Knockdown of Cyclin-dependent Kinase Inhibitors Induces Cardiomyocyte Re-entry in the Cell Cycle

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Proliferation of mammalian cardiomyocytes stops rapidly after birth and injured hearts do not regenerate adequately. High cyclin-dependent kinase inhibitor (CKI) levels have been observed in cardiomyocytes, but their role in maintaining cardiomyocytes in a post-mitotic state is still unknown. In this report, it was investigated whether CKI knockdown by RNA interference induced cardiomyocyte proliferation. We found that triple transfection with p21Waf1, p27Kip1, and p57Kip2 siRNAs induced both neonatal and adult cardiomyocyte to enter S phase and increased the nuclei/cardiomyocyte ratio; furthermore, a subpopulation of cardiomyocytes progressed beyond karyokinesis, as assessed by the detection of mid-body structures and by straight cardiomyocyte counting. Intriguingly, cardiomyocyte proliferation occurred in the absence of overt DNA damage and aberrant mitotic figures. Finally, CKI knockdown and DNA synthesis reactivation correlated with a dramatic change in adult cardiomyocyte morphology that may be a prerequisite for cell division. In conclusion, CKI expression plays an active role in maintaining cardiomyocyte withdrawal from the cell cycle.

The adult mammalian heart has very limited regenerative capacity (1). In fact, cardiomyocytes divide extensively during development and lose proliferative capacity shortly after birth in rodents and at ~7 months of age in humans, when they withdraw from the cell cycle and remain in G0 stage of the cell cycle indefinitely (2–5). Permanently differentiated cardiomyocytes lose their ability to divide and switch from hyperplastic to hypertrophic growth in response to mitogenic stimuli (5–8). This transition is characterized by a marked increase in myofibril density, the appearance of mature intercalated discs, and the formation of binucleated cardiomyocytes (9).

Cell cycle progression is positively modulated by cyclin-cyclin-dependent kinase (CDK)2 complexes. Specifically, cyclin D-CDK4–6 and cyclin E-CDK2 complexes phosphorylate protein kinases of the retinoblastoma (pRb) family, releasing E2F transcription factors (3, 10, 11). Indeed, pRb phosphorylation is a prerequisite to initiate a new round of cell cycle activity allowing the cell to enter the S phase of the cell cycle (12). The expression of cyclin and CDK genes is down-modulated in neonatal hearts and further decreases in the adult. Other proliferation-promoting genes, such as E2F1 and Myc family members are similarly repressed, as demonstrated both in vitro and in vivo (13, 14). pRb and pRb family proteins also have a critical role in regulating cardiac cell cycle both in the developing and in the adult heart. pRb is barely detectable in proliferating fetal cardiomyocytes, its expression is up-regulated progressively during neonatal development, and pRb is the predominant pocket protein expressed in terminally differentiated adult cardiomyocytes. The accumulation of pRb plays a critical role in regulating cell cycle arrest associated with terminal cardiac muscle differentiation, as also observed in other in vitro culture systems, including skeletal muscle, adipocytes, and macrophages, suggesting that this may be a general phenomenon (5, 15, 16). However, other mechanisms are at play as well, as pRb phosphorylation by cyclin D/CDK4 in differentiated cardiomyocytes isolated from neonatal rat induces their hypertrophic growth but not their proliferation (17). Cyclin-CDK complexes are regulated by two structurally defined classes of inhibitors (CKIs): INK4 and CIP/KIP families. The INK4 family, which includes p15INK4B, p16INK4A, p18INK4C, and p19INK4D, specifically inhibits CDK4/6, preventing their heterodimerization with D cyclins. The CIP/KIP family comprises p21CIP1 (p21), p27KIP1 (p27), and p57KIP2 (p57). These molecules display lower specificity, as they can bind and inhibit all cyclin/CDKs (5, 18). However, CIP/KIP CKI also have a positive role in the modulation of CDK activity, as they facilitate the assembly of cyclin D-CDK4 complexes (19, 20).

The expression of all CKIs is detectable during embryonic development. At later stages, the progressive withdrawal of maturing cardiac myocytes from the cell cycle coincides with increased levels of both p21 and p27, whereas p16INK4A, and p18INK4C levels are low or undetectable (9, 16, 21). Specifically, p27 seems to be critical for controlling the exit from the cell cycle, whereas p21 may maintain cell cycle arrest and prevent re-entry into cell cycle (22). In fact, p21 null mice do not show developmental defects or elevate tumor incidence. Conversely, p27 null mice are predisposed to pituitary tumors and display generalized hyperplasia with a 20% increase in heart weight.
The CKI expression pattern observed in humans is very similar to that found in rodents (30); p21 is detectable during fetal development and its level increases in post-natal life; p27 expression increases by 25 weeks of fetal life and remains constant thereafter; p57 expression decreases during development and only low levels of p57 protein are present in the adult human heart. However, in both acute and chronic heart failure, p57 increases, whereas p21 and p27 expression decreases (30, 31). Cardiomyocyte terminal differentiation may also involve other factors that can create a barrier to proliferation beyond the immediate perinatal period (31), such as telomerase reverse transcriptase (TERT) down-regulation and the resulting loss of telomerase activity (32–35). Indeed, in contrast to high activity detected in the immediate perinatal period such that p57 protein is not detectable at all other stages (31), cardiomyocyte terminal differentiation may also involve other factors that can create a barrier to proliferation beyond the immediate perinatal period (31), such as telomerase reverse transcriptase (TERT) down-regulation and the resulting loss of telomerase activity (32–35). Indeed, in contrast to high activity detected in the immediate perinatal period such that p57 protein is not detectable at all other stages (31), cardiomyocyte terminal differentiation may also involve other factors that can create a barrier to proliferation beyond the immediate perinatal period (31), such as telomerase reverse transcriptase (TERT) down-regulation and the resulting loss of telomerase activity (32–35). 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cytes/sample and 50–100 adult cardiomyocytes/sample. To compare the results of different experiments, data were expressed as % of control. Images were acquired by a fluorescence microscope (Axioplan2; Carl Zeiss). Images were analyzed by IAS software (Delta Sistemi), processed, and overlaid using Adobe Photoshop CS2 (Adobe). Cells were counted by two blinded readers obtaining similar results.

Apoptosis Assay—Apoptosis was assessed by measuring the amount of cytoplasmic nucleosomes generated during the apoptotic fragmentation of cellular DNA by Cell Death Detection ELISA (Roche Applied Science), according to the manufacturer’s instructions.

Statistical Analysis—Variables were analyzed by Student’s t test and one-way analysis of variance. A value of p ≤ 0.05 was deemed statistically significant. Results are reported as mean values ± S.E.

RESULTS
p21 and p27 Knockdown Induces Neonatal Cardiomyocyte Progression into S Phase—To investigate the functional role of CKIs in cardiomyocyte withdrawal from the cell cycle, neonatal mouse cardiomyocytes were transfected with siRNA targeting p21, p27, or both, as well as with a control sequence. To minimize the number of residual proliferating cardiomyocytes and of contaminating fibroblasts, cell cultures were treated with AraC, which kills proliferating cells selectively (49). AraC was added to cultures for 2 days, followed by a 3-day recovery time before transfection. Western blotting analysis of the transfected cells confirmed a p21 and p27 knockdown efficiency of >70% 2 days after transfection (Fig. 1A). To assay the cells that entered S phase upon CKI RNAi, BrdU was added at the time of transfection, and its incorporation was assayed 2 days later. Fig. 1, B and C, show that both single and double CKI knockdown significantly increased DNA replication. Interestingly, double p21 and p27 silencing was the most effective and almost half of the cardiomyocytes entered S phase. Thus, double CKI silencing was adopted for the following experiments.

p21 and p27 Knockdown Induces Neonatal Cardiomyocyte Proliferation—The increase in S phase induced by CKI knockdown was associated with a 2.7± 0.5-fold increase of bi-nucleated cardiomyocytes (CKI RNAi, 17.3± 3.2; control, 6.4± 1.7; p < 0.006, n = 4), which could account for the observed increase in DNA synthesis only in part. Hence, these data suggested that some cardiomyocytes had undergone successful cytokidosis. Thus, we assessed whether p21 and p27 knockdown stimulated not only S phase entrance but also the progression through the following phases of the cell cycle. To this aim, histone H3 phosphorylation at Ser10 (H3P), a mitotic marker, was assayed by immunofluorescence staining. Down-modulation of CKI expression more than tripled H3P-positive cardiomyocytes, which reached >30% 2 days after transfection (Fig. 2, A and B). In keeping with these findings, mitotic figures were observed in p21 and p27 knockdown cardiomyocytes (Fig. 2C) but not in controls (data not shown).

Aurora B kinase plays a role in chromosomal condensation by phosphorylating histone H3. Indeed, Aurora B associates with centromeric heterochromatin early in mitosis (prometaphase and metaphase). Subsequently, Aurora B relocates to the central spindle during anaphase and then to the contractile ring and the mid-body during telophase representing a marker of cytokinesis (50–52). To determine whether CKI knockdown promoted cardiomyocyte cell division, we measured Aurora B by immunofluorescence staining. We found that ~5% of the p21 and p27 knockdown cardiomyocytes displayed an Aurora B-positive mid-body (Fig. 2, D and E), whereas these structures were not present in control transfected cells.

To confirm these results, cardiomyocyte number was determined at different times after transfection. We found that the number of α-sarcymetic actinin-positive cells significantly increased 1.7± 0.1-fold at day 2 (n = 9; p < 0.0001).

FIGURE 1. p21 and p27 knockdown induces neonatal cardiomyocyte DNA synthesis. Neonatal cardiomyocytes were transfected with p21, p27, or control (Cnt) siRNAs. A, cell lysates were prepared 2 days after transfection. Western blot analysis demonstrates efficient p21 and p27 single and double knockdown; β-tubulin shows equal loading in each lane. B and C, neonatal cardiomyocytes were transfected with p21, p27, or control siRNA. Nontransfected (NT) cells were used as additional control to exclude potential effects of the control siRNA. Then, BrdU was added. Two days later, BrdU-incorporating cells were detected by immunofluorescence using a FITC-conjugated specific antibody, whereas cardiomyocytes were stained using a Texas Red-conjugated antibody to α-sarcymetic actinin. Nuclei were detected using Hoechst 33342 (blue). B, bar graph indicating the percentage of BrdU-positive cardiomyocytes (n = 4; *, p < 0.003; †, p < 0.00001). C, representative picture of two cardiomyocytes, one BrdU-positive and one -negative, at 2 days after transfection with p21 and p27 siRNAs. The left panel shows an α-sarcymetic actinin/Hoechst 33342 overlay; the right panel shows an α-sarcymetic actinin/BrdU overlay of the same field. Scale bar, 20 μm.
Cell Cycle Progression Induced by CKI Knockdown Does Not Cause DNA Damage and Apoptosis—CKI knockdown in skeletal muscle myotubes is associated with induction of aberrant mitoses and apoptosis (43). However, most mitotic figures we observed in dividing cardiomyocytes were morphologically normal (Fig. 2C), and p21 and p27 double knockdown was not associated with a significant increase of cell apoptosis as assessed by TUNEL assay (supplemental Fig. S3A).

To further explore this issue, cell micronucleation was assayed by Hoechst 33342 staining in cardiomyocytes that were cultured in the presence of BrdU to monitor the cells that entered S-phase and replicated their genome over a period of 48 h. Significantly, we found that BrdU-positive cardiomyocytes displaying overt micronucleation were <2% and that the micronucleation rate did not increase following CKI knockdown (supplemental Fig. S3, B and C).

Aberrant mitosis is associated with DNA double-strand brakes (53). Thus, DNA damage in neonatal cardiomyocytes was tested measuring the formation of γH2AX foci in cells cultured in the presence of BrdU from the time of transfection. We found that, albeit virtually all γH2AX-positive cells were BrdU-positive as well, CKI interference did not increased the number of foci-positive nuclei compared with control cells (supplemental Fig. S4A). Moreover, when the percentage of γH2AX-negative cells was calculated among BrdU+ cardiomyocytes, it was found that up to 70% of the CKI knockdown cardiomyocytes underwent DNA replication in the absence of overt DNA damage (supplemental Fig. S4B). These data confirm that proliferation is compatible with cardiomyocyte differentiation program.

p57 Helps Regulating Neonatal Cardiomyocyte Proliferation—When we assayed the number of cardiomyocytes following p21 and p27 knockdown, we found that after the initial increase displayed at day 2, no further increase was observed afterward (Fig. 3A), despite sustained knockdown of both p21 and p27 (Fig. 3B). To test whether this was due to growth arrest, BrdU incorporation was assayed after an 8-h labeling pulse. It was found that, although >20% of the cardiomyocytes were BrdU-positive 2 days after p21 and p27 knockdown, DNA synthesis rate declined afterward and was very close to control at 6 days after transfection (Fig. 3C). These results suggest that p21 and p27 knockdown efficiently triggered one or few neonatal cardiomyocyte cell divisions, followed by a second arrest of the cell cycle.

To determine whether other CKIs may contribute to limit cardiomyocyte proliferation upon p21 and p27 knockdown, their
level was measured by qRT-PCR. We found that, although the p15, p16, p18, and p19 were not affected significantly (supplemental Fig. S5), p57 was induced over time and peaked at 6 days after transfection when it increased more >7-fold (Fig. 3D).

To investigate the role played by p57, its levels were knocked down, alone or in combination with p21 and p27. Fig. 3D shows p57-siRNA targeting efficiency. p21, p27, and p57 triple knockdown more than doubled the percentage of BrdU-incorporating cardiomyocytes at 4 and 6 days after transfection compared with p21 and p27 double interference, whereas p57 knockdown alone was not effective (Fig. 3C). In keeping with these findings, p21, p27, and p57 triple siRNA further increased cardiomyocyte number at 4 and 6 days after transfection (Fig. 3A).

Cardiomyocyte terminal differentiation is associated not only with CKI induction, but also with cyclin down-modulation and consequent pRb hypophosphorylation (5, 9). Thus, we measured whether neonatal cardiomyocyte proliferation induced by CKI knockdown was associated with the reactivation of the cyclin/CDK pathway.

We found that, although the expression of cyclins D (D1, D2, and D3) was not affected (data not shown), CKI double or triple knockdown was associated with the induction of cyclin A, E1, and E2 (Fig. 4, A–C). In keeping with these findings, pRb hyperphosphorylation was induced, as assessed by SDS-PAGE mobility up-shift in Western blotting experiments (Fig. 4D). The phosphorylation of the crucial threonine 826 residue (54) increased as well and was more intense and permanent following p21, p27, and p57 triple knockdown compared both to p21 and p27 double knockdown and to control siRNA transfection. Furthermore, global pRb protein levels increased upon CKI knockdown, in keeping with negative pRb regulation of its own expression (55). Finally, we also found that, similarly to double CKI silencing, triple CKI knockdown also did not increase DNA damage, as assessed measuring the number of γH2AX foci (supplemental Fig. S4).

p21, p27, and p57 Knockdown Promotes Proliferation of Adult Cardiomyocytes—Previous studies indicate that rat cardiomyocyte proliferation arrests soon after birth (9). To evaluate the functional consequences of CKI knockdown in adult cardiomyocytes, ventricular cardiomyocytes were isolated from 8–12-week-old rats. Cardiomyocytes were transfected with siRNAs targeting p21, p27, and p57 or control. The efficiency of double and triple CKI knockdown was assayed by qRT-PCR (Fig. 5A). As observed in neonatal cardiomyocytes, p21 and p27 double knockdown up-regulated p57 expression. However, this increase was efficiently prevented when a siRNA to p57 was co-transfected (Fig. 5A). Furthermore, both double and triple CKI RNAi were accompanied by the induction of cyclin A (Fig. 5B).

To determine whether double or triple CKI interference promoted DNA synthesis in adult cardiomyocytes even in the absence of growth factor stimulation, cells were cultured in serum-free medium (56, 57) for 10 days after transfection and then assayed for BrdU incorporation after a 24-h pulse. Double p21 and p27 CKI interference induced BrdU incorporation in >12% of the adult cardiomyocytes, whereas no BrdU-positive cells were observed among the controls (Fig. 6, A and B). However, unlike neonatal cardiomyocytes, no significant increase of BrdU incorporation was observed when p57 was silenced as well (Fig. 6A). Intriguingly, mitotic figures (Fig. 6C) and cardiomyocytes with four nuclei (Fig. 6D) were observed only in CKI knockdown cultures.
The increase in S phase induced by CKI knockdown was associated with a significant increase of cardiomyocytes displaying more than two nuclei (supplemental Table S1). However, this increase could explain the observed induction in DNA synthesis only in part. To learn whether adult mammalian cardiomyocytes can undergo cytokinesis, cardiomyocytes exhibiting an Aurora B-positive mid-body staining were evaluated (Fig. 7, A and B). Both double and triple CKI interference induced an increase in cytokinesis compared with control. Cardiomyocytes were also counted to measure cell proliferation. We observed that both double and triple CKI knockdown induced a small but reproducible cardiomyocyte number increase (Fig. 7C). Taken together, these results indicate that CKIs are involved in the control of adult cardiomyocyte cell cycle withdrawal.

**TERT Overexpression Associated with CKI Knockdown Further Increases Proliferation in Neonatal but Not in Adult Cardiomyocytes**—Down-regulation of TERT and the resulting loss of telomerase activity represents one mechanism that can cooperate together with activators and inhibitors of the CDKs to create a barrier to cardiomyocytes proliferation beyond the immediate perinatal period (32, 33). Indeed, it has been shown that TERT and telomerase activity are down-regulated in adult mouse myocardium (38).

To assess whether TERT overexpression can increase cardiomyocyte proliferation in association with CKI triple interference, neonatal and adult cardiomyocytes were infected with adenovirus expressing either human TERT (Ad-hTERT) or LacZ (Ad-LacZ, negative control). Eight hours later, the cells were transfected with siRNAs to CKIs (p21, p27, and p57) or control. Both neonatal and adult cardiomyocytes were efficiently transduced as assessed by qRT-PCR to hTERT and CKIs (data not shown).

We observed that hTERT expression further increased both neonatal cardiomyocyte S phase entry and proliferation induced by CKI knockdown (supplemental Fig. S3, A and B). However, this additional induction exerted by hTERT was very...
modest and was not found in adult cardiomyocytes (supplementary Fig. S6, C and D).

Moreover, it has been shown that Notch1 overexpression can induce the proliferation of neonatal cardiomyocytes (58–60). However, we found no additional increase of adult cardiomyocytes proliferation when CKI knockdown was associated with Notch pathway stimulation obtained by overexpressing Notch1 intracellular domain (data not shown).

**CKI Knockdown Induces Neonatal Gene Expression**—We observed that CKI knockdown induced a drastic change of adult cardiomyocyte morphology. Fig. 6B shows that, although control transfected cells retained principally the characteristic rod shape, CKI siRNA-transfected cardiomyocytes assumed a stellate morphology resembling that of neonatal cardiomyocytes. Also myofibrils organization was disarrayed, assuming a configuration reminiscent of neonatal cardiomyocytes.

Thus, the expression of a subset of genes that are differentially expressed in fetal, neonatal, and adult cardiomyocytes was measured. We found that CKI knockdown sharply decreased the levels of adult genes mRNA Glut4 (glucose transport 4) and myoglobin (61, 62) and induced the expression of neonatal genes, tubulin β5, matrix metalloproteinase 2 (MMP2), p100 co-activator, ZnT4, and IGF-1 (Insuline-like Growth Factor-1) receptor (62). Conversely, the expression of fetal/hypertrophic markers glucose transport 1 (Glut1) (61, 63), nkx2.5, and mef2C (myocyte enhancer factor 2C) was unaffected, whereas GATA4 and atrial and B-type natriuretic factors ANP and BNP displayed only minor increases (Fig. 8).

**DISCUSSION**

Cardiomyocyte terminal differentiation makes the heart a particularly vulnerable organ, especially toward ischemic, toxic, and inflammatory events. Thus, following massive injury, the adult heart is unable to replace necrotic or damaged tissue. Indeed, cardiac regeneration granted by endogenous cardiac stem cells and other regenerative mechanisms is limited and, in
most circumstances, is insufficient to replace a large myocardial tissue loss (64, 65). Therefore, reinduction of cell cycle progression in terminally differentiated cardiomyocytes may represent an attractive approach to treat heart disease (4).

Although cardiomyocyte terminal differentiation has long been regarded as a condition of irreversible proliferation arrest or postmitotic state (66), the molecular bases of such a condition has remained unclear. High CKI expression levels have been regarded as a condition of irreversible proliferation arrest (66), the molecular bases of such a condition have mostly been correlative. On the other side, convincing evidence indicates that adult cardiomyocytes still hold signaling pathways capable of efficiently resuming their proliferative potential (57, 68–70). In this study, we found that cardiomyocyte postmitotic state depends on the constant expression of CKIs, demonstrating that permanent proliferation arrest cannot be maintained in their absence. These results are in keeping with a previous study of our group, demonstrating a critical requirement of CKI in sustaining skeletal muscle myofibers terminal differentiation (43). Thus, cardiomyocyte growth arrest should not be regarded simply as the consequence of the absence of proliferation-promoting regulators (cyclin/CDKs). On the contrary, it is an active state that requires continuous CKI expression to be maintained.

A clear efficiency difference in the proliferation rate was observed between neonatal and adult cardiomyocytes following CKI knockdown. Indeed, >20% of the neonatal cardiomyocytes and >10% of the adults displayed DNA synthesis upon CKI knockdown. This massive effect is unlikely due to the potential presence of small stem-like populations in the culture. Thus, CKIs seem to play an active role in maintaining the permanent withdrawal from the cell cycle in both neonatal and adult cardiomyocytes, paving the way to the investigation of further anti-proliferative mechanisms acquired during development. Indeed, we found that pRB phosphorylation decreased over time, despite sustained CKI knockdown, suggesting the presence of additional CDK regulatory mechanisms.

We found CKI down-modulation increased both cyclin E and A levels, whereas cyclin D levels were not affected. Although this result may seem surprising, it should be considered that D cyclins are not strictly required for the proliferation of most cell types. In fact, mice lacking D cyclins or CDK4/6 display relatively mild phenotypes. Triple D cyclin mice KO die in utero at embryonic day 17.5. Heart development is aberrant, but myocytes are clearly present, indicating that cyclins E and A may be sufficient to allow cardiomyocyte proliferation (71, 72).

One possible mechanism restraining cardiomyocyte proliferation was represented by TERT down-modulation and the resulting loss of telomerase activity after the immediate perinatal period (31). To test this idea, we associated CKI knockdown to TERT overexpression. Although a small increase in cell cycle induction was observed in neonatal cardiomyocytes, no difference was observed in adults, possibly owing to the absence of other telomere-associated factors in adult cardiomyocytes (73).

Cardiomyocytes are polyploid and polynucleated cells. Although ploidy was not measured, we found that most neonatal cardiomyocytes were mononucleated, and the majority of the adult cardiomyocytes were bi-nucleated, as observed previously in rodents (74). As expected for a proliferation promoting intervention, CKI knockdown increased the nuclei/cardiacmyocyte ratio in both neonatal and adult cells. However, at least a sub-population of the cardiomyocytes further progressed after karyokinesis, as assessed by the detection of mid-body structures and by straight cardiomyocyte counting.

Intriguingly, neonatal cardiomyocyte proliferation induced by CKI knockdown was not associated with massive increase of DNA damage, micronucleation, and apoptosis, in sheer contrast with the skeletal muscle, where CKI knockdown induces DNA replication followed by mitotic catastrophe (43). Although the molecular mechanism underpinning this difference is not clear, it is worth noting that CKI knockdown...
and DNA synthesis reactivation correlated with a change in adult cardiomyocyte morphology that may be a prerequisite for division. CKI knockdown in serum-free medium induced a neonatal-like morphology and the re-expression of neonatal genes in adult cardiomyocytes, suggesting the involvement of CKIs in cardiomyocyte maturation. Intriguingly, well-established hypertrophy markers such as ANP and BNP were induced only marginally, and no overt reactivation of fetal gene program was observed.

Intriguingly, it has been shown that serum stimulation of adult cardiomyocyte promotes cell spreading and loss of sarcomeric structure, resembling a spontaneous dedifferentiation to a neonatal-like state (75–77). One may speculate that, in the serum-free culture conditions we adopted, CKI knockdown may, at least in part, be a substitute for growth factor stimulation. Further studies are needed to better assess the consequences of cell cycle reactivation on genome integrity in both neonatal and adult cardiomyocytes.

Our findings are in keeping with other studies showing that cell cycle molecular machinery of the cardiomyocytes can be successfully reprogrammed, such that partial cell cycle reactivation and, less frequently, full cell cycle reactivation is observed (78). In some instances, for example upon overexpression of E2F1 or E2F3, reactivation of the cell cycle was observed, but it was followed by apoptosis (14). Overexpression of c-Myc, E2F2, or D-type cyclins promotes cell cycle reactivation in neonatal cardiomyocytes and hypertrophic growth in adult cardiomyocytes (13, 14, 79, 80). Moreover, reinitiation of cell cycle in cardiomyocytes promoted by hCDC5, jumonji (JMJ), or E1A/E1B, E2F, or growth factor stimulation (5, 57, 81, 82). Conversely, cyclin A2 overexpression seems to be sufficient to induce cardiomyocyte proliferation and induces cardiac regeneration after myocardial infarction (83, 84). Among several possible mechanisms of action, one may envision that CKI knockdown leads to a derepression of cyclin A2 activity. In a reciprocal perspective, it is also possible that cyclin A2 overexpression induced p21 family CKI titration, yielding an intracellular milieu similar to that induced by CKI knockdown.

The therapeutic potential of the cell cycle machinery manipulation still needs to be assessed. However, it is worth noting that RNAi therapeutics of cardiac diseases has gained considerable momentum, being burdened by lower safety concerns compared with other gene therapy approaches (85). Furthermore, one may consider that an attractive in vivo cell target is represented by cardiac stem cells or newly formed cardiomyocytes derived from the stem cells, whose amplification may be increased by CKI knock down.

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