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Mechanisms of Cardiac Repair and Regeneration

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Abstract

Cardiovascular regenerative therapies are pursued on both basic and translational levels. Although efficacy and value of cell therapy for myocardial regeneration can be debated, there is consensus that profound deficits in mechanistic understanding limit advances, optimization, and implementation. In collaboration with the Transnational AllianCe for Regenerative Therapies In Cardiovascular Syndromes (TACTICS), this review overviews several pivotal aspects of biological processes impinging upon cardiac maintenance, repair, and regeneration. The goal of summarizing current mechanistic understanding is to prompt innovative directions for fundamental studies delineating cellular reparative and regenerative processes. Empowering myocardial regenerative interventions, whether dependent upon endogenous processes or exogenously-delivered repair agents, ultimately depends upon mastering mechanisms and novel strategies that take advantage of rather than being limited by inherent myocardial biology.

Keywords

heart; regeneration; repair; mechanism; stem cell; proliferation; signaling

Introduction

Myocardial repair and regeneration is an important area of research motivated by increasing occurrence and expanding distribution of heart failure and cardiac-related diseases. Desperate unmet need for novel interventional strategies to treat cardiovascular disease prompted rapid implementation of clinical approaches to promote myocardial regeneration

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M.A. Sussman and K.M. Broughton have a significant interest in CardioCreate, Inc.

and repair, but outcomes thus far have been modest at best. To promote comprehensive discussion of cardiovascular regenerative medicinal products, an international research collaboration group was formed known as TACTICS (Transnational AllianCe for Regenerative Therapies In Cardiovascular Syndromes). TACTICS recently produced a consensus statement prelude to topic-specific manuscripts asserting need for a thorough understanding of mechanisms involved in myocardial repair and regeneration¹. Serving this purpose, we offer an overview of key mechanisms involved in cardiac repair and regeneration including: 1) survival and protection, 2) inflammation reduction, 3) cell-cell communication, 4) angiogenesis/vascularization, 5) cardiomyogenesis, 6) molecular regulation of proliferation and cell cycle, and 7) cardiac aging associated with impairment of reparative and regenerative potential. These mechanisms act both independently and collectively to influence cardiac regeneration (Figure 1). Clearly there is still much that we do not understand, significant obstacles to be overcome, and boundless room for improvement. Nevertheless, amazing progress has been made in a relatively short time and the excitement that myocardial regenerative research has created will undoubtedly continue.

1. Survival and Protection

Myocardial preservation post-injury and throughout remodeling mitigates long-term damage and cardiac dysfunction. Myocardial infarction (MI) affects left ventricular structure associated with loss of functional tissue leading to complications of ischemic (angina, additional infarctions, infarct extension), mechanical (heart failure, cardiac hypertrophy, valve dysfunction, aneurysms and cardiac rupture¹, or arrhythmic (ectopic heart rhythm, sinus node dysfunction and atrioventricular node dysfunction²⁻⁵) nature. Preservation of tissue and cellular function is imperative to maintain functionality of the heart, with multiple signaling pathways involved that influence survival.

Cardiomyocyte survival is critical for preservation of myocardium for ongoing hemodynamic performance and limitation of remodeling with concomitant fibrosis and scarring. Multiple signaling pathways are involved in cardiomyocyte survival⁶⁻⁸ that operate through mechanism associated with inhibition of senescence, reduction of inflammation, allowing for generation of new myocytes, and angiogenesis and vascularization of healthy cardiac tissue. One of the most extensively studied survival signaling pathways focuses upon Akt, a Ser/Thr kinase with three known isoforms in mammals (Akt1/PKB α , Akt2/PKB β , Akt3/PKB γ) activated by several well-characterized growth factors, cytokines, mitogens and hormones⁹. Akt signaling pathways promote cardiomyocyte survival in zebrafish¹⁰, mice¹¹, rat¹², rabbit⁹, canine¹³, swine¹⁴ and in human cells¹⁵. Akt induces multiple downstream targets in cardiomyocytes including Phosphoinositol 3-Kinase (PI3K) via Ras or ERK1/2¹⁶, mTOR¹⁷, v-Raf1 Murine Leukemia Viral Oncogene Homolog-1 (Raf1)¹⁸, CAMP Response Element-Binding Protein (CREB)¹⁹, Cyclin Dependent Kinase Inhibitor p21 (p21CIP1)²⁰, forkhead box O (FoxO)²¹, proto-oncogene proviral integration site for moloney murine leukemia virus (PIM) kinase -1 (PIM-1)²², insulin-like growth factor 1 (IGF-1)²³, but even this accounting falls short of a comprehensive list as previously reviewed²⁴⁻²⁶. Clearly Akt serves as a nodal master regulator on multiple levels of cell biological activity including survival.

Cardiomyocyte survival after initial injury is also enhanced through autophagy by activation of BNIP3, regulating opening of the mitochondrial permeability transition pore (MPTP)^{27,28}. Ischemia initially increases the ratio of AMP/ATP, resulting in activation of AMP activated protein kinase (AMPK) along with inhibition of mTOR activity⁸ to stimulate autophagy while Beclin-1 initiates autophagy during reperfusion^{29,30}. Beclin-1, autophagosome formation, and autophagic flux are increased by ischemia/reperfusion and oxidative stress in mice³¹. Beclin-1 is inhibited by the anti-apoptotic protein Bcl-2, which contributes to regulation of cell survival:cell death signaling ratio after initial injury³².

Along with autophagy, anti-apoptotic signaling promotes cardiomyocyte survival. Apoptosis peaks within hours after an ischemic event and is induced by both extrinsic and intrinsic pathways³³. For example, Tumor Necrosis Factor alpha (TNF- α) is an extrinsic molecule increased by congestive heart failure³⁴ as part of the inflammatory response (discussed below). TNF- α activity increases by immune cell release of the stress protein high-mobility group box 1³⁵ that amplifies apoptotic effects in cardiomyocytes. Intrinsically, pro-apoptotic Bcl-2-associated-X protein (BAX) and BH3-only proteins may amplify the apoptotic cascade to destabilize the outer mitochondrial membrane and release additional pro-apoptotic molecules to activate caspases³⁶. Suppression of BAX and p38 mitogen-activated protein kinase phosphorylation increases Akt-Bcl-2 and reduces apoptosis in the ischemic myocardium³⁷. Another important regulator of cardiomyocyte apoptosis is p53, such that reduction in p53 levels decreases apoptosis and promotes myocyte survival during ischemia³⁸ and complete deletion of p53 also decreases apoptosis in cardiomyocytes after myocardial infarction³⁹. Despite decades of cumulative, compelling, and often elegant detailed studies the topic of anti-apoptotic signaling as related to cardiomyocyte survival and preservation of function continues to be an actively studied and central theme of myocardial biology^{6-8,40,41}.

2. Inflammation Reduction

Inflammation naturally occurs in response to injury and is prerequisite for remodeling, regeneration and scar formation. Infarction injury triggers inflammation extending myocardial damage, triggering healing and remodeling responses. Inflammatory responses in injured myocardium include mononuclear cell infiltration, interleukins that modulate inflammation, mast cell accumulation in the healing scar, fibroblast and ECM remodeling, and temporal regulation of angiogenesis in the evolving and subsequently healing infarct. Cardiac healing involves multiple waves of interstitial cell types, initiated with infiltration of mononuclear cells and mast cells responding to multiple cytokine and growth factors including C5a, TGF β 1, MCP-1, IL-8, histamine, TNF α , IL-6 and ICAM-1⁴². After monocyte recruitment to infarcted regions, these cells mature and differentiate into macrophages driven by cytokines including macrophage colony-stimulating factor (M-CSF)⁴³. Lymphocytes in ischemic and reperfused myocardium express IL-10, contributing to the healing process by inducing TIMP-1 expression⁴⁴. IL-10 simultaneously suppresses inflammation through inhibition of multiple cytokines (IL-6, IL-8, IL-12, TNF α , IL-1 α and IL-1 β). Mast cell accumulation in fibrotic regions of infarction⁴⁵ is stimulated by chemoattractants including SCF, PDGF, VEGF and β FGF⁴⁵⁻⁴⁹. Myofibroblasts accumulate within a week after infarction in the ischemic and border zone with structural and

phenotypic characteristics of smooth muscle cells⁵⁰. Myofibroblasts express a homologue of frizzled 2 and are involved with spatial control of cardiac healing to thereafter undergo apoptosis in the mature scar⁵¹. Inflammatory response factors, such as VEGF, IL-8 and β FGF promote angiogenesis in the healing myocardium⁵².

Cellular mediated inflammatory response includes neutrophil infiltration in the reperfused myocardial infarct region, neutrophil–endothelial interactions, neutrophil rolling and activation of selectins, leukocyte β 2 integrins, chemotaxis and chemokines. Neutrophils release oxidants, proteases, and autacoids that collectively trigger additional cell recruitment^{53,54}. The large and stiff morphology of neutrophils is thought to promote adherence to capillary endothelium and impair reperfusion. Neutrophil rolling leads to upregulation of selectin family members including cell-surface molecules L-selectin, E-selectin and P-selectin^{55–57}. L-selectin is expressed in various circulating cell types (e.g. neutrophils, monocytes, T cells and B cells) and is released from the surface of these activated cells⁵⁵. E-selection is expressed following activation of endothelial cells by cytokines such as TNF α or IL-1 β ^{56,58}. P-selectin is induced by agents such as thrombin, histamine, complement fragments, and oxygen-derived free radicals⁵⁷. Integrins mediate selectin-dependent adhesion of neutrophils, particularly through activation of β 2 integrins⁵⁹. Neutrophils are also attracted to Interleukin-8 (IL-8)⁵² and sequential release of chemoattractants such as C5a in the interstitial fluid after IL-8 production⁶⁰.

Although neutrophils are attracted to and activated in the ischemic and reperfused myocardium to assist in recovery, neutrophils can perpetuate damage in the injured myocardium. Neutrophil-induced myocardial injury, opposed to neutrophil assisted repair, occurs from adhesion-dependent cytotoxicity and ICAM-1 activation⁶¹. ICAM-1 mRNA circulates in both normal as well as ischemic regions in the heart; however, ICAM-1 protein resides in ischemic tissue areas approximately 3-6 hours after injury^{62,63}. High levels of ICAM-1 mRNA in the viable border zone region of an infarction is also where highest amounts of neutrophil infiltration occur. Likewise, in the ischemic region where ICAM-1 mRNA expression is elevated, IL-6 mRNA expression is also elevated. IL-6 serves as a nitric oxide-dependent cardiac depressant associated with stunned myocardium⁶⁴. As inflammation and damage progresses with cellular infiltrates, the stage is being set for transition toward myocardial healing.

Humoral inflammatory responses include complement activation, increased ROS, and multiple cytokines. Complement cascade activation (C1, C2, C3 and C4) occurs through cardiomyocyte necrosis and release of cardiolipin⁶⁵. Interference with the complement system, such as infusion of human complement receptor type 1 (sCR1) decreases infarction size⁶⁶ and may reduce myocardial injury. ROS inhibit cardiomyocyte function⁶⁷, activate the complement cascade⁶⁸, induce P-selectin expression⁶⁹, chemokine upregulation⁷⁰ and increase the ability of ICAM-1 to bind to neutrophils⁷¹. Cytokines, such as TNF α , IL-1 β and IL-6 trigger inflammatory responses after myocardial ischemia^{58,72}. Together with these aforementioned humoral agents, antibody-mediated immune responses represent another facet of coordinated inflammation reaction.

3. Cell-Cell Communication

Efficient cellular cross-talk ensures proper myocardial development, homeostasis, and initiation of adaptive responses to injury and stress. Cell-cell communication within or between organs allows exchange of electrical and chemical signals through gap junctions and secretome signaling. Transfer of secreted factors is mediated by apoptotic bodies (1-5 μ m in diameter), microvesicles (100-1000nm in diameter) and exosomes (40-100nm in diameter)⁷³. Additional “outside-in” and “inside-out” signaling within tissue occurs between cells and the extracellular matrix (ECM) to adjust myocardial mechanical force, thereby preserving cardiac function during homeostasis and in response to myocardial injury. Critical players in cell-ECM communication primarily produced by fibroblasts include integrins, metalloproteinases, and ECM proteins such as collagen, laminin, fibronectin, osteopontin, thrombospondins, tenascins and periostin. Myocardial tissue stiffness triggers focal adhesion kinases (FAKs) as well as yes-associated protein (YAP)/transcriptional coactivator with PDZ-binding motif (TAZ) leading to interstitial cell activation⁷⁴.

Cardiomyocytes communicate through multiple routes including gap junctions, adhesion complexes, and secretome factors. The cardiomyocyte secretome influences hemodynamics by regulating vascular tone and blood flow through vasoconstriction (endothelin-1 (ET-1)), vasodilation (urocortin)⁷⁵, or development and long term growth of coronary vessels (VEGF, angiopoietins, HGF, FGF, PDGF and TGF β)^{76,77} as adaptation to stress⁷⁸. Additionally, endothelium influences cardiomyocyte response via paracrine signals including neuregulin to protect from ischemia/reperfusion damage⁷⁹. Direct interaction via connexin family gap junction proteins, allows passage of ions and small solutes controlling impulse conduction. Gap junctions undergo remodeling during myocardial hypertrophy characterized by phosphorylation changes and lateralization from intercalated discs⁸⁰.

Interstitial cells residing between cardiomyocytes are highly contributory to intercellular communication, response to environment, ECM, and remodeling stimuli. A primary example are cardiac fibroblasts that sense and respond to biomechanical stress with autocrine/paracrine factors including ET-1, TNF α , IL1 β , ATP, IGF1 FGF2, AngII (and downstream target TGF β), collectively leading to fibrosis⁸¹⁻⁸³. Additional fibroblasts are derived from the endothelial cell pool via TGF β -mediated endothelial-mesenchymal transformation (EndMT) consequential to fibrosis⁸⁴. TGF β -induced fibroblast proliferation also promotes ECM production and downstream Connective Tissue Growth Factor (CTGF) action⁸⁵. CTGF is negatively regulated by miRNAs produced by fibroblasts (miR-30) and cardiomyocytes (miR-133) acting through neighboring fibroblasts⁸⁶. In return, fibrosis promotes cardiomyocyte hypertrophy through TGF β -mediated activation of Smad and TGF β -activated kinase 1 (TAK1)⁸⁷. Fibroblasts are also a major source of secreted frizzled-related protein 2 (Sfrp2) that may blunt myocardial injury responses^{88,89}. Fibroblast-produced IL-33 is another putative cardiomyocyte-protective paracrine signal in pressure overload and MI mouse models⁹⁰. Interestingly, in contrast to the adult myocardium where cardiomyocyte-fibroblast communication generally prompts hypertrophy, fibroblasts mediate cardiomyocyte proliferation via ECM-mediated β 1-integrin signaling during embryonic development⁸⁷. Communication from cardiac fibroblasts to activate resident c-Kit⁺ cardiac progenitor cells (CPCs) is mediated by a secreted factor called high mobility

box 1 protein (HMGB1)⁹¹. CPCs also contribute to intercellular communication and significantly expand in heart failure patients⁹². Exosomes derived from resident c-Kit⁺ CPCs or cardiosphere-derived cells (CDCs) reduce cardiomyocyte apoptosis, increase viable mass and improve cardiac function *in vivo*⁹³ mediated in part by miR-146a, miR-210 and miR-132^{93–95}.

4. Angiogenesis / Vascularization

Vessels deliver oxygen and metabolites and export waste products and new blood vessel formation is essential for recovery of tissue function after ischemia or tissue injury. Impaired circulatory flow correlates with a poor prognosis in patients suffering from depressed coronary flow reserve after acute MI⁹⁶ as well as compromised microcirculatory response with dilated cardiomyopathy⁹⁷. Similarly, infarct expansion in mice is determined by capillary density in the border zone region⁹⁸. Disruption of angiogenic response and angiocrine signaling from endothelial cells promotes heart failure in mice after transaortic constriction^{99,100}, whereas overexpression of angiogenic VEGF rescued hibernating myocardium¹⁰¹. As such, vasculogenic action is inextricably linked to preservation and repair of injured myocardium. Ischemic damage in rodent hindlimb models promotes tissue revascularization by “arteriogenesis” through collateral formation wherein remodeling of existing arterioles to larger conduction arteries re-establishes blood flow¹⁰². Unfortunately, myocardial arteriogenesis is inefficient with huge heterogeneity of collateral vessel formation in myocardial ischemia patients¹⁰³.

Endothelial progenitor cells (EPC) incorporate into newly formed capillaries and promote revascularization of ischemic tissues. Endothelial progenitor cells translocate from bone marrow to the vascular injury sites, playing an important role in vascular restitution and functional repair after MI^{104,105}. Angiogenic incorporation and maturation of EPCs into functionally active endothelial cells has been debated, but lineage tracing experiments show bone marrow-derived cells expressing the EPC marker endothelial NO-synthase (eNOS) contribute to recovery after acute MI¹⁰⁶. Benefits from EPC activity may derive from physical formation of new capillaries that subsequently regress during resolution of inflammation and wound healing or paracrine action regulating angiogenesis and vessel maturation¹⁰⁷. Surely, paracrine mechanisms augment pro-angiogenic activity of EPCs as well as other progenitor and inflammatory cells such as adipose tissue-derived hematopoietic progenitor cells, bone marrow- and tissue-derived mesenchymal cells, monocytes and T-cell subpopulations (reviewed in ¹⁰⁸). Paracrine factors such as VEGF alone efficiently foster angiogenesis, but of questionable therapeutic value for long term vascularization since mature vessels fail to materialize post-treatment. Cellular cues for angiogenesis inferred from zebrafish as well as postnatal retina models indicate initiation by an endothelial “tip cell” followed by migrating and proliferating “stalk cells”¹⁰⁹. Subsequent pericyte recruitment together with mechanical activation of endothelial cells by blood flow promotes vessel maturation. Thus, it seems that multiple concurrent processes might be essential for neoangiogenic activity to be persistent and functional.

5. Cardiomyogenesis

Two primary hypotheses of cell source that contribute to new cardiomyocyte formation are from resident stem and progenitor cell pool differentiation and from pre-existing cardiomyocytes dedifferentiation followed by cell cycle re-entry. By all accounts, resident cardiomyocyte mitotic activity remains a rare event in late postnatal and adult mice and degree of myocardial regeneration from pre-existing cardiomyocytes is functionally insignificant^{110,111}. A fate-mapping study combining two different pulse-chase approaches concluded that pre-existing cardiomyocytes are the primary source of cardiomyocyte replacement after injury¹¹². Similar findings of α MHC-expressing cardiomyocytes as the origin of rare postnatal cardiomyogenesis events were reported from *in vivo* clonal analysis and fate mapping at single-cell level based on the mosaic analysis with double markers (MADM) model¹¹³. An “instant” lineage tracing strategy based upon tracking of c-kit antigen expression asserted that detection of fluorescent positive cardiomyocytes within a 48 hour time window implicates pre-existing cardiomyocyte rather than derived from stem cells through lineage conversion, although transient expression of c-kit within cardiomyocytes was not ruled out by the analyses¹¹⁴. All these genetic lineage-tracing studies share common outcomes of severely limited cardiomyocyte formation from pre-existing cardiomyocyte pool as a rare event. Cardiomyocyte mitosis during first year of life in humans contributes to 0.04% of total cardiomyocytes present at birth and dropping to 0.009% in a 20 year-old young adult heart as assessed by phospho-Histone3 immunolabeling¹¹⁵. Annual human cardiomyocyte turnover rates over lifespan are calculated to be 1.9% (adolescent), 1% (middle age), and 0.45% (old age), and by age 50 the cardiomyocytes remaining from birth are ~55% while 45% are generated later in life¹¹⁶. There are clearly substantial limitations of endogenous regenerative mechanisms from cardiomyocytes in adult mammalian heart. Although the adult mammalian heart possesses an extremely limited capacity for cardiomyocyte renewal, understanding mechanism(s) of cardiomyocyte proliferation and cell cycle arrest are fundamental to develop strategies to stimulate turnover and promote cardiac regeneration.

Cardiomyocyte dedifferentiation and proliferation is well known in lower vertebrates (e.g. zebrafish and newts¹¹⁷⁻¹¹⁹) as the primary mechanism of heart regeneration¹²⁰, but the early postnatal mammalian cardiomyocytes also capable of considerable plasticity. Within the first week of postnatal life, neonatal mouse heart can fully regenerate after partial surgical resection, and this regenerative response was characterized by cardiomyocyte proliferation^{121,122}. Neomyogenesis is unique to neonatal heart repair, as this critical feature is lost after postnatal day 7 and in the adult mouse heart¹²². Perinatal persistence of cardiomyocyte proliferation in mice may also occur, with a burst of cardiomyocyte mitosis at postnatal day 15¹²¹. In contrast, adult murine cardiomyocytes dedifferentiate, proliferate, and partially exhibit properties of CPCs including c-Kit expression and multipotency¹²³. Cardiomyocyte dedifferentiation changes in structural and fetal protein expression levels¹²⁴ and is a primary cellular response in response to stresses such as atrial fibrillation and hibernating myocardium¹²⁵. Molecular mechanism(s) of cardiomyocyte dedifferentiation and proliferation involve epigenomic reprogramming with downregulation of cardiac structure and function genes as opposed to induction of cell cycle re-entry and proliferation genes¹²⁶. Dedifferentiation increases in human and mouse cardiomyocytes treated with

osteopontin (OSM, a member of the IL-6 inflammatory cytokines)¹²⁷ as well as P130 and retinoblastoma protein¹²⁸. Despite these initial observations, cardiomyocyte dedifferentiation in adult mammalian heart remains a rare and inefficient process, with poorly understood underlying molecular mechanisms requiring further characterization.

Self-renewing, clonogenic, and multipotent CSCs have been extensively studied as a potential source to promote myocardial repair and regeneration through direct engagement with tissue as well as indirect actions to activate endogenous myocardial cells in studies of mice, rats, dogs, swine and humans^{129–135}. CSC derived from postnatal hearts express stem cell surface markers (c-Kit/Sca-1) and stem cell phenotypic function (colonogenicity/sphere formation ability)^{130,136,137}. Activation by environmental stimuli such as infarction injury prompts CSCs that normally reside in cardiac niches to divide, migrate, undergo lineage commitment, and mitigate pathological damage¹³⁸. CSCs lineage determination in the adult mammalian heart demonstrate presence of Ca²⁺ oscillations as well as Numb, α -adaplin, and Notch-1 signaling as regulators of symmetric versus asymmetric division that influence fate decisions^{139,140}. Asymmetric division of CSCs leading to lineage commitment is promoted by ATP released from dying cells that increases Ca²⁺ oscillations through IP3 pathway in the endoplasmic reticulum (ER)¹⁴¹.

Bone marrow-derived cells have also been intensively assessed for capacity to mediate myocardial repair and regeneration as documented in many basic and clinical studies^{142–149}. c-Kit⁺ bone marrow cells (BMCs) injected directly into the local injury site exhibit lineage commitment toward myocytes and vessels, mitigating damage from coronary artery disease¹⁴³. Bone marrow-derived mesenchymal stromal cells (MSCs) function in myocardial regeneration and survival primarily by influencing resident cardiac cells. MSC-mediated cytokine release regulates cardiac cells behavior through multiple signaling pathways^{150,151}. Observation of male cells presence in the female hearts transplanted into the male patients¹⁵², raised the possibility of bone marrow derived progenitor cells differentiation into cardiac cells, evaluated by the presence of Y-chromosome in cardiomyocytes. BMCs migration to the damaged tissue (facilitated through cytokines paracrine system) has led to a significant magnitude of myocardial remodeling and functional repair^{145,146}. However, outcomes of multiple clinical trials involving treatment of heart failure patients with bone marrow-derived cells has settled into a consensus of safe, albeit very modest, improvements in myocardial structure and function. Overall, the poor retention and survival of these and other adoptively transferred stem cell types into damaged myocardium remains a major limitation for clinical implementation.

6. Molecular Regulation of Proliferation, Cell Cycle, and Commitment

Genetic triggers for cell cycle reactivation to drive cardiomyocyte mitosis in the adult heart have been advanced as potential therapeutic targets for heart regeneration. For example, Hippo-Yap signaling is critical for cell intrinsic regulation of cardiomyocyte proliferation. Hippo-deficient mouse embryos develop cardiomegaly with robust cardiomyocyte proliferation and potentiated canonical Wnt/ β -catenin signaling^{153,154}. Loss of cardiac-specific Yap and Taz (cKO) impairs heart development and knockout mice suffer progressive dilated cardiomyopathy^{155,156}. Yap-cKO neonatal hearts failed to regenerate after MI at P2,

displayed extensive fibrotic infarct scar and deleterious loss of healthy myocardium, whereas control mice effectively recovered from resection challenge¹⁵⁶. Constitutive Yap activation in adult heart significantly enhanced cardiac regeneration, improved cardiac function, and promoted cardiomyocyte proliferation by 2.5-folds after infarction challenge^{155,156}. Regenerative activity of Yap is partially associated with stimulating insulin-like growth factor (IGF), phosphorylation of Akt, and inhibition of GSK3 β which could enhance β -catenin nuclear recruitment^{154,156}.

Additional molecular candidates for regulation of cardiomyocyte proliferation include Meis1 and neuregulin (Nrg1). Meis1 is a homeodomain transcription factor essential for normal cardiogenesis and embryonic hematopoiesis. Meis1 deletion in adult hearts increased number of cardiomyocytes prompted to enter cell cycle with concomitant increases in cytokinesis, while Meis1 cardiomyocyte overexpression induced premature cell cycle arrest in neonatal hearts¹⁵⁷. Inhibitory actions of Meis1 upon proliferation are hypothetically mediated through transcriptional activation of CDK inhibitors p15, p16, and p21^{157,158}. (Nrg1) purportedly induces mature mononucleated cardiomyocyte cell division through ErbB4/ErbB2 receptor leading to enhanced myocardial regeneration^{159,160}. Transient induction of constitutively active ErbB2 may trigger cardiomyocyte dedifferentiation and proliferation upon MI, followed by redifferentiation and regeneration^{159,161}. Administration of recombinant Nrg1 rescues cardiomyocyte depletion in pediatric heart disease and stimulates generation of new myocardium in neonatal mice after injury¹⁶². Cardiomyocyte cell cycle re-entry stimulated by rNrg1 administration in human infants with congenital heart disease at <6 months of age could provide therapeutic opportunity for treatment prior to surgical repair for developmental defects¹⁶². Despite such intriguing observations, relevance of these type of molecular intervention candidates adult human myocardium remains both questionable and unknown, as studies were predominantly performed in the context of postnatal or developing myocardium that exhibits increased cardiomyocyte proliferative plasticity similar to lower vertebrates.

Multiple additional paracrine factors and associated signal transduction pathways contribute in the process of myocardial repair. For example, FGF is essential for recruitment of receptors and coordinating epicardial and myocardial activities into regenerating muscle, improving angiogenesis and myocyte survival in acutely injured mammalian hearts¹⁶³. FGF induces downstream PI3 activity correlated with cardiac mitosis during development that is blunted by p38 MAPK. Inhibition of p38 induces DNA synthesis and G2/M transition in cardiomyocytes, acting synergistically with FGF1 signaling mediated by PI3K and Akt^{164,163}. Thyroid hormones (THs) regulate diverse developmental processes as both hyper- and hypothyroid conditions detrimentally impact upon myocardial growth and maturation. Tri-iodo-L-thyronine (T3) serves as the primary driver of maturation and suppresses proliferation of near-term fetal ovine cardiomyocytes *in vitro*¹⁶⁵ by upregulating p21 and downregulating cyclin D1^{165,166}. Preadolescent T3 surge in mice coincides with a brief cardiomyocyte proliferative burst mediated by IGF-1/Akt pathway that may dictate binuclear cardiomyocyte number¹²¹.

During cardiac regeneration, fibroblasts can play an important role, via myofibroblast trans-differentiation through WNT signaling pathway. Stress fiber formation and contractile

proteins expression are the hallmarks of fibroblast trans-differentiation¹⁶⁷. Several attempts at genetically engineered induction of fibroblast differentiation into cardiomyocytes and CPCs using defined regulatory factors such as selected miRNAs or JAK inhibitor I tout the possibility of *in vivo* trans-differentiation of cardiac fibroblasts into cardiomyocytes through cellular reprogramming^{168–171}. However, extrapolation of any of these putative mechanisms in service of therapeutically relevant regeneration in the human (or large animal) context remains dubious due to low conversion efficiency and will require further exploration.

Fundamental biological differences between lower vertebrates and humans remain problematic for extrapolation of findings to promote myocardial repair and regeneration. For example, centrosomes of cardiomyocytes in rat hearts disassemble shortly after birth but remain intact in adult zebrafish and newts¹⁷². Loss of centrosome integrity in postnatal cardiomyocytes coincides with cell-cycle arrest, revealing a potential mechanism underlying the post-mitotic state of mammalian adult cardiomyocytes¹⁷². Mps1 protein kinase is a mitotic checkpoint kinase in cell cycle regulation via its function in centriole assembly and centrosome duplication in mouse and human cells¹⁷³. In zebrafish, Mps1 mutants fail to regenerate and restore ventricular wall, instead, the injured heart developed large connective-tissue scars 3 weeks after ventricular resection, suggesting a critical cell-cycle regulatory role of Mps1 in proliferation^{173,174}. Similarly, inhibition of the cell-cycle regulator Plk1 drastically inhibited the heart regeneration in zebrafish, resembling the regeneration perturbation seen in zebrafish Mps1 mutants in zebrafish. This inhibition was not due to cardiomyocytes apoptosis increase, but primarily by loss of mitotic cardiomyocytes^{175,117}.

Despite decades of targeted and focused efforts the adult mammalian cardiomyocyte remains remarkably refractory to molecular interventions intended to promote re-entry into cell cycle and proliferation. Clearly, neonatal and lower vertebrate myocardium is a very different molecular and cellular milieu for manipulation of cardiomyocyte proliferation relative to the adult mammalian heart¹⁷⁶. Although research continues to identify novel and provocative avenues for influencing cardiomyocyte proliferation, the implementation of any such maneuvers in any therapeutic context continues to be hampered by low efficiency and debatable reproducibility. Lastly, almost all experimental models involve use of lower vertebrate species and relatively young animals, both of which are problematic for translational relevance to the aged human population most likely to require regenerative therapy. Indeed, the reparative potential of myocardium inexorably declines over time and is clearly impaired in aged individuals at the time and place where it is most needed by several intractable factors as described in the following section.

7. Cardiac aging associated with impairment of reparative and regenerative potential

Cardiac aging is a heterogeneous process characterized by increased levels of reactive oxygen species (ROS), genomic DNA damage, epigenetic modifications and telomere shortening. Consequences of aging pursuant to these deleterious changes include defective protein homeostasis, progressive loss of quality control processes and accumulation of dysfunctional organelles that directly impact cardiomyocyte, fibroblast and stem cell populations. Such stochastic insults initiated by both extrinsic and intrinsic stimuli

eventually lead to impaired contractile function, lower hemodynamic performance as well as defective regenerative responses to injury and stress stimuli.

Need for ongoing myocardial reparative and regenerative maintenance is due in part to deterioration of cardiomyocyte biological and functional competency as a consequence of aging. A hallmark of cardiac aging is decreased total number of cardiomyocytes by apoptotic or necrotic cell death. Multiple growth and anti-apoptotic molecules including survivin, protein kinase C (PKC), protein kinase B (PKB/Akt), STAT3 and metallothioneins are downregulated with age¹⁷⁷⁻¹⁷⁹. Although levels of some apoptotic markers decrease with age, there is a clear shift towards pro-death signals in aging cardiomyocytes, hence, a consensus that apoptosis is a major contributor to deteriorating function in aging hearts¹⁸⁰. Cell death-induced inflammation¹⁸¹, leads to oxidative stress and damage through the generation of ROS that interact with and alter the properties of biological molecules including nucleic acids, proteins, lipids and sugars¹⁸². In turn, oxidative damage induces inflammation creating a vicious cycle in the aging myocyte. Oxidative stress-induced damage and gradual deterioration of cardiomyocyte function also can be mediated by extrinsic environmental factors such as exposure to ionizing radiation or chemicals through ingestion, inhalation or skin contact^{183,184}. Cardiomyocyte mechanics, calcium dynamics and contractile potential are also significantly altered during aging process¹⁸⁵. Upregulation of cytoskeletal proteins and a transcriptional switch of contractile protein from fast to slow myosin are evident during aging¹⁸⁶. Simultaneous deposition of extracellular matrix (ECM) proteins, mainly collagen, upregulation of matrix metalloproteinases (MMPs) and downregulation of tissue inhibitor of metalloproteinase (TIMP) result in increased stiffness¹⁸⁷⁻¹⁸⁹. This is associated with defective myocyte activation, contraction and relaxation. Age-related dysregulation of contractile machinery has primarily attributed to decreased responsiveness of β -adrenergic signaling, the most important regulatory mechanism of cardiovascular performance, possibly due to reduced β -adrenergic receptor density and some defects involving the adenylyl cyclase¹⁹⁰. Additionally, defective myocyte activation results from downregulation of sarcoplasmic reticulum Ca^{2+} -ATPase (SERCA2) and $\text{Na}^+/\text{Ca}^{2+}$ exchanger¹⁹¹. Altered calcium transients during aging also arise from deposition of cellular garbage such as lipofuscin due to impaired autophagy¹⁹². Autophagy is a major quality control degradation pathway primarily regulated by mammalian target of rapamycin (mTOR). Interestingly, activation of autophagy through administration of rapamycin (mTOR inhibitor) or caloric restriction preserves cardiac function with age in rodent animal models^{193,194}. Indeed, electrical coupling and cell-cell communication are also substantially impaired as a consequence of aging in large animal models where downregulation and misdistribution of connexin 43, a critical gap junction protein, occurs in guinea pig sinoatrial node and rabbit hearts, respectively^{195,196}. Overall, defective cardiomyocyte contractile function in addition to global cardiomyocyte loss lead to enlargement of the remaining cardiomyocytes leading to LV hypertrophy to preserve cardiac function.

Cardiac progenitor cells (CPCs) are severely impacted by aging and undergo drastic phenotypic and functional alterations. Stem cell niches are the microenvironment where CPCs reside in a quiescent metabolically dormant state of reversible cell cycle arrest awaiting injury stimulus to be activated, undergo proliferation and/or lineage commitment in

turn of the century and has provided a wealth of return on investment with expanding knowledge and innovative approaches. The pressure for deliverables has been intense, leading to clinical testing that some have dubbed as premature and of questionable value. However, conclusions drawn from experience in the clinical arena are quite contrary to the view of skeptics and demonstrate not only safety, but also modest (albeit variable) efficacy. These initial forays into treatment also have highlighted theoretical and practical challenges that need to be conquered to improve upon pioneering cell therapy trials. The future belongs to optimists who recognize and acknowledge limitations of current approaches while promoting their view that “If we can all agree that regeneration occurs, no matter how inefficiently, then we should stop debating relevance for cardiac biology and concentrate instead upon improving the process and outcome.”

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Nonstandard Abbreviations and Acronyms

(TACTICS)	Transnational AllianCe for Regenerative Therapies In Cardiovascular Syndromes
(MI)	Myocardial Infarction
(ECM)	Extracellular Matrix
(EPC)	Endothelial Progenitor Cells
(MADM)	Mosaic Analysis with Double Markers
(EPC)	Cardiac Progenitor Cell
(CSC)	Cardiac Stem Cell
(MSCs)	Mesenchymal Stromal Cells
(THs)	Thyroid Hormones
(ROS)	Reactive Oxygen Species

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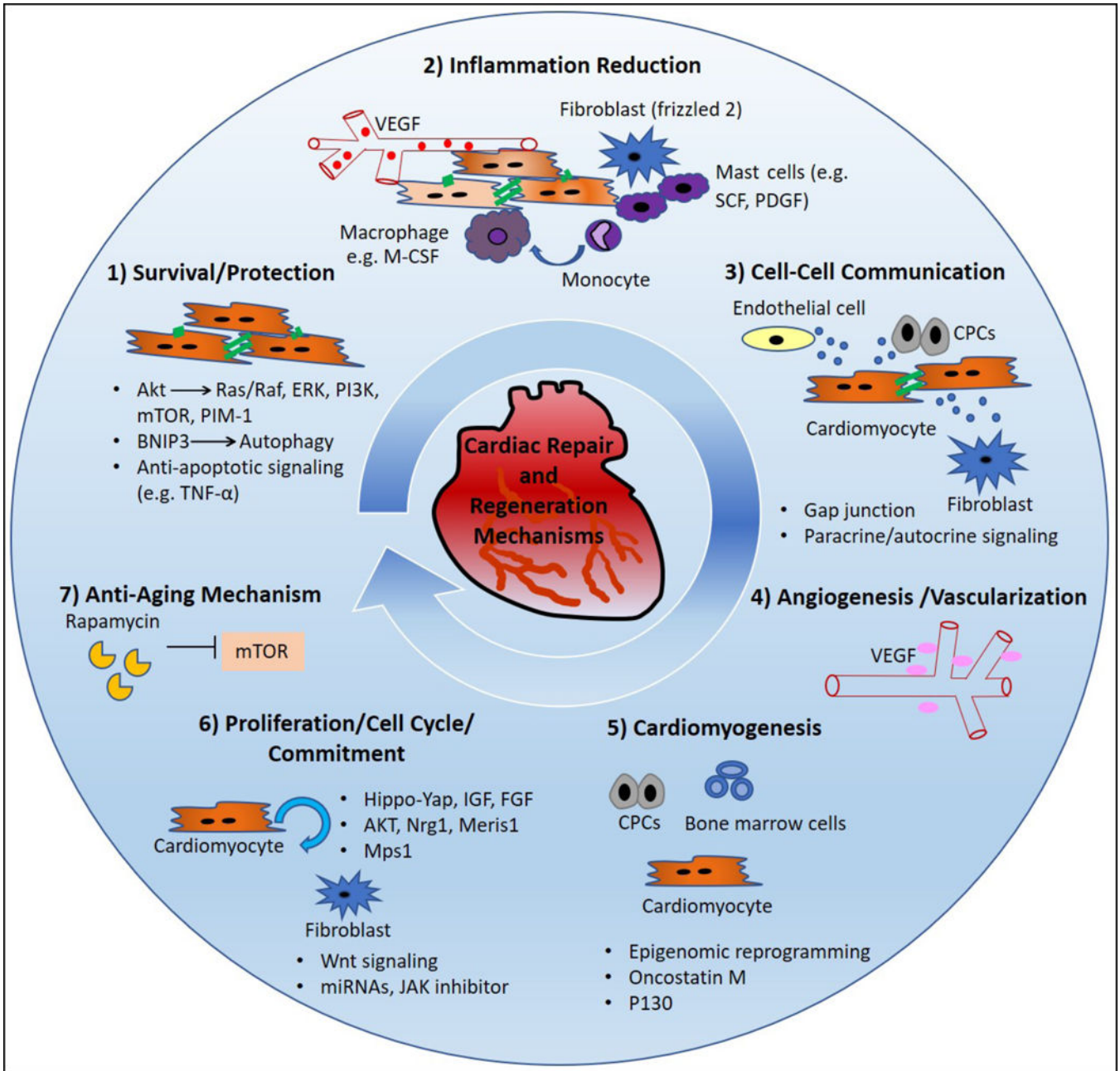


Figure 1. Cardiovascular repair and regeneration involves multiple mechanisms
 Representative categories and selected examples of processes to enhance heart repair and regeneration covered in this review. Mechanisms work independently on a molecular level to collectively mediate concurrent cellular actions of survival, repair and regenerative responses.