Normal human mammary epithelial cells proliferate rapidly in the presence of elevated levels of the tumor suppressors p53 and p21$^{WAF1/CIP1}$

Lily I. Huschtscha[1,3], Jonathan D. Moore[1,3], Jane R. Noble[1,3], Hamish G. Campbell[1,3], Janice A. Royds[2], Antony W. Braithwaite[1,2,3] and Roger R. Reddel[1,3,*]

[1]Children's Medical Research Institute, Westmead, New South Wales 2145, Australia
[2]Department of Pathology, University of Otago, Dunedin, New Zealand
[3]University of Sidney, New South Wales 2006, Australia
*Author for correspondence (rreddel@cmri.usyd.edu.au)

Accepted 12 May 2009
Journal of Cell Science 122, 2989-2995 Published by The Company of Biologists 2009
doi:10.1242/jcs.044107

Summary

In normal cells, p53 protein is maintained at low levels, but the levels increase after stress or inappropriate growth signals to coordinate growth arrest or apoptosis. Human mammary epithelial cells (HMECs) are unusual in that they exhibit two phases of growth. The second growth phase, referred to as post-selection, follows a period of temporary growth arrest and is characterized by the absence of p16^{INK4a} (also known as CDK4I and p16-INK4a) expression. Previously, we observed that post-selection HMECs have elevated levels of p53. Exogenous p16^{INK4a} expression decreased levels of both p53 transcript and protein, and this effect was inhibited by nutlin-3a, indicating that p16^{INK4a} can regulate p53 expression by affecting both *p53* transcription and Mdm2-dependent degradation of p53. The p53 in post-selection HMECs was wild type and, as expected, increased p53 expression was associated with elevated p21$^{WAF1/CIP1}$ and Mdm2 levels; the p53 response to DNA damage seemed normal. Despite elevated levels of wild-type p53 and p21$^{WAF1/CIP1}$, post-selection cells grew more rapidly than their pre-selection HMEC precursors. We found that the post-selection HMECs contain a truncated Mdm2 protein (p60), which presumably lacks the p53 ubiquitylation domain. We propose that the increased levels of p53 in post-selection HMECs are due to the presence of an Mdm2 fragment that binds p53 but does not result in its degradation.

Supplementary material available online at
http://jcs.biologists.org/cgi/content/full/122/16/2989/DC1

Key words: p53, p21$^{WAF1/CIP1}$, Mdm2, Human mammary epithelial cells, Nutlin-3a

Introduction

The tumor-suppressor proteins p16^{INK4a} (also known as CDK4I and p16-INK4a) and p53 play key roles in the control of cellular replicative life span. The retinoblastoma protein (pRB)-p16^{INK4a} and p53 pathways are both activated at senescence and, when cells become immortalized, both pathways are usually inactivated by mutation or viral oncoproteins (reviewed by Huschtscha and Reddel, 1999; Reddel, 2000). p16^{INK4a} is an active inhibitor of cell-cycle progression. It binds and inhibits the activity of cyclin-dependent kinases, Cdk4 and Cdk6, which prevents the hyperphosphorylation of pRB and thus prevents entry into the cell cycle (reviewed by Jimenez et al., 1999; Sharpless, 2005). p53 is a transcription factor that coordinates the cellular response to stress resulting in temporary or permanent (senescence) growth arrest, or apoptosis (reviewed by Braithwaite et al., 2005; Woods and Vousden, 2001). Inevitably, p53 is kept under tight control and at least ten feedback loops (seven negative and three positive) have been described (Harris and Levine, 2005). Of these, six are known to act through the negative regulator of p53, Mdm2, which mediates ubiquitylation and degradation of p53, thus maintaining p53 at low levels in unstressed cells (Moll and Petrenko, 2003; Kubbutat et al., 1997; Honda and Yasuda, 1999). Upon stress, the p53-Mdm2 interaction is disrupted and p53 becomes stabilized. This allows p53 to induce growth arrest by upregulation of p21$^{WAF1/CIP1}$, which inhibits the activity of cyclin-E–Cdk2 and cyclin-A–Cdk2 complexes, both of which would otherwise hyperphosphorylate pRB, leading to activation of E2F-responsive genes and cell-cycle progression. Alternatively, this stabilization of p53 can lead to apoptosis by transactivation of pro-apoptotic genes.

Unlike other human diploid cells, human mammary epithelial cells (HMECs) exhibit two phases of growth when cultured using standard serum-free conditions (Stampfer, 1985). In the first phase of growth, referred to as pre-selection, HMECs grow for five to seven population doublings (PDs), after which they cease growth temporarily. During this period of growth arrest, called 'selection' or 'stasis', colonies of small and rapidly proliferating cells (referred to as post-selection or variant HMECs) appear that can grow for another 40 to 50 PDs, before they eventually become senescent. This extended post-selection phase of rapid growth is associated with hyper-methylation of the *p16^{INK4a}* promoter and loss of p16^{INK4a} expression (Brenner et al., 1998; Foster et al., 1998; Huschtscha et al., 1998), and, paradoxically, with elevated levels of wild-type p53 (Stampfer et al., 2003; Delmolino et al., 1993).

We report here our investigations of the relationship between p53 and p16^{INK4a} in several pre- and post-selection HMEC cultures derived from the same donors. We have confirmed the reciprocal relationship between p16^{INK4a} and p53 levels, and also confirmed the results of a previous study showing that p53 is activated normally in post-selection HMECs in response to genotoxic stress (Zhang et al., 2006). The post-selection cells grew

rapidly in the presence of elevated p53 and $p21^{WAF1/CIP1}$ levels, but did not depend on the elevated level of p53 for their growth. We obtained evidence that $p16^{INK4a}$ can regulate p53 expression both by decreasing transcription of the gene encoding p53 (*TP53*) and by increasing Mdm2-dependent degradation of p53. Most notably, we found that the increased levels of p53 in post-selection cells were associated with an excess of a 60-kDa Mdm2 protein (hereafter referred to as Mdm2 p60) in post-selection HMECs; we propose that this protein can bind p53 but not mediate its degradation by ubiquitylation.

Results

Rapidly dividing post-selection HMECs with undetectable $p16^{INK4}$ have elevated levels of wild-type p53

As previously observed (Stampfer, 1985; Brenner et al., 1998; Foster et al., 1998; Huschtscha et al., 1998), all of the HMEC strains used in this study exhibited two phases of growth in standard serum-free medium (Fig. 1A), with the prolonged post-selection growth phase being associated with undetectable levels of $p16^{INK4a}$ expression (Fig. 1B). The post-selection cells grew more rapidly than their pre-selection counterparts (26 vs 52 hours doubling time for Bre-16; data not shown). This rapid growth is further exemplified by the ability of early-passage post-selection HMECs to complete five PDs within 5 days. The $p16^{INK4a}$-negative post-selection cells had levels of p53 that were two- to six-fold greater than the $p16^{INK4a}$-positive pre-selection HMECs (Fig. 1B). Elevated levels of p53 were consistently observed in matched pre- and post-selection cultures from six individual donors. Moreover, the level of p53 in post-selection HMECs (Bre-16, PD 19) was 10- to 30-fold higher than in skin keratinocytes (Kre-16, PD 3) and dermal fibroblasts (Fre-16s, PD 15) from the same donor (both of which exhibit a single growth phase) (Fig. 2A). As is seen in many early-passage fibroblast cultures, Fre-16s cells at PD 15 (approximately 25% of its total life span) had virtually undetectable $p16^{INK4a}$ expression (Barrett et al., 1994; Alcorta et al., 1996).

Elevated levels of p53, which are found in many human cancers, mostly result from mutations (Hollstein et al., 1991) that interfere with Mdm2-mediated degradation of p53. To determine whether the *p53* gene in post-selection HMECs is mutated or not, we analyzed the sequence of exons 2-11 from four donors: Bre-16, Bre-24, Bre-28 and Bre-80. In each case, the sequence was found to be wild type (data not shown). Therefore, post-selection HMECs are able to proliferate rapidly despite having elevated levels of wild-type p53.

Stability of p53 in post-selection HMECs is due to altered Mdm2 regulation

Next we investigated the mechanism of p53 stability in post-selection HMECs. Zhang et al. suggested that this was due to increased levels of $p14^{ARF}$ (Zhang et al., 2006), which inhibits the ability of Mdm2 to promote p53 proteolysis (Sharpless, 2005), because they found elevated $p14^{ARF}$ mRNA in post-selection HMECs. We examined $p14^{ARF}$ mRNA and protein levels in pre- and post-selection HMECs. We also found elevated levels of $p14^{ARF}$ mRNA of 7- to 15-fold in post-selection HMECs, but this was not reflected in $p14^{ARF}$ protein levels (supplementary material Fig. S1). The ratio of $p14^{ARF}$ protein relative to the actin loading control, and normalized to pre-selection cells (set to a value of 1.0), in this experiment is 1.01 for post-Bre-28 and 0.92 for post-Bre-38. Similar results were obtained in a further three separate experiments. These results confirm our previous findings (Huschtscha et al., 2001) and suggest that the elevated p53 levels in post-selection HMECs cannot be explained by increased levels of $p14^{ARF}$ protein. We therefore studied the role of Mdm2 directly in post-selection HMECs. In the first instance, we monitored the levels of Mdm2 by western blotting in pre- and post-selection HMECs from the Bre-16 donor. Mdm2 levels were increased in post-selection HMECs compared with their pre-selection counterparts by more than threefold (Fig. 2A, compare lanes 1 and 2). Isogenic keratinocytes retained moderate levels of Mdm2, whereas, in skin fibroblasts Mdm2 was undetectable. We also analyzed $p21^{WAF1/CIP1}$ expression levels in these cells. Compared with pre-selection HMECs, the levels of $p21^{WAF1/CIP1}$ were highly elevated in post-selection HMECs (Fig. 2A). Isogenic keratinocytes and skin fibroblasts also exhibited higher levels of $p21^{WAF1/CIP1}$. Therefore, in post-selection HMECs when $p16^{INK4a}$ expression is lost, we observe an increase not only in p53 levels, but also in the expression of the p53 target genes $p21^{WAF1/CIP1}$ and *Mdm2*.

To investigate the role of Mdm2 in regulating p53 levels in post-selection HMECs, we used nutlin-3a, a molecule that specifically disrupts p53 from its complex with Mdm2, thus causing p53 stabilization and activation of p53-responsive genes (Vassilev, 2004). Both pre- and post-selection HMECs were treated with nutlin-3a for 24 hours and the levels of p53 and Mdm2 were

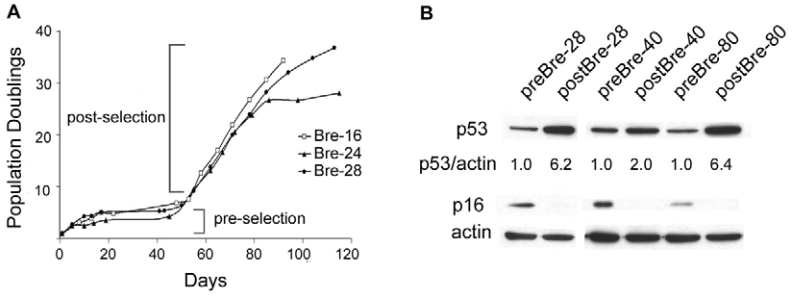

Fig. 1. Inverse pattern of p53 and $p16^{INK4a}$ expression in rapidly proliferating HMECs. (A) Growth properties of HMECs. Growth curves are shown for Bre-16, Bre-24 and Bre-28, and are representative of all the HMEC strains. HMECs grown in serum-free medium exhibited two phases of growth, referred to as pre- and post-selection. The growth of three post-selection HMEC cultures was more rapid than their respective pre-selection counterparts. (B) p53 and $p16^{INK4a}$ expression in HMECs. Pre- and post-selection HMECs (Bre-32 at PD 5 and 41, Bre-40 at PD 5 and 16, and Bre-80 at PD 3 and PD 11) were analyzed for p53 and $p16^{INK4a}$ expression by Western blotting as described in the Materials and Methods. Loss of $p16^{INK4a}$ expression was associated with a two- to six-fold increase in p53 expression. p53 expression levels were measured by densitometry and compared to actin expression. p53 and actin levels were normalized to each pre-selection HMEC strain. The results shown are representative of data from paired pre- and post-selection HMECs obtained from six individuals.

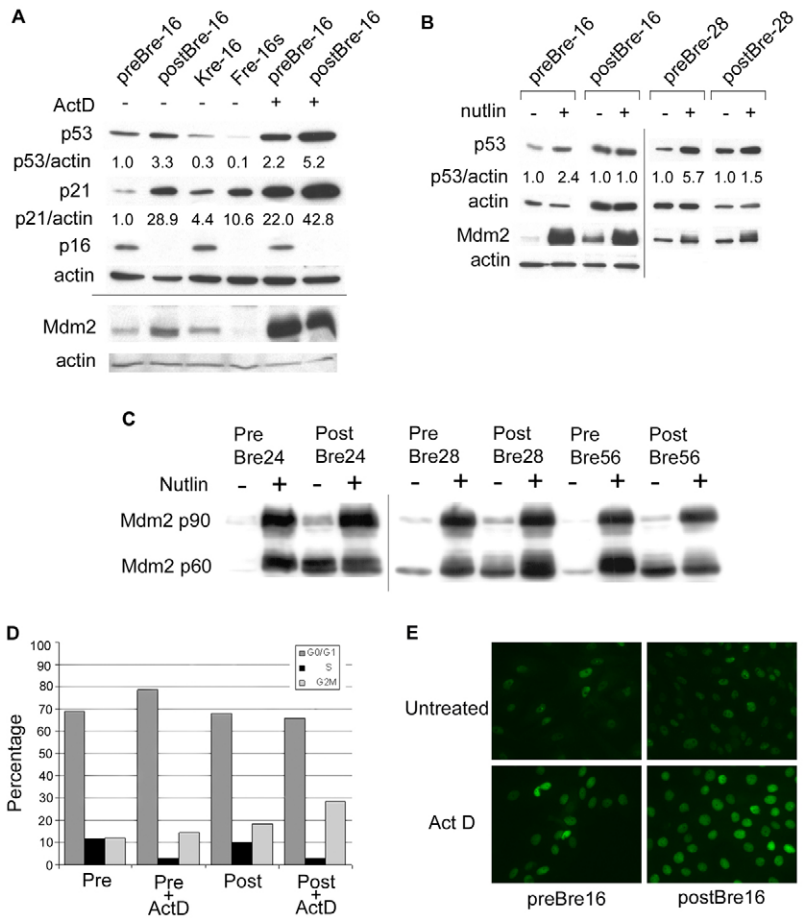

Fig. 2. Post-selection HMECs exhibit normal p53 responses to DNA damage and nutlin-3a exposure. (A) p53 (*TP53*) and p53-target gene expression in different cells. Expression levels of p53, p16^{INK4a}, p21$^{WAF1/CIP1}$ and Mdm2 were compared by western blotting from various cell types obtained from a single donor. HMECs: pre- and post-selection Bre-16 cells (PD 3 and 19, respectively), with (+) and without (–) treatment for 24 hours with 7.5 nM Act-D; skin keratinocytes: Kre-16 (PD 3); and dermal fibroblasts: Fre-16s (PD 15). (B) Effect of nutlin-3a on p53 levels in HMECs. Cells (pre-selection Bre-16, PD 3; post-selection Bre-16, PD 15; and pre-selection Bre-28, PD4, and post-selection Bre-28, PD 14) were treated for 24 hours with 10 μM nutlin-3a. Analysis of p53 levels by western blotting showed that p53 levels increased in pre-selection, but not in post-selection, cells. (C) Pre- and post-selection HMECs from three donors, Bre-24 (PD4 and PD16), Bre-28 (PD4 and PD14) and Bre-56 (PD4 and PD12), were treated with nutlin-3a and the levels of both full-length Mdm2 and Mdm2 p60 were monitored by western blotting using the SMP-14 monoclonal antibody. All post-selection HMECs showed an increase in Mdm2 p60 when compared with their respective pre-selection counterparts. Both forms of Mdm2 were observed to increase after nutlin-3a treatment for all HMEC donors. (D) Cell-cycle arrest after DNA damage in pre-and post-selection Bre-38 HMECs. Flow cytometry for DNA content was carried out before and after treatment with 7.5 nM Act-D for 24 hours. The cell-cycle phases are presented as a histogram. Act-D treatment reduced the proportion of cells in S phase, whereas only the G1 phase was increased in pre-selection HMECs and the G2 phase for post-selection HMECs. (E) Normal DNA-damage response in both pre-and post-selection HMECs. Indirect immunofluorescence was used to visualize the levels of p53 for both pre- and post-selection HMECs treated with Act-D for 24 hours. The levels of p53 were increased after drug treatment for both pre- and post-selection Bre-16 HMECs compared with untreated HMECs. Untreated pre- and post-selection Bre-16 HMECs exhibited heterogeneous expression of p53, but there were considerably more cells expressing high levels of p53 in post-selection Bre-16 HMECs. The same exposure was used for all four images; 400× magnification.

determined by western blotting. Nutlin-3a increased the p53 levels in pre-selection HMECs by two- to five-fold depending on the experiment, but did not alter p53 levels significantly in post-selection cells (Fig. 2B). Similar results were obtained in three separate experiments with paired pre- and post-selection HMEC cultures from two individuals. Mdm2 levels also increased substantially in both pre- and post-selection HMECs treated with nutlin-3a (Fig. 2B). Because nutlin-3a allows p53 to transactivate Mdm2 efficiently in both pre- and post-selection cells, but only stabilizes p53 in pre-selection cells, the data suggest that much of the p53 in post-selection HMECs is not susceptible to Mdm2-mediated degradation, and this, at least in part, might account for the increased levels of p53 in post-selection HMECs.

Previous studies have shown the existence of several Mdm2 protein products, one of which, the p60 fragment, is the predominant species in some tumor lines and in a subset of breast and lung tumors (Pochampally et al., 1998; Pochampally et al., 1999). Because Mdm2 p60 does not retain the C-terminus but can still bind p53 and inhibit its transcriptional activity, Mdm2 p60 could potentially contribute to stabilization of p53 in post-selection HMECs. To explore this possibility, we determined by western blotting the relative levels of both full-length Mdm2 (90 kDa) and Mdm2 p60. Results showed substantially more Mdm2 p60 in post-selection HMECs when compared with their pre-selection counterparts for three donors (Fig. 2C, compare lanes 1 and 3, 5 and 7, and 9 and 11). Nutlin-3a treatment of pre- and post-selection donors increased both forms of Mdm2 – p90 and p60 (Fig. 2C). These results indicate that the increased levels of p53 in post-selection HMECs could be due to an excess of Mdm2 p60.

p53 DNA-damage response is retained in post-selection HMECs

To investigate whether the stable p53 in post-selection HMECs is functional, cells were treated with the DNA-damaging agent actinomycin D (Act-D) for 24 hours. Results for pre-selection cells showed a normal DNA-damage response (Braithwaite et al., 2005; Woods and Vousden, 2001), namely, growth arrest as indicated by a marked decrease of cells in S-phase (Fig. 2D) and upregulation of p53 protein (Fig. 2A), which was accompanied by a very substantial increase in expression of the downstream targets p21$^{WAF1/CIP1}$ and Mdm2 (Fig. 2A, compare lanes 1 and 5). When post-selection HMECs were treated with Act-D, growth arrest also occurred (Fig. 2D), with a further increase in p53 (Fig. 2A, compare lanes 2 and 6) and, again, an increase in p21$^{WAF1/CIP1}$ and Mdm2. These data indicate that p53 in post-selection HMECs is able to maintain a normal damage response by activation of the p53 pathway. Immunofluorescence studies further confirmed that post-selection HMECs are able to initiate a normal DNA-damage response: the levels of p53 in post-selection HMECs were elevated after Act-D treatment in a manner similar to pre-selection HMECs (Fig. 2E). It is noteworthy that the distribution of p53 expression in cells within untreated pre- and post-selection HMEC cultures is heterogeneous, reflecting a pattern observed for wild-type p53 (Lahav et al., 2004). Mutant p53, by contrast,

is distributed evenly throughout the whole cell population (Vojtesek and Lane, 1993).

Proliferation of post-selection HMECs is not dependent on p53

We next investigated whether reduction of p53 protein levels in post-selection HMECs would affect their growth rate. Early-passage post-selection Bre-16 at PD 8 were stably transfected with either a vector control, pMSCV, or a short hairpin (sh)-p53 vector, p1018shp53. After puromycin selection, eight and fifteen clones were isolated for pMSCV and p1018shp53 vectors, respectively. p53 levels were determined for each of these cell clones by western blotting, and each clone showed a different degree of silencing (Fig. 3A). p1018shp53 clones expressing high or low levels of p53 were selected for growth studies. These were clones B, E and G, with p53 expression levels similar to the vector control, pMSCV-C, and clones, A, C and I that had 80-90% reduction of p53 compared with controls (Fig. 3A). However, despite the wide variation in p53 levels, there was no significant difference ($P>0.3$) in growth rates for the clones over 8 days (Fig. 3B) or in PDs completed within a 6-day period (Fig. 3C). No growth studies could be done on the control-vector clones because they all senesced soon after expanding into standard flasks (at 26.4-34.9 PDs). By comparison, the p1018shp53 clones exhibited a longer life span (31.3-44.9 PDs; $P\leq0.00005$), as expected, because loss of p53 is well known to contribute to lifespan extension (Duncan and Reddel, 1997; Braithwaite and Edwards, 2003). These data indicate that the proliferation of post-selection HMECs is neither inhibited by, nor dependent on, the elevated levels of p53.

Forced expression of $p16^{INK4a}$ in post-selection HMECs induces both Mdm2-mediated degradation of p53 and decreased p53 transcription

The data illustrated in Figs 1 and 2 indicate that post-selection HMECs have elevated levels of p53 that is protected from Mdm2-mediated degradation. To examine whether this is causally related to loss of $p16^{INK4a}$ expression, two strains of post-selection HMECs were transfected with a $p16^{INK4a}$ expression plasmid with or without simultaneous nutlin-3a treatment and western blotting was carried out 24 hours later. $p16^{INK4a}$ overexpression was found to lower p53 levels two- to threefold (Fig. 4A), similar to the levels observed in pre-selection cells, and treatment with nutlin-3a prevented this reduction of p53 (Fig. 4A). Therefore, expression of $p16^{INK4a}$ in post-selection cells lowers p53 protein levels because the p53 becomes susceptible to Mdm2-mediated degradation.

In addition to the effect of Mdm2 on p53 stability, the mechanisms that modulate p53 levels also include control of *TP53* gene transcription. We therefore investigated the possibility that $p16^{INK4a}$ might also control p53 levels via an effect on its transcriptional regulation. Quantitative reverse-transcriptase (RT)-PCR was used to measure *TP53* mRNA levels in $p16^{INK4a}$-expressing post-selection HMECs. Post-selection HMECs showed a twofold reduction in *TP53* mRNA after transfection with a $p16^{INK4a}$ expression plasmid when compared with vector controls (Fig. 4B). Together, the data indicate that the reduction of p53 evident in post-selection HMECs after re-expression of $p16^{INK4a}$ is caused by a combination of both transcriptional repression and Mdm2-mediated degradation.

Discussion

In response to stress or inappropriate growth signals, levels of the tumor suppressor p53 protein become elevated owing to stabilization, and this might result in either growth arrest or apoptosis (reviewed by Braithwaite et al., 2005; Woods and Vousden, 2001). In this study, we have shown that post-selection HMECs that have lost expression of $p16^{INK4a}$ display two- to six-fold elevated levels of wild-type p53, in accord with the results of other investigators (Stampfer et al., 2003; Delmolino et al., 1993; Zhang et al., 2006), but proliferate at least twice as fast as their pre-selection precursors. Strikingly, rapidly growing post-selection HMECs harbor levels of p53 that are higher than those observed in growth-arrested pre-selection HMECs treated with a DNA-damaging agent, Act-D (Fig. 2A, compare lanes 2 and 5). Post-selection HMECs also exhibit elevated levels of $p21^{WAF1/CIP1}$ and Mdm2, and are able to mount a normal DNA-damage response as efficiently as pre-selection HMECs: after Act-D treatment, post-selection HMECs were growth arrested, and the levels of p53 and its downstream targets, $p21^{WAF1/CIP1}$ and Mdm2, were further elevated (Fig. 2A,D,E). A normal DNA-damage response was also observed after γ irradiation in post-selection HMECs (Zhang et al., 2006).

On the basis of the observation that proliferation of post-selection HMECs increased following transduction of GSE22, a genetic suppressor element that acts as a p53 dominant-negative (Ossovskaya et al., 1996), it has been suggested that growth of these cells is inhibited by increased p53 activity (Zhang et al., 2006). By contrast, we found that downregulation of p53 in post-selection

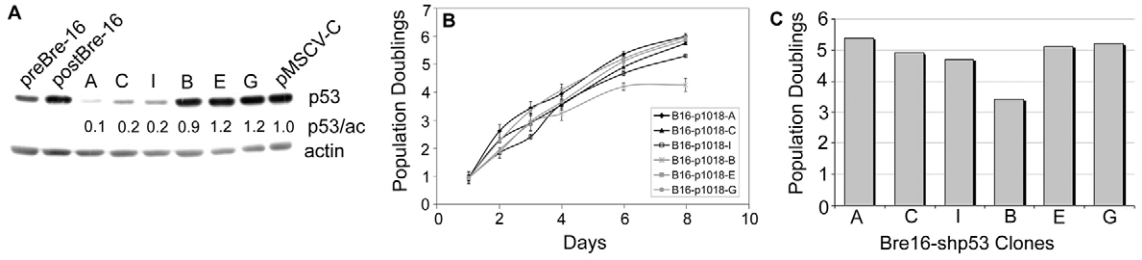

Fig. 3. Reduced levels of p53 do not affect the proliferation rate of post-selection HMECs. Early-passage post-selection HMECs (Bre-16 at PD 8) were transfected with vector-control pMSCV and a sh-p53 vector p1018shp53. Stable clones for each vector were selected from each vector construct and grown in 0.6 μg/ml puromycin to the end of their life span. (A) The levels of p53 were determined in six p1018shp53 clones by western blot analysis. Three low-expressing clones, A, C and I, which had a reduction in p53 levels of about 80-90% were compared with three high-expressing clones, B, E and G, which had levels of p53 similar to the vector control. Pre- and post-selection Bre-16 HMECs and one vector-control plasmid clone pMSCV-C were included as a reference. p53 and actin levels were normalized to the vector-control plasmid clone Bre16 pMSCV-C. (B,C) Cells were seeded and viable-cell counts made over 8 days (results at day 6 are shown in C), and cumulative PDs determined. Error bars denote the standard deviation.

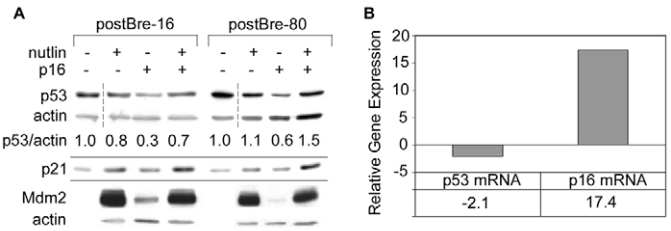

Fig. 4. p16INK4a affects p53 stability and transcription in post-selection HMECs. (A) Post-selection HMECs (Bre-16 PD 13 and Bre-80 PD 14) were treated with nutlin-3a (10 μM) and transfected with p16INK4a expression plasmid or control plasmid. After 24 hours, the levels of p16INK4a, p53, Mdm2 and p21WAF1/CIP1 were determined by western blotting. Data shown are representative of two separate experiments, each with two HMEC strains. Lanes shown for each western blot are from the same autoradiograph of the one gel and the grey dashed lines indicate that an irrelevant lane has been removed to maintain the original sequence. p53 and actin levels were normalized to each post-selection HMEC strain. (B) Post-selection HMECs (Bre-80 PD 21) were transfected with a p16INK4a expression plasmid or control plasmid as in Fig. 2A and, 24 hours later, RNA was isolated for quantitative RT-PCR. A two-step real-time RT-PCR reaction was performed using cDNA reverse transcribed from RNA primed with oligo(dT). Relative gene expression was determined using GAPDH, β2M or HPRT1 as house-keeping-gene controls. The samples were measured on three separate occasions.

HMECs using RNA interference had no statistically significant effect on cell proliferation (Fig. 3B,C), indicating that the rapid growth of post-selection HMECs does not require increased p53. The reasons for the differences are not clear, but it could be that GSE22 does more than just inhibit p53 function, because it also causes a transformed phenotype (Ossovskaya et al., 1996), whereas the shRNA we used is specific to p53.

Several investigators have observed elevated expression of p53 in cells with a disrupted pRB-p16INK4a pathway. Cells exhibit increased p53 expression when the pRB pathway is disrupted by the E7 oncogene of the high-risk human papillomavirus strain HPV-16; these cells proliferate rapidly and have an extended life span (Bischof et al., 2005). In addition, fibroblasts that overexpress the cyclin-dependent kinase Cdk4 recapitulate this phenotype (Ramirez et al., 2003). Finally, elevated levels of wild-type p53 in the absence of p16INK4a expression, which is associated with extended proliferative life span in culture, have been observed in atypical dysplasia of oral epithelium, in contrast to typical dysplasia in which the levels of these tumor-suppressor genes are more normal (Muntoni et al., 2003). Post-selection HMECs display a similar association between disruption of the pRB-p16INK4a pathway, high levels of p53 and increased proliferation. Thus, it would seem that disruptions to this checkpoint pathway in general render cell-proliferation controls refractory to elevated levels of growth suppressors such as p53 and p21WAF1/CIP1.

p16INK4a seems to decrease p53 levels by at least two mechanisms. On the basis of the observation that nutlin-3a, which specifically inhibits the binding of p53 and Mdm2, was able to rescue p16INK4a-induced destabilization of p53 (Fig. 4A), it can be deduced that p16INK4a affects the Mdm2-p53 interaction in post-selection HMECs. Re-introduction of p16INK4a into post-selection HMECs also reduced the level of *TP53* mRNA (Fig. 4B), suggesting that p16INK4a can affect *TP53* transcription as well. Although unusual, it has been shown that p16INK4a can repress transcription of cyclin D1 (D'Amico et al., 2004), thus providing a precedent for the transcriptional component of the mechanism observed here.

It was previously found that silencing of p16INK4a in pre-selection HMECs led to an increase in *p14ARF* mRNA levels (Zhang et al., 2006) and it was therefore suggested that this would result in a reduced ability of Mdm2 to cause proteolytic degradation of p53. We also found that post-selection HMECs have substantially increased *p14ARF* mRNA levels compared with pre-selection cells but, in confirmation of our previous results (Huschtscha et al., 2001), there was no difference in p14ARF protein levels. It therefore seems unlikely that p14ARF plays a substantial role in the stabilization of p53 in post-selection cells. We therefore examined the levels of Mdm2 p60. This form of Mdm2 does not retain the C-terminus, and therefore might be expected to be defective in mediating the ubiquitylation and degradation of p53 (Pochampally et al., 1998; Pochampally et al., 1999), but does retain its ability to bind p53 and inhibit its transcriptional activity. We found the levels of Mdm2 p60 to be markedly elevated in post-selection HMECs (Fig. 2C) compared with their pre-selection counterparts. It will clearly be of interest to determine whether expression of exogenous p16INK4a is able to restore the Mdm2 p60:p90 ratio to that of pre-selection cells. Our preliminary data (not shown) suggest that this is not the case,

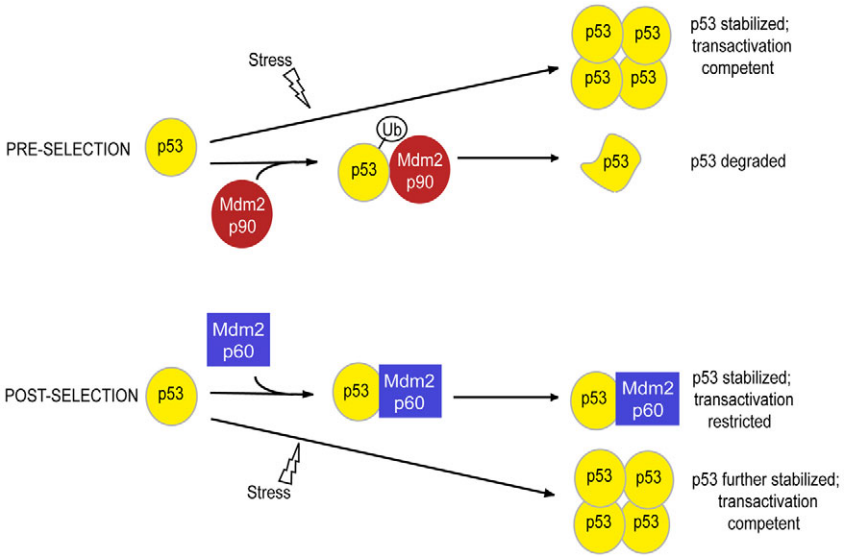

Fig. 5. Model for increased stabilization of p53 in post-selection HMECs. In pre-selection HMECs, the full-length form of Mdm2, p90, is the major species detected, whereas the shorter form, p60, is the predominant species in post-selection HMECs. The p53 levels in pre-selection HMECs are kept low by the interaction between p53 and Mdm2 p90, which results in the ubiquitylation and degradation of p53. In post-selection HMECs, the p53 levels are higher because Mdm2 p60 cannot ubiquitylate p53. However, post-selection HMECs continue to proliferate rapidly in the presence of high levels of p53 because p53–Mdm2-p60 binding partly inhibits p53 transactivation. After stress, the p53 response is normal in both pre- and post-selection HMECs owing to its disassociation from Mdm2 p90 and p60, respectively.

and we note that loss of $p16^{INK4a}$ expression is not the only significant epigenetic change in post-selection cells (Huschtscha et al., 2001; Hinshelwood et al., 2007).

We propose (Fig. 5) that the increased levels of p53 observed in post-selection HMECs are due to Mdm2 p60 being the major form of Mdm2 in these cells, which would permit high levels of wild-type p53 to be present as post-selection HMECs proceed through the cell cycle. This model is compatible with the normal response of p53 to stress that is observed in post-selection HMECs (Fig. 2A,D,E), because the p53–Mdm2-p60 interaction would dissociate upon a damage signal to permit the transcription of the appropriate p53 target genes (Fig. 5). This model is consistent with the many tumor cell lines that retain elevated wild-type p53, including those of the breast and lung, that frequently harbor Mdm2 p60 as the dominant species of Mdm2 (Bueso-Ramos et al., 1996; Gorgoulis et al., 1996; Landers et al., 1997). Our investigations into how p53 is stabilized in post-selection HMECs might well provide an explanation as to how some tumor lines can proliferate rapidly in the presence of high levels of wild-type p53 protein.

Materials and Methods

Cell culture and treatments

HMEC cultures were prepared as described (Huschtscha et al., 1998) from breast tissue removed at reduction mammoplasty with institutional ethics committee approval and informed donor consent. HMEC strains studied were Bre-16, Bre-24, Bre-28, Bre-32, Bre-38, Bre-40, Bre-56 and Bre-80 isolated from donors aged 37, 42, 32, 22, 29, 32, 39 and 36 years, respectively; the Bre-40 and Bre-80 strains have been described previously (Huschtscha et al., 1998). HMECs were cultured as previously described (Huschtscha et al., 1998) in MCDB-170 medium (Gibco, Invitrogen Corporation, Carlsbad, CA) containing gentamicin (50 μg/ml) at 37°C in 5% CO_2 + 95% air. Isogenic dermal fibroblasts (Fre-16s) and skin keratinocytes (Kre-16) were isolated from skin of the Bre-16 mammoplasty specimen and grown in DMEM supplemented with 10% fetal bovine serum or Keratinocyte-SFM (Gibco, Invitrogen), respectively.

Cells were transfected when at 40-60% confluence as described previously (Huschtscha et al., 2001), with the following expression vectors: pcDNA-3 control vector (Invitrogen Corporation), pcDNA-3/p16 (Huschtscha et al., 2001), pMSCV control vector (from Graham Mann, Westmead Millennium Institute, NSW, Australia) and pMSCV-p1018shp53 (from Scott Lowe, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY). For transfections, 4 μg DNA/10-cm dish (unless otherwise stated) and FuGENE 6 transfection reagent (Roche Diagnostics Corporation, Indianapolis, IN) were used for 24 hours. The transient transfection efficiency was about 80-90% for post-selection HMEC strains. For stable transfections, post-selection Bre-16 HMECs were transfected at PD 8 and selected in 0.6 μg/ml puromycin. HMECs were treated for 24 hours with either 10 μM nutlin-3a (Sigma-Aldrich, St Louis, MO) dissolved in DMSO or 7.5 nM Act-D (Sigma-Aldrich) dissolved in 100% ethanol. Cells were harvested for protein- and RNA-analysis 24 hours after transfection or drug treatment.

Western blot analysis

Western analysis was performed as previously described (Toouli et al., 2002). Antibodies were obtained from the following sources: mouse monoclonal antibodies for p53 (DO1 and DO7+BP53-12) and $p16^{INK4a}$ (Ab-1) from Neomarkers (LabVision, Fremont, CA), actin (A2066) from Sigma-Aldrich, $p21^{WAF1/CIP1}$ (DCS60) from Cell Signaling Technology (Beverly, MA), Mdm2 (2A9C1.18) from Calbiochem (EMDBiosciences, La Jolla, CA) and SMP14 from Santa Cruz Biotechnology (Santa Cruz, CA). Western blot analyses were quantitated using a calibrated densitometer (GS-800; Bio-Rad Laboratories, Hercules, CA).

Quantitative real-time PCR

A two-step real-time RT-PCR reaction was performed using cDNA reverse transcribed from RNA by Superscript III (Invitrogen Corporation), primed with oligo(dT). The real-time PCR reaction was prepared using Platinum SYBR Green qPCR Supermix (Invitrogen Corporation) with primers for p53 and $p16^{INK4a}$ as described (Miyajima et al., 2001; Takasaki et al., 2003). The reactions were performed on an ABI PRISM 7000 SDS instrument (Applied Biosystems, Foster City, CA). Relative gene expression was determined by the Relative Expression Software Tool (Pfaffl et al., 2002) using glyceraldehyde phosphate dehydrogenase (GAPDH), β-microglobulin (β-2M) or hypoxanthine guanine phosphoribosyl transferase-1 (HGPRT-1) as controls.

Flow cytometry

Cell-cycle analysis was performed on pre- and post-selection HMECs with and without Act-D. Single-cell suspensions were stained according to the method of Smyth et al. (Smyth et al., 1993). Briefly, approximately 1×10^6 cells in 0.05 ml PBS were added to 0.1 ml 5% Triton X-100 (BDH, Merck), 0.5 mg ribonuclease A (Sigma-Aldrich), 25 μg propidium iodide (Sigma-Aldrich) and made to a final volume of 0.3 ml. After incubation for 1 hour on ice, the cells were analyzed on a FACSCanto (BD Biosciences, San Jose, CA).

Immunofluorescence

The distribution of p53 staining in pre- and post-selection HMECs treated with and without Act-D was detected by indirect immunofluorescence staining using a mouse monoclonal antibody against p53, DO1 Ab-6 (Neomarkers), followed by a goat anti-mouse IgG Alexa-Fluor-488 secondary antibody (Invitrogen Corporation). Pre- and post-selection HMECs were seeded into two-well chamber slides (Nunc, Roskilde, Denmark) and, after Act-D treatment for 24 hours, slides were washed three times in PBS before fixation in methanol at –20°C for 10 minutes. The cells were blocked in 3% BSA/PBS for 10 minutes before incubation with the primary antibody (1:300) for 1 hour in a humidified chamber, followed by further incubation with the secondary antibody (1:500) for 30 minutes. Slides were mounted with DABCO (Sigma-Aldrich) anti-fade and examined on a Leica DMLB fluorescence microscope. Images were captured with a cooled CCD camera (SPOT2, Diagnostic Instruments).

Statistical analysis

Statistical analysis of differences in lifespan and proliferation rates between clones treated with either pMSCV or p1018shp53 were calculated with a two-tailed Student's *t*-test assuming equal variance.

We thank Scott Lowe (Cold Spring Harbor Laboratory) and Graham Mann (Westmead Millennium Institute) for providing plasmids, Christine Smyth for analyzing and performing DNA flow cytometry, and Clare Fasching and Daniel Speidel for comments on the manuscript. We are grateful for the cooperation of the plastic surgeons Cholmundeley Williams and Graham Sellars (Sydney Adventist Hospital, Australia) and Paul Curtin (Westmead Private Hospital, Australia), who have provided us with reduction mammoplasty tissues. This work was supported by a Cancer Council NSW Program Grant (R.R.R.), a Cancer Institute NSW Program Grant (A.W.B., H.G.C.) and a Health Research Council of New Zealand Program Grant (A.W.B., J.A.R.). The authors have no conflicting interests.

References

Alcorta, D. A., Xiong, Y., Phelps, D., Hannon, G., Beach, D. and Barrett, J. C. (1996). Involvement of the cyclin-dependent kinase inhibitor p16 (INK4a) in replicative senescence of normal human fibroblasts. *Proc. Natl. Acad. Sci. USA* **93**, 13742-13747.

Barrett, J. C., Annab, L. A., Alcorta, D., Preston, G., Vojta, P. and Yin, Y. (1994). Cellular senescence and cancer. *Cold Spring Harb. Symp. Quant. Biol.* **59**, 411-418.

Bischof, O., Nacerddine, K. and Dejean, A. (2005). Human papillomavirus oncoprotein E7 targets the promyelocytic leukemia protein and circumvents cellular senescence via the Rb and p53 tumor suppressor pathways. *Mol. Cell. Biol.* **25**, 1013-1024.

Braithwaite, A. W. and Edwards, S. J. (2003). *The p53 Pathway, Cancer and Aging* (ed. S. C. Kaul and R. Wadhwa), pp. 121-149. The Netherlands: Kluwer.

Braithwaite, A. W., Royds, J. A. and Jackson, P. (2005). The p53 story: layers of complexity. *Carcinogenesis* **26**, 1161-1169.

Brenner, A. J., Stampfer, M. R. and Aldaz, C. M. (1998). Increased *p16* expression with first senescence arrest in human mammary epithelial cells and extended growth capacity with *p16* inactivation. *Oncogene* **17**, 199-205.

Bueso-Ramos, C. E., Manshouri, T., Haidar, M. A., Yang, Y., McCown, P., Ordonez, N., Glassman, A., Sneige, N. and Albitar, M. (1996). Abnormal expression of MDM-2 in breast carcinomas. *Breast Cancer Res. Treat.* **37**, 179-188.

D'Amico, M., Wu, K., Fu, M., Rao, M., Albanese, C., Russell, R. G., Lian, H., Bregman, D., White, M. A. and Pestell, R. G. (2004). The inhibitor of cyclin-dependent kinase 4a/alternative reading frame (INK4a/ARF) locus encoded proteins p16INK4a and p19ARF repress cyclin D1 transcription through distinct cis elements. *Cancer Res.* **64**, 4122-4130.

Delmolino, L., Band, H. and Band, V. (1993). Expression and stability of p53 protein in normal human mammary epithelial cells. *Carcinogenesis* **14**, 827-832.

Duncan, E. L. and Reddel, R. R. (1997). Genetic changes associated with immortalization: a review. *Biochemistry (Mosc.)* **62**, 1263-1274.

Foster, S. A., Wong, D. J., Barrett, M. T. and Galloway, D. A. (1998). Inactivation of p16 in human mammary epithelial cells by CpG island methylation. *Mol. Cell. Biol.* **18**, 1793-1801.

Gorgoulis, V. G., Zoumpourlis, V., Rassidakis, G. Z., Karameris, A., Rassidakis, A. N., Spandidos, D. A. and Kittas, C. (1996). A molecular and immunohistochemical study of the MDM2 protein isoforms and *p53* gene product in bronchogenic carcinoma. *J. Pathol.* **180**, 129-137.

Harris, S. L. and Levine, A. J. (2005). The p53 pathway: positive and negative feedback loops. *Oncogene* **24**, 2899-2908.

Hinshelwood, R. A., Huschtscha, L. I., Melki, J., Stirzaker, C., Abdipranoto, A., Vissel, B., Ravasi, T., Wells, C. A., Hume, D. A., Reddel, R. R. et al. (2007). Concordant

epigenetic silencing of transforming growth factor-β signaling pathway genes occurs early in breast carcinogenesis. *Cancer Res.* **67**, 11517-11527.

Hollstein, M., Sidransky, D., Vogelstein, B. and Harris, C. C. (1991). p53 mutations in human cancers. *Science* **253**, 49-53.

Honda, R. and Yasuda, H. (1999). Association of p19^{ARF} with Mdm2 inhibits ubiquitin ligase activity of Mdm2 for tumor suppressor p53. *EMBO J.* **18**, 22-27.

Huschtscha, L. I. and Reddel, R. R. (1999). p16^{INK4a} and the control of cellular proliferative life span. *Carcinogenesis* **20**, 921-926.

Huschtscha, L. I., Noble, J. R., Neumann, A. A., Moy, E. L., Barry, P., Melki, J. R., Clark, S. J. and Reddel, R. R. (1998). Loss of p16^{INK4} expression by methylation is associated with lifespan extension of human mammary epithelial cells. *Cancer Res.* **58**, 3508-3512.

Huschtscha, L. I., Neumann, A. A., Noble, J. R. and Reddel, R. R. (2001). Effects of simian virus 40 T-antigens on normal human mammary epithelial cells reveal evidence for spontaneous alterations in addition to loss of p16^{INK4a} expression. *Exp. Cell Res.* **265**, 125-134.

Jimenez, G. S., Khan, S. H., Stommel, J. M. and Wahl, G. M. (1999). p53 regulation by post-translational modification and nuclear retention in response to diverse stresses. *Oncogene* **18**, 7656-7665.

Kubbutat, M. H., Jones, S. N. and Vousden, K. H. (1997). Regulation of p53 stability by Mdm2. *Nature* **387**, 299-303.

Lahav, G., Rosenfeld, N., Sigal, A., Geva-Zatorsky, N., Levine, A. J., Elowitz, M. B. and Alon, U. (2004). Dynamics of the p53-Mdm2 feedback loop in individual cells. *Nat. Genet.* **36**, 147-150.

Landers, J. E., Cassel, S. L. and George, D. L. (1997). Translational enhancement of *mdm*2 oncogene expression in human tumor cells containing a stabilized wild-type p53 protein. *Cancer Res.* **57**, 3562-3568.

Miyajima, K., Tamiya, S., Oda, Y., Adachi, T., Konomoto, T., Toyoshiba, H., Masuda, K. and Tsuneyoshi, M. (2001). Relative quantitation of p53 and MDM2 gene expression in leiomyosarcoma; real-time semi-quantitative reverse transcription-polymerase chain reaction. *Cancer Lett.* **164**, 177-188.

Moll, U. M. and Petrenko, O. (2003). The MDM2-p53 interaction. *Mol. Cancer Res.* **1**, 1001-1008.

Muntoni, A., Fleming, J., Gordon, K. E., Hunter, K., McGregor, F., Parkinson, E. K. and Harrison, P. R. (2003). Senescing oral dysplasias are not immortalized by ectopic expression of hTERT alone without other molecular changes, such as loss of INK4A and/or retinoic acid receptor-β: but p53 mutations are not necessarily required. *Oncogene* **22**, 7804-7808.

Ossovskaya, V. S., Mazo, I. A., Chernov, M. V., Chernova, O. B., Strezoska, Z., Kondratov, R., Stark, G. R., Chumakov, P. M. and Gudkov, A. V. (1996). Use of genetic suppressor elements to dissect distinct biological effects of separate p53 domains. *Proc. Natl. Acad. Sci. USA* **93**, 10309-10314.

Pfaffl, M. W., Horgan, G. W. and Dempfle, L. (2002). Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. *Nucleic Acids Res.* **30**, e36.

Pochampally, R., Fodera, B., Chen, L., Shao, W., Levine, E. A. and Chen, J. (1998). A 60 kd MDM2 isoform is produced by caspase cleavage in non-apoptotic tumor cells. *Oncogene* **17**, 2629-2636.

Pochampally, R., Fodera, B., Chen, L., Lu, W. and Chen, J. (1999). Activation of an MDM2-specific caspase by p53 in the absence of apoptosis. *J. Biol. Chem.* **274**, 15271-15277.

Ramirez, R. D., Herbert, B. S., Vaughan, M. B., Zou, Y., Gandia, K., Morales, C. P., Wright, W. E. and Shay, J. W. (2003). Bypass of telomere-dependent replicative senescence (M1) upon overexpression of Cdk4 in normal human epithelial cells. *Oncogene* **22**, 433-444.

Reddel, R. R. (2000). The role of senescence and immortalization in carcinogenesis. *Carcinogenesis* **21**, 477-484.

Sharpless, N. E. (2005). INK4a/ARF: a multifunctional tumor suppressor locus. *Mutat. Res.* **576**, 22-38.

Smyth, C. M., Helmer, M. A., Dalla-Pozza, L. and Rowe, P. B. (1993). Flow cytometric DNA analyses of frozen samples from children's solid tumors. *Pathology* **25**, 388-393.

Stampfer, M. R. (1985). Isolation and growth of human mammary epithelial cells. *J. Tissue Cult. Methods* **9**, 107-115.

Stampfer, M. R., Garbe, J., Nijjar, T., Wigington, D., Swisshelm, K. and Yaswen, P. (2003). Loss of p53 function accelerates acquisition of telomerase activity in indefinite lifespan human mammary epithelial cell lines. *Oncogene* **22**, 5238-5251.

Takasaki, Y., Yamada, Y., Sugahara, K., Hayashi, T., Dateki, N., Harasawa, H., Kawabata, S., Soda, H., Ikeda, S., Tomonaga, M. et al. (2003). Interruption of p16 gene expression in adult T-cell leukaemia/lymphoma: clinical correlation. *Br. J. Haematol.* **122**, 253-259.

Toouli, C. D., Huschtscha, L. I., Neumann, A. A., Noble, J. R., Colgin, L. M., Hukku, B. and Reddel, R. R. (2002). Comparison of human mammary epithelial cells immortalized by simian virus 40 T-Antigen or by the telomerase catalytic subunit. *Oncogene* **21**, 128-139.

Vassilev, L. T. (2004). Small-molecule antagonists of p53-MDM2 binding: research tools and potential therapeutics. *Cell Cycle* **3**, 419-421.

Vojtesek, B. and Lane, D. P. (1993). Regulation of p53 protein expression in human breast cancer cell lines. *J. Cell Sci.* **105**, 607-612.

Woods, D. B. and Vousden, K. H. (2001). Regulation of p53 function. *Exp. Cell Res.* **264**, 56-66.

Zhang, J., Pickering, C. R., Holst, C. R., Gauthier, M. L. and Tlsty, T. D. (2006). p16INK4a modulates p53 in primary human mammary epithelial cells. *Cancer Res.* **66**, 10325-10331.