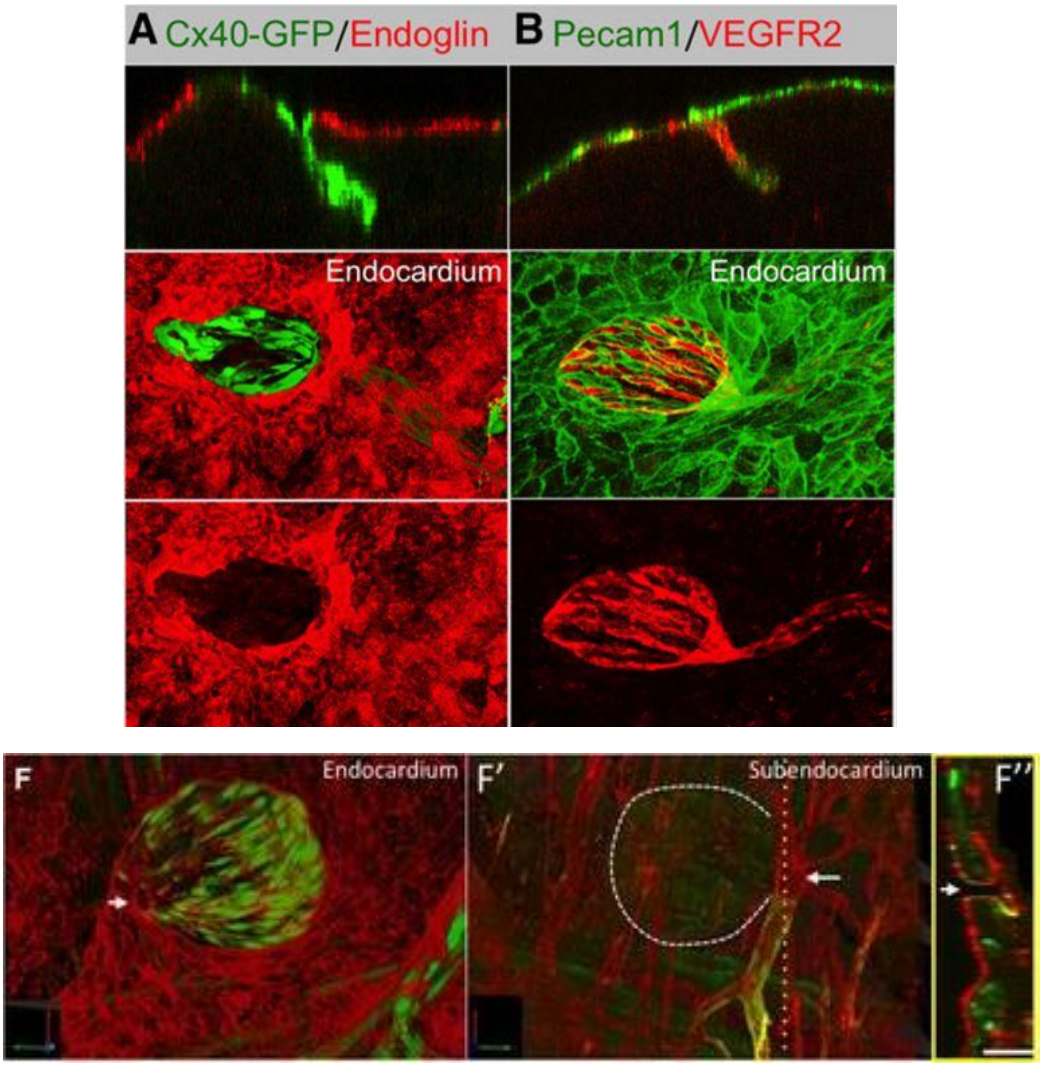


Figure 6. the molecular properties of endocardial flowers in post-myocardial infarction hearts were investigated by wholemount coimmunofluorescence. (A and B) show the 3D reconstruction of an endocardial flower. The endocardial flowers possess a distinct endothelial phenotype to surrounding endocardium. (F) Pecam1 labelleing showing elongated endothelial cells within an endocardial flower, compared with hexagonal cells everywhere else in the endocardium. (F') shows the connecting vessels in the subendocardium, in adult mice heart post myocardial infarction. This figure shows that endocardial flowers, do indeed contribute to new coronary arteries post myocardial infarction. (Adapted from Miquerol *et al*., 2015).

2.4.4 Endocardial marker expression

The endocardium expresses lineage-specific markers in a pattern distinct from other endothelial cell types. Studies have shown genetic factors that control the development of endothelial cells residing in the endocardium are highly related but still distinct from the endothelial cells located elsewhere in the embryo. For example, Nuclear factor of activated T cell cytoplasmic 1 (Nfatc1) is a transcription factor specifically expressed in the endocardium, with high expression during the initiation of endocardial differentiation in the primary heart forming field and at the linear heart tube stage. The activity of Nfatc1 is also critical during later cardiac development and valvulogenesis (de la Pompa *et al*., 1998). In addition, GATA4 is a zinc-finger transcription factor and whole embryo in situ analysis of *Xenopus* embryos shows that the GATA4 gene is transcribed in the cardiac ventral mesoderm at the time when the cardiac tube forms, therefore it is the earliest marker of cardiogenesis. Later on in embryo development, GATA4 is seen to be expressed in the endocardial layer and not the myocardium, hence, it can be used to study the formation of the cardiovascular system and specification of the endocardial lineage (Kelley *et al*., 1993). Also, GATA4 is an upstream regulator of the *Erbb3-Erk* pathway, which is necessary for EMT, and also acts to promote cushion mesenchyme growth and remodelling (Rivera-Feliciano *et al*., 2006). Furthermore, Zhang *et al*., 2016 also stained the coronary vascular endothelial cell specific marker, fatty acid binding protein 4 (FABP4) and found that Nfatc1 expressing cells contribute to FABP4 subepicardial endothelial cells and intramyocardial coronary vessels. FACS and single-cell gene expression analysis has revealed that natriuretic peptide receptor 3 (NPR3) is a novel endocardial gene, expressed in the entirety of the endocardium including the ventricular endocardium (VE), but not in the sinus venosus (SV). Further whole mount and *in-situ* hybridisation also showed that NPR3 was highly expressed in the atrial endocardium and VE; the same findings were observed when NPR3-GFP heart sections were stained in mice. Also, at stage E14.5, NPR3 expression was only seen in the endocardium and not in the coronary vascular ECs of the compact myocardium. Overall, findings based on immunostaining, *in-situ* hybridisation, and single-cell quantitative polymerase chain reaction analysis show that NPR3 is a specific endocardial marker, as it's only expressed in the endocardium and not in the SV or coronary vessels of the heart (Zhang *et al*., 2016). Both endocardial cells and coronary ECs are two types of endothelial cells, however their transcriptional profile is different. Studies have revealed that endocardial cells express high levels of NPR3 and Nfatc1, whereas coronary ECs express FABP4 and APLN (He *et al*., 2014). Generating reporter lines and immunostaining studies further confirmed the presence of these genes in endocardium and coronary endothelium. On the other hand, even though NPR3 is the most specific endocardial marker, it is still expressed at low levels in other cell types, such as, kidney, epicardium, aortic endothelium, granulosa cells, and pancreas. Similarly,

APLN is expressed at low levels in the brain, and FABP4 in adipocytes and the colon (Proteinatlas.org, 2019). However, when used in conjunction with other endocardial or coronary endothelial cell markers, they can specifically label those cell types. Another key gene in the endothelium is CDH5, a pan-endothelial-specific transmembrane component of the adherens junction complex (Nyqvist *et al*., 2008). This adhesion molecule is located at junctions between endothelial cells and is a master regulator of endothelial cell-cell adhesive properties and is crucial for the maturation, extension and remodelling of vessels which are essential for angiogenesis (Crosby *et al*., 2005). Overall, the information on these markers for the endocardium and coronary endothelium will give us valuable knowledge about their expression, role, and functionality in the body, and hence allow us to exploit this information for use in cardiovascular therapies (Zhang *et al*., 2016).

2.5 Natriuretic peptide receptor 3 in the cardiovascular system

Natriuretic peptide receptor 3 (NPR3) is a gene which encodes one of the three natriuretic peptide receptors. These natriuretic peptides regulate blood volume and pressure, pulmonary hypertension, and cardiac function, alongside some growth processes (Pereira *et al*., 2013). In addition, the peptides play a key role in modulating the cardiac structure and function by affecting the renin-angiotensin-aldosterone system, sodium and water excretion, and vasomotor tone (Boerrigter *et al*., 2009). NPR3 is a single pass type I membrane protein with a 450 amino acid extracellular ligand-binding domain and a transmembrane region, with its cytoplasmic tail containing 37 amino acid residues. Its main function is the clearance of natriuretic peptides through the process of internalisation and degradation, with further roles in signalling function (Potter., 2011). Furthermore, through single-cell gene expression analysis, NPR3 has been identified as a specific endocardial marker. To confirm the expression of NPR3, *NPR3-GFP* reporter mouse lines were generated in which a GFPCre fusion cDNA was knocked into the 3' untranslated region of NPR3 through homologous recombination. The NPR3-GFP heart sections were stained with a GFP antibody, and this revealed NPR3 is expressed in the atrial endocardium and ventricular endocardium, but not in the coronary vascular ECs in the myocardium. This study reveals that NPR3 is specifically expressed in the endocardial cells within the endocardium, and not in the sinus venosus or the coronary vessels. Fate mapping approaches and immunostaining E9.5 and E10.5 embryonic hearts showed that NPR3 is expressed in almost all Nfatc1 positive endocardial cells, which again suggests that NPR3 is an endocardial cell marker (figure 7). Studies revealed that Nfatc1 is expressed in the VE and SV and is expressed in a subset of coronary vascular ECs, which shows that Nfatc1 is a marker of the coronary endothelium. Hence, NPR3 was tested to see if it can label the endocardium specifically. Immunostaining studies revealed that NPR3 is expressed in the atrium and VE but not the SV. It was also expressed in the endothelial cells of the dorsal aorta and a subset of

epicardial cells. However, the main conclusion from this study is that NPR3 is expressed in the VE but not SV (figure 8); this allows us to distinguish VE-derived coronary vessels from SV-derived coronary vessels which is essential in exploring the role of NPR3 in the cardiovascular system, specifically, within the endocardium (Zhang *et al*., 2016). Some hearts were tested at a later embryonic stage to see the contribution of NPR3 VE to coronary ECs and found that NPR3 positive ventricular endocardial cells contribute to coronary vessels in the ventricular septum and the most ventral part of the ventricular free wall that connects to the ventricular septum (Wu *et al*., 2012). Overall, these studies reveal that NPR3 is an endocardial cell marker and hence is really useful in studying the biology of the endocardium in embryonic hearts as well as in adult hearts. NPR3 also contributes to the formation of coronary vessels in the ventricular septum, in embryonic hearts, so we can study this mechanism further and implement it in adult hearts to induce cardiovascular regeneration post disease.

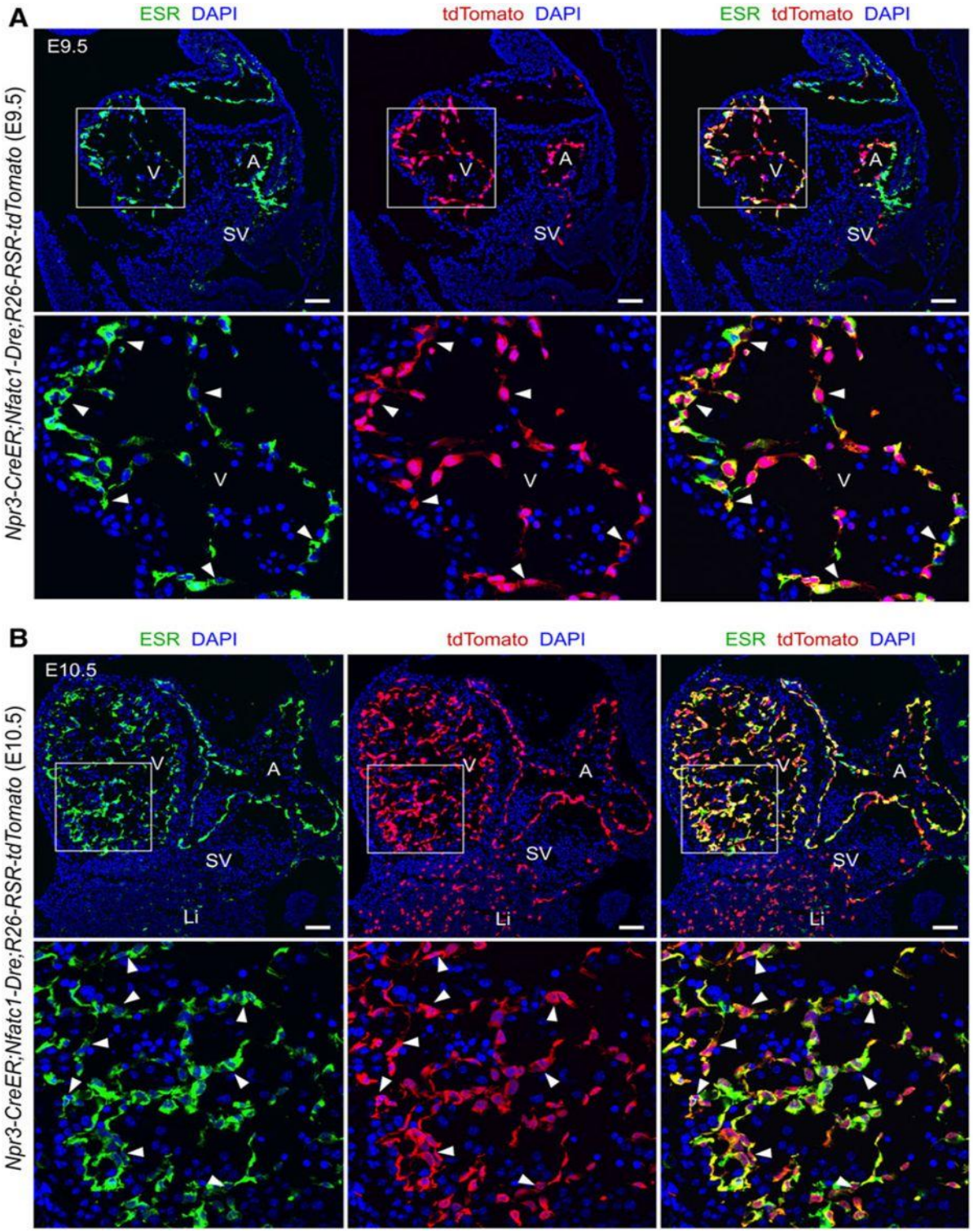


Figure 7. Figure showing that natriuretic peptide receptor 3 (NPR3) is expressed in Nfatc1 ventricular endocardial cells. A and B: immunostaining of Estrogen receptor (ESR) and tdTomato on heart sections of E9.5 and E10.5 embryos, respectively. ESR is a surrogate for NPR3 and Nfatc1 endocardial cells are shown as tdTomato cells. White arrowheads show the Nfatc1-NPR3 endocardial cells, hence proving the presence of NPR3 in the endocardium (Zhang *et al*., 2016).

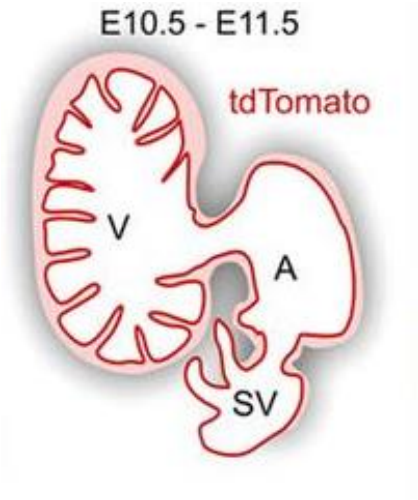


Figure 8. animated figure showing that natriuretic peptide receptor 3 (NPR3) labels the endocardium of the ventricle (v) and atrium (A), but not the sinus venosus (SV), in a E10.5-E11.5 embryonic heart (adapted from Zhang *et al*., 2016).

2.6 Apelin (APLN) in the cardiovascular system

The human apelin (APLN) gene is localised on the chromosome Xq25-26.1 and its sequence contains three exons and two introns, which encode a 77 amino acid propeptide: pre-apelin, that has a hydrophobic N-terminal region which is a signal sequence, and a C-terminal that has several biological activities and regions that bind to the apelin receptor (APJ) (Lee *et al*., 2001). Furthermore, APLN is part of the family A of G-protein coupled receptors (GCPRs) and is involved in a range of physiological and pathological functions, such as, fluid homeostasis, depression, cardiovascular diseases, metabolic disorders, along with, roles in cardiac contractility, angiogenesis, cell proliferation, regulation of blood pressure, etc. APLN works by interacting with other GCPRs to form heterodimers which modulate distinct intracellular signal transduction pathways. APLN also exerts a positive inotropic effect on the heart, so the drugs targeting the APLN/APJ system provide a range of therapeutic options for patients with congestive heart failure (Hu *et al*., 2021). Immunohistochemistry was used to reveal the tissue expression of APLN in humans and it was revealed that APLN is highly expressed in endothelial cells and cardiomyocytes within the heart (De Falco *et al*., 2002).

In normal conditions, APLN is expressed in cardiac myocytes, and has a positive inotropic effect along with roles in lowering arterial blood pressure and improving cardiac output (Japp *et al*., 2010). Foldes *et al*., 2003 found the first connection between APLN and cardiovascular pathology; they showed higher expression levels of APLN mRNA in human hearts undergoing cardiac failure, compared to normal tissue, thus suggesting its role in pathophysiology of heart failure. In patients

with heart failure, APLN also increases cardiac output, lowers blood pressure, and lowers peripheral vascular resistance (Japp *et al*., 2010). In addition, it was shown that APLN has a cardioprotective effect against myocardial infarction, as when APLN was injected into a rat model of myocardial infarction, it decreased the infarct size, increased heart rate, and increased the serum nitric oxide levels (Azizi *et al*., 2013). Also, an APLN-knockout study in mice revealed that the loss of APLN increased myocardial infarction mortality, infarct size and inflammation, with a reduction of the prosurvival pathway (Wang *et al*., 2013). A meta-analysis of several studies had shown that APLN might be a prominent athero-protective marker against the development of coronary artery diseases, such as myocardial infarction, and hence it can be used as a biomarker for cardiovascular diseases (Chen et al., 2017). It has also been shown APLN signalling is essential for angiogenesis promotion, and that the administration of APLN-13 to rats with ischemic stroke led to increased formation of new blood vessels (Chen *et al*., 2015), hence suggesting the role of APLN in cardiac regenerative therapies. Furthermore, a study on isolated perfused rat hearts revealed that APLN exerts the most potent, dose dependent positive inotropic effect, and causes a significant increase in heart contractility, with the maximal response at APLN concentration of 1 nmol/L, thus, highlighting its role in the regulation of cardiac function (Szokodi *et al*., 2002). Lastly, studies have shown that APLN is a sprouting marker of the coronary vessels in the embryo, and that coronary vessels are formed by vascular sprouting from the sinus venosus and the endocardium (Red-Horse *et al*., 2010). This quality allows scientists to create APLN-CreER mouse lines to study the development of coronary vessels. In a study, APLN-CreER mouse line was created, which labelled subepicardial endothelial cells and all their progenies, which revealed that subepicardial ECs migrate into the myocardium of the embryonic ventricle free walls and give rise to coronary veins and intramyocardial coronary arteries and capillaries. This verified that APLN expression is specific to subepicardial ECs and not in endocardial ECs, thus making APLN a specific marker for coronary vascular ECs (figure 9); scientists can utilise this specificity of APLN and use it to study coronary vessel behaviour during postnatal growth, as well as in adults during injury, regeneration, and cardiovascular diseases (Tian *et al*., 2013).

2.7 CDH5 in the cardiovascular system

Endothelial cells form the vasculature and regulate the exchange of solutes and fluids between the blood and tissue. The endothelium is also the site for angiogenesis, which involves the extension and remodelling of blood vessels, and for its functioning, it is essential for the endothelial cells to properly regulate cell-cell adhesions between themselves and neighbouring cells. Vascular endothelial (VE)- cadherin 5, also known as CD144 or CDH5, is a pan-endothelial-specific transmembrane component of the adherens junction complex (Nyqvist *et al*., 2008). This adhesion

molecule is located at junctions between endothelial cells and is a master regulator of endothelial cell-cell adhesive properties. CDH5, through its cytoplasmic domain associates directly with β-catenin, plakoglobin and p120, and indirectly with α-catenin to form a ternary complex (Yamanda *et al*., 2005). To show the importance of CDH5 in endothelial cell biology, in a mouse model, the CDH5 gene was inactivated, and the deficient mice died mid-gestation as a result of major defects in vascular remodelling. The primitive vascular plexus initially forms, however, beyond E9.25 the vessels regress and disintegrate, leading to embryonic lethality (Crosby *et al*., 2005). This shows that VE-cadherin is crucial for the maturation, extension and remodelling of vessels which are essential for angiogenesis. Another study analysing the need of VE-cadherin for maintenance of vascular integrity in adults, found that gene inactivation of VE-cadherin caused vascular leaks for plasma protein in the heart and lungs (Frye *et al*., 2015). In addition, intraperitoneally injecting anti VE-cadherin hybridoma cells into adult mice caused severe venous stasis and subcutaneous bleeding, eventually leading to death. These findings are evidence of an essential role of CDH5 in the regulation of vascular endothelial cell-cell adhesion in vivo (Matsuyoshi *et al*., 1997). Several other in vitro studies have also shown that CDH5 plays a crucial role in other aspects of endothelial cell biology as well, such as, regulation of cell adhesion, cell proliferation, cell survival, cell shape, cell motility, modulation of signalling pathways, and transcriptional gene regulation (Dejana and Vetweber, 2013).

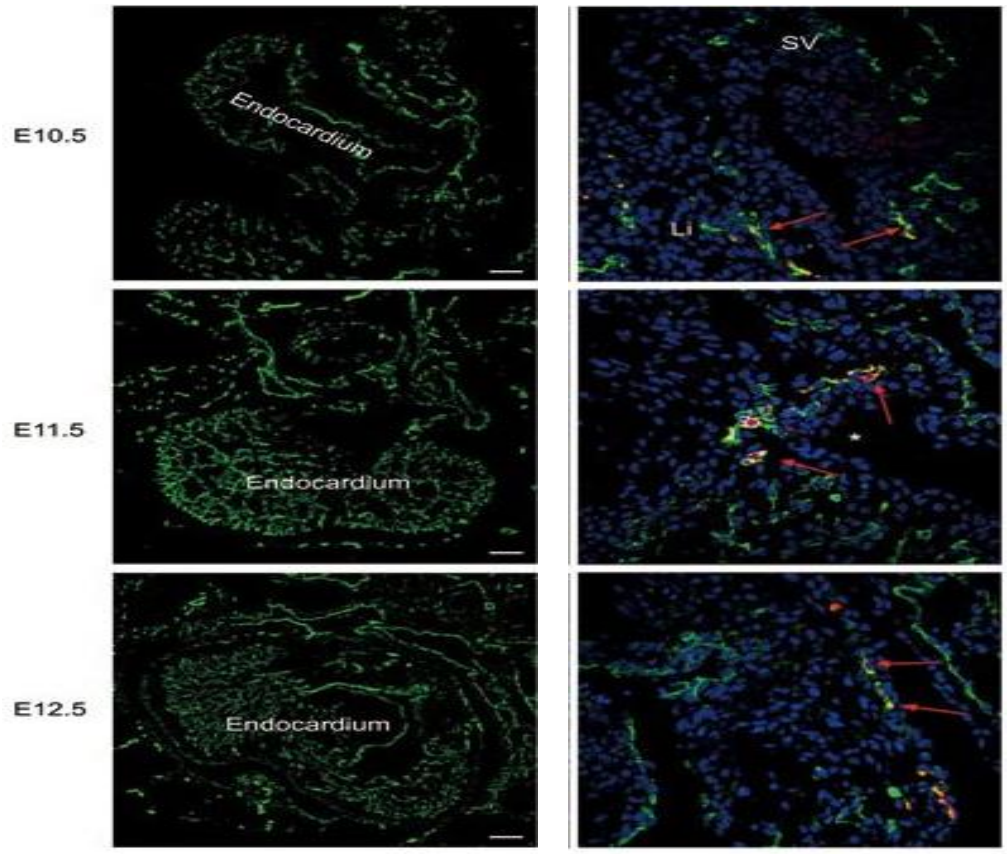


Figure 9. APLN expression map from E10.5 to E12.5. APLN is seen to be expressed in vascular endothelial cells (ECs), but not in endocardial ECs within the endocardium. Red arrows indicate the APLN positive vascular ECs (adapted from Tian *et al*., 2013).

2.7 Using induced pluripotent stem cells to model endocardial cells and study cardiovascular disease

As mentioned above, the adult heart has a very limited, next to none, regenerative capacity, so different cardiac and non-cardiac derived cells have been used as a cell-based therapy to restore cardiac function, in patients post cardiovascular disease. However, as mentioned, both of these therapies have a very low efficacy in improving cardiac function, cell retention is very low, the functionality of transplanted cells is reduced, they do not mimic the endogenous activity of cardiomyocytes, and there are still safety concerns about administering these cells into the body, and their possible side effects (Smith *et al*., 2014, Chugh *et al*., 2012, Mathiasen *et al*., 2015, Okano *et al*., 2013). Therefore, new cardiac regenerative therapies need to be made, using an endocardial-like cell population, that can be translated into the clinic. These therapies can be used in several

medical applications, such as, lineaging tracing experiments in human engineered heart tissue and *in vivo* animal models, to facilitate disease modelling, inducing revascularisation post myocardial infarction, improvement of cardiac function, cellular transplantation, drug screening, etc. However, one of the biggest hurdles in creating endocardial-like cell population is that there are no endocardial cell lines available to research on. Studies are being conducted to explore the potential of induced pluripotent stem cells (iPSCs) and use them to create endocardial cell lines, and eventually introduce cardiac regenerative therapies into the clinic.

iPSCs are adult-derived somatic cells that have been reprogrammed using different transcription factors. In 2006, Takahashi and Yamanaka introduced the concept of iPSCs and used four reprogramming factors to create iPSCs, including, Oct4 (octamer binding transcription factor-4), Sox2 (sex determining region Y), Klf4 (Kruppel Like Factor-4), and c-Myc. Embryonic stem cells (ESCs) are also a potential cell source to model development, generate disease models, differentiate into many cell lineages, however, their use in medical applications is limited, as there are several ethical issues regarding the use of embryos in research and clinics, there are chances of immune rejection in transplantation of these cells into the recipient, etc. (Singh *et al*., 2015). To overcome all of these hurdles, iPSCs can be used as they are made specifically for individual patients, eliminating the chances of immune rejection along with any ethical issues. iPSCs can be used to understand developmental biology and how different tissues are differentiated, helping us understand the genetic processes of different cell lineages, along with their ability to self-renew, differentiate into almost all cell types in the body, be used in gene editing, model disease, create *in vitro* human tissues for drug testing, etc. (Yokoo *et al*., 2009). Therefore, iPSCs are an excellent tool to study disease mechanisms, generate cell types of interest, and be useful in creating cellular therapies, such as cardiac regenerative therapies, in our case.

2.8 Role of iPSCs in cardiac tissue engineering

iPSCs have also emerged as a key component of cardiac tissue engineering, studying cardiovascular disease mechanisms, drug responses, along with, studying developmental processes in human 3D tissue models (figure 13). Several studies have been done where iPSC-derived cardiac spheroids, organoids, transplantable cardiac patches, 3D- bioprinted hearts, and heart-on-a-chip models have been developed, and technologies are constantly being developed to take these approaches further and build *in vitro* cardiac muscle tissue (Ogle *et al*., 2016 and Weinberger *et al*., 2017). Scientists have exploited the features of iPSCs and developed differentiation protocols for several major cardiovascular cell types, including iPSC cardiomyocytes, iPSC endothelial cells, iPSC fibroblasts, and iPSC vascular smooth muscle cells (Roberts *et al*., 2016). The advantages of using this technique is

that more physiological constructs can be made showing improved structural and functional maturity of multi-cell type microtissues, which enables researchers to investigate the developmental pathways of these cells, pathogenic mechanisms, drug responses in a cell-type specific manner, examination of intracellular communication mechanisms using isogenic lines, which eliminates the cell-line dependence and species-specificity of cells, hence, demonstrating the key advantages of creating iPSC cells lines (Giacomelli *et al*., 2020). Based on these advantages, engineered 3D heart tissues are replacing 2D monolayers for modelling of complex diseases, such as mitochondrial cardiomyopathy, Barth syndrome, etc. (Hinson *et al*., 2016). In addition, these 3D tissues can accentuate disease phenotypes that are unobservable in 2D cultures, for example, 3D cardiac microtissues revealed contractility defects that were undetectable in cells cardiomyocytes cultured in 2D (Hinson *et al*., 2015 and Hashimoto *et al*., 2018). Therefore, to harness all these advantages of iPSC derived cell lines, the Rana lab has developed a PSC differentiating protocol to make endocardial cells (iPSC-Endoc), and a further transdifferentiating protocol to transdifferentiate these into coronary-like endothelial cells (cECs), that can be used to study the endocardium, the developmental processes and signalling pathways within the endocardium, development of coronary endothelial-like cells, screening for therapies and drugs to improve cardiac function and revascularisation post myocardial infarction, etc. To create endocardial cells in around 10 days, the Rana lab combined two protocols: the protocol to generate endothelial cells and one to create cardiomyocytes. Within the protocol, pluripotent stem cells are first differentiated towards anterior mesoderm, then cardiac mesoderm, which expresses both first and second heart field markers, and then finally towards an endothelial cell fate. The Rana lab has also validated this endocardial differentiation protocol in several pluripotent cell lines, including iPSC lines and human embryonic stem cell lines (figure 10). To further validate the PSC derived coronary-like ECs, the Rana lab looked at the cell line's ability to transdifferentiate into coronary ECs, and found that they expressed APLN and FABP4, which are both markers for coronary ECs, along with downregulated NPR3 expression, which is a specific endocardial marker, hence showing the successful creation of coronary-like ECs (figure 11). The lab also looked at the ability of iPSC-endocardial cells to improve the maturation of cardiomyocytes and their effect on the functioning of human engineered heart tissue (figure 12). The results showed that the addition of iPSC-endocardial cells indeed increased cardiomyocyte maturation, elongation, and contractility.

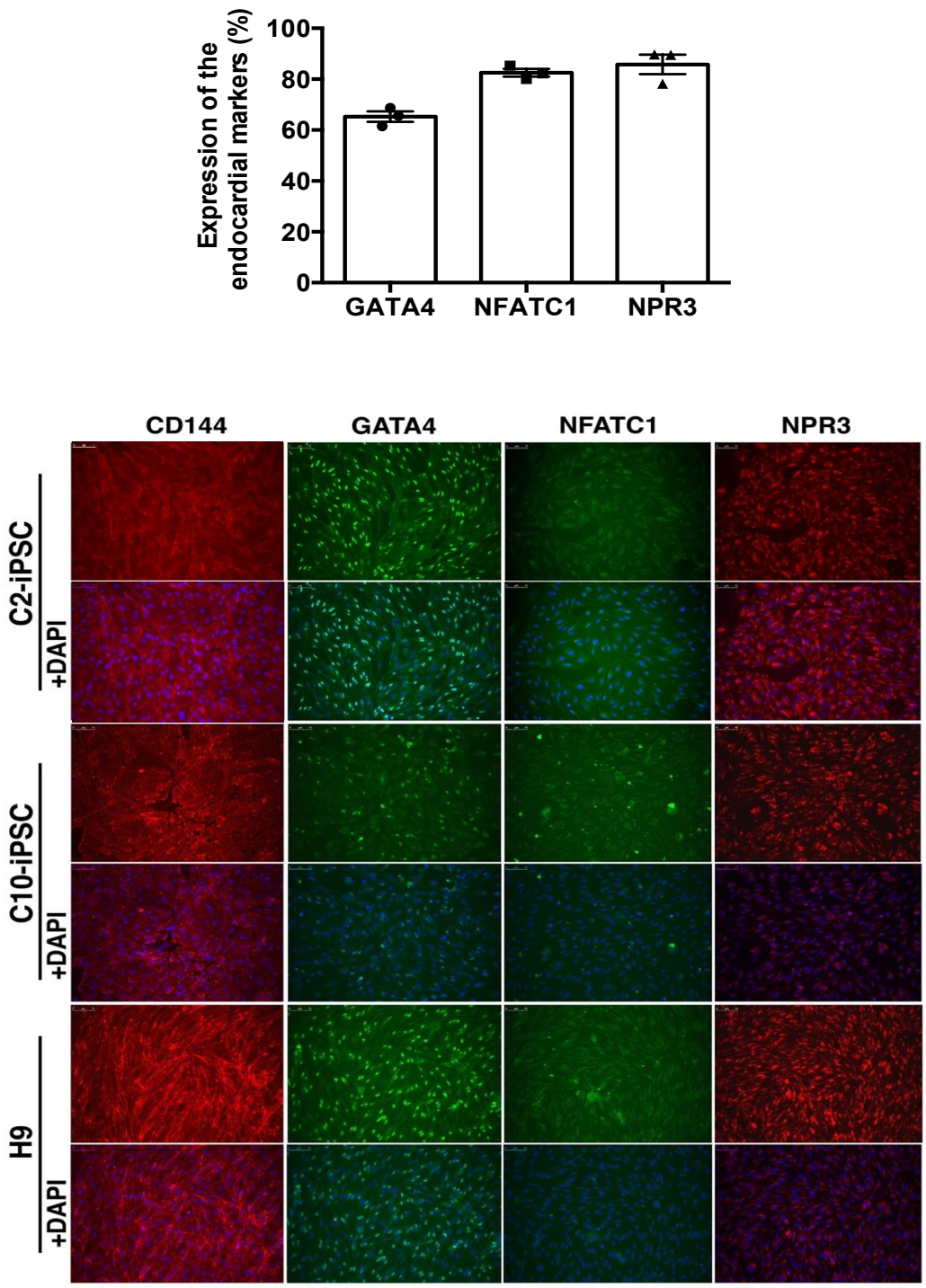


Figure 10. iPSC-derived endocardial cells being expressed in iPSC lines and human embryonic stem cell lines to validate the endocardial differentiation protocol. The cell lines show around 65.3% GATA4 expression, 82.6% NFATC1, and 85.8% NPR3 expression, which are all endocardial markers, hence proving that the cells produced using the differentiation protocol are indeed endocardial cells.

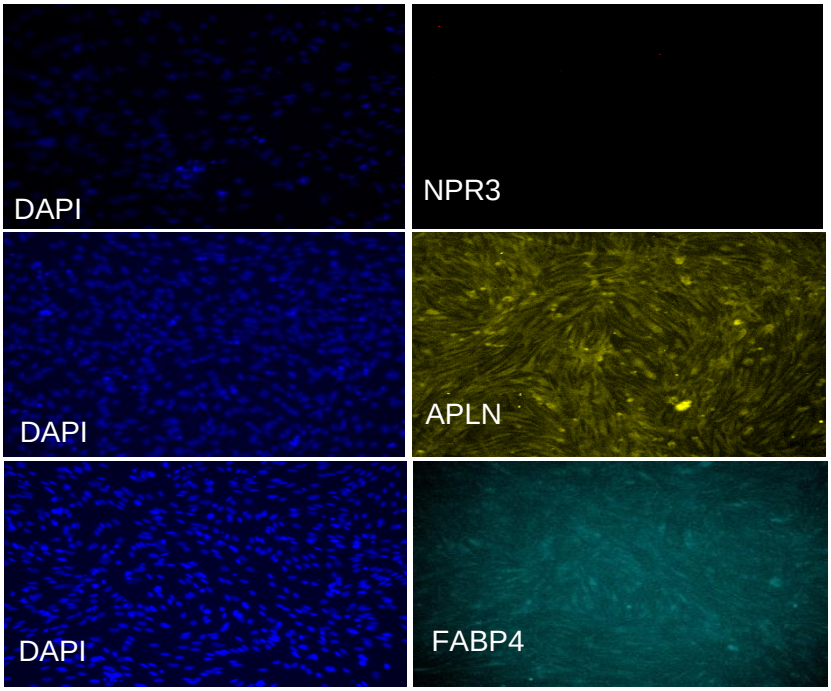


Figure 11. Transdifferentiation of Endocs to coronary EC-like cells using developmental and regenerative cues. There is increased expression of APLN and FABP4, which are both coronary EC markers, and no expression of NPR3, which is a specific endocardial marker.

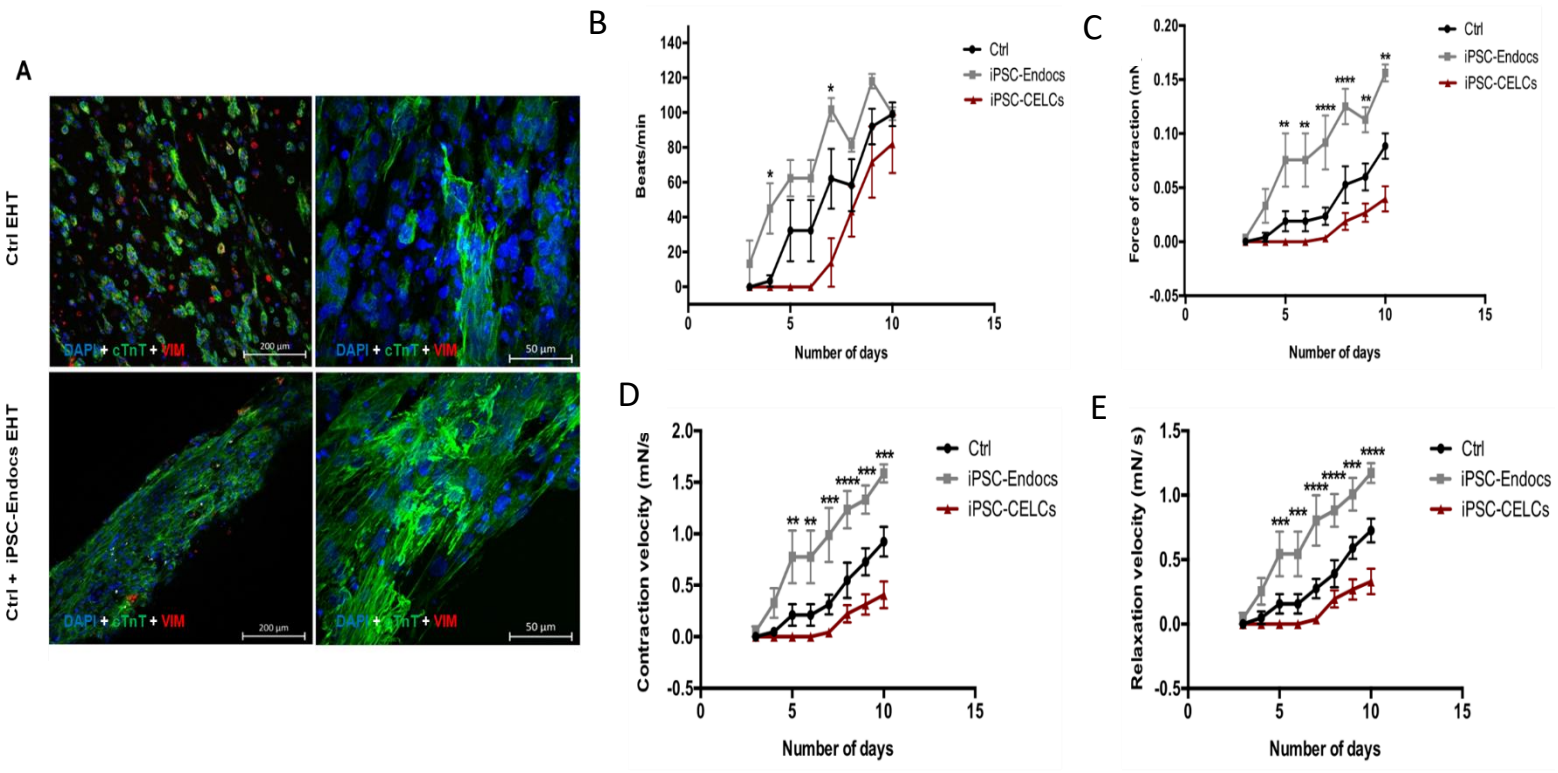
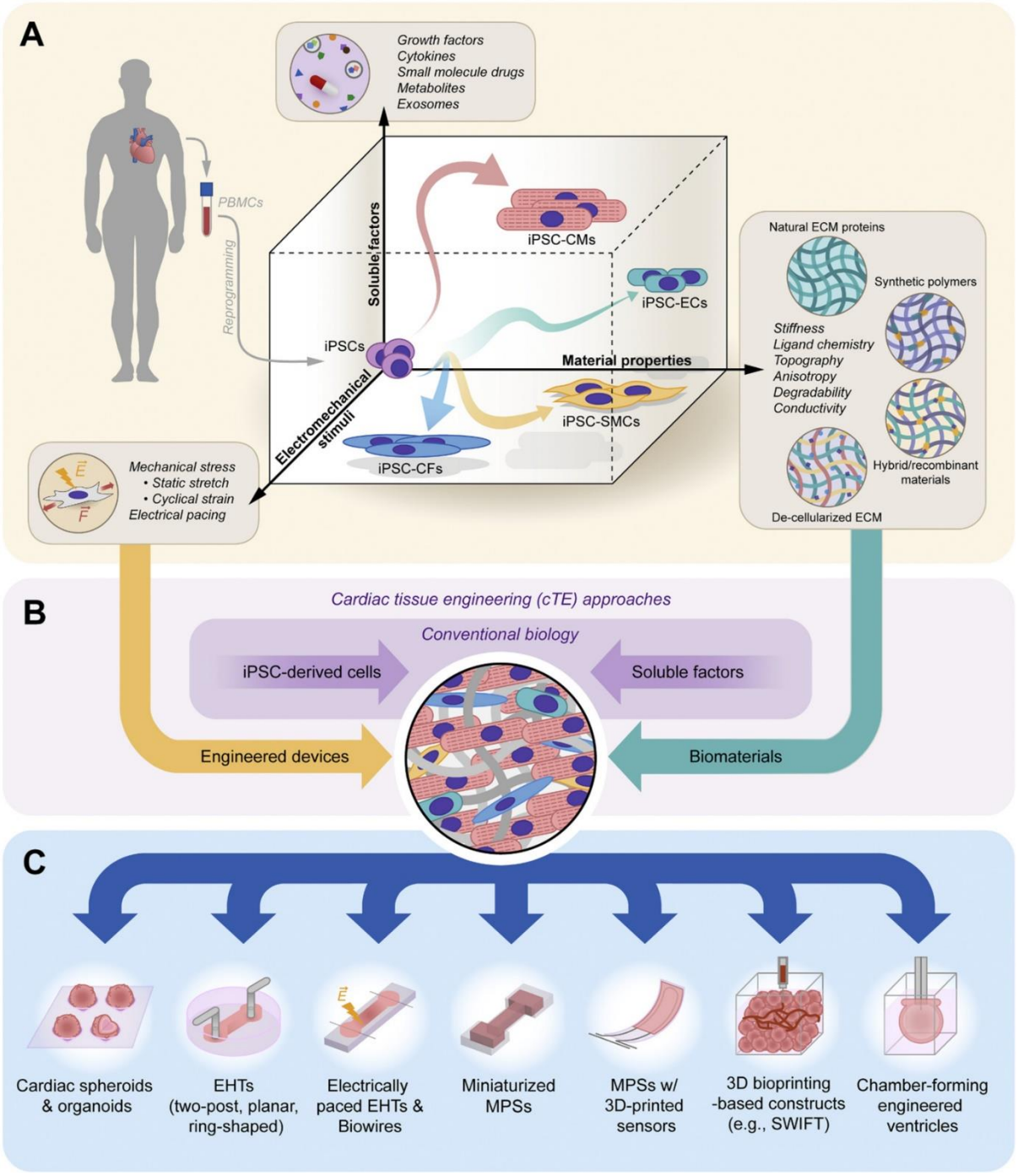


Figure 12. Effect of iPSC-endocardial-like cells on the maturation of cardiomyocytes and function on human engineered heart tissue (EHT). (A) in control EHT, the cardiomyocytes are very short and rounded- similar to foetal cardiomyocytes, whereas, in iPSC-Endoc EHT, the cardiomyocytes have a much more elongated rod-like morphology- similar to mature cardiomyocytes. In (B, C, D, and E), the addition of PSC-endocardial cells increases cardiomyocyte elongation and contractility, along with, force of contraction, contraction velocity, and relaxation velocity, respectively.

1.


A
Growth factors
Cytokines
Small molecule drugs
Metabolites
Exosomes
PBMCs
Reprogramming
Soluble factors
iPSC-CMs
iPSC-ECs
iPSCs
Material properties
iPSC-SMCs
iPSC-CFs
Electromechanical stimuli
Mechanical stress
• Static stretch
• Cyclical strain
Electrical pacing
Natural ECM proteins
Synthetic polymers
Stiffness
Ligand chemistry
Topography
Anisotropy
Degradability
Conductivity
Hybrid/recombinant materials
De-cellularized ECM
B
Cardiac tissue engineering (cTE) approaches
Conventional biology
iPSC-derived cells
Soluble factors
Engineered devices
Biomaterials
C
Cardiac spheroids & organoids
EHTs (two-post, planar, ring-shaped)
Electrically paced EHTs & Biowires
Miniaturized MPSs
MPSs w/ 3D-printed sensors
3D bioprinting -based constructs (e.g., SWIFT)
Chamber-forming engineered ventricles

2.

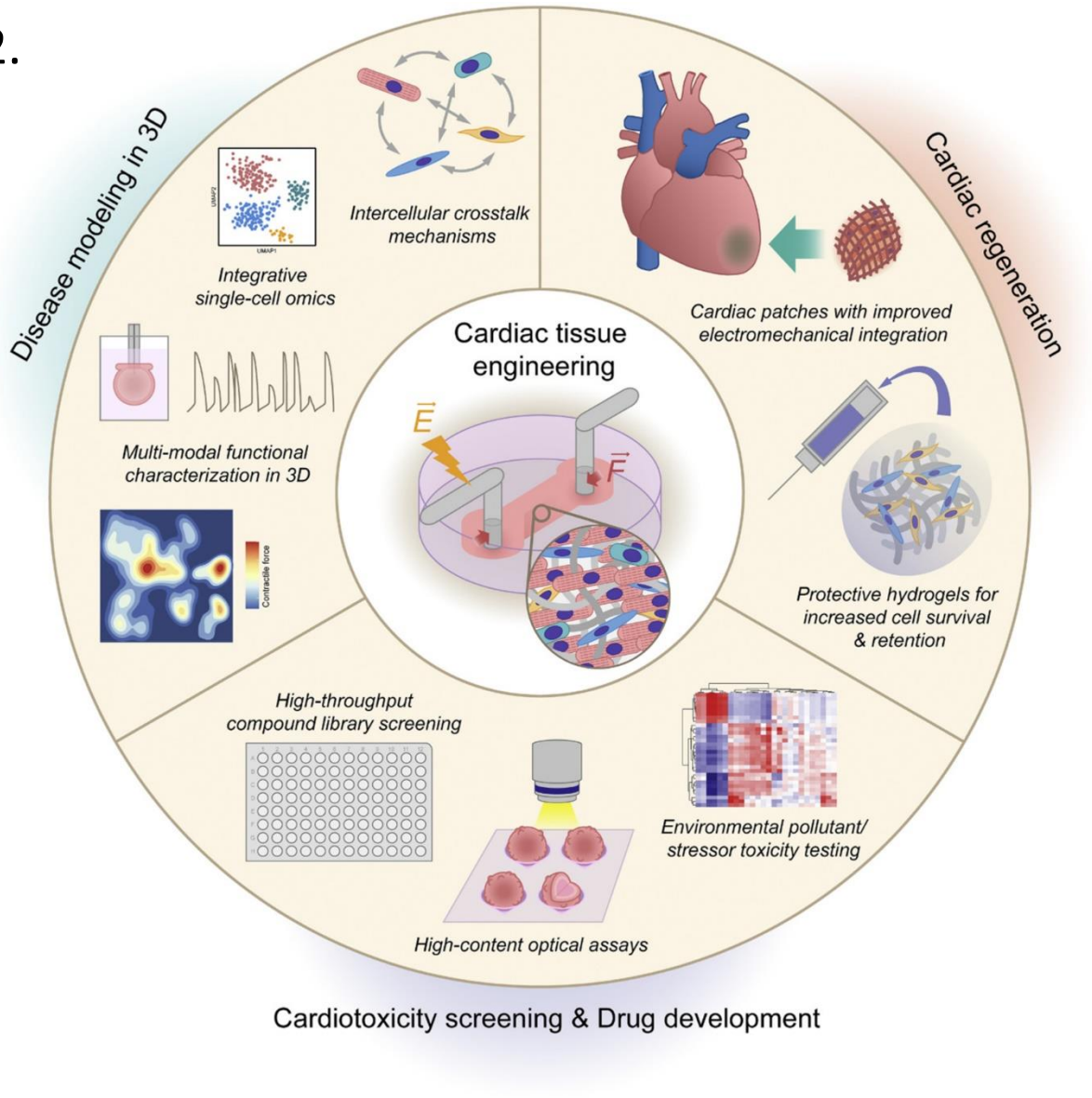


Figure 13. both figures 1 and 2 show the applications of cardiac tissue engineering using iPSCs. (1A) shows the differentiation of patient-specific iPSCs into different cardiovascular lineages, including iPSC-CMs, iPSC-ECs, iPSC-SMCs, and iPSC-CFs. (1B) shows that maturation of differentiated cells to near-adult state requires signals derived from biochemical factors, biophysical stimuli, along with material properties of surrounding extracellular matrix, biomaterials, and engineered devices to provide cells with the appropriate microenvironmental cues to generate physiological 3D tissues. (1C and 2) show the applications of cardiac tissue engineering in creating, cardiac organoids, engineered heart tissues, miniaturised microphysiological systems (MPS), chamber-forming engineered ventricles, cardiac patches, protective hydrogels, etc. Continued improvements in development of cardiac tissues will enable researchers to model complex diseases in vitro to unmask pathological phenotypes that cannot be observed in 2D models, high-throughput screening of tissue-level responses to drugs, and the development of cardiac regenerative therapies with improved cell retention, safety, functionality, and electromechanical integration. (figure adapted from Cho *et al*., 2021).

3. Aims

The aim of this project was to generate fluorescent protein expressing reporter constructs for key endocardial and coronary EC markers and test their expression in endothelial cells. To achieve this, I had to:

1. Clone and introduce GFP:polyA or mCherry:polyA cassette into a destination vector that also carries a PGK:Neo cassette
2. Identify the promoter regions and sequences of the human NPR3, APLN and CDH5 genes
3. Design primers to clone promoter regions, PCR amplify the regions and clone them into the destination vector upstream of the fluorescent reporter gene cassette- to create a fluorescent protein expressing reporter construct
4. Express the construct in endothelial cells to analyse the gene's expression, and also in non-endothelial cells where the genes should not normally be expressed, as a control

4. Methods and Materials

4.0 Overview of cloning strategy

The aim of this study was to generate fluorescent reporters for specific genes to study their expression and function in the heart. These fluorescent reporters were created by inserting the GFP:polyA and mCherry:polyA cassettes into the LND4 vector, along with the minimal promoter region of NPR3, APLN, and CDH5, upstream of the fluorescent protein. The LND4 vector is a previously in-house modified bluescript vector with an altered multiple cloning site that carries a phosphoglycerate kinase promoter which drives the neomycin resistance gene (PGKNeo) expression cassette, for positive selection, along with 6 bp and 8 bp cutter restriction enzyme sites, to aid cloning. Bluescript are cloning vectors designed to simplify commonly used cloning and sequencing procedures. This reporter construct will fluoresce whenever the gene of interest is being expressed, hence allowing us to evaluate its expression within the tissue. An overview of this cloning strategy is shown in figure 14.

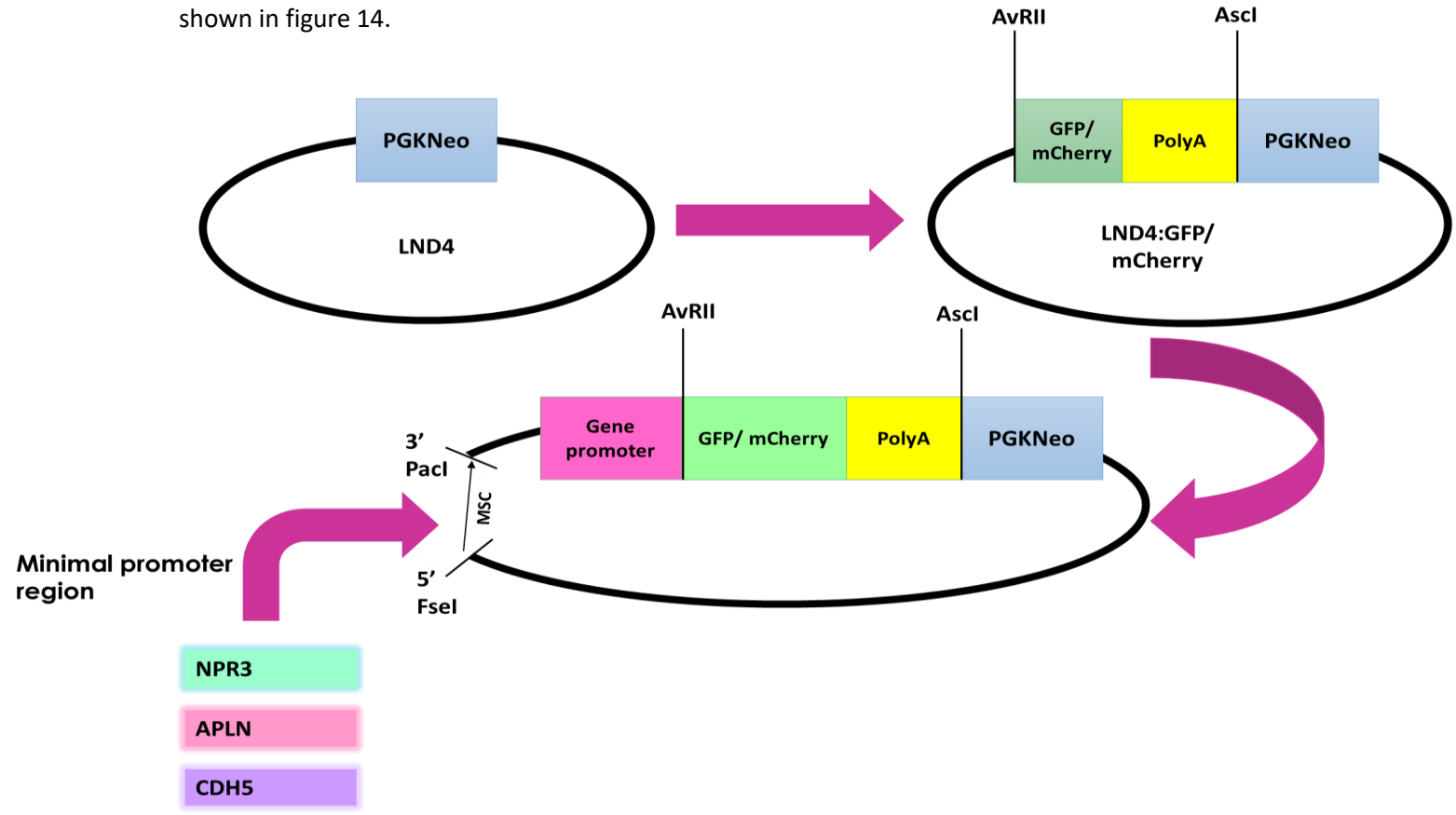


Figure 14. diagram outlining the cloning strategy. Step one is to insert a fluorescent protein, attached to a polyA tail into the LND4 vector. Second step is where the gene minimal promoter is inserted into this vector, upstream of the fluorescent protein, to create a final cloning vector consisting of LND4:PGKNeo:PolyA:GFP/mCherry:gene promoter. GFP and mCherry are green and red fluorescent proteins, respectively. The lines show the restriction enzyme cut sites: AvRII and AscI are cut sites for GFP and mCherry, and PacI and FseI are for the regulatory regions of NPR3, APLN, and CDH5.

4.1 Bioinformatics

To explore the structure of our genes NPR3, APLN, and CDH5 we used the University of California, Santa Cruz (UCSC) and Ensembl genome browsers, and this helped us identify the regulatory regions of the gene, its exons, transcriptional start, CpG islands, acetylation levels, cis-regulatory elements, enhancer regions, promoter regions, evolutionary conservation between species, etc. The minimal promoter regions identified were exported into Benchling where NCBI primer blast was used to generate forward and reverse primers for each gene, as well as adding restriction enzyme cut sites that will be used later on in the study to amplify, digest, and ligate the desired gene regions into the LND4:GFP and LND4:mCherry destination vectors.

4.2 Amplification and cloning of regulatory regions

4.2.1 PCR of regulatory regions

The potential minimal promoter regions were amplified through polymerase chain reaction (PCR), using the primers mentioned in table 6. The PCR reactions were set up following the Invitrogen ™ Platinum ™ SuperFi ™ II DNA Polymerase protocol (Catalogue number: 12368010). The PCR for the minimal promoter region for each gene was setup as shown in table 1. The PCR running program is shown in table 2.

Table 1. table showing the components, their concentration and volumes that were used to set up a 25 µl PCR reaction to amplify the regulatory regions of our genes.

Component	Final concentration	Volume for 25 µl reaction
2X Platinum ™ SuperFi ™ II PCR Master Mix	1X	12.5 µl
Forward primer	0.5 µM	1.5 µl
Reverse primer	0.5 µM	1.5 µl
Template DNA	300 ng genomic DNA	3 µl
Nuclease free water	-	6.5 µl

Table 2. table showing the PCR cycle that was used for the amplification of regulatory regions.

Cycle step	Temperature (°C)	Time	Cycles
Initial denaturation	98	30 seconds	1
Denaturation Annealing Extension	98 60 72	5-10 seconds 10 seconds 15-30 seconds per 1 kb	25-35
Final extension	72 4	5 minutes Hold	1 -

4.2.2 Gel electrophoresis

Agarose gel electrophoresis was used to confirm the presence of the right sized amplicon for each gene. The first step was to make a TAE buffer, we combined 900 ml of distilled water to 100 ml of 1X TAE and mixed well. Then, to make a 1% agarose gel, we combined 1 g of agarose and 100 ml TAE buffer into a conical flask, heated it up in a microwave till the agarose dissolved, cooled it down till it's warm enough to touch, and poured it into a gel tank with 5 µl of 0.5 µg/ml ethidium bromide. Once the gel was set, we loaded the first lane with 4 µl of ThermoFisher Scientific™ GeneRuler 1 kb Plus DNA ladder (catalogue number: SM1333), then the subsequent lanes were loaded with the PCR reactions. Each well had 9 µl of PCR product combined with 1 µl of BlueJuice™ 10X Gel loading buffer (catalogue number: 10816015). The gel was run at 120 V for 40 minutes and then analysed under UV light.

4.2.3 Preparing Lucia Broth (LB) Agar plates

In order for our *E.coli* cells to grow into bacterial colonies, we made LB-Agar plates, with kanamycin and ampicillin used as positive selection markers. LB is a nutrient rich broth that is a standard broth for culturing E.coli, as it allows for quick bacterial growth and a higher yield of colonies. Kanamycin was used on cells within the TOPO vector only, whereas ampicillin was used when our reporters were ligated to the LND4 vector. To make 50 µg/ml kanamycin, we dissolved 0.5 g kanamycin powder into 10 ml sterile water, to create a 1000x stock. On the other hand, to make 100 µg/ml ampicillin, we dissolved 1 g ampicillin powder into 10ml of sterile water, to create a 1000x stock. Then, to make 40 LB-Agar plates, we added 15 g of agar powder to 1 L of LB broth and put it in the autoclave machine for 2 hours, in order to sterilise the broth and kill any infectious agents. Once the LB-Agar was out of the autoclave, we allowed it to cool down to room temperature, before adding the antibiotics, to prevent the antibiotics from denaturing at a higher temperature. For kanamycin/ ampicillin-LB-Agar

plates, we added 1 ml of the 1000x antibiotic stock, to 1 L of LB-Agar solution, and mixed well. A 25 ml sample of the kanamycin/ ampicillin-LB-Agar solution was poured into each sterile Petri dish and allowed to cool so the agar sets properly. This produced around 40 plates which were then stored at 4°C.

4.2.4 Preparing LB medium with antibiotics

After picking bacterial colonies from the LB-Agar plates, we cultured the colonies in LB-antibiotic medium to allow the bacteria to grow and yield a high concentration of DNA, once purified. To make the medium, 5 ml of liquid LB was added to a 50 ml centrifuge tube, along with 5 µl of kanamycin (50 mg/ml stock) or 5 µl of ampicillin (100 mg/ml), depending on which medium you want to make. Bacterial colonies can then be put into this medium, and incubated overnight at 37 °C in, to allow the bacteria to grow.

4.2.5 TOPO cloning of PCR products into the TOPO vector and transformation into competent cells

The TOPO cloning of the PCR products and their transformation into competent cells was done following the protocol of the Invitrogen™ Zero Blunt™ TOPO™ PCR cloning kit with One Shot™ TOP10 Chemically Competent E.coli cells (catalogue number: K280020). This kit provides a one-step cloning strategy for the direct insertion of blunt-ended PCR products into a plasmid vector: the pCR™ Blunt II-TOPO™ vector, which is 3519 bps in size. The first step was to set up the TOPO® cloning reaction following the procedure shown in table 3. Then this reaction was mixed up gently and incubated for 5 minutes at room temperature (~22°C) to allow for the cloning reaction to happen. We extended this 5-minute incubation period to 30 minutes, to yield a higher number of colonies. Then we proceeded to chemically transform the One Shot® competent *E.coli* cells with this TOPO® reaction. In this, 2 µl of the TOPO® cloning reaction was added to a vial of One Shot® chemically competent *E.coli* cells and mixed very gently, to avoid the cells breaking, as the cells become very fragile after thawing and can break open easily, so won't be able to clone the products into themselves. This reaction was then incubated on ice for 30 minutes, and then heat shocked for 30 seconds at 42°C to allow the transformation to happen. These cells were immediately transferred to ice and 250 µl of room temperature S.O.C. medium was added to the vial. These tubes were placed into a shaking incubator and shaken horizontally (200 rpm) at 37°C for 1 hour. From each transformation, 50 µl was spread on a prewarmed agar plate with Kanamycin for positive selection, and incubated overnight at 37°C. Then, 8-12 colonies were picked from each plate and cultured overnight in a shaking incubator at 37°C and 180 rpm, in LB medium containing 50 µg/ml Kanamycin.

Table 3. procedure shown to set up the TOPO® cloning reaction, to a final volume of 6 µl.

Reagent	Volume
Fresh PCR product	2 µl
Salt solution	1 µl
pCR™ Blunt II- TOPO™ vector	1 µl
Water	2 µl

4.2.6 Isolating plasmid DNA

The next step was to isolate plasmid DNA from the cloning reaction, and this was done by following the protocol of the QIAprep® Spin Miniprep Kit (catalogue number: 27106X4). The QIAprep 2.0 Spin Columns contain a silica membrane that binds DNA in the presence of a high concentration of chaotropic salt and allows elution in a small volume of low-salt buffer. In this protocol we put 2 ml of overnight bacterial culture from the previous step into an Eppendorf tube and centrifuged it at 8000 rpm for 3 minutes, to pellet the cells. These pelleted bacterial cells were resuspended in 250 µl of buffer P1 and transferred to a microcentrifuge tube, and further 250 µl buffer P2 was added, and the tube was mixed thoroughly to combine all the reagents. 350 µl buffer N3 was added to this mixture to bind all the DNA to the column, and this was centrifuged at 13,000 rpm for 10 minutes. 800 µl supernatant was applied into a spin column and centrifuged for 60 seconds, and the flow through was discarded. To wash the column, first 0.5 ml buffer PB was added and centrifuged and then 0.75 ml buffer PE added, centrifuged, and flow through discarded. The spin column was then placed into a clean microcentrifuge tube, and to elute DNA, 50 µl buffer EB was added to the centre of the column, and we let it stand for 5 minutes to bind the maximum amount of DNA, and then centrifuged for 1 minute. The tube now has extracted plasmid DNA in it. The concentration of this DNA was measured using the Nanodrop spectrophotometer.

This DNA was sent for sequence verification to ensure that we were cloning the desired regulatory regions of our genes. Sequences were verified using the mix2seq sequencing service from Eurofins Genomics.

4.3 Building the LND4:GFP and LND4:mCherry vector

4.3.1 Amplification of LND4 vector

The LND4 vector was transformed into One Shot® chemically competent *E.coli* cells, using the transformation method mentioned above, and the transformation reaction was spread on agar plates with ampicillin for positive selection. The plates were incubated overnight at 37°C. Then, 8-12 colonies

were picked from each plate and cultured overnight in a shaking incubator at 37°C and 180 rpm, in LB medium containing Ampicillin. The next step was to isolate and purify this LND4 vector DNA from the cloning reaction, and this was done by following the protocol of the QIAprep® Spin Miniprep Kit, mentioned in 4.2.6. We now have the LND4 vector in its purified form.

4.3.2 PCR to amplify GFP and mCherry amplicons

To amplify GFP+SV40 polyA tail (using Cer0.4-EGFP as a template) and mCherry + β globin polyA tail (using pClCherry v2 as a template), we designed forward and reverse primers mentioned in table 5, with AvRII and AscI restriction enzyme sites added. These primers were used to set up PCR reactions following the Invitrogen ™ Platinum ™ SuperFi ™ II DNA Polymerase protocol (Catalogue number: 12368010), mentioned on 4.2.1. Then, agarose gel electrophoresis was used to run the PCR products on a 1% agarose gel, as mentioned in 4.2.2 and confirm the presence of the right sized amplicon.

4.3.3 TOPO cloning of GFP and mCherry PCR products into the TOPO vector and transformation into competent cells

The TOPO cloning of the PCR products and their transformation into competent cells was done following the protocol of the Invitrogen™ Zero Blunt™ TOPO™ PCR cloning kit with One Shot™ TOP10 Chemically Competent E.coli cells (catalogue number: K280020). The first step was to set up the TOPO® cloning reaction following the procedure shown in table 3. Then this reaction was mixed up gently and incubated for 5 minutes at room temperature (~22°C) to allow for the cloning reaction to happen. We extended this 5-minute incubation period to 30 minutes, to yield a higher number of colonies. Then we proceeded to chemically transform the One Shot® competent *E.coli* cells with this TOPO® reaction. In this, 2 µl of the TOPO® cloning reaction was added to a vial of One Shot® chemically competent *E.coli* cells and mixed very gently, to avoid the cells breaking. This reaction was then incubated on ice for 30 minutes, and then heat shocked for 30 seconds at 42°C to allow the transformation to happen. These cells were immediately transferred to ice and 250 µl of room temperature S.O.C. medium was added to the vial. These tubes were placed into a shaking incubator and shaken horizontally (200 rpm) at 37°C for 1 hour. From each transformation, 50 µl was spread on a prewarmed agar plate with Kanamycin for positive selection, and incubated overnight at 37°C. Then, 8-12 colonies were picked from each plate and cultured overnight in a shaking incubator at 37°C and 180 rpm, in LB medium containing Kanamycin.

4.3.4 Isolating plasmid DNA from the cloning reaction

The next step was to isolate plasmid DNA from the cloning reaction, and this was done by following the protocol of the QIAprep® Spin Miniprep Kit (catalogue number: 27106X4). In this protocol we put

2 ml of overnight bacterial culture from the previous step into an Eppendorf tube and centrifuged it at 8000 rpm for 3 minutes, to pellet the cells. These pelleted bacterial cells were resuspended in 250 µl of buffer P1 and transferred to a microcentrifuge tube, and further 250 µl buffer P2 was added, and the tube was mixed thoroughly to combine all the reagents. 350 µl buffer N3 was added to this mixture to bind all the DNA to the column, and this was centrifuged at 13,000 rpm for 10 minutes. 800 µl supernatant was applied into a spin column and centrifuged for 60 seconds, and the flow through was discarded. To wash the column, first 0.5 ml buffer PB was added and centrifuged and then 0.75 ml buffer PE added, centrifuged, and flow through discarded. The spin column was then placed into a clean microcentrifuge tube, and to elute DNA, 50 µl buffer EB was added to the centre of the column, and we let it stand for 5 minutes to bind the maximum amount of DNA, and then centrifuged for 1 minute. The tube now has extracted plasmid DNA in it. The concentration of this DNA was measured using the Nanodrop spectrophotometer.

4.3.5 Restriction Digestion of TOPO® vector, LND4, GFP, and mCherry

To set up a TOPO® vector digest reaction, in an Eppendorf tube we added 10 µg of plasmid DNA (from the minipreps performed above), 8 U of AvRII, 6 U of AscI, 8 µl of 10x rCutSmart® buffer, and nuclease free water to make the final reaction volume to 80 µl. These reactions were incubated overnight at 37°C. Then, to digest the LND4 vector with AvrII and AscI, we used the same protocol. These reactions were then ran on an agarose gel, following the protocol mentioned in 4.2.2.

4.3.6 Gel extraction and purification

After running the agarose gel with the restriction digest reactions, we cut out the correct sized bands, extracted them, and purified them using the QIAquick Gel Extraction kit (catalogue number: 28706X4) protocol. This kit uses a bind-wash-elute procedure which enables the removal of nucleotides, enzymes, salts, agarose, ethidium bromide, and other impurities from samples, to recover up to 80% of DNA. In this protocol, the gel slice is excised from the agarose and mixed with three times the volume of buffer QG that has a pH indicator in it. This gel slice is completely melted by incubation at 50 °C for 10 minutes, and one gel volume of isopropanol is added. The mixture is applied to a QIAquick spin column, and centrifuged. The nucleic acids absorb to the silica membrane in the high-salt conditions provided by the buffer. Then, the impurities are washed away by the addition of 750 µl buffer PE, and the column is centrifuged for one minute. The QIAquick column is placed into a clean microcentrifuge tube, and 30 µl of buffer EB (low salt buffer) is added to the centre of the tube, incubated at room temperature for 5 minutes, and then centrifuged. The purified DNA is now in the microcentrifuge tube, ready to be used for further applications. The concentration of this DNA was measured using the Nanodrop spectrophotometer.

4.3.7 DNA ligation of GFP and mCherry into LND4

To create the final destination vector, GFP and mCherry (inserts) were ligated into LND4 (vector). The ligation reactions were set up using a 3:1 molar ratio of insert: vector, and this ratio was calculated using the NEBioCalculator® ligation calculator. For these ligation reactions, the Invitrogen® T4 DNA ligase buffer (catalogue number: 46300018) was used, which catalyses the formation of phosphodiester bonds in the presence of ATP between double-stranded DNA with 3' hydroxyl and 5' phosphate termini. Two reactions were set up, one for the ligation of LND4:GFP and the other for LND4:mCherry. For the LND4:GFP reaction, 50 ng of LND4, 21.5 ng of GFP, 2 µl of T4 DNA ligase buffer, 1 U T4 DNA ligase, and nuclease free water up to 20 µl, was all added into a microcentrifuge tube. Then, for the LND4:mCherry reaction, 50 ng of LND4, 20.7 ng of mCherry, 2 µl of T4 DNA ligase buffer, 1 U T4 DNA ligase, and nuclease free water up to 20 µl, was all added into a microcentrifuge tube. These reactions were incubated for 1 hour at 22°C, and then the T4 DNA ligase was heat inactivated at 65°C for 10 minutes to improve the electrotransformation efficiency. From each of these reactions, 5 µl of the sample was taken out to transform One Shot® chemically competent *E.coli* cells, and spread onto LB agar + ampicillin plates, and then the colonies were picked from these plates, using the protocol mentioned in 4.3.3. To isolate plasmid DNA, we used the protocol in 4.3.4. A test digest of the ligations was performed and ran on an agarose gel to confirm that GFP and mCherry were ligated into LND4 successfully. We now had the destination vectors ready, LND4:GFP and LND4:mCherry.

4.3.8 Glycerol stocks

Glycerol stocks of the destination vector were created, to stabilise the frozen bacteria, preventing damage to cell membranes and keeping the cells alive, so that they can be stored for use in later experiments. To make these glycerol stocks, 500 µl of the bacterial culture was added to 500 µl of 50% glycerol in water, in a screw-cap tube, and stored at -80°C.

4.4 Constructing the final destination vector with gene promoter regions, LND4:GFP and LND4:mCherry

4.4.1 Digestion of minimal promoter regions of NPR3, APLN and CDH5

Following the isolation of plasmid DNA for NPR3, APLN, and CDH5 in the TOPO® vector, in 4.2.6, we digested out the TOPO® vector, using the restriction enzymes FseI and PacI. In this reaction, 10 µg of the TOPO® vector was added to 8 U of FseI, 6 U of PacI, 8 µl of 10x rCutSmart® buffer, and nuclease free water to make the final reaction volume to 80 µl. These reactions were incubated overnight at 37°C. These reactions were then ran on an agarose gel, following the protocol mentioned in 4.2.2, to confirm the presence of the right sized bands.

4.4.2 Ligating NPR3, APLN and CDH5 into LND4:GFP and LND4:mCherry

To construct the final fluorescent reporters, NPR3, APLN and CDH5 (inserts) were ligated into LND4:GFP and LND4:mCherry (vector), the ligation reactions were set up using a 3:1 molar ratio of insert: vector, and this ratio was calculated using the NEBioCalculator® ligation calculator. For these ligation reactions, the Invitrogen® T4 DNA ligase buffer (catalogue number: 46300018) was used. To make the ligation reaction, 20-100 ng of linear vector DNA was added to 3:1 molar ratio insert DNA, 2 µl of T4 DNA ligase buffer, 1 U T4 DNA ligase, and nuclease free water up to 20 µl, was all added into a microcentrifuge tube.

These reactions were incubated for 1 hour at 22°C, and then the T4 DNA ligase was heat inactivated at 65°C for 10 minutes to improve the electrotransformation efficiency. From each of these reactions, 5 µl of the sample was taken out to transform One Shot® chemically competent *E.coli* cells, and spread onto LB agar + ampicillin plates, and then the colonies were picked from these plates, and cultured in LB+ Ampicillin, using the protocol mentioned in 4.3.3. To isolate plasmid DNA, we used the protocol in 4.3.4. A test digest of the ligations was performed and ran on an agarose gel to confirm that NPR3, APLN, and CDH5 have successfully been inserted into our destination vectors.

At this point, all our fluorescent reporter constructs were successfully created, NPR3:LND4:GFP, NPR3:LND4:mCherry, APLN:LND4:GFP, APLN:LND4:mCherry, CDH5:LND4:GFP, and CDH5:LND4: mCherry. Glycerol stocks of all of these constructs was made using the protocol in 4.3.8, so that they can be stored and used in future experiments.

4.5 Electroporation of reporter constructs into human adult endothelial cells

Due to time constraints, we were only able to transfect the CDH5:LND4:GFP construct into cells and image them. Endothelial progenitor cells were cultured in Lonza EGM-2MV growth medium without heparin until 70-80% confluent. Cells were trypsinised and 500,000 cells were electroporated, using the Amaxa Nucleofector II ®, according to the manufacturer's instructions, with 2 ng of CDH5:GFP reporter DNA plasmid produced by Qiagen QIAprep® Spin Miniprep Kit (catalogue number: 27106X4). Cells were imaged on a Liece DMi8 24 hours after electroporation.

5. Results

5.1 Amplification of GFP and mCherry

Primers, shown in table 5, were designed around the minimal promoter region of GFP, using Cer0.4-EGFP as a template, and mCherry using pClCherry v2 as a template, and these were then amplified through PCR, and run on an agarose gel, to determine the band sizes of PCR products (figure 15). The desired amplicon size for both GFP and mCherry is ~1 kb. The PCR products were then cloned into the TOPO vector. Next, this plasmid was transformed into OneShot®*E.coli* cells and plated onto LB plates with Kanamycin to positively select the cells which have a successful insertion of the vector. The colonies were picked from the plate and grown in LB + Kanamycin liquid culture, and then miniprepped and nano-dropped. At this point, we have the fluorescent protein cloned into a TOPO vector.

Table 5. Sequences of primers made for GFP and mCherry, capturing their minimal promoter regions, with the polyA tail and AvrII and AscI restriction enzyme sites attached, and the primer direction (forward or reverse primers) mentioned, along with the amplicon size.

Primer name	Primer sequence	Amplicon size (bp)
GFPbGpA-F1-AvrII	5'- AAAAAACCTAGGATCTACAGCATCAGCAGGACC -3'	1002
GFPbGpA-R1-AscI	5'- AAAAAAGGCGCGCCAACGCTTACAATTTACGCCT -3'	
mChbGpA-F1-AvrII	5'- AAAAAACCTAGGGAAAGGTACACGTGTCCATGG -3'	996
mChbGpA-R1-AscI	5'- AAAAAAGGCGCGCCCTCCCATATGTCCTTCCGAG -3'	

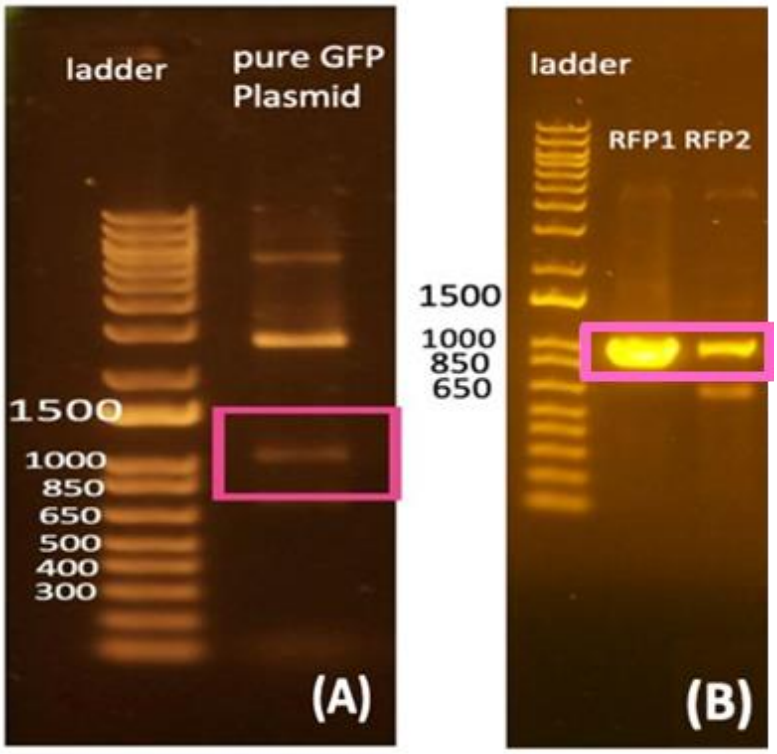


Figure 15. PCR gel electrophoresis of GFP and mCherry amplification, with a 1 kb plus DNA ladder. (A) GFP PCR product shows a band around 1000 bp (highlighted in the pink box), which is our desired amplicon size. (B) RFP PCR products were loaded into two lanes and both the lanes have a band around 1000 bp (highlighted in pink box), which is the correct sized band, as our desired amplicon size was 996 bp. These results showed that our GFP and mCherry amplifications were both successful.

5.2 Generating the LND4:GFP and LND4:mCherry destination vector

The aim of this step is to create the destination vector into which the gene promoters will be inserted into (figure 16). This vector is composed of the LND4 vector and the GFP/mCherry poly A cassettes. These two products were ligated together, cultured, and then gel electrophoresis was conducted to confirm the presence of the fluorescent proteins in the LND4 vector. The gel images are shown in figure 17 and figure 18, for LND4:GFP and LND4:mCherry, respectively.

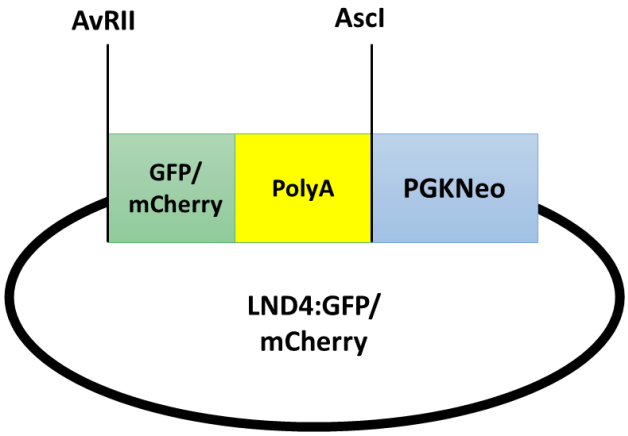


Figure 16. diagram showing the destination vector composed of the LND4 vector with GFP or mCherry attached with a PolyA tail and PGKNeo cassette, inserted into it. This is the vector into which the gene promoter regions will be cloned into.

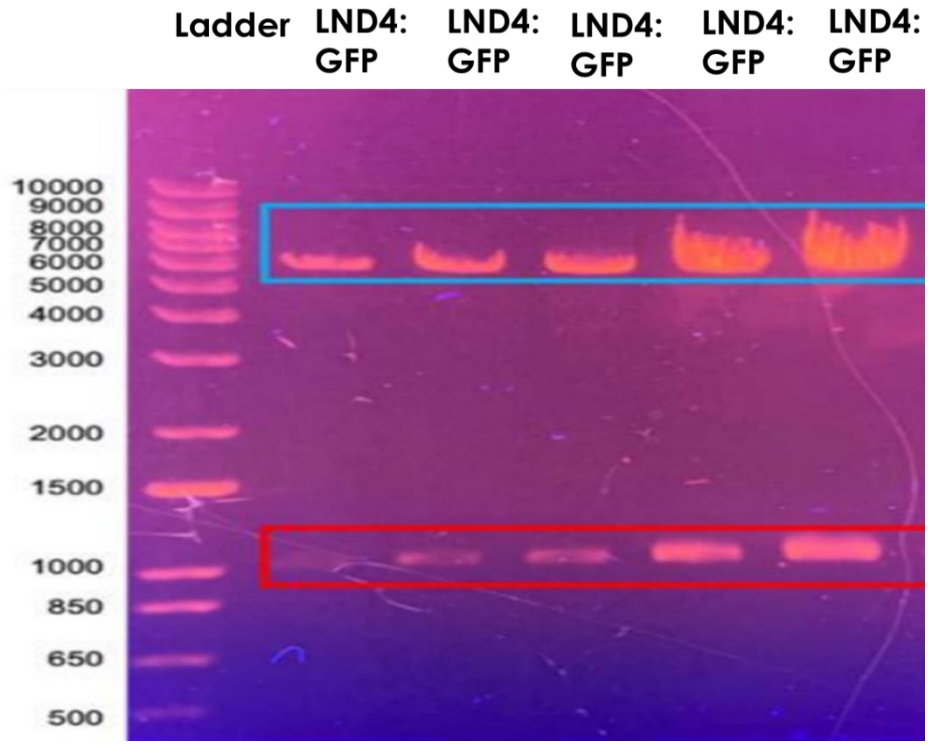


Figure 17. 1% agarose gel electrophoresis of the final destination vector, with a 1 kb plus DNA ladder. The gel shows the successful insertion of GFP (1002 bp) into LND4 (6000 bp). The correct sized bands are highlighted in the red and blue boxes for GFP and LND4, respectively.

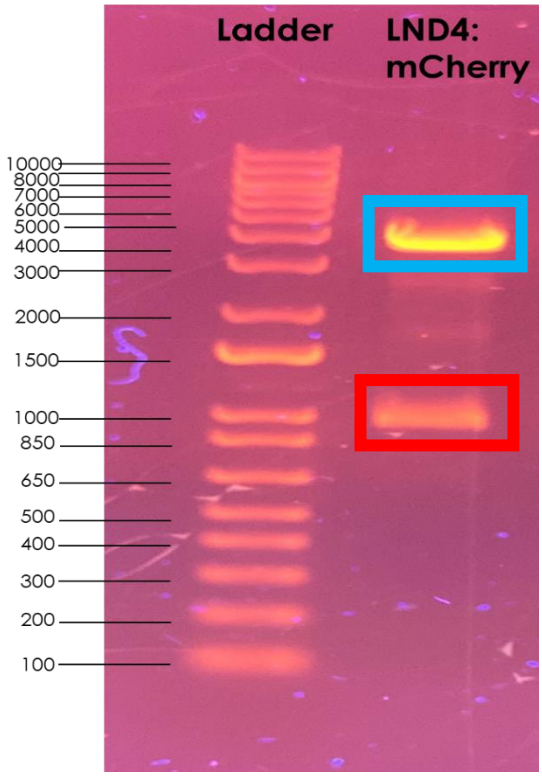


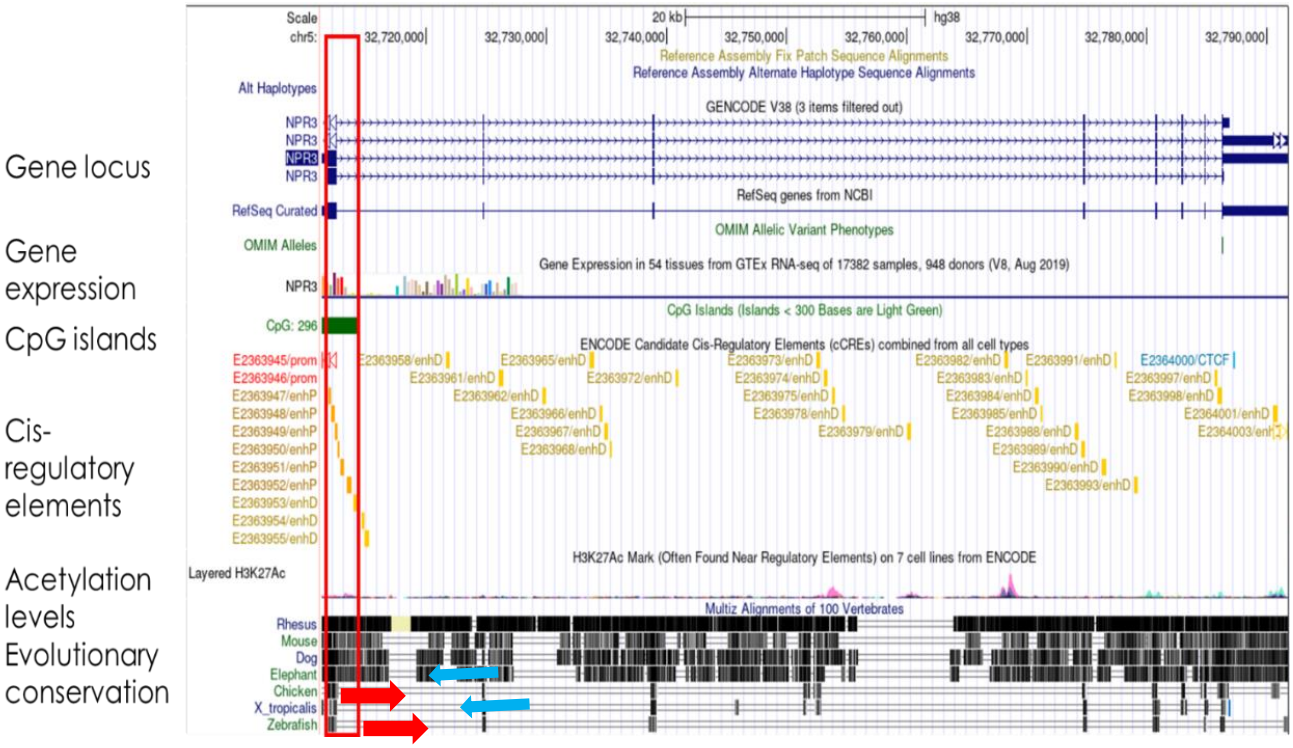
Figure 18. 1% agarose gel electrophoresis of the final destination vector, with a 1 kb plus DNA ladder. The gel shows the successful insertion of mCherry (996 bp) into LND4 (6000 bp). The correct sized bands are highlighted in the red and blue boxes for mCherry and LND4, respectively.

5.3 Bioinformatics

The UCSC genome browser was used to explore the structure of our genes: NPR3, APLN, and CDH5, and identify their regulatory regions, particularly the minimal promoter region for our study. To identify these regions, we applied specific tracks on the genome browser, which characterise gene regulatory regions. The tracks we focused on were, CpG islands which are DNA methylation regions present in the promoter sites, known to regulate gene expression through transcriptional silencing of the corresponding gene, then the cis-regulatory elements which are cis-acting noncoding DNA regions that regulate gene transcription, and key cis-regulatory elements include, promoters, enhancers, silencers, etc. In addition to these tracks, we also focused on H3K27ac which is an epigenetic modification to Histone H3, a DNA packaging protein, and is associated with higher activation of transcription, therefore, must be present in the promoter regions of the genes. We also focussed on regions with high sequence conservation between species. Overall, we selected the regions at the start of the gene with the highest levels of CpG islands, cis-regulatory elements, H3K27ac levels, and highest conservation between species, as all these characteristics should be

present in the promoter region, where the process of transcription is initiated in a gene. Figure 19 demonstrates our approach into selecting the promoter regions for NPR3, APLN, and CDH5.

A.



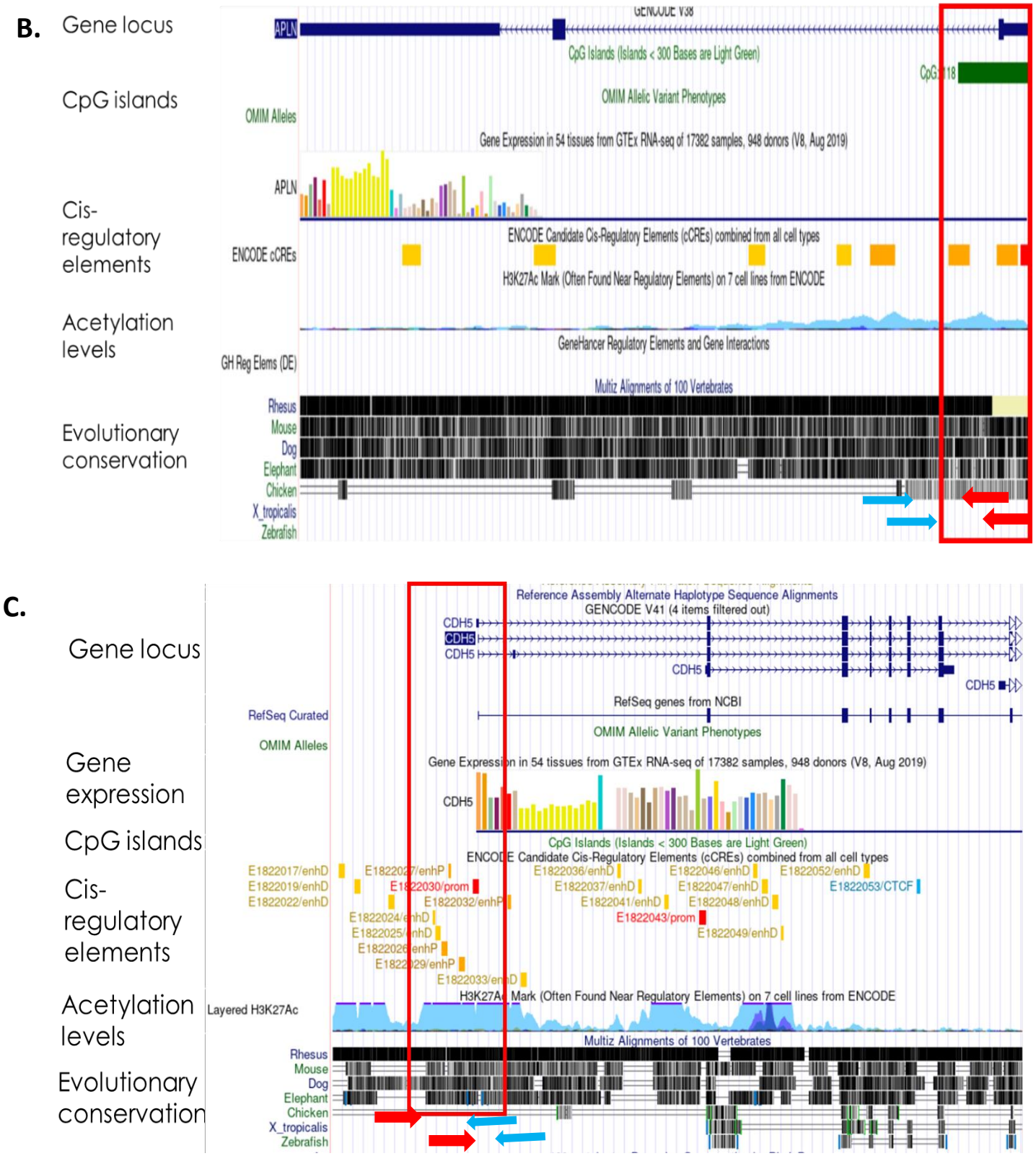


Figure 19. Screenshot of the UCSC genome browser showing the gene structure of (A) NPR3, (B) APLN and (C) CDH5. The red boxes show where we identified the minimal promoter region of the gene and designed the primers. The red arrows show the approximate location of forward primers, blue arrows for reverse primers. These regions are selected based on the highest levels of CpG islands, cis-regulatory elements, and H3K27ac acetylation levels present, along with strong evolutionary conservation between species.

5.4 Designing primers for NPR3, APLN and CDH5 minimal promoter regions

After the minimal promoter region was identified, we designed primers to amplify this region using PCR. The primers designed were between the range of 20-23 bps, with a Tm of around 60-65 °C to ensure they work optimally with the polymerase we used. After designing the primers, a polyA tail was attached to them to make the primers more stable and prevent degradation, along with adding restriction enzyme sites to aid with the digestion and ligation of the products into the LND4:GFP or LND4:mCherry destination vector. The primers designed are shown in table 6.

Table 6. Table showing the forward and reverse primers designed to amplify the minimal promoter regions of NPR3, APLN, and CDH5 with the polyA tail and restriction enzyme sites added.

Primer name	Primer sequence	Amplicon size (bp)
NPR3- F1- FseI	5'- AAAAAAGGCCGGCCTTCTAGAACCATCCCTTTCCC -3'	1700
NPR3- F2- FseI	5'- AAAAAAGGCCGGCCCACGTTTCCTTTTTTGAAGCC -3'	
NPR3- R1- PacI	5'- AAAAAATTAATTAACCAAAAGATAGAGAGGAAGC -3'	
NPR3- R2- PacI	5'- AAAAAATTAATTAACACTCTTCTCTCCAAGGTCCC -3'	
APLN- F1- PacI	5'- AAAAAATTAATTAATTGGTTGTCTTGACCAAACCC -3'	1696
APLN- F2- PacI	5'- AAAAAATTAATTAATGAGAAAGGGTTAAGTGTGCC -3'	
APLN- R1- FseI	5'- AAAAAAGGCCGGCCGCGAGCTCTTTCTTAGCG -3'	
APLN- R2- FseI	5'- AAAAAAGGCCGGCCCGAGCTCTTTCTTAGCGG -3'	
CDH5- F1- FseI	5'- AAAAAAGGCCGGCCTCCAGCCTCAGCGTCACTGT -3'	924
CDH5- R1- PacI	5'- AAAAAATTAATTAACCGACCGTCTTTGGAGGGGA -3'	

5.5 Amplification of regulatory regions

To amplify the promoter region of each gene, we did PCR reactions, using the primers specific to each gene, and then ran the PCR products on agarose gel electrophoresis to confirm the presence of a right sized band, indicating that the desired region has been amplified. The expected amplicon size for both NPR3 and APLN is around 1700 bps, and 924 bps for CDH5, which can be seen in the gel electrophoresis image in figure 20.

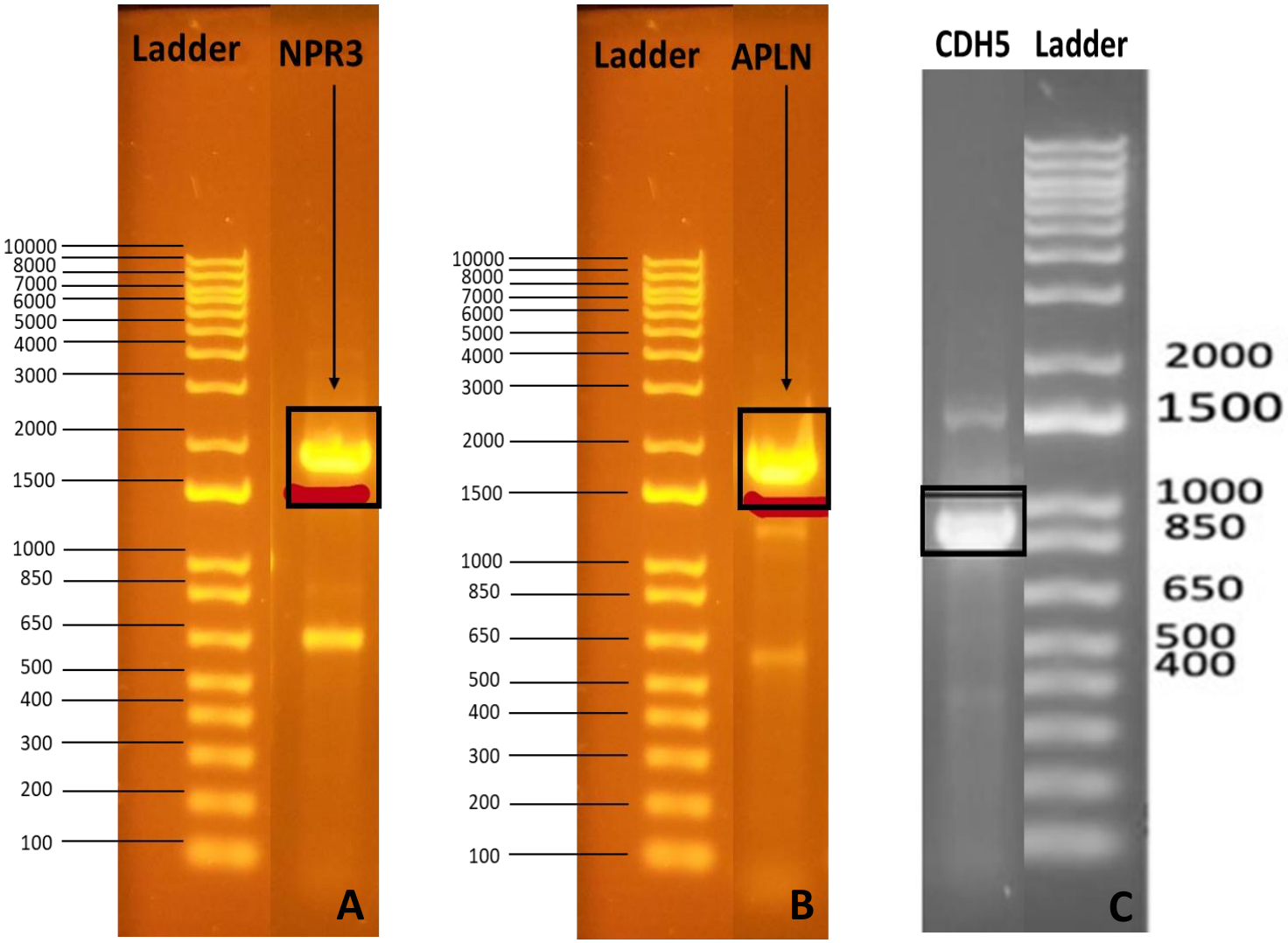


Figure 20. 1% agarose gel electrophoresis of the PCR products, with a 1 kb plus DNA ladder. The gel shows that there is a band present for (A) NPR3 at 1700 bps, (B) APLN at 1696 bps, and (C) CDH5 at 924 bp, which are the expected amplicon sizes, hence indicating that the right regions have been amplified. *** all of these gel images have been taken from larger gels with multiple lanes, but cropped for the desired lanes, for the ease of visualisation.

5.6 Cloning, digestion and ligation of regulatory regions into the final vector

At this stage, these PCR products were TOPO cloned, transformed into competent *E.coli* cells, from which the cells that have taken in the product were positively selected, then cultured in liquid LB + Kanamycin, and miniprepped to isolate plasmid DNA. Next, these samples were digested using FseI and PacI restriction enzymes, which can be seen in figure 21.

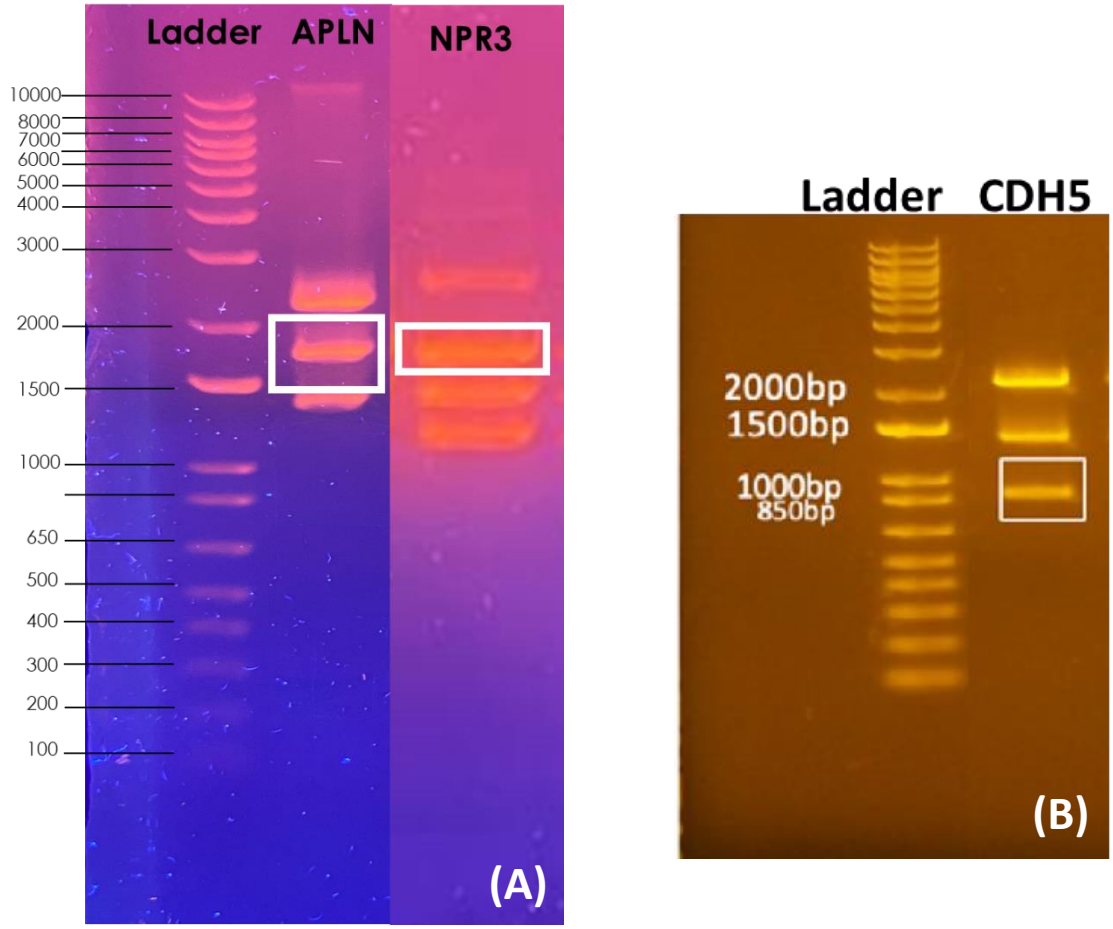


Figure 21. 1% agarose gel electrophoresis of the digest products, with a 1 kb plus DNA ladder. (A) The gel image shows that there are bands present for both APLN (lane 2) and NPR3 (lane 3) at ~1700 bps, and (B) gel image shows a band present for CDH5 at ~924 bp, which is the desired product size for our genes, indicating successful digestion. The correct sized bands are highlighted in white boxes in both A and B gel images. *** all of these gel images have been taken from larger gels with multiple lanes, but cropped for the desired lanes, for the ease of visualisation.

These bands were extracted from the gel, using the gel purification kit, and ligated into the final destination vectors, LND4:GFP and LND4:mCherry. The gel image showing the successful ligation of our NPR3, APLN and CDH5 minimal promoter regions into the destination vector is shown in figure 22. After the confirmation of successful ligation, these samples were cultured and frozen in the form of glycerol stocks, to be used for future experiments.

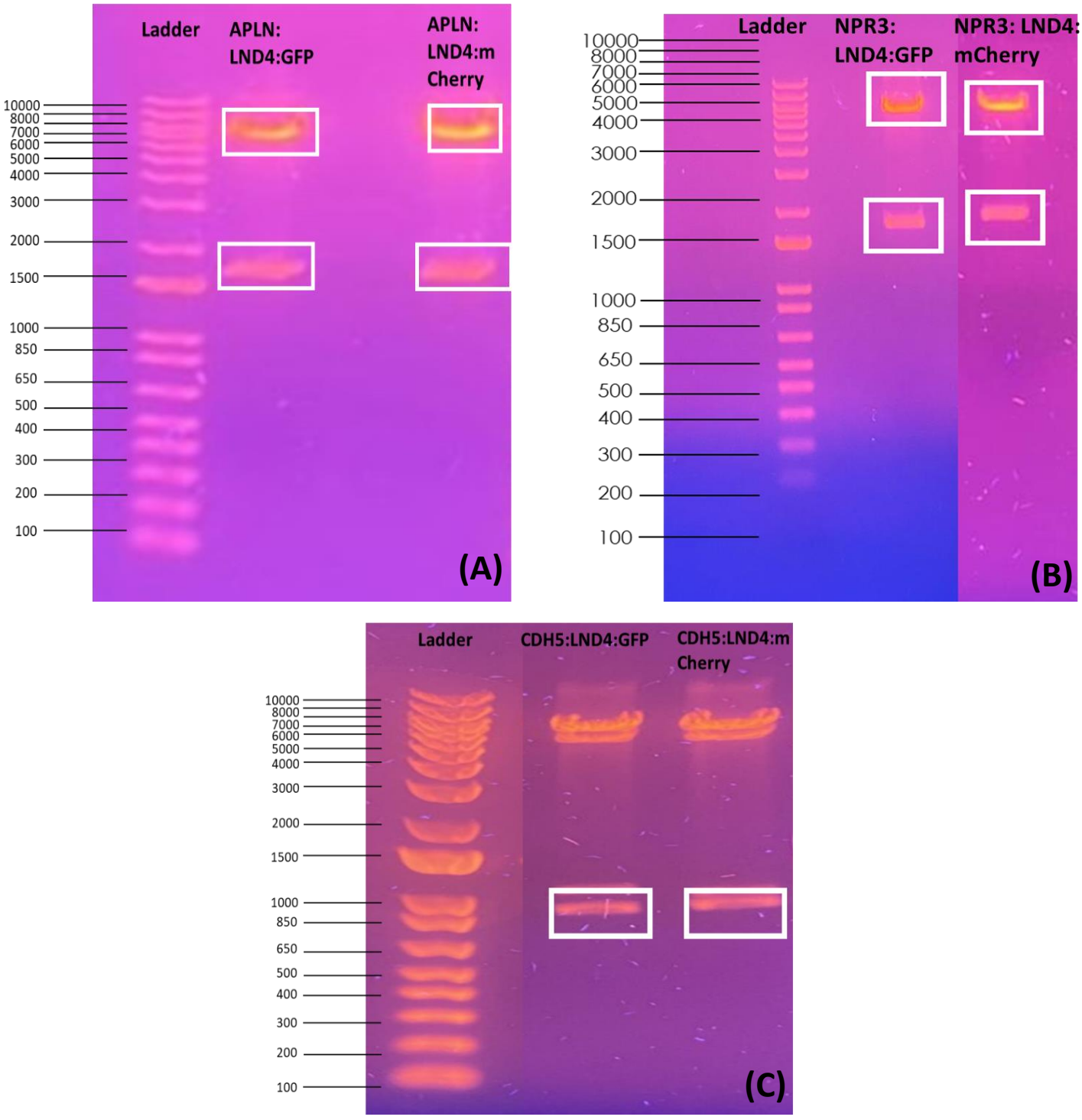


Figure 22. 1% agarose gel electrophoresis of the ligation of APLN, NPR3 and CDH5 inserts into our destination vectors, with a 1 kb plus DNA ladder. (A) Lanes 2 and 3 show the successful ligation of APLN into LND4:GFP and LND4:mCherry destination vectors, as the bands are present at 1700 bps for APLN and 7000 bp for LND4:GFP and LND4:mCherry, which are the desired band sizes. (B) Lanes 2 and 3 show the successful ligation of NPR3 into LND4:GFP and LND4:mCherry destination vectors, as the bands are present at 1700 bps for NPR3 and 7000 bp for LND4:GFP and LND4:mCherry, which are the desired band sizes. (C) Lanes 2 and 3 show successful ligation of CDH5 into LND4:GFP and LND4:mCherry destination vectors, as the bands are present at ~924 bp for CDH5 and 7000 bp for LND4:GFP and LND4:mCherry. These results show that we have successfully ligated APLN, NPR3, and CDH5 into both of our destination vectors. *** all of these gel images have been taken from larger gels with multiple lanes, but cropped for the desired lanes, for the ease of visualisation.

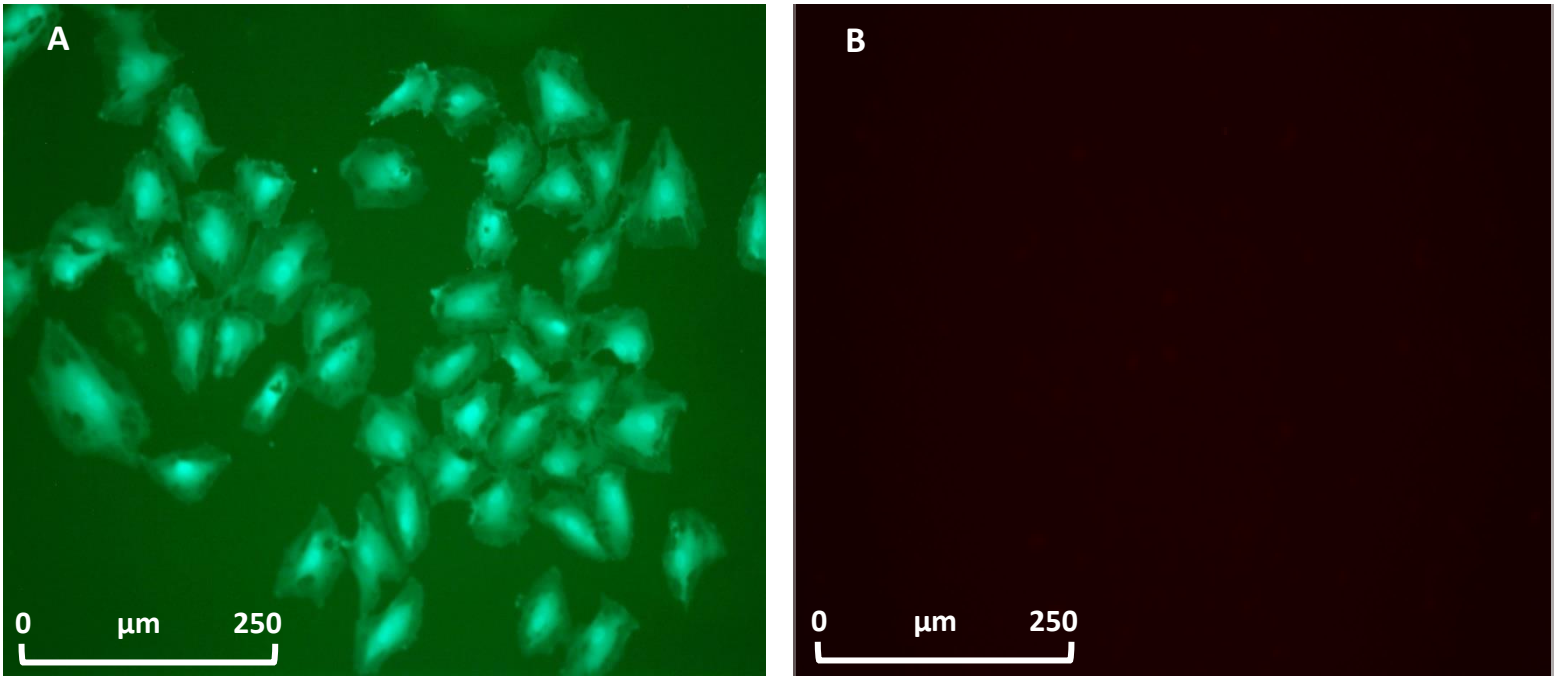


Figure 23. Fluorescence microscope image of adult endothelial progenitor cells, (A) transfected with the CDH5:LND4:GFP reporter construct, fluorescing green, highlighting the successful expression of the CDH5:LND4:GFP reporter construct in endothelial cells *in vitro*. (B) adult endothelial progenitor cells transfected with LND4:GFP vector, as a negative control, showing no fluorescence. Scale bars shown on the images.

5.7 Transfection of reporter construct into adult endothelial cells

We aimed to transfect all of our reporter constructs into adult endothelial cells, however, due to time constraints, we were only able to electroporate the LND4:GFP and CDH5:LND4:GFP into adult endothelial progenitor cells (figure 23). The LND4:GFP vector electroporated cells did not fluoresce, however, in the image below, we can see GFP fluorescence within our cells electroporated with CDH5:LND4:GFP, hence, indicating that our reporter construct was successfully transfected into our cells. CDH5 is an endothelial specific marker, so seeing its expression in endothelial cells shows that the correct gene was cloned into our construct. However, further validation still needs to be done to confirm this conclusion as we did not electroporate the non-endothelial cells with our reporter, due to time constraints.

6. Discussion

In this project, our aim was to generate fluorescent reporter constructs for NPR3, APLN, and CDH5 in order to study their role in the cardiovascular system and their potential in cardiac regenerative therapies. Fluorescent reporter constructs are a powerful tool for measuring gene expression in individual cells or in population of cells and has been extensively used in several studies. The fluorescent reporters work by cloning the reporter gene downstream of gene regulatory region of interest, either in the genome or on a plasmid that can be transformed into the host cell (Zaslaver *et al*., 2006). The intensity of light emitted by a cell, or a population of cells provides an indication of the quantity of fluorescent protein present and therefore tells the quantity of gene of interest present in the cells, its transcriptional activity, and the temporal-spatial localisation. The reporter construct we created was made by cloning the minimal promoter region of NPR3, APLN and CDH5 upstream of a reporter gene, which in our case was GFP and mCherry (figure 14). This type of fluorescent reporter construct can be inserted into cells and stably expressed to create a promoter-driven reporter line (Hartogh and Passier, 2016). Furthermore, we used LND4 as a vector to insert our regulatory region and fluorescent protein into; LND4 vector is a previously in-house modified bluescript vector with an altered multiple cloning site that carries a phosphoglycerate kinase promoter which drives the neomycin resistance gene (PGKNeo) expression cassette, for positive selection. Bluescript are cloning vectors designed to simplify commonly used cloning and sequencing procedures, and hence were really useful in our study. Overall, we managed to successfully create fluorescent reporter constructs for our genes, NPR3, APLN and CDH5: NPR3:LND4:GFP, NPR3:LND4:mCherry, APLN:LND4:GFP, APLN:LND4:mCherry, CDH5:LND4:GFP, and CDH5:LND4:mCherry.

Although NPR3, APLN and CDH5 were the main focus of my study, the Rana lab also generated fluorescent reporters for other key endocardial and endothelial cell markers, that play an essential role in the cardiopulmonary system, which I also contributed to. Some of these genes included, GATA4 which is a key marker for endocardial cells and a transcription factor with a key role in cardiac development, function, and morphogenesis. It is transcribed in the cardiac ventral mesoderm at the time when the cardiac tube forms, therefore it is the earliest marker of cardiogenesis. Later on in embryo development, GATA4 is seen to be expressed in the endocardial layer and not the myocardium, hence, it can be used to study the formation of the cardiovascular system and specification of the endocardial lineage (Kelley *et al*., 1993). Also, GATA4 is an upstream regulator of the *Erbb3-Erk* pathway, which is necessary for EMT, and also acts to promote cushion mesenchyme growth and remodelling (Rivera-Feliciano *et al*., 2006). In addition, we focused on

platelet endothelial cell adhesion molecule-1 (PECAM1) which is expressed at the cell boundaries of endothelial cells, with an important role in cell-cell contacts, maintenance of endothelial monolayer, endothelial cell barrier functionality, thrombosis, etc. (Privratsky and Newman, 2014). Fatty acid binding protein 4 (FABP4), which is a vascular endothelial cell marker, with roles in lipid mediated transcriptional regulation, neointima formation after vascular injury, its elevated levels can predict the development of cardiovascular diseases, etc. (Fuseya *et al*., 2017). Lastly, we made constructs for MYH11 and CNN1, which are both key markers for vascular smooth muscle cells, with roles in maintaining stability of vascular walls, cytoskeleton interactions, and their levels are significantly reduced in conditions including vessel remodelling (Zhu *et al*., 2007). The overexpression of CNN1 showed that it acts as an antagonist for injury induced neointimal formation in the arteries, preventing the vessels from regenerating themselves (Long *et al*., 2011), therefore, altering the levels of MYH11 and CNN1 might also be a therapeutic approach to treat CVDs. Overall, we have made these constructs: NPR3:LND4:GFP, NPR3:LND4:mCherry, APLN:LND4:GFP, APLN:LND4:mCherry, CDH5:LND4:GFP, CDH5:LND4:mCherry, GATA4:LND4:GFP, GATA4:LND4:mCherry, PECAM1:LND4:GFP, PECAM1:LND4:mCherry, FABP4:LND4:GFP, FABP4:LND4:mCherry, MYH11:LND4:GFP, MYH11:LND4:mCherry, CNN1:LND4:GFP, and CNN1:LND4:mCherry. All of these essential reporter constructs will allow us to further understand the biology of endocardial and endothelial cells, their role in development of the heart, and their role in cardiopulmonary disease, and cardiac regenerative therapies.

Reporter constructs are an invaluable tool for studying the regulation of gene expression, both by gene regulatory elements and transcription factors or exogenous regulators; these reporter genes allow the use of tissue-specific or pathway-specific gene promoters as biomarkers for specific event processes in the body. Majority of reporter constructs have a fluorescent protein within the construct, that can be used to measure gene expression in individual cells or a population of cells. When illuminated with certain wavelengths of light, these proteins emit fluorescent light of a specific colour that can be detected, which correlates with the amount of reporter gene being expressed in the body (Soboleski *et al*., 2005). There are several different methods to create reporter constructs and each has its own advantages and disadvantages. The type of reporter construct used in this study is called a transcriptional reporter, in which the promoter region from a gene of interest drives the expression of a fluorescent protein (GFP/ mCherry). The promoter regions of a few kilobases are immediately upstream of the start codon and contain a high number of cis-regulatory elements that regulate the transcription of genes, high number of CpG islands, which are DNA methylations regions located in the promoter region, known to regulate gene expression through transcriptional silencing of the corresponding gene. DNA methylation at CpG islands is

crucial for gene expression and tissue-specific processes (Lim *et al*., 2019). Lastly, they have high H3K27Ac levels as it is associated with the higher activation of transcription and therefore defined as an active enhancer marker (Creyghton *et al*, 2010). These promoter regions are then fused upstream of a fluorescent protein within our construct (figure 14). Green fluorescent protein (GFP) is excellent in visualising the spatial and temporal patterns of gene expression *in vivo*, studying protein localisation and trafficking, and as a quantitative reporter of gene expression, as GFP fluorescence increases in direct proportion to the number of cells with the GFP gene in it, and that GFP fluorescence is also proportional to the GFP mRNA abundance in cells. GFP also has excellent sensitivity as even if 1% of the cells in a population express GFP reporter gene, the quantity of GFP fluorescence is not diluted into the other 99% of cells that are GFP negative, therefore, reporter gene expression can also be detected successfully in experiments in which only a small number of cells are expected to express the reporter gene (Dundr *et al*, 2002). The same advantages apply to other fluorescent proteins, such as the red fluorescent protein, mCherry that was also used in this study. Apart from this huge advantage in our reporter constructs, these constructs are also relatively easy to prepare and have low cellular toxicity. However, the disadvantage is that only short transcriptional regions of a promoter can be studied, so we might not have captured the desired regions, so the reporter may not be expressed in the same way as the endogenous gene. Also, the fluorescence of cells can decrease over time. Another approach to create reporter constructs is by creating a fusion protein. The fusion protein is created by cutting the target gene between its regulatory region and coding region, and the same is done with the reporter gene. Then the regulatory region of the gene of interest is joined to the coding region of the reporter gene (figure 24). Gene fusions have the same use as transcriptional reporters as they are used to study gene expression and determine the level of gene expression within cells. Gene fusions are particularly useful in monitoring genes whose products are difficult to assay. Another advantage is that gene fusions can be used to test for possible effects of regulatory genes on gene expression, where a mutation that inactivates a regulatory gene can be introduced into the gene fusion carrying cells, and then measure the expression. Then, a series of regulatory genes suspected of controlling the target gene can be rapidly surveyed with this approach (Clark, Pazdernik and McGehee, 2019). However, there are disadvantages, such as, the fusion protein interfering with the protein's interaction with other molecules and impairing gene function. Another major disadvantage of this method is that there can be an overexpression of the gene of interest which would have produced different results than that of an endogenous gene, and hence would fail to replicate the original expression of the gene, therefore, researchers must delete the wild-type version of the gene from

the chromosome before the gene fusions are used, which requires extra time and hence we didn't use this strategy in our study (Terpe, 2003).

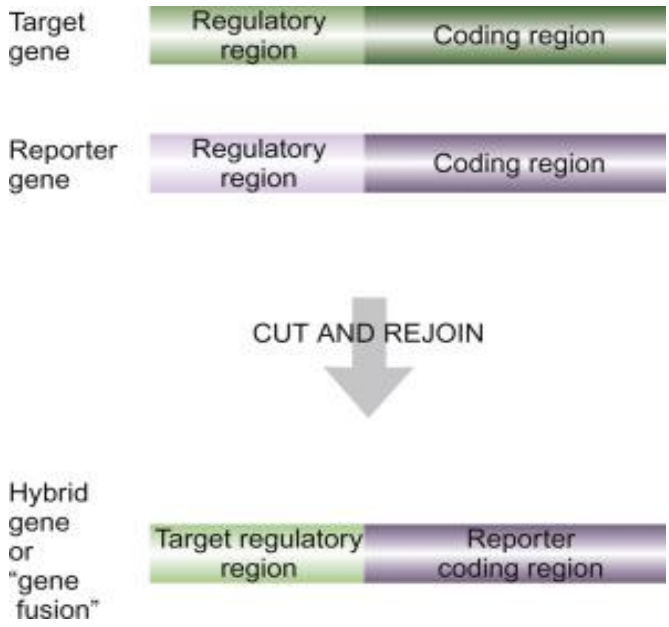


Figure 24. the method to create gene fusions to study gene regulation in cells. In this, the regulatory region of the target gene is joined to the coding region of the reporter gene. Adapted from (Clark, Pazdernik and McGehee, 2019).

Lastly, to overcome the limitations of transcriptional reporters, gene fusions and other ways to construct reporters, a knock-in reporter strategy can be used, which is carried out using the CRISPR-Cas9 system. Clustered regularly interspaced short palindromic repeats (CRISPR)- Cas (CRISPR-associated) system has two main components: RNA-guided Cas9 endonuclease which has two nuclease domains (HNH and RuvC) that each cleave one strand of the target double-stranded DNA, and a single-guide RNA (sgRNA) that binds to Cas9 to form a ribonucleoprotein which binds and cleaves the specific DNA target Chen *et al*., 2014). In addition, a protospacer adjacent motif (PAM) sequence is also required for the Cas9 protein to bind to the target DNA (Ran *et al*., 2013). During genome editing, sgRNA recruits Cas9 endonuclease to a specific site in the genome to generate a double-stranded break (DSB) which can be repaired by endogenous self-repair mechanisms: NHEJ or HDR (figure 25). Non-homologous end joining can introduce random insertions or deletions into the cleavage sites, and cause frameshift mutations or premature stop codons with open reading frames, thereby inactivating the target gene. On the other hand, homology directed repair introduces precise genomic modifications at the target site by using a homologous DNA repair template (Patsali *et al*., 2019). NHEJ is active in 90% of the cell cycle and hence is much more efficient than HDR. Since

its introduction in 2013, the CRISPR-Cas9 system has become a very common genome editing tool as it has several advantages, such as its simple design, high efficiency, excellent repeatability, short cycle, and low cost, and can modify genomic DNA at a specific target site in almost all cell types and organisms. Due to its advantages, it has been used in several species, including, bacteria, zebrafish, *Xenopus laevis*, monkey, etc., along with human cells lines, tumour cells, adult cells, and stem cells. One of its biggest applications is to develop cell models of human diseases, including gene knockout and exogenous knock-in models, which is what we also plan to do with this technology (Zarei *et al*., 2019, and Rodriguez-Rodriguez *et al*., 2019). However, there are some disadvantages of the CRISPR-Cas9 system such as off-target effects caused by sgRNAs mismatching with non-target DNA sequences and introducing unexpected gene mutations, problems with the delivery of CRISPR-Cas components to specific cells such as safety issues in viral vectors, immunogenicity, ethical issues, etc. Despite all these issues, CRISPR-Cas9 is still a very useful genome editing tool, and by combining iPSC and CRIPSR-Cas system, researchers have developed several disease models with isogenic background and provided new solutions for patient-specific cellular therapy, disease model establishment, drug discover, etc. (Hotta *et al*., 2015, and, Xu and Li, 2020). We also aimed to use this technology in our study, but due to time constraints and unavailability of the CRISPR kits, this was not possible. We aim to use this technology in the very near future, to create gene knock-in constructs.

6.1 Future Directions

Now that we have finished creating reporter constructs for our desired genes, the next step will be to validate the constructs and see if their expression matches with the endogenous gene expression. To do this, we will transiently express our reporter constructs into adult coronary endothelial cells and smooth muscle cells, through electroporation, and if we see green or red fluorescence under the microscope, it will show that our constructs were successfully made. APLN and CDH5 will be tested in adult cells initially, however, this cannot be done with NPR3, as that is a specific endocardial marker, and there are no adult endocardial cell lines available, therefore, NPR3 will be introduced into iPSCs to observe whether our construct fluoresces or not. The reporter constructs will also be introduced into *Xenopus laevis* and chick embryos, to analyse the expression of our reporters *in vivo*, as well as giving information on where our genes are expressed in the embryo and at which stage of development, to help us better understand their role in developmental biology. However, in transiently transfected cells, the foreign DNA does not integrate into the host genome and hence doesn't replicate, so it's eventually lost through cell cycle divisions over a period of few days; therefore, we will aim to generate stable cell lines, where the foreign gene becomes a part of the host genome and is replicated (Stepanenko and Heng, 2017). We only managed to transfect CDH5

reporter construct into adult endothelial cells, which showed fluorescence, indicating that the reporter is expressing the gene successfully. However, due to time constraints we could not transfect any other reporters into cells. Once all the constructs have been validated, they will be linearised by digesting them with RsrII, and then transfected into iPSC-Endocs, through electroporation. The cells which express the reporter constructs will be positively selected by using a selection marker, such as neomycin. These reporter PSCs will be differentiated into PSC-endothelial cells, PSC-endocardial cells, and PSC-coronary endothelial cells (figure 26), using the differentiation protocols produced by the Rana lab. I then aim to assess if the identity and expression of these reporter cells in the same in both reporter and wild-type line at the same stages of differentiation. In addition, throughout our study we will also work to optimise the endocardial differentiation and cEC transdifferentiation protocol. Through this we will have validated reporters and reporter lines suitable for studying endothelial cell identity and biology in cardiopulmonary disease and development, and improved model of human endocardial and coronary cells, along with the potential identification of pathways that regulate coronary endothelial cell (cEC) regeneration and enhance revascularisation post myocardial infarction. The improved model of endothelial and cEC's will help us to identify druggable pathways to treat disease.

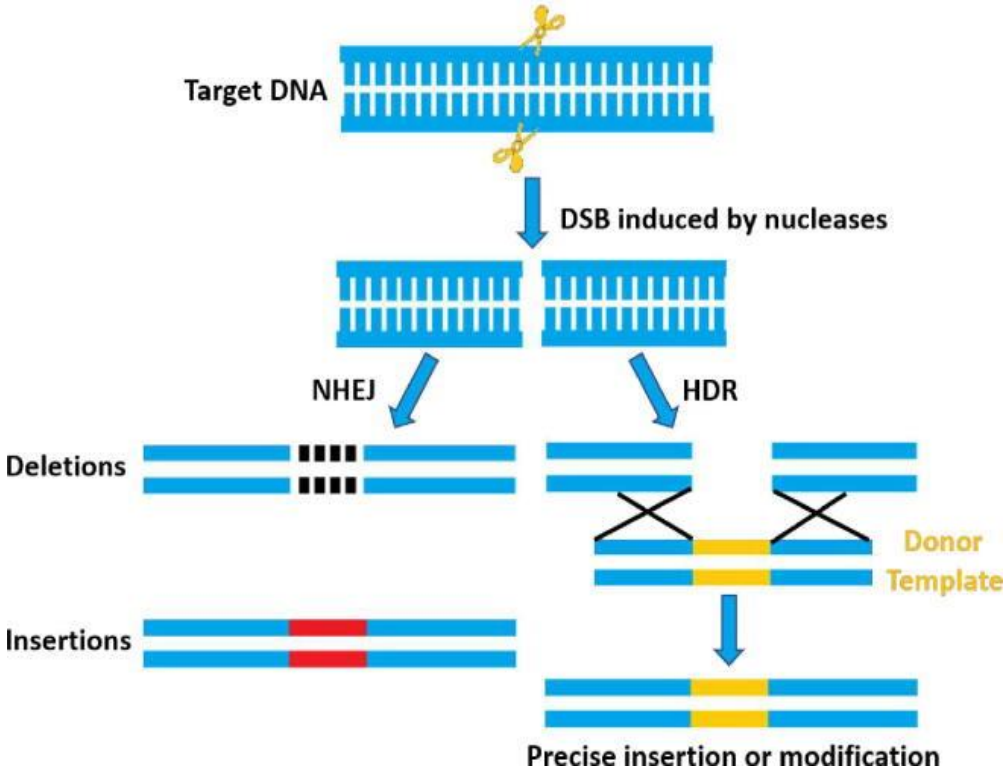


Figure 25. the genome-editing mechanism by which CRISPR-Cas9 works. The Cas9 endonuclease generates a double-stranded break (DSB), which is repaired by non-homologous end joining (NHEJ) or homology-directed repair (HDR). NHEJ introduces random deletions or insertion at the site of DSB, whereas HDR introduces precise genomic modifications at the target site by using a homologous DNA donor template.

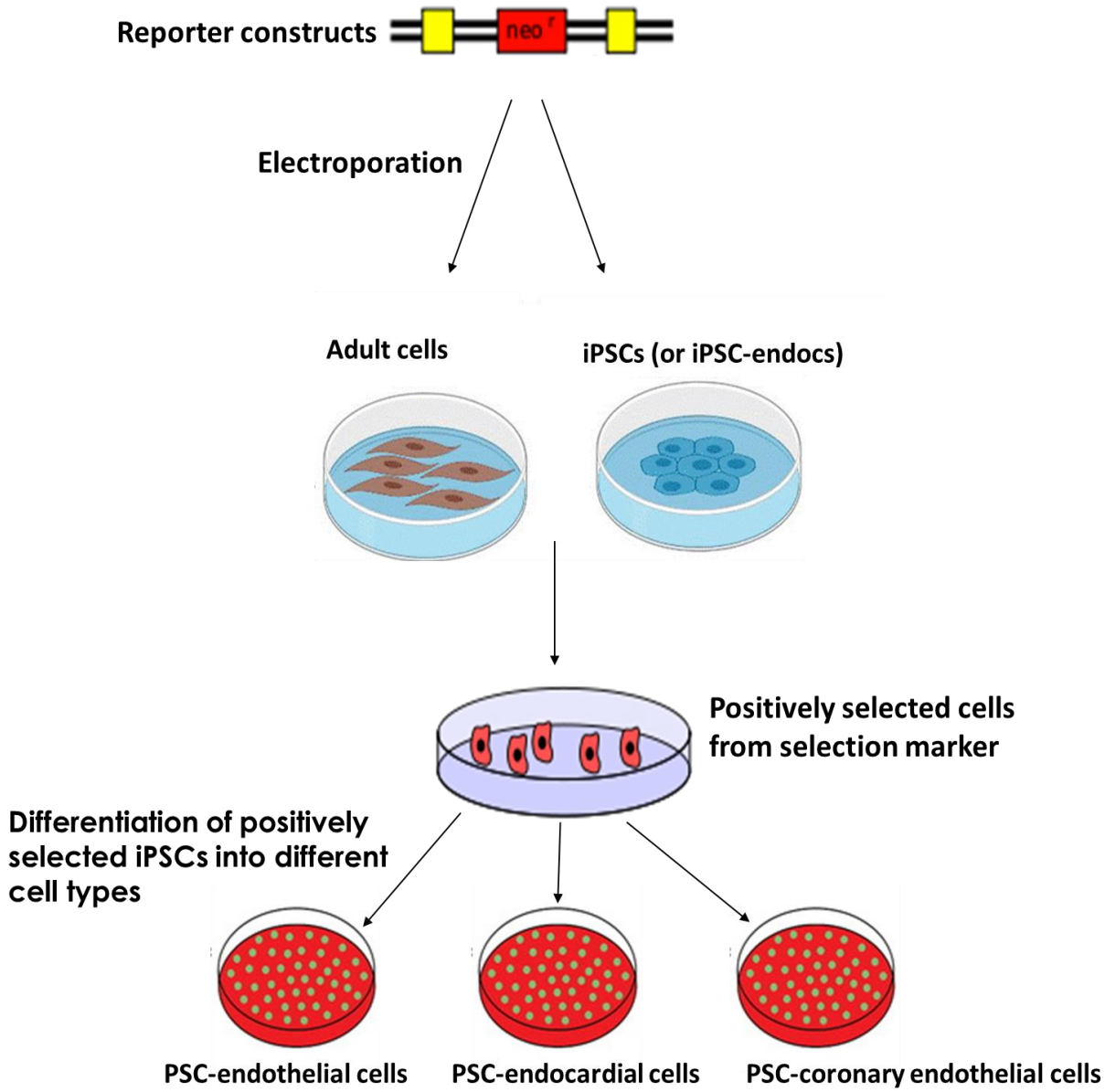


Figure 26. flow chart of how we will introduce our reporter constructs into cells to generate reporter lines. The reporter constructs will be transiently expressed into adult coronary endothelial cells and smooth muscle cells, through electroporation, and if we see green or red fluorescence under the microscope, then we'll know that our reporter constructs are successfully expressing the genes. Once all the constructs have been validated, they will be transfected into iPSCS and iPSC-Endocs, through electroporation. The cells which express the reporter constructs will be positively selected by using a selection marker, such as neomycin. These reporter PSCs will be differentiated into PSC-endothelial cells, PSC-endocardial cells, and PSC-coronary endothelial cells. This applies to all reporter constructs, except NPR3, as that is a specific endocardial marker, and there are no adult endocardial cell lines available, therefore, NPR3 will be introduced into iPSCs directly.

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