Monolayer differentiation of human induced Pluripotent Stem Cells (hiPSC) into cardiomyocytes

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Biotechnology

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Abstract

The following work aims at the establishment of a robust protocol for the monolayer differentiation of human induced pluripotent stem cells (hiPSC) into cardiomyocytes in xeno- and serum-free conditions for future large scale production of human cardiomyocytes to be potentially used in further drug screening and Regenerative Medicine applications. Three hiPSC cell lines were characterized to ensure their stability on the expression of pluripotency markers when cultured on matrigel and mTeSR1 medium prior commitment into the cardiac lineage. The efficiency of several recently published protocols of monolayer cardiac differentiation of hiPSC was evaluated in different hiPS cell lines and the optimal conditions to induce cardiac differentiation based on the addition of growth factors (Activin A and BMP4) and/or small molecules (CHIR99021 and IWP4) were studied. Quantification of direct-cardiac differentiation efficiency via growth factors addition in iLB-c1-30m-r12 cells showed low expression of late cardiac markers cTnT and α-actinin (less than 3%). However, cardiac differentiation via small molecule modulators of Wnt signaling proved to efficiency differentiate iPS-DF6-9-9T.B cells into cardiomyocytes, when adding 5 µm CHIR to the culture on day 0, showing up to 70% α-actinin+ cells after 18 days of differentiation. Additionally, the final yield of hiPSC-Cardiomyocytes produced based on the small molecule modulators of Wnt signaling protocol was increased by three fold by re-plating cells on the 6^{th} day of differentiation. When 10 ng/mL of VEFG were added to the culture just on day 6 and then cells were re-plated on laminin, fibronectin and vitronectin, this method proved to efficiently produce an actively beating monolayer of cardiomyocytes throughout the entire well, expressing up to 73% α-actinin+ cells. The use of biochemically defined coatings such as laminin, fibronectin and vitronectin as substrates for the cardiac commitment of hiPSC contributed to the establishment of a direct-cardiac differentiation protocol completely free of xenogeneic contaminants. This in combination with the use of small molecules to direct cardiac differentiation of hiPSC turns this culture system much more cost-effective compared to growth factors-based protocols which gives this method a unique potential for the large scale production of human cardiomyocytes for future clinical purposes.

Resumo

O trabalho experimental desenvolvido no âmbito desta tese teve como objectivo o estabelecimento de uma plataforma quimicamente definida, livre de contaminantes xenogénicos e robusta para permitir a diferenciação cardíaca de hiPSC em monocamada, para futura produção em larga escala de cardiomiócitos humanos e potencial uso no *screening* de novos fármacos e em Medicina Regenerativa. Três linhas de hiPSC (iLB-c1-30m-r12, iPS-DF6-9-9T.B e iPS-DF19-9-11T.H) foram caracterizadas para assegurar a estabilidade da expressão de marcadores de pluripotência antes dos estudos de diferenciação cardíaca. A eficiência de vários protocolos recentemente publicados para a diferenciação cardíaca de hiPSC foi avaliada em diferentes linhas e as condições óptimas para indução de comprometimento cardíaco com base na adição de factores de crescimento (Activina A e BMP4) e/ou pequenas moléculas (CHIR99021 e IWP4) foram estudadas. A quantificação da eficiência de diferenciação cardíaca via adição de factores de crescimento em células iLB-c1-30m-r12 revelou baixa expressão dos marcadores cardíacos tardios cTnT e α-actinina (inferior a 3%). Contudo, diferenciação cardíaca via pequenas moléculas modeladoras da sinalização Wnt demonstrou eficiente diferenciação de células iPS-DF6-9-9T.B em cardiomiócitos, ao adicionar 5 µm CHIR à cultura no dia 0, revelando até 70% de células α-actinin+ após 18 dias de diferenciação. Adicionalmente, o rendimento final de cardiomiócitos produzidos a partir de hiPSC foi aumentando em cerca de três vezes através de re-plaqueamento de células diferenciadas ao dia 6 em laminina, vitronectina e fibronectina. A adição de 10 ng/mL de VEGF à cultura apenas no dia 6 de diferenciação, às células re-plaqueadas demonstrou produção eficaz de uma monocamada de cardiomiócitos com batimento activo, expressando até 73% de células α-actinin+. O uso de substractos bioquimicamente definidos como a laminna, fibronectina e vitronectina para o comprometimento cardiaco de hiPSC contribui para o estabelecimento de um protocolo de diferenciação cardíaca directa inteiramente livre de contaminantes xenogénicos. Este último em combinação com o uso de pequenas moléculas para a diferenciação cardíaca de hiPSC torna este sistema de cultura muito mais eficiente a nível de custo comparando com protocolos com base na adição de factores de crescimento, o que confere a este método um potencial único para a produção em larga escala de cardiomiócitos humanos para futuras aplicações clínicas.

Keywords

Human induced Pluripotent Stem Cells
Cardiac commitment
Monolayer differentiation
Growth factors
Small molecules
Wnt signaling modulation
Serum-free culture

Palavras-Chave

Células Estaminais Pluripotentes induzidas humanas
Comprometimento cardíaco
Diferenciação em monocamada
Factores de crescimento
Pequenas moléculas
Modelação da via Wnt
Cultura sem soro

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List of Abbreviations

AA	Activin A
ALK	Activin receptor-Like Kinase
ALS	Amyotrophic Lateral Sclerosis
BMP	Bone Morphogenic Protein
BSA	Bovine Serum Albumin
DAPI	4',6-Diamidino-2-Phenylindole
DMEM	Dulbecco's Modified Eagle's Medium
EBs	Embryoid Bodies
ECM	Extracellular Matrix
EDTA	Ethylenediamine Tetraacetic Acid
EmGFP	Emeral Green Fluorescent Protein
ESCs	Embryonic Stem Cells
FBS	Fetal Bovine Serum
FGF	Fibroblast Growth Factor
hESCs	human Embryonic Stem Cells
hiPSC	human induced Pluripotent Stem Cells
hPSC	human Pluripotent Stem Cells
iPSC	induced Pluripotent Stem Cells
IWP	inhibitor of Wnt production
KO-DMEM/SR	KnockOut DMEM complete medium
KO-SR	KnockOut Serum Replacement
MEFs	Mouse Embryonic Fibroblasts
MLC	myosin light chain
NGS	Normal Goat Serum
PAS	Peptide-Acrylate Surfaces
PBS	Phosphate Buffered Saline
PFA	Paraformaldehyde
PMEDSAH	Poly[2-(methacryloyloxy)ethyl dimethyl-(3-sulfopropyl)ammonium hydroxide]
PSCs	Pluripotent Stem Cells
ROCK	Rho-associated Kinase
SIP1	Smad-Interacting Protein 1
SMAD	Smad and mothers against decapentaplegic related protein
SSEA	Stage Specific Embryonic Antigens
TGF-β	Transforming Growth Factor beta
TRA	Tumour Rejection Antigens
WNT	Wingless-type mmtv integration site family members

I. Aim of Studies

Until very recently, the induction of cardiac differentiation from hPSC relied on embryoid body (EB) formation, however, this culture method is highly variable between hiPSC lines and is associated with relatively poor efficiencies (less than 10%). New approaches are now emerging in the field and one of the most promising starts with cells cultured as monolayers. This culture method takes in advantage a relatively uniform monolayer of cells without the diffusional barriers present in EBs. In this way, the application of growth factors and other molecules can theoretically be more controlled, homogeneous and reproducible. Current protocols for monolayer differentiation of hiPSC into cardiomyocytes are based in the addition of growth factors, Activin A and BMP4, or in the addition of small molecule modulators of Wnt signaling. The few existing protocols for cardiomyocyte generation were published very recently and therefore, there is still the need to fully optimize the conditions for the generation of functional and mature cardiomyocytes for future applications. Additionally, significant differences can occur between different hiPSC lines depending on the tissue of origin, which turns these protocols to be highly variable between cell lines.

The present work aims to establish and optimize a reproducible culture system for robust monolayer differentiation of hiPSC into cardiomyocytes under xeno- and serum-free conditions, in order to perform the initial step towards the establishment of cultures under GMP conditions that could be used for clinical applications. For that purpose, three hiPSC lines available at the Stem Cell Bioengineering Lab (IBB-IST) were characterized to ensure their stability prior cardiac commitment. Monolayer direct cardiac differentiation was induced by two main approaches: via growth factors (Activin A/BMP4) or small molecules (CHIR/IWP4). Different concentrations of these molecules were tested to efficiently induce cardiac differentiation in different hiPSC lines. Finally, the increase on the yield of obtained cardiomyocytes was attempted by taking in advantage the proliferative capacity of cardiac progenitor cells.

II. Introduction

II.1. The Differentiation Potential of Stem Cells

II.1.1. Basic concepts and definitions

Stem cells are characterized by two important properties: they are unspecialized cells able to self-renew, which means they can generate a daughter cell with identical potential; and they can differentiate into specific functional phenotypes, under specific physiologic or experimental conditions[1]. According to development potentials, stem cells can be classified in four main different categories: totipotent, pluripotent, multipotent and unipotent cells (figure II-1)[2].

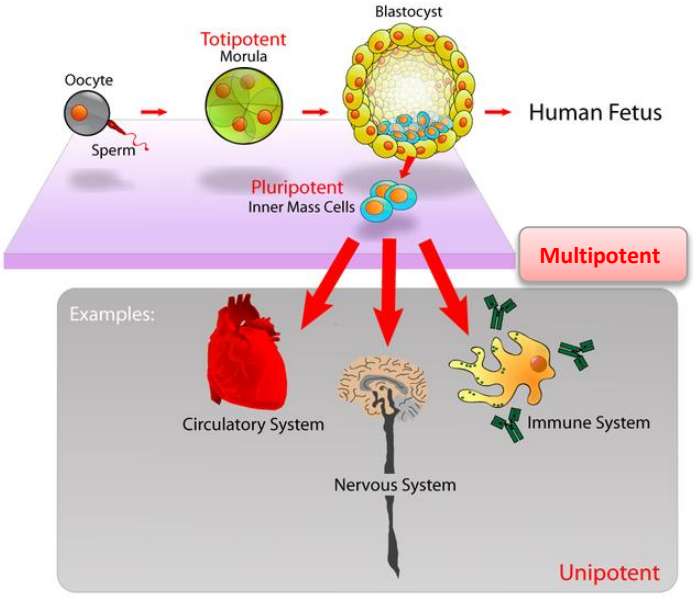

Figure II-1 – Plasticity and differentiation potential of stem cells in the development of the three embryonic germ layers (ectoderm, mesoderm and endoderm).[3]

Totipotent- the fertilized egg is said to be totipotent because it has the potential to generate all the cells and tissues that make up an embryo and also support its development in utero. The fertilized egg divides and differentiates to produce a mature organism consisting of more than 200 types of cells[2]. These include nerve cells (neurons), muscle cells (myocytes), skin cells (epithelial), blood cells (erythrocytes, monocytes, lymphocytes, etc.), bone cells (osteocytes), cartilage cells (chondrocytes), among others. Other cells that are generated from the totipotent cell include the extra-embryonic tissues, placenta and umbilical cord, which are essential for embryonic development but are not incorporated into the body of the embryo[2].

Pluripotent- most scientists use the term pluripotent to describe stem cells that can give rise to cells derived from all three embryonic germ layers—mesoderm, endoderm and ectoderm —

with the exception of the extra-embryonic tissues[2]. On the fourth day of development, the embryo forms into two layers, an outer layer which will become the placenta and an inner cell mass which will form all tissues derived from the three germ layers (Table 1). These inner cells are not totipotent, but pluripotent. As these pluripotent stem cells continue to divide they become specialized cells that make up the human body[2].

Multipotent- These cells are less plastic and more differentiated stem cells. Multipotent stem cells are found in adult tissues and are essentially committed to produce specific cell types within a tissue. For example, hematopoietic stem cells can develop into erythrocytes, leucocytes and platelets but not other cells[4]. It is thought that they are in most body organs, where they replace diseased or aged cells. This process allows a steady state of self-renewal for the tissue, whenever the replacement of a specific cell type is required[5].

Unipotent- a term that is usually applied to a cell derived from a multipotent cell in adult organisms capable of differentiating in only one type of cell. Skin cells are one of the examples of unipotent stem cells. These cells must readily undergo rapid cell division to replace damaged cells[6].

Table II-1 – Embryonic Germ Layers From Which Differentiated Tissues Develop

Embryonic Germ Layer	Differentiated Tissue
Endoderm	Thymus, thyroid, parathyroid glands Larynx, trachea, lung Urinary bladder, vagina, urethra Gastrointestinal organs (liver, pancreas) Lining of the GI tract Lining of the respiratory tract
Mesoderm	Bone marrow (blood) Adrenal cortex Lymphatic tissue Skeletal, smooth, and cardiac muscle Connective tissues (including bone, cartilage) Urogenital system Heart and blood vessels (vascular system) Skin
Ectoderm	Neural tissue (neuroectoderm) Adrenal medulla Pituitary gland Connective tissue of the head and face Eyes Ears

II.1.2. Pluripotent Stem Cells versus Adult Stem Cells

In 1998, for the first time, investigators were able to isolate pluripotent stem cells from early human embryos and grow them in culture[7]. In the few years since this discovery, these stem cells proved to be capable of becoming almost all of the specialized cells of the body and, thus, may have the potential to generate replacement cells for a variety of tissues and organs, such as the heart, the pancreas, and the nervous system[2].

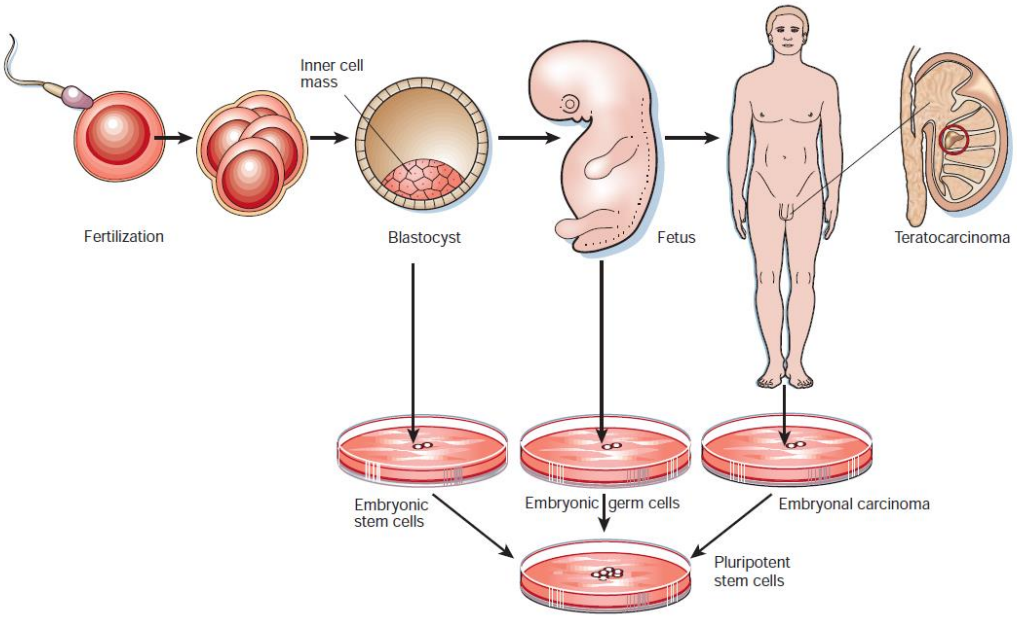

Figure II-2 – Origin of human pluripotent stem cells. Embryonic stem (ES) cells are derived from the inner cell mass of the pre-implantation embryo. Embryonic germ (EG) cells are derived from primordial germ cells (PGCs) isolated from the embryonic gonad. Embryonal carcinoma (EC) cells are derived from PGCs in the embryonic gonad but usually are detected as components of testicular tumours in the adult [8].

The first pluripotent stem cell was first recognized in teratocarcinomas (figure II-2). These are gonadal tumours containing a wide variety of tissues derived from the three germ layers that make up an embryo. These tumours contain several tissue types including cartilage, squamous epithelia, primitive neuroectoderm, muscle and bone. The differentiated cells of the tumour are formed from pluripotent **embryonic carcinoma (EC) cells** present in the tumour[8].

In contrast, the **embryonic stem cell** is defined by its origin— it is derived from the blastocyst stage, one of the earliest stages of the development of the embryo. More specifically, embryonic stem cells are derived from the inner cell mass (ICM) of the blastocyst at a stage before uterine wall implantation [9].

Finally, **embryonic germ (EG) cells** are derived from cultured PGCs, the same cells from which EC cells are derived. PGCs isolated directly from the embryonic gonad onto feeder

layers will, in the presence of serum and certain growth factors, form colonies of cells that seem morphologically indistinguishable from EC cells or ES cells grown on feeder layers[8].

New information is now emerging about a different class of stem cells - **adult stem cells**. An adult stem cell is an undifferentiated cell that is found in a differentiated tissue in the adult. It can renew itself for the lifetime of the organism and become specialized in different types of cells[1]. During the past decade, scientists have been discovering adult stem cells in tissues that were previously not thought to contain them. Sources of adult stem cells have been found in the bone marrow[10], cornea and retina of the eye[11], the dental pulp of the tooth[12], heart[13], skin[14], gastrointestinal tract and pancreas[15]. Their primary function is to maintain the steady-state of a tissue— called homeostasis—and, with certain limitations, to replace cells that die because of injury or disease[2]. Adult stem cells were found to be very rare in the body. For example, only an estimated 1 in 10,000 to 15,000 cells in the bone marrow is a hematopoietic stem cell (HSC)[16]. Adult stem cells are dispersed in tissues throughout the mature animal and behave very differently, depending on their local environment. For example, HSCs are constantly being generated in the bone marrow where they differentiate into mature types of blood cells[16]. In contrast, stem cells in the small intestine are stationary, and are physically separated from the mature cell types they generate[15]. Unlike embryonic stem cells, there is no evidence until now, of isolated adult stem cells that are capable of forming all cells of the body.

II.1.3. Induced Pluripotent Stem Cells (iPSC)

In 2006, Takahashi and Yamanaka generated the first iPSC lines by co-transduction with viruses expressing 24 different factors into mouse fibroblasts[17]. Subsequent experiments narrowed the required factors down to four: Oct4, Sox2, Klf4, and c-Myc, with Oct4 proving to be the most important. The expression of Oct4 is highly specific for pluripotent stem cells, whereas the other three factors are expressed in other cells (Sox2 in neural stem and progenitor cells; Klf4 in skin, stomach, intestine, and skeletal muscle; c-Myc is ubiquitously expressed).

In 2007, human iPS cells were first generated, by the transduction of either the same set of transcription factors (c-Myc, Oct4, Sox2, Klf4) or another set of transcription factors (Oct4, Sox2, Nanog, Lin28) into human fibroblasts[18]. These human iPS cells are similar to human embryonic stem cells in their morphology, gene expression, and the epigenetic status of pluripotent cell-specific genes and they can differentiate into the cell types of the three germ layers *in vitro* and *in vivo* (figure II-3). They express human embryonic stem cell-specific surface antigens, including SSEAs (Stage-Specific Embryonic Antigen), TRAs (Tumor Rejection Antigen), and NANOG protein[18]. In addition to fibroblasts, human iPS cells have been generated from hepatocytes, epithelial cells, pancreatic cells, neural stem cells and B-

lymphocytes. These data demonstrated that iPS cells can be generated not only from mouse, but also from human cell cultures for different types of cells with the same defined factors.

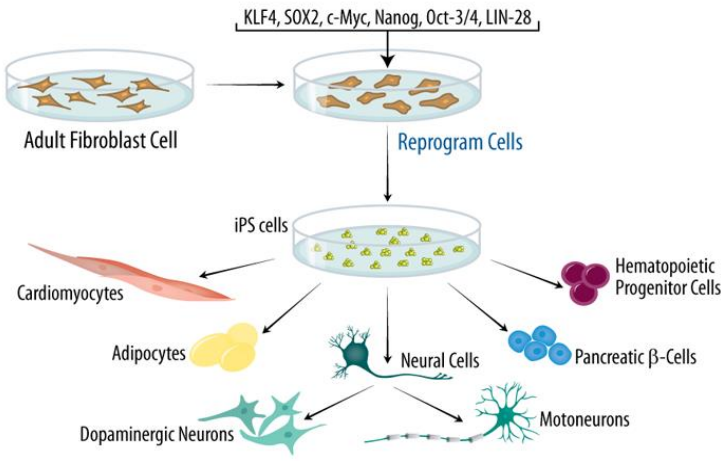

Figure II-3 – Directed Differentiation of iPS Cells. iPS cells are generated by reprogramming adult somatic cells. Expression of vectors encoding transcription factors associated with pluripotency triggers a gradual process of silencing markers of the differentiated phenotype and inducing markers of the pluripotent state in some cells. As pluripotent cells, iPS cells have the ability to generate all cell types found in the body [19].

II.1.3.1. Applications of iPSC technology

Organ transplantation among non-related individuals is complicated due to the limited availability of compatible tissues and the requirement for life-long treatment with immunosuppressive drugs. Human iPSC might potentially avoid these problems, as they could be differentiated into the desired cell types that would already be genetically matched with the patient. Recently, a number of studies have reported the successful generation of patient-specific iPSC lines from individuals with different diseases. In 2008, researchers succeeded in generating iPS cells from various patients, including those suffering from amyotrophic lateral sclerosis (ALS), Parkinson's disease, muscular dystrophy, and type I diabetes[20]. These cells can provide great opportunities to understand how these diseases develop, to screen effective drugs, and to predict both side effects and toxicity. One of the challenges in these kinds of *in vitro* applications is the establishment of methods to recapitulate the pathogenesis in somatic cells derived from the patient's own iPS cells. This might be more difficult in diseases like ALS, in which it takes 10 or more years before patients develop symptoms[1]. In addition, creation of iPS cell lines from patients with single-gene disorders allows experiments on disease phenotypes *in vitro*, and an opportunity to repair gene defects *ex vivo*[20].

II.1.3.2. Issues facing the use of iPSC

The iPS cell technology potentially could overcome two important obstacles associated with human embryonic stem cells: immune rejection after transplantation and ethical concerns regarding the use of human embryos[21]. However, the clinical application of iPS cells also faces many obstacles, some shared with embryonic stem cells. The first common obstacle is teratoma formation. Even a small number of undifferentiated cells can result in the formation of teratomas (germ cell tumors comprising several cell types), so a key goal is to induce differentiation of human iPS cells into the required cell type while leaving no undifferentiated cells behind[21]. Another key issue is the presence of transgenes in iPS cells. Most iPS cells are generated by transduction of somatic cells with retroviruses or lentiviruses carrying transgenes, which are integrated into the host cell genome[18]. Transgenes are silenced in iPS cells, but the reactivation of such transgenes, especially the transgene encoding c-Myc, could lead to tumorigenesis. Leaky expression of these transgenes may also inhibit complete iPS cell differentiation and maturation, leading to a greater risk of immature teratoma formation[22]. Also, with cells derived from diseased individuals for an autologous treatment, there is the concern that the genetic cause of the disease remains in the iPSC and will manifest in the same way[20]. However, recent studies proved that is possible to use a targeted gene correction approach to correct the genetic defect causing single-gene disorders in reprogrammed hiPSCs, this can be particularly difficult in the correction of the vast majority of diseases that are polygenic and show more complex genetic patterns[20].

Another problem that has not significantly changed since the beginning of iPSC research is the low efficiency of reprogramming, usually less than 0.1%[18]. Some researchers hypothesize that the addition of other factors would aid the reprogramming process and rate of iPSC production[23]. An alternate possibility for the reprogramming methods of iPSC is transdifferentiation. It may not always be necessary to reprogram cells to their most primitive pluripotent stem cell state, and instead reprogram one type of adult somatic tissue directly into a different type, bypassing the complete reprogramming and subsequent differentiation. For example, in theory fibroblasts that can be easily and safely obtained from a patient's skin could be converted into neurons or heart muscle cells without ever passing through a pluripotent stage. This would have advantages not only in saving time and resources but also for safety, once transdifferentiation theoretically overcomes the risk of tumorgenesis since cells never are pluripotent[21].

II.2. Heart: a target for Regenerative Medicine

II.2.1. Cardiogenesis: lessons from development biology

Pioneering work in lower vertebrate species such as frog and chick have given the blueprint for modern cardiac developmental biology by identifying the mesoderm as the germ layer responsible for mammalian cardiogenesis[24]. The heart forms soon after gastrulation in a specific region of the anterior mesoderm adjacent to the endoderm while blood cells arise from the posterior mesoderm[25]. The specification of the cardiogenic mesoderm cells is induced by the endoderm through BMP signaling. BMPs are expressed in the lateral endoderm along the entire anterior-posterior axis of the embryo (figure II-4). To restrict heart induction to the anterior mesodermal region, inhibitory signals prevent heart formation where it should not occur: the notochord secretes Noggin and Chordin, blocking BMP signaling in the center of the embryo, and Wnt proteins from the neural tube, inhibit heart formation but promote blood formation. The anterior endoderm, however, produces Wnt inhibitors such as Cerberus, Dickkopf and Crescent, which prevent Wnts from binding to their receptors[26]. In this way, cardiac precursor cells are specified in those places where BMPs and Wnt antagonists overlap.

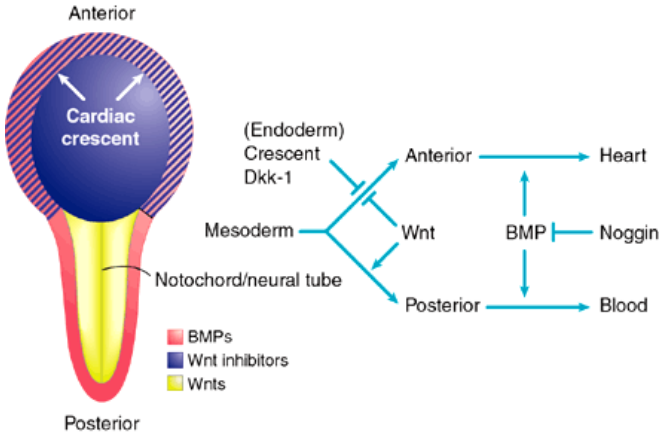

Figure II-4 – Model of inductive interactions involving BMP and Wnt pathways that form the boundaries of the cardiogenic mesoderm [26].

In order to prevent the heart cells from being re-specified, one of the first proteins made in the cardiac field is Mesp1, a transcription factor that activates the *Dickkopf* gene in these cells[25]. Precursors for heart-forming cells in the mesoderm transition from expressing Brachyury T, a T-box transcription factor, to expressing Mesp1 when they enter the pre-cardiac mesoderm stage of development. Mesp1+ cells comprise all cardiac progenitor cells and the expression of Mesp1 is turned off as they migrate away from the primitive streak[27]. During their migration, cardiac precursor cells expand rapidly to form the anterior and lateral plate mesoderm where they eventually generate a crescent-shaped structure called the cardiac crescent (figure II-5).

Mesp1+ cells can also give rise to derivatives of the paraxial mesoderm and skeletal muscle of the head and neck. It is at the cardiac crescent stage that heart precursor cells commit irreversibly to the cardiac lineage and become cardiac progenitor cells expressing Nkx2.5 and Isl-1. Genetic marking techniques have helped to identify that cells in the first heart field (marked by the expression of Tbx5 or the first wave of Nkx2.5) give rise to the left ventricle and portions of the right and left atrium, whereas cells in the second heart field (marked by the expression of Isl-1 or the second wave of Nkx2.5) contribute to the right ventricle, the outflow tract, and portions of both atria[28].

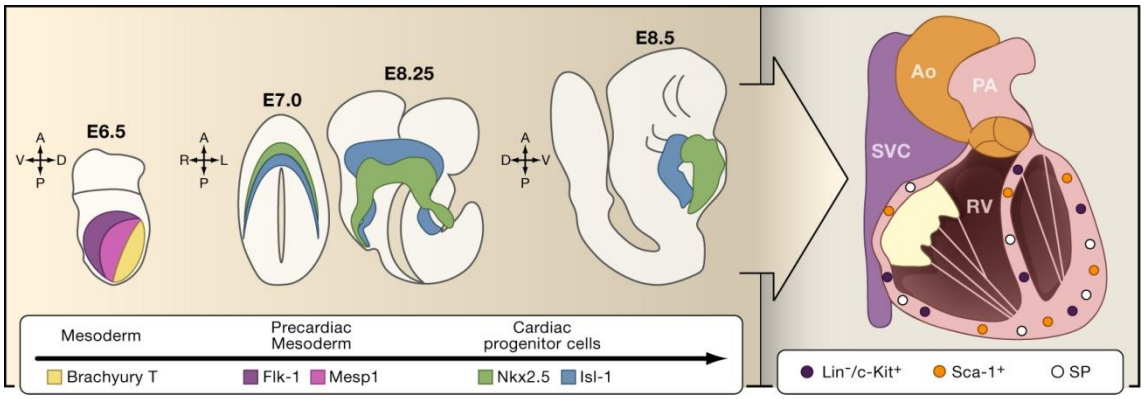

Figure II-5 – Embryonic development of heart-forming cells. Cells from the mesoderm of the embryo are marked by the expression of Brachyury T (yellow). As they transition into pre-cardiac mesoderm, they start to express Mesp1 (pink) and Flk-1 (purple). As these cardiac precursor cells reach the anterior and lateral plate mesoderm, they commit irreversibly to become cardiac progenitor cells by expressing Nkx2.5 (green) or Isl-1 (blue). Within the adult heart reside several different populations of cardiac stem cells including side population (SP) cells and cells that express c-Kit or Sca-1. (Ao, aorta; SVC, superior vena cava; PA, pulmonary artery; RV, right ventricule) [28].

II.2.2. Heart regenerative capacity

The heart is one of the least regenerative organs in the body and only a limited number of species (e.g. zebrafish) are capable of renewing cardiac tissue[29]. The adult human heart has a very limited regenerative capacity and consequently any significant heart cell loss or dysfunction may lead to the development of progressive heart failure[30]. Acute myocardial infarction (MI) is a leading cause of death worldwide, typically caused by coronary artery occlusion and ischemia[31]. For those who can survive MI, necrotic myocardium might represent one billion loss of heart muscle cells, known as cardiomyocytes, leading to an inflammatory response and a recruitment of local fibroblasts. In few weeks, a large, collagen-rich scar tissue forms in its place. The scar is a rapid solution, however, it is not contractile which weakens the heart while also increases the susceptibility to additional MI events. The persistence of scar

tissue following myocardial infarction suggests that the heart has little if any capacity to generate new cardiomyocytes[32].

Shortly after birth, myocardial growth transitions from a hyperplastic to a hypertrophic phase. This transition gave rise to the notion that adult cardiomyocytes are incapable of proliferating; that is, they are terminally differentiated (figure II-6). Cardiomyocytes in fetal humans and mice typically have a single nucleus with a diploid genome (2n) and increase in mass through cell division. Human cardiomyocytes can proliferate for the first few months after birth but are believed to lose this capacity early in life. These cells undergo DNA replication without karyokinesis or cytokinesis, which result in mononucleated cardiomyocytes with tetraploid (4n) or higher DNA content. Murine cardiomyocytes can divide robustly until the first few days after birth, after which most withdraw from the cell cycle. These cells undergo DNA replication with karyokinesis but not cytokinesis, which results in binucleated cardiomyocytes that are diploid (2n) in each nucleus. By contrast, most cardiomyocytes in zebrafish are mononucleated with a diploid genome (2n) throughout life and have significant proliferative capacity [32].

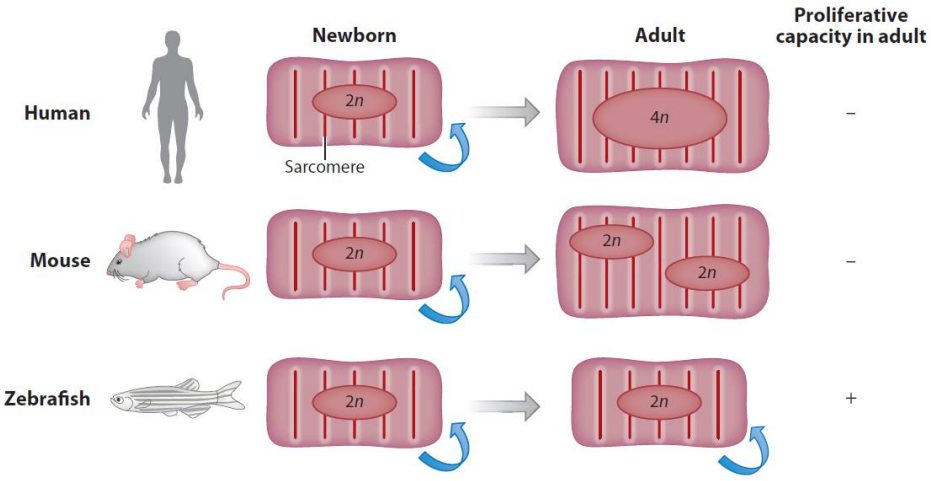

Figure II-6 – Nuclear dynamics and proliferative capacity of cardiomyocytes during growth.[32]

II.2.3. Cell-based therapies

It is estimated that heart failure is responsible for more hospitalizations than all forms of cancer combined[31]. Despite advances in surgical procedures, mechanical assistance devices, drug therapy, and organ transplantation, more than half of patients with congestive heart failure die within five years of initial diagnosis[29]. With lack of donors limiting the number of patients who can benefit from heart transplantations, development of new therapeutic strategies have become urgent. Researchers are now trying to explore ways to save lives by using replacement cells for dead cells so that the heart muscle can recover[33]. The recent advances in the areas of stem cell biology and tissue engineering have provided scientists with potential tools to develop

novel strategies for myocardial repair. The cell replacement approach is based on the assumption that myocardial function may be improved by re-populating diseased areas with a new pool of functional cells[2]. One important type of cell that can be developed is the cardiomyocyte, the heart muscle cell that contracts to eject the blood out of the heart. Two other cell types are important to a properly functioning heart: the vascular endothelial cell (EC), which forms the inner lining of new blood vessels, and the smooth muscle cell (VSMC), which forms the wall of blood vessels[34]. The heart has a large demand for blood flow, and these specialized cells are important for developing a new network of arteries to bring nutrients and oxygen to the cardiomyocytes after the heart has been damaged.

Cell-based cardiac repair began with the transplantation of autologous skeletal muscle satellite cells (commonly referred to as myoblasts), progenitor cells that mediate regeneration of skeletal muscle[35]. Although some investigators initially hoped that engrafted myoblasts would transdifferentiate into cardiomyocytes, multiple evidences indicate that these cells remain committed to a skeletal muscle fate[35]. Mature skeletal muscle cells do not express the adhesion or gap junction proteins required to electromechanically couple with one another or with host myocardium and available physiological data suggest that these grafts do not beat in synchrony with the rest of the heart. Because these transplanted populations do not generate new cardiomyocytes, the improvement may be due to paracrine effects that lead to enhanced vascularization[29].

II.2.4. Cellular sources for cardiac muscle regeneration

Human cardiomyocytes can be isolated from heart biopsies, but the access to human heart tissue is very limited, and the procedure is complicated; it is difficult to obtain viable cell preparations in large quantities, and the cells obtained do not beat spontaneously[36]. One potential source of these cells is the adult heart, in the form of **Cardiac Progenitor Cells (CPCs)**[37]. The notion that the adult mammalian heart may harbor stem cells with replicative and regenerative capacity was suggested initially by a study in patients with myocardial infarction[38]. This study showed an increased number of immature cardiomyocytes with the capacity for mitotic division in the infarct border zone that may have originated from a circulating or endogenous stem cell pool. The same investigators subsequently isolated a lineage-negative, c-Kit-positive (Lin−, Kit +) cell population from adult mice that was clonogenic, self-renewing and capable of differentiating into cardiomyocytes, vascular smooth muscle cells and endothelial cells[13]. In 2005, Laugwitz et al. reported that Isl-1 (an LIM homeodomain transcription factor), which is known as a secondary heart field marker, can also be used as a cardiomyocyte progenitor marker in the postnatal heart[39]. Isl-1 is expressed at the early stages of human cardiogenesis in a multipotent primordial progenitor that gives rise to more than two-thirds of the heart and to the heart's three major cell types: cardiomyocytes, VSMCs and ECs

(Figure II-7)[40]. The generation of these cardiovascular cell types might be a result of sequential decisions that increase the restriction of multipotent cardiovascular progenitors to specific intermediates and their differentiated derivatives. As such, understanding cardiogenesis at the level of specific decisions that are made by specific heart cell lineages requires the identification of pathways that are responsible for critical steps in the formation, renewal, specification and differentiation of the hierarchy of cardiac multipotent progenitors[41].

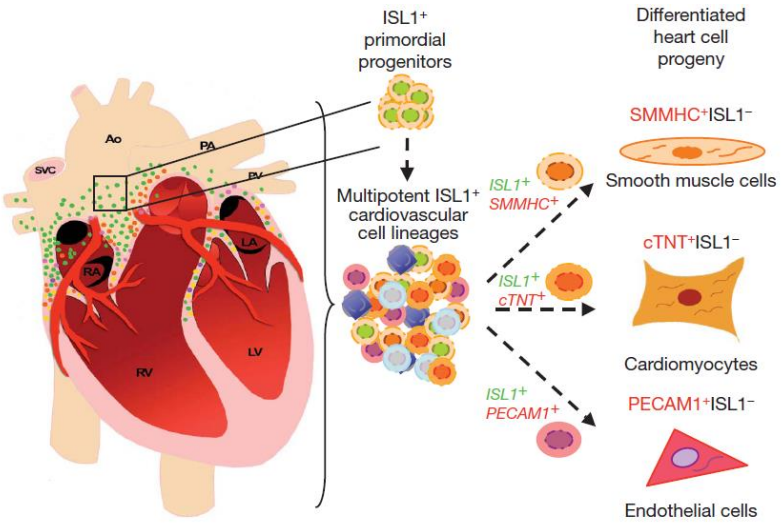

Figure II-7 – Model proposing a primordial ISL1+ progenitor generating a family of human multipotent cardiovascular lineages (adapted from Lei Bu et al. 2009).

With the discovery of human embryonic stem cells (hESCs)[7] and more recently, human induced pluripotent stem cells (hiPSC)[18], many investigators have focused their efforts on developing strategies to efficiently direct stem cell differentiation to the cardiovascular lineage. Since the initial demonstration that contracting cardiomyocytes can be generated from both types of **human pluripotent stem cells** (hPSC)[42], great efforts have been made to improve the efficiency and reproducibility of differentiation, while progressing to more defined conditions and producing cells on a clinically relevant scale. In this context, the demonstration of direct reprogramming of human skin fibroblasts to iPSC are particularly exciting once these cells have the potential to develop into cardiomyocytes and replace damage cells in the heart and restore its function to people with congestive heart failure. If derived from patients carrying gene mutations affecting the cardiovascular system, it should also be possible to obtain cardiac progenitors with the same mutations. This may allow pathogenesis to be followed at the cellular level “in a dish” and should enable molecular and genetic screens to find drugs or reverse the disease phenotype[43].

II.3. Main Signaling Pathways of Cardiomyocytes Differentiation

In hPSC, as in embryos, differentiation is triggered by developmental cues such as morphogens or cytokines that are present in the extracellular space. These morphogens or cytokines bind to their plasma membrane-bound receptors and activate specific signaling pathways inside the cell[44]. Activation of signaling pathways involves a sequence of phosphorylation events that eventually result in the regulation of specific transcription factors. These transcription factors, in turn, can recruit other co-factors and directly cause transcription of downstream genes. Furthermore, transcription factors can recruit histone modifying and chromatin remodeling enzymes to reshuffle the epigenetic structure, such that pluripotency genes become inaccessible for transcription and are repressed, whereas lineage-specific genes become accessible and are activated[44]. This sequence of events finally leads to expression of lineage-specific proteins such as transcription factors and structural proteins, causing a morphological change in the cell. Also, pluripotency associated transcription factors and other pluripotency-associated genes are permanently repressed, thereby completing the process of differentiation. Thus, the process of differentiation is a rather complex cascade of events, controlled by signaling pathways, transcription factors, epigenetic factors and lineage-specific proteins[44]. The development of cardiac muscle during embryogenesis requires extracellular instructive signals that are regulated precisely in time and space. Among the frequent players are Wnt proteins, fibroblast growth factors and many members of the transforming growth factor (TGF)-β superfamily, including bone morphogenetic proteins (BMPs), Nodal and Activin[45]. The key features of each major signaling cascade are shown schematically in figure II-8.

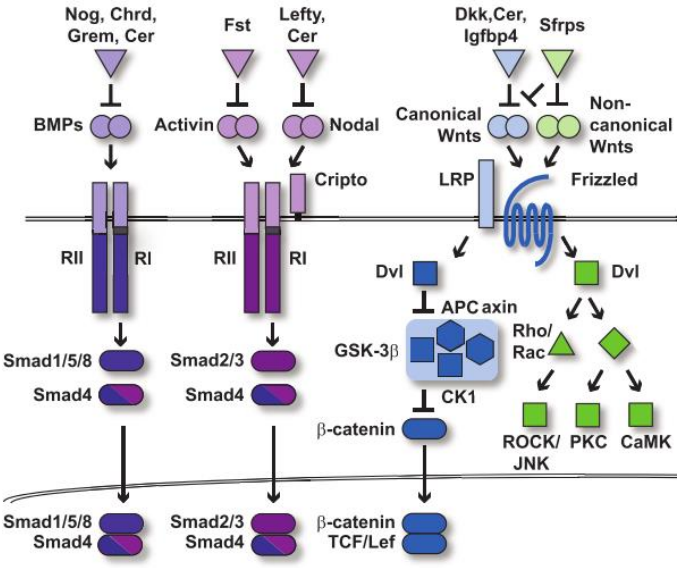

Figure II-8 – Major signaling cascades controlling cardiogenesis. From left to right are the BMP, Activin/Nodal, canonical Wnt, non-canonical Wnt. Circle indicates ligand; inverted triangle, ligand antagonist; rectangle, receptor ligand-binding and signaling domains (light and dark, respectively); square, protein kinase; triangle, G-protein; hexagon, scaffold protein; pentagon, protease; oval, transcription factor[45].

II.3.1. Canonical Wnt pathway

Wnt proteins are growth factors that function during embryonic development and in the adult organism by regulating diverse cellular processes such as gene transcription and cell proliferation, migration, polarity, or division[46]. Wnt proteins are also involved in cardiac development and differentiation[47]. The classic canonical Wnt signaling pathway involves the multifunctional protein β-catenin (figure II-9). β-catenin is known for its function in cell adhesion through interaction with transmembrane cadherins but also functions as a signaling molecule. In the absence of Wnt signals, β-catenin that is not bound to cadherins is captured by a degradation complex containing GSK3β, Axin/Conductin, CK1α and APC and is targeted to degradation. In the nucleus, the transcriptional inhibitor Groucho binds to LEF/TCF (lymphoid enhancer factor/T-cell factor) transcription factors and inhibits transcription of Wnt target genes. In the presence of Wnt ligands that are secreted from neighboring cells (secretion and post-translational modifications of Wnt ligands are accomplished by molecules, such as Porcupine, a membrane-bound O-acyltransferase[48]), LRP5/LRP6 receptors are phosphorylated and GSK3β and Dishevelled (DVL) molecules are recruited to the plasma membrane which leads to inactivation of the degradation complex and accumulation of β-catenin, which translocates to the nucleus. In the nucleus, β-catenin forms a transcriptionally active complex with LEF and TCF by displacing Groucho and regulates target gene transcription[49]. Through particular target genes such as c-myc and cyclinD1, the canonical Wnt/β-catenin pathway is also linked to cell proliferation control[25]. Well-known extracellular inhibitors of the Wnt/β-catenin signaling are Dkk1 and Crescent[50,49].

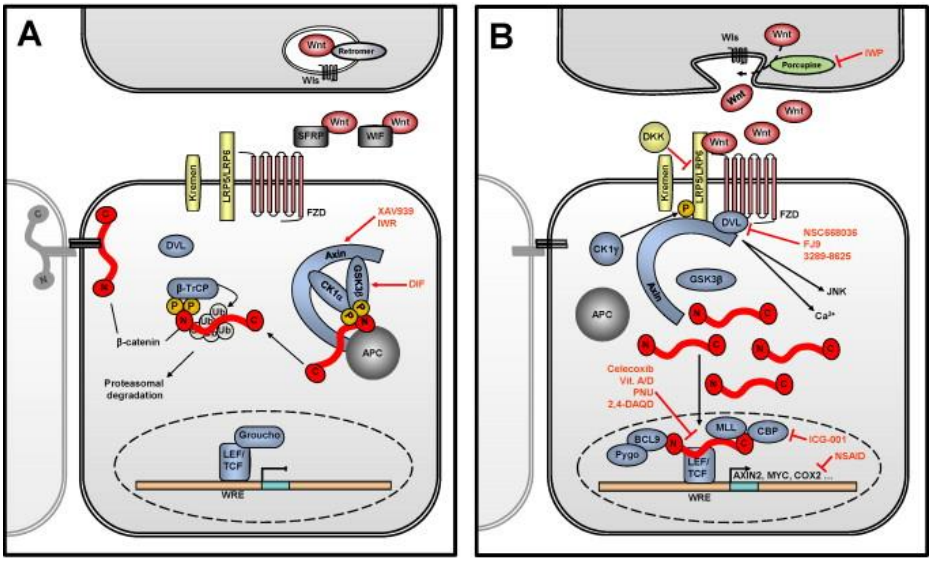

Figure II-9 – Scheme of the canonical Wnt pathway. (A) In the absence of Wnt signals, **(B)** In the presence of Wnt signals.[49]

II.3.1.1. The biphasic role of Wnt signaling

Diverse roles of Wnt/β-catenin signaling have been determined by the observation that Wnt signaling can have different effects depending on the time of action. Many studies have shown that the formation of mesoderm is dependent on canonical Wnt signaling[50,51,52]. The knockout mouse of β-catenin, fails to generate mesodermal tissue[50]. Also the loss of Wnt3a function leads to an absence of mesoderm-specific marker genes. Inhibition of canonical Wnt at an early time point blocks the expression of mesoderm specific marker genes and leads to an inhibition of cardiac differentiation and a reduction in contractile colonies[51,52]. These data indicate a strong requirement of canonical Wnt signaling during mesoderm induction in the early embryo, a prerequisite for the formation of cardiac progenitor cells. In accordance with the inhibitory role of Wnt/β-catenin signaling during cardiac development, it has recently been shown that β-catenin negatively regulates the expression of GATA6, one of the major early cardiac transcription factors[53]. These findings are furthermore in line with the observation that the canonical Wnt inhibitor Dkk1 can improve the differentiation of Mesp1-induced cardiomyogenesis. In ES cells overexpressing Mesp1, Dkk1 is upregulated, whereas in Mesp1$^{-/-}$ /Mesp2$^{-/-}$ embryos, Dkk1 expression is dramatically downregulated[54].

II.3.2. TGF-β pathway

Activin and Nodal, like BMPs, are members of the TGF-β family but unlike BMPs, signal via Smad2/3 not Smad1/5/8. Activin, Nodal and BMP signal via the same type of receptor, an Activin type II receptor (ActRII or ActRIIB), but Nodal effects require a co-receptor of the EGF family, named Cripto. Activin A has been used successfully, together with other factors, especially BMPs, to induce cardiac myogenesis gene expression, such as Mesp1, Gata4, Nkx2.5, Isl1 in both hESCs and human iPSC[55].

Human embryonic stem cells rely on Activin-Nodal signaling to maintain their pluripotency, however Activin-Nodal signaling is also known to induce mesendoderm differentiation. The mechanisms by which Activin-Nodal signaling can achieve these contradictory functions are still unclear. However, Chng et al. have recently revealed the mechanism by which Activin-Nodal signaling acts through Smad-Interacting protein 1 (SIP1) to regulate the cell-fate decision between neuroectoderm and mesendoderm in the progression from pluripotency to primary germ layer differentiation[56] (figure II-10). They observed by knockdown and overexpression analysis that SMAD2, NANOG and OCT4 repress SIP1 expression, whereas SOX2 activates its expression (all these bind to the same region in SIP1 promotor). Importantly, only SOX2 binds the SIP1 promotor during differentiation of neuroectoderm lineage, suggesting that SOX2 favors neuroectoderm differentiation through the activation of SIP1 expression.

In pluripotent hESCs, the transcriptional regulation of SIP1 by Activin-Nodal signaling is implemented through repressive effects (NANOG and OCT4) and activating effects (SOX2) that act in opposition to each other, thereby resulting in a tight regulation of SIP1 expression. SIP1 balances opposing tendencies toward neuroectodermal and mesendodermal differentiation, thus preserving the pluripotent state. This limits the capacity of SMAD2/3 to activate mesendoderm markers, but does not block the pluripotency-maintaining effects of Activin-Nodal signaling. Mesendoderm specification is triggered by an increase in Activin-Nodal signaling. The consequent increase of NANOG and OCT4 expression releases SOX2 from the SIP1 gene. SIP1 expression is thus repressed and increases the mesendoderm inducing effects of BMP signaling. This allows the mesendodermal cell fate to prevail over neuroectodermal fates. Together, these results suggest that in hESCs, SMAD2/3 regulate the expression of SIP1, an important factor required to block the expression of mesendoderm markers in pluripotent stem cells and to protect neuroectoderm differentiation against inhibition by Activin-Nodal and BMP signaling cascades.

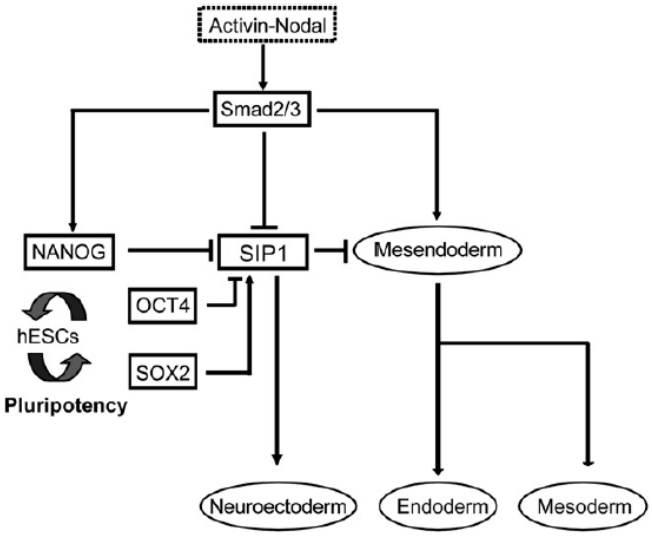

Figure II-10 – SIP1 mediates differentiation of hESCs, favoring neuroectoderm and inhibiting mesendoderm [56]**.**

II.4. Cardiac Differentiation of human Pluripotent Stem Cells *in vitro*

II.4.1. Culture methods

The 3 major approaches for differentiation of human pluripotent stem cells (hPSC) to cardiomyocytes are: Embryoid bodies (EB), monolayer culture and inductive co-culture (figure II-11). For both EBs and monolayer approaches, stage-specific application of key growth factors (GFs) in defined media are required, although some protocols use small molecules to induce cardiogenesis. Coculture of hPSC with endodermal-like END2 cells takes advantage of cell signaling from END2 cells to promote cardiogenesis.[57]

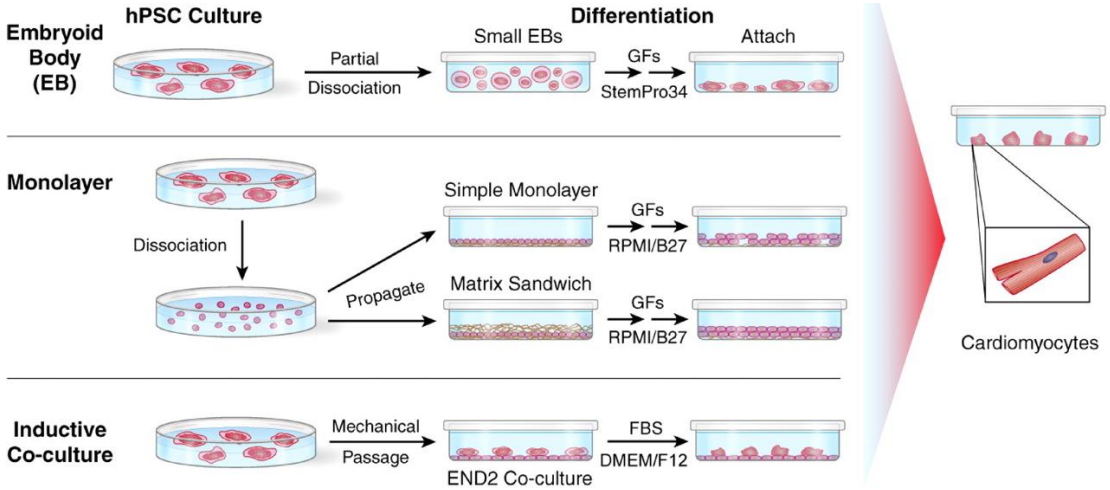

Figure II-11 - Current methods for cardiac differentiation of human pluripotent stem cells.

II.4.1.1. Embryoid Body Formation

The initial observation that hESCs could mature into spontaneously contracting cardiomyocyte-like cells was reported ten years ago when hESCs were cultured in suspension and formed three-dimensional aggregates called embryoid bodies. Within the embryoid body, derivatives of the three germ layers (ectoderm, endoderm and mesoderm) develop spontaneously[58]. Cardiomyocytes are one of the first cell types induced from pluripotent stem cells in embryoid bodies, once cell to cell interactions stimulate the expression of markers for mesodermal and early cardiac cell lineages. Typically, the embryoid bodies are plated on a matrix-coated tissue culture plate for further differentiation, and within a few days after plating, contracting areas can be observed[59]. Regarding human iPS cells, cardiomyocyte induction using embryoid body method was reported for the first time in 2009[60]. The spontaneous differentiation of cardiomyocytes from pluripotent stem cells in embryoid bodies is considered to be very inefficient usually under 10% and is highly cell line dependent. Cardiomyocyte induction

in the embryoid body-based differentiation system has also proved to be highly variable due to the heterogeneity of the aggregates that may differ in size and morphology. At the time when embryoid body cardiac differentiation method was introduced, limited information regarding the mechanisms underlying cardiogenesis in human pluripotent stem cells was available. Later, it has become evident that the differentiation process can be manipulated by the addition of growth factors, morphogenes, or by transgenic modifications to direct the pluripotent stem cells toward the cardiac cell fate[36].

II.4.1.2. Co-culture of Pluripotent Stem Cells with Cardio-inductive Cell Types

Another approach for cardiac differentiation was inspired by developmental studies indicating the important role of anterior endoderm in the cardiac induction of adjacent mesodermal structures and has been applied to induce cardiomyocyte differentiation *in vitro*. The method is based on co-culture of pluripotent stem cells with a visceral endoderm-like cell line (END-2), derived from mouse P19 embryonal carcinoma (EC) cells, which results in the formation of beating clusters of cells[61,62]. This differentiation method was found to be effective as well with iPS cell lines. An END-2 conditioned media (END-2-CM) system has also been demonstrated to induce robust differentiation into cardiomyocytes[63]. Based on cardiomyocyte phenotype and electrophysiology, the majority of pluripotent stem cell- derived cardiomyocytes are similar to human fetal ventricular cardiomyocytes[61]. Based on the knowledge from developmental biology, the cardio-inductive signals are thought to originate from direct cell to cell contact and/or secreted factors from the END-2 cells which stimulate the presence of embryonic endoderm. This END-2 co-culture protocol has been reported to result in about 20%–40% cardiomyocytes[62]. Identifying further cardiomyocyte inducing factors from the END-2 cells may provide opportunities to develop defined and more efficient processes of differentiation for the induction of stem cells to cardiomyocytes and cardiac progenitors[36].

II.4.1.3. Monolayer differentiation to cardiomyocytes

An alternative approach for differentiation of hPSC starts with cells grown as monolayers. This culture method takes in advantage a relatively uniform monolayer of cells without the diffusional barriers presents in EBs. In this way, the application of growth factors and other molecules can theoretically be more controlled and reproducible. The monolayer protocol also do not require the re-plating steps of EBs protocols, thus reducing the number of procedure steps and the use of tissue culture supplies[64].

a. Cardiomyocytes differentiation via Activin A and BMP4 signaling

In 2007, Laflamme et al. first described a differentiation protocol with H7 hESCs on Matrigel as a monolayer in the presence of mouse embryonic fibroblast-conditioned media[65]. To direct cardiac differentiation, cells are treated sequentially with Activin A for 24 hours followed by BMP4 in a serum -free RPMI medium plus a B27 supplement. The growth factors are then removed, and the cells are maintained in the serum-free medium for 2-3 weeks in the absence of exogenous growth factors (figure II-12). This protocol for H7 hESCs resulted in >30% CMs, and with a Percoll gradient centrifugation step, the CMs were enriched to >70%, which was a great improvement compared with the EB method for this cell line. Both hESCs and hiPS cells have been differentiated to cardiomyocytes by using this approach [13-21]. However, the efficiency of the Activin A and BMP4 monolayer differentiation protocol can be highly variable between cell lines and experimental repeats[66].

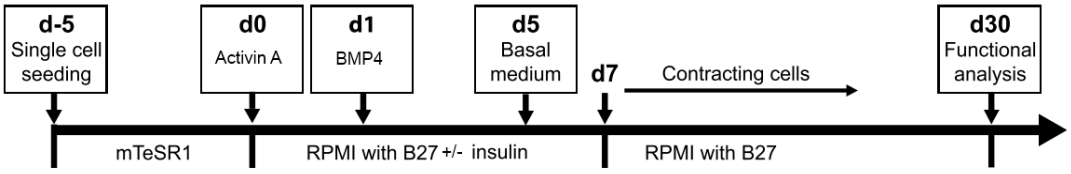

Figure II-12 – Schematic of protocol for hPSC differentiation to cardiomyocytes via Activin A and BMP4.[52]

b. Matrix Sandwich Approach

A recent innovation for monolayer protocols uses extracellular matrix in combination with growth factors, because ECM proteins play fundamental roles in development and can complement responses to soluble cytokines[67]. For this matrix sandwich approach, hPSC are seeded as single cells on Matrigel and cultured in serum-free mTeSR1 media. When cells reach 90% of confluence after 2 to 4 days, a matrix overlay is applied on the monolayer of cells by mixing the Matrigel with mTeSR1 media. When cells reach 100% confluence, then Activin A with Matrigel is added to the basal media RPMI/B27 minus insulin supplement for 24 hours, followed by BMP4 and basic FGF for 4 days in the same basal medium (figure II-13). Flow cytometry analysis by troponin T labeling has demonstrated high purity (40-90%) of CMs for a range of hESCs and hiPSC lines. Thus, the use of ECM to supplement soluble growth factors signaling can enable reproducible and robust cardiac differentiation in multiple hESCs and hiPSC generating relatively high purity of CMs[67]. The ECM effect likely reflects the behavior of cells during normal development in which dynamic changes in both ECM and growth factors are critical. However, the detailed mechanisms by which the Matrigel promotes cardiogenesis will require future investigations focused on the individual components of this complex ECM mixture and potential cellular receptors and signaling pathways.

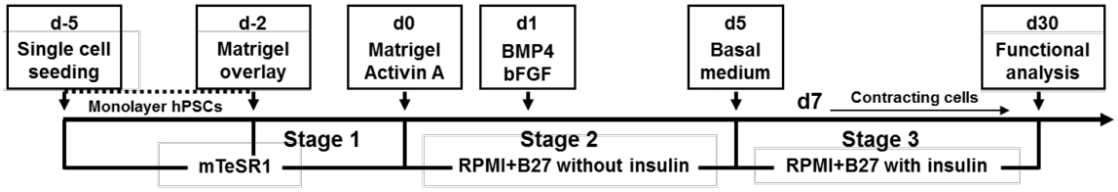

Figure II-13 – Schematic of the matrix sandwich protocol for efficient cardiogenesis of PSCs lines.[67]

c. Temporal modulation of canonical Wnt signaling

- **Induction of TGF-β superfamily signaling via Gsk3 inhibitors**

An alternative monolayer protocol has also been described that also involves the TGF-β family members, Activin A and BMP4, and also exploits the important roles of canonical Wnt signaling in cardiogenesis. Lian et al. have recently reported that the pre-treatment at the undifferentiated hPSC expansion stage with the GSK3 inhibitor CHIR, leads to nuclear accumulation of β-catenin, which associates with T-cell factor/lymphoid enhancer-binding factor (Tcf/Lef) and activates gene transcription[51]. This method showed to enhance cardiomyocyte generation, producing an average of 50% spontaneously contracting cardiac troponin T (cTnT)-labeled cells[51]. However, the use of growth factors makes these approaches costly. Thus, it is of interest to develop novel applicable, efficient strategies to induce cardiac differentiation of human ES cells as well as iPS cells based on cost-effective culture systems.

- **Cardiac-directed differentiation via small molecule modulators of Wnt signaling**

Chemical biology offers alternative opportunities for discovering novel cellular signaling molecules mediating pluripotent stem cell cardiogenesis. As a consequence, high-throughput molecular screening technology has been used to search for compounds with the cardiomyogenesis potential *in vitro*. To date, a few studies have published results from such screening approaches, describing the identification of novel small molecules that appear to stimulate the generation of cardiomyocytes from pluripotent stem cells[36]. Chen et al. recently discovered two small molecule Wnt inhibitors: IWR (inhibitor of Wnt response) and IWP (inhibitor of Wnt production 1). IWP showed to prevent palmylation of Wnt proteins by Porcupine, thereby blocking Wnt protein secretion and activity[48].

In 2012, Lian et al. have reported that appropriate temporal modulation of regulatory elements of Wnt signaling alone is sufficient to differentiate multiple hPSC lines to cardiomyocytes very efficiently[51,68]. Canonical Wnt signaling was found to have stage-dependent effects on cardiac differentiation: it is first required for mesoderm induction but must be inhibited later for the induction of pre-cardiac mesoderm. This modulation can be achieved by either two methods: genetic modification by shRNA inhibition of β-catenin or using only small

molecule inhibitors of mediators of canonical Wnt signaling, such as CHIR99021 and IWP. Although shRNA protocol provides specific and easy temporal regulation of canonical Wnt signaling, this method requires genetic modification of the hPSC line, reducing its utility for potential clinical applications. However, the use of small molecules instead of growth factors could allow cost-effectivei and reproducible generation of human cardiomyocytes in completely chemically defined conditions, facilitating the use of these cells to high throughput screening applications or regenerative therapies. Accordingly to the small molecule protocol, first hPSC are maintained in mTeSR1 on Matrigel for 5 days; then the medium is switched to RPMI/B27-insulin containing 12 µM CHIR. At a later stage (day 3 of differentiation) IWP4 or IWP2 are used to block Wnt signaling, via Porcupine inhibition (figure II-14). It was demonstrated that it is possible to generate populations consisting of up to 98% cardiomyocytes from hPSC lines without any enrichment and/or purification step only via temporal modulation of regulatory elements of Wnt signaling. Therefore, this protocol showed to be a robust, inexpensive, completely defined, growth factor-free, scalable method of producing cardiomyocytes from hPSC.

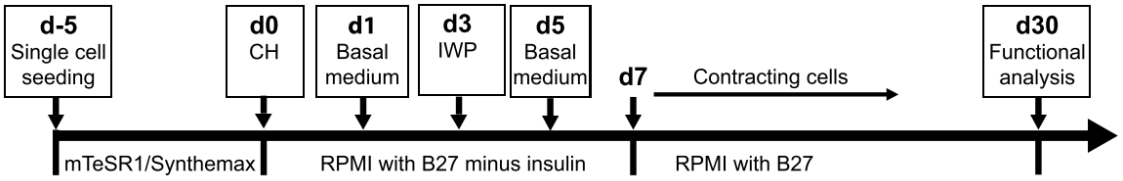

Figure II-14 – Schematic of protocol for fully defined, growth factor-free differentiation of hPSC to cardiomyocytes via treatment with small molecules.[51]

II.5. Characteristics of hPSC-Derived Cardiomyocytes

Determining the success of differentiation protocols requires detailed characterization of the resulting cardiomyocytes. Typically, the first sign of successful differentiation to CMs is the appearance of contractile colonies in the culture. Although early studies using EB differentiation protocols usually reported the percentage of contracting EBs as a measure for cardiac differentiation, more recent studies have described the proportion of CMs, estimated by intracellular flow cytometry for a cardiac-specific protein, such as cardiac troponin T (cTnT). This reflects the efficiency of the protocol with much more precision. To better characterize and quantify the resulting CMs, cell analysis has been based on the expression of specific molecular markers for cardiomyocytes, structural architecture, and functionality[57].

II.5.1 Structural Organization

At the structural level pluripotent stem cell-derived cardiomyocytes show sarcomeres structure with A, I, and Z bands and intercalated discs with gap juctions and desmosomes. These cells share similarities with adult cardiomyocytes although the myofibrillar and sarcomeric organization indicate an immature phenotype in the stem cell-derived population [69,70]. In addition to structural proteins including sarcomeric proteins α-actinin, cardiac troponins T, and I, sarcomere myosin heavy chain (MHC), atrial- and ventricular myosin light chains (MLC-2A and MLC-2V), desmin, and tropomyosin, gap junction proteins are also expressed (figure II-15)[61,71]. The presence of other cardiac and muscle-specific proteins including atrial natriuretic peptide (ANP), creatine kinase-MB and myoglobin has also been described[69]. Troponin complex is located on the thin filament of striated muscles and regulates muscle contraction in response to alterations in intracellular calcium ion concentrations. As in mature cardiomyocytes, the trigger for contraction in pluripotent stem cell-derived cardiomyocytes is a rise in intracellular calcium[61,62]. However, the regulation of intracellular calcium has been shown to differ between pluripotent stem cell-derived cardiomyocytes and mature adult cardiomyocytes, most likely due to the apparently immature sarcoplasmic reticulum which has been suggested to be caused by lack of expression of phospholamban and calsequestrin, which are two of the main intracellular calcium regulators [60].

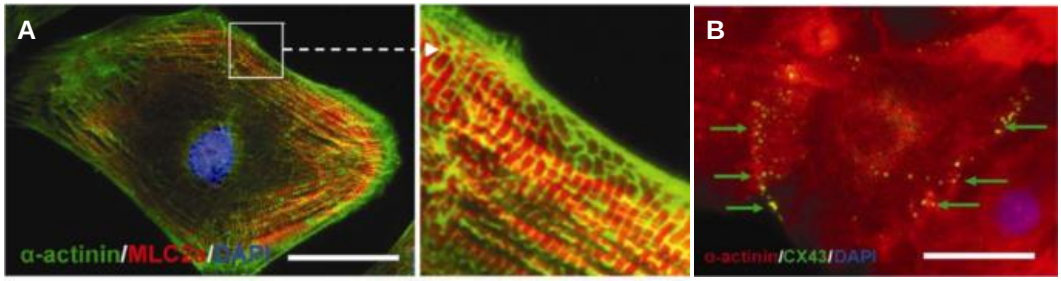

Figure II-15 – (A) Immunostaining for α- actinin (green) and MLC2a (red) shows sarcomere organization and (B) connexin 43 (green) and α-actinin (red) immunostaining shows gap junction formation between neighboring cardiomyocytes. Scale bar = 50 µm.[52]

II.5.2 Molecular Markers

On the molecular level, several markers expressed by cardiomyocytes are also expressed by pluripotent stem cell-derived cardiomyocytes, including transcription factors, structural proteins, hormones, ion-channels, and tight junction proteins [61,72]. By following the progress from undifferentiated hESCs towards cardiomyocytes in vitro, molecular studies have shown that during the initial phase of the differentiation process the expression of early mesoderm-like genes, such as Brachyury T, gradually increases followed by the expression of cardiac mesoderm markers such as Mesp-1, Isl-1 and Flk-1, which are considered to be the first markers expressed in the cardiac progenitor population as referred previously. Later in cardiac specification, the full committed cardiac progenitors are characterized by the expression of the

early cardiac-specific transcription factors GATA-4, Nkx2.5, Tbx-5, Tbx-20, and Mef2c[71,59]. Mature cardiomyocytes are characterized by the expression of cardiac structural proteins, such as the cardiac troponins (cTnT), α-actinin and myosins (MHC and MLC), which confirms the cardiac phenotype and the beating capacity of these cells (figure II-16)[34].

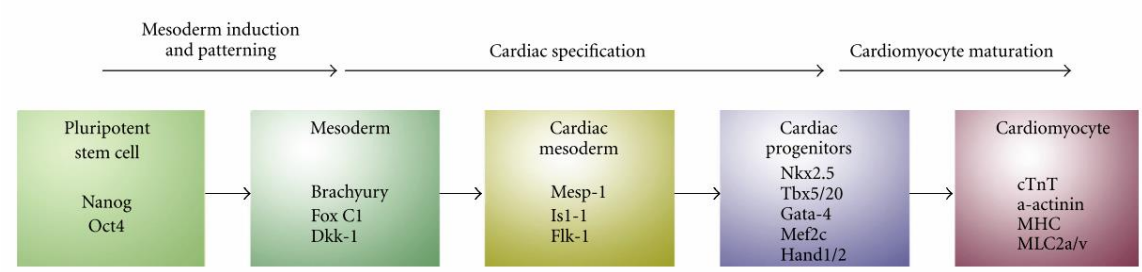

Figure II-16 – Diagram depicting sequential steps required for obtaining cardiomyocytes from pluripotent stem cells. Early mesoderm differentiates via cardiac mesoderm and committed cardiac progenitors further to functional beating cardiomyocytes. Typical markers for each step are indicated.[36]

II.5.3 Electrophysiology

A key property of CMs is generation of action potentials (APs) necessary for propagation of the electric signal in the myocardium and for triggering intracellular Ca^{2+} release, resulting in mechanical contraction. Properties of the AP such as the AP amplitude, spontaneous depolarization duration, maximum diastolic potential and rate of the AP can provide information regarding the type and maturity of the CM[60]. The majority of CMs display ventricular-like APs, but atrial-like and nodal-like APs are also detected (figure II-17). The properties of the APs suggest that the hPSC-derived CMs are relatively immature in phenotype compared with adult CMs[57].

Several studies have demonstrated that pluripotent stem cell-derived cardiomyocytes exhibit spontaneous contractile activity that could be modulated by drugs such as isoproterenol and carbachol, and thus these cells respond to alpha/beta-adrenergic- and muscarinic stimuli indicating that these cells express specific surface membrane receptors coupled to a signaling pathway that activate ion channels, membrane transporters, and myofilament proteins[73]. Furthermore, cardiomyocyte induction from pluripotent stem cells results in mixtures of ventricular-like, atrial-like, and pacemaker-like cells defined by intracellular electrophysiological measurements of action potentials (APs)[57]. Interestingly, different differentiation protocols seem to affect the ratios of these cardiac cell types. While most differentiation protocols based on embryoid bodies result in more or less equal numbers of ventricular- and atrial-like cells, cardiac induction by END-2 co-culture generally results in a majority of the cardiomyocytes displaying ventricular-like phenotype[62]. Intracellular Ca^{2+} can also be monitored with fluorescent Ca^{2+} indicators to determine whether cardiac-typical transients are present. Overall, is important to

note that molecular assays alone are not sufficient to confirm CM identity, functional assays are also essential.

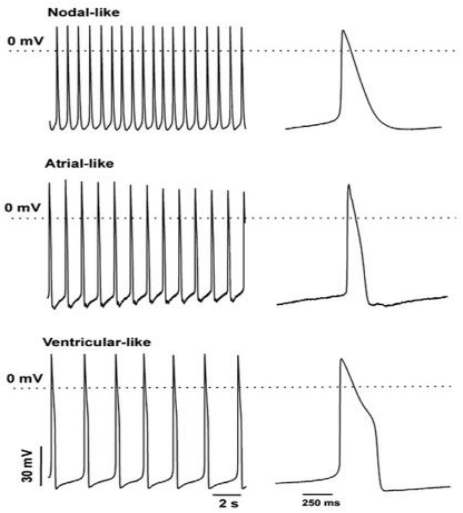

Figure II-17 - Action potential (AP) morphologies of hPSC-CMs. Intracellular recording from 3 different iPS cell-derived CMs demonstrating the 3 major types of AP: nodal-like (top), atrial-like (middle) and ventricular-like (bottom). [60]

In summary, based on the gene expression profile and the structural, electrophysiological and pharmacological properties, pluripotent stem cell-derived cardiomyocytes possess immature phenotype and appear to have an immature sarcoplasmic reticulum function[74]. The cardiac phenotype of hiPS cell-derived cardiomyocytes seems to be comparable to that of hESC-derived cardiomyocytes[57]. Pluripotent stem cell-derived cardiomyocytes have been shown to mature over time in culture but without carrying through to full maturity (figure II-18)[74]. Thus, additional research is needed to find novel strategies to mature pluripotent stem cell-derived cardiomyocytes *in vitro*. Nevertheless, even if some functional properties of pluripotent stem cell-derived cardiomyocytes apparently differ from the mature cells present in adult myocardium, the *in vitro* generated cardiomyocytes have a basal functionality and can still provide a useful model for molecular cardiology. The electrophysiology properties are particular relevant in investigations evaluating basic mechanism of arrhythmia, such as recent studies using patients-specific hiPSC to study inherited long QT syndrome[75].

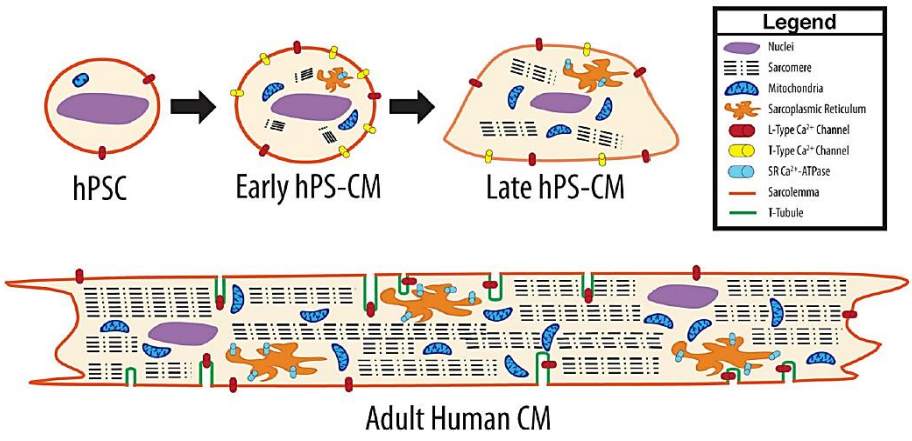

Figure II-18 – Comparison of early hPS-CM, late hPS-CM and adult CM morphology. Late hPS-CM differ from early hPS-CM with respect to shape, sarcomeric area and receptor expression. Adult CM are larger, with multiple nuclei, large sarcomeric area, and large numbers of mitochondria[74].

II.6. Cardiomyocytes Enrichment and Selection

Although cardiomyocytes can be generated using various differentiation culture systems, there is the need to develop strategies to increase the purity of the generated cardiomyocytes for clinical purposes. A selection strategy is required to increase the number of cardiomyocytes and to avoid the presence of other cell derivatives or remaining pluripotent stem cells, and thus to prevent the generation of teratomas[57]. There are a number of different approaches being tested to select cardiomyocytes from mixed cell populations. In some cases, fluorescent reporters have been attached to cardiomyocyte-associated promoters and used to produce transgenic reporter lines. The yield of cardiomyocytes after selection can be >90%, but requires genetic manipulation that has not been successful in all hESC lines and might not be feasible for routine use in hiPSC. Another approach uses Percoll gradient centrifugation to isolate the cardiomyocytes physically, on the basis of their larger size compared with most other cells[72]. A third approach was recently reported by Hattori et al. and is based in a non-toxic dye TMRM (tetramethylrhodamine methyl ester perchlorate) that reversibly labels the mitochondria[76]. Labelling a mixed culture of cells produces three fractions, a high fluorescent fraction with >99%cardiomyocytes, an intermediate fraction with other viable cells and a low fraction which contained dead or blood cells (figure II-19). As this technique depends on FACS purification, there is a scale limitation due to cell sorting capacity. A forth method selects cardiomyocyte progenitors using the cell-surface proteins. Recently, it has been discovered a novel cell surface molecule for human cardiomyocytes, VCAM1 (vascular adhesion molecule 1 also called CD106)[78]. Approximately 95-98% of cardiomyocytes were purified from a mixed population after *in vitro* differentiation, proving that VCAM1 is a robust marker for cardiomyocytes from various human ESC and iPSC lines. This method demonstrates to be easily scalable and selective for clinical purposes. Other less invasive methods of purification rely on metabolic differences between hPS-CMs and pluripotent stem cells. hPS-CM have higher expression of oxidative phosphorylation genes and proteins compared to hPSC and can use lactate as a source of energy unlike hPSC. hPSC are in constant division and need high energetic availability depending almost exclusively on glycolysis. Due to these metabolic differences between hPSCs and hPS-CM, when glucose is absent in the medium and high concentration of lactate (4Mm) are presence, hPSCs die within 12 hours whereas hPS-CMs are residually affected (10%).[79] In this way, it is not only possible to increase the purity of the cardiomyocyte culture but also avoid the risk of teratoma formation *in vivo.*

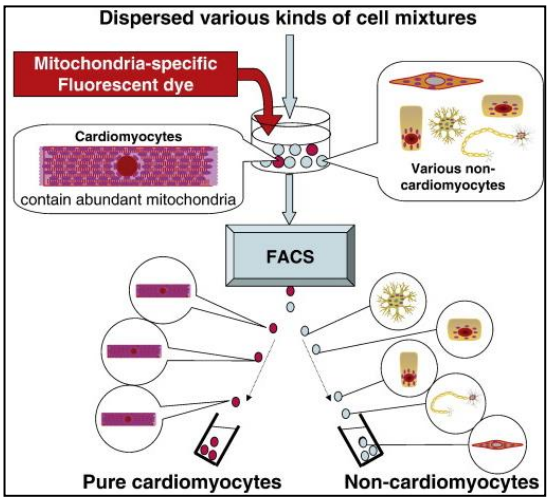

Figure II-19 – Scheme of mitochondrial method for cardiomyocytes purification.[76]

II.7. From non-defined to defined conditions for hPSC culture

The development of synthetic coatings and bioreactors that support hPSC expansion and self-renewal within defined culture conditions that are free from xenogeneic contamination has been one important focus of hPSC research[80]. The establishment of defined culture conditions and synthetic matrices will facilitate studies focusing the molecular basis of pluripotent stem cell self-renewal and differentiation (figure II-20). When combined with three-dimensional cultures in bioreactors using microcarriers to support cell adhesion, these systems will also enable large-scale expansion for future clinical applications.

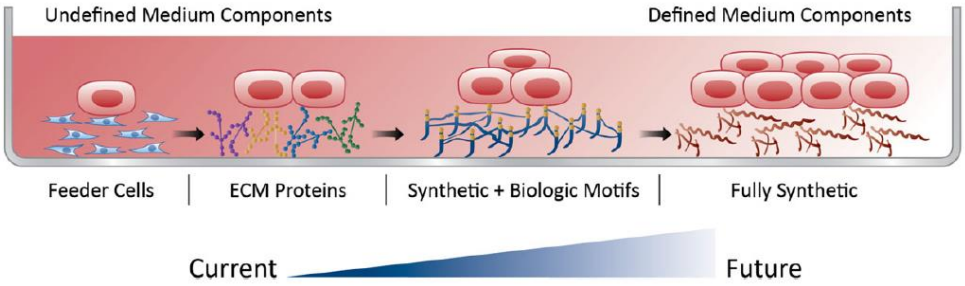

Figure II-20 – Evolution of human pluripotent stem cell (hPSC) culture. Progression (left to right) from co-culture with feeder cells (fibroblasts represented in blue) and serum-containing medium, to feeder-independent cultures in chemical-defined medium. The gradient transition from dark to light red color (left to right) indicates the complexity of the culture medium, from undefined to defined components.[80]

II.7.1. Feeder layer culture systems

Current practices to maintain human pluripotent stem cells (hPSC), which include induced pluripotent stem cells and embryonic stem cells, in an undifferentiated state typically depend on the support of feeder cells such as mouse embryonic fibroblasts (MEFs)[80]. Feeder cells such as MEFs define support the self-renewal of hPSC by the secretion of essential growth factors, such as TGFβ, activin A, laminin-511 and vitronectin. However, culture conditions that depend on this undefined system limit the ability to interpret mechanistic studies of how hPSC interact with their extracellular environment to remain in an undifferentiated state and to make fate-changing lineage decisions[80]. Feeder cells and their products can also be a source of pathogens for hPSC. For example, in the co-culture of hESCs and MEFs with animal-derived serum replacements, the detection of an immunogenic sialic acid (Neu5Gc) has been reported[81]. This is of particular concern because the presence of non-human sialic acid may induce an immune response upon transplantation of hPSC derivatives. Xenogeneic feeder cells and serum are also a common source of mycoplasma contamination. Because mycoplasma compete with host cells for essential nutrients, mycoplasma contamination of cultured cells may affect diverse aspects of cell physiology such as cell growth, phenotype, karyotype, and induction of cytokine expression[80].

II.7.2. Feeder-free culture systems

One of the first examples of alternatives to feeder-dependent PSC cultures was the demonstration of long-term culture of hESCs on tissue culture plates coated with Matrigel in combination with MEF-conditioned medium[82]. Matrigel is composed mainly of laminin, collagen IV, heparin sulfate proteoglycans, entactin, and growth factors[80]. However, Matrigel is derived from Engelbreth-Holm-Swarm mouse sarcomas, and thus, exhibits lot-to-lot variability and can introduce unwanted xenogeneic contaminants[80]. Therefore, Matrigel is not an ideal substrate for feeder-free culture of hPSC if the primary objective is to culture these cells for eventual human therapies. Nevertheless, Matrigel remains one of the most commonly used substrates and has served as an important starting point to define the requirements for hPSC growth and differentiation.

Alternatively, Laminin-coated surfaces have demonstrated to support the growth of hESCs[82]. In contrast, when cultured on fibronectin and collagen IV, the self-renewal of hESCs is compromised[82]. It has also been reported that the specific laminin isoforms -111, -332, and -511 support the adhesion and proliferation of undifferentiated hESCs, while isoforms -211 and 411 do not[80]. Following this hypothesis, vitronectin has also been shown to support hESC self-renewal via integrin aVb5[83]. Similarly, E-cadherin, which mediates cell-cell interactions and has been involved in hESC colony formation and self-renewal, has been used as a substrate for long-term culture of hPSC[80].

As mentioned previously, the secretion of soluble factors by feeder cells influences the fate of PSCs in self-renewal and differentiation. Consequently, conditioned medium from feeder cells has been commonly not only used in presence of feeder layers but also used to culture hPSC in feeder-free conditions. However, due to its undefined characteristics, variability, and the risks associated with contaminants, new practices include the use of chemically defined culture medium. Several culture media formulations, such as mTeSR1 and E8[83], have been developed to support the culture of hPSC in feeder free conditions. The combination of chemically defined medium and xenogeneic-free biological substrates represents an important progress in the generation of clinical-grade culture systems for hPSC. Nevertheless, for large-scale expansion of hPSC in chemically defined and clinically compliant conditions, biological substrates have some disadvantages. These include factors such as batch-to-batch variability, limited scalability, difficultly in isolation, expense to manufacture and the need to ensure pathogen-free conditions. As important alternatives, synthetic substrates that support the proliferation of undifferentiated hPSC have been developed[84]. These synthetic environments exhibit little batch-to-batch variation, are defined, are reproducible and are stable. Due to the nature of hPSC, synthetic substrates must allow cell adhesion, spreading, self-renewal and subsequent colony formation of undifferentiated hPSC[84]. Furthermore, hPSC cultured on synthetic substrates must retain pluripotency and must not develop chromosomal and genomic abnormalities. In addition, synthetic coatings should demonstrate efficacy in the long-term

culture of multiple stem cell lines/types, be cost-effective and have the potential to be scaled-up for commercial purposes.

Several defined synthetic substrates that allow the long-term propagation of hPSC have been developed, such as peptide or protein-based systems, polymers, and polymers in conjunction with biomolecules. Among the peptide-based systems is the heparin-binding peptide GKKQRFRHRNRKG conjugated to an alkanethiol self-assembled monolayer for the long-term culture of hESCs[80]. Similarly, arrays of laminin-derived peptides have also been shown to support hESCs. Polymer-based substrates include the hydrogel poly2-(methacryloyloxy)ethyl dimethyl-(3-sulfopropyl) ammonium hydroxide (PMEDSAH). The surface coatings composed of PMEDSAH are fully synthetic and are extremely effective in sustaining the long-term expansion of hESCs and hiPSC[82]. Other polymers coatings for hPSC include polymer Hit9 and the aminopropylmethacrylamide (APMAAm) and Poly(methyl vinyl ether-alt-maleic anhydride) (PMVE-alt-MA)[80]. Other research groups have taken a hybrid approach, using polymers as the base substrate and then modifying the surface with biomolecules. For example, Synthemax (Corning), an acrylate polymer modified with amino-containing peptides, has been shown to be effective in the prolonged culture of multiple hESC lines[84]. To date, all these substrates have been used in the culture of multiple hESC and hiPSC lines.

Biomedical and research products such as culture plates to expand PSCs must be sterilized before use and thus it is important for synthetic substrates to be compatible with common sterilization techniques such as electron beam and c-radiation. However, substrates with biologic components such as proteins and peptides cannot be sterilized because the sterilization treatment may denature or degrade them[82]. At this time, only Synthemax and PMEDSAH have shown compatibility with common sterilization methods[84]. Another disadvantage for the inclusion of peptides and proteins in stem cell culture substrates is that these surfaces do not allow to be reused once biomolecules are known to undergo degradation from metalloproteases secreted by cultured cells, which for future clinical applications increases significantly the costs[80]. Due to the large number of stem cells that will likely be needed for cell-based regenerative therapies, it will be important to use processes to generate substrates that can be scaled-up from the bench top to high-throughput manufacturing processes[43].

II.8. Scaling Up the production of hPSC-CMs

The left ventricle of the human heart contains about $5.8x10^9$ cardiomyocytes. Given the fact that 25% of the cells are lost during a typical myocardial infarction leading to heart failure, cell transplantation strategies to regenerate the myocardium would require several hundred millions of cells[30]. Additionally, transplantation of an even greater number of cells may be required to replace this cell loss because of the relatively low survival of the grafted cells. Therefore, a major barrier for the clinical use of hPSC-CMs is the generation of sufficient

numbers of cardiomyocytes[34,85]. A scalable platform for human pluripotent stem cell (hPSC)-derived cardiomyocyte production can provide a fast source of CMs not only for cell therapies but also for drug screening and cardiotoxicity tests[86] (figure II-21). Given this fact, tissue engineering may provide an alternative to organ and tissue transplantation by developing cultured functional tissues *in vitro* for subsequent implantation. Engineering tissue is generally achieved by seeding autologous cells into a three-dimensional scaffold/matrix followed by *in vitro* culture. Once implanted, the seeded scaffold starts to remodel and integrate with the surrounding tissue. The development of dynamic systems for *in vitro* investigation of three-dimensional cardiovascular cell growth is an essential aspect of tissue engineering[87].

The main advantage of a dynamic cell culture environment is that experimental parameters can be precisely controlled. To study and achieve this goal, various forms of bioreactors have been developed. A bioreactor is a system that simulates the physiological environments for the creation, physical conditioning and testing of cells, tissues, support structures and organs *in vitro*[87]. Fundamentally, a bioreactor for cardiovascular applications tries to mimic the parameters that exist *in vivo*. In the design of a bioreactor, both the biomechanical and biochemical controls are essential in the creation of a simulated physiological environment for cell and tissue growth. Pulsatile forces, pressure, flow rate, compression, expansion, shear stress, frequency, stroke rate and stroke volume are extremely important factors to be considered. The biochemical environment is also important for cellular proliferation and tissue development, such as the transfer of nutrients and the removal of waste products[87].

To design bioprocesses capable of producing clinical-grade stem cell-derivatives at this scale for numerous patients in a cost effective, pathogen-free, and reproducible manner, it is essential that materials used for stem cell culture and differentiation are fully defined (synthetic or recombinant produced), with no feeder cell layers, conditioned media, or animal or human-derived serum or proteins[88].

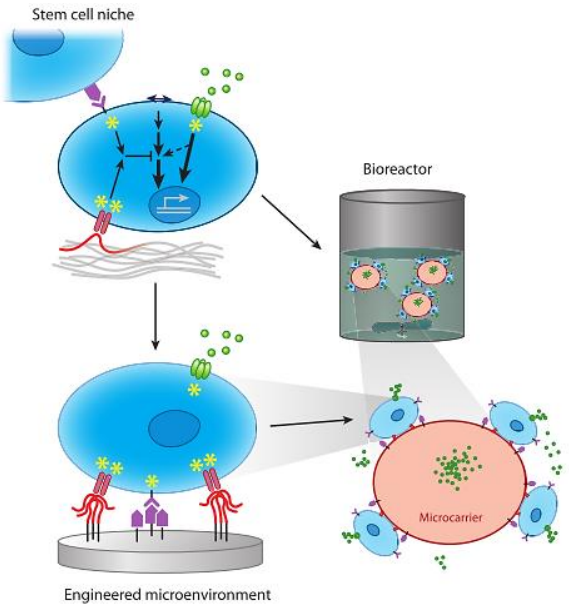

Figure II-21 – Engineering approaches for stem cell biology and therapeutics. The study of the regulatory factors involved in microenvironment signaling can be applied in engineering synthetic microenvironments to study and control stem cell behavior *ex vivo*. This will facilitate the design of bioreactors for large-scale and clinical-grade stem cell therapeutics (adapted from Ashton, R. S. et al., 2011).

II.8.1. Large-Scale expansion of Pluripotent Stem Cells

Significant advancements have occurred in single-phase stirred suspension bioreactors (SSBs), which generally contain only culture media and cells, however the heterogeneity of cell aggregates, such as embryoid bodies (EBs), remains the main obstacle for producing homogenous cultures of undifferentiated cells[88,89]. Therefore, is still uncertain if the appropriate control of hESC or hiPSC, can be achieved using the standard single-phase SSB.

Several groups have utilized ROCK inhibition with single-phase SSB culture to expand hPSC, using media supplemented with animal-derived ECM proteins as well as defined culture conditions[90]. However, 30–50% of the cell culture is lost during sub-culturing, which must be performed periodically to limit the development of larger cell aggregates (>500 μm in diameter) and thus avoid spontaneous cell differentiation and cell death due to limited oxygen and nutrient diffusion. The use of microcarriers for the expansion of undifferentiated hESC can be an alternative strategy and has been described by several groups[91,92]. These studies have demonstrated that hESC grown on microcarrier-based platforms achieved higher cell densities than those grown on tissue cultures plates while maintaining all the characteristics of undifferentiated hESC. However, similarly to single-phase SSBs, two-phase microcarrier SSBs also suffer from loss of significant cell numbers during sub-culturing. Development of microcarriers with novel 2-D synthetic culture surfaces that improve the microcarrier seeding protocols may improve the development of fully defined two-phase microcarrier SSBs[93].

Although EB and microcarrier culture systems have been widely investigated for PSC culture in SSBs, these methods expose the cells to shear stress and conditions that may permit aggregation of cell clusters. Increased shear stress or cell cluster aggregation can negatively affect proliferation rates, but reducing the agitation speed to avoid this problem can compromise optimal gas and nutrient transfer rates[94]. As an alternative to cell adhesion to the exterior of solid carriers, several studies have explored cell microencapsulation within hydrogels to physically ‘protect’ proliferating cell clusters from the bioreactors fluidic environment. Using conditioned medium, static cultures with hyaluronic acid–based hydrogels were found to limit EB formation and actively promote the pluripotent state of encapsulated hESCs[95]. Also in static culture, alginate-based hydrogels were able to limit cluster size and maintain the pluripotency of encapsulated hESCs over extended periods in defined culture conditions[95]. In SSBs using conditioned medium, agarose hydrogels supported the expansion of encapsulated hPSC. Alternatively, alginate-poly-L-lysine hydrogels permitted hPSC expansion while limiting cell aggregate size to increase within the hydrogel capsule[88]. The choice of microencapsulation technique may vary for different processes, nevertheless, hydrogel pore size, porosity, and mechanical properties will have to be engineered to achieve the desired hPSC growth profiles.

II.8.2. Large-Scale Differentiation of Pluripotent Stem Cells

Although stem cells can be effectively differentiated in 2-D static culture, bioreactor protocols for derivation of therapeutic progeny from hPSC typically use EB cultures in SSBs because these are more ready to be scalable due to their aggregate conformation[58]. However, EBs can become resistant to morphogenic diffusion and as a result, hPSC in EBs can spontaneously differentiate into cell derivatives of the three embryonic germ layers (endoderm, mesoderm and ectoderm), such as neural, hematopoietic, endothelial, cardiac, or pancreatic cells.

Differentiation protocols using two-phase microcarrier SSBs are a viable alternative to monolayer culture and EB protocols due to their scalability and efficiency. Two-dimensional monolayer cultures offer the benefit of exposing cells to more uniform conditions, and differentiation of hPSC on microcarriers may combine this advantage with scalability. A recent microcarrier SSB protocol have been reported to differentiate hPSC into cardiomyocytes[91]. In this study, a common improvement observed in all the types of the microcarriers tested was a higher cell expansion fold compared to EB cultures. The cell expansion fold for the EB cultures was about 1.5, whereas in microcarrier cultures it ranged between 2.3- and 3.4-fold[91]. This difference can be attributed to the surface area provided by the microcarriers for the hESC growth. The main factor that affected hESC aggregate formation and their size distribution was the microcarrier shape and size. Modification of microcarrier surfaces with synthetic polymers known to support hPSC culture could further enhance the scalability of microcarrier bioreactors for differentiation of hPSC.

For clinical applications, differentiated CMs must be separated from the microcarriers. The aggregates can be disaggregated into single cells by an enzymatic treatment (for example, collagenase and trypsin)[91]; and then, the CM can be purified from the suspension of cells and microcarriers by either using antibodies to CM surface markers or, alternatively, by loading on a density gradient (like Percoll gradient)[72].

Finally, hydrogel microencapsulation SSBs may increase control of hPSC fate because hydrogels can be engineered to present fate-instructive ligands to encapsulated cells in 3-D microenvironment. Differentiation of hPSC encapsulated in alginate, hyaluronic acid, and agarose hydrogels into cardiomyocytes (in SSB), has also been demonstrated[88].

II.8.3. 3-D Scaffolds

As previously referred, designing a tissue-engineered scaffold requires the consideration of a large number of variables: material; porosity; pore size; mechanical stability; degradability; biocompatibility; hydrophobicity; and biodegradability. Scaffolds are typically fabricated by either natural materials or synthetic materials. These scaffolds can be engineered with the desirable shape and micro-architecture (pore size and porosity), as well as being modified to have desired bioactive properties that will improve cellular growth in a biomimetic system. Polymeric scaffolds should possess a network of channels and interconnected pores to facilitate transport of nutrients and metabolites to allow the growth of large numbers of cells and the formation of cell to cell interactions. Pore size and high surface area to volume ratio are also key parameters for cell spreading and expansion[95]. Pore size affects cellular adhesion, distribution and formation of an ECM by specific cell types; it has been suggested that the pore size in scaffolds can be used to preferentially support or prevent the growth of specific cells. A high surface area to volume ratio is necessary for in vitro cell attachment, proliferation and subsequent matrix deposition[96].

Dawson et al. have recently reported the efficient differentiation of mouse ES-derived EBs into beating cardiomyocytes on collagen scaffolds[97]. The cardiomyocytes contained sarcomeric components, including organized bundles of myofibrils and Z bands as expected for embryonic stem cell-derived cardiomyocytes. It has also been described the use of chitosan nanofiber scaffolds as a 3-D cardiac culture model, using both electrospinning[98] and photolithography techniques[99]. Chitosan is a natural polysaccharide biomaterial that is biocompatible, biodegradable, nontoxic, and cost effective. The chitosan was used to provide a structural scaffold similar to the extracellular matrix (ECM) *in vivo*. Interestingly, the cultures demonstrated synchronized contractions involving large tissue-like cellular networks and exhibited expression of cardiac Troponin I (figure II-22). These results provided the first evidences that 3-D chitosan nanofibers and patterned cultures can be used as a potential scaffold that can retain cardiomyocyte morphology and function.

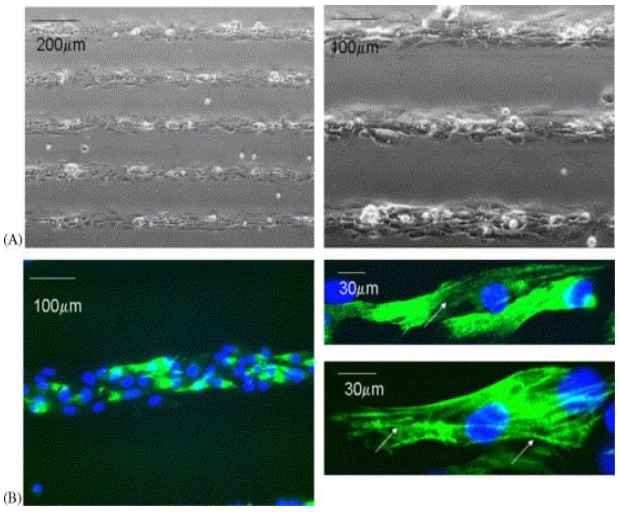

Figure II-22 – Patterning of cardiomyocytes by photolithography. (A) Neonatal rat cardiomyocytes were seeded on chitosan patterned glass surfaces at 8 days of culture. The cells adhered to glass exhibited spontaneous contractions. (B) Patterned cardiomyocytes express cardiac troponin I (green) and exhibit a developed contractile apparatus (arrows).[99]

II.9. Future applications and challenges of *de novo* cardiomyocyte production

One of the main long-term goals of *de novo* cardiomyocyte production is to provide a source of donor cardiomyocytes for cell replacement in damaged hearts[33] (figure II-23). Many forms of heart disease, including congenital defects and acquired injuries, are irreversible because they are associated with the loss of non-regenerative, terminally differentiated cardiomyocytes[8]. In cases of myocardial infarction, 1 billion cells would potentially need to be replaced[30], highlighting the need for fast, scalable and reproducible methodologies for *de novo* cardiomyocyte production. A major challenge in this field is to establish the most efficient way for the transplantation of these cells. Transplantation of single cell suspensions is easy, but engraftment of 3-D engineered scaffolds may be the best approach for replacing scar tissue. In addition, concerns over cell survival, immune rejection, electrical maturation, electrical coupling, arrhythmia, and whether autologous hiPSC possess immune privileges still need to be addressed[33].

A second application lies in novel cardiac drug discovery, development, and safety testing, a process that is expensive and lacks of reliable methods to accurately mimic the human cardiac physiology. Many drug discovery programs have failed because targets validated in animal models proved to be unreliable in humans. The pharmaceutical industry currently invests approximately $1.5 billion to successfully develop a candidate drug from primary screening to market[33]. Among the drugs that ultimately make it to market, many are later removed due to side effects associated with electrophysiological alterations of the heart. The use of *de novo* human cardiomyocytes offers the pharmaceutical industry a tool for pre-clinical screening of candidate drugs to treat arrhythmia and heart failure. Studies have already demonstrated that hiPSC-derived cardiomyocytes will react to cardioactive drugs with the expected response, indicating that these cells can be used in the context of larger predictive toxicology screens[73].The development of new screens using human cardiomyocytes will allow time saving and will reduce the cost of bringing new drugs to the market[33].

A third application is in developmental biology, disease modeling and personalized medicine. Deriving hiPSC from patients with specific cardiac diseases, differentiating them to cardiomyocytes, and then performing electrophysiological and molecular analyses may provide a unique tool to understand the molecular mechanisms of a disease. By generating beating cardiomyocytes from iPS cells derived from these sensitive individuals, candidate drugs could be tested *in vitro*. Some studies have tried to recapitulate genetic disease phenotypes *in vitro*, such as long QT syndromes[75]. The combination of novel drug discovery and efficacy testing with cardiomyocytes derived from patient-specific hiPSC is a potentially option for personalized medicine.

In addition to poor differentiation yields and the tumorogenic risk associated with the use of retroviruses or lentiviruses to generate hiPSC, most importantly, there is still a great inconsistency in differentiation efficiency between various hiPSC lines[100]. This variability is likely due to genetic and epigenetic differences between hPSC lines that directly impact their cardiac differentiation capacity. Therefore, there is the need for a reproducible, fully optimized and universally applicable differentiation system capable of overcoming the interline variability that commonly exists amongst human pluripotent stem cells. The development of a universal cardiac differentiation protocol that can be used in multiple pluripotent stem cell lines allows immediate application to genetically diverse hiPSC lines created from patients with cardiac related diseases[101]. The uniformity of the electrophysiological profiles gives these cells the potential for future high-throughput cardiotoxicity testing and novel drug development.

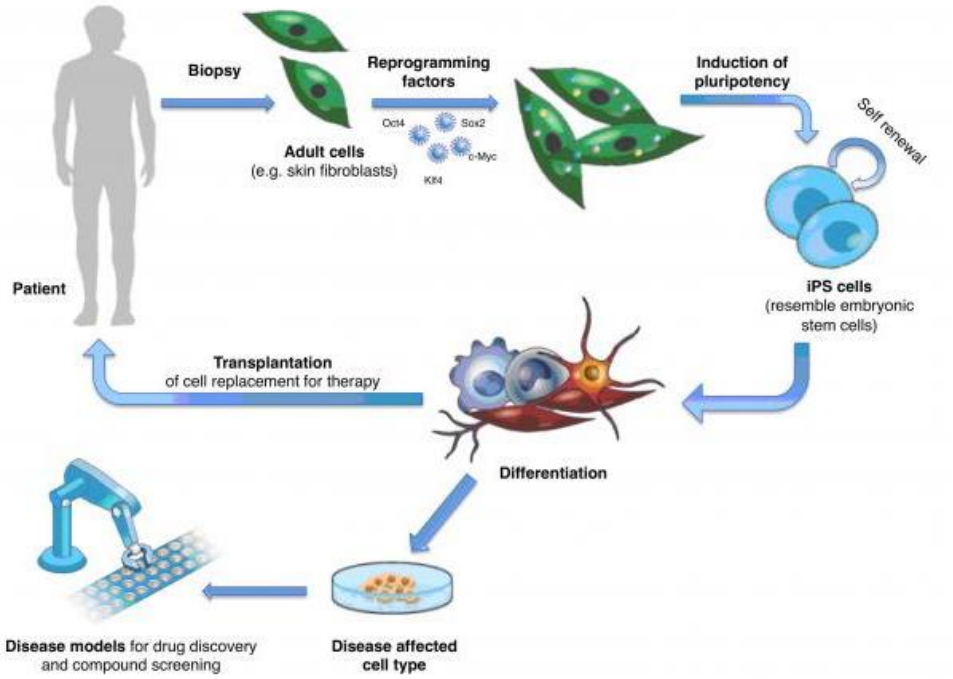

Figure II-23 – Future applications of human iPS cells: creation of human models of human disease *in vitro* for studying the molecular mechanisms of disease, for screening drug candidates, assessing drug safety and toxicity and transplantation of cells in the patient[43].

III. Materials and Methods

III.1. Substrate preparation

III.1.1. Gelatin

Gelatin 0.1% (v/v) was diluted from 2% stock solution (Sigma®) in phosphate-buffered saline (PBS; Gibco®). The culture plates were incubated for at least 30 minutes at 37ºC, guaranteeing that the whole surface is covered, and the gelatin was removed prior to cell seeding.

III.1.2. Matrigel

A 200 µl Matrigel™ (BD Biosciences) aliquot of stock solution (1.5 mg protein/aliquot) was thawed overnight in ice, at 4ºC. Then, it was diluted in a proportion of 1:30 on ice cold Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM-F12; Gibco®) and the diluted solution was used for covering the bottom of the wells and let to incubate for at least two hours at room temperature. Matrigel™ was removed prior to cell seeding and replaced by cell culture medium.

III.1.3. Human Recombinant Laminin-511

Prior to the laminin coating, the well surface (12-well plate) was covered with 1 mL of poly-ornithine solution and incubated at 37ºC for 30min or, optionally, overnight. After slowly thawing the recombinant laminin-511 (Sigma®) at 4ºC, it was diluted with 1x sterile PBS to obtain a final concentration of 30 µg/mL of laminin coating solution (LCS). Afterwards, the poly-ornithin solution was removed and the LCS was distributed on the wells (500µL/well) and the plates were incubated at 37ºC, 5% CO_2 for 2 hours. Before cell seeding, LCS was removed and the coated wells were washed twice with 1x PBS.

III.1.4. Human Recombinant Fibronectin

A human recombinant fibronectin (Sigma®) aliquot of stock solution (1mg/mL) was slowly thawed at 4ºC. 72 uL of the solution was diluted with 3 mL of 1x sterile PBS. The diluted solution was used to cover the bottom of 6 wells of a 12-well plate (3 ug human recombinant fibronectin per cm^2). The plate was left to incubate at room temperature for at least 60 minutes prior cell seeding.

III.1.5. Human Recombinant Vitronectin

One or two aliquots of 60 uL vitronectin (StemCell™ Technologies) were taken from -80°C and put on ice. Upon thawing, each aliquot of vitronectin was resuspended in 6ml cold sterile PBS. The vitronectin solution was plated into each well of a 12-well plate (500µL/well).

Vitronectin coating should cover the entire well surface. The plate was left inside the hood for at least 60 minutes.

III.2. Culture Media preparation

III.2.1. mTeSR1 medium

mTeSR1 medium (StemCell™ Technologies) is a commercially-available, and fully-defined serum-free medium for the maintenance of both hESCs and hiPSC under feeder-independent conditions. The medium is formulated for use with Matrigel™ coating matrix and is composed by a basal medium and a supplement. The complete medium provides a variety of growth factors or proteins, such as gamma-aminobutyric acid (GABA), bFGF, TGF-β1, human insulin and bovine serum albumin (BSA), in addition to a variety of amino acids, vitamins and other components (Thomson, J.A. and Ludwig, T. 2010). The medium was prepared according to the manufacturer's instructions.

III.2.2. Cardiac differentiation basal medium

The basal medium used in the first 5 days (Lian et al. protocol) or 7 days (Laflamme et al. protocol) of cardiac differentiation of hiPSC was prepared by adding 2% (v/v) of 1x B27 without insulin (Invitrogen™), that were previously thawed at 4ºC, to RPMI1640 (Invitrogen™). After this period of 5 or 7 days, the medium was changed to RPMI1640 supplemented with 2% (v/v) of 1x B27 with insulin (Invitrogen™).

III.2.3. Neural Differentiation medium

The medium used for neural commitment of hiPSC is based on KO-DMEM/SR medium, which comprises a base of Knock-Out DMEM (KO-DMEM; Gibco®) supplied with Knockout-Serum Replacement (KO-SR; Invitrogen™), MEM-non essential amino acids (Gibco®), L-glutamine (Gibco®) and β-Mercaptoethanol (Sigma®). The quantitative composition of the medium is described in Table III-1.

Table III-1- Composition of KO-DMEM/SR culture medium.

Component	Concentration
KO-DMEM	~ 79% (v/v)
KO-SR	~ 20% (v/v)
MEM-non essential amino acids	~ 1% (v/v)
L-glutamine	1 mM
β-Mercaptoethanol	0.1 mM

Before the addition of the culture medium to the wells, it was supplemented with 10 µM of the activin/nodal inhibitor (SB-431542, Sigma®) from a 10mM stock solution and the antagonist of BMP signalling, the small molecule inhibitor of BMP receptors, LDN-193189 (StemGent™) at 100 nM from a 10 mM stock solution.

III.2.4. N-2 medium

N-2 medium, used in hiPSC neural commitment, is composed by the basal medium DMEM/F-12, GlutaMAX™ (Gibco®) and the chemically-defined and serum-free N-2 Supplement (Invitrogen™), D-(+)-glucose (Sigma®) and human recombinant insulin (Sigma®). This medium appears to be selective for neural cells. The complete formulation of the medium is described in Table III-2.

Table III-2 - Composition of N-2 medium.

Component	Concentration
DMEM/F-12, GlutaMAX	97% (v/v)
N-2 supplement	1% (v/v)
Glucose	1,6 g/L
Insulin	20 µg/mL

III.2.5. hPSC Washing medium

The composition of hPSC washing medium is similar to the composition of KO-DMEM/SR culture medium, however the percentage of KO-DMEM is 88% and the percentage of KO-SR is 10%. hPSC washing medium is also supplemented with 1% Penicilin/Streptomycin.

III.3. Cell dissociation reagents

III.3.1. EDTA dissociation solution

EDTA dissociation solution was prepared by adding 1000 µL of 0.25M EDTA (Sigma) and 0.9 g NaCl (Sigma) into 500 mL PBS (Gibco®). The prepared solution was sterilized by filtration and stored at 4°C.

III.3.2. Accutase™

Accutase™ (Sigma®) is a cell dissociation solution composed by proteolytic and collagenolytic enzymes, allowing cell-cell and cell-ECM dissociation with no mammalian- or bacterial-derived products.

III.4. Cell Culture

III.4.1. Cell lines

The human induced pluripotent stem cell line **iLB-c1-30m-r12** used in differentiation experiments was kindly provided by Professor Oliver Brüstle from the Institute of Reconstructive Neurobiology, LIFE & BRAIN Center, University of Bonn, Germany. These cells were reprogrammed from human foreskin fibroblasts that were consensually collected from healthy donors and reprogrammed using defined factors by retrovirus transduction.

Commercially obtained hiPSC used in cardiomyocyte differentiation, **iPS-DF19-9-11T.H** and **iPS-DF6-9-9T.B**, were derived from human foreskin fibroblasts by a single transfection with oriP/EBNA1 (Epstein-Barr nuclear antigen-1)-based episomal vectors, which included OCT4, SOX2, NANOG, LIN28, c-Myc, KLF4, and to counteract the possible toxic effects of c-Myc expression, it was included the SV40 large T gene, SV40LT. These cell lines were acquired from the Laboratory of Dr. James Thomson, University of Wisconsin, United States of America.

III.4.2. Thawing of hiPSC to Matrigel

Cryovials (BD Biosciences) of frozen hiPSC stocks were directly taken from liquid nitrogen and immersed in a 37°C water bath during approximately 10 seconds. In this step, cells should not be thawed completely. Within the hood, 1mL of warm washing medium with 10 µM ROCK inhibitor (Y-27632; StemGent^{TM}) was carefully added, drop by drop, to the cryovial. The cell suspension was transferred to a 15mL Falcon tube with 4mL of warm washing medium also with 10 µM ROCKi and centrifuge at 1000rpm for 5 minutes. The pellet was resuspended in mTeSR1 (StemCell™ Technologies) supplemented with 10 µM ROCKi. The cell suspension was transferred to matrigel-coated well and incubated at 37°C, 5% CO_2. After 24 hours, the exhaust medium was removed and replaced by fresh mTeSR1 medium, without ROCKi. The medium was changed daily until the next passage.

III.4.3. Passaging of hiPSC with EDTA dissociation solution

hiPSC maintained on Matrigel and mTeSR1 were dissociated with an EDTA solution to remove magnesium and calcium from the medium. When rising, the EDTA solution was added and aspirated immediately without incubating. When washing with EDTA, the solution should be added to the wall of the well slowly to avoid washing the colonies off the plate too early. This washing step was repeated twice to ensure that all magnesium and calcium is removed, however in the last step, EDTA was let it sit for 5 min at room temperature within the hood. Cells were recovered with mTeSR1 medium, which should be added rapidly to wash the colonies off the plate and disperse the colonies as quickly as possible. The magnesium and calcium in the medium will neutralize any remaining EDTA. This rising step was repeated at least once in order to recover all cells from the plate. Cells were seeded at $2x10^5$ cells/well in a matrigel and

vitronectin-coated 12-well plate in mTeSR1 medium and incubated at 37°C, 5%CO_2. After 24 hours, the exhaust medium was removed and replaced by fresh mTeSR1 medium. The medium was changed daily until the next passage. At each passage, viable and dead cell numbers were determined by counting in a hemocytometer under an optical microscope using the trypan blue dye exclusion test (Gibco®).

III.4.4. Cryopreservation of hiPSC

hiPSC maintained on Matrigel and mTeSR1 were pre-incubated for 60 minutes with washing medium containing 10 µM of ROCK inhibitor (Y27632). Colonies in the plate were dissociated into single cells with Accutase (Sigma®) at 37°C for 7 minutes. Cells were gently detached from the plate by using a 5mL pipette, with washing medium and collected to a 15mL Falcon tube. The rising step was repeated at least once in order to recover all cells from the plate. Cells were centrifuged at 1000 rpm for 5 minutes. The pellet was gently resuspended in freezing medium (90%KO-SR +10%DMSO (Sigma®) at a concentration of at least $4x10^6$ cells/mL. 250 µM of the cell suspension was transferred into each cryovial. The cryovials were placed at 80°C overnight. The next day, the cryovials were transferred to liquid nitrogen until further use.

III.4.5. Neural Commitment of hiPSC

For neural commitment, hPSC were first inoculated as single-cells (100.000 cells/cm2), in mTeSR1 medium and allowed to expand for 3/4 days. The neural commitment was performed using a BMP inhibitor, the small molecule inhibitor of BMP receptors, LDN-193189 (StemGent™), at 100 nM from a 10 mM stock solution.

Day 1 of neural commitment was initiated by replacing mTeSR1 medium for neural differentiation medium and supplemented with 10 µM of SB-431542 and 500 ng/mL Noggin or 100 nM LDN-193189. The medium was changed daily until day 4. Upon day 5 of differentiation, the TGF-β (activin/nodal) inhibitor was withdrawn and increasing amounts (25%, 50% and 75% (v/v)) of N-2 medium were added to the differentiation medium every two days while maintaining the same BMP inhibitor concentration. On day 11, the medium was replaced by 100% N-2 medium with the same concentration of BMP inhibitor.

III.4.6. Cardiac Commitment of hiPSC

III.4.6.1. Cardiac-Directed Differentiation via Activin A and BMP4

i. Laflamme et al. protocol

hiPSC maintained on Matrigel in mTeSR1 were dissociated with EDTA solution and seeded onto a Matrigel-coated 12-well plate at $1x10^5$cells/cm^2 in mTeSR1, which was changed daily. When cells achieved 100% confluence, cells were treated with 100 ng/mL activin A (R&D) in RPMI/B27-insulin. After 24 h, the medium was changed to RPMI/B27-insulin supplemented with 10 ng/mL BMP4 (R&D) for another 4 days. At day 5, the medium was changed to RPMI/B27-insulin. At day 7 the cells were transferred to RPMI/B27, and medium was changed every 3 days.

ii. GiAB protocol

The GiAB protocol is a modified version of the monolayer directed differentiation protocol reported by Laflamme et al., with Gsk3 inhibitor pre-treatment of undifferentiated cells to provide more robust cardiac differentiation in multiple hPSC lines. When Gsk3 inhibitors were used to stimulate cardiomyocyte differentiation, cells were cultured in mTeSR1 containing 3 μM CHIR99021 from day −3 to day 0.

iii. Matrix sandwich protocol

hiPSC maintained on Matrigel and cultured in mTeSR1 medium were dissociated with EDTA solution and seeded onto a Matrigel-coated 12-well plate at the density of 100,000 cells/cm^2. The medium was changed daily, and after 3-4 days when the monolayer of cells reached 80-90% confluence, a thin layer of Matrigel was overlaid by freshly mixing 0.3 mg Matrigel in 8 ml ice cold mTeSR1 medium and replacing the medium in each well with 1 ml of Matrigel containing mTeSR1. Cells were cultured in mTeSR1 medium for another 3 days until the cells were 100% confluent, which is referred to as day 0 when the medium was replaced with 1 ml of RPMI/B-27 without insulin supplement containing Activin A (100 ng/ml) and Matrigel (0.3 mg Matrigel/8 mL fresh medium). After 24 hours, the medium was changed with the same medium as day 0 (1.5 ml/well) without Matrigel but supplemented with BMP4 (5 ng/ml) and bFGF (10 ng/ml, Invitrogen™) for another 4 days without medium change. At day 5, the medium was changed to RPMI plus B27 complete supplement, and the medium was changed every 3 days.

III.4.6.2. Cardiac-direct differentiation via GSK3 inhibitor and Wnt inhibitor (GiWi) protocol

hiPSC maintained on Matrigel in mTeSR1 were dissociated with EDTA and seeded onto a Matrigel-coated cell-culture plate at $1x10^5$cells/cm^2 in mTeSR1, which was changed daily. When cells achieved 100% confluence, cells were treated with 12 µM CHIR99021 (StemGent™) in 2mL RPMI supplemented with B27-insulin for 24 hours. After this period of incubation, the medium was changed to RPMI/B27-insulin. On day 3 of differentiation (72h after addition of CHIR99021), 1 mL of medium was collected from the each well and mixed with fresh RPMI/B27-insulin in a 15mL Falcon tube. To the combined medium was added 2uL of 5mM IWP4 (final concentration is 5uM). The remaining medium in the plate was aspirated and replaced by the prepared medium. On day 5, the medium was changed to 2mL of fresh RPMI/B27-insulin. On day 7 and every 3 days thereafter, the medium was changed to 2mL of RPMI/B27.

III.5. Characterization of hiPSC

III.5.1. Detection of AP using the Alkaline Phosphatase Staining Kit (Stemgent™)

III.5.1.1. Reagents

- **PBST**

10 ml of 1X PBS were mixed with 5 µl of Tween20* for a final concentration of 0.05%. The solution was well mixed and stored at room temperature.

*Tween® is a registered trademark of ICI America, Inc

- **AP Staining Solution**

For one well of a 12-well plate, 0.5 ml of AP Staining Solution A was mixed with 0.5 ml of AP Staining Solution B. The solution was prepared just before the initiation of the Alkaline Phosphatase Staining Protocol.

III.5.1.2. Alkaline Phosphatase Staining Protocol

The culture medium was removed and cells were washed twice with 1 ml of PBST (12-well plate). Then, 1 ml of Fix Solution was added and cells were incubated at room temperature for 1 to 2 minutes. The Fix Solution was removed and cells washed with 1 ml of 1X PBST. The 1X PBST was changed with 1 ml of freshly prepared AP Staining Solution. Cells were incubated in the dark at room temperature between 10 and 20 minutes until the color turned purple. AP expression results in a red or purple stain, while the absence of AP expression results in no

stain. The reaction was stopped by removing the AP Staining Solution and wells were washed twice with 1 ml of 1X PBS.

III.5.2. Immunostaining

III.5.2.1. Intracellular markers of hiPSC, hiPSC-CMs and hiPSC-NSCs

Expression of pluripotency markers of hiPSC and early and late markers of hiPSC-CMs and hiPSC-NSCs were accessed by immunocytochemistry. Cells were fixed with paraformaldehyde (PFA) 4% (Sigma®) for 10 minutes at room temperature and washed twice with PBS (Gibco®). To prevent non-specific binding of antibodies, cells were incubated for 60 minutes at room temperature with a blocking solution (0.1% Triton X-100 (Sigma®) in PBS with 10% normal goat serum NGS (Sigma®). Primary antibodies were diluted with a staining solution (0.1% Triton X-100 and 5% NGS) and incubated at room temperature for 120 minutes. Cells were washed twice with PBS and incubated with a secondary antibody for 1 hour at room temperature and protected from the light. Cells were washed three times with PBS and incubated with DAPI nucleic acid stain (1.5 µg/mL in PBS) for 2-3 minutes at room temperature. Finally, cells were washed three times with PBS and the stained cells were visualized under a fluorescence microscope (Leica DMI 3000B), using the software Nikon-AcT1. Primary and secondary antibodies and corresponding dilutions can be visualized in table III-3 and table III-4, respectively.

Table III-3 - Primary antibodies and corresponding features and working dilutions used in intracellular immunostaining.

Marker	Brand	#CAT	Host	Storage	Working dilution
Sox2	R&D Systems	MAB2018	mouse IgG	-20°C	1:1000
Oct4	Millipore	MAB4419	mouse IgG	4°C	1:750
Brachyury T	R&D Systems	#AF2085	goat IgG	-20°C	1:10
Nkx-2.5	Santa Cruz	sc-14033	rabbit IgG	4°C	1:250
α-Actinin	Sigma	AA7732	mouse IgG	-20°C	1:400
cTnT	Thermo Scientific	MA5-12960	mouse IgG	4°C	1:100
Pax6	Covance	PRB-278P	rabbit IgG	-20°C	1:1000
Foxg1	Abcam	ab18259	rabbit IgG	-20°C	1:200
Nestin	R&D Systems	MAB1259	mouse IgG	-20°C	1:1000
a-SMA	R&D Systems	MAB1420	mouse IgG	-20°C	1:1000
Sox17	R&D Systems	MAB1924	mouse IgG	-20°C	1:1000

Table III-4 - Secondary antibodies and corresponding features and working dilutions used in intra- and extracellular immunostaining.

Marker	Brand	#CAT	Host	Storage	Working dilution
anti-mouse IgG Alexa 488	Invitrogen	A11029	Goat	4°C	1:500
anti-mouse IgG Alexa 546	Invitrogen	A11029	Goat	4°C	1:1000
anti-rabbit IgG Alexa 546	Invitrogen	A11034	Goat	4°C	1:500
anti-mouse IgM	Invitrogen	A21045	Goat	4°C	1:500
anti-goat IgG Alexa 488	Invitrogen	A11055	Donkey	4°C	1:500

III.5.2.2. Extracellular markers of hiPSC

Culture medium was replaced by primary antibody solution in KO-DMEM/SR medium, according to the dilutions in Table III-5 and the cells were incubated for 30 minutes at 37ºC. After washing the cells three times with medium, they were incubated for 30 minutes with the secondary antibody solution, according to the dilutions in Table III-4. Finally, the cells were washed another three times with medium and analyzed under a fluorescence optical microscope (Leica DMI 3000B, Germany) and a digital camera (Nikon DXM 1200 F).

Table III-5 - Primary antibodies and corresponding features and working dilutions used in extracellular immunostaining.

Marker	Brand	#CAT	Host	Storage	Working dilution
SSEA4	StemGent	09-0006	mouse IgG	4°C	1:135
SSEA1	StemGent	09-0005	mouse IgM	4°C	1:135
TRA-1-60	StemGent	09-0010	mouse IgM	4°C	1:135
TRA-1-81	StemGent	09-0011	mouse IgM	4°C	1:135

III.5.3. Flow cytometry

III.5.3.1. Intracellular markers of hiPSC and hiPSC-CMs

Eppendorf tubes were coated with 1% Bovine Serum Albumin (BSA; Invitrogen™) solution for 15 minutes. The samples of cell suspension previously stored in 2% PFA were centrifuged at 1250rpm, for 5 minutes and washed twice with 1%NGS, centrifuging both times. Cells were resuspended in 3% NGS, guaranteeing a minimum of $5x10^5$ cells per condition. After removing the BSA solution from the Eppendorf tubes, the cell suspension was equally distributed in each tube, and once again centrifuged at 1000 rpm (Hermle-Z-300k) for 3 minutes. The cell membrane was then permeabilized using a solution 1:1 of 3% NGS and 1% saponin (Sigma®) for 15 minutes, at room temperature. Following a wash with 3% NGS and centrifugation, the obtained pellet was resuspended in primary antibody solution and incubated for 90 minutes in the dark, at room temperature. Afterwards, it was centrifuged and washed twice for 45 minutes in the dark with the secondary antibody solution, including the negative control. After another wash with 1% NGS, cells were resuspended in PBS and analyzed in the FACSCalibur flow cytometer (Becton Dickinson). Primary and secondary antibodies and corresponding used dilutions can be visualized in table III-6 and table III-7.

Table III-6 - Primary antibodies and corresponding features and working dilutions used in intracellular FC.

Marker	Brand	#CAT	Host	Storage	Working dilution
Sox2	R&D Systems	MAB2018	mouse IgG	-20°C	1:500
Oct4	Millipore	MAB4419	mouse IgG	4°C	1:300
Nanog	Millipore	MAB9220	rabbit IgG	-20°C	1:5000
Nkx-2.5	Santa Cruz	sc-14033	rabbit IgG	4°C	1:200
α-Actinin	Sigma	AA7732	mouse IgG	-20°C	1:100
cTnT	Thermo Scientific	MA5-12960	mouse IgG	4°C	1:200

Table III-7 - Secondary antibodies and corresponding features and working dilutions used in intracellular FC.

Marker	Brand	#CAT	Host	Storage	Working dilution
anti-mouse IgG Alexa 488	Invitrogen	A11029	Goat	4°C	1:300
anti-rabbit IgG Alexa 488	Invitrogen	A11034	Goat	4°C	1:300

III.5.3.2. Extracellular markers of hiPSC

The samples of cell suspension previously stored in PFA were centrifuged at 1000 rpm, for 5 minutes and washed twice with PBS, centrifuging both times. Cells were resuspended in 100 µl of FACS buffer, guaranteeing a minimum of $2x10^5$ cells per condition. The primary antibody was added to the FACS tubes and incubated for 15 minutes in the dark, at room temperature. Afterwards, cells were washed twice with PBS and centrifuged both times at 1000 rpm for 5 minutes. Cells were resuspended in FACS buffer with the secondary antibody solution and incubated in the dark for 15 minutes, including the negative control. Cells were washed twice with PBS and centrifuged for 5 minutes at 1000 rpm. Finally, stained cells were resuspended in PBS and analyzed in the FACSCalibur flow cytometer. Antibodies and corresponding used dilutions can be visualized in table III-8.

Table III-8 - Surface antibodies and corresponding features and working dilutions used in extracellular FC.

Marker	Brand	#CAT	Host	Storage	Working dilution
SSEA4-PE	StemGent	09-0003	mouse IgG	4°C	1:10
SSEA1-PE	Biolegend	'125606	mouse IgM	4°C	1:10
TRA-1-60-PE	StemGent	09-0009	mouse IgM	4°C	1:10
TRA-1-81-PE	StemGent	09-0011	mouse IgM	4°C	1:10

IV. Results and Discussion

IV.1. Characterization of human induced Pluripotent Stem Cells

One of the challenges of hiPS cell research is to ensure that cells maintained in culture for long periods of time have not become unstable through chromosomal loss or duplication, or have suffered changes in the expression of the factors necessary to maintain pluripotency. In that manner, regular characterization of stem cell cultures is necessary to guarantee that pluripotent stem cells are as stable as possible and preserve their characteristics: self-renewal, maintenance of pluripotency markers without spontaneous and uncontrolled differentiation and ability to differentiate into the three germ layers[102]. As recommended by the International Stem Cell Banking Initiative (ISCBI)[103], several tests can be performed. These tests include: (i) pluripotency tests; (ii) differentiation tests both *in vitro* and *in vivo*; (iii) karyotype analysis; (iv) gene expression profiling which detects the expression of a common set of pluripotency genes expressed in undifferentiated cells that are down-regulated upon differentiation; and (v) a microbiological test to ensure that the cultures are free of any contaminants.

The objective of this study was to evaluate the characteristics of three hiPS cell lines available at the Stem Cell Bioengineering Lab (iLB-c1-30m-r12, iPS-DF6-9-9T.B and iPS-DF19-9-11T.H) to ensure the stability of hiPSC prior to monolayer cardiac-direct differentiation. In this characterization study, several tests were performed such as intra- and extracellular immunostaining and flow cytometry analysis of pluripotency markers, alkaline phosphatase staining, karyotype analysis and *in vitro* differentiation into the neural lineage by dual inhibition of SMAD signaling[104]. This chapter will comprise 3 main sections, each one corresponding to one hiPS cell line.

hiPSC were cultured on Matrigel and mTeSR1 medium and passaged every 4/5 days with EDTA dissolution solution.

IV.1.1. iLB-c1-30m-r12 cell line

Intracellular staining of pluripotency markers OCT4 and SOX2 demonstrated that iLB-c1-30m-r12 cells at passage 42 maintained a pluripotent state throughout the entire area of the colonies (figure IV-1). iLB-c1-30m-r12 cells maintained also the expression of the pluripotency extracellular markers SSEA-4, TRA-1-60 and TRA-1-81 (figure IV-2). The pluripotency state of iLB-c1-30m-r12 cells was also evaluated using the Alkaline Phosphatase Staining Kit. With this kit is it expected that undifferentiated cells appear red or purple, whereas differentiated cells appear colorless. After staining, iLB-c1-30m-r12 colonies revealed a purple color indicating high alkaline phosphatase activity (figure IV-3).

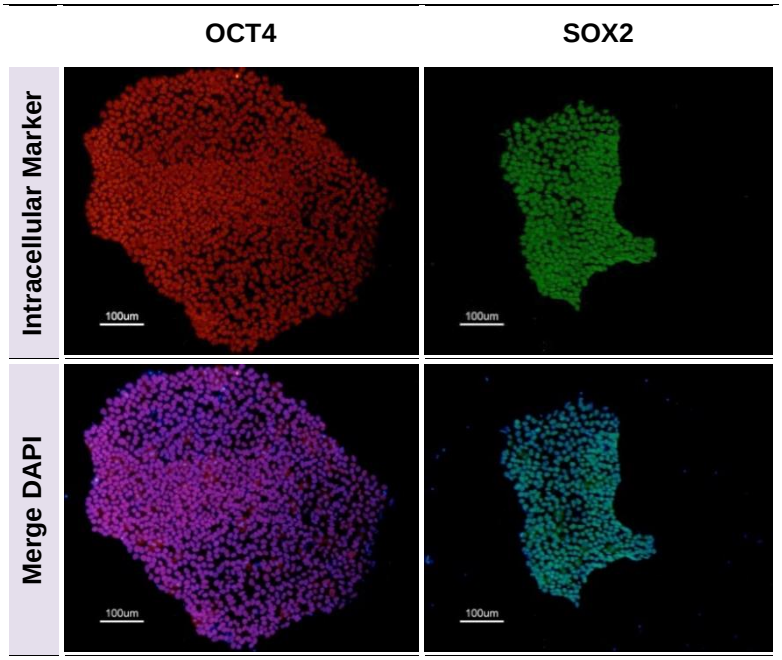

Figure IV-1- Intracellular immunostaining of iLB-c1-30m-r12 cells at passage 42 on Matrigel in mTeSR1 medium. Staining of the transcription factor OCT4 (right) and SOX2 (left) and merge with nuclear staining with DAPI (below). Scale: 100µm.

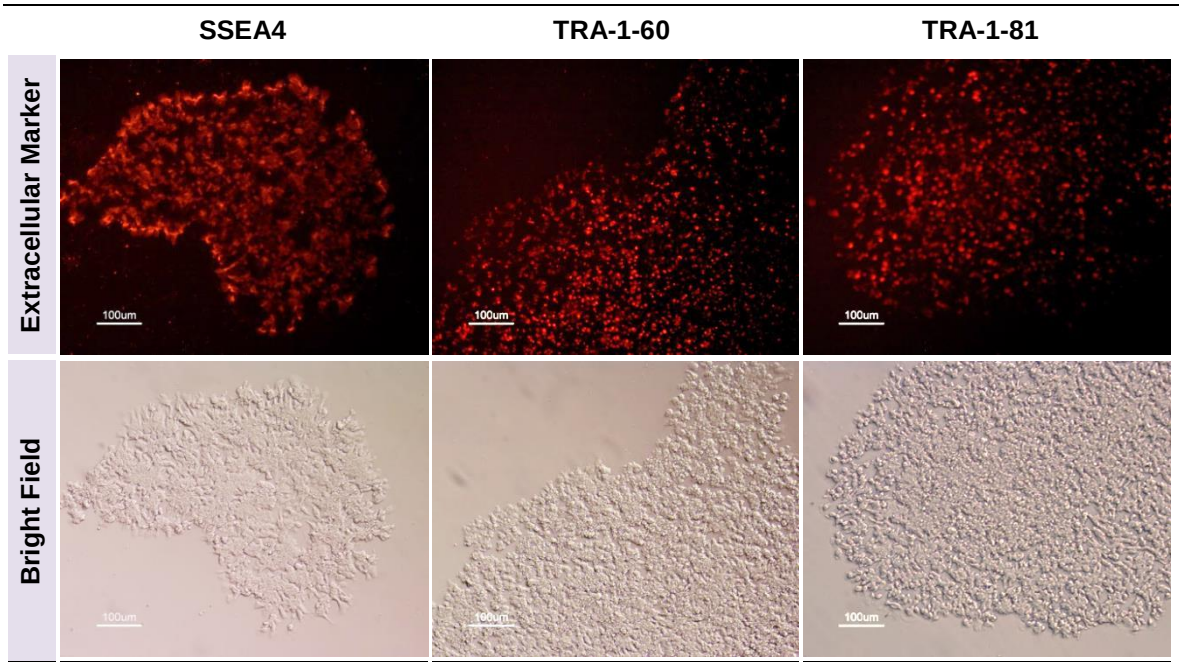

Figure IV-2 - Extracellular immunostaining of iLB-c1-30m-r12 cells at passage 42 on Matrigel in mTeSR1 medium. Staining of the surface marker SSEA-4 (left) ,TRA-1-60 (center) and TRA-1-81 (left) and respectively bright field images (below). Scale: 100µm.

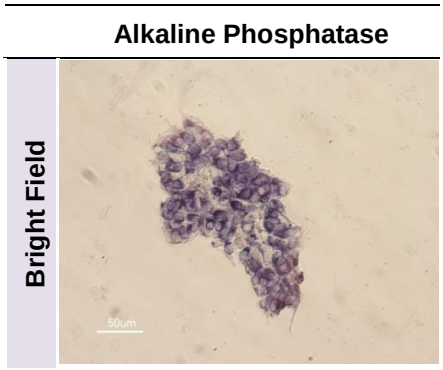

Figure IV-3- Phenotypic assessment of iLB-c1-30m-r12 cells at passage 42 using the Alkaline Phosphate Staining kit. Scale: 50µm.

To confirm these results, iLB-c1-30m-r12 cells were further analyzed by flow cytometry at passage 49 for the intracellular markers SOX2 and OCT4, the extracellular marker TRA-1-60 and the early differentiation extracellular marker SSEA-1 (figure IV-4). This analysis revealed high expression of all pluripotency markers and no expression of SSEA-1, indicating preservation of the expression of pluripotency markers and no spontaneous differentiation within the population. The observed lower expression of TRA-1-60 compared to other markers can be due to inherent limitations of the extracellular staining protocol used for flow cytometry analyses. TRA-1-60 is a carbohydrate epitope present on the cell surface of pluripotent stem cells and thus, when Accutase is used to enzymatically individualize cells by degrading the components of the extracellular matrix and cell-to-cell interaction proteins, it can degrade some of the cell surface proteins, especially with increasing enzyme exposure, reducing the expression of extracellular markers measured by flow cytometry.

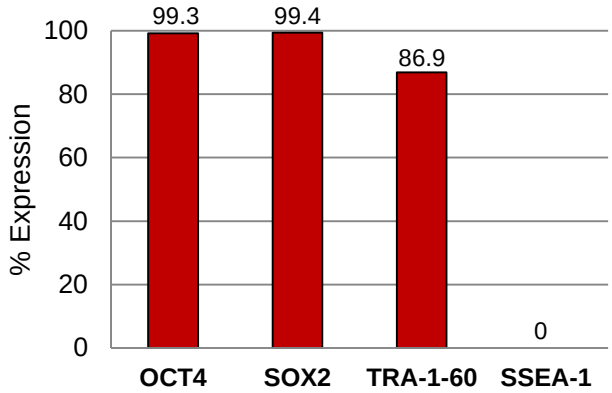

Figure IV-4 - Flow cytometry analysis of iLB-c1-30m-r12 at passage 49 for the pluripotency markers Oct4, Sox2, Tra-1-60 and the early differentiation marker SSEA-1.

To test the differentiation potential of these cells, iLB-c1-30m-r12 cells were differentiated into the neural lineage through the dual inhibition of SMAD signaling. Neural induction was monitored after 12 days by expression of early markers of neuroectodermal differentiation FOXG1, PAX6 and NESTIN, (figure IV-5). Intracellular immunostaining revealed positive staining for all neural stem cells markers. Thus, iLB-c1-30m-r12 cells demonstrated capacity to differentiate into the neural lineage upon TGF-β inhibition with very high efficiency proving the ability as pluripotent stem cells to differentiate *in vitro.*

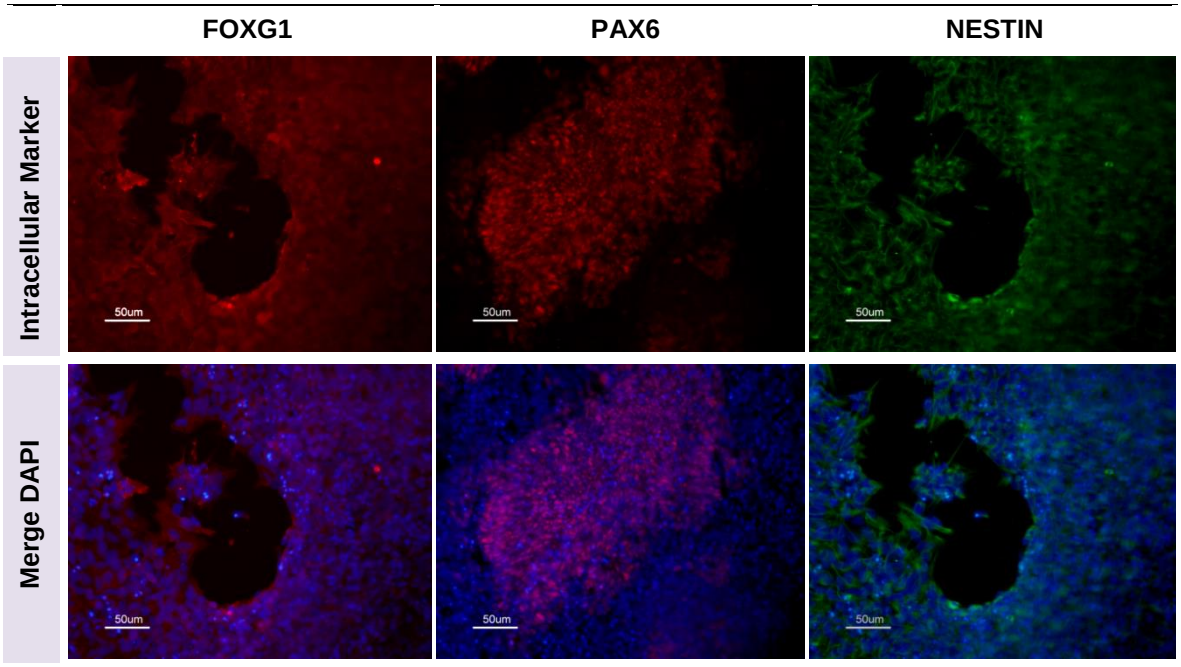

Figure IV-5 – Intracellular immunostaining of neural differentiated cells from iLB-c1-30m-r12 cells. Staining of FOXG1 (left), PAX6 (center) and NESTIN (right) and respectively merge with nuclear staining with DAPI (below). Scale: 50µm.

There was also the opportunity to perform a karyotype analysis of this cell line at passage 51 at the Instituto Português de Oncologia - Centro de Investigação de Patologia Molecular. One hour of incubation with Colcemid, a mitotic inhibitor that depolymerizes microtubules and inactivates spindle fiber formation, thus arresting cells in metaphase, produced around 46% of metaphase events. The karyotype established for iLB-c1-30m-r12 cells at passage 51 was the following: 47,XY,+12 [cp5] / 93,XXYY,+12 [1] / 46,XY [4] / 92,XXYY [2]. Meaning that in about 50% cells karyotyped it was possible to identify a trisomy in the chromosome 12. The remaining cells analyzed presented a normal karyotype of 46 chromosomes with no chromosomal abnormalities (figure IV-6).

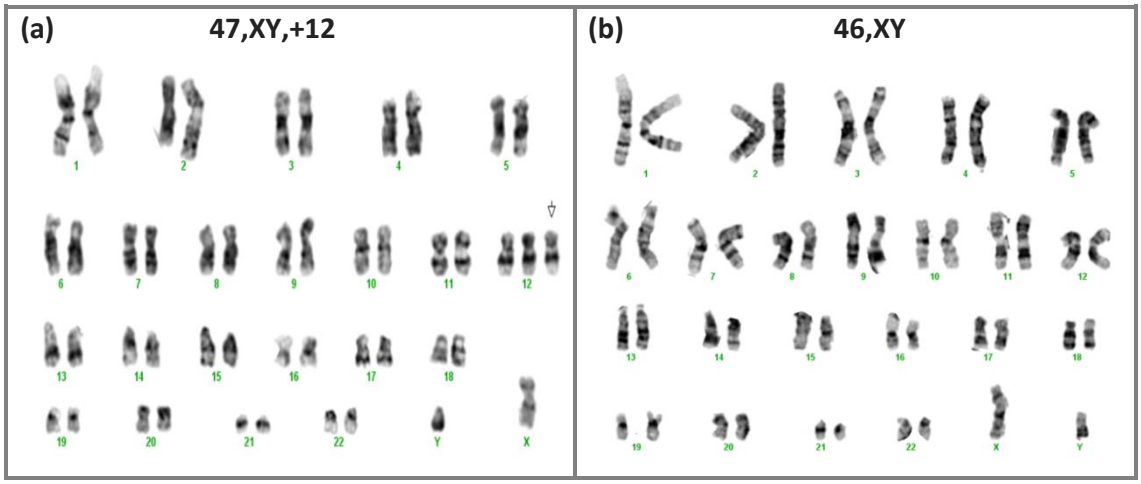

Figure IV-6 – Karyotype analysis of iLB-c1-30m-r12 cells at passage 51. (a) Abnormal karyotype showing a trisomy 12 and (b) Normal karyotype.

A recent study published in *Cell Stem Cell* indicates that chromosomal aberrations in hiPSC are, in fact, more common than thought and are directly related with increasing time in culture[105]. In hiPSC, trisomy of chromosome 12 is one of the most frequently found. Both NANOG and GDF3, which are involved in maintaining pluripotency, reside on chromosome 12p and are overexpressed as a consequence[105]. As previous findings revealed from chromosomal aberrations in hESCs, the rapid rate at which this trisomy appears and remains in culture demonstrates that this gene expression alteration confers selective growth advantage to the cells that carry them[106]. Also, the high occurrence of aneuploidy in hiPSC could be a side effect of integrating viral vectors that are often used in the reprogramming process. Viral integrations have been previously shown to cause chromosomal aberrations in a proximity to the site of integration[107]. Together, these results suggest that iLB-c1-30m-r12 cells, cultured on Matrigel in mTeSR1 medium, are able to preserve the pluripotency state and ability to differentiate *in vitro* upon stimuli, without spontaneous differentiation for several passages. However, the stability of this cell line was compromised during prolonged culture. These findings indicate that hiPSC need to be carefully monitored in culture for morphologic and genomic changes, so in this way, we can correctly interpret their biological properties and responses to culture changes.

IV.1.2. iPS-DF6-9-9T.B cell line

Intracellular staining of OCT4 and SOX2 demonstrated that iPS-DF6-9-9T.B cells at passage 38 maintained a pluripotent state throughout the entire area of the colonies (Figure IV-7). iPS-DF6-9-9T.B maintained also the expression of the pluripotency extracellular markers SSEA-4, TRA-1-60 and TRA-1-81 (figure IV-8).

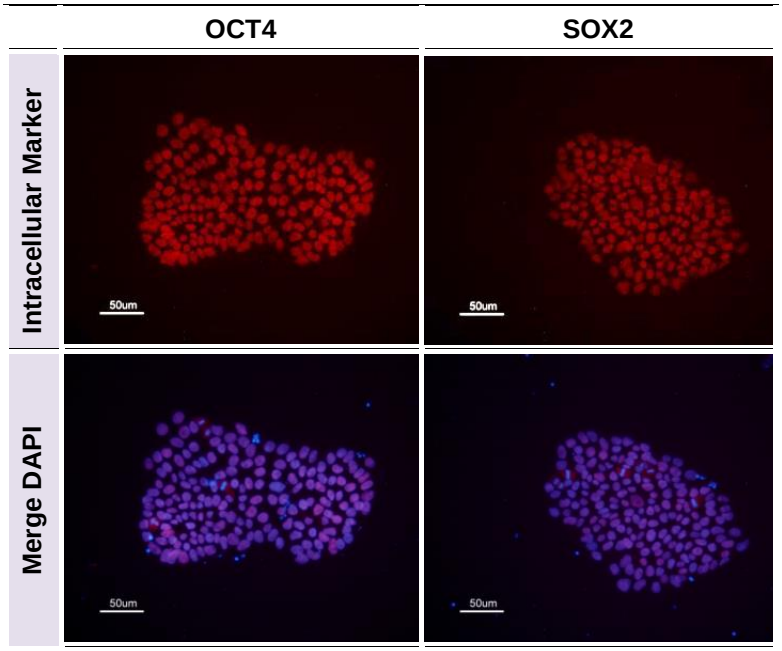

Figure IV-7 - Intracellular immunostaining of iPS-DF6-9-9T.B cells at passage 38 on Matrigel in mTeSR1 medium. Staining of the transcription factor OCT4 and SOX2 and respectively merge with nuclear staining with DAPI. Scale: 50µm.

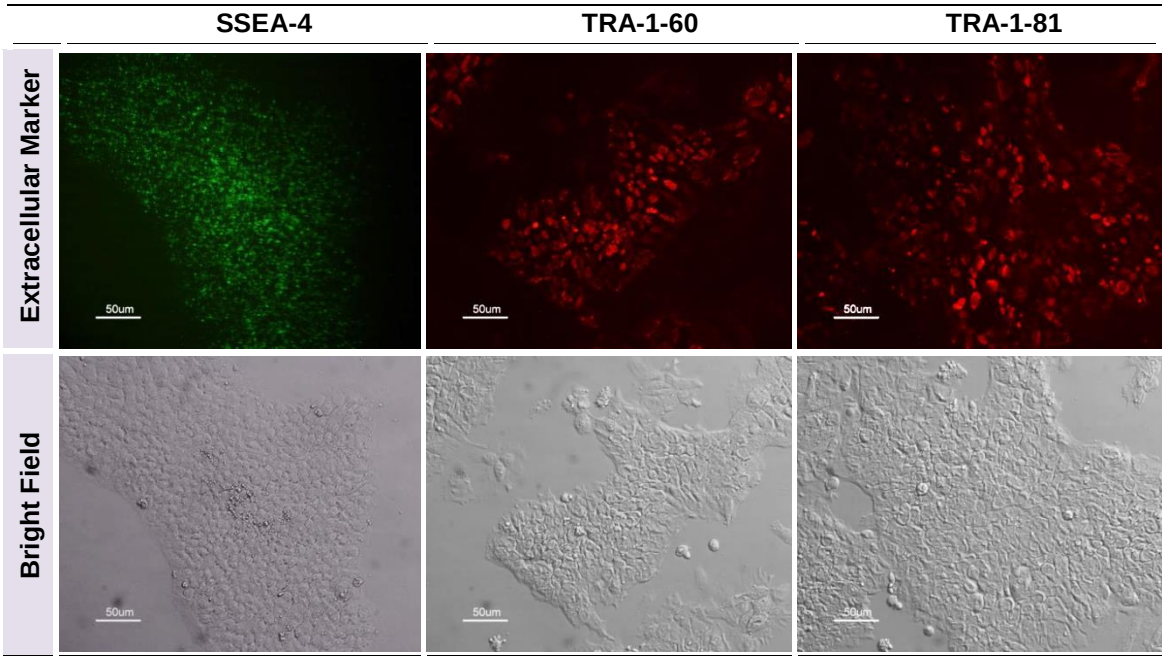

Figure IV-8 - Extracellular immunostaining of iPS-DF6-9-9T.B cells at passage 38 on Matrigel in mTeSR1 medium. Staining of the surface marker SSEA-4 (left) and TRA-1-60 (center) and TRA-1-81 (left) and respectively bright field images (below). Scale: 50µm.

The pluripotency state of iPS-DF6-9-9T.B cells was also evaluated using the Alkaline Phosphatase Staining Kit. After staining, iPS-DF6-9-9T.B colonies revealed a purple color indicating high alkaline phosphatase activity (figure IV-9).

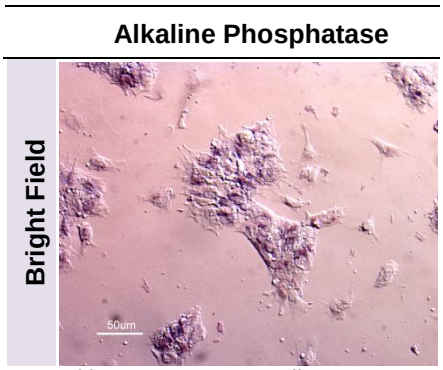

Figure IV-9 - Phenotypic assessment of iPS-DF6-9-9T.B cells at passage 38 using the Alkaline Phosphate Staining Kit. Scale: 50µm.

Flow cytometry analysis for expression of the pluripotency markers NANOG, OCT4, SOX2, TRA-1-60, SSEA-4 and the early differentiation extracellular marker SSEA-1 was performed to confirm these results (Figure IV-10). Flow cytometry results showed high expression levels of all pluripotency markers. In agreement with this result, the early differentiation marker SSEA-1 was not present. However, the extracellular marker TRA-1-60 showed a lower expression within the population compared with the other markers. As previously referred in section IV.1.1, a possible explanation for this result can be the associated limitations of the extracellular staining protocol used for flow cytometry. Additionally, it is also worth mention the fact that these cells have two more passages and were taken for flow cytometry analysis when the culture was almost confluent. As pluripotent stem cells tend to spontaneous differentiate with relative frequency, especially with increasing colony size[108], these cells could be already starting to lose their pluripotency. A solution for this problem is to carefully look for changes in the morphology of the cultured cells and passage cells when cell confluence is around 70% of well occupancy.

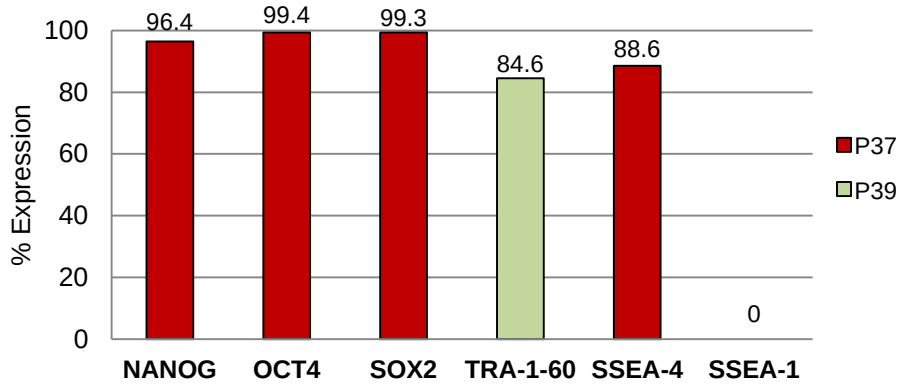

Figure IV-10 - Flow cytometry analysis of iPS-DF6-9-9T.B at passage 37 (dark red) and 39 (light green) for the pluripotency markers NANOG, OCT4, SOX2, TRA-1-60, SSEA-4 and the early differentiation marker SSEA-1.

To test the differentiation potential of these cells, iPS-DF6-9-9T.B cells were committed into the neural lineage by dual inhibition of SMAD signaling. After 12 days of differentiation, cells were fixed and stained for the pluripotency marker OCT4 and early differentiation neural markers FOXG1 and NESTIN.

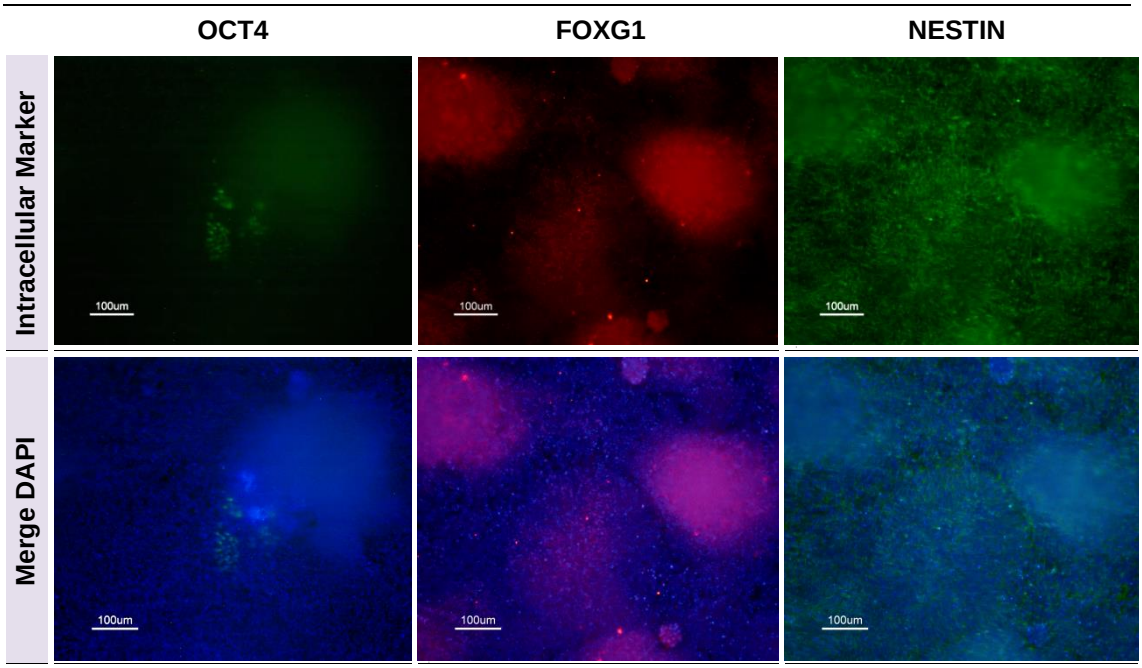

Figure IV-11 – Intracellular immunostaining of neural differentiated cells from iPS-DF6-9-9T.B cells. Staining of OCT4 (left), FOXG1 (center) and NESTIN (right) and respectively merge with nuclear staining with DAPI. Scale: 100µm.

Results revealed positive staining for both neural stem cells markers, with few remaining pluripotent cells in culture (figure IV-11). Thus, iPS-DF6-9-9T.B cells demonstrated high potential to differentiate into the neural lineage with relatively high efficiency proving their capacity as pluripotent stem cells to differentiate *in vitro*. Together, these results suggest that iPS-DF6-9-9T.B cells have the potential to be cultured on Matrigel and mTeSR1 culture medium and preserve their pluripotency state and ability to differentiate *in vitro* into the neural lineage. Additionally, iPS-DF6-9-9T.B cells did not revealed uncontrolled and spontaneous differentiation when grown in relatively small colonies (less than ~70% well occupancy).

IV.1.3. iPS-DF19-9-11T.H cell line

Intracellular staining of OCT4 and SOX2 in mTeSR1 demonstrated that iPS-DF19-9-11T.H at passage 40 maintained a pluripotent state throughout the entire area of the colonies with some few exceptions. hiPSC showed negative OCT4 staining regions indicating spontaneous differentiation (figure IV-12). SOX2 staining also showed a weaker fluorescence signal compared to the previous studied cell lines. iPS-DF19-9-11T.H cells maintained the

expression of the pluripotency extracellular markers SSEA-4, TRA-1-60 and TRA-1-81 (figure IV-13).

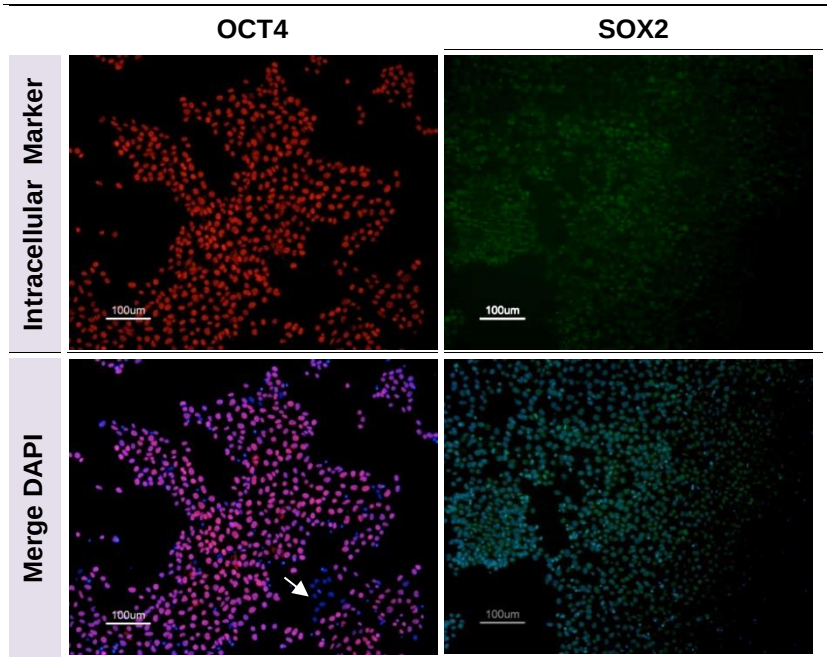

Figure IV-12- Intracellular immunostaining of iPS-DF19-9-11T.H cells at passage 40 on Matrigel in mTeSR1 medium. Staining of the transcription factor OCT4 (right) and SOX2 (left) and merge with nuclear staining with DAPI (below). The arrow on the OCT4 staining indicates differentiated cells. Scale: 100µm.

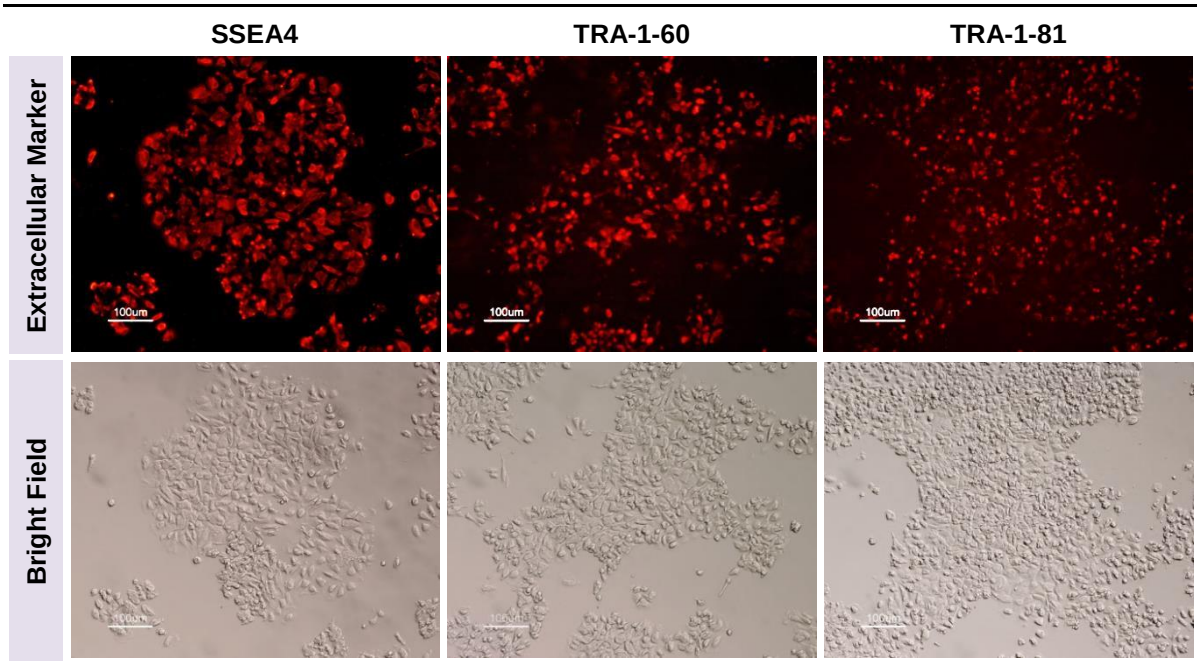

Figure IV-13 - Extracellular immunostaining of iPS-DF19-9-11T.H cells at passage 40 on Matrigel in mTeSR1 medium. Staining of the transcription factor SSEA-4 (left) and TRA-1-60 (center) and TRA-1-81 (left) and respectively bright field images (below). Scale: 100µm.

The assessment of pluripotency state by the presence of alkaline phosphatase activity of iPS-DF19-9-11T.H cells also pointed to the undifferentiated state of these cells (figure IV-14). To confirm these results, cells were analyzed by flow cytometry to evaluate the expression of the pluripotency markers OCT4, SOX2, TRA-1-60 and SSEA-4. Additionally, cells at passage 41, which presented morphology changes with very elongated cells present throughout the culture, were also analyzed by flow cytometry (Figure IV-15).

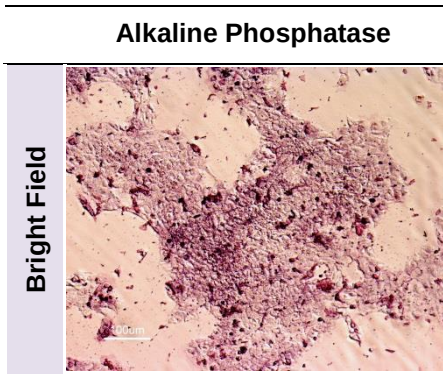

Figure IV-14 - Phenotypic assessment of iPS-DF6-9-9T.B cells using the Alkaline Phosphate Staining Kit. Scale: 50µm.

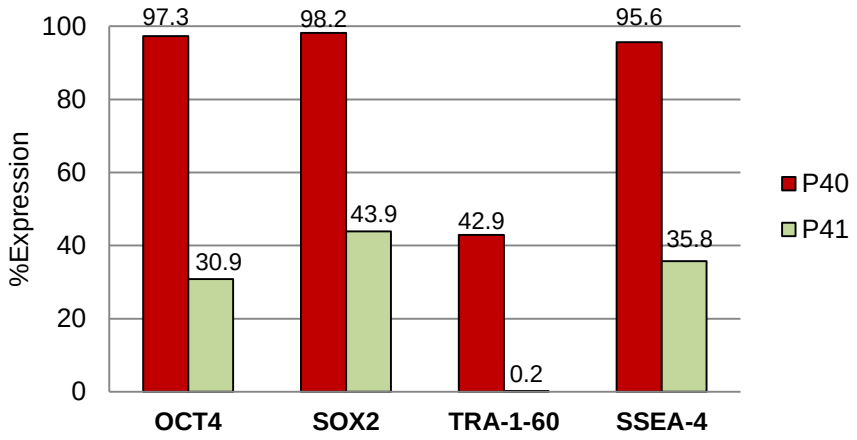

Figure IV-15 - Flow cytometry analysis of iPS-DF19-9-11T.H at passage 40 (dark red) and 41 (light green) for the pluripotency markers OCT4, SOX2, TRA-1-60 and SSEA-4.

Flow cytometry results of iPS-DF19-9-11T.H cells at passage 40 showed high expression levels of all pluripotency markers tested with the exception of TRA-1-60. iPS-DF19-9-11T.H cells at passage 41 that presented an elongated morphology in culture showed a 55-65% reduction in the expression of all pluripotency markers. To evaluate the differentiation potential of iPS-DF19-9-11T.H cells at passage 41, which presented low levels of pluripotency markers, cells were differentiated into the neural lineage by dual inhibition of SMAD signaling. After 12 days of differentiation, cells were fixed and stained for the pluripotency marker OCT4 and early neural markers PAX6 and NESTIN (figure IV-16).

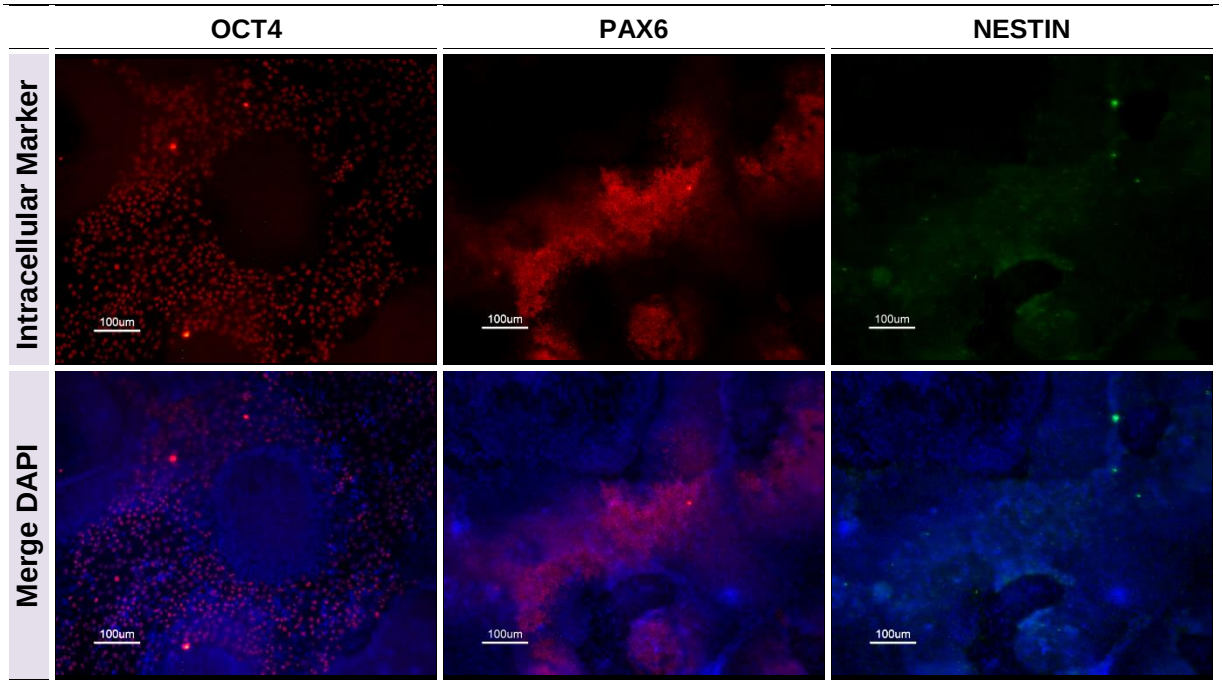

Figure IV-16 – Intracellular immunostaining of neural differentiated cells from iPS-DF19-9-11T.H cells. Staining of OCT4 (left), PAX6 (center) and NESTIN (right) and respectively merge with nuclear staining with DAPI. Scale: 100µm.

Intracellular immunostaining revealed positive staining for the early neuroectoderm markers, however with a high number of remaining pluripotent cells in culture. NESTIN and PAX6 staining also showed a weaker fluorescence signal compared to the previous studied cell lines. Together, these results showed that iPS-DF19-9-11T.H cells seem to be more unstable in culture, having a higher tendency to spontaneously differentiate in culture when compared with iLB-c1-30m-r12 and iPS-DF6-9-9T.B cell lines. Several factors can trigger this spontaneous differentiation. As previously referred, allowing cells to become over confluent will cause differentiation, and passaged before confluence will avoid this. However, another reason that should be taken in mind to understand the differences shown in the characterization study of the 3 hiPS cell lines is that iPSC can be, in fact, very different from each other. They are differently derived and the genetic and epigenetic profile of each cell line is not exactly equal although they all are functionally characterized as pluripotent. Some cell lines can be more stable and maintain their pluripotency in culture without extreme levels of attention, while others do not. The instability evident in iPS-DF19-9-11T.H and iLB-c1-30m-r12 cell lines needs to be carefully controlled to ensure that they can differentiate into the required tissue type efficiently and without tumorigenic consequences. In general, it is recommended that cell lines be used within 30 passages for experimental purposes. Continuing with cell culture until achieving very high numbers of passage can prolong cultures with unstable profiles of pluripotency[106]. For that reason, it is also highly advised to do a small bank of early passage cells prior to initiating experiments. If this is done, then cultures can be "retired" at a particular age and fresh thaws obtained, when higher passage numbers start to become unstable.

IV.2. Monolayer Differentiation of hiPSC into cardiomyocytes *in vitro*

Efficient production of functional cardiac cells from hiPSC is required for cell-based therapy. Adherent monolayer cultures are now being preferred to obtain more homogenous differentiation to avoid the variability associated with embryoid body cultures. Additionally, several monolayer cardiac differentiation methods have been described and the majority of these procedures makes use of feeder cells, fetal bovine serum (FBS) or various growth factors [29,36]. However, the use of growth factors such as Activin A and BMP4 is not cost effective for large-scale production, and the use of serum needs to be avoided for clinical purposes due to the potential risk of immunogenic responses. The use of small molecules has great potential as substitutes of growth factors and they are suitable for making defined media for large-scale culture. Despite recently published protocols are very promising in the production of cardiomyocytes from hiPSC in serum-free conditions, they show high variability between hiPS cell lines, proving that we are still in the beginning of full optimization of these protocols. In this chapter, the efficiency of monolayer cardiac-direct differentiation of hiPSC via growth factors and small molecules addition, in xeno- and serum-free conditions, was evaluated for two hiPS cell lines.

IV.2.1. Comparison of direct cardiac differentiation via Activin A and BMP4 addition; by induction of TGF-B signaling via Wnt activation; by sandwich matrix approach; and via small molecule modulators of Wnt signaling

To induce monolayer differentiation of hiPSC into cardiomyocytes *in vitro* four recently published protocols were tested: 1) cardiac differentiation via Activin A and BMP4 signaling (Laflamme et al. protocol); 2) induction of TGF-β superfamily signaling via Gsk3 inhibitors (GiAB); 3) matrix sandwich approach based on the addition of the TGF-β members Activin A and BMP4, in a 3-D microenvironment; and 4) cardiac differentiation via small molecule modulators of Wnt signaling (GiWi). The same cell line (iPS-DF19-9-11T.H) was used in all protocols at the same passage (P36). The cardiac commitment was followed exactly until the 31st day of differentiation.

None of the four protocols tested revealed morphological evidences on the formation of beating colonies. To identify the type of differentiated cells present in culture, cells were re-plated on the 31st day of differentiation on 0.1% gelatin and stained for the endoderm marker sox17; the late cardiac markers, α-actinin and cardiac Troponin T; the early cardiac markers, Nkx-2.5 and Brachyury T; the smooth muscle cells marker, α-smooth muscle actin and the pluripotency marker, OCT4. The only marker with positive staining was α-SMA in the four protocols tested (figure IV-17).

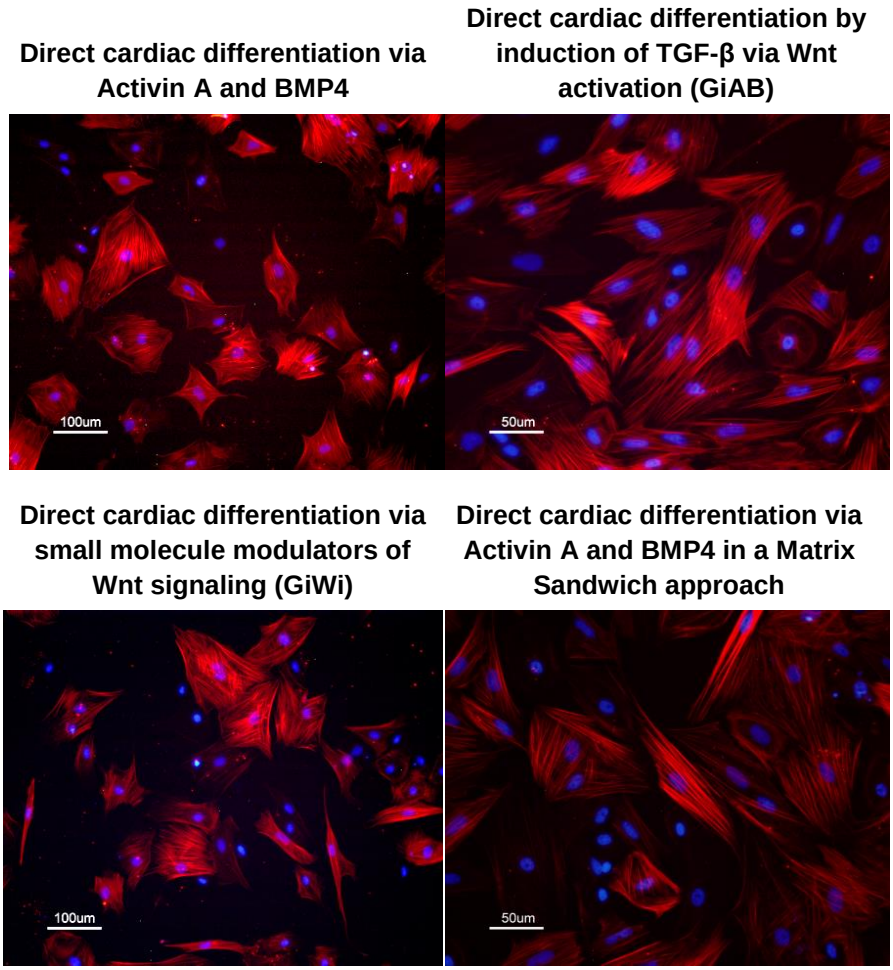

Figure IV-17 - Intracellular immunostaining of α-smooth muscle actin and nuclear staining with DAPI of differentiated iPS-DF19-9-11T.H cells at passage 36 on the 31st day, on 0.1% gelatin, accordingly with four cardiac differentiation protocols. Scale: left- 100 µm; right- 50 µm.

Vascular smooth muscle cells are one of the 3 main cell types derived from multipotent cardiac progenitors which indicate that these protocols could effectively differentiate iPS-DF19-9-11T.H cells into mesoendoderm and the pre-cardiac lineage, however, did not produce beating cardiomyocytes. This might indicate that the concentration of the factors used should be optimized to the specific cell line used and must be fine-tuned to specifically direct the commitment into cardiomyocytes. Another possibility to the verified inefficiency of these protocols can be the initial stability and pluripotent state of this cell line that could be already bias to produce a specific cell type. To exclude this last possibility, the pluripotency state of this hiPS cell line was evaluated. As mentioned in the previous chapter, iPS-DF19-9-11T.H cells revealed spontaneous differentiation in culture and low expression of pluripotency markers. Therefore, the following cardiac differentiation studies that were performed excluded this cell line.

IV.2.2. Cardiac-direct differentiation via Activin A and BMP4 signaling

IV.2.2.1. Factorial design-based approach for the optimization of growth factors concentration

The main objective of this experiment was to determine the conditions that could successfully induce cardiac differentiation of iLB-c1-30m-r12 cells at P45 based on Laflamme et al. protocol (that originally produced ~30% of cardiomyocytes in different hiPSC cell lines). The concentration of Activin A was ranged from 3 to 250 ng/ mL, with fixed concentrations of BMP4: 10 and 100 ng/ ml. It was also tested, based on GiAB protocol[51], if Wnt activation by CHIR99021 (3 µM) two days prior Activin A addition could increase cardiac differentiation efficiency in low doses of BMP4 (10 ng/ml). The tested culture conditions are described in (table IV-1).

Table IV-1 – Conditions tested in order to obtain cardiac differentiation based on the addition of the growth factors Activin A and Bmp4.

	10 ng/ml BMP4				**100 ng/ml BMP4**	
Activin A	**Without CHIR activation**		**With CHIR activation**		**Without CHIR activation**	
	A1: 3ng/ml	**A2**: 6ng/ml	**B1**: : 3ng/ml	**B2**: 6ng/ml	**C1**: 6ng/ml	**C2**: 25ng/ml
	A3: 10ng/ml	**A4**: 25ng/ml	**B3**: 10ng/ml	**B4**: 25ng/ml	**C3**: 100ng/ml	**C4**: 250ng/ml
	A5: 100ng/ml	**A6**: 250ng/ml	**B5**: 100ng/ml	**B6**: 250ng/ml		

It was possible to observe that on the 14th day of differentiation, the B6 condition formed beating colonies throughout the well surface. The next day, it was possible to identify in the B4 condition five small beating colonies. On day 16, B3 condition showed one beating colony in culture (figure IV-18).

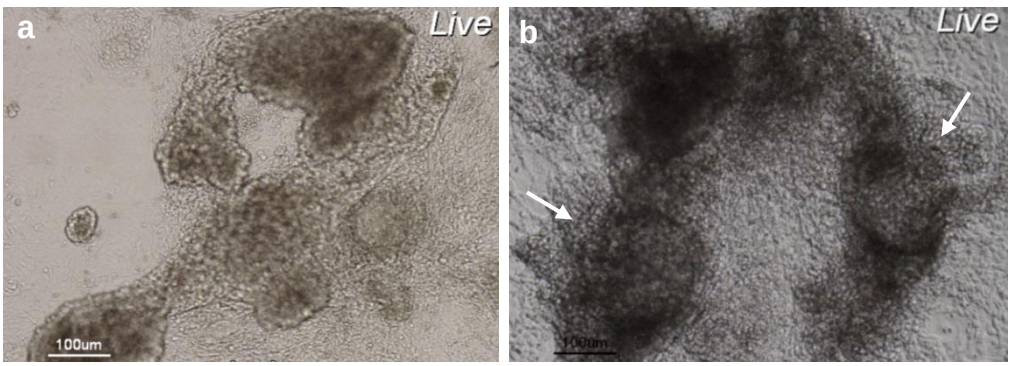

Figure IV-18– Live bright field images of differentiated beating colonies after Wnt activation with CHIR and Activin A and BMP4 addition in (a) B6 (250 ng/ml AA; 10 ng/ml BMP4) on day 14 and (b) B4 (25 ng/ml AA; 10 ng/mL BMP4) on day 15. Arrows in (b) indicate independent beating colonies.

To evaluate the evolution on the number of beating areas in the different conditions tested, regions with synchronized and independent contractile activity were counted and registered from day 15 until the 20th day of differentiation (figure IV-19).

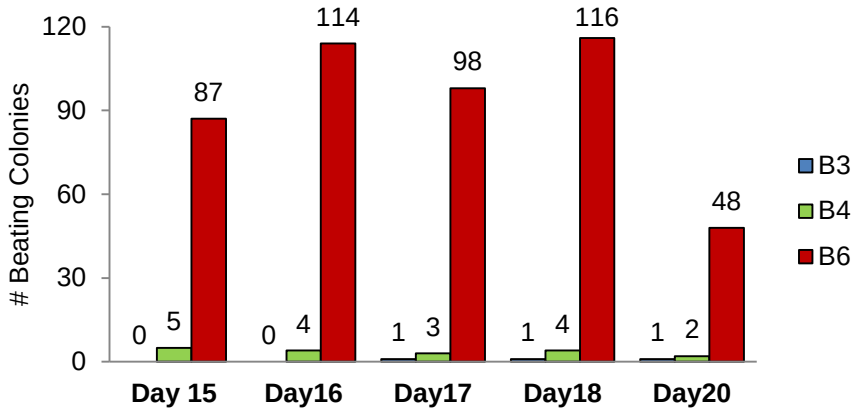

Figure IV-19 - Number of beating colonies counted in B3 (10ng/mL AA; 10ng/ml BMP4), B4 (25ng/mL AA; 10ng/ml BMP4) and B6 (250 ng/mL AA; 10ng/ml BMP4) conditions from day 15th to 20th of differentiation.

The number of beating colonies declined after the 18th day of differentiation. Based on the daily observation of culture progression, two possible reasons that can explain this decrease are: 1) adjacent small beating colonies that were beating independently started to beat synchronously as the culture matured and formed one large contracting area; 2) the proliferation of cells below and in the surrounding of the beating aggregates started to overcome the proliferation of cardiomyocytes, decreasing drastically its beating capacity and, in some cases, abrogated completely the beating activity of the smallest colonies.

Due to the full confluence state of the culture on 21st day of differentiation and to obtain a more reliable intracellular staining, A4, A6, B1, B2, B4, B5, C3 and C4 conditions were split in the ratio 1:4, with EDTA dissociation solution, and re-plated on 0.5% gelatin-coated plates. However, re-plated cells were still very confluent and so, B6 condition, which was re-plated on day 22, was split in the ratio 1:8. Three days after re-plating, cells were stained for the late markers of cardiac differentiation, cTnT and α-actinin; early cardiac marker, Nkx-2.5; endothelial marker, sox17 and smooth muscle marker, α-SMA. Although B6 condition showed the highest number of beating colonies in culture, cells did not stain positively for cardiac markers. This could be due to the high ratio of split 1:8, which may have caused the loss of the beating aggregates that couldn't re-attach to the gelatin coating in the re-plating process. On the contrary, the split ratio 1:4 used in the B4 condition preserved the aggregate structure of some beating colonies. Three days after re-plating one of those aggregates recovered its beating capacity and stained positively for cTnT (figure IV-20). B4 (25ng/mL AA; 10ng/mL BMP4) was the only condition that was positive for cTnT.

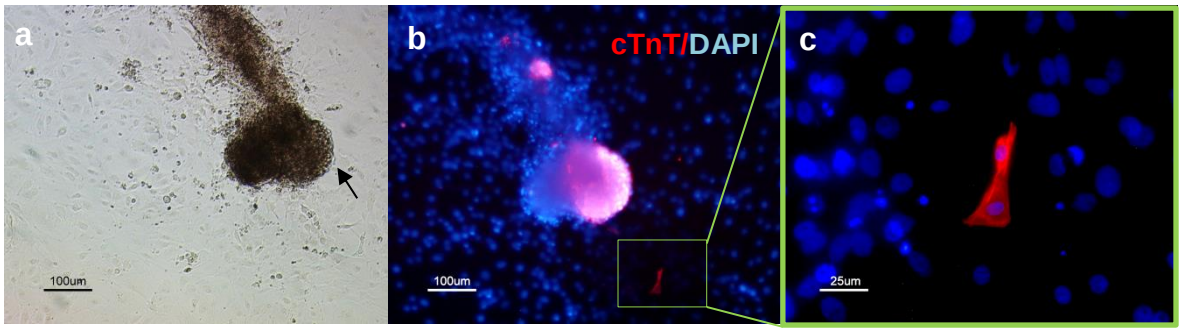

Figure IV-20– (a) Bright field image of a beating colony derived from B4 condition after re-plating on 0.5% gelatin. (b) Merge staining of cTnT with nuclear staining with DAPI and (c) amplification of isolated cardiomyocytes. Black arrow in bright field image indicates the region with contractile activity. Scale: 100 µm; amplified image: 25 µm.

All conditions were positive for α-SMA staining (C4 condition given as example in figure IV-21) and negative for the remaining markers. This result suggests that cardiac-direct differentiation via Activin A and BMP4 signaling induces differentiation of vascular smooth muscle cells in a wide range of Activin A and BMP4 concentrations. On the contrary, cardiac differentiation seems to be depend on Wnt activation with CHIR99021, low dosages of BMP4 (10ng/mL) and high dosages of Activin A (250ng/ml).

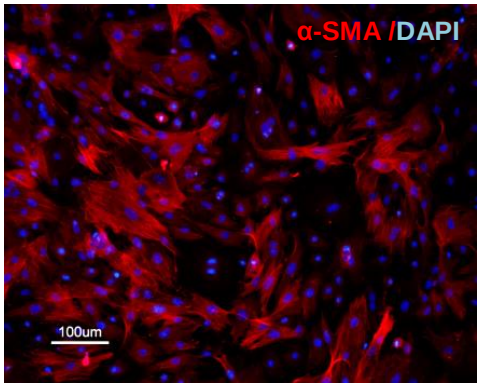

Figure IV-21 – Immunostaining of C4 condition (250ng/mL AA; 100 ng/mL BMP4), after re-plating on 0.5% gelatin. Merge staining of α- SMA with nuclear staining with DAPI. Scale: 100 µm.

IV.2.2.2. Quantification of hiPSC cardiac differentiation efficiency when using the GiAB protocol

To quantify the efficiency of cardiac differentiation performed according to the GiAB method, iLB-c1-30m-r12 cells at passage 50 were differentiated until the 15th day of differentiation. Taking into account the previous results, it was added to the culture 250 ng/mL Activin A on day 0 and 10 ng/mL BMP4 on day 5. Cells were cultured in mTeSR1 containing 3 µM CHIR99021 from day −3 to day 0. On the 9th day of cardiac differentiation it was evident the formation of two small beating colonies in culture (figure IV-22). Until the 15th day of differentiation no aditional beating colonies were formed.

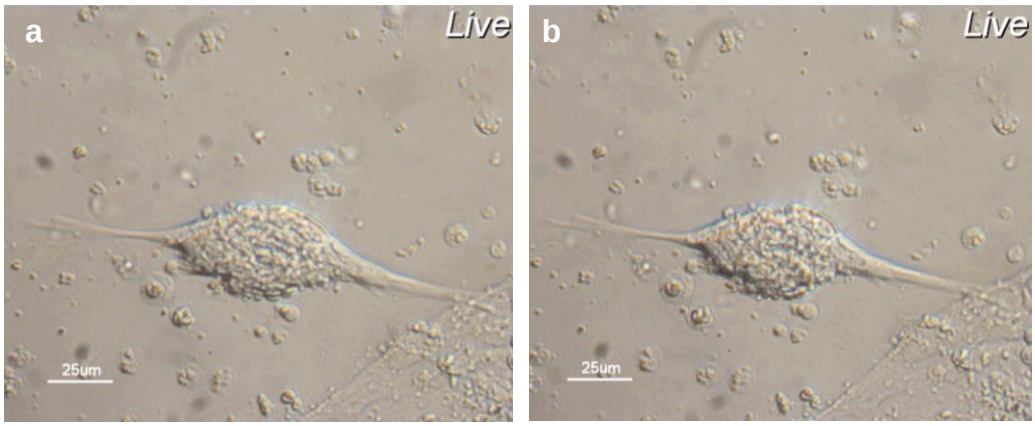

Figure IV-22 - Live bright field images on the 15th day of differentiation of a beating colony after Wnt activation with CHIR and addition of 250 ng/mL Activin A and 10 ng/mL BMP4. (a) Extended form and (b) contracted form of a beating colony in culture. Scale: 25 µm.

The expression of the pluripotent marker OCT4 and cardiac markers Nkx-2.5, α-actinin and cTnT were quantified by flow cytometry analysis (figure IV-23). Results revealed low expression of all markers, revealing low efficiency of cardiac differentiation of hiPSC, as expected. However, the low amount of OCT4+ cells in culture indicates a commitment of these cells into another direction. As previously demonstrated by intracellular immunostaining, this protocol is able to easily produce vascular smooth muscle cells. These can be the major "cell contaminant" on the culture. Although, it was not possible to measure the number of vascular smooth muscle cells in culture and determine if, in fact, these cells are the ones being generated preferentially to cardiomyocytes.

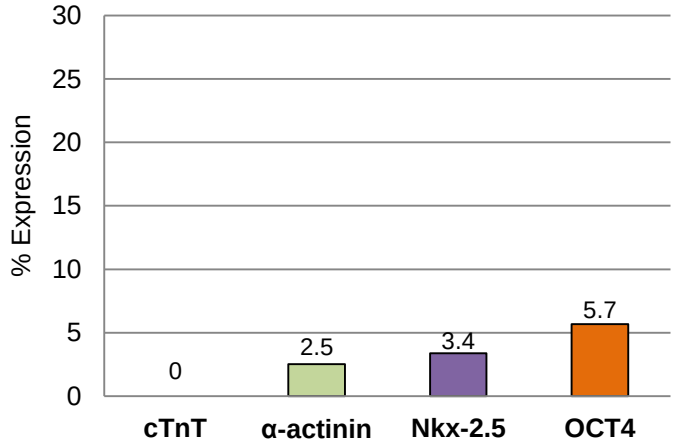

Figure IV-23 – Flow cytometry analysis of iLB-c1-30m-r12 cells differentiated into the cardiac lineage accordingly with GiAB protocol after 15 days in culture for the pluripotency marker OCT4 and cardiac markers cTnT, α-actinin and Nkx-2.5.

These results suggest that cardiac-direct differentiation via Activin A and BMP4 signaling seems to be highly variable in the successful generation of cardiomyocytes. Cardiac differentiation of iLB-c1-30m-r12 cells at passage 45 produced more than one hundred beating areas, while iLB-c1-30m-r12 cells at passage 50 produced only two, in the same culture conditions. A possible explanation for this experimental outcome can be the different genetic and epigenetic background of these cells at these different passages. Moreover, as mentioned in section IV.1.1, iLB-c1-30m-r12 cells analyzed at P51 showed a trisomy 12 in about 50% of the population. This chromosomal abnormality may have appeared/increased in culture between P45 and P50, affecting the starting population to commit efficiently into the cardiac lineage and generate cardiomyocytes. However, further studies should be performed to confirm this hypothesis, such as karyotyping of iLB-c1-30m-r12 cells at lower passages to ensure the stability of this cell line and repeat cardiac-direct differentiation in the same conditions.

IV.2.3. Cardiac differentiation via small molecule modulators of Wnt signaling

IV.2.3.1. Factorial design-based approach for the optimization of small molecules concentration

A recent protocol published in PNAS in 2012, proved that modulation of Wnt pathway with the use of small molecules induces effectively cardiac differentiation in different hiPSC lines. Wnt activation by the addition of 12 µM CHIR and Wnt inhibition by the addition of 5 µM IWP4 produced ~98% cardiomyocytes without any enrichment and/or purification step[51]. As this protocol is growth factor-free has the advantage to be much more cost-effective and completely defined method to produce cardiomyocytes from hiPSC.

In several experimental attempts to differentiate iLB-c1-30m-r12 and iPS-DF19-9-11T.H cell lines, the addition of 12 µM CHIR and 5 µM IWP4 did not produce beating cardiomyocytes. To determine the conditions that could successfully induce cardiac differentiation of iLB-c1-30m-r12 cells at P44, the concentration of CHIR was ranged from 3 to 20 µM, with fixed concentrations of IWP4: 5 and 10 µM. 72 hours exactly after CHIR addition, half of the medium was changed and IWP4 was added to the culture, accordingly with GiWi protocol.

On the 11th day of differentiation, the condition using 6 µM CHIR/5 µM IWP4 formed a monolayer of actively beating cardiomyocytes. The condition of 3 µM CHIR/5 µM IWP4 and 8 µM CHIR/5 µM IWP4 produced a low number of small beating colonies, similar to the structures obtained with the Activin A/BMP4 protocol (figure IV-24). All other conditions tested did not reveal any beating activity.

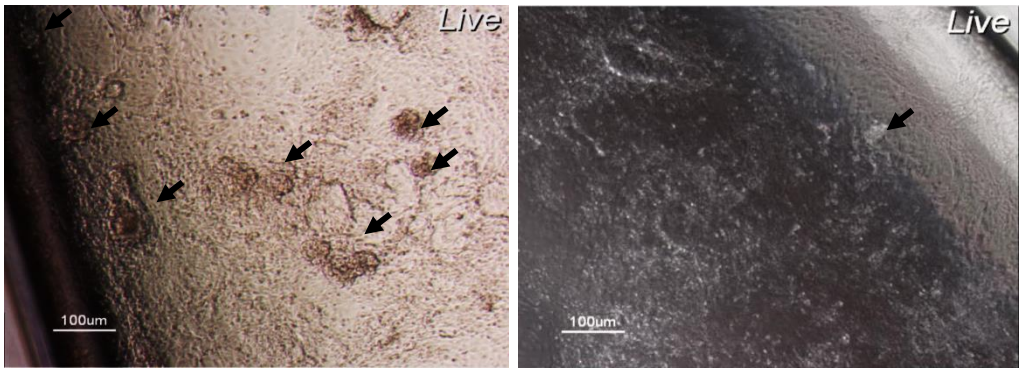

Figure IV-24 - Live bright field images of differentiated beating colonies after Wnt modulation with CHIR and IWP4 in (a) 3 µM CHIR/5 µM IWP4 and (b) 8 µM CHIR/5 µM IWP4 on the 11th day of cardiac differentiation. Black arrows indicate independent beating colonies.

After 23 days of differentiation, both conditions of 6 µM CHIR/5 µM IWP4 and 6 µM CHIR/10 µM IWP4 (used as positive and negative conditions of cardiac differentiation, respectively) were fixed and stained for the late cardiac marker cTnT and analyzed by fluorescence microscopy (figure IV-25). As expected, the 6 µM CHIR/5 µM IWP4 condition stained positively for cTnT, while the 6 µM CHIR/10 µM IWP4 condition did not.

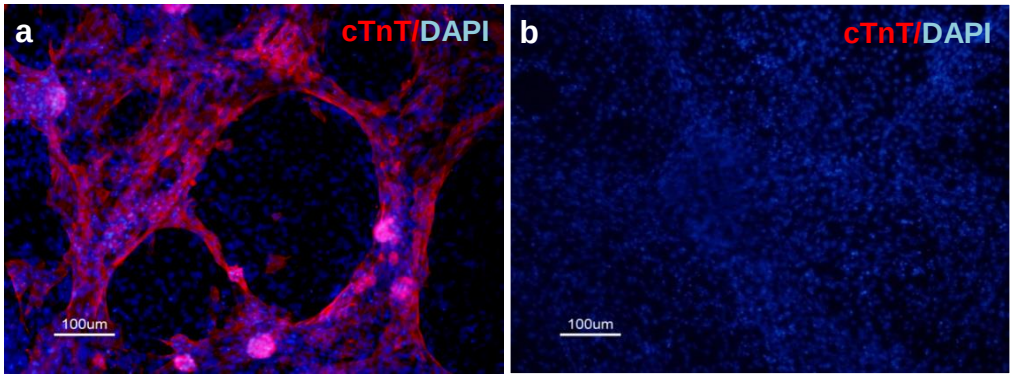

Figure IV-25 – Immunostaining of (a) 6 µM CHIR/5 µM IWP4 and (b) 6 µM CHIR/10 µM IWP4 conditions.

These results showed that Wnt activation with 6 µM CHIR, which is half of the concentration used by Lian et al., and Wnt inhibition with 5 µM IWP4 was the optimal condition to successfully induce monolayer differentiation of iLB-c1-30m-r12 cells into cardiomyocytes. Additionally, the addition of 10 µM IWP4 seems to be inhibitory of cardiac differentiation. Interestingly, concentrations above 8 µM CHIR did not induce cardiac differentiation for both 5 and 10 µM IWP4 conditions. One possible explanation for this result is the apoptotic effect of CHIR addition: increasing CHIR concentrations led to increasing cell death. In fact, 24 hours after the addition of 10, 12 and 14 µM CHIR, almost all cells in culture died (figure IV-26). Although, the 10 µM CHIR/5 µM IWP4 condition achieved confluence after 5 days of CHIR addition, it did not induce cardiomyocytes differentiation.

Association between the Wnt signaling pathway and apoptosis has been established through recent reports in the literature. In fact, it has been reported that activation of Wnt/β-

catenin signaling in Hematopoietic Progenitor Cells *in vitro* elevates the activity of caspases 3 and 9 and induces the mitochondrial apoptotic pathway[109]. It was also found that fibroblasts, corneal fibroblasts, corneal epithelia, and several carcinoma cell lines overexpressing beta-catenin all undergo apoptosis[110].

Together these results suggest that the initial confluence state of the culture seems to be an important factor on the efficiency of cardiac induction. If full confluence is not restored before IWP4 addition, cardiac differentiation seems to be completely abrogated (figure IV-27). The confluence state of the culture results in the establishment of a microenvironment of cell-to-cell interactions that, subsequently, will trigger a signaling cascade that results in the activation of commitment-related genes and repression of pluripotency genes[55]. This continues in time as more lineage-specific genes are activated and others are repressed. If the environment stimuli are not sufficient and exact in time (e.g. cell interactions and factors concentration) the culture may not achieve the required threshold to further commit into the desired population. Thus, the culture will fail to differentiate and successfully generate mature cardiomyocytes.

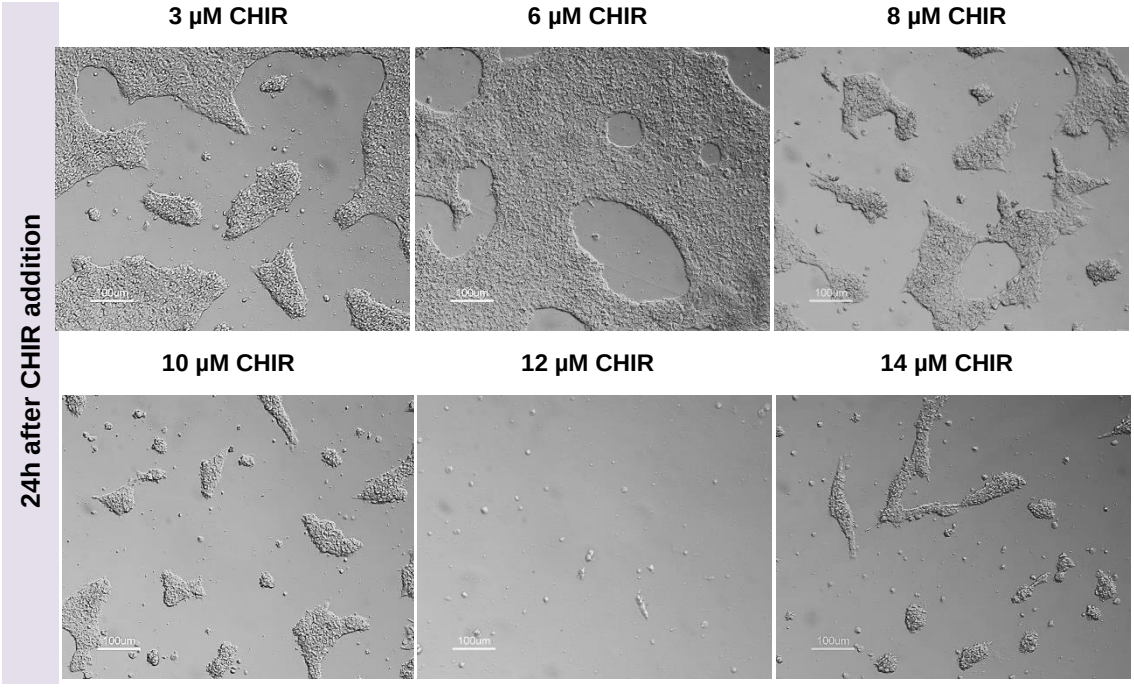

Figure IV-26 - Bright field images of the 3 to 14 µM CHIR/5 µM IWP4 conditions, 24h after CHIR addition. Scale: 100 µm.

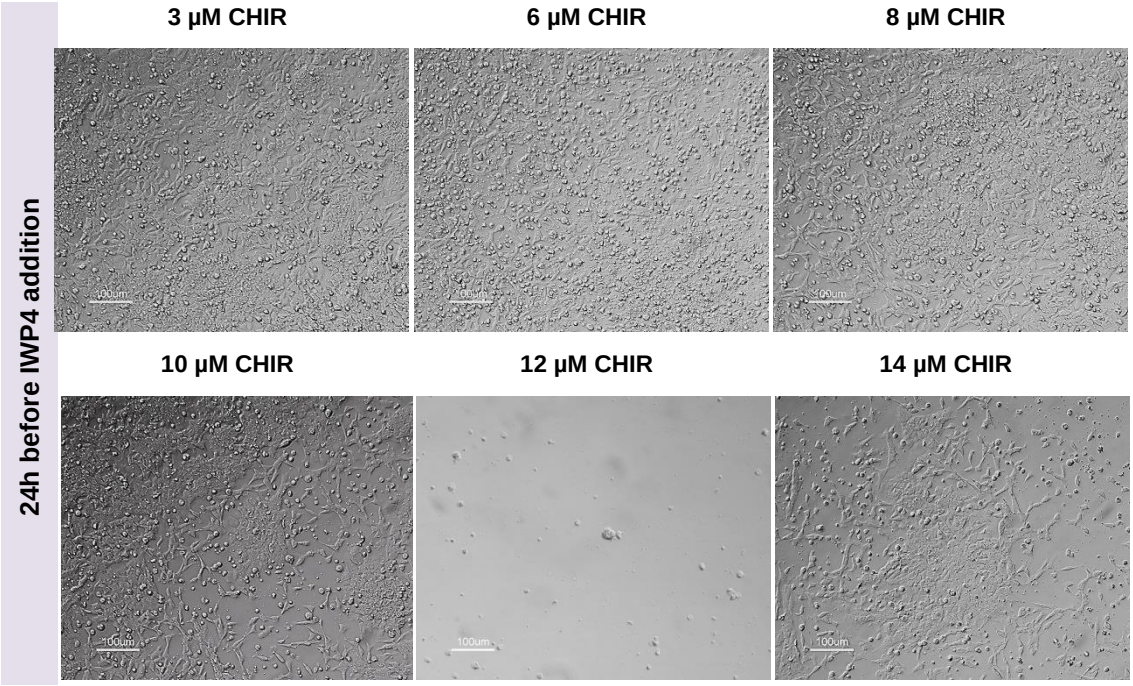

Figure IV-27 – Bright field images of the 3 to 14 µM CHIR/5 µM IWP4 conditions, 24h before CHIR addition. Scale: 100 µm.

IV.2.3.2. Temporal variation of Wnt signaling inhibition by IWP4

To determine if the time period of Wnt pathway inhibition had an improvement effect in the efficiency of cardiomyocytes differentiation, the exact time in which IWP4 is added to the culture was varied. Thus, 5 µM IWP4 was added 24h, 48h, 72h and 96h exactly after CHIR addition. Taking in account previous results, the addition of 3, 6 and 8 µM CHIR was tested for all time periods. This experiment was performed for both iLB-c1-30m-r12 and iPS-DF6-9-9T.B cell lines.

i. iLB-c1-30m-r12 cell line at P50

The cardiac-direct differentiation of iLB-c1-30m-r12 cells at passage 50, via GSK3 inhibitor and Wnt inhibitor protocol (GiWi) was followed exactly until the 24th day, with the variations above mentioned. The results are resumed on table IV-2.

Table IV-2 – Results on cardiac differentiation of iLB-c1-30m-r12 cells based on GiWi protocol with variation of the time of IWP4 addition in different CHIR concentrations. (++: monolayer beating in more than 70% of the well; +: beating aggregates in less than 70% of the well; - : no beating colonies).

		Time of 5 µM IWP4 addition after CHIR			
		24h	**48h**	**72h**	**96h**
CHIR	**3 µM**	**A1: ++ monolayer**	**B1: + monolayer**	**C1: ++ monolayer**	**D1: ++ monolayer**
	6 µM	**A2: + aggregates**	**B2: ++ monolayer**	**C2: ++ monolayer**	**D2: -**
	8 µM	**A3: - Cell death**	**B3: ++ monolayer**	**C3: -**	**D3: -**

On day 10 of cardiac differentiation A1, A2 and B1 conditions showed beating activity. A1 condition produced a beating monolayer showing a fast pace comparing to B1 condition. The A2 condition showed similar rhythm as A1 however the beating structure produced was more aggregate-like rather than in monolayer. The next day, B3 and C1 conditions showed a monolayer of slow beating cardiomyocytes. On the 12th day of differentiation, B2 and D1 showed slow and similar beating activities. On the 13th day, A1, C1, C2 and B3 were the conditions that showed the fastest rhythm, beating every 1 second, B2 and D1 showed contraction every 3 seconds and B1 every 5 seconds. Interestingly, D1 condition produced a branch-like structure that start losing robust beating capacity over time (figure IV-28). Not all conditions produced a beating monolayer throughout the entire well, with some conditions showing only few regions with beating capacity. A3, C3, D2 and D3 conditions did not show any beating activity until the end of the experiment.

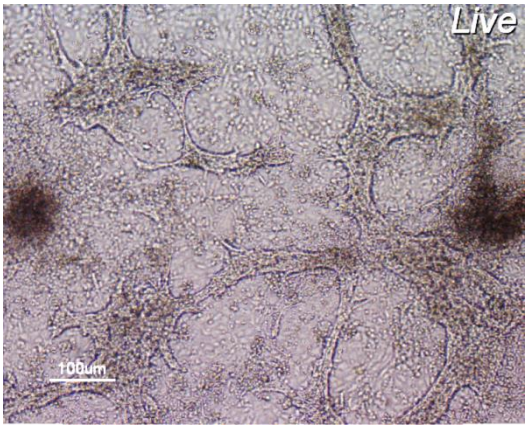

Figure IV-28 – Live bright field image of actively beating monolayer in D1 condition (3 µM CHIR plus 5 µM IWP4 added 96h after CHIR).

Gathering these results, the conditions that produced a actively beating monolayer throughout the entire well with no loss of robust beating capacity during cardiac differentiation were A1, C1, C2 and B3 conditions which indicates that high concentrations of CHIR combined with both extremes of IWP4 addition (24h and 96h after CHIR) may not efficiently direct hiPSC differentiation into cardiomyocytes. Interestingly, some conditions presented a fastest beating pace while others showed a slower contraction. This might suggest that the concentration of CHIR together with the time period in which Wnt signaling is being inhibited by IWP4 may influence the myofibrillar and sarcomeric organization as well the formation of gap junction proteins that regulate muscle contraction. The fine adjustment of these two factors might allow the production of cardiomyocytes with specific physiological properties and direct cardiac differentiation into a specific cardiomyocyte population - ventricular-like, atrial-like and pacemaker-like cells – depending on the final application. Fast pace cardiomyocyte populations may possibly be associated with the production of pacemaker-like cells while slower beating cultures might be associated to ventricular-like cells. However, this might be a possibility; the functional characterization of differentiated cardiomyocytes would be required to confirm this hypothesis.

ii. iPS-DF6-9-9T.B cell line at P40

The cardiac-direct differentiation of iPS-DF6-9-9T.B cell line at passage 40, accordingly with GiWi protocol, was followed exactly until the 18th day, with the variations previously mentioned.

Table IV-3 – Results on cardiac differentiation of iPS-DF6-9-9T.B cells based on GiWi protocol with variation of the time of IWP4 addition in different CHIR concentrations. (++: monolayer beating in more than 90% of the well; +: beating aggregates in less than 90% of the well; - : no beating colonies).

		Time of 5 µM IWP4 addition after CHIR			
		24h	**48h**	**72h**	**96h**
CHIR	**3 µM**	**A1: + monolayer**	**B1: ++ monolayer**	**C1: ++ monolayer**	**D1: + monolayer**
	6 µM	**A2: + monolayer**	**B2: + monolayer**	**C2: + monolayer**	**D2: + monolayer**
	8 µM	**A3: - Cell death**	**B3: + monolayer**	**C3: + monolayer**	**D3: -**

On the 9th day of cardiac differentiation all conditions showed beating activity, with the exception of A2, A3, C2 and D3 conditions. A2 and A3 conditions revealed increased cell death over time. Interestingly, the B3 condition reproduced the fast beating rhythm and the A3 condition the extended cell death as previously seen for the iLB-c1-30m-r12 cell line. Until the end of the experiment, A3 and D3 did not reveal morphological evidences in the formation of cardiomyocytes.

iPS-DF6-9-9T.B cells demonstrated a large range of conditions to effectively produce a monolayer of beating cardiomyocytes, however, the beating monolayer extension in culture as well the beating rhythm observed varied between conditions. B1 and C1 were the only conditions that produced a beating monolayer in more than 90% of the well area; B2 and C1 showed the fastest pace; and D2 the slowest. Again, as previously observed in iLB-c1-30m-r12 cells, high concentrations of CHIR combined with either early (24h after CHIR) or later (96h after CHIR) inhibition of Wnt signaling by IWP4 were inefficient in production of monolayer cultures of beating cardiomyocytes. Intermediate times of Wnt inhibition between 48h and 72h after CHIR, seems to be the ideal to obtain high cardiac differentiation efficiency when low concentrations of CHIR are used.

IV.2.3.3. Quantification of hiPSC cardiac differentiation efficiency when using the GiWi protocol

i. Cell line iLB-c1-30m-r12 at P44

To quantify the efficiency of cardiac differentiation of the GiWi protocol, A1 - 3 µM CHIR plus 5 µM IWP4 added 24h after CHIR- and an intermediate condition between C1 and C2 conditions- 5 µM CHIR plus 5 µM IWP4 added 72h after CHIR- were selected to determine the expression of cardiomyocyte markers by flow cytometry. In a second optimization step of the culture conditions for the efficient differentiation of iLB-c1-30m-r12 cells into cardiomyocytes, the decrease of 6 µM to 5 µM CHIR plus 5 µM IWP4 addition at 72h after CHIR, proved to produce similar or even higher extension of a beating monolayer of cardiomyocytes in culture when compared to condition 6 µM CHIR plus 5 µM IWP4 added 72h after CHIR. For this reason, this condition was selected to quantify the efficiency of cardiac differentiation. A1 condition was also chosen based on the robust cardiac differentiation observed in culture, as previosuly reported in the section IV.2.3.2.

iLB-c1-30m-r12 cells at passage 44 were differentiated until the 18th day and stained for the pluripotency marker OCT4; the late cardiac markers, cTnT and α-actinin; and the early cardiac marker, Nkx-2.5. As negative control, iLB-c1-30m-r12 cells expanded in on matrigel and mTeSR1 were also stained for the same markers. hiPSC expanded in mTeSR1 showed residual expression for all cardiac markers. This percentage of expression was subtracted to the values of expression obtained under differentiation conditions.

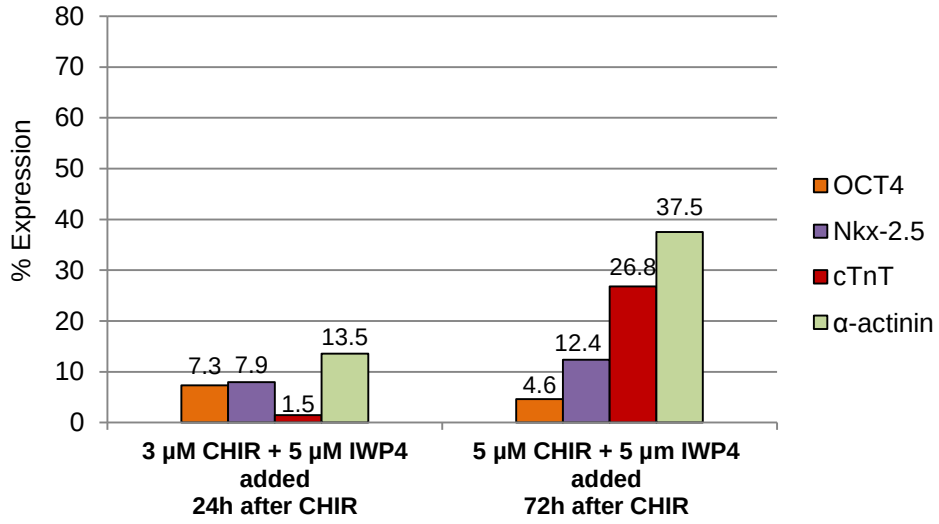

Figure IV-29 – Flow cytometry analysis of iLB-c1-30m-r12 cells committed into the cardiac lineage, accordingly with GiWi protocol after 18 days in culture, for the pluripotency marker OCT4 and cardiac markers Nkx-2.5, cTnT and α-actinin. (n=1; 6 replicates)

From the analysis of the flow cytometry results (figure IV-29) it can be observed that the expression of late cardiac markers cTnT and α-actinin was increased in condition 5 µM CHIR plus 5 µM IWP4 added at 72 hours, showing about 38% of α-actinin+ cells and 27% of cTnT+ cells. The difference observed between the two conditions indicate that the addition of 5 µM CHIR plus 5 µM IWP4 added at 72 hours after CHIR seems to be more close to the optimal condition for efficient differentiation of this cell line into cardiomyocytes. However, the expression of late cardiac markers is still reduced indicating that further optimization studies of the culture conditions for cardiac differentiation would be needed to achieve robust production of cardiomyocytes from iLB-c1-30m-r12 cells. Additionally, the percentage of Nkx-2.5+ cells observed in both conditions indicates the presence of differentiated cells in a early stage of cardiac differentiation within the population. Moreover, OCT4 expression in both conditions indicates the presence of a subpopulation of pluripotent stem cells within the differentiated culture.

ii. Cell line iPS-DF6-9-9T.B at P42

The expression of cardiomyocyte markers by flow cytometry was quantified in order to obtain the efficiency of cardiac differentiation of iPS-DF6-9-9T.B cells at passage 42. Cells were differentiated accordingly with GiWi protocol until the 18th day of differentiation. The same two conditions used for the iLB-c1-30m-r12 cell line were tested: 3 µM CHIR plus 5 µM IWP4 added at 24h and 5 µM CHIR plus 5 µM IWP4 added at 72h. On the 18th day, cells were fixed and stained for the pluripotency marker OCT4; the late cardiac markers, cTnT and α-actinin; and the early cardiac marker, Nkx-2.5. As negative control, iPS-DF6-9-9T.B cells expanded in mTeSR1

and on matrigel were also stained for the same markers. The residual expression of hiPSC expanded in mTeSR1 was subtracted to the values of expression obtained under differentiation conditions for all markers.

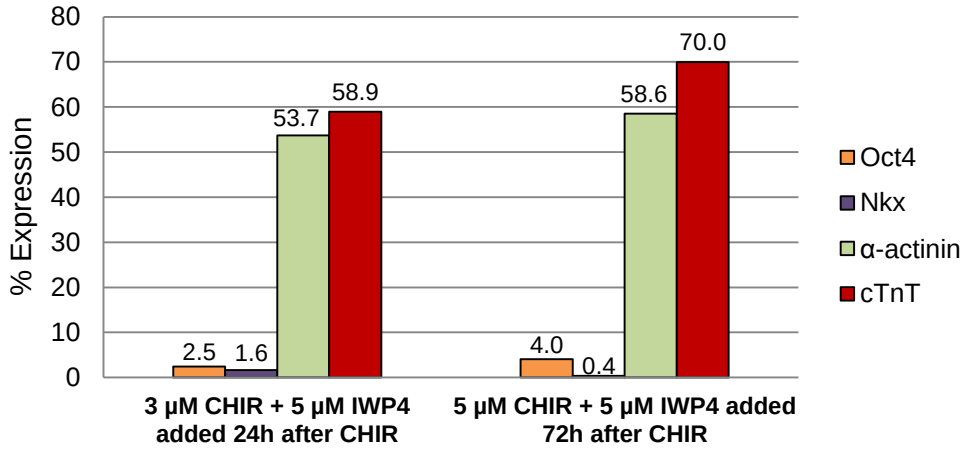

Figure IV-30 - Flow cytometry analysis of iPS-DF6-9-9T.B cells committed into the cardiac lineage, accordingly with GiWi protocol after 18 days in culture, for the pluripotency marker OCT4 and cardiac markers Nkx-2.5, cTnT and α-actinin. (n=1; 6 replicates)

Similar to the iLB-c1-30m-r12 cell line, differentiated iPS-DF6-9-9T.B cells showed higher expression of the late cardiac markers cTnT and α-actinin in 5 µM CHIR plus 5 µM IWP4 added at 72h after CHIR. Moreover, iPS-DF6-9-9T.B cell line demostrated much higher efficiency of cardiac differentiation for the same conditions, expressing 70% of cTnT+ cells in the condition 5 µM CHIR plus 5 µM IWP4 72h after CHIR and 59% cTnT+ cells in the condition 3 µM CHIR plus 5 µM IWP4 added 24h after CHIR. In agreement, the expression of the early cardiac marker Nkx-2.5 was residual in the population. In addition, the percentage of OCT4+ cells was less than 4%, indicating a low number of undiferentiated cells in culture after 18 days of cardiac differentiation. One possible explanation for the significant differences observed between iLB-c1-30m-r12 and iPS-DF6-9-9T.B cell lines may be due to the increased expression of the pluripotency markers NANOG and GDF3 associated to the extra chromosome 12 found in the iPS-DF6-9-9T.B cell line. This increased expression might turn the induction of Wnt pathway by CHIR addition insufficient to counteract the expression of pluripotency genes and fully induce the expression of pre-cardiac genes.

IV.2.3.4. Re-plating Cardiac Progenitor Cells

Cardiomyocytes derived from hiPSC can have several applications; however, the low proliferative capacity of mature cardiomyocytes is still an obstacle to obtain a sufficient number of cells for those applications. The main objective of this experiment was to increase the yield of the obtained cardiomyocytes for the 3 µM CHIR; 5 µM 72h IWP4 condition in the iLB-c1-30m-r12 cell line at P47, by splitting cells to a fresh and new coated surface. Exploiting the high proliferative capacity of cardiac progenitor cells, cells were re-plated at an early stage of differentiation (day 6). Accordingly with gene expression studies, performed during cardiac differentiation of hiPSC accordingly with the GiWi original protocol, on day 6 of differentiation there is an increased expression of the early cardiac genes Nkx-2.5, GATA4 and TBX5, indicating that a population of cardiac progenitor cells is presence in culture at this stage of cardiac commitment[52]. In an attempt to increase the generation of cardiac progenitor cells, 10 ng/mL of vascular endothelial growth factor (VEGF) was added to the culture on day 5 and 6 or only on day 6 of cardiac differentiation. VEGF is known as a key molecule in the promotion of proliferation and differentiation of the endothelial lineage from the earliest stages of development[25]. Several developmental studies have shown the important role of endoderm signaling in the early cardiovascular development *in vivo*[111,112]. Based on these reports, different groups have been recently studying the effect of co-culturing pluripotent stem cells with endoderm-like cell lines and the addition of VEGF on cardiac development *in vitro.* Recently Ye L. et al. reported a novel differentiation method that combined Activin-A and BMP-4, followed by VEGF treatment for cardiac mesodermal commitment[112]. VEGF has shown to promote KDR+ cardiovascular progenitor cell development from hiPSC, by up-regulating KDR and PDGFRa expression, and consequently producing a higher percentage of cardiomyocytes (<85%) compared to the original Activin A/BMP4 protocol (<30%). Until now, it was not reported the effect of VEGF combined with the small molecules modulation of Wnt signaling protocol.

Additionally, to evaluate the optimal coating surface for the adherence of differentiated cells, wells were coated with laminin, vitronectin and fibronectin. Cells were gently dissociated with EDTA solution on day 6 and split at a 1:3 ratio. After 3 days of re-plating (9th day of differentiation), all conditions showed monolayer spontaneous beating activity.

The expression of the late cardiac markers α-actinin and cTnT was measured by flow cytometry. On the 18th day of differentiation, cells were collected from each culture condition and each sample was divided in three, including the negative control, goat anti-mouse IgG, and two samples for each cardiac marker. The two markers, which are expressed in the same stage of cardiac differentiation, were used as a staining control once their expression is usually similar within the same population. Having this in mind, two culture conditions showed unexpected results: the culture condition exposed to VEGF at day 6 on laminin concerning the expression of α-actinin and the culture condition exposed to VEGF at day 5 plus day 6 on fibronectin concerning the expression of cTnT. The first culture condition should be expressing around 60%

α-actinin+ cells and the second culture condition around 35% cTnT+ cells. Interestingly, re-plated VEGF-untreated cells revealed low expression of the 2 markers (less than 19%). Additionally, the addition of VEGF on the day prior to re-plating showed less than 41% expression of cTnT+ and α-actinin+ cells. However, the addition of VEGF on day 6 revealed high expression of the two cardiac markers, showing up to 73% of α-actinin+ cells and 67% cTnT+ cells (figure IV-31). Moreover, the expression of the late cardiac markers appeared to be increased when cells were re-plated on vitronectin compared to laminin and fibronectin coatings, in all conditions.

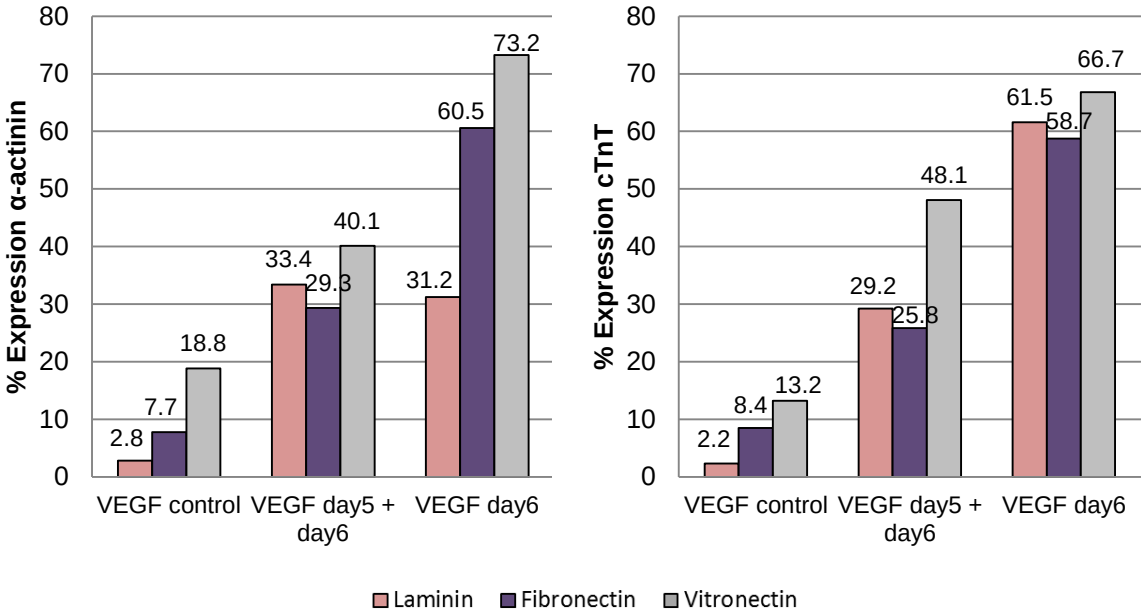

Figure IV-31- Flow cytometry analysis of iLB-c1-30m-r12 cells, committed into the cardiac lineage accordingly with GiWi protocol (3 µM CHIR plus 5 IWP4 added 72h after CHIR**)**, re-plated on day 6 on laminin, fibronectin and vitronectin coatings, for the cardiac markers cTnT and α-actinin, after 18 days in culture. VEGF was added to the culture on day 5 and 6 or just on day 6 of differentiation. Additionally, VEGF-untreated cells were used as control condition. (n=1; 2 replicates)

The pre-treatment of differentiated cells with VEGF one day prior re-plating improved cardiac differentiation compared with VEGF-untreated cells, however it revealed about half the expression of the late cardiac markers cTnT and α-actinin, present in cells treated with VEGF just on day 6. A possible explanation for this result can be the effect of VEGF on the proliferation of endothelial cells in the early mesoendoderm stage of differentiation. Continuous treatment with VEGF on day 5 and day 6 may have caused preferentially differentiation of some cells into the endothelial lineage over the production of cardiomyocytes. In fact, Pasha et al. have reported that hiPSC pre-treated with VEGF (50ng/ml) for 5 days revealed a high percentage of endothelial cells expressing CD31 (85%) and VE-cadherin (47%)[113]. The addition of VEGF just on day 6 may have been sufficient to induce the required signaling to increase cardiac genes expression and improve cardiac commitment, while maintaining the number of

endothelial cells at residual levels. However, it would be necessary to repeat the re-plating experiment in the same conditions and measure the expression of endothelial markers to further confirm this hypothesis.

As previously shown in section IV.2.3.3, the quantification of cardiac differentiation efficiency of iLB-c1-30m-r12 cells at P44, accordingly with GiWi protocol, showed only up to 37% α-actinin+ cells. In this study, the combination of VEGF (10 ng/mL) with Wnt signaling modulators (3 µM CHIR and 5 µM IWP4 added at 72h) proved to increase the yield of generated cardiomyocytes while increasing the efficiency of cardiac differentiation up to 73%. Typically, cardiomyocytes generated by Wnt modulation achieve on day 15 a total density equal to 0.8-1.3 million cardiomyocytes per cm^2 (80-98%cTnT+)[114]. By re-plating cells on day 6 at 1:3 ratio in a 12-well plate, we can largely estimate the production of around 9 million cardiomyocytes from an input of 400.000 hiPSC.

V. Conclusions

Cardiac differentiation of human induced pluripotent stem cells (hiPSC) offers a potentially unlimited source of cardiomyocytes for novel drug discovery, regenerative medicine, and the study of human cardiac development and diseases. Multiple approaches have been described for directed and efficient cardiac differentiation of hiPSC. These methods include co-culture with END2 (mouse visceral endoderm-like cell) stromal layers[62], differentiation of hiPSC in monolayer culture with high levels of Activin A and bone morphogenetic protein 4 (BMP4) which yielded 30% cardiomyocytes[65], and the formation of human embryoid bodies (hEB) with growth factor supplementation. These techniques are all limited in their capacities for scaling-up due to inherent low-throughput, poor differentiation yields, and the use of expensive reagents. Most importantly, there is great inconsistency in differentiation efficiency between various hiPSC lines. This variability is likely a function of genetic and epigenetic differences that directly impact their cardiac differentiation capacity[101].

In this work, several culture protocols were evaluated with the final aim of achieving a serum-free system that is capable of differentiating two different hiPS cell lines into cardiomyocytes with high efficiency while maintaining them in a monolayer culture. Although it was not possible to establish a differentiation system completely free of xenogenic contaminats mainly due to the use of matrigel coating as substrate for cell adherence, several advances were made in the establishment of biochemically defined coatings (laminin, fibronectin and vitronectin) from the day 6 of cardiac commitment.

The major part of this work relied on the optimization and efficiency quantification of the Activin A and BMP4 protocol and the small molecule modulators of Wnt signaling protocols for cardiac commitment of hiPSC. The concentration of 250 ng/mL Activin A (2.5 times higher the concentration used in the original method reported by Laflamme et al.[65]) and pre-treatment with 3uM CHIR for 2 days showed to be the best culture condition to produce spontaneous beating colonies. However, repetition of these culture conditions with the same cell line at passage 50 showed less than 3% expression of cardiac markers. This might be due to the chromosomal abnormality found in this cell line at P51 or to high inherent heterogeneity in cardiomyocyte differentiation yield associated with this protocol. Interestingly, it was also shown a high efficiency in the generation of vascular muscle cells following the Activin A/BMP4 protocol, for a wide range of growth factor concentrations. In near the future, it would be interesting to explore the production of vascular muscle cells, which play an important role in vascular homeostasis and disease, once the current protocols rely on the use of FBS, MEF-conditioned medium and a complex cocktail of growth factors[115], making it expensive and unsuitable for clinical applications.

Additionally, optimization of the recently published cardiac differentiation method based on the use of small molecule modulators of Wnt signaling protocol (GiWi), showed that the

addition of a low concentration of CHIR, 5 µM, 72 after CHIR, is the optimal value among the ones tested to efficiently produce a monolayer of actively beating cardiomyocytes. The original protocol reports the use of 12 µM CHIR[68], however in this study, that concentration led to complete cell death, 24 hours after CHIR addition in different hiPS cell lines. In addition, the GiWi protocol proved to efficiency differentiate iPS-DF6-9-9T.B cells, when adding 5 µm CHIR to the culture medium on day 0, showing up to 70% α-actinin+ cells after 18 days of differentiation.

The evaluation of the time period of Wnt induction in two hiPS cell lines showed that the timing in the addition of IWP4 to the culture can produce monolayers of actively beating cardiomyocytes with different paces. This result is very interesting in the way that the conjugation of different CHIR concentration and time of Wnt induction may lead to the production of different types of cardiomyocytes in different stages of maturation. This may allow the derivation of specific populations of ventricular-like, auricular-like or pacemaker-like cells to be engraft in different regions of the human heart or to test new drugs for the treatment of cardiac diseases with more reliable results.

Finally, by taking in advantage of the proliferative capacity of cardiac progenitor cells it was possible to increase the yield of the obtained cardiomyocytes by 3 fold by re-plating cells to fresh laminin-, fibronectin- and vitronectin-coated plates. By adding 10 ng/mL of VEGF to the culture on an early stage of differentiation, re-plated cells could proliferate, achieve confluence and differentiate into cardiomyocytes with very high efficiency (up to 73% α-actinin+ cells). Vitronectin showed to be a promising define substitute of matrigel for cardiomyocyte differentiation in fully xeno- and serum-free culture conditions.

Overall this work allowed the establishment and optimization of a serum-free method for the production of cardiomyocytes from hiPSC in a monolayer culture based on the GiWi protocol, which turns this method much more cost-effective than Activin A/BMP4 protocol and with great potential to be further used in the large scale production of cardiomyocytes for several applications such as cell-replacement therapies for heart regeneration after myocardial infarction, toxicological tests to evaluate the optimal concentration to be used in the treatment of specific patients or to model the evolution of cardiac diseases *in vitro* to further understand the molecular mechanisms of the disease.

VI. Future Work

This work was the first step given towards the successful production of cardiomyocytes from hiPSC in chemically defined conditions at the Stem Cell Bioengineering Lab (IBB-IST). However, future work is still needed in order to become closer to a major goal, the creation of hiPSC-CMs based therapies at a clinical scale. According to this, future work will consist in studying other conditions improving the differentiation of hiPSC.

A limited number of experiments was performed due to the time constrains of this Master project. For that reason, all experiments should be repeated in order to verify the results obtained and evaluate their statistical significance.

In order to further characterize the efficiency of hiPSC cardiac differentiation, the expression of specific markers already analyzed by immunocytochemistry and flow cytometry will also need to be confirmed by RT-PCR. For a complete characterization of hiPSC-derived cardiomyocytes, an electrophysiology analysis will also be important.

The next step will consist on scaling-up the production of cardiomyocytes from hiPSC, in a xeno-free microcarrier-based culture system, in serum-free conditions, using the protocol based on the use of the small molecule modulators of Wnt signaling. Additionally, it would be interesting to evaluate the combination of different biomaterials to improve cardiac maturation and sarcomere organization such as chitosan nanofiber scaffolds as a 3-D cardiac culture model. This may reduce population heterogeneity and improve the quality of final cardiomyocytes[98]. To use cardiomyocyte for regenerative medicine purposes it will be also necessary, in a near future, to establish a non-invasive and scalable purification system to obtain pure culture of cardiomyocytes such as the addition of high dosages of lactate in the absence of glucose to specifically select cardiomyocytes over pluripotent stem cells[79].

References

1. Lanza, R. *et al. Essentials of Stem Cell Biology*. (Academic Press, 2009). at <http://books.google.pt/books/about/Essentials_of_Stem_Cell_Biology.html?id=auyfanDzHlIC&redir_esc=y>

2. *Stem Cells: Scientific Progress and Future Research Directions*. (Department of Health and Human Services, 2001).

3. Jones, M. Stem cells diagram. (2006). at <http://en.wikipedia.org/wiki/Cell_potency>

4. Wagers, A. J., Sherwood, R. I., Christensen, J. L. & Weissman, I. L. Little Evidence for Developmental Plasticity of Adult Hematopoietic Stem Cells. *Science* **297 ,** 2256–2259 (2002).

5. Simons, B. D. & Clevers, H. Strategies for homeostatic stem cell self-renewal in adult tissues. *Cell* **145,** 851–862 (2011).

6. Slack, J. M. W. Stem cells in epithelial tissues. *Science* **287,** 1431–1433 (2000).

7. Thomson, J. A. *et al.* Embryonic Stem Cell Lines Derived from Human Blastocysts. *Science* **282 ,** 1145–1147 (1998).

8. Donovan, P. J. & Gearhart, J. The end of the beginning for pluripotent stem cells. *Nature* **414,** 92–7 (2001).

9. Ohtsuka, S. & Dalton, S. Molecular and biological properties of pluripotent embryonic stem cells. *Gene therapy* **15,** 74–81 (2008).

10. Jiang, Y. *et al.* Pluripotency of mesenchymal stem cells derived from adult marrow. *Nature* **418,** 41–49 (2002).

11. Tropepe, V. *et al.* Retinal stem cells in the adult mammalian eye. *Science* **287,** 2032–2036 (2000).

12. Gronthos, S., Mankani, M., Brahim, J., Robey, P. G. & Shi, S. Postnatal human dental pulp stem cells (DPSCs) in vitro and in vivo. *Proceedings of the National Academy of Sciences* **97,** 13625–13630 (2000).

13. Beltrami, A. P. *et al.* Adult cardiac stem cells are multipotent and support myocardial regeneration. *Cell* **114,** 763–76 (2003).

14. Toma, J. G. *et al.* Isolation of multipotent adult stem cells from the dermis of mammalian skin. *Nature cell biology* **3,** 778–784 (2001).

15. Barker, N. *et al.* Identification of stem cells in small intestine and colon by marker gene Lgr5. *Nature* **449,** 1003–1007 (2007).

16. Domen, J. & Weissman, I. L. Self-renewal, differentiation or death: regulation and manipulation of hematopoietic stem cell fate. *Molecular Medicine Today* **5,** 201–208 (1999).

17. Takahashi, K. & Yamanaka, S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell* **126,** 663–76 (2006).

18. Takahashi, K. *et al.* Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell* **131,** 861–72 (2007).

19. Amabile, G. & Meissner, A. Induced pluripotent stem cells: current progress and potential for regenerative medicine. *Trends in molecular medicine* **15,** 59–68 (2009).

20. Park, I.-H. *et al.* Disease-Specific Induced Pluripotent Stem Cells. *Cell* **134,** 877–886 (2008).

21. Yamanaka, S. A fresh look at iPS cells. *Cell* **137,** 13–7 (2009).

22. Okita, K., Ichisaka, T. & Yamanaka, S. Generation of germline-competent induced pluripotent stem cells. *Nature* **448,** 313–7 (2007).

23. O'Malley, J., Woltjen, K. & Kaji, K. New strategies to generate induced pluripotent stem cells. *Current Opinion in Biotechnology* **20,** 516–521 (2009).

24. Rawles, M. The heart-forming areas of the early chick blastoderm. *Physiol Zool* 22–42 (1943).

25. Gilbert, S. F. *Developmental Biology*. (Sinauer Associates, 2010). at <http://books.google.pt/books/about/Developmental_Biology.html?id=CL2DSQAACAAJ&redir_esc=y>

26. Olson, E. N. The Path to the Heart and the Road Not Taken. *Science* **291 ,** 2327–2328 (2001).

27. Bondue, A. & Blanpain, C. Mesp1: a key regulator of cardiovascular lineage commitment. *Circulation research* **107,** 1414–27 (2010).

28. Wu, S. M., Chien, K. R. & Mummery, C. Origins and fates of cardiovascular progenitor cells. *Cell* **132,** 537–43 (2008).

29. Laflamme, M. A. & Murry, C. E. Heart regeneration. (2011). doi:10.1038/nature10147

30. Laflamme, M. A. & Murry, C. E. Regenerating the heart. **23,** 845–856 (2005).

31. Mensah, G. A. & Brown, D. W. An Overview Of Cardiovascular Disease Burden In The United States. *Health Affairs* **26 ,** 38–48 (2007).

32. Kikuchi, K. & Poss, K. D. Cardiac regenerative capacity and mechanisms. *Annual review of cell and developmental biology* **28,** 719–41 (2012).

33. Burridge, P. W., Keller, G., Gold, J. D. & Wu, J. C. Review Production of De Novo Cardiomyocytes : Human Pluripotent Stem Cell Differentiation and Direct Reprogramming. *Stem Cell* **10,** 16–28 (2011).

34. Capi, O. & Gepstein, L. Myocardial regeneration strategies using human embryonic stem cell-derived cardiomyocytes. *Journal of Controlled Release* **116,** 211–218 (2006).

35. Devarajan, K., Staecker, H. & Detamore, M. S. A Review of Gene Delivery and Stem Cell Based Therapies for Regenerating Inner Ear Hair Cells. *Journal of Functional Biomaterials* **2,** 249–270 (2011).

36. Rajala, K., Pekkanen-Mattila, M. & Aalto-Setälä, K. Cardiac differentiation of pluripotent stem cells. *Stem cells international* **2011,** 383709 (2011).

37. Vincent, S. D. & Buckingham, M. E. How to make a heart: the origin and regulation of cardiac progenitor cells. *Current topics in developmental biology* **90,** 1–41 (2010).

38. Beltrami, A. P. *et al.* Evidence That Human Cardiac Myocytes Divide after Myocardial Infarction. *New England Journal of Medicine* **344,** 1750–1757 (2001).

39. Laugwitz, K.-L. *et al.* Postnatal isl1+ cardioblasts enter fully differentiated cardiomyocyte lineages. *Nature* **433,** 647–653 (2005).

40. Bu, L. *et al.* Human ISL1 heart progenitors generate diverse multipotent cardiovascular cell lineages. *Nature* **460,** 113–7 (2009).

41. Laugwitz, K.-L., Moretti, A., Caron, L., Nakano, A. & Chien, K. R. Islet1 cardiovascular progenitors: a single source for heart lineages? *Development* **135 ,** 193–205 (2008).

42. Boheler, K. R. Differentiation of Pluripotent Embryonic Stem Cells Into Cardiomyocytes. *Circulation Research* **91,** 189–201 (2002).

43. Passier, R., van Laake, L. W. & Mummery, C. L. Stem-cell-based therapy and lessons from the heart. *Nature* **453,** 322–9 (2008).

44. Kallos, M. S. *Embryonic Stem Cells- Differentiation and Pluripotent Alternatives*. (InTech, 2011).

45. Noseda, M., Peterkin, T., Simões, F. C., Patient, R. & Schneider, M. D. Cardiopoietic factors: extracellular signals for cardiac lineage commitment. *Circulation research* **108,** 129–52 (2011).

46. Nusse, R. Wnt signaling and stem cell control. *Cell research* **18,** 523–7 (2008).

47. Blauwkamp, T. a, Nigam, S., Ardehali, R., Weissman, I. L. & Nusse, R. Endogenous Wnt signalling in human embryonic stem cells generates an equilibrium of distinct lineage-specified progenitors. *Nature communications* **3,** 1070 (2012).

48. Chen, B. *et al.* Small molecule-mediated disruption of Wnt-dependent signaling in tissue regeneration and cancer. *Nature chemical biology* **5,** 100–107 (2009).

49. Wend, P., Holland, J. D., Ziebold, U. & Birchmeier, W. Wnt signaling in stem and cancer stem cells. *Seminars in Cell & Developmental Biology* **21,** 855–863 (2010).

50. Gessert, S. & Kühl, M. The multiple phases and faces of wnt signaling during cardiac differentiation and development. *Circulation research* **107,** 186–199 (2010).

51. Lian, X. *et al.* Robust cardiomyocyte differentiation from human pluripotent stem cells via temporal modulation of canonical Wnt signaling. *Proceedings of the National Academy of Sciences of the United States of America* **109,** E1848–57 (2012).

52. Lian, X., Zhang, J., Zhu, K., Kamp, T. J. & Palecek, S. P. Insulin Inhibits Cardiac Mesoderm, not Mesendoderm, formation during Cardiac Differentiation of Human Pluripotent Stem Cells and Modulation of Canonical Wnt Signaling Can Rescue this Inhibition. *Stem cells (Dayton, Ohio)* (2012). doi:10.1002/stem.1289

53. Blauwkamp, T. a, Nigam, S., Ardehali, R., Weissman, I. L. & Nusse, R. Endogenous Wnt signalling in human embryonic stem cells generates an equilibrium of distinct lineage-specified progenitors. *Nature communications* **3,** 1070 (2012).

54. Rai, M., Walthall, J. M., Hu, J. & Hatzopoulos, A. K. Continuous antagonism by Dkk1 counter activates canonical Wnt signaling and promotes cardiomyocyte differentiation of embryonic stem cells. *Stem cells and development* **21,** 54–66 (2012).

55. Guo, X. & Wang, X.-F. Signaling cross-talk between TGF-beta/BMP and other pathways. *Cell research* **19,** 71–88 (2009).

56. Chng, Z., Teo, A., Pedersen, R. a & Vallier, L. SIP1 mediates cell-fate decisions between neuroectoderm and mesendoderm in human pluripotent stem cells. *Cell stem cell* **6,** 59–70 (2010).

57. Mummery, C. L. *et al.* Differentiation of Human Embryonic Stem Cells and Induced Pluripotent Stem Cells to Cardiomyocytes: A Methods Overview . *Circulation Research* **111 ,** 344–358 (2012).

58. Itskovitz-Eldor, J. *et al.* Differentiation of human embryonic stem cells into embryoid bodies compromising the three embryonic germ layers. *Molecular medicine (Cambridge, Mass.)* **6,** 88–95 (2000).

59. Niebruegge, S. *et al.* Generation of human embryonic stem cell-derived mesoderm and cardiac cells using size-specified aggregates in an oxygen-controlled bioreactor. *Biotechnology and bioengineering* **102,** 493–507 (2009).

60. Zhang, J. *et al.* Functional Cardiomyocytes Derived From Human Induced Pluripotent Stem Cells. *Circulation Research* **104 ,** e30–e41 (2009).

61. Mummery, C. *et al.* Differentiation of human embryonic stem cells to cardiomyocytes: role of coculture with visceral endoderm-like cells. *Circulation* **107,** 2733–40 (2003).

62. Fujiwara, M. *et al.* Induction and enhancement of cardiac cell differentiation from mouse and human induced pluripotent stem cells with cyclosporin-A. *PloS one* **6,** e16734 (2011).

63. Ojala, M. *et al.* Culture conditions affect cardiac differentiation potential of human pluripotent stem cells. *PloS one* **7,** e48659 (2012).

64. Moon, S.-H. *et al.* Development of a novel two-dimensional directed differentiation system for generation of cardiomyocytes from human pluripotent stem cells. *International journal of cardiology* (2012). doi:10.1016/j.ijcard.2012.09.077

65. Laflamme, M. a *et al.* Cardiomyocytes derived from human embryonic stem cells in pro-survival factors enhance function of infarcted rat hearts. *Nature biotechnology* **25,** 1015–24 (2007).

66. Osafune, K. *et al.* Marked differences in differentiation propensity among human embryonic stem cell lines. **26,** 2007–2009 (2008).

67. Zhang, J. *et al.* Extracellular Matrix Promotes Highly Efficient Cardiac Differentiation of Human Pluripotent Stem Cells: The Matrix Sandwich Method. *Circulation research* (2012). doi:10.1161/CIRCRESAHA.112.273144

68. Lian, X. *et al.* Directed cardiomyocyte differentiation from human pluripotent stem cells by modulating Wnt/β-catenin signaling under fully defined conditions. *Nature protocols* **8,** 162–175 (2012).

69. Snir, M. *et al.* Assessment of the ultrastructural and proliferative properties of human embryonic stem cell-derived cardiomyocytes. *American Journal of Physiology - Heart and Circulatory Physiology* **285 ,** H2355–H2363 (2003).

70. Liu, J., Fu, J. D., Siu, C. W. & Li, R. A. Functional Sarcoplasmic Reticulum for Calcium Handling of Human Embryonic Stem Cell-Derived Cardiomyocytes: Insights for Driven Maturation. *STEM CELLS* **25,** 3038–3044 (2007).

71. Xu, C. Differentiation and enrichment of cardiomyocytes from human pluripotent stem cells. *Journal of molecular and cellular cardiology* **52,** 1203–12 (2012).

72. Xu, C., Police, S., Rao, N. & Carpenter, M. K. Characterization and Enrichment of Cardiomyocytes Derived From Human Embryonic Stem Cells. *Circulation Research* **91 ,** 501–508 (2002).

73. Tanaka, T. *et al.* In vitro pharmacologic testing using human induced pluripotent stem cell-derived cardiomyocytes. *Biochemical and biophysical research communications* **385,** 497–502 (2009).

74. Robertson, C., Tran, D. D. & George, S. C. Concise Review: Maturation Phases of Human Pluripotent Stem Cell-Derived Cardiomyocytes. *Stem Cells* N/A–N/A (2013). doi:10.1002/stem.1331

75. Moretti, A. *et al.* Patient-Specific Induced Pluripotent Stem-Cell Models for Long-QT Syndrome. *New England Journal of Medicine* **363,** 1397–1409 (2010).

76. Hattori, F. *et al.* Nongenetic method for purifying stem cell-derived cardiomyocytes. *Nature methods* **7,** 61–66 (2010).

77. Rust, W., Balakrishnan, T. & Zweigerdt, R. Cardiomyocyte enrichment from human embryonic stem cell cultures by selection of ALCAM surface expression. *Regenerative Medicine* **4,** 225–237 (2009).

78. Uosaki, H. *et al.* Efficient and Scalable Purification of Cardiomyocytes from Human Embryonic and Induced Pluripotent Stem Cells by VCAM1 Surface Expression. *PLoS ONE* **6,** e23657 (2011).

79. Tohyama, S. *et al.* Distinct metabolic flow enables large-scale purification of mouse and human pluripotent stem cell-derived cardiomyocytes. *Cell stem cell* **12,** 127–37 (2013).

80. Villa-Diaz, L. G., Ross, a M., Lahann, J. & Krebsbach, P. H. The Evolution of Human Pluripotent Stem Cell Culture: From Feeder Cells to Synthetic Coatings. *Stem cells (Dayton, Ohio)* 1–7 (2012). doi:10.1002/stem.1260

81. Martin, M. J., Muotri, A., Gage, F. & Varki, A. Human embryonic stem cells express an immunogenic nonhuman sialic acid. *Nature medicine* **11,** 228–232 (2005).

82. Xu, C. *et al.* Feeder-free growth of undifferentiated human embryonic stem cells. *Nature biotechnology* **19,** 971–4 (2001).

83. Chen, G. *et al.* Chemically defined conditions for human iPSC derivation and culture. *Nature methods* **8,** 424–9 (2011).

84. Melkoumian, Z. *et al.* Synthetic peptide-acrylate surfaces for long-term self-renewal and cardiomyocyte differentiation of human embryonic stem cells. *Nature biotechnology* **28,** 606–10 (2010).

85. *Stem Cell and Gene-Based Therapy: Frontiers in Regenerative Medicine*. 422 (Springer, 2007). at <http://books.google.com/books?id=VMojvOvOTjAC&pgis=1>

86. Niebruegge, S. *et al.* Cardiomyocyte production in mass suspension culture: embryonic stem cells as a source for great amounts of functional cardiomyocytes. *Tissue engineering. Part A* **14,** 1591–601 (2008).

87. Lyons, E. & Pandit, A. in *Topics in Tissue Enginneering* (Ashammakhi, N. & Reis, R. L.) (2005).

88. Ashton, R. S., Keung, A. J., Peltier, J. & Schaffer, D. V. Progress and prospects for stem cell engineering. *Annual review of chemical and biomolecular engineering* **2,** 479–502 (2011).

89. Kehoe, D. E., Jing, D., Lock, L. T., Tzanakakis, E. S. & Ph, D. Scalable Stirred-Suspension Bioreactor Culture. **16,** (2010).

90. Amit, M. *et al.* Suspension Culture of Undifferentiated Human Embryonic and Induced Pluripotent Stem Cells. *Stem Cell Reviews and Reports* **6,** 248–259 LA – English (2010).

91. Reuveny, S., Ph, D. & Oh, S. Scalable Platform for Human Embryonic Stem Cell Differentiation to Cardiomyocytes in Suspended Microcarrier Cultures. **16,** (2010).

92. Rodrigues, C. a V, Fernandes, T. G., Diogo, M. M., da Silva, C. L. & Cabral, J. M. S. Stem cell cultivation in bioreactors. *Biotechnology advances* **29,** 815–29 (2011).

93. Karam, J.-P., Muscari, C. & Montero-Menei, C. N. Combining adult stem cells and polymeric devices for tissue engineering in infarcted myocardium. *Biomaterials* **33,** 5683–95 (2012).

94. Ulloa-Montoya, F., Verfaillie, C. M. & Hu, W.-S. Culture systems for pluripotent stem cells. *Journal of bioscience and bioengineering* **100,** 12–27 (2005).

95. Synthetic, P., Pore, P. & Engineering, T. Polymer Scaffolds processing . Biodegradable polymers for tissue engineering Cell and Tissue Engineering Biomaterials : 67–118

96. Placzek, M. R. *et al.* Stem cell bioprocessing: fundamentals and principles. *Journal of the Royal Society, Interface / the Royal Society* **6,** 209–32 (2009).

97. Dawson, J. *et al.* Collagen scaffolds with or without the addition of RGD peptides support cardiomyogenesis after aggregation of mouse embryonic stem cells. *In Vitro Cellular & Developmental Biology - Animal* **47,** 653–664 LA – English (2011).

98. Hussain, A., Collins, G., Yip, D. & Cho, C. H. Functional 3-D cardiac co-culture model using bioactive chitosan nanofiber scaffolds. *Biotechnology and bioengineering* **110,** 637–47 (2013).

99. Karp, J. M. *et al.* A photolithographic method to create cellular micropatterns. *Biomaterials* **27,** 4755–4764 (2006).

100. Zwi-Dantsis, L. & Gepstein, L. Induced pluripotent stem cells for cardiac repair. *Cellular and molecular life sciences : CMLS* **69,** 3285–99 (2012).

101. Burridge, P. W. *et al.* A Universal System for Highly Efficient Cardiac Differentiation of Human Induced Pluripotent Stem Cells That Eliminates Interline Variability. **6,** (2011).

102. Martí, M. *et al.* Characterization of pluripotent stem cells. *Nature protocols* **8,** 223–53 (2013).

103. Crook, J., Hei, D. & Stacey, G. The International Stem Cell Banking Initiative (ISCBI): raising standards to bank on. *In Vitro Cellular & Developmental Biology - Animal* **46,** 169–172 (2010).

104. Chambers, S. M. *et al.* Highly efficient neural conversion of human ES and iPS cells by dual inhibition of SMAD signaling. *Nat Biotech* **27,** 275–280 (2009).

105. Mayshar, Y. *et al.* Identification and classification of chromosomal aberrations in human induced pluripotent stem cells. *Cell stem cell* **7,** 521–31 (2010).

106. Olariu, V. *et al.* Modeling the evolution of culture-adapted human embryonic stem cells. *Stem cell research* **4,** 50–6 (2010).

107. Kadaja, M., Isok-Paas, H., Laos, T., Ustav, E. & Ustav, M. Mechanism of Genomic Instability in Cells Infected with the High-Risk Human Papillomaviruses. *PLoS Pathog* **5,** e1000397 (2009).

108. Bauwens, C. L. *et al.* Control of Human Embryonic Stem Cell Colony and Aggregate Size Heterogeneity Influences Differentiation Trajectories. *STEM CELLS* **26,** 2300–2310 (2008).

109. Ming, M. *et al.* Activation of Wnt/β-Catenin Protein Signaling Induces Mitochondria-mediated Apoptosis in Hematopoietic Progenitor Cells. *Journal of Biological Chemistry* **287 ,** 22683–22690 (2012).

110. Kim, K., Pang, K. M., Evans, M. & Hay, E. D. Overexpression of β-Catenin Induces Apoptosis Independent of Its Transactivation Function with LEF-1 or the Involvement of Major G1 Cell Cycle Regulators. *Molecular Biology of the Cell* **11 ,** 3509–3523 (2000).

111. Kattman, S. J. *et al.* Stage-specific optimization of activin/nodal and BMP signaling promotes cardiac differentiation of mouse and human pluripotent stem cell lines. *Cell stem cell* **8,** 228–40 (2011).

112. Ye, L. *et al.* Effective Cardiac Myocyte Differentiation of Human Induced Pluripotent Stem Cells Requires VEGF. *PLoS ONE* **8,** e53764 (2013).

113. Pasha, Z., Haider, K. H., Li, N. & Ashraf, M. VEGF Signaling Enhances Endothelial Differentiation of Induced Pluripotent Stem Cells: In vitro and in vivo Characterization. *Circulation* **124,** (2011).

114. Lian, X. *et al.* Directed cardiomyocyte differentiation from human pluripotent stem cells by modulating Wnt/β-catenin signaling under fully defined conditions. *Nature protocols* **8,** 162–75 (2013).

115. Bajpai, V. K., Mistriotis, P., Loh, Y.-H., Daley, G. Q. & Andreadis, S. T. Functional Vascular Smooth Muscle Cells Derived From Human Induced Pluripotent Stem Cells Via Mesenchymal Stem Cell Intermediates. *Cardiovascular Research* (2012). doi:10.1093/cvr/cvs253