cAMP-mediated Induction of Cyclin E Sensitizes Growth-arrested Adipose Stem Cells to DNA Damage-induced Apoptosis

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The differentiation capacity of mesenchymal stem cells has been extensively studied, but little is known on cell cycle-related events in the proliferation and differentiation phases of these cells. Here, we demonstrate that exposure to cAMP-increasing agents inhibits proliferation of adipose stem cells (ASCs). This antiproliferative effect is associated with both reduced cdk2 activity and pRB phosphorylation. Concomitantly, however, the level of cyclin E markedly increases upon cAMP induction, indicating that cyclin E may have cdk2-independent functions in these cells besides its role as a cdk2 activator. Indeed, we found indications of a cdk2-independent role of cyclin E in DNA damage-induced apoptosis. 8-CPT-cAMP sensitizes ASCs to γ -irradiation-induced apoptosis, an effect abolished by knockdown of cyclin E. Moreover, cAMP induces early activation of ERK, leading to reduced degradation of cyclin E. The cAMP-mediated up-regulation of cyclin E was blocked by knockdown of ERK or by an inhibitor of the ERK kinase MEK. We conclude that cAMP inhibits cdk2 activity and pRB phosphorylation, leading to reduced ASC proliferation. Concomitant with this growth inhibition, however, cyclin E levels are increased in a MEK/ERK-dependent manner. Our results suggest that cyclin E plays an important, cdk2-independent role in genotoxic stress-induced apoptosis in mesenchymal stem cells.

INTRODUCTION

Human mesenchymal stem cells (MSCs) have been shown to differentiate not only into mesenchymal lineages (such as osteogenic, chondrogenic, adipogenic, and myogenic), but also into neurogenic, angiogenic, and hepatic lineages (Zuk *et al.*, 2002; Baksh *et al.*, 2004; Boquest *et al.*, 2006a; Schaffler and Buchler, 2007). The pluripotent nature of MSCs makes them potentially ideal candidates for tissue engineering, and they have already demonstrated some efficacy in cell therapy and regenerative medicine (Kassem *et al.*, 2004; Kassem, 2004; Leo and Grande, 2006; Mimeault and Batra, 2006). Adipose tissue contains a supportive stroma that can be easily isolated and provides a rich source of MSCs (Zuk *et al.*, 2002; Boquest *et al.*, 2006a). Thus, adipose tissue represents a valuable source of multilineage stem cells. It is therefore important to understand the biology of adipose stem cells (ASCs).

Cyclic AMP (cAMP) is a ubiquitous second messenger that regulates a number of different cellular processes, including metabolism, growth, differentiation, and gene regulation (Krebs and Beavo, 1979; McKnight, 1991; Vossler *et al.*, 1997; Daniel *et al.*, 1998; Kim *et al.*, 2005). Most effects of cAMP are mediated through activation of protein kinase A

(PKA) or the Epac (exchange protein directly activated by cAMP) family of exchange proteins (Walsh *et al.*, 1968; Bos, 2003). Elevation of intracellular cAMP has both proliferative and antiproliferative effects depending on the cell type. For instance, cAMP stimulates the proliferation of thyroid cells, neurons, and Swiss 3T3 cells, while inhibiting the proliferation of lymphocytes, fibroblasts, and adipocytes (Rozengurt *et al.*, 1981; Blomhoff *et al.*, 1987; Burgering *et al.*, 1993; Sevetson *et al.*, 1993; Dugan *et al.*, 1999; Iacovelli *et al.*, 2001). One target of cAMP that regulates cell proliferation is the extracellular signal-regulated kinase (ERK; also called mitogen-activated protein kinase, or MAPK) cascade. Several studies have demonstrated a cross-talk between the cAMP signaling pathway and the Ras-Raf-MEK-ERK pathway (Stork and Schmitt, 2002; Dumaz and Marais, 2005). In a cell-specific manner, cAMP can either inhibit (e.g., astrocytes and adipocytes) or activate (e.g., neurons and bone marrow-derived MSCs) ERK (Sevetson *et al.*, 1993; Jaiswal *et al.*, 1994; Stork and Schmitt, 2002; Kim *et al.*, 2005).

Control of the G1/S phase transition at the restriction point plays a crucial role in the regulation of cell proliferation (Zetterberg *et al.*, 1995; Bartek *et al.*, 1996). At the restriction point, cyclin/cdkb complexes inactivate retinoblastoma protein (pRB) by hyperphosphorylation, resulting in E2F release and transcription of S-phase genes (Weinberg, 1995). D-type cyclins are the first group of cyclins to be expressed in response to growth or mitotic signals (Sherr, 1993). They assemble with either cdk4 or cdk6 to promote G1 progression by partial inactivation of pRB, resulting in E2F-mediated transcription of cyclin E, and by titrating the cdk inhibitors p21^{Cip1} and p27^{Kip1} to prevent them from inactivating cyclin E/cdk2 complexes (Sherr, 1996; Sherr and Roberts, 1999;

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Cheng *et al.*, 1999). Cyclin E/cdk2 complexes then complete pRB inactivation leading to S-phase entry (Zarkowska and Mittnacht, 1997; Lundberg and Weinberg, 1998). pRB remains in a hyperphosphorylated state in S phase by the combined actions of cyclin E/cdk2 and cyclin A/cdk2 complexes (Mittnacht, 1998).

Each phase of the cell cycle contains checkpoints that allow cell cycle arrest and activation of repair mechanisms when a defect is detected. If repairs cannot be made, for instance due to a large amount of DNA damage, the apoptotic cascade is activated, leading to programmed cell death (Rowinsky, 2005; Siegel, 2006). Thus, apoptosis is an element of cell cycle checkpoints, allowing for the removal of damaged and abnormal cells. Besides the key regulatory function of cyclin E in the G1/S transition and the initiation of DNA replication, cyclin E also plays an important role in apoptosis of tumor cells of hematopoietic origin. Mazumder *et al.* (2000) demonstrated that genotoxic stress, such as γ -irradiation, increased the levels of cyclin E, leading to amplification of apoptosis through activation of the caspase cascade.

The differentiation capacity of MSCs, including ASCs (Boquest *et al.*, 2005), has been widely studied, yet little is known about the cell cycle-related events controlling proliferation and differentiation of these cells. As cAMP is one of the established differentiation factors of MSCs, we unraveled the cell cycle-related events downstream of cAMP signaling in ASCs. In the present study, we demonstrate that elevation of intracellular cAMP strongly inhibits proliferation of ASCs and that this antiproliferative effect is associated with a reduction in both cdk2 activity and pRB phosphorylation. Surprisingly however, the level of cyclin E is increased under these conditions, indicating that cAMP leads to uncoupling of cyclin E from cdk2 activity and cell cycle progression. This suggests that cyclin E may have other functions in ASCs besides its role as a cdk2 activator. Indeed, we show that cAMP promotes a cdk2-independent effect of cyclin E in DNA damage-induced apoptosis of MSCs and that cyclin E is induced in an ERK-dependent manner.

MATERIALS AND METHODS

Reagents and Antibodies

Forskolin, prostaglandin E_2 (PGE_2), and U0126 were obtained from Calbiochem (La Jolla, CA). 8-CPT-cAMP and 6-Bnz-cAMP were from Biolog (Hayward, CA). Cycloheximide, 5-bromo-2-deoxyuridine (BrdU), NaF, Na_3VO_4 , β -glycerophosphate, and antibiotics against Neurofilament 200 (NF200) were from Sigma (St. Louis, MO). Monoclonal anti-cyclin D1 (DCS-6), cyclin D2 (DCS-3), and cyclin D3 (DCS-22) were purchased from Medical and Biological Laboratories (Woburn, MA). Antibodies against cyclin E (HE12; for Western blot analysis, HE11; for immunoprecipitation), cdk2 (M2), actin (C-2), p27^{Kip1} (C-19), p21^{Cip1} (C-19), cyclin A (C-19), and Lamin A/C (sc-7292) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-pRB (14001A) and FITC-conjugated anti-BrdU (556028) antibodies were obtained from PharMingen (San Diego, CA). Antibodies against p44/42 MAP kinase (9102), phospho-p44/42 MAP kinase (Thr202/Tyr204; 9101), and Bcl-2 (50E3) were from Cell Signaling Technology (Beverly, MA). Cy3-conjugated antibodies were from Jackson ImmunoResearch Laboratories (West Grove, PA).

Cell Culture and Treatment

Adipose tissue was obtained by liposuction from abdominal, hip, and thigh regions of healthy female donors after formal consent, and the stromal vascular portion was used for subsequent isolation of ASCs essentially as previously described (Boquest *et al.*, 2006b). Briefly, lipoaspirate (300–400 ml) was washed and digested with 0.2% collagenase (Sigma) for 2 h at 37°C with shaking. Floating adipocytes were separated from the stromal vascular fraction by centrifugation. After lysis of erythrocytes and sedimentation, the cellular pellet was resuspended and strained through 100- and 40- μ m sieves. Magnetic beads were used to remove CD45⁺ and CD31⁺ cells, and as previously reported, the remaining stem cells expressed a

CD45[−]CD31[−]CD34⁺CD105⁺ phenotype (Boquest *et al.*, 2005). Immediately after separation, cells were washed and resuspended in DMEM/F12 supplemented with 20% fetal bovine serum (FBS), antibiotics, and 2.5 μ g/ml amphotericin B. After 7 d in culture, attached cells were passaged by trypsinization and cultured further in DMEM/F12 supplemented with 20% FBS and antibiotics. ASCs (polyclonal) were cultured at a density of 2000–6000 cells/cm² and passaged at 70–80% confluency. To induce proliferation, treatment was carried out in high-glucose DMEM (4.5 g/l; Invitrogen-BRL, Carlsbad, CA) containing 10% FBS, 10 ng/ml epidermal growth factor (EGF; Sigma, St. Louis, MO), 20 ng/ml basic fibroblast growth factor (bFGF; Sigma), B27 (1:50; Invitrogen), and antibiotics. Cells at passages 5–12, i.e., in the log expansion phase, were used in all experiments.

Cell Proliferation and BrdU Incorporation

Cell proliferation was determined by measuring the incorporation of [³H]thymidine (Amersham Biosciences, Piscataway, NJ) into DNA and by counting the number of viable cells. For the [³H]thymidine assay, ASCs were seeded in 25-cm² flasks at a density of 4×10^4 cells/flask. Cells were pulsed with 0.2 mCi of [³H]thymidine for the last 20 h of a 72-h incubation. Cells were harvested by trypsinization and collected by centrifugation ($300 \times g$ for 10 min). After transferring to a microtiter plate, cells were harvested on a cell harvester and counted in a liquid scintillation counter (Topcount; Packard Instrument, Meriden, CT), according to the manual of the instruments. For direct cell counting, ASCs were seeded in 75-cm² flasks at 1.5×10^5 cells/flask. After 4 d, cells were collected before counting using a Bürker chamber. For the BrdU incorporation study, ASCs were seeded in 150-cm² flasks at 3×10^5 cells/flask. Cells were pulse-labeled with BrdU (10 μ M) for the last 90 min of a 72-h incubation, harvested by trypsinization, and analyzed for BrdU incorporation by fluorescence-activated cell sorting (FACS) as described by Naderi *et al.* (2005).

TUNEL Assay

The in situ cell death detection kit fluorescein (Roche Diagnostics, Alameda, CA) was used to detect DNA strand breaks generated during apoptosis. TUNEL was performed as recommended by the manufacturer and the proportion of TUNEL-positive cells was determined by fluorescence microscopy.

Immunoblot Analysis

ASCs (400,000 cells/175-cm² flask) were lysed in RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% NP-40, 0.1% SDS, 0.5 mM EDTA, 50 mM NaF, 1 mM Na_3VO_4 , 10 mM β -glycerophosphate, 0.2 mM PMSF, 10 μ g/ml leupeptin, and 0.5% aprotinin) before protein concentration was determined using the Bradford method (Bio-Rad, Richmond, CA). Equal amounts of protein (50 μ g) were subjected to SDS-PAGE and transferred to nitrocellulose (Amersham Biosciences) using a semidry transfer cell (Bio-Rad). Proteins were detected using appropriate primary antibodies and the enhanced chemiluminescence detection system (ECL Plus, Amersham Biosciences).

Subcellular Fractionation

Nuclear and cytoplasmic extracts from 1.0×10^6 ASCs were prepared using the Nuclear Extract Kit (Active Motif, Carlsbad, CA; Cat. No. 40010) according to the manufacturer's instruction. Equal amounts of protein (50 μ g) were then subjected to SDS-PAGE and examined by Western blotting.

Immunoprecipitation and Kinase Assay

ASCs (400,000 cells/175 cm² flask) were lysed in Triton X-100 lysis buffer (20 mM Tris-HCl, pH 7.5, 250 mM NaCl, 0.1% Triton X-100, 10 mM NaF, 5 mM β -glycerophosphate, 0.1 mM Na_3VO_4 , 0.2 mM PMSF, 10 μ g/ml leupeptin, and 0.5% aprotinin) for 1 h on ice with occasional vortexing, followed by sonication. After centrifugation, total cell lysate (300 μ g) was incubated with anti-cdk2 or anti-cyclin E antibodies (2 μ g) overnight at 4°C with rotation. A 1:1 slurry of protein G Sepharose beads (30 μ l; Upstate Biotechnology, Lake Placid, NY) was added to the lysate, and the incubation was continued for another hour at the same conditions. For immunoprecipitation, the resulting immune complexes were washed three times in lysis buffer before they were resuspended in 1 $\times$ SDS sample buffer, boiled for 10 min, and subjected to Western blot analysis. For detection of cdk2 activity, the immune complexes were washed twice in lysis buffer and once in kinase buffer (50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 1 mM DTT, 2 mM EGTA, 1 mM NaF, 5 mM β -glycerophosphate, and 0.1 mM Na_3VO_4) before they were resuspended in 20 μ l kinase buffer containing 30 μ M ATP, 5 μ g histone H1 (Upstate Biotechnology) and 10 μ Ci of [γ -³²P]ATP (Amersham Biosciences). After 15 min at 30°C, the reactions were terminated by adding 10 μ l of 3 $\times$ SDS sample buffer, followed by boiling for 5 min. Samples were subjected to SDS-PAGE, and the gels were dried before phosphorylated histone H1 was detected by autoradiography.

Immunocytochemistry

For immunocytochemistry, ASCs were plated onto coverslips at $1\text{--}1.5 \times 10^4$ cells/coverslip into six-well plates. After 2 d (for detection of cyclin E) or 5 d (for detection of NF200) of culture, cells were fixed with 3% paraformaldehyde

Figure 1. cAMP inhibits proliferation of ASCs. (A) ASCs (40,000 cells) were treated with increasing concentrations of 8-CPT for 72 h, before DNA synthesis was measured as uptake of [³H]thymidine (see *Materials and Methods*). Data are expressed as mean $\pm$ SD ($n = 4$) and as percentage of control. * $p \leq 0.001$. (B) ASCs were grown for 4 d in the presence or absence of 8-CPT (250 μ M). The cells were collected, and the number of cells was determined using a Bürker chamber. The cell counts are presented as mean fold induction $\pm$ SD ($n = 4$). * $p \leq 0.001$. (C) ASCs were treated with or without 8-CPT (250 μ M) for 72 h. BrdU was added at a final concentration of 10 μ M, 90 min before harvesting. The cell cycle distribution was determined by flow cytometry after staining cells with FITC-conjugated antibodies against BrdU and with propidium iodide (PI). Percentages of cells in G1, S, and G2 phases are indicated. One representative of three independent experiments is shown. (D) ASCs were grown in the presence or absence of 8-CPT (250 μ M). After 3 d, cells were harvested, washed, and replated. Control cells (40,000 cells; C) were grown for additional 3 d, whereas 8-CPT-treated cells were either treated with 8-CPT for additional 3 d (40,000 cells; 8-CPT) or kept untreated (40,000 cells; 8-CPT removed). (E) ASCs were treated with or without 250 μ M 8-CPT, 50 μ M forskolin (F), 0.33% ethanol (EtOH), 500 μ M 6-Bnz, or 100 μ M prostaglandin E₂ (PGE₂) for 72 h. DNA synthesis was measured as uptake of [³H]thymidine as described in *Materials and Methods*. Data are expressed as mean $\pm$ SD ($n = 3$ in D; $n = 4$ in E) and as percentage of control. * $p \leq 0.001$.

hyde in phosphate-buffered saline (PBS) for 15 min, washed three times with PBS followed by permeabilization with PBS containing 0.1% Triton X-100 for 15 min. After washing, the cells were incubated with blocking solution (PBS containing 0.01% Tween-20 and 2% BSA) for 15 min, before incubation with cyclin E antibodies (1:50 dilution in blocking solution overnight at 4°C) or NF200 antibodies (1:200 dilution in blocking solution for 30 min). The cells were washed four times with blocking solution and then further incubated with goat anti-rabbit or goat anti-mouse antibodies conjugated to Cy3 (1:300 in blocking solution) for 30 min. Cells were washed twice in PBS followed by DNA-staining with 0.25 μ g/ml DAPI for 10 min. After washing twice with PBS and once with dH₂O, coverslips were mounted and observed with an Olympus BX51 microscope (Melville, NY) using Analysis (Soft Imaging Systems, Münster, Germany), or an Olympus Fluoview 1000 laser scanning confocal microscope (60 $\times$ objective).

siRNA and Transient Transfection

Stealth RNA interference (RNAi) duplex oligoribonucleotides (siRNA) for human cyclin E1 (HSS101458 and HSS101460) and ERK/MAPK (12935-025), and control small interfering RNAs (siRNAs; 12935-200 and 12935-300, respectively) were purchased from Invitrogen. Stealth RNAi oligos are 25-mer double-strand RNA (dsRNA) molecules with chemical modifications that increase stability and reduce off-target effects by limiting sense strand activity. Two nonoverlapping Stealth RNAi duplexes of each siRNA were used in our experiments. The plasmids encoding cdk2 and cdk2-D146N (dominant negative cdk2) were constructed by Dr. Sander van den Heuvel (Utrecht University, The Netherlands; van den Heuvel and Harlow, 1993) and obtained from Addgene (Addgene plasmids 1884 and 1885, respectively). The siRNAs/plasmids were transiently transfected into ASCs using the Human MSC Nucleofector Kit (Amaxa Biosystems, Gaithersburg, MD). For each nucleofection sample, 5×10^5 cells were subjected to nucleofection with 5 μ g of siRNA/plasmid according to the manufacturer's protocol. The nucleofection was carried out under the program U-23 of the Nucleofector device, and after

nucleofection the cells were incubated in DMEM/F12 supplemented with 20% FBS and antibiotics for 24–40 h before treatment.

RESULTS

cAMP Inhibits Proliferation of ASCs

The effect of cAMP on proliferation of ASCs was determined. As shown in Figure 1A, elevation of intracellular cAMP levels with the cell-permeable cAMP analog 8-CPT-cAMP (8-CPT) inhibited DNA synthesis in a dose-dependent manner, as determined by [³H]thymidine incorporation. Growth inhibition was confirmed by cell counting, because the fold induction of cells at day 4 was reduced from 5 to 1.6 with 8-CPT (Figure 1B). We also examined the cell cycle distribution by FACS after incorporation of BrdU with or without 8-CPT. Consistent with the above results, 8-CPT reduced the number of cells in S phase from 26 to 5%, whereas the number of cells in G1 and G2 phases increased (Figure 1C).

Reduced [³H]thymidine incorporation and cell number by 8-CPT could be due to apoptosis. To investigate this possibility, viability of ASCs was determined by a propidium iodide (PI) exclusion test, and disruption of mitochondrial membrane potential was monitored by JC-1 staining. 8-CPT did not induce apoptosis in ASCs (data not shown), a conclusion supported by the reversibility of the 8-CPT-induced cell cycle arrest. Removal of 8-CPT by washing after 72 h

Figure 2. Effect of cAMP on the expression of cell cycle proteins. (A) ASCs were treated with or without 250 μ M 8-CPT, 50 μ M forskolin (F), 0.33% ethanol (EtOH), 500 μ M 6-Bnz, or 100 μ M prostaglandin E₂ (PGE₂) for 48 h. (B) ASCs were treated with 8-CPT (250 μ M) for the indicated time periods. Total cell lysates were prepared in RIPA buffer before equal amounts of protein (50 μ g) were subjected to Western blot analysis using antibodies against the indicated proteins. Actin is shown as loading control. The blots represent one of three reproducible experiments.

reversed the antiproliferative effect of cAMP ($p = 0.001$), measured by incorporation of [³H]thymidine (Figure 1D). Taken together, these results indicate that cAMP inhibits the proliferation of ASCs without affecting viability.

To examine whether other inducers of intracellular cAMP had the same effect as 8-CPT on proliferation of ASCs, we tested the effects of PGE₂, forskolin, and the PKA-specific cAMP analog 6-Bnz-cAMP (6-Bnz). The PGE₂ receptors EP2 and EP4 activate G-proteins, which in turn stimulate adenylate cyclase activity (Regan, 2003), whereas the diterpene forskolin directly activates the catalytic subunit of adenylate cyclase (Seamon and Daly, 1981). As shown in Figure 1E, all cAMP-elevating agents were able to inhibit DNA synthesis. Similar observations were made in a polyclonal culture of ASCs from three different donors (data not shown).

Effect of cAMP on Protein Expression of Cell Cycle-regulating Genes

To study the mechanisms underlying growth inhibition of ASCs by cAMP, we analyzed the expression of various cell cycle-regulating proteins after 48 h of treatment with cAMP inducers. As shown in Figure 2A, pRB was highly phosphorylated in stimulated ASCs. Addition of different cAMP-increasing agents resulted in a prominent inhibition of pRB phosphorylation, consistent with the observed cAMP-induced growth arrest. The changes in pRB phosphorylation appeared after 12 h of treatment with 8-CPT (Figure 2B). Reduced phosphorylation of pRB was associated with reduced levels of cdk2, especially of the lower band, and reduced expression of cyclin A was also observed (Figure 2A). No or minor effects were seen on the levels of D-type cyclins and of the two cyclin-dependent kinase inhibitors p21^{Cip1} and p27^{Kip1}. Interestingly however, the level of cyclin E markedly increased upon cAMP induction despite reduced phosphorylation of pRB. The time-dependent effect of 8-CPT on cdk2 expression correlated temporally with the kinetics of pRB phosphorylation, whereas the effect on cyclin E was noted at 3 h of treatment (Figure 2B).

cAMP Inhibits cdk2 Activity

The activity of cdk2 is known to be modulated by a variety of mechanisms, including the levels of cyclin E, cyclin A, and cdk2 itself, by binding of cyclin-dependent kinase inhibitors

(CKIs), and by phosphorylation (Morgan, 1997). Because we have shown that cAMP reduced the expression of cdk2 and cyclin A, concomitant with an increase in cyclin E (see Figure 2A), we determined the effect of cAMP on cdk2 activity. This was addressed by analyzing anti-cdk2- and anti-cyclin E immunoprecipitates from stimulated ASCs for cdk2 activity using histone H1 as substrate (Figure 3). Cdk2 activity was significantly reduced by 8-CPT and PGE₂, and this could be explained by the reduced cdk2 level observed in IPs of cdk2 (Figure 3, top panels). Reduced kinase activity was also observed when cyclin E-associated kinase activity was measured, and in IPs of cyclin E the cdk2 level in 8-CPT-treated cells was reduced relative to cyclin E (Figure 3, middle panels). Western blot analysis of the same lysates confirmed the cAMP-induced down-regulation of cdk2, inhibition of pRB phosphorylation, and up-regulation of cyclin E (Figure 3, bottom panels). The same effects of cAMP on the cell cycle machinery were observed in a polyclonal culture of ASCs from three different donors (data not shown). We conclude therefore that in ASCs, cAMP leads to reduced proliferation, reduced cdk2 activity, and reduced pRB phosphorylation, despite markedly induced levels of cyclin E.

cAMP Changes the Subcellular Localization of Cyclin E

The reduced protein levels of cdk2 and cyclin A could possibly explain the cAMP-induced decrease in cdk2 activity. Nevertheless, because the activity of cdk2 reduced concomitant with increased cyclin E expression, we also investigated the subcellular localization of cyclin E upon 8-CPT treatment. Cyclin E has previously been shown to be localized in the nucleus (Ohtsubo *et al.*, 1995). However, a recent report showed that cyclin E shuttles between the nucleus and cytoplasm and that its apparent nuclear localization is a reflection of slow nuclear export compared with a more rapid import (Jackman *et al.*, 2002). Therefore, we explored the possibility that cAMP might affect this shuttling, leading to translocation of cyclin E from the nucleus. Immunofluorescence staining of cyclin E in stimulated ASCs treated with or without 8-CPT showed that in control cells, staining of cyclin E was predominantly nuclear (Figure 4AI). In contrast, no nuclear staining was observed in cells treated with 8-CPT (Figure 4AII), suggesting that cyclin E was shuttled to the cytoplasm. In line with this assumption, Western blot

Figure 3. cAMP inhibits cdk2 activity. ASCs were grown for 40 h in the presence or absence of 250 μ M 8-CPT or 100 μ M prostaglandin E_2 . Total cell lysates were prepared in Triton X-100 lysis buffer, immunoprecipitated with cdk2 (top panels) or cyclin E (middle panels) antibodies, and then assayed for activity using Histone H1 (2 μ g) as substrate or subjected to Western blot analysis using antibodies against cyclin E or cdk2. Phosphorylation of histone H1 was monitored by autoradiography. Fifty micrograms of whole cell extract was also subjected to Western blot analysis using antibodies against the indicated proteins (bottom panels). One representative experiment of three is shown.

analysis of the cytosolic fraction confirmed up-regulation of cyclin E in the cytoplasm after 8-CPT-treatment (Figure 4B). The expected reduced nuclear expression of cyclin E upon cAMP treatment was probably masked by contamination of

cytosolic cyclin E in the nuclear fraction, because the cytoplasmic marker was expressed in both fractions. When cells were analyzed by immunofluorescence staining, we did not detect increased staining of cyclin E in the cytoplasm upon cAMP induction. The weak cytoplasmic staining in Figures 4A, I and II, seemed to be background staining, because similar staining was detected in cells transfected with cyclin E siRNA (see Figure 6). No translocation of cdk2 from the nucleus was observed upon 8-CPT treatment (Figures 4A, III and IV). Therefore, the results indicate that cAMP induces a shift in the subcellular localization of cyclin E. This might explain why elevation of cyclin E seems to be uncoupled from cdk2 activation in ASCs.

cAMP Induces Differentiation of ASCs into Neuron-like Cells

Next, we examined the functional significance of cyclin E induction in 8-CPT-treated ASCs. In agreement with previous studies on bone marrow-derived MSCs (Kim *et al.*, 2005), cAMP induced differentiation of ASCs into neuron-like cells with distinct changes in cell morphology (Figure 5A; compact cell bodies and long processes) and with increased levels of the neuron-specific protein NF200 (Figure 5B). After 4 d of treatment, ~30% of the cells treated with 8-CPT expressed NF200, compared with 8% in control cells. Having shown that cyclin E was induced by cAMP in ASCs and apparently was uncoupled from cdk2 activation, we postulated that cyclin E might have cdk2-independent function in these cells. Cyclin E has been shown to promote development of the CNS in *Drosophila melanogaster* (Berger *et al.*, 2005); thus we investigated whether cyclin E could play a role in the observed cAMP-induced differentiation of ASCs. Expression of cyclin E was silenced by transient transfection with siRNA targeted to cyclin E, and as shown in Figure 6, A and B, cyclin E levels were markedly reduced. In accordance with its role as a regulator of the G1/S transition, knocking down cyclin E in the control cells reduced [3 H]thymidine incorporation to 34% compared with the mock-transfected cells (Figure 6C). However, no effect of cyclin E siRNA was noted on the cAMP-mediated growth-inhibition and on cAMP-mediated differentiation, as demonstrated by NF200 staining (Figure 6D) and by morphology (data not shown).

Figure 4. cAMP changes the subcellular localization of cyclin E. (A) ASCs were plated onto coverslips at a density of 1.5×10^4 cells/coverslip into six-well plates. After 48 h of treatment with or without 8-CPT (250 μ M), cells were fixed, stained for cyclin E (I and II), cdk2 (III and IV), or nuclei (DAPI), and then analyzed by confocal microscopy (60 $\times$ objective). One representative experiment of three is shown. (B) ASCs were treated with or without 250 μ M 8-CPT. Subcellular fractionation was performed as described in *Materials and Methods*, before equal amounts of protein (50 μ g) were subjected to Western blot analysis using antibodies against the indicated proteins.

Figure 5. cAMP induces differentiation of ASCs into neuron-like cells. ASCs were grown for 5 d in the presence or absence of 250 μ M 8-CPT. (A) Cell morphology was then monitored by phase-contrast microscopy (magnification $\times$ 200). (B) For immunocytochemistry, ASCs were plated onto coverslips at a density of 1.5×10^4 cells/coverslip into six-well plates. After 5 d of treatment with or without 8-CPT (250 μ M), cells were fixed, stained with NF200 antibodies, and then observed with an Olympus BX51 microscope (40 $\times$ objective). One representative experiment of three is shown.

Cyclin E Sensitizes ASCs to Radiation-induced Apoptosis

A cdk2-independent role of cyclin E in sensitization to apoptotic stimuli has previously been demonstrated (Leonce *et al.*, 2001). Further, recent investigations have revealed that genotoxic stress increases the levels of cyclin E in hematopoietic cells, leading to amplification of apoptosis (Mazumder *et al.*, 2000, 2002, 2004). To study whether the cAMP-mediated high levels of cyclin E sensitized ASCs to genotoxic stress, cells were first treated with 8-CPT for 24 h to induce cyclin E expression and then were exposed to 25 Gy γ -irradiation for 48 h. Apoptotic cells were assessed by TUNEL assay. Treatment with 8-CPT alone did not induce apoptosis (Figure 7A), consistent with the other viability tests that we performed (see above). However, 8-CPT-treated ASCs were sensitized to γ -irradiation-induced apoptosis, revealing a sevenfold induction in the number of TUNEL-positive cells upon irradiation compared with un-

treated cells (Figure 7A). No effect of γ -irradiation was noted on the 8-CPT-induced up-regulation of cyclin E (Figure 7A, bottom panels); however, when cyclin E was knocked down by siRNA, the proportion of TUNEL-positive cells after γ -irradiation was markedly reduced compared with mock-transfected cells (Figure 7, B and C) as well as cells transfected with control siRNA (data not shown). Furthermore, reducing the activity of cdk2 by transfecting the cells with a construct of dominant negative cdk2 did not affect cAMP-mediated induction of apoptosis (Figure 7D). Taken together, these results indicate that cyclin E plays an important cdk2-independent role in γ -irradiation-induced apoptosis in ASCs and that this effect is promoted by cAMP, through elevation of cyclin E levels.

ERK Is Involved in cAMP-mediated Induction of Cyclin E

We next investigated the mechanisms whereby elevation of cAMP induces cyclin E. cAMP activates ERK (also referred

Figure 6. The effect of knockdown of cyclin E on cAMP-induced growth inhibition and differentiation. ASCs were mock-transfected or transfected with 8 μ g of cyclin E siRNA using the Human MSC Nucleofector Kit as described in *Materials and Methods*. The transfected cells were then treated with or without 8-CPT (250 μ M) for 72 h. (A) Total cell lysates were prepared in RIPA buffer before equal amounts of protein (50 μ g) were subjected to Western blot analysis. Actin is shown as loading control. (B) ASCs plated onto coverslips were fixed and stained for cyclin E and nuclei (DAPI) before examination by immunofluorescence (see *Materials and Methods*). (C) DNA synthesis was measured as uptake of [3 H]thymidine as described in *Materials and Methods*. (D) ASCs plated onto coverslips were fixed, stained for the neuron-specific protein NF200, and examined by immunocytochemistry, before differentiation was measured as number of cells expressing NF200. The data represent one of three reproducible experiments.

Figure 7. Cyclin E sensitizes ASCs to DNA damage-induced apoptosis. ASCs were plated onto coverslips at a density of 1.5×10^4 cells/coverslips into 35-mm culture plates (for TUNEL assays) or grown in 10-cm culture plates ($1\text{--}1.5 \times 10^5$ cells/plate; for Western blot analysis). After 40 h of treatment with or without 8-CPT (250 μM), the cells were irradiated with 25 Gy and then grown for additional 40 h. (A) Cells were subjected to TUNEL assay followed by fluorescence microscopy. Apoptosis was determined by counting the number of TUNEL-positive cells, and the results are presented as mean fold induction of control $\pm$ SEM ($n = 4$). Total cell lysates were prepared in RIPA buffer and then subjected to immunoblot analysis with antibodies against cyclin E. Actin is shown as loading control. The blot is representative of three independent experiments. (B) ASCs were mock-transfected or transfected with 8 μg of cyclin E siRNA using the Human MSC Nucleofector Kit as described in *Materials and Methods*. The transfected cells were then stimulated with or without 8-CPT and irradiated as described above. Cells were subjected to TUNEL assay and analyzed for apoptosis by counting the number of TUNEL-positive cells (the vertical bars indicate range of two experiments), or total cell lysates were prepared in RIPA buffer. Equal amounts of protein (50 μg) were subjected to immunoblot analysis with antibodies against cyclin E. Actin is shown as loading control. (C) Visualization of apoptotic (TUNEL-positive) cells and nuclei (DAPI). The cells were observed with an Olympus BX51 microscope (40 $\times$ objective). (D) ASCs were mock-transfected or transfected with 5 μg cdk2-DN using the Human MSC Nucleofector Kit as described in *Material and Methods*. The transfected cells were then stimulated with or without 8-CPT and irradiated as described above, before they were subjected to TUNEL assay. Apoptosis was determined by counting the number of TUNEL-positive cells, and the results are presented as fold induction of control. To measure cdk2 activity, total cell lysates of untreated transfected cells were immunoprecipitated with cdk2 antibodies and assayed for activity using Histone H1 (2 μg) as substrate.

to as MAPK) in several neuronal and endocrine cell types (Dugan *et al.*, 1999; Grewal *et al.*, 1999; Iacovelli *et al.*, 2001). Because many targets of the MEK-ERK pathway are cell cycle regulatory proteins (Meloche and Pouyssegur, 2007), we assessed the possible involvement of this pathway in cAMP-mediated induction of cyclin E in stimulated ASCs. Pretreatment with the MEK inhibitor U0126 markedly reduced the 8-CPT-induced elevation of cyclin E expression (Figure 8A). The inhibitor did not reverse the effect of 8-CPT on any of the other cell cycle parameters and rather inhibited the levels of cdk2 alone. To confirm the involvement of the MEK-ERK pathway in cAMP-mediated signaling in ASCs, we directly analyzed the activity of ERK. The level of phosphorylated (activated) ERK was analyzed by Western blotting using phospho-specific anti-

bodies (Thr202/Tyr204-phosphorylated p44/p42 MAPK). As shown in Figure 8B, cAMP markedly induced phosphorylation (activation) of ERK, and the effect was noticeable by 3 h of treatment (Figure 8C). As a control of equal loading, the lysates were also analyzed for expression of total ERK (Figure 8, B and C, bottom panels). The time-dependent effect of cAMP on cyclin E expression (see Figure 2B) closely resembled that of ERK phosphorylation, supporting a causative relationship between these events. To further demonstrate the requirement of ERK for the cAMP-mediated up-regulation of cyclin E, ERK was knocked down by siRNA. Indeed, reducing the levels of ERK prevented the induction of cyclin E upon 8-CPT treatment and totally prevented the induction of apoptosis (Figure 8D).

Figure 8. ERK is involved in cAMP-mediated elevation of cyclin E levels in ASCs. (A) ASCs were pretreated with the MEK inhibitor U0126 (10 μ M) for 40 min before treatment with 8-CPT (250 μ M) for 48 h. (B) ASCs were grown for 48 h in the presence or absence of 250 μ M 8-CPT, 50 μ M forskolin (F), 0.33% ethanol (EtOH), 500 μ M 6-Bnz, or 100 μ M prostaglandin E2 (PGE₂). (C) ASCs were treated with 8-CPT (250 μ M) for the indicated time periods. Total cell lysates were prepared in RIPA buffer before equal amounts of protein (50 μ g) were subjected to Western blot analysis using antibodies against the indicated proteins. Actin (A) and total levels of ERK (B and C) are shown as loading controls. The blots are representatives of three reproducible experiments. (D) ASCs were transfected with 8 μ g of cyclin E siRNA or control siRNA using the Human MSC Nucleofector Kit as described in *Materials and Methods*. At 20 h after transfection, the cells were treated with or without 8-CPT for 40 h, before they were irradiated with 25 Gy and then grown for additional 40 h. Cells were subjected to TUNEL assay and analyzed for apoptosis by counting the number of TUNEL-positive cells, or total cell lysates were prepared in RIPA buffer. Equal amounts of protein (50 μ g) were subjected to immunoblot analysis with antibodies against total ERK and cyclin D3.

Given the role of ERK in cAMP-induced neuronal differentiation (Vossler *et al.*, 1997; Kim *et al.*, 2005; Jori *et al.*, 2005), we also assessed the role of MEK-ERK pathway in cAMP-mediated differentiation of ASCs into NF200-positive cells. However, because pretreatment of ASCs with U0126 did not reduce the number of NF200-positive cells induced by elevation of cAMP (data not shown), we concluded that the MEK-ERK pathway does not play a role in cAMP-mediated neuronal differentiation of ASCs. This conclusion was supported by the inability of siRNA against the ERK-induced cyclin E to prevent differentiation of ASCs by cAMP (Figure 6).

cAMP Inhibits the Degradation of Cyclin E

To further investigate the mechanisms whereby cAMP up-regulates cyclin E expression, we analyzed whether cAMP affects the levels of cyclin E mRNA. RT-PCR analysis showed that 8-CPT did not affect the steady state level of cyclin E mRNA (data not shown), indicating that cAMP regulates expression of cyclin E posttranscriptionally. Cyclin E is an unstable protein, and its expression is also regulated by ubiquitin-dependent proteolysis (Hwang and Clurman, 2005). To address whether cAMP affected the rate of cyclin E degradation, ASCs pretreated with or without 8-CPT for 48 h were treated with the protein synthesis inhibitor cycloheximide

in a time-course experiment. Western blot analysis revealed that cyclin E was rapidly degraded in control cells, whereas 8-CPT completely stabilized cyclin E protein levels (Figure 9), indicating that cAMP prevents degradation of cyclin E.

DISCUSSION

One of the factors known to regulate differentiation of MSCs is cAMP (Strickland *et al.*, 1980; Zhao *et al.*, 2006; Wang *et al.*,

Figure 9. cAMP inhibits the turnover of cyclin E. ASCs were treated with or without 300 μ M 8-CPT for 40 h before incubation with cycloheximide (CHX; 25 μ g/ml) for the indicated time periods. The cells were lysed, and equal amounts of protein (50 μ g) were subjected to Western blot analysis using antibodies against cyclin E. Actin is shown as loading control. The blots are representatives of three independent experiments.

2006), and activation of the cAMP/PKA pathway has been shown to drive these cells in neurogenic direction (Rydel and Greene, 1988; Deng *et al.*, 2001; Kim *et al.*, 2005; Shi *et al.*, 2006; Wang *et al.*, 2007). We have identified a hitherto unknown role and regulation of cyclin E downstream of cAMP signaling in ASCs. Thus, although the cells were markedly growth inhibited by cAMP, cyclin E levels were potently induced and rendered the cells susceptible to DNA damage-induced apoptosis.

The antiproliferative effect of cAMP was associated with a reduction in both cdk2 activity and pRB phosphorylation. Induced expression of cyclin E was therefore unexpected, given the critical role of cyclin E in cell cycle progression. However, induction of cyclin E concomitant with reduced DNA synthesis has previously been demonstrated in HT29 human colon carcinoma cells (Leonce *et al.*, 2001), as well as in growth-arrested hepatocytes and bone marrow-derived MSCs (Oliva *et al.*, 2003; Mullany *et al.*, 2007). In the latter studies, the growth arrest was explained by increased levels of cdk inhibitors, whereas in our experiments, treatment with cAMP-increasing agents did not change the expression of cdk inhibitors. Instead, the reduced activity of cdk2 and thereby reduced proliferation could be the result of the reduced protein levels of cdk2 and cyclin A. The discrepancy between cyclin E induction and reduced cdk2 activity could further be explained by the decreased nuclear localization of cyclin E in response to elevated levels of cAMP, leading to different subcellular localization of cyclin E and cdk2 and thereby making cyclin E unable to activate cdk2.

The apparent uncoupling of cyclin E from cdk2 activity and cell cycle function in cAMP-treated ASCs lead us to postulate that cyclin E might have cdk2-independent functions in these cells. Knockout experiments have shown that cdk2^{-/-} mice are viable, whereas cyclin E1^{-/-}, E2^{-/-} mice die in utero, suggesting that cyclin E may have cdk2-independent roles (Berthet *et al.*, 2003; Geng *et al.*, 2003; Ortega *et al.*, 2003; Parisi *et al.*, 2003). In fact, recent reports have demonstrated kinase-independent functions of cyclin E in cell cycle progression, as well as in other cellular processes (Mazumder *et al.*, 2002; Matsumoto and Maller, 2004; Geng *et al.*, 2007). Thus, cyclin E can act as a cell fate determinant in the developing CNS of *D. melanogaster* (Berger *et al.*, 2005), and cyclin E2 is required for embryogenesis in *Xenopus laevis* (Gotoh *et al.*, 2007). Given the role of cyclin E in differentiation and because cAMP induced differentiation of ASCs into neuron-like cells, the possibility existed that the role of cAMP-induced elevation of cyclin E expression was to drive differentiation of ASCs into neurogenic direction. However, knocking down cyclin E by siRNA did not inhibit the cAMP-mediated neurogenic differentiation of the cells, ruling out this possibility.

Cyclin E has also been reported to play a role in apoptosis (Leonce *et al.*, 2001; Mazumder *et al.*, 2004). We did not observe any induction of apoptosis in response to 8-CPT alone, a result supported by the fact that the growth-inhibitory effect of cAMP on ASCs was reversible. It was still possible, however, that the increased level of cyclin E rendered the cells susceptible to apoptosis-inducing agents, because this would be in accordance with reports showing that cyclin E plays a critical role in amplification of genotoxic stress-induced apoptosis of hematopoietic cells (Mazumder *et al.*, 2000, 2002). To address a possible role of cyclin E in DNA damage-induced apoptosis, ASCs pretreated with 8-CPT to induce cyclin E expression were exposed to 25 Gy γ -irradiation for 48 h. Indeed, elevated levels of cAMP enhanced the number of TUNEL-positive cells upon irradiation, thus sensitizing these cells to DNA damage-induced

apoptosis. This sensitization by cAMP was dependent on cyclin E expression, thus we concluded that cyclin E induction seems to play an important role in γ -irradiation-induced apoptosis of ASCs. The proapoptotic effect of cyclin E was apparently independent of cdk2 activity, which is in accordance with other reports (Leonce *et al.*, 2001; Mazumder *et al.*, 2007). Cyclin E expression has been shown to decline late in the apoptotic process, due to caspase-dependent proteolysis of cyclin E (Mazumder *et al.*, 2002). Thus, cyclin E induction is likely to play an important role in the initiation phase of cell death. The mechanism behind cyclin E-mediated amplification of DNA-damage-induced apoptosis in adipose stem cells is the subject of further studies.

Although expression of cyclin E usually depends on the pRB/E2F pathway (Ohtani *et al.*, 1995; Le *et al.*, 1999; Moroy and Geisen, 2004), cyclin E may also be a direct target of extracellular signals, such as the MEK/ERK pathway (Nourse *et al.*, 1994; Mohapatra *et al.*, 2001). Several studies have demonstrated a cross-talk between the cAMP signaling pathway and the Ras-Raf-MEK-ERK pathway (Stork and Schmitt, 2002; Dumaz and Marais, 2005). In a cell-specific manner, cAMP can either inhibit or activate ERK, depending on the relative amounts of Rap1 (a GTPase of the Ras superfamily) and the Raf isoforms B-Raf and Raf-1 expressed by the cell (Stork and Schmitt, 2002). In B-Raf-positive cells, such as neurons, Rap1 associates with B-Raf, leading to activation of ERK. Activation of ERK by cAMP has also been reported for bone marrow-derived MSCs (Kim *et al.*, 2005). Thus in these cells, cAMP induced neuronal differentiation due to PKA/B-Raf-mediated activation of ERK. To examine the possibility of ERK being involved in cAMP-mediated up-regulation of cyclin E, ERK was knocked down by siRNA or cells were pretreated with the MEK inhibitor U0126 before addition of 8-CPT. Both the siRNA and the inhibitor markedly reduced the induction of cyclin E upon 8-CPT treatment, suggesting the involvement of the Ras-Raf-MEK-ERK pathway in this process. This was further supported by the enhanced activity of ERK in response to increased levels of intracellular cAMP. Moreover, the time-dependent effect of cAMP on ERK closely resembled that on cyclin E, supporting a causative relationship between these events. Our results are consistent with the recent finding that treatment of rat mesangial cells with the MEK inhibitor U0126 completely inhibited the stimulation-induced cyclin E expression in these cells (Bokemeyer *et al.*, 2007).

Expression of cyclin E is primarily regulated at the level of transcription or by ubiquitin-dependent proteolysis (Ohtani *et al.*, 1995; Le *et al.*, 1999; Moroy and Geisen, 2004). As expected, because cdk2 activation and pRB phosphorylation were reduced in 8-CPT-treated ASCs, we did not observe transcriptional induction of cyclin E. Instead, we demonstrated that cAMP induced a pronounced stabilization of cyclin E at the protein level. Cyclin E is an unstable protein that is degraded by two distinct pathways: 1) ubiquitination of monomeric cyclin E (by the Cul-3 ubiquitin-ligase) and 2) ubiquitination of phosphorylated cyclin E in complex with cdk2 (by the SCF-Fbw7 ubiquitin-ligase; Clurman *et al.*, 1996; Singer *et al.*, 1999; Welcker *et al.*, 2003; Hwang and Clurman, 2005). We are currently investigating which of these pathways are disrupted by cAMP induction in ASCs.

In conclusion, we have demonstrated that cAMP induction in ASCs leads to uncoupling of cyclin E from its established role as an obligate driving force in cell cycle progression and that cAMP-signaling instead directs cyclin E to render these cells sensitive to DNA damage-induced apoptosis. It is well established that stem cells in general are resistant to such apoptosis (Guo *et al.*, 2006; Hong *et al.*,

2006), and this might be important for preserving a pool of self-renewing stem cells. However, by promoting differentiation into neuron-like cells, it is possible that cAMP also must ensure that resistance to DNA damage-induced apoptosis is relieved. Thus, it might be important for neuron-like cells to more readily undergo cell death when exposed to threatening DNA-damaging agents.

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