Establishment of endothelialized cardiac tissue using human induced pluripotent stem cells generated cardiomyocytes

Etablierung eines endothelalisierten kardialen Gewebes mittels Kardiomyozyten, differenziert aus induzierten pluripotenten Stammzellen

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Abstract:

Cardiovascular diseases are considered the leading cause of death worldwide according to the World Health Organization. Heart failure is the last stage of most of these diseases, where loss of myocardium leads to architectural and functional decline.

The definitive treatment option for patients with CVDs is organ or tissue transplantation, which relies on donor availability. Therefore, generating an autologous bioengineered myocardium or heart could overcome this limitation. In addition, generating cardiac patches will provide ventricular wall support and enable reparative stem cells delivery to damaged areas. Although many hurdles still exist, a good number of researches have attempted to create an engineered cardiac tissue which can induce endogenous cardiac repair by replacing damaged myocardium.

The present study provided cardiac patches in two models, one by a detergent coronary perfusion decellularization protocol that was optimized, and the other that resulted in a 3D cell-free extracellular matrix with intact architecture and preserved s-glycosaminoglycan and vasculature conduits. Perfusion with 1% Sodium dodecyle sulfate (SDS) under constant pressure resulted in cell-free porcine scaffold within two and cell-free rat scaffold in 7 days, whereas scaffold perfused with 4% sodium deoxycholate (SDO) was not able to remove cells completely. Re-reendothelialization of tissue vasculature was obtained by injecting human microvascular endothelial cell and human fibroblast in 2:1 ratio in a dynamic culture. One-week later, CD31 positive cells and endothelium markers were observed, indicating new blood lining. Moreover, functionality test of re-endothelialized tissue revealed improvement in clotting seen in decellularized tissues. When the tissue was ready to be repopulated, porcine induced pluripotent stem cells (PiPSc) were generated by transfected reprogramming of porcine skin fibroblast and then differentiated to cardiac cells following a robust protocol, for an autologous cardiac tissue model. However, due to the limitation in the PiPSc cell number, alternatively, human induced pluripotent stem cells generated cardiac cells were used.

For reseeding a coculture of human iPSc generated cardiac cells, human mesenchymal stem cells and human fibroblast in 2:1:1 ratio respectively were used in a dynamic culture for 6-8 weeks. Contractions at different areas of the tissue were recorded at an average beating rate of 67 beats/min. In addition, positive cardiac markers (Troponin T), Fibroblast (vemintin), and mesenchymal stem cells (CD90) were detected. Not only

that, but by week 3, MSC started differentiating to cardiac cells progressively until few CD90 positive cells were very few by week 6 with increasing troponin t positive cells in parallel. Electrophysiological and drug studies were difficult to obtain due to tissue thickness and limited assessment sources. However, the same construct was established using small intestine submucosa (SISer) scaffold, which recorded a spontaneous beating rate between 0.88 and 1.2 Hz, a conduction velocity of 23.9 ± 0.74 cm s−1, and a maximal contraction force of 0.453 ± 0.015 mN. Moreover, electrophysiological studies demonstrated a drug-dependent response on beating rate; a higher adrenalin frequency was revealed in comparison to the untreated tissue and isoproterenol administration, whereas a decrease in beating rate was observed with propranolol and untreated tissue.

The present study demonstrated the establishment of vascularized cardiac tissue, which can be used for human clinical application.

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Abbreviations:

2D	Two-dimension
3D	Three-dimension
AFP	Alpha-fetoprotein.
AV	Atrioventricular
bFGF	Basic Fibroblast growth factor.
BMP	Bone morphogenetic proteins
c-Myc	bHLH transcription factor
CBM	Cardiac basal media.
CD31	Cluster of differentiation 31
CD34	Cluster of differentiation 34
CD90	Cluster of differentiation 90
CDM	Cardiac differentiation media.
cDNA	Complementary deoxyribonucleic acid
CHIR99021	6-[[2-[[4-(2,4-Dichlorophenyl)-5-(5-methyl-1H-imidazol-2-yl)-2-
CM	Cardiomyocytes.
cTNC	Cardiac Troponin C.
cTNT	Cardiac Troponin T.
DAB	3,3’- diaminobenzidine.
DAPI	4’,6-Diamidin-2-phenylindol, C16H15N5·2HCl.
DAPI	4’,6-Diamidin-2-phenylindol, C16H15N5·2HCl
DMEM	Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12
DMSO	Dimethyl sulfoxide.
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
EB	Embryoid bodies.
ECM	Extracellular cellular matrix
EDTA	Ethylenediaminetetraacetic acid
EG	Embryonic germ
ERK	Extracellular regulated kinase.
ES	Embryonic stem
FACS	Fluorescence-activated cell sorting.
FCS	Fetal calf serum.
FGF	Fibroblast growth factor
FITC	Fluorescein isothiocyanate.
GFP	Green fluorescence protein
GSK3	Glycogen synthase kinase 3
HEK	Human embryonic kidney cells.
hFib	Human fibroblast.
HMVEC	Human microvascular endothelial cells.
HRP	Horseradish peroxidase.
HSC	Hematopoietic stem cell

iPSc	Induced pluripotent stem cells
iPSc-CM	iPSc generated cardiomyocytes
ISL1	Insulin gene enhancer protein
JNK	c-Jun N-terminal kinase
Klf4	Kruppel like factor 4
LIF	Leukemia inhibitor factor.
Lin29	Lineage transcriptional factor
MCH	Histocompatibility complex
MEF	Mouse embryonic feeder
MEK	Mitogen activated kinase.
MMPs	Matrix metalloproteinases
mRNA	Messenger RNA
MSC	Mesenchymal stem cells
NADPH	Nicotinamide adenine dinucleotide phosphate.
NANOG	NANOG homeobox
Nkx2.5	NK2 homeobox 5
OCT	Octamer binding transcriptional factor
OSKM	OCT4, SOX2, KlF4, c-Myc
PBS	Phosphate balanced salt
PBST	Phosphate balanced salt tween
PCL	Poly-L-Milchsäure (poly L lactic acid)
PCR	Polymerase chain reaction
PE	Phycoerythrin
PFA	Paraformaldehyde
PGA	Polyglycolide
PiPSc	Porcine induced pluripotent stem cells
PKC	Protein kinase C
PLLA	Poly (L-lactide)
	pyrimidinyl]amino]ethyl]amino]-3-pyridinecarbonitrile
RNA	Ribonucleic acid
ROCK	Rho-associated, coiled-coil containing protein kinase
RPMI	Roswell Park Memorial Institute
RT-PCR	Real time PCR
SA	Sinoatrial
SDO	Sodium deoxycholate
SDS	Sodium dodecyle sulfate
SEM	Scanning electron microscope
sGAG	Sulfated glycosaminoglycan
SIS	Small intestine submucosa
SMA	Smooth muscle actin
SOP	Standard operation procedure
SOX	Sex determining region Y-box
SSEA	Stage specific embryonic antigen
TERM	Tissue engineering and regenerative medicine

TGF	Transforming growth factor
TRA-60	Keratan sulfate antigen 1-60
TUBB3	Tubulin beta-3 chain.
VEGF	Vascular endothelial growth factor
vWF	von Willebrand factor
WNT	Wingless/INT
XAV939	WNT inhibitor
Y27632	ROCK inhibitor

Chapter (1)

Introduction

1- Introduction

1.1: The human heart:

The heart is a complex muscular structure, responsible for pumping and delivering oxygenated blood and nutrients to different body parts through blood vessels by rhythmic beating and contractions. This involuntary muscle organ is composed of four champers and has its own electrical conducting system to create spontaneous contraction with a constant heart rate that can be manipulated by hormonal and nervous stimulus. There are two atria and two ventricles which represent the heart chambers: the right atrium, which receives deoxygenated blood from the body parts though superior and inferior vena cava, whereas the left atrium receives oxygenated blood from the lungs through the pulmonary vein. The right and the left ventricles reap blood from both atria in order to pump it out of the heart; the right ventricle pumps deoxygenated blood to the pulmonary circuit via the pulmonary artery and the left ventricle pumps blood out of the heart via the aorta. The heart has four valves, two which are located between each atrium and ventricles on both sides of the heart (Atrioventricular valves) to ensure blood flow in one direction from each atrium to the ventricle, and two additional valves (semilunar valves) which are located between each ventricle and the respective artery leaving the heart. During the relaxation phase or diastole, the right (AV) valve opens and allows blood to flow from right atrium to right ventricle, and when the heart contracts or enters the systolic phase, the atrium pushes blood to the ventricle until the pressure of the ventricle is raised, and once it exceeds the atrium pressure, the valve snaps shut (78,79). The heart lining layers are composed of different cell population: endocardium, the innermost layer, is populated with endothelial cells, whereas the myocardium has mostly contracting cardiac muscle cells, and epicardium, the outer layer is made of connective tissue and fat (78,80). The heart generates its spontaneous beating by two major biological pacemakers that are regulated by the autonomic nervous system and circulating adrenalin. Sinoatrial (SA) node, the primary pacemaker, is located in the right atrium and comprises a bundle of modified cardiac cells that discharge impulses at 70 times/minute, triggering cardiac contraction. Post receiving impulses from atrium, the Atrioventricular (AV) node conducts the electrical impulses from atrium to ventricle. Through bundle of His, these impulses are transferred to the apex at fascicular branches where they reach the Purkinjie fibers, which innervate the ventricles, causing them to contract in a paced interval (78,79, 80).

For such a complex and precise function many chemical, electrical, and mechanical signaling pathways are involved to achieve an efficient response. When stimulation occurs, an intracellular signal is generated, which is in turn interpreted differently in different cardiac cells. The heart is composed of several cell types, including: (i) conductive and neuronal cells, such as pacemaker cells, Purkinje cells, cardiac neuronal cells, Schwann cells, and satellite cells; (ii) Vasculature cells, such as endothelial cells, fibroblasts, smooth muscle cells, and pericytes; and (iii) Immune

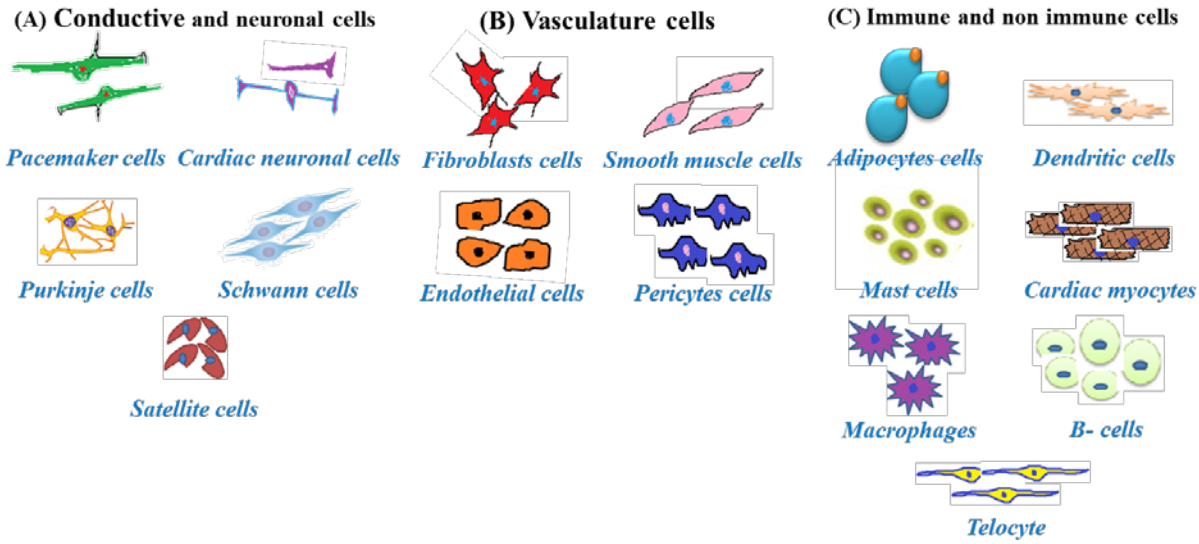

Figure 1: *Sketch of different types of cells present in human Heart*

and non-immune cells present in atria and ventricles, such as B cells, mast cells, macrophages, adipocytes, dendritic cells, telocytes, and cardiac myocytes (76, 80,81,82).

Cell proportion and distribution differ among species (83). For example, larger mammals would have a larger radius of curvature of left ventricle and extracellular matrix than small mammals, thus an increased wall tension is achieved. This explains the big proportion of Fibroblasts, as they are the main source of collagen; however, fibroblasts don't contribute the majority of cells in the heart. While cardiomyocytes account for 20-35% of heart cells, a lack of consensus regarding the percentage of endothelial cells and fibroblasts within the heart still exists Fig (1). Some studies calculated the endothelial cells to be three-times the number of cardiomyocytes among the heart (81,85), while others found the fibroblasts to be the majority of heart cells (86,87). Each cell in the heart possesses its function by releasing a specific protein and growth factor to interact with the surrounding cells or structure. Myofibroblasts, which

represent 27% of the heart, contribute to cardiac repair through the release of growth factors and extracellular matrix component (81,85). Endocardial and myocardial endothelial cells (three times the number of cardiomyocytes) work together with cardiomyocytes (20-35% of the heart) also and play a role in cardiac modeling. Vascular endothelial cells, on the other hand, control vasomotor tone and coronary blood supply without any close contact with cardiomyocytes. The rest of the vascular fibroblasts and smooth muscles work in auto- and paracrine fashion to control blood supply to the beating heart. Moreover, pericytes, found in arterioles, capillaries, and postcapillary venules are involved in angiogenesis, coagulation, inflammation, and immune response by making numerous tight junctions with other cells namely the telocytes (87,88). Cardiac adipocytes, which cover 80% of the heart, are also found within the epicedial fat and are responsible for heart's protection (89,90). Finally, the cardiac immune cells, including macrophages, mast cells, T cells, B cells, and dendritic cells are all maintained through in proliferation (91,92). The extracellular matrix, where the cardiac cells are lined, comprises fibrous protein needed for tensile strength and elasticity, and proteoglycans for enzymatic activity and hydration. The extracellular matrix is controlled by a number of matrix metalloproteinases (MMPs) and tissue metalloproteinases with controlled activation and inhibition manipulated by physiological changes, such as cardiac hypertrophy, dilation, and failure (93).

1.2 Tissue Engineering:

Although tissue and organ transplantation have improved remarkably in the last decades, but with limited biocompatibility and biofunctionality (94,95), many problems in transplantation, such as: (i) end stage organ failure, (ii) shortage of organ donation, and (iii) immune rejection still exist, eventhough immunosuppressive therapy has improved. A new paradigm has emerged alternatively to reconstruct tissues, termed "Tissue Engineering". The feature of this approach is to regenerate a patient's own tissues to overcome current hurdles in organ transplantation (94, 95). Earlier, in the 1980s, the surgical manipulation of tissue and the use of prosthetic devices were loosely termed as tissue engineering without focusing on the use of biologics and scaffold for new tissue generation. In 1991, a published article "Functional Organ Replacement: The New Technology of Tissue Engineering" came out to be the first of its kind,(94). Earlier, in the 1970s, W. T. Green, M.D, generated a new cartilage in nude mice using chondrocytes seeded onto spicules bone, and concluded that with biocompatible

material, one could generate new tissue when using an appropriately configured scaffold. Later, Burke and Yannas in the Massachusetts general hospital and M.I.T. generated tissue-engineered skin using a collagen matrix to support the growth of dermal fibroblasts. Possibly, tissue engineering field was born in the mid-1980s when Dr. Vacanti and Dr. Langer approached the idea to design appropriate scaffolding to deliver cells, instead of seeding cells onto available, naturally occurring scaffolds, which cannot be manipulated. To define the potential of this field, a number of centers have been established in the U.S. and Europe. Among the first significant centers were the Pittsburgh Tissue Engineering Center in the 1990s by Dr. Peter Johnson, the cardiovascular Tissue Engineering by Dr. Nerem at Georgia Tech, and Laboratories by Drs. Antonios Mikos and Larry McIntire at Rice University in Houston. In Europe, Dr. Julia Polak, a stem cell biologist in London, developed a British-based society associated with tissue engineering in Boston. In the late 1990s, Dr. Una Chen conducted tissue engineering and stem cells studies at Giessen, Germany, and Dr. Wolfgang P. Wulacher opened a laboratory for tissue engineering in Innsbruck at the Leopold Institute. In Asia, Dr. Ueda established a tissue-engineering laboratory in Japan and organized the first meeting on tissue engineering 1997 at Nagoya. By the late 1990s, tissue engineering centers were springing up in virtually every developed country (94). In 1998, a new term ‘Regenerative Medicine” appeared when Thomson and Shamblott established a human embryonic stem cell and an embryonic germ cell line, to which the researchers’ focus shifted.

The three keys in tissue engineering are: (i) cells, which synthesize the tissue, (ii) fabricated scaffolds to provide the appropriate environment for cell growth (Figure 2), (iii) growth factors facilitating cells to regenerate the new tissue, and (iv) provide sufficient oxygen level to ensure diffusion through dense tissue layers. Nevertheless, numerous studies have been carried out for different tissue regeneration, and there are still many critical issues that are needed to be modified, such as: cell source and culturing conditions, matrix production analysis, and functional and mechanical properties of the construct and animal models. However, still there is a possibility in the future to use the patient’s own cells, propagate, seed in a 3D scaffold, and used them for the same patient.

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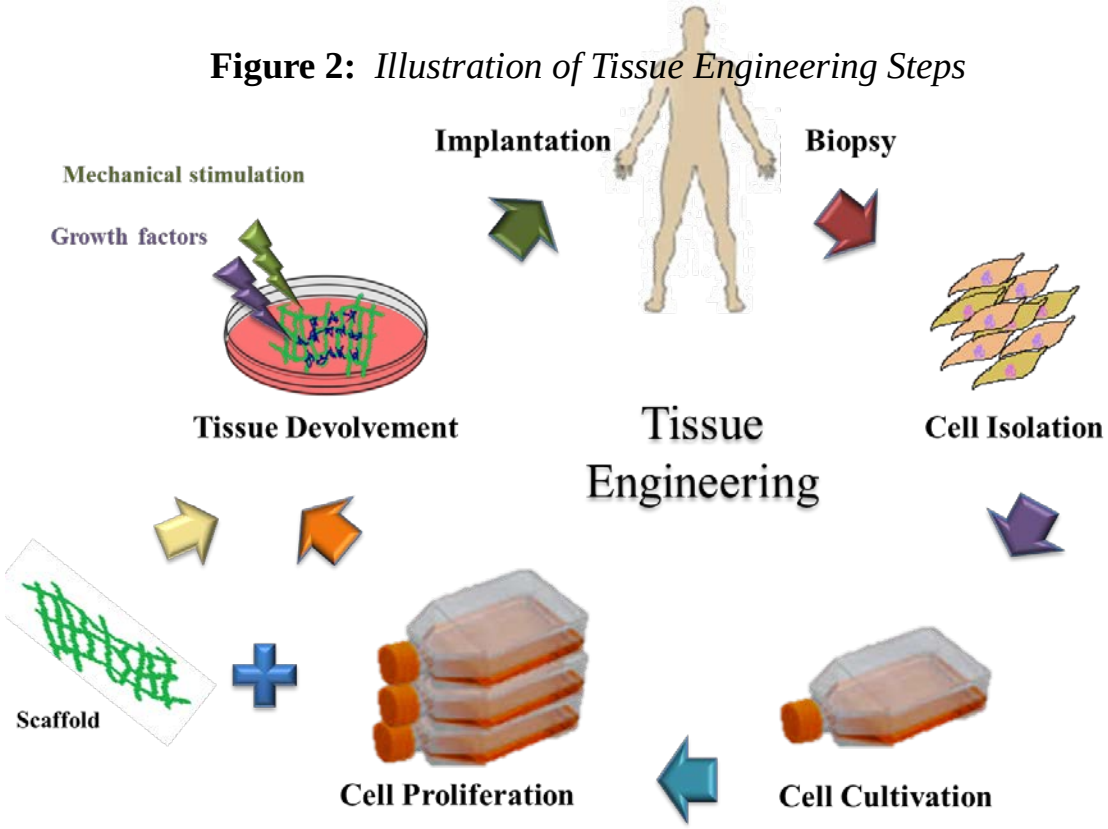
Figure 2: *Illustration of Tissue Engineering Steps*



1.2.1 Cells:

Cell sources are very important for the success of tissue regeneration. Depending on the donor, cells can be classified as: (i) autologous (patient's own), (ii) allogenic (human other than patient) and, (iii) xenogeneic (animal origin). The most successful transplant comprises autologous cells, since no immunosuppressant is needed post transplantation as in the other two sources (94). Difficulties that arise with allogeneic cells' use are the large numbers of cells needed, well-controlled harvesting conditions, and maintenance in animal serum free conditions to avoid viral infection. The extent of differentiation is another factor cells can be classified under: embryonic stem cells (ES) and embryonic germ cells (EG) both are able to differentiate into all kinds of cells; that is why these cells receive much attention in the field of tissue engineering. However, many problems are still to be solved before using them for patients or including them in clinical studies. Stem cells exist and can be differentiated into different lineage under suitable conditions; e.g.: mesenchymal stem cells (MSC), which can be differentiated into connective tissue cells. Hematopoietic stem cell (HSC), found in the bone marrow (98,99) or adipose tissue (102), provide macrophages, eosinophils, B-cells, T cells, and

erythrocytes. In addition, some tissues contain progenitor cells that can differentiate into organ-specific cell types.

The MSC is the most studied class of cells because of their capability to differentiate into various tissues, including adipose, blood cells, nerve, skin, bone, and cartilage. Therefore, it attracts the attention of tissue engineering researchers. The advantage when using these cells over ES is that ES tend to form teratoma when transplanted in animals before differentiation (96) in contrast to MSC that show no tumorigenesis and are safe to be used clinically. Moreover, MSC regenerate only the tissue specific to the site of transplantation and can go under trans differentiation if exposed to the right condition into any cell type (96, 97, 100).

The other type of cell which has a great potential in tissue engineering, is induced pluripotent stem cells (iPSc) that provide a unique platform from which to evaluate potential drug therapeutics and to gain mechanistic insight into a variety of diseases (101). Despite considerable difficulties, generating patient-specific iPSc through reprogramming has been a long-standing goal in tissue engineering. In 2009, researchers harnessed iPSc to establish familial dysautonomia disease and drug screening model. iPSc cells were generated from patients with the disorder and produced central and peripheral nervous system precursors (102), then found three disease phenotypes, thus validating the disease cell types that could reflect pathogenesis in vitro (102). Other group differentiated iPSc from individuals with long QT syndrome into cardiomyocytes in which it showed an increased susceptibility to catecholamine-induced tachycardia and response to B-adrenergic blockers (103).

Compared to embryonic stem cells (ESC), induced pluripotent stem cells (iPSc) can be obtained easily by fibroblast reprogramming through transfecting with transcriptional factors (Oct4, Sox2, Klf4, and c-Myc) from somatic cells without facing hurdles of ESC, such as ethical concerns. Moreover, iPSc can committ to differentiate to several lineages; therefore, it has a tremendous potential in tissue engineering (104). Applications of iPSc also go further to successfully repair primate cardiac infarction by allogeneic transplant of iPSc-CMs from histocompatibility complex (MCH) haplotype (HT4) homozygous Monkey into HT4 heterozygous monkey with myocardial infarction. Twelve weeks post transplantation, the grafted cells survived, improving cardiac contractility, indicating the feasibility of iPSc transplant for repairing clinical diseases (105). Recently, Zhong et al. successfully differentiated human iPSc and recreated internal retinal structure where retinal development was observed, forming

three dimensional-cups with mature photoreceptors (106). Furthermore, Cyranoski implanted retinal pigment epithelial cell sheet generated from autologous age-related macular degeneration (AMD) patient's iPSc into an eye and recovered the vision of the patient (107). Generally, iPSc brings an encouraging tool for cell therapies with careful evaluation of safety and effectiveness. The main safety issue that has been addressed frequently is the tumorigenesis due to the use of retrovirus or adenovirus producing the cells. However, following the new free exogenous DNA integration reprogramming protocol, this risk has decreased (108).

1.2.2: Scaffolds:

In contrast to cell therapy, tissue engineering allows the replacement of a defective organ with a 3D structure injected with appropriately needed cells. In reality, most organs have a 3D architecture, and for any transplanted cells to start the regenerating process, it needs a supportive structure called scaffold or artificial extracellular matrix. The major function of scaffolds resembles the natural extracellular matrix; mainly to assist proliferation, differentiation, and biosynthesis of cells. Scaffolds should meet a number of requirements to fulfill their regenerative function; first, it should have interconnected micropores, with optimal pore size of 100 and 500 um to facilitate cell seeding, proliferation, and migration. Furthermore, it should be supplied with adequate nutrients and should have an optimal mechanical strength. Second (95), scaffolds' absorption kinetics profoundly affect tissue engineering success rate. Depending on the regenerated tissue, scaffold degradation could be slow as in the case of skeletal system regeneration, or fast as in the case of skin regeneration (95). The degradation occurs through non-enzymatic hydrolysis, in contrast to naturally occurring biomaterials, such as as collagen, hyaluronic acid and chitin, which undergo enzymatic hydrolysis. Poly (a-hydroxyacid)s have been widely used in scaffold fabrication (109), as well as polyglycolide (PGA) and its polymer (PLGA) which rapidly degrade as their tensile strength decreases within two weeks. In contrast, poly (L-lactide) (PLLA) degrades slowly over a period of 3-6 years for resorption. In bone regeneration, inorganic as well as polymeric scaffolds have been used after being mixing with soft material, such as polymers, to yield a tough structure that is stable for implantation (110). Different methods have been used to fabricate porous scaffolds, such as freeze-dry and porogen leaching (111). In addition to recent sophisticated methods, including solid-free prototype and electrospinning, one of the most promising techniques in tissue

fabrication is electrospinning, as it shows advanced progress in producing material with a fixable fiber arrangement that is very close to biological tissue, thus there are less chances of rejection (112). Electrospinning material has been used in different tissue implantations, including skin, cartilage, bone, vascular, nerve, and cardiac tissue with the appropriate cells growing and proliferating. In cardiac graft fabrication (113), polycaprolactone (PCL) is electrospun onto a wire ring of 15-um diameter and then coated with type I collagen to promote cell attachment. Cells were cultured on scaffold, and three days later cardiomyocytes' contraction was observed with tight intracellular contact throughout the mesh.

Application of the cell-scaffold construct can be carried out either by placing the construct in a bioreactor to reconstruct an engineered tissue (in vitro) or by implanting the construct in the body (in vivo) until new tissue is regenerated. In vitro, tissue engineering has showed a great potential because of the production of multiple tissues from a single-cell. On the other hand, in vivo, tissue engineering application is limited to a single patient, who has his own personalized cells on implanted construct. Two points must be taken into consideration for clinical application in tissue engineering: first, standardizing the cell-scaffold construct, which will be difficult as it contain human cells; and second, neovascularization formation to ensure oxygen and nutrient delivery to the cell-scaffold construct and promote proliferation, and differentiation. In contrast, in vivo engineering will induce neovascularization of the construct, providing adequate supplement to the surrounding tissues (Figure 3).

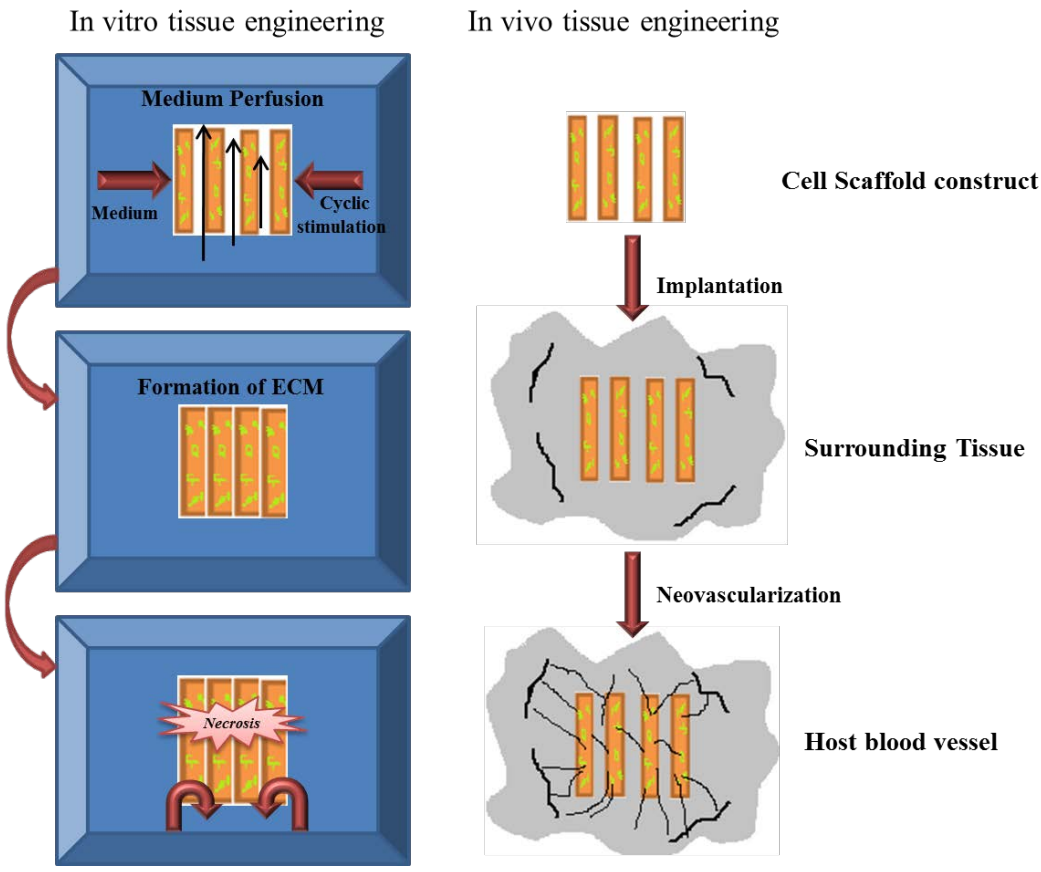


Figure 3: *Differences in biological reaction between in vivo and in vitro tissue engineering*

1.2.3 Growth factors: To proliferate and differentiate, any cell in human body requires proteins that are secreted by itself in the body (autocrine) or secreated by others (paracrine), these proteins are called growth factors. The same applies when tissue is implanted. Growth factors, such as as bone morphogenetic proteins (BMPs), basic fibroblast growth factor (bFGF or FGF-2), and vascular epithelial growth factor, and transforming growth factor-b (TGF-b) are used to enhance regeneration. In particular, BMP and bFGF alone can induce regeneration of vascular tissue and bone, respectively, without the assistance of scaffold or seeded cells. Apparently, by adding proper growth factor further promotion of tissue regeneration will take place. Commercially available carriers are acidic or basic; for example, acidic gelatin forms ionic complex with basic bFGF, in which it is released from the ionic complex formed when implanted in the body after enzymatic degradation of gelatin (114,115,116). Application of growth factors in tissue engineering has attracted the attention of researchers lately to induce

neovascularization through nutrient cell supplement; this will be enhanced once growth factors become less expensive and widely available.

Tissue engineering, like any new technology, should be examined for safety on animal model before clinical application, in skeletal system, Lamerights et al. (2000) established a bone model to study the effect of load on bone formation using tissue engineered construct by applying controlled pressure on material inserted in distal femur defect (117). In another example, in cardiac system, Shum-Tim et al. (1999) isolated a mixture of cell population of endothelial cells, smooth muscles, and fibroblasts from ovine arteries and then seeded them onto PGA–polyhydroxyalkanoate blended tubular scaffolds. Later, the construct was implanted in lamb aorta and survived for five months. In addition, collagen and DNA continents meet the native level (118). Further work on cardiac tissue engineering by Carrier et al. (2002a) was carried out when they cultured a fibrous resorbable scaffold construct of 9.5 mm diameter and 2 mm thickness, seeded it with neonatal cardiac myocytes, and then cultured it by direct perfusion, improved cells distribution and enhanced cardiac marker expression were observed (119, 120).

Bone marrow transplantation is an example of the most successful-evolved therapy for over than 20 years in a variety of malignant diseases. In 1994, an articular chondrocyte was used to reconstruct small defects in knee articular cartilage by Brittberg et al. (121). A few years later, in a trial study, another marked step was taken by Rosenthal (1998) when she injected fetal human mesencephalic cells into the striatum of a Parkinson's patient. Cells differentiate into dopaminergic neurons, making synaptic connections with host neurons and restoring striatopallidothalamic output pathway to normal (122). Decellularized submucosa of small intestine produced collagen-based matrices has been used in tissue regeneration and in some clinical applications (123,124). This was confirmed clinically in patients with hypospandiashypospadias, an anatomical abnormality in the urethral, when a decellularized submucosa was used for urethral repair by anastomosing the matrix to the urethral plate, and 4-7 years later, the patients showed successful outcomes (124). Another group in Hanover used the same decellularized porcine proximal jejunum seeded with a patient's autologous muscle cells and fibroblast to create an airway patch, which was successfully transplanted and caused improvement in patient condition (124). Clinical application of tissue engineering showed good success in myocardial muscles when Menashe (2002) transplanted satellite cells into damaged human hearts of 72 patients suffering from

severe congenital heart failure. Six months later, a dramatic improvement of patients' symptoms was observed (125). Besides the mentioned trials, others reported a significant progress in the following tissues: finger bone (126), jawbone (127), maxillary sinus augmentation (128), metacarpophalangeal joints (129), osteoarthritic knee cartilage (130), myocardial infarction (131), and peripheral nerve (132). Although huge progress has been made in tissue engineering clinical applications, without enough collaboration with clinical doctors among different fields, it would be difficult to meet the expectations of diseased organ patients.

1.3: Decellularization: Tissue and organ engineering had a recent innovation technique by which the whole organ was decellularized and resulted in cell, nucleic acid, and antigenic epitope removal, leaving 3D scaffold ECM with all its vascular content preserved(133). The technique depends on antegrade or retrograde perfusion through vasculature network using detergent and/or another chemical for complete cell removal. The resultant scaffold will have the natural biological properties of the ECM that will support cell proliferation, differentiation, and the architectural properties of the organ (133,134,135).

1.3.1: Extracellular matrix:

The ECM is composed of structural and functional molecules, which are arranged in a tissue-specific 3D ultrastructure that suits the same harvested organ. Not only that, the structure's compassion changes with the tissue's mechanical demands and resident cell metabolic activity. The advantage of biological ECM scaffolds over synthetic materials is due to the dynamic reciprocity between cell population and ECM (140), by it exerting a gene expression influence via transmembrane proteins and cytoskeletal components. Generally, ECM from any organ is composed primarily of type I collagen, glycosaminoglycan, laminin, fibronectin, and a variety of growth factors. ECM composing biological scaffolds is essential to facilitate tissue remodeling in animal and human studies. These scaffolds can be harvested from a variety of tissues including small intestinal submucosa (136), urinary bladder (138), blood vessels, skin (137), and heart valves (139). There is no evidence or proof yet whether ECM-specific composition or 3D ultrastructure is more important with regard to cell growth or/and differentiation. However, recent studies demonstrate the importance of ECM ligand landscape for cells by supporting their attachment and differentiation through what is

called zip code (133,141,142). Moreover, it was shown that the ECM basal membrane complex plays an important role in regulating cell growth, differentiation, and migration during reconstruction and development of tissues as it contains laminin, collagens IV, and VII, nidogens, perlecan, agrin, and other macromolecules that help in direct the growth of selected cell population in tissue engineering (143). In addition to the biological ECM scaffolds' structural functionality, biological signaling activities result from ECM degradation, such as soluble molecules, which are found to have a marked effect on the host remodeling post implantation (133). This includes all processes such as cell proliferation, migration, and processes like angiogenesis. In conclusion, ECM of biological scaffold composition and ultrastructure characteristic are highly complex. They can provide important micro environmental cues to support cell growth, proliferation, and differentiation if prepared properly. 3D scaffolds composed of decellularized organ-specific ECM have been established in heart (146), liver (144), and lungs (145) to date.

1.3.2: Decellularization methods:

Although allogeneic and xenogeneic (natural anti-galactose alpha1,3 galactose) antigens are usually recognized by a recipient as a foreign body which may elicit an immune rejection, however, anti-galactose alpha1,3 galactose antibodies, expressed in response to α-gal antigens on ECM, are widely conserved among species. In fact, lower species are well-tolerated by xenogeneic recipients as they do not express the α-gal antibodies due to the α1,3 galactosyltransferase gene, which catalyzes the attachment of α-gal to proteins and lipids (147). For example, galactosyl Galα galactose moiety that is known to be associated with hyperacute rejection of xenogeneic whole-organ transplant, fail to bind to IgM antibody or activate complement because of their scattered distribution and in a small amount of antigen on ECM (148). Moreover, studies have shown that the effect of xenogeneic ECM response would specifically elicit M2 macrophages, which in turn support a scaffold-constructive remodeling (149). In order to prepare a three-dimensional whole organ scaffold from a mammalian organ, different processes for cell removal from an organ, while retaining the native structure of ECM are required. This involves the exposure of the organ parenchymal cells to a chemical, a detergent, or enzymes by perfusion through the vasculature. A recent study cleared out objective criteria for decellularization efficacy in three points: (i) negative staining with hematoxylin, eosin and DAPI to confirm the absence of nuclei, (ii)

quantitative DNA measurement at less than 50 ng/mg dry tissue weight, and (iii) DNA fragment size < 200bp. Therefore, the ultimate aim of decellularization is to remove all cellular material without adversely affecting mechanical integrity of the remaining 3D matrix, while avoiding excusive or massive antigenic epitopes associated with cell membrane removal. Decellularization processes can be performed by using chemical/or enzymatic agents, as well as physical stimulation such as sonication, freezing and thawing with agitation to disrupt cell membrane and facilitate the rinsing of cell remnants from ECM. These steps should be performed in the beginning of decellularization protocol to enhance transportation of cellular residue out of the tissue in future. Agitation and perfusion help to facilitate decellularization solution transportation to the tissue and clear the cellular debris out of the tissue. It's difficult to completely decellularize a tissue or an organ, and most decellularized ECM scaffold retains residual DNA and other cytoplasmic material (150). Different approaches can be combined to maximize the efficacy of decellularization for each tissue or organ individually with the consideration of minimizing the use of detergents and chaotropic agents, such as Triton X, sodium dodecyl sulfate, and deoxycholate to avoid ultrastructural and compositional damage to the native ECM (151). The choice of the technique to use depends on tissue characteristic; agitation is commonly used for tissues with difficult approachable vasculature tissues, such as trachea and small intestine submucosa (SIS), whereas tissues with clear approachable vasculature, such as heart and lung perfusion would be optimum to use.

Most decellularization protocols include more than one chemical used alternatively in a cycle to increase the efficacy of each and minimize its time of exposure. Detergent is one chemical used in decellularization in order to rupture cell membrane. It can be classified according to its ionic status: non-ionic detergent, such as Triton-X 100 is the most desirable, while ionic detergent, such as SDS is the harsher one to use. Nevertheless, this classification is still controversial among researchers. Ultimately, there is no single detergent that would decellularize all tissues with the same outcomes and effects. Acidic and alkaline solutions, such as peracetic acid, are sometimes employed in low concentrations to decellularize more complex tissues, for instance; however, it was shown in some cases in complete decellularization and, in others, a provoking effect of ECM desirable immune response (150). Enzymes are a third class of chemicals used in decellularization, as they disrupt interactions between ECM and cells. Trypsin is commonly used but with minimal exposure time to avoid collagen

destruction and the loss of mechanical strength of ECM. Nucleases, in particular DNase, are used in decellularization in order to break nucleic acid sequence within tissue and facilitate their removal. Regardless of the chemical used in decellularization, effective removal of residues within tissue after treatment is essential. If any persist during the rinsing step, the chemical will remain in the tissue and affect new cells' attachment and migration. Therefore, efficient assays to detect that before recellularization steps must be carried out (135).

1.4: Recellularization Methods:

Whole-organ scaffold recellularization methods are integrated from a wide range of procedures, including tissue culture, cell seeding, and cell-transplantation therapies. Recellularization can be achieved in two steps: cell seeding, followed by culture perfusion to ensure cell function in vivo. The first challenging step in reseeding a decellularized scaffold is cell type and cell number, and then the placement of cells in scaffold to match native tissue distribution as much as possible.

Endothelial cells are important when reseeded on any decellularized tissue to ensure adequate blood flow in vivo for cell survival (151,152). Many promising attempts have been made showing endothelial cell attachment (153), yet long-term endothelialization needs to be improved (154). However, some studies showed that natural re-endothelialization could occur after in vivo transplantation of decellularized tissues, such as in skin (155). Another non-parenchymal cell type that contributes an important role in tissue engineering is fibroblast, which supports and remodels ECM and improves parenchymal cell function in vivo. In addition, fibroblasts are proven to facilitate remodeling and cellular organization in vitro organ culture as well. Recent studies have demonstrated the involvement of fibroblasts in myocardium electrical properties by coupling with myocytes and contributing to electrical stimuli propagation (156). In general, co-culturing mixed cell type is limited to the use of fibroblast and endothelial cell along with specific non-parenchymal cell (156).

The number of seeded cells is an important parameter in recellularization and depends on the target tissue to be reseeded. For example, tissues with biomechanical demands, such as those of heart and lung require whole organ implantation; hence, a high percentage of original cell types and number need to be immediately available. In contrast, organs with metabolic functions, such as pancreas and liver, can be deployed with small percentage of cell mass. Taking that into consideration, the use of stem-

cell-based technique may be limited, especially with organs demanding a high number of cells, such as heart and lung.

There are two main techniques for seeding a scaffold: intramural injections or infusion of cells though vasculature. In a rat whole heart reseeding report, cardiomyocytes were injected intramurally in left ventricle wall, which yielded 50% cell seeding efficacy (15). Continuous infusion technique, on the other hand, reported 95% seeding of hepatocytes which were infused into the portal vasculature, followed by continuous perfusion (144). Still, as a whole organ, vascularization is a new approach to tackle and optimize in order to obtain maximum seeding efficacy.

Cellular component of the engineered whole organ is quite large in mass and size, although not as dense as native, yet it still needs adequate delivery of nutrients to the cells inside the scaffold, and removal of waste products (157). Indeed, this issue has been a bottleneck in the development of vascularized tissue in vitro and survival of none in vivo. The need for such supplement of nutrients renders perfusion the method of choice for in vitro culture of vascularized organ; this can be achieved by the use of bioreactors, suitable perfusion medium, oxygen carrier, and appropriate biophysical stimulation (158). Normothermic perfusion by machine has been used for a decade as an isolated organ model and for preservation before cold storage solutions, which is currently receiving a renewed interest. The existing systems are providing availability of all components at mouse until human scale, yet highly vascularized organ preservation is still limited in duration.

Temperature is an important parameter in 3D ECM organ scaffold recellularization. The optimum temperature would be the physiological 37°C; for instance, this temperature was tested by a group of researchers for kidney perfusion to ensure cell attachment and self-assembly (159). The results were encouraging, as the warm perfusion restored functions in severe ischemically damaged kidneys. Common methods, such as positive-displacement and centrifugal pumps are used for organ-perfusion system, and in some cases a ventilator is needed in a lung recellularization system (145). Perfusion flow for 3D ECM culture is mostly at constant mode, unless pulsatile perfusion flow is needed, with careful consideration of the flow rate and sheer stress. One of the hurdles in tissue engineering is how to provide a highly vascularized and actively metabolic organ with sufficient oxygen level to ensure diffusion through dense tissue layers. Therefore, gassing the perfusion medium with oxygen/carbon

dioxide mixture in a membrane oxygenator, which probably would be a part of the bioreactor, is essential in tissue engineering.

1.5: Induced pluripotent stem cells:

1.5.1: Reprogramming of Fibroblast into induced pluripotent stem cell:

Reprogramming entails the expression of the core of pluripotency-transcription factors: OCT4, SOX2, KLF4, and c-MYC (OSKM) in a somatic cell. The resultant compact colonies resemble ESCs morphologically, phenotypically, and molecularly (160,161). Induced pluripotent stem cells are used in a wide range of diseases, and drug and cell therapies. In order to serve these different proposes, a variety of reprograming protocols have to be established using different donor species (162), with variable number, types, and carriers of transcriptional factors (163).

1.5.1.1 Cell Donor: Reprogramming efficacy and kinetics differ depending on cell donor type. For example; it takes a shorter time for mouse embryonic fibroblast reprogramming compared to human fibroblasts, which are used more in reprogramming. The efficacy, as well as the variety among cell types compared to fibroblast and keratinocytes reprogramming with OSKM is twofold and 100 times more efficient. The endogenous levels of certain reprogramming factors also attribute the increase or decrease in reprogramming efficacy, in which it obviates their expression state during reprogramming (164). Cell differentiation state when starting reprogramming matters in resultant colonies number; for instance, hemopoietic stem cells and progenitor cells yield 300 times more iPSc colonies than B and T cells do (164).

1.5.1.2 Reprogramming Cocktail: One important factor that influences the efficacy of reprogrammed cells is the reprogramming cocktail. Many of the factors used in reprogramming are genes involved in early development that maintain pluripotency potential for cells of inner mass (ICM). This is true for OCT4, NANOG, and SOX2. For instance, when NANOG are co-expressed with YAMANAKA (OSKM) cocktail in mouse B cells, the time for colony formation is reduced compared with that by OSKM alone (165). NANOG ectopic expression enables the establishment of embryonic like cascade that is stabilized by endogenous core pluripotency reactivation. Telomerase reverse transcriptase and SV40 T antigen enhance proliferation by increasing ESC-like colonies' appearance once combined with OSKM (166). MicroRNAs also increase the reprogramming efficacy tenfold when combined with OSK compared with OSK alone

(167) and when introduced into OCT4-GFP reporter MEFs. Both reprogramming speed and efficacy are enhanced by barriers to inhibition, such as apoptosis and senescence, through p53 blocking (168). Compounds such as 5-azacytidin alter DNA methylation and chromatin modification through inhibition of methyltransferase, thus improving MEFs reprogramming (169).

1.5.1.3 Culture condition: one needs to consider the culture condition of reprogrammed cells, such as media composition, supporting cells co-cultured and factors that could modulate reprogramming efficacy. Studies have shown that reprogramming under hypoxic condition has a positive effect on reprogramming efficacy by 40 folds (172). Marson et al. (170) demonstrate the promoting effect on iPSc generation when adding conditioned media containing WNT3a in MYC absence. Moreover, Okada et al. (171) found that iPSc can be obtained earlier if cultured media was serum free (KK20).

1.5.1.4: Integrative delivery system: It is a viral delivery system of reprogramming transcriptional factors (OSKM) into a human or mouse fibroblast, allowing gene delivery into the genome of dividing cells. The genome-integrating viral vector transgenes are integrated into the host genome together with viral vector backbone at multiple sites. The exogenous transgenes' expression is then silenced when reprogramming is achieved.

1.5.1.4.1: Retrovirus delivery system***:*** Mouse and human fibroblast transfection was originally achieved by delivering transcription gene (OSKM) usingthe Moloney murine leukemia virus (MMLV) retroviruses (173). These victors deliver the genes into dividing cells genome with cloning capacity of around 8kb and are usually silenced in ESC immature cells (174, 175). Reprogramming state of iPSc is related to their retrovirus expression; Class I iPSc are partially reprogrammed and are permissive for retroviral expression (mouse Fbx15-iPS cells), whereas class II iPSc are fully reprogramed and repressive for retroviral expression (mouse Nanog-iPS cells) (197).

1.5.1.4.2: Lentivirus delivery system: This type of delivery virus has also been used successfully, expressing reprogramming factors in somatic cells (176). In contrast to retrovirus, they infect both dividing and non-dividing cells genome with cloning capacity of 8-10kb, with a higher efficacy (177). Although viruses used in reprogramming are reproducible and efficient, it entails harmful viral production that expresses potent oncogenes (178). Virus silencing can result by deacylation of histone

(H3), DNA methylation, and chromatin rearrangement of linker H1 (177). Even if perfectly silenced, transgenes can be reactivated during iPSc differentiation or animal transplantation, leading to tumor which results from basal MYC transgenes' expression. Although using Cre-detectable or inducible lentiviruses solves this problem (179), yet viral system still lacks therapeutic application safety.

1.5.1.4.3: Transfection of linear DNA: This is an alternative method to avoid the use of viral vectors, based on liposomes or electroporation to transfect DNA, however, with much lower efficiency. A designed polycistronic vector, which allows several cDNA expressions from the same promoter, meets this approach (181). One group was able to reprogram mouse embryonic fibroblasts (MEF) successfully by using a linearized 2A peptide polycistronic vector flanked by loxP sites. In order to delete the reprogramming construct, they induced recombination between the loxP sites by transiently expressing the Cre recombinase. Compared to viral transfection, this method has the advantage of its ability to delete reprogrammed cDNAs in iPSc, which would improve differentiation and avoid reactivation of reprogramming factors, thus reducing oncogenic potential. Some researchers try to include the polycistronic vectors in integrative virus vectors by including loxP sites which in turn induce transgene-free iPSc from different donors with high transduction efficiencies (180).

1.5.1.5: Non-integrative approaches: To overcome hurdles facing clinical application of iPSCs, a non-integrative approach was established. This system works by the deletion of Cre-deletable viral vectors, naked DNA transgenes, or non-conservative transposon remobilization from the integration of classic retrovirus vector and its permanent genetic modification. .

1.5.1.5.1: Integration-defective viral delivery: It is one of the first attempts reported in the use of replication-defective adenoviral vector, pHIHG-Ad2 (182). Cloning of OSKM set as a single factor into pHIHG-Ad2 resulted in transgene free iPSc. However, the efficiency of this system is three times lower than the retrovirus-based system. Terminally differentiated T cells and human fibroblasts were successfully reprogrammed using F-deficient Sendai viral vectors. Fusaki et al. (183) generated iPSc line using Sendai viral vectors expressing OSKM; the resultant colonies were viral RNA free.

1.5.1.5.2: Transient episomal delivery: This strategy depends on the direct delivery of replicated or non-replicated episomal vectors. Through serial transfection of two plasmids expressing OSK and MYC, respectively. By using this method, iPSCs that

were generated from MEFs showed no sign of plasmid integration, but a low percentage of cells (33.91%) (184).

1.5.1.5.3: RNA delivery: If a complete elimination of plasmid or viral vectors is desired, this is the method to choose. One study (185) established a system that allows the efficient conversion of human somatic cells into iPSCs using synthetic mRNA direct delivery in just 17 days. Once delivered, synthetic RNAs encoding OSKM and Lin29 reprogramming will be achieved. This system is extremely appealing; however, the high reprogramming factors, such as gene dosages from direct mRNA delivery may contribute an oncogenic risk owing to high Myc expression that affects genomic stability.

1.5.1.5.4: Protein delivery: When proteins directly delivered into cells in vivo or in vitro, after it fused with peptides mediating their transduction; for instant HIV transactivator of transcription (Tat) and poly- arginine. Proteins then expressed in Escherichia coli in inclusion bodies, and were then solubilized, refolded and further purified, by serial transduction of Oct4–GFP reporter MEFs with OSKM or OSK recombinant proteins (186). Kim et al. (187) fused OSKM factors individually with Myc tag and a nine-arginine tract. This resulted in the generation of stable HEK293 cell lines expressing the four-reprogramming factors, which were used on human neonatal fibroblasts. iPSc colonies were generated after dissociation and MEF feeder cell re-plating.

1.5.2: iPSc cardiac differentiation:

1.5.2: iPSc cardiac differentiation:

Cardiac progenitor cells are localized in the mid-streak during embryonic cardiac development. Adjacent cells send signals and promote cardiac mesoderm and endoderm development, which have a major role in cardiogenesis. Three important protein growth factor families are involved in controlling the early stage of mesoderm formation and cardiogenesis: bone morphogenetic proteins (BMPs), member of transforming growth factor β superfamily, fibroblast growth factors (FGFs), and Wingless/INT proteins (WNTs). BMP signaling promotes cardiogenesis, while WNTs are involved in cardiac specification through their complex role. Depending on the spatiotemporal context pathway they act on, WNTs could inhibit (canonical signaling pathway acting via β-catenin/GSK3) or promote (non-canonical pathway acting via PKC/JNK) cardiogenesis (188). Later, combinations of transcriptional factors activate

mesodermal precursor cells to express T-box factor Brachyury (T) and the homeodomain protein, Mixl1. In turn, these cells transiently activate the basic helix-loop-helix transcription factor mesoderm posterior 1 (Mesp1) to enter a precardiac mesoderm stage of development. Consequently, Mesp1+ cell population begins expressing early cardiac lineage markers, such as homeodomain transcription factor Nkx2-5, the T-box protein Tbx5 and Isl1, an LIM homeodomain transcription factor (189). Thus, a complex interaction between this highly conserved gene network manipulates the initial cardiac differentiation and maturation (Fig. 4). To induce cardiogenesis, most of cardiac differentiation protocols are established in serum free media (190,192).

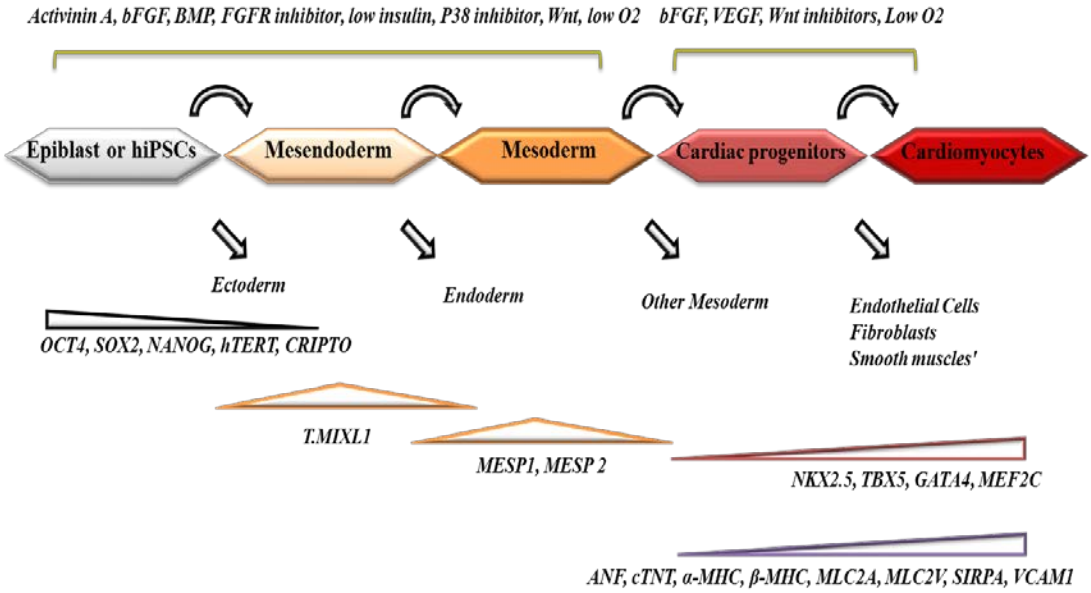


Figure 4*: Cardiomyocytes' differentiation pathway, temporal regulation of molecules associated with each step that allow understanding factors involved in cardiogenesis and designing the proper differentiation approach*

A study demonstrated that when coculture ESC was in serum-free media, it exhibited a 20% increase in beating cells comparing to serum-containing media. Moreover, this percentage increased to 40% when ascorbic acid was added to the culture media, and concomitantly, the cardiac marker IsL1 expression increased as well at mRNA and protein levels (191). Growth factors that are implicated in normal cardiac development, including BMP4, Wnt agonists and antagonists, Activin A, FGF2, and VEGE induce cardiogenesis thorough their activation or inhibition (figure. 4). When cells are differentiated in BMP4 alone, their fate is biased to posterior mesodermal derivatives,

such as primitive hematopoietic precursors, while, when differentiated in the presence of high concentration of Activinin their fate moves towards endodermal differentiation (191). Furthermore, growth factors like Wnt need to be activated at early stages of differentiation and inhibited in later ones, with consideration of concentration and time of addition. Therefore, it's very important to titrate growth factors' concentration and time of use to optimize the yield of generated cardiac cell. Moreover, line-to-line reprogramming method variability used and iPSc quality could result in different gene expression among treated cell lines with the same differentiation protocol (193); Katman et al. demonstrated different requirements of Activinin A and BMP4 concentrations for efficient cardiomyocyte generation among pluripotent cell lines (194). Since Wnt pathway is one of the major pathways in cardiogenesis, it was found that small molecule WNT inhibitors increase embryoid body (EB) cardiac generation in a monolayer culture (195). Moreover, Cao et al. found that adding ascorbic acid increases cardiac differentiation by its effect on MEK/ERK pathway (196). Therefore, a combination treatment of BMP4 and CHIR99021 was used by Kadari et al. (2015) followed by enrichment using Lactic acid addition, which enhanced the generation of cardiomyocytes to 90%. With all the above different approaches to generate cardiomyocytes, an efficient cardiac protocol should always be tailored and optimized for each individual cell line.

1.6: Cardiac Patch:

Limitations in cardiac cell therapy due to poor engraftment and significant cell death encouraged researchers to shift to ex vivo tissue engineering as a tool for enhancing cell retention and survival. An approach of combining procedures aiming at both extracellular matrix and cellular regeneration has been addressed. Thus, hybrid therapies, including biomaterials and cells developed a promising solution for repairing myocardial tissue. R. Gaetani et al. used alginate tissue printing technology with cardiomyocytes progenitor cells to create a cardiac patch, with a précise microstructure that allows better cell viability for a long time (197). However, the reduced size of the scaffold limited its application. A group in Hanover created one possible solution by which they developed a therapeutic relevant 3D structure of a combination of gel-based cardiac construct with porcine decellularized small intestinal submucosa (198). However, generating a proper supply with essential nutrients and oxygen into cell

aggregates was limited to only a small area. On the other hand, a study highlighted the advantage of using SIS scaffold seeded with MSC to repair infarction in a rabbit model. They detected improvement in left ventricular function, increase in vascular density, induction of MSC differentiation, and attenuation of pathological changes. Yet, the study was for a short period of 4 weeks, which is not enough to confirm functionality for clinical application (199). Schurlein et al. (2017) used the same concept of SIS in cardiac patch development but with advanced improvements; they re-vascularized the SIS scaffold with endothelial cells prior to the introduction of iPSc generated cardiac cell, fibroblasts, and endothelial cells. Two weeks later, positive cardiac markers and physiological function were observed. Moreover, beating rate and response to drugs and electrical stimulation were also detected. Since the culture was in a physiologically conditioned bioreactor, long-term cultures of up to four months were facilitated.

Because native human heart extracellular matrix provides the best physiological micro and macro architecture for bioartificial myocardium, researchers started developing strategies to decellularized native heart from rat, porcine, and finally humans through vascular perfusion (12,14,15,16) in order to serve as a foundational scaffold for myocardium engineering. Guyette et al. and his group were able to generate human myocardial tissue based on decellularized ECM from humans and human iPSc-derived cardiac myocytes in an attempt to bring this technology closer to future clinical application (14). However, no matter how elegant and functional this construct was, the problem with neovascularization remains to be solved. Undoubtedly, many obstacles, such as different cell types were also needed to be addressed in order to repopulate the whole structure, including the valvular apparatus to regenerate the whole heart.

Chapter (2)

Materials

2. Materials

2.1 Equipment:

Table 2.1: *List of equipment and devices:*

Equipment	Manufacturer
Aspiration pump	INTEGRA Biosciences GmbH, Biebertal, Germany
Autoclave	Systec GmbH, Linden, Germany
Balance	Kern & Sohn, Balingen, Germany
Bioreactors and incubator board	Chair of Tissue Engineering & Regenerative Medicine, Wurzburg, Germany.
Cell freezing Chambers	VWR, Darmstadt, Germany
Cell Incubator (37C, 5% CO2)	Heracus, Hanau, Germany
Centrifuges: Centrifuge 5417R Multifuge X12	 Eppendorf, Hamburg. Germany Thermo Fisher Scientific, Dreiech, Germany
Cold Storage Room (4C)	Genheimer, Hochberg, Germany
Digital Camera	Canon, Krefold. Germany
Embedding Cassette printer VSP-5001	Vogel, Medizintechnik, Giessen, Germany
Freezers: -20C -80C	 Liebherr-International Deutschland GmbH, Biberach an der Riß, Germany Thermo Fisher Scientific, Langenselbold, Germany
Fume hood	Prutscher Laboratory system, Germany
Ice Machine AF-80	Scotsman, Gelato, Milano, “Italy”
Laminar flow cabinet Safe 2020	 Thermo Fisher Scientific, Dreieich, Germany
Liquid Nitrogen Storage Tank MVE 815 P190	German-Cryo, Juchen, Germany
Magnetic stirrer with hot plate	VWR, Darmstadt, Germany
Microscopes: Bright field (Axio Lab. A1) Fluorescence (BZ-9000)	 Carl Zeiss Microscopy. GmbH. Göttingen. Germany Keyence, Neu-Isenburg. Germany
Multichannel pipette plus	Eppendorf, Hamburg. Germany
Neubauer cell counting chamber (hemocytometer)	Marienfeild GmbH & Co. KG, Lauda-Konigshofen. Germany
Objective slides printer VSP- 5001	Vogel, Medizintechnik, Giessen. Germany

Equipment	Manufacturer
PH- Meter	Mettler, Toledo, Geissen. Germany
Pipettes: 0.5-10µL, 10-100µL, 100-1000µL	Eppendorf, Hamburg. Germany
Pipetting aid (Accu-jet - pro)	Brand, Wertheim. Germany
Pressure sensor: Reusable transducers	HJK Sensoren + System GmbH & Co KG, Merching. Germany
Pump Tubing Cassette	Ismatec, Wertheim- Mondfeld. Germany
Refrigerator (Midiline)	Liebherr, Biberach a.d Riss. Germany
Rocking platform shaker	VWR, Darmstadt. Germany
Rotating Mixer	NeoLab, Heidelberg. Germany
Sliding Microtome (Leica SM 2010 R)	Leica, Wetzler. Germany
Spin Tissue Processor (Microm STP 120)	Thermo Fisher Scientific, Dreieich. Germany
Steamer (MultiGaurmet)	Braun, Kronberg/Taunus. Germany
Thermo-mixer	Eppendrof, Hamburg. Germany
Timer	Carl Roth GmbH, Karlsruhe. Germany
Tissue drying over TDO66	Medite GmbH, Brugdorf. Germany
Tissue Float bath GFL1052	GFL Gesellschaft Fur Labortechnik GmbH, Burgwedel. Germany
Vortex	Carl Roth GmbH, Karlsruhe. Germany
Water Bath 37C^{o}	Memmert GmbH + Co. KG, Schwabach, Germany
Orbital shaker KM-2 AKKU	Edmund. Buhler GmbH, Hechingen. Germany
Paraffin Embedding Module EG1150H	Leica, Wetzlar, Germany
Peristaltic pump	Ismetic, Wertheim-Mondfeld. Germany

Continue table 2.1…..

2.2 Consumables:

Table 2.2: *List of consumable Materials:*

Material	Manufacturer
Cell culture flasks (150 Cm^2, 75 Cm^2, 25Cm^2)	TPP Techno Plastic Products AG, Transadingen, (CH)
Cell Culture Multi-well Plates (6-well, 12-well, 24-well, 96-well)	TPP Techno Plastic Products AG, Transadingen, (CH)

Cell Culture 6-well plate (Nunc Delta™ surface)	Thermo Fisher Scientific, Dreiech. Germany # 140675
Cell scraper	Sarstadt, Numbercht. Germany
Cell strainer (70 µm, 100 µm)	BD Bioscience, Heidelberg. Germany

Continue table 2.2….

Material	Manufacturer
Centrifuge tubes (15 mL, 50 mL)	Greiner Bio-one, Frickenhausen, Germany
Chamber slides (8-well glass)	Nunc, Wiesbaden. Germany
Cover slips for objective slides (24X60 mm)	Menzel-Glaser Braunschweig. Germany
Cryo tubes (1.8 mL)	Nunc, Wiesbaden. Germany
Disposable pipettes (5 ml, 10 ml, 25 ml, 50 ml)	Greiner Bio-One , Frikenhausen. „Germany“
Embedding Cassettes	Klinipath, Duiven. (NL)
Gloves	Cardinal Health, Kleve. Germany
Grease pencil	DAKO, Hamburg. Germany
Objective slides Uncoated (26X16X1 mm) Polysine (25X75X1 mm)	Menzel-Glaser Braunschweig. Germany
O-rings (sealing rings)	Dichtelemente areas GmbH, Seevetal. Germany
Parafilm	Carl Roth GmbH, Karlsruhe. Germany
Pasteur pipettes	Brand, Wertheim. Germany
Petri dishes (145X20 mm, 6X20mm)	Greiner Bio-One, Frikenhausen, Germany
Pipettes tips (0.5-10 µl, 10-100 µl, 100-1000 µl)	Eppendorf, Hamburg. Germany
Pressure Dome 844-28	Memscape AS, the Netherlands
Pump Tubing	Ismatec, Wertheim- Mondfeld. Germany
Scalpel blades	Bayla, Tuttlingen. Germany
Sterile Filters disposable (50 mm, 0.2 µm	Sartorius Stadium Biotech, Göttingen. Germany
Syringes	BD Bioscience, Heidelberg. Germany
Weighting dishes	Hartenstein, Wurzburg. Germany
Whatman filter Paper	Hartenstein, Wurzburg. Germany

2.3: Laboratory Materials

Table 2.3: *List of Laboratory Materials:*

Material	Manufacturer
Beakers (250ml, 500ml, 1L, 2L, 5L)	Schott, Mainz. Germany
Bioreactor small accessories	Nordson Medical, Loveland. "USA"
Cell crowns for two-dimensional culture	Chair of Tissue Engineering & Regenerative medicine, Wurzburg." Germany".
Funnels	Hartenstein, Wurzburg. Germany.

Continue table 2.3…

Material	Manufacturer
Humidity chamber	Chair of Tissue Engineering & Regenerative medicine, Wurzburg. Germany.
Glass Pipettes (2ml, 5ml, 10ml, 25ml)	Mercaleo, Munich, Germany
Laboratory Glass Bottles (50ml, 100ml, 250ml, 500ml, 1000ml)	Brand, Wertheim. Germany
Magnetic stirring bars	Hartenstein, Wurzburg. Germany
Magnetic stirring bar retrieval	Hartenstein, Wurzburg. Germany
Measuring cylinders (50ml, 100ml, 250ml, 500ml, 1000ml) Plastic Glass	Vitlab GmbH, Grossostheim. Germany Brand, Wertheim. Germany
Objective slide rack	Mercaleo, Munich, Germany
Protective goggles	Neolab, Heidelberg. Germany
Scalpel blade handles	Bayha, Tuttlingen. Germany
Spatula	Hartenstein, Wurzburg. Germany
Stainless steel tissue embedding molds	Labonord, Monchengladbach. Germany
Tweezers	Assistant, Sondheim. Germany
Volumetric Flasks with lid (250ml, 500ml, 1000ml, 2l, 5l)	Hirschmann Laborgerate GmbH. Eberstadt. Germany

2.4 Chemicals & Solutions:

2.4.1 General chemical and solutions:

Table 2.4: *General Chemicals and Solution:*

Chemical/Solution	Manufacturer	Catalog No.
2-Propanol	Sigma-Aldrich Chemie GmbH, Munich, Germany	33539-2.5-GL-R-D

3-(4,5 Dimethylthiazol-2, 5-diphenyltetrazolium-bromie (MTT)	Serva, Heidelberg. Germany	20395.01
4,6-Diamidino-2-phenyl-indoldihydrochloride (DAPI)	Sigma-Aldrich, Munich. Germany	D9542
β-Mercaptoethanol	Sigma-Aldrich, Munich. Germany	M7522
Accutaze	Sigma-Aldrich, Munich. Germany	A6964
Acetone (≥99.5%)	Carl Roth GmbH, Karlsruhe. Germany	5025.5
Activin A	Thermo scientific, Germany.	PHG9014
Adenosine	Sigma-aldrich, Munich. Germany	A4036-5G

Continue table 2.4…

Chemical/Solution	Manufacturer	Catalog No.
Ascorbic Acid	Laborbedarf Schneller M. GmbH & Co. KG, Estenfeld, Germany	A36040250
Albumin Factor V (BSA)	Carl Roth GmbH, Karlsruhe. Germany	90604-29-8
Alcian Blue solution (1%, pH 2 with acetic acid	Morphiso, Frankfurt, Germany	10126,00500
Ammonium persulfate	Carl Roth GmbH, Karlsruhe. Germany	9592.1
B27 supplement 50X	Gibco® Life Technologes™, Darmstadt. Germany	17504-044
B27 supplement minus insulin	Gibco® Life Technologes™, Darmstadt. Germany	A1895506
Basic fibroblast growth factor (bFGF)	PeproTech Hamburg, Germany,	100-18B
BMP4	Gibco® Life Technologes™, Darmstadt. Germany	PHC9534
BSA protein standard (2mg/ml)	Sigma-aldrich, Munich. Germany	P083410x1ML
Calcium chloride (CaCl2)	VWR, Darmstadt. Germany	1.02391.1000
CHIR99021	Biomol GmbH , Germany	Cay13122-5
Chloroform	Sigma-aldrich, Munich. Germany	372978-1L
DAPI Fluoromount-G™	Southern Biotech, Birmingham. USA	SBA-0100-20
Descosept® AF	Dr. Schumacher GmbH, Melsungen. Germany	00-311-050
Deoxycholic acid sodium salt (≥98%)	Carl Roth GmbH, Karlsruhe. Germany	34842

Deoxycycline hyclate	Sigma-aldrich, Munich. Germany	D9891
Dimethyl sulfoxide (DMSO)	Sigma-aldrich, Munich. Germany	D2438-50ML
Dispase	Sigma-aldrich, Munich. Germany	8418-100ml
Deoxyribonuclease I (DNase I)	Roche, Penzberg Germany	10104159001
Donkey serum	BIOZOL Diagnostica Vertrieb GmbH, Eching. Germany.	ECL-ECS0217D
Dulbecco's Modified Eagle Medium (DMEM) (4.5g/l D-Glucose)+ GlutaMax ™ -1	Gibco® Life Technologes™, Darmstadt. Germany	32430-027
Dulbecco's Modified Eagle Medium (DMEM) high Glucose	Gibco® Life Technologes™, Darmstadt. Germany	11965-092

Continue table 2.4…

Chemical/Solution	Manufacturer	Catalog No.
Dulbecco's Modified Eagle Medium/F-12 (DMEM/F-12) No Glutamine	Gibco® Life Technologes™, Darmstadt. Germany	21331-046
Dulbecco's Modified Eagle Medium/F-12 (DMEM/F-12)+ GlutaMax ™ -1	Gibco® Life Technologes™, Darmstadt. Germany	31331-028
Eosin Y	Sigma-aldrich, Munich. Germany	E4009-5G
Ethanol: Absolute 96% denatured	Carl Roth GmbH, Karlsruhe. Germany	 9056-4 T171-4
Ethylenediaminetetraacetic acid (EDTA)	Sigma-aldrich, Munich. Germany	E5134-1KG
Fetal Calf Serum (FSC):	Lonza, Koln, Germany Bio N sell, Feucht, Germany	Lot No. 8SB016 Lot No: BS196368 BS196369 BS210601
(FSC) continued	PAN-Biotech GmbH, Aldenbach. Germany	Lot No. P150508
Formaldehyde, Phosphate buffer (4%, pH7)	Carl Roth GmbH, Karlsruhe. Germany	P087.3
Gelatin	Sigma-Aldrich, Munich. Germany	G-1890
Heparin injection	Ratiopharm GmbH, Ulm, Germany.	25000 I.E/5mL

Hydrogen peroxide (30%)	Sigma-Aldrich, Munich. Germany	216763-500mL
Hematoxylin acid (HCL)	Carl Roth GmbH, Karlsruhe. Germany	3816.1
Hydrochloric acid (HCL) 37% 1M volumetric standard solution	Carl Roth GmbH, Karlsruhe. Germany	4625.2 K025.1
IWR1	Sigma-Aldrich, Munich. Germany	10161-5MG
KnockOut™ Serum replacement	Gibco® Life Technology™, Darmstadt, Germany	10828-028
L-Cysteine hydrochloride	Carl Roth GmbH, Karlsruhe. Germany	3468.1
L-Glutamine	Sigma-Aldrich, Munich. Germany	7513-100ml
Leukemia inhibitory factor (LIF)	Millipore, Germany	ESG1107
Magnesium chloride	Carl Roth GmbH, Karlsruhe. Germany	HN03.3

Continue table 2.4…

Chemical/Solution	Manufacturer	Catalog No.
Matrigel	Omni lab	FALC356231
Methanol	Sigma-Aldrich, Munich. Germany	34860-2 5R
Mounting Media: Entellan® Mowiol® 4-88	Merck Chemicals GmbH, Darmstadt. Germany Sigma-Aldrich, Munich. Germany	107960 81381
mTeSR™1, defined, feeder free maintenance media	Stem cell Technologies	85850
Non-essential amino acids (NEAA)	Invitrogen, Darmstadt, Germany	11140-035
Papain	R&D System	P4762
Papain Extraction Buffer; -Sodium acetate 2,87gm -L-Cysteihydrochloride 0.394gm. -EDTA-Na2 salt.$2H_2O$ 4,653gm Dissolve all in deionized water and pH to 6	Carl Roth GmbH, Karlsruhe. Germany Carl Roth GmbH, Karlsruhe. Germany Sigma. Germany	6773 3468.1 E5134-1KG
Paraffin	Carl Roth GmbH, Karlsruhe. Germany	6642-6
PD0325901 (N-[(2R)-2,3-Dihydroxypropoxy]-3,4-difluoro-2-[(2-fluoro-4-iodophenyl)amino]-benzamid	Sigma-Aldrich, Munich. Germany	PZ0162
Penicillin/Streptomycin 100X concentrated	Sigma-Aldrich, Munich. Germany	P4333

Potassium Chloride	Merck Chemicals GmbH, Darmstadt. Germany	1049361000
Roswell Park Memorial Institute Medium 1640 (RPMI 1640)+ GlutaMax™	Gibco® Life Technology™, Darmstadt, Germany	61870-010
RPMI 1640 minus insulin	Gibco® Life Technology™, Darmstadt, Germany	11879-020
Sodium acetate	Carl Roth GmbH, Karlsruhe. Germany	6773
Sodium chloride	Carl Roth GmbH, Karlsruhe. Germany	HN00.3
Sodium deoxycholate (SDO)	Sigma-Aldrich, Munich. Germany	D6750
Sodium dodecyl sulfate (SDS)	Carl Roth GmbH, Karlsruhe. Germany	CN30.3
Sodium Hydroxide (NaOH) pellets: 1M volumetric Solution 5M volumetric Solution	Carl Roth GmbH, Karlsruhe. Germany	6771.3 K021.1 KK71.1
Sodium-L-Lactate	Sigma-Aldrich, Munich. Germany	71718

Continue table 2.4…

Chemical/Solution	Manufacturer	Catalog No.
Thrombin	Sigma-Aldrich, Munich. Germany	T4393
Triton™ X-100	Sigma-Aldrich, Munich. Germany	X100
Trypan Blue 0.4%	Sigma-Aldrich, Munich. Germany	P7949
VascuLife®	Cell Systems Biotechnologie Vertrieb GmbH, Troisdorf, "Germany"	LL-0005
Versene 100	Thermo Fisher Scientific, Dreiech. Germany	15040066
XAV 939 (3,5,7,8-Tetrahydro-2-[4-(trifluoromethyl)phenyl]-4H-thiopyrano[4,3-d]pyrimidin-4-one)	Sigma-Aldrich, Munich. Germany	X3004
Xylene	Carl Roth GmbH, Karlsruhe. Germany	9713.3
Y-27632	R&D	1254/10

2.4.2: Cell Culture Buffers and Solutions:

Table 2.5: *Cell Culture buffers and solution:*

Media/ Buffer	Composition
0.05% Trypsin/EDTA working solution	10ml Trypsin/EDTA 0.5% (10x) +90ml PBS solution stored at 4°C
Cardiac Basal medium	RPMI 1640 500ml contains; 2% B27 Supplement (50x) 1% Penicillin/Streptomycin, 0.4% Ascorbic acid (25 mg/ml), 0. 2% Mercaptoethanol (50 mM).
Cardiac Differentiation medium	RPMI 1640 500ml contains; 2% B27 Supplement (50"x") insulin minus, 1 % Penicillin/Streptomycin, 0.4% Ascorbic acid (25 mg/ml), 0,2% Mercaptoethanol (50 mM)
Dispase solution	100mg Dispase + 50ml DMEM/F12 minus Glutamine
Endothelial cells culture medium	VascuLife Basal medium 475 ml include; rh bFGF 5 ng/ml – Ascorbic acid 50 µg/ml – Hydrocortisone Hemisuccinate 1 µg/ml -L-Glutamine 10 mM– rh IGF-1 15 ng/ml – rhEGF 5 ng/ml – rh VEGF 5 ng/ml – Heparin sulfate 0,75 U/ml –FBS 5%. Store in 4C°.
Fibroblast culture medium	10% (V/V) FBS 1% Penicillin/Streptomycin DMEM /Glutamax , Store in 4C°

Continue table 2.5…

Media/ Buffer	Composition
MEF cells medium	DMEM high Glucose 500ml contains; FBS 94 ml, 2% Pen/strep, 6.25ml 200mM glutamine, 6.25 ml MEM nonessential amino acid.
MSC medium	DMEM/F12 500ml contains; 10% FCS, 50µg/ml ascorbic acid, 1% Pen/Strep.
mTeSR ™1 medium	80% mTeSR™1 medium + 20% supplements.
MTT reagent (3mg/ml)	3-(4,5-Dimethylthiazol-2-yl)-2,5- diphenyltetrazoliumbromide (MTT) dissolved in ultrapure water (MilliQ®)
MTT solution (1 mg/ml)	MTT reagent (3 mg/ml) (1 volume) Cell specific culture medium (3 volumes)
PBS^-/EDTA	PBS solution without Ca^{2+}, Mg^{2+} (500ml) ,0.5MNa_2-EDTA·2H_2O (500µl)
Porcine iPSc medium (I)	DMEM/F12 400ml contains; Knockout Serum replacement 100 ml, 1% L-Glutamine, 1% Pen/Strep, 3,5µl β-Mercaptoethanol, 5-10 µg human bFGF
Porcine iPSc medium (II)	DMEM/F12 400ml contains; Knockout Serum replacement 100 ml, 1% L-Glutamine, 1% Pen/Strep, 100µM β-Mercaptoethanol, 1% NEAA, 10ng/ml LIF, 1µM PD0325901, 3µM CHIR99021, 2µg/ml doxycycline.

Porcine iPsc embryoid body differentiation medium	DMEM/F12 400ml contains; Knockout Serum replacement 100 ml, 1% L-Glutamine, 1% Pen/Strep, 100µM β-Mercaptoethanol, 1% NEAA, 2µg/ml deoxycyclin.
Reprogramming media	DMEM/F12; 20% Knock out serum, 1% L-Glutamine, 1% Pen/Strep, 100µM β-Mercaptoethanol, 1% NEAA, 10ng/ml basic fibroblast growth factor.

2.4.3: Histology Buffers and Solutions:

Table 2.6: *Histology buffers and solutions:*

Buffer/ Solution	Composition
Antibody diluent	Bovine serum albumin (BSA) in PBS 5% (W/V) Filtered and stored in 4C°.
Blocking solution	Donkey serum in Antibody diluent freshly 5% (v/v) prepared prior to use
Citrate buffer stock solution (10x concentrated)	42 g/l Citric acid monohydrate 17.6 g/l NaOH pellets in Ultrapure water pH 6.0 (stored at RT)
Citrate buffer working solution	Citrate buffer stock solution 10% (v/v) in Ultrapure water (MilliQ®)

Buffer/ Solution	Composition
Embedding solution (aqueous)	0.1% DAPI in Mowiol® 4-88(stored at -20C°)
Eosin	10mg/ml Eosin Y in Ultrapure water (stored at RT)
Hemalum	(1.2g/L) Hematoxylin, (0.2g/L) $NaIO_3$, (20g/L) Potassium alum, (20g/L) Chloral hydrate, (1g/L) Citric acid monohydrate in Ultrapure water (MilliQ®) used after four weeks of maturation (stored at RT).
HCl-Ethanol (HE staining)	6.85% (v/v) HCl 1M in Ethanol 50% (stored at RT)
Hydrogen peroxide 3 % (H_2O_2)	10% (v/v) H_2O_2 30% in Ultrapure water prepared freshly before use.
Permeabilization solution	0.2% (v/v) Triton™X-100 in PBS (stored at RT) .
Tris-EDTA buffer stock solution (10x concentrated)	12.11 g/l Tris base 3.72 g/l Na2-EDTA in Ultrapure water pH 9.0 (stored at RT).
Tris-EDTA buffer working solution	10% (v/v) Tris-EDTA stock solution in Ultrapure water (store at RT).

Washing buffer (PBS-T)	10% (v/v) Washing buffer stock solution 0.5% (v/v) Tween® -20
Washing buffer stock solution (PBS, 10x concentrated)	1370 mM NaCl 26.8 mM KCl 14.5 mM KH_2PO_4 64.6 mM $Na_2PO_4 \cdot 2H_2O$ in; Ultrapure water (MilliQ®) pH 7.2, autoclaved before use (stored at RT).

Continue table 2.6…

2.5 Kits:

Table 2.7: *Table of kits used:*

Kit	Supplier/Catalog No	Supplier/Catalog No
Alkaline Phosphatase Detections kit.	Fast Red Violet-solution (0,8 g/l) Naphthol AS-BI Phosphate-solution (4 mg/ml) in AMP-buffer (2 mol/l), pH 9,5	Merck Millipore Germany SCR004
BD Cytofix/ Cytoperm Fixation/ Permeabilization Kit (FACS-staining)	BD Perm/Wash™ Buffer (100 ml) Fixation/Permeabilization -Solution (125 ml)	BD Biosciences Germany 554714
Blyscan™Glycosaminoglycan assay	Dye reagent Sodium Nitrite solution Dissociation reagent Standard 100µg/mL Acetic acid solution Ammonium sulfamate.	Biocolor. (UK) B1000
cDNA-iScript Kit (cDNA-	5 × Reactions mix for	Bio-Rad Laboratories

Kit	Supplier/Catalog No	Supplier/Catalog No
SsoFast EvaGreen Supermix (RT-qPCR)	2 × Real time PCR-Mix: dNTPs EvaGreen $MgCl_2$ Sso7d fusion Polymerase Stabilisatoren	Bio-Rad Laboratories GmbH Germany 172-5202
Synthisase)	the Reverse Transcription iScript reverse Transcriptase nuclease free Water	GmbH Germany 170-8891
CellTiter-Glo® Luminescent Cell Viability Assay	CellTiter -Glo® buffer CellTiter-Glo substrate	Promega. Germany G7570
IHC-Kit DCS SuperVision 2 HRP	DCS polymer enhancer. DCS polymer Reagent. DAB Concentrate solution. DAB Substrate buffer	DCS Innovative Diagnostic-System GmbH & Co. KG, Hamburg, Germany,PD000KIT
Pico Green dsDNA Assay	Quantiti-T™ picogreen dsDNA reagent. TE-buffer 20X Lambda DNA slandered 100ug/mL	Thermo Fisher Scientific GmbH Germany PT11496
RNeasy Micro Kit (mRNA-Isolation)	Eppendorff tubes (1,5 ml) Eppendorff tubes (2,0 ml) Carrier RNA, poly-A – RDD-buffer RLT-buffer RNase-free DNase I (lyophilized) RNase-free water RNeasy MiniElute® Spin Columns RPE-buffer (concentrate) RW1-buffer	QUIAGEN Germany 74004

Continue table 2.7…

2.6: Antibodies:

Table 2.8: *List of Antibodies used in histology and immunofluorescence (IF), immunoperoxidase staining with DAB (DAB):*

Antibodies / Isotype	Host	Dilution	Supplier/Catalog No
Alexa Fluor® 488 anti-Mouse IgG (H+L)	Goat	1:200	Thermo Fisher Scientific GmbH 35503
Alexa Fluor® 488 anti-Mouse IgG (H+L)	Donkey	1:400	Thermo Fisher Scientific GmbH A21202
Alexa Fluor® 488 anti-Rabbit IgG (H+L)	Donkey	1:400	Thermo Fisher Scientific GmbH A21206
Alexa Fluor® 555 anti-Rabbit IgG (H+L)	Donkey	1:400	Thermo Fisher Scientific GmbH, A-31572
Alexa Fluor® 555 anti-Mouse IgG (H+L)	Donkey	1:400	Thermo Fisher Scientific GmbH, A-31570
Alexa Fluor® 647 anti-Mouse IgG (H+L)	Donkey	1:400	Thermo Fisher Scientific GmbH, A-31571
Alexa Fluor® 647 anti-Rabbit IgG (H+L)	Donkey	1:400	Thermo Fisher Scientific GmbH, A-31573
Alpha Fetoprotein (AFP)	Rabbit	1:100	DAKO, Germany. A-0008
Alpha Actinin / IgG1	Rabbit	1:500-1:1000	Sigma Aldrich Germany HPA006035-100UL
Alpha smooth muscle actin / IgG2a	Mouse	0.1 - 0.5 µg/ml	Abcam, Cambridge, UK ab7817
Cardiac Troponin C / IgG	Mouse	1:100	Santa Cruz Biotechnology, Inc., Heidelberg, Germany sc-48347
Cardiac Troponin T / IgG1	Rabbit	1:1000	Sigma-Aldrich Chemie GmbH, München, Germany HPA0015774

Continue table 2.8…

Antibodies / Isotype	Host	Dilution	Supplier/Catalog No
Cardiac Troponin T / IgG1	Mouse	1:100	Abcam, Cambridge, UK ab8295
CD31 / IgG1	Mouse	1:100	Abcam, Cambridge, UK ab24590
CD31 / IgG	Mouse	1:5000	Antibodies online GmbH, Aachen, Germany M0823
CD34/ IgG	Rabbit	1:50	Abcam, Cambridge, “UK”.

Antibodies / Isotype	Host	Dilution	Supplier/Catalog No
β Tubulin Isotype III (TUBB) / IgG2b	Mouse	1:400	Sigma Aldrich, Munich. Germany. T8660
VE-Cadhrine/ IgG	Mouse	1:50	Abcam, Cambridge, "UK" ab7047
Vemintin	Rabbit	1:100	Abcam, Cambridge, "UK" EPR3776
vWF/ IgG	Rabbit	1:200	Abcam, Cambridge, "UK" ab6994
			ab110643
CD90 / IgG	Rabbit	1:200	Abcam, Cambridge, "UK" ab92574
Collagen III / IgG	Mouse	1:200	Abcam, Cambridge, "UK". ab23746
Connexin 43 / IgG1	Mouse	1:1000	Sigma Aldrich, Munich, Germany MAB3068
Elastin /IgG	Rabbit	1:100	Abcam, Cambridge, "UK" ab21610
Fibronectin / IgG	Rabbit	1:100	Abcam, Cambridge, "UK" ab23750
NANOG / IgG2a	Goat	1:100	Thermo Fisher scientific GmbH PAS-18406
SSEA-4 / IgG3k	Mouse	1:100	Stem cell Technology 60062, clon Mc-813-70
TRA1-60 / IgM	Mouse	1:250	Millipor, Germany MAB4360
β Tubulin Isotype III (TUBB) / IgG2b	Mouse	1:400	Sigma Aldrich, Munich. Germany. T8660
VE-Cadhrine/ IgG	Mouse	1:50	Abcam, Cambridge, "UK" ab7047
Vemintin	Rabbit	1:100	Abcam, Cambridge, "UK" EPR3776
vWF/ IgG	Rabbit	1:200	Abcam, Cambridge, "UK" ab6994
Fibronectin / IgG	Rabbit	1:100	Abcam, Cambridge, "UK" ab23750
NANOG / IgG2a	Goat	1:100	Thermo Fisher scientific GmbH PAS-18406
SSEA-4 / IgG3k	Mouse	1:100	Stem cell Technology #60062, clon Mc-813-70
TRA1-60 / IgM	Mouse	1:250	Millipor, Germany MAB4360

Continue table 2.8…

Table 2.9: *List of control Isotype Used:*

Isotype	Host	Dilution	Producer
IgG	Rabbit	5mg/mL	Abcam, Cambridge "UK". 172730
IgG2a-FITC	Rat	1:25	eBioscience Germany. 53-4321-80
IgG2a-PE	Mouse	1:10	Miltenyi Biotec GmbH Germany. 130-091-835
IgG3, k-PE	Mouse	1:25	BD Pharmingen Germany. 559926
IgM-PE	Mouse	1:25	BD Pharmingen Germany. 555058

2.7: Software:

Software	Version	Using for	Company
Bio-Rad CFX Manager	3.0	qRT-PCR (CFX96)	BioRad, Germany
GraphPad Prism	6.07	Statistics and graphs	GraphPad prism Co, Germany
Keyence BZ II Analyzer	2.1	Fluorescence figures	Keyence. Germany
Keyence BZ II Viewer	2.1	Fluorescence Figures Figures	Keyence. Germany
LAS X		Luminescence (Tecan infinite 200)	Leica Microsystem, Germany
Tacan iControl	2.11		Tecan GmbH, Germany

2.8: Biological Materials:

Table 2.10: *List of cell lines and other biological material:*

Cell Line
ARiPS (Human iPSc)

CF-1MEF 4M Mito
IMR90-4 (Human iPSc)
Human Mesenchymal stem cells (MSC)
Human skin microvascular endothelial cells
Human skin Fibroblast
Porcine skin Fibroblast
psPAX2 plasmid
pMD2 G plasmid
STEMCCA lentivirus vector

Chapter (3)

Method

3. Methods:

3.1: Decellularization of Cardiac tissue:

The protocol for decellularization was adapted from Ott et al. which describes (1) rat tissue and (3) porcine tissue. All animals were treated in compliance with Care and the use of Laboratory animals Guide published By the National Institution of Health (NIH) and the approval of Institutional board of animal protection and the German society for animal care (section §4 Abs. 3).

For Rats: 10-12-week-old Sprague Dawley Rats were anesthetized by 100mg/kg Ketamine followed by 10mg/Kg Xylazine injected intraperitonially after systemic heparinization. The pericardium was exposed after median sternotomy and a cut was made at the descending Aorta with clamping all three brachiocephalic branches. The Aorta was ligated using 1.8 mm cannula, and retrograde coronary perfusion started under a constant pressure of 77mm Hg (Fig.5). Heparinized PBS^- containing 10µM adenosine was used for 15 min to flush out any residual blood in the heart followed by 1% SDS in MilliQ water for 12 hours, then 15 min of water, and 30 min of 1% Triton X 100 in MilliQ water. The scaffold was then flushed using PBS/ antibiotics (100U streptomycin, 100U penicillin) for additional 124 hours. Tissue was then sent for Gamma sterilization at a dose of 25 gray.

For Porcine*:* Similar protocol was followed with a minor diversion by using different detergent SDO for decellularization in parallel to SDS. The piglets (3 months old, 15–25 kg) were anesthetized using trapanal Fentanyl, heparinized (1000UE/Kg) and sacrificed using (T61®). A median sternotomy cut was made to expose the pericardium and ligate the heart through the ascending Aorta using reducing connector to fit around the Aorta. The heart was then placed in a 5-liter chamber and retrograde coronary perfusion was started with heparinized PBS under constant pressure of about 60 mmHg for 4-5 hours (Fig. 5). Following the previous protocol: (1) 4%SDO in MilliQ water was used for seven days of decellularization, followed by 3 hours of MilliQ water, then one day with 1% Triton X-100 in milliQ water. The heart was then flushed for another seven days with PBS^-+antibiotic (100U Penicillin, 100U streptomycin) solution; and (2) 1% SDS in MilliQ water was used to decellularized the heart for 3

days, followed by 3 hours of MilliQ water rinsing, and one day of 1% Triton X 100 in MilliQ water. The scaffold later flushed with PBS-/antibiotics solution, then send to gamma irradiation sterilization.

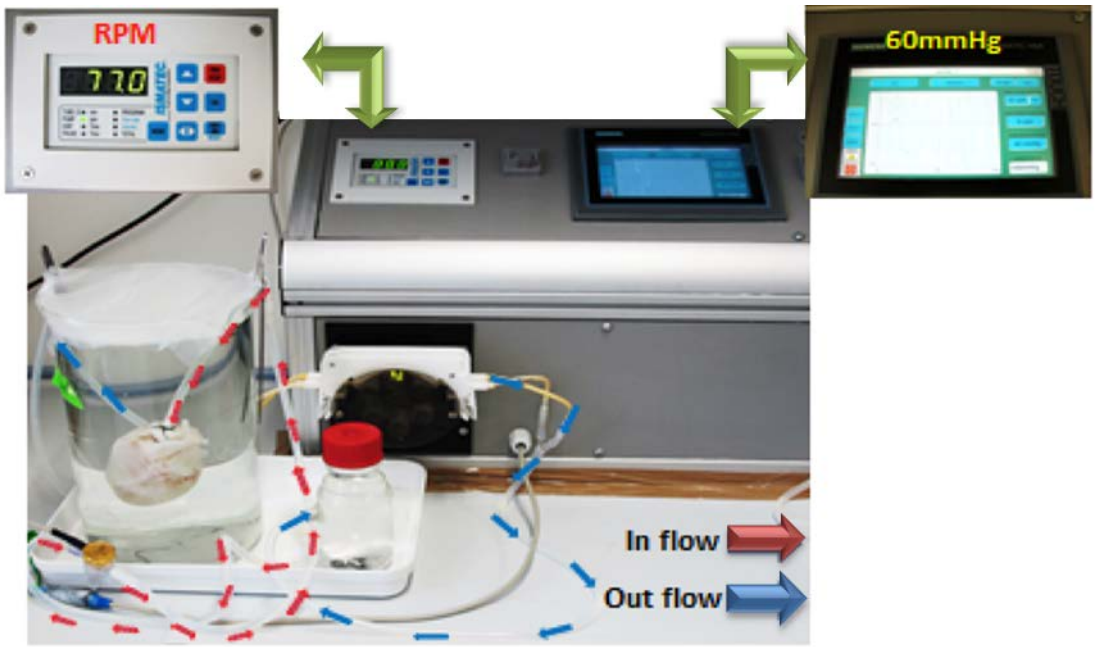


Figure (5)*:* *Setting of the system used for rat/porcine heart decellularization. The system include peristaltic pump, designed to correlate with pressure sensor and adjust flow rate according to the desired pressure set in the apparatus. This allows controlled parameters throughout the process.*

3.2 Cell isolation and preparation:

3.2.1 Human Primary cells: In the recellularization of rat/Porcine heart scaffolds, three different types of cells were used: human fibroblast (h fibs), human microvascular endothelial cells (HMEC), Human Mesenchymal stem cells (h MSC), and human cardiac cells derived from induced pluripotent stem cells (hiPSc-CM). All cell biopsies were done with the approval of ethical committee of the University of Wurzburg (vote 182/10). Written consents were obtained from either the patients or their families.

3.2.1.1: Isolation and propagation of Fibroblast and endothelial cells from skin biopsy:

Following a Standard operating procedure (SOP), a protocol was established at the department of tissue engineering; skin biopsy was transferred to a petri dish containing PBS^- and rinsed three times. A sample from the tissue-transferred media was collected and incubated at 37C° in a T25 flask for sterility control. Using a scalpel, fat and connective tissue was removed and washed again three times with PBS^-. Then the biopsy was cut in to 2-3 mm stripes and incubated for 16-18 hours in dispase (2U/ml). In the following day tissue was transferred in to a petri dish filled with PBS^- after the dispase was aspirated. With forceps the epidermal from dermal was detached and dermal part used for fibroblast isolation. The dermal parts were cut further without chopping, rinsed with PBS^- and transferred in a 50mL tube containing pre-warmed (37C°) 10 mL collagenase (500U/mL). Tissue was incubated for 45 minutes at 37C° and then centrifuged at 1,200 rpm for 5 minutes. The supernatant was aspirated and re-suspended in 2mL DMEM/ 10% FSC media. On the following day 6 mL of media was added to the flask without disturbing the floating pieces. Media was changed after five days and then every 2 days. Cells split when they reached 80-90% confluency.

For Microvascular Endothelial cells isolation, the dermal pieces were washed with 10 ml Versene, then transferred to a 50mL tube containing pre-warmed (37C°) Trypsin/EDTA 0.05%. Tissue was Incubated at 37C° for 40 minutes and then the reaction was stopped by adding 1 mL FCS. Tissues then transferred in to a petri dish containing pre-warmed Vasculife™ media, using forceps cells were squeezed out, then passed through a strainer and centrifuged for 5 minutes at 1200 rpm. Media was changed after 2-3 days, then washed with versine and incubated for 15 min at 37C°, followed by PBS^- wash, and then the cell culture media was added for propagation.

3.2.1.2: Isolation of mesenchymal stem cells from spongiosa,

Human bone spongiosa was provided by Uni Klinikum hospital. The biopsy was placed in 50mL centrifuge tube, with 20 mL pre-warmed PBS--/EDTA. Then the sample was vigorously shaken to wash out blood. Supernatant was aspirated carefully and fresh pre-warmed PBS^-/EDTA added. This step was repeated until the biopsy turned white, then centrifuged at 300g for 5 minutes. Supernatant was aspirate and pallet re-suspended with 25 ml pre-warmed Mesenchymal stem cell media and plated in a T150 flask with tissue chunks. Culture was untouched for three days to allow cell attachment. Later, media with unattached cells was aspirated and fresh MSC media added.

3.2.1.3: Cell Number Calculation,

Cell number and viability can be determined by using hemocytometer counting chamber and trypan blue dye. Cell sample is mixed with 0.4% Trypan blue and 10µl of this is added beneath the chamber and the coverslip at the edge, allowing the solution distribution on the chamber. Cells are then counted on the four quadrants of the chamber. Living cells will appear shiny and colorless, whereas dead cells will appear stained blue.

Using special equation the number can be estimated.

$$\bar{x}_{LC} \cdot V_{CF} \cdot DF = CC$$

$\bar{x}_{LC}$ = Average number of cells in the four quadrants.

V_{CF} = Chamber Factor (10^4/ml).

DF = Dilution factor with trypan blue.

CC = Cell count.

Total cell viability can be calculated as the Number of live cells to the total cell count.

3.2.1.4: Passaging cells,

When cells reached 80-90% confluency, they had to be split and passaged to avoid overgrowing. Culture media was aspirated, and then the cells were washed with PBS^- /EDTA twice to remove any residual FSC. 2ml of 0.05% Trypsin/EDTA was added and the flask was then placed at 37C° for 3 minutes to allow cell detachment, and 4mL of complete media or FCS was then added to stop reaction. Detached cells were collected and centrifuged at 1200rpm for 5 min, then re-suspended and counted as mentioned in (sec. 3.2.1.3) and cultured to the desired number. Media was changed two days later.

3.2.1.5: Freezing and Thawing Cells,

Cell lines are always frozen at lower passages. First, cells are detached by Trypsinization as mentioned (section 3.2.1.4) and counted (section 3.2.1.3). Then, the cell number was adjusted to one million cell/mL in Freezing Media (10% DMSO in complete media or FSC) in a cryo tube. Cells were then quickly placed in a freezing chamber at -80C° for 24 hours before being transferred into a liquid nitrogen Tank.

To start cells from Liquid nitrogen; Cells quickly transferred and thawed in 37C° water bath by shaking for two min then 1mL of FSC added to stop DMSO effect. Then 3mL of culture media was added to the cells and spun at 1200rpm for 5 minutes. The pallet was re-suspended in complete media and cell count and viability determined as in

section (3.2.1.2). Cells cultured according to the desired number, and media was changed the following day.

3.2.2: Human Induced pluripotent Stem Cells:

Two induced pluripotent cell lines were used to be differentiated into cardiac cells: (1) ARiPS, and (2) IMR-90 4. Both needed special care and maintenance requirements.

3.2.2.1: Matrigel™ plate preparation,

To achieve good cell attachments in Culture a special six-well plate (Nucleon™ delta Surface) was used with 0.5mg Matrigel™ per 12 mL of DMEM/F12 media, without Glutamine for each six-well plate. The Matrigel™ dissolved on ice with cold media, and 2mL per well was prepared and kept at room temperature for an hour before use. When the plate was not used directly, 1 mL of media was added an hour later, and the plate was sealed with parafilm, labeled, and stored at 4C°. The plate can be used until two weeks from preparation.

3.2.2.2: Induced pluripotent stem cell maintenance,

For both iPSc lines, mTeSR™1 media was used for daily maintenance as it contains supplements that preserve the pluripotency characteristics on feeder free culture. The media was freshly prepared and used for no longer than two days. It was changed on cells everyday 2ml/well.

3.2.2.3: Induced pluripotent stem cell passaging:

Induced pluripotent stem cells need a careful passaging protocol, depending on cells' morphology, colony size, and density. If the field had 10% or more differentiated cells, then picking was required before enzymatic splitting. For enzymatic splitting, dispase 2mg/ml was used for IMR-90 cells and accutase® ARiPs cells. The media was aspirated from 6-well plates, and 2 mL of dispase solution added after being freshly prepared by mixing with 2mg/ml DMEM/F12 without glutamine. Plates were then placed in 37C° incubator for 7 minutes. Plates were then removed from the incubator, and when the detached colonies' edges were observed under ?, dispase was aspirated and wells rinsed with pre-warmed DMEM/F12 media without glutamine (1mL per each well). Cells were collected using 1000ul pipet tip by vigorously re-suspending. The rinsing step was repeated three times to ensure that all cells were collected. Cells were then spinet at 200g for 3 minutes, and re suspended in mTeSR™ media with 10uM ROCK inhibitor Y27632. IMR-90 cells usually split in 1:6 or 1:8 ratios depending on cells' growth rate. Plates were placed in incubator and media changed the following day with mTeSR™

without ROCK inhibitor. For ARiPs cells, media was aspirated and 2mL of accutase® added in each well, then plate was incubated at 37C° for 6 minutes. One mL of pre-warmed DMEM/F12 no Glutamine was added into each well to stop enzyme reaction. Using Eppendorff 1000ul tip, cells were re-suspended and collected in a 50 mL Falcon tube. This step was repeated twice to ensure collecting all cells, before centrifuged at 200g for 3 minutes. Cells were then cultured in mTeSR™ media with 10uM Rock inhibitor Y27632 and media was changed the following day without ROCK inhibitor addition. The splitting ratio of ARiPs depends on growth rate and proliferation ratio is 1:6 to 1:8.

3.2.2.4: Thawing and Freezing of Induced pluripotent stem cells,

Freezing of iPSc started by enzymatic detaching of cells as described in section 3.2.2.3. Cells were then centrifuged at 200g for 5 minutes and re-suspended in 1mL freezing media (90% Knock out serum and 10% DMSO). Cells were then transferred into a cryo-vial and placed at -80C° for 24 hours before being stored in a liquid nitrogen tank.

For thawing the cells from liquid nitrogen, the cells were quickly transferred on ice and thawed in 37C° water bath by shaking for 2 minutes. Once cells thawed, 1mL of mTeSR™ media was added with 10uM ROCK inhibitor, and the cells were re-suspended, and cultured in 3-4 wells of 6-well plate. Media was changed the following day without ROCK inhibitor addition.

3.2.2.5: Differentiation of human induced pluripotent stem cells to cardiac cells,

Differentiation of iPSc cells to cardiac cells depends on systemic manipulation of BMP and Wnt signaling pathways as described in (4) (Fig 6). The protocol was applied on both cell lines, IMR-90 and ARiPs. When cells reached 80% confluency, differentiation started with the addition of BMP4 10ng/ml and CHIR990221 5uM in RPMI 1640 containing B27 supplement 2%, ascorbic acid 50μg/mL, β-mercaptoethanol 50mM, and penicillin/streptomycin 1%. 24 hours later, the BMP4 was omitted and cells were incubated for 24 hours further with the same media. On day 2, cells were kept in RPMI 1640 media with B27 minus insulin [cardiac differentiation media] for additional 24 hours. On days 3-7, cells were incubated in cardiac differentiation media with the addition of 10μM of XAV939 Wnt inhibitor. Afterwards, cells were cultured back in cardiac basal media with B27 for two days before the enrichment media started, RPMI without glucose but contained 4mM sodium L-lactate, for 4-5 days. Thereafter, cells were cultured in cardiac basal media (Fig.6).

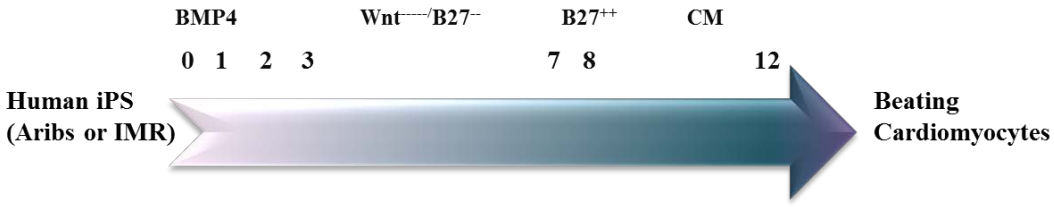


Figure (6): *Day by day steps of human iPSc of cardiac differentiation protocol*

3.2.3: Re-programming of porcine skin Fibroblast into Porcine induced Pluripotent Stem Cells (p iPSc),

The reprogramming protocol was adapted from the protocols described in (5,6) (Fig.7). Porcine skin fibroblasts were plated at a density of $1X10^5$ cells on a gelatin coated 35mm plate or in one well of a 6-well plate in 10% FSC DMEM media (5,6). The next day, cells were infected with human STEMCAA-IoxP lentivirus that had the four genes: OCT4, Sox2, klf-4, and c-myc. In S2 culture hood, 4 mL of the virus was prepared and mixed with 4ul of the polybrene then added to the culture media, plate then incubated for 16-20 hours. Media was then changed with complete cardiac basal media (CBM). Two days later, cells were cultured on mitomycin treated mouse embryonic fibroblast (MEF) in reprogramming media. Three weeks further, colonies of iPSc cells started growing and were ready to be picked individually and expanded on Matrigel™. When cells started losing the pluripotency characters, they were cultured back on MEF cells. Media was changed on daily basis and split when cells reached 70% confluency.

Figure (7)*: Reprogramming steps of skin fibroblast to induce porcine iPSc formation.*

3.2.3.1: Preparing Mouse embryonic fibroblast (MEF) plates,

Porcine iPSc were always cultured on MEF cells in a special media (porcine iPSc media

II) to preserve the pluripotency characters of the cells. As it showed that Matrigel™ and mTeSr™ media were not the best conditions for these cells in term of pluripotency preservation, six-well plates were coated with 2% gelatin and incubated for 2 hours at 37C°. MEF cells were thawed, re-suspended in 4 ml MEF media, and centrifuged at 1200rpm for 4 minutes. Pallet then re-suspended in MEF media and cultured at a density of $2x10^6$ cell per 6-wells, incubated and used after 24 hours.

3.2.3.2: Splitting Porcine Induced pluripotent stem cells (piPSc),

Porcine iPSc has to be split when it is 70-80% confluent. As described in section 3.2.2.3, dispase enzyme 2mg/ml was used. When cells detached, they were collected in a 50mL tube and centrifuged at 200g for 3 minutes. Since the culture was mixed with MEF cells, an optional extra step was performed to separate cells. The mixture of cells was cultured on a 2% gelatin coated plate and incubated for an hour at 37C° to allow MEF attachment. Then non-attached cells (piPSc) were collected, and plated in new 6-well plates on fresh MEF cells and piPSc media II with ROCK inhibitor 10µg/ml. Media then changed the following day with piPSc II media without ROCK inhibitor. Split ratio of the cells was 1:6-1:10 depending on proliferation rate.

3.2.3.3: Freezing and thawing porcine induced Pluripotent stem cells:

To freeze the piPSc, cells were detached with dispase 2mg/mL as described in 3.2.2.3. Cells were then centrifuged at 200g for 3minutes, then re-suspended in I ml freezing media; (90% knock out serum and 10% DMSO); cell number was $1X10^6$ cells per cryo vial. Quickly freezing chamber placed in -80C° for 24 hours then transfer into liquid nitrogen. To thaw cells, cryo vial brought to a 37C° water bath, and shacked for 2 minutes, then 1 ml of piPSc II media added with 10µg/mL ROCK inhibitor to collected cells. Centrifuge at 200g for 3 minutes and pallet was re suspended with 8 ml of media then cultured in 4-wells of MEF 6-well plate. The next day, the media was changed with ROCK inhibitor free media.

3.2.3.4: Differentiation of porcine induced pluripotent stem cells into cardiac cells,

Porcine iPSc have different morphology and characteristics from Human iPSc. For this reason, various differentiation protocols were applied, one of which is that applied on human iPSc described in section 3.2.2.5 (4). Since these cells had common properties and morphology with embryonic stem cells (ECS), a protocol that was suitable for ESC differentiation to cardiac cells was used. The protocol, as described in (7), started when cells detached (as in section 3.2.3.2) using 2mg/mL dispase enzyme. Cells then plated on Matrigel™ coated plate at 100,1000 cell/cm^2 density. MEF-CM (MEF condition

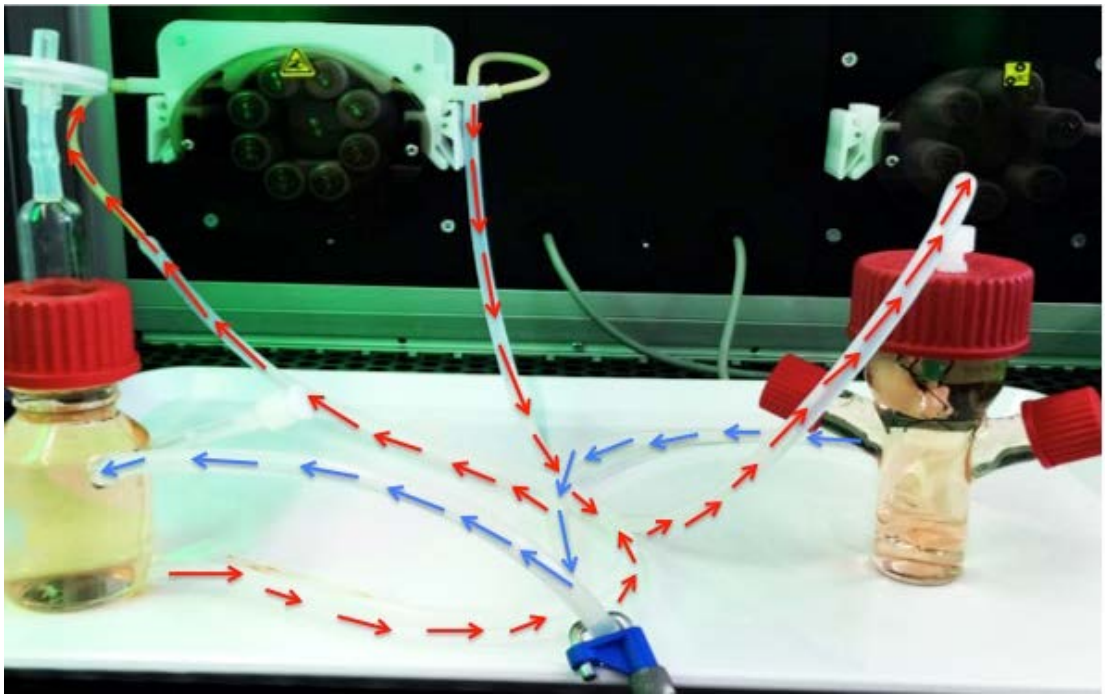
media), was collected on a daily basis from MEF cells culture, and 8ng/ml of bFGF was used on cells for 6 days. The differentiation started on day 7 when MEF-CM was replaced by RPMI 1640 with B27 supplemented and 100ng/ml human activin A for 24 hours. Then 10ng/ml of human BMP-4 was used for 4 days. The cells were then switched to a cardiac basal media (CBM) for 2-3 weeks.

3.3: Recellularization of cardiac scaffolds:

3.3.1: Rat Cardiac Scaffold Re-seeding 3D dynamic culture,

The heart was mounted in a special designed bioreactor established in TERM chair for this purpose (Fig.9). Following the Mathew et al. (8) protocol, a flow of vascolife endothelial cell special media was started for 24 hours at a rate of 5ml/min in 37C° 5% CO_2 incubator. Human microvascular endothelial cells (hMVEC) were injected through the brachiocephalic artery and the inferior vena cava vain at total number of $2X10^7$cells on each in 500µl media. The scaffold was then placed back in the incubator, with no flow on, to allow cells' attachment overnight. Next day, the flow started on scaffold at 1ml/min for the first day to be increased gradually till it reached 3ml/min. The culture was incubated for 7 -10 days with regular pressure measurement thoughout the experiment, with media being changed every 5 days. Media cell perfusate was collected at different point times of experiment for cell viability testing. The second part of re-seeding was a co-culture of human fibroblast cells (hFibs), human mesenchymal stem cells (hMSC), and iPSc-CM in a ratio of 1:1:2 as mentioned in (9,10,11). It was observed that co-culture of the three cell types helps cardiac cells integrate and distribute evenly rather than aggregate when cultured alone on the scaffold. Regarding cell number, it was negotiable as mentioned in (9), and starting with high number of cells doesn't ensure better cell survival. Therefore, a decision was to have a total number of $5X10^6$ cell/cm^{-2} of tissue on the ratio mentioned above, followed by a series of cell number standardization experiments. Cells were injected in few intramural injections in different spots of the left and right ventricles, in a total of 800ul media. The scaffold was afterwards incubated overnight in a static culture in the bioreactor in the incubator; with media flow started the next day at rate of 3ml/min and pulsatile pressure with maximum 10±2 mmHg (Fig.8). The culture media used for cells is a mixture of Vascolife media, MSC media, and CBM in ratio of 1:1:1. The culture was run for 6 weeks, and the sample was fixed by 4% PFA for histological

assessments.



Figure(8): *Setting of 3D dynamic culture of Rat Heart scaffold with special designed bioreactor the re-seeded heart scaffold mounted in the bioreactor at a flow rate 3mL/min and maximum pressure of 10±2mmHg.*

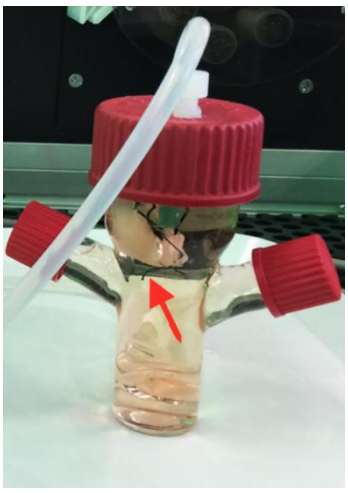


Figure (9): *Custom-made mini bioreactor for Rat heart recellularization with two convenient arms allows media changing and sampling without affecting the tissue position.*

3.3.2. Cell viability assay: MTT & Cell Titer GloCell,

Viability during experiment was checked in different time points. In order to do so, perfusate sampling was frequently collected from bioreactor and saved at -20C° for

assay later. Perfusate 1 was at 48 hours after dynamic culture started, 2 was collected at day 6 and, 3 were at day 10. This test depends on color change of tetrazolium salt from yellow to purple due to a metabolic reduction of formalin by NADPH–enzyme in the endoplasmic reticulum or by succinate dehydrogenase of the mitochondria. In parallel, the collected perfusate were used to culture endothelial cells in 2D 6-well plates for two days to observe cell viability and survival as well. MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) working solution was prepared 1mg/ml by mixing stock solution with culture media 1:3. Aspirate cell culture medial and add 1.5 ml of this solution then incubate for 4 hours at 37C° and 5%CO_2. Media was aspirated and 1 ml of extraction solution was added to each well, and then incubated on an orbital shaker for 30 minutes at room temperature. The extraction step could be repeated till cells become white in color. The extract is then mixed, and 200µL is pipetted in each well of 96-well reading plate in triplet, then absorbance measurement is determined using TECAN reader at 570nm. Blank was measured in parallel using Isopropanol 0.04N HCl.

3.3.2: Porcine Cardiac Scaffold Re-seeding in 2D static and 3D dynamic culture,

For Porcine scaffold, the first plan was to be re-seeded with porcine cells in an attempt to establish a clinical model. For that purpose, porcine iPSc line was established, characterized, and differentiated, as well as porcine aortic endothelial cells and fibroblasts. However, yield of the cardiac cells' differentiation was limited to start with. Therefore, alternatively, the human cells were used instead, as in the rat model. Porcine heart scaffold was cannulated through the left coronary artery by an 18G cannula and secured by surgical sutures. A section of the tissue was cut in the area around the artery, including parts of left ventricle and right ventricle with the coronary artery in the middle. The tissue was 9 cm in length approximately (Fig.10). Next, the tissue was mounted in a bioreactor to start the 3D dynamic culture. Flow started at a rate of 5ml/min using

Figure (10) *Steps out line of porcine scaffold re-endothelialization process***:** *Explaining the cutting of tissue section 9 cm in length around the left coronary artery including part of right and left ventricles with a width of 2cm from each side. Cells hFibs and HMVEC 1:2 ratios then injected through catheter and mount in the bioreactor for 3D Culture.*

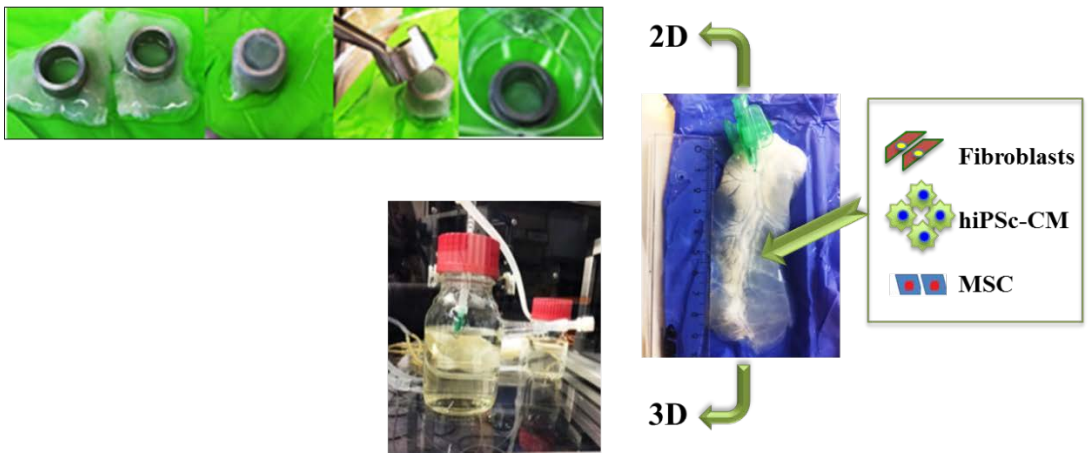


Figure (11): *2D and 3D Culture of porcine cardiac tissue**;** illustration of static and dynamic co-culture of hFibs, hMSC, and hiPSC-CM in 1:1:2 ratios. Metal cell crowns were used for 2D culture to mount different parts of seeded porcine cardiac scaffold. In dynamic culture the whole section in incubated in the bioreactor at pulsatile flow rate of 5ml/min and a maximum 20±5 mmHg pressure.*

vascolife endothelial cells media and DMEM contained 15% FSC media at 1:1 ratio overnight at 37C° 5%CO^2 incubator. Human microvascular endothelial cells and fibroblasts cells were injected in 2:1 ratio through the cannula with a total number of $2X10^8$ cells in 1.5 ml vascolife media/DMEM 15% FSC media. The culture was incubated overnight in static condition to allow cell attachment. Then, the flow was started gradually, at 1ml/min the first day, and then increased gradually by 1ml until it reached 3ml/min, with media being changed every 5 days for two weeks. Coagulation test was performed at this point on the construct before proceeding to ensure new blood vessel functionality. Later, a co-culture of hFibs, hMSC, and hiPSc-CM at ratio of 1:1:2 at $6X10^6$ cell/cm^{-2} was started. Cells were injected in different areas on right and left ventricle, in a total volume of 2ml cell and media. The tissue was mounted back into the bioreactor for 3D culture at flow rate of 5ml/minutes Fig (10). In parallel, some constructs were cultured in 2D culture by tissue placed on a metal cell crown, which was established at TERM for that purpose Fig (10). Both 3D and 2D cultures were run for 6-8 weeks and then fixed with 4%PFA for histological tests.

3.3.3: Coagulation Assay,

This assay was carried on re-endothelialized porcine scaffold to check the effect of endothelial cells' re-introduction on reversing or minimizing coagulation that occurs when decellularized scaffold is injected with peripheral blood plasma (PBP). When porcine heart is decellularized, all cells including endothelial cells and smooth muscle cells in the vasculature responsible of anticoagulation function are lost. Therefore, coagulation happened up on human plasma injection preformed, with high pressure inside construct observed.. Human Blood 10ml was collected at university clink of Würzburg and was centrifuged at 2500 rpm for 30 minutes to separate plasma. Plasma 2ml was then injected in the coronary artery and incubated for 10 min to allow coagulation (this time was calculated based on a standardized curve of plasma coagulation time established already). Flow was then started, and pressure was recorded accordingly for 4 hours.

3.4: Characterization of Decellularized Cardiac scaffolds:

To ensure cells free scaffolds with intact ECM various tests were done after the completion of decellularization process. This was crucial for new cells' adaptation and homing.

3.4.1: Double stranded deoxyribonucleic acid (dsDNA) Picogreen Quanti-T assay,

This assay helps determine the quantity of dsDNA in a sample through colorimetric reaction. Following the kit protocol, dsDNA was isolated from decellularized and native heart. The concentration of the samples was adjusted before assay and was within the slandered curve dilutions. Lambda DNA slandered stock solution was prepared in 2mg/ml as high-range and 50ng/ml as low-rate standard curve TE buffer provided used as diluent. The test was carried out in a 96-well plate black in color, samples were mixed (10µl) with (100µl) of picogreen reagent and incubated in the dark for 2-5 minutes. Samples were measured at Excitation of 480nm and Emission 525nm). Samples were prepared in triplets.

3.4.2: Histological Staining:

3.4.2.1: Fixation of Sample processing and microtome cutting,

Tissues and cells fixation is an important process for preserving internal structure over a long period of time, and it has to be performed before any histological test. There are many different types of fixatives that could be used depending on the followed test requirement. In the fixation process, the fixative reacts with the proteins, lipid, amino acids, carbohydrates, or other cell and tissue structure and forms cross-links to preserve their structure from deteriorating by autolysis by the internal enzymes or by providing stabilization of cells and tissue structures. Following SOP protocol established at TERM department, tissues were rinsed twice with PBS^- then fixed with 4% paraformaldehyde (PFA) for overnight on slow shaker. Tissues were then cut and placed in the embedding cassettes for processing as follows:

Step	Solution	Time (hours)
Washing out Fixative	Deionized water	1
Dehydration Ascending alcohol gradient	Ethanol 50%	1
	Ethanol 70%	1
	Ethanol 80%	1
	Ethanol 96%	1
	Isopropanol I	1
	Isopropanol II	1
Washing out Alcohol	Isopropanol/Xylene (1:1)	1
	Xylene I	1
	Xylene II	1
Paraffin Tissue infiltration	Paraffin I	1.5
	Paraffin II	1.5

Using manual sliding microtome, tissue sections were cut into 3μm thickness if possible or maximum 5μm thickness. Sections were then placed in a 50°C tissue bath, to help sections surface smoothen, then mounted on a microscope slide and placed in (37°C) oven for overnight.

3.4.2.2: Gradient De-paraffinization of tissue sections,

Before staining, tissue sections were de-paraffinized and re-hydrated as indicated in the table below:

Step	**Solution**	**Time (minute)**
Melting Paraffin at 60C°		30-60
De-Paraffinization	Xylene I	10
	Xylene II	10
Step	Solution	Time (minute)
Rehydration descending alcohol gradient	Ethanol 96%I	Dunk up and down 2-3X
	Ethanol 96% II	Dunk up and down 2-3X
	Ethanol 70%	Dunk up and down 2-3X
	Ethanol 50%	Dunk up and down 2-3X
	Deionized water	Dunk up and down till become clear.

3.4.2.3: Hematoxylin-Eosin staining (H&E),

It is one of the major stains in tissue characterization and clinical biopsies as it is described as the basic structure of the cell. The hematoxylin reacts with basophilic structures as nuclei (blue), whereas Eosin Y reacts with acidophilic structures as extracellular matrix and collagen fibers. The steps are summarized as bel

Step	Solution	Time (minutes)
Nuclei Staining	Hematoxylene	6-8
	Deionized water	Till water become clear
Hematoxylene Differentiating (optional)	HCL-Ethanol	Dip 3-4X
	Deionized water	Dip 3-4X
Hematoxylene Color stabilization	Tap water	5
Staining cytoplasm	Eosin Y	6
	Deionized water	Till water is clear
Ascending alcohol Dehydration	Ethanol 70%	Dip 3-4X
	Ethanol 96%	2
	Isopropanol I	5
	Isopropanol II	5
	Xylene I	5
	Xylene II	5
Mounting section	Entellan®	

3.4.2.4: Masson Trichrome staining,

This stain was used to stain collagen fibers, fibrin, and muscles. The name indicates three colors' involvement: Biebrich scarlet acidic dye, which reacts with acidophilic component and turns it into blue color (Collagen and muscle), phosphor acid dye, which gives red color to less permeable structure and binds collagen to aniline blue third dye to have blue color. The sections were processed at the pathology department laboratory at King Feisal hospital via automated staining machine.

3.4.2.5: Immunoperoxidase staining with DAB,

The staining principle is based on the reaction between HRP enzyme and 3,3'-diaminobenzidine (DAB)**,** which results in brown color. Sections were deparaffinized as described in (3.3.2.2), then antigen retrieval was performed to allow the exposure of antigen epitopes, and can freely react with the primary antibody. This can be done enzymatically or by heat, depending on the antibody. 1"x" citrate buffer (pH= 6) was used and slides were boiled in the solution for 20 minutes, and then washed with PBS^- /Tween buffer. Sections were then incubated in primary antibodies: Collagen III (1:200), Fibronectin (1:100), and Elastin (1:100) for overnight in a humidified chamber at 4C°. For negative controls, antibody diluent solution was applied instead of primary antibody, and Isotypes of primary antibody were used as well, at similar concentrations of experiment. Quenching endogenous peroxidase is an important step to do after

primary antibody to avoid DAB-peroxidase reaction and hence false color appearance. Therefore, sections were incubated with 3% hydrogen peroxide as indicated in steps below;

Step	Solution	Time (minutes)
Peroxidase Blocking	3% H_2O_2	10
Washing	PBS-T	5
Primary antibody	As needed	Over night
Washing (3X)	PBS-T	5
Secondary antibody	DCS enhancer	10
Washing (3X)	PBS-T	5
Polymer incubation	DCS polymer	20
Washing (3X)	PBS-T	5
DAB detection	DAB solution	3-5
Washing (3X)	PBS-T	3-5X dip
Nuclei counter staining	Hematoxylin	1
Blue color fixing	Tap water	1-1.5
Ascending alcohol	Described section 3.3.2.2	
Mounting	Entellan®	

3.4.2.6: Blyscan™ GLYCOSAMINOGLYCAN Assay,

Blyscan is a quantitative colorimetric assay which helps to identify total sulfated Glycosaminoglycan (sGAG) in a sample. Where 1,9-dimethylmethylene blue labeled sulfated polysaccharide component of proteoglycans or the protein free sulfated glycosaminoglycan chains under certain conditions. Before started tissue samples were digested by papain first, as per SOP established protocol in TERM chair. 1% of papain enzyme (0.56U/ml) was mixed with papain digestive buffer, and 1 ml of the mix was pipetted on a dried tissue in a 2ml tube. Later, tubes were incubated in 60C° thermo-mixer overnight to allow tissue digestion. The following day, samples were collected and used or stored at -20 C°. To start the test, a serial volumes of samples was used, between 10µl-100µl with a total volume of 100µl to be completed with distilled water. 1 ml of dye reagent was then pipetted, and tubes placed on a shaker for 30 minutes. Samples were centrifuged at 12000 rpm for 10 minutes, supernatant decanted, and pallet allowed to dry. 0.5ml of dissociation reagent was then added, mixed and incubated for 10 minutes at room temperature. In a 96-well plate, 200µl of sample, blank or standard was pipetted in triplicate, and then measured at 656nm wavelength. Total sGAG concentration values were calculated in reference with standard curve.

3.4.2.7: Scanning Electron microscopy (SEM),

This type of scanning allows a beam of electrons to move across the sample, creating an image of the surface of the sample. This image is a result of detecting the release of secondary electron from the sample, as high-energy molecules primary electrons scan it. Samples were fixed using preferably 2.5% glutaraldehyde solution, dehydrated through ethanol gradient, and then dried at critical point method to avoid sample distortion due to drying tensions. Samples were then mounted on specific metal holder with adhesive, coated with 40-60nm metal (gold) and observe in the microscope. Samples were scanned using SEM at Pathology and histology department at King Faisal hospital and research center, Riyadh, Saudi Arabia.

3.5: Characterization of Human iPSc differentiation:

3.5.1: Gene expression throughout hiPSc differentiation in to Cardiac cell:

Gene expression of human iPSc was detected by real time polymerase chain reaction test (RT-PCR). Cells from variable time frame during differentiation were collected. RNA was isolated and then after cDNA to start RT-PCR using below primers;

Primers	Tm(C°)
Oct4-F: aac ctg gag ttt gtg cca ggg ttt Oct4-R: tga act tca cct tcc ctc caa cca	60
Brachyury-F: tgt ccc agg tgg ctt aca gat gaa Tbrachyury-R: ggt gtg cca aag ttg cca ata cac	60
ISL1-F: cac aag cgt ctc ggg att gtg ttt ISL1-R: agt ggc aag tct tcc gac aa	60
Nkx2.5-F: gcg att atg cag cgt gca atg agt Nkx2.5-R: aac ata aat acg ggt ggg tgc gtg	60
cTNT-F: ttc acc aaa gat ctg ctc gct cTNT-R: tta ctg gtg tgg agt ggg tgt gg	60
β-actin (BA)-F: ttt gaa tga tga gcc ttc gtc ccc β-actin (BA)-R: ggt ctc aag tca gtg	

tac agg taa gc	

3.5.1.1: RNA Isolation:

Cells were collected from Day 0 of differentiation until Day 20 for RNA preparation using RNeasy® Micro Qiagen kit. Cells (maximum $5X10^5$ cell/ml) were lysed with 350μl of RLT buffer containing 1%β-mercaptoethanol. Cells were then scraped off with cell scraper, transferred into 1.5μl Eppendorf tube and placed in -20C° or proceeded with RNA isolation. Lysate was then transferred in to a cell shredder provided with kit, and spun for 2 min at 20,000 rpm. 70% ethanol (350μl) was added to lysate and mixed by pipetting. Out of the mixture 700μl was transferred to a spin column and centrifuged for 15 seconds at 10,000 rpm, flow was discarded out and column placed on a 2ml tube. Next 250μl RW1 buffer was added and centrifuged for 15 second at 10.000 rpm to wash out columns. 10μl of DNase-I mixed with 70μl RDD buffer and dropped in the center of the column then left in room temperature for 15 minutes. Later 359μl of RW1 buffer added to the column then centrifuged for 15 second at 10.000 rpm and flow was discarded. Next 500μl of RPE buffer was added and centrifuged at 10.000 rpm for 15 seconds, then placed in a new 1.5ml tube. Later, 30-50μl of RNase-free water was added and spun for 1 minute at 10.000rpm before RNA was eluted.

3.5.1.2: cDNA synthesis,

For RT-PCR, cDNA synthesis from total RNA was completed first using cDNA-iScript™ kit. Steps are summarized in the table below, mix and place in thermocycler for the following program; *Table 2.17: cDNA synthesis:*

Buffer used	Volume (μL)
5X iScriptTM Reaction Mix iScriptTM Reverse Transcriptase 1 RNase-free Water RNA-Template	4 1 X X
Total Volume	20

5 min at 25C, 30min at 42C°, and 5 min at 85C°, 4C° (optional). Quickly dilute cDNA and place at -80C°.

3.5.1.3: Real Time Polymerase chain reaction,

It is a widely used technique to detect and amplify certain nucleic acids based on polymerase chain reaction. Samples were heated and cooled to a precise temperature to promote nucleotide denaturing, annealing, and polymerase-mediate reaction for DNA amplification rounds. Because of the presence of DNA specific pro dye, fluorescence intensity measurement allows detecting the target gene of interest. The software in turn, is responsible for interpretation of results. Each cycle includes the denaturing of nucleotides double chain at 95 C°, then the binding of specific primers (Forward and Reverse) with DNA template at 50-60 C°, and finally the polymerization by DNA polymerase. This process is repeated 25-50 times. To start a mix of cDNA, Primers and PCR Super mix was prepared first as following;

Table 2.18: Real Time Polymerase chain reaction protocol:

Reagent	Concentration	Dilution factor	Volume
Forward primer Reverse primer	4pmol/µl	1:25	2µl
cDNA	4pmol/µl	1:25	2µl
	------	1:25-1:5	1µl
DNase free water	-------	--------	5µl
BioRad-Supermix	---------	---------	10µl

Reagents were kept on ice while pipetting, and the Super mix was thawed once and then placed in 4C°. Because it is light sensitive, it should always be wrapped with aluminum foil. First the Master mix was prepared (with one sample extra) as calculated in the table above. The mix was then pipetted in a 96-well plate, in a duplicate. Negative control was run along as well, which was DNA free water instead of cDNA. Seal plate with adhesive foil and place in the cycler then on the desired protocol;

Temperature [C°]	Cycle Time [s]	Number of cycle	Step
95	180	1"x"	Double strands separation
95	10	40"x"	Double strands separation
			Activation of Polymerase

60	30	40”x”	Primers Annealing
72	30	40”x”	Stabilization, Elongation, and florescence
95	10	1”x”	detection
65-95	5	1”x”	Curve analysis

The results then were analyzed by the cycler software or statistically using graph prism software.

Table 2.19: Real time PCR program used:

3.5.2: Immunofluorescence Staining;

It is a technique used to detect specific antigen in a tissue or cell by using primary antibody, which is in turn specific to this antigen. The primary antibody can be conjugated to a fluorescence dye, or unconjugated and needs a specific secondary antibody that is labeled with the fluorescence dye. For hiPSc-CM, Cells were grown on round coverslips in 6-well plate and in where the differentiation took place. Once cardiac cells are fully differentiated, it was fixed with 4%PFA for 10 minutes then washed twice with PBS-T. Cells then incubated with 0.02% Triton® X-100 for cells permeabilization for 5 minutes followed by blocking with 5% Donkey serum (as its the secondary antibody species) for an hour before incubated with primary antibodies: α-smooth muscle actin (1:100), Troponin C (1:100), Troponin T (1:1000), and Connexin 43 (1:1000). Plates were incubated at 4C° overnight with slight rocking and shaking. The next day, cells were washed three times with washing buffer PBS-T on a shaker for 5 minutes each wash. Later, cells were incubated with appropriate secondary antibodies at 1:400 dilutions with dilution buffer for an hour at room temperature in the dark (plate wrapped with aluminum foil). The antibody was then decanted and plate washed with washing buffer on a shaker three times each for 10 minutes. Cover slips were mounted (upside down) each on a microscope slide after adding 30 µl of 4-88® 0.1% DAPI in beneath.

Table2.20: Summary of Immunofluorescence staining steps,

Step	Solution	Time (min)
Blocking to mask any background	5% Donkey serum	20
Incubation with primary antibody	Diluted primary AB	O/N (4C^{o})
Washing (3”x”)	PBS-T	5
	Diluted secondary AB	60
	PBS-T	10

Incubation with secondary antibody Washing (3"x") Mount cover slips on slides	Mowiol- 4-88® 0.1% DAPI	

3.6: Characterization of Porcine iPSc:

Porcine induced pluripotent stem cells resulted from reprogramming need to be tested and characterized to demonstrate their pluripotent efficacy. Out of reprogramming experiment, four different colonies were picked (1,2,3,4). Colonies 1,2 were expanded on Matrigel using mTeSr 1 media and passaged two passages before characterization. Colonies 3,4 were propagated on MEF cell using special media containing LIF. The first test used was the alkaline phosphatase that indicated the pluripotency character of cells. The second test was embryoid body three-germ differentiation: ectoderm, mesoderm, and endoderm.

3.6.1: Alkaline phosphatase test,

This test is used as one of the indications for pluripotency markers in human and mouse EC and iPSc (13). The test is based on the expression of alkaline phosphatase by the cells, which vary depending on cell growing stage. For the test, cells were cultured on glass cover slips for five days before test. On day five, cells were fixed with 4% PFA for 1-2 minutes only. Then, cells were washed with PBS^{-} and the dye mixture was added to cells and incubated in the dark for 15 minutes. Cells were then washed in PBS^{--}.and observed in fluorescent BZ-9000 BIOREVO System.

3.6.2: Characterization of the common pluripotency markers,

There are common pluripotency markers used to characterize human and mouse stem cells, could be intracellular markers as OCT4, SOX2, and NANOG, these are important for pluripotency maintenance of stem cells. The other markers are located on cell surface as TRA1-80, TRA1-61, SSEA1, and SSEA4, which are expressed in teratoma cells and embryonic germ cells. Because of species' antibodies limitation, there was a difficulty in finding most of the cell markers. However, some of the available ones, such as NANOG, SSEA4 and OCT4, were used for immunofluorescence staining following the protocol indicated in section 3.5.2. Cells were fixed with 4% PFA 15 minutes and permeabilized using 0.2% Triton X 3-5 minutes before. Dilutions of

primary and secondary antibodies are mentioned in table 2.8. Primary antibodies were incubated overnight in humidified chambers at 4C°, and secondary antibodies were incubated at room temperature on a shaker for an hour.

3.6.3: Three-Germ layers differentiation via embryo bodies,

Porcine iPSc clone3a, 3c, and clone4a,4b were dissociated with Dispase 2mg/ml and cultured in low attachment binding plate in DMEM/F12 media with;.20% Knock out serom,1% penicillin /streptomycin (pen/strep), 1% non-essential amino acids, 100µl β-mercabtoethanol, and 2µg/ml doxycycline for 4 days. On Day 5 doxycycline was removed, and on day 7 embryoid bodies (EBs) were plated on 0.1% gelatin coated plates, in DMEM 1% pen/strep, 10% FBS, and 1% glutamine was added fresh every time media was changed. Three weeks later, after differentiation, cells were tested for: lineage-specific protein expression by immunofluorescence for TUBB3 (ectoderm), AFP (endoderm), and α-SMA (mesoderm). Dilutions of antibodies mentioned in antibody table 2.8. The staining protocol is described above in section 3.5.2. . Primary antibodies were incubated overnight in humidified chambers at 4C°, and secondary antibodies were incubated at room temperature on a shaker for an hour.

3.6.4: Porcine iPSc cardiac differentiation characterization,

For cardiac differentiation characterization, cells were fixed with 4% PFA for 10 minutes, and permeabilized with 0.2% Triton X 100 for 3-5 minutes. Then cells washed with PBS^- before primary antibody incubation (Trop c, α-smooth muscle actin). Dilutions were mentioned in table 2.8 and the protocol described in section 3.5.2. . Primary antibodies were incubated overnight in humidified chambers at 4C°, and secondary antibodies were incubated at room temperature on a shaker for an hour.

3.7: Characterization of re-seeding of cardiac scaffolds:

3.7.1: Rat scaffolds:

Rat heart was fixed after 8 weeks culture with 4% PFA. Solution was run through the aorta for internal tissue fixation, and then the whole tissue placed in fixative for overnight on a shaker at 4C°. Tissue then cut in to three sections as illustrated below and placed in embedding cassettes Fig (12). Paraffin sections were then cut in 3µm sections, using microtome as described before.

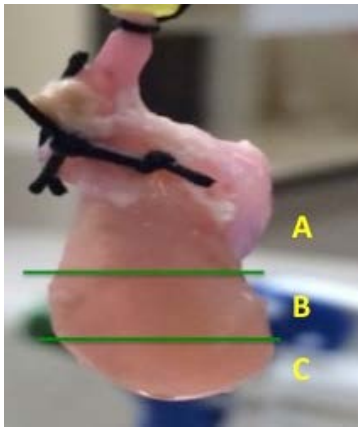


***Figure (12*)**: *Sectioning of re-seeded rat heart for histological studies*

For immunofluorescence staining: (1) endothelial cells, the following markers was used; VE-cadherin, von Willebrand factor, CD31, and CD34, (2) cardiac markers; Troponin t was used. Dilutions of primary antibodies are listed in table 2.8, using dilution buffer. Sections then were incubated over night at 4C^{o} in a humidified chamber. Later three times wash with PBST for five minutes on a rotating shaker was performed. Secondary antibodies were incubated at room temperature on shaker for one hour in the dark. Followed by PBST washes three times for 10 minutes each. Slides then mounted with DAPI-Mowiol 40µl, and cover slip placed carefully to avoid air bubbles beneath. Slides then placed in the dark to dry.

3.7.2: Porcine cardiac tissue,

Porcine tissue was removed from 3D dynamic culture and 2D cell crown cultures after 8 weeks. For the 3D culture tissue, fixative was run through the coronaries to ensure the fixation of internal areas and blood vessels. Then the whole tissue was sectioned as illustrated in figure (13),

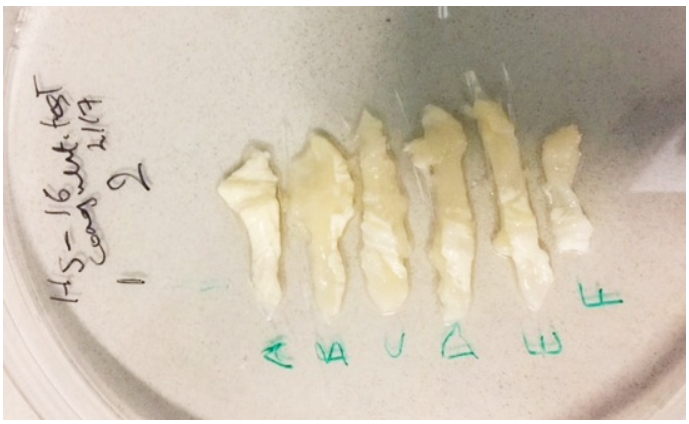


***Figure (13*)**: *Sectioning of porcine heart tissue for histological studies*

then placed in falcon tubes filled with fixative solution 4% PFA for 24 hours on shaker at 4C°. 2D cell crown tissues were fixed in 4% PFA for 6-8 hours on shaker at room temperature. Tissues placed for paraffin embedding cassettes and later were cut at 3µm thickness sections for slides preparation. Slides were then stained by H&E, Trichrome (described 3.4.2.3, and 3.4.2.4), and Van Gison staining.

Immunofluorescence staining on sections preformed as described before. First sections were blocked with Donkey serum 5% in PBS-T for an hour at room temperature, then primary antibodies; CD31 (for EC), CD90 (hMSC), Vemintin (fibroblast), and Troponin T (cardiac cells), incubated in humidified chamber at 4C° for overnight. All antibodies dilutions were mentioned in table 2,8. The next day, after 3 times washes with PBST 5 minutes each, incubation with secondary antibodies for an hour at room temperature in the dark took place. Later three washed with PBST 10 minutes each was performed before sections were mounted with Mowiol-DAPI.

3.7.2: Van Gison Staining,

Von Gison stain is used to differentiate between Collagen fibers and other connective tissues. It is composed of mixture of two dyes; Picric Acid and Acid Fuchsin, when combined picric acid small molecules penetrate all structures. However only closed textures retain the dye, red blood cells, and muscles. Larger molecules of Ponceau S displace picric acid molecules from collagen fibers, because they have a larger pore size. After de-paraffinization of slides and rehydration, staining was performed. Steps for Von Gison stain are summarized in the table below:

Table 2.21: Von Gison staining steps;

Time	Solution	Step
5 min	Xylene	Deparaffinization
5 min	Xylene	Deparaffinization
2 min	96% Ethanol	Rehydration

2 min	80% Ethanol	Rehydration
2 min	70% Ethanol	Rehydration
15 min	Resorcinol-Fuchsin	Elastin staining
1 min	Tap water running	Washing
5 min	Weigert's Elsenhematoxylin Solution A+B 1:1	Nuclei Staining
10 min	Tap water running until water is clear	Washing
10 sec	1% Hydrochloric acid	Blue color gaining
5 sec	Deionized water	Rinsing
2 min	Von Gison stain	Staining
5 sec	Deionized water	Washing
2 min	96% Ethanol	Dehydrating
2 min	96% Ethanol	Dehydrating
2 min	Isopropanol	Dehydrating
5 min	Xylene	Dehydrating
5 min	Xylene	Dehydrating

Mount slides with few drops of Entellan then place cover slip avoid bubbles in between section and cover slips.

Chapter (4)
Results

4: Results:

4.1: Decellularization of Heart:

4.1.1 Rat: The decellularization protocol adopted from Ott et al. (151) was performed on tissues obtained from 32 Sprague Dawely rats at ages between 11-14 weeks. The protocol aims to decellularize the heart by removing cells, DNA, and intracellular structure proteins. Moreover, essential extracellular matrix glycoproteins should be preserved. The decellularization of the tissue was accomplished by the use of 1% SDS for 12 hours and 1% Triton X 100 for 30 minutes in a retrograde langendorff flow through the aorta. During the process, a constant inlet pressure throughout the experiment was ensured and measured. This allowed flow rate adjustment accordingly. The decellularization started at the outer border areas on the scaffolds, progressing toward the middle with time (Fig. 14b). As the flow was through the coronary tree, it resulted in a whitening of the scaffold after 5 hours from the sides, indicating cell removal process. Ten hours later, the whole scaffold turned into white, resulting in a transparent construct with an increased inflow rate (Fig.14c). H&E staining showed that the scaffolds were free from cells, nuclei, and contractile elements (Fig.15). DNA double strand measurements in both cadaveric and decellularized samples confirmed that DNA remnants in the decellularized samples were less than 4 ng/µl whereas cadaveric tissue contained 70 ng/µl. Staining of decellularized and cadaveric tissue for elastin (n = 5), showed a partial loss of elastin. Yet, a good percentage was preserved that is enough to accommodate new cells re-seeding and provide elastic properties to the new seeded tissues. Staining was also carried out to investigate the presence of collagen fibers in decellularized scaffold using Trichrome collagen satin. The staining revealed intact collagen fibers with small lacunae within which cells may be deposted. In addition, SEM imaging of scaffold confirmed at a focused angle the retention of collagen alignments and bundling, which is important for new cells' reseeding and deposition (Fig.16).

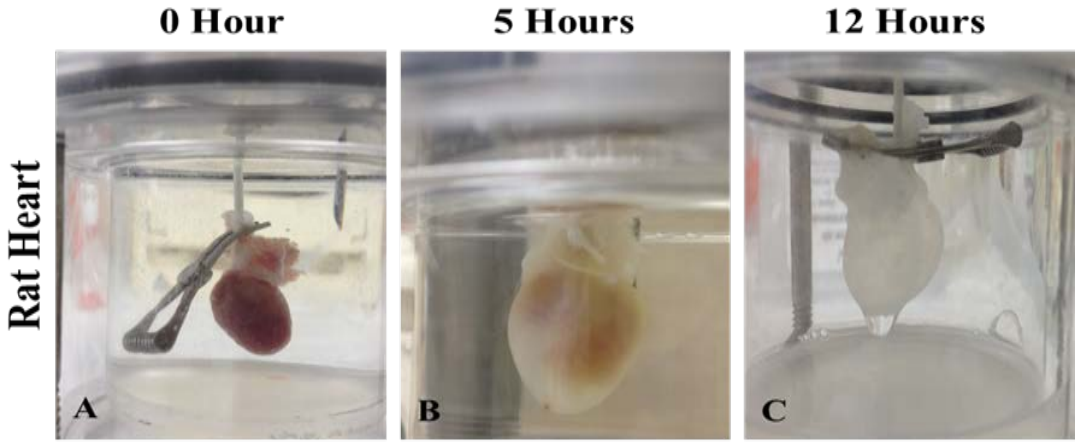


Figure (14): *The decellularization of rat heart, retrograde flow started through the aorta with 1% SDS under constant pressure 77mmHg. (A) Starting point with 1% SDS, (B) five hours later, most of the heart was decellularized, (C) 12 hours later the complete heart was transparent and cell free.*

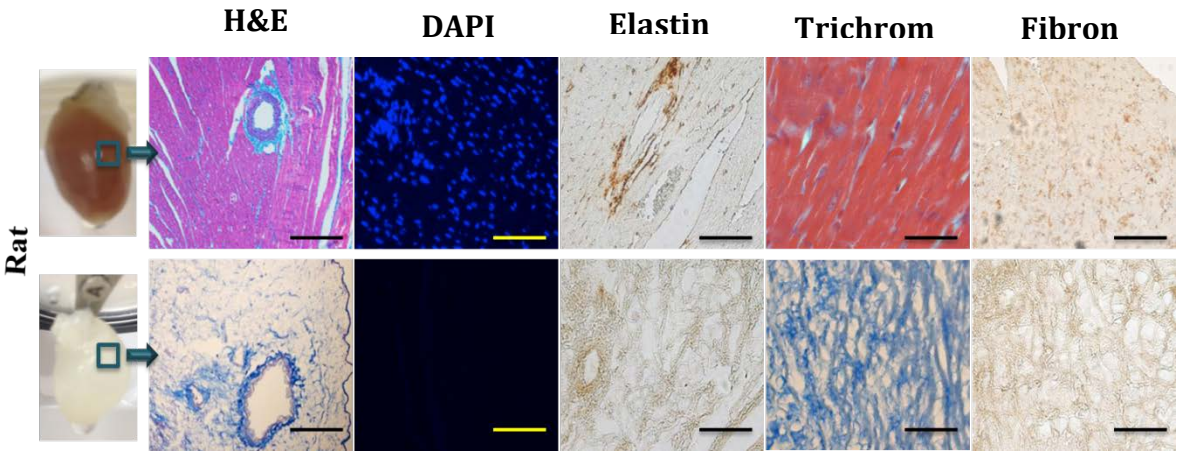


Figure (15): *Histological Staining of cadaveric and decellularized rat heart: (H&E) indicate the removal of cells nuclear substance. DAPI fluorescence staining was negative in decellularized tissue. Trichrome, elastin, and fibronectin staining showed the preservation of ECM proteins.*

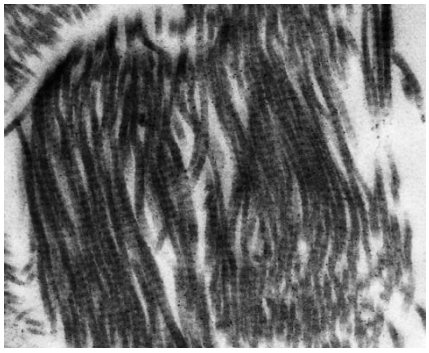


***Figure (16)*:** SEM scanning indicating an intact aligned collagen fiber in decellularized rat heart scaffold.

4.1.2 Porcine Scaffold: Once the parameters of the decellularization protocol was standardized on Rat heart model; the same were used to decellularize porcine heart. In addition, 4% SDO was used as a decellularization detergent in parallel with 1% SDS to compare outcomes of both in term of cell removal and on extracellular matrix proteins content preservation. Retrograde flow at 60 mmHg through the aorta via the coronary blood vesicle was started, with clamping the three branches of brachiocephalic arties using 4% SDO for 7days followed by 24 hours of 1% Triton X 100 then 7 days of PBS^{--}/antibiotics. At day 7 the heart was partially decellularized, with no further removal process even after one week later. The resultant scaffold was only 60% decellularized with light pinkish color. On the other hand, almost complete removal of cells accomplished after only 3 days of 1% SDS treatment followed by 24 hours of TritonX-100, then 10 days washing with PBS^{--}/ antibiotic to flush out SDS and cell debris. Scaffold turned white with transparent structure.

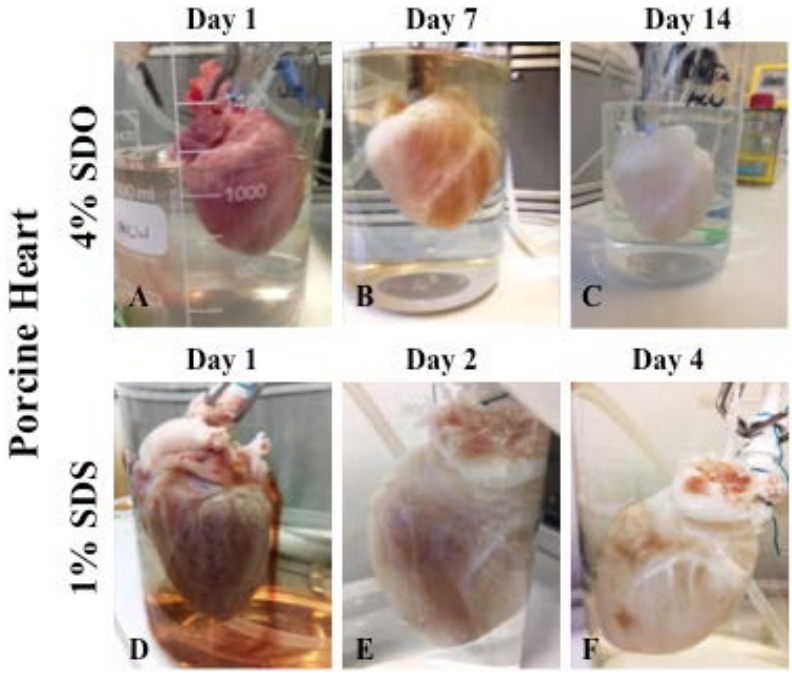


Figure (17): *Decellularization of porcine heart under constant pressure 60mmHg using two different detergent protocols; (1) SDO 4% for 7 days followed by 1%Triton-X for 24 hours then 7 days rinsing with PBS, (A) day 1, (B) day 7 30% of the heart was decellularized, (C) day 14 only part of the heart was decellularized. (2) 1% SDS use for 3-4 days followed by 1% Triton-X for 24 hours, then excessive rinsing with PBS for 10 days; (A) day 1 blood is flushed out thoroughly, (B) day 2 most of the heart was decellularized, (C) by day 4 the whole heart was completely white, indicating decellularization of the tissue.*

Flow rate was recorded throughout the decellularization process; with using 4% SDO, flow rate was not changed significantly, and was recorded to be between 50-68 rpm/min for the 14 days. When 1%SDS was used, the flow rate was increased by time, and proportional to cell removal process. Starting at a flow rate of 60ml/minute and reaching a maximum of 160 mL/minuet at day 8, then continued until day 14 Fig.(17). In a decellularized scaffold, measured DNA reflects a decrease in concentration of less than 4ng/µl compared to almost 80ng/µL in cadaveric samples fig (19). While cells were removed leaving a compressed thin cardiac scaffold, essential extracelluar proteins collagen I, Fibronictin and Elastin remined intact and sufficient to support new cell re-seeding fig (21), with preservation of fibers' composition and orientation..

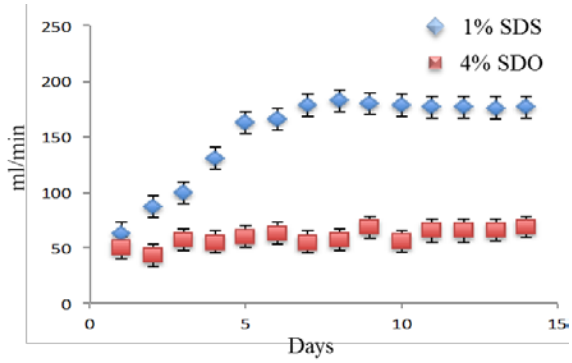


Fig (18): Recorded flow rate through out the decellularization day by day, which was adjusted according to the measured pressure inside the heart champers.

Fibers within the aortic wall and and aortic valve were preserved as well after exposure to 60-70mmHg pressure during decellularization for consecutive 14 days. In addition, blood vessel structures were completely intact in the decellularized scaffold with out any endothelial cells or smooth mucsle cells in them. This was observed after injecting the scaffold with phenol red dye through coronaries to light up only the blood vessicles without any leaking in to the construct chambers. Quantitative Assay for total glycosaminoglycan in both SDO and SDS decellularize samples showed close levels of total sGAG with no significant difference, between SDO (0.66 µg/mL), and SDS (0.56µg/mL) compared to cadaveric concentration, total sGAG was reduced by

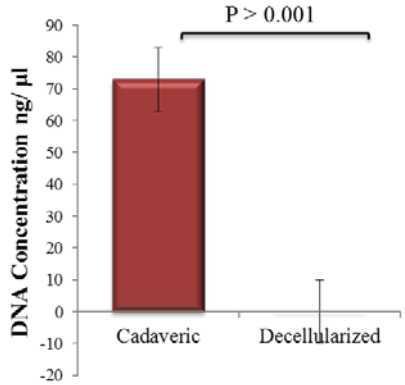


Figure (19): *Measured DNA concentration in cadaveric (75ng/µl) and decellularized tissue (4ng/µl).*

30% for (4% SDO) and 45% for (1% SDS) fig (20).

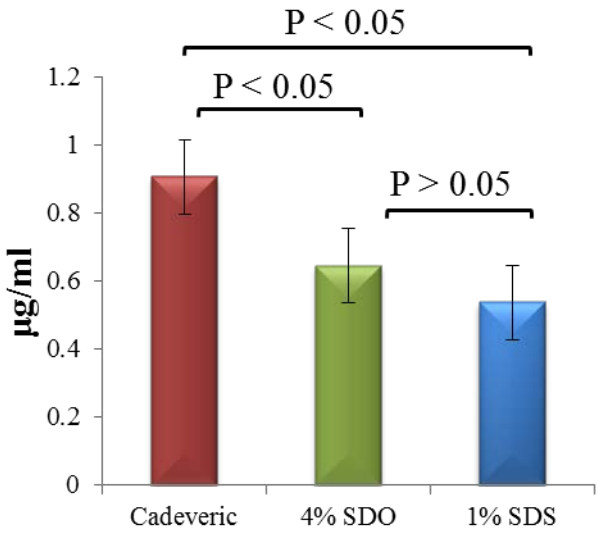


Figure (20): *Total GAGs concentration in cadaveric (0.90µg/mL), decellularized scaffold by SDO 4% (0.646µg/mL), decellularized scaffold by SDS 1% (0.54088µg/mL).*

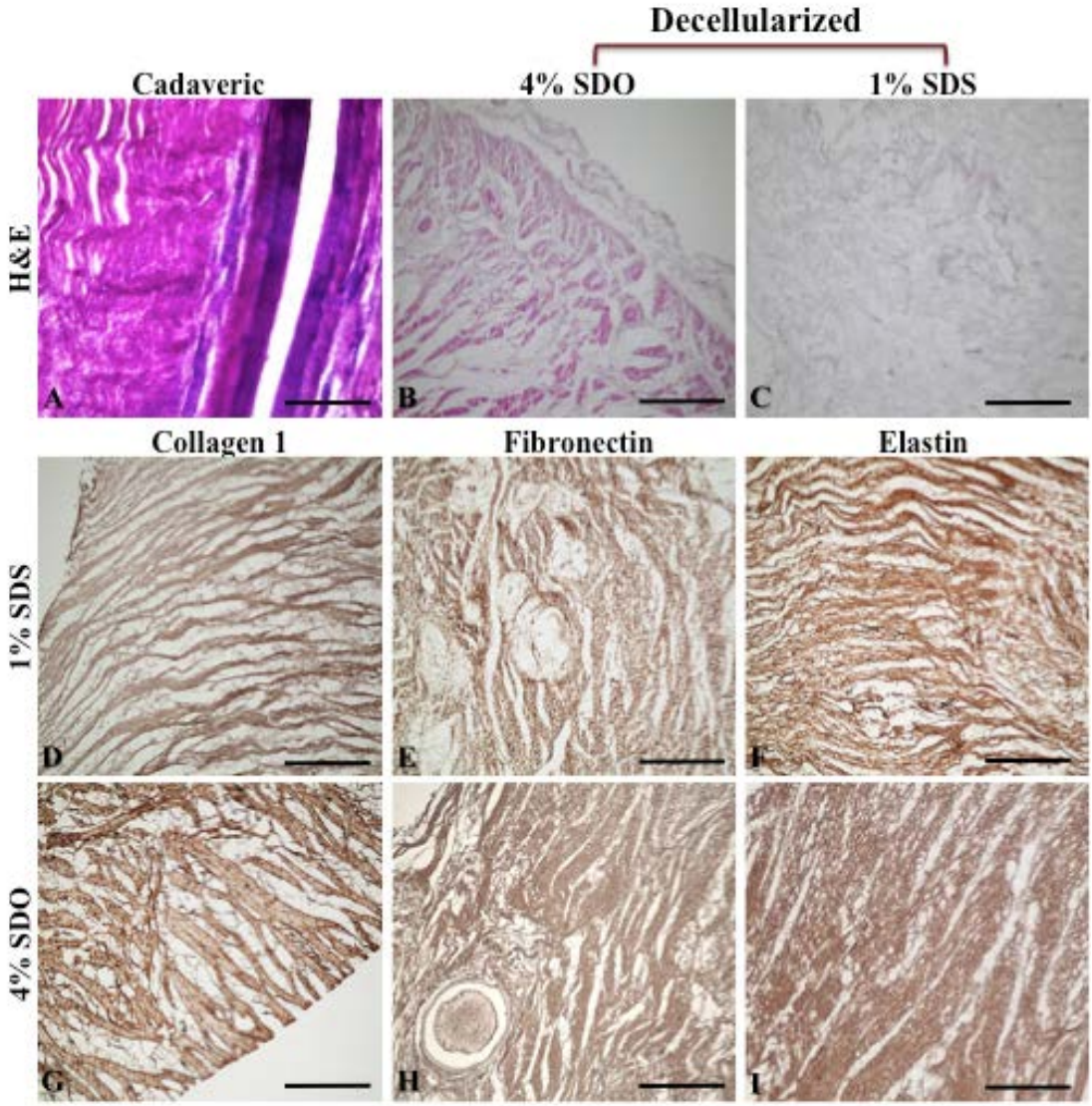


Figure (21): *Histological staining for GAGs qualification in cadaveric and decellularized.* ***H&E****; SDS 1% showed more cell removal(C) than scaffold treated with SDO 4%. Extracellular Matrix indicated the preservation of collagen (D) and (G), Fibronectin (E) and (H), and Elastin (F) And (I) in both SDS and SDO treated scaffolds.*

4.2: Differentiation of hiPCs to cardiac cells:

Two human iPS cell lines; ARiPS, and IMR-90 were successfully differentiated into cardiac cells with a spontaneous rhythmic beating that started at day 8 or10 at rate of 40-50 beats/minute respectivly. Cardiac differentiation was confirmed by immunofluorescence staining; for IMR-90 differentiated cells (n=5), which was positive for cardiac marker, troponin t, cardiac protein that regulates calcium between actin and myosin fibers. Also, Troponin c was labeling of cell, a protein present in striated muscles and control contraction by calcium level modification. α-SMA (85%), protein that controls contractions and gets activated during stem cells' cardiac differentiation (Clement et al. 2005). For ARiPs differentiated cells, the staining was positive for Troponin t, troponin C and α-SMA fig (22). Some cells appear with two nuclei, indicating cell division and proliferation.

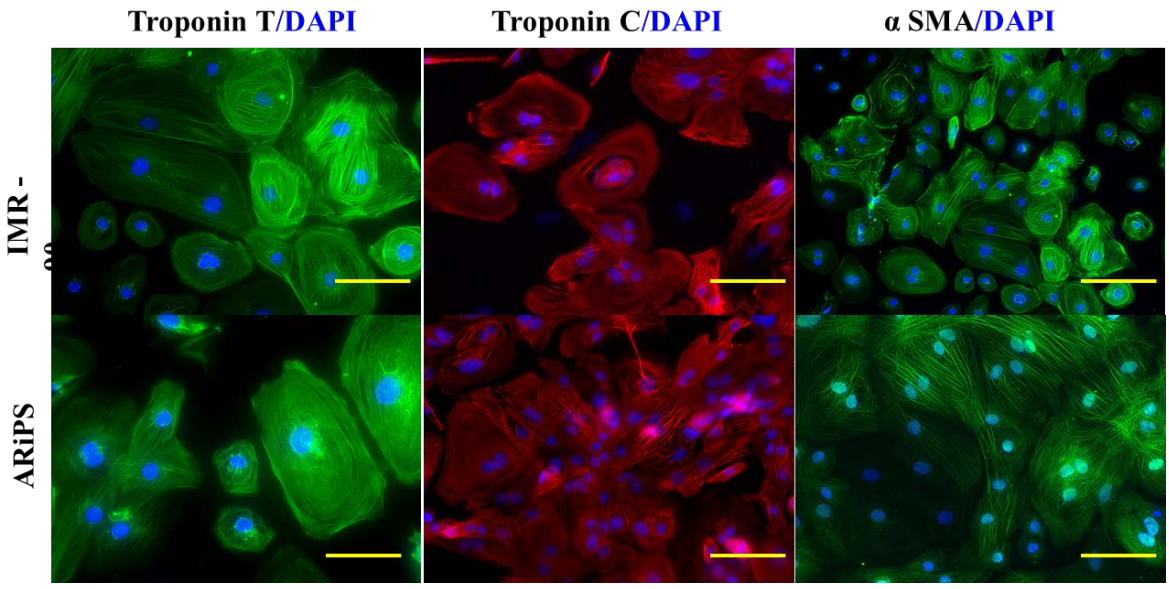


Figure (22): *Immunofluorescence staining for cardiac markers of both IMR and ARIPs generated cardiac cells. (1) Troponin T, (2) Troponin C, and (3) α-Smooth muscle actin.*

Quantitative RT PCR showed varied levels of genes expression at a different time during cardiogenesis. Cardiac differentiation is based on the activation and inhibition of WNT pathway (31). The activation is achieved by CHIR99021 at day 1 - 2, and the inhibition is by small molecules XAV939 between days 3-8, at day 5 the early cardiac marker ISL1 is expressed (Fig23). Cardiac induction is mainly due to BMP 4 growth factor along with CHIR99021 this is reflected by the expression of T-brachyury gene at day 2 and ISL1 gene at day 5. Complete differentiation is achieved by day 12 with the expression of both genes Nkx2.5 and cTnT. Gene expression was confirmed by q-RT-PCR for both cell lines, Fig (23). Oct4 was expressed on days 0-2 as cells are still preserving their pluripotent characters, T- brachyury at days 2-4 indicating mesoderm induction, ISL 1 at days 4-5 for cardiac progenitor cells formation, Nkx2.5 at days 7-8 indicating early cardiac cells development, and Troponin t at days 9-10 reflecting the complete cardiac cell maturation.

On the other hand, FACS analysis showed 90.5% cTnT positive IMR-90 induced cardiac cells, whereas only 38% cTnT positive induced cardiac cells. Although it was confirmed (31) that treating ARiPs cells with enrichment media at day 12 boosts the percentage of cTnT positive cells to around 90%. However, because cells were subjected to different treatments during differentiation, adding further enrichment treatment would cause more cell damage. This was confirmed by the observation of percentage of cells death, between days 3 and 5 when cells are cultured in insulin minus media.

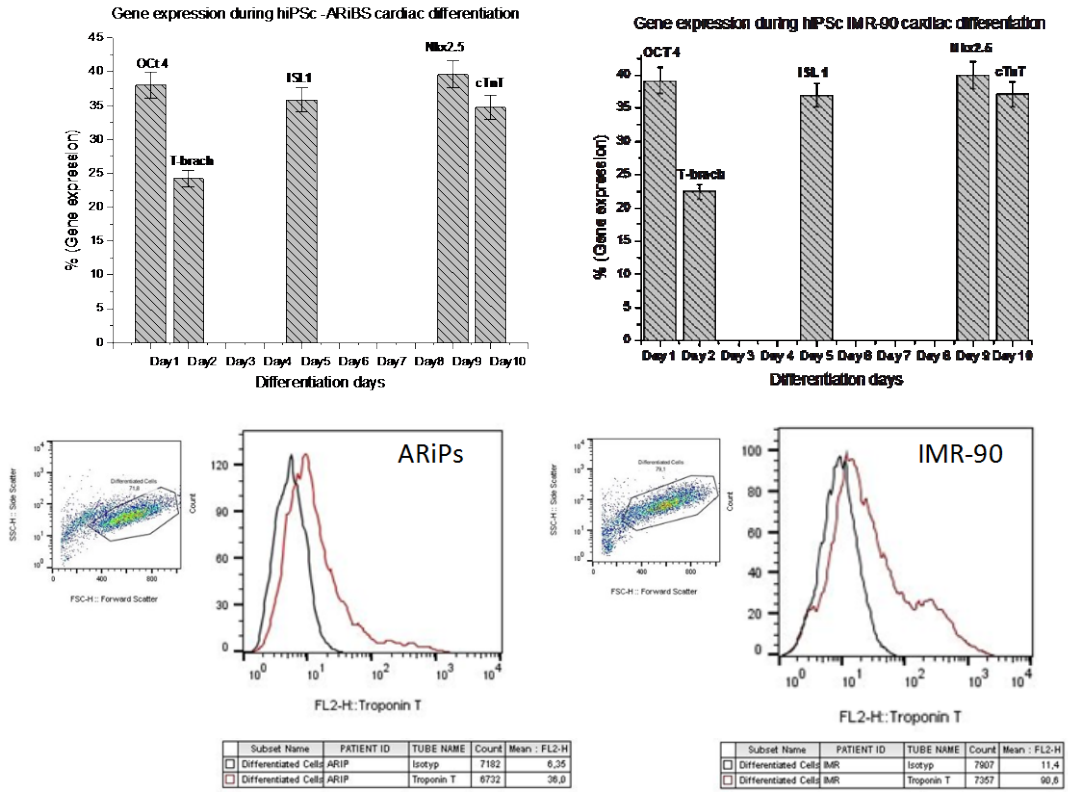


Figure (23) G*ene expression during cardiac differentiation. (1) OCT4 expressed between day 0- 2. (2) T-brachy expressed* in *day 2-4. (3) ISL 1 expressed in day 6. (4) Nkx2.5 expressed in day 8. (5) Troponin T Expressed at day 10.*

4.3: Reprogramming of porcine fibroblasts into porcine iPS cells:

The reprogramming protocol adapted from Kadari et al. (30) was depending on the use of lentivirus vector STEMCCA to generate induced pluripotent stem cells from porcine fibroblasts. This technique needs no repeated transfection or viral preparation and ensures transgene free clone production. Two clones were obtained and expanded for few passages before starting characterization tests. Half of each clone was propagated on either feeder free conditioned (mTeSr media and Matrigel) or on MEF cells and LIF conditioned media. It was noticed that about 50% of cells, which were propagated on MEF free conditions, start changing their morphology (spike elongated shape) and lose some of their pluripotent characters. Whereas, cells that were propagated on MEF and LIF conditioned media; preserve their original colonies shape (round sharp bordered cells) for until higher passage (p16) . This implies that keeping cells in the Feeder conditions is mandatory for maintaining their pluripotent state and avoid cells to go

through differentiation. .Fig (24)

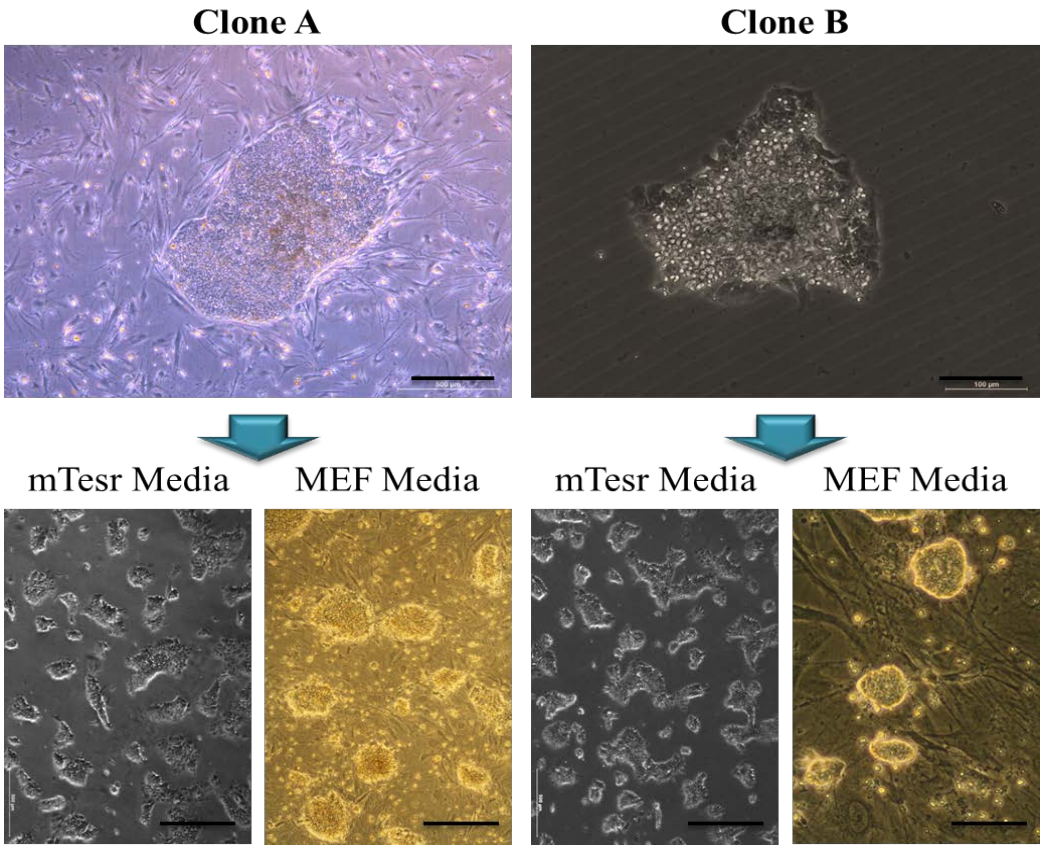


Figure (24): *Two different clones of porcine iPSc were propagated on different conditions; Feeder free culture mTeSr media and Matrigel, or MEF (mouse embryonic fibroblast) culture.*

4.4: Characterization of piPS:

Characterization of pluripotency markers of piPS cell, started after passage 12. Although it was challenging to find antibodies that reacted with porcine cells, two antibodies were found to be compatible. These antibodies indicate positive immunofluorescence staining, for SSEA4 (surface marker), NANOG, and OCT4 (intracellular markers) Fig (25). Stage specific embryonic antigen 4 (SSEA4), is a cell surface glycoprotein that is expressed in early embryonic development and stem cells. NANOG and oct4 are intracellular markers that are working together

in stem cell renewal and stabilization in undifferentiated state. These two intracellular proteins were positive in immunofluorescence staining of porcine reprogrammed iPSc at a percentage of 75% for NANOG and 80% for Oct4, whereas 60% for SSEA4.

Alkaline phosphatase assay was used as well to test pluripotency state of cells, giving faint red color after staining. Fig (25) however, the color intensity was not too strong as expected. The pluripotency state was confirmed, by the successful differentiation of the reprogrammed porcine iPSc cells into three germ layers; ectoderm, mesoderm, and endoderm from Embryoid bodies (EBs). Fig (25) Embryoid bodies start forming after culturing cells in low attachment surface 6-well plate. Aggregates start 24 hours later, average diameter of colonies were ~100±35µm. Differentiation started after transferring colonies to 12 well gelatin-coated plates. A few days' later aggregates started form different cell type with different morphology. Immunofluorescence staining for the three germ layers markers was positive; Alpha-fetoprotein (AFP) for endoderm, beta-II tubulin (TUBB) for ectoderm, and α-smooth muscle actin (αSMA) for mesoderm.

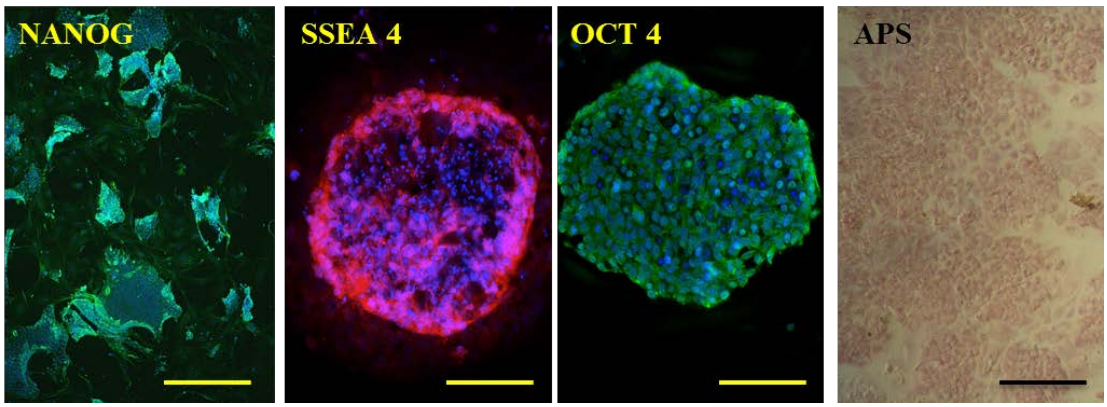


Figure (25): *Pluripotency characterization by immunofluorescence staining of surface markers NANOG and SSEA4, intracellular marker OCT4, and alkaline phosphatase (APS).*

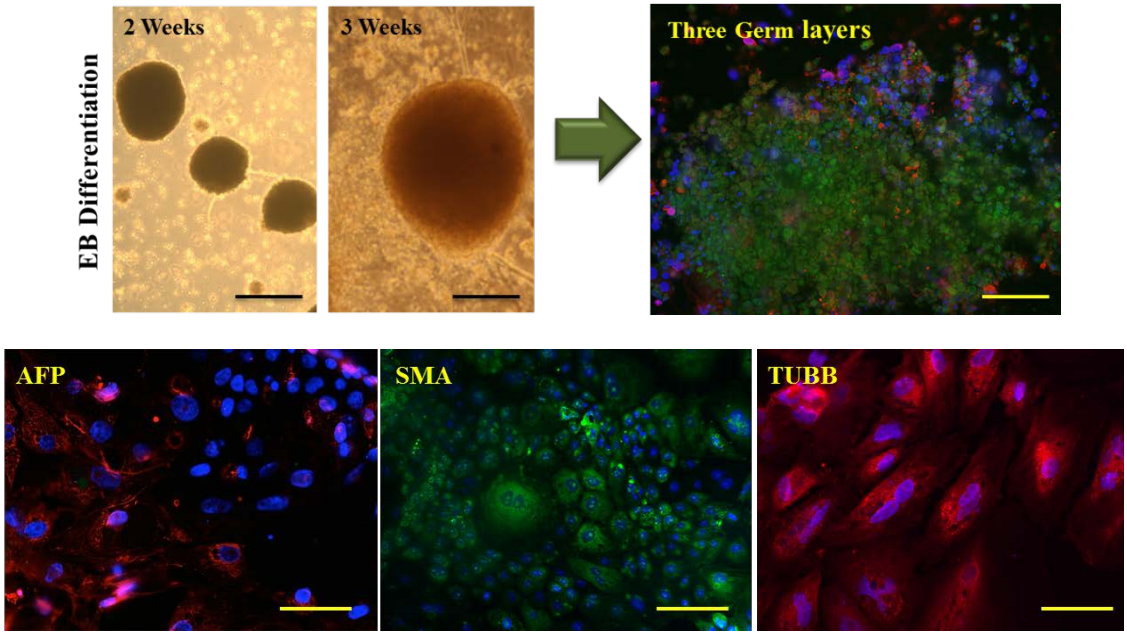


Figure (26): *Embryoid body formation at week 2, and three-germs layer differentiation of porcine iPSc at week 3; mesoderm (SMA), endoderm (AFP), and ectoderm (TUBB).*

Overall, these tests give a conclusion that the reprogrammed PiPSc have a great potential of pluripotent characters, if maintained under specific conditions; MEF cells plated culture and conditioned media that contains LIF and some essential growth factors. This will help in preserving cells in a stable pluripotent state during culturing and propagation.

4.5: Differentiation of piPSc into Cardiac cells:

The two different protocols used for differentiation were leading to the same results. The resulted cells were with positive cardiac markers, and morphologically identical to those results from hiPSc cardiac differentiation. However, piPSc-CM cells failed to accomplish cardiac rhythmic beating, which was achieved by hiPSc cardiac differentiation. Three different cell lines from two different colonies were used for cardiac differentiation, yet none has reached the complete phase of rhythmic beating. Although calcium content of culture media was adjusted to meet the optimal concentration needed for cardiac cell survival and functioning, yet normal beating

expected was not achieved.

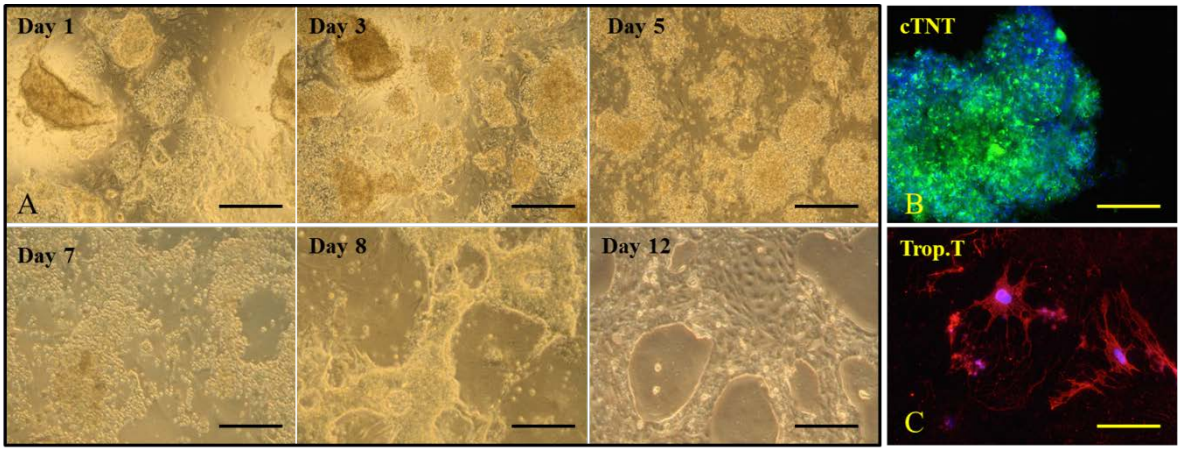
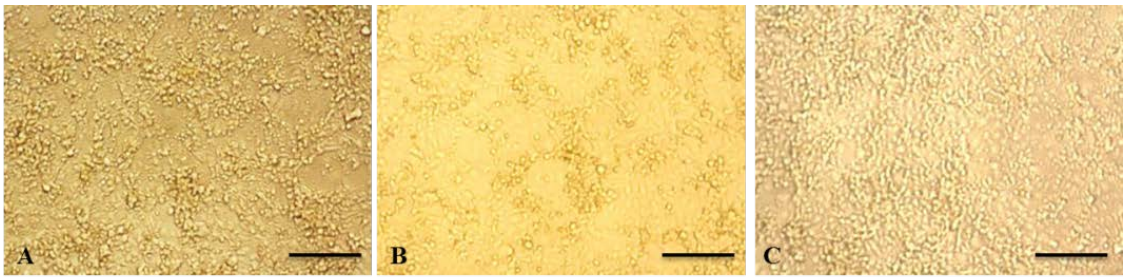


Figure (27): *Morphological changes of piPSc during cardiac differentiation; day (1) cells start aggregated, then at day (3-5) cells start forming monolayer, and by day (7) tubules formation followed by myoblast formation by day (12). (B) Troponin T expressed in piPSc cardiac generated cells at day 18. (C) Troponin C expressed at day 14.*

4.6: Recellularization of Cardiac Scaffolds:

4.6.1: Rat scaffold Re-seeding:

MMT viability test reflects cells' survival, by the number of viable cells per mL of hMVEC cultured in 2D static culture, using cell perfusate collected from 3D dynamic culture. Cells cultured in P1 (perfusate 1), was collected from 3D culture 48 hours post recellularization, had cell survival number of approximately $15X10^5$ cell/mL. Cells cultured with P2 (perfusate 2), which was collected 6 days post recellularization, had cell survival of $15X10^5$ cells/mL. P3 (perfusate 3) culture had a cell survival of $11X10^5$ cells/mL. Compared to hMVEC cultured in Normal media, $15.5X10^5$ cell/mL, P1 and P2 cell survival rate was almost similar, whereas cells cultured in P3 showed a slight decrease in survival rate compared to normal culture by 25%. In all three experiments, cell morphology and culture appearance were stable.



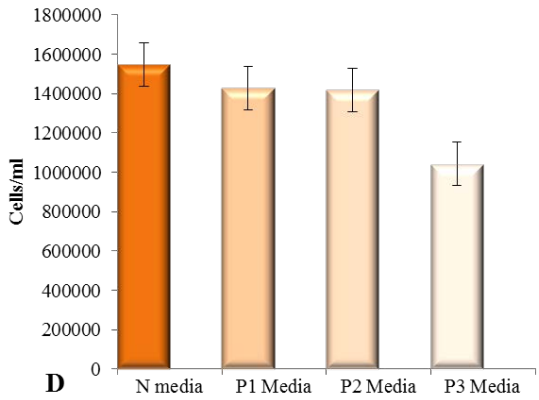


Figure (28): *Cell viability was measured in culture in three different perfusates from the revascularization culture. (A) cells culture in perfusate (1) which was collected 48-hour post revascularization culture. (B) cells culture in perfusate (2) which was collected 6 days post reseeding.(C) cells cultured in perfusate (3) which was collected 10 post reseeding. (D) comparison among three cultures indicates cell survival in three different perfusate. P value >0.05*

Histological tests (H&E) for recellularized scaffold, demonstrate cells distribution throughout the tissue with new large and small blood vessel regeneration. These vessels varied in diameter between 10-20 micron. In addition, it was observed that the vessels were lined with hMVEC. Cell distribution among the scaffold varied between, 40% in left ventricle 30% in apex area and 20% in right ventricle. Transversal sectioning of the scaffolds to three parts A, B, and C as in fig (12), confirmed cell distribution to be mostly in the left side of the heart 70%.

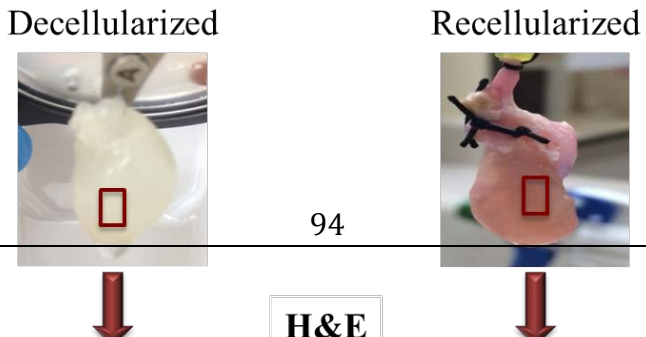


Figure (29): *H&E staining in decellularized and revascularized scaffolds. While decellularized scaffold showed no cells left, reseeded scaffold showed new vascularization with different size of blood vessels 20-50 micron.*

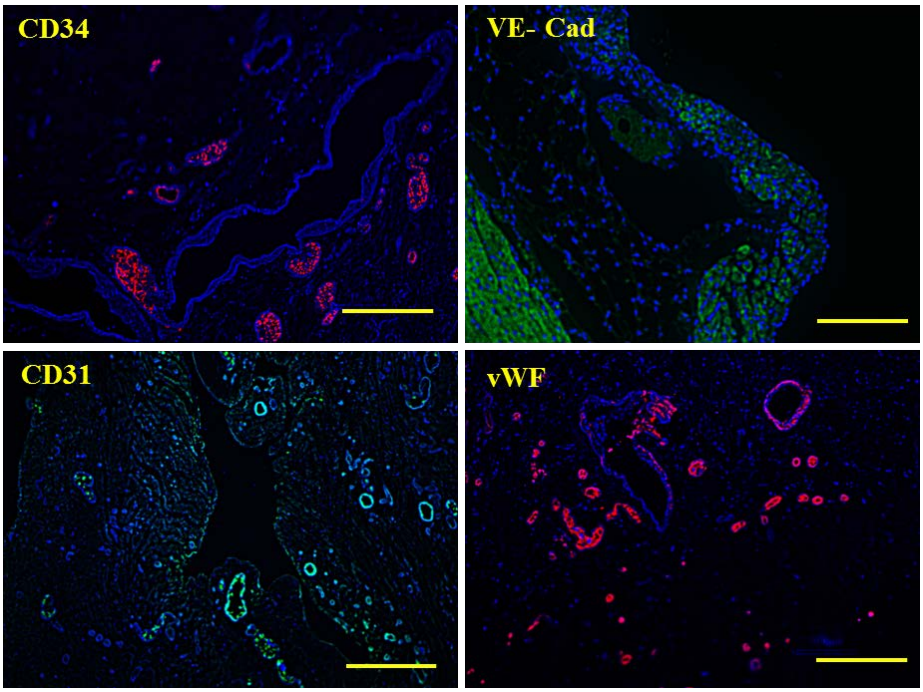


Figure (30): *Endothelialization markers in rat scaffold of 3D dynamic culture; CD31 positive cells were around blood vessels at cell junction. VE cadherin the second marker was positive and found at cell junction area. vWF was labeling endothelium areas as well in blood vessels. CD34 cells were located in the endothelium of small blood vessels.*

When recellularized rat scaffold stained by Immunofluorescence staining for endothelial cell markers, scattered areas on tissue were positive for CD31/PCAM (platelet endothelial cell adhesion molecule) specially surrounding the lumen of blood

vessels and mostly at cells intracellular junctions stained positive for von Willebrand factor (VWF) and as expected it was mostly localized in the endothelium area. The third marker was calcium dependent cell adhesion protein, VE Cadherin, which is usually found in cell junction areas, and it was covering 50% of the left ventricle area. In order to confirm new blood vessel generation, CD34 antibody was used. This marker is for endothelial progenitor cell and is believed to play a role in endothelium formation in new blood vessels. This marker was positive as well and located in the endothelium of small blood vessels.

To test the ability of a decellularized scaffold to generate cardiac tissue, 2D culture started by using section part of cardiac scaffold and re-seed with IMR- generated cardiac cells, human fibroblasts and human mesenchymal stem cells, in 2:1:0.5 ratio. As shown in figure (30) using cell crowns, construct start showing rhythmic beating at day 9 with 40 beat per minutes for 5 weeks when beating start slowing down at week 7 to be 25 beat per minute. H&E staining of cardiac section showed cells distribution along the matrix. Beside, immunofluorescence staining of cardiac tissue using cardiac marker troponin t confirmed cell-matrix interaction by the presence of troponin positive cells along the tissue. Once the culture parameter was standardized, 3D dynamic culture using bioreactor started. Although beating was not observed in the dynamic culture tissue, when the construct was placed under a microscope, small areas demonstrated slow contractions of 35 beat per minutes. Moreover, cell contraction was reduced, perhaps due to keeping tissue longer outside 37 C incubator, thus temperature inside the construct declined. This observation was true for cardiac tissue cultured in 2D culture as well.

Histological staining of 3D culture was more encouraging comparing to 2D one, as more cells interact with and attached to matrix 5-6 times more, with better distribution and matrix areas lining Fig (31). As expected, most of those cells were troponin t positive cells. Results of both 2D static and 3D dynamic cultures, confirm cells-matrix interaction with spontaneous beating. However, dynamic cultures consistently display more cells growth compared to static culture.

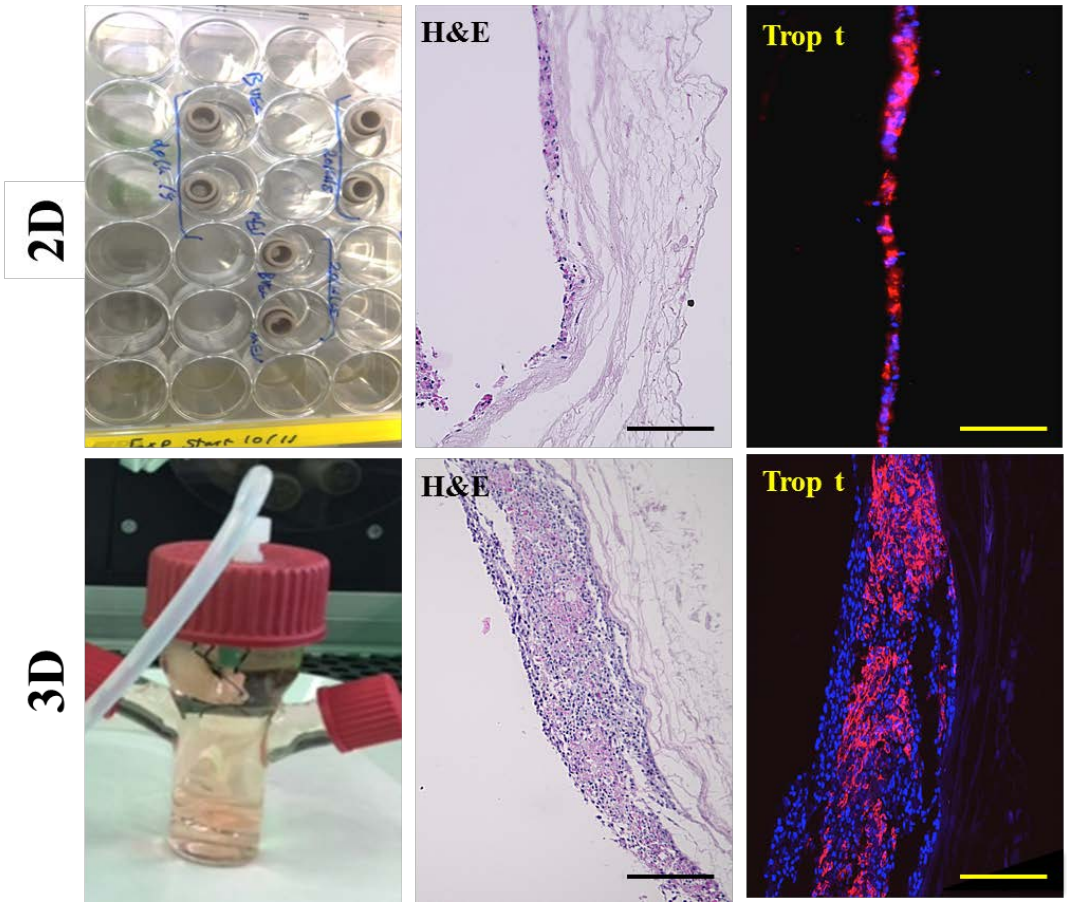


Figure (31): *2DStatic culture and 3D dynamic culture of cardiac generation. 2D culture with help of cell crown to place tissue around, and 3D using bioreactor flow system. H&E and Immunofluorescence staining (Trop t) for 2D & 3D Culture.*

4.6.2: Reseeding of Porcine cardiac scaffold:

Porcine cardiac tissue was re-endothelialized with hMVEC only or with hFibs in 2:1 ratio. Immunofluorescence using vWF marker demonstrated more blood vessels and endothelial cells in cardiac tissue with co-culture of EC and Fib (4 times more) compared to cardiac tissue cultured with only hMEC. Further specific endothelialization staining confirms new blood vessels generation in cardiac tissue. H&E staining of cardiac tissue, indicated endothelialization at different part of tissue with generation of different size of new blood vessels in different areas Fig (32). Compared to the decellularized tissue, Van Gison staining highlighted the new generated blood vessels by red color of the surrounding border. This was not observed with decellularized tissue. In addition, Pentachromic staining of re –endothelialized cardiac tissue labeled smooth muscle lining of new vessels, which was not the case with decellularized tissue. As coagulation is an expected to be faced in decellularized tissue due to endothelium absence, coagulation assay (n=4) was performed on decellularized,

re-endothelialized, and cadaveric cardiac tissues, to assess the ability of re-seeded tissue with hMVEC on reversing coagulation caused by peripheral blood plasma introduction. Standardized coagulation assay indicate a clot formation and coagulation after 10 minutes from plasma injection. This time was used as standard for three experiments to compare coagulation occurrence. In cadaveric tissue, when injecting plasma and waiting 10 minutes before starting flow, the pressure fluctuated throughout three hours experiment between 15-35mmHg. On the other hand, decellularized cardiac tissue, record a high-pressure range throughout the experiment after injecting plasma to be between 200-580mmHg. This may indicate coagulation in the vasculature tree that cause increasing in internal pressure. When the pressure reached 580 the cannula disconnected from the tissue as resistance was reaching its maximum limit. The re-endothelialized cardiac tissue however, recorded pressure was varied between 50-130 mmHg indicating resistance of coagulation and increased pressure but not as high as decellularized tissue. Fig(33). Immunofluorescence staining of porcine re-seeded cardiac tissue using three different markers was carried out on different time during 6 weeks. At week one after re-seeding scaffold with hiPSc-CM, hFibs, and hMSC, three markers were used Vimentin; showed minimum labeling (10%), CD90 positive cells were 35%, and troponin t positive cells were 40%. At week 3, vimentin labeled more cells (20%) compared to the past weeks. While CD90 positive cells were decreasing to 20% at week 3 after re-seeding, Troponin t positive cells were increasing to be more than 50%. By week 6, vimentin showed more positive cells (30%) with more aligned layers of cells, CD90 positive cells were almost vanishing to less than 5%. Troponin t cells on the cardiac tissue were almost covering 75% of the cultured scaffold areas. Fig (34).

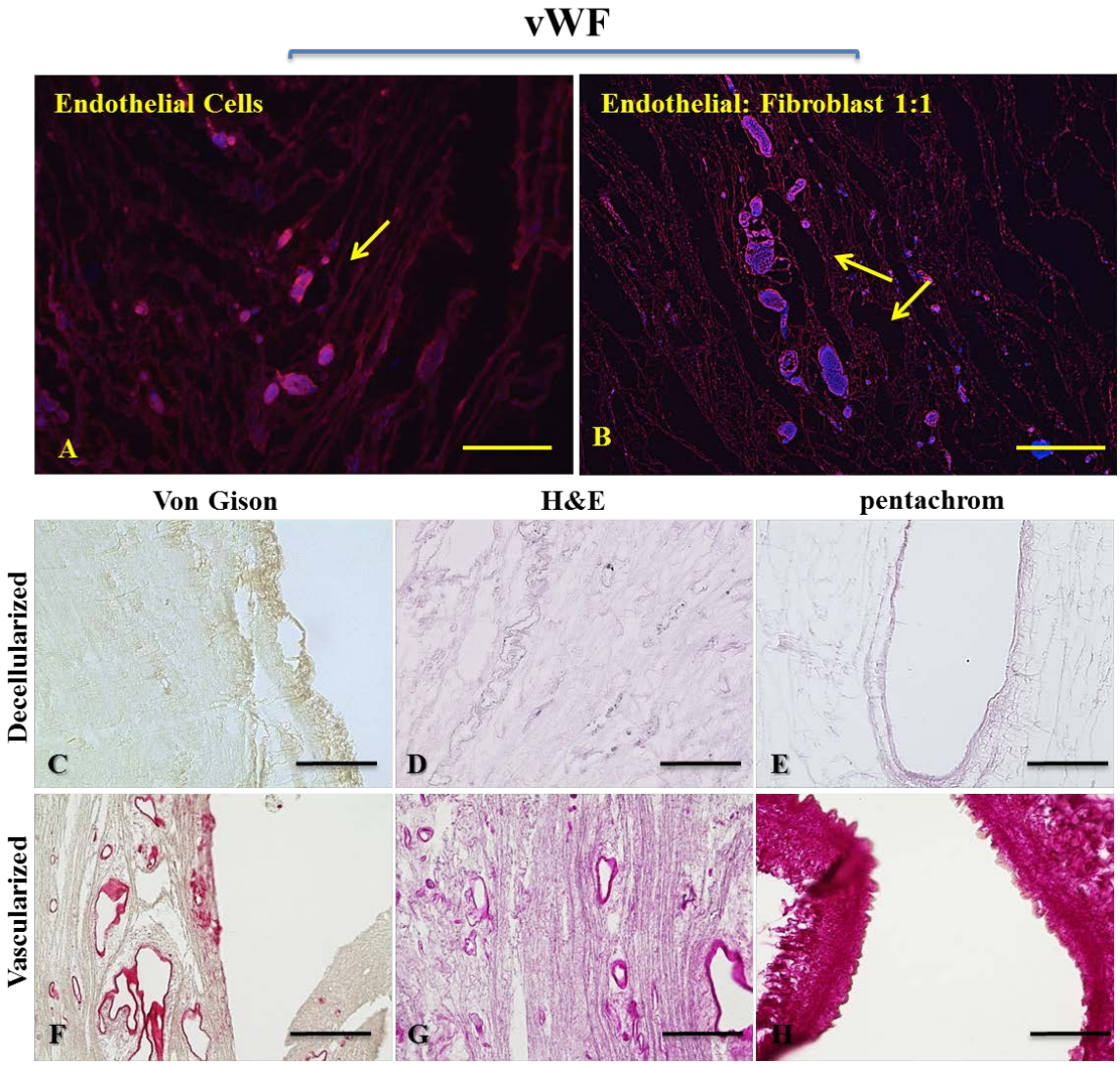


***Figure (32)**: (A, B) vWF staining of re-endothelialization Porcine scaffold using hMVEC alone or in coculture with fibroblasts at ratio of 1:1. (C, F) Von Gison staining for collagen fibers in both decellularized and re-seeded scaffolds. (D, G) H&E staining of decellularized and re-seeded scaffolds. (E, H) Pentachrom staining of decellularized and reseeded scaffolds.*

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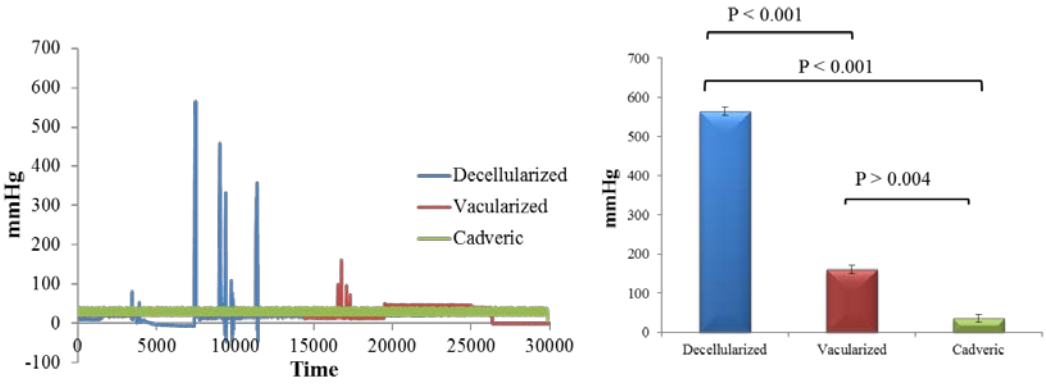

Figure (33): *functional coagulation assay for porcine re-endothelialized tissue Preferral blood plasma was injected in the vasculature of decellularized and in re-vascularized tissue. Right side graph showed changes in pressure through out the experiment, in decellularized, re-endothelialized tissues and cadaveric.*

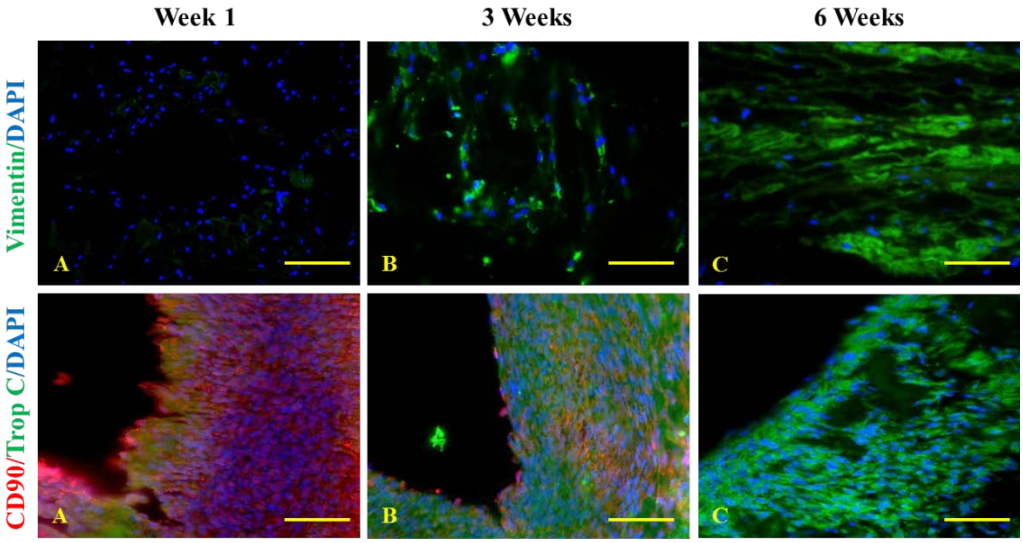

Figure (34)*: Immunofluorescence staining of re-seeded cardiac tissues with CD90 (MSC marker), vimentin (fibroblast Marker), Troponin t (cardiac marker) along a period of 6 weeks culture. Upper line, vimentin showed minimum labeling at week 1 , which was significantly increased by week 3, and at week 6 was more intense. Lower line represent MSC fate throughout the 6-week culture, indicating gradual differentiation toward cardiac cells as troponin t increases at week 3 and doubles at week 6.*

Chapter (5)
Discussion

Discussion:

According to word health organization (WHO), global death is attributed to cardiovascular diseases yearly (1). Worldwide it's the number one cause of death, especially in low and middle-income countries. Heart failure (HF) is one of the most common heart diseases as it's estimated that 38 million of global population with increased age (>65 years old) are suffering from HF. These patients suffer poor quality of life, and frequent requirement of medical care involvement that causes billions of dollars a year (1,2). While some cases of heart failure could be resolved by artificial mechanical instrument, the majority still required heart transplantation with long waiting list for a donor. Even with the donor availability, the recipient will face problems such as rejection and lifelong immunosuppressant intake. Alternatively, the bioengineered cardiac tissue or myocardium, using the patient own stem cells provides a solution to overcome transplantations hurdles. In heart failure, the myocardium progressively loses its contractile function due to fibrosis, necrosis and extracellular matrix degradation and remodeling (3). Many researchers attempt to reverse this remodeling partially, by injecting contracting cardiac cells and biomaterial (2,3). In 2014 Chong and Murray showed that human embryonic stem cell- generated cardiomyocytes can re-muscularized a lesion in monkey infarcted heart for three months however, ventricular arrhythmia on the engrafted primate was observed later (3). In another study, researchers injected alginate biomaterial (as Matrigel) intracoronary in small animal models, but with limited myocardium remodeling results (2). Under the same principal, many scaffold material and different cells have been used to regenerate myocardium tissue, yet it fails to fulfill the successful tissue engineering requirements; biocompatibility with cells, biodegradability in host tissue allowing new cells growing, consistent mechanical properties, and right scaffold architecture (5,7). Although, some artificial scaffold with anisotropic behavior was used with cardiac cells to regenerate myocardium tissue, it fails to match the native cardiac fiber architect. Native myocardium is composed of alignment of collagen fibers and myosin that possess mechanical and electrical anisotropic differences across the heart depending on wall thickness (7). Since this property clear to be important for functional cardiac tissue, it's important to replicate this feature in any replacement engineered myocardium (6). In that path a group in Harvard medical school established a honeycomb porous synthetic scaffold seeded with rat neonatal myocytes to overcome

the structural limitations by other scaffolds (4). The resultant product was able to closely match the native myocardium properties in term of alignments, stiffness and mechanical properties, but with limitation in scaffold thickness manipulation. In most of the above studies, the resultant contractile structure last for short period of time, due to poor neovascularization, thus less nourishment to meet cardiomyocytes demand and survival (8). Artificial scaffolds constrained by long term functional graft, as it lack the ideal architecture and mechanical properties of physiological scaffolds. For successful myocardium generation and clinical application, it's important that scaffolds establish the physiological tissue mechanical properties and shape to allow immediate perfusion upon implantation. Many studies explained the optimal mechanical scale and physiological environment for cardiomyocytes survival and functionality (9,10). Most of which comply with physiological scaffolds characteristics and dimensions, in term of micro and macro architecture, ECM content, and perusable vasculature bed (13)

5.1 Decellularization:

In this study, an optimization of decellularization protocol was carried out to establish a bio-artificial cardiac patch. Therefore, (i) decellularization of a native heart tissue from rat and porcine using standardized protocol was preformed. (ii) Scaffold quality, ECM content and structure was analyzed. (iii) Porcine iPSc line was establishment, differentiated toward cardiac cells using established protocol, and differentiation of human iPSc to cardiac cells using protocol provided by Kadari et.al (2015), (iv) Finally, re-vascularization using co-culture of endothelial cells and fibroblasts allowed (v) testing the functionality of vascularization using coagulation assay showed a reduction in coagulation and clotting. (vi) Re-seeding the cardiac scaffold with three different cell lines facilitate the generating of contractile cardiac patch.

In a small-scale rat model, following a decellularization protocol established by ott.et.al 2008 (15), hearts were decellularized successfully resulting in cells, and nucleic acids-free scaffold. Thereby a biocompatible three-dimensional structure with intact perfusable vasculature was generated. Aortic ligation of hearts is a key step for successful decellularization as it is important to ensure the flow through coronaries in order to facilitate that decellularizing solution reaches all scaffold areas. This explains the decellularization and cells removal of peripheral parts of the heart in the first 2 hours, proceeding towards center with time. Extracellular matrix content was the main concern

for many researchers, when choosing the appropriate decellularization detergent type and exposure time (11,12). Since ECM represents the secreted product of cells and provides cues for cells proliferation, migration and differentiation, the used decellularizing agent should exerts a maximum cell removal with minimum ECM composition and ultrastructure damage as possible in order to result in a functional scaffold (13). Choosing a suitable agent and tailoring the protocol and exposure time are requirements to achieve a cell-free scaffold with minimized ECM disruption. Although sodium dodecyle sulfate (SDS) showed an ultrastructure disruption and some elimination of growth factors, it was found to be the most effective anionic detergent in removal of nuclei material from dens tissues and organs such as kidneys and heart compared to Triton X 100. Moreover, SDS was more effective in removing cell residuals compared to other detergents.

In an attempt to compare alternative less toxic detergent, sodium deoxycholate was used in porcine heart decellularization in parallel with SDS, following the standardized parameters of rat tissue. With SDO decellularized tissue, cell removal was very slow and partial, followed by a steady state of no further cells disruption or removal, explaining light pinkish color appearance of scaffold after 14 days of decellularization. Furthermore, recorded flow rate throughout the procedure, also reflects a steady state range between 50-68 ml/minutes indicating a pressure building inside the vessels due to cells blocking the flow. In addition, H&E staining indicated that only 60 % of cells were removed, with some cells and nuclei retention in different areas. On the other hand, SDS decellularized hearts, showed rapid cell removal and faster changes in tissue appearance and complete whitening by day 3 indicating a successful decellularization. This was confirmed by the increased recorded flow rate, which reached maximum of 160 mL/minutes at day 10, that suggested continued cell removal and the decrease of internal pressure and resistance inside the heart chambers. In addition, H&E staining for both rat and porcine scaffolds showed an almost complete cells removal by the absence of cells and nuclei compared to cadaveric tissues. DNA quantification demonstrated the loss of more than 95 % of DNA in decellularized scaffold. Although TritonX-100 was used along with SDO in decellularization, yet this has not enhanced cell removal process. This meant that the key in choosing a decellularizing agent for an organ as the heart, is using a powerful cell membrane disruption agent to ensure good decellularizing process as mentioned in (13) The effect of SDS on ECM ultrastructure was considered as well; therefore, a series of different exposure times

was tested to identify the optimum outcome with less exposure time compared to SDS. In Ott protocol (2008), SDS was perfused in the heart scaffold for 6-7 consecutive days followed by TitonX-100 for 24 hours and PBS- for 5 days (16). Here, the SDS was used for only 3-4 days followed by TritonX-100 for 24 hour and PBS- for 7 days without affecting the quality of the resultant scaffold. In conclusion, with thick tissues like myocardium, SDS was solely the most effective detergent to remove cellular component (antigenic proteins, DNA, lipids) and conserving extracellular matrix. With slight manipulation in detergent tissue exposure time, more ECM proteins and essential growth factors can be revived. This was the baseline to decellularized myocardium in many studies, beside other techniques to alternate with, such as tissue freeze and thaw that allowed cell membrane disruption or alternating washes with water to create tissue hypotonic shock, thus less SDS-tissue exposure time (17).

5.2 Extracellular Matrix:

Extracellular matrix examination by histological staining showed the conservation of the major histoarchitecture. In addition, a detailed structure of collagen fibrils and their alignment was observed using SEM (fig 16). Collagen and trichrome staining reflected a homogenous surface of collagen structure, which was devoid of cell. Also staining for fibronectin and elastin showed a homogeneous surface of both with lower intensity compared to cadaveric, this was in parallel with other studies (17, 16, 19), which confirmed the preservation of most important matrisome proteins as fibrillin-1, collagen IV, collagen VI, and elastin. However, contradictory to studies by Weymann and Akhyari (20,21) that showed heterogeneous structure of collagen in tissue treated with 1% SDS while homogeneous structure in tissue treated with 4% SDO. Surprisingly, in the same study, quantitative analysis, revealed a higher amount of elastin, laminin and GAGs in tissues treated with 1% SDS compared to those treated with 4% SDO. In the present study, when comparing total sGAG in cadaveric, SDO-, and SDS-treated tissue, a reduction in about 30% of total sGAG content in SDO treated tissues, and 40% in SDS treated tissue compared to cadaveric was observed (fig. 20).). In Mirsadraee et al., SDS concentration was reduced 10 times then used in decellularizing a porcine myocardium along with protease inhibitors, nucleases and hypotonic buffer sequentially with agitation. The resultant scaffold indicated

cell-free areas with retained cells basal membranes covering the surface, collagen and sGAG were also preserved (19). On the other hand, Weymann et al. used 4% SDS alone to avoid any physical, chemical, and enzymatic treatments, as it has been reported that these treatments caused disruption of normal ECM structures. (20). Therefore, it's important not to use multimodal decellularization protocols to preserve mechanical stability for future cells seeding. True other detergents and protocols preserve more sGAG, and ECM essential proteins (18), yet on the coast of less decellularization efficacy. As long as there is uniform structure of collagen, laminin and elastin, to home and surrogate new cells, decellularization protocol should be approved. In general, there is no decellularization protocol that doesn't cause ECM disruption, with complete cell and DNA removal, but choosing the (i) right solution (ii) at the right concentration (iii) on the right tissue is important to take into account. For each tissue or organ and species, a customized protocol must be established or tailored and must be optimized to yield a biocompatible scaffold for future seeding and clinical application.

5.3 Differentiation of hiPSc in to Cardiac cells:

In 2006, Takahashi & Yamanaka reprogrammed mouse somatic cells and tail fibroblast using transcription factors, namely Oct3/4, Sox2, c-Myc, and Klf4, and produced induced pluripotent stem cells. Later in 2007, they reprogrammed human skin fibroblast the same way and produced human induced pluripotent stem cells that can be differentiated into different lineages (22,23). Induced pluripotent stem cells hold huge promises in clinical applications, and cardiac regeneration. In myocardial infarction models, cardiomyocytes derived from induced pluripotent stem cells generated were used to treat infarcted areas, leading to improved cardiac function, enhanced cardio protection and provided homing and survival of infarcted tissue (24,25,26). Along with this research area, myocardium tissue engineering made good progress in employing induced pluripotent stem cell cardiomyocytes to repopulate different scaffold material in order to create functional cardiac tissue (27,28,29). In the present study, combined approaches of creating porcine cardiac scaffold and the use of either differentiated human iPSc or porcine iPSc to cardiac cells in order to establish cardiac patch were focused on. Two human IPSc cell lines were subjected to cardiac differentiation; IMR-90-4 commercial cell line and ARiPs cell line, which was established by Edenhoffer

group through cellular reprogramming (30). The differentiation protocol adapted from Kadari.et.al (31) is based on manipulation of canonical Wnt/β-catenin signaling of IMR-90 or/ ARiPs in 2D culture that yield to approximately $1X10^7$ cardiac cells, starting with $1.8X10^6$ iPSc. At day0, cells were expressing Oct4 gene indicating pluripotency state, when treated with BMP4/CHIR this resulted in up-regulation of mesodermal marker T-brachyury, for cardiac mesodermal specification, when cells were treated with Wnt inhibitor (XAV), early and late cardiac precursors markers ISL1 and Nkx2.5, expressed respectively. Subsequently, cells were matured to beating cardiac cells (for 160 days) expressing cTnT cardiac marker. The effect of insulin on cardiac differentiation was a concern in some studies, as it showed to slow down the effect on stage-specific action, namely mesoderm but not mesoendoderm (32). Therefore, insulin was omitted from day 1 for mesoderm induction and cardiac specification, then added back at day 7. Whereas, in the present protocol, insulin was not excluded during mesodermal induction, up until cardiac specification step, as it was shown that media with no insulin to start with would stress cell before cardiac specification step (31). This could explain the modest expression of T-brachyury.at day 2, and the requirements of enrichment step for boosting the percentage of cardiac cells. This was by suppressing the growth of other cells through omitting glucose from culture media and substitute with Lactate (31). FACS analysis showed 90% cTnT-positive cells when enrichment media was used, whereas only 39% cTnT positive cell were observed if no enrichment media was added. This was the case in both cell line IMR-90 and ARiPs and parallel to. Findings were supported by immunostaining of cell using cardiac markers; Troponin t, Troponin c, and α Smooth muscle actin. The question whether to include or omit insulin in the beginning of differentiation remains unclear, as n enrichment step found to subject cells to further stress, specially post insulin minus steps. Yet, the used protocol for differentiation (31) was successful to a big extent in our case and intensions. Moreover, it was applied on hiPSc in suspension culture (aggregates) and was able to yield beating aggregate within 7-10 days. Cardiac cells generated from hiPSc following the same differentiation protocol were used in re-seeding biological (small intestine submucosa) and 3D beating cardiac patch that possessing physiological functionality successfully (33). In addition, the same cardiac cells were employed in the development of a flexible, porous 3D electrode (34) designed for future clinical applications. Overall, the hiPSc were successfully differentiated into a beating cardiac cells, which is found to be suitable for tissue

engineering purpose. In future, some modulation on differentiation protocol steps could be helpful to increase resultant cardiac cells number and specificity; cardiac cell type.

5.4 Reprogramming of porcine fibroblast and iPSc generation

The second approach in cardiac patch generation was the use of porcine induced pluripotent stem cell generated cardiac cells to repopulate porcine myocardium and establish allograft model. No doubt that porcine is an outstanding model for most of biochemical studies and transplantation, as it's very close to human among other species. For the same reason porcine iPSc would be an excellent source for creating clinical model, to be up scaled later into human. For this aim, reprogramming of porcine skin fibroblast generated a porcine induced pluripotent stem cell. Initial protocols of reprogramming involved retroviruses as a mode of transgene delivery (22,23), however, the produced iPSc were clinically restricted because of integrated transgenes that have the risk of tumor formation (38) or insertional mutagenesis (39). In addition, the negative effect of transgene re-expression on differentiation (40) potential and pluripotency (37). Alternative to these virus based protocol, other method for iPSc generation has been introduce to circumvent the genetic modification effect such as the use of microRNAs [44], Sendai virus (45), transposons [41], episomal plasmids [42], synthetic mRNA (43). However, these non-integrating strategies have limitation in terms of low efficiency, slow reprogramming kinetics, and poor reproducibility (46). Still, retrovirus and lentivirus are considered as the best methods in iPSc generation. Later the method of using one vector that carries the four transcriptional factors in one caste for transfection and iPSc production was established, and showed low risk of insertional mutagenesis (47). Thus, a method based on the same principle was used in this study to generate porcine iPSc. Following Kadari.et.al protocol (30), (37) that based on the use of single polycistronic excisable lentiviral stem cell cassette (STEMCCA) encoding four reprogramming factors Oct4, Sox2, Klf4, and c-Myc through and producing porcine iPSc.

Herein, porcine iPSc were successfully produced in a serum-free system and satisfying the criteria of multipotency state predominantly naïve-state, and few prime-state. In 2007, Brons et.al (49) isolated the first epiblast stem cells from mammalian embryos. Unlike ESC, these cells grew as flat compact colonies, and they were positive for Oct 4, Nanos, and SSEA1. In addition, the cells were passaged by enzymatic single cell dissociation, showing good growing rate and were LIF dependent. Later, another study

classified these epiblast stem cell in to two types depending on their pluripotency state; (i) Prime state, or (ii) Naïve state (51). Primed state cells displayed flattened colonies and expressed SSEA4, TRA-80, and TRA-61 markers, whereas the naïve state cells had round dome shape and express the SSEA1 surface marker. Both types were positive for Oct 4, and NANOG (50). In the present study, , cells displayed naïve state characteristics, morphology, and surface markers expression. When colonies were dissociated as single cells and propagated, part of the cells were on feeder free culture, particularly Matrigel, the other part was cultured on a feeder cells using LIF 2i media. The behavior of cells was contradictory among the two cultures as indicated in (53), as in feeder-free culture, cells started losing their pluripotency features expressing a flat shape and spikes that indicate the start of differentiation. Whereas, in feeder LIF 2i culture, cells appeared in a round dome shape with defined border line indicating their preservation of pluripotency state. This shape resembles mouse ESC in features and culture condition, and as indicated before, naïve-state pluripotent stem cells. This agreed with most of the porcine iPSc in other studies (48,49). Zhang et Al. were able to generate porcine iPSc by reprogramming porcine adipose tissue stem cells using drug-inducible expression of (Oct4, Sox2, c-Myc and Klf4), and the resultant cell line were naïve–like cells in a feeder and serum independent system (35). Here, cells were cultured all time on MEF cells in LIF 2i conditioned media except when cells were differentiated. When they were cultured on Matrigel, cells started losing their pluripotency features. This was not because of MEF-free culture only, perhaps also because of the absence of LIF. Since it has not been cultured in LIF 2i media (35), one cannot definitely claim that these cells were Feeder dependent but for sure LIF dependent, as its one of the hallmark of naïve state cells (53). Pluripotency markers of the current established porcine iPSc, were also similar to Naïve-state cell; as they were positive to OCt4, NANOG, and SSEA4 but negative to TRA-80, and TRA-61. These results were similar to most studies that established porcine iPSc lines. Some has more markers expressed (52) as TRA-80, TRA-61, and SSEA1 in addition to the above markers, however it was not clear whether their cells follow the prime state or naïve state criteria. Others classified their cells as prime-state cells, which explain their expression to SSEA1, TRA-80, and TRA-61, as it's the classic marker for prime state cells (50). In a third study, produces porcine iPSc line, which has both the prime-state and Naïve state characteristics; positive for NANOG, Oct4, Sox2, and weak or negative for SSEA4, TRA-80, and TRA-61 48). The diversity observed among studies, in

porcine iPSc surface markers expression; SSEA1, SSEA4, TRA-80, and TRA-61 can be explained by differences between antibodies used, and embryo developing stage at which marker expressed among different species. For example, in the mouse, SSEA1 is expressed in the inner mass cell, whereas in human it is absence, but cells are positive for SSEA4, TRA-80, and TREA-61 (55,56). Because limited research focused on this point, this remains unclear. To add more complexity, those markers expressed in inner mass cell and in trophectoderm in bovine, the reason why these markers couldn't be used to identify bovine ESC.

Pluripotency of cells was demonstrated also by a positive Alkaline phosphatase test, and by their ability to differentiate to embryoid bodies and consequently up regulate genes corresponding to trophoectoderm or three germ layers; α-Fetoprotein (endoderm), Beta –II tubulin (ectoderm), and α-smooth muscle actin (mesoderm). The results were encouraging to go further and test the ability of those cells to commit toward cardiac cell. Despite the fact that different groups established porcine iPSc lines, none have achieved a full differentiated cardiac cells from 2D culture. Most of these studies had attempt of cardiac differentiation through embryoid bodies' formation (56). The cardiac regeneration in porcine model was focused on one path, which is injecting human iPSc (57) or porcine stem cells (58) into an infarcted ventricle to promote cardiogenesis through differentiation in vivo, mostly to endothelial cells. Herein, cardiac differentiation was induced following the same pathway as in human iPSc cardiac induction; first stimulating Wnt pathway by BMP4 and CHIR then inhibiting by XAV 939. Morphological change progression through differentiation resembles the human iPSc. In addition, cells were positive for the cardiac marker Troponin T, but no beating was observed indicating either immature state of cardiogenesis or that the formed myoblasts-like cells were fused and form myotubes and skeletal cells. Calcium concentration in the differentiation media also might not be enough to elicit beating or there was incomplete calcium transporter or regulator gene expression during differentiation. Further investigation would be required in that direction to overcome this obstacle.

5.5 Re-endothelialization of cardiac scaffolds:

One of the most important points in regenerating a tissue is to provide a sufficient nutrient supply for the reseeded cells to ensure survival and differentiation (59). This is achieved by re-endothelialization of decellularized tissue before introducing other

tissue-specific cells. Scaffold re-seeding started by delivering human endothelial cells in manner to ensure their deposition on the vascular conduit surfaces and the ventricular cavity, not to the parenchyma of the scaffold. It was demonstrated by one study that introducing rat endothelial cells through arterial and venous pathways in decellularized heart model, results in a good lining of coronary vessel conduits and proliferation along the vessel conduit walls (59). The same was observed in this study on rat model reflected by H&E staining and endothelial cell markers CD31, VE-Cadherin, and vWF. Moreover, due to retrograde perfusion a good endothelial cell survival rate was detected throughout the experiment with reduced apoptosis rate. Due to the fact that the big surface area of porcine heart and large cell number needed for endothelialization only part of the heart tissue was used. As many studies confirmed the advantage of co-culture of fibroblast with endothelial cells, porcine decellularized cardiac scaffold was endothelialized in that manner (60). Fibroblasts found to support tubules formation during vascularization and fibroblast-derived proteins and growth factors promote endothelial cells sprouting, capillaries-like networks in vitro and maintain vascular integrity (61). This was emphasized in the current study, when fibroblasts co-cultured with endothelial cells, more new blood vessel had regenerated compared to monoculture of endothelial cells (Fig.33). In addition, with a decellularized scaffold, fibroblast augment the endothelial cells-lumen formation, by increasing the stiffness of extracellular matrix as noticed in (62). Positive endothelial cell makers and cells phenotype had to be confirmed by a coagulation reduction efficacy test, which reflected the growing of new boold vessels and contribute anticoagulation effect. Therefore, it was important to establish an assay to determine the thrombogenicity of scaffold after recellularization. In a re-endothelialized rat heart study, a thrombomodulin measurement assay was used for that purpose (59). This assay depends on colorimetric measurement of protein C activation and consequently thrombomodulin expression, hence anticoagulation stimulation (63). As pressure was determined thorough out decellularization and recellularization processes, it was a parameter to count on for coagulation measurement in that test. Pressure was measured after injecting peripheral blood plasma in to the vascular tree of decellularized, reendothelialized and native tissues allowing 10-12 min coagulation time, which was standardized in control experiment. Highest pressure recorded in the decellularized tissue was reaching above 500 +/- 4.55 mmHg after plasma injection, and because of that the cannula ligated the tissue was forced out indicating blockage. On the other hand, the recellularized tissue

recorded a maximum pressure around 160+/- 16.44 mmHg compared to cadaveric, which reached maximum pressure of 35 mmHg. These results refer to the reverse proportionality between the presence of endothelium and maximum pressure recoded, with the normal endothelium as in cadaveric recorded the lowest pressure. Although pressure was still high in the re-endothelialized tissue, the construct was able to prevent clotting to some extent compared to decellularized tissue, when plasma was injected in the vasculature. In future, thromodulin measurements; vessel anticoagulant efficiency marker, and/or protein C and other coagulation metabolite will be considered.

5.6 Re-seeding of cardiac scaffolds using human induced pluripotent stem cells generated cardiac cells, human endothelial cells, and Fibroblasts:

The last part of cardiac tissue engineering was to deliver suitable cardiac cells. Cardiac regeneration in the past decade depended on stem cells injection into infarcted areas in an attempt to regenerate the damage cardiac tissue. While the potential of ESC and iPSc to differentiate into cardiac cell is undisputable, their origin presents challenges (65). ECS originate from inner mass cells that might consider an allogeneic when transplanted and may require immuno suppressants. iPSc originated from fibroblast reprogramming that can be used in an autologous fashion. While, the reprogrammed virus could integrate in the host raising the possibility of neoplastic transformation (65,66). Recent studies refine the generation of iPSc and overcome this risk by the use of episomal gene delivery, excisable transgenes, synthetic mRNA or cell-permeant recombinant proteins (66). Because of the large number of cells required for this purpose and the limited outcomes, PiPSc cardiac differentiated cells was eliminated from cardiac repopulation step. This was one aim of this study to create an active cardiac tissue within the same species (autogenic) and provide a line of clinical model to use a personalized iPSc for treatment. Despite modest literature studies, time limitation and obstacles faced in generating beating porcine cardiac cell, it's believed that a protocol fine-tuning of PiPSc cardiac differentiation would overcome this problem. Alternatively, hiPSc generated cardiac cells were used in a step toward allogenizing a xenograft by repopulating porcine cardiac tissue and successfully create a cell-matrix coupling with rhythmic beating. When this model was established first, it will set and standardized different culture issues and cell adaptation between two different species before moving further for auto transplantation within the same species. Although it was difficult to observe that in three-dimensional model because of tissue thickness, it was detected in certain tissue areas under microscope. Nevertheless, in two dimensional cell crowns culture 95% of the repopulated tissue area was conducting an unsynchronized beating that last up to 6 weeks at rate that varied between 30-95 beat/ minutes. The reason for this wide range in beating areas among 2D and 3D cultures is might be the scaffold surface geometry and folding, as cells in 3D scaffold were injected intramural, find their position and anchored on the first suitable contact point of ECM. Whereas, in 2D culture, because scaffolds were stretched around cell

crowns, this allow the complete exposure of ECM area with cell lacuni ready for new cells to be deposit and find the right tissue site. In both cases, human induced pluripotent stem cell-generated cardiac myocytes (hiPSc-CM) were growing in aggregates throughout the scaffold, which is not the case in a physiological cardiac tissue. This may explain weak contraction forces in some areas of tissue and the diminishment of others. Another explanation could be the limited CM number, lack of cell gap junction and lack of cardiac conduction system. Because cardiac fibroblasts has a crucial role in regulating electrical cardiac conduction and myocardial contraction response, co-culturing of fibroblast with iPSc-CM was interesting to test in an attempt to improve construct electrical propagation and mechanical force.

Not only fibroblast, but also mesenchymal stem cells were shown to improve ventricular function if injected in an infarcted lesion (66). This was not because of their trans differentiation to cardiac cells, but rather by their action thorough paracrine release of kinases or long-term inhibitory effect of Wnt signaling pathway that promotes antiapoptotic effect (69). Another hypothesis suggested that mesenchymal stem cells stimulate the proliferation and differentiation of stem as part of their regenerative repertoire (64). Because of the clinical relevance of fibroblasts and MSC in cardiac repair, co-culturing of both fibroblasts and MSC along with iPSc-CM was important to be considered, for optimizing active cardiac function of the reseeded cardiac scaffold. The resulted construct started unsynchronized beating rhythm after two days of 40+/- 1.3 beat/minute, which then synchronized within two weeks of 40+/- 1.1-85+/- 1.4 beat/ minutes. Moreover, histological studies showed alignment of cells in dense tissue architecture, in contrast to monoculture, which showed cell aggregate formation. These results confirm the positive effect of fibroblasts and MSC in cardiac repair in agreement to previous studies (66,67,68), for example, infusing bone marrow MSC into an increase in left ventricular ejection fraction and reduced volume expansion cardiac lesion (70). This was believed that it is due to fibrosis reduction, and cardiac differentiation enhancement. Results of the current study demonstrated the disappearance of MSC by time post reseeding on scaffold; at week 3 about 20%, week 6 less than 5% CD90 positive cells. Moreover, Troponin-t-positive cells reversibly increased with time to be 75% at week 6 in culture indicating the cardiac differentiation of MSC. These finding agree with the above studies confirming the role of MSC in cardiac repair.

Fibroblast inclusion in culture helps not only in improving systolic function (71), but also in neovascularization stimulation. This was the case here during the vascularization step, as hMVEC mono culture showed less neovascularization compared to a co-culture with fibroblast that indicate more positive endothelial cell markers and new vessels generation. In addition, fibroblasts thought to release cytokines, which in turn enhance tissue repair by diminishing cardiomyocytes' death and augmenting new vessel formation.

5.7 Assessment of cardiac construct function:

Functional test for the cardiac tissue were challenging, as thickness was a barrier to set for measurements in both measuring instruments; multichannel multielectrode array (MEA), and organ bath culture (DMT Myograph PL3504B16). However, in future new software and more sensitive electrode of MEA system will allow the measurements through the tissue. So far, tissue under the microscope was observed on a daily basis for spontaneous beating monitoring and progression throughout the culture (Movie 1,2). Nevertheless, in parallel cardiac construct were established using standardized Biovasc scaffold that was vascularized with hMVEC then co-cultured with same cell types under same culturing condition. The resultant construct could be characterized easily since it was detectable by MEA electrodes. A recorded spontaneous beating rate between 0.88 and 1.2 Hz, a conduction velocity of 23.9 ± 0.74 cm s−1, and a maximal contraction force of 0.453 ± 0.015 mN were detected (n = 7). Moreover, QT-interval, which is an important cardiac parameter, was characterized and drug respond test was carried out. Adrenalin recorded higher beating frequency comparing to normal tissue, whereas propranolol, showed a decreased beating frequency indicating resemblance with normal cardiac drug response (schurline.et.al).

Conclusion:

In the present study, perfusion decellularization of porcine hearts resulted in acellular scaffolds, using optimized decellularization method based on a

constant fixed pressure. The protocol was tailored to minimize the exposure time of tissue to decellularized detergent SDS; moreover, excessive washing after treatment to ensure scaffold free of any chemicals or cell debris. Major extracellular matrix components, such as collagens, elastin, and glycosaminoglycans, were preserved which is in line with similar data published in other species (Ott. et.al, Guyette et al.). Proteomics will be carried out next not only for identification of remaining proteins preserved, but also identifying further cues and growth factors within ECM that may play an important role in recellularization process. Perfusion decellularized porcine heart matrices, contained less DNA than the currently accepted standard for implants, and could therefore be compatible with clinical transplantation. However, further studies on the absence of porcine leukocyte antigens, macrophage-response and chronic inflammation in the decellularized scaffold should be checked.

The engineered porcine cardiac scaffold has an advantage of pre-repopulation of the vasculature with CD31-positive cells, lining the walls of the scaffolds microvasculature, that makes it comparable to the outcomes when a graft transplanted in human. In addition, the neovasculature helps in reducing coagulation and enhance another cell's survival within the construct. The absence of endothelium in reengineered tissue may provoke coagulation once blood is introduced. Therefore, establishing suitable microenvironment and vasculature is critical for enhancing the tissue survival and cells nourishment. Vukadinovic–Nikolic et al. generated vascularized cardiac tissue, however due to the use of neonatal cardiomyocytes the tissue exhibit high beating rate of 200 beats/minutes (73). Herein, hiPSc generated cardiac cells were used instead in a co-culture with MSC and Fibroblast yield in a rhythmic beating close to human physiological rate 65-70 beats/minute. Drugs and other physiological characterization for the construct functionality using the suitable devices and software for such a thick cardiac scaffold will allow the consideration of clinical application and toxicological studies.

Outlook:

Future studies will be focused on the role played by ECM remaining after

decellularization in cell proliferation, migration, differentiation, and behavior, as it's the source for all biomedical and biophysical cues needed. These cues are very important for directing specific tissue gene expression encounter for tissue engineering proposes. It will be relevant to study the effect of single protein matrix components on hiPSc proliferation and differentiation and other cells long-term survival, post scaffold reseeding. Some studies demonstrate the effect of ECM on cardiac progenitor cells differentiation in general (75), others study the diversity among different tissue ECMs composition and how they can affect cells behavior in a different ways (74), yet, few showed a focused study on specific ECM components; normally collagen iV, Fibronectin, Laminin, or collagen III on cells differentiation and fate. Conducting such a protein specific hiPSc cardiac differentiation will improve the outcome of the cardiac tissue as well as guaranteed successes in functionality. It will be wise also, to sort different types of cardiac cells by hiPSc, and weather it represents the different cell types in human heart. Optimal heart function depends on many signaling moieties released in controlled manner by several cardiac cells (76). For instance, myofibroblasts secrete growth factors and ECM components to maintain cardiac muscle post cardiac overload, ventricular and atrial cardiomyocytes, and possess different calcium homeostasis and excitation contraction coupling. Therefore, identifying cardiac cell types of differentiated iPSc is necessary to generate more functional cardiac tissue that can be used in pharmaceutical studies and also can be included in clinical studies. Beside, understanding cardiac cellular composition will allow better therapeutic development and enhances cardiac repair therapy. Although many attempts to survey heart cellular content in rat and mouse models have hindered with difficulties, recent techniques and genetic tests were successful to certain extent to analyze and identify cells using specific cell markers.

1- *Go AS, Mozaffarian D, Roger VL, Benjamin EJ, Berry JD, Blaha MJ, Dai S, Ford ES, Fox CS, Franco S, Fullerton HJ, Gillespie C, Hailpern SM, Heit JA, Howard VJ, Huffman MD, Judd SE, Kissela BM, Kittner SJ, Lackland DT, Lichtman JH, Lisabeth*

LD, Mackey RH, Magid DJ, Marcus GM, Marelli A, Matchar DB, McGuire DK, Mohler ER 3rd, Moy CS, Mussolino ME, Neumar RW, Nichol G, Pandey DK, Paynter NP, Reeves MJ, Sorlie PD, Stein J, Towfighi A, Turan TN, Virani SS, Wong ND, Woo D, Turner MB. (2014). Heart disease and stroke statistics--2014 update: A report from the American heart association. Circulation.

2- *Jonathan Leor, Shmuel Tuvia, Victor Guetta, Ferenc Manczur, David Castel, Udi Willenz, Örs Petneha´zy, Natali Landa, Micha S. Feinberg, Eli Konen, Orly Goitein, Orna Tsur-Gang, Mazal Shaul, Lea Klapper, Smadar Cohen. (2009) ,Intracoronary Injection of In Situ Forming Alginate Hydrogel Reverses Left Ventricular Remodeling After Myocardial Infarction in Swine. Journal of the American College of Cardiology.*

3- *Chong JJ1, Yang X, Don CW, Minami E4, Liu YW, Weyers JJ, Mahoney WM, Van Biber B, Cook SM, Palpant NJ, Gantz JA, Fugate JA, Muskheli V, Gough GM7, Vogel KW, Astley CA, Hotchkiss CE, Baldessari A, Pabon L, Reinecke H, Gill EA, Nelson V, Kiem HP, Laflamme MA, Murry CE. (2014). Human embryonic-stem-cell-derived cardiomyocytes regenerate non-human primate hearts. Nature.*

4-*George C. Engelmayr Jr., Mingyu Cheng, Christopher J. Bettinger, Jeffrey T. Borenstein, Robert Langer, and Lisa E. Freed, (2008). Accordion-Like Honeycombs for Tissue Engineering of Cardiac Anisotropy. Nat Mater.*

5-*Fergal J. O'Brien. (2011). Biomaterial and scaffolds for tissue engineering. Materials today*

6- *Kevin D. Costa, Ph.D., Eun, Jung Lee, M.S., and Jeffrey W. Holmes, M.D., Ph.D. (2003). Creating Alignment and Anisotropy in Engineered Heart Tissue: Role of Boundary Conditions in a Model Three-Dimensional Culture System. Tissue Eng.*

7-*Zimmermann WH, Melnychenko I, Eschenhagen T. (2004). Engineered heart tissue for regeneration of diseased hearts. Biomaterials.*

8- *Coulombe KL, Bajpai VK, Andreadis ST, Murry CE. (2014). Heart regeneration with engineered myocardial tissue. Annu Rev Biomed Eng*

9- *Engler AJ, Carag-Krieger C, Johnson CP, Raab M, Tang HY, Speicher DW, Sanger JW, Sanger JM, Discher DE. (2008). Embryonic cardiac myocytes beat best on a matrix with heart-like elasticity: Scar-like rigidity inhibits beating. Journal of cell science.*

10- *Buckberg G, Hoffman JI, Mahajan A, Saleh S, Coghlan C. Cardiac mechanics revisited: (2008). The relationship of cardiac architecture to ventricular function Circulation.*

11-Wainwright JM, Czajka CA, Patel UB, Freytes DO, Tobita K, Gilbert TW, Badylak SF. Preparation of cardiac extracellular matrix from an intact porcine heart. Methods. Tissue engineering. Part C. (2010).

12- *Badylak, S.F., Freytes, D.O., and Gilbert, T.W. Extracellular matrix as a biological scaffold material: structure and func- tion. (2009).Acta Biomater.*

13- *Peter M. Crapo, Ph.D., Thomas W. Gilbert, Ph.D., and Stephen F. Badylak, D.V.M., Ph.D., M.D. (2011). An overview of tissue and whole organ decellularization processes. Biomaterials.*

14- *Jacques P. Guyette l, Jonathan M Charest, Robert W Mills, Bernhard J. Jank, Philipp T. Moser, Sarah E. Gilpin, Joshua R. Gershlak, Tatsuya Okamoto1, Gabriel Gonzalez David J. Milan Glenn R. Gaudette, and Harald C. Ott. (2016). Bioengineering Human Myocardium on Native Extracellular Matrix. Circulation research.*

15- Harold C Ott, T.S.M., Saik-Kia Goh , Lauren D Black Stefan M Kren, Theoden I Netoff& Doris A Taylor., Perfusion-decellularized matrix: using nature's platform to engineer a bioartificial heart .Nature.(2008).

16- *Jacques P Guyette, S.E.G., Jonathan M Charest, Luis F Tapias, Xi Ren, & Harold C ott. (2014). Perfusion decellularization of whole organs. Nature protocol.*

17- *Matthew J. Hodgson, Christopher C. Knutson, Nima Momtahan, and Alonzo D. Cook. (2017) Extracellular Matrix from Whole Porcine Heart Decellularization for Cardiac tissue Engineering.*

18- *Francesco Moroni, Teodelinda Mirabella. (2014). Decellularized matrices for cardiovascular tissue engineering. American journal stem cells.*

19- *Saeed Mirsadraee, Helen E. Wilcox, Sortiris A. Korossis, John N. Kearney, Kevin G. Watterson, F.R.A, John Fisher, and Eileen Ingham. (2006). Development and Characterization of an Acellular Human Pericardial Matrix for Tissue Engineering. Tissue Eng.*

20-– *Alexander Weymann, Sivakkanan Loganathan, Hiroaki Takahashi, Carsten Schies; Benjamin Claus, Kristóf Hirschberg, Pál Soós, Sevil Korkmaz, Bastian Schmack, Matthias Karck, Gábor Szabó, (2010). Development and Evaluation of a Perfusion Decellularization Porcine Heart Model– Generation of 3-Dimensional Myocardial Neoscaffolds. Circulation journal.*

21- *Payam Akhyari, Hug Aubin, Patricia Gwanmesia, Mareike Barth, Stefanie Hoffmann, Jo ̈rn Huelsmann, Karlheinz Preuss, and Artur Lichtenberg. The Quest for an Optimized Protocol for Whole-Heart Decellularization: (2011) A Comparison of Three Popular and a Novel Decellularization Technique and Their Diverse Effects on Crucial Extracellular Matrix Qualities. Tissue Eng Part C Methods.*

22- *Akahashi K, Tanabe K, Ohnuki M, Narita M, Ichisaka T, Tomoda K, Yamanaka S. (2007). Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell.*

23- *Zhang J, Wilson GF, Soerens AG, Koonce CH, Yu J, Palecek SP, Thomson JA, Kamp TJ. . (2009). Functional cardiac myocytes derived from human induced pluripotent stem cells. Circulatory Research.*

24- *Citro L, Naidu S, Hassan F, Kuppusamy ML, Kuppusamy P, Angelos MG, Khan M. (2014). Comparison of human induced pluripotent stem cell derived cardiac myocyteswith human mesenchymal stem cells following acute myocardial infarction. PLoS One.*

25- *Carpenter L, Carr C, Yang CT, Stuckey DJ, Clarke K, Watt SM. Efficient differentiation of human induced pluripotent stem cells generates cardiac cells that provide protection following myocardial infarction in the rat. (2012). Stem cells and*

development.

26- *Malecki M, Putzer E, Sabo C, Foorohar A, Quach C, Stampe C, Beauchaine M, Malecki R, Tombokan X, Anderson M. (2014). Directed cardiomyogenesis of autologous human induced pluripotent stem cells recruited to infarcted myocardium with bioengineered antibodies. Molecular and cellular therapies.*

27- *Kawamura M, Miyagawa S, Fukushima S, Saito A, Miki K, Ito E, Sougawa N, Kawamura T, Daimon T, Shimizu T, Okano T, Toda K, Sawa Y. (2013) Enhanced survival of transplanted human induced pluripotent stem cell-derived cardiac myocytes by the combination of cell sheets with the pedicled omental flap technique in a porcine heart. Circulation.*

28- *Lu TY, Lin B, Kim J, Sullivan M, Tobita K, Salama G, Yang L. (2013) Repopulation of decellularized mouse heart with human induced pluripotent stem cell-derived cardiovascular progenitor cells Nature Communication.*

29- *Kawamura M, Miyagawa S, Miki K, Saito A, Fukushima S, Higuchi T, Kawamura T, Kuratani T, Daimon T, Shimizu T, Okano T, Sawa Y. (2012) Feasibility, safety, and therapeutic efficacy of human induced pluripotent stem cell-derived cardiac myocyte sheets in a porcine ischemic cardiomyopathy model. Circulation.*

30- *Asifiqbal Kadari, Min Lu, Ming Li , Thileepan Sekaran, Rajkumar Thummer, Naomi Guyette, Vi Chu and Frank Edenhofer (2014).Excision of viral reprogramming cassettes by Cre protein transduction enables rapid, robust and efficient derivation of transgene-free human induced pluripotent stem cells . Stem Cell Res Ther.*

31- *Asifiqbal Kadari & SubbaRao Mekala & Nicole Wagner & Daniela Malan & Jessica Köth & Katharina Doll & Laura Stappert & Daniela Eckert & Michael Peitz & Jan Matthes & Philipp Sasse & Stefan Herzig & Oliver Brüstle & Süleyman Ergün & Frank Edenhofer. (2015) Robust Generation of Cardiomyocytes from Human iPS Cells Requires Precise Modulation of BMP and WNT Signaling. Stem Cell Rev.*

32- Lian, X., Zhang, J., Zhu, K., Kamp, T. J., & Palecek, S. P. (2013). Insulin inhibits cardiac mesoderm, not mesendoderm, formation during cardiac differentiation of human pluripotent stem cells and modulation of canonical Wnt signaling can rescue

this inhibition. Stem Cells, 31, 447–457.

33- *Sebastian Schürlein, Reem Al Hijailan, Tobias Weigel, Asifiqbal Kadari, Christoph Rücker, Frank Edenhofer, Heike Walles, Jan Hansmann. (2017). Generation of a Human Cardiac Patch Based on a Reendothelialized Biological Scaffold (BioVaSc). Advnced biosystem.*

34- *Tobias Weigel, Tobias Pfister, Tobias Schmitz, Maren Jannasch, Sebastian Schürlein, Reem Al Hijailan, Heike Walles, Jan Hansmann. (2017) Flexible tissue-like electrode as a seamless tissue-electronic interface. BioNanoMaterials.*

35- *Yu Zhang., Chao Wei., Pengfei Zhang, Xia Li, Tong Liu, Yong Pu, Yunsheng Li, Zubing Cao, Hongguo Cao, Ya Liu, Xiaorong Zhang*, Yunhai Zhang.(2014), Efficient Reprogramming of Na ̈ıve-Like Induced Pluripotent Stem Cells from Porcine Adipose-Derived Stem Cells with a Feeder-Independent and Serum-Free System. PLoS One.*

36- *Jacob H. Hanna, Krishanu Saha1, and Rudolf Jaenisch, (2010) Somatic cell reprogramming and transitions between pluripotent states: facts, hypotheses, unresolved issues. Cell.*

37- *Somers, J.C. Jean, C.A. Sommer, A. Omari, C.C. Ford, J.A. Mills, L. Ying, A. Sommer Gianotti, J.M. Jean, B.W. Smith, R. Lafyatis, M.F. Demierre6, D.J. Weiss, D.L. French, P. Gadue, G.J. Murphy, G. Mostoslavsky, and D.N. Kotton. (2010). Generation of transgene-free lung disease-specific human iPS cells using a single excisable lentiviral stem cell cassette. Stem Cells*

38- *Okita K, Ichisaka T, Yamanaka S: (2007) Generation of germline-competent induced pluripotent stem cells. Nature.*

39- *Mikkers H, Berns A: Retroviral insertional mutagenesis: tagging cancer pathways. (2003) Advance Cancer Research.*

40- *Kopp JL, Ormsbee BD, Desler M, Rizzino A: (2008) Small increases in the level of Sox2 trigger the differentiation of mouse embryonic stem cells. Stem Cells.*

41-*Kaji K, Norrby K, Paca A, Mileikovsky M, Mohseni P, Woltjen K (2009): Virus-free*

induction of pluripotency and subsequent excision of reprogramming factors. Nature.

42- *Chou BK, Mali P, Huang X, Ye Z, Dowey SN, Resar LM, Zou C, Zhang YA, Tong J, Cheng L (2011), Efficient human iPS cell derivation by a non-integrating plasmid from blood cells with unique epigenetic and gene expression signatures. Cell Research.*

43- *Warren L, Manos PD, Ahfeldt T, Loh YH, Li H, Lau F, Ebina W, Mandal PK, Smith ZD, Meissner A, Daley GQ, Brack AS, Collins JJ, Cowan C, Schlaeger TM, Rossi DJ (2010). Highly efficient reprogramming to pluripotency and directed differentiation of human cells with synthetic modified mRNA. Cell Stem Cell.*

44-*Anokye-Danso F, Trivedi CM, Juhr D, Gupta M, Cui Z, Tian Y, Zhang Y, Yang W, Gruber PJ, Epstein JA, Morrisey EE: (2011) Highly efficient miRNA-mediated reprogramming of mouse and human somatic cells to pluripotency. Cell Stem Cell.*

45- *Fusaki N, Ban H, Nishiyama A, Saeki K, Hasegawa M (2009),: Efficient induction oftransgene-free human pluripotent stem cells using a vector based on Sendai virus, an RNA virus that does not integrate into the host genome. Proc Jpn Acad Ser B Phys Biol .*

46- *Gonzalez F, Boue S, Izpisua Belmonte JC: (2011) Methods for making induced pluripotent stem cells: reprogramming a la carte. Nat Rev Genet.*

47- *Carey BW, Markoulaki S, Hanna J, Saha K, Gao Q, Mitalipova M, Jaenisch R (2009): Reprogramming of murine and human somatic cells using a single polycistronic vector. Proc Natl Acad Sci.*

48- *Telugu BP, Ezashi T, Roberts RM (2010). Porcine induced pluripotent stem cells analogous to naive and primed embryonic stem cells of the mouse. International Journal of Developmen.*

49- *Gabrielle M. Brons, Lucy E. Smithers, Matthew W. B. Trotter, Peter Rugg-Gunn, Bowen Sun, Susana M. Chuva de Sousa Lopes, Sarah K. Howlett, Amanda Clarkson, Lars Ahrlund-Richter , Roger A. Pedersen & Ludovic Vallier (2007). Derivation of pluripotent epiblast stem cells from mammalian embryos. Nature.*

50- *Jin-Kyu Park, Hye-Sun Kim, Kyung-Jun Uh, Kwang-Hwan Choi, Hyeong-Min*

Kim1, Taeheon Lee, Byung-Chul Yang, Hyun-Jong Kim, Hak-Hyun Ka, Heebal Kim, Chang-Kyu Lee1 (2013). Primed Pluripotent Cell Lines Derived from Various Embryonic Origins and Somatic Cells in Pig. PLoS one.

51- *Jennifer Nichols and Austin Smith (2009). Naive and Primed Pluripotent States. Cell stem cell.*

52- *De Cheng, Yanjie Guo, Zhenzhen Li, Yajun Liu, Xing Gao, Yi Gao, Xiang Cheng, Junhe Hu, Huayan Wang (2012).. Porcine Induced Pluripotent Stem Cells Require LIF and Maintain Their Developmental Potential in Early Stage of Embryos. PLoS one.*

53- *Shuh-hei Fujishiro, Kazuaki Nakano, Yoshihisa Mizukami, Takuya Azami, Yoshikazu Arai, Hitomi Matsunari, Rikiya Ishino, Takashi Nishimura, Masahito Watanabe, Tomoyuki Abe, Yutaka Furukawa, Kazuhiro Umeyama, Shinya Yamanaka, Masatsugu Ema, Hiroshi Nagashima, and Yutaka Hanazono (2013). Generation of Naive-Like Porcine-Induced Pluripotent Stem Cells Capable of Contributing to Embryonic and Fetal Development. Stem Cells Dev.*

54- *Xiaorong Li. Fengxiang Zhang. Guixian Song, Weijuan Gu, Minglong Chen, Bing Yang, Dianfu Li, Daowu Wang, Kejiang Cao (2013).. Intramyocardial Injection of Pig Pluripotent Stem Cells Improves Left Ventricular Function and Perfusion: A Study in a Porcine Model of Acute Myocardial Infarction. PLos one.*

55- *Sasitorn Rungarunlert, Nuttha Klincumhom, Theerawat Tharasanit, Mongkol Techakumphu, Melinda K. Pirity, and Andras Dinnyes (2013). Slow Turning Lateral Vessel Bioreactor Improves Embryoid Body Formation and Cardiogenic Differentiation of Mouse Embryonic Stem Cells. Cell Reprogram.*

56- *Chakritbudsabong a, Ladawan Sariya b, Sirikron Pamonsupornvichit b, Rassmeepen Pronarkngver b, Somjit Chaiwattanarungruengpaisan b, Joao N. Ferreira c, Piyathip Setthawong d, Praopilas Phakdeedindan e, Mongkol Techakumphu d, Theerawat Tharasanit d, Sasitorn Rungarunlert f (2017), Generation of a pig induced pluripotent stem cell (piPSC) line from embryonic fibroblasts by incorporating LIN28 to the four transcriptional factor-mediated reprogramming. Stem Cell Res.*

57- *CellsYe L, Chang YH, Xiong Q, Zhang P, Zhang L, Somasundaram P, Lepley M, Swingen C, Su L, Wendel JS, Guo J, Jang A, Rosenbush D, Greder L, Dutton JR, Zhang J, Kamp TJ, Kaufman DS, Ge Y, Zhang J (2015). Cardiac Repair in a Porcine Model of Acute Myocardial Infarction with Human Induced Pluripotent Stem Cell-Derived Cardiovascular. Cell Stem Cell.*

58- *Xiaorong Li., Fengxiang Zhang., Guixian Song, Weijuan Gu, Minglong Chen, Bing Yang, Dianfu Li, Daowu Wang, Kejiang Cao (2013). Intramyocardial Injection of Pig Pluripotent Stem Cells Improves Left Ventricular Function and Perfusion: A Study in a Porcine Model of Acute Myocardial Infarction. PLOS one.*

59- *Matthew J. Robertson Jessica L. Dries-Devlin, Stefan M. Kren, Jana S. Burchfield, Doris A. Taylor (2014). Optimizing Recellularization of Whole Decellularized Heart Extracellular Matrix. PLOS one.*

60- *Raquel Costa-Almeida, MSc, Maria Gomez-Lazaro, Carla Ramalho, Pedro L. Granja, Raquel Soares, and Susana G. Guerreiro (2015)., Fibroblast-Endothelial Partners for Vascularization Strategies in Tissue Engineering. Tissue Eng Part A.*

61- *J Michael Sorrell, Marilyn A. Baber BA,Arnold . Caplan (2008). Human dermal fibroblast subpopulations; differential interactions with vascular endothelial cells in coculture: Non-soluble factors in the extracellular matrix influence interactions. Wound Repair Regeneration.*

62- *Newman, A.C., Nakatsu, M.N., Chou, W., Gershon, and Hughes, C.C.W (2011).. The requirement for fibroblasts in angiogen- esis: fibroblast-derived matrix proteins are essential for endo- thelial cell lumen formation. Mol Biol Cell*

63- *David S. Calnek1Brian W.Grinnell (1998). Thrombomodulin-Dependent Anticoagulant Activity Is Regulated by Vascular Endothelial Growth Factor. Exp Cell Res.*

64- *Konstantions E. Hatzistergos,, Henry Quevedo, Behzad N. Oskouei, Qinghua Hu, Gary S. Feigenbaum, Irene S. Margitich, Ramesh Mazhari, Andrew J. Boyle, Juan P. Zambrano, Jose E. Rodriguez, Raul Dulce, Pradip M. Pattany, David Valdes, Concepcion Revilla, A.W. Heldman, I, McNiece, and Joshua M. Hare (2010). Bone*

marrow mesenchymal stem cells stimulate cardiac stem cell proliferation and differentiation. Circulatory Research.

65- *Michael A. Laflamme and Charles E. Murry (2011). Heart Regeneration. Nature.*

66- *Lu T-Y, Lin B, Kim J, Sullivan M, Tobita K, Salama G, Yang L (2013). Repopulation of decellularized mouse heart with human induced pluripotent stem cell-derived cardiovascular progenitor cells. Nature Communication.*

67- *Murry CE, Soonpaa MH, Reinecke H, Nakajima H, Nakajima HO, Rubart M, Pasumarthi KB, Ismail Virag J, Bartelmez SH, Poppa V, Bradford G, Dowell JD, Williams DA, Field LJ (2004). Haematopoietic stem cells do not transdifferentiate into cardiac myocytes in myocardial infarcts. Nature.*

68- *Balsam LB, Wagers AJ, Christensen JL, Kofidis T, Weissman IL, Robbins RC (2004). Haematopoietic stem cells adopt mature haematopoietic fates in ischaemic myocardium. Nature.*

69- *Maria Mirotsou, Zhongyan Zhang, Arjun Deb, Lunan Zhang, Massimiliano Gnecchi, Nicolas Noiseux, Hui Mu, Alok Pachori, and Victor Dzau (2007). Secreted frizzled related protein 2 (Sfrp2) is the key Akt-mesenchymal stem cell-released paracrine factor mediating myocardial survival and repair. Proc Natl Acad Sci.*

70- *Pedro L. Sanchez, M. Eugenia Fernandez-Santos, Salvatore Costanza , Andreu M. Climent , Isabel Moscoso. Angeles Gonzalez-Nicolas, Ricardo Sanz-Ruiz, Hugo Rodríguez , Stefan M. Kren , Gregorio Garrido ,Jose L. Escalante, Javier Bermejo, Jaime Elizaga , Javier Menarguez , Raquel Yotti , Candelas Perez del Villar , M. Angeles Espinosa, María S. Guillem ,James T. Willerson , Antonio Bernad , Rafael* Matesanz, Doris A. Taylo, Francisco Fernandez-Aviles (2015). Acellular human heart matrix: A critical step toward whole heart grafts. Biomaterials.

71- *Emmanouil Chavakis, Masamichi Koyanagi, Stefanie Dimmeler (2010), Enhancing the Outcome of Cell Therapy for Cardiac Repair Progress From Bench to Bedside and Back. Circulation.*

72- *Jun Fujita, Yuji Itabashi, Tomohisa Seki, Shugo Tohyama, Yuichi Tamura, Motoaki Sano, and Keiichi Fukuda (2012). Myocardial cell sheet therapy and cardiac function. Am J Physiol Heart Circ Physiol.*

73- *Z. Vukadinovic-Nikolic, B. Andree, S. E. Dorfman, M. Pflaum, T. Horvath, M. Lux, L. Venturini, A. Bar, G. Kensah, A. R. Lara, I. Tudorache, S. Cebotari, D. Hilfiker-Kleiner, A. Haverich, A. Hilfiker (2014), Tissue Eng.,*

74- *Agmon G1, Christman KL1. (2016) . Controlling stem cell behavior with decellularized extracellular matrix scaffolds. Curr Opin Solid State Mater Sci.*

75 French KM1, Boopathy AV, DeQuach JA, Chingozha L, Lu H, Christman KL, Davis M (2012). A naturally derived cardiac extracellular matrix enhances cardiac progenitor cell behavior in vitro. Acta Biomater.

76- *Al-Mohanna. F (2016). The Cardiokines: An expanding family of the heart secretome. Endocrinology of the heart in health and disease,*

77- *Alexander R. Pinto, Alexei Ilinykh, Malina J. Ivey, Jill T. Kuwabara, Michelle L. D'Antoni, Ryan Debuque, Anjana Chandran, Lina Wang, Komal Arora, Nadia Rosenthal, and Michelle D. Tallquist (2016). Revisiting Cardiac Cellular Composition. Circ Res.*

78- *Anderson, RM. (1993). The Gross Physiology of the Cardiovascular System.*

79- *Tortora, G. & Grabowski, S. (2000) Principles of Anatomy & Physiology. Wiley & Sons. Brisbane, Singapore & Chichester.*

80- *Van De Graaff, Kent M (2002).. Human Anatomy. McGraw Hill Publishing, Burr Ridge, IL.*

81- *Dirk L. Brutsaert. Cardiac Endothelial-Myocardial Signaling: Its Role in Cardiac*

Growth, Contractile Performance, and Rhythmicity (2003). Physiol Rev.

82- *Nees S, Weiss DR, Senftl A, Knott M, Forch S, Schnurr M, Weyrich P, Juchem G. (2012) Isolation, bulk cultivation, and char- acterization of coronary microvascular pericytes: the second most frequent myocardial cell type in vitro. Am J Physiol Heart Circ Physiol.*

83- *Stephan Nees & Dominik R. Weiss & Gerd Juchem. Focus on cardiac pericytes. Pflugers Arch. (2013)*

84- *Indroneal Banerjee, John W. Fuseler, Robert L. Price, Thomas K. Borg, and Troy A. Baudino (2007). Determination of cell types and numbers during cardiac development in the neonatal and adult rat and mouse. Am J Physiol Heart Circ Physiol.*

85- *Hsieh PC, Davis ME, Lisowski LK, Lee RT. Endothelial-cardiomyocyte interactions in cardiac development and repair. Annu Rev Physiol. (2006)*

86- *Nag AC. Study of non-muscle cells of the adult mammalian heart: A fine structural analysis and distribution. Cytobios. (1980)*

87- *Bergmann O, Zdunek S, Felker A, Salehpour M, Alkass K, Bernard S, Sjostrom SL, Szewczykowska M, Jackowska T, Dos Remedios C, Malm T, Andra M, Jashari R, Nyengaard JR, Possnert G, Jovinge S, Druid H, Frisen J (2015). Dynamics of cell generation and turnover in the human heart. Cell.*

88- *D. Cretoiu. Hummel, H. Zimmermann c, M. Gherghiceanu , L. M. Popescu. Human cardiac telocytes: 3D imaging by FIB-SEM tomography (2014). J Cell Mol Med.*

89-. *S,w, Rabkin. Epicardial fat (2008): proparties, function,and relationship with obesity Obesity review.*

90- *Samanta R, Pouliopoulos J, Thiagalingam A, Kovoor P (2016). Role of adipose tissue in the pathogenesis of cardiac arrhythmias. Heart Rhythm.*

91-. *Slava Epelman, Peter P. Liu and Douglas L. Mann (2015).. Role of innate and adaptive mechanisms in cardiac injury and repair. Nature review immunology.*

92- *Yasmine Zouggari, Hafid Ait-Oufella, Philippe Bonnin, Tabassome Simon, Andrew*

P Sage, Coralie Guérin, José Vilar, Giuseppina Caligiuri, Dimitrios Tsiantoulas, Ludivine Laurans, Edouard Dumeau, Salma Kotti, Patrick Bruneva, Israel F. Charo, Christoph J. Binder, Nicolas Danchin, Alain Tedgui, Thomas F. Tedder, Jean-Sébastien Silvestre, Ziad Mallat (2013). B lymphocytes trigger monocyte mobilization and impair heart function after acute myocardial infarction. Nat Med.

93- *Mohammed Quttainah, Reem Al-Hejailan, Soad Saleh, Ranjit Parhar, Walter Conca, Bernard Bulwer, Narain Moorjani, Pedro Catarino, Raafat Elsayed, Mohammed Shoukri, Mansour AlJufan, Maie AlShahid, Abderrahman Ouban, Zohair Al-Halees, Stephen Westaby, Kate Collison, Futwan Al-Mohanna' (2015). Progression of matrixin and cardiokine expression patterns in an ovine model of heart failure and recovery. International journal of cardiology.*

94- *Vacanti CA The history of tissue engineering (2006). J Cell Mol Med*

95- *Ikada. Challenges in tissue engineering (2006).. J R Soc Interface.*

96- *Toma C1, Pittenger MF, Cahill KS, Byrne BJ, Kessler PD (2002). Human mesenchymal stem cells differentiate to a cardiomyocyte phenotype in the adult murine heart. Circulation.*

97- *McCloskey KE, Gilroy ME, & Nerem RM Use of embryonic stem cell-derived endothelial cells as a cell source to generate vessel structures in vitro (2005)., Tissue engineering.*

98-. *Trivanovic D. (2013). Mesenchymal stem cells isolated from peripheral blood and umbilical cord Wharton's jelly. Srp Arh Celok Lek*

99- *Martin DR, Cox NR, Hathcock TL, Niemeyer GP, & Baker HJ (2002). Isolation and characterization of multipotential mesenchymal stem cells from feline bone marrow., Exp Hematol .*

100- *Baksh D, Song L, & Tuan RS (2004). Adult mesenchymal stem cells: characterization, differentiation, and application in cell and gene therapy., J Cell Mol Med.*

101- *Robinton DA & Daley GQ (2012), The promise of induced pluripotent stem cells in research and therapy. Nature.*

102- *Lee G, (2009) . Modelling pathogenesis and treatment of familial dysautonomia using patient-specific iPSCs. Nature.*

103- *Itzhaki I, (2011). Modelling the long QT syndrome with induced pluripotent stem cells. Nature.*

104- *Yi-Chen Li, Kai Zhu, Tai-Horng Young (2017), Induced pluripotent stem cells, form in vitro tissue engineering to in vivo allogeneic transplantation. J Thorac Dis.*

105- *Shiba Y, Gomibuchi T, Seto T. Allogeneic transplantation of iPS cell-derived cardiomyocytes regenerates primate hearts. Nature (2016).*

106- *Zhong X, Gutierrez C, Xue T. Generation of three- dimensional retinal tissue with functional photoreceptors from human iPSCs. Nat Commun (2014)*

107- *Reardon S, Cyranoski D (2014). Japan stem-cell trial stirs envy. Nature*

108- *Zhou H, Wu S, Joo JY (2009). Generation of induced pluripotent stem cells using recombinant proteins. Cell Stem Cell*

109- *Morita, S. & Ikada, Y. Lactide (2002) Copolymers for scaffolds in tissue engineering. In Tissue engineering and biodegradable equivalents: scientific and clinical applications, 1rst edition.*

110- *Laurencin, C. T., Lu, H. H. & Khan, Y. Processing of polymer scaffolds: polymer–ceramic composite forms (2002. In Methods of tissue engineering (ed. A. Atala & R. P. Lanza)*

111- *Whang, K. & Healy, K. (2002) EProcessing of polymer scaffolds: freeze-drying. In Methods of tissue engineering. (ed. A. Atala & R. P. Lanza)*

112- *Teo WE, He W, & Ramakrishna S (2006). Electrospun scaffold tailored for tissue-specific extracellular matrix. Biotechnol J*

113- *Shin, M., Ishii, O., Sueda, T., Vacanti, J. P (2004), Contractile cardiac grafts using a novel nanofibrous mesh. Biomaterials.*

114-*Urist, M. R. Bone (1965): formation by autoinduction. Science*

115- *Wozney, J. M., Rosen, V., Celeste, A. J., Mitsock, L. M., Whitters, M. J., Kriz, R. W., Hewick, R. M. & Wang, E. A. (1988) Novel regulators of bone formation: molecular clones and activities. Science.*

116-*Yamamoto, M., Takahashi, Y. & Tabata, Y (2003). Controlled release by biodegradable hydrogels enhances bone formation of bone morphogenetic protein. Biomaterials.*

117- *Lamerigts, N. M., Buma, P., Huiskes, R., Schreurs, W., Gardeniers, J. & Slooff, T. J (2000). Incorporation of morsellized bone graft under controlled loading conditions. A new animal model in the goat. Biomaterials.*

118- *Shum-Tim, D. et al. Tissue engineering of autologous aorta using a new biodegradable polymer. (1999) Ann. Thorac.*

119- *Carrier, R. L., Rupnick, M., Langer, R., Schoen, F. J., Freed, L. E. & Vunjak-Novakovic, G (2002). A Perfusion improves tissue architecture of engineered cardiac muscle. Tissue Eng.*

120- *Carrier, R. L., Rupnick, M., Langer, R., Schoen, F. J., Freed, L. E. & Vunjak-Novakovic, G. (2002) Effect of oxygen on engineered cardiac muscle. Biotechnol. Bioeng.*

121- Brittberg, M., Lindahl, A., Nilson, A., Ohlsson, C., Isaksson,O. & Peterson, L. 1994 Treatment of deep cartilage defects in the knee with autologous chondrocyte transplantation. N. Engl. J. Med. 331, 889–895

122- *Rosenthal, A (1998). Auto transplants for Parkinson's disease? Neuron.*

123- *Paolo Macchiarini, MD, PhD,a,b Thorsten Walles, MD,a,b Christian Biancosino,b and Heike Mertsching, PhD (2004). First human transplantation of a bioengineered airway tissue. J Thorac Cardiovasc Surg.*

124- *Atala, A. (1999) Engineering tissues and organs. Curr. Opin. Urol.*

125- *Menashe, A (2002)Autologous skeletal myoblast transplan- tation for ischemic*

cardiomyopathy: first clinical case. Cardiac Vasc. .

126- *Vacanti, C. A., Bonassar, L. J. (2001), Vacanti, M. P. & Shufflebarger, J. Replacement of an avulsed phalanx with tissue-engineered bone. N. Eng. J. Med.*

127- *Kinoshita, Y. & Amagasa, T (2002) Jaw bone. In Methods of tissue engineering .(ed. A. Atala & R. P. Lanza),*

128- *Rodriguez, A., Anastassov, G. E., Lee, H., Buchbinder, D. & Wettan, H (2003). Maxillary sinus augmentation with deproteinated bovine bone and platelet rich plasma with simultaneous insertion of endosseous implants. J. Oral Maxil lofac. Surg.*

129- *Honkanen, P. B., Kellomaki, M., Lehtimaki, M. Y., Tormala, P., Makela, S. & Lehto, M. U. (2003) Bioreconstructive joint scaffold implant arthroplasty in metacarpophalangeal joints: short-term results of a new treatment concept in rheumatoid arthritis patients. Tissue Eng.*

130- *Wakitani, S., Imoto, K., Yamamoto, T., Saito, M., Murata, N. & Yoneda, M. (2002) Human autologous culture expanded bone marrow mesenchymal cell transplantation for repair of cartilage defects in osteoarthritic knees. Osteoarthritis Cartilage*

131- *Wollert, K. C. (2004) Intracoronary autologous bone- marrow cell transfer after myocardial infarction: the BOOST randomized controlled clinical trial. Lancet.*

132- *Weber, R. A., Breidenbach, W. C., Brown, R. E., Jabaley, M. E. & Mass, D. P. (2000) A randomized prospective study of polyglycolic acid conduits for digital nerve reconstruction in humans. Plast. Reconstr. Surg.*

133- *Badylak, S.F., Taylor, D., and Uygun, K (2011). Whole-organ tissue engineering: decellularization and recellularization of three- dimensional matrix scaffolds. Annu Rev Biomed Eng*

134- *He M & Callanan (2013) A Comparison of methods for whole-organ decellularization in tissue engineering of bioartificial organs. Tissue Eng Part B Rev.*

135- *Gilbert, T.W (2012). Strategies for tissue and organ decellularization. J Cell Biochem.*

136- *Badylak SF, Lantz GC, Coffey A, Geddes LA. (1989). Small intestinal submucosa as a large diameter vascular graft in the dog. J. Surg. Res.*

137- *ChenRN, Ho HO, Tsai YT ,Sheu MT (2004). Process development of an acellular dermal matrix(ADM) for biomedical applications. Biomaterials.*

138- *Gilbert TW, Stolz DB, Biancaniello F, Simmons-Byrd A, Badylak SF (2005) Production and character- ization of ECM powder: implications for tissue engineering applications. Biomaterials.*

139- *Bader A, Schilling T, Teebken OE, Brandes G, Herden T(1998). Tissue engineering of heart valves—human endothelial cell seeding of detergent acellularized porcine valves. Eur. J. Cardiothorac. Surg.*

140- *BissellMJ,AggelerJ (1987). Dynamic reciprocity :How do extracellular matrix and hormones direct gene expression? Prog. Clin. Biol. Res.*

141- *Cortiella J, Niles J, Cantu A, Brettler A, Pham A. (2010) Influence of acellular natural lung matrix on murine embryonic stem cell differentiation and tissue formation. Tissue Eng. Part A*

142- *Sellaro TL, Ranade A, Faulk DM, McCabe GP, Dorko K, (2010) Maintenance of human hepatocyte function in vitro by liver-derived extracellular matrix gels. Tissue Eng. Part A.*

143- *Brown B, Lindberg K, Reing J, Stolz DB, Badylak SF(2006).. The basement membrane component of biologic scaffolds derived from extracellular matrix. Tissue Eng.*

144- Uygun BE, Soto-Gutierrez A, Yagi H, Izamis ML, Guzzardi MA(2010). Organ reengineering through development of a transplantable recellularized liver graft using decellularized liver matrix. Nat. Med.

145- Petersen TH, Calle EA, Zhao L, Lee EJ, Gui L (2010) .Tissue-engineered lungs for in vivo implantation. Science .

146- Wainwright JM, Czajka CA, Patel UB, Freytes DO, Tobita Kl (2010). Preparation of cardiac extracellular matrix from an intact porcine heart. Tissue Eng. Part C

147- Raeder RH, Badylak SF, Sheehan C, Kallakury B, Metzger DW (2002). Natural anti-galactose alpha1,3 galactose antibodies delay, but do not prevent the acceptance of extracellular matrix xenografts. Trans- plant. Immunol.

148- Daly K, Stewart-Akers A, Hara H, Ezzelarab M, Long C (2009). Effect of the alphaGal epitope on the response to small intestinal submucosa extracellular matrix in a nonhuman primate model. Tissue Eng. Part A.

149- Badylak SF, Valentin JE, Ravindra AK, McCabe GP, Stewart-Akers AM. (2008)Macrophage phenotype as a determinant of biologic scaffold remodeling. Tissue Eng. Part A

150- Gilbert TW, Freund JM, Badylak SF. Quantification of DNA in biologic scaffold materials. J. Surg. Res. 1. (2009) 52:135–39

151- Vunjak-Novakovic G, Tandon N, Godier A, Maidhof R, Marsano A (2010).Challenges in cardiac tissue engineering. Tissue Eng. Part B Rev .

152- NahmiasY,BerthiaumeF,YarmushML(2007)..Integration of technologies for hepatic tissue engineering. Adv. Biochem. Eng. Biotechnol.

153-Uygun BE, Soto-Gutierrez A, Yagi H, Izamis ML, Guzzardi MA. (2010) Organ reengineering through development. Nat Med.

154-Lovett M, Lee K, Edwards A, Kaplan DL (2009). Vascularization strategies for tissue engineering. Tissue Eng. Part B Rev

155- Schechner JS, Crane SK, Wang F, Szeglin AM, Tellides G. (2003) Engraftment of a vascularized human skin equivalent. FASEB J.

156- Kamkin A, Kiseleva I, Lozinsky I, Scholz H. (2005). Electrical interaction of mechanosensitive fibroblasts and myocytes in the heart. Basic Res. Cardiol.

157- Kulig KM, Vacanti JR. (2004) Hepatic tissue engineering. Transplant. Immunol

158- Martin I, Wendt D, Heberer M. (2004). The role of bioreactors in tissue engineering. Trends Biotechnol.

159- Brasile L, Stubenitsky BM, Booster MH, Lindell S, Araneda D (2002), Overcoming severe renal ischemia: the role of ex vivo warm perfusion. Transplantation .

160- Maherali, N. (2007) Directly reprogrammed fibroblasts show global epigenetic remodeling and widespread tissue contribution. Cell Stem Cell.

161- Wernig, M. (2007).. In vitro reprogramming of fibroblasts into a pluripotent ES-cell-like state. Nature .

162- Aasen, T. (2008). Efficient and rapid generation of induced pluripotent stem cells from human keratinocytes. Nature Biotech.

163- Nakagawa, M. (2008) Generation of induced pluripotent stem cells without Myc from mouse and human fibroblasts. Nature Biotech.

164- Kim, J. B. (2009). Oct4-induced pluripotency in adult neural stem cells. Cell .

165- Hanna, J. (2009). Direct cell reprogramming is a stochastic process amenable to acceleration. Nature.

166- Park, I. H. (2008). Reprogramming of human somatic cells to pluripotency with defined factors. Nature.

167- Judson, R. L., Babiarz, J. E., Venere, M. & Blelloch, R (2009). Embryonic stem cell-specific microRNAs promote induced pluripotency. Nature Biotech.

168- Banito, A. (2009). Senescence impairs successful reprogramming to pluripotent stem cells. Genes Dev.

169- Huangfu, D. (2008) Induction of pluripotent stem cells by defined factors is greatly improved by small-molecule compounds. Nature Biotech.

170- Marson, A.(2008).. Wnt signaling promotes reprogramming of somatic cells to pluripotency. Cell Stem Cell

171- Okada, M., Oka, M. & Yoneda, Y (2010).. Effective culture conditions for the induction of pluripotent stem cells. Biochim. Biophys. Acta

172- Yoshida, Y., Takahashi, K., Okita, K., Ichisaka, T. & Yamanaka, S (2009).. Hypoxia enhances the generation of induced pluripotent stem cells. Cell Stem Cell.

173- Takahashi, K. (2007). Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell .

174- Jahner, D.(1982) . De novo methylation and expression of retroviral genomes during mouse embryogenesis. Nature.

175- Hotta, A. & Ellis, J (2008). Retroviral vector silencing during iPS cell induction: an epigenetic beacon that signals distinct pluripotent states. J. Cell. Biochem.

176- Blelloch, R., Venere, M., Yen, J. & Ramalho-Santos, M (2007). Generation of induced pluripotent stem cells in the absence of drug selection. Cell Stem Cell.

177- Yao, S. (2004).. Retrovirus silencing, variegation, extinction, and memory are controlled by a dynamic interplay of multiple epigenetic modifications.Mol. Ther.

178- Varas, F. (2009) Fibroblast-derived induced pluripotent stem cells show no common retroviral vector insertions. Stem Cells.

179- Brambrink, T. (2008). Sequential expression of pluripotency markers during direct reprogramming of mouse somatic cells. Cell Stem Cell.

180- Rodriguez-Piza, I(2010). Reprogramming of human fibroblasts to induced pluripotent stem cells under xeno-free conditions. Stem Cells.

181- Kaji, K. (2009). Virus-free induction of pluripotency and subsequent excision of reprogramming factors. Nature.

182- Stadtfeld, M., Nagaya, M., Utikal, J., Weir, G. & Hochedlinger, K (2008). Induced pluripotent stem cells generated without viral integration. Science.

183- Fusaki, N., Ban, H., Nishiyama, A., Saeki, K. & Hasegawa, M (2009). Efficient induction of transgene-free human pluripotent stem cells using a vector based on Sendai virus, an RNA virus that does not integrate into the host genome. Proc. Jpn Acad. Ser. B Phys. Biol. Sci.

184- González, F (2009). Generation of mouse-induced pluripotent stem cells by

transient expression of a single non-viral polycistronic vector. Proc Natl Acad Sci.

185- Warren, L (2010). Highly efficient reprogramming to pluripotency and directed differentiation of human cells with synthetic modified mRNA. Cell Stem Cell.

186- Inoue, M. et al. p53 protein transduction therapy: successful targeting and inhibition of the growth of the bladder cancer cells. Eur. Urol. (2006) 49, 161–168.

187- Kim, D. (2009) Generation of human induced pluripotent stem cells by direct delivery of reprogramming proteins. Cell Stem Cell.

188- Olson EN, Schneider MD (2003). Sizing up the heart: Development redux in disease. Genes Dev.

189- Bondue A, Tannler S, Chiapparo G, Chabab S, Ramialison M, Paulissen C, Beck B, Harvey R, Blanpain C (2011). Defining the earliest step of cardiovascular progenitor specification during embryonic stem cell differentiation. J Cell Biol.

190- Elliott DA, Braam SR, Koutsis K, Ng ES, Jenny R, Lagerqvist EL, Biben C, Hatzistavrou T, Hirst CE, Yu QC, Skelton RJ, Ward-van Oostwaard D, Lim SM, Khammy O, Li X, Hawes SM, Davis RP, Goulburn AL, Passier R, Prall OW, Haynes JM, Pouton CW, Kaye DM, Mummery CL, Elefanty AG, Stanley EG (2011). Nkx2-5(egfp/w) hescs for isolation of human cardiac progenitors and cardiomyocytes. Nat Methods.; 8:1037–1040

191- Kubo A, Shinozaki K, Shannon JM, Kouskoff V, Kennedy M, Woo S, Fehling HJ, Keller G. (2004) Development of definitive endoderm from embryonic stem cells in culture. Development.

193- Osafune, K., Caron, L., Borowiak, M (2008), Marked differences in differentiation propensity among human embryonic stem cell lines. Nature Biotechnology.

192- Passier, R., Oostwaard, D. W., Snapper, (2005). Increased cardiomyocyte

differentiation from human embryonic stem cells in serum-free cultures. Stem Cells,

194- Sa, S., & McCloskey, K. E. (2012). Stage-specific cardiomyocyte differentiation method for H7 and H9 human embryonic stem cells. Stem Cell Reviews.

195- Ren, Y., Lee, M. Y., Schliffke, S. (2011) Small molecule Wnt inhibitors enhance the efficiency of BMP-4-directed cardiac differ- entiation of human pluripotent stem cells. Journal of Molecular and Cellular Cardiology.

196- Cao, N., Liu, Z., Chen, Z.. (2011) Ascorbic acid enhances the cardiac differentiation of induced pluripotent stem cells through promoting the proliferation of cardiac progenitor cells. Cell Research.

197- Hotta, A. & Ellis (2008)., J. Retroviral vector silencing during iPS cell induction: an epigenetic beacon that signals distinct pluripotent states. J. Cell. Biochem.

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