

Enhancing cardiomyogenesis in stem cells with the use of small molecules

By

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## **Abstract**

Cardiovascular diseases contribute a large amount of morbidity and mortality in developing and developed countries all around the world. In conditions such as the myocardial infarction, a significant amount of cardiomyocytes die leading to an impaired function of the heart. One promising method for replacing these cardiomyocytes would be with the use of cardiomyocytes derived from embryonic stem cells. However, a large number of cardiomyocytes and a highly efficient method for obtaining cardiomyocytes are needed. Using the principles of small molecule treatment to induce differentiation in a serum-free based differentiation protocol, I have demonstrated that the induction of canonical Wnt signalling via CHIR 99021, and subsequent addition of bone morphogenetic protein 4 was best able to induce cardiomyogenesis in mouse embryonic stem cells. While improvements in efficiency are still required, the manipulation of the Wnt and BMP4 signalling pathways hold great promise in improving cardiomyogenesis in mESCs.

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## Abbreviations

- AHF: Anterior heart field
- APC: Adenomatosis polyposis coli
- AR: Androgen receptor
- BMP: Bone Morphogenetic Protein
- BMP4: Bone Morphogenetic Protein 4
- cDNA: complementary deoxyribonucleic acid
- CHIR: CHIR99021
- c-Kit: Tyrosine protein kinase kit A.K.A. CD117
- CK1: Casein kinase
- CSC: Cardiac stem cell
- Co-SMAD: Common mediator Smad
- DAPI: 4', 6-Diamidino-2-Phenylindole, Dihydrochloride
- DMEM: Dulbecco's Modified Eagle Medium
- DMSO: Dimethyl sulfoxide
- E: Embryonic development day
- EB: Embryoid body
- EC: Embryonal carcinoma cells
- ECL: Enhanced chemiluminescence reagent
- ESC: Embryonic stem cell
- FBS: Fetal bovine serum
- FGF: Fibroblast growth factor
- Fz: Frizzled receptor

GATA4: GATA binding protein 4

GSK3 $\beta$ : Glycogen synthase kinase 3  $\beta$

hESC: human embryonic stem cell

HRP: Horseradish peroxidase

IF: immunofluorescence

Int1: Integration 1

iPSC: induced pluripotent stem cell

Isl1: Isl LIM homeobox domain 1

LIF: Leukemia inhibitory factor

LRP5/6: Lipoprotein receptor-related protein -5/6

Mef2C: Myocyte-specific enhancer factor 2C

mESC: Mouse embryonic stem cell

MHC: Myosin heavy chain

MHC7: Myosin heavy chain 7 ( $\beta$ -myosin heavy chain)

MI: Myocardial infarction

Mixl 1: Mix-like 1

mRNA: Messenger RNA

mTORC1: Mammalian target of rapamycin complex 1

Nkx2.5: Nk2 homeobox 5 protein

PBS: Phosphate buffered saline

PVDF: Polyvinylidene fluoride

QPCR: Quantitative polymerase chain reaction

R-SMAD: Receptor regulated Smad

SDS: Sodium dodecyl sulfate

sPBS: Stockholm's phosphate buffered saline

Tbx5: T-box protein 5

TC: Tissue culture

TCF/LEF: Transcription factor/lymphoid enhancer-binding factor

TGF- $\beta$ : Transforming growth factor  $\beta$

TBST: Tris buffered saline-tween

TF: Transcription factor

Wg: Wingless

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## **Contributions**

### **Project Overview**

Dr. Ilona Skerjanc has devised the experimental ideas portrayed in the research conducted.

### **Experiments**

All of the experiments were performed by Neven Bosiljcic except the following. The serum based differentiations of the mESCs, were performed by visiting Ph.D. candidate Vinicius Melo and Neven Bosiljcic. M.Sc. candidate Avetik Kocharyan helped collect protein lysates used in the immunoblot analysis and lab technician Rashida Rajgara (M.Sc.) assisted Neven Bosiljcic with the quantification of MHC+ cells.

### **Thesis Writing**

The Thesis was written by Neven Bosiljcic with additional editing and guidance performed by Dr. Alexandre Blais.

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## **1. Introduction**

### **1.1. Cardiovascular disease**

Cardiovascular disease is a term that describes a multitude of clinical problems associated with the heart and supporting blood vessels (1). These cardiovascular diseases are also the world's leading cause of mortality, contributing to about 30% of all deaths, and are subsequently a leading cause of morbidity throughout the world (2, 3). A common cardiovascular disease that contributes to significant number of morbidity and mortality is the heart attack or myocardial infarction. In this situation, the muscle cells of the heart, the cardiomyocytes die due to a lack of oxygenated blood which is caused by an obstruction of one or more arteries that supply blood to the heart (4). The resultant heart is thus in a compromised state with the damaged muscles and this may ultimately lead to other cardiovascular diseases including heart failure (5, 6). When heart failure is established, the heart of the patient is unable to pump the blood throughout the body, ultimately contributing to as much as 10% mortality in individuals over the age of 65, and is usually caused by underlying coronary heart disease (7, 6). Unfortunately, there exists no cure for either the myocardial infarction or heart failure, and although improvements have been made in preventative medicine such as lifestyle choices and diets, there still exists a substantial problem for individuals afflicted. Current therapies for the myocardial infarction and heart failure include managing the disease by utilising medications and selective rehabilitation techniques to improve the quantity and quality of life (4). Alternatively, transplantation of the organ is another option for heart attack and heart failure patients, however, the effectiveness is limited based on the availability of organs (8). In the transplantation route, as with the case of other organ transplantations, a suitable donor with the same blood type and

leukocyte antigen must be found (9). Furthermore, the long term survival after a heart transplant is currently not at optimal levels; there is a 54% -69% survival rate 10 years after the transplant depending on risk factors (10). Additionally, both heart failure and myocardial infarctions have major implications on the health care system in terms of the financial aspect. It has been recently reported that the cost treating patients with heart failure was \$37 billion only in the United States (11) while the median costs for dealing with myocardial infarction patients are about \$20,000 per patient (12). Up until the most recent few decades, cardiomyocytes (and the heart as a whole) were thought to be terminally differentiated and were thought to be able to only undergo hypertrophy or death, a theory which was established nearly 100 years ago (13, 14, 15). Research during these last few decades however, shows that cells that are Lin<sup>-</sup> (without the presence of lineage marker proteins) and c-kit<sup>+</sup> (also referred to as cluster of differentiation [CD117]) (16) have stem cell properties in self-renewal and multipotent differentiation, thus helping establish a relatively new theory which claims that the cardiomyocytes may in fact be able to differentiate and proliferate which was not previously considered (17, 18). Despite these novel concepts, the notion of the cardiac stem cell has many difficulties to overcome. For instance, the niche of these cells is poorly understood (13). The niche is the microenvironment which gives support both physically and chemically to the stem cells and thus plays an important role in regulating its fate. In the case of cardiac stem cells, the niche of these undifferentiated c-kit<sup>+</sup> cells is thought to include both the atria and ventricles and this niche has been postulated to include other cells including endothelial cells, fibroblasts and smooth muscle cells (14). More recently, researchers did prove that the c-kit<sup>+</sup> cells were able to contribute to a percentage of cardiomyocytes in mice but that percentage was very low (19). Despite the potential

presented with the cardiac stem cells, a more practical and preferred option for the treatment of a myocardial infarction may lie with the better understood embryonic stem cell.

Embryonic stem cells can be differentiated into the cardiomyocyte lineage and can then theoretically be used to replace the dead and damaged cell in the afflicted patient with the hope of improving both the survival time and quality of life. While there has been progress in the stem cell field, there are still many hurdles to overcome. It is hoped that closely mimicking embryonic heart development and studying its formation, proteins, genes, and pathways will lead to improvements in the efficacy of the differentiation of embryonic stem cells into the cardiac lineage.

## **1.2. Embryonic stem cells**

Embryonic stem cells are derived from the inner cell mass from the blastocyst of a developing embryo (E3.5 in the mouse) and have pluripotent differentiation potential (20, 21, 22). The pluripotent nature of embryonic stem cells allows them to differentiate into almost any tissue of the three germ layers with the exception of the extra embryonic fluid and umbilical cord (23). Consequently, there has been much attention given to embryonic stem cells given this capability as their differentiation into many cell types can be useful in many medical scenarios. Initially, the study of stem cells came to widespread use in the early 1970's with the use of embryonal carcinoma (EC) cells (24). These cells, which are teratocarcinoma cancer cells, were able to differentiate into all three of the germ layers. As the cells were relatively simple to culture they started becoming prominent in developmental studies in the 1970's and gained widespread use in years and decades that followed (24, 23, 25, 26). The next step forward in the evolution of stem cell research came with mouse

embryonic stem cells (mESCs) which appeared first in the early 1980's (22, 27). These cells were indeed derived from the inner cell mass of a developing mouse blastocyst, and were proven to be able to propagate in *in vitro* scenarios and showed their pluripotency by demonstrating teratoma formation, and thus the ability to develop into multiple germ layers, when injected in a mouse (23). The isolation of human embryonic stem cells (hESCs) from the blastocyst and their proof of teratoma formation was observed in the year 2000 (19, 24, 29). Many current studies focus on the usage of human embryonic stem cells due to their clinical significance; human embryonic stem cells, not other species stem cells, would be used for human treatments. However, just as the animal models serve the purpose to provide experiments before moving on to the human model, mouse embryonic stem cells (mESCs) are easier to manipulate and are more affordable to maintain than hESCs (22). For instance, in the case of hESCs, the cells require the support of feeder layers and the culture of the cells remains "difficult, slow, and labor intensive" (30). Conversely, mESCs can remain undifferentiated with just the addition of LIF (Leukemia Inhibitory Factor) and without the use of a feeder layer (30). More importantly, the murine model is the most commonly used animal model to study human diseases, and is amongst the easiest of animal models to manipulate for myocardial damage (31, 32). Cardiomyocytes obtained from differentiated mESCs have been transplanted to the hearts of mice in previous studies (33). While this has not yet been the case for hESCs and human recipients, hESC derived cardiomyocytes were transplanted on a rat based model with some success; the hESC derived cardiomyocytes were able to engraft, proliferate, and improve cardiac remodelling of rats which had experienced myocardial infarctions (34). Further encouragement is demonstrated with the engraftment of hESC derived cardiomyocytes in the primate (macaque) host. The stem cell

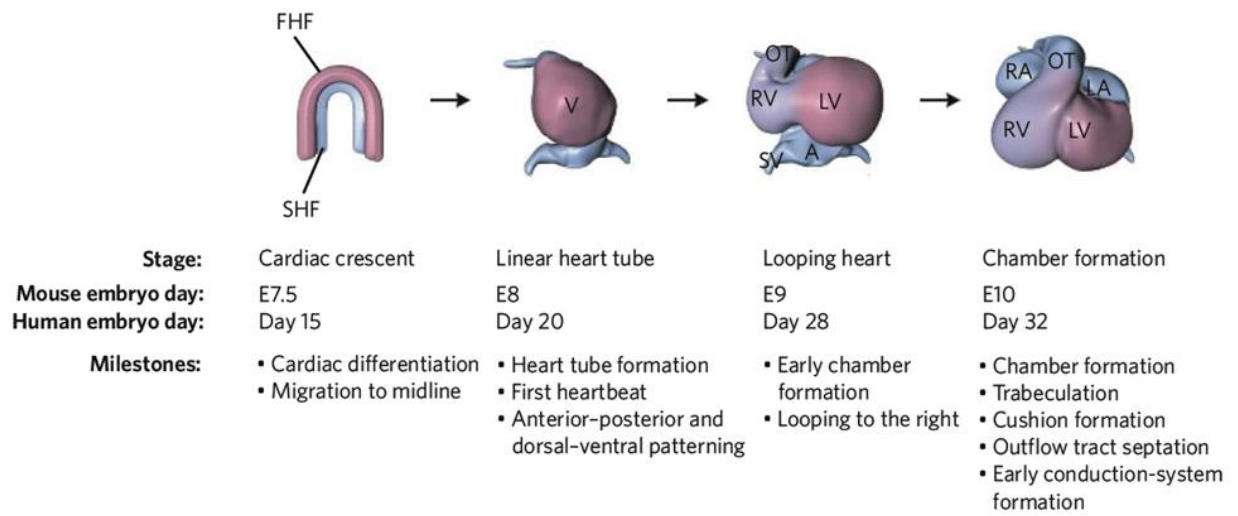
derived cardiomyocytes were able to re-muscularize the damaged areas of the heart with minimal presence of rejection (35). Although heart mass and function, in addition to engrafted cell maturity were observed, new complications such as ventricular arrhythmias arose as well (35). Embryonic stem cells also show greater differentiation potential compared to other stem cell alternatives such as adult stem cells. Adult stem cells, for example mesenchymal stem cells, have a much more restrictive differentiation potential (35, 36). Thus, a majority of the work related to cardiomyogenesis differentiation is performed with the use of embryonic stem cells, despite the fact that other stem cells can also differentiate into the cardiac lineage. For instance, adult stem cells are able to differentiate into cardiomyocytes from a bone marrow derived side population (38). Two of the outstanding problems faced with embryonic stem cells however still deal with the issues regarding cell purity and differentiation efficiency. Before ESCs can be used in a therapeutic setting, there needs to be a high purity of cardiomyocytes derived from ESCs that is both free of contamination from other cell lineages and one with a high yield of cardiomyocytes. Though the pluripotent capability of embryonic stem cells is attractive, it nonetheless carries some drawbacks; embryonic stem cells can also spontaneously differentiate into many other lineages as hepatocytes, neurons, and cardiomyocytes, amongst other cells which are observed in several *in vitro* studies (38, 39, 40, 41). Subsequently, it is essential that these factors be met before any major work and progress on clinical trials can be achieved. Understandably the *in vivo* aspects of the developing heart must be understood and applied to the ESCs in order to achieve the best results possible.

### **1.3. Mammalian heart development and cardiomyogenesis**

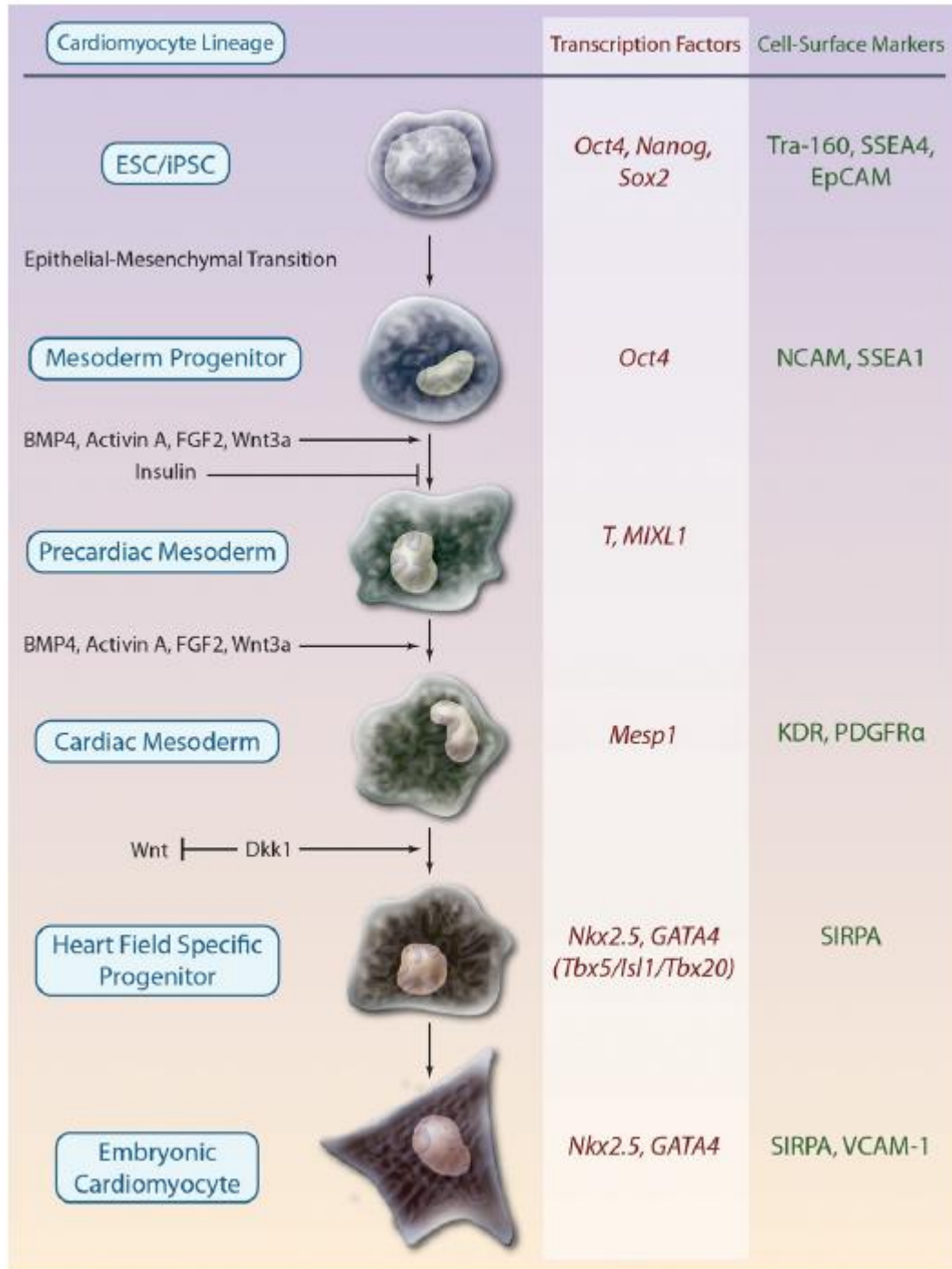
During development in vertebrates, the heart is the first major organ to be formed (43). The heart arises from the middle of the germ layers, the mesoderm, and the formation of the heart initiates from the cardiac crescent and then continues to the linear heart tube, through to the looped heart, ultimately ending with the mature heart (Figure 1) (44). During the process of gastrulation early on in development, the primitive streak is formed. The primitive streak creates and defines the anterior-posterior axis and causes the epiblast to transform into the mesoderm and endoderm lineage (45). Additionally, the primitive streak initiates germ cell formation and causes the mesoderm precursors to migrate to their proper locations (46). Cells expressing the factors Brachyury-T and Mixl 1 give rise to the mesodermal precursor population (47). Brachyury-T is a T-box transcription factor and is an essential gene necessary for mesoderm formation as mice with homozygous knock-out of the *brachyury* gene fail to pass day 10 of gestation as a consequence of the lack of mesoderm formation amongst others developmental issues (including notochord and allantois defects) (47, 48). Mixl 1 is also required for mesoderm formation in ECs as cells with disrupted Mixl 1 are known to have defects in heart tube formation (50). With regards to the cells destined to comprise the heart lineage, these mesodermal cells expressing the factor Mesp-1 migrate antero-laterally away from the primitive streak and lead to the formation of the cardiac crescent stage of heart development (E7.5 in mouse developments and approximately 2 weeks into human development) (51). The expression of Mesp1 is a critical point of cardiac development as its expression not only up-regulates cardiac progenitor markers, but also down-regulates mesodermal markers (52). At the level of the cardiac crescent, the cells are exposed to the major regulatory roles of the BMP, FGF, and Wnt regulatory pathways (which will be examined in more detail later) (53). More recent research has also identified

two separate mesodermal pools, the primary heart field and the anterior heart field, also named the secondary heart field (54). While the primary heart field leads to the development of the left ventricle, and parts of the atria, the anterior heart field leads to the development of the outflow tract, right ventricle and certain parts of the atria (54, 55). The LIM homeobox transcription factor *Islet1* (*Isl1*) is a marker for the AHF; in the murine model, the animals which lack *Isl1* in the heart have a missing outflow tract, right ventricle and a large portion of the atria (57). *Isl1* positive cells in fact contribute to the cardiomyocyte population. Concurrent expression of *Isl1* along with *Brachyury-T* in a cell mark the cardiac mesoderm (53, 57). *Isl1* expression along with *Nkx2.5* also marks the cardiac progenitor stage which ultimately contributes to most of the cardiomyocytes in the heart (58, 59). The heart tube is then formed with the fusion of the first and secondary heart field (60,61). It is worth mentioning that in the cardiac crescent stage of development, the critical cardiac progenitor markers *Nkx2.5*, *GATA4*, and *Tbx5* are all expressed and are required for proper heart development (63). The combined effort of *Brachyury-T*, *Mesp1* and *Islet1* (*Isl1*) however, is needed for the formation of early cardiac mesoderm and the expression of these genes (64). *Nkx2.5* has been described an essential factor for the formation of the heart, and along with *GATA4*, is crucial for development of the cardiac progenitor stage (64, 65). *Nkx2.5* mouse models where the gene is knocked out are embryonically lethal due to failed looping of the heart (67). *Nkx2.5* also represses *Isl1* at the cardiac progenitor stage to ensure that the ventricular cardiomyocyte lineage is achieved (68). Similarly to what was observed with *Nkx2.5*, mice which lack *GATA4* do not survive past day E8.5 due to failure of heart tube formation and failure of ventral morphogenesis (69). *GATA4* is highly expressed in the cardiomyocyte lineage and regulates terminal markers of cardiomyocyte differentiation such

as both isoforms ( $\alpha$  and  $\beta$ ) of the (MHC) myosin heavy chain protein (70). Nkx2.5 and GATA4 also have a major impact with each other's expression, as a down-regulation of Nkx2.5 is associated with a down-regulation of GATA4 (71). Expanding on that concept, it is actually a combination of factors, especially Nkx2.5, GATA4, Mef2C, and Tbx5 that are needed to have correct cardiac development (69). During the heart tube stage of development, the endothelial cells on top of a myocardial cellular layer elongate which causes the entire tube to elongate leading to the looping stage (72). The looping heart advances to chamber stage where the 2 atria and 2 ventricles are defined. Ultimately, the definitive structure of the heart is obtained when proper septation occurs separating both the atria and ventricles and leading to the functional 4 chambered heart (72). Over the years, research has shown that the concept of deriving cardiomyocytes from stem cells follows a similar developmental pathway. Undifferentiated stem cells proceed through the mesoderm progenitor, pre-cardiac mesoderm/mesoderm (Brachyury-T), cardiac mesoderm, cardiac progenitor (Nkx2.5, GATA4 later, Isl1 earlier) and cardiomyocyte stage (Nkx2.5, GATA4) (Figure 2) (47). Critically, the process of cardiomyogenesis in the stem cells follows the embryonic cardiomyogenesis closely and the cardiomyocytes obtained from this differentiation are similar to the cardiomyocytes observed in the primitive heart tube and early myocardium based on gene expression and electrophysiological analyses (72, 73). However, to maximize the potential in differentiating cardiomyocytes, the knowledge regarding signalling pathways regulating cardiomyogenesis will be required.



**Figure 1. The progression stages of heart development.** The progress of heart development is intricate and regulated by a multitude of different factors and signaling pathways. However, general landmarks in the physiological appearance of the developing heart, such as the cardiac crescent, the linear heart tube, the looping heart, and the formation of the chambers can be used to recognize the milestone in development. Figure reproduced from Bruneau, B.G. (2008). The developmental genetics of congenital heart disease. *Nature*, **451**, 943-948 with permission from Nature © 2008.



**Figure 2. The stages depicting the differentiation of an embryonic stem cell into the cardiac lineage.** Pluripotent stem cells differentiate initially into the mesodermal lineage before being further focused into the cardiac lineage leading to the fully differentiated cardiomyocyte. Critical cell signaling pathways in addition to the transcription factors and cell surface markers help identify the progress of the differentiation. Figure reproduced from Mummery, C. L., *et al.* (2012). Differentiation of human embryonic stem cells and induced pluripotent stem cells to cardiomyocytes: a methods overview. *Circ Res.* **111**(3), 344-58 with permission from Circulation Research © 2012.

#### 1.4. Wnt signalling

The Wnt signalling pathway holds importance in a number of biological processes. First discovered by Roel Nusse and Harold Varmus in 1982 within the context cancer research, Wnt was initially discovered as a mouse proto-oncogene that was named int1 (integration 1) at the time of discovery (75). Previous studies in the *D. melanogaster* had identified the Wingless (Wg) gene and after the discovery of Int1, it was demonstrated that Wg was in fact the int1 orthologue as int1 was demonstrated to be a highly conserved gene amongst many species from the animal regime all the way from the simple metazoans to the highly complex mammals (75, 76). As there were several other int genes related to int1, which may or may not have had the same viral type of integration as the original int1 was first demonstrated to display in the original cancer studies, an amalgamation of the two gene names thus resulted into the name of that we now know as Wnt to avoid any complications and confusions (78). The Wnt signalling transduction can occur via a canonical and a non-canonical signalling pathway. There are currently 19 known mammalian Wnt family members, with Wnt1, Wnt 3a, and Wnt 8 being involved in the canonical Wnt signalling pathway (79). While in the canonical pathway, the Wnts bind to the transmembrane Frizzled (Fz) receptor to transduce their signal, the non-canonical Wnt signalling pathway does not involve the Wnt/Fz mediation and has diverse mechanisms of signal transduction such as the Wnt-atypical protein kinase C pathway; however, their mechanisms are less understood as compared to the canonical Wnt signalling pathway (79). In the canonical Wnt signalling pathway there are two possible outcomes, with the pathway being either activated or not. The start of the Wnt signalling begins when the Wnt protein binds to the Frizzled receptor and the co-receptors LRP5, or LRP6 (lipoprotein receptor related protein 5 and 6). LRP6

then gets phosphorylated (by CK1 and GSK3 $\beta$ ) which induces the recruitment of axin and the destruction complex to the membrane bound protein complex consisting of Fz and Wnt (77).  $\beta$ -catenin can accumulate in the cytoplasm and then translocates to the nucleus where it interacts with the TCF LEF factors and allows for the transcription of Wnt signalling targets (77). The Wnt signalling pathway plays major roles in cell proliferation, migration, polarity and division (80). Additionally, Wnt signalling has been shown to be of importance in heart development and stem cell biology (79, 80). Mutations or defects in the canonical Wnt signalling pathway play a role in many developmental and non-developmental diseases including problems associated with bone formation, eye defects, kidney defects and heart defects just to name a few (82). In relation to stem cell regulation, canonical Wnt signalling is important for their self-renewal. The loss of the tumour suppressor APC, which negatively regulates Wnt signalling, promotes the undifferentiated state of mESCs (82, 83). With respect to heart development, the deletion of the  $\beta$ -catenin gene within the mouse embryo endoderm results in embryos being formed with several hearts (85). Additionally, Wnt signalling and  $\beta$ -catenin are noted to be required for cardiac looping, proper epicardial development, and heart valve formation amongst other processes (85, 86, 87). What is of particular interest is the effect Wnt has on the formation of cardiomyocytes from stem cells. Wnt signalling is both required to be expressed or repressed depending on the temporal stage of the differentiation. Most importantly, the activation of Wnt signalling is beneficial early in the differentiation to the cardiac lineage (Figure 3). To begin with, Wnt signalling was seen to be required in committing undifferentiated stem cells to differentiate to the mesodermal lineage. In mice studies with  $\beta$ -catenin knockout, there is no mesodermal tissue that is formed (89). This is not surprising considering the fact that the *brachyury* gene is a direct

target of Wnt/B-catenin and that Wnt3a signalling via Brachyury-T has been established for proper mesoderm formation (89, 90). The mesodermal marker gene Brachyury-T, in fact requires Wnt signalling for its expression (92). The addition of a GSK3 $\beta$  inhibitor such as CHIR, gives rise to approximately 91% of hESCs expressing a positive signal for Brachyury-T staining (93). CHIR 99021, simply referred to as CHIR, is a potent GSK3 $\beta$  inhibitor that was first discovered in diabetes and insulin research in 2003 (94). Research relating to GSK3 $\beta$  inhibitors however dates back to the use of lithium. As lithium is a prevalent treatment choice for bipolar disorder, a large effort on researching its roles in the context of the disease led to the conclusion that it acts through GSK3 $\beta$  inhibition (95). Since then, a vast array of specific inhibitors of GSK3 $\beta$  inhibitors, like CHIR have been developed (96). Wnt/ $\beta$ -catenin, as mentioned, is important for early activation of cardiomyogenesis and this is observed as cells treated with another GSK3 $\beta$  inhibitor, BIO [(2'Z,3'E)-6-Bromoindirubin-3'-oxime], results in nearly 80% of the embryoid bodies showing spontaneous contractions with initial cardiac progenitor marker (Nkx2.5 and Tbx5) expression being antagonized when cells are treated early with a Wnt inhibitor (97). The same group of researchers also noticed that Wnt signalling in latter stages of cardiomyogenesis results in suppressed cardiac troponin T expression as well as reduced MHC positive cells (97). Wnt/ $\beta$ -catenin was also determined to interact with and down-regulate important cardiomyogenic factors such as GATA4 which could be the reason for the inhibitory effects of Wnt signalling when added late to the cardiomyogenic protocols (98). Combining these two ideas, the group of Sean Palecek was able to obtain 80-90% functional cardiomyocytes from hESCs where initial Wnt activation via GSK3 $\beta$  inhibition was done from days 0 to day 3 and Wnt inhibition via an IWP factor was done from days 3-5 (99). It

was concluded that the manipulation of the Wnt signalling pathway would be an adequate starting point for devising a new protocol for cardiomyogenic differentiation in mESCs. While several potent GSK3 $\beta$  inhibitors have been used to demonstrate a high level of efficacy in Wnt signalling, the one that has been commonly used in differentiation protocols is CHIR. This small molecule is particularly attractive to use in cell culture as it is still highly specific and the least toxic to use with mESCs as compared to some of its contemporaries, which had problems with either being more toxic, less specific, or both (100). Furthermore, the inhibitors of the Wnt pathway, such as IWP4 also contribute to cardiomyogenesis. IWP4 is a small molecule inhibitor of the Wnt pathway [MW 496.62 g/mol] which acts by blocking the palmitoylation, which in turn inhibits the secretion of Wnt by Porcupine. Additionally IWP4 acts by blocking the phosphorylation of LRP6 receptor preventing  $\beta$ -catenin accumulation and is commonly used in the directed differentiation protocols due to its efficacy in blocking canonical Wnt signalling as demonstrated in the works of Burridge, *et al* and Lian, *et al.*, (52, 98).

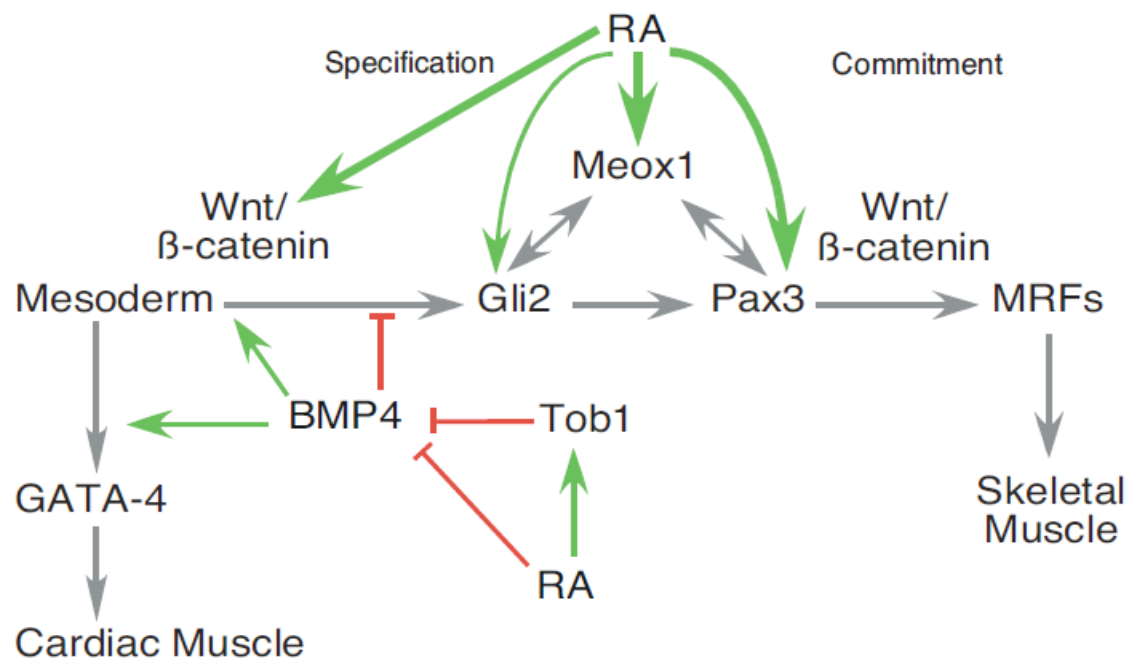
### **1.5. BMP4 signalling**

Along with the Wnt signalling pathway, the TGF- $\beta$  (transforming growth factor  $\beta$ ) family of proteins and the TGF- $\beta$  signal transduction pathway are also involved in the regulation of many biological processes including, but not limited to, proliferation, differentiation and cell migration (100, 101). The members of this super-family are extracellular cytokines that can be subdivided into families including TGF- $\beta$ 's, activins, inhibins, and the bone morphogenetic proteins (103). The variety of the cytokines is high, however, the basic principle of the TGF- $\beta$  signalling pathway remains highly conserved. The

cytokines are in fact ligands for the type I and type II receptors. These ligands bind to type I and type II receptors, resulting in their dimerization which precedes the phosphorylation of the type I receptor by the type II receptor, ultimately leading to the recruitment of the R-SMAD (receptor regulated SMAD) (102). The SMAD family is a group of proteins that are the intracellular mediators of the TGF- $\beta$  signalling pathway, taking the nomenclature from the SMA homolog from the worm, and the MAD homolog from the fly (102). Upon phosphorylation by the type 1 receptor, the R-SMAD forms a complex with the Co-SMAD (Common mediator SMAD) that can translocate to the nucleus where they act as transcription factors, binding to DNA and interacting with other DNA binding proteins and transcription factors and allow them to transduce the message by increasing or decreasing transcription of select genes (102). Due to its prevalence in a myriad of roles in the cell, the TGF- $\beta$  signalling pathway has implications with regards to disease as well. When looking at cardiovascular disease for instance, TGF- $\beta$  plays a role in heart failure, cardiac fibrosis, cardiomyocyte hypertrophy, and cardiac neovascularisation (104). The TGF- $\beta$  ligands known as the bone morphogenetic proteins (BMPs) have an especially prominent role in heart molecular biology. There are more than 30 isoforms of BMPs that have been discovered to date (104, 105). BMPs were first discovered in the late 1800's but in 1965 their addition was observed to result in bone formation (106, 107). In the years since, BMPs have been discovered to have roles in cell growth, apoptosis, differentiation and in several maladies including impaired fertility, defects in kidney function, impaired formation of glucocorticoid hormones, and heart problems just to name a few (109). As they are ligands in the TGF- $\beta$  pathway, the BMPs are capable of binding to the type I or type II receptors on the cell membrane and these include BMPR-II and ALK3.6 for BMP2, 4, and 7, in addition

to the receptors, ActR-II/B and ALK3.6 for BMP4, and the ActR-IIB, and ALK2 for BMP7 (104, 109). The R-SMADs that are then phosphorylated include SMAD 1,5, or 8 and they lead to the activation, or inhibition of the Co-SMAD, SMAD 4, allowing the SMAD complex to localize in the nucleus thereby exerting the effects of the signalling to approximately 500 target genes (104, 109). The BMP4 isoform is important in mice, as the knockout of this gene is embryonically lethal due to the fact that there is no mesoderm formation and there are no primordial germ cells (110, 108). Additional studies demonstrate that knocking out the BMP4 isoform can result in problems with digit patterning, problems with the mandible, as well as problems with the lens of the eye (109). Importantly for this study, BMP4 is required for proper mesoderm and heart formation. In fact, issues with BMP4 have led to medical conditions such as hypertension (present with BMP4 infusion in animal models), and myocardial ischemia (prevented by TGF- $\beta$  inhibition and down-regulated BMP4 levels) (112). In hESCs, short applications of BMP4 (24 hours) at the beginning of differentiation results in mesoderm formation (113). BMP4 expression, with respect to the heart and cardiovascular system, thus has both positive and negative implications. BMP4 is expressed in the myocardium of the outflow tract which initiates at E8.5 and continues through development (113, 114). Similarly, in experiments in which BMP4 is conditionally knocked-out, it is shown that BMP4 is necessary in the myocardium or pharyngeal endoderm for proper septum separation and formation (116). Application of BMP2 and BMP4 ectopically also results in an increase in Nkx2.5 and GATA4 expression which, as mentioned, are both critical genes in cardiac development (117). On the other hand, BMP4 can also induce pathological cardiomyocyte hypertrophy and cardiomyocyte death by apoptosis, and is capable of inducing cardiac fibrosis (106). Constant addition of

BMP4 to differentiating mESCs retards the expression of cardiac progenitor markers and terminal differentiation markers (118). The usage of dorsomorphin, a BMP pathway inhibitor from day 0 to day 3 of differentiation in ESCs is able to induce cardiomyogenesis to a greater extent than the control method as an approximate 20-fold increase was observed in beating cardiomyocytes (119). Similar results can be obtained with the use of noggin, another inhibitor of BMP pathway signaling, when applied at the same time points as dorsomorphin (120). Evidently, BMP4 expression and signaling have proven to be essential in cardiomyogenic differentiation, but the role they play seem to be determined based on the time of its expression. As noted by Witty *et al.*, BMP4 is necessary for induction of cardiomyogenesis as it is able to specify cardiomyocytes from the mesodermal lineage but it is needed to be repressed in the already differentiating cardiomyocytes (121). As mentioned previously, when used early in differentiation (days 0-4 in hESCs) BMP4, along with Activin A, is able to induce cardiomyogenesis which is demonstrated by the 35-40% of Nkx2.5 positive cells observed and by the 30% of the cardiomyogenic differentiation efficiency (122). In P19 EC, differentiation is usually induced by the addition of 1% DMSO in the medium, however, the addition of soluble BMP4, in lieu of DMSO does give rise to the cardiac lineage as identified by the increase in Nkx2.5 transcript levels (123). Nkx2.5, as mentioned, is an essential factor for the commitment of the mesodermal cells to cardiac lineage/fate, and helps highlight the necessity of BMP4 in cardiomyogenesis. Thus BMP4 when used in the right temporal frame can be used to increase the efficiency of cardiomyogenesis from hESCs. From the studies listed, BMP4 is needed for commitment of mesodermal cell to the cardiac lineage but is not required at early stages of differentiation (Figure 3).



**Figure 3. Wnt signaling and BMP4 are required to obtain cardiac muscle.** A plethora of pathways and proteins interplay in differentiation pathways, but canonical Wnt signalling along with BMP4 are necessary for obtaining cardiac muscle. Figure reproduced from Kennedy, K. A. M., *et al.* (2009). Retinoic acid enhances skeletal muscle progenitor formation and bypasses inhibition by bone morphogenetic protein 4 but not dominant negative  $\beta$ -catenin. *BMC Biology*, **7**(67), 1741-7007/7/67. with permission from BMC Biology © 2009.

## 1.6. Testosterone

The androgen and male sex hormone, testosterone is most commonly associated with the development of secondary sex characteristics in the male body. Testosterone is produced by the Leydig cells of the male testis and is a requirement for the formation of the penis, prostate and scrotum, as well as secondary sex characteristics (123, 124). In addition to the well described role in sexual development, testosterone also plays a crucial role in the physiology of many other organs, including muscle tissue. The use of testosterone and androgens has been clearly documented to increase muscle mass and help reduce fat at the same time in skeletal muscle, but in fact, cardiac muscle shows an elevated level of androgen concentration, comparable in fact to the testosterone levels observed in skeletal muscle (126). Cardiomyocytes possess a receptor for androgen molecules and can bind both testosterone and the chemically reduced, and more biologically active form of testosterone, dihydrotestosterone (127). In response to high levels of the androgens, hypertrophy can occur both in *in vivo* and *in vitro* models (with the activation of the mTORC1 pathway being critical), and can result in a medical condition known and documented as left ventricle hypertrophy (127, 128, 129, 130). In hypertrophy, the cardiomyocytes increase in length and width (132). Pathological and physiological are two types of hypertrophy that differ in the sense that myocyte width increases to a larger degree than myocyte length in pathological hypertrophy, while in physiological hypertrophy, the opposite occurs (132). In the case of pathological hypertrophy, it is a malady that can precede a multitude of cardiac conditions which can ultimately lead to a myocardial infarction and heart failure (132). In addition to its well documented roles in cardiac hypertrophy, testosterone plays other roles in cardiac biology, which are both beneficial and harmful depending on the levels and the length of

exposure to the hormone. At abusive levels, anabolic androgenic steroids, including testosterone, can lead to complications such as cardiomyopathy, heart attacks, and the aforementioned hypertrophy, in addition to the induction of apoptosis in cardiomyocytes (133). On the other hand, at a physiologically relevant concentration, testosterone actually demonstrates anti-apoptotic effects (133). Deficiencies in androgenic hormones like testosterone result in a reduced lifespan and an overall increase in risk of developing cardiovascular disease (128). Besides its role in apoptosis within cardiomyocyte populations, previous studies demonstrate that testosterone improves the differentiation of mESCs into cardiomyocytes (134). Research conducted in the Skerjanc lab has also revealed that the addition of testosterone to both P19 EC cells and mESCs causes an up-regulation in important transcription factors for cardiomyogenesis that included GATA4, Mef2c, Nkx2.5 and MHC (135). ChIP studies demonstrate that the androgen receptor (AR) is able to bind to regions of the Mef2C and HCN4 genes in the presence of testosterone suggesting a potential mechanism of action for the testosterone (135). Mef2C, along with GATA4 and Nkx2.5 is another of the essential cardiac transcription factors (136). Mice with null Mef2C deletion suffer from vascularization malformations in the heart (137). HCN4 (hyperpolarization-activated cyclic nucleotide-gated cation channel 4) on the other hand is predominantly found in the sinoatrial node and is a requirement for the pacemaker action potentials of the heart (138). Consequently, it is hoped that the addition of testosterone can also improve on the efficiency of cardiomyogenic differentiation.

## **1.7. Current methods of differentiation**

The standard differentiation protocol previously used to differentiate mESCs into cardiomyocytes has been the serum-based hanging drops differentiation. In this method, first published by Wobus *et al.*, in 1991, mESCs are initially dissociated with trypsin and then put into single cell suspension. Following this, they are allowed to aggregate creating the hanging drops from days 0 to 2 (139). Subsequently, the embryoid bodies that form are allowed to aggregate further together in suspension on agarose coated petri dishes before being plated down on gelatin coated tissue culture dishes on day 7, though the Skerjanc lab has been able to plate down the cells on day 5 with success (140). Utilising the hanging drops approach, cardiomyocytes can be observed as early as day 7 of the differentiation (141). Despite the efficacy of the hanging drops approach there are limitations with this type of differentiation protocol; for instance, the technique is limited to experiments that do not require large scale amounts of cells (142). With large scale experiments there are several variables that make the usage of hanging drops ineffective. As the cells need to be initially suspended in hanging drops, this generates a few problems. The first problem is that to get the hanging drops, the petri plate cover needs to be flipped, and the hanging drops need to be individually placed on the inside of the plate. Though the use of a multichannel pipette is beneficial, for large scale experiments, a high number of plates is needed. Similarly, the amount of media and petri plates that are required are also high. The amount of labour is also high on day 2 of differentiation, when the hanging drops need to be placed in suspension. The hanging drops need to be transferred to agarose coated tissue culture dishes which is not only challenging to perform but also affects the yield as some of the drops stick on the plate or the pipette. Another issue with the hanging drops method of differentiation is the low efficiency of differentiation. The hanging drops method has been previously reported to only

give rise to about 1-5% cardiomyocytes in mESCs (142, 21) and about 8% efficient in hESCs (144). Similarly, the Skerjanc lab has demonstrated that using the hanging drop method of differentiation of mESCs leads to about 5% efficiency for cardiomyogenesis (145). Additionally, the hanging drops method has the complication that it also leads to skeletal myogenesis if the differentiation lasts longer and furthermore, the neuronal lineage can also be obtained relatively easy. Understandably, the ESCs can differentiate into the almost any cell lineage, but in the context of therapeutics and medicine, a homogenous population of differentiated cells would be required. To relieve some of the shortcomings of the hanging drops method, improvements using small molecules to help direct differentiation have been developed. Using the hanging drops method supplemented with dorsomorphin, an inhibitor of the BMP4 pathway, can increase the number of beating EB's almost 20 fold as compared to the number of beating EB's in the hanging drops control (DMSO vehicle) (119). Similarly the addition of 5-azacytidine, a de-methylating agent that can be used to differentiate mesenchymal stem cells into cardiomyocytes, also increases the number of observed beating EB's and the up-regulation of certain cardiac specific genes (146). In conjunction with the hanging drops approach there also has been several other approaches to differentiation into the cardiomyocyte lineage (especially when concerned with hESCs) with those being the monolayer based approach and inductive co-culture methods (47). To combat unwanted contamination with other lineages, recent protocols have been using small molecules to manipulate signalling pathways to obtain a higher percentage of a single lineage differentiation. The more successful differentiations also placed special emphasis on the Wnt signalling and BMP4 pathways due to their importance in cardiomyogenesis (as mentioned in the previous sections). For instance, manipulation of the Wnt pathway in

hESCs, can lead to a cardiomyocyte efficiency of differentiation of up to 80% (147). Similarly, using the combination of Activin A and BMP4 results in up to 50% cardiomyocytes observed in hESC differentiation (122) while the use of the small molecule family of cardiogenols can also lead to greater than 50% expression of MHC staining after 7 days of differentiating R1 mESCs (148). The cardiogenols are a group of diaminopyrimidines that were determined to be the most potent out of 100,000 compounds tested in P19 EC inducing MHC expression (148). In addition, these advances in efficiency using the monolayer approach have come with a serum-free based approach. This is an attractive approach as the use of serum as a media supplement was required for the hanging drop method and is one that has some ethical concerns (both moral and scientific) (149) and is an option that is more expensive than serum-free media. The fetal bovine serum (FBS) is obtained from bovine fetus and requires that pregnant cows be slaughtered in scenarios that are likely painful and considered inhumane (149). On the scientific side, the use of FBS is also not ideal as it is known to sometimes carry contaminations which can include viruses, bacteria, and prions to name a few, all coming from a non-human origin (148, 149). The use of fetal bovine serum in differentiating cells has also been a cause for variability in the cardiomyogenic differentiation in p19 EC cells. The variability of the different serum lots also had implications that had an effect on the quality of differentiation. Different serum lots make an impact on the expression of Nkx2.5 and Mef2C, both of which are genes critical in cardiomyogenesis (151). Using the approach with serum-free media and small molecules to better control the efficiency of cardiomyocyte differentiation and reduce differentiation into unwanted cell lineages we hope to obtain a high percentage differentiation that is both

simple to perform and is highly reproducible. Of course, there is room for improvements to existing protocols as the process of cardiomyogenesis is one that is very complex.

### **1.8. Rationale**

Currently, there are many protocols working on directed differentiations to derive cardiomyocytes from stem cells. In this approach, differentiation of stem cells into a specific lineage is achieved with chemically defined and highly reproducible conditions (152). With the use of small molecules in directed differentiation protocols, researchers have experienced a considerable amount of success in developing protocols that are more efficient than the hanging drops method. In some cases, small molecules were added in conjunction to the hanging drops method while in other cases, a monolayer protocol using serum-free media was used. Many times, the most dramatic increase in cardiomyocyte efficiency was obtained with the use of Wnt signalling activation and inhibition or BMP4 addition in a serum-free scenario. That being said, much of the improvements in cardiomyogenic efficiency were observed only in the hESCs model and using a combination of media that have differences to the E6 media used in this study, and used to success in previous research conducted by the Skerjanc lab. While there have been great advances in efficient mESC cardiomyogenic differentiation, there still remains improvements to be made. As mentioned, the hanging drops provide a relatively inefficient platform (with a couple of exceptions, such as the work by Yuasa, et al (153)). Additionally, the laborious nature and the ethical concerns with the usage of serum based media in the embryoid body/hanging drops method provide positive influences for the development of a different protocol. It is hoped by using a serum free and monolayer, directed differentiation method similar to the successful protocols developed for

the hESCs that a new and efficient cardiomyogenic protocol would be obtained for mESCs. Since the mouse and other animal models will be important in several application whether it be clinical or pharmaceutical application of cardiomyocytes derived from stem cells, there is still a need for a robust, simple, and efficient protocol which will a pure population cardiomyocytes derived from mESCs.

### **1.9. Hypothesis**

My hypothesis was that with the use of small molecules controlling and manipulating the Wnt and BMP4 signalling pathways at specific developmental time points in a serum-free and monolayer protocol, an efficient and robust cardiomyocyte differentiation protocol can be developed.

### **1.10. Objectives**

Thus, with all of the knowledge and success observed with Wnt manipulation and BMP4 manipulation I am hoping to come up with a protocol for mESCs that will not only be more efficient in differentiating into the cardiomyocyte lineage, but also be serum-free based, and monolayer based. I predict that this protocol will be easier to manipulate and have lower variability with the use of these small molecules in a monolayer. Additionally, the ethical concerns regarding fetal bovine calf serum will not be a hindering factor. The Skerjanc lab has a lot of relevant experience regarding this situation as in a recent publication the lab has demonstrated that using the GSK3 $\beta$  inhibitor CHIR, Wnt signalling was induced resulting in a large proportion of mesodermal lineage cells. Furthermore, the CHIR, combined with other factors and small molecules ultimately resulted in a highly

efficient skeletal muscle protocol in both the hESCs and mESCs (93). I am aspiring to develop a similar based protocol as both the heart and skeletal muscle are derived from the mesodermal lineage. Since this was to be a new protocol developed from the ground up, much of the focus has been given to early stage cardiomyogenesis. My objectives will thus be to first examine the mesoderm formation after the use of CHIR. After successful mesoderm induction, the cells will be treated BMP4, as well as the Wnt inhibitor IWP4, the BMP inhibitor Noggin, and testosterone in hopes of obtaining cardiomyocytes. Gene expression and protein levels will be monitored to assess progress of differentiation.

## **2. Materials and Methods**

### **2.1. mESCs culture and differentiation**

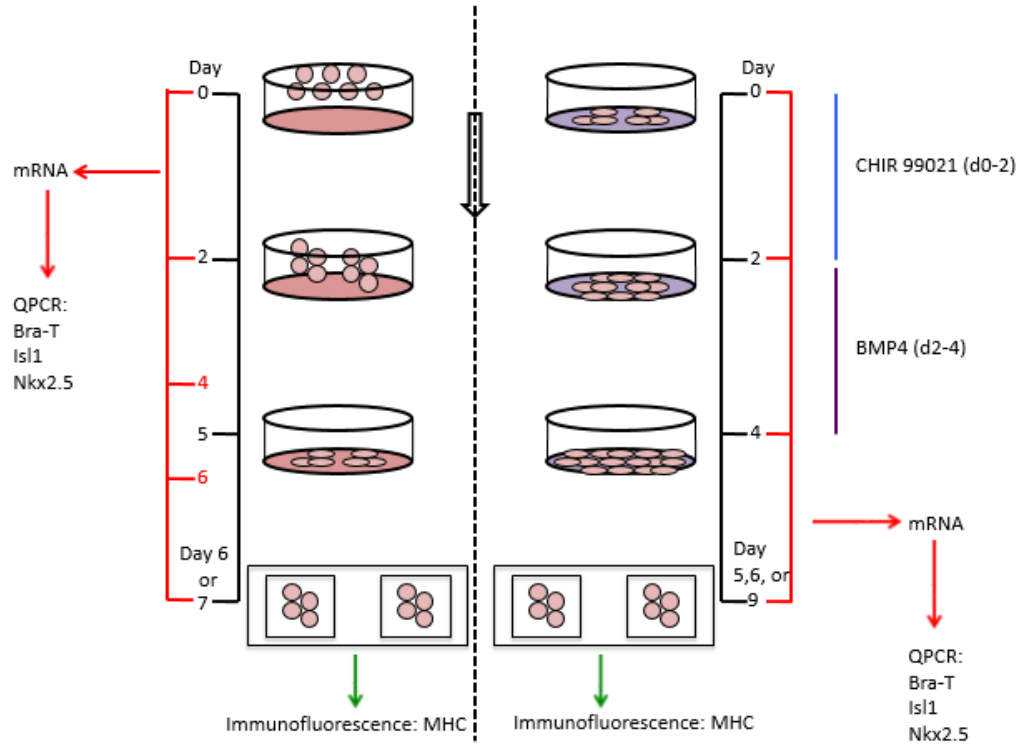
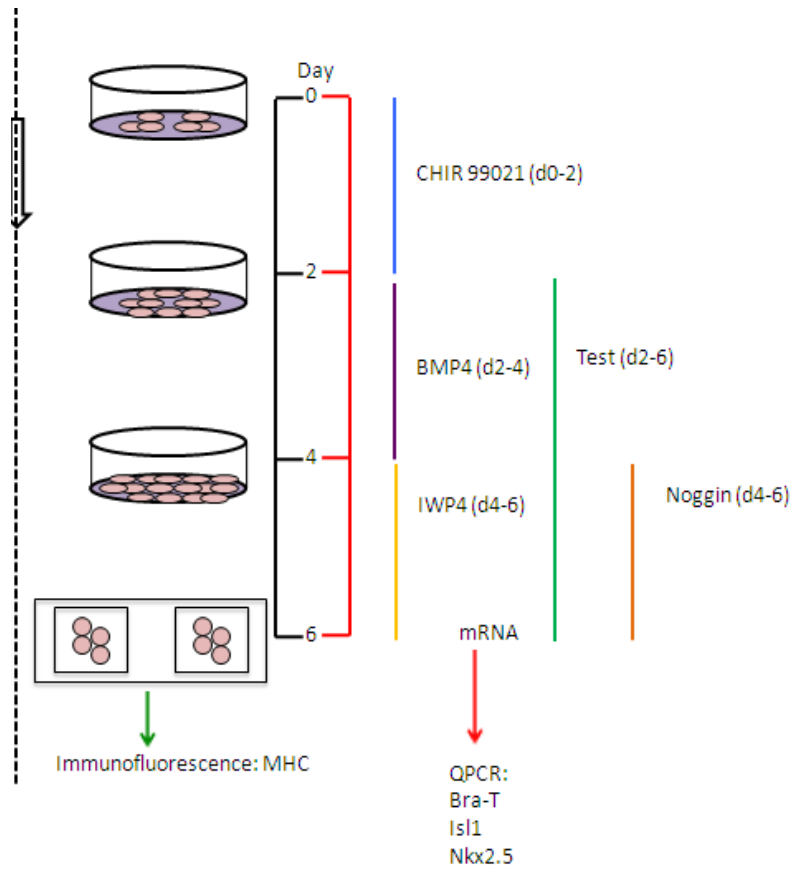
#### **2.1.1. New method of differentiation (monolayer, serum-free, small molecules based)**

R1-TCF LEF (TCF LEF) mESC were maintained in high glucose DMEM (Gibco), which was supplemented with 12.5 % fetal bovine serum (Wisent), non-essential amino acids (Gibco), penicillin streptomycin (Invitrogen), and  $\beta$ -mercaptoethanol (J. T. Baker) with leukemia inhibitory factor (LIF) (Millipore). Cells were trypsinised and passed every two days and were cultures in conditions at 37 °C and 5% CO<sub>2</sub>. When Cells were trypsinized for the differentiations KO media (KO DMEM and KOSR- Knockout supplement reagent (Gibco)) was used to stop trypsin activity.

To induce differentiation, cells were plated on 12 well or 24 well dishes (Corning) coated with matrigel (BD Scientific) or coverslips (Fisher Scientific) coated with matrigel at a density of 500,000 cells/3.8 cm<sup>2</sup> (unless stated otherwise) in serum-free media. The media, termed E6, was DMEM/F12 based (1:1) (Gibco), and was supplemented with ascorbic acid (Sigma Aldrich), insulin (Wisent), sodium selenite (Sigma-Aldrich), human transferrin (Sigma-Aldrich), sodium bicarbonate (Sigma Aldrich), and gentamicin (Gibco). Media was changed every day and was supplemented with CHIR99021 (Tocris) from days 0-2 or its vehicle DMSO (Sigma Aldrich), BMP4 (R&D Systems) from days 2-4, or its vehicle (4mM HCl, 0.1% BSA), IWP4 from days 4-6, or its vehicle (DMSO), Noggin from days 4-6, or its vehicle (0.1% BSA in PBS), or testosterone from days 2-6 (Sigma Aldrich) or it's vehicle (DMSO) (Figure 4).

### **2.1.2. Established method of differentiation (hanging drops, embryoid body formation, serum based)**

The D3 mESCs were maintained in the same conditions as described above for the mESCs TCF LEF but there was a difference in the differentiation protocol. The cells were differentiated with the embryoid body/hanging drops protocol. The cells were first trypsinized and were suspended in hanging drops at a concentration of 40,000 cells/mL on petri dishes (Fisher Scientific) for 2 days (days 0-2). The aggregated embryoid bodies were then transferred to 1% agarose coated petri dishes (Fisher Scientific) and were allowed to further aggregate for an additional 3 days (days 2-5). On day 5, the aggregates were transferred to tissue culture dishes (Corning) or microscope coverslips (Thermo-Scientific) treated with 0.1% gelatin and remained there until day 7, the last day of observed differentiation. The mESC media without LIF was used throughout the process and the media was changed every 2 days (Figure 4).

**A****B**

**Figure 4. Comparison and analysis of the methods of differentiation.** **A)** Initial side by side comparison of the hanging drops, serum based method (left of the divider) and the initial version of the new serum-free based (right of the divider) method using directed differentiation from CHIR and BMP4. Cells in both protocols were generally most often differentiated to day 6, and mRNA was usually taken on days 0, 2, 4, and 6 and assessed for Brachyury-T, Isl1, and Nkx2.5. Assessment of MHC staining by immunofluorescence was performed on day 6 unless noted otherwise. **B)** The revisions of the new serum-free protocol included additions of IWP4, noggin, and testosterone in efforts to improve the differentiation efficiency.

## 2.2. Immunofluorescence

Myosin heavy chain staining was performed at the day 4, 5, 6, and 9 time points. Differentiating mESCs were fixed with -20°C methanol (Fisher) for 5 minutes, and were rehydrated using sPBS (Stockholm's phosphate buffer), which was followed by the addition of the MF20 primary antibody (154) 1:1 with sPBS and was incubated overnight at 4°C. The samples were then washed three times with sPBS and the secondary antibody, Cy3 (goat or donkey anti mouse) (Jackson ImmunoResearch) diluted 1:100 in sPBS was applied for one hour room temperature. Coverslips were mounted in 1:1 mixture of sPBS and glycerol with 1:100 Hoechst's dye.

Brachyury-T and Nkx2.5 expression were visualized on days 2 and 4 respectively. Differentiating mESCs cells were fixed in 3.7% formaldehyde for 20 minutes at room temperature, permeabilized with Triton x-100 for 15 minutes, and blocked with donkey block (donkey serum and BSA (Jackson ImmunoResearch)) for 1 hour. Brachyury-T specific (Abcam 1:50) or Nkx2.5 specific (1:50 dilution) (Santa Cruz) primary antibodies were then incubated overnight at 4°C (diluted in donkey block). After 3 sPBS washes, secondary antibodies (Cy3 Goat ant-Rabbit IgG for Brachyury-T, and Alexa 488 donkey anti goat for Nkx2.5 (Jackson ImmunoResearch)) were added in diluted in sPBS and incubated for 1 hour. The coverslips were mounted using Vectashield mounting media (Vector Laboratories)

All of the visualization of the immunofluorescence specimens was performed using the Leica DMI6000B inverted microscope (Leica Microsystems). Images were analyzed and processed using Volocity 4.3.2 (Perkin Elmer). Myosin heavy chain positive cells were quantified by taking the amount of cells appearing positive for MHC and dividing by the total number of nuclei. Error bars represent the standard error of the mean (SEM) utilising at

least 3 biological replicates (10 images from each condition in each replicate) and significance was determined using the student's t-test.

### **2.3. Quantitative PCR**

Differentiating mESCs were subjective to RNA extraction on each specific time points of differentiation. A minimum of  $1 \times 10^5$  cells or  $5 \times 10^5$  cells were collected and the total RNA was extracted using either the RNeasy micro kit or Total RNA kit (Omega Bio-tek) respectively. The extracted RNA was reverse-transcribed into cDNA, using a minimum of at least 100 ng of RNA with the QuantiTect Reverse Transcription Kit (Qiagen). For the QPCR reaction, a mixture was prepared which consisted of 1/40<sup>th</sup> of the cDNA generated, validated primers sets, and KAPA SYBR master mix (KAPA Biosystems) and were run for 50 cycles to assess the transcript levels of the genes of interest. Data was processed and obtained using the Eppendorf Realplex II QPCR machine. Genes were normalized to  $\beta$ -actin or 18s and the analysis was performed using a comparative delta delta CT method as previously described in (155). Error bars represent the standard error of the mean (SEM) utilising at least 3 biological replicates and significance was determined using the student's t-test.

### **2.4. Immunoblot analysis**

Total protein was extracted from mESC R1 differentiating cells (following the new serum free based protocol) on days 0, 2, 4, 6 using radioimmunoprecipitation (RIPA) buffer [50 mM Tris pH 7.5, 150 mM NaCl, 0.2% NP-40, 2 mM EDTA, 1X PIC (Roche), 0.5 mM PMSF (Sigma-Aldrich)]. The lysates were collected after the extracts were spun down at

16000g for 30 minutes. A total of 10 µg of protein from each condition was obtained and they were eluted by boiling for 10 minutes in 2x sample buffer (Sigma Aldrich). The samples were then run using a 4-12% gradient bis-tris polyacrylamide gel (NuPAGE, Invitrogen) with accordance to the manufacturer's protocol. The proteins were then transferred onto a PVDF membrane (Bio-Rad), and subsequently blocked using 5% milk in TBST. GATA4 specific (Santa Cruz) and  $\alpha$ -Tubulin specific antibodies were used to probe for the proteins (Sigma Aldrich) with the membrane being stripped with re-blot plus mild stripping buffer (Millipore) between each antibody incubation. The protein signals were detected using horseradish peroxidase (HRP) conjugated secondary antibodies including anti-goat (Santa Cruz) and anti-mouse (Cell signaling). Chemiluminescence was visualized using ECL substrates on the membrane (Fisher Scientific and Thermo-Scientific).

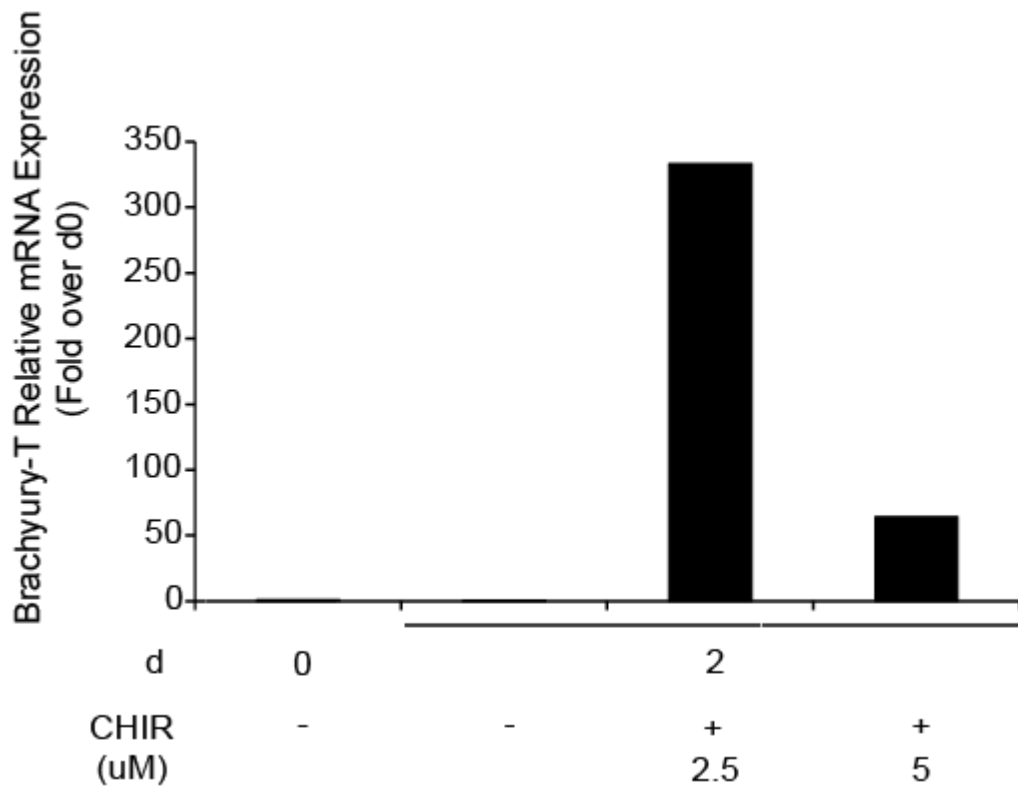
## **2.5. Subcellular localization analysis**

Undifferentiated cells from day 0, as well as differentiating cells from day 2 (DMSO treated and CHIR treated) and day 4 (DMSO/4mM HCl 0.1% BSA, CHIR, BMP4 and CHIR + BMP4) were collected and assessed for their nuclear and cytoplasmic expression of Nkx2.5. Cells were collected and the cytoplasmic and nuclear extracts were obtained as per the REAP protocol (156). One modification from the original protocol is that the nuclear pellet was resuspended in 20 mM Tris pH 6.8, 6M urea, and 0.1% SDS. Nuclear fractions and whole cell extracts were sonicated at 10% intensity for 1 round (whole cell extracts) or 2-3 rounds (nuclear fractions). Subsequent nuclear and cytoplasmic extracts were run on a 4-12% bis-tris NuPage gel, as previously mentioned in the immunoblot materials and methods section.

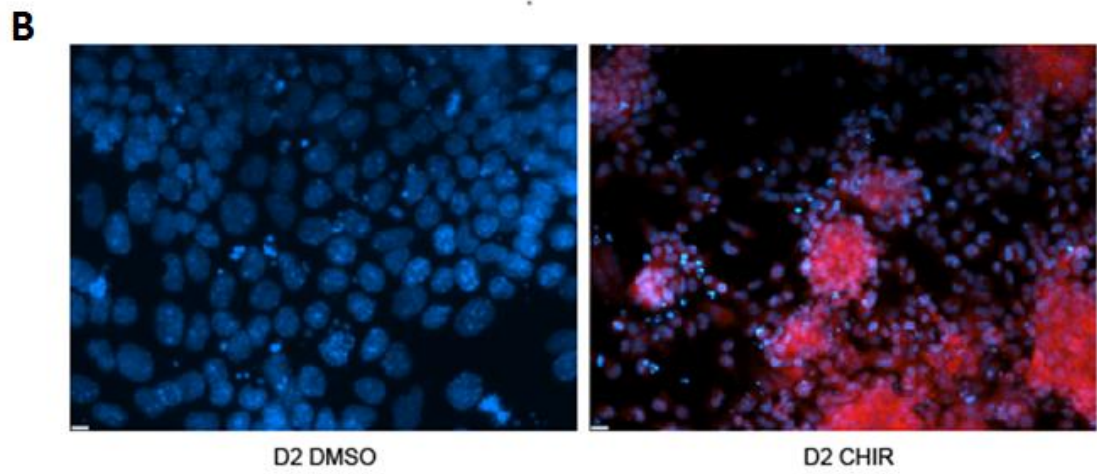
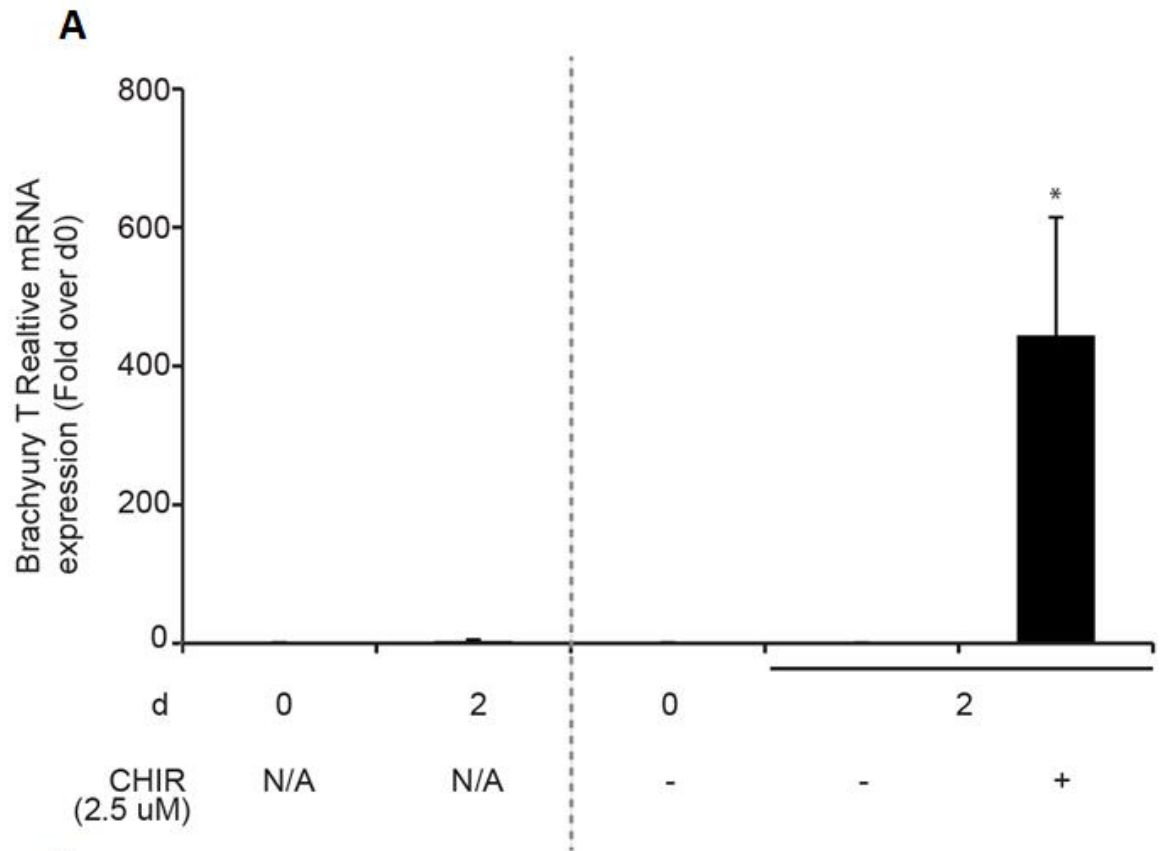
### **3. Results**

#### **3.1. CHIR induces mesoderm formation**

To determine the optimal concentration of CHIR to induce mesoderm and to see if the new protocol induced mesoderm better than the hanging drops protocol, the mesodermal marker Brachyury-T was assessed using QPCR analysis. From days 0 to 2 of differentiation, 2.5 or 5  $\mu\text{M}$  of CHIR or the vehicle (DMSO) were added. The resulting expression of Brachyury-T (Figure 5) demonstrated that both 2.5 and 5  $\mu\text{M}$  CHIR resulted in an increase in Brachyury-T gene expression with 2.5  $\mu\text{M}$  having about 105 fold induction and 5  $\mu\text{M}$  CHIR having approximately 64 fold induction over day 0 baseline expression. Brachyury-T levels with the DMSO control were at negligible levels. As a result of this experiment, the concentration of 2.5  $\mu\text{M}$  CHIR was used for further analysis. Using 2.5  $\mu\text{M}$  CHIR, it was determined that Brachyury-T expression was statistically up-regulated on day 2 (approximately 444 fold over day 0) compared to that of the DMSO vehicle and day 2 of the old hanging drops protocol (only about 3.30 fold increase over day 0) (Figure 6a). To validate the gene expression of Brachyury-T, the protein levels were also observed on day 2 of differentiation where Brachyury-T levels were present in the CHIR treated sample but not with the DMSO vehicle control (Figure 6b).



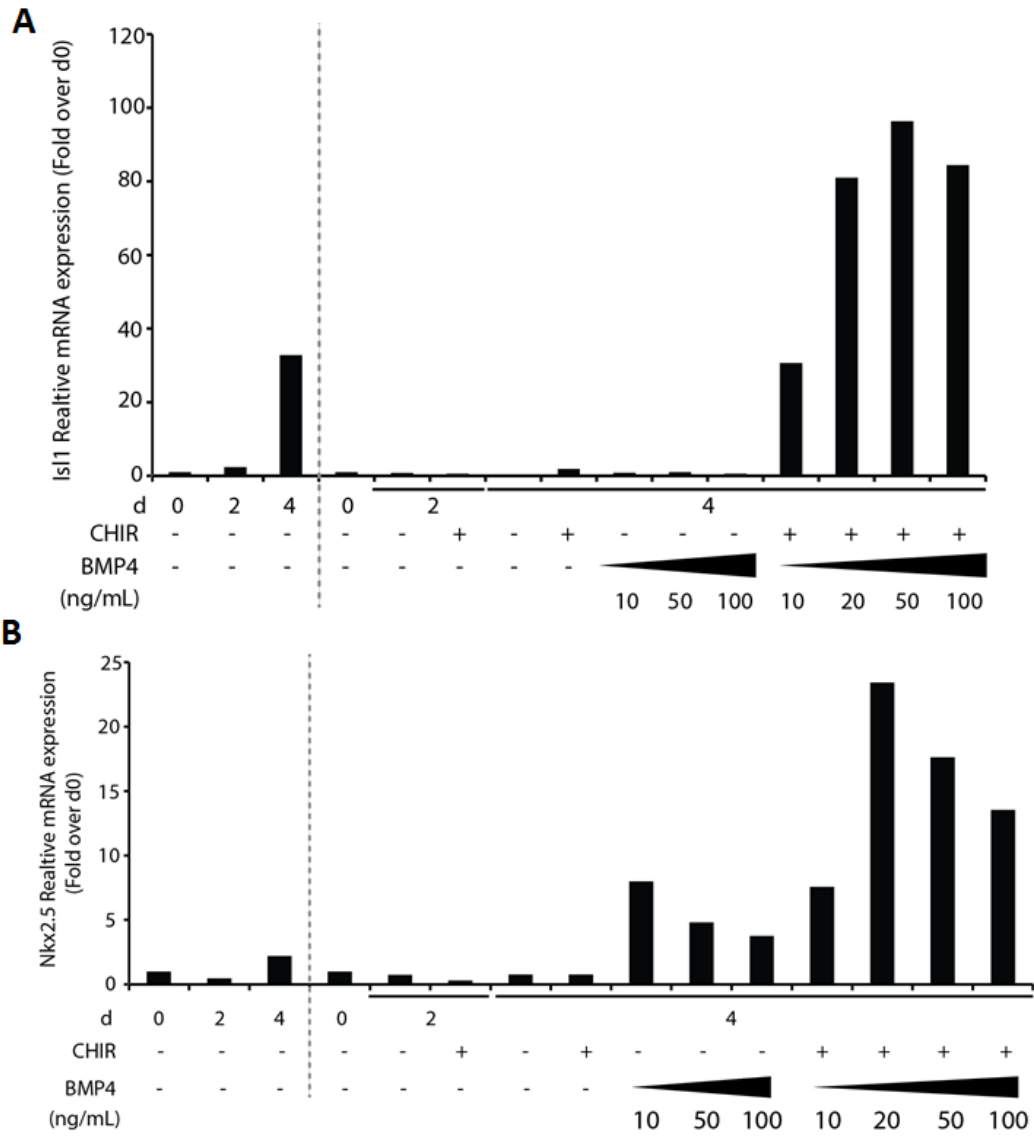
**Figure 5. The use of CHIR has a positive impact on Brachyury-T levels on day 2 of differentiation.** The mESCs (TCF LEF) were differentiated via the serum-free approach with small the molecule CHIR administered from days 0 to 2. The mRNA was analyzed on days 0, and 2 with QPCR. Gene expression was normalized to the day 0 control. n=1.



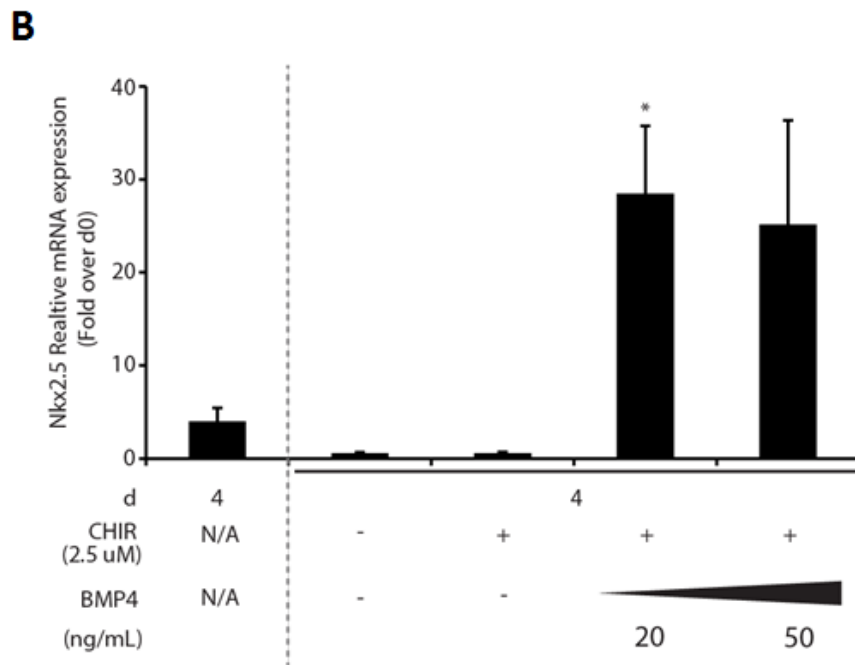
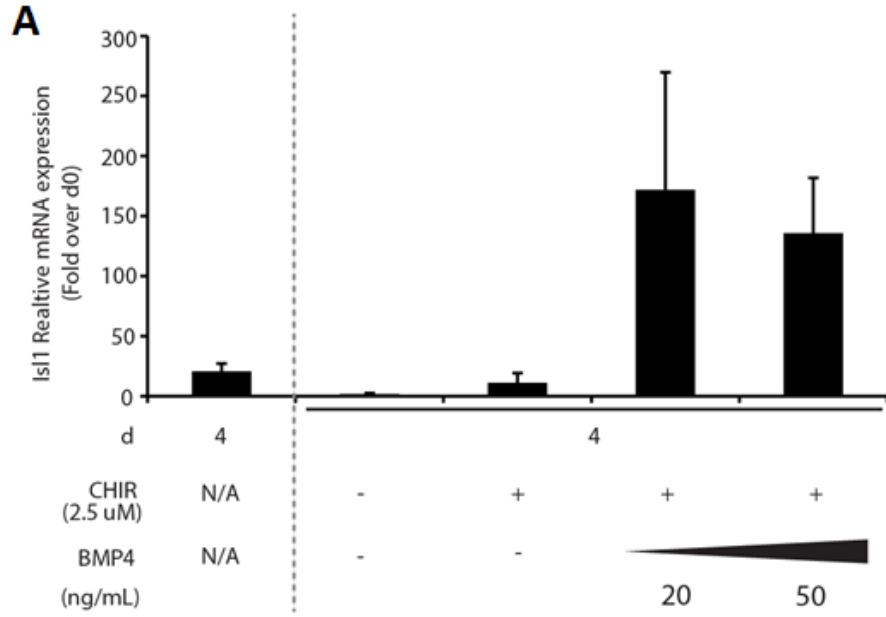
**Figure 6. The addition of CHIR significantly increases Brachyury-T transcript levels on day 2 of differentiation of mESC.** **A)** The mESCs (TCF LEF) were differentiated via the hanging drops, serum based method (left side) and the monolayer, serum-free approach with CHIR (right side). The mRNA was analyzed on days 0, and 2 with QPCR. Gene expression was normalized to each respective methods day 0 control. Error bars represent + SEM from n=3 (old method), or n=5 (new method) biological replicates. \*p<0.05 (day 2 CHIR treated as compared to day 2 of the old protocol, and day 0, 2 of the monolayer protocol without CHIR). **B)** mESCs (TCF LEF) cells were differentiated using the monolayer serum-free approach with CHIR at 2.5  $\mu$ M until day 2. Cells were then fixed with 3.7 % formaldehyde, permeabilized and stained for Brachyury-T expression (Red). The Hoechst dye (blue) represents the nuclei, while the red represents Brachyury-T staining. Images were taken at 20x magnification with the scale bar representing 20  $\mu$ m.

### **3.2. BMP4 addition after CHIR treatment results in a significant increase in Nkx2.5 transcript levels and a trend in increase of Isl1 levels.**

CHIR treatment from days 0-2 of differentiation resulted in successful mesoderm formation. Because of its importance in cardiac development, the cells were subjected to the addition of BMP4 from days 2-4, after the treatment of the cells with CHIR (2.5  $\mu$ M from days 0-2) to help induce cardiomyocyte formation from the mesodermal precursors. As was the case for CHIR, the amount of BMP4 was also titrated in different concentrations to observe the best results (Figure 7a,b). The QPCR was performed on day 4 of differentiation and thus the gene expression of the cardiac precursor marker *Isl1* was used as well as the cardiac progenitor marker *Nkx2.5* as these two genes would be up-regulated in the early stages of cardiomyogenesis. It was determined that both 20 ng/mL and 50 ng/mL of BMP4 were the most beneficial in up-regulating the gene expression of *Isl1* and *Nkx2.5* so a further 2 biological replicates were performed to see if one concentration of BMP4 would yield statistically improved results than the other (Figure 8a,b). Adding the CHIR and BMP4 (both 20ng/mL and 50ng/mL of BMP4) collectively caused an increase in *Nkx2.5* transcript levels. More specifically, with the collective addition of CHIR and 20ng/mL BMP4, there was a statistically significant increase in *Nkx2.5* transcript levels on day 4 compared to the vehicle treated, CHIR treated, and hanging drop method. A similar result was obtained for *Isl1* transcript levels but the results were not significant. With the new method of differentiation the best results were obtained using 20 ng/mL of BMP4 which when combined with CHIR resulted in 172 fold and 28 fold of *Isl1* and *Nkx2.5* respectively compared to day 0.



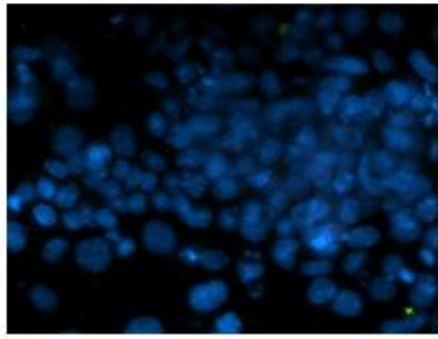
**Figure 7. CHIR and BMP4 are able to induce the expression of Isl1 and NKx2.5 transcript levels to higher level than the control or individually.** The mESCs (TCF LEF) were differentiated via the serum based hanging drops method, or the serum-free approach with small molecules CHIR administered from days 0-2 (noted with the + sign) and the sequentially with BMP4 from days 2-4. The mRNA was analyzed on days 0, and 4 with QPCR for **A)** Isl1 and **B)** Nkx2.5 with the left side representing the hanging drops method and the right side depicting the new serum free based method. Gene expression was normalized to each respective methods day 0 control. n=1.



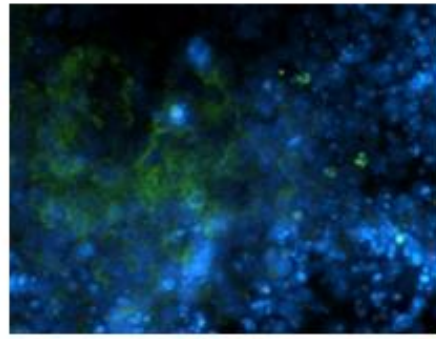
**Figure 8. Collective addition of CHIR and BMP4 result in an increase of Isl1 and Nkx2.5 expression with the new approach compared to the old approach on day 4.** The mESCs (TCF LEF) were differentiated via the serum based hanging drops method, or the serum-free approach with small molecules CHIR administered from days 0-2 (noted with the + sign) and the sequentially with BMP4 from days 2-4. The mRNA was analyzed on days 0 and 4 with QPCR for **A)** Isl1 and **B)** Nkx2.5 with the left side representing the serum based hanging drops method and the right side depicting the new serum free based method. Gene expression was normalized to each respective methods day 0 control. Error bars represent  $\pm$  SEM from n=3. \*p<0.05. Nkx2.5 was statistically upregulated with CHIR and BMP4 (20 ng/mL) compared to either CHIR alone or control conditions on day 4 of the monolayer method, and day 4 of the hanging drops approach.

### **3.3. The addition of CHIR or BMP4 results in predominately cytoplasmic Nkx2.5 localization while the sequential addition of CHIR + BMP4 resulted in predominately nuclear localization of Nkx2.5**

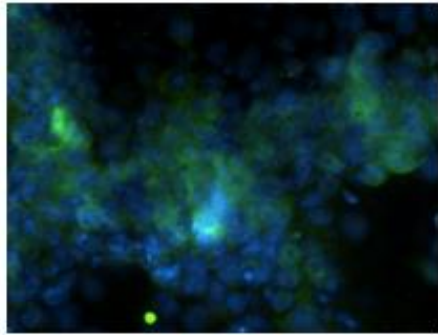
In addition to the QPCR gene level analysis of Nkx2.5, the protein levels of Nkx2.5 were also visualized by immunofluorescence on day 4 of differentiation (Figure 9). Nkx2.5 specific antibodies determined that the treatment of the cells either with CHIR or BMP4 resulted in Nkx2.5 expression being seen in the cytoplasm of the cell. Interestingly, combining the CHIR and BMP4 treatment resulted in a majority of the Nkx2.5 signalling to be localized to the nucleus (though it appears to have less Nkx2.5 signal overall). This subcellular localization was also attempted to be shown by immunoblot but the immunoblotting for Nkx2.5 was inconclusive due to the lack of any specific band on the developed film (data not shown).



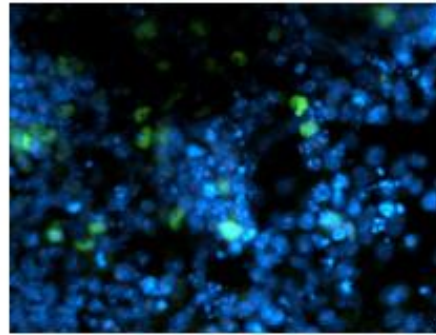
D4 DMSO



D4 CHIR



D4 BMP4



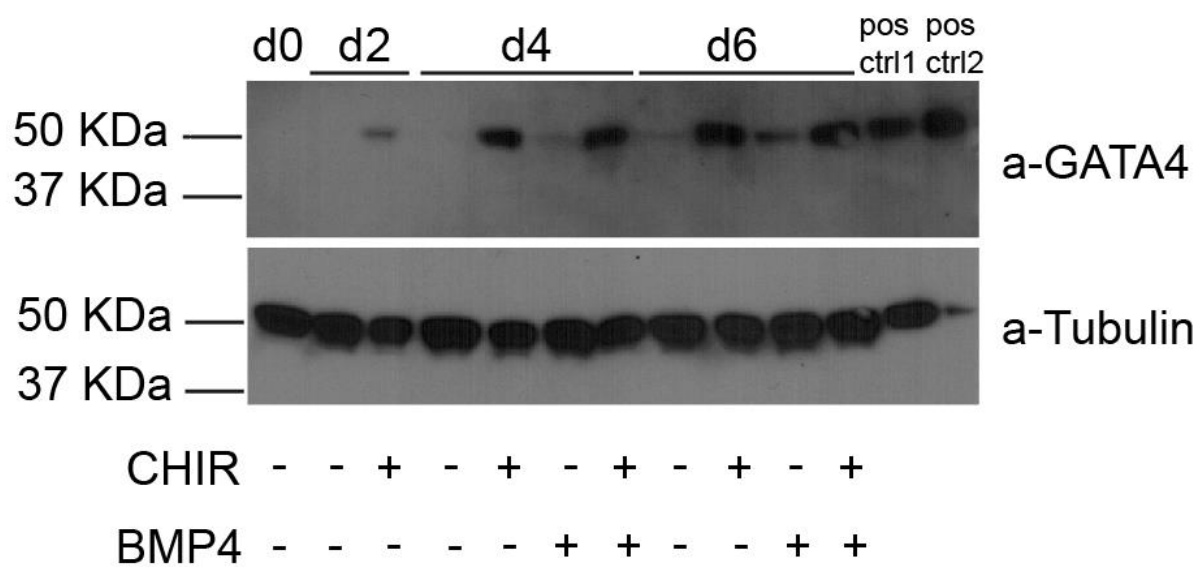
D4 CHIR + BMP4

**Figure 9. Addition of CHIR and BMP4 results in an increase of Nkx2.5 expression. A)**

TCF LEF mESCs were differentiated according to the new monolayer protocol with the sequential addition of CHIR (2.5  $\mu$ M) from days 0-2, and BMP4 (20 ng/mL) from days 2-4, and were evaluated for Nkx2.5 expression on day 4 of differentiation. Cells were fixed with 3.7 % formaldehyde, permeabilized, and stained for Nkx2.5 expression (green). The Hoechst dye (blue) represent the nuclei, while the green represents Nkx2.5 staining. Images were taken at 40x magnification with the scale representing 20  $\mu$ m.

### **3.4. The addition of CHIR or CHIR+BMP4 results in the up-regulation of GATA4 protein levels.**

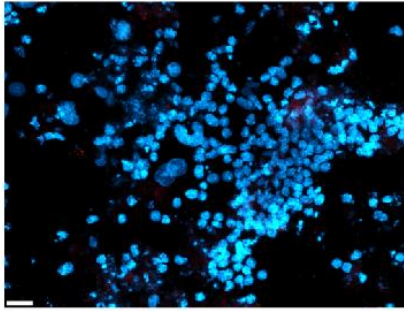
While an increase in cardiac progenitor Nkx2.5 and a trend in increase for cardiac precursor Isl1 were observed, we wanted to further see if other critical markers for cardiac formation were also up-regulated. A time course immunoblot was performed including days 0, 2, 4 and 6 under all experimental conditions and the GATA4 protein levels were observed (Figure 10). From the immunoblot, it was observed that even as early as day 2 under the CHIR treatment, GATA 4 was already being expressed. Similarly, that expression further increased on days 4 and 6. GATA4 expression levels remained highest with CHIR treated or CHIR and BMP4 treated cells. As was witnessed with the Nkx2.5 QPCR, BMP4 by itself was also able to induce a slight increase in GATA4 expression levels over the DMSO controls, but not as much as CHIR or the CHIR + BMP4 combination. The high levels of GATA4 expression observed on day 6 of differentiation with CHIR and CHIR+BMP4 were similar to those observed with day 6 hanging drops (1<sup>st</sup> positive control) and the day 6 hanging drops nucleus (day 6 hanging drops, nuclear extraction).



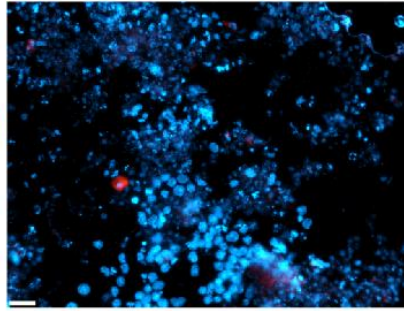
**Figure 10. The treatment of differentiating mESCs with CHIR individually or with combination of BMP4 results in the induction of GATA4 expression.** The protein lysates from differentiating TCF LEF mESCs were taken on days 0, 2, 4, and 6 under the directed differentiation of the sequential addition of the small molecules CHIR (administered from days 0-2 at 2.5  $\mu$ M), and BMP4 (administered from days 2-4 at 20ng/mL). Positive control used were d6 whole cell lysate from hanging drops protocol (1<sup>st</sup> positive control) and d6 hanging drops nuclear extraction from hanging drops protocol (2<sup>nd</sup> positive control). Proteins were probed with the  $\alpha$ -GATA4 or  $\alpha$ -Actin antibodies (loading control), and for visualization, HRP-conjugated secondary antibodies were added.

### **3.5. The combination of both CHIR and BMP4 induces greater cardiomyocyte formation as compared to their individual effects.**

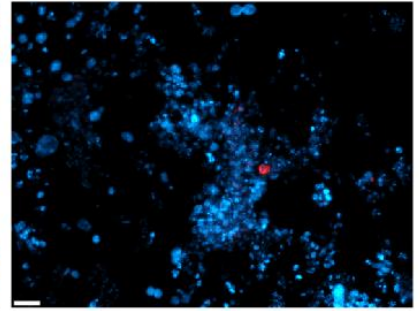
After the positive increases seen with Isl1, GATA4 and Nkx2.5, the terminal cardiac differentiation marker of MHC was assayed for (Figure 11). Using immunofluorescence, to detect MHC expression, the vehicle control and BMP4 resulted in low observable cardiomyocytes (about 2%). Conversely, CHIR by itself and CHIR+BMP4 were able to induce cardiomyogenesis. The treatment of the mESCs with CHIR led to about 7% observable MHC-positive cells while for CHIR+BMP4, that number was approximately 9%. In fact, the CHIR + BMP4 was the only combination where there was a statistical increase in cardiomyocytes observed compared to the DMSO control (Figure 12). While not statistically significant different, there was also a trend in the increase in number of cardiomyocytes in the CHIR+BMP4 condition as compared the CHIR by itself.



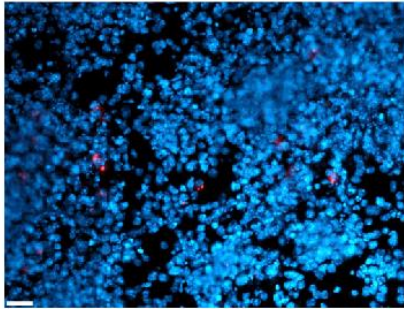
D6 DMSO, 4mM HCL 0.1% BSA



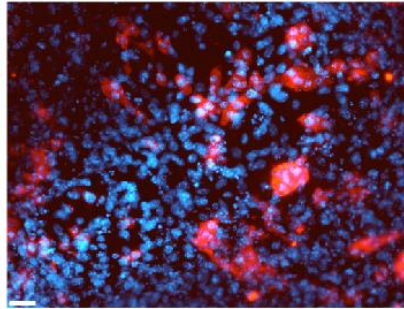
D6 BMP4 (20 ng/mL)



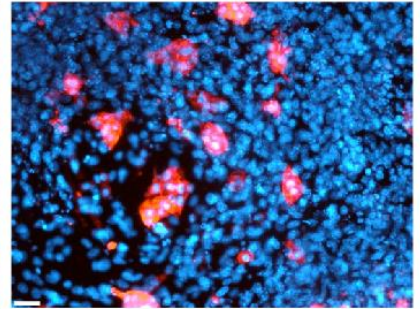
D6 BMP4 (50 ng/mL)



D6 CHIR

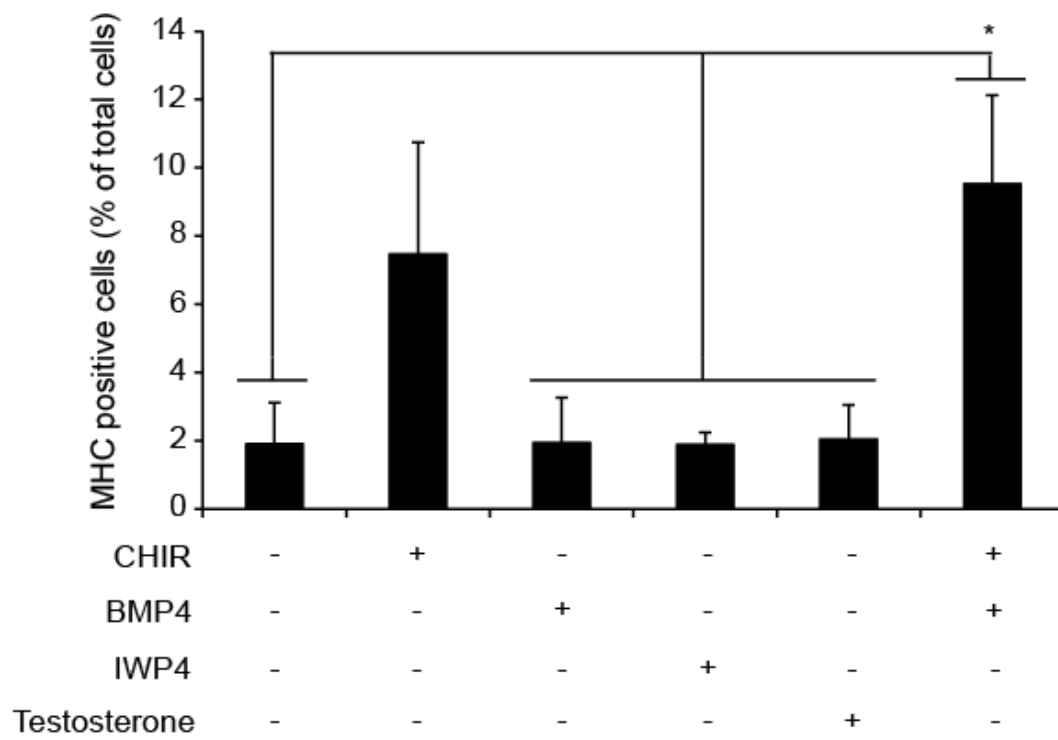


D6 CHIR+BMP4 (20 ng/mL)



D6 CHIR+BMP4 (50 ng/mL)

**Figure 11. CHIR or CHIR+BMP4 addition during differentiation in mESC leads to cardiomyogenesis.** The mESCs (R1- TCF-LEF) were analyzed on day 6 after monolayer based differentiation either treated with control (DMSO, HCl, BSA), with CHIR (2.5  $\mu$ M added days 0-2), with BMP4 (20 or 50 ng added days 2-4) or with CHIR (2.5  $\mu$ M) and BMP4 (20 or 50ng), with the molecules being added sequentially. Cells were visualized using indirect immunofluorescence. The Hoechst dye (blue) represents the nuclei, while the MF20 (red) represents MHC staining. Images were taken at 20x magnification with the scale representing 20  $\mu$ m.



**Figure 12. The addition of CHIR+BMP4 results in an increase in cardiomyogenesis as compared to the individual small molecules and control tested but not compared to the CHIR alone condition.** The percentage of MHC cells was quantified by individually counting the MHC-positive cells, and dividing them over the total number of cells (nuclei) quantified. Error bars represent  $\pm$  SEM from n=3. \*p<0.05.

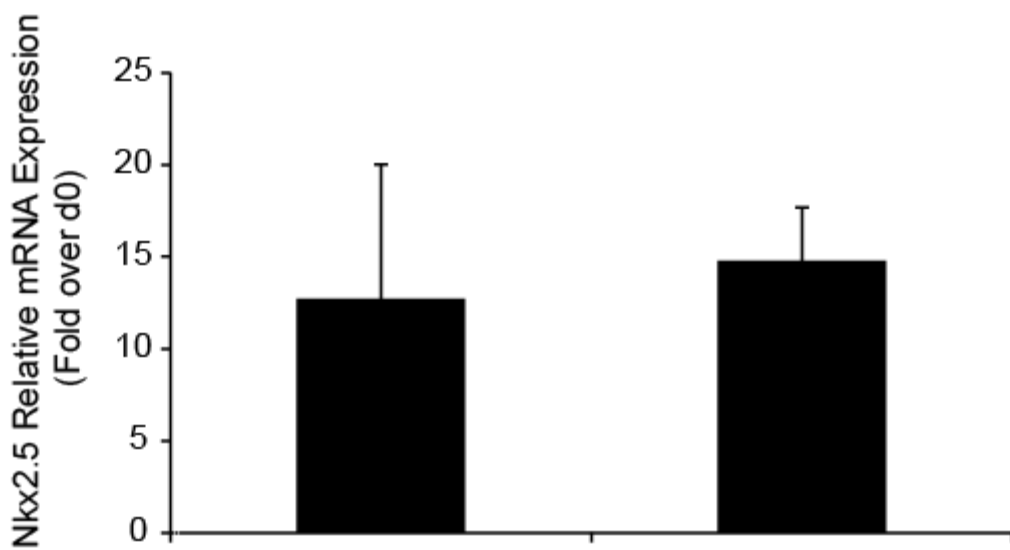
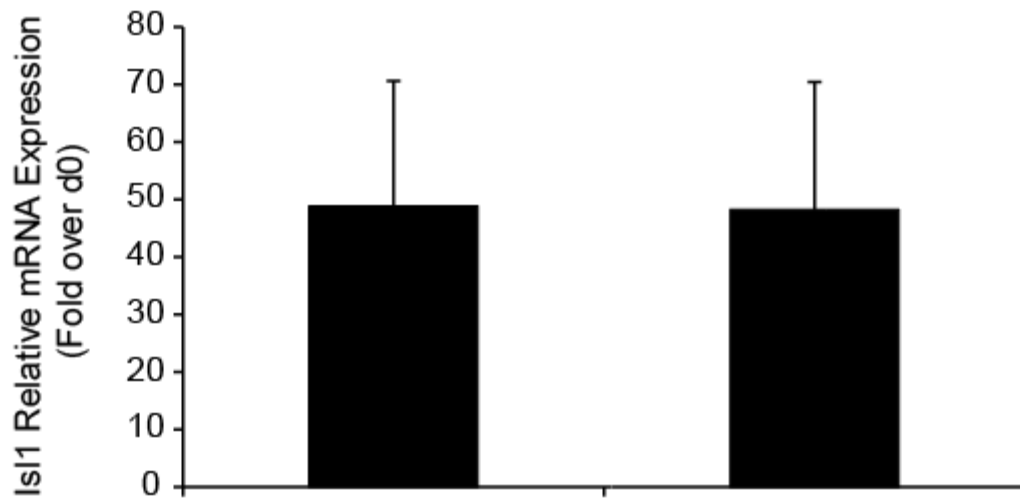
**3.6. Further manipulation of Wnt and BMP4 signalling by their inhibition or the addition of testosterone resulted in no statistical change to the amount of cardiomyocytes observed.**

In order to improve on the CHIR and BMP4 combination, the next step to try was to inhibit both the Wnt signalling and BMP4 signalling. This was performed as based on previous results showing an increase in cardiomyogenesis yield when these two pathways were inhibited (155,118). Initially, the small molecule inhibitor of Wnt (IWP4) was used as it has been observed to increase cardiomyogenesis in hESCs. IWP4 concentrations were titrated and after observing the expression of select genes, 2.5  $\mu$ M was deemed to be the most effective added during days 4-6 after CHIR, and BMP4 addition as similar gene expressions of Nkx2.5 were observed from 2.5, 5, and 10  $\mu$ M of IWP4(results not shown). Analysis of the Isl1 and Nkx2.5 expression levels yielded no differences between CHIR+BMP4 and CHIR+BMP4+IWP4 (Figure 12). Additionally, the terminal differentiation marker MHC was also assessed and no differences were present in the differentiating conditions (Figure 13). To see if there was a difference in cardiomyocytes observed, quantification was once again performed (Figures 12, 17). Quantification of the effects of IWP4 alone and in conjunction with other small molecules however revealed that there was no significance in MHC positive cells as compared to the CHIR+BMP4 combination (Figures 12, 17).

In addition to the inhibition of the Wnt signalling pathway, BMP4 inhibitors have also demonstrated improved cardiomyogenesis (153). To accomplish the BMP4 inhibition, the cells were treated with the BMP4 inhibitor noggin from days 4-6 at several concentrations, a time point after the CHIR and BMP4 addition as BMP4 is needed to be

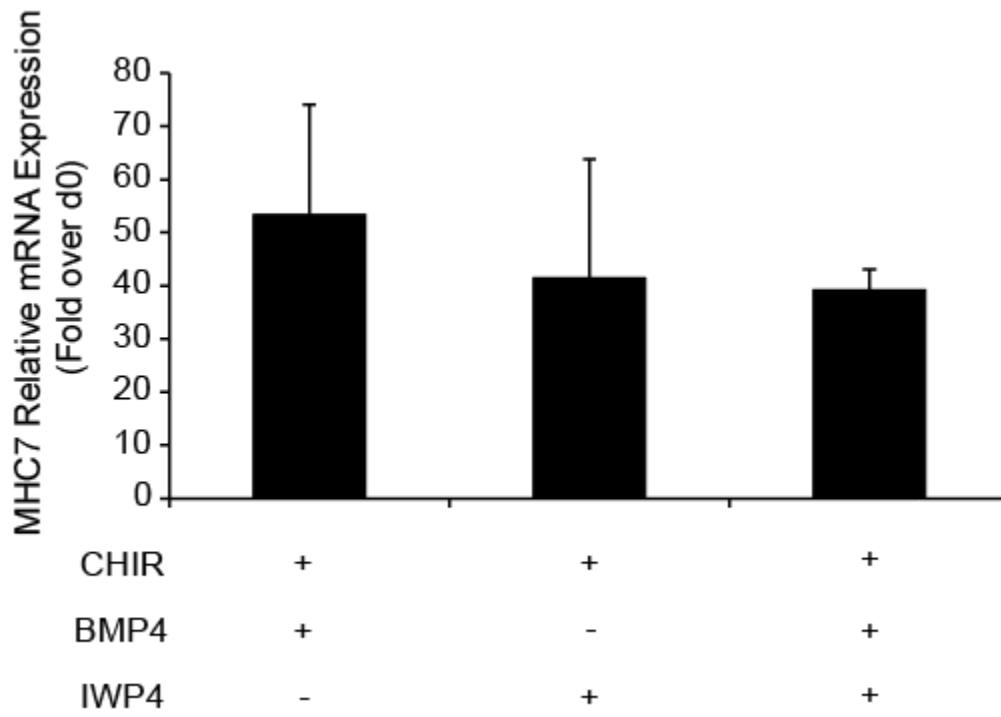
repressed in differentiated cardiomyocytes (158). A prior study has reported that a concentration of 150 ng/mL of noggin was effective in increasing cardiomyogenesis so that concentration was set as a middle value. From the MHC staining on day 6, it was apparent that noggin did not result in an increase in cardiomyogenesis at any tested concentration and no further pursuit into its effect on cardiomyogenesis was done (Figure 16).

As our lab has had success with testosterone increasing cardiomyogenesis, testosterone was also added to the combination of Wnt and BMP4 signalling to observe if any improvements would be seen. After trying out several concentrations shown to be successful in previously induced increase in cardiomyogenesis in mESCs (135), a final testosterone concentration of 1  $\mu$ M was the concentration used as half and double the concentrations did not seem to give more beneficial results (data not shown). As per the work of Al Madhoun *et al.*, testosterone was added from day 2 onward until the end of differentiation (135). The testosterone was added concurrently with the other small molecules from day 2 onward. While initial observation of MHC staining looked promising with the testosterone in combination with CHIR, BMP4, and IWP4, the number of MHC positive cells in the different conditions were not significantly different than the CHIR+BMP4 combination regardless of the combination testosterone was used with (Figures 12, 15, 17).

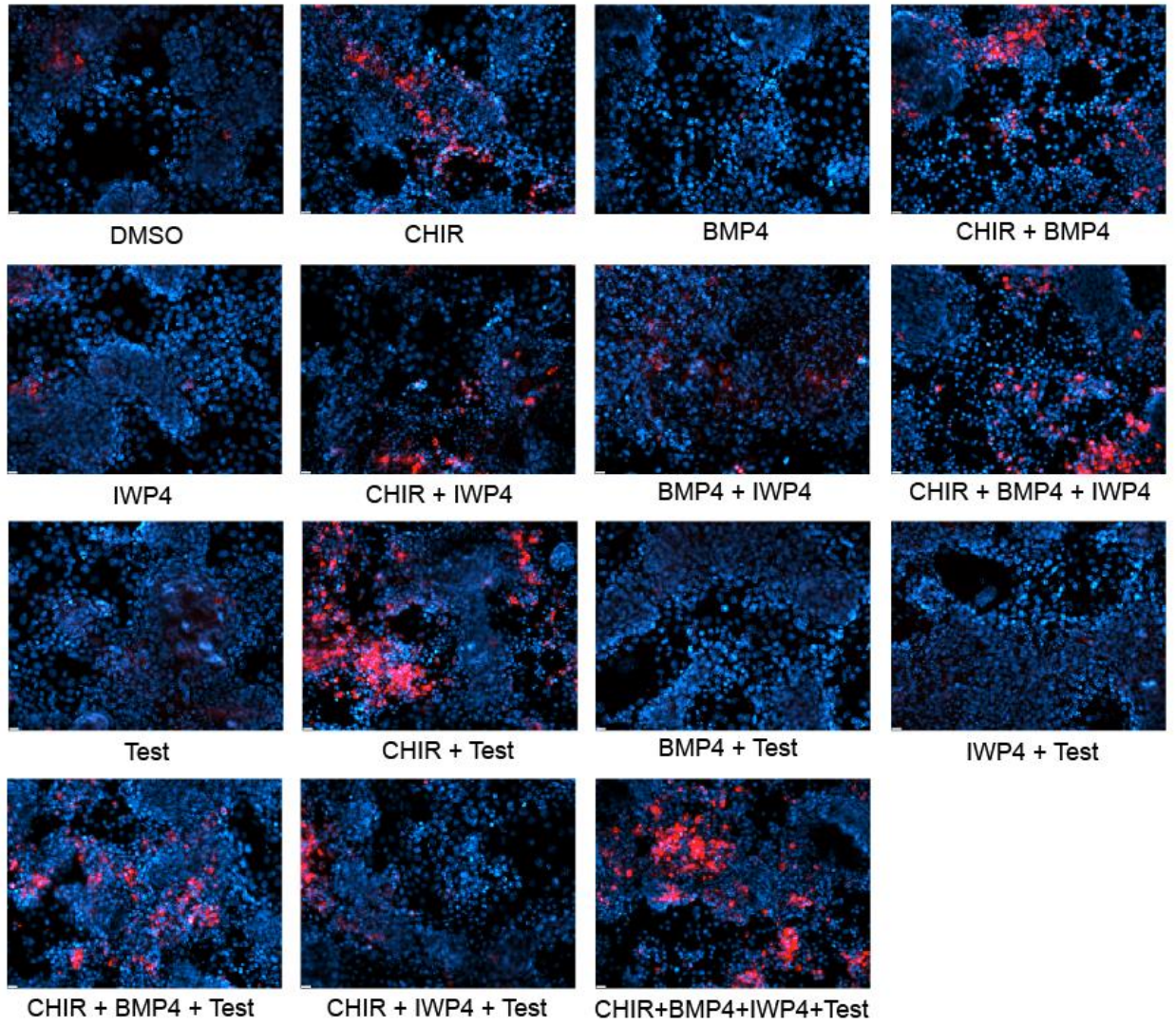


CHIR	+	+
BMP4	+	+
IWP4	-	+

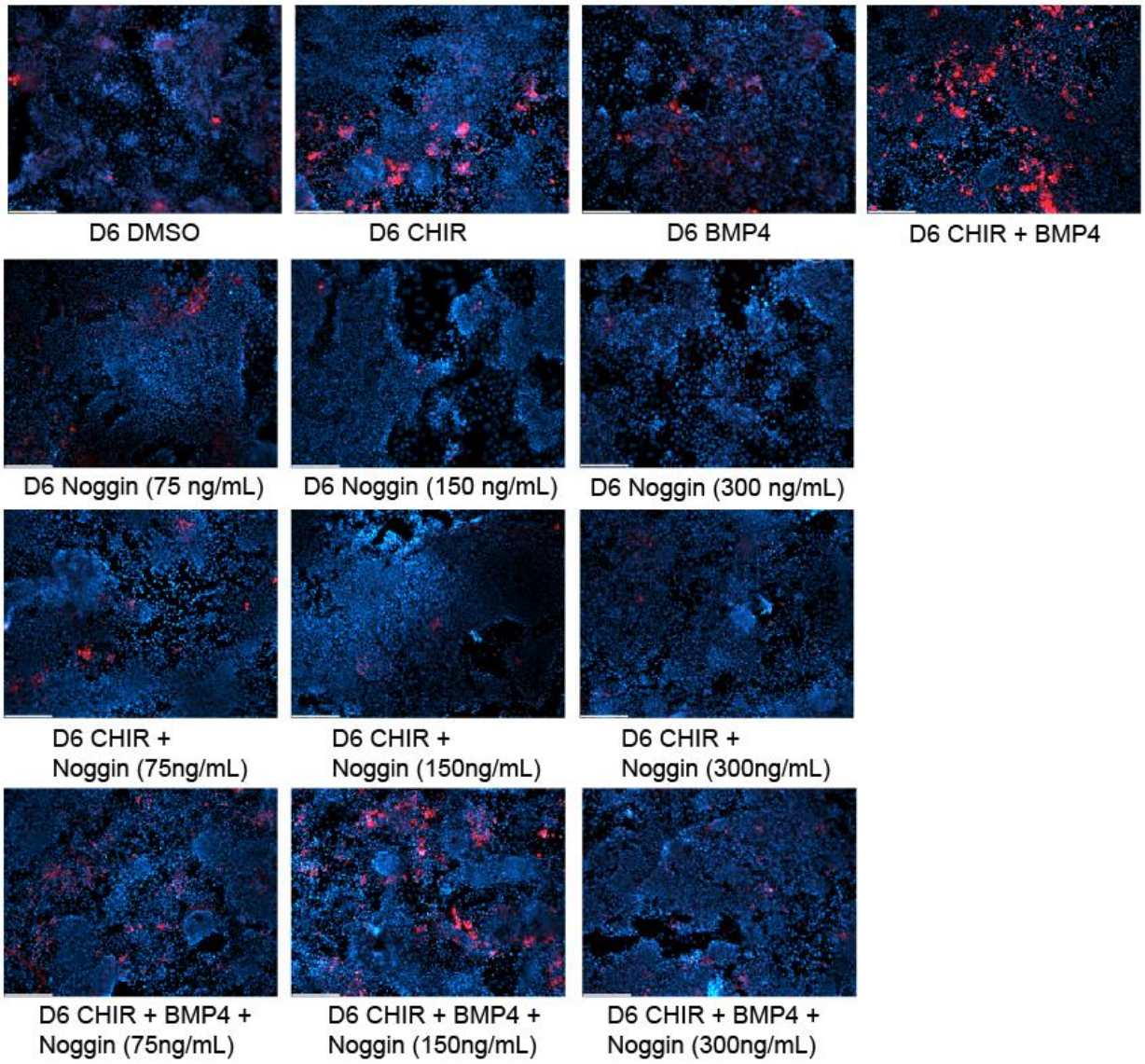
**Figure 13. The addition of IWP4 to the CHIR+BMP4 combination does not lead to an increase in Isl1 or Nkx2.5 expression.** TCF LEF mESCs were differentiated according to the monolayer protocol with the sequential addition of CHIR (added day 0-2 at 2.5  $\mu$ M), BMP4 (added days 2-4 at 20 ng/mL), and IWP4 (added day 4-6 at 2.5  $\mu$ M). The mRNA was analyzed on day 6 with QPCR. Gene expression was normalized to each respective methods day 0 control. Error bars represent  $\pm$  SEM from n=3.



**Figure 14. The addition of IWP4 to the CHIR+BMP4 combination does not lead to an increase in MHC7 expression.** TCF LEF mESCs were differentiated according to the monolayer protocol with the sequential addition of CHIR (added day 0-2 at 2.5  $\mu$ M), BMP4 (added days 2-4 at 20 ng/mL), and IWP4 (added day 4-6 at 2.5  $\mu$ M). The mRNA was analyzed on day 6 with QPCR. Gene expression was normalized to each respective methods day 0 control. Error bars represent  $\pm$  SEM from n=3.

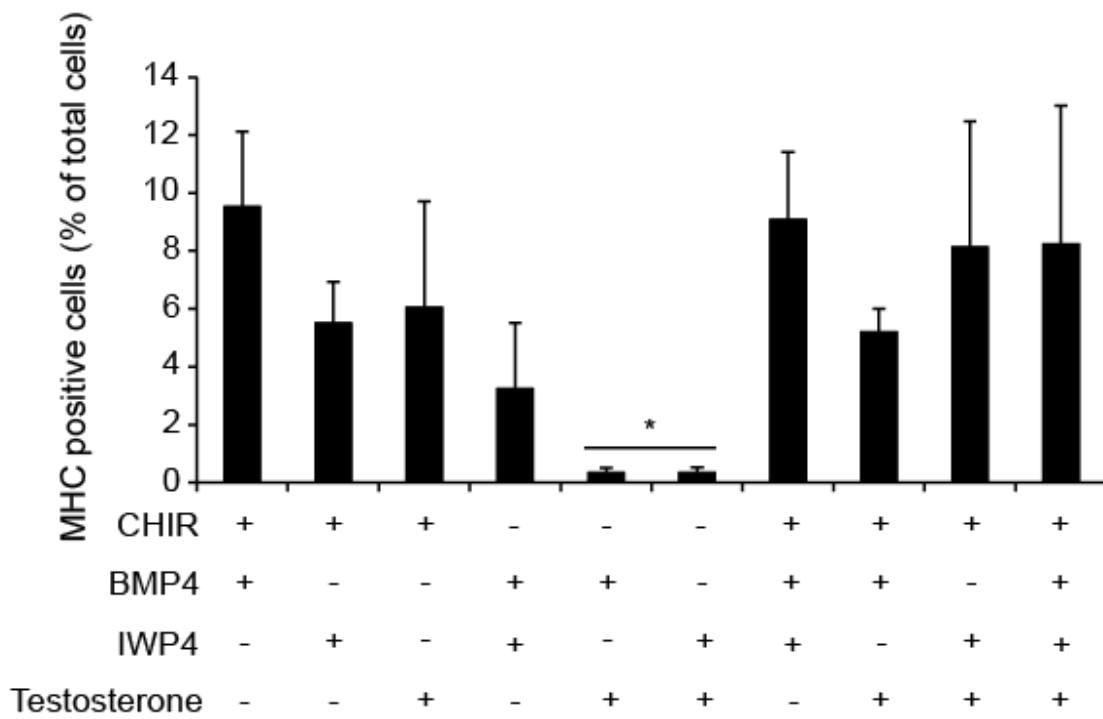


**Figure 15. IWP4 and testosterone do not increase the number of cardiomyocytes on day 6 of differentiation.** Using the monolayer based approach, mESC TCF-LEF cells were differentiated in the presence of small molecules added collectively and sequentially. The small molecules used included CHIR (added days 0-2 at 2.5  $\mu$ M), BMP4 (added days 2-4 at 20 ng/mL), IWP4 (added days 4-6 at 2.5 $\mu$ M), and testosterone (1 $\mu$ M added days 2-6 at  $\mu$ M). The Hoechst dye (blue) represent the nuclei, while the red represents MHC staining. Images taken at 20x magnification with the scale representing 20  $\mu$ m.



**Figure 16. Noggin induces cardiomyogenesis but not as efficiently as CHIR + BMP4.**

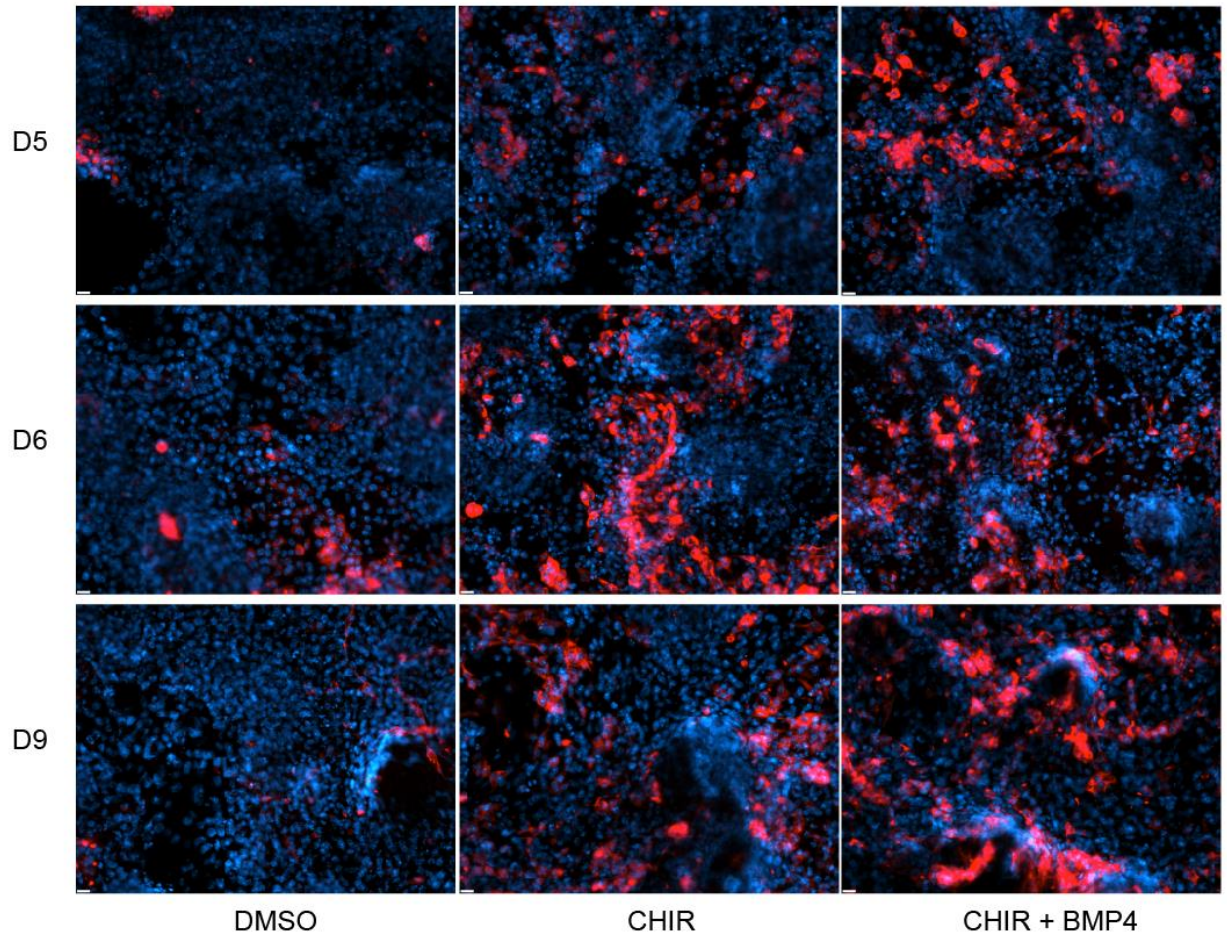
Using the monolayer based approach, mESC TCF-LEF cells were differentiated with the sequential addition of small molecules including CHIR (added days 0-2 at 2.5  $\mu$ M), BMP4 (added days 2-4 at 20 ng/mL), and noggin (added days 4-6 at the concentrations listed). The Hoechst dye (blue) represents the nuclei, while the red represents MHC staining. Images were taken at 10x magnification with the scale representing 200  $\mu$ m. n=1.



**Figure 17. The combinations of IWP4 and testosterone with CHIR and/or BMP4 do not lead to an increased number of cardiomyocytes as compared to the CHIR+BMP4 combination.** The percentage of MHC cells was quantified by individually counting the MHC-positive cells, and dividing them over the total number of cells (nuclei) quantified. Error bars represent  $\pm$  SEM from n=3. \*p<0.05.

### **3.7. The addition of CHIR and BMP4 induce cardiomyogenesis as early as day 5 and seems to increase cardiomyogenesis on time points after d6.**

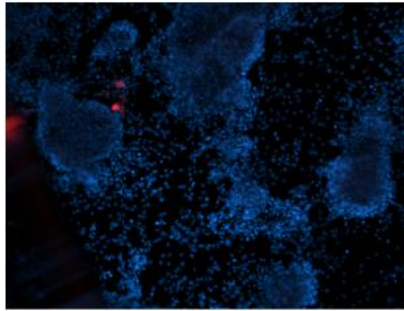
With the notion observed with the increase in Nkx2.5 and GATA4 transcript and protein levels on day 4 compared to the hanging drops method where high levels of cardiac progenitor markers were observed later on at day 6, there is a possibility that the CHIR+BMP4 combination results in accelerated cardiomyogenesis. Here cardiomyocytes can be seen already on day 5 (Figure 18). Cells expressing MHC were also observed on day 6 and day 9 under the CHIR or CHIR+ BMP4 treated conditions. While not yet determined, it appears as though there is a greater number of MHC positive cells as the time-course of differentiation is increased in length however, this needs further verification and replicates to be performed as the data is preliminary.



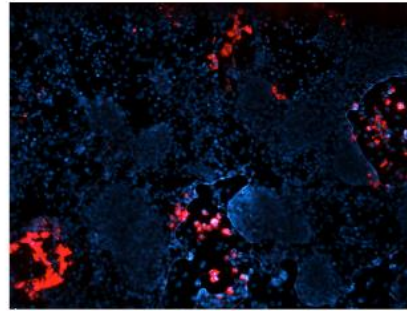
**Figure 18. Increased duration of differentiation may lead to a higher percentage of cardiomyocytes.** Using the monolayer based approach, mESC TCF-LEF cells were differentiated in the presence of small molecules which included CHIR (added days 0-2 at 2.5  $\mu$ M), BMP4 (added days 2-4 at 20 ng/mL), which were added sequentially. Cells were fixed on days 5, 6, and 9 with MeOH. The Hoechst dye (blue) represents the nuclei, while the red represents MHC staining. Images were taken at 20x magnification with the scale representing 20  $\mu$ m. n=1.

### **3.8. Plating the cells at different densities affects cardiomyogenesis.**

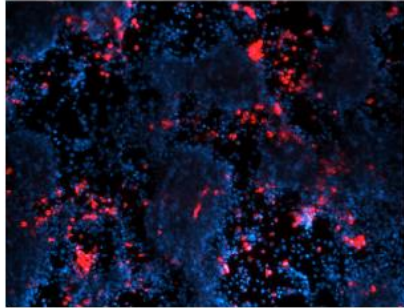
To observe if the initial cell density affects the differentiation efficiency, mESCs were plated at densities ranging from 100K, 300K, 500K, 750K, and 1 million cells per a 3.8 cm<sup>2</sup> well each with 1 mL of E6 media. It was interesting to see that 500K cells was deemed to look the best with MHC positive cell visualization while both the extremes, the 100K and 1 million cells/3.8 cm<sup>2</sup> gave what appeared to be the least amount of cardiomyocytes (Figure 19). In terms of MHC expression, there was a very strong trend in decreased MHC positive cells in the 150K cells/3.8cm<sup>2</sup> density as opposed to number of MHC positive cells at the 500K cells/3.8cm<sup>2</sup> density but the result was not significant (Figure 19). Confirming what was observed with the immunofluorescence and MHC quantification, a QPCR was run on the Brachyury-T, Isl1 and Nkx2.5 genes comparing both 500K and 150K cells/3.8cm<sup>2</sup> (150K cells was used as this number was previously successful in a mESCs skeletal muscle protocol (93) (Figure 20). Both the mesoderm marker, Brachyury-T, and cardiac progenitor marker Nkx2.5 were down-regulated with the 150K cells/3.8 cm<sup>2</sup> condition as compared to the 500K cells/3.8 cm<sup>2</sup> condition. Although not significant, a similar down-regulation was also observed for Isl1.



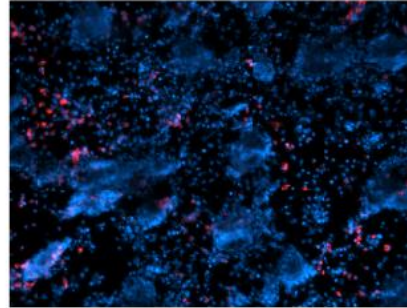
D6 CHIR + BMP4 100K cells/3.8 cm<sup>2</sup>



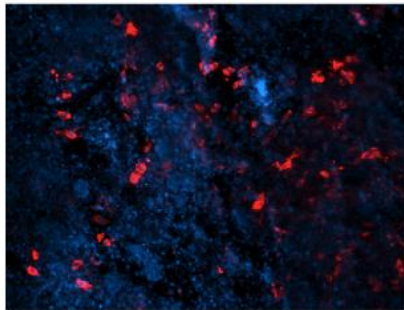
D6 CHIR + BMP4 300K cells/3.8 cm<sup>2</sup>



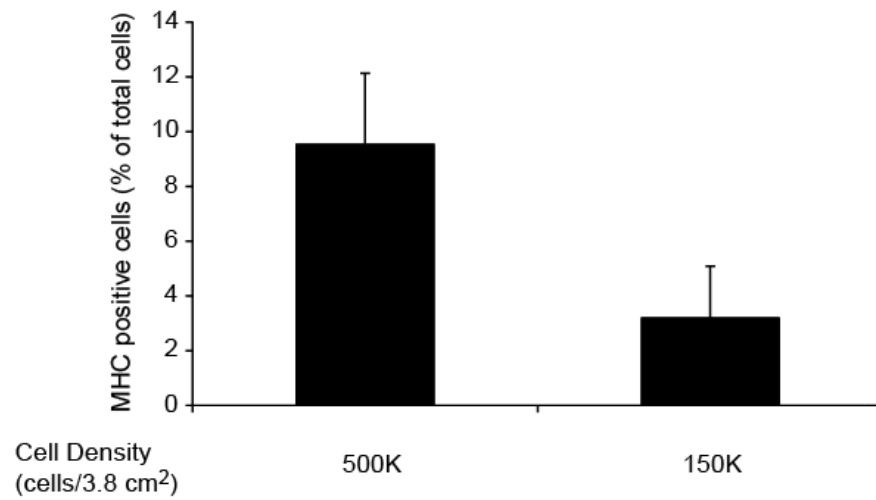
D6 CHIR + BMP4 500K cells/3.8 cm<sup>2</sup>



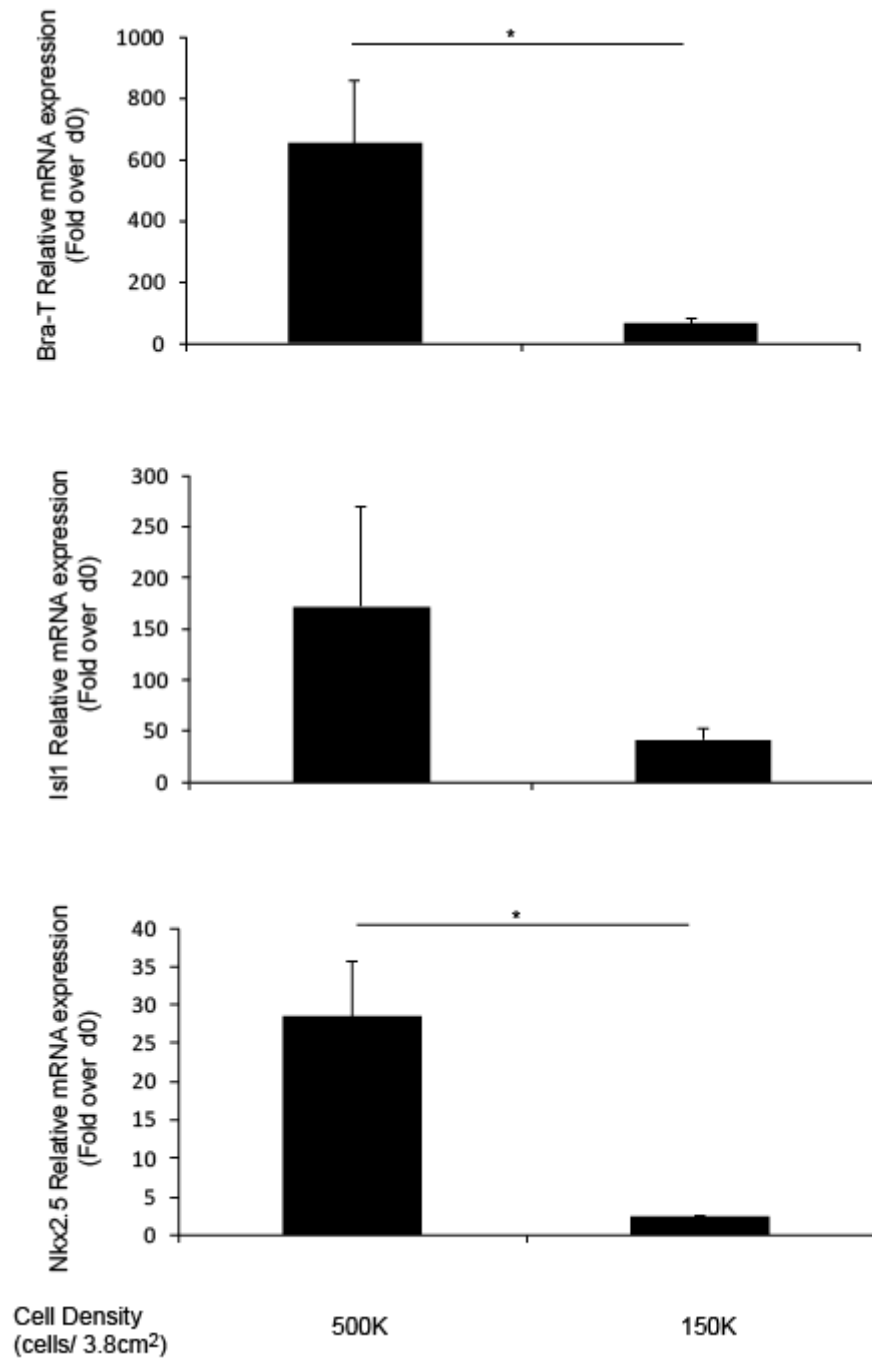
D6 CHIR + BMP4 750K cells/3.8 cm<sup>2</sup>



D6 CHIR + BMP4 1 million cells/3.8 cm<sup>2</sup>



**Figure 19. Initial cell plating density impacts cardiomyogenesis.** Using the monolayer based approach, mESC TCF-LEF cells were differentiated in the presence of small molecules which included CHIR (added days 0-2 at 2.5  $\mu$ M), BMP4 (added days 2-4 at 20 ng/mL). Cells were initially plated at the indicated densities in 3.8 cm<sup>2</sup> surface area dishes with 1 mL of E6 media on day 0. The Hoechst dye (blue) represents the nuclei, while the red represents MHC staining. Images were taken at 10x magnification with the scale representing 200  $\mu$ m. The percentage of MHC cells was quantified by individually counting the MHC-positive cells, and dividing them over the total number of cells (nuclei) quantified. Error bars represent + SEM from n=3.



**Figure 20. Initial plating density impacts mesodermal and cardiac progenitor markers.**

TCF LEF mESCs were differentiated according to the monolayer protocol with CHIR (added day 0-2 at 2.5  $\mu$ M), and BMP4 (added days 2-4 at 20 ng/mL). Cells were initially plated at the indicated densities in 3.8 cm<sup>2</sup> surface area dishes with 1 mL of E6 media on day 0. The mRNA was analyzed on day 2 for Brachyury-T and day 4 for Is11 and Nkx2.5 with QPCR. Gene expression was normalized to each respective methods day 0 control. Error bars represent + SEM from n=3. \*p<0.05.

#### **4. Discussion.**

Since the heart is derived from the mesoderm lineage, the first goal of obtaining a more efficient protocol was to get the pluripotent mESCs into the mesoderm lineage which would then be followed by the induction of the cells into the cardiac lineage.

Several studies have reported that the mesodermal marker Brachyury-T is induced by the canonical Wnt signalling pathway (91). In previous work performed by the Skerjanc lab using mESCs, Brachyury-T was present at day 2 of differentiation either with the treatments consisting of BMP4/INHBA (the combination of BMP4 and Activin A) or with CHIR, the same concentration of CHIR that was used in my study, although with a slightly altered protocol. In the published paper, the cells were aggregated 2 days before treatment which was not the situation with my work (93). With the results I have obtained with Brachyury-T (Figure 6a), there is an approximately 300 fold difference between the CHIR treated samples and the DMSO control, while in the research conducted in aforementioned study, the difference was less drastic; the control conditions yielded a Brachyury-T level of about 40% that of the CHIR treated samples on day 2 while the INHBA treated samples had less Brachyury-T levels than the control (93). On the other hand, the approximate 300 fold up-regulation of Brachyury-T in my studies was limited compared to the approximate 5000 fold up-regulation observed with CHIR treatment in hESCs obtained with a protocol much more similar to what I have proceeded with as it was entirely monolayer based (93). More importantly, the levels of Brachyury-T were also statistically up-regulated compared to the hanging drops method. This high level of Brachyury-T mRNA expression also co-related with the high protein expression of Brachyury-T in the CHIR treated conditions (Figure 6b). Therefore, the use of CHIR resulted in the successful induction of mesoderm from

undifferentiated mESCs and provided a foundation for further experimentation into obtaining a better mESCs cardiomyocyte protocol. Alternatively, additional mesoderm markers such as Foxc1 or Foxc2 could have also been looked at to further demonstrate mesoderm formation (159).

I next sought to see if the cardiac lineage could be further obtained from the mesodermal cells produced with the use of BMP4. To monitor the effects of the assayed BMP4 titration concentrations, a time course differentiation was done assessing the Isl1 and Nkx2.5 genes as these markers both serve to identify the cardiac population at early stages of differentiation (59). From this initial experiment, it was clearly demonstrated that the BMP4 addition in conjunction with CHIR treatment resulted in the highest expressions of both Isl1 and Nkx2.5. While the concentrations of BMP4 increased Isl1 and Nkx2.5 mRNA levels in a dose-dependent manner, the addition of 20 or 50ng/mL of BMP4 (with the combination of CHIR) lead to the most prominent up-regulations. As expected the vehicle control lacking the CHIR and BMP4 additions resulted in essentially no expression of the genes, while the addition of CHIR by itself resulted in an only modest increase (Figure 7, 8) over the control. This was anticipated: in the case of the vehicle control, the undifferentiated cells are not driven to the mesodermal lineage and thus are not capable of going into the cardiac lineage either. Initial observations with the QPCR results also demonstrated that BMP4 addition by itself was able to induce Nkx2.5 expression on day 4 as well, though not as high of a degree as within the presence of CHIR+BMP4. This could be explained as BMP4 is shown to regulate Nkx2.5 levels, in addition to inducing mesoderm levels (123). As the main goal was to improve on the standard hanging drops protocol used, I once again wanted to see if there were improvements in the transcript of the early cardiac progenitor genes using the two best

concentrations of BMP4. On day 4, it was established that Nkx2.5 was up-regulated to statistically significant levels compared to the hanging drops protocol on the same day, while for Isl1, there was a strong trend in increase with the new protocol (Figure 8). While Isl1 was not significantly up-regulated, there was still a high induction of the gene over day 0. What would be interesting to observe is if these differentiating cells are expressing Isl1 and Nkx2.5 concurrently in the same cell. This would be beneficial to see as the expression of both of these factors is known to contribute to the majority of the cardiomyocytes population, with some contribution to the smooth muscle and endothelium lineages as well (157, 158, 58). To observe this, the cells could be probed with antibodies specific to both Isl1 and Nkx2.5 and visualized with immunofluorescence. With comparison again to the Skerjanc's lab previously shown results, the CHIR+BMP4 combination led to a greater induction of Nkx2.5 gene expression on day 4 of differentiation compared to that of the BMP4/INHBA or CHIR alone which resulted in little or no increase over day 0 expression levels, however that data was presented on day 6 of differentiation (93). Work with mESCs and the BMP4 inhibitor dorsomorphin also yielded less Nkx2.5 transcript levels, approximately 10 fold, as compared to the new protocol (119).

Immunofluorescence was also performed for Nkx2.5 expression. Consistent with the QPCR expression, the vehicle control resulted in no observable Nkx2.5 signal. What was exciting however, were the CHIR, BMP4, and CHIR+BMP4 treated samples. With individually CHIR or BMP4 treated samples, the results from the immunofluorescence were similar. Both conditions resulted in observable Nkx2.5 that was predominately cytoplasmic. On the other hand, the treatment of CHIR+BMP4 resulted in a majority of the Nkx2.5 signalling to be nuclear (Figure 9). As Nkx2.5 is a transcription factor this brings up the

potential for a mechanism. Perhaps, the collective addition of CHIR+BMP4 results in localization of Nkx2.5 to the nucleus whereby it can act in increasing cardiomyogenesis. To determine the importance that Nkx2.5 has on the differentiation protocol, there could be several next steps that could be taken. It would be of value to see what would happen if the Nkx2.5 could be excluded from or targeted to the nucleus on a controlled basis. One strategy that could be employed would be to mutate the nuclear localization sequence of Nkx2.5. If the signal is mutated, then Nkx2.5 is not be able to localize to the nucleus, and in this case, it would be valuable to compare the transcript and protein levels of Nkx2.5 and other downstream targets important in cardiomyogenesis like GATA4 and Nppa (64, 159). If cardiac genes are not up-regulated, one might expect to see results of those similar to the individual CHIR or BMP4 conditions. In those situations, the majority of the Nkx2.5 is cytoplasmic and the Nkx2.5 transcript levels are lower than the levels observed for the CHIR+BMP4 nuclear localized Nkx2.5. A similar experiment could be conducted with the binding of Mypt1 (myosin phosphatase targeting subunit 1) and Nkx2.5. If the affinity Nkx2.5 has on binding Mypt1 can be increased by a certain molecule or if an over-expression of Mypt1 can be induced, then the Nkx2.5 proteins could be sequestered with the Mypt1 which would prevent the translocation of Nkx2.5 into the nucleus and would not allow it to act as a transcriptional factor (145). On the opposite end of the spectrum, the Nkx2.5 protein can also be maintained to nuclear localization. While the use of leptomycin has been used to block nuclear export, this compound is non-selective and blocks nuclear export of all proteins, not just Nkx2.5 (160, 161). There is however, a CSX-Nkx2.5 associated LIM protein (Cal) that interacts with Nkx2.5 via the double zinc finger LIM domain which is thought to play a role in transporting Nkx2.5 to the nucleus (165). As

increased levels of intracellular Ca<sup>2+</sup> causes nuclear localization of Cal, this treatment could be used to increase the nuclear localization of Nkx2.5 via the Cal shuttling (165). In this instance, I would expect to see other cardiac genes and downstream targets of Nkx2.5 to be up-regulated. There is a possible mechanism for the nuclearly localized Nkx2.5 protein under the CHIR+BMP4 condition; the initial induction of Wnt results with the activation of GATA4 (98), and then the addition of BMP4 activates SMAD1/4 which allows the two cofactors to activate Nkx2.5 as previously noted (166).

The observed increase in Nkx2.5 was predicted as it is a known target of BMP4 signalling, but I also wanted to observe if there were similar changes in the other necessary cardiac transcription factors, such as GATA4. The later time points, days 4 and 6 with CHIR+BMP4 appeared to have the highest level of GATA4 protein as determined by the immunoblot analysis (Figure 10). This is in line with expectations as undifferentiated cells should not express GATA4. It was interesting that CHIR treatment resulted in GATA4 being detected on day 2 of differentiation. This observation is a surprise; the *Isl1* and *Nkx2.5* transcript levels observed only started to get visibly up-regulated at day 4 of differentiation, and the CHIR treatment by itself did not result in any significant increase with regards to *Isl1* and *Nkx2.5*. This is somewhat unexpected as previous studies demonstrate that *Nkx2.5* directly controls GATA4 expression. Knock-down of *Nkx2.5* results in decreased GATA4 protein levels. The *Nkx2.5* and GATA4 are directly related as an increase in antisense RNA targeting *Nkx2.5* results in decreased GATA4 (71). Additionally, the activation of Wnt signalling decreases the amount of GATA4 observed and GATA4 is in fact a downstream target of the Wnt signalling (97, 164). However additional research demonstrates that GATA4, *Nkx2.5* and *Mef2C* in fact have a regulatory loop and regulate each other (168).

Furthermore, Nkx2.5 is proven to be under the control of the cooperative functions of both GATA4 and SMAD1/4, the primary transducer of the BMP signalling pathway (166). This very well could be the situation here; GATA4 may be expressed first and then with the SMAD1/4 (activated by the addition of BMP4 from days 2-4) is able to induce Nkx2.5 expression which is seen highest on day 4 of differentiation.

In agreement with the QPCR results observed for the cardiac progenitor markers, the CHIR+BMP4 combination (using 20ng/mL of BMP4) initially looked to have led to the most cardiomyocytes as viewed by the MHC staining (Figure 11). The addition of BMP4 and CHIR individually, also resulted in some cardiomyocytes being formed, especially in the case of the CHIR alone condition, which provides further claim that it is indeed able to induce GATA4 by itself. While the number of MHC positive cells were quantified, the results obtained were relatively poor. Although the CHIR+BMP4 efficiency is statistically greater than the vehicle or BMP4 individually, and showed a positive trend as compared to the CHIR treated count, it is not an improvement on the older protocols. A reason for the lack of efficient cardiomyogenic differentiation with just the CHIR+BMP4 pathway could have been due to overly-active Wnt signalling. The levels of Wnt may have still been high when the BMP4 treatment began, and at this stage, the Wnt signalling would decrease cardiomyogenesis.

I next sought to further continue to manipulate the Wnt and BMP4 pathways by trying to inhibit them at specific times in hopes of improving the cardiomyogenic differentiation efficiency. As Wnt was needed to be inhibited after the mesoderm induction and increased cardiac gene expression, it was decided to use IWP4 to block Wnt from day 4 to day 6. This was chosen due to the reason that Isl1, Nkx2.5 and GATA4, were starting to

become highly expressed thus marking the cardiac progenitor stage. In hESCs, a similar approach was used in the sense that CHIR was initially used, then, two days passed until the addition of IWP4 was performed (169). *Isl1* and *Nkx2.5* transcript levels were assessed (Figure 13), and there were no observable differences between CHIR+BMP4 and CHIR+BMP4+IWP4 conditions. By observing the immunofluorescence and transcript levels of MHC7, the cells conditioned with CHIR+BMP4+IWP4, and CHIR+IWP4 yielded no statistical increase in either the quantification of MHC positive cells or MHC7 transcript levels (Figures 12, 17). This was not expected as the inhibition of Wnt signalling is a critical component in an hESCs protocol that yields over 80% cardiomyocytes (170). One would expect at least a slight improvement in the efficiency. In the protocol derived from the research in hESCs, Wnt inhibition is applied 3 days after the treatment of cells with CHIR (169), which is similar to the timeframe that I used on the conducted research. One major difference however, is that the use of the IWP4 in the hESCs is done before the induction of *Isl1* (169) whereas on day 4, where I use the IWP4, *Isl1* is already highly expressed, but not to statistically significant level. It would be of value also to observe how effective the IWP4 is in inhibiting Wnt signalling. Ideally, different concentrations and time points of IWP4 addition should be done and then further monitored by QPCR and immunoblot techniques specifically assaying for  $\beta$ -catenin or TCF LEF factors.

I also decided to use testosterone to see if an increase in cardiomyocytes would result by its addition. When used in my monolayer protocol, initially there appeared to be a slightly increase of MHC staining in the CHIR+BMP4+testosterone and the CHIR+BMP4+IWP4+testosterone combinations, however, the quantification of these conditions, amongst the others with testosterone addition, did not result in any statistical

increase in cardiomyocytes (Figures 15, 17). While this was not expected, perhaps the differences are in the cardiomyocytes themselves. As testosterone is known to cause hypertrophy in cardiomyocytes, it could be that the cardiomyocytes have increased in cell area, as is noted by the work of Altamirano *et al.*, whereby the use of a testosterone for just 48 hours results in a 26% increase in the cell surface area of cardiomyocytes (171). The surface area measurements were not performed due to difficulties in measuring confluent microscope slides, however, that particular experiment would be interesting to look at in the future.

While it was disappointing that the combinations of CHIR, BMP4, IWP4 and testosterone were not able to sufficiently generate a large percentage of cardiomyocytes, one further class of small molecule was used to see if it could help out with the low differentiation efficiency. Even though BMP4 has been shown to be necessary for obtaining the cardiomyocyte lineage, it is repressed in already differentiating at the cardiomyocyte time point (105). For this reason, I used the BMP inhibitor noggin, from days 4-6 as the cardiac progenitor genes are prominently present on day 4 (Figure 16). The concentrations used were based on a previous finding showing the impact of noggin on cardiomyogenesis in mESCs (120). The initial experiment I performed resulted in poor differentiation of the mESCs into the cardiac lineage, and in fact, all of tested concentrations of the noggin actually appeared to inhibit cardiomyogenesis as noted to a reduced amount of MHC-positive cells observed. Consequently, the use of noggin was not further evaluated after the preliminary results. One thing to consider however is that BMP inhibition early on in differentiation did seem to improve cardiomyogenesis (153), so it is possible that noggin followed by CHIR treatment would then be the scenario to test.

Despite the difficulties associated with the new protocol, one other area of importance in differentiation efficiency was the actual duration of the differentiation. While my protocol focused until the 6<sup>th</sup> day of differentiation, the majority of the protocols used focused on termination of the protocol at a later time point even though in the hanging drops protocol, cardiomyocytes have been seen as early as day 5 of differentiation in the Skerjanc lab. Furthermore, differentiation protocols using small molecules to enhance the efficiency of differentiation have protocols that go well past the day 6 mark I have studied. Our lab's assessment of testosterone results in mESCs being differentiated until day 10 as was the case with the group showing the importance of dorsomorphin (134, 118). In other work with mESC differentiation with Wnt modulation, the differentiations are carried out until day 14 (172). To observe if extending the differentiation period had an effect on the quality of differentiation, a preliminary experiment was conducted whereby the MHC staining was assessed on day 5, and day 9, in addition to the day 6 I have been testing (Figure 18). From the n=1 experimental run, there was not an observable difference in the number of cardiomyocyte at first glance from the day 6 samples. Despite that, the cardiomyocytes did appear to have a more defined shape and a higher level of physical definition when observed through the microscope. Additionally, while the red cy3 channel remained on the same setting, the day 9 cardiomyocytes also did have a brighter immunofluorescence signal. Another interesting observation was the fact that even day 5 staining revealed what appeared to be a competitive number of cardiomyocytes in comparison to day 6, and day 9. While the protocol I have tried to develop has not demonstrated a high percentage of differentiation efficiency, it is possible, that there could be an accelerated phenotype. It would be useful to see if cardiomyocytes are observed on day 4 or even earlier. Once the earliest observable

cardiomyocytes are detected, perhaps the temporal additions of CHIR and BMP4 modulating small molecules can be better optimised leading to a higher efficiency in differentiation.

The hanging drops protocol, as well as the majority of other differentiation protocols have also had optimized cell numbers and densities for obtaining cardiomyocytes. With the struggles I have had with the efficiencies, I have also looked into the potential differences that may arise with the cellular densities. As the new protocol was developed for monolayer use, the initial plating density was varied. While 500K cells per 3.8 cm<sup>2</sup> was used (in 12 well dish diameter) initially, densities ranging from 100K, 300K, 500K, 750K, and 1 million cells/3.8 cm<sup>2</sup> were also tested (Figure 19). While only a single biological replicate was performed, from the MHC staining it was deduced that the 500K, (or even 300K cells/3.8 cm<sup>2</sup>) was the best option as this condition had the most MHC staining. Both tails of the extremes, the 100K and 1 million cells/3.8 cm<sup>2</sup> had the fewest cardiomyocytes observed. In fact, a previous protocol was used in our lab where 150K cells/3.8 cm<sup>2</sup> was tested and was compared to the 500K cells/3.8 cm<sup>2</sup> density that I used (Figures 19, 20). With the MHC quantification, there was a strong trend in a decrease in the number of MHC positive cells with the 150K cells/3.8 cm<sup>2</sup> condition as compared to the 500K cells/3.8 cm<sup>2</sup> condition. Likewise, I decided to compare the Brachyury-T, Isl1 and Nkx2.5 genes as well which indicated that the lower cell density also led to significantly less expression of Brachyury-T on day 2 and significantly down-regulated Nkx2.5 expression of Nkx2.5 on day 4. Furthermore, there was also a strong trend in down-regulation with regards to the Isl1 transcript levels. This is not surprising given the importance regarding initial cell density and differentiation efficiency. In a study using various lines of hESCs, the optimal plating density used for cardiomyocyte differentiation is 500K cells/3.8 cm<sup>2</sup> (12 well dish) and is

determined to be optimal, though different cell lines required different densities (169). Despite the changes, literature does suggest that a higher cell plating density, up to certain point, does indeed favour a higher percentage of differentiation in other stem cells as well. For instance, higher cell density is shown to be important in the umbilical cord stem cell osteodifferentiation (173).

Considering that the overall goal of my research was to develop a more efficient protocol for differentiating mESCs in cardiomyocytes than the current hanging drops protocol used, the best differentiation percentage that I obtained was around 9%, which was not a major improvement on the 5% previously observed in our lab with the use of mESCs (145). In fact, what I have obtained from the protocol was less than the higher percentage differentiations obtained with the hanging drops protocols using some variations (around 30% cardiomyocytes for the BMP4/Activin A) and was highly reduced in efficiency as compared to the hESCs protocols where nearly 90% of cells were differentiated into the cardiac lineage (121,171,172). Looking at the differentiation, there are several areas that need to be further investigated. While the induction of the mesoderm from undifferentiated stem cells was successful, perhaps the additional quantification of the Brachyury-T staining should be performed. The necessity of deriving the mesoderm is essential for proper cardiomyogenesis, however, it is possible that there are some drawbacks with the mesoderm induction used in my protocol. One reason and explanation leading to the low cardiac differentiation efficiency just might be from the high levels of Brachyury-T and CHIR that defined the high levels of mesoderm induction. Wnt signalling results in increased Brachyury-T expression. However, Brachyury-T also has a positive impact on Wnt signalling as the Brachyury-T zebrafish orthologue Ntl, is proven to induce the Wnts that are

used in the canonical Wnt signalling (i.e. Wnt3a) helping establish mesodermal progenitors (176). It is possible that even though CHIR is added from days 0-2 to induce the mesoderm, and high Brachyury-T expression is observed on day 2, that the Brachyury-T expression remains at high enough levels until later points of differentiation and induces Wnt signalling enough to repress cardiomyogenesis. However, this would not explain why the addition of the Wnt inhibitor IWP4 did not enhance cardiomyogenesis. More likely, the difficulty with the efficiency of the differentiation of my protocol lies from the mesoderm to the cardiac progenitor stager, and/or the cardiac progenitor to the terminally differentiated cardiomyocyte stage. It would be advantageous to monitor the expression levels of Tbx5, Mef2C, in addition to GATA4. These three factors are needed in proper stoichiometric coefficients to obtain proper and efficient cardiomyogenesis in reprogrammed fibroblasts (177). Another important variable to consider would be the state of the chromatin during the differentiation. For instance, an increase in Nkx2.5 is attributed to the down regulation of HDAC1 via Wnt signalling (178). Indeed, it would be worthwhile to see if this occurs during the differentiation protocol as I would expect to see since Wnt is being induced by CHIR. While the general roles of Wnt signalling has been mentioned in a number of situations (i.e. Wnt is needed for ESCs to mesoderm conversion but is has to be repressed in mesoderm to cardiomyocyte differentiation), it is important to consider that Wnt signalling is necessary for cardiomyocytes to increase proliferation (179). Considering this piece of information, an alternate approach to yield a high number of cardiomyocytes could be to isolate the MHC positive cells after the CHIR+BMP4 treatment by a method such as FACS and condition them with media and Wnt to induce further cardiomyocyte proliferation.

Other factors that may have impacted the differentiation include the media and matrigel used. The serum free media dubbed E6, was a DMEM/F12 based media with six factors previously used to success in the Skerjanc's lab serum-free based skeletal muscle protocol. One of the components in the media, insulin, was one shown to have an impact on cardiac differentiation. As a matter of fact, insulin is shown to have a dose dependent relation in down-regulating both the number of beating embryoid bodies as well as Nkx2.5 and MHC genes in cardiac differentiation in hESCs (180). It is thought that insulin inhibits cardiomyogenesis in the early stages by decreasing the expression of mesoderm expression (decreased Brachyury-T), and cardiac mesoderm (GATA4 and Nkx2.5) and is capable of actually redirecting the lineage from cardiac mesoderm to the neuroectoderm lineage and thought to be mediated by the IGF-1R, the Insulin receptor (181). It might be useful to observe if neuroectoderm markers such as NF160 are expressed during the differentiation (181). Additional research demonstrates that insulin does in fact inhibit cardiomyogenesis when the cardiomyogenesis is initiated with BMP4 and or Activin A treatment, with one hypothesis being that insulin causes the GSK3 $\beta$  to be phosphorylated thus leading to increased Wnt signalling (182). This proposed hypothesis is observed with the CHIR+BMP4 results obtained in the research I have performed. When treated with CHIR, the insulin and CHIR can both activate Wnt signalling leading to increased mesoderm formation while the addition of BMP4 does not induce cardiomyogenesis based on the fact that insulin is still used in the media, and is thus continuing to activate the Wnt signalling repressing cardiomyogenesis via blocking the expressions of GATA4 and Nkx2.5 (183). Nonetheless, this contradicts the fact that insulin also decreases mesoderm levels as well as the observation that the modulation of Wnt signalling with GSK3 $\beta$  and Wnt inhibitors are not

affected by the insulin (182). If this is the case, then the protocol I am developing should have at least showcased some improvement in cardiomyocyte number with the CHIR+IWP4 combination. The use of BMP4 in a different manner could be another route to follow for improvements in cardiomyocyte number. Increased efficiency of differentiation is also confirmed when cardiac progenitors positive for *Isl1* are treated with BMP4 for an extended period of time (6 days at 25ng/mL) (184). This results in nearly a two-fold increase in cardiac troponin T expression. Consequently, it is possible that increasing the duration of BMP4 exposure to the mESCs would increase the cardiomyogenic efficiency as the preliminary results also seem to favor longer differentiation times for more cardiac muscle (Figure 18). The monolayer protocol was set up with cells being cultured on matrigel coated TC dishes. The matrigel used might have also had a detrimental impact on the cardiomyogenesis. With the comparison of matrigel, SNL mouse fibroblasts layer, and MEF feeder layers, it is demonstrated that the matrigel results in the least efficient cardiomyogenesis, and demonstrates significant numbers of cells committing to the neural lineage (185). However, there were limitations in this study as there was variability in the cell lines and the media being utilised. Even though the matrigel used was growth factor depleted, the matrigel used in my study contains growth factors, including ones which have an impact on differentiation such as TGF $\beta$  and IGF-1. That being said, the levels of these growth factors should be low, but an experiment that can be proposed would be to test out matrigel depleted of growth factors and compare the differentiation with the matrigel I used. Ideally, additional feeder layers should be tested as well and the one with the best cardiomyogenic differentiation would be considered for use in the new protocol, however, this is not the priority since feeder layers can also be a source of contamination (186).

Preceding experiments by the Skerjanc's lab have also used the familiar RPMI media with CHIR and IWP4 however, no substantial increase in cardiomyogenesis was observed (unpublished data). I have also attempted a protocol with CHIR being used in the hanging drops. Two experimental conditions were used; hanging drops were done with the serum based DMEM and CHIR and with the serum free based E6 and CHIR, along with the control, which was just the hanging drops without CHIR. After the initial 2 days of differentiation, it was observed that the E6 based experiment with CHIR yielded no viable cells. The experiment with CHIR and serum based media did yield embryoid bodies and the differentiation was continued either with or without the addition of the BMP4. On day 6, cells were fixed and stained for MHC and comparing to the control hanging drops method, no visible differences in the amount of cardiomyocytes were observed. Despite these being only preliminary results, this protocol was abandoned and no additional testing was performed. Interestingly, there is data to support the notion that the embryoid body hanging drops method is superior to the monolayer based method of differentiation and that serum restricted the efficiency of cardiomyogenesis (187). So the proper approach might just be to use the serum-free based method but using a more defined media for differentiation. Despite that scenario, serum, with the addition of BMP4 may have an effect in cardiomyogenesis as evidenced with the original hanging drops protocol. Using a serum free approach just with BMP4 or vehicle results in no cardiomyogenic differentiation; however, the cardiomyocytes were present when serum is added back to the differentiation media (118).

Notwithstanding the shortcomings of the differentiation efficiency obtained, an alternative approach could be to see if in fact the new protocol results in differentiation into another cardiac lineage. The increase in *Isl1* and *Nkx2.5* levels are a strong starting point in

the search for an improved protocol. Isl1 cardiac progenitors can make up almost two-thirds of the heart, but that being said, it is possible that cell I derived from the protocol are not cardiomyocytes, as this is only one of the cell types Isl1 progenitor cells give rise to with the other being smooth muscle cells, and endothelial cells (181, 157, 158). Additional experiments can be performed to see if maybe these other cardiac cell types are produced. This could be achieved with QPCR, looking specifically at endothelial or smooth muscle marker. Similarly, the protein levels of these markers could also be assayed using Western blot or immunofluorescence. If the presence of these unwanted differentiation lineages is obtained and confirmed, the next steps would be to see how they can be repressed and hopefully with that knowledge, it would lead to a more efficient protocol for generating cardiomyocytes.

In conclusion, mESCs were able to be differentiated into cardiomyocytes with the use of small molecules first with treatment of a GSK3 $\beta$  inhibitor CHIR and then with BMP4, however, the efficiency of differentiation was not higher than the established method I sought to replace. Nevertheless, the results obtained with the small molecule manipulators of the Wnt and BMP4 signalling pathways, in addition to the testosterone provided a good foundation for further experimentation which could lead to an improved cardiomyogenic differentiation efficiency in mESCs.

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## 6. Appendix.

### 6.1 Appendix A. List of the primers used to assess genes during QPCR experiments

Target Gene	Primer Direction	Sequence
$\beta$ -actin	Forward	AAATCGTGCGTGACATCAAA
	Reverse	AAGGAAGGCTGGAAAAGAGC
18s	Forward	CGCCGCTAGAGGTGAAATC
	Reverse	CCAGTCGGCATCGTTTATGG
Brachyury-T	Forward	CTGGACTTCGTGACGGCTG
	Reverse	TGACTTTGCTGAAAGACACAGG
Isl1	Forward	AAGCAGCTGCACACCTTGC
	Reverse	CATGGGAGTTCCTGTCATCC
Nkx2.5	Forward	AAGCAACAGCGGTACCTGTC
	Reverse	GCTGTGCTTGCACCTTGTAG
MHC7	Forward	ACTGTCAACACTAAGAGGGTCA
	Reverse	TTGGATGATTTGATCTTCCAGGG

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### **6.3. Appendix C. CV Curriculum Vitae**

#### **Personal/Contact information**

Name: Neven Bosiljcic

#### **Academics**

2013 – present Masters of Science – Biochemistry (Supervisor: Dr. Ilona Skerjanc),  
University of Ottawa, Canada K1N 6N5

2008 - 2013 Honor's Bachelor of Science, Specialization in Biochemistry, University of  
Ottawa (Summa Cum Laude), Ottawa, Ontario, Canada K1N 6N5

2004 – 2008 High School Diploma, Earl of March Secondary School, Kanata, Ontario,  
Canada K2K 2B6

#### **Awards**

2014 – 2015 – CIHR Banting and Best Master's Scholarship

2014 – 2015 – OGS scholarship

2014 – 2015 – University of Ottawa Excellence Scholarship

2013 – present - University of Ottawa Admission Scholarship (Graduate level)

2008 – 2012 University of Ottawa Registrar Special Scholarship  
(with renewal)

2008 – 2012 University of Ottawa Admission Scholarship  
(with renewal)

2008 – present Dean's Honour List

#### **Work/Volunteer Experience**

2014 – present – Volunteer – Geriatric rehabilitation Elisabeth Bruyere Hospital, Ottawa,  
Ontario

2014, 2015 – Teaching assistant for BCH2333 Biochemistry Laboratory 1, University of  
Ottawa, Ottawa, Ontario

2013, 2014 – Teaching assistant for BCH4300 – selected topics in biochemistry, University  
of Ottawa, Ottawa, Ontario

2012, 2013 - Summer Student, Dr. Iлона Skerjanc lab, University of Ottawa, Department of Biochemistry, Microbiology, Immunology, Ottawa, Ontario

2011 – 2012 Tutor for Organic Chemistry II (CHM2120) and Introduction to Biochemistry (BCH2333), Ottawa, Ontario

2010 –present - Volunteer – Various activities, Canadian Aviation and Space Museum, Canadian Science and Technology Museum, and Canadian Agricultural Museum, Ottawa, Ontario

2010 - Volunteer - Guitar Raffle, Ottawa Bluesfest, Ottawa, Ontario

2010 - Volunteer - Information Desk, Ottawa General Hospital, Civic Campus, Ottawa, Ontario

2006-2008 - Volunteer - Children's Crafts, Ottawa Public Library, Beaverbrook Branch, Kanata, Ontario

2006 - Volunteer - Ticket sales, High School Music Festival, Earl of March S.S., Kanata, Ontario

### **Publications (refereed articles)**

Voronova, A., Coyne, E., Al Madhoun, A., Fair, J. V., **Bosiljic, N.**, St-Louis, C., Li, G., Thurig, S., Wallace, V. A., Wiper-Bergron, N., Skerjanc, I. S. (2013). Hedgehog Signalling Regulates MyoD Expression and Activity. *The Journal of Biological Chemistry*. **288**(6), 4389-404.