# MOLECULAR MECHANISM OF CARDIAC REGENERATION

by

Galip Servet Aslan

Submitted to Graduate School of Natural and Applied Sciences
in Partial Fulfilment of the Requirements
for the Degree of Master of Science in
Biotechnology

Yeditepe University

2017

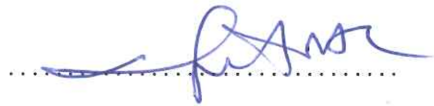
MOLECULAR MECHANISM OF CARDIAC REGENERATION

APPROVED BY:

Assist. Prof. Dr. Fatih Kocabaş
(Thesis Supervisor)

Assist. Prof. Dr. Esra Çağavi

Assist. Prof. Dr. Hüseyin Çimen



DATE OF APPROVAL: ..../..../2017

# ACKNOWLEDGEMENTS

First of all, I would like to thank and express my special gratitude to my thesis supervisor Dr. Fatih Kocabaş who always motivated an supported me with his knowledge, guidance and leadership throughout my study. Thank you for giving me the opportunity to work in the field of cardiac regeneration and hematopoiesis. Working in YUREG laboratory has provided a great contribution and perspective to my academic journey. I would like to thank Dr. Esra Cagavi and Dr. Hüseyin Çimen for separating their time to be on my thesis committee and revising my work. I also would like to thank to The Scientific and Technological Research Council of Turkey (TUBITAK) for supporting me with the graduate scholarship during my study through TÜBİTAK 1001 program [grant number 115S185]. Thanks to my lab partners Yazgı Alyazıcı, Batuhan Kalkan, Doğacan Yücel, Pınar Siyah, Merve Uslu, Dolay Damla Çelik, Esra Albayrak, Dilek Turan, Merve Aksöz, Dr. Dilvin Güney for their friendship and support. Working with Semih Arbatlı and Zeynep Çokçeken in an harmony was an excellent experience so I would like to express my gratitude to them.A special thanks goes to my friends Merve Seven, Ezgi Türksever, Burcu Gür and Görkem Cemali for their friendship. Finally, my deepest heart-felt gratitude is to my family, Servet Aslan, Nazire Aslan, Nükhet Ülker and Selçuk Doğan Aslan who have been always with me supporting with their uninterruptible love and concern.

# ABSTRACT


## MOLECULAR MECHANISM OF CARDIAC REGENERATION

Neonatal mammalian heart has recently been shown to possess the capacity to regenerate completely after injury. This remarkable regenerative capacity is lost after postnatal day 7. This transition has been marked with cardiomyocyte cell cycle arrest and induction of fibrotic response similar to what occurs after myocardial infarction in adults. We have recently outlined the function of Meis1 as a new cardiogenic factor that plays a pivotal role in neonatal cardiac regeneration. However, underlying molecular mechanisms of neonatal cardiac regeneration and other cardiogenic factors remained elusive. Here, we investigated the involvement of novel putative cardiogenic factors in neonatal cardiac regeneration and cardiomyocyte cell cycle withdrawal. We have confirmed their expression in the heart and cardiomyocytes. We have shown that expressions of Inpp5d, Dnmt3a, c-Myc and Cbl are significantly downregulated during neonatal cardiac regeneration process. Intriguingly, these four factors are upregulated in non-regenerative period of 7-day old mice after injury. Taken together, our findings suggest that Inpp5d, Dnmt3a, c-Myc and Cbl may be involved in the regulation of cardiomyocyte cell cycle progression and represent new targets for induction of cardiac regeneration.

# ÖZET

## KALP REJENERASYONUN MOLEKÜLER MEKANİZMALARI

Yeni doğan memeli kalbinin oluşturulan hasar sonrası rejeneratif özelliğe sahip olduğu yakın zamanda gösterildi. Öne çıkan bu çalışmada, rejeneratif kapasitenin doğumdan sonraki 7. günden itibaren kaybolmaktadır. Bu geçiş evresinin, miyokardiyal infraksüyona benzer şekilde kardiyomiyosit hücre siklüs aresti ve fibrotik doku oluşumu ile tespit edilmektedir. Yakın zamanda yapılan çalışmalarda yeni doğan kardiyak rejenerasyonunda öncül bir rol oynayan Meis1'ın fonksiyonunu vurgulanmıştır. Fakat, yenidoğan faredeki kardiyak rejenerasyonunun altında yatan diğer moleküler mekanizmaların ve diğer kardiyojenik faktörler hala bilinmektedir. Burada, farklı olarak kardiyojenik faktörlerin yeni doğanda kardiyak rejenerasyonuna etkisini ve hücre döngüsündeki gerilemesini araştırdık ve bu faktörlerin kalpteki ve kardiyomiyositlerdeki ekspresyonunu doğruladık. Lnpp5d, Dnmt3a, c-Myc ve Cbl faktörlerinin ekspresyonunun yeni doğanda kardiyak rejenerasyon sürecinde önemli ölçüde baskılandığını gösterdik. İlginç bir şekilde, rejeneratif olmayan zaman diliminde (doğumdan sonra 7. gün) yapılan cerrahi müdahaleden sonra, bu dört faktöründe arttığı gözlemlenmiştir. Hepsi birlikte ele alındığında, bulgularımız Lnpp5d, Dnmt3a, c-Myc ve Cbl faktörlerinin kardiyomiyosit hücre döngüsü regülasyonunda etkili olabileceğine işaret etmekte ve kardiyak rejenerasyonun indüksiyonu için yeni hedefler sunmaktadır.

# TABLE OF CONTENTS

ACKNOWLEDGEMENTS ........ iii

ABSTRACT ........ iv

ÖZET ........ v

LIST OF FIGURES ........ viii

LIST OF TABLES ........ ix

LIST OF SYMBOLS/ABBREVIATIONS ........ x

1. INTRODUCTION ........ 1

1.1. Heart Failure ........ 1
1.2. Heart Regeneration ........ 2
1.3. Heart Regeneration In Zebrafish ........ 3
1.4. Heart Regeneration In New Newt ........ 5
1.5. Heart Regeneration In Mammals ........ 5
1.6. Heart Regeneration In Neonatal Mouse ........ 8
1.7. Modulators Of Cardiomyocyte Renewal ........ 9
1.8. Cardiogenic Factors Of Cardiomyocytes ........ 10
1.9. Cardiogenic Modulation Of Stem And Progenitor Cells ........ 12
1.9.1. Cardiac Stem Cells And Induced Cardiomyocytes ........ 13
1.9.2. Bone Marrow Derived Stem Cells In Myocardial Regeneration ........ 14

2. MATERIAL AND METHODS ........ 19

2.1 Animal Information ........ 19
2.2 Neonatal Mice Surgery Operation ........ 19
2.3 Immunofluorscence Detection Of Putative Regulators ........ 19
2.4 siRNA Knockdown ........ 20
2.5 Immunofluorscence Detection Of Cardiomyocytes Proliferation ........ 20
2.6 RT-PCR analysis of cDNA samples ........ 20
2.7 Isolation of adult cardiomyocytes ........ 20
2.8 Western Blot Analysis of Tissue Samples ........ 22
2.9 Statistical Analysis ........ 23

3. RESULTS ........................................................................................................ 26

3.1. Immunofluorscence Detection Of Putative Regulators Confirmed Protein Level In Adult Heart Myocardium ........................................................................................ 26

3.2. Profile Of mRNA Expression In Cardiomycotes And Fibroblast Revealed Cardiomyocyte Specific Regulators ........................................................................ 27

3.3. Western Blot Analysis Corralted With RT-PCR And Indicated High Expression Of Cbl In Heart ................................................................................................... 29

3.4. Neonatal Surgery Model On Neonatal Mouse Showed Negative Regulator Of Regenerative Response In Mouse ............................................................................ 30

3.5. siRNA Knockdown Of Putative Regulators Indicated Negative Effect On Cardiomyocyte Proliferation ................................................................................... 33

4. DISCUSSION .................................................................................................... 36

REFERENCES ........................................................................................................ 40

# LIST OF FIGURES

Figure 1.1. Different strategies to achieve cardiac regeneration in adults ............................ 2

Figure 1.2. Response to various types of injuries in the mammalian heart .......................... 9

Figure 1.3. Mechanisms of cardiomyocyte renewal ......................................................... 10

Figure 3.1.Detection of putative regulators of cardiomyoycte cell cycle modulators ......... 20

Figure 3.2.Differential expression of putative regulators in cardiomyocytes fibroblast ..... 21

Figure 3.3. Relative mRNA expression of putative regulators in adult mouse heart. ......... 22

Figure 3.4. Western Blot image of putative regulators in different tissue. ......................... 23

Figure 3.5.Quantification of Western Blot ........................................................................ 24

Figure 3.6. RT-PCR analysis of operated mouse at P3 and P7 .......................................... 25

Figure 3.7. RT-PCR analysis of operated mouse at P3 and P7 .......................................... 26

Figure 3.8. RT-PCR analysis summary of upregulation- downregulation of regulators. .... 27

Figure 3.9. Cell count after siRNA treatment. ................................................................... 28

Figure 3.10.Representative image of IF of neonatal rat cardiomyocytes. ........................... 29

# LIST OF TABLES

Table 1.1. Various cardiac injury methods in different organisms and their regenerative.... 3

Table 1.2. Unstimulated rate of cardiomyocyte in different species. ................................... 7

Table 1.3. Major factors involved in cardiomyocyte proliferation..................................... 15

Table 1.3. Major factors involved in cardiomyocyte proliferation..................................... 19

Table 2.1. Primers used to detect putative regulators……………………………………19

Table 2.2. Reagents for isolation of adult mouse cardiomyocytes…………………………21

Table 2.3. Reagents for preparation of western blot gels………………………………….22

# LIST OF SYMBOLS/ABBREVIATIONS

| | |
|---|---|
| BrdU | Bromo deoxyuridine |
| CDKI | Cyclin dependent kinase inhibitor protein |
| CSCs | Cardiac stem cells |
| ESC | Embryonic stem cells |
| FGF | Fibroblast growth factor |
| GATA | Globin transcription factor |
| GFP | Green fluorscent protein |
| HF | Heart failure |
| iCMs | Induced cardiomyocytes |
| LAD | Left anterior descending artery |
| µL | Microliter |
| µg | Microgram |
| mL | Microliter |
| Ph3 | Phospho histone protein 3 |
| RT-PCR | Real time polymerase chain reaction |
| siRNA | small interfering RNA |

# 1. INTRODUCTION

## 1.1. HEART FAILURE

Heart Failure (HF) is a complex clinical syndrome associated with decreased function of ventricle to fill or eject blood and affecting more than 23 million people in worldwide [1]. Unfortunately heart transplantation in the presence of appropriate donor remains only definitive treatment against HF [2].

Cardiac regeneration is multidisciplinary research area comprising physiology, stem cell, developmental biology and tissue engineering, and has ultimate goal of achieving to reverse heart failure in the context of regenerative medicine (Figure 1.1). Over the past decade, there have been mounting evidences demonstrating that the heart is certainly not a terminally differentiated organ. There is a constant cardiomyocyte turnover within the mammalian, and the human heart throughout life [3-6]. Evidence for role of cardiac resident stem cell, cardiomyocyte proliferation and contribution of exogenous stem cells were identified in back to back reports [7-10].

However revealing cardiac regenerative capacity of human heart and designing robust therapeutical strategies requires learning more about molecular mechanism of cardiac regeneration. Due to the complexity of heart organ, different populations and regulators within the heart have been recently identfied and indicated a complex system involving in regenerative capacity of heart. Moreover, studies on model organism including zebrafish, newt and murine heart giving great opportunity to enlighten underlying mechanism and recipe for cardiac regeneration. In the ligh of findings from those organism human based cardiovascular therapies are currently being developed and utilized in clinical trials to be used.

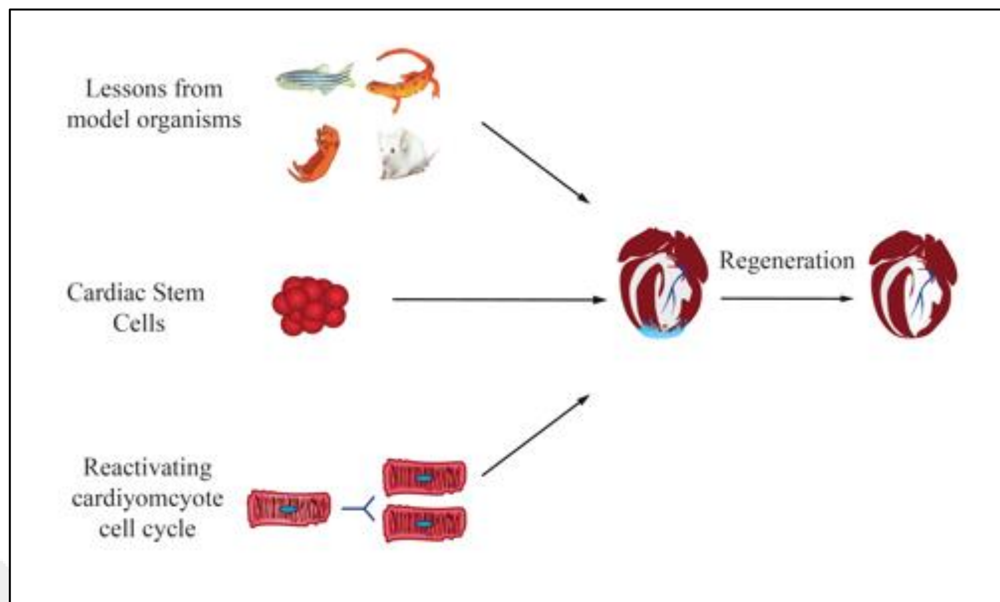



Figure 1.1. Different strategies to achieve cardiac regeneration in adults

## 1.2. HEART REGENERATION

Heart regeneration in lower vertebrates has been intensively studied. The regenerative potential of vertebrate hearts including amphibian, axolotls and newts was identified in early reports [11-13]. Using electron microscopy in 1974, Oberpriler et al. (1974) demonstrated the prospect of cardiac regeneration in newt. Later, Witman et al. (2011) reported that adult newt is able to completely regenerate its heart after a basal resection [14]. Zebrafish (*Danio rerio*) is a tropical fresh water fish that has been widely used in many different regenerative studies including heart [7, 8, 15], kidney [16], central nervous system [17] and others. The complete regeneration of zebrafish heart after amputation of the ventricular apex has already been reported [15] and led to new studies to understand the mechanisms of cardiac regeneration [7, 8, 18, 19]. On the other hand, the utilization of a recently developed cardiac injury model in 1-day old neonatal mice revealed that the neonatal mouse heart is also capable of regeneration after apical resection of 15% of the ventricular apex [20, 21]. Many different studies regarding the mechanism of cardiac regeneration in mammals were reported to enlighten underlying mechanisms of heart regeneration. Here, we review studies regarding cardiac regeneration in zebrafish, newt and neonatal mice (Table 1.1).

Table 1.1. Various cardiac injury methods in different organisms and their regenerative

| Organism | Zebrafish | Newt | Neonatal Mice | Adult mice | Human |
|---|---|---|---|---|---|
| Injury model | Apical Resection | Apical Resection | Apical Resection | Apical Resection | - |
| | Cryoinjury | Basal Resection | Cryoinjury | Cryoinjury | LVAD |
| | Genetic ablation | - | Ischemic MI | Ischemic MI | Ischemic MI |
| Response and time. | Regeneratio n in 60 days | Incomplete regeneration | Regenerati-21 days | No regeneration | - |
| | Regeneratio > 130 days | Regeneratio n 60 days | Regeneration 21 days | No regeneration | No regeneration |
| | Regeneratio n in 30-45 days | - | Regeneration in 21 days | Fibrosis & No regeneration | Fibrosis & No regeneration |

## 1.3. HEART REGENERATION IN ZEBRAFISH

Heart regeneration has been observed in non-mammalian vertebrates' hearts such as in salamanders and zebrafish [15, 22-25]. Zebrafish have become one of the major model organisms to study cardiac regeneration over a last decade. This is largely due to the visibility of structures during development, easy access to the heart to perform surgical operations, large number of offspring and low cost of maintenance. In addition, an intact cardiovascular system in a zebrafish embryo is not required, which allowed the investigation of cardiac regeneration studies without causing the death of zebrafish embryos [26]. Poss et al. (2002) demonstrated zebrafish cardiac regeneration following an amputation of up to 20% of the ventricle by surgical resection [15]. This operation initially led to fibrosis, followed by the complete regeneration of lost tissue in 60 days. In addition to the resection of the heart, other means of injury in zebrafish have been shown to provide tools to study different aspects of heart regeneration with different degrees of regenerative response [27, 28].

The cryoinjury method, for instance, depends on the induction of injury by a liquid nitrogen probe on the heart. When this procedure was performed in zebrafish, it led to heart regeneration over a longer time period (more than 130 days) [28, 29]. Wang et al. (2011)

applied a genetic ablation injury to extrapolate the regenerative response of zebrafish hearts. They have selectively expressed diphtheria toxin gene A (DTA) in cardiomyocytes by Cre-loxp mediated recombination, which is under control of a cardiac myosin light chain 2 promoter (cmclc2). This led to the genetic ablation and death of cardiomyocytes, albeit it resulted in complete regeneration of the heart in 45 days [27]. These findings prompted studies regarding the identification of the origin of newly formed cardiomyocytes.

The source of newly grown cardiomyocytes following myocardial injury in zebrafish has been a subject of debate. Many researchers used to think that new cardiomyocytes following cardiac injury in zebrafish were originated from progenitor cells instead of pre-existing cardiomyocytes [30]. However, two landmark studies provided evidence that newly formed cardiomyocytes in the cardiac regeneration process were largely derived from pre-existing cardiomyocytes [7, 8]. Integration of the Cre recombinase system as well as the GFP inducible genetic lineage tracing method in zebrafish led to tracking the origin of newly formed cardiomyocytes in the apical injury model following the removal of 20 % of the ventricular apex [7, 8]. Expression of GFP in newly formed cardiomyocytes provided that they arise from pre-existing ones. Furthermore, this study suggested a dedifferentiation mechanism, which has been measured by the disassembly of sarcomeric organization of cardiomyocytes, and initiates cardiomyocyte cell cycle progression and proliferation. Besides, this cell cycle progression has been regulated by polo like kinase1 (*plk1),* an important cell cycle modulator [7].

Kikuchi *et al.* (2010) has provided further evidence on the origin of newly formed cardiomyocytes, which has relied on GATA4 expression. GATA4 has been expressed in cardiomyocytes of the subepicardial ventricular layer and proliferating cells near the site of injury [31-33]. Kikuchi *et al.* (2010) utilized *gata4*-EGFP and demonstrated that myocyte marker carrying cells expressed GFP. On the other hand, cells with epicardium marker did not express GFP two weeks post-injury. These results suggested that cardiomyocytes existing near the injury site are triggered to express GATA and reactivated the cardiomyocyte cell cycle and proliferation [8].

## 1.4. HEART REGENERATION IN NEWT

*Notophthalmus viridescens*, a kind of red-spotted newt, is classified in the urodele amphibians and is commonly accepted as the champion of regeneration. Regenerative biology studies in newt have established the extraordinary regenerative ability of various organs and tissues including limbs, tail, lenses, spinal cord and heart [14]. The newt heart is located close to the skin and made of three chambers (two atria and one ventricle). Trabeculae form the cardiac ventricle of newt, where a single layer of epicardial cells surrounds myocytes, fibroblasts and nerve fibers [34]. Initial reports on newt cardiac regeneration date back to the 1970s. In 1974, Oberpriller and colleagues showed the mitotic response of the newt heart and the possibility of newt cardiac regeneration. Although underlying studies did not report the complete regeneration of the newt heart after amputation of one-eighth of the ventricular apex, they led to increased mitosis in the heart [35, 36]. Thus, studies were rather performed with a modified apical resection model, in which the ventricular cavity was left intact and investigated for a longer period to assess regenerative response. In this case, complete cardiac ventricular regeneration has been observed in 60 days along with an increased expression of cardiac specific transcription factors such as GATA4, Nkx-2.5**,** GATA5, Islet1, and HAND2 at the peak of proliferation. This indicates that a proliferative response is achieved by a coordinated expression of transcription factors [14]. In addition, newt change the expression of extra cellular matrix (ECM) related genes instead of metabolic or inflammation related ones following cardiac injuries. The expression of ECM genes, such as collagen III and tenascin-C, increases just after amputation or injury of the ventricular apex. This suggested that reorganization of the ECM was involved in the replenishment of the lost cardiomyocytes [37, 38]. Moreover, Mercer et al. (2013) reported that tenascin-C significantly increases the reentry of cardiomyocytes into the cell cycle *in vitro* [37].

## 1.5. HEART REGENERATION IN MAMMALS

It was believed that the total number of cardiomyocytes in a mammalian heart is set at birth and does not change through life. However, it has been demonstrated over the past decade that the heart is certainly not a terminally differentiated organ [3-6]. A number of recent

studies provided strong evidence for the fact of cardiomyocyte renewal in human heart (Bergmann et al., 2012b; Laflamme et al., 2002; Quaini et al., 2002; Kajstura et al., 2010). Bergmann et al. (2009) utilized an elegant approach to determine the age of cardiomyocytes, thus determining if any cardiomyocyte turnover occurs in the human heart. Pulse-chase conditions of $^{14}C$ level in the atmosphere due to the testing of nuclear weapons during cold war enabled to measure the age of cardiomyocytes in human subjects (older than 20 years). Analysis of the $^{14}C$ content and turnover in cardiomyocytes indicated that cardiomyocytes were younger than expected, which suggested that they are not set at birth. Further analysis and mathematical modeling indicated that about 1% of cardiomyocytes are renewed per year at the age of 20, and 0.4% at the age of 75. This provided an estimation of 40-50% cardiomyocyte renewal in an average human lifespan [3].

Kajstura and colleagues (2010) have provided further evidence of cardiomyocyte renewal by utilizing samples from post-mortem hearts of thymidine analogue iododeoxyuridine (IdU) treated cancer patients. Analysis of IdU incorporation and turnover in the heart indicated the presence of 22 % cardiomyocyte turnover per year, which is higher than the estimations of Bergmann and colleagues [5]. Discrepancy between these two studies has been thought to be due to the age and distribution of human subjects. Mollova et al. (2013) recently outlined that there are distinct, age-dependent cardiomyocyte division rates (0.016% in 0-1 year old, 0.01% in 2-10 year old and 0.005% in 10-20 year old) [39]. In another study, male patients who received heart from female donor were subjected for chimerism of transplanted heart. Depending on Y chromosome analysis and immunolabeling of recipient primitive cells, contribution of primitive cells migration from recipient to graft area thus chimerism of heart was reported [40].

Moreover, Shiba et al. (2012) reported that integrated cardiomyocytes, which derived from human embryonic stem cells, could be used against arrhythmias in Guiana pig model. Beside previous findings suggested that transplantation of fetal cardiomyocytes can improve the function of infarcted hearts this was a landmark study regarding cell replacement therapies for cardiovascular disease [41-44]

Studies showed that the DNA damage response due to a high oxygen environment during postnatal mice exposure has been shown to be an important mechanism in cardiomyocyte cell cycle arrest [45]. A recent study investigated the effect of the left ventricular assist device (LVAD) on mitochondrial content and cardiomyocyte proliferation [46]. By

comparing pre-LVAD and post-LVAD patients, they have demonstrated that prolonged mechanical unloading causes to % 60 decreased mitochondrial mass and ROS as well as about three fold increased cardiomyocyte proliferation [46].

Examination of the regenerative capacity of the mouse heart took cardiac regeneration studies a step further. Mouse cardiomyocytes are highly proliferative during embryogenesis. After 4 days from birth (postnatal day 4), cardiomyocytes dramatically lose this proliferative capacity. Cardiomyocytes undergo karyokinesis without the cytokinesis step thus resulting in binucleation of 90% of adult cardiomyocytes [47-49]. Mammals differ from other vertebrates in terms of cardiac regeneration capacity by possessing mostly binucleated cardiomyocytes, greater heart volume, four chambered hearts, and high pressure containing blood flow and an associated complex genome [50]. Similar to zebrafish, one of the debated issues was the source of newly formed cardiomyocyte in mammals that was recently enlightened by rodent and human studies [21, 51]. Mice possess a low rate of cardiomyocyte turnover confirmed by various studies and different approaches. BrdU incorporation and quantification by an anti-BrdU antibody were utilized to assess cell proliferation in the mouse heart. Similarly, a thymidine analog (thymidine [$^3$H]) was incorporated into newly formed DNA strands as the cell cycle progress and used as a marker for cardiomyocyte proliferation. Another approach utilized the incorporation of $^{15}$N, and coupled to multi-isotope imaging mass spectrometry (MIMS) to assess proliferating cells in the heart. These studies indicated that an adult mouse shows 0.74 - 4.5 % of an unstimulated rate of cardiomyocyte renewal (Table 1.2) [48, 52-54].

Table 1.2. Unstimulated rate of cardiomyocyte renewal in different species

| Species | Estimated cardiomyocyte renewal/year | Methods | Sources of newly formed cardiomyocytes | References |
|---|---|---|---|---|
| **Zebra fish** | ND | Not determined | Largely cardiomyocytes but it doesn't not exclude the involvement of cardiac progenitors | [7, 8] |
| **Mouse** | 0.74-4.5% | BrdU, $^{15}$N, imaging mass spectrometry, | Largely cardiomyocytes but it doesn't exclude the | [52, 53, 55] |

| | | | | |
|---|---|---|---|---|
| | | $[^3H]$ thymidine | cardiac progenitors | |
| **Human** | 0.04-40% | $^{14}C$, accelerator mass spectrometry, Ki67, phospho-H3, Aurora B, and IdU | Largely cardiomyocytes but it doesn't exclude the cardiac progenitors | [5, 39, 56] |

The source of newly formed cardiomyocytes in mammals have been the subject of debate for years [57]. Recent studies demonstrated that proliferation of pre-existing cardiomyocytes occurs in mice after myocardial infarction and reported that cardiac progenitor cells have a modest effect as suggested in previous studies [51, 52, 58]. However, the possibility of a progenitor or stem cell population to involve in cardiac regeneration following injury through differentiation has not been excluded. Along with the resident capacity to replenish cardiomyocytes, recent studies especially in neonatal animals suggest that mammalian hearts possess a hidden regeneration potential.

### 1.6. HEART REGENERATION IN NEONATAL MOUSE

Compared to zebrafish and newt, the adult mammalian heart has a limited capacity for cardiomyocyte renewal following injury and responds to cardiac tissue damage by scar formation. As we have explained earlier, mouse cardiomyocytes undergo dramatic changes during the first week of life as marked with the expression of adult isoforms of contractile proteins, and the induction of DNA synthesis without cytokinesis resulting in binucleation and cell cycle arrest at $G_0/G_1$ [48]. A recent landmark study accomplished a similar injury model of zebrafish in mouse and demonstrated that 1-day old neonatal mouse is capable of heart regeneration [21]. One study reported the complete regeneration of neonatal mouse heart without a visible scar and fibrosis following amputation of 15 % of the ventricle [21]. Another study permanently ligated the left anterior descending (LAD) coronary artery of 1-day-old mice, thus it induced an ischemic myocardial infarction [59]. This study further provided that a neonatal mouse following LAD ligation could regenerate the heart in as short as 21 days without obvious fibrosis and scar formation [59]. In addition, a Cre-lox inducible genetic fate mapping approach has been utilized to address the source of regenerated cardiomyocytes, and showed newly formed cardiomycoytes arise from the pre-existing

cardiomyocytes after the apical resection of the neonatal myocardium [59]. Moreover, a cyroinjury model was used to study the regenerative response of neonatal mice hearts. One study indicated that cardiac function was not recovered after a transmural (severe) cyroinjury [60]. In contrast, although cardiomyocyte proliferation was not robust, non-transmural (mild) cyroinjury allowed for complete recovery of cardiac function [60]. These cardiac regeneration models (Figure 1) allowed identification of the cardiomyocyte cell cycle modulators as well as prospective targets to induce heart regeneration.

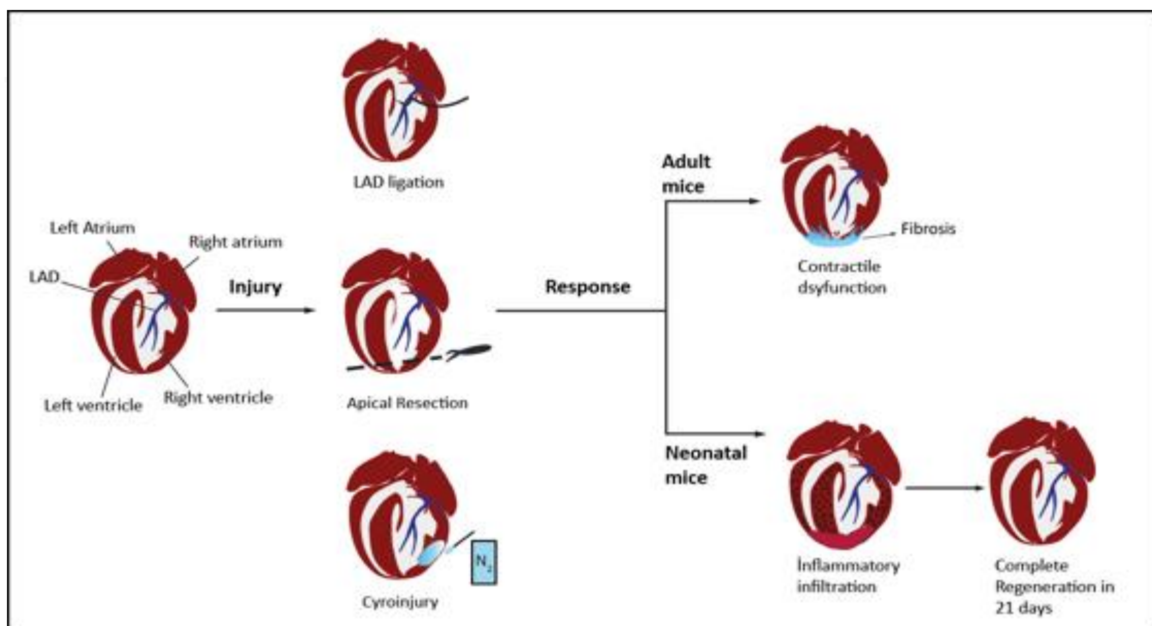



Figure 1.2. Response to various types of injuries in the mammalian heart. Different injury models utilized to measure regenerative response of the mammalian heart. Left anterior descending arteria (blue) has been permanently ligated in the LAD ligation model. The apical resection injury model leads to an amputation of up to 15% of the ventricular apex. On the other hand, the cyroinjury model induces cardiac damage on the ventricular apex by a pre-cooled probe (cooled by liquid nitrogen).

## 1.7. MODULATORS OF CARDIOMYOCYTE RENEWAL

Myocardial infarction leads to loss of substantial amount of cardiomyocytes, which negatively influence cardiac function. This urges therapeutic approaches that either preserve

existing cardiomyocytes or increase the number of functional cardiomyocytes following myocardial injuries.

In this frontier, studies to uncover the mechanism of functional cardiomyocytes renewal became a major point of focus in the context of cardiac therapies. Development of an injury model in zebrafish [15] and neonatal mouse [21] provided a great opportunity to investigate these mechanisms and factors involved in heart regeneration. Over the last decade, contribution of different mechanisms including transdifferentiation [61, 62], dedifferentiation [7, 8], proliferation of pre-existing cardiomyocytes [7], the contribution of both cardiac resident stem cells [10], and bone marrow derived stem cells [5] were suggested to be plausible in treatment of cardiovascular disorders (Figure 2). These studies revealed some of the important factors that modulate cardiac progenitors or the cardiomyocyte cell cycle.

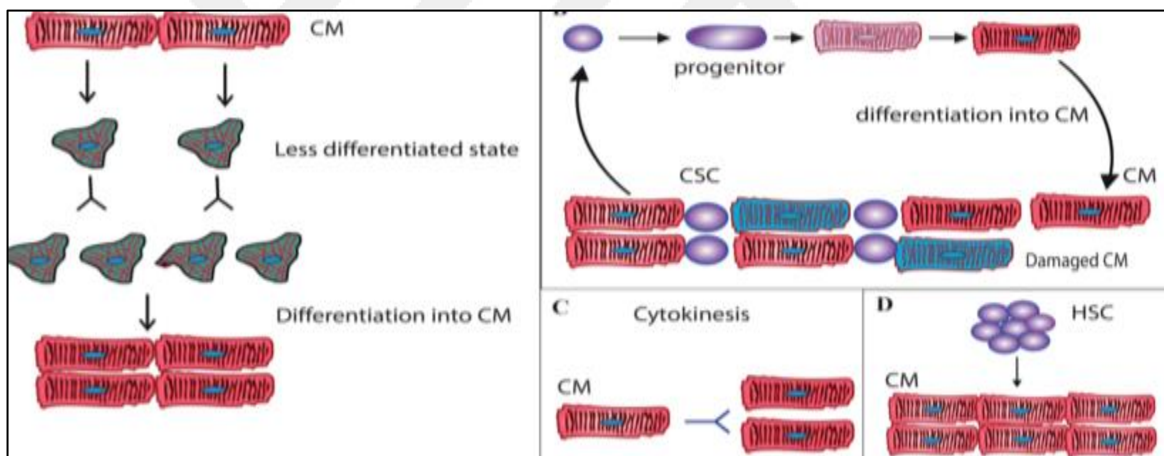



Figure 1.3. Mechanisms of cardiomyocyte renewal. a) Dedifferentiation initiated by detachment of cardiomyocytes followed by differentiation back into cardiomyocytes. b) Cardiac resident stem cells give rise to new cardiomyocytes through differentiation. c) Proliferation of pre-existing cardiomyocytes. d) Transdifferentiation of bone-derived hematopoietic stem cells into cardiomyocytes

## 1.8. CARDIOGENIC FACTORS OF CARDIOMYOCYTES

Various molecular intervention approaches have been utilized to manipulate cardiomyocyte proliferation (Table 3) [55, 63-66]. Jackson et al. (1990) overexpressed c-myc during

embryogenesis of mice and demonstrated both increased cardiomyocyte number (almost 2 fold) and heart weight [65]. In another study, adenoviral overexpression of oncogene E1A in cardiomyocytes resulted in induced cardiomyocyte cycling followed by apoptosis [67]. Overexpression of cell cycle regulatory proteins has been also tested to see if they enhance cardiomyocyte proliferation. Pasumarthi et al. (2005) overexpressed cyclin D1, cyclin D2 and cyclin D3, which are considered as positive regulators of G2/M transition state. They reported that neither cyclin D1 nor cyclin D3 increased proliferation of cardiomyocyte after myocardial infraction; however, cyclin D2 overexpression resulted in promoted cardiomyocyte proliferation in adult transgenic mice [66]. Successive studies including forced expression of cyclin B1-CDC2 and transgenic expression of cyclin A2 have resulted in an increased percentage of cardiomyocytes in G2/M *in vitro*, increased cardiomyocyte proliferation and induced myocardial regeneration in adult mice [63, 68]. Other studies investigated the downregulation of cell cycle inhibitors such as cyclin-dependent kinase inhibitors (CDKIs) ($p21^{Waf1}$, $p27^{Kip1}$, and $p57^{Kip2}$) due to their high expression in neonatal and adult heart and reported an increased cardiomyocyte number [69].

Over the past decade, a number of transcription and growth factors involved in the modulation of the cardiomyocyte cycle were identified [70-74]. Development of the neonatal mouse cardiac regeneration model gave an opportunity to investigate inhibitors of mammalian cardiac regeneration being activated after the neonatal period [20, 21, 59, 75]. Thus, we successfully used this model to identify Meis1, one of the key regulators of neonatal cardiac regeneration and reported that Meis1 inhibits cardiomyocyte proliferation through transcriptional activation of CDKIs p15, p16 and p21 [75].

Signaling pathways have been intensively studied to understand and overcome the limited regenerative capacity of the heart [76]. In this frontier, Neuregulin, a ligand for neuregulin/ErbB2/ErbB4 signaling cascade, was revealed as a positive regulator of cardiomyocyte proliferation [77] both in overexpression [18] and recombinant protein administration studies [78-81]. In addition, administration of recombinant oncostatin M, TWEAK, FGF10, periostin and co-administration of FGF1 with p38 inhibitor resulted in cardiomyocyte proliferation after myocardial infraction [72, 73, 82-84]. Several studies on the Hippo signaling pathway demonstrated its prospect in cardiac regeneration [85]. Overexpression of one of the key component of The Hippo pathway, yes-associated

protein [85] and knockdown of an upstream effector element of the Hippo pathway resulted in an increased cardiomyocyte number and a thickening of the myocardial wall [86-90].

Nerves have been known to guide to organ regeneration. However, their function in cardiac regeneration was not determined until recently [91]. In this frontier, Mahmoud et al. (2015) reported that pharmacological inhibition of cholinergic nerve formation in zebrafish and newborn mice reduce cardiomyocyte proliferation following injury thus suggesting that innervation is crucial for heart regeneration [92]. Moreover, mechanical inhibition of innervation (left vagotomy surgical operation) decreases cardiac regenerative response in newborn mice that could be rescued by recombinant neuregulin 1 (NRG1) and nerve growth factor (NGF) administration. In addition, they reported that immune response and inflammatory associated genes are downregulated following denervation, which shows denervation impairs heart regeneration through down regulating immune response mechanism [92].

## 1.9. CARDIOGENIC MODULATION OF STEM AND PROGENITOR CELLS

A number of studies attempted to modulate resident or non-resident progenitor and stem cells to induce new cardiomyocyte formation following myocardial infarctions. Embryonic stem cells (ESCs) demonstrate a capacity to differentiate into beating cardiomyocytes *in vitro*. Thus, ESCs are considered to be an option to use towards regenerative cardiology. However, several drawbacks regarding teratoma formation following transplantation, immune reactions and ethical concerns made them unsuitable for use in clinical studies [93]. The recent discovery of induced pluripotent stem cells (IPSCs) provided new angles to overcome these issues and eliminated major ethical concerns [94, 95], IPSCs have been shown to share many characteristics of ECSs and they have been successfully generated from various somatic cells [96, 97]. In addition, differentiation of IPCSs into specific cells including cardiomyocytes were reported [98-101]. Although there are no clinical trials yet using IPSCs cells for myocardial regeneration, intense research is currently focused on the discovery of new methods for safer cellular reprogramming methods and induction of lineage specific differentiation of iPS cells to prevent teratoma formation.

Skeletal myoblasts (satellite cells) are also thought to be a potential source for cardiac therapies. *In vitro* expansion of skeletal muscle cells derived from skeletal muscle biopsies

favored them for autologous transplantation and their ischemia-resistant property made them among the first cells tried in cellular therapies in the treatment of cardiac failure. Engraftment of skeletal myoblasts into damaged myocardium resulted with improved cardiac function [102, 103] and prevented progressive ventricular dilatation [104]. Although several studies suggest that engrafted skeletal myoblast cells do not transdifferentiate into functional cardiomyocytes [105] and electrophysiologically differ from resident cardiomyocytes [106, 107], clinical trials are still proceeding to test the potential of these cells in cardiac regenerative therapy.

### 1.9.1. Cardiac stem cells and induced cardiomyocytes

Cardiac stem cells (CSCs) are widely investigated in the treatment of cardiovascular disorders (reviewed in [108]. The discovery of c-kit$^{+}$ CSCs followed the identification of other CSCs including epicardial progenitors, Isl1$^{+}$ cardiovascular progenitors, side population progenitors, Sca1$^{+}$ progenitors, heterogeneous progenitors containing cardiospheres and cardiac mesenchymal stem cells [10, 109-113]. Following identification of these progenitors, factors involved in differentiation into cardiomyocytes started to emerge. Oxytocin treatment on Sca-1$^{+}$-CD45$^{-}$ cells has been shown to induce differentiation into spontaneously-beating cardiomyocytes [114]. Furthermore, transplantation of these cells into necrotic myocardium increased cardiac function [112]. In another study, intramyocardial injection of HGF-cMet and IGF-1 factors after induction of myocardial injury resulted in an increased number of cardiac resident stem cells [115]. Qyan et al. (2007) reported that cardiac mesenchymal cells regulate the renewal and differentiation of cardiac *Isl1*$^{+}$ cardiovascular progenitors via the Wnt/β-catenin pathway [116]. In addition, chemical inhibition of glycogen synthase kinase-3 (GSK-3) resulted in a 2 fold-increased number of cardiac Isl1$^{+}$ cardiovascular progenitors. Cardiospheres, which were derived from endomyocardial biopsy specimens, also have a potential use in cardiac stem cell therapy. The existence of different progenitors and differentiated cells within cardiospheres mimic the stem cell niche existing in the heart thus it can take a step further in cardiac stem cell studies [117].

A recent study reported the direct reprogramming of cardiac fibroblasts into cardiomyocytes, which has provided an alternative source of cells to trigger heart regeneration [99]. Stable integration of cardiac specific markers Gata4, Tbx5 and Mef2x led to transform 20% of

cardiac fibroblast into induced cardiomyocytes (iCMs), which have similar epigenetic states and gene expression as in normal cardiomyocytes [118, 119]. Integration of different factors (Oct4, Sox2, Klf4 and c-Myc) by Efe et al. (2011) supported the reprogramming of mouse embryonic fibroblast into beating cardiomyocytes in 11-12 days, which was shorter when compared to the study by Ieda et al. (2010) [120]. A decreased trend towards tumor formation and the ability to reprogram a large number of a patient's fibroblast into cardiomyocytes made iCMs an alternative for cardiac therapies. Even though there are many advantages of iCMs, it requires further investigations to effectively use endogenous fibroblast cells in the repair of damaged myocardium before proceeding into clinical trials.

### 1.9.2. Bone marrow derived stem cells in myocardial regeneration

The bone marrow contains heterogonous cell populations. Investigations on bone marrow cells (BMC) and hematopoietic stem cells (HSCs) in the induction of myocardial regeneration date back to the early 2000s. Orlic and colleagues (2001) showed that bone marrow derived cells acquire a cardiomyocyte-like phenotype and provide a functional recovery following myocardial infarction [9]. Further clinical studies with BMCs demonstrated the prospect of human heart regeneration [121-123]. Studies based on bone marrow derived mesenchymal stem cells (MSC) indicated the ability of MSCs to induce proliferation and differentiation of resident cardiac stem cells [124, 125] Many different mechanisms have been proposed to explain the effect of BMC cells in myocardial regeneration [126, 127]. A recent study showed that bone marrow c-kit$^{+}$ cells but not MSCs stimulate an endogenous pool of cardiac progenitors that dilute the pool of cardiomyocyte specific GFP expression thus they improve cardiac function [128]. In addition, induction of a number of growth factors including hepatocyte growth factor (HGF), insulin-like growth factor (IGF-1), vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) were reported just after treatment with MSCs or multipotent human BM stem cells (hBMSCs) (Yoon et al., 2005). In another study, TGF-β and bone morphogenetic protein (BMP)-2 increased the expression of cardiac transcription factors in a paracrine manner. In addition, it was reported that periostin and neuroglin administration induce cardiomyocyte proliferation. However, the source of these ligand receptor interactions (existing in both cardiomyocytes and HSCs) remain undetermined. The paracrine effect could be the

underlying mechanism giving rise to a modest improved diastolic function following BMC derived stem cell treatment.

Table 1.3. Major factors involved in cardiomyocyte proliferation

| **Cardiogenic factors and manipulations** | **Fold change in proliferating cardiomyocytes** | **Reference** |
|---|---|---|
| **Meis1 knockout** | 9 (pH3 + CMs, Aurora B + CMs) | [75] |
| **GSK-3 inhibition** | 5 (pH3 + CMs) | [129] |
| **Foxo1 dominant negative** | 2.5 (pH3 + CMs) | [130] |
| **miR-133a knockout** | 2.5 (pH3 + CMs) | [131] |
| **Jumonji knockout** | 2.2 (pH3 + CMs) | [132] |
| **$p27^{KIP1}$ knockout** | 2-3 (troponin I + CMs) | [133] |
| **Constitutively active ERBB2** | >12 (Ki67 + CMs, pH3 + CMs, Aurora B+ CMs) | [77] |
| **Nrg1 (or FGF1, periostin) treatment** | >4 (BrdU + CMs, Aurora B + CMs, pH3 + CMs,) | [71] [18] |
| **Activated Yap1** | >7 (Ki67 + CMs, pH3 + CMs, Aurora B+ CMs) | [89] |
| **Salv knockout** | >4 (pH3 + CMs) | [88] |
| **IL13 (or IL3, CTGF, Nrg1) treatment** | >1.5 (3H Thymidine CMs, Ki67 + CMs, BrdU + CMs) | [134] |
| **Oncostatin M treatment** | >2 (EdU + CMs) | [83] |
| **TWEAK treatment** | 6.2 (BrdU + CMs) | [84] |

| | | |
|---|---|---|
| **C3orf58 treatment** | >2 (Ki67 + CMs, BrdU + CMs, Aurora B + CMs) | [135] |
| **Periostin treatment** | >5 (BrdU + CMs, pH3 + CMs, Aurora B+ CMs) | [72] |
| **FGF10 treatment** | 2 (Ki67 + CMs, pH3 + CMs,) | [73] |
| **Cyclin D2 overexpression** | >5 (MHC-nLAC + CMs) | [66] |
| **Cyclin B1-CDC2 or cyclin a2 overexpression** | >1.4 (CMs) | [63] [64] |
| **Activated Notch** | >7 (Ki67 + CMs, BrdU + CMs, Aurora B + CMs) | [136] |
| **c-myc or E1A overexpression** | 2 (CMs) | [65-67] |

### 1.9.3 Putative regulators of cardiac regeneration

Recent findings in the last decade led to identify major factors involved in cardiomyocyte proliferation (Table 1.3). Different effect of those regulators chanlged dogma that heart is terminally differentiated organ. Moreover, recent studies in different quiscent cell type also enabled scientist to monitor their effect on other quiscent cells including but not limited to cardiomycocte. To this end, one of the important regulator, meis1, which is a homeodomain transcription factor, was reported to be important for HSCs development, and recently identified as major regulator of HSCs quiescence [142]. Morevover, recent study also revealed Meis1 in cardiac regeneration, and suggested that Meis1 also negatively regulates cardiomycote proliferation similar its effect on shifting HSCs quiscence [75]. This findign led to identify another meis1 like regulators to be screened in cardiac regeneration. To this end, carefully investigation of literature indicated six diferen regulator to be important for cardiac regeneration (APPENDIX C).

The role of different transcription factors have been suggested to be important for HSCs differentiation. The Growth factor independence 1 (Gfi1), a member of zinc finger proteins, reported to be major regulator of of transcriptional network of hematopoiesis required for

HSCs maintenance. Moreover, in a recent study, role of Gfi1b for erythroid and megakaryocytic development, thus HSCs. The effect of Gfi1b in HSCs was suggested to depend on increased level of ROS due to activation of those developmental pathways and ultimately resulted with expansion of HSCs [143].

Another putative regulator of cardiac regeneration, SHIP1 (inositol-5-phosphatase) belongs SH2 domain containing protein family, suggested to be a negative regulator of hematopoiesis. This action of mechanism in HSCs expansion was reported to be formation of tryosine residues which lead to a novel signalling complex with DOK1 (p62) and CRK (p38) [144].

ITCH, E3 ubiquitin ligase, transcriptional corepressor of p45/NF-E2 and another negative regulator of HSCs expansion. In a recent study, *Itch*$^{-/-}$ mice lead to increased number of HSCs and increased long term repopulating activity of HSCs which indicate multiple effect of Itch on HSCs. Moreover, *Itch*$^{-/-}$ mice resulted with increased proliferation of HSCs with decrease in progentior characteristics. Moreover, notch signalling pathway was suggested to reverse this increased number of HSCs proliferation which explained the mechanism of action of Itch on HSCs biology troguh regulating notch pathway [145, 141].

Dnmt3a, NA (cytosine-5)-methyltransferase 3A, enzyme responsbile to transfer methyl groups to specific CpG regions in DNA, which is defined as DNA methylation. Depending on study using conditional ablation strategy, it was reported abnormality in HSCs differentation and impaired HSCs over serial transplantation while expanding HSCs numbers in bone marrow. Moreover, Dnmt3a$^{-/-}$ mice resulted with substantial CpG hypermethlyation and altered HSCs multipotency genes while downregulate differentation factors. These findings established Dnmt3a as a critical regulator of epigenetic mechanism of HSCs which enabling efficient differentiation and expand HSCs [146].

Study regarding to another putative regulator, Depending on conditional elimination of of c-Myc protein in HSCs, its unexpected function on HSCs homeostasis was reveleaed. Moreover, conditional knock out of cmyc transcriptional activity in bone marrow lead to cytopenia and accumlation of HSCs in situ. Furthermore, observed abnormality in differentation capacity of c-myc knock out mice HSCs was correlated with up-regulation of several adhesion receptors, suggesting that release of HSCs from the stem cell niche requires c-Myc activity. Collectively, these findings revealed that c-Myc controls the balance

between stem cell self-renewal and differentiation, trough controling HSCs and their niche in transcriptional manner [147].

The final putative regulator of cardiac regenration, Cbl, is proto-oncogene encoding RING finger E3 ubiquitin ligase which is required to target substrates to be degraded by proteasome. Cbl is ubiquiting conjugating enzyme (E2) to substrates. Moreover, N-terminal phosphotyrosine binding domain led to Cbl enzyme to interact with several tyrosine-phosphorylated substrates. Moreover, Cbl reported to regulator of cellular activation pathways. Cbl mutations, for instance, are associated with myeloproliferative disorders (MPD). Moreover, in a recent study, link between HSC expansion and MPD development upon combined Cbl and Cbl-b deletion were reported. Depending on cell cycle analysis, $Cbl^{-/-}$ mice HSCs showed reduced quiescence. Moreover, $Cbl^{-/-}$ mice showed a sustanied c-Kit and FLT3 signalling which promoted loss of colony forming potential [138].

# 2. MATERIAL AND METHODS

## 2.1 ANIMAL INFORMATION

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 NEONATAL MICE SURGERY OPERATION

Apical resection and LAD surgeries were performed on neonatal mice (Balbc) at on post-natal day 3 (P1) and day 7 (P7). Considering that neonates up to one 1 week can endure and survive in hypothermic conditions, mice were anesthetized on ice bath approximately for 5 minutes. Additionally, this anesthesia method prevent extreme bleeding by decreasing blood flow during injury. Lateral thoracotomy was performed in order to heart take out. Left anterior descending artery was ligated using 7-0 non absorbable silk suture. Skin sore closed up by suturing and using skin adhesive. For sham group, only chest cavity was opened mice and skin sore closed up immediately. Hearts were harvested 4 days after surgery. Harvested hearts were washed in PBS solution for a short time and stored at -80 until use.

## 2.3 IMMUNOFLUORSCENCE DETECTION OF PUTATIVE REGULATORS

After antigen retrieval step using EDTA Buffer (1mM EDTA, 0.05% Tween 20, pH 8.0) in boiling water for approximately 40 min, paraffin sections were permeabilized with 0.25% triton X/PBS for 10 minutes and then blocked with 1% BSA for 30 minutes at incubator. Primary antibodies against (Gfi1b (Sigma, HPA007012), Inpp5d (SCBT, sc-8425), ITCH (Sigma, HPA021126), Dnmt3a (Sigma, HPA026588), c-myc (SCBT, sc-789), CBL (Sigma, HPA027956) were incubated for overnight at 4 Celsius and secondary antibodies for detection (Life technologies, Alexa Fluor 488 anti mouse; Anti-Mouse anti rabbit) were done incubatin for 45 minuts at incubator. Afterwards, sections were washed with PBS for three

times and detection under immunofluorescence microscope under 40X magnification was performed.

## 2.4 IN VITRO siRNA KNOCKDOWN

In vitro siRNA knockdown approach was utilized on cultured rat neonatal cardiomyocytes. To this end, cardiomyocytes were cultured on 48-well plates at 70-75% confluency in the presence of fibroblast inhibitor factor (BrdU), and triplicates per group was done. Diluted lipofectamine (invitrogen) and and siRNA (20 µM) in OPTIMEM media was given to culture and incubated for 6- hours. Afterwards, cells were either fixed or used for RNA or protein isolation.

## 2.5 IMMUNOFLUORSCENCE DETECTION OF CARDIOMYOCYTES PROLIFERATION

Immunostaining of siRNA treated neonatal cardiomyocytes were performed after fixation step using paraformaldehyde. To this end, cells were incubated in paraformaldehyde for 10 minutes at incubator. Coming after, to provide cell membrane permeability 0.1% Triton-X in 1X PBS for 10 minutes at incubator. Than blocking step was done by %1 BSA-tween 20 solution for 30 minutes at incubator. Fixed cells were incubated with TnnT (1:200) overnight at +4$^{0}$C. The day after cells were incubated with PH3(1:200) for 2 hours at incubator. Following, cells are treated with suitable secondary antibodies containing 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 fluorescent imaging. Each PH3 positive cardiomyocyte cells were counted by 40X objective.

## 2.6 RT-PCR ANALYSIS OF cDNA SAMPLES

Total RNA was isolated using TRIzol reagent according to manufacturer's instructions. Briefly, 1ml TRIzol reagent was used for 1 millon cells and RNA was precipitated using % 100 propanol, and washed out using %75 alchool. cDNA sysnthesis was performed using 5µg of RNA using NEB cDNA synthesis kit (NEB, protoscript). Indicated primers (Table

2.1 ) were ordered from Sentegen Technologies. RT-PCR was performed using BioRad Fx96 system and using SyberGreen (Thermoscientifi). Results were normalized according to the either GAPDH or actin gene expression by using ΔΔCt method.

Table 2.1. Primers used to detect putative regulators

| Primer Oligo ID | Primer 5' to 3' |
|---|---|
| mGfi1b-F | CTAGAAAGGACCGTGGCATT |
| mGfi1b-R | CAGGGACAGTGTGGAGGTTC |
| mInpp5d-F | GTGAAGAACCTCATGGGGAC |
| mInpp5d-R | GCTGTTCCGGAATTGTGTTT |
| mItch-F | CCTTATGTAGAAGTCACAGTAG |
| mItch-R | TCACCTACAAGCTGCAAAGTCA |
| mDnmt3a-F | TACATCAGCAAACGGAAACG |
| mDnmt3a-R | CCTCCTCCACCTTCTGAGACT |
| mCbl-F | TTCCAGCACTTCTCCACCAT |
| mCbl-R | GATCGGGCTCATGAAGGAC |
| mMyc-F | TGAAGTTCACGTTGAGGGG |
| mMyc-R | AGAGCTCCTCGAGCTGTTTG |

## 2.7 ISOLATION OF ADULT MOUSE CARDIOMYCOCYTES

Isolation of adult mouse cardiomyocytes were performed by previously defined protocol [140]. Briefly; buffer and media were prepared according to Table 2.2. Perfusion system was set on to 37°C and pump was started on lowest setting (15 RPM, 6 ml/min). Perfusion system was sterilized by flushing with %70 ethanol then remove ethanol traces by flushing with distilled water. The digestion buffer was loaded into perfusion system and was let to circulate

until all air bubbles are removed. Secondly, Heart cannulation step was performed. To this end, ice cold perfusion buffer circulated for several times to heart from aorta by using 18G blunt needle. Heart was fixed into system using 18G blunt needle by catgut, then strap the two ends of the catgut to ensure stability of the heart during perfusion and was let the system running until the heart begin slouching as the collagen is degraded and it loses mechanical support. For Mechanical Dissociation and Purification step, 7.5 ml of digestion buffer was collected from the heat jacket of the perfusion system and was placed back to a petri dish then add 2.8 µl 1M $CaCl_2$ was added. Heart was removed from system was triturated in digestion buffer added petri dish. Petri dish was placed in to incubator 37°C with 5% $CO_2$ was agitated every two 2min until collecting sufficient number of cardiomyocytes. For the termination of isolation step, 7.5 mL of stop buffer was added to digestion buffer. Following that, digested tissues was filtered using sterile gauze bandage. It was added 7.5 mL of digestion buffer and procedure was applied to for remaining tissue again. Collected supernatants were let to settle the cells by gravity for 15 minutes. This step was repeated for 4-5 times. Afterwards, collected cells were resuspended in media and 7,500 cells/well were cultured on gelatine coated well plates. Cells were characterized using immunofluorescence staining method against to Troponin T.

Table 2.2. Reagents for isolation of adult mouse cardiomyocytes

| **Cardiomyocyte isolation buffer (CIB)** | |
|---|---|
| Component | 1X (g/L) |
| NaCl ($M_w$: 58.44 g/mole) | 7.013 |
| KCl ($M_w$: 74.55 g/mole) | 0.403 |
| $Na_2HPO_4.2H_2O$ ($M_w$: 177.99 g/mole) | 0.058 |
| $MgSO_4.7H_2O$ ($M_w$: 246.48 g/mole) | 0.123 |
| Taurine ($M_w$: 125.1 g/mole) | 3.753 |
| BDM ($M_w$: 101.1 g/mole) | 1.011 |
| HEPES ($M_w$: 238.3 g/mole) | 5.958 |

| | |
|---|---|
| Glucose ($M_w$: 180.16 g/mole) | 3.964 |
| Adjust the pH to 7.1 with NaOH. | |
| Adjust the volume to 1L with ultrapure water. | |
| 0.088 g of $Na_2HPO_4.2H_2O$ ($M_w$: 268.07 g/mole) can be used instead of $Na_2HPO_4.2H_2O$. | |
| **Perfusion Buffer (for 200ml)** | |
| CIB | 200 ml |
| EGTA (0.4M) | 200 µl |
| **Digestion Buffer (for 15ml)** | |
| CIB | 15ml |
| $CaCl_2$ (1M) | 4.5µl |
| Collagenase II | 36 mg |
| **Stop Buffer (for 15ml)** | |
| Perfusion buffer | 14.25 ml |
| FBS | 750 µl |
| $CaCl_2$ (1M) | 22.5 µl |
| **Culture media (for 100ml)** | |
| MEM media | 90ml |
| FBS | 10ml |
| Penicillin Streptomycin | 0.2 ml |

## 2.8 WESTERN BLOT ANALYSIS OF TISSUE SAMPLES

All the western blot equipment's and glasses were sterilized using %70 alcohol. %10 APS solution was prepared by dissolving 0.05 g of APS in 500 µl. Separating gel was prepared according to Table 2.3. Prepared gels were poured into cassette and was incubated for gel solidification. Afterwards, stacking gel was prepared by adding 0.65 ml %30 acrylamide, 1.25 ml 4x Tris-Cl/SDS pH 6.8, 3.05 ml distilled water, 0.05 ml %10 APS and 0.02 ml TEMED. The comb was placed and it was waited again for solidification of gel. Running buffer was prepared by diluting 10x running buffer into 1x. To prepare 10x running buffer, 15.14 g of Tris BASE and 72.06 g of Glycine and add 400 ml of distilled water were mixed. Samples diluted with Laemmli buffer were prepared with addition ß-mercaptoethanol. Samples were heated at 95°C for 5 minutes. After loading samples into the wells, gel was run at 70V for 10 minutes and voltage was increased to 110 V. Gel was run until staining was completely disappear. For transfer step, gel was stacked into whatman paper and nitrocellulose membrane. Afterwards, gel was transferred into nitrocellulose membrane on 350 mA currency for an hour in 1x transfer buffer. Blocking step was accomplished using %5 non-fat milk with 50 ml of 1X TBS-T and was incubated in this solution for 1.5 hour by shaking at 35 rpm. Primary antibody against putative regulators were incubated for over weekend at 4 °C. Then, primary antibody was removed ant was kept at -20°C for further use and membrane was washed for three times (15 minutes) with 1X TBS-T on the shaker. Second antibody against to primary antibody (Cell signaling, Cat No: 7074S) was incubated for 2 hours in 35 rpm shaker. After washing the membrane with 1X TBS-T for 3 times for 10 minutes in the shaker imaging step was performed using imaging solution kit according to manufacturer instructions (Cell signaling, Cat No:6883S).

Table 2.3. Reagents for preparation of western blot gels

| **Concentration (%)** | **6** | **7** | **7,5** | **8** | **9** | **10** | **12** |
|---|---|---|---|---|---|---|---|
| **%30 acrylamide** | 3 | 3,5 | 3,75 | 4 | 4,5 | 5 | 6 |
| **Tris-Cl/SDS pH 8.8** | 3,75 | 3,75 | 3,75 | 3,75 | 3,75 | 3,75 | 3,75 |
| **H2O** | 8,25 | 7,75 | 7,5 | 7,25 | 6,75 | 6,25 | 5,25 |

| **%10 APS** | 0,05 | 0,05 | 0,05 | 0,05 | 0,05 | 0,05 | 0,05 |
|---|---|---|---|---|---|---|---|
| **TEMED** | 0,02 | 0,02 | 0,02 | 0,02 | 0,02 | 0,02 | 0,02 |

## 2.9 STATISTICAL ANALYSIS

Results are expressed as mean ± SEM and a 2-tailed Student *t* test was used to determine the level of significance. $p<0.05$ was considered statistically different.

# 3. RESULTS

## 3.1. IMMUNOFLUORSCENCE DETECTION OF PUTATIVE REGULATORS CONFIRMED PROTEIN LEVEL IN ADULT HEART MYOCARDIUM

Immunofluorscence detection of putative regulators of cardiac regeneration using primary antibodies against Gfi1b (Growth factor independent 1B), Inpp5d (Inositol polyphosphate), ITCH (E3 ubiquitin ligase), Dnmt3a (DNA (cytosine-5)-methyltransferase 3A), c-myc (Myc protooncogene), Cbl (E3 protein ubiquitin ligase) and secondary antibodies for detection (Life techonolgies, Alexa Fluor 488 anti mouse; Anti-Mouse anti rabbit) were performed.

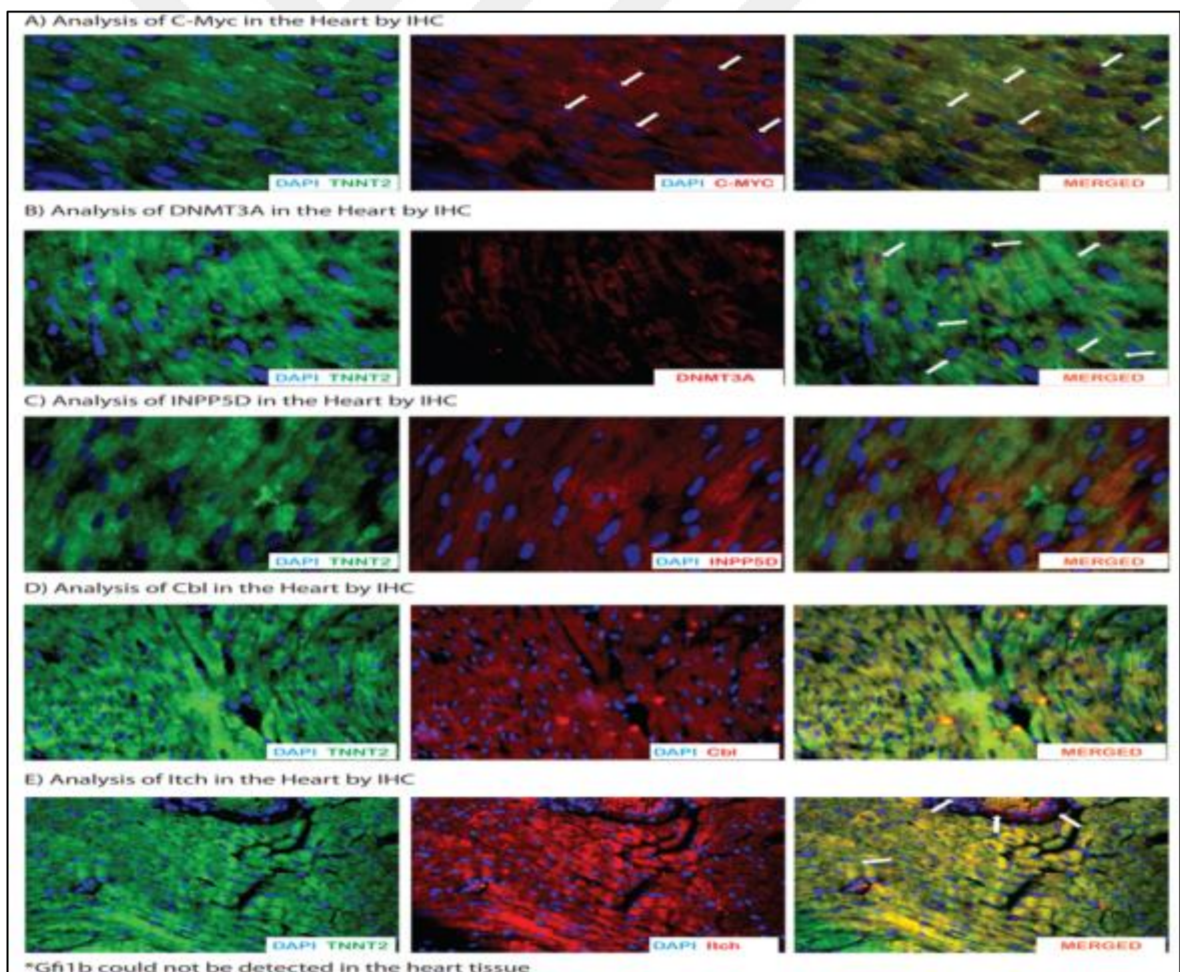



Figure 3.1. Detection of putative regulators of cardiomyocyte cell cycle modulators using immunofluorsence method. a) C-myc, b)Dnmt3a, c) INPP5D, d)Cbl, e) Itch immunostainings were shown.

The considarable level of protein of each putative regulators were observed in almost all samples expect in GFi1b. The findings indicated that these regulators could be important repressor regenerative capacity in adult myocardium (Figure 3.1).

### 3.2. PROFILE OF mRNA EXPRESSION IN CARDIOMYOCYTES AND FIBROBLAST REVEALED CARDIOMYOCYTE SPECIFIC REGULATORS

To identify cardiomyocyte specific putative regulator, relative mRNA expressions were measured and expression of putative regulators were analyzed. To this end, landgerdorf apparatus was utilized digest adult heart tissue which enabled to collect cells within heart (Figure A.1). Afterwards, RNA isolation and RT-PCR analysis were performed to identify mRNA expression level. Results indicated that  Itch, Dnmt3a, C-myc and Cbl were mostly expressed in cardiomycotes rather than fibroblast which also suggested that these regulators could have more important role in cardiomyocyte prolifration and could be cardiomyocyte specific genes.

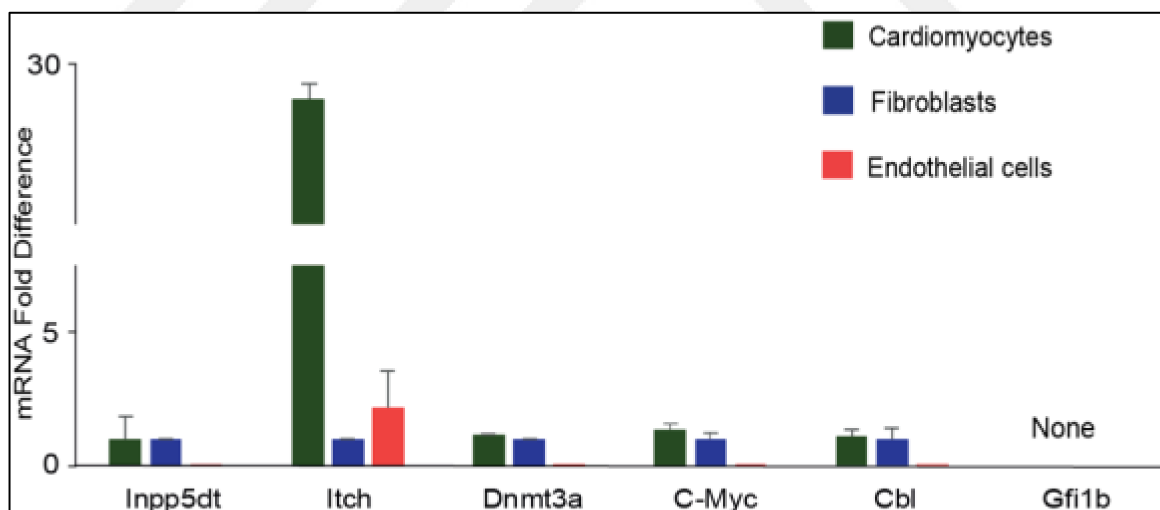



Figure 3.2. Differential expression of putative regulators in cardiomyocytes and fibroblast. Seperated cells utilized for RT-PCR to measure mRNA fold difference. ITCH, Dnmt3a, C-myc and CBL were mostly expressed in cardiomycotes.

Next, relative mRNA expressionprofile of putative regulators of cardiac regeneration were detected in whole adult mouse heart. To this end, RNA extraction and RT-PCR was utilized from homogenized adult heart myocardium and relative mRNA expression profile was detected using ΔΔCT method. (Figure 3.3). CBL was detected to have the highest mRNA expression in heart among the others. Since higher expression of CBL in cardiomycote was already detected in previous data (Figure 3.2), the highest global expression in heart tissue indicated its important role within the heart tissue.

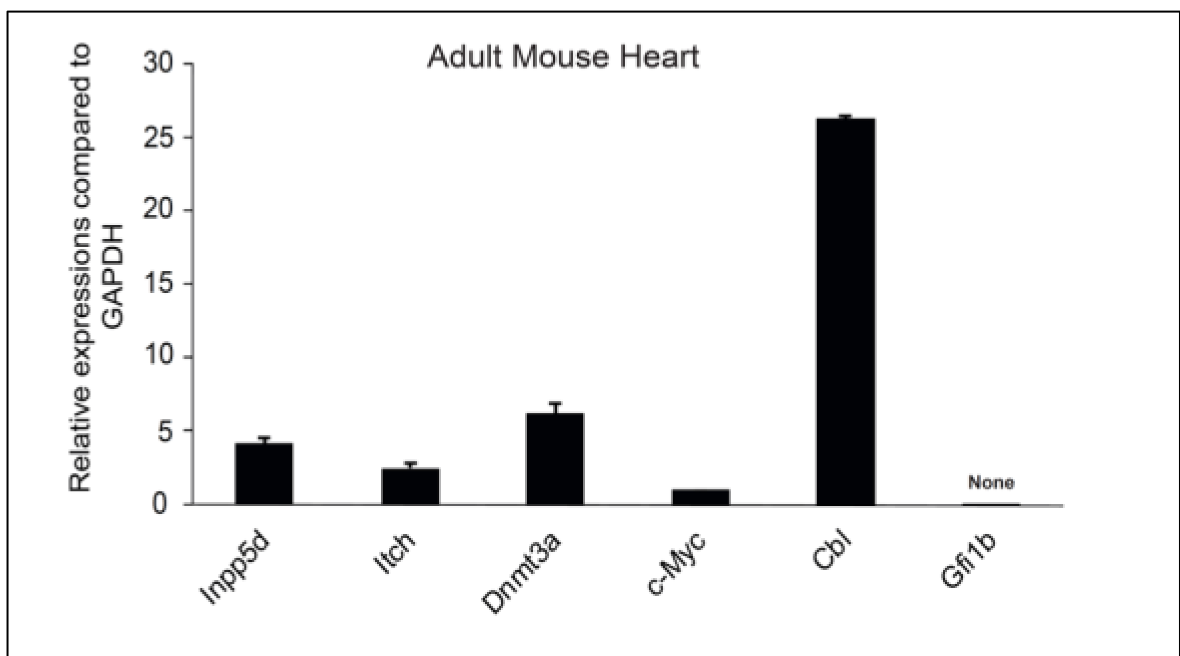



Figure 3.3. Relative mRNA expression of putative regulators in Adult mouse heart. CBL is mostly expressed among the other regulators in adult mouse heart.

Findings in RT-PCR utilized for cardiomycotes and fibroblast, and adult mouse heart indicated potential role of CBL in heart tissue (Figure 3.2 and Figure 3.3). The reveal the role of CBL in heart tissue in more deeply different strategies and experiments were designed to be utilized.

## 3.3. WESTERN BLOT ANALYSIS CORRELATED WITH RT-PCR AND INDICATED HIGH EXPRESSION OF CBL IN HEART

To identify protein level of putative regulators in different organs western blot method was utilised and detection of relative protein level of each regulators were identified (Figure 3.4.). Western blot analysis indicated that high expression of CBL, Dnmt3a and relatively high level of of c-myc in heart tissue.

Moreover, protein level of CBL in heart tissue was highest among the others which also correlates with previous RT-PCR data and enabled to confirm its expression both in RNA and protein level.

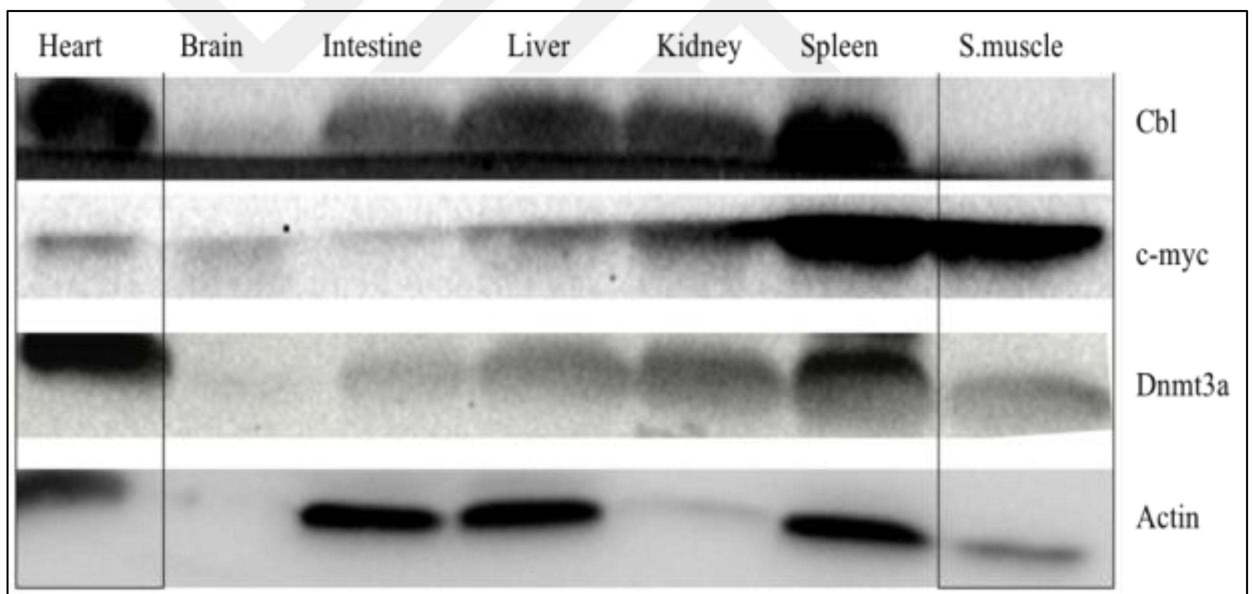



Figure 3.4. Western blot image of putative regulators in different tissue. High expression of Cbl, Dnmt3a and relatively highg expression of c-myc was shown

.

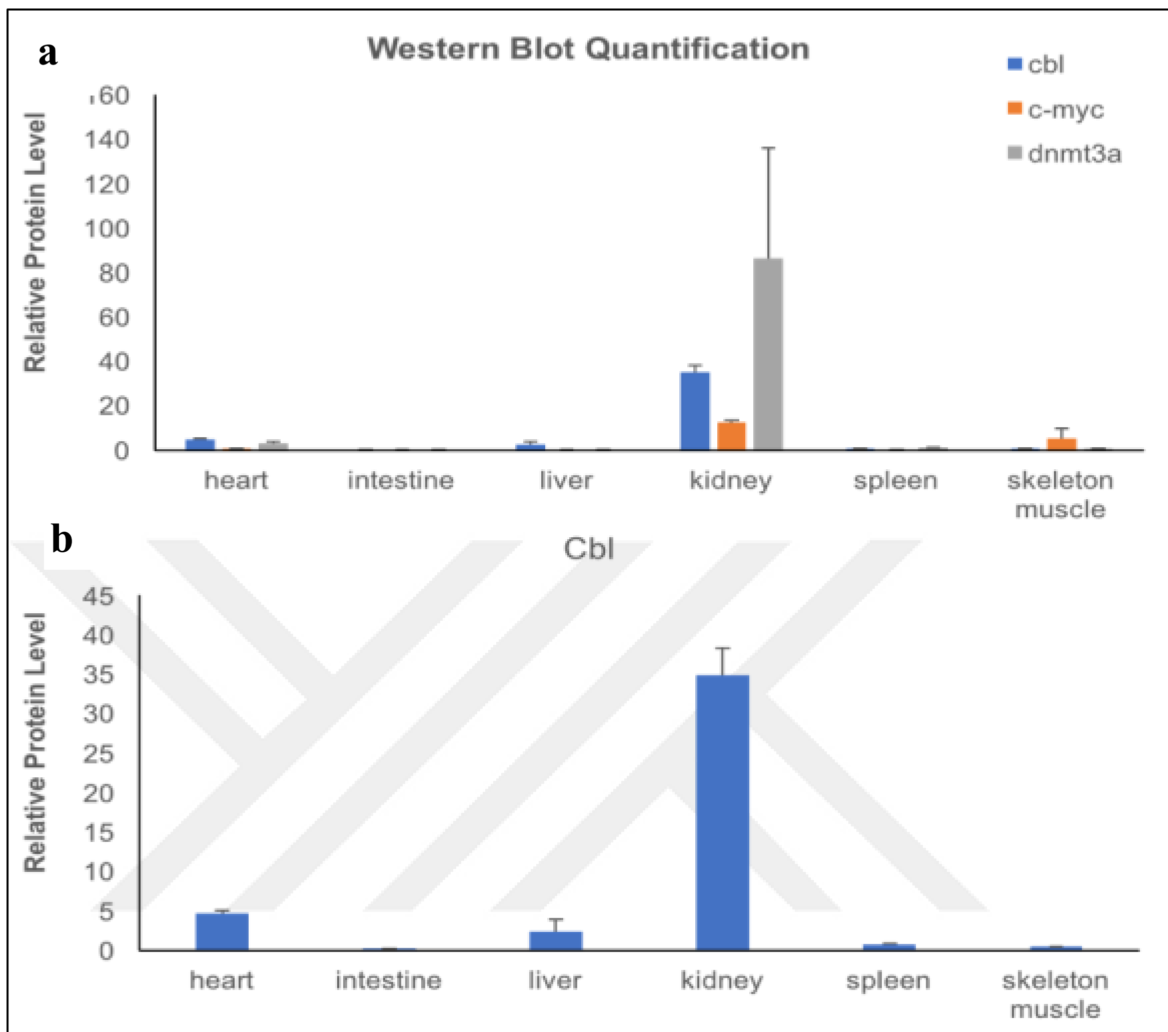



Figure 3.5. Quantification of Western Blot data. Indicating relative protein level in different tissueof adult mouse.

## 3.4. NEONATAL SURGERY MODEL ON NEONATAL MOUSE SHOWED NEGATIVE REGULATOR OF REGENERATIVE RESPONSE IN MOUSE

To test whether these regulators of neonatal mouse heart regeneration are negatively or positively affecting regenerative response of myocardium upon a injury, we performed apical resection and ligation LAD surgery model on neonatal mouse (Figure A.2). To this end, new born mice were anaesthesiad on ice bath and followed by lateral thoracotomy was

applied to open chest cavity which enabled to perform indicated surgery model.

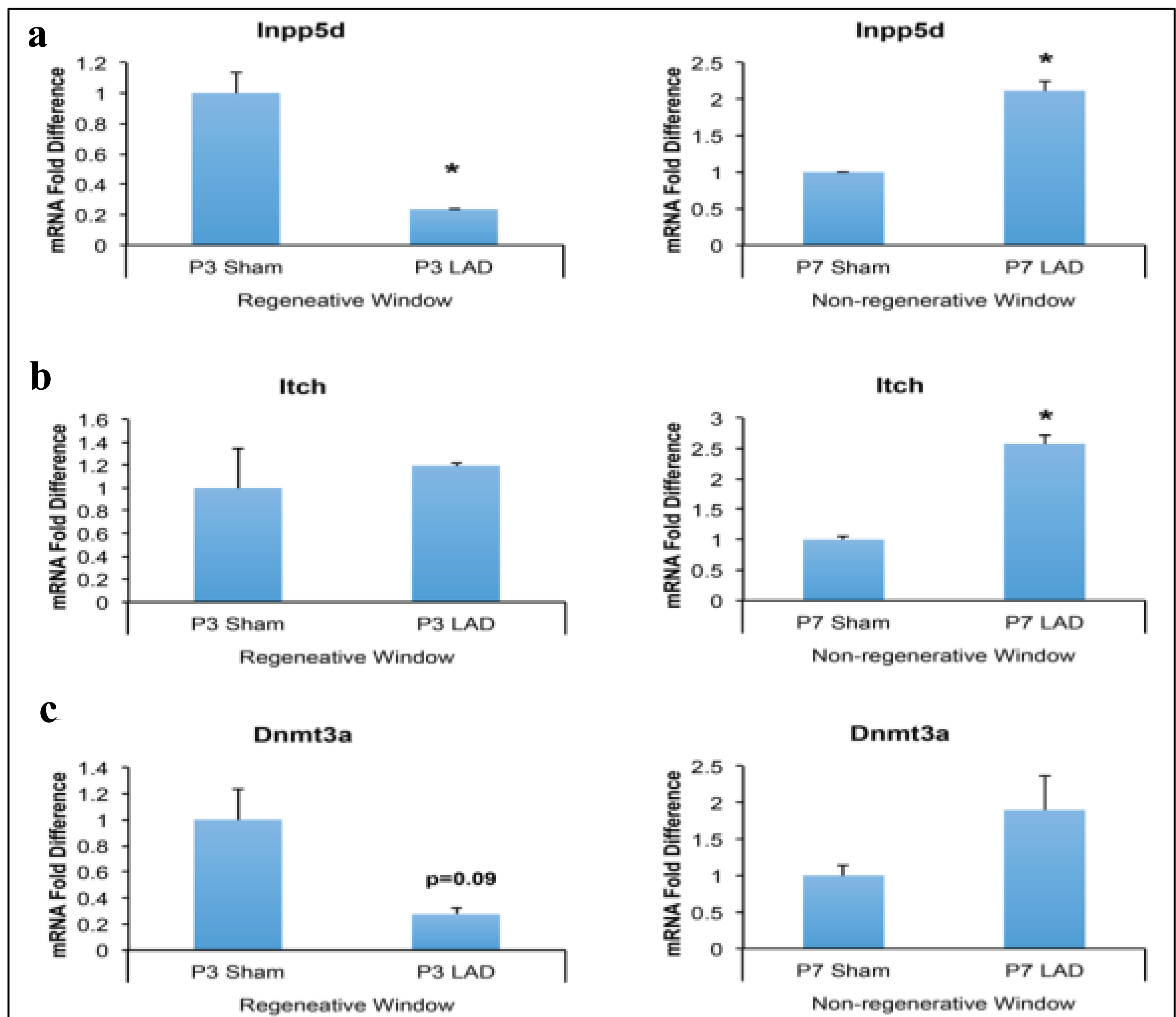



Figure 3.6. RT-PCR analysis of operated mouse at P3 and P7. Sham refers to control group. a)Expression profile of Inpp5d , b)Itch and c) Dnmt3a, * $p < 0.05$. n=3

All the surgery operation was carried on day 3 (p3) after birth and day7 (p7) in which myocardium is regenerative and non regenerative, respectively. Samples were collect after 4 days of neonatal surgery operation in order to analyse RNA and protein change in heart tissue. The surgery model enabled us to have regenerative myocardium between P3-P7 and non regenerative myocardium between P7-P11 which was used to identify negative and positive effect of these putative regulators (Figure 3.4 and Figure 3.5).

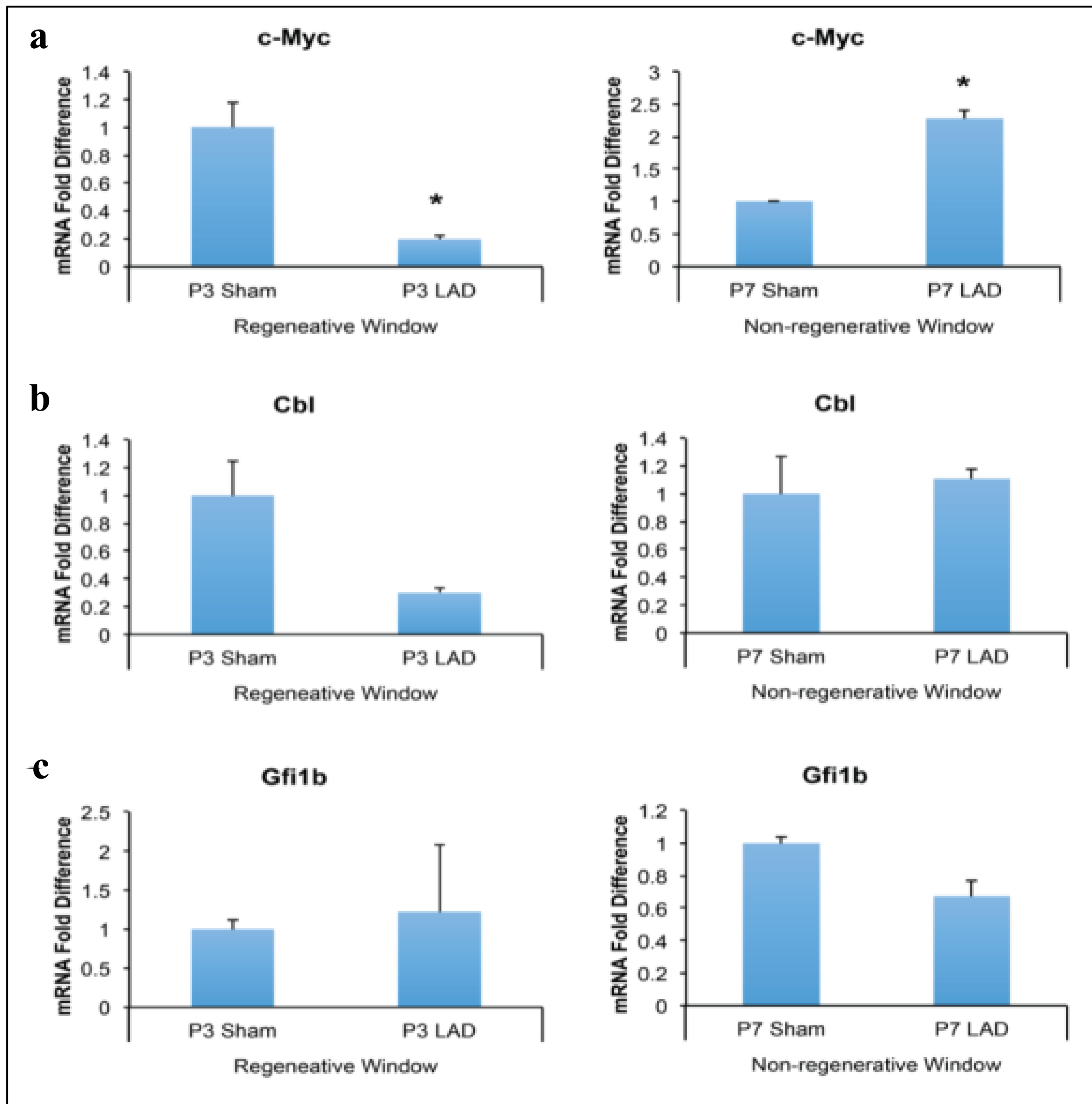



Figure 3.7. RT-PCR analysis of operated mouse at P3 and P7. Sham refers to control group. A)Expression profile of c-Myc , B)Cbl and C) Gfi1b, * $p < 0.05$. n=3

Depending RT-PCR data, downregulation and upregulation of putative regulators were shown (Figure 3.6 and Figure 3.7) and summarized (Figure 3.10). Since operated mouse at P3 have regenerative myocardium, down regulation in this sample suggested to increase this capacity trough negatively regulating proliferation of cells in heart. Moreover, up regulation of these putative regulators in operated mice at P7, confirm their negative effect of

regenerative response and cell proliferation since it was observed significant decrease in their mRNA expression (Figure 3.7.).

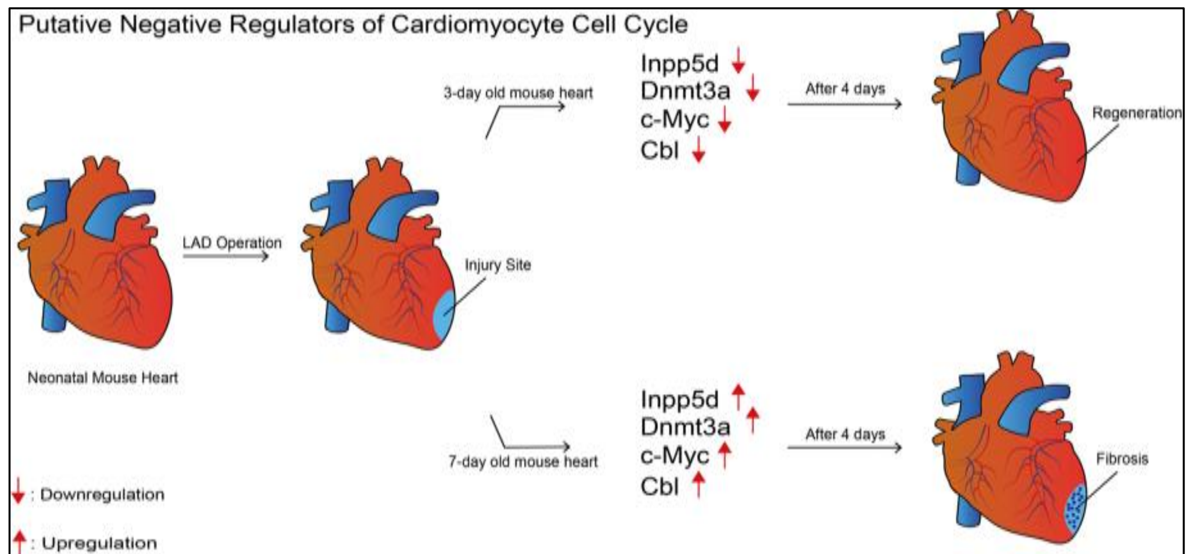



Figure 3.8. RT-PCR analysis summary for upregulation and downregulation of regulators. Inpp5d, Dnmt3a, c-Myc and Cbl down regulated in regenerative myocardium (operated at P3) and upregulated in non regenerative myocardium (operated at P7).

## 3.5. SIRNA KNOCKDOWN OF PUTATIVE REGULATORS INDICATED NEGATIVE EFFECT ON CARDIOMYOCYTE PROLIFERATION

To test effect of putative regulators on cardiomyocyte proliferation siRNA knockdown study was performed. To this end, cells were incubated with siRNAs according protocol mentioned. Following this, cells were fixed and immunofluorescence detection of proliferating cardiomycotes and fibroblast were done using Anti Tnnt and Anti-pH3 (mitosis marker) primary antibodies conjugated with Alexa Fluor 488 and Alexa Fluor 555 secondary antibodies (Figure 3.10). Cbl siRNA group showed a significant increase in cardiomyocyte proliferation. Moreover, ITCH and and Dnmt3a also showed an increase in cardiomyocyte proliferation (Figure 3.9A). In order to check effect of these knockdown on fibroblast, we also count proliferating fibroblast and observed that CBL also increased

proliferating fibroblasts, but not as much as in cardiomyocyte (Figure 3.9B). In the light of findings above and here, we confirmed a negative role of CBL, ITCH, and c-Myc in cardiomyocyte proliferation, thus neonatal mouse cardiac regeneration.

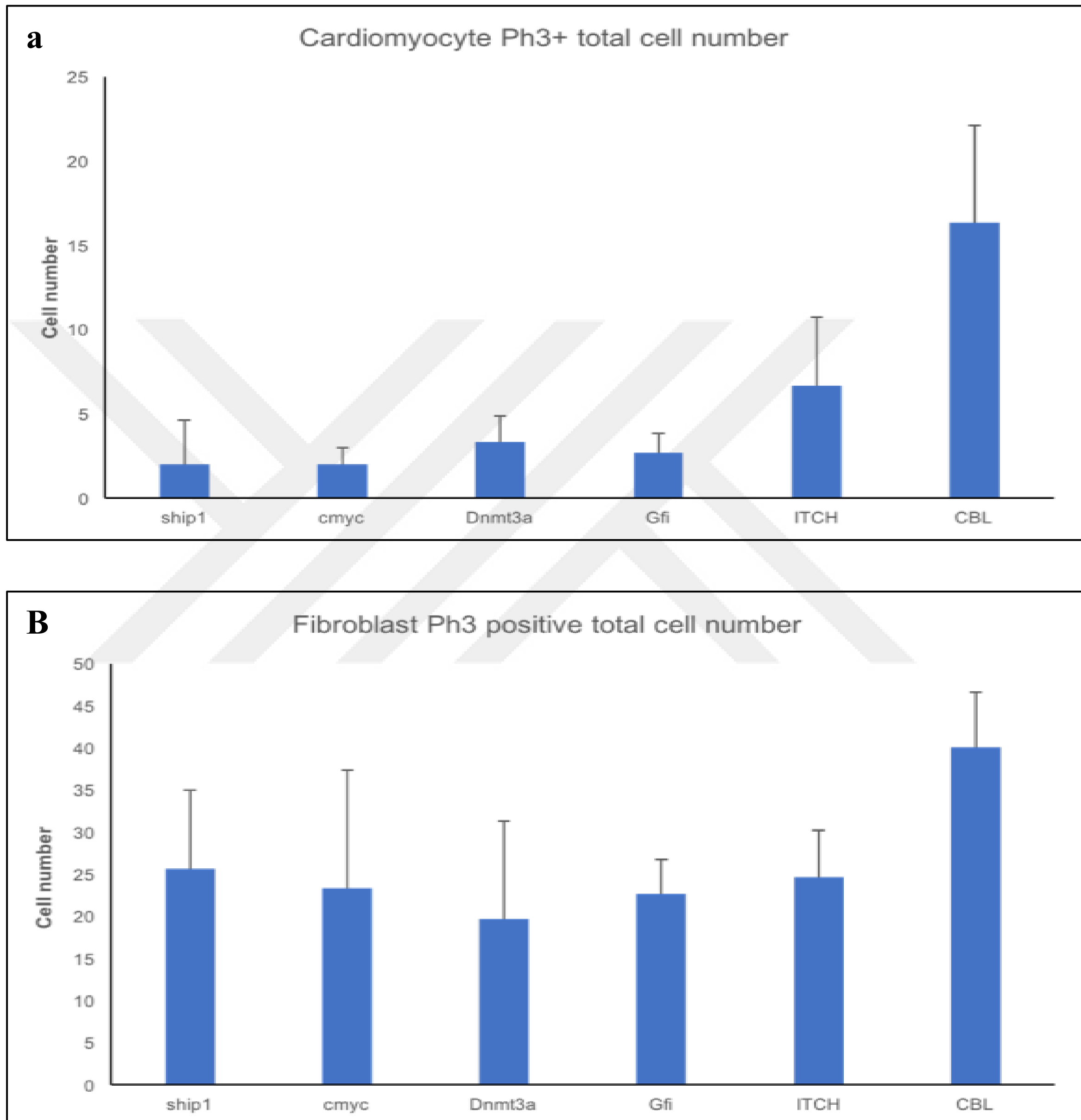



Figure 3. 9. Cell count after siRNA treatment. siRNA against to putative regulators of cardiac regeneration. A) Cardiomycocyte and Ph3 positive cells. B) Fibroblast and Ph3 positive cells.

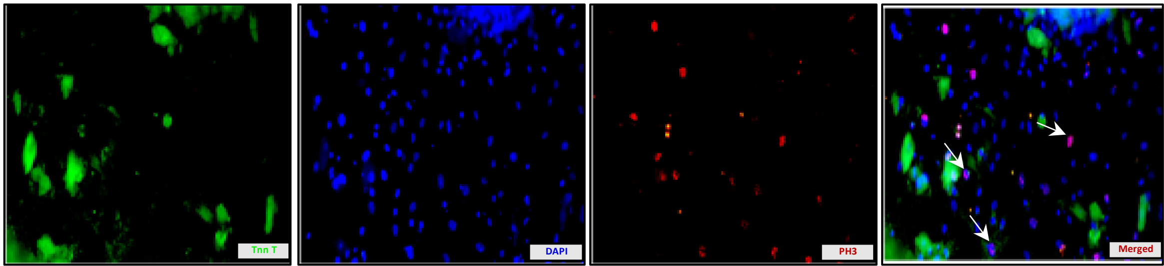



Figure 3.10. Representative image of immunofluorescence of neonatal rat cardiomyocytes under 40x magnification. Stained against Tnnt (cardiomyocyte marker) and pH3 (mitosis marker). a) Cardiomyocyte and pH3 positive cells. b) Fibroblast and pH3 positive cells

# 4. DISCUSSION

Recent studies in different animal models of cardiac injury shed light on the underlying mechanisms and prospects of heart regeneration. The presence of barriers to rejuvenate lost cardiomyocytes such as high levels of cell cycle inhibitors and fibrosis, and the lack of factors to stimulate cardiomyocyte proliferation and stem cell differentiation into cardiac cells following myocardial injuries are among the major issues. De novo CM proliferation and differentiation are thought to be a prospect for cardiac regeneration. Manipulations used for CM cell cycle modulation have yielded DNA synthesis, karyokinesis and cytokinesis in the heart to some extent [51, 56]

Inducible knockout systems used in adult mouse models further demonstrated that CM cell cycle re-entry may be achieved in adult mammalian heart. Discovery of small molecules that trigger and promote differentiation of stem cells into CMs and induce CM cell cycle re-entry brought further excitement for development of therapies targeting MI and HF [75, 137]. Overall, studies have proven feasibility of resident CMs and stem cell recruitment following therapeutic stimulation in heart regeneration.

CM renewal, for instance, has been documented in adult mammalian heart, albeit inadequate for restoration of cardiac function following cardiac injury [51, 52]. Cardiac regeneration in zebrafish, newt, and neonatal mouse is associated with reactivation of CM cell cycle. Discovery of CM cell cycle modulators provided a new platform for development of cardiovascular therapeutics targeting CM cell cycle. Use of different approaches including miRNA, small molecules or other factors to stimulate cardiac cell proliferation brings up questions regarding their utilization. More studies are needed to identify suitable strategies and targets to achieve neonatal mouse cardiac regeneration.

Cardiac regeneration in human have been reported in many different reports which enabled to this field to develop different strategies in order to find out novel factors important for human cardiac regeneration field [4, 57, 108]. To this end, role of many different regulators were suggested to be important in cardiac regeneration field [18, 75, 77, 87, 135]. The negative or positive effect of those regulators on cardiomycote prolfieration were reported in back to back report and enabled to develop robust therapeutical stratgeis against to heart failure. To this end, neonatal mouse surgery model was established which facilitate to

identify negative or positive effect of factors in mouse cardiac regeneration [20, 21]. Different surgery model were reported in last years and enabled to screen regulators using different model to reveal robust results[60]. One of the negative regulator of this response, Meis1, was identified using this model in the last year and promised for future therapeutical agent [75]. Mahmoud et al. (2013) identified role of Meis1, in that study, the negative role of Meis1 on cardiomycoyte proliferation was identified using LAD ligation surgery model and many different stratgies. The work presented here also utilize similar approach to idenfity the role of six different putative regulators of neonatal mouse cardiac regeneration. The differntial role of these candidates and suggested role cell proliferation (summarizd in Table A.1) were collected and included in this study in order to reveal their effect. Moreover, their mRNA expression and protein level was confirmed using different databases (BIOGPS, Human Protein Atlas) prior to research. The initial detection of these putative regulators were analyzed using immunofluorscence method on paraffin embedded sections derived from adult mouse heart organ. This enabled us to confirm databases and observe their expression in non regenerative myocardium (Figure 3.1). Following that, their mRNA expression in cardiomycote and fibroblast indicated that they could be important for both of cell type residing in the heart. Higher mRNA expression of ITCH, c-Myc, Cbl, and Dnmt3a in cardiomyocytes rather than fibroblast suggested that they could be cardiomyocyte specific gene and could specifically induce their proliferation.

To test, which one of those regulators are expressed in adult heart organ in mRNA level, we also utilized another RT-PCR analysis, and reported that Cbl is expressed highest among the others (Figure 3.3). These findings enabled us to analysis to measure relative mRNA level of those regulators and decide which one could be more important for neonatal mouse cardiac regeneration. In order to confirm their protein level as alternative to immunofluroscence method, western blot analysis was utilized to measure relative protein content of those regulators in different tissues of adult mouse. Similar to RT-PCR result, Cbl was expressed greater than other regulators in adult mouse heart tissue and confirmed by other experiments.

Next, neonatal mouse surgery model was utilized to identify downregulation or upregulation of those candidates upon an injury (Figure A.2). The method enabled us to observe change of mRNA level in regenerative (P3) and nonregenerative myocardium (P7) similar to conducted by Mahmoud et al. (2015) [75]. It was observed downregulation of Inpp5d,

Dnmt3a, c-Myc and Cbl after operation on P3 neonates, but upregulation on P7 neonates (Figure 3.8). This enabled us to suggest that, dowregulation of these regulators on P3 neonates which have regenerative myocardium enabled heart to increase regenerative response, and their upregulation on P7 neonatas which have non regenerative myocardium enabled heart to decrease regenerative response upon an injury. ). To test wheter this suggestion also work in vitro, siRNA knockdown method was applied to cultured neonatal rat cardiomyocytes. After sucesfull isolation of neonatal rat cardiomyocytes, siRNA treatment was applied to culture and cells were fixed at the day of 4 after culture (Figure 3.10.). Immunostaining againist to Tnnt and Ph3 (mitosis marker) was utilized and used to detect proliferating cardiomyocytes (Figure 3.10). Results indicated that Cbl, ITCH, and Dnmt3a siRNA treatment signifcanlty increased proliferating cardiomyocytes. Moreover, this increase was highest in Cbl group, which was also highly expressed regulator both in RNA and protein level.

Similar findings regarding Cbl and c-myc resulted with increase in cell proliferation was reported in another reports [138, 139]. The similarty of Hematopoeitic Stem Cells (HSCs) quiscence with cardiomyocyte cell cycle arrest could be directed into similar results in terms of cell cycle stimulation for Cbl [138]. In that study, knock out profile of Cbl in HSCs triggered cell cycle and increased population of HSCs existing in bone marrow [138]. Moreover, in another study, the knock out of cmyc resulted with decreased heart fibrosis which also correlates finding in this study [139]. Since decreased heart fibrosis could directly contribute to cardiac regeneration, the results obtained from neonatal heart surgery (Figure 3.7.) could be reason why decrease in cmyc expression enabled to heart to regenerate [139]. Similar to cmyc and cbl, ITCH showed a negative effect on neonatal mouse cardiac regeneration and its siRNA knockdown showed a considarable increase in proliferating cardiomycocytes. Moreover, ITCH, E3 ubiquitin ligase, was suggested to be important negative regulator of homeostasis and function of HSCs in a recent study which also correlates with our findings and indicate dual effect of ITCH on two different quiscent cells [141].

Taking all findings into consideration, cmyc, ITCH, and Cbl indicate a potential role in the mouse cardiac regeneration and promise as future therapeuticall agents. In this study indicated role of putative regulators on neonatal mouse cardiac regeneration. Previous reports regarding their role and importance in many different pathways also affect cardiac

regeneration in neonatal mouse. Their negative effect on cardiomyocyte proliferation and cardiac regenerative response could be a valuable targetting strategy to develop therapies againist cardiovascular diseases.

# REFERENCES

1. Jessup M, Abraham WT, Casey DE, Feldman AM, Francis GS, Ganiats TG, et al. 2009 focused update: ACCF/AHA Guidelines for the Diagnosis and Management of Heart Failure in Adults: a report of the American College of Cardiology Foundation/American Heart Association Task Force on Practice Guidelines: developed in collaboration with the International Society for Heart and Lung Transplantation. Circ Res.119(14):1977-16.2009

2. Jessup M, Brozena S. Heart failure. N Engl J Med.348(20):2007-18.2003

3. Bergmann O, Bhardwaj RD, Bernard S, Zdunek S, Barnabé-Heider F, Walsh S, et al. Evidence for cardiomyocyte renewal in humans. Science.324:98-102.2009

4. Bergmann O, Zdunek S, Frisén J, Bernard S, Druid H, Jovinge S. Cardiomyocyte Renewal in Humans. Circ Res.110:e17-e8.2012

5. Kajstura J, Urbanek K, Perl S, Hosoda T, Zheng H, Ogórek B, et al. Cardiomyogenesis in the adult human heart. Circ Res.107:305-15.2010

6. Laflamme MA, Myerson D, Saffitz JE, Murry CE. Evidence for cardiomyocyte repopulation by extracardiac progenitors in transplanted human hearts. Circ Res.90:634-40.2002

7. Jopling C, Sleep E, Raya M, Martí M, Raya A, Belmonte JCI. Zebrafish heart regeneration occurs by cardiomyocyte dedifferentiation and proliferation. Nature.464:606-9.2010

8. Kikuchi K, Holdway JE, Werdich AA, Anderson RM, Fang Y, Egnaczyk GF, et al. Primary contribution to zebrafish heart regeneration by gata4(+) cardiomyocytes. Nature.464:601-5.2010

9. Orlic D, Kajstura J, Chimenti S, Jakoniuk I, Anderson SM, Li B, et al. Bone marrow cells regenerate infarcted myocardium. Nature.410(6829):701-5.2001

10. Beltrami AP, Barlucchi L, Torella D, Baker M, Limana F, Chimenti S, et al. Adult cardiac stem cells are multipotent and support myocardial regeneration. Cell.114(6):763-76.2003

11. Rumyantsev PP. Post-injury DNA synthesis, mitosis and ultrastructural reorganization of adult frog cardiac myocytes. An electron microscopic-autoradiographic study. Z Zellforsch Mik Ana.139:431-50.1973

12. Rumyantsev PP. Autoradiographic study on the synthesis of DNA, RNA, and proteins in normal cardiac muscle cells and those changed by experimental injury. Folia Histochem Cytochem.4:397-424.1966

13. Sulima VI. On the regeneration of the myocardium in various injuries to the cardiac wall of reptiles. Arkhiv anatomii, gistologii i émbriologii.55:56-63.1968

14. Witman N, Murtuza B, Davis B, Arner A, Morrison JI. Recapitulation of developmental cardiogenesis governs the morphological and functional regeneration of adult newt hearts following injury. Dev Biol.354:67-76.2011

15. Poss KD, Wilson LG, Keating MT. Heart Regeneration in Zebrafish. Science.298:2188-90.2002

16. Sander V, Davidson AJ. Kidney injury and regeneration in zebrafish. Sem Nephrol.34:437-44.2014

17. Becker CG, Becker T. Adult zebrafish as a model for successful central nervous system regeneration. Restor Neurol Neurosci.26:71-80.2008

18. Gemberling M, Karra R, Dickson AL, Poss KD. Nrg1 is an injury-induced cardiomyocyte mitogen for the endogenous heart regeneration program in zebrafish. eLife.4:e05871.2015

19. Wills AA, Holdway JE, Major RJ, Poss KD. Regulated addition of new myocardial and epicardial cells fosters homeostatic cardiac growth and maintenance in adult zebrafish. Development.135(1):183-92.2008

20. Mahmoud AI, Porrello ER, Kimura W, Olson EN, Sadek HA. Surgical models for cardiac regeneration in neonatal mice. Nat Protoc.9:305-11.2014

21. Porrello ER, Mahmoud AI, Simpson E, Hill JA, Richardson JA, Olson EN, et al. Transient regenerative potential of the neonatal mouse heart. Science.331:1078-80.2011

22. Flink IL. Cell cycle reentry of ventricular and atrial cardiomyocytes and cells within the epicardium following amputation of the ventricular apex in the axolotl, Amblystoma mexicanum: confocal microscopic immunofluorescent image analysis of bromodeoxyuridine-label. Anatomy and embryology.205:235-44.2002

23. Neff AW, Dent AE, Armstrong JB. Heart development and regeneration in urodeles. Int J Dev Biol.40:719-25.1996

24. Oberpriller JO, Oberpriller JC. Response of the adult newt ventricle to injury. J Exp Zool.187:249-53.1974

25. Raya A, Koth CM, Büscher D, Kawakami Y, Itoh T, Raya RM, et al. Activation of Notch signaling pathway precedes heart regeneration in zebrafish. Proc Natl Acad Sci U S A.100 Suppl:11889-95.2003

26. Pelster B, Burggren WW. Disruption of hemoglobin oxygen transport does not impact oxygen-dependent physiological processes in developing embryos of zebra fish (Danio rerio). Circ Res.79:358-62.1996

27. Wang J, Panáková D, Kikuchi K, Holdway JE. The regenerative capacity of zebrafish reverses cardiac failure caused by genetic cardiomyocyte depletion. Development.138(16).2011

28. González-Rosa JM, Martín V, Peralta M, Torres M, Mercader N. Extensive scar formation and regression during heart regeneration after cryoinjury in zebrafish. Development 138:1663-74.2011

29. Chablais F, Veit J, Rainer G, Jaźwińska A. The zebrafish heart regenerates after cryoinjury-induced myocardial infarction. Bmc Dev Biol.11:21.2011

30. Lepilina A, Coon AN, Kikuchi K, Holdway JE, Roberts RW, Burns CG, et al. A dynamic epicardial injury response supports progenitor cell activity during zebrafish heart regeneration. Cell.127:607-19.2006

31. Molkentin JD, Lin Q, Duncan SA, Olson EN. Requirement of the transcription factor GATA4 for heart tube formation and ventral morphogenesis. Genes Dev.11:1061-72.1997

32. Pu WT, Ishiwata T, Juraszek AL, Ma Q, Izumo S. GATA4 is a dosage-sensitive regulator of cardiac morphogenesis. Dev Biol.275:235-44.2004

33. Zeisberg EM, Ma Q, Juraszek AL, Moses K, Schwartz RJ, Izumo S, et al. Morphogenesis of the right ventricle requires myocardial expression of Gata4. J Clin Invest.115:1522-531.2005

34. Singh BN, Koyano-Nakagawa N, Garry JP, Weaver CV. Heart of newt: a recipe for regeneration. J Cardiovasc Transl Res.3(4):397-409.2010

35. Bader D, Oberpriller J. Autoradiographic and electron microscopic studies of minced cardiac muscle regeneration in the adult newt,Notophthalmus viridescens. J Exp Zool.208:177-93.1979

36. Borchardt T, Braun T. Cardiovascular regeneration in non-mammalian model systems: what are the differences between newts and man? Thromb Haemost.98(2):311-8.2007

37. Mercer SE, Odelberg SJ, Simon H-GG. A dynamic spatiotemporal extracellular matrix facilitates epicardial-mediated vertebrate heart regeneration. Dev Biol.382:457-69.2013

38. Piatkowski T, Mühlfeld C, Borchardt T, Braun T. Reconstitution of the myocardium in regenerating newt hearts is preceded by transient deposition of extracellular matrix components. Stem Cells Dev.22:1921-931.2013

39. Mollova M, Bersell K, Walsh S, Savla J, Das LT, Park S-YY, et al. Cardiomyocyte proliferation contributes to heart growth in young humans. P Natl Acad Sci Usa.110:1446-51.2013

40. Quaini F, Urbanek K, Beltrami AP, Finato N, Beltrami CA, Nadal-Ginard B, et al. Chimerism of the transplanted heart. N Engl J Med.346(1):5-15.2002

41. Li RK, Jia ZQ, Weisel RD, Mickle DA, Zhang J, Mohabeer MK, et al. Cardiomyocyte transplantation improves heart function. Ann Thorac Surg.62(3):654-60; discussion 60-1.1996

42. Laflamme MA, Chen KY, Naumova AV, Muskheli V, Fugate JA, Dupras SK, et al. Cardiomyocytes derived from human embryonic stem cells in pro-survival factors enhance function of infarcted rat hearts. Nat Biotechnol.25(9):1015-24.2007

43. Caspi O, Huber I, Kehat I, Habib M, Arbel G, Gepstein A, et al. Transplantation of human embryonic stem cell-derived cardiomyocytes improves myocardial performance in infarcted rat hearts. J Am Coll Cardiol.50(19):1884-93.2007

44. Shiba Y, Fernandes S, Zhu WZ, Filice D, Muskheli V, Kim J, et al. Human ES-cell-derived cardiomyocytes electrically couple and suppress arrhythmias in injured hearts. Nature.489(7415):322-5.2012

45. Puente BN, Kimura W, Muralidhar SA, Moon J, Amatruda JF, Phelps KL, et al. The oxygen-rich postnatal environment induces cardiomyocyte cell-cycle arrest through DNA damage response. Cell.157:565-79.2014

46. Canseco DC, Kimura W, Garg S, Mukherjee S, Bhattacharya S, Abdisalaam S, et al. Human ventricular unloading induces cardiomyocyte proliferation. J Am Coll Cardiol.65:892-900.2015

47. Li F, Wang X, Capasso JM, Gerdes AM. Rapid transition of cardiac myocytes from hyperplasia to hypertrophy during postnatal development. J Mol Cell Cardiol.28:1737-46.1996

48. Soonpaa MH, Kim KK, Pajak L, Franklin M, Field LJ. Cardiomyocyte DNA synthesis and binucleation during murine development. Am J Physiol.271:9.1996

49. Walsh S, Pontén A, Fleischmann BK, Jovinge S. Cardiomyocyte cell cycle control and growth estimation in vivo--an analysis based on cardiomyocyte nuclei. Cardiovasc Res.86:365-73.2010

50. Fishman MC, Olson EN. Parsing the heart: genetic modules for organ assembly. Cell.91 153-6.1997

51. Senyo SE, Steinhauser ML, Pizzimenti CL, Yang VK. Mammalian heart renewal by pre-existing cardiomyocytes. Nature.493(7432):433-36.2013

52. Malliaras K, Zhang Y, Seinfeld J, Galang G, Tseliou E, Cheng K, et al. Cardiomyocyte proliferation and progenitor cell recruitment underlie therapeutic regeneration after myocardial infarction in the adult mouse heart. EMBO Mol Med.5:191-209.2013

53. Senyo SE, Steinhauser ML, Pizzimenti CL, Yang VK, Cai L, Wang M, et al. Mammalian heart renewal by pre-existing cardiomyocytes. Nature.493:433-6.2013

54. Soonpaa MH, Field LJ. Assessment of cardiomyocyte DNA synthesis in normal and injured adult mouse hearts. Am J Physiol.272:6.1997

55. Soonpaa MH, Koh GY, Pajak L, Jing S, Wang H, Franklin MT, et al. Cyclin D1 overexpression promotes cardiomyocyte DNA synthesis and multinucleation in transgenic mice. J Clin Invest.99(11):2644-54.1997

56. Bergmann O, Zdunek S, Frisén J, Bernard S. Cardiomyocyte renewal in humans. Circ Res.324:98-102.2012

57. Laflamme MA, Murry CE. Heart regeneration. Nature.473:326-35.2011

58. Hosoda T, D'Amario D, Cabral-Da-Silva MC, Zheng H, Padin-Iruegas ME, Ogorek B, et al. Clonality of mouse and human cardiomyogenesis in vivo. Proc Natl Acad Sci U S A.106:17169-174.2009

59. Porrello ER, Mahmoud AI, Simpson E, Johnson BA, Grinsfelder D, Canseco D, et al. Regulation of neonatal and adult mammalian heart regeneration by the miR-15 family. P Natl Acad Sci Usa.110:187-92.2013

60. Darehzereshki A, Rubin N, Gamba L, Kim J. Differential Regenerative Capacity of Neonatal Mouse Hearts after Cryoinjury. Dev Biol.399:91-9.2014

61. Orlic D, Kajstura J, Chimenti S, Bodine DM, Leri A, Anversa P. Bone marrow stem cells regenerate infarcted myocardium. Pediatr Transplant.7-Suppl 3:86-8.2003

62. Yeh ET, Zhang S, Wu HD, Körbling M, Willerson JT, Estrov Z. Transdifferentiation of human peripheral blood CD34+-enriched cell population into cardiomyocytes, endothelial cells, and smooth muscle cells in vivo. Circulation.108:2070-3.2003

63. Bicknell Katrina A, Coxon Carmen H, Brooks G. Forced expression of the cyclin B1–CDC2 complex induces proliferation in adult rat cardiomyocytes. Biochem J.382:411-16.2004

64. Cheng RK, Asai T, Tang H, Dashoush NH, Kara RJ, Costa KD, et al. Cyclin A2 Induces Cardiac Regeneration After Myocardial Infarction and Prevents Heart Failure. Circ Res.100:1741-48.2007

65. Jackson T, Allard MF, Sreenan CM. The c-myc proto-oncogene regulates cardiac development in transgenic mice. Mol Cell Biol.10(7):3709-716.1990

66. Pasumarthi KBS, Nakajima H, Nakajima HO, Soonpaa MH, Field LJ. Targeted Expression of Cyclin D2 Results in Cardiomyocyte DNA Synthesis and Infarct Regression in Transgenic Mice. Circ Res.96:110-8.2005

67. Liu Y, Kitsis RN. Induction of DNA synthesis and apoptosis in cardiac myocytes by E1A oncoprotein. J Cell Biol.133:325-34.1996

68. Chaudhry HW, Dashoush NH, Tang H, Zhang L. Cyclin A2 mediates cardiomyocyte mitosis in the postmitotic myocardium. J Biol Chem.279(34).2004

69. Di Stefano V, Giacca M, Capogrossi MC, Crescenzi M, Martelli F. Knockdown of cyclin-dependent kinase inhibitors induces cardiomyocyte re-entry in the cell cycle. J Biol Chem.286(10):8644-654.2011

70. Agah R, Kirshenbaum LA, Abdellatif M, Truong LD, Chakraborty S, Michael LH, et al. Adenoviral delivery of E2F-1 directs cell cycle reentry and p53-independent apoptosis in postmitotic adult myocardium in vivo. TL - 100. The Journal of clinical investigation.100 VN -:2722-8.1997

71. Bersell K, Arab S, Haring B, Kühn B. Neuregulin1/ErbB4 signaling induces cardiomyocyte proliferation and repair of heart injury. Cell.138:257-70.2009

72. Kühn B, Monte Fd, Hajjar RJ, Chang Y-S, Lebeche D, Arab S, et al. Periostin induces proliferation of differentiated cardiomyocytes and promotes cardiac repair. Nat Med.13:962-9.2007

73. Rochais F, Sturny R, Chao CM. FGF10 promotes regional foetal cardiomyocyte proliferation and adult cardiomyocyte cell-cycle re-entry. Cardiovasc Res.104:432-42.2014

74. Tanaka M, Lörchner H, Schimanski S, Szibor M. Oncostatin M is a major mediator of cardiomyocyte dedifferentiation and remodeling. Cell Stem Cell.9(5).2011

75. Mahmoud AI, Kocabas F, Muralidhar SA, Kimura W, Koura AS, Thet S, et al. Meis1 regulates postnatal cardiomyocyte cell cycle arrest. Nature.497:249-53.2013

76. Xin M, Olson EN, Bassel-Duby R. Mending broken hearts: cardiac development as a basis for adult heart regeneration and repair. Nat Rev Mol Cell Bio.14:529-41.2013

77. D'Uva G, Aharonov A, Lauriola M, Kain D, Yahalom-Ronen Y, Carvalho S, et al. ERBB2 triggers mammalian heart regeneration by promoting cardiomyocyte dedifferentiation and proliferation. Nat Cell Biol.5:627-38.2015

78. Gassmann M, Casagranda F, Orioli D, Simon H, Lai C, Klein R, et al. Aberrant neural and cardiac development in mice lacking the ErbB4 neuregulin receptor. Nature.378:390-4.1995

79. Lai D, Liu X, Forrai A, Wolstein O, Michalicek J, Ahmed I, et al. Neuregulin 1 sustains the gene regulatory network in both trabecular and nontrabecular myocardium. Circ Res.107:715-27.2010

80. Lee KF, Simon H, Chen H, Bates B, Hung MC, Hauser C. Requirement for neuregulin receptor erbB2 in neural and cardiac development. Nature.378:394-8.1995

81. Liu J, Bressan M, Hassel D, Huisken J, Staudt D, Kikuchi K, et al. A dual role for ErbB2 signaling in cardiac trabeculation. Development.137:3867-75.2010

82. Engel FB, Hsieh PCH, Lee RT. FGF1/p38 MAP kinase inhibitor therapy induces cardiomyocyte mitosis, reduces scarring, and rescues function after myocardial infarction. Proc Natl Acad Sci U S A.103 (42):5546–51.2006

83. Kubin T, Pöling J, Kostin S, Gajawada P, Hein S, Rees W, et al. Oncostatin M Is a Major Mediator of Cardiomyocyte Dedifferentiation and Remodeling. Cell Stem Cell.9.2011

84. Novoyatleva T, Diehl F, Amerongen MJv, Patra C, Ferrazzi F, Bellazzi R, et al. TWEAK is a positive regulator of cardiomyocyte proliferation. Cardiovasc Res.2010

85. Sdek P, Zhao P, Wang Y, Huang C-JJ, Ko CY, Butler PC, et al. Rb and p130 control cell cycle gene silencing to maintain the postmitotic phenotype in cardiac myocytes. J Cell Biol.194 (3):407-23.2011

86. Halder G, Johnson RL. Hippo signaling: growth control and beyond. Development.138:9-22.2011

87. Xiao F, Kimura W, Sadek HA. A hippo "AKT" regulates cardiomyocyte proliferation. Circ Res.116:3-5.2015

88. Heallen T, Zhang M, Wang J, Bonilla-Claudio M, Klysik E, Johnson RL, et al. Hippo pathway inhibits Wnt signaling to restrain cardiomyocyte proliferation and heart size. Science.332:458-61.2011

89. von Gise A, Lin Z, Schlegelmilch K, Honor LB, Pan GM, Buck JN, et al. YAP1, the nuclear target of Hippo signaling, stimulates heart growth through cardiomyocyte proliferation but not hypertrophy. P Natl Acad Sci Usa.109:2394-9.2012

90. Xin M, Kim Y, Sutherland LB, Qi X, McAnally J, Schwartz RJ, et al. Regulation of insulin-like growth factor signaling by Yap governs cardiomyocyte proliferation and embryonic heart size. Sci Signal.4.2011

91. Kumar A, Brockes JP. Nerve dependence in tissue, organ, and appendage regeneration. Trends Neurosci.35(11):691-9.2012

92. Mahmoud AI, O'Meara CC, Gemberling M, Zhao L, Bryant DM, Zheng R, et al. Nerves Regulate Cardiomyocyte Proliferation and Heart Regeneration. Dev Cell.34(4):387-99.2015

93. Kehat I, Kenyagin-Karsenti D, Snir M, Segev H, Amit M, Gepstein A, et al. Human embryonic stem cells can differentiate into myocytes with structural and functional properties of cardiomyocytes. J Clin Invest.108:407-14.2001

94. Takahashi K, Tanabe K, Ohnuki M, Narita M, Ichisaka T, Tomoda K, et al. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell.131:861-72.2007

95. Takahashi K, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell.126:663-76.2006

96. Hossini AM, Megges M, Prigione A, Lichtner B, Toliat MR, Wruck W, et al. Induced pluripotent stem cell-derived neuronal cells from a sporadic Alzheimer's disease donor as a model for investigating AD-associated gene regulatory networks. BMC genomics.16:84.2015

97. Lowry WE, Richter L, Yachechko R, Pyle AD, Tchieu J, Sridharan R, et al. Generation of human induced pluripotent stem cells from dermal fibroblasts. P Natl Acad Sci Usa.105(8):2883-888.2008

98. Gai H, Leung EL, Costantino PD, Aguila JR, Nguyen DM, Fink LM, et al. Generation and characterization of functional cardiomyocytes using induced

pluripotent stem cells derived from human fibroblasts. Cell Biol Int.33(11):1184-93.2009

99. Ieda M, Fu J-DD, Delgado-Olguin P, Vedantham V, Hayashi Y, Bruneau BG, et al. Direct reprogramming of fibroblasts into functional cardiomyocytes by defined factors. Cell.142:375-86.2010

100. Nelson TJ, Martinez-Fernandez A, Yamada S, Perez-Terzic C, Ikeda Y, Terzic A. Repair of acute myocardial infarction by human stemness factors induced pluripotent stem cells. Circulation.120:408-16.2009

101. Zwi L, Caspi O, Arbel G, Huber I, Gepstein A, Park IH, et al. Cardiomyocyte differentiation of human induced pluripotent stem cells. Circulation.120(15):1513-23.2009

102. Menasché P. Skeletal muscle satellite cell transplantation. Cardiovasc Res.58:351-7.2003

103. Taylor DA, Atkins BZ, Hungspreugs P, Jones TR, Reedy MC, Hutcheson KA, et al. Regenerating functional myocardium: improved performance after skeletal myoblast transplantation. Nat Med.4(8):929-33.1998

104. Pouly J, Hagège AA, Vilquin J-TT, Bissery A, Rouche A, Bruneval P, et al. Does the functional efficacy of skeletal myoblast transplantation extend to nonischemic cardiomyopathy? Circulation.110:1626-31.2004

105. Reinecke H, Poppa V, Murry CE. Skeletal muscle stem cells do not transdifferentiate into cardiomyocytes after cardiac grafting. J Mol Cell Cardiol.34:241-9.2002

106. Leobon B, Garcin I, Menasche P, Vilquin J-TT, Audinat E, Charpak S. Myoblasts transplanted into rat infarcted myocardium are functionally isolated from their host. Proc Natl Acad Sci U S A.100:7808-11.2003

107. Murry CE, Wiseman RW, Schwartz SM, Hauschka SD. Skeletal myoblast transplantation for repair of myocardial necrosis. J Clin Invest.98:2512-523.1996

108. Bernstein HS, Srivastava D. Stem cell therapy for cardiac disease. Pediatr Res.71(4 Pt 2):491-9.2012

109. Chong JJ, Chandrakanthan V, Xaymardan M, Asli NS, Li J, Ahmed I, et al. Adult cardiac-resident MSC-like stem cells with a proepicardial origin. Cell Stem Cell.9(6):527-40.2011

110. Messina E, De Angelis L, Frati G, Morrone S, Chimenti S, Fiordaliso F, et al. Isolation and expansion of adult cardiac stem cells from human and murine heart. Circ Res.95(9):911-21.2004

111. Bu L, Jiang X, Martin-Puig S, Caron L, Zhu S, Shao Y, et al. Human ISL1 heart progenitors generate diverse multipotent cardiovascular cell lineages. Nature.460(7251):113-17.2009

112. Oh H, Bradfute SB, Gallardo TD, Nakamura T, Gaussin V, Mishina Y, et al. Cardiac progenitor cells from adult myocardium: homing, differentiation, and fusion after infarction. Proc Natl Acad Sci U S A.100(21):12313-8.2003

113. Smith RR, Barile L, Cho HC, Leppo MK, Hare JM, Messina E, et al. Regenerative potential of cardiosphere-derived cells expanded from percutaneous endomyocardial biopsy specimens. Circulation.115(7):896-908.2007

114. Matsuura K, Nagai T, Nishigaki N, Oyama T, Nishi J, Wada H, et al. Adult cardiac Sca-1-positive cells differentiate into beating cardiomyocytes. J Biol Chem.279(12):11384-91.2004

115. Linke A, Müller P, Nurzynska D, Casarsa C, Torella D, Nascimbene A, et al. Stem cells in the dog heart are self-renewing, clonogenic, and multipotent and regenerate infarcted myocardium, improving cardiac function. P Natl Acad Sci Usa.102:8966-971.2005

116. Qyang Y, Martin-Puig S, Chiravuri M, Chen S, Xu H, Bu L, et al. The Renewal and Differentiation of Isl1+ Cardiovascular Progenitors Are Controlled by a Wnt/β-Catenin Pathway. Cell Stem Cell.1:165-79.2007

117. Leri A, Kajstura J, Anversa P. Role of cardiac stem cells in cardiac pathophysiology: a paradigm shift in human myocardial biology. Circ Res.109(8):941-61.2011

118. Ieda M, Fu JD, Delgado-Olguin P, Vedantham V, Hayashi Y, Bruneau BG, et al. Direct reprogramming of fibroblasts into functional cardiomyocytes by defined factors. Cell.142(3):375-86.2010

119. Passier R, Mummery C. Getting to the heart of the matter: direct reprogramming to cardiomyocytes. Cell Stem Cell.7(2):139-41

120. Efe JA, Hilcove S, Kim J, Zhou H, Ouyang K, Wang G, et al. Conversion of mouse fibroblasts into cardiomyocytes using a direct reprogramming strategy. Nat Cell Biol.13:215-22.2011

121. Orlic D, Kajstura J, Chimenti S, Bodine DM, Leri A, Anversa P. Transplanted adult bone marrow cells repair myocardial infarcts in mice. Ann N Y Acad Sci.938:221-30.2001

122. Perin EC, Dohmann HF, Borojevic R, Silva SA, Sousa AL, Mesquita CT, et al. Transendocardial, autologous bone marrow cell transplantation for severe, chronic ischemic heart failure. Circulation.107:2294-302.2003

123. Strauer BE, Brehm M, Zeus T, Köstering M, Hernandez A, Sorg RV, et al. Repair of infarcted myocardium by autologous intracoronary mononuclear bone marrow cell transplantation in humans. Circulation.106(15):1913-918.2002

124. Chen SL, Fang WW, Ye F, Liu YH, Qian J, Shan SJ, et al. Effect on left ventricular function of intracoronary transplantation of autologous bone marrow mesenchymal stem cell in patients with acute myocardial infarction. Am J Cardiol.94(1):92-5.2004

125. Hatzistergos KE, Quevedo H, Oskouei BN, Hu Q, Feigenbaum GS, Margitich IS, et al. Bone marrow mesenchymal stem cells stimulate cardiac stem cell proliferation and differentiation. Circ Res.107(7):913-22.2010

126. Kajstura J, Rota M, Whang B, Cascapera S, Hosoda T, Bearzi C, et al. Bone marrow cells differentiate in cardiac cell lineages after infarction independently of cell fusion. Circ Res.96:127-37.2005

127. Matsuura K, Wada H, Nagai T, Iijima Y, Minamino T, Sano M, et al. Cardiomyocytes fuse with surrounding noncardiomyocytes and reenter the cell cycle. J Cell Biol.167:351-63.2004

128. Loffredo FS, Steinhauser ML, Gannon J, Lee RT. Bone marrow-derived cell therapy stimulates endogenous cardiomyocyte progenitors and promotes cardiac repair. Cell Stem Cell.8(4):389-98.2011

129. Tseng AS, Engel FB, Keating MT. The GSK-3 inhibitor BIO promotes proliferation in mammalian cardiomyocytes. Chem Biol.13(9):957-63.2006

130. Evans-Anderson HJ, Alfieri CM, Yutzey KE. Regulation of cardiomyocyte proliferation and myocardial growth during development by FOXO transcription factors. Circ Res.102(6):686-94.2008

131. Liu N, Bezprozvannaya S, Williams AH, Qi X, Richardson JA, Bassel-Duby R, et al. microRNA-133a regulates cardiomyocyte proliferation and suppresses smooth muscle gene expression in the heart. Genes Dev.22(23):3242-54.2008

132. Jung J, Kim TG, Lyons GE, Kim HR, Lee Y. Jumonji regulates cardiomyocyte proliferation via interaction with retinoblastoma protein. J Biol Chem.280(35):30916-923.2005

133. Poolman RA, Li JM, Durand B, Brooks G. Altered expression of cell cycle proteins and prolonged duration of cardiac myocyte hyperplasia in p27KIP1 knockout mice. Circ Res.85(2):117-27.1999

134. O'Meara CC, Wamstad JA, Gladstone RA, Fomovsky GM, Butty VL, Shrikumar A, et al. Transcriptional reversion of cardiac myocyte fate during mammalian cardiac regeneration. Circ Res.116(5):804-15.2015

135. Beigi F, Schmeckpeper J, Pow-Anpongkul P, Payne JA, Zhang L, Zhang Z, et al. C3orf58, a novel paracrine protein, stimulates cardiomyocyte cell-cycle progression through the PI3K-AKT-CDK7 pathway. Circ Res.113(4):372-80.2013

136. Campa VM, Gutierrez-Lanza R, Cerignoli F, Diaz-Trelles R, Nelson B, Tsuji T, et al. Notch activates cell cycle reentry and progression in quiescent cardiomyocytes. J Cell Biol.183(1):129-41.2008

137. Aslan GS, Dudu GM, Kocabas F, Underlying mechanisms and prospect of heart regeneration, Turkish J. Biol, 40: 276-289. 2015

138. An W, Nadeau SA, Mohapatra BC, Feng D, Zutshi N, Storck MD, Arya P, Talmadge JE, Meza JL, Band V, Band H., Loss of Cbl and Cbl-b ubiquitin ligases abrogates hematopoietic stem cell quiescence and sensitizes leukemic disease to chemotherapy, Oncotarget, 6:10498-10509. 2015

139. Hofmann JW, Zhao X, De Cecco M, Peterson AL, Pagliaroli L, Manivannan J, Hubbard GB, Ikeno Y, Zhang Y, Feng B, Li X, Serre T, Qi W, Van Remmen H, Miller RA, Bath KG, de Cabo R, Xu H, Neretti N, Sedivy JM, Reduced Expression of MYC increases longevity and enhances Healthspan, Cell, 160(3):477-88, 2015

140. Judd J, Lovas J, Huang GN, Isolation, Culture and Transduction of Adult Mouse Cardiomyocytes, J. Vis. Exp. (114), e54012

141. Rathinam C, Lydia E Matesic & Richard A Flavell, The E3 ligase Itch is a negative regulator of the homeostasis and function of hematopoietic stem cells, Nature Immunology, 12: 399–407 (2011)

142. Kocabas F, Zheng J, Thet S, Copeland N. G, Jenkins A, DeBerardinis R, Zhang C, Sadek H. A, Meis1 regulates the metabolic phenotype and oxidant defense of hematopoietic stem cells, Blood, 120:25

143. Khandanpour C, Sharif-Askari E, Vassen L, et al. Evidence that growth factor independence 1b regulates dormancy and peripheral blood mobilization of hematopoietic stem cells, Blood 2010;116(24):5149-5161

144. Sattler M., Verma S., Pride Y. B., Salgia R., Rohrschneider L. R., Griffin J. D. (2001) SHIP1, an SH2 domain containing polyinositol-5-phosphatase, regulates migration through two critical tyrosine residues and forms a novel signaling complex with DOK1 and CRKL. J. Biol. Chem, 276:2451–2458

145. X. Chen, S. Wen, M.N. Fukuda, N.R. Gavva, D. Hsu, T.O. Akama, et al. Human ITCH is a coregulator of the hematopoietic transcription factor NF-E2 Genomics, 73 (2001), pp. 238-241

146. G.A. Challen, D. Sun, M. Jeong, M. Luo, J. Jelinek, J.S. Berg, C. Bock, A. Vasanthakumar, H. Gu, Y. Xi, et al. Dnmt3a is essential for hematopoietic stem cell differentiation Nat. Genet., 44 (2012), pp. 23-31

147. A. Wilson, M.J. Murphy, T. Oskarsson, K. Kaloulis, M.D. Bettess, G.M. Oser, A.C. Pasche, C. Knabenhans, H.R. Macdonald, A. Trumpp c-Myc controls the balance between hematopoietic stem cell self-renewal and differentiation Genes Dev., 18 (2004), pp. 2747-276

# APPENDIX A: LANGERDORF APPARATUS

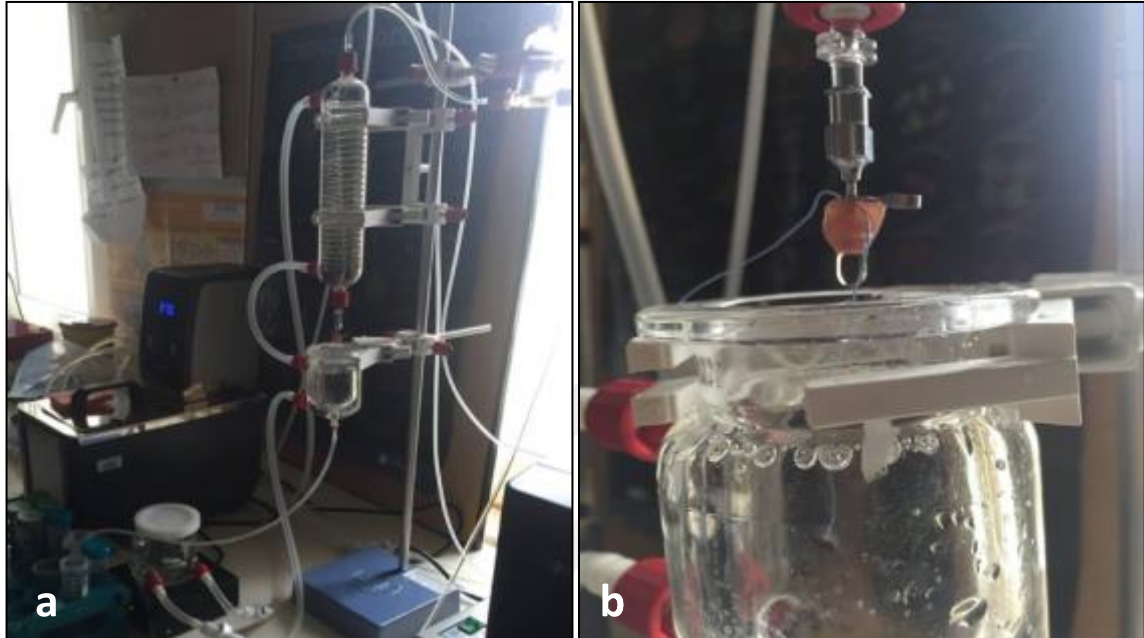



Figure A.1. Langendorff apparatus to digest heart tissue. A) Complete picture of langendorff system which include heated jacket, isolation unit and enzyme container. B) Heart at the tip of langendorff apparatus.

# APPENDIX B: NEONATAL MOUSE SURGERY

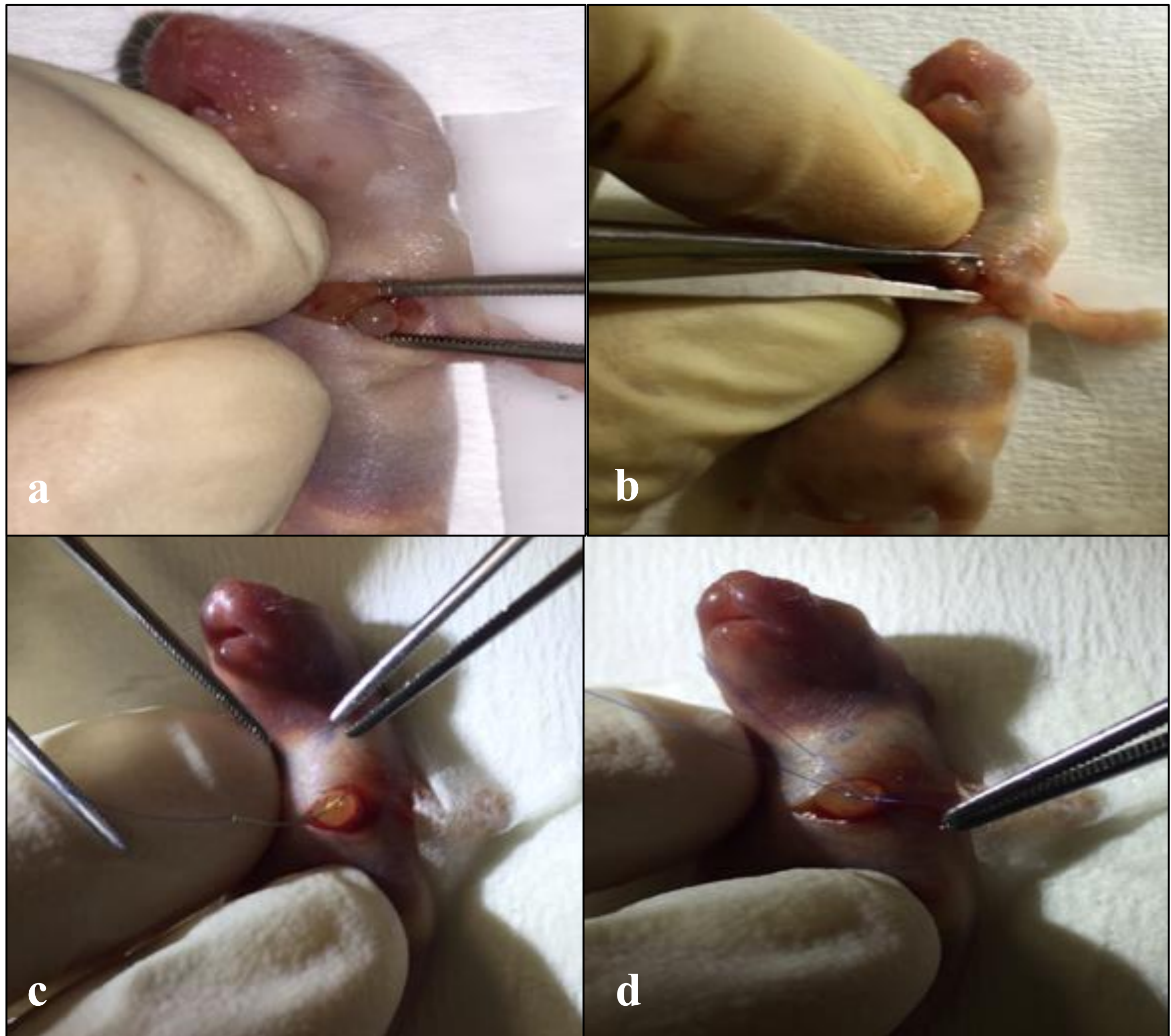



Figure B.1. Lateral throacatmoy and heart surgery operation on neonatal mouse. a-b) Apical Resection surgery model. c-d) Ligation of LAD surgery model on neonatal mouse

# APPENDIX C: SUMMARY FOR PUTATIVE REGULATORS

Table C.1. Summary of previous reports regarding role of putative regulators of cardiac regeneration.

| **Putative Regulato rs** | **Protein Type** | **Type of mouse (deleter)** | **Change in HSc number** | **Criteria used for HSC phenotype definition** | **Fold Differe nce in HSC :%** | **IHC Resul ts (HPA )** |
|---|---|---|---|---|---|---|
| **Gfi1b** | Transcriptio n repressor | cKO | + | LSK CD150+CD4 8– | 39 | Mode rate |
| **Inpp5d** | DNA methyltransf erase | cKO | + | LSK Thy1-lo | 5.7 | Weak |
| **Itch** | DNA methyltransf erase | KO | + | LSK CD150+CD4 8– | 5 | Mode rate |
| **Dnmt3a** | RNA binding protein | KO | + | LSK CD34- Flk2- | 4.5 | Mode rate |
| **Meis1** | Transcriptio n factor | cKO (Scl- Cre) | + | LSK Flk2- CD34- | 4.5 | Weak |
| **c-Myc** | TPO receptor | KO | + | LSK Flk2- | 3.6 | Mode rate |
| **Cbl** | Transcriptio n factor | KO | + | LSK CD150+CD4 8− | 3.1 | Mode rate |
| **hoxa9** | Transcriptio n factor | cKO | + | LSK CD150+ | 2.75 | No info |