Efficient immortalization of primary human cells by p16^{INK4a}-specific short hairpin RNA or Bmi-1, combined with introduction of hTERT

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(Received August 31, 2006/Revised October 11, 2006/Accepted October 15, 2006/Online publication December 13, 2006)

Activation of telomerase is sufficient for immortalization of some types of human cells but additional factors may also be essential. It has been proposed that stress imposed by inadequate culture conditions induces senescence due to accumulation of p16^{INK4a}. Here, we present evidence that many human cell types undergo senescence by activation of the p16^{INK4a}/Rb pathway, and that introduction of Bmi-1 can inhibit p16^{INK4a} expression and extend the life span of human epithelial cells derived from skin, mammary gland and lung. Introduction of p16^{INK4a}-specific short hairpin RNA, as well as Bmi-1, suppressed p16^{INK4a} expression in human mammary epithelial cells without promoter methylation, and extended their life span. Subsequent introduction of hTERT, the telomerase catalytic subunit, into cells with low p16^{INK4a} levels resulted in efficient immortalization of three cell types without crisis or growth arrest. The majority of the human mammary epithelial cells thus immortalized showed almost normal ploidy as judged by G-banding and spectral karyotyping analysis. Our data suggest that inhibition of p16^{INK4a} and introduction of hTERT can immortalize many human cell types with little chromosomal instability. (*Cancer Sci* 2007; 98: 147–154)

Normal human cells undergo a limited number of divisions in culture and then enter a non-dividing state termed replicative senescence.[1,2] In some cases, like BJ foreskin fibroblasts and RPE-340 retinal pigment epithelial cells, telomerase activity is sufficient for immortalization.[3] However, this is not the case for other cell types such as HDK, HMEC and HPrEC.[4,5] Even fibroblasts derived from lung, such as the WI38 line, can not be immortalized with hTERT alone[6] and additional inactivation of the p16^{INK4a}/Rb pathway by E7 or downregulation of p16^{INK4a} expression by promoter methylation is required for efficient immortalization.[4,6–8] HMEC appear to senesce in two stages: an initial growth arrest stage (M0),[7] and a subsequent growth arrest stage (M1)[9] or agonescence.[10] M0 occurs after approximately 20 PD and is apparently controlled by the p16^{INK4a}/Rb pathway, because M0 can be prevented by E7,[7] and spontaneous reduction in p16^{INK4a} expression due to promoter methylation has been documented.[4,8,11] M1 can be bypassed by E6 or induction of telomerase activity.[4,9] In most human somatic cells, telomerase activity is too low to maintain telomere length in dividing cells, and is induced by transcriptional activation of the human telomerase reverse transcriptase gene, *hTERT*.

A number of Myc binding sites are located in the promoter and introns of *hTERT*, and introduction of c-Myc results in activation of telomerase in some cell types.[12] The polycomb group gene *Bmi-1* was first isolated as an oncogene that could cooperate with c-Myc in inducing lymphomas in Eμ-Myc transgenic mice.[13] Overexpression of Bmi-1 downregulates p16^{INK4a} and p19^{ARF} expression in mouse embryonic fibroblasts, and results in their immortalization.[14] These findings led us to consider that Bmi-1 might cooperate with hTERT in immortalization of human epithelial cells. Indeed, exogenous expression of Bmi-1 was reported to extend the life span of primary human fibroblasts[6] and to immortalize human cells in cooperation with hTERT.[15,16] However, as exogenous expression of Bmi-1 was reported to immortalize primary (pre-M0) HMEC by activating telomerase,[17] it is not clear whether the Bmi-1 inhibition of p16^{INK4a} or p14^{ARF} is important for immortalization of HMEC. To address this question, we here examined the effects of Bmi-1 and inhibition of p16^{INK4a} on immortalization of primary human cells from several tissues, including (pre-M0) HMEC.

Materials and Methods

Cell culture. HMEC cultures were derived from reduction mammoplasty specimens as described previously.[8,11] HMEC were cultured in DFCI-1 medium.[18] HBEC, HDK and HDPC were purchased from Cell Applications (San Diego, USA), and HSAEC, HMSC and HPrEC were from Cambrex (Walkersville, USA). HDPC, HMSC and HPrEC were cultured in PCGM (Toyobo, Osaka, Japan), MSCGM and PrEGM (Cambrex), respectively, according to the supplier's instructions. HDK, HBEC and HSAEC were cultured in keratinocyte serum-free medium (Invitrogen, USA). This study using primary human cells from patients with written consent was approved by the Ethics Committee of the National Cancer Center.

Vector construction and retroviral infection. Construction of the destination vectors pDEST-CLXSN and pDEST-CMSCVpuro, and the expression vectors pCMSCVpuro-16E7 and pCLXSN-hTERT was described previously.[19,20] The HPV16 E6E7 segment and human Bmi-1 cDNA were similarly cloned into the destination vectors. Briefly, after cloning segments of HPV16 E6E7 (16E6E7) and a splice donor site-mutant version of HPV16 E6 (16E6SD)[21] into pDONR201 (Invitrogen), these segments were recombined into retroviral vectors by LR reaction (Invitrogen) to generate pCMSCVpuro-16E6E7 and pCMSCVpuro-hBmi-1. Construction of the destination vector pDEST-CL-SI-MSCVpuro (designated as pSI-CMSCVpuroDEST previously), for retroviral expression of shRNA, pCL-SI-MSCVpuro-p53-shRNA (designated as pSI-CMSCVpuro-p53Ri previously) and the entry vector pENTR-H1R-stuffer was

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Abbreviations: HBEC, human bronchial epithelial cells; HDK, human dermal keratinocytes; HDPC, human dermal papilla cells; HFK, human foreskin keratinocytes; HMEC, human mammal epithelial cells; HMSC, human mesenchymal stem cells; HPrEC, human prostate epithelial cells; HSAEC, human small airway epithelial cells; hTERT, telomerase catalytic subunit; M0, mortality stage 0; M1, mortality stage 1; PCR, polymerase chain reaction; PD, population doublings; shRNA, short hairpin RNA; SKY, spectral karyotyping; TRAP, telomere repeat amplification protocol.

doi: 10.1111/j.1349-7006.2006.00373.x

described previously.[21] To generate p16shRNA expression vectors pSI-CMSCVpuro-H1R-p16shRNA1, 6 and 8, 5′-AAC GCA CCG AAT AGT TAC G-3′, 5′-GGA CGA AGT TTG CAG GGG A-3′ and 5′-GCC CAA CGC ACC GAA TAG TTA CGG TC-3′, respectively, were chosen as the targeted sequences. Production of recombinant retroviruses was as described earlier.[22] Briefly, the retroviral vector and the packaging construct pCL-10A1 were cotransfected into 293T or 293FT (Invitrogen) cells using TransIT-293 (Mirus Co., Madison, USA) according to the manufacturer's instructions, and culture fluid was harvested 48–72 h post-transfection. Titers of the recombinant viruses were greater than 2×10^5 drug-resistant colony forming units/mL with HeLa cells. Following addition of 0.5 mL of the recombinant viral fluid to primary human cells seeded on six-well dishes in the presence of polybrene (4 μg/mL), infected cells were selected in the presence of 0.5 μg/mL puromycin or 50–200 μg/mL G418. For combinations of retroviral infections, cells were first transduced with Bmi-1 or $p16^{INK4a}$ shRNA, and then with hTERT.

Immunoblotting analysis. Whole-cell lysates were prepared by washing cells with phosphate-buffered saline, and collected in lysis buffer (50 mM Tris-HCl at pH 7.5, 250 mM NaCl, 1% Nonidet P-40, 20% glycerol, 1 mM dithiothreitol, and 5% (v/v) protease inhibitor cocktail [Nakarai, Japan]). Lysates were then sonicated and clarified by centrifugation. Protein concentrations were determined by using the DC protein assay (Bio-Rad). The same amounts of the proteins (generally 20 μg/lane) were electrophoresed on sodium dodecylsulfate–polyacrylamide gels and transferred to Immobilon-P membranes (Millipore, Billerica, MA, USA). Antibodies against $p16^{INK4a}$, (clone G175-405; PharMingen), San Jose, CA, USA, p53 (Ab6; Oncogene Research Products, Cambridge, MA, USA), p21 (WAF1 Ab1; Oncogene Research Products), $p14^{ARF}$ (sc-8613; Santa Cruz, CA, USA), β-actin (sc-1616; Santa Cruz) and Bmi-1 (5G4, monoclonal antibody raised against carboxy-terminal 99 amino acids)[23] were used as probes, and horseradish peroxidase-conjugated anti-mouse (Jackson Immunoresearch Laboratories, West Grove, PA, USA) or anti-goat (sc-2033; Santa Cruz) IgG were used as secondary antibodies. The LAS3000 CCD-Imaging System (Fujifilm Co., Tokyo, Japan) was used for detection and quantification of proteins visualized by Lumi-light Plus immunoblotting substrate (Roche, Indianapolis, IN, USA).

Telomerase activity. Telomerase activity was detected using a non-radioisotopic method with a TRAPeze telomerase detection kit (Intergen, Purchace, USA), according to the manufacturer's instructions. One microgram of cell protein lysed in CHAPS buffer was used for the assay. PCR products separated on 12.5% polyacrylamide gels were stained with SYBR Green I (Cambrex Co., New York, USA), and visualized with the LAS3000 CCD-Imaging System (Fujifilm Co.) on an ultraviolet transilluminator.

INK4a promoter methylation analysis. PCR-based assays for CpG island methylation of the INK4a promoter region were carried out as described previously.[11] Briefly, after sodium bisulfite treatment, the INK4a promoter region was amplified by PCR with primers 5′-TTT TTA GAG GAT TTG AGG GAT AGG-3′ (−159 to −136) and 5′-CTA CCT AAT TCC AAT TCC CCT ACA-3′ (+209 to +233).[24] The PCR product contained 35 CpG and 48 non-CpG cytosines internal to the primers. Reaction conditions were as described previously,[11] except with a 59°C annealing temperature. PCR were carried out with a thermal cycler (Takara Bio, Otsu, Japan) and the resulting PCR products were purified using a PCR purification kit (Qiagen, Valencia, CA, USA) and TA cloned with the pGEM-T easy plasmid vector (Promega, Madison, WI, USA). Individual clones were sequenced with the T7 primer on an ABI PRISM 310 DNA sequencer (Applied Biosystems, Foster City, CA, USA) using a dye terminator cycle sequencing kit and AmpliTaq DNA polymerase (Applied Biosystems).

G-banding and spectral karyotyping analysis. Metaphase spreads were prepared from cells treated with Colcemid (2.8 μg/mL for 4 h; Demecolcin; WAKO, Osaka, Japan). The karyotype analysis was carried out using standard G-banding and multicolor SKY according to the manufacturer's instructions (ASI; Carlsbad, USA) and a previously published method.[25] At least 50 and five metaphase spreads for each population were analyzed by G-banding and SKY, respectively.

Results

$p16^{INK4a}$ expression level is upregulated in several human primary cultured cells. Several human primary cells were examined for $p16^{INK4a}$ (p16) expression during serial passages of culture, and were found to senesce in accordance with p16 elevation (Fig. 1A,B,D) and reduction of the phosphorylated form of Rb (Fig. 1B for HFK). The only exception among those so far examined proved to be human foreskin fibroblasts, which expressed little p16 throughout serial passage until reaching replicative senescence (Fig. 1C), and could be immortalized by hTERT transduction alone (data not shown) in accordance with previous results.[3] Interestingly, the p16 levels that presumably induced senescence were very similar among different cell types, ranging from about a quarter (HDPC) to a half (HFK) of those in HeLa cells (Fig. 1A, data not shown for HSAEC, HPrEC and HFK). These results not only confirmed previous findings for epithelial cells[4,5] but also suggest many human cell types in culture similarly undergo senescence associated with activation of the p16/Rb pathway (Fig. 1D).

Extension of the life span of epithelial cells and downregulation of p16 expression by introduction of Bmi-1. To examine whether Bmi-1 can extend the life span of human epithelial cells, Bmi-1 was introduced into HDK and HSAEC as well as HMEC. Premature senescence of HMEC, or M0, was clearly bypassed by Bmi-1 as well as by E7, as reported previously (Fig. 2A).[7] As with HMEC, the life span of HDK and HSAEC was also extended by introduction of Bmi-1 as well as by E7, and E6 plus E7 (Fig. 2B,C). As expected, low-level expression of p16 was observed in HMEC transduced with Bmi-1 (Fig. 2D), though that of control cells was lower probably because of the complete growth arrest, which can be also observed in HFK (Fig. 1B). Introduction of Bmi-1 remarkably inhibited the expression of p16 in HDK and HSAEC (Fig. 2E,F). However, none of them could be immortalized by introduction of Bmi-1 alone (Fig. 2A–C). These results not only confirmed the previous result with HMEC that Bmi-1 can extend the life span of human epithelial cells, but also suggest that inhibition of p16 expression by Bmi-1 is the common mechanism that extends the life span of cells.

Weak activation of telomerase by Bmi-1 in HMEC but not in HDK and HSAEC. As Bmi-1 was reported to activate telomerase in HMEC and immortalize them without introduction of hTERT, telomerase activity was measured by TRAP assay at different PD in our three cell types (Fig. 3A,B). Interestingly, only HMEC expressing Bmi-1 showed weak but significantly elevated telomerase activity, whereas HDK and HSAEC did not, though very weak telomerase activity was detectable in HDK. Unlike the previous reports, no increase in activity with passage of cells was observed in our experiment (Fig. 2A). These results further support the notion that the inhibition of p16 expression by Bmi-1 is a common mechanism that extends the life span of cells. It is possible that the weak activation of telomerase by Bmi-1 is involved in the extended life span of HMEC to some extent.

Efficient immortalization of HDK and HSAEC by the combination of Bmi-1 and hTERT. Additional introduction of hTERT into HDK, and HSAEC transduced with Bmi-1 led to their efficient immortalization without any growth retardation, whereas the control vector resulted in complete growth arrest around PD = 70 and 33, respectively (Fig. 4A,B). Interestingly,

doi: 10.1111/j.1349-7006.2006.00373.x

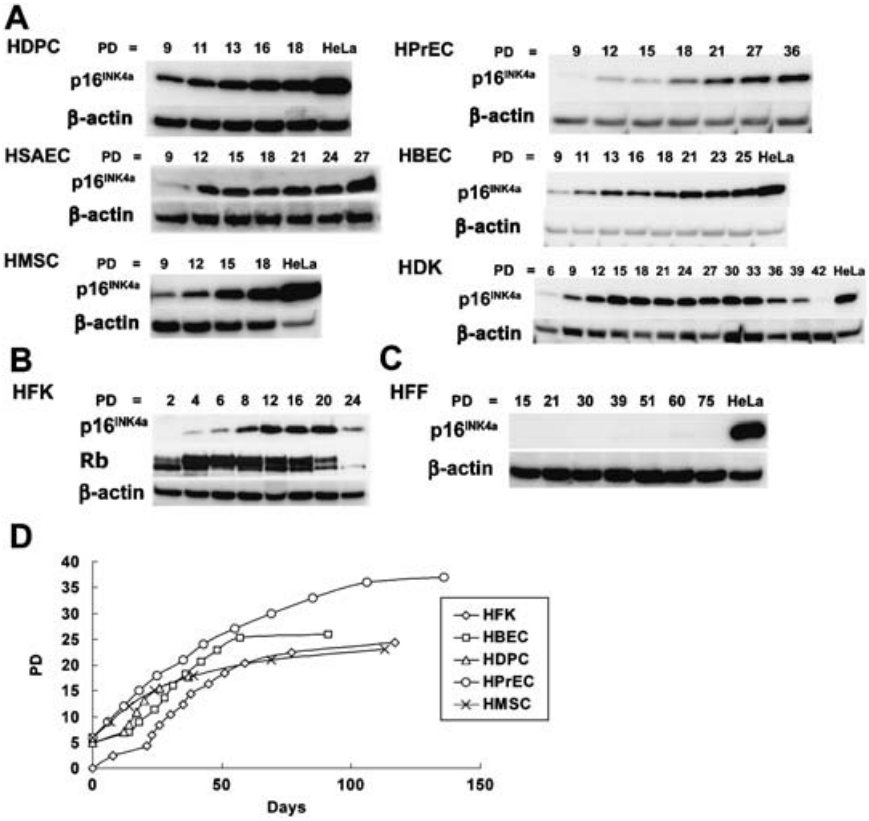

Fig. 1. Elevation of p16 during serial passage of several primary human cell types but not of neonatal skin fibroblasts. Whole-cell protein extracts of human dermal papilla cells (HDPC), human small airway epithelial cells (HSAEC), human mesenchymal stem cells (HMSC), human prostate epithelial cells (HPrEC), human bronchial epithelial cells (HBEC), human dermal keratinocytes (HDK), human foreskin keratinocytes (HFK) and human foreskin fibroblasts (HFF) were collected at the indicated population doublings (PD). Expression levels of (A,C) p16 together with (B) Rb were analyzed by immunoblotting. HeLa cell extract was used as a positive control for p16 in some blots. β-Actin was used as a loading control. (D) Growth curves for the different cell types.

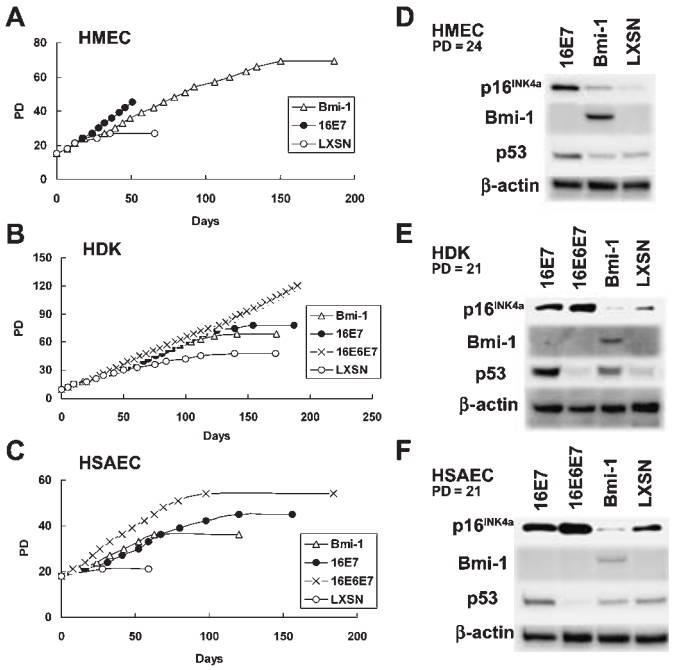

Fig. 2. Bmi-1 extends the life span and downregulates p16 of human mammary epithelial cells (HMEC), human dermal keratinocytes (HDK) and human small airway epithelial cells (HSAEC) as well as E7. (A,D) HMEC were infected at population doublings (PD) = 15, designated as day 0. Infected cells were selected in the presence of 200 μg/mL G418 and harvested at PD = 24. (B,E) HDK were infected at PD = 12, designated as day 0. Infected cells were selected in the presence of 100 μg/mL G418, and harvested at PD = 21. (C,F) HSAEC were infected at PD = 15, designated as day 0. Infected cells were selected in the presence of 50 μg/mL G418 and harvested at PD = 21. Expression levels of Bmi-1, p16 and p53 as well as β-actin were analyzed by immunoblotting.

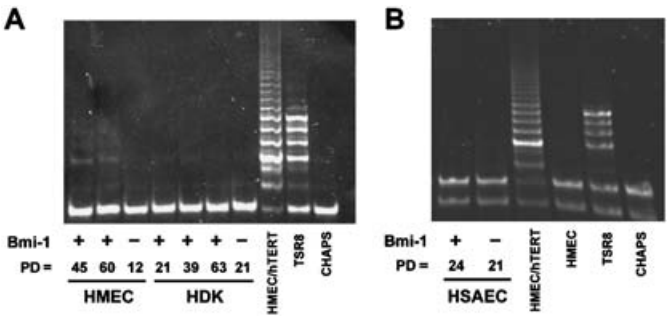

Fig. 3. Weak activation of telomerase by Bmi-1 in human mammal epithelial cells (HMEC) but not in human dermal keratinocytes (HDK) and human small airway epithelial cells (HSAEC). Telomerase activity of (A) HMEC, HDK and (B) HSAEC transduced with Bmi-1 was analyzed by TRAP assay. Those without Bmi-1 and HMEC transduced with the telomerase catalytic subunit hTERT, as well as eight tandem repeats of telomeric sequence (TSR8) and CHAPS buffer alone were used as controls.

introduction of c-Myc could substitute for hTERT in both activation of telomerase (Fig. 4C) and efficient immortalization of Bmi-1-expressing HDK (Fig. 4A). These results suggest that Bmi-1 can allow these cell types to bypass senescence mainly by inhibiting p16 expression, and additional introduction of hTERT is sufficient for efficient immortalization. The activation of telomerase by Bmi-1 observed in HMEC appeared insufficient to maintain telomere length and overcome M1.

Inhibition of p16 by Bmi-1 or p16shRNA allows HMEC to bypass M0 but not M1. To further analyze the molecular mechanisms of immortalization, we chose HMEC, which have been well characterized. To examine whether the extended life span with Bmi-1 was solely dependent on downregulation of p16, we first introduced three shRNA targeting different regions of the *INK4a* gene, or the control vector MSCVpuroH1R into early passage

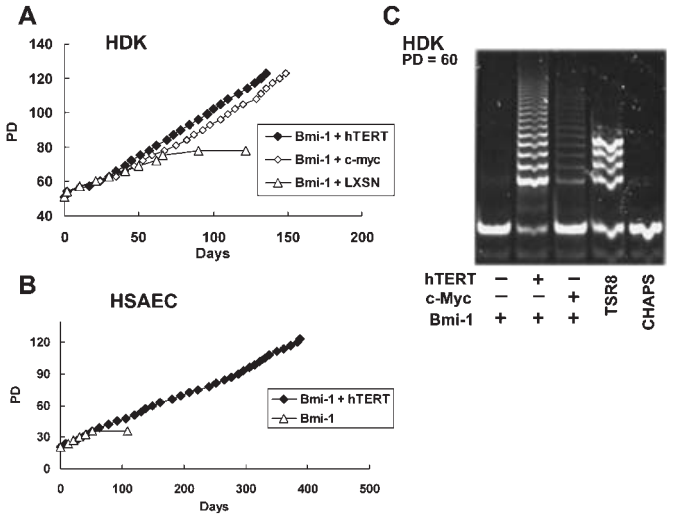

Fig. 4. Efficient immortalization of human dermal keratinocytes (HDK) and human small airway epithelial cells (HSAEC) by combination of Bmi-1 and the telomerase catalytic subunit hTERT. (A) Bmi-1 HDK were infected with hTERT, c-Myc or control vector at PD = 51. Three days after infection, hygromycin (5 μg/mL) was added for selection. (B) Bmi-1 HSAEC were infected with hTERT at population doublings (PD) = 18. Two days after infection, puromycin (0.5 μg/mL) was added for selection. (C) Telomerase activity of Bmi-1 HDK after hTERT or c-Myc transduction was analyzed as in Fig. 3. TSR8, eight tandem repeats of telomeric sequence.

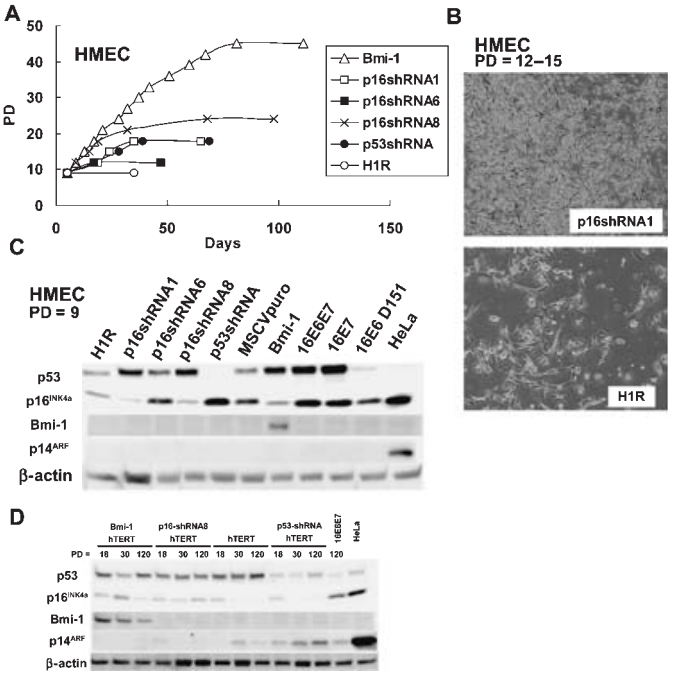

Fig. 5. Expression of p16-specific short hairpin RNA (shRNA) 1 and 8 and Bmi-1 as well as E7 allows human mammal epithelial cells (HMEC) to bypass mortality stage 0 (M0). (A) Growth of HMEC introduced with each retroviral construct. HMEC were infected at population doublings (PD) = 9, designated as day 0. Two days after infection, puromycin (0.5 μg/mL) was added to the medium for selection. H1R indicates HMEC infected with the MSCVpuroH1R control vector. (B) Phase contrast image of HMEC transduced with p16 shRNA1 (upper) or H1R (lower) at PD = 12–15. (C) Downregulation of p16 expression by p16 shRNA1, p16 shRNA8 and Bmi-1. At PD = 12, whole-cell protein extracts were analyzed by immunoblotting. Three different target sequences (1, 6 and 8) were set up, and were assessed. H1R indicates MSCVpuroH1R control vector-infected. (D) Expression of $p14^{ARF}$ was clearly detectable at later passage in HMEC expressing p53 shRNA plus the telomerase catalytic subunit hTERT. Whole-cell protein extracts of each immortalized HMEC at PD = 18, 30 and 120 were analyzed by immunoblotting.

(PD = 9) HMEC cells. Among them, only shRNA1 and shRNA8 effectively repressed p16 expression. Like HMEC expressing E7, cells expressing Bmi-1 or p16 shRNA1 and shRNA8 bypassed M0 and grew exponentially until PD = 18–24 (Fig. 5A) with retention of the morphology of early passage cells (Fig. 5B top), whereas in control cells infected with p16 shRNA that were not effective, MSCVpuro or MSCVpuroH1R, almost all senesced around PD = 9–15 (Fig. 5A) and most became morphologically enlarged (Fig. 5B bottom). Expression of p16 was also very low in these latter post-M0 cells, as analyzed by immunoblotting (Fig. 5C), whereas expression of E7 or E6E7 was associated with high levels of p16.

As Bmi-1 is also reported to downregulate $p19^{ARF}$ in mouse embryonic fibroblasts, we assessed $p14^{ARF}$ expression in HMEC. Because endogenous levels of $p14^{ARF}$ were much lower in HMEC than HeLa cells (Fig. 5C), we could not formally exclude the possibility that repression of $p14^{ARF}$ by Bmi-1 is involved in extended life span of HMEC. Interestingly, p53 levels in cells where the p16/Rb pathway was inhibited either by p16 shRNA, Bmi-1 or E7 expressed higher levels of p53 (Fig. 5C), and p53 shRNA-transduced HMEC expressed detectable levels of $p14^{ARF}$, which increased through passages (Fig. 5D). It has been reported that $p14^{ARF}$ is not repressed by overexpression of Bmi-1 in a HMEC strain (76 N).[17] From these results, it seems likely that the extended life span of HMEC expressing Bmi-1 depends primarily on repression of p16 and possibly in part on weak activation of telomerase.

hTERT cooperates with p16shRNA or Bmi-1 in immortalization of HMEC. When HMEC expressing p16 shRNA1 and 8 or Bmi-1 were introduced with hTERT, they bypassed M1, reached PD = 120 without any sign of senescence (Fig. 6A–C), and demonstrated telomerase activity in a TRAP assay (Fig. 6E). In contrast, Bmi-1 or p16 shRNA1 and 8 cells transduced with control vector senesced at PD = 40 and PD = 20, respectively (Fig. 6A–C), and showed weak or no TRAP activity (Fig. 6E). Mild growth retardation was observed around day 60–70 or PD = 40–50 (Fig. 6A–D), but this was not the case for cells expressing E7 plus hTERT, E6E7 plus hTERT, or p53 shRNA plus hTERT (data not shown), indicating an involvement of the p53/p21 pathway. Mild increase of p53 and p21 around PD = 40–50 in cells expressing p16shRNA8 plus hTERT or hTERT alone, and slight decrease of Bmi-1 around the same period in cells expressing Bmi-1 plus hTERT might be involved in the observed growth retardation (Fig. 6F,G).

Bmi-1 and p16 shRNA repress expression of p16 without promoter methylation. To examine whether reduction of p16 levels due to Bmi-1 expression might be associated with promoter methylation, the Na-bisulfite method was applied. The promoter of HMEC (PD = 90) with hTERT alone was heavily methylated as expected,[4,8] whereas those of both p16 shRNA1 and p16 shRNA8 cells were scarcely methylated (Fig. 7A), in line with almost complete inhibition and weak expression of p16, respectively (Fig. 6F,G). The promoter regions in HMEC exposed to Bmi-1 and hTERT were methylated heterogeneously, perhaps because expression levels of Bmi-1 were not sufficient in some populations to sufficiently suppress the p16 expression. However, some promoter regions were completely unmethylated in two independent cultures, indicating that Bmi-1 can repress p16 expression without causing methylation of the promoter. In accordance with the methylation status, treatment with a DNA methylase inhibitor, 5-aza-2′-deoxycytidine, did not increase the expression of p16 in cells treated with p16 shRNA, but clearly restored it in cells fully immortalized with hTERT after spontaneously bypassing M0 (Fig. 7B).

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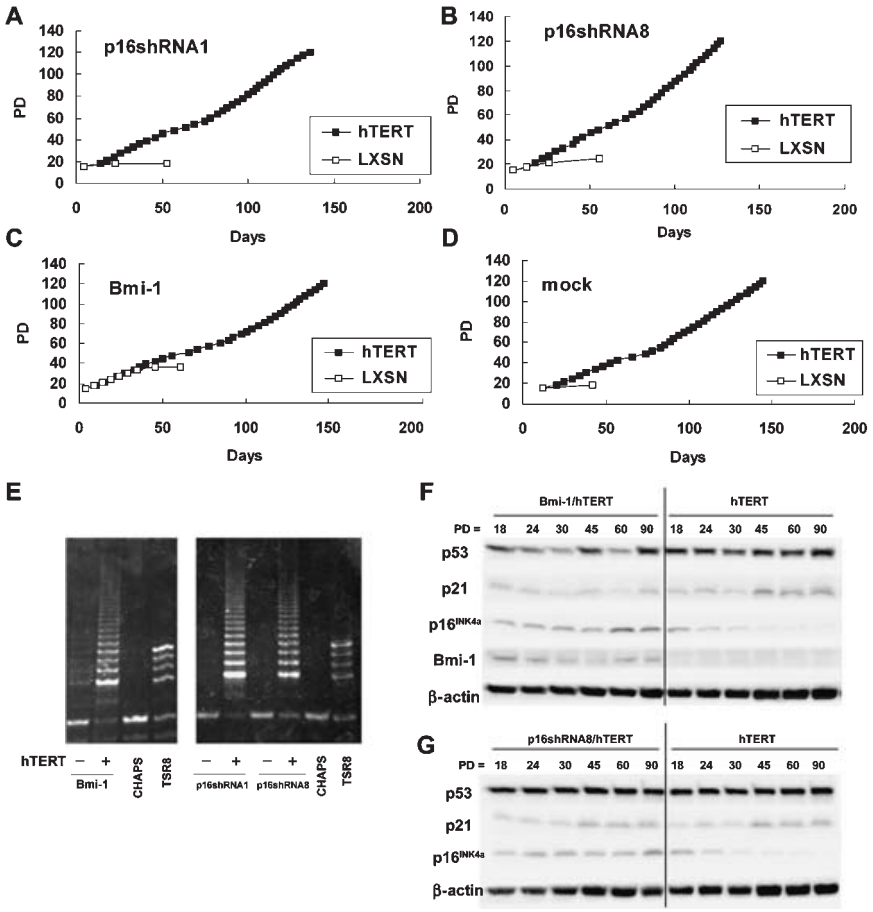

Fig. 6. The telomerase catalytic subunit hTERT overcomes mortality stage 1 (M1) and induces immortalization of human mammal epithelial cells (HMEC). (A–D) Growth curve of the post-mortality stage 0 (M0) HMEC with or without introduction of hTERT. Post-M0 HMEC transduced with (A) p16 shRNA1, (B) p16 shRNA8 and (C) Bmi-1, or (D) those that spontaneously escaped from M0 were infected with LXSN-hTERT or LXSN retrovirus at population doublings (PD) = 15. Two days after infection, G418 (50 μg/mL) was added to the medium for selection. (E) TRAP assays with various post-M0 HMEC after transduction of hTERT (+) or LXSN (–). (F,G) Expression levels of several cell cycle-associated proteins in post-M0 HMEC at the indicated PD were measured by immunoblotting. HMEC expressing (F) Bmi-1 plus hTERT and (G) p16 shRNA8 plus hTERT were analyzed with the same samples from those expressing hTERT alone. Note that a mild increase of p16 levels together with some fluctuation of Bmi-1 levels were observed in HMEC expressing Bmi-1 plus hTERT at later passages, whereas there was a constant decrease of p16 levels in control cells (F). Also, a mild increase of p21 levels was observed in HMEC expressing p16 shRNA8 plus hTERT and hTERT alone at PD = 45 and later passages (G).

Normal ploidy in HMEC immortalized by hTERT and p16 shRNA or Bmi-1. Though normal ploidy of fibroblasts immortalized by hTERT alone has been reported, both E6 and E7 can induce chromosomal instability. Therefore, we examined karyotypes of HMEC immortalized by E6E7 or combinations of hTERT and p16 shRNA, Bmi-1 or E7 at late passage (PD = 120–135). SKY analysis was used to detect minimal changes of chromosomal integrity. In the first experiment, five randomly selected cells were analyzed from each population of immortalized cells (Table 1). With E6E7, two translocations were found in two cells, whereas no translocation was detected in any cells immortalized by hTERT plus Bmi-1 or hTERT alone. Cells immortalized by hTERT plus Bmi-1 and cells immortalized with hTERT alone were almost normal diploid with or without trisomy of chromosome 20, which was also observed commonly in cells immortalized by other combinations of genes. In the second set of experiments, we detected more chromosomal abnormalities on average by SKY analysis (Table 2), and also applied G-banding to analyze the karyotype of the cells (Tables S1 and S2). Though translocations were detected in all of the populations, chromosomal numbers were near diploid (46–49) except that two cells near tetraploid were detected in those immortalized with E7 plus hTERT (Table S1). The majority of the cells immortalized by hTERT alone, or p16 shRNA8 plus hTERT were near diploid (47 or 48) and had no translocations, in clear contrast to the case with E6 and E7.

Discussion

Many lines of evidence suggest that activation of telomerase is not sufficient for immortalization of cell types other than human neonatal fibroblasts and retinal pigment cells under commonly used culture conditions. We earlier showed that both inactivation of the p16/Rb pathway and telomerase activation may be required for immortalization of human epithelial cells such as HMEC and HDK,[4] and here demonstrated elevation of p16 levels through passage that could be bypassed by overexpression of Bmi-1. Furthermore, immortalization proved possible when this treatment was combined with introduction of hTERT.

Polycomb group genes have been shown to function as repressor complexes of *HOX* genes. Among them, Bmi-1 and CBX7 were reported to repress p16 expression.[26,27] However, the mechanisms remain largely unknown. Here, we showed Bmi-1 to readily repress p16 expression in HMEC, HDK and HSAEC (Figs 2D–F and 5C), and this repression could be maintained without hypermethylation of the promoter in the HMEC case (Fig. 7A), which is invariably observed in cells spontaneously escaping from premature senescence or M0.[8,11] The repression of p16 by Bmi-1 could naturally have been responsible for the observed lack of senescence. However, as Bmi-1 has other functions, independent of its transcriptional repression[28] and activation of telomerase,[17] we directly knocked down p16 expression with specific shRNA in HMEC, in which two-step immortalization is well characterized. The results support the notion that inhibition of p16 by Bmi-1 is indeed one reason for extension of the life span but expression of Bmi-1 appeared more effective than the p16-specific shRNA tested. We confirmed telomerase activation by Bmi-1,[17] and this might have played a role to some extent. However, this was relatively weak and proved insufficient for efficient immortalization of HMEC in our assay (Figs 2A,3A,5A,6E). Bmi-1 did not activate telomerase in HDK or HSAEC (Fig. 3A,B), but repressed p16 expression

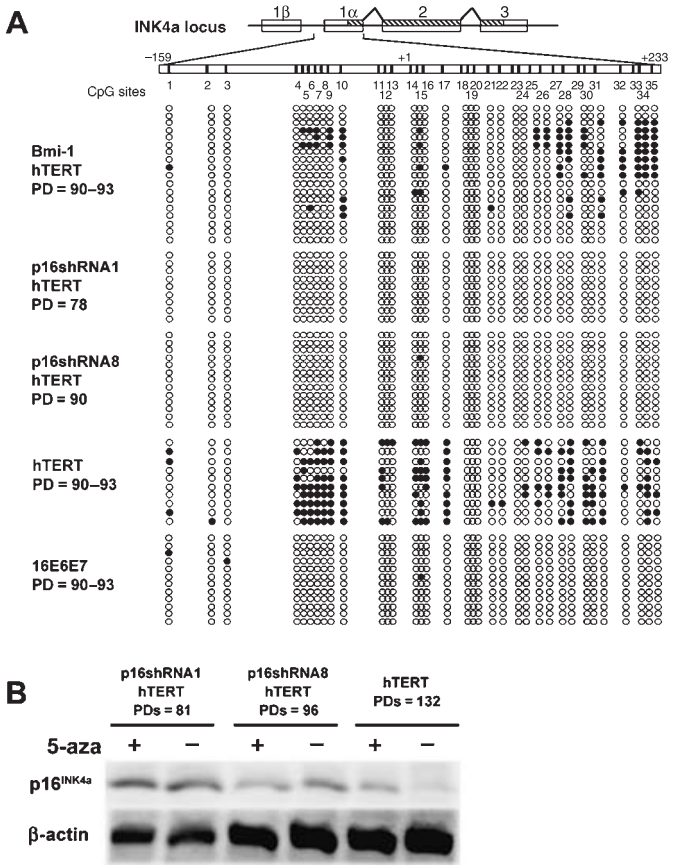

Fig. 7. Repression of p16 by Bmi-1 and p16 shRNA without promoter methylation. (A) Genomic map of the 5′-CpG islands of the *INK4a* gene. The bar under the *Ink4a* locus presents a magnification of the region, from −159 to +233, analyzed in this study. The 35 CpG sites in this region are numbered according to their 5′ to 3′ order in the *INK4a* genomic sequence and positioned based on their location within the genomic sequence. This region was amplified from bisulfite-treated DNA. White and black circles represent unmethylated and methylated CpG sites, respectively. For each cell type, 8–19 clones were analyzed. (B) 5-Aza-2′-deoxycytidine treatment does not induce p16 re-expression in human mammal epithelial cells (HMEC) expressing p16 shRNA plus hTERT. HMEC at population doublings (PD) = 81–132 were treated with 5-aza-2′-deoxycytidine (5 μM) for 72 h and whole-cell protein extracts were analyzed by immunoblotting.

Table 1. Karyotype analysis of each transduced primary human mammary epithelial cells by fluorescence *in situ* hybridization (1st time)

Cell type	Chromosome number	Karyotype	Cell number
Bmi-1/hTERT	47	+20	5
hTERT	47	+20	4
	46		1
16E6E7	47	+18, +20, t(X; 12), der(X; 13)	1
	46	der(X; X), der(8; 8)	1
	46		3
16E7/hTERT	47	+20	1
	47	+20, der(3; 10)	1
	46	+20, der(3; 10), del(4)	1
	46		2

der, derivative chromosome; hTERT, telomerase catalytic subunit; t, translocation.

Table 2. Karyotype analysis of each transduced primary human mammary epithelial cells by fluorescence *in situ* hybridization (2nd time)

Cell type	Chromosome number	Karyotype	Cell number
p16shRNA1/hTERT	49	+i(1), +7, i(8), +20	3
	49	+i(1), +7, i(8), +20, inv(1)	1
	49	+i(1), +7, i(8), +20, der(5; 6)	1
p16shRNA8/hTERT	47	+20	2
	48	+20, +7	3
Bmi-1/hTERT	47	+20, add(11)	5
hTERT	47	+20	1
	48	+20, +7	4
16E6E7	47	+20, der(22; 8)	3
	47	+20, der(22; 8), del(13)	1
	46	+20, der(22; 8), t(13; 21)	1
16E7/hTERT	47	+20	2
	46	der(8; 8)	1
	46		2

add, additional material of unknown origin; der, derivative chromosome; der(A; B), B is added to A partially; del, deletion; hTERT, telomerase catalytic subunit; i, isochromosome; inv, inversion; shRNA, short hairpin RNA; t, translocation.

and extended their life span, strongly suggesting that the inhibition of p16 by Bmi-1 is the common and primary mechanism for the extended life span. In accordance with this notion, we and others have also succeeded in extending the life span of human myoblasts, human bone marrow-derived mesenchymal stem cells and bovine cementoblast progenitor cells using Bmi-1 plus hTERT.[15,16,23]

Expression of p16 is induced by certain oncogenic signals and DNA damage or other stress, and functions in telomere-independent senescence of not only human epithelial cells[4,29] but also human fibroblasts not derived from skin, such as the WI38 and IMR90 strains.[6,30] Recently, normal but not hTERT-immortalized human foreskin fibroblasts were reported to be resistant to activated Ras-induced senescence.[30] We found here that most primary human cells showed elevation of p16 levels on serial passage but that human neonatal skin fibroblasts are exceptional in this regard. Stress can induce p16 even in human neonatal skin fibroblasts immortalized by hTERT expression.[31] As elevation of p16 levels in mammary epithelial cells and keratinocytes is reported to be alleviated by cocultivation with mouse fibroblasts as feeder cells,[32,33] other factors do have an impact. Recently, another group suggested that a single genetic event (c-myc) can immortalize human epithelial cells (HPrEC) with p16 accumulation.[34] Though we do not know the precise mechanism of the immortalization, it is clear that c-Myc can somehow suppress the p16 Rb pathway in HPrEC in addition to activating telomerase, as the immortalized cells proved insensitive to p16 overexpression and Ras-induced senescence. However, immortalization by c-Myc alone could be cell type specific. Indeed, we failed to extend life span of HFK by c-Myc alone. Such cell type specificity might be explained by another known function of c-Myc as a potent inducer of apoptosis.[14]

Finally, we showed normal or near normal diploidy of the cells immortalized by hTERT plus Bmi-1 or p16 shRNA (Tables 1 and 2 and Tables S1 and S2), in contrast to cells immortalized with viral oncogenes E6 and E7, which showed a tendency for chromosomal abnormalities, including translocations and tetraploidy.[35,36] However, the majority of cells immortalized by E7 plus hTERT had no translocations and were also diploid, only trisomy 20 being commonly detected (Tables 1 and 2). We and others have reported similar observations for

doi: 10.1111/j.1349-7006.2006.00373.x

preadipocytes,[37] bone marrow stromal cells[23] and ovarian surface epithelial cells[38] immortalized by introduction of E7 and hTERT. In these cases, functional p53 was induced by E7, probably through upregulation of $p14^{ARF}$, and could have prevented chromosomally abnormal cells from emerging and proliferating. Recently, p16 in cooperation with p21 was reported to prevent centrosome dysfunction and genomic instability in primary cells under conditions that trigger inhibition of the DNA replication cycle.[39] This might also explain the chromosomal stability of cells immortalized by E7 plus hTERT, in which p16 as well as p53 is elevated (Fig. 2). In cells immortalized by E6 and E7, inactivation of p53 by E6 can result in abnormal centrosome amplification[40] and abolish the DNA damage checkpoint and p53-dependent apoptosis, and E7 can induce centrosome duplication error[36] and abolish spindle checkpoint induced by nocodazol.[41] It seems likely that tetraploid cells induced by E7, as detected in G-banding, were induced by the colcemid treatment and naturally eliminated by active p53 in cells immortalized by E7 plus hTERT. Chromosomal stability of cells immortalized by hTERT and inhibition of p16, either by Bmi-1 or p16shRNA, is not surprising given the fact that p16 expression is not detected throughout developmental stages of the mouse, except in the thymus,[42] neither is the finding that Bmi-1 is expressed predominantly in hematopoietic stem cells and proliferating cerebellar precursor cells.[43,44] Inhibition of p16 should have milder effects than E7 as it just inactivates a Rb pathway through activation of Cdk4–Cyclin D complexes. The method described here could be applied widely to immortalization of normal human cells to establish chromosomally stable, virtually normal human cell lines, suitable for research in a wide range of fields.

Many lines of evidence suggest that premature senescence induced by the p16/Rb pathway is a tumor suppressor mechanism[45] or a mechanism to limit keratinocyte proliferation in the process of wound healing,[46] even though its abrogation is not a requirement for experimental immortalization *in vitro*.[33,45] Bmi-1, which can inhibit the transcription of p16,[26] was first described as a cooperating oncogene in Eμ-*Myc* transgenic mice,[13] and recently amplification and overexpression of Bmi-1 have been reported in several human malignancies including non-small cell lung, breast, penile and colorectal cancers, as well as Hodgkin's lymphomas, and a reverse correlation with p16 and $p14^{ARF}$ expression was evident in some of these cases.[47–52] Cells immortalized with Bmi-1 or p16 shRNA plus hTERT could be clearly useful for studying multistep carcinogenesis for human malignancies.

Acknowledgments

We would like to express our sincere thanks to Takako Ishiyama for providing expert technical assistance. This work was supported in part by the Grant-in-Aid for Cancer Research from the Ministry of Health Labor and Welfare, and the Grant-in-Aid for Scientific Research on the Priority Area 'Cancer' from the Ministry of Education, Culture, Sports, Science, and Technology of Japan to T. K.

References

1 Hayflick L, Moohead PS. The limited *in vitro* lifetime of human diploid cell strains. *Exp Cell Res* 1965; **37**: 614–36.
2 Campisi J. Cellular senescence as a tumor-suppressor mechanism. *Trends Cell Biol* 2001; **11**: S27–31.
3 Bodnar AG, Ouellette M, Frolkis M *et al*. Extension of life-span by introduction of telomerase into normal human cells. *Science* 1998; **279**: 349–52.
4 Kiyono T, Foster SA, Koop JI, McDougall JK, Galloway DA, Klingelhutz AJ. Both Rb/p16INK4a inactivation and telomerase activity are required to immortalize human epithelial cells. *Nature* 1998; **396**: 84–8.
5 Jarrard DF, Sarkar S, Shi Y *et al*. p16/pRb pathway alterations are required for bypassing senescence in human prostate epithelial cells. *Cancer Res* 1999; **59**: 2957–64.
6 Itahana K, Zou Y, Itahana Y *et al*. Control of the replicative life span of human fibroblasts by p16 and the polycomb protein Bmi-1. *Mol Cell Biol* 2003; **23**: 389–401.
7 Foster SA, Galloway DA. Human papillomavirus type 16, E7 alleviates a proliferation block in early passage human mammary epithelial cells. *Oncogene* 1996; **12**: 1773–9.
8 Foster SA, Wong DJ, Barrett MT, Galloway DA. Inactivation of p16 in human mammary epithelial cells by CpG island methylation. *Mol Cell Biol* 1998; **18**: 1793–801.
9 Shay JW, Wright WE, Brasiskyte D, Van der Haegen BA. E6 of human papillomavirus type 16 can overcome the M1 stage of immortalization in human mammary epithelial cells but not in human fibroblasts. *Oncogene* 1993; **8**: 1407–13.
10 Romanov SR, Kozakiewicz BK, Holst CR, Stampfer MR, Haupt LM, Tlsty TD. Normal human mammary epithelial cells spontaneously escape senescence and acquire genomic changes. *Nature* 2001; **409**: 633–7.
11 Wong DJ, Foster SA. Galloway DA, Reid BJ. Progressive region-specific *de novo* methylation of the p16 CpG island in primary human mammary epithelial cell strains during escape from M(0) growth arrest. *Mol Cell Biol* 1999; **19**: 5642–51.
12 Wang J, Xie LY, Allan S, Beach D, Hannon GJ. Myc activates telomerase. *Genes Dev* 1998; **12**: 1769–74.
13 van Lohuizen M, Verbeek S, Scheijen B, Wientjens E, van der Gulden H, Berns A. Identification of cooperating oncogenes in E mu-myc transgenic mice by provirus tagging. *Cell* 1991; **65**: 737–52.
14 Jacobs JJ, Scheijen B, Voncken JW, Kieboom K, Berns A, van Lohuizen M. Bmi-1 collaborates with c-Myc in tumorigenesis by inhibiting c-Myc-induced apoptosis via INK4a/ARF. *Genes Dev* 1999; **13**: 2678–90.
15 Saito M, Handa K, Kiyono T *et al*. Immortalization of cementoblast progenitor cells with Bmi-1 and TERT. *J Bone Miner Res* 2005; **20**: 50–7.
16 Cudre-Mauroux C, Occhiodoro T, Konig S, Salmon P, Bernheim L, Trono D. Lentivector-mediated transfer of Bmi-1 and telomerase in muscle satellite cells yields a duchenne myoblast cell line with long-term genotypic and phenotypic stability. *Hum Gene Ther* 2003; **14**: 1525–33.
17 Dimri GP, Martinez JL, Jacobs JJ *et al*. The Bmi-1 oncogene induces telomerase activity and immortalizes human mammary epithelial cells. *Cancer Res* 2002; **62**: 4736–45.
18 Band V, Sager R. Distinctive traits of normal and tumor-derived human mammary epithelial cells expressed in a medium that supports long-term growth of both cell types. *Proc Natl Acad Sci USA* 1989; **86**: 1249–53.
19 Kyo S, Nakamura M, Kiyono T *et al*. Successful immortalization of endometrial glandular cells with normal structural and functional characteristics. *Am J Pathol* 2003; **163**: 2259–69.
20 Takeda Y, Mori T, Imabayashi H *et al*. Can the life span of human marrow stromal cells be prolonged by bmi-1, E6, E7, and/or telomerase without affecting cardiomyogenic differentiation? *J Gene Med* 2004; **6**: 833–45.
21 Sawada M, Kiyono T, Nakashima S *et al*. Molecular mechanisms of TNF-α-induced ceramide formation in human glioma cells: p53-mediated oxidant stress-dependent and -independent pathways. *Cell Death Differ* 2004; **11**: 997–1008.
22 Naviaux RK, Costanzi E, Haas M, Verma IM. The pCL vector system: rapid production of helper-free, high-titer, recombinant retroviruses. *J Virol* 1996; **70**: 5701–5.
23 Mori T, Kiyono T, Imabayashi H *et al*. Combination of hTERT and bmi-1, E6, or E7 induces prolongation of the life span of bone marrow stromal cells from an elderly donor without affecting their neurogenic potential. *Mol Cell Biol* 2005; **25**: 5183–95.
24 Herman JG, Graff JR, Myohanen S, Nelkin BD, Baylin SB. Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. *Proc Natl Acad Sci USA* 1996; **93**: 9821–6.
25 Schrock E, Veldman T, Padilla-Nash H *et al*. Spectral karyotyping refines cytogenetic diagnostics of constitutional chromosomal abnormalities. *Hum Genet* 1997; **101**: 255–62.
26 Jacobs JJ, Kieboom K, Marino S, DePinho RA, van Lohuizen M. The oncogene and Polycomb-group gene bmi-1 regulates cell proliferation and senescence through the ink4a locus. *Nature* 1999; **397**: 164–8.
27 Gil J, Bernard D, Martinez D, Beach D. Polycomb CBX7 has a unifying role in cellular lifespan. *Nat Cell Biol* 2004; **6**: 67–72.
28 Cohen KJ, Hanna JS, Prescott JE, Dang CV. Transformation by the Bmi-1 oncoprotein correlates with its subnuclear localization but not its transcriptional suppression activity. *Mol Cell Biol* 1996; **16**: 5527–35.
29 Rheinwald JG, Hahn WC, Ramsey MR *et al*. A two-stage, p16 (INK4A)- and p53-dependent keratinocyte senescence mechanism that limits replicative potential independent of telomere status. *Mol Cell Biol* 2002; **22**: 5157–72.

30 Benanti JA, Galloway DA. Normal human fibroblasts are resistant to RAS-induced senescence. *Mol Cell Biol* 2004; **24**: 2842–52.
31 Benanti JA, Williams DK, Robinson KL, Ozer HL, Galloway DA. Induction of extracellular matrix-remodeling genes by the senescence-associated protein APA-1. *Mol Cell Biol* 2002; **22**: 7385–97.
32 Herbert BS, Wright WE, Shay JW. p16 (INK4a) inactivation is not required to immortalize human mammary epithelial cells. *Oncogene* 2002; **21**: 7897–900.
33 Ramirez RD, Morales CP, Herbert BS *et al*. Putative telomere-independent mechanisms of replicative aging reflect inadequate growth conditions. *Genes Dev* 2001; **15**: 398–403.
34 Gil J, Kerai P, Lieonart M *et al*. Immortalization of primary human prostate epithelial cells by c-Myc. *Cancer Res* 2005; **65**: 2179–85.
35 Hashida T, Yasumoto S. Induction of chromosome abnormalities in mouse and human epidermal keratinocytes by the human papillomavirus type 16, E7 oncogene. *J General Virol* 1991; **72**: 1569–77.
36 Duensing S, Lee LY, Duensing A *et al*. The human papillomavirus type 16, E6 and E7 oncoproteins cooperate to induce mitotic defects and genomic instability by uncoupling centrosome duplication from the cell division cycle. *Proc Natl Acad Sci USA* 2000; **97**: 10 002–7.
37 Darimont C, Zbinden I, Avanti O *et al*. Reconstitution of telomerase activity combined with HPV-E7 expression allow human preadipocytes to preserve their differentiation capacity after immortalization. *Cell Death Differ* 2003; **10**: 1025–31.
38 Maeda T, Tashiro H, Katabuchi H *et al*. Establishment of an immortalised human ovarian surface epithelial cell line without chromosomal instability. *Br J Cancer* 2005; **93**: 116–23.
39 McDermott KM, Zhang J, Holst CR, Kozakiewicz BK, Singla V, Tlsty TD. p16 (INK4a) prevents centrosome dysfunction and genomic instability in primary cells. *PLos Biol* 2006; **4**: e51.
40 Fukasawa K, Choi T, Kuriyama R, Rulong S, Vande Woude GF. Abnormal centrosome amplification in the absence of p53. *Science* 1996; **271**: 1744–7.
41 Thomas JT, Laimins LA. Human papillomavirus oncoproteins E6 and E7 independently abrogate the mitotic spindle checkpoint. *J Virol* 1998; **72**: 1131–7.
42 Nielsen GP, Stemmer-Rachamimov AO, Shaw J, Roy JE, Koh J, Louis DN. Immunohistochemical survey of p16INK4A expression in normal human adult and infant tissues. *Lab Invest* 1999; **79**: 1137–43.
43 Park IK, Qian D, Kiel M *et al*. Bmi-1 is required for maintenance of adult self-renewing haematopoietic stem cells. *Nature* 2003; **423**: 302–5.
44 Leung C, Lingbeek M, Shakhova O *et al*. Bmi1 is essential for cerebellar development and is overexpressed in human medulloblastomas. *Nature* 2004; **428**: 337–41.
45 Ishikawa F. Cellular senescence, an unpopular yet trustworthy tumor suppressor mechanism. *Cancer Sci* 2003; **94**: 944–7.
46 Natarajan E, Omobono JD 2nd, Jones JC, Rheinwald JG. Co-expression of p16INK4A and laminin 5 by keratinocytes: a wound-healing response coupling hypermotility with growth arrest that goes awry during epithelial neoplastic progression. *J Invest Dermatol Symp Proc* 2005; **10**: 72–85.
47 Kim JH, Yoon SY, Jeong SH *et al*. Overexpression of Bmi-1 oncoprotein correlates with axillary lymph node metastases in invasive ductal breast cancer. *Breast* 2004; **13**: 383–8.
48 Kim JH, Yoon SY, Kim CN *et al*. The Bmi-1 oncoprotein is overexpressed in human colorectal cancer and correlates with the reduced p16INK4a/p14ARF proteins. *Cancer Lett* 2004; **203**: 217–24.
49 Lessard J, Sauvageau G. Bmi-1 determines the proliferative capacity of normal and leukaemic stem cells. *Nature* 2003; **423**: 255–60.
50 Vonlanthen S, Heighway J, Altermatt HJ *et al*. The bmi-1 oncoprotein is differentially expressed in non-small cell lung cancer and correlates with INK4A-ARF locus expression. *Br J Cancer* 2001; **84**: 1372–6.
51 Bea S, Tort F, Pinyol M *et al*. *BMI-1* gene amplification and overexpression in hematological malignancies occur mainly in mantle cell lymphomas. *Cancer Res* 2001; **61**: 2409–12.
52 Raaphorst FM, van Kemenade FJ, Blokzijl T *et al*. Coexpression of BMI-1 and EZH2 polycomb group genes in Reed–Sternberg cells of Hodgkin's disease. *Am J Pathol* 2000; **157**: 709–15.

Supplementary Material

The following supplementary material is available for this article:

Table S1. Counting chromosomes of each transduced primary HMECs by G-banding analysis.

Table S2. Karyotype analysis of each transduced primary HMECs by G-banding.

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doi: 10.1111/j.1349-7006.2006.00373.x