Contributions of differential p53 expression in the spontaneous immortalization of a chicken embryo fibroblast cell line

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Abstract

Background: The present study was carried out to determine whether the p53 pathway played a role in the spontaneous immortalization of the SC-2 chicken embryo fibroblast (CEF) cell line that has been in continuous culture for over three years.

Results: The SC-2 cell line emerged from an extended crisis period with a considerably slower growth rate than primary CEF cells. The phenotype of the SC-2 cells changed dramatically at about passage 80, appearing smaller than at earlier passages (e.g., passage 43) and possessing a small, compact morphology. This morphological change coincided with an increase in growth rate. Passage 43 SC-2 cells expressed undetectable levels of p53 mRNA, but by passage 95, the levels were elevated compared to primary passage 6 CEF cells and similar to levels in senescent CEF cells. However, the high level of p53 mRNA detected in passage 95 SC-2 cells did not correlate to functional protein activity. The expression levels of the p53-regulated p21WAF1 gene were significantly decreased in all SC-2 passages that were analyzed. Examination of the Rb pathway revealed that E2F-1 and p15INK4b expression fluctuated with increasing passages, with levels higher in passage 95 SC-2 cells compared to primary passage 6 CEF cells.

Conclusion: The present study suggests that altered expression of genes involved in the p53 and Rb pathways, specifically, p53 and p21WAF1, may have contributed to the immortalization of the SC-2 CEF cell line.

Background

Normal cells cultured in vitro undergo a characteristic number of divisions before entering a non-dividing state termed senescence [1]. Most cells are unable to overcome senescence to continue dividing unless key tumor suppressor pathways are first altered. Cellular immortalization has been achieved by the inactivation of the p53 and Rb pathways or by the activation of telomerase [2,3]. While spontaneous immortalization is an extremely rare event in human cells [4], rodent cells spontaneously immortalize at much greater rates [2]. For avian cells, spontaneous immortalization also has been very rare, with only two other spontaneously immortalized chicken cell lines reported (DF-1 [5-8] and SC-1 [9]).
The tumor suppressor gene, p53, has often been referred to as the ‘cellular gatekeeper’ or the ‘guardian of the genome’ because of its role in cell cycle arrest and apoptosis in response to cellular damage [10,11]. The p53 protein is dysfunctional in most human cancers, with p53 itself being mutated in about half of these cancers. In the other half of cancers when p53 itself is not mutated, it is inactivated indirectly as a result of alterations in the gene products that interact with p53 or transmit information to or from p53 [12]. In normal cells, the main negative regulator of p53, MDM2, maintains a relatively short p53 half-life by targeting it for degradation, thus preventing it from exerting antiproliferative effects [13]. The MDM2 and p53 proteins operate in a feedback loop whereby MDM2 degrades p53 while p53 activates transcription of MDM2, thereby helping to maintain low p53 levels in normal cells [14]. Another important gene involved in the p53 pathway is ARF, which binds directly to MDM2, protecting p53 from degradation and allowing for the increased expression of p53 [15,16]. One study showed that senescence requires an increase in the levels of ARF and that conversely, fibroblasts lacking ARF proliferate indefinitely [17]. Many signals activate p53 such as DNA damage, telomere shortening, hypoxia, and aberrant proliferative signals. Upon activation, p53 induces a G1/S phase cell cycle arrest that is mediated by p21WAF1 [18,19], which functions by inhibiting the CDKs (cyclin-dependent kinases) during the G1 phase of the cell cycle, thereby preventing cell cycle progression [20-22]. Consistent with this, the levels of p21WAF1 have been reported to be elevated in senescent cells [23].

The other main tumor suppressor mechanism involves the Rb pathway, and alterations in this pathway have been shown to lead to tumorigenesis [24]. Throughout the cell cycle Rb undergoes changes in phosphorylation, with a hyperphosphorylated form predominant in proliferating cells and a hypophosphorylated form predominant in quiescent or differentiating cells [25]. As Rb is hyperphosphorylated by CDK-cyclins in the mid-to-late G1 phase of the cell cycle, Rb is unable to bind and inhibit the actions of E2F-1, allowing E2F-1 to promote cell cycle progression from the G1 phase of the cell cycle into the S phase [26]. Senescent cells express high levels of the CDK-inhibitor, p16INK4a, which prevents CDK-cyclins from phosphorylating Rb, keeping E2F-1 bound to Rb and unable to promote entry into the S phase of the cell cycle [27,28]. The p16INK4a gene is inactivated in many tumor cells lines [29-31], while overexpression of p16INK4a results in G1 arrest [32].

In the present study, mRNA and protein expression levels of various genes involved in the p53 and Rb pathways were measured in multiple passages of a spontaneously immortalized CEF cell line, SC-2. By analyzing the expression of important cell cycle regulatory genes at various stages of the immortalization process it was observed that the SC-2 cells underwent an initial crisis period between passages 18 and 33 and another transition period at about passage 80. From this data, it has been hypothesized that following the initial crisis period, changes in the expression of important cell cycle regulatory genes allowed the cells to continue growing. It is quite possible that additional coordinated fluctuations in gene expression allowed the cells to increase their growth rate at approximately passage 80 and to become fully immortal.

Results and discussion

Growth rate and morphological analysis

In the present study the third reverse transcriptase (RT)-negative spontaneously immortalized chicken cell line (SC-2) was established. Data were obtained from many different passages of SC-2 cells, but only shown for passages 43 and 95, since they represent the major transition phases that occurred in the SC-2 cells. An examination of this type has been performed for another spontaneously immortalized cell line (SC-1) [9], but the expression of most cell cycle regulatory genes were very different compared to the SC-2 cells.

The SC-2 cell line was derived from primary CEF cells that were thawed at passage 4. Between passages 5 and 17 the growth rate of the CEF cells averaged 1.03 population doublings per day (pd/d). The cells entered a crisis period between passages 18 and 33, during which their growth rate was negligible except for measurable growth between passages 20 and 21. During this crisis period, cells were refed every 2–3 days and passaged until small foci of cells appeared. At about passage 33 a subpopulation of cells emerged that was able to overcome cellular senescence and continue growing when other cells in the population succumbed to cell death. These cells were considered to have an extended lifespan and as such were designated the SC-2 cell line. The SC-2 cells continued to grow slowly between passages 33 and 80 averaging 0.12 pd/d. Passage 43 SC-2 cells were flattened and enlarged compared to the presenescent CEF cells. The phenotype of the SC-2 cells changed dramatically at about passage 80, appearing smaller than observed earlier at passage 43 and possessing a small, compact morphology, similar to DF-1 cells (Fig. 1A). Coinciding with the dramatic morphological change at passage 80, the growth rate of the SC-2 cells increased to an average of 0.60 pd/d after passage 80 (Fig. 1B). The SC-2 cells are currently at passage 120 and have maintained the improved morphology and increased growth rate since passage 80. The SC-2 cells displayed contact inhibition when grown to confluence, did not show evidence of multi-layered growth, and did not form colonies when grown in a semi-solid agarose suspension (data not shown), suggesting that these cells lacked many of the...
properties of transformed cells. Karyotypic analysis (data not shown) of the SC-2 cells indicated that they displayed a normal diploid karyotype. The SC-2 cells were analyzed twice for RT activity and mycoplasma contamination and found to be negative for both (data not shown).

FACS analysis showed that SC-2 cells possessed a distinct cell cycle distribution compared to either primary passage 6 CEF cells, senescent passage 18 CEF cells, passage 43 SC-2 cells, passage 95 SC-2 cells and immortal passage 280 DF-1 cells. B) Growth curve of SC-2 cells. SC-2 cells were plated at a density of 3 × 10⁵ cells/10 cm dish. At 80% confluency cells were trypsinized, counted and the number of population doublings per day calculated.

Figure 1
Morphology and growth curve of primary and immortal CEF cells. A) Representative photomicrographs of primary passage 6 CEF cells, senescent passage 18 CEF cells, passage 43 SC-2 cells, passage 95 SC-2 cells and immortal passage 280 DF-1 cells. B) Growth curve of SC-2 cells. SC-2 cells were plated at a density of 3 × 10⁵ cells/10 cm dish. At 80% confluency cells were trypsinized, counted and the number of population doublings per day calculated.

Cell growth rates were compared as shown in Figure 2B. Passage 43 SC-2 cells had a very slow growth rate, with only 0.17 pd/d after six days, while passage 95 SC-2 cells showed increased growth rates (0.60 pd/d) that were more similar to primary CEF cells (0.72 pd/d). The DF-1 cells displayed the highest growth rate at 1.33 pd/d after four days.

Analysis of p53 and p53-regulated genes
Because of the importance the p53 pathway plays in controlling the cell cycle, an examination of p53 and its related genes was performed during the conversion process in the SC-2 cell line using RT-PCR and Southern blot analysis. Like many other studies, an increase in p53 mRNA expression (25%) was observed in normal senescent passage 18 CEF cells compared to primary passage 6 CEF cells (Fig. 3A). The decreased p53 mRNA expression in passage 43 SC-2 cells agrees with other studies that have observed decreased expression of p53 in immortal cells
[8,33]. Unexpectedly, increased p53 levels were observed in passage 95 SC-2 cells that were similar to levels in senescent passage 18 CEF cells. One possible explanation for this observation is the functional inactivation of p53 via deletion or point mutations. However, the nucleotide sequence of p53 mRNA in the SC-2 cells was identical to

Figure 2
FACS analysis and growth rates of primary and immortal CEF cells. A) Cell cycle analysis of pre-senescent passage 6 CEF, passage 95 SC-2, and immortal passage 280 DF-1 cells was performed using fluorescence-activated cell sorting (FACS) of propidium iodide-stained cells. B) To determine growth rates, primary CEF (passage 6) and immortal CEF (passage 43 SC-2, passage 95 SC-2, and passage 280 DF-1) cells were plated at a density of $1 \times 10^5$ cells/10 cm dish using conditions as described in Materials and Methods. Both primary and immortal CEF cells were counted each day for up to 6 days to determine cell numbers. The immortal DF-1 cells are only shown through day 4 due to their relatively higher cell numbers.
Analysis of the p53 pathway using RT-PCR and a p53-Luciferase reporter assay. A) Relative levels of mRNA expression for genes involved in the p53 pathway determined by RT-PCR. For each gene the expression level of passage 6 CEF cells was set to 1.0. The levels for other samples were then adjusted accordingly. A minimum of three independent RT-PCR experiments were analyzed using the NIH image software program and normalized using the expression levels of GAPDH. Differences \( \leq 0.05 \) were considered significant. Different letters indicate significant differences. B) p53 and p21\textsuperscript{WAF1} mRNA expression levels were measured by RT-PCR in passage 43 and 95 SC-2 cells as well as in passage 280 DF-1 cells ± the protein synthesis inhibitor CHX. C) p53 protein levels in primary passage 6 CEF and passage 95 SC-2. A minimum of three independent Western blot experiments were analyzed using the NIH image software program and normalized using the expression levels of actin. Differences \( \leq 0.05 \) were considered significant. D) Altered biological activity of p53 in immortal DF-1 and SC-2 cells using the luciferase reporter gene assay. All cells were cotransfected with a control reporter plasmid (Con-Luc) or p53 reporter plasmid (p53-Luc) and pcDNA3.1-LacZ plasmid. The \( \beta \)-lactamase (LacZ) gene was amplified by PCR to normalize transfection efficiency and the GAPDH gene was amplified by RT-PCR to account for loading differences. Levels of luciferase activity were determined using the Luciferase Assay System (Promega) following the manufacturer’s instructions. Luciferase activity in the cells transfected with the Con-Luc plasmid were set to 1.0. The normalized values shown are relative expression levels with s.d. Differences \( \leq 0.05 \) were considered significant. Different letters indicate significant differences.
Another important target of p53 is p21 WAF1, a gene that
takes part in a feedback loop. The related gene p27Kip1, which belongs to the same fam-
ily as p21 WAF1 (the Kip/Cip family), is a CDK inhibitor that prevents transition from the G1 to S phase of the cell cycle. In order to assess whether or not the fluctuating p53 levels were functional, mRNA expression of p21 WAF1 was measured. Similar to other studies [36,37], levels of p21 WAF1 dramatically increased in senescent passage 18 CEF cells (82%; Fig. 3A). Significant down-regulation of p21 WAF1 mRNA was observed in all passages of SC-2 cells compared to primary passage 6 CEF cells. Since the p21 WAF1 gene is up-regulated by p53 in response to DNA damage [19], the fact that the SC-2 cells possessed elevated levels of p53 mRNA it was not surprising that CHX had no effect on p53 expression (data not shown). However, in cells that contained depressed levels of p53 (passage 43 SC-2 and DF-1 cells), CHX treatment resulted in an upregulation of p53 mRNA expression (Fig. 3B). This suggested that both the passage 43 SC-2 cells and DF-1 cells contained a p53-regulatory element that blocked expression of p53 resulting in a 5- and 2.5-fold increase in mRNA expression following CHX treatment, respectively. Removal of this putative p53 inhibitor via CHX allowed expression levels of p53 mRNA to increase. However, CHX had no effect in passage 95 SC-2 cells, which already possessed elevated levels of p53 mRNA compared to primary passage 6 CEF cells. An examination of the effect of CHX on the expression of p21 WAF1 would help substantiate the functional status of p53 in the passage 95 SC-2 cells. However, treatment with CHX had no effect on the expression of p21 WAF1 mRNA expression in SC-2 cells (Fig. 3B). This indicates that the p53 mRNA levels in passage 95 SC-2 cells and the CHX-restored levels in passage 43 SC-2 cells were functionally inactive.

Thus far, indirect assessment of the functional activity of p53 has been demonstrated by measurement of the mRNA levels of p53 in CEF cells and in immortalized (10)3 and (10)7 murine embryonic fibroblast (MEF) cells expressing constitutively low levels of p53 mRNA [8]. We reasoned that a translation inhibitor such as CHX should block the function of translation-dependent p53 regulatory elements that may be present, resulting in increased p53 mRNA levels. Since primary passage 6 CEF cells possessed normal levels of p53 mRNA it was not surprising that CHX had no effect on p53 expression (data not shown). However, in cells that contained depressed levels of p53 (passage 43 SC-2 and DF-1 cells), CHX treatment resulted in an upregulation of p53 mRNA expression (Fig. 3B). This suggested that both the passage 43 SC-2 cells and DF-1 cells contained a p53-regulatory element that blocked expression of p53 resulting in a 5- and 2.5-fold increase in mRNA expression following CHX treatment, respectively. Removal of this putative p53 inhibitor via CHX allowed expression levels of p53 mRNA to increase. However, CHX had no effect in passage 95 SC-2 cells, which already possessed elevated levels of p53 mRNA compared to primary passage 6 CEF cells. An examination of the effect of CHX on the expression of p21 WAF1 would help substantiate the functional status of p53 in the passage 95 SC-2 cells. However, treatment with CHX had no effect on the expression of p21 WAF1 mRNA expression in SC-2 cells (Fig. 3B). This indicates that the p53 mRNA levels in passage 95 SC-2 cells and the CHX-restored levels in passage 43 SC-2 cells were functionally inactive.

Thus far, indirect assessment of the functional activity of p53 has been demonstrated by measurement of the
expression of p53-regulated genes. Since the ability of p53 to activate or repress transcription of downstream genes is important for cell cycle control, the protein level of p53 and the direct functional activity also were measured. Unlike the mRNA data that showed an increase in p53 mRNA expression in passage 95 SC-2 cells, Western blot analysis showed a slightly decreased level of p53 protein in passage 95 SC-2 cells (Fig. 3C). To determine whether the protein levels of p53 in the passage 95 SC-2 cells were functional, direct activity was measured after transfection of a p53 consensus binding sequence promoter/luciferase reporter plasmid construct. It was discovered that the functional protein activity of p53 was 2.8-fold less than that of primary passage 6 CEF cells (Fig. 3C). The functional inactivation of p53 protein in the passage 95 SC-2 cells likely allowed the cells to escape the growth constraints imposed by p53. In the DF-1 cells the functional p53 activity was greatly decreased compared to the level in primary passage 6 CEF cells, which is in agreement with the lower mRNA expression levels observed in the DF-1 cells.

Expression analysis of genes involved in the Rb pathway

Another tumor suppressor that plays a fundamental role in controlling cell cycle progression and senescence is Rb. Alterations in this pathway are second only to alterations in the p53 pathway in human cancers [43]. Surprisingly, an up-regulation of Rb expression (~40%) was observed in the SC-2 cells compared to primary passage 6 CEF cells (Fig. 4A). However, this is in agreement with the results of a previous study that showed up-regulated levels of Rb mRNA in the immortal DF-1 cell line [44]. The up-regulation of Rb expression that was observed in all passages of SC-2 cells examined may be the result of a functionally inactive p53, as others have shown that Rb mRNA expression is repressed by p53 in mammalian cells [45].

The tumor suppressor p16INK4a, a key regulator of Rb that maintains Rb in a hypophosphorylated state, is inactivated in many transformed cell lines [29-31]. Since p15INK4b assumes the role of p16INK4a in chickens [46], its vated in many transformed cell lines [29-31]. Since maintains Rb in a hypophosphorylated state, is inacti-

To determine whether the increased levels of p15INK4b mRNA were translating normal protein levels, Western blot analysis was used to determine the levels of p15INK4b protein in the SC-2 cells. As shown in Fig. 4B the p15INK4b protein levels also were increased (30%) in the SC-2 cells compared to primary passage 6 CEF cells.

The p15INK4b alternate reading frame sequence (ARF), is encoded by the same genetic locus as p15INK4b, but in a different reading frame, resulting in two completely different genes. The chicken ARF gene was recently isolated, and it was determined that chicken ARF binds to MDM2 and stabilizes p53, just as it does in human and mouse [59]. To verify the role of ARF in the immortalization process of chicken cells, levels were compared in primary and immortal CEF cells. As predicted, the levels of ARF were elevated in senescent passage 18 CEF cells and decreased in passage 43 SC-2 cells (Fig. 4A). Although increased expression of ARF has been shown to induce cell cycle arrest [47], this was not observed in the passage 95 SC-2 or DF-1 cells. It has been shown that E2F-1 (an important gene in the Rb pathway) directly activates the expression of ARF, establishing a link between the Rb and p53 pathways [48]. E2F-1 is one of the growth-regulatory genes that is suppressed in senescent human cells [49,50] and can induce senescence when over-expressed in normal human fibroblasts via ARF. This induced senescence is dependent upon the presence of a functional p53 gene [51]. In the present study, elevated levels of E2F-1 were observed in immortal SC-2 and DF-1 cells, which is similar to other immortal chicken cell lines developed in our lab [8]. The study by Bates et al. [48] proposed that induction of ARF by E2F-1 would stabilize p53 unless there existed a mutation in ARF or in p53 itself, again supporting our hypothesis that p53 was inactive in the SC-2 cells.

Expression analysis of various cell cycle regulatory genes

Since the cell cycle also is regulated by the various cyclins [43], we examined mRNA expression levels of multiple chicken cyclin genes (Fig. 5). As expected, decreased expression for most of the cyclins was observed in senescent passage 18 CEF cells compared to primary passage 6 CEF cells. The only cyclin that was up-regulated was cyclin D2, which has been shown to increase its expression in growth-arrested cells [52]. In passage 43 SC-2 cells, the expression levels of most of the cyclins also were decreased, except for cyclin B3, which had similar levels compared to passage 6 CEF cells. However, by passage 95 the expression of cyclins B2, B3 and D1 were increased, while the expression of cyclins A, C, D2, and E were decreased compared to primary passage 6 CEF cells. Although unexpected, the decreased expression of certain cyclins in the SC-2 cells was also found in the fully immortal DF-1 cells (Fig. 5).
Analysis of the Rb pathway

Figure 4

A) Levels of mRNA expression for genes involved in the Rb/p16INK4a pathway measured by RT-PCR. For each gene, the expression level was set to 1.0 for passage 6 CEF cells. The levels for other samples were then adjusted accordingly. A minimum of three independent RT-PCR experiments were analyzed using the NIH image software program and normalized using the expression levels of GAPDH. Differences ≤ 0.05 were considered significant. Different letters indicate significant differences.

B) p15INK4b protein levels in primary passage 6 CEF, and passage 95 SC-2 cells. A minimum of three independent Western blot experiments were analyzed using the NIH image software program and normalized using the expression levels of actin. Differences ≤ 0.05 were considered significant.
Levels of mRNA expression for the various cyclins

Figure 5

Levels of mRNA expression for the various cyclins. For each gene, the expression level was set to 1.0 for passage 6 CEF cells. The levels for other samples were then adjusted accordingly. A minimum of three independent RT-PCR experiments were analyzed using the NIH image software program and normalized using the expression levels of GAPDH. Differences ≤ 0.05 were considered significant. Different letters indicate significant differences.
Telomere regulation
Telomerase activation has been shown to be an important step in the immortalization of normal fibroblasts [53]. However, in the current study we were unable to show telomerase activation using the TRAP assay. Previous attempts to show telomerase activity in other immortal CEF cells [8,9], including the fully immortal DF-1 cell line (passage 280) have proven unsuccessful as well. Although telomerase activity was undetectable in the immortal SC-2 or DF-1 cells, telomere length did not decrease, even in senescent passage 18 CEF cells (data not shown). These results suggested that perhaps alterations in the p53 and/or Rb pathways may be more important than telomerase activation for the immortalization of CEF cells, which was in agreement with our other spontaneously immortalized cell lines (SC-1 and DF-1). The question still remains as to how chicken cells maintain telomere length without detectable telomerase activity. It is possible that sufficient telomerase activity is present in immortal CEF cells to maintain telomere length, but that this level is not high enough to be detected by the TRAP assay. It also is possible that telomere length is maintained in CEF cells through alternative mechanisms, as has been proposed in a number of human cells lines that do not display telomerase activity [54,55]. Although telomerase activity was undetectable, mRNA expression of TRF2, which is involved in the maintenance of telomeres, was elevated in passage 95 SC-2 cells (data not shown) suggesting that TRF2 may participate in the stabilization of telomere length in SC-2 cells.

Conclusion
Unlike other studies that have introduced exogenous genes into cells in attempt to achieve immortalization or else have characterized a spontaneously immortalized cell line after it had been established, the spontaneous immortalization of the SC-2 cell line was analyzed as it transitioned from a life-span extended population of cells into a fully immortal cell line. Our results indicated that an initial down-regulation of p53 allowed the SC-2 cells to bypass senescence. The elevated levels of p53 mRNA in passage 95 SC-2 cells were shown to be partially non-functional using the luciferase reporter assay, and also verified by the significantly down-regulated p21\(^{WAF1}\) expression that was observed. In fact, it has been shown that the loss of p21\(^{WAF1}\) allowed cells to bypass senescence in spite of p16\(^{INK4a}\) expression [56], which is what was observed in the SC-2 cells. The inactivation of p53, and consequently p21\(^{WAF1}\), likely contributed to the SC-2 cells becoming a fully immortal cell line.

Methods

Cells and culture conditions
Primary and immortal CEF cells were grown in Dulbecco’s Modified Eagle Medium (DMEM) high glucose enriched with 10% fetal calf serum (FCS), 1% penicillin-streptomycin, and 2 mM L-glutamine. All cell culture reagents were purchased from Invitrogen (Carlsbad, CA). The spontaneously immortalized DF-1 CEF cell line was derived from East Lansing Line 0 (ev-0) leghorn layer embryos [Avian Disease and Oncology Laboratory, East Lansing, MI] [5,6,7,8]. The spontaneously immortalized SC-2 CEF cell line was derived from SPAFAS eggs (specific pathogen free avian supply, Charles River Laboratories, North Franklin, CT). For the inhibition of translation studies, primary and immortal cells were incubated in 10% FCS-DMEM supplemented with 10 \(\mu\)g/ml of cycloheximide (CHX; Sigma, St. Louis, MO) for 4 h.

Cell growth and cell cycle analysis
To determine growth rates, primary (passage 6 and 18) and immortal (DF-1 and SC-2) CEF cells were plated at a density of 3 \(\times\) 10\(^5\) cells/10 cm dish using culture conditions as described above. At 80% confluency, cells were trypsinized, counted, and the number of population doublings per day (pd/d) calculated. Cell growth also was determined by plating cells at a density of 1 \(\times\) 10\(^5\) cells/10 cm dish using conditions as described above, and counting cells each day for up to 6 days to determine cell numbers. Cell cycle analysis was carried out using FACS of propidium-iodide-stained cells and a Cell Quest software program (Becton Dickinson, Franklin Lakes, NJ) as described previously [57].

Cell line characterization
Karyotypic analysis of cells was conducted by the Veterinary Diagnostic Laboratory at the University of Minnesota. The SC-2 cells were analyzed twice for reverse transcriptase (RT) activity using the EnzCheck RT Assay kit (Molecular Probes/Invitrogen Inc., Eugene, OR) according to the manufacturer’s instructions. Mycoplasma contamination was analyzed using the Mycoplasma Plus™ kit (Stratagene, La Jolla, CA) according to the manufacturer’s instructions.

RT-PCR analysis
For semi-quantitative RT-PCR, 3 \(\mu\)g of DNase I-treated RNA was converted to cDNA using Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA) following the manufacturer’s instructions. A portion (1 \(\mu\)l) of the reverse transcription reaction was used to amplify cDNA fragments with chicken-specific primers (information regarding primers and PCR conditions used in RT-PCR will be provided upon request). All semi-quantitative cDNA fragments were amplified using Takara Ex Taq™ (Intergen, Burlington, MA). PCR products were verified to be in the linear range and visualized by ethidium bromide staining. Images were processed using the Eagle Eye II still video system (Stratagene, La Jolla, CA). To validate the above quantitation method, PCR products also were amplified
for 15 cycles (undetectable by ethidium bromide staining) and detected by standard Southern blot hybridization using their corresponding [α-32P]-labeled cDNA probes prepared by RT-PCR (data not shown). A minimum of three independent RT-PCR experiments were analyzed using the NIH image software program and normalized using the expression levels of GAPDH.

**Analysis of p53 and p15INK4b proteins**

Cell lysate protein (30 μg; determined using the Bio-Rad protein reagent) was separated by SDS-PAGE with precast 4%-20% gradient gels (Bio-Rad, Hercules, CA). Gels were electro-transferred to nitrocellulose paper, and proteins were detected with antibodies specific for p53 (SC-99, Santa Cruz Biotechnology, Santa Cruz, CA) and p15INK4b (a gift from Gordon Peters), followed by horseradish peroxidase-labeled secondary antibody and either chemiluminescence or colorimetric detection systems.

**p53 functional assay**

A p53-Luciferase reporter plasmid was constructed in the pGL3 Luciferase reporter vector (Promega, Madison, WI) using double-stranded oligonucleotides corresponding to tetrans of the p53 consensus binding sequence (5'-AGGCAATGCCCT-3') as described previously [44]. Transient transfection was carried out using electroporation (1100 V, 25 µF, 275 V) as previously described [58]. For transfection, 20 µg of promoter reporter plasmids (p53-Luc) or 20 µg of control reporter plasmids (Con-Luc) [44] were cotransfected with 5 µg of pcDNA3.1-Z plasmid. Transfection efficiency was normalized using the β-oxidase-labeled secondary antibody and either chemiluminescence or colorimetric detection systems.

**Authors’ contributions**

S.A.C. carried out isolation of the cells, cell culture, cell growth analysis, cell cycle analysis, RT-PCR analysis, p53-functional assay, conceived of the study, participated in the design and coordination of the study, and drafted the manuscript. B-W.K. performed the Western blot analysis and participated in the design and coordination of the study. M.M.L. helped perform some of the RT-PCR experiments. H.K. participated in the design and coordination of the study. D.N.F. participated in the design and coordination of the study and helped draft the manuscript. All authors read and approved the final manuscript.

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**References**

1. Hayfllick L, Moorehead PS: The serial cultivation of human diploid cell strains. Exp Cell Res 1961, 25:585-621.
2. Sherr C, DePinho RA: Cellular senescence: mitotic clock or culture shock? Cell 2002, 109:207-210.
3. Campisi J: From cells to organisms: can we learn about aging from cells in culture? Exp Gerontol 2001, 36:607-618.
4. Campisi J: Replicative senescence and immortalization. In The Molecular Basis of Cell Cycle and Growth Control Edited by: Stein G, Baserga R, Giordano A, Deinhardt D, New York: Wiley-Liss; 1999:348-373.
5. Himly M, Foster DN, BottoI I, Lacovoni JS, Vogt PK: The DF-I chicken fibroblast cell line: Transformation induced by diverse oncogenes and cell death resulting from infection by avian leukosis viruses and vectors. Virology 1999, 248:305-311.
6. Schaefer-Klein J, Givol I, Barsov EV, Whitcomb JM, VanBrocklin M, Foster DN, Federspiel MJ, Hughes SH: The EV-O-derived cell line DF-I supports the efficient replication of avian leukosis-sarcoma viruses and vectors. Virology 1999, 248:295-408.
7. Kim H, You S, Kim HJ, Farris J, Foster LK, Foster DN: Increased mitochondrial-encoded gene transcription in immortal DF-I cells. Exp Cell Res 2001, 265:339-347.
8. Kim H, You S, Foster LK, Farris J, Foster DN: The rapid destabilization of p53 mRNA in immortal chicken embryo fibroblast cell line. Oncogene 2001, 20:5118-5123.
9. Christman SA, Kong B-W, Landry MM, Kim H, Foster DN: Fluctuations in the p53 and Rb/p16INK4a pathways contribute to the spontaneous immortalization of a chicken embryo fibroblast cell line. FEBS Lett 2005, 579:6705-6715.
10. Lane DP: p53, guardian of the genome. Nature 1992, 358:15-16.
11. Levine AJ: p53, the cellular gatekeeper for growth and division. Cell 1997, 88:23-31.
12. Vogelstein B, Lane D, Levine AJ: Surfing the p53 network. Nature 2000, 407:307-310.
13. Loehrma MAE, Vousden KH: Regulation and activation of p53 and its family members. Cell Growth Differ 1999, 11:1162-1168.
14. Wu XW, Bayle JH, Olson D, Levine AJ: The p53 mdm-2 autoregulatory feedback loop. Genes Dev 1993, 7:1126-1132.
15. Sharpless NE, DePinho RA: The INK4a/ARF locus and its two gene products. Curr Opin Genet Dev 1999, 9:22-30.
16. Sherr CJ: The INK4a/ARF network in tumour suppression. Nat Rev Mol Biol 2001, 2:731-737.
17. Kamiyto T, Zindy F, Rouas MF, Quelle DE, Downing JR, Ashmun RA, Grant-Spence G, Sherr CJ: Tumor suppression at the mouse INK4a/ARF locus mediated by the alternative reading frame product p19ARF. Cell 1997, 91:649-659.
18. Bi-Dery WS, Tokiio ET, Veluclescu V, Levy D, Parsons R, Trent J, Lin D, Mercer E, Kinzler K, Vogelstein B: WAF1, a potential mediator of p53 tumor suppression. Cell 1993, 75:817-825.
19. Bi-Dery WS, Harper JW, O’Connor PM, Veluclescu VE, Canman CE, Jackman D, Pietenpol JA, Burrell M, Hill DE, Wang Y, Wisman KG, Mercer WE, Kastan MB, Kohn KW, Ellodge SJ, Kinzler KW, Vogelstein B: WAF1/CIP1 is induced in p53-mediated G1 arrest and apoptosis. Cancer Res 1994, 54:1669-1674.
20. Gu Y, Tureck C, Morgan D: Inhibition of CDK2 activity in vivo by an associated 20 K regulatory subunit. Nature 1993, 366:707-710.
21. JW Harper, Adami G, Wei N, Keyomarsi K, Ellodge S: The p21 cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases. Cell 1993, 75:805-816.
22. Xiong Y, Hannon G, Zhang H, Casso D, Kobayashi R, Beach D: p21 is a universal inhibitor of cyclin kinases. Nature 1993, 362:701-704.
23. Stein GH, Drullinger LF, Souland A, Dulevic V: Differential roles for cyclin-dependent kinase inhibitors p21 and p16 in the mechanisms of senescence and differentiation in human fibroblasts. Mol Cell Biol 1999, 19:2109-2117.
24. Huang JJ, Yue JK, Shew CJ, Chen PL, Bookstein JR, Friedman T, Lee EY, Lee WH: Suppression of the neoplastic phenotype by replacement of the RB gene in human cancer cells. Science 1988, 242:1563-1566.
25. Chen PL, Scully P, Shew JY, Wang JY, Lee WH: Phosphorylation of the retinoblastoma gene product is modulated during the cell cycle and cellular differentiation. Cell 1989, 58:1193-1198.
26. Qin XQ, Livingston DM, Kaelin WG, Adams PD: Derepressed transcription factor E2F-1 expression leads to S-phase entry and...
p35-mediated apoptosis. Proc Natl Acad Sci USA 1994; 91:10918-10922.

27. Parry D, Barrett D, Mann DJ, Peters G: Lack of cyclin D-Cdk complexes in RB-negative cells correlates with high levels of p16INK4/MTS1 tumour suppressor gene product. EMBO J 1995, 14:503-511.

28. Serrano M, Hannon G, Beach D: A new regulatory motif in cell-cycle control causing specific inhibition of cyclin D/Cdk4. Nature 1993, 366:704-707.

29. A Kamb, Grulis MA, Weaver-Reildhaus J, Liu Q, Harshman K, Tavtigian SV, Stockert E, Day RS III, Johnson BE, Skolnick MH: A cell cycle regulator potentially involved in genesis of many tumor types. Science 1994, 264:436-440.

30. Nobori T, Miura K, Wu DJ, Lois A, Takabayashi K, Carson DA: Deletions of the cyclin-dependent kinase-4 inhibitor gene in multiple human cancers. Nature 1994, 368:753-756.

31. Oliner JD, Kinzler KW, Meltzer PS, George DL, Vogelstein B: Cloning of p57 Kip2, a cyclin-dependent kinase inhibitor family is a candidate tumor suppressor gene. Proc Natl Acad Sci USA 1995, 92:8507-8511.

32. Williams BO, Remington L, Albert DM, Mukai S, Bronson RT, Jacks T: Nature Genet 1994, 7:480-484.

33. Biem B, Zaluzt-Hourri R, Givol D, Oren M: Analysis of the gene encoding the murine cellular tumor antigen p53. EMBO J 1993, 12:2179-2183.

34. Oliner JD, Kinaler KW, Meltzer PC, George DL, Vogelstein B: Amplification of a gene encoding a p53-associated protein in human sarcomas. Nature 1992, 358:80-83.

35. Tonehara H, Sato E, Noda A, Ide T: Increase in expression level of p21WAF1/CIP1 with increasing division age in both normal and SV40-transformed human fibroblasts. Oncogene 1995, 10:835-840.

36. Wyllie F, Haughton M, Bartek J, Rowson J, Wynford-Thomson D: Mutant p53 can delay growth arrest and loss of CDK2 activity in senescing human fibroblasts without reducing p21WAF1 expression. Exp Cell Res 2003, 285:236-242.

37. Matsuo S, Edwards M, Bai C, Parker S, Zhang P, Baldini A, Harper-JW, Elledge J. p57kip1, a structurally distinct member of the p21/Cdk inhibitor family is a candidate tumor suppressor gene. Genes Dev 1995, 9:650-662.

38. Lee M, Reynolds D, I Massague J. Cloning of p57kip1, a cyclin-dependent kinase inhibitor with unique domain structure and tissue distribution. Genes Dev 1995, 9:639-649.

39. Ohtsubo K, Nakamura K, Ishihara K, Nakano H, Saito K, Taupin V, Hara K, Shiosaka S, Taniyama Y, Tsujimoto Y, et al.: The telomerase-related gene POT1 is a candidate suppressor gene for apoptosis. Genes Dev 2001, 15:2543-2549.

40. Wyllie F, Haughton M, Bartek J, Rowson J, Wynford-Thomson D: Mutant p53 can delay growth arrest and loss of CDK2 activity in senescing human fibroblasts without reducing p21WAF1 expression. Exp Cell Res 2003, 285:236-242.

41. Dynlacht BD, Ngwu C, Winston J, Swindell EC, Elledge SJ, Harlow E, Hannon JG: Purification and analysis of CIP/KIP proteins. Methods Enzymol 1997, 283:230-244.

42. Yelding NM, Procopio VN, Rehman MT, Lee WMF: c-myc mRNA Is Down-regulated during Myogenic Differentiation by Accelerated Decay That Depends on Translation of Regulatory Coding Elements. J Biol Chem 1998, 273:15749-15757.

43. Sherr CJ: Cancer Cell Cycles. Science 1996, 274:1672-1677.

44. Kim H, You S, Kim IJ, Foster LF, Farris J, Ambody S, Ponce de Leon FA, Foster DN: Altemations in p53 and E2F-1 function common to immortalized chicken embryo fibroblasts. Oncogene 2003, 22:5238-5251.

45. Ko L, Prives C: p53: puzzle and paradigm. Genes Dev 1996, 10:1054-1072.

46. Kim S-H, Mitchell M, Fuji H, Llanos S, Peters G: Absence of p53-INK4a and truncation of ARF tumor suppressors in chicken. Proc Natl Acad Sci USA 2003, 100:211-216.

47. Quelle DE, Zindy F, Ashmun RA, Sherr CJ: Alternative reading frames of the INK4a tumor suppressor gene encode two unrelated proteins capable of inducing cell cycle arrest. Cell 1995, 83:993-1000.

48. Bates S, Phillips AC, Clark PA, Stott F, Peters G, Ludwig RL, Vousson KH: p14ARF links the tumor suppressors RB andp53. Nature (Lond) 1998, 395:124-125.

49. GP Dimri, EE Hara, J Campisi: Regulation of two E2F-related genes in presenescent and senescent human fibroblasts. J Biol Chem 1994, 269:16180-16186.

50. Saunders NA, Smith RJ, Jetten AM: Regulation of proliferation-specific and differentiation-specific genes during senescence of human epidermal keratinocytes and mammary epithelial cells. Biochem Biophys Res Commun 1994, 197:46-54.

51. Dimri GP, Itahana K, Acosta M, Campisi J: Regulation of a senescence checkpoint response by the E2F1 transcription factor p14ARF tumor suppressor. Molec Cell Biol 2000, 20:273-285.

52. Meyyappan M, Wong H, Hull C, Riabowol KT: Increased expression of cyclin D2 during multiple stages of growth arrest in primary and established cells. Mol Cell Biol 1998, 18:3163-3172.

53. Bodnar AG, Ouellette M, Frolikis M, Holt SE, Shiu CP, Morin GB, Harley CB, Shay JW, Lichtsteiner S, Wright WE: Extension of life-span by introduction of telomerase into normal human cells. Science 1998, 279:349-352.

54. Bryant TM, Englezou A, Gupta J, Bacchetti S, Reddel RR: Telomere elongation in immortal human cells without detectable telomerase activity. EMBO J 1995, 14:2420-2428.

55. Bryant TM, Marusici L, Bacchetti S, Namba M, Reddel RR: The telomere lengthening mechanism in telomerase-negative immortal human cells does not involve the telomerase RNA subunit. Hum Mol Genet 1997, 6:921-926.

56. Brown JP, Wei W, Sedivy JM: Bypass of senescence after disruption of p21CIP1/WAF1 gene in normal diploid human fibroblasts. Science 1997, 277:831-834.

57. Felsher DW, Bishop JM: Transient excess of MYC activity can elicit genomic instability and tumorigenesis. Proc Natl Acad Sci USA 1999, 96:3940-3944.

58. Rodriguez MS, Desterrro JM, Lain S, Midgley CA, Lane DP, Hay RT: SUMO-1 modification activates the transcriptional response of p53. EMBO J 1999, 18:6453-6461.

59. Kim SH, Rowe J, Fujii H, Jones R, Schmierer B, Kong BW, Kuchler K, Foster D, Ish-Horowicz D, Peters G: Upregulation of chicken p53INK4b at senescence and in the developing brain. J Cell Sci 2006, 119:2435-2443.