Endothelial cell dysfunction and cytoskeletal changes associated with repression of p16\(^{\text{INK4a}}\) during immortalization

C-Y Kan\(^{1,4}\), VW Wen\(^{1,4}\), E Pasquier\(^2\), K Jankowski\(^1\), M Chang\(^2\), LA Richards\(^1\), M Kavallaris\(^{2,3}\) and KL MacKenzie\(^1\)

The immortalization process is a fundamental step in the development of most (if not all) human cancers, including the aggressive endothelial cell (EC)-derived malignancy angiosarcoma. Inactivation of the tumor suppressor p16\(^{\text{INK4a}}\) and the development of multiple chromosomal abnormalities are features of angiosarcoma that are recapitulated during telomerase-mediated immortalization of human ECs in vitro. The present study used a panel of telomerase-immortalized bone marrow EC (BMEC) lines to define the consequences of inactivation of p16\(^{\text{INK4a}}\) on EC function and to identify molecular changes associated with repression of p16\(^{\text{INK4a}}\). In a comparison of two immortalized BMEC mass cultures and six clones, the cell lines that repressed p16\(^{\text{INK4a}}\) showed a higher rate of proliferation and an impaired ability to undergo morphogenic differentiation and form vessel-like structures in vitro. Proteomic comparison of a p16\(^{\text{INK4a}}\)-negative and a p16\(^{\text{INK4a}}\)-positive BMEC mass culture at early- and late-passage time points following transduction with telomerase reverse transcriptase (hTERT) revealed altered expression of cytoskeletal proteins, including vimentin and α-tropomyosin (αTm), in the immortal cells. Immunoblot analyses of a panel of 11 immortal clones showed that cells that lacked p16\(^{\text{INK4a}}\) expression tended to accumulate more dramatic changes in these cytoskeletal proteins than cells that retained p16\(^{\text{INK4a}}\) expression. This corresponded with aberrant cytoskeletal architectures among p16\(^{\text{INK4a}}\)-negative clones, which featured thicker actin stress fibers and less fluid membrane ruffles than p16\(^{\text{INK4a}}\)-positive cells. A direct link between p16\(^{\text{INK4a}}\) repression and defective EC function was confirmed by analysis of normal cells transfected with small interfering RNA (siRNA) targeting p16\(^{\text{INK4a}}\). siRNA-mediated repression of p16\(^{\text{INK4a}}\) significantly impaired random motility and vessel formation in vitro. This report is the first to demonstrate that ECs that repress the expression of p16\(^{\text{INK4a}}\) are prone to defects in motility, morphogenesis and cytoskeletal organization. These defects are likely to reflect alterations that occur during the development of EC-derived malignancies.

Oncogene (2012) 31, 4815–4827; doi:10.1038/onc.2011.645; published online 6 February 2012

Keywords: endothelial cell; p16\(^{\text{INK4a}}\); TERT; immortalization; cytoskeleton; morphogenesis

INTRODUCTION

Endothelial cell (EC)-derived neoplasias range from benign hemangiomas and intermediate-grade Kaposi’s sarcoma to aggressive angiosarcomas, which respond poorly to current treatments.\(^1,2\) These malignancies are characterized by EC hyperproliferation and development of tumor masses composed of poorly formed vascular structures. Silencing of the gene encoding p16\(^{\text{INK4a}}\) (CDKN2A) by promoter methylation or deletion has been identified as one of the most commonly occurring molecular aberrations in angiosarcoma, affecting approximately 70% of cases.\(^3,4\) p16\(^{\text{INK4a}}\) is expressed at low levels in normal ECs and accumulates to very high levels during senescence.\(^5\) It functions as a cyclin-dependent kinase inhibitor that enforces cell-cycle arrest through activation of the retinoblastoma tumor-suppressor protein (pRB).\(^6\) It is broadly accepted that repression of the CDKN2A gene contributes to tumorigenesis by enabling cell-cycle progression and promoting excessive proliferation. However, there is also evidence indicating that inactivation of p16\(^{\text{INK4a}}\) and/or deregulation of pRB contributes to other aspects of the malignant phenotype, such as impaired differentiation, defective mitosis and development of chromosomal abnormalities.\(^7-10\)

Cellular immortalization is a fundamental step in the development of most, if not all, cancers.\(^11\) In 80–90% of human cancers, cellular immortality is underpinned by activation of the telomere maintenance enzyme telomerase. In contrast to immortal cancer cells, most normal somatic cells, including mature ECs, express very little telomerase and have a limited replicative lifespan as a result of progressive telomere shortening.\(^12-15\) Critically short telomeres activate a TP53-mediated DNA damage signal, which together with concurrent upregulation of p16\(^{\text{INK4a}}\), induces cell-cycle arrest and senescence.\(^16-19\) However, in cells that have silenced p16\(^{\text{INK4a}}\), cell-cycle arrest may be compromised, resulting in outgrowth of cells with dysfunctional telomeres that promote the development of karyotypic abnormalities.\(^20-22\)

Reconstitution of telomerase activity by constitutive expression of telomerase reverse transcriptase (hTERT) enables normal human ECs to proliferate beyond senescence.\(^23\) However, reactivation of telomerase does not appear to be sufficient for immortalization of certain types of ECs.\(^13,24\) Our past studies...
have shown that hTERT-transduced, bone marrow-derived microvascular ECs (BMECs) were immortalized with low efficiency and/or following a lag period (crisis), whereas the combination of hTERT and SV40T early region, which disables both the p16\(^{N\text{KDA}}\)/pRB and p53 tumor-suppressor pathways, very efficiently immortalized both BMECs and mammary ECs.\(^{13,25}\) Many of the BMEC clones that were transduced with hTERT alone and were eventually immortalized were shown to have spontaneously inactivated p16\(^{N\text{KDA}}\) by methylation of CpG islands.\(^{27}\) Complex karyotypes developed in the clones that silenced p16\(^{N\text{KDA}}\) during immortalization as an apparent consequence of telomere dysfunction. It was notable that the karyotypic abnormalities frequently involved loci that are rearranged in angiosarcoma.\(^{4,25-31}\) Karyotypic complexity, together with observations of frequent CDKN2A promoter methylation in hTERT-immortalized BMECs, highlight the relevance of this immortalization model to the development of EC malignancies.

The present study used a panel of hTERT-immortalized BMECs to investigate the way repression of p16\(^{N\text{KDA}}\) may contribute to EC dysfunction in vascular neoplasias. The results revealed altered expression of cytoskeletal proteins, morphologic changes, impaired motility and defective vessel formation in association with suppression of p16\(^{N\text{KDA}}\) in human BMECs.

RESULTS

Increased rate of proliferation associated with repression of p16\(^{N\text{KDA}}\) during BMEC immortalization

Immortalization of the hTERT-transduced BMEC (BMhTERT) lines used in this study was described previously.\(^{13,25}\) Among this panel of cell lines, expression of p16\(^{N\text{KDA}}\) mRNA and protein was repressed owing to methylation of the CDKN2A promoter, or genetic deletion in 1 of 2 mass cultures and 5 out of 12 BMhTERT cultures\(^{25}\) (Figure 1). Irrespective of the status of p16\(^{N\text{KDA}}\) expression, immortalized BMhTERT cells were not capable of anchorage-independent growth and did not form tumors when \(\sim 10 \times 10^6\) cells were injected into the hind flank of Balb/c Nude mice.\(^{13}\) The present study therefore examined other aspects of EC function that may be altered in association with loss of p16\(^{N\text{KDA}}\) expression and that may less overtly contribute to a neoplastic phenotype.

Over the course of the immortalization process there was no overall pattern of proliferation that distinguished the p16\(^{N\text{KDA}}\)-positive and p16\(^{N\text{KDA}}\)-negative populations (examples provided in Figure 2a). However, among the populations that surpassed 75 population doublings (PDs), which represents greater than twofold lifespan extension, the rate of proliferation of clones that repressed p16\(^{N\text{KDA}}\) (0.69 \(\pm\) 0.04 PDs per day) was significantly higher than that of clones that continued to express p16\(^{N\text{KDA}}\) (0.46 \(\pm\) 0.04 PDs per day) \((P < 0.0005,\) unpaired Student's t-test) (Figure 2b). The greater proliferative rate of the p16\(^{N\text{KDA}}\)-negative clones was also reflected by higher mitochondrial activity, as determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Figure 2c). Thus, although loss of p16\(^{N\text{KDA}}\) expression was not associated with a particular growth pattern during immortalization, it did correlate with an increased rate of proliferation in some immortalized populations.

The MTT assay was also used to compare the proliferation of p16\(^{N\text{KDA}}\)-positive and p16\(^{N\text{KDA}}\)-negative BMhTERT cells in the absence of recombinant human fibroblast growth factor-\(\beta\) and vascular EC growth factor (VEGF), with or without 5% bovine serum (Figure 2d). In serum-supplemented medium, withdrawal of human fibroblast growth factor-\(\beta\) and VEGF had very little impact on normal BMECs or BMhTERT clones. However, serum starvation resulted in significantly lower MTT values for each of the three p16\(^{N\text{KDA}}\)-positive and p16\(^{N\text{KDA}}\)-negative BMhTERT cell lines that were assayed, as well as normal BMECs. Overall the data indicated no apparent impact of p16\(^{N\text{KDA}}\) silencing on the mitogenic requirements of BMhTERT cells.

Defective vessel formation by p16\(^{N\text{KDA}}\)-negative BMhTERT cells

To investigate the impact of p16\(^{N\text{KDA}}\) repression on the unique ability of ECs to undergo morphogenic differentiation and form vessels, parental BMECs and representative p16\(^{N\text{KDA}}\)-positive and p16\(^{N\text{KDA}}\)-negative BMhTERT cell lines were cultured on Matrigel for 6 h. The relatively short duration of this assay was sufficient for control BMECs to undergo morphogenic differentiation and reorganize to form enclosed polygonal structures with anastomosing cords (representing vessel formation), yet was brief enough to be independent of the influence of proliferative rate and increasing cell numbers. In this assay, all four p16\(^{N\text{KDA}}\)-positive BMhTERT immortal cell lines formed vessel-like structures that were morphologically indistinguishable from the structures formed by parental BMECs (Figure 3a). There was no significant difference in the number of enclosed structures generated by immortal BMhTERT cell lines that retained p16\(^{N\text{KDA}}\) expression compared with control BMECs (Figure 3b). By contrast, 3 out of 4 p16\(^{N\text{KDA}}\)-negative BMhTERT cell lines (BMhTERT-2L mass culture, BM2B8 and BM2B12) were ineffective in the vessel formation assay, producing significantly fewer vessels than normal BMECs and congregating as clumps \((P < 0.01\) for each cell line compared with BMECs; Dunnett’s multiple comparison test). One p16\(^{N\text{KDA}}\)-negative BMhTERT clone that retained angiogenic potential, BM2A9, was an exception in this assay (discussed further below).
Figure 2. Immortal BMhTERT cells lacking p16\(^{\text{INK}4a}\) expression have a higher proliferative rate than BMhTERT cells that retained p16\(^{\text{INK}4a}\) expression. (a) Long-term proliferation of BMhTERT-1 mass culture, and representative BMhTERT clones that retained p16\(^{\text{INK}4a}\) expression (BM2A2, BM2A6 and BM2A11) and clones that repressed p16\(^{\text{INK}4a}\) expression during immortalization (BM2A9, BM2B12 and BM2A4).

(b) Average rate of proliferation of normal BMECs, BMhTERT-1 mass culture and representative BMhTERT clones. The values are mean PDs per day ± s.e.m. calculated from at least six time points between 75 and 100 PDs. The P-value was generated by comparison of the p16\(^{\text{INK}4a}\)-positive and p16\(^{\text{INK}4a}\)-negative BMhTERT cultures by unpaired Student’s t-test. (c) BMhTERT cells and BMECs were seeded at 3000 cells per well and mitochondrial activity was measured by MTT assay. Values are means ± s.e.m. normalized to the 24-h time point and reflect the proliferation of each cell line. (d) BMECs and BMhTERT cells were seeded in 96-well plates in full-factor EGMT-2-MV medium, including 5% fetal bovine serum, recombinant human fibroblast growth factor-β and VEGF. Twenty-four hours later, the medium was changed to either fresh full-factor medium (control) or medium with or without recombinant human fibroblast growth factor-β and VEGF. Twenty-four hours later, the medium was changed to either fresh full-factor medium (control) or medium with or without recombinant human fibroblast growth factor-β and VEGF. Means and s.e.m. were calculated from duplicates in three independent experiments. *P < 0.05; **P < 0.01; ***P < 0.001, determined by two-way analysis of variance with comparison to control conditions using Bonferroni post tests.
Molecular comparison of p16\(^{INK4a}\)-positive and p16\(^{INK4a}\)-negative BMhTERT cells

To identify molecular alterations associated with loss of p16\(^{INK4a}\) expression, a four-way comparison of the proteomes of BMhTERT-1 (retained p16\(^{INK4a}\) expression) and BMhTERT-2 (repressed p16\(^{INK4a}\)) mass cultures at early and late stages of the immortalization process were performed using two-dimensional difference in gel electrophoresis (2D DIGE). Protein samples labeled with Cy3- and Cy5-fluorescent dyes, and mixed with a Cy2-labeled internal standard, were separated in six 2D-PAGE gels (Table 1, and Figures 4a and b). Protein spots with differential abundance of at least 1.6-fold were organized into a Venn diagram (Figure 4c).

Substantially more proteins were found to be differentially expressed during immortalization of the BMhTERT-2 culture, which repressed p16\(^{INK4a}\), compared with the BMhTERT-1 culture that retained p16\(^{INK4a}\) expression. Whereas 43 proteins were differentially expressed in late- versus early-passage BMhTERT-2 cells (BMhTERT-2E versus BMhTERT-2L; Figure 4c, yellow circle), only seven differentially expressed proteins were identified in the comparison of early- and late-passage BMhTERT-1 cells (BMhTERT-1E versus BMhTERT-1L; Figure 4c, red circle). Thirty-two of the 43 proteins that were altered during immortalization of the BMhTERT-2 culture, which repressed p16\(^{INK4a}\), were differentially expressed in late- versus early-passage BMhTERT-2 cells (BMhTERT-2E versus BMhTERT-2L; Figure 4c, yellow circle), only seven differentially expressed proteins were identified in the comparison of early- and late-passage BMhTERT-1 cells (BMhTERT-1E versus BMhTERT-1L; Figure 4c, red circle). Thirty-two of the 43 proteins that were altered during immortalization of the BMhTERT-2 mass culture (BMhTERT-2E versus BMhTERT-2L) also differed in the comparison of the two immortal mass cultures (BMhTERT-1L versus BMhTERT-2L) (Figure 4c, intersection of blue and yellow circle), indicating a specific association between these 32 proteins and immortalization of the BMhTERT-2 culture, which repressed p16\(^{INK4a}\) expression.

After staining with SYPRO Ruby (Figure 4b), proteins of interest were excised from gels under ultraviolet light and analyzed by matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS). Among the 32 proteins that were exclusively altered with immortalization of BMhTERT-2 and repression of p16\(^{INK4a}\), 10 were successfully identified by MALDI-MS (Table 2). These included four cytoskeletal proteins (vimentin #413, macrophage capping protein #599, \(\alpha\)-tropomyosin (\(\alpha\)Tm) #732 and myosin regulatory light chain 2 #1064), two metabolic proteins (L-lactate dehydrogenase B chain and glutathione-S-transferase P (GSTPi)) and four additional proteins with various known and unknown functions (hematopoietic lineage cell-specific protein (HCLS1), testis-specific Y-encoded protein 1 (TSPY1), charged multivesicular body protein 4b and Annexin A4).

Three protein alterations were identified that were commonly altered during immortalization of both BMhTERT-1 and BMhTERT-2 mass cultures (Figure 4c, intersection of red and yellow circles).
Figure 4. Proteomic alterations during immortalization of BMhTERT cells. Protein lysates from early- and late-passage BMhTERT-1 and BMhTERT-2 cultures were labeled with Cy3 and Cy5 fluorescent dyes. Combinations of the labeled samples, and a Cy2-labeled internal control, were run on six 2D DIGE gels (Table 1). (a) A representative 2D DIGE gel (gel #4 in Table 1) showing total cellular proteins in BMhTERT-1L and BMhTERT-2L labeled with Cy5 and Cy3 fluorescent dyes, respectively, and an internal standard labeled with Cy2. Labeled protein spots were detected using the Typhoon imager. (b) A representative 2D DIGE gel (gel #4) stained with SYPRO Ruby showing total cellular protein from late-passage BMhTERT-1 and BMhTERT-2 mass cultures, and an internal control. Protein spots of interest (boxed) were excised from SYPRO Ruby-stained gels and identified by MALDI-MS. Protein size estimates in kDa are indicated on the right of the gel. (c) A Venn diagram highlighting proteomic changes that occurred during immortalization of the BMhTERT-1 and BMhTERT-2 mass cultures. Statistical analysis of differential protein abundance was calculated using the DeCyder 2D software V4.0, using the Cy2-labeled reference sample as a normalization control. The diagram shows identification numbers of proteins with an abundance of more than 1.6-fold compared with at least one of the other samples. The colored circles delineate the sample comparisons indicated in the legend.
However these proteins could not be identified by MS, presumably owing to their low abundance. In addition to these three proteins, a vimentin isoform (#502) also appeared to be downregulated in both BMhTERT-1L and BMhTERT-2L, although the values did not reach statistical significance (Table 2). The other vimentin isoform identified in these analyses (#413), which had a molecular mass approximating that of the most studied form of vimentin (57 kDa), was of relatively low abundance in the

| Spot ID | Gene name | Protein name | Sequence coverage (%) | MW (kDa) | P | Primary accession number | BMhTERT-1 | BMhTERT-2 | Functional classification | Locus |
|---------|-----------|--------------|-----------------------|----------|---|--------------------------|-----------|-----------|---------------------------|-------|
| 922 UCHL1 | Ubiquitin carboxy-terminal hydrolase isoenzyme L1 | 56 | 25 | 5.3 | 0.095 | 0.075 | 0.16 | 0.18 | Protein degradation | 4p14 |
| 1211 TUBB6 | Tubulin β-6 chain (tentative match) | 13 | 50 | 4.8 | Q9BUFS | -0.26 | -0.25 | 0.08 | 0.5 | Cytoskeleton regulation | 18p11.21 |
| 502 VIM | Vimentin | 73 | 53 | 5.1 | P08670 | 0.11 | -0.19 | 0.13 | -0.04 | Cytoskeleton regulation | 17q12.3 |
| 666 TALDO1 | Transaldolase | 27 | 38 | 6.4 | P37837 | -0.13 | 0.08 | 0.01 | 0.01 | Pentose phosphate pathway enzyme | 11p15.5-p15.4 |
| 835 GSTO1 | Glutathione transferase o-1 | 40 | 28 | 6.2 | P78417 | -0.13 | 0.09 | 0.02 | 0.09 | Cytoskeleton regulation | 10q25.1 |
| 487 EEF1G | Elongation factor-1 γ | 31 | 50 | 6.3 | P26641 | 0.05 | -0.12 | 0.02 | -0.3 | Translation elongation | 11q12.3 |
| *216 HCLS1 | Hematopoietic lineage cell-specific protein | 28 | 54 | 4.7 | P14317 | -0.13 | -0.13 | 0.05 | 0.3 | Signal transduction, gene regulation | 3q13 |
| *413 VIM | Vimentin | 64 | 53 | 5.1 | P08670 | 0.11 | 0.1 | -0.07 | -0.34 | Cytoskeleton regulation | 17q12.3 |
| *599 CAPG | Macrophage capping protein | 45 | 38 | 5.9 | P40121 | -0.05 | 0.08 | 0.08 | -0.22 | Cytoskeleton regulation | 2p11.2 |
| *631 TSPY1 | Testis-specific Y-encoded protein 1 | 30 | 35 | 5.2 | Q01534 | -0.15 | -0.07 | -0.15 | 0.36 | Unknown | Yp11.2 |
| *688 LDHB | L-lactate dehydrogenase B chain | 44 | 36 | 5.7 | P07195 | 0.05 | 0.05 | 0.03 | -0.15 | Metabolism | 12q12.2-p12.1 |
| *694 CHMP4B | Charged multivesicular body protein 4b | 35 | 25 | 4.8 | Q9H444 | -0.06 | -0.06 | -0.06 | 0.32 | Protein transport | 20q11.22 |
| *732 TPM1 | Tropomyosin 1 (α) | 41 | 33 | 4.7 | P09493 | -0.17 | -0.31 | -0.27 | 0.6 | Cytoskeleton regulation | 15q22.1 |
| *762 ANXA4 | Annexin-A4 | 61 | 36 | 5.9 | P05952 | -0.04 | 0.04 | 0.04 | -0.18 | Exocytosis regulation | 2p13 |
| *977 GSTP1 | Glutathione-S-transferase P | 56 | 23 | 5.4 | P09211 | -0.01 | -0.01 | -0.01 | 0.21 | Metabolism | 11q13 |
| *1064 MRLC2 | Myosin regulatory light chain 2, non-sarcomeric | 52 | 20 | 4.7 | P19105 | 0.06 | 0 | 0 | 0.27 | Cytoskeleton regulation | 18p11.31 |

Abbreviations: BMhTERT, hTERT-transduced BMEC; MALDI-MS, matrix-assisted laser desorption ionization mass spectrometry. *Spot numbers were generated by the DeCyder 2DDIGE analysis software. **Sequence coverage % is the proportion of amino-acid sequence matched to the indicated protein. ***Theoretical molecular weight (MW) and isoelectric point (PI) of the identified protein in the database. ****SwissProt accession number, accessible at www.expasy.org. **Protein abundance value was determined using the DeCyder image analysis program.
Figure 5. Alterations in vimentin and αTm isoforms, and the altered cell cytoskeleton, of p16<sup>INK4a</sup>-negative immortal BMhTERT cells. (a) Immunoblot of BMECs, early- and late-passage BMhTERT-1 and BMhTERT-2 mass cultures, as well as 11 immortal BMhTERT clones. The top panel shows hybridization with antibodies for vimentin and the bottom panel shows hybridization with an antibody specific for αTm. Actin was used as a loading control for both gels. PDs at the time the cells were assayed are indicated above the gels. (b) Immunofluorescence staining with paxillin antibody for visualization of focal adhesions (green), phalloidin-Alexa 568 (red) for actin and DAPI (blue). The stained cells were photographed using a ×63 objective.

© 2012 Macmillan Publishers Limited
Alterations in vimentin and Tm isoforms in p16INK4a-negative BMhTERT cell lines

Where suitable antibodies were available, immunoblot analysis of 11 BMhTERT clones, as well as early- and late-passage BMhTERT-1 and BMhTERT-2 mass cultures, was performed to validate the 2D DIGE proteomic analysis. Immunoblot analysis of GSTPI and HCLS1 (spot # 216 and 977, respectively) corresponded with the 2D DIGE protein abundance data, showing moderate elevation in late-passage BMhTERT-2 cells relative to early-passage BMhTERT-2 (Supplementary Figure 1 and Table 2). However, whereas 2D DIGE indicated that vimentin approximated 57 kDa, which corresponded with spot #413 on the 2D DIGE gel. However, whereas 2D DIGE indicated that vimentin #413 was downregulated in BMhTERT-2 cells (Table 2), the immunoblot showed downregulation of the 57-kDa species in the BMhTERT-1 mass culture and not in BMhTERT-2. Nevertheless, the overall results for the immunoblot analysis across the panel of cell lines was consistent with the tendency shown by the proteomics for downregulation of vimentin isoforms, particularly lower molecular weight isoforms, in immortal cells relative to early-passage BMhTERT cultures and normal BMECs. In total, at least 8 out of 13 immortal cultures showed lower expression of one or more vimentin isoforms, with vimentin barely detectable in 4/5 p16INK4a-negative cell lines (BM2A9, BM10B1, BM288 and BM2B12) and 2/8 p16INK4a-positive cell lines (BM2A6 and BM10A3) (Figure 5a, upper panel).

Immunoblot analysis of αTm confirmed the 2D DIGE results, showing downregulation of an αTm isoform to very low levels during immortalization of the BMhTERT-1L culture and robust upregulation during immortalization of BMhTERT-2L cells (Figure 5a, lower panel, and Table 2). Consistent with the immortalized BMhTERT-2L cells, two strong bands were evident in the immunoblot of each of the four immortal BMhTERT clones that repressed p16INK4a. The αTm isoform represented by the upper band was strong in only 1 out of 8 BMhTERT cell lines that retained expression of p16INK4a (BM2A7). Together, the immunoblot results for vimentin and αTm highlight the tendency for deregulation of cytoskeletal proteins in p16INK4a-negative cells and are in general concordance with the proteomic findings.

Cytoskeletal changes in immortal BMhTERT cells that repressed p16INK4a

In light of the deregulated expression of cytoskeletal proteins in immortalized BMhTERT cells, immunofluorescence staining was performed to visualize the cytoskeletal architecture and morphology of BMhTERT and BMECs. Phalloidin and paxillin antibodies were used to stain the actin cytoskeleton and focal adhesions, respectively (Figure 5b). Fluorescence microscopy showed no substantial difference in the cytostructure of early-passage BMhTERT-2 cells and immortal p16INK4a-positive BMhTERT-1 cells compared with normal BMECs (Figure 5b, top three panels). These cells all had a fine meshwork of actin fibers, diffusely stained focal adhesions and actin-rich membrane ruffles with a fluid appearance, which was suggestive of motility (Figure 5b). This morphology was also seen in the two p16INK4a-positive clones that were examined by immunofluorescence staining (BM2A7 and BM2A2) (Supplementary Figure 2, top two panels). By contrast, the p16INK4a-negative immortalized BMhTERT-2 cells had a dense network of thick actin cables, prominent focal adhesions and blunt membrane edges (Figure 5b, bottom panel). The morphologic features of immortalized BMhTERT-2 cells are consistent with a more stable cytoskeleton that is less likely to be amenable to motility and the morphogenic changes required for vessel formation, compared with normal BMECs and p16INK4a-positive BMhTERT cells. The p16INK4a-negative BMhTERT clones BM288 and BM2B12 also had thick actin stress fibers relative to normal BMECs (Supplementary Figure 2, bottom two panels, compared with Figure 5b, top panel). Interestingly the BM2A9 cell line, which was the only p16INK4a-negative clone to form vessels in the Matrigel assay (Figure 3), showed a morphology that was more consistent with the p16INK4a-positive cells. BM2A9 cells had fine actin-rich membrane ruffles and relatively few dense stress fibers (Supplementary Figure 2, middle panel). Overall, the results from the immunofluorescence staining demonstrate that immortal BMhTERT cells that continue to express p16INK4a have retained the cytoskeletal features of normal BMECs and early-passage BMhTERT cells, whereas immortal BMhTERT cells that repressed p16INK4a had a propensity to undergo cytoskeletal changes.

siRNA-mediated repression of p16INK4a in normal BMECs impairs cell motility and vessel formation in vitro

To determine whether the impaired capacity of p16INK4a-negative BMhTERT cells to undergo morphogenesis and form vessels was directly linked to repression of p16INK4a, normal BMECs were transiently transfected with small interfering RNA (siRNA) targeting p16INK4a. Repression of p16INK4a in the transfected cells was confirmed by quantitative real-time PCR and immunoblotting (Figures 6a and b). By 24 h after transfection, p16INK4a mRNA levels were depleted to ~10% of the level detected in BMECs transfected with a scrambled siRNA (Sc) (Figure 6a). Protein levels were also very low at the 24-h time point and remained suppressed until 48 h after transfection (Figure 6b). Fluorescence-activated cell sorting analysis confirmed that siRNA-mediated repression of p16INK4a did not alter the expression of VEGF receptors (VEGFR1 and VEGFR2) or the EC adhesion molecules PECAM-1 (CD31) and ICAM-1 (CD54) (Supplementary Table 1).

The ability of the BMECs to form vessels in Matrigel was measured 24 h after siRNA transfection. Microscopic examination and quantitation of the transfected cultures in four independent experiments confirmed that repression of p16INK4a dramatically impaired the ability of BMECs to form vessel-like structures in this assay (Figure 6c). The number of vessels per field was approximately threefold reduced in cultures of p16INK4a siRNA-transfected BMECs when compared with Sc-transfected cultures (P <0.05, paired Student’s t-test) (Figure 6d). Indeed these experiments also revealed a direct correlation between the level of p16INK4a expression and the number vessels formed in this assay (P <0.01, Pearson’s correlation) (Figure 6e). Overall these data confirm that loss of endogenous p16INK4a expression impairs the ability of BMECs to undergo morphogenetic differentiation and form vessels. As the defect in vessel formation by p16INK4a-repressed cells could not be attributed to a decreased rate of proliferation or response to mitogens (Figures 2b–d), we investigated the effects...
of p16<sup>INK4a</sup> repression on random cell motility, a property required by ECs to explore and colonize new areas during the process of angiogenesis. Live-cell imaging over a 6 h period revealed a clear motility defect in BMECs transfected with p16<sup>INK4a</sup> siRNA. The p16<sup>INK4a</sup>-transfected cells covered a distance that was approximately 30% less than the controls in each of three independent
These results indicate that defective vessel formation associated with repression of p16\(^{INK4a}\) was likely due (at least in part) to an impaired ability to move and explore the surrounding environment.

**DISCUSSION**

This report reveals novel functional and molecular alterations associated with repression of p16\(^{INK4a}\) in human ECs. Using a panel of hTERT-immortalized BMECs, it was shown that the majority of the cell lines that spontaneously repressed p16\(^{INK4a}\) were unable to form vessel-like structures in vitro. The causal role of p16\(^{INK4a}\) silencing in this defect was supported by analysis of normal BMECs subject to siRNA-mediated repression of p16\(^{INK4a}\), which confirmed that vessel formation inversely correlated with the level of p16\(^{INK4a}\) expression. Time delay video microscopy revealed that p16\(^{INK4a}\)-siRNA-transfected BMECs also had compromised motility, which would have contributed to the impaired function of these cells in the vessel formation assay. BMhTERT cells that silenced p16\(^{INK4a}\) during immortalization had a propensity to accumulate cytoskeletal alterations that were also likely to impact on vessel formation.

Prior to the present study, it was shown that overexpression of p16\(^{INK4a}\) in human umbilical vein and microvascular ECs decreased proliferation, impaired migration and abrogated vessel formation in vitro.\(^{32}\) The phenotype described in the report by Alhaja et al.\(^{32}\) illustrates the impaired function of ECs undergoing senescence, a process others have shown to be efficiently induced by over-expression of p16\(^{INK4a}\) in human umbilical vein ECs.\(^{33}\) Independent investigators have also demonstrated very high levels of endogenous p16\(^{INK4a}\) and EC dysfunction in ECs subject to stress-induced or replicative senescence.\(^{5,14,34,35}\) As the level of p16\(^{INK4a}\) expressed by p16\(^{INK4a}\)-positive immortal BMhTERT cells approximated that of early-passage BMECs, our data do not conflict with the report by Alhaja et al.\(^{32}\) Instead the current report, taken together with previous studies of EC senescence, highlight the importance of low levels of p16\(^{INK4a}\) for normal EC function and illustrate how either loss of p16\(^{INK4a}\) during carcinogenesis or accumulation of p16\(^{INK4a}\) during replicative aging may result in EC dysfunction. The effect of p16\(^{INK4a}\) expression on EC function is most likely mediated by pRB, which impacts on diverse cell functions by regulating a suite of eight E2F transcriptional repressors and activators, as well as numerous other interacting proteins.\(^{36}\)

As remodeling of the cytoskeleton has a crucial role in EC motility and morphogenic differentiation,\(^{37}\) it was notable that the immortalized BMhTERT-2 cell line, which had repressed p16\(^{INK4a}\) expression and failed to form vessel structures, had deregulated expression of several cytoskeletal proteins, including vimentin and high-molecular-weight αTm isoforms. Vimentin is an intermediate filament that is required for cytoskeletal stability, and has a key role in cell adhesion and motility.\(^{38,39}\) The detection of multiple vimentin variants in this study was consistent with vimentin’s known propensity for post-translational modification and cleavage.\(^{40,41}\) The two isoforms detected by MALDI-MS in the current study were consistent with vimentin proteins detected by 2D proteomic analysis of an irradiated EC line.\(^{42}\) Our proteomic analyses showed that the lower molecular weight isoform (\(\sim 48\) kDa) was reduced during immortalization of both mass cultures, whereas the higher molecular weight isoform (\(\sim 57\) kDa) was less abundant in the mass culture that repressed p16\(^{INK4a}\). The apparent ambiguity between the proteomic and immunoblot analyses was most likely a consequence of the different solutions used to isolate and fractionate proteins in the 2D verses one-dimensional gel formats, and/or a reflection of the epitopes detected by the antibody. Irrespective of these differences, our results revealed a general tendency for downregulation of experiments (Figures 7a and b) \((P < 0.001, \text{paired Student's } t\text{-test})\).
vimentin isoforms in most immortalized BMhTERT cell lines, which is consistent with the maintenance of a 'young' phenotype in TERT-immortalized ECs, relative to the extreme abundance of this protein in senescent cells.43,44 Demonstration of downregulation of vimentin during EC immortalization is also a notable finding in relation to the use of this protein as a diagnostic marker for EC tumors and cancers of unknown primary sites.45

Tm is a highly conserved actin-binding cytoskeletal constituent that functions in the stabilization of stress fibers in non-muscle cells. The zTm gene encodes at least 10 different isoforms that show varied patterns of expression among different tissues and during development.46 To date, very little has been published on the regulation of Tms in ECs, although post-translational modifications have been observed in human umbilical vein ECs subject to oxidative and shear stresses.47,48 The current study demonstrated upregulation of a high-molecular-weight zTm gene product in each of the five p16^INK4a-negative immortal BMhTERT cell lines analyzed in this study. These results are striking in relation to studies that have shown high-molecular-weight Tms have tumor-suppressor properties and are down-regulated in experimentally transformed fibroblasts and various human malignancies.49-54 However, as p16^INK4a-negative BMhTERT cells are non-tumorigenic and retain p53 function,49-54 our results may have more relevance to a study that showed an association between expression of high-molecular-weight Tms and retarded cell motility in cells and non-transformed, immortal breast epithelial cells.55 Also relevant to the current report, previous studies implicated zTm as a mediator of anti-angiogenic signals.56,57 Further studies will be required to fully characterize Tm isoforms expressed in human ECs and to delineate their functions.

BMhTERT cells that silenced p16^INK4a during immortalization tended to have more dramatic changes in cytoskeletal proteins as well as cytoarchitectures that had a more rigid appearance compared with normal BMECs and immortal cells that retained p16^INK4a expression. While each of the p16^INK4a-negative cell lines showed abnormalities in both vimentin and zTm, concurrent alteration of these proteins was not detected in any of the p16^INK4a-positive cell lines, which all retained normal cell morphology. However, the observed alterations in vimentin and zTm did not appear to be directly linked to p16^INK4a expression, as changes in either vimentin or zTm were observed in some p16^INK4a-positive clones and these proteins were not altered by siRNA-mediated downregulation of p16^INK4a in normal BMECs (additional data not shown). Paxillin and phalloidin staining confirmed that there were also no morphologic changes immediately induced by siRNA-mediated repression of p16^INK4a in BMECs (data not shown). One possible explanation for the accumulation of cytoskeletal changes in BMhTERT clones that silenced p16^INK4a by methylation, and the absence of these changes from BMECs transfected with siRNA targeting p16^INK4a, is that some of the cytoskeletal alterations were a consequence the epigenetic event(s) that orchestrated the methylation of the CDKN2A promoter in BMhTERT cells. The in silico analysis of vimentin and zTm expression among the p16^INK4a-negative cells may also reflect a process of selection during immortalization. Based on a previous report that illustrated the importance of an appropriate EC cytoskeletal structure for G1-to-S-phase progression,59 we hypothesize that the compound changes in vimentin and zTm may not be permissive for normal cell-cycle progression in cells with the G1/S checkpoint intact. However, the uncoupling of this checkpoint in p16^INK4a-negative BMhTERT cells may provide a mechanism for the continued proliferation and eventual immortalization of cells that harbored these cytoskeletal changes. Alternatively, as we have shown that repression of p16^INK4a directly impacted on normal BMEC function (motility), it is also possible that the observed alterations in vimentin and/or zTm were compensatory changes required for stability and continued proliferation of BMhTERT cells that lacked expression of p16^INK4a. In summary, this report is the first to demonstrate the essential role of low levels of p16^INK4a in the maintenance of normal EC function and morphology. This study shows that ECs that repress expression of p16^INK4a are prone to defects in motility and morphogenesis, as well as cytoskeletal alterations. As the immortalization process is an essential step in tumorigenesis and p16^INK4a is frequently silenced in angiosarcomas, the results from this study provide valuable insight to mechanisms that may contribute to the pathogenesis of aggressive EC-derived malignancies, which feature methylation and/or deletion of the p16^INK4a locus.54

MATERIALS AND METHODS

Cell culture and siRNA transfections

The establishment and immortalization of BMhTERT clones and mass cultures were described previously.13,25 For all experiments except the growth factor withdrawal study, BMECs and BMhTERT cells were maintained in EC growth medium at 37 °C in a humidified incubator with 5% CO₂.13 For growth factor withdrawal experiments, the cells were grown using the EGMT-2-MV BulletKit (Lonza, Mt Waverly, VIC, Australia). siRNA targeting exon-1 (106-126) of the CDKN2A gene was designed using the HiPerformance siRNA Design Algorithm (licensed by Novartis, Basel, Switzerland and made available by Qiagen, Doncaster, VIC, Australia). The sequence of siRNA targeting p16^INK4a was as follows: sense: 5'-CGUGC CAACGGCCAGCAAAtt-3'; antisense: 5'-UAUUGGGCUUGGUGGCAcgC-3'. The sequence of the control siRNA (Sc) was sense: 5'-CACUGCGGUAUUCGUUGG-3'. For siRNA transfections, 2.0 × 10⁴ BMECs were suspended in 100 µl of Nucleofector solution (Lonza) and transfected with 150 pmole of siRNA (Sigma-Aldrich, St Louis, MO, USA) suspended in Tris-EDTA buffer using program X001 on an Ammax Biosystems Nucleofector II (Lonza). Transfected BMECs were immediately returned to culture in EC growth medium and incubated at 37 °C under humidified air and 5% CO₂.

MTT proliferation assay

Cells were plated at 3000 per 100 µl EC growth medium per well in a 96-well plate and MTT was added 24 h later. The reaction was stopped 4 h later by addition of 10% sodium dodecyl sulfate (Ameresco, Framingham, MA, USA) in 0.01 M HCl. Optical density was measured at 570 nm using an Amersham Biotrak II Plate Reader (GE Healthcare, Piscataway, NJ, USA).

In vitro vessel formation and random motility assays

Matrigel (BD Biosciences, North Ryde, NSW, Australia) was diluted 1:1 in M199 (Life Technologies, Mulan, VIC, Australia; 0.1% fetal calf serum, 20 µg/ml Endothelial Cell Growth Factor (Roche, Mannheim, Germany), 10 mg/ml penicillin (Invitrogen, CA, USA), 10 mg/ml streptomycin (Invitrogen) and 29.2 mg/ml glutamine mix (Invitrogen, Mulgrove, VIC, Australia). The Matrigel solution was dispensed into a 24-well plate and incubated for 1 h at 37 °C. BMECs were seeded to the Matrigel at 7.5 × 10⁴ cells per well and incubated at 37 °C for 6 h. Vessel-like structures (enclosed polygons) were photographed using the ×5 objective of an Axiovert 200M fluorescent microscope coupled to an AxioCamMR3 camera (Carl Zeiss, Munich, Germany). Ten fields of view were photographed for each condition and the number of vessel-like structures was quantified using the Zeiss Axiosvision 4.7 Image Analysis software.

Random motility was assessed 24 h after BMEC transfection by time-lapse microscopy as described previously.25 Briefly, BMECs were seeded at 2 × 10⁴ cells/ml in 3 ml of EC growth medium per well in 0.1% gelatin-coated six-well plates and incubated at 37 °C under 5% CO₂. Images of at least two fields of view were captured every 5 min for 6 h using the microscope and camera setup described above. At least 25 cells per field of view were tracked over 6 h. Cells undergoing division or apoptosis were
excluded from analyses. Cell motility was quantified using the tracking module of the AxioVision 4.7 software. The persistent random-walk model was used to quantify motility of individual cells.61

Quantitative real-time PCR
RNA was extracted using the Qiagen RNaseasy Mini kit (Qiagen), followed by reverse transcription using SuperScript III reverse transcriptase (Invitrogen), according to the manufacturer’s instructions. Real-time reverse transcription–PCR was performed using the iQ SYBR Green Supermix (Bio-Rad, Hercules, CA, USA), using the MyiQ Real Time PCR Machine (Bio-Rad), applying the following cycling conditions: 95 °C for 10 min; followed by 40 cycles of 95 °C for 30 s, 64 °C for 30 s, 72 °C for 1 min; and a final 10-min extension step at 72 °C. The primer sequences were as follows: p16INK4a forward 5'-ATGGAGCCTTCGGCTGACTGGC-3', p16INK4a reverse 5'-CTGCCCA TACATGTACGCTGGA-3'; β2-microglobulin forward 5'-CTCCGCGTACTCT CTTCTTC-3', β2-microglobulin reverse 5'-TACATGTCTCGTATCCACTTAA CTAT-3'.

2D DIGE and MALDI-MS analysis
The methods used for 2D DIGE and identification of differentially expressed proteins by MALDI-MS were described previously.44 Briefly, mixtures of 50 μg of Cy3- and Cy5-labeled cell lysates, plus 50 μg of a Cy2-labeled internal standard (comprised of mixtures of each sample), were run on six 2D DIGE gels (Table 1) using the Ettan IPGphor IEF system (GE Healthcare, Giles, UK), using Immobiline DryStrips (pH 4–7). The first-dimension gels were run at 300 V for 3 h, 100 V for 6 h, 8000 V for 3 h and 8000 V for 4.4 h. The samples were then run in the second dimension on 12.5% polyacrylamide gels using an Ettan DALT six apparatus (GE Healthcare, Hong Kong, China) at 1.5 W. Protein spots were detected using the Typhoon imager (GE Healthcare) and statistical analysis of differential protein abundance was performed using the DeCyder 2D software v4.0, using the Cy2-labeled reference sample used as a normalization control. Protein spots of interest were excised from gels stained with SYPRO Ruby. MALDI-MS analysis was performed using an Applied Biosystems 4700 Proteomics Analyzer at the Australian Proteome Analysis Facility. MS data were exported to the Mascot database search program (Matrix Science Ltd, London, UK) and searched against Homo sapiens entries in the SwissProt database.

Immunoblot analysis
Proteins were extracted in buffer containing 10 mM Tris (pH 8.0), 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40 and 1% protease inhibitor (Sigma-Aldrich) for 30 min, then separated on a NuPAGENovexBlis–Tris 4–12% 1.0 Mini Gel (Invitrogen) and transferred onto a polyvinylidene fluoride membrane (Millipore, Kilsyth, VIC, Australia). The membranes were blocked with 5% skim milk in Tris-buffered saline and hybridized with primary antibodies, followed by a horseradish peroxidase-labeled secondary antibody (GE Healthcare) and visualized using the Pierce ECL Western Blotting Substrate (Thermo-Fisher Scientific, North Ryde, NSW, Australia). The primary antibodies were p16INK4a (BD Biosciences), vinculin (V9; Chemicon, Boronia, VIC, Australia), GSTPI (2G6-F6; Abnova, Taipei, Taiwan), HSC11 (clone 3DS; Abnova Taipeii), αTm exon-9 (provided by Dr Galina Schevzov, University of New South Wales, Australia)13 and β-actin (Sigma-Aldrich).

Immunofluorescence staining
BMECs and BMhTERT cells were seeded at 2.5 × 105 cells/ml on 0.1% gelatin-coated chamber slides. Twenty-four hours later, the cells were stained with paxillin antibody for visualization of focal adhesions (green), phallolidin–Alexa 568 (red) for actin and DAPI (4',6-diamidino-2-phenylindole) (blue). The stained cells were photographed under oil immersion using the ×63 objective of an Axiovert 200M fluorescent microscope coupled to an AxioCamMR3 camera (Carl Zeiss).

CONFLICT OF INTEREST
The authors declare no conflict of interest.

Oncogene (2012) 4815 - 4827

ACKNOWLEDGEMENTS
We thank Dr Galina Schevzov and Professor Peter C Dunn for provision of the Tm antibody and helpful discussion. Children’s Cancer Institute Australia for Medical Research is affiliated with the University of New South Wales and Sydney Children’s Hospital. This research was facilitated by access to the Australian Proteome Analysis Facility established under the Australian Government’s Major National Research Facilities Program. Funding: National Health and Medical Research Council (KLH Career Development Award no. 510378; MK Senior Research Fellowship ID no. 586611), New South Wales Cancer Council (KLH ID no. 07-14) and Cancer Institute New South Wales (KLH Career Development and Support Fellowship ID no. 07-CDF-1/23; EP Early Career Research Fellowship ID no. 07/ECF-1-21).

REFERENCES
1 Bell CD. Endothelial cell tumors. Micros Res Tech 2003; 60: 165 - 170.
2 Young RJ, Brown NJ, Reed MW, Hughes D, Wolf PJ. Angiosarcoma. Lancet Oncol [Research Support, Non-US Gov’t; Review] 2010; 11: 983 – 991.
3 Tannapel A, Weihrauch M, Benicke M, Ullmann D, Hassu W, Wiblitzyk R et al. p16INK4A alterations in primary angiosarcoma of the liver. J Hepatol 2001; 35: 62 – 67.
4 Weihrauch M, Markwart A, Lehnert G, Wittekind C, Wiblitzyk R, Tannapel A. Abnormalities of the ARF-p53 pathway in primary angiosarcomas of the liver. Hum Pathol 2002; 33: 884 – 892.
5 Wagner M, Hampel B, Bernhard D, Mala Z, Zwerschke W, Jansen-Durr P. Replicative senescence of human endothelial cells in vitro involves G0 arrest, polyploidy and senescence-associated-phenotype. Exp Gerontol 2001; 36: 1327 – 1347.
6 Serrano M, Hannon GJ, Beach D. A new regulatory motif in cell-cycle control causing specific inhibition of cyclin D/CDK4. Nature [Research Support, Non-US Gov’t; Research Support, US Gov’t, PHS] 1993; 366: 704 – 707.
7 Spike BT, Drielam A, dibbling BC, Marvin J, Williams BO, Jacks T et al. The Rb tumor suppressor is required for stress erythropoiesis. EMBO J [Research Support, Non-US Gov’t; Research Support, US Gov’t] 2004; 23: 4319 – 4329.
8 Miliyavsky M, Shats I, Chalosty A, Broski R, Buganim Y, Weiss L et al. Inactivation of myocardin and p16 during malignant transformation contributes to differentiation defect. Cancer Cell 2007; 11: 133 – 146.
9 Hernandez E, Nahle Z, Jaskudzisz G, Ocampo-Diaz, Alaminos M, Hennam et al. Rb inactivation promotes genomic instability by uncoupling cell cycle progression from mitotic control. Nature 2004; 430: 797 – 802.
10 Urashima M, Teoh G, Akiyama M, Yuzu Y, Anderson KC, Maekawa K. Restoration of p16INK4A protein induces myogenic differentiation in RD rhabdomyosarcoma cells. Br J Cancer [Research Support, Non-US Gov’t; Review] 1999; 79: 1032 – 1036.
11 Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell [Research Support, NIH, Extramural; Review] 2011; 144: 666 – 674.
12 Hsiou R, Sharma HW, Ramakrishnan S, Kerr E, Narayanan R. Telomerase activity in normal human endothelial cells. Anticancer Res 1997; 17: 827 – 832.
13 MacKenzie KL, Franco S, Naiyer AJ, May C, Sadelain M, Ralfi S et al. Multiple stages of malignant transformation of human endothelial cells modelled by co-expression of telomerase reverse transcriptase, SV40T antigen and oncogenic N-ras. Oncogene 2002; 21: 4200 – 4211.
14 Yang J, Chang E, Cherry AM, Bangs CD, Oei Y, Bodnar A et al. Human endothelial cell life extension by telomerase expression. J Bio Chem [Research Support, Non-US Gov’t; Research Support, US Gov’t, PHS] 1999; 274: 26141 – 26148.
15 Chang E, Harley CB. Telomere length and replicative aging in human vascular tissues. Proc Natl Acad Sci USA 1995; 92: 11190 – 11194.
16 d’Adda di Fagagna F, Reaper PM, Clay-Farace L, Fiegler H, Carr P, Von Zglinicki T et al. A DNA damage checkpoint response in telomere-initiated senescence. Nature 2003; 426: 194 – 198.
17 Alcorta DA, Xiong Y, Phelps D, Hannon G, Beach D, Barrett JC. Involvement of the cyclin-dependent kinase inhibitor p16INK4a in replicative senescence of normal human fibroblasts. Proc Natl Acad Sci USA 1996; 93: 13742 – 13747.
18 Hara E, Smith R, Panzy D, Tahara H, Stone S, Peters G. Regulation of p16CDKN2 expression and its implications for cell immortalization and senescence. Mol Cell Biol 1996; 16: 859 – 867.
19 Jacobs JJ, de Lange T. Significant role for p16INK4a in p53-independent telomere-directed senescence. Curr Biol 2004; 14: 2302 – 2308.
20 Crawford YG, Gauthier ML, Joublé A, Mantel K, Kozakiewicz K, Afshari CA et al. Histologically normal human mammary epithelia with silenced p16INK4a overexpress COX-2, promoting a premalignant program. Cancer Cell [Research Support, Non-US Gov’t; Research Support, US Gov’t, Non-PHS; Research Support, US Gov’t, PHS] 2004; 5: 263 – 273.
21 Romanov SR, Kozakiewicz BK, Holst CR, Stumper MR, Haupt LM, Tlsty TD. Normal human mammary epithelial cells spontaneously escape senescence and acquire genomic changes. Nature 2001; 409: 633 – 637.

© 2012 Macmillan Publishers Limited
Supplementary Information accompanies the paper on the Oncogene website (http://www.nature.com/onc)