Generation of new cardiomyocytes after injury: *de novo* formation from resident progenitors *vs*. replication of pre-existing cardiomyocytes

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Heart failure is the number one killer among all the cardiovascular diseases (1). Indeed, mortality of heart failure patients is high at first diagnosis and, sadly, poorer at 3-5 years after its onset (1). This negative outlook has been worsened by the increasing yearly incidence of new onset heart failure, which has now become truly epidemic worldwide (1). All heart failure treatments in use aim at improving symptoms with the exception of heart transplantation, the only curative treatment, with all its logistic and insurmountable limitations (1). Thus, it is not surprising that regenerative biology and medicine have focused on the search for an effective and broadly applicable replacement of contractile muscle mass to compensate for cardiomyocyte loss, deficit and dysfunction (2). Indeed, the main proximate cause of heart failure is ischemic cardiac disease, in particular myocardial infarction, in which functional tissue is replaced by scar with little or no significant anatomically and functionally effective myocardial regeneration. Even when heart failure is of non-ischemic origin, as is the case of the many forms of structural, infectious and toxic cardiomyopathies, the key issue is also the failure of the affected myocardium to generate a sufficiently robust and functional cardiomyocyte replacement. This is the problem creatively addressed by the paper of Shapiro *et al*., prompting this Editorial, which using a relevant pre-clinical animal model provides the design and development of a cardiac regenerative therapy based on fostering new myocyte formation through the regulation of the cell cycle (3).

In the adult, most mammalian somatic cells are highly differentiated to carry out specific functions and, with the exception of the skin, lining of the gut and bone marrow, are static cells permanently withdrawn from the cell cycle or elementi perenni, like neurons. On the other hand, quiescent cells in G_0 upon appropriate stimulation can re-enter the cell cycle and undergo mitosis to generate more cells with similar characteristics. Hepatocytes represent a good example of a specialized parenchymal cell type able to regenerate a cohort of similar cells in response to diverse physiological needs (4). On the contrary, the myocardial response to increased load or loss of contractile power is determined by the fact that, early in the postnatal period, mammalian cardiomyocytes become incapable of undergoing mitosis and, therefore, of replicating themselves (5). This process, termed terminal differentiation, represents an irreversible commitment to the differentiated phenotype and permanent cell cycle arrest, despite the fact that these terminally differentiated cells continue to express functional growth factor receptors, as shown by their ability to respond to different ligands by induction of immediate early response genes (5,6).

The molecular mechanism(s) responsible for terminal differentiation of mammalian cardiac myocytes early in post-natal life and, in particular, their stubborn block to re-enter a productive cell cycle has been intensively investigated. The search to re-activate mitotic competence

has been stimulated, at least in part, by the prospect that such re-activation could result in cardiomyocyte regeneration in the adult (6). Studies in skeletal muscle have shown that a muscle lineage determination gene, namely MyoD (a transcription factor of the basic helix-loop-helix family and the myogenic factors subfamily), directly interacts with the retinoblastoma protein (Rb) in its under- or un-phosphorylated form to produce and maintain the terminally differentiated state (7). Indeed, disruption of the MyoD-Rb mitotic block protein assembly renders terminal-differentiated skeletal myocytes responsive to serum growth factor stimulation to re-enter the cell cycle showing that inactivating Rb can reverse terminal differentiation (7).

Cardiac muscle cells on the surface appear very similar to skeletal muscle, especially because they share expression of a large numbers of structural and contractile and excitation-contraction coupling proteins. However, cardiac myocytes have a different embryological origin, biology and regulatory gene networks (8). First and foremost in the context of this Editorial, cardiomyocyte specification and capacity for hyperplasia are not mutually exclusive. The heart, the first functional organ to develop in embryonic life, grows dramatically in size and functional capacity in all mammals so far studied, including human, mainly via myocyte cell division until shortly after birth (8). Contrariwise, in most skeletal muscle, these two processes, differentiation and ability to undergo mitosis, are mutually exclusive. That is, cycling myoblasts do not express much of a differentiated phenotype and differentiated mature skeletal myocytes are permanently withdrawn from the cell cycle (9). Second, cardiac muscle cells have not been shown to express any so-called master determination gene like MyoD (10). This latter difference is exemplified by the need to activate multiple genes for cardiomyogenic specification in embryonic stem cells (11), direct reprogramming of fibroblasts (12) and post-natal cardiomyocytes (13,14) into regenerating myocytes. Accordingly, Rb knock-out is not sufficient to un-lock and reverses the terminally differentiated state of cardiomyocytes. p130 and Rb double-knock-out results in cardiomyocyte disarray, apoptosis and cardiac function impairment (15). Accordingly, Shapiro *et al.*, in this issue explore protocols to potentially get around this problem.

Cardiomyocytes terminally differentiate starting in late fetal life and up to the neonatal period [by postnatal day 5 (P5) in the mouse and 1 month in the human] with a reported time-limited burst of cell division in early preadolescence (P15 in the mouse) after which expression of the genes responsible for cell-cycle entry, S phase, mitosis, and cytokinesis fall dramatically. These changes are accompanied between P5 and P14 by extensive binucleation of the cardiomyocytes, a widely accepted, but not strictly required, hallmark of terminal differentiation (16). Despite the abundant and widely confirmed molecular and cellular evidence of cardiomyocyte permanent withdrawal from the cell cycle, recent evidence has indisputably proved that new cardiomyocytes are added in the post-neonatal mammalian heart, including the human, either in response to physiological tissue wear and tear or different forms of myocardial injury (17-19). The rate of this constant and reparative myocyte replenishment seems to be specific for each species and it has been highly debated. Most reports have extrapolated an annual rate of about 1% myocyte formation in adult healthy individuals (20). This replenishing rate increases significantly after injury but its precise quantification is even more passionately argued than the existence of the phenomenon itself (20). Over and above the noise of the controversy, it should be remembered that if the nay-sayers were correct that no new myocytes are formed after the replicative post-natal period, the heart would be the only known parenchymal mammalian organ devoid of parenchymal cell replenishment. Thus, the heart will be caught in an irreversible downward spiral of continuously decreasing cell number from cradle to grave. Nevertheless, independently of the controversy, there is an obvious consensus on the fact that, whatever the nature and robustness of the regenerative response, it is insufficient to counteract the myocyte loss and dysfunction which occurs after myocardial infarction and in heart failure. Therefore, it is reasonable to expect and predict that robust and functional myocyte replenishment may be obtained by better understanding the cellular and molecular basis of cardiomyocyte generation in the adult heart as addressed by Shapiro *et al.* (3).

A main problem besieging cardiac regeneration has been and remains the controversy about the origin of the myocytes which are born post-natally; that is, after most or all cardiomyocytes are terminally differentiated and permanently withdrawn from the cell cycle. Most, if not all, adult mammalian tissues contain a small pool of resident tissue-specific progenitor cells whose functional differentiation is responsible for the maintenance of tissue/organ cell homeostasis and replacement throughout the lifespan of the organism. The mammalian heart, including the human, also contains a pool of resident tissue specific cardiac stem/progenitor cells (CSCs) (21),

capable to differentiate *in vivo* and *in vitro* into the four main myocardial cell types: myocytes, endothelial and smooth vascular cells and connective tissue cells. Recent evidence has shown by a variety of well-accepted cellular, genetic and molecular means that CSCs are necessary and sufficient for myocardial cell homeostasis, repair and regeneration (22). Contrariwise, it has been argued, based on the identification of cells expressing cardiac specific gene markers undergoing DNA replication and cytokinesis that post-natal cardiomyocyte replenishment derives not from the CSCs but mainly from division of pre-existing adult cardiomyocytes (23). Obviously, taken at face value, these two viewpoints are antithetical and the second would require that cardiomyocyte division occur in otherwise terminally differentiated cardiomyocytes. However, as often is the case in science when the energy is used to generate heat instead of light, the discordance between the two viewpoints might be more apparent than real and due mostly to incomplete data sets and misinterpretation of the data available.

Robust evidence has not been yet presented that terminally differentiated cardiomyocytes can really re-enter the cell cycle and undergo productive cytokinesis. Obviously, there is also no evidence of further rounds of cell division by the daughters of the small number of myocyte mitosis so far identified. Aside from the recent data that terminal differentiation of post-natal cardiomyocytes in the mouse might happen a bit later than previously believed (24), all available evidence of myocyte division in adulthood has been shown to occur in small-sized mononuclear myocytes (20), while in adult mice/rats >80% of the myocytes are bi-nucleated (24). The rare spotting of cardiac cells expressing contractile protein genes undergoing mitosis have prompted the hypothesis that there exists a small pool of adult cardiomyocytes retaining proliferative competence (25) or, even more speculatively, of a stochastic phenomenon occurring in adult cardiomyocytes that would very rarely favour replicative competence over terminal differentiation. These assertions of what we would provocatively name the "UFO hypothesis of cardiomyocyte regeneration" and its apparent contradictions with the "stem/precursor cell hypothesis of cardiomyocyte replenishment" need to be interpreted in light of the well-established facts in adult stem cell and regenerative biology. Bona fide adult resident stem cells in healthy tissues seldom divide and the tissue parenchymal cell types are mainly replaced by the expansion of transient amplifying cell intermediates (progenitors/precursors) derived from the symmetric or asymmetric division of the more primitive stem cells (26). The main difference between homeostasis in healthy tissue and repair after damage is that in the latter a significantly higher number of stem cells become activated and amplify to generate a larger cohort of progenitor/precursors which, in turn, commit and differentiate into the specific cell lineages of the tissue/organ (27). Viewed from this perspective, it is clear that the hypothesis of mature myocyte division as source of the myocytes born in the mature myocardium requires to be convincingly documented showing that the dividing small mononucleated cardiomyocytes are/were fully mature and fully differentiated pre-existing myocytes that have re-entered the cell cycle and NOT transient amplifying myocytes derived from the differentiation of resident stem/progenitor cells as previously hypothesized and reported (28). Because, except for binucleation (or polyploidy), there are no known cellular or molecular markers to identify mature terminally differentiated myocytes from those that have not yet reached terminal differentiation, it is clearly a tall hurdle to ascertain that mature myocytes have re-entered the cell cycle.

Importantly, the two antithetical positions as to the source of new myocyte formation in homeostasis and repair are not necessarily mutually exclusive, and theoretically both could participate in the generation of new cardiomyocytes *in vivo* and *in vitro*. This open-minded position is wisely discussed in the paper by Shapiro *et al*. (3). However, it is worth remembering that aside from pluripotent stem cell myogenic differentiation so far the only solidly documented adult source of well differentiated cardiomyocytes *in vivo* and *in vitro* is the CSCs, even when starting from a single cell derived clone (21,22,29).

Neonatal myocyte division competence followed by terminal differentiation early in life in mammals together with the ability of adult cardiomyocytes from certain non-mammalian vertebrate species to replicate and repair myocardial loss throughout the lifespan of the organism are currently widely investigated. It was recently also shown that, in early postnatal stages, the mouse [and also the human as shown in rare cases of severe myocyte loss in early infancy as consequence of anomalous origin of a coronary artery from the pulmonary artery (30)] possesses a transient and robust regenerative capacity in response to injury (31) comparable to that shown by certain fish and amphibians, which can regenerate their myocardium throughout life (32,33). The ultimate goal of most of this research is to identify the mechanisms that govern the

postnatal loss of cardiomyocyte proliferation in mammals and those responsible for the persistence of myocyte mitotic competence in non-mammalian species. It is expected that this knowledge will allow manipulation of these pathways to reactivate cardiomyocyte proliferation in the injured adult mammalian heart (6).

Despite the appeal of the biology involved, there are many practical and conceptual problems with the attempts to extrapolate the biology of the newborn mammalian heart and the regenerative capacity of certain fishes and amphibians to the adult mammalian heart in general and the human in particular. There is no doubt that understanding the basis for neonatal myocyte division is of paramount importance to identify the mechanism(s) responsible for terminal differentiation. This could also help to properly instruct pluripotent or tissue adult stem cells to undergo efficient lineage specification and maturation into functional contracting myocytes that contribute to ventricular performance *in vivo*. However, it is a risky leap into the unknown to use this information to attempt *in situ* adult myocardial regeneration. At present there is not a single piece of experimental evidence that the genetic program regulating late fetal and neonatal mammalian myocyte division and maturation has any relevance for the phenomenon of myocyte replenishment in the adult. Notwithstanding the elegant studies documenting that adult cardiomyocytes divide in some vertebrate species other than mammals, there is no basis to extrapolate this biology to the adult mammalian heart. These species can also regenerate lost limbs and despite this uncontested evidence no one is attempting to extrapolate this property to the mammals. Furthermore, the myocardium is a privileged tissue with a very low incidence of neoplasias. Attempts to induce replication in the cohort of terminally differentiated cardiac cells might entail a Faustian bargain with the risk of awakening the sleeping genie with the result of trading a myocyte deficit for uncontrolled cell proliferation.

Despite the pitfalls above-mentioned, mammalian neonatal myocyte proliferation and myocyte replenishment in amphibians have raised the provocative possibility that a single gene mutation could un-block cardiomyocyte mitotic arrest and create a permissive environment for a functional robust regenerative process (34). Unfortunately, however, most attempts to re-activate mitotic competence in terminally differentiated murine myocytes through myocyte-restricted gene modulation have resulted in increased polyploidy or myocyte death by apoptosis (6). Cyclin A2 (Ccna2), a key cell cycle regulator that complexes with its cyclin-dependent kinase partners to mediate both the G1-S and G2-M transitions of the cell cycle, is silenced shortly after birth in mammalian cardiomyocytes (35). In two small-animal models of myocardial infarction (MI), Hina W. Chaudhry and colleagues demonstrated that myocyte-specific transgenic expression of Ccna2 ameliorates cardiac repair along with an increased number of cardiomyocyte mitoses after MI (35,36). The same group have now reported elegant and clinically translatable results in the pig (3). Delivery of Ccna2 to the infarcted myocardium produces a robust regenerative response in pigs. One week after myocardial infarction produced by balloon occlusion of left anterior descending coronary artery, an adenovirus carrying cDNA encoding CCNA2 or an empty adenovirus were intra-myocardially injected into the peri-infarct zone through a left thoracotomy. Six weeks after treatment, a multimodality imaging approach including magnetic resonance imaging, documented a 20% increase in ejection fraction in Ccna2-treated pigs while there was 4% decrease in the control animals. Myocardial histology 7 weeks post-MI showed improved myocardial remodelling in the CCNA-2 treated animals with decreased reactive myocyte hypertrophy, attenuated myocardial fibrosis and almost a two-fold increase in cardiomyocyte number per unit area in the infarct zone. Interestingly, a significant increase in mitotic myocytes was detected in the infarcted myocardium from 0.5% in the control animals to 2% in the CCNA-2 treated animals. No significant improvement in vascular density was observed with CCNA-2 treatment. Finally, CCNA-2 was transfected into adult porcine cardiomyocytes *in vitro*, showing cytokinesis in some transfected cells by time-lapse microscopy. However, despite the predicted high efficiency of the adenovirus transfection, CCNA-2 up-regulation induced cytokinesis in less than 3% of the transfected cells.

A significant strength of the Shapiro *et al*. report is that the study was performed in a relevant pre-clinical experimental setting and in an animal model that closely mimics the human in size, anatomy and physiology. As well pointed out by the authors, large-animal models of cardiovascular disease are of paramount importance on the road to clinical translation of successful experimental therapies. Indeed, in a mouse model sparing or generating of a few mg of myocardial tissue is physiologically significant while irrelevant in a large-animal or human heart where only gram amounts of functional tissue will impact function. Accordingly, it remains to be proven that an any regenerative approach effective in replacing the ~1 million of cardiomyocytes lost after an MI in mice

can be scaled up to regenerate the billions of myocytes lost after a human or large animal MI. Shapiro *et al.*, should also be commended for using different, up-to-date and clinically relevant cardiac imaging techniques to measure the functional effect of the Ccna2 treatment compared to the control. Not surprisingly, because of the translational experimental approach used, many questions about the cellular and molecular mechanisms of the beneficial therapeutic effect are left unanswered: it would be important and illuminating to know whether or to what extent Ccna-2 up-regulation in a sub-acute MI setting favourably impacted the survival of pre-existing cardiomyocytes; histology at 7 weeks post-injury is a snapshot of the final outcome but, without DNA labelling during follow-up, the cumulative myocyte formation over the course of the experiment remains unknown. Accordingly, as also pointed out by the authors, their study could not determine the contribution, if any, of resident cardiac stem/progenitor cells in the regenerative response in the Ccna-2 treated pigs. In this regard, in their previous rodent study, the authors demonstrated that Ccna2 myocardial overexpression increased resident cardiac progenitor cell activation after injury (36). Finally, no matter how undisputable the detection of mitotic myocytes in the infarcted hearts, the available data does not and cannot determine whether these cycling myocytes are pre-existing terminally differentiated cardiomyocytes that have re-entered the cell cycle or myocytes derived from stem/progenitor cells that have yet to be permanently withdrawn from the cell cycle, an interpretation we favour. In this respect it is noticeable that the available representative figures and videos of mitotic myocytes *in vivo* and *in vitro* show small-sized mononucleated contractile gene-expressing cells. Nevertheless, these issues do not undermine the undisputable beneficial effect of the single gene transfection strategy on myocardial remodelling and function after MI.

In conclusion, Shapiro *et al.* have provided another exciting instalment of the evolving cardiac regeneration field. As is the case for any scientific research field with a high potential to positively impact human health, new results lead to new questions that can only be answered by additional experimental work.

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