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Proliferation of cardiomyocytes: a question unresolved

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1. ABSTRACT

In the past, human heart has been considered a post-mitotic organ formed by cells, like adult cardiomyocytes, terminally differentiated and incapable of proliferation. This paradigm has been shaken by recent works indicating that postnatal adult cardiomyocytes are able to re-enter in the cell cycle and proliferate. In this view, the understanding of main candidates regulating cardiomyocyte cell cycle is of vital importance for future clinical approach.

2. INTRODUCTION

Highly differentiated mammalian cells, like adult cardiomyocytes, are thought to be incapable of proliferation; therefore, it is not surprising that complex and often overlapping systems have evolved to regulate their growth (1, 2). In the past, the heart has been considered as a terminally differentiated organ unable to replace working cardiomyocytes (3). In addition the concept was that the heart reacts to an increase in workload only by hypertrophy of the existing cardiomyocytes during

postnatal maturation, adulthood, and senility (3). 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 cardiomyocytes. Subsequent reports from 1921 to 1925 questioned the ability of cardiomyocytes to proliferate, suggesting that the increase in cardiac muscle mass in the pathologic heart was the result of pure cellular hypertrophy (4). 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 cardiomyocytes in humans should be terminally differentiated cells, and their lifespan corresponds to that of the individual (3). The number of cardiomyocytes attains an adult value a few months after birth (4), and the same cardiomyocytes 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 cardiomyocytes are immortal, functionally and structurally (3). Since cardiomyocyte cell death occurs with age, the chronic loss of cells in the absence of cardiomyocyte duplication would result in the disappearance of the entire organ over a period of a few decades (3). Recently, a new concept is prevailing over the solely cardiomyocyte hypertrophy: during normal cardiac growth to adulthood, new cardiomyocyte generation predominates over cardiomyocyte death and contributes significantly to the organ growth (5). In the course of normal aging, when this balance is altered and cardiomyocyte formation is overtaken by cell death, the number of ventricular cardiomyocytes decreases, cardiomyocyte hypertrophy becomes apparent, and with time, chronic heart failure supervenes (5). New cardiomyocyte generation, together with cardiomyocyte apoptosis and necrosis are major determinants of the evolution of pressure and volume overload hypertrophy pointing to common mechanisms in the response of the heart to workload and damage (5). Failure of new cardiomyocyte generation and hypertrophy to compensate for extensive cell dropout may be the most relevant factor in the onset of heart failure in the elderly (5). The finding that most cardiomyocytes irreversibly withdraw from the cell cycle soon after birth (5, 6) and the failure to develop cell culture system capable of supporting cardiomyocyte replication, further reinforced the notion that all adult cardiomyocytes are terminally differentiated (5). The strongest argument in favour of new cardiomyocyte formation in the adult heart is the increase in cardiomyocyte number from birth to young adulthood in both animals and humans (4). These data, obtained by morphometric analysis (4, 5), are consistent because they show that the increase in cardiac mass during normal growth cannot be accounted solely by cardiomyocyte hypertrophy (5). These results strongly suggest that cardiomyocyte renewal occurs throughout life in the myocardium and it is part and parcel of cardiac homeostasis (5). The recognition that cardiomyocytes are continuously replaced in adulthood and senescence, and that cell regeneration is enhanced by hemodynamic overloads (5, 7-10) and ischemia (11), indicates that cells of different ages are present during the entire life of the non-diseased and diseased heart (5). These distinct cell populations differ in their ability to react to growth stimuli. The history of a cell conditions the type and magnitude of its growth response and its capacity to succumb or survive apoptotic and necrotic death signals (5). Large cardiomyocytes are old, do not react to growth stimuli, are more prone to activate the cell death pathway and these features increase with age. Moreover, large cardiomyocytes express more inhibitors of the cell cycle (11) and, in response to work overload, are unable to activate the program responsible for the quantitative and qualitative changes in gene expression characteristic of cardiomyocyte hypertrophy (5, 12). Small cardiomyocytes are younger, retain the ability to divide and possess the ability to become hypertrophic and are less susceptible to cell death. The smallest cells have been born only recently and still can undergo a limited number of cell cycles (5). This subpopulation of cycling cardiomyocytes whose presence throughout life continuously changes the proportion of young and old cells in the heart, confers to myocardium a significant regenerative capacity. Now, the adult heart should not be considered a postmitotic organ (5).

How can cardiomyocytes proliferate? Heart development must be considered a complex multistep process that involves highly precise spatiotemporal changes in gene expression (13). Combinatorial interactions involving multiple transcriptional regulators control the various genetic program required for proper cardiomyocytes proliferation, differentiation and survival (13). In the past years, different critical cardiac regulators have been identified. In particular, in 1970's several investigators demonstrated by live cell imaging that neonatal cardiomyocytes divide *in vitro*, albeit rarely (14). From then on, considerable effort has been invested to accomplish mammalian cardiomyocytes *in vivo*.

In contrast with to adult cardiomyocytes, mammalian cardiomyocytes normally proliferate during foetal development. Analysis of cardiomyocyte proliferation during mouse development determined that cardiomyocytes DNA synthesis occurs in two distinct phases (15): the first occurring during foetal life is characterized by matching of karyokinesis and cytokinesis, resulting in cardiomyocyte proliferation (2); the second phase occurring early in the neonatal period, with a peak at day 4-6 postnatally, is characterized by the fact that karyokinesis occurred in the absence of cytokinesis, resulting in binucleation of the ventricular cardiomyocytes (2). However, before terminal withdrawal from the cell cycle, cardiomyocytes undergo a final round of incomplete cell division, resulting in binucleated cardiomyocytes (2). In humans, the species with the highest percentage of mononucleated cardiomyocytes and the degree of binucleation estimates ranged from 25 to 57% (16, 17). The physiological significance of having cells that are binucleated is unclear, but it has been postulated to be an adaptative response in metabolically active cells where the capacity to generate twice the RNA for protein synthesis might be advantageous (2).

In perinatal period, mammalian cardiomyocytes down-regulate cell-cycle-perpetuating factors like cyclin A

and cdk2 (1). The loss of proliferation capacity coincides with increased levels of the cell cycle inhibitors p21, p27 and Rb, similar to those seen in skeletal muscle (1, 2, 18-22). Typically, adult cardiomyocytes do not re-enter the cell cycle when exposed to growth signals, and further increases in cardiac mass are achieved through an increase in cell size or hypertophy (2). It appears that cardiomyocytes have acquired several mechanisms that prevent proliferation (14). Several possibilities may explain the observed limited proliferation potential of adult cardiomyocytes. Mature cardiomyocytes contain highly ordered structures, called sarcomeres, which contain contractile proteins required for force generation. This structure is incompatible with cytokinesis. Thus, primary adult mammalian cardiomyocytes are thought to be incapable of cytokinesis (1). Secondly, adult cardiomyocytes must continuously beat to sustain cardiac output, and it was not clear how they could simultaneously divide and contract. Thirdly, cardiomyocytes are often binucleated or polyploid (14).

In general, there is an inverse relationship between proliferation and differentiation (23), and molecules that promote differentiation may also repress cell cycle re-entry. However, the mechanism that regulates cell cycle exit of cardiomyocytes is unknown. This might be the reason why most studies trying to induce cardiomyocytes proliferation have focused on inducers of proliferation, namely growth factors, cell cycle genes, and oncogenes (14, 24-30).

Cell cycle progression entails the tightly regulated transduction of mitogenic signals to cyclically expressed proteins known as cyclins and to their catalytically active targets, the cyclin-dependent protein kinases (cdks) (2). To ensure proper progression through each phase cells have developed a series of orchestrated checkpoints that govern the different cyclin-cdk complexes required for distinct cell cycle events (2). Key regulators in the major cell cycle checkpoint in late G1, known as the restriction point in mammalian cells, include cdk4 and cdk6, which preferentially assemble into holoenzymes with cyclin D1, D2 or D3 (31). Specifically, cyclin D1 and other D-type cyclins accumulate in response to mitogenic stimulation and assemble with their catalytic partners, cdk4 and cdk6 (29). Cyclin D1 plays an important role in promoting G1-to-S phase progression by inactivating the action of the retinoblastoma protein (Rb) through phosphorylation and leading to the release of E2F transcription factors (2, 29). Cyclin E is mainly expressed at the G1-S transition where it enters into active complexes with its catalytic partner cdk2 to accelerate Rb protein phosphorylation (2). Moreover, cyclin A and cdk2 complexes play a major role in S phase, while cyclin B and cdc2 are required during G2/M phase. E2F is required for the transcription of genes involved in mediating G1 exit and DNA synthesis (2, 32).

The embryonic heart exhibits high levels of expression of cyclins involved in G1, S, G2 and M-phase like D1, D2, D3, A, B1, and E (2, 33-36). Moreover, other genes required for DNA replication such as proliferating cell nuclear antigen (PCNA) and cdc2, cdk2, cdk4 and cdk6 are also highly expressed along with their associated kinase activities (2, 33-36). Although the relative importance of these factors remains largely unknown, development of embryonic ventricle appears to be critically dependent on cyclin D expression (2). Mice deficient for all three D-cyclins died at mid/late gestation secondary to heart abnormalities (37). Mutant embryos displayed severely thinned ventricular walls, mainly affecting the compact zone and large ventricular septal defects (2). On the contrary, overexpression of each cyclin D in adult myocardium exhibited elevated rates of cardiomyocytes DNA synthesis at baseline in the adult hearts (38). Mice deficient for cdk2 and cdk4 died during embryogenesis as a result of heart defect (39). The loss of cdk2 and cdk4 caused hypophosphorylation of Rb, which in its turn led to repression of E2F target genes, like cdc2 and cyclin A2. Double-mutant mice displayed hearts with globally reduced size, enlargement of atria, and thin ventricular walls (2).

The withdrawal of postnatal cardiomyocytes from the cell cycle is linked with a change in the expression pattern of many cell cycle regulatory molecules (2). The protein expression profiles of cyclins D1, D2, D3, A, B1, and E and their associated kinases are significantly down-regulated in cardiomyocytes after birth compared with the levels observed in the embryonic heart (2). Moreover, other than down-regulation of cyclins D, it has been demonstrated that in postmitotic cardiomyocytes, the nuclear import of the cyclin D1/cdk4 complex is tightly prevented (29). Furthermore, overcoming of this inhibitory mechanism is sufficient to induce cell cycle progression leading to cell division of neonatal cardiomyocytes (29). So, in this view, the nucleocytoplasmic transport machinery of cyclin D1 plays a critical role for determining proliferative capacity of cardiomyocytes (29). Nucleocytoplasmic transport pathways of proteins are selectively regulated by various transport receptors and signals, such as nuclear localization signals (NLSs) and nuclear export signals (NESs), and are involved in various cell functions including cell cycle control (29, 40, 41). D-type cyclins and cdk4 lack consensus NLSs, and p21 and p27, which contain NLSs, promote the assembly and nuclear localization of the cyclin D1/cdk4 complex (29, 42-44). In postmitotic cardiomyocytes, cyclin D1/cdk4 complex was formed but remained in the cytoplasm. In addition, the cytoplasmic cyclin D1/cdk4 complex associated with p21 and ectopic expression of p21 or p27 did not promote nuclear localization of cyclin D1/cdk4 (29). The subcellular localization of cyclin D1 is also regulated by the phosphorylation of cyclin D1 at threonine286 by glycogen synthase kinase 3-beta (GSK3-beta) that promotes the nuclear export and degradation of cyclin D1 during S phase (29, 45-48). Therefore it is possible that the GSK3-beta-mediated nuclear export of cyclin D1 may contribute to the cytoplasmic sequestration of cyclin D1 in postmitotic cardiomyocytes (29). Tamamori-Adachi et al. (29) have demonstrated that the majority of D1NLS protein was still detected in the cytoplasm of cardiomyocytes supporting the notion that postmitotic cardiomyocytes have machinery aimed at positively preventing cyclin D1 nuclear localization,

resulting in significant sequestration of D1NLS protein in the cytoplasm (29). Thus the inhibition of cyclin D1 nuclear accumulation is likely to be a critical barrier for maintaining cardiomyocytes tightly in the postmitotic state. In addition prevention of nuclear import of cyclin D1/cdk4 complex is unlikely to be a common feature in cells reversibly arrested in G0 phase and it appears to be a characteristic of postmitotic cardiomyocytes which may play a critical role in switching from a proliferating state to a terminally differentiate state in cardiomyocytes (29). On the other hand it has been demonstrated that nuclear import of cyclin D1/cdk4 induces the cell cycle re-entry of a number of cardiomyocytes in the adult heart as demonstrated by expression of the Ki-67 nuclear antigen and BrdU, markers associated with cell cycle entry. Moreover, Ki-67 expression was only detected in a small population of cardiomyocytes in regions adjacent to the infarcts suggesting that the nuclear import of cyclin D1/cdk4 complex may potentiate the ability of cardiomyocytes that retain proliferative capacity (29). It is also possible that their nuclear import might promote the cell cycle re-entry of a population of cardiomyocytes that otherwise do not enter into the cell cycle in the adult heart (29).

The down-regulation in expression of cyclins and cdks during normal development of cardiomyocytes has been shown to parallel a reciprocal up-regulation of cdk-inhibitors (cdkI). Two protein families exist to specifically inhibit cdks: one family of cdk inhibitors is specific for cdk4/6 (the INK4 family, comprising p15, p16, p18 and p19); a second, the Cip/Kip family (p21, p27 and p57), has much broader activity inhibiting cdk4/6 as well as cdk2 and cdc2 (31). It has been reported that p16 and p18 are expressed in the embryonic heart but their levels in young adult hearts are low or undetectable (2, 20, 49, 50). It has been suggested that there is a progressive increase in the percentage of p16 positive cardiomyocytes with age, which may not represent a quiescent status but instead a marker of cardiomyocyte senescence (2, 11, 51). In contrast, expression of Cip/Kip family, p21 and p27, increases in cardiomyocytes in the perinatal period and reaches high levels in adult myocardium (2, 50, 52). However, postmitotic cardiomyocytes still retain the capacity to respond to mitogenic stimulation, which includes hypertrophic cell growth and up-regulates expression of cyclins and cdks, including cyclin D1 and cdk4 (29, 53, 54).

The primary target of G1 cdks is the product of the retinoblastoma susceptibility gene (Rb). This protein along with p107 and p130 comprises a family of proteins often called pocket proteins (55). All three family members are expressed in developing myocardium, although the temporal pattern diverges considerably. Rb is scant or undetectable in fetal mouse myocardium at age E12.5 but it is up-regulated by the neonatal stage, and in adult, terminally differentiated cardiac tissue becomes the predominant pocket protein expressed (56, 57). p107 is expressed in a pattern reciprocal to Rb, highest in the embryonic heart and lowest in adult. p130 expression peaks in the neonatal period and is subsequently down-regulated and expressed at low levels in adult myocardium. These proteins are best known for their roles in inhibiting cell cycle progression through the regulation of E2F-responsive genes (58). In their hypophosphorylated form, Rb proteins bind to E2F complexes, recruiting transcriptional repressors such as histone deacetylases (HDACs) or the Jumonji, a repressor that plays a critical role in embryonic heart development (59). Phosphorylation of Rb and its relatives by cdk2 and -4 results in the release of E2F complexes, enabling them to activate transcription and to trigger the expression of genes required for DNA synthesis and other cell cycle regulating molecules such as cyclin E, cyclin A, the mitotic kinase Cdc2 (p34/Cdk1), and E2F-1 itself. Previous studies have shown that activation of the Rb pathway by forced expression of adenovirus E1A or E2F1, a downstream target of Rb, can induce DNA synthesis but results in apoptotic cell death in cardiomyocytes (29, 60, 61). There is an accumulating evidence that Rb proteins play a critical role in regulating cell cycle exit and possibly cardiac muscle differentiation. Rb-null embryos died at day 14.5 post coitum with widespread cell death and aberrant cell cycling in a variety of tissues (2, 62-64) but apparently normal hearts. It was reported recently the Rb_/_ embryonic stem cells displayed a delay in the expression of cardiac-specific transcription factors and subsequently cardiac differentiation. It was shown by other investigators that LEK1, a murine homolog of the cardiomyogenic factor 1, interacts with Rb, inhibiting its activity and allowing cardiomyocytes to proliferate despite the presence of Rb proteins during development (65).

Unlike Rb-deficient mice, p107 and p130 nullizygous mice were initially reported as viable and phenotypically normal (66, 67) but these mice developed a thin-walled, hypoplastic myocardium with defective looping and chamber formation (68). It has been demonstrated that Rb and p130 have overlapping functional roles *in vivo* to suppress cell cycle activators and maintain quiescence in postnatal cardiac muscle. Mice that are deficient in both Rb and p130 demonstrated a threefold increase in the heart weight-to-body weight ratio and showed increased numbers of BrdU- and phosphorylated histone H3-positive nuclei, consistent with persistent cardiomyocyte cycling (57). These data support the notion that Rb family members are critical for normal cardiomyocyte cell cycle exit but do not address the issue of whether they also mediate the inability of adult cardiomyocytes to divide in response to growth stimuli.

In mammalian heart mitogen-activated protein kinase (MAPK) signaling pathways have been hypothesized to regulate cardiomyocyte growth in response to diverse developmental signals (69). The MAPK signaling pathways consist of at least three prominent phosphorylation cascades terminating in the activation of extracellular signal regulated kinases (ERK), c-Jun NH2-terminal kinases (JNK), or p38 MAPKs. In cardiomyocytes the ERK cascade is thought to be primarily activated in response to tyrosine kinase receptor and G protein-coupled receptor (GPCR) activation, while the JNK and p38 cascades are activated by both GPCR activation and stress signals. Of the four different p38 isoforms that have been

identified, the predominant isoform expressed in the adult heart is p38-alpha, while p38-beta and p38-gamma are expressed at low levels, and p38-delta is not expressed in the heart (1, 69, 70). The major upstream activators of p38 MAPKs are MAPKKs including MKK3, MMK4, and MKK6, which directly phosphorylated the dual site in p38 MAPKs (Thr-Gly-Tyr). Activated p38 phosphorylates downstream signaling molecules important for cardiomyocytes differentiation and hypertrophy (1, 69). Substrates of p38 MAPKs include mainly other protein kinases and a growing list of transcription factors that includes MEF2, MAPKAPK2 and -3, ATF-2, ELK-1, Chop, TEF-1, C/EBP-beta, and Max (71, 72). Depending on the cell type and stimulus, p38 MAPKs can have either a positive or negative influence on cell cycle progression (73).

Recently p38 MAP kinase has been identified as a major negative regulator of cardiomyocytes proliferation during development *in vivo* (1). p38 activity is inversely correlated with cardiac growth during development and its overexpression blocks proliferation of foetal cardiomyocytes *in vitro* (1). In particular it has been demonstrated that activation of p38 *in vivo* by MKK3bE reduces foetal cardiomyocytes proliferation whereas cardiac-specific *p38-alpha* knockout mice, along with growth factor stimulation, show an increase in neonatal cardiomyocytes mitoses together with a cell cycle reentry of cultured adult cardiomyocytes (14). Simultaneous stimulation with growth factors, like FGF1 and inhibition of p38 MAP kinase resulted in transient dedifferentiation of adult cardiomyocytes by eliminating sarcomeric structures and completion of cytokinesis indicating that the inhibitory effects of p38 on cardiomyocytes proliferation are reversible and that post-mitotic, differentiated cells are capable of proliferation (1). So it has been demonstrated that both mono- and binucleated fully matured mammalian cardiomyocytes, can divide *in vitro* (14).

Moreover, it has been demonstrated that in p38-alpha knockout hearts, BrdU incorporation was increased 20-fold, indicating that DNA synthesis in adult cardiomyocytes is enabled by the absence of p38. However, p38 inhibition by itself is not sufficient to induce cardiomyocyte mitosis or cytokinesis. Specific growth factors, not present in the healthy adult heart, are also required (1). It is not clear how p38 inhibition enables cardiomyocyte proliferation. Previous studies have shown that p38 activation inhibits S phase, M phase and cytokinesis in many cells by modulating cell cycle proteins like p21, cyclin D1, cdc25B, cyclin B and cdc2 (1, 74-76). In addition p38 is required for differentiation of several cell types, including skeletal myoblasts (1, 73, 77). p38 promotes stabilization and enhanced translation of mRNAs, increases protein stability by phosphorylation, and is involved in chromatin remodelling (1, 78-80). In cardiomyocytes Engel et al. (1) have shown up-regulation of cdc2, cdc25B, cyclin D and cyclin B, all factors required for cell cycle progression. Therefore, p38 could regulate cardiomyocyte proliferation by modulating important cell cycle factors (1). In particular these authors have supposed that p38 inhibits the transition from S phase to mitosis by down-regulating mitotic genes including cyclin A and cyclin B (1, 81). In addition p38 inhibition acts synergistically with FGF1 to promote cell cycle progression, possibly through molecules like phosphoinositide-3-kinase (PI3 kinase) (1). In its turn, FGF1 up-regulates foetal cardiac genes inducing dedifferentiation independently of p38 (1). Moreover, FGF1 regulates genes involved in apoptosis, and this effect is also enhanced by p38 inhibition. Finally, p38 activity prevents up-regulation of factors required for karyokinesis and cytokinesis confirming a role for p38 in G2/M checkpoint control (1). In addition, when p38 inhibitor is removed from culture media after induction of DNA synthesis, cardiomyocytes fail to progress through G2/M and cytokinesis indicating that p38 inhibition is required for growth-factor-mediated induction of all phases of the cell cycle and substantially enhances the proliferative capacity of mammalian cardiomyocytes (1). Complicating the interpretation of p38-alpha's role in adult myocardium there is a report that cardiac-specific transgenic mice expressing a dominant negative mutant p38-alpha generate a hypertrophic rather than proliferative response in adult hearts (2, 82).

3. PERSPECTIVES

Understanding the regulation of cardiomyocyte proliferation is biologically important and clinically relevant (14). The dogma that adult cardiomyocytes are unable to proliferate has been definitively knocked down. Thus, the identification of principal regulators of cardiomyocytes re-entry in the cell cycle must be the main effort of the next future in order to attempt effective treatments of heart disease.

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Abbreviations: Cyclin-dependent protein kinases (cdks), Nuclear localization signals (NLSs), Nuclear export signals (NESs), Glycogen synthase kinase 3-beta (GSK3-beta), Cdk-inhibitors (cdkI), Retinoblastoma gene (Rb), Histone deacetylases (HDACs), Mitogen-activated protein kinase (MAPK), Extracellular signal regulated kinases (ERK), c-Jun NH2-terminal kinases (JNK), G protein-coupled receptor (GPCR), Phosphoinositide-3-kinase (PI3 kinase).

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