toward SDF-1 and efficient engraftment by converted $CD34^+CD38^{-/low}CXCR4^+$ cells, properties that were similar to those of the original migrating fraction (M) (Fig. 4A).

Self-renewal of stem cells can only be determined by their ability to also repopulate secondary transplanted recipients with high numbers of both myeloid and lymphoid cells. Consistent with previous studies, secondary transplanted mice that received untreated human cells showed little engraftment (Fig. 4B, panel a) (*22*). Human interleukin-6 (IL-6) synergizing with SCF induced high levels of CXCR4 expression on $CD34^+$ cord blood cells (Fig. 4C). Incubation of bone marrow cells from primary transplanted mice with SCF and IL-6 for 48 hours resulted in up-regulation of surface CXCR4 expression (Fig. 4B, panel c) and increased migration of human progenitor cells to SDF-1 in vitro (Fig. 4B, panel d). Transplantation of similar numbers of human cells from the bone marrow of primary transplanted mice after treatment with these cytokines resulted in higher engraftment levels in secondary transplanted mice compared with mice transplanted with untreated cells (Fig. 4B, panel b versus panel a). Thus, by up-regulating surface CXCR4 expression on primitive cells, the population of self-renewing $CD34^+CD38^{-/low}$ SRC stem cells could be increased.

Our data provide evidence that CXCR4-dependent migration to SDF-1 is essential for human stem cell function in NOD/SCID mice. We characterized SRCs further as $CD34^+CD38^{-/low}CXCR4^+$ stem cells and showed that $CD34^+CD38^{-/low}CXCR4^{-/low}$ cells can be converted into functional $CXCR4^+$ stem cells by cytokine treatment. This suggests that migration to SDF-1 is associated with localization of stem cells in the bone marrow, permitting differentiating cells with reduced migration levels to exit into the blood circulation. In conclusion, our findings define human $CD38^{-/low}CXCR4^+$ cells as stem cells endowed with migration and repopulation potential and provide insights into human stem cell biology.

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23. Human cells were obtained after informed consent according to procedures approved by the Weizmann Committee. In all experiments, samples of the same initial cell pool were compared. Differences in the results are due to the different $CD34^+$ cell sources (cord blood, bone marrow, and mobilized peripheral blood). $CD34^+$ enrichment, flow cytometry, and fluorescence-activated cell sorting (FACS) were performed as previously described (*5*, *6*). SDF-1 (125 ng/ml, R&D Systems) transmigration assays were done as previously described (*13*) with 2×10^5 $CD34^+$ cells. Percents in the results represent percent of initial 2×10^5 cells in the migrating and nonmigrating cell fractions. The sources for the reagents are as follows: PMA (100 ng/ml), was purchased from Sigma, stem cell factor (SCF) and IL-6 (50 ng/ml) from R&D Systems, and antibodies to CXCR4 from Pharmingen {12g5 monoclonal antibody [immunoglobulin G2a (IgG2a)]} or R&D Systems [MBA171 monoclonal antibody (IgG2a)] (10 μg per 2×10^5 cells). CXCR4 expression was always analyzed by double staining with anti-CD34. Polyclonal anti–SDF-1 (10 μg per mouse, R&D Systems) was injected intravenously with the cells (2×10^5 cells per mouse) and 24 hours later injected again intraperitoneally. Control cells were incubated with anti-CD34 [IgG1, Becton Dickinson, 10 μg per 2×10^5 cells]. Human lymphoid and myeloid cells were immunostained with anti-CD45 (Immuno Quality Products, Groningen, Netherlands), anti-CD19, and anti-CD56 (Coulter). Natural killer cells differentiated into mature $CD56^+$ cells after incubation with human SCF (100 ng/ml) and human IL-15 (100 ng/ml, R&D Systems) for 10 days. NOD/SCID, and NOD/SCID β_2-microglobulin knockout (*20*) mice were bred and maintained under defined flora in intraventilated cages and transplanted by injection into the tail vein after sublethal (375R) irradiation according to established protocols (*5*, *6*) approved by the Weizmann animal ethics committee. Southern (DNA) blot analysis with a human-specific α satellite probe and human-specific progenitor assays were done as previously described (*5*, *6*). Percent engraftment always indicates the percent of either human DNA or of human CD45 cells in the mouse bone marrow. The levels of engraftment were dependent on the injected cell dose, the duration of the experiment, and the source of human $CD34^+$ cells. Cells were cultured either in serum-free media as previously described (*6*) or in media supplemented with 10% fetal calf serum.

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CD3- and CD28-Dependent Induction of PDE7 Required for T Cell Activation

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Costimulation of both the CD3 and CD28 receptors is essential for T cell activation. Induction of adenosine 3′,5′-monophosphate (cAMP)–specific phosphodiesterase-7 (PDE7) was found to be a consequence of such costimulation. Increased PDE7 in T cells correlated with decreased cAMP, increased interleukin-2 expression, and increased proliferation. Selectively reducing PDE7 expression with a PDE7 antisense oligonucleotide inhibited T cell proliferation; inhibition was reversed by blocking the cAMP signaling pathways that operate through cAMP-dependent protein kinase (PKA). Thus, PDE7 induction and consequent suppression of PKA activity is required for T cell activation, and inhibition of PDE7 could be an approach to treating T cell–dependent disorders.

Activation of peripheral T cells in vivo by an antigen-presenting cell is a result of the engagement of both the T cell receptor–CD3 complex (TCR-CD3) and the CD28 costimulatory receptor. When both receptors are occupied by their appropriate ligands, T cells are stimulated to proliferate and produce interleukin-2 (IL-2), whereas occupation of the T cell receptor alone favors T cell anergy or apoptosis (*1*). Occupation of the CD28 receptor alone appears to have no obvious effect on T cells; nevertheless, CD28 costimulation is required for full activation of CD4 T helper cells, if not all T cells (*2*). Why is CD28 costimulation required for T cell activation? One possible reason has been suggested by

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the observation that ligation of TCR-CD3 initiates not only stimulatory signal transduction pathways, such as the MAP kinase (mitogen-activated protein kinase)–dependent signaling pathways (*3*) and the NFAT (nuclear factor of activated T cells)–dependent signaling pathways (*4*), but also inhibitory signal transduction pathways such as the PKA (the cAMP-dependent protein kinase)–dependent signaling pathway (*5*). PKA can inhibit proliferation in many cell types, including fibroblasts (Rat-1 and NIH 3T3 cells), smooth muscle cells, and adipocytes (*6*), as well as T cells (*7*). Phosphorylation of NFAT by PKA can abolish the translocation of NFAT from the cytoplasm to the nucleus (*8*); and phosphorylation of Raf kinase, again by PKA, can block the MAP kinase–dependent signaling pathway (*9*). Both the translocation of NFAT and the MAP kinase–dependent signal are essential for IL-2 gene expression (*3*, *4*). It is plausible, therefore, that to become fully activated, T cells require additional TCR-independent signals to overcome PKA inhibition.

Because phosphodiesterases (PDEs) play a major role in down-regulation of PKA activity by hydrolyzing cAMP in many cell types (*10*), and PDE7 protein appears to be detected only in lymphocytes (*11*), we reasoned that PDE7 might play a role in regulating T cell activation. To test this hypothesis, we first analyzed Northern (RNA) blots to determine the tissue distribution of PDE7. Consistent with previous work demonstrating that PDE7 contributed up to 40% of total PDE activity in several T cell lines (*11*), PDE7A1, one of the two RNA splicing variants of PDE7, is predominantly expressed in human lymphoid tissues (Fig. 1B). However, the PDE7 protein was barely detectable in isolated peripheral T cells (Fig. 2A), but PDE7 activity was increased by costimulation of T cells with antibodies to CD3 and CD28 (Fig. 2B); whereas the activity of PDE4, the other major isoform of cAMP-specific PDE in T cells, remained unchanged (*12*). Very high doses of antibody to CD3 (anti-CD3) or antibody to CD28 (anti-CD28) alone could induce PDE7 (*12*). However, at lower doses that are more likely to mimic the normal physiological state (0.2 ng of anti-CD3 per milliliter and 0.2 μg of anti-CD28 per milliliter), only the combination of the two antibodies increased the amount of PDE7 protein (Fig. 2A) and its activity (Fig. 2B), decreased cAMP levels (Fig. 2C), increased IL-2 expression (Fig. 2D), and promoted proliferation (Fig. 2E), in isolated peripheral T cells.

To investigate whether induction of PDE7 was not merely correlated with but essential for T cell proliferation, we tested the effect of blocking PDE7 expression on T cell proliferation as well as on IL-2 production. Because no PDE7-specific inhibitor is yet available, we used PDE7 antisense oligonucleotides to block PDE7 expression. Several nonoverlapping antisense oligonucleotides were tested. In addition, a series of control oligonucleotides that shared the same nucleotide composition with their corresponding antisense oligonucleotide but had a reversed sequence order were also

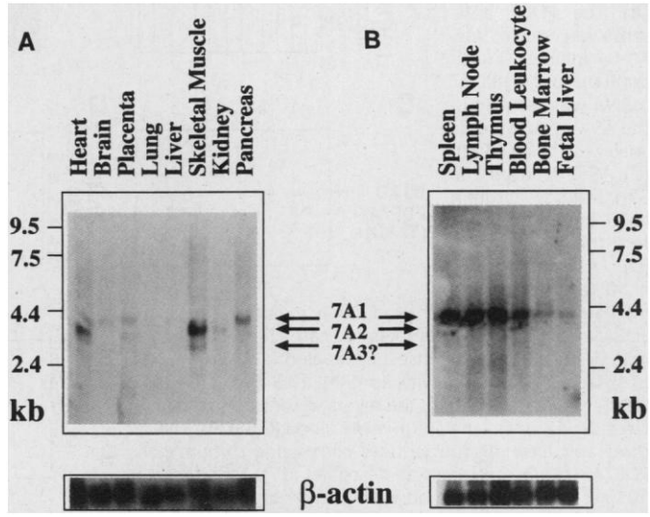

Fig. 1. Northern blot analysis of PDE7A1 and PDE7A2. (**A**) PDE7A2 is expressed in heart and skeletal muscle, whereas PDE7A1 is expressed in the pancreas and the placenta. (**B**) PDE7A1 is predominantly expressed in lymphoid tissues. Northern blots of multiple human tissues [sample number 7760-1 in (A) and 7768-1 in (B)] (Clontech, Palo Alto, CA) were hybridized with a ^{32}P-labeled, 1.2-kb, Not 1–Eco RI fragment of human PDE7 cDNA, which can hybridize with both PDE7A1 and PDE7A2. ExpressHyb solution (Clontech) was used for both prehybridization and hybridization performed at 60°C. The ^{32}P-labeled probe, together with salmon sperm DNA (0.1 mg/ml), was added directly to the pre-hybridization solution for hybridization. Membranes were washed twice in 2× SSC [3 M sodium chloride and 0.3 M sodium citrate (pH 7.0)] and 0.1% SDS at room temperature for 10 min, then once in 0.1× SSC and 0.1% SDS at 60°C for 20 min. β-actin cDNA (Clontech) was used as a control probe for the mRNA quantity of the Northern blots. Relative molecular weights are indicated. 7A1 stands for the 4.2-kb mRNA of PDE7A1 (*15*) and 7A2 for the 3.8-kb mRNA of PDE7A2 (*11*, *15*). 7A3? indicates a possible third splice variant, PDE7A3.

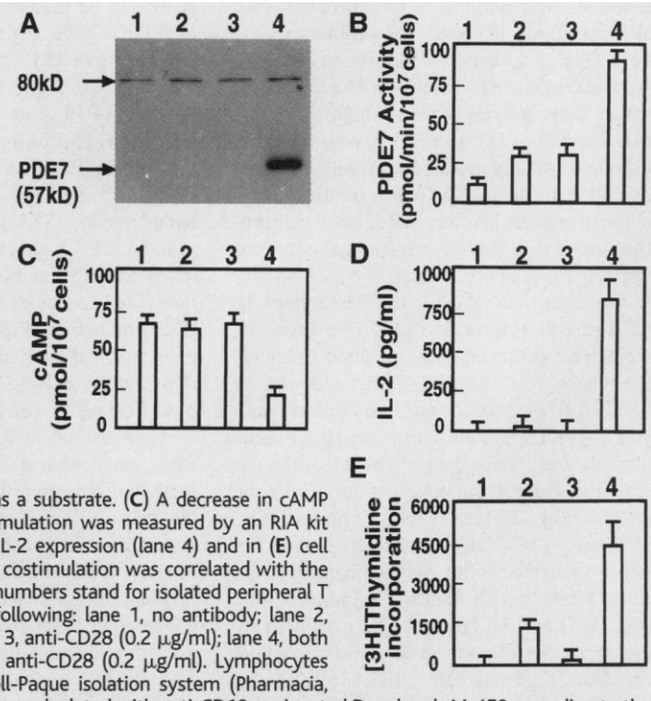

Fig. 2. Induction of PDE7 in peripheral T cells by anti-CD3 and anti-CD28 costimulation. (**A**) Protein immunoblot analysis of PDE7 protein. The 57-kD band (lane 4) is the predicted size of the PDE7A1 protein. The 80-kD band, which cross-reacts with PDE7 antibodies, is an unknown protein present in the particulate fraction and appears to have no PDE activity. (**B**) PDE activity in the immunoprecipitated protein of PDE7-specific antibodies was measured using 1 μM cAMP as a substrate. (**C**) A decrease in cAMP levels (lane 4) upon costimulation was measured by an RIA kit (*15*). (**D**) The increase in IL-2 expression (lane 4) and in (**E**) cell proliferation (lane 4) after costimulation was correlated with the PDE7 induction. The lane numbers stand for isolated peripheral T cells incubated with the following: lane 1, no antibody; lane 2, anti-CD3 (0.2 ng/ml); lane 3, anti-CD28 (0.2 μg/ml); lane 4, both anti-CD3 (0.2 ng/ml) and anti-CD28 (0.2 μg/ml). Lymphocytes were isolated with a Ficoll-Paque isolation system (Pharmacia, Uppsala, Sweden). B cells were depleted with anti-CD19 conjugated Dynabeads M-450 according to the protocol provided (Dynal, Lake Success, New York). Isolated peripheral T cells were plated into a six-well plate precoated with goat antibodies to mouse IgG in RPMI 1640 containing 10% fetal calf serum (5 × 10^6 cells per well). The cells were then stimulated with anti-CD3 alone, anti-CD28 alone, or a combination of both. Eight hours after incubation, the supernatant of the cell culture was collected for assay of IL-2 with an enzyme-linked immunosorbent assay (ELISA) kit (Endogen, Woburn, Massachusetts), and the cells were harvested for protein immunoblot analysis, immunoprecipitation, radioimmunoassay of cAMP, and cell proliferation (*16*).

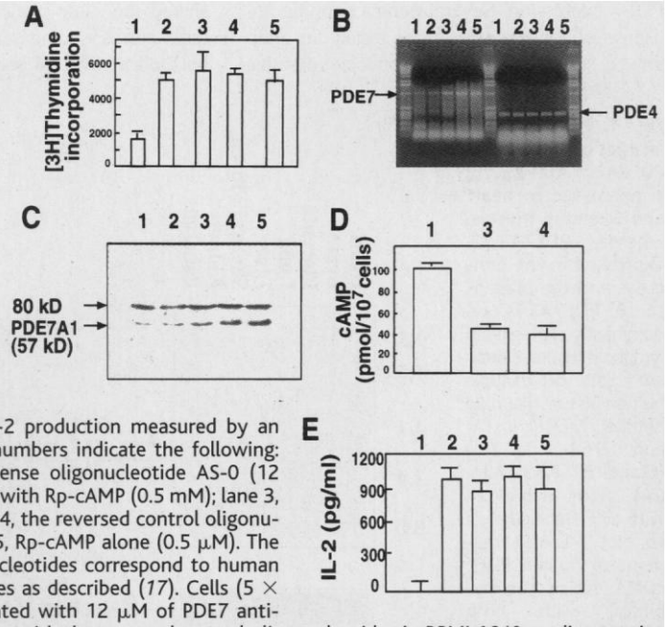

Fig. 3. The effect of a PDE7 antisense oligonucleotide on proliferation and IL-2 production in Hut78 T cells. (**A**) The PDE7 antisense oligonucleotide, AS-0, inhibits T cell proliferation. (**B**) PDE7 mRNA was reduced in the AS-0–treated cells analyzed by RT-PCR. (**C**) AS-0 reduced the 57-kD PDE7 protein band but did not affect the 80-kD band. (**D**) AS-0 increased cAMP levels in the cell. (**E**) AS-0 attenuated IL-2 production measured by an ELISA assay. The lane numbers indicate the following: lane 1, the PDE7 antisense oligonucleotide AS-0 (12 μM); lane 2, AS-0 along with Rp-cAMP (0.5 mM); lane 3, RPMI-1640 media; lane 4, the reversed control oligonucleotide (12 μM); lane 5, Rp-cAMP alone (0.5 μM). The three antisense oligonucleotides correspond to human PDE7A1 cDNA sequences as described (*17*). Cells (5×10^6 per well) were treated with 12 μM of PDE7 antisense oligonucleotides or with the reversed control oligonucleotides in RPMI 1640 media containing 10% fetal calf serum. To test the effect of PKA inhibitors on cell proliferation, 0.5 mM Rp-cAMP (BioLOG, La Jolla, California) was added together with the oligonucleotide. Twenty-four hours after incubation, the cells were labeled with 1 μCi of [^{3}H]thymidine per well for 20 hours and then harvested for scintillation counting. For RT-PCR analysis, total RNA was isolated with a TRIZOL RNA isolation kit (Gibco-BRL). cDNA was synthesized with the SuperScript II reverse transcription system (Gibco-BRL). One-tenth the volume of the reverse-transcribed cDNA was amplified with PDE7- or PDE4-specific primers (*17*). The products were subjected to electrophoresis on 2% agarose gels.

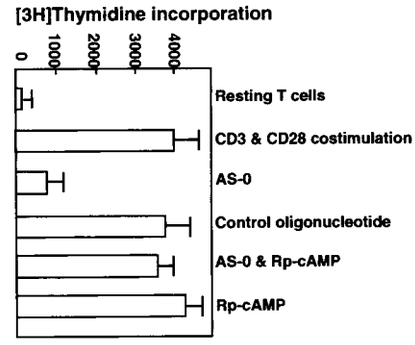

Fig. 4. Inhibition of primary CD4 T cell proliferation by PDE7 antisense oligonucleotides. Peripheral T cells were isolated with anti-CD4–conjugated Dynabeads M-450 according to the provided protocol (Dynal). The isolated CD4 T cells were activated by anti-CD3 (0.2 ng/ml) together with anti-CD28 (0.2 μg/ml), with or without 12 μM of PDE7 antisense AS-0 or the reverse control oligonucleotides, in a 96-well plate precoated with goat antibodies to mouse. Rp-cAMP (0.5 mM) was added along with the oligonucleotides in the defined wells. After 24 hours of incubation, [^{3}H]thymidine was added and the cells were incubated for another 24 hours. Cell proliferation was measured as described above.

tested. As shown in Fig. 3, 12 μM of the AS-0 oligonucleotide inhibited cell proliferation by 70% (Fig. 3A, lane 1) and attenuated IL-2 production (Fig. 3E, lane 1) in Hut78 T cells, which constitutively express high levels of PDE7 activity (*11*); incubation with the other two antisense oligonucleotides resulted in 20% inhibition or less (*12*). None of the control oligonucleotides had any effect on T cell proliferation and IL-2 production at this concentration (Fig. 3, A and E, lane 4).

To determine whether AS-0 treatment indeed caused a decrease in PDE7 expression, we performed reverse transcription polymerase chain reaction (RT-PCR) analysis of PDE7 mRNA and protein immunoblot analysis of PDE7 protein from the Hut78 cells treated with antisense oligonucleotides or with the control oligonucleotides. Both the mRNA (Fig. 3B, lane 1) and the protein (Fig. 3C, lane 1) of PDE7 were decreased in the AS-0–treated cells but were unchanged in the cells treated with control oligonucleotide (Fig. 3, B and C, lane 4). The inhibition by the antisense oligonucleotide was selective for PDE7, because the PDE4 mRNA remained unchanged in all the cells tested (Fig. 3B, right panel). cAMP concentration in the AS-0–treated cells was increased 2.5 times as compared with cells treated with the control oligonucleotides (Fig. 3D). Therefore, inhibition of T cell proliferation and IL-2 production by PDE7 antisense oligonucleotide appeared to be mediated through the targeting of PDE7 gene expression and consequent increases in cAMP and PKA activity. If this were the case, one would predict that suppression of PKA activity should reverse the inhibition of proliferation and IL-2 production by the PDE7 antisense oligonucleotide. As shown in Fig. 3, Rp-cAMP (0.5 mM Rp-adenosine 3′,5′-cyclic monophosphorothioate), a PKA-specific inhibitor, completely reversed both the inhibition of cell proliferation (Fig. 3A, lane 2) and the attenuation of IL-2 production (Fig. 3E, lane 2) in Hut 78 cells that had been caused by treatment with AS-0, whereas Rp-cAMP itself had no obvious effect on the cell (Fig. 3, A and E, lane 5).

Conclusions based on studies with growth factor–independent T cell lines do not necessarily hold true in primary T cells. To confirm the role of PDE7 in T cell activation, we tested the effect of the PDE7 antisense oligonucleotide on isolated peripheral CD4 T cells that had been activated by anti-CD3 and anti-CD28. As expected, the PDE7 antisense oligonucleotide AS-0 inhibited T cell proliferation by 80%, whereas the reverse control oligonucleotide showed no inhibition (Fig. 4). The PKA inhibitor Rp-cAMP blocked the inhibition of proliferation in the PDE7 antisense AS-0–treated cells (Fig. 4). Therefore, induction of PDE7 is essential for T cell activation.

It is still not clear how PDE7 protein and activity are increased upon activation of T cells. Easily detectable amounts of PDE7 mRNA were constitutively expressed in resting T cells (Fig. 1B). Therefore, the increase in PDE7 protein amount and activity may result from an up-regulation of protein translation or a decrease in degradation.

It has been reported that PDE4 inhibitors can also be effective in blocking T cell proliferation and that PDE3-inhibitors potentiate this blocking effect (*13*). None of the inhibitors used in those studies are thought to inhibit PDE7, thus raising the question of how all three of these PDE isozymes could be involved in T cell proliferation. This issue remains unresolved, but it is perhaps worth noting that in other cell types, different PDEs have been shown to be differentially localized within the cell (*10*). As a result of this localization, the regulation (*14*) and function of different PDEs may vary accordingly (*10*). It is also possible that changes in cAMP modulated by different PDE isozymes work at different phases of the cell cycle. In either of these cases, selective inhibitors of specific PDEs might be expected to inhibit the overall process of cell proliferation.

Our data provide evidence for a mechanism of CD28-dependent costimulation during T cell activation. The data also suggest that PDE7 may be a good target for selective therapeutic modulation of T cell responsiveness.

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16. For the protein immunoblot analysis, 20 μg of total protein was loaded in each lane and separated with 8% SDS–polyacrylamide gel electrophoresis. Affinity-purified antibody 6858 to PDE7, diluted with phosphate-buffered saline (PBS) containing 5% nonfat milk, was used for protein immunoblot analysis. Specific protein bands were revealed by the ECL system (Pierce, IL). For the immunoprecipitation and PDE assay, 10 million isolated peripheral T cells were homogenized with 1 ml of 40 mM tris-HCl buffer (homogenizing buffer) containing 1 mM EDTA, 5 mM dithiothreitol, 1 μM pepstatin (Sigma), and 10 μM leupeptin (Sigma). After centrifugation for 20 min at 14,000 rpm (Microcentrifuge), the supernatant was saved and was incubated overnight at 4°C with 100 μl of affinity-purified antibody 6858. Protein A–Sepharose (200 μl of a 5% suspension) was then added, and the mixture was incubated for 3 hours at 4°C. The protein A–bound proteins were washed twice with PBS and resuspended with the homogenization buffer for measurement of PDE activity, using 1 μM cAMP as a substrate. For the radioimmunoassay of cAMP, cells were homogenized in 5% trichloroacetic acid according to the protocol provided with the radioimmunoassay system (NEN, Boston, MA). To measure proliferation, cells (10^5 cells per well) were plated in a 96-well plate precoated with goat antibodies to mouse IgG and were incubated with anti-CD3 or anti-CD28 or both for 8 hours. One microcurie of [^{3}H]thymidine was then added per well. Sixteen hours later, cells were harvested for scintillation counting.
17. The three PDE7 antisense oligonucleotides were as follows: from position 1 to 24 (AS-0: 5′-CGGCAGCTGCTAACACACTTCCAT); from position 708 to 728 (AS-708: 5′-CAGTGCATGGCCTGAGTAAC); and from position 937 to 959 (AS-959: 5′-GGCAGATGTGAGAATAAGCCTG). For RT-PCR analysis, PDE7-specific primer pairs were as follows: 5′-GATATTTGTAACCCATGTCGGACG-3′ and 5′-AAAGCTTGGCGGTACTCTATCGAT-3′. PDE4A-specific primer pairs were as follows: AAGAGGAGGAAGAAATATCAATGG and TTACAGCAACCACGAATTCCTCC.
18. We thank L. H. Li for help with RT-PCR analysis and D. Stenger for comments on the manuscript. This research is supported by NIH grant DK21723 to J.A.B. and by a grant from the Ono Pharmaceutical Company. C.Y. is the recipient of a Career Award from the Burroughs Wellcome Fund.

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Requirement of Cdk2–Cyclin E Activity for Repeated Centrosome Reproduction in *Xenopus* Egg Extracts

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The abnormally high number of centrosomes found in many human tumor cells can lead directly to aneuploidy and genomic instability through the formation of multipolar mitotic spindles. To facilitate investigation of the mechanisms that control centrosome reproduction, a frog egg extract arrested in S phase of the cell cycle that supported repeated assembly of daughter centrosomes was developed. Multiple rounds of centrosome reproduction were blocked by selective inactivation of cyclin-dependent kinase 2–cyclin E (Cdk2-E) and were restored by addition of purified Cdk2-E. Confocal immunomicroscopy revealed that cyclin E was localized at the centrosome. These results demonstrate that Cdk2-E activity is required for centrosome duplication during S phase and suggest a mechanism that could coordinate centrosome reproduction with cycles of DNA synthesis and mitosis.

In animal cells, the interphase centrosome reproduces or duplicates only once per cell cycle, thereby ensuring a strictly bipolar mitotic spindle axis (*1*). Because there is no cell cycle checkpoint that monitors the number of spindle poles (*2*), uncontrolled duplication of the centrosome can contribute to genomic instability through the formation of multipolar mitotic spindles. Indeed, many human tumor cells, including those lacking the tumor suppresser protein p53 (*3*), have abnormally high numbers of centrosomes (*4*).

Studies of sea urchin and *Xenopus* embryos and clam oocyte lysates have revealed that the centrosome cycle can be regulated solely by cytoplasmic mechanisms (*5*–*8*): The repeated duplication of the centrosome proceeds in the complete absence of either a nucleus (*7*) or protein synthesis (*8*). In theory, the cyclical rise and fall in the activity of one or more cyclin-dependent kinases (Cdks) could be the cytoplasmic mechanism that coordinates centrosome reproduction with cell cycle progression. However, the fact that centrosomes repeatedly duplicate in the complete absence of protein synthesis indicates that the activities of those Cdks that are dependent on the translation of their cyclin subunits during each cell cycle (that is, Cdk1–cyclin A or –cyclin B or both) do not regulate centrosome reproduction or assembly (*8*). Nevertheless, Cdk2–cyclin E (Cdk2-E) remains a potential candidate to control centrosome duplication and coordinate it with nuclear events during the cell cycle (*6*, *9*, *10*). Cdk2-E activity drives the transition from G_1 to S phase in somatic cells (*11*), which is the time during the cell cycle when daughter centrosome assembly is thought to begin (*12*). Importantly, in early *Xenopus* embryos, Cdk2-E activity is not dependent on the synthesis and degradation of the cyclin E subunit, as the amount of cyclin E remains constant until the mid-blastula transition (MBT) (*13*).

To investigate whether Cdk2-E activity regulates centrosome duplication, we developed an S phase–arrested *Xenopus* egg extract that supports repeated centrosome reproduction in vitro. We used an S phase extract because centrosomes will undergo multiple rounds of duplication during S phase arrest in both zygotes and somatic cells (*6*, *8*, *14*, *15*). Unlike cycling extracts, Cdk2-E activity can be inhibited in S phase–arrested extracts without the concern that this inhibition will block cell cycle progression at a point before centrosomes are normally scheduled to reproduce. To make these extracts, we prepared a cycling *Xenopus* egg extract (*16*, *17*) and then added aphidicolin, an inhibitor of α-DNA polymerase (*18*), and demembranated *Xenopus* sperm nuclei (*19*). Histone H1 kinase activity in control extracts cycled at least twice with a cell cycle time of ~50 min; in contrast, H1 activity in aphidicolin-treated extracts remained at a constant, low amount for 6 hours (*20*, *21*). Time-lapse videomicroscopy of aphidicolin-treated extracts revealed that nuclear envelope breakdown did not occur during the 6-hour experiment (*20*). Thus,

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