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CARDIOVASCULAR GENOMIC MEDICINE **Viewpoint and Commentary**

Cardiac Regeneration

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The role and even the existence of new myocyte formation in the adult heart remain controversial. Documentation of cell cycle regulators, deoxyribonucleic acid synthesis, and mitotic images has only in part modified the view that myocardial growth can be accomplished exclusively from hypertrophy of an irreplaceable population of differentiated myocytes. However, myocyte regeneration and death occur physiologically, and these cellular processes are enhanced in pathologic states. These observations have challenged the view of the heart as a postmitotic organ and have proposed a new paradigm in which parenchymal and non-parenchymal cells are continuously replaced by newly formed younger populations of myocytes as well as by vascular smooth muscle and endothelial cells. Heart homeostasis is regulated by a stem cell compartment characterized by multipotent cardiac stem cells that possess the ability to acquire the distinct cell lineages of the myocardium. Similarly, adult bone marrow cells are able to differentiate into cells beyond their own tissue boundary and create cardiomyocytes and coronary vessels. This process has been termed developmental plasticity or transdifferentiation. Because of these properties, bone marrow cells and cardiac stem cells have been employed experimentally in the reconstitution of dead myocardium after infarction. These cell classes hold promise for the treatment of heart failure in humans. (J Am Coll Cardiol 2006;47:1769–76) © 2006 by the American College of Cardiology Foundation

THE HEART AS A POST-MITOTIC ORGAN: THE CONTROVERSY

A fundamental issue concerning the ability of the heart to sustain cardiac diseases is whether myocardial regeneration occurs in the adult organ or whether this growth adaptation is restricted to prenatal life, limiting the response of the heart to pathologic loads. The concept of the heart as a terminally differentiated organ unable to replace working myocytes has been at the center of cardiovascular research and therapeutic developments for the past 50 years (1). The accepted view has been and remains that the heart reacts to an increase in workload only by hypertrophy of the existing myocytes during postnatal maturation, adulthood, and senility. When myocardial hypertrophy is exhausted, ventricular dysfunction supervenes. In the past three decades, the focus of molecular cardiology has been the identification of the signaling pathways regulating the activation and depression of genes implicated in the hypertrophic reaction of myocytes in physiologic development and aging or following abnormal pathologic states (2).

The possibility that the heart renews its parenchymal cells was dismissed, and even today myocardial repair is viewed with suspicion and trepidation. The engrained paradigm that promotes a rather uninteresting biologic perspective of the maturing, old, or diseased heart has been shaken by studies from our laboratory indicating that myocyte regeneration occurs in humans and animals after infarction (3–5), after prolonged pressure overload (6), and in the senescent decompensated heart (7). Although some of these studies were published almost 20 years ago (8) and have continued to appear through the past two decades, the traditional establishment rejected this alternative notion of cardiac biology, defending a territory that was considered unwavering and immovable. The conviction that nothing could be done to generate new myocardium was so strong that even the documentation that multipotent bone marrow cells (BMCs) reconstitute dead myocardium after infarction (9) was immediately challenged (10–12). Before discussing the controversy about myocardial regeneration with exogenous or endogenous undifferentiated cells, some comments about the history of the heart as a postmitotic organ are relevant for understanding the shift in paradigm required for the implementation of the novel field of regenerative cardiology.

Numerous studies of the human heart from 1850 to 1911 held the view that myocardial hypertrophy was the consequence of hyperplasia and hypertrophy of existing myocytes. Subsequent reports from 1921 to 1925 questioned the ability of myocytes to proliferate, suggesting that the increase in cardiac muscle mass in the pathologic heart was the result of pure cellular hypertrophy (13). The concept that myocytes cannot divide originated from difficulty in identifying mitotic figures within these cells. This conviction gained support from autoradiographic analysis of thymidine incorporation in hearts of animals during postnatal growth and after conditions of overload (14). Deoxyribonucleic acid

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Abbreviations and Acronyms

BMC = bone marrow cell
CSC = cardiac stem cell
EGFP = enhanced green fluorescent protein
GFP = green fluorescent protein

synthesis in myocyte nuclei either was not detected or was found to be negligible. The dogma was then introduced that the heart survives and exerts its function until death of the organism with the same or lesser number of cells that are present at birth. Accordingly, ventricular myocytes in humans are terminally differentiated cells, and their lifespan corresponds to that of the individual. The number of myocytes attains an adult value a few months after birth (13), and the same myocytes are believed to contract 70 times per minute throughout life. Because a certain fraction of the population reaches 100 years of age or more, an inevitable consequence of this paradigm is that cardiac myocytes are immortal, functionally and structurally. This assumption contradicts the concept of cellular aging and apoptotic cell death as well as the logic of a slow turnover of cells with time in the heart. Myocyte death occurring with age and the chronic loss of cells in the absence of myocyte multiplication would result in the disappearance of the entire organ over a period of a few decades.

In spite of the obvious facts and findings documenting activation of the cell cycle machinery, karyokinesis, and cytokinesis in a subpopulation of myocytes (3,6,15), the resistance to a shift in paradigm has been enormous. Several reports have provided evidence that myocytes die (16) and that new ones are constantly being formed in the heart at all ages in animals and humans (13). Both processes are markedly enhanced in the presence of disease states, and the imbalance between cell growth and cell death is a critical determinant of cardiac decompensation and its evolution to congestive heart failure and death of the organism. The expression of nuclear proteins typical of dividing cells and measurements of myocyte mitotic index were rejected as proofs of myocyte formation and considered inconclusive or the product of incorrect methodology (17,18).

The criticisms of this work varied from the assumption that proliferating endothelial cells and fibroblasts were confused with cycling myocytes to the belief that nuclei of dividing endothelial cells and fibroblasts traversed the myocyte cytoplasm and reached the position of myocyte nuclei to give the erroneous image of dividing myocytes (17). The nature of these comments is better appreciated when the methodology employed in the collection of the data supporting myocyte regeneration is considered. Confocal microscopy was invariably used because of its high resolution and its ability to identify structural proteins (Fig. 1). By this approach, thick histologic sections can be viewed with a degree of resolution that was previously restricted to semithin sections of the myocardium. These images can be analyzed three-dimensionally, excluding misinterpretation of endothelial cells and fibroblasts as myocytes.

The most relevant observation that dramatically challenged the old paradigm of the heart was the identification of male cells in female hearts transplanted in male recipients (19). In these cases of sex-mismatched cardiac transplants, the female heart in a male host had a significant number of Y-chromosome–positive myocytes and coronary vessels. Although discrepancies exist among groups in terms of the degree of cardiac chimerism (19–24), these results raised the possibility that these male cells colonized the female heart and differentiated into myocytes and vascular structures. The presence of male cells in the female heart was consistent with the contention that stem-like cells can migrate to the cardiac allograft and give rise to cardiac cell progenies. Primitive cells that expressed c-kit, stem cell antigen 1-like, and multidrug resistance 1 were identified in control and transplanted hearts (19). The recognition of these undifferentiated cells together with early committed cells was suggestive of a true cardiac stem cell (CSC) as the critical modulator of the homeostasis of the normal and stressed myocardium. These data were the foundation of the work that led to the identification of a resident CSC pool in the adult heart (25).

The possibility that a multipotent progenitor cell resides in the heart was received with great enthusiasm by some (26,27) and great skepticism by others (22,28). The high degree of myocyte chimerism found in our study by confocal microscopy (Fig. 2) was interpreted as further demonstration of myocyte turnover and convincing proof of the formation of new myocytes in the human heart (19). Conversely, the low extent (20) or absence (22) of myocyte chimerism seen in other reports by light microscopy was used to question the concept of myocyte replication, supporting the view that the myocardium is a terminally

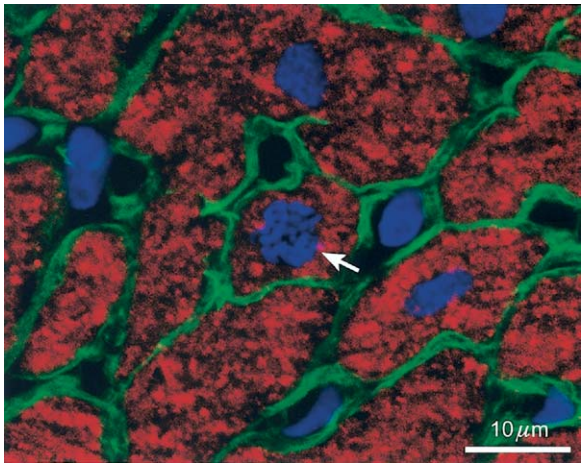

Figure 1. Myocyte proliferation in humans. A small dividing myocyte (alpha-sarcomeric actin, **red**) with metaphase chromosomes **(arrow)** is present in the left ventricular myocardium of a patient affected by chronic ischemic cardiomyopathy. Nuclei and metaphase chromosomes are labeled by propidium iodide **(blue)**. Laminin defines the boundary of the cells **(green)**.

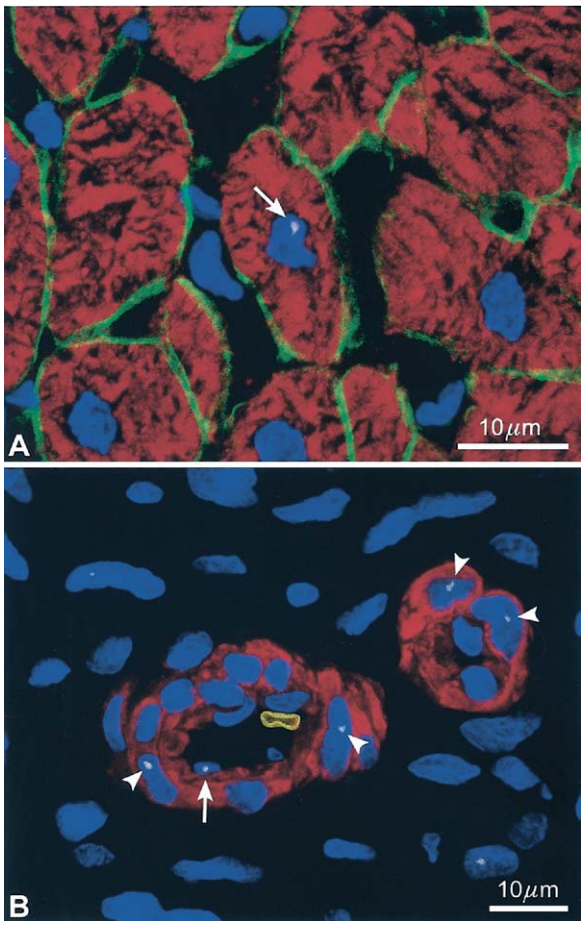

Figure 2. Chimerism of the transplanted female human heart. The localization of the Y-chromosome in the nucleus of a myocyte (**A**; alpha-sarcomeric actin, **red**; **arrow**), endothelial cell **(B, arrow)**, and smooth muscle cells (**B**; α-smooth muscle actin, **red**; **arrowheads**) is illustrated in the left ventricle of a female heart transplanted in a male recipient. Laminin defines the boundary of the cells **(A, green)**. A red blood cell is present in the lumen of the coronary arteriole (**B**; glycophorin; **A, yellow**).

differentiated tissue incapable of undergoing regeneration. An editorial was published stating that light microscopic images can document artifacts inherent in confocal microscopy and therefore myocyte chimerism did not occur (28). However, if a result is questioned, the challengers must use a technique and an approach that are at least as sensitive as those employed to obtain the information being challenged.

Over the years, several lines of evidence have suggested that the heart is a dynamic organ: Myocyte-restricted overexpression of insulin-like growth factor-1 (13), telomerase (29), cyclin D (18), bcl-2 (30) or cdk2 (31) is associated with an increase in the number of cardiomyocytes and higher tolerance to pathologic conditions. Additionally, the number of myocytes in the myocardium increases several-fold from birth to adulthood, and BrdU, MCM5, Ki67, or thymidine labeling of myocytes persists throughout life in rodents, indicating a continuous generation of parenchymal cells (13). Similarly, heart failure in large mammals is characterized by up-regulation of telomerase activity, stimulation of cyclins and cyclin-dependent kinases, and enhanced myocyte karyokinesis and cytokinesis (32). Myocyte regeneration in humans is significant acutely and chronically after infarction, dilated cardiomyopathy, and aortic stenosis (3,4,6,15). These results were expected to promote a change in paradigm, pointing to a more realistic view of the potential mechanism of cardiac growth. Unfortunately, review articles and editorials published in 2002 to 2004 tried to reestablish the dogma that the heart becomes terminally differentiated one day after birth (33). It is emblematic that the ability of the heart for proliferative growth was defined as "on shadings between none and almost none (18)."

PLASTICITY OF BONE MARROW PROGENITOR CELLS AND MYOCARDIAL REGENERATION

During prenatal life, undifferentiated cells undergo a hierarchical progressive restriction of developmental options, and this mechanism of embryonic specification was thought to be irreversible and inviolable in adulthood. However, this notion has been challenged by several examples of transition from one cell type to another or, more unexpectedly, from one cell lineage to another lineage (34). The ability of adult stem cells to generate cells beyond their own tissue boundary constitutes a process called developmental plasticity. Currently, the terms plasticity and transdifferentiation are used as synonyms, though transdifferentiation belongs to a broader class of cell transformation called metaplasia. Moreover, the reintroduction of the notion of cellular fusion, extremely popular in the 1980s, has created uncertainty about stem cell plasticity. Cellular and/or nuclear fusion requires the merge of two distinct cells with the formation of a hybrid. The growth of the heterokaryon depends on the nucleus of the undifferentiated cell, and the destiny of the heterokaryon is regulated by the differentiated cell (35).

The most versatile cell is the BMC, which is the best-characterized cell in terms of surface antigens and growth properties in vitro and in vivo. The focus of plasticity and fusion was therefore directed to BMCs to identify therapeutic strategies for tissue regeneration. A number of studies suggested that injury to a target organ promotes alternate stem cell differentiation, raising the possibility that BMCs have the ability to restore dead myocardium after infarction. For this purpose, BMCs were injected in the border zone of a myocardial infarct or were mobilized systemically into the circulation with cytokines. Both interventions led to the repair of the injured tissue and the formation of functionally competent myocardium in mice (9,36). Clinicians rapidly implemented BMCs in the management of ischemic and nonischemic cardiomyopathy in humans. Several clinical trials have been completed demonstrating that the administration of BMCs is safe and therapeutically promising (37). Double-blind clinical trials are ongoing in Europe, Asia, and the U.S., and, before their

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completion, it is difficult to establish the actual therapeutic efficacy of BMCs for the diseased heart.

Recently, two studies (10,11) and two commentaries (33,38) have presented negative results, criticizing the early experimental data and clinical trials. The main criticism was that the documentation of new myocytes derived from BMCs injected in the infarcted heart (9) was the result of autofluorescence and, thereby, a product of unspecific labeling detected by confocal microscopy (10,11,33,38). The possibility of autofluorescence artifacts was initiated by the erroneous interpretation of poor fixation of skeletal muscle made in Goodell's laboratory (39). The uneven distribution of green fluorescence detected in frozen sections of skeletal muscle was considered equivalent to fluorescence resulting from labeling of cells positive for enhanced green fluorescence protein (EGFP) with GFP primary and secondary antibody. The autofluorescence that is generated by the cross-linking of skeletal muscle proteins during aldehyde fixation by immersion of muscle samples has nothing to do with the detection of EGFP in cardiomyocytes with anti-GFP antibody. The intensity of the actual fluorescent signal is at least 20- to 30-fold stronger than background fluorescence (40). Similarly, the use of light microscopy (10) or confocal microscopy and frozen sections (11) has severe limitations; they involve quality of the sections, immunolabeling, and microscopic resolution. With this approach, major difficulties exist in the identification of small tissue structures; myocytes derived from BMCs have a diameter of 3 to 5 μm and a volume of <500 μm^3 (Fig. 3). Transdifferentiation of human BMCs into myocytes has been confirmed by others in vivo (41–43).

The notion of BMC transdifferentiation has also been questioned on a different ground, claiming that cardiac repair is not a primary event but a secondary process. Fusion of BMCs with preexisting parenchymal cells that subsequently reenter the cell cycle and lead to the formation of a large progeny has been proposed as an alternative mechanism of tissue repair (12). However, whether BMCs transdifferentiate or fuse, growth activation occurs, numerous cells are created and the diseased organ phenotype is largely rescued (44). These findings should have minimized the biologic controversy between these two distinct forms of growth, because from a clinical standpoint the intervention was successful, regardless of the mechanism(s) involved.

Unfortunately, the concept of cell fusion was introduced in an attempt to challenge once more the existence of stem cell plasticity and the prospective therapeutic efficacy of BMCs for the human disease (45). Complex animal models were introduced (10–12,46) and their limitations ignored (47) to address a question that can easily be answered with more direct and simpler approaches. They include the analysis of the karyotype and the detection of the number of sex chromosomes in the nuclei (40). Surprisingly, these fundamental determinations were not performed in studies claiming the presence (46) or absence (48) of fusion events. Currently, fusion of BMCs with cardiomyocytes remains an

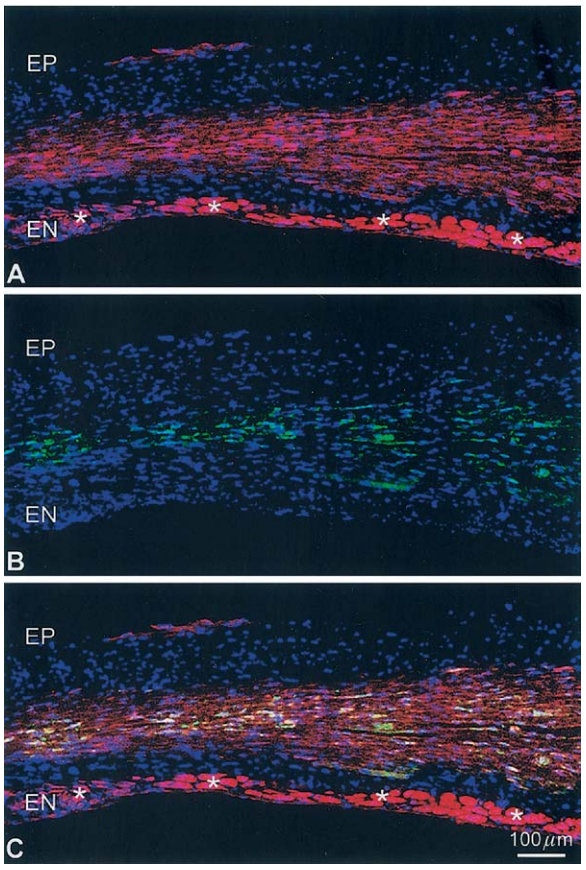

Figure 3. Bone marrow cells promote myocardial regeneration after infarction. The intramyocardial injection of enhanced green fluorescent protein (EGFP)-positive blood marrow cells in mice acutely after infarction induced the formation of new myocardium. In the infarcted region, the newly formed myocytes are small (**A**; alpha-sarcomeric actin, **red**) and show EGFP in the cytoplasm **(B, green)**. **(C)** This merged image of **A** and **B**. The regenerated myocytes express both α-sarcomeric actin and EGFP **(yellow-green)**. These myocytes are $\sim$400 μm^3 in volume and, because of their size, cannot be the product of cell fusion. Nuclei are labeled by propidium iodide **(blue)**. *Spared myocytes in the subendocardium. EP = epicardium; EN = endocardium.

in vitro phenomenon (41), although occasional examples have been reported in the normal heart. The several million myocytes formed in the infarcted mouse heart by injection of BMCs are the product of BMC transdifferentiation and not cell fusion (9,40). New myocytes have a volume $\sim$20-fold smaller than the remaining cells and contain a number of chromosomes that excluded cell fusion (Fig. 4). This is an important principle, because fusion of BMCs with adult mature myocytes cannot induce multiplication of terminally differentiated cells and result in significant myocyte formation and tissue reconstitution.

The reports and editorials discussed above have challenged an earlier study (9). It is unfortunate that these differences were not resolved by conducting parallel experiments and exchanging reagents and protocols among the diverging groups. The suggestion that laboratories with different results become engaged in a collaborative effort to clarify the dispute has to be followed to settle confusion and debate and to promote clarity and understanding. In this

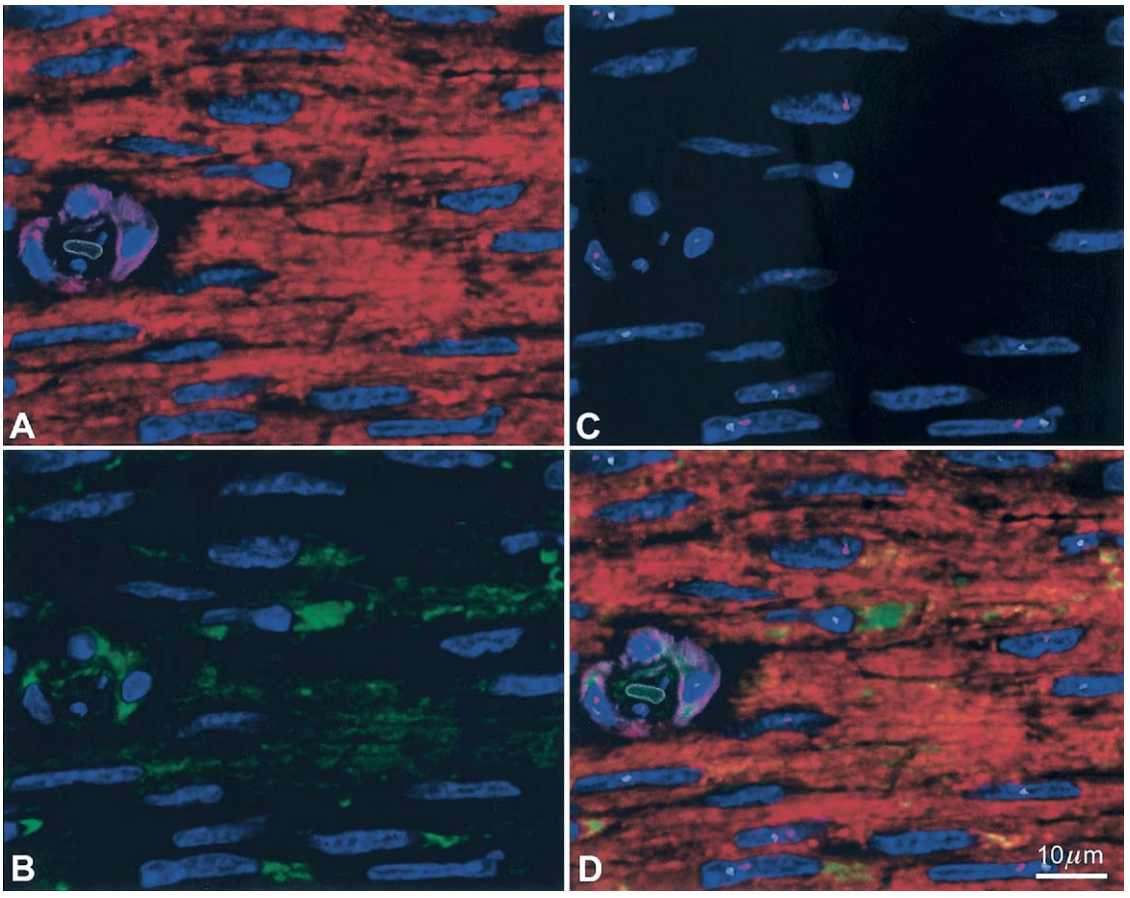

Figure 4. Myocyte regeneration by bone marrow cells does not involve cell fusion. The intramyocardial injection of enhanced green fluorescent protein (EGFP)-positive male blood marrow cells in female mice acutely after coronary artery ligation induced the formation of male myocytes in the infarcted region. The newly formed myocytes are small (**A**; alpha-sarcomeric actin, **red**) and express EGFP in the cytoplasm **(B, green)**. A small developing arteriole is also present (**A**; alpha-smooth muscle actin, **yellow**). The nuclei (**C**; propidium iodide, **blue**) contain at most one Y-chromosome **(white dots)** and one X-chromosome **(magenta dots)**, indicating the male phenotype of these cells and therefore excluding cell fusion. **(D)** The merged images of **A**, **B**, and **C**. The regenerated myocytes express alpha-sarcomeric actin, EGFP **(yellow-green)**, and one X- and one Y-chromosome.

manner, the controversy, discomfort, and uncertainty that have been generated in the scientific and clinical community may be overcome to help heart failure patients in facing a dramatic decision as never before.

In summary, BMCs appear to have the ability to generate new myocardium independently of cell fusion. If negative results would have been more cautiously interpreted, it is likely that the actual role that BMCs play in cardiac repair would have been better understood and appreciated. The heated controversy about BMC plasticity will only be resolved when laboratories with conflicting results are willing to work together and amicably resolve their differences instead of perpetuating a futile debate.

CARDIAC PROGENITOR CELLS AND MYOCARDIAL REGENERATION

The most common comment made against myocyte replication is that the heart does not regenerate itself after infarction (18). Even if a few myocytes are created, the growth reserve of the heart is severely limited and the intrinsic mechanisms of repair are inadequate for reconstitution of the injured myocardium. There is validity in this definition because it describes the evolution of the infarcted heart. It establishes the basis for cell therapy and the search for the most appropriate cell for the restoration of the necrotic myocardium. The inability to rebuild an infarct is not restricted to the heart but it is a general characteristic of all organs regardless of whether their cells proliferate or not. The outcome of infarction is identical whether it affects heart, brain, liver, kidney, testis, skin, or intestine. Resident stem cells do not spontaneously migrate and home to the damaged area where they can grow and mature to replace the dead tissue. Healing occurs, a scar is formed, and regional function is permanently impaired.

The unsuccessful repair of the infarcted heart does not necessarily indicate that the growth reserve of the surviving myocardium is insufficient to reconstitute the amount of mass lost after ischemia. The magnitude of growth that the human heart can achieve in response to a chronic increase in pressure and/or volume load is enormous. Hearts weighing more than two pounds and containing two to three times the number of myocytes present in normal hearts have repeatedly been de-

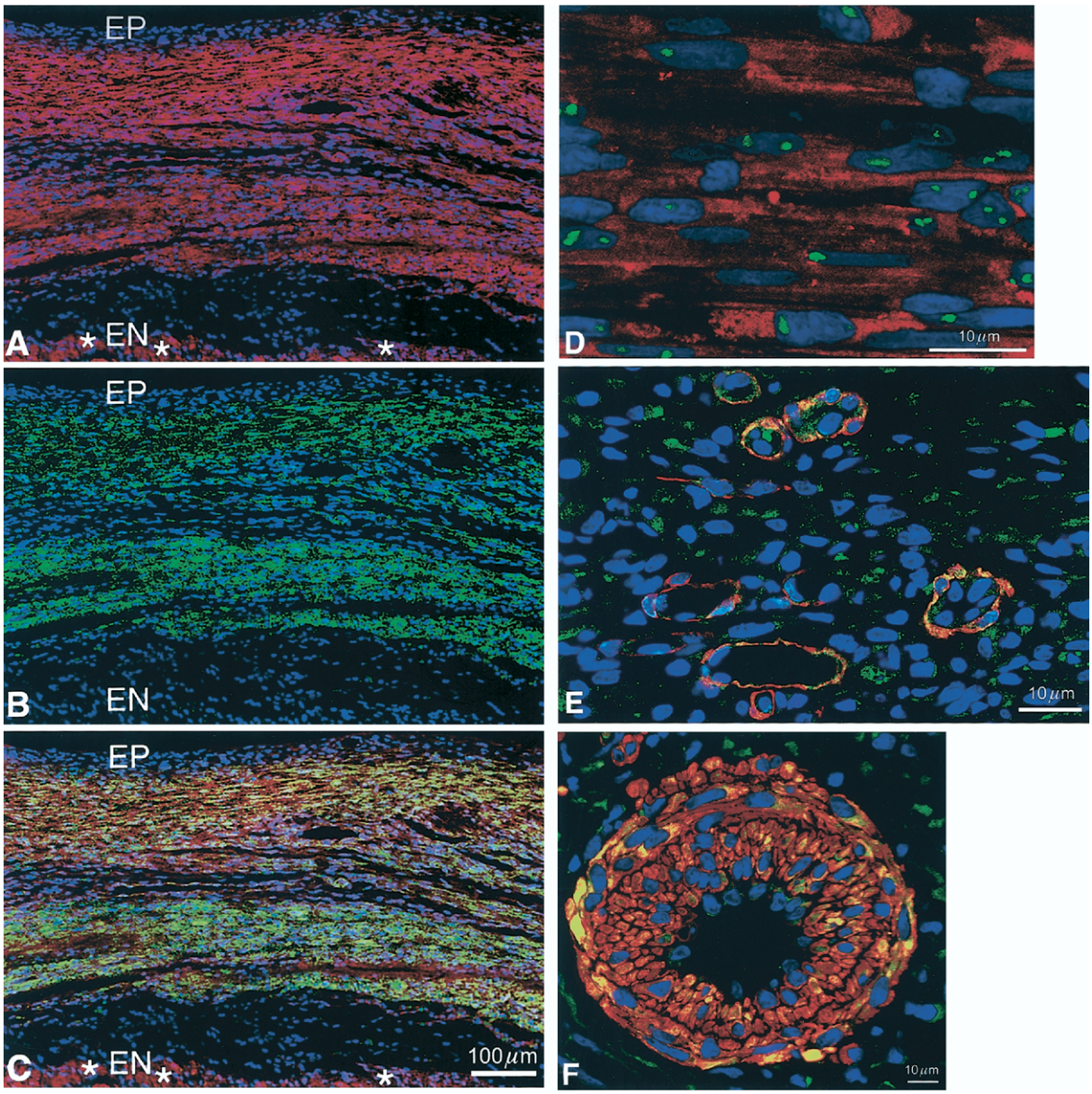

Figure 5. Cardiac stem cells (CSCs) and myocardial regeneration after infarction. The intramyocardial injection of enhanced green fluorescent protein (EGFP)-positive CSCs in syngeneic rats acutely after infarction induced the formation of myocardium. The new myocytes express alpha-sarcomeric actin **(A, red)** and EGFP in the cytoplasm **(B, green)**. **(C)** The merged images of **A** and **B**. The regenerated myocytes show both α-sarcomeric actin and EGFP **(yellow-green)**. Nuclei are labeled by propidium iodide **(blue)**. **(D)** The formed myocytes are small and carry at most two chromosomes 12 **(green dots)**. Therefore, myocyte regeneration does not involve cell fusion. Laminin is distributed between myocytes **(white lines)**. **(E, F)** Regenerated coronary arterioles that are positive for alpha-smooth muscle actin and EGFP **(yellow-red)**. *Spared myocytes in the subendocardium. EP = epicardium; EN = endocardium.

scribed in humans (13,49). The issue at hand is whether strategies can be developed to modulate the regenerative potential of the human heart. Interventions have to promote translocation of CSCs from the site of storage to the infarct, their activation and differentiation into myocytes and coronary vessels, ultimately, mending the "broken heart."

Maturation and survival of myocytes invading the infarct is dependent on the availability of oxygen in the area undergoing repair. There are two prerequisites for successful integration of cells in the ischemic region. Coronary arterioles and capillary structures have to be formed in order to bridge the dead tissue and establish communication with the normally perfused vessels of the viable myocardium (Fig. 5). Additionally, the new vascular supply has to permeate the engrafted myocytes to preserve their survival, and favor their growth, differentiation, and contractile function. There is an orderly organization of myocytes within the myocardium and a well-defined relationship between the parenchymal cells and the capillary network (13). This proportion is altered with cardiac pathology, and the goal of cell therapy is the reconstitution of the heart with its physiologic and structural properties.

Several strategies have been implemented experimentally to repair the infarcted heart. They include fetal cardiomyocytes, skeletal myoblasts, embryonic-derived endothelial cells, bone marrow-derived immature myocytes, fibroblasts, smooth muscle cells, endothelial progenitor cells, and BMCs (50). These approaches had a rather uniform outcome that consisted of variable degrees of improvement in cardiac performance. This was most likely due to the formation of a passive graft that reduced negative remodeling by decreasing the stiffness of the scarred portion of the wall. An active graft, which dynamically contributes to myocardial contractility, has been observed in only a few cases (9,36,40,41,43). However, the implanted cells may exert a paracrine effect activating a growth response of resident progenitor cells (41,51).

Recently, a CSC has been identified and characterized in the heart of rats (25), mice (46,52,53), and dogs (5). It is intuitively apparent that resident CSCs are the preferential cells to be tested for cardiac repair, because these cells are programmed to make myocytes and vascular structures. The c-kit–positive cells possess the fundamental properties of stem cells: They are self-renewing, clonogenic, and multipotent (5,25). The intramyocardial injection of c-kit–positive CSCs or their local activation by growth factors results in significant reconstitution of the infarcted heart (5,25,54). A more limited impact on myocardial regeneration was obtained with the intravenous delivery of stem cell antigen 1-positive cells following ischemia-reperfusion injury (46). Whether the less impressive outcome was related to the route of administration, the animal model, or the distinct progenitor cell is unclear.

The Isl-1–positive cell has been improperly presented as a new CSC and claimed to be important for the reconstitution of the adult damaged heart (48). It has been known for quite some time that the Isl-1 transcription factor is present in cells that are implicated in the morphogenesis of the embryonic mouse heart (55). The homozygous deletion of Isl-1 results in developmental defects of the right ventricle, atria, and outflow tract. It is surprising that Isl-1–positive cells have been interpreted as a distinct population of CSCs, because the expression of Isl-1 corresponds to the onset of myocyte commitment; Isl-1, together with GATA-4, is a transcriptional activator of the myocyte transcription factor MEF2C. Moreover, the expression of Isl-1 in progenitor cells clustered in the niches or scattered throughout the atrial and ventricular myocardium of the adult mouse heart is, at best, extremely rare. Similarly, Isl-1–positive cells have not been detected in the failing human heart, calling into question the role of these cells in cardiac pathology. Even during development, Isl-1–positive cells are not implicated in the formation of the left ventricle. Thus, there is no basis for the conviction that Isl-1–positive cells are the "true" CSCs or are relevant for treatment of the diseased human heart (48).

Myocardial repair requires the formation of myocytes and coronary vessels, and it cannot be accomplished by a cell already committed to the myocyte lineage. Myocytes would not grow or survive in the absence of vessels. Similarly, the utilization of cells capable of creating exclusively coronary vessels cannot result in significant tissue regeneration. In spite of an unsubstantiated and rather popular belief (33), vessels alone do not generate force in an akinetic scarred region of the ventricular wall. Myocardial regeneration necessitates the administration of a more primitive cell that is multipotent and can differentiate into the main cardiac cell lineages: myocytes, vascular smooth muscle cells, and endothelial cells.

In conclusion, the demonstration that the heart harbors stem cells capable of creating myocardium points to novel strategies for a safe and robust regenerative response of the failing infarcted and noninfarcted human heart. Cardiac stem cells may be coaxed in vivo to home to damaged regions to promote the formation of functionally competent myocytes and coronary vasculature. A rapid and efficient restoration of lost myocardium is often crucial for the survival of the organ and organism. This clinical necessity has its dramatic overtone in patients with large myocardial infarcts in which the immediate reduction of infarct size is critical for survival. The CSC offers an alternative or complementary therapeutic approach to exogenous cells. The extraordinary clinical potential of myocardial repair makes the dissection of the biology of the CSC a challenging and exciting endeavor.

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