The Orchestra of Myocardial Regeneration

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Het Orkest der Regeneratie van het Myocard
(met een samenvatting in het Nederlands)

Proefschrift

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“You have to learn the rules of the game.
And then you have to play better than anyone else”

Albert Einstein

To those without opportunity……..

Contents

General Introduction

A glimpse on previous and current literature ignites the recognition of the luxurious era that cardiac science has reached. In particular, the past fifteen years have provided tremendous advancements in the field of myocardial biology with the characterization of cardiac stem cells, reprogramming of somatic cells, microRNA discovery, exosome-protocols and imaging modalities. In addition, conventional and outdated biological processes such as myocyte metabolism, cell cycle, and senescence are revisited with fresh perspectives. This phylogeny represents a foundation that provides a broad range of possibilities and directions that can be pursued. While preceding science evolved around characterization of peculiar topics, perhaps contemporary science postulates an orchestrated approach where molecular biological conglomerates are deciphered and connections are made in a panoramic manner. Similar to a musical composition; molecular biological systems demand comprehensive knowledge of distinct notes or molecules that are then orchestrated in a timely and harmonious fashion. This composition-based demeanor, as symbolized by a portrait of the legendary composer Ludwig van Beethoven, concurrently calls for contemplation of the frame-works and restrictions that are imposed by innate properties of a system. The current thesis is a compilation of assorted topics that may appear heterogeneous at first but are related in the harmony of 'myocardial regeneration composition'.
The perspective of myocardial regeneration consists of two main branches: Myocyte-mediated regeneration and Stem Cell mediated regeneration.

Part I. Upon perpetual attempts of studying myocyte cell cycle and manipulating myocyte division, the field of myocyte-mediated regeneration seemed somewhat discouraged and appeared rather sedentary for a while. From characterization of cyclins, cyclin-dependent-kinases (CDKs), cyclin-dependent kinase-Inhibitors (CDKIs) and pocket proteins[1-7] to overexpression of telomerase[8] and major oncogenes such as c-myc[9] were proven ineffective in combating myocyte resistance to division. Although myocytes carry the potential to proliferate during early development, the transition to proliferative arrest upon maturation still leaves scientists mystified. One of the factors that make myocyte cell cycle so perplex is the fact that common cell cycle knowledge from other cell types cannot be extrapolated to a myocyte context effortlessly. General knowledge on cell cycle from other cell types soon get blurred by two main specific myocyte attributes of hypertrophic growth and bi-and multinucleation. These growth processes require proteins and systems that are present and active during the cell cycle. Thus, myocyte cell cycle requires a disruption of the common belief that myocyte cell cycle is an extension of any other cell cycle. Myocyte cell cycle ought to be considered as a separate arena where, perhaps, processes and cell cycle phases must be characterized from scratch. The first part of this thesis is a work in progress on this particular topic. A reporter mouse is generated that provides distinction between myocytes in G1-phase of the cell cycle and in S/G2/M-phase of the cell cycle. A combination of this reporter system and detection of

ploidy is expected to provide more insight in specific phases of a myocyte cell cycle. Once knowledge and expertise is gained on these distinct phases and breaks at the checkpoints, more doors may open for manipulation of myocyte division for the future. Of note is that cell cycle progression and completion is an energy consuming process that requires vitality of the cell.

Part II. Research on myocyte senescence has gained momentum over the past years due to the confrontational statistics on population longevity worldwide. Cardiac patients not only suffer from age-related general deterioration but are also victims of previous cardiac events that leave the heart damaged. In order for regeneration to occur, myocyte environment must be hospitable to newcomers. The common assumption is that the opposite of senescence is rejuvenation and myocytes and stem cells ought to be *'rejuvenated'*. This however, tempers the extent of the impact of senescence on a myocyte and the environment. Perhaps the opposite of senescence should be referred to as *vitality.* Reversal of senescence and vitalization of myocytes and stem cells require insight in the process of premature aging and senescence[10-13]. The second part of this thesis focuses on pathological mechanisms underlying various types of senescence. One of the proteins that has been described in other cell types as a mediator of proliferation and pluripotency, Nucleostemin, is characterized in the heart during development and post pathologic injury. As a marker of pluripotency in border zone myocytes. Nucleostemin resembles aspects of a recent study where transient dedifferentiation of borderzone myocytes was introduced as an initial protective response to damage[15]. Natural protective pathways and mechanisms in myocytes can be useful tools in vitalization and reversal of senescence in aged and damaged hearts. In an attempt towards vitalization, another pro-proliferative and pro-survival protein, Pim-1 kinase has been studied rigorously in the heart which is summarized in the final section of part two. Cardiac senescence is not limited to the myocyte population[12, 16-18]. As a biological protective mechanism against oncogenesis, senescence carries the role of informing the environment of potential "danger". Indeed senescent cells are characterized by proactively secreting the so called, senescence-associated secretome[16], where neighboring cells are dragged along towards a less vital condition. This hegemonic propagation of senescence, in turn, affects upon stem cell proliferation and quality of progeny[17].

Part III. The final section of this compilation is focused on Stem cell-mediated myocardial regeneration. It wouldn't be novel to point out the promptness in the progress of cardiac stem cell regeneration and therapy. Within a decade, cardiac stem cells migrated from the first characterization and isolations[19-21] in the laboratories to three currently ongoing clinical trials[22-24]. However, the more the field evolves, the more insight is gained in challenges that are faced for broad applicability of cardiac cell therapy. The final part of this thesis focuses on pointing out various cell types that are currently used in

clinical practice, the limitations and the future of myocardial regeneration in aged and damaged heart.

Overall, the current work may represent certain controversies and even attempts to challenge particular common beliefs. However, in this era of accelerated phylogeny and the extent of knowledge generated on a daily basis, perhaps new scientists ought to question certain existing criteria and definitions from time to time. After all, a common criteria for a successful composer might be ‘a crystal clear sense of hearing’ and yet, while composing some of his legendary compositions, Ludwig van Beethoven was deaf!

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Part One: Myocyte Division Requires Insight in Myocyte Cell Cycle

Chapter 1

The heart: Mostly Postmitotic or Mostly Premitotic? Myocyte Cell Cycle, Senescence & Quiescence

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Abstract

The concept of myocyte division and myocyte-mediated regeneration has re-emerged in the past five years through development of sophisticated transgenic mice and carbon-dating of cells. Although, recently, a couple of studies have been conducted as an attempt to intervene in myocyte division, the efficiency in adult animals remains discouragingly low. Re-enforcing myocyte division is a vision that has been desired for decades, leading to years of experience in myocytes resistance to pro-proliferative stimuli. Previous attempts have indeed provided a platform for basic knowledge on molecular players and signaling in myocytes. However, natural biological processes such as hypertrophy and binucleation provide layers of complexity in interpretation of previous and current findings. A major hurdle in mediating myocyte division is a lack of insight in the myocyte cell cycle. To date, no knowledge is gained on myoycte cell cycle progression and/or duration. The current review will provide an overview of previous and current literature on myocytes cell cycle and division. Furthermore, this overview will point-out the limitations of current approaches and focus on re-igniting basic questions that may be essential in understand myocardial resistance to division.

Expansion means complexity and complexity decay...

Parkinson's third Law

The complexity of myocyte cell cycle regulation is manifest. Efforts to sort out conundrums in the concept of myocyte division have suffered from the tendency to extrapolate cell cycle knowledge from other fields to a myocyte context. The term *cell cycle* refers to occurrence of subsequent events leading to cell duplication and generation of progeny. However, certain fundamental attributes of a cardiac myocyte do not integrate seamlessly with our general comprehension of cell cycle biology such as hypertrophy and physiologic binucleation. In addition, cell cycle terminology has migrated to the field of 'myocyte cell cycle' without explicit insight into the substantive meaning of those processes in a cardiac myocyte context. This disparity has led to a certain degree of dispersion for explanations of myocyte cell cycle in the scientific community. Cumulative experience in myocardial biology has prompted re-examination of previous assumptions, some of which may turn out to be inaccurate with regards to myocyte cell cycle control. Revisiting such long held assumptions based on newly built knowledge will redefine new directions ultimately culminating with a new appreciation for the potential for myocardial regeneration. One such common belief up for challenge today is that "terminal differentiation is an irreversible withdrawal from the myocyte cell cycle"[1-3]. Cell division is not the sole function of myocyte cell cycle, which may also involve biological processes such as hypertrophy[4-7] and DNA-repair[8-12] requiring participation of proteins and processes that are present and active during the cell cycle. The presumption of "terminal differentiation representing an irreversible withdrawal from cell cycle" implies that such cells are incapable of cell division as well as blocked from undergoing general biological processes that require cell cycle activity. Furthermore, references to *withdrawal from the cell cycle* and *cell cycle arrest* are often used interchangeably and carelessly[1]. Specifically, withdrawal from cell cycle indicates a G0-arrest caused by nutrient and mitogens deprivation, while non-G0-arrest (G1 and G2) is characterized by high levels of cyclins, CDKs and other growth stimuli that promote cellular growth[13]. Hypertrophic growth has been described as non-G0 arrest[13, 14] that is reversible, but a prolonged state of growth can push a hypertrophic cell into senescence[13, 14]. Senescence is irreversible cell cycle arrest decision point executed by a cell in response to very specific triggers. For example, DNA damage activates the DNA-Damage Response at cell cycle checkpoints intended to execute DNA-repair[15, 16]. If DNA-repair is successful then the check-point arrested cells resume cell cycle progression, but if DNA-damage is not sufficiently resolved the cell becomes irreversibly arrested and is now a senescent cell[15-17]. Senescent cells do not contribute to tissue homeostasis and may eventually undergo apoptotic cell death. There are multiple types of cellular senescence; replicative senescence is caused by critical telomere shortening, premature senescence occurs as a response to exposure to reactive oxygen species and DNA-damage and hypermitogenic arrest as a protective mechanism to oncogenic stimuli[16, 17]. Senescent cells are not only irreversibly arrested in the cell cycle, but also detrimentally affect the environment via their senescence-associated secretory phenotype (SASP)[16, 17]. SASP is crucial in the context of normal myocytes since neighboring senescent cells adversely impact upon regenerative and reparative potential.

Myocyte division has recently received renewed attention as a candidate for myocardial regeneration, driving the recent spate of studies that are redefining understanding of myocyte cell cycle and revisiting previous definitions. Distinctions between myocyte cell cycle processes, senescence and quiescence will have important consequences for future interventional approaches. Comprehension and interpretation of myocyte cell cycle has been extremely challenging due to technical limitations in the field. Unlike other organs, an adult heart is a difficult platform for myocyte cell cycle studies due to scarcity of the number of proliferating cells. Neonatal hearts have been studied rigorously in their proliferative potential; however, data and mechanisms studied in a young heart do not extrapolate to adult myocytes in any straightforward fashion. Culture of adult myocytes *in vitro* is possible, but technically challenging and accompanied by a

plethora of biological changes prompted by isolation and artificial culture conditions that cannot faithfully recapitulate the *in vivo* contextual environment. Molecular pathways of myocyte proliferation in neonatal hearts resemble hypertrophic signaling in an adult heart. The overlap in signaling between these two processes and the ultimate phenotypic differences requires expertise in both developmental growth and pathological remodeling to decipher relationships between time-points and specific proteins that interact in these distinct juvenile versus adult processes. In most cell types, measurements of ploidy provides insight into the stage of cell cycle; however, binucleation and multinucleation of myocytes lead to ploidy ranging from 2N to 16N or more. Binucleation or endoreplication on a molecular level shares certain aspects with cell cycle signaling and intracellular remodeling, thereby necessitating a cautious and circumspect approach to interpretation of myocyte proliferation studies.

One distinct luxury of living in the 21st century is the advances in civilization and associated increase in longevity. Unfortunately, longevity does not necessarily come with more youth but rather extended old age. The worldwide cardiac disease pandemic has prompted attempts to instigate myocardial regeneration via a variety of approaches. From clinical trials using stem cells, generation of tissue engineered patches, gene therapy clinical trials, to reprogramming somatic cells in an effort to intervene in cardiac disease. Unquestionably, an ideal approach would be to manipulate innate myocyte properties for proliferation when possible and promote myocytes to provide a natural replacement mechanism for the scarred ventricle with newly formed young competent myocytes. Fortunately, despite inherent technical limitations, the field has gained a considerable amount of knowledge on molecular pathways involved in myocyte cell cycle. This initiates a shift towards the second part of Parkinson's law: *complexity decay* wherein elucidation of distinct processes ultimately provides sufficient understanding of the system in order to deconstruct and simplify mysterious fundamentals that governs myocyte division and myocardial regeneration. The ultimate challenge is to assimilate our collective knowledge in order to define caveats and processes that could be used as a mechanistic foundation for future interventions. This review summarizes cell cycle pathways that have been studied in myocytes, the process of terminal cell cycle arrest; senescence and one particularly neglected aspect of cell cycle in the context of myocardial regeneration: quiescence.

Myocyte Cell Cycle

Adult cardiac myocytes possess an intricate almost crystalline-like myofibrillar structure required for the demanding job of providing contractile force. During fetal life myocytes undergo cell division that, soon after birth, seems to cease for all practical terms of mediating reparative processes. Although cell division is blocked, myocytes continue to increase in cell size in the form of hypertrophic growth and DNA-content in the form of bi-and multinucleation[18, 19]; two processes that require cell cycle activity but do not lead to cytokinesis. Molecular regulators involved in myocyte cell cycle progression can be subdivided in 'cytokines and growth factors', 'transcription factors' and 'cell cycle regulators' and 'pocket proteins'.

Growth Factors. The role of cardiomyokines is distinct from physiologic cardiac development and cardiac pathologic growth and is therefore beyond the scope of this review and is discussed elsewhere[20-24]. Fibroblast growth factors (FGFs) are proteins of ~150–300 amino acids playing multiple roles in development and metabolism. Neonatal and embryonic cardiomyocyte proliferation is stimulated by FGF *in vitro*[25] and inhibited by blocking FGF signaling *in vivo*[26]. Although FGF-2 receptor has been suggested to be cardiac specific, early genetic deletion of FGFR1 and FGFR2 leads to a hypoplastic ventricle and dilated atria implying an important role of the FGF-pathway in myocyte proliferation[25]. Similarly, ligand activation of IGF-1 stimulates myocyte proliferation in culture[27], while IGF-1 inhibition causes a decrease in growth, nuclear mitosis and DNA synthesis[27]. Overexpression of IGF-1 in transgenic mice causes an increase in body weight that is accompanied with a hyperplastic cardiac phenotype, implicating an increased myocyte division rate[28]. Although IGF-1 has been shown to play a primary role in myocyte proliferation rather than hypertrophy, downstream signaling and consequences

of IGF-1 overexpression seem to play an important role in the equilibrium of myocyte division versus hypertrophy. A major downstream effector of IGF-1 signaling is Akt, which when overexpressed at non-physiologic levels increases cardiac size by hypertrophy[29]. However, targeting Akt to the nucleus of myocytes more faithfully recapitulates the normal biological behavior of the kinase leads to a hyperplastic phenotype with an increased number of young myocytes[30]. Since genetic inactivation of Akt during early development does not lead to lethality in mice, a prominent role for Akt in myocyte division remains questionable. With the nascent advent of stem cell therapy, the role of nuclear targeted Akt could be revisited in the context of endogenous stem cell activation and stem cell mediated myocyte regeneration, thereby causing a hyperplastic phenotype in part via paracrine signaling to enhance cell survival and the growth milieu[31]. IGF-1 has also been associated with antagonism of senescence, increasing telomerase activity and contributing to preservation of cardiac stem cell pool, thereby implying a general beneficial effect of IGF-1 on both myocytes and stem cells[32]. That interaction, by itself, can be beneficial in mediating terminal growth arrest through senescence as discussed later in this review.

Cyclins, Cyclin-Dependent Kinases and Cyclin-Dependent Kinase Inhibitors. Cyclin dependent kinases (CDKs) are serine-threonine kinases, which become enzymatically active upon formation of a complex with the corresponding Cyclin. The regulatory role of Cyclins and CDKs are very well studied since their function has been evolutionary conserved[33]. Cell cycle progression requires accurate orchestration of Cyclins and CDKs at the corresponding checkpoints to assure specific events at very specific phases. The CDKs that are the most crucial at the G1/S-phase transition are CDK4 and CKD6, which bind and regulate the Cyclin D-family[34]. These complexes in turn lead to phosphorylation of Rb-proteins thereby modifying E2F activity and subsequent gene expression and cell cycle progression[34]. Accelerated phosphorylation of Rb is mediated during the S-phase by the active complex of Cyclin E and CDK2[35]. Further into the cell cycle at the G2/M transition, the main players are Cyclin E and CDC2[35]. CDK-Activating Kinase (CAK) and Cyclin-Dependent Kinases-Inhibitors (CDKIs) are positive and negative regulators of CDKs, respectively. Not surprisingly, Cyclins and CDKs are present in the embryonic heart in conjunction with other proteins involved in transcription and DNA-replication. Cardiac development seems to be affected the most by deletion of the Cyclin-D family. Although genetic deletion of single Cyclin-Ds does not cause cardiac phenotype, triple deletion is lethal, partially due to cardiac defects[36]. Mutant CyclinD mice reveal a hypoplastic ventricle and ventricular septal defects. Overexpression of Cyclin-D family members lead to increased DNA-synthesis at baseline in an adult heart[37]. Another finding is the knockout of CDK2 in conjunction with CDK4 that is similarly embryonic lethal due to cardiac defects. CDK2 and CKD4-deletion leads to hypophosphorylation of Rb that in turn affects E2F and downstream E2F targets[34]. Double-mutant mice reveal a hypoplastic ventricle, dilated atria and ventricular wall thinning. Upon cardiac growth and maturation, the expression pattern of these cell cycle regulators is altered. Whether downregulation of these regulators cause the decrease in myocyte proliferation after birth or vice versa remains a mystery. Upon birth, Cyclin D, A, B1 and E and the corresponding kinases are significantly downregulated. Temporal studies reveal that downregulation of cell cycle proteins is accompanied by an upregulation of CDKIs[35, 38, 39]. CDKIs consist of two major protein families that are structurally and functionally different; the INK4 family (p15, p16, p18 and p19) and the Cip/Kip-family (p21, p27, p57). INK4-family CDKIs are selective inhibitors of CDK4 and CDK6 thereby blocking complex formation with Cyclin D and subsequent enzymatic activity. Since, Rb is an essential downstream effector of CDK4/6, INK4-family mediated inhibition of cell cycle progression requires a responsive Rb-protein. The Cik/kip-family of CDKIs, are more effective in inhibition of CDK2, which forms a complex with cyclin E and reinforces progression through the S-phase[40]. In addition, the members of cik/kip-family of CDKIs inhibit cdc2 and Cyclin A activity, thereby playing a broader role in inhibition of cell cycle progression throughout mitosis[40]. In the heart, expression of CDKIs has been studied during development as well as upon pathologic challenge. Although the expression of p16 has been suggested to be low in young adult hearts and increase in aged hearts, p16 and p18 are predominantly present during the embryonic development of the heart[20].

Expression of the Cip/kip family of CDKIs are undetectable during embryonic development, increase in the perinatal phase and peak in adult myocytes. Both, p21 and p27 are downregulated during cardiac injury[20]. Although not very well understood, this could potentiate increased DNA-synthesis that is required to support the metabolic need during a hypertrophic response.

Transcription Factors. An important transcription factor in cell cycle progression of a variety of cell types is E2F. E2F represents a family of eight members from which some function as transcription initiators and others transcription inhibitors. The main targets for E2F members are Cyclins, DNA-repair genes, checkpoint genes and apoptosis genes[10]. Due to the multitude of family members, studying the effects of specific E2F members is rather flawed, due to their compensatory tendencies. Specific roles of distinct E2F family members in the heart haven't been provided as yet, and expression patterns of E2F during cardiac development yield confusing and sometimes overtly contradictory results thus far[41]. Although deletion of E2F3 is mostly embryonic lethal and leaves survivors with early congestive failure, knock-out of other members do not cause an evident phenotype, presumably due to the functional redundancy of the family members[42, 43]. Overexpression of E2F1-4 in cultured neonatal rat cardiac myocytes increases the rate of S-phase entry, while E2F1 and 3 induce apoptosis[43]. Overall, an accurate role of E2F family members in cardiac proliferation remains unresolved. Nonetheless, since E2F-family members are key regulators of cell cycle progression, extensive and comprehensive studies regarding the regulation of the G1/S-phase is mandatory in understanding and manipulation of myocyte division. Another rather well studied transcription factor of myocyte proliferation is c-myc[44-49]. The myc family of transcription factors consists of three main members, N-myc, L-myc and c-myc, which regulate transcription by forming a heterodimer with the protein Max. C-myc has a long history of understanding in the oncology field where it is predominantly associated with excessive cellular proliferation. G1 exit is mediated by c-myc by various mechanisms including upregulation of Cdk4, cyclin D1 and D2, Cdc25A, cyclin E, and cyclin A[50]. In addition, it antagonizes the action of at least one Cdk inhibitor, p27[51]. Transgenic mice lacking c-myc expression do not survive post early embryonic stage and c-myc-null mice displayed a general developmental retardation that cannot be attributed to the heart[52]. Overexpression of c-myc mRNAcauses a hyperplastic ventricle during early postnatal development[53]. This increase in proliferation does not continue throughout the processes of maturation and aging. In an adult heart, overexpression of this proto-oncogene translates into a hypertrophic growth phenotype[53]. Although there are clues for a role of c-myc in myocyte proliferation the mechanisms for c-myc action in myocytes remain multifaceted and complex. Another, interesting, transcription factor in myocyte division is HIF1alpha. HIF1alpha knockout mice developed a hyperplastic phenotype, causing an obstruction of the outflow tract and subsequent complications[54, 55]. In addition, hyperplastic growth was not complimented by increased angiogenesis due to a lack of VEGF, which is naturally induced by HIF1alpa[55]. Mechanistically, HIF1 is a known antagonist of c-myc, hereby explaining the overall phenotype of HIF1 knockout mice[56]. Future studies on myocyte transcription factors require rigorous mechanistic hypothesis on whether such manipulations should be aimed at 'inducing proliferation' or 'inhibiting a checkpoint inhibition'.

Pocket Proteins. The pocket protein family consists of three proteins involved in regulation of CDKs in the G1-phase of the cell cycle; Rb, p107 and p130. During development, levels of Rb increase in myocytes, while p130 has the opposite expression pattern. Pocket proteins are well known for regulating E2F-effector genes thereby regulating cell cycle progression at the G1/S-transition[57]. In its active form, Rb is hypophosphorylated that allows binding to E2F and subsequent recruitment of transcription repressors and inhibiting cell cycle progression. Upon phosphorylation, Rb in incapable of binding to E2F, thereby enabling active transcription of genes crucial for cell cycle progression[58]. Major Rb phosphorylating kinases are CDK2 and CDK4. Rb plays an important role in cell cycle exit and myocyte differentiation[59]. Rb-deficient mice are lethal[60], however, animals deficient in Rb and p130 have an increased heart-to-body weight and show enhanced BrdU-incorporation and pH3-staining, indicating persistent myocyte division[61]. Although,

the role and pattern of pocket proteins require more elucidation, there are hints towards a role of these proteins in myocyte cell cycle progression.

One significant drawback of molecular studies regarding myocyte cell cycle is the necessity of using transgenic mouse models where cell cycle regulators are constitutively manipulated. The cell cycle is regulated in a tremendous dynamic fashion, where timely degradation of one protein in necessary for functionality of the next regulatory protein. This temporal oscillation is crucial for accurate regulation of cell cycle progression. Although conditional transgenic mice are available for c-myc and Cyclin D, the duration and intensity of cell cycle regulators in these model systems is anything but normal or physiologic so that insight and knowledge derived from such investigations likely only provides a jaded and biased glimpse of the functional properties of these important regulators in normal growth and proliferation. Unfortunately, to date, no knowledge is available on the temporal regulation for “molecular clocks” that time the distinct phases of a myocyte cell cycle. Thus, successful manipulation of cardiomyocyte proliferation versus hypertrophic growth remains rather complex. Recently, studies conducted on myocyte turnover in an adult heart using transgenic mice models[62] and carbon isotope-labeling[63] indicate that myocytes do possess potential for division. These studies were followed by overexpression of miRNA’s[64] and knock-out of meis1[65] in an attempt to further promote myocyte division. Although these initial probing exploratory approaches are acceptable for the current state of affairs, the efficiency of myocyte division remains poor. Ultimately, we still await new techniques and models that not only provide a system to be manipulated but will also provide insight into distinct phases of the cell cycle, the kinetics of molecular signaling in real-time, and the balancing act between stimulatory and inhibitory pathways.

Myocyte Senescence

Cellular senescence is a mechanism of protective irreversible cell cycle arrest in the face of threats stemming from DNA-damage and potential oncogenic risk. Cellular senescence can be induced by genetic and or epigenetic abnormalities. In addition, telomere shortening and/or oxidative stress-mediated metabolic changes can prompt acquisition of a senescent phenotype. A major initiator of senescence is the DNA-damage response (DDR) initiating cell cycle arrest. Persistent DDR activity presents as nuclear foci with high levels of DDR-proteins[16, 17]. Re-initiating or resuming cell cycle progression for a senescent cell is an uphill battle that would need to overcome high expression of p16 and p53, upregulation of pH2AX and increased lysosomal content. Inability of senescent cells to continue cycle progression consequently leads to lack of senescent cell contribution to maintenance of tissue homeostasis. Noteworthy is the fact that senescence is not a developmental process, but rather a degenerative process that predominantly is forced upon myocytes by the environment and demands adaptations that ultimately lead to a twilight existence typified by marginal function that can only end in death. Senescent cells can withdraw from cell cycle and hold up in G0 if cellular damage is detected during G1-phase (e.g telomere erosion). Detection of DNA-damage at a DNA-checkpoint at G2/M (where ATM/ATR is activated) does not lead to cell cycle withdrawal but a permanent cell cycle arrest at the checkpoint[13, 14, 66]. Although these concepts are used interchangeably in the literature, the interpretation of different types of cell cycle disruptions in a senescent cell is crucial for manipulation and reversal of such phenotypes.

Myocyte senescence is associated with mitochondrial dysfunction. Oxidative stress and nutrient deprivation result in excessive amount of reactive oxygen species (ROS) production in the mitochondria as a by-product of oxidative phosphorylation processes. Oxidized proteins can aggregate and interfere in biological function. Simultaneously, ROS attack mitochondrial membranes and cause additional damage to mitochondrial DNA and oxidizing enzymes that, in turn, contributes to additional ROS production. Concurrently with these changes, mitochondrial biogenesis can be hampered due to lack of energy substrates as well as loss of intact mitochondrial DNA[67, 68]. Mitochondria carry their own telomeres[69], and telomeric attrition within the mitochondria themselves presumably compromises their capacity for self-biogenesis. Collectively, the accrual of these aforementioned adverse events leaves a rather cytotoxic

internal milieu. Mitochondrial preservation as a strategy for staving off aging has been shown by overexpression of mitochondrial catalases as well as deletion of p66shc[70]. Thus, mitochondrial functional impairment serves as a primary inciting stimulus for transition into senescence.

Another major contributor to myocyte senescence is chronic adrenergic signaling. Although a powerful compensatory system in the short run, hyper-activation of the renin-angiotensin-aldosterone signaling axis inflicts damage directly upon myocytes. In particular, angiotensin II has a detrimental effect upon myocytes through downregulation of SERCA2 and diminished calcium homeostasis[71, 72]. Mice with local cardiac overexpression of AngII developed dilated cardiomyopathy and an aged phenotype[73]. Consistent with these findings in experimental models, patients with dilated cardiomyopathy are now regularly treated with angiotensin-converting enzyme-inhibitors to blunt ongoing cardiac remodeling[72].

And finally, as described in multiple other systems, the mTORC pathway is involved in myocyte senescence and inhibition of mTORC1 has been linked to blunting of senescence phenotype acquisition[74] consistent with caloric restriction diets that prolonged lifespan in rodents[75]. Overstimulation of mTORC1 pathway through growth factors promotes cardiac senescence[76]. Typically, inhibition of mTORC1 has been performed pharmacologically with rapamycin, but side-effect toxicity of the drug limits clinical utilization. Alternatively, a molecular interventional approach to selective mTORC1 inhibition and diversion toward mTORC2 has been studied by our group using PRAS40 in the myocardial context, where overexpression of PRAS40 ameliorates hypertrophy and prevents development of diabetic cardiomyopathy in rodents.[77-79]

Any possibility for molecular interventional strategies toward senescence reversal will require understanding the underlying molecular cues responsible for prompting and maintaining the senescent state. Antagonism of replicative senescence caused by telomere attrition is a feasible target for reversal as previously performed in murine and human cardiac stem cells[80-82]. Similarly, metabolic and catecholamine-based senescence could be amenable to manipulation and subsequent rejuvenation[83]. However, a judicious approach is mandatory in reversal of senescence in systems with DNA-damage and cellular senescence due to excessive DNA-damage. Reinforcing cell cycle progression in cells that show evidence of “non-reliable” DNA is probably not worth the risk.

Myocyte Quiescence

Quiescence (Latin: quiescere = to rest) refers to a state of quietness and rest. The role of quiescence is crucial in phylogeny. Exponential growth is historically shown to inevitably surmount the supplies required for survival of species, as would be the case for organisms and their constituent cells that may be challenged to survive under nutrient deprivation or other compromised environments. Thus, growth arrested state is biologically reasonable if not mandatory for adaptation to stress. Long-standing presumptions asserted that cells become quiescent in response to external stimuli, but recent insights suggest that cells carry an autonomous propensity for quiescence as a mechanism to preserve their fundamental biological characteristics. Quiescent cells reside in the G0 phase of the cell cycle and therefore posses ability to re-enter the cell cycle upon normal physiological stimulation[84, 85]. Thus, although quiescence refers to a state of growth arrest, this type of withdrawal from cycling does not involve a prolongation of the G1-phase. Actually, the G1-phase of cell cycle possesses a restriction point that can serve to determine cell fate. Cells can exit G1 before the checkpoint, in mammals called the “restriction-point” and become quiescent, but passage through restriction-point is considered irrevocable commitment toward replication and division[84, 85]. A unique constellation of a phenotypic signature for quiescent cells is lacking, but is slowly emerging based upon investigation of genetics, epigenetic and transcriptional profiling of the resting state. On the molecular level, a major player of quiescence is the previously mentioned tumor suppressor Rb. Indeed, the quiescent population in hematopoietic stem cells vanishes if all three Rb family proteins are genetically

deleted[84-86]. Consistent with a cell cycle arrest phenotype, p21, p27 and p57 are upregulated in quiescent cells. Prevailing thinking posited that quiescent cells ought to maintain expression of key cell cycle regulators in preparation to enter the cell cycle in response to appropriate inductive stimulation. Expression and synthesis of cell cycle regulators requires energy consumption from the cells, thus implies increased metabolic activity. This metabolic demand is contradictory to the main quiescence attribute of low energy consumption. Indeed, quiescent cells regulate cellular processes in a less energy demanding fashion, miRNA-mediated regulation of transcription, in particular the microRNA 16 family[87, 88]. Since miRNA families target multiple genes in common pathways, miRNA's have been proposed as major regulators of quiescence. Transcriptional regulation of a quiescent phenotype has been extensively studied in multiple types of stem cells[89], revealing a "blueprint" for creating senescence involving down regulation of genes promoting cell cycle progression, DNA-replication and metabolic pathways. Specifically, major cyclins (B1, A2 and E3)[84-86, 90, 91], Survivin[92] and cytochrome c are suppressed in conjunction with up-regulation of genes important for differentiation and fate decision-making (FOXO3 and EZH1)[84-86, 90, 91]. Observing that epigenetic modification influences gene expression, epigenetic profiling of quiescence has begun in earnest. Studies conducted in embryonic stem cells reveal that presence of bivalent domains in the proximity of transcriptional sites are key regulators of gene expression. Two such domains have been shown to be relevant in a quiescent phenotype, namely H3K4me3 and H3K27me3. In muscle stem cells and human fetal stem cells manipulation of a great number of genes are marked by H3K4me3, which indicates active transcription sites in a phenotype with low transcriptional activity[93].

Overall, the black box of quiescence is slowly revealing the mysteries hiding within, offering new hope for comprehensive understanding of myocyte quiescence that will provide exciting opportunities for manipulation of cell cycle entry and division to enhance myocardial function and regeneration. Unlike senescent cells, quiescent cells provide a tremendous platform for new cell formation, since the cells retain origin and lineage commitment, lack hurdles such as DNA-damage and risks for adverse effects and essentially require a push in the right direction. Unfortunately, quiescence has never been rigorously studied in (cardio)myocytes. The term "myocyte quiescence" in the literature is based upon a lack of DNA-synthesis or incorporation of BrdU. However, a distinction between myocytes in G1 versus G0 has never been possible due to lack of accurate myocyte models that provide distinction between these phases.

If you can't explain it easily, then you don't understand it well enough

Albert Einstein

For decades, the heart was considered a postmitotic or recently a *mostly* postmitotic organ. A postmitotic cell refers to a cell that has completed mitosis, not surrended the capacity for cell cycling activity. Since processes such as hypertrophic growth and DNA-synthesis and repair require cell cycle proteins and machinery, these features could be interpreted as indicative of the myocardium being in an ongoing state of "premitotic" life and the heart as a "premitotic" rather than "postmitotic". Comprehending the distinction between possibilities of premitosis versus postmitosis are crucial for defining targets to manipulate in order to promote myocyte division.

The ever-elusive goal of myocardial regeneration is closer today than ever and currently enjoying a renaissance with the discovery of new rules for the reparative and regenerative potential of the heart. However, ambition and enthusiasm surrounding myocardial regeneration seem to be exacerbating an intellectual disconnect between "myocyte division" and "myocyte cell cycle". Currently, the field lacks straightforward mechanistic postulates on how the number of dividing myocytes can be increased. If the aim is to force a binucleated myocyte to divide, what exactly is it that we want such a myocyte to do? If the aim is to predominantly focus on mononucleated myocytes, then are we aiming for quiescent

mononucleated cells, mononucleated cells that are presumably arrested in G1 or do we want to rejuvenate senescent cells and reinforce division (mitosis)? If a mononucleated myocyte population is in G1, is it due to a checkpoint inhibitory block or a lack of stimulatory regulators? Are these two pathways additive or is one superior to the other? Overall, after all our pretentions to mastery of myocardial biology fall away, we must confess that we *can't easily explain regulation of the myocyte cell cycle* just yet. Efficient and successful manipulation of myocyte division requires insight in all phases and processes of the myocyte cell cycle; quiescence, G1, S, G2/M, checkpoints, senescence and perhaps most importantly a temporal assessment of the duration of a myocyte cell cycle. Future studies must be aimed at generating reporter models that provides real-time distinction between the different stages of the cell cycle, duration of these phases in conjunction with ploidy and regulatory pathways.

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Chapter 2

Myocyte Specific Fluorescent Ubiquitination Cell Cycle Indicator allows Myocyte Cell Cycle Detection and Progression

Work in Progress

Introduction

An aura of mystery surrounds the division of myocytes due to remarkable myocardial resilience to proliferative stimuli. Since the 1980s, scientists have endeavored to interfere in the myocyte cell cycle to force proliferation. Proteins such as Telomerase Reverse Transcriptase (TERT)[1], c-Myc[2], IGF[3], c-Fos[4], Cyclin D[5, 6] and Akt[7-9] appear to enhance myocyte proliferation during fetal development but fail to persist upon maturation and, ultimately, lead to hypertrophy in the adult heart. The manipulation of myocyte division requires a rigorous understanding of innate myocyte proliferation and a distinction between cell cycle phases to clarify where and how possibilities for interventions appear. Myocyte turnover in an adult heart has been reported to vary from less than 1%[10-12] to 40%[13, 14] per year. The basis for this expansive range lies in the methodological challenges associated with studying a slow growing and heterogeneous organ with cells at diverse stages of development and cell cycle. Studies have frequently used halogenated nucleotide analogues and radiolabeled thymidine but these cause tissue toxicity, thereby limiting experiment duration, and appear to interfere with normal cellular biology by inducing cell cycle arrest.[15-18] Confocal microscopy is regularly used to detect myocyte cell cycle stages, however accurate cardiomyocyte identification requires both cytoplasmic and nuclear markers to exclude proximate non-myocyte nuclei thus limiting the available staining palette. Convenient myocyte cell cycle reporter systems that can provide insight without tissue toxicity or interference with native cell biology are needed.

Here, we describe a myocyte specific Fluorescent Ubiquitination Cell Cycle Indicator (FUCCI) reporter system that permits the detection of cell cycle phases. The concept of FUCCI is based upon the oscillation of two cell cycle proteins that play a role in replication origin licensing, Chromatin licensing and DNA replication factor 1 (Cdt1)[19] and Geminin[19]. As direct substrates of SCF^{skp2} [20] and APC^{cdh21}, respectively, protein levels of Cdt1 and Geminin oscillate inversely through the stages of the cell cycle.

Eukaryotic cells have developed accurate control mechanisms to assure that cells undergo only one round of DNA replication per cell cycle to maintain the correct karyotype through multiple rounds of division. DNA replication initiates at the origins of replication, requiring the assemblage of protein complexes dictating the process. Transitions of these protein complexes are very tightly and accurately regulated throughout the cell cycle to avoid re-replication and cellular transformation. Cdt1 plays a major role in determining when licensing takes place. During G1, the origin recognition complex binds to the origin

and Cdt1 (and Cdc6). This pre-replication complex recruits the Mini-Chromosome Maintenance (MCM) proteins 2-7.[22-26] This complex then serves as a helicase in unwinding the double-stranded DNA. Upon loading DNA-polymerase, Cdt1 is released from the complex. This subsequently results in the disassembly of the complex causing the cell to exit from G1. At the onset of S-phase, Cdt1 is degraded through ubiquitination proteolysis. From S-phase through the end of mitosis, the Cdt1 inhibitor Geminin is expressed, serving to limit Cdt1 activity strictly to the G1-phase of the cell cycle. At the end of mitosis, Geminin is degraded through ubiquitination proteolysis and Cdt1 assembly is initiated. Once Cdt1 has accumulated sufficiently a another round of DNA replication may begin again during the G1 phase.[22, 27] When exiting the cell cycle in response to growth arrest and entering a quiescent state by resting in G0, the licensing machinery is lost and nuclei fail to replicate their DNA.[28] Protein levels of Cdt1 and Geminin have been shown to oscillate predictably during G1 and S/G2/M-phase of the cell cycle and are down regulated in quiescent cells.[27, 29, 30]

The FUCCI-reporter technique tracks this dynamic physiological turnover by fluorescent labeling of Cdt1 and Geminin. A truncated form of human Cdt1 is labeled red and indicates the G1-phase, while truncated human Geminin is labeled green indicating S/G2/M.[19] Cells in transition from G1 to S-phase present in yellow and cells in G0 are colorless. The FUCCI-system has been used regularly *in vitro* to study HeLa cell response to hypoxia and cell cycle arrest agents.[31-35] Visualization of neural tissue *in vivo* has been demonstrated using a global FUCCI-transgenic mouse with ubiquitous expression of Cdt1 and Geminin that provides insight in proliferation patterns during development.[19] In addition, FUCCI-zebrafish have been generated and used in elucidating cell proliferation in whole fish embryos.[36] Recently, cardiac myocyte transgenic zebrafish were developed to study myocyte proliferation and identify chemical modifiers.[37] Although zebrafish are ideal for high throughput screening, the highly regenerative potential of a zebrafish heart does not serve as a basis for insight into the mammalian myocyte cell cycle. In this manuscript, we took advantage of the FUCCI-system by generating an alpha-myosin heavy chain promoter driven transgenic mouse model to study Cdt1 and Geminin oscillation and demonstrate the validity of this system for future interventional studies on the myocyte cell cycle.

Results

Cdt1-mKO and Geminin-AzG are expressed in myocytes and indicate distinct phases of the cell cycle.

To validate the expression specificity in myocytes of the FUCCI reporter constructs, Cdt1-mKO and Gemenin-AzG (Gem-AzG), hearts from adult FUCCI-mice were isolated and stained for a cardiac specific cytoplasmic (tropomyosin) marker in addition to the nuclear marker Gata-4. Gem-AzG protein is present exclusively in cells that are positive for tropomyosin and Gata-4 (Fig 1), indicating that the reporter construct is only present in myocytes and absent in cardiac stromal cells.

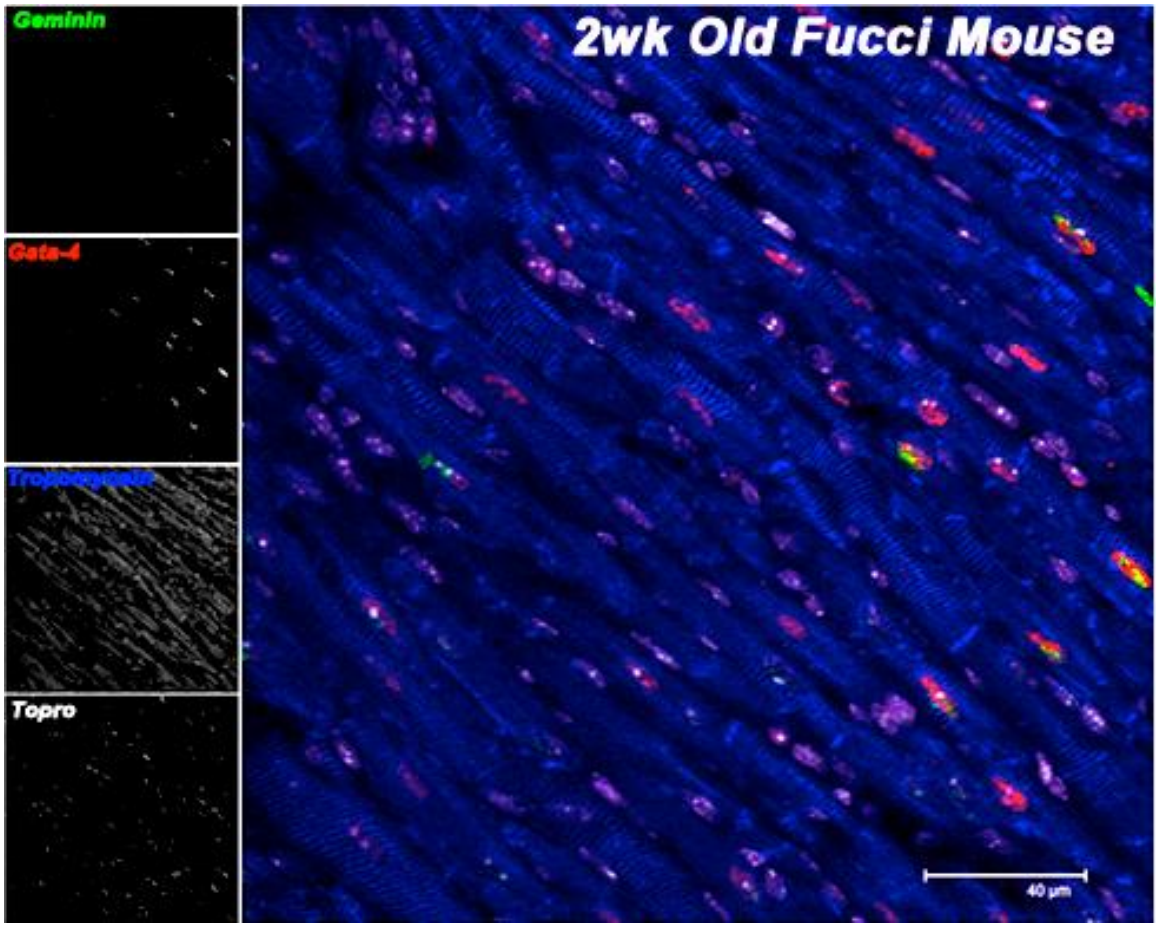


Fig 1. Fucci construct expression is restricted to myocytes. Confocal image of a 2 week old Fucci heart representing colocalization of Geminin (Green), Gata-4 (Red) and tropomyocin (blue). Nulei are represented by Topro staining (white).

To test whether levels of Gem-AzG in myocytes correlates with distinct phases of the cell cycle, two cell cycle proteins were chosen: a long-lived protein that persists throughout the entire cell cycle but peaks in the S-phase, PCNA, and a mitotic marker phosphor-Histone3 (pH3). Our results demonstrate that Gem-AzG protein is colocalized with PCNA

in cardiac myocytes (Fig 2 left). Although some PCNA+ cells are detected that are Gem-AzG negative (indicative of cells G1) all Gem-AzG+ cells are PCNA positive at 7 days of age. In addition, Gem-AzG colocalizes with pH3 in myocytes of a 7 day old FUCCI-heart (Fig 2 right) indicating that Gem-AzG levels in myocytes are representative of the S/G2/M-phases of the cell cycle.

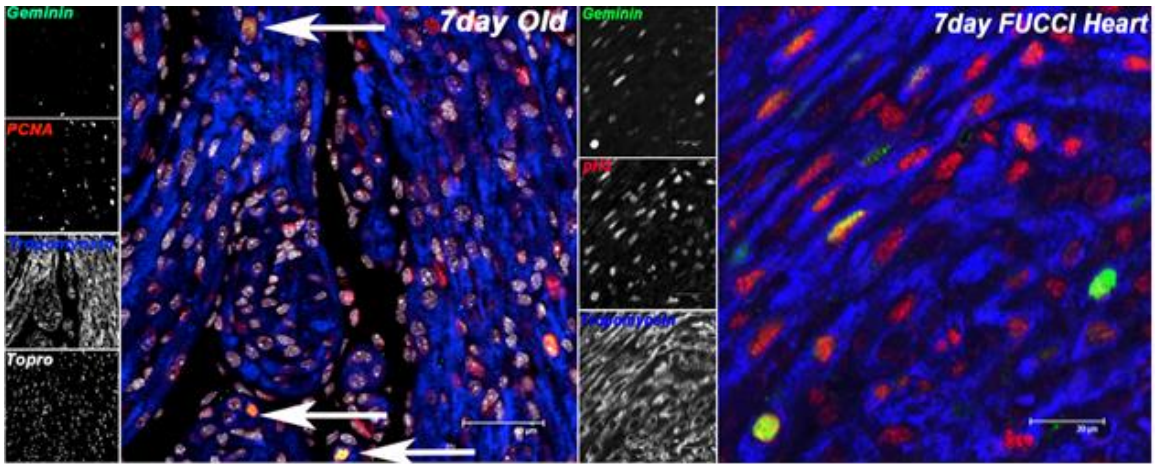


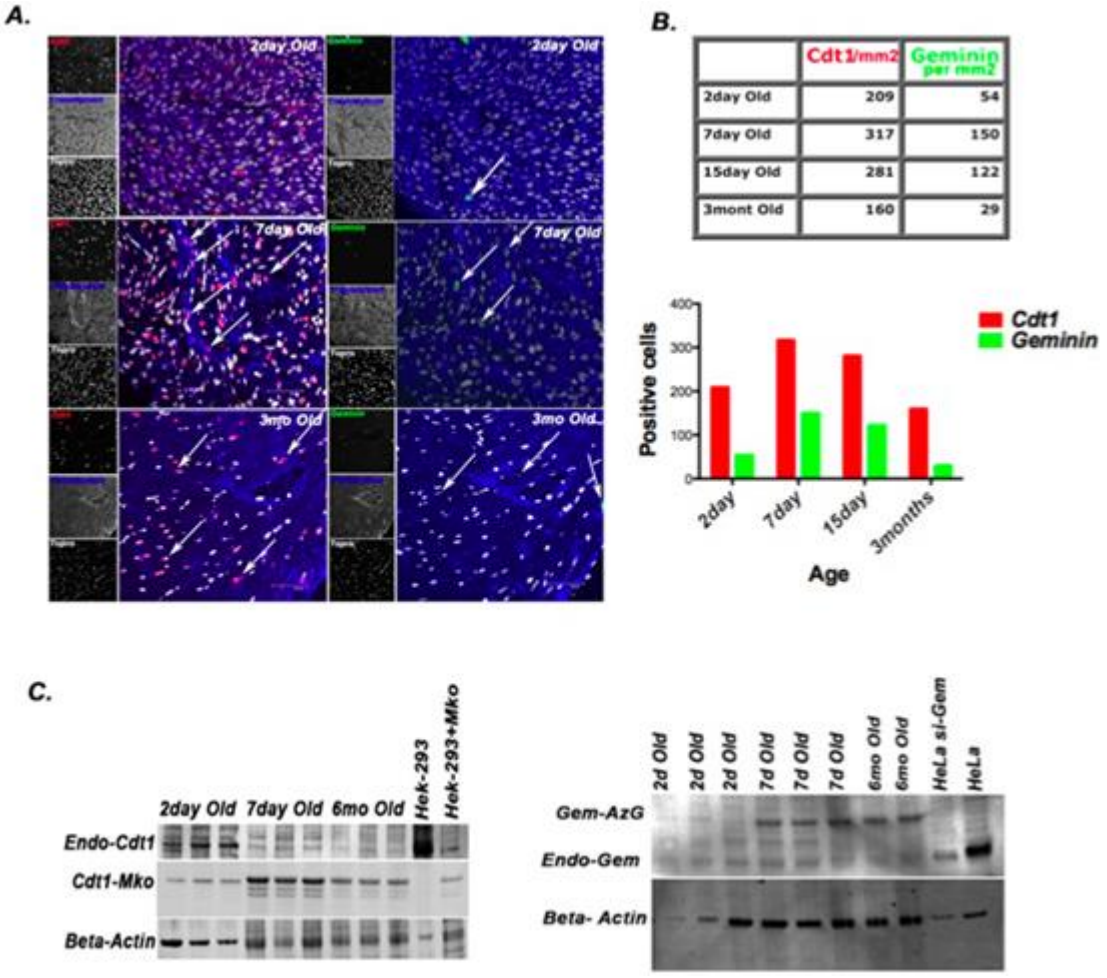
Fig 2. Geminin expression in myocytes indicates S/G2/M-phases of a myocyte cell cycle. Confocal images of 7 day Old Fucci hearts demonstrating colocalization of Geminin (green) with PCNA (Red) (Left pannel). (Right pannel), colocalization of Geminin (green) with phosho-H3 (red).

Cdt1-mKO and Gem-AzG levels oscillate and decrease upon age in myocytes during development

To determine whether levels of Cdt1-mKO and Gem-AzG oscillate during development and correlate with the proliferative state of the heart, Fucci-hearts were stained at different time-points during development for Cdt1-mKO and Gem-AzG. Both Cdt1-mKO and Gem-AzG are expressed in myocytes 2 days postnatal, increase at 7 days and decrease upon maturation in an adult heart (Fig 3A). Although the total number of Cdt1-mKO+ cells is higher at each time-point than the number of Gem-AzG+ cells, as detected by cell counts, the overall trend and pattern remains similar (Fig 3B). Similarly, protein levels of both Cdt1-mKO and Gem-AzG are detectable 2 days postnatal, increase at 7 days and decrease in an adult heart (Fig 3C).

To further validate the findings, and to avoid concerns regarding antibodies and autofluorescence, FUCCI hearts were studied using flow cytometry by detecting the original fluorescent tags, mKO and AzG without addition of antibody against Cdt1 or Geminin.

Fig 3. mKO and Gem decrease upon age and correspond with endogenous expression of Cdt1 and Geminin, respectively. A. Confocal images of 2-day, 7-day, 15-day and 3-month old Fucci mouse demonstrating Cdt1-mKO in myocytes (red) and Gem-AzG in myocytes (green). B. Quantification of cell counts of 2-day, 7-day, 15-day and 3-month old Fucci mouse, demonstrating a decrease upon maturation. C. Western Blot analysis of Fucci-construct and endogenous Cdt1 and Geminin proteins demonstrating a decrease in protein levels upon maturation.



Myocytes isolated from Fucci hearts at various developmental time-points were viable and capable of expressing the Fucci construct post isolation (Fig 4). To be able to select myocytes, the mononuclear cell population was stained specifically for alpha-sarcomeric actin. The alpha-sarcomeric actin population was selected and gated upon for further analysis strictly limited to the myocyte population. Age-matched non-transgenic mice were used as controls for auto-fluorescence.

Four distinct populations were characterized and quantified: Cdt1-mKO, double-positive cells, Gem-AzG cells and negative myocytes (Fig 5/6). FACS analysis reveal that Gem-

Fig 4. Isolated myocytes from Fucci hearts are viable and express the Fucci-construct. Isolated myocytes from a zero day old Fucci heart (upper left), 20 day (upper right) and 4 week old (bottom) Fucci hearts.

AzG positive cells occur at 2% at 2 days of age, peak to 12% between 15 and 20 days of age and decrease precipitously upon maturation to 1.5% in the adult heart. Thus the maximum number of myocytes in S/G2/M-phase of the cell cycle occur between 15-20 days, in line with prior literature indicating a peak in binucleation. Cdt1-mKO positive cells occur at 9% in 2 day old hearts and peak at 20 days reaching 29%. Thus the maximum number of myocytes in G1-phase of the cell cycle occur at 20 days, possibly indicating maturation and hypertrophic growth. Similarly, the number of double-positive cells reach a maximum level of 5% at 20 days (Fig 5/6 Red).

The population selected by sarcomeric staining negative for both Cdt1-mKO and Gem-AzG are myocytes in G0 (Fig 5/6 Blue). This population occurs at 88% in a 2 day old, decreasing to 54% in a 20 day old, then increasing to 95% in an adult heart. This indicates that the majority of myocytes in an adult heart are quiescent. The negative myocytes

population may also contain senescent myocytes, an intriguing possibility beyond the scope of this study.

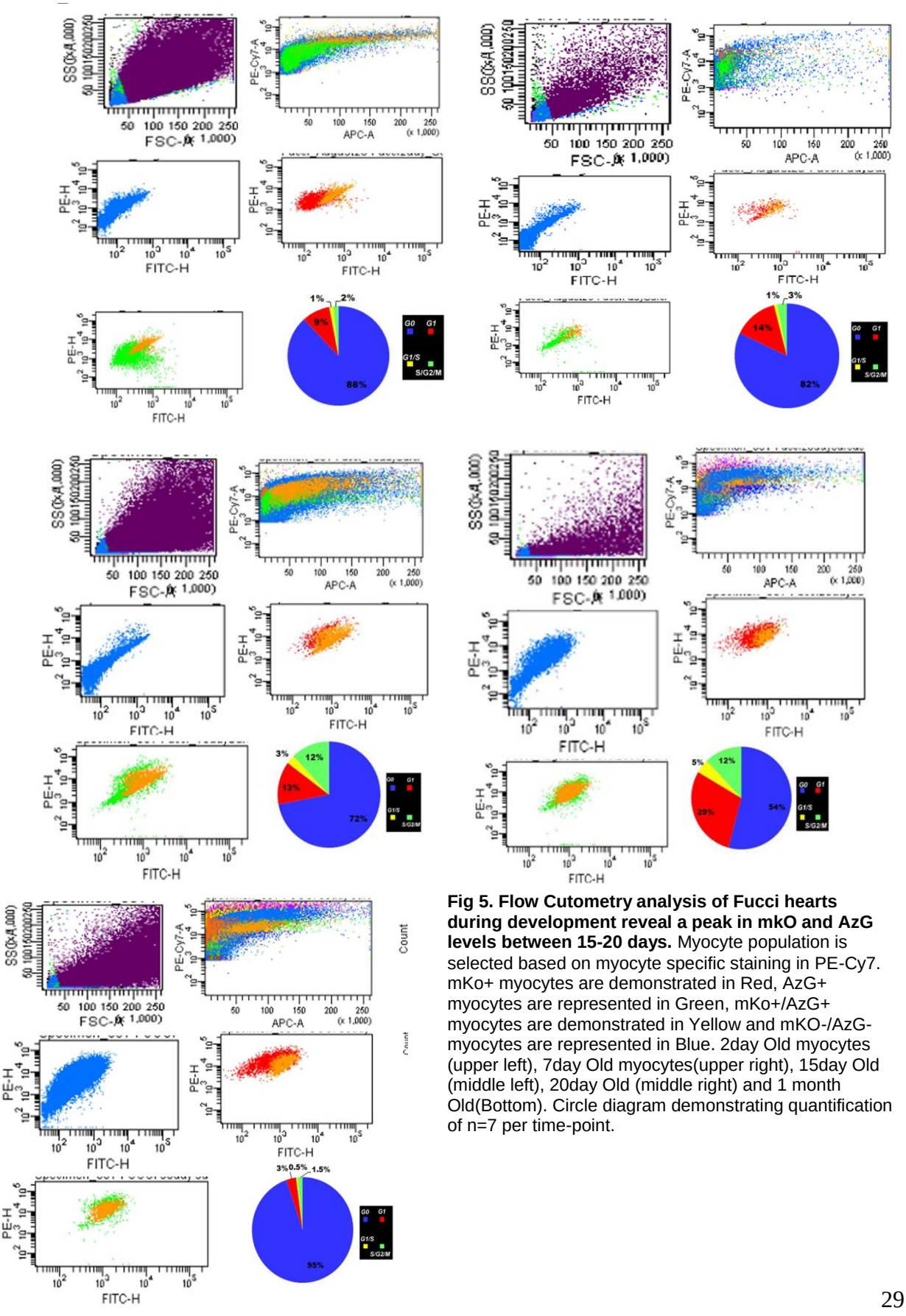
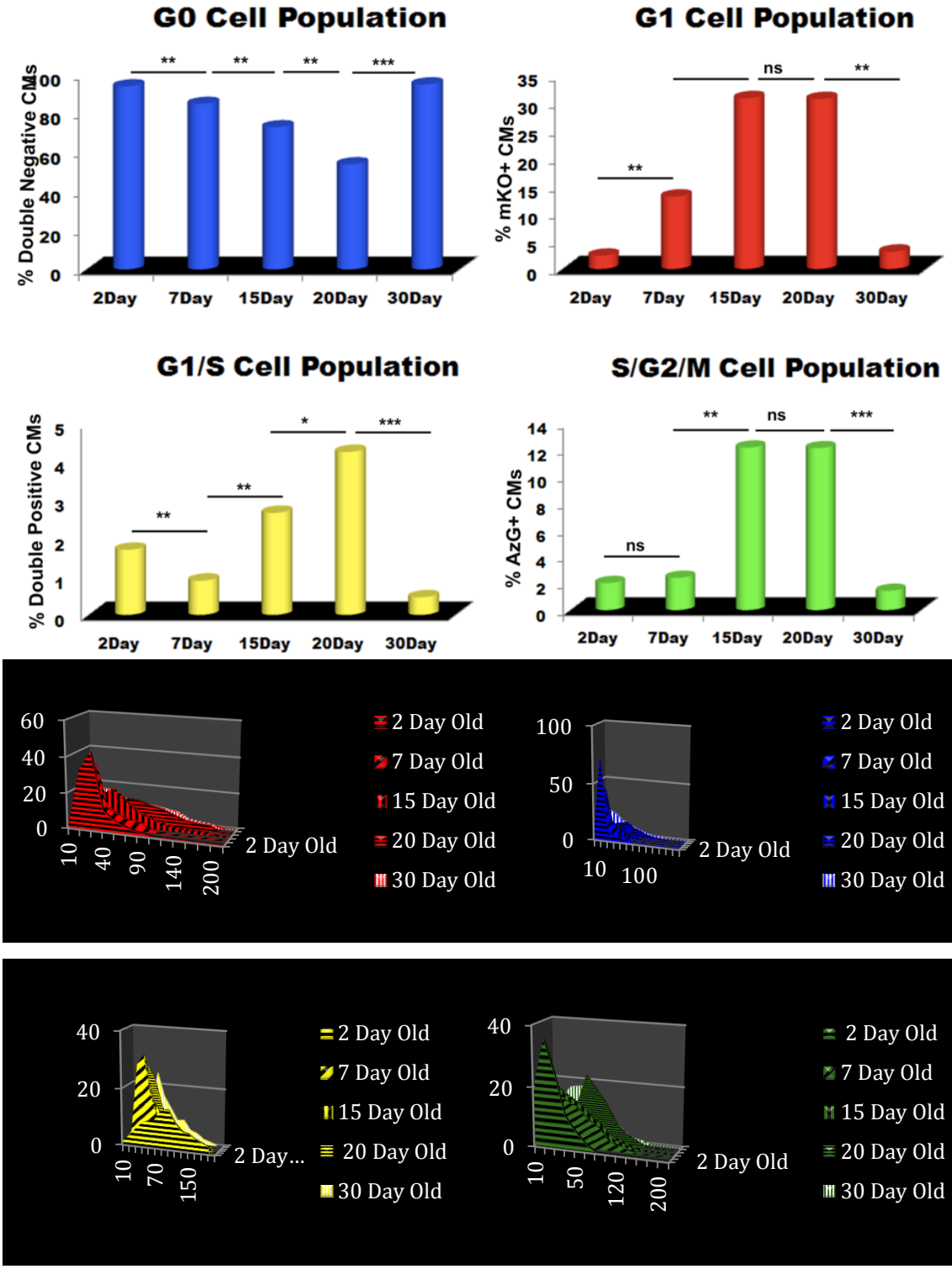


Fig 5. Flow Cutometry analysis of Fucci hearts during development reveal a peak in mkO and AzG levels between 15-20 days. Myocyte population is selected based on myocyte specific staining in PE-Cy7. mKo+ myocytes are demonstrated in Red, AzG+ myocytes are represented in Green, mKo+/AzG+ myocytes are demonstrated in Yellow and mKO-/AzG- myocytes are represented in Blue. 2day Old myocytes (upper left), 7day Old myocytes(upper right), 15day Old (middle left), 20day Old (middle right) and 1 month Old(Bottom). Circle diagram demonstrating quantification of n=7 per time-point.

Fig 6. Increase in levels of mKO and AzG between 7 days and 20 days correspond with an increase in total amount of DNA. FACS quantification of the number of cells in the cell cycle and quantification of Topro staining for subsequent ploidy determination showing an overall increase in DNA content between 7and 20 days of age.

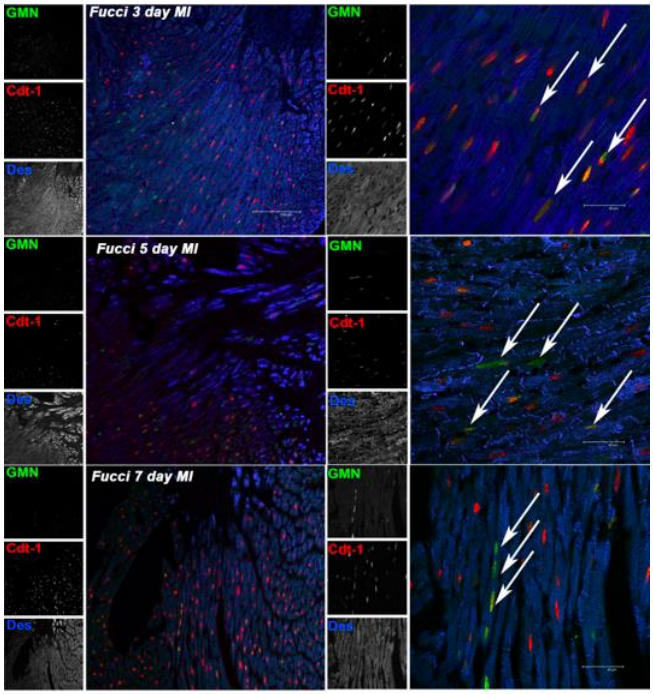


Gem-AzG levels 15-20 days postnatal indicate an increase in myocyte ploidy and binucleation

Myocytes isolated from Fucci hearts were fixed with ethanol and stained for Topro to study myocyte ploidy. A DNA average for various time points during development reveal a shift in the ploidy curve 15 days postnatal that is maintained upon further maturation (Fig 6 Bottom). The double-positive population (Fig 6 yellow) shows an initial increase in ploidy at 2 days, then decreasing at 7 days indicating the occurrence of cytokinesis between 2-7 days.

Cdt1-mKO & Gem-AzG increase upon myocardial injury

To study myocyte cell cycle re-entry after injury, myocardial infarction was induced in adult FUCCI-hearts. Cdt1-mKO levels increase after pathologic injury in the border zone and remote region from 5 days post MI to 10 days post MI, peaking day 7. Gem-AzG, however, is higher in the remote region and remains low in the border zone of the infracted wall (Fig 7).



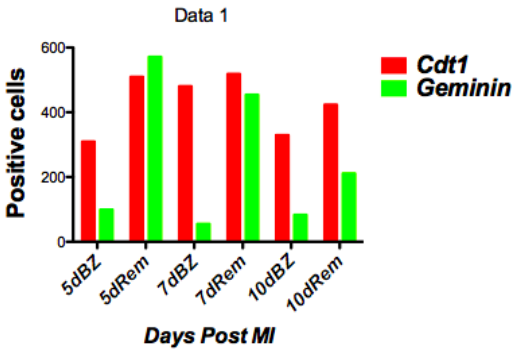


Fig 7. Myocytes re-enter the cell cycle upon pathologic challenge. Confocal images of mKO and AzG stainings in FUCCI mice at 3 days, 5 days and 7 days post myocardial infarction. Quantification of cell counts reveal a peak increase.

Cdt1-mKO & Gem-AzG levels do not indicate hypertrophy or repair

Myocyte entry into the cell cycle does not necessarily indicate proliferation as processes like DNA-repair or hypertrophy could also lead to DNA-synthesis and positivity for S-phase markers. To exclude this, infarcted FUCCI-hearts were stained for ANP (hypertrophy) and

the well-known DNA-repair protein 53BP1 (Fig 8). In both the border zone and remote region of infarcted FUCCI hearts, Cdt1-mKO and Gem-AzG did not colocalize with the conventional hypertrophy marker ANP (Fig 9), indicating that the entry of these myocytes into the cell cycle is not due to a hypertrophic response. The same heart sections were stained for 53BP1 to detect a potential repair response. Our findings reveal that Cdt1-mKO and Gem-AzG did not colocalize with 53BP1 in border zone and remote region at different time-points after infarction (Fig 9) suggesting that myocyte expression of Gem-AzG does not represent a DNA-repair process or a hypertrophic response.

Fig 8. Gem-AzG and Cdt1 does not indicate a repair process.
Fucci heart sections stained for 53BP1 (red) and Gem (Green) and Cdt1 showing no colocalizati**on.**

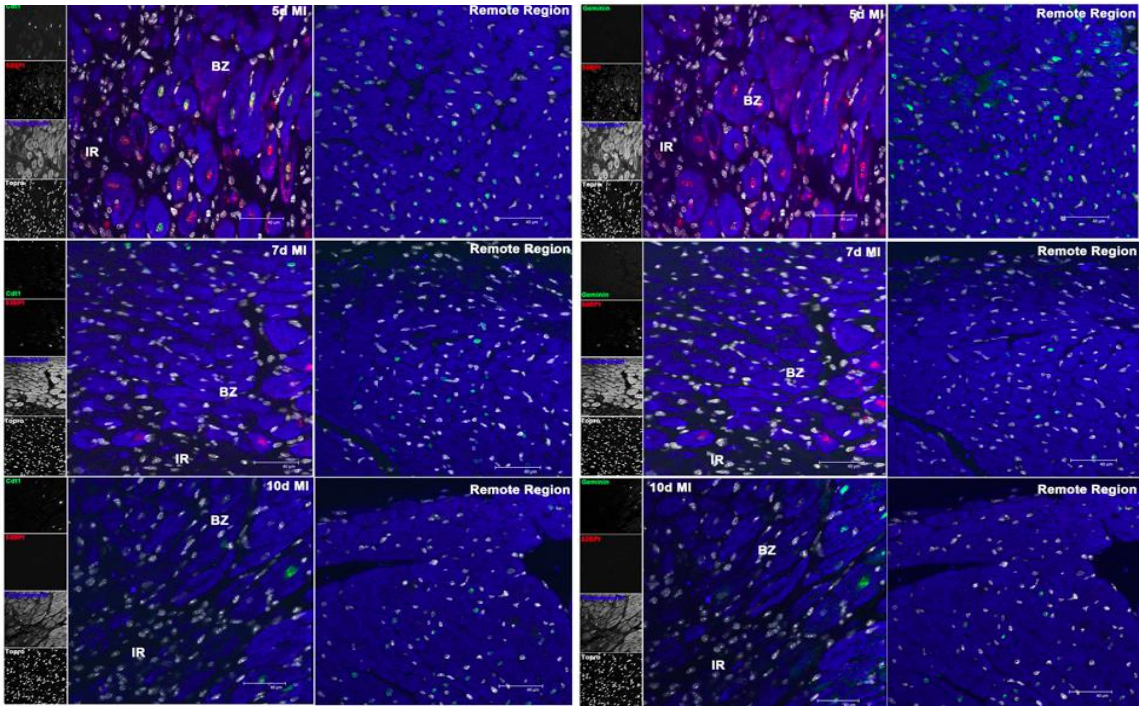
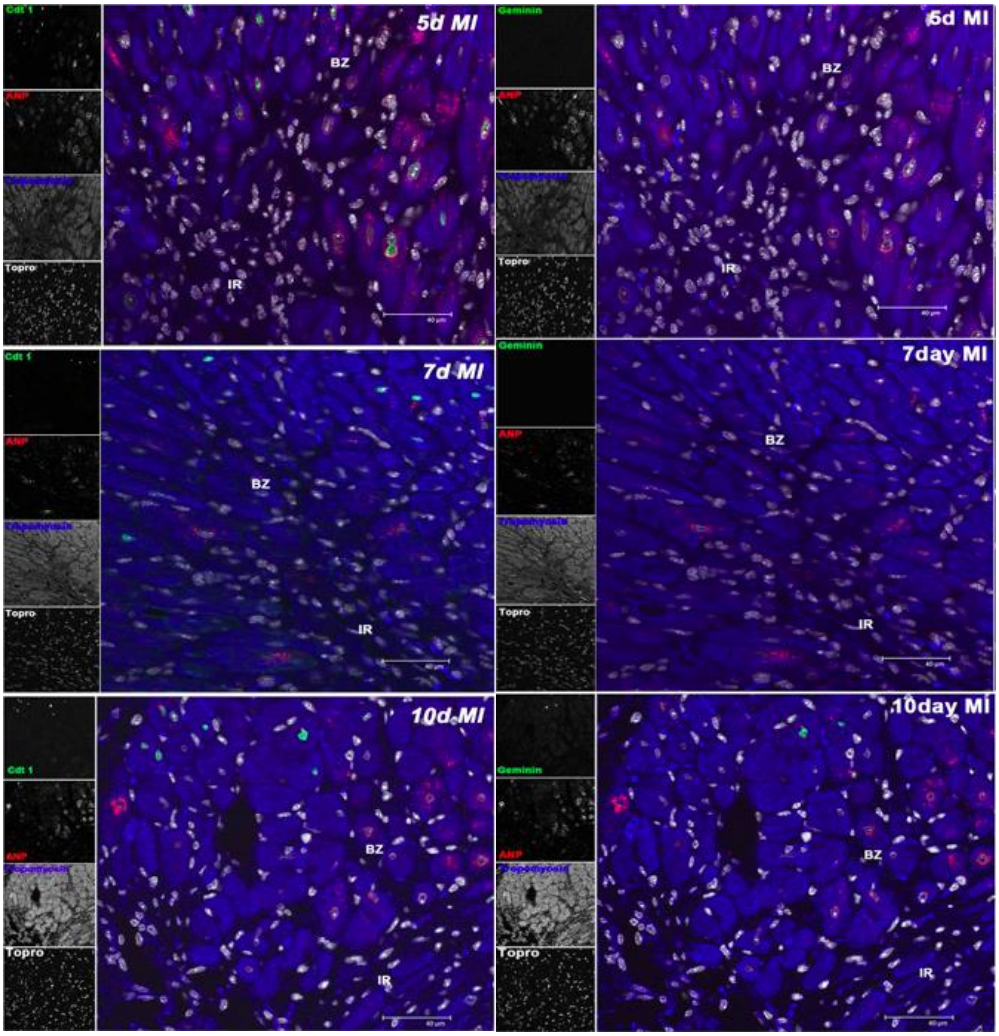


Fig 9. Geminin and Cdt1 expression do not indicate a hypertrophic response. Confocal images of Fucci hearts stained for ANP (red) and Gem/Cdt1 (green), showing no consistent colocalization.



Discussion

The past two years have been most successful for the field of myocyte division. Sophisticated mouse models and carbon dating have been used to estimate myocyte turnover.[12, 38] Recently, two elegant studies demonstrated interference in myocyte cell cycle and successful division of myocytes by knock down of Meis1[39] and over expression of microRNA-590 and microRNA-199a.[40] The current consensus is that myocytes certainly are capable of division; however the rate at which this occurs is very limited, despite manipulation through Meis1 and/or microRNAs, for example. Manipulation of myocyte division has been proven most successful during the fetal development.[1, 2, 9, 39, 40] However, the precise instance of transition to myocardial resistance to division remains unknown. The technique described in this manuscript represents a step forward in observing this complex division machinery. FUCCI allows myocyte cell cycle detection without incorporation of radiolabeled thymidine or halogenated nucleotides and with or without using confocal microscopy. To our knowledge, this is the first technique that allows distinction between, G1, S/G2/M-phase of the cell cycle and the quiescent population in G0. By using FACS analysis, insight can be gained by determining the ploidy status of myocytes in conjunction with the phases of the cell cycle. Future research may take advantage of this system by studying multi-nucleation to a greater degree. Though the Fucci system alone cannot reveal the exact phase of the cell cycle from S-phase on, the system can serve as a useful tool in double transgenic mice where the cell cycle is manipulated and progression is studied. Previous studies have revealed myocyte cell cycle re-entry post injury mediated repair and enhanced cardiac function.[5, 6] In addition to myocyte cell division, processes such as cell cycle re-entry mediated repair mechanisms dictate further intensive research using reporter systems.

The Fucci-system reveals the number of cells entering the cell cycle and G0. Different systems will be needed to analyze the senescent cell population. The phases of a myocyte cell cycle are informative but do not offer insight into the origin of these cells – whether they are stem cell or myocyte derived. Double-transgenic mice using Fucci and cardiac stem cell tracking mice may provide further understanding of the source and efficacy of myocardial regeneration. Doing so will represent a great step toward the manipulation of myocyte division and repair.

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Part Two: Survival Signaling Pathways in Myocardial Reversal of Senescence

Chapter 3

Cardiac Hegemony of Senescence

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Abstract

Cardiac senescence and age-related disease development have gained general attention and recognition in the past decades due to increased accessibility and quality of health care. This advancement in global civilization is complementary to concerns regarding population aging and development of chronic degenerative diseases. Cardiac degeneration has been studied rigorously in the past. The molecular mechanisms of cardiac senescence are on multiple cellular levels and hold a multilayer complexity level thereby hampering development of unambiguous treatment protocols. In particular, the synergistic exchange of the senescence phenotype through a senescence secretome between myocytes and stem cells appear complicated and of great future therapeutic value. The current review article will highlight hallmarks of senescence, cardiac myocyte and stem cell senescence and the mutual exchange of senescent secretome. Future cardiac cell therapy approaches require comprehensive understanding of myocardial senescence to improve therapeutic efficiency as well as efficacy.

Introduction

The word senescence derives from the Latin *senescere* that translates literally as “to grow old”. Aging and inter-species differences in longevity have fascinated scientists and philosophers since the beginning of recorded history with ancient Egyptians. Aristotle’s *Parva Naturalia* (350BC) called attention to organism aging and degenerative conditions. However, in more recent times 40 years ago, initial insight was achieved in the cellular biology of senescence by Hayflick’s description of senescence as ‘a process that limits proliferation’[1]. Subsequent decades witnessed increasing cumulative knowledge on the causes and nature of senescence and organ-specific effects of senescence.

Aging and senescence are often used interchangeably and synonymously, but the evolutionary and biological basis of these processes and underlying mechanisms show deviations. Aging refers to biological, behavioral and social variability occurring over the course of life that does not necessarily increase the risk of death. Senescence, however, is a biological process of inexorable dysfunctional alterations leading to reduced probability of reproduction and an increased susceptibility to death[2].

Intermingling of aging and senescence as related phenomena stems from aging being an indisputable major risk factor for promotion of senescence. Recent statistics by the United Nations Fund for Population Activities (UNFPA) from 2012 report the number of “old” people has increased to 810 million, is projected to reach 1 billion in less than 10 years, and will reach 2 billion by 2050. Longevity goes hand in hand with prolonged exposure to additional risk factors such as high cholesterol, hypertension, obesity, diabetes and high levels of stress. Collectively, age-related alterations coincident with exposure to risk factors that adversely affect vitality leaves our current population with substantially increasing risk for senescence and progression of degenerative diseases. Furthermore, medical advances have replaced early mortality with increased chronic morbidity. Just to highlight a few out of many such examples, 1) developments in chemotherapeutics have turned selected fetal cancers from postnatal lethality into chronic disease, 2) implementation of PTCA in clinics transformed lethal myocardial infarctions into chronic conditions, 3) management of diabetes has improved, and 4) many veterans survive injuries that would have killed them in the past wars leaving veterans with polytrauma rather than costing their lives[3]. A military journal report estimates that veterans (and other war survivors worldwide) will require decades of health care with risks for a variety of chronic and degenerative diseases[3]. Thus, the perspective on aging and senescence will inevitably shift from a “luxury problem” of a select few lucky individuals to a legitimate global concern affecting both bourgeois and proletariat alike and requiring an effective remedy plan. The fundamental basis for combating senescence originates in comprehensive understanding of underlying mechanisms in order to design interventional strategies to alter mechanisms and complement longevity with improved quality of life.

Cellular senescence refers to a permanent arrest of cell division functionally linked with deterrence of potential maladaptive threats stemming from oncogenic stress or DNA-damage. In other words, stringent cell cycle arrest in senescent cells enforces a safety mechanism to defeat potential development of cancer. Cellular senescence can be induced by genetic damage and/or epigenetic disruption. The major initiator and facilitator of senescence is the DNA-Damage

Response (DDR) that causes permanent cell cycle arrest. Telomere erosion, nontelomeric DNA damage, DNA-double strand breaks, histone deacetylase inhibitors that modify chromatins and strong mitogenic stimuli that cause misfired replication origins initiate persistent DDR that culminates in a senescent phenotype by inducing and maintaining cell cycle arrest. Continual DDR activity in a senescent cell is reflected by distinct nuclear foci containing DDR proteins and phospho-ATM/ATR substrates designated as DNA **s**egments with **c**hromatin **a**lterations **r**einforcing **s**enescence (DNA-SCARS)[4-8].

The major difference between a senescent and a quiescent cell is that, under physiological conditions, quiescent cells can re-enter the cell cycle while senescent cells require rigorous non-physiologic interventions that reinforce cell cycle entry (e.g. inactivation of p53). The majority of senescent cells express markers of cell cycle inhibition such as p16INK4a and p53 and have increased lysosomal content detected by senescence-associated beta-galactosidase activity[9-12]. Unlike quiescent cells, permanent cell cycle arrest increases cell size and metabolic activity by more than twofold compared to healthy cells. Perhaps the most important hallmark of a senescent cell is *inability* to contribute in tissue repair and regeneration and maintenance of normal homeostasis. In addition, senescent cells possess the peculiar *ability* to propagate their senescent phenotype through proactive secretion of proteases, growth factors and cytokines that ultimately lead to the **s**enescence-**a**ssociated **s**ecretory **p**henotype (SASP)[13-19]. Senescent cells are involved in multiple degenerative diseases, and this review is focused predominantly on the process of senescence in the heart, the hegemonic perpetuation of this phenotype throughout the myocardium, and approaches for reversing a senescent phenotype and rejuvenation of the myocardium.

Cardiomyocyte Senescence

Aging and cardiovascular disease are positively correlated with cardiac diseases reaching epidemic proportions in elderly. With progression of aging, the myocardium undergoes degenerative alterations and processes that ultimately lead to myocyte death. Pathologic changes in expression of contractile protein isoforms (versus -MHC) and altered levels of calcium-handling proteins lead to gradual but increasing deterioration of contractile function[20, 21]. Longitudinal echocardiographic assessments reveal prominent age-related hypertrophy and decrease in diastolic function[22, 23]. In a compensatory effort, remaining myocardium undergoes structural and functional alterations culminating in reduced myocardial performance measured by MPI (myocardial performance index)[23]. Age-related cardiac histopathologic alterations present as interstitial and subendocardial fibrosis, hyaline cytoplasmic changes, vacuolization, collapsed sarcomeres, etc[24]. Morphometeric measurements of aging hearts reveal myocyte hypertrophy, increased myocyte apoptosis and increased fibrosis and amyloid deposition. The mechanistic basis for accrual of myocyte age-related changes and senescence involves multiple pathways influenced by multiple levels in the cells[25].

Myocyte Senescence and Mitochondria*:* Cardiac aging and susceptibility to senescence are associated with mitochondrial function. During oxidative phosphorylation, mitochondria generate high levels of reactive oxygen species (ROS) as a byproduct of electron transfer. Cellular nucleic acids and proteins are influenced by ROS since protein oxidation alters signaling. In addition, oxidized proteins accumulate in insoluble protease-resistant aggregates and interfere with cellular

function. Ironically, ROS also attack mitochondrial nucleic acid, lipids, and proteins that culminate in mitochondrial DNA mutations and dysfunction by damaging respiratory enzymes. Defects in respiratory enzymes further the increase in ROS generation, causing decreased mitochondrial function that promotes cell and organ dysfunction. High metabolic demands of the heart and ROS-production as a byproduct of oxidative phosphorylation renders the myocardium prone to oxidative damage and gradual organ malfunction[26, 27]. Initial evidence for the role of mitochondrial ROS in cardiac aging were gathered from overexpression of mitochondria-specific catalase in mice[28]. Unlike overexpression of catalase in the nucleus, mitochondrial catalase increased lifespan and animals were resistant to phenotypic changes indicative of cardiac aging. The rescue of premature aging senescent cardiac phenotype in hearts of mice by mitochondrial-specific overexpression of catalase supports the premise that ROS production, DNA-damage and biogenesis are contributors of a vicious circle of ROS-induced ROS-release leading inevitably to functional deterioration. Damaged mitochondria undoubtedly increase metabolic stress upon cells that, in turn, stimulates mitochondrial biogenesis through PGC-1 mediated signaling. Indeed, PCG-1 knock-out mice exhibit decreased mitochondrial biogenesis and reduced cardiac energy production[29, 30]. Although cells naturally posses the ability to produce additional mitochondria to avoid metabolic stress, the protection of mitochondrial biogenesis can be disrupted by damaging effects of ROS. Mitochondrial biogenesis requires well-orchestrated expression of mitochondrial DNA and transcription factors. ROS-mediated mitochondrial DNA-damage and mutations are thought of as a major cause for reduced mitochondrial biogenesis in senescent hearts. Mutation of mitochondrial polymerase- prompts increased oxidative stress with cardiomyopathic changes in middle-age mice[31]. Aged heart cells have a reduced threshold for ROS-induced ROS-release and thus are more sensitive to mitochondrial permeability transition pore (MPTP) induction. MPTP opening leads to mitochondrial swelling and collapse, leading to ATP-deprivation, oxidative stress and apoptosis.

Further validation of the role played by mitochondria in cardiac aging was derived from a mutant mouse with alteration of p66shc, a mitochondrial redox enzyme localized in the intermembranous space that forms ROS using electrons resulting in H_2O_2 production[32]. Additionally, p66shc accumulates within mitochondria and activates mitochondrial calcium responses to subsequently induce apoptosis. Genetic mutation p66shc increases life span and decrease cardiac functional deterioration through decreased oxidative damage in transgenic mice[32]. Along with the natural process of aging, “authentic” cardiac risk factors such as smoking, diabetes, high levels of homocysteine are additional inducers of oxidative stress and contribute to furthering the vicious cycle of mitochondrial deterioration. In addition, paracrine effectors such as Insulin-like Growth Factor (IGF-1)-1 induce ROS production and modulate cellular signaling via regulation of redox reactions[33].

Collectively, these findings illustrate a prominent role of increased mitochondrial ROS-production and decreased mitochondrial biogenesis upon aging and organ deterioration. As a coincident process developing with advanced age, suboptimal mitochondrial function alone may not necessarily appear problematic. However, imposition of additional risk factors, stress, or pathologic challenges such as myocardial infarction where substrate availability decreases in a highly energy demanding environment, places demands upon mitochondrial energy production

and biogenesis that ultimately fails leading to senescence and severe functional deterioration upon time.

Myocyte Senescence and Adrenergic signaling: Chronic activation of □-adrenergic signaling is deleterious for cardiac function[34]. Increases of heart rate, contractility, peripheral vasoconstriction, and wall stress are tied to heightened catecholamine-driven increases cardiac metabolic demand when oxygen availability is limited in conditions such as coronary atherosclerosis. Stimulation of □-adrenergic receptor decreases anti-apoptotic and anti-oxidative stress signaling, contributing to senescence and functional deterioration in a cascade starting with adenylate cyclase 5 activation with downstream induction of cyclic-AMP and PKA that act as inhibitors of the cardioprotective Raf/MEK/Erk pathway[35]. Genetic deletion of adenylate cyclase 5 increases lifespan through upregulation of Raf/Erk-pathway and increased anti-apoptotic signaling and stress resilience in knockout mice[36, 37]

Myocyte Senescence and Renin-Angiotensin-Aldosteron System (RAAS): RAAS is another critical player for regulation of cardiac function regulated through Angiotensin II (Ang II). RAAS normally acts as a protective mechanism to increase blood pressure following disturbance of systemic pressure sensed by renal artery flow. Ang II is a vasoconstrictor and plays a crucial role in fluid balance. Persistent RAAS activation with chronic Ang II exposure of myocytes promotes development of cardiac failure[38]. More so than the systemic production of Ang II, local Ang II increases particularly detrimental for cardiomyocytes[39] prompted worldwide usage of Ang II blockers for prevention and treatment of cardiac remodeling. Detrimental effects of Ang II on cardiac myocytes have been studied in transgenic mice that exhibit cardiac-specific elevation of Ang II production. Mice with cardiac-overexpression of Ang II reveal a dilated cardiomyopathic and aged phenotype in addition to increased mortality. The major cause for dilated cardiomyopathy occurred due to induced down-regulation of the sarcoplasmic reticulum pump (SERCA2) and diminution of Ca2+ transients, indicative of disruption on calcium homeostasis[40]. Along with effects on vasoconstriction and fluid balance, Ang II plays an important role in cardiac contractility by increasing cardiac pump function independent of systemic blood pressure that with persistent stimulation will exacerbate cardiac dysfunction. Cardiac tissue Ang II is significantly elevated in aged rodents, presumably in conjunction with elevation of Angiotensin Converting Enzyme. Although more studies are needed to fully decipher the mechanisms involved in elevation of cardiac Ang II, cumulative evidence clearly shows Ang receptor inhibition and disruption decreases age-related cardiac pathology and prolongs lifespan in rodents[41, 42].

Myocyte Senescence and Mammalian Target of Rapamycin Complex1(mTORC1): The serine/threonine protein kinase mTORC 1 acts as the cellular sensor for nutrients, stress, cellular growth and metabolism and is a major player in cellular aging, metabolic disorders and cancer. The relationship of mTORC1 to longevity is established in a variety or organisms[43]. Pharmacologic inhibition or genetic inactivation of mTORC1 increase longevity of rodents[44]. Additionally, suppression of mTOR 1 through restriction of caloric intake antagonizes aging in monkeys, suggestive of a potential role for mTOR1 in human longevity. Cardiac aging correlates with maladaption to stress, hypertrophy and cardiac failure, so a causal link with mTORC1 is plausible. Cardiac pathologies involving mTORC1 link synergistic increases of stress-induced protein synthesis with decreased protein degradation due to impaired autophagy. Overstimulation of mTORC1 by excessive growth hormone (Ang II, IGF-1, or catecholamines) promotes cardiac

senescence, with IGF-1 hyperactivity in particular leading to insulin resistance through phosphorylation of Insulin receptor-1 (IRS-1)[45] that furthers myocardial risk to pathologic changes. Consolidation of all mTORC 1 activities in cardiac aging has provided insight and understanding in a new cluster of potential targets for cardiac rejuvenation.

Senescence of Cardiac Stem cells: Stem cells contribute in tissue repair and regeneration following damage. As primitive and naïve cells, stem cells are highly susceptible to signals and stimuli from their habitat and systemic environment. Thus, stem cells from an unexploited niche are presumably exposed to a "younger" and healthier environment than stem cells from an overly actively proliferating niche. Essentially, the natural process of aging is detrimental for cardiac stem cell quality. Age-related changes such as DNA-damage, impaired catabolism, altered epigenetics and exposure to environmental stress factors contribute to deterioration of stem cell function and negative signaling from the niche towards stem cells that culminates in a vicious cycle of unfavorable effects on tissue repair and regeneration. Unlike myocytes, stem cells posses a relatively high capacity for proliferation, leaving stem cells with increased susceptibility to single and double DNA-breaks, chromosomal translocation, telomere erosion and additional types of mutations that ultimately lead to replicative senescence. In addition, cardiac stem cells in a pathologic heart are exposed to chronic elevated levels of Ang II and sympathetic hyperactivity that contribute to initiation and maintenance of senescence. Cardiac stem cells and particularly c-kit+ human cardiac stem cells (hCSCs) undergo senescence in aged and diseased hearts as measured by p16 expression. Stem cells derived from myocardial tissue of chronic heart failure patients show increased numbers of dual positive c-kit+/p16+ hCSCs with concurrent evidence of telomeric shortening[46, 47]. Further elucidation of this mechanism in hCSCs revealed that hCSCs derived from pathologically injured aged hearts have reduced telomerase activity, increased dysfunction foci, and elevated expression of p16 and p21[48]. The molecular pathways studied in cardiac stem cell senescence appear to those previously identified described in cardiomyocytes. hCSCs senescence is influenced by the IGF-2/IGF2R, HGF/c-Met and RAAS-activated Angiotensin II pathways. Protective IGF-1 increases telomerase activity, maintains telomere length, blocks occurrence of replicative senescence and preserves a functional population of cardiac stem cells capable of tissue repair and regeneration, whereas IGF-2 appears to promote adverse effects. HGF-production decreases upon age, inhibiting migratory capability of CSCs in response to tissue damage and external stimuli[49]. Addition of HGF to rescue migratory potential of CPCs indeed increases activity, tissue repair, and regeneration[48]. Additionally, hCSCs are also sensitive to local Ang II and increasingly express AT1R with age. Ang II induces oxidative stress through increased ROS generation. The lifespan of aged hCSCs was improved through use of ACE-inhibitors, demonstrating the detrimental effect of Ang II exposure for stem cell functional capacity[50].

Myocyte & CSC-senescence synergy; the role of SASP

The environment within tissue is characterized by the collective phenotypes of constituent cells in the contiguous area and their chemical properties as revealed by chemokines, cytokines and growth factors. In addition, cells communicate and interact with each other in a dynamic network that forms and alters their microenvironment. Simultaneously, extracellular signals impinging upon cell behavior promote additional phenotypic changes such that the collective population of

cells as a whole are intimately related and exhibit solidarity in maintaining tissue homeostasis. The presumptive role of senescent cells to function primarily in suppression of tumor formation through irreversible cell cycle arrest renders them as guardians that notify their local environment when potential replicative errors appear within the population. Indeed, senescent cells profoundly alter their transcriptome to provide protection from uncontrolled cell growth through propagation of permanent cell cycle arrest throughout the environment in a hegemonic fashion by development of SASP. Existence of a senescent secretome was initially demonstrated in fibroblasts undergoing replicative senescence as revealed by array analysis of the classic wound healing response in surrounding senescent fibroblasts[51, 52] SASP factors can be generally subdivided in three major categories: 1) soluble signaling factors, 2) secreted proteases, and 3) insoluble proteins/extracellular matrix component. IL-6, IL-1, chemokines (e.g. IL-8 and GRO□□, as well as IGF or colony stimulating factors are examples of soluble SASP factors that affect neighboring cells through cell-surface receptors. Additionally, select extracellular proteases are known SASP agents including matrix metalloproteinases and urokinases upregulate in multiple cell types upon progression to senescence[19]. Although a wide range of SASP factors are upregulated during senescence, the severity of phenotype and mechanistic basis of senescence appear to be influential in determination of patterns and levels of secreted factors. Replicative senescence, telomere erosion, DNA-damage and chromatin disruption display a closely related pattern of SASP factors. However, p16-mediated senescent cells do not exhibit any potential for secretion of SASP factors[53]. Another attribute of the SASP is a temporal development of intensity, with progression and severity of SASP driving higher levels of SASP factor production.

Although SASP displays a markedly beneficial role by empowering one particular cell to prevent progression of tumor growth in oncogenesis, the dark side of SASP becomes apparent when provoked in the myocardium where exposure of myocytes to risk factors and/or pathologic challenge ultimately culminates in myocyte senescence. In a proliferative organ that exhibits a high regenerative potential, restricted induction of senescence in troublesome cells and their neighboring brethren may not necessarily lead to dramatic consequences. However, the limited regenerative potential of the heart means that myocyte losses from death or the twilight functional consequences of senescence cannot be adequately compensated by generation of new cardiomyocytes. Additionally to a limited baseline regenerative capacity within the myocytes themselves, the cohabitating hCSC are similarly susceptible to risk factors and pathologic injury leading to conversion into senescence over time. Notably, as post-mitotic myocytes undergo senescence as a result of combined effects of metabolic disruptions, RAAS, catecholamines and DNA-damage, bystander hCPCs must cope not only with their chronological aging and premature senescence, but also undergo *replicative* senescence. Considering the prominent role of SASP in maintaining tissue similitude, SASP factors secreted by cardiomyocytes influence other cardiac cell types including hCSC. Thus, already impaired hCSC are further vanquished by SASP factors from myocytes that accelerate deterioration of cardiac regenerative and reparative potential. As the dominos fall, the hCSCs in turn are stimulated to secrete stem-cell specific SASP factors and influence other cell types in the heart, propagating a vicious circle culminating in the spread of senescence throughout the entire myocardium. Thus, cardiac senescence is not solely a “one cell type concern”. Once the process of senescence is initiated, it develops into hegemony of cellular deterioration that ultimately dominates the cardiac function and leaves the heart impaired. Considering the multilayer extent of senescence in the heart, efforts are underway to approaches

to intervene in this senescence-driven downward spiral and inhibit progression of global cardiac deterioration.

Senescence Reversal

If spreading senescence is ultimately detrimental to myocardial performance, then reasonable molecular interventional strategies could be valuable in slowing or reversing the acquisition of a senescent phenotype. Two areas that have been explored in cardiomyocytes include mTORC1 or telomerase manipulation[44, 54-56]. Correlations that link metabolic activity to aging and senescence have prompted approaches to restrict caloric intake to 30% of typical daily intake levels to study effects on the cardiovascular system in rodents and humans. Caloric restriction findings reveal a very powerful ability to prevent structural detrimental changes in the cardiovascular system, partially through reduction of ROS. Inhibition of mTORC1 as a target for longevity and reversal of senescence has also been studied using Rapamycin, resulting in reduced cardiac inflammation, increased cardiac ejection fraction, decreased ANP levels and overall reduction of hallmarks associated with myocardial hypertrophy[54, 55]. Alternatively exploring the influence of telomere length on senescence, hTERT expression in human somatic cells maintains telomere length and antagonizes reaching their replicative limit. In addition to elongation of telomeres, these cells displayed a more “youthful” phenotype defined by their gene expression pattern. Although telomerase expression does not possess the ability to create new myocytes through initiation of myocyte proliferation from a quiescent state, evidence from mouse studies supports a role for telomerase overexpression as an inducer of hypertrophy and protector from cardiac failure through inhibition of apoptosis. Telomerase expression and activity in the heart is an intriguing field to explore, but it is important to be mindful that telomerase can perpetuate cell immortalization *in vitro* with evidence of associated karyotypic abnormalities. Cautious and rigorous studies are required to asses safety and further application of telomerase manipulation in the heart in a feasible and safe fashion.

In the context of stem cells, molecular pathways associated with senescence include IGF-2 signalling, mTOR/autophagy, NO/telomerase/Sirtuins and p38MAPK[50]. Despite this roster of potential signaling players, interference with pathways promoting senescence in hCPCs remains essentially unexplored. Recently, our group implemented a novel strategy for rejuvenation of hCPCs by overexpression of the proto-oncogene Pim-1. Pim-1 is a serine/threonine kinase that autophosphorylates and plays an important anti-apoptotic and pro-proliferative role in hCPCs. Working with hCPCs isolated from terminal heart failure patients undergoing left ventricular assist device (LVAD) placement implantation, adoptive transfer studies proved hCPCs engineered for Pim-1 overexpression displayed a remarkable increase in cell survival, engraftment, as well as contributing to durable improvement of cardiac function up to 6 months[57]. As might be expected, hCPCs derived from failing hearts are exposed to an environmental milieu that would place the heart under severe stress and likely accelerate development of senescence characteristics. Indeed, subsequent phenotypic characterization of hCPCs isolated from LVAD patients revealed a senescent phenotype characterized by shortened telomeres, high levels of p16 and p53, and limited proliferation capacity. Importantly, modification of hCPCs using Pim-1 ameliorated senescent characteristics with restoration of youthful telomeric length, enhanced replicative capacity, and decreased levels of p16 and p53[58]. To date, Pim-1 engineering of hCPCs has been performed using a lentiviral delivery approach and further safety testing is warranted. However, f

note in the recent study is the fact that even Pim-1 engineered hCPCs underwent replicative senescence *in vitro* at comparable cell passaging to youthful hCPC controls isolated from fetal hearts[58]. The efficacy for enhancing myocardial regeneration together with molecular antagonism of senescence makes Pim-1 a promising candidate for future interventional approaches to combat loss of functional and reparative potential in the failing heart.

Conclusions

Human beings have the unusual characteristic of remembering the past and worrying about the future, which sets us apart from other organisms, Thus, knowing statistics from the past and predictions about the future; aging has become a contemporary earnest discussion in today's scientific community. It is worth emphasizing that chronologic aging does not necessarily lead to cellular senescence under normal healthy conditions. However, older age does provide a protracted platform for exposure to harmful factors that lead to older-age-mediated pathologic alterations and occurrence of senescence. As a mechanistic basis for development of degenerative diseases, the cardiac field has been fascinated by senescence and cardiac age-related pathologies for decades now. Molecular studies on multiple levels of cellular biology on myocyte aging ascertain the complexity of senescence in the context of the myocardium. In addition to intracellular biology, the paracrine aspect of senescent cells in a multi-cellular organ with limited regenerative potential contributes to the complexity of senescence mediated cardiac degeneration. Although other fields are more skilled and experienced in interpretation of senescence and primitive cells, the concept of stem cell mediated repair and regeneration of the heart is, relatively, juvenile. On one hand, the recognition of the concept of myocardial stem cell mediated regeneration has gained momentum, on the other hand, understanding of myocardial senescence had gained an additional layer of complexity by situating a new cell type to the equation of age-mediated functional deterioration. In particular, stem cell mediated SASP, perhaps, carries the potential of inducing permanent cell cycle arrest in remaining myocardial stem cell population leaving the heart with a further diminished regenerative potential. Not only is this phenomenon concerning in the context of endogenous myocardial repair and regeneration but it also reveals the reality that isolated CSCs from a senescent heart for future adoptive transfer may not represent a "primitive" and highly proliferative cell type that is capable of combating a senescent environment in addition to regenerating new healthy myocytes. Collectively, in the treatment of heart failure requires a stringent remedy plan against myocardial senescence. Future therapeutic interventions not only require rejuvenation of myocytes and the myocardium but also rejuvenation of isolated CSCs with interventional strategies currently under investigation, such as Pim-1. The cardiac field has a tremendous basis of expertise in protective signaling cascades. Perhaps, it is time to utilize the built up knowledge and proactively encounter senescence using gene therapy, modified-cell therapy and other timely approach.

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Chapter 4
Myocardial Induction of Nucleostemin in Response to Postnatal growth and Pathologic Challenge

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Abstract

Stem cell–specific proteins and regulatory pathways that determine self-renewal and differentiation have become of fundamental importance in understanding regenerative and reparative processes in the myocardium. One such regulatory protein, named nucleostemin, has been studied in the context of stem cells and several cancer cell lines, where expression is associated with proliferation and maintenance of a primitive cellular phenotype. We find nucleostemin is present in young myocardium and is also induced following cardiomyopathic injury. Nucleostemin expression in cardiomyocytes is induced by fibroblast growth factor-2 and accumulates in response to Pim-1 kinase activity. Cardiac stem cells also express nucleostemin that is diminished in response to commitment to a differentiated phenotype. Overexpression of nucleostemin in cultured cardiac stem cells increases proliferation while preserving telomere length, providing a mechanistic basis for potential actions of nucleostemin in promotion of cell survival and proliferation as seen in other cell types.

Introduction

Cellular-based myocardial regeneration depends on tightly regulated signaling cascades that control survival and proliferation. In the case of stem cell populations, these signaling pathways have been predominantly defined by decades of study in hematopoietic[1–4] and developmental contexts.[5–7] The relatively recent advent of myocardial adult stem cells and their distinctive characteristics has prompted reexamination of the operational definition of "stem cells" and "stemness."[8,9] The traditional view of stem cell behavior as derived from classic lineage studies may not appropriately reflect the biology of stem cells in tissues characterized by slow cellular turnover such as the myocardium. For example, activation of signaling typically associated with regulation of proliferation and survival in stem cells is also observed in combination with partial or fully committed cellular pheno- types following tissue injury.[10–12] These revelations have prompted dissolution of long-standing assertions related to "stem cell–associated" signaling, now viewed as regulation of tissue repair and regeneration or, in some, cases oncogenic transformation.[13–16]. Nucleostemin is found at high levels in various stem cells and human cancers,[17] where it has been associated with maintenance of proliferation.[17–20] Expression of nucleostemin drops precipitously durin differentiation[21,22] and genetic deletion of nucleostemin results in embryonic lethality at approximately day 4 postcoitum with blastocysts comprised of cells that fail to enter S phase.[23]

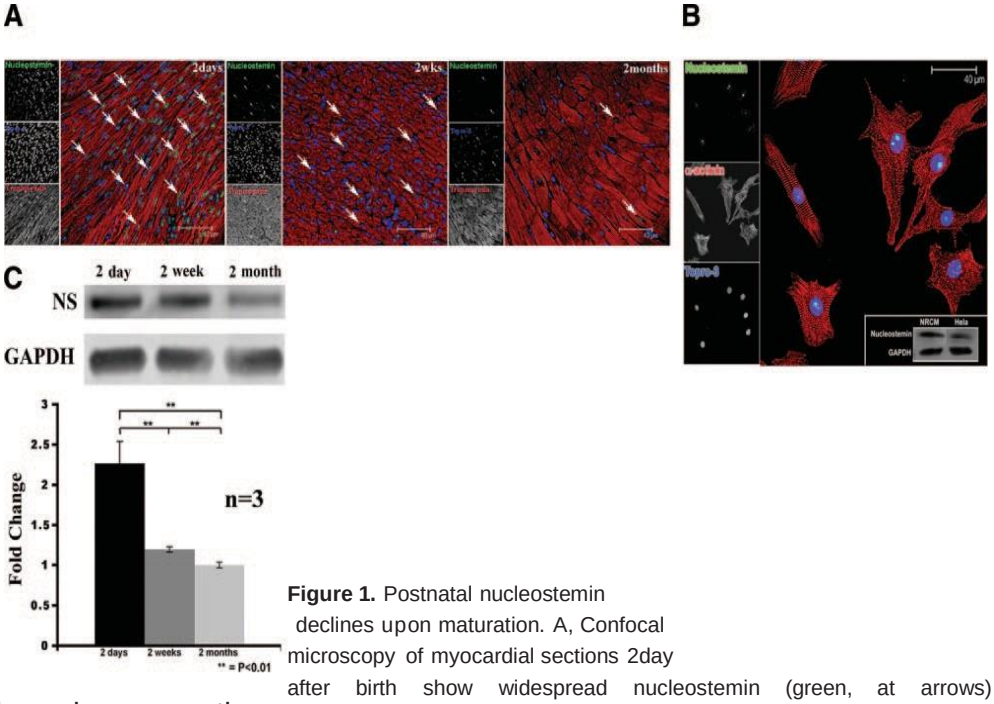

Figure 1. Postnatal nucleostemin declines upon maturation. A, Confocal microscopy of myocardial sections 2day after birth show widespread nucleostemin (green, at arrows) immunoreactivity relative to sections from hearts at 2 weeks or 2 months after birth. Tropomyosin (red) labels sar- comeric structure and nuclei are labeled with Topro-3 stain (blue). B, Confocal microscopy and immunoblot (inset, lower right) of nucleostemin expression in cultured neonatal rat cardiomyocytes. Nucleostemin is predominately nucleolar. Immunoblot shows nucleostemin expression relative to Hela cell lysate–positive control. C, Decline in nucleostemin expression after birth assessed by quantitative immunoblot analyses. A significant ($P<0.01$) decrease in nucleostemin occurs between 2 days and 2 weeks until 2 months after birth.

Similar arrest in G_0/G_1 phase of cell cycle was observed in HeLa cells if nucleoste- min was eliminated by RNA interference.[20] Nucleostemin has been purported to mediate cellular dedifferentiation and regenerative processes in newts.[24] Although the molecular basis of nucleostemin-mediated actions remains controver- sial, evidence supports mechanisms related to inhibition of p53[17] or telomeric repeat-binding factor 1 (TRF1) that nega- tively regulates telomere length.[25] Collectively, these charac- teristics point to a pivotal role for nucleostemin in maintenance of cell survival, antagonizing senescence, and promotion of regenerative potential.

Participation of nucleostemin in myocardial repair and regeneration has no precedent in the literature. Our findings establish a role for nucleostemin in response to pathological injury and demonstrate biological properties of nucleostemin expression in cardiac stem cells (CSCs), postnatal development, and response to paracrine fibroblast growth factor (FGF) treatment, as well as induction by Pim-1 kinase activity. Beneficial action depends on enhanced cell proliferation coupled with maintenance of telomeric length, which is preserved in c-kit$^+$ CSCs by nucleostemin overexpression. Therefore, nucleostemin is a novel marker of protective signaling in the myocardium that, together with established links to stem cells, point to a role in myocardial repair and regeneration.

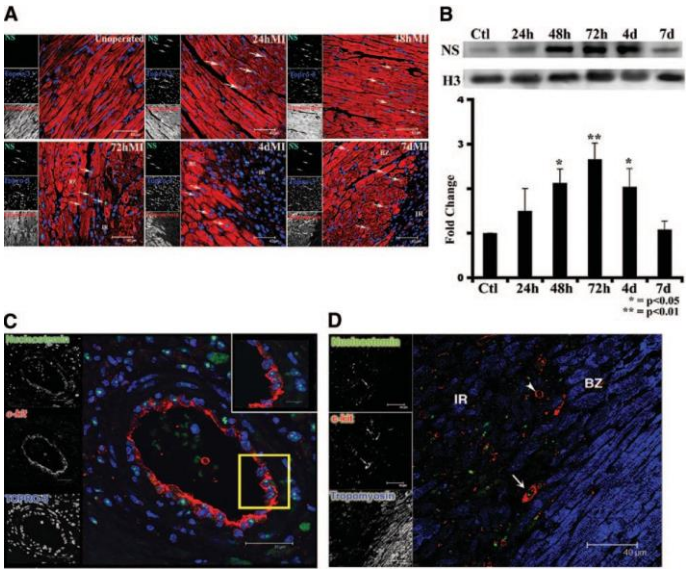

Figure 2. Nucleostemin expression is induced by myocardial infarction. A, Confocal microscopy of myocardial sections at various time points following myocardial infarction. Nucleostemin expression (green, at arrows) is observed in surviving cardiomyocytes within the border zone surrounding the infarct. Tropomyosin (red) labels sarcomeric structure and nuclei are labeled with Topro-3 stain (blue). B, Immunoblot shows time course of nucleostemin expression after myocardial infarction peaking at 72 hours postinduction. C, Confocal microscopy show- ing coincidence of nucleostemin (green) and c-kit (red) expression in cells lining a vessel proximal to the region of injury at 4 days postinfarc- tion. The inset at the upper right shows the boxed region (yellow) at higher magnification. Nuclei are labeled with Topro-3 stain (blue). D, Confocal microscopy showing nucleostemin (green) and c-kit (red) expression coincident in a small cell (arrow) at the interface between the border zone (BZ) and infarct region (IR). A cell expressing c-kit but lacking nucleostemin is also shown (arrowhead). Single-channel scans that were used for creation of the color overlays are shown to the left of each image.

Nucleostemin Expression Declines Rapidly After Birth

Nucleostemin is detectable within nuclei of cardiomyocytes in sections of neonatal mouse myocardium, as well as cultured neonatal rat cells (Figure 1). Nucleostemin expression diminishes rapidly within weeks after birth evidenced by fewer positive nuclei with lower intensity immunofluores- cence in sections of older hearts relative to postnatal sections (Figure 1A). Nucleoli of cultured cardiomyocytes are labeled consistent with nucleostemin localization (Figure 1B).[32] Progressive loss of nucleostemin correlated with increased age in myocardial sections (Figure 1A) and lysates showing significant ($P<0.01$) decreases in nucleostemin protein (Figure1C). These results indicate nucleostemin association with young myocytes possessing proliferative potential during early postnatal growth.[33–36] Exposure of neonatal rat cardio- myocytes to doxorubicin significantly decreases nucleoste- min protein levels (Figure IA in the online data supplement), indicating cardiotoxic effects of doxorubicin may impair proliferation of young myocytes through antagonizing nucleostemin. However, nucleostemin overexpression is ineffective at

antagonizing p53 protein in this system (supple- mental Figure IB).

Nucleostemin Is Induced Following Pathological Challenge

Relatively low-level nucleostemin expression in adult myocardium is markedly increased by acute pathological chal- lenge or chronic heart failure (Figure 2). Myocardial infarc- tion prompts nucleostemin expression in nuclei of cardiomyocytes primarily localized to the border zone adja- cent to the ischemic region (Figure 2A). Immunoblot analyses of excised border zone/infarct regions reveal nucleostemin is increased at 24 hours after induction of myocardial infarction, with significant elevation of protein level within 48 hours that peaks at 72 hours. After 96 hours, expression of nucleostemin decreases from peak levels and returns to basal levels within 1 week (Figure 2B). In addition to cardiomyocyte expression, nucleostemin is also expressed in c-kit$^+$ cells observed 4 days postinfarction. Two areas of enrichment for these c-kit$^+$/ nucleostemin$^+$ cells were the endothelial layer of healthy vessels in proximity to the infarct (Figure 2C) and individual cells in proximity to the border zone of damaged tissue (Figure 2D, at arrow). Observations of nucleostemin expression in pathologically challenged myocardium were extended to include additional models of cardiac stress characterized by heart failure or pressure overload hypertrophy. The tropomodulin-overexpressing transgenic (TOT) mouse model is a well- characterized model of chronic dilated cardiomyopathy de- veloped by our group.[37–39] TOTs show nucleostemin expression throughout the myocardium by confocal micros- copy (Figure 3A) and elevated protein level by immunoblot (Figure 3B). In comparison, pressure overload–induced hy- pertrophy also induced increased nucleostemin immunoreactivity in sections prepared from mice subjected to transaortic constriction. Areas of nucleostemin reactivity are restricted to cells neighboring and comprising large vessels such as endothelium lining the interior as well as cardiomyocytes surrounding vessels (Figure 3C). Quantitative immunoblot analysis of TAC-induced nucleostemin expression in the vasculature is not practical because of comparatively restricted regionalization of protein expression around large vessels relative to the whole heart.

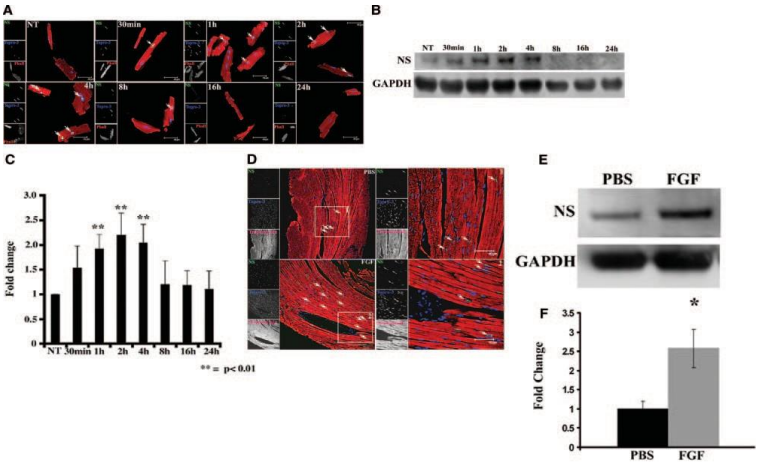

Figure 4. Nucleostemin expression is upregulated by FGF in vitro and in vivo. A, Adult car- diomyocytes at various time points following treatment with FGF-2. Nucleostemin expression (green, at arrows) in treated cardiomyocytes within 30 minutes after exposure. Phalloidin (red) labels sarcomeric structure and nuclei are labeled with Topro-3 (blue). Single-channel scans used for creation of the color overlays are shown to the left of each image. B, Immunoblot demonstrates a 2- to 4-hour peak in nucleostemin expression following FGF treatment.
C. Quantitation from adult cardiomyocyte cultures shows a significant increase in nucleostemin expression between 2 to 4 hours after FGF treatment. D, Myocardial sections from mice implanted with osmotic pumps filled with vehicle (PBS) or FGF-2 (FGF) shows immunoreactivity for nucleostemin (green, at arrows) intensified by FGF-2 exposure. E, Immunoblot quantitation shows significant increase in myocardial nucleostemin protein level accompanies after 3 days of FGF-2 exposure.

Nucleostemin Expression Is Induced by FGF

At present, relatively little is known about inductive signals that mediate nucleostemin expression, but FGF-2 increases nucleostemin in adult bone marrow stem cells.[21] Similarly, treatment of cultured adult mouse cardiomyocytes with FGF-2 prompts induction of nucleostemin immunoreactivity (Figure 4A). Immunofluorescence microscopy of FGF-2–treated cells show relatively preserved rod-shaped morphology of the FGF-treated cultures compared to vehicle-treated cells (Figure 4A). Immunoblot analyses demonstrate signifi- cant elevation of nucleostemin protein expression that peaks within 2 hours posttreatment but returns to basal levels after 8 hours (Figure 4B and 4C). In vitro findings were validated in vivo using systemic FGF-2 delivery by osmotic pump. Myocardial sections show increased nucleostemin immuno- reactivity in cardiomyocytes of mice receiving osmotic pumps with FGF-2 compared to control samples (Figure 4A). This increase in myocardial nucleostemin is significant as assessed by quantitative immunoblots (Figure 4E and 4F).

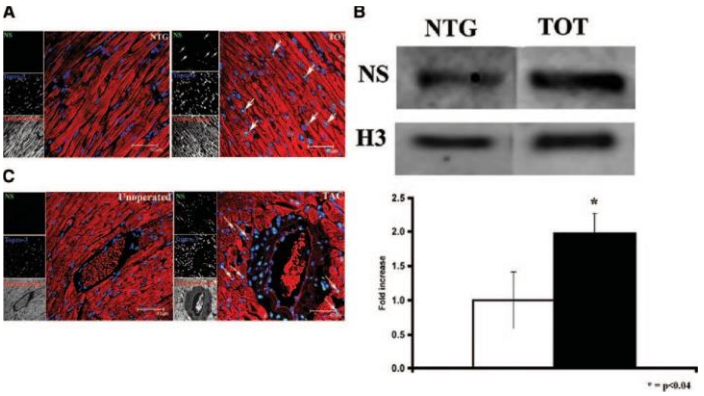

Figure 3. Nucleostemin expression is increased by pathlogical stress. A. Confocal microscopy shwing increased nucleostemin expression (green at arrow) in myocardial sections from tropomodulin overexpressing transgenic (TOT) mice experiencing chronic dilated cardiomyopathy. Tropomyosin (red) labels sarcomeric structure and nuclei are labeled with Topro-3 stain (blue). Single-channel scans that were used for creation of the color overlays are shown to the left of each image. B, Immunoblot and quantitation of nucleostemin protein expression in lysates prepared from nontransgenic (NTG) or TOT hearts show a significant increase in protein associated with the heart failure phenotype. C, Confocal microscopy showing increased nucleostemin immunoreactivity (green, at arrows) proximal to a large vessel in myocardial sections from mice subjected to pressure overload hypertrophy by transaortic constriction at 4 days after banding.

Nucleostemin Is Expressed in Cardiac Stem Cells and Declines on Differentiation

Established association of nucleostemin with stem cells[17,21] and c-kit$^+$ cells in the myocardium (Figure 2C and 2D) prompted further assessment of nucleostemin expression in CSCs. Neonatal mouse myocardium, which is enriched for c-kit$^+$ cells,[26] shows colocalization between c-kit and nu- cleostemin immunoreactivity (Figure 5A). Furthermore, cultured CSCs express high levels of nucleostemin as observed by immunohistochemistry (Figure 5B), as well as immunoblot (Figure 5C). Expression of nucleostemin in CSCs is associated with maintenance of an undifferentiated pheno- type. When induced to lineage commitment by exposure to dexamethasone,[40] CSCs show a precipitous decline in nucleostemin expression that is statistically significant (Figure5D), along with increased labeling for GATA-4 (Figure 5B) and loss of c-kit expression (data not shown).

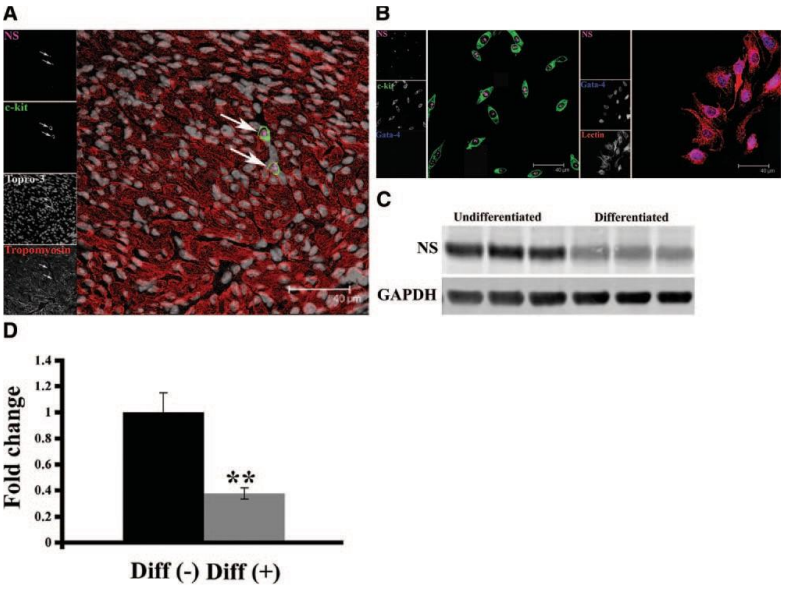

Figure 5. Nucleostemin expression in CSCs declines on differentiation. A, Myocardial section from a mouse at 2 days after birth shows c-kit$^+$ cells (green) with coincident expression of nucleostemin (magenta) at arrows. Sarcomeres are labeled with tropomyosin (red), and nuclei were detected with Topro-3 (white). Single-channel scans used for cre- ation of overlays are shown on the left of each panel. B, Cultured car- diac c-kit$^+$ cells (green) express high levels of nucleostemin (magenta). Cardiac stem cell cultures induced to differentiate by dexamethasone treatment show decreased nucleostemin expression and increased labeling for GATA-4 (blue). Lectin (right) is used as a cytoplasmic marker because of loss of c-kit expression. C, Decreased nucleoste- min expression in CSC culture following dexamethasone treatment. D, Quantitation demonstrates a significant decrease in expression of nucleostemin in CSC cultures following dexamethasone treatment. Whole cell lysates are normalized to GAPDH to correct for minor variation in protein

Nucleostemin Expression Is Associated With Pim-1 Kinase Activity

Recent studies from our group have identified Pim-1 kinase as an essential regulator of cell survival downstream of Akt.[31] Pim-1 is associated with cell proliferation and survival in the hematopoietic system[41]; therefore, experiments were per- formed to assess the relationship between Pim-1 activity and nucleostemin expression in

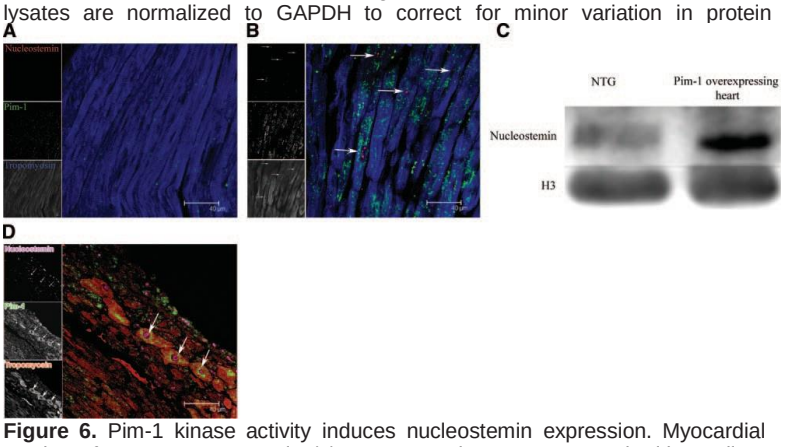

Figure 6. Pim-1 kinase activity induces nucleostemin expression. Myocardial sections from a nontransgenic (A) or transgenic mouse created with cardiac-specific expres- sion of Pim-1 kinase (B). Single-channel scans shown to the left of each micrograph correspond to color overlays representing merged images of nucleostemin (red), Pim-1 kinase (green), or tropomyosin (blue) scans. Nucleostemin is evident in nuclei of cardiomyocytes as indicated (arrows in B). C, Immunoblot of lysates created from a nontransgenic or transgenic mouse created with cardiac-specific expression of Pim-1 kinase shows increased nucleostemin with histone bands shown to demon- strate comparable loading of protein samples. D, Myocardial section from infarcted mouse heart showing colocalization of Pim-1 and nucleostemin (at arrows) in surviv- ing myocytes. Single-channel scans shown along left of micrograph corresponding to color overlays representing merged images of nucleostemin (magenta), Pim-1 kinase (green), or tropomyosin (red) scans.

myocardium. Normal mice show minimal levels of Pim-1 or nucleostemin expression (Figure6A), whereas sections from transgenic mice created to over- express Pim-1 kinase show accumulation of nucleostemin in cardiomyocyte nuclei (Figure 6B, at arrows). Induction of nucleostemin expression is also demonstrable by immunoblot analyses of lysates created from Pim-1– overexpressing trans- genics relative to nontransgenic controls (Figure 6C). Fur- thermore, colocalization is observable in myocardial sections from mice at 4 days after infarction challenge, where surviv- ing myocytes in the border zone coexpress both Pim-1 and nucleostemin (Figure 6D, at arrows).

Nucleostemin Increases TERT and Telomere Regulatory Protein Expression

The molecular basis for nucleostemin effects on telomere regulatory components was assessed by immunoblot analyses of CSC culture lysates. Nucleostemin overexpression prompted concomitant increases in levels of telomere- associated regulatory proteins TERT, TRF1, and TRF2 (supplemental Figure II). These results are consistent with preservation of telomeric length mediated by nucleostemin overexpression in cultured CSCs.

Nucleostemin Increases Cardiac Stem Cell Proliferation While Preserving Telomere Length

Effects of nucleostemin overexpression on c-kit$^+$ cultured CSCs were studied to assess consequences for cell prolifer- ation and preservation of telomeric length. Increased nucleostemin expression was readily detected in the CSC cultures following infection with the adenoviral vector by immunoblot (supplemental Figure III). Nucleostemin overex- pression in CSC promotes increased 5-bromodeoxyuridine labeling of nuclei indicative of DNA synthesis, as well as increased cell cycle progression, as demonstrated by a greater percentage of cells labeled by Ki67 (Figure 7). The number of CSCs with telomerase detectable by immunolocalization was significantly increased following nucleostemin overexpres- sion, corresponding with a higher percentage of proliferative cells within the telomerase positive CSC population when nucleostemin is overexpressed. Despite enhanced CSC pro- liferation resulting from nucleostemin overexpression, aver- age telomeric length in the CSC population was preserved and remained unchanged relative to normal control CSCs that undergo proliferative growth at a lower rate (supplemental Figure III).

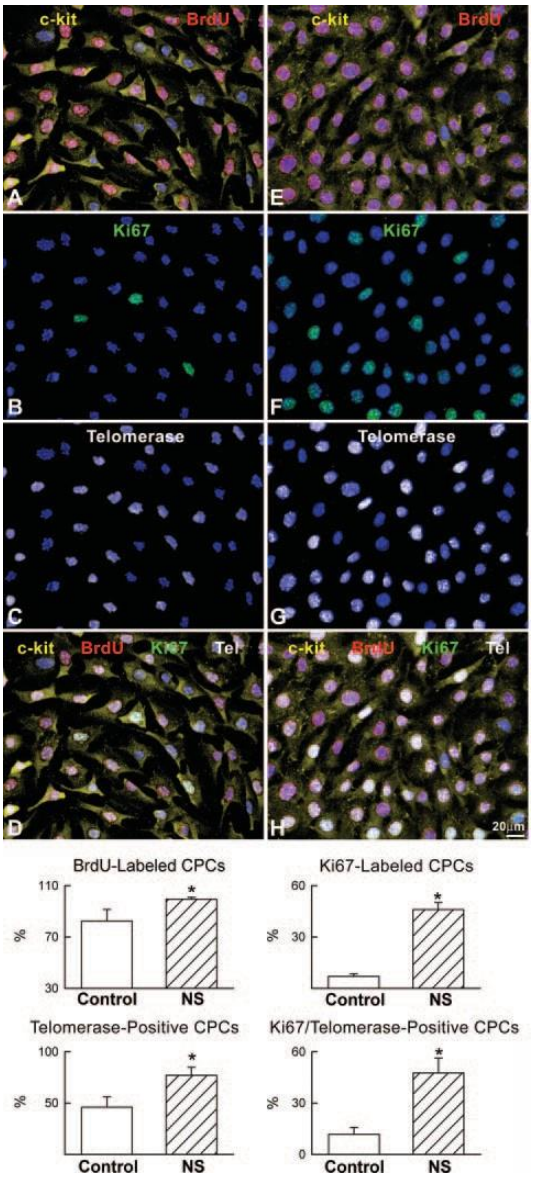

Figure 7. Cardiac progenitor cell proliferation is increased by nucleostemin. A through H, Left and right images correspondto control and nucleostemin-overexpressing CPCs, respectively. CPCs (c-kit, yellow) (A and E) incorporate 5-bromodeoxyuridine (red) (A and E) and express the cell cycle protein Ki67 (green) (B and F) and the catalytic subunit of telomerase (white) (C and G). D and H, Merge of stainings. I, Results are mean±SD.*P<0.05 vs control.

Methods

Details regarding nucleostemin cDNA, adenovirus, FGF treatments, antibodies, and immunoblotting are provided in the expanded Materials and Methods section in the online data supplement, available at http://circres.ahajournals.org. Immunohistochemistry and confocal microscopy, including cell proliferation and telomere length measurements, were performed as previously described,[26,27] with details in the online data supplement. Stem cell and adult cardiomyocyte cultures were performed as described previously,[28,29] with details in the online data supplement. Murine surgical procedures were performed as previously described,[30] with details provided in the online data supplement. Pim-1– overexpressing transgenic mice were created as previously described.[31] All data are expressed as means±SEM. Differences in variables examined by Student *t* test. $P<0.05$ was considered significant. Statistical data analyzed using Microsoft Excel software.

Discussion

Traditional categorizations of stem cell associated molecules, such as nucleostemin, are being redefined as thesesignaling cascades are discovered in partially committed or fully differentiated cells and tissues.[30] Because nucleostemin is associated with cellular proliferation, it is not surprising that this pathway is activated in response to postnatal growth or pathological injury. Initially, our intent was to demonstrate expression of nucleostemin in regenerative processes associ- ated with cardiac stem and progenitor cell populations. However, in addition to observing associations between nucleostemin and c-kit$^+$ cells, we noted profound increases in nucleostemin activation in neonatal and pathologically chal- lenged myocardium within cardiomyocytes, prompting addi- tional studies to understand the role of nucleostemin signaling in response to myocardial injury and survival signaling. Since the discovery of nucleostemin 5 years ago[17] subse- quent literature has focused predominantly on aspects of cancer,[18–20,42,43] stem cells,[21,22,44,45] and developmental biology.[23] Previous studies of nucleostemin establish the connection between nucleostemin and proliferative populations, either in the form of stem cells[21,46] or cancer.[20] In this context, nucleostemin appears to be a consistent marker for mainte- nance of a proliferative state, because expression is rapidly lost on commitment to a differentiated postmitotic pheno- type[22] and depletion of nucleostemin leads to cell cycle arrest.[47] Conversely, nucleostemin expression is rapidly in- duced in response to regenerative growth in the newt[24] and is required for embryonic development because nucleostemin null mice die in blastocyst stage approximately 4 days after fertilization.[23] Loss of nucleostemin apparently renders cells incapable of DNA synthesis completion in S phase for HeLa cell cultures.[20] In the context of myocardium, nucleostemin is enriched in postnatal myocytes, as well as cultured neonatal rat cardiomyocytes, and is downregulated in adult heart (Figure 1) or in CSCs induced to differentiation (Figure 5). These findings are consistent with expression of nucleostemin in a proproliferative state as neonatal myocytes are capable of limited mitotic activity. Although nucleostemin overexpression does not stimulate proliferation or hypertro- phy in cultured adult cardiomyocytes (data not shown), induction of nucleostemin expression may be useful for antagonizing telomeric shortening associated with cardio- myocyte senescence and death.[48,49] In addition, increased

TERT expression following nucleostemin expression in CSCs may promote cell proliferation similar to that observed for hair follicle stem cells.[50] Thus, in CSCs, the presence of nucleostemin may serve important roles in maintaining pro- liferative potential, as well as antagonizing telomeric short- ening associated with enhanced mitotic activity (Figure 7 and supplemental Figure II).

Functionally competent telomerase is restricted to a few cells in adult organism, germ cells, and stem/progenitor cells.[51] In telomerase-competent cycling cells, detection of TERT in combination with markers of the cell cycle indicates that telomerase is active and prevents telomeric shortening. TERT expression is higher in nucleostemin-overexpressing CPCs than in control CPCs (Figure 7 and supplemental Figure II). Additionally, TERT and Ki67 colocalize in CPCs (Figure 7). Cycling CPCs that express TERT represent morphological counterparts of telomerase activity detectable with PCR-based methods. Importantly, the fraction of telomerase-competent cycling CPCs was higher in nucleostemin-infected CPCs, indicating that nucleostemin promotes CPC proliferation without affecting telomere length. In fact, by upregulating TERT expression, nucleoste- min allows CPCs to undergo multiple divisions opposing telomere attrition. It is not surprising that length of telomeres did not differ in noninfected and nucleostemin expressing CPCs. Although nucleostemin overexpressing CPCs showed higher levels of TERT (Figure 7 and supplemental Figure II), control CPCs also possess telomerase. In physiological con- ditions, the function of telomerase is not to elongate telo- meres beyond their physiological length but to prevent telomeric shortening. Finally, 3 to 5 days in culture is a very short time interval for the control cells that would not be expected to show detectable erosion of telomeres caused by rounds of replication.

TRF1 and TRF2 are 2 telomere-related protein components of a multiple protein complex, shelterin, that control ho- meostasis of telomeres by modulating access of telomerase to telomeres.[52] In this regard, decreased TRF1 binding to telo- meres reduce the affinity of telomerase to telomeres.[53] TRF1 and TRF2 promote formation of T loops in which the telomere terminus is concealed to prevent its recognition as DNA strand break by DNA damage/repair machinery.[54] This particular conformation of telomeres is nonaccessible to telomerase, thereby blocking telomere elongation. TRF1 and TRF2 are abundant in long telomeres but are absent in short telomeres, allowing telomerase to act only on short telomeres to prevent further erosion.[55] Increases in TRF1 and TRF2 protein resulting from nucleostemin overexpression (supple- mental Figure II) may indicate that 3 telomere termini are sequestered within the T loops opposing telomerase- dependent elongation of telomeres of normal length. TRF1 and TRF2 are critical for T-loop formation, and maintenance of this specific telomere-associated molecular structure is essential for continued cellular proliferation and prevention of senescence.Because telomerase activity is critical for maintenance of cardiac structure and function,[56] nucleostemin may act to fine-tune endogenous telomerase activity and promote maintenance of telomere length as well as inhibit p53-associated signaling resulting from shortened telomeres. These postu- lates would be consistent with diminution of nucleostemin following exposure of cultured cardiomyocytes to doxorubi- cin (supplemental Figure I), and lack of nucleostemin over- expression affecting p53 levels in this context may be explained by inhibition of MDM2 resulting from aberrant nucleostemin levels.[57] Under normal circumstances where telomeric shortening is linked to senescence and possibly apoptosis,[56,58,59] nucleostemin accumulation may serve to antagonize these processes in injured or aging myocardium, as implicated by increases in nucleostemin resulting from cardiomyopathic injury (Figures 2 and 3). In the case of stem cells, nucleostemin may enable cell cycling, as would be desirable in regenerative processes resulting from tissue injury or stress as supported by association of nucleostemin with c-kit$^+$ cells in the myocardium and cultured CSCs under proliferative conditions (Figure 5).

Signal transduction controlling nucleostemin expression is not well documented, but FGF-2 induces nucleostemin ex- pression in bone marrow stem cells.[21] Interestingly, FGF-2 exerts prosurvival effects in myocardium, is a potent angio- genic molecule, and is a crucial factor for proliferation and maintenance of several cell types, including stem cell popu- lations.[60] Interestingly, FGF-2 promotes differentiation of resident cardiac precursors into functional cardiomyocytes,[61] which would seem at odds with maintenance of a prolifera- tive state unless the action of FGF-2 occurs at an early stage of commitment, when limited mitotic activity occurs in concert with lineage specification. FGF-2 stimulates Akt activity that could account for prosurvival and proprolifera- tive effects,[62–64] and Akt activation lies upstream from Pim-1 induction in cardiomyocytes.[31] Induction of nucleostemin expression by Pim-1 activity (Figure 6) is without precedent in the literature and reveals an important mechanistic basis for Pim-1–mediated promotion of proliferation in the myo- cardium that will require further investigation.

The expression of nucleostemin in proliferative neonatal cardiomyocytes and CSCs, together with reemergence of this protein in damaged myocardium, opens up a new facet of our understanding of reparative and regenerative signaling in the heart. Nucleostemin may be useful as a molecular interven- tional tool for antagonizing cellular senescence, as well as maintaining proliferation. Alternatively, nucleostemin in ma- ture postmitotic cells such as cardiomyocytes may represent part of the reversion to a fetal or embryonic gene expression profile associated with cardiomyopathic challenge or stress. For the emerging field of CSCs, nucleostemin could be useful as a marker for identification of activated stem cells in the heart and provide a valuable marker of cellular proliferative state similar to Ki67.[26,37,65] Future studies are needed to expand on these intriguing seminal observations and define relationships between nucleostemin and cell status, as well as functional effects, in myocardial cells of both multipotent and lineage-committed cell types.

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Chapter 5

Cell and Gene Therapy for Patients with Severe Heart Failure: the Time and Place for Pim-1

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Abstract

Regenerative therapy in severe heart failure patients presents a challenging set of circumstances including a damaged myocardial environment that accelerates senescence in myocytes and cardiac progenitor cells. Failing myocardium suffers from deterioration of contractile function coupled with impaired regenerative potential that drives the heart toward decompensation. Efficacious regenerative cell therapy for severe heart failure requires disruption of this vicious circle that can be accomplished by alteration of the compromised myocyte phenotype and rejuvenation of progenitor cells. This review focuses upon potential for Pim-1 kinase to mitigate chronic heart failure by improving myocyte quality through preservation of mitochondrial integrity, prevention of hypertrophy and inhibition of apoptosis. In addition, cardiac progenitors engineered with Pim-1 possess enhanced regenerative potential, making Pim-1 an important player in future treatment of severe heart failure.

Introduction

Optimal care for a cardiac patient requires a dual approach: on one hand limiting damage and salvaging viable myocardium and on the other hand replacing dead myocardium with newly formed force-generating myocytes. To date, the majority of therapeutic options seem to prefer one side of this dichotomy: salvaging jeopardized cells. Clinical trials using Bone Marrow-Derived Stem Cells (BMSCs) were first initiated more than a decade ago. In retrospect, after hundreds of preclinical studies and over a dozen BMSC-clinical trials, the meta-analysis of collective findings shows the approach to be safe with beneficial reduction of infarct size without consistent marked improvement in Left Ventricular Ejection Fraction (LVEF)[1-6]. Outcomes with use of BMSC prompt the prevailing interpretation that BMSCs may be able to limit myocardial damage and potentially recruit endogenous stem cells in a paracrine fashion[7]. Lack of improvement in LVEF remains a point of concern as an important clinically relevant endpoint for efficacy, making more enduring cell-based therapeutic interventional strategies mandatory. Sustainable improvement in LVEF will inevitably require regeneration, prompting incorporation of newer stem cell types cardiac-lineage differentiation potential into the clinic as exemplified by the SCIPIO (cardiac **S**tem **C**ell **I**nfusion in **P**atients with **I**schemic **C**ardiomyopathy) trial[8]. SCIPIO Phase 1 provided initial proof of concept, safety and optimistic sustainable improvement with regards to LVEF and cardiac scar reduction[8]. In comparison, conclusions from an alternative approach as represented by the CADUCEUS (Cardiosphere-Derived Autologous Stem Cells to Reverse Ventricular Dysfunction) trial appear to recapitulate findings from the aforementioned BMSC trials insofar as the major reported benefit was myocardial scar-reduction without evidence of increased LVEF[9]. Although these Phase 1 trials are indeed primarily designed to assess safety rather than efficacy, the good news is that cardiac progenitor cell (CPC)-mediated therapy has ripened from a proof of concept at laboratory benches to therapeutic reality at a patient's bedside. Currently, while we await further expansion into Phase 2 trials for both SCIPIO and CADUCEUS, enthusiasm and optimism is tempered by circumspection of how broadly applicable such cell-based interventions will be to a broader patient population. Patients enrolled in the SCIPIO-trial were selected within criteria for being less than 75 years of age together with baseline LVEF<40%. Similarly, patients with New York Heart Association Class IV were excluded from the CADUCEUS trial. These rational and legitimate criteria for the initial clinical assessment of stem cell treatment have, by design, excluded a substantial patient population that desperately needs regenerative therapy: severe heart failure patients currently on a Left Ventricular Assist Device (LVAD) who cling to life as cardiac transplant candidates.

Terminal heart failure is a chronic disease involving progressive organ damage that clearly demonstrates myocardial regenerative capacity as insufficient to mediate functionally meaningful repair[10]. Aging, oxidative stress, DNA damage, and inflammation contribute to development of replicative and premature cellular senescence with subsequent secretome-mediated tissue impairment that drags neighboring cells into

senescence[11-18]. Severe heart failure cardiac patients predominantly correlate with a plethora of cardiovascular disease-associated risk factors such as smoking, excess caloric intake, or alcohol abuse that all participate in acceleration of telomere erosion and descent into cellular senescence[19-22]. Thus, cardiac stem cells residing within the embattled environment of severe heart failure may not necessarily reflect the reparative potential of cells employed in clinical trials to date. Collectively, the very target population of aged and infirmed patients destined to be at the forefront of interventional therapy are also likely to possess the most compromised stem cell population in terms of functional capacity and regenerative potential. Therefore, a reasonable question to ask is whether "youthful vigor" could be restored in these aged and pathologically-embittered stem cells without altering programming for context-dependent recognition of the environment and appropriate integration into the local environment in a delicate fashion (as is problematic for current inducible pluripotent stem cell-based approaches). In this special report, we will elaborate upon the role of the Serine/threonine kinase Pim-1 as a "rejuvenating" approach in cardiac stem cell therapy and myocardial regeneration. In addition, the potential role of Pim-1 as a gene therapy target in salvaging damaged myocardium will be discussed.

Pim-1

The proto-oncogene *Pim-1* gene displays characteristics of *primary response genes* that are induced by activation of transcription factors downstream of growth factor signaling such as Akt, Jak-STAT and NF-κB[23-25]. Pim-1 mRNA has a very short half-life due to presence of the destabilizing AUUU (A) sequence in the 3' UTR. The 5' UTR sequences of Pim-1 mRNA contains a GC-rich region representing a 'weak transcript', thereby imposing a cap-dependent translation[26, 27]. Due to presence of alternative translation initiation sites, two isoforms of this calcium/calmodulin-regulated kinase family member are produced (44kDa and 33kDa). Pim-1 protein is known to autophosphorylate, thereby being constitutively active[27, 28]. Once activated, the kinase has a half-life time of 10 minutes, indicative of a tightly regulated production/degradation process. Indeed, Pim-1 has been shown to physically interact with the prolyl-isomerase Pin-1, which allows interaction with protein phosphatase 2A leading to dephosphorylation, ubiquitinylation and subsequent proteosomal degradation[29, 30]. In addition, Hsp90[31] and Hsp70[32] have been correlated with regulation of Pim-1 stability and degradation respectively. Expression and activity of Pim-1 is induced by multiple growth factors, mitotic stimuli and cytokines in various cell types. In most cells, Pim-1 activity is associated with cell survival and proliferation.

Although belonging to different kinase families, Pim-1 is a downstream target of Akt kinase and shows similar substrate specificities. In fact, Akt-dependent survival signaling is attributed in part to physical interaction of Pim-1 with Bad, a major apoptotic initiator[33, 34]. Phosphorylation and inactivation of Bad leads to increased levels of pro-survival proteins Bcl2 and Bcl-xl in various cell types. In addition, Pim-1 has been reported as anti-apoptotic, independent of Akt, via phosphorylation of p38 MAPK in hematological cells[35].

The well-accepted role of Pim-1 in fostering cell cycle progression occurs in conjunction with phosphorylation of the phosphatase Cdc25A, a positive regulator of G1-phase of the cell cycle[36]. Increased phosphatase activity leads to amplification of Cdc25A and increased G1-S-phase transition rate. In addition, the main inhibitory protein of G1-S-phase transition, p21, is phosphorylated and inactivated by Pim-1 resulting in an increased cellular proliferation[37]. In addition, the pro-proliferative function of Pim-1 is not restricted to cell cycle progression. NuMa protein, responsible for organization of the spindle apparatus in the M-phase, is regulated by Pim-1 phosphorylation[38]. Similarly, Pim-1 mediates C-TAK1 and Cdc25C phosphorylation[39, 40]. Pim-1-dependent regulation of c-Myc transcription and protein levels have been demonstrated in multiple tumor types. Both c-Myc transcription and protein stabilization by Pim-1 have been reported, contributing to the perception of Pim-1 as a protein with proto-oncogenic activity[41, 42].

Pim-1: The *"P"* as a Proto-oncogene, *"M"* as a Mediator

Aforementioned molecular mechanisms of Pim-1 expression and activity have prompted indictment of Pim-1 as an instigator of cellular transformation in the field of oncology, based largely upon detection of increased Pim-1 levels in various hematological and solid tumors. In a group of malignancies, high Pim-1 protein level is associated with poor prognosis (e.g gastric cancer, head and neck tumors)[43-46]. Intriguingly, a major study on 2000 tumor samples reported an inverse correlation of Pim-1 levels and tumor recurrence rate[47]. Consistent with this counter-culture viewpoint, Pim-1 overexpression in prostate cancer, pancreatic ductal carcinoma and non-small cell lung carcinoma has been reported to be correlative with favourable prognosis[48-50] emphasizing the *cell-dependent/context dependent* role of Pim-1. Appreciation of the dogmatic perspective that Pim-1 contributes to cancer requires consideration of the cellular context for Pim-1-associated oncogenic behavior. In most tumors, Pim-1 is overexpressed in conjunction with increases in c-Myc levels. High level c-Myc activity has been correlated with induction of apoptosis, necessitating a molecular compensatory response by cells to preserve survival and potentially promote oncogenesis. Indeed, Pim-1 counteracts apoptosis in c-Myc–transformed cells[51, 52]. Similarly, high Pim-1 activity has been reported in K-Ras-mutation-based-transformed pancreatic malignancies[53]. Unlike many types of cellular transformation in tumor specimens characterized by gene rearrangement or dysregulated amplification for typical oncogenes, Pim-1 overexpression is thought to rest with altered transcriptional regulation. To our knowledge, the only cases of Pim-1 hypermutation occur in a select few lymphoma subtypes (Hodgkin, DLBCL and MALT). In these lymphoma, Pim-1 mutational hyperactivity is accompanied by simultaneous mutation in other genes such as c-Myc[51, 54]. Taken as a whole, oncology literature would seem to implicate Pim-1 as a 'proto-oncogenic-mediator' rather than an 'oncogenic-initiator'.

Another facet of consideration in the context of this myocardial-centric exposition is the decades of incontrovertible experience with the heart as an organ virtually refractory to oncogenic transformation. Cardiac-specific overexpression of canonical oncogenes such as c-myc and c-fos during mouse development increase cardiac myocyte number in

early stages after birth without persistent myocyte proliferation[55]. In fact, c-myc-driven increases in myocyte proliferation during development do not lead to abnormal myocyte formation in adulthood[55]. Even forced expression of telomerase by cardiac-specific transgenesis in mice causes hyperplastic heart development followed by overriding mitotic arrest and typical hypertrophic growth in adolescence, underscoring the legendary resistance of myocardium to transformation. The mechanism of mature myocardial resistance to proliferation is yet to be elucidated, but it is worth contemplating that cardiac myocytes possess shortened telomeres, particularly after pathologic injury. The concept of Oncogene Induced Senescence (OIS) or a 'hypermitogenic arrest' is based upon failure of oncogene-mediated transformation due to eroded and shortened telomeres. Thus, proto-proliferative proteins display distinct and unique phenotypic consequences in the heart relative to other cells or organs that retain mitotic activity as part of their normal homeostasis in adult life.

Taking our interpretation of Pim-1 mechanism in oncology together with an established legacy of non-transformation using cardiac-specific overexpression for otherwise canonical oncogenes in the heart, we now propose that Pim-1 can be reasonably and safely considered as an important target in rejuvenating aged stem cells for cardiac cell therapy of severe heart failure patients.

Pim-1 as a Rejuvenating-tool for CPCs

Pim-1 overexpression in CPCs leads to elongation of telomeres that is tightly regulated as evidenced by normalization of telomere length with prolonged cell passage, although during the transient phase of telomere elongation the CPCs exhibited a youthful phenotype characterized by higher proliferation rate and metabolic activity. In addition, Pim-1 overexpression in CPCs does not inhibit the capacity for cardiac lineage commitment[56], as has been observed for stem cells modified by activated Akt[57]. Chromatid segregation in CPCs is non-random (also referred to as asymmetric) ensuring that one daughter cell receives an enriched number of "immortal" chromosomes associated with preservation of stemness, whereas the other daughter cell will possess a disproportionate number of newly synthesized chromosomes that are thought to promote lineage commitment and cellular differentiation. Asymmetric chromosome distribution is important to create daughter cells participating in tissue regeneration, a phenomenon linked to expression of Pim-1 in CSC that increases asymmetric chromosome segregation by nearly twofold[58]. Similarly, transgenic mice with cardiac-specific overexpression of Pim-1 exhibit significantly higher number of assymmetric dividing CPCs as compared to their normal control brethren[59]. CPCs overexpressing Pim-1 and co-cultured with neonatal rat cardiac myocytes (NRCMs) show normal acquisition of I_{ca} current and Ca^{2+} signaling consistent with cardiac lineage[60], electrical connections through Cx43 gap junctions, and an authentic response to paracrine signals from NRCMs[60]. Collectively, these observations highlight expansion of *progeny-targeted* cardiogenesis mediated by Pim-1 activity as opposed to dysregulated and unproductive CPC-pool expansion.

In the setting of myocardial infarction injury, intramyocardial adoptive transfer of c-kit(+) BMSC modified to overexpress Pim-1 (BMSC-Pim) at time of infarction supported enhanced anterior wall dimension thickening and blunting of left ventricle dilation compared with hearts treated with vehicle alone[61]. Early recovery of cardiac function conferred by BMSC-Pim facilitated modest improvements in hemodynamic function up to 12 weeks after infarction between cell-treated groups and persistence of BMSC-Pim was improved relative to BMSC-GFP. The number of recruited endogenous c-kit(+) cells mobilized to the site of infarction injury was increased with BMSC-Pim compared to BMSC-GFP[61]. Interestingly, the paracrine effects of BMSC in these mouse studies promoted cellular hypertrophy in the border and infarcted regions coupled with an upregulation of hypertrophic genes[61]. Although the conclusion using BMSC-Pim supported a net improvement in structural remodeling relative to BMSC-GFP, the lack of functional commitment of BMSC-Pim echoed the limited efficacy of BMSC clinical trials. The need to use a more specialized and better adapted CPC cell population was reinforced by these results.

Fischer et al. conducted the first *in vivo* study using CPCs engineered to express Pim-1 (CPCeP) in the context of myocardial infarction, demonstrating significantly higher hemodynamic performance and LVEF as compared to CPC expressing green fluorescent protein (CPCeG) as controls that persisted for up to 32 weeks post-injection[62]. The persistent functional improvement was attributed to increased *do novo* myogenesis, neovascularization, engraftment of CPCeP relative to CPCeG[62]. The stage was now set to move toward translational studies by incorporating human-derived CPCs into the next set of studies.

Rejuvenating CPCs by overexpression of Pim-1 was, for the first time, extrapolated to human CPCs (hCPCs) in 2012[63]. The experimental study was designed specifically for the clinically-relevant patient population with severe heart failure in mind. Mohsin *et al.* isolated CPCs from tissue samples harvested from patients undergoing an LVAD-placement procedure. Stem cells were isolated and selected on the expression of stem cell surface marker c-kit. hCPCs were then lentivirally engineerd with Pim-1 or a GFP-control. Similar to prior findings by Fischer *et al*. using the mouse CPCs, these human CPCs expressing Pim-1 (hCPC-eP) showed enhanced proliferation, metabolic activity and telomerase activity relative to human CPCs expressing GFP control (hCPC-eG)[63]. The aged CPCs in this possessed a normal karyotype even while concomitantly exhibiting their boosted proliferation rate, negating a potential safety issue that has plagued the embryonic stem cell field for years. Long-term *in vivo* assessment of heart function upon myocardial infarction and hCPC-eP delivery to immunocompromised SCID-mice showed significant improvement in cardiac function within 6 weeks after cell injection relative to the hCPC-eG control group. Interestingly, differences between hCPC-eP versus hCPC-eG groups became increasingly evident with the ensuing months, where salutary effects of hCPCeP remained at a superior level for 20 weeks relative to hCPCeG[63]. Enhanced hemodynamic performance in hCPCeP-treated hearts correlated with prolonged persistence and evidence of cardiogenic lineage commitment, increased *de novo* myocyte formation and neovascularization[63, 64].

Sustained improvement of LVEF along with findings of *de novo* myocyte formation and neovascularization seem a desirable cocktail for the Holy Grail of myocardial regeneration. Realization of enduring improved cardiac function using HCPCeP isolated from an aged and severely diseased population is highly encouraging. However, confounding factors of patient clinical variability, inter-individual inherent stem cell differences and myocardial response to cell therapy remain as important and as yet unresolved uncertainties. At this point, ensuring the future success of myocardial cell therapy may very well require patient-specific assessment of inherent regenerative potential and endogenous stem cell exhaustion. Recent results combining hCPCeG and bone marrow mesenchymal stem cells (MSCs) advocate that a blend of cell types is more beneficial than CPCs only or MSCs only[65]. Future combinatorial avenues are mind-boggling when one considers the plethora of potential stem cells candidate types for adoptive transfer therapy. The major relevant aspect in salvaging jeopardized cells and myocardial regeneration is *time*. Rescue-time for myocytes in the wake of acute injury is severely limited and requires a ready to go 'off-the-shelf' product. On the other hand, autologous CPC-growth and associated regeneration will inevitably require a more protracted interventional time course to isolate, expand, and eventually reintroduce the donated cell population. Together with cell therapy, the field of gene therapy has witnessed major progress in development of broadly applicable minicircle plasmids, site-specific gene insertions using lentiviral construct and a great level of experience and expertise in the field of Adeno-Associated Viruses (AAV). In fact, current clinical trials are ongoing using AAV6 as a vector for cardiac gene therapy. Indeed, gene therapy appears a pragmatic and tractable 'off-the-shelf' approach to rescue myocytes at time of an interventional procedure such as percutaneous transluminal coronary angioplasty (PTCA). However, a heart failure patient presents distinct challenges not amenable to an acute interventional strategy. The complexity of underlying etiology, pervasiveness of degenerative changes, and deterioration of structural and functional characteristics in the failing myocardium will likely require a variety of combinatorial strategies to reverse cellular losses and combat accumulation of senescent underperforming cells. Conceptual fusion of genetic engineering to potentiate myocardial repair with *ex vivo* manipulation of stem cells offers distinct advantages: controlled manipulation of donated cells without concerns related to *in vivo* gene therapy issues of delivery and cell targeting.

Pim-1 as a Target for Gene Therapy

Pim-1 is abundantly expressed in neonatal hearts and decreases upon aging. Postnatal expression levels of Pim-1 declines but remains significantly elevated until 8 weeks of age when protein becomes comparable to 7 months of age[66]. Subcellular localization of Pim-1 switches from predominantly nuclear in neonates to cytosolic in early adulthood when protein levels start decreasing. In failing mouse and human hearts, Pim-1 expression re-emerges and is localized in the nucleus of myocytes. After acute pathologic injury, Pim-1 is reactivated to play a cardioprotective role in the cytosol of borderzone myocytes. Cardiac-specific overexpression of Pim-1 results in higher levels

of anti-apoptotic Bcl-XL and Bcl-2 compared to samples from normal control hearts. Genetic ablation of Pim-1 does not provoke an overt cardiac phenotype under physiologic conditions presumably due to compensatory upregulation of Pim-2 and Pim-3, but an impaired compensatory cardiac phenotype becomes evident upon pathologic challenge[66]. Cardiac-specific overexpression of Pim-1 (Pim-WT) in transgenic mice exhibit a 33% higher number of myocytes reflected in decreased average myocyte size relative to wild-type controls[66]. The preponderance of smaller, more numerous myocytes in Pim-WT hearts results in a hyperdynamic myocardium with an enhanced cellular reserve to cope with pathologic challenge, without abnormal myocyte formation, transformation or tumorogenesis. In fact, Pim-1 overexpression actually inhibits hypertrophy induced by endothelin-1 in neonatal rat cardiac myocytes. Pim-1 overexpression in cultured neonatal rat cardiac myocytes is characterized by enhanced calcium reuptake and decreased relaxation period with increasing sarcomeric shortening and SERCA expression. These cardioprotective actions extend to preservation of cardiac structure and function *in vivo* following hypertrophic challenge in Pim-WT hearts, which exhibit blunted hypertrophic remodeling under pressure overload challenge and preservation of functional output as evidenced by increased anti-hypertrophic signaling, decreased pro-hypertrophic proteins and increased hemodynamic function[67]. The cellular basis of response to hypertrophic stimulation in the Pim-WT heart consists of increased cellular proliferation and decreased apoptosis. Moreover, coping capacity of the Pim-WT heart in response to myocardial infarction (MI) is also superior to identically challenged nontransgenic controls with a 40% decrease in infarct size. Although, baseline hemodynamic performance of NTG and Pim-WT mice was the same, Pim-WT mice show higher contractile function after MI than NTG-controls[66]. Biochemical, molecular, and microscopic analyses have demonstrated beneficial effects of Pim-1 upon mitochondrial integrity[68]. Pim-1 levels increase in the mitochondrial fraction with a corresponding decrease in the cytosolic fraction of myocardial lysates from hearts subjected to 30 minutes of ischemia followed by 30 minutes of reperfusion. In response to oxidative stress, Pim-1 preserves inner mitochondrial membrane potential ($\Delta\Psi m$)[68]. Mitochondrial ultrastructure is maintained by Pim-1 activity, preventing swelling-induced calcium overload. Finally, mitochondria isolated from Pim-WT mice show inhibition of cytochrome *c* release triggered by a truncated form of pro-apoptotic Bad. In addition to preservation of mitochondrial integrity, Pim-1 also serves to blunt mitochondrial fission through inhibition of Drp-1[69]. High glucose treatment of adult rat cardiomyocytes leads to cell death. Increased level of Pim-1 mitigates high glucose induced cell death by increased survival signaling[70]. The common, non-ischemic/hypertensive, diabetic cardiomyopathy progresses from diastolic dysfunction to cardiac failure. Pim-1 expression is decreased in diabetic transgenic mice along with an increase in protein phosphatase 2A. In addition, diabetic hearts show low levels of anti-apoptotic proteins and increased caspase-3 activity. Adeno-Associated-Virus9 mediated delivery of Pim-1 in diabetic mouse hearts, has been shown to improve cardiac function and prevent cardiac failure, symbolizing Pim-1 cardioprotection on a mitochondrial level[70]. Collectively, this constellation of cardioprotective and anti-hypertrophic properties exhibited by cardiac-specific Pim-1 activity stands in stark contrast to forced expression

of typical oncogenic mediators such as Ras or myc that induce and contribute to progression of hypertrophy, making Pim-1 an effective genetic modification to promote stem cell-mediated regeneration and preserve myocardial structure / function in the pathologically injured heart. Combinatorial therapy using Pim-1 as a myocardial genetic approach and CPCs-engineered with Pim-1 captures advantages of both sides of the *stem cell / myocyte environment* dichotomy **(Fig. 1).**

Expert Commentary: Over a century ago the American writer and philosopher Elbert Hubbard wrote: "Optimism is a kind of heart stimulant - the digitalis of failure." Unfortunately, in the intervening 100 years neither optimism nor digitalis has provided any substantive progress for the prognosis or outcome of severe heart failure patients. Instead, alternative approaches for treatment of severe cardiac failure patients are required now more than ever before. Severe cardiac failure patients are challenged by a seemingly intractable combination of chronic stress, debilitating conditions and/or premature and replicative cellular senescence of the myocardium. All this, together with the high probability of compromised regenerative potential incapacitates the relatively modest ability of the heart to ameliorate or prevent further progression of cardiac failure. In retrospect, experience and expertise from studies using BMSCs and CPCs suggest that priming myocardial environment and myocyte regeneration could serve as a complementary approach. However, combinatorial-based cell therapy will require substantial investigation involving "trial and error" to sort through the cornucopia of various cell types, their respective combinations, relative percentages, and preparation / delivery protocols through rigorous testing and mechanistic studies prior to reaching a tractable clinical platform. Uncertainties of the innate stem cell biology for therapeutic use are further complicated by inter-patient variability, individual differences in stem cell behavior, and inherent deficiencies in myocardial responsiveness. Patient-specific assessment and choice of interventional time-line remains a critical unresolved area of investigation and requires expert assessment of the physician depending on patient-etiology and medical history. While one group of heart failure patients may require advanced environment remodeling to improve outcome prior to cell-based therapy, others might require a one-time procedure receiving a stem cell cocktail in combination with the gene therapy vector. In addition, manipulation of the pathologically damaged heart by preemptive gene therapy would allow much needed time and opportunity for longitudinal assessment of the myocardial environment to improve survival and persistence prior to cell delivery for regenerative treatment.

In order to figuratively "level the playing field" in this daunting task of regenerative therapy for heart failure we as researchers and implementers of technology must begin to reduce the number of possible approaches and optimize characteristics of the cells involved in order to standardize treatment protocols. "Rejuvenating" the patient's own stem cells with a validated and safe *ex vivo* genetic modification for autologous therapy avoids many pitfalls currently plaguing regimens implementing embryonic stem cells, inducible pluripotent stem cells, or allogeneic stem cells. Furthermore, this genetic engineering strategy should ideally confer improved survival and functional characteristics not only to the stem cell population, but also to their daughter progeny

responsible for the arduous task of rebuilding the damaged myocardium. At present, Pim-1 is the only genetic modification to our knowledge that has been comprehensively studied and proven effective in both stem cell engineering as well as preserving/upgrading of myocyte quality. Ultimate success for myocardial regenerative treatment involving severe cardiac failure will likely require teamwork from multiple areas of investigation involving cell biology, gene therapy, and the clinical practitioners responsible for turning optimism into reality.

Five-year view: The outcome of ongoing and planned clinical trials for adoptive cell transfer therapy and gene therapy for heart failure will provide a foundation for development and implementation of clinical cardiac cell- and gene therapy protocols worldwide. Thereby, further improvement of issues such as efficiency, broadening of the patient population and modification of compromised stem cells and myocytes will be essential for ongoing success in the clinical arena given the inherent variability of the target patient population. Overall, the evolution of cell therapy in the field of cardiology is likely to follow the fundamental clinical influences as the first cardiac transplant procedures, LVADs, beta-blocker application and other critical milestones. However, impatience of a desperate public, pressure from political concerns, and the often over-hyped allure of translational medicine tempers the reality that ongoing CPC- and gene therapy trials result from many years of incremental scientific discovery and testing that culminate in a clinical "breakthrough." Pressures for interventional "deliverables" from scientists and physicians has prompted an atmosphere of "translation-centric" and patient-oriented focus that sometimes can be dismissive of the importance for basic mechanistic understanding and relatively incremental, but necessary, slow progress. In the scientific "Maslow's hierarchy of needs", clinical trials require a foundation in basic scientific and mechanistic knowledge. The natural shift in focus towards translational medicine and subsequent mechanistic arrears could conceivable lead to a new dichotomy: either the clinics will be confronted with a protracted waiting time for major future therapeutic discoveries or patients will be confronted with premature clinical application of certain drugs or interventions with the subsequent consequences for the patients themselves and the scientific fields. Cell signaling studies *in vitro,* studies on epigenetics, molecular mechanistic cell cycle studies in all stem cell types (adult, ES, iPS, etc…), calcium-handling studies, and much more might not be directly clinically applicable in the short term but nevertheless remain crucial in delineation of knowledge and expertise essential for development of major discoveries such as cell or gene therapy.

Key Issues
CPCs have entered clinical practice for patients with moderate heart failure.
Severe heart failure patients may not be good candidates for early stage clinical trials due to impairment of their endogenous regenerative responses.
Heart failure patients suffer from aged-senescent myocytes and CPCs.
Therapy for heart failure requires a dual approach; modification of the environment through reversal of senescence in myocytes and reversal of senescence in CPCs.
Pim-1 has been proven to upgrade myocyte quality through prevention of hypertrophy, increased mitochondrial integrity and quality, less apoptosis and subsequent improved contractile function.
Pim-1 rejuvenates CPCs through telomere elongation, increased proliferation & metabolic activity and higher regenerative capability without alteration of cardiac-lineage commitment.
Optimal Pim-1 mediated therapy for severe heart failure patients will require Pim-1 gene therapy for priming the environment and application of "rejuvenated" CPCs for new myocyte formation.

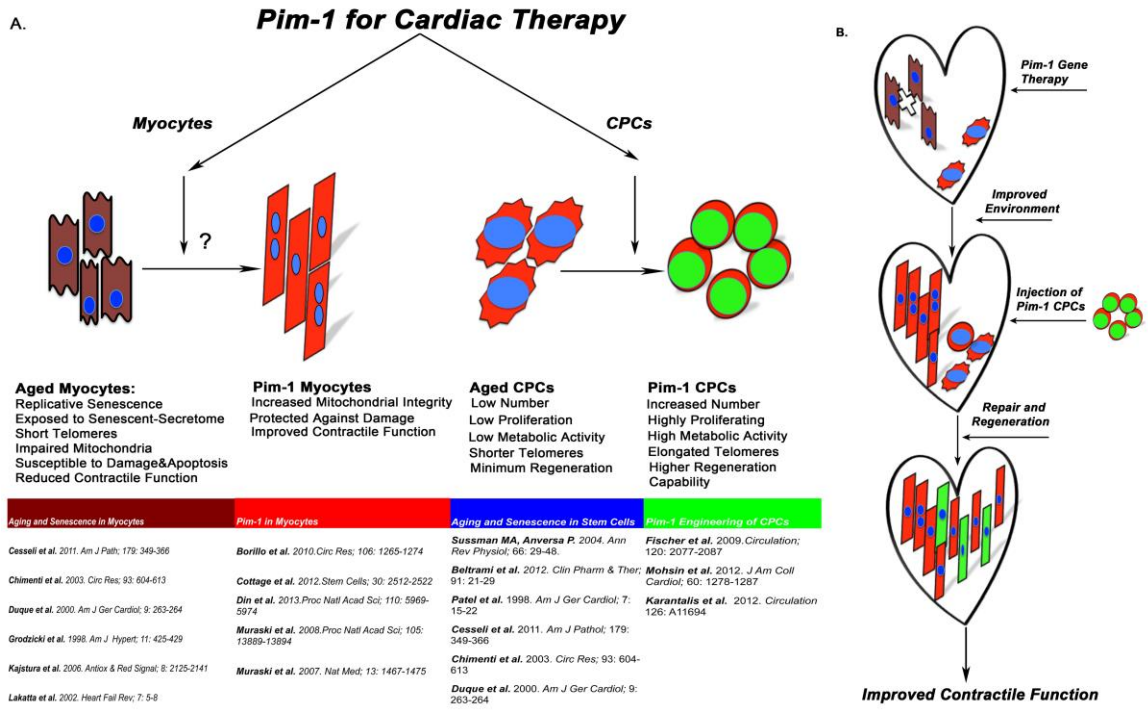

Figure 1. Application of Pim-1 genetic engineering for cardiac progenitor cells, cardiomyocytes, and the pathologically damaged myocardium of severe heart failure. ***A) 1***. Myocytes suffering from replicative senescence, exposure to the senescent "secretome", with shortened telomeres and impaired mitochondrial function exhibit reduced contractile function. ***2.*** Myocytes "rejuvenated" by Pim-1 overexpression possess heightened survival and metabolic activity together with increased contractile function. ***3.*** CPCs with compromised proliferative capability, short telomeres as a result of replicative and premature senescence, exhibiting compromised regenerative potential. ***4.*** CPCs engineered with Pim-1 recover proliferative potential, higher metabolic activity and elongated telomeres, thereby having an increased regenerative potential. ***B)*** Conceptual representation of Pim-1 mediated molecular interventional strategy to treat severe heart failure. Decompensated heart suffering from replicative and premature senescence with aged/damaged myocytes and senescent stem cells (top). Time and place for Pim-1 gene therapy intervention is indicated by the arrow (upper panel). Pim-1 mediated priming of the environment results in improved myocyte quality with subsequent beneficial paracrine effect on the endogenous CPCs (middle of panel). Delivery of Pim-1 "rejuvenated" CPCs into a modified environment is indicated by the arrow (middle panel). Injection of Pim-1 engineered CPCs results in myocardial regeneration and improved contractile function (lower panel) in mouse CPCs, human CPCs in mouse and human CPCs in swine (*Fischer et al., Mohsin et al.*, *Karantalis et al.)*

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Part Three: Cardiac Cell Therapy: How to Get to Rome?

Chapter 6

The optimal Cell Type for Myocardial Regeneration

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Introduction

Regeneration refers to a process of sustainable development. The word 'process' in the definition of regeneration is crucial for scientific intentions of cardiac therapy. In retrospect, the past decade has witnessed major advances in the field of myocardial regeneration by studying a variety of cell types, in a broad range of animal species and patients, at various time-points after injury, using multiple imaging techniques for assessment and visualization of cells and experimenting with delivery techniques. The more the field has accomplished, the more insight is gained on the complexity and extend of hurdles that sustainable myocardial improvement has to combat. Clinical trials using cells from the bone marrow are ongoing for a protracted amount of time now. More recently, isolation of stem cells from cardiac specimens has been brought to realization with an accelerated phylogeny towards clinical application exemplified by three major cardiac stem cell clinical trials currently ongoing. In addition, the field of pluripotent stem cells has gained momentum over the past years with the perspective for future clinical application. The element for optimism is the degree and promptness of developments in the field of regeneration. The element for realism remains that, unlike other fields such as hematology and oncology, the field of myocardial cell mediated regeneration is rather juvenile and immature. While animal studies on myocardial regeneration are ongoing worldwide, human cardiac cell therapy carries additional layers of complexity that are not effortlessly deciphered. Patient populations represent a natural inter-individual variability in medical history, exposure to risk factors, medication etc. This variability cannot be normalized for in standardized animal strains and require studies using and characterizing individual human stem cells. With longevity reaching the peak in history, population aging leaves scientists with an "aged" cell source what, unmistakably, hampers the quality of cell therapy. In addition, since the implementation of PTCA's in clinical practice and other medical advances, cardiac patients survive longer with a prolonged history of cardiac damage. Damaged myocardium not only represents an impaired stem cell source but also an environment that is less hospitable to naïve cells and subsequent new cell formation. Overall, specification of an optimal cell type for regeneration is affirmable from experience and conclusions at the end of this journey. At this point, the subject matter is not what the *optimal cell is* but criteria for what an *ideal cell would do* in order to promote regeneration to a degree that translates to cardiac functional improvement in a patient.

New cell formation in an organ that consists of such highly specialized structure and function is a high demanding task for naïve cells such as stem cells. Proper maturation of cells requires an assembly of factors and circumstances that ought to align accurately and timely. First, stem cells should survive the arrival and remain alive in a foreign environment that appears hostile. Since, cell quantity is essential for final outcome, proliferation of stem cells on the ground is consequential. Upon acclimatization, cells are required to take charge of committing to a cardiac lineage and initiating the process of differentiation towards a cardiac cell. One of the major challenges in building cells in a "failed" environment is the biological force to ascertain endurance of the initiated

commitment process to fully functionally differentiation towards myocytes and vessel cells. Considering cardiac structure and the inter-myocyte connections and communication that are required for cardiac synchrony, new myocyte members are required to fit in and harmonize with the existing myocytes to a degree that cardiac synchrony is preserved. Engraftment indeed is crucial not only for cardiac synchrony but also for significant contribution to contraction that ultimately translates to functional improvement and enhancement of quality of life. The concept of cell mediated therapy for cardiac regeneration gained an additional layer of enthusiasm by identification of the endogenous cardiac stem cell niches. The hart possesses an innate population of stem cells that are designated for myocardial regeneration and myocyte formation. However, due to the low turnover of myocytes in adult hearts, the proliferative potential of these cardiac stem cells appear insufficient for compensating for massive cell loss. It was soon hypothesized that intervening and manipulating the endogenous cardiac stem cell niches might provide a convenient source for mediating endogenous cell repair and regeneration. Added to the specific criteria defined to promote regeneration, the general criteria of cell safety are the building foundation for clinical cell therapy.

In the journey of endogenous regeneration and adoptive transfer using various cell types, much knowledge is gained on a variety of cell sources. This review is an assembly of cell types that are studied in animals and clinical trials, with the emphasis on advantageous and disadvantageous of each cell type and their position on the regenerative platform.

Bone Marrow-derived Stem Cells

In 1978, Schofield et el. introduced the concept of a bone marrow niche; the so called, hematopoietic stem cell niche (HSCN)[1]. The niche was defined as an expedient environment where stem cell maturation is prevented and the stemness attributes are maintained. The HSCN regulates biological processes such as quiescence, proliferation, differentiation, cell localization and mobilization. The niche consists of cells from the osteolineage, endothelial cells, mesenchymal stem cells, neurons and a significant amount of extracellular matrix networks[2]. Through the developmental phase, BMSCs are predominantly localized in the aorto-gonad-mesonephros region from where they mobilize and accumulate in fetal liver[2]. Upon birth, these cells migrate to the calvarium bone marrow and spread further. Bone marrow is a highly vascularized and extensively innervated organ from where hematopoiesis occurs throughout the entire human existence[3]. The extracellular networks of bone marrow are dominated by fibronectin, collagen I and IV, laminin, hyaluronan, heparine sulfate, glycosaminoglycans and a supply of growth factors and cytokines that determine cell fate[3-6]. Circadian rhythm driven sympathetic modulation regulates trafficking of BMSCs between the niche and the systemic circulation. Thus adrenaline and beta-adrenergic signaling have been shown to play a crucial role in mobilization of BMSCs from the niche[7].

Bone Marrow Mononuclear Cells (BM-MNC). The mononuclear cell population from the bone is a pragmatic approach of isolating an unfractionated and heterogeneous population of cells that include HSCs, endothelial progenitor cells (EPCs), mesenchymal stem cells (MSCs) and a few side populations[8].

On March 30th 2001, the first case of BM-MNC injection was reported in a 46-year old patient with ventricular failure after myocardial infarction[9, 10]. Subsequent years have witnessed thousands of patient injections in a dozen clinical trials. The rational for these trials stem, predominantly, from the hypothesis that BMSCs provide paracrine and or merocrine assistance to the damaged myocardium. The two influential trials in the early 2000s were the BOOST (BOne marrOw transfer to enhance ST-elevation infarct regeneration)[11-14] and REPAIR-AMI (Reinfusion of Enriched Progenitor cells and Infarct Remodeling in Acute Myocardial Infarction)[15-22] which both demonstrated a remarkable improvement of Ejection Fraction at 4 (6.7%) and 6 months (5.5%) after cell delivery in addition to increased event-free survival a year after treatment. However, no significant differences were measured as far as end systolic volume, maximal oxygen consumption or myocardial perfusion. In chronic ischemic cardiomyopathy, BM-MNCs have been studied in the FOCUS-CCTRN phase II-trial[23] and CELL-WAVE-trial[24]. Once again, these trials revealed an increase in stroke volume and ejection fraction but no significant enhancement of end systolic volume or other hemodynamic parameters. The FOCUS-CCTRN trial however, revealed an interesting finding that the functional improvement in cardiac function was correlated with enrichment for CD133+ and CD34+ cells[23], which was the first indication for advocating that composition of unfractionated bone marrow might be of clinical value and importance. Subsequent studies and trials focused on singular cell types obtained from bone marrow samples.

Mesenchymal Stem Cells (MSCs). MSCs have been shown and suggested to be a lead candidate from the bone marrow population, partially due to the prospect of their ability to inhibit T-cell mediated inflammation and cytokine balance thereby affecting the atrocious process of remodeling[25-27]. MSCs have the expertise to differentiate into osteocytes, chondrocytes and adipose tissue[28]. Differentiation of MSCs in to a "cardiomyocyte-phenotype" after infarction has been reported previously[29-33], which unfortunately is contradicted by additional studies that reveal a temporary functional improvement without signs of myocyte differentiation[34, 35]. This intricate topic is a matter of perpetual debate and controversies and the consensus is yet to be achieved. Differentiation of MSCs in endothelial cells and thereby promotion of angiogenesis has been discussed and studied in the past[36, 37]. This latter phenomenon, however, is slightly decipherable to the field considering the embryonic development of the coronary arteries and neoangiogenesis in tumorogencity through the well-known process of Mesenchymal Endothelial Transition (MET). The convenient isolation procedure and beneficial biological properties of MSCs were encouraging in conducting clinical trials using, solely, MSCs for cardiac therapy. The major trial, TAC-HF (Transendocardial Autologous Cells in ischemic Heart Failure)[38] is a phase II trial currently ongoing, which is taking advantage of MSCs in treatment of cardiac patients. The patients show an impressive safety profile and the preliminary results are promising. MSCs are propositioned to carry immunosuppressive attributes due to a lack of Major Histocompatibility Class II antigen expression and the B7 and CD40 ligand co-stimulatory units, which point toward a possibility of allogeneic applicability. Indeed, so far two clinical trials have been initiated using and comparing safety and efficacy profiles of allogeneic MSCs. The POSEIDON-trial is a direct comparison between autologous

and allogeneic MSCs, and reveals that both autologous and allogeneic MSC delivery is safe without any adverse effects[38]. However, the POSEIDON-trial is lacking a placebo group, which calls for caution regarding conclusions as far as efficacy matters. A second, double-blinded, randomized and placebo-controlled trial of 220 patients using allogeneic MSCs reveals no cases of toxicity, fewer arrhythmogenecity and overall improvement of clinical status at 6 months post cell delivery. In addition, patients with major myocardial infarctions showed an increase in Ejection Fraction. An additional ongoing multicenter clinical trial using mesenchymal precursor cell has been initiated with initial promising results (AMICI)[39]. Despite the astonishing findings and safety profile of MSC treatments so far, two major hurdles remain to be overcome; although initial functional improvements are observed, the results and findings of long-term persistent functional improvement and safety have not yet been fully elucidated. In addition, studies using murine BM-MSCs have revealed a propensity towards occurrence of mutations upon passaging *in vitro* and risks of ectopic tissue formation. Although these findings have not been reported in trials so far, they remain crucial and essential determinant for long-term future MSC- studies and clinical trials.

Hematopoietic Stem Cells (HSCs). HSCs account for 1-3% of the bone marrow mononuclear fraction and are characterized by the cell surface markers CD34, CD45 and CD133[6]. However, upon time, the HSC surface markers seem to overlap with some other cell types from the bone marrow, which contributes to the complexity of elucidating the HSC distinct mechanism of action. Clinicians have decades of experience in isolation and application of HSC for treatments of leukemic patients, since HSC carry an excessive potential to give rise to all subsets of peripheral blood. A sporadic study of HSC transdifferentiation and engraftment in the myocardium has been reported[40]. Unfortunately, the majority of laboratories worldwide have not succeeded in recapitulating these findings[41], which restricts the applicability of HSC for that purpose to a few laboratories and is limiting for implantation as standard protocol. Since, functional improvement using HSC is temporary, it is hypothesized that HSCs secrete an effective paracrine cocktail that lead to stimulation of angiogenesis, recruitment of cardiac resident progenitor cells, inhibition of apoptosis and modulation of extracellular matrix composition. So far, to our knowledge, no clinical trial using HSC, solely, have been finalized or initiated.

Endothelial Progenitor Cells (EPC). The EPC population comprise 0.1-0.4% of the mononuclear fraction of bone marrow and are also detectable in peripheral blood. As the name implies, EPC have the potential to incorporate into damaged regions and contribute to neoangiogenesis and vessel formation[6]. EPCs have been shown to be highly proliferative, however, cell numbers and proliferative profile are, presumable, negatively correlated with risk factors for vascular disease and have been shown to be upregulated in clinical cases of coronary artery disease. In addition, multiple studies have described blood EPC-levels as an independent predictor of cardiovascular disease[42]. These findings point toward a correlation of systemic disease and quality deterioration of EPCs. In particular, patients with coronary artery disease require healthy and highly proliferative cells. Thus, future studies on EPC application require rigorous characterization of cellular biology and proliferative potential prior to clinical-applicability.

Excellent previous reviews have focused on creating extensive overviews of BMSC-clinical trials. Further coverage of the trials is beyond the scope of this review.

Overall, the safety profile of BMSCs has been proven excellent over the past decades. In addition, BMSCs have been shown to be effective and increase cardiac function and patient quality of life. However, the functional improvement appears to be temporary in repetitive attempts and studies. This impermanent aspect of BMSC mediated cell therapy is contradictory to the sustained process of regeneration. As anticipated, BMSCs are naturally preprogrammed to give rise to components of the blood and bone marrow. So, differentiation of BMSCs to a cardiac lineage would be a cellular "metamorphosis" which is an ambitious expectation. Appreciably, a sporadic case of BMSC transdifferentiation to a cardiac lineage remains a possibility, however, this phenomenon does not meet the degree of efficiency that is required to contribute to sustained enhancement of cardiac function. Multiple studies are ongoing on comparison of cell combinatorial effect of BMSCs, titrations and repetitive injections without significant improvements in durability of functional improvement. These observations, however, must not temper the clinical value of BMSCs. The variability in cardiac patient population provides room and audience for every possible approach. Terminal heart failure patients with co-morbidities may not serve as candidates for invasive procedures required for achieving 'regeneration' and may be very helpfully served by an intervention that provides temporary mobilization for daily life. In addition, BMSCs have an advantageous paracrine profile that seems protective to vulnerable myocytes and or stimulatory to the endogenous cardiac stem cells to promote regeneration. These attributes can be utilized in a combinatorial fashion with other cell types to promote and "prime" myocardial environment, increase activation of the endogenous niches and perhaps, in the future, decrease risks of host versus graft rejections by using MSCs. Duran et al. published a study regarding isolation of bone-derived stem cells and cardiac progenitor cells. Although the results are interesting, the study is conducted in a time-range of 6 weeks and, unfortunately, does not provide rigid conclusive elements. A major concern remains the variability in clinical trials using BMSCs. Major clinical trials such as the BAMI-trial[43] and equivalents are necessary for solid conclusions in he field of BMSC therapy that can serve as a foundation for future combinatorial therapy. Although, many large animal studies are still conducted every year for elucidating mechanisms for regeneration, the outcome seems evident. BMSCs are advantageous but do not, effectively, participate on the regenerative platform. Future BMSCs studies should rather focus on identifying factors that provide consistency in the current protocols. The issues may not root in small number of animals (mostly pigs) or number of patients but in innate cellular variability. BMSCs are, so far, not rigorously studied on their cellular biological characteristics and variability within a culture. The ratios of quiescent versus proliferating cells may be an indicator for variability in outcome. BMSC senescence is not studied extensively and screened for in cell therapy approaches and the injection sites in the niche are not identified. These biological characteristics of BMSCs can provide insight in BMSCs-trial variability and can be studied in affordable small animals. These fundamental studies are required for broad utilization of BMSCs as

a standard clinical protocol for certain patient populations and are essential for future combinatorial therapy approaches.

Embryonic Stem Cells and Induced Pluripotent Stem Cells

Murine embryonic stem cells were discovered for the first time in 1981[44] and the concept of using human embryonic stem cells followed 17 years later (1998). Human embryonic stem cells (hESCs) are obtained from the inner cell mass of a 5-day-old embryo at the blastocyst stage[45]. ESCs are then propagated on murine embryonic fibroblasts where they remain pluripotent and self-renewal properties. ESCs divide symmetrically to create progeny identical to the daughter cell[46]. The cell cycle of ESCs is significantly shorter than the division time of an adult stem cell, predominantly due to an incomplete and a lack of G1-phase and G2-phase, respectively. ESCs, thus lack the major DNA checkpoints stages[47]. The differentiation phase requires ESC growth in suspension where embryoid bodies are formed and contain cells of all 3 germ layers. The general concept is to recapitulate the embryonic development of the heart *in vitro*, which requires titrated application of specific factors that are involved in cardiopoeisis. Ever since 1998, scientists worldwide are investigating various differentiation protocols of cardiac differentiation from ESCs[48]. The pluripotent attribute of ESCs becomes more evident *in vivo* where injection of ESCs in SCID mice, consistently, leads to teratoma formation[49]. Similarly *in vitro*, the ESC culture permanently consists of the so-called, non-responders, ESCs that appear resistant to differentiation stimuli and remain pluripotent in a mixture of differentiation-stimulated cells. The suggested purification protocols are based on antibiotic resistance and treatment of cells with suicide genes. These genetic manipulations of ESC add an additional layer of complexity and risk for tumorogenesis[50, 51]. Numerous animal studies reveal the potential of ESCs to differentiate into a cardiac lineage. In fact, the differentiated ESC-derived cells contribute to cardiac function[52-54], however, the unpurified cultured conditions and cases of tumorogenesis remain a major safety hurdle in ESC-mediated cell therapy. Due to the perpetual ethical discussions regarding the use of ESCs, alternative approaches were desired and looked for.

In 2006, Yamanaka and Takahashi announced the development of iPSCs by overexpressing four pluripotent transcription factors; Oct3/4, Sox2, KLF4 and c-Myc[55]. In 8 years, the number of iPSCs based scientific papers increased to 1675. iPSCs have been generated from a variety of somatic cell sources including fibroblasts, hair follicles, bone marrow, liver cells and so on[56]. A major initial hurdle in generation of iPSCs was the delivery technique used for overexpression of pluripotent transcription factors, which carry the risk of random insertion sites and potential tumorogenicity. Although that issue is being overcome by plasmid-mediated overexpression[57-60], the reprogramming efficiency remains a major hold up. iPSCs are phenotypically resembling hESCs as far as morphology, pluripotency state, gene expression and differentiation ability *in vitro* and *in vivo*. The animal studies using iPSCs and iPSCs-derived cardiomyocytes, thus far, reveal a similar pattern of functional improvement and tumorogencity as the ESCs.

While embryonic stem cells have a natural process of transcriptional silencing prior to the blastocyst stage, iPSCs have an epigenetic memory and provide a “package” of

epigenetic modifications, depending on the cell source. This epigenetic memory provides an additional layer of complexity in assessment and comprehension of tumorogenicity tendency in this cell population. One of the great advantageous of iPSCs over ESCs was previously suggested to be the lack of an immune rejection due to use of autologous somatic cells. However, recent reports have raised questions and suggest that the reprogramming process could perhaps induce expression of antigens that are considered "foreign" to the immune system[61, 62]. The major supremacy of iPSCs relative to ESC is the fact that iPSCs provide a highly profitable platform for patient specific disease studies and drug testing. In less than a decade, iPSCs have been used in studying mechanisms underlying dilated cardiomyopathy, hypertrophic cardiomyopathy, mutations in sarcomeric proteins, calcium channel disorders, rare arrhythmogenecity diseases and many more[63-66]. At this point, iPSCs-mediated cardiac regeneration seems rather far away. Similar to the ESC-field, iPSC-mediated cell therapy remains disputable and calls for caution. Meanwhile, more insight can be generated in specific disease, drugs mechanism of action and rare hereditary disorders and preventions. This has, however, been proven to be a field of high-speed improvement of protocols, advancements and overcoming of substantial hurdles.

Cardiac Resident Stem Cells

Similar to bone marrow, cardiac stem cells reside in particular stem cell niches that provide an ideal environment for stem cells to maintain stemness, remain quiescent and get mobilized upon stimulation, the so called Cardiac Stem Cell Niche (CSCN)[67]. Typically, the niches are characterized as peculiar structures in the interstitium where stem cells and early committed cells inhabit. CSCNs are detected as clusters of CSCs and early committed cells that are in direct contact with the surrounding myocytes and fibroblasts through gap-junctions (connexins) and calciumdependent transmembrane adhesion proteins (cadherins)[67]. The two main extracellular matrix components of a CSCN are fibronectin and laminin with integrin as the most prevalent surface protein within the niche. Inside the CSCN, cells divide symmetric or asymmetric, thereby maintaining homeostasis of the niche and generating progeny. In the year 2002, a first report of a "stem-cell like" side population (SP) cells was published where SP-cells were characterized by expression of the ATP-binding cassette transporter Abcg2[68]. These cells seemed capable of differentiating into myocytes *in vitro* and represented 1% of the total number of cardiac cells. The subsequent years revealed comprehensive characterization of cardiac resident stem cell population from murine hearts based on the 3 surface markers c-Kit, Sca-1 and MDR-1[69, 70]. This discovery, indeed, was pathbreaking to the field of cardiac cell therapy since, analogous to BMSC's ability to give rise to all blood components, CSCs have the natural predisposition to generate the cardiac lineages and thus provides a major platform for studies aimed to regenerate the myocardium. The knowledge and insight on CSC soon propagated to various species such as rodents, canine and porcine. CSCs are self-renewal, clonogenic and multipotent[71]. Upon injection *in vivo*, CSCs differentiate into all 3 cardiac lineages; cardiomyogenic, smooth muscle and endothelial lineage[72, 73]. The current understanding of CSCs reveals a physiologic role in inherent aging and gradual

replacement of myocytes upon natural apoptosis. This given justifies why endogenous repair process does not suffice in combating massive injury post myocardial infarction. The knowledge on CSCN and cell characteristics were soon extrapolated to human hearts where, similar to previous data from mice, clusters of CSC were defined (hCSCs) in samples isolated from cardiac patients[73, 74]. hCSCs appear in niche like structures and are in direct contact with myocytes and cardiac fibroblasts. hCSCs have been isolated from human hearts based on the surface marker c-Kit (CPCs) and Sca-1 (CMPCs). Unlike perpetual attempts towards regeneration using BMSCs, CSCs, repetitively and in multiple species, lead to a persistent functional improvement and have the ability to give rise to al 3 cardiac lineages; smooth muscle cells, endothelial cells and cardiomyocytes. The new myocytes appear to engraft and express markers of gap-junctions and cadherins on the surface. CSC-mediated cell therapy has been proven safe without adverse effects of tumorogencity or arrhythmias. A simple literature comparison reveals that CSCs used for cardiac therapy are by far better characterized than BMSCs used in clinical trials. CSCs have been studied extensively as far as their proliferative capacity, differentiation potential *in vitro*, telomere length and telomerase activity and expression of surface markers. After multiple validation processes of myogenic differentiation of CSCs in rodents, the first clinical trial using CSCs was initiated in August 2011. The SCIPIO-trial is a randomized phase I/II-clinical trial that is aimed for studying safety and efficacy of CSCs in patients with moderate ischemic cardiomyopathy[75]. So far, the safety record of the SCIPIO-trial is remarkable with no adverse effects after intracoronary injection of CSCs. In addition, patients treated with CSC show an 8.5 unit increase in LVEF at 4 months that further increases to 12.3 units at 1 year. Nevertheless, at 4 months and 1 year of follow-up, MRI measurements of the hearts treated with CSCs reveal a significant decrease in infarct size. The SCIPIO-trial is currently in progress and the initial 1year results are encouraging and call for great optimism for patients eligible for regenerative treatment of cardiac diseases. An additional method of hCSC isolation is established where large amounts of cardiac surgical specimens are minced and put in primary culture[76, 77]. Upon placement in suspension, the cells develop spherical clusters, the now called cardiospheres (CSps). Initial animal studies using CSps seemed promising, however the cardiospheres appeared to be too large for coronary infusion. This limitation opened the field of Cardiosphere-derived Cells (CDCs) where cells derived from CSps are minced and cultured in monolayers for application[78, 79]. The first clinical trial using CDCs, the CADUCEUS-trial, is currently ongoing. This randomized clinical trial is based on intracoronary injection of CDCs isolated from myocardial biopsies of patients with moderate ischemic cardiomyopathy and recent myocardial infarction. The initial outcomes of the CADUCEUS-trial reveal a substantial scar-reduction and increase in viable myocardium, however, no significant enhancement of LVEF or significant changes in ventricular volumes are detected[80]. Over the past decades, animal studies showing regeneration reveal a significant increase in Ejection Fraction and improvement of ventricular volumes, even using CDCs[81]. This discrepancy remains to be elucidated. In addition, application of CDCs requires a particular range of cell quantity that is safe.

CDCs are 20micron in diameter and the diameter of a coronary capillary is 7 micron , which brings limitations to the number of cells that can be safely injected in patients.
The most recent and preliminary cardiac derived stem cells-trial (ALCADIA) is an open label non-randomized combinatorial treatments of CSC and a hydrogel with gradual secretion of bFGF[82]. The primary outcomes are safety and efficacy. The initial steps appear promising, however, the low number of patients and the preliminary state of the trial requires long-term comprehensive evaluation.

Overall, CSCs are by and large the most prominent cell type for regeneration. The consistency in animal studies and the outcomes of the clinical trials, realistically, are promising and a favourable advancement. Given the fact that isolation of CSCs requires myocardial biopsies or auricle tissue isolation during open cardiac procedures, the patient population eligible for CSC-trials requires an appropriate health qualification. Specifically, CSC-trial patients should be capable of undergoing a CABG-procedure or a myocardial biopsy, which requires accurate health and risk benefit ratio assessment by physicians involved. Unlike the BMSC-field, the CSC field has reached a point where issues such as retention, efficiency and delivery techniques have become crucial and require not only basic mechanistic studies but also large animal protocols and funding redistribution

Expert Commentary

Based on the experiences from the past decade, the optimal cell type for cardiac regeneration appears to have a cardiac source. Cells from the bone marrow seem clinical valuable but are not designed for cardiac regeneration. However, BMSCs can be used as additives to CSC interventions in an attempt to increase efficiency by priming the environment and modulating local inflammation. Pluripotent stem cells carry the potential to differentiate into a cardiac lineage, however, lack the foundation for clinical applicability as far as safety concerns. Pluripotent stem cells have been proven valuable in drug testing and screening for genetic diseases but application of these cells into patient subjects must not be rushed. The magnitude of this responsibility goes beyond convincing the field and scientists and physicians involved. It requires rigid validation and certainties that these cells can be safe for the sake of individual patients and the general public hope and trust in reputation of stem cell therapy.
Although CSC-clinical trials seem promising as far as myocardial regeneration, the inclusion and exclusion criteria are very rigidly defined for selecting the fitting patient population. This given, raises questions regarding generalization of these affirmative findings to patients with other types of pathologies e.g sever ischemic cardiomyopathy, dilated cardiomyopathy and hypertrophic cardiomyopathy. The field of cardiac senescence is gaining more attention than ever before due to the fact that CSCs may be affected differently in different environments and time-range of exposure to a senescent environment. CSC-treatment of each patient population requires rigorous characterization of the isolated cells as far as characterization of the population, proliferative potential, potency, viability and differentiation ability. Stem cells from severely injured hearts may require manipulation, and empowerment as previously described using Pim-1 in CSCs isolated from terminal heart failure patients that undergo

a Left ventricular Assist Device placement. Pim-1 modified CSCs appear superior to unmodified CSCs as far as survival and proliferation *in vitro* and *in vivo*[83]. In addition, overexpression of Pim-1 does not seem to interfere with the differentiation potential of CSCs as detected by sustained increase in Ejection Fraction and detection of myocytes specific to a human origin in murine hearts. Alternative approaches to modifying CSCs for improved regeneration may be a combinatorial therapy using 2 or more specific cells with very specific purposes e.g CSC with MSCs and/or EPCs for promoting regeneration in an allogeneic fashion with simultaneous stimulation of angiogenesis. Since an optimal cell is currently non-existing, future studies should focus on outside the box ideas of manipulating the cells in a way that represent the general criteria for optimal regeneration. The field of tissue engineering has been successful in the past by forming patches and hydrogel mediated delivery of cells where survival and persistence are stimulated. Another alternative approach may be fusion of different cell types in order to *create* a perfect cell based on the currently defined criteria e.g fusion of CSC with MSCs where a cardiac stem cell is formed with MSC immune-modulatory attributes. Or fusion of CSCs with EPCs where an overly angiogenic stem cell is generated. These out side the box alternative approaches are required for taking myocardial regeneration to a higher level.

Five-Year Perspective

The five-year perspective depends on what distinct fields choose to invest their focus, efforts and funding in. The field of BMSCs has the potential of becoming a standard clinical protocol for severely ill cardiac patients. However, that requires a shift of focus from large animal studies to decreasing trial variability by conducting small animal consistency studies and further characterization of the cells. ESCs and iPSCs may appear to have clinical application but require years of studies regarding safety. A 5-year risk for this field is premature clinical application with subsequent negative consequences for the patients, the scientists and funding organizations.

The approach of CSC-mediated regeneration has the potential of evolving and spreading very rapidly to multicenter trials and perhaps multi-nation trials. A great population of cardiac patients will be served in a significant and sustainable fashion. However, as mentioned previously, so far, the patient population is defined in a rather narrow range. The coming 5 years will focus more on expanding the inclusion and exclusion criteria to reach a broader range of patients. That maneuver introduces a field of patient specific cell therapy that requires characterization of patient specific human CSCs and subsequent application or manipulation of the cells. Recently, the field of CSC-mediated regeneration has gained an encouraging competition field of myocyte division. C14 labeling has now shown the potential of adult myocytes to undergo division and generate new myocytes. The evolvement of this field is eagerly awaited since that could introduce an entire new dynamic of *stem cell-myocyte-mediated regeneration* of the myocardium that with multi-disciplinary collaborations and efforts can take myocardial regeneration to a new level.

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Chapter 7

Empowering Adult Cardiac Stem Cells for Myocardial Therapy

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Abstract

Treatment strategies for heart failure remain a high priority for ongoing research due to the profound unmet need in clinical disease coupled with lack of significant translational progress. The underlying issue is the same whether the cause is acute damage, chronic stress from disease, or aging: progressive loss of functional cardiomyocytes and diminished hemodynamic output. To stave off cardiomyocyte losses, a number of strategic approaches have been embraced in recent years involving both molecular and cellular approaches to augment myocardial structure and performance. Resultant excitement surrounding regenerative medicine in the heart has been tempered by realizations that reparative processes in the heart are insufficient to restore damaged myocardium to normal functional capacity and that cellular cardiomyoplasty is hampered by poor survival, proliferation, engraftment, and differentiation of the donated population. To overcome these limitations, a combination of molecular and cellular approaches must be adopted involving use of genetic engineering to enhance resistance to cell death and increase regenerative capacity. This review highlights biological properties of approached to potentiate stem cell–mediated regeneration to promote enhanced myocardial regeneration, persistence of donated cells, and long-lasting tissue repair. Optimizing cell delivery and harnessing the power of survival signaling cascades for ex vivo genetic modification of stem cells before reintroduction into the patient will be critical to enhance the efficacy of cellular cardiomyoplasty. Once this goal is achieved, then cell-based therapy has great promise for treatment of heart failure to combat the loss of cardiac structure and function associated with acute damage, chronic disease, or aging.

Prologue

Perplexity is the beginning of knowledge.

- Khalil Gibran

Substantial resources have been expended over the last decade in pursuit of interventional strategies to treat the unmet need of heart failure patients to restore their myocardial structure and function. In the wake of thousands of research reports and hundreds of clinical studies we remain perplexed, which is reassuring in the context of the Gibran quote that begins this review. Although we have a lot to learn, knowledge is coalescing into understanding that, in turn, refines the search for answers into ever more fruitful directions of investigation. One fact that has become abundantly clear from both clinical and basic research studies is that regenerative medicine for myocardial damage will not be enacted simply by delivery of the stem cell types we currently have at our fingertips. This review will endeavor to summarize the run up to current understanding, where road is blocked or splits apart, and how the utilization of enhanced stem cells may provide the means to overcome current barriers that limit efficacious implementation of regenerative cell therapy for heart failure.

Part 1: In the beginning there were a couple ideas.

Ideas are like rabbits. You get a couple and learn how to handle them, and pretty soon you have a dozen.

- John Steinbeck

Today in a new age of enlightenment, students and trainees regard their mentors with bemused incredulousness when told that, until recently, the prevailing dogma held the myocardium as a fully post-mitotic tissue incapable of regeneration. At the turn of this century, cell therapy approaches were essentially limited to adoptive transfer of various non-cardiac cell types into the pathologically injured heart in the hopes of stimulating chimeric engraftment and some modicum of repair[1-5]. The transplantation of skeletal myoblasts into the myocardium of a patient with severe ischemic heart failure in 2001 and subsequent arrythmogenic complications raised concern over the safety of adoptive transfer cell therapy[6]. Despite this setback the concept of adoptive cell transfer remained an attractive one, especially in a tissue considered post-mitotic. Finding a cell type that was safe, efficacious, and durable for mediating repair remained the holy grail of cardiac regenerative medicine. Coincidentally, while skeletal myoblast transfer studies stalled in 2001, a new era was concurrently dawning with the advent of bone marrow adoptive cell transfer for repair of the infarcted heart[7, 8] Regardless of the maelstrom of debate which ensued about the findings of these seminal studies[9, 10] there is no question that these publications represented a turning point in the perspective of how myocardial repair could be effected. The following decade witnessed numerous clinical trials with bone marrow and bone marrow derived cells to assess the clinical application of stem cells as summarized in excellent reviews and meta-analyses[11-14]. In brief, cardiac clinical trials from the past decade have mainly

been based on different cell subsets of autologous bone marrow. The general conclusion of these clinical studies declare that bone-marrow stem cell therapy is safe and is associated with a moderate (1.93%- 5.40%) increase in ejection fraction. This improvement appears to be temporary[12] presumably due to limitation of remodeling or relief of angina through paracrine effects, rending this approach possibly efficacious in biologically old patients but a suboptimal choice for the majority of the mid-life patient population. Long-term functional improvement requires application of stem cells possessing true cardiomyogenic and vascular differentiation potential and contributing to new cell and vessel formation in the myocardium. This rationale underpinned the announcement that resident cardiac progenitor cells (CPC) derived from human samples capable of generating myocardium and vasculature[15] had been isolated and, as a consequence of experimental studies and published reports now numbering in the thousands, the reputation of the heart as an organ incapable of cell regeneration has been transformed[16, 17]. No longer slumbering in post-mitotic quiescence, the heart is a dynamic organ capable of repair, cellular replacement over aging, and a fertile milieu for the panoply of stem cells, sourced from adults, embryos, and induced fibroblasts. With subtypes of each cell category seemingly multiplying like proverbial rabbits, the field has morphed from a lack of suitable regenerative cell populations to an overabundance of possibilities. A brief examination of the embryonic / inducible pluripotent camp versus adult cells is in order to understand the empowerment issues involved.

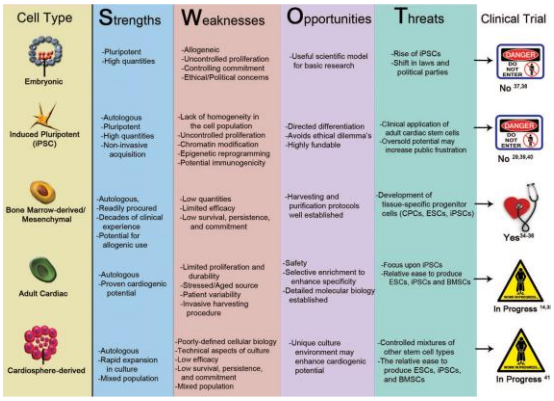

Figure 1. SWOT analysis of different stem cells and their possible clinical application. Matrix assessment delineating a SWOT analysis (Strengths, Weaknesses, Opportunities, and Threats) of various stem cell types and their clinical implementation.

With a goal of recreating tissue in mind, employment of cells that give rise to all the tissue types in our bodies in early development seems a logical and promising choice. Indeed, embryonic stem cells (ESC) derived from human blastocysts have been around since the end of the last century[18]. These pluripotent cells exhibited normal karyotypes, very high telomerase activity and expressed cell surface markers that characterize embryonic stem cells and not any early lineages. This scientific revolution was received with simultaneous healthy doses of scientific optimism and ethical skepticism. Human embryonic stem cells (hESCs) can, conceptually, give rise to cells in any somatic cell line. Differentiation of hESCs can be regulated by different culture conditions and growth factor[19, 20]. Animal studies using ESCs have demonstrated restoration of cardiac function but teratoma formation and immunological rejection will restrict the utility of this cell type, in addition to ethical considerations[21, 22]. Tumorigenic potential of ESCs persists in various differentiated stages regardless of cell population leading to teratoma formation[23] which clearly illustrates safety concerns associated with purportedly “differentiated” hESC-derived material intended for clinical application. Although hESC are the most primitive cell type, chromosomal instability has been reported in later passages of these

cells in culture[24]. Human induced pluripotent cells (hiPSC) are similar to embryonic cells in morphology, proliferation, surface antigens, gene expression, epigenetic status of pluripotent cell specific genes, telomerase activity[25] and cardiac potential[26]. Along with these attributes, hiPSCs also show similarities with hESCs regarding teratoma formation and tumorogenicity. In addition, incomplete reprogramming or accumulation of genetic abnormalities during the iPSC derivation process may render even autologous iPSC lines immunogenic[27]. iPSC co-culture studies with various cell types have revealed that until now, no cell type has been able to generate the cell type of interest with higher than 95% purity. Until the discovery of an accurate cell purification protocol, scientist are dealing with a heterogeneous cell population, which in turn raises the risks of cellular transdifferentiation and susceptibility to teratomogenesis. The dilemma of heterogeneity does not stop in the pre-differentiation stage. iPSCs know heterogeneous maturation stages[27, 28]. Cardiomyocytes driven from iPSCs can display different maturation levels[28]. This latter is of major concern, in particular, in delicate organs such as the heart and the brain where synchrony and structure is of fundamental importance. In summary, at the time of this review, clinical cardiac application of ESC and hiPSC populations must traverse a deep chasm that can only be bridged by harnessing overenthusiastic proliferative potential, gaining control over cell fate determination signals, and coping with issues of allogenic rejection for the ESC. As such, these cell types have yet to make an appearance in a clinical trial for treatment of heart failure.

Safety concerns over the utilization of ESC or hiPSC contrasts vividly with the lack of adverse events associated with adult stem cell therapy with cells derived from bone marrow or cardiac tissue explants. Although ontogeny of adult cardiac stem cells remains unresolved, collective findings from multiple laboratories validate the cardiogenic potential of these cells[29-32]. The presumption for the presence of tissue resident adult stem cells is their participation in normal cellular renewal due to consequences of aging over the lifetime of an organism. Therein lies the crux of the problem, since the resident adult cell population never evolved for rapid creation of new tissue in the wake of injury. The positive aspect of an adult stem cell's limited proliferative potential is the fact that not a single incidence of oncogenic transformation has been documented, and this distinction from their embryonic brethren has enabled clinical trials with adult stem cells to move forward. In the SCIPIO trial (cardiac Stem Cell Infusion in Patients with Ischemic cardiOmyopathy), cardiac stem cells are isolated from patients undergoing a coronary artery bypass grafting (CABG) procedure for autologous reintroduction following expansion in culture when they are percutanously infused into the scar tissue four months after CABG. Although the SCIPIO trial is mainly based on determining the feasibility and safety of harvesting adult cardiac progenitors for autologous reintroduction, there is also optimism toward obtaining functional hemodynamic improvement.

The good news is that clinical utilization of adult stem cells is a reality today and the results appear promising as well as safe. The caveat is that whereas embryonic or induced pluripotent cells possess an inherently youthful phenotype, heart failure patients who provide tissue for autologous stem cells isolation are usually above the age of sixty years and suffer from coronary occlusions, possibly multiple events, and previous cardiac procedures. Indeed, aging may be, in part, a "stem cell disease" characterized by the ravages of time upon the resident adult cell population that renders them increasingly stressed in the progressively dysfunctional tissue

environment of aging myocardium. Stem cells would be well suited for regeneration if they clung to the exuberance of youth while also maintaining self-control that comes with maturity.

Part 3: Getting older, not necessarily better

By the time we've made it, we've had it.

- Malcom Forbes

In adult mammalian tissue, stem cells participate in normal tissue homoeostasis through repair and regeneration upon damage[33]. Stem cell niches are profoundly affected by signals and growth factors from the local and systemic environment[34]. Thus, a younger niche is exposed to a different local milieu than an older or injured niche. Since normal regeneration is a function of local stem cell niches, the accretion of age–related changes such as DNA damage, impaired catabolism, altered epigenetics, and environmental stress prompt decline in stem cell function. In the process of DNA replication, alterations such as single- and double-strand DNA breaks, chromosomal translocations, telomere shortening, and single base mutations[35-37] can occur and lead to a process referred to as replicative cellular senescence[38-41]. In addition to replicative senescence, adult stem cells in the heart are susceptible to chronological aging, reflected by aggregation of damaged proteins, lipids and other macromolecules due to a decrease in cellular autophagy[42]. Inefficient catabolism leads to accumulation of dysfunctional organelles and cellular substructures over time, which in turn reduces quality and efficiency of cellular and molecular biological processes required to maintain homeostasis and survival[42-44]. As an organ matures, the well-orchestrated regulation of sequential expression timing and intensity for genes such as Wnt, Notch and Hedgehog in the stem cell pool can be epigenetically disrupted leading to changes cell progeny[45]. Disturbance of gene expression cascades into production of misprogramed daughter cell progeny that fail to maintain tissue structure and function. The accumulation of aberrant cells can be significant with advancing age, as predictive calculations reveal the entire myocyte compartment is replaced 15 times in women and 11 times in men from 20 to 100 years of age, meaning an average of 13 replications in 80 years. As an indication of repetitive rounds of replication, shortening of telomeres in the adult cardiac stem cell pool was paralleled by appearance of myocytes with

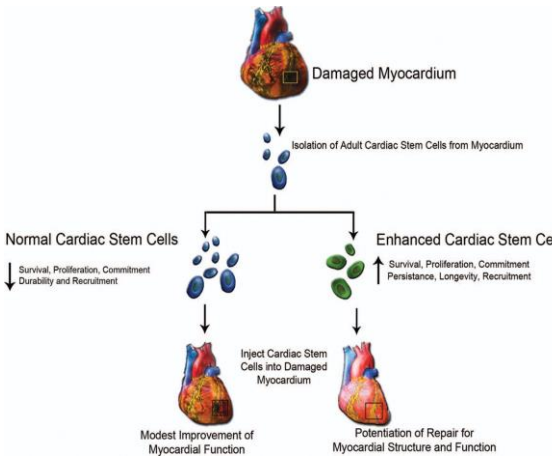

Figure 2. Adult cardiac stem cell requires empowerment. Schematic representation of enhanced cardiac stem cells (CPCs) and their potentiation for repair to damaged myocardium relative to normal CPCs.

severe telomere attrition[46] suggesting that older CPCs are the likely source for phenotypically old myocyte progeny. Last, but not least, in this cavalcade of detrimental insults are the exogenous stresses that stem cells endure in a pathologically compromised heart. For example, cardiac stem cells from a CABG patient have not only likely suffered from replicative and chronological aging, but have also been forced to persevere in a genotoxic environment of reactive oxygen species and chemical substances, promoting a process called *stress induced premature senescence*[44]. Stress induced premature senescence, in turn, leads to DNA damage and mitochondrial DNA destruction, which ultimately influences stem cell replicative capacity and progeny[44]. All cardiac patients suffer from hypertension and hyperactivation of the sympathetic nerve system. Chronic exposure of cells to angiotensin II through the renin-angiotensin-aldosterone system (RAAS) promotes premature senescence[47-50]. The majority of the target patient population for stem cell therapy suffers from sympathetic hyperactivity and carries a stem cell pool that is chronically exposed to the adverse repercussions of RAAS. The emerging paradigm of cellular senescence also portrays senescent cells as active participants in communicating their decrepitude by profoundly affecting their microenvironment in a paracrine fashion through an altered secretome that inhibits proliferation and modulates immune responses[51, 52]. These processes initiate a vicious circle of negative events on stem cell function and progeny, ultimately compromising the regenerative potential of the tissue as a whole.

Taken collectively, the evidence indicates that adult stem cells are unlikely to be equivalent in their regenerative potential. Moreover, the very target population of aged and infirmed patients destined to be at the forefront of interventional therapy also possess the most compromised stem cell population in terms of functional capacity and regenerative potential. Like so many biological problems, the solution is conceptually simple but fraught with technical challenges. Simply put, we would want to metaphorically “turn back the clock” on aged adult stem cells and empower them with the phenotypic characteristics of youthful vigor while not obviating their programming for context-dependent recognition of the environment and appropriate integration into the local environment in a salubrious fashion.

Part 3: May-December wedding between science and stem cells

You've got to go out on a limb sometimes because that's where the fruit is.

- Will Rogers

As researchers pursue the ultimate goal of therapeutic implementation for regenerative medicine, the journey slowly yields hard won fruits of knowledge gathered through innovation and creativity. Transformational ideas alter longstanding paradigms and redefine approaches to creating and delivering stem cells, but major issues concerning the therapeutic application of stem cells still remain unresolved. Success of adoptively transferred adult stem cells remains modest primarily as a consequence of three factors: poor survival, marginal proliferation, and limited functional engraftment / commitment within the host tissue. Adoptively transferred stem cells need to be primed against apoptotic, necrotic and hypoxic conditions prevalent within the damaged tissue. Furthermore, the aforementioned deterioration of proliferative capacity in old age adversely affects the stem cell regenerative capacity. Finally, if cells persist and even proliferate but are functionally incapable of appropriate lineage commitment and functional

integration, then the end result is a cell predisposed to oncogenic transformation. Therefore, combating a constellation of negative factors affecting stem cell mediated regeneration must be balanced against the need for restraint and appropriate participation in direct or indirect tissue repair. Threading this figurative "eye of the needle" is the purview of stem cell empowerment as detailed in the remainder of the review wherein current concepts, research efforts and problems associated with stem cell modification to enhance function are enumerated.

Survival. Poor survival and marginal retention of adoptively transferred cells into the pathologically challenged heart is widely accepted as a significant barrier to enhancing efficacy of regenerative therapy, and one doesn't need a doctoral education to appreciate that live cells do a better job of mediating biologically relevant effects than dead ones. And yet, researchers readily acknowledge massive losses of donated stem cells and failure to engraft in the damaged organ takes place within the first few days after delivery [53] [54]. If most of the effects we observe are mediated by cells that disappear within a week, then imagine the possibilities for enhanced repair if the donated population persisted for weeks, months, or even became incorporated permanently into the heart tissue? Clearly this is one of the front lines in the battle to enhance efficacy of adoptive transfer cell therapy. Stem cell survival is influenced by a number of factors such as ischemic conditions, inflammatory response[55] and quality of donor cells [56], and research has focused on enhancement of stem cell survival within host environment to augment repair.

"Preconditioning" in the context of stem cells refers to treatment with growth factors, hypoxic shock, or anti-aging compounds for augmentation of stem cell potency. Preconditioning promotes cyto-protection that enhances resistance stem cell survival against oxidative stress *in vitro* and *in vivo*[57] [58] [59] [60] as well as promotes migration and recruitment to ischemic myocardium [59] . Cytokines and chemokine preconditioning strategies augment stem cell recruitment to injured tissue after intra-cardiac delivery of erythropoietin[61], hepatocyte growth factor (HGF), or vascular endothelial growth factor (VEGF) [62]. Other growth factors used to enhance stem cell function included BMP-2, IGF-1 FGF-2[63], HGF, Hsp70 and atorvastatin[64] [65] [66] [67] [68]. Similarly, mesenchymal stem cells treated with hypoxia exhibit increased proliferation and differentiation[69] associated with pro survival[45] and pro angiogenic signaling[46]. The mechanistic signal transduction basis for these preconditioning effects promoting cell survival involves activation of PI3K/AKT and p42/44 MAPK signal transduction and activation of STAT3[55] as well as scenarios involving ERK1/2 expression[70]. Preconditioning can be initiated by multiple different cytokines that differentially influence downstream targets; therefore, multiple signaling pathways participate in mediating stem cell survival. The advantage of preconditioning is that the treatments are often simple, take advantage of cellular endogenous responses, and do not depend upon genetic manipulation that is time consuming and introduces foreign DNA into the treatment regimen. The duration of the protective response is a significant limitation of the preconditioning approach, as cell surface receptors are down-regulated, desensitized, or internalized in response to stimulation. Therefore, protection afforded by *ex vivo* preconditioning treatment prior to delivery will likely improve donated cell survival, but only by hours to days.

Alternative to preconditioning, genetic modification of stem cells to express pro-survival factors also enhances endurance of stem cells in the hostile environment of a pathologically damaged heart. Moreover, genetic manipulation allows for cells to serve as a source of growth factors that initiate intracrine, autocrine and paracrine effects, which augment activity of the donated population, endogenous cells, and their local environment. The candidates molecules employed

for genetic modification of cells include canonical mediators of cell survival in the context of cardiomyocytes or oncogenically transformed cells and will be briefly delineated in the next few paragraphs.

Apoptosis is a serious threat faced by transplanted cells into a hostile environment, so modifying stem cells to circumvent apoptotic signaling increases cell survival. The Bcl-2 protein family regulates caspase activation and mitochondrial integrity through dual actions of anti- and pro-apoptotic members. Bcl-2 engineering of mesenchymal stem cells increases survival after acute myocardial infarction[71]. Bcl-2 modified mesenchymal stem cells ameliorated LV remodeling and improved LV function. Exogenous delivery of Bcl-2 in MSCs activates a survival pathway that is sufficient to suppress hypoxia induced apoptosis[71] and adenoviral Bcl-2 transgene expression attenuated early donor cell death in cardiomyoblast transplantation[72]. Heme oxygnase-1 (HO-1) is an anti-apoptotic stress-inducible enzyme with anti-oxidant cytoprotective activity under ischemic conditions[73]. Overexpression of HO-1 in mesenchymal stem cells promotes angiogenesis and reduces fibrotic area [73] after transplantation in ischemic myocardium. Transplantation of survivin-engineered mesenchymal stem cells also enhanced cellular survival after transplantation [74]. Similarly, other survival molecules including SDF-1[75], Ang-1[76] and CXCR4[77] significantly improve survival of transplanted cells.

This approach has proven successful with mesenchymal stem cells expressing myristolated AKT that augments heart function resulting in significant infarct size reduction[78] and inhibition of ventricular remodeling 72 hrs after transplantation[79] despite the fact that donated cells did not significantly contribute to formation of new myocardium [80]. Paracrine effects of these AKT-expressing modified cells were postulated to play an important role in protection, with identification of genes including VEGF, FGF-2, HGF, IGF, and notably thymosin β4 that complexes with PINCH and integrin-linked kinase (ILK) resulting in the activation of AKT within cardiomyocytes of the border zone. Secreted frizzled related protein 2 (Sfrp 2) was also identified as a key paracrine factor mediating myocardial survival and repair after ischemic injury since protection of injured myocardium by AKT-modified mesenchymal stem cells was lost following suppression of Sfrp2 [75].

Proliferation. Another important factor for consideration to improve the efficacy of cellular therapy is to augment the rate of proliferation of transplanted adult stem cells, which leads to persistence and expansion of the donated cell population and increases the number of cells available for engraftment. Combined with enhanced survival, increasing proliferation can serve as a powerful combinatorial approach to expand the impact of donated stem cells, as shown in studies using cardiac progenitor cells modified to express Pim-1 kinase[32] [81] Similarly, over expression of nucleostemin in cultured cells cardiac stem cells increased proliferation accompanied by preservation of telomere length[83]. However, an important caveat is that enhancing proliferation at the expense of lineage commitment and functional engraftment may not provide significant long term benefits, as was the case when cardiac progenitor cells were modified to express nuclear-targeted Akt resulting in expansion and persistence of the donated cells[84]. This study points out the importance of balancing the trifecta of desirable stem cell properties judiciously, as the optimal outcome can only be effected when appropriate cell phenotypic properties accompany enhanced survival, proliferation, and commitment to cardiogenic fate.

Commitment. Ideally, donated stem cells will ultimately participate directly in repair of damaged tissue by becoming new myocardium through synthesis of *de novo* myocytes, vessels, and

endothelium. Regulatory pathways involved in embryonic stem cell differentiation to cardiomyocytes provide insight into how such cell fate decisions might be controlled and influenced[85-87]. A commonly employed pharmacologic strategy to promote differnetiation is exposure to the DNA demethylation reagent 5-azacytidine as performed upon mesenchymal stem cells, bone marrow derived stem cells [88-90], or cardiac progenitor cells[91]. Long term stimulation of cardiac stem cells with TGF-β1 also favors acquisition of a cardiomyocyte phenotype[92]. Such approaches are unlikely to have significant clinical implications due to regulatory concerns about the effects of such treatments upon stem cells, but examining molecular processes induced by such treatments facilitates unraveling the pathways involved in optimizing cardiac differentiation of transplanted cells. An interesting alternative approach is the delivery of cardiac transcription factors as chimeric proteins fused to cell penetrating peptides to promote differentiation into cardiac phenotypes[93, 94]. Paracrine factors secreted by adoptively transferred mesenchymal stem cells may play an important role in orchestrating recruitment and lineage commitment of endogenous responses by promoting vasculogenesis and inhibiting apoptosis via vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), Hepatocyte growth factor (HGF), Angiotensin- (Ang-1), GATA4, or angiopoetin [76, 95-98]. However, at present the most intriguing multifaceted player in the signaling cascade of cardiac stem cell myogenic determination is Notch, which regulates commitment[99] as well as survival[100] . Notch is also a key regulator in smooth muscle differentiation as noted in epicardium derived cells [101]. Therefore, manipulating stem cells with Notch seems a likely avenue for enhancing stem cell commitment and persistence. Similarly, GSK3-β induce cardiomyocyte differentiation, myocardial injection of bone marrow mesenchymal stem cells over expressing GSK3-β induce cardiomyocyte differentiation and angiogenesis [102].

*Rejuvenation***.** One additional consideration alluded to earlier in this review is the problem of stem cell exhaustion due to aging. Autologous cell therapy on an aging target population will likely be hampered by the biological limitations of endogenous stem cells and the advent of senescence in the myocardial cell pool. Ideally, empowering the explanted stem cell population requires attention to antagonizing senescence and "turning back the clock". While relatively little has been accomplished in the myocardial context, there are signaling pathways that seem connected to reversing the passage of time. For example, experimental activation of Notch restored "youthful" myogenic responses to satellite muscle cells isolated from 70-year-old humans rendering them similar to cells from 20-year-old humans[103]. Declining proliferation in hepatic progenitor cells has been ascribed to formation of a complex involving cEBP-a and the chromatin remodelling factor brahma (Brm) that inhibits the regenerative capacity of aged liver[104]. The mTOR pathway has been studied in the context of hematopoietic stem cells where rapamycin increased life span and restored self-renewal and hematopoiesis in aged mice, implicating mTOR signaling in aging and showing the potential of mTOR inhibitors to restoring hematopoiesis in the elderly[105]. Manipulation of telomere-telomerase axis was suggested in 1998 when two different human cell lines; retinal pigment epithelial cells and foreskin fibroblasts were transfected with vectors encoding for human telomerase catalytic subunit. Overexpression of telomerase resulted in elongated telomeres, vigorous cell division and reduced expression of senescence markers[106]. Allsopp et al. demonstrated a marked increase in the level of telomerase activity in antigenic stimulated T-cells derived from serially transplanted HSCs. The increase in telomerase activity

resulted in an elongation of the telomeres to a size similar to that observed in T-cells isolated from young mice[107].

Genetic modulation for guidance and trafficking. Stem cell homing through injured myocardium represents another relevant key facet for furthering stem cell based regeneration for both donated as well as endogenous cell populations. Multiple molecular players are involved in the journey from a niche or injection site to the battleground of border zone or infarct region. Adhesion molecules such as integrins [108] as well as proteases work in concert to facilitate migration of stem cells through damaged tissue. Several integrins have been identified on stem cells and found to be involved in the recruitment, mobilization and homing of stem cells to the site of injury [109, 110]. Directional motility for mesenchymal stem cells was enhanced by engineered expression of the SDF-1/ CXCR4 axis [111]. Similarly, endothelial progenitor cells recruitment into injured myocardium is promoted by CD8/ ICAM after myocardial infarction[112]. Similarly, MCP-3-CCR1/2 axis also demonstrated increase stem cell homing a month later myocardial infarction – this is an incomplete thought / sentence[110]. Selected proteases and their inhibitors that influence stem cell trafficking have been touted as candidates for genetic modulation such as PAI-1, a protease inhibitor that blunts trafficking of mobilized CD34+ bone marrow cells and influences ventricular remodeling [109]. Similarly, endothelial nitric oxide synthetase (eNOS) activity increases MMP-9 leading to enhanced stem cell homing after acute myocardial infarction [113], with enhanced eNOS transcription promoting SDF-1 mediated stem cell migration [114]. Thus, engineering of stem cells to induce expression of stem cell mobilization and homing factors can augment recruitment and retention of prodigal stem cells in their effort to find the right place to exert their reparative effects.

Collectively, the information in this section of the review shows that modification of adult stem cells can adopt many forms and vary in method of implementation, but always shares the singular goal of enhancing regeneration. Optimization of stem cell modification will depend upon an approach or combination of approaches that maximizes all aspects of the regenerative process encompassing survival, proliferation, trafficking, lineage commitment, and functional engraftment. Published results using Pim-1 kinase support the premise that engineering of stem cells is a viable option to enhance the reparative process, and Pim-1 is unique among the molecules used thus far as a combinatorial mediator of enhanced survival, proliferation, lineage commitment, and functional engraftment (Muraski, Borillo, Cottage, Fischer Circulation, Fischer JMCC review). Some might argue that using such powerful molecular interventions with adult stem cells is going out on a limb and taking a risk, but cellular reprogramming by genetic engineering yielded inducible pluripotency that is unquestionably one of the greatest advances of stem cell biology. And, just like inducible pluripotent cells, the challenge is not in seeing the destination for where adult stem cell engineering needs to go, but rather how to get there as quickly and safely as possible.

Part 4: Clinical implementation and the challenge of stem cell empowerment

If you find a path with no obstacles, it probably doesn't lead anywhere.

- Anthony Michael Hall

The primary hurdle in empowering stem cells for clinical application does not rest primarily with lack of knowledge on molecular mechanisms and pathways, but rather how best to deliver the engineered solution to the stem cell population in an acceptable and feasible solution. Traditional gene delivery relies upon recombinant protein expression through viral vectors which possess the inherently desirable characteristics of easy cell delivery of the engineered construct, use of replication deficient vectors and cell-type specific vectors to limit spread and target delivery, and the persistent expression of introduced genetic material by incorporation into the genome or maintained episomal presence in non-dividing cells. Adenoviral, adeno-associated, lentiviral, and retroviral vectors are widely employed for gene delivery in the experimental setting and to a limited extent in clinical trials[115-119]. Each type of delivery vector has a different set of strengths and weaknesses in the context of empowering stem cells.

Lentiviruses of the Retroviridae family have efficiency of myocardial transfection is similar to adenoviruses but with longer duration of gene expression. Lentiviruses have the ability to infect non-dividing cells, whereas retroviruses express in a proliferative cell population. The primary advantage of lentiviral and retroviral-based engineering is the persistent incorporation of the viral genome (and with it the gene of interest) into the host genome so that the genetic modification can be selected for and propagated in daughter cell progeny. Although incorporation of the transgene into the host cell genome makes these vectors an excellent choice for engineering cells, risk of insertional mutagenesis and difficulty in regulating expression of the introduced gene limits utilization of these vectors in the clinical setting. Ongoing research is focused upon addressing these issues[120-123] in an effort to make the lentiviral and retroviral vectors more palatable to regulatory agencies.

Adenoviruses deliver their genomes to the nucleus of both dividing and non-dividing cells, are relatively cheap to produce in high titers and have a broad tropism to target cells especially within the cardiovascular system, which makes them widely used in myocardial gene therapy[124] However, virus-specific cellular immune responses eventually lead to destruction of the adenoviral genetically modified cells[125] that can provoke adenoviral-induced myocarditis[126]. As such the temporal expression of adenoviral-encoded proteins is relatively short lived (10-14 days). As of May 2001, 532 adenoviral gene therapy protocols had been approved for evaluation in clinical trials conducted predominantly in oncologic patients; however, only five of these trials had been evaluated in phase III testing. Multiple side effects including fever, chills, shivering, myalgias and even death were reported in these clinical trials[127]. As long as the inherent problem of high immunogenicity of these vectors remains unsolved, their production and application will remain restricted essentially to experimental and academic purposes.

The contemporary virus of choice is the adeno-associated virus. Adeno-associated virus (AAV) is a member of the parvovirus family, a single-stranded DNA virus that requires a helper virus such as adenovirus or HSV for replication. Although wild-type AAV is able to infect non-dividing human cells and stably integrate into a specific locus on chromosome 19[128] no pathologic consequences have been reported with infection by AAV. The many distinct AAV serotypes bear the advantage of increased tissue-specific tropism[129]. AAVs have been employed in numerous clinical trials[130-133] including treatment of heart failure by increasing cardiac myocyte contractility in 2007[134]. However, on the downside, AAV vectors carry a small and restricted amount of DNA

(low capacity), are more challenging to produce in high titer than other viral vector types, and their viral backbones render them susceptible to gradual epigenetic modification.

Development of minicircles for gene delivery show promise as a viable option for DNA delivery in engineering of stem cells[135-137] Minicircles are episomal DNA vectors produced as circular expression cassettes devoid of any bacterial plasmid DNA backbone. Their smaller molecular size enables more efficient transfections and offers sustained expression over a period of weeks as compared to standard plasmid vectors that only work for a few days. By virtue of the production methodology in minicircle creation, the expression plasmid no longer contains the bacterial origin of replication or the antibiotic resistance markers. Thus, delivering only the minicircles to cells lengthens the expression of the transgene over traditional transient transfections of plasmids. For dividing cells, expression of the minicircles lasts up to 14 days. For non-dividing cells, expression drops slightly after the first week, but then can continue expressing the transgenes for months. The lack of a bacterial backbone, the small size of the vector, potential expression duration of months, lack of genomic integration, and low cost of production make this delivery technique superior to viral delivery methods for *ex vivo* gene delivery involved in autologous stem cell modification. Moreover, the ability to produce minicircle vectors in bacterial expression systems devoid of animal by-products such as serum together with the ability to perform high quality good manufacturing practice to control for batch-to-batch quality makes them attractive from a regulatory perspective.

Epilogue

By prevailing over all obstacles and distractions, one may unfailingly arrive at his chosen goal or destination.

- Christopher Columbus

Success in the future of stem cell therapy for currently incurable conditions rests primarily in maintaining the unshakable faith espoused by Columbus. Advances in the field of regenerative medicine are coming fast and furious, both in the figurative and literal sense of those words. Controversies and disagreements are to be expected in any endeavor as complex and perplexing as stem cell research, especially in view of the high stakes placed on funding the “magic bullet” that will hit the target of clinically relevant intervention. This review has focused predominantly upon adult stem cells simply because from a clinical perspective that population is far more advanced than ESC or iPS cell types, not because adult-derived cells are the “best” type of cell. With decades of successful bone marrow reconstitution procedures now commonplace in hospitals it is clear that adult stem cell therapy works, but can it work in a structurally complex tissue such as the heart where proliferative activity is so limited? That question lies at the root of a massive international effort to understand the signals and cues necessary to coax stem cells into functionally relevant cardiac engraftment now entering the second decade of study.

The journey to a New World of regenerative medicine has been phenomenally productive as evidenced by a quick scan of the more than 7,500 references available today in a PubMed search for the keywords “cardiac, stem cell, heart”, with almost 1,800 of those references being review articles to summarize our current understanding. All this for a field of research that was

essentially non-existent a little over a decade ago. In view of this overwhelming body of literature it seems pointless to debate whether cardiac regeneration occurs, as that question has now been asked and affirmatively answered in lower vertebrates[138] the neonatal heart[139] and even in adult hearts in response to injury[140]. While scientists in the laboratory benches unravel molecular pathways and mechanistic basis for curing the basis of heart disease, experts at bedside think in terms of individual patient indications, specific disease stages and co-morbidities. Zealous advocates and confirmed skeptics agree that the endogenous regenerative potential in adult human myocardium alone is not capable of mediating recovery from acute pathologic injury or long-term chronic stress. Toward that goal, empowering normal biological process of regeneration by potentiating stem cells to enhance repair works and provides improvement that is both structurally measurable and clinically relevant over non-primed cells. Thus, next question to be asked and answered is whether such enhancement can be done safely, reproducibly, and efficiently. Many alternatives have been presented in this review, yet we are still on the tip of the proverbial iceberg in terms of the possibilities and their implementation. We are in the Golden Age of translational stem cell research and achieving our shared goal of translational implementation looks more promising today than at any time in history.

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Chapter 8

Summary & Future Perspective

Summary

Myocardial regeneration appears to be a multifaceted endeavor. Both myocytes and Cardiac Stem Cells (CSC) were shown to have the fundamental potential of forming new myocytes. However, experience in the area of CSC as well as myocytes unravel additional layers of complexity. Myocyte division efficiency, at this point, is extremely poor at baseline as well as upon manipulation. In addition, the myocyte cell cycle field is rather scattered, where definitions and hypothesis are not consistent and no consensus has been reached as far as 'proliferation' versus processes such as hypertrophy and binucleation. Myocyte function and in particular proliferative potential is affected by age-related adverse adaptations and premature senescence due to previous cardiac events. The propagating senescence course of action is detrimental to the heart and requires remedy, perhaps prior to modulation of the system for regeneration. The good news is that more and more proteins are characterized as targets for reversal of senescence and "priming" of the myocardial environment, including Nucleostemin and Pim-1. Considering the developments in the field of gene therapy and clinical application of gene therapeutic targets, reversal of senescence seems more feasible now than before. Disruption of the senescence vicious cycle is crucial for Stem Cell-mediated regeneration. Stem cells are naïve cells that are reliant on signals and directions from the environment. A damaged environment will, conceivably, not be hospitable to these primitive cells. This will have consequences, not only for the patients involved, but also for the progression of the cardiac stem cell field. Currently, the field is susceptible and overly absorbent of findings and conclusions from clinical trials. Application of stem cells in a harmful environment does not necessarily indicate the lack of functionality of the cells since they may be very effective in a different patient population.

Part I. Myocyte cell cycle requires rigorous studies on distinct phases of the cell cycle. The cardiac specific FUCCI-system is an increment in deciphering details and specifics of a myocyte cell cycle. Geminin and Cdt1 are expressed in myocytes during early postnatal life and decrease upon maturation. FUCCI constructs correspond to other markers of the cell cycle, specifically to distinct cell cycle stages. Geminin and Cdt1 levels correspond to earlier findings in the literature regarding binucleation and increase in ploidy. In addition, cardiac specific FUCCI system allows detection of quiescent myocytes. 'Quiescent myocytes' has been a neglected population in the past. Unlike senescent cells, quiescent myocytes provide a platform for myocyte division, since the cells are preprogrammed to form myocytes and are capable of re-entering the cell cycle. The final results regarding the number of quiescent cells versus cells in G1 and S/G2/M are probably going to be consequential for future interventions towards myocardial regeneration. Current and future studies using the FUCCI system will provide insight in the number of quiescent cells, thereby defining a potential target population for enforcing division. Furthermore, FUCCI system can be utilized as a read-out system where the outcome of manipulations of myocyte cell cycle can be tracked and studied on a 'cell cycle-phase' level thereby providing understanding of mechanisms and ultimately contributing to reproducibility and consistency of interventions in future myocytes mediated regeneration.

Part II. Myocyte senescence can be triggered by a plethora of triggers such as myocardial infarction[1], prolonged over-activity of the adrenergic system[2] and increased mTORC1

activity[3]. This assembly of factors covers a major cardiac patient population. Thus, senescence is no longer an “aging” or luxury problem; it has maneuvered to a legitimate global concern. Occurrence of senescence in myocytes is a downhill process where healthy myocytes declare solidarity and respond to the senescent secretome by turning senescent and dragging the entire environment to a detrimental place. Pim-1 has been shown to play a role in reversal of senescence through elongation of telomeres, increasing mitochondrial biogenesis, inhibiting apoptotic cell death and proliferation of stem cells [4-7]. Pim-1 has been shown protective both in myocytes, using transgenic mouse models, and cardiac progenitor cells, using a lentiviral mediated overexpression system[4-7]. Similarly, Nucleostemin is a pro-proliferative protein that plays a role in maintaining pluripotency[8-10]. Nucleostemin is expressed in myocytes early after birth and decreases precipitously upon maturation. Thereby, indicating a role for Nucleostemin in myocyte proliferation during early life. Upon myocardial infarction and in response to pressure overload, Nucleostemin is expressed in myocytes surrounding the damaged area and in the myocytes around the vasculature in a pressure overload model. Unlike other fetal genes and embryonic markers that are expressed in response to damage, the expression of Nucleostemin is restricted to the myocytes in proximity of damaged areas.Thereby, indicating a peculiar role of Nucleostemin in borderzone myocytes in the face of pathology. Recently, the mechanism of pluripotent markers in borderzone myocytes was described and attributed to Oncostatin[11]. Pluripotent marker expression in the borderzone indicates a temporary protective state of dedifferentiation that is beneficial in the short run, but is associated with dilated cardiomyopathy on the long run[11]. Interestingly, while Nucleostemin is expressed solely in the borderzone after infarction, myocytes in tropomodulin-overexpressing transgenic mice, a cardiomyopathic model, reveal high levels of Nucleostemin expression throughout the entire myocardium. Nucleostemin has been shown to delay cellular senescence by negatively regulating trf1. In addition, p53 has been shown to be antagonized by physical interaction with Nucleostemin[8-10]. Although, the mechanism for Nucleostemin upregulation in the heart remains unknown, the compensatory upregulation of this pluripotent marker might be an attempt to prevent further trf1 and p53-mediated deterioration and senescence mediated apoptotic cell death.

Part III. While major progress is made in the field of Stem Cell mediated regeneration, discussions regarding the optimal cell type have not ceased. One of the main hurdles in Stem Cell mediated regeneration is a lack of solid proof for new myocyte formation in patients. However, so far the improvements in cardiac function and scar reduction appear to be sustained at 2 years of follow-up. While a select group of patients are being served successfully, the inclusion and exclusion criteria of current clinical trials are very rigid[12-14]. This raises questions regarding broad applicability of these interventions to a general cardiac population. Severe heart failure patients, who are probably the most eligible for myocardial regenerative therapy, are by design excluded from current trials. It is speculated that stem cells from severely damaged hearts of patients undergoing a Left Ventricular Assist Device placement, are probably more hampered and less capable of successful regeneration. That is a major concern and requires remedy when true. Multiple pathways and proteins are described so far that are beneficial in stem cell behavior and can be used as “rejuvenation/vitalization” targets for diseased stem cells. Revitalization of these stem cells will end up requiring a patient specific

approach of characterization and manipulation of stem cells before reapplication and seems rather far away. However, it is worth mentioning that myocardial regeneration is not a solitary option in helping a cardiac patient. Severe heart failure patients may benefit from developments in the field of bone marrow derived stem cells transplantation, where significant functional improvements are achieved. Although the benefits are not persistent, the increase in quality of life might just be what a severe heart failure patient calls for.

Future Perspectives

Overall, the field of cardiac regeneration promises a bright future for cardiac patients. Stem Cell mediated improvements in cardiac performance have been brought to realization as evidenced by three currently ongoing clinical trials[12,13,4]. However, several challenges remain unresolved and require investigation in the coming years. Cardiac patient population that requires cell therapy is variable on multiple levels, from age to gender, exposure to risk factors, and history of previous cardiac events. These factors play a role in general “cardiac health” and presumably influence the quality of Cardiac Stem Cells as well. Since the current Cardiac Stem Cell clinical trials are based on a very narrow range of cardiac patients, extrapolation of the findings from these trials becomes challenging and requires caution in interpretation and enthusiasm. Future steps towards broadening this patient population requires patient specific “quality assessment” of the Cardiac Stem Cells. Ageing and senescence are examples of processes that can be studied and modified in these cells. Future protocols require not only knowledge of Stem Cell ageing but ways of manipulating these Stem Cells towards empowering and revitalizing, thereby generating a healthy young cell population that carries the capability of proliferation, survival and differentiation to promote new cell formation upon injection into the heart. An additional future step in Cardiac Stem Cell therapy remains reduction of common divergence in the field as far as isolation protocols, markers used for Stem Cell characterization and the source of these primitive cells in the heart. So far, different markers have been used in isolation of Cardiac Stem Cells and all groups provide convincing evidence for regeneration in animal models. However, in order for the field to move forward, rigorous comparison studies of “different” Cardiac Stem Cell populations are necessary and called for. Last but not least, the efficiency of Stem Cell mediated cardiac regeneration remains poor. Although more and more sophisticated delivery techniques are being developed, the retention and survival of Stem Cells remain discouragingly low. This major hurdle remains despite major research efforts. An important future step may be, involving and involvement of the field of cardiac surgery. Cardiac surgeons appear somewhat under-represented in the field of cardiac Stem Cell therapy, while that is a group of technical physicians who work on development of surgical techniques regularly and approach the heart from a hands-on interventional eye on a daily basis. A multidisciplinary approach where knowledge of cell biology, physiology and surgical technicality come together may provide a very promising platform for improving Stem Cell-mediated regeneration.

The future of myocyte mediated myocardial regeneration is an issue of the future. In the current state-of-the-art, myocyte division requires basic insight of *myocyte cell cycle duration and progression*, processes that are unknown so far. Premature studies of manipulating myocyte division may appear glamorous and promising at first, but will likely backfire on the long run

where issues of consistency and reproducibility become relevant in a patient-related context. For a hopeful long-term future, the current field of *myocyte-mediated regeneration* must be transformed to the *field of myocyte cell cycle*, where men focus on the basics of the system rather than being overly ambitious just too early. Although hypotheses are worshiped, successful division of myocytes in the future requires current non-hypothesis- based descriptive systems such as reporter animals and constructs in which the dynamics and oscillation of biological processes are elucidated. Only then, can myocyte-mediated regeneration be considered as a future perspective.

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