E2F-1 Overexpression in Cardiomyocytes Induces Downregulation of $p21^{CIP1}$ and $p27^{KIP1}$ and Release of Active Cyclin-Dependent Kinases in the Presence of Insulin-Like Growth Factor I

Rüdiger von Harsdorf, Ludger Hauck, Felix Mehrhof, Ursula Wegenka, M. Cristina Cardoso, Rainer Dietz

Abstract—The heart is a postmitotic organ unable to regenerate after injury. The mechanisms controlling cell cycle arrest in cardiomyocytes are still unknown. Adenoviral delivery of E2F-1 to primary rat cardiomyocytes resulted in an increase in the expression of key cell cycle activators and apoptosis in >90% of the cells. However, insulin-like growth factor I (IGF-I) rescued cardiomyocytes from E2F-1–induced apoptosis. Furthermore, overexpression of E2F-1 in the presence of IGF-I induced the specific downregulation of total $p21^{CIP1}$ and $p27^{KIP1}$ protein levels and their dissociation from cyclin-dependent kinases (cdks). In contrast, $p16^{INK4}$ and $p57^{KIP2}$ protein levels and their association with cdks remained unaltered. The dissociation of $p21^{CIP1}$ and $p27^{KIP1}$ from their cdk complexes correlated well with the activation of cdk2, cdk4, and cdk6 and the release from cell cycle arrest. Under these circumstances, the number of cardiomyocytes in S phase rose from 1.2% to 23%. These results indicate that IGF-I renders cardiomyocytes permissive for cell cycle reentry. Finally, the specific downregulation of $p21^{CIP1}$ and $p27^{KIP1}$ further suggests their key role in the maintenance of cell cycle arrest in cardiomyocytes. **(*Circ Res*. 1999;85:128-136.)**

Key Words: cell cycle ■ E2F-1 ■ cardiomyocyte ■ apoptosis ■ insulin-like growth factor I

Shortly after birth, cardiomyocytes stop dividing and succumb to cell cycle arrest, resulting in the loss of their regenerative capacity and making the heart a particularly vulnerable organ, especially toward ischemic, toxic, and inflammatory events. However, in contrast to skeletal muscle in which differentiation and cell division are mutually exclusive, cardiac muscle, in principle, is able to differentiate and divide simultaneously, as it happens throughout the fetal period of life.[1] Therefore, reinduction of cell cycle in cardiomyocytes even in the terminally differentiated state is a conceivable and attractive approach to treat heart diseases. In fact, several reports have shown that overexpression of a variety of cell cycle factors may result in the induction of S phase in cardiomyocytes.[2–8]

The cell cycle is controlled by a complex interaction and stoichiometrically balanced equilibrium of cell cycle activators and inhibitors. In particular, cyclin-dependent kinases (cdks) are among the critical regulators of cell division in eukaryotic cells. The sequential activation of individual members of this family and the subsequent phosphorylation of critical substrates order progression through the cell cycle.[9] Cdks can form quaternary protein complexes consisting of a cdk, a cyclin, proliferating cell nuclear antigen (PCNA), and a member of the family of cdk inhibitors. The enzymatic activity of a cdk is regulated at 3 different levels: cyclin activation, subunit phosphorylation, and association with cdk inhibitors.[10] The complexes formed by cdk4 or cdk6 with D-type cyclins and cdk2 with cyclin A or cyclin E are critical for the progression into S phase. These cyclin/cdk complexes are regulated by 2 families of inhibitors, the INK4 family and the CIP/KIP family.[11] INK4 proteins and CIP/KIP proteins are structurally very distinct and interact with cyclins and cdks in different ways. The INK4 family members bind specifically to cdk4 and cdk6. In contrast, CIP/KIP proteins, including $p21^{CIP1}$, $p27^{KIP1}$, and $p57^{KIP2}$, bind to a variety of cyclin/cdk complexes, including cyclin D/cdk4 or cyclin D/cdk6, cyclin E/cdk2, during G1 phase, and cyclin A/cdk2, which are active at the G1/S transition and during S phase.[9]

Very little is known about the cell cycle regulatory mechanisms acting at different phases of differentiation in cardiomyocytes. The cell cycle block correlates with loss of activity and coordinated disappearance of most cyclins and cdks,[12–16] as well as with an increase in some cdk inhibitors.[16,17] The obvious therapeutic potential of regenerative cardiomyocyte growth in repairing myocardial lesions after injury has prompted a search for strategies to revert cardiomyocyte cell

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From the Department of Cardiology (R.v.H., L.H., F.M., U.W., R.D.), Franz Volhard Clinic, Humboldt University; the Max Delbrück Center for Molecular Medicine (M.C.C.), Berlin, Germany.

Correspondence to Rüdiger v. Harsdorf, MD, Franz-Volhard-Klinik, Medizinische Fakultät der Charité, Humboldt-Universität, Wiltbergstr 50, 13125 Berlin. E-mail rharsdo@mdc-berlin.de

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cycle arrest. Indeed, a recent study showed that viral delivery of E2F-1 to cultured rat neonatal cardiomyocytes can overcome their cell cycle arrest.[4] As with the adenoviral E1A expression, massive apoptosis was the main consequence and was also prevented by coexpression of the viral E1B protein. E2F-1–induced apoptosis occurred also in vivo and was not dependent on p53, because myocyte death was not prevented in p53−/− mice.[2]

To elucidate the molecular mechanism underlying this phenomenon, ie, reversal of cardiomyocyte cell cycle withdrawal and apoptosis and how the latter is prevented, we used an alternative approach in which E2F-1 overexpression was performed in the presence of insulin-like growth factor I (IGF-I). We show that IGF-I efficiently rescues cardiomyocytes from E2F-1–induced apoptosis. We then investigated in this system the effect of E2F-1, in the presence or absence of IGF-I, on the regulatory machinery controlling cell cycle in cardiomyocytes, and we identified $p21^{CIP1}$ and $p27^{KIP1}$ as key factors that maintain cardiomyocyte cell cycle arrest.

Materials and Methods

Rat Primary Cardiomyocyte Cultures

Animals were obtained from Moellegard, Schoenwalde, and their care and use were in accordance with approved animal care guidelines of the Max-Delbrück-Center. Ventricular cardiomyocytes from 1- to 3-day postnatal Wistar rats were isolated and cultivated as described with minor modifications.[18] Briefly, hearts were dissected, minced, and trypsinized. The dissociated cells were preplated for 1 hour in the presence of 0.1 mmol/L BrdU (Sigma) to selectively enrich for cardiomyocytes. The resultant cell suspension (4×10^6 cells) was plated onto 60-mm collagen I (Gibco)–coated culture dishes in culture medium. More than 90% of the cells were myocytes, as evaluated by indirect immunofluorescence staining with a monoclonal antibody to sarcomeric tropomyosin (CH1, Sigma).

Recombinant Adenoviral Constructs and Infection

The adenoviral constructs Ad-p21, Ad-Luc, and Ad-β-Gal were provided by Michael Strauss (Max Delbrück Center, Berlin, Germany). The recombinant Ad-p21 contained a cytomegalovirus (CMV) promoter driving the human p21 cDNA. The recombinant Ad-E2F-1 was constructed using an adenovirus vector containing a CMV promoter[19] (gift from Robert Gerard, Leuven, Belgium) driving the human E2F-1 cDNA (gift from Martin Lipp, Max Delbrück Center, Berlin). HEK293 cells (American Type Culture Collection, Manassas, Va) were used for homologous recombination and packaging. The virus titer was determined through direct immunofluorescence staining for adenovirus hexon protein (Imagen Adenovirus, DAKO). Cardiomyocyte cultures were infected with Ad-E2F-1, Ad-Luc, or Ad-p21 at 20 plaque-forming units (pfu)/cell for 1.5 hours. Infected cells were incubated in culture medium in the presence and absence of IGF-I (50 ng/mL, lot 14848300; Boehringer Mannheim) for 24 hours. Infection efficiency of ventricular cardiomyocyte cultures was >90% as determined by Ad-β-Gal infection (10 to 50 pfu/cell) and β-galactosidase assay.

Immunofluorescence, In Situ Apoptosis Assay, and In Situ DNA Synthesis Assay

All manipulations were performed at room temperature, and solutions were made in PBS with 1.5 mmol/L $MgCl_2$ and 1 mmol/L $CaCl_2$ (pH 7.2). Cardiomyocytes, cultivated on collagen-coated coverslips, were fixed in 3.7% formaldehyde for 10 minutes. For detection of exogenous E2F-1, cells were permeabilized with 0.2% Triton X-100 for 15 minutes, blocked for 15 minutes with 5% goat serum and 0.2% Tween 20, and incubated with 10 μg/mL rabbit polyclonal anti–E2F-1 antibody (KH95, Santa Cruz) for 1 hour. For identification of cardiomyocytes, both TUNEL- and E2F-1–stained immobilized cells were incubated with antisarcomeric tropomyosin antibody (CH1, Sigma, diluted 1:50) for 1 hour. Samples were incubated with TRITC-conjugated secondary goat anti-mouse antibody (Dianova, diluted 1:50) and FITC-conjugated secondary goat anti-rabbit antibody (Dianova, diluted 1 to 50) for 30 minutes. Slides were then mounted and examined by fluorescence microscopy.

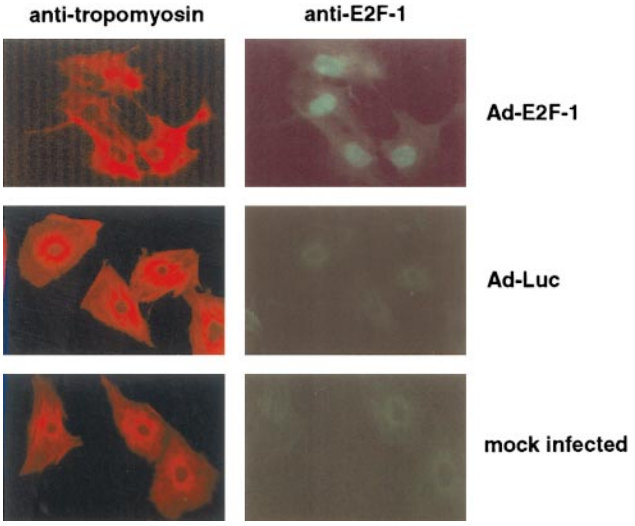

Figure 1. Expression and nuclear localization of adenoviral-delivered E2F-1 in rat neonatal cardiomyocytes that were infected at 20 pfu/cell with Ad-E2F-1, Ad-Luc, or mock-infected as indicated. After cultivation for 24 hours, cells were fixed, permeabilized, and costained by indirect immunofluorescence with rabbit polyclonal anti–E2F-1 antibody (green) and cardiac-specific mouse monoclonal antisarcomeric tropomyosin antibody (red).

For in situ detection of fragmented genomic DNA, a TUNEL assay was used according to the manufacturer's instructions (ApopTag, Amersham). For detection of DNA synthesis, adenoviral-infected cells (20 pfu/cell) were labeled with 30 μmol/L BrdU (Sigma) for 4 hours, fixed in 3.7% formalin, and stained with FITC-conjugated anti-BrdU antibody (B44; Becton Dickinson) according to the manufacturer's instructions. For quantification of BrdU-positive cardiomyocytes, ≈200 cells double stained by tropomyosin were counted in a random field.

[^{3}H]-Methyl-Thymidine Incorporation

At 24 hours after infection, 2.5 μCi/mL [^{3}H]-methyl-thymidine (247.9 GBq/mmol, NEN) was added to cardiac myocyte cultures for 6 hours. Cells were then extracted with 15% trichloracetic acid. The precipitate was solubilized with 0.5 mL 1 mol/L NaOH and neutralized with 0.5 mL HCl. The radioactivity was counted in a liquid scintillation counter.

Cell Cycle and Apoptosis Analysis by Flow Cytometry

At 24 hours after infection, cells were labeled with 30 μmol/L BrdU (Sigma) for 4 hours and stained with FITC-conjugated anti-BrdU antibody (B44; Becton Dickinson) according to the manufacturer's instructions. Samples (10^4 cells) were analyzed with a flow cytometer (Coulter Epics). Cell cycle analysis was performed using Multicycle Software (Coulter).

For detection of apoptotic cells by annexin-V-FLUOS (Boehringer Mannheim) staining, preparation and labeling of cells were performed according to the supplier's instructions for fluorescence-activated cell sorter (FACS) analysis.

Preparation of Whole-Cell Extracts

Cells were lysed in a buffer composed of 50 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 0.5% (vol/vol) Triton X-100, 5 mmol/L

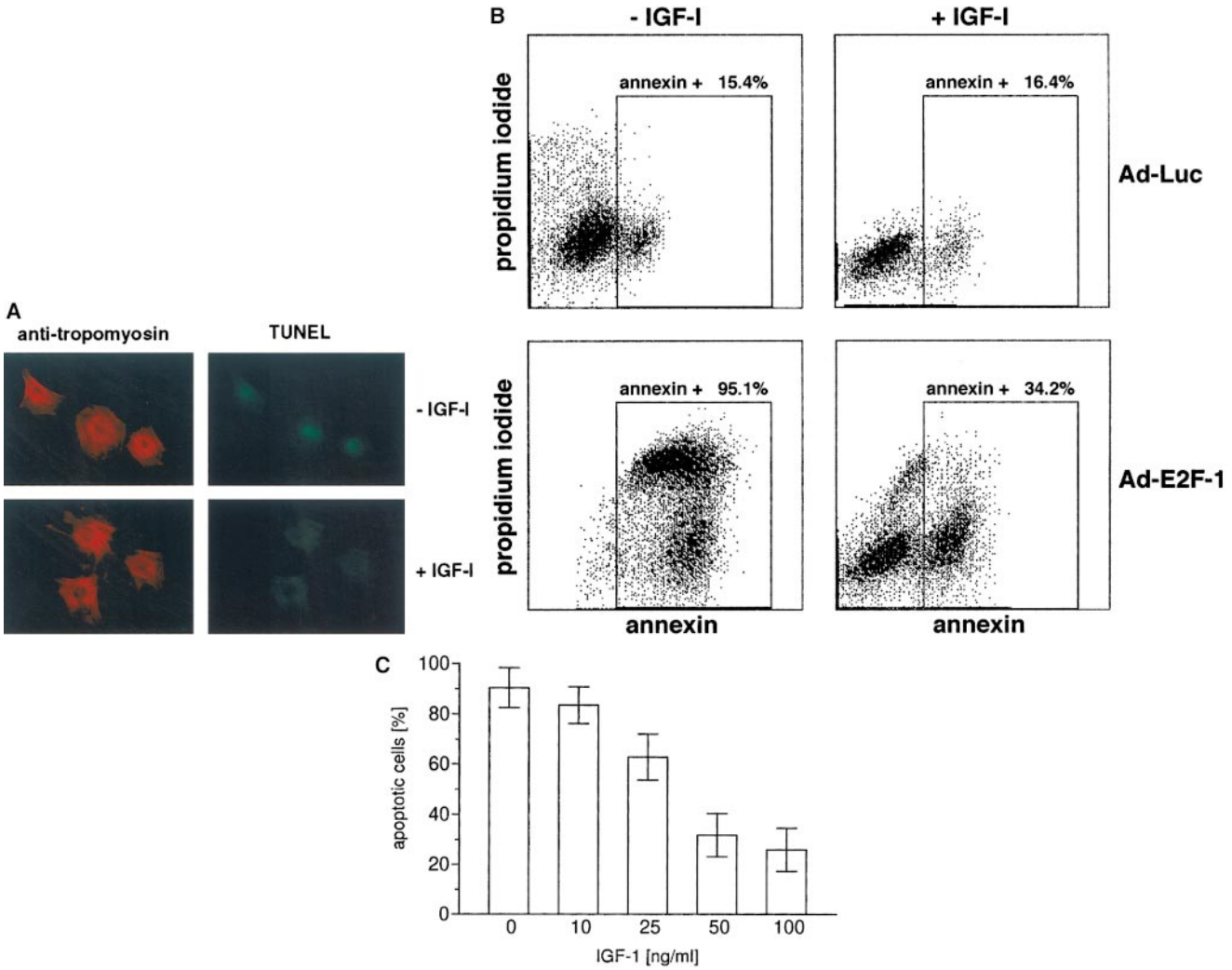

Figure 2. E2F-1–mediated apoptosis is inhibited by IGF-I in rat neonatal cardiomyocytes. Cultures were infected with Ad-E2F-1 and cultivated in the presence or absence of IGF-I for 24 hours. A, Apoptotic cell death was detected using TUNEL of fragmented nuclear DNA and FITC-conjugated antidigoxigenin antibody (green). Cardiomyocytes were identified by costaining with antisarcomeric tropomyosin antibody (red). B, Trypsinized, unfixed cells were stained with annexin-V-FLUOS and propidium iodide, and the percentage of apoptotic annexin–positive cells was quantified by FACS as indicated. The result of one representative experiment is shown. C, Mean±SEM of 3 independent annexin-V-FLUOS experiments is presented, showing the dose-response curve of the IGF-I effect on E2F-1–induced apoptosis.

EDTA, 5 mmol/L DTT, deoxyribonuclease I (50 U/mL), ribonuclease A (50 U/mL), phenylmethylsulfonylfluoride (1 mmol/L), aprotinin (0.3 mmol/L), leupeptin (1 μmol/L), pepstatin (1 μmol/L), NaF (25 mmol/L), Na_3VO_4 (0.1 mmol/L, all from Sigma), and trypsin inhibitor from soybean (100 μg/mL, Boehringer Mannheim). Cellular extracts were centrifuged twice for 30 minutes at 18 000*g*, 4°C. The protein content was determined with the Bradford protein assay (Bio-Rad).

Immunoprecipitation and Immunoblotting

The following antibodies were used: anti-pRb (IF8), anti-E2F1 (KH95), anti-DP1 (K-20), anti-PCNA (PC10), anti-$p16^{INK4}$ (M-156), anti-$p21^{CIP1}$ (C-19), anti-$p27^{KIP1}$ (C-19), anti-$p57^{KIP2}$ (E-17), anti-cyclin A (H-432), anti-cyclin D1 (HD11, 72-13G), anti-cyclin E (M-20), anti-cdc2 (PSTAIRE), anti-cdk2 (M2), anti-cdk4 (C-22), anti-cdk6 (C-21, all from Santa Cruz), and antisarcomeric tropomyosin antibody (CH1, Sigma).

Cell extracts (500 μg total protein; 1.0 mg total protein for cyclin E) were precleared with protein G–agarose beads (Boehringer Mannheim) and incubated with antibody (1 to 2 μg/mL). Coimmunoprecipitation studies were performed with 2.0 mg protein in 1.0 mL lysis buffer. The immune complexes were then collected with 20 μL protein G–agarose for 2 hours and washed 3 times with lysis buffer for 10 minutes, and 30 μL SDS sample buffer (10 mmol/L Tris-HCl [pH 8.0], 1 mmol/L EDTA, 1% wt/vol DTT, 2% wt/vol SDS, and 0.01% wt/vol bromophenol blue) was added. Samples were electrophoretically separated, transferred to polyvinylidene difluoride (PVDF) membranes, blocked, and incubated with primary antibody (0.5 to 5.0 μg/mL). They were subsequently probed with secondary HRP-conjugated anti-mouse or anti-rabbit IgG antibodies (diluted 1:5000, Amersham). For normalization of equal loading, a smaller aliquot (50 μg total protein) was directly resolved by SDS-PAGE and probed with antisarcomeric tropomyosin antibody. Detection was carried out using the enhanced chemiluminescence assay (Amersham).

Assays for Protein Kinase Activity

Immune complexes were washed 3 times with ice-cold kinase buffer (50 mmol/L Tris-HCl [pH 7.5], 10 mmol/L $MgCl_2$, and 1 mmol/L DTT) and resuspended in a mixture containing 5 μg lysine-rich histone H1 (type IIIS, Sigma), 1 μmol ATP, 3 μmol $MgCl_2$, 10 μmol cAMP inhibitor (Santa Cruz), 20 μCi (γ-^{32}P)ATP (111 MBq/mmol,

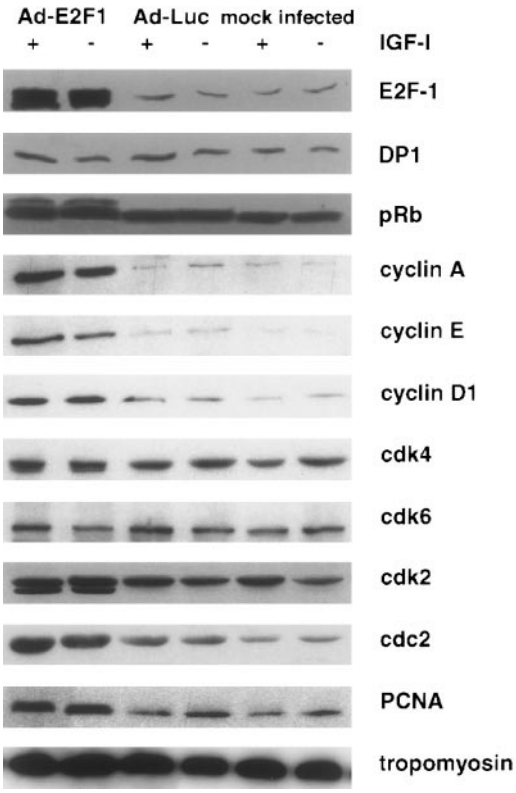

Figure 3. E2F-1 induces the expression of important cell cycle regulatory factors in cardiomyocytes. Rat neonatal cardiomyocytes were treated as described in Figure 2. Aliquots of whole-cell extracts (500 μg total protein) were immunoprecipitated with antibodies to the proteins indicated, separated by denaturing PAGE, and immunoblotted with the same antibodies. For normalization, a smaller aliquot (50 μg total protein) was directly resolved by SDS-PAGE and probed with antisarcomeric tropomyosin antibody. Representative blots of at least 3 independent experiments are shown.

New England Nuclear), and kinase buffer in a total volume of 50 μL. For cdk4 and cdk6 activity, 5.0 μg of recombinant Rb protein (amino acids 769 to 921, Santa Cruz) were used. After 30 minutes of shaking at 30°C, the reaction was stopped by addition of 25 μL 2× SDS sample buffer. Samples were subjected to SDS-PAGE and the amount of incorporated radioactive label was quantitated using a phosphorimager (Fuji) and the TINA software program (Raytest).

Results

IGF-I Blocks E2F-1–Dependent Cardiomyocyte Apoptosis

To test the protective effect of IGF-I toward the E2F-1–dependent apoptosis, rat neonatal cardiomyocytes were infected with Ad-E2F-1 or Ad-Luc (20 pfu/cell) in the presence or absence of IGF-I (50 ng/mL). The expression and nuclear localization of exogenous E2F-1 in cardiomyocytes were confirmed by double immunostaining with anti–E2F-1 antibody and antisarcomeric tropomyosin antibody (Figure 1). After 24 hours, E2F-1 overexpression resulted in massive apoptotic cell death in cardiomyocytes, as demonstrated by TUNEL assay, whereas IGF-I was capable of rescuing Ad-E2F-1–infected cardiomyocytes from apoptosis (Figure 2A).

For quantitative assessment of apoptotic cells, we used annexin-V-FLUOS staining followed by flow cytometry. The translocation of phosphatidylserine from the inner side to the outer side of the plasma membrane occurs at early stages in the apoptotic process. Annexin-V binds to phosphatidylserine with high affinity and thus is a very sensitive method for the detection of apoptotic cells. The cardiomyocyte population infected with Ad-E2F-1 in the absence of IGF-I displayed high numbers of annexin-positive cells (Figure 2B). In contrast, in a dose-dependent manner, the addition of IGF-I to the culture medium was followed by a decrease of the percentage of Ad-E2F-1–infected annexin-positive cells (Figure 2C).

E2F-1 Overexpression Leads to the Upregulation of Cyclins and cdks and pRb Phosphorylation

To study the effect of IGF-I and E2F-1 on the phosphorylation state of pRb, cell lysates from cardiomyocytes were subjected to immunoprecipitation. In control infected cells, Rb protein was detected as one band representing the hypophosphorylated growth-suppressive form (Figure 3). In contrast, E2F-1 overexpression led to the appearance of a second Rb protein variant with a slower electrophoretic mobility corresponding to the inactivated, hyperphosphorylated pRb variant. Interestingly, this band shift occurred irrespective of the addition of IGF-I to the culture medium. Furthermore, E2F-1 overexpression was accompanied by the induction of cell cycle regulatory cdc2, cyclins D1, E, and A, as well as of the replication-associated factor PCNA and a small increase in cdk2 and cdk4 (Figure 3). Notably, the elevated protein content of these endogenous cell cycle regulatory components was also independent of the presence of IGF-I. Previously, it has been demonstrated that coexpression of DP1 along with E2F-1 in quiescent REF52 cells does not alter the ability of E2F-1 to activate downstream target genes.[20] This shows that the endogenous levels of DP1 (Figure 3) are sufficient to mediate the transcriptional activation by E2F-1.

In the Presence of IGF-I, E2F-1 Induces Downregulation of $p21^{CIP1}$ and $p27^{KIP1}$

The cdk inhibitors $p16^{INK4}$, $p57^{KIP2}$, $p27^{KIP1}$, and $p21^{CIP1}$ are expressed in neonatal cardiomyocytes (Figure 4). To elucidate the effect of E2F-1 in conjunction with IGF-I on cell cycle inhibitors in cardiomyocytes, the association of $p16^{INK4}$, $p57^{KIP2}$, $p27^{KIP1}$, and $p21^{CIP1}$ with cdks was investigated by coimmunoprecipitation. The amount of cdk inhibitor bound to the kinase complexes was determined by immunoblotting of anti-cdk precipitates with anti-cdk inhibitor antibodies. Immunoblot analysis of total cell lysates with anti-$p16^{INK4}$ and anti-$p57^{KIP2}$ antibodies revealed that the levels of these cdk inhibitors did not change under the various culture conditions. In relation to the other cdk inhibitors, $p16^{INK4}$ levels were very low. The addition of IGF-I to Ad-E2F-1–infected cell cultures also did not change the amount of $p16^{INK4}$ bound to cdk4, cdk6, nor the association of $p57^{KIP2}$ with cdk2, cdk4, and cdk6.

The results of the immunoprecipitations indicate a higher saturation of cdk4 and cdk6 with $p27^{KIP1}$ than of cdk2. Reportedly, $p27^{KIP1}$ displays a higher affinity in vitro for cdk4 than for cdk2.[21] On infection with Ad-E2F-1 in the presence of IGF-I, the total $p27^{KIP1}$ protein level declined abruptly. Accordingly, the $p27^{KIP1}$ protein pool bound to cdk2, cdk4, and cdk6 under the same conditions decreased markedly.

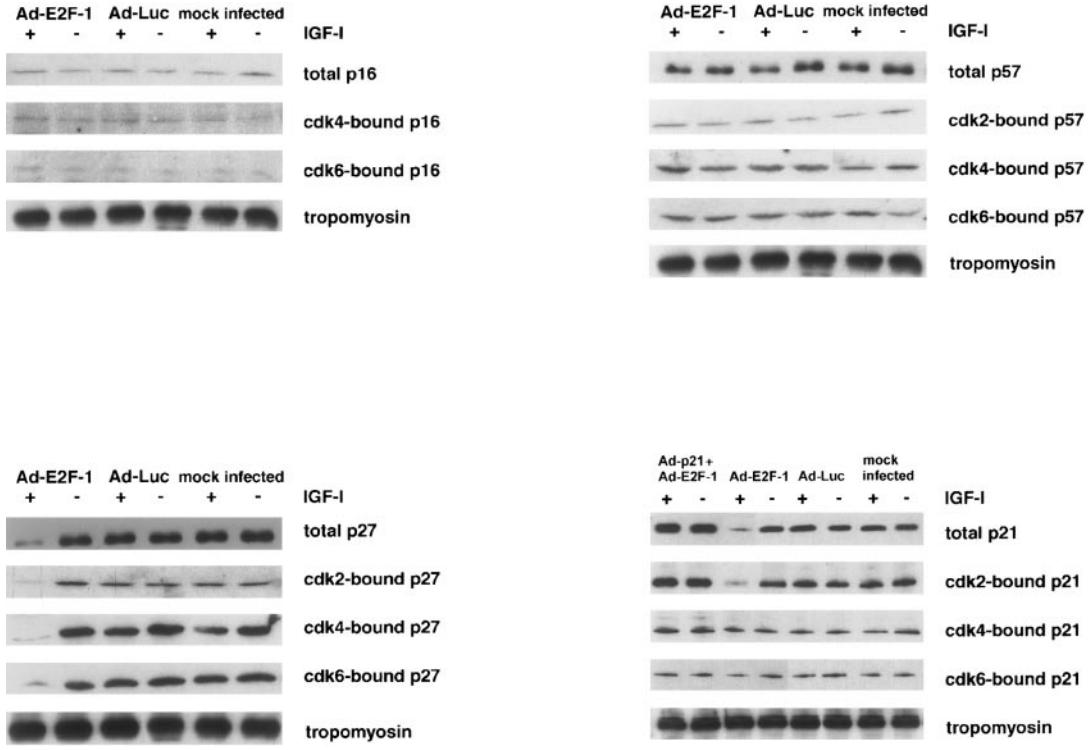

Figure 4. E2F-1 and IGF-I lead to the specific downregulation of $p27^{KIP1}$ and $p21^{CIP1}$. The effect of E2F-1 and IGF-I on the expression of the cell cycle inhibitors $p16^{INK4}$, $p57^{KIP2}$, $p27^{KIP1}$, and $p21^{CIP1}$ and their interaction with cdk2, cdk4, and cdk6 kinase complexes were assessed. Isolated cardiomyocytes were treated as indicated in Figure 2. Total cdk inhibitor protein levels were determined by subjecting whole-cell extracts (50 μg total protein) to SDS-PAGE and probing with the respective antibodies as indicated. For analysis of inhibitor protein levels associated with cdks, cellular extracts from the various conditions containing 2 mg of total protein were immunoprecipitated with anti-cdk2, anti-cdk4, and anti-cdk6 antibodies as indicated. After electrophoretical separation and transfer to PVDF membranes, the blots were probed with the respective cdk inhibitor specific antibody. Evaluation of equal loading of bands was done as in Figure 3. Representative blots of at least 3 independent experiments are shown.

Anti-cdk4 and anti-cdk6 immunoprecipitates probed with anti-$p21^{CIP1}$ antibody revealed comparably low levels of bound $p21^{CIP1}$, indicating that in cardiomyocytes, $p21^{CIP1}$ is mainly associated with cdk2. The protein level of total $p21^{CIP1}$ decreased significantly after treatment of Ad-E2F-1–infected cardiomyocyte cultures with IGF-I. This effect of IGF-I could be reversed by the concomitant overexpression of $p21^{CIP1}$. In addition, the amount of $p21^{CIP1}$ associated with cdk2 under these conditions was barely detectable.

Downregulation of $p21^{CIP1}$ and $p27^{KIP1}$ on E2F-1 Overexpression in the Presence of IGF-I Releases Active Cyclin-cdk Complexes

Because progression through G1 and entry into S phase are tightly regulated by the enzymatic activity of cdk2, cdk4, and cdk6, the phosphotransferase activity of these cdks was investigated via immune complex in vitro kinase assays. The infection of cardiomyocytes with Ad-E2F-1 did not lead to an increase in cdk4- and cdk6-associated activity (Figure 5). In contrast, E2F-1 overexpression in the presence of IGF-I induced cdk4 and cdk6 activities 6.2-fold and 5.5-fold, respectively. The release of $p27^{KIP1}$ protein from cdk4 and cdk6 complexes paralleled the induction of their respective kinase activity. The cdk2-associated histone H1 kinase activity in E2F-1–infected cultures supplemented with IGF-I was stimulated by 4.7-fold compared with E2F-1–infected cultures in the absence of IGF-I (Figure 5). The gain of cdk2 activity is probably due to the downregulation of $p21^{CIP1}$, because the concomitant overexpression of $p21^{CIP1}$ could reverse the effect of IGF-I on activation of cdk2 (Figure 5). The absolute level of cdk2 activity was significantly higher than cdk4 and cdk6 activities, which could explain why overexpression of $p21^{CIP1}$ was able to almost completely reverse the effect of IGF-I, because it preferentially binds to cdk2. In the presence of IGF-I, overexpression of E2F-1 similarly led to an increase in the corresponding kinase activity associated with cyclin E, cyclin A, and cdc2 (data not shown).

In the Presence of IGF-I, E2F-1 Induces S Phase in Cardiomyocytes

We determined whether in cardiomyocytes the effect of E2F-1 and IGF-I on cell cycle regulatory factors is accompanied by cell cycle reentry as indicated by the induction of DNA synthesis. [^{3}H]-methyl-thymidine incorporation was measured to quantify the effect of IGF-I on DNA synthesis in E2F-1–overexpressing cardiomyocytes. As shown in Figure 6A, there was an IGF-I dose-dependent increase of [^{3}H]-methyl-thymidine incorporation in E2F-1–overexpressing cardiomyocyte cultures that could be prevented almost completely by the concomitant overexpression of $p21^{CIP1}$.

To test whether DNA synthesis was being induced specifically in cardiomyocytes, we performed double immunofluorescence staining to detect incorporated BrdU indicating

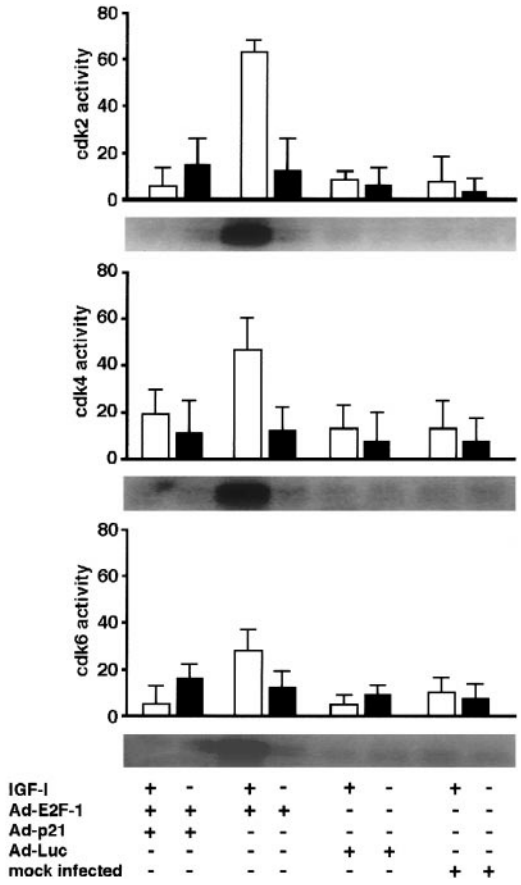

Figure 5. IGF-I induces kinase activity of cdk2, cdk4, and cdk6 in E2F-1–overexpressing cardiomyocytes. Cellular extracts (500 μg total protein) were subjected to immunoprecipitation with anti-cdk2, anti-cdk4, and anti-cdk6 antibodies. Kinase activities were determined using histone H1 (for cdk2) and recombinant Rb protein (for cdk4 and cdk6) as substrate. Amount of incorporated radioactive label was determined by SDS-PAGE and analyzed in a phosphorimager using TINA software. Results of one representative experiment are shown together with the quantitative assessment pooling 3 independent experiments (mean±SEM), *y*-axis values in cpm$\times 10^{-3}$. Black bars, without IGF-I; open bars, with IGF-I.

DNA synthesis in cardiomyocytes identified by antisarcomeric tropomyosin staining (Figure 6B). Adenoviral gene delivery of E2F-1 was not sufficient to trigger DNA synthesis in cardiomyocytes at 24 hours after infection. Five percent of mock-infected cells, 3% of Ad-Luc–infected cells, and 6% of Ad-E2F-1–infected cardiomyocytes were BrdU positive. In contrast, the combined action of E2F-1 and IGF-I led to induction of DNA synthesis, resulting in 20% of BrdU-positive cardiomyocytes. However, at no time were we able to detect cardiomyocytes exhibiting typical mitotic figures or other signs of cytokinesis. Overexpression of $p21^{CIP1}$ was able to inhibit the effect of IGF-I on cell cycle activation as indicated by a reduction of the number of BrdU-positive cells from 20% to 4% of cardiomyocytes.

To assess the distribution of cells at different stages of the cell cycle, FACS analysis of cells stained with BrdU and propidium iodide was used. No significant difference in the cell cycle distribution of cells was observed in Ad-Luc–infected cultures in the presence or absence of IGF-I (Figure 6C). In contrast, the number of cells in S phase in Ad-E2F-1–infected cultures supplemented with IGF-I rose to 23%, and the peak of apoptotic cells containing $<2n$ DNA content (sub–G1 fraction) disappeared. Overexpression of $p21^{CIP1}$ led to a decrease of apoptosis in Ad-E2F-1–infected cardiomyocytes by one third, which is in agreement with the observation that $p21^{CIP1}$ is able to block apoptosis in C2C12 myoblasts.[22] In addition, $p21^{CIP1}$ was able to inhibit the effect of IGF-I on cell cycle activation almost completely, as reflected by a decrease of S-phase cells from 23% to 1.9%.

Discussion

The present study shows that E2F-1 in combination with IGF-I induces cell cycle in postmitotic primary cardiomyocytes. This is accompanied by the specific downregulation of $p21^{CIP1}$ and $p27^{KIP1}$ and release of active cdk complexes, indicating an important role of these 2 cell cycle inhibitors in the maintenance of cell cycle arrest in differentiated cardiomyocytes.

The ability to block cardiomyocyte apoptosis induced by E2F-1 or E1A by overexpression of the viral protein E1B has been shown previously.[3–5] However, until now, it was unknown whether any physiological factors exist allowing cell cycle in postmitotic cardiomyocytes and as such may serve as attractive tools for future interventional studies aimed at the induction of cardiac regeneration on a molecular level. In this regard, our data provide a molecular basis of the effect of IGF-I, because it has been suggested to be the mediator responsible for the beneficial effect observed in patients with congestive heart failure treated with growth hormone.[23]

Our results demonstrate that in postmitotic cardiomyocytes, IGF-I alone neither has an effect on the expression and activity of cell cycle activating factors, nor does it lead to DNA synthesis (see Figures 3 through 6). This is in contrast to other studies suggesting that IGF-I might have the capacity to directly induce DNA synthesis in cardiomyocytes.[24,25] The discrepancy between these studies and our results is not clear. However, Kajstura et al[24] observed an increase from 1% to 6% in BrdU-positive cells when cardiomyocytes were exposed to IGF-I, values all of which are well within the percentage of cells incorporating BrdU under unstimulated control conditions in our experiments. Also, no quantitative or functional data regarding molecular markers of cell cycle control are presented in the present study, which would corroborate their conclusion. This also holds true for the study published by Reiss et al,[25] in which the authors created transgenic mice overexpressing IGF-I locally in the heart. Although this was a well-performed study, a model of high and continuous overexpression of IGF-I within the heart may have only limited implications for the effect of physiological serum levels of IGF-I on cardiomyocytes. Finally, our data are supported by other recent studies indicating a lack of effect of IGF-I, IGF-II, or the intracellular downstream mediator of IGF-I H-Ras on the cell cycle machinery in cultured rat neonatal cardiomyocytes.[26–28] It would be of interest to test other known cardiomyocyte growth factors for their ability to interact with apoptotic and mitogenic signaling pathways in cardiomyocytes. In this regard, it is noteworthy that angiotensin II has been shown to fail to induce DNA

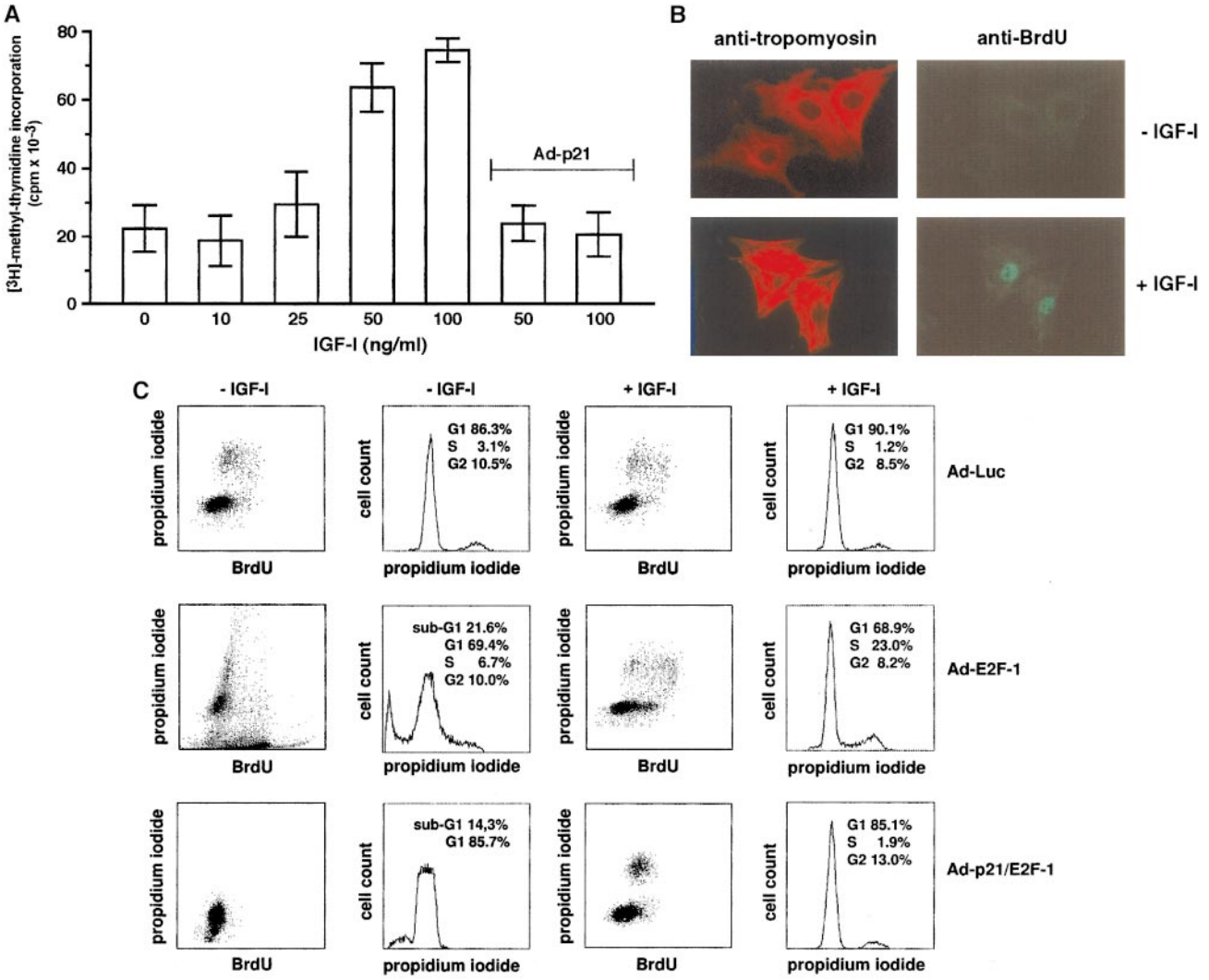

Figure 6. E2F-1 and IGF-I induce cell cycle reentry in rat neonatal cardiomyocytes. Cell cultures were infected with Ad-E2F-1 and were treated as described in Figure 2. A, [^{3}H]-methyl-thymidine incorporation at the indicated doses of IGF-I in E2F-1–overexpressing cardiomyocytes (mean±SEM of 3 independent experiments). B, Cell cultures were pulse-labeled with BrdU for 4 hours at 24 hours after infection. Cells on slides were then prepared for immunofluorescence microscopy by staining with FITC-labeled anti-BrdU antibody (green) and cardiac-specific antisarcomeric tropomyosin antibody (red). C, Cells were trypsinized and fixed with 70% ethanol. For determination of cell cycle distribution, cells were subsequently stained with propidium iodide and analyzed by FACS. By using Multicycle software (Coulter), the percentage of cells in different cell cycle stages was determined and is indicated. The result of one representative experiment is shown.

synthesis in cultured cardiomyocytes, despite the fact that it induced a transient expression and activation of cyclins and cdks.[29]

It would be of interest to know through which intracellular signaling pathways IGF-I exerts its antiapoptotic effect on cardiomyocytes. Several studies indicate a role for protein kinase B/Akt in the antiapoptotic effect of IGF-I in PC12 pheochromocytoma cells[30] or in Rat-1 fibroblasts.[31] Notably, there appear to exist other protein kinase B/Akt-independent antiapoptotic pathways activated by IGF-I,[32] one of which may be a mitogen-activated protein kinase pathway.[30] Recently, it was shown that in fetal rat cardiomyocytes, IGF-I attenuates the induction of Bax and the activation of caspase 3 induced by serum withdrawal or incubation with doxorubicin.[33] However, the underlying signaling cascade leading to the IGF-I–dependent prevention of apoptosis in cardiomyocytes is yet unknown, and future studies dedicated to unravel this important issue are urgently needed.

One might argue that cell cycle control in neonatal cardiomyocytes may differ from fully differentiated adult cardiomyocytes on the basis that in vivo the former can still undergo DNA synthesis and karyokinesis.[14,34,35] Although this may hold true for in vivo conditions, this is certainly not the case under in vitro conditions as applied in our study. DNA content and nucleotide incorporation assays (see Figure 6C) clearly show the extremely low percentage of cultured neonatal rat cardiomyocytes entering S phase (<5%). This is in agreement with other studies, which also have shown that neonatal[29] and even fetal[5,27] cardiomyocytes lose their proliferative capacity as soon as they are taken into culture.

Our data show that E2F-1 overexpression leads to apoptosis. This observation is in agreement with the results of the

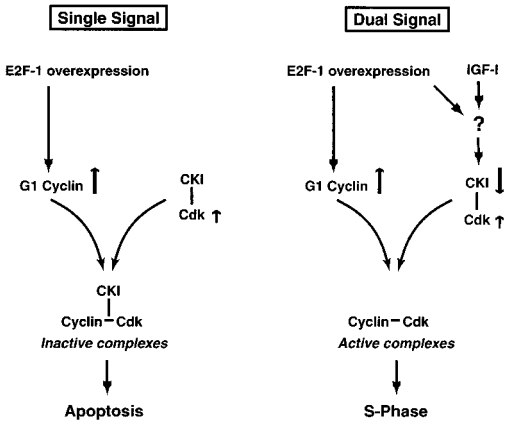

Figure 7. Model for E2F-1 effect in cardiomyocytes in the absence or presence of IGF-I.

phenotype of the E2F-1 null mice,[36] transgenic mice overexpressing E2F-1,[37] and in vitro studies of E2F-1 overexpression in fibroblast cell lines,[20] all showing that E2F-1 leads to the induction of apoptosis. Recently, two studies were published indicating the induction of apoptosis even in cardiomyocytes by overexpression of E2F-1.[2,4] As in our study, the concomitant presence of an antiapoptotic factor (E1B or Bcl-2) was necessary to prevent apoptosis and to achieve DNA-synthesis. Although there are no qualitative differences between these studies and ours, there are several reasons explaining the obvious quantitative differences, including the different methods used to detect apoptosis and DNA synthesis, the different time points chosen to assess cell cycle activation, and the different models used to infect cardiomyocytes with adenoviral constructs.

The induction of apoptosis can be modified dramatically by the action of cytokines, as well as other factors. Previous studies have shown that c-*myc*–induced apoptosis can be blocked by a second signal mediated by cytokines, in particular IGF-I.[38] Furthermore, different studies using activated T lymphocytes have established the requirement for an additional signal rescuing these cells from apoptosis and leading to S-phase reentry.[39,40] Our results and other studies have shown that these signals converge in the downregulation of cdk inhibitors, in particular of $p27^{KIP1}$.[39,41–45] The downregulation of cdk inhibitors allows accumulation of active cdk complexes thereby eliciting cell cycle progression (see model in Figure 7). In this regard, our model also provides a mechanism explaining previous results showing E1A induced S-phase reentry in cardiomyocytes,[3,5] because the viral oncoprotein E1A directly binds to and blocks the inhibitory activity of $p27^{KIP1}$.[46]

In summary, our results implicate the cdk inhibitors $p21^{CIP1}$ and $p27^{KIP1}$ as key regulators of cell cycle arrest in postmitotic cardiomyocytes and furthermore emphasize the importance of their abundance in modulating the apoptotic versus mitogenic response in these cells.

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