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Stem cells and regenerative medicine for congenital heart disease.

Mohammed Salah ElSaid Debes

A dissertation submitted to the University of Bristol in accordance with the requirements for award of the degree of PhD in the Faculty of Bristol Medical School.

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Abstract

Congenital heart diseases are the most common congenital defects worldwide. Materials used for corrective surgery such as the biologic scaffold cormatrix are inherently non-contractile and cannot meet the dynamic growth of the heart. Seeding cormatrix with cells showed superior results but this approach is limited by short durability of cells in-vivo which is not well understood.

We investigated the effect of seeding cormatrix with triad of cells including Wharton jelly mesenchymal stem cells (WjMSCs), human umbilical vein endothelial cells (HUVECs) and human cardiac fibroblasts (HCFs). Then to examine their mutual interaction to understand and also anticipate their in-vivo behaviour.

The three cell phenotypes were characterised using morphology and expression of specific markers in addition to wjMSCs trilineage differentiation. Then conditioned media (CdM) were used to examine the mutual cell interaction. We used immunofluorescence to assess protein expression, qPCR for mRNA, flowcytometry for apoptosis/cell death assay and BrdU assay to assess cell proliferation.

The CdM effects were quite variable. It did enhance pro-survival and angiogenic factors of wjMSCs, did not support survival or angiogenic profile of HUVECs while mostly neutral effect on HCFs.

We attempted to boost the patch's cellular properties using small molecules. So, we trialled to differentiate wjMSCs to cardiomyocytes using 5-azacitidine. This trial failed to show any cardiac differentiation. Then, we examined effects of the GSK-3 inhibitor CHIR99021 on the triad of cells and extended the assessment to the combined effect of CHIR99021 and CdM (CHIR/CdM) which showed better survival of wjMSCs and HUVECs while still neutral effect on HCFs. Finally, cormatrix uptake of this combination of cells was tested using H&E, cells viability using live/dead cell staining and surface topography using scanning electron microscope. Also effect on mechanical properties of the patch was examined using tensile stress machine. Cormatrix proved biocompatible with the triad of cells and maintained its mechanical elasticity.

The next step would be to test this patch in clinically relevant large animal model.

Covid-19 statement

This project was interrupted by the pandemic restrictions. I had more work to be done including more factors to be interrogated and more qPCR experiments. Indeed, I had several flasks ready for image capture and also plans to further expand the biologic replicates.
With the pandemic lockdown and further restrictions, I was advised to submit the available data as this situation could potentially take long and it is unpredictable.
I did highlight these drawbacks at the relevant areas in the body of the thesis.

Author's declaration

I declare that the work in this dissertation was carried out in accordance with the requirements of the University's *Regulations and Code of Practice for Research Degree Programmes* and that it has not been submitted for any other academic award. Except where indicated by specific reference in the text, the work is the candidate's own work. Work done in collaboration with, or with the assistance of, others, is indicated as such. Any views expressed in the dissertation are those of the author.

SIGNED: Mohammed S. Debes DATE: 15/3/2021

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Abbreviations and acronyms

A

ASCs: Adult stem cells.

5-az: 5-azacitidine.

B

BI: branching index.

BIO: 6-bromoindirubin-3′-oxime.

Bp: base pair.

C

CdM: conditioned media.

C-dK: cyclin dependent kinase.

CHD: congenital heart disease.

CMC: cardiomyocyte.

CNC: cardiac neural crest.

CSCs: cardiac stem cells.

D

DMSO: dimethyl sulfoxide.

E

ECs: endothelial cells.

EGM: endothelial cell growth medium.

EMT: epithelial-mesenchymal transformation.

ET: endothelin 1.

EVG: Verhoeff's Van-Gieson.

F

FACS: flow cytometry assisted cell sorting.

FCM: flow cytometry.

FHF: first heart field.

G

H

HCF: human cardiac fibroblasts.

H&E: Haematoxylin and Eosin.

HUVECs: human umbilical cord endothelial cells.

I

ICC: immunocytochemistry.

ICM: inner cell mass.

Id: integrated density.

ISCT: International Society for Cellular Therapy.

J

K

L

LIF/Stat3: Leukaemia inhibitory factor/signal transducer and activator of transcription factor.

LV: left ventricle.

M

MACS: magnetic activated cell sorting.

MHC: major histocompatibility.

MSCs: mesenchymal stromal cells.

N

NK cells: natural killer cells.

NO: nitric oxide.

O

OFT: outflow tract.

P

PCR: polymerase chain reaction.

P/S: penicillin/streptomycin.

PS: primitive streak.

R

Rb: retinoblastoma.

ROS: reactive oxygen species.

RT: room temperature.

RV: right ventricle.

S

SCID: severe combined immune deficient.

SHF: second heart field.

SMCs: smooth muscle cells.

T

TEM: transmission electron microscope.

TF: transcription factor.

TLR: Toll like receptor.

U

UC: umbilical cord.

V

W

WB: western blot.

WJ: wharton jelly.

Y

YM: Young's Modulus.

Chapter I

Introduction

1.Introduction

1.1.Cardiovascular system and circulation

The cardiovascular system comprises heart and blood vessels. The heart has four chambers including left and right atria separated by the interatrial septum, left and right ventricles separated by interventricular septum in addition to the atrio-ventricular valves between atria and ventricles. In continuity with the heart there are major blood vessels including aorta, pulmonary artery, vena cavas and pulmonary veins.

The left atrium (LA) receives oxygenated blood from the pulmonary veins and passes it through the mitral valve to the left ventricle (LV) to get pumped to the systemic circulation through the aorta and its downstream branches. Blood flows through the aorta and its branches which get gradually smaller from arteries to arterioles to capillaries where the actual tissue perfusion takes place. Then, the returning deoxygenated blood drains through the venous vessels which get gradually bigger from venules to veins and large veins until superior and inferior vena cava to the right atrium (RA). The RA passes blood through the tricuspid valve to the right ventricle (RV). RV pumps blood through the pulmonary artery to the pulmonary circulation in order to get oxygenated in the lungs and get rid of the CO_2. Then blood drains via pulmonary veins to the LA to start another cycle (1).

This summarised overview illustrates how intact anatomical status of the heart and its connections with major blood vessels both arterial and venous is quite crucial for the integrity of the heart function and adequate circulation to sustain the whole organism.

1.2.Cardiogenesis

Heart is the first organ to form and function during embryogenesis (2, 3) in order to support fetal development. Indeed, heart beating is the first indicator of fetal life (4). Therefore, embryonic cardiac defects represent a major cause of foetal mortality due to failure of distribution of oxygen and other essential nutrients (5).
The cardiogenesis journey starts from gastrulation and mesoderm formation (6). All cardiac cell lineages have common ancestor cells that proliferate and gradually differentiate into endocardial, myocardial and smooth muscle cell phenotypes (7). The precursors of progenitor cardiac cells reside anteriorly in the primitive streak (PS) and ingress early from the PS in preparation to form the cardiac crescent (3) but still at this phase cells are not fully committed to cardiac fate (2). Cells would ingress from the lateral mesoderm plate to form the first heart field (FHF) which populates the LV and sinus venosus while cells for the RV and outflow tract (OFT) would originate from the second heart field (SHF) which forms posterior to the FHF (8). Then, cells would expand antero-laterally to form the cardiac crescents (2, 9). During this stage cardiac markers start to express (2). It is noteworthy that this phase would be orchestrated via endodermal signals to control migration of cardiac lateral plate mesoderm toward the midline (10). This is a mandatory step for single heart tube formation in addition to other critical steps such as cell migration, polarity and ECM formation (10).

This takes place at day E7.5 in mouse and 2 weeks gestation in human (2, 3). This gradual commitment into cardiac fate which is a further step during the differentiation journey toward the cardiac fate would take place under effect of several extracellular signals such as BMPs, FGFs and TGF-β (6). For instance,

bone morphogenetic protein (BMP) is critically involved in Isl1 positive cells which dominate the SHF and deletion of BMP 1a receptor results in defects in SHF derivatives (11). Also, FGF is involved in SHF fate defining and deletion of the receptors FGFr1 and FGFr2 results in outflow defects and cells cannot support ECM production (11).

Then, by day E8 in mouse/3weeks in human the cardiac crescents fuse at the midline to start forming the linear heart tube that is derived from the FHF which eventually undergoes further growth and rightward looping around E8.5 in mouse/4weeks in human (9). During this phase the first sign of contractile motion would start to show (8).

Cells of the linear heart tube continue to proliferate and other cells would add to the growing heart from the SHF that originates from the pharyngeal mesoderm and contribute to progressive growth of the linear heart tube through adding to arterial and venous poles (3, 9). By E10.5 in mouse/E32 in human the heart becomes fully septated and by E14.5 in mouse/7weeks in human there will be well defined chambers in which the FHF gives rise to the LV and the SHF would eventually contribute to the RV, atria and outflow tract (OFT) (3, 9).

During the late stages of cardiac development one of the most important steps would take place which includes proper positioning of the aorta and pulmonary artery in relation to the LV and RV respectively under control of Isl1 expressing cells and defects in this phase represent one of the most common causes of CHDs (11).

It is notable that all through embryonic life the CMCs undergo hyperplastic proliferation while after birth this would cease and cells would undergo a final phase of karyokinesis without actual cell division resulting in binuclear CMCs which transit to a post-mitotic nature and exit the cell cycle (12).

Table 1-1. Sources of different parts of the heart during cardiogenesis (3).

Source	**Fate**
Cardiac Mesoderm	➢ Myocardium in ventricles, atria and OFT.
Proepicardium	➢ Epicardium and cardiac fibroblasts. ➢ SMCs and ECs of coronary vessels. ➢ Myocytes in atrio-ventricular septum.
Cardiac Neural Crest	➢ Aorto-pulmonary ridge. ➢ Cardiac autonomic nervous system. ➢ Distal smooth muscle cells of the OFT.

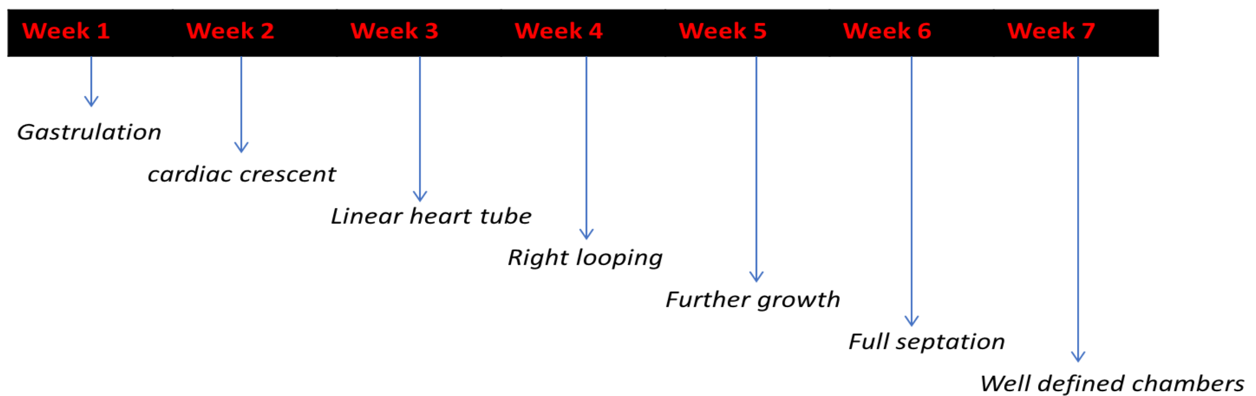

Figure 1-1.Timeline of embryonic cardiac development in human.

Progress of embryonic heart from first gestational week with gastrulation until fully septated by week 7 gestation.

1.3.Cardiac tissue

1.3.1. Introduction

The heart tissue is composed of the cellular pool including the permanent cells cardiomyocytes (CMCs), vascular smooth muscle cells (VSMCs), endothelial cells (ECs) as well as fibroblasts in addition to the non-permanent cells such as mast cells, lymphocytes and macrophages (13). In addition to the non-cellular pool which is the extracellular matrix (ECM) (14).

The myocardial tissue is a highly organised network of inter-connected/communicating cells enmeshed in the dynamic pool of ECM and its development and performance under physiologic as well as during pathologic events is quite dependent on efficient communication between all these components (14, 15).

The myocardial tissue is organised as laminae of cellular and acellular components consisting of layers each about 2-5 cells in thickness embedded in a collagen network (14, 16). There is close inter-relation between different cellular components to build up functioning units as CMCs are aligned in layers and fibroblasts are interspersed between these layers so that almost every cardiomyocyte has some contact with a fibroblast (16).
Within the ECM mesh, distribution of cardiac cells is not homogenous but there are anatomical variations according to physiological demands such as variations between atria and ventricles (13). Also, the alignment of cells and ECM fibrils in layers with specific orientation generates significant anisotropic mechanical and electrical properties (17). Linearity and orientation of cardiac cells is quite crucial for its efficient pumping function therefore, disruption by angulation of cells or fibrosis is actually a feature of failing heart (11).
Other cell types mainly ECs and VSMCs are restricted to the vascular tree (14) which is diffusely spread to meet the high metabolic demands. Indeed, cardiac metabolism is crucially dependent on oxidative energy sources and its oxygen consumption is about 20 folds that of resting skeletal muscles (18).

Therefore, it is supported by extensive vascular network in addition to the highly controlled coronary blood flow (18).

Also, volume of cardiac cells undergo dynamic changes during the different phases of fetal life, neonatal period and adult life (13). Also, the various constituents of the myocardium interact in a sensitive dynamic manner to maintain adequate cardiac function and to react efficiently to various physiological and pathological stressors (13).

1.3.2.Cardiac cells

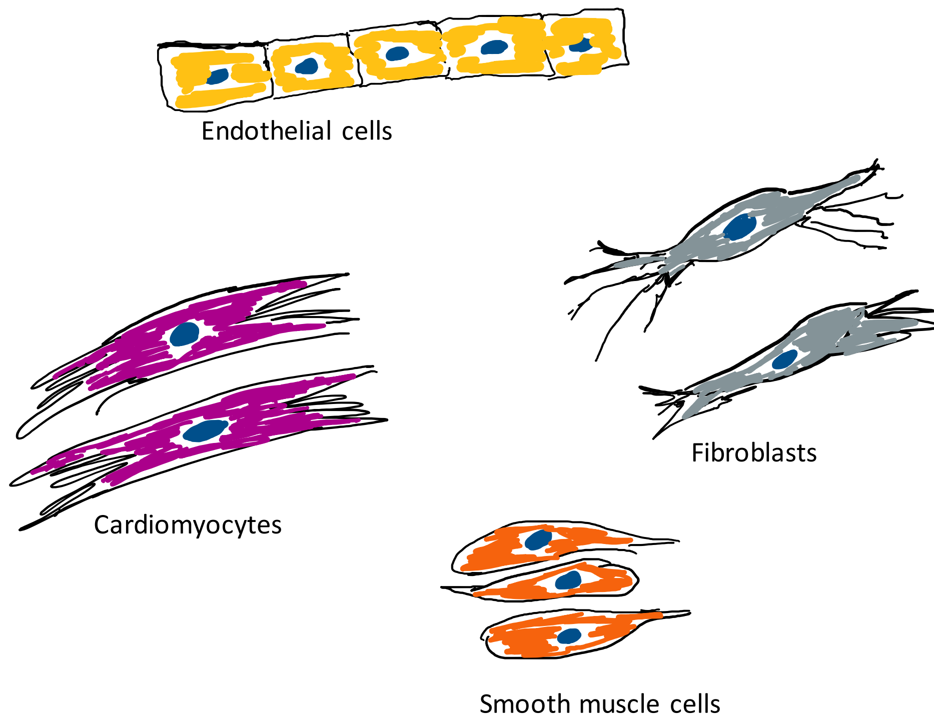

Figure 1-2. The four main cell phenotypes in the myocardium.

1.3.2.1.Cardiomyocytes (CMCs)

CMCs are the main functioning cells in the myocardium and despite they form significant portion of the myocardial volume (14) they only represent 25- 35% of its cellular pool while the rest are non-myocytes (19).

CMCs have two distinct phases of growth, the hyperplastic/mitotic embryonic phase that could extend to early neonatal life and the hypertrophic/post-mitotic post-natal phase (20). During embryonic development CMCs proliferate and increase in number while after birth the DNA replicates without actual cell division ending up with two distinct nuclei which is a characteristic of mature CMCs while during adult life and in response to work load CMCs increase in size but not in number and in absence of

pathologic cell loss, CMCs number remains stable throughout life and its turnover rate is about 0.5-2% annually (21).

Considering these phases of growth, the adult heart was believed to be a post mitotic organ as CMCs are locked at G_0 and are terminally differentiated cells with no proliferation potential (22). This view was adopted due to absent mitotic nuclei and lack of DNA replication as shown in the 3H-Thymidine incorporation studies (23). However, this was changed in 2001 by the work of Beltrami et al. (16) who demonstrated that CMCs could re-enter the cell cycle to divide and proliferate again in addition to CMC progenitors known as the cardiac stem cells (CSCs) which still hold proliferation potential (5, 24). Nevertheless, this is not enough to replace the big volume of cells lost during a cardiac event such as myocardial infarction and still transplantation is the only cure for advanced heart failure (14).

CMCs work in a highly organized manner so that the myocardium of each chamber will contract as one unit to generate enough force to eject blood and maintain the circulation (20) which mandates meticulous anatomical and physiological co-ordination. Therefore, CMCs are aligned in layers within the ECM in close contact with fibroblasts and capillaries with very efficient communication channels including adherens junctions, gap junctions and desmosomes located at the intercalated discs (25).

Further to their contractile function, CMCs share in cross talk between each other through several mechanisms such as autocrine route, paracrine and endocrine factors as well as other modes of communication such as propagation of depolarization waves, gap junctions and adhesion complexes (18). Also interact with other cells via secreting several factors including angiogenic factors such as VEGF which activates VEGFR2 on ECs to activate angiogenesis in addition to other factors such as bFGF, HGF, PLGF and angiopoietin as well as ECM peptides and cytokines to regulate cardiac function (26). In addition, CMCs interact with the ECM via integrin-B1 that connects with the intracellular sarcomeric contractile proteins and under mechanical load it would activate intracellular signals to induce CMCs hypertrophy (18).

1.3.2.2.Cardiac fibroblasts(CFs)

Fibroblasts are widely distributed connective tissue cells that could be defined as "*cells of mesenchymal origin that produce different components of the extracellular matrix such as collagens and fibronectin*" (13). They have characteristic filamentous morphology with branching processes and oval nucleus with extensive granular material in the cytoplasm but do not have basement membrane (14, 16).

Fibroblasts were believed to be uniform cells in all tissues however, recently they are recognised to have diverse phenotypes with functional heterogeneity that is displayed in different anatomical and physiological circumstances to optimise morphogenesis, mechanical properties and therefore function of the hosting tissue (13, 27).

During embryonic development, CFs originate from the epicardium and play crucial rule in heart development and formation of fully functioning heart as well as epithelial-mesenchymal transformation (EMT) during valves formation and also play role in CMCs proliferation via fibronectin-collagen-I integrin-β1 pathway (28). Then after birth the number of cardiac fibroblasts doubles to prepare the heart to withstand the postnatal load (16, 27).

In the adult heart CFs were believed to be the most common cell phenotype accounting for about 50% of all cardiac cells (27). Recently this belief was challenged and found to be outnumbered by ECs (19). However, CFs represent a pretty dynamic cell pool contributing to mechanical, chemical and electrical properties of the myocardium (16). The mechanical rule is mainly via secretion of the ECM which organises the architecture of the cardiac tissue (29) and form highly organised network of interconnected cells within the ECM to facilitate transmission of mechanical forces (14, 20). Also they are involved in regulation of ECM composition via secreting the degradation factors matrix metalloproteases (MMPs) as well as the tissue inhibitors of MMPs (TIMPs) (30).
CFs also sense dynamic changes in wall tension through several pathways such as integrins, ion channels as well as second messengers (16, 20) and secrete pro-inflammatory cytokines in response to myocardial stress (14, 16). Furthermore, CFs make mechano-electrical coupling through Na^+, K^+ and Ca^{2+} channels (16) and communicate with each other and with CMCs via gap junctions such as connexin43 (Cx43) and connexin45 (Cx45) (31) to facilitate synchronized electrical transduction (32).

When fibroblasts were cultured with CMCs, they did organise electrical conduction and synchronised contractions which seems to be related to synchronous fluctuation of membrane potential in CMCs and fibroblasts and indicates electrical coupling (32). This was also proved by altered electrical properties of CMCs in culture upon receive of fibroblast graft (33)

So, CMCs and fibroblasts hold short distance communication via gap junctions as well as long distance transmission of impulse and therefore fibroblasts have a role to synchronise intracardiac electrical activation and contractility under physiologic conditions in addition to their role under pathologic conditions as they could induce arrhythmias post infarction (32).

Indeed, CFs are quite plastic cells that could adopt different functional rules in different circumstances (34). They have pro-inflammatory function as they could secrete cytokines and chemokines and recruit leucocytes in response to ischemic events and limit cardiomyocytes loss after ischemia reperfusion (35). Also HCFs help to clear the infarct area post MI via phagocytosis of the apoptotic cells and exert anti-inflammatory effect as well (36). Also, during cardiac events such as myocardial infarction the CFs role dominates the scene as the main players in cardiac remodeling (37). They would differentiate to myofibroblasts that are quite active phenotype of HCFs to produce more ECM proteins leading to fibrosis of the area which despite being associated with deteriorating cardiac function due to myocardial stiffness (34) but it is a protective process to guard against cardiac rupture.

Another interesting finding is that gene profile analysis of short term cultured cardiac fibroblasts revealed significant number of cardiac muscle genes expression as compared with non-cardiac fibroblasts which could indicate that cardiac fibroblasts hold the potential to differentiate into CMCs (38) and this could be part of the biologic regenerative reserve of the heart.

It is noteworthy that some CHDs would ensue secondary to genetic disorder(s) in the CFs such as mutations of GATA4 family of transcription factors in CFs leading to some disorders such as double outlet RV, myocardial hypoplasia and common AV canal (39) and the TBX20 mutation which leads to septal defects as well as compromised valve formation (38). This reflects the crucial rule of CFs for adequate development of the heart.

1.3.2.3.Endothelial cells (ECs)

ECs represent the innermost lining of blood vessels and they share common functions such as control of coagulation, extravasation of inflammatory cells, barrier function and signaling to immune cells in addition to angiogenesis to promote regeneration and tissue perfusion. Nonetheless, they are not all uniform, indeed they hold some regional differences in order to meet various anatomical and physiological needs in a tissue signature based manner (40). For instance, ECs In the myocardium in addition to their in-vessel role also play indispensable functional role through their interaction with CMCs to sustain and regulate myocardial function (41).

Due to the fact that the myocardium is in a continuously active and crucial energy demanding status therefore, it is highly vascularized tissue to the point that ECs to CMCs ratio is about 3:1 with almost every CMC is bounded by a vascular capillary (42). This means quite dense capillary network. It was demonstrated that myocardial capillary density is about 3000-4000/mm^3 which is almost five fold higher than in skeletal muscles (43). This myocardial high vascularity is reflected on ECs number which represents the highest percent of non-myocyte cells in the heart (19).

ECs in the heart are distributed between micro and macro-vascular areas. The microvascular ECs are the major pool of cardiac ECs and Further to their vasculo-structural role they share in intercellular communication via paracrine, juxtacrine and autocrine factors through which cross talk instructions would ensue (44). On the other hand, the coronary ECs represent the macrovascular cardiac ECs pool and despite they represent a minor proportion but they have their important role to control contractility of underlying smooth muscles (43).

Cardiac microvascular ECs have a well characterised secretome profile that is indispensable for cardiac tissue homeostasis such as nitric oxide (NO), endothelin-I (ET-1), neuregulin-1 (NRG1), Angiotensin II and prostacyclin. NO plays role in control of cardiac contractility as well as protective effect against pathologic remodeling and also to enhance angiogenesis (26, 45, 46). Its secretion is controlled by changes in blood flow and contraction-relaxation cycles. In case of dysfunction of ECs, the NO secretion decreases significantly leading to myocardial hypertrophy and stiffness leading to heart failure due to diastolic dysfunction (47). While endothelin-I (ET-1) is a vasoconstrictor with positive inotropic and anti-apoptotic effect on CMCs (44, 48) and its long term up-regulation leads to cardiac hypertrophy. Therefore, it was considered to play role in heart failure pathogenesis (12). However, trials on ET-1 blockers in heart failure patients did not show significant results (49). NRG1 activates ErbB2 and ErbB4 receptors to induce CMCs regeneration which indicates its cardio-protective effect and its potential to modulate cardiac remodeling (26, 45, 50). This clearly exemplifies for the beneficial effects of the ECs secretome.

Furthermore, ECs secrete other factors such as prostaglandins and microRNAs which share in control of myocardial function. Therefore, ECs-CMCs interaction is considered mandatory for optimal cardiac function and during pathologic events as well (41, 51). In addition, ECs have other functions such as secreting some ECM proteins such as fibronectin (12) and act as sensors to detect and react to external triggers including hemodynamic changes, chemical, neuronal and hormonal stimuli (44, 52).

This exemplifies for the importance of the secretome profile of ECs in addition to their structural role and reflects the indispensable role of ECs for cardiac tissue viability and functionality.

1.3.2.4.Extracellular matrix (ECM)

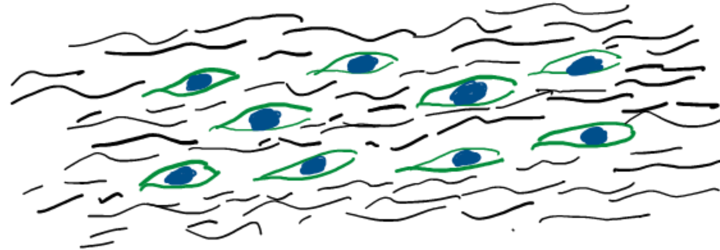

Figure 1-3. Extracellular matrix and cells enmeshed in its network.

ECM is the non-cellular compartment of all tissues. It represents the 3D matrix which holds cells and organises tissue morphogenesis and therefore it is responsible for shaping the tissues and determining their mechanical properties such as elasticity and tensile strength (53, 54). Further to its structural role the ECM has functional role as it facilitates cellular communication via transport of factors and initiation of chemical and biomechanical stimuli (54, 55).

Due to the dynamic nature of the myocardium its ECM is indeed one of its indispensable pillars during health and disease alike, as it forms the network to organise cellular alignment, distributes the mechanical load and fluid transport which is crucial for cell perfusion, transport of metabolites and facilitates contraction of various layers of the myocardium (56). It Has several components such as proteoglycans, fibrous proteins and glycosaminoglycans and hosts several physiologically active factors such as growth factors and cytokines (56, 57) while during pathologic events the ECM would undergo dynamic changes to adjust inflammation and support repair (58).

Collagens are the major structural components of ECM. In the myocardium collagen-I constitutes about 80% and collagen III about 10% (58) of all collagens in the myocardial ECM. It is notable that these two types of collagen can self-assemble to make fibrils (59). These main collagens in the myocardium are produced mainly by fibroblasts according to tissue needs and act mainly to provide ECM with its tensile strength as well as support cellular adhesions and migration (54). In addition, there is less contribution of collagens IV and VI synthesised by myocytes (60) .

It is also noteworthy that ECM is not a static domain, indeed, it is subject to continuous remodelling via enzymatic and non-enzymatic factors to accommodate various physiological and anatomical demands of the hosting tissue (53, 54). This dynamicity is facilitated by its proteoglycan and glycoprotein content (57).

Also, ECM is not entirely homogenous in all cardiac areas but varies from region to another such as between atria and ventricles. In addition to variability in its density which affects its functions that would reflect on control of cell migration, expression of cell receptors and fluid diffusion within the ECM which are all guided by the physiological demands (56).

1.4.Congenital heart diseases (CHDs)

1.4.1. Introduction

The old concept of considering the heart as a muscular pump was dominant in the cardiovascular field for quite long time. This concept was dramatically altered to multicellularity structure and multifunctionality of different components including cardiac cells, fibroblasts, smooth muscle cells, extra cellular matrix, valvular tissue, conductive system as well as the vascular network (11).
All these items should integrate and align according to a predefined program to form a fully functioning heart. In case a developmental defect takes place this would lead to a congenital cardiac/vascular deficit.
Congenital heart diseases (CHDs) are the most common congenital defects worldwide and the second leading cause of death in the first year of life (61). They account for about 20% of perinatal mortality and 50% of childhood mortality due to congenital anomalies (62) and also count for almost double mortality caused by all cancers combined (63).
In 1971 Mitchell et al. defined CHD as "*a gross structural abnormality of the heart or intrathoracic great vessels that is actually or potentially of functional significance*" (64). This means that CHDs would extend beyond the heart to include the great vessels lesions.
CHDs could be in the form of valvular disorder, cardiac or vascular malformation such as coarctation of the aorta and patent ductus arteriosus, septum defects, cardiomyopathic disease or congenital arrhythmia disorder (65).
Incidence of CHDs is quite variable between different studies from 4/1000 to 50/1000 (66). Even in some studies the incidence of CHDs was reported as 1% and taking congenital bicuspid aortic valve into account the incidence will rise to 4% (67). The distribution of CHDs is almost comparable between different populations with some exceptions such as the higher incidence of right sided CHDs in Asians (67, 68).

This incidence variability between different reports is due to several factors including better diagnosis with non-invasive tools such as echocardiography in addition to different scopes of studies (66). Some studies focused on CHDs in infants (69), others looked at congenital defects that would show at adult life (56) while other groups considered the CHD(s) once diagnosed regardless the age of diagnosis (70). Ultimately, it is estimated that 2.5 million children around the globe are borne with CHD(s) and many of them are candidate for deteriorating cardiac function and heart failure (66, 71).

This reflects how common CHDs are and the extent of its burden with all social and economic consequences (72). For example, in the USA in 2004, the cost of hospital care for all birth defects was 2.6 billion USD while the cost of care for cardiovascular birth defects was 1.4 billion which exceeds 50% of the overall expenditure (73).

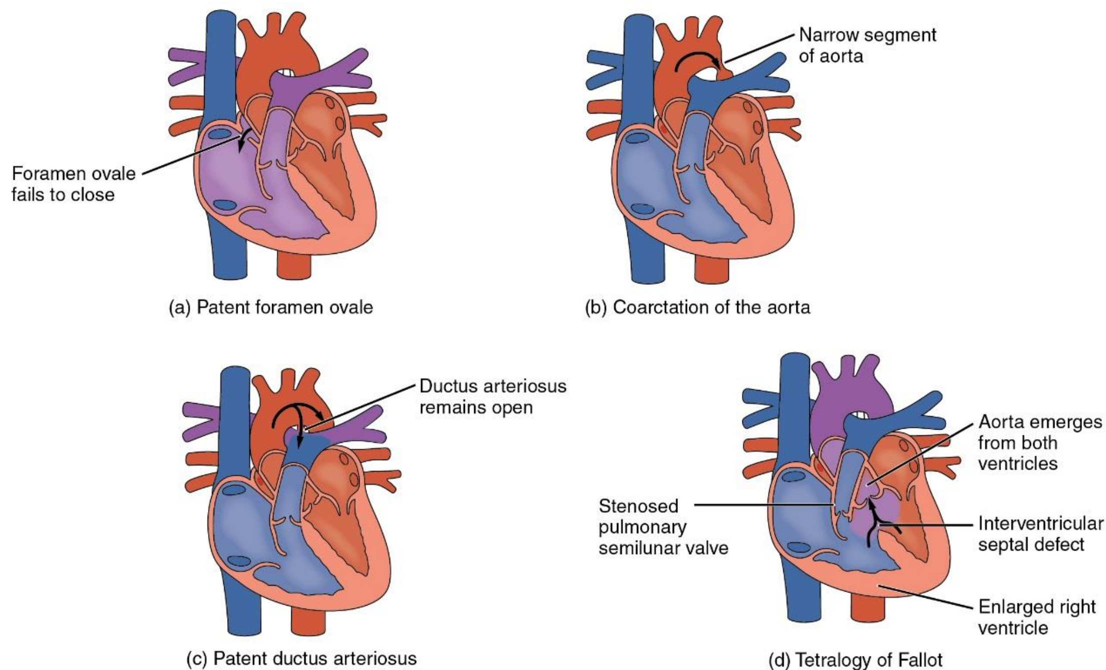

Figure 1-4. Examples of congenital heart diseases.

Source: Anatomy & Physiology, Connexions Web site. http://cnx.org/content/col11496/1.6/, Jun 19, 2013, no modification.

1.4.2. Aetiology of CHDs

The heart is the first organ to form and its proper embryonic development is indispensable for proper structure and function in later life. CHDs would develop due to error(s) in the development of heart/blood vessel.

Some studies suggest a multifactorial aetiology for CHD(s) including environmental teratogens, maternal infections such as rubella infection mainly within the first trimester (74) , maternal age (75), maternal excess alcohol intake, vitamin deficiencies, paternal factors and genetic defect(s) (61, 76, 77).

The notation of environmental factors as contributor to CHDs aetiology was based on epidemiological studies and most probably would incur risk rather than a sole aetiology (7).

Recently, genetic defects are increasingly recognised as the underlying cause(s) for CHDs. The genetic defect could be familial or de novo mutation (61, 67, 78). Better understanding of the CHDs genetics is more important recently due to the fact that the prognosis of CHDs became significantly better after introduction of corrective surgery. This led to more children with CHD(s) surviving into adulthood to initiate families with the potential to pass any underlying genetic defect(s) to next generation(s) (61).

The concept of genetic aetiology for CHDs came from the observation that CHDs run in families, but the complexity of the underlying defect is not well understood (67). It was linked to certain mutational syndromes such as microdeletions when some genes in one chromosome are deleted (7) and the

prototype for this defect is the 22q11 deletion that was found in about half of cases with interrupted aortic arch, one third of cases with truncus arteriosus and about 16% of cases with Fallot's tetralogy (79).

Also, it could be precipitated by a mendelian mutation such as the autosomal dominant Noonan syndrome and Holt-Oram syndrome (67). In addition to single nucleotide polymorphisms (SNPs) and other mutational forms such as translocation or aneuploidy (76). However, it was not clear why members of same family have different forms of CHDs and why a single form of CHD could have different underlying genetic anomalies (3, 70).

Other developmental errors could result in CHD such as fusion of the bilateral cardiac primordia which is one of the critical events to form single cardiac tube and if this did not take place it may lead to cardia bifida and death (10). Also, early cells ingressing from the primitive streak would form the cardiac mesoderm and genes controlling this process include Mesp1 and Nap1 which if deleted would lead to cardia bifida (10).

Even further to this unclear correlation between the genetic defects and the resulting CHD, the background genetic set up of the experimented animal models did make difference as regard the resulting deficit. For example, fibronectin null mice would show cardia bifida and intrauterine death in 129S4 mice while single heart tube would develop in C57BL/6J mice (10) which means same genetic defect but different phenotype result.

In the comprehensive Danish study that analysed a nationwide registry, the percent of cases of CHD(s) with chromosomal anomalies was 41.1%. The relative risk in twins was 15.17 and 3.33 for monozygotic and dizygotic respectively and paternal consanguinity increased the risk by 2-3 folds while risk in families with first degree relative was about 3.3 (70) which also supports the concept of genetic factors as a major drive for CHDs.

It is worth note that a wide spectrum of CHDs are associated with chromosome number variants like trisomy 21 in which about half cases would develop atrio-ventricular canal defects without or with Fallot's tetralogy (67), trisomy 18, trisomy 13 and monosomy X in which the CHD would be part of a syndromic presentation (80).

Also, single gene defect(s) may lead to CHD(s) which could be associated with other anomalies such as in Marfan syndrome (FBN1 defect), Alagille syndrome (JAG1 defect) with RV outflow obstruction, conotruncal or ventricular septal defect and Holt-Oram syndrome (TBX5 defect) with ASD, VSD or both defects, Noon syndrome with variable cardiac defects. On the other hand the CHD(s) may be non-syndromic when the defect is associated with cardiac defect only with no other defects (7, 70).

In addition, the genetic defect(s) could be secondary to interaction of two or more genes with their downstream effects such as interaction of TBX5 with NKX2-5 and GATA4 interaction with NKX2-5 (7).

Indeed, the genetic studies revealed that CHDs could be related to altered gene-protein dosage, mutations affecting several factors controlling cardiac development in addition to possible higher order interaction of factors orchestrating embryonic cardiac formation (81).

Table 1-2. Examples of gene defects leading to CHD (67).

Defective Gene	***Syndrome***
NKX2-5	ASD, Fallot tetralogy, conotruncal defects, left hypoplastic heart syndrome, conduction defect.
TBX5	ASD, VSD, Holt-Oram syndrome
GATA4	ASD, VSD
TBX1	DiGeorge syndrome
FBN1	Marfan syndrome
JAG1	Alagille syndrome
ELN	Aortic pathology in William's syndrome, familial supravalvular stenosis.

1.4.3. Presentation and classification

CHD is a broad term with a wide spectrum of presentations that range from asymptomatic to heart failure shortly after birth (68). In addition, it may manifest with nonspecific symptoms such as fatigue and exertional shortness of breath (81), even in some cases CHD would be diagnosed at autopsy (82).

In the study of Wren and his colleagues routine clinical examination of neonates was able to pick up 45% of cases with CHD while some new borne babies passed the initial examination but were diagnosed later when started to develop symptoms as early as before discharge from hospital. About 1/3 of cases were diagnosed within 6 weeks post-partum while more than half at about 3 months and still these figures could be higher if echo was used for assessment of suspected cases as early as 4 weeks post-partum (82).

Others reported 40-50% of CHDs diagnosed within one-week age and up to 60% within the first month post-partum (83). It is noteworthy that the early diagnosis is a key factor for better prognosis and therefore in some developed countries pre-natal diagnosis is in practice to pick up CHD cases before birth while unfortunately some developing countries suffer from lack of resources and so surgical management is not an option in some cases due to late diagnosis despite the fact that prevalence of CHDs is comparable with Caucasian population (83, 84) and CHDs represent an important underlying cause for heart failure in some of these cases.

In a prospective study on an African cohort with heart failure CHDs were the most common underlying etiology as they counted for 35% of cases (85) which indicates the caliber of the CHDs and its deleterious sequelae. Even another study in different African area reported CHDs prevalence to outnumber rheumatic heart disorders (86).

In the study of Mocumbi and colleagues on another African cohort, 33.5% were diagnosed between the age of 2 to 17 years old while 13.7% were diagnosed after the age of 18 years old and left-right shunt represented the most common deficit. Unfortunately, about 29% of cases presented with complications such as pulmonary hypertension, stroke, endocarditis, heart failure and polycythemia. Within the cohort of this study 90% of cases had indication for surgery either corrective or palliative (83).

Classification of CHDs is rather difficult due to the marked diversity of the phenotypes and also the background aetiology as well as their variable associations (87).
CHDs could be classified into cyanotic when there is right to left shunt such as in transposition of great vessels and acyanotic in absence of peripheral cyanosis such as in aortic coarctation (88).
Another classification done by a group of investigators who did review a French cohort of 2867 patients and organised a classification for CHDs into 10 categories with 23 subcategories based on anatomical distribution, echocardiographic features as well as therapeutic modalities but they did not consider developmental background due to the blurred causality association between the defects and the resulting phenotypes (87).
The classification included Heterotaxy with isomerism and mirror images defects, anomalies of venous return, anomalies of atria and inter-atrial communication, anomalies of atrioventricular junctions, complex atrioventricular junctions, uni-ventricle, ventricular septal defects, anomalies of ventricular outflow, extra-pericardial arteries and anomalies of the coronary arteries (87).
Another more comprehensive classification was adopted in the Danish study of Nina et al in which they divided the CHDs into 17 different categories considering different parameters such as right or left defect, intracardiac or vessel related, atrial or ventricular septal defects, outflow or venous return vessel disorder, preterm or full term at birth in addition to the extent of the defect whether confined to the cardiovascular system or associated with extracardiac anomalies and furthermore they extended their classification to the underlying defect if chromosomal or non-chromosomal (70).

1.4.4. Management

Diagnosis of CHDs could start from the fetal life using intrauterine doppler echocardiogram in families with risk factors such as family history of CHD, as well as postnatal assessment that would start from the clinical examination, echocardiography and MRI in addition to other assessment modalities such as ECG and blood tests (65).
In view of the nature of CHDs as structural defect(s) that could result in altered cardiovascular hemodynamic status with potential significant short and long-term sequelae, management could be medical via pharmacologic support or interventional that could be catheter mediated especially in acyanotic cases or surgical intervention which is indicated in a substantial percent of cases (89, 90).

Medical management includes clinical follow up with periodic assessment, pharmacologic therapy such as beta blockers, digoxin and diuretics and prophylactic antibiotics in case of bacteraemia producing procedures (90). Transcatheter intervention would be indicated in some cases such as pulmonary and aortic stenosis, once cases met intervention criteria such as presence of symptoms, peak gradient and ECG changes (90).

CHD patients would need a series of investigations and interventions which may need to start early to ensure adequate growth and development physically and mentally (63) .
Despite the benefits of early intervention but still there are some draw backs such as exposure to risks of surgery in addition to another risk of HLA sensitisation that would-in theory- hinder future plans for transplant (91).

Start of surgical intervention goes back to the 1930s with ligation of a patent ductus arteriosus (68). With time the field achieved great advancement with increasing experience and better technical facilities to support the circulation in particular with the start of cardiopulmonary bypass era which allowed for intracardiac surgery (68, 92). This was reflected on patient survival with marked reduction of

mortality (93, 94). Indeed, recently about 85% of CHD patients would survive into adult life (56) and this would lead to more adult population with CHD than paediatrics (94).However, still CHD cases have higher mortality relative to general population (95).

Nevertheless, due to the fact that 50% of surgeries are performed in the first year of life (96) while the graft lacks growth potential (63) and therefore, surgery going through palliation followed by corrective surgery in a later age is sometimes unavoidable. This is due to heart/vessel growth while the patch is inherently static in addition to possible graft failure due to other reasons such as immune reaction to allo/xeno-grafts (97, 98).
For instance, one of the severe forms of CHDs is the mono-ventricle anomaly. Surgical reconstruction to establish separate systemic and pulmonary circulations is essential to support survival. This goes through serial surgeries known as Fontan operation to maintain efficient circulation. This procedure was first described by Fontan and his team in 1971 for treatment of tricuspid atresia and despite further refinement of the procedure it still carries his name (63, 99, 100).

Indeed, Fontan operation is one of the most commonly performed surgeries for CHDs as this procedure revolutionised the care of patients with single ventricle and significantly improved the survival (101). In this procedure there is direction of venous return to the pulmonary artery via connecting the superior vena cava to the PA followed by connecting the inferior vena cava to the PA via establishing a vascular conduit between these vessels allowing for passive flow, then the oxygenated blood would flow through the single ventricle to the systemic circulation (63, 99).

It is noteworthy that the underlying aetiology would play a determinant role for perioperative prognosis as it was noticed that perioperative outcome using same approach for same CHD could have different outcome due to different underlying pathogenesis. For instance, 22q11.2 chromosomal microdeletion was associated with higher risk of neurodevelopmental deficits (102, 103) and cases with complex congenital disorders including extracardiac deficits showed higher mortality (104). Despite the underlying pathophysiology is not well elaborated but the perioperative genomics science is expected to shade light on this perioperative outcome variability which could include the effect of genetic polymorphism on humoral inflammatory mediators such as IL-6, IL-10 and TNF-α as well as the platelets glycoprotein receptors (103). This indicates that despite the importance of modifiable factors such as the surgical approach to help predict the operative outcome but still underlying pathologic aetiology should be considered as part of the management algorithm.

This would emphasise on the importance of understanding the genetic backgrounds of the CHDs as it would impact the prognosis as well next generations.

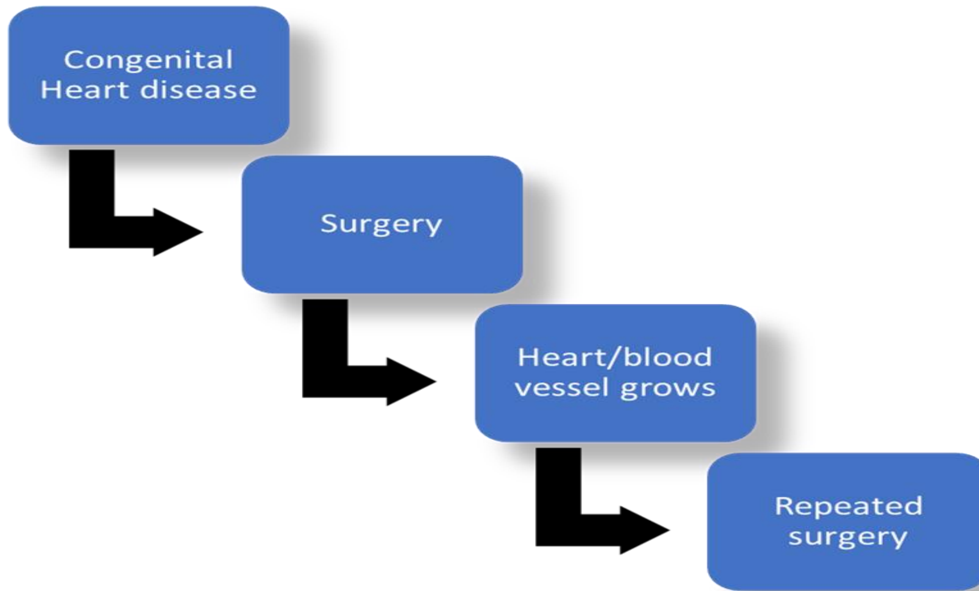

Figure 1-5. *Surgery and repeated surgeries in congenital heart disease.*

Progressive growth of the heart and blood vessels leading to graft failure with need for repeated surgery.

This illustrates the current challenges in management of CHD cases due to the need for surgery and repeated surgeries with all social and economic burden. Therefore, there is need for grafts that can offer growth potential to avoid or at least reduce the frequency of repeated surgeries. In this regard tissue engineering can offer a potential solution to address this requirement.

1.5.Tissue engineering

Regeneration potential in adult mammals is limited to certain tissues such as the intestinal mucosa, bone marrow and superficial skin layers while other tissues react to injury via reactive inflammatory and scarring phenomenon (105). The regeneration of lost tissue could be through two main pathways, the first consists of blastema formation which means local recruitment of stem cells that migrate, proliferate and gradually differentiate to construct tissue similar to the lost part and this type of blastema based regeneration occurs in Salamander while the other pathway which occurs in adult mammals involves local cells dedifferentiation and proliferation, cells trans-differentiation and/or gradual differentiation of resident stem/progenitor cells (105, 106).

Human body has its own regenerative/healing properties however, sometimes the extent of tissue injury/loss goes beyond these inherent abilities (107). Indeed, native tissue regeneration in adult mammals is quite limited to certain tissues such as bone marrow and superficial skin layers while the typical tissue reaction to injury is through inflammatory and scarring process which would result in functional impairment to variable degrees and therefore there is need for external means to regenerate the lost tissue and recover its function. Indeed, this is the scope of tissue engineering and regenerative medicine which would utilise cells, scaffold and/or bioactive materials (108) to support function and/or enhance tissue regeneration.

The borders between cell therapy and tissue engineering (scaffold based) are to some extent blurred. The core of cell therapy is to administer dissociated cells into the injured area via direct injection or systemic administration (109). This would demand an intact biological support in the form of stromal connective tissue (110). However, in case of extensive damage or scarring the cells would lose the necessary cues for migration, proliferation and differentiation in addition to lack of adequate perfusion resulting in failure (110). While the tissue engineering operates at the interphase between biological science and materials science to support organ, recover function or restore lost tissue/organ using biomaterial(s) +/- cells (111).

Some authors consider both approaches under the same umbrella and define tissue engineering as using cells, biomaterials and biological factors single or combined (110). While others would consider it as evolution of the field and scaffold based approach is the second generation of cell therapy (109).

The principle of tissue engineering was in use for long time (112) but the terminology was adopted in 1988 during the National Science Foundation Workshop when the term tissue engineering was formally defined as *“the application of principles and methods of engineering and life sciences toward the fundamental* ***understanding of structure-function relationships*** *in normal and pathological mammalian tissues and the development of biological substitutes to restore, maintain or improve tissue function”* (113).

So, in principle, tissue engineering aims to regenerate/replace lost tissues through different combinations of cells, materials such as biologic scaffolds and biochemicals like angiogenic and growth factors to build up a tissue like structure to replace diseased/lost tissue (112). In addition to these pillars other tools such as bioreactor could be utilised to optimise the uptake and configuration of the tissue engineered structure (114). So, scaffolds, cells, chemical factors and physical factors are the fundamental pillars for tissue engineering.

Despite lapse of decades with tissue engineering in experimental and application life but still it is considered premature as some basic concerns not yet fully addressed such as the best cell source and optimal material for specific application in addition to further development of more specialised equipment for complex anatomy configuration (115). In addition to a major challenge which is the retention of cells in-vivo as transplanted cells mostly died and washed out and very minimal residue could be detected in the infarct area. This hindered the progress of cell therapy to move forward to the standard of practice (109).

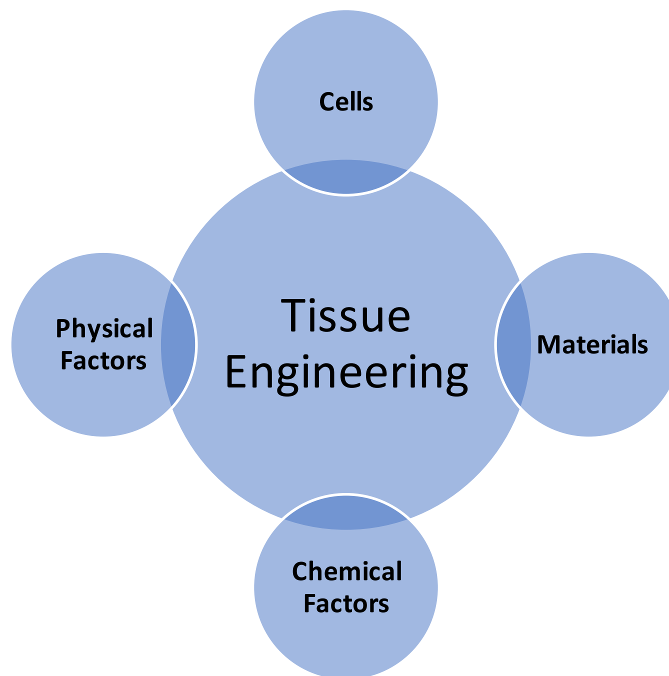

Figure 1-6. *Tissue engineering as a core with its* four pillars: cells, materials, chemical factors and physical factors.

1.5.1.Scaffolds

Use of biomaterials for medical purposes such as implants in human body is known for long time (116) and even found in ancient times (117). So, the concept of biomaterials is quite old but the terminology is recent. A biomaterial in medicine is defined as *"Any natural or synthetic material intended for introduction into living tissue especially as part of a medical device or implant" (117).*

Based on this definition biomaterials have a wide variety of medical applications to the point that they became indispensable in our daily medical practice. Another more comprehensive definition was adopted in 1982 by the NIHCD (The National Institute of Health Consensus Development Conference) which defined a biomaterial as "*any substance (other than a drug) or combination of substances, synthetic or natural in origin which can be used for any period of time as a whole or as a part of a system which treats, augments or replaces any tissue, organ or function of the body*" (118).

On daily basis we use cannulas, needles, tracheal tubes, chest tubes and many more devices and implants. All these applications revolutionized medical practice and patient care with better outcome, less disability and better functional capacity.

Materials used as scaffolds could be synthetic or natural. Synthetic scaffolds such as hydroxyapatite and ceramics offer good mechanical integrity and are therefore suitable for certain applications such as orthopaedic applications (113, 119) but lack elasticity and therefore not suitable for cardiovascular applications. While natural scaffolds would offer the mechanical integrity, the biocompatibility and some elasticity (120) and therefore considered more suitable for cardiovascular applications but still have limitations such as the potential immune reaction.

Biologic scaffolds for cardiovascular applications could be autologous such as homo pericardium which provides the necessary compliance and favorable hemodynamics but limited with the availability and the need for further processing. Also, xeno grafts such as porcine pericardium which also provides good hemodynamics and the necessary cues for cell integration and the processed ECM produced by resident cells mainly fibroblasts (121, 122) that could be extracted from different organs like intestine and bladder and its main constituent is collagen (123, 124).

As previously illustrated, ECM is an integral component of all tissues due to its structural and functional roles as a mechanical support as well as allowing cell migration, proliferation and differentiation (125). Therefore, it is more natural to use a biologic scaffold as it would offer the biocompatibility and reasonable mechanical integrity and there is a good body of evidence from preclinical and clinical studies utilising biologic scaffolds and showed promising results for good constructive remodelling with good uptake, repopulation, vascularisation and tissue remodelling (109, 123, 124, 126). However, still bioprosthetic materials are limited with deterioration and calcification that occurs at a faster pace in children and limits their durability (127) while processing such as cross linking with Glutaraldehyde would offer protection against enzymatic degradation and increase tensile strength but would increase the biomaterial stiffness in addition to higher risk of calcification (122).

As of November 2019, there were 428 clinical trials registered on ClinicalTrials.gov investigating scaffolds in different clinical settings. This reflects the importance of biomaterials and tissue engineering as potential solution for many medical applications in general and the cardiovascular in particular. Also, there are tens of FDA approved bio-scaffolds consisting of ECM derived from different tissues for a wide

variety of clinical applications (128). The choice between these bio-materials will be based on several factors such as the intended application with its mechanical requirements, required longevity, risk of contamination in addition to the financial aspects (128).

1.5.1.1.Requirements of biomaterials

Use of biomaterials in tissue engineering aims to provide the architectural support to native cells to migrate, proliferate and interact to regenerate and support tissues. Therefore, for a biomaterial to be suitable for in vivo use it should meet some criteria including biocompatibility, biodegradability and mechanical integrity (figure 1.6).

1.5.1.1.1.Biocompatibility

Biocompatibility could be defined as " *Ability of a material to exist in harmony within a biologic system*" (129). So, for a biomaterial to be biocompatible it should meet some basic requirements defined by the ISO 10993 including being *" non-toxic, non-thrombogenic, non-antigenic, non-carcinogenic and non-mutagenic"* (118).

This means to be biocompatible the material should be cell friendly allowing cells to adhere onto its surface so that they can establish the proper cellular environment and produce ECM to support their proliferation and function (130). In order to achieve cell adhesion to the scaffold surface, ligands would be required such as the ligands Arg-Gly-Asp (RGD) which are present in a hydrophilic loop of some of the ECM proteins such as fibronectin and vitronectin and would act to facilitate cell adhesion via interaction with integrins (113). In addition, the biomaterial should be immune inert to avoid inflammatory reactions (113, 119).

Therefore, biological scaffolds are considered the first choice but still have some limitations. For instance, homo-grafts are limited with availability in addition to potential fibrosis while xeno-grafts such as bovine pericardium and porcine small intestinal submucosa which hold the cues for cell adhesion and have hemodynamic performance close to native tissues still lack growth potential, also durability is limited especially in children with faster calcification and degeneration (122).

1.5.1.1.2.Biodegradability

Biodegradability could be defined as "*Ability of material to decompose within a biologic system"* (131). Ideally the biomaterial should not last for ever unless necessary for the specific application.

It should give the physical configuration of the functioning tissue and support cell migration and proliferation so that it will be repopulated with native cells. Then the attached cells will lay their own ECM and end up with functioning tissue that is entirely of the host cellular origin. In order to accomplish this task the scaffold should degrade gradually and meanwhile gets replaced with native cellular and extracellular components (113, 119). On the other hand, the biodegradable products should be non-toxic and amenable for clearance via normal metabolic pathways over a period that could range from months to years (122).

Indeed, biodegradability is one of the defining factors for tissue reaction to biologic ECM based scaffolds. The degradation products were reported to hold chemotactic effects to recruit stem cells for local regeneration as well as activating mononuclear cell infiltration that is dominated with M2 macrophages that would support constructive remodelling (126). While in case of chemically cross linked ECM scaffolds the mononuclear infiltrate is dominated with the pro-inflammatory M1 macrophages that would support inflammation and scarring process (126).

1.5.1.1.3.Mechanical integrity

This is a critical requirement for the biomaterial so that it would tolerate the mechanical load of surgery followed by taking the intended load of the normal tissue function. In cardiovascular applications the implant should be able to tolerate the shear stress of blood flow and to offer some elasticity with the cardiac contraction/relaxation cycles (130).

For obvious reasons, if the scaffold fails to tolerate this stress and comes to mechanical fatigue/rupture then it cannot go safely to clinical practice.

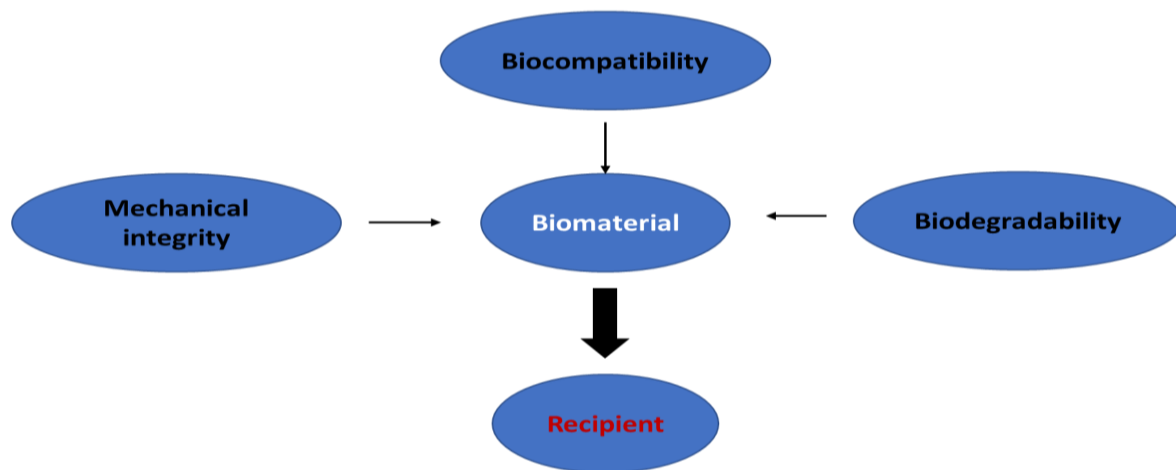

Figure 1-7. Requirements of biomaterials to be compatible for in-vivo applications.

1.5.1.2.Types of biomaterials

Biomaterials could be broadly classified into synthetic and natural (130). Also could be divided based on the application into hard tissue/load bearing biomaterials intended for orthopaedic and dental applications and non-load bearing biomaterials for soft tissue engineering purposes (132).

1.5.1.2.1.Synthetic biomaterials

Synthetic biomaterials including metals and polymers have the advantage of mechanical integrity and durability but lack functionality.

1.5.1.2.1.1.Metals

Metals used as biomaterials include stainless steel, titanium and their alloys and cobalt alloys (132).They are characterised by high compression strength and therefore more suitable for load bearing applications such as joint replacement. However, they are limited by lack of bioactivity in addition to potential toxic metabolic end-products (115).

Still metals have some applications in cardiovascular field such as steel and titanium stents as they can offer the necessary mechanical integrity but recently they are over-favoured by alloys because of better mechanical properties. Also, lifelong valves are made of steel and titanium which offer the necessary durability but with the drawback of lifelong anticoagulation and its potential side effects in particular major bleed (130).

1.5.1.2.1.2.Polymers

Start of synthetic materials as vascular grafts could be tracked back to 1906 by the work of Carrel (133). Also Blakemore used Vinyon-N-fibre as vascular grafts in 1954 but it did not demonstrate satisfactory durability and did not support tissue ingrowth (134, 135). Currently other polymers are used for cardiovascular applications such as expanded Polytetrafluoroethylene (ePTFE) and Polyethylene Terephthalate (Dacron). However, they are limited to vascular grafts of size less than 6 mm due to risk of thrombosis and compliance mismatch (135).

ePTFE was first used for vascular prosthesis in 1972 and since that time it is considered one of the most commonly used vascular prosthesis (136). It has the commercial name Goretex and it has favourable properties including good strength to weight ratio with reasonable dilatation resistance, low thrombogenicity and less calcification (130). In addition, it has less incidence of restenosis with better success rate than Dacron (63). Consequently it has many applications in cardiovascular medicine including corrective surgery and to construct shunts. Nonetheless, as a synthetic material it could trigger immune reaction and due to incomplete endothelialisation still the graft is amenable for thrombosis which indicates anticoagulant prophylaxis with its lifelong risk of bleeding (130, 137).

Also, its porosity and low thrombogenic potential raised some concerns about exacerbated suture line bleeding which would prolong time of surgery (138). Other reported complications include leakage, pseudo aneurysm formation, diffuse inflammation and infection (136). In addition, it showed limited infiltration with ECs or collagen which was generally limited to the periphery of the graft but increased gradually with time (136).

Another polymer in use is the Dacron which is a thermoplastic polymer used for synthesis of vascular grafts as it has the advantage of enhancing endothelialisation with no evidence of higher calcification rate. However, still can induce immune reaction as a foreign body in addition to the potential thrombosis (130). In 1986 Weinberg et al used Dacron mesh to design a vascular patch consisting of multilayers of endothelial cells and smooth muscle cells integrated with collagen. Despite the model did not last for long but it is one of the earliest trials to attempt multilayers culture of different phenotypes on synthetic scaffold (135, 139).

1.5.1.2.1.3.Ceramics

Ceramics are a group of natural and synthetic materials such as calcium phosphate, bioactive glass and glass ceramics that are characterised by biocompatibility, osteo-conductivity with reasonable resistance to corrosion as well as its hard load bearing surface. These characteristics make bio-ceramics excellent choice for orthopaedic and dental applications (140). However, bio-ceramics have poor elasticity and therefore not suitable for cardiovascular applications.

1.5.1.2.2.Natural biomaterials

Natural biomaterials are derived from native tissues that could be of auto, allo or xeno origin (130). These materials would offer biocompatibility and degradability but with less favourable mechanical properties and possibility to trigger immune reaction (130).

Autograft is favourable as no immune reaction concern but clinical application is limited by the availability. On the other hand, allografts and xenografts are readily available, however, they are ECM based and need certain processing prior to in-vivo use including decellularisation +/- cross linking to enhance its mechanical properties (126).

Xenografts such as bovine pericardium is in use since Ionescu et al reported using it for replacement of a cardiac valve in 1970s (141) and since then it was used for other applications such as closure of ASD, VSD and reconstruction of right ventricle outflow tract (142).

Nonetheless, still there are drawbacks such as mechanical dehiscence, shrinkage, thrombosis, infection and calcification which are at least in part secondary to toxic effects of glutaraldehyde residues in addition to risk of immune reaction (142). Also, use of bovine pericardium for aorta repair was reported to offer about 16 folds of stiffness while the autologous pericardium would offer about 7 folds only, which indicates compliance mismatch and incurs risk of distortion and stenosis (143).

These scaffolds did definitely help many patients with CHDs to survive to adult life including those with severe forms that would not previously survive. Despite these positives However, they have drawbacks such as structural disruption including stenosis and aneurysm formation among others in addition to lack of growth and poor adaptability (142) which means that still the optimal graft does not exist.

1.5.1.2.3.Composite biomaterials

A composite material is a term coined to the combination of two or more constituents at a scale higher than atomic scale that significantly alter the net product characteristics. Therefore fibreglass and reinforced plastics are considered as composite materials such as biopolymers and bio-ceramics while alloys are not (144). Formation of composite materials could be via physical combination, polymerization, coating and multilayering (130). This combination could include both synthetic and natural materials aiming to boost each type with the positives of the other.

1.5.1.2.4. Injectable Biomaterials

Injectable biomaterials include hydrogels that may contain fibrin, chitosan, collagen, alginate or matrigel (130). In addition to nanofibers which are self-assembling peptides used to provide 3D network to assemble and deliver cells and growth factors (15).

This approach proved beneficial in heart failure models to support stem cells survival, differentiation and electromechanical integration in addition to angiogenesis (15). Also proved advantageous in animal studies to enhance neovascularisation and regeneration with better recovery of cardiac function in myocardial infarction(MI) models (130). These effects were attributed to reducing remodelling and enhancing CMCs survival (15).

So, this approach offers the advantages of non-invasive delivery and biocompatibility however, the effect to ameliorate cardiac remodelling is limited to the short term post MI phase and would fade with longer observation (145). On the other hand, this approach is more suitable for heart failure and MI models to reduce remodelling, reduce cell loss and enhance recovery but it is not suitable for CHD(s) cases that mandate reconstructive approaches.

1.5.1.3. Biomaterials for congenital heart diseases (CHDs)

With introduction of corrective cardiac surgery to CHDs patients there was a need for compatible material to close a defect or reconstruct a malformed part of the heart/great vessel.

Several materials have been used such as autologous pericardium, allografts from pulmonary artery and aorta, xenografts such as the bovine pericardium and small intestinal submucosal extracellular matrix (cormatrix) in addition to synthetic materials like ePTFE and Dacron (146).

Despite the used materials could withstand the mechanical load and proved reasonably durable however, the patch is inherently non-functioning and lacks growth in parallel with the rest of the heart/blood vessel. In addition, there are other drawbacks such as provoking inflammatory response, fibrosis, stenosis and variable degrees of degeneration with time (146). This means anatomical success but physiological failure.

1.5.1.3.1. Pericardium

The pericardial tissue has been used for cardiac bio-prosthesis due to its favourable properties such as easy handling with reasonable pliability that would facilitate adjustment for different anatomical configurations. Furthermore, it holds favourable surgery properties such as suture retention and resistance to infection (130).

Xenografts of bovine, porcine and equine pericardium were used for cardiac reconstruction surgeries (130, 147, 148) however, the xeno-pericardium would need processing to remove its antigenicity via a decellularization process. This is done using different methods such as detergents, enzymatic decellularisation and chemicals (149). This approach despite its advantages is limited by calcification and mechanical failure (150).

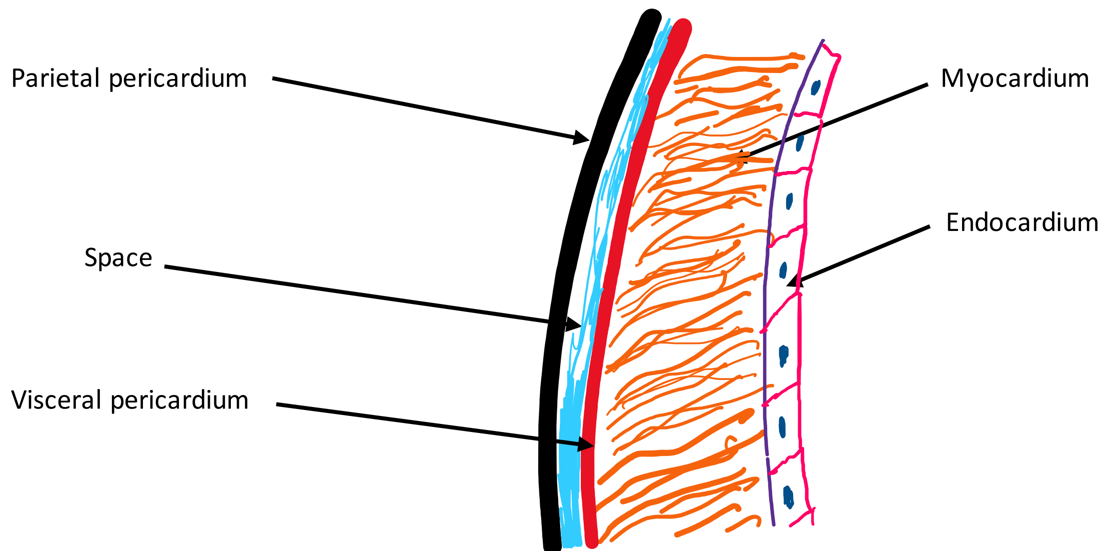

Figure 1-8. Pericardium (outer layer of the heart)

1.5.1.3.2. Extracellular matrix (ECM)

ECM is integral component of all tissues to support structure as well as function. It works in a fluidic manner to support cells, maintain cellular homeostasis, cell migration, proliferation and differentiation as well as its role for tissue morphogenesis, mechanical properties and post injury healing (108). ECM composition is conserved among different species and therefore it is feasible to utilise xenogeneic ECM in clinical applications.

Cormatrix is a decellularized porcine small intestinal submucosal extracellular matrix composed mainly of collagens, fibronectin and glycosaminoglycans with high content of growth factors (146, 151, 152). It is also approved by the FDA as biologic scaffold for cardiac surgery (146, 151).

Animal studies using cormatrix for corrective cardiac surgery showed promising results with good uptake, repopulation with native cardiac cells, vascularisation and contractility with reasonable mechanical integrity as well (124). Unfortunately, human studies failed to show comparable results. Despite the pliability and durability of the cormatrix patches there was lack of repopulation with any well organised tissue in addition to inflammatory cell infiltrate and degenerative changes as well (151).

In a prospective study cormatrix patches were implanted as part of hemi-Fontan operation then taken out as part of the standard of care and analysed after 18-26 months in-vivo which is a reasonable time to demonstrate behaviour of the patch as well as the body reaction if any (151). All explanted patches failed to show repopulation with any meaningful organised cell type or tissue pattern with no vascularisation demonstrated (151). Indeed, all patches showed foreign body reaction, inflammation and fibrosis to variable extent and this was consistent with other groups' findings (146, 151).

In the study of Woo et. Al and despite its limitation with the small number as they analysed 11 explanted patches out of 532 cases received cormatrix patch but it demonstrated almost comparable findings with inflammation, foreign body reaction, fibrosis and degeneration to variable degrees and the most important finding was failure of repopulation with any native myocardial tissue. These results were comparable between cases with clinical graft failure and those that dictated patch removal for other reasons (146).

The contradiction between animal studies and human findings are attributed at least in part to the fact that in animal studies the patches were introduced to normal hearts after a full thickness excision which promotes inflammatory reaction that would enhance cell proliferation (151). Furthermore, the animals received cormatrix were normal animals which means no underlying genetic defect(s) so that once the cells are released from contact inhibition this will activate division, proliferation and migration of cells to fill the gap. Then the scaffold would support this process which is not the case in CHD cases.

Also, cormatrix is assumed to be immunologically inert as it is processed to remove all antigenic epitopes such as cell membranes, nuclei and other intracellular antigenic components to get pure extracellular matrix components such as collagen, chondroitin sulphate and hyaluronic acid (146). However, still sometimes staining of cormatrix shows cellular nuclei which means incomplete decellularization that could contribute to the inflammatory and foreign body reactions found in the explants. Indeed, the host reaction to ECM would vary according to efficacy of decllularisation, chemical cross linking and also age of the source animal (126). In addition, cormatrix is of porcine origin which expresses gal epitope while in human anti-gal antibodies are expressed and this could play role in immunogenic reactions found in cormatrix explants (146, 153).

Even though, still cormatrix has a good clinical record including significant reduction of post-operative complications when used for pericardial closure after CABG (154).

1.5.2. Stem cells for tissue engineering

Cells and their interaction with the biomaterial are integral part of tissue engineering. Cells could be native or non-native seeded in-vitro on a biomaterial for in-vivo implantation (155).

Cells used for in-vitro seeding of a biomaterial should adhere to the scaffold, proliferate to reasonable volume and ideally to be of same phenotype as the target tissue, have the potential to differentiate into the target tissue cells phenotype(s) or can support the target tissue through various aspects such as trans differentiation and/or paracrine effects. All these requirements can be met in the stem cells.

The fundamental criteria of stem cells include self-renewal and pluripotency (125, 156). Self-renewal means ability for continuous proliferation over long duration while pluripotency refers to the potential to differentiate into all cell phenotypes. For tissue engineering purposes these properties are quite favourable as the cells can be expanded in-vitro to produce the required volume of cells and can differentiate into relevant cell phenotypes as well.

Recently, there is better understanding of the molecular mechanisms for cellular differentiation and trans-lineage transformation including cardiomyocytes and therefore stem cells research holds great potential for applications in corrective cardiac surgery on once done basis.

The start of stem cells research focused on embryonic stem cells (ESCs) (157) which are isolated from the pre-implantation inner cell mass (ICM) of the early embryo (158). For obvious reasons, this approach raised lots of ethical concerns as the research material is indeed an early embryo which would be damaged by this research (159). Consequently, efforts made to circumvent this problem by finding alternative sources of cells with pluripotent potential such as reprogramming somatic cells to resume stemness criteria which is known as the induced pluripotent stem cells (IPSCs) (159). In addition, the utilisation of mesenchymal stem cells (MSCs) which are known to be multipotent (160-162) and represent a realistic source of stem cells.

1.5.2.1. Molecular basis of stemness

Cellular stemness depends on balance between several factors including pluripotency and differentiation factors. Indeed, the pluripotency is a net result of a complex network of pluripotency factors that enhance pluripotency genes and repress the differentiation genes in a balanced manner and the stemness factors Oct4, Sox2 and Nanog are the main players that act in collaboration to maintain the stemness signature (163, 164).

It is noteworthy that the pluripotency potential of stem cells is due to an innate program that is independent of extrinsic signals and therefore, their self-renewal potential is eventually part of their latent carcinogenicity potential (165). These TFs are indispensable for self-renewal and pluripotency of stem cells (166) but time of expression of these factors would vary considerably.

Oct4 starts its effect during early embryonic life to maintain pluripotency (166). It is considered indispensable during this early phase as its complete deletion was incompatible with life. Embryos with Oct4-/- failed to develop pluripotent inner cell mass (ICM) in vivo and could not develop ESc colonies in vitro (167, 168) and also lost paracrine signalling effect of the ICM stem cells on the trophectoderm (167). Despite Oct4 rule in pluripotency is quite central however it is not enough and still needs to act as a partner with other stemness factors such as the sox2 and Nanog to establish the basis for self-renewal (169).

Balanced expression of Oct4 in ESCs was found crucial for normal development as manipulation of its expression has different consequences. Under-expression of Oct4 would favour trophoectoderm differentiation while upregulation would drive ESCs differentiation into endoderm and mesoderm (170, 171). In addition, Oct4 expression is not unique all through but there are spatiotemporal variations. Its expression was found higher in the ICM of the early blastocyst than in trophectoderm as the ICM and epiblast are critically dependent on Oct4 for cell lineage specification. Also, there is transiently high Oct4 expression by late blastocyst phase when Oct4 expression is higher in the primitive endoderm than in ICM (170). This indicates the direct role of Oct4 in early extra embryonic tissues development (168).

Sox2 expression starts at the morula phase. It gradually gets localised to the ICM of the blastocyst as well as the epiblast and germ cells to control pluripotency. Also acts in collaboration with Oct4 to establish Oct4/Sox2 code and activate its enhancers to stabilise pluripotency (172) and control commitment of the cells to the trilineage. Contrary to Oct4, it is also expressed by the multipotent cells of the extra-embryonic ectoderm (168, 173).

Then the crucial Sox2 role would start in the chorion post day 7.5 post-coitum (167, 168) where it would be diffusely expressed in the preimplantation cells including the extra embryonic cells as well as the ICM and Epiblast. Cell lineage commitment at this phase would depend on the combined effect of Oct4 and Sox2 (158, 168). At this point the embryonic development is crucially dependent on sox2 as proved by failure of implantation in Sox2 deficient embryos (173). With progress of the differentiation journey of cells, the role of Sox2 would step down and its expression will down regulate (168) but still expressed during somitogenesis in neurectoderm as well as primitive streak ectoderm and gut endoderm (174).

It is noteworthy that Oct4 and Sox2 collaboration control other factors such as Fgf4 and Nanog as well as working in an autocrine manner to regulate their own genes (158).

The third TF Nanog was discovered 2003 by Chambers et al. as a pluripotency factor in embryonic stem cells but not expressed in differentiated cells (175). Nanog exerts It's effect in combination with the other two key factors, Oct4 and Sox2 to establish the stemness signature of ESCs as well as the variable expression pattern in ESCs to specify the heterogeneity of these stem cells for further differentiation and cell fate control (176).
Overexpression of Nanog resulted in loss of ESCs differentiation in response to retinoic acid (175) and enhanced the ESCs pluripotency and enhanced their self-renewal independent of other effectors such as growth factors (163). On the other hand, Nanog -/- embryos had pluripotent ESCs but could not sustain their self-renewal and eventually differentiated into extra-embryonic endoderm (177). Alike to Sox2 and Oct3/4, Nanog would down regulate during differentiation of ESCs (175).

Kruppel like factor (Klf4) is another TF that potentiates self-renewal of ESCs (178). It belongs to the KLF family of conserved zinc finger TFs (163) that is expressed in several tissues and acts to regulate proliferation, differentiation and apoptosis (163).

KLF4 expression is quite high in ESCs and decreases significantly in differentiated cells (163, 179). Its functions would vary depending on the target genes as it can activate genes involved in cell-cycle and acts as oncogene while in another context works as a tumour suppressor and represses cell cycle (163, 179).

1.5.2.2. Types of stem cell

Stem cells could be natural or induced. The naturally occurring stem cells include embryonic and adult stem cells. Embryonic stem cells (ESCs) are isolated from the inner cell mass (ICM) of blastocyst (180) while adult stem cells are tissue specific and more specialised than ESCs as they give rise to tissue specific cells but do not go beyond the boundaries of their hosting tissue such as hematopoietic stem cell and liver stem cells (181, 182). Furthermore, the MSCs that can be isolated from various tissues such as bone marrow, adipose tissue, dental pulp in addition to perinatal sources such as the umbilical cord, placenta and amniotic fluid (183).

While the artificially induced stem cells are already differentiated somatic cells that underwent artificial induction of stemness via forced expression of pluripotency factors. These cells are known as the induced pluripotent stem cells (IPSCs)

Table 1-3. Degrees of cells potency and examples (184, 185)

Term	**Definition**	**example**
Totipotent	Can give rise to the whole embryo in addition to extra-embryonic tissues.	The zygote.
Pluripotent	Develops into any cell type within the embryo proper but not extra-embryonic tissues.	ESCs. IPSCs.
Multipotent	Can give rise to several cell phenotypes.	MSCs.
Unipotent	Can develop into only one cell phenotype.	Skin cells and cardiac progenitor cells.

1.5.2.2.1. Embryonic stem cells (ESCs)

Start of ESCs era was in 1981 when it was first isolated from mouse blastocyst and cultured in medium conditioned by teratocarcinoma stem cells. It was 17 years later before the ESCs were isolated from human blastocyst by Thomson and co-workers (157).

The embryonic development journey starts with fertilisation and formation of the totipotent zygote. Then it would undergo a series of sequential divisions forming distinct phases known as the morula followed by blastomere. Then the blastomere would cavitate to form blastocyst which contains the inner cell mass(ICM). Then the ICM which is the source of ESCs would develop into the embryo proper (158).

ESCs are pluripotent cells with short life span as they gradually differentiate according to a pre-set genetic program to give rise to the embryo proper (180). Therefore, ESCs in the laboratory are not normally existing cells, indeed, they are the result of processing ICM cells. In order to support their in-vitro proliferation in undifferentiated state they would need to enhance proliferation and repress differentiation with cytokines as well as growth factors such as using the Leukaemia inhibitory factor (LIF) to activate STAT3 in addition to the BMP to repress the differentiation factors (165, 186).

Human ESCs have high nucleus to cytoplasm ratio with prominent nucleoli and they express surface markers of undifferentiated state such as SSEA-3, SSEA-4, TRA-1-60, TRA-1-81 and alkaline phosphatase as well as high levels of telomerase. In addition, they can form teratoma when implanted in vivo which indicates their trilineage potential (157).

The most important characteristics of ESCs is self-renewal which means their ability to proliferate indefinitely while maintaining their undifferentiated state and being pluripotent so can differentiate into any cell phenotype (157).

Despite these promising features there are some limitations that hindered ESCs moving forward toward clinical applications including the ethical/legal concern and safety concern as well.

In human, ESCs were isolated from spare embryos of in-vitro fertilisation (187) which raised lots of ethical and legal concerns about this kind of research on human embryos. After announcement of the ESCs research it became a hot topic because the source of these embryonic cells is the inner cell mass which is indeed the early days of an embryo and in order to obtain these cells the ICM would be destroyed. This means the research is destroying a potential human life for the sake of helping others. So, the important question was: is it acceptable and legal to proceed with such research or it must stop because every human life is protected?

As expected in such question there will be a debate with no final right answer and it will continue to be a matter of debate because different people will look at different angles. Some look at the possible outcome and the potential of saving many lives and therefore they support this kind of research. This view has some religions to support such as Judaism which considers the embryo in these early days is not a human being. Others will look at the other angle and see that embryo from the first start of conception is a human life and must be protected so they will be totally against this sort of research and those have also some religions to support their vision such as catholic and conservative orthodox (158).

Then second and quite important concern is the risk of carcinogenesis. ESCs are not normally existing out of their embryonic milieu and their use for experimental/clinical applications with their self-renewal

and pluripotency criteria would carry risk of tumour formation (188). Actually, early discovery and research of stem cells started from embryonic carcinoma cells isolated from teratocarcinoma (189).

It is worth note that ESCs share some features with malignant cells such as fast proliferation with loss of contact inhibition (188) in addition to some common markers (189) as well as higher expression of some genes (190). For instance, the main regulators of stemness/pluripotency are either established oncogenes such as the MYC and KLF4 or linked to malignancy such as Oct4, Sox2 and Nanog (189). Furthermore, the key genes for induction of stemness namely Oct4, Sox2, Nanog and c-MYC are more frequently expressed in poorly differentiated tumors in comparison to the well differentiated tumors (191). Also, the process of ICM manipulation to isolate ESCs could be a part of the carcinogenic induction of the ESCs due to possible epigenetic alteration (189).

So, in view of all these concerns there was a need for alternative sources of stem cells.

1.5.2.2.2. Induced pluripotent stem cells (IPSCs)

IPSCs are terminally differentiated somatic cells that underwent forced expression of stemness factors to induce pluripotency (186) and regain the main stem cell characteristics including self-renewal and pluripotency. This approach circumvents ESCs ethical concerns and being autologous would eliminate the immune reaction concern (159).

The IPSCs era started with the work of Takahashi and Yamanka who successfully reprogrammed mouse fibroblasts into stem cells using defined factors. This was published 2006 and Yamanaka was granted Nobel prize for his work in 2012. It is also worth mention that Thomson et al did reach same results independently using different combination of factors (192)

Before IPSCs breakthrough, others could characterise the main transcription factors necessary for pluripotency which include the pluripotency genes Oct4, Sox2 and Nanog that co-occupy promoters of a large scale of genes and act in concert to maintain stemness of ESCs (193).

After a series of trials using large number of factors in different combinations they came up with the successful regimen which is oct4, sox2, klf4 & C-Myc in Yamanaka's model (194) and Oct4, Sox2, Lin28 and Nanog in Thomson's model (195). Both regimens could reprogram terminally differentiated somatic cells to stem cells with pluripotency features.

Early IPSCs experiments used virus mediated transfection to reprogram fibroblasts (194, 196). Later on, several ways found to work for induction of IPSCs. Some methods include genomic integration with the vector and therefore known as integration method while the non-integration methods if no genomic integration encountered (197).

Despite the great potential of IPSCs but there are some limitations including the efficiency and the safety. The efficiency of IPSCs induction is considered quite limited (198, 199).

There is also a safety concern as using viral vectors for induction in early regimens carries risk of mutagenesis. Also, using C-Myc in some regimens in order to enhance the efficiency raised a safety concern as it is known as an oncogene (200). Indeed, Yamanaka group demonstrated about 20% tumor

formation rate in the offspring of IPSCs clones passed from first to second generation. The culprit was most probably reactivation of C-Myc (201). Nevertheless, IPSCs induction is still possible without C-Myc but with lower efficiency rate and longer duration but is considered much safer because of lower tumour rate (196, 202). Therefore, non-virus mediated and oncogene free regimens should be considered for safe clinical applications (201). In addition, the immune competence which was considered as a major advantage of IPSCs was challenged in another study with evidence of inflammatory T-cell mediated response and even immune rejection of IPSCs when injected into their parent mice (203).

1.5.2.2.3. Adult stem cells (ASCs)

ASCs are resident in specialised micro-environments known as stem cell niche. Stem cell niche has several crucial functions as it supports stem cell survival, defines their role and regulates their differentiation. Therefore, the niche must have certain anatomic and physiologic criteria for successful function as stem cells out of their appropriate niche lack their regenerative value (204, 205).

ASCs are more specialised than ESCs. They are present in different tissues and work as backup to replace-within limits- any cellular loss such as neural stem cells in brain and the hematopoietic stem cells in bone marrow which hold the most prevalent clinical experience until now (206-208).

Some of the notable criteria of ASCs is that they are prone to metabolic stress and their self-renewal potential decreases with age (206). On transplantation to myocardial infarction area, cells from young and elderly donors -despite the volume was comparable- there was better differentiation of the young than their counterparts from elderly. This indicates a negative effect of aging on their regenerative potential (209). Nonetheless, the number of stem cells is ultimately affected by disease status. It was demonstrated that ASCs number in fat of diabetics was significantly lower than in non-diabetics which reflects the effect of metabolic stress on stem cells availability (206).

1.5.2.2.4. Mesenchymal stem cells (MSCs)

MSCs are a subtype of stem cells. They hold self-renewal potential and are multipotent cells that can differentiate into several cell phenotypes such as chondrocytes, adipocytes, osteoblasts and CMCs among others. However, their multi-potency is not comparable with the pluripotency of ESCs and IPSCs which limits their potential in regenerative medicine (210). However, despite their less potency they hold another advantage relative to the ESCs which is the genetic stability with less liability for mutagenesis and carcinogenesis (211).

MSCs were first isolated from the bone marrow. With time, extensive preclinical and clinical experience utilising MSCs in various cell therapy and regenerative trials were built up (210, 212, 213). Lately, MSCs could be isolated from almost all tissues and perinatal tissues as well (214).

After birth the frequency of MSCs in tissues decreases gradually from its peak in the neonate until almost 50% by the age of 80 years (215). In different tissues, MSCs are resident in a reservoir known as the perivascular niche (215, 216) while the yield of MSCs isolation from peripheral blood is very low if any (216, 217).

In addition to adult tissues, the perinatal sources like amniotic fluid, placenta and umbilical cord (218) are rich sources of MSCs with the extra advantage of being autologous with no immune rejection or ethical concerns. Also, they are considered medical waste and therefore to employ them for regenerative purposes is quite cost effective.

Amniotic fluid derived MSCs express embryonic cell markers and have high renewal potential with low antigenicity. Also, they did not induce teratomas when transplanted into animals (219). So, sounds attractive for cell replacement purposes but the main obstacle that would hinder their use on wide scale is the invasive nature of amniocentesis and risk of miscarriage (220).

MSCs derived from amnion, chorion and placenta, regardless gestational age could differentiate into several cell types (221). Practically speaking, placenta seems a good source of MSCs but chorionic villi apparently impractical source of stem cells for therapeutic purposes due to the invasive nature of obtaining samples with risk of miscarriage and even developmental abnormalities of the developing foetus (220).

The umbilical cord is a rich and realistic source of MSCs. It is a medical waste, readily available and easy to handle without the ethical concerns related to use of ESCs (222). There are two main sources for stem cells in the cord including the Wharton jelly and the cord blood. The Wharton jelly was first described by Thomas Wharton in 1656 (223). It composes the gelatinous core of the umbilical cord encasing the umbilical vessels and it is considered a rich source of MSCs (218, 222, 224). In addition, the umbilical cord blood derived MSCs are also multipotent and their proliferation potential is comparable with bone marrow derived MSCs (225).

1.5.2.2.4.1. How to identify MSCs

The Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy (ISCT) adopted criteria to define the MSCs including: MSCs must be plastic-adherent when maintained in standard culture conditions, they must express CD105, CD73 and CD90 and lack expression of CD45, CD34, CD14 or CD11b, CD79alpha or CD19 and HLA-DR surface molecules and must differentiate to osteoblasts, adipocytes and chondroblasts in vitro (226) (figure 1.7).

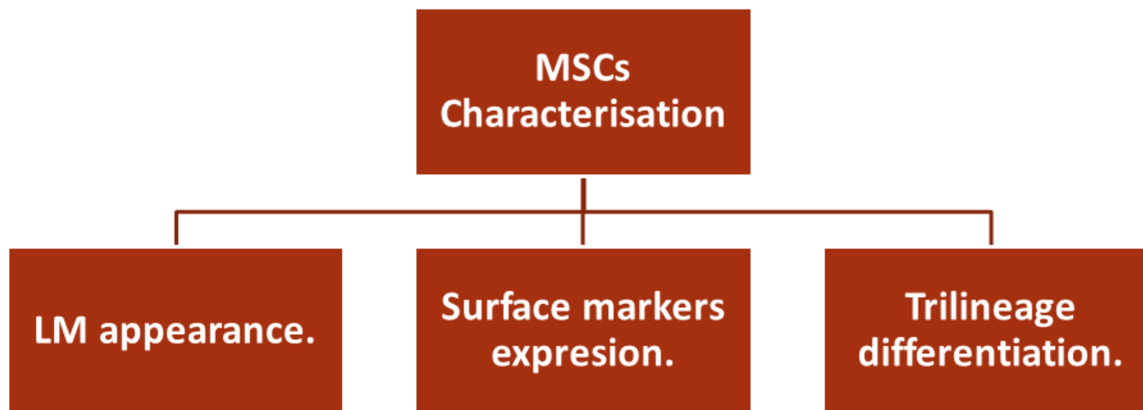

Figure 1.9. Hierarchy to demonstrate the three major criteria for MSCs characterisation.

Criteria including appearance (adherence to floor of plastic flask), characteristic markers profile and trilineage differentiation potential.

1.5.2.2.4.2.MSCs and cardiovascular medicine

MSCs are quite attractive phenotype of cells due to several advantages such as being easy to obtain and harvest in reasonable number for implantation from different sources, hold good potential for repair of injury without fibrous tissue formation as they home to sites of injury and help repair through secretion of cytokines, chemokines and extracellular matrix proteins (177, 227, 228). In addition they have immune suppressive/evasive properties which allows allogenic transplantation in addition to a seriously important criterion which is their lower tumorigenicity as compared to IPSCs (229).

Due to these advantages it is quite notable the surge of clinical trials utilizing MSCs at different clinical areas. On review of *ClinicalTrials.gov.* there were no registered clinical trials using MSCs before 1995 then within the next five years only 3 trials registered while by November 2019 there were a total of 1017 registered studies in different clinical areas. Also, trials progressed from preclinical to phase III clinical trials which means they passed the safety and efficacy assessment already.

Trials recruiting MSCs in cardiovascular medicine relative to the total number of clinical trials utilising MSCs since 1995 was 8.61% and this includes trials recruiting MSCs for heart as well as peripheral vascular disease but does not include other organ specific ischemic events or vascular diseases such as renal artery stenosis, stroke or ischemic retinal events (figure 1.8).

This reflects the promising results of this modality of treatment in addition to the challenges the field faces currently and the urgent need for radical treatment that can avoid organ transplantation which is the only radical cure for terminal organ failure until now.

For instance, Mandani et al in 2007 compared the New York heart association (NYHA) class and single photon emission CT (SPECT) scan of patients receiving standard of care PCI or CABG as a control versus the test group who received same therapy in addition to MSCs injection intramuscular and intra-coronary. The cell therapy group showed significantly better functional status and SPECT imaging (230). This study despite the small volume -8 patients each group- showed clear safety and efficacy of the MSCs based cell therapy in established ischemic heart patients.
The first double blind trial started 2009 and did demonstrate good tolerance of intravenous administration of allogenic MSCs with better left ventricle ejection fraction (LVEF) with less arrhythmias which pointed to safety and efficacy of this approach (8).

Currently, there is ongoing research aiming to optimise the mode of administration of MSCs whether intravenous, intramyocardial or trans-endocardial via catheter or intracoronary. This indicates that MSCs based therapy is quite close to move to standard medical care (8).

It is noteworthy that the start of the MSCs clinical trials was dominated by autologous MSCs however, trialling allogenic MSCs is gradually increasing until it became almost equal by 2014 and this is due to the immune modulatory effect of MSCs (8). Indeed, this is another advantage as allogenic MSCs are more readily available and could be standardised and optimised for transplantation.

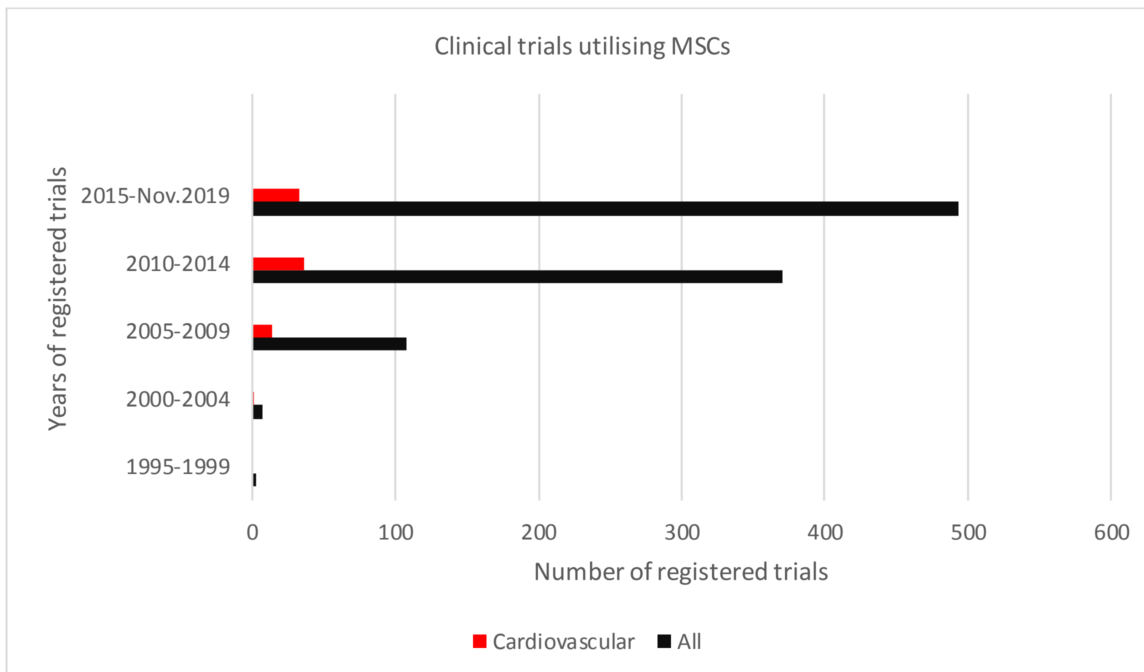

Figure 1-10. Clinical trials registered on clinicalTrials.gov utilizing MSCs in five yearly intervals.

The graph shows the great increment of volume of clinical trials utilizing MSCs based therapy and the relative volume of trials in cardiovascular field relative to total volume.

1.5.2.2.4.3. Therapeutic effects of MSCs

Experiments utilizing MSCs in cardiovascular research are quite promising with good body of evidence about their efficacy to restore cardiac function and reduce remodeling (8, 231, 232).
Nevertheless, it is notable that the physical engagement of cells or myogenic trans-differentiation is unlikely to justify for these significant results given the volume of cells and the short time window to achieve the observed results. Also, the fact that comparable results were obtained using conditioned media from MSCs rather than physical existence of the cells (233).
Further to these observations, administration of certain factors did achieve significant results such as intracoronary injection of stroma derived factor-1 (SDF1) which could ameliorate the LV function post induced ischemia as early as 3 hours (11).
Also overexpression of protein kinase B (Akt) in MSCs could prevent ventricular remodeling and restored cardiac function within about 3 days of induced MI which is not fully explained by the cells trans-differentiation (234). In the Akt over-expressing cells, other factors were also identified to be up-regulated such as VEGF, bFGF and HGF (235) which could explain the growth of endothelial cells (ECs) and smooth muscle cells (SMCs) when treated with MSCs conditioned media and the effect was dose dependent.
Furthermore, angiogenesis was enhanced by MSCs via secretion of angiogenic factors such as VEGF, bFGF, monocyte-chemotactic protein 1 (MCP1), SDf-1 and nitric oxide (NO). These factors play invaluable role for angiogenesis and network formation. In addition to hypoxia induced factor-1 (HIF-1) which is secreted under hypoxic conditions to stimulate ECs proliferation and migration in addition to the counterbalancing apoptosis to adjust new-vessel lumen formation (11).

MSCs also secrete anti-apoptosis factors such as Akt and B-cell lymphoma (Bcl-2) which inhibits caspase activation and renders cells more resistant to apoptosis signals and supports tissue regeneration. This is in addition to action of other survival factors such as VEGF which enhances survival of ECs after serum starvation via up-regulating Bcl-2 and induces phosphorylated activation of focal adhesion kinase (FAK) that suppresses the p53 and enhances survival (236).

Taken these observations together indicates that the regenerative/therapeutic effects of MSCs are most probably via paracrine factors such as secreting angiogenic factors in addition to immune-modulatory and anti-apoptotic effects rather than trans-differentiation (11).

1.5.2.2.4.4. Immune-modulatory effect

Immune modulatory effect of MSCs was first reported by Bartholomew et al who reported significant reduction of lymphocytes proliferation after being stimulated with allogenic antigen and significantly more durable skin graft in-vivo (237). This effect was also demonstrated in the trans-well model despite no physical contact of cells which indicates that the effect is mediated by secreted factors rather than cell engagement. Also, MSCs did enhance transit of pro-inflammatory macrophages M1 to the anti-inflammatory form M2, activate expression of suppressive cytokines such as IL-10 (228) and down-regulated INF-ɣ and IL-12. This could suppress lymphocytes inflammatory function and their effect was dose dependent. In addition to the immune-suppressive effect of MSCs on T-lymphocytes and natural killer (NK) cells and the inhibitory effect on maturation and function of monocyte derived dendritic cells and lower expression of MHC (11).

Due to the ability of MSCs to suppress immune response via suppressing T-cell proliferation and activity (238, 239) so, this would facilitate to circumvent immune rejection and allow for allogenic transplantation (237).

Another good point is that MSCs maintained their immune modulatory effect with increasing passage number (240). This means it is feasible to make reasonable in-vitro expansion to get the desired volume of cells for the intended application before transplantation without losing their immune-modulatory activity.

In one study, it was reported that MSCs were still detectable long time after allogeneic transplantation which indicated they could overcome immune clearance (228). However, this finding was challenged in another study (241) and the investigators considered MSCs to exert their effect through "hit and run" phenomenon and this means that MSCs are no longer immune privileged but rather considered according to Ankrum et al. as "immune evasive" (242).

Also, the previous concept of being immune inert and not stimulating allo-rejection was challenged (243). Some investigators classified MSCs into MSC1 which are Toll-like receptor4 (TLR4) primed and those are pro-inflammatory and MSC2 which are TLR3 primed and those are immunosuppressive (227). So, MSCs are not considered as one solution for everything but rather they should have their rule that fits their properties.

1.5.2.2.4.5. Senescence as drawback of MSCs

Expansion of MSCs is mandatory to meet the number requirements of cells for clinical applications (244, 245). This carries risk of cellular senescence with more chances of genetic and epigenetic alteration in addition to the potential of MSCs differentiation (246).

Replicative senescence occurs after certain number of proliferation/division cycles when cells cease to replicate. Restriction of the number of mitotic cycles that the cells can undertake is known as the Hayflick limit (247). Indeed, this mitotic clock (23) is a multifactorial phenomenon that involves progressive telomere shortening, increased expression of cyclin dependent kinases (CDKs) inhibitors particularly P16, hypo-phosphorylation of retinoblastoma (Rb) gene product in addition to the effect of oxidative stress (244, 248-250).

Nevertheless, replicative senescence of MSCs is a preset program that switches on with progressive passaging of MSCs during in-vitro expansion with subsequent alteration in differentiation potential, proliferation rates, global gene expression patterns and DNA methylation profiles (245). This was described by Wagner et al. as a continuous process that starts with the first passage (249).

1.6. Summary of background and study aims

1.6.1.Introduction

Congenital heart diseases (CHDs) are the most common congenital anomalies worldwide and surgery is indicated in substantial percent of cases. The currently available materials for reconstructive surgery lack growth potential which necessitates recurrent surgery.

Cormatrix is a biologic scaffold with good animal results while in humans and despite good durability, studies showed clear contradictory findings with no organised tissue or repopulation with native cells which means anatomical success but physiological failure.

In this project we are working on the physiological aspect of the patch aiming to enhance its in-vivo uptake, repopulation and vascularisation in addition to growth potential.

The plan is to seed cormatrix with triple cell phenotypes: wjMSCs, HUVECs and cardiac fibroblasts (HCFs). Then would study the potential interaction of the cells.

In other words, there are three cellular pillars to the project including wjMSCs, HUVECs and HCFs. We would dissect this triad and examine each pillar separately. The aim is to understand the cells' reaction to this combination, likelihood of successful maintenance of the cells as well as anticipate the in vivo behaviour of the cells.

Every cell has relevant functional/physiologic role and the whole triad would be supported by cormatrix as a biologic scaffold.

1.6.2.Facts and Concerns

Animal studies showed promising results of cormatrix in terms of uptake, repopulation, vascularisation and regaining some contractile function (124). Also, seeding cormatrix with MSCs showed even superior results relative to the acellular one (251, 252).

However, MSCs do not last for long. Indeed, they disappear from the implant quite shortly after transplantation. Also, HUVECs undergo rapid apoptosis when transplanted in-vivo. This means that cells will be shortly cleared from the scene which is not fully understood (251) and the effects are most probably related to their paracrine effects.

In human, there were clear discrepancy between animal studies and human findings. The most notable finding in human studies is that cormatrix was disproved to have the potential for repopulation with any organised tissue like structure (151).

So, the hypothesis here is that in order to boost cormatrix with a functional element and growth potential when implanted in a human with CHD(s), we need the seeded cells to maintain and integrate with the surrounding myocardium rather than being washed out.

Therefore, the seeded cells should meet two main conditions. First, to fulfill the main tissue requirements of cellular heterogeneity rather than single cell phenotype. Second, the cellular reaction/interaction should be favorable for the myocardial milieu.

In theory, this triad of cells would fulfil the first requirement. Nevertheless, the combination of cells is not natural and it is not clear how they will react/interact when combined together. So, we would analyse the cells' reaction to this combination to find out to which extent this combination would meet the second condition.

In the next chapters we would characterise the cells and study their mutual interaction using conditioned media (CdM). Also, would examine cormatrix uptake of cells, viability of cells on its surface and if any effect on its mechanical properties.

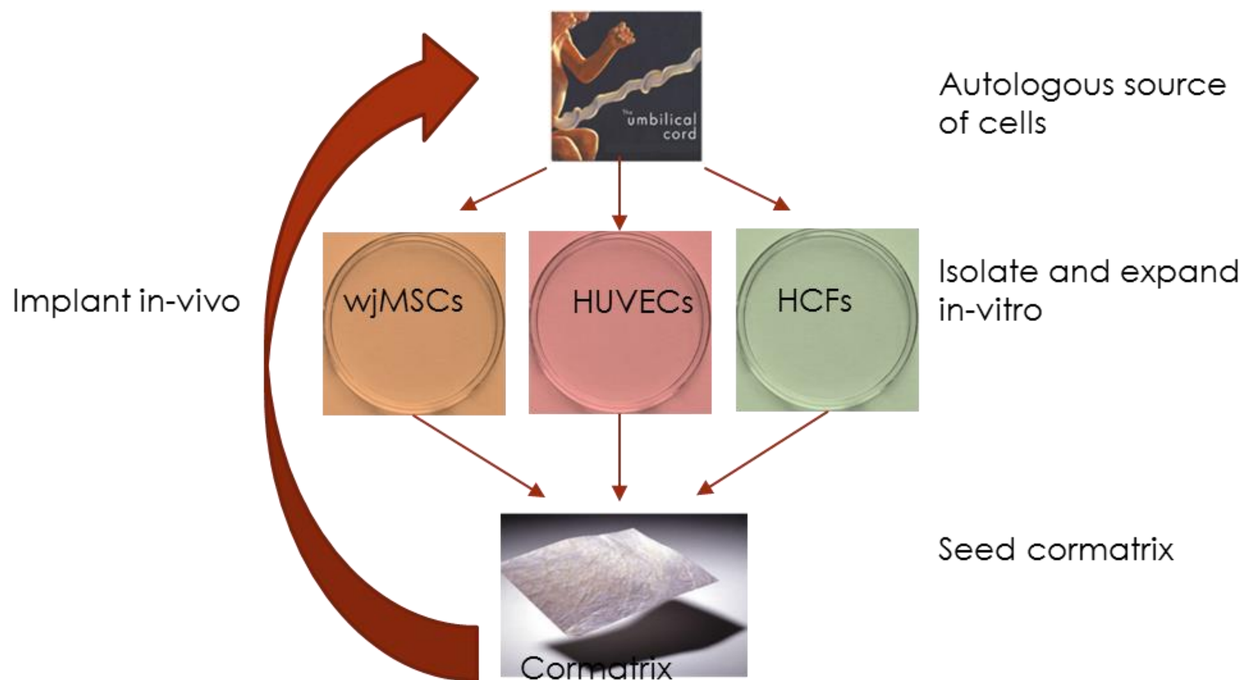

Figure 1-11.Overview of the project.

Autologous cells extracted from the baby including wjMSCs, HUVECs and CFs to be used for seeding cormatrix then implanted in-vivo for reconstructive cardiac surgery.

The original plan included testing the patch in-vivo. However, due to facility/time constrain this could not be done.

CHAPTER II

MATERIALS AND METHODS

2.1. Solutions

2.1.1. Endothelial cell growth media (EGM)

Sterile EGM with supplement was purchased from PromoCell. The supplement contains as final concentration FCS 0.05 ml/ml, EGF (recombinant human) 5 ng/ml, bFGF (recombinant human) 10 ng/ml, IGF (Long R3 IGF) 20 ng/ml, VEGF 165 (recombinant human) 0.5 ng/ml, ascorbic acid 1 ug/ ml and hydrocortisone 0.2 ug/ml. This supplemented medium was used for culture and expansion of wjMSCs, HUVECs and co-cultures of HUVECs-wjMSCs and HUVECs-HCFs.

2.1.2. Fibroblast growth media

FGM 3 with supplement was purchased from PromoCell. The supplement contains as final concentration FCS 0.1 ml/ml, bFGF (recombinant human) 1 ng/ml, Insulin (recombinant human) 5 ug/ml. This medium was used for culture of HCFs and co-culture of HCFs-wjMSCs.

2.1.3. Dulbecco's Modified – Eagle's Medium (DMEM)

High glucose DMEM supplemented with glutamine, pyruvate, 10% foetal bovine serum (FBS) and 1% penicillin/streptomycin all were purchased from Thermofischer. This supplemented medium was used for suspension of wharton jelly pieces to allow outgrowth of wjMSCs for initial isolation of MSCs and also for subsequent culture and expansion of wjMSCs. Low glucose DMEM was used as well but high glucose was offering apparently faster growth. In addition,

2.1.4. Paraformaldehyde (PFA)

Paraformaldehyde 4% was used as fixative for histology samples. All PFA used was premade in-house using PFA powder from Sigma (40 grams added to 800 ml of PBS, warmed to 58-60 c then NaOH 1 M from Sigma added drop by drop until the solution clears then adjust volume to one litre and PH to 6.9).

2.1.5. Phosphate buffered saline (PBS)

Sterile Dulbecco's phosphate buffered saline without calcium or magnesium was purchased from life technologies was used for cell culture purposes while for non-sterile uses PBS (0.137 M NaCl, 0.0027 M KCl, 0.01 M Na_2HPO_4 KH_2PO_4) was prepared by mixing 5 PBS tablets from Sigma Aldrich in one litre distilled water to get 1 X solution of PBS then autoclaved. Also, lately PBS buffer prepared by adding PBS tablets from Thermofischer, (2 tablets) to one litre of distilled water and then filtered through 0.2 um filters, was used.

2.1.6. Flow cytometry buffer

The buffer used for wash steps in flow cytometry was 0.5% fetal bovine serum (FBS) in PBS.

2.1.7. Alpha Modified – Eagle's Medium (α-MEM)

α-MEM purchased from life Technologies was used as basal medium for initial culture of wjMSCs for differentiation as recommended by manufacturer.

2.2. Ethics

All human samples were collected after informed consent from mothers giving birth at St. Michael's hospital, Bristol Royal Infirmary, University Hospital of Bristol according to hospital ethical regulations.

2.3. Cells

2.3.1 Wharton jelly mesenchymal stem cells (wjMSCs)

Wharton jelly was obtained from umbilical cords collected from mothers with normal pregnancy attended for delivery at St Michael's Hospital in Bristol who did not have any of the exclusion criteria.
Exclusion criteria

- Termination of pregnancy.
- New-borns with significant perinatal asphyxia.
- New-borns with major malformation.
- Not capable to understand information sheet and consent process.
- Any abnormal pregnancy at the 36-week visit.

2.3.1.1. Isolation of mesenchymal stem cells from umbilical cord wharton jelly

Cords were processed within the first 24 hours post-delivery after 3X washes in PBS with 1% supplemented with 1% penicillin/streptomycin. The explant method was used to extract wjMSCs.

The overlying layer of the cord was removed as well as the umbilical cord vessels and the Wharton jelly minced into small pieces about 2-8 mm diameter and plated in T75 flask in high glucose DMEM (Thermofischer), supplemented with glutamine and pyruvate in addition to 10% foetal bovine serum FBS (Thermofischer) and 1% penicillin/streptomycin (Thermofischer) and Kept in humidified incubator at 37 ^{o}C with 5% Co_2 for 10-12 days. Media was half refreshed every 2-3 days. When confluence reaches about 80% cells were passaged.

2.3.1.2. Culture and expansion of Wharton jelly MSCs

In order to harvest the cells, medium was aspirated and cells were washed with PBS then 5-10 ml of accutase solution (Sigma-Aldrich) was added and left for 5-10 minutes to detach the cells from the flask base then the cell suspension was collected using PBS in a falcon tube and centrifuged at 1500 rpm for 5 minutes. The supernatant was discarded and 1 ml of fresh media added to the cell pellet and cells counted.

Cells were plated in T75 flask in high glucose DMEM with 10%FBS and 1% P/S in a humidified incubator with 5% CO_2 at 37 ^{o}C and the media was changed every 2-3 days. Low glucose DMEM was sometimes used but it was notable that the growth rate is a bit slower. In low glucose DMEM cells reach 90-100% confluence within about 4-6 days while in high glucose DMEM same confluence is achieved within 2-4 days.

With advance of the project and considering the fact that wjMSCs are planned to be seeded with HUVECs, EGM MV2 with supplement mix (PromoCell) was used as it was found to be quite suitable for wjMSCs growth and expansion.

2.3.2. Human umbilical vein endothelial cells (HUVECs)

2.3.2.1. Isolation of HUVECs

For HUVECs isolation, umbilical cords were processed within 24 hours after birth. Pieces of 3-6 cm of umbilical cord were used. The cord was washed 3X in warm PBS (Thermofischer) supplemented with 1% penicillin/streptomycin (Thermofischer) to get rid of blood and eliminate any contamination.

Then umbilical vein identified by being single and large pore and going inward in contrast to two umbilical arteries with small pore and lumen bulging outward. The vein was cannulated and gently washed once with pre-warmed PBS to get rid of any blood residues. Then the cord was clamped from both ends while keeping the cannula in and injected with pre-warmed sterile collagenase IV 0.2% (Stem Cell Technologies) and kept in a beaker in warm PBS at 37 ^{o}C for 15 minutes. Then, distal clamp was released and the vein was washed gently with warm PBS and out-coming fluid was collected in a Falcon tube and centrifuged at 1500 rpm for 5 minutes and supernatant was discarded and cells were resuspended in EGM MV2 with supplement mix (PromoCell).

2.3.2.2. Culture and expansion of HUVECs

Cells were plated in uncoated T75 flask in EGM with supplement mix (PromoCell) and kept in a humidified incubator at 37 ^{o}C with 5% Co_2. The cells reached confluence of 80-90% within 3-5 days then passaged following same protocol described for wjMSCs.

2.3.3. Human cardiac fibroblasts (HCFs)

1.3.2.1. Source of HCFs

HCFs isolated from adult heart ventricle, were purchased from PromoCell.

2.3.2.2. Culture and expansion of HCFs

HCFs were cultured in FGM (PromoCell) supplemented with fetal calf serum 0.1 ml/ml, bFGF (recombinant human) 1 ng/ml and insulin (recombinant human) 5 ug/ml. Medium was changed every 2-3 days until reaching 90-100% confluence then HCFs were passaged following same protocol described for MSCs.

2.4. Trilineage differentiation of wjMSCs

2.4.1. Osteogenic differentiation

2.4.1.1. Protocol for osteogenic differentiation

Following the manufacturer's recommendations, completed osteogenic medium was prepared by adding osteogenic differentiation supplement (R&D) to the base medium at 1:20 final dilution.

Cells were harvested from culture flask by removing media then washing once with PBS then adding trypsin 0.5% (Thermofischer) for 1-2 minutes to detach cells from flask floor. Alpha MEM with 10% FBS

was then added to stop trypsin activity and cells' suspension was collected and centrifuged at 1500 rpm for 5 minutes. The supernatant was discarded, cells were resuspended in alpha MEM with 1% P/S and counted using haemocytometer. About $4.3x10^3$ MSCs were plated in 2-3 mL of the pre-warmed base media in 6 well plate and the cells were followed until reaching about 50% confluence then complete osteogenic media warmed to 37 ºC was added to the 50% confluent cells. Medium was replaced every 3-4 days. After 14 days cells were stained for alkaline phosphatase using BCIPI/NBT (Sigma-Aldrich) and after 19-21 days they were stained for calcium deposits using Alizarin red to confirm osteogenic differentiation.

2.4.1.2. Staining to confirm differentiation

Medium was aspirated and the well rinsed with 500 ul PBS then cells were fixed for 1 hour with ice cold ethanol 70% at 4 ºC. Then fixative was aspirated and cells were stained with 500 ul Alizarin red for 5 minutes at room temperature. Then stain was aspirated and the well was washed 5x with PBS.

To stain for alkaline phosphatase one tablet BCIPI/NBT (5-Bromo-4-chloro-3-indolyl phosphate/Nitro blue tetrazolium; Sigma-Aldrich) was dissolved in 10 ml of dH2o (should be kept in dark and used within two hours). The used washing buffer was 0.05% Tween 20 in PBS (w/o Ca^{++}/Mg^{++}). The medium was aspirated and cells were washed with PBS followed by fixation with ice cold ethanol 70% (at 4 ºC) for 60-90 seconds. The fixative was aspirated and cells were washed with washing buffer. Then washing buffer was aspirated and BCIP/NBT solution was added at a volume enough to cover the whole well. Staining was checked within 5-10 minutes then examined using Zeiss microscope using colour camera to assess for red staining.

2.4.2. Chondrogenic differentiation

2.4.2.1. Protocol for chondrogenic differentiation

Following the manufacturer recommendations**,** human chondrogenic supplement (R&D) was added at 1:100 dilution to basal media to form completed Chondrogenic differentiation media.

Cells were harvested from culture flask as described above and counted using haemocytometer. Then $2X10^5$ MSCs were plated in a well of 96 well plate in a 200 ul of α-MEM basal media. Then 30-60 minutes later 200 ul of completed chondrogenic differentiation medium was added and medium was changed every 3-4 days.

2.4.2.2. Staining to confirm differentiation

Alcian blue solution 1% prepared by dissolving 1 gm Alcian blue powder in 100 ml of acetic acid 3% and PH was adjusted at 2.5 by adding acetic acid. Cells were washed in PBS twice then fixed in PFA 4% for 20 min at RT. Alcian blue (400 ul) was added to the cells and left overnight at RT protected from light. Then cells were washed once with acetic acid 3% followed by wash with PBS 3 times. Then cells were examined using Zeiss microscope colour camera to assess for staining.

2.4.3. Adipogenic differentiation

2.4.3.1. Protocol for Adipogenic differentiation

Following the manufacturer's recommendations, adipogenic supplement (R&D) was added at 1:100 dilution to basal media to form completed adipogenic media. Cells were harvested from culture flask as described above then counted with haemocytometer. Cells were plated at $2.5x10^5$ per well in a 6-well plate in basal media and followed daily until 90-100% confluence was reached. Once target confluence was achieved, completed adipogenic differentiation medium was added to the cells and changed every 3-4 days. After 14 days, adipocytes induced cells were stained with oil red.

2.4.3.2. Staining to confirm differentiation

Oil red was used to stain lipid vacuoles in order to confirm adipocyte differentiation.

Medium was aspirated and cells were rinsed once with PBS and 500 ul PFA 4% was added at RT for 30 minutes. The fixative was aspirated and cells were washed with PBS and 60% isopropanol then stained with 500 ul Oil red for 30 minutes at RT. Oil red stain was aspirated and cells were washed briefly with 60% isopropanol. Then cells were examined using Zeiss microscope colour camera to assess for staining.

2.5. Flow cytometry

2.5.1. Cells preparation

Cells were harvested from the flask (as described in the isolation and cultivation paragraph) and washed once in 10 ml FCM buffer then resuspended in 1 ml of buffer and counted. Then about $2x10^5$ cells were transferred into each FCM tube in 200 ul of buffer.

2.5.2. Staining for surface markers expression

The primary antibody was added at 1:100 dilution and kept in the dark for 45 minutes. Cells treated with conjugated antibodies were washed in 2 ml buffer and centrifuged for 5 minutes at 1500 rpm then supernatant discarded and pellet resuspended in 200 ul buffer for analysis.

Cells treated with unconjugated antibodies had a similar wash step in 2 ml buffer and the pellet was resuspended in 200 ul buffer. The secondary antibody was added at 1:200 dilution in the dark for 15-30 minutes at room temperature then cells were washed again with 2 ml buffer and the supernatant was discarded then the pellet was resuspended in 200 ul buffer and taken for analysis.

Table 2-4. Antibodies for flow cytometry

Primary antibody	**Species**	**Stain**	**Company**
CD105	Mouse anti-human	PE	R&D
CD90	Mouse anti-human	PE	R&D
CD73	Mouse anti-human	Unconjugated	BD Biosciences
CD45	Mouse anti-human	PE	R&D
CD34	Mouse anti-human	Unconjugated	GeneTex
CD31	Mouse anti-human	FITC	Sino Biological
CD19	Mouse anti-human	FITC	Sino Biological
CD14	Mouse anti-human	Unconjugated	GeneTex
HLA-DR	Mouse anti-human	Unconjugated	Sigma-Aldrich

In addition, PE (catalogue:) and FITC (catalogue:) IgG control purchased from R&D.

2.5.3. Flow cytometry surface markers expression analysis

Analysis was done using NovoCyte flow cytometry machine in the biomedical school, University of Bristol using NovoCyte 3000 from Acea Biosciences, Inc. (San Diego, CA). with software NovoExpress 1.2.4.

2.6 Immunofluorescence

2.6.1. Cells preparation

Cells were harvested from flask (as described above) and seeded in 96 well plate at 30,000-50,000 cells/well for at least 24 hours to reach confluence of about 90%.

2.6.2. Protocol for staining

Cells were washed twice with PBS then fixed with pre-cooled methanol (Sigma Aldrich) at about -10 ^{o}C for 5-10 minutes. Then methanol was aspirated and cells were allowed to air dry. Cells were then blocked with Ultra Cruz blocking agent (Santa Cruz) for 30 minutes. Then primary antibody in Ultra Cruz blocking agent was added at a dilution 1:200 and left overnight at 4 ^{o}C.

Primary antibody suspension was aspirated and cells were washed 2-3 times then the secondary antibody suspension was added (in case of unconjugated primaries). Secondary antibodies used include goat anti-mouse AF488 from R&D and AF488 conjugated binding protein (BP) from Santa-Cruz and AF596 conjugated BP from Santa-Cruz.

The secondary antibody/conjugated BP was added to cells in Ultra Cruz blocking agent at 1:500 dilution for 1 hour at RT.

Secondary antibody/conjugated BP was aspirated and DAPI diluted in PBS at 1:500 dilution was added to cells for 5 minutes then aspirated and cells were washed 2-3 times with PBS then keep in PBS and examine under fluorescence microscope using Zeiss Axio Observer Z1 with Zen Blue software (Zeiss).

2.6.3.Imaging and analysis of immune-flourescence

For the quantitative imaging, images were captured using incucyte system in Wolfson imaging unit in the biomedical school, Bristol University. The incucyte is an automated system that captures 9 images/well of a 96 well plate. Quantification of protein expression was done in terms of mean integrated density (Id) using image-J.

Table 2-5. Immune-fluorescence primary antibodies.

Primary antibody	Species	Stain	Company
AKT	Mouse anti-human	Unconjugated	Santa-Cruz
ITG	Mouse anti-human	Unconjugated	Santa-Cruz
NRG1	Mouse anti-human	Unconjugated	Santa-Cruz
Collagen I	Mouse anti-human	Unconjugated	Santa-Cruz
Collagen III	Mouse anti-human	Unconjugated	Santa-Cruz
Fibronectin	Mouse anti-human	Unconjugated	Santa-Cruz
Angiopoietin	Mouse anti-human	Unconjugated	Santa-Cruz
bFGF	Mouse anti-human	Unconjugated	Santa-Cruz
HGF	Mouse anti-human	Unconjugated	Santa-Cruz
PDGF	Mouse anti-human	Unconjugated	Santa-Cruz
VEGF	Mouse anti-human	Unconjugated	Santa-Cruz
FLK1 (VEGFR2)	Mouse anti-human	Unconjugated	Santa-Cruz
FLT1 (VEGFR1)	Mouse anti-human	Unconjugated	Santa-Cruz

B-catenin	Mouse anti-human	Unconjugated	Santa-Cruz/ R&D
N-cadherin	Mouse anti-human	Unconjugated	Santa-Cruz
MCP-1	Mouse anti-human	Unconjugated	Santa-Cruz
SDF-1	Mouse anti-human	Unconjugated	Santa-Cruz
BCl-2	Mouse anti-human	Unconjugated	Santa-Cruz
C. troponin	Mouse anti-human	Unconjugated	Santa-Cruz
MHC	Mouse anti-human	Unconjugated	R&D
Cx43	Mouse anti-human	Unconjugated	Santa-Cruz
MMP	Mouse anti-human	Unconjugated	Santa-Cruz
TIMP	Mouse anti-human	Unconjugated	Santa-Cruz

2.7. Live/dead staining

In order to examine viability of seeded cells, Calcein and Ethidium live cell assay; (R&D) were used to stain live and dead cells, respectively. Cells were seeded on cormatrix in 48 well plate and kept for 4-7 days with media refreshed every 2-3 days. Calcein and Ethidium were added to the well at concentration of 2 ul/ml and kept in incubator for 30 minutes then examined under fluorescence microscope using Zeiss Axio Observer Z1 with Zen Blue software (Zeiss) using Calcein and Ethidium channels.

2.8.Apoptosis and cell death assays

For assessment of apoptosis and cell death, Caspase 3/7 (from ThermoFischer) was used. The kit contains caspase 3/7 to detect apoptotic cells and sytosol to detect dead cells. However, during the FCM experiments there was bleeding and overlap between the two assays that could differentiate between the two events. Therefore, we did use caspase 3/7 to detect apoptotic cells and DRaq7 (from Biostatus).

The manufacturer's recommendatons were followed. After cells were collected from the flask they were washed in PBS with 0.5% FBS then caspase was added at 1 ul/ml then kept for about 30 minutes in incubator or 45 minutes at RT. Shortly before running the FCM experiment DRaq7 was added at 0.5 ul/200 ul and run the FCM using Novocyte FCM machine.

2.9. qPCR

qPCR was used to assess mRNA expression of different genes.

2.9.1. RNA extraction

RNA extraction was carried out using miRNeasy kits, Qiagen, following the manufacturer's recommendations.

In summary, media was aspirated from flask and cells were washed with PBS. Then 350 ul of cell lysis substrate supplemented with 1% Mercaptoethanol was added and cells were scraped off the flask floor and collected into 1.5 ml tube. Cells were homogenized using bench rotator and then centrifuged at 11000/min for 3 minutes. The supernatant was transferred to another 1.5 ml tube and one volume of ethanol 70% biology grade was added and mixed via pipetting before transferring to a miRNeasy column. The column was washed twice and RNA was eluted in 30 ul of RNAse free water and kept immediately on ice then stored at -20 ^{o}C.

2.9.2. Reverse transcription

For cDNA synthesis Qiagen kit was used and the manufacturer's recommendations were followed. In summary, 200 ug of RNA was used and 1 ul of gDNA clearing was added and then a volume of RNAse free water was added to complete 14 ul, then heated at 42 ^{o}C for 2-3 minutes followed by immediate transfer to ice. Then cDNA primers as well as polymerase were added and heated again at 42 ^{o}C for 15 minutes then transferred to 95 ^{o}C for 3 minutes to stop the reaction.

2.9.3. Rt-PCR

For the Rt-PCR the SYBR green Qiagen kit was used and the manufacturer's protocol was followed.

In summary: 10 ul of SYBR green dye, 0.5 ul of forward primer and 0.5 ul of reverse primer and 8 ul of RNAse free water and 1 ul of cDNA all mixed. The reaction was run in capillary tubes that were purchased from Roche. The capillary tubes fitted in the round holder and kept within the processing chamber of a Roche PCR machine.

2.9.4. DNA oligos

The oligos were designed using the NCBI software and made by Sigma Aldrich.

The following are the primer sequence used

18S was used as the house keeping gene, purchased from Qiagen.

Table 2-6. qPCR primers

Gene	Forward sequence	Reverse sequence
ANP	CAAGCTGGAGCGGAACTGCTACTT	GCTCGCACACATGATCACCACTCT
Desmin	TCAACGTGAAGATGGCCCTGGATG	CTGGTTTCTCGGAAGTTGAGGGCA
GJA1	GGTCTGAGTGCCTGAACTTGCCTT	GCCTGGGCACCACTCTTTTGCTTA
NKX2.5	GAGCCGAAAAGAAAGAGCTGTGCG	ATAGACCTGCGCCTGCGAGAAGAG
MHC	GTTTGGGACTGAGGCGCTGGATCT	ATCCAAAAGCAATTGCCTCTTCAGCC
TPM	TCCAACTGAAAGAGGCCAAGCACA	CTCGGCACATTTGCCTTCTGAGAG
cTPN	CTCCATCCTCTGCCTCACCCAGTC	TGGTCTCTGCTCTCCCTCAGAACA
Akt	CTGTCAGCTGGTGCATCAGAGGCT	GCCAACCCTCCTTCACAATAGCCA
NRG1	AGAGGCAAAGGGAAGGGCAAGAAG	TTTCAATCGGGGAGGCAAGGCTGG
Col1A1	GTCCTCCAGGTGAAGCAGGCAAA	GGAAACCTCTCTCGCCTCTTGCTC
Col3A1	GGACCTCCTGGCAAAGATGGAACC	GGGGAGCCCTCAGATCCTCTTTCA
Fibronectin	GAACATCCCTGACCTGCTTCCTGG	GCATCAGGCGCTGTTGTTTGTGAA
Angiopoietin	CCACAACCTTGTCAATCTTTGCACT	AAAACACCTTTTTGGGTTCTGGCA
bFGF	CTGTACTGCAAAAACGGGGGCTTC	TTGTAGCTTGATGTGAGGGTCGCT
HGF	AAGGACGCAGCTACAAGGGAACAG	GGCAAAAAGCTGTGTTCGTGTGGT

PDGF	GTCTCTCTGCTGCTACCTGCGTCT	AACTCGGCCCCATCTTCCTCTCC
VEGF-A	GAAAGCGCAAGAAATCCCGTCCCT	AACGCGAGTCTGTGTTTTTGCAGG
FLK1	AAAGCGGGGCATGTACTGACGATT	CTGGGGTGGGACATACACAACCAG
FLT1	GGCTGTGAAAATGCTGAAAGAGGGG	TTCAGATGGTGGCCAATGTGGGTC
B-catenin	AGACGGAGGAAGGTCTGAGGAGCA	CAAATACCCTCAGGGGAACAGGCTC
VE-cadherin	CAGGCCAGGTATGAGATCGTGGTG	TGTACTTGGTCTGGGTGAAGAAGG
N-cadherin	GTAGAGGCTTCTGGTGAAATCGCA	TGCAGTTGCTAAACTTCACATTGAG
ITG	ACGCCGCGCGGAAAAGATGAATTT	CCCACAATTTGGCCCTGCTTGTAT

2.10. Statistical analysis

T-test was used for quantitative analysis.

Unless otherwise specified, data are presented in terms of mean +/-SEM and difference was considered significant when $p < 0.05$.

One way analysis of variance (ANOVA) with post hoc Tukey test was used where appropriate for three groups analysis.

CHAPTER III

ISOLATION, EXPANSION AND CHARACTERISATION OF CELLS.

3.1. Introduction

CHDs are the most common congenital anomalies around the globe and substantial percent of cases would require surgical intervention at some point. Current practice is limited with the fact that all materials in use for corrective cardiovascular surgery despite meeting the structural demands but still cannot offer any growth advantage in addition to lack of contractility of the patch. This means anatomical success but physiological failure.

In this project we are working on the physiologic aspect of the patch. We aim to engineer a patch for corrective cardiac surgery with better chance for uptake and vascularisation that would also support contractility in addition to potential growth.

After review of the various components of the cardiac tissue and the functional as well as the structural role of each element, it is clear that every single component of the cardiac tissue is quite important and should be considered in order to optimise any replacement/reconstruction of a cardiac defect. Therefore, the hypothesis of this project is to design a patch for corrective cardiac surgery with better properties and potential for growth.

In order to accomplish this task we are planning to seed the biologic scaffold cormatrix with three different cell phenotypes aiming to bring the patch a step forward closer to natural tissue. The cellular pillars for seeding would be Wharton jelly mesenchymal stem cells (wjMSCs), human umbilical vein endothelial cells (HUVECs) and human cardiac fibroblasts (HCFs).

Each phenotype of this cellular triad has relevant role. WjMSCs have paracrine, anti-apoptotic and immune modulatory properties with potential to transdifferentiate to cardiomyocytes (253), HUVECs would boost the patch with pillars for angiogenesis and HCFs to produce ECM in addition to their role as mechanosensors and electrical coupling with CMCs to organise myocardial contractility this is in addition to the mechanical support of cormatrix.

In this chapter would focus on isolation of the cells and to validate their identity via different characterisation parameters.

3.2. Wharton jelly MSCs

3.2.1. Introduction

In human, Wharton jelly is the gelatinous core of the umbilical cord covered with amniotic membrane and encases two arteries and one vein with no other blood or lymph vessels and no innervation and is quite enriched with MSCs (21). It is an attractive source of stem cells as it is rich in wjMSCs and the cord is a medical waste so its utilisation for regenerative purposes is a cost-effective approach with no ethical concerns as well as being autologous with no immune reaction concern. Also, the wjMSCs hold an immune modulatory effect which would support better tolerance in allo-transplantation.

WjMSCs are multipotent cells that can trans-differentiate into other cell phenotypes such as osteoblasts, adipocytes, chondrocytes, and cardiomyocytes (253). Furthermore, wjMSCs have less carcinogenicity potential as compared to ESCs and IPSCs in addition to their paracrine, anti-oxidative and anti-apoptosis effects and they have reasonable proliferation potential which is promising for decent expansion in-vitro to meet the requirements for in-vivo transplantation.

Also, there is a good body of evidence for MSCs cardioprotective effect in myocardial infarction models as well as improving cardiac performance in cardiac failure(231, 254, 255).
Also, our group have good experience using MSCs from different sources such as thymus and cord blood to seed scaffolds for in-vivo cardiac/vascular surgery. So, we opted to use wjMSCs to seed the patch in combination with other cell phenotypes aiming to benefit from their peculiar cellular profile.

3.2.2. Objectives

To isolate and expand MSCs from Wharton jelly using explant method. Then to characterise the isolated cells as MSCs using ISCT criteria including morphology under Light microscope and adherence to floor of plastic flasks under normal culture conditions, surface markers analysis using flow cytometry (FCM) and trilineage differentiation including chondrogenic, adipogenic and osteogenic differentiation of wjMSCs in addition to assess wjMSCs proliferation potential and calculate their doubling time.

3.2.3 Results

3.2.3.1. Isolation and expansion of MSCs from Wharton Jelly

All umbilical cords collected from mothers giving birth at St. Michael hospital, Bristol Royal Infirmary NHS foundation trust after informed written consent according to the University of Bristol ethical regulations.

After processing the umbilical cord as described in the materials and methods section, MSCs were noticed to start migrating out of Wharton jelly pieces within 5-8 days and achieved confluence of 70-90% within 10-12 days. Then cells were harvested and passaged (as described earlier) to establish wjMSCs cell line (figure 3.1).

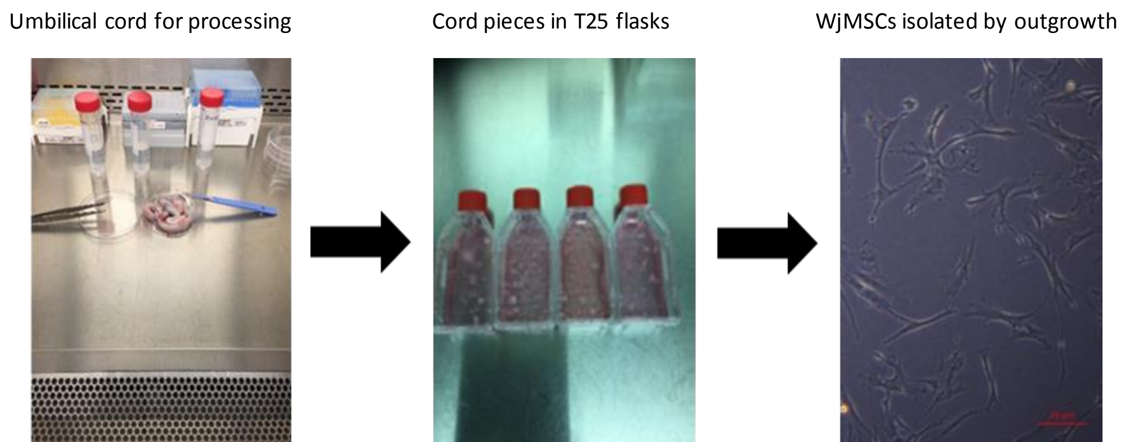

Figure 3-1. WjMSCs extraction from umbilical cord via explant method

(left) piece of umbilical cord in dish plate under laminar flow hood for processing. (middle) minced Wharton jelly pieces plated in DMEM* in T25 flasks. (right) wjMSCs extracted via outgrowth under light microscope with scale bar 20 um.

*DMEM: Dulbecco modified eagle's medium.

3.2.3.2. Characterisation of wjMSCs

After isolation of cells from the Wharton jelly and in order to confirm their nature as MSCs the cells were tested against criteria adopted by the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy (ISCT) to define MSCs.

3.2.3.2.1 Morphology

When assessed under light microscope the isolated cells were adherent to the plastic floor of the flask after maintaining in standard culture conditions with branching fibroblast like morphology (figure 3.2).

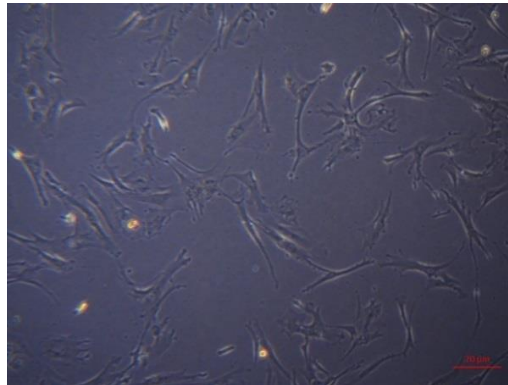

Figure 3-2. WjMSCs under light microscope.

Cells are adherent to plastic floor of the flask under normal culture conditions with branching fibroblast like morphology in keeping with MSCs appearance. Scale bar 20 um.

3.2.3.2.2 Surface markers analysis using flow cytometry (FCM)

In order to examine the outgrowing cells against the characteristic panel of surface markers adopted by the ISCT, the cells were tested using FCM for expression of CD105, CD90, CD73, CD45, CD34, CD19, CD14 and HLA-DR. Cells used at passages 2-4 were highly positive for CD73, CD90 and CD105 while there was very low expression of CD45, CD34, CD19, CD14 and HLA-DR which is consistent with MSCs nature of these cells (figures 3-3 & 3-4).

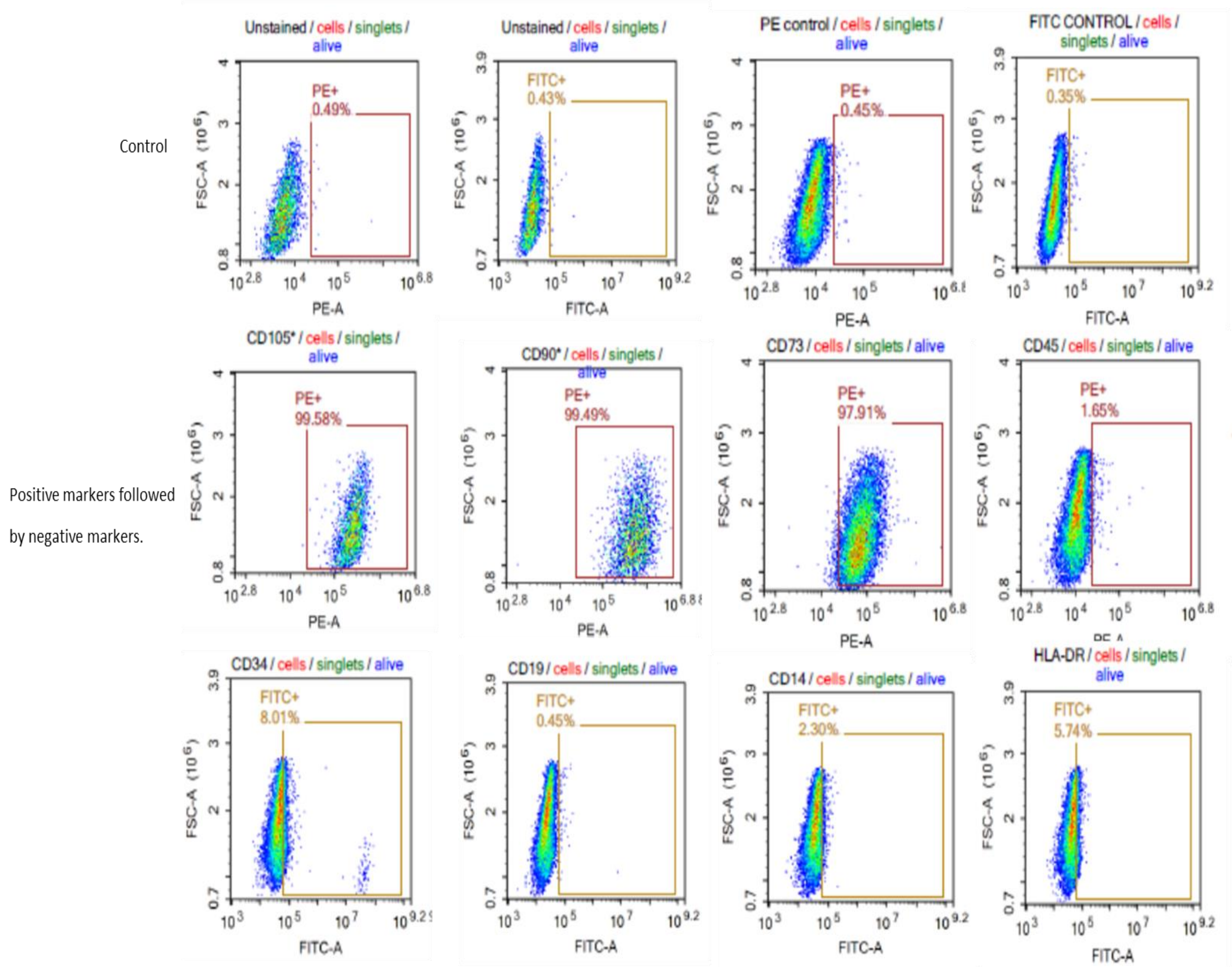

Figure 3-3. Representative images of FCM results for WjMSCs surface markers expression.

Upper row shows the control: (left to right) unstained PE, unstained FITC, PE control and FITC control respectively. Second and third rows show the wjMSCs highly positive for CD105, CD90 and CD73 followed by the negative markers CD45, CD34, CD19, CD14 and HLA-DR respectively. This is in keeping with the MSCs nature of these cells.

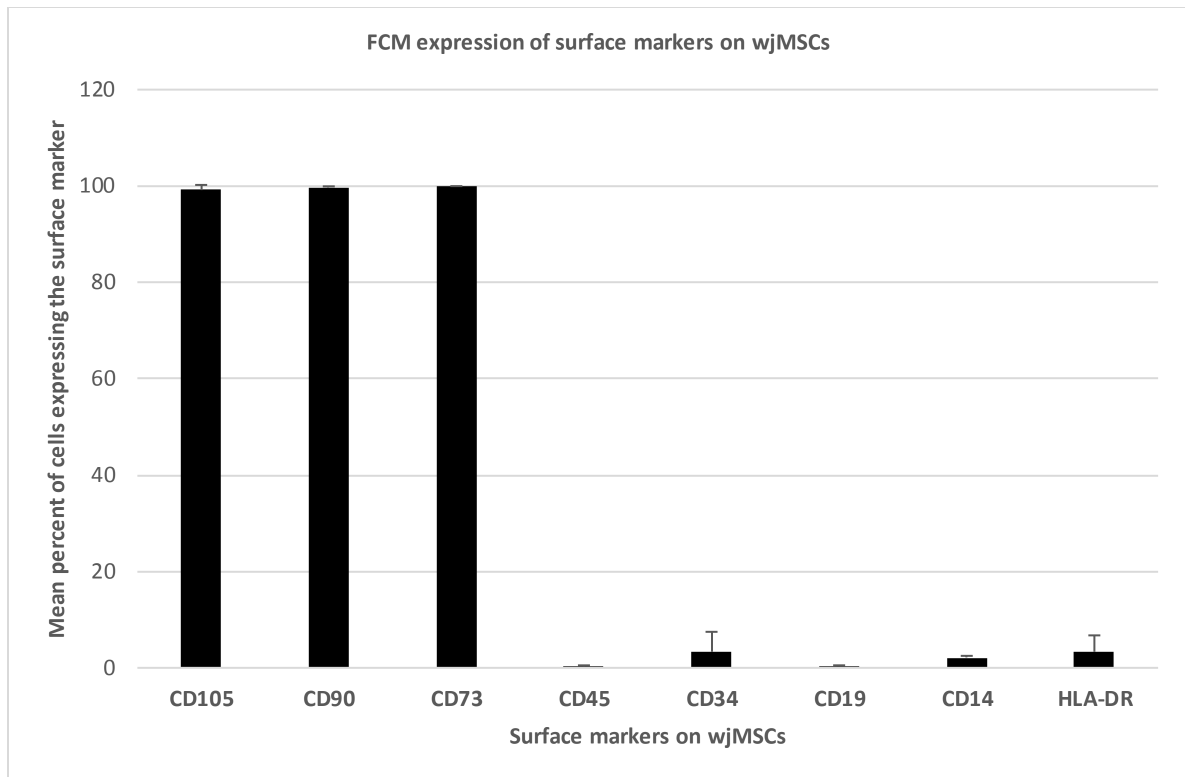

Figure 3-4. WjMSCs surface markers expression via flow cytometry

FCM Highly positive for CD105, CD90 and CD73 while negative for CD45, CD34, CD19, CD14 and HLA-DR respectively. Graphs are the mean of 3 independent experiments and error bars for SD.

3.2.3.2.3 Trilineage differentiation of wjMSCs

The trilineage differentiation potential of the wjMSCs is an integral part of their characterisation criteria (226). So, in order to confirm that cells fullfilled all criteria including the differentiation potential into adipo, osteo and chondrocytes the outgrowing cells were treated with the relevant differentiation media as described earlier. After appropriate time intervals the cells were examined using relevant stains to assess their differentiation.

For chondrogenesis the cells were treated with chondrogenic differentiation media as described earlier. Cells started to clumb forming a micromass as early as 24 hours (not shown) then after 12 days the cells were fixed and stained with Alcian blue. There was clear blue/violet staining as compared with light blue stain of the control which indicates collagen synthesis and confirms differentiation into chondrocytes (figure 3.5-A&B).

For adipogenesis the cells were treated with adipocyte differentiation media and morphologic changes were observed around day 7. Cells noted to change from branching fibroblast like appearance to polygonal non-branching morphology, then by the day 12-14 intracellular lipid vacuoles were noticed (not shown). Adipogenic differentiation was confirmed with oil red staining that showed red/brown staining of the lipid vacuoles as compared with the control cells which denotes lipid droplets formation and indicates differentiation to adipocytes (figure 3.5-C&D).

For osteogenesis the cells were treated with osteogenic differentiation media as described earlier. The cells continued to proliferate all through the treatment period and noted to show the characteristic filament like elongated morphology with central nucleus which indicates gradual differentiation (not shown). After 21 days the cells were fixed and stained with Alizaren red which showed red-orange staining of extracellular calcium deposits (figure 3.5-E&F) and on staining with BCIP/NBT (5-Bromo-4-chloro-3-indolyl phosphate/Nitro blue tetrazolium) they showed violet blue staining denoting high alkaline phosphatase activity (figure 3.5-G&H) as compared with the control which indicates osteogenic differentiation.

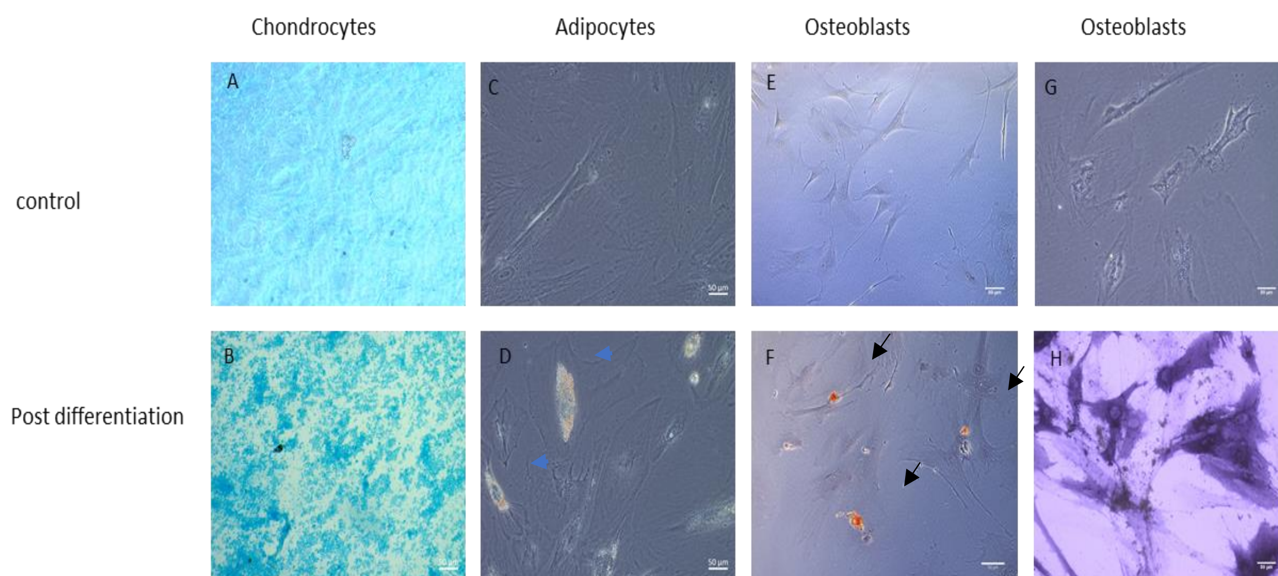

Figure 3-5. Representative images of wharton jelly extracted cells after treatment with differentiation media then staining to confirm differentiation.

(A) Non treated wjMSCs stained with Alcian blue as control. (B) WjMSCs treated with chondrocyte differentiation media for 12 days showing the intense bluish discolouration as compared with the control which denotes extracellular collagen synthesis an d indicates differentiation into chondrocytes. (C) Non treated wjMSCs stained with oil red as control. (D) WjMSCs treated with adipocyte differentiation media for 14 days showing the lipid droplets stained red (blue arrow heads) which indicates differentiation to adipocytes. (E) Non treated wjMSCs stained with Alizaren red as control. (F) WjMSCs post exposure to osteoblast differentiation media showing the calcium deposits staining orange-red (black arrows) which indicates differentiation to osteoblasts. (G) Non treated wjMSCs stained with BCIP/NBT as control. (H) WjMSCs post exposure to osteoblast differentiation media showing the violet blue discolouration which denotes high alkaline phosphatase levels and indicates successful differentiation into osteoblasts. Scale bar 50 um.

3.2.3.3. Doubling time (DT)

The DT of wjMSCs is expressed as the mean of 4 experiments according to the equation

DT = duration X log (2)/ (log final concentration- log initial concentration)

DT=2.17 ds.

3.3. Human umbilical cord endothelial cells (HUVECs)

3.3.1. Introduction

Tissue perfusion is a mandatory requirement for viability of any tissue. Therefore, in the design of this patch endothelial cells were included to boost the patch with the necessary angiogenic pillars.

The umbilical cord has three blood vessels including two arteries and a vein. The vein is easy to identify with its bigger orifice and central position as compared with the smaller bilateral arteries. Further to easy identification of the umbilical vein it is also easy to cannulate in order to detach HUVECs.

HUVECs hold several advantages such as being convenient source of endothelial cells from a readily available source, easy to isolate and expand to achieve adequate volume for in-vivo applications as well as being autologous so no rejection concern. With literature review, it is quite obvious that HUVECs are a well-recognised prototype of endothelial cells and are used for long time to study endothelial cells properties and angiogenesis which built a wealth of knowledge and expertise regarding their behaviour and potential uses. Therefore, HUVECs were our endothelial cell phenotype of choice to include in the planned cardiac patch design.

3.3.2. Objectives

To isolate endothelial cells lining the umbilical vein and expand them in vitro to establish cell line. Then to characterise HUVECs via morphology under light microscope, Immunocytochemistry (ICC) and flow cytometry (FCM) to examine expression of characteristic proteins and surface markers. And to assess HUVECs proliferation via calculating their doubling time.

3.3.3. Results

3.3.3.1. Isolation and expansion of HUVECs

The first step was to identify the umbilical vein. The Wharton jelly holds three umbilical vessels, two arteries and one vein. The vein is characterised by central position, bigger lumen with outward protruding margins while both arteries have apparently small equal lumen with inward directed openings. Cord pieces used were in the range of 4-6 cm long.

HUVECs were isolated from the umbilical vein using enzymatic detachment as described earlier and cultured in EGM. Within 24 hours they appear in the flask as sparse colonies each has few cells then rapidly proliferate to achieve 90-100% confluence within 2-5 days (figure 3.6). Once confluent in the flask HUVECs could be passaged 1:4 almost every 3-5 days, up to passage 10.

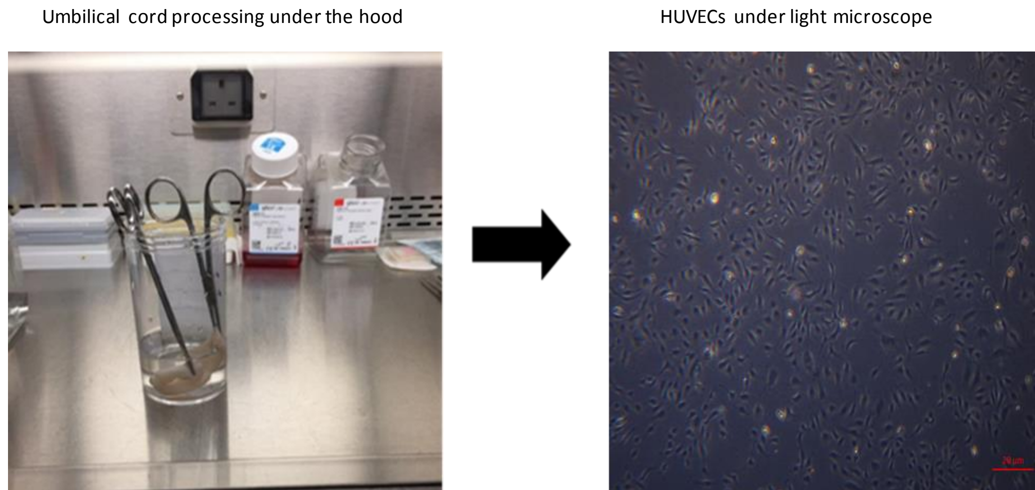

Figure 3-6. HUVECs extraction from umbilical cord.

(left) Piece of umbilical cord in warm PBS clamped both sides and collagenase IV injected into the umbilical vein under laminar flow hood. (Right) LM characteristic cobble stone picture of HUVECs extracted from the umbilical vein and cultured in flask, scale bar 20 um.

3.3.3.2. Characterisation of HUVECs

In order to confirm their endothelial nature, the cells were tested for tube formation, morphology and specific protein expression.

3.3.3.2.1. Morphology under light microscope and propensity for tube formation

Under inverted light microscope the cells appeared small with the characteristic cobble stone appearance (figure 3.6) which is quite typical for endothelial cells. The cells had the propensity to get end to end contact and form tube like extensions in culture (not shown) and when seeded on basement membrane matrix (Geltrex) they showed a characteristic network appearance (3.7).

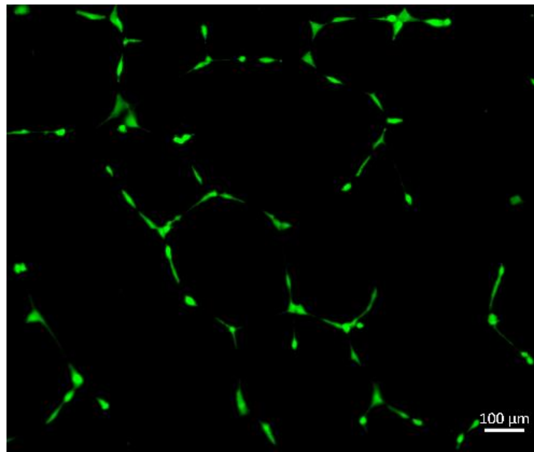

*Figure 3 7. Representative i*mage for HUVECs network formation.

HUVECs stained with Calcein (live imaging) under fluorescence microscope showing the characteristic contact end to end forming network like appearance. Scale bar 100 um.

3.3.3.2.2. Immunocytochemistry (ICC)

Further to the LM appearance, the cells were stained with antibodies against Isolectin B4, VEGF, VE-cadherin and vWF. Cells were examined under fluorescence microscopy for expression of this panel of proteins. The cells showed positive expression of all proteins in keeping with their nature as endothelial cells (figure 3.8).

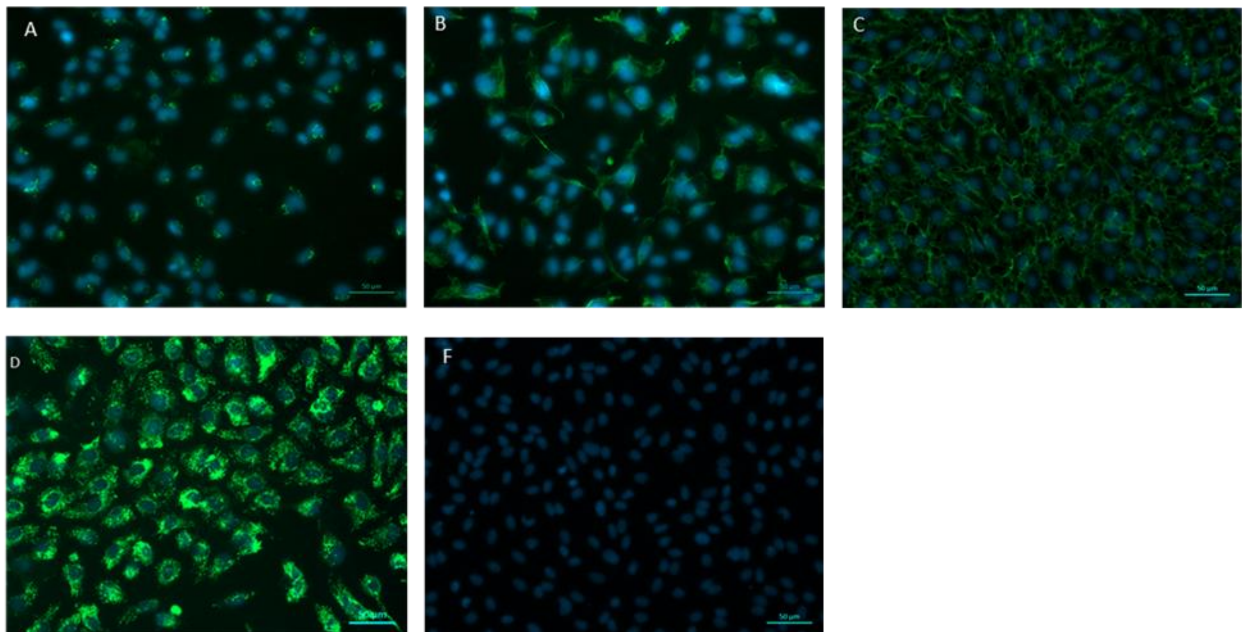

Figure 3-8. Representative ICC images of cells extracted from the umbilical vein to confirm their endothelial nature. Scale bar 5o um.

HUVECs under fluorescence microscope stained for (A) Isolectin B4. (B) VEGF. (C) VE-cadherin. (D) vWF.(F) Control. Nuclei counterstained with DAPI. All proteins are expressed in keeping with their endothelial nature.

3.3.3.2.3. Flow cytometry (FCM)

In addition, the cells were tested via FCM for expression of the endothelial cells surface markers CD31 and CD105.The cells were highly positive for both markers and the mean percent of cells 99.82% and 99.88% respectively which is consistent with their endothelial nature (figure 3.9 - 3.10).

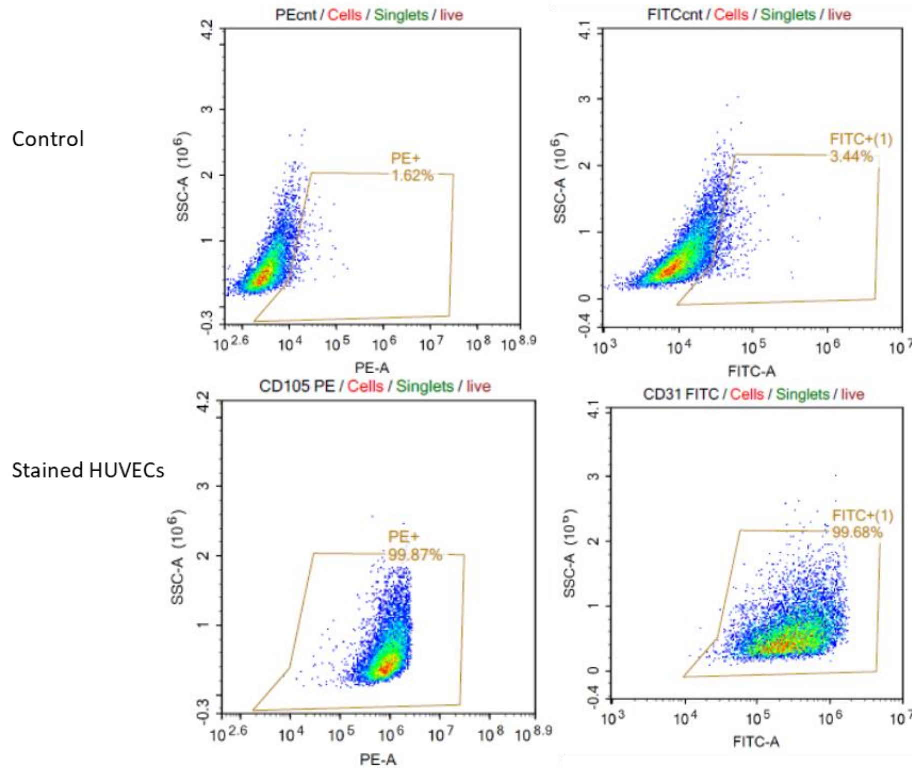

Figure 3 9. Representative image of flow cytometry results for surface markers expression on HUVECs.

Image demonstrates high expression of the endothelial markers CD105 and CD31 on HUVECs.

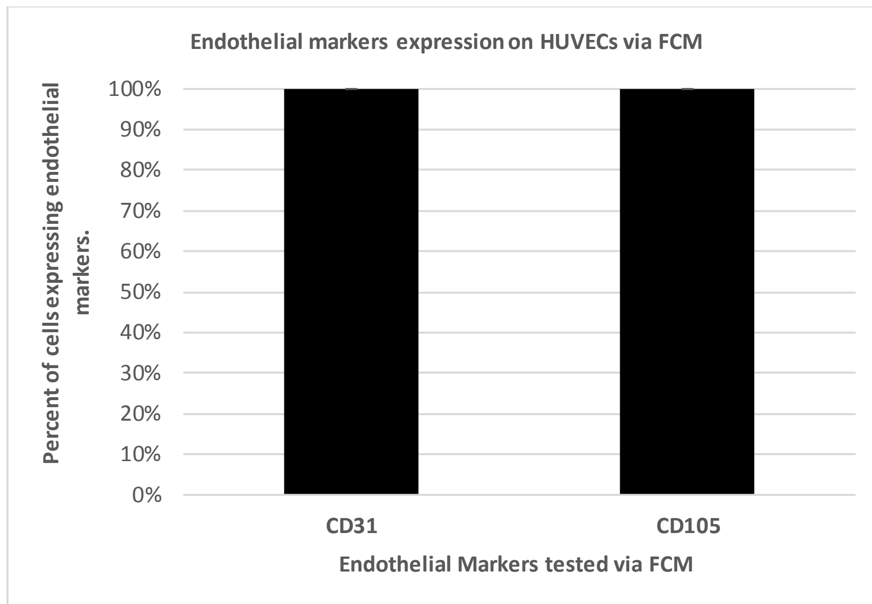

Figure 3 10. Graph to represent the percent of cells extracted from the umbilical vein expressing the endothelial markers. The graph represents the mean of 3 independent experiments and error bars for SD.

3.3.3.3. Doubling time (DT)

The doubling time for HUVECs is expressed as the mean of three independent experiments and calculated according to the equation:

DT= duration X log (2)/ (log final concentration- log initial concentration)

=2.35 ds.

3.4. Human cardiac Fibroblasts (HCFs)

3.4.1. Introduction

Fibroblasts are widespread phenotype in many tissues but there are variations between fibroblasts in different tissues to accommodate anatomical and physiological demands.

In the myocardium fibroblasts are the most common stromal cells (256). They are widely spread and are aligned in intimate contact with CMCs. They produce the ECM and share in electrical coupling with CMCs as well as working as mechanosensors to interact with mechanical stresses. Furthermore, the cardiac fibroblasts share some genetic set up with CMCs and so they are claimed to have potential to transdifferentiate to CMCs as well.

In case of cell loss such as during myocardial infarction the fibroblasts would dominate the area and differentiate to myofibroblasts to overproduce ECM and replace the damaged area with fibrous tissue which is a non-functioning area and may negatively impact the cardiac function but on the other hand it would guard against the more serious myocardial rupture. So, it is quite important phenotype that cannot be underestimated in any regenerative approach and therefore in the design of the patch cardiac fibroblasts were chosen to support the patch with this invaluable phenotype.

HCFs in this study were isolated from adult ventricular fibroblasts.

3.4.2. Objectives

To characterise HCFs via morphology as well as their expression of characteristic proteins. And to assess proliferation potential of HCFs and calculate their doubling time (DT).

3.4.3. Results

3.4.3.1. Characterisation of HCFs

Validation of CFs was based on the morphology and specific protein expression profile.

3.4.3.1.1. Morphology under light microscope

Shortly after culturing the cells in fibroblast growth medium (FGM) they showed characteristic appearance under light microscope with branching filamentous morphology in keeping with fibroblast nature (figure 3.11).

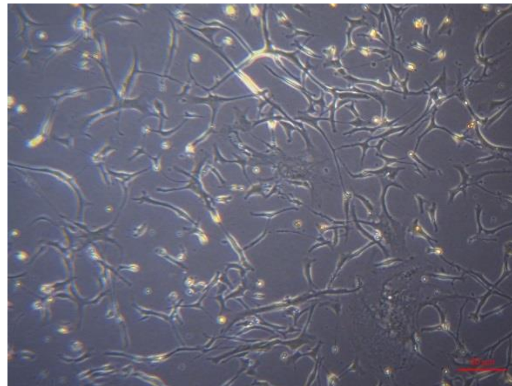

Figure 3 11. Representative image of cardiac fibroblasts under light microscope.

Cardiac fibroblasts showing the characteristic filament like branching morphology. Scale bar 20 um.

3.4.3.1.2. Immunocytochemistry (ICC)

Then the cells were examined via ICC for expression of a panel of proteins including Fibronectin, vimentin, DDR2, collagen I, collagen III and α-SMA. The cells showed positive expression of all these proteins in keeping with their nature as cardiac fibroblasts (figure 3.12).

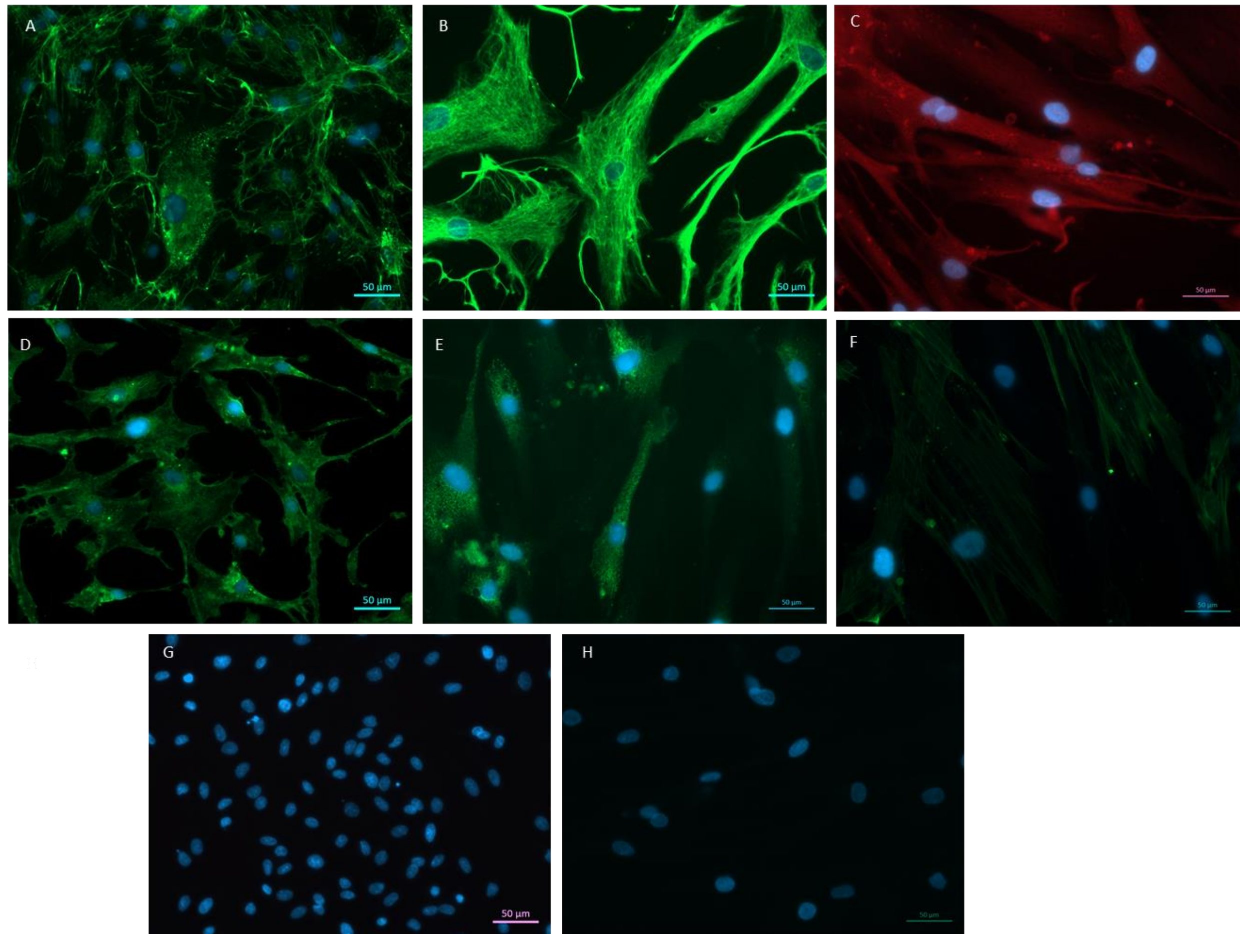

Figure 3 12. Representative images of HCFs ICC results.

ICC images to illustrate the expression of a panel of proteins by the cells which confirms their nature as HCFs. (A) Fibronectin. (B) Vimentin. (C) DDR2. (D) Collagen I. (E) Collagen III. (F) α-SMA. (G) HUVECs control for DDR2. (H) HCFs control. Nuclei are counterstained with DAPI. Scale bar 50 um.

3.4.3.2. Doubling time

Doubling time DT of the cardiac fibroblasts presented as the mean of 3 experiments, calculated according to the equation:

- duration X log (2)/ (log final concentration- log initial concentration)
- DT=2.34 ds. (mean of 3 experiments).

3.5. Discussion

3.5.1. Introduction

Congenital heart diseases are the most common congenital defects worldwide with considerable morbidity and mortality. The outcome dramatically improved after introduction of corrective surgery. Recently it is estimated that majority of children with CHD(s) would survive to adult life. This is expected to reflect on the next generations as patients with CHD(s) of a genetically driven aetiology would pass the genetic defect(s) to their children.

Corrective surgery started about 8-9 decades ago and gradually achieved great forward steps to improve care of CHD cases. Different biomaterials and approaches are used including auto, allo and xeno grafts for reconstruction of cardio-vascular defects. The grafts showed variable degrees of durability and anatomical success but still all available patches lack functionality and are hindered by their inherent no-growth nature. Therefore, still the ideal patch does not exist.

Ideally, a patch used for correction of a structural cardiac defect in an infant should have the necessary mechanical integrity as well as the elasticity to tolerate the load of surgery and pressure of the continuous contraction/relaxation cycles. In addition, it should integrate with native cardiac tissue, repopulate with myocardial cells to share in myocardial contractility, promote angiogenesis for proper perfusion to maintain its viability and to grow in parallel with the rest of heart to avoid repeated surgeries. This means that the ideal patch is indeed natural or near natural tissue which is far from our current position. However, tissue engineering offers a hope to accomplish some of these targets.

Our hypothesis is to utilise a biologic scaffold and seed it with relevant cells aiming to make use of the mechanical integrity of the scaffold in addition to the biologic properties of the cellular components. This would include physical engagement with the native myocardium, potential of trans-differentiation, paracrine effects as well as the proliferation potential.

Therefore, we aim to seed the biologic scaffold cormatrix with a triad of autologous cells including wjMSCs, HUVECs and HCFs. Each cell phenotype is meant to offer a pillar and support the viability of the patch to improve its chances for uptake, repopulation, vascularisation and provide it with the growth potential as well. In theory, through this approach we did supplement the patch with a diversity of relevant cells and we are moving forward and getting a step closer to the natural tissue and to the best of our knowledge this approach was not done before.

As a general rule of thumb, in any cellular therapy several factors should be considered including feasibility and safety of the approach, potential immunogenicity of cells, the carcinogenic potential, potential of migration and ectopic tissue formation, their in-vitro as well as in-vivo trans-differentiation potential and the biosafety of the culture medium as well (257).

As regard the safety of the approach of cells combination and transplant of non-native cardiac cells, there was a concern about arrhythmias induced by skeletal myoblast transplantation to the heart (258). However, the study of combination of MSCs and ECs progenitors for cardiac transplantation via intracoronary administration did not show any arrhythmogenic potential for up to 3 years follow up. Indeed, the participants in the study had some recorded ventricular arrhythmias before the cell therapy while after this therapy no arrhythmias were reported over 3 years of follow up and eventually showed

better left ventricle (LV) wall motion (259) which indicates that the approach of multicellular transplantation is still valid and reasonable to be experimented further.

So, the starting point was to isolate and characterise the triad of cells. Isolation of the wjMSCs and HUVECs was done in house while HCFs were purchased from PromoCell. Characterisation was based on approved criteria for each cell phenotype. The triad of cells did meet all criteria for characterisation which was promising to move forward in the project.

3.5.2. Wharton jelly mesenchymal stromal cells (wjMSCs)

The first cellular pillar is MSCs. After isolation of wjMSCs we did test them against criteria of the International society for cell therapy (ISCT) including their light microscope appearance, adherence to floor of plastic flask, pattern of expression of surface markers as well as their differentiation potential. The wjMSCs did satisfy all ISCT criteria which confirmed their MSCs nature.

MSCs are non-hematopoietic multipotent cells, present in almost all tissues (260) as well as some perinatal tissues (183). They reside in the perivascular niche to be released in response to tissue injury as a back-up to enhance regeneration (260).

The attention to MSCs goes back to the 1960-70s by the work of Friedenstein and colleagues on a subset of non-hematopoietic bone marrow cells and then started to gain attention in human in 1980s (261). This was followed by a surge in researchers' interest due to unmasking their favourable criteria and with time they were found to reside in other tissues and eventually nominated as one of the most common stromal cells in almost all tissues as well as perinatal tissues such as the amnion, placenta and umbilical cord which is our interest in this project.

Bone marrow is the most studied source of MSCs (BM-MSCs) however, isolation of cells is considered invasive procedure with low yield per aspirate and further purification steps are needed to establish pure cell line in addition to the negative effect of the donor's age on properties of BM-MSCs (262). On the other hand the umbilical cord is a readily available source, easy to handle and isolate MSCs in reasonable volume from the Wharton jelly in addition to the MSCs immune-modulatory properties which would allow for allo-transplant.

Human umbilical cord is a medical waste and making use of its derivatives is a good investment. It holds several advantages such as circumventing any ethical concerns, no immune rejection issue as well as being abundant source of cells with reasonable proliferation and differentiation potential and also easy to handle. In addition, wjMSCs represent a realistic non-controversial source of stem cells suitable for autologous cell therapy and banking for allogeneic transplantation due to their immune modulatory effects (263).

The acronym (MSCs) in the literature points to mesenchymal *stromal* cells and mesenchymal *stem* cells, however, it is more prudent to adopt the stromal nomination given the heterogeneity nature of these cells and not all of them meet the stemness criteria of self-renewal and multipotency. Therefore, as a precautionary step it is recommended for accuracy purposes to specify the origin of cells as well as the

species, given the fact that the surface markers as well as their differentiation potential would vary significantly between sources and species (261, 264).

Indeed, the ISCT adopted this approach and referred to the MSCs as multipotent mesenchymal stromal cells and the committee did stress on reserving the term stem cells to cells that would meet the stemness criteria (110, 226, 264).

Several methods were reported for wjMSCs isolation such as enzymatic digestion using enzymes like collagenase, hyaluronidase and trypsin in different combinations to release the cells from their Wharton jelly mesh (265). However, the enzymatic method is time consuming and more costly, so it is less convenient for processing large number of samples. Therefore, In this study the explant method was adopted for extraction of wjMSCs as it is easy to use with high yield and it was reported to offer higher proliferation rate (266). Contrary to what other groups used to do for explant method as they attach small pieces of Wharton jelly to floor of plastic dish, in this study the Wharton jelly pieces were just suspended in DMEM supplemented with FBS 10% and P/S 1% without attachment to flask floor and still the cells were able to outgrow, proliferate and further expanded up to passage 10 and characterised using the conventional criteria.

All parts of the umbilical cord were considered good source of MSCs including near mother, central/middle part and near infant part (267) and in our study any part of the cord was used without selectively looking for certain part.

Also, the disinfection via immersion in ethanol 70% for 30 seconds was trialled as it was reported by another group (268), however, just several washes in PBS with 1% P/S were enough with no notable difference in incidence of contamination (own observation).

As aforementioned, wjMSCs characterisation was based on criteria approved by ISCT for defining MSCs. These criteria were published in 2006 to standardize defining MSCs and facilitate comparing results between different studies (226). These criteria include adherence to floor of plastic flasks under normal culture conditions, being highly positive for surface markers CD105, CD90 and CD73 while negative for CD45, CD34, CD19, CD14 and HLA-DR in addition to their ability to differentiate into osteocytes, adipocytes and chondrocytes.

Our results with cells outgrowing from the Wharton jelly pieces were in keeping with these criteria and consistent with results of other research groups (263). The cells demonstrated adherence to floor of plastic flask and were highly positive for CD105, CD90 and CD73 while very low expression of CD45, CD34, CD19, CD14 and HLA-DR using FCM. Also, their multi-potency was proved by positive differentiation into chondrocytes, adipocytes and osteoblasts which confirms their nature as MSCs and prepares going forward with the project.

During the journey of this project and after processing more than 20 umbilical cords it was notable that the cells are quite generous and offer very high proliferation rate at early passages, mainly passage 1 (P1) when the doubling time (DT) was about 19 hours. Also, at this phase the cells had small size with high nucleus to cytoplasm ratio. With later passages, the cells get larger size with lower nucleus to cytoplasm ratio, slow down their proliferation rate and the DT increases to days. This was not universal between all wjMSCs cell lines but it was notable in at least some of them (own observation). This would indicate some heterogeneity of these cells and needs further studies to figure out the underlying

mechanisms and the most reliable criteria for best therapeutic outcome in case of planned allogenic transplantation. This is quite important due to the surge of MSCs utilisation in different clinical settings and the variable outcome reported that could be in part due to the non-standardised cells used and missing proven potency/regeneration criteria (269).

These observations are consistent with other reports such as the report of Rebekah et al about MSCs potency (270).They reported variable proliferation rates of bone marrow derived MSCs between different donors and divided MSCs into high-growth capacity and low-growth capacity. The difference between the two types of growth potential was not based on ISCT criteria. Indeed, both groups were comparable as regard the ISCT criteria, however, the high-growth capacity MSCs were of smaller size with longer telomere and showed higher expression of stromal precursor antigen-1 (STRO-1) and platelet derived growth factor receptor-α (PDGFR-α) (269).

So, despite the role of the ISCT criteria to establish standardised validation criteria for MSCs but still the criteria are not able to pinpoint the most promising cell lines for therapeutic applications. This could be part of the explanation about variability in outcome between studies. For instance, Qian et al reported successful differentiation of wjMSCs to cardiomyocytes using 5-azacitidine (271, 272) but Rendon et al did challenge these results and demonstrated that 5-azacitidine could induce differentiation of bone marrow derived MSCs into CMCs but at low yield while umbilical cord derived MSCs failed to show any cardiomyocyte differentiation (273).

In addition, ISCT criteria cannot be used to define "the functional capacity" of MSCs. Indeed, the therapeutic efficacy of MSCs did fluctuate between suboptimal to truly significant (260). This significant variability is most probably reflection of the heterogeneous nature of MSCs as regard their multipotency and the paracrine profile which were not predictable based on the ISCT criteria. Indeed, all studied cells did meet the same defining criteria (269) but the outcome was quite variable.

Also, there was notable discrepancy in results between animal studies and clinical trials (260). This could be in part due to the inherent limitations of animal models due to the differences in the genetic set up of animals in addition to the immune status. But also could be partly explained by the variability in paracrine potential between MSCs of different origins including tissue and species.

In addition, clinical applications would require in-vitro expansion to achieve the required volume of cells for clinical utilisation which was estimated in the range of 10^6 cells/kg (274). This means long time in culture with potential down regulation of chemokine receptors expression, less homing potential, less differentiation capacity and higher cell death rate (260).

So, the ISCT criteria should be utilised as the minimal identification criteria (231) but still need subdivision to cover the functional/therapeutic potential of MSCs. This is due to the fact that the ISCT criteria ignore several crucial elements such as the biological background, clonogenicity of the cells, differentiation potential and therefore their applied therapeutic values in addition to other fine differences such as the genomic differences and surface epitopes (275).

These differences were also recognised between MSCs from different sources such as bone marrow and adipose tissue and even between MSCs from same tissue but different locations (275). Indeed, this is consistent with our observation of the MSCs heterogeneity and also to emphasise on the fact that MSCs are not same population everywhere but rather there are appreciable differences that would reflect on

their therapeutic applicability. For instance, adipose tissue derived MSCs (Ad-MSCs) are more collagen producers including collagens I,II and III while bone marrow derived MSCs (BM-MSCs) hold higher angiogenic and anti-inflammatory profile (231).

In the study of Leor et al. they did compare BM-MSCs with MSCs from the right atrium, pericardial and epicardial fat. The surface markers expression showed interesting finding as the cardiac marker c-kit expression was only found in the right atrium derived MSCs which could reflect commitment to their hosting tissue (275).

Also, there was appreciable difference in the proliferation rate, paracrine profile such as HGF, bFGF and PDGF in addition to differences in other immune modulating factors such as TNF-a, IL-6 and IL-10 as well as the macrophage polarization potential. Also, the pro-inflammatory MSCs1 did enhance inflammatory changes and negatively affected the LV remodeling while MSCs2 had better outcome (275).

This indicates the variability in angiogenic and immune modulatory effects in addition to different in-vivo angiogenic and remodeling potential among these MSCs based only on the parent tissue (275).

Furthermore, MSCs being quite abundant type of stromal cells in almost all tissues, they were linked to the embryonic development of the hosting tissues and this could reflect on their antigenic expressions, differentiation potential and regenerative capacity (276).

Indeed, even at local level of the hosting tissue, MSCs would vary in response to various insults and their secretome also would display different profiles and variable immune modulating effects as well (276).

Nevertheless, despite these facts, because our aim here is to seed the cormatrix with autologous cells so it was not within the scope of this study to assess the potency of every cell line to pick up the most promising ones. But this sort of potency-based selection criteria still valid for cell banking and allogenic use rather than autologous transplantation purposes.

Within this heterogeneity of MSCs, a portion of them hold the advantage of having the multipotent potential (277) as they can differentiate into chondrocytes, adipocytes, osteoblasts, smooth muscle cells (278), neurones (279) and cardiomyocytes (280). This multipotency supports their use for tissue engineering purposes as they offer flexibility of trans-differentiation and could support cardiovascular regeneration via differentiating into cardiomyocytes as well as endothelial cells (281).

WjMSCs hold another positive criterion which is being of low tumorigenicity potential as compared with embryonic stem cells (ESCs) and induced pluripotent stem cells (IPSCs) which hold significant carcinogenic risk (282). Actually, MSCs possess anti-neoplastic activity and their conditioned media could repress some neoplastic cells such as ovarian and breast cancer cells (263). In addition, there is good body of evidence for MSCs favourable cardioprotective potential mainly via paracrine effects as well as their angiogenic, anti-inflammatory, anti-fibrotic (283, 284), anti-oxidative (285) and anti-apoptotic effects (233, 277, 286). In fact, MSCs moved already from bench side experiments to bed side clinical trials. As of December 2019 there were total of 1017 registered clinical trials on the *ClinicalTrials.gov* utilising MSCs while before 2000 there were only three registered trials utilising MSCs. This clearly reflects the current interest in MSCs properties and the promising results of these cells in the field of regenerative medicine.

3.5.3. Human umbilical vein endothelial cells (HUVECs)

The second pillar of the cellular triad is the HUVECs. Isolation of HUVECs was done in-house as described earlier. Characterisation of HUVECs was based on characteristic light microscope appearance and characteristic surface markers as well as protein expression pattern. Indeed, HUVECs met all characteristics and confirmed their endothelial cell nature.

In theory, HUVECs would provide the patch with the basic units for angiogenesis to support tissue perfusion in-vivo as a prerequisite for survival, proliferation and functionality and to meet the tissue metabolic demands via adequate blood supply.

HUVECs are considered a good model for study of ECs in vitro as well as for in-vivo applications (287). This is due to several factors such as being easy to isolate and handle as well as their good proliferation potential and therefore could establish a good volume of cells for experimental as well as therapeutic applications. Further to its in-vitro and preclinical studies, HUVECs also moved to clinical trials phase and as of December 2019 there were 13 clinical trials registered on *ClinicalTrials.gov* using HUVECs for clinical experimentation. Also, on PubMed by end of 2019 there were 18,520 papers dealing with HUVECs going back to 1986.

Adequate tissue perfusion is critically dependent on capillary network within a maximum distance of few hundred micrometres. So, in order to enhance the patch sustainability in-vivo we need to support angiogenesis. Indeed, several models have been constructed to study the process of angiogenesis and HUVECs were the prototype of choice in many of these studies (288).

Angiogenesis is the formation of new vessels from pre-existing vessels through outgrowth of endothelial cells (289). It is quite crucial process under physiologic conditions such as normal development as well as pathologic conditions such as tumorigenesis (290).
There are two types of angiogenesis, sprouting and intussusceptive.
Sprouting angiogenesis consists of outgrowing endothelial cells that invade new areas and migrate under effect of angio-tropism of angiogenic factors. The process starts when the parenchymal cells sense oxygen requirement and secrete angiogenic factors. Then the capillary basement membrane would degrade and ECs proliferate and migrate with new vessel lumen formation followed by recruitment of pericytes to stabilise the new vessel wall (291).
While intussusceptive angiogenesis takes place when the vessel wall extends to split the lumen and form new vessels from existing vessel. It is more prominent in-utero as a fast and efficient process but still could take place during adult life (290).
Some investigators put criteria for the source of HUVECs such as certain gestational age, weight and delivery method (287). This approach was an attempt to standardise the results between different studies as some papers reported variability of HUVECs behaviour secondary to some factors such as medical background of the mother and smoking status (292, 293). However, due to the fact that our study is aiming to seed a biologic scaffold with HUVECs from the infant’s umbilical cord so we did not restrict the source of umbilical cord with selection criteria apart from the following exclusion criteria (i)termination of pregnancy, (ii)new-borns with significant perinatal asphyxia, (iii)new-borns with major malformation, (iv)not capable to understand information sheet and consent process and (V)any abnormal pregnancy at the 36-week visit. So, we did not specify based on maternal age or smoking status of the mother.

HUVECs isolation here followed generally the same principle as other groups. Started with cannulation of the umbilical vein, wash to get rid of blood followed by enzymatic detachment of ECs (294). However, collagenase IV was used rather than collagenase I or trypsin. In fact, it was quite effective with no concerns as regard cell characterisation or subsequent expansion of HUVECs. Also, HUVECs were cultured in regular uncoated flasks without any specific handling such as adding fibronectin (294) and it was quite successful to proliferate and expand HUVECs up to passage 10.

Characterisation of HUVECs here was based on a panel of criteria including cobble stone morphology under LM which is typical for ECs, network formation in culture as well as when seeded on basement membrane matrix as the cells formed clear network which is another feature for endothelial cells that reflects their angiogenic potential (295). Indeed, this characteristic is used for functional assessment of HUVECs under different experimental conditions (287, 296, 297).

This is in addition to characteristic expression of a panel of proteins that are collectively characteristic for endothelial cells (298, 299) including Isolectin B4, VEGF, vWF and VE-cadherin.

These proteins were tested via ICC and the cells showed highly positive expression of these proteins in addition to the highly positive expression of CD31 and CD105 via FCM which confirms their endothelial nature. Actually CD31 - platelet derived cell adhesion molecule (PECAM)- is considered one of the earliest markers for endothelial cells during embryonic life (300) and CD105 – Endoglin - is also mainly expressed in ECs (301) in addition to high level of expression in MSCs however, differentiation is quite feasible by morphologic features as well as other characteristics for MSCs.

As mentioned earlier, we did not specify certain part of the cord. Both ends of the cord and central part as well were used with no preference. Also, did not specifically look for particular sex for the cells, appreciating the sex difference that was reported in the study of Roberta et al. (287) as they demonstrated sex differences in HUVECs such as significantly higher proliferation rate of HUVECs from females than males, in addition to some ultra-structural differences between male and female HUVECs in terms of abundance of pinocytic vacuoles and their distribution. Also, significant differences in H2O2 expression and NOS3 while Akt and mToR expression was comparable between both male and female cells and also no viability difference (287). Despite these differences, we did not consider them as relevant in our project because the intention is to utilise HUVECs from self-cord and therefore, no indication to subdivide our cells based on sex differences.

3.5.4. Human cardiac fibroblasts (HCFs)

The third cellular pillar is the cardiac fibroblasts. HCFs were purchased from PromoCell. Characterisation was based on light microscope appearance in addition to a panel of proteins that are characteristically expressed by cardiac fibroblasts. HCFs did express all characteristic panel of proteins.

Previously, fibroblasts were considered as "*biological glue*" and did not get enough attention in the active research. Recently this view was dramatically changed as fibroblasts were realised to hold key roles in both physiological and pathological situations alike (302). CFs reside in the cardiac valves and septa as well as the myocardial interstitium and have close contact and cross talk with CMCs and ECs (302). Therefore, they play invaluable structural as well as functional role in the myocardium and can influence CMCs activity via direct and paracrine effects (303).

In this project we considered CFs as one of the pillars to optimise the cellular pool of the patch. CFs from adult human ventricle were used at passages 3-8. In early passages the cells showed fast proliferation rate with the characteristic filament like morphology. At later passages that is usually beyond passage 10 however, sometimes starts as early as passage 6, they started to show spindle like projections with large cytoplasm to nucleus ratio and notably slower proliferation rate.

Previously, fibroblasts were believed to be uniform in all tissues. However, recently phenotypic and functional heterogeneity were identified between different tissues which reflects their dynamicity to support specific functions in their hosting tissues (13, 27). In the study of Astrid et al they did analyse the proteomic profile of fibroblasts from skin, lung and bone marrow. The results showed characteristic tissue based proteomic profile and this also extended to the inflammatory response as well as specific tissue related tumours (304).

Furthermore, fibroblasts are involved in the inflammation process as they recruit immune cells to the injury area via secreting cytokines and also mediate clearance of remnant immune cells. In fact, this is a balanced process and if interrupted such as when fibroblasts maintain immune cells or inhibit their apoptosis this would lead to switching acute inflammation to chronic phase (304).

In the myocardium, fibroblasts were considered the most common phenotype among cardiac cells (27). This concept was recently challenged and CFs were found to be over-numbered by endothelial cells (19). Indeed, CFs represent a dynamic cellular pool and are indispensable for integrity and functionality of the myocardium as they hold several crucial roles including their contribution to the mechanical, chemical and electrical properties of the myocardium (16).

On the other hand, the distribution of CFs in different cardiac areas is quite heterogeneous. This is due to the fact that they are influenced by the anatomical and physiological needs (13). This reflects the nature of CFs as quite active/inter-active cells and normal myocardial function is critically dependent on proper interaction of fibroblasts with other cells particularly the CMCs (305).

As CFs represent a dynamic pole of cardiac cells, their number would increase after birth with subsequent increase of ECM secretion in a crucial step for the postnatal heart to accommodate changes in blood pressure and maintain adequate circulation (306).

HCFs mechanical rule is quite invaluable through secretion of the extracellular matrix (ECM) which organises the architecture of the cardiac tissue (29) and this is a lifelong role. In addition, CFs would maintain integrity of ECM via a continuous build and degradation processes mastered by the CFs via secreting collagens, MMPs and TIMPs (305). Furthermore, CFs form highly organised network with CMCs within the ECM. So, the mechanical forces will be transmitted to fibroblasts and CMCs through the ECM with subsequent organised cross talk to accommodate different stresses (14, 20).

Under physiologic conditions cardiac fibroblasts are organised in layers and oriented parallel to the muscle fibres to maintain intercellular connections and maintain homeostasis of ECM via balanced secretion of factors that build up or degrade ECM such as MMPs and TIMP in addition to cytokines and growth factors (307). While during pathologic conditions fibroblasts would be the main players in the remodelling process as they differentiate into myofibroblasts which are more readily proliferating and more active phenotype to lay more ECM and maintain myocardial wall integrity (308).

CFs are non-excitable cells however, they can adjust electrophysiologic response of CMCs via electrical coupling with cardiomyocytes (309) via junctions such as cadherins and connexins. Also, CFs secrete pro-inflammatory cytokines such as TFN-α, IL-1 and IL-6 in response to stress which means quite focal interaction and modification at the level of the micro-environment (14, 16). These cytokines act at paracrine and autocrine scales. This demonstrates the CFs anatomical, physiological and immune modulatory function as well.

In addition, analysis of CFs genomic profile as compared with non-cardiac fibroblasts revealed significant cardiac specific genes including genes that underpin some CHDs such as GATA4 and TBx20. This would reflect the cardiac identity of CFs (302) as well as their potential role in the pathogenesis of some CHDs.

Therefore, inclusion of CFs in the design of a patch for corrective cardiac surgery is considered the most relevant type of fibroblasts that could optimise the cellular pool.

There are many markers for fibroblast identification but specificity is limited (307) due to the wide heterogeneity of fibroblasts in different tissues. Characterisation would start from the appearance as they hold spindle filamentous shape, adhere to floor of plastic flasks and express the mesenchymal marker vimentin (256). In addition, cardiac fibroblasts could be further characterised based on ECM proteins that indicate their synthetic capacity as demonstrated by Howard et al in their seminal study of fibroblasts that did show clear spatiotemporal as well as functional identity of fibroblasts including their tissue specific identity such as specific ECM protein synthesis and regulation in a site specific pattern that can be considered as tissue signature (256).

So, characterisation of HCFs here was based on expression of the characteristic markers vimentin, fibronectin, DDR2, collagen I, collagen III and α-SMA (34, 307, 310, 311).

Vimentin is an intermediate filament protein that was considered a reliable marker for HCFs (307). However, vimentin is also detected in other cells such as endothelial cells which was evident during this project work but differentiation was possible based on cell morphology.

Also, the collagen receptor Discoidin Domain Receptor-2 (DDR2) is a tyrosine kinase receptor and is another specific marker for cardiac fibroblasts (13). In fact, its specificity for CFs was challenged by some investigators who reported its expression in other cells such as fibrocytes from bone marrow and hepatic stellate cells however, among cardiac cells, DDR2 expression is limited to CFs (16, 312, 313). Also, DDR2 is involved in CFs proliferation, migration as well as differentiation (14).

α-SMA was used as a marker for HCFs. Indeed, α-SMA is a marker of myofibroblasts which are differentiated fibroblasts (314) and its expression during this work was prominently higher at late passages.

Previously, the fibroblast specific protein (FSP) was considered as specific marker but this was challenged when it was demonstrated that it is also expressed by other cell phenotypes such as leucocytes and some cancerous cells as well (13).

CD90 expression in HCFs was also examined using FCM (not shown) as one of the CFs markers (310). It was expressed in about 40% of HCFs and with later passages the level of expression was going down (15% at passage 9). This gradual step down of expression of this marker indicates that it was not specific enough to include in the characterisation panel.

In this project HCFs isolated from adult ventricle were used. Planning for the future, HCFs would be isolated from cardiac tissue of children with CHD(s) that undergo cardiac surgery. During surgery a small biopsy can be taken or just part of the left over tissue can also be used.

3.5.5. Conclusion

So, in the design of this patch the umbilical cord which is a medical waste could be used to isolate two cellular pillars to seed the patch. Obviously this is quite convenient with no ethical concerns. In addition, the cells are autologous which rules out any immune rejection concerns. Also, cardiac fibroblasts were included as a dynamic cardiac cell phenotype to support and enhance the patch functional and mechanical properties.

It is noteworthy that the three cell phenotypes have very close doubling times which is promising as it indicates comparative proliferation potential and unlikely for one cell line to overpopulate the others.

Supporting the patch with this triad of cells is expected to offer reasonable growth potential in parallel with the overall heart growth as well as the necessary pillars for angiogenesis and extracellular matrix synthesis.

In the next chapters we will analyse the four components of the patch including the three cell phenotypes and the cormatrix to find out their potential interaction and discuss the results relative to the other laboratories work.

Due to the sudden unexpected shut of the University I was advised to present the available data. Therefore, I have biologic replicates n=3 in wjMSCs experiments while experimental replicates of 4 but biologic replicates of 2 in HUVECs and experimental replicates of 4 with biologic number n=1 in HCFs experiments.

So, some of the data are more of indicative rather than finalised conclusions.

CHAPTER IV

Effect of CdM and CHIR99021 on WjMSCs survival, proliferation and paracrine profile.

4.1. Introduction

As aforementioned, this project aims to engineer a patch for corrective cardiac surgery with better properties to support regeneration and offer growth potential as well. Therefore, we hypothesised that we can accomplish this task via seeding the biologic scaffold cormatrix with wjMSCs, HUVECs and HCFs. This combination would benefit from the mechanical integrity, pliability and biocompatibility of coramtrix and would utilise the cellular properties for growth, ECM production and angiogensis in addition to their paracrine effects.

Cell therapy including MSCs represents a potential therapeutic modality in cardiovascular medicine (315). MSCs did demonstrate some positive effects when transplanted into ischemic myocardium as evidenced by enhanced myogenesis and angiogenesis (281). However, MSCs have short lifetime in-vivo which hindered its progress in clinical trials. Also, this phenomenon is not well understood.

In our study to tissue engineer a patch for reconstructing the pulmonary artery. We did seed cormatrix with MSCs and non-seeded cormatrix was used as control. The cellularised patch performed significantly better with superior results of all pre-set assessment parameters (316). In this study we used MSCs from male donor to seed cormatrix and implanted in a female recepient so that we can track the cells using the Y chromosome as a marker. MSCs were washed out and could not be detected in the graft after just few weeks. Nonethless, still there was good evidence of superior regenerative and functional results in the cellularised patch compared to the acellular one which is against the hypothesis that cellular engagement or trans-differentiation underpin the regenerative effects.

This was consistent with reports from other groups with same observation that MSCs disappear from the scene shortly after transplantation despite better regenerative results (281). Indeed, this was reflected on clinical applications as MSCs survival post MI was quite limited which is considered as a limiting factor for usage of MSCs in this application (317).

Furthermore, MSCs conditioned media did give comparable results to the MSCs. Even some investigators reported that MSCs had similar effects in trans-well models which rules out any physical contact of the cells.

Taking these observations together, we can conclude that the paracrine effects are the main players of the MSCs regenerative and cardioprotective effects. However, the underlying molecular mechanisms for these effects are not yet elucidated (316). Indeed, it was not analysed in depth due to the complex nature of the in-vivo environment.

In addition, given the fact that this combination of cells is not natural as these cells do not exist in this combination in a native tissue, therefore, we do not know at the molecular level how they will behave and how this combination would affect their survival, proliferation and regenerative potential.

Also, no guarantee about balanced growth and there is a theoretical concern about one cell phenotype particularly fibroblasts to overpopulate the other two phenotypes and end up with fibrotic patch if fibroblasts dominate the scene. Obviously, this may lead to deviating from balanced multi-cellularised environment with subsequent loss of the targeted functionality and proliferative potential of the patch.

The interaction of the cells at the molecular level was analysed using conditioned media (CdM) as a convenient method. CdM from co-culture of every two cell phenotypes was used to assess their effect on the third one. In other words, this triad of cells was dissected and the effect of every two cells on the

third one was analysed. So, we can understand the mutual effects and predict their in-vivo behaviour. Keeping in mind the complexity of the in vivo environment that can not be mimicked in vitro.

Assessment done to examine the effects of CdM at several domains to ensure and optimise the patch properties in-vitro before proceed to in-vivo experiments.

Table 4-7. Areas of assessment of CdM effect on cells and its rationale

Assessment area	**Rationale**
Anti-apoptosis/prosurvival factors	To examine the effect of this combination on scells' survival.
ECM* proteins	To assess if any extra secretion of ECM that would raise concern of excessive fibrotic changes of the patch.
Paracrine/angiogenic factors	To examine the potential effect of this combination of cells on angiogenesis.
Adhesion/communication factors	To examine the effect on these factors in view of the fundamental role of intercellular communication for functionality of tissues.
Cell proliferation	To assess if any potential for one cell phenotype to overpopulate the patch.
Cell death/apoptosis	To ensure cells' viability is well supported and therefore the patch cellularity would be maintained.

*ECM: extra-cellular matrix.

4.2. Objectives

To analyse the possible reactions of wjMSCs when combined with HUVECs and HCFs. The CdM of HUVECs and HCFs co-culture was used and its effects were assessed as follows.

Table 4-8. Assessment of CdM effect on cells.

Assessment domain	Assessed factors	Assessment level	Technique used
Antiapoptosis/prosurvival	AKT BCl-2 NRG1	-Protien expression -mRNA level (apart from BCl-2)	ICC* (using image j) qPCR
ECM** proteins	Collagen I Collagen III Fibronectin	-Protien expression -mRNA level	ICC (using image j) qPCR
Paracrine/angiogenic factors	Angiopoietin bFGF HGF VEGF MCP-1 SDf-1	-Protien expression -mRNA level (apart from MCP-1 and SDf-1)	ICC (using image j) qPCR
Adhesion/communication factors	B-catenin Endoglin N-cadherin Connexin-43	Protien expression mRNA level	ICC (using image j) qPCR
Cell viability	Caspase/Draq7	Apoptosis and cell death	FCM***
Cell proliferation potential		BrdU assay	ELISA

*ICC: immune-cytochemistry.

**ECM: extracellular matrix

***FCM: flow cytometry.

4.3. Materials and Methods

4.3.1. Conditioned media (CdM) preparation

CdM was prepared from co-culture of HUVECs with HCFs. Cells from each phenotype were plated in endothelial growth media (EGM) at about $5x10^5$ in T25 or $1x10^6$ in T75 flasks and kept in humidified incubator at 37 ^oC with 5% Co_2.

After about 48 hours 70-80% confluence was achieved. Media was taken out and cells were washed with prewarmed PBS then addedd plain EGM (serum and growth factors free) and kept in incubator under same conditions for 24-48 hours. Then media was aspirated and filtered through 0.2 um filter for immediate use or kept at-20 ^oC for use within two weeks or at -80 ^oC for longer storage.

4.3.2. Culture in CdM

Before induction, the CdM was thawed in water bath at 37 ^oC then filtered through 0.2 um filters and mixed with plain EGM (serum and growth factors free) at 1:1 ratio for immediate use.

Media aspirated and cells were washed with prewarmed PBS then the prepared CdM was added and kept in humidified incubator at 37 ^oC with 5% Co_2 . WjMSCs were kept in CdM for 48-72 hours before further analysis.

Due to the fact that serum is a good stimulus for cell growth and collagen deposition and contains several factors that can influence the cells' reaction such as ANG and catecholamines (318). In order to avoid the confounding effects of serum and other growth factors, plain media were used as the control media. So, wjMSCs cultured in plain EGM (serum and growth factor free) were used as control.

4.3.3. Images and analysis of ICC

After culturing cells in the CdM, cells were fixed and stained (as described earlier). At the start images were taken in-house using the Zeiss fluorescence microscope. It was very time consuming. Hence I opted to use an automated system. So, all images analysed here are captured using incucyte system in Wolfson imaging unit in the biomedical school, Bristol University.

Incucyte is an automated system that captures 9 images per well of a 96 well plate. Quantification of protein expression was done in terms of mean integrated density (ID) using image-J software.

4.3.4. Statistical analysis

T-test was used for quantitative analysis.

All data described in terms of mean +/-SEM and difference was considered significant when $p <0.05$.

Afterwards, will refer to the protein expression under CdM by (**Protein-CdM**) and the control will be (**Protein-cnt**). The protein expression was assessed in terms of mean integrated density and was quantified relative to the control +/-SEM.

So, protein expression in the control cells (basal level) was considered the unit. Then protein expression in the treated cells was quantified relative to the basal level in the control.

One way analysis of variance (ANOVA) with post hoc Tukey test was used where appropriate for three groups analysis.

4.3.5. Apoptosis and cell death assays

For assessment of cell apoptosis and death Caspase 3/7 (from ThermoFischer) was used. The kit contains caspase 3/7 to detect apoptotic cells and Sytox to detect dead cells. However, during the FCM experiments there was overlap between the two assays that hindered the ability to differentiate between the two events. Therefore, we did use caspase 3/7 to detect apoptotic cells ad DRaq7 (from Biostatus) to detect the dead cells.

The manufacturer's recommendatons were followed. After cells collected from the flask they were washed in PBS with 0.5% FBS then caspase was added at 1 ul/ml then kept for about 30 minutes in incubator or 45 minutes at RT. Shortly before running the FCM experiment DRaq7 was added at 0.5 ul/200 ul and the assessment done using incucyte FCM machine.

4.4. Results

The protein expression was assessed using ImageJ software and expressed in terms of mean integrated density (ID). We got nine images per well. The mean ID of the nine images/well was calculated and taken forward for statistical analysis relative to the control.

Unless otherwise specified, all experiments of wjMSCs are done with 3 independent cell lines.

4.4.1. CdM effect on WjMSCs

In order to assess the effect of the combination of HUVECs & HCFs on wjMSCs, conditioned media (CdM) from co-culture of HUVECs/HCFS was used. Expression of pro-survival and anti-apoptosis factors, ECM proteins expression, paracrine/angiogenic factors, cell adhesion/communication factors in addition to the cell survival and proliferation potential were assessed.

4.4.1.1. CdM enhances WjMSCs expression of Akt but non-significant effect on BCl-2 and NRG1

Here we did attempt to examine the effect of CdM on expression of the survival promoting factors Akt,BCl-2 and NRG1 in wjMSCs. So, wjMSCs were cultured in CdM for 48-72 hours and wjMSCs cultured in plain media for same duration were used as control. Cells were washed, fixed and stained for the Akt, BCl-2 and NRG1 and images were captured using the incucyte system (as described earlier).Protein expression was assessed using imageJ software, expressed in terms of mean integrated density (ID).

Akt is a serine/threonine kinase that acts as a common point of action for survival factors both upstream and downstream. It regulates a plethora of cellular metabolic activities in addition to its survival promoting effect (319). So, it acts as a cytoprotective factor with anti-apoptosis effects and is considered as one of MSCs paracrine factors (233).

Akt overexpression in MSCs significantly enhanced recovery of cardiac function and reduced size of infarct area when transplanted to myocardial infarction in porcine model and also enhanced CMCs viability after cryoinjury (320). So, we did attempt to examine the effect of CdM of HUVECs/HCFs co-culture as this would be expected to reflect on viability of cells on the patch. Also, expected to extend to potential in-vivo support to CMCs.

WjMSCs expression of Akt-CdM (M= 1.02,SEM= 0.01) relative to the control Akt-cnt was consistently higher and statistically significant, p=.018.

BCl2 is another antiapoptotic factor and one of the MSCs paracrine factors that enhances survival post transplantation in myocardial infarction model (233, 236). Expression of BCl2-CdM (M=1.102,SEM=0.12) relative to the control Bcl-2-cnt was statistically comparable, p=0.45.

NRG1-ERBB4 is a signal pathway that could activate cardiomyocytes to exit their locked- in status and resume mitosis and proliferation potential (317). NRG1-CdM (M=1.01,SEM= 0.04) relative to control NRG1-cnt was also statistically comparable, p= .7, figure (4-1,4-2).

This indicates that the combination of HCFs and HUVECs would have positive impact on wjMSCs in terms of Akt overexpression with its pro-survival effect. However, the effect is tiny and therefore its biologic impact is still not clear to which extent it may reflect on wjMSCs survival or their paracrine effect.

Also, the effect on BCl-2 was non-significant which means wjMSCs in this combination are not expected to gain extra advantage of BCL-2 as anti-apoptotic factor and also NRG1 expression in wjMSCs is still comparable with the control.

These results despite the positive effect on Akt would be limited with the non-significant effect on BCl-2 which acts as a mediator in the downstream effects of Akt (321). So, survival of wjMSCs is not expected to be supported by this combination of cells but still expected to support the patch's effect on the surrounding myocardium.

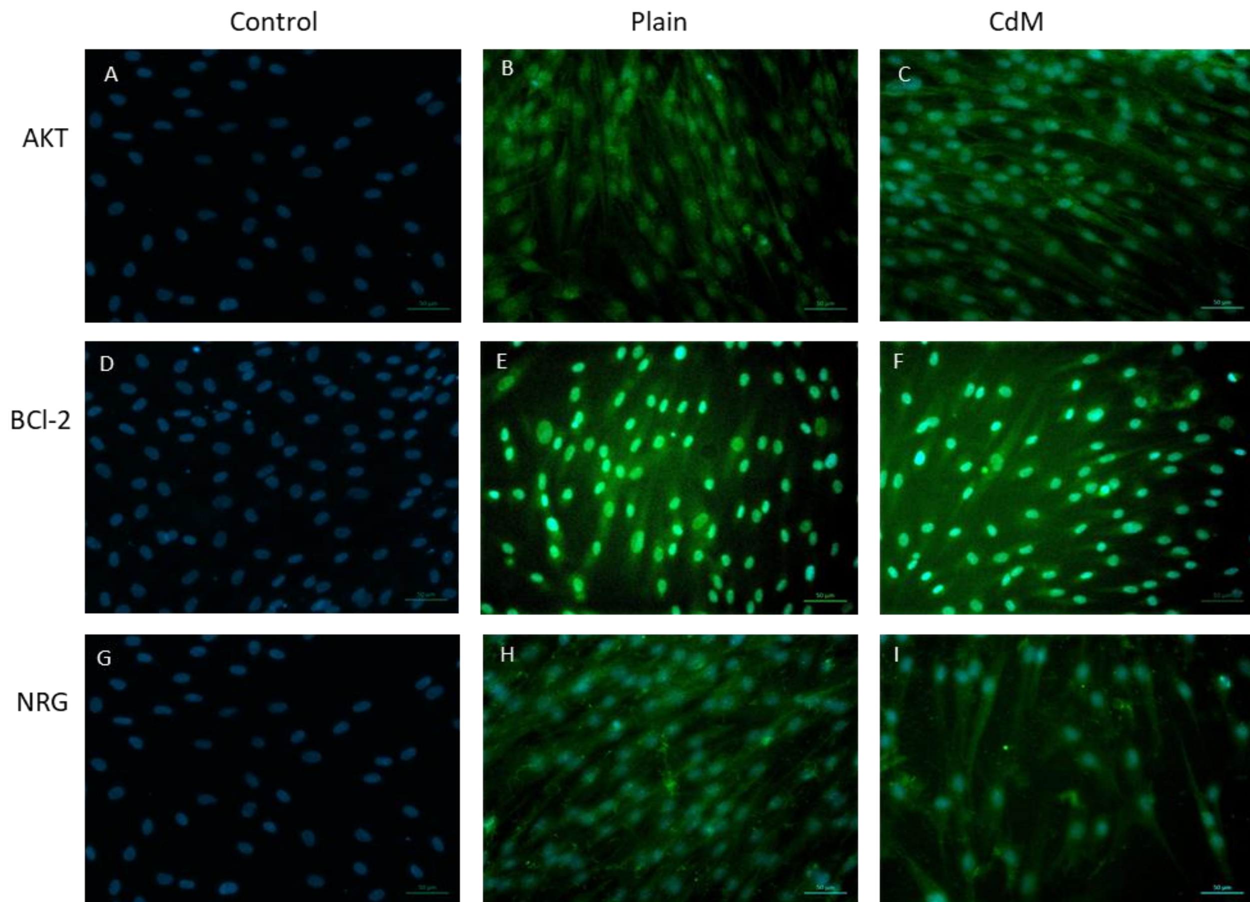

Figure 4-1. Akt, BCl-2 and NRG1 expression in wjMSCs under effect of CdM.*

Effect of CdM on wjMSCs pro-survival factors Akt, BCl2 and NRG1 demonstrate significant upregulation of Akt while non-significant effect on BCl2 and NRG1. WjMSCs cultured in plain media were used as control. (A) Control. (B) Akt in plain media. (C) Akt in CdM. (D) Control. (E) BCl-2 in plain media. (F) BCl-2 in CdM. (G) Control. (H) NRG1 in plain media. (I) NRG1 in CdM. Akt in wjMSCs was significantly up-regulated under effect of CdM as compared to wjMSCs in plain media, p=0.018 while BCL-2 and NRG1 expression did not show significant difference, p=0.45 and 0.71, respectively. N=3. Scale bar 50 um.

*CdM: conditioned media from co-culture of HUVECs and HCFs.

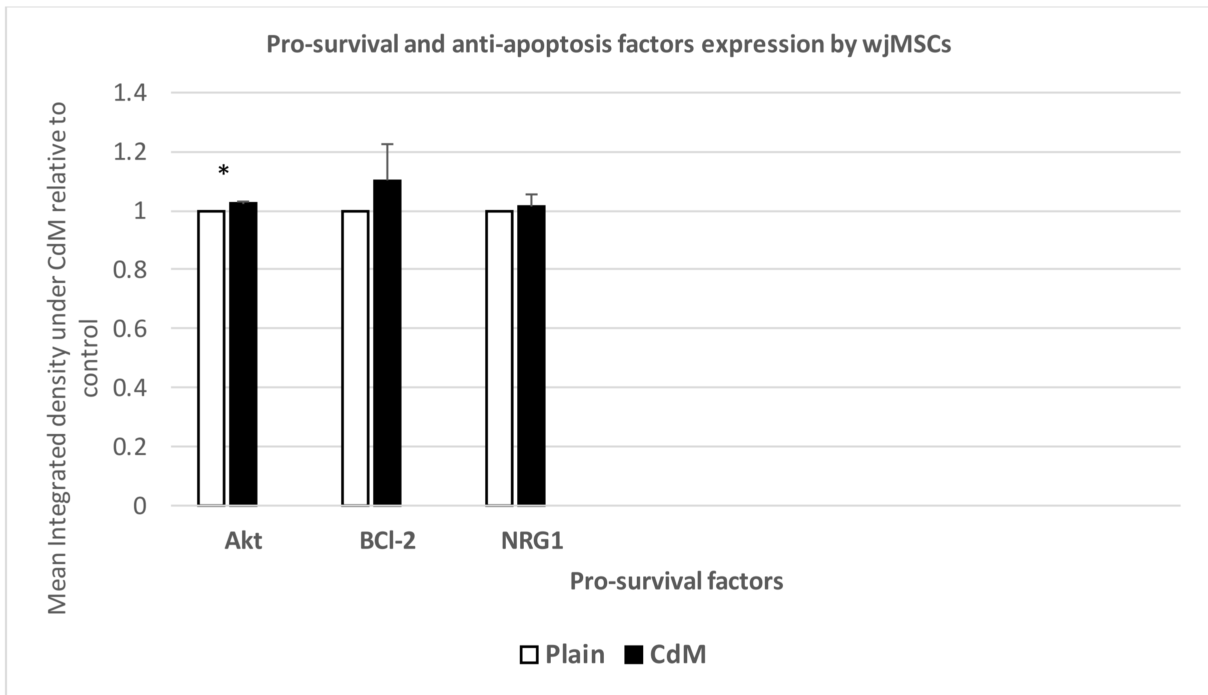

Figure 4-2. CdM effect on wjMSCs expression of Akt, BCl-2 and NRG1.

Effect of CdM on wjMSCs pro-survival factors Akt, BCl2 and NRG1 demonstrate significant upregulation of Akt while non-significant effect on BCl2 and NRG1. Plain media used as control. The graph represents the expression of these proteins under CdM effect relative to plain media. Akt is significantly up-regulated, p=0.018 while BCL-2 and NRG1 expression did not show significant difference, p=0.45 and 0.71, respectively. N=3, expressed as mean+/- SEM.

4.4.1.2. CdM decreases Collagen III expression by WjMSCs but no significant effect on collagen I and fibronectin

ECM is a crucial pillar in any tissue with a diversity of functions including but not limited to, enmesh the cells and organise their polarity and orientation, organise the tissue morphogenesis and maintain mechanical properties, support cellular perfusion and communication and also active rule to initiate signals in a cross talk with cells to maintain local homeostasis in addition to its role in remodeling (57).

Collagen is the main component of ECM. The type of collagen would differ from tissue to another according to its anatomical and physiological circumstances. In the myocardium, collagen I and collagen III are the main collagens and fibronectin is one of the main non-collagen components of ECM. Therefore, we did attempt to examine the CdM effect on wjMSCs expression of collagen I, collagen III and fibronectin as the main myocardial ECM proteins.

In order to assess the effect of CdM on wjMSCs expression of these ECM proteins we did culture wjMSCs in CdM for 48-72 hours. Then, cells were washed, fixed and stained with antibodies for collagen I, collagen III and fibronectin and images were captured using the incucyte system (as described earlier). WjMSCs cultured in plain media for same duration and under same conditions except the culture media were used as control.
The expression of collagen-I and fibronectin under CdM effect was statistically comparable with the control. Collagen I-CdM (M=1.04,SEM= 0.02) relative to collagen I-cnt was non-significant statistically, p= .08. Also, fibronectin-CdM (M=1.01,SEM= 0.007) relative to fibronectin-cnt was statistically comparable, p= 0.15, figure (4-3,4-4).
While there was significant reduction of collagen III expression. Collagen III-CdM was (M=0.89,SEM= 0.002) relative to collagen III-cnt, $p < .00001$, figure (4-3,4-4).
Indeed, these results are consistent with the earlier results of upregulation of Akt which was found to reduce post ischemic inflammation as well as reducing remodeling via reduction of collagen deposition (235).

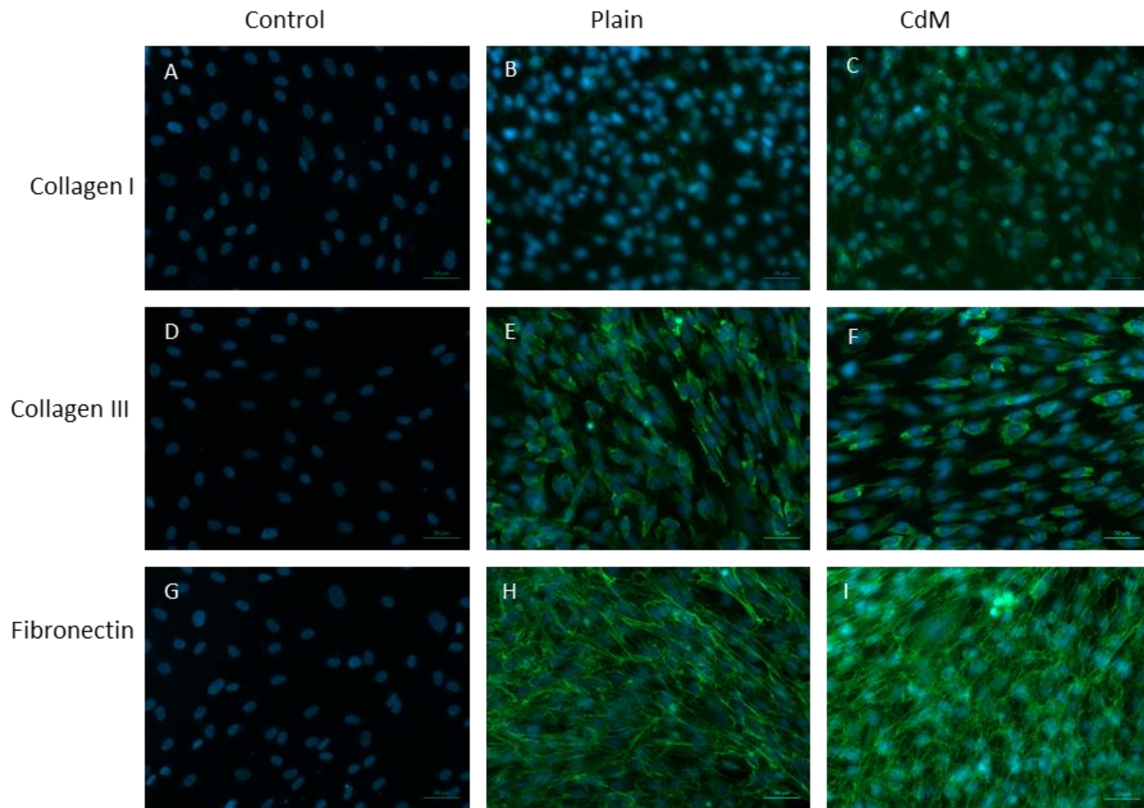

Figure 4-3. ECM proteins- Collagen I, Collagen III and Fibronectin- expression in wjMSCs under effect of CdM.

WjMSCs expression of the ECM proteins collagen I, collagen III and fibronectin under effect of CdM demonstrates significant downregulation of collagen III while non-significant effect on collagen I and fibronectin. WjMSCs cultured in plain media were used as control. (A) Control. (B) Collagen I in plain media. (C) Collagen I in CdM. (D) Control. (E) Collagen III in plain media. (F) Collagen III in CdM. (G) Control. (H) Fibronectin in plain media. (I) Fibronectin in CdM. Collagen III is downregulated, $p<0.0001$ while no significant change of collagen I and fibronectin, $p=0.08$ and 0.15, respectively. N=3. Scale bar 50 um.

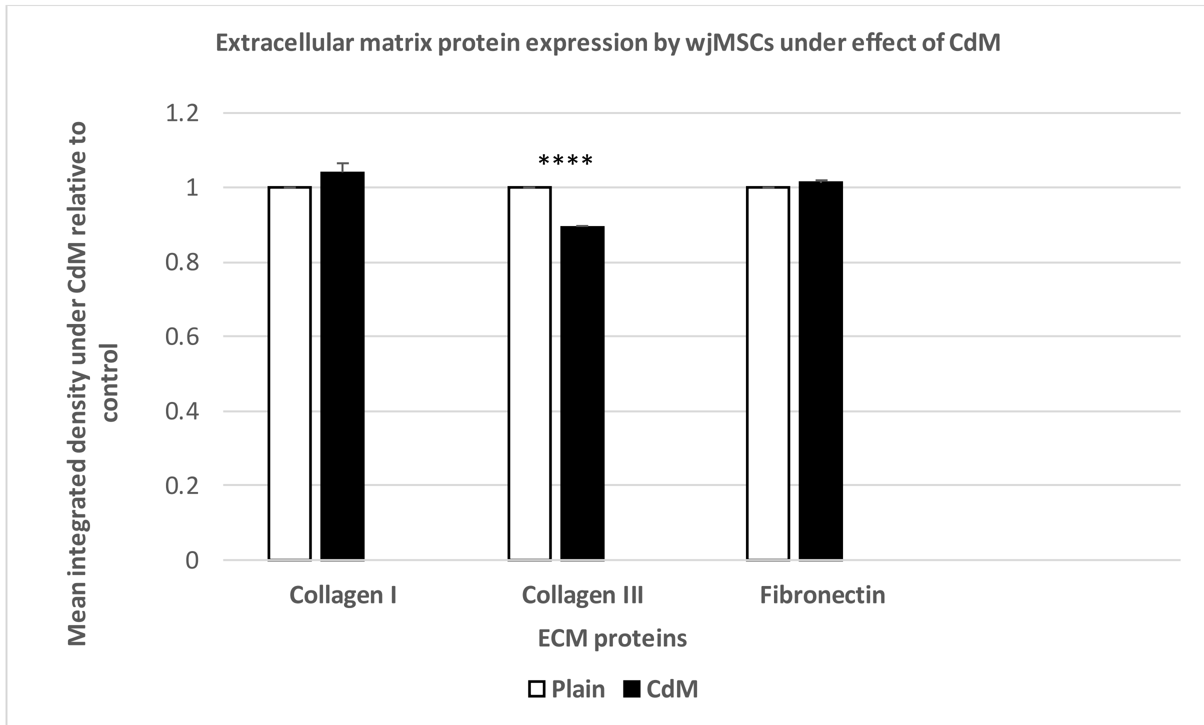

Figure 4-4. ECM proteins- Collagen I, Collagen III and Fibronectin- expression in wjMSCs under effect of CdM**.*

Effect of CdM on wjMSCs expression the ECM collagen I, collagen III and fibronectin demonstrates Collagen III is downregulated, p<0.0001 while no significant change of collagen I and fibronectin, p=0.08 and 0.15, respectively. The graph represents the wjMSCs expression of ECM proteins under CdM effect relative to the control. N=3. Expressed as mean +/- SEM.

*ECM: extracellular matrix.

**CdM: conditioned media from co-culture of HUVECs and HCFs.

4.4.1.3. CdM enhances WjMSCs expression of HGF, VEGF and MCP1 while does not have significant effect on Angiopoietin, bFGF & SDF1

Next assessment domain is the angiogenic profile of wjMSCs. The advantageous effects of MSCs in-vivo are mostly explained by their paracrine effects and one of the most important effects would be to support angiogenesis. So, assessment of angiogenic profile of MSCs would indicate their potential regenerative effects in-vivo. The proangiogenic properties of MSCs were demonstrated with higher capillary density when transplanted into infarct area and better wound healing (322).

The angiogenic properties of MSCs are supported by secretion of several angiogenic factors that would enhance angiogenesis via supporting ECs survival, proliferation, migration as well as maturation of neovessel (233, 323).

So, we did examine the CdM effect on wjMSCs angiogenic factors expression including angiopoietin1, bFGF, HGF, VEGF, SDf1 and MCP1 due to their potential influence on the patch's behaviour in-vivo. WjMSCs in plain media were used as control.

In order to assess the effect of CdM on wjMSCs expression of these angiogenic factors we did culture wjMSCs in CdM for 48-72 hours. Then, cells were washed, fixed, stained with antibodies for angiopoietin1, bFGF, HGF, VEGF, SDf1 and MCP1. Then images were captured using the incucyte system (as described earlier). WjMSCs cultured in plain media for same duration and under same conditions except the culture media were used as control.

Expression of Angiopoietin-CdM (M= 1.006, SEM= 0.013) relative to Angiopoietin-cnt was statistically comparable, *p*= 0.6. Also, bFGF-CdM (M=1.013,SEM= 0.016) relative to bFGF-cnt showed no significant difference, *p*= 0.4.
In addition, expression of SDF1-CdM (M=1.02,SEM= 0.01) relative to SDF1-cnt demonstrated no significant difference, *p*= 0.2.
But expression of HGF-CdM (M= 1.045,SEM= 0.01) relative to HGF-cnt showed significant up regulation , *p*= .03. Also, VEGF-CdM (M= 1.029,SEM= 0.005) relative to VEGF-cnt demonstrated significant up regulation, *p*= .003. And expression of MCP1-CdM (M= 1.015,SEM= 0.003) compared to MCP1-cnt showed up regulation and results were statistically significant, p= 0.005.
These data show that expression of the angiogenic factors under CdM effect demonstrated significant upregulation of HGF, VEGF and MCP-1 while non-significant effect on angiopoietin, bFGF and SDf1. This would indicate the positive angiogenic potential of wjMSCs when combined with HUVECs and HCFs due to the significant angiogenic effects of HGF, VEGF and MCP-1 with still maintained baseline level of angiopoietin, bFGF and SDf1 as there was no significant down regulation of any of these factors.

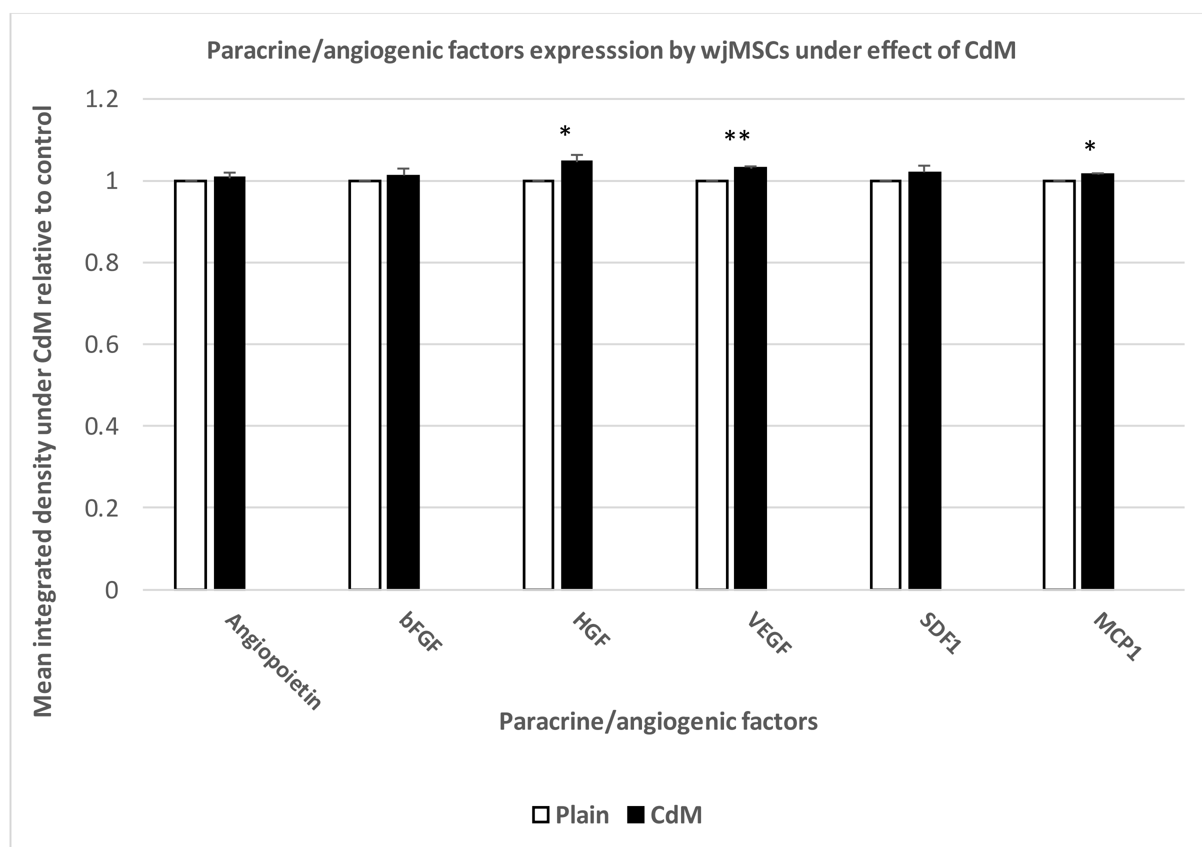

Figure 4-5. Angiopoietin I, bFGF, HGF, VEGF, SDF1 and MCP1 expression by wjMSCs under CdM effect.*

CdM effect on wjMSCs expression of paracrine factors revealed variable results. HGF, VEGF and MCP1 showed significant upregulation, p=0.03, 0.003 and 0.005 respectively while Angiopoietin, bFGF and SDf1 did not show significant change, p=0.68, 0.47 and 0.26, respectively. N=3. Expressed as mean +/- SEM..

*CdM: conditioned media from co-culture of HUVECs and HCFs.

4.4.1.4. CdM enhances WjMSCs expression of N-cadherin but no significant effect on β-catenin, endoglin and Cx43

Cellular adhesion and communication with surrounding milieu including neighbouring cells as well as the ECM is crucial function to maintain tissue integrity. Therefore, we did consider assessment of various factors that would affect the on-patch as well as in-vivo integration and communication of cells.

In order to make assessment of wjMSCs expression of adhesion and communication factors under CdM effect we did examine expression of ITG, β-catenin, N-cadherin, endoglin and Cx43. The ITG cell surface receptors have their physiological role as part of the cell adhesion complexes and act as mechano-sensors involved in interaction with ECM. Unfortunately the ITG antibody did not work and therefore could not quantify its expression.

In order to assess the effect of CdM on wjMSCs expression of these factors we did culture wjMSCs in CdM for 48-72 hours. Then, cells were washed, fixed, stained with antibodies for β-catenin, N-cadherin, endoglin and Cx43.
Then images were captured using the incucyte system (as described earlier). WjMSCs cultured in plain media for same duration and under same conditions except the culture media were used as control.

The only statistically significant upregulated protein was the N-cadherin while other proteins did demonstrate higher mean of expression but it did not meet statistical significance most probably due to small number and scattered data, figure (4-6).

N-cadherin-CdM (M=1.078,SEM= 0.028) relative to N-cadherin-cnt was significantly upregulated, p = .02.
β-catenin-CdM (M= 1.073,SEM= 0.057) relative to β-catenin-cnt did not show significant difference, p = 0.2.
Endoglin-CdM (M= 1.08,SEM=0.04) relative to Endoglin-cnt was not significantly altered, p= 0.06.
And Cx43-CdM (M= 1.042,SEM=0.064) relative to Cx43-cnt was not significantly changed as well, p= 0.4.
These results show that N-cadherin was the only factor that demonstrated statistically significant upregulation while all other factors including b-catenin, Endoglin and connexin43 did show higher mean of expression but did not achieve statistical significance. This would suggest positive potential for intercellular communication as the N-cadherin was upregulated while others did not show any down-regulation which means still the cells are expected to support cellular communication via same mediators but at the basal level rather than any extra benefit.

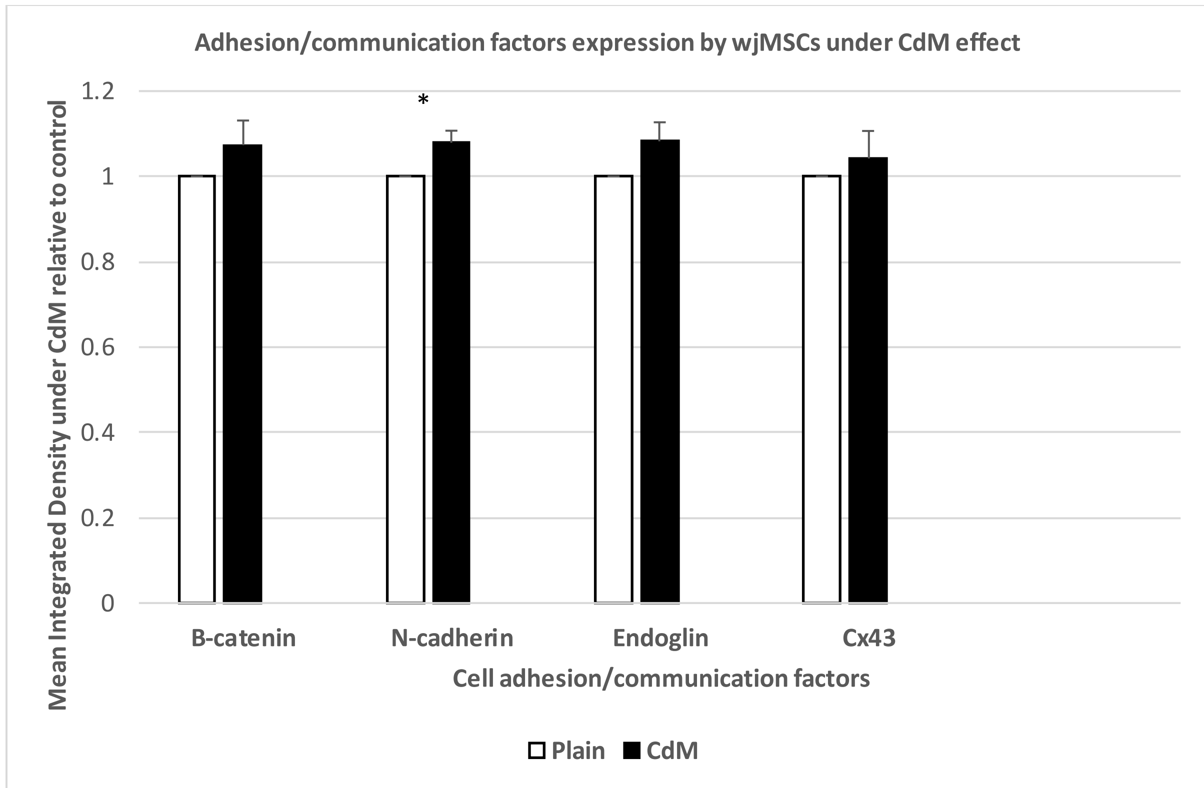

Figure 4-6. Expression of B-catenin, N-cadherin, endoglin and connexin43 by wjMSCs under CdM effect.*

CdM effect on wjMSCs revealed significant upregulation of N-cadherin while non-significant change in expression of B-catenin, Endoglin and Cx43. N-cadherin was significantly upregulated with p=0.024 while β-catenin, Endoglin and Cx43 did not meet statistical significance, p=0.27, 0.069 and 0.47, respectively. N=3. Expressed as mean+/-SEM.

*CdM: conditioned media from co-culture of HUVECs and HCFs.

4.4.1.5. mRNA change in wjMSCs under CdM effect

In order to find out the effect of CdM on gene expression of the assessed factors and to get insight about the changes in protein levels if it is transcription dependent or not. We used qPCR to assess the mRNA change in response to CdM effect and got the following results.

4.4.1.5.1. CdM downregulates mRNA levels of NRG1 in wjMSCs while does not have significant effect on Akt mRNA

To find out if the mRNA of Akt and NRG1 in wjMSCs would be influenced by CdM effect we did examine its levels using qPCR. After culture of wjMSCs in CdM for 48-72 we did process the cells to isolate RNA (as described earlier). mRNA in wjMSCs cultured in plain media for same duration and under same conditions was used as control.

The CdM did downregulate mRNA of NRG1 in wjMSCs as compared with the control while mRNA of Akt did not show any significant alteration.

mRNA fold change of Akt-CdM (M= 0.92, SEM= 0.209) was statistically comparable with the control, p= 0.7.
While fold change for mRNA of NRG1 under CdM effect (M=0.46, SEM=0.21) demonstrated significant down-regulation, p=0.02.
These results suggest that Akt and NRG1 proteins expression change within this time window were not transcription dependent.

Unfortunately, we could not proceed for the BCl-2 mRNA assessment due to the facility constrain during the pandemic.

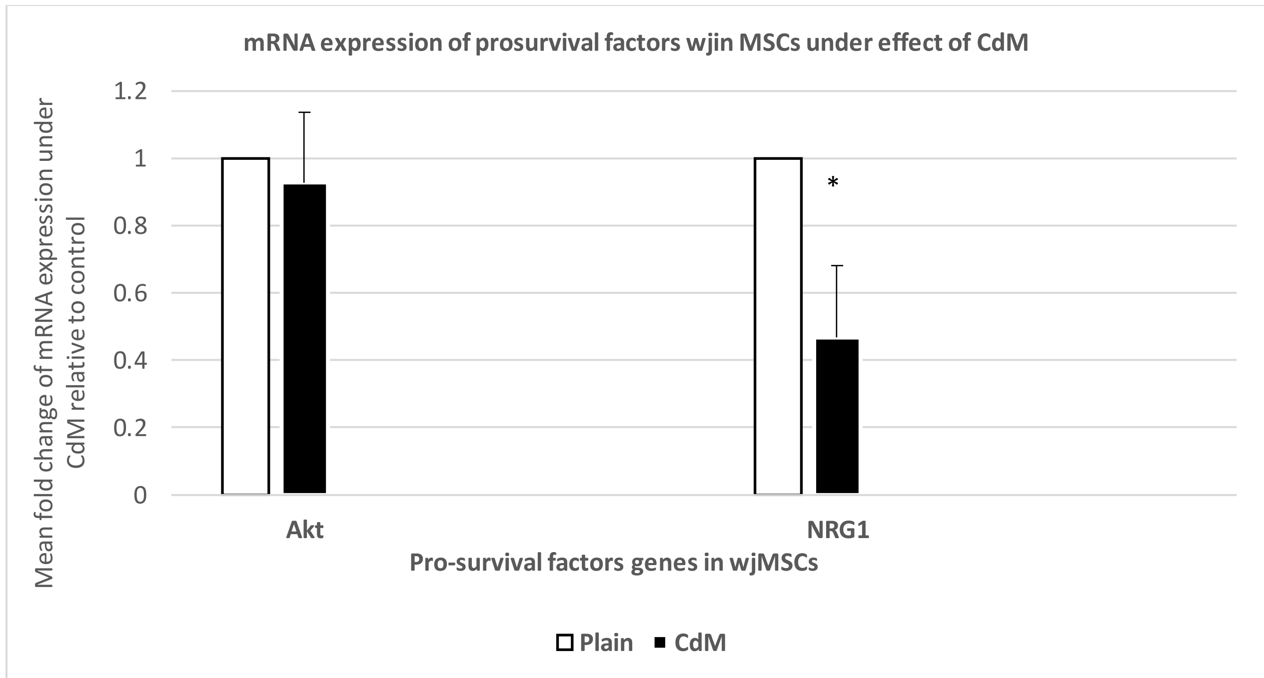

Figure 4-7.Fold changes of mRNA levels for Akt and NRG1 in wjMSCs under effect of CdM.*

CdM effect on mRNA level of Akt and NRG1 revealed significant downregulation of NRG1 mRNA while Akt mRNA was statistically comparable with the control. Akt fold change is comparable with the control, p=0.7 while NRG1 mRNA was significantly downregulated, p=0.02. N=3. Expressed as mean+/-SEM.

*CdM: conditioned media from co-culture of HUVECs and HCFs.

4.4.1.5.2. CdM up-regulates mRNA of HGF and VEGF

To find out if the mRNA of the angiogenic factors angiopoietin, bFGF, HGF and VEGF in wjMSCs would be influenced by CdM effect we did examine its levels using qPCR. After culture of wjMSCs in CdM for 48-72 we did process the cells to isolate RNA (as described earlier). mRNA in wjMSCs cultured in plain media for same duration and under same conditions was used as control.

mRNA of HGF and VEGF showed significant upregulation while angiopoietin despite getting numerically higher mean of expression but did not achieve statistical significance.

Angiopoietin-CdM (M=2.017, SEM=1.16) relative to the control showed non-significant difference, p=0.5.
bFGF-CdM (M=0.67, SEM=0.409) relative to the control showed non-significant difference, p=0.4.
HGF-CdM (M=5.12 ,SEM=1.7) relative to the control showed statistically significant upregulation, p=0.03.
VEGF-CdM (M= 2.63,SEM= 0.15) relative to the control demonstrated significant upregulation, p=0.004. However, here n=2 so it is more suggestive rather than consolidated result.

These results are leaning toward the same change of angiogenic proteins expression. Despite VEGF data are based on n=2 but still suggestive of transcription dependent upregulation of VEGF protein.

Unfortunately, we could not proceed for the SDf1 and MCP1 mRNA assessment due to the facility constrain during the pandemic.

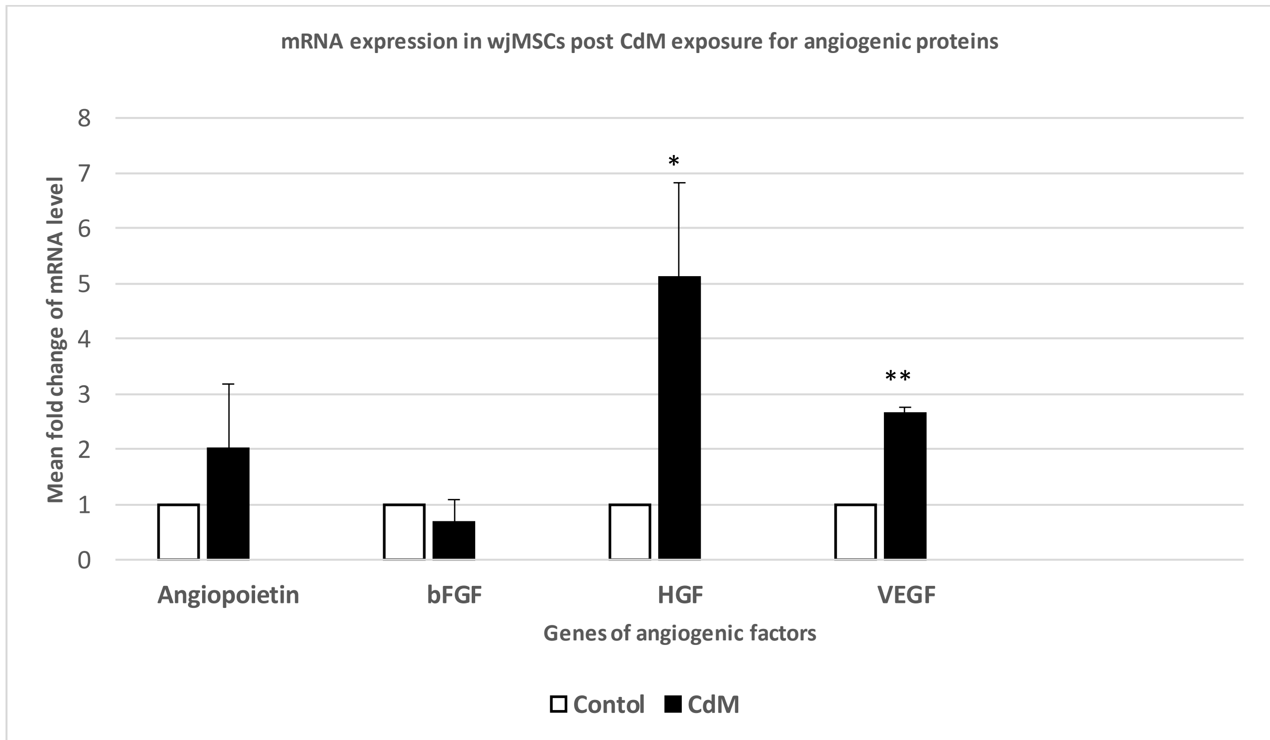

Figure 4-8. mRNA levels of angiogenic factors in wjMSCs under effect of CdM. mRNA levels of angiogenic factors in wjMSCs showed significant upregulation of both HGF and VEGF while statistically non-significant change of Angiopoietin and bFGF. Angiopoietin and bFGF fold change was comparable with the control, p=0.54 and 0.4 respectively while HGF and VEGF mRNA fold changes were significantly upregulated with P= 0.036 and 0.004, respectively. N=3 except VEGF n=2. Expressed as mean+/- SEM.*

*CdM: conditioned media from co-culture of HUVECs and HCFs.

4.4.1.5.3. CdM downregulates mRNA of ITG

To find out if the mRNA of the integrin in wjMSCs would be influenced by CdM effect we did examine its levels using qPCR. After culture of wjMSCs in CdM for 48-72 we did process the cells to isolate RNA (as described earlier). mRNA in wjMSCs cultured in plain media for same duration and under same conditions was used as control.

Fold change of mRNA of ITG under CdM effect (M=0.51, SEM=0.19) demonstrated significant down-regulation as compared with the control, p=0.03.

Unfortunately, we could not proceed for examining mRNA of the rest of factors (β-catenin, N-cadherin and Endoglin) assessment due to the facility constrain during the pandemic.

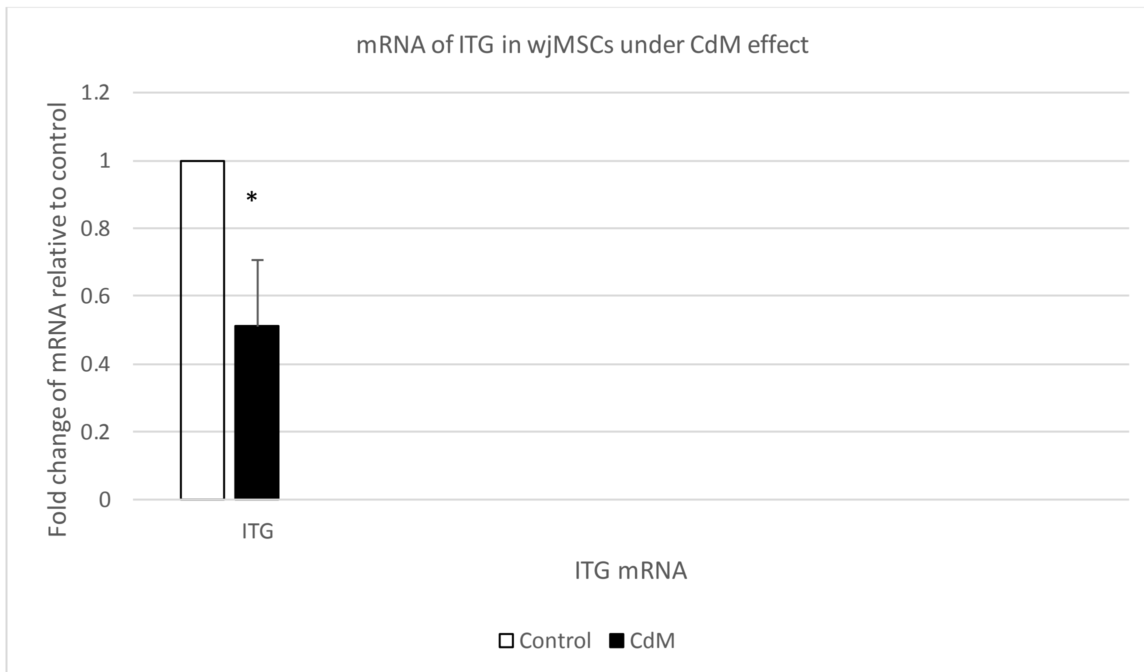

Figure 4-9. ITG mRNA fold change in wjMSCs under effect of CdM**.*

ITG mRNA is significantly downregulated relative to the control, p=0.03. N=3. Expressed as mean+/-SEM.

*ITG: integrin.

**CdM: conditioned media from co-culture HCFs & HUVECs.

4.4.1.6. CdM does not support wjMSCs survival

In order to assess potential of the combination of HCFs and HUVECs to support wjMSCs survival we did examine CdM effect on wjMSCs apoptosis and death. WjMSCs were cultured for up to 48 hours in CdM then tested using the caspase/DRaq7 (apoptosis/dead cell) assay via flowcytometry (FCM) and wjMSCs in plain media were used as control.

Apoptotic cells under CdM effect (M=3.73%, SEM= 0.19) were significantly higher than the control (M=2.066, SEM=0.48), p=0.04.

And dead cells under CdM effect (M=4.33,SEM= 1.35) also were significantly higher than the control (M=1.72,SEM= 0.13), p= 0.042.

These data are limited by the fact that N=3 for the control group while only 2 for the CdM condition. However, it is suggestive of no good support for wjMSCs survival as within 48 hours the cells are leaning toward apoptosis and cell death, figure (4-10, 4-11).

Our earlier data showed higher level of Akt however, the non-significant change of BCl-2 and NRG1 in addition to other apoptosis control factors that could not be examined due to the facility constrain would underpin this result. Indeed, this is more in keeping with the experimental findings that revealed early loss of cells post in-vivo transplant (281, 324).

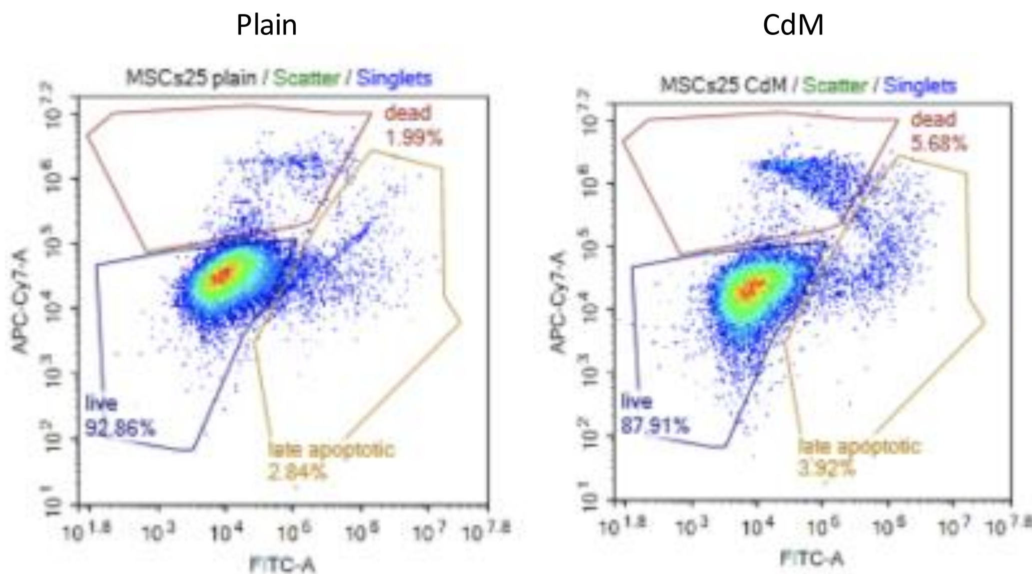

Figure 4-10. Representative images of Caspase/DRaq7 assays of wjMSCs under effect of CdM**.*

WjMSCs survival under effect of CdM. Apoptosis and cell death significantly higher in the CdM condition relative to the control and results were statistically significant with p=0.04 and 0.042 respectively.

*Caspase/DRaq7 assay: apoptosis/cell death assays

**CdM: conditioned media from co-culture HCFs & HUVECs.

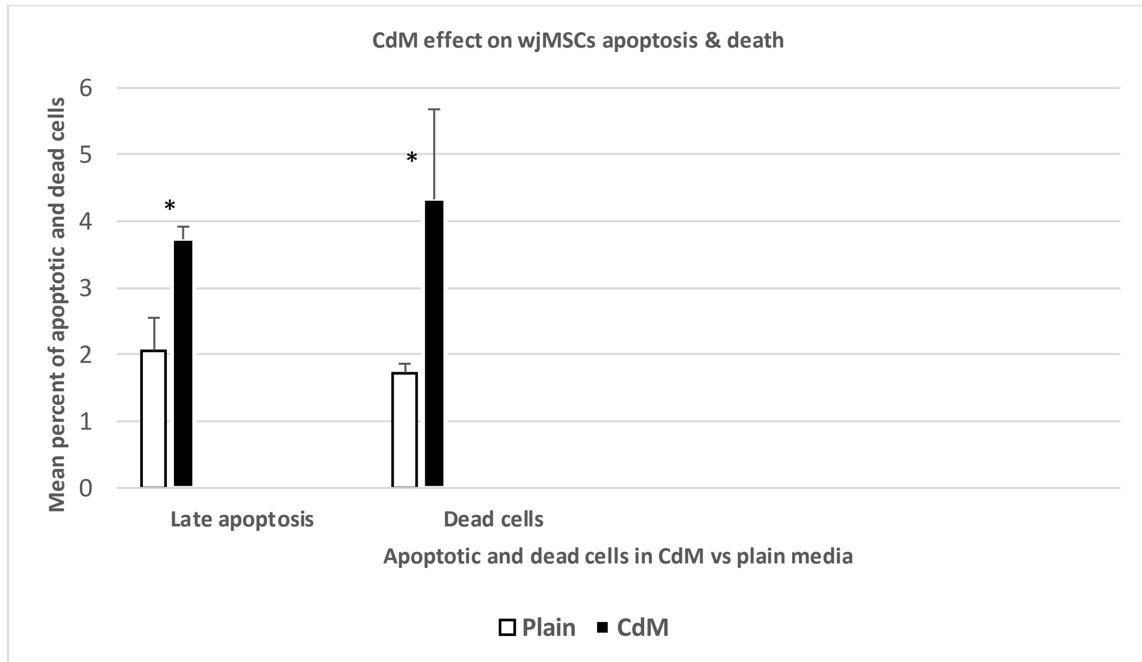

Figure 4-11. The CdM does not offer survival benefit to wjMSCs.*

WjMSCs survival under effect of CdM. Apoptosis and cell death significantly higher in the CdM condition relative to the control and results were statistically significant with p=0.04 and 0.042 respectively. N=3 for the control but 2 for CdM. Expressed as mean+/-SEM.

*Caspase/DRaq7 assay: apoptosis/cell death assays

**CdM: conditioned media from co-culture HCFs & HUVECs.

4.4.1.7. CdM does not enhance wjMSCs proliferation potential

In order to find out if the combination of cells would enhance wjMSCs proliferation which could raise concern about over-populating the other two cell phenotypes. We used the BrdU assay to assess the wjMSCs proliferation potential under effect of CdM.

The BrdU assay for wjMSCs cultured in CdM for 24-48 hours was compared with wjMSCs cultured in plain media for same duration. The BrdU reading under CdM effect (M= 0.11, SEM= 0.01) was statistically comparable with the control wjMSCs (M= 0.11, SEM= 0.008) and no significant difference could be elicited, p= 0.8.
This result eliminates the concern of wjMSCs over-populating the other cells in the patch and is considered as positive result in terms of balanced growth.

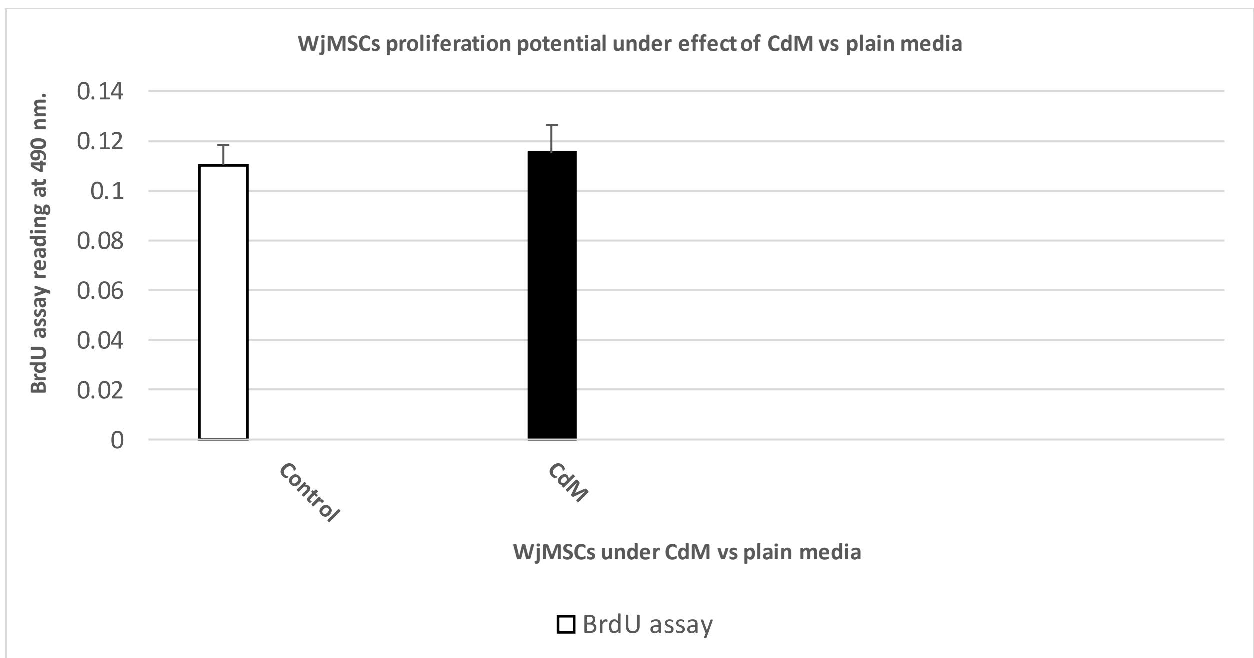

Figure 4-12. WjMSCs proliferation potential under CdM effect compared with plain media.*

CdM does not affect the proliferation rate of wjMSCs. Results are statistically insignificant, p=0.4. The results represented are the mean of 3 independent cell lines for the plain media control but two cell lines for the CdM condition, expressed as mean+/-SEM.

*CdM: conditioned media from co-culture HCFs & HUVECs.

4.5.Trial to differentiate wjMSCs into cardiomyocytes using 5-azacitidine

4.5.1.Introduction

After this initial assessment of CdM effect on wjMSCs with some promising results of positive reaction of wjMSCs to the HCFs/HUVECs combination, we did attempt to boost functional properties of the cells via treatment with small molecules.

Some groups reported succesful MSCs differentiation into CMCs using the demthylating agent 5-azacitidine (272). We hypothesised that this would be a valuable addition to the patch if we could seed it with cardiomyocytes from inexhaustible source such as the umbilical cord. In addition to the fact that drug induced transdifferentiation seems quite convenient.

4.5.2.Material and methods

5-Azacitidine purchased from Sigma Aldrich was used at 5 uM. It was prepared as per manufacturer's recommendation. In summary, 1.2 mg diluted in 10 ml DMEM for immediate use or stored for a maximum of 7 days at 4 ^{o}C.

The cells were washed with pre-warmed PBS then fresh media added (DMEM with 10% FBS and 1% P/S) and 5-azacitidine was added to a dilution of 5 uM and cultured in humidified incubator at 37 ^{o}C before change media after 48-72 hours. Cells cultured in DMEM with 10% FBS and 1% P/S were used as control.

4.5.3. Results

Assessment of the effect of 5-azacitidine revealed

4.5.3.1. WjMSCs do not express cardiac proteins after 5-azacitidine treatment

In order to assess if any cardiac proteins would express in wjMSCs post treatment with 5-Azacitidine, wjMSCs were examined via ICC after about 2 weeks of treatment. ICC done for MHC, α-sarcomeric actin, cardiac troponin and connexin43. ICC failed to show expression of any specific cardiac proteins. The only result was upregulation of Connexin 43 expression (data not shown).

4.5.3.2. 5-azacitidine does not upregulate mRNA of cardiac genes in wjMSCs

Also, we did assessment of mRNA fold change after treatment with 5-Azacitidine to find out if any cardiac gene mRNA upregulation that would indicate successful differentiation. Four independent cell lines were treated with 5-Azacitidine for 6-10 days and wjMSCs cultured in regular media were used as control.

The mRNA of genes for atrial natriuretic peptide (ANP), Connexin43 (GJA1), Desmin, NKX2.5, myosin heavy chain (MHC) and tropomyosin (TPM) were assessmed.

ANP-Az (M= 0.96,SEM= 0.63) relative to the control, p= 0.9,
Desmin-Az (M= 0.62,SEM=0.25) relative to the control, p= 0.2,
GJA1-Az (M= 6.76,SEM= 0.39) relative to the control, p=0.003,
NKX2.5-Az (M=0.79,SEM=0.502) relative to the control, p= 0.7,
MHC-Az (M= 0.96, SEM= 0.404) relative to the control, p=0.9 and
TPM-Az (M=0.929, SEM=0.18) relative to the control, p=0.7.

So, Connexin 43 gene (GJA1) was the only gene that showed significant upregulation of its mRNA, p=0.003, which is consistent with the ICC results while the rest did not show any significant change.

These results do not support any significant potential of wjMSCs to differentiate into CMCs.

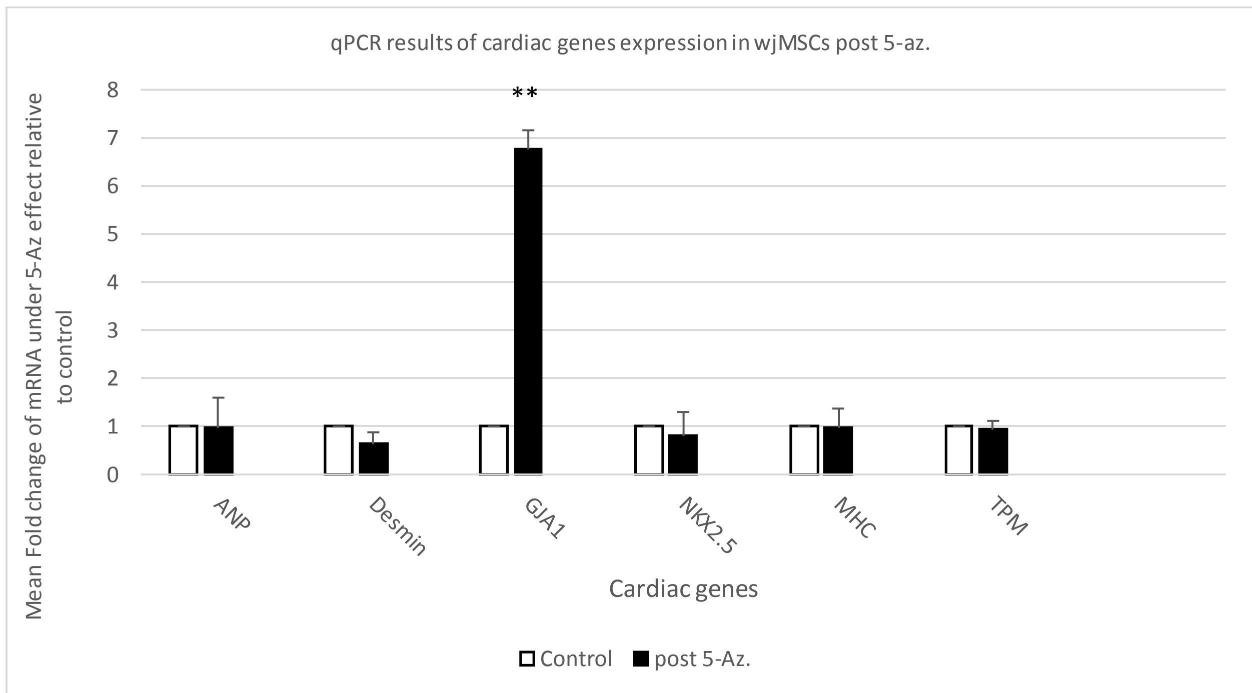

Figure 4-13. fold change of mRNA expression for cardiac genes in wjMSCs treatment with post 5-Azacitidine at 5 uM for about 7 days.

GJA1 was significantly upregulated in wjMSCs after treatment with 5-azacitidine, p=0.003. mRNA of other genes did not show any significant change. N=3 except GJA1, n=2. Expressed as mean+/-SEM.

This attempt was a trial to boost the patch with functioning CMCs. After spending about 12 months with many trials to optimise the protocol and look for signs of differentiation it all proved unsuccessful. Several trials reported successful differentiation of MSCs into cardiomyocytes in-vivo (325, 326) and in-vitro. For instance, the report of Shinji et al (271) who reported successful differentiation of bone

marrow MSCs to cardiomyocytes using the demethylating agent 5-azacytidine (271, 327). This report gave a credit to the MSCs as a potential source for cardiomyocyte replacement with a quite convenient and cost effective approach.

These results are not consistent between different laboratories and differentiation of wjMSCs into CMCs using 5-Azacitidine became a matter of debate in the literature.

Some groups confirmed successful differentiation of wjMSCs into cardiomyocytes (CMCs) (328). Even they figured out the underlying pathways for this trans-differentiation and reported it via the extracellular signal related kinases (ERK) (272). However, others challenged this result and reported that 5-Azacytidine does differentiate bone marrow MSCs into CMCs (329, 330) with a low yield in the range of 0.07% while the wharton jelly MSCs do not have the potential for transdifferentiation into CMCs (273).

In fact, after handling many independent cell lines we came to the conclusion that wjMSCs are quite heterogenous cells with variable differentiation potential. The only positive result that was consistent among majority of wjMSCs cell lines was the upregulation of connexin 43 that was also consistent with the PCR results while other cardiac proteins did not show any consistent results.

Furthermore, in an attempt to optimise the approach and get consistent results we did assess the surface markers' expression on wjMSCs post 5-Azacitidine treatment. There was significant CD90 downregulation in keeping with differentiation (331) but obviously does not indicate differentiation into cardiomyocytes. Believing that the CD90 -ve cells are the differentiated cells so we focused on this group of cells as the target for further analysis.

So, we attempted to sort the CD90 -ve cells via MACS microbeads followed by analysis of the CD90-ve cells via qPCR, ICC and WB (data not shown). The results again failed to show any consistently positive cardiac differentiation or consistent cardiac gene upregulation.

This variable response of different cell lines to 5-azacytidine with lack of standardisation is not reliable for clinical applications. Therefore, we did deviate from this approach as it is unreliable and clearly non-reproducible.

4.6. Treatment of wjMSCs with CHIR99021

4.6.1. Introduction

After the initial attempt with 5-Azacitidine that failed to show reliably reproducible results we did start to assess the possible advantageous addition of using the GSK-3 inhibitor CHIR99021 (afterwards will be referred to as CHIR).

CHIR treatment of MSCs was reported to enhance the trans differentiation potential of MSCs including differentiation to cardiomyocytes with maintained multipotency and the HLA-DR negative status which is a key factor for immune privilege of MSCs (210). Also, BIO which is another GSK inhibitor demonstrated a positive effect on MSCs. It did up-regulate the stemness genes, proliferation potential in addition to maintaining the MSCs differentiation potential (332).

This indicates that GSK inhibition via small molecules holds favourable effect on MSCs. Indeed, this effect is of clinical interest to optimise the MSCs proliferation and differentiation potential while maintaining their immune modulatory properties.

The effect of CHIR on wjMSCs was assessed as regard same domains of concern including anti-apoptotic factors, ECM proteins, paracrine/angiogenic factors, adhesion/communication factors, survival and cell proliferation potential. Cells in plain media were used as control. Also, because CHIR is delivered in a powder form and DMSO was used to dissolve it therefore, all experiments included an extra step which is to add a DMSO containing media as a vehicle control (333).

Before assessment of the effect of CHIR on the cells we did test for the appropriate dose. Ideally, will use same dose for the three cell phenotypes as our target is to combine the triad of cells on the surface of cormatrix. We did test the dose on HUVECs as the most vulnerable/demanding cell phenotype. This was obvious from the experiments on HUVECs as they could not tolerate more than 24 hours in serum free media and the level of apoptosis was quite high.

We did test CHIR effect on HUVECs at 15 uM, 10 uM and 5 uM. The 15 uM dose was clearly unsuitable as most cells died and could not survive even the first 24 hours. The 10 uM did upregulate the mRNA of the stemness genes OCT4 and Nanog and enhanced HUVECs tube formation but the 5 uM results were statistically comparable with the 10 uM effect with better angiogenic profile (data not shown). So, we opted to use the 5 uM in all experiments.

Afterwards and unless otherwise specfified the term CHIR would refer to the 5 uM dose.

4.6.2. Materials and methods

CHIR99021 purchased from StemCell Technology was handled according to manufacturer's recommendation. It was dissolved using well sealed DMSO from Sigma Aldrich. Aliquoted and stored at -20 ^{o}C. For the application, it was taken out of freezer for immediate use. For the control wells an amount of DMSO similar to CHIR containing solution was added. Plain media (serum and growth factor free) was used as a hosting solution for the culture as well as the control wells/flasks in order to eliminate any confounding effects from serum or additional growth factors that could interact with DMSO or CHIR and alter the results.

4.6.3. Objectives

CHIR is a GSK-3 inhibitor and its effects on bone marrow derived MSCs were assessed before (334). Here we are making assessment with focus on the relevant areas that would reflect on the patch's behaviour and sustainability in-vivo. So, in this section we aim to assess the effects of CHIR on wjMSCs in the following domains

- ✓ Pro-survival/anti-apoptosis factors.
- ✓ ECM proteins.
- ✓ paracrine/angiogenic factors.
- ✓ Adhesion/communication factors.
- ✓ Cells survival.
- ✓ Cells proliferation potential.

4.6.4.Results

After treatment of wjMSCs with CHIR we followed same sequence of domains for assessment as done for the CdM and got the following resluts

4.6.4.1.CHIR does not have significant effect on wjMSCs expression of Akt, BCl-2 or NRG1

We started with the effect on the pro-survival factors Akt, BCl-2 and NRG1 to find out if CHIR at 5 uM would affect their expression in wjMSCs. Indeed, the assessment revealed non-significant alteration of any of these factors. The expression was compared with expression in wjMSCs in plain media as well as under DMSO effect as a carrier control.
The expression of AKT-CHIR (M=0.99, SEM= 0.006) compared to AKT-DMSO (M=1.028, SEM= 0.027) relative to AKT-cnt demonstrated no significant difference, p= 0.3.

Also, expression of BCl 2-CHIR (M=1.07, SEM= 0.074) compared to BCl 2-DMSO (M=1.014, SEM= 0.05) relative to BCl 2-cnt demonstrated no significant difference, p= 0.6.

And expression of NRG1-CHIR (M=1.03, SEM= 0.057) compared to NRG1-DMSO (M=1.032, SEM= 0.039) relative to NRG1-cnt demonstrated no significant difference, p= 0.6.

This reflects fairly neutral effect of CHIR at this dose on wjMSCs as regard the survival factors. Therefore, it would be prudent to anticipate neutral effect on survival as well.

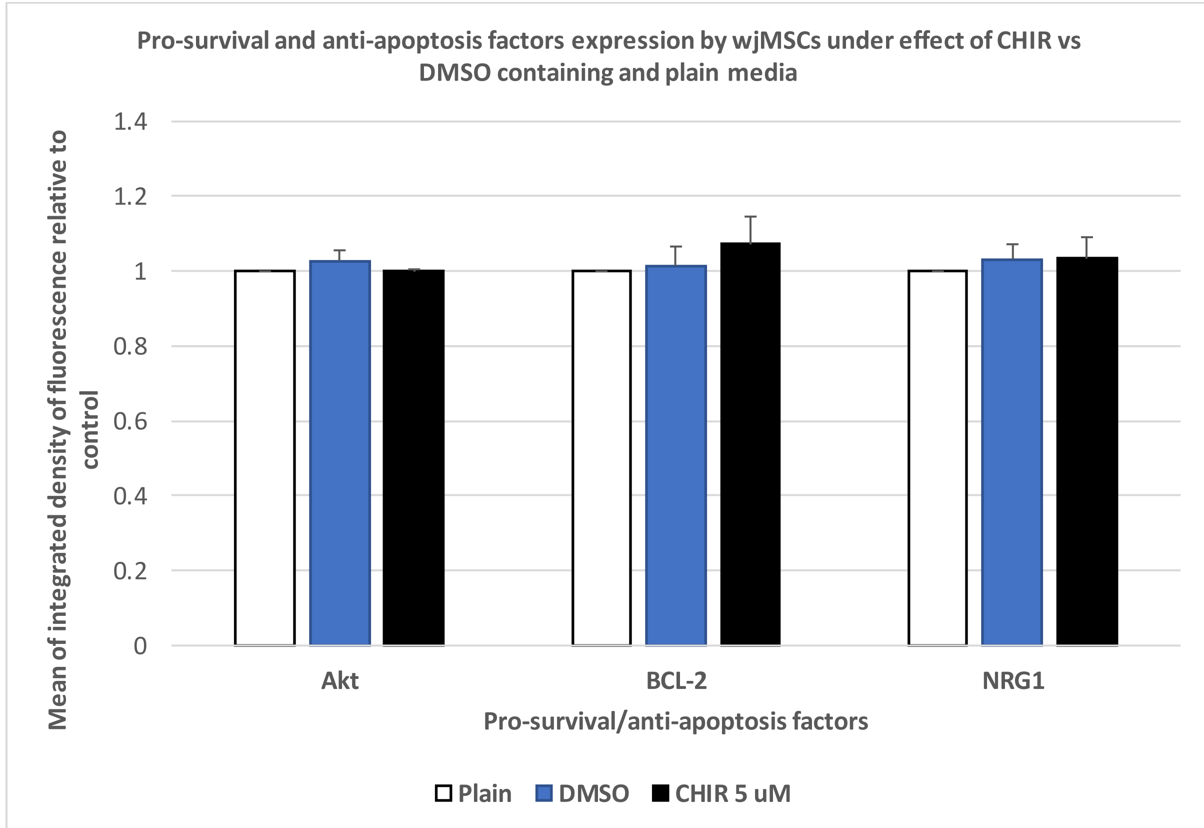

Figure 4-14. Effects of CHIR99021 at 5 uM on wjMSCs expression of AKt, BCl-2 and NRG1.

Assessment of CHIR effect on expression of the pro-survival factors Akt,BCl2 and NRG1 expression by wjMSCs and compared to effect of DMSO (carrier control) and plain media. All factors showed comparable results with no statistically significant difference, p=0.36, 0.61 and 0.65, respectively. N=3 . Expressed as mean +/-SEM.

4.6.4.2. CHIR does not have significant effects on wjMSCs secretion of collagen I, collagen III and fibronectin

To assess the effect CHIR on the wjMSCs expression of the ECM proteins collagen I and III as well as the glycoprotein fibronectin, we did examine their expression (as described earlier). The levels of these ECM proteins was compared with their expression in wjMSCs in plain media as well as under DMSO effect as a carrier control. There were no significant change in the expression of these ECM under CHIR effect.

Collagen I-CHIR (M=1.035, SEM= 0.023) compared to Collagen I-DMSO (M=0.99, SEM= 0.026) relative to Collagen I-cnt demonstrated no significant difference, p=0.4.

Collagen III-CHIR (M=0.9, SEM= 0.045) compared to Collagen III-DMSO (M=1.05, SEM= 0.03) relative to Collagen III-cnt demonstrated no significant difference, p= 0.2.

Fibronectin-CHIR (M=1.045, SEM= 0.034) compared to fibronectin-DMSO (M=1.01, SEM= 0.002) relative to fibronectin-cnt demonstrated no significant effect, p= 0.4.

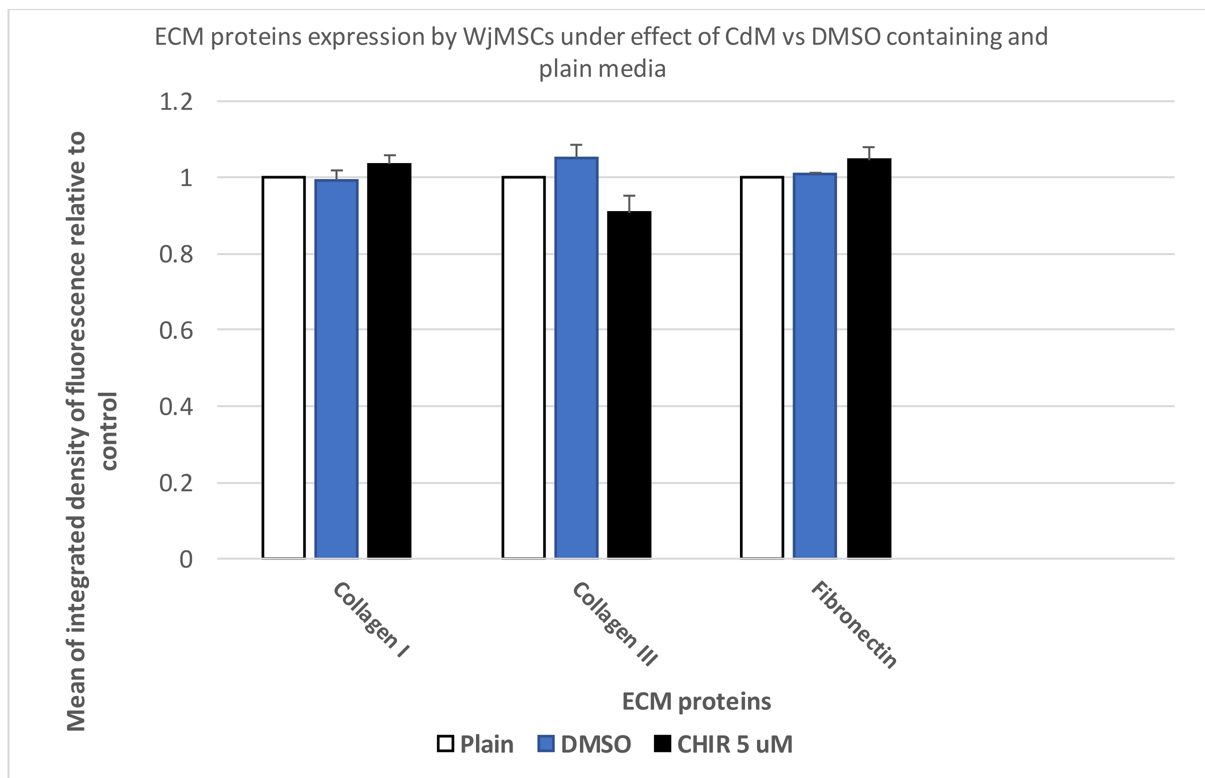

Figure 4-15. Effect of CHIR99021 at 5 uM on wjMSCs expression of extracellular matrix proteins; collagen I, collagen III and fibronectin.

WjMSCs treated with CHIR were examined for ECM proteins and DMSO as well as plain media treated cells used as control. No statistically significant difference between the CHIR group and their control, p=0.4, 0.2 and 0.4, respectively. N=3. Expressed as mean +/- SEM.

4.6.4.3. CHIR upregulates HGF while no significant effect on angiopoietin, bFGF, VEGF, SDf1 and MCP1 in wjMSCs

When examined the effect of CHIR on wjMSCs expression of the angiogenic factors we found HGF to be the only factor significantly upregulated while no significant effect on the rest of examined factors.

Angiopoietin-CHIR (M= 1.067, SEM= 0.04) compared to Angiopoietin-DMSO (M= 1.01, SEM= 0.02) relative to Angiopoietin-cnt demonstrated no significant difference, p= 0.2.

bFGF-CHIR (M= 0.99, SEM= 0.003) compared to bFGF-DMSO (M=1.004, SEM= 0.026) relative to bFGF-cnt demonstrated no significant effect, p= 0.8.

HGF-CHIR (M= 1.033, SEM=0.007) compared to HGF-DMSO (M=1.016, SEM= 0.009) relative to HGF-cnt demonstrated significant change, p= 0.03. Further analysis revealed upregulation of CHIR group relative to plain control, p=0.02.

VEGF-CHIR (M= 1.048, SEM= 0.03) compared to VEGF-DMSO (M= 1.026, SEM=0.017) relative to and VEGF-cnt demonstrated no significant difference, p= 0.3.

SDf1-CHIR (M=1.058, SEM= 0.05) compared to SDf1-DMSO (M=1.044, SEM= 0.05) relative to SDf1-cnt demonstrated no significant difference, p= 0.6.

MCP1-CHIR (M=1.008, SEM= 0.01) compared to MCP1-DMSO (M=1.001, SEM= 0.004) relative to MCP1-cnt demonstrated neutral effect, p= 0.6.

Despite the limited positive value here but still HGF is quite valuable angiogenic factor. This is considered a positive result as there was no downregulation of any factor in addition to the significant upregulation of the HGF.

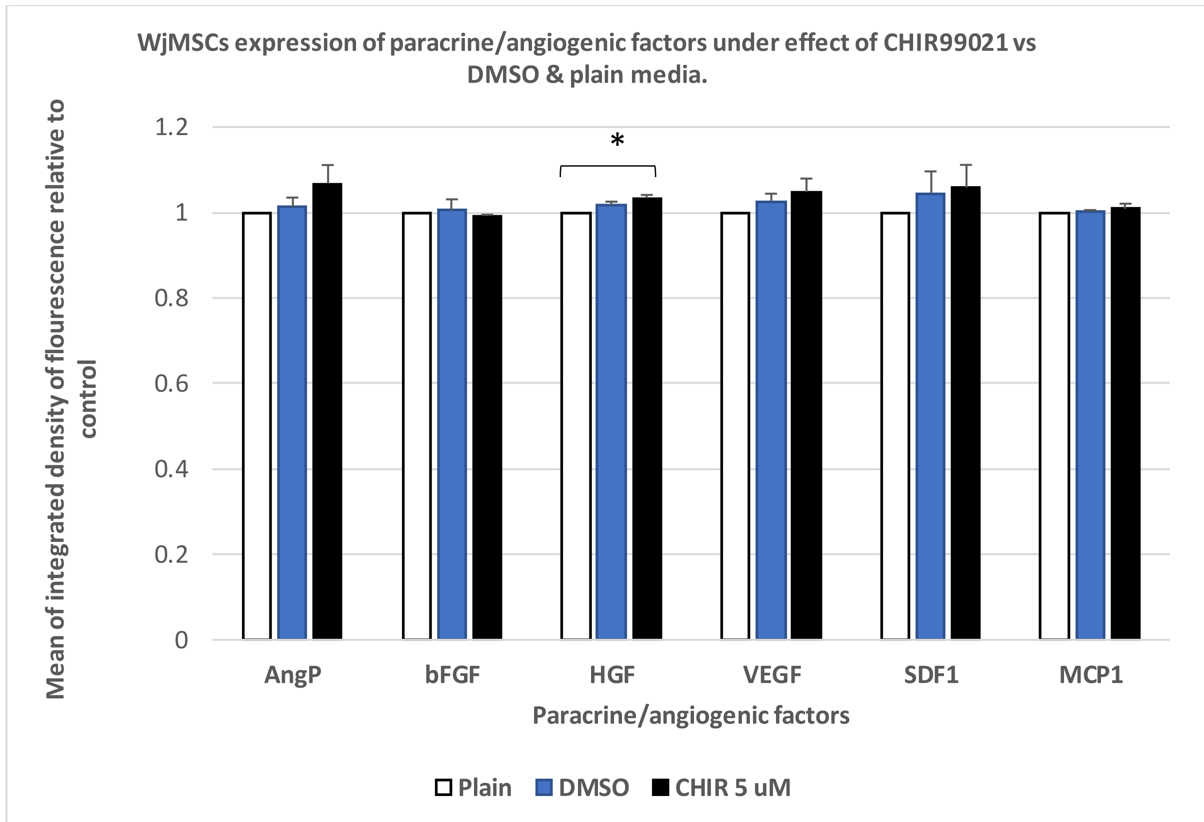

Figure 4 16. Effect of CHIR99021 at 5 uM on wjMSCs expression of paracrine/angiogenic factors.

CHIR effect on wjMSCs secretion of angiogenic factors was examined and compared with effect of DMSO as well as plain media as control. Results revealed that HGF showed significant upregulation in CHIR treated cells relative plain media cells, p=0.02 while Angipoietin, bFGF, VEGF, SDf1 and MCP1 factors demonstrated no statistically significant difference between the CHIR group and the control, p=0.2, 0.8, 0.3, 0.6 and 0.6 respectively. N=3. Expressed as mean +/-SEM.

4.6.4.4. CHIR upregulates N-cadherin while did not have statistically significant effect on β-catenin, Endoglin or connexin43 in wjMSCs

On assessment of CHIR effect on wjMSCs expression of β-Catenin and other adhesion/communication factors, the N-Cadherin was the only factor significantly upregulated while the rest showed non-significant change.

β-catenin-CHIR (M= 1.073, SEM= 0.046) compared to β-catenin-DMSO (M= 1.028, SEM= 0.0069) relative to β-catenin-cnt demonstrated no significant change, p= 0.2.

N-cadherin-CHIR (M= 1.06, SEM= 0.015) compared to N-cadherin-DMSO (M= 1.035, SEM= 0.007) relative to N-cadherin-cnt demonstrated significant change, P= 0.01. Post hoc Tukey analysis revealed significant upregulation in the CHIR group relative to plain media control, p= 0.01.

Endoglin-CHIR (M= 1.06, SEM= 0.02) compared to Endoglin-DMSO (M= 1.029, SEM= 0.036) relative to Endoglin-cnt demonstrated no significant difference, P= 0.2.

Cx43-CHIR (M= 1.047, SEM= 0.03) compared to Cx43-DMSO (M=1.034, SEM= 0.014) relative to Cx43-cnt demonstrated no significant change, P= 0.2.

Despite the N-cadherin was the only statistically upregulated factor, however, other factors did not show any down regulation which means still the cells would maintain their basal level of these factors and would not predict negative impact.

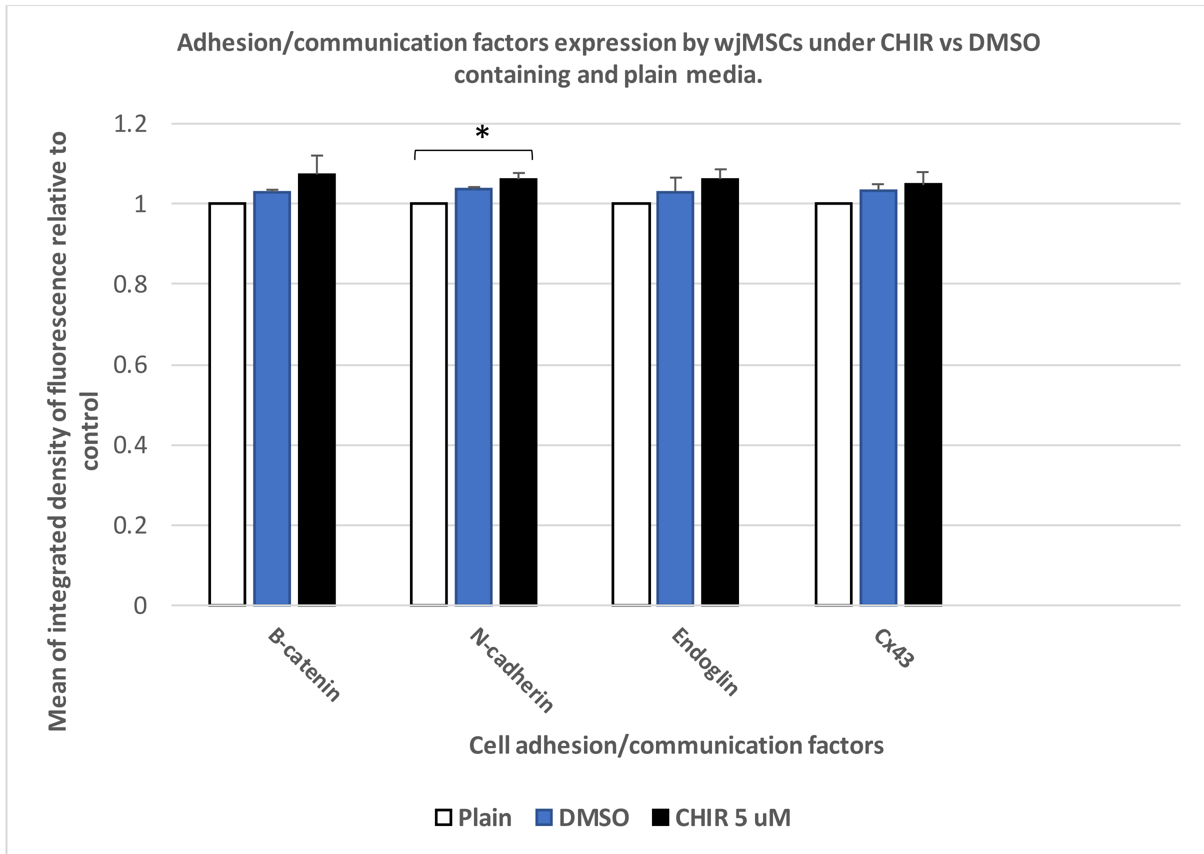

Figure 4-17. Effect of CHIR99021 at 5 uM on wjMSCs expression of adhesion/communication factors.

CHIR effect on wjMSCs was examined as regard the adhesion/communication factors and wjMSCs incubated with DMSO as well as plain media were used as control. N-cadherin showed significant upregulation in CHIR treated cells relative plain media cells, p=0.01 while β-catenin, Endoglin and Cx43 demonstrated no statistically significant difference between the CHIR group and their control, p=0.2, 0.2 and 0.2 respectively. N=3. Expressed as mean +/- SEM.

4.6.4.5. CHIR does not support wjMSCs survival

In order to find out the effect of CHIR on wjMSCs survival. The caspase/DRaq7 assays were used. The apoptotic wjMSCs under the effect of CHIR were comparable with the control in plain and DMSO containing media. WjMSCs apoptotic percent: CHIR (M= 3.08,SEM=0.13), DMSO (M= 2.64,SEM=0.48) and plain (M= 2.005,SEM=0.39), p=0.9. Apparently, apoptosis in the CHIR treated wjMSCs was higher but it did not meet statistical significance.
However, there was significant difference in the percent of dead WjMSCs. CHIR (M=4.52, SEM= 0.6), DMSO (M=1.35,SEM=0.12) and plain (M= 1.76,SEM=0.1), p=0.01. The post Hoc analysis revealed that the CHIR condition was significantly higher relative to both control conditions.
Indeed, these results are consistent with the effect on the pro-survival/anti-apoptotic factors Akt, BCl-2 and NRG1 as it did not significantly affect these proteins and therefore it was expected that survival will not be enhanced. Also, in keeping with the report of Qiu et al. who noticed more dead cells in the CHIR treated cells as well as their results that demonstrated more nuclear accumulation of phosphorylated p53 in the CHIR treated cells. Unfortunately, we could not analyse the p53 response in our project (despite it was already on our radar) due to the shut down situation. However, it is more likely that the cells' survival was negatively impacted via a p53 dependent mechanism (333).

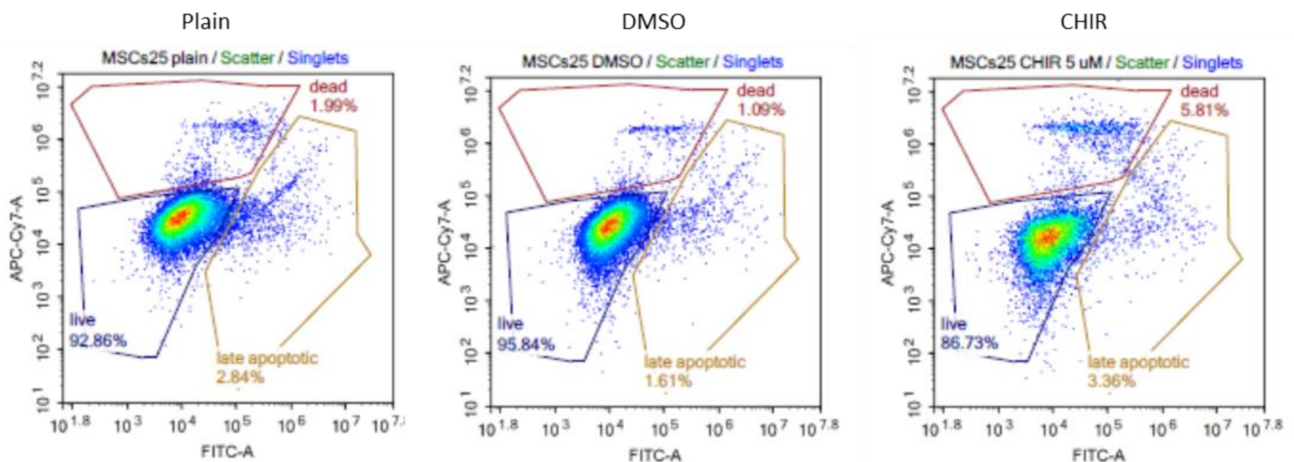

Figure 4-18.*Representative images* of Caspase apoptosis and DRaq7 dead cell assays of wjMSCs.

Effect of CHIR99021 at 5 uM on wjMSCs survival as compared with DMSO and plain media. The percent of apoptotic cells was comparable between the three conditions, p= 0.9 while the percent of dead cells was higher in the CHIR condition, p=0.01. N=3.

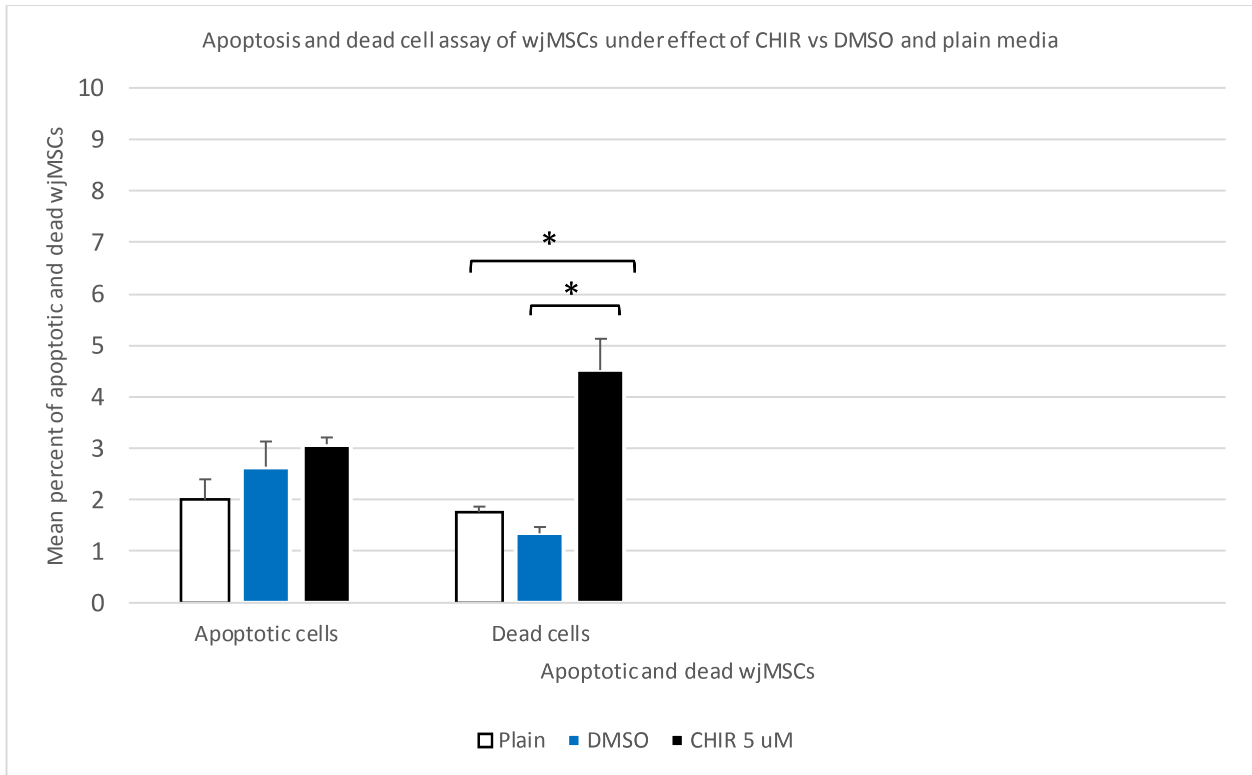

Figure 4-19. *Caspase apoptosis and DRaq7 dead cell assays of wjMSCs under effect of CHIR99021 5 uM.*

Effect of CHIR99021 at 5 uM on wjMSCs survival as compared with DMSO and plain media. The percent of apoptotic cells was comparable between the three conditions, p= 0.9 while the percent of dead cells was higher in the CHIR condition, p=0.01. N=3, expressed as mean +/-SEM.

4.6.4.6. CHIR does not affect wjMSCs proliferation

As mentioned earlier, we plan to seed the patch with a triad of cells that do not exist in native tissue. So, we had a concern about one cell phenotype to overpopulate the other two cell lines. During assessment of CdM effect on wjMSCs proliferation the results showed non-significant difference which was quite assuring.

So, in order to find out if CHIR treatment would influence wjMSCs proliferation BrdU assay was used. There was no significant difference in wjMSCs proliferation potential between the 3 conditions; CHIR (M=0.11, SEM=0.01), DMSO (M=0.11, SEM=0.01) and plain media (M= 0.11, SEM= 0.008), p= 0.9. This indicates no significant difference between the 3 conditions and is a positive sign of balanced growth.

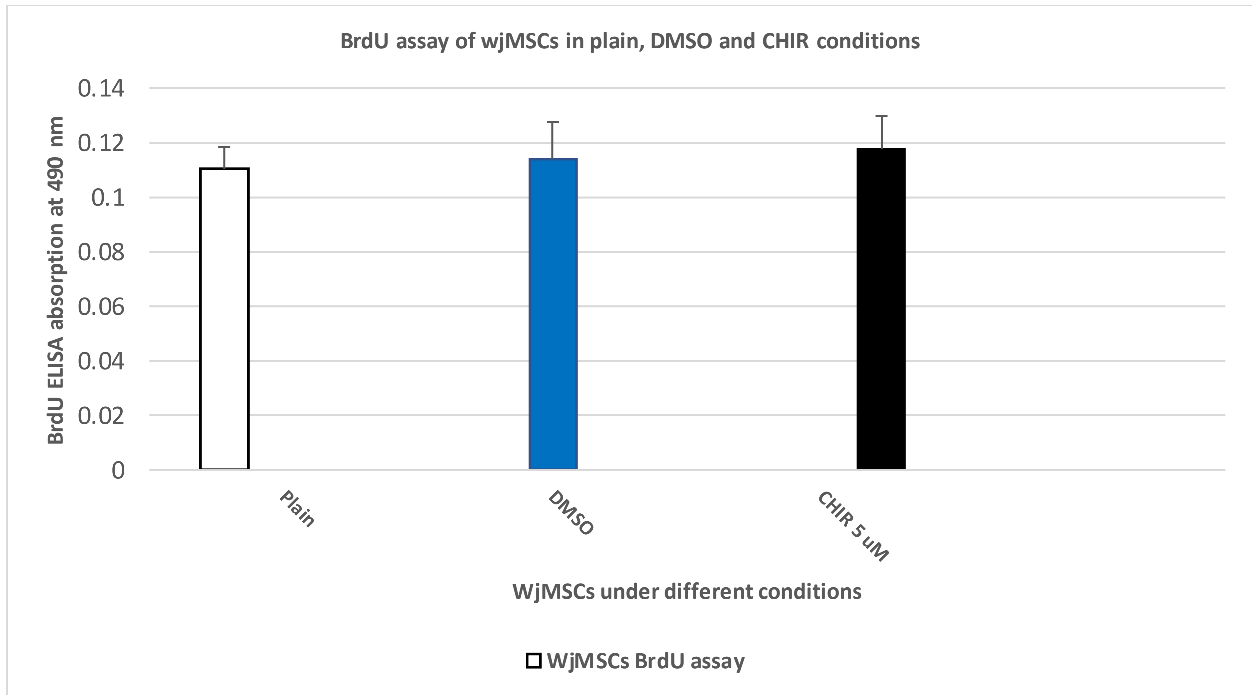

Figure 4-20. Effect of CHIR99021 5 uM on wjMSCs proliferation using BrdU assay.

WjMSCs proliferation was assesed under effect of CHIR, DMSO as well as plain media. No significant difference in cell proliferation between the 3 conditions, p=0.9. N= 3, expressed as mean+/- SEM.

4.7.Combined effect of CHIR and CdM on WjMSCs

Initially, we tested the effects of CdM on wjMSCs at six relevant domains. CdM effects were promising with positive effects mainly on the angiogenic profile. Then, we tested the effect of CHIR99021 considering the same domains. Apart from the negative impact on cells' survival it did not seem to have other negative impacts. Indeed, it was fluctuating between neutral effects on most preset parameters to limited positive effects which was still supportive to go forward with this approach.

So, here we attempt to examine the net effect of CHIR99021 at 5 uM when combined with CdM. Indeed this is our target as we plan to treat the cells with CHIR 5 uM then combine them together on patch.

Afterwards, will refer to the combined effect of CHIR99021 at 5 uM with conditioned media as CHIR/CdM. Cells in CdM were used as control as the aim is to find out any significant difference if we treat the cells with CHIR versus if we just seed them without any treatment.

4.7.1.Objectives

To assess the combined effects of CHIR/CdM on wjMSCs with focus on survival factors, ECM proteins, angiogenic factors, cell acommunication factors, cell apoptosis/death and proliferation.

4.7.2.Results

After treatment of wjMSCs with CHIR/CdM for 48-72 hours and wjMSCs cultured in CdM were used as control we got the following results

4.7.2.1.CHIR/CdM does upregulate Akt but no significant effect on BCl-2 and NRG1 in wjMSCs relative to CdM

The first domain for assessment is the pro-survival factors Akt, BCl-2 and NRG1. As explained earlier, we had the pro-apoptotic factors Bax and (BAD) (335, 336) and the tumour suppressor p53 as one of the apoptosis control factors (337) on our list for assessment to get better overview but the sudden suspension of our laboratory operations prevented from moving forward with the rest of factors. There was significant upregulation of Akt in wjMSCs under the combined effect of CHIR/CdM relative to Akt expression in wjMSCs under CdM effect. Akt-CHIR/CdM (M= 1.016, SEM=0.009) compared to Akt-CdM revelaed significant upregulation, p= 0.02 which indicates positive survival effect as well as its paracrine effect.

However, the expression of BCl2-CHIR/CdM (M= 1.008, SEM=0.059) compared to BCl2-CdM revelaed no significant difference, p= 0.3. Also, NRG1-CHIR/CdM (M= 1.03, SEM=0.009) compared to NRG1-CdM revelaed no significant difference, p= 0.9.

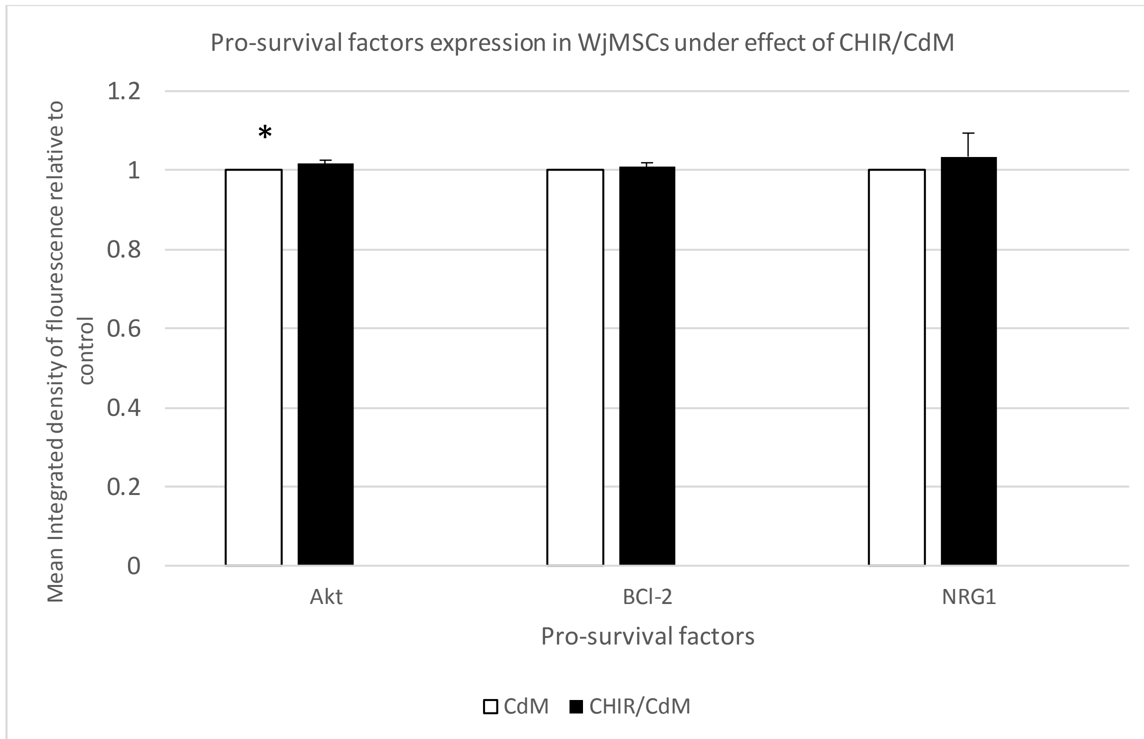

Figure 4-21. Expression of prosurvival factors Akt, BCl-2 and NRG1 in wjMSCs under effect of CHIR/CdM relative to CdM**.*

The combined effect of CHIR/CdM on wjMSCs compared with the effect of CdM showed that Akt was significantly upregulated, p=0.02 while BCl-2 and NRG1 did not demonstrate significant difference between the two conditions, p=0.3 and 0.9 respectively. N= 3, expressed as mean +/-SEM.

*CHIR/CdM: combination of CHIR99021 at 5 uM with conditioned media.

**CdM: conditioned media from co-culture of HUVECs and HCFs.

4.7.2.2.CHIR/CdM does not affect ECM proteins expression by wjMSCs relative to CdM

The next assessment domain was the wjMSCs expression of ECM proteins to find out any significant change of ECM production. Expression of these ECM proteins under effect of CHIR/CdM was comparable with their levels under CdM effect.

The expression of collagen I-CHIR/CdM (M= 0.97, SEM=0.029) compared to Collagen I-CdM revelaed no significant difference, p= 0.8.

Also, collagen III-CHIR/CdM (M= 0.97, SEM=0.03) compared to Collagen III-CdM revelaed no significant difference, p= 0.7.

And, fibronectin-CHIR/CdM (M= 0.99, SEM=0.01) compared to fibronectin-CdM revelaed no significant difference, p= 0.8.

This non-significant effect eliminates the concern of significant fibrotic changes that would negatively impact the patch's elasticity and functionality.

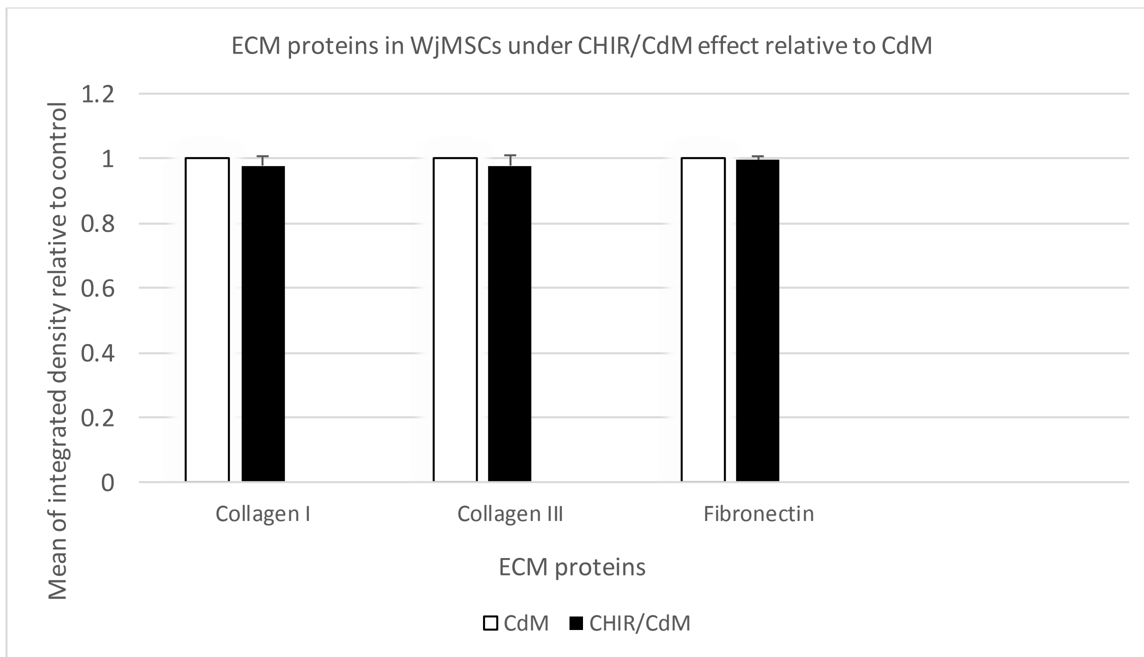

Figure 4-22.Expression of ECM proteins in wjMSCs under effect of CHIR/CdM** relative to CdM***.*

Assessment of the effect of CHIR/CdM relative to CdM on wjMSCs demonstrated that expression of collagen-I, collagen-III and fibronectin all were statistically comparable and did not demonstrate significant difference between the two conditions, p=0.8, 0.7 and 0.8 respectively.N=3.Expressed in mean +/-SEM.

*ECM: extracellular matrix.

**CHIR/CdM: combination of CHIR99021 at 5 uM with conditioned media.

***CdM: conditioned media from co-culture of HUVECs and HCFs.

4.7.2.3. CHIR/CdM does not have significant effect on angiogenic factors relative to CdM in wjMSCs

The next assessment domain was the angiogenic factors. Expression of Angiopoietin, bFGF, HGF, VEGF, SDF1 and MCP1 under effect of CHIR/CdM was comparable with their expression under CdM effect.

Expression of Angiopoietin-CHIR/CdM (M= 0.99, SEM=0.006) compared to Angiopoietin-CdM revelaed no significant difference, p=0.06. bFGF-CHIR/CdM (M= 1.03, SEM=0.01) compared to bFGF-CdM revelaed significant upregulation, P= 0.2.

HGF-CHIR/CdM (M= 0.99, SEM=0.02) compared to HGF-CdM revelaed no significant difference, P=0.8. VEGF-CHIR/CdM (M= 1.045, SEM=0.05) compared to VEGF-CdM revelaed no significant effect, p=0.5. SDf1-CHIR/CdM (M= 1.058, SEM=0.07) compared to SDf1-CdM revelaed no significant effect, p=0.4. And MCP1-CHIR/CdM (M= 1.015, SEM=0.007) compared to MCP1-CdM revelaed no significant difference, p=0.05.

These data indicate that the addition of CHIR would not significantly affect the angiogenic profile of wjMSCs. However, they would still maintain their level of secrection post CdM exposure. Considering the previous superior results of CdM effects so, this is expected to be maintaned post CHIR treatment with no inferiority at the angiogenic profile level.

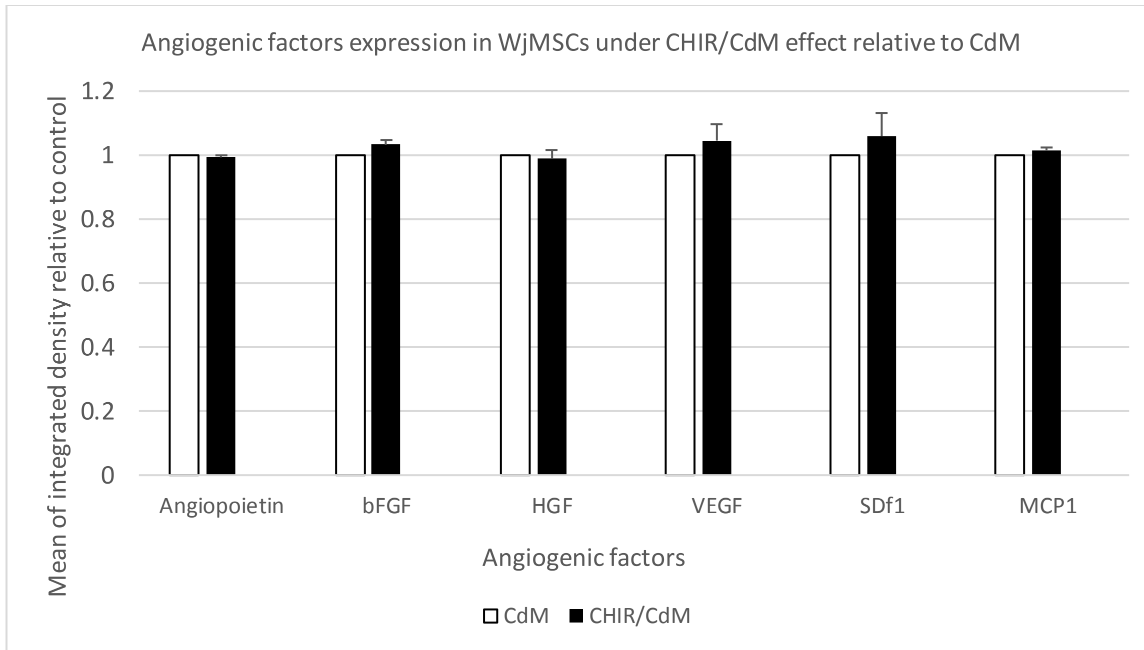

Figure 4-23.Expression of angiogenic factors in wjMSCs under effect of CHIR/CdM relative to CdM**.*

The angiogenic profile of wjMSCs under effect CHIR/CdM was compared to effect of CdM and revealed that WjMSCs expression of Angiopoietin, bFGF, HGF, VEGF, SDf1 and MCP1 all were statistically comparable and did not demonstrate significant difference between the two conditions, p=0.06, 0.2, 0.8, 0.5, 0.4 and 0.05 respectively. N=3. Expressed as mean +/- SEM.

*CHIR/CdM: combination of CHIR99021 at 5 uM with conditioned media.

**CdM: conditioned media from co-culture of HUVECs and HCFs.

4.7.2.4. CHIR/CdM upregulates β-catenin and Connexin 43 (Cx43) while comparable effects on N-cadherin and endoglin relative to CdM in wjMSCs

Then, assessment of wjMSCs expression of the adhesion/communication factors under the combined effect of CHIR/CdM revealed upregulation of β-catenin and Cx43 as compared with their expression under CdM effect while expression of N-cadherin and Endoglin was comparable in both conditions.

β-catenin-CHIR/CdM (M= 1.035, SEM=0.004) compared to β-catenin-CdM revelaed significant upregulation, p=0.001.
Also, Cx43-CHIR/CdM (M= 1.062, SEM=0.02) compared to Cx43-CdM revelaed significant upregulation, p= 0.01.
While, effect on N-Cadherin and Endoglin was non significant. N-cadherin-CHIR/CdM (M= 1.0029, SEM=0.04) compared to N-cadherin-CdM revelaed no significant difference, p=0.6.

And Endoglin-CHIR/CdM (M= 1.05, SEM=0.048) compared to Endoglin-CdM revelaed no significant difference, p=0.1.

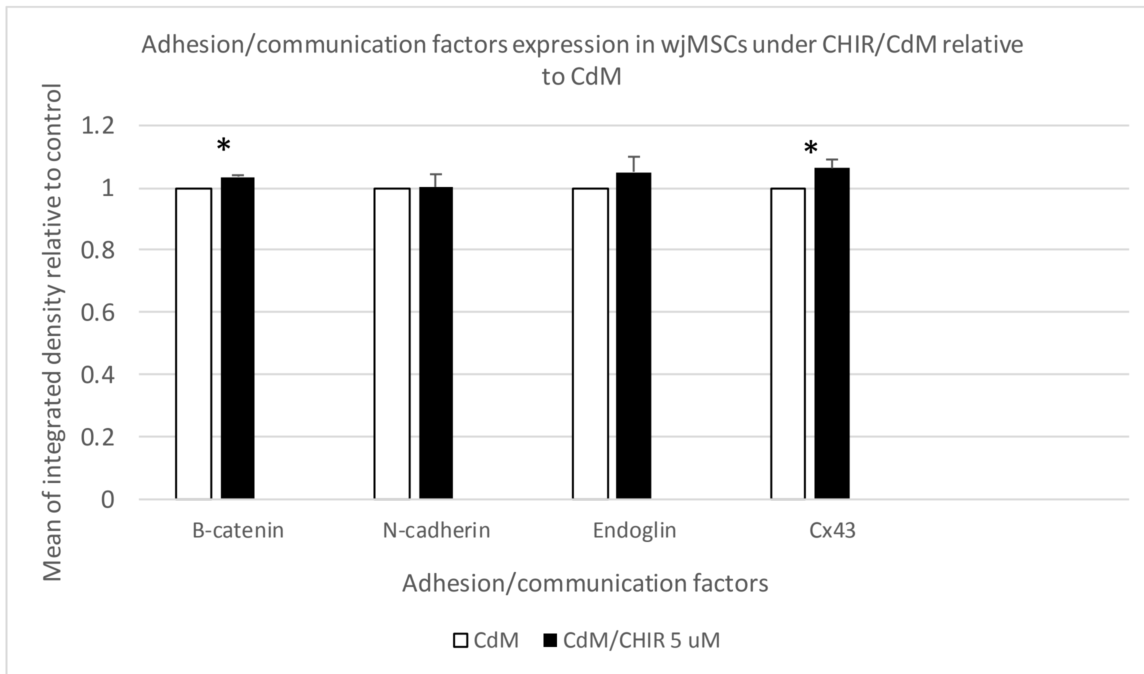

Figure 4-24. Expression of adhesion/communication factors in wjMSCs under effect of CHIR/CdM relative to CdM**.*

Effect of CHIR/CdM on wjMSCs compared with effect of CdM revealed that B-catenin and Cx43 were significantly upregulated, p=0.001 and 0.01 respectively. While N-cadherin and Endoglin were statistically comparable and did not demonstrate significant difference between the two conditions, p=0.6 and 0.1 respectively. N=3, expressed as mean +/- SEM.

*CHIR/CdM: combination of CHIR99021 at 5 uM with conditioned media.

**CdM: conditioned media from co-culture of HUVECs and HCFs.

4.7.2.5. CHIR/CdM enahances wjMSCs survival.

After the initial results with negative impact on wjMSCs survival in both CdM as well as CHIR, here we attempt to examine if the combination of CHIR/CdM would offer influence survival. We used caspase apoptosis and DRaq7 dead cell assay.

Apoptotic- CHIR/CdM (M=1.73, SEM=0.62) was signficantly lower than apoptotic-CdM (M=3.73, SEM=0.19), p= 0.04.

While Dead-CHIR/CdM (M=2.87, SEM=0.72) was statistically comparable to dead-CdM (M=4.33, SEM=1.35), p= 0.3, which seems to be secondary to the small number and scattered data.

These data are limited by the N=2 for the CdM samples, however it is quite supportive to adopt this approach as it suggests significant survival benefit. This effect is consistent with other results of significant Akt upregulation (324). Also, in keeping with the upregulation of β-catenin that was reported to enhance MSCs survival and engraftment (338).

CdM CdM/CHIR

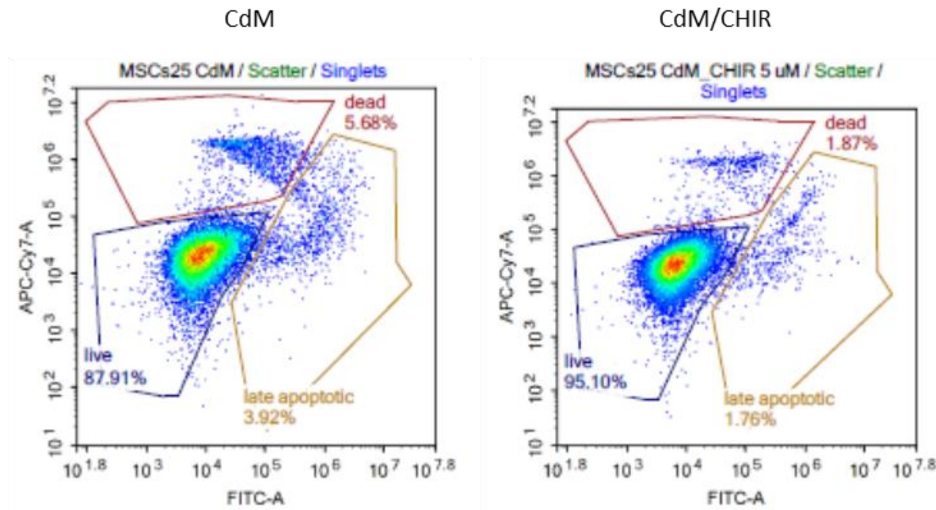

Figure 4-25.Representative images of FCM results of caspase apoptosis and DRaq7 dead cell assay of wjMSCs in CdM** and CHIR/CdM***.*

The combination of CHIR/CdM had significantly lower apoptosis rate as compared with CdM effect.

*FCM: flow cytometry.

**CdM: conditioned media from co-culture of HUVECs and HCFs.

***CHIR/CdM: combination of CHIR99021 at 5 uM with conditioned media.

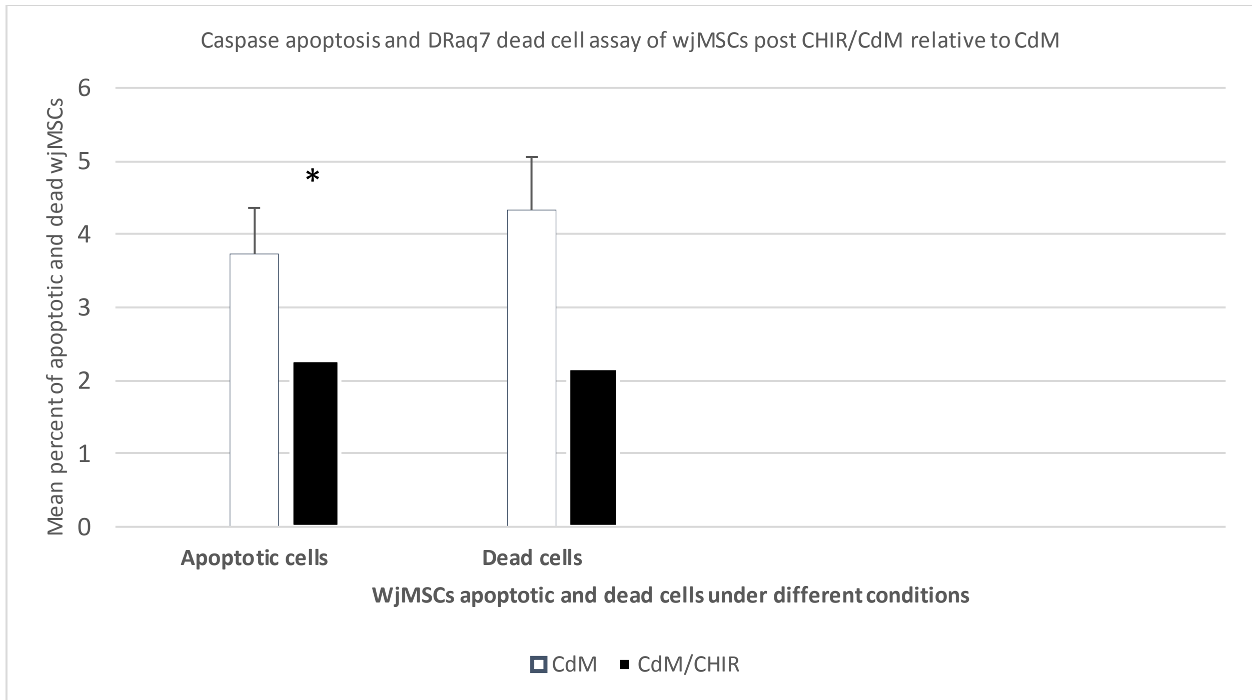

Figure 4-26.FCM results of caspase apoptosis and DRaq7 dead cell assay of wjMSCs in CHIR/CdM* and CdM**.*

Effect of CHIR/CdM on wjMSCs survival revealed significantly lower apoptosis rate relative to CdM. N=3 for the CHIR/CdM but N=2 for the CdM. Expressed as mean +/- SEM.

*FCM: flow cytometry.

**CdM: conditioned media from co-culture of HUVECs and HCFs.

***CHIR/CdM: combination of CHIR99021 at 5 uM with conditioned media.

4.7.2.6. CHIR/CdM does not affect wjMSCs proliferation potential as compared to CdM

In order to find out if the combination of CHIR/CdM would influence wjMSCs proliferation, BrdU assay was used.
The wjMSCs Brdu assay in CdM (M= 0.115, SEM= 0.01) was statistically comparable with the CHIR/CdM (M= 0.11,SEM= 0.004), p= 0.8.
This indicates no significant difference between the 2 conditions and assures about balanced growth with no concern about overpopulating the other two cell lines.

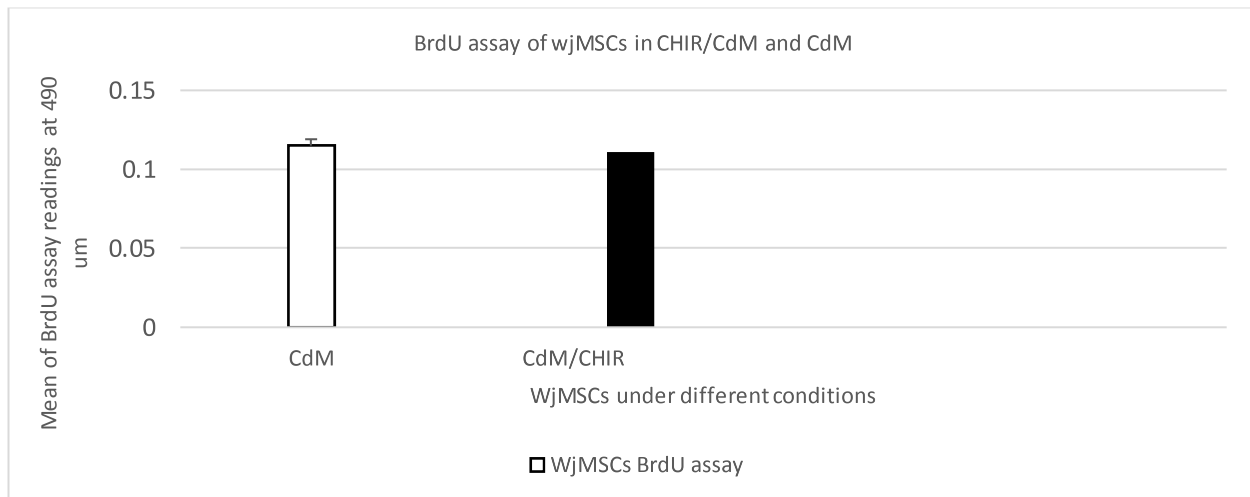

Figure 4-27.BrdU assay to assess wjMSCs proliferation potential under CHIR/CdM effect in comparison to CdM**.*

WjMSCs proliferation in both conditions was comparable with no significant difference. N=2.Expressed as mean +/- SEM.

*CHIR/CdM: combination of CHIR99021 at 5 uM with conditioned media.

**CdM: conditioned media.

4.8.Discussion

4.8.1.Introduction

CHDs are the most prevalent congenital anomalies world wide and the most common cause of birth defects related death (339). Also the burden of CHDs goes beyond the pediatric age to later life. It was estimated that about 500,000 adults in the USA have some form of CHD (81) and significant percent of cases would require surgery. Surgical intervention sometimes need redo(s) due to the mismatch between the static graft size and the dynamic growth of the heart/blood vessels with all subsequent social and financial burden (339) which is a significant drawback of the current practice.

Several materials were used for reconstructive cardiac surgery including the biologic scaffold cormatrix which has long clinical record with proved durability but still has some limitations. One of these limitations is its inherent no-growth potential. In addition, the human studies failed to demonstrate any evidence of repopulation with native cells or meaningful tissue like pattern of cells (151). This drawback means repeated surgeries with all subsequent cost and hazards.

Our group trialled to overcome this drawback through seeding cormatrix with MSCs as monocellular approach and this proved superior to the acellular cormatrix in porcine models (340). However, it was notable that cells do not last for long. Indeed, they disappear from the scene within short period and therefore the positive results are most probably secondary to other factors rather than cellular engagement. This is actually consistent with reports from other groups and still the underlying mechanisms are not fully understood (324).

In this project we did modify our approach of monocellular seeding to multicellular seeding. Also, tried to figure out the possible underlying mechanisms that would govern the MSCs effects. In addition, we trialled to enhance the properties of the patch via treatment of cells with small molecules in an attempt to acquire better properties and better survival that is expected to reflect on the patch's uptake and functionality.

So, the objectives of this chapter were to gain the following insights (i) how WjMSCs would react when combined with HUVECs and HCFs ?, (ii) is it possible to differentiate WjMSCs to CMCs using 5-azacitidine?, (iii) what are the effects of CHIR99021 at 5 uM on WjMSCs as regard the domains of interest?, and (iv) what would be the net effect of CHIR99021 at 5 uM on WjMSCs when combined with HUVECs and HCFs ?

Answering these questions would highlight the on-patch behaviour of wjMSCs. Also, would help to predict their potential in-vivo behaviour as well. Obviously, this would be within the limits of uncomaparable complexity of the in-vivo environment.

After implanting the patch in-vivo it is expected to undergo a process quite close to wound healing in order to get integrated into neighbouring myocardium. This would require cellular proliferation, migration, neovascularisation as well as ECM production (341). Therefore, assessment of celular behaviour is invaluable to anticipate their suitability for transplantation.

Assessment was done at six domains including pro-survival/anti-apoptosis factors, ECM proteins, angiogenic/paracrine factors, cellular adhesion/communication factors, cell survival and cell proliferation.

4.8.2.CdM effect on wjMSCs

4.8.2.1.Survival factors

The first domain of assessment was the survival factors including Akt, BCl-2 and NRG1 which represent pro-survival, anti-apoptotic and trophic factors, respectively. Bak, Bax and P53 were on our radar for assessment but due to the sudden unexpected shut down of the University it was not feasible to be examined.

Our results showed that Akt was significantly upregulated under effect of CdM while BCL-2 and NRG1 did not show any signifcant alteration under effect of CdM.

Protein kinase B (Akt) is a serine threonine kinase that is considered a main player in several cellular processes such as survival, proliferation, differentiation as well as neovascularisation and acts downstream of the IGF1/PI3K pathways (317, 321, 342-344). Akt holds survival provoking effect through activation of growth factors and inactivation of pro-apoptotic factors. So, Akt null cells are more vulnerable to apoptosis with less stress resistance and shorter lifespan (343, 345). Akt survival effects are mediated via downstream factors including BCl-2 and there is evidnce that inhibition of Akt leads to BCL-2 downregulation (321, 346, 347).

The PI3kα/Akt signaling is expressed in the CMCs and was proven to enhance post natal CMCs growth and survival (348). Activation of Akt was associated with less atherosclerotic changes and better survival and metabolism of cardiac cells to resist the oxidative stress and support survival of CMCs as well as VSMCs. On the other hand, absence of Akt was associated with enhanced atherosclerosis and lower ECs viability with less NO production (343).

The study done by Mangi and colleagues was quite informative about the potent survival effect of Akt. They did over-express Akt in MSCs and transplanted into infarct area in a rat model. The MSCs over-expressing Akt significantly reduced post ischemia inflammation with collagen deposition and demonstrated better recovery of myocardial function with reduced remodelling (235). These findings are indeed consistent with the study of Sang lim and colleagues to emphasise on the cytoprotective effect of Akt (281). In this study, Sang and colleagues did transduce MSCs with Akt gene to over-express Akt and then transplanted via intracoronary root into infarct area in porcine model. The follow up done with echocardiography as well as SPECT to assess the effect on LV function as well as the infarct area. The results showed significant recovery of LV EF and consistent reduction of infarct area size in the group transplanted with the Akt over-expressing MSCs.

Our results showed that Akt was significantly upregulated under effect of CdM. This indicates positive reaction to the combination with HCFs/HUVECs with the potential of this treatment to enhance its cardioprotective effects.

The second assessed factor was the B cell lymphoma (BCl-2) factor. BCl-2 is a key regulator of apoptosis pathways to inhibit cell death (349). Also acts as a mediator for Akt to maintain cell survival and down-regulate apoptosis (321). It is expressed in cells with long lifespan such as neurones and cells with self-renewal via mechanisms involving apoptosis such as colonic epithelium (350). In the heart, it is not normally expressed, but was demonstrated to be expressed in case of inflammatory or stress conditions such as ischemia, arrhythmia and in post-transplant hearts which could represent a cytoprotective mechanism (351). When expressed, it would salvage myocardium via repressing CMCs apoptosis through regulating mitochondrial function in case of ischemic events and limits its degradation (352).

MSCs overexpressing BCl-2 did show increased viability in-vivo with higher density of neovascularisation and better functional outcome when transplanted in myocardial infarction area (236). In the study done by Wenzhong and colleagues, they did over-express BCl2 in MSCs and transplanted to myocardial infarct area in a rat model. There was significant retention of MSCs post-transplant which reflects better survival with less infarct size. Also better vascularisation demonstrated by significantly higher capillary density in the MSCs-BCl2 transplanted group. These findings indicate better survival as well as superior angiogenic profile of MSCs with upregulation of BCl2 (236).

Our data show that Bcl2 expression under CdM effect was comparable with the control. This indicates no extra BCl-2 dependent survival advantage would be offered by the wjMSCs when combined with HCFs and HUVECs. However, still its baseline production is there and expected to offer a basal level of survival support via apoptosis inhibition when seeded in vivo.

The third factor in this domain was the Neuregulin (NRG1). NRG1 is one of the epidermal growth factors family (353) which is expressed by several cell phenotypes including the endothelial cells in the adult heart. NRG1 and its receptors ERbB family of tyrosine kinases are crucial players during cardiovascular development as well as to maintain functional status of the adult heart via acting as paracrine factors to adjust cell-cell interaction (354). NRG1 has the potential to activate myocardial regeneration via promoting cardiomyocytes to re-enter the cell cycle and resume proliferation potential (51, 355). Therefore, NRG1 is a pro-survival factor for CMCs and is one of the MSCs paracrine profile.

Our data show that Its expression by wjMSCs was not significantly affected by CdM. Also, its mRNA level was downregulated under CdM effect. These results suggest NRG1 protein expression within this time window is not transcription dependent. In addition, wjMSCs when seeded in combination with HUVECs and HCFs are not expected to give extra NRG1 based support to the neighbouring myocardium. However, still the basal level of wjMSCs support via BCl2 and NRG1 are maintained as the results did not demonstrate any down regulation of these proteins and neighbouring CMCs are expected to benefit from this paracrine support.

Despite WjMSCs expression of Akt was significantly elevated, it did not reflect on cells survival. Indeed, apoptosis and cell death of wjMSCs in CdM were significantly higher as compared with the control. First, we have to admit that the data are limited with N=2 in the CdM samples. Nevertheless, this could be explained by the fact that the BCl-2 which is an Akt mediating factor as well as NRG1 did not show any significant change in wjMSCs-CdM. Also, other apoptosis regulating factors were not examined.

On the other hand, mere Akt upregulation is a promising result. It indicates that the combination of wjMSCs with HUVECs and HCFs would be expected to support survival of CMCs in neighbouring myocardium. Also, AKt acts as a downstream effector for VEGF and angiopoietin and enhances NO production and therefore, it is expected to enhance ECs survival and angiogenesis in-vivo (356). So, in addition to its predicted support to CMCs survival, it is also expected to enhance angiogenesis and reduce remodelling as well (357).

Our data are suggestive of no extra survival support would be offered to the wjMSCs when co-seeded with HUVECs and HCFs. This could explain the early loss of MSCs when transplanted in vivo as they would be exposed to stress of inflammatory effects of surgery as well as the oxidative stress in case of ischemia reperfusion and would not hold clear protective mechanisms.

4.8.2.2.ECM proteins

The second assessment domain consisted of the ECM proteins collagen I, collagen III which are the main collagens in the myocardial ECM (358). Also, the glycoprotein fibronectin that acts as one of the mediators in attachment of collagen to sarcolemma (359). In addition, it is a fibrous protein that helps to organise the ECM and facilitates cell adhesion (53).
Collagen III showed significant downregulation under CdM effect while collagen I and fibronectin expression was comparable with the control.

Collagens have crucial structural role in the myocardium under physiologic conditions. They determine the tissue architecture and geometry of cardiac chambers with significant spatiotemporal variability and flixible levels of expression to accommodate different demands (360). In addition to their role in pathologic conditions including the remodelling process which underpins the pathology of heart failure. Indeed, cardiac remodelling is mainly a fibrotic process with gradual deposition of collagens (359). After ischemic event, the ECM secretion is markedly increased with deposition of collagen III initially which is replaced at a later stage with collagen I. Then gradually the collagens get mature with cross-linking to enhance their tensile strength and achieve full maturity by 8 weeks (361).

Since over secretion of ECM proteins is a feature of fibrotic changes in stressed myocardium such as in hypertensive patients and post infarction as part of the remodeling process. Also it is a feature of myocardial aging that is associated with increased stiffness with less contractility (359). Therefore, ECM protein expression by wjMSCs was assessed and we focused on wjMSCs expression of the two main collagens and fibronectin to find out if any potential overproduction as an indicator of remodeling and scarring or still balanced secretion.
Therefore, assessment of MSCs production of ECM is important as it would influence the mesh that holds the cells and reflect on the integrity as well as the functionality of the patch (362). In addition, due to the dynamic nature of the heart function, the ECM production by cells used for any cardiac tissue engineering should hold some dynamic response to variable degrees of stress.

In the study of Yacoub and co-workers they did expose MSCs to mechanical stretch at different degrees and estimated the collagen deposition relative to the native aortic valve cells. MSCs did demonstrate dynamic response to mechanical stress and their ECM production was found comparable to valve interstitial cells at 14% degree of stretch. This indicated positive potential for MSCs to accommodate and favourably react to mechanical stretch and supports use of MSCs as reasonable cell phenotype for cardiac tissue engineering purposes (362). The interesting result in this study was the close similarity between MSCs and aortic valve interstitial cells in terms of ECM production and interaction with the applied forces which indicates suitability of MSCs to support tissue engineering of a cardiac valve (362).

Our data demonstrate that expression of collagen I by wjMSCs was comparable in both CdM and control conditions. On the other hand, expression of collagen III, which is the second most common collagen in the myocardium, by wjMSCs under CdM effect was significantly downregulated.

These results are considered positive findings as no extra collagen production. Also, indicates better remodeling profile hence down-regulation of collagen production is considered as part of the favorable MSCs cardioprotective effects (232). Also, collagen I production is expected to continue at the basal level to support cells and maintain integrity of the patch with less collagen III production which rules out concerns about fibrotic changes of the patch. And still expected to hold dynamic response to mechanical stress in-vivo to upregulate collagen to levels comparable to native cells.

Indeed, these data are consistent with the other reports that did correlate overexpression of Akt in MSCs, which was evident in our earlier results, with favourable sequelae in terms of less collagen deposition, repression of remodeling and better recovery of cardiac function after in-vivo transplantation (357).

4.8.2.3.Angiogenic factors

The third domain for assessment was the angiogenic factors. This is an important area to examine as tissue perfusion is a pre-requisite for tissue viability. Therefore, angiogenesis is one of the main processes for reliable and durable cell survival and tissue functionality. So, angiogenic factors expression by wjMSCs is considered a key area for assessment.
Angiogenesis is the formation of new vessels from pre-existing vessels and the process is dependent on angiogenic factors including angiopoietin, bFGF, HGF, VEGF (363) in addition SDf-1and MCP-1 (322).

Our data showed significant upregulation of HGF, VEGF and MCP-1 while non-significant change inexpression of angiopoietin, bFGF and SDF-1 under CdM effect.

Angiopoietin1 is a member of the Angiopoietins family of angiogenic factors. It acts via phosphorylation of tie2 receptors to enhance maturation of neovessels (341). In the study of Yunling and colleagues, they did generate MSCs over-expressing Angiopoietin1 using Adenovirus transduction and examined its effect on wound healing. The MSCs over-expressing Angiopoietin1 did enhance angiogenesis and promoted wound healing with denser capillary network relative to the control (341). This would indicate the rule of Angiopoietin1 as one of the paracrine secretome profile of MSCs to support its regenerative effects via enhancing angiogenesis. Also, bFGF is an angiogenic factor with pro-inflammatory potential (364) and one of the paracrine factors secreted by MSCs to enhance angiogenesis (365).

HGF is another angiogenic factor that is secreted in ischemic myocardium and enhances angiogenesis in addition to anti-apoptotic and anti-fibrotic effects as well (366, 367). In the study of Sullivan et al. they demonstrated that its overexpression in addition to SDf1 by MSCs did enhance survival of CMCs under oxidative stress (367).

VEGF is a dimeric glycoprotein which mainly affects the vascular endothelium and exerts its effects through binding to two receptors VEGFR1 and VEGFR2. It enhances angiogenesis during normal development as well as under pathologic conditions such as ischemic events and wound healing (368). VEGF acts to enhance proliferation, migration and repress apoptosis of EPCs and ECs via several pathways including the PI3k/Akt pathway (369). VEGF is considered quite potent angiogenic factor and plays a key rule to maintain vascular integrity. It’s withdrawal leads to vascular regression of retinal vessels via selective apoptosis of ECs in addition to its positive effect on myogenesis to recover cardiac injury (370).

Also, it is a key factor during vascular remodelling as it represses cell-cell contact with local degradation of the ECM to allow ECs proliferation and migration for neo-vessel formation (371). This action would be followed by effect of TGF-B1 and PDGF-B to stabilise the vascular network via enhancing ECM production and to recruit pericytes as well as SMCs to enclose and support the neovessel resulting in maturation of vascular plexus (371).

Indeed, VEGF rule is quite critical for normal development. Heterozygous deletion of VEGF gene led to embryonic death secondary to vascular malformations (368). On the other hand, when over-expressed in MSCs through exposure to hypoxic conditions, it did significantly enhance neovascularisation in the infarct area, reduced apoptosis, reduced remodelling of LV post infacrtion and promoted durability of cells (372). This indicates the positive effects of VEGF upregulation in response to hypoxia and their beneficial effects on infarcted myocardium (372). In addition, VEGF has a vasodilatory effect mainly via NO as well as prostacyclyin (373). This effect is exerted via the PI3K/Akt pathway to enhance endothelial nitric oxide synthase (eNOS) and to promote vascular permeability (373).

SDf1 (stroma derived factor 1) is a chemokine factor that is involved in angiogenesis during fetal as well as adult life (369). Its gene transcrption is enhanced by the transcrption factor hypoxia induced factor-1 (HIf-1) and was also detected in endothelial cells in-vivo in ischemic areas in proportion to oxygen depletion (374). It acts to recruit stem and progenitor cells to hypoxic areas (374). Also considered a prosurvival factor and its overexpression would enhance HGF expression (367).

MSCs secretion of SDf1 was reported to promote cardiac regeneration secondary to SDf1 interaction with CXCR4 which enhances survival of progenitor cells. However, its effects on the myocardium were attributed to preserve rather than to enhance regeneration of CMCs (375). This indicates its role as a conservative rather than regenerative factor and its expression is mostly HIf-1 driven secondary to oxygen deprivation.

MCP1 (monocyte chemoattractant protein-1) is one of the C-C chemokine family (376). It holds vasculogenic effects. On experimenting its vascular effects in-vivo, the MCP-1 eluting scaffolds did demonstrate vasculogenic potential comparable with bone marrow mononuclear cells (BMCs) seeded scaffolds. Also, MCP1 could enhance recruitment of host monocytes and maintain patent vascular conduit (377).

Our data show that the angiogenic profile of wjMSCs under CdM effect did fluctuate between neutral and positive while no downregulation to any factor. There was significant upregulation of HGF, VEGF and MCP-1 which indicates favourable paracrine reaction of wjMSCs. While non-significant change in the expression of angiopoietin, bFGF and SDf1.

Upregulation of the VEGF in the CdM treated MSCs indicates that wjMSCs are expected to enhance angiogenesis when combined with HUVECs and HCFs on the patch. This is expected to enhance angiogenesis, myogenesis and survival of cells on the patch. Therefore, VEGF upregulation would be anticipated to reflect on the regenrative effects in-vivo. Also, the combination of HGF and VEGF were shown to act synergistically to enhance angiogenesis (378). While VEGF which is known as a potent in-vivo angiogenic factor was not sufficient to support tubulogenesis in-vitro.

For instance, in the VIVA study, the effect of VEGF was comparable with placebo up to 60 days post infusion, while at 120 days only the high dose VEGF showed significantly better angina profile and exercise tolerance (379, 380). This indicates the important role of HGF to facilitate VEGF effect. Therefore, upregulation of both HGF and VEGF is expected to reflect on in-vivo recruitment of myocardial ECs for angiogenesis. This is also boosted by the upregulation of MCP1 with its potential vasculogenic effects.

While Angiopoietin1 expression was stable all through with no significant alteration by CdM. These results in addition to the non significant change in collagen are in agreement with other studies that showed more collagen deposition when Angiopoietin1 was overexpressed (341).

Also, CdM did not significantly enhance Sdf1 expression which means the combination of HUVECs and HCFs would not significantly affect SDf1 expression. This could be due to missing hypoxia as a provoking condition. However, still its basal expression level is maintained as no underexpression was demonstrated.
So, overall our data indicate that wjMSCs in this combination of cells would enhance favorable angiogenic profile and would support recruitment of ECs for angiogenesis and the patch perfusion.

Furthermore, the angiopoietin, bFGF, HGF and VEGF results were consistent with the mRNA change which indicates transcription dependent protein expression and anticipates longer effect in vivo.

4.8.2.4. Adhesion/Communication factors

The next area for assessment included cellular adhesion and communication factors. Obviously, cellular adhesions and communication is a crucial part for integrity and functionality of any tissue. The direct intercellular communication depends on several channels including adherence and gap junctions and β-catenin in addition to interaction with ECM via integrins (ITG). So, for seeding a patch with multicellular phenotypes there should be effective way of cellular adhesions and communication to ensure adequate functional properties of the patch in-vivo.

Therefore, we did assess for the β-catenin, N-cadherin, Endoglin,Connexin43 and integrin. This is due to the invaluable role of these factors.

The wjMSCs under CdM effect demonstrated upregulation of N-cadherin while non-significant effect on β-catenin, Endoglin,Connexin43 and ITG was not assessed.

Integrins (ITG) are adhesion molecules in all mammals. They are considered the most dominant surface receptors to communicate with ECM. ITGs act as receptors to connect ECM with the cell cytoskeleton using linking proteins such as talin, vinculin, α-actinin and paxilin (59). Therefore, they facilitate cellular communication with surrounding micro-environment in order to adapt to signals from extracellular milieu for spread, polarity and differentiation (381). In the study of Leda et al. β1-integrin knock down in adult CMCs resulted in heart failure and fibrosis at 6 months in murine model. Also, the embryonic fibroblasts secretome did support CMCs proliferation via a β1-integrin dependent mechanism which indicates the crucial developmental as well as regenerative rule of integrins (382).

Unfortunately, the antibody for ITG did not work and therefore could not make objective assessment of its expression.

Other factors on our list included β-catenin, N-cadherin, Endoglin and Cx43.

β-catenin exists in the cell membrane to participate in cell-cell adhesion and communication and may also exist in the nucleus when it enhances transcription/activation of target genes and also could exist in a free non-cytoskeletal form in the cytoplasm where its degree correlates with dedifferentiation status (383).

N-cadherin is one of the cadherin group which have trans-membrane as well as cytoplasmic location. It promotes cell migration and adhesion as well as suppresses cell growth via prolonging the G2/M phase of cell cycle and induces β-catenin mediated expression of the cyclin dependent kinase (CdK) P21 (384).

Endoglin is a trans-membrane glycoprotein expressed mainly by endothelial cells and reacts to signals by many members of TGF-β family (385). It acts as a receptor for TGF-β superfamily to support

angiogenesis, and it was found essential for normal vascular development (301). It is a crucial mediator of the TGF-B action to mature neo-vessels and establish fully functioning network and to establish arteriovenous identity (386). Deficiency of endoglin did impair arteriovenous characterisation with poor recruitment of VSMCs to variable degrees. This would range from marked impairment in homozygous absence to aberrant recruitment with resultant fragile vessels in heterozygous cases.
For instance, hereditary haemorrhagic telangiectasia type I (HHT1) is an autosomal dominant vascular disorder characterised by nasal and gastrointestinal telangiectasia with recurrent bleeding as well as vascular malformations in other organs such as brain and lung (387, 388). Mutation of Endoglin gene was found to underpin its pathogenesis (386) which indicates its importance for maintaining integrity of the vascular plexus.
Endoglin expression is upregulated in concordance with upregulated TGF-B such as during angiogenesis, wound healing and inflammation. On the other hand, its absence led to intra-uterine death due to failure of maturation of vascular plexus with subsequent loss of osmotic balance as well as poor cardiovascular development. Indeed, this was quite similar to the effect of TGF-B I and TGF-B II knockout models (387).

The last factor on this list is the Cx43. Connexins are gap junctions that represent universal intercellular communication channels. They are aggregates of intercellular channels that facilitate exchange of messengers, ions and matabolites between neighbouring cells (389). This is in addition to their role for transcription regulation of other intercellular communication factors such as the role of Cx43 in transcription regulation of N-cadherin (389). They were proved invaluable for normal heart development (18). Connexin43 null mice did not survive beyond the neonatal phase due to conotruncal malformation and right ventricular outflow tract (RVOT) obstruction (390). Also, CMCs specific homozygous Cx43 deletion caused neonatal death. However, heterozygous deletion did not show significant developmental alteration which indicates some redundancy between different connexins (391).

They also have important role for adequate cardiac function. Indeed, they are mandatory for normal conductivity of the heart and if disrupted, the heart would suffer from arrhythmias secondary to altered coupling of CMCs (392). In addition, their role is quite remarkable in case of injury as they activate fibroblasts and facilitate ECM modulation via collagen deposition (393).

Cx43 is the main connexin isoform in the myocardium. It gets downregulated in case of injury and its down-regulation features remodelling and predisposes to arrhythmias (392).
It represents a gap junction that would facilitate channel mediated communication between endothelial and mesenchymal cells during angiogenesis. In the study of Hirschi and colleagues, endothelial cells (ECs) were co-cultured with Cx43-/- mesencymal progenitors and were compared with ECs co-cultured with Cx43+/+ cells. It was evident that Cx43-/- did lag to undergo mural differentiation while their differentiation ability could recover with re-expression of Cx43. Also, ECs could establish communication channels with the Cx43+/+ and secreted TGF-β to facilitate the angiogenesis process (323). This study did conclude the important role of gap junctions particularly Cx43 to establish the intercellular communication during angiogenesis and support maturation of newly formed vascular plexus.

Our results demonstrate that CdM upregulated N-cadherin while it was fairly neutral with no significant alteration in the expression of other factors.

Up-regulation of N-cadherin under CdM effect indicates enhanced migration and invasion potential. Also shades light on the proliferation potential of wjMSCs as N-cadherin represses proliferation via prolonging the G2/M phase of the cell cycle . So, proliferation of wjMSCs did not demonstrate any significant difference relative to the control which is in part mediated via N-cadherin/β-catenin complex. Also it is expected to enhance MSCs retention in-vivo (394) which is in favor of longer paracrine effect and higher potential for integration. Also, would be expected to enhance MSCs trans-differentiation into cardiomyocytes (395). In the study of Ishimine et al. the upregulation of MSCs showed significantly higher ability to differentiate into cardiomyocytes (395) which is quite interesting result to support our approach.

Also, our data demonstrated Cx43 was fairly stable under effect of CdM as well as pure CHIR treatment. However, it was significantly upregulated under CHIR/CdM effect which indicates better potential for intercellular communication and supports the presumed role of wjMSCs to facilitate the angiogenesis process.

4.8.2.5. Proliferation and survival

The next area of assessment was the wjMSCs proliferation. Our data show comparable proliferation potential under effect of CdM. This would argue against the potential of overpopulating the other cells on the patch and indicates the basal level of proliferation with no enhanced proliferation rate which is one of the favourite results as it indicates balanced proliferation.

The last domain of assessment was the wjMSCs potential survival. We did examine the wjMSCs apoptosis and death as this would reflect to which extent wjMSCs will be supported when combined with HUVECs and HCFs. As expected after BCl-2 and NRG1 expression that did not demonstrate any significant change from the control and therefore it was clear that wjMSCs are not extra supported in this combination. Indeed, the results show that the rate of apoptotic as well as dead cells was significantly higher in CdM relative to plain media. which means that not only this combination of cells would not offer better survival benefit, but actually the survival is negatively impacted.

So, it is clear that the reaction of wjMSCs when combined with HUVECs and HCFs would mostly vary between neutral to positive. They would up-regulate Akt, HGF, VEGF, MCP-1 as well as N-cadherin which have positive effect on survival, angiogenesis and cellular communication while will down-regulate collagen III which is considered a positive effect as it would argue against any extra fibrotic changes and is consistent with modified remodelling. In addition, the rest of factors are just comparable with the control which indicates that wjMSCs would still hold their baseline supportive paracrine profile and did not lose any of its items.

4.8.3. Effect of CHIR99021

After these promising results in addition to the multi potency of MSCs and in order to optimise the patch's functionality we did attempt to enhance the MSCs properties using small molecules.

Due to the fact that in order to meet clinical needs there is need for big volume of cells for transplantation (332). Also, MSCs expansion is limited by senescence that usually happens above passage 8.

Several studies looked at the possibility to enhance the proliferation potential of MSCs to meet clinical requirements using several growth factors such as bFGF, BMP, TGF-b, VEGF and PDGF (332). This approach had also some draw backs such as compromised differentiation potential and increased heterogeneity of cells with still potentially progressive telomere shortening and senescence (332).

The other approach was to utilise small molecules such as the Wnt/β-Catenin agonists CHIR99021 and BIO as these factors are not substrates for protease degradation and could be manufactured via standard chemical industry (332).

CHIR is a potent selective GSK-3β inhibitor that promotes activity of Wnt pathway (210). The Wnt signal is a part of the pluripotency molecular circuitry. It is upregulated in undifferentiated ESCs and gets downregultaed with differentiation. Its upregulation via GSK-3 inhibition was associated with maintained pluripotency factors Oct3/4, Rex-1 and Nanog expression and promoted pluripotency of ESCs (396). Another study reported that the intrinsic pluripotency program of ESCs could be enhanced by GSK-3 inhibition as it represses inhibition of the pluripotency factors (165, 397). So, CHIR holds the ability to maintain pluripotency of ESCs and IPSCs as it inhibits the GSK-3β and activates the Wnt pathway (210).

The dose of CHIR is one of the parameters to determine its effect. In the differentiation experiments of MSCs to hepatocytes, CHIR at 2 uM was enough to upregulate the mRNA of endoderm specific genes as well as endoderm specific markers and it did enhance differentiation into hepatocyte like cells (398). While Xiao et al used 12 uM to optimise ESCs differentiation into cardiomyocytes (333). And Kavitha et al experimented CHIR at 10 uM to enhance the MSCs differentiation potential (210).

It is not clear if mere dose difference could have significant directing effect on either differentiation or pluripotency because we do not have a dose based comparative study. Nevertheless, we can expect that different doses potentially have different results, in addition to the time window used for assessment. As shown by Xiao et al, the viability of CHIR treated ESCs did not show significant change with Rapamycin until the third day and higher cell density achieved (333).

The potential for pluripotency versus differentiation status is also dependent on epigenomic changes. The CHIR treated MSCs showed significantly higher levels of the enhancing H3K4Me3 and H3K36Me3 while stable levels of repressing H3K27Me3 and H3K9Me3 (210).

GSK-3β has several target factors including the β-catenin which has different location dependent rules. Upon inhibition of the GSK-3β, cytoplasmic β-catenin would be relaesed from degradation, accumulate in the cytoplasm and transfer to the nucleus where it would activate the Wnt depnedent genes and on the other hand β-catenin acts as a partner to the transmembrane cadherins involved in cell-cell adhesion (399).

As wjMSCs are quite plastic cells and hold a multipotent potential with no ethical concerns around its isolation in addtion to its relative immune innert properties and being available from abundant source all these factors make them ideal for tissue engineering and regenrative purposes (210).

Treatment of ESCs with CHIR and its vehicle control DMSO resulted in high apoptosis rate and this was due to the progressive increment of P53 expression in addition to significant increase of intracellular ROS (333). This could explain the high rate of apoptosis in CHIR treated MSCs despite we could not examine the p53 expression.

Glycogen synthase kinase-3 (GSK-3) is a quite unique kinase as it gets inhibited rather than stimulated upon response to its main upstream pathways including insulin and Wnt. Thereafter, it acts to orchestrate action of tens of substrates that should get phosphorylated with another kinase prior to action of GSK3 (400). It is a multi-functional kinase (400) involved in regulation of several tasks such as apoptosis, proliferation, glucose metabolism, cell signalling and intracellular communication signals (401). Therefre, it is a therapeutic target in several pathologic conditions such as cardiac hypertrophy, some neuropsychiatric disorders as well as some neoplasms (400).
Wnt signalling is widely expressed in metazoan organisms and has a wide variety of crucial actions such as its role in cell fate determination, organ formation, cell polarity and cellular communications and also involved in pathogenesis of some pathological conditions such as neoplasm and osteoporosis (402)

The Wnt has two distinct pathways, canonical and non-canonical (403). Canonical Wnt activation would release β-catenin from phosphorylation by GSK-3β. This would accumulate β-catenin and allows its translocation to the nucleus to activate targets of Wnt gene (404). In absence of canonical Wnt activation, cytoplasmic B-Catenin gets phosphorylated by GSK-3b and degraded by the ubiquitin proteasome system (405).
β-catenin is a part of the cadherin-based adherens junctions. It is the main player to affect nuclear canonical Wnt signalling which is deeply involved during fetal development for axis determination as well as organs formation. It's effect would continue during adult life as a key player in several functions such as homeostatsis and regeneration (406).

CHIR99021 is a potent selective GSK-3 inhibitor and therefore was elected to enhance the wjMSCs hoping to accomplish higher proliferation potential with maintained self renewal, multipotency and favourable paracrine profile. Its effects at 5 uM concentration on wjMSCs were tested but results were fairly neutral with quite little significant effect.

It did not have significant effect on Akt, BCl-2 or NRG1 which was reflected on the survival experiments. Indeed, its survival effect was negative which could be explained by the unmeasured p53 accumulation as well as intracellular ROS (333). Also, it did not affect the ECM protein production and therefore we expect the wjMSCs to continue producing their own ECM and will not lose its integral role by over or under production. While at the level of angiocrine factors, it did significantly upregulate the HGF which is a good positive effect as it would offer angiogenic support while other angiogenic factors still maintain their basal angiogenic support but we can not expect extra support from this prospective.

Cell adhesion/communication factors assessment revealed statistically significant upregulation of N-cadherin while the rest did not achieve statistical significance. Actually, β-catenin was expected to upregulate under effect of CHIR. This could be due to the need for some extra factors to fully function as what was already reported by other laboratories with another GSK-3 inhibitor known as BIO.

BIO could not establish positive effects on HUVECs until supplemented with VEGF (404) which could also apply for CHIR effect on wjMSCS. Also, it could be related to the dose as we used 5 uM while others used 10 uM in addition to the media and other additional factors to induce upregulation of β-catenin (210). Indeed, we opted for the 5 uM based on its more favourable effect on HUVECs so that we use stable dose for all cell phenotypes. In addition, CHIR effect was examined in plain media with no additional factors.

4.8.4.Combined effect of CdM and CHIR on WjMSCs

After combining CHIR and CdM it sounds that the combination has some few but still relevant advantageous results.

It did significantly upregulate Akt which is expected to reflect on their cytoprotective effects. Still did not meet statistically significant effect on BCl-2 and NRG1, however, the survival experiments did demonstrate positive advantage with significantly lower rate of apoptotic wjMSCs under CHIR/CdM effect which indcates clear survival benefit from this combination. Also, this is expected to reflect on the longevity as well as paracrine profile of MSCs in-vivo.

The ECM and angiogenic factors expression by wjMSCs under CHIR/CdM effect were in the neutral zone relative to CdM but still wjMSCs did not lose any of their potential with this combination. Considering the already established HGF, VEGF and MCP-1 upregulation under CdM effect which is still maintained under CHIR/CdM effect so, we can conclude overall positive paracrine influence that is expected to enhance the angiogenic in-vivo effect.

Furthermore, CHIR/CdM significantly upregulated β-catenin which was missing as a pure effect of CHIR on its own but seems to gain benefit from the CdM and expected to enhance MSCs differentiation potential (210).

Upregulation of β-catenin would indicate better maintenance of self renewal capacity of MSCs as it was shown to upregulate expression of Nanog in both mouse and human (407). Also, its upregulation in the context of GSK inhibition would correlate with significantly better MSCs survival (324).

In the study of Brunt et al. they looked at the effect of aging on MSCs differentiation. They found that MSCs differentiation potential was adversely affected by aging. While β-catenin upregulation was able to resume MSCs differentiation potential (408) and there was some interdepnednce between β-catenin and telomere transcriptase to maintain telomere length and guard against cellular senescece which would support the juvenisation effect of β-catenin upregulation (408).

However, in the study of Zhang et al. there was positive correlation between β-catenin upregulation and senescence. They examined the effect of old rat serum (ORS) on MSCs. ORS induced senescence of MSCs which correlated with Wnt/β-catenin activation and could be reveresed by repression of Wnt/β-catenin (405).

Considering the differences between the two studies as the Zhang et al experimented bone marrow MSCs (BM-MSCs) from rats aged 12-14 weeks while Brunt et al. isolated human BM-MSCs from two groups of patients undergoing cardiovascular surgery with mean age of (56.1+/-4.8 years) in the young group and (73.3+/-7.1 years) in the old group, however, still the Wnt/β-catenin system effects are quite different. This reflects the complexity of the survival and aging processes and the potential for other factors to get involved with resultant different effects.

In fact, the outcome of cellular therapy would potentially vary dpending on the cell's background. For instance, in the study of Leor and coworkers they did compare MSCs from right atrium, epicardial fat and subcutaneous fat with bone marrow derived MSCs for their angiogenic profile as well as their in-vivo effect of LV remodelling post ischemia. Interestingly, the right atrium and epicardial fat MSCs did demonstrate higher angiogenic profile but also higher pro-inflammatory profile and the net result was inferior remodelling results relative to the subcutaneous fat derived MSCs (275). Also, the inflammatory

changes in -vivo were more dominant in the right atrium and epicardial fat arm. This study did shade light on the variable outcome of this modality of cellular therapy based on the biological background of the transplanted cells.

In our study, the combined CHIR/CdM did demonstarte significantly lower apoptosis rate relative to CdM treated wjMSCs. This result correlated with further Akt upregulation and also with upregulated β-catenin. This would strongly support the CHIR treatment approach as it is expected to enhance survival and durability of wjMSCs in-vivo.

Then, pure CHIR treatment did demonstrate significantly higher rate of apoptosis while dead cells were higher but did not achieve statistical significance most probably due to small number and scattered data. And the most interesting results are in fact the significant reduction of apoptosis rate when CHIR combined with CdM.

Indeed, survival of MSCs is a multifactorial complex process (324). Our data show that earlier Akt upregulation was not enough to support MSCs survival, also pure CHIR at 5 uM negatively impacted MSCs survival. This could indicate other apoptosis factors such as nuclear accumulation of phosphorylated p53 under CHIR effect that would enhance apoptosis .

These negative results were reversed when Akt was further upregulated under combined effect of CHIR/CdM which could indicate certain threshold for the Akt to hit apoptosis repression. Also, this was consistent with β-catenin upregulation that could not be achieved by pure CHIR treatment despite same dose. This would indicate other unmeasured factors that would influence its effect, in addition to other apoptosis factors that could not be assessed due to facility constrain.

Indeed, the limited in-vivo durability of MSCs is considered as a limiting factor that did hinder its widespread clinical utilisation (324).

As previously illustrated MSCs have a good body of evidence to emphasise on their cardioprotective effects in animals with variable results in clinical studies. However, most of the available data are in the context of myocardial infarction (MI) models in animals. Nevertheless, still their wide scale use is limited by the short in-vivo durability which is not fully explored (281).

Our previous results using MSCs seeded on biologic scaffold in large animal models were quite promising but again limited with the fact that animals experimented were normal animals which means they have normal genetic setup. Therefore, the native cellular reaction to graft implantation after full thickness excision did demonstrate normal reaction to injury. The native cells utilized the biologic scaffold cormatrix to migrate and proliferate in order to repopulate the graft and organise spatially in a 3D tissue architecture including its neo-vasculature.

This was clearly enhanced by the cellular component of seeded grafts which demonstrated superior results relative to the acellular patch. However, the cells were mostly washed out within weeks which did point to paracrine effects rather than cellular engagement or trans-differentiation.

The case is quite different in human cases with CHD(s). These cases have developmental error(s) that underpins the defect(s). So, anatomical correction/support is not expected to promote normal cellular reaction in terms of proliferation, migration and angiogenesis even if supported with some paracrine factors. Therefore, there is need for the patch to be cellularised with relevant cells and the cells to hold

the potential to stay in-vivo and get integrated with the surrounding myocardial milieu hoping to offer trans-differentiation into functional cells in-vivo rather than to get washed from the scene.

So, wjMSCs are considered crucial pillar in the design of this patch and our data here are promising about favorable reaction and paracrine profile modification when combined with HUVECs and HCFs in addition to further positive survival advantage via CHIR99021 addition.

4.9.Conclusion

WjMSCs reaction to the combination with HCFs/HUVECs would hold positive effects on the angiogenic profile but the cells’ survival is negatively impacted. However, addition of CHIR99021 at 5 uM would be expected to salvage the cells and enhance their survival and retention.

CHAPTER V

Effect of CdM and CHIR99021 on HUVECs survival, proliferation and paracrine profile

5.1.Introduction

For the design of a patch for corrective cardiovascular surgery we did consider the multicellular nature of native tissues. So, we considered to utilise HUVECs as the pillars for angiogenesis which is indispensable for perfusion.

The angiogenic process is a complex process that requires successful integration of several factors mainly effective angiogenic factors, ECs proliferation and survival. Also, other factors are needed to orchestrate the angiogenic process such as the effect of Wnt/β-catenin to facilitate cell survival, proliferation and orientation (409).

Previous reports raised concern about HUVECs rapid apoptosis in-vivo during study of angiogenesis models. This would raise concern that HUVECs may not engage in-vivo. So, the reaction of HUVECs to conditioned media (CdM) extracted from co-culture of wjMSCs/HCFs was analysed at the relevant domains to find out their potential behaviour and if suitable to go for in-vivo applications.

Then would assess the effects of CHIR99021 at 5 uM on HUVECs and finally would examine the combined effect of CHIR/CdM relative to CdM.

5.2. Treatment of HUVECs with CdM

5.2.1. Objectives

In order to analyse the possible HUVECs reaction to the combination of wjMSCs and HCFs, conditioned media (CdM) from co-culture of wjMSCs and HCFs was used and its effects were assessed as follows.

Table 5-1. Assessment of CdM effect on HUVECs.

Assessment domain	Assessed factors	Assessment level	Technique used
Antiapoptosis/prosurvival	AKT NRG1	Protien expression mRNA level	ICC* (using image j) qPCR
ECM** proteins	Fibronectin	Protien expression mRNA level	ICC (using image j) qPCR
Paracrine/angiogenic factors	Angiopoietin bFGF HGF PDGF VEGF	Protien expression mRNA level	ICC (using image j) qPCR
Adhesion/communication factors	B-catenin Endoglin VE-cadherin PECAM FLK1 (VEGFR2) FLT1 (VEGFR1) ITG	Protien expression mRNA level	ICC (using image j) qPCR
Cell viability	Caspase/Draq7	Apoptosis and cell death	FCM***
Cell proliferation potential		BrdU assay	ELISA

*ICC: immune-cytochemistry.

**ECM: extracellular matrix

***FCM: flow cytometry.

5.2.2. Materials and Methods

The following materials and methods used for preparation of CdM.

5.2.2.1.Conditioned media (CdM) preparation

CdM was prepared from co-culture of wjMSCs with HCFs. Cells from each phenotype were plated in FGM at about $5x10^5$ in T25 or $1x10^6$ in T75 flasks and kept in humidified incubator at 37 ^oC with 5% Co_2.

After about 48 hours 70-80% confluence was achieved. Media was taken out and cells were washed with prewarmed PBS then addedd plain EGM (serum and growth factors free) and kept in incubator under same conditions for 24-48 hours. Then media was aspirated and filtered through 0.2 um filter for immediate use or kept at-20 ^oC for use within two weeks or at -80 ^oC for longer storage.

5.2.2.2.Culture in CdM

Before induction, the CdM was thawed in water bath at 37 ^oC then filtered through 0.2 um filters and mixed with plain EGM (serum and growth factor free) at 1:1 ratio for immediate use. Media aspirated and cells were washed with prewarmed PBS then the prepared CdM was added and kept in humidified incubator at 37 ^oC with 5% Co_2 .

HUVECs could not tolerate more than 24 hours. Beyond this time limit there was dramatic loss of cells and therefore effect of CdM on HUVECs was limited to 24 hours only. After culturing cells in CdM, cells were fixed and stained (as described earlier). Images were captured using incucyte system which is an automated system that captures 9 images per well of a 96 well plate in Wolfson imaging unit, biomedical school, University of Bristol. Cells cultured in plain EGM (serum and growth factor free) were used as control.

5.2.2.3.Images and analysis of Immune-flourescence

All images analysed here are captured using incucyte system in Wolfson imaging unit in the biomedical school, Bristol University. Quantification of protein expression was done in terms of mean integrated density (Id) using image-J.

5.2.2.4.Statistical analysis

T-test was used for quantitative analysis.

Afterwards, will refer to the protein expression under CdM by (**Protein-CdM**) and the control will be (**Protein-cnt**). The protein expression was done in terms of mean integrated density $\pm$ SEM and $p<0.05$ was considered statistically significant.

One way analysis of variance (ANOVA) with post hoc Tukey test was used where appropriate for three groups statistical analysis.

5.2.2.5.Apoptosis and cell death assays

For assessment of HUVECs apoptosis Caspase 3/7 (from ThermoFischer) was used. The kit contains caspase 3/7 to detect apoptotic cells. The manufacturer's recommendatons were followed. After cells collected from the flask they were washed in PBS with 0.5% FBS then caspase added at 1 ul/ml then kept for about 30 minutes in incubator or 45 minutes at RT. There was overlap between the caspase labelled cells and cells labelled with sytox and was not able to clearly differentiate apoptotic cells from dead cells and therefore DRaq7 was used to label the dead cells.

5.2.3.Results

Assessment of CdM effects on HUVECs was limited to 24 hours because beyond this limit there was massive cell loss. After the CdM treatment, HUVECs were fixed and stained (as described earlier). Images captured with the incucyte system. HUVECs cultured in plain media with same other conditions were used as control.

Analysis done using ImageJ to measure the protein expression in terms of integrated density and got the following results.

NB: Due to the laboratory shut down, the data presented here are the results of four experimental replicates but only two cell lines.

5.2.3.1.CdM does not induce significant effect on AKt or NRG1.

Assessment started with the pro-survival factors Akt and NRG1 and could not go further for Bcl2, Bax, Bad and p53 due to the facility constrain.

Akt and NRG1 are recognised as cytoprotective and survival factors for HUVECs (368, 410). So, we attempted to examine if their expression in HUVECs would be altered by CdM as this is expected to reflect on HUVECs survival and durability.

HUVECs expression of Akt and NRG1 under effect of CdM was comparable with the control.
Akt-CdM (M= 0.96, SEM= 0.02) relative to Akt-cnt demonstrated no significant difference, p= 0.2.
Also, NRG1-CdM (M= 0.98,SEM= 0.026) relative to NRG1-cnt did not show significant difference, p= 0.6.
These results suggest that HUVECs when combined with wjMSCs and HCFs are not expected to gain extra survival advantage. This may be reflected on HUVECs survival and raises concern about sustainability of HUVECs in the patch.

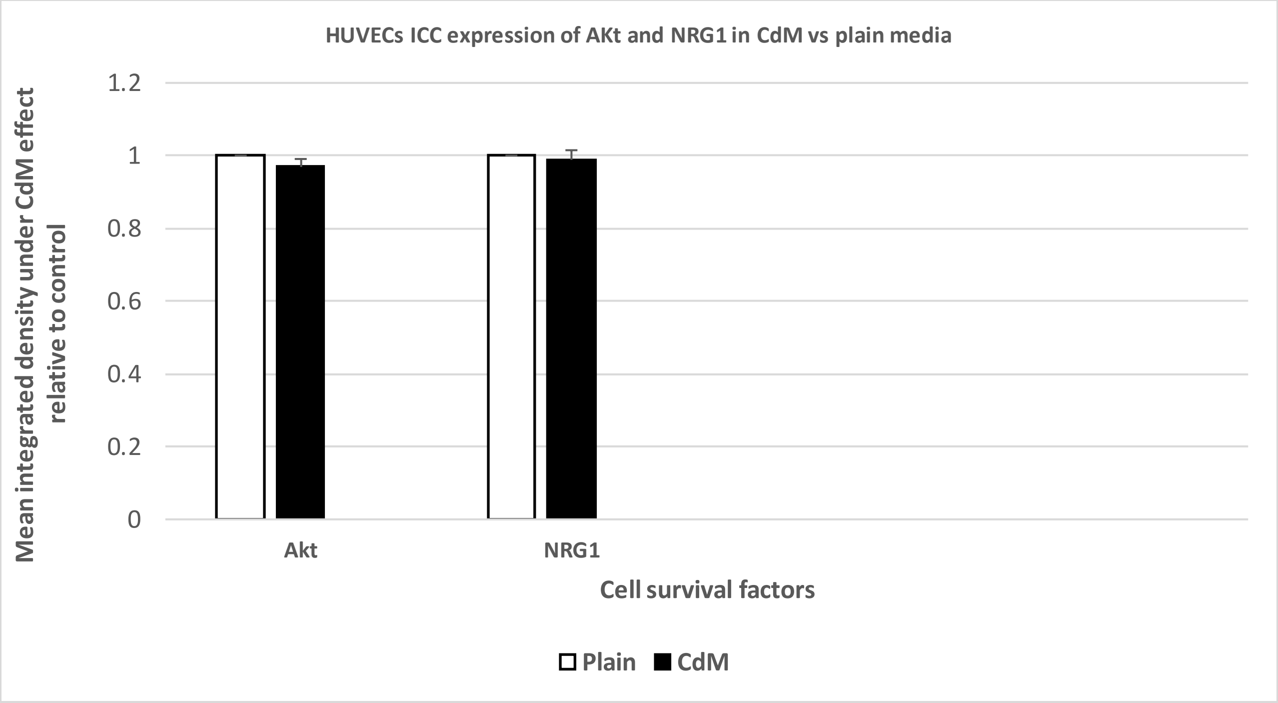

Figure 5-1.HUVECs expression of AKt and NRG1 under CdM.*

Akt and NRG1 levels in HUVECs under effect of CdM were statistically comparable with the control, p=0.2 and 0.6, respectively. N= 4 experiments but 2 cell lines. Expressed as mean+/-SEM

*CdM: conditioned media from co-culture of wjMSCs and HCFs.

5.2.3.2.CdM does not have significant effect on fibronectin production

Due to the invaluable role of ECM to activate, support and adjust neovessel formation (411). So, we did attempt to assess CdM effect on production of ECM proteins by HUVECs. We did examine for the main ECM proteins collagen I, collagen III and fibronectin. Only fibronectin was expressed by HUVECs while collagen I and collagen III did not express.

Fibronectin plays role for ECs proliferation, migration and adhesion in addition to enhancing ECs survival which are crucial componenets of the angiogenesis process (411).

Expression of fibronectin-CdM (M=1.02,SEM= 0.028) relative to fibronectin-cnt showed comparable results which suggests no significant change under CdM effect, p= 0.5.
So, non-significant change of fibronectin expression by HUVECs is considered as a negative promise for angiogenesis.

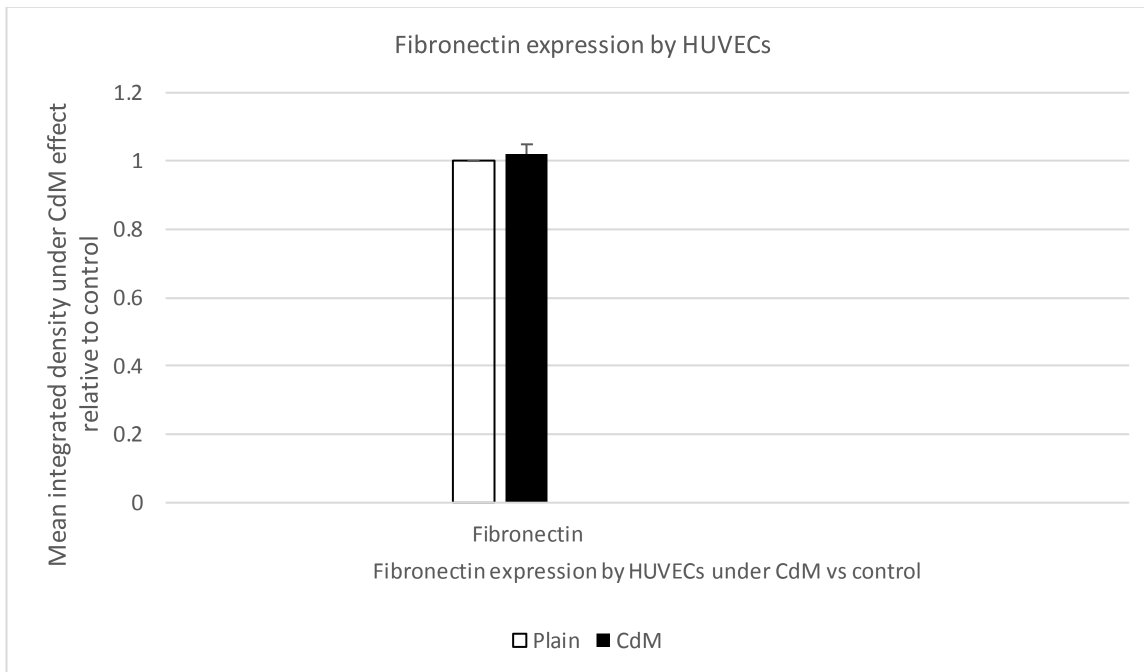

Figure 5-2.HUVECs expression of fibronectin under CdM effect.*

Fibronectin expression by HUVECs under effect of CdM was statistically comparable with the control, p=0.5.N= 4 experiments but 2 cell lines. Expression as mean+/-SEM.

*CdM: conditioned media from co-culture of wjMSCs and HCFs.

5.2.3.3.CdM downregulates bFGF and HGF with no significant effect on Angiopoietin and VEGF in HUVECs

The next and most important assessment domain was the angiogenic factors. These factors are quite critical to determine the ECs angiogenic potential due to their fundamental role during the angiogenesis process.

Assessment of the CdM effect on angiogenic factors revealed unfavourable profile. There were significant downregulation of bFGF as well as HGF under CdM effect while Angiopoietin and VEGF expression were comparable between CdM and control.

Angiopoietin- CdM (M= 1.006,SEM= 0.006) relative to Angiopoietin-cnt showed comparable results, p= 0.3.
bFGF-CdM (M= 0.96,SEM= 0.01) relative to bFGF-cnt showed significant down-regulation, P=0.02.
HGF-CdM (M= 0.98,SEM= 0.004) relative to HGF-cnt showed also significant down-regulation, p= 0.002.
And VEGF-CdM (M=1.004,SEM= 0.01) compared to VEGF-cnt demonstrated comparable results, p=0 .7.
This would indicate that this combination is not clearly supportive for angiogenic process. Indeed, it seems HUVECs are losing some support by down-regulation of bFGF and HGF which would raise concern about their durability and angiogenic potential in the patch.

Unfortunately, the PDGF antibody did not work and its results were omitted.

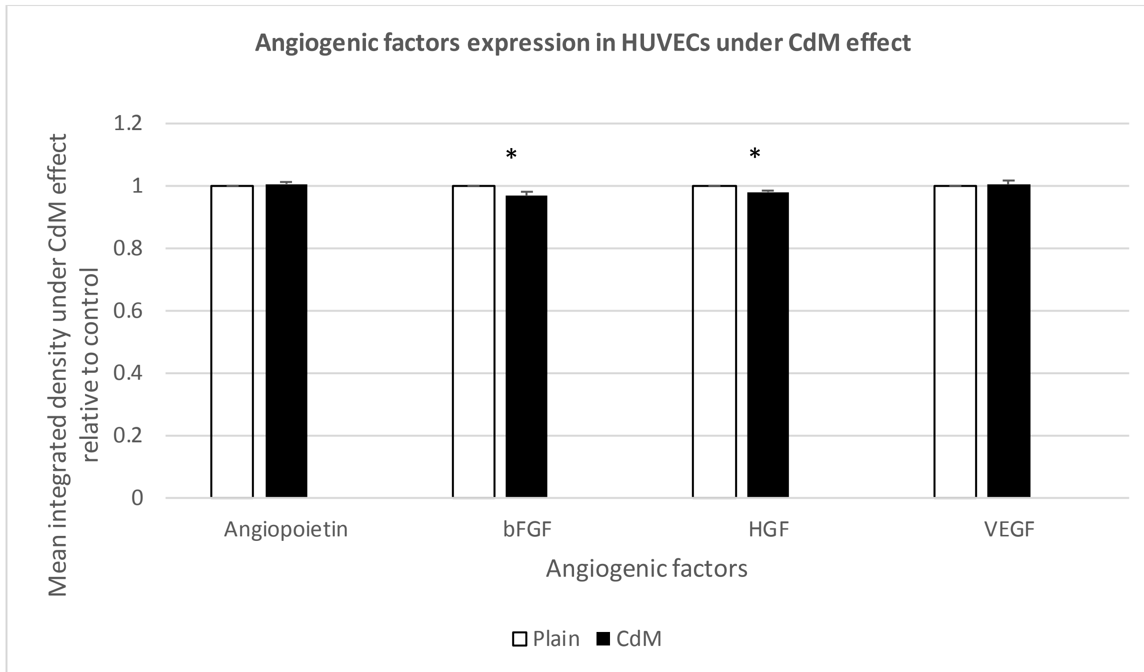

Figure 5-3.Angiopoietin, bFGF, HGF, PDGF & VEGF expression in HUVECs under CdM effect.*

bFGF and HGF are significantly down-regulated with p=0.02 and 0.002 respectively while angiopoietin and VEGF expressions were statistically comparable with the control, p=0.3 and 0.7, respectively. N= 4 experiments but 2 cell lines. Expression as mean+/-SEM.

*CdM: conditioned media from co-culture of wjMSCs and HCFs.

5.2.3.4.CdM upregulates Endoglin and β-catenin while no significant effect on VE-cadherin, FLK1 and PECAM in HUVECs

Due to the crucial rule of the cellular adhesion and communication factors in stability and functional status of the patch, so we did assess these factors.

HUVECs expression of Endoglin and β-catenin showed upregulation under CdM effect as compared with the control, while VE-cadherin, PECAM and FLK1 did not show any significant alteration.

Endoglin-CdM (M=1.017,SEM=0.007) relative to Endoglin-cnt showed significant upregulation, p= 0.03.
β-catenin-CdM (M=1.019,SEM=0.007) relative to β-catenin-cnt showed significant upregulation, p= 0.04.
VE-Cadherin-CdM (M=1.02,SEM=0.02) relative to VE-Cadherin-cnt showed no significant change, p= 0.6.
PECAM-CdM (M= 1.011,SEM=0.02) relative to PECAM-cnt demonstrated no significant change, p= 0.6.
FLK1-CdM (M= 1.024,SEM= 0.026) relative to FLK1-cnt showed no significant effect, p= 0.3.
This upregulation of the surface receptors endoglin and β-catenin is in favour of better interaction with the intended seeding in combination with wjMSCs and HCFs. Expression of the rest of surface receptors lean toward neutral effect.

NB: FLT1 (VEGFR1) and integrin (ITG) were also attempted but the antibodies did not work, so results were omitted.

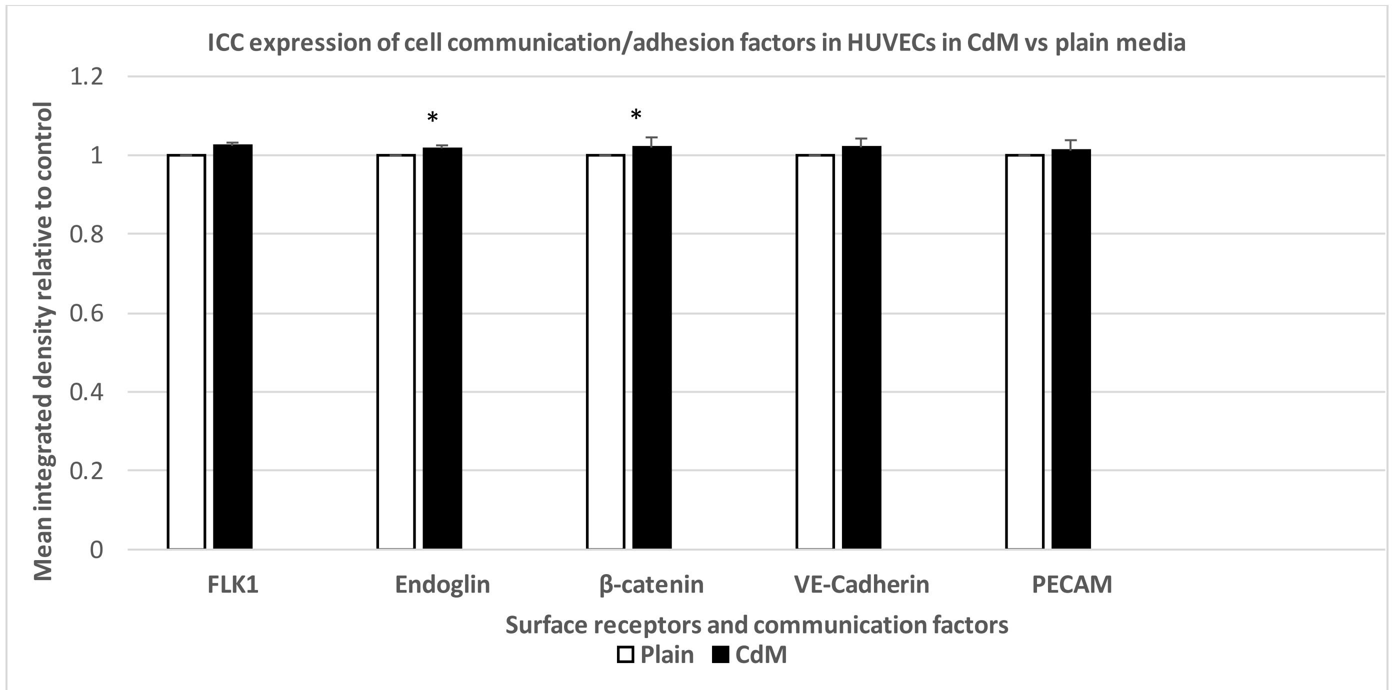

Figure 5-4.HUVECs expression of FLk1, Endoglin, β-catenin, VE-cadherin and PECAM.

HUVECs expression of β-catenin, VE-cadherin, FLK1, Endoglin and PECAM in CdM compared with plain media. β-catenin and Endoglin were significantly upregulated, p=0.04 and 0.03 respectively . No significant change in expression of VE-cadherin, FLK1 and PECAM, p=0.6, 0.3,0.6, respectively.N= 4 experiments but 2 cell lines. Expression as mean+/-SEM.*

*CdM: conditioned media from co-culture of wjMSCs and HCFs.

5.2.3.5.CdM does not alter HUVECs proliferation potential

In order to assess the proliferation potential of HUVECs when seeded in combination with wjMSCs and HCFs, the BrdU assay was used.

The BrdU assay of HUVECs in CdM for 24 hours (M=0.13, SEM= 0.0038) compared to HUVECs in plain media for same duration (M=0.14, SEM= 0.005) demonstrated non-significant difference results, p= 0.1. This means the combination of cells does not offer a proliferation advantage to HUVECs. Indeed, it seems to reflect the high apoptosis level of HUVECs under both conditions and again emphasises on the potential short durability on-patch and in-vivo.

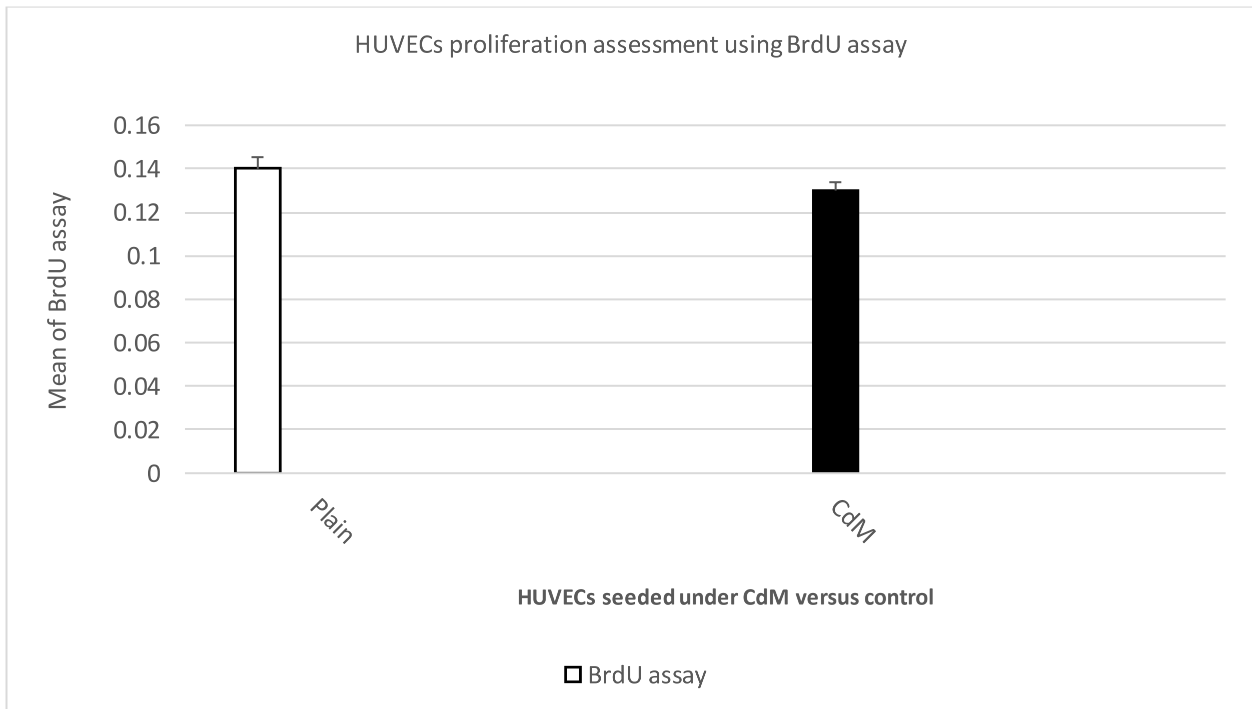

Figure 5-5.Proliferation potential of HUVECs under CdM effect.

Proliferation of HUVECs in CdM for 24 hours was comparable with HUVECs in plain media for same duration. Results are the mean of 3 experiments and are statistically comparable, p= 0.1. N=3. Expressed as mean+/-SEM.

5.3. Treatment of HUVECs with CHIR99021

5.3.1. Introduction

GSK-3 inhibition by BIO (which is a GSK-3 inhibitor) was reported to induce dedifferentiation of HUVECs and enhanced HUVECs migration as well as tube formation properties of HUVECs (404). However, BIO effect was in need for VEGF supplement. Here, we did use CHIR as another GSK inhibitor and its effects were assessed without the VEGF supplement.

5.3.2. Objectives

To assess the CHIR 5 uM effects on HUVECs as regard the six relevant domains that would impact the patch including survival/anti-apoptosis factors, ECM protein, angiogenic factors, cellular adhesion/communication factors, cell survival and proliferation. DMSO containing media (vehicle control) as well as plain media were used as control.

Afterwards, will refer to CHIR99021 5 uM as CHIR.

5.3.3. Materials and methods

5.3.3.1. Preparation of CHIR99021

CHIR99021 (purchased from StemCell Technology) was used according to the manufacturer's recommendation. It was delivered in powder form and was dissolved in DMSO which was purchased from Sigma Aldrich, then was aliquoted and kept at -20 ^{o}C . It was taken out of freezer and diluted in relevant media to dilution 5 uM for immediate use.

5.3.3.2. Treatment of HUVECs with CHIR

After achieving confluence about 90%, HUVECs were washed with pre-warmed PBS and added the plain (serum and growth factor free media) endothelial growth media (EGM) containing CHIR to dilution 5 uM. Similar volume of DMSO solution was added to plain EGM and added to control flasks/wells and also same volume of plain EGM was added to control flasks/wells. So, we had the CHIR treated HUVECs as well as the DMSO containing media and plain media as control.

HUVECs were not able to tolerate serum and growth factor deprived environment for long and our cut point was 24 hours.

5.3.4.Results

At about 24 hours limit the HUVECs were fixed, stained and images analysed (as described earlier) for the main domains of survival factors, ECM proteins, angiogenic factors, cell survival as well as HUVECs proliferation and got the following results

5.3.4.1. CHIR does not have significant effect on HUVECs expression of Akt and NRG1.

The first assessment domain was the survival factors Akt and NRG1. The expression of both factors remained fairly stable under effect of CHIR and comparable with the control.

HUVECs expression Akt-CHIR (M= 0.97, SEM= 0.021) and Akt-DMSO (M= 0.97, SEM= 0.014) relative to Akt-cnt demonstrated no significant effect, p= 0.4.
Also, expression of NRG1-CHIR (M= 0.98 ,SEM= 0.025) and NRG1-DMSO (M= 0.98,SEM= 0.022) relative to NRG1-cnt demonstrated no significant effect, p= 0.8.
This indicates that pure CHIR treatment would not offer a survival support with respect to expression of Akt or NRG1.

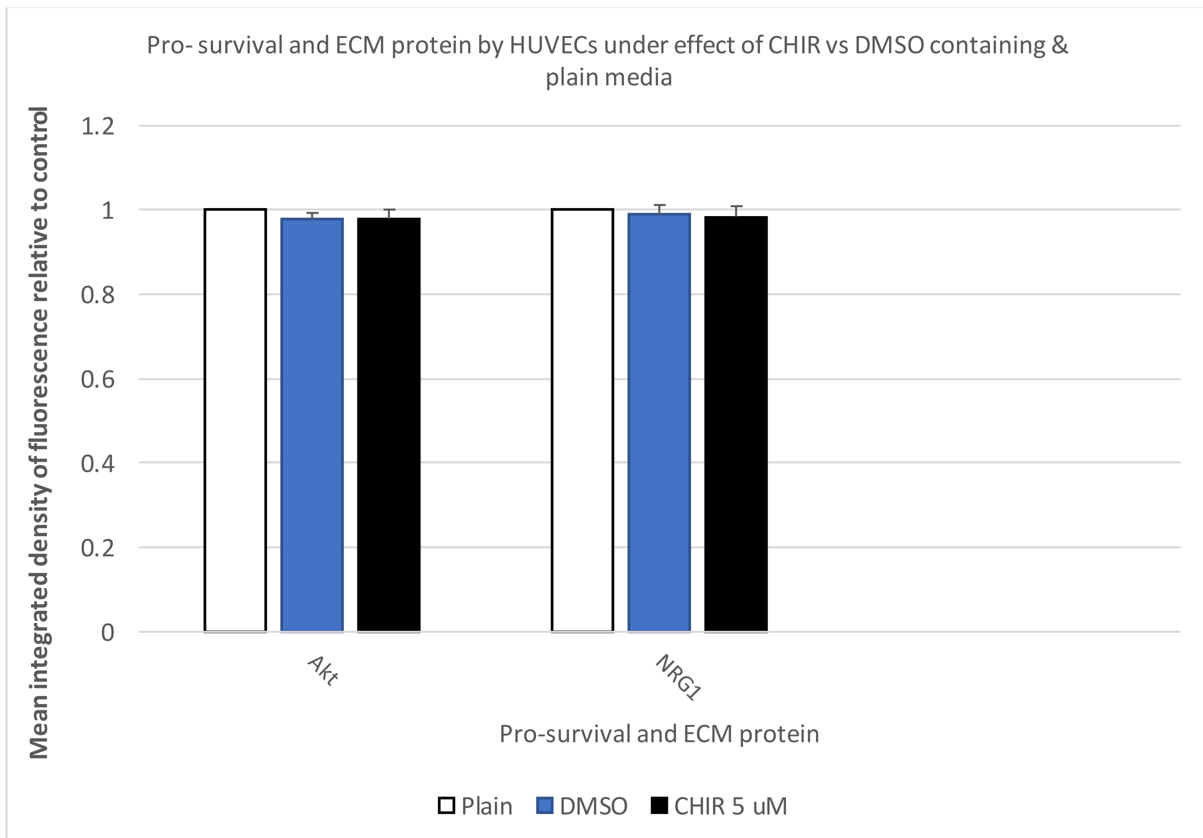

Figure 5-6.Effect of CHIR on HUVECs expression of Akt and NRG1.

CHIR does not indiuce significant change of Akt or NRG1 in HUVECs relative to the control (DMSO containing and plain media) HUVECs, p=0.4 and 0.8, respectively.N= 4 experiments but 2 cell lines. Expressed as mean+/-SEM.

5.3.4.2.CHIR does not have significant effect on HUVECs expression of fibronectin

Then assessment of fibronectin expression by HUVECs under effect of CHIR was examined. Thre was no significant change in the expression of fibronectin in HUVECs under effect of CHIR.

Fibronectin-CHIR (M= 1.06, SEM= 0.078) and fibronectin-DMSO (M= 1.022,SEM= 0.048) relative to fibronectin-cnt demonstrated no significant effect, p= 0.7.

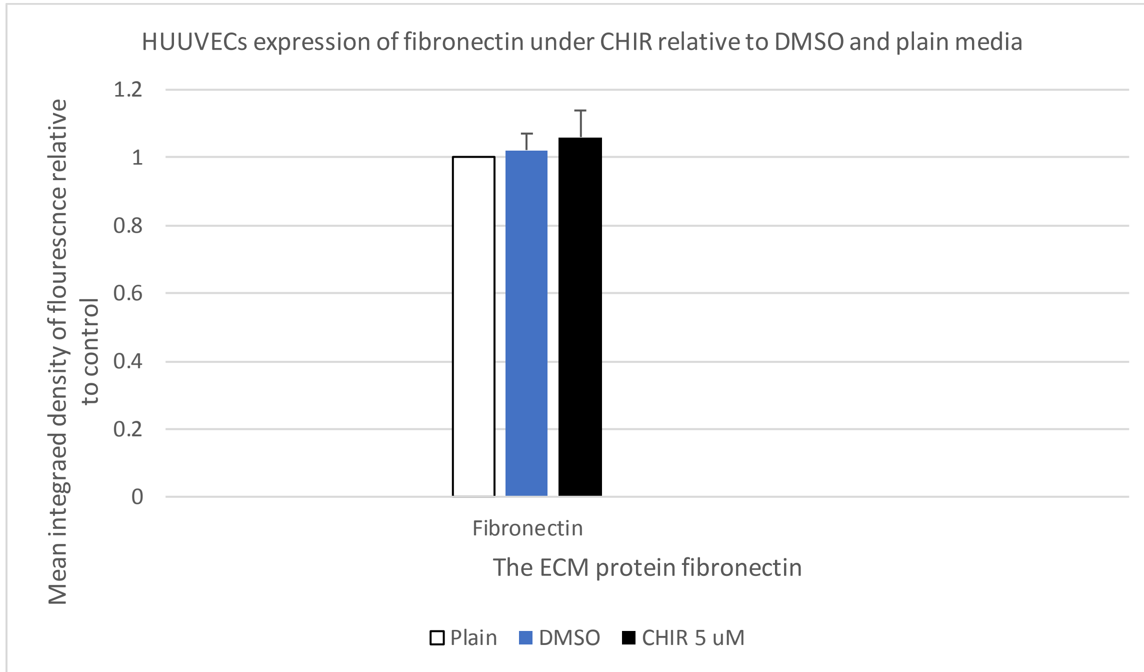

Figure 5-7.Effect of CHIR on HUVECs expression of fibronectin.

CHIR did not make significant change of fibronectin expression relative to. DMSO containing and plain media treated control HUVECs, p=0.7. N= 4 experiments but 2 cell lines. Expressed as mean+/-SEM.

5.3.4.3. CHIR does not influence significantly HUVECs expression of angiogenic factors

The next domain to assess was the angiogenic profile. HUVECs expression of angiogenic factors under effect of CHIR revealed significant downregulation of HGF expression while other factors remained fairly stable with no significant change.

Expression of Angipoietin-CHIR (M=1.013, SEM= 0.011) compared to Angiopoietin-DMSO (M= 0.99, SEM= 0.005) relative to Angiopoietin-cnt demonstrated no significant effect, p= 0.3.

bFGF-CHIR (M= 0.97, SEM= 0.014) compared to bFGF-DMSO (M=0.97, SEM= 0.013) relative to bFGF-cnt demonstrated no significant effect, p= 0.2.

HGF-CHIR (M= 0.96, SEM= 0.012) compared to HGF-DMSO (M= 0.96, SEM= 0.01) relative to and HGF-cnt demonstrated very close to significant effect, p= 0.06. But post HOC analysis revealed significant down-regulation of the DMSO group, p= 0.04.

VEGF-CHIR (M= 1.006, SEM= 0.01) compared to VEGF-DMSO (M= 1.017, SEM= 0.012) relative to VEGF-cnt demonstrated no significant effect, p= 0.4.

These results indicate non-significant effect of CHIR at 5 uM on HUVECs expression of these angiogenic factors. Also, the downregulation of HGF was notable under effect of DMSO rather than CHIR.

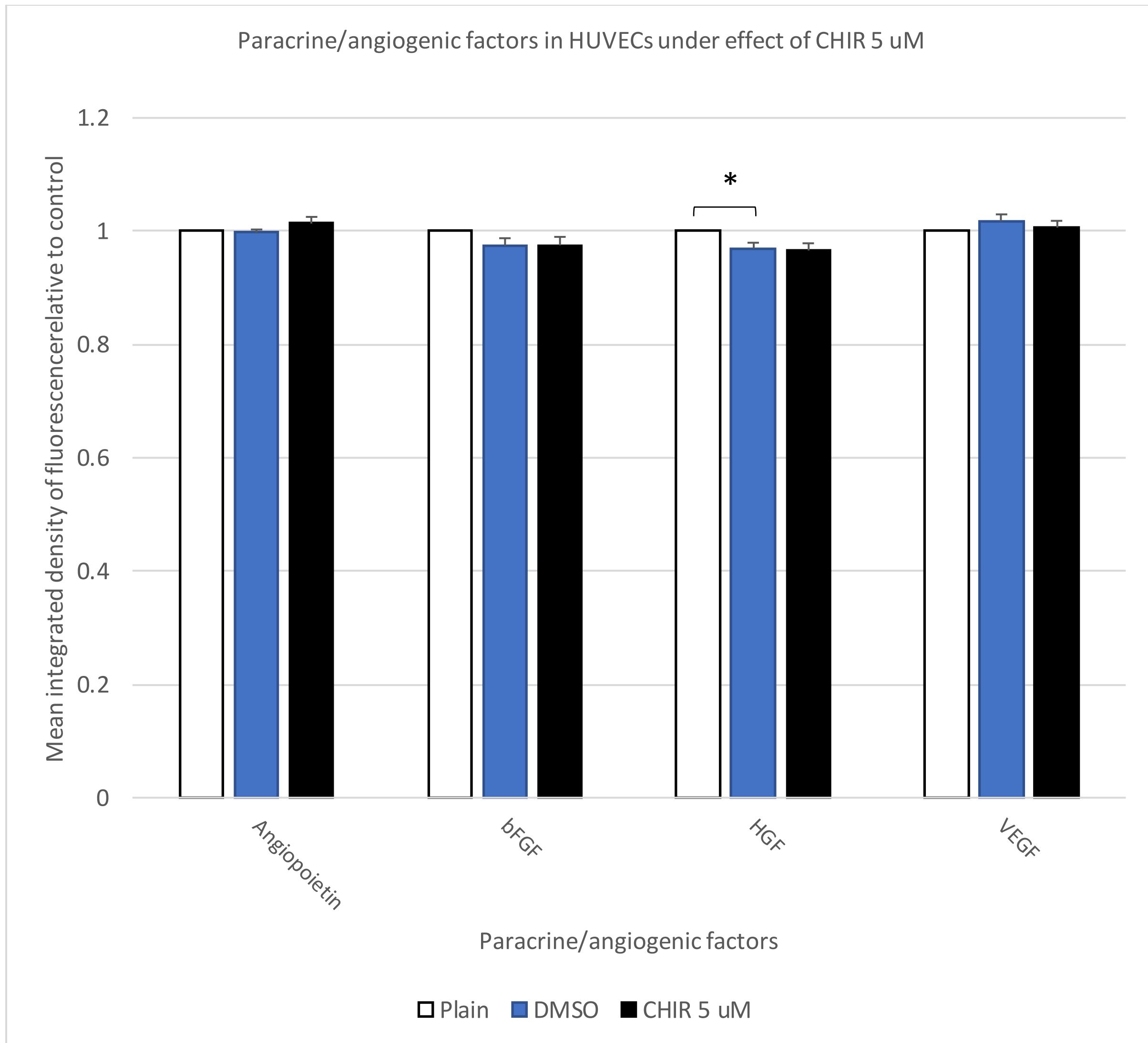

Figure 5-8.Effect of CHIR on HUVECs expression of angiogenic factors.

HUVECs expression of the angiogenic factors, angiopoietin, bFGF, HGF and VEGF under effect of CHIR was comparable with the control, p=0.3, 0.2, 0.06 and 0.4 respectively. N= 4 experiments but 2 cell lines. Expressed as mean+/-SEM.

5.3.4.4. CHIR upregulates β-catenin while no significant effect on Endoglin, VE-cadherin and FLK1 in HUVECs

Then assessment of the adhesion/communication factors revealed that HUVECs expression of β-catenin was significantly upregulated under CHIR effect while Endoglin, VE-cadherin and FLK1 expression remained fairly stable.

FLK1-CHIR (M= 1.016,SEM= 0.013) compared to FLK1-DMSO (M= 0.99,SEM= 0.016) relative to FLK1-cnt demonstrated no significant effect, p= 0.5.

Endoglin-CHIR (M= 1.03, SEM= 0.011) compared to Endoglin-DMSO (M= 1.006, SEM0.017) relative to Endoglin-cnt demonstrated no significant effect,p= 0.2.

β-catenin-CHIR (M= 1.019, SEM= 0.008) compared to B-catenin-DMSO (M= 1.01,SEM= 0.003) relative to β-catenin-cnt demonstrated no significant effect, p= 0.07. But inter-group analysis revealed significant upregulation of β-catenin relative to plain media control, p= 0.04.

VE-Cadherin-CHIR (M= 1.02, SEM= 0.02) compared to VE-Cadherin-DMSO (M= 1.017, SEM= 0.01) relative to VE-Cadherin-cnt demonstrated no significant effect, p= 0.7.

PECAM-CHIR (M= 0.96,SEM= 0.01) compared to PECAM-DMSO (M= 0.98, SEM=0.01) relative to PECAM-cnt demonstrated no significant effect, p= 0.1.

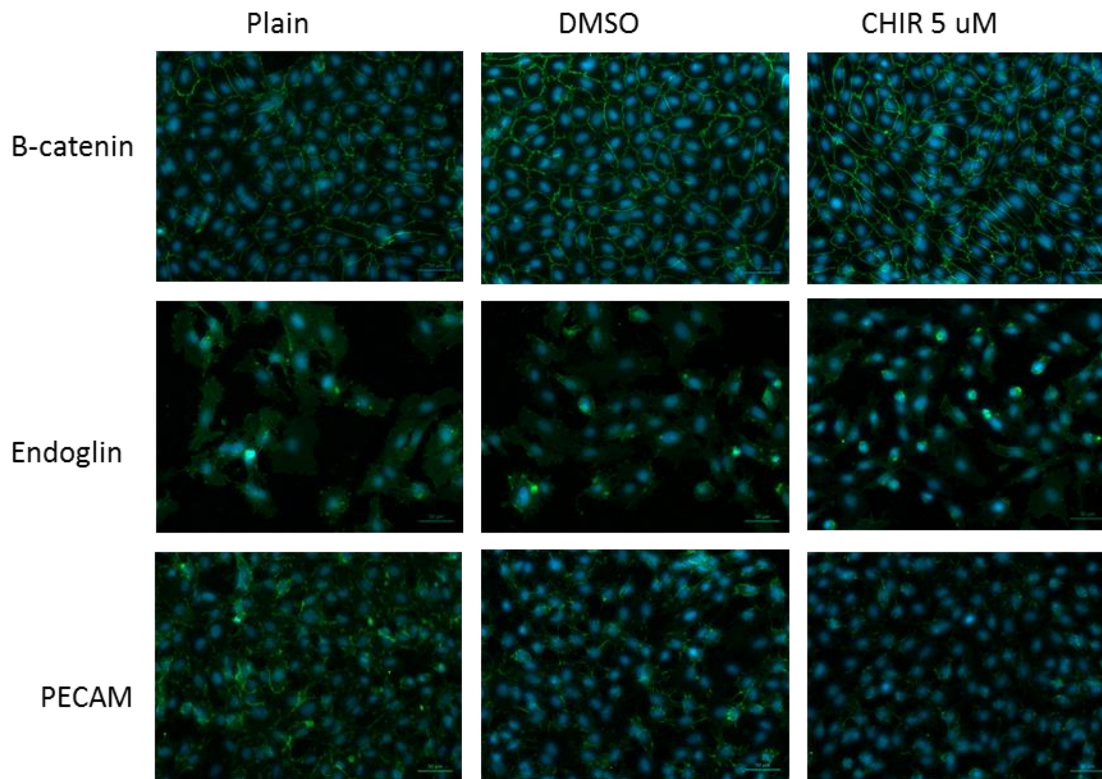

Figure 5-9.Representative ICC images of β-catenin, Endoglin and PECAM expression by HUVECs under effect of CHIR99021 at 5 uM vs DMSO and plain media control.

CHIR did upregulate β-catenin in HUVECs, p=0.04 relative to plain media while HUVECs expression of FLK1, Endoglin, VE-cadherin and PECAM was statistically comparable with the control, p=0.5, 0.2, 0.7 and 0.1, respectively.Scale bar 50 um.

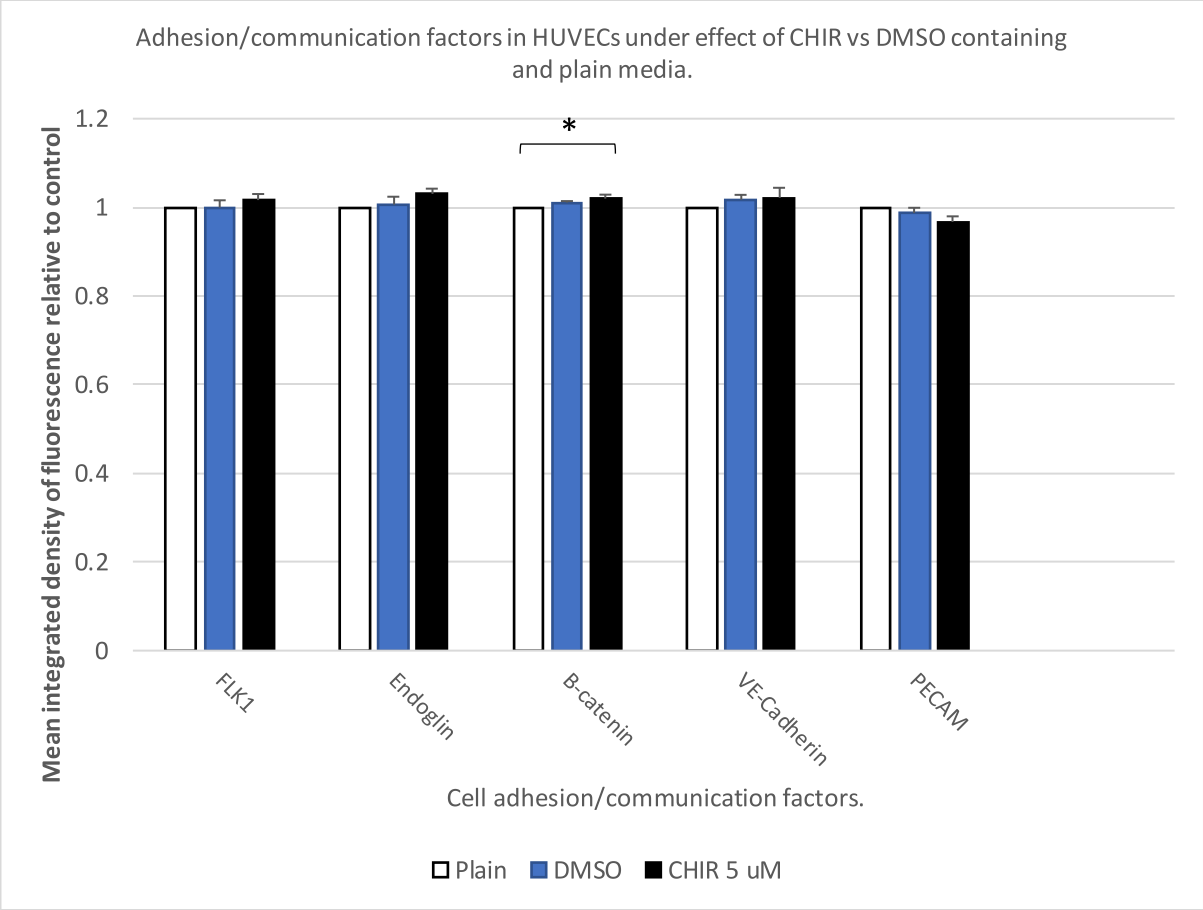

Figure 5-10. Effect of CHIR99021 at 5 uM on HUVECs expression of cell adhesion/communication factors.

CHIR did upregulate β-catenin in HUVECs, p=0.04 relative to plain media while HUVECs expression of FLK1, Endoglin, VE-cadherin and PECAM was statistically comparable with the control, p=0.5, 0.2, 0.7 and 0.1, respectively. N= 4 experiments but 2 cell lines. Expressed as mean+/-SEM.

5.3.4.5.CHIR 5 uM does not affect survival of HUVECs

In order to find out if CHIR would influence HUVECs survival, Caspase assay was used. CHIR did not show significant effect on survival of HUVECs in comparison with HUVECs in plain and DMSO containing media. Apoptotic volumes of cells were statistically comparable between the three conditions in keeping with its effect on Akt and NRG1.

Tukey HSD Post-hoc Test

- Plain vs DMSO: Diff=-2.65, 95%CI=-27.4 to 22.09, p=0.9
- Plain vs CHIR : Diff=21.54, 95%CI=-3.2 to 46.29, p=0.08
- DMSO vs CHIR : Diff=24.2, 95%CI=-0.55 to 48.9, p=0.05

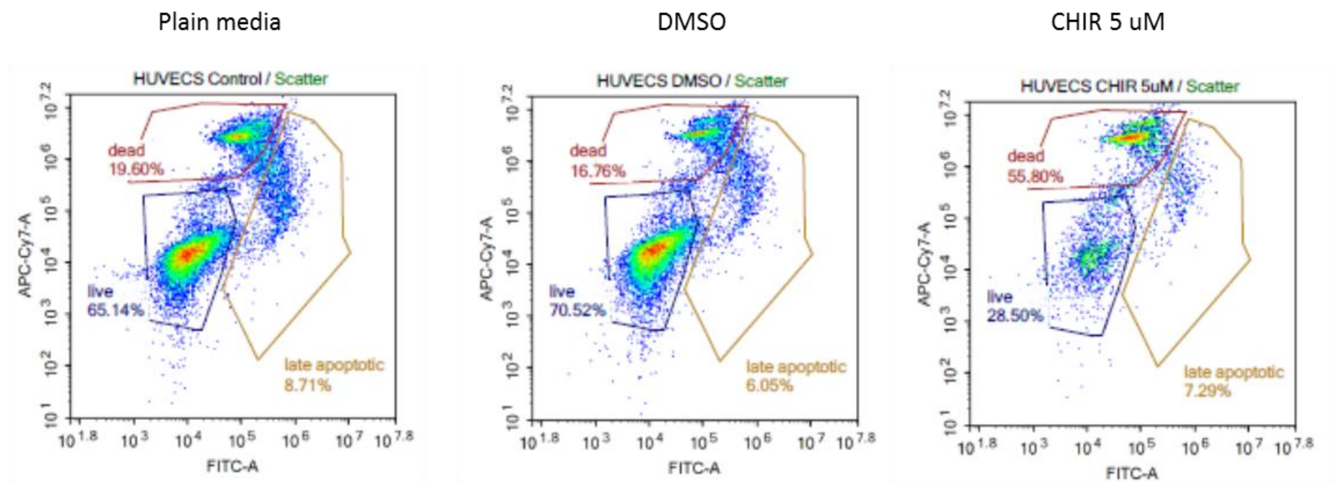

Figure 5-11.Representative images of FCM results of HUVECs survival under CHIR effect relative to control.

Effect of CHIR99021 at 5 uM, DMSO and plain media on HUVECs apoptosis and cell death.Percent of apoptotic and dead HUVECs was comparable between the three different conditions.N=3.

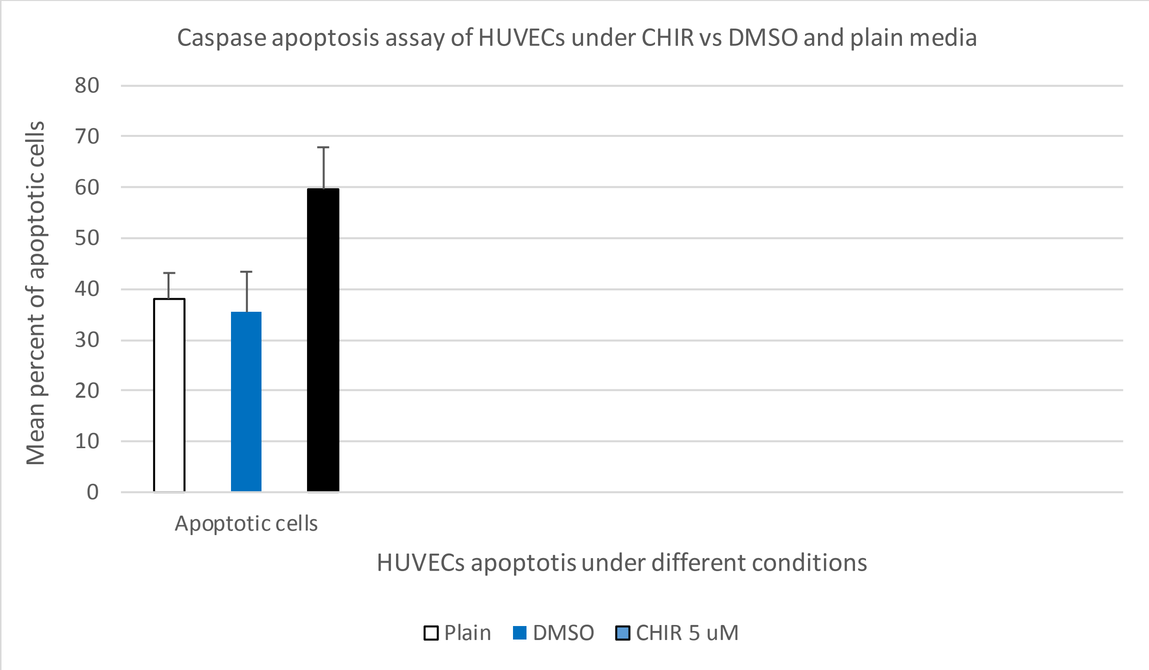

Figure 5-12.FCM results of HUVECs survival under CHIR effect relative to control.

Effect of CHIR99021 at 5 uM on HUVECs survival using caspase apoptosis assay in comparison with DMSO and plain media. All conditions were comparable with no statistical significance. Expressed as mean 3 experimental replicates +/-SEM.

5.3.4.6. CHIR/DMSO repress HUVECs proliferation

In order to to find out if CHIR treatment would influence HUVECs proliferation the BrdU assay was used. HUVECs cultured for up to 24 hours under CHIR, DMSO and plain media were examined and one way ANOVA with post HOC test was used to analyse the results.

There was significant difference with p=0.003. Then Tukey Post-hoc analysis revealed that HUVECs proliferation is comparable under DMSO and CHIR conditions while both significantly repressed HUVECs prolifearion relative to plain media.

Indeed, this could indicate DMSO effect rather than CHIR related effect.

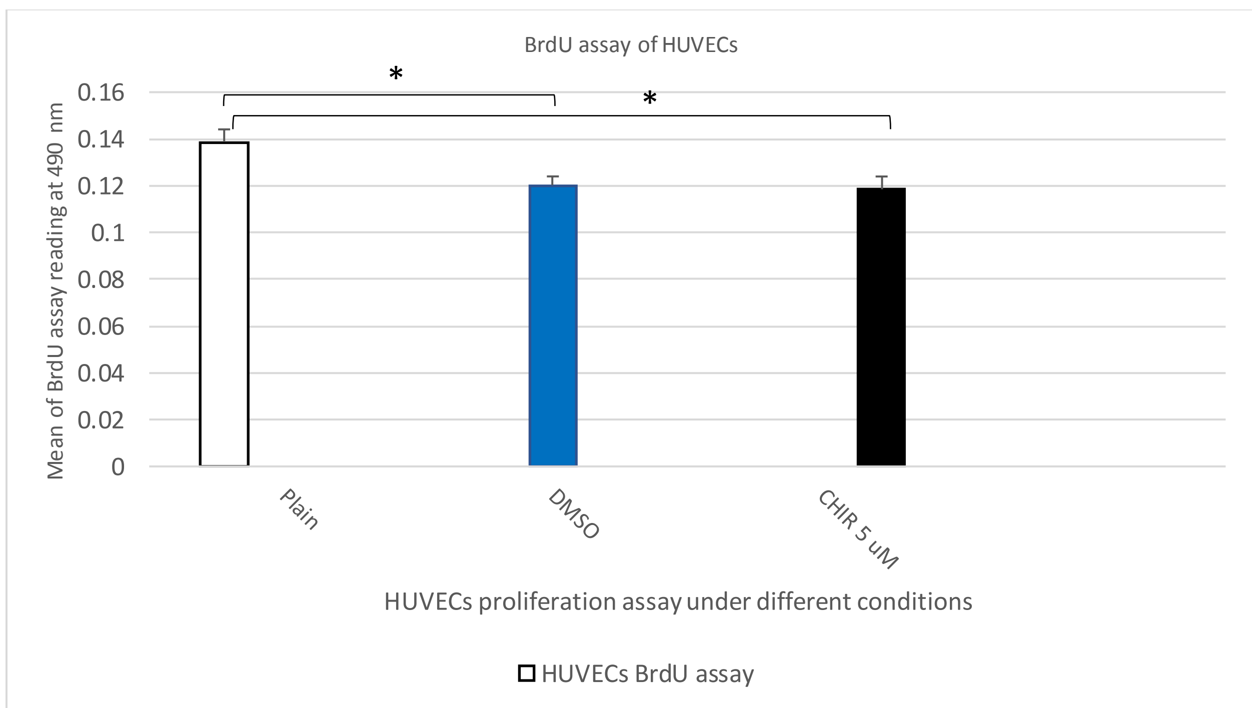

Figure 5-13.Proliferation potential of HUVECs under effect of CHIR relative to control.

Results of BrdU assay of HUVECs cultured for up to 24 hours to demonstrate effect of CHIR99021 at 5 uM on HUVECs proliferation in comparison with DMSO and plain media. HUVECs proliferation was repressed under effect of CHIR and DMSO relative to plain media. Expressed as the mean of 3 experimental replicates +/- SEM.

5.4. Combined effect of CHIR and CdM on HUVECs

5.4.1. Introduction

The initial assessment of the effect of CdM from co-culture of wjMSCs and HCFs on HUVECs was not promising. Indeed, it was more of unfavourable effect. It did downregulate bFGF and HGF with also high apoptosis rate. Then, we assessed the effect of CHIR99021 at 5 uM at the same assessment domains which did upregulate B-catenin while mostly neutral effect at other areas.

Here we plan to examine the combined effects of CHIR99021 at 5 uM and CdM to find out the potential behaviour of HUVECs after treatment with CHIR99021 when combined with HCFs and wjMSCs.

5.4.2. Objectives

To assess the effects of the combination of CHIR99021 at 5 uM in addition to CdM and compare it with just CdM to find out if treatment with CHIR99021 at 5 uM would have significant advantage relative to just co-seed. The focus of assessment was on HUVECS expression of survival factors, ECM proteins, angiogenic/paracrine factors, cell apoptosis/death and cell proliferation.

Afterwards, the combination of CHIR99021 at 5 uM in addition to CdM will be referred to as CHIR/CdM.

5.4.3.Results

After treatment of HUVECs with CHIR/CdM for up to 24 hours, cells were fixed, stained and images captured using the incucyte automated system and analysed using imageJ (as described earlier) and we got the following results.

HUVECs treated with CdM were used as control.

5.4.3.1.CHIR/CdM does not significanly affect Akt and NRG1 in HUVECs relative to CdM.

Firstly we assessed the survival factors Akt and NRG1 in HUVECs under CHIR/CdM effect relative to CdM. Expression of both factors under combined effect of CHIR/CdM was comparable with the CdM effect.

Akt-CHIR/CdM (M= 0.99, SEM= 0.01) relative to Akt-CdM revealed no significant difference, p= 0.65. Also,NRG1-CHIR/CdM (M= 0.99,SEM= 0.02) relative to NRG1-CdM revealed no significant difference,p= 0.9.

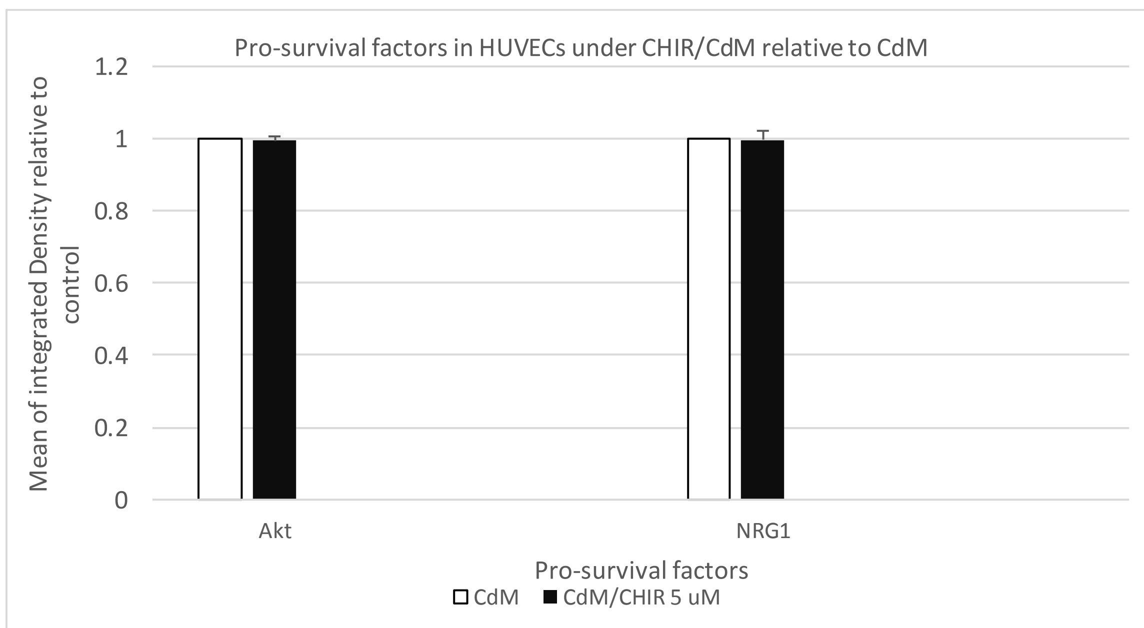

Figure 5-14.Expression of prosurvival factors Akt and NRG1 in HUVECs under CHIR/CdM effect in comparison to CdM**.*

HUVECs expression of Akt and NRG1 under CHIR/CdM was statistically comparable with CdM, p=0.6 and 0.9, respectively. N= 4 experiments but 2 cell lines. Expressed as mean+/-SEM.

*CHIR/CdM: combination of CHIR99021 at 5 uM with conditioned media.

**CdM: conditioned media from co-culture of wjMSCs and HCFs.

5.4.3.2. CHIR/CdM effect on HUVECs expression of fibronectin is comparable with CdM

Next assessment domain was the ECM protein fibronectin. HUVECs expression of Fibronectin-CHIR/CdM (M= 0.97,SEM= 0.014) relative to fibronectin-CdM revealed no significant difference, p= 0.1.

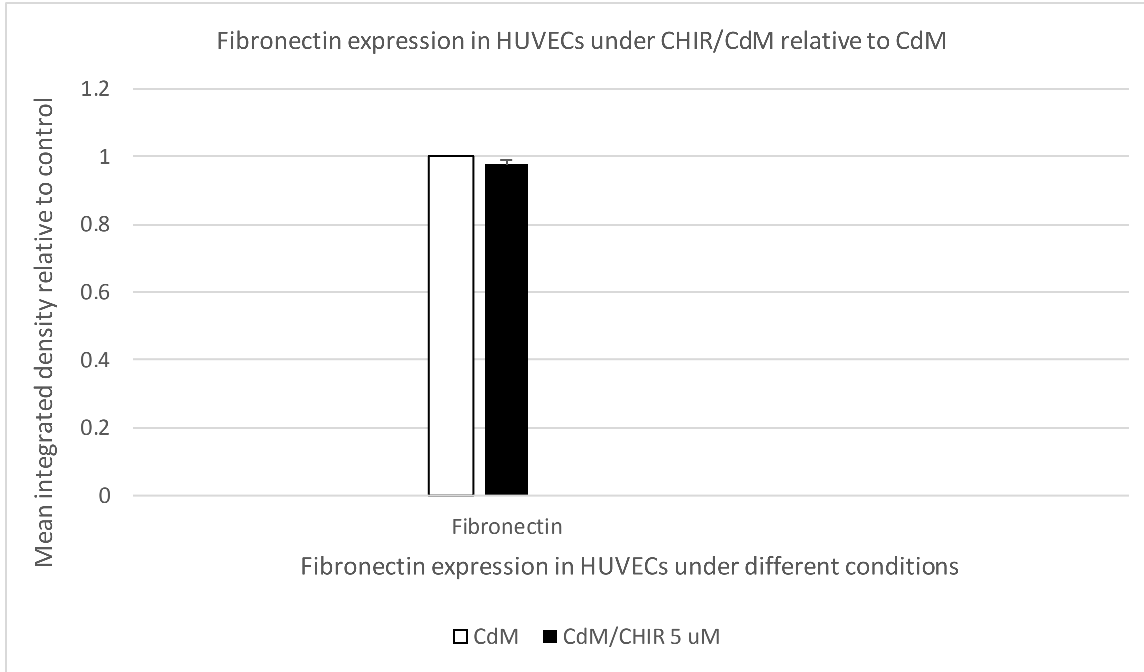

Figure 5-15. Expression of fibronectin in HUVECs under CHIR/CdM effect in comparison to CdM**.*

HUVECs expression of fibronectin under CHIR/CdM was comparable with CdM with no significant difference, p=0.1. N= 4 experiments but 2 cell lines. Expressed as mean+/-SEM.

*CHIR/CdM: combination of CHIR99021 at 5 uM with conditioned media.

**CdM: conditioned media from co-culture of wjMSCs and HCFs.

5.4.3.3. CHIR/CdM downregulates angiopoietin and bFGF in HUVECs but no significant effect on HGF and VEGF relative to CdM

The next assessment domain was the effect on angiogenic factors. HUVECs angiogenic profile under effect of CHIR/CdM relative to CdM revealed downregulation of Angiopoietin and bFGF while non-significant effect on expression of HGF and VEGF.

Angiopoietin-CHIR/CdM (M= 0.98, SEM= 0.003) relative to Angiopoietin-CdM showed significant downregulation, p= 0.001.

bFGF-CHIR/CdM (M= 0.98, SEM= 0.005) relative to bFGF-CdM revealed significant downregulation, p= 0.01.

HGF-CHIR/CdM (M= 0.99, SEM= 0.006) relative to HGF-CdM revealed no significant difference, p= 0.08.

VEGF-CHIR/CdM (M= 1.011, SEM= 0.01) relative to VEGF-CdM revealed no significant difference, p= 0.3.

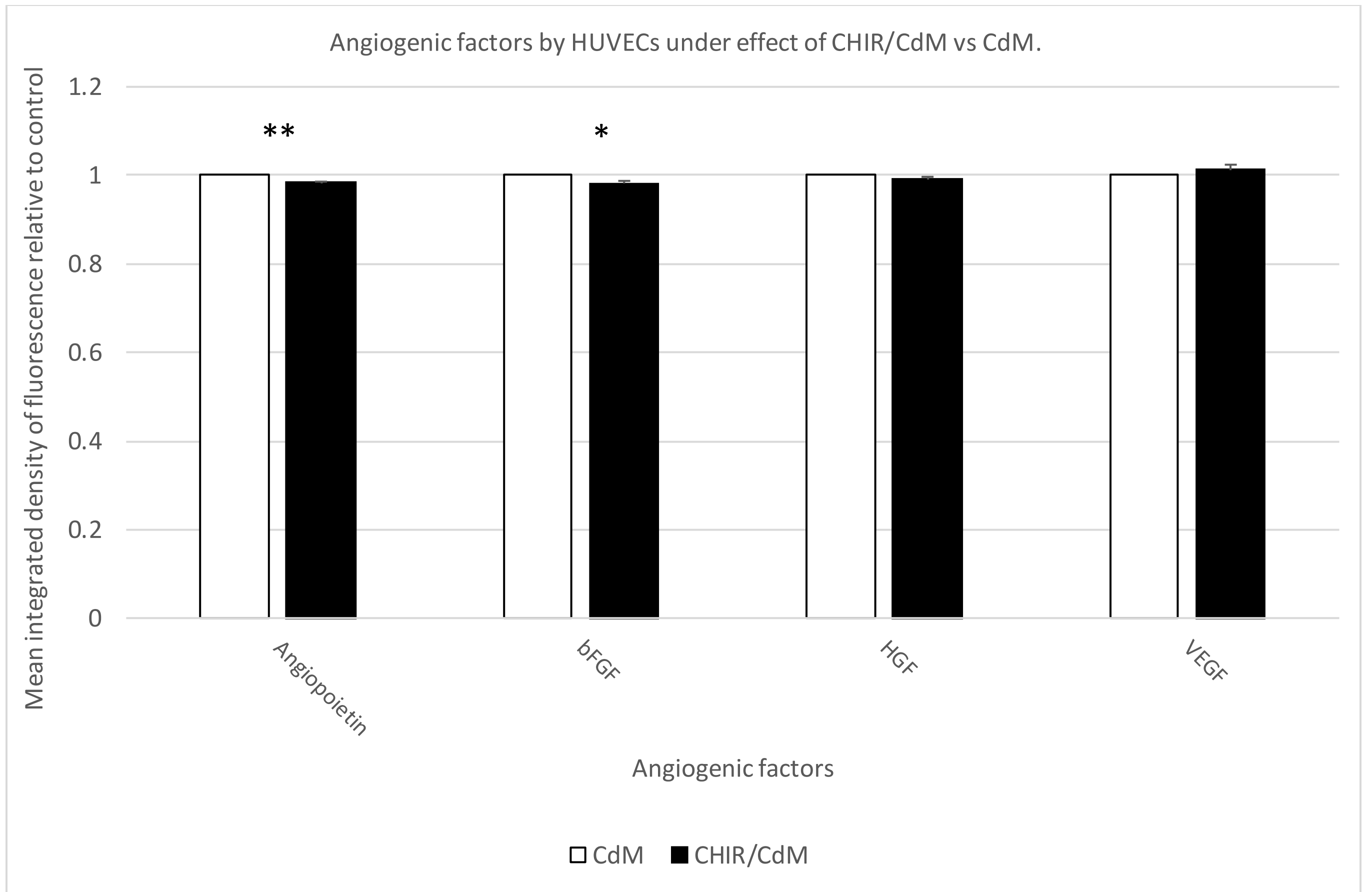

Figure 5-16.ICC expression of angiogenic factors in HUVECs under CHIR/CdM effect in comparison to CdM**.*

Angiopoietin and bFGF were significantly downregulated, p= 0.0018 and 0.01, respectively. While expression of HGF and VEGF in both conditions were comparable with no significant difference, p=0.08, 0.5 and 0.3, respectively. N= 4 experiments but 2 cell lines. Expressed as mean+/-SEM.

*CHIR/CdM: combination of CHIR99021 at 5 uM with conditioned media.

**CdM: conditioned media from co-culture of wjMSCs and HCFs.

5.4.3.4. CHIR/CdM downregulates PECAM in HUVECs relative to CdM but comparable effect on FLK1,Endoglin, β-catenin and VE-cadherin

The next assessment domain was the adhesion/communication factors. Assessment of HUVECs expression revealed significant downregulation of PECAM while non-significant effect on expression of FLK1, Endoglin, β-catenin and VE-cadherin.

FLK1-CHIR/CdM (M= 1.0039, SEM= 0.007) relative to FLK1-CdM revealed no significant difference, p= 0.8.

Endoglin-CHIR/CdM (M= 0.99, SEM= 0.016) relative to Endoglin-CdM revealed no significant difference, p= 0.7.

β-catenin-CHIR/CdM (M= 0.997, SEM= 0.01) relative to β-catenin-CdM revealed no significant difference, p= 0.8.

VE-Cadherin-CHIR/CdM (M= 1.001737, SEM= 0.008435) relative to VE-cadherin-CdM revealed no significant difference, p= 0.805117.

PECAM-CHIR/CdM (M=0.955, SEM= 0.018) relative to PECAM-CdM revealed downregulation, p= 0.02.

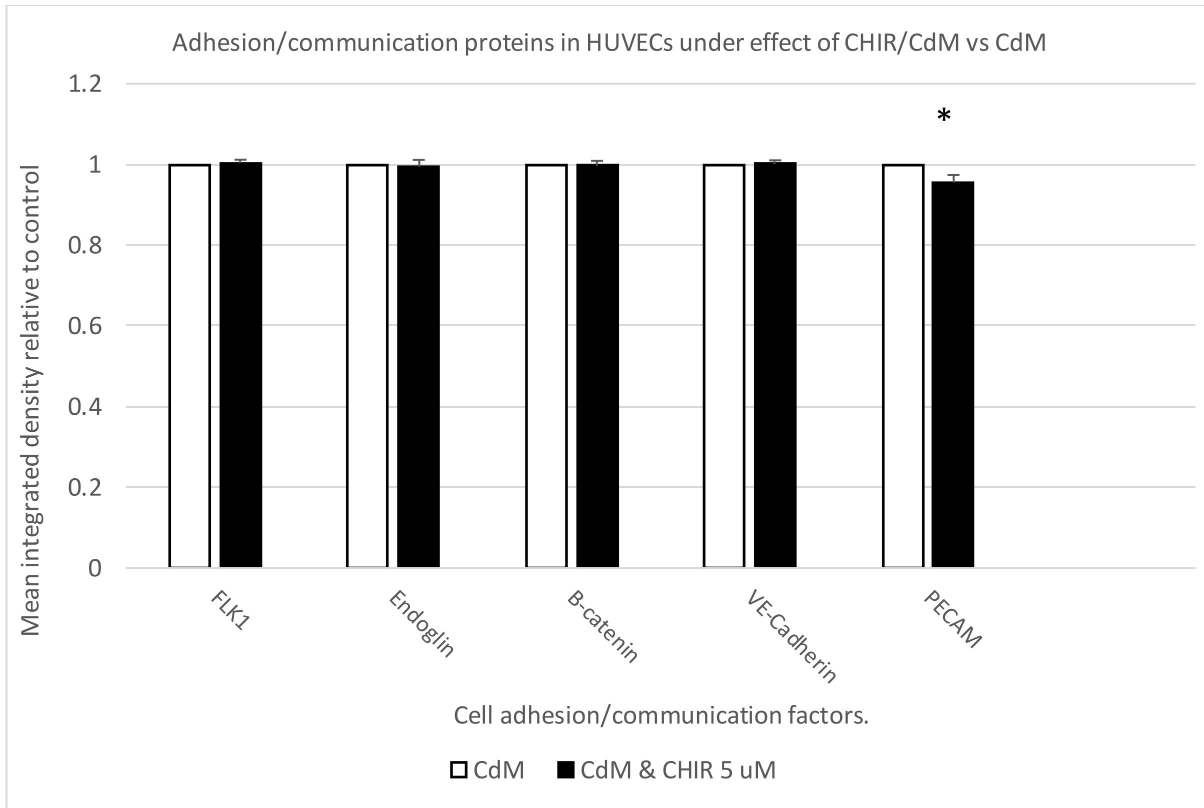

Figure 5-17.Expression of adhesion/communication factors in HUVECs under CHIR/CdM effect in comparison to CdM**.*

PECAM was significantly downregulated, p= 0.02, while FLK1, Endoglin, β-catenin and VE-cadherin in both conditions were comparable with no significant difference, p=0.8, 0.7, 0.8 and 0.8, respectively. N= 4 experiments but 2 cell lines. Expressed as mean+/-SEM.

*CHIR/CdM: combination of CHIR99021 at 5 uM with conditioned media.

**CdM: conditioned media from co-culture of wjMSCs and HCFs.

5.4.3.5.CHIR/CdM supports HUVECs survival

In order to assess CHIR/CdM effect on HUVECs survival, caspase apoptosis assay was used. After culture of HUVECs in CdM/CHIR for 24 hours cells were collected and Caspase assay was used to detect the apoptotic cells. HUVECs in CdM were used as control.

HUVECs apoptosis-CHIR/CdM (M=26.13,SEM=9.75) compared to apoptosis-CdM (M=60.43,SEM=10.44) showed significantly reduced incidence of apoptosis, p=0.01. Which means significant survival advantage.
Indeed, this result is quite supportive to adopt this treatment as it would significantly enhance survival of HUVECs which is expected to reflect on angiogenesis and sustainability of the patch.

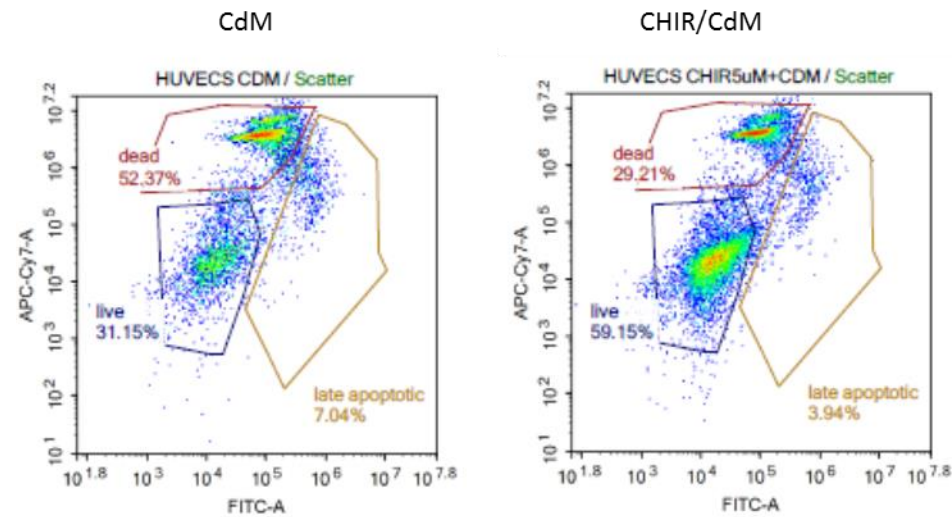

Figure 5-18.Representative images of FCM results for caspase apoptosis assay of HUVECs under effect of CHIR/CdM and CdM**.*

HUVECs apoptosis under effect of CHIR/CdM showed significantly lower rate under as compared with CdM effect, p=0.01.

*CHIR/CdM: combination of CHIR99021 at 5 uM with conditioned media.

**CdM: conditioned media from co-culture of wjMSCs and HCFs.

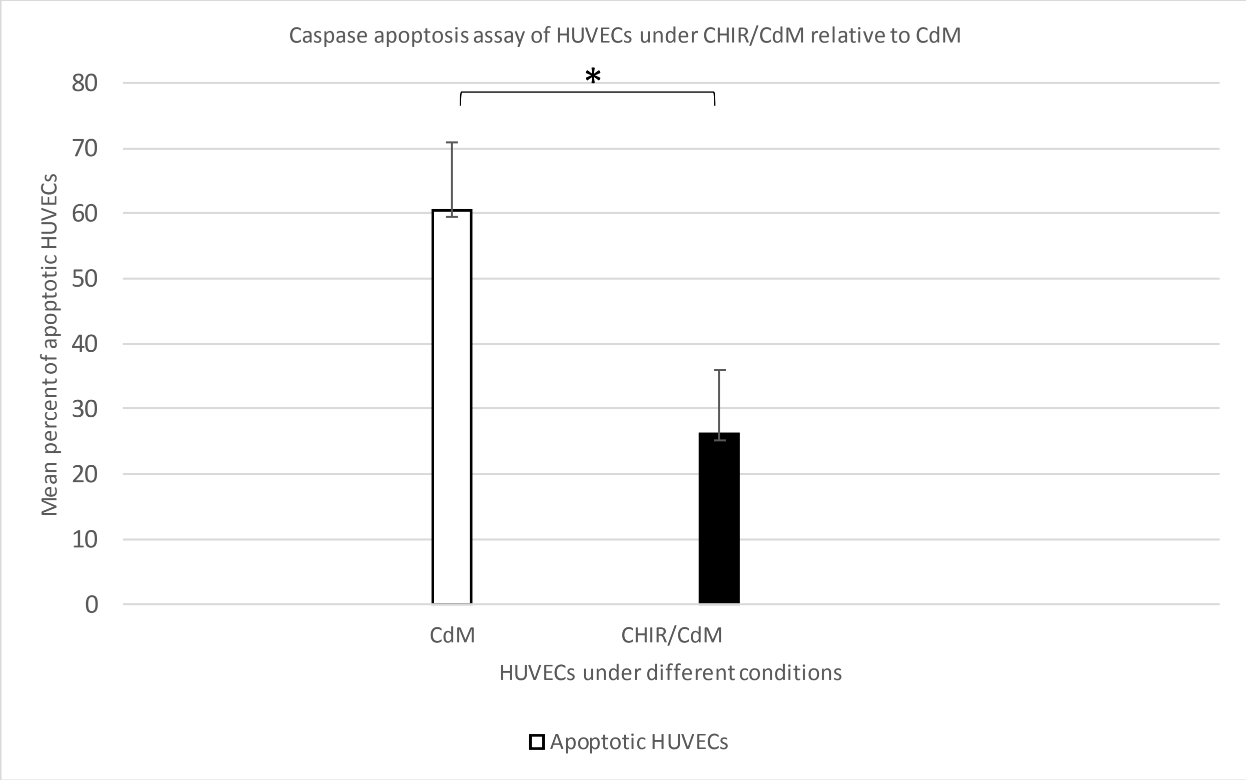

Figure 5-19.FCM results for caspase apoptosis assay of HUVECs under effect of CHIR/CdM and CdM**.*

HUVECs apoptosis under effect of CHIR/CdM was significantly lower than in CdM, p=0.01.N=2. Expressed as mean+/-SEM.

*CHIR/CdM: combination of CHIR99021 at 5 uM with conditioned media.

**CdM: conditioned media from co-culture of wjMSCs and HCFs.

5.4.3.6. CHIR/CdM does not affect HUVECs proliferation

In order to assess the CHIR/CdM potential influence on HUVECs proliferation BrdU assay was used. The results showed no significant difference of the proliferation potential.

CHIR/CdM (M=0.119,SEM=0.004) compared to CdM (M=0.117, SEM=0.013) showed statistically comparable results, p=0.9, which indicates to significant influence on HUVECs proliferation.

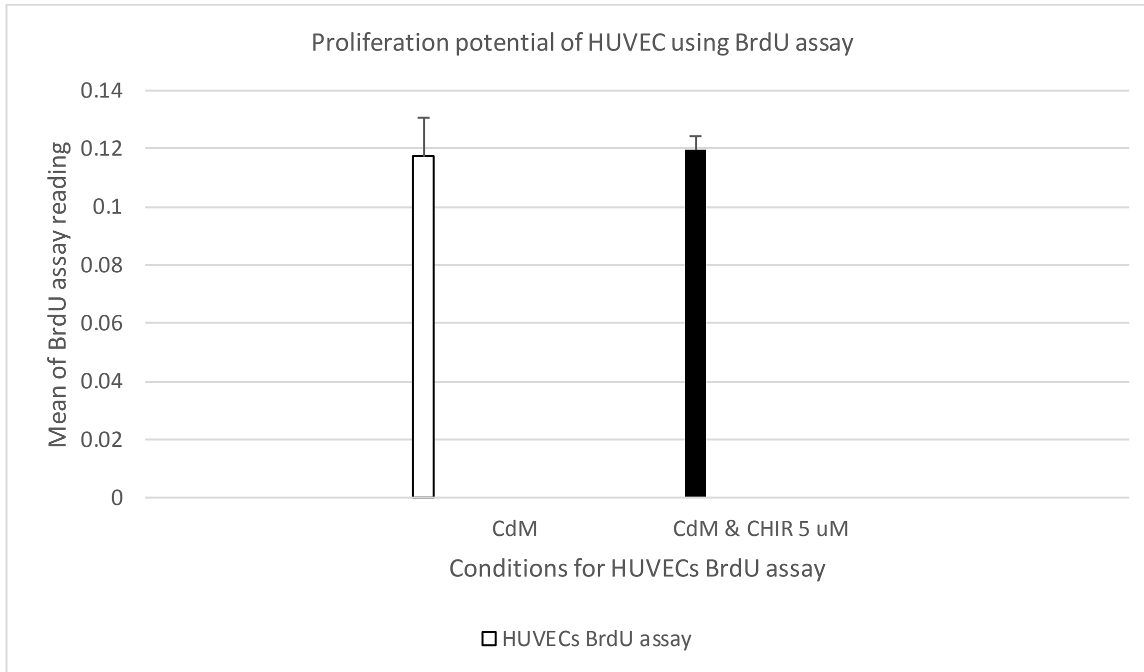

Figure 5-20.BrdU assay of HUVECs proliferation potential under Effect of CHIR/CdM as compared with CdM**.*

No signficant difference could be elicited, p=0.9 . N=3. Expressed as mean+/- SEM.

*CHIR/CdM: combination of CHIR99021 at 5 uM with conditioned media.

**CdM: conditioned media.

5.5. Discussion

5.5.1. Introduction

Perfusion is indispensable for any viable tissue and it is considered one of the challenges in any grafting therapy (412). Hence in the design of this patch we did consider HUVECs as pillars for angiogenesis as our target is to get them integrated with the neighbouring myocardial ECs to establish a proper perfusion network. Use of HUVECs was supported by their favourable properties such as autologous nature, easy isolation and handling in addition to quite extensive record of knowledge about in-vitro and in-vivo utilisation of HUVECs. Indeed, there are thousands of papers published on PubMed that vary between primary studies or reviews about HUVECs.

Blood vessels formation comes under two major formation processess, vasculogenesis and angiogenesis. Vasculogenesis refers to the initial formation of blood vessel but not from any previously established vessel. So, it starts from differentiation of precursor cells into ECs which integrate into the primordial vascular plexus while angiogenesis is extension of already estabsihed vessel (413). Vasculogenesis dominates during embryonic development to establish the vascular network. While angiogenesis would take the upper hand during post-natal life to maintain tissues perfsion, support growth in addition to acting as a back up during tissue damage to facilitate the healing process (371).

Angiogenesis is a multifactorial process controlled by balance between pro-angiogenics and anti-angiogenic factors such as angiostatin 1 and thrombospondin (371) as well as apoptosis signals to limit the angiogenesis to the demand and establish vascular lumen. This process is mainly required during normal development but still periodically active in adult life such as during wound healing as well as in pathologic conditions like tumour formation.

Angiogenesis would start with the angiogenic signals such as growth factors that would cause downstream cascade of events including to enhance gene transcription to initiate angiogenesis. This would continue until balanced by anti-angiogenic signals to resume the quiescent state. During this process, the microenvironment acts to guide the angiogenic process. The family of VEGFs bind surface receptors to enhance angiogenesis. When VEGF-A binds FLK1 it activates non-receptor tyrosine kinases such as Src-family kinases and focal adhesion kinases (FAK) (414). Src-kinase would phosphorylate VE-cadherin and disrupt endothelial cell junctions and induce vascular permeability (415). This would increase the concentration of angiogenic factors locally due to slow down of blood flow. And Src-kinases induced phosphorylation of FAK leads to disrupted ECs junctions (416). And PI3K activation would signal GTPases of the Rho family to initiate MAPK activation and cell proliferation (417).
The ECM would degrade and intercellular connections between ECs would loosen to allow ECs proliferation and migration to form the primordial vascular plexus. Then ECs would secrete TGF-B to recruit mesenchymal progenitor cells which would differentiate into pericytes and SMCs to enclose and support the neovessel with further maturation of the vascular network (323, 371, 418).

ECs migration would start with integrin activation of MAPK downstream of Src, FAK and Ras. This pathway would support ECs survival and angiogenesis and indicates the crucial role of MAPK as a main angiogenic signal pathway as the target for bFGF and VEGF via Ras and Raf (419).
Therefore, the integrins profile is not stable all through. Indeed, it undergoes a dynamic alteration during the angiogenesis process to modify the interaction with ECM which releases the ECs from quiescence phase to re-enter the cell cycle. On the other hand, it would maintain the balance through up-regulating apoptosis genes as well (420). Therefore, during angiogenesis integrins are considered as

dependence receptors and their upregulation without ligand would enhance apoptosis (421). This indicates that apoptosis of ECs would be influenced by integrins as well as the microenvironment (422). So, ECs would support angiogenesis as structural pillars to integrate into newly formed capillaries as well as their paracrine effects. This is via secreting several angiogenics such as angiopoietin, bFGF, HGF, PDGF and VEGF (412, 423).

It is noteworthy that despite angiogenesis is mainly ECs dependent however, ECs cannot establish mature vessel independently. Indeed, further cell recruitment including pericytes in small vessels as well as VSMCs in larger vessels to enclose the neovessel is indispensable for maturation of the neovessls. This process is partially mediated via action of TGF-B on Endoglin as co-receptors in addition to other factors such as the PDGF and Ang-1/Tie-2 with support of the extracellular matrix secreted by mural cells (300, 386).
As aforementioned, we aim in this project to combine different cell phenotypes on cormatrix for corrective cardiac surgery. In view of previous reports including our group experience with short in vivo durability of seeded or transplanted cells, so we did focus on examining the mutual reaction of each cell phenotype toward this combination and also aimed to boost the cellular properties via treatment with the small molecule CHIR99021 as a GSK-3 inhibitor.

5.5.2. CdM effect on HUVECs

In this chapter we did examine HUVECs as the main pillars for the patch angiogenesis. Despite the limitation here with two biologic replicates (despite 4 experimental replicates) but still assessment of the reaction of HUVECs to CdM from co-culture of wjMSCs and HCFs did not seem promising. It did not support survival and its angiogenic effect was not clearly positive. Indeed, it was leaning toward the negative effect which would raise concern about their angiogenic potential in this combination as the ECs survival is quite crucial for neovessel integrity (368).

5.5.2.1.Survival factors

We did start assessment by the survival factors Akt and NRG1. As explained before, we had a plan to examine other survival factors but due to the facility constrain we could not proceed.
Akt is an ECs survival promoting factor but its activation in ECs is depnedent on combination of VEGF as well as the anchorage based signaling pathways (368). Also, involved as downstream for other factors involved in angiogenesis and ECs survival such as bFGF and VEGF (348).

Our results showed that anti-apoptosis factor Akt as well as NRG1 did not show significant difference which suggests that HUVECs in this combination would lack any extra Akt and NRG1 survival support. These results are consistent with the apoptosis assay which showed statistically comparable results between cells in CdM and those in plain media (data not shown). Also, Akt non-significant change here is also consistent with the non-significant change of VEGF and downregulation of bFGF which holds survival effect for ECs.

The factors bFGF, VEGF and PDGF activate the PI3kα signalling in cardiac ECs and fibroblasts (348). Also PI3k/Akt expression in HUVECs is affected by these angiogenic factors in addition to the VE-cadherin/β-catenin complex which are involved in regulation of HUVECs proliferation, migration and survival (424).

Eventually, the apoptosis assay was clear reflection of lack of support and the percent of apoptotic cells in CdM was comparable with plain media (data not shown). which could be part of the downregulation effect of the angiogenic factors as well as non-significant effect on the anti-apoptosis Akt and effect on VE-cadherin which means loss of the Akt/VE-cadherin survival effect.

Indeed, HUVECs survival is a multifactorial complex process regulated by multiple inputs incldDuing the PI3k/Akt which is a key signal invloved in ECs proliferation, apoptosis and senescence (425). It's activation represses caspases 3,9 and 12 in HUVECs (425). On the other hand, it's activation would promote downstream signals including GSK-3α/β phosphyraltion with upregulation of β-catenin and VE-cadherin. This is still influenced by co-activators inclding bFGF and VEGF in addition to other downstream signals such as BCl-2, caspase3 and caspase9.

Another factor here is that HUVECs were demonstrated to undergo rapid apoptosis in 3D collagen-I mesh (379). Given the fact that both wjMSCs and HCFs produce collagen-I, so, this could be part of the unfavourable environemnt for HUVECs that would promote the apoptosis process.

This is an important concern here, because we know from previous experience that HUVECs undergo rapid apoptosis in-vivo (423), and in view of these results we expect HUVECs to undergo more rapid apoptosis and would disappear even earlier from the scene.

5.5.2.2. ECM proteins

Due to the critical rule of the ECM for integrity of both mature and evolving blood vessels, so we did examine HUVECs for production of ECM proteins including collagen-I, collagen-III and fibronectin.

Our results show that Collagen-I and collagen III did not express in HUVECs which is consistent with other reports (426) while Fibronectin was the only positively expressed by HUVECs and its expression by HUVECs under CdM effect was again suggestive of neutral effect.

Further to being one of the ECM proteins, fibronectin was found to initiate signal for ECM deposition. Indeed, it precedes collagen IV for ECM deposition during network formation (426). These results would raise another concern about to which extent the potential of HUVECs to actively share in angiogenesis in this combination of cells.

5.5.2.3. Angiogenic factors

The next assessment domain was the angiogenic factors which are indispensable for angiogenesis including the angiopoietin, bFGF, HGF, PDGF and VEGF.

Our data showed that HUVECs under CdM effect down-regulated bFGF and HGF while neutral effect on angiopoietin and VEGF and PDGF was not assessed.

The bFGF and VEGF are considered the main pillars for angiogenesis. They are secreted by ECs under control of an autocrine mechanism. ECs produce bFGF which up-regulates VEGF secretion which in turn mediates bFGF trophic effects on endothelial cells (427).
PDGF is a mitogen and motility promoting factor that acts on several cells inclduing endothelial cells (428). During angiogenesis, PDGF-B expression on ECs is not uniform. It varies from highest concentration at the foremost ECs of the sprouting neovessel with gradually lower expression in the more proximal ECs while pericytes recruitment lags behind the sprout to stabilise the neovessel (418). Furthermore, PDGF secreted by ECs acts as a chemoattractant to facilitate recruitment of mural

progenitor cells which woud differentiate to mural cells fate upon contact with ECs (323) and this maturation step is also supported by activation of TGF-B (429).

Assessment of the angigenic factors expression by HUVECs under CdM effect demonstrated down-regulation of bFGF and HGF while its effect was more of neutral effect on angiopoietin and VEGF. Unfortunately the PDGF antibody did not work, so it's data were omitted.

These data suggest reduction of the angiogenic effect of bFGF and HGF and loss of the synergistic angiogenic effect between HGF and VEGF (430). In fact this would flag more concerns about the stability of HUVECs within this combination and their angiogenic potential. Also the results are consistent with the migration experiments that seemed to be negatively affected (data not shown) and again raises a concern about fate of HUVECs in this triad of cells and to which extent it would support angiogenesis in-vivo.

5.5.2.4. Adhesion/communication factors

Due to the crucial role of inter endothelial cells communication in adjusting angiogenesis and vascular permeability (431) so we did examine expression of the adhesion and communication factors. The panel examined here included β-catenin, Endoglin and VE-cadherin, PECAM as well as VEGFR1, VEGFR2 and ITG.

Our results demonstrated upregulation of Endoglin and β-catenin while VE-cadherin, VEGFR1 and PECAM expression was comparable with the control under effect of CdM. ITG and VEGFR2 were not assessed.

Unfortunately, VEGFR1 and ITG antibodies did not seem to work so their results were omitted.

One of the β-catenin effects is to promote the angiogenic properties of HUVECs and enhance tube formation via upregulation of VEGF. However, β-catenin acts via a downstream signalling pathway that involves the Akt and so despite its upregulation was a promising result but still in absence of effective downstream signals its effects are still quiestionable. Our data demonstrate that B-catenin was up-regulated under CdM effect while survival was not supported and the apoptosis rate was still quite high.

Endoglin is a membrane glycoprotein that is mainly expressed on endothelial and hematopoetic cells in addition to other cells such as the pro-imflammatory macrophaes (432) and MSCs (226). It acts as a co-receptor for some of the TGF-B superfamily (432). Its role is quite crucial to support and maintain integrity of vascular plexus as it enhances proliferation and migration of ECs. Indeed, Endoglin is mainly a co-receptor that modulates response rather than to initiate a downstream cascade of signals (301, 433). Its expression on ECs is influenced by certain factors such as TGF-B superfamily in particular BMP-9 and TGF-B1 as well as hypoxia (388), in addition to its respons to other factors such as cleavage from membrane bound position via matrix metalloprotienase-14 (MMP-14) and MMP-12 (432, 434).

Endoglin action is mainly to facilitate maturation of primordial vascular plexus during angiogenesis. Therefore, it gets upregulated during active angiogenesis such as in wound healing as well as during inflammation (435).

VE-cadherin (CD144 or cadherin 5) acts as a regulator of endothelial permeability (436) as Its facilitates loosening the cell-cell adhesion in established vessels to allow permeability as well as sprouting for further angiogenesis. It acts downstream of VEGFR2 which are in turn under control of VEGF (431).

Our data demonstrate upregulation of Endoglin while VE-cadherin expression was comparable with the control under effect of CdM. Despite Endoglin upregulation sounds promising for support of angiogenesis, however, still these results should be interpreted with caution especially in view of other negative angiogenic results.

ECs interaction with the ECM via integrins is another key factor for maintainance of vessel integrity. If it gets disrupted, the ECs apoptosis would ensue with subsequent detachment from surrounding matrix in a process known as anoikis (368).

VEGFR1 (Flt1-fms-like tyrosine kinase 1) is expressed on endothelial cells as well as hematopoietic stem cells, monocytes and macrophages. Also, VEGFR2 (flk1-fetal liver kinase 1) is expressed on endothelial cells both vascular and lymphatic (373). Unfortunately, Flt1 antibody did not work so it was not included in the data but FLk1 did show basal level of expression and was not upgraded at any point.

VEGFR-2 is one of the earliest markers expressed on ECs as well as hematopouetic cells during embryonic life (300). Its expression is upregulated during active vasculogenesis/angiogenesis under physiologic conditions such as during embryonic life as well as pathologic conditions such as tumour formation (437). In the study of Shalaby et al. they did generate Flk1-/- embryonic stem cells. The embroys died between days 8.5- 9.5 inutero due to failure of development of ECs with absent signs of any organised blood vessels in the embryo or the yolk sac and marked reduction of hemangioblasts (437) which indicates the essential role of VEGFR-2 to facilitate proper vasculo/angiogenesis.

Our data show that CdM effect on HUVECs expression of surface receptors VEGFR2 was suggestive of neutral effect which is in keeping with the neutral effect on VEGF (431). So, HUVECs expression of β-catenin and endoglin were the only significantly upregulated factors while the rest of factors did not achieve statictical significance. Unfortunately, these results in addition to the apoptosis data do not seem in favour of angiogenesis in-vivo esepcially in the context of other negatively regulated angiogenic factors.

The platelet endothelial cell adhesion molecule PECAM-1 (CD31) is a transmembrane glycoprotein and one of the ECs adhesion molecules that acts to maintain the integrity of the vascular barrier (438) and also involved in cell migration and angiogenesis (439).

Also, PECAM-1 was reported to play role in cell survival (440). This report is not consistent with our data that showed significant survival benefit despite down-regulation of CD31 under combined effect of CHIR/CdM. However, the study of Sardjono and colleagues that did demonstrate the CD31 cytoprotective effect was in platelets (440). PECAM physiologic role was further characterised by Duncan and colleagues who generated CD31-/- mice. The mice survived the in-utero period and borne healthy with no clear vascular abnormality which proves dismissible role for ECs survival (441). Nevertheless, the abnormality elicited in the CD31 deficient mice was poor trans-vascular migration of leucocytes when triggered by inflammation (441). On the other hand, Tsuneki et al. reported CD31 down-regulation to correlate with reduced apoptosis (442) which is consistent with our data.

Indeed, CD31 and VE-cadherin are considered as junctional molecules that also act downstream of other factors such as CD44 to mediate its effects. They are multi-function factors as they mediate cell-cell interaction, junctional integrity as well as their role in ECs proliferation and migration (442).

5.5.2.5. Proliferation potential

For assessment of HUVECs proliferation potential we used the BrdU assay. Despite HUVECs proliferation is expected to be facilitated by β-catenin up regulation, the results showed that proliferation was comparable between CdM and plain media. Indeed, this is consistent with loss of the proliferation support of bFGF and VEGF (443). This is in keeping with the observation of other groups who reported that Wnt/β-catenin proliferative effects are dependent on other angiogenic factors such as the bFGF (409).

5.5.3. Effect of CHIR99021 on HUVECs

Then, we did attempt to enhance the properties of the patch via upgrading the properties of the cells using small molecules as a convenient way to boost the HUVECs properties using the GSK-3 inhibitor CHIR99021 (CHIR).

The GSK-3β pathway is a vital signalling pathway for HUVECs angiogenic behaviour. It is inversely related to the HUVECs migration and response to chemotaxis of bFGF and VEGF. Therefore, its inhibition is expected to positively reflect on HUVECs angiogenic potential (444).

GSK-3 inhibition in HUVECs was reported with another laboratory using BIO. They reported partial dedifferentiation indicated by upregulation of the stemness genes Oct4 and Nanog. This effect had some positive impact on HUVECs functional assays including migration and tube formation. However, BIO was not able to achieve significant effects untill boosted with VEGF (404).
Here, we did experiment CHIR99021 without any additional growth factors. It did upregulate Oct4 and nanog but there was no effect on Sox2 mRNA (data not shown) which is keeping with same dedifferentiation effect. However, the pure effect of CHIR at 5 uM on other relevant domains was still mostly neutral. This could indicate the need for further factors to accomplish its effects.

The starting point reveals that CHIR at 5 uM does not significantly affect the expression of Akt or NRG1.

Despite data are non-conclusive but it gives impression of neutral effect on HUVECs survival which was actually seen in the apoptosis experiments as the level of apoptosis was numerically higher in the CHIR group but still statistically comparable between the CHIR treated HUVECs and its control. This could be related to the p53 upregulation as well as the increased intracellular ROS in the CHIR treated cells (210).

The fairly neutral effect still applies to the fibronectin and angiogenic factors expression by HUVECs which did not demonstrate any significant alteration relatve to control. However, CHIR has some favourable effects in terms of upregulation of β-catenin.

In addition, it did demonstarte downregulation of the proliferation potential which was comparable between the DMSO and CHIR groups and gives the impression of DMSO effect rather than CHIR induced effect.

5.5.4. Combined effect of CdM and CHIR99021 on HUVECs

Then, we attempted to examine the combined effect of CHIR in addition to conditioned media from co-culture of wjMSCs and HCFs (CdM/CHIR) to find out the net effect and if it would offer any positive effect relative to the CdM.

Indeed, the combination of CdM/CHIR revealed significant effect on HUVECs survival which seems the most important and relevant effect as it could address the concern of rapid HUVECs apoptosis. The

combination of CdM/CHIR did reduce HUVECs apoptosis by about 57%. This could be due to some other pro-survival factors that were not examined here.

This is quite significant and promising result that this approach could salvage HUVECs when combined with the other cells and also promising to support retention of HUVECs on patch as well as in-vivo.

Despite the data show downregulation of the angiogenic factors angiopoietin and bFGF while the rest of angiogenic factors expression were comparable. This may flag a concern about the angiogenic support that would be available for HUVECs once treated with CHIR and seeded in combination with wjMSCs and HCFs. However, further experiments are required to assess to which extent CHIR would support HUVECs to engage with angiogenesis so that we can assess if the higher survival outweighs the risk of angiopoietin and bFGF downregulation.

The effect of CHIR/CdM on cellular communication factors was mostly neutral with the exception of significant reduction of PECAM. Also, Effect on fibronectin as ECM protein was also non significant and effect on HUVECs proliferation was also neutral.

5.6. Conclusion

HUVECs reaction to the combination of wjMSCs/HCFs has negative impact with poor survival support and down regulation of some angiogenic factors. This could explain the rapid wash out of HUVECs after transplant in-vivo and would argue against the use of HUVECs in the design of the patch. However, survival of HUVECs could be partly salvaged with the addition of CHIR at 5 uM. This seems promising and supports to get it experimented in-vivo.

CHAPTER VI

Effect of CdM and CHIR99021 on HCFs survival, proliferation and paracrine profile

6.1.Introduction

HCFs represent a main cell line in the myocardium. They have indispensable roles during development. In addition to their invaluable role for adequate function under physiologic conditions as well as adaptation to response to pathologic stress.

Cardiac fibroblasts have the most important structural role via secreting the ECM. ECM represents the mesh that contains cells, connects them and maintains organ integrity and function. Also, fibroblasts in the myocardium sense mechanical signals and make electrical coupling with CMCs to facilitate electric transmission which is a prerequisite for contraction of each champer as one unit.

During inflammatory stress such as ischemic event, the CFs would dominate the scene to offer anti inflammatory effect and limit the cellular loss. Also, would differentiate into myofibroblasts with higher ECM production to seal the gap and protect against the more serious event which is myocardial rupture.

Therefore, CFs are quite mandatory and is expected to add extra value to the patch as one of the cellular triad.

In the next chapter would attempt to examine HCFs reaction when combined with HUVECs and wjMSCs via testing the effect of CdM. Then would examine their reaction to CHIR99021 at 5 uM, and finally would examine the net effect of CHIR/CdM.

6.2. Effect of CdM

6.2.1. Introduction

Here we would attempt to examine the HCFs reaction to the combination with wjMSCs and HUVECs via analysing the effect of conditioned media (CdM) from co-culture of wjMSCs with HUVECs. Assessment done to examine the effects of CdM at several relevant domains to ensure the patch's properties in-vitro before proceed to in-vivo experiments.

6.2.2. Objectives

The CdM from co-culture of wjMSCs and HUVECs was used to study the effect of the combination of cells on HCFs . And the effect was tested at the following domains

- Anti-apoptosis and prosurvival: to ensure the combination would support survival of cells.
- ECM proteins expression: to assess if any extra secretion of ECM that would raise concern of excessive fibrotic changes of the patch.
- Cell adhesion/communication factors: in view of the fundamental role of intercellular communication for functionality of tissues.
- Cell survival: to ensure cells viability is well supported and therefore the patch cellularity would be maintained.
- Cell proliferation potential: to assess if any potential for HCFs to overpopulate the patch.
  HCFs cultured in plain media were used as control.

6.2.3. Materials and Methods

The following materials and methods were used for preparation of CdM.

6.2.3.1. Conditioned media (CdM) preparation

CdM was prepared from co-culture of wjMSCs with HUVECs. Cells from each phenotype were plated in endothelial growth media (EGM) at about $5x10^5$ in T25 or $1x10^6$ in T75 flasks and kept in humidified incubator at 37 ^oC with 5% Co_2.

After about 48 hours 70-80% confluence was achieved. Media was taken out and cells were washed with prewarmed PBS then addedd plain EGM (serum and growth factors free) and kept in incubator under same conditions for 24-48 hours. Then media was aspirated and filtered through 0.2 um filter for immediate use or kept at -20 ^oC for use within two weeks or at -80 ^oC for longer storage.

6.2.3.2. Culture in CdM

Before induction, the CdM was thawed in water bath at 37 ^{o}C then filtered through 0.2 um filters and mixed with plain EGM (serum and growth factors free) at 1:1 ratio for immediate use.

Media was aspirated and cells were washed with prewarmed PBS then the prepared CdM was added and kept in humidified incubator at 37 ^{o}C with 5% Co_2 . After culturing cells in CdM for 48-72 hours, cells were fixed and stained (as described earlier). Images were captured using incucyte system automated system in Wolfson imaging unit, biomedical school, University of Bristol.

Cells cultured in plain EGM (serum and growth factor free) were used as control.

6.2.4. Results

Analysis done using ImageJ to measure the protein expression in terms of integrated density and got the following results.

Due to the sudden suspension of all university facilities so we could not run three biologic replicates and HCFs experiments are done at 4 experimental replicates but one cell line.

6.2.4.1. CdM effect on HCFs proteins expression

6.2.4.1.1. CdM downregulates Akt but does not significantly affect NRG1

The first assessment area was the pro-survival factors Akt and NRG1.

HCFs expression of Akt-CdM (M=0.958,SEM=0.01) relative to AKT-cnt did show statistically significant downregulation, p= 0.004. While NRG1-CdM (M=1.0064,SEM=0.01) relative to NRG1-cnt demonstrated no significant effect, p=0.6, figure (6-1).
This suggests HCFs in this combination would lose some of their survival enhancing factors. However, still we do not know if this would reflect negatively on survival or not given the fact that other factors were not examined.

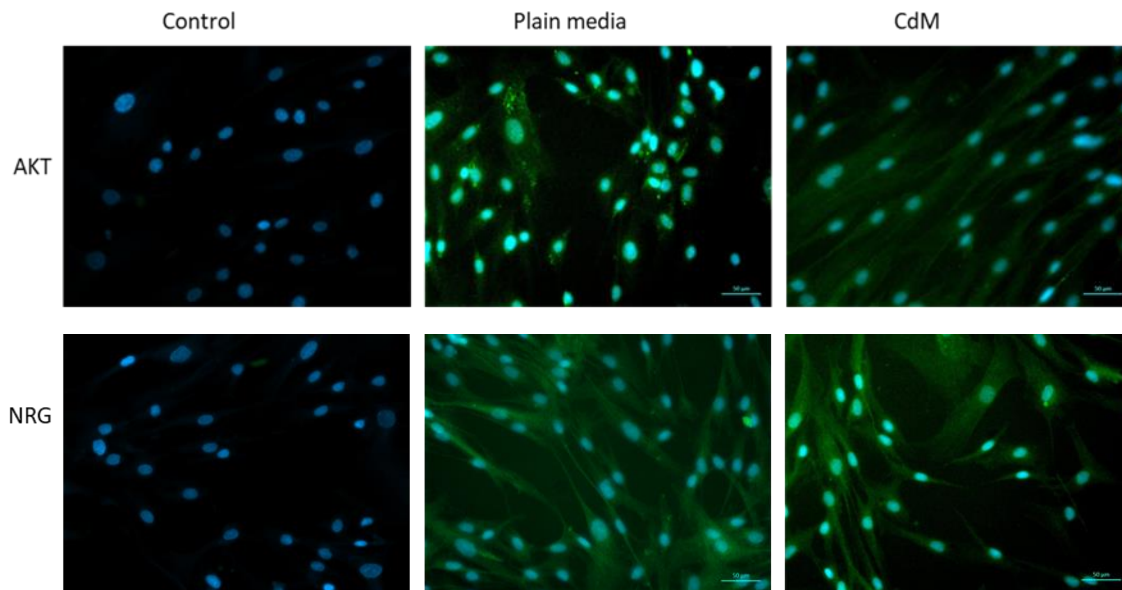

Figure 6-1. Representative images of HCFs expression of Akt and NRG1 in CdM vs control.*

HCFs expression of Akt was downregulated in under effect of CdM relative to control, p=0.004 while NRG1 expression in HCFs under effect of CdM was comparable with the control,p=0.6. N=5 experimental replicates . Scale bar 50 um.

*CdM: conditioned media for HCFs from co-culture of wjMSCs and HUVECs.

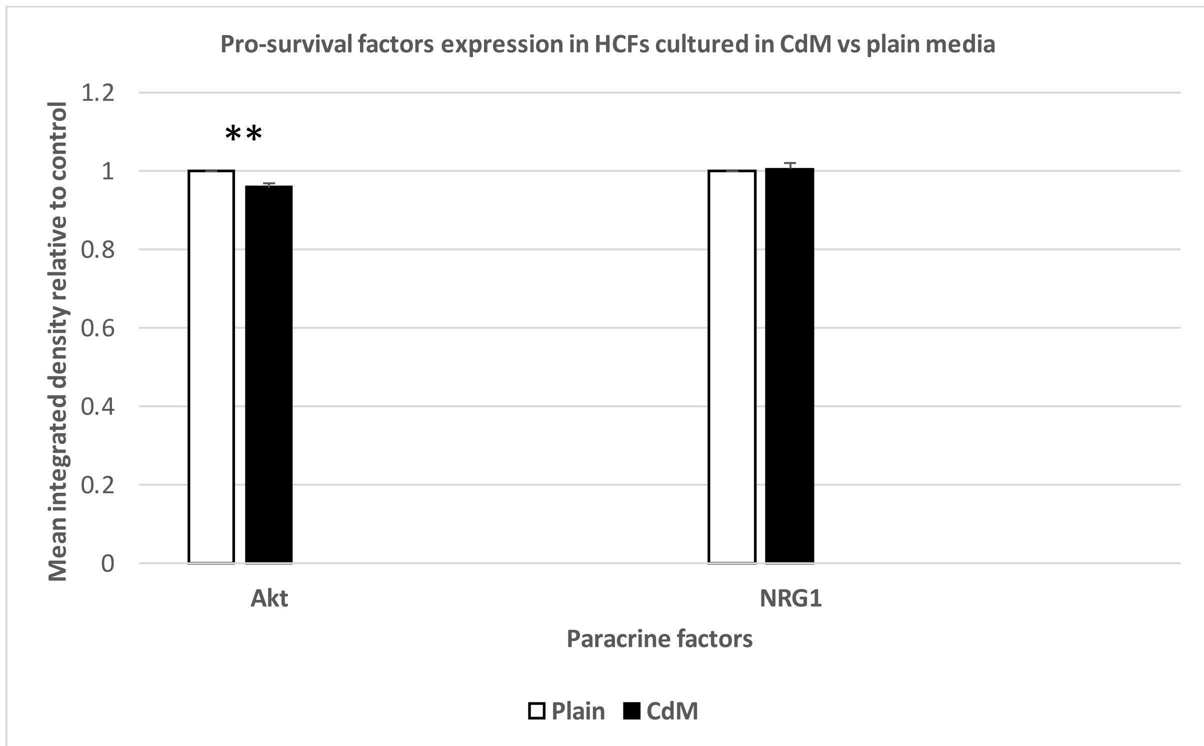

Figure 6-2.HCFs expression of AKt and NRG1 in CdM vs control.

HCFs expression of Akt was downregulated in under effect of CdM relative to control, p=0.004 while NRG1 expression in HCFs under effect of CdM was comparable with the control,p=0.6. N=5 experimental replicates.Expressed as mean+/-SEM.

*CdM: conditioned media for HCFs from co-culture of wjMSCs and HUVECs.

6.2.4.1.2.CdM has neutral effect on ECM proteins in HCFs

The next assessment domain was the ECM proteins. We did examine the effect of CdM on HCFs secretion of collagens I and III as well as fibronectin. Indeed, HCFs are the main ECM producing cells in the myocardium. They produce ECM at basal levels, but once activated, they differentiate into myofibroblasts with more enhanced rate of ECM production which is a feature of remodeling with all its sequelae ncluding myocardial stiffness with poor contractility and poor prognosis. Therefore, it is quite crucial to ensure balanced ECM production with no extra ECM deposition. So, we did assess for the main components including collagen I,collagen III and the glycoprotein fibronectin.

Expression of these ECM proteins in HCFs under effect of CdM was comparable with the control.

Collagen I-CdM (M= 0.996,SEM= 0.02) compared to Collagen I-cnt demonstrated non-significant difference, p=0.8.
Collagen III-CdM (M= 0.99,SEM= 0.006) compared to Collagen III-cnt demonstrated non-significant difference, p=0.2.
Fibronectin-CdM (M= 1.004,SEM= 0.012) compared to Fibronectin-cnt demonstrated non-significant difference, p=0.7.

These data suggest quite assuring results as fibroblasts are the main producers of ECM and as long as the ECM proteins are not upregulated under effect of CdM, so it is expected that the patch would not suffer from ECM over-production. This stands against the concern of massive fibrotic changes of the patch and also no under-production to give concern about loss of ECM structural and functional role.

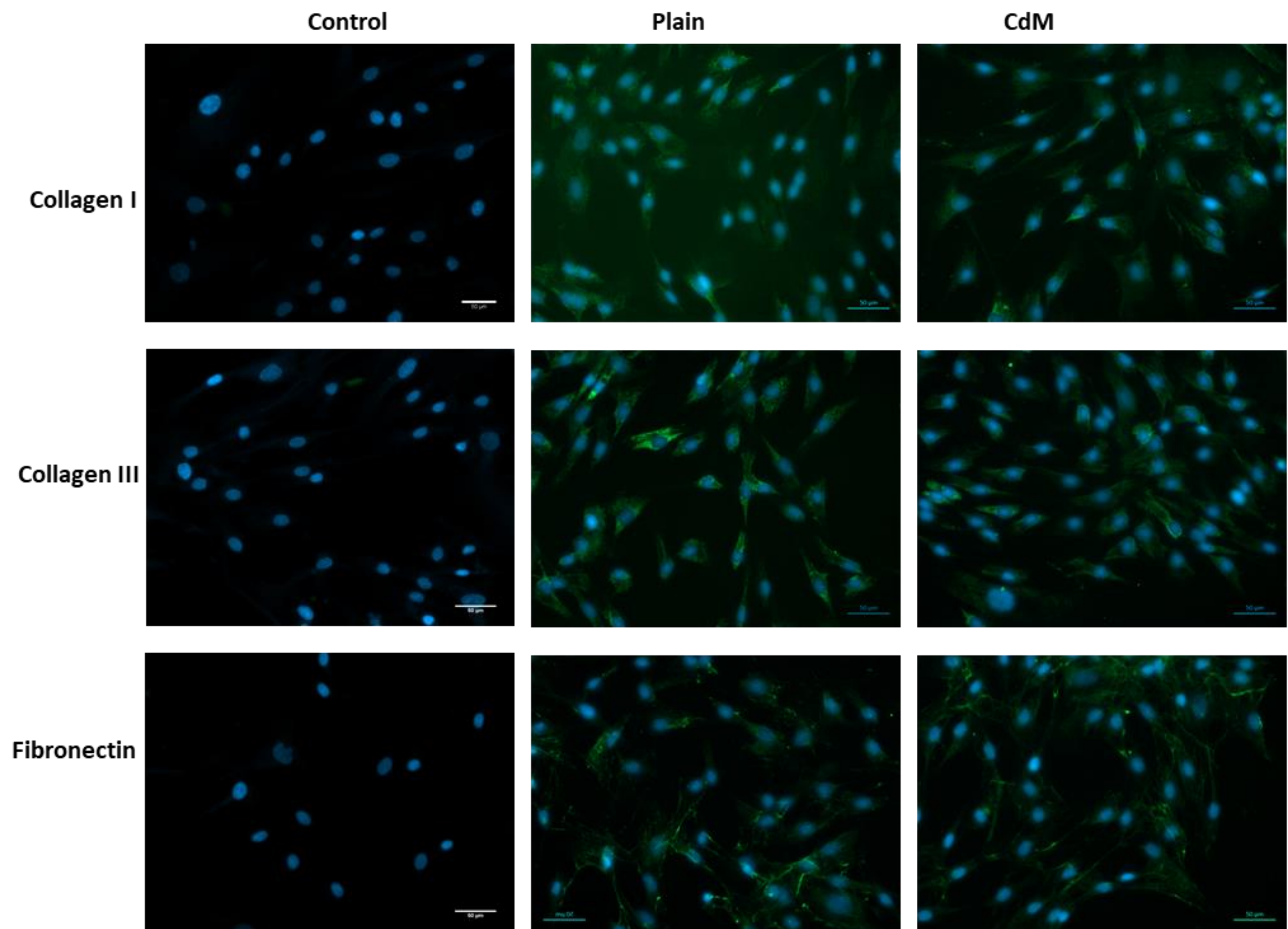

Figure 6-3.Representative image of HCFs expression of ECM proteins under CdM** effect.*

The ECM proteins expression in HCFs under effect of CdM was comparable with the control. Collagen I, p=0.8, collagen III, p= 0.2 and fibronectin, p=0.7. N=5 experimental replicates but fibronectin N=4. Scale bar 50 um.

*ECM: extracellular matrix.

**CdM: conditioned media for HCFs from co-culture of wjMSCs and HUVECs.

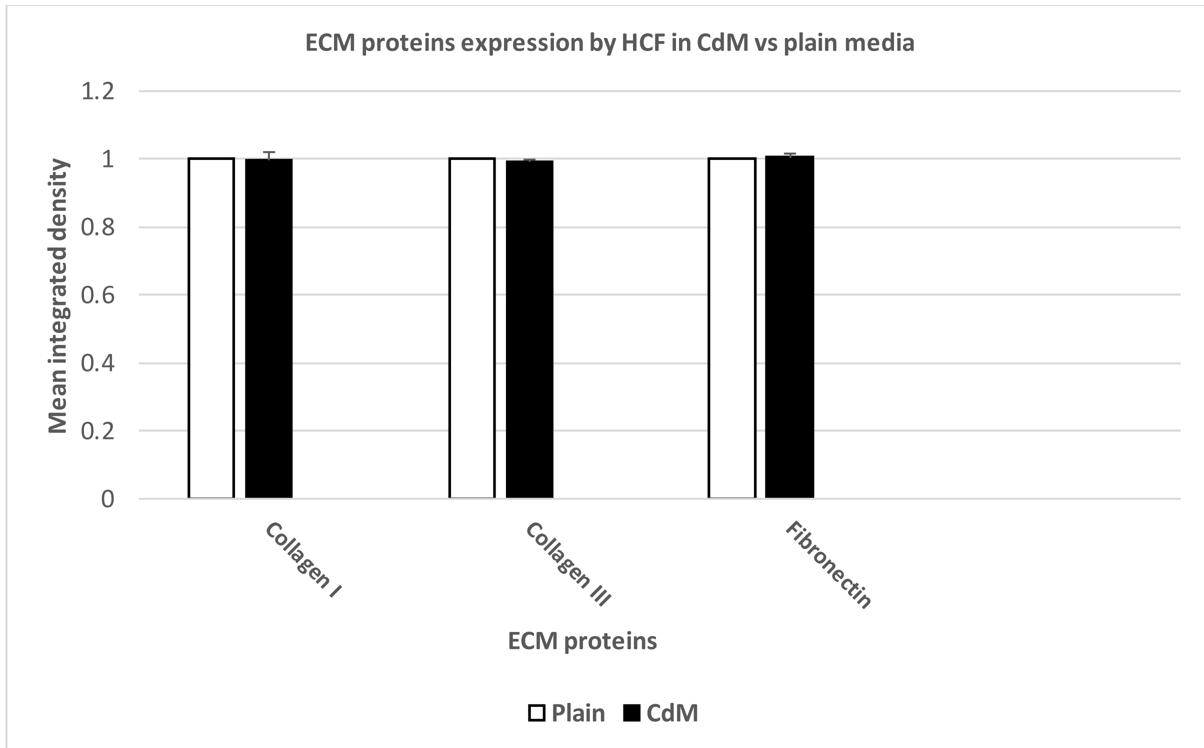

Figure 6-4.HCFs expression of ECM proteins under CdM effect.*

The ECM proteins expression in HCFs under effect of CdM was comparable with the control. Collagen I, p=0.8, collagen III, p= 0.2 and fibronectin, p=0.7. N=5 experimental replicates but fibronectin N=4. Expressed as mean+/-SEM.

*ECM: extracellular matrix.

**CdM: conditioned media for HCFs from co-culture of wjMSCs and HUVECs.

6.2.4.1.3. CdM does not significantly affect B-catenin, upregulates N-cadherin and does not promote HCFs differentiation to myofibroblasts

The next assessment domain was the adhesion/communication factors as well as the α-SMA as a marker of HCFs differentiation into myofibroblasts.

β-catenin-CdM (M=1.0089,SEM= 0.018) relative to β-catenin-cnt demonstrated non-significant difference, $p=0.5$.
But N-cadherin-CdM (M=1.028,SEM= 0.007) compared to N-cadherin-cnt demonstrated significant up-regulation, $p= 0.001$.
Further to the communication factors, we did assess the HCFs for the potential of differentiation to myofibroblasts. Myofibroblasts are more active and produce more ECM which would raise concern of fibrotic changes of the patch. We did the assessment using α-SMA as an indicator of myofibroblast differentiation.

α-SMA-CdM (M=0.95,SEM= 0.058) compared to α-SMA-cnt demonstrated no significant difference, $p=0.5$.
So, α-SMA expression was comparable between CdM and control. This result argues against the concern of potential fibroblast differentiation into myofibroblasts which are more active in ECM production. Also, these data are consistent with the previous results of neutral effect of CdM on HCFs secretion of collagens and fibronectin.

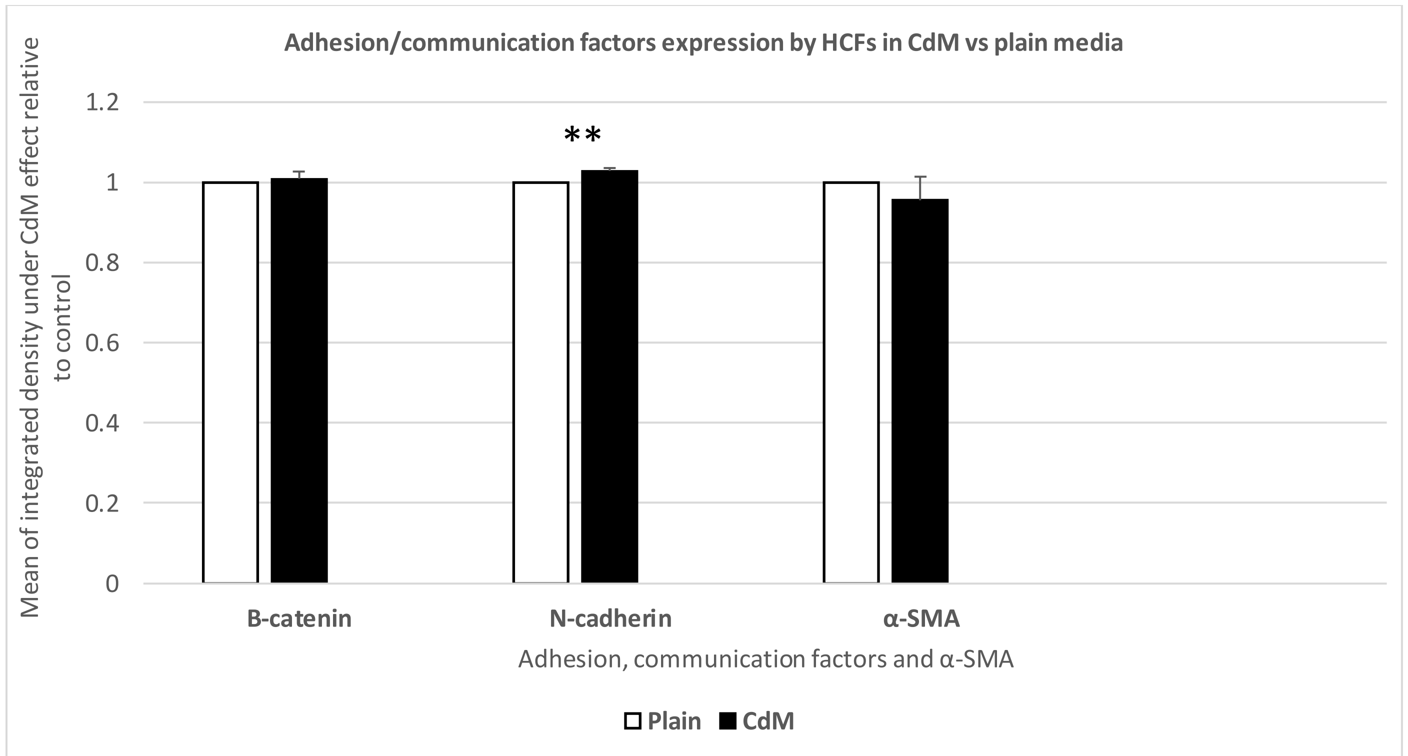

Figure 6-5. HCFs expression of β-catenin, N-cadherin and α-SMA by HCFs under CdM effect.*

N- Cadherin was significantly up-regulated under CdM effect while b-catenin and a- SMA expression were comparable between the CdM and control conditions with the p=0.001, 0.5 and p=0.5 respectively. N=4 experiments. Expressed as mean+/-SEM.

*CdM: conditioned media for HCFs from co-culture of wjMSCs and HUVECs.

6.2.4.2.mRNA levels assessment via qPCR

In order to assess the effect of CdM on HCFs at level of mRNA and to which extent the relevant genes are affected by the CdM, we did use qPCR and got the following results

6.2.4.2.1.HCFs level of mRNA for Akt and NRG1.

The mRNA levels for AKt and NRG1 was apparently higher in the CdM cells as compared with plain media, however, none achieved statistical significance.

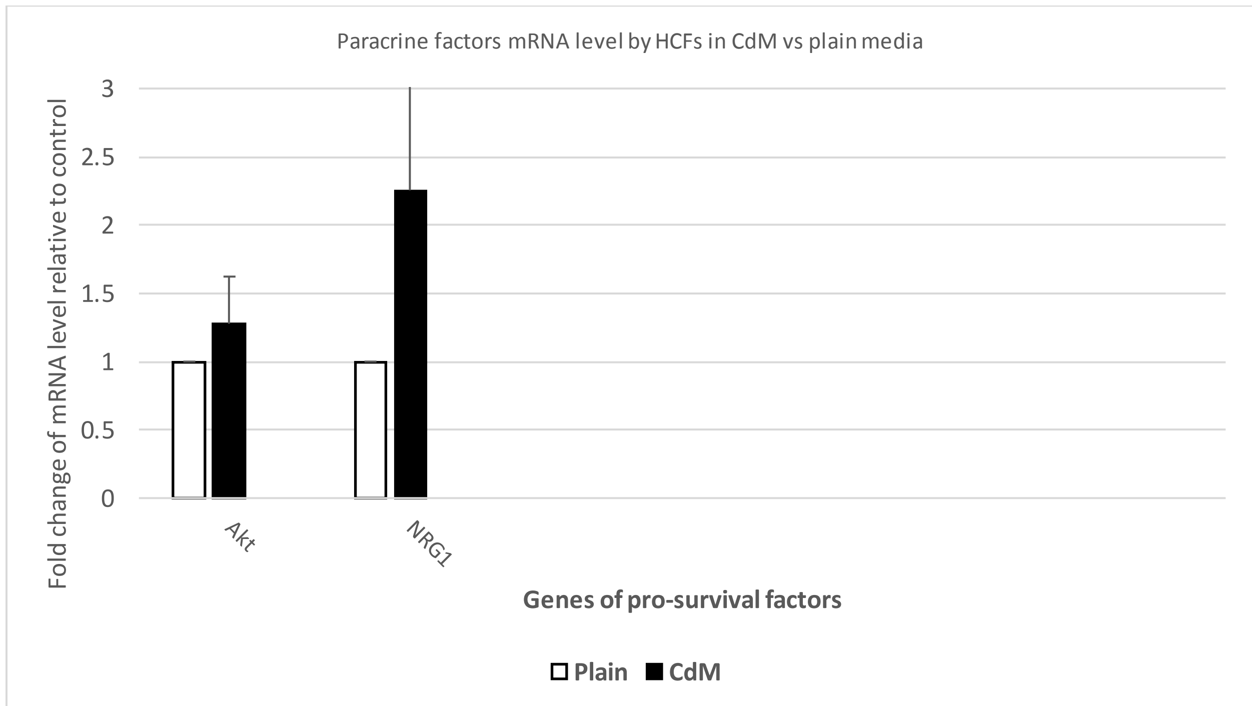

Figure 6-6.mRNA of Akt and NRG1 in HCFs under CdM effect.*

No significant statistical difference could be elicited.

*CdM: conditioned media for HCFs from co-culture of wjMSCs and HUVECs.

6.2.4.2.2.Effect of CdM on HCFs mRNA of genes for ECM, differentiation and cell adhesion/communication factors

mRNA levels of collagen I and fibronectin were significantly upregulated in HCFs-CdM in comparison with the HCFs-cnt while mRNA for collagen III, β-catenin, MMP and TIMP did not demonstrate statistical significane.

Despite the limitation with lack of biologic replicates but these data suggest upregulation of collagen I and fibronectin genes and in view of the ICC results of non-significant change of colagen I and fibronectin so most probably these proteins are not transcriptionally dependent within this time window.

The mRNA for MMP and TIMP did not demonstrate any significant alteration under CdM effect. Their proteins were attempted in the ICC experiments but the antibodies did not work so can not draw any conclusions about transcription correlation.

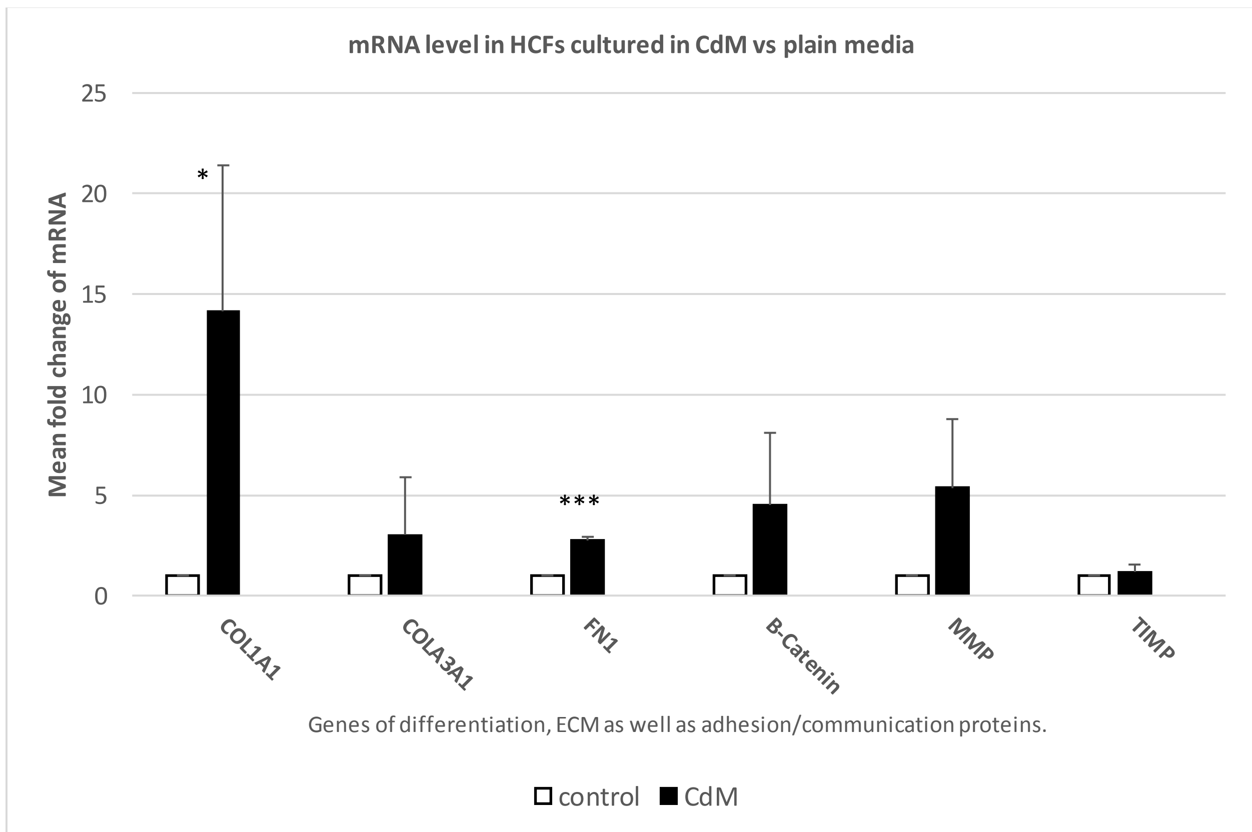

Figure 6-7.mRNA for ECM, differentiation and cell adhesion/communication factors in HCFs under CdM effect.*

Collagen I and fibronectin were significantly upregulated while all the rest of factors were comparable with the control.

*ECM: extracellular matrix.

**CdM: conditioned media for HCFs from co-culture of wjMSCs and HUVECs.

6.2.4.3. CdM does not enhance HCFs survival

In order to assess the effect of the combination of cells on HCFs survival, caspase apoptosis and DRaq7 dead cell assay were used via FCM.

The caspase/DRaq7 assays did not show any survival benefit. The percent of apoptotic and dead cells were comparable in HCFs cultured in CdM for 48-72 hours and HCFs cultures in plain media for same duration, p= 0.2.

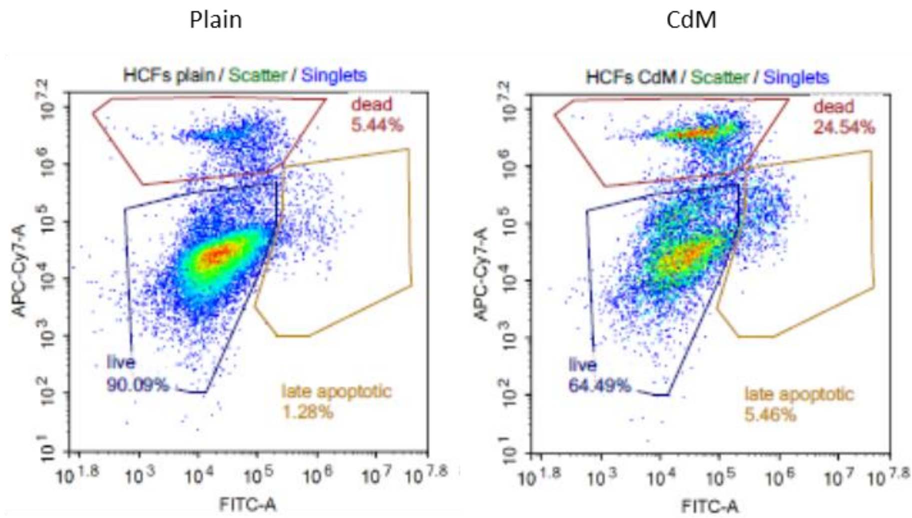

Figure 6-8.Representative image of HCFs apaoptosis/cell death under CdM vs control.*

Volume of apoptotic HCFs was comparable between CdM and plain media, p=0.2. N=3

*CdM: conditioned media for HCFs from co-culture of wjMSCs and HUVECs.

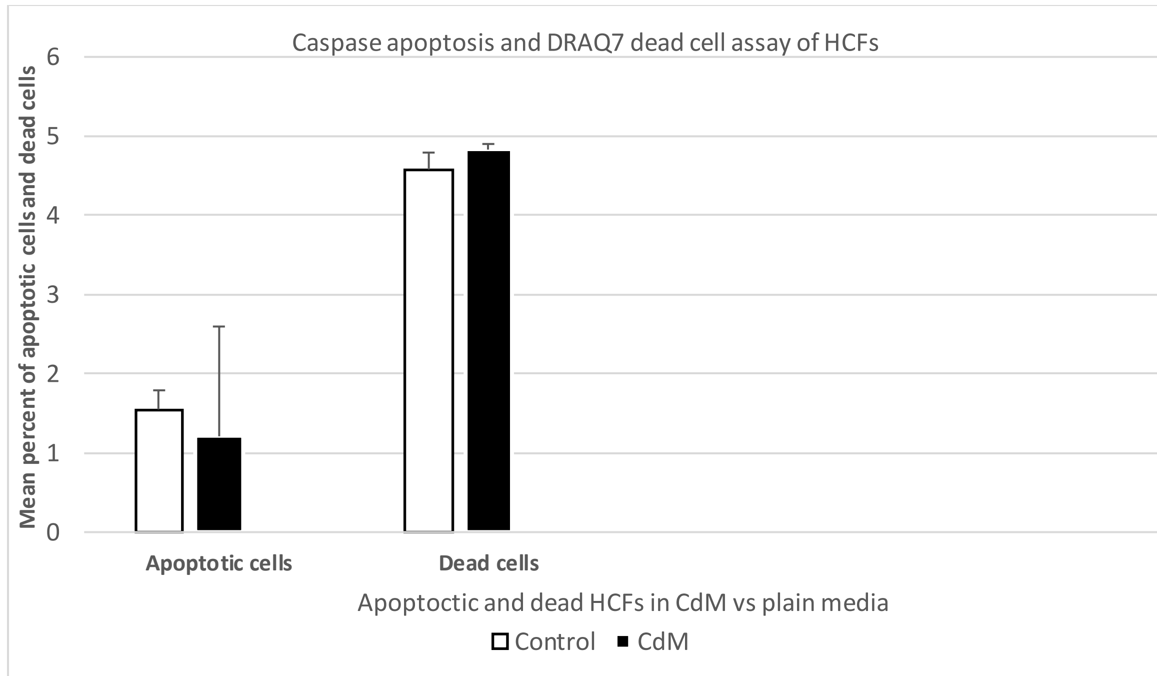

Figure 6-9.HCFs apaoptosis/cell death under CdM vs control.

CdM does not offer survival advantage to HCFs. Volume of apoptotic HCFs was comparable between CdM and plain media, p=0.2. N=3.Expressed as mean+/-SEM.

*CdM: conditioned media for HCFs from co-culture of wjMSCs and HUVECs.

6.2.4.4. CdM does not alter HCFs proliferation

In order to assess the effect of the combination of cells on HCFs proliferation potential, BrdU assay was done and experiments repeated three times.

Proliferation of HCFs cultured in CdM for 24-48 hours as demonstrated by the BrdU assay readings was (M= 0.083, SEM= 0.002) and the control HCFs cultured in plain media (M=0.096, SEM=0.0059) for same duration and results were statistically non-significant, p= 0.1.

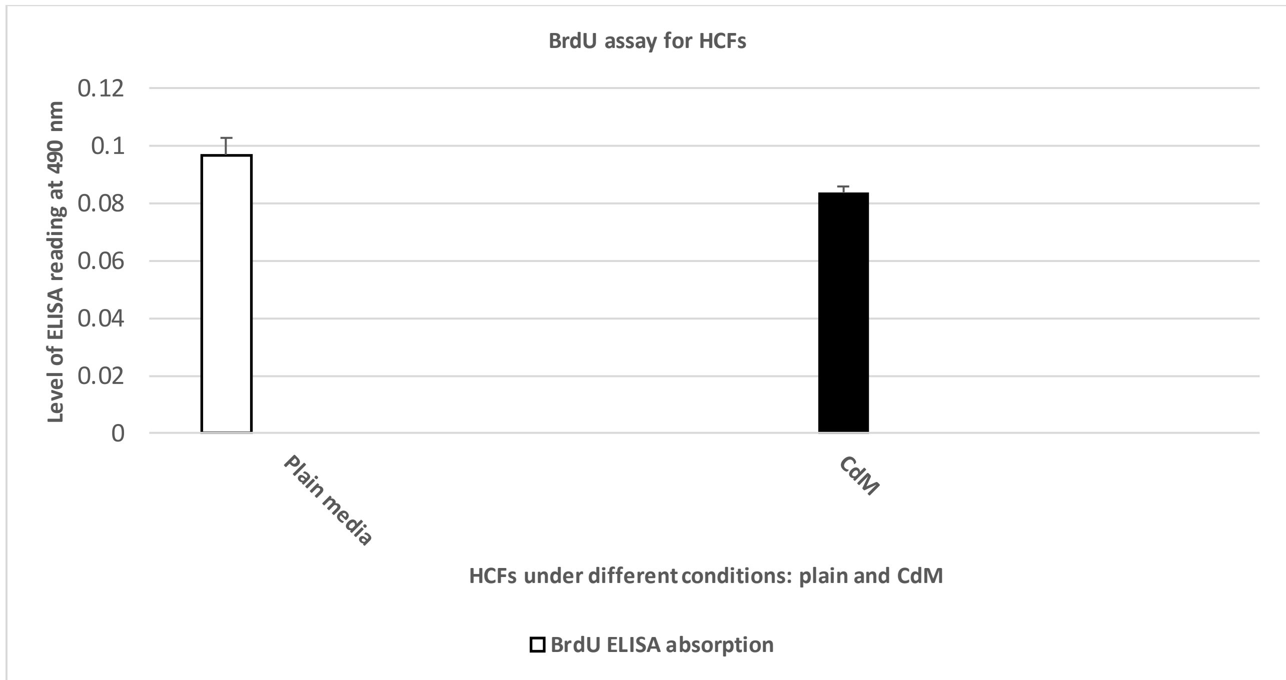

Figure 6-10.BrdU assay to reflect HCFs proliferation potential under CdM vs control.

Prolifeartion potential of HCFs under effect of CdM was comparable with the control.It did not show significant difference between the two conditions. N=3 experiments, expressed as mean+/-SEM.

*CdM: conditioned media for HCFs from co-culture of wjMSCs and HUVECs.

6.3.Effect of CHIR on HCFs

6.3.1. Introduction

Due to the fact that we aim to co-seed HCFs with HUVECs and wjMSCs and the plan would be to treat this combination with CHIR99021 at 5 uM while co-seeded. So, we aim here to assess the effects of CHIR99021 at 5 uM on HCFs. As this dose was found the most tolerated by HUVECs. So, would assess the HCFs reaction to it prior to examining the combined effects of CHIR and CdM.

6.3.2. Objectives

To examine the effect of CHIR on HCFs as regard

- Survival/anti-apoptosis factors.
- ECM proteins.
- Cellular adhesion/communication factors.
- HCFs survival.
- HCFS proliferation potential.

DMSO containing media (vehicle control) as well as plain media were used as control.

6.3.3. Results

After treatment of HCFs with CHIR 5 uM and using DMSO containing media as well as plain media as control we got the following results.

It is worth note that the results here are based on four experimental replicates but still one cell line.

This limitation is due to the sudden unexpected shut of all University premises including our laboratory and other facilities due to the COVID-19 pandemic. So, the results here are more of indicator rather than final consolidated results.

6.3.3.1. CHIR does not significantly influence Akt or NRG1 expression by HCFs

The starting point was to find out if CHIR would have significant effect on HCFs expression of Akt and NRG1. Indeed, expression of both factors did not show any alteration and results were comparable under effect of CHIR with the control.
Akt-CHIR (M= 0.973,SEM= 0.01) and Akt-DMSO (M= 0.98, SEM= 0.014) relative to Akt-cnt demonstrated no significant effect, P=0.2.

While NRG1-CHIR (M= 1.018,SEM= 0.015) and NRG1-DMSO (M= 1.019,SEM= 0.01) relative to NRG1-cnt demonstrated non-significant difference, P=0.3.

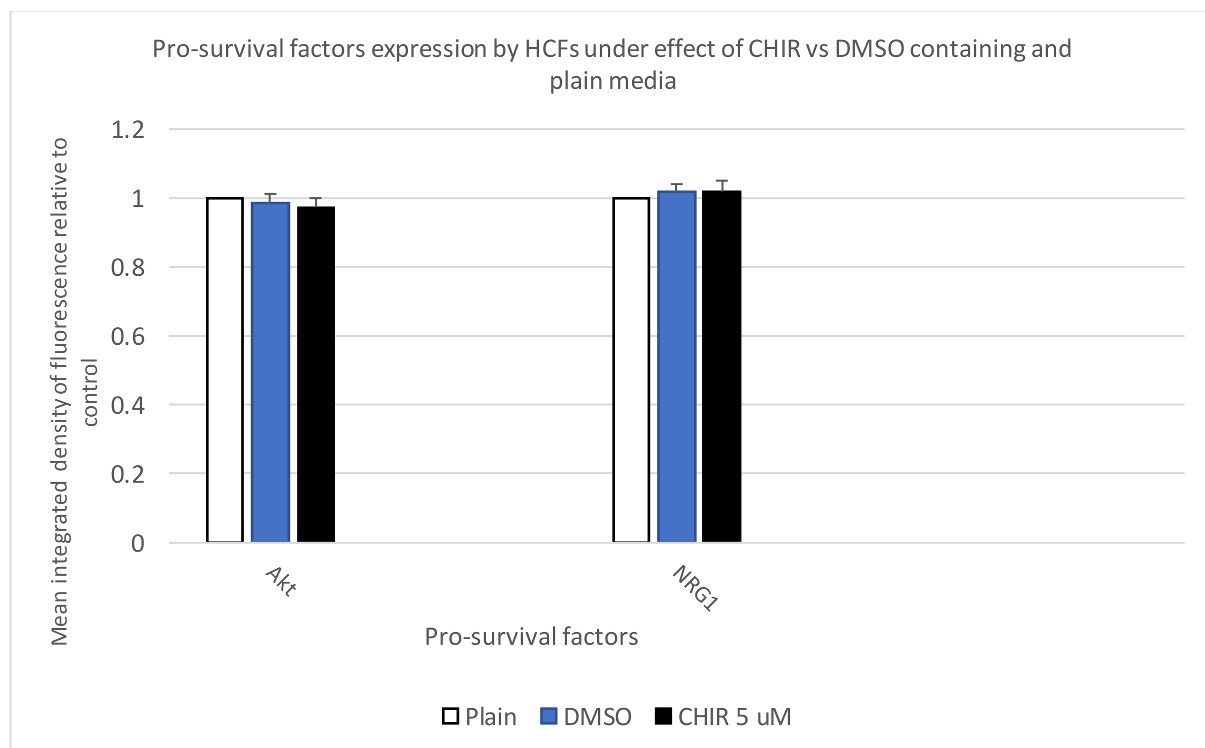

Figure 6-11. Effect of CHIR99021 at 5 uM on HCFs expression of Akt and NRG1.

Both showed statistically comparable levels of expression with the control, p=0.2 and 0.3 respectively. N= 4 experimental replicates. Expressed as mean+/-SEM.

6.3.3.2. CHIR has neutral effect on HCFs expression of ECM proteins

Then we did examine the HCFs expression of the ECM proteins collagen I, collagen III and fibronectin under effect of CHIR. Expression of the three proteins remained fairly stable with no significant change. Collagen I-CHIR (M= 0.99,SEM=0.01) and collagen I-DMSO (M=1.0069 ,SEM=0.024) relative to collagen I-cnt demonstrated neutral effect, p=0.4.
Collagen III-CHIR (M=0.99, SEM=0.026) and collagen III-DMSO (M=1.01, SEM=0.02) relative to collagen III-CNT demonstrated neutral effect. P=0.7.

Fibronectin-CHIR (M=1.032, SEM=0.027) and fibronectin-DMSO (M=1.16, SEM=0.099) relative to fibronectin-cnt demonstrated non significant difference, P=0.1.

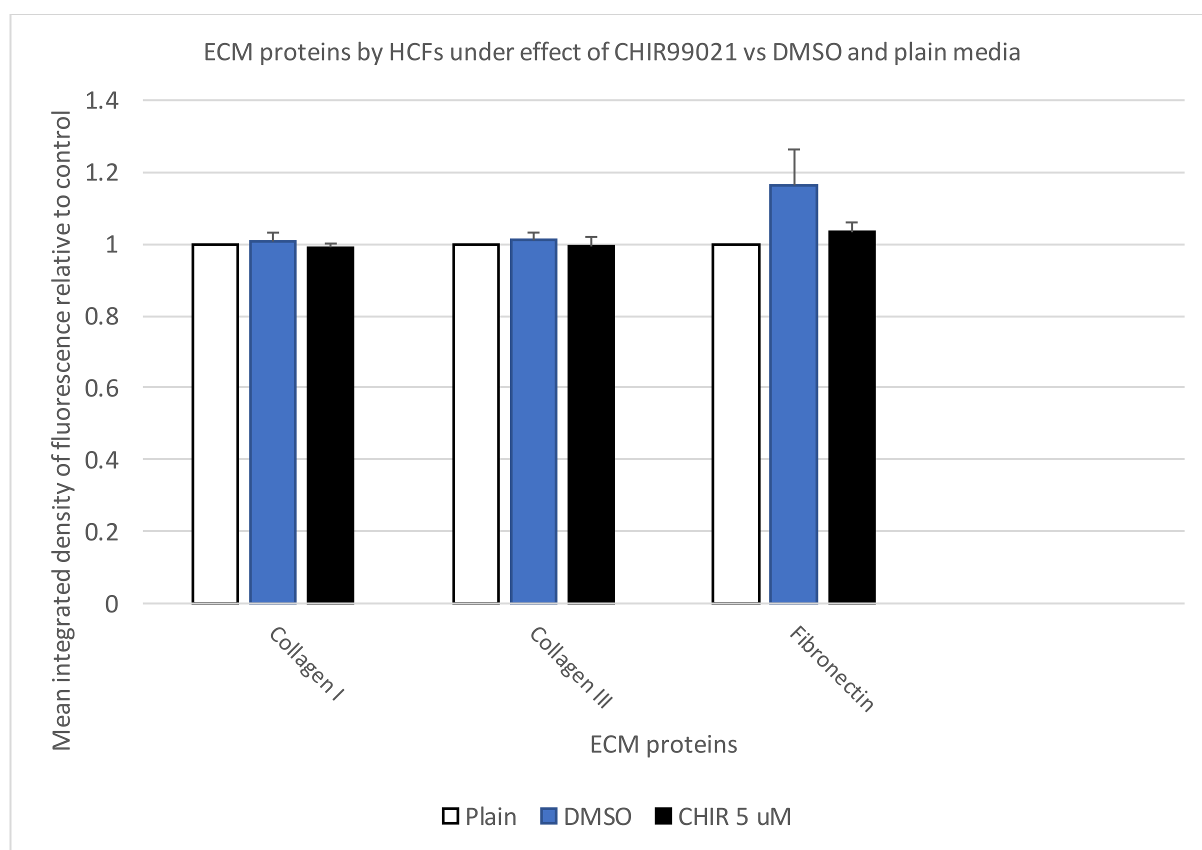

Figure 6-12.Effect of CHIR99021 at 5 uM on HCFs expression of the ECM proteins collagen I, collagen III and fibronectin.

All showed statistically comparable levels of expression with the control, p=0.4 , 0.7 and 0.1 respectively. N= 4 experimental replicates. Expressed as mean+/-SEM.

6.3.3.3. CHIR did not significantly affect β-catenin, N-cadherin or α-SMA in HCFs

Then we did examine the effect of CHIR on HCFs expression of β-catenin, N-cadherin as well as α-SMA. Here also no significant change of expression of any of these factors was encountered and their levels were comparable with the control.
β-catenin-CHIR (M= 1.041,SEM= 0.021) and β-catenin-DMSO (M= 1.019, SEM= 0.015) relative to β-catenin-cnt demonstrated no significant difference, p=0.1.

N-cadherin-CHIR (M= 1.026, SEM= 0.019) and N-cadherin-DMSO (M= 1.043, SEM= 0.022) relative to N-cadherin-cnt demonstrated no significant, p=0.1.

α-SMA-CHIR (M= 0.888, SEM= 0.11) and α-SMA-DMSO (M= 0.942, SEM= 0.059) relative to α-SMA-cnt demonstrated no significant difference, p= 0.6.

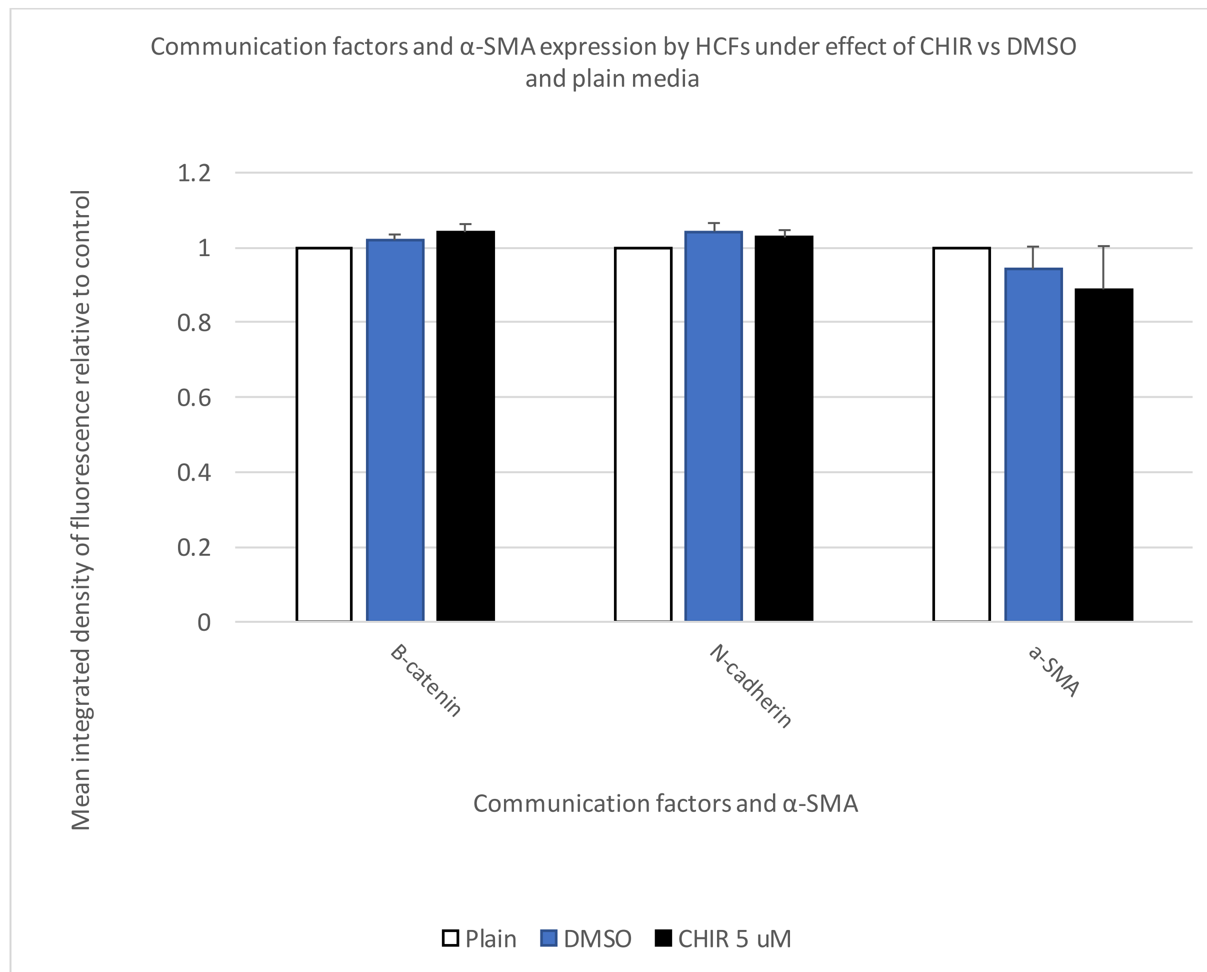

Figure 6-13.Effect of CHIR99021 at 5 uM on HCFs expression of the adhesion/communication factors as well as α-SMA.

All showed statistically comparable levels of expression with the control, p=0.1, 0.1 and 0.6 respectively. N= 4 experimental replicates. Expressed as mean+/-SEM.

6.3.3.4. CHIR does not affect Effect HCFs survival

In order to assess if CHIR has any effect on HCFs apoptosis and/or death we did use Caspase apoptosis and DRaq7 cell death assay via FCM. The percent of apoptotic and dead cells were comparable in all groups with no significant difference.

Tukey HSD Post-hoc Test:

- Plain vs DMSO, p=0.9
- Plain vs CHIR, p=0.2
- DMSO vs CHIR, p=0.2

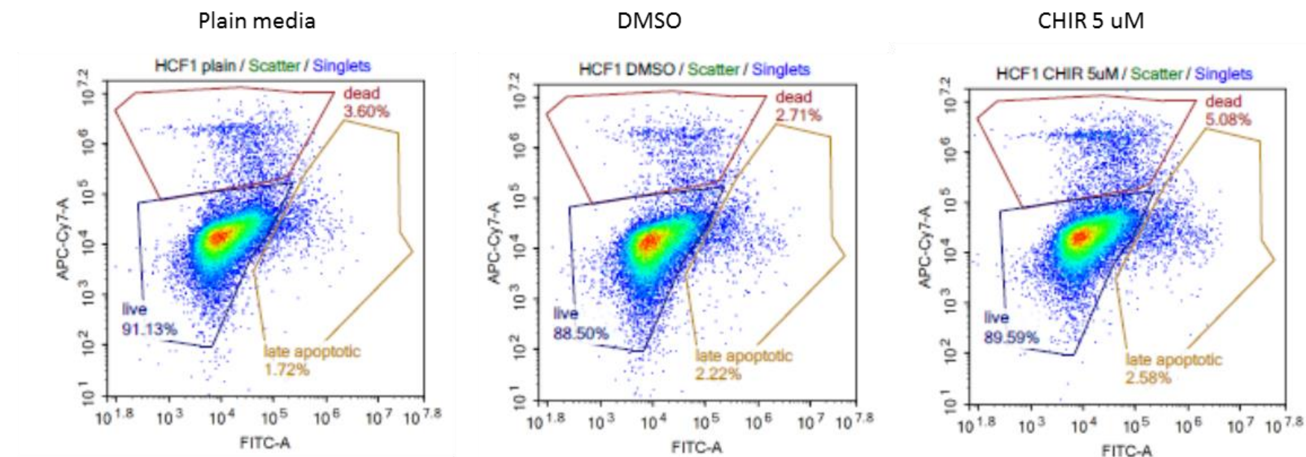

Figure 6-14. Representative images of FCM results for caspase apoptosis and DRaq7 dead cell assay.

Survival of HCFs under effect of CHIR compared to DMSO and plain media. Results were statistically comparable, n=3.

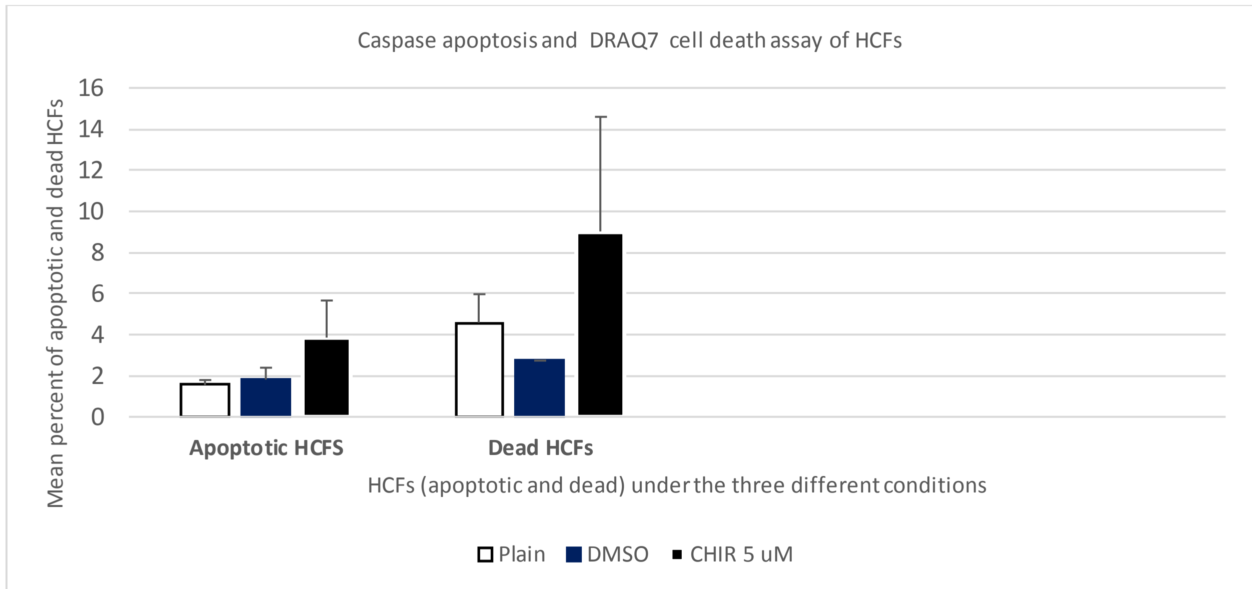

Figure 6-15.FCM results of caspase apoptosis and DRaq7 dead cell assay of HCFs.

CHIR effect on HCFs survival was assessd by Caspase/DraQ7 assay. HCFs cultured in CHIR containing media at 5 uM were tested. HCFs cultured in DMSO containing and plain media under same conditions were used as control. No significant difference in proliferation potential between the three conditions. One way ANOVA used foranalysis. N=3 experiments, expressed as mean+/-SEM.

6.3.3.5. CHIR does not affect HCFs proliferation

In order to assess if CHIR would affect HCFs proliferation potential, the BrdU ssay was used. HCFs-CHIR (M=0.084, SEM=0.0008) while HCFs-DMSO (M=0.097, SEM=0.007) and HCFs-cnt (M=0.096, SEM=0.005). One-way ANOVA was used for analysis and it revealed non-significant difference, p=0.2. So, proliferaton of HCFs was comparable in the three conditions which indicates neutral effect.

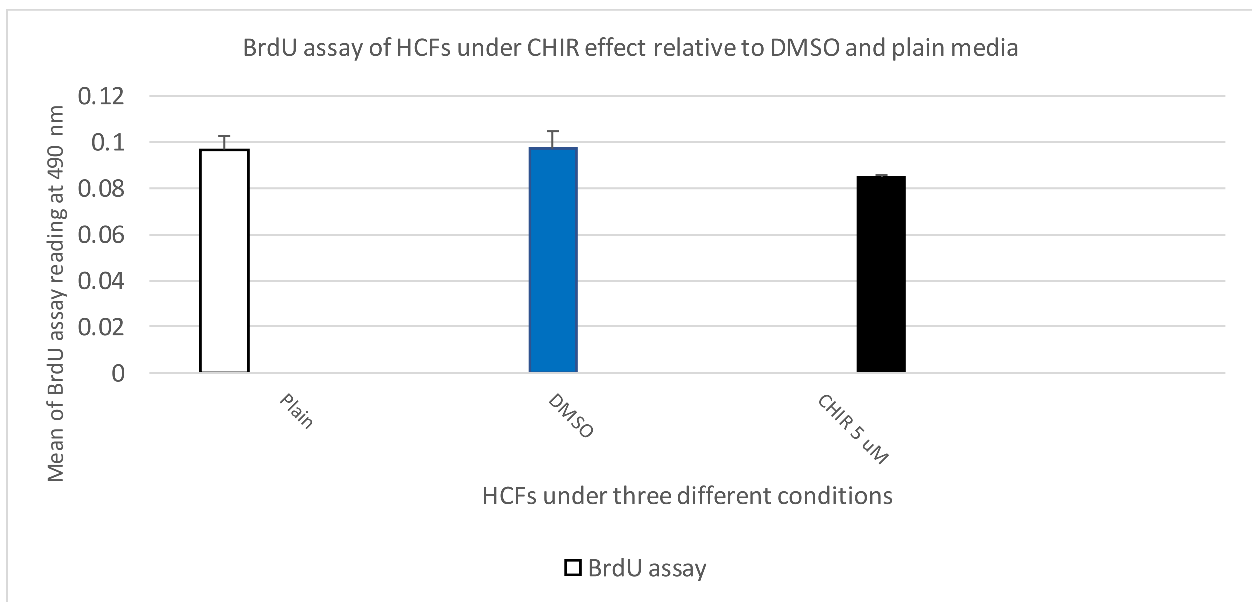

Figure 6-16.BrdU assay of HCFs cultured in plain media, DMSO and CHIR99021 at 5 uM concentration.

HCFs proliferation potential as assessd by BrdU assay. HCFs cultured in CHIR containing media at 5 uM were tested. HCFs cultured in DMSO containing and plain media under same conditions were used as control. No significant difference in proliferation potential between the three conditions. One way ANOVA used foranalysis, p=0.2. N=3 experiments, expressed as mean+/-SEM.

6.4. Combined effect of CHIR/CdM on HCFs

6.4.1. Introduction

Here we aim to examine the combined effects of CHIR99021 at 5 uM and CdM on HCFs. This would reflect the behaviour of HCFs treated with CHIR99021 when combined with wjMSCs and HUVECs.

6.4.2. Objectives

To examine the effects on CHIR and CdM at the same six relevant domains.

- ✓ Pro-survival factors.
- ✓ ECM proteins.
- ✓ Adhesion/communication factors.
- ✓ HCFs survival.
- ✓ HCFs proliferation.

6.4.3. Results

Due to the sudden unexpected shut of all university premises, so, results here are based on four experimental replicates (done from different flasks and different passages at different times) but only one cell line.

6.4.3.1. CHIR/CdM has non significant effect on Akt and NRG1 relative to CdM

After the initial assessment revealed significant down-regulation of Akt while no significant effect on NRG1 under CdM effect while CHIR effect was non-significant on both factors. So, to find out the net effect of the combination CdM and CHIR, we did culture HCFs with CdM and CHIR. HCFs cultured in CdM under same conditions were used as control.

HCFs expression of both factors under effect of CHIR/CdM was comparable with the control with no significant change.
The Akt-CdM/CHIR (M= 1.0178,SEM= 0.02) relative to AKT-CdM demonstrated comparable results, p= 0.3.

And NRG1-CdM/CHIR (M= 0.979, SEM= 0.016) relative to NRG1-CdM demonstrated no significant difference, p= 0.1.
This indicates no extra survival benefit. Indeed, given the initial result of Akt down-regulation with CdM so we expect still same down regulation of Akt applies with the combination of CdM/CHIR.

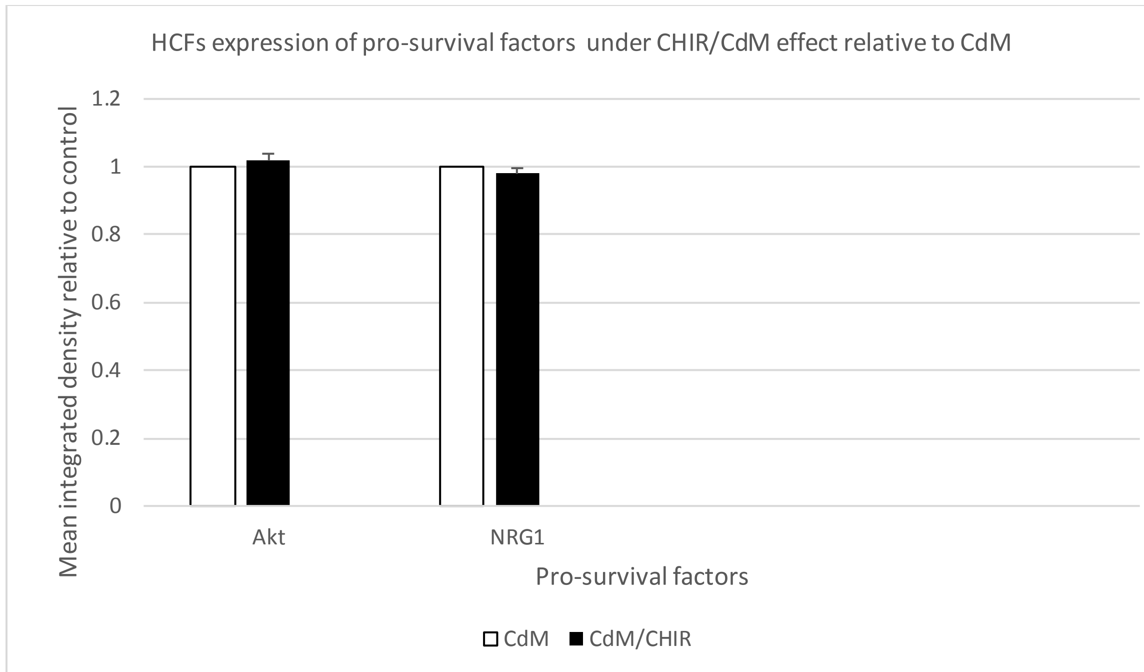

Figure 6-17.HCFs expression of Akt and NRG1 under effect of CHIR/CdM and CdM**.*

Akt and NRG1 expression by HCFs under combined effect of CdM/CHIR as compared with CdM. No significant difference between the two conditions, p=0.3 and 0.1 respectively. N=4 experiments. Expressed as mean+/-SEM.

*CHIR/CdM: combination of CHIR99021 at 5 uM with conditioned media.

**CdM: conditioned media.

6.4.3.2. CdM/CHIR does not influence ECM proteins relative to CdM

Further to the previous results of non-significant change in the HCFs expression of the ECM proteins collagen I, collagen III and fibronectin under effect CdM and CHIR. Here, we did examine the combined effect of CdM/CHIR to find out if any significant change relative to CdM. Expression of the three ECM proteins in HCFs under combined effect of CHIR/CdM remained statistically comparable with the control with no significant change.

Collagen I-CdM/CHIR (M= 0.983,SEM= 0.01) relative to Collagen I-CdM demonstrated comparable results, p= 0.2.
Collagen III-CdM/CHIR (M= 1.001,SEM= 0.025) relative to Collagen III-CdM demonstrated comparable results, p= 0.9.
Fibronectin-CdM/CHIR (M= 0.989,SEM= 0.007) relative to fibronectin-CdM demonstrated comparable results, p= 0.1.
These results are actually quite assuring of no significant over secretion of the examined ECM proteins. So, no concern about fibrotic changes that would hinder the intended target from cellularization of the patch.

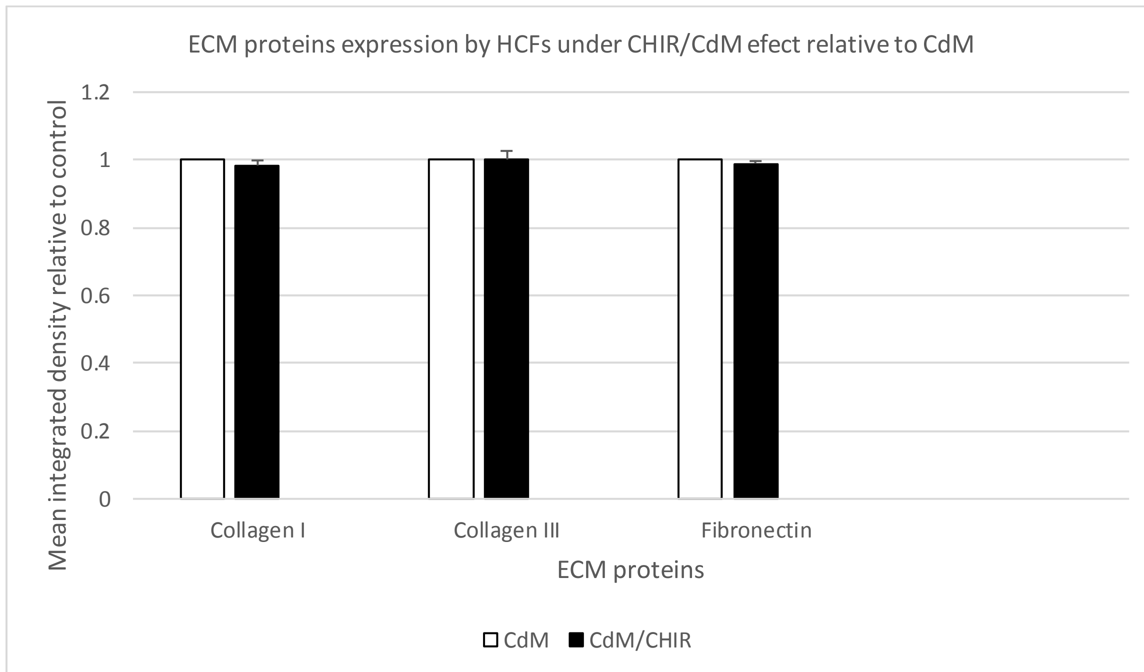

Figure 6-18.ICC results for HCFs expression of the ECM proteins collagen I, collagen III and fibronectin under effect of CHIR/CdM and CdM**.*

Collagen I, collagen III and fibrnectin expression by HCFs under effect of CdM/CHIR as compared with CdM. There were no significant differences between the two conditions, p=0.2, 0.9 and 0.1 respectively. N=4 experiments. Expressed as mean+/-SEM.

*CHIR/CdM: combination of CHIR99021 at 5 uM with conditioned media.

**CdM: conditioned media.

6.4.3.4. CdM/CHIR effect on β-catenin and N-cadherin was comparable with the CdM

Initially, the CdM did up-regulate N-cadherin while CHIR had fairly neutral effect. So, here we did go further to examine the net effect of the combination of CdM/CHIR relative to CdM. Our results still show statistically comparable results which indicates most probably maintained upregulation of N-cadherin while still neutral effect on B-catenin.

β-catenin-CdM/CHIR (M= 0.995, SEM= 0.01) relative to β-catenin-CdM demonstrated non-significant difference, p= 0.7.

Also, N-cadherin-CdM/CHIR (M= 0.996, SEM= 0.005) relative to N-cadherin-CdM demonstrated non-significant change, p=0.5.

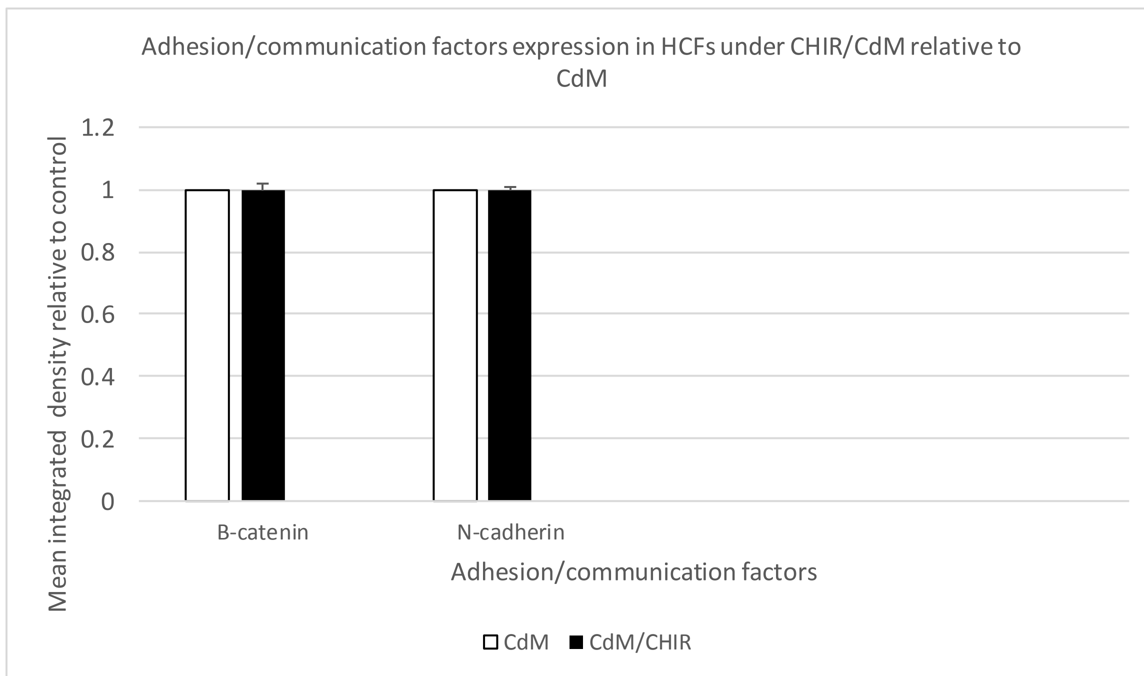

Figure 6-19.ICC results for HCFs expression of the adhesion/communication factors β-catenin, N-cadherin and ITG under effect of CHIR/CdM and CdM**.*

β-catenin and N-cadherin expression by HCFs under effect of combined effect of CdM/CHIR relative to CdM. Both did not show significant difference between the two conditions, p=0.7 and 0.5 resectively. N=4 experiments. Expressed as mean+/-SEM.

*CHIR/CdM: combination of CHIR99021 at 5 uM with conditioned media.

**CdM: conditioned media.

6.4.3.5. CdM/CHIR effect on α-SMA is comparable with the CdM

As previously illustrated, HCFs are the main producers of ECM. They produce ECM at basal level while they are under quiescent state. Then when activated under pathologic stressors such as ischemia, they differentiate into myofibroblasts with higher ECM production. Indeed, this is the pathologic ground of the remodelling process which is despite its functional deleterious effects on the cardiac performance including impaired contractility with subsequent heart failure and overall poor prognosis. However, still it is quite protective process to guard against the more catastrophic event of myocardial rupture in case of transmural infarction.

The main indicator of HCFs differentiation into myofibroblast is the expression of α-SMA. Initially, we found α-SMA expression did not alter under CdM effect as well as under CHIR effect. Here we did assess the combined effect of both CdM/CHIR and it did show non-significant change.

α-SMA-CdM/CHIR (M= 0.92,SEM= 0.06) relative to α-SMA-CdM demonstrated no significant difference, p= 0.3.

These results are actually promising as no concern about extra fibrotic changes of the patch. Also, these results are consistent with the initial results of fairly static level of collagens production.

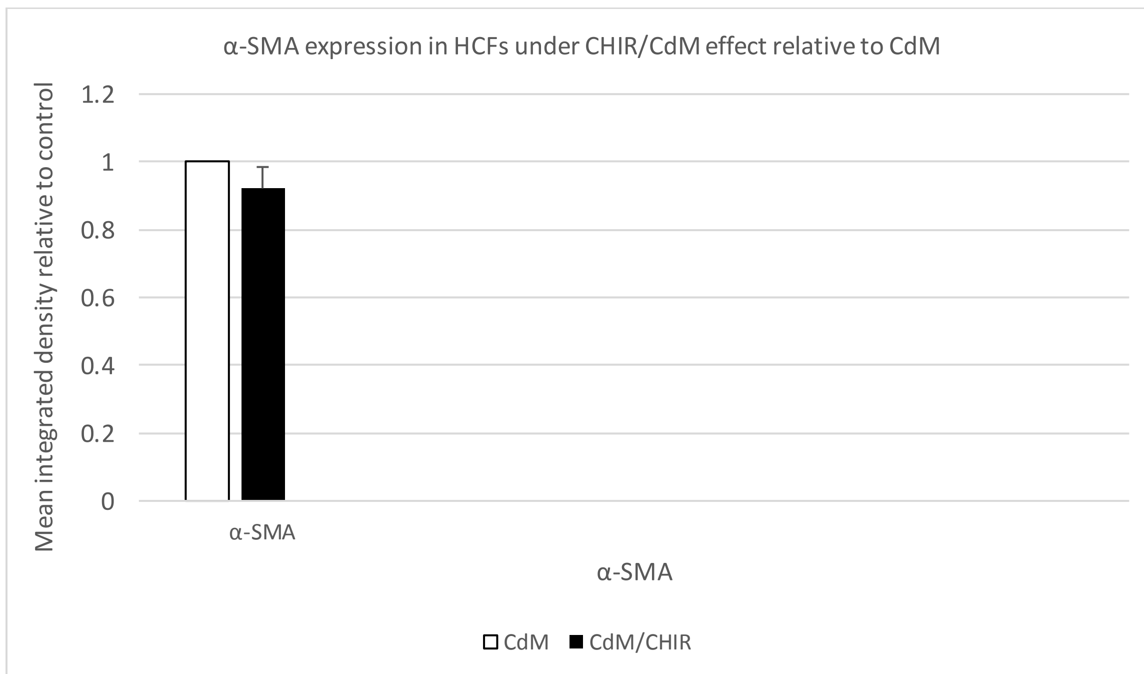

Figure 6-20.ICC results for HCFs expression of α-SMA under effect of CHIR/CdM and CdM**.*

α-SMA expression under combined effect of CdM/CHIR relative to CdM. It dd not show significant difference between the two conditions, p=0.3. N=5 experiments. Expressed as mean+/-SEM.

*CHIR/CdM: combination of CHIR99021 at 5 uM with conditioned media.

**CdM: conditioned media.

6.4.3.6. CHIR/CdM does not influence HCFs survival relative to CdM

In order to find out if HCFs survival would be influenced by the combination CHIR and CdM relative to CdM. We used caspase apoptosis and DRaq7 cell death assay via FCM. After culture of HCFs in CdM/CHIR for 48 hours the cells were collected and incubated with Caspase antibody (as previous illustrated) then run the assay using the Novocyte machine for FCM. HCFs cultured with CdM under same conditions were used as control. The results were comparable between both conditions

Both assays showed comparable results which is in keeping with neutral effect.

Apoptotic HCFs-CdM (M=1.21,SEM=0.12) and HCFs-CHIR/CdM (M=1.58,SEM=0.26) and p=0.2.
Dead HCFs-CdM (M=4.83,SEM=0.04) and HCFs-CHIR/CdM (M=5.76,SEM=0.54) and p=0.1.

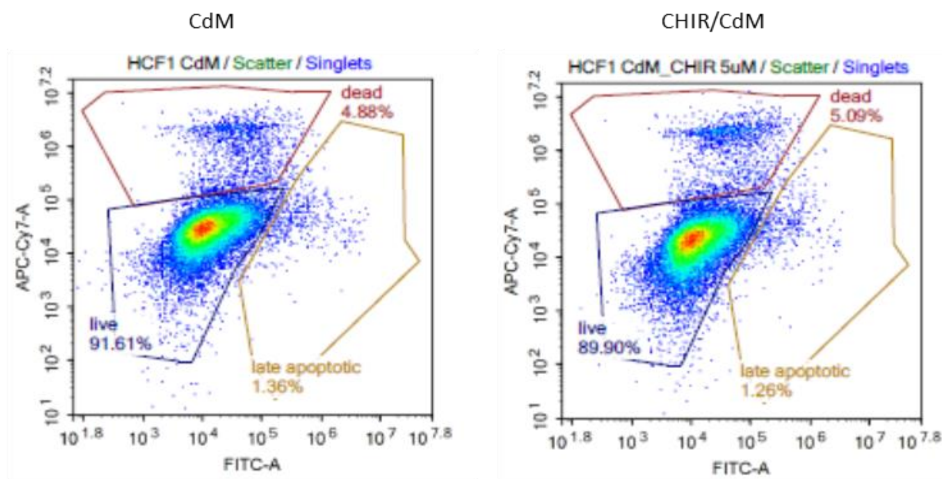

Figure 6-21.Representative images of FCM results for Caspase apoptosis assay and DRaq7 dead cell assay of HCFs in CdM and CHIR/CdM.

No significant difference could be elicited.N=3.

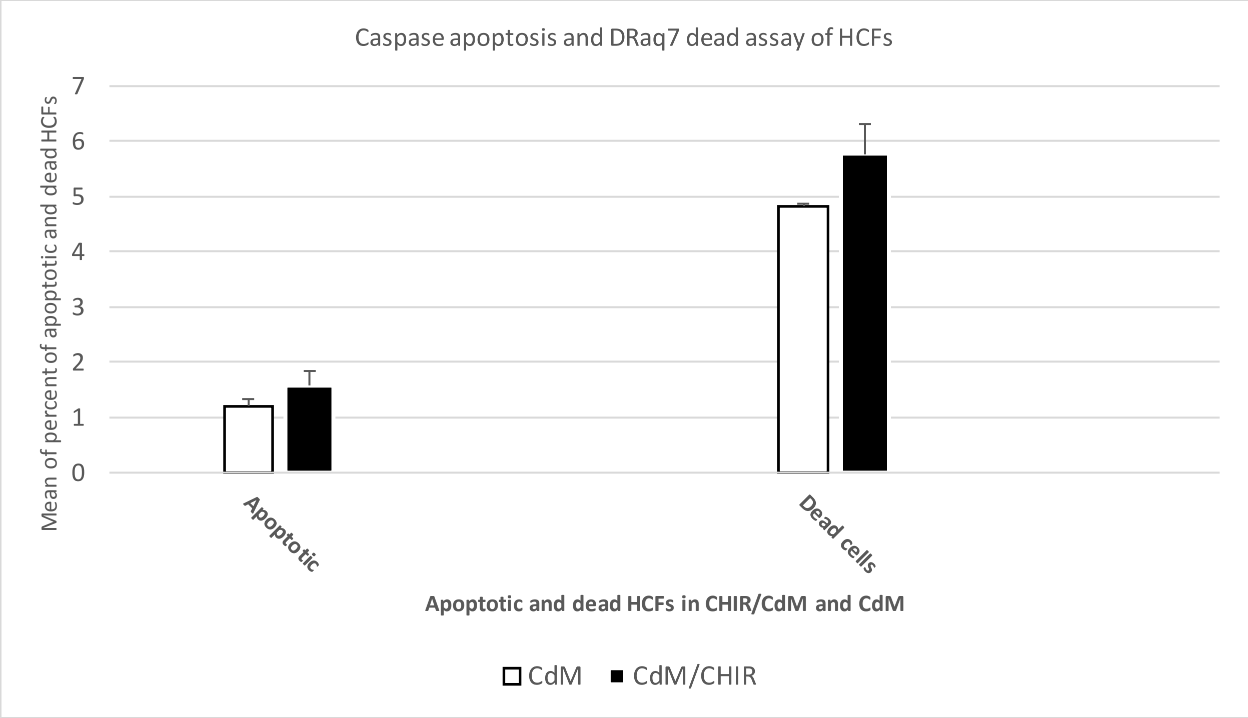

Figure 6-22.FCM results for HCFs apoptosis and cell death under effect of CHIR/CdM and CdM**.*

It dd not show significant difference between the two conditions. N= 3 experiments, expressed as mean+/- SEM.

*CHIR/CdM: combination of CHIR99021 at 5 uM with conditioned media.

**CdM: conditioned media.

6.4.3.7. CHIR/CdM does not influence HCFs proliferation potential relative to CdM

In order to examine if the combination of CHIR and CdM would significantly influence the HCFs proliferation, BrdU assay was used. The results showed statistically comparable between the two conditions, which indicates comparable proliferation potential under both conditions.

HCFs-CdM/CHIR (M= 0.082, SEM= 0.0002) while HCFs-CdM (M= 0.083, SEM= 0.002) and p=0.5, which indicates non-significant difference.
These data are in keeping with the non-significant alteration of α-SM which is associated with higher proliferation rate and indicates that HCFs are still in quiescent state.

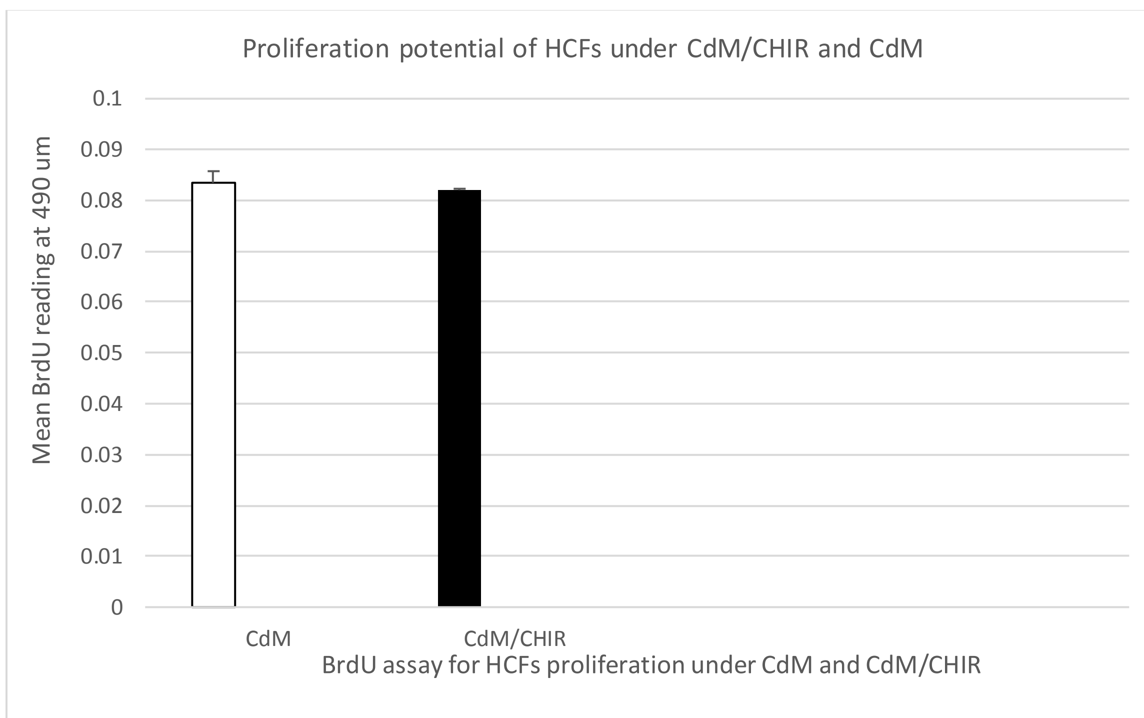

Figure 6-23.BrdU results for HCFs proliferation potential under effect of CHIR/CdM and CdM**.*

Prolifeartion potential of HCFs under effect of CdM/CHIR compared to CdM. It dd not show significant difference between the two conditions. N=3 experiments, expressed as mean+/-SEM.

*CHIR/CdM: combination of CHIR99021 at 5 uM with conditioned media.

**CdM: conditioned media.

6.5. Discussion

6.5.1. Introduction

HCFs are indispensable for myocardial function under physiologic conditions as well as during remodelling in diseased myocardium. They are the main ECM producers and the main ECM regulators via secreting its components, degradation factors and their inhibitors to maintain balanced composition and efficiently working ECM. ECM represents the network that connects cells, organise orientation and transmit mechanical forces from myocytes to ensure organised contraction during systole as well as elastic recoil during diastole (318).
Despite this crucial function they still go beyond it to share in electrical coupling with CMCs via cell-cell contact (445) and therefore HCFs are considered as CMCs partners. In addition, they act as mechanosensors, secrete growth factors and cytokines and also promote angiogenesis (13). Furthermore, HCFs support myocardial regeneration via ECM remodelling in addition to activating CMCs alignment and elongation (30). Therefore, HCFs were considered as strong pillar in the design of the patch.
In this project we aim to co-seed HCFs with HUVES and wjMSCs. Here, we did examine the HCFs reaction to CdM induced by co-culture of wjMSCs and HUVECs to find out how HCFs will behave when co-seeded with these cells. Assessment included survival factors, main cardiac ECM proteins, cellular communication factors, HCFs survival and prolifeartion. Then we did assess the effect of CHIR99021 at 5 uM on HCFs and the last area was to examine the combined effect of CHIR and conditioned media of HUVECs/wjMSCs co-culture as regard the same domains.

Assessment was done at these relevant areas to explore if HCFs reaction would be favourable to its target as an in-vivo patch with better chances for engagement with neighbouring myocardium.

There were some concerns here about the fibroblasts as rapidly proliferating cells if they would overpopulate the other cells in the patch. Also, if they would differentiate into myofibroblasts which have higher rate of proliferation as well as more ECM production. Because if this happens, it would threaten the patch to lose its balanced cellular compnenets and hold features of hypertrophied hearts which is associated with poor prognosis (314). Obviously, this may deviate from the target of potentially functioning patch and end up with fibrotic patch mainly dominated with myofibroblasts.

Here it is really prudent to emphasise on the fact that data are based on four experimental replicates but only one cell line. Experiments were done using different passages and different flasks but still this is considered as a limitation. This happened due to the sudden unexpected shut down of the whole university premises including our laboratory and the medical school facilities during the COVID-19 pandemic, so it was impossible to go ahead with any more experiments. Therefore, the results are more of suggestive value and not final consolidated results.

Cardiac fibroblasts are quite dynamic cells. They sense and react to various stressors such as pressure load of high blood pressure and hemodynamic changes caused by valvular heart diseases. They proliferate with more ECM deposition leading to cardiac hyper-trophy and stiffness which is a feature of cardiac remodelling and heart failure. Indeed, this is a leading cause of mortality worldwide (314).

6.5.2. CdM and CHIR Effects on HCFs

6.5.2.1. Survival factors

The starting point was to assess the effect on the pro-survival/anti-apoptosis factors Akt and NRG1.

Akt in HCFs is involved in the differentiation process into myofibroblasts via the Akt/GSK-3 signalling pathway with subsequent extra ECM production which is a key process in cardiac hypertrophy (314).

Akt expression did significantly down regulate under effect of CdM as compared with the control. These results are consistent with the other findings of non significant change in expression of α-SMA which reflected non significant differentiation to myofibroblasts and also steady level of ECM production.

NRG1 enhances HCFs survival via PI3k/Akt pathway (446). Our data demonstrate fairly neutral effect of CdM with no significant alteration of its expression. This is expected to reflect on survival of HCFs which does not seem to be supported by this combination but on the other hand it did not demonstrate significant down regulation or enhanced apoptosis which indicates most probably neutral effect, however still the results are non conclusive and need to be empowered with the necessary biologic replicates.

The CHIR effect as well as the combined CHIR/CdM did not show any significant difference on both Akt and NRG1 which is also in keeping with the rest of other results that showed mostly neutral effect.

6.5.2.2. ECM proteins and α-SMA expression by HCFs

The next assessment domain was the ECM proteins collagen I and III as the main cardiac ECM collagens as well as the glycoprotein fibronectin which shares in connecting collagen to the sarcolemma (359). Collagen is normally secreted as procollagen which undergoes a series of processing events ending with mature collagen that holds the self assembly capacity. CFs are the main producers of ECM proteins and also they are the producers of the main regulators of ECM. These include the matrix metalloprotienases (MMPs) which are a family of endopepetidases that act to degrade ECM. In addition, CFs produce MMPs counter-regulators known as tissue inhibitors of metaloproteinases (TIMPs) that inhibit the MMPs (359). Unfortunately, antibodies for MMP and TIMP did not work, so could not include any quantitative data about their expression.

Our data show that levels of collagen I, collagen III and fibronectin were comparable with the control with no significant alteration under effect of CdM, CHIR and combined CHIR/CdM.

However, mRNA levels of collagen I and fibronectin showed significant upregulation under CdM effect which indicates that collagen I and fibronectin expression within this time window were not transcriptionally depnedent.These data are consistent with the non-significant alteration of α-SMA expression by HCFs.

This was considered as a good point as it suggests that no extra ECM production and unlikely to lead to fibrotic changes of the patch. On the other hand ECM secretion is still comparable with the control which would provide the patch cellular compartment with the necessary matrix for cells communication and homeostasis. On the other hand it would indicate that HCFs within this time window are still keeping quiescent state but can not draw predictions about longer time windows.

It is of note here that α-SMA expression was comparable with the control under effect of CdM, CHIR as well as the combination of CHIR/CdM. α-SMA is a contractile protein secreted by myofibroblasts (359) and is considered as an indicator of fibroblast differentiation into myofibroblasts. Myofibroblasts are present in normal myocardium but they dominate the field during remodelling such as after ischemic event or in heart failure (447) as part of the remodelling process.

There are two types or phases of myofibroblasts, the proto-fibroblasts characterised by expression of microfilaments (stress fibres) and the mature myofibroblasts which express α-SMA (448). Fibroblast differentiation occurs on exposure to static pressure via interaction of transfroming growth factor β1 (TGF-β1) and fibronectin leading to upregulation of α-SMA with resultant generation of contractile force and altered tissue tension with subsequent ECM collagen reorganisation that leads to stiffness which is a poor prognostic event (448).

After a myocardial injury with significant cell loss, the HCFs would differentiate to myofibroblasts which are more rapidly proliferating with more ECM secretion. Indeed, this is the core of the remodelling process. Therefore, we had the concern about the HCFs seeded in the patch if they would lean toward differentition to myofibroblasts. Our data argue against this behaviour and give assurance from this perspective that the patch's cellular environment does not support a remodelling like process as the α-SMA was not significantly changed and its mRNA was non-significantly altered.

These results indicate that HCFs are leaning toward quiescent state rather differentiating to myofibroblasts. These results are also consistent with stable ECM protein production and is considered as a promising feature as it indicates that unlikely for the triple combination of cells to lead to fibrotic patch. Indeed, this is in favour of biologically active cells and potential engagement with neighbouring myocardium and also in keeping with the results of neutral effect on HCFs proliferation which is also a good result that the patch will not be over-populated by HCFs.

6.5.2.3. Adhesion/communication factors

The next area of assessment was the cellular adhesion/communication factors. Our results showed neutral effect on β-catenin while N-cadherin was upregulated under CdM effect. ITG could not be assessed.

Cellular adhesions are indispensable for integrity of any tissue. This takes place via adhesion factors including the cadherins which are calcium dependent homophilic cell-cell adhesion molecules that play role in regulating adhesion and migration of cells. Therefore, they are involved in reorganising the tissue morphogenesis (449, 450).

Cadherins are a family of proteins that act as mechanical linkers between cells (451).
Expression of cadherins varies between tissues. For instance, skeletal myocytes express R-Cadherin, M-Cadherin and N-Cadherin while in the myocardium N-Cadherin is the main cadherin subtype (449). N-cadherin expression in the heart is mainly found in cardiomyocytes and quiescent fibroblasts (451). The cadherin molecule comprises single transmembrane protein which connects with the actin cytoskeleton via interacting with catenins including β-catenin that acts to bridge the cytoplasmic domain of cadherin with the actin (450).

In addition to the β-catenins role as a connecting arm between cadherin and actin it also plays central physiologic role as a co-activator for transcription under control of Wnt signals (450). This is in addition to its developmental role as the Wnt/β-catenin signalling pathway is involved in cardiac morphogenesis of first heart field under control of FGFs (452).
So, we did examine for β-catenin, N-cadherin and ITG. Unfortunately, the ITG antibody did not work properly so it was omitted from analysis. HCFs expression of both β-catenin did not show significant alteration under effect of CdM, CHIR as well as the combination of CHIR/CdM. This indicates that HCFs when combined with wjMSCs and HUVECs would maintain levels at its basal levels with no extra expression and in keeping with the quiescent state.

Furthermore, CHIR treatment did not make significant difference of both β-catenin and N-cadherin. It was expected that β-catenin will be upregultaed however, this result could be due to the need for different dose or need for extra factor to induce its full Wnt inhibiting effect or even both factors needed.

Wnt/β-catenin plays important role in the myocardium response to pressure overload with subsequent CFs differentiation to myofibroblasts and excess collagen deposition as well as myocytes hypertrophy (453. In the study of Xiang et al. they did generate B-catenin deficient CFs using Cre inducible gene knock down model and examined the myocardium response to pressure overload in rats via transaortic obstruction for 8 weeks. Blunting the action of Wnt/b-catenin did significantly down-regulate collagen deposition and myocytes hypertrophy and preserved cardiac function {Xiang, 2017 #800). Also did down-regulate collagen 1 and 3 gene expression (453).
In addition, the same study reported that CFs survival and proliferation were not affected by β-catenin blunting (453). The authors concluded regulatory role for β-catenin on collagen secretion by CFs during the fibrosis remodelling process.
While CdM did significantly upregulate N-cadherin which is also consistent with the non-significant change of α-SMA and in keeping with the quiescent state of HCFs.

6.5.2.4. Survival and proliferation

Then we did examine the HCFs survival under effect of the CdM using the apoptosis/cell death assay. Also, in keeping with neutral effect the HCFs apoptosis/death was comparable between CdM and control. This neutral effect extended to the effect of CHIR as well as the combination of CHIR/CdM which did not make any significant difference on HCFs survival. This neutral effect also noticed in the HCFs proliferation experiments which showed proliferation potential comparable under CdM effect as well as under CHIR and combined CHIR/CdM.

Indeed, it is quite clear that HCFs maintained their quiescent state all through with non-significant change at most domains. The only concern here is the time window of the experiments that could be too short to reflect on the HCFs behaviour. However, longer time window was not possible within the facility constrain.

6.6. Conclusion

Despite the limitation of one biologic cell line but still the results from 4 experimental replicates showed fairly neutral effect. The survival factors, ECM and communication factors were mostly comparable with the control with quite limited significant differences. Within the limitation of missing biologic replicates these results are more suggestive of quiescent state of the HCFs with no over proliferation, differentiation or ECM production. Therefore, still the patch would utilise their basal structural and functional contribution.

Chapter VII

Cormatrix seeding and mechanical properties

7.1. Introduction

As explained before our aim is to design a patch that is as close to natural myocardium as possible making use of the principles of tissue engineering. So, we planned to seed cormatrix (CorMatrix Cardiovascular, Inc., Roswell, GA, USA) (454) with a triad of relevant cells. Cormatrix is non-crosslinked porcine small intestinal submucosa extracellular matrix (455).

Aiming to offer biologic advantage in terms of integration with neighbouring myocardium, vascularisation via angiogenesis utilising the seeded HUVECs and angiogenic factors secreted by wjMSCs as well as potential of trans differentiation of wjMSCs and growth as an inherent character of the seeded cells. Also, to promote native cells to migrate and populate the patch as well.

7.2. Objectives

To assess the potential of the triad of cells (HCFs, wjMSCs and HUVECs) to adhere and spread on the surface of cormatrix using histologic staining, cells survival on cormatrix after seeding using live and dead assay, surface topography using SEM and mechanical properties of the patch after seeding using the tensile strength machine.

7.3. Materials and Methods

7.3.1. Seeding

The cells were collected from the flask using accutase (Sigma Aldrich) as described earlier. Then the cells were counted and seeded on cormatrix at density of 5-10 x 10^5 cells/cm^2. Then kept in humidified incubator at 37 ^oC with 5% Co^2 and media changed every 2-4 days.

7.3.2. Live/dead assay

Live/dead cell assay (Thermofischer) were used. Calcein (stains green for live cells) and Ethidium (stains red for dead cells) were used.

5-8 days after seeding, Calcein was added at 0.2 ul/100 ul and Ethidium was added at 0.2 ul/100 ul and left in the incubator for 30 minutes then examined under fluorescence microscope.

7.3.3. Mechanical properties

To assess the mechanical properties of the patch the tensile strength machine, Instron 3343B machine (Instron, Norwood, Massachusetts) with pneumatic grips and 100-N cell load was used.

The patch dimensions were measured using ruler and thickness was measured using a micrometre. Then the tensile strength (force per unit area in MPa) as well as the Young's modulus (stress/ strain) as a measure of elasticity were used as indicators for the patch's mechanical integrity as well as elasticity and recorded from load/elongation curve using Blue hill software (Instron).

7.4. Results

7.4.1. Histology of cormatrix mono and tri-seeded

We started with seeding wjMSCs as single cell phenotype to assess the adherence and spread of these cells on the cormatrix surface. After seeding cormatrix with cells, it was kept static for 5-7 days. The medium changed every 2-3 days. Then fixed with PFA 4% and processed for histology staining as previously described. Then, further assessment to examine tri-seeding of cormatrix with the combination of wjMSCs, HUVECs and HCFs was done.

Examination under light microscope using H&E stain demonstrated that the seeded patches showed successful uptake of cells on surface of cormatrix scaffold. Histological slides showed up to 3 layers of cells on the Cormatrix surface with good attachment and alignment of cells. While in the control slides there were pure longitudinally aligned collagen fibres (figure 7.1).

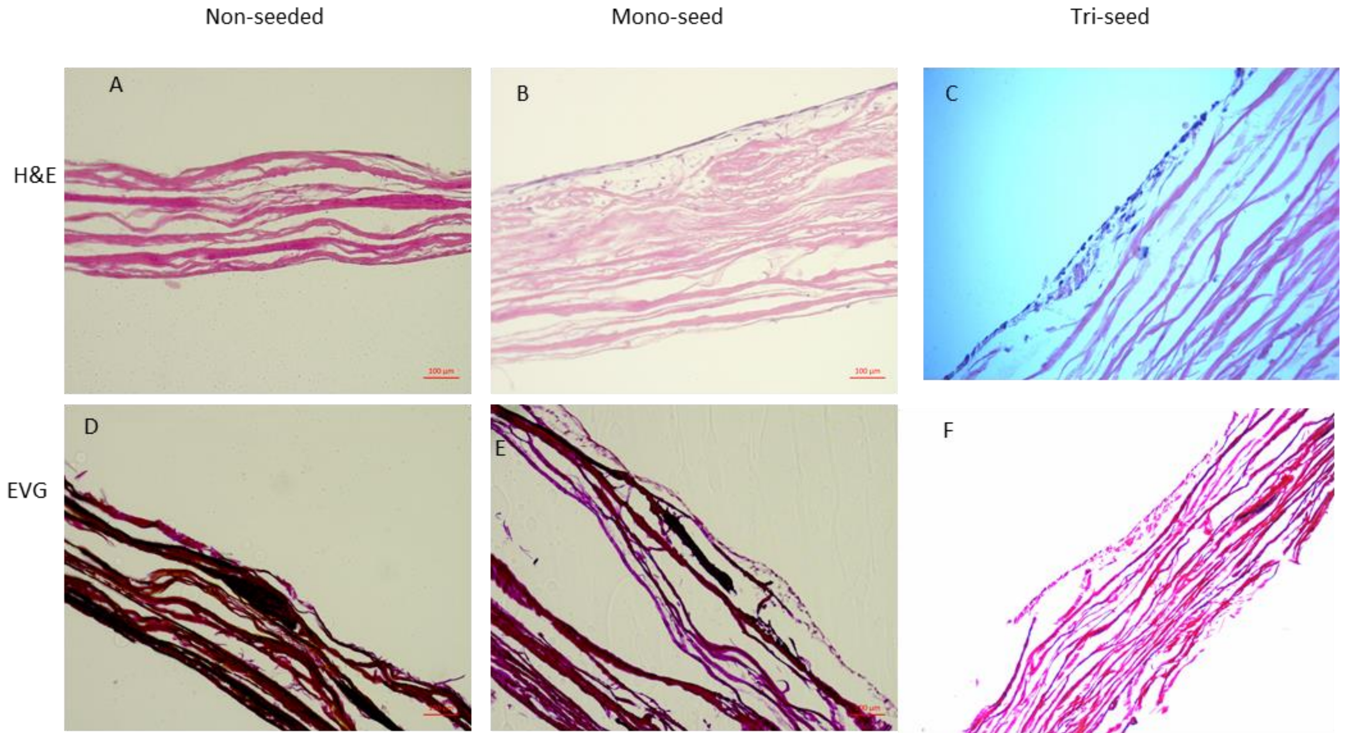

Figure 7-1. Representative images of Cormatrix with mono and tri-seed stained with H&E and EVG.

Seeding of cormatrix with wjMSCs as mono-cell and wjMSCs, HUVECs and HCFs as tri-cellular seed demonstrated successful uptake, adhesion and spread over the cormatrix surface. (A)Non-seeded cormatrix stained with H&E as control. (B) Cormatrix seeded with wjMSCs as mono-seed stained with H&E. (C) Cormatrix seeded with wjMSCs, HUVECs and HCFs as tri-seed stained with H&E. (D) Non-seeded cormatrix stained with EVG as control. (E) Cormatrix seeded with wjMSCs as mono-seed stained with H&E. (F) Cormatrix seeded with wjMSCs, HUVECs and HCFs as tri-seed stained with EVG. The seeded cormatrix demonstrates successful adherence of cells to the surface of cormatrix at mono and tri-seed. Scale bar 100 um.

7.4.2. Live/dead cell imaging

In order to confirm viability of the attached cells, Calcein and Ethidium assays were used as described before. It did demonstrate good viability of cells on surface of cormatrix (figure 7.2). However, it was notable that dead cells in the tri-seed patches were more frequent (own observation) that would be in keeping with the survival experiments.

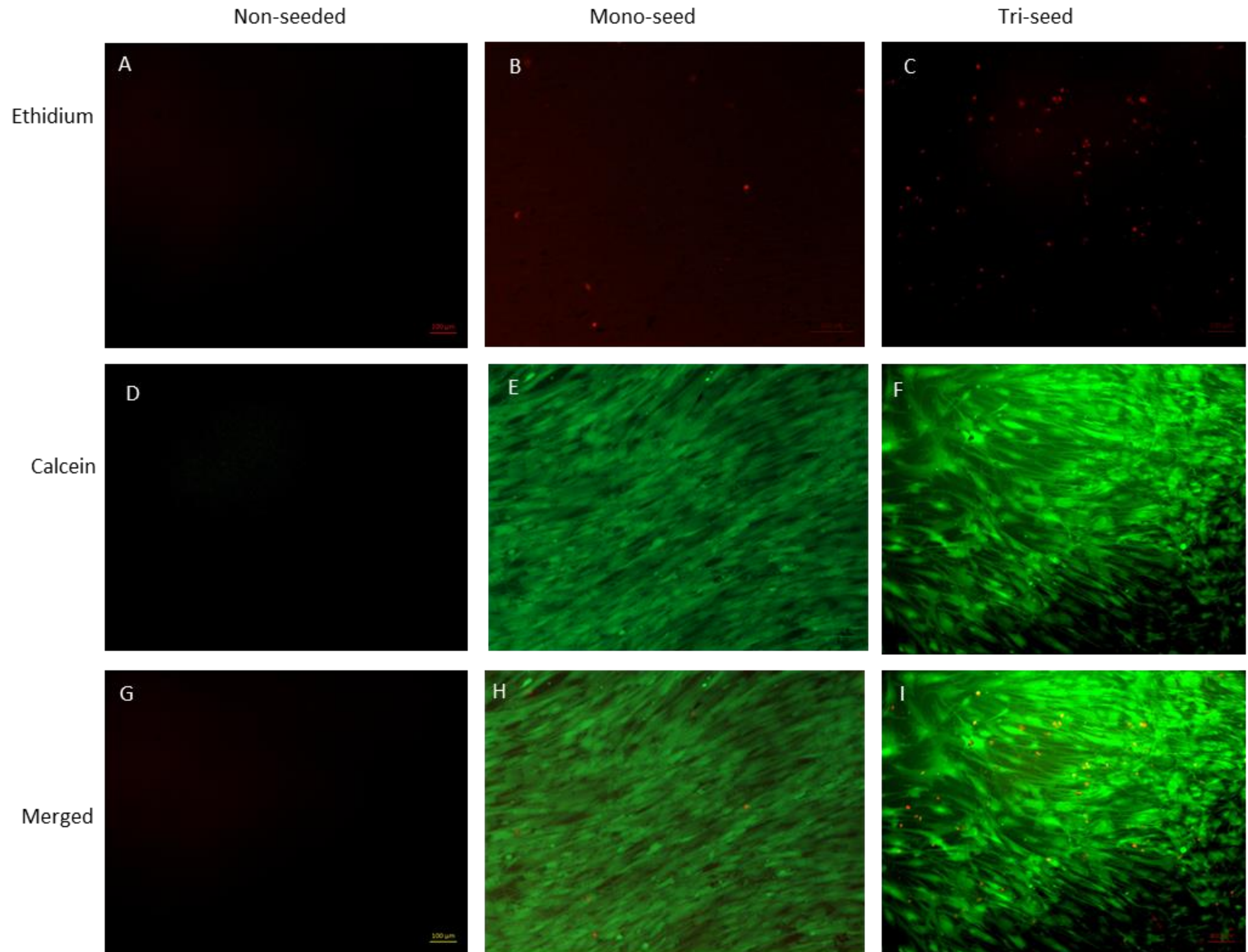

Figure 7-2.Live/dead cell imaging of wjMSCs as mono-seed as well as the (wjMSCs, HCFs and HUVECs) as tr-seed on cormatrix. Scale bar 100 um.

Cormatrix seeded with wjMSCs as mono-seed and cormatrix seeded with wjMSCs, HUVECs and HCFs as tri-seed. Viability of seeded cells assessed using Calcein and Ethidium assays demonstrating good viability of attached cells with apparently more dead cells in the tri-seed patches. (A) Non-seeded cormatrix exposed to Ethidium as control. (B) Cormatrix with mono-seed and stained with Ethidium. (C) Cormatrix with tri-seed** and stained with Ethidium. (D) Cormatrix non-seeded stained with Calcein. (E) Cormatrix with mono-seed stained with Calcein. (F) Cormatrix with tri-seed stained with Calcein. (G) Merged A&D. (H) Merged B&E. (I) Merged C&F. Scale bar 100 um.*

*Mono-seed: seeded with one cell phenotype (wjMSCs).

**Tri-seed: seeded with three cell phenotypes (wjMSCs, HUVECs and HCFs).

Calcein: stains live cells with green.

Ethidium: stains dead cells with red.

7.4.3. Scanning electron microscope assessment

Further assessment of the seeded patches done to assess the surface topography using scanning electron microscope (SEM). About 5-7 days after seeding we did fix the patch and processed as previously described. The seeded cormatrix showed good alignment of wjMSCs as mono-seed on its surface which indicates reasonable uptake and spread of cells on cormatrix (Figure 7.3).

The tri-seed SEM images are comparable with the mono-seed (not shown due to missing comparable image dimensions).

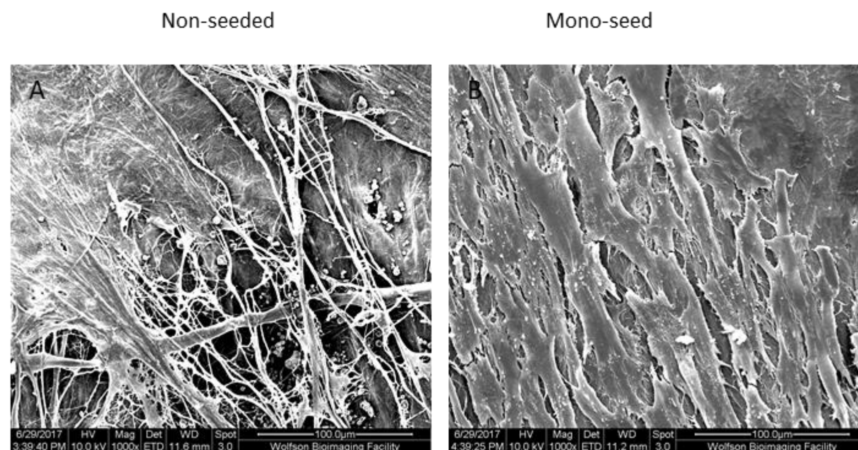

Figure 7-3.Representative SEM images of cormatrix after seeding with wjMSCs alone and in combination with HUVECs and HCFs.

Assessment of cormatrix surface after seeding with wjMSCs shows good alignment over cormatrix surface.

(A) Cormatrix non-seeded. (B) Cormatrix mono-seeded.*

**Mono-seed: seeded with one cell phenotype (wjMSCs).*

7.4.5. Mechanical properties of the patch

Due to the inherent nature of the heart as a pump for blood with rhythmic contraction relaxation cycles with subsequent dynamic pressure and shear stress. So, any patch for constructive cardiac surgery should be mechanically competent to withstand stress of surgery as well as the subsequent load.

Also, it is expected that cells seeded on cormatrix surface would lay their own extracellular matrix that may reflect on the mechanical properties of the patch. Therefore, it is quite crucial to assess mechanical properties of the patch after seeding to ensure mechanical competence.

Assessment included elasticity expressed as Young's modulus (YM) which is the ratio of stress to strain and non-seeded cormatrix was used as control.

Young's modulus is also known as the elastic modulus. It is used to describe the material resistance to gets deformed under stress (456). So, YM= Stress/strain
Stress is the force applied per area while strain is the ratio of elongation of the material in response to the stress to its original length dL/L, and therefore it is a dimensionless quantity (456). This means the higher the YM the stiffer is the material.
The YM of cormatrix after tri-seed (M=19.73, SEM=0.98) compared to non-seeded cormatrix (M=25.73, SEM=3.19) but still did not demonstrate a significant difference, p=0.1. Also, the maximum load of cormatrix after tri-seed (M= 17.46, SEM= 2.58) compared to non-seeded cormatrix (M= 12.36, SEM= 3.61) demonstrated non-significant difference, p=0.3, figure (7-4).
These data indicate maintained mechanical elasticity of cormatrix after seeding which is reasonably assuring for safe application as regard the mechanical perspectives.

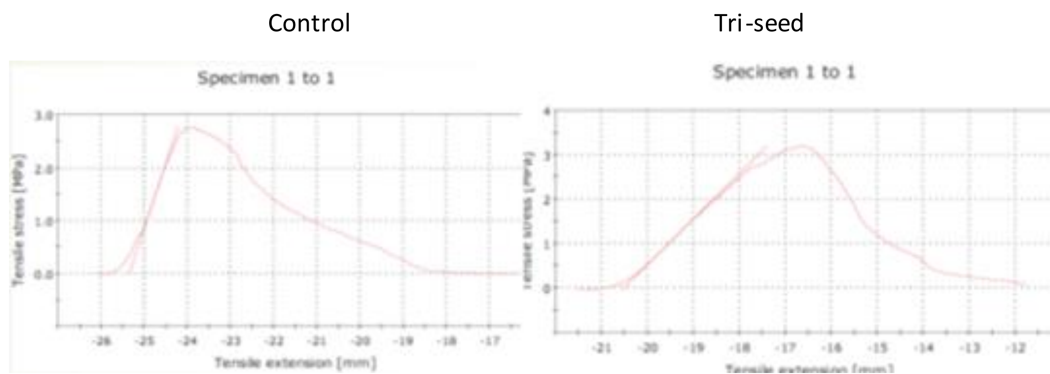

Figure 7-4. Sample of Cormatrix tensile strength assessment results.

Assessment of mechanical properties of cormatrix post tri-seed relative to the non-seeded cormatrix showed comparable results with no significant change of the patch mechanical elasticity after seeding.*

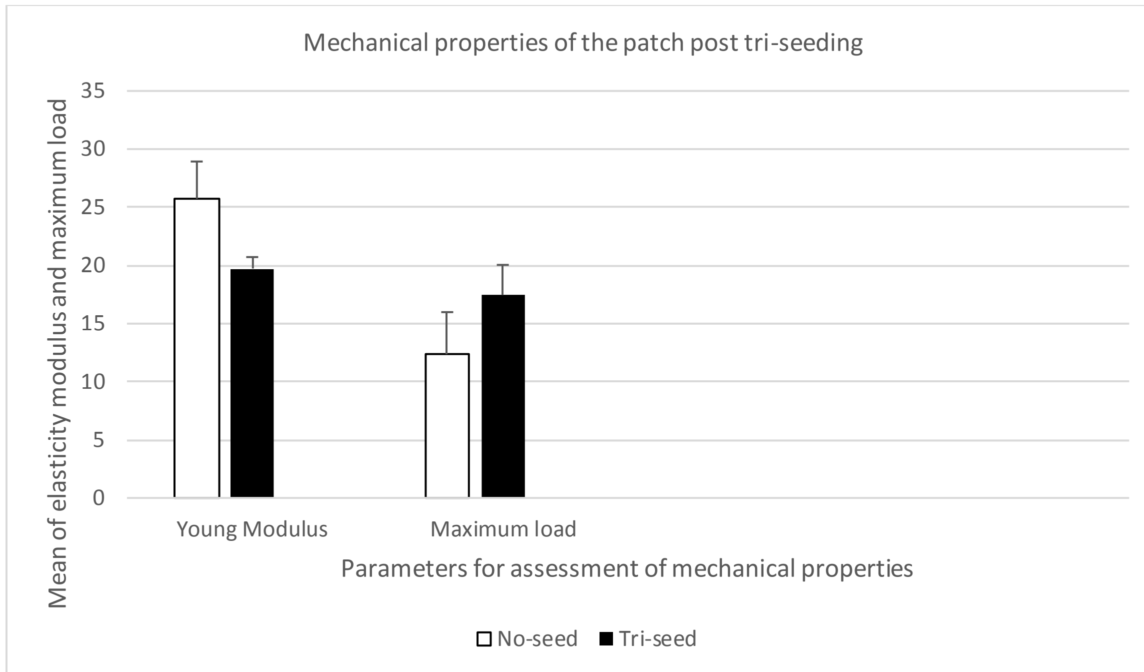

Figure 7-5. Assessment of mechanical properties of cormatrix post tri-seed relative to the non-seeded cormatrix.*

*Both YM** and maximum load were comparable between tri-seed and non-seed, p=0.1. N=3, expressed as mean +/-SEM.*

*Tri-seed: cormatrix seeded with the three cell phenotypes wjMSCs, HUVECs and HCFs.

**YM: Young's modulus.

7.5. Discussion

7.5.1. Introduction

Cormatrix is a decllularised porcine small intestinal submucosal extracellular matrix (457) which is commercially available and FDA approved (151). It would offer a scaffold for seeding cells and provide the patch with the necessary mechanical integrity. The clinical use of cormatrix started in 2006 (316) and as of December 2019 there were 12 trials registered on *ClinicalTrials.gov* using cormatrix in the cardiovascular field including ischemic heart disease, heart failure and peripheral arterial diseases and in addition, there are 119 papers on PubMed related to cormatrix use.

This reasonable record for in-vivo use of cormatrix showed pliability and durability in animals as well as humans which is quite promising. However, in terms of repopulation and vascularisation of the implant, several studies showed clear contradictory findings between animal and human studies.

Animal studies demonstrated good uptake, repopulation with native myocardial cells and vascularisation in addition to its functional element in terms of contractility of the patch that achieved up to 70% contractility relative to the adjacent native myocardium (124). However, human studies failed to demonstrate this favourable outcome. Indeed, despite cormatrix remained pliable but it did activate inflammatory reaction and infiltrate with eosinophils, plasma cells and other inflammatory cells in addition to fibrotic changes which led to hemodynamic instability in some cases (458) with no evidence of uptake or significant repopulation or vascularisation. Also, it was notable that the patch's thickness 9 months post implant was comparable with the pre-implant thickness which would raise concern about its biocompatibility in-vivo (459).

Use of decllularised xenogeneic porcine extracted ECM as a biologic scaffold offers the advantage of reduced immune reaction due to the conserved structure of the matrix molecules between different species (460). Also, ECM based scaffolds contain growth and angiogenic factors such as TGF-β, bFGF and VEGF that are maintained even with long shelf life in addition to the metabolically active degradation products of ECM that would support the constructive remodelling process (461, 462).

In the design of a cellularised graft for corrective cardiovascular surgery there are some key factors that should be considered. These include the mechanical integrity of the scaffold to withstand the surgical handling during implantation and retain sutures as well as the functional element of rhythmic contraction relaxation cycles, the shear stress of blood flow with the variability of pressure between systole and diastole. Also, to hold favourable microenvironment with the necessary ligands to accommodate seeding with cells and allow cell proliferation and differentiation (463). In addition, it hold the potential for repopulation with native cells when implanted in-vivo. Also, to be immune inert to avoid immune reaction that may lead to patch rejection and failure.

7.5.2. Post-seeding

The objectives of this chapter included to find out the potential of cormatrix to accept the triad of cells in terms of adherence to the surface, alignment and maintaining their viability in addition to assessment of any alteration of its mechanical properties post seeding. In other words, in this chapter we aimed to examine part of the biocompatibility arm of the cormatrix in addition to its mechanical integrity.

Our results demonstrate good compatibility of cormatrix with the triad of cells in addition to maintained mechanical integrity.

The post seeding assessment revealed good adherence of wjMSCs to cormatrix surface with reasonable widespread across the surface with no clumping of cells. Also, seeding with the triad of wjMSCs, HUVECs and HCFs did demonstrate same behaviour with reasonable spread and alignment on the surface of cormatrix. This is promising for smooth lining of the cormatrix and proper attachment of the lining cells to the neighbouring myocardial cells once the patch is implanted in-vivo. Unfortunately due to the facility constrain with the pandemic we could not proceed with the IHC experiments to demonstrate the triad of cells in combination on the cormatrix surface. Also, could not complete the dynamic seeding experiments as was planned. Indeed, we planned to seed the cormatrix with the triad of cells in static conditions for 3-5 days then proceed to bioreactor culture for 7 days then further experiment all aspects such as the histology, IHC, SEM and mechanical properties, but this all was suspended.

The second point of concern was the viability of the cells post seeding. For obvious reasons viability of cells is crucial for the patch's applicability and so we did examine post seeding viability but only in static culture conditions. We did use Calcein/Ethidium live/dead staining assay and then examined under immunofluorescence microscope. The assay demonstrated that majority of seeded cells are alive after about 3-5 days, however, it was notable that dead cells are more frequent in the tri-seed patches. Most probably the dead cells were HUVECs as expected from the prior survival experiments.

During handling the cormatrix and seeding with cells either mono or tri-seed, there was a concern about cells orientation. Despite the histology and SEM did not reveal any clumping of cells, however, still the cells are not in a clear smooth orientation relative to each other (own observation). Indeed, they showed some whirling appearance with frequent crossings. In theory, mal-aligned cells would not be ideal for smooth mono-directional conductivity and contractility. This concern should be addressed before going further with the patch into in-vivo assessment.

As explained before, the plan was to make dynamic seeding of the cormatrix using the bioreactor. So that after seeding the cormatrix with the triad of cells in static culture conditions, will fix the patch to the axis of bioreactor which would keep rotating in one direction for a week. In theory, this would generate some shear stress and enhance cells to orient/align in a smooth parallel manner. Unfortunately, this did not happen due to the University closure.

Then last step of assessment included the mechanical properties of the patch. Our group have already good experience of cormatrix use in-vivo (252, 316) with good integrity and durability. However, post seeding with this untested triad of cells still we need to be properly assess for any alteration of the cormatrix mechanical properties.

YM is a property of the material (160). It represents the relation of stress/strain and therefore the higher the YM the stiffer will be the material.

In biologic tissues, YM ranges from 0.2 MPa in granulation tissue up to 6000 MPa in mature bone (463).

Our results showed that the YM of cormatrix after the tri-seed was comparable with the control. Also, the maximum tolerated load which is the maximum load applied before breakdown and rupture happens, this was also comparable between the two conditions.

This indicates that mechanical properties of cormatrix would not be affected by seeding and it will maintain its elasticity and load tolerance.

In the study of Neethling and colleagues, they did assess elasticity of cormatrix in comparison with bovine pericardium after different treatments such as cross linking with glutaraldehyde and dye mediated photo-oxidised. Cormatrix was found to hold the highest stiffness indicator. This could be related to its nature of origin as porcine intestinal submucosal ECM as well as the longitudinal alignment of its fibres that would enhance its mechanical stiffness (122).

Use of ECM as a biologic scaffold for regenerative purposes to enhance local constructive tissue processes is well experimented in various anatomical areas but results are variable (460). Post ECM implantation, the host reaction would vary from favourable constructive remodelling to unfavourable pro-inflammatory reaction. The constructive remodelling takes place when the ECM is gradually degraded and replaced with host tissue. This variance would depend on the processing of ECM including efficacy of decllularisation, presence or absence of chemical cross linking and also the implantation procedure including contact to healthy tissues (126). As the implant would undergo initial phase of neutrophils infiltrate followed by infiltrate with mononuclear cells dominated with M2 macrophages. This reaction would favour constructive remodelling in addition to recruiting progenitor cells to gradually culminate into host tissue regeneration (126).

While pro-inflammatory reaction mechanisms could be related to host tissue interaction with the ECM ligands as well as ECM degradation products (126). So, the pro-inflammatory reaction would ensue if ECM is processed in an inappropriate manner such as incomplete decllularisation with residual antigenic epitopes and chemical cross linking to prevent macrophage induced degradation. This would enhance pro-inflammatory M1 macrophage phenotypes infiltrate leading to scar formation. In addition to possible inappropriate implantation such as lack of healthy vascularised tissue. All these factors would activate inflammatory process with loss of constructive properties (126).

During graft implantation in-vivo, the host reaction to biologic materials did vary considerably. In a clinical study using ECM for oesophageal circumferential engraftment for Barrett oesophagus using porcine small intestinal ECM, it did show good uptake and repopulation with oesophageal squamous epithelium within as early as four months post endoscopic surgery (460). Another study experimented use of ECM based scaffold for musculotendinous connection after full thickness tissue resection of the gastrocnemius muscle. The scaffold showed good uptake, repopulation and vascularisation with a functional recovery about half of the contralateral contractility force (464).

While these studies were quite promising, another study using 8-ply SIS for genitourinary reconstructive surgery showed good initial results while with long term follow up, further morbidity and chronic inflammatory reactions were elucidated (465). Another study used SIS for hernia repair in a rat model revealed lytic lesions and inflammatory reactions (466).

The underlying mechanism for modifying the tissue reaction to the biologic scaffold from inflammatory to reconstructive is not fully elucidated (460). Some preclinical studies reported heavy mononuclear cell infiltrate with dominant M2 macrophages in the non-cross linked small intestinal submucosa (SIS) within the first 4 weeks that could enhance constructive remodelling phenomenon by 16 weeks post implant. On the other hand the cross linked SIS did demonstrate dominant M1 macrophages that hold pro-inflammatory effect resulting in chronic inflammatory changes by 16 weeks (467). In addition, the

products of ECM degradation would recruit stem and progenitor cells and enhance ECs migration and angiogenesis which would support constructive remodelling (462). This was illustrated in the study of Badylak and colleagues as they examined SIS graft in a canine model of tendon Achilles resection. The SIS showed degradation by 8 weeks post implant with residual constructive remodelling as it did support local regeneration (468).

7.6.Conclusion

Cormatrix is biocompatible with the triad of cells wjMSCs, HUVECs and HCFs and its mechanical elasticity post seeding would not be altered by seeding of these cells.

Chapter VIII

Discussion and conclusions

8.1. Discussion

Cardiovascular diseases are the most common cause of mortality world wide. Current pharmacologic as well as interventional therapy still limited to maintenance of available cells and to minimise cellular loss while still heart transplant is the only cure for terminal heart failure (17).
On the other hand, CHDs are the most common birth defects worldwide (71). Millions of new-borns are affected around the globe and the majority of patients would survive to adult life. It was estimated that 1.2 million in Europe have some form of CHD(s) with substantial percent of these cases would need surgery at some point (89, 469).

Actually, management of CHDs represents the main bulk of scope of the paediatric cardiologists' practice. Substantial percent of the paediatric age patients with heart defects would require some intervention including surgery with all its draw backs as an invasive approach with cost and potential complications (470). Surgery could be corrective or palliative to alleviate symptoms and support growth followed by another corrective surgery at later stage (470).

Currently and despite the higher calibre of health care and marked advancement in interventional procedures including transcathere as well as surgical interventions, still surgical correction of CHDs is limited with the fact that available materials for reconstructive surgery are non-viable materials. This would lead to size mismatch with progressive child growth. This is in addition to inefficient remodeling as well as other complications such as aneurysmal dilatation, stenosis, infection, thromboembolism and calcification (377, 471). This means they would offer anatomical support but missing the physiologic element in terms of lack of growth and lack of contractility. So, it is anatomical success but physiological failure.

The heart has about 2-3 x 10^9 CMCs which represent almost 30% of its cellular pool while the rest are non-myocytes (18). Despite CMCs are the actual functioning cells but collaboration and proper communication with other cellular and non-cellular pools is indispensable for cardiac muscle integrity and function.

During development, tissues attain their morphogenesis from the intricate interactions of different cell phenotypes that would gradually shape the tissue structure and function. This process is tightly controlled by cell-cell interaction in addition to the spatio-temporal regulation of cell proliferation and differentiation (382). In the myocardium, cells communicate through direct physical contact in addition to gap junctions such as connexins which are expressed on CMCs, ECs and fibroblasts. Cellular communication would facilitate electrical coupling and therefore organised signal spread through the myocardium (26). Therefore, for organised cardiac function the cellular pillars of the myocardium must come into harmonised communication (42). And every cell phenotype within this multicellular pool has its role to support cardiac anatomical and physiological integrity.

Some groups including our group attempted to tackle the physiologic aspect of cardiovascular grafts to enhance the graft with a growth potential via seeding with cells. This approach did demonstrate positive results in animal studies but still limited with short durability of cells in-vivo. Therefore, the general concensus is that the positive results are secondary to paracrine factors. However, these paracrine effects as well as the short in-vivo durability of cells are not fully understood.

This is considered a lag between bench side studies and clinical studies. Indeed, clinical studies should stand on solid grounds of basic research which should ideally predate the clinical arm to make good standardisation of items of research and make reasonable anticipation of outcome.

Therefore, our target here is to tackle the physiologic arm to boost the patch with potential growth and contractility utilising the principles of tissue engineering via seeding with a triad of relevant cells and also to establish a new paradigm that is to the best of our knowledge the first time to be proposed to understand the reaction of cells to this combination and if the combination of cells would support survival and paracrine profile of cells.

Cell based therapy is a new approach for treatment of cardiac impairment to restore cardiac performance. This includes transplantation with cells of various phenotypes such as MSCs, skeletal myoblasts, ESCs and IPSCs derived CMCs among others (236). However, transplantation of cardiomyocytes revealed that they do not align and maintain poor differentiation (472).

In the study of Zimmermann and coworkers, they did attempt to engineer a cellularised cardiac patch using native cardiac cells and enriched the patch with CMCs. The multicellular approach proved superior with better contractility and conductivity as well as vascularisation which would support in-vivo uptake (473). Also, this would emphasise on the crucial rule of non-myocyte cells for optimum myocardial function.

Nonethelss, the experiments including combination of cells were carried out in order to gain extra advantage of the different cellular potentials but still the approach is quite limited with limited durability of transplanted cells in-vivo (236) which is not fully understood.

Also, several models of contractile in vitro patches that could be termed “myocardial equivalents” have been trialled such as seeding of biologic fibrin gel with myocytes and seeding matrix with cells (17). Bursac and colleagues did seed neonatal rat ventricular myocytes on polymeric scaffold and cultured in a bioreactor for one week. The assessment revealed organised tissue like structure and achieved thickness 5-70 um of myocytes layers with some electrophysiologic properties which was quite promising but still inferior to the native ventricular myocardium (474). Also, this study was designed to assess electrophysiology in vitro and did not address the multicellularity requirements of the tissue.

Leor and his colleagues did trial seeding porous alginate scaffold with fetal cardiac cells and implanted in vivo. The seeded scaffolds enhanced neovascularisation with better LV function relative to the control (475). However, this study did trace the effect of the seeded cells which still could be a paracrine effect while did not trace the cells in vivo and still did not address the multicellularity approach.

In the study of Radisic and co-workers, they did trial seeding neonatal rat ventricular myocytes on ultra-foam collagen sponges and exposed the construct to cyclic electric stimulation to build up a biomimetic to the myocardial contractile tissue (472). Despite the results were positive in terms of inducing contractile constructs but still it is mono-cellular seed and clearly missing the other pillars of cellular heterogeneity in a tissue.

Also Shimizu and colleagues did lay multiple layers of cardiomyocytes on a temperature responsive polymer sheet that could degrade and detach cells with only temperature manipulation. This approach did prove in vivo longevity up to 12 weeks. However, they did experiment it under the skin in nude rats,

so the approach is missing again the concept of multicellularity and also its longevity was assessed in an immune inert and physically non stressful environment (476).

In this project we planned to design a multi-cellularised cardiac patch utilising cormatrix as a biologic scaffold and to seed it with a triad of relevant cell phenotypes including wjMSCs, HUVECs and HCFS.

Considering the fact that the embryogenesis of various tissues builds tissue specific genetic identity that imprints on the harmonic behaviour of various cell phenotypes. Several studies elaborated the spatio-tempral unique identity of different cells which have same phenotype but different genomics based on different hosting tissues such as fibroblasts and endothelial cells and this is expected to apply to other cell phenotypes (477). This was elaborated in the experiment of Jen et al to figure out any difference between ECs in different anatomical areas. They did demonstrate clear tissue specific expression patterns of microvascular ECs that would contribute to local homeostasis of their hosting tissues (478).

So, during embryonic development various tissues have their own homeostatic setup that would accommodate different cell phenotypes in a friendly cross talk. This includes different cell phenotypes within same tissue to support cells' survival and their physiologic roles as well.

Therefore, non-native cells are not in a full concert. This could explain why cells disappear from the scene after in-vivo implantation even if no immune rejection concern is there.

This is quite relevant here as we attempt to co-seed three different cell phenotypes while this triad is not existing in a normal tissue and therefore the combination could have positive, negative or neutral effects at different aspects.

The objectives of this project went beyond the design of a multicellularised patch using principles of tissue engineering to examine the reaction of the three cell phenotypes when combined together so that we can assess their on-patch interaction and predict their potential in-vivo behaviour. Assessment was done at six domains including pro-survival/anti-apoptosis factors, ECM proteins, angiogenic/paracrine factors, cellular adhesion/communication factors, cell survival and cell proliferation.

It is quite important to stress on the point of mutlicellularity and interdependence of cells for successful function under physiologic conditions as well as appropriate reaction to pathologic events to sustain cardiac function (14).

As we did illustrate earlier regarding multicellularity of the myocardium, as every cell phenotype has its indispensable rule that can not be underestimated in any design for a biologic substitute. This is quite evident from experiments modifying behaviour of myocardial cells such as transgenic studies which resulted in global myocardial events due to the effects that would go beyond single cell phenotype to the whole tissue effects secondry to altered intercellular communication (14).

Indeed, uptake of the patch would need fine tuned integration of several biologic and molecular processes such as cell proliferation and migration as well as angiogenesis and ECM deposition (479). So, here we aim to dissect the relevant processes and examine each individually.

From the definition of tissue engineering (TE) as *" the application of principles and methods of engineering and life sciences toward the fundamental understanding of* <u>*structure-function relationships*</u> *in normal and pathological mammalian tissues and the development of biological substitutes to restore,*

maintain or improve tissue function " (113). So, understanding these interactions falls under the scope of TE.

The current practice in surgical management of CHDs consists of repeated palliative surgeries at different time intervals (340) until final corrective surgery is done when the body reaches plateau growth. Obviously, this approach incurs more economic burden in addition to the potential risk and adverse events associated with surgery such as the perioperative mortality which was estimated in a German study as 3.4%. Importantly, mortality rate was higher in palliative surgery than in corrective surgery which emphasises on the importance of minimising the number of surgeries to reduce risk of complications (469).

The prototype of surgery for a CHD is the Fontan operation. Currently, Fontan operation is one of the most commonly performed surgeries for single functioning ventricle anomaly (63). In this procedure the venous blood is redirected from the systemic venous drainage to the pulmonary artery directly. This would bypass/replace the missing right ventricle. The operation did dramatically improve the patients' survival; however it needs to start with palliative procedure which is believed to be better delayed until age of 2-4 years due to the fact that in this age the IVC diameter would reach about 60-80% of the adult IVC. Realistically speaking, this is not always feasible as there is risk of other complications with delayed surgery due to long standing volume overload that may lead to heart failure with subsequent impairment of physical and mental growth of the child in this crucial development age. On the other hand the approach to fit a graft that would over-size the actual need in order to avoid being overgrown may lead to turbulent flow in addition to thrombotic complications (63, 480).
Grafts used for this procedure include PTFE which holds good properties such as less thrombotic tendency and higher success rate relative to Dacron. Also, autografts were used but limited with availability. In addition, synthetic grafts such as PTFE and Dacron would need long term anticoagulation to reduce incidence of thromboembolism while tissue engineered grafts could go safely without anticoagulant after the first 6 months postoperative (471).

This exemplifies for the major limitation in the current practice. The available materials used for correction of CHDs are non-viable and cannot cope with the normal growth curve of the heart/blood vessels from neonate to adult life (65) which dictates for repeated surgeries to cope with overall organ growth.
Also these grafts are non-contractile which means the patch area is a non-functioning area and therefore not expected to share in contractile function which is the core of cardiovascular performance. Furthermore, the cardiac cells have limited proliferation potential (271, 481) which means that even if a scaffold is offered to reconstruct a defect still the native cells are not expected to repopulate it and therefore we can state that anatomical support is not enough.
This was evident in different studies and also in our group experience with in-vivo implantation that did demonstrate that a scaffold to reconstruct a malformed chamber did not initiate proper repopulation with native cells while seeding with cells proved significantly superior even if the cells did not last for long which is most probably secondary to paracrine effects rather than physical engagement of cells (151, 316).
In the study of Sugiura and colleagues, they did use a scaffold of woven fabric of poly-l-lactide acid or polyglycolic acid and an equal percent of poly (l-lactic-co-ε-caprolactone) copolymer and seeded the graft with autologous bone marrow mononuclear cells (BM-MNCs). The grafts were used for correction of univentricular anomaly in children with mean age of 5.5 years. The follow up continued for about 11 years and showed good durability with no reported graft related mortality, no rupture, aneurysmal

dilatation, infection or calcification. Stenosis was the main complication, reported in about 28% of cases which is more than in cases received artificial grafts (480) but dilatation via balloon angioplasty was feasible. One patient out of 25 participants had thrombosis that could be treated with anticoagulant. The notable advantage was that anti-platelet and anticoagulant were stopped 6 months postoperative which would improve quality of life and reduce cost and complications (471).

Indeed, this study is one of the earliest (482) and even longest studies to present data about tissue engineered vascular graft with less anticoagulation requirement. However, it did not present any control cases to enable for clear comparison. Also, there is lack of histology and further tissue assessment to demonstrate clear uptake and repopulation with native cells. Also, the methodology used is questionable in terms of seeding cells and if they were successfully up taken and adhered to the graft surface. The investigators did aspirate bone marrow and seeded the graft with mononuclear cells for two hours then straight to surgical implantation with no way to track these cells or comment on their viability, uptake in-vivo or any interaction with the cardiac milieu.

The assumption was that the stem cell population from bone marrow derived cells would proliferate and differentiate on the scaffold and eventually integrate with the neighboring milieu to form neovessel (377) but this did not prove correct. Indeed, studies on BM-MSCs demonstrated that their differentiation in-vivo is almost negligible (377) and the positive effects are most probably due to a paracrine effect to support angiogenesis and enhance cell survival.

The preclinical study to assess the approach of seeding a biodegradable scaffold that was manufactured similar to the clinical study, with BM-MNCs did demonstrate complete wash out of the seeded cells few days after in-vivo implantation. Roh and colleagues did examine implantation of a biodegradable scaffold seeded with BM-MNCs in a SCID mouse model. The seeded cells did not seem to integrate and the neo-vessel formation was achieved via invading host monocytes through inflammation enhanced vasculogenesis and it was evident that the role of BMCs is rather paracrine effect to secrete MCP-1 which would enhance monocyte migration, proliferation and neovessel formation (377).

The scaffold did demonstrate remodeling to a neovessel that is morphologically comparable with native vessels including ECs lining the innermost layer, supported by SMCs and adventitia like layer formed of collagen to support the vessel while the scaffold was gradually degrading and the seeded cells could not be tracked beyond the first week of implantation despite they were implanted in SCID mice and the authors concluded an inflammation mediated process to recruit host monocytes and activate a vascular remodeling to replace the biodegradable scaffold with a neovessel (377).

In fact, animal studies demonstrated significant improvement of cardiac function with cellular therapy such as stem cells derived CMCs (483). For instance, Chen et al. reported better left ventricular function after implantation of a patch seeded with multi-layers of MSCs derived from bone marrow (484). Another group used injection of biomaterials to enhance replacement of failing myocardium after infarction (117).

The evidence until now supports stem cell therapy to ameliorate cardiac function (485) and the delivery method of cellular therapy seems to make difference. For instance, cells injection resulted in

arrhythmias and did not clearly integrate into myocardium while pre-aligned cells on tissue engineered patches showed good uptake and neovascularisation with no reported arrhythmias (486).

However, clinical trials of MSCs transplantation showed modest effect (126) as it has been limited by lack of effective uptake and short in-vivo durability (486).

Several clinical trials did assess stem cells potential to enhance cardiac function (26). Also, multi-lineage transplantation with IPS derived CMCs in addition to ECs and SMCs fabricated into a 3D patch proved beneficial for recovery of cardiac function and integration into myocardium with proper vascularization with no appreciable arrhythmias (487). Also, Engineering a patch with CMCs boosted with ECs proved to be superior to the non-ECs containing patch with better uptake and vascularisation which was also reflected on the overall cardiac function post-transplant (488).

The concern here is that these promising results came from experiments on normal animals with expected normal genetic set up. Obviously, the situation is different in CHD cases with underlying genetic error and so the approach should emphasise on the uptake of cells for longer lasting effects. Therefore, we aim to establish sustainability of cells in-vivo and minimise their wash out.

However, still tissue engineering with in-vivo implantation of a patch seeded with cardiac cells has limitations including death of cells and wash out of cells which would limit its capability to enhance further cardiac tissue regeneration and this could be due to unfavorable milieu and poor perfusion of the patch (42). The cells have short in-vivo durability but they exert their effect via paracrine factors secretion in addition there was a concern about ventricular arrhythmias that would hinder their use in standard practice (26).

In general, implantation and successful integration of a biomaterial within a biologic system with repopulation and vascularisation is a multifactorial complex process (412) that needs high degree of biocompatibility of the biomaterial as regard its interaction with native cells/seeded cells, any immune/inflammatory reaction in addition to the proper cellular cues for cell adhesion and spread on its surface. While in the cardiovascular area engineering a graft needs to consider that the myocardium is a highly dynamic tissue with multiple cell phenotypes and dense vasculature in addition to quite dynamic ECM and its efficient rhythmic contractility is critically dependent on proper function and interaction of all these components and it is expected as a general rule of thumb that implant success would critically depend on integration as well as vascularisation (412).

After implantation, a series of events would start including inflammatory reaction and cellular proliferation and start of angiogenesis in order to initiate a healing phase which includes remodelling process.

Regeneration of fully structured and functioning cardiac tissue is the dream of cardiovascular regeneration scientists and despite it is still a dream but there are some forward steps taken already.

In 2006 Guo et al successfully generated in-vitro beating cardiac patch. They did differentiate embryonic stems cells (ESCs) into cardiomyocytes (CMCs) using the embryoid body (EB) approach as the ESCs would spontaneously differentiate into tissue like spheroids and mixed with collagen-I and matrigel to build up a cardiac tissue like patch. However, in this study and despite they did try to purify CMCs cell population but still there was a concern about non-cardiac cells resulting from the EBs and even the investigators admitted for the limitation of lack of vascularisation and the potential of teratoma formation (489).

Another group trialed injectable hydrogel composite of oligo-(poly-ethylene-glycol) fumarate as a carrier for ESCs into MI model. This approach showed better vascularisation, better retention of cells and less remodelling relative to the control (490).

Despite the promising results of these approaches but our target patients are different. We target the CHDs cases which are indeed cases with defective/erroneous growth. So, we aim to reconstruct a malformed chamber rather than to supplement the heart with multicellularised patch in order to integrate with neighbouring eu-genetic myocardium.

Ideally a graft for reconstructive cardiovascular surgery should offer the necessary mechanical integrity and functionality as well as the more important requirement which is to expand with time in coping with the natural growth of the heart in order to avoid repeated surgeries. This looks difficult task to accomplish but tissue engineering offers hope to accomplish this task.

Tissue engineering is the area of interest of our group and we have previous experience with successful tissue engineering projects in animal models ((251, 491, 492). Also, we did provide evidence that seeding cormatrix with cells gives better outcome in terms of the degree of repopulation and vascularisation as compared with the non-seeded patches (252).

We used mesenchymal stem cells from different sources such as MSCs from thymus and from cord blood to seed natural grafts for in-vivo testing. In this project we planned to establish natural graft seeded with MSCs derived from umbilical cord Wharton jelly in addition to cardiac fibroblasts which play pivotal role in the myocardium and to add HUVECs to supplement our patch with the necessary pillars for vascularisation.

Here we describe a new approach to seed cormatrix as a biologic scaffold with a triad of autologous cells. The aim is to combine the multicellularity which is a basic requirement for any tissue in addition to the mechanical integrity of the scaffold. The design of this patch was sparked from the belief that natural tissue is the best, so we should get closer to the natural pattern to achieve better outcome. Natural tissue would have two major constituents, stroma and parenchyma. The parenchyma is the actual functioning cells that carry on the tasks of the tissue while stroma includes the supporting cells.

So, for the patch to be a realistic solution we included several relevant cell phenotypes namely wjMSCs, cardiac fibroblasts and endothelial cells and these cells could be extracted from the patient so there is no immune rejection concern.

The properties of the patch will be the net result of its whole components. This includes the cells viability and their secretome profiles that would be reflection of each cell reaction to the new milieu and would also indicate their potential effect on neighbouring native cells.

We did dissect this triad of cells into individual components and examined the potential reaction of each cell to the other two cells using conditioned media followed by examining the scaffold in terms of seeding, uptake, viability of cells as well as its mechanical properties.

Indeed, in-vitro studies utilising culture systems under different conditions is invaluable tool for developmental, physiologic, pharmacologic as well as pathological studies in a controlled environment that would circumvent the complexity of the in vivo environment (474).

Assessment of the potential reaction of each cell phenotype was done at the relevant domains that are expected reflect on the patch cellularity and in-vivo behaviour. Also to try to find out the cause for rapid wash of cells seeded on biologic scaffolds shortly after in vivo implantation.

Our results demonstrate no survival advantage would be offered by the combination of cells to any of the three phenotypes of cells and HUVECs were the most affected with quite high apoptosis rate. This is consistent with other group findings (473) who reported a high degree of cell loss in the first few days in culture with mostly apoptotic cells despite the combination was indeed native cardiac cells and there was need for survival supporting factors to maintain cell survival.

In our design, MSCs were chosen as a multipotent cell phenotype with well reported cardioprotective effect via enhancing myogenesis as well as angiogenesis (281). In addition to their immune modulatory effect as they do not express HLA-II which allows allogenic transplantation as well as non-teratoma formation in contrast to the more potent ESCs and IPSCs which is an important safety privilege for MSCs use in-vivo (210).

Indeed, MSCs are one of the most commonly used cells for cell therapy for preclinical and clinical studies (493). However, experiments in large animals showed just limited success mostly due to loss of majority of cells early after transplant (236, 281).

Wei et al trialled to seed an electrospun poly (ξ-caprolactone) (PCL) graft with MSCs extracellular vesicles which contain vasculogenic factors such as VEGF and miRNAs like miRNA126 and miRNA145 and tested it as a vascular graft in a rat aorta model. The study demonstrated better endothelialisation with less thrombogenesis and calcification. In addition, the study showed positive immune modulatory effect as they switched the pro-inflammatory M1 macrophages to the anti-inflammatory phenotype M2c (494).

Another point of concern was the risk of malignant transformation that is linked with any cell therapy particularly the stem cells. However, MSCs potential for neoplasia is quite low as compared with ESCs and IPSCs (282).

Bernardo et al did address this concern for bone marrow derived MSCs (BM-MSCs). They did examine the BM-MSCs at high passages. 80% of cell lines underwent senescence with progressive passages and were further observed for 8-12 weeks while the remaining reached P25. Analysis done for telomerase activity, telomerase reverse transcriptase transcripts and if any alternative telomere lengthening mechanisms.

They reported significant variabilities in MSCs proliferation potential which is consistent with our observation. The most important finding was that MSCs did not exhibit chromosomal abnormalities, in fact, they did show progressive telomere shortening with no telomerase activity, telomerase reverse transcriptase transcripts or alternative telomere lengthening mechanisms (495). These results would support safety of this approach as no evidence of malignant transformation.

Some reports raised concern about potential of MSCs to emerge from senescence and form malignant cells (282). This was reported by two groups, however, one of these papers was withdrawn later due to failure to reproduce same results and the authors admitted for cross-contamination (496).

So, MSCs are safe for clinical use but the most suitable window for transplantation is before start of senescence as within this window the probability of malignant transformation is quite low (282).

WjMSCs have the ability to self-renew and hold multi-lineage differentiation potential (497) including cardiomyocytes. Also, several experiments reported positive impact on cardiac function when tested in-vivo (255, 277, 498, 499). This is in addition to their availability and easy handling with no ethical concerns as the umbilical cord is considered a medical waste. Therefore we opted to use them for seeding the patch. However, still there are concerns about limited durability of cells in-vivo that was reported in some studies to be as short as few days (325). In our experience, within few weeks after transplantation the cells were not detectable. However, we have to admit that we did not attempt to examine the cells persistence at scheduled time intervals and therefore the cells may have been washed out of the scene even within shorter duration.

Assessment of wjMSCs started with the survival factors and the plan was to assess for Akt,BCl-2, Bad, Bax,P53 and NRG1. However, we could only examine for Akt, BCl-2 and NRG1 due to the pandemic restrictions.

WjMSCs have a well characterised secretome profile including Akt and BCl-2 as anti-apoptosis factors in addition to NRG1 as pro-survival factor that can enhance CMCs to re-enter cell cycle and proliferate. The Akt inhibits apoptosis through activating mediators including the anti-apoptosis factor BCl-2.

Our results show that CdM did up-regulate Akt in wjMSCs but this did not extend to BCl-2 and wjMSCs survival was not enhanced. However, still Akt upregulation is considered as a positive finding as it would have paracrine effect and expected to support the in-vivo interaction of the patch with its neighbouring myocardium. Then, on treatment of wjMSCs with CHIR, expression of both Akt and BCl-2 was comparable with the control. However, when combined CHIR and the CdM this resulted in further significant upregulation of Akt but not BCl-2. This was reflective on the wjMSCs survival that was significantly higher in the CdM/CHIR arm and it is a good indicator of positive paracrine survival support to neighbouring myocardial cells once implanted in-vivo. As it was shown that Akt overexpression in MSCs did demonstrate better functional recovery and reduced infarct size(234). So, in this design Akt overexpression was already evident in-vitro and expected to have positive effect in-vivo.

Here it is prudent to emphasise on the need for balanced secretion of angiogenic/paracrine factors as a crucial requirement for healthy tissues and adequate organ functions. For instance, Akt short term expression induced physiological hypertrophy with appropriate angiogenesis while long term expression went beyond this and caused hypertrophy with inadequate angiogenesis and led to failure (18). So, in our co-seed model the Akt upregulation is considered as a physiologic phenomenon and expected to boost the myocardium with relevant cues but long-term effects would require to be experimented in-vivo.

NRG1 is a member of the EGF family (500). NRG-1/ErbB2 role is crucial for heart development and it acts in a paracrine way (500). Its role in adult heart was discovered after the ErbB2 inhibitor trastuzumab used for treatment of mammary carcinoma was noticed to cause cardiomyopathy in these patients (18).

NRG1 is one of the MSCs paracrine factors that would enhance CMCs proliferation and boost the myocardial regeneration post infarction through phosphatidylinositol-3-kinase (PI3K/Akt) pathway. This effect was enhanced with overexpression of ERbB4 via an autocrine loop leading to NRG-1 upregulation

(317, 355). It promotes CMCs to re-enter the cell cycle and resume proliferative potential and also exerts anti-apoptotic effect on CMCs via Akt-dependent mechanism (501). This indicates that Akt upregulation even with neutral NRG1 is expected to offer survival and regenerative support to native CMCs.

NRG-1/ERbB expression is upregulated early during pressure overload but decreases afterwards if the myocardium starts to fail (18) which could reflect the mechanism for NRG1 upregulation that is more pressure induced and this is missing here in the co-seeding model but still NRG1 expression in wjMSCs did not down-regulate which is more important as we expect to benefit from its basal level of expression but cannot expect more.

The next assessment point included the ECM proteins. We did focus on the collagen I and collagen III as the main collagens in the myocardium in addition to fibronectin. Our data demonstrate that wjMSCs produce the three types of proteins and the CdM did down-regulate expression of collagen III while its effect on collagen I and fibronectin was comparable with the control.

Collagen III is a fibrillar collagen that is involved in different inflammatory processes such as pulmonary, hepatic, renal and vascular disorders (502) . Collagens in the myocardium play crucial structural role due to their physical properties. They hold tensile strength which guards against myocardial rupture and offer stiffness to resist progressive dilatation of the various chambers and also their elasticity helps diastolic suction (503).

If myocardial collagen content is altered it may lead to adverse effects including potential arrhythmias as well as potential uncoupling of CMCs as well as altered stiffness and filling/contraction functions (503).

In addition, the paracrine profile of MSCs includes angiogenic factors and this is part of its regenerative effects to enhance angiogenesis via its paracrine factors including bFGF, HGF, VEGF, SDf1 and MCP-1.

Our data demonstrate CdM would enhance HGF, VEGF and MCP-1 expression by wjMSCs while no significant effect on angiopoietin, bFGF and SDf1. These results indicate that angiogenesis would be promoted by MSCs secretome and the non-enhanced factors are still expected to exert effect at their basal level such as the bFGF which in addition to its angiogenic and growth properties also shares in vascular tone control (391).

Indeed, some of the factors are crucial for normal cardiac function such as VEGF that was proved necessary for coronary microvascular tree development and overall functional capacity and stress tolerance (504). Also, clinical trials showed significant upregulation of exercise tolerance post VEGF administration (40).

Last point included the assessment of wjMSCs proliferation. Our results demonstrate that proliferation potential of wjMSCs under CdM effect as well as CHIR/CdM were all comparable with the control with no significant change which eliminates the concern of overpopulating the patch and gives impression of balanced proliferation of all cell phenotypes.

Considering the immune modulatory effects of MSCs as they enhance M2 macrophage phenotype and repress the pro-inflammatory M1 macrophages which in the context of ECM based scaffold would degrade in-vivo with release of cryptic peptide degradation products that promotes M2 macrophage

infiltrate, therefore, both the seeded MSCs as well as its ECM scaffold would enhance the constructive remodeling by M2 macrophages and support its in-vivo biocompatibility (126, 505).

As the rapidly oscillating contraction/relaxation cycles are the basic set up of the heart function, the cardiac muscle proved to be quite efficient for oxygen extraction as it can extract up to 70-80% and this is more obvious if compared with skeletal muscles that extract only 30-40% and this high energy demands are supplemented with the dense capillary network in the myocardium (18). So, we considered to include ECs in the design of the patch so that we ensure the patch has the necessary pillars for angiogenesis but the behavior of HUVECs with this combination raised some concerns.

Angiogenesis is the formation of new blood vessels from existing vessels. ECs, ECM and their crosstalk are the dynamic pillars for angiogenesis as they initiate and guide ECs proliferation and also initiate apoptosis signals to maintain the balance and avoid over-vascularisation. Normally, ECs of blood vessels have tight intercellular junctions and adherence to basal lamina. ECs under these conditions are in a quiescent state with resistance to apoptosis (414) and their cell-cell and cell-ECM communication maintains the integrity of the new forming vessels (506).

Endothelial cells (ECs) are the innermost cells in all blood vessels . They are quite active cells and play crucial functional roles such as nutrient and fluid exchange, regulation of vessel tone and blood flow as well as angiogenesis and immunity amongst other functions (287). On the other hand the myocardium is one of the highly vascularised tissues with high metabolic demands and therefore adequate vascularisation is indispensable for myocardial function (507). Therefore, the endothelial cells would be an invaluable pillar in the patch.

The concept of seeding a biomaterial with ECs is already well known but there are some conflicting data about the efficacy of this approach. For instance, seeding ePTFE which is considered the most commonly used vascular graft due to its favorable properties but seeding with ECs was not consistently promising (136). It was reported by some laboratories as non-effective while others reported better patency of the ePTFE graft after seeding with ECs (508). In addition, others reported that ECs could facilitate the vascularisation process and enhance organisation of CMCs in-vitro (42).

Inclusion of HUVECs as autologous endothelial cells in the design of the patch was based on the hypothesis that they would boost the patch with reliable pillars for angiogenesis. However, assessment of HUVECs reaction to the CdM was non-promising.

Our data would shade light on ECs behavior when combined with other cells. Considering the angiogenic factors that control vascular development and function include angiopoietin, bFGF, HGF, PDGF, VEGF, MCP-1 and TGF-B family (18). The HUVECs reaction to the combination of wjMSCs and HCFs did down-regulate bFGF and HGF expression with no significant effect on other angiogenic factors.

The interaction between mesenchymal cells and ECs is a multifactorial complex process (323). The key factors assessed here would support the role of wjMSCs while the HUVECs reaction was not quite positive in particular the high apoptosis rate. However, CHIR use did demonstrate significantly better survival rate with reduction of the apoptosis rate by about 57%. This was also noted in the wjMSCs survival experiments as the CdM/CHIR arm showed significantly less apoptosis rate which indicates the valuable role of CHIR treatment to enhance cells survival which is expected to reflect on cells retention in-vivo.

The fibroblasts are the most common stromal cells (256) and they are readily available in various tissues with a reasonable proliferation potential so that we can expand a good volume of fibroblasts for seeding. However, fibroblasts are not unique in all tissues. Indeed, they have a tissue dependent identity. This was evidenced by transcriptomic analysis done by independent laboratories which showed that fibroblasts have diversity of phenotypic and functional characteristics such as ECM production, migration and immune modulatory effects that are more of tissue specific character (477) in addition to distinct response to inflammatory cytokines (509).

Even more, fibroblasts have spatio-temporal specificity as they hold specific functional characteristics and homeobox (HOX) gene expression pattern that are diverse even in the same tissue but different locations as well as between fetal and adult life which is termed HOX code (256).

In this project we started with cardiac fibroblasts as the most relevant and more of tissue specific fibroblast type cell to support our multicellular patch. Our data demonstrate that HCFs reaction was fairly neutral all through including the reaction to CHIR. This is still considered positive as the cells kept their quiescent state which indicates they would maintain their basal functional levels as regard their intended target to support the patch with the ECM.

The Cormatrix has its limitations as a non-growing patch in addition to the recent reports of discrepancy between animal studies and findings in human studies (151, 510). However, there are good points as it is a biologic scaffold that can support cell adhesion to its surface which was shown in the histology sections as well as the SEM images. Another advantage of cormatrix is that it can tolerate the mechanical load of surgery as well as the dynamic nature of the cardiovascular system.

Recent reports show failure of cormatrix to be repopulated with any myocardial cells or organised tissue and absent vascularisation in human cases. This could be due to the fact that in humans with CHD there is underlying developmental error that prevented the cells to go through the correct pathway from the start and that is why they will not proliferate and fill the gap even if they have a scaffold to support their growth. This is because the scaffold will offer anatomical but not physiological support. Also, in animal studies the patches were sutured after full thickness section which will induce inflammatory response and release the cells from the contact inhibition so that they proliferate using the scaffold support as these cells do not have underlying genetic errors but this is not the case in human cases with CHD.

Interaction of embryonic Fibroblasts/CMCs during embryonic development promotes myocytes proliferation that could be secondary to collaboration of fibronectin, collagen and heparin binding EGF-like GF that would support CMCs proliferation via B1-integrin signaling (28).

The timing of cell transplantation could be also an influential factor. After MI, inflammatory process starts with fibrin dependent ECM formation to dominate the field in addition to other inflammatory changes preparing to the remodelling process including angiogenic factors upregulation. Therefore, MSCs transplantation around 3 days post MI was elected to avoid adverse involvement of transplanted MSCs in the inflammatory process that would have deleterious effects on them. However, in our target population this kind of timing is not feasible as the patch is intended to be used for corrective/reconstructive surgery and so it would be sutured to a well-established myocardium without

diffuse inflammatory cascade which is promising that cells will not suffer from diffuse inflammatory process effects (281).

Also, the ECM degradation products were shown to hold chemotactic effect to attract stem cells, promote inflammatory reaction and enhance regeneration. In the study of Agrawal and colleagues, they did examine injection of ECM degradation products into amputated digit. The ECM injected digits had significant infiltrate with mononuclear cells relative to control group which stained positive for stem cell markers such as Sca1, Sox2 and Rex1 and demonstrated differentiation potential toward both ectodermal and mesodermal lineage while cells infiltrating the control group were able to differentiate into mesodermal lineage only. This could point to recruitment of stem cells via chemotaxis or local cell dedifferentiation. This would demonstrate that ECM degradation products hold stem cell chemotactic potential (105) and this regenerative potential of SIS was also reported in a canine model of tendon Achilles resection (468). These results all in support of cormatrix utilisation for the patch design.

8.2. Conclusions and future perspectives

In this study we did attempt to design a patch for corrective cardiac surgery with better chances for uptake, vascularisation and growth. The hypothesis of the project was to seed cormatrix with three cell phenotypes wjMSCs, HUVECs and HCFs. In theory, this triad of cells on a biologic scaffold would offer the necessary pillars for tissue regeneration and to the best of our knowledge this approach was not done by any laboratory before.

Due to the fact that theories should be based on facts as well as primary assumptions. Also, theories are not proven true but they can be proven wrong. So, we did establish a paradigm for proper assessment of this hypothesis prior to in-vivo experimentation. In addition, we wanted to expand our previous experience with mono-cellular seed of cormatrix to multi-cellular seed and also to fill the gap in our knowledge about the cells behaviour and rapid disappearance from the scene shortly after transplantation.

The study demonstrates feasible co-culture and co-seed of the three cell phenotypes wjMSCs, HUVECs and HCFs. Their mutual interaction showed some favourable results particularly of the wjMSCs while mostly neutral at HCFs area and to some extent negatively impacting HUVECs.

HUVECs apoptosis rate was quite high which anticipates fast wash out of HUVECs post implantation. However, treatment with CHIR99021 5 uM did significantly reduce rate of apoptosis of wjMSCs as well as HUVECs which is expected to offer better sustainability of these cells in-vivo.

Cormatrix is biocompatible with the co-seed of this triad of cells with good adherence to its surface, spread with no clumping as well as viability of cells with maintained mechanical integrity of the patch.

The future directions of this study is to go for the in-vivo assessment in large animal model. Indeed, this was the original plan but was not possible at this phase.

8.3. Limitations of the study

The study was limited with some factors including the lack of biologic replicates at the HUVECs and HCFs levels, missing to interrogate some factors such as Bak, Bax and p53 to complement our apoptosis/anti-apoptosis profile for better understanding of the survival results. Also, I was keen to make more qPCR experiments and IHC but all these targets were not feasible by any means due to the restrictions imposed by the pandemic.

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