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Reduction of Cdc25A contributes to cyclin E1-Cdk2 inhibition at senescence in human mammary epithelial cells

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Replicative senescence may be an important tumor suppressive mechanism for human cells. We investigated the mechanism of cell cycle arrest at senescence in human mammary epithelial cells (HMECs) that have undergone a period of 'self-selection', and as a consequence exhibit diminished $p16^{INK4A}$ levels. As HMECs approached senescence, the proportion of cells with a 2N DNA content increased and that in S phase decreased progressively. Cyclin D1-cdk4, cyclin E-cdk2 and cyclin A-cdk2 activities were not abruptly inhibited, but rather diminished steadily with increasing population age. In contrast to observations in fibroblast, $p21^{Cip1}$ was not increased at senescence in HMECs. There was no increase in $p27^{Kip1}$ levels nor in KIP association with targets cdks. While $p15^{INK4B}$ and its binding to both cdk4 and cdk6 increased with increasing passage, some cyclin D1-bound cdk4 and cdk6 persisted in senescent cells, whose inhibition could not be attributed to $p15^{INK4B}$. The inhibition of cyclin E-cdk2 in senescent HMECs was accompanied by increased inhibitory phosphorylation of cdk2, in association with a progressive loss of Cdc25A. Recombinant Cdc25A strongly reactivated cyclin E-cdk2 from senescent HMECs suggesting that reduction of Cdc25A contributes to cyclin E-cdk2 inhibition and G1 arrest at senescence. Although ectopic expression of Cdc25A failed to extend the lifespan of HMECs, the exogenous Cdc25A appeared to lack activity in these cells, since it neither shortened the G1-to-S phase interval nor activated cyclin E-cdk2. In contrast, in the breast cancer-derived MCF-7 line, Cdc25A overexpression increased both cyclin E-cdk2 activity and the S phase fraction. Thus, mechanisms leading to HMEC immortalization may involve not only the re-induction of Cdc25A expression, but also activation of this phosphatase. *Oncogene* (2000) **19,** 5314–5323.

Keywords: senescence; cell cycle; Cdc25A; $p15^{INK4B}$; cyclin E-cdk2; HMEC

Introduction

The proliferative potential of normal cells in culture is constrained to a finite number of population doublings, a phenomenon known as cellular senescence (Hayflick, 1965). Senescence may function as a tumor suppression mechanism since the proliferative constraint imposed by senescence limits the accumulation of genetic errors in the progeny of a single cell (Vojta and Barrett, 1995). The progressive reduction in telomere length that occurs with successive cell divisions may govern the point at which cells enter replicative senescence (Bodnar *et al*., 1998; de Lange, 1998). Cultured human fibroblasts show a predominantly 2N DNA content at senescence, reflecting cell cycle arrest in G1 phase (Sherwood *et al*., 1988). The present study addresses cell cycle regulation during population aging and senescence in post-selection human mammary epithelial cells (HMECs).

Movement through the cell cycle is governed by cyclic dependent kinases (cdks) (Morgan, 1995; Sherr, 1994). The D-type and E-type cyclins activate G1-to-S phase cdks and contribute to phosphorylation of the retinoblastoma protein (pRb), while cyclin A-cdk2 activity is required for S phase entrance and progression. The accumulation of predominantly hypophosphorylated pRb in senescent human fibroblasts reflects loss of these cdk activities (Stein *et al*., 1990).

Cdk activity is also regulated by two families of cdk inhibitors (Sherr and Roberts, 1995, 1999) and by phosphorylation (Solomon and Kaldis, 1998). Members of the kinase inhibitor protein (KIP) family (consisting of $p21^{Cip1}$, $p27^{KIP1}$ and $p57^{KIP2}$), and the inhibitor of cdk4 family (INK4) (consisting of $p15^{INK4B}$, $p16^{INK4A}$, p18 and p19) bind and regulate the cdks that govern the G1 to S phase transition (Sherr and Roberts, 1995, 1999). For full activation, cdk2 must be phosphorylated by the cdk activating kinase (CAK) at threonine 160 (Thr 160) (Gu *et al*., 1992). Phosphorylation of Thr 14 and/or tyrosine 15 residues by homologs of the wee 1 kinase inactivates cdk1, and homologous sites exist on cdk2 and cdk4 (Gould and Nurse, 1989; Krek and Nigg, 1991; McGowan and Russell, 1995; Parker *et al*., 1995). Cdc25 phosphatase family members act at discrete times during the cell cycle to remove these inhibitory phosphates (Draetta and Eckstein 1997). Cdc25A expression peaks at the G1/S transition and targets cyclin E- and cyclin A-bound cdk2 (Blomberg and Hoffmann, 1999; Hoffmann *et al*., 1994; Jinno *et al*., 1994). Cdc25A is itself activated by phosphorylation and this may involve Raf1 (Galaktionov *et al*., 1995) and cyclin E-cdk2 (Hoffmann *et al*., 1994).

Most previous investigations of cell cycle arrest in senescence have used fibroblasts (Afshari *et al*., 1993;

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Alcorta *et al*., 1996; Dulic *et al*., 1993; Hara *et al*., 1996). In humans, malignant tumors of fibroblast origin occur infrequently, while carcinomas of epithelial origin are the most common form of human cancer. While spontaneous immortalization rarely occurs in normal human cells, immortal cell lines can be derived from advanced carcinomas with a predictable frequency (Campisi, 1996). Thus, overcoming senescence may be necessary for malignant progression (Vojta and Barrett, 1995). A better understanding of cell cycle arrest at senescence may inform the study of malignant transformation.

Investigation of the senescence checkpoint in fibroblasts has shown a role for cdk inhibitors in this process. $p21^{Cip1}$ was cloned as a gene whose expression is upregulated in senescent fibroblasts (Noda *et al*., 1994). Loss of $p21^{Cip1}$ through homologous recombination was sufficient to extend the lifespan of human diploid fibroblasts in culture (Brown *et al*., 1997) but did not induce full immortalization. $p16^{INK4}$ protein and its association with target cdks are elevated at senescence in both human and murine fibroblasts, in endothelial cells and in prostatic epithelial cells (Alcorta *et al*., 1996; Hara *et al*., 1996; Palmero *et al*., 1997; Reznikoff *et al*., 1996; Sandhu *et al*., 2000; Zindy *et al*., 1997). Moreover, many human tumors and immortal cell lines show an absence of p16 protein (Hunter and Pines, 1994; Kamb *et al*., 1994) and embryonic fibroblasts from p16 knockout mice undergo spontaneous immortalization (Serrano *et al*., 1996). Thus, in these cell types, G1 arrest in senescence is mediated in part by the action of $p16^{INK4A}$.

HMECs grown in serum free culture medium have two phases of growth. Upon dissociation of normal breast tissue, HMECs will undergo approximately 20 population doublings (6–8 passages), following which they exhibit a flattened morphology (Stampfer, 1985; Stampfer and Bartley, 1992) and a reduced percentage S phase (Foster and Galloway, 1996). This period is referred to as self-selection and has been called arrest at 'early senescence' (Brenner *et al*., 1998) or, under somewhat different growth conditions, mortality stage 0 (MO) (Foster and Galloway, 1996). This first growth phase of pre-selection cells is limited by the pRb/p16 pathway, but not by telomere shortening (the mean TRF of cells arrested at this point is 7–8 Kb) (Brenner *et al*., 1998; Garbe *et al*., 1999; Foster *et al*., 1998; Kiyono *et al*., 1998).

HMECs grown in serum free medium that emerge spontaneously post-selection have reduced p16 due to methylation of the p16 promoter (Brenner *et al*., 1998) and can undergo a further 25–45 population doublings (8–15 passages) (Foster *et al*., 1998; Stampfer, 1985). Telomere shortening is appreciable only during this post-selection phase of proliferation (the HMEC lifespan investigated in this study). Senescent post-selection HMECs lack telomerase activity and exhibit a TRF of 4–5 Kb (Stampfer *et al*., 1997), similar to other cell types at replicative senescence. hTERT overexpression fails to rescue pre-selection HMECs from the M0 or early proliferative arrest, but does abrogate senescence arrest in post-selection cells (Kiyono *et al*., 1998; Wang *et al*., 1998). Similarly, overexpression of genes that upregulate hTERT (c-*myc* and HPV-E6) can immortalize post selection HMECs (Perez-Roger *et al*., 1999; Wang *et al*., 1998).

In this study, we show that terminal cell cycle arrest at senescence in post-selection 184 HMECs is not a consequence of increased cdk association with either $p21^{Cip1}$ or $p27^{Kip1}$. Although $p16^{INK4A}$ is not detectable in these cells, $p15^{INK4B}$ is substantially elevated and plays a role in cdk inhibition at senescence. In senescent 184 HMECs, cyclin E-cdk2 inhibition is due, at least in part, to the accumulation of inhibitory cdk2 phosphorylation resulting from reduced Cdc25A expression.

Results

The proportion of senescent cells increases with increasing population age

Post-selection 184 HMECs at mid-lifespan, or passage 10 (P10) have already undergone approximately 30–40 population doublings, and senescence occurs at around P22, after approximately 60–80 population doublings (one passage represents three population doublings). The aging of the 184 HMEC population was accompanied by a gradual decrease in the proliferative fraction as shown by flow cytometry in Figure 1a. Cells were deemed senescent when the culture remained subconfluent for more than 1 month. The proportion of cells in S phase was highest at P10 (54%) and

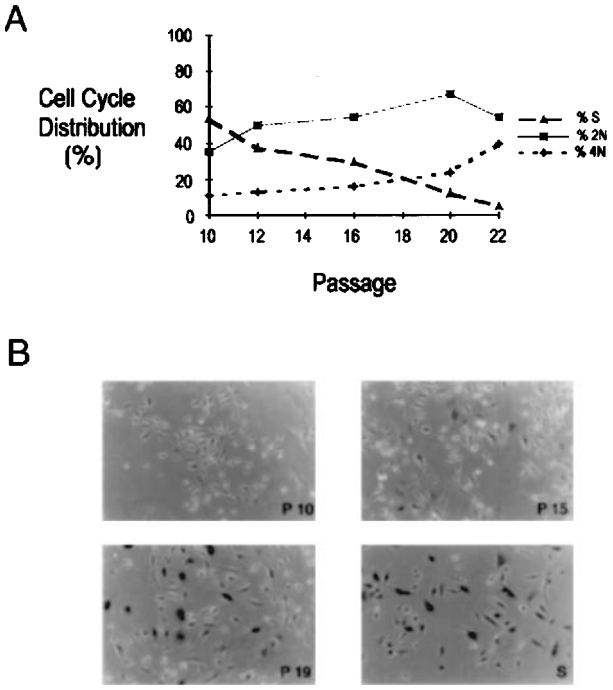

Figure 1 Gradual increase in senescent cells with increasing passage. (**a**) DNA profile of HMEC from passage 10 (P10) to senescence. Asynchronously growing cells of increasing passage were recovered for flow cytometric analysis. Cells were pulse labeled with 10 μM BRDU for 2 h and counterstained with propidium iodide. The proportion of cells with 2N, 4N or S phase DNA content were plotted as a function of passage number. Results are representative of two different biologic assays of HMEC progression from P10 to senescence and at least three flow cytometric assays per time point. (**b**) Senescence associated β-galactosidase expression in cells of increasing passage. Asynchronously growing HMEC of increasing passage were stained for acid stable β-galactosidase activity. Cells were fixed and incubated with X-gal. Blue staining indicates the presence of senescence associated β-galactosidase

decreased steadily with successive passage as the proportion of cells with a 2N DNA content increased.

Although a majority of senescent cells had a 2N DNA content, the percentage of cells with a 4N DNA content was increased in late passages and at senescence. These 4N cells could represent cells arrested in G2/M or G1 arrested tetraploid cells. To distinguish between these two possibilities, metaphase chromosome spreads were analysed at different passages. The proportion of cells in metaphase fell with increasing passage number, consistent with a reduction in the number of cells entering M phase with increasing population age. The proportion of total metaphases exhibiting tetraploidy increased with increasing population age, from 7% at P10, to 16% at P15 and 24% at P17. Thus, the increased proportion of cells with a 4N DNA content at senescence reflects the progressive appearance of tetraploid cells arrested at the G1 checkpoint during senescence.

The staining for senescence-associated β-galactosidase, another senescence marker, also rose with increasing 184 HMEC passage, confirming the gradual onset of senescence (Figure 1b). β-galactosidase activity was not observed in late passage 184A1, an immortally transformed line derived from the finite lifespan 184 HMECs (Stampfer and Bartley, 1985) (data not shown). Senescent HMECs were larger and had an increased ratio of cytoplasmic to nuclear volumes.

Cdk activities decrease with increasing population age

Asynchronously growing 184 HMECs showed a gradual loss of cyclin E-, A- and D1-associated kinase activities as cells progressed from P10 to senescent arrest at P22 (see Figure 2). The pRb protein was largely phosphorylated at P10 and hypophosphorylated in senescent cells (Figure 3).

The decrease in G1 cyclin-cdk activity at senescence could not be attributed to reduced cyclin or cdk proteins. Cyclin E, cyclin D1, cdk4 and cdk6 levels did not change during the aging of 184 HMEC (Figure 3). While total cdk2 decreased (Figure 3), the proportion of total cdk2 showing faster mobility, the CAK-activated form of cdk2 (Gu *et al*., 1992) was similar at early passage and at senescence. Cyclin A, cyclin B and cdk1 levels decreased progressively with increasing passage (not shown), consistent with cell cycle arrest in G1.

Of the cdk inhibitors, p21 or p27 levels did not increase, while p15 levels did, with maximal p15 in senescent HMECs. Low levels of p18 and p19 were detected, but showed no increase with increasing HMEC passage (not shown). Equal protein loading was verified by blotting for β-actin (not shown). Data from Figures 1–3 was representative of repeat assays (see Materials and methods).

Cyclin E-cdk2 inhibition is not due to increased KIP association

Although KIP levels did not rise, increased KIP binding to cyclin E-cdk2 could play a role in kinase inhibition at senescence. Immunoblotting of cyclin-E associated proteins showed no loss of cyclin E-cdk2 complex formation, nor any increase in either p21 or p27 binding (Figure 4a). The proportion of total cyclin

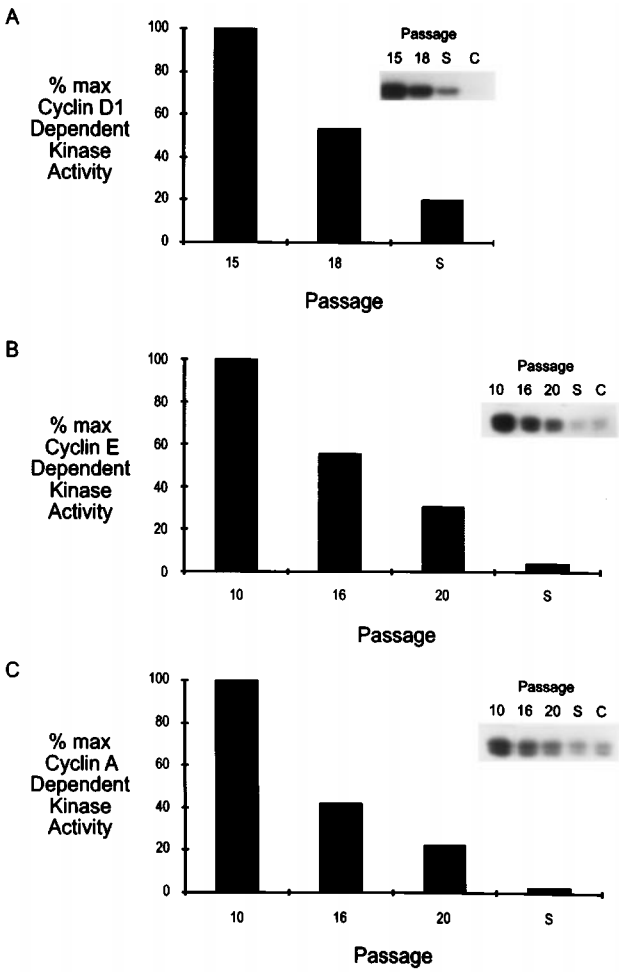

Figure 2 Cyclin dependent kinase activities. Cyclin associated kinase activities were assayed in cyclin immunoprecipitates recovered from cell lysates from P10 to senescence. Reaction products were resolved by SDS–PAGE, dried and autoradiographed. Phosphorylation of substrate was quantitated by phosphoimager and the mean results of 2–3 different assays graphed as a percentage maximum of early passage kinase activity. (**a**) Cyclin D1- associated kinase activity. The C-terminal domain of pRb was used as substrate in cyclin D1-associated kinase assays. (**b**) Cyclin E-associated kinase activity. (**c**) Cyclin A-associated kinase activity. For (**b**) and (**c**), histone H1 kinase activity was assayed in cyclin E or cyclin A immunoprecipitates recovered from increasing passages

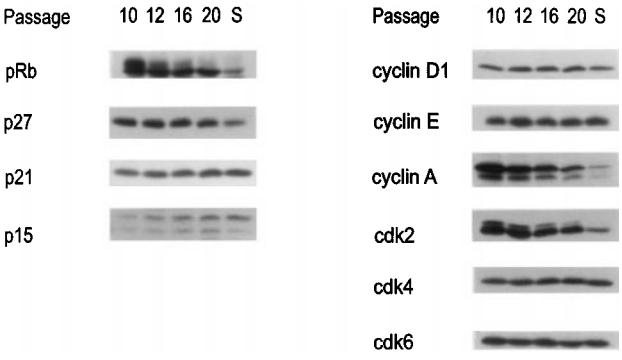

Figure 3 Levels of G1/S associated cell cycle regulators during the aging of the HMEC population. Lysates were collected from 184 HMEC between P10 and senescence (S). Cell lysates from the indicated passage numbers were resolved by SDS–PAGE and immunoblotted with the indicated antibodies

E-associated cdk2 activated by Thr 160 phosphorylation was constant (as assessed by cdk2 mobility). p57 was not reliably detected in these HMECs and was not detected in cyclin E immune complexes (data not shown). Thus, cyclin E-cdk2 inhibition at senescence was not caused by increased p21 or p27 binding, nor by inhibition of CAK.

p15 association with cdk4 and cdk6 complexes increases with increasing population age

In addition to the notable rise in p15 levels, p15 binding to cdk4 and cdk6 complexes increased significantly by six and fourfold respectively, between mid-passage and senescence. Cdk6-bound cyclin D1 and p21 fell slightly, with a more notable loss of p27 binding (Figure 4b). However, no loss of cyclin D1, p21 or p27 from cdk4 complexes was noted with increasing passage (see cdk4 IP in Figure 4a). Moreover, cyclin D1 immunoprecipitates showed no increase in associated p21 or p27, and no loss of cdk4 and cdk6 with increasing passage, and p15 was not detected in these complexes (data not shown).

To determine whether the p15 associated with cdk4 in senescent cells was present in cyclin D1/cdk4/p15 complexes, or if the p15-bound cdk4 and cdk6 complexes were devoid of associated cyclin D1, senescent cell lysates were serially immunodepleted of cyclin D1, p27 and then p21 (see Figure 5). Cyclin D1 complexes contained either p27 or p21, but no associated p15. p27 and p21 immunoprecipitates from the cyclin D1-depleted lysate also contained no detectable p15. p15 was abundantly detected in the remaining cdk4 and cdk6 immunoprecipitated from cyclin D1- and KIP-depleted senescent cell lysates. Thus, with increasing 184 HMEC passage, p15 appears to inhibit a subpopulation of cdk4 and cdk6 molecules. Neither the increased p15 nor an increase in KIP binding could account for the inhibition of the cyclin D1-associated cdk4 or cdk6 remaining in senescent cells.

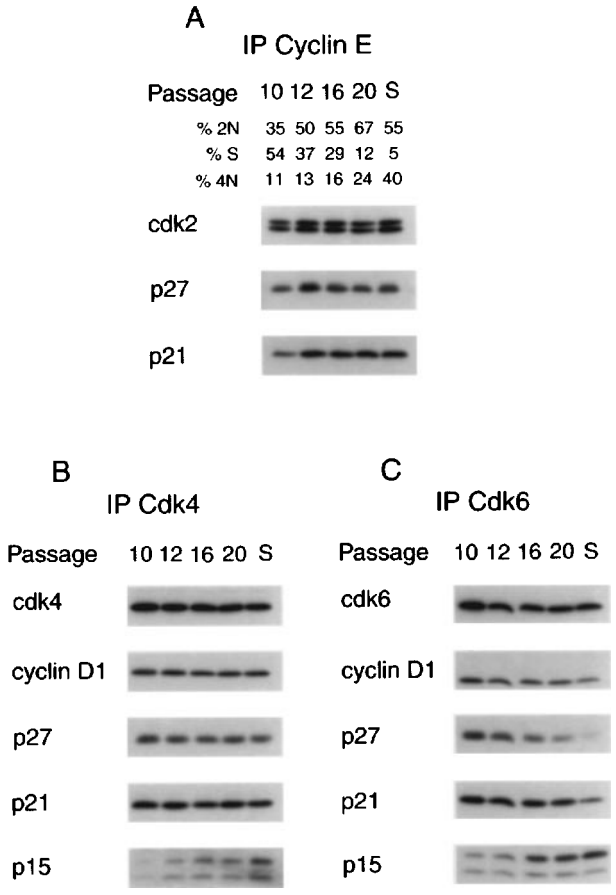

Figure 4 Cyclin/cdk inhibitor complexes in HMECs from P10 to senescence. Cdk or cyclin immunoprecipitations from cell lysates at the indicated passages were carried out as described in Materials and methods (**a**–**c**). Complexes were resolved and immunoblotted for associated cdk, cyclin and cdk inhibitors. Results are representative of repeat assays. (**a**) Cdk4 binding to cyclin D1 and cdk inhibitors p15, p21 and p27. (**b**) Cdk6 binding to cyclin D1 and cdk inhibitors p21, p27 and p15. (**c**) Cyclin E binding to cdk2 and cdk inhibitors p21 and p27

Cdc25A is reduced in senescent HMEC

Cyclin E-cdk2 inhibition at senescence could reflect the presence of an inhibitor or the absence of an activating factor. To test for an inhibitory factor in senescent 184 HMECs, active cyclin E-cdk2 was immunoprecipitated from mid-passage, proliferating HMECs (P10) and then incubated with cyclin E-depleted lysate from senescent cells under conditions allowing KIP association (Slingerland *et al*., 1994). The senescent cell lysate did not inhibit cyclin E-cdk2 activity from mid-passage cells (data not shown), excluding the presence of an excess KIP-like inhibitory factor in senescent cells. To test whether an essential cdk activator might be lost at senescence, cyclin E-cdk2 complexes from senescent cells were incubated with cyclin E-depleted lysate from proliferating P10 cells. As shown in Figure 6a, inactive

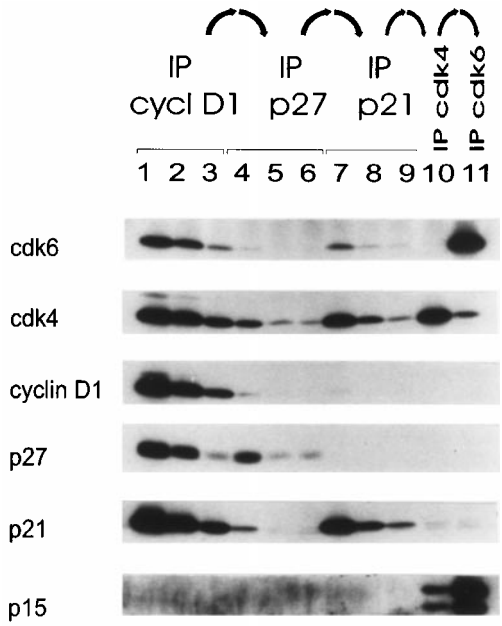

Figure 5 p15 is not detectable in cyclin D1 complexes. Serial immunodepletions of cyclin D1, p27 and p21, respectively, were performed to identify the p15-associated proteins. Five hundred μg of lysate from senescent 184 HMEC was serially immunodepleted three times with cyclin D1 antibody (lanes 1–3), followed by three immunodepletions with p27 antibody (lanes 4–6) and finally three immunodepletions with p21 antibody (lanes 7–9). The immunodepleted lysate was then used to immunoprecipitate cdk4 (lane 10) and then cdk6 (lane 11). The immune complexes were resolved by SDS–PAGE and immunoblotted with the indicated antibodies

cyclin E-cdk2 from senescent cells (lane 1) was activated by the addition of cyclin E-depleted lysate from P10 cells (lane 2), revealing a cyclin E-cdk2 activating factor in earlier passage cells. The cyclin E-depleted P10 lysate itself contained no cyclin E-cdk2 activity (lane 3).

Progression from G1 into S phase requires Cdc25A activity (Hoffmann *et al*., 1994; Jinno *et al*., 1994). We investigated whether Cdc25A might be the cyclin E-cdk2 activating factor lacking in senescent HMEC. Cdc25A protein and mRNA were abundantly expressed in HMECs at P10, but reduced significantly in senescent 184 HMECs (Figure 7a,b respectively). It is noteworthy that HMECs immortalized by constitutive hTERT expression maintained a higher Cdc25A protein level than that in early passage 184 cells (Figure 7a). Similarly, a benzo-a-pyrene immortalized derivative of 184 HMECs, 184A1, showed abundant Cdc25A protein (not shown).

Reactivation of senescent cyclin E-cdk2 by recombinant Cdc25A

While Cdc25A was reduced in senescent cells, it was necessary to establish that this was relevant to cyclin E-cdk2 inactivation. Incubation of recombinant GST-Cdc25A with cyclin E-cdk2 immunoprecipitated from senescent 184 HMEC reactivated this kinase (>70-fold). Cdc25A incubation with cyclin E-cdk2 from asynchronously growing P10 cells caused only a two-fold increase in kinase activity (Figure 6b). Results graphed are the mean of 3–5 repeat assays. Phosphotyrosine blotting showed an increase in tyrosine phosphorylation of cyclin E-bound cdk2 between mid-passage and senescence (Figure 6c). Thus, cyclin E-cdk2 inhibition in senescent cells is, at least in part, a consequence of increased inhibitory phosphorylation of cyclin E-associated cdk2 due to loss of the activating phosphatase, Cdc25A.

Attempts to assay the phosphotyrosine content of cyclin D1 associated cdk4 and cdk6 by anti-phosphotyrosine blotting were not successful. Furthermore, GST-Cdc25A failed to activate cdk6 from senescent cells and caused little or no activation of this kinase in early passage cell lysates (not shown).

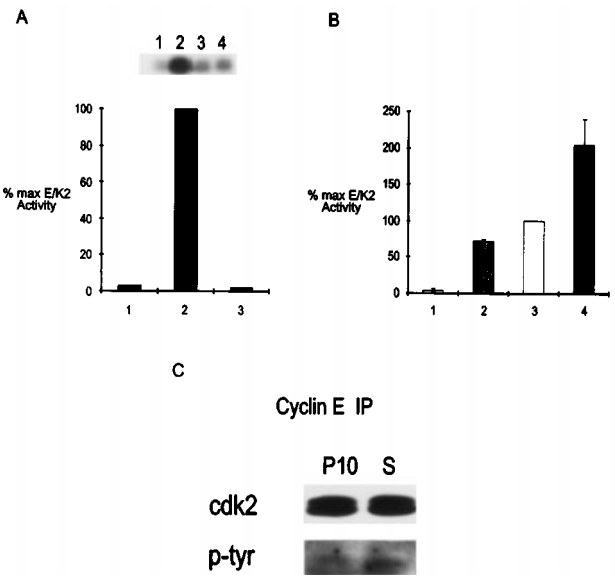

Figure 6 Loss of Cdc25A in senescent HMECs contributes to cyclin E-cdk2 inhibition. (**a**) Early passage cells contain an activator of senescent cyclin E-cdk2. The addition of cyclin E-depleted P10 lysate to cyclin E complexes immunoprecipitated from senescent cells revealed a kinase activating factor in P10 cells. Lane 1 shows the kinase activity of senescent cyclin E complexes. In lane 2, cyclin E depleted lysate from P10 was incubated with the senescent cyclin E-cdk2 complex prior to assaying kinase activity. Lane 3 shows that cyclin E depleted P10 lysate was devoid of cyclin E-cdk2 kinase activity. Lane 4 shows the activity in a control immunoprecipitate using non-specific antibody. Repeat assays confirmed results shown. (**b**) Reactivation of senescent cellular cyclin E-cdk2 by recombinant Cdc25A. Lanes 1 and 3 show cyclin E-cdk2 activities in senescent and P10 cells, respectively. Recombinant GST-Cdc25A was incubated with senescent cyclin E-cdk2 immunoprecipitates in lane 2 and with cyclin E-cdk2 from P10 in lane 4. Graphed results are the mean of at least three different assays. (**c**) Phospho-tyrosine content of cyclin E-associated cdk2 in P10 and senescent HMEC. Cyclin E immunoprecipitates were recovered from early passage and senescent cells, resolved and associated cdk2 was immunoblotted with either anti-cdk2 (top) or anti-phospho-tyrosine (bottom) antibodies

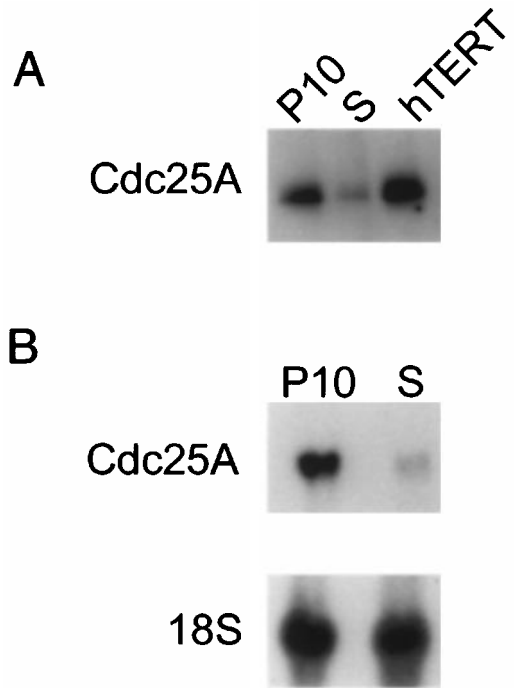

Figure 7 Loss of Cdc25A expression from mid-passage to senescence. (**a**) Loss of Cdc25A protein in senescent HMECs. Cdc25A was immunoprecipitated from P10 (lane 1), senescent cells (lane 2) and hTERT immortalized cells (lane 3). Immunoprecipitates were resolved and immunoblotted with Cdc25A polyclonal antibodies. Results are representative of repeated assays. (**b**) Loss of Cdc25A mRNA in senescent HMECs. Northern analysis shows loss of Cdc25A expression between P10 and senescence. Equal loading of the RNA was verified by reprobing the blot with an 18S rRNA probe

Constitutive expression of Cdc25A failed to extend HMEC lifespan

The influence of constitutive Cdc25A expression on HMEC lifespan was assayed by retroviral transfection of *CDC25A* into early and late passage 184 HMEC. Consistent with the results of Wang *et al*. (1998), overexpression of Cdc25A failed to extend the lifespan of 184 HMECs in culture (not shown). Moreover, Cdc25A overexpression did not shorten the G1-to-S phase interval (not shown), nor did it change the cell

cycle profile of asynchronously growing 184 cells (Figure 8a).

To ensure the potential activity of the Cdc25A encoded by the retroviral construct, the *CDC25A* cDNA sequence was verified. In addition, the retroviral vector was also used to transform the breast cancer derived line, MCF-7. Three independent pools of *CDC25A* transformed MCF-7 showed a reduced per cent G1 and an increased per cent S phase cells (mean per cent S of 36% in MCF-7 and mean per cent S of 61% in the three MCF7-*CDC25A* populations). The cell cycle profiles of the parental or vector alone controls and individual CDC25A overexpressing 184 HMEC and MCF-7 populations are shown in Figure 8a. Three independent Cdc25A transformations of each of the 184 HMECs and MCF-7 cells yielded similar results.

Although Cdc25A protein was increased in the *CDC25A* transformed 184 HMECs, there was no corresponding increase in the cyclin E-cdk2 activity. In contrast, a similar increase in Cdc25A expression in MCF7-*CDC25A* yielded a significant increase in cyclin E-cdk2 activity (Figure 8b). The endogenous Cdc25A proteins in 184 and MCF-7 cells differed in electrophoretic mobility on immunoblotting (Figure 8c). The predominant Cdc25A isoform in MCF-7 had an increased mobility on SDS–PAGE compared to that in 184 cells. Thus, post-translational regulation of Cdc25A may differ between normal and malignantly transformed human breast epithelial cells.

Discussion

Studies of mechanisms of cellular aging and immortal transformation have provided strong support for the hypothesis that replicative senescence in normal human cells limits the potential for malignant transformation (Greider, 1998; Rohme, 1981). If tumorigenicity were to develop without augmentation of lifespan, a human tumor could not expand beyond a limited size (1 cm^3) (Vojta and Barrett, 1995). Abrogation of senescence may be required for continued tumor growth and metastatic progression. The investigation of the effectors of cell cycle arrest at senescence in epithelial cells may identify molecular targets whose deregulation is required for immortal transformation and cancer progression.

While HMEC arrest at self-selection, MO, or early senescence is dependent on p16, the senescence arrest of post-selection HMEC, which lack p16, appears to involve, in part, an increase in p15 and the reduction of Cdc25A with accumulation of inhibitory phosphotyrosylation of cyclin E-bound cdk2. Human breast cancers show frequent loss of p16, due to genetic changes or promoter hypermethylation (Brenner and Aldaz, 1995; Herman *et al*., 1995; Wong *et al*., 1997), in addition to Cdc25A overexpression (Cangi *et al*., 2000) and telomerase activation (Kim *et al*., 1996). Malignant mammary epithelial transformation may require abrogation of both the early self-selection (or MO) checkpoint and the later replicative senescence observed in post-selection HMEC.

We observed a gradual loss of proliferative activity and a progressive increase in senescence associated β-galactosidase staining with successive HMEC passage. Human fibroblasts also show a progressive loss of proliferative activity with increasing population age (Cristofalo and Sharf, 1973). As has been observed in aging fibroblast populations (Sherwood *et al*., 1988), we noted an increased incidence of tetraploidy in late passage HMECs. The accumulation at senescence of HMEC populations with 2N and 4N DNA contents reflects the G1 arrest of cells with normal and tetraploid DNA content, respectively.

Senescence arrest in HMEC was not attributable to loss of G1 cdks or cyclins nor to loss of cdk2

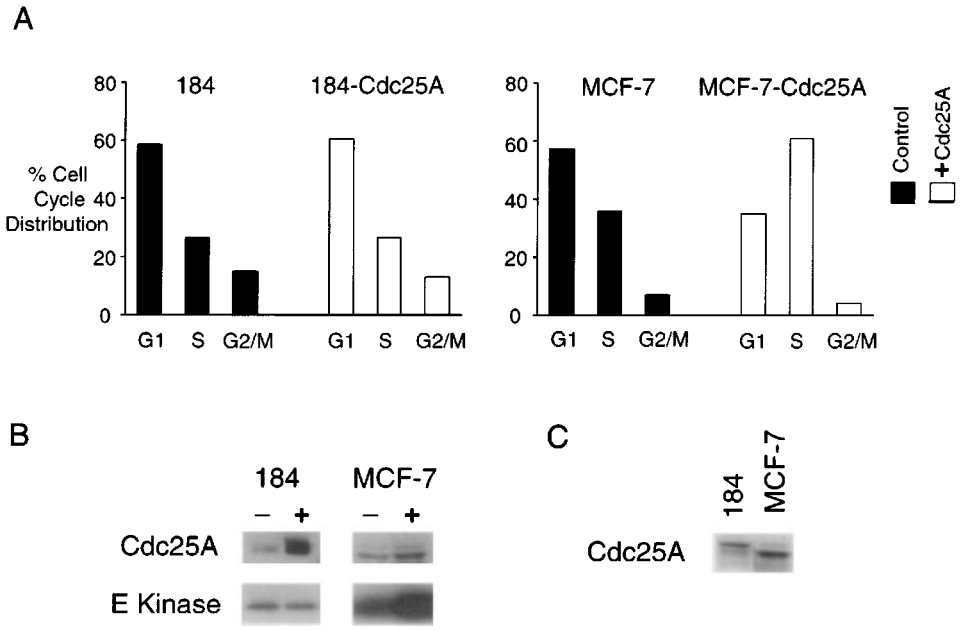

Figure 8 Constitutive overexpression of Cdc25A has different effects in normal and malignantly transformed HMECs. Three independent Cdc25A transformations of each of the 184 HMECs and MCF-7 cells yielded similar results. (**a**) DNA profiles of asynchronously growing 184 HMECs and MCF-7 cells infected with control or Cdc25A overexpressing retroviral vector. (**b**) Cyclin E dependent kinase activity and Cdc25A immunoblot of control (−) and Cdc25A infected (+) 184 and MCF-7 cells. (**c**) Immunoblot of Cdc25A from MCF-7 and 184 HMECs

activation by CAK. While senescent human fibroblasts show elevated cyclin D1 and cyclin E levels (Dulic *et al*., 1993; Lucibello *et al*., 1993), these cyclins were not elevated in senescent HMECs. In both aging fibroblasts and in our aging HMECs, cyclin A levels decrease steadily (Afshari *et al*., 1993; Dulic *et al*., 1993; Lucibello *et al*., 1993), likely reflecting cyclin E-cdk2 inhibition and loss of the stimulatory effect of cyclin E/cdk2 via E2F on cyclin A gene transcription (DeGregori *et al*., 1995; Schulze *et al*., 1995).

It is perhaps not surprising that replicative senescence in post-selection HMECs involves different effectors of cell cycle arrest than in senescent fibroblasts. Epithelial cells and fibroblasts differ not only in their potential for malignant transformation in humans, but also in growth regulation of cytokines and other factors (Parkinson and Balmain, 1990; Roberts *et al*., 1985). In contrast to senescent rodent and human fibroblasts, where p21 plays a critical role in cdk inhibition (Brown *et al*., 1997; Noda *et al*., 1994), p21 does not appear to be an effector of G1 arrest at senescence in HMECs. Human prostate epithelial cells also fail to upregulate p21 during replicative senescence (Sandhu *et al*., 2000). Differences in senescence effector mechanisms between fibroblasts and epithelial cells may ultimately prove relevant to the differing frequencies with which these two cell types give rise to malignancies in humans.

p16 plays a role in cdk inhibition in the pre-selection arrest of HMECs and in human fibroblasts senescence (Alcorta *et al*., 1996; Brenner *et al*., 1998; Hara *et al*., 1996; Kiyono *et al*., 1998). In post-selection HMECs, which lack p16, p15 accumulates in cdk4 and cdk6 at senescene. The increase in p15 levels may reflect the loss of a negative transcriptional feedback loop driven by diminishing pRb levels, as reported by the Peters group for p16 in senescent human fibroblasts (Hara *et al*., 1996). While p15 inhibits a subset of cdk4 and cdk6 complexes at senescence, the mechanism of inhibition of remaining cyclin D1-bound cdks remains unclear. Mechanisms other than the loss of Cdc25A must contribute to the inactivation of cyclin D1-dependent kinases, since cyclin D1-cdk6 complexes from senescent 184 HMEC cells were not activated by recombinant Cdc25A. This is consistent with recent reports that cyclin E- and cyclin A-cdk2 complexes are the major targets of Cdc25A (Blomberg and Hoffmann, 1999; Cangi *et al*., 2000).

Our investigation has revealed a role for the Cdc25A phosphatase in cyclin E-cdk2 inhibition at senescence. The cyclin E-cdk2 activating factor, detected in earlier passage cells, is likely to be Cdc25A based on the following evidence. Senescent 184 HMECs had a substantially reduced expression Cdc25A phosphatase, while hTERT immortalized HMECs showed elevated Cdc25A. Cyclin E-cdk2 from senescent cells was activated by GST-Cdc25A. Moreover, cyclin E-associated cdk2 from senescent cells was more phosphotyrosylated than that from earlier passage. That GST-Cdc25A failed to fully restore cyclin E-cdk2 activity to the level seen in early passage cells suggests that other factors may also inhibit this kinase in senescence. Since loss of Cdc25A is sufficient to inhibit S phase entrance (Hoffmann *et al*., 1994; Jinno *et al*., 1994; Cangi *et al*., 2000), the reduced Cdc25A expression likely contributes to cyclin E-cdk2 inhibition in senescent HMECs.

We next addressed whether overexpression of Cdc25A could abrogate senescence arrest in 184 HMECs. Constitutive Cdc25A expression failed to stimulate cyclin E-cdk2 activity, did not shorten the G1 to S phase interval and did not extend the finite lifespan of 184 HMEC. In contrast, increased Cdc25A expression in the malignantly transformed MCF-7 cells resulted in a dramatic elevation of both cyclic E-dependent kinase activity and the fraction of cells in S phase (as has been observed with Cdc25A overexpression in other cell types (Blomberg *et al*., 1999)). Thus, MCF-7 cells differed from finite lifespan 184 HMEC in their ability to activate ectopically expressed Cdc25A. The different electrophoretic mobilities of the endogenous Cdc25A from 184 HMEC and MCF-7 cells may also reflect differences in the post-translational regulation of Cdc25A in these finite lifespan versus cancer-derived mammary epithelial cells.

To date, cyclin E-cdk2 and Raf-1 have been implicated as activators of Cdc25A phosphatase (Galaktionov *et al*., 1995; Hoffmann *et al*., 1994). MCF-7 cells have a higher basal cyclin E-cdk2 activity than do 184 HMECs (data not shown), which could account for the greater activation of overexpressed Cdc25A in MCF-7. It is noteworthy that transformation with either *hTERT* or c-*myc* can lead to immortalization of post-selection HMECs (Kiyono *et al*., 1998; Wang *et al*., 1998). Since overexpression of Cdc25A was not sufficient to abrogate HMEC senescence, HMEC immortalization by c-*myc* and by hTERT must not only restore Cdc25A expression but also activate this phosphatase. Elucidation of the differences in post-translational regulation of Cdc25A between finite lifespan and cancer-derived HMEC and the mechanisms whereby c-*myc* overexpression leads to Cdc25A activation may yield important insights into the process of mammary carcinogenesis.

In this study, we show that Cdc25A expression is downregulated at senescence in HMECs. It is tempting to speculate that telomere shortening and/or genetic damage such as the accumulation of tetraploidy may reach a critical point in late passage HMECs and activate a DNA damage checkpoint leading to reduced Cdc25A, contributing to cdk inhibition in senescence. In two different immortal derivatives of 184 HMEC, the 184A1 cell line, and hTERT immortalized HMEC, Cdc25A is abundantly expressed. Reinduction and activation of Cdc25A may be required during immortalization or tumor progression. This notion is supported by recent data showing overexpression of Cdc25A in head and neck cancers (Gasparotto *et al*., 1997) and in up to 60% of primary human breast cancers (Cangi *et al*., 2000).

Materials and methods

Cell culture

Normal finite lifespan 184 HMECs were obtained from reduction mammoplasty tissues of a 21-year-old woman (Stampfer, 1985). These HMECs were passaged at a 1 : 8 split and thus each passage represents three population doublings. When grown in serum containing medium, these cells lose proliferative potential after 20–25 population doublings (Stampfer *et al*., 1992). When grown in serum free MCDB 170 medium (Clonetics Corporation, San Diego, CA,

USA), these cells undergo a period of self-selection around population doubling 10–20, in which most cells cease proliferation. This initial period of reduced proliferation is not accompanied by telomere shortening (Garbe *et al*., 1999). This self-selection is followed by the outgrowth of a population with a basal mammary epithelial phenotype (Stampfer *et al*., 1992). These post-selection cells no longer express p16, and undergo approximately 20–22 passages or 80 population doublings in total (Stampfer, 1985), during which telomere length is progressively reduced (Garbe *et al*., 1999; Hammond *et al*., 1984). In the present study, HMECs were grown in MCDB 170 medium as described and cell cycle parameters assayed in post-selection cells of similar confluency from passage 10 to replicative senescence at about passage 22. Cells were harvested for flow cytometry and for assays of cell cycle regulators at intervals from P10 to senescence on two occasions and all assays were repeated at least three times. The immortally transformed line, 184A1, was derived from 184 HMEC following exposure to the chemical carcinogen, benzo-α-pyrene (Stampfer and Bartley, 1985). The MCF-7 cells were provided by Dr K Osborne and were grown in I-modified essential medium supplemented with 5% fetal calf serum and insulin (Soule *et al*., 1973).

Flow cytometric analysis

At different passages, cells were pulse-labeled with 10 μM bromodeoxyuridine (BrdU) for 2 h. Cells were then harvested, fixed with 70% ethanol, treated with 0.1 N HCl and heated for 10 min at 90°C to expose the labeled DNA. Cells were then stained with anti-BrdU-conjugated FITC (Becton Dickinson) and with propidium iodide. Cell cycle analysis was carried out on a Becton Dickinson FACScan, using Cell Quest software.

Karyotype analysis

Metaphase chromosomes were prepared from passage 10, passage 15 and passage 17 HMEC, and grown on glass slides. Cells were treated for 34 min with colcemid, incubated in hypotonic solution (1% Na Citrate), fixed with methanol/acetic acid (3 : 1) and stained with Giemsa. One hundred metaphase spreads from cells at each of passages P10, P15 and P17 were counted and scored for tetraploid by a registered cytogenetic technologist.

β-galactosidase staining

HMECs from increasing passages were washed with PBS and fixed for 5 min at room temperature with 3% formaldehyde. The cells were stained in 1 mg/ml of X-gal (5-bromo-4-chloro-3-indolyl β-D-galactosidase) in dimethylformamide, 40 mM citric acid/Na phosphate buffer (pH 6.0), 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 150 mM NaCl and 2 mM MgCl. Cells were left in staining solution for 12 h at 37°C and then washed with PBS and photographed (Dimri *et al*., 1995).

Antibodies

Antibodies to the retinoblastoma protein, cdk2, cyclins A and D1, and p21 were obtained from Pharmingen or Santa Cruz Biotechnology. Cyclin E antibodies (mAbs E12 and E172) (Lees *et al*., 1992) were from E Lees and E Harlow (Mass. General, MA, USA). Monoclonal PSTAIRE antibody (Yamashita *et al*., 1992) was a gift from S Reed (The Scripps Research Institute, CA, USA), and cyclin D1 antibody, DCS-11, was purchased from Neomarkers. Cyclin A monoclonal mAb E67 was provided by J Gannon and T Hunt (ICRF, UK). Monoclonal p27 antibody was purchased from Transduction Labs. Cdk4 and cdk6 polyclonal sera were provided by G Hannon and D Beach (CSH Labs, NY, USA). A monoclonal antibody, JC-6, which recognizes the third ankyrin repeat of human p16 (Enders *et al*., 1995) and cross reacts with human p15, was used for immunoblotting of p15 in these studies. β-actin antibody was purchased from Sigma. Monoclonal p19 antibody was obtained from Neomarkers. Polyclonal p18 antibody was kindly provided by Y Xiong (Chapel Hill, NC, USA).

Derivation of Cdc25A antibody

Monoclonal and polyclonal antibodies were raised against a Cdc25A specific peptide, DYEQPLEVKNNSNLQRMG, derived from the N-terminal region of the protein. The peptide was synthesized as an amide containing an additional C-terminal cysteine for coupling with the malemide activated KLH (Pierce). The peptide was purified by HPLC and sequence verified by mass spectroscopy and amino acid analysis. Both polyclonal and monoclonal antibodies were purified using an IgG purification kit (Pierce). The Cdc25A monoclonal antibodies showed complete specificity for Cdc25A. The Cdc25A monoclonal antibodies failed to recognize up to 30 μg of GST-Cdc25B or GST-Cdc25C on Western analysis. A minor cross reactivity of the Cdc25A polyclonal antibody with Cdc25B or Cdc25C was seen, but only at supraphysiological expression levels. The polyclonal antibody detected Cdc25A with a 10-fold greater sensitivity than it did Cdc25B or Cdc25C. Preincubation of both polyclonal and monoclonal antibodies with a 30-fold molar excess of the immunizing peptide abolished recognition of Cdc25A.

RNA analysis

Total cellular RNA from early passage and senescent cells was prepared using Trizol (Canadian Life Technology). Samples of 20 μg of total RNA were separated by 1% agarose-formaldehyde gel electrophoresis and blotted onto Zeta-Probe (Biorad). ^{32}P-labeled probes for Northern blotting were generated using a random priming kit (Boehringer Mannheim) and full-length human Cdc25A cDNA. The hybridizations were carried out in 0.5 M sodium phosphate (pH 7.2), 7% SDS and 1 mM EDTA at 65°C. The blots were washed three times for 15 min in 40 mM sodium phosphate (pH 7.2) and 1% SDS at 65°C. Blots were stripped by incubating them twice in 0.1× SSC and 0.1% SDS at 100°C for 5 min, and subsequently re-probed with a ^{32}P-labeled 18S rRNA probe to verify loading (Florenes *et al*., 1994).

Immunoblotting

Cells were lysed in ice cold NP-40 lysis buffer (0.1% NP-40, 50 mM Tris, pH 7.5, 150 mM NaCl, 1 mM phenyl methyl sulfonyl fluoride (PMSF), and 0.02 mg/ml each of aprotinin, leupeptin and pepstatin). Lysates were sonicated and clarified by centrifugation. Protein was quantitated by Bradford analysis. Fifty or 100 μg protein was loaded in each lane and resolved by SDS-polyacrylamide gel electrophoresis (SDS–PAGE). Transfer and blotting was as described (Dulic *et al*., 1992). Equal loading of the lanes was verified using a β-actin antibody. For detection of cdk4 associated proteins by immunoprecipitation/Western analysis (IP/Western), cdk4 was immunoprecipitated from 200 μg of protein lysate, complexes resolved, blotted and the blot reacted with either cdk4, cyclin D1, p21 or p27 antibodies. For immunoblotting of cdk4 associated p15, 500 μg of protein lysate was used to immunoprecipitate cdk4. To verify the identity of associated proteins, control cyclin D1, p15, p21, and p27 immunoprecipitations were resolved along side the cdk4 immunecomplexes (not shown). Similar methods were used to detect cdk6 and cyclin E associated proteins. For detection of Cdc25A, 600 μg of protein was immunoprecipitated with a monoclonal

anti-Cdc25A and immunoblots reacted with a polyclonal anti-Cdc25A.

Protein expression in bacteria

A pGEX vector containing full-length human Cdc25A (kindly provided by H Piwnica-Worms) was transformed into *E. Coli* DH5α. Cells were grown to an O.D.^{695} of 0.8 and then induced for 7 h at 25°C with 0.4 mM IPTG. Bacteria were recovered, washed with PBS and lysed in lysis buffer containing 1% NP40, 20 mM TrisCl pH 8.0, 100 mM NaCl, 1 mM EDTA, 1 M DTT and protease inhibitors (1 mM PMSF and 0.02 mg/ml of aprotinin and leupepsin). Lysates were sonicated, clarified by centrifugation and recombinant Cdc25A was recovered on glutathione-sepharose columns. The columns were washed in 50 mM TrisCl, 2 M NaCl, 1 mM DTT and 1 mM EDTA and then in wash buffer containing decreased NaCl (150 mM NaCl). Cdc25A was eluted with 50 mM TrisCl pH 8.0, 50 mM NaCl, 1 mM DTT, 1 mM EDTA and 50 mM glutathione. Recombinant protein was then concentrated using centricon C-10 columns (Chemicon).

pRb substrate for cyclin D1 associated-kinase assays was generated from a pGEX vector containing the C-terminus of pRb (amino acids 729–928) fused to GST (kindly provided by J Zhao and E Harlow). Bacteria were lysed in PBS containing 1% Triton X-100, 1 mg/ml lysozyme and protease inhibitors, and lysates were clarified by centrifugation. The pRb fragment was isolated by incubating bacterial extract with glutathione beads for 1 h. Beads were then washed three times with PBS 1% Triton X-100 and then with PBS alone. The pRb fragment was eluted in 20 mM glutathione PBS at pH 7.8.

Cyclin-dependent kinase assays

For cyclin D1 associated kinase assays, cells were lysed in 50 mM HEPES, 150 mM NaCl, 1 mM EDTA pH 8.0, 2.5 mM EGTA pH 8.0, 10% glycerol, 10 mM β-glycerophosphate, 1 mM NaF, 0.1% Tween-20, 0.1 mM Na_2VO_4, 1 mM AEBSF, 0.5 mM DTT and 1 mg/ml of both leupeptin and aprotinin. Cyclin D-associated kinase assays were performed using 200 μg protein lysate following the method of LaBaer *et al.* (1997) using cyclin D1 antibody DCS11 for immunoprecipitation and a carboxyl-terminal fragment of pRb as substrate. Quantitation of radioactivity was performed using a Molecular Dynamics PhosphorImager and ImageQuant software. Cyclin E-associated kinase assays were performed as described (Slingerland *et al.*, 1994). Graphed results represent the mean values from repeat experiments.

Reactivation of cyclin E-cdk2 by Cdc25A

Reactivation of cyclin E-cdk2 from senescent cells by cyclin E-depleted early passage lysates was performed as follows. Cyclin E complexes were immunoprecipitated from senescent cell lysates, washed twice with 0.1% NP40 lysis buffer and then incubated with early passage lysate from which cyclin E had been serially immunodepleted four times. The buffer was brought to 10 mM DTT, 1 mM EDTA and 15 mM $MgCl_2$ and incubated for 30 min at 37°C. Cyclin E-associated kinase activity was then assayed. Reactivation of cyclin E-cdk2 by recombinant Cdc25A was performed by washing cyclin E immunoprecipitated complexes three times with Cdc25A buffer (50 mM Tris HCl pH 8.0, 50 mM NaCl, 1 mM DTT, 1 mM EDTA) followed by incubation with GST-Cdc25A for 2 h at room temperature. For assays of Cdc25A's potential to reactive cyclin D1-cdk6, cdk6 complexes were immunoprecipitated using the polyclonal anti-cdk6 serum of Meyerson *et al.* (provided by the Harlow Lab). Cdk6 immunoprecipitates were washed with Cdc25A buffer followed by incubation with GST-Cdc25A for 30 min at 30°C and kinase activities assayed as described above.

Retrovirus-mediated gene transfer

Retroviral plasmids were used to introduce full-length cDNA for human telomerase (hTERT) or Cdc25A into the HMEC. hTERT cDNA was provided by the Geron Corporation (Nijjar *et al.*, 2000), and the full-length human Cdc25A cDNA (provided by Mitotix) was subcloned into the *Bam*HI and *Hind*III sites of the pLNC3 vector. Phoenix Amphotrophic packaging cells were used for retroviral production.

Phoenix cells were plated onto 10 cm dishes and transfected with 20 μg of retroviral plasmid by calcium-phosphate transfection. The transfected cells were incubated for 12 h at 37°C with 25 μM chloroquine (Sigma). Phoenix cells were washed and replenished with MCDB 170 media and incubated for an additional 6 h at 30°C. Viral supernatant was recovered from Phoenix cells and supplemented with 0.2 μg/ml of polybrene (Sigma). The polybrene containing viral supernatant was filtered and added to HMEC cultures for 3 h at 30°C. HMECs were then washed and fresh media applied. Infected cells were selected with 100 μg/ml G418 (Gibco) starting 24 h post infection. The transformed cells were maintained in 25 μg/ml G418 to sustain selection pressure.

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