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Mechanisms of Myocardial Regeneration

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Abstract

Traditionally, the adult heart has been viewed as a terminally differentiated post-mitotic organ in which the number of cardiomyocytes is established at birth and these cells persist throughout the lifespan of the organ and organism. However, the discovery that cardiac stem cells (CSCs) live in the heart and differentiate into the various cardiac cell lineages has dramatically changed our understanding of myocardial biology. But deciphering the biological processes that lead to myocyte renewal is a challenging task. Cardiac regeneration may be accomplished by: 1. commitment of multipotent stem cells that generate all specialized lineages within the parenchyma; 2. activation of unipotent progenitors with restricted differentiation potential; 3. replication of pre-existing differentiated cells; 4. transdifferentiation of exogenous progenitors that undergo plastic conversion into cells different from the organ of origin; and 5. dedifferentiation of cardiomyocytes that reenter the cell cycle and divide. These multiple mechanisms of cell growth may act in concert to regenerate complex structures and restore the function of the target organ.

Introduction

The magnitude of myocyte renewal in the adult mammalian heart remains a matter of intense debate in the scientific community, although the implementation of different methodologies has provided clear evidence of the dynamic state of the normal and pathologic heart (Leri et al. 2011). During the entire lifespan of the organ, a sub-population of cardiomyocytes express functionally active telomerase, synthesizes DNA incorporating BrdU, show positive labeling for the cell proliferation markers cdc6, Ki67, MCM5 and cyclin B1, and possess all the molecular components required for the entry and progression through the cell cycle (Leri et al. 2005, Boström et al. 2010). The most impressive event within the cell cycle is represented by the mitotic phase, characterized by the assembly of the mitotic spindle and the subsequent formation of the actomyosin contractile ring, which constricts and pinches off the membrane in the region of cytoplasmic separation (Beltrami et al. 2001, Kajstura et al. 2010). The recognition of karyokinesis and cytokinesis in cells with myofibrillar structures (Figure 1A and 1B) has documented unequivocally that myocyte replication occurs in the adult heart. More recently, retrospective ¹⁴C birth dating of human cardiomyocytes has confirmed that these cells are replaced during an individual's lifespan albeit at a very low pace (Bergmann et al. 2009). Collectively, these findings pose the fundamental question concerning the identification of the cellular mechanisms responsible for the generation of new myocytes; myocyte formation may occur by activation and

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commitment of resident stem cells, transdifferentiation of hematopoietic stem cells that home to the heart via the systemic circulation, or through dedifferentiation of post-mitotic myocytes that acquire a replicating/progenitor state.

Understanding the biological process that leads to myocyte renewal is a challenging task that cannot be resolved by restricting observations to events occurring at the level of the myocyte population, a limitation that unfortunately applies to most, if not all, fate-mapping-based studies; the use of reporter genes driven by myocyte specific promoters excludes a priori the critical role that endogenous and exogenous stem cells may have in the process. A rigorous analysis has to occur at the single cell level in which the phenotypic properties of the primitive cells and those of the derived progeny are characterized. By this approach only, it is possible to discriminate whether myocardial regeneration is accomplished by: **a)** commitment of multipotent stem cells that generate all specialized lineages within the parenchyma; **b)** activation of unipotent progenitors with restricted differentiation potential; **c)** replication of pre-existing differentiated cells; **d)** transdifferentiation of exogenous progenitors that undergo plastic conversion into cells different from the organ of origin; and **e)** dedifferentiation of cardiomyocytes that reenter the cell cycle and divide. These multiple mechanisms of cell growth may act in concert to regenerate complex structures and restore the function of the target organ.

Newly-formed cardiomyocytes provide important information that aids in recognizing, retrospectively, the founder mother cell. Replicating cardiomyocytes are typically mononucleated, have a 60–80% smaller volume than non-cycling myocytes, and show a disorganized contractile apparatus with modest accumulation of myofibrils in the subsarcolemmal region (Urbanek et al. 2003). With rare exceptions (Figure 1C) (Limana et al. 2002), only small mononucleated myocytes undergo karyokinesis and cytokinesis; this pattern of myocyte growth is incompatible with the notion that mature cardiomyocytes correspond to a pool of functionally homogenous cells that, upon need, reenter the cell cycle and divide. According to basic principles of cellular biology, this unrealistic process would imply that terminally differentiated cardiomyocytes, $\sim 25,000 \mu\text{m}^3$ in volume, double in size, $\sim 50,000 \mu\text{m}^3$, prior to undergoing cytokinesis and forming two daughter cells, each $\sim 25,000 \mu\text{m}^3$ in size. This sequence of events has never been shown physiologically, or following forced reentry of post-mitotic myocytes into the cell cycle by overexpression of cyclin D (Rubart and Field, 2006), or intramyocardial injection of neuregulin (Bersell et al. 2009), and periostin (Kuhn et al. 2007). The attempts made to coax cell division of post-mitotic myocytes result consistently in abortive mitosis with the formation of anaphase bridges and apoptotic cell death (Agah et al. 1997). Thus, the possibility that mature myocytes re-enter the cell cycle and form two daughter myocytes is biologically unsound, suggesting that other mechanisms of cardiomyogenesis have to be considered during the homeostatic control of the organ and in the presence of myocardial injury.

Cardiac Progenitor Cells

The existence of a pool of small cycling cardiomyocytes is suggestive of a model of organ growth in which a restricted number of stem cells generate a large compartment of parenchymal cells. Replicating myocytes represent a short-lived cell pool that is continuously replenished by commitment of primitive cells and, concurrently, is constantly transitioning to the terminally differentiated phenotype (Leri et al. 2011). Rarely dividing stem cells give rise to oligolineage progenitors that lose self-renewal ability and form rapidly proliferating transit amplifying cells, which undergo a finite number of divisions and, eventually, withdraw from the cell cycle, reaching full maturation. In this scenario, the number of dividing myocytes reflects the rate of activation and lineage specification of stem/

progenitor cells, like circulating reticulocytes constitute the biomarker of the extent of activation of hematopoietic stem cells (HSCs) in the bone marrow.

The notion that the heart harbors populations of progenitor/stem cells capable of creating functionally-competent cardiomyocytes and coronary vessels is consistent with the robust regenerative response observed after acute and chronic myocardial infarction in humans (Beltrami et al. 2001, Urbanek et al. 2005). A variety of surface antigens, transcription factors and functional assays have been employed to define different classes of progenitor cells (Leri et al. 2011); whether these cell subsets represent independent categories of undifferentiated cells with diverse functional import, or distinct phenotypical aspects of a common progenitor is currently unknown. The first myocardial progenitor was identified on the basis of its ability to expel toxic compounds through an ATP-binding cassette transporter (Hierlihy et al. 2002). Following this initial report, ABC-transporter activity was documented repeatedly by employing the DNA-binding dye Hoechst 33342 to sort progenitor cells that occupy a side position in the FACS profile, i.e., side population cardiac cells (Oikonomopoulos et al. 2011). Additionally, the expression of Sca1, a member of the Ly-6 family of proteins, has identified cell classes, which share the Sca1 antigen, but do not possess the functional properties of stem cells. Sca1-positive cells express multiple mesenchymal markers, including CD29, CD44, CD90, and CD106 (Li et al. 2009), that question their cardiac origin. Recently, a population of cardiac colony-forming units-fibroblasts residing in a perivascular niche has been described (Chong et al. 2011). These cells, of proepicardial origin, have the broad trans-germ layer potency that characterizes mesenchymal stromal cells (MSCs) in the bone marrow and other organs.

Nearly 10 years ago, the Isl1 transcription factor was shown to be associated with a pool of cardiac progenitor cells that condition heart morphogenesis in the mouse embryo. However, Isl1-positive cells become undetectable shortly after birth in the atrial and ventricular myocardium of the mouse and rat heart or in the decompensated human heart (Bu et al. 2009). Importantly, Isl1-positive cells lack the essential properties of stem cells: they are not self-renewing, clonogenic, and multipotent (Leri et al. 2011). Additionally, the functional import of Isl1-positive cardioblasts in vivo has never been shown. Fibronectin-coated dishes have been used for large-scale amplification of cardiosphere-derived cells (Smith et al. 2007). The cells located in the center of the cardiosphere express the stem cell antigen c-kit and are surrounded by an outer layer composed of cells positive for CD105, a membrane glycoprotein commonly expressed in bone marrow MSCs. Collectively, these observations indicate that a compartment of cells with some characteristics of progenitor cells is present in the myocardium. However, their role in cardiac homeostasis and their regenerative potential following myocardial damage has only been partially defined.

c-kit-Positive Cardiac Stem Cells

The c-kit receptor tyrosine kinase was detected originally in a class of murine HSCs with long-term bone marrow reconstituting ability in irradiated recipients (Orlic et al. 1993). Recently, c-kit has been found in stem cells of the adult lung and heart (Beltrami et al. 2003, Bearzi et al. 2007, Kajstura et al. 2011). Several lines of evidence have been accumulated in favor of the notion that cardiac c-kit-positive cells are bona fide stem cells. To establish a lineage relationship between the ancestor and its progeny, retrospective analyses are commonly performed. As in a family tree, the phenotype of the descendants allows to define the characteristics of the founder cell. In vitro clonogenic and differentiation assays serve this purpose. c-kit-positive cardiac stem cells (CSCs) form in vitro large clones composed of a high number of identical undifferentiated daughter cells (Figure 2A). Sub-clones can also be obtained upon seeding of individual clonal daughter cells; these growth properties

strengthen the notion that the parental c-kit-positive cell is a true stem cell (Beltrami et al. 2003).

The multipotentiality of c-kit-positive CSCs has been documented repeatedly by exposing clonal cells to differentiation media; myocytes, endothelial cells, smooth muscle cells, and fibroblasts, in various proportions, are easily obtained (Figure 2B). However, differentiation assays of stem cell clones in vitro have inherent limitations including the possibility that culture conditions result in the preferential or biased acquisition of a selective lineage phenotype, masking the in vivo potential of the founder cell. Similarly, the identification in vivo of multiple phenotypes in the progeny of transplanted non-clonal stem cells does not provide direct evidence of the multipotentiality of each delivered cell. This problem has been overcome by transplantation of clonal human CSCs in infarcted animals (Figure 2C) (Bearzi et al. 2007) and by viral tagging and genetic marking of CSCs (Hosoda et al. 2009).

Clonal c-kit-positive CSCs differentiate in vivo into a progeny with the electrical, mechanical and calcium transient characteristics of differentiated myocytes. The generation of cardiomyocytes is impressive and markedly exceeds the number of cells lost by myocardial infarction. Additionally, a large number of resistance coronary arterioles and a relatively low number of capillary structures develop, resulting in the formation of new myocardial tissue that resembles structurally and functionally the parenchyma of the neonatal heart. The hyperplastic phenotype of the new myocytes and their small size indicate clearly that cardiac repair follows a pattern of growth that reiterates the expected evolutionary changes coupled with the activation of a stem cell.

Viral transduction for clonal tracking of individual mouse and human CSCs has been employed to determine their fate in vivo under physiological conditions and after myocardial injury (Hosoda et al. 2009). The analysis of the clonality of CSCs and myocyte turnover cannot be performed in humans since it requires genetic tagging of the undifferentiated cells so that the clonal marker of individual mother cells is traced in the specialized progeny. c-kit-positive CSCs located in the niches of the atrioventricular groove and apex of the mouse heart were infected with a lentivirus carrying EGFP and the destiny of the tagged cells was determined 1–6 months later. Genetic marking of CSCs in situ allowed us to assess the behavior of resident stem cells in the non-injured heart. Although the extent of myocyte turnover in the intact heart is slower than the rapid pace at which cells renew themselves in the presence of damage, the intrinsic properties of CSCs are better characterized in the absence of tissue injury. A common integration site was identified in isolated c-kit-positive CSCs, cardiomyocytes, ECs and fibroblasts, documenting the multipotentiality of CSCs and the clonal origin of the differentiated cells (Hosoda et al. 2009). During a 6 month-period, each EGFP-positive CSC divided ~8 times giving rise to 230 cardiomyocytes. These findings, together with data obtained with BrdU pulse-chase assays, indicate that activation and differentiation of CSCs is an ongoing process which results in a significant renewal of cardiomyocytes in the young-adult mouse heart.

Similarly, myocyte regeneration in the physiologically aging human heart takes place at previously unexpected levels in both women and men. In the female heart, myocyte replacement occurs at a rate of 10%, 14%, and 40% per year at 20, 60, and 100 years of age, respectively. Corresponding values in the male heart are 7%, 12%, and 32% per year, documenting that myocyte turnover involves a large and progressively increasing number of parenchymal cells with aging (Kajstura et al. 2010). Importantly, these growth processes are potentiated in the presence of pathologic states.

The high level of myocyte turnover documented in the mammalian heart is at variance with lineage tracing studies suggesting that very little replacement of cardiomyocytes occurs with

age in the absence of pathologic states (Hsieh et al. 2007). However, the young adult mouse heart typically shows a degree of myocyte apoptosis of ~300 cells per million or one thirtieth of 1% (Torella et al. 2004). Although this may be seen as inconsequential physiologically, the duration of this form of cell death is at most 4 hours (Kajstura et al. 2010). Thus, ~1,800 myocytes/ 10^6 cells are lost per day in the heart implying that, over a period of 6 months, ~1.1 million myocytes are lost and have to be replaced by new cells to preserve cardiac function. Based on this premise, caution has to be exercised in the interpretation of fate mapping strategies (Hsieh et al. 2007). Genetic manipulations may provide incorrect understanding of myocardial biology. Facts may be obscured by technical artifacts.

Reprogramming, transdifferentiation, and dedifferentiation

The HSC was the first identified tissue-specific adult stem cell (McCulloch and Till 1960, Becker et al, 1963). In addition to the three fundamental attributes of self-renewal, clonogenicity and multipotentiality, HSCs were defined as essential components of a hierarchically structured system. Based on this principle, stem cell-regulated organs may adhere to a model in which stem cells attain first a committed state, and then undergo progressive maturation, ultimately, reaching terminal differentiation. This modality of organ growth has been challenged, raising the possibility that adult stem cells may not be viewed as discrete “fixed” entities but rather as functional units. A bidirectional shift between more primitive and more differentiated cells may also occur. The latter requires the concept of reversibility, a notion that contrasts the hierarchical model of cell growth.

Reprogramming, transdifferentiation, transdetermination, and dedifferentiation represent multiple aspects of the same phenomenon, i.e., the conversion of one cell type into another. These cell transformations include the switch from one differentiated cell to another differentiated cell type, the developmental plasticity of stem cells that may cross tissue boundaries committing to lineages different from the organ of origin, and regression of a mature cell to a proliferative or progenitor cell phenotype.

Reprogramming reverts somatic differentiated cells into pluripotent stem cells that have the ability to generate the large majority of cell types in the organism. Although reprogramming occurs naturally during fertilization to produce totipotent cells, this process is not a component of the physiological regenerative response of adult organs, questioning its clinical relevance. In vitro reprogramming of somatic cells requires complex interventions including forced overexpression of transcription factors, or insertion of synthetic modified mRNA, recombinant proteins, microRNAs, and mini-circle plasmids (Barrilleaux and Knoepfler 2011). In all cases, nuclear reprogramming is coupled with chromatin rearrangements that are needed for the acquisition of embryonic stem cell-like properties. However, a residual epigenetic memory of the original parental cell leads to biased differentiation of the biologically modified progeny. Incomplete erasure of tissue-specific methylation and aberrant de novo methylation are the molecular component of incomplete epigenetic transformation (Kim et al. 2011). These currently unresolved issues raise serious concerns on the validity of induced pluripotent stem (iPS) cells as a platform for human disease modeling and drug screening (Martinez-Fernandez et al. 2011). Additionally, the tumorigenic potential of iPS cells makes their clinical implementation unlikely.

Transdifferentiation is not a rare event if we consider the nearly infinite number of permutations that occur in each individual during prenatal development and postnatal life. The presence of foreign tissue in ectopic sites, i.e., metaplasia, is more easily recognized in pathologic states than in physiological conditions. Transdifferentiation may be implicated in cell turnover, although this process remains one of the most questioned mechanisms of cell

growth and tissue repair in adult organs. Originally, transdifferentiation was defined as an irreversible switch from one differentiated cell to another differentiated cell type, while transdetermination was viewed as the commitment of stem cells towards closely related lineages. The term transdetermination is rarely used and transdifferentiation or developmental plasticity indicates the process by which adult stem cells are capable of generating mature cells beyond their own tissue boundaries. Typically, HSCs migrate to sites of injury in damaged organs, promoting tissue repair (Anversa and Nadal-Ginard 2002). c-kit-positive HSCs injected in the border zone of a myocardial infarct, or mobilized systemically into the circulation by cytokines, lead experimentally to the regeneration of the necrotic myocardium with the formation of functionally-competent cardiomyocytes and coronary vessels (Orlic et al. 2001, Rota et al. 2007). These observations, which were confirmed in multiple laboratories (Sussman and Murry 2008), argue in favor of the differentiation of HSCs into the myogenic and vascular cell phenotypes, representing an important mechanism of the structural and functional recovery of the injured heart. Various molecular and genetic tools have been employed to obtain unequivocal evidence of HSC transdifferentiation (Rota et al. 2007). These findings and the paucity of effective new drugs for the treatment of heart failure, has prompted cardiologists to the rapid implementation of bone marrow-derived cells in the management of the acutely infarcted human heart. However, a mixed population of bone marrow cells rather than a highly purified stem cell pool has commonly been used clinically. Thus far, c-kit-positive HSCs have not been used in clinical trials.

If c-kit-positive HSCs attain the properties of CSCs when they home to the myocardium and give rise to cardiomyocytes and coronary vessels, the concept of transdifferentiation has to be accepted. Thus, understanding the difference between CSCs and HSCs becomes extremely important. Hypothetically, stem cells that reside in the heart should be more effective in making new myocardium than stem/progenitor cells from other organs including the bone marrow. CSCs are programmed to generate cardiac structures and, upon activation, can rapidly form parenchymal cells, resistance arterioles, and capillary profiles possibly rescuing the failing heart (Leri et al. 2011). Conversely, HSCs have to acquire a different phenotype which necessitates chromatin remodeling and reprogramming of genes before they transdifferentiate and create functionally-competent cardiac cells. To date, the HSC appears to be the most versatile stem cell in crossing lineage boundaries and the most prone to break the law of tissue fidelity.

In contrast to transdifferentiation, dedifferentiation refers to regression of a mature cell within its own lineage; this process is coupled with the acquisition of the proliferative cell phenotype. Dedifferentiation is considered the natural regenerative response to injury in zebrafish, newts, salamanders, and planaria (Straube and Tanaka 2006). Following disassembly of the sarcomeric contractile apparatus, which physically impedes cytokinesis, cardiomyocytes replicate to reconstitute up to 20% of the zebra fish ventricle after resection (Jopling et al. 2010). By cre/lox cell lineage protocols, the newly-formed structure appears to derive primarily from a subpopulation of GATA4-positive cardiomyocytes rather than from a non-myocyte, primitive cell pool. The poorly organized sarcomeres of replicating GATA4-positive cells has led to the conviction that dedifferentiation constitutes the critical regenerative step (Kikuchi et al. 2010). However, failure to express the early myocyte commitment genes *Nkx2.5* and *Hand2* in dividing myocytes indicates that the cells of origin underwent limited dedifferentiation, or replicating GATA4-positive cardiomyocytes may correspond to the later phases of CSC commitment. In the adult mouse heart, *Nkx2.5* precedes GATA4 expression in transit amplifying myocytes derived by the lineage specification of CSCs (Boni et al. 2008).

A comparable regenerative response occurs after surgical resection of the apex of the left ventricle in the neonatal mouse heart (Porrello et al. 2011). Again, cardiomyocyte proliferation was invoked as the mechanism responsible for cardiac repair. Genetic fate mapping strategies have been employed to distinguish the contribution of preexisting myocytes and stem/progenitor cells to the regenerative process in the zebrafish and mouse heart. Unfortunately, this approach is inadequate to answer the question raised. CSCs engineered with fluorescent constructs placed under the control of promoters encoding myocyte-specific transcription factors and sarcomeric proteins show transgene protein expression (Bailey et al. 2009). Although the GATA4 and α -MHC promoters are generally considered to be active primarily in cardiomyocytes, the expression of the reporter gene in c-kit-positive cells reflects very early stage of cardiogenic commitment of CSCs. These observations support the notion that myocyte regeneration in the zebrafish and neonatal mouse heart may be mediated by CSC specification.

Thus far, the evidence of myocyte dedifferentiation is based exclusively on cellular morphology. Myocytes with characteristics intermediate between an early and a fully mature phenotype have been interpreted arbitrarily as the product of cell dedifferentiation. However, the lack of specific markers makes it impossible to define *in vivo* whether the cell of interest is undergoing differentiation or whether it is in the process of reverting to an earlier immature state.

The possibility of dedifferentiation as a major component of the regenerative process was raised by observations conducted in vertebrates with exceptional growth reserve. This conclusion, however, has been challenged. During salamander limb regeneration, cells from muscle, bone, cartilage, nerve sheath, and connective tissues are believed to dedifferentiate into a pool of proliferating cells known as the regeneration blastema (Nacu and Tanaka 2001). Unfortunately, it remains to be determined whether pre-existing unipotent or multipotent stem cells constitute the pool of proliferating blastemal cells, questioning the notion that dedifferentiation and subsequent re-differentiation is the repair modality in limb reconstitution (Nacu and Tanaka 2001).

In mammals, liver and pancreas regenerations after partial surgical resection are viewed as typical examples of cell dedifferentiation. Cell cycle reentry of mature hepatocytes and the reversal of pancreatic duct cells to an immature phenotype constitute the cellular adaptations triggered by the loss of parenchymal mass (Yanger and Stanger 2011). Similarly, myocyte dedifferentiation with amplification of the reprogrammed progeny has been claimed to play a critical role in the regenerative response of the heart to pathologic stimuli (Morrisey 2011). In addition to sarcomeric disorganization, changes in the molecular fingerprint have been considered indicative of myocyte dedifferentiation. Although a large subpopulation of post-mitotic cardiomyocytes shows a nuclear localization of GATA4, the presence of this transcription factor in myocytes with myofibrillar disassembly has inexplicably been interpreted as reexpression of an immature marker. This possibility is in contrast with findings indicating that GATA4 is necessary and sufficient to induce cardiac hypertrophy in which enlarged myocytes are characterized by accumulation of myofibrils (Oka et al. 2006). Importantly, the activation of the fetal gene program has traditionally been viewed as an adaptation of the myocardium to an increased workload, but questions can be raised concerning the origin of these immature cells (Leri et al. 2011).

The myocardium in rodents is composed mainly of binucleated cardiomyocytes. Surprisingly, whether dedifferentiation of mouse and rat cardiomyocytes is accompanied by expulsion or selective apoptosis of one of the two nuclei has not been addressed. Starting cultures of myocytes prior to oncostatin M stimulation are particularly enriched in mononucleated cells (Kubin et al. 2011). In non-genetically manipulated and transgenic

mice, it has been shown repeatedly that adult myocyte proliferation in vitro and in vivo is restricted to small mononucleated cells that correspond to the pool of transit amplifying myocytes derived from lineage determination of CSCs. Thus, future work will have to decipher whether dedifferentiation is an independent growth process or myocyte regeneration mediated by activation and commitment of CSCs constitutes the predominant mechanism of cell renewal and tissue repair in the adult heart.

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Figure 1.

Myocyte cytokinesis. **Panels A and B:** Chromosomes (blue: PI, propidium iodide) are labeled by the cell cycle protein Ki67 (yellow) in cardiomyocytes (cardiac myosin heavy chain, MHC, red). Arrows point to cytokinesis. **Panel C:** Mitotic figure in an isolated binucleated mouse myocyte (α -sarcomeric actin, α -SA, red).

Figure 2.

Cardiac stem cells and their progeny in vitro and in vivo. **Panel A:** Phase contrast microscopy. Single cell-derived clones from c-kit-positive human CSCs. **Panel B:** c-kit-positive human CSCs exposed in vitro to differentiation medium form myocytes (α-SA, red), endothelial cells (von Willebrand factor, vWf, yellow) and smooth muscle cells (α-smooth muscle actin, α-SMA, green). **Panel C:** Human CSCs regenerate infarcted myocardium in an immunodeficient mouse heart 21 days after infarction and cell delivery. Human myocardium (arrowheads) is present within the infarct (MI). BZ, border zone. Areas in rectangles are shown at higher magnification below. Human myocytes are α-SA (red) and *Alu* probe (green) positive. *Alu* documents human DNA sequences in the newly-formed myocardium. Asterisks, spared myocytes.