SMALL MOLECULES IN CARDIAC REGENERATION

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SMALL MOLECULES IN CARDIAC REGENERATION

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So long and thanks for all the fish.

ABSTRACT

SMALL MOLECULES IN CARDIAC REGENERATION

The common prevalence of heart failure and limitations in its treatment are leading cause of attention and interest towards the induction of cardiac regeneration with novel approaches. Recent studies provide growing evidence regarding bona fide cardiac regeneration post genetic manipulations, administration of stimulatory factors and myocardial injuries in animal models and human studies. To this end, small molecules of different sources have been tested to treat heart failure for the development of cellular therapies. Amongst all molecules tested, p300/CBP Inhibitor VI ($C_{15}H_{15}NO_5S$), c-Myc Inhibitor II ($C_{12}H_{11}NOS_2$), Trp/p53 Pifithrin-α ($C_{16}H_{18}N_2OS \cdot HBr$) and Tauroursodeoxycholic Acid ($C_{16}H_{14}O_7$) have the potential to induce cardiac regeneration and reduce fibroblast proliferation.

ÖZET

KALP REJENERASYONUNDA KÜÇÜK MOLEKÜLLERİN KULLANILMASI

Kalp krizi tedavisindeki prevalans ve limitasyonlar, kardiyak rejenerasyonun indüklenmesini hedefleyen çalışmaların ortaya çıkmasını sağlamıştır. Yapılan güncel çalışmalar, bona fide kardiyak rejenerasyonun, post genetik manipülasyonlar, stimülatör faktörlerin administrasyonu ve iskemik miyokardiyal hayvan modellerinin kullanılmasını içermektedir. Bu sebeple yapılan çalışmada farklı kaynaklardan elde edilen küçük moleküllerin kardiyak rejenerasyon üzerindeki etkisi test edilmiş ve kalp krizine yönelik terapötik bir strateji geliştirmek amaçlanmıştır. Test edilen moleküller arasında p300/CBP Inhibitor VI ($C_{15}H_{15}NO_5S$), c-Myc Inhibitor II ($C_{12}H_{11}NOS_2$), Trp/p53 Pififthrin-α ($C_{16}H_{18}N_2OS \cdot HBr$) ve Tauroursodeoxycholic Acid ($C_{16}H_{14}O_7$) kardiyak rejenerasyonun indüklenmesi ve fibroblast proliferasyonun durdurulması açısından en yüksek potansiyele sahiptir.

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BrdU	5-bromo-2-deoxyuridine
CSC	Cardiac stem cell
CM	Cardiomyocyte
CDKI	Cyclin dependent kinase inhibitor protein
DAPI	4',6-diamidino-2-phenylindole
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic Acid
ESC	Embryonic stem cell
FB	Fibroblast
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
GATA	Globin transcription factor
GCP	Glycolytic cardiac proge
pH3	Phospho histone protein 3
RNA	Ribonucleic Acid
SCA1	Stem Cell Antigen 1
SP	Side population
SSEA1	Stage-specific embryonic antigen 1
TnnT	Troponin T
TWEAK	TNF-related weak inducer of apoptosis
mL	Mililiter
µL	Microliter
µg	Microgram
µM	Micromolar

1. INTRODUCTION

1.1. HEART FAILURE

Heart failure affects more than 23 million people worldwide. Heart transplantation is still the only available treatment, provided that an appropriate donor is present [1,2]. Several strategies and mechanisms haven been reported to be important to achieve cardiac regeneration as an alternative approach to support cardiomyocyte renewal post cardiovascular issues including but not limited to small molecule inhibitors of proteins, noncoding RNAs and stem cell administration [3–5]. However, it is still unclear whether newly formed cardiomyocytes derived from preexisting cardiomyocytes or cardiac stem cells [6]. In addition, in recent years, several clinical investigators and researchers have proposed the induction of cardiomyocyte proliferation as a plausible strategy for the development of novel cardiac regenerative approaches [7].

1.2. EVIDENCE OF CARDIAC REGENERATION AT BASELINE

Adult heart, historically, was considered as terminally differentiated and suggested as a non-regenerative organ. However, intensive studies on lower vertebrate and mammalian indicated that heart is not a post mitotic organ and there exists a constant cardiomyocyte turnover [8–11]. Cardiac regeneration studies in different organisms including zebrafish, amphibian, newt, human and neonatal mice indicated regenerative response of heart [8,12–15]. Using electron microscopy, Oberpriller et al. (1974) demonstrated that newt is capable to regenerate myocardium. Poss et al. (2002) reported that zebrafish could regenerate myocardium after amputation of 20% of ventricular apex of heart [8]. In 2000s, evidences for mammalian heart regeneration were reported in successive studies. In a landmark study, proliferative time period of neonatal mice were reported. It was shown that neonatal mice capable to regenerate myocardium until postnatal day 7 (P7) [14]. Moreover, using this model, we successfully identified Meis1 as one of the important regulator of this regenerative response [16]. In the last decade, different modulators of cardiac regeneration were reported and suggested to be important to reactivate cardiomyocyte cell cycle. In study

regarding the administration of cardiogenic factors such as IL3, FGF10, C3orf58, Oncostatin M, TNF-related weak inducer of apoptosis (TWEAK) and periostin increased cardiomyocyte number [17–22]. In gene manipulation studies, knockout profile of p27KIP1, mir-133a and salvador homolog (Salv) resulted with increased cardiomyocyte proliferation determined by mitosis-specific marker phospho-histone 3 (pH 3) [23–25]. Moreover, overexpression studies regarding c-myc, E1A, cyclin B1-CDC2, cyclinA2, cyclin D2, Notch signaling pathway, YAP and ERBB2 induce cardiomyocyte proliferation determined by mitosis markers (pH 3, Ki67 and aurora B) [26–32]. Kajstura et al. (2010) and Bergmann et al. (2009) reported that cardiomyogenesis occurs in adult heart depending on analysis of post mortem heart samples obtained from thymidine analogue iododeoxyuridine (IdU) treated cancer patients and 14C labeled human subject due to nuclear weapon test during Cold War, respectively [9,33]. In recent study, Canseco et al. (2015) reported adult heart cardiomyocytes proliferate response to injury due to integration of left ventricular assist device (LVAD) [34]. However, it is still unclear whether these newly formed cardiomyocytes derived from preexisting cardiomyocytes or cardiac stem cells.

1.3. STEM CELLS IN CARDIAC REGENERATION

After successive discovery of multiple cardiac progenitor and stem cells, cardiac clinical stem cell research area generated hope for their use in heart failure. Despite the fact that reactivating cardiomyocyte proliferation is one of the prominent approaches in cardiac regeneration, many researchers investigated embryonic stem cells (ESCs), bone marrow derived stem cells (BMCs), cardiac resident stem cells (CSCs) and skeletal myoblast cells [35,36]. It was suggested that embryonic stem cells (ESCs), derived from inner cell mass of blastocysts, could differentiate into beating cardiomyocytes. Induced pluripotent stem cells (IPSCs) created a new aspect in stem cell field and made possible to establish patient. specific cardiomyocyte differentiation studies [37]. ESCs are derived from the inner cell mass of an early stage embryo called as blastocyst [38]. ESCs are pluripotent and give rise to three different types of primary germ layers, which are ectoderm, endoderm and mesoderm. They represent a renewable progenitor cell source. The differentiation of ESCs into cardiomyocytes includes different stages: First step starts with induction and stepwise differentiation into mesoderm followed by differentiation into cardiosphere (expressing GATA4 and Nkx2.5 transcription factors). Cardiomyocyte differentiation occurs with the

expression of MHC, cTNI, α-actinin and other proteins inducing contraction [39,40]. ESCs can differentiate into almost all cell lines. However, there have not been any clinical studies regarding use of embryonic stem cells in myocardial regeneration. Beside the ethical issues, teratoma formation and the possibility of immunologic rejection might be the major limitations to utilize ESCs [41]. Takahashi and Yamanaka discovered induced pluripotent stem cells (IPSCs), which are differentiated from somatic cells into embryonic like pluripotent state. IPSCs can differentiate into different type of cells including but not limited to blood cells, islet cells, neurons and muscle cells [37]. Differentiation into IPSCs starts with retroviral introduction of the transcription factors Oct4, Sox2, cMyc and Klf4 and process takes 3–4 weeks for human cells [42]. Several groups reprogrammed the human skin fibroblasts into iPSCs. In a study, fully functional cardiomyocytes were derived from these fibroblasts [43–45]. In two other recent studies, injection of IPSCs to the infarct area resulted with in vivo differentiation into the cardiac cells [46,47]. Moreover, in vitro differentiation of IPSCs into cardiomyocytes, endothelial cells and smooth muscle cells were reported [48]. However tumorigenic property and difficulties in obtaining IPSCs are two major problems complicating use of IPSC [49]. Skeletal myoblast cells (SMCs) are the first cell types utilized in cardiac regeneration studies. Besides their contraction ability, SMCs can resist hypoxic environment and they can endure several hours of severe ischemia without being irreversibly injured [50,51]. However, injections of SMCs into patients with depressed Left Ventricular (LV) function failed to improve heart function in the MAGIC clinical study (Multicenter randomized myoblast autologous grafting in ischemic cardiomyopathy) [52]. Various factors were reported to lead the inefficient integration of injected myoblasts. It was suggested that adhesion proteins involved in myoblast integration could be an important factor [51]. Moreover, the intravenous injection of granulocyte colonystimulating factor (G-CSF) for the mobilization of bone marrow stem cell to myocardium was reported. However, no significant difference was observed with the G-CSF treatment [53]. The bone marrow derived mononuclear cells (MNCs), hematopoietic stem cells (HSCs), and mesenchymal stem cells (MSCs) have been investigated in regenerative cardiology. MNCs are classified as single nucleated cells in bone marrow and mostly contain differentiated cells with a less number of HSCs [3]. A study conducted by Orlic et al. (2001a, b) shows that intravenous transplantation of isolated HSCs to infarcted left ventricle (LV) could increase cardiomyocyte and vasculature renewal [36,54]. However further studies focusing on HSC transplantation shows that the regeneration capacities of HSCs are limited for a clinical

benefit [55]. Bone marrow MSCs are also promising for the cardiac regenerative therapies. MSCs are found in many different tissue types including adipose tissue and cord blood. Besides MSCs also can be differentiated in to vascular endothelial cells in vitro and in vivo. It has been shown that MSCs could be differentiated into cardiomyocytes to some extent [56,57]. However, applicability of MSCs post-acute myocardial injuries and heart failure is still investigated.

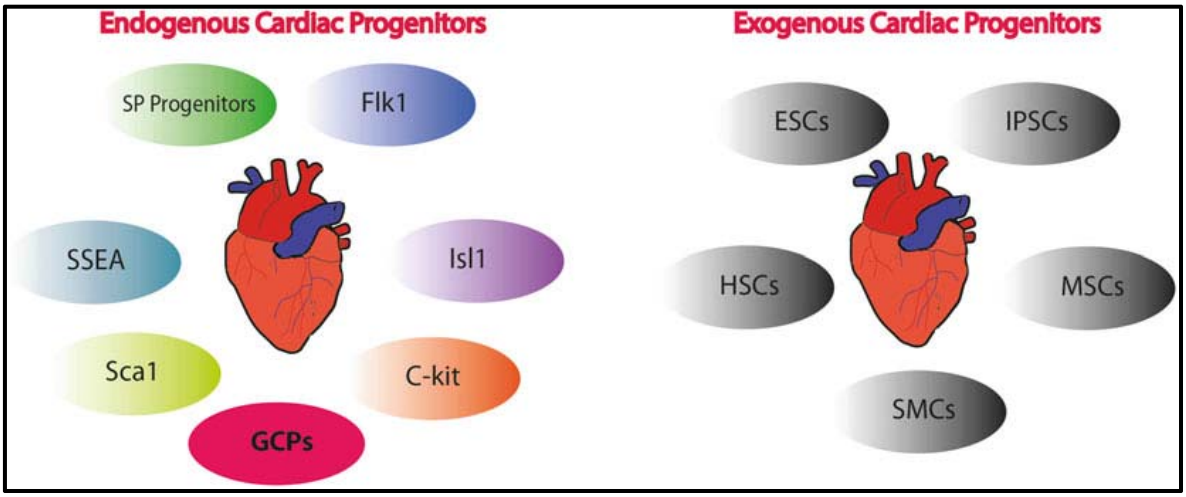

Figure 1.1. Endogenous and exogenous cardiac progenitor and stem cells studied in cardiac regeneration. SP progenitors: Cardiac Side Population Cells, Sca1: Sca1+ Stem Cells, c-Kit: Lin-, c-Kit + Stem Cells, Isl1: Isl1+ Progenitor Cells, SSEA: SSEA-1+ Progenitor Cells, Flk1: Flk1+ Progenitor Cells, GCPs: Glycolytic Cardiac Progenitors, ESC: Embriyonic stem cells, IPSCs: Induced pluripotent stem cells, HSCs: Hematopoietic stem cells, MSCs: Mesenchymal stem cells, SMCs: Skeletal myoblast cells.

1.4. ENDOGENOUS PROGENITOR & STEM CELLS IN CARDIAC REGENERATION

1.4.1. Resident Cardiac Stem Cells

Resident cardiac stem cells (CSCs) are probably the most promising cell types in the context of cardiac regeneration. After the discovery of stem cell-like cell populations in heart, successive studies showing the contribution towards cardiac regeneration were reported. Beltrami et al. (2003) discovered the c-Kit+ CSCs through the analysis of c-Kit receptor expression in myocardium [58]. Depending on the surface marker such as Sca1 (Morrison et al. 1997), Abcg-2 and Flk-1 [59,60], successive identification of different types of CSCs have been reported. Furthermore, Menasche (2004) reported the clonogenicity of resident CSCs in vivo and in vitro [61]. CSCs exist in supportive cell niches and their ratio in the heart tissue is reported to be approximately 1/30.000 [58,62,63]. In addition, their important roles in regeneration of myocytes, fibroblasts, smooth muscle cells and endothelial cells were reported in back to back reports [58,62–64]. After characterization of different CSCs including c-Kit+, Isl1 progenitors epicardial progenitors, side population (SP) progenitors, Stem cell antigen-1 (Sca1+) progenitors, cardiac mesenchymal stem cells were classified into different subgroups of CSCs [65], [66]. In another study regarding characterization of a population of epicardium-derived multipotent cardiac progenitor cells (cCFU-Fs) were established. Moreover, it was suggested that these cells resemble MSCs and may participate in cardiac development, homeostasis, and repair [67].

1.4.2. Side Population Progenitor Cells

Side population (SP) progenitors exist in a variety of organs such as brain, lungs, skin, bone marrow, liver and skeletal muscle. SP progenitors were firstly isolated from bone marrow and constitute 0.03–3.5% of mononuclear cardiac cells [68]. SP progenitor cells specifically defined as their ability to efflux the DNA binding Hoechst dye by ATP binding cassette. Moreover, SP progenitors are easily isolated by Fluorescent Activated Cell Sorting (FACS) technique [69]. It was reported that bone marrow SP progenitors differentiate into endothelial cells and cardiomyocytes in vitro [70]. In addition, heart originated cardiac SP

progenitors demonstrate Sca1+, CD34-, c-Kit- markers and express MEF2c, GATA4, Nkx2-5 transcription factors (Table 1). Moreover, SP progenitors can differentiate into cells expressing sarcomeric proteins like troponin and cardiac α-actinin [71,72].

1.4.3. Flk1+ Progenitor Cells

Studies in mouse cardiac development showed that the endocardium and a population of the myocardium develop from an intermediate population, which expresses Fetal liver kinase1 (Flk1) [73,74]. Yamashita et al. (2005) showed that cardiomyocytes could be generated from Flk1+ progenitors isolated from ES cell differentiation cultures and early embryos [75]. The Flk1+ cells exit the primitive streak and migrate for the formation of cardiac crescent [74]. The results support the idea that the myocardial and endothelial lineages develop from a common Flk1+ progenitor [76]. Moreover, multipotent Flk1+ progenitors can give rise to cardiomyocyte, endothelial and vascular smooth muscle lineages. A recent study revealed that CD45-, Flk1+ cells are present in adult human circulation and increase with acute myocardial infarction [77]. Flk1+ cells might be assigned for improvement of LV systolic function after myocardial infarction.

1.4.4. Isl1+ Progenitor Cells

First evidence related to Insulin gene enhancer protein1+ (Isl1+) progenitor cell population in the anterior heart field of mouse was identified by Kelly and Buckingham. (Kelly and Buckingham 2002). The study based on Isl1 mutants suggested that Isl1-expressing cells might contribute to the outflow tract of the heart. Laugwitz et al. (2005) identified Isl1+ cardiac progenitor cell population in neonatal heart [65]. The Isl1+ progenitors isolated after pediatric surgery were cultured on cardiac mesenchymal feeder layers, which are positive for Nkx2.5 and GATA-4 markers. Although Isl1+ cardiac progenitors may not necessarily have the same molecular identity, they are able to differentiate into mature cardiomyocytes. Unfortunately, Isl1+ cells are detected only in neonates. The absence of Isl1+ progenitors in adult heart suggests that cardiac progenitors identified during later stages of life might arise de novo from epicardium [78]. Later studies show that Isl1 is not a marker for cardiac progenitors in adult heart albeit it is a marker of the adult sinoatrial node [79]. Moreover,

another study showed that Isl1 also labels cardiac the neural crest besides the second heart field progenitors in adult murine and human hearts [80]. It has been recently shown that by Bone Morphogenic Protein 4 (BMP4) signal activation, functional cardiomyocytes can be differentiated from Isl1+ CSC population [81]. The first clinical case study was conducted by Menasche et al. (2015) and demonstrated improvement to some extent in short term [82].

1.4.5. c-Kit+ Cardiac Stem Cells

c-Kit is a tyrosine kinase receptor factor (CD117) and c-Kit+ cells are well identified population existing in heart. c-Kit is one of the intensively studied cell surface marker and it was reported that the intracoronary delivery of isolated murine heart c-Kit+ cells to the infarcted rat hearts improved LV function at 35 d. Due to low retention and rapid disappearance of CSCs from the recipient heart, authors suggested that paracrine effects could be the underlying mechanism in this recover process [83]. These cells have the mesenchymal origin and it has been shown that c-Kit+ cells migrate through the infarct zone and may give rise to cardiomyocytes in vivo [84]. Another study reveals that the infusion of c-Kit+ CSCs reduce the rate of oxidative stress and apoptosis in both cardiomyocyte and non-cardiomyocyte cell populations [85]. c-Kit+ cells also express Nkx2-5, GATA4 and GATA5 transcription factors. Tillmanns et al. (2008) indicated that the myocardial injection of IGF1 and hepatocyte growth factor treated c-Kit+ cells increase the regeneration capacity of myocardium [86]. Moreover, Choi et al. (2013) reported that c-Kit+ CSCs proliferate in vitro provided that optimal culture and enzymatic isolation methods maintained [87].

1.4.6. Sca1+ Cardiac Stem Cells

Stem cell antigen1 (Sca1) expressing cardiac stem cells, which are characterized by CD45 , CD34-, Flk1- markers as well, comprise differentiation capability into myocardium, smooth muscle cells and endothelial vascular cells [64,69]. Sca1+ cells can differentiate into cardiomyocytes through formation of cardiospheres. In a study conducted by Matsuura et al. (2004) Sca1+, CD45- mice cells were exposed to oxytocin. Oxytocin leads to the expression of cardiac transcription factors and contractile proteins. Thus, it leads to the contraction of Sca1+ CSCs in-vitro [88]. Moreover, intramyocardial injection of these Sca1+ CSCs after,

Table 1.1. Classification and characteristics of resident CSCs, *; low amount of identification, N/A; Not available, MR; Myocardial regeneration

Cell Types	(+)	(-)	Species	In vivo MR	In vitro Differentiation	References
	Markers	Markers				
Cardiac SP Cells	ABCG2, Sca1, C-kit*, CD34*	CD31	Adult mouse	N/A	Co culture with cardiac cells	[88]
Sca1+ Stem Cells	Sca1	c-Kit, CD34, CD45, Lin, Flk1	Adult mouse	Yes	5-Azacytidine or Oxytocine treatment	[89], [90]
c-Kit+ Stem Cells	c-Kit, Nkx2.5, GATA4, GATA5	CD34, CD45, Lin	Adult Rat, mouse, dog, porcine, human	Yes	Medium (MEM+10%, FCS+10nM)	[58]
Isl1+ Progenitor Cells	Isl-1, GATA4	Sca1, c-Kit	Neonatal mouse, rat and human	N/A	Co culture with neonatal cardiomyocytes	[65]
SSEA-1+ Progenitor Cells	Nkx2.5, GATA4, Oct 3/4	c-Kit, Sca1, TnnT	Adult rat, Neonatal rat, human	Yes	5-Azacytidine treatment	[91]
Flk1+ Progenitor Cells	Flk1,	CD45	Adult human	N/A	Collagen type IV	[92]
Glycolytic Cardiac Progenitors	Hif-1α,	CD45, Sca1, c-Kit, CD31, FSP1	Adult mouse	N/A	Co culture with neonatal cardiomyocytes, 5-Azacytidine treatment, serum withdrawal, Endothelial differentiation medium	[93]
(GCPs)	Nkx2.5, GATA4, WT-1, Tbx 18, preferential cytoplasmic glycolysis					

the infarction increased cardiac function [89]. Another study revealed that Sca1 knockout mice lead to myocardial contraction dysfunction [90]. In another study, Sca1+ cells were

identified in the stromal area of the myocardium [91]. The location and relationship of these Sca1+ cells are behaviorally like skeletal muscle stem cells, which are located in below the basal lamina and containing laminin. Moreover, studies regarding laminin showed that the Sca1+ located under the basal lamina and tightly associated with cardiomyocytes [91]. Their easily accessible nature and optimized culturing conditions makes Sca1+ CSCs an ideal candidate to study and research cardiac tissues [92].

1.4.7. SSEA+ Cardiac Progenitors

Stage-specific embryonic antigen1+ (SSEA1+) cardiac stem cell population has been isolated from neonatal and adult rat hearts [93]. Adult rat heart SSEA1+ cells are characterized by OCT3/4+, c-Kit-, and Sca1+ surface markers. Moreover, neonatal rat heart SSEA1+ cells express Nkx2.5, GATA4 and cardiac myosin heavy chain indicating their cardiomyogenic differentiation potential. Moreover, in the case of co-culture with primary cardiomyocytes, SSEA1+ progenitor cells differentiate and express α-sarcomeric actin or cardiac myosin heavy chain, leads to formation of beating colonies. In addition, transplantation of SSEA1+ cells into an infarcted rat heart (106 cells injected intromyocardially) induce myocardial regeneration and functional improvement (LVEF ¼ 57% in treated vs LVEF ¼ 28% in control at 3 weeks compared to 2-week baseline LVEF ¼ 36%) [93]. Ott et al. (2007) suggested that the SSEA1 identifies the most primitive cardiac stem cells present in the adult mammalian heart suggesting that c-Kit, Sca-1 and Abcg2 identify cardiac stem cells at later stages of cardiac-specific differentiation [93]. In another study, SSEA1 surface marker was used for the isolation of CPCs from human embryonic hearts isolated neonatal human SSEA1+ cells also express Oct4, Nkx2.5, Isl1 and Tbx5 at the mRNA level. However, they did not express troponin T (TnnT) which is defined as mature cardiomyocyte marker. Moreover, human neonatal SSEA1+ cells co-express the mesenchymal stem cell markers such as CD105, CD166, CD73, CD59 and CD44 but not the hematopoietic markers (CD45, CD133 and CD34).

1.4.8. Glycolytic Cardiac Progenitors

Over the last decade, CSC niches were suggested to be more complicated than other epithelial organs owing to basal and apical morphology of heart [94]. Utilization of different anatomical methods on epicardial region revealed numerous different cardiac niches [95–100]. Moreover, it was reported that CSC niches not only existed in sub-epicardium, but also differentially disturbed in myocardium [101]. A recent study, demonstrated an epicardial/ subepicardial microenvironment with characteristics of low oxygen tension. This was evident by low capillary density compared to myocardium and endocardium. In addition, they have shown that epicardial/subepicardial microenvironment houses hypoxia inducible 1 alpha (Hif-1α) positive cells. Hif-1α gene is constitutively expresses but it has been known to be stabilized at protein level only in hypoxic (<2% O2) microenvironments. These suggests that 3–7 cell layer of epicardial/subepicardial microenvironment could be a niche for long term maintenance of cardiac stem and progenitors. Indeed, Kocabas et al. (2012) shows that they could metabolically profile and isolate cells from adult mouse heart with low mitochondrial potential (Low MP) using FACS. These Low MP cells, named glycolytic cardiac progenitors (GCPs) express bone fide cardiac progenitor markers Nkx2.5, GATA4, WT1, Tbx18. GCPs could be differentiated into endothelial, smooth muscle and cardiac lineages [100]. In addition, GCPs are clonogenic and could be maintained in cell culture up to 60 passages in ES medium. GCPs marked with HIF-1α protein stabilization even in normoxic (21% O^2) cell culture conditions. GCPs drastically resistant to anoxia and hypoxia with concomitant higher survival rates, which might be related to their localization to hypoxic cardiac epicardial/ subepicardial microenvironment. GCPs [100].

1.5. SMALL MOLECULES IN CARDIAC REGENERATION

Cardiac regenerative medicine aims to induce proper tissue development and enhance regeneration of injured heart. By their ability to conveniently pass through the cells without triggering immune response, and their ability to induce or reduce targeted types of cells, small molecule treatments can revolutionize the field of cardiac regeneration [102].

Despite multipotent cells have a potential in the aspect of cardiac regeneration, favorable tissue regeneration relates to the broad control of multipotent cell differentiation to targeted

cell type and integration to the delivered area to constitute a functional structure. Each step of this process governed by the essential signals of proteins and small molecules [102]. Therefore, identification of the essential signals is a foremost important objective of cardiac regeneration.

Prostaglandins are a class of small molecule (fatty acid derivatives) which are responsible from various physiological effects. Specially Prostaglandin I2 and E2 have a regenerative effect on ischemic myocardium. The studies show that the Prostaglandin I2 and E2 may have a therapeutic potential post myocardial infarction. Prostaglandin I2 is a anti coagulant agent having the approval of FDA to be used as a therapeutic agent on hypertension disorders. Prostaglandin I2 also have a short half-life period in vivo. Therefore it is decreased in the case of myocardial infarction [103]. In a study conducted on rodent models reveals that Prostaglandin I2 is effective on the upregulation of HGF,VEGF,G-CSF and DSF-1 cardioprotective factors [104].

The studies show that the Prostaglandin E2 also have a regenerative role on ischemic myocardium. In the presence of Sca1(+) cells the level of Prostaglandin E2 is increased and regulated their potential for cardiac differentiation. It can be suggested that the Prostaglandin E2 can activate the endogenous CSC populations. In a study conducted on adult mouse hearts reveals that the Prostaglandin E2 treatment refreshes the ability of cardiomyocytes at the infarct border [105]. Prostaglandin E2 is also an FDA approved molecule for their effect of induction of translational potential.

Pyrvinium Pamaoate is an anthelminic drug which is responsible from the ihibiton of NADH fumarate reductase activity. In a study conducted by Murakoshi et al. the administration of Pyrivinium Pamaoate to the myocardial scar after infarct produces differential cytotoxic effects on fibroblasts which are responsible from the acumulation to the scar area and therefore formation of excessive fibrotic tissue [106]. The study reveals that that the administration of Pyrvinium Pamaoate significantly reduces the fibroblast formation in the infarct and border regions of LAD ligated mouse hearts (176).

Dipeptidylpeptidase IV (DPP-IV) is a peptidase that bounds to membrane and responsible from the cleavage of SDF-1. The inhibition of DPP-IV resulted with stabilization of myocardial SDF-1 after myocardial infarction and by that increasing the level of recruitment of CXCR4(+) CSCs to induce regenerative response. In a study conducted by Zaruba et al.

the administration of DPP-IV inhibitor Diprotin A resulted with the mobilization of circulating progenitors and DPP-IV inhibition [107]. The study shows that CXCR4(+) level increased and neovascularization occurred on infarct area. Moreover, the study reveals that the administration of DPP-IV inhibitor also enhanced myocardial function and therefore increases the survival rate [107].

This study aims to identify novel therapeutic effect of the small molecules on neonatal rat cardiomyocytes and fibroblasts. The small molecules were selected by their ability to induce proliferation of ex-vivo hematopoietic stem cells. In this study, over 30 small molecules were tested in 3 different concentrations to achieve the dose dependent manner effect on cardiomyoctes and fibroblasts.

2. METHODS

2.1. ANIMAL INFORMATION

Wistar rat pups and adult Balb-C mice have been used in this study, and animal studies were used under decision number 417 which approved by the Institutional Clinical Studies Ethical and the Institutional Animal Care and Use Committee of Yeditepe University (YUDHEK, decision number 417).

2.2. ADS BUFFER PREPARATION AND MEDIA CONDITIONS

10x ADS buffer was prepared by addition of 68gr of Sodium Hydroxide, 47,6 gr HEPES ($C_8H_{18}N_2O_4S$), 1,2g Sodium dihydrogen Orthophosphate (NaH_2PO_4), (1.37g $NaH_2PO_4.H_2O$), 10g Glucose, 4g Potassium Chloride, 1g Magnesium Sulfide ($MgSO_4$), (1.97g $MgSO_4$.7H2O), 900ml of dH^2O into 1L autoclaved beaker with stirrer bar and stirred until dissolvation process (pH: 7,35). Solution filtered 0,2 μM filters. Growth media conditions were %10HBS, %10FBS, %1 Penicillin/Streptomycin in DMEM.

2.3. NEONATAL RAT HEART TOTAL CELL ISOLATION

Neonatal rat pups were taken at P1 and hearts (10 hearts approximately) were extracted after decapitation and were maintained in petri dish with 5ml of 1xADS buffer. Each heart sample were cut into 4 or 5 pieces. After the washing step, samples were taken with 1000 ml filter tip which was cut with scissors to prevent the physical deformation of the samples and inserted into the MagnaFlask with the addition of 5 ml of collagenase II and pancreatin solution (Figure 2.1). The MagnaFlask was placed onto the heater with magnetic stirrer (37^0C, 180rpm) for 20 min. The supernatant were slowly poured into 15ml falcon tube with the addition of 1 ml of FBS while paying attention to not to take any parts from the sample. The solution was centrifuged for 10 minutes at 1800 rpm, 4^0C. The supernatant was disposed and 1 ml of cold complete media was added into 15ml falcon tube. The tubes were maintained on ice while the procedure proceeds. After the decomposition of all of the heart

samples, all tubes were combined together in 50 ml falcon tube and centrifuged for 10 minutes at 1800 rpm 4^0C. 2 ml 1xADS buffer added onto the pellet and pipetaged. The procedure continued with the percoll gradient step.

Figure 2.1. Representative image of MagnaFlask cell isolation apparatus on heater with magnetic stirrer.

2.4. NEONATAL RAT HEART CARDIOMYOCYTE ISOLATION

Top (13.5ml Percoll Stock + 16.5ml 1xADS Buffer) and Bottom (13ml Percoll Stock + 7ml 1xADS Buffer) percoll gradient were prepared. 4ml of top gradient was poured into a 15ml falcon tube. 3ml of bottom gradient was inserted into the bottom of the tube underlaying top gradient. 2ml of the isolated heart cell sample in 2ml 1xADS were inserted at the top of falcon tube (Figure 2.2). The centrifuge was prepared (acceleration :1, deceleration:0). The sample tube was centrifuged at 3000rpm for 30 min. The sample tube was taken and the cells on 3 ml band were taken and inserted into a different falcon tube to wash cells. The cells were washed with cold 1x ADS buffer and 1ml of complete media were poured onto the pellet for further cell counting with hemocytometer. Approximately 10.000 cells were inserted into each 96 well plate with 0,1ml of complete media.

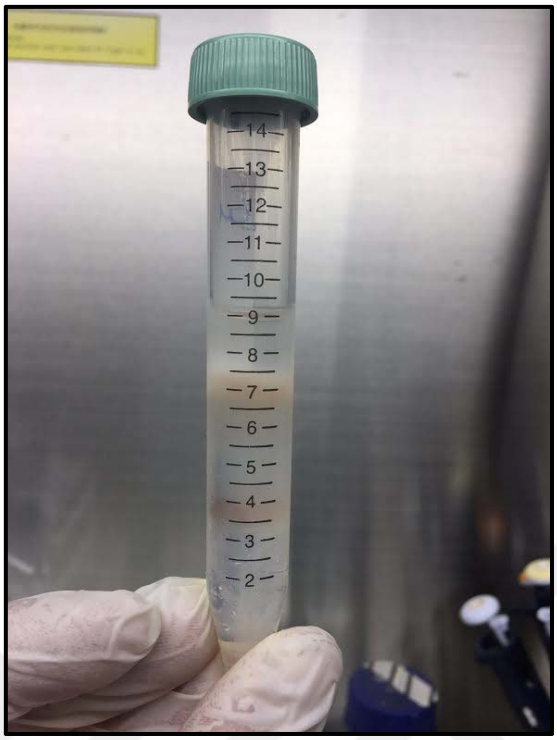

Figure 2.2. Percoll gradient seperated cardiomyocytes and fibroblasts observed as different cell populations on varied levels.

2.5. SMALL MOLECULE INSERTION

The small molecules were dissolved in DMSO and diluted in PBS for 1mm, 0,1mm and 0,01mm concentrations. Each small molecule concentration added to a well of 96 wellplate and labeled. %1 DMSO were inserted into control wells. The 96 wellplate was incubated at 37^0C for 4 and 7 days respectively for the analysis at different time points. Final small molecule concentration was %1 for each well.

2.6. IMMUNOFLUORESCENCE OBSERVATION OF NEONATAL RAT CARDIOMYOCYTES PROLIFERATION AND CELL COUNTING

Paraformaldehyde was used for the fixation of the cardiomyocyte cultures. Cells were incubated with paraformaldehyde for 10 min in incubator (37^0C, %5 CO^2). %0,1 Triton-x in 1xPBS was inserted onto each well and incubated for 10 min in incubator. %1 BSA added

onto each well for blocking and incubated for 30 min in incubator. For the immunostaining. Tnnt (1:200) antibody was inserted to the wells and incubated in refrigerator overnight (+4^{0}C). Next day, cells were incubated with pH3 for 2 hours at incubator. Furthermore, cells are treated with Alexa Fluor® 488 Donkey Anti-Mouse IgG (H+L) Antibody and Alexa Fluor® 555 Donkey Anti-Rabbit IgG (H+L) Antibody, Hoechst 33342 staining and each pH3(+) CMs were counted by 40X fluorescence microscope.

2.7. MTS CELL VIABILITY ASSAY

Promega MTS substance was used in this experiment. MTS substance with 1/10 ratio mixed with complete medium (0,1 µL MTS dissolved in complete medium). Cells were incubated for 3 hours in incubator. The plate was then analyzed with spectrophotometer at 490 nm. Blank subtracted and results were normalized to the DMSO control wells.

3. RESULTS AND DISCUSSION

3.1. LIST OF SMALL MOLECULES AND PATHWAYS TESTED IN THIS STUDY

Table of small molecules that have been tested in this study and their targets have been listed below (Table 3.1).

Table 3.1 Complete list of tested small molecules in this study

NO	Gene Symbol	Ex vivo (or in vivo) Expansion	Drugs Selected
1	PTPMT	Blood paper	Alexadine Dihdrochloride
2	HAT (p300&CBP Inhıbitor IV)	Not Yet Tested	HAT Inhibitor - 505298 / p300&CBP Inhıbıtor VI
3	Inppp5d		AS1949490
4	Dnmt3a	Not Yet Tested	RG 108
5	HAT Inhıbıtor	YES	HAT Inhibitor - Garcinol and derivatives
6	GSK-3	Not Yet Tested	CHIR-99021
7	Puma (Bbc3)	Not Yet Tested	ZINC 19882186 - C#7
8	c-Myc	Not Yet Tested	c-Myc inhibitor II
9	Tet2	Not Yet Tested	SC1 (pluripotin)
10	Trp53 or p53	Not Yet Tested	Pifithrin-α
11	II2	Not Yet Tested	Ro 26-4550 trifluroacetate
12	Gli1	Not Yet Tested	GANT 61
13	Hif1a	Not Yet Tested	400083 I HIF-1 Inhibitor
14	AhR	YES	StemReginin 1, 182706 I AhR Antagonist II, SR1
15	Cdc42	Not Yet Tested	CASIN
16	Cxcr4	Yes	CXCR4 Antagonist !, AMD3100
17	Id1	Not Yet Tested	Cannabidiol (CBD)
18	Runx1	Not Yet Tested	219506 I CFB-β-Runx1 Inhibitor II, Ro5-3335
19	Skp2	Not Yet Tested	SKP2-C25, 506305 I SKP2 E3 Ligase Inhibitor III
20	Pten	Not Yet Tested	bpV (Hopic)
21	Gjb1 (Cx32)	Not Yet Tested	2-aminoethoxydipenyl-borade (2APB)
22	Sirt1	YES	EX 527 & Nicotinamide
23	Unidentified	YES	Tauroursodeoxycholic acid (TUDCA)
24	p38	YES	SB203580 and SB239063, a p28 MAPK inhibitor

25	Unidentified	YES	α-Tacopherol
26	iNOS	YES	L-N&-(1-iminoethyl)-lysine hdrochloride (L-NIL)
27	Unidentified	Not Yet Tested	BIO (6-bromoindirubin-30-oxime)
28	Unidentified	Not Yet Tested	SKF 96395 hdrochlorine
29	Unidentified	Not Yet Tested	Mdivi-1
30	Unidentified	Not Yet Tested	(5Z)-7-Oxozeaenol
31	Unidentified	Not Yet Tested	SB203580 and p38 MAPK inhibitor
32	Unidentified	Not Yet Tested	trans-2-phenylcyclopropyşamine hdrochloride
33	Unidentified	Not Yet Tested	Tetraethylammonium chloride
34	Unidentified	Not Yet Tested	K252c
35	Unidentified	Not Yet Tested	BML-260
36	Unidentified	Not Yet Tested	N-4-Tosyl-L-arginine methyl ester hydrochloride

3.2. SCION IMAGE CELL COUNTING ANALYSIS

Scion Image cell counting program was used to observe and count the DAPI stained cells to obtain total cell number in a CM-FB mixed culture. The images were turned into black and white via Scion Image and automatically counted. The small molecules were labeled with numbers to ease data analysis. Afterwards, hit substances were selected (marked with arrows) for further experiments (Figure 3,1, Figure 3,2, Figure 3,3).

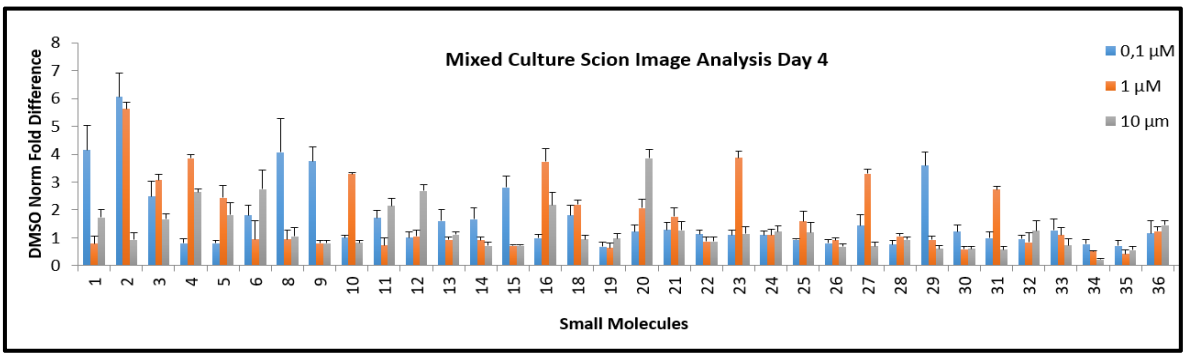

Figure 3.1. CM and FB mixed culture cell count with Scion Image analysis at day 4 after small molecule treatment at different doses.

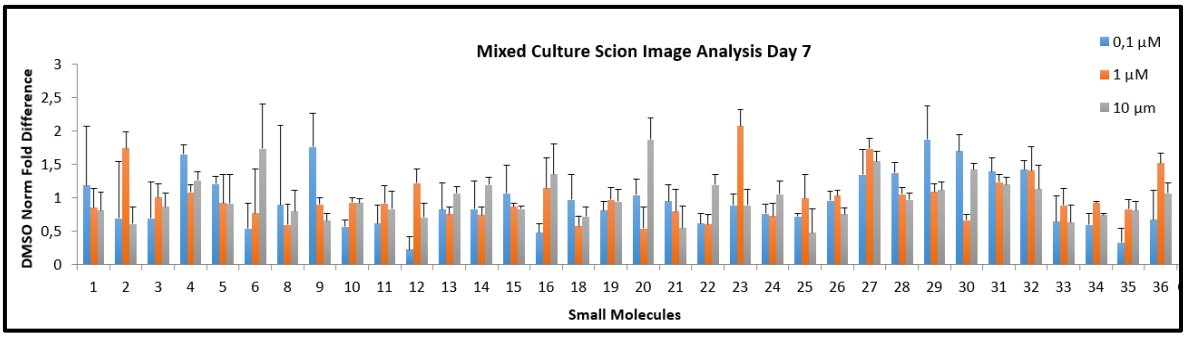

Figure 3.2 CM and FB mixed culture cell count with Scion Image analysis at day 7 after small molecule treatment at different doses.

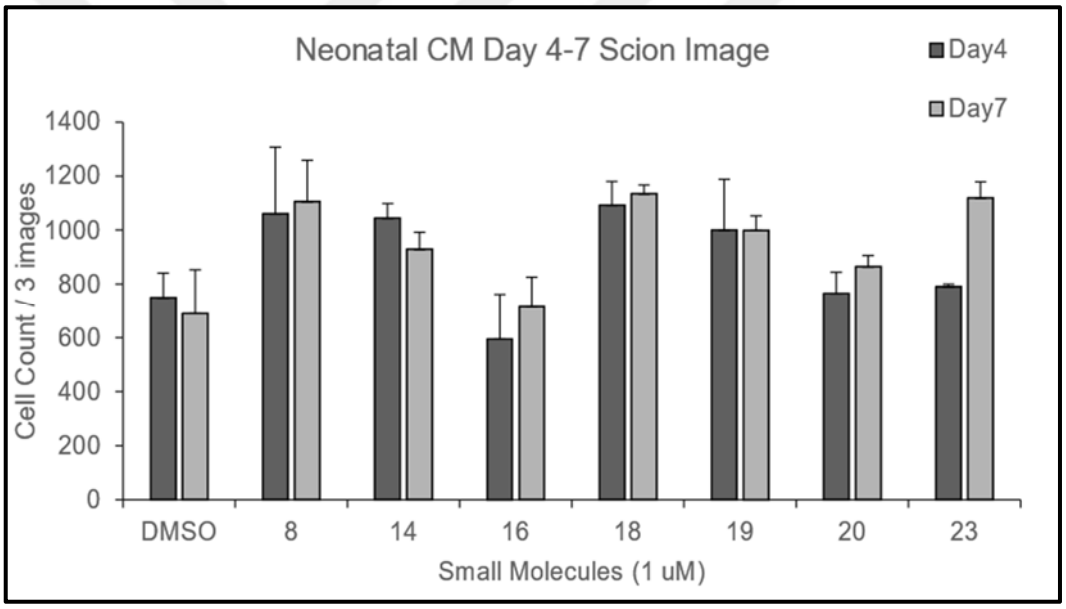

Figure 3.3. CM and FB mixed culture cell count with Scion Image analysis of selected molecules at day 4 and day 7 after small molecule treatment at 1µM.

3.3. MTS CELL VIABILITY ASSAY ANALYSIS

MTS cell viability assay was conducted at day 4 and day 7 after small molecule treatment. Hit molecules were marked with arrows for further analysis (Figure 3,4, Figure 3,5, Figure 3,6).

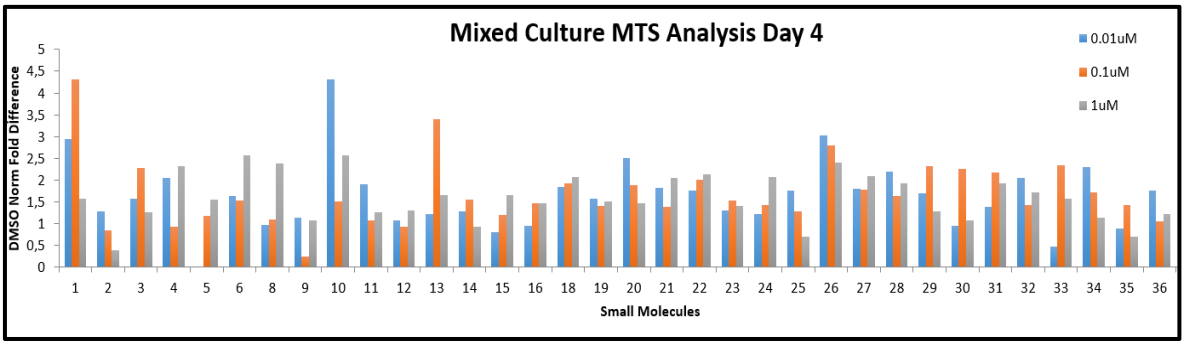

Figure 3.4. MTS Cell viability assay results of CM and FB mixed culture at day 4 after small molecule treatment at different doses.

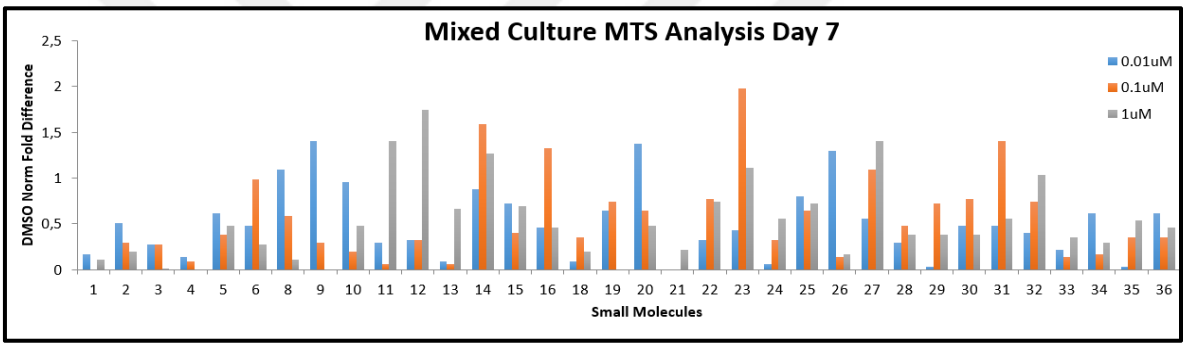

Figure 3.5. MTS Cell viability assay results of CM and FB mixed culture at day 7 after small molecule treatment at different doses.

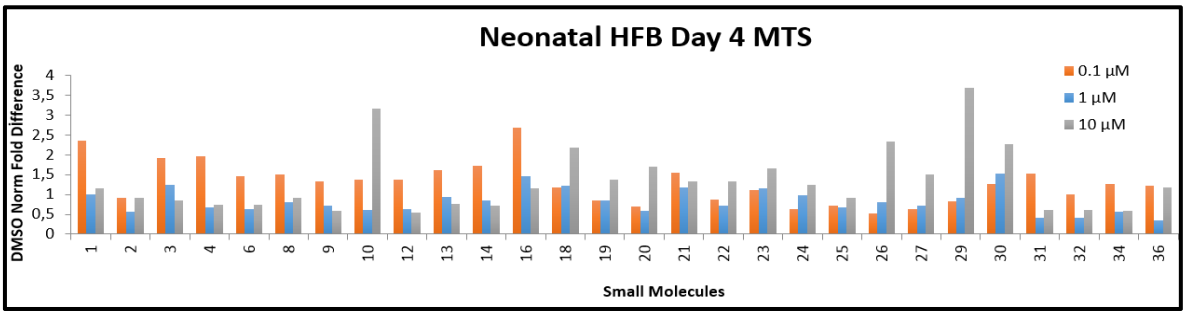

Figure 3.6. MTS Cell viability assay results of Neonatal Heart FB at day 4 after small molecule treatment at different doses.

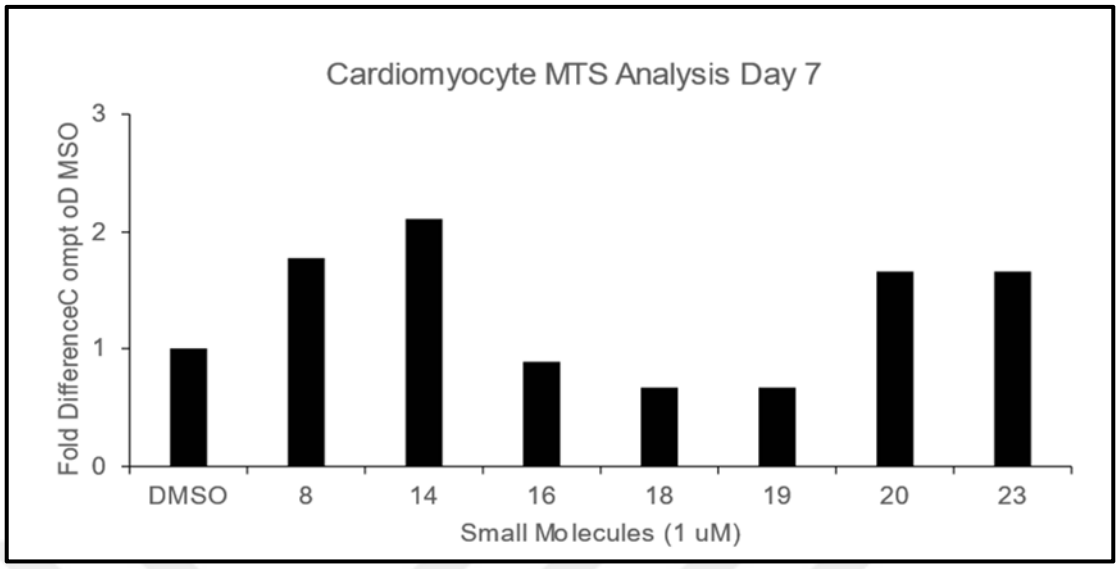

Figure 3.7. MTS Cell Viability assay analysis of selected molecules.

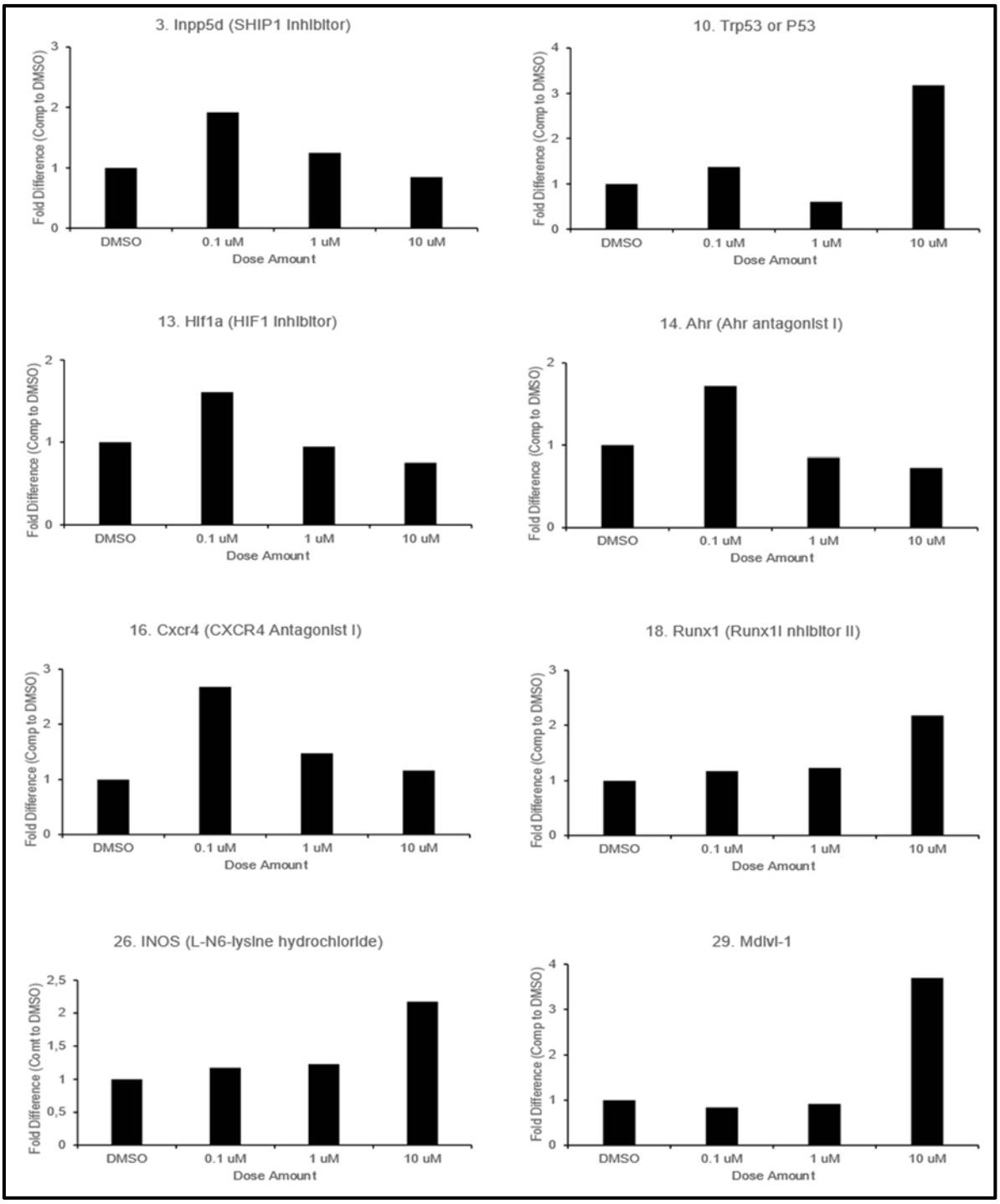

Figure 3.8. Dose dependent MTS analysis of selected molecules on Neonatal Heart FBs.

3.4. IMMUNOSTAINING PH3 POSITIVE CARDIOMYOCYTE CELL COUNTING

Immunostaining procedure was conducted and images were taken with fluorescence microscopy using 20x, 40x and 63x lenses (Figure 3,9). Neonatal heart CMs and FBs have been observed and pH3+ cells were counted from each sample containing well.

Primary molecule selection was based on the positive feedback of the molecules in their experimental setup. The secondary selection is based on their effect on 5 or more different studies. Tertiary selection was done by excluding FB based studies and focus on CM and mixed culture related studies (Figure 3,14, Figure 3,15, Figure 3,16).

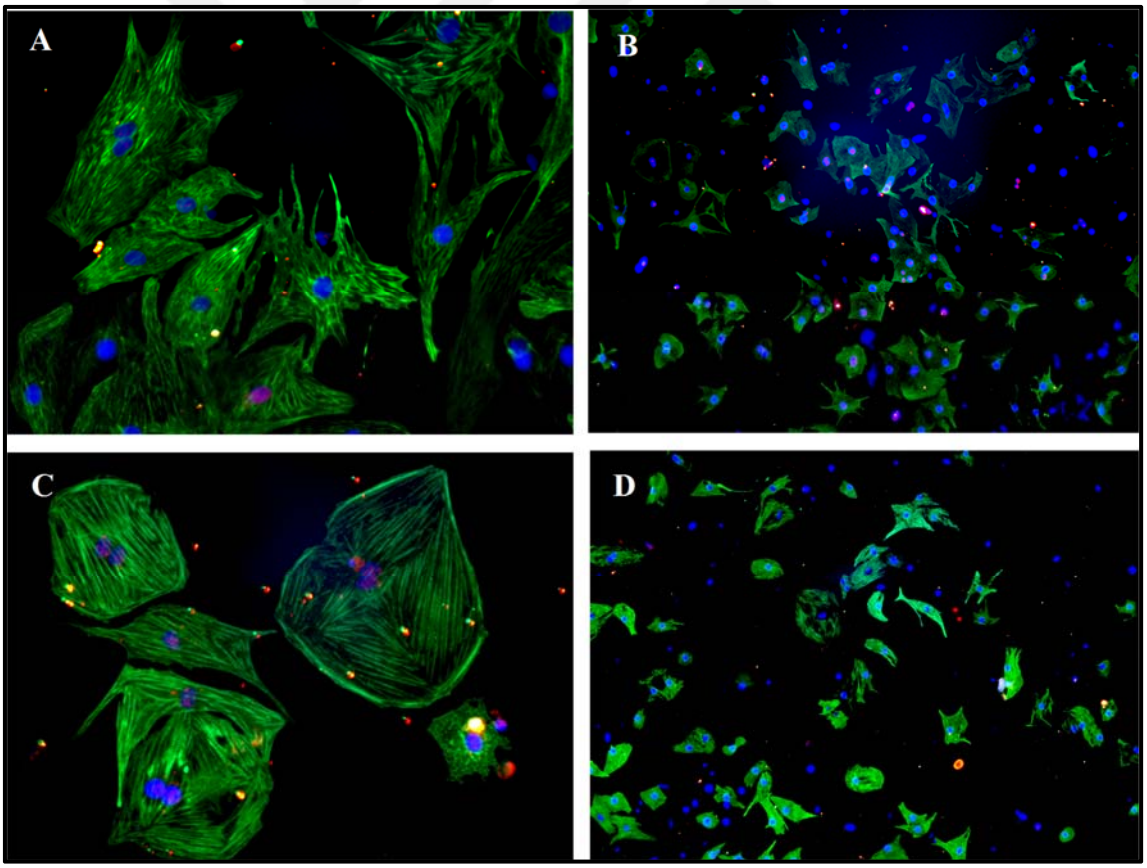

Figure 3.9. Representative image of Tnnt (Troponin T) and pH3 (Phospho Histone 3) immunostained Neonatal CMs. A) pH3 positive CM cells at 40x B) CM and FB coculture pH3 positive cells at 20x C) pH3 positive cardiomyocyte cells at 63x D) pH3 postive

cardiomyocyte cells at 20x (Tnnt cardiomyocyte marker: green, pH3 proliferation marker: red, DAPI nucleus marker: Blue).

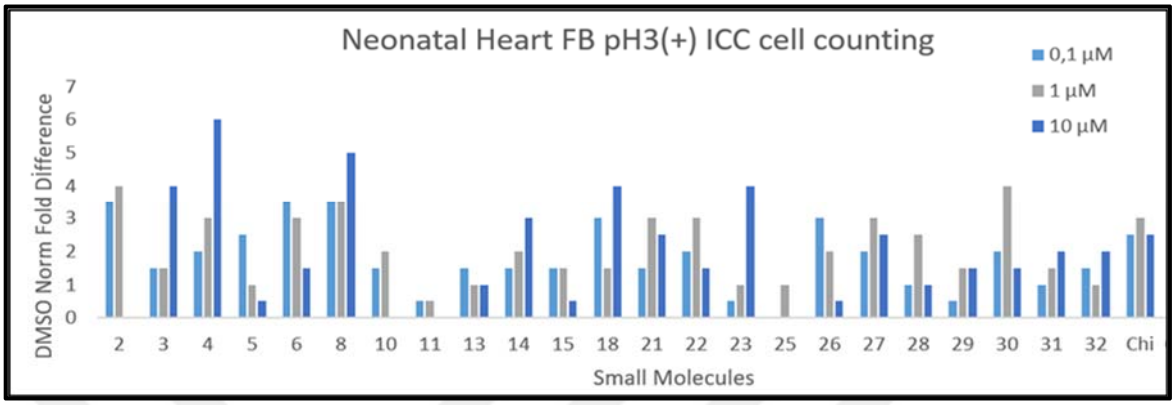

Figure 3.10. pH3(+) Neonatal Heart FB cell count with fluorescence microscopy. 5 photographs taken from each well randomly using 3 different filters. ZEN software have been used to merge images taken from DAPI, TnnT and pH3. pH3(+) CMs were counted and the data was normalized to the cell count value of DMSO control wells.

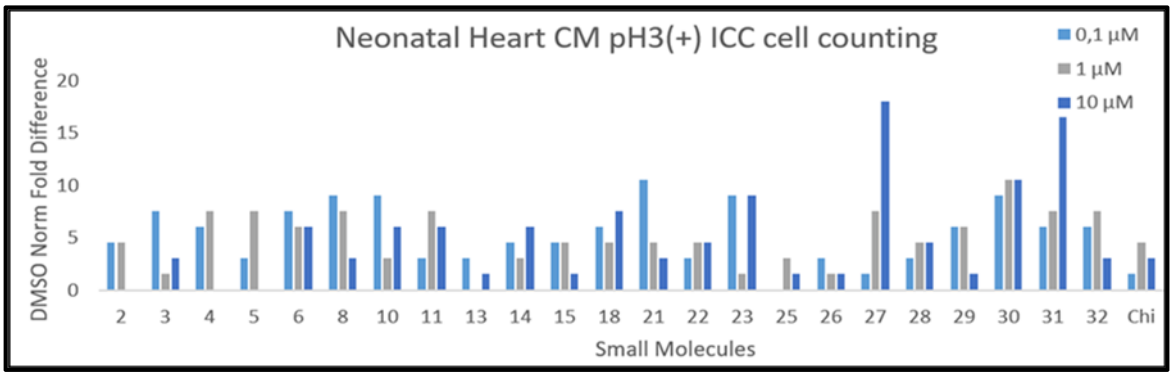

Figure 3.11. pH3(+) Neonatal Heart CM cell count with fluorescence microscopy. 5 photographs taken from each well randomly using 3 different filters. ZEN software have been used to merge images taken from DAPI, TnnT and pH3. pH3(+) CMs were counted and the data was normalized to the cell count value of DMSO control wells.

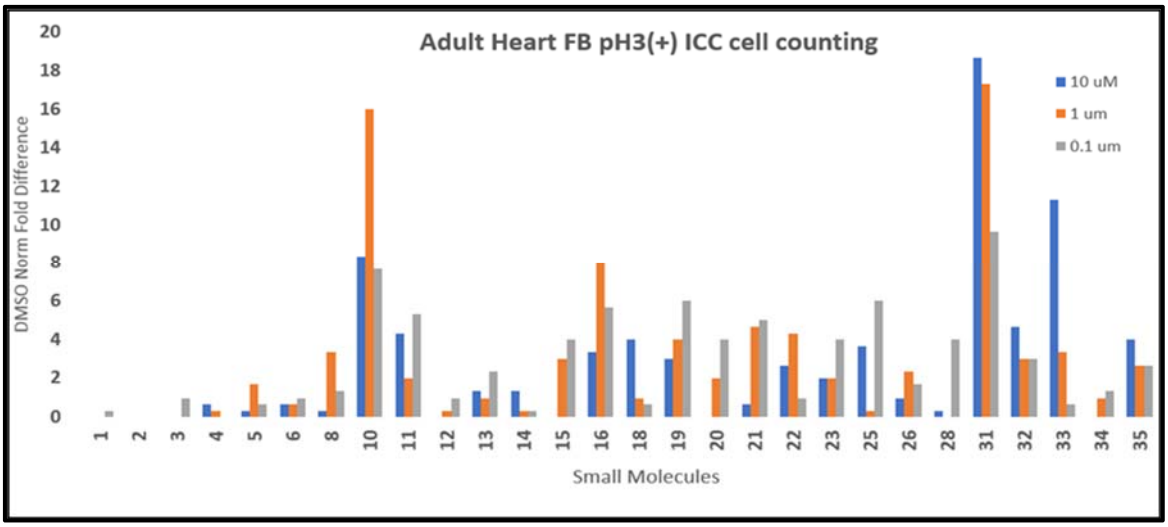

Figure 3.12. pH3(+) Adult Heart FB cell count with fluorescence microscopy. 5 photographs taken from each well randomly using 3 different filters. ZEN software have been used to merge images taken from DAPI, TnnT and pH3. pH3(+) CMs were counted and the data was normalized to the cell count value of DMSO control wells.

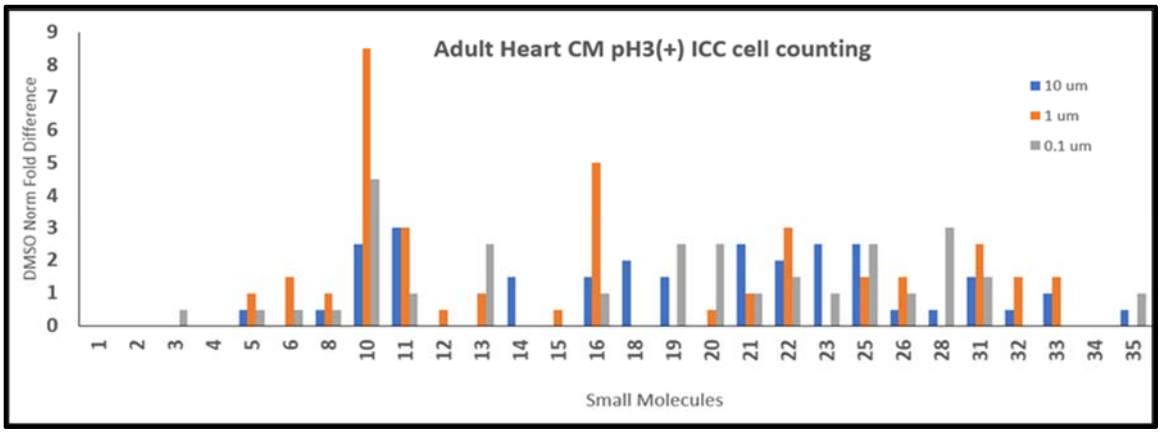

Figure 3.13. pH3(+) Adult Heart CM cell count with fluorescence microscopy. 5 photographs taken from each well randomly using 3 different filters. ZEN software have been used to merge images taken from DAPI, TnnT and pH3. pH3(+) CMs were counted and the data was normalized to the cell count value of DMSO control wells.

Name of the study	Small Molecules												
Neonatal CM ICC	2	4	6	8	10	18	21	23	27	30	31		
Neonatal FB ICC	5	10	11	13	15	18	21	23	25	26	29	30	31
Adult CM ICC	10	11	13	16	22	25	31						
Adult FB ICC	1	2	4	6	8	12	13	14	20	26			
Mixed Scion Day 4	1	2	8	10	12	16	20	23	21	29			
Mixed Scion Day 7	2	6	12	16	23	27	29	30					
Mixed Scion Selected	8	14	18	19	23								
Mixed Culture MTS 4	1	2	3	6	10	13	18	20	26	29	30		
Mixed Culture MTS 7	9	10	11	12	14	16	20	23	27	31			
Neo HFB MTS 4	2	6	8	9	12	25	31	34					
CM MTS Selected	8	14	20	23									

Figure 3.14. Selected hit molecules amongst all concluded screenings.
The molecules were selected due to their activity in Neonatal CM ICC, Neonatal FB ICC, Adult CM ICC, Adult FB ICC, Mixed Scion Day 4, Mixed Scion Day 7, Mixed Scion Selected molecules, Mixed Culture MTS Day 4, Mixed Culture MTS Day 7, CM MTS Selected molecules respectively.

Name of the study	Small Molecules												
Neonatal CM ICC	2	4	6	8	10	18	21	23	27	30	31		
Neonatal FB ICC	5	10	11	13	15	18	21	23	25	26	29	30	31
Adult CM ICC	10	11	13	16	22	25	31						
Adult FB ICC	1	2	4	6	8	12	13	14	20	26			
Mixed Scion Day 4	1	2	8	10	12	16	20	23	21	29			
Mixed Scion Day 7	2	6	12	16	23	27	29	30					
Mixed Scion Selected	8	14	18	19	23								
Mixed Culture MTS 4	1	2	3	6	10	13	18	20	26	29	30		
Mixed Culture MTS 7	9	10	11	12	14	16	20	23	27	31			
Neo HFB MTS 4	2	6	8	9	12	25	31	34					
CM MTS Selected	8	14	20	23									

Figure 3.15. Selected hit molecules after the primary elimination.
The molecules were selected due to their positive response in more than 5 concluded studies naming Neonatal CM ICC, Neonatal FB ICC, Adult CM ICC, Adult FB ICC, Mixed Scion Day 4, Mixed Scion Day 7, Mixed Scion Selected molecules, Mixed Culture MTS Day 4, Mixed Culture MTS Day 7, CM MTS Selected molecules respectively.

Name of the study	Small Molecules										
Neonatal CM ICC	2	4	6	8	10	18	21	23	27	30	31
Adult CM ICC	10	11	13	16	22	25	31				
Mixed Scion Day 4	1	2	8	10	12	16	20	23	21	29	
Mixed Scion Day 7	2	6	12	16	23	27	29	30			
Mixed Scion Selected	8	14	18	19	23						
Mixed Culture MTS 4	1	2	3	6	10	13	18	20	26	29	30
Mixed Culture MTS 7	9	10	11	12	14	16	20	23	27	31	
CM MTS Selected	8	14	20	23							

Figure 3.16. Selected hit molecules after the secondary elimination.
The molecules were selected due to their positive response in more than 4 concluded CM and CM, FB mixed culture studies naming Neonatal CM ICC, Adult CM ICC, Mixed Scion Day 4, Mixed Scion Day 7, Mixed Scion Selected, Mixed Culture MTS Day 4, Mixed Culture MTS Day 7 and CM MTS Selected molecules respectively.

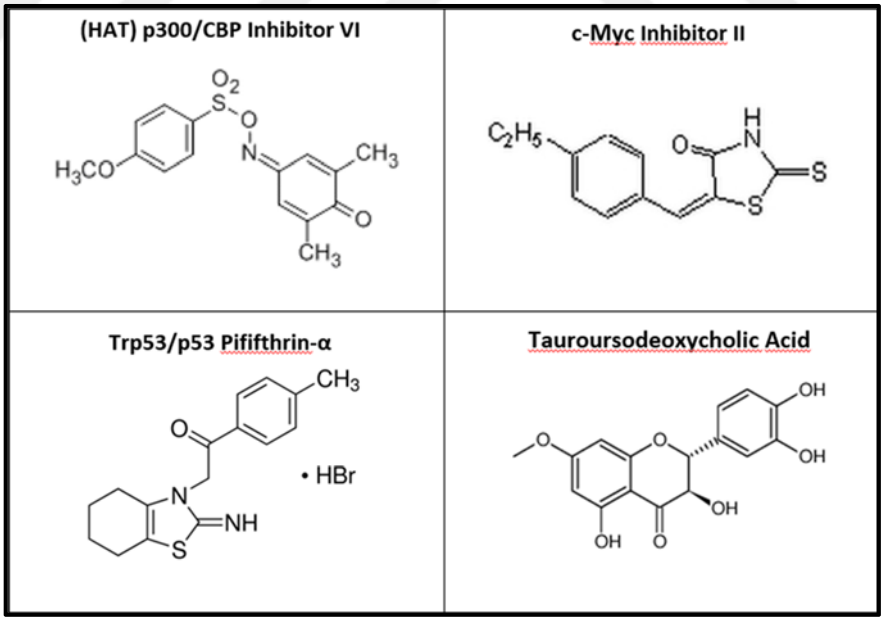

Figure 3.17. Chemical structures of hit small molecules.
A) Chemical structure of p300/CBP Inhibitor VI ($C_{15}H_{15}NO_5S$), B) Chemical structure of c-Myc Inhibitor II ($C_{12}H_{11}NOS_2$), C) Chemical structure of Trp/p53 Pifithrin-α ($C_{16}H_{18}N_2OS \cdot HBr$), D) Chemical structure of Tauroursodeoxycholic Acid ($C_{16}H_{14}O_7$).

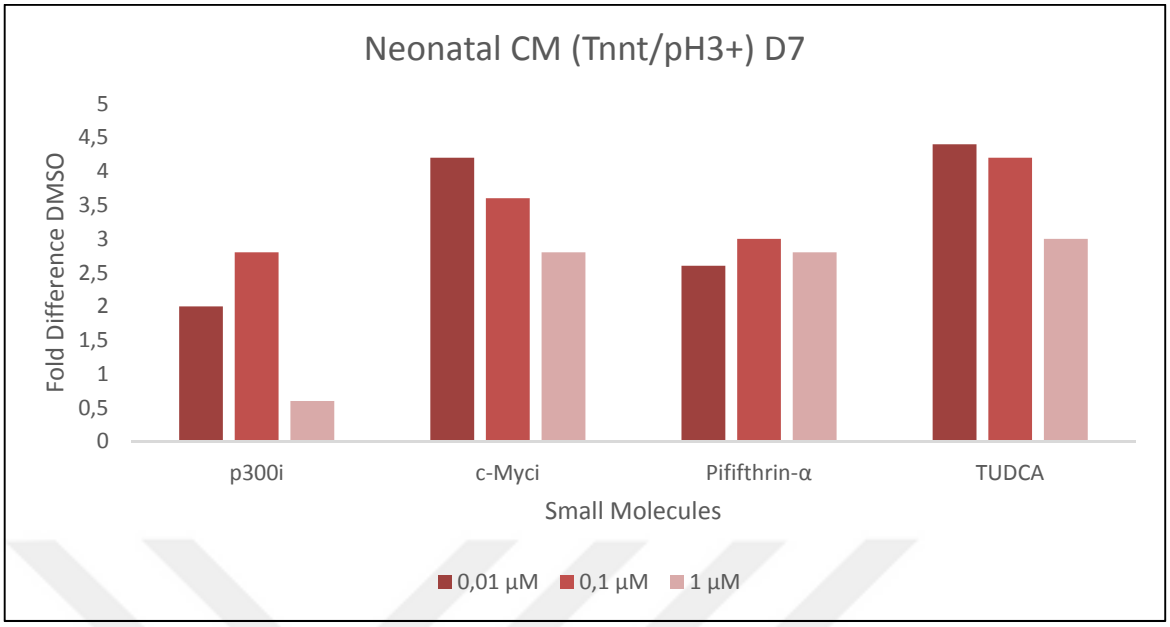

Figure 3.18. Final immunostaining results of selected molecules (p300/CBP Inhibitor VI ($C_{15}H_{15}NO_5S$), c-Myc Inhibitor II ($C_{12}H_{11}NOS_2$), Trp/p53 Pifithrin-α ($C_{16}H_{18}N_2OS \cdot HBr$), Tauroursodeoxycholic Acid ($C_{16}H_{14}O_7$)) pH3+ CMs

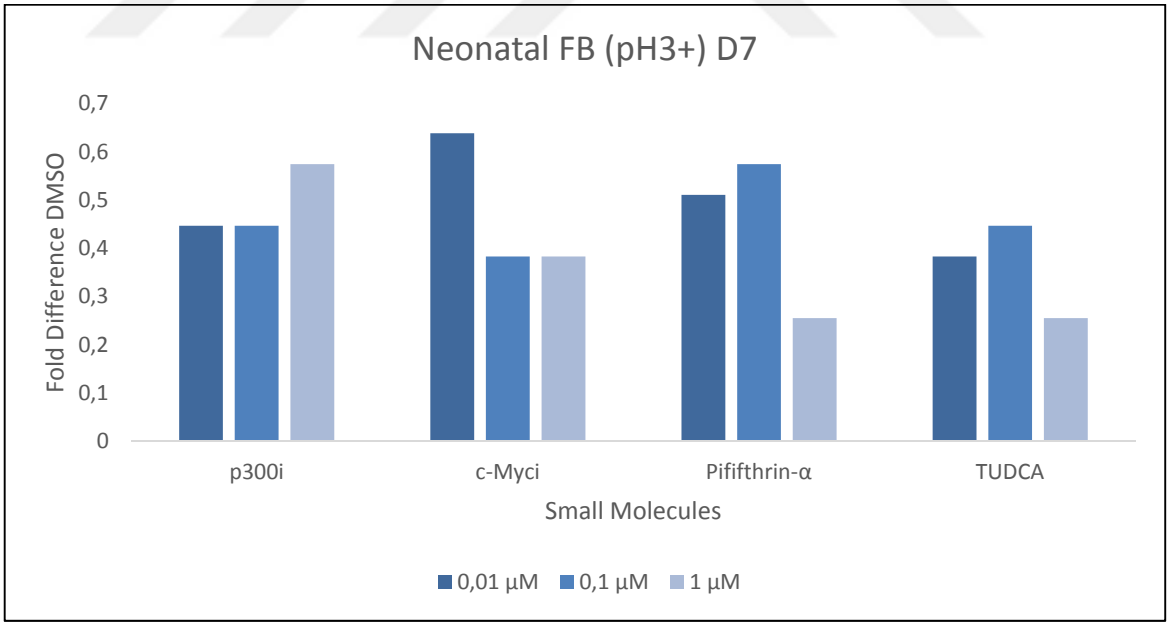

Figure 3.19. Final immunostaining results of selected molecules (p300/CBP Inhibitor VI ($C_{15}H_{15}NO_5S$), c-Myc Inhibitor II ($C_{12}H_{11}NOS_2$), Trp/p53 Pifithrin-α ($C_{16}H_{18}N_2OS \cdot HBr$), Tauroursodeoxycholic Acid ($C_{16}H_{14}O_7$)) pH3+ FBs

4. CONCLUSIONS AND RECOMMENDATIONS

Recent involvements in cardiac regeneration field challenged that heart is not terminally differentiated organ and suggested that there exist cardiomyogenesis in adult heart. Up to know, different strategies have been utilized which have ultimate goal of reversing the heart failure trough inducing progression of cell cycle of different cells residing in the heart [22]. Moreover, investigation of cardiomyocyte biology and identification of regulators of cardiomyocyte proliferation revealed many different mechanisms including dedifferentiation of pre-existing cardiomyocytes, transdifferentiation of other cells and role of CSCs.

Although reactivation of pre-existing cardiomyocytes proliferation has been intensively researched, the impact of resident CSCs and exogenous stem cells became hot topic research areas in the context of cardiac regeneration. Utilization of exogenous stem cells including ESCs, IPSCs, SMCs and MNCs to repair cardiac injury gave promising results which led later on clinical trials in this field [43]. However, their insufficient transdifferentiation capacity into cardiomyocyte or inefficiency of integration to injury area became major obstacles to this end. Moreover, ethical and immunologic rejection issues involving in utilization of IPSCs and ESCs debated their utilization in therapeutic strategies [121].

In the meantime, the effect of CSCs challenged the cardiac regeneration field. Due to complexity of heart organ, different population of those stem cells in different sites of heart have been recently characterized and different progenitors within heart tissue including SP progenitors, Flk1, SSEA, ISL1, Sca1, GCPs and c-Kit have been reported in successive studies [49]. In different reports, the differential expression of markers led to identify those progenitors and their location in heart suggested to be important to for their function.

Construction of new myocardial tissue and replacement of lost structures in cardiac tissues are designated as the definition of heart regeneration, where in fact that the definition of cardiac repair is used to point restoring of the original structures such as rejuvenating of a scar and deposition of collagen. By anticipating this fact, repair mechanism is only contributed by the process of regeneration.

Re-entering the cardiomyocyte cell cycle is shown to be possible in adult mammalian heart tissue by using inducible knockout systems used in adult mouse models. Differentiation of stem cells into cardiomyocytes is shown to be triggered and promoted by certain small molecules by inducing cardiomyocyte cell cycle re-entry. The discovery of such small molecules awakened further enthusiasm to advance therapies targeting myocardial infarction and heart failure. A number of approaches are being studied and developed to induce heart repair and regeneration. Small molecule drugs which can be chemically synthesized are proved to be sufficiently effective in specific context. The existence of predictive preclinical models are crucial needs for development of suitable therapies such as small molecule introduction.

In this study, a pool of small molecules were tested to identify the cardiac proliferative purposes and induction of cardiac regeneration. To this end neonatal rat and adult mice heart CMs and FBs were cultured and small molecues were introduced in a dose dependent manner. The molecules were selected by their ability to induce CM proliferation and FB growth inhibition. To observe the behaviour of CMs in their unique environment, FB, CM mixed cell culture was applied.

Amongst more than 30 small molecules have been tested and it has been observed that the p300/CBP Inhibitor VI, c-Myc Inhibitor II, Trp/p53 Pififthrin-α and Tauroursodeoxycholic Acid have the highest potential to increase CM proliferation meanwhile reducing the growth of FBs. However comprehensive studies should be carried out regarding to the identification of correct doses and therefore mixing up the proliferative small molecules to achieve the therapeutically favorable drug discoveries.

Myocyte enhancer factor-2 (MEF2) is the one of the most important targets of Histone deacetylases (HDACs) in muscle cells. However, in adult heart, the activity of MEF2 proteins are on basal level. MEF2 activity enhanced by calcium dependent stress signals that cause hypertrophy [108]. This response is due to the phosphorylation dependent dissociation of HDACs and recruitment of Histone acetyltransferases (HATs) by MEF2.

The HAT p300 collaborates with MEF2 and GATA4 transcription factors that are responsible of fetal cardiac gene regulations and the level of transcriptional activity [109].
It has been shown that expression of mutant (signal resistant) HDACs in CMs resulted with the insensitivity to hypertrophic antagonists [110]. Moreover, HDAC9 knockout mice shown

high sensitivity to stress signals. In another study it has been shown that the overexpression of p300 increases the level of hypertrophy on CMs [111].Therefore p300 inhibitor have been tested.

C-Myc is a well known transcription factor for their role in differentiaton, proliferation and regulation of many types of the cells [112,113]. Several studies have shown the importance of c-Myc for the preservation and pluripotency of adult HSC and ESCs [114,115]. C-Myc also have been tested to observe its potential on cancer stem cells [116,117]. Moreover c-Myc is a common and important tool in the treatment of heart failure via cell mediated therapies [118]. Recent studies have shown that the downregulation of c-Myc by ischemic injury have a major role on the impairment of the regenerative potential on the cardiac progenitor cells. Therefore c-Myc inhibitor II have been tested in this study.

Transcription regulator protein 53 (Trp53)/p53 Pififthrin-α is regulatory element of diverse cellular stresses, induction of cell cycle arrest, DNA repair or metabolical changes. p53 protein is expressed at high level in transformed cell lines whereas it remains at low level in normal cells [119]. It has been suggested that it contributes to transformation and malignancy. In a study conducted on canine models reveals that the expression of ventricular p53 were upregulated and relatedly Bax level also upregulated [120]. Another study reveals that the p53 transfected (ventricular) myocytes resulted with the induction of apoptosis by initiation of mitochondria dependent (apoptotic) pathways [121]. Therefore, it can be suggested that p53 might have a significant role on cardiac apoptosis. Trp53 inhibitor also have been tested in this study.

Ursodeoxycholic Acid (UDCA) is a substance that reduced hepatocyte apoptosis by controlling the mitochondrial membrane permeability [122]. Conjugate (taurine) of UDCA, tauroursodeoxycholic acid (TUDCA) have been related with the mitochondrial associated permeability. Another study show that the subsequent apoptosis was reduced up to %75 on apoptosis induced rat brain models [123]. Moreover it has been shown the inhibitory effect of TUDCA on cytochrome-c efflux of mitochondria. Also it inhibits the Bax pore formation and relatedly reduces apoptosis [124].

Finally Cytell cell imaging instrument have been used for the observation of proliferative response on the cardiomyocyte and fibroblast cultures. To test this, triple stained (Tnnt, DAPI, pH3) cardiomyocte and fibroblast cultures were analyzed with Cytell. The instrument

having the ability to take 51 pictures from each 96 well plate with 3 filters. Therefore it allows us to observe the complete surface area of each well plate and consecutively having a much more accurate results.

Heart failure still remains leading cause of death worldwide and recent research in this field revealed the possibility of reversing the heart failure. Although many of clinical trials have been resulted with promising discoveries, robust and well designed future studies could be more effective to finalize development of human cardiac therapies trough utilization of small molecules.

REFERENCES

1. Jessup M. and Brozena S., Heart failure, *N. Engl. J. Med*., vol. 348, no. 20, p. 2007 18., 2003.

2. Mozaffarian D. et al., Heart disease and stroke statistics-2015 update : A report from the American Heart Association, *Circulation*, vol. 131, no. 4, pp. e29–e39, 2015.

3. J. Garbern C. and Lee R. T., Cardiac stem cell therapy and the promise of heart regeneration, *Cell Stem Cell*, vol. 12, no. 6. pp. 689–698, 2013.

4. Boon R and Dimmeler S, Reviews: MicroRNAs in myocardial infarction, *Nat. Rev. Cardiol*., pp. 135–142, 2014.

5. Boon R. A., Jae N, Holdt L., and Dimmeler S., Long Noncoding RNAs from Clinical Genetics to Therapeutic Targets, *J. Am. Coll. Cardiol*., vol. 67, no. 10, pp. 1214–1226, 2016.

6. Arbatlı S, Aslan G. S., Kocaba F., Arbatlı S,Aslan G. S, and Kocaba F., Stem Cells in Regenerative Cardiology, *Adv Exp Med Biol* – Cell Biol. Transl. Med.

7. Laflamme M. A. and Murry C. E., Heart regeneration, *Nature,* vol. 473, no. 7347. pp. 326–335, 2011.

8. Poss K. D., Heart Regeneration in Zebrafish, *Science* (80-.)., vol. 298, no. 5601, pp. 2188–2190, 2002.

9. Kajstura J. et al., Cardiomyogenesis in the adult human heart, *Circ. Res.,* vol. 107, no. 2, pp. 305–315, 2010.

10. Bergmann O., Zdunek S., Frisn J., Bernard S., Druid H., and Jovinge S., Cardiomyocyte renewal in humans, *Circulation Research*, vol. 110, no. 1. 2012.

11. Senyo S. E. et al., Mammalian heart renewal by pre-existing cardiomyocytes, *Nature*, vol. 493, no. 7432, pp. 433–436, 2013.

12. Rumyantsev P. P., Post-injury DNA synthesis, mitosis and ultrastructural reorganization of adult frog cardiac myocytes - An electron microscopic-autoradiographic study, *Zeitschrift für Zellforsch. und Mikroskopische Anat.*, vol. 139, no. 3, pp. 431–450, 1973.

13. Oberpriller J. O. and Oberpriller J. C., Response of the adult newt ventricle to injury, *J. Exp. Zool.*, vol. 187, no. 2, pp. 249–259, 1974.

14. Porrello E. R. et al., Transient regenerative potential of the neonatal mouse heart., *Science*, vol. 331, no. 6020, pp. 1078–80, 2011.

15. Witman N., Murtuza B., Davis B., Arner A., and Morrison J. I., Recapitulation of developmental cardiogenesis governs the morphological and functional regeneration of adult newt hearts following injury, *Dev. Biol.*, vol. 354, no. 1, pp. 67–76, 2011.

16. Muralidhar S. A. and. Sadek H. A, Meis1 regulates postnatal cardiomyocyte cell cycle arrest, in Etiology and Morphogenesis of Congenital Heart Disease: From Gene Function and Cellular Interaction to Morphology, 2016, pp. 93–101.

17. Kühn B. et al., Periostin induces proliferation of differentiated cardiomyocytes and promotes cardiac repair, *Nat. Med.*, vol. 13, no. 8, pp. 962–969, 2007.

18. Novoyatleva T. et al., TWEAK is a positive regulator of cardiomyocyte proliferation, *Cardiovasc. Res.*, vol. 85, no. 4, pp. 681–690, 2010.

19. Kubin T. et al., Oncostatin M is a major mediator of cardiomyocyte dedifferentiation and remodeling, *Cell Stem Cell*, vol. 9, no. 5, pp. 420–432, 2011.

20. Beigi F. et al., C3orf58, a novel paracrine protein, stimulates cardiomyocyte cell-cycle progression through the PI3K-AKT-CDK7 pathway*, Circ. Res.*, vol. 113, no. 4, pp. 372–380, 2013.

21. Rochais F. et al., FGF10 promotes regional foetal cardiomyocyte proliferation and adult cardiomyocyte cell-cycle re-entry, *Cardiovasc. Res.*, vol. 104, no. 3, pp. 432–442, 2014.

22. O'Meara C. C. et al., Transcriptional reversion of cardiac myocyte fate during mammalian cardiac regeneration, *Circ. Res.*, vol. 116, no. 5, pp. 804–815, 2015.

23. Jung J., Kim T. G., Lyons G. E.,. Kim H. R. C, and Lee Y., Jumonji regulates cardiomyocyte proliferation via interaction with retinoblastoma protein, *J. Biol. Chem.*, vol. 280, no. 35, pp. 30916–30923, 2005.

24. Liu N. et al., microRNA-133a regulates cardiomyocyte proliferation and suppresses smooth muscle gene expression in the heart*, Genes Dev.*, vol. 22, no. 23, pp. 3242–3254, 2008.

25. Heallen T. et al., Hippo Pathway Inhibits Wnt Signaling to Restrain Cardiomyocyte Proliferation and Heart Size, *Science* (80-.)., vol. 332, no. 6028, pp. 458–461, 2011.

26. Jackson T., Allard M. F., Sreenan C. M., Doss L. K., Bishop S. P., and Swain J. L., The c-myc proto-oncogene regulates cardiac development in transgenic mice., *Mol. Cell. Biol.*, vol. 10, no. 7, pp. 3709–16, 1990.

27. Li R. K.et al., Cardiomyocyte transplantation improves heart function*, Ann. Thorac.* Surg., vol. 62, no. 3, pp. 654–661, 1996.

28. Bicknell K. A., Coxon C. H., and Brooks G., Forced expression of the cyclin B1-CDC2 complex induces proliferation in adult rat cardiomyocytes., *Biochem. J.*, vol. 382, no. Pt 2, pp. 411–6, 2004.

29. Pasumarthi K. B. S., Nakajima H., Nakajima H. O., Soonpaa M. H., and Field L. J., Targeted expression of cyclin D2 results in cardiomyocyte DNA synthesis and infarct regression in transgenic mice*, Circ. Res.*, vol. 96, no. 1, pp. 110–118, 2005.

30. Cheng R. K. et al., Cyclin A2 induces cardiac regeneration after myocardial infarction and prevents heart failure*, Circ. Res.*, vol. 100, no. 12, pp. 1741–1748, 2007.

31. Campa V. M. et al., Notch activates cell cycle reentry and progression in quiescent cardiomyocytes*, J. Cell Biol.*, vol. 183, no. 1, pp. 129–141, 2008.

32. D'Uva G. et al., ERBB2 triggers mammalian heart regeneration by promoting cardiomyocyte dedifferentiation and proliferation, *Nat. Cell Biol.*, vol. 17, no. 5, pp. 627–638, 2015.

33. Bergmann O. et al., Evidence for Cardiomyocyte Renewal in Humans*, Science* (80-.)., vol. 324, no. 5923, pp. 98–102, 2009.

34. Canseco D. C. et al., Human ventricular unloading induces cardiomyocyte proliferation, *J. Am. Coll. Cardiol.*, vol. 65, no. 9, pp. 892–900, 2015.

35. Murry C. E., Wiseman R. W., Schwartz S. M., and Hauschka S. D., *Skeletal myoblast transplantation for repair of myocardial necrosis*, J. Clin. Invest., vol. 98, no. 11, pp. 2512–2523, 1996.

36. Orlic D., Kajstura J., Chimenti S., Bodine D. M., Leri A., and Anversa P., *Transplanted adult bone marrow cells repair myocardial infarcts in mice*, Ann. N. Y. Acad. Sci., vol. 938, pp. 221-229-230, 2001.

37. Takahashi K. and Yamanaka S., *Induction of Pluripotent Stem Cells from Mouse Embryonic and Adult Fibroblast Cultures by Defined Factors,* Cell, vol. 126, no. 4, pp. 663–676, 2006.

38. Bishop A. E., Buttery L. D. K., and Polak J. M., *Embryonic stem cells*, J. Pathol., vol. 197, no. 4, pp. 424–429, 2002.

39. Kehat I. et al., Human embryonic stem cells can differentiate into myocytes with structural and functional properties of cardiomyocytes., *J. Clin. Invest.*, vol. 108, no. 3, pp. 407–14, 2001.

40. Beqqali A., Kloots J., Ward-van Oostwaard D., Mummery C., and Passier R., Genome-Wide Transcriptional Profiling of Human Embryonic Stem Cells Differentiating to Cardiomyocytes, *Stem Cells*, vol. 24, no. 8, pp. 1956–1967, 2006.

41. Oettgen P., Cardiac stem cell therapy: Need for optimization of efficacy and safety monitoring, *Circulation*, vol. 114, no. 4. pp. 353–358, 2006.

42. Zhou H. et al., Generation of induced pluripotent stem cells using recombinant proteins, *Cell Stem Cell*, vol. 4, pp. 381–384, 2009.

43. Gai H. et al., Generation and characterization of functional cardiomyocytes using induced pluripotent stem cells derived from human fibroblasts, *Cell Biol Int*, vol. 33, no. 11, pp. 1184–1193, 2009.

44. Zhang J. et al., Functional cardiomyocytes derived from human induced pluripotent stem cells, *Circ. Res.*, vol. 104, no. 4, 2009.

45. Zwi L. et al., Cardiomyocyte differentiation of human induced pluripotent stem cells, *Circulation*, vol. 120, no. 15, pp. 1513–1523, 2009.

46. Kensah G. et al., Murine and human pluripotent stem cell-derived cardiac bodies form contractile myocardial tissue in vitro, *Eur. Heart J.*, vol. 34, no. 15, pp. 1134–1146, 2013.

47. S. P. Yu, Z. Wei, and L. Wei, Preconditioning Strategy in Stem Cell Transplantation Therapy, *Translational Stroke Research*, vol. 4, no. 1. pp. 76–88, 2013.

48. Lee J. H. et al., Somatic transcriptome priming gates lineage-specific differentiation potential of human-induced pluripotent stem cell states, *Nat. Commun*., vol. 5, 2014.

49. Dixit P. and Katare R., Challenges in identifying the best source of stem cells for cardiac regeneration therapy, *Stem Cell Res. Ther*., vol. 6, no. 1, p. 26, 2015.

50. Marelli D., Desrosiers C., El-Alfy M., Kao R. L., and Chiu R. C. J., Cell transplantation for myocardial repair: An experimental approach, *Cell Transplant*., vol. 1, no. 6, pp. 383–390, 1992.

51. Reinecke H., Poppa V., and Murry C. E., Skeletal Muscle Stem Cells Do Not Transdifferentiate Into Cardiomyocytes After Cardiac Grafting, *J. Mol. Cell. Cardiol*., vol. 34, no. 2, pp. 241–249, 2002.

52. Menasché P. et al., The myoblast autologous grafting in ischemic cardiomyopathy (MAGIC) trial: First randomized placebo-controlled study of myoblast transplantation," *Circulation*, vol. 117, no. 9, pp. 1189–1200, 2008.

53. Zimmet H. et al., Short- and long-term outcomes of intracoronary and endogenously mobilized bone marrow stem cells in the treatment of ST-segment elevation myocardial infarction: a meta-analysis of randomized control trials, *Eur. J. Heart Fail*., vol. 14, no. 1, pp. 91–105, 2012.

54. Orlic D. et al., Bone marrow cells regenerate infarcted myocardium., *Nature*, vol. 410, no. 6829, pp. 701–705, 2001.

55. Murry C. E. et al., Haematopoietic stem cells do not transdifferentiate into cardiac myocytes in myocardial infarcts, *Nature*, vol. 428, no. 6983, pp. 664–668, 2004.

56. Pittenger M. F. et al., Multilineage potential of adult human mesenchymal stem cells., *Science*, vol. 284, no. 5411, pp. 143–7, 1999.

57. Fatkhudinov T. K., G. B. Bol'Shakova, D. V. Goldshtein, and G. T. Sukhikh, Mechanisms of therapeutic activity of multipotent cells in heart diseases, *Bull. Exp. Biol. Med*., vol. 156, no. 4, pp. 535–543, 2014.

58. Beltrami A. P. et al., Adult cardiac stem cells are multipotent and support myocardial regeneration, *Cell*, vol. 114, no. 6, pp. 763–776, 2003.

59. Iida M., Heike T., Yoshimoto M., Baba S., Doi H., and Nakahata T., Identification of cardiac stem cells with FLK1, CD31, and VE-cadherin expression during embryonic stem cell differentiation, *FASEB J*, vol. 19, no. 3, pp. 371–378, 2005.

60. Maher T. J. et al., ATP-Binding Cassette Transporter Abcg2 Lineage Contributes to the Cardiac Vasculature After Oxidative Stress, *Am. J. Physiol. Heart Circ. Physiol*., vol. 306, no. 12, pp. H1610-8, 2014.

61. Menasché P., Embryonic stem cells pace the heart, *Nature Biotechnology*, vol. 22, no. 10. pp. 1237–1238, 2004.

62. Bearzi C. et al., Human cardiac stem cells, *Proc Natl Acad Sci U S A*, vol. 104, pp. 14068–14073, 2007.

63. Bearzi C. et al., Identification of a coronary vascular progenitor cell in the human heart., *Proc. Natl. Acad. Sci. U. S. A.*, vol. 106, no. 37, pp. 15885–15890, 2009.

64. Linke A. et al., Stem cells in the dog heart are self-renewing, clonogenic, and multipotent and regenerate infarcted myocardium, improving cardiac function, *Proc. Natl. Acad. Sci*., vol. 102, no. 25, pp. 8966–8971, 2005.

65. Laugwitz K. L. et al., Postnatal isl1+cardioblasts enter fully differentiated cardiomyocyte lineages, *Nature*, vol. 433, no. 7026, pp. 647–653, 2005.

66. Leri A., Kajstura J., and Anversa P., Role of cardiac stem cells in cardiac pathophysiology: A paradigm shift in human myocardial biology, *Circulation Research*, vol. 109, no. 8. pp. 941–961, 2011.

67. Chong J. J. H. et al., Adult cardiac-resident MSC-like stem cells with a proepicardial origin, *Cell Stem Cell*, vol. 9, no. 6, pp. 527–540, 2011.

68. Goodell M. A., Brose K., Paradis G., Conner A. S., and Mulligan R. C., Isolation and functional properties of murine hematopoietic stem cells that are replicating in vivo., *J. Exp. Med*., vol. 183, no. 4, pp. 1797–806, 1996.

69. Unno K., Jain M., and Liao R., Cardiac side population cells: Moving toward the center Stage in cardiac regeneration, Circulation Research, vol. 110, no. 10. pp. 1355–1363, 2012.

70. Gussoni E. et al., Dystrophin expression in the mdx mouse restored by stem cell transplantation, *Nature*, vol. 401, no. 6751, pp. 390–394, 1999.

71. Pfister O. et al., CD31- but not CD31+ cardiac side population cells exhibit functional cardiomyogenic differentiation, *Circ. Res*., vol. 97, no. 1, pp. 52–61, 2005.

72. Liang S. X., Tan T. Y. L., Gaudry L., and Chong B., Differentiation and migration of Sca1+/CD31- cardiac side population cells in a murine myocardial ischemic model, *Int. J. Cardiol*., vol. 138, no. 1, pp. 40–49, 2010.

73. Motoike T., Markham D. W., Rossant J., and Sato T. N., Evidence for novel fate of Flk1+ progenitor: Contribution to muscle lineage, *Genesis*, vol. 35, no. 3, pp. 153–159, 2003.

74. Ema M., Takahashi S., and Rossant J., Deletion of the selection cassette, but not cis-acting elements, in targeted Flk1-lacZ allele reveals Flk1 expression in multipotent mesodermal progenitors, *Blood*, vol. 107, no. 1, pp. 111–117, 2006.

75. Yamashita J. K. et al., Prospective identification of cardiac progenitors by a novel single cell-based cardiomyocyte induction., *FASEB J.*, vol. 19, no. 11, pp. 1534–1536, 2005.

76. Kattman S. J., Huber T. L., and Keller G. M. M., Multipotent Flk-1+ Cardiovascular Progenitor Cells Give Rise to the Cardiomyocyte, Endothelial, and Vascular Smooth Muscle Lineages, *Dev. Cell*, vol. 11, no. 5, pp. 723–732, 2006.

77. Suresh R. et al., CXCR4+ and FLK-1+ identify circulating cells associated with improved cardiac function in patients following myocardial infarction, *J. Cardiovasc. Transl. Res.*, vol. 6, no. 5, pp. 787–797, 2013.

78. Wu S. M., Chien K. R., and Mummery C., Origins and Fates of Cardiovascular Progenitor Cells, *Cell*, vol. 132, no. 4. pp. 537–543, 2008.

79. Weinberger F. et al., Localization of islet-1-positive cells in the healthy and infarcted adult murine heart, *Circ. Res.*, vol. 110, no. 10, pp. 1303–1310, 2012.

80. Engleka K. A. et al., Islet1 derivatives in the heart are of both neural crest and second heart field origin, *Circ. Res.*, vol. 110, no. 7, pp. 922–926, 2012.

81. Cagavi E. et al., Functional cardiomyocytes derived from isl1 cardiac progenitors via Bmp4 stimulation, *PLoS One,* vol. 9, no. 12, 2014.

82. Menasche P. et al., Human embryonic stem cell-derived cardiac progenitors for severe heart failure treatment: First clinical case report, *Eur. Heart J.*, vol. 36, no. 30, pp. 2011–2017, 2015.

83. Hong K. U. et al., c-kit+ cardiac stem cells alleviate post-myocardial infarction left ventricular dysfunction despite poor engraftment and negligible retention in the recipient heart, *PLoS One*, vol. 9, no. 5, 2014.

84. Sullivan K. E., Burns L. J., and Black L. D., An in vitro model for the assessment of stem cell fate following implantation within the infarct microenvironment identifies ISL-1 expression as the strongest predictor of c-Kit+ cardiac progenitor cells' therapeutic potential, *J. Mol. Cell. Cardiol*., vol. 88, pp. 91–100, 2015.

85. Kazakov A. et al., C-kit+ resident cardiac stem cells improve left ventricular fibrosis in pressure overload, *Stem Cell Res*., vol. 15, no. 3, pp. 700–711, 2015.

86. Tillmanns J. et al., Formation of large coronary arteries by cardiac progenitor cells, *Proc Natl Acad Sci U S A*, vol. 105, no. 5, pp. 1668–1673, 2008.

87. Choi S. H., Jung S. Y., Suh W., Baek S. H., and Kwon S. M., Establishment of isolation and expansion protocols for human cardiac C-kit-positive progenitor cells for stem cell therapy, *Transplant. Proc*., vol. 45, no. 1, pp. 420–426, 2013.

88. Hierlihy A. M., Seale P., Lobe C. G., Rudnicki M. A., and Megeney L. A., The post-natal heart contains a myocardial stem cell population, *FEBS Lett*., vol. 530, no. 1–3, pp. 239–243, 2002.

89. Oh H. et al., Cardiac progenitor cells from adult myocardium: Homing, differentiation, and fusion after infarction, *Proc. Natl. Acad. Sci*., vol. 100, no. 21, pp. 12313–12318, 2003.

90. Matsuura K. et al., Adult Cardiac Sca-1-positive Cells Differentiate into Beating Cardiomyocytes, *J. Biol. Chem*., vol. 279, no. 12, pp. 11384–11391, 2004.

91. Ott H. C. et al., The adult human heart as a source for stem cells: Repair strategies with embryonic-like progenitor cells, *Nat. Clin. Pract. Cardiovasc. Med*., vol. 4, no. SUPPL. 1, 2007.

92. Ishitobi H. et al., Molecular basis for Flk1 expression in hemato-cardiovascular progenitors in the mouse, *Development*, vol. 138, no. 24, pp. 5357–5368, 2011.

93. Kocabas F. et al., The hypoxic epicardial and subepicardial microenvironment, *J. Cardiovasc. Transl. Res.*, vol. 5, no. 5, pp. 654–665, 2012.

94. Tateishi K. et al., Clonally amplified cardiac stem cells are regulated by Sca-1 signaling for efficient cardiovascular regeneration, *J. Cell Sci.*, vol. 120, no. 10, pp. 1791–1800, 2007.

95. Bailey B. et al., Sca-1 knockout impairs myocardial and cardiac progenitor cell function, *Circ. Res.*, vol. 111, no. 6, pp. 750–760, 2012.

96. Uchida S. et al., Sca1-derived cells are a source of myocardial renewal in the murine adult heart, *Stem Cell Reports*, vol. 1, no. 5, pp. 397–410, 2013.

97. Jha A. K. et al., Enhanced survival and engraftment of transplanted stem cells using growth factor sequestering hydrogels, *Biomaterials*, vol. 47, pp. 1–12, 2015.

98. Leri A., Rota M., T. Hosoda, P. Goichberg, and P. Anversa, Cardiac stem cell niches, *Stem Cell Research*, vol. 13, no. 3. pp. 631–646, 2014.

99. Limana F. et al., Identification of myocardial and vascular precursor cells in human and mouse epicardium, *Circ. Res.*, vol. 101, no. 12, pp. 1255–1265, 2007.

100. Zhou B. et al., Epicardial progenitors contribute to the cardiomyocyte lineage in the developing heart, *Nature*, vol. 454, no. 7200, pp. 109–113, 2008.

101. Di Meglio F. et al., Localization and origin of cardiac CD117-positive cells: Identification of a population of epicardially-derived cells in adult human heart, *Ital. J. Anat. Embryol.*, vol. 115, no. 1–2, pp. 71–78, 2010.

102. Di Meglio F. et al., Epithelial-mesenchymal transition of epicardial mesothelium is a source of cardiac CD117-positive stem cells in adult human heart, *J. Mol. Cell. Cardiol.*, vol. 49, no. 5, pp. 719–727, 2010.

103. Smart N. et al., De novo cardiomyocytes from within the activated adult heart after injury, *Nature*, vol. 474, no. 7353, pp. 640–644, 2011.

104. Urbanek K. et al., Cardiac stem cells possess growth factor-receptor systems that after activation regenerate the infarcted myocardium, improving ventricular function and long-term survival, *Circ. Res.*, vol. 97, no. 7, pp. 663–673, 2005.

105. Antos C. L. et al., Dose-dependent blockade to cardiomyocyte hypertrophy by histone deacetylase inhibitors, *J. Biol. Chem.*, vol. 278, no. 31, pp. 28930–28937, 2003.

106. Ishimaru K. et al., Synthetic prostacyclin agonist, ONO1301, enhances endogenous myocardial repair in a hamster model of dilated cardiomyopathy: A promising regenerative therapy for the failing heart, *J. Thorac. Cardiovasc. Surg.*, vol. 146, no. 6, pp. 1516–1525, 2013.

107. Hastings C. L., Roche E. T., Ruiz-Hernandez E., Schenke-Layland K., Walsh C. J., and Duffy G. P., Drug and cell delivery for cardiac regeneration, *Advanced Drug Delivery Reviews*, vol. 84. pp. 85–106, 2015.

108. Hsueh Y. C., Wu J. M. F., Yu C. K., Wu K. K., and Hsieh P. C. H., Prostaglandin E2 promotes post-infarction cardiomyocyte replenishment by endogenous stem cells, *EMBO Mol. Med.*, vol. 6, no. 4, pp. 496–503, 2014.

109. Murakoshi M., Saiki K., Urayama K., and Sato T. N., An anthelmintic drug, pyrvinium pamoate, thwarts fibrosis and ameliorates myocardial contractile dysfunction in a mouse model of myocardial infarction, *PLoS One*, vol. 8, no. 11, 2013.

110. Zaruba M.-M. et al., Synergy between CD26/DPP-IV inhibition and G-CSF improves cardiac function after acute myocardial infarction., *Cell Stem Cell*, vol. 4, no. 4, pp. 313–323, 2009.

111. Passier R. et al., CaM kinase signaling induces cardiac hypertrophy and activates the MEF2 transcription factor in vivo., *J. Clin. Invest*., vol. 105, no. 10, pp. 1395–406, 2000.

112. Leor J. et al., Gene transfer and cell transplant: An experimental approach to repair a 'broken heart, *Cardiovascular Research*, vol. 35, no. 3. pp. 431–441, 1997.

113. Zhang C. L., McKinsey T. A., Chang S., Antos C. L., Hill J. A., and Olson E. N., Class II histone deacetylases act as signal-responsive repressors of cardiac hypertrophy, *Cell*, vol. 110, no. 4, pp. 479–488, 2002.

114. Gusterson R. J., Jazrawi E., Adcock I. M., and Latchman D. S., The transcriptional co-activators CREB-binding protein (CBP) and p300 play a critical role in cardiac hypertrophy that is dependent on their histone acetyltransferase activity, *J. Biol. Chem*., vol. 278, no. 9, pp. 6838–6847, 2003.

115. Dang C. V. et al., Function of the c-Myc Oncogenic Transcription Factor, *Exp. Cell Res*., vol. 253, no. 1, pp. 63–77, 1999.

116. Pelengaris S. and Khan M., The many faces of c-MYC, *Archives of Biochemistry and Biophysics*, vol. 416, no. 2. pp. 129–136, 2003.

117. Wilson A. et al., c-Myc controls the balance between hematopoietic stem cell self-renewal and differentiation., *Genes Dev*., vol. 18, no. 22, pp. 2747–63, 2004.

118. Cartwright P., LIF/STAT3 controls ES cell self-renewal and pluripotency by a Myc-dependent mechanism, *Development*, vol. 132, no. 5, pp. 885–896, 2005.

119. Ashihara E., Takada T., and Maekawa T., Targeting the canonical Wnt/β-catenin pathway in hematological malignancies, *Cancer Sci*., vol. 106, no. 6, pp. 665–671, 2015.

120. Zhang C. L., McKinsey T. A., Chang S., Antos C. L., Hill J. A., and Olson E. N., Class II histone deacetylases act as signal-responsive repressors of cardiac hypertrophy, *Cell*, vol. 110, no. 4, pp. 479–488, 2002.

121. Yoshida Y. and Yamanaka S., IPS cells: A source of cardiac regeneration, *Journal of Molecular and Cellular Cardiology*, vol. 50, no. 2. pp. 327–332, 2011.

122. Liu X. et al., Pifithrin-alpha protects against doxorubicin-induced apoptosis and acute cardiotoxicity in mice., *Am. J. Physiol. Heart Circ. Physiol*., vol. 286, no. 3, pp. H933–H939, 2004.

123. Leri A. et al., Pacing-induced heart failure in dogs enhances the expression of p53 and p53-dependent genes in ventricular myocytes., *Circulation*, vol. 97, pp. 194–203, 1998.

124. Regula K. M. and Kirshenbaum L. A., P53 Activates the Mitochondrial Death Pathway and Apoptosis of Ventricular Myocytes Independent of De Novo Gene Transcription., *J. Mol. Cell. Cardiol*., vol. 33, no. 8, pp. 1435–45, 2001.

125. Rodrigues C. M. P., Fan G., Ma X., Kren B. T., and Steer C. J., A novel role for ursodeoxycholic acid in inhibiting apoptosis by modulating mitochondrial membrane perturbation, *J. Clin. Invest*., vol. 101, no. 12, pp. 2790–2799, 1998.

126. Rodrigues C. M. P., Stieers C. L., Keene C. D., and Ma X., Tauroursodeoxycholic acid partially prevents apoptosis induced by 3-nitropropionic acid: Evidence for a mitochondrial pathway independent of the permeability transition, *J. Neurochem*., vol. 75, no. 6, pp. 2368–2379, 2000.

127. Rodrigues C. M. P., Solá S., Sharpe J. C., Moura J. J. G., and Steer C. J., Tauroursodeoxycholic acid prevents Bax-induced membrane perturbation and cytochrome c release in isolated mitochondria, *Biochemistry,* vol. 42, no. 10, pp. 3070–3080, 2003.

APPENDIX A: LANGENDORFF APPARATUS FOR ADULT HEART DIGESTION

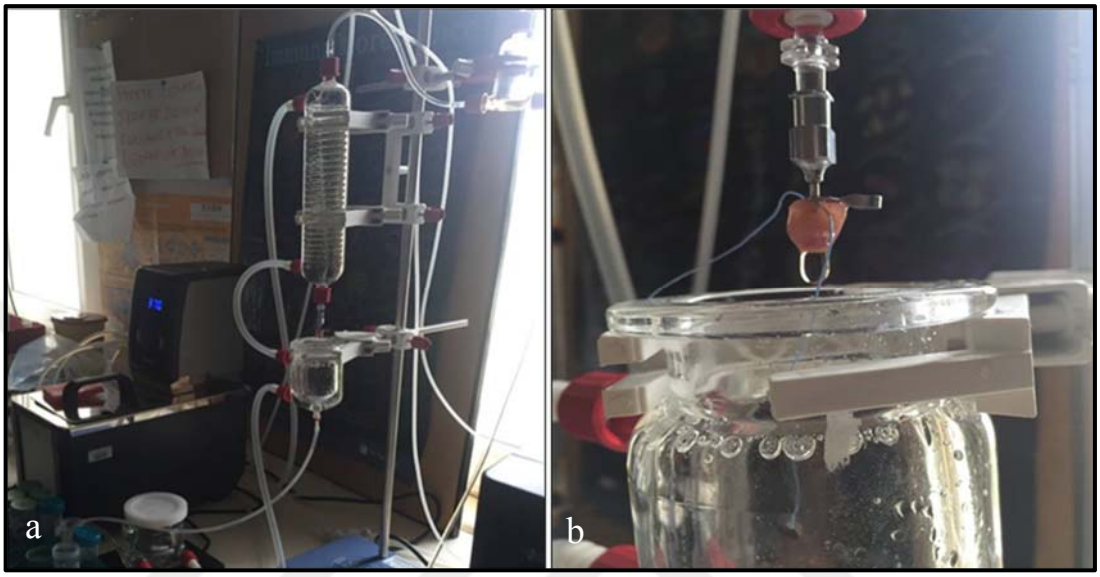

Figure A.1. Image of Langendorff Apparatus used for adult mouse heart digestion a) Complete picture of the Langendorff Apparatus b) Heart sample was cut and the tip of the apparatus inserted into the aorta for succesfull digestion.