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Enhancing cell therapy for ischemic cardiomyopathy.

Michael J. Book
University of Louisville

ENHANCING CELL THERAPY FOR ISCHEMIC CARDIOMYOPATHY

By

Michael J. Book
B.S., A.A., Indiana University, 2011
M.S., University of Louisville, 2015

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by the following Dissertation Committee

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Dissertation Director
Dr. Kyung Hong

_____

Dr. Yiru Guo

_____

Dr. Claudio Maldonado

_____

Dr. Suresh Tyagi

_____

Dr. Dale Schuschke

DEDICATION

This dissertation is dedicated to my wife and family who have always supported me along my journey, regardless of where the path has taken me.

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I'd like to thank my primary mentor, Dr. Kyung Hong, for his invaluable guidance. He has taught me more than I ever imagined I would learn. I would also like to thank my secondary mentors both Dr. Roberto Bolli and Dr. Yiru Guo for their tremendous support. Without their leadership, my pursuit of this work would not have been possible. They have both provided me with countless opportunities to expand my knowledge. I would also like to extend my gratitude to Dr. Liang Tang, Dr. Steve Jones, Dr. Joseph Moore, Dr. Marcin Wysoczynski, Dr. Qianhong Li and the rest of the Institute of Molecular Cardiology for their input and direction as I navigated through my work at the University of Louisville. None of it would have been possible without the collaboration of the entire department and I will be forever grateful to be a part of such an incredible team. Last, but certainly not least, I would like to thank the members of my committee, Dr. Maldonado, Dr.Schushke and Dr. Tyagi, for their guidance towards the success of this project. I hope that my future and remainder of my career will reflect the amazing collection of experience and leadership I have received at the University of Louisville. I could not be more excited for the road ahead!

ABSTRACT

ENHANCING CELL THERAPY FOR ISCHEMIC CARDIOMYOPATHY

Michael J. Book

May 13, 2017

Cardiac cell therapy using cardiac mesenchymal cells (CMC) significantly reduces ventricular dysfunction in patients with ischemic cardiomyopathy. Despite the improvement in function, a modest number of CMCs survive in the heart post-transplantation. In this study, we sought to improve the survival and retention of transplanted CMCs to prolong the therapeutic benefits afforded by cardiac cell therapy. To do this, we targeted the enzyme telomerase (TERT), known to be active in some highly proliferating cells (e.g. germ, stem). TERT is responsible for preventing telomere attrition, thereby allowing continued proliferation. TERT has also been shown to be protective, improve cell migration and stimulate angiogenesis. These actions make the expression of TERT an ideal target. In this study, we overexpressed TERT in CMCs using a lentiviral vector. While TERT overexpression immortalized other cell types, in our hands TERT overexpression did not result in increasing CMC lifespan. Also, overexpression did not improve migration or oxidative stress resistance in CMCs. While TERT is commonly believed to be silent in somatic cells, it has been shown to be detected in the heart. So, we also attempted to exhaustively identify endogenous TERT expression in CMCs, but expression in CMCs could not be detected. To go a step further, we then attempted to

identify TERT expression in a highly proliferative subpopulation of clonogenic CMCs. Clonogenicity is a known characteristic of TERT expressing cells, but we were unable to detect TERT in clonal CMCs. Although we did not find TERT expression and overexpression was fruitless, we did identify more resilient, proliferative CMCs using the cloning technique. We successfully identified CMC clones with differential stress resistance, paracrine stimulation and growth rate that appear morphologically and genetically distinct. Furthermore, we found that transplanting clonogenic CMCs with reduced stress resistance does not ameliorate cardiac function in chronically infarcted rat hearts. However, transplanting unsorted CMCs caused a modest, but significant, improvement of cardiac function. These results suggest that subpopulations exist within the unsorted CMCs that provide no therapeutic benefit. It also suggests a correlation between in vitro stress resistance and reduction in ventricular impairment.

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CHAPTER I

GENERAL INTRODUCTION

The Heart

The heart is a four-chambered organ made up of two atria and two ventricles. The right side of the heart is the low-pressure pump that is responsible for pumping blood through the pulmonary vasculature to oxygenate the blood [1]. The left side of the heart is the high-pressure pump that is responsible for pumping the oxygenated blood to the systemic circulation, including the heart itself. Specifically, the heart provides most of the nutrients to itself by blood passing through the coronary circulation. The coronary circulation is divided into the right main coronary artery and left main coronary artery, which is further divided into left anterior descending (LAD) and circumflex branches. Both the left and right coronary arteries emanate from the base of the aorta and lie on the surface of the heart with the smaller vasculature penetrating the muscular walls of the heart. The left main coronary artery supplies the blood to the anterior and left lateral portion of the heart whereas the right main coronary artery supplies blood to the right ventricle and the posterior left ventricle. In the resting person, about 5% of the total cardiac output flows through the coronary vasculature.

In the normal, resting state, the heart consumes about 70% of the oxygen supplied by the blood in the coronary arteries. This leaves very little oxygen reserve if there is an increased demand. This means that flow of blood through the heart is coupled with oxygen demand and metabolism of the heart. In the normal heart, as activity increases

and demand for oxygen increases there is almost a proportionate increase in coronary blood flow [1]. It is easy to understand how a reduction of blood flow to the heart tissue could have devastating effects.

Pathological Heart

Heart disease is the leading cause of death claiming 25% of all deaths in the U.S. [2, 3], more lives than all forms of cancer combined. The magnitude of burden placed on the globe is tremendous. With the rise in prevalence of poor diet, obesity, sedentary lifestyle, diabetes and excessive alcohol use, the problem will continue to escalate. The most common, coronary artery disease, costs the U.S. over $100 billion and results in over 700,000 heart attacks each year. That equates to someone having a heart attack about every 34 seconds. A heart attack occurs when the flow of oxygen-rich blood to a section of the heart becomes blocked [4].

The blockage begins as a buildup in the walls of the coronary arteries resulting in thickening and stiffening of the arterial walls [1]. When the arterial endothelial cells encounter one or a combination of a diverse variety of bacterial products or risk factors, vasoconstrictor hormones, glycoxidation products, or proinflammatory cytokines, the endothelial cells modify the expression of adhesion molecules [5]. Adhesion molecules, such as VCAM1, promote the sticking of monocytes to the inner surface of the arterial wall causing the monocytes to roll along the endothelial wall and transmigrate at the site of the insult [6]. Monocytes begin to cross the endothelial wall, enter the intima layer of the coronary vessel and differentiate into macrophages. The macrophages release factors that stimulate inflammation; smooth muscle cell migration and proliferation from the tunica

media to the tunica intima; and fibrosis [5]. The macrophages also digest the accumulated lipoproteins and turn into what is commonly called “foam cells” which aggregate in the intima of the vessel forming a lipid-rich necrotic lesion [7]. The preliminary lesion is called a intima xanthoma eventually becoming a fibroatheroma [6].

Over time, the lesion will cause either positive or negative remodeling of the vessel. Positive remodeling is an outward compensatory remodeling that maintains the size of the lumen whereas negative remodeling results in shrinkage of the diameter of the lumen, reducing the flow of blood [8]. Sometimes the negative remodeling can become so severe that it totally occludes the vessel. Covering the plaque of the atheroma in the damaged vessel is a fibrous cap that can vary in thickness. The fibrous cap covered plaque can be referred to as a thick- or thin-cap fibroatheroma. Depending on the fragility of the fibrous cap, the level of stenosis and the lipid core and plaque dimensions, the lesion can weaken the vessel and result in the rupture of the plaque of the fibroatheroma forming a thrombus. Plaque ruptures occur in a lesion with a large necrotic core with an overlying thin disrupted fibrous cap, measuring <65 μm and is heavily infiltrated by macrophages and T-lymphocytes [6]. Atherosclerotic plaque rupture and thrombosis is the primary cause of most acute coronary syndromes and sudden coronary death [6]. The thrombus causes a sudden blockage of blood flow to the area beyond the occlusion resulting in ischemia referred to as an infarct precipitating what is known as a myocardial infarction. If blood flow is not restored quickly there is a perfusion imbalance between the supply and demand, causing myocardial tissue death [4, 9].

The myocardium consists of 3 primary components: myocytes, extracellular matrix and capillary microcirculation [10]. The myocytes are the contractile cells of the heart that

are supported by the stress-tolerant extracellular matrix which maintains the spatial relationship between the myofilaments and the microcirculation. The framework of the extracellular matrix, consisting primarily of collagen, helps to optimize the force generated by the myocytes and distribute it across the tissue. After the onset of ischemia, cell death isn't immediate [9]. There is finite amount of time before cell death leading to complete necrosis causing a loss of myocardium and an abrupt change of the loading conditions of the heart. To accommodate the change, there is a remodeling of the heart that is divided into early and late stage remodeling [10].

Occurring within hours of the infarct, early stage remodeling is the expansion of the infarct [10]. This expansion is highlighted with myocyte death, intermyoctye collagen degradation and a thinning of the myocardial wall. Wall thinning leads to the deformation and dilation of the ventricle, as seen in Figure 1, and an increase in systolic and diastolic

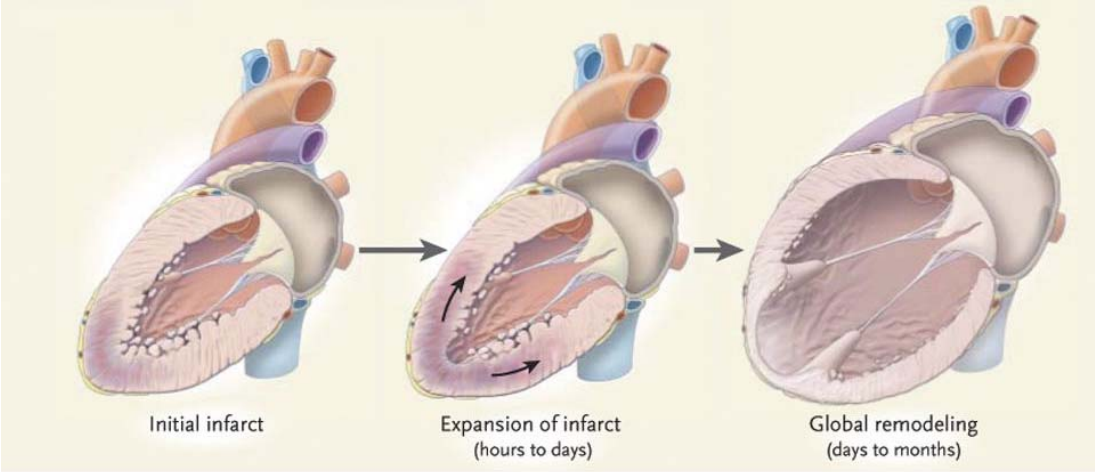


Figure 1 - The phenotypic change of the heart after an infarct. From the left, an occlusion results in blockage of blood flow to the apical region of the heart resulting in an infarct. In the middle, early stage remodeling expands the infarct with increased tissue necrosis. As myocardium is lost, on the right, wall thinning causes deformation & dilation of the ventricle.

stress. Infarct expansion causes the deformation of the border zone and remote myocardium, which alters Frank/Starling relations and augments shortening [10]. The increase in stress stimulates a compensatory hypertrophy in the non-infarcted region of the heart to preserve stroke volume leading into late stage remodeling. In late stage remodeling, a collagen scar is formed to attenuate further deformation of the ventricle and additional myocyte hypertrophy occurs to adapt to the increased load. This pathological state is referred to as ischemic cardiomyopathy, the most common cause of heart failure [11].

Treatment of Ischemic Cardiomyopathy

The treatment of ischemic cardiomyopathy has continued to evolve as our understanding of cardiac physiology has progressed. Prior to the 1980s, treatment of heart failure was largely non-pharmacologic with treatments focusing on changes or limitations of lifestyle. Then a variety of pharmacologic agents began to be introduced including vasodilators, angiotensin-converting enzyme inhibitors, angiotensin-receptor blockers, beta-blockers and many others which primarily acted as a way to manage heart failure [12]. Advances in technology introduced several devices to manage the disease including stents, defibrillators, pacemakers, left ventricular assist devices and even a total artificial heart. However, in many cases these devices simply act as a bridge to heart transplant. Each of these treatments have their own problems, but none of them address the problem of loss of cardiac function.

It was previously believed that the adult human heart was unable to repair itself because it was terminally differentiated [13]. It was believed that cardiomyocytes were unable to reenter the cell cycle, synthesize DNA and undergo mitotic division [14]. The

general contention was that in neonatal life cardiomyocytes lose their ability to proliferate and progress to hypertrophic growth [15]. This belief was supported by the lack of myocyte regeneration following a cardiac insult and the low frequency of cardiac tumors. It was later found that mitotic cells could occasionally be detected in the pathologic heart, but at the time it was thought to be irrelevant [14]. It was then discovered that cell death occurs with age in the human heart inferring a turnover of cells [16] and subsequently found that cardiomyocytes can re-enter the cell cycle and could be detected in the heart undergoing mitosis [14].

This lead to the discovery of cardiac cells that have a regenerative capacity that could be used for cardiac cell therapy [17]. Cardiac cells used for therapy have been identified with antigenic selection (e.g. c-kit) [18] or by cardiosphere-expansion [19]. Our group performed the first Phase I trial utilizing antigenic-selected c-kit-positive cardiac cells called the SCIPIO trial [18]. In this trial, we collaborated with Dr. Piero Anversa at Harvard University who isolated c-kit-positive adult cardiac cells from the patient's heart. C-kit is a receptor tyrosine kinase that is known to be stimulated by Stem Cell Factor (SCF) to activate several different signaling pathways [20]. It has been shown to be important in regulating proliferation, survival and vital functions in hematopoietic cells.

In the SCIPIO trial, c-kit-positive cardiac cells were isolated from and transplanted back in to the damaged heart of patients with ischemic cardiomyopathy using a catheter in combination with a balloon occluder. It was found that cardiac cell therapy using c-kit sorted cardiac cells resulted in a significant sustained improvement of ventricular function and quality of life [18]. However, it has now been discovered that sub-selection of cardiac cells for cell therapy may not be required to achieve ventricular functional improvement.

Cardiac cells harvested directly from the initial outgrowth from cardiac tissue have shown similar therapeutic benefits [21]. These cardiac cells, deemed as cardiac mesenchymal/stromal cells (CMC), are largely made up of mesenchymal cells and fibroblasts [21, 22]. This simplified method alleviates the need for arduous selection processes and greatly increases the efficiency of isolation of cardiac cells for cell therapy, but also indicates the possibility that an optimal sub-population of cardiac cells has yet to be defined.

Regardless of the cell type used for cardiac cell therapy, currently only a modest number of cells survive transplantation and the precise mechanism of ventricular functional improvement is unknown. In mice with a chronic MI, our group found that only ~2% of the successfully transplanted cardiac cells survive after 35-days by using quantitative RT-PCR to calculate the number of cells that were retained in the heart [23]. Thus far, data suggests paracrine mechanisms have a greater importance due to cells exhibiting poor survival and differentiation when transplanted in the infarcted heart [22-24]. A recent study further supported this by revealing that co-transplantation of CMCs with circulatory angiogenic cells resulted in a greater ventricular functional improvement without an increase in cell retention compared to CMCs alone [25]. It is believed that the synergy of these individual therapies was mediated by complimentary paracrine profiles. Unfortunately, identifying the precise combination of cells or paracrine factors that provide this therapeutic phenomenon would result in chasing an endless number of possibilities.

However, it has also been shown that prolonging the retention of transplanted cells in the infarcted heart results in greater ventricular functional improvement as well. Dr. Mark Sussman's group overexpressed Pim-1 kinase in cardiac progenitor cells [26].

Overexpression of Pim-1 kinase in cardiac progenitor cells resulted in a significantly greater number of cells retained in the heart post-transplantation and an improvement in left ventricular function compared to unaltered cardiac progenitor cells. This suggests that CMCs can be optimized to increase therapeutic efficacy. Furthermore, the poor survival of the transplanted CMCs could indicate that a therapeutic sub-population exists that better survives transplantation into the ischemic heart. In this study, we sought to identify "superior" CMCs with elevated survival and proliferation characteristics to prolong the beneficial effects resulting from cardiac cell therapy which we believe will result in a greater functional improvement.

CHAPTER II

CMCs EXPRESSING TELOMERASE FOR CELL THERAPY

Introduction

Ischemic cardiomyopathy is a heart condition that leads to cardiomyocyte loss, scar formation and heart failure [11], one of the world's leading causes of death. In recent years, treatment of ischemic cardiomyopathy with cardiac cell therapy has had salutary, albeit modest, therapeutic benefits [18]. Regardless of the selected cells used for transplantation into the damaged heart, few cells (2-3%) are retained in the heart one month post-transplantation [23]. We believe that by improving the survival and retention of transplanted CMCs we can cause a greater attenuation of ventricular dysfunction. To improve survival and retention of transplanted CMCs, we sought to identify and establish human CMCs with enhanced proliferation and survival characteristics by 1) identifying telomerase reverse transcriptase (TERT)-expressing cells among CMCs and 2) targeting the expression of TERT to CMCs.

TERT is the active subunit of telomerase holoenzyme that works in conjunction with an essential RNA subunit, known as TERC, and several species-specific proteins to maintain the ends of the DNA called the telomere [27]. Telomeres are DNA-protein structures found at both ends of each chromosome that protect the genome from nucleolytic degradation, unnecessary recombination, repair, and interchromosomal fusion [28]. Telomerase acts to prevent attrition of the telomeres to preserve the information in the genome as cells divide, permitting continued cell proliferation [29]. During mitosis, the

DNA polymerase cannot fully replicate the 3' end of the lagging strand of DNA creating an overhang [30]. If the telomerase holoenzyme is present, it finds this overhang and extends the 3' end to maintain the telomere as illustrated in Figure 2. If telomerase is not present, the telomeres will progressively shorten at a rate of 50-100 base pairs of the terminal telomeric sequence (TTAGGG) with each population doubling [31] causing a limitation in the number of times the cell can divide, a phenomenon referred to as the Hayflick Limit [32]. Over time, the telomeres shorten and reach a critical length and induce either replicative senescence (Figure 2), apoptosis or continued proliferation accompanied by genomic instability [33].

Although telomerase expression has been detected in neonatal human somatic cells [34], most adult somatic cells do not express telomerase or maintain the telomere length during proliferation (Figure 3) [31]. The Hoffman group performed an extensive study in

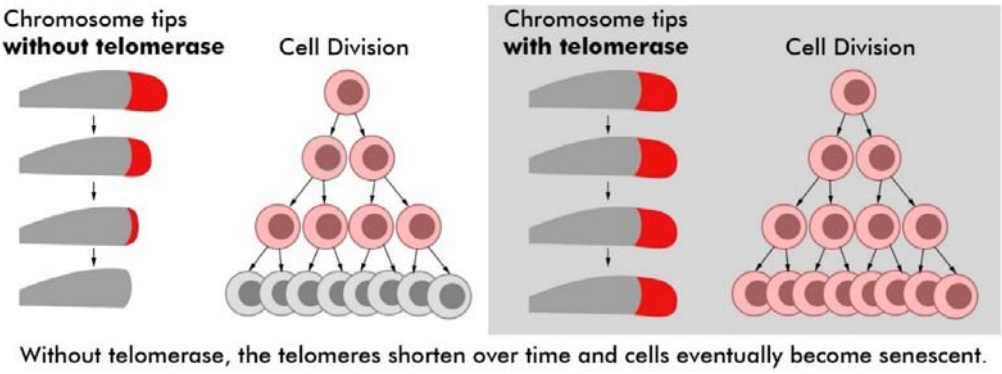


Figure 2 – Without telomerase activity, the end of the DNA, called the telomere, progressively shortens as the cell continues to divide. The telomere will reach a length called the Haflick limit that will result in the cells becoming senescent. However, telomere attrition is circumvented with the activity of telomerase allowing cells to continue to divide.

1998 looking at TERC expression, TERT mRNA expression and TERT activity throughout 21 weeks of gestation of various human tissues, including the heart. In the heart, it was found that TERT activity could be detected until the 11th gestational week, but found that

TERC was detected at all time points. These results suggest that telomerase is developmentally regulated and that TERT is the limiting subunit of telomerase activity in the heart, providing a rationale to focus our attention on TERT in the current study.

However, it has also been observed that telomerase can be re-activated in quiescent cells such as contact inhibited fibroblasts using growth factors indicating the possibility of recrudescence of telomerase activity [35]. As previously mentioned, the terminally differentiated adult heart does not express TERT and lacks telomerase activity, but in the

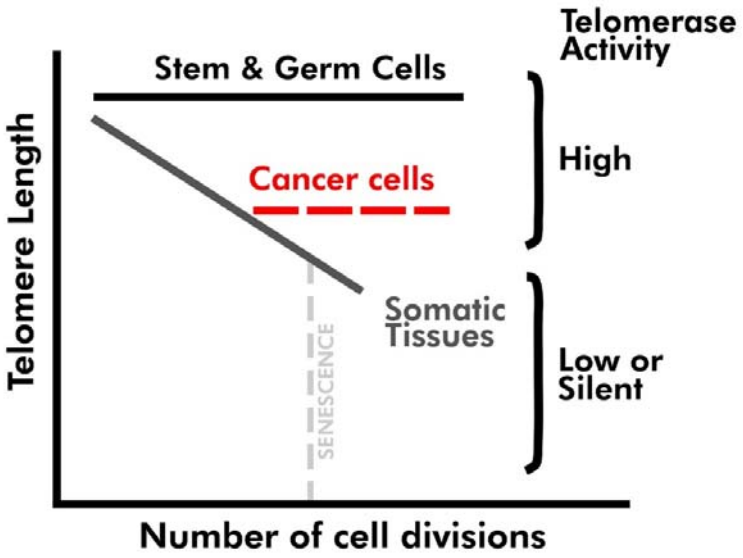


Figure 3 - This figure demonstrates the association of telomere length with telomerase activity as cells continue to divide. The lines on the graph represent the progression of different types of cells in relation to their telomere length and telomerase activity.

pathological heart cardiomyocyte hypertrophy and non-cardiomyocyte cell hyperplasia occurs, which may present the possibility of a re-activation of TERT. In a study performed by Richardson et al., a transgenic mouse was created in which the TERT promoter drove GFP expression in all cells [36]. Following cryoinjury, the authors detected an increased percentage of TERT expressing cells was detected in injured transgenic mouse hearts. In addition, the number of GFP expressing, TERT positive cells was also examined in non-

injured 3- and 12-month-old mice. Counter to others findings that show no TERT expression in adult somatic cells, TERT could be detected in less than 0.03% of cardiac cells in the non-injured heart which may indicate that previous studies did not have enough sensitivity to detect the low level of TERT present in the heart. The researchers then attempted to characterize these cells and found that the TERT expressing were made up of a heterogenous mix of cells. It was further speculated that TERT-expressing cells represent a rare subpopulation of cardiomyocytic, endothelial and fibroblast lineage cells. Anversa's group also found TERT expression in the injured hearts of dogs [37]. However, TERT expression was found to be isolated to cardiomyocytes. Taken together, these findings reveal that TERT may be present in the heart at very low levels making detection possible, but challenging. Results also indicate that TERT is expressed heterogeneously in the cardiac cells which could mean that TERT is also expressed in some CMCs. Data suggests that TERT may be reactivated in the injured heart, which may be critical to the survival and/or protection of cardiac cells.

The protective characteristics of TERT are supported by evidence that TERT overexpression causes cardioprotection [38, 39]. Cardioprotection is defined as "all mechanisms and means that contribute to the preservation of the heart by reducing or even preventing myocardial damage" [40]. To test this, a transgenic mouse model was created to overexpress TERT in the heart [38]. It was found that transgenic overexpression of TERT resulted in a 50% reduction in apoptosis after myocardial infarction (MI) and an infarct reduction of 25% compared to transgenic mice with catalytically inactive TERT. However, the normal, non-infarcted hearts were concentrically hypertrophic in both ventricles suggesting that infarct reduction could be a result of hypertrophy of the heart. In

an attempt to circumvent hypertrophy observed by this group, Maria Blasco's group overexpressed TERT in the whole mouse heart using a cardiospecific adeno-associated virus of serotype 9 (AAV9) [39]. Unlike the transgenic mouse model, an alteration of heart morphology was not observed in the normal heart. Consistent with the transgenic model, after MI, a reduction in scar size, a reduction in fibrosis and a significant improvement in ejection fraction was also observed in mouse hearts treated with AAV9 compared to untreated hearts. Although TERT overexpression preferentially targeted cardiomyocytes in the heart, neither study ruled out the potential effects of non-targeted TERT expression in other cardiac cell types so it is unclear what cardiac cells benefit from hTERT overexpression. The Blasco study does provide evidence that cardiac fibroblasts are not transduced, but failed to address other cell types present in the heart. It is notable that the AAV9 also preferentially targets hepatocytes, but the efficiency is about ten-fold lower than cardiomyocytes. In both overexpression studies, there is uncertainty of whether TERT expression is protective or reparative. This indicates that it may be possible to prolong the survival and/or reparative characteristics of cells used in cell therapy by introducing TERT expression to them.

To explore TERT overexpression of cultured cells, Armstrong et al. overexpressed TERT in embryonic stem (ES) cells and found that overexpressed ES cells were more resistant to stress [41], which is consistent with the protective characteristics of TERT overexpression previously discussed. While ES cells proliferate indefinitely, the ES cells with TERT overexpression also had an increase in the rate of population doubling. And TERT overexpression improved the proclivity of ES cells to differentiate into a hematopoietic lineage, indicating the possibility that TERT expression may help overcome

the differentiation deficiency observed in other studies transplanting CMCs [42]. Consistent with Armstrong's findings in vitro, the Xu group overexpressed TERT in human nucleus pulposus cells (HNPC), a chondrocyte-like cell, and witnessed improved proliferation of the cells [43]. Moreover, it was found TERT-expressing HNPCs experienced less apoptosis and were protected from cell cycle arrest under serum starvation. It was speculated that that serum starvation-induced apoptosis and cell cycle G1 arrest is regulated by the gene expression of p53, CCNE1, Fas, and Caspase 3 in the HNPCs overexpressing hTERT, which they found to be upregulated. In an in vivo study by Madonna et al., bone marrow mesenchymal stem cells were overexpressed with TERT and Myocardin and then transplanted into a ligated mouse hind limb [44]. After 15 days, there was a significant improvement in blood flow recovery in the ligated leg compared to PBS and Mock treated groups. It was also found that TERT overexpressing cells, without Myocardin overexpression, had an elevated paracrine activity as indicated by a significant increase in VEGF secretion into the cell supernatants. Paracrine stimulation by transplanted cells is speculated to be one of the causes of improvement in cardiac cell therapy [45], making this feature of TERT overexpression particularly interesting. It is also important to mention that bone marrow-derived mesenchymal cells do not endogenously express active TERT [46] which may be synonymous to cardiac-derived mesenchymal cells.

In another in vivo study, Mark Sussman's group transplanted cardiac progenitor cells overexpressing Pim-1 kinase into the infarcted heart [26]. The Pim-1 kinase overexpressed cells transplanted into the infarcted heart resulted in improved retention of transplanted cells and a greater improvement of ventricular function compared to GFP transduced progenitor cells [26]. It was also found that Pim-1 kinase overexpression

indirectly increased TERT expression in the cardiac progenitor cells. Although it is possible that ancillary TERT expression from Pim-1 kinase overexpression had little to do with the experimental results since several other protective mechanisms are also upregulated, the results follow the same trend as other TERT overexpression studies.

Disregarding TERT overexpression in the Pim-1 kinase study, the findings do confirm that improvement of retention of transplanted cells leads to improvement of ventricular function in the infarcted heart. These results support our overall goal of the current study to prolong the survival of transplanted CMCs to ameliorate cardiac dysfunction. The combined results from these experiments suggest that overexpression of TERT may improve cardiac cell therapy through enhancing proliferation and survival of CMCs. Furthermore, overexpressing TERT in CMCs may lend to improved tissue repair via paracrine stimulation of the endogenous cardiac cells.

It is also worth considering that there are secondary mechanisms of TERT, outside of telomere maintenance, that could be responsible for therapeutic potential of TERT. There are less-understood telomere-independent actions of TERT that make it an interesting target for cardiac cell therapy as well. For example, Zhou et al. observed a positive correlation between TERT expression and vascular endothelial growth factor (VEGF) promoter activity and expression in Hela cells, independent of telomerase activity [47]. To examine this, Hela cells were transiently transfected with TERT overexpression. A concomitant increased expression of VEGF was seen in TERT overexpressed Hela cells. To explore promoter activity, the group co-transfected Hela cells with TERT overexpression and a luciferase reporter driven by the VEGF promoter. A four-fold increase in the VEGF promoter activity was found when co-transfected with TERT

compared to the control vector. The finding is consistent with the results seen by Madonna et al. in the TERT and Myocardin overexpression study which observed an increased expression of VEGF, previously discussed [44]. In another study, TERT was revealed to promote U2OS cell invasion through an up-regulation of matrix metalloproteinase-9 (MMP9), again independent of telomerase activity [48]. To perform this, U2OS cells were transfected with either a TERT overexpression vector or a catalytically inactive TERT vector. It was found that both the TERT and catalytically inactive TERT caused an increase in invasion through a Matrigel barrier and an upregulation of MMP9 compared to untransfected cells. Taken together, there is evidence that TERT is not only important in cell proliferation, but also plays a role in angiogenesis, cell differentiation and cell migration [49]. If TERT expression is effective in CMCs, the action of TERT could include a greater migration of transplanted CMCs into the damaged myocardium by upregulation of MMP9; an increase in paracrine stimulation and enhancement of blood flow to the ischemic zone through VEGF stimulated angiogenesis; an increased proclivity to differentiate similar to what was seen in ES cells; and improved survival and an increase in proliferation of the transplanted CMCs within the infarcted heart to prolong the therapeutic effects of cardiac cell therapy. It should be noted that all qualities of TERT expression discussed are also important for the progression of cancer. 85-90% of human tumors constitutively express TERT [50] (Figure 3), but many studies have shown that TERT itself is not tumorigenic [51-53].

In our study, we sought to identify if TERT expression in CMCs has proliferative advantages and assessed if these cells present any ancillary benefits of TERT, such as protection and migration, *in vitro*. We hypothesized that TERT expression in CMCs

(endogenous or exogenous) would improve the survival and proliferation of human CMCs and prolong their therapeutic potential. We also believed that based on previous findings by other groups, TERT expression would result in enhanced migration of CMCs into the damaged myocardium, improved paracrine stimulation of endogenous cardiac cells resulting in angiogenesis, a reduction in damaged myocardial tissue and ultimately an improvement in ventricular function (Figure 4). While previous studies reveal TERT expression as valuable in other cells, TERT expression in CMCs has yet to be examined. As previously stated, it is commonly observed that TERT is not expressed or active in somatic, human cells [54], but was later shown to be expressed at very low levels in the normal heart and upregulated in the pathological heart [36, 37]. Until now, it had not been determined if CMCs being isolated for cell therapy endogenously express TERT. First, we attempted to identify and isolate human CMCs that endogenously express TERT. Additionally, we exogenously overexpressed TERT in CMCs, which had not been tested previously. After performing these experiments, our results show a lack of endogenous TERT expression and activity in CMCs. Unexpectedly, overexpression of active TERT in CMCs did not improve proliferation, survival or migration of CMCs in vitro, giving little

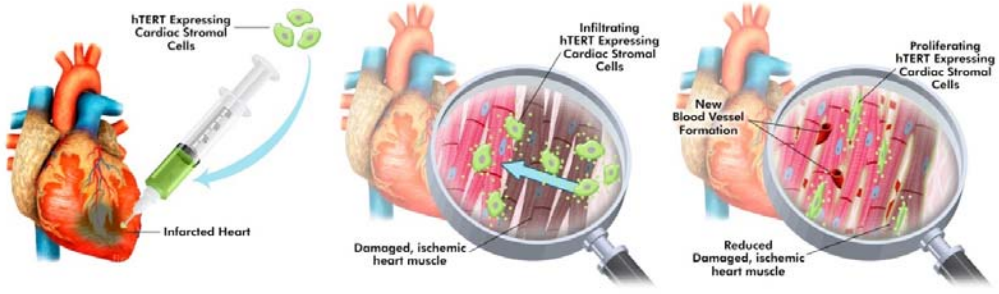


Figure 4 - Intramyocardial transplantation of TERT-expressing CMCs into the border zone of the infarct. We hypothesized a greater infiltration and paracrine stimulation of TERT-expressing CMCs in the infarcted heart. We believed this would improve angiogenesis, reduce the damaged, ischemic heart muscle and improve cardiac function.

evidence or justification that TERT expression alone in CMCs would improve cardiac cell therapy.

Materials & Methods

Explanting CMCs

Human right atrial appendage was collected from patients during coronary artery bypass graft or valve replacement at Jewish Hospital by University of Louisville. A written consent was provided for the collection of the right atrial appendage following the protocol approved by the Institutional Review Board at the University of Louisville. To process the appendage tissue, all extraneous tissue was first removed (i.e. adipose tissue, etc) and then tissue was thoroughly cleaned using sterile 1X PBS. Then, tissue was finely minced with sterile surgical scissors and then minced tissue was digested.

Collagenase digestion was prepared by adding 25 mg of collagenase crystals (Worthington Labs, Lot#421T3577, 320 Units/mg) to 20 mL of 1X PBS to achieve a final concentration of 2000 Units/mL. Solution was vortexed until crystals dissolved and then filtered by a 0.2 μm cellulose acetate syringe filter (VWR). Collagenase solution was added to the minced heart tissue, vortexed and incubated at 37 ºC on an agitator for 30 minutes, vortexing every 10 minutes. Sample was removed from the incubator and the volume raised to 50 mL with 1X DMEM media containing 20% fetal bovine serum (FBS). It was centrifuged at 600 G for 10 minutes after which the supernatant was aspirated. The pelleted cells & tissue was resuspended in complete media (CM). Digested tissue was incubated in the CM at 37 ºC with 5% CO_2, changing the media every 2 days and allowed to adhere to the plate. The outgrowth of adherent cells was harvested 7-10 days later. Human CMCs

complete media (CM) contained: Ham's F12 (Gibco) containing 10% FBS (Seradigm), 10 ng/mL bFGF (Peprotech), 0.005 U/mL human erythropoietin (Sigma) and 0.2 mM L-Glutathione (Sigma) and 100 U/mL Penicillin-Streptomycin (ThermoFisher).

It should be mentioned that an alternative method of digestion was also tested in which Collagenase was replaced with TrypLE (ThermoFisher). The digestion protocol was followed in the same manner as above. Additionally, some plates were coated in fibronectin (Sigma), while other plates were not coated. No difference was detected between methods the different techniques examined.

Quantitative PCR Analysis

mRNA was harvested from cells, treated with DNase (Qiagen) and purified using the RNeasy Kit (Qiagen) following the manufacturers protocol. cDNA was synthesized from 250 ng of mRNA using the AffinityScript qPCR cDNA Synthesis Kit (Agilient). qPCR analysis was then performed on samples in duplicate. The primer pairs used include:

Human Primers

Marker	Primers
TERT	CCTCACCCACGCGAAAACCT, TGGGCCGGCATCTGAACAAA
THY1	GCACTCCTCGGCAGGCATGG, TGGGCCAAACCCTGTGCAGC
FSP1	TGGTTTGATCCTGACTGCTGTCATG, CTCCCGGGTCAGCAGCTCCT
VE CAD	ACAGCATCTTGCGGGGCGAC, CCCGCGGGAGGGCTCATGTA
KDR	AGCTCAAGGCTCCCTGCCGT, GCGGGGTGAGAGTGGGTTGG

SM22α	GCCTTCTTTCCCCAGACATGGCCAA, TGGTTTGATCCTGACTGCTGTCATG
αSMA	AGCGACCCTAAAGCTTCCCAGACT, CGGGGGCTGTTAGGACCTTCCC
TNNT2	AGAAGGCCAAGGAGCTGTGGCA, CCAGCGCCCGGTGACTTTAGC
MEF2C	CAGGAATTTGGGAACTGAGCTGTGC, CGGCTCTCATGCGGCTCGTT
GATA4	CGGCGAGGAGGAAGGAGCCA, TGGGGGCAGAAGACGGAGGG
cKIT	TGGGCCACCGTTTGGAAAGCT, AGGGTGTGGGGATGGATTTGCTCT
DDR2	GGGCAGTGCTCCCTATCCGCT, CATGGCCAGGCACTGACAGCA
NG2	GCGATGCCTTCTCGCTGGAT, CCGTCATGCACGTAGCGGAT
CD146	CGTCTGTGCCCAGCATACCC, TGGTGTTTTTGCCCAGGTCGT
PW1	CCCTTCCTGTGGTGGCGAAA, GATGAGTGGCCCTGCGTCAT
CD31	TCCCAGGAGCACCTCCAGCC, TGGACCTCATCCACCGGGGC
GFP	ACCCTGACCTACGGCGTGCA, TAGCCTTCGGGCATGGCGGA
β-ACTIN	GCAGTCGGTTGGAGCGAGCA, ATCACCTCCCCTGTGTGGACTTGG

Table 1: This table provides a detailed list of primers designed for humans and their specific bases.

Results were analyzed for selected marker expression using SYBR Green Master Mix (Applied Biosystems) and 7900HT Fast Real Time PCR System with SDS version 2.4.1 (Applied Biosystems). Dissociation curves were compared to controls for accuracy and CT values were compared among duplicates. If dissociation curves did not align with

the control, the results were deemed as undetected. If CT values were dramatically different between duplicates, the experiment was repeated for validation.

hTERT Reporter

An hTERT (human TERT) reporter construct was created using a 455 bp hTERT promoter, cut from a pGEM-hTERT construct [55], that drives EGFP and puromycin resistance (Figure 5). The construct was cloned and amplified using NEB Stable Competent High Efficiency E. coli (New England Biolabs) and was harvested using HiPure Plasmid Filter Midiprep Kit (Invitrogen). The reporter was validated by sequencing. Lipofectamine Plus (Invitrogen) transfection was performed in mouse myoblasts (C2C12 - known TERT

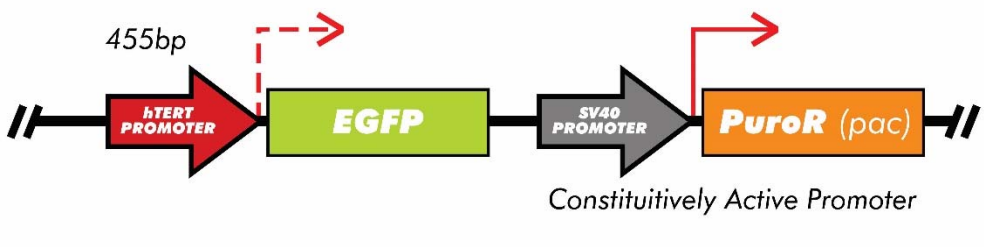


Figure 5 - The hTERT reporter construct consisted of a 455bp hTERT promoter driving EGFP expression in hTERT positive cells. It was followed by a constitutively active SV40 promoter driving Puromycin Resistance expression to protect successfully transduced cells. Successful transduction would yield puromycin resistant cells that were both GFP+ and GFP-. If TERT was expressed in the transduced cells, GFP was translated.

positive) and Normal Human Dermal Fibroblasts (NHDFs – known TERT negative). Cells were co-transplanted with CMV-Histone H2B-mCherry. GFP & mCherry fluorescence was confirmed with epifluorescence microscopy using an EVOS FL microscope.

hTERT Overexpression Vector

The 3.4 kb hTERT transcript was cloned into a pLVX-Puro lentiviral vector followed by a GFP reporter (Figure 6). The hTERT, Puromycin resistance and GFP

reporter transcripts were separated by a self-cleaving peptide (i.e. E2A, T2A), as seen in . Additionally, an empty vector that lacks hTERT was produced as a control. Again, the constructs were cloned and amplified using NEB Stable Competent High Efficiency E. coli (New England Biolabs) and was harvested using HiPure Plasmid Filter Midiprep Kit (Invitrogen). The vectors were validated by sequencing the construct. Lipofectamine Plus (Invitrogen) transfection was performed on C2C12 cells and GFP expression was

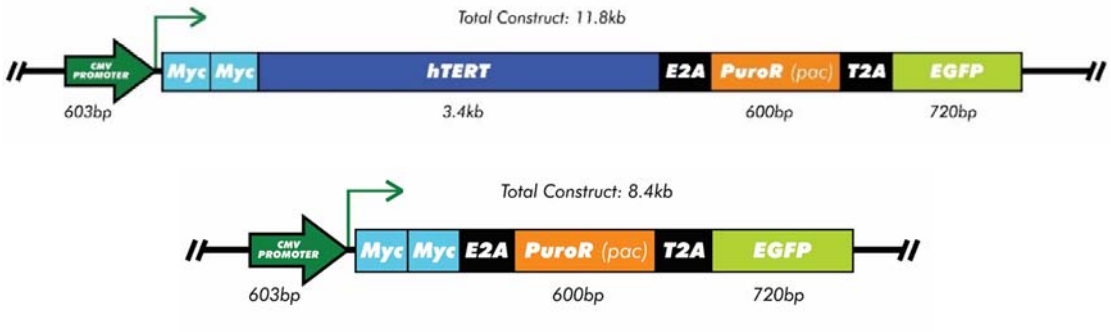


Figure 6 – Top: A constitutively active CMV promoter is driving 2 Myc tags followed by hTERT overexpression transcript. It is followed by Puromycin resistance and GFP expression that is separated by self-cleaving peptides to provide a 1:1:1 expression. Bottom: The empty vector is similar to the hTERT overexpression vector, but it lacks hTERT.

visualized with epifluorescence. The presence of hTERT was then verified in these transfected cells by Immunocytochemistry and Western Blot using an anti-TERT antibody (Rockland, 600-401-252S) [15].

Immunocytochemistry

Cells were plated on a coverslip in the well of a 6-well plate and allowed to incubate overnight at 37 °C in CM. The next day, cells were washed in 1X PBS and then fixed using 3.7% Formaldehyde for 15 minutes at room temperature (RT). Cells were then washed twice with 1X PBS and permeabilized with 0.25% Triton X-100 for 10 minutes at RT. After being permeabilized, cells were washed twice with 1X PBS and then blocked with

5% Bovine Serum Albumin (BSA) for 30 minutes at RT. We then diluted the primary antibody, Rabbit anti-TERT (Rockland, 600-401-252S), in a 1:150 dilution with 5% BSA and incubated the cells in the primary antibody solution. Next, the solution was decanted and the cells washed with 1X PBS three times, letting the last wash incubate for 15 minutes. During that time, we diluted the secondary antibody, Alexa Fluor 555 Goat Anti-Rabbit IgG (Invitrogen), by 1:1000 and incubated the cells in the secondary antibody solution in the dark for 1 hour. The cells were again washed with 1X PBS three times and then counterstained with 1X DAPI staining solution for 1 minute in the dark, followed by being washed with 1X PBS three times. All excess 1X PBS was removed from the slips. A drop of mounting medium was added to a glass slide and the coverslip carefully placed on the slide. After the medium dried, the edges were sealed with clear nail polish. Results were observed with epifluorescence microscopy.

Lentivirus Production, Transduction and Puromycin Selection

Lentivirus was produced using the ViraPower Lentiviral Expression System (Invitrogen) per the manufacturer's protocol. Briefly, on the first day of virus production, 293T Cells (Clontech) in a 100 mm dish were cultured in 8 mL of Opti-MEM (Gibco) containing 3 μg of DNA, 9 μg of Packaging Mix (Invitrogen) and 360 μL of Lipofectamine 2000 (Invitrogen) and then incubated overnight. The next day, the media was changed to antibiotic-free media and incubated another day. On the 3rd day, we checked the virus titer with Lenti-X Go Stix (Clontech). Once the titer gave a positive result, the virus was harvested and then concentrated using Lenti-X Concentrator (Clontech) overnight. Concentrated virus was then diluted 10X and stored in aliquots at

-80 ºC for later use. On the day of transduction, the virus was thawed and human CMCs were transduced with the vector of interest (previously described) and 6 μg/mL of polybrene (Sigma).

The next day, the transduction media was replaced with fresh CM. After 2-5 days, transduction was verified using epifluorescence microscopy to identify GFP expressing cells. CMCs were then treated with 1.5 μg/mL of Puromycin (Thermofisher) in CM to select for the puromycin resistant cells. The optimal Puromycin treatment concentration was determined prior to beginning this experiment. After selection, GFP was again verified by epifluorescence microscopy. Selected CMCs were then maintained in 0.5 μg/mL of Puromycin supplemented CM to prevent growth of cells not expressing Puromycin resistance.

Flow Cytometry & MoFlo Cell Sorting

To check the percentage of GFP expression in transduced CMCs and NHDFs, flow cytometry was used to count the number of GFP positive cells relative to GFP negative cells using the BD LSR II and FACSdiva software. Briefly, data was acquired from untransduced cells. P1, P2 and P3 gating was established in the FACSdiva software which identified the cells expressing GFP. Once gating was set, we ran the experiment on the transduced cells. This data determined the percentage of GFP positive cells within the transduced cells.

Additionally, we collaborated with Dr. James McCracken to perform MoFlo (modular flow) cell sorting using a MoFlo XDP system (Beckman Coulter). The MoFLo system is a high-speed cell sorting system that precisely sorts rare cell populations. Using

this system, we sorted out the top 5% and bottom 5% of GFP expressing cells within the transduced population. We captured these cells in 1.5 mL Eppendorf tubes and immediately harvested the RNA using the RNeasy Mini Kit (Qiagen) to perform qPCR (previously described).

Western Blot Analysis

Protein was harvested from CMCs using a Laemmli buffer and then heated at 100 ºC for 10 minutes. A bicinchoninic acid assay (BCA) kit (Thermo Scientific) was used to measure the protein concentration of each sample, per the manufacturer's protocol. Once measured, 3 µL of β-mercaptoethanol per 100 µL of sample was added to an Eppendorf tube and then heated at 100 º C for 3 minutes. Using the concentration acquired from the assay, 50 µg of protein was loaded on the NuSep precast gel (VWR) alongside a PageRuler Plus Prestained Protein Ladder (ThermoFisher). The gel underwent electrophoresis at 100 V until the ladder was 1 cm from the bottom of the gel. The separated proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore) and the transfer was confirmed with Ponceau (Mallinckrodt Chemicals) staining. The membrane was then blocked with 5% skim milk for 1 hour. Proteins were probed with a primary antibody (1:1000 dilution) overnight at 4 º C. The next day, the primary antibody solution was decanted, the membrane was washed with Tris-Buffered Saline Tween 20 and then incubated with HRP-conjugated secondary antibody (1:10000 dilution) for 1 hour. The results were developed using an Amersham ECL Prime Western Blot Detection Reagent (GE Healthcare) purchased through VWR and visualized with MyECL Imager

(ThermoFisher). The primary antibody anti-hTERT (Rockland, 600-401-252S) was used to detect TERT and the secondary Anti-body was Goat Anti-Rabbit HRP (Pierce).

Telomeric Repeat Amplification Protocol (TRAP)

To measure telomerase activity, a TeloTAGGG Telomerase PCR ELISA Kit (Roche) was utilized based on the manufacturers protocol. Briefly, the TeloTAGGG kit uses PCR and biotin-labelled primers to amplify the product produced by telomerase. The amplified product is then immobilizeded to a 96-well ELISA plate and hybridized with a digoxigenin (DIG)-labeled, telomeric repeat-specific detection probe. An anti-DIG antibody conjugated to peroxidase produces a colored reaction and is used to translate the quantity of product into absorbance. The more telomerase activity present resulted in a higher absorbance. The results of the kit were displayed as absorbance at 450nm and compared to an RNase treated isolate as a negative control. Human CMCs transduced with hTERT were compared to cells transduced with an empty vector in the same cell line. This was performed in triplicate and compared to NHDFs, a known hTERT negative cell line, and HeLa Cells, a known hTERT positive cell line. The kit also included a positive control cell extract as a reference.

Population Doubling Time

Population doubling time, in the simplest terms, is the time is takes for a population of cells to double in the number of cells. In this study, population doubling of hTERT overexpressed CMCs were compared to CMCs transduced with an empty vector. Cells were grown to 90% confluence and then passaged (as previously described). CMCs were

resuspended in CM and counted using a hemocytometer (Bright-Line). $1.5x10^6$ CMCs were plated on 100 mm dishes after each passage. Passaging was repeated until CMCs appeared senescent. To calculate doubling time, the number of CMCs counted was divided by the number of cells plated to arrive at value A. Then the number of days between passaging was divided by A to enumerate the number of days it takes the double the population of CMCs. Population doubling level (PDL) was calculated by the equation PDL=X+3.322(log Y - log I) where X is the current doubling level, Y is the yield of cells produced and I is the initial number of cells plated. Doubling was performed in biological replicates. The mean doubling rate was observed to identify if hTERT overexpression resulted in an increase in proliferation rate while population doubling level was used to identify if hTERT overexpression immortalized the CMCs.

Oxidative Stress Induced by DMNQ

To test hTERT overexpressed CMCs resistance to oxidative stress, $1x10^4$ hTERT overexpressed CMCs and control CMCs were plated in triplicate on a 96-well cell culture plate. Varied concentrations of DMNQ (dimethoxy-naphthoquinone; Sigma) was added to CM to final concentrations of: 0.0 μM, 0.25 μM, 0.5 μM, 0.75 μM, 1 μM, 1.25 μM, and 1.5 μM. The next day, a colorimetric cell viability reagent called PrestoBlue (Invitrogen) was used following the manufacture's protocol. Viability of the CMCs was measured by absorption at Ex/Em 560/590 nm on a Biotek Synergy system. The absorption results were graphed as a percentage of the absorbance of the 0.0 μM DMNQ treated wells to determine the fraction of remaining viable cells.

Of note, H_2O_2 was also tested as a method of inducing oxidative stress. However, colorimetric results were inconsistent between experiments making H_2O_2 induced oxidative stress in CMCs less reliable. While the experiments were performed, the results from oxidative stress induced by H_2O_2 treatment were not used in this study.

Cell Migration Assay

To test migration, $1x10^4$ hTERT expressing CMCs and control CMCs were plated on Boyden chambers containing transwell membranes with a 8 μm pore size (Corning). After 24 hours, the media in the upper chamber was replaced with non-supplemented, serum-free Ham's F12 media (Gibco) and the bottom chamber was replaced with Ham's F12 media (Gibco) containing only 2% FBS (Seradigm). 24 hours later, cells that migrated to lower surface of the transwell membrane were fixed with 3.7% formaldehyde for 10 minutes. Cells remaining in the upper chamber were removed using a cotton tip. Fixed cells were washed in 1X PBS for 10 minutes and then incubated with 50 μg/mL of propidium iodide solution for 10-15 min. Migrated CMCs were quantified by counting the number of cells within four different fields taken with the EVOS FL Cell Imaging System (Life Technologies).

Results

Endogenous hTERT Detection

To begin, we explanted CMCs from human right atrial appendage, as described in the methods. Briefly, atrial appendage tissue was digested with collagenase and then plated on a 100 mm plate. The media was changed every 2 days and digested tissue was incubated at 37 ^oC with 5% CO_2 for 7-11 days. The adherent CMC outgrowth was harvested and

expanded in CM. CMCs were transferred to a 6-well plate and the next day immunocytochemistry was performed to detect endogenous hTERT using an Anti-TERT primary antibody from Rockland. The antibody was selected because previous studies showed successful detection of hTERT both in immunocytochemistry and Western blot [56]. In our hands, the anti-hTERT antibody produced an indistinguishably low-level of fluorescence among Hela, NHDFs and CMCs (Figure 7). Since Hela cells (positive control) had undetectable results, the low level of fluorescence among all cells was interpreted as inconclusive. Later, we overexpressed hTERT in C2C12 cells and again used the Rockland antibody to perform immunocytochemistry. In this experiment, the Rockland anti-hTERT antibody convincingly detected hTERT in C2C12 cells overexpressing hTERT, leading us to conclude that the antibody was not able to detect endogenous hTERT in Hela, NHDFs and CMCs (Figure 7).

Next, we harvested mRNA using the RNeasy Kit (Qiagen) following the manufacturers protocol. cDNA was synthesized from 250 ng of mRNA using the AffinityScript qPCR cDNA Synthesis Kit (Agilient). We then ran quantitative RT-PCR using the primers previously described to detect hTERT and other cardiac cell markers. For a positive control for hTERT, we used mRNA harvested from the thymus (Clontech) because the thymus had been previously observed to express hTERT [57]. While we detected hTERT expression in the thymus, we were unable to detect hTERT in CMCs (Figure 8A) from nine different patients. hTERT was also undetected in a whole heart sample, purchased from Clontech.

Despite the lack of hTERT detected in human CMCs from 9 patients, we were able to do a preliminary characterization of the CMC populations using a selection of other

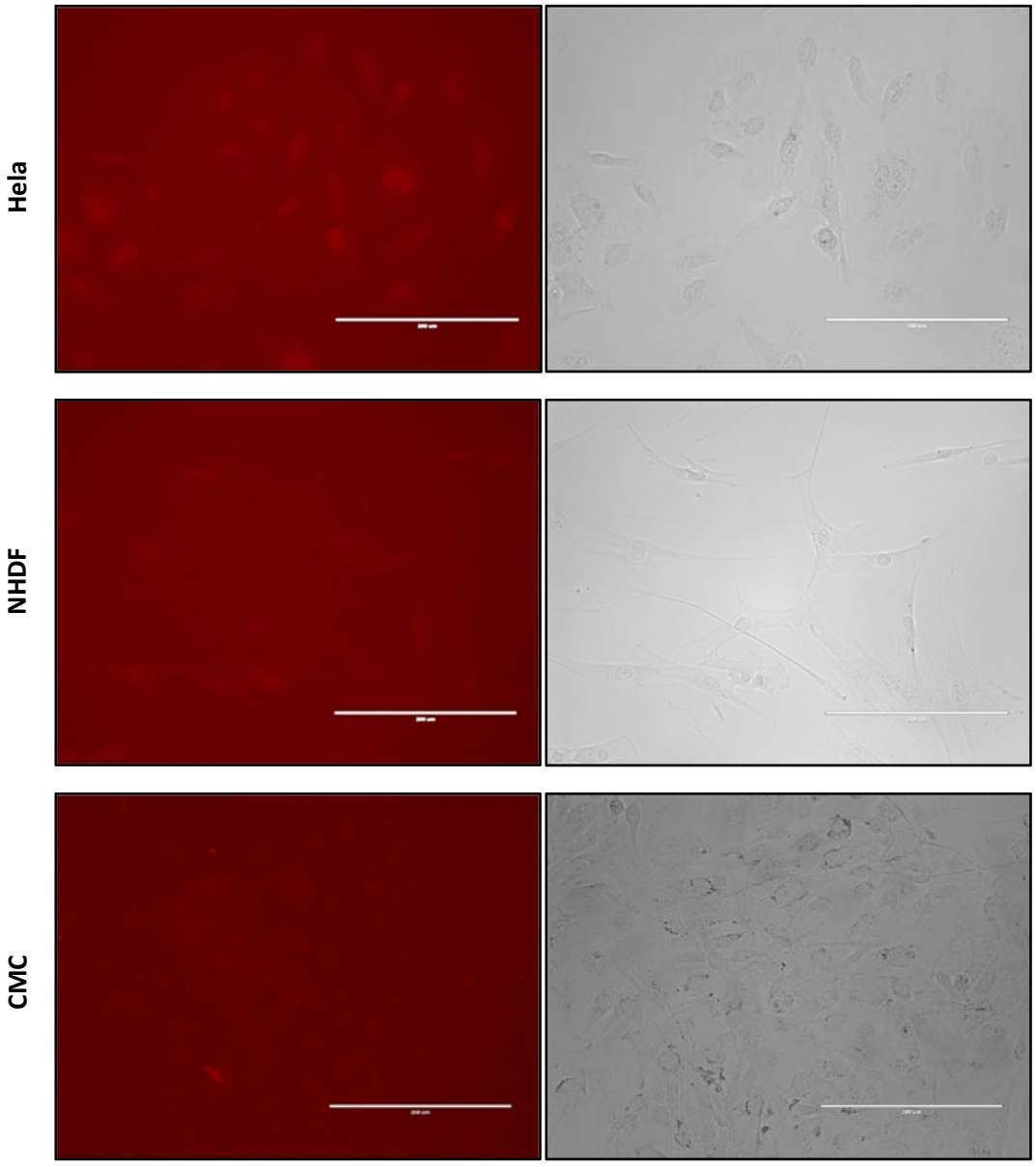


Figure 7 - Immunocytochemistry was performed on Hela, NHDFs and CMCs. Hela is known to be hTERT positive while NHDF is known to be hTERT negative, acting as controls to compare CMC hTERT detection. On the left is the epifluorescent images of the anti-hTERT antibody and on the right is a phase contrast image of the same field to provide a reference of the cells present. Detection of hTERT with the antibody among all cell types appeared indistinguishable between each cell type and results were deemed inconclusive.

cardiac cell markers (Figure 8, Figure 9). From those results, we concluded that within the human CMCs we have no myocyte marker expression (Figure 9B) and very little

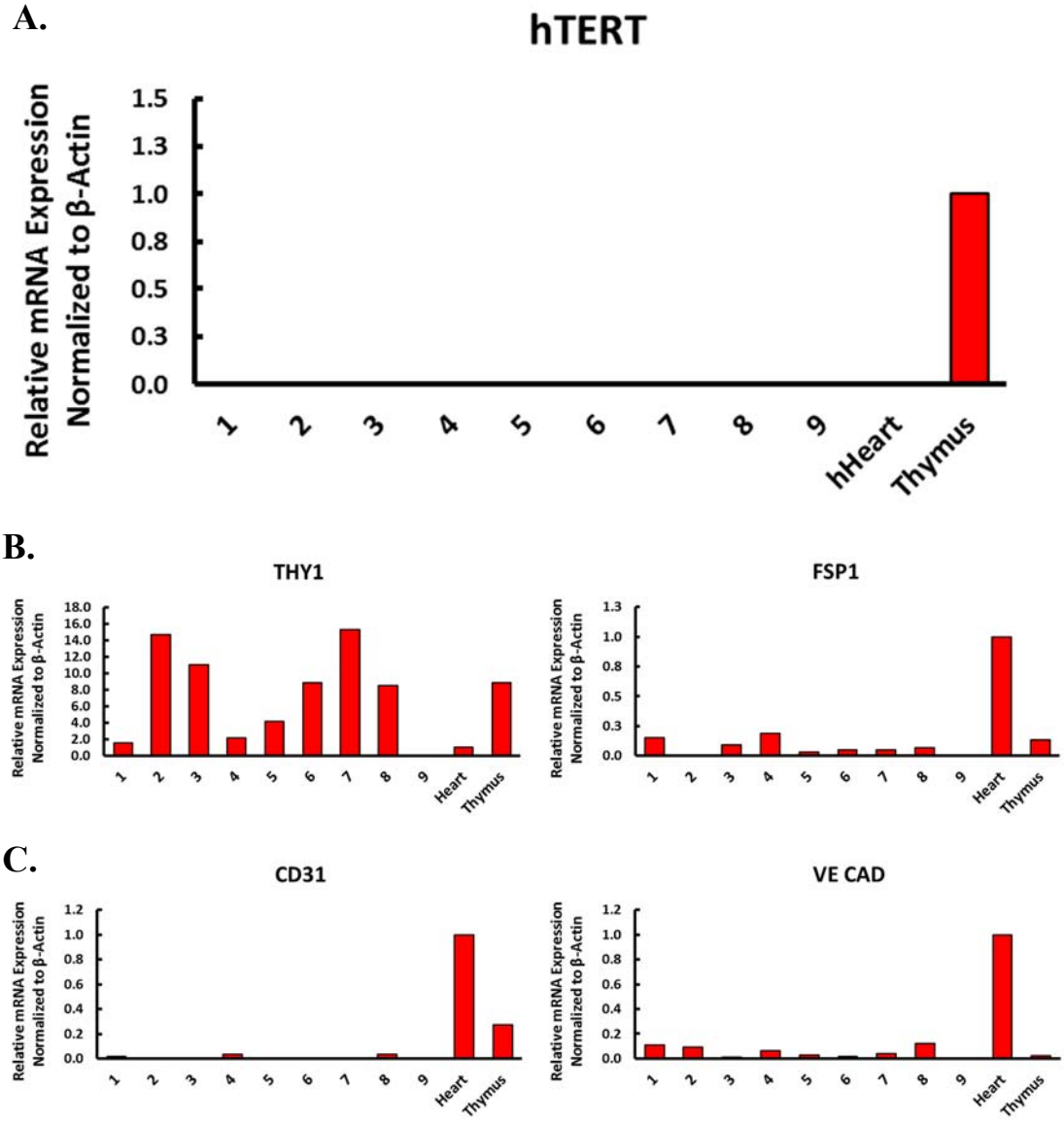


Figure 8 - Using quantitative RT-PCR, we tested expression of a variety of cardiac markers (previously outlined) among 9 different patients. All samples were normalized to β-Actin, a ubiquitously expressed transcript. **A.** hTERT expression was compared to the thymus, a known hTERT positive tissue in humans. Other cardiac markers were compared to whole heart (Clontech) expression. **B.** Fibroblast Markers, THY1 & FSP1, were expressed at varying levels. **C.** Endothelial Markers, CD31 & VE CAD, were expressed at low levels relative to the whole heart.

endothelial cell marker expression (Figure 8C, Figure 9A) suggesting that these cell populations do not contain cardiomyocytes and very few endothelial cells. Likewise, developmental markers appear to have a low expression in human CMCs (Figure 9D).

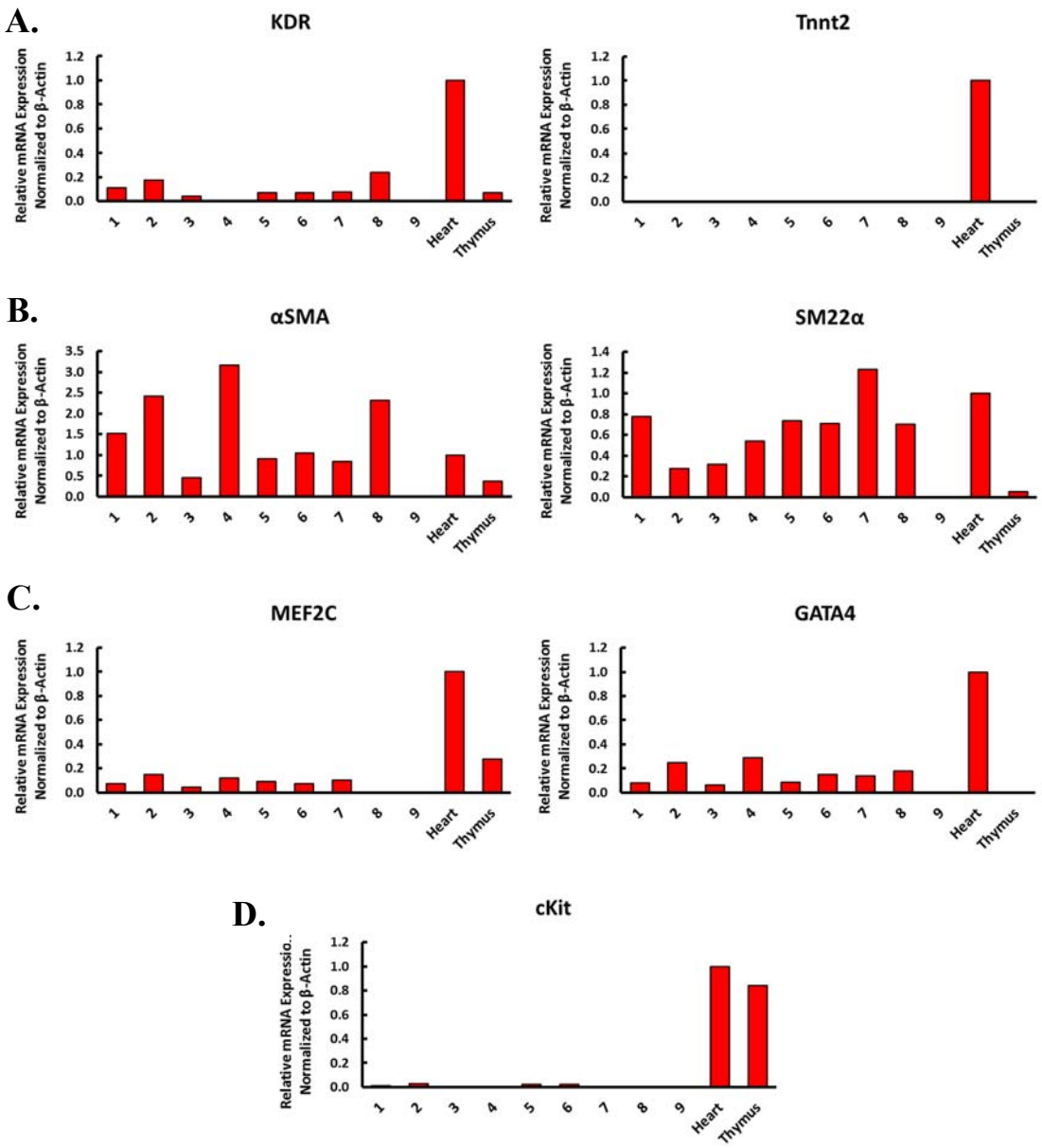


Figure 9 - Using quantitative RT-PCR, we tested expression of a variety of cardiac markers (previously outlined) among 9 different patients. All samples were normalized to β-Actin, a ubiquitously expressed transcript. **A.** Endothelial Marker, KDR, was expressed at low levels relative to the whole heart, similar to CD31 & VE CAD. **B.** Myocyte Marker, Tnnt2, was undetectable. **C.** Smooth Muscle Markers, aSMA & SM22a, had variable levels of expression. **D.** Development Markers, MEF2C, GATA4 & c-Kit, are all expressed at relatively low levels in all 9 patients. C-Kit was undetectable in nearly all cases.

However, we did observe variable fibroblast (Figure 8B) and smooth muscle cell marker expression (Figure 9C) in the CMCs, which in some cases appear to be expressed more

highly than in the whole heart sample. While at this point there are no surface markers that have been exclusively associated with mesenchymal cells, the initial expression results from human CMCs in the current study are consistent with a mesenchymal cell profile further confirming the identity of the CMC population harvested in this study [58].

To exhaust our search for endogenous hTERT in CMCs, we created an hTERT reporter vector (Figure 5). Briefly, in the construct a 455 bp hTERT promoter was driving GFP expression to label any CMC that expressed hTERT. Also included, the construct had Puromycin resistance being driven by a constitutively active CMV promoter. Only CMCs expressing TERT within the Puromycin selected population would express GFP, allowing us to test transduction efficiency as well as isolate a rare TERT-expressing population. After sequencing the hTERT reporter, we performed a co-transfection of C2C12 and NHDF with the hTERT-reporter and a constitutively promoted mCherry construct. From this, we expected to observe cells that were either mCherry+/GFP- (TERT negative), or mCherry+/GFP+ (TERT positive). We observed that NHDFs had mCherry+/GFP-,

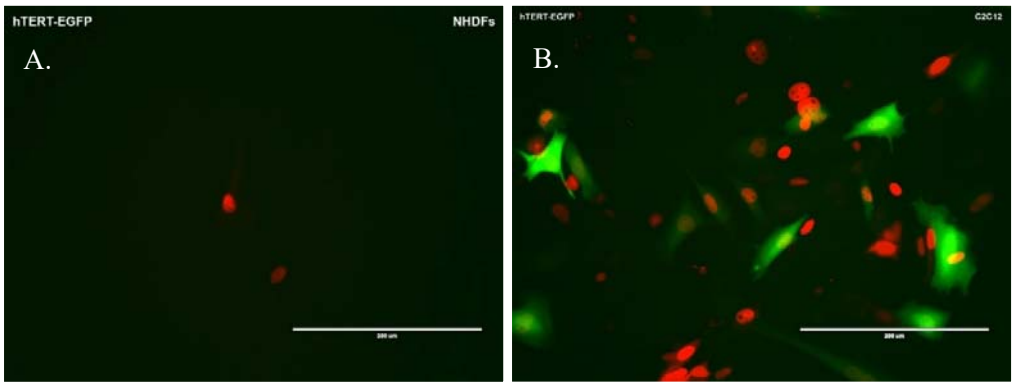


Figure 10 – NHDFs & C2C12 cells were co-transfected with the hTERT reporter and CMV-Histone H2B-mCherry. **A.** The NHDF cells are mCherry+/GFP- indicating a successful transfection of TERT- cells. **B.** C2C12 cells were both mCherry+/GFP+ and mCherry+/GFP-, again indicating a successful transfection, but showing both TERT+ and TERT- cells within the C2C12 population.

indicating successful transfection with no TERT expression (Figure 10A). On the other hand, C2C12 cells had a mix of mCherry+/GFP+, mCherry+/GFP- cells, indicating a successful transfection and that TERT expression was present in only some of the C2C12 cells (Figure 10B).

After successful hTERT reporter transfection, we produced the lentivirus containing the reporter. We tittered the virus and CMCs were successfully transduced. After 4 days, we used epifluorescence microscopy to observe GFP expression in the CMCs (Figure 11A) and NHDFs cells (not shown). Again, only cells that express hTERT should be GFP+, therefor we anticipated the NHDFs would have no detectable GFP and CMCs would have rare GFP expression. Upon visual inspection, a surprising number of CMCs and NHDFs expressed GFP. For this reason, we performed flow cytometry on the hTERT reporter transduced CMCs and NHDFs to determine the number of GFP-positive cells. It was found that in some cases, nearly 80% of the hTERT reporter transduced CMCs expressed GFP (Figure 11B). Counter to the hTERT reporter transfection results, nearly 40% of NHDFs were GFP+. We concluded that our hTERT reporter failed to detect hTERT expression or there was no hTERT expression in the CMCs and NHDFs. It is possible that GFP expression was being driven by some non-specific promoter activity. To test if there was a failure to detect or lack of hTERT expression, we arbitrarily sorted out the top 5% and bottom 5% of GFP+ CMCs and NHDFs using MoFlo. It is possible that some of the GFP+ cells may indeed be hTERT positive, but was hidden by a discrepancy with the reporter virus. After successfully sorting the top and bottom 5% of GFP+ cells, we immediately harvested the mRNA from each aliquot of cells and synthesized cDNA to perform quantitative RT-PCR.

In the GFP sorted CMCs and NHDFs, we confirm GFP gene expression using quantitative RT-PCR in CMCs and NHDFs in both the top 5% and bottom 5% of GFP expressers (Figure 12A, B, C). However, we did not detect hTERT in any of the cells (Figure 12D). The lack of detection of hTERT gene expression in CMCs and NHDFs was more consistent with our previous results. These results indicate that the lentiviral hTERT

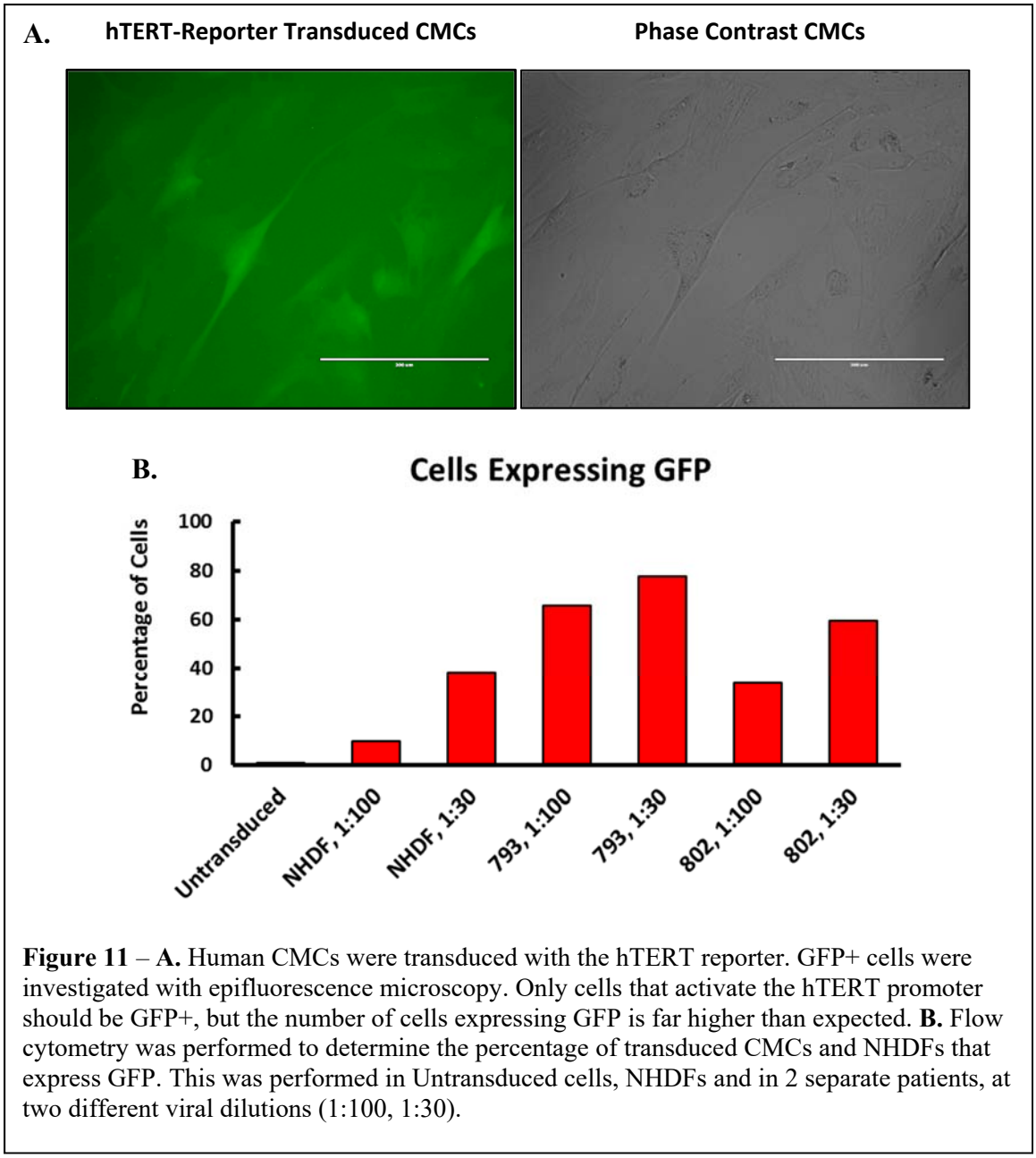


Figure 11 – **A.** Human CMCs were transduced with the hTERT reporter. GFP+ cells were investigated with epifluorescence microscopy. Only cells that activate the hTERT promoter should be GFP+, but the number of cells expressing GFP is far higher than expected. **B.** Flow cytometry was performed to determine the percentage of transduced CMCs and NHDFs that express GFP. This was performed in Untransduced cells, NHDFs and in 2 separate patients, at two different viral dilutions (1:100, 1:30).

reporter was not detecting hTERT, at any level, in the CMCs. We concluded that the GFP in our hTERT reporter was being non-specifically activated and was not sufficient to detect hTERT. We also concluded that, at this point, endogenous hTERT could not be detected in CMCs.

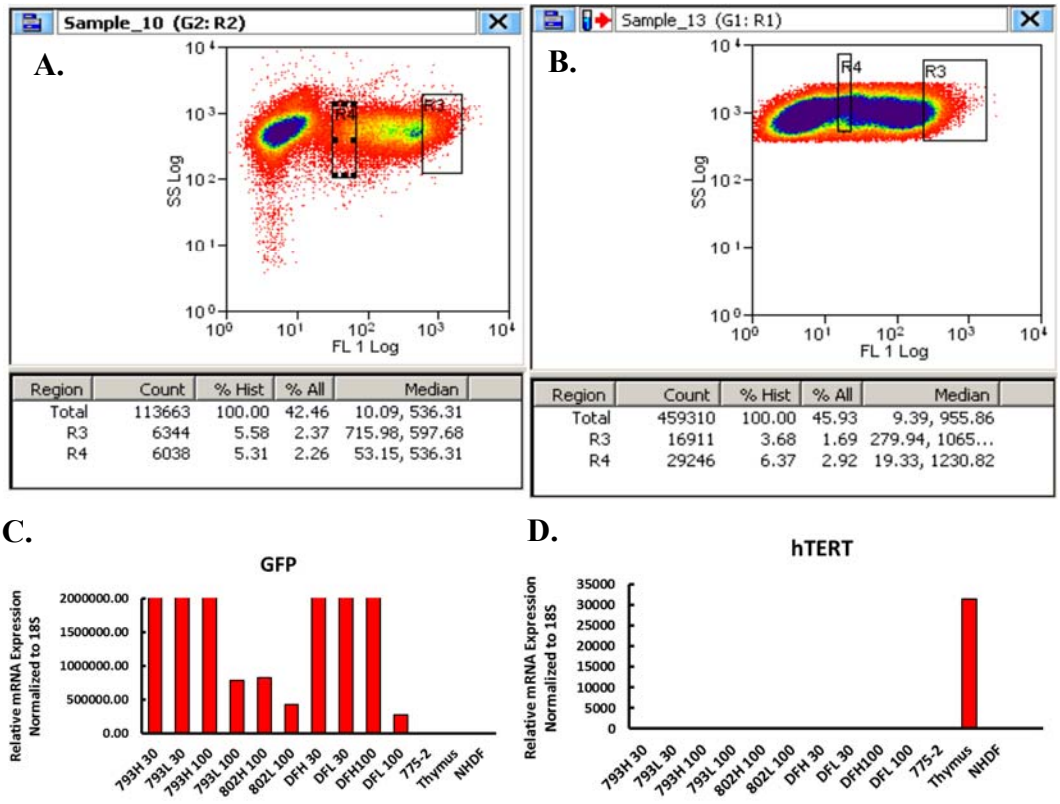


Figure 12 – hTERT reporter transduced CMCs (A.) and NHDFs (B.) were arbitrarily sorted for the Top 5% of GFP expressing cells (R3) and the bottom 5% of GFP expressing cells (R4). RNA from the sorted cells was immediately harvested and processed. C. Quantitative RT-PCR was performed, confirming GFP expression in the Top 5% (H) and bottom 5% (L) in two different patients (793 & 802) and NHDF (DFH) cells compared to untransduced NHDFs, patient (775) and Thymus. D. In all samples, compared to the Thymus, TERT was not detectable.

hTERT Overexpression

Up to this point, endogenous hTERT was undetectable in CMCs. However, a secondary aim of this study was to exogenously overexpress hTERT in CMCs, thereby

overcoming a lack of endogenous hTERT. We again utilized a lentiviral delivery system, producing a constitutively active construct that expressed hTERT, Puromycin resistance and GFP, separated by self-cleaving peptides (Figure 6). We also created a transduction control vector that lacked hTERT (Figure 6). After confirming the sequencing, we again used Lipofectamine to transfect C2C12 cells to examine the efficacy of the vectors. For this experiment, we not only looked at GFP expression driven by hTERT overexpression, but we also performed immunocytochemistry using the anti-hTERT antibody (Rockland) to detect the presence of hTERT. We confirm that C2C12 cells transfected with the hTERT overexpression vector are GFP+ and the presence of hTERT can be detected using immunocytochemistry (Figure 13). Then, protein was harvested from hTERT transfected C2C12 cells to perform Western Blot. We confirmed that transient overexpression of hTERT in C2C12 cells did indeed translate detectable levels of hTERT compared to the control vector (Figure 14). Based on the results, transfection of C2C12 cells with the hTERT overexpression vector corresponds with GFP expression, detection of hTERT using immunocytochemistry (Figure 13) and the presence of translated hTERT using Western Blot (Figure 14). Whereas the control vector had GFP expression, but lacked hTERT detection with immunocytochemistry and Western blot.

Therefore, we moved forward with lentivirus production of the hTERT overexpression vector and control vector. Lentiviruses containing the vectors were produced using the ViraPower Lentiviral Expression System (Invitrogen) so that overexpression could be integrated into CMCs. Then, human CMCs were transduced with either pLVX-hTERT (hTERT overexpression) or empty pLVX (control) (Figure 15).

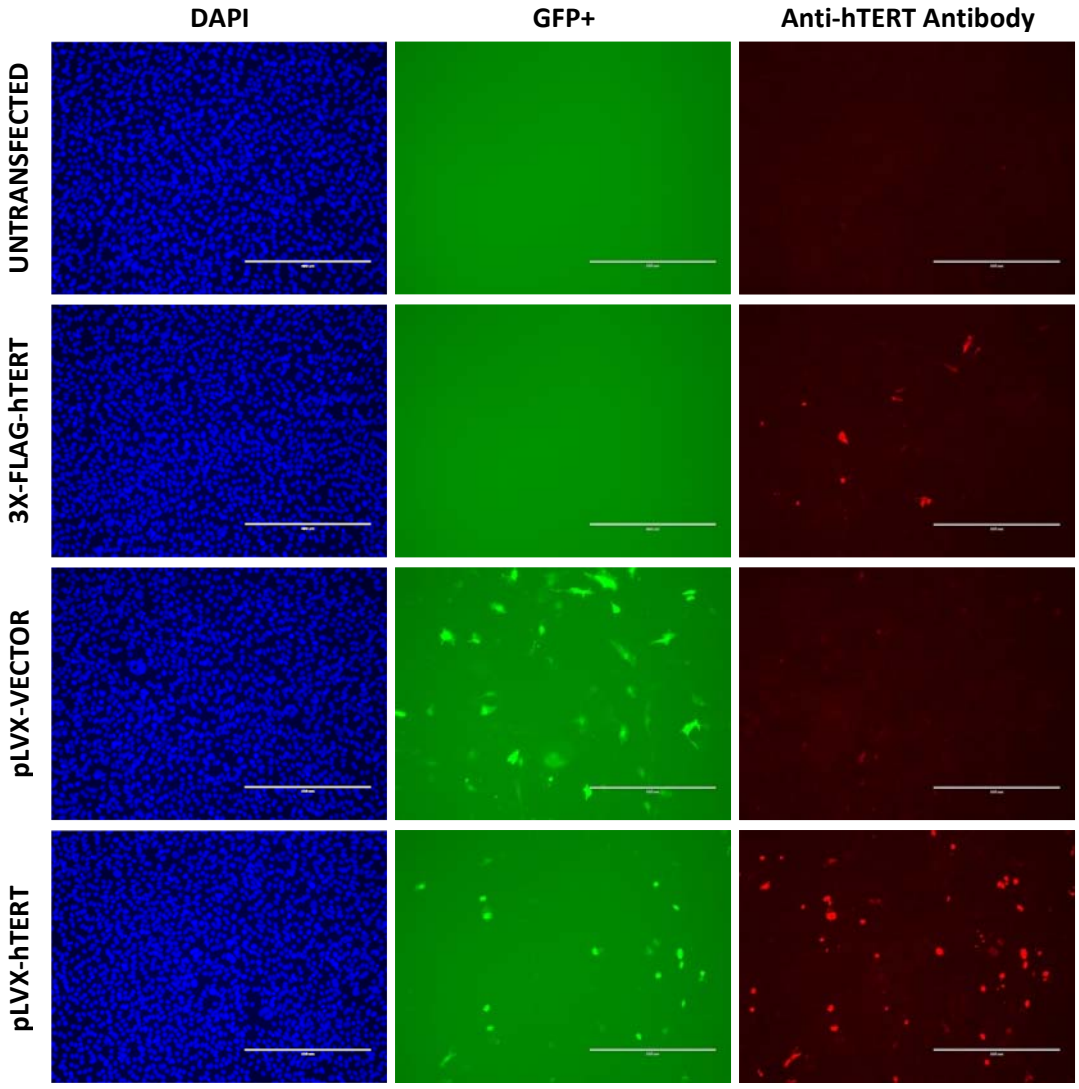


Figure 13 – This figure demonstrates overexpression of hTERT in C2C12 cells using a Lipofectamine transfection. The left column is the DAPI stain of the cells in the field. The middle column is GFP expression detected in the transfected cells. In the right column, an Anti-hTERT antibody (Rockland) was used to detect hTERT expression using Immunocytochemistry. The first row of cells are untransfected C2C12 cells, which are GFP-/hTERT-. The second row is an hTERT overexpression vector lacking GFP expression transfected cells, which is confirmed by GFP-/TERT+. The third row of cells are empty vector transfected cells which have GFP expression and lacks hTERT, confirmed by GFP+/hTERT-. Finally, the last row is the hTERT overexpression vector transfected cells, confirmed by GFP+/hTERT+.

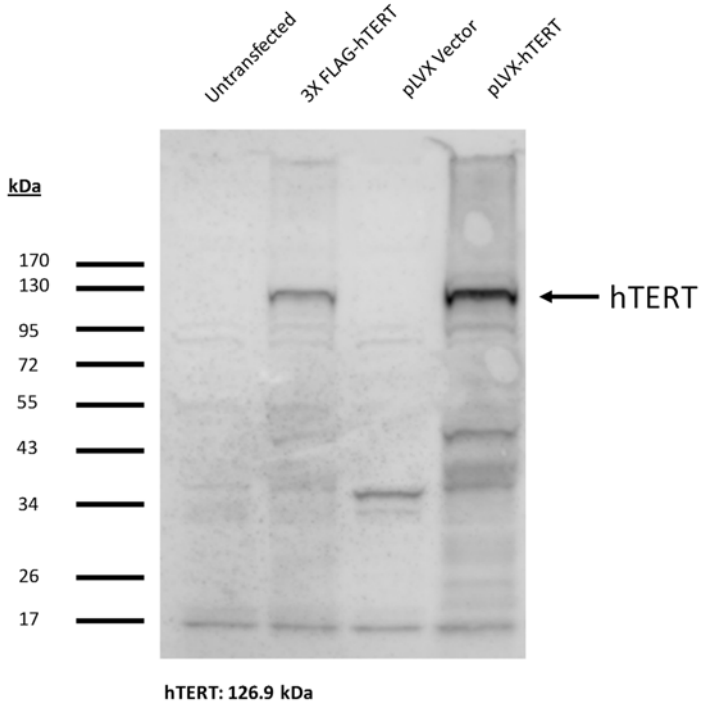


Figure 14 – Protein was harvested from C2C12 cells transfected with either hTERT Overpression (3X-FLAG-hTERT, pLVX-hTERT) or with the control vector (pLVX-Vector), compared to untransfected C2C12 cells. hTERT Overexpression could be identified by the presence of a band 126.9 kDa in size.

Transduced CMCs were then treated with 1.5 µg/mL of Puromycin to eliminate CMCs not expressing Puromycin resistance (Figure 15). After selection, surviving CMCs were maintained in 0.5 µg/mL of Puromycin. Results show that Puromycin resistant CMCs were also GFP-positive, thus far confirming the construct is working as designed (Figure 15). For the final test to examine if our construct is working, we then verified hTERT overexpression in CMCs by harvesting mRNA from the transduced, selected CMCs. cDNA was synthesized and quantitative RT-PCR was performed. hTERT overexpressed CMCs had a 50-300-fold increase in hTERT expression compared to control treated CMCs (Figure 16A). We also harvested protein from transduced CMCs to perform Western Blot. We observed a significant band at 126.9 kDa in hTERT overexpressed CMCs compared to

the control (Figure 16B), which had no detection of hTERT. From this, we concluded that the pLVX-hTERT vector was successful at expressing and translating hTERT in CMCs. Additionally, the transduced CMCs could successfully be selected and verified by GFP expression (Figure 15). We also further support our findings that hTERT is not endogenously present in CMCs in two additional patients by qPCR, as well as Western Blot (Figure 16).

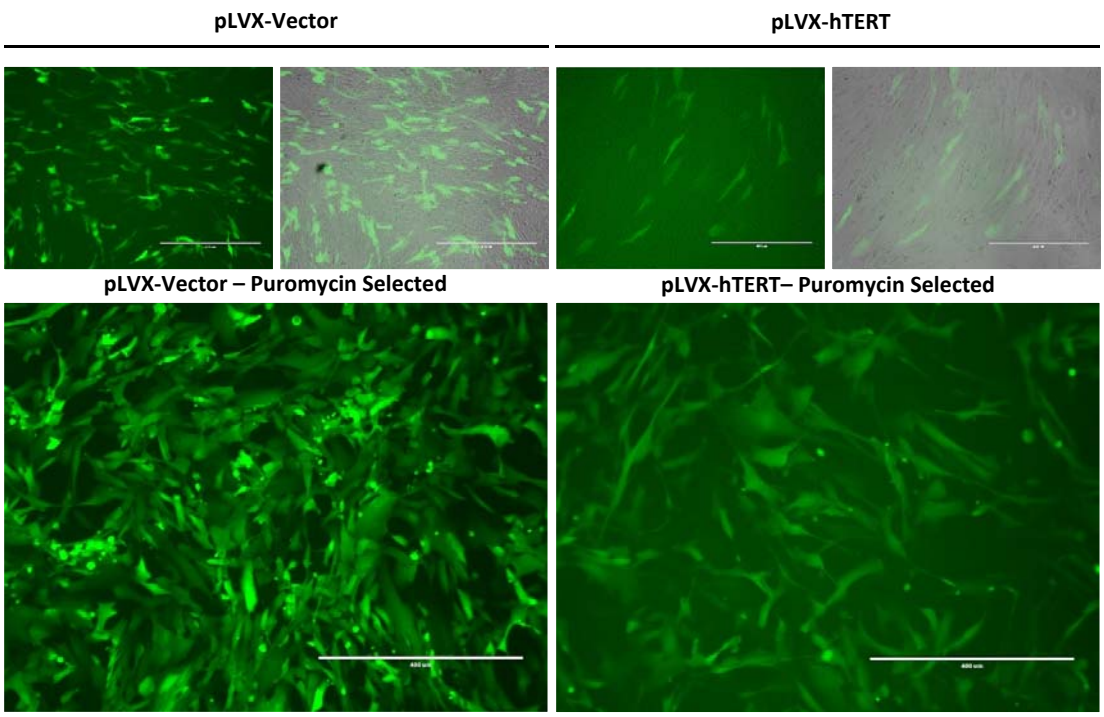


Figure 15 – Human CMCs were transduced with either pLVX-Vector control or pLVX-hTERT overexpression vectors. 4-days after transduction, epifluorescence was performed to visualize the GFP expression (Top). Transduced CMCs were then treated with 1.5 µg/mL of Puromycin to select for the Puromycin resistant cells. Cells were maintained for 3-weeks in 0.5 µg/mL of Puromycin in CM (Bottom).

After revealing that the hTERT overexpression was successful at expressing and translating hTERT in CMCs, we then wanted to verify hTERT activity in the CMCs. To do this, we performed a TeloTAGGG Telomerase PCR ELISA assay (Roche). Briefly, this assay uses PCR and ELISA to detect telomeric activity. Transduced CMCs are harvested and treated

with biotinylated synthetic primers. If telomerase is present in the sample, it will add repetitive sequences to the 3′-end of the labelled primer. Then, the telomerase elongated products are amplified by PCR. The product is then hybridized with a detection probe and immobilized by the biotin-label. The immobilized PCR product is detected with an anti-digoxigenin antibody conjugated to peroxidase, which metabolizes TMB to form a colored reaction measured as absorbance. Therefore, the more absorbance measured equates to more telomerase activity. We observed significantly more absorbance in CMCs overexpressing hTERT than in the empty vector transduced CMCs (Figure 17). In fact, the empty vector transduced cells had virtually no detectable level of activity, consistent with the lack of endogenous hTERT (Figure 16). Results from these experiments confirm that we successfully overexpressed an active form of hTERT in human CMCs.

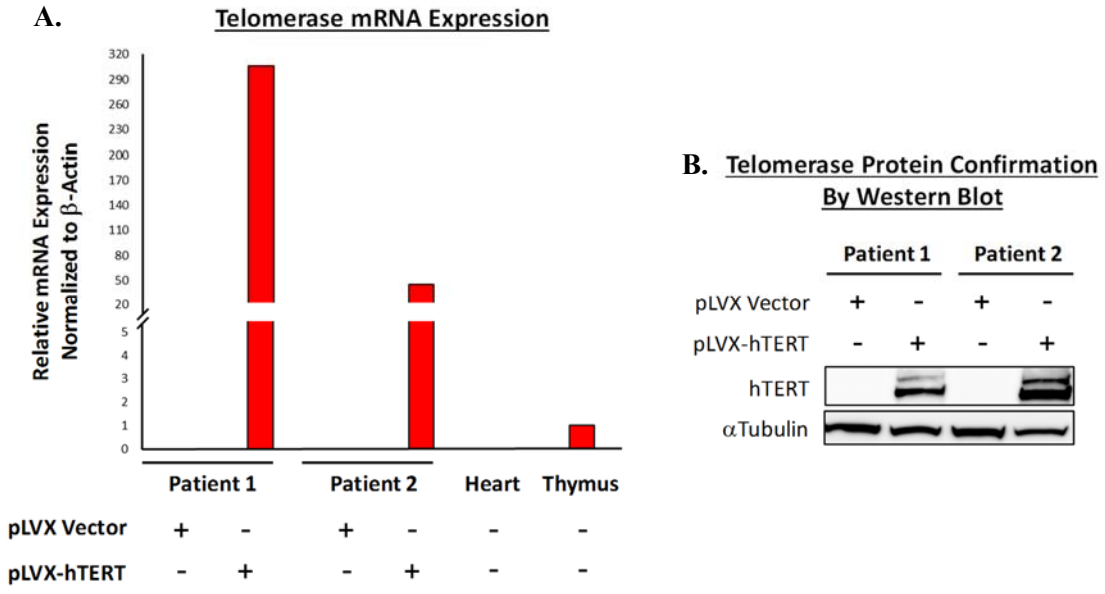


Figure 16 – A. mRNA was harvested from hTERT overexpressed and control transduced CMCs. cDNA was synthesized and quantitative RT-PCR was performed in two separate patients compared to the whole heart and thymus (Clonetech). The hTERT overexpressed CMCs show a 50-300-fold increase in hTERT expression. B. Protein was harvested from the transduced CMCs in two patients. hTERT was detected in hTERT overexpressed CMCs, whereas hTERT was not detected in control vector transduced CMCs.

hTERT Activity in CMCs

Once active hTERT overexpression was confirmed in pLVX-hTERT tranduced CMCs, we then studied the characteristics of the TERT overexpressed CMCs. First and foremost, we maintained transduced CMCs (both hTERT and control) for a duration of over 50 days to test their proliferative capacity. Based on previous studies, hTERT

overexpression immortalizes cells and allows for continued proliferation [59]. However, after growing the transduced CMCs for over 50 days, the CMCs were in poor health and appeared mostly senescent. We repeated with transduced CMC aliquots frozen at earlier passages and the same result occurred, only this time at a faster rate. With the concern of virus toxicity and/or contamination, we produced a new lentivirus and repeated the experiment at different viral dilutions with freshly created virus only to find the same

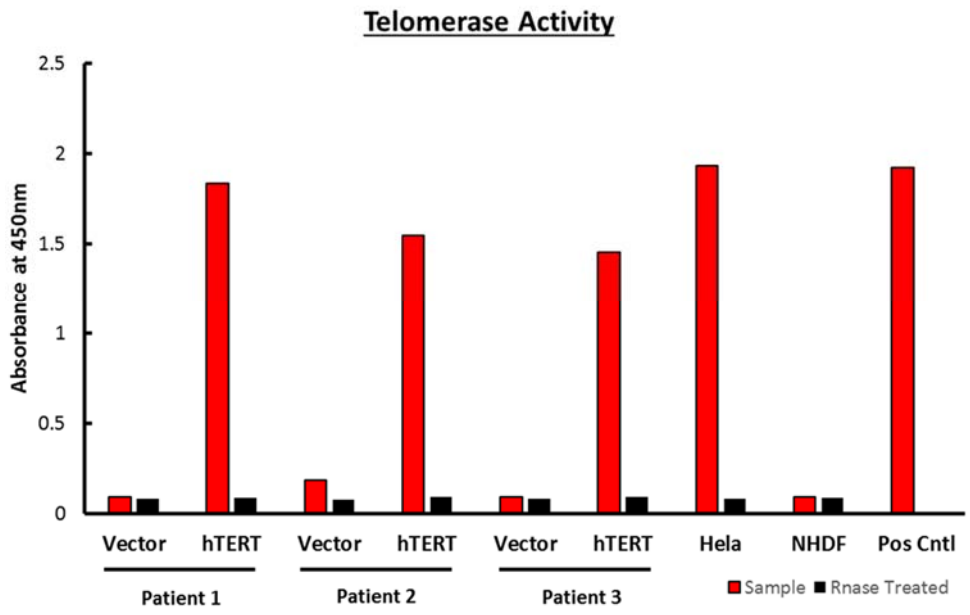


Figure 17 - TeloTAGGG Telomerase PCR ELISA was performed on hTERT overexpressed CMCs compared to the empty vector transduced CMCs. Transduced CMCs were compared to Hela (known hTERT-positive), NHDF (known hTERT-negative) and a positive control included with the assay. One lane of each cell type was also treated with RNase to inhibit the telomerase activity as an additional control.

outcome. During the time that we tracked the traduced CMCs in culture, we found no change in the rate of doubling between the hTERT overexpressed CMCs compared to control vector transduced CMCs (Figure 18A). We also found that overexpressing hTERT in CMCs did not prolong the proliferation of CMCs compared to the control. In fact, the population doubling level in the hTERT transduced CMCs begin to plateau prior to the empty vector transduced cells (Figure 18B). Although active hTERT can be overexpressed in CMCs, hTERT alone does not improve the proliferative capacity of CMCs in culture.

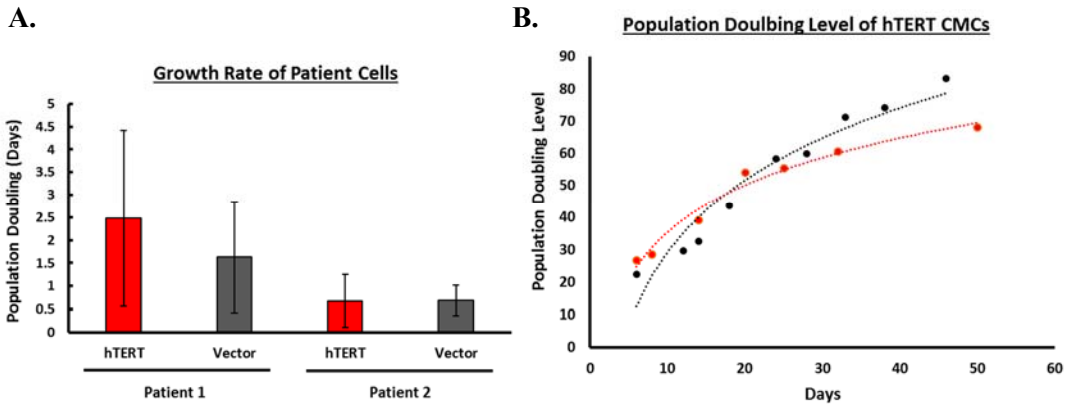


Figure 18 – **A.** Proliferation rate of the hTERT overexpressed CMCs was compared to vector transduced CMCs in two different patients in triplicate. No significant difference was detected in the growth rate. **B.** Population Doubling Level of the hTERT overexpressed CMCs (red) and compared to the control CMCs (black). The results displayed are Patient 1 CMCs. Patient 2 CMC population doubling level followed a similar trend (not shown).

The phenomenon of immortalization caused by hTERT overexpression does not occur in CMCs in our hands.

Despite the mortality observed in hTERT overexpressed CMCs, we continued to test some of the secondary characteristics of hTERT in CMCs. It is possible that the functionality of hTERT in CMCs differs from what has been reported in other cell lines. For this set of experiments, we subjected hTERT overexpressing CMCs to oxidative stress induced by DMNQ. Prior to beginning, we tested a range of DMNQ concentrations to find

the LC50 of CMCs when treated with DMNQ. Then, transduced CMCs were plated in triplicate and treated with varying concentrations of DMNQ, up to the LC50 concentration. CMCs were incubated in DMNQ for 24 hours and then cell viability was measured using PrestoBlue, per the manufacturer's protocol. We observed that hTERT CMCs were no more resistant to oxidative stress than vector transduced CMCs after treating with DMNQ for 24 hours (Figure 19A). Again, this differed from the results observed in other cell types. We then examined if hTERT overexpression in CMCs provided any migratory advantages.

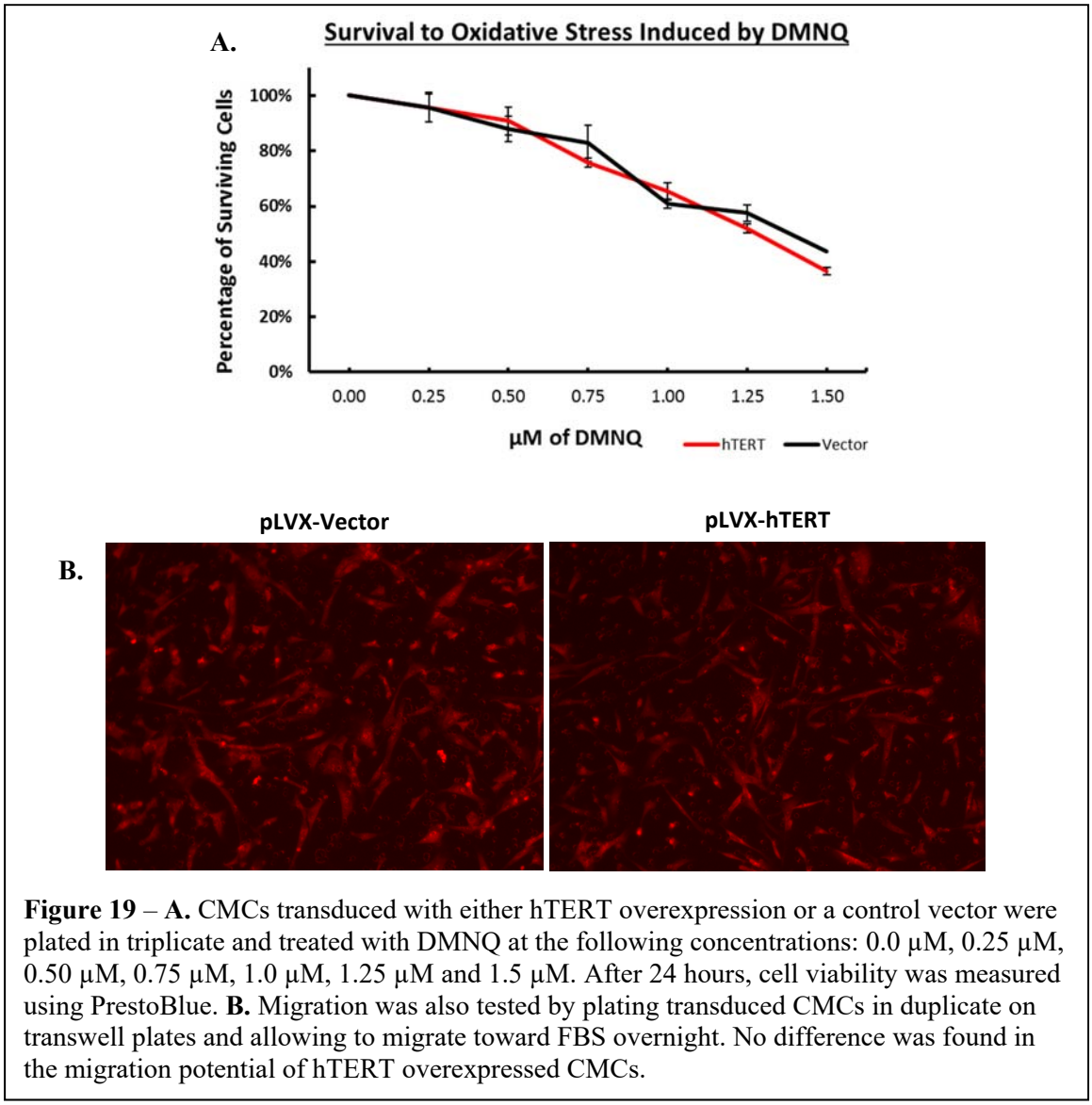


Figure 19 – **A.** CMCs transduced with either hTERT overexpression or a control vector were plated in triplicate and treated with DMNQ at the following concentrations: 0.0 µM, 0.25 µM, 0.50 µM, 0.75 µM, 1.0 µM, 1.25 µM and 1.5 µM. After 24 hours, cell viability was measured using PrestoBlue. **B.** Migration was also tested by plating transduced CMCs in duplicate on transwell plates and allowing to migrate toward FBS overnight. No difference was found in the migration potential of hTERT overexpressed CMCs.

To do this, we performed a Boyden chamber assay. Briefly, transduced CMCs were plated on the semi-permeable membrane of a transwell plate. The CMCs were incubated in CM overnight. The next day, the media in the top well of the transwell plate was changed to serum-free, basal media. The media in the bottom well was changed to Ham's F12 containing 2% FBS. Cells were incubated overnight. Prior to beginning this study, we examined different durations of migration to identify an optimal migration time for CMCs. It was found that overnight migration (18 hours) provided the most robust results. We found that migration of CMCs toward a chemoattractant (2% FBS) was not improved by the overexpression of hTERT (Figure 19B). Results of some of hTERT's secondary characteristics suggest that hTERT expression in CMCs does not improve resistance to stress or improve migration, in vitro (Figure 19). Taken together, our observations indicate that overexpression of hTERT in CMCs has no observed therapeutic advantages in vitro.

Discussion

In this study, we comprehensively determined that CMCs lack endogenous expression of hTERT. Furthermore, we elucidated that the lifespan of explanted CMCs is not singularly reliant on the expression of hTERT. While we did look at the presence of hTERT and identify that hTERT was active in the CMCs overexpressing hTERT, we did not confirm that hTERT activity resulted in telomere maintenance. To perform this, a Southern Blot would need to be performed and probed to detect the segments of the telomeres compared to normal CMCs. If it was found that overexpression of hTERT does not maintain telomere length, it is possible that other factors are inhibiting the activity of the hTERT, allowing for telomere attrition. One possible example is the presence and

activity of the Shelterin complex [60]. If the Shelterin complex, or one of its six subunits, were not present and/or working as it should then maintenance of the telomeres could not be regulated by hTERT. POT1, one of the Shelterin subunits, has single-stranded-DNA binding activity that has been shown to be able to block telomerase from gaining access to the telomere [61]. In fact, introducing a mutant form of POT1 causes a loss of telomerase regulation and allows for telomeres to be overextended [62]. It is possible that POT1 could be highly active in CMCs, preventing hTERT access to the telomere. Of course, this is speculative and just one potential inhibitor of hTERT activity.

It is also possible that the CMCs represent a population of cells that do not depend on hTERT for the maintenance of telomeres. Approximately 30% of in vitro immortalized human cell lines do not express hTERT [63]. CMCs may rely on an Alternative Lengthening of Telomere (ALT) pathway to prevent telomere attrition [64]. Despite the reliance on ALT for telomere maintenance, the Wen group found that in some cases that ALT and hTERT can co-exist in cells to maintain the telomeres. In other words, overexpression of hTERT in cells relying on ALT can enhance telomere maintenance. However, they did identify that TERC must be present as well as hTERT. Although we overexpressed hTERT, we did not also verify the presence of TERC which could be an inhibitor of the hTERT activity. In any case, it would be challenging to identify the precise cause for the lack of telomere lengthening in cells overexpressing hTERT and is a project that is outside of the scope of the current study.

Additionally, it is possible that the telomere length is independent of proliferation of CMCs explanted from atrial appendage tissue. Several studies have reported that mammary epithelial cells and keratinocytes cannot be immortalized by hTERT

overexpression [65, 66]. In these studies, an inactivation of p16 along with hTERT overexpression was necessary to immortalize the cells, which presents the question of whether telomere length is the sole factor of senescence. Another group introduced *ras* into rodent cells and induced senescence, despite having sufficiently long telomeres [67]. Regardless, the hypothesis of this project was that expression of hTERT in CMCs would prolong the proliferation and longevity of these cells thereby improving cell therapy. Unfortunately, that was not found to be the case in our hands. Pinpointing the specific reason hTERT does not have the expected hTERT overexpression phenotype and the solution to that problem is beyond the scope of this project. This project simply sought to identify if hTERT alone could improve CMCs therapeutic potential, which we believe we have sufficiently addressed.

Even though CMCs overexpressing hTERT did not exhibit therapeutic potential, it is worth considering that CMCs are not the optimal cardiac cell population to achieve the benefits of hTERT overexpression. In a study performed by Leri et al. hTERT expression was detected exclusively in cardiomyocytes in the injured heart [37]. On the other hand, Richards et al. found hTERT expression was present in a heterogenous mix of cells in the non-injured heart [36]. This could indicate that the optimal target of hTERT overexpression in the heart has yet to be examined. Although CMCs weren't fruitful for this project, it would be interesting to shift the approach to identify the cell lines in the heart that do express hTERT that could be used for cardiac cell therapy. For instance, it is possible that hTERT expression is indeed important in the cardiomyocytes similar to what was seen in the Leri et al. study. To test hTERT's importance in cardiomyocytes, Zhang et al. reversibly overexpressed hTERT in cardiomyocytes so that cardiomyocytes could be expanded and

then “turned off” using Cre recombinase [68]. Unlike CMCs, they observed that cardiomyocytes could be controllably immortalized with hTERT expression. It will be interesting to see the results of future experiments utilizing these cells in vivo for cardiac cell therapy. It would be of added interest to determine if hTERT expression in endogenous cardiomyocytes is enhanced after treatment with CMCs and if so, would blocking the stimulation of hTERT in cardiomyocytes eliminate the ventricular functional improvement observed. This again is beyond the scope of the current project. It was our objective to improve the therapeutic potential of CMCs used for cell therapy by expression of hTERT. Despite the negative results observed in hTERT overexpression of CMCs in this study, hTERT remains an intriguing target for the advancement of cardiac cell therapy in the future.

CHAPTER III

CLONOGENIC CARDIAC MESENCHYMAL CELLS FOR CELL THERAPY

Introduction

Clonogenicity, a known stem cell characteristic [69, 70], is the ability of a single cell to survive and expand into a large colony of monoclonal cells and retain self-renewal qualities [71]. The cloning technique was developed in the 1950s [72] and was used to examine the effects of radiation on cells [73, 74]. While other cells can proliferate, clonogenic cells must first survive the stress of self-preservation without becoming senescent or dying and then maintain the ability of continued proliferation. Later, Barrandon and Green classified 3 types of colonies formed by cloning which were termed holoclones, meroclones and paraclones using keratinocytes [75]. These classifications are derived from cells within the parent population that have different proliferative capacities. Holoclones form large, rapidly proliferating colonies with less than 5% of the colonies terminally differentiating. Paraclones have limited growth and form small, terminally differentiated colonies. And finally, Meroclones form both proliferating and terminally differentiated colonies. In the current study, we performed a limited dilution and cloning cylinder assay, similar to that described in Puck, Marcus & Cieciura 's seminal paper [72], to select out individual colonies that had varying capacity to grow and survive. The limited dilution technique selected clones that would most appropriately be deemed as a Meroclone. Then, we performed the cloning cylinder assay and isolated clones from the Meroclone, separating them into either Holoclone or a Paraclone colonies, the difference

being undefined. By definition, some clones will be more robust than others which we believe could provide a method of teasing out more resilient, proliferative cells residing in the human CMCs.

Due to the inherent qualities of clonogenicity and our interest in telomerase, we initially believed that cloning would select out telomerase-expressing human CMCs. Telomerase is a holoenzyme consisting of a cellular reverse transcriptase (TERT, telomerase reverse transcriptase) capable of preventing telomere attrition through de novo addition of TTAGGG repeats onto the chromosome ends, called the telomere, by using an associated RNA subunit as a template (TERC, telomerase RNA component) [76]. It has been found by Blasco's group that increased TERT expression positively correlates with clonogenic potential making it possible for the TERT-expressing CMCs to have a predisposition to survive the cloning assay [76]. However, we found that human clonogenic CMCs did not have detectable expression of hTERT, which was consistent with our previous hTERT findings in human CMCs.

Despite the lack of hTERT detection in human clonogenic CMCs, we believed that isolated clonogenic CMCs alone would be an interesting target for cardiac cell therapy since clonogenic CMCs fundamentally exhibit enhanced survival and proliferation characteristics compared to non-clonogenic CMCs (parent). An advantage to utilizing this simplified methodology would eliminate the need of genetic modification to bolster CMCs for cardiac cell therapy, allowing us to achieve the same goal of improved survival and proliferation of CMCs in an unbiased approach. To our knowledge, this was the first time a clonogenic assay was used to select proliferative, resilient rat CMCs for cardiac cell therapy.

Although clonogenicity itself is a differentiating characteristic, because of the expected variability between isolated CMC clones (i.e. holocone, paraclone or meroclone) we further screened clonal rat CMCs. The initial screening tested the resistance of clonogenic rat CMCs against oxidative stress. Oxidative stress resistance was selected as characteristic because it is known that the environment of the ischemic heart contains high levels of reactive oxygen species (ROS) [77], where transplanted rat CMCs would be required to survive. Zweier et al. performed several studies measuring free radicals in the ischemic heart using electron paramagnetic resonance spectroscopy directly measuring free radical generation in perfused rabbit hearts [78, 79]. It was found that the total free radical concentration of the control was 4.7 ± 0.5 μM; after 10 minutes of ischemia concentration was 6.1 ± 0.4 μM, and after 10 seconds of reperfusion the concentration was 11.4 ± 0.6 μM. While this free radical increase was observed during ischemia and reperfusion, the duration of ischemia and reperfusion differs from a chronic ischemia/reperfusion model. However, more recent studies performed by Hill and Singal have consistently provided substantial evidence that oxidative stress is increased in heart failure following myocardial infarction and contributes to development and progression of the disease [80, 81], thus supporting the concept to investigate oxidative stress resistance of clonogenic rat CMCs.

Furthermore, one of the major problems identified with the efficacy of cell therapy is insufficient resistance of transplanted cells to oxidative and inflammatory stresses leading to poor survival. To investigate this, Zeng et al. pre-treated human umbilical cord derived mesenchymal cells (HUCMC) with edaravone and diethyl maleate and observed an increased resistance to oxidative and inflammatory stress and a greater amelioration of

hepatic injury compared to untreated HUCMC in an acute liver failure model in mice [82]. Of additional interest, it has been shown that oxidative stress-induced cells secrete more exosomes [77], which some believe to be implicated in ventricular functional improvement of the infarcted heart. If oxidative stress induces the release of therapeutic exosomes from rat clonogenic CMCs and these CMCs are also resistant to the oxidative stress, this would result in prolonging the efficacy of cardiac cell therapy. Interestingly, we discovered rat CMC clones that had a range of resistance to oxidative stress compared to the parental rat CMC population. We selected the three most and least resistant individual colonies (holoclones & paraclones), a batch of mixed clonogenic rat CMCs (meroclone) and parental rat CMCs for further experimentation. It is also possible that oxidative stress resistance in vitro has no correlation in vivo, making this selection process somewhat arbitrary.

Since many factors contribute to cell death outside of the lack of oxygen in the ischemic zone, we performed an additional screening of the rat CMC clones. Other contributors of cell death include deprivation of: nutrients, growth factors and survival factors. An interesting study in 2005 compared the effects of hypoxia and serum deprivation on bone marrow derived mesenchymal stem cells [83]. It was found that in all cases, serum deprivation resulted in a greater level of caspase-3 activity and more apoptosis than mesenchymal stem cells treated with hypoxic conditions. These results suggest that mesenchymal stem cells are far more sensitive to nutrient deprivation than reduced oxygen in vitro. They also performed a combined treatment of hypoxia and serum deprivation on mesenchymal stem cells and observed a modest additive effect on apoptosis and caspase-3 activity. Therefor in the current study we investigated if there was a correlation between

the results we observed in oxidative stress resistance and serum starvation in clonogenic rat CMCs.

Because we were selecting for proliferative rat CMCs, we calculated the growth rate of individual rat colonies. If resistance to oxidative stress or serum starvation is found to be important in vivo, then it is possible that growth rate could affect the composition of the parental CMCs that had previously shown to improve ventricular function [21]. For example, if clonogenic CMCs that are less resistant to oxidative stress do not improve ventricular function and have a faster growth rate compared to more resistant CMC clones, then it is possible that the parental CMCs have a larger concentration of cells that do not improve cardiac function, which could aid in explaining the low survival rate [23]. This would support the hypothesis that a subpopulation of CMCs exist that has a greater therapeutic efficacy.

In consideration that the therapeutic effects of cardiac cell therapy may be a result of paracrine stimulation, we also collected conditioned media from each of the selected CMC clones to test the stimulatory characteristics of the paracrine milieu released from each clone. In an interesting study in Nature Medicine, conditioned media from Akt-1 overexpressed mesenchymal stem cells (MSC) was collected and injected into the infarcted rat heart [84]. Compared to media collected from MSCs transduced with GFP, the Akt-1 overexpressed MSC conditioned media significantly reduced the size of the infarct. The same group saw the same benefit by transplanting Akt-1 overexpressed MSCs into the infarcted heart, supporting the possibility that paracrine stimulation is the source of action of cardiac cell therapy. While ventricular function is not addressed in this study, the reduction of infarct size warrants testing of conditioned media collected from clonogenic

CMCs. Finally, we also examined various cell markers with quantitative RT-PCR to discern any potential similarities between the selected CMCs.

In the current study, we also performed an in vivo experiment to compare ventricular functional improvement resulting from treatment of chronically infarcted rat hearts with either mixed clonogenic CMCs, Parental CMCs or PBS. Echocardiography was performed prior to occlusion, before transplant and 30-days after transplantation. After the 30-day echocardiography was completed, we then performed hemodynamics and euthanized the rats per the approved IACUC protocol.

We postulated that survival, proliferation and paracrine secretion would be markedly enhanced in mixed clonogenic CMCs and result in a significant improvement in ventricular function when transplanted into the chronically infarcted rat heart. Our results reveal significant cardiac functional improvement in rats treated with unsorted, parental CMCs, but it was found that rats treated with mixed clonal CMCs had no significant functional improvement. Furthermore, we observed that the mixed clonal CMCs from the selected rat were less resistant to oxidative stress, less resistant to serum starvation and produced a less stimulatory conditioned media compared to the unsorted, parental CMCs. These findings revealed that there are clonal CMCs within the parental CMCs that alone have reduced therapeutic value. Additionally, there is an implication of a positive correlation between our in vitro and our in vivo results. It is possible this correlation may pinpoint clonal CMCs within the parental CMC population that provide the therapeutic benefits of cardiac cell therapy. If CMC clones are discovered that have a greater therapeutic advantage, we can then look for unique identifying markers that enable us to more quickly harvest the beneficial CMCs in the future.

Materials and Methods

Explanting CMCs

To explant CMCs, we used the methodology previously described in the TERT experiments. Briefly, cardiac tissue was finely minced and then digested. The digested tissue was plated on a 100mm dish and allowed to adhere to the plate. We then harvested cells that grew out from the tissue on the plate which we used for these experiments. A similar methodology was used for CMCs harvested from rat tissue. Rat tissue was also incubated in complete media, but the media differed in composition.

Rat CMC complete media contained: Ham's F12 (Gibco) with 10% FBS (Seradigm), 5% Horse Serum (Gibco), 10ng/ml bFGF (Peprotech), 0.005 U/ml human erythropoietin (Sigma), 0.2 mM L-Glutathione (Sigma), 50 µg/mL Porcine gelatin (Sigma), 10 ng/mL Leukemia Inhibitor factor (Millipore) and 100 U/mL Penicillin-Streptomycin (ThermoFisher).

Clonogenic Assay

Explanted rat and human CMCs were grown to ~90% confluence and then passaged. Plates were washed with PBS and then TrypLE Express (ThermoFisher) was added to the plate. Plates were then incubated for 2-5 minutes at 37 ºC in 5% CO_2. A pipet was used to add CM and remove the media from the plate. The mix was transferred to a conical tube and then plunged with the pipet to help breakdown the remaining bonds between the cells. The mix was centrifuged at 900 g for 3 minutes. The supernatant was

removed and the cells were resuspended in 10 mL CM. Using a hemocytometer, the cell concentration was counted to determine the number of cells per mL.

The cloning density was established by performing a limited dilution technique [85]. Briefly, 1 mL of the resuspension was added to 9 mL of fresh CM in a separate conical tube and mixed well to provide a 1/10 dilution. This was repeated with each subsequent dilution to achieve a 1/100, 1/1000 and 1/10000 dilution. Cell dilutions were plated on 100mm plates and incubated at 37 ºC in 5% CO_2, allowing single cells to expand to large colonies, changing CM every 2-3 days. It was found that diluting the cells to 1000 cells per 100mm was the optimal number of cells for the cloning technique. Going forward, only 1000 cells were plated on a 100 mm plate to achieve the results of limited dilution.

Colonies formed were harvested using two separate methods (Figure 20). The first method was the mixed clonogenic population in which we allowed the plated cells that formed colonies to fully propagate the 100 mm dish. This dish consisted of all the cells that successfully formed colonies. We then continued to expand these cells and deemed them the “mixed” clonogenic CMCs. The second method was to isolate individual colonies formed from the clonogenic assay using a cloning ring technique [85].

To do this, individual colonies were identified using microscopy by marking the colony location and borders on the underside of the plate with a marker. Then we coated the edge of autoclaved glass rings with sterile vacuum grease (Dow Corning) and then pressed the greased edge firmly down on the plate so that it surrounded an individual colony (Figure 20). This was repeated for 20-30 colonies. The media within the rings was aspirated and the colonies harvested by washing with PBS and treating with TrypLE Express (as previously described). Cells within the rings were transferred to their own well

to continue expansion. Upon expansion, each surviving colony received an ID. All rat colonies were photographed to compare morphology between individual colonies. Additionally, cloning efficiency was calculated by (# of cells counted/(Volume plated*dilution of the volume))*100 to achieve the value for Colony Forming Units (i.e. the percentage of cells plated that form a colony). Furthermore, colonies were separated by size: Small (3-20 cells), Medium (21-50 cells) and Large (>50 cells) using a previously defined method [86].

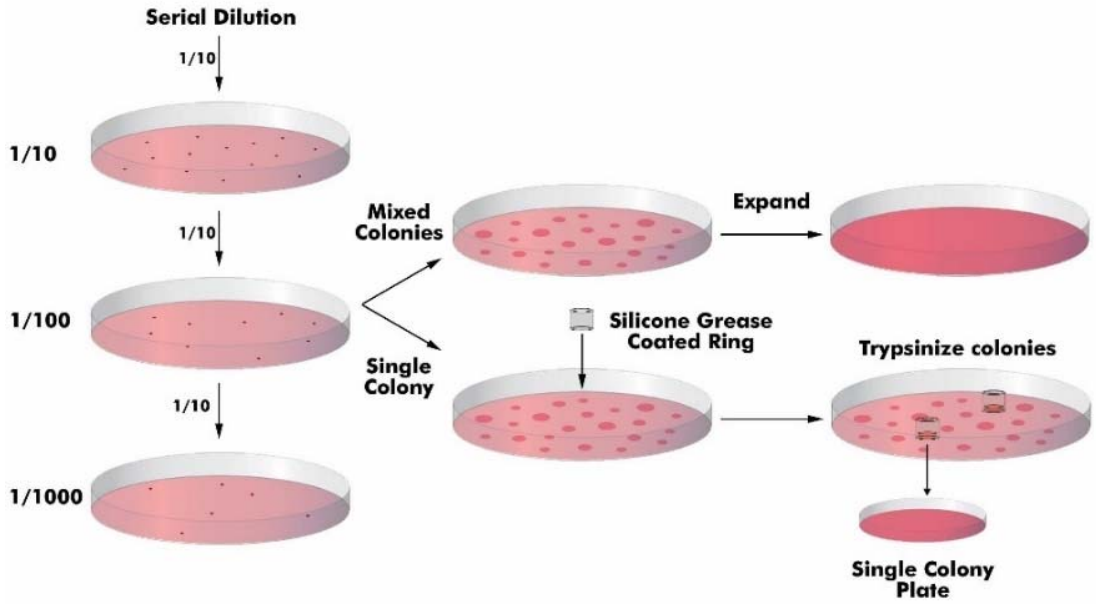


Figure 20 – We performed cloning in two different ways. First, we performed a serial dilution to identify the number of cells required for cloning. It was found that 1000 cells plated on a 100 mm dish was ideal for the cloning technique. Then, we had either Mixed Colonies or Single Colonies. For the Mixed Colony, or Meroclone, we plated 1000 cells and then allowed the surviving colonies to expand to confluence. For the single colonies, clones were grown and then a glass cloning cylinder coated with silicone vacuum grease was used to trypsinize and separate the colonies.

Oxidative Stress by DMNQ

To test resistance of oxidative stress on CMC clones, $1x10^4$ cells from each clone isolated from the parental CMCs, the mixed clonal CMCs and the parental CMCs were plated in quadruplicate on 96-well cell culture plates. DMNQ was added to complete media to a final concentration of 1.5 μM (per the determined IC50 concentration). The next day, PrestoBlue (Invitrogen) was used following the manufacture's protocol and viability of the CMCs was measured by absorption at Ex/Em 560/590 nm on a Biotek Synergy system. The results were graphed as a percentage of the 0.0 μM well to determine the percentage of remaining viable cells.

Population Doubling Time

Population doubling time was performed using a similar strategy used in the TERT project. Selected clonogenic CMCs were grown to 90% confluence and then passaged (as previously described). After resuspension of the pellet of cells, CMCs were counted using a hemocytometer (Bright-Line). After counting, $1x10^6$ CMCs were plated on 100mm dishes. This was repeated three times. To calculate doubling time, the number of CMCs counted was divided by the number of cells plated, then the number of days between passaging was divided by this number to provide the number of days it takes to double. The doubling time for each was averaged over 3 passages to provide the mean doubling time.

Serum Deprivation Assay

We then wanted to test the clonogenic CMC's resistance to nutrient deprivation. For this protocol, we plated $1x10^5$ cells in 6-wells of a 6-well plate and allowed them to adhere to the wells overnight. The next day, 1 well of cells was passaged and counted with a hemocytometer to verify accuracy and efficiency of cells plated per well. In 3 of the other wells, the media was changed to basal media. The remaining well was maintained in CM. Cells were incubated in the basal media at 37 ºC in 5% CO_2 for 3 days. Cells were then passaged and counted using a hemocytometer in triplicate. The average number of cells surviving were compared between clones.

Conditioned Media Production

To examine the paracrine milieu produced by the clonogenic cells, we plated $1x10^6$ of each of the selected clones on a 100mm plate. 24hr later, the media was aspirated from the plate and the plate was washed with 1X PBS. Then, 5mL of basal media was added to each plate. Cells were incubated at 37ºC in 5% CO_2 for 24 hours. The media was then harvested and placed in a 15mL conical tube and labelled respectively. We performed a bicinchoninic acid assay (Pierce), following the manufacturer's protocol, to determine the protein concentration of the conditioned media to verify that there was no significant difference in concentration of media produced. Media was stored at 4 ºC for later use.

Endothelial Tube Formation Assay

An Endothelial Tube Formation Assay (Invitrogen) was performed per the manufacturers protocol. Briefly, cryopreserved Human Umbillical Vein Cells (HUVEC)

cells were plated in a 75 cm^2 culture flask and allowed to expand to ~80% confluence (about 5-6 days) in EBM-Plus media (Lonza). Geltrex (Gibco) was then thawed overnight on ice in the 4 ^oC refrigerator. The next day, 100-150 µl of Geltrex was added to each well of a 24-well plate and incubated at 37 ^oC for 30min to allow it to solidify. After 30 minute, $8x10^4$ cells suspended in 250 µl of media were added to each well. The media used for resuspension of HUVECs included Complete Media as a positive control, F12 basal media (Gibco) as a negative control and media from each of the selected clones. After plating the cells on Geltrex-coated wells, the cells were incubated at 37 ^oC in 5% CO_2 for 4 hours. Photos of tube formation were taken using an Epifluorescence microscope. Tube formation was assessed by counting the number of tubes formed in four fields to arrive at the mean tube formation stimulated by the respective media.

HUVEC Migration Assay

To test migration, $1x10^4$ HUVEC cells were plated on Boyden chambers containing transwells with 8 µm pore size (Corning). After 24 hours, the media in the upper chamber was replaced with non-supplemented, serum-free Ham's F12 media (Gibco) and the bottom chamber was replaced with either: Ham's F12 media (Gibco) containing only 2% FBS (Seradigm) as a positive control; Ham's F12 basal media as a negative control; and conditioned media from the selected colonies for the experimental groups. This was performed in duplicate. After 24 hours, cells that migrated to lower surface of the transwell membrane were fixed with 3.7% formaldehyde for 10 min. Cells remaining in the upper chamber were removed using a cotton tip. Fixed cells were permeablized with 0.25% Triton X-100 in 1X PBS for 10 min and then incubated with 50ug/ml of propidium iodide

solution for 10-15 min. Images were taken using the EVOS FL Cell Imaging System (Life Technologies). Migrated HUVECs were quantified.

Quantitative PCR Analysis

mRNA was harvested from cells, treated with DNase (Qiagen) and purified using the RNeasy Kit (Qiagen) following the manufacturers protocol. cDNA was synthesized from the mRNA using the AffinityScript qPCR cDNA Synthesis Kit (Agilient). qPCR analysis was then performed on samples in duplicate. The human primers used in this study were previously described in the TERT study. The rat primer pairs used include:

Rat Primers

Marker	Primers
Tert	AGCCTTTCTCAGCACCCTGGTC, GCCGGTCTCCACAGGGAAGT
αSma	AGAAGCCCAGCCAGTCGCCAT, CTGCGCTTCGTCCCCCACAT
Sm22α	AGGTGCCTGAGAACCCGCCC, GCCGGGGTCGCCCATAGC
Thy1	GGGTGCAGCAACCAGAGGCG, AAGGTTGACGCGGGAGCGG
Fsp1	GGCGAGACCCTTGGAGGAGG, TCCGGGGCTCCTTATCTGGGC
Ddr2	CCATGCAGGGGGTCATGGCA, TGCCCAGCGGGTGCATTGTA
Vegf	AAAACACAGACTCGCGTTGC, ACTCCCTAATCTTCCGGGCT
Kdr	AAAGAGAGGGACTTTGGCCG, GTCGCCACTTGACAAAACCC
Tnnt2	AAGCTCTGTTCCTTGCCTGTGC, CGTGGTGTGGGCATAGGGGT
Myh6	GCGGGCCAAGAGCCGTGAC, GCGAGGCTCTTTCTGCTGGACAG
18S	GGAAGGGCACCACCAGGAGT, TGCAGCCCCGGACATCTAAG

Table 2: This table provides a detailed list of primers designed for rats and their specific bases

Results were analyzed using SDS 2.4 software. Dissociation curves were compared to controls for accuracy and CT values were compared among duplicates. If dissociation curves did not align with the positive control, the results were deemed as undetected. If CT values were dramatically different between duplicates, the experiment was repeated for validation.

Experiment Myocardial Infarction and Cell Injection

This portion of the study was performed in collaboration with Dr. Xian-Liang Tang. The methods were like those described previously (Tang *et al., Circulation 2010*). Female Fischer 344 rats (3-4 months of age) were anesthetized with ketamine (37 mg/kg) and xylazine (5 mg/kg), intubated, and ventilated with a rodent respirator (Harvard Apparatus). Anesthesia was maintained with 1% isoflurane inhalation and body temperature was closely monitored and maintained at 37 °C with a heating pad. After administration of antibiotics, the chest was opened and the heart exposed. All rats underwent a 2-hr occlusion of the left anterior descending coronary artery followed by reperfusion. Thirty days after surgery, the animals were randomly allocated to vehicle control group or wild-type CMC-treated group or mixed clonogenic CMC-treated group. Rats were reanesthetized, the chest was reopened, and rats received 6 intramyocardial injections of either vehicle, wild-type CMC, or clonogenic CMC ($1x10^6$ cells suspended in 300 µL of PBS, 50 µL administered in each injection) around the infarct borders using a 30G needle. Rats were euthanized 35 days after injection.

Echocardiography and Hemodynamic Measurement and Analysis

These experiments were performed in collaboration with Dr. Xian-Liang Tang. Serial echocardiograms were obtained at baseline (three days before coronary artery occlusion), 30 days after MI (before treatment), and 35 days after treatment using Vevo2100 Imaging System equipped with a 20 MHz transducer. Before echocardiography, rats were lightly anesthetized with 3% isoflurane. The anterior chest was shaved and the animals placed in the left lateral decubitus position. A rectal temperature probe was inserted, and the body temperature carefully maintained between 37.0 °C to 37.5 °C with a heating pad throughout the study. Anesthesia was maintained with 1% isoflurane inhalation. The parasternal long-axis and parasternal short-axis views were used to obtain 2D and M-mode images for the measurement of the LV mass, LV wall diastolic and systolic thickness of the interventricular septum (IVSd and IVSs, respectively) and posterior (PWd and PWs), Infarcted and posterior LV wall thickening fraction (IWThF and PWThF), end-diastolic and end-systolic volume (LVEDV and LVESV), stroke volume (SV), cardiac output (CO), ejection fraction (EF), and fractional area change (FAC). All measurements were performed in a blinded fashion per the American Society for Echocardiology, and averaged over 3 consecutive cardiac cycles.

The hemodynamic studies were performed 35 days after treatment, just before euthanasia. Rats were anesthetized with ketamine (37 mg/kg) and xylazine (5 mg/kg), intubated, and mechanically ventilated. Anesthesia was maintained with 1% isoflurane and the core temperature kept at 37.0 °C with a heating pad throughout the study. A 2 F microtip pressure-volume (PV) catheter (SPR-869, Millar Instruments) was inserted into the right carotid artery and advanced into the LV cavity. The right jugular vein was

cannulated for fluid administration. After 20 min of stabilization, the PV signals were recorded continuously with an ARIA PV conductance system (Millar Instruments) coupled with a Powerlab/4SP A/D converter (AD Instruments), stored, and displayed on a personal computer. PV relations were assessed by transiently compressing the inferior vena cava with a cotton swab. Parallel conductance from surrounding structures were calculated by injecting a small bolus of 15% NaCl through the jugular vein. Hemodynamic indexes were calculated using the PVAN 3.2 software (Millar Instruments).

Results

Isolation of Clonogenic Cells

We successfully isolated 23 clones from human CMCs and 20+/- clones from CMCs of three different rats. A Mixed Clonal population was created in all three rats. Photographs were taken at different time points throughout the cloning process (Figure 21). All surviving clones were expanded and photographed so that morphology could later be compared. Then, clonogenic CMCs were passaged and suspended in freezing media containing 10% DMSO and then placed in a CoolCell (VWR) in the -80ºC freezer. 24 hours later, the frozen cells were transferred to liquid nitrogen and thawed as needed.

The efficiency of our cloning process was determined by plating 1000 cells on a 100 mm dish. We allowed the colonies to grow 7-10 days prior to fixing them with 3.7% formaldehyde and stained them with crystal violet. We counted the colonies and categorized them by size [86]. Since the colonies isolated with the cloning ring were

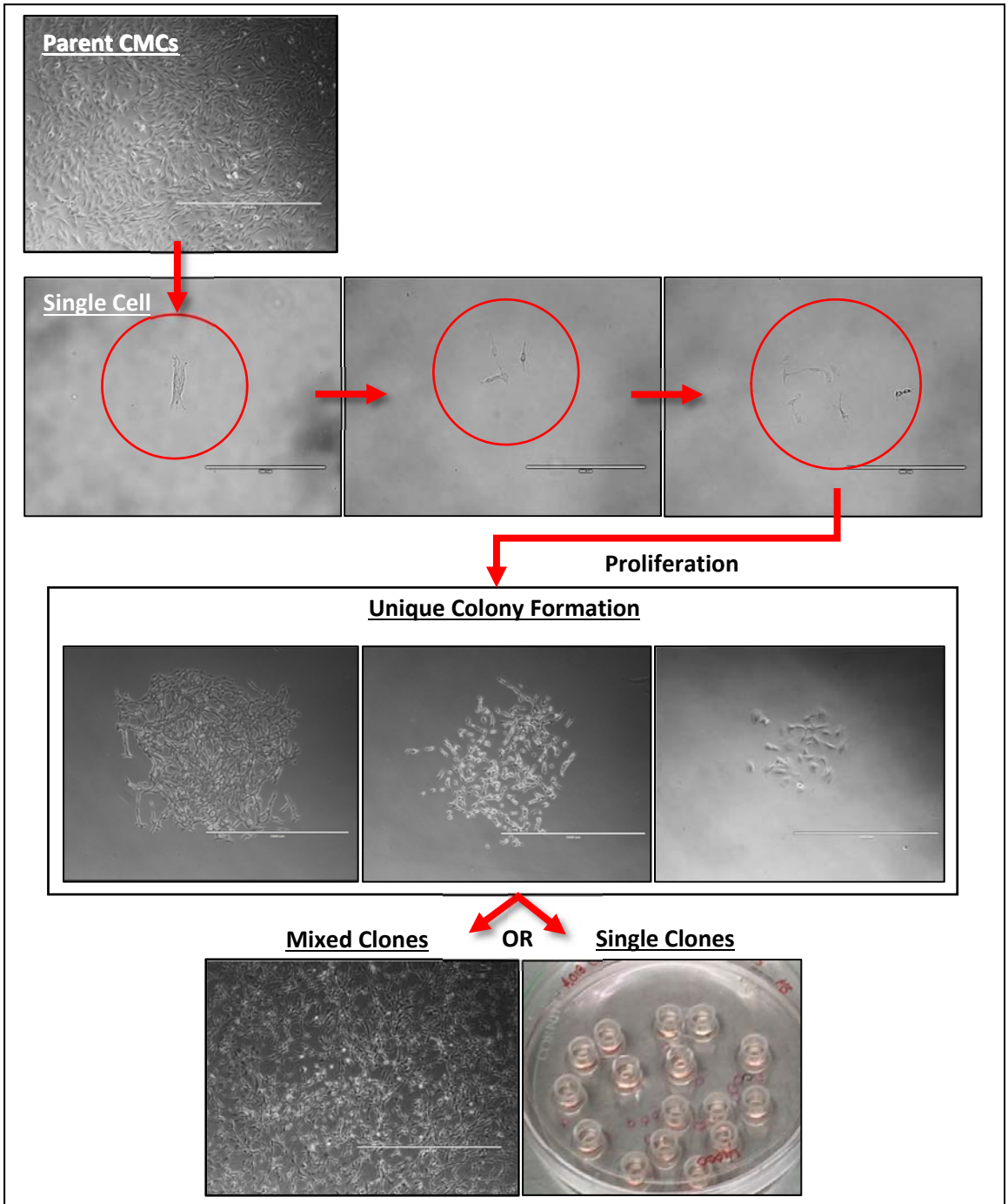


Figure 21 – This figure illustrates the cloning technique. We started with the Parent CMCs, freshly explanted from the tissue. Cells were diluted so that single cells adhered to the plate. Only the clonogenic cells proliferated into colonies, while non-clonogenic cells die. The colonies are either allowed to expand to a Mixed Clone plate or individual colonies were isolated using cloning cylinders, as seen in the bottom set of images.

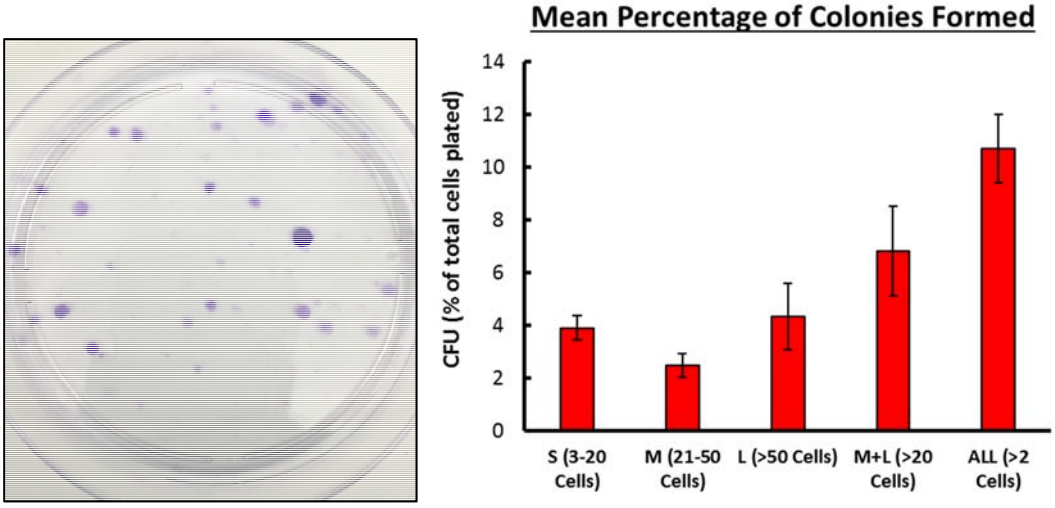


Figure 22 - Cloning efficiency was then calculated by plating 1000 cells and allowing them to form colonies. Colonies were then fixed to the plate with 3.7% formaldehyde and stained with crystal violet (left). Colonies were quantified by size in three rats and then provided as a Colony Forming Unit mean. Colonies were divided into sizes S (3-20 cells), M (21-50 cells) and L (>50 cells). Clones selected by the cloning cylinder were primarily M+L in size.

medium or large, we concluded that our efficiency was around 8% (Figure 22). Our efficiency was found to be consistent with other studies [87].

Human Clonogenic CMC Gene Expression

As previously stated, it was believed that if a sub-population of cells expresses

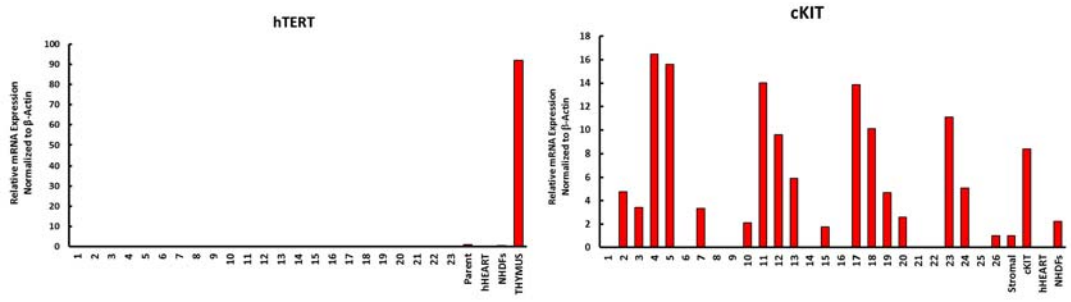


Figure 23 - Human Clonogenic CMCs from a single patient were analyzed using quantitative RT-PCR. hTERT was not detected among the human clones (left). C-kit was detected at varying levels among human CMC clones (right). Only 23 clones were investigated for hTERT, whereas 3 additional clones were investigated for other markers.

TERT it would likely be represented in the clonogenic population. Consistent with our previous study, we did not detect hTERT in human clonogenic CMCs (Figure 23). Other markers confirmed that these clones represented unique populations and are from a mesenchymal stromal cell lineage (Figure 23, Figure 24, Figure 25).

Since hTERT was not detected in the human clones, our testing of clonogenic cells shifted to rat isolates. Survival of the clonogenic assay alone is a selection for CMCs with enhanced survival characteristics. For that reason, we expedited an in vivo study of clonogenic CMCs in the rat model. Thus, we performed an in vivo study and in vitro studies in rat clonogenic cells simultaneously.

Resistance to Oxidative Stress Induced by DMNQ in Rat Clonogenic CMCs

The first of our in vitro experiments in the rat clonogenic CMCs was to test their resistance to oxidative stress. We initially induced oxidative stress by treating cells with varying concentrations of H_2O_2. However, we found the results to be inconsistent among the replicates of the same cell line. Therefore, we used a cell-permeable, non-alkylating redox cycling quinone called 2,3-Dimethoxy-1,4-naphthoquinone (DMNQ). DMNQ works by generating intracellular reactive oxygen species. We found our results with DMNQ to be far more consistent among replicates providing a higher level of confidence in the data.

Among rat clonogenic CMCs, it was found that clones have varying levels of resistance to oxidative stress induced by 1.5 μM DMNQ (Figure 26). Compared to the parental CMCs (non-clonogenic cells), some clones exhibited significantly higher resistance to oxidative stress while others had significantly lower resistance. Interestingly,

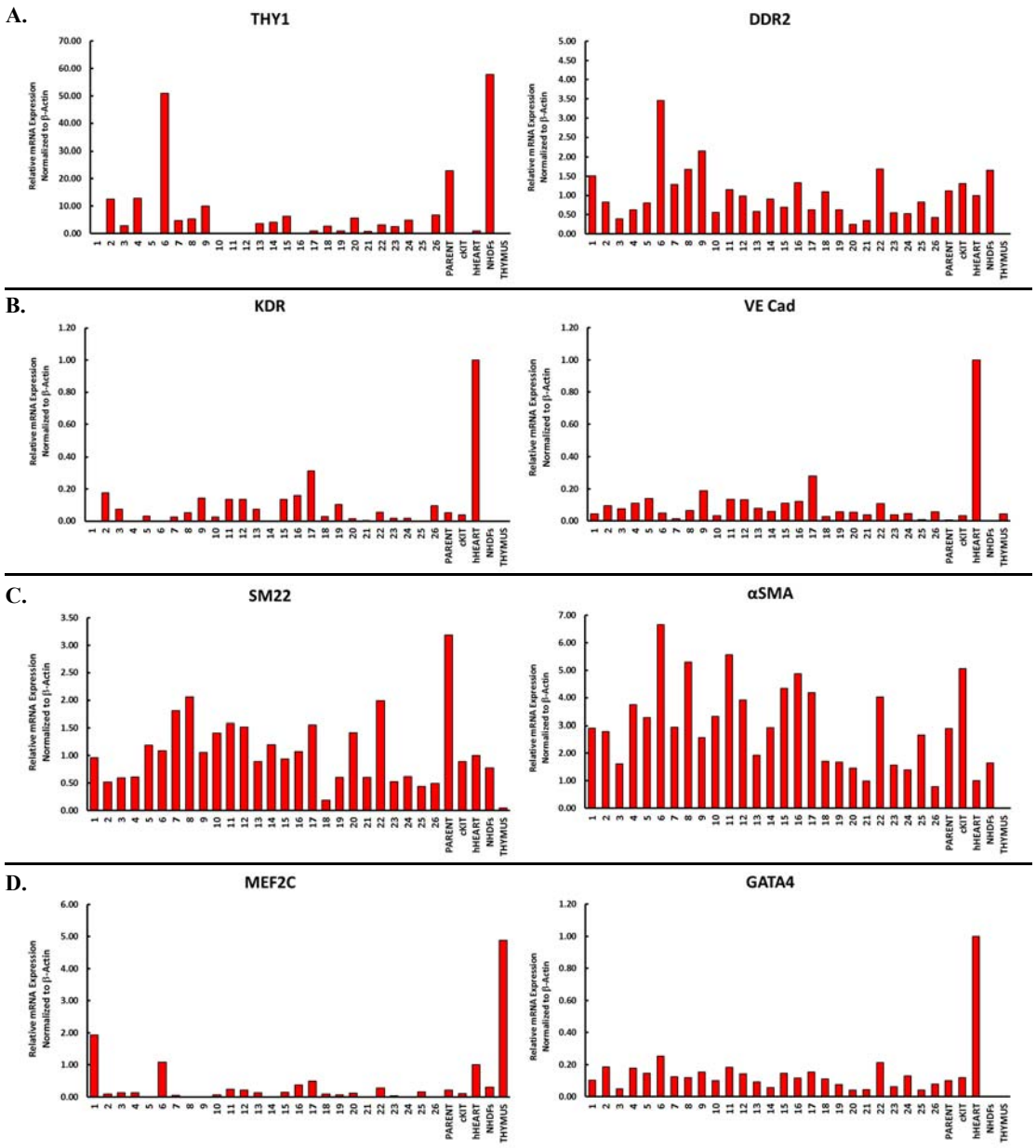


Figure 24 – Quantitative RT-PCR was performed on 26 different clones and compared to mRNA harvested from the whole heart. **A.** Varying levels of fibroblast markers were detected among clones. #6 seems to have a high expression compared to the others CMC clones. **B.** Endothelial cell markers were expressed at low levels compared to the whole heart. **C.** Smooth muscle cell markers are also variable and appear to express higher levels than the whole heart. **D.** Developmental markers have low expression.

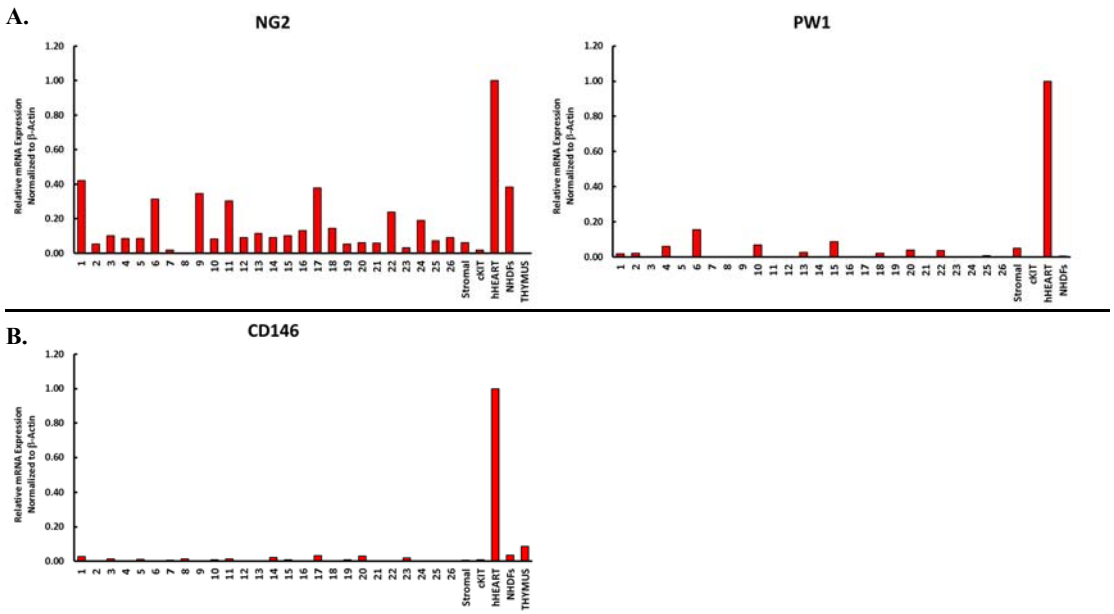


Figure 25 - **A.** Pericyte marker (left) and progenitor cell marker (right) were detected at very low levels in clonogenic CMCs. **B.**CD146, another progenitor marker, was also expressed at very low levels in clonogenic CMCs.

the mixed clonogenic CMCs from the R18 rat, which also happened to be the clonogenic CMCs selected for the in vivo study, had a significantly lower resistance to oxidative stress compared to the parent CMCs. The R19 and R20 rat mixed clones were significantly more resistant, suggesting that the mixed cloning technique may not be a reliable method of selection. The R18 rat results will be discussed in more detail later in this this study. We then selected the three most resistant and three least resistant clones from each rat for further experimentation. Although clones were selected as "least resistant," this did not always mean clonal CMCs were less resistant to oxidative stress than parental CMCs.

Growth Rate in Rat Clonogenic CMCs

After selecting the three clones that were most and least resistant to oxidative stress,

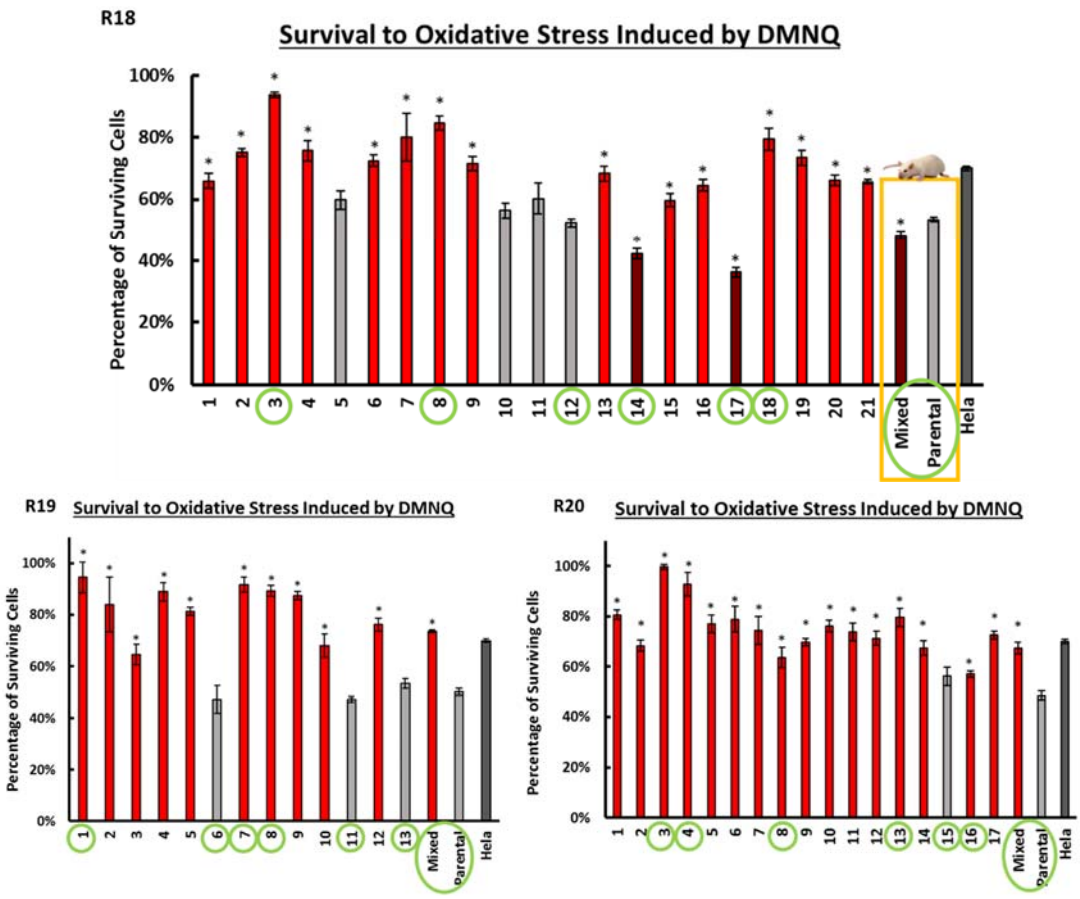


Figure 26 - Rat clones from three different rats were subjected to oxidative stress induced by 1.5 µM DMNQ. Each figure is labelled in the top left corner respectively, they include R18, R19 and R20. There is a variety of resistance to oxidative stress. The clones circled were selected for additional experiments. These clones represent the 3 most and least resistant CMC colonies. In the R18, the Mixed Clone and Parental Clone were used for in vivo experimentation.

we calculated the growth rate of the selected CMCs over three passages. Generally, there was no difference in the growth rate observed between clones. Although, one of the most resistant clones in the R18 rat and the R20 rat had a significantly slower growth rate than the parental CMCs (Figure 27A). It was also observed that in many cases, the lease resistant clones trended toward having a faster growth rate. This is important because if oxidative stress resistance correlates with in vivo functional improvement, then this would mean that cells with the least resistance make up a larger percentage of the parental population

because the calls can proliferate at a greater rate. Therefore, only a small fraction of CMCs would exist that result in the therapeutic benefits observed implying that transplanting a greater quantity of only this subpopulation of cells would result in a greater functional improvement.

Serum Deprivation in Rat Clonogenic CMCs

Next, we subjected the selected clones to serum starvation to determine if resistance to oxidative stress correlated with survival to nutrient deprivation. It was found that selected clones had similar viability after being subject to serum deprivation for 3 days (Figure 27B). It was also found that the mixed clonal CMCs from the R18 rat were significantly more sensitive to serum starvation than the parental CMCs, but this seemed coincidental. There was no noticeable viability trend among the clonal populations. In fact, one of the least resistant clones in the R18 rat was significantly more sensitive to serum starvation. At the same time, one of the least resistant clones in the R19 rat was significantly less sensitive to serum starvation.

Gene Expression in Rat Clonogenic CMCs

Quantitative RT-PCR was performed on the selected clones to identify any potential trends between populations of clonal CMCs. It was found that in every case, the least resistant CMC clone selected from each rat expressed no smooth muscle cell markers (Figure 28) and very little fibroblast markers compared to other CMC clones (Figure 29, Figure 30). It was also found that all of the least resistant CMC clones among all rats had similar levels of expression of all markers examined (Figure 28 Figure 29 Figure 30).

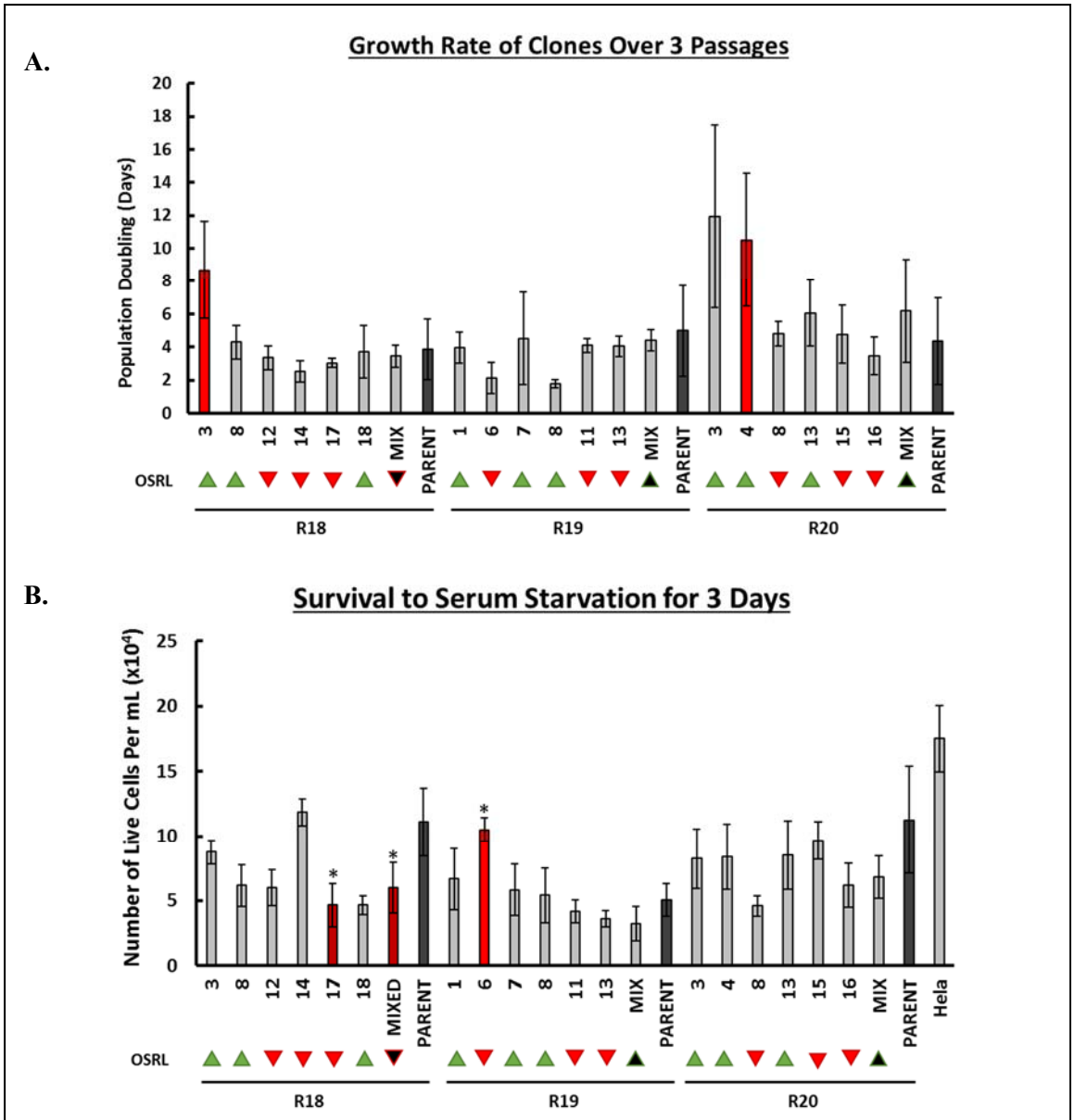


Figure 27 - In both A & B, the Oxidative Stress Resistance Level (OSRL) is indicating the level of resistance to oxidative stress by which the clone was selected. Up = Most resistant, Down = Least **A.** Population doubling (growth rate) was tracked over three passages in the selected clones from all 3 rats (R18, R19, R20). The R18 #3 clone and the R20 #4 clone doubled significantly slower than the parental cell line. **B.** Selected clones from all three rats were also subjected to serum deprivation. After 3 days of serum starvation, the remaining, viable cells were quantified by counting the number of cells using a hemocytometer. In the R18 rat, the #17 and & Mixed clone were significantly less resistant to serum starvation. Whereas in the R19, the #6 clone was significantly more resistant to serum deprivation.

Additionally, the mixed clonal CMC populations had similar expression and in some cases was very similar to the least resistant CMC clones. Other CMC clones had varying levels of expression with no apparent trend. If resistance to oxidative stress does correlate with ventricular functional improvement, this may help us to define the specific sub-population of CMCs that provide therapeutic benefit. If we can identify a unique set of markers with the most resistant CMCs, this would eliminate the need for the arduous selection process.

Endothelial Tube Formation Assay with CMC Conditioned Media

After culturing one million clonal CMCs in basal media overnight, the conditioned media was collected. This was performed in each of the selected CMCs from all three rats. The conditioned media from the R18 rat was used in an endothelial tube formation assay to determine if any of the CMC clones produced a more stimulatory conditioned media. Because of the sensitivity of the HUVEC cells, this assay was performed multiple times. We first examined the quantity of protein in the conditioned media to verify that the differences observed were not simply a result of a variation in the quantity of factors released from the CMCs. Our BCA assay revealed that there was no significant difference in protein concentration between the conditioned media produced by each CMC clone (Figure 31).

Once no difference in protein concentration in collected media was confirmed, we proceeded forward to test stimulation of endothelial tube formation using the conditioned media (Figure 32). Most of the clonal CMCs did not cause a significant increase or decrease in endothelial tubes formed, but this lack of significance may have been a result of too few fields analyzed (only 2 fields in this experiment). Despite that, the #8 CMC clone did

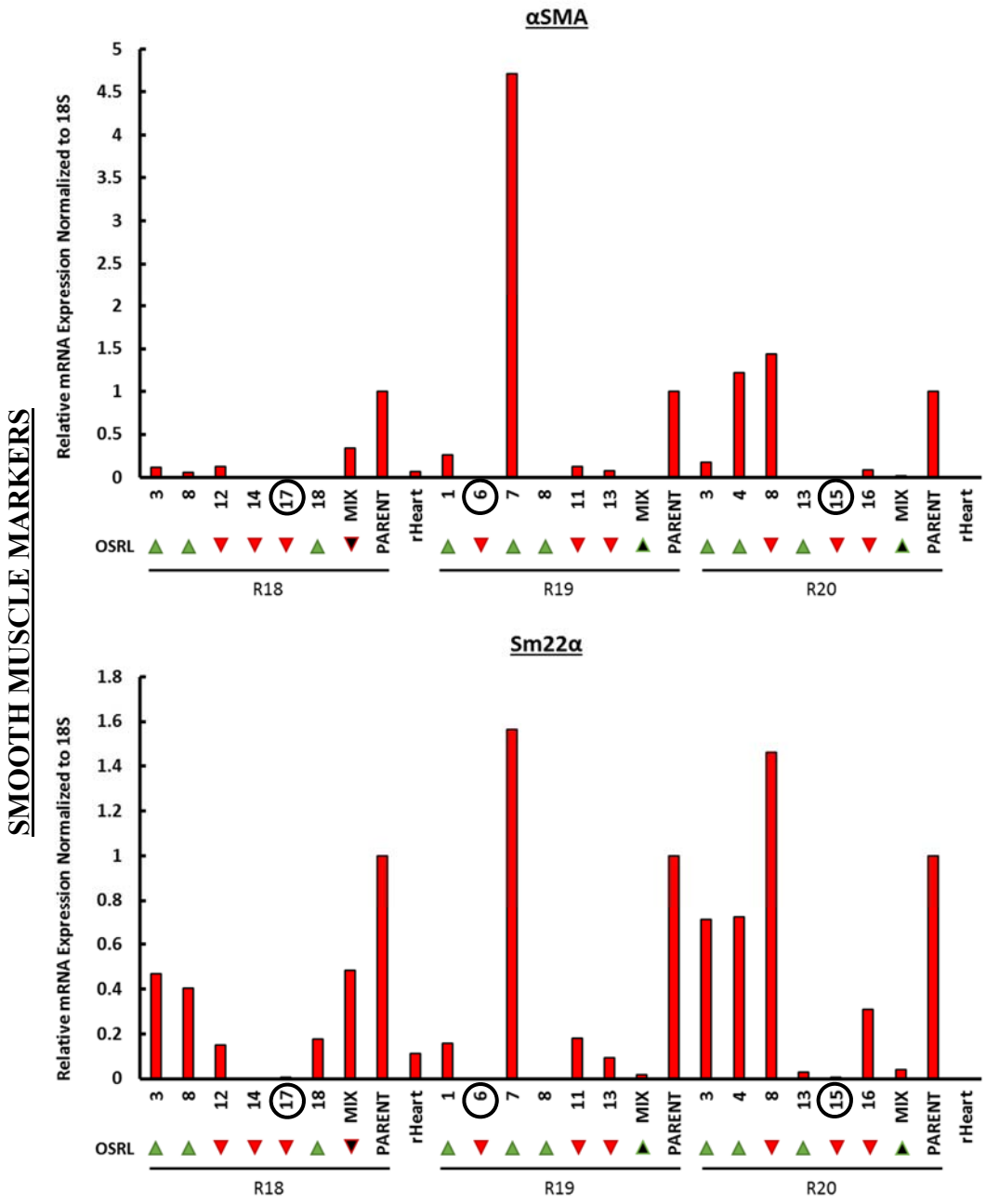


Figure 28 - Smooth muscle markers were variable among CMC clones in 3 rats (R18, R19 & R20). The least resistant CMC clone in each rat (circled) consistently had incredibly-low/undetectable levels of smooth muscle markers. Oxidative Stress Resistance Level (OSRL) is indicating the level of resistance to oxidative stress by which the clone was selected. Up = Most resistant, Down = Least

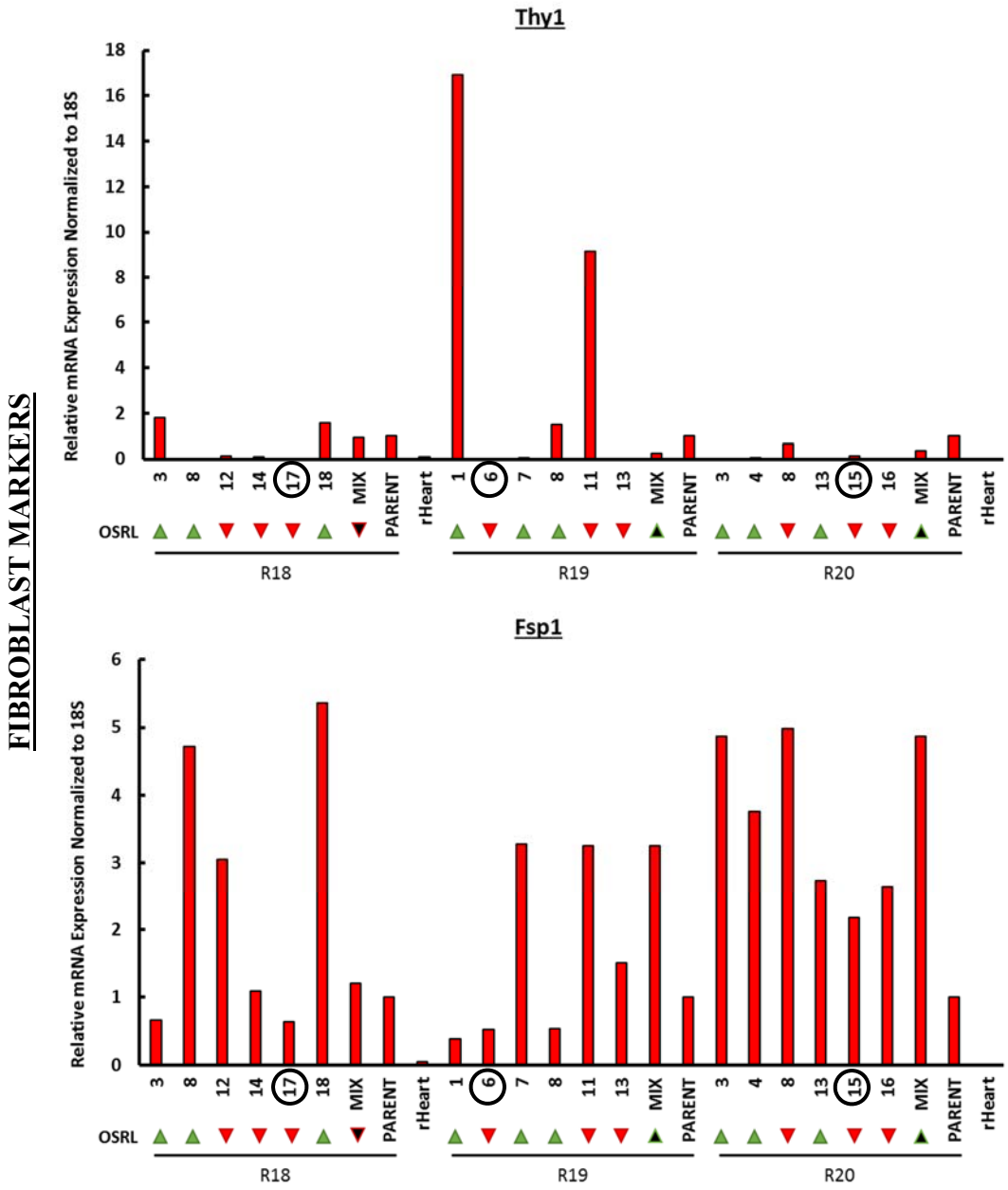


Figure 29 - Fibroblast markers were variable among CMC clones in all rats (R18, R19, R20). The least resistant clone in each rat (circled) consistently had similar detection of Thy1. Unlike fibroblast markers Thy1 of DDR2 (Figure 30), Fsp1 had variable levels of detection. Oxidative Stress Resistance Level (OSRL) is indicating the level of resistance to oxidative stress by which the clone was selected. Up = Most resistant, Down = Least

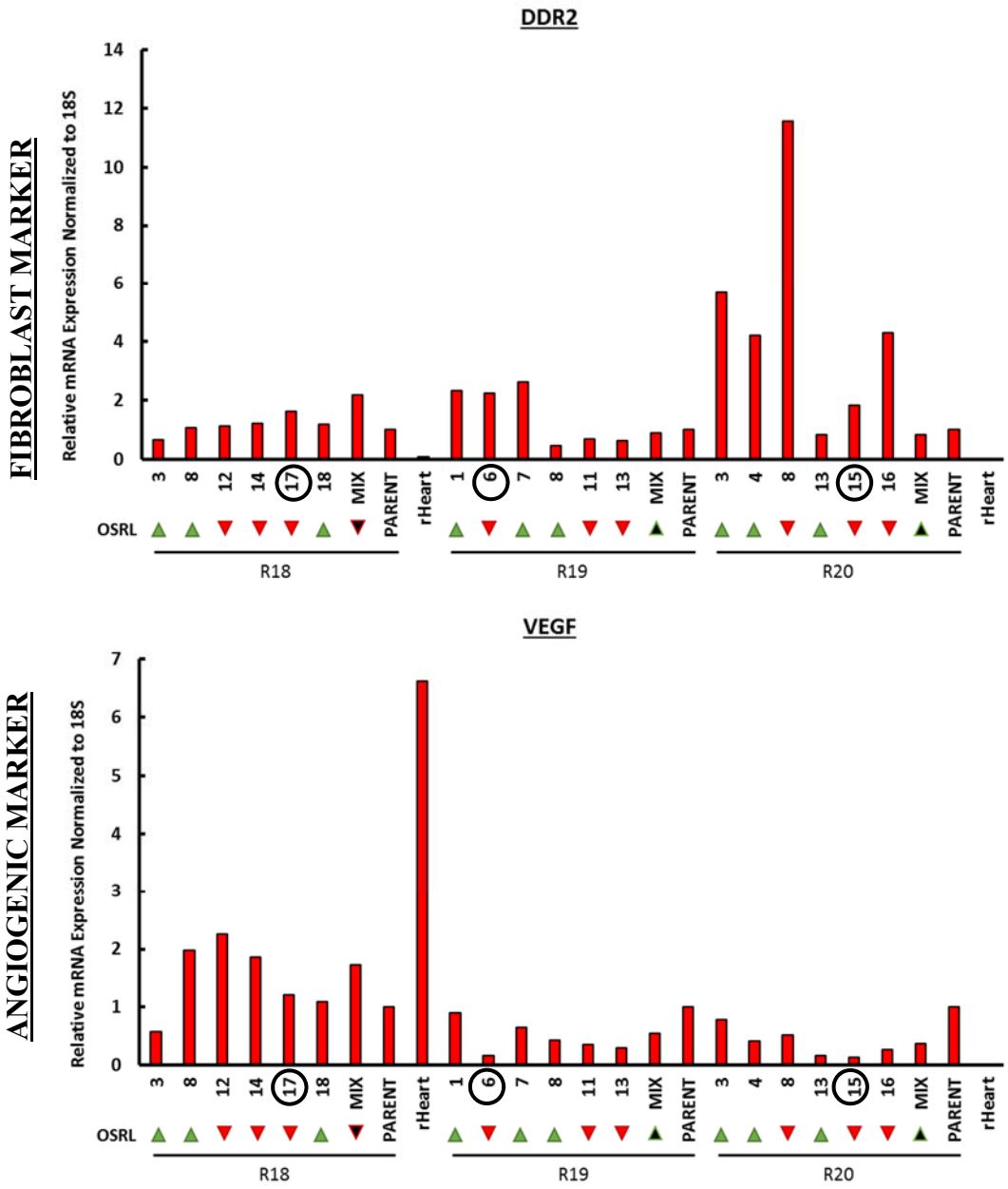


Figure 30 – The least resistant clone in each rat (circled) consistently had similar detection of DDR2. Angiogenic marker VEGF was relatively similar among all clones, but the least resistant in all rats (circled) had a lower detection of VEGF. Oxidative Stress Resistance Level (OSRL) is indicating the level of resistance to oxidative stress by which the clone was selected. Up = Most resistant, Down = Least

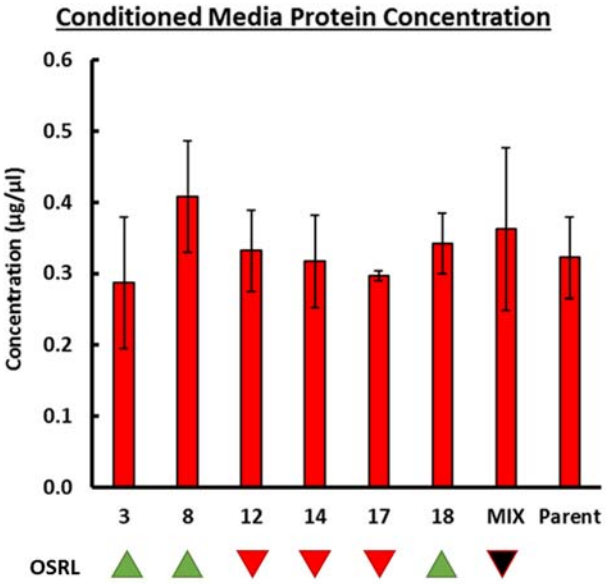


Figure 31 - Conditioned media was collected from each of the selected R18 CMC clones. The protein concentrations of the collected conditioned media were measured using a BCA assay. No significant difference in concentration was detected between the conditioned media from the selected CMC clones. Oxidative Stress Resistance Level (OSRL) is indicating the level of resistance to oxidative stress by which the clone was selected. Up = Most resistant, Down = Least

cause significantly more endothelial tube formation than the parental CMCs (Figure 33). Of added interest, the Mixed CMC Clone was close to being significantly less stimulatory. For that reason, we repeated the experiment specifically looking at the Mixed Clone compared to the parental cell line. It was found that the Mixed Clone did indeed significantly stimulate the formation of fewer endothelial tubes (Figure 34). This again suggests that the Mixed Clonal CMC population in the R18 rat are CMCs. The results indicate that subpopulations do exist in the CMCs that have differential paracrine milieu, identifying another potential distinguishing factor between clones.

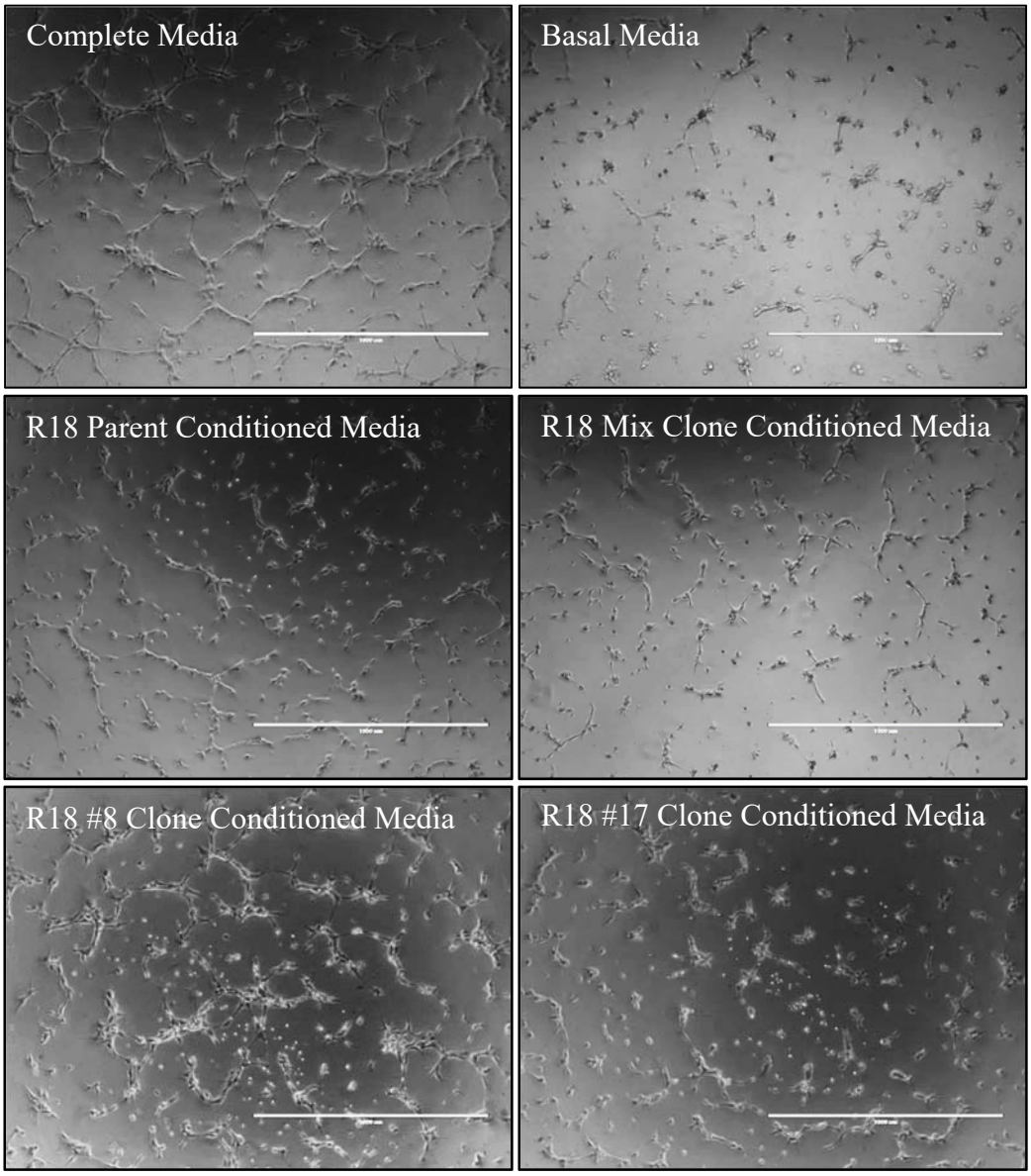


Figure 32 - HUVEC cells plated on geltrex-coated plates were incubated in conditioned media from selected CMC clones for 4 hours. Then, representative photos were taken from different fields for each well treated with conditioned media, compared to Complete Media and Basal Media. These images are representative of the variation of tube formation results observed.

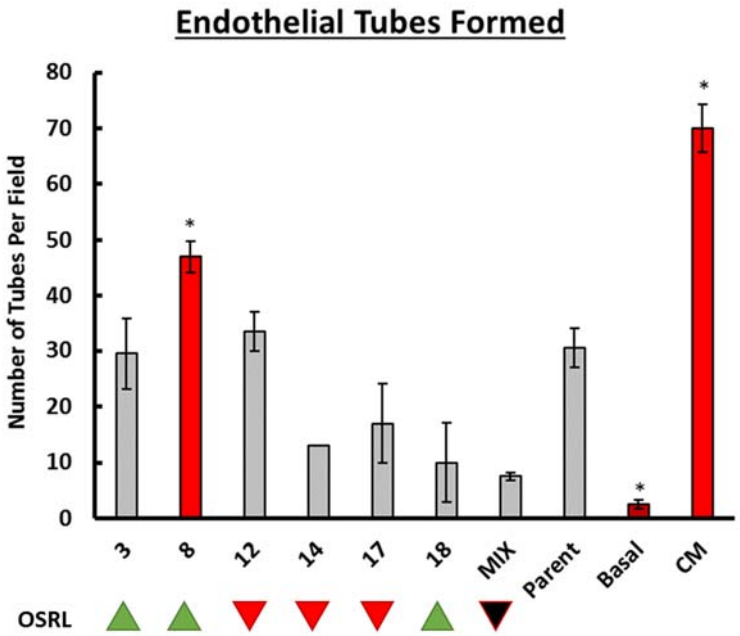
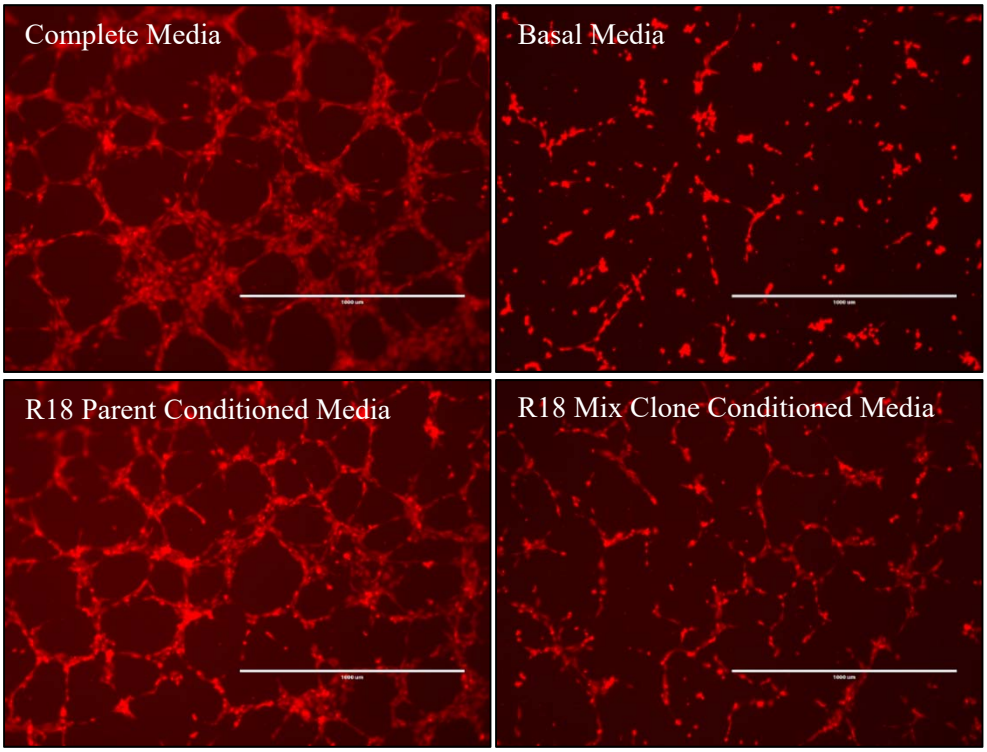


Figure 33 - Tube formation was quantified by counting the number of tubes formed in two separate fields (n=2). The #8 clone had significantly more tubes formed compared to parental media. Basal media is growth factor (GF) free media while Complete Media (CM) had GFs. Oxidative Stress Resistance Level (OSRL) is indicating the level of resistance to oxidative stress by which the clone was selected. Up = Most resistant, Down = Least

HUVEC Migration Assay

To further examine the conditioned media, we performed a Boyden chamber assay. We plated HUVEC cells on the semi-permeable membrane of a transwell plate and a tested the ability of conditioned media from the R18 selected clones to stimulate migration across the membrane. In most cases, there was no significant difference in migration compared to the parental CMC conditioned media (Figure 35). The R18 #18 CMC clone conditioned media did cause significantly more migration than the parental CMC conditioned media (Figure 36). Coincidentally, the R18 #18 CMC clone was also selected as one of the most resistant CMC clones. Other conditioned media from the most resistant clones appeared to be trending toward having more migration, but it was not significant compared to



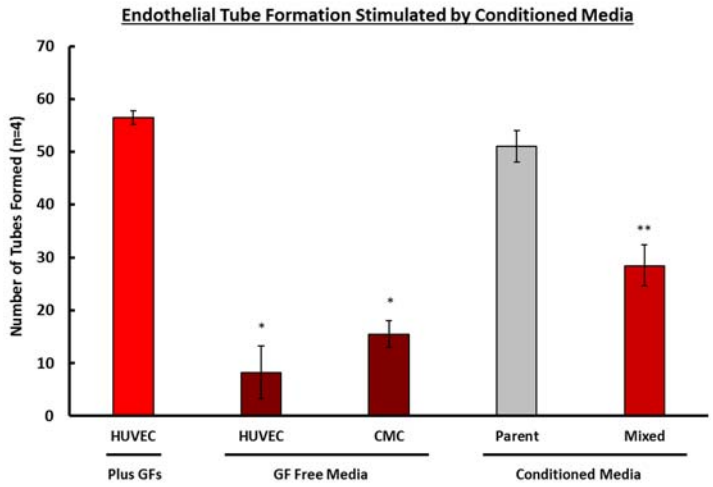


Figure 34 – The endothelial tube formation assay was repeated. HUVEC media with GF was used as a positive control and GF-free media were the negative controls. Mixed CMC clonal conditioned media formed significantly less tubes than the parental cell media. In this experiment, we analyzed 4 fields per sample (n=4).

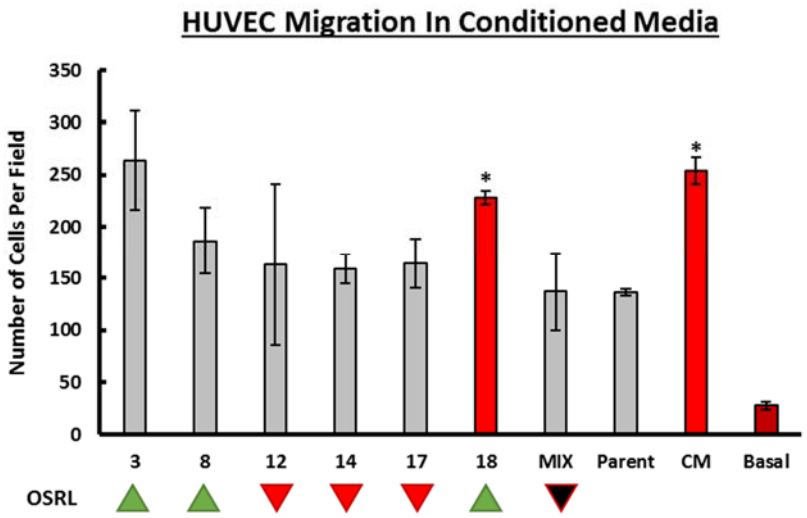


Figure 35 - HUVEC cells were plated on a transwell plate. Conditioned media from the R18 selected CMC clones was used an attractant and CMCs were allowed to migrate overnight. There was no significant difference among most R18 CMC clones, but the #18 CMC clone had significantly more migration. The #3 CMC clone trended toward having more, but lacked significance. OSRL = Oxidative Stress Resistance Level

parental CMC conditioned media. It was also found that the conditioned media from the mixed CMC clones had a similar stimulation for migration as the parental CMC conditioned media. This lacks consistency from the other findings in which the parental CMC population outperforms the mixed clonal CMCs in the R18 rat.

CMC Clone Morphology

While it may have been purely coincidental, we also made note of some interesting morphological differences between the clones. It was found that the clones most resistant to oxidative stress in each rat had a very similar morphology. These clones were larger, rounded and flattened with more surface area in contact with the dish. By comparison, the clones with the lease resistance to oxidative stress were elongated and had a narrow spindle shape. This may provide a crude method to quickly identify the cells of interest for cell

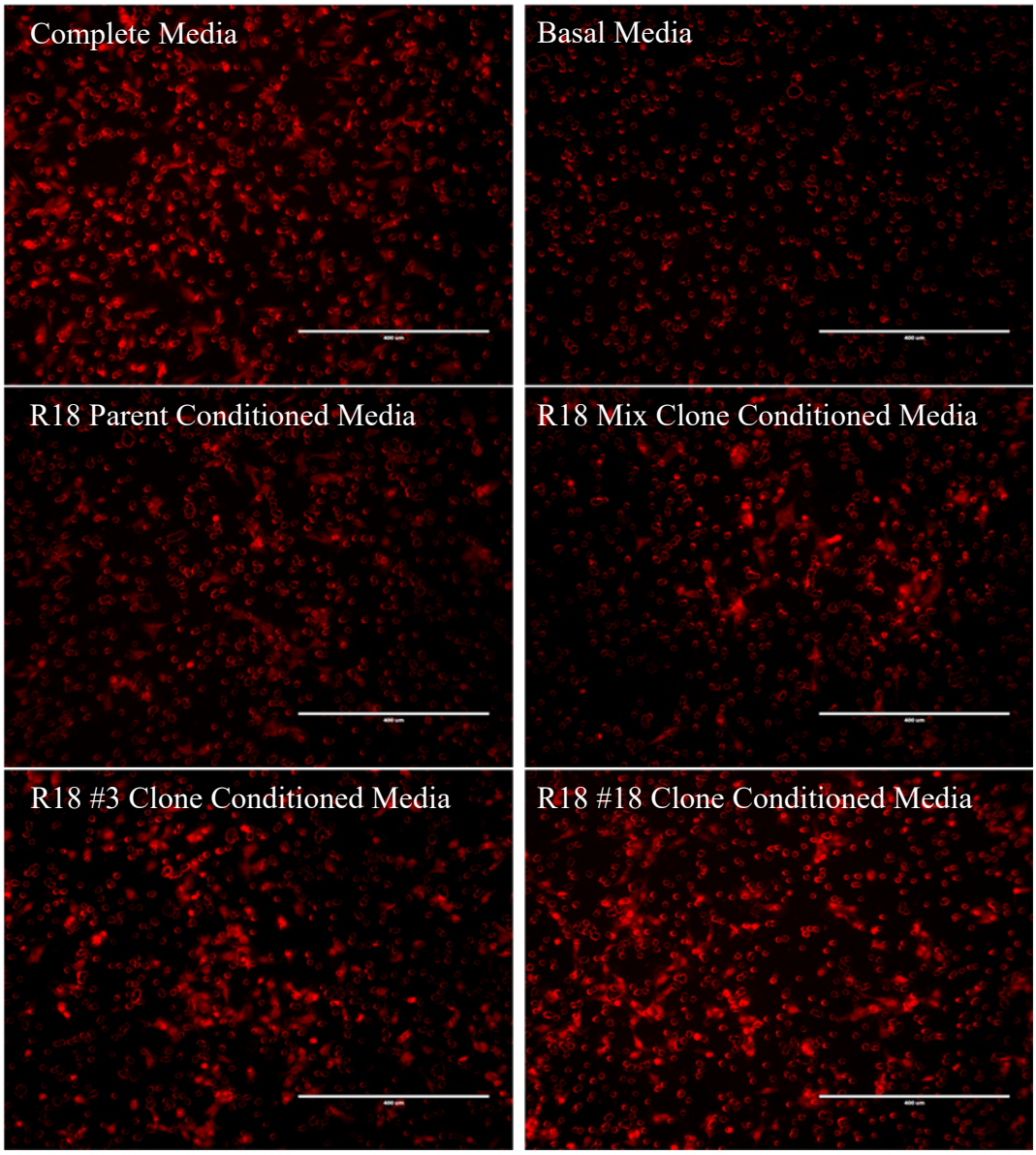


Figure 36 – HUVEC cells were plated on a semi-permeable transwell plate. The next day, the media top well of the plate was changed to basal media and the bottom well was changed to the media of interest. Cells were incubated overnight and then fixed with 3.7% formaldehyde, stained with Propidium Iodide and then representative images were taken from 4 fields in each treatment group (n=4). Cells were then quantified.

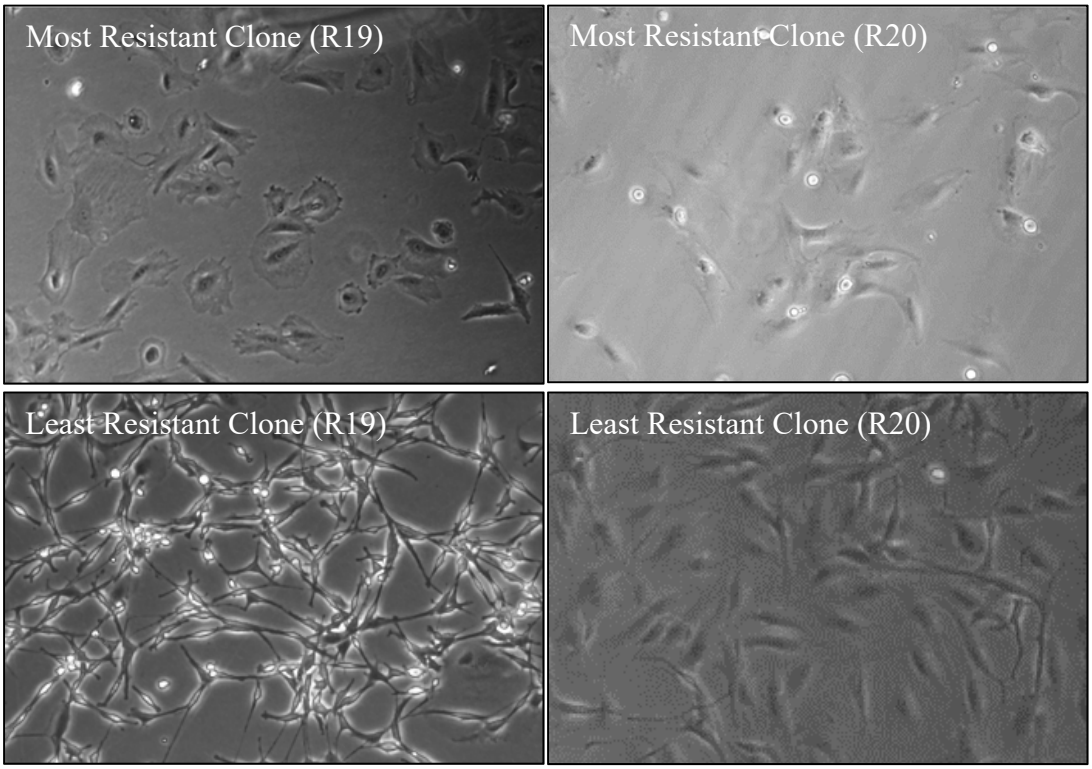


Figure 37- Cell morphology images were captured in all clones from all three rats. Shown here are the most and least resistant to oxidative stress from the R19 & R20 rats. The quality of the R18 photos was poor, so they were not included. Despite that, the most resistant clones in all rats had similar phenotypes. The most resistant were very large, flattened cells. When compared to known cardiac cell types, the most resistant resembled endothelial cells and the least resistant were similar to mesenchymal cells.

therapy if a correlation can be made between in vitro and in vivo data.

Transplantation of Mixed Clonogenic CMCs into the Chronically Infarcted Rat Heart

Female Fischer rats (n=24) underwent ischemia/reperfusion surgery (Figure 38). After reperfusion, rats were re-anesthetized and had the treatment transplanted around the border zone of the infarcted heart. Treatment included: 1 million R18 Mixed Clonal CMC (n=8), 1 million R18 Parent CMC (n=8) or PBS (n=8). CMCs were suspended in 300 µl of PBS. 35 days after transplantation, ventricular function was measured and rats were euthanized. Mortality within treated rats was very low, only two rats died during this study

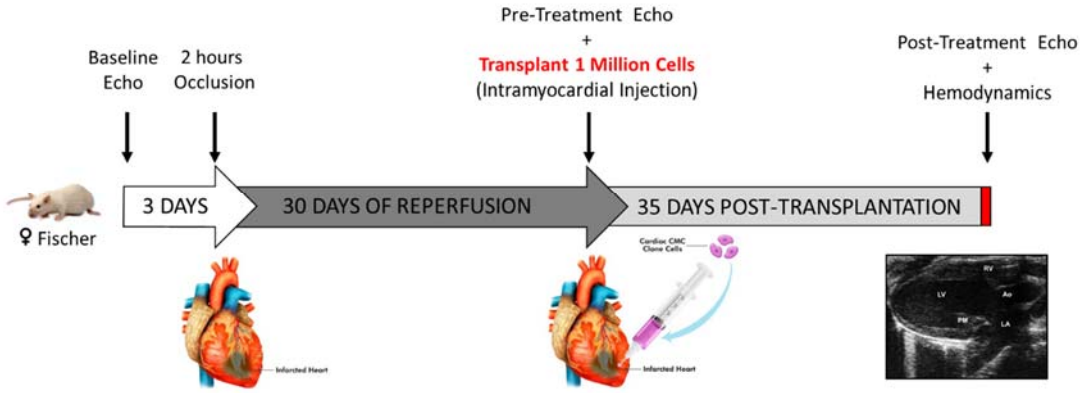


Figure 38 - This is a graphical representation of the surgical protocol. Female Fischer rats received baseline echocardiography. 3 days later they underwent 2 hours of occlusion followed by 30 days of reperfusion. Pre-treatment echocardiography was performed and then then treatment was administered to the damaged heart. Treatment included: 1 million R18 Mixed Clonal CMC (n=8), 1 million R18 Parent CMC (n=8) or PBS (n=8). CMCs were suspended in 300 µl of PBS. After 35 days, echocardiography was again performed along with hemodynamics.

(1 PBS treated rat, 1 Parental CMC treated rat). Upon analyzing echocardiography, it was found that rats treated with parental CMCs from the R18 rat exhibited significant improvement in ejection fraction and fractional shortening compared to PBS treated rats (Figure 39). However, the rats treated with Mixed Clonal CMCs from the R18 rat show no improvement in ventricular function. Hemodynamic data varied slightly, showing an improvement in end-systolic volume and pressure in the parental CMCs, but oddly did not show a significant improvement in ejection fraction (Figure 40). Overall the results show a significant, albeit modest, improvement in ventricular function in parental CMCs and no change in the Mixed Clonal CMC treated rats. This revealed that there are CMCs that do provide a therapeutic benefit and that there are subpopulations of cells that do not provide a functional improvement. This implies that there are subpopulations, whether it be a single cell type or combination of cell types, of CMCs within the parental CMCs that are more

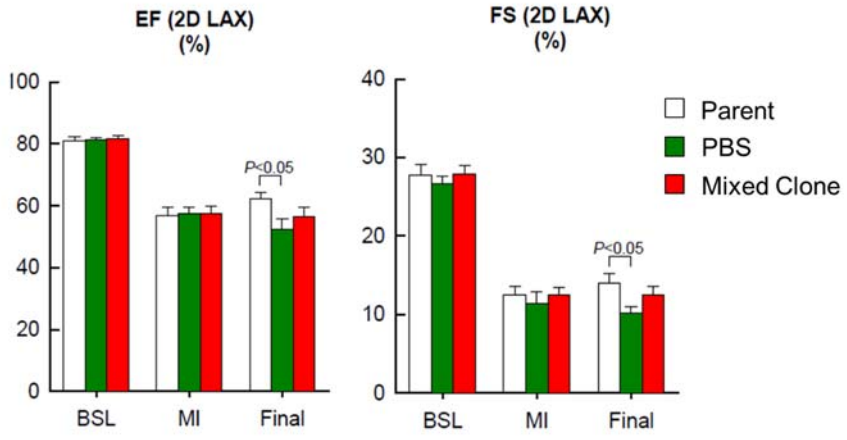


Figure 39 - Ejection fraction and fractional shortening was found to be significantly improved in the parental CMC treated group (white bar) compared to the PBS treated group (green bar). The Mixed Clone CMC treated group (red bar) was no different than the PBS or Parental CMC treated groups.

therapeutically advantageous. Concurrently, the in vitro findings with the Mixed Clonal CMCs from the R18 rat appear to positively correlate with the in vivo results.

Discussion

For the first time in our hands, we confirm that unsorted rat CMCs can improve cardiac function in the chronically infarcted rat heart. Our data reveals cell therapy using mixed clonal CMCs from the R18 rat does not attenuate heart failure in the chronically infarcted heart. We also saw that the mixed clonal CMCs from the R18 rat performed poorly in vitro. Results show that the R18 mixed clonogenic CMCs were less resistant to oxidative stress, less resistant to serum starvation and produced a less stimulatory paracrine milieu. In contrast, mixed CMC clones from R19 & R20 rats performed better in vitro compared to the R18 mixed CMC clonal population, implying that the mixed clonal CMCs are an unreliable source of clonogenic CMCs. Additionally, it was found that individual clones in the R18 rat outperformed the mixed clonal CMCs in vitro. Since our in vitro data

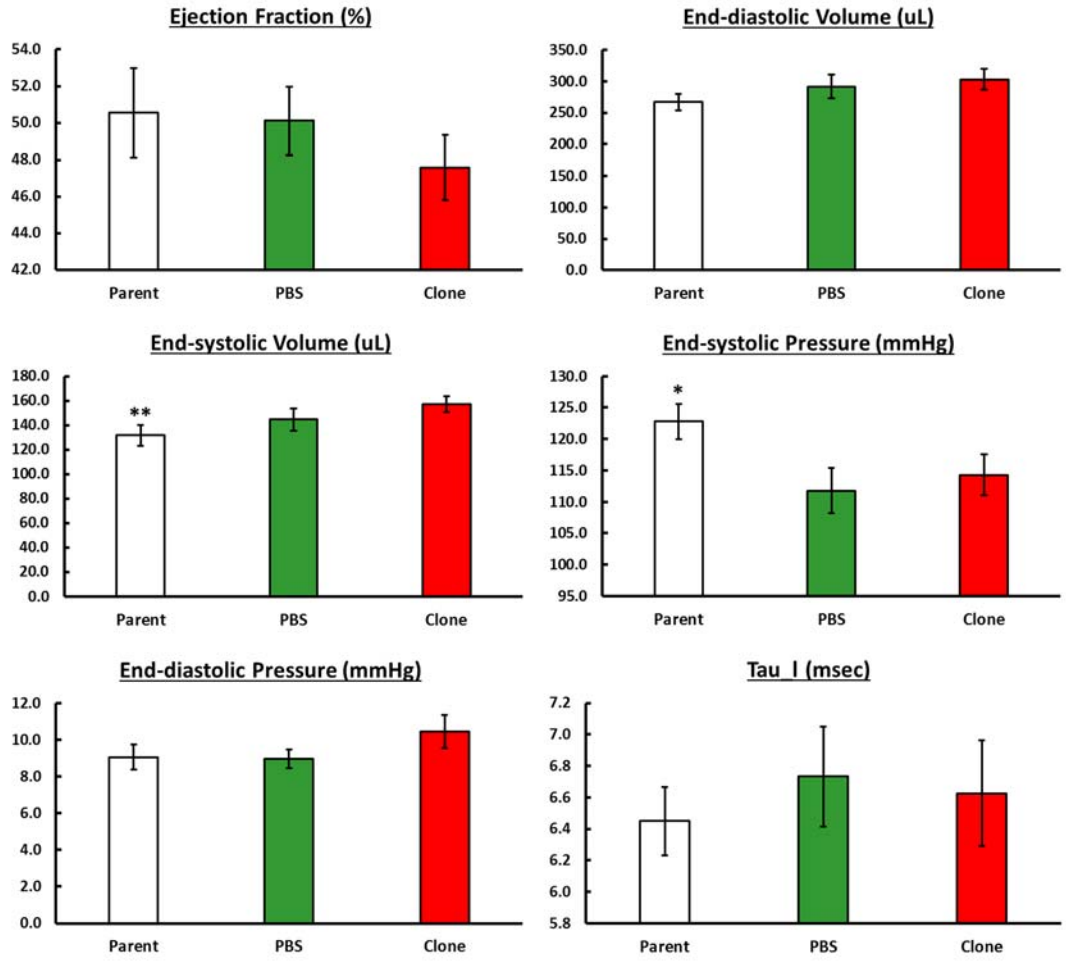


Figure 40 - Hemodynamics data was less convincing than echocardiography, but trended toward similar results. End-systolic volume was significantly improved in parental CMC treated groups compared to the Mixed clonal CMC treated group. End-systolic pressure in Parental CMC treated rats was significantly improved compared to the PBS treated group. However, there was no difference in ejection fraction among any groups.

for the R18 mixed clonal CMCs correlates with our in vivo data, it supports the rationale that transplanting CMCs with "superior" in vitro results would yield a greater improvement in ventricular function in the chronically infarcted rat heart.

Until now, cells transplanted into the heart have been a heterogenous mix, even in sorted cells. Heterogeneity is further amplified in co-transplantation studies showing a synergistic improvement in ventricular function [25, 88]. The benefit of our approach is

that we are reducing CMCs down to a single cell type and expanding more homogenous colonies. If we can identify which specific cell type in the CMC population is the most advantageous, we can then begin to identify why certain CMCs or combinations of CMCs are more valuable for cell therapy.

Of course, it is possible that a combination of CMCs is required to improve cardiac function. It may be that the heterogenous mix of parental CMCs (or other cells used for cardiac cell therapy) is essential to achieve the therapeutic benefits observed. The reduced heterogeneity may explain why the R18 mixed clonal CMC population was ineffective. However, the argument could be made that mixed clonal CMCs themselves are heterogenous CMCs. Although, it is important to recall that the mixed CMC population in the R18 rat was less viable than other mixed clonal CMC populations from other rats. The individual R18 clones, which make up the mixed population, also had a larger variation in resistance. It could be that the R18 CMC clones with significantly less resistance make up a greater concentration of the mixed clonal CMC population thereby driving down their therapeutic potential.

Regardless of rejecting our hypothesis, the preliminary results confirm that populations of CMCs exist that afford a differential therapeutic potential. The current correlation of in vitro and in vivo data is promising and may provide us with the methodology to identify sub-populations of CMCs that are responsible for the ventricular functional improvement observed. We are currently underway with a secondary in vivo study that tests if a clone more resistant to oxidative stress delivers a greater improvement in ventricular function compared to a clone with less resistance. Since we saw a variation in gene expression, we are also currently performing a microarray of the mRNA to attempt

to tease out unique markers that identify the “superior” clones. The outcomes of our ongoing studies are sure to address these important questions.

CHAPTER IV

CONCLUSIONS AND FUTURE DIRECTIONS

In these studies, our goal was to identify a method of prolonging the therapeutic benefit of cardiac cell therapy to generate a greater improvement in heart function. Our first solution was to locate CMCs that endogenously expressed telomerase (TERT). We also artificially overexpressed TERT in CMCs using lentiviral transduction. In other cell types, TERT expression has been shown to immortalize the cells [41, 43, 44] , but expression in cardiac mesenchymal cells had not yet been addressed. While we were successful in achieving TERT expressing CMCs, we found that TERT expression did not immortalize or offer any secondary protective or migratory characteristics in CMCs. We concluded that TERT is not singularly responsible for prolonging the proliferation or survival of CMCS. In a last-ditch effort, we tried to narrow our search for TERT in CMCs by looking for expression in clonogenic CMCs, but expression was again not detected.

While we believe that the novelty of exploiting TERT for cardiac cell therapy has yet to be pinpointed, the premise of clonogenic CMCs has led us to some interesting results. In this study, we identified clonogenic sub-populations of CMCs that exhibited survival and paracrine characteristics that had potential therapeutic implications. We then show for the first time in our hands that we can attenuate heart failure with transplantation of unsorted CMCs. We also saw that mixed clonal CMCs with reduced survival and paracrine characteristics in vitro correlated with no enhancement of cardiac function in vivo. Results confirmed that cells exist in the CMC population that serve little benefit for cardiac cell

therapy. This suggested the possibility that clonal CMCs with "superior" characteristics would provide an improvement in ventricular function. In fact, we are currently underway with another in vivo experiment in rats to support this hypothesis. We are also running a Microarray on mRNA harvested from select CMC clones to find any coinciding data that would help us determine a marker(s) to quickly identify "superior" CMCs. Evidence of finding these unique markers was promising from the gene expression results found in this study.

If we are successful, "superior" CMCs used for cardiac cell therapy will have been identified without prejudice and optimized without the need for exogenous modification (i.e. TERT overexpression). If an improvement in function is observed, we would then need to elucidate the mechanism. We would first identify if there was an improvement of survival by utilizing the methodology developed in our lab to precisely quantify the surviving CMCs [23]. We would also examine if CMCs functionally integrate into the damaged myocardium. Although, thus far no cell type has convincingly proven to replenish the cardiomyocytes in the heart so it would be unreasonable to expect otherwise with the "superior" clonogenic CMCs [89].

However, it is possible that select clonal populations of CMCs ameliorate cardiac function to a greater extent by paracrine signaling [90]. One study has shown that c-kit+ sorted cardiac cells (CSC) preconditioned with exosomes released from bone marrow-derived mesenchymal cells stimulated proliferation, migration and tube formation of the CSCs [91]. The researchers also observed a greater improvement in ventricular function after transplantation of these preconditioned CSCs into the heart. This suggests the possibility that potent paracrine factors released from CMCs could stimulate endogenous

CSCs to generate a greater protection and/or improvement of function. In other words, it may be possible that the paracrine factors released from certain sub-populations of CMCs could provide the optimal support for endogenous CSCs to alleviate pathological dysfunction. If the oxidative stress resistant clonogenic CMCs survive longer, it is possible that ROS present in the ischemic heart stimulates a prolonged release of paracrine factors and therefore yields a greater functional improvement [92]. Again, it was previously shown by Mohsin et al. that prolonging the survival of transplanted cells resulted in a greater improvement of ventricular function [26]. Of course, paracrine stimulation also insinuates a mediated immune response which is known to be important in remodeling the damaged heart [93, 94].

In fact, many believe that the immunologic response may be complemented by cell therapy [95]. It has been shown that immune activation is required for the repair of the damage by clearing debris and stimulating remodeling of the damaged heart. It is thought that immunity aids in providing an appropriate microenvironment for the survival, development and function of the transplanted cells. While it has not been unequivocally proven, the immune system is also believed to initiate regeneration of the damaged tissue dependent upon the availability of progenitor cells. It is possible that there are clonal CMCs that provide a more complementary interaction with immunity, which may explain variances in outcome that have been observed to this point. By utilizing more homogenous "superior" populations of CMCs resistant to stress, we may be able to expose CMCs that synergize better with the immune system.

If resistance to oxidative stress correlates to improved function, it could also be the result of elevated levels of intracellular antioxidants compared to the less resistant CMCs.

In muscle-derived stem cells, it was found that antioxidant depletion caused a significant reduction is cell survival to oxidative and inflammatory stress [96]. The study also discovered a reduced survival of cells transplanted into the infarcted heart and significantly less improvement of ventricular function. From what we have found, antioxidants in CMCs have yet to be investigated. The mRNA microarray may reveal expression of intracellular antioxidants of interest that we could then overexpress to confirm protection of the CMCs.

Within that realm, it is possible that transplanted CMCs somehow aid in reducing oxidative stress on surviving cardiomyotcytes. During ischemia, the cardiac tissue shifts to anaerobic metabolism decreasing the pH of the cell [97]. To combat it, the sodium-hydrogen ion exchanger excretes excess hydrogen ions causing an increase of sarcoplasmic sodium. There is also a depletion of ATP which results in a reduction of calcium ion uptake by the sarcoplasmic reticulum. This causes an increase in mitochondrial permeability and further reduces ATP production. In our model, ischemia is followed by reperfusion which restores delivery of oxygen and nutrients for aerobic metabolism and washes out the hydrogen ions to restore the extracellular pH. However, reperfusion itself causes injury. Some of the causes of reperfusion injury include the generation of ROS, calcium overload and an inflammatory response [97]. The ROS can target the L-type calcium channels and suppress the calcium ion current [98]. It can also reduce the activity of calcium-ATPase on the sarcoplasmic reticulum, shown to affect contractility [99]. ROS has also been shown to influence contractility by depressing myofilatment sensitivity to calcium [100]. If the transplantation of cells helps to reduce the oxidative stress on the surviving cells, it could aid in restoring the appropriate flow of calcium ions and improve contractility of the

cardiac cells. This may result in a modest improvement of cardiac function without the functional integration of transplanted cells.

Of course, most of this is conjecture until a correlation between oxidative stress and improved ventricular function is found. It is very possible that the results thus far have merely been coincidental. It is also possible that we will find that to achieve functional improvement we need a combination of cells. It may be that no unique cell type is individually important, which would give greater support to the paracrine hypothesis.

However, our discussion of the various potential outcomes provides a road map of different directions this project could take in the future. It is easy to see that there is much that can be done to continue exploration of these results. Indeed, it is unknown what the future may hold for the project. What is known is that we have observed that a sub-population of cells within parental CMCs, that alone provide cardiac improvement, provided no therapeutic benefit in vivo and correlated with our in vitro findings. Finding the reasoning for this difference observed is certain to lead to some incredibly important findings for the field of cardiac cell therapy.

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CURRICULUM VITAE

MICHAEL J BOOK, PhD

michaeljbook@gmail.com – 502.681.4146 – Louisville, KY
https://www.linkedin.com/in/michaeljbook

EDUCATION

PhD, Physiology & Biophysics
University of Louisville, Louisville, KY, April 2017

Master of Science, Physiology & Biophysics
University of Louisville, Louisville, KY, August 2015

Bachelor of Science with Distinction, Biology, Minor in Microbiology
Indiana University, New Albany, IN, May 2011

Associate of Arts with Distinction, Chemistry
Indiana University, New Albany, IN, May 2011

Associate of Science with Honors, Industrial Design Technology
Art Institute of Pittsburgh, Pittsburgh, PA, June 2002

PROFESSIONAL EXPERIENCE

PhD Fellow, University of Louisville – *Full time position*
August 2013-April 2017, Louisville, KY

- Manage laboratory stock and purchase orders
- Perform all lab management duties
- Manage required laboratory paperwork
- PCR, qPCR, colony PCR
- RNA Isolation & cDNA synthesis
- Aseptic cell culture techniques
- Microscopy, Epifluorescence
- Primer design
- Immunocytochemistry
- Lentiviral vector design & virus production
- Viral Transduction
- Lipofectamine Transfection
- Cloning: Limited Dilution & Cloning Ring
- Protein Isolation, BCA Assay, Western Blot
- Plasmid Preparation
- ELISA
- Flow cytometry & MOFLO Cell Sorting
- Transwell Migration Assays
- Oxidative Stress Assays
- Telomeric Repeat Amplification Protocol
- Data/statistical analysis and figure preparation
- Seminar & Meeting Presentations
- Extensive knowledge of SigmaPlot, EndNote, Adobe Creative & more

Research Technologist II, University of Louisville – *Full time position*
December 2011-August 2013, Louisville, KY

Work centered on cardiac cell therapy in the murine model.

- Perform and assist advanced murine surgery
  - Ischemia/reperfusion model
- Tissue microscopy, photography and analysis
  - Developed updated computerized analysis methodology
- Perform and analyze echocardiography
- Data/statistical analysis and figure preparation
- Perform and assist in heart tissue staining
- Manage laboratory stock and purchase orders
- Perform all lab management duties
- Coordinate laboratory inspections and new product demonstrations
- Member of the Recruiting/Hiring Committee
- Create Postdoctoral candidate itineraries, facilitate laboratory tours and provide performance analyses
- Manage laboratory paperwork for animal models, controlled substances and etc.
- Write detailed protocols of laboratory procedures
- New staff training
- Assist in preparation and writing of manuscripts
- Assist in peer reviews of manuscripts

Research Assistant, Indiana University – *Part time position*
May 2009-May 2011, New Albany, IN

- Physiological experiments on Marsh Rice Rats (*Oryzomys palustris*)
- Performed surgical procedures including implants & castrations
- Dissection, blood and tissue collection
- Administered medication and/or treatment
- Collected and analyzed experimental data
- Facility maintenance and animal care

Laboratory Assistant, Indiana University - *Part time position*
August 2007-August 2008, New Albany, IN

- Assisted with chemistry laboratory class
- Solution and chemical preparation
- Handled hazardous chemicals
- Set-up laboratory class room experiments
- Graded laboratory reports

OTHER EXPERIENCE

Manuscript Reviewer, December 2016, April 2017

- PLoS One

Judge of Louisville Regional Science & Engineering Fair, Spring 2016

- High school student presentations of biological sciences

Echocardiography Training, Spring 2012

- Washington University at Saint Louis, Saint Louis, MO

National Science Foundation: Becoming the Messenger Workshop, Spring 2013

- University of Louisville, Louisville, KY

AWARDS & RECOGNITION

- **IPIBS Fellowship**, University of Louisville, Fall 2013
- **Outstanding Staff Member**, UofL, Winter 2012
- **Dr. Claude D Baker Scholarship**, Spring 2010
- **Bill Forsyth Memorial Scholarship**, Spring 2009
- **Public Speaking Award**, Spring 2009
- **Academic Full-Time Scholarship**, Fall '07, '08

PROFESSIONAL & HONOR SOCIETY MEMBERSHIPS

- American Heart Association Member 2014
- Better Business Bureau Member, 2011
- Alpha Chi Honor Society, Inducted Spring 2007
- National Technical Honor Society, 2002

EXTRACURRICULAR ACTIVITIES

Black Orchard, January 2017 - Current

- Owner, Operator

Legend at Pope Lick, September 2016 – Current

- Owner, Operator

Louisville Halloween Parade & Festival, October 2015 – Current

- Owner, Operator

Louisville Halloween, July 2010 – Current

- Co-Chair, Owner

Danger Run, January 2009 – Current

- Owner, Operator

Starlight Strawberry Festival, Spring 2013 - Current

- Advertising Design, Internet Marketing, Website Design & Management

Kosair Charities, Fall 2014

- Coordinating a fall fundraiser raising over $3,000

Children's Miracle Network, Fall 2012

- Coordinating a fall fundraiser raising $1,600

American Red Cross Blood Drive, Fall 2010-2012

- Coordinated multiple blood drives responsible for bringing in hundreds of donations

Special Needs, Winter 2005-2011

- Assist with holiday party management, ornament presentation, and more

VistaCare Hospice, Spring 2006-Fall 2008

- Food Drives and created ceramic painting parties to produce gifts for patients

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PEER REVIEWED PUBLICATIONS

1. Wysoczynski M, Guo Y, Moore J, Muthusamy S, Li Q, Nasr M, Li H, Nong Y, Wu W, Tomlin A, Zhu X, Hunt G, Gumpert A, **Book MJ**, Khan A, Tang X, Bolli R. A New Population of Cardiac Mesenchymal Cells Isolated on the Basis of Adherence: Phenotype and Reparative Properties. Journal of American College of Cardiology Volume 69, Issue 14, April 2017. DOI: 10.1016/j.jacc.2017.01.048.
2. Guo Y, Wysoczynski W, Nong Y, Tomlin A, Zhu X, Gumpert A, Nasr M, Muthusamy S, Li H, **Book MJ**, Khan A, Hong K, Li Q, Bolli R. Repeated doses of cardiac mesenchymal cells are therapeutically superior to a single dose in mice with old myocardial infarction. Basic Research in Cardiology. 2017 March;112(2):18. PMID: 28210871

3. Cai C, Guo Y, Teng L, Nong Y, Tan M, **Book MJ**, Zhu X, Wang XL, Du J, We WJ, Xie W, Hong KU, Li Q, Bolli R. Preconditioning Human Cardiac Stem Cells with HO-1 Inducer Exerts Effects After Cell Transplantation in the Infarcted Murine Heart. *Stem Cells*. December 2015. Volume 33, Issue 12, 3596-3607.

4. Hong KU, Guo Y, Li Q, Cao P, Al-Maqtari T, Vajravelu B, Du J, **Book MJ**, Zhu X, Nong Y, Bhatnagar A, Bolli R. c-KIT+ Cardiac Stem Cells Alleviate Post-Myocardial Infarction Left Ventricular Dysfunction Despite Poor Engraftment and Negligible Retention in the Recipient Heart. *PLoS One*. 2014. 9(5): e96725. doi:10.1371/journal.pone.0096725

5. Guo Y, Tukaye DN, Wu W-J, Zhu X, **Book M**, Tan W, Jones SP, Rokosh G, Narumiya S, Li Q, Bolli R. The COX-2/PGI_2 receptor axis plays an obligatory role in mediating the cardioprotection conferred by the late phase of ischemic preconditioning. *PLoS One*. 2012;7(7):e41178. Epub 2012 Jul 23. PubMed PMID: 22844439; PubMed Central PMCID: PMC3402528.

ABSTRACTS

1. **Book MJ,** Nakamura S, Tang X, Wsyoczynski M, Wu W, Stowers H, Hong K, Bolli R. Clonogenic Cardiac Cells Exhibit Improved Stress Resistance: Implication in Cardiac Cell Therapy. *American Heart Association Scientific Sessions*. November 2016.

2. **Book MJ,** Nakamura S, Tang X, Wsyoczynski M, Wu W, Stowers H, Hong K, Bolli R. Clonogenic Cardiac Cells Therapeutic Implications Exhibited in Clonogenic Cardiac Mesenchymal Cells. *Research!Louisville*. October 2016.

3. **Book MJ**, Al-Maqtari T, Vajravelu B, Cao P, Guo Y, Bolli R, Hong KU. Identification and Isolation of Cardiac Cells Expressing Telomerase. *Research!Louisville.* September 2014.

4. Obal D, Katragadda K, Tomlin A, **Book MJ**, Bhatnagar A, Guo Y, Bolli R, Rokosh G. Caesin Kinase 1α Gene Therapy Facilitates Cardiac Regeneration After Myocardial Infarction in Mic. *American Heart Association Scientific Sessions.* November 2016.

5. Al-Maqtari T, Vajravelu B, Moktar A, Cao P, **Book MJ**, Bhatnagar A, Bolli R, Hong KU. Gata4 Overexpression to Solve the Dilemma of Limited Differentiation of Human c-kit+ Cardiac Stem Cells Used in Heart Repair. *Research!Louisville.* September 2014.

6. Al-Maqtari T, Vajravelu B, Moktar A, Cao P, **Book MJ**, Bhatnagar A, Bolli R, Hong KU. Solving the Dilemma of Limited Differentiation of c-kit+ Cardiac Stem Cells Used in Heart Repair. *Ohio Valley Society of Toxicology.* August 2014.

7. Obal D, Brittian K, **Book MJ**, Bhatnagar A, Guo Y, Bolli R, Rokosh G. Cardiomyocyte Specific Conditional Overexpression Of Stromal Cell Derived Factor 1 Facilitates Cardiac Regeneration After Permanent Coronary Artery Ligation In Mice. *Basic Cardiovascular Science Conference.* 2014; 115:A132
8. Lefer D, Jones S, Steenbergen C, Kukreja R, Guo Y, Tang XL, Li Q, Ockaili R, Salloum F, Kong M, Polhemus D, Bhushan S, Goodchild T, Chang C, **Book MJ**, Du J, Bolli R. Sodium Nitrite Fails to Limit Myocardial Infarct Size: Results from the CAESAR Cardioprotection Consortium (LB645). Abstract. The FASEB Journal vol. 28 no.1 Supplement LB645. April 2014.

9. Kukreja R, Tang XL, Lefer D, Steenbergen C, Jones S, Guo Y, Li Q, Kong M, Stowers H, Hunt G, Tokita Y, Wu W, Ockaili R, Salloum F, **Book MJ**, Du J, Bhushan S, Goodchild T, Chang C, Bolli R. Administration of Sildenafil at Reperfusion Fails to Reduce Infarct Size: Results from the CAESAR

Cardioprotection Consortium (LB650). Abstract. The FASEB Journal vol. 28 no.1 Supplement LB650. April 2014.

10. Guo Y, **Book MJ**, Du J, Wu WJ, Zhu X, Li Q, Bhatnagar A, Bolli R. The Effect of Early Phase of Ischemic Preconditioning on the Leptin Resistant Diabetic Heart. *American Heart Association Scientific Sessions.* November 2013. APS.514.03

11. Cummins T, Li Q, Guo Y, Du J, **Book MJ**, Zhu X, Wu WJ, Salabei J, Haberzettl P, Jones SP, Bolli R, Bhatnagar A, Hill B. Diabetes Promotes a Warburg-like Metabolic Phenotype in Cardiac Stem Cells. *American Heart Association Scientific Sessions.* November 2013. Abstract 17898.

PUBLISHED ACKNOWLEDGEMENTS

1. Guo Y, Flaherty MP, Wu W-J, Tan W, Zhu X, Wang T, Li Q, Bolli R. Genetic background, gender, age, body temperature, and arterial blood pH have a major impact on myocardial infarct size in the mouse and need to be carefully measured and/or taken into account: results of a comprehensive analysis of determinants of infarct size in 1,074 mice. *Basic Res Cardiol*. 2012 Sep;107(5):288. Epub 2012 Aug 3. PubMed PMID: 22864681.

2. Edmonds K. Melatonin, But not Auxin, Affects Postnatal Reproductive Development in the Marsh Rice Rat (*Oryzomys palustris*). *Zoological Science*. 2013. 30(6): 439-445.