Abrogation of oncogene-induced senescence by PI3K pathway activation contributes to human nevus-to-melanoma progression

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Abrogation of oncogene-induced senescence by PI3K pathway activation contributes to human nevus-to-melanoma progression

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ABSTRACT

Human melanocytic nevi (moles) are benign neoplasms harboring an activated oncogene, most commonly BRAF or NRAS. Although initially, these oncogenes act mitogenically, eventually oncogene-induced senescence (OIS) ensues. This acts as a potent fail-safe mechanism, which usually persists for decades. Nonetheless, malignant melanoma may emerge within a nevus. However, formal proof for nevus-to-melanoma progression is lacking and the underlying mechanism is unclear. We identified identical driver (BRAF or NRAS) mutations in 10 out of 17 laser capture-microdissected nevi and their contiguous melanomas. The PI3K pathway was commonly induced in melanomas relative to nevi, either by a decrease in PTEN and/or an increase in AKT3 levels. RNAi depletion of PTEN was sufficient to abrogate OIS in cultured melanocytes. These observations suggest that PI3K pathway activation serves as a rate-limiting event in human melanoma progression, acting at least partly by abrogating OIS.

INTRODUCTION

Although the nevus (mole) is considered to be a precursor of melanoma, a highly aggressive type of (skin) cancer, relatively little is known about the mechanism underlying progression from nevus to melanoma. We and others have previously hypothesized that abrogation of OIS of nevus cells acts as a rate-limiting event in melanomagenesis (Bennett, 2003; Mooi and Peeper, 2006). Favoring this model, nevi and melanomas are commonly, and significantly, histologically associated (Stolz et al., 1989; Smolle et al., 1999; Bevona et al., 2003; and references therein). One of the most important molecular engines driving nevogenesis as well as melanomagenesis

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is the activation of the ERK pathway, mainly due to oncogenic BRAF (Davies et al., 2002; Pollock et al., 2003) or NRAS mutations (Raybaud et al., 1988; van ‘t Veer et al., 1989). Other frequent genetic events during melanoma progression include the loss of CDKN2A and ARF (Kamb et al., 1994; Nobori et al., 1994). Loss of p16\(^{INK4a}\) has long been suspected to play a critical role in the abrogation of OIS. However, although its involvement in melanomagenesis is undisputed (Curtin et al., 2005; COSMIC database) and a role in replicative senescence has been reported (Gray-Schopfer et al., 2006), there is little evidence to support a non-redundant role for p16\(^{INK4a}\) in BRAF\(^{E600}\)- or NRAS\(^{K61}\)-induced senescence, either in vitro (Michaloglou et al., 2005; Denoyelle et al., 2006; Haferkamp et al., 2009) or in vivo (Dhomen et al., 2009). Another common genetic event in melanoma is the activation of the PI3K pathway, which is seen in ~60% of cases (Stahl et al., 2004; Dhawan et al., 2002; reviewed in Wu et al., 2003). This can be achieved by loss of PTEN expression (by mutation or epigenetic silencing; Birck et al., 2000; Mirmohammadsadegh et al., 2006), by increased AKT3 activity owing to overexpression or mutation (Stahl et al., 2004; Davies et al., 2008), or, to a lesser extent, by mutations in PIK3CA (Omholt et al., 2006). Interestingly, some 20% of melanomas show concurrent mutation in BRAF and diminished expression of PTEN (Tsao et al., 2004; Daniotti et al., 2004; Lin et al., 2008). Therefore, we investigated whether activation of the PI3K pathway corresponds to a rate-limiting step in cellular senescence and human melanoma progression.

RESULTS AND DISCUSSION

Activation of the PI3K pathway abrogates BRAF\(^{E600}\)-induced senescence in primary human cells

To investigate the possible role of PI3K pathway activation in evasion of BRAF\(^{E600}\)-induced senescence, we first focused on any effect of PTEN loss. As expected, PTEN depletion from primary human fibroblasts (HDF) resulted in an increase in phosphorylated AKT (P-AKT), a critical downstream effector (Figure 1a). Consistent with our previous results (Michaloglou et al., 2005), ectopic expression of BRAF\(^{E600}\) in HDF induced a robust cell cycle arrest, along with induction of p16\(^{INK4a}\) (Figure 1a and b). To determine whether activation of the PI3K pathway antagonizes the cytostatic action of BRAF\(^{E600}\), we depleted PTEN. This led to an effective abrogation of BRAF\(^{E600}\)-induced arrest (Figure 1b), indicating a causal role for the PI3K signaling route in the growth-inhibitory action of BRAF\(^{E600}\), consistent with previous results by Cichowski and co-workers (Courtois-Cox et al., 2006). The increase in cell number as seen in proliferation assays was not due to decreased apoptosis, but reflected increased DNA replication as a function of PTEN depletion, as evident from BrdU incorporation assays (Figure 1c). As an alternative way to activate the PI3K pathway, we ectopically expressed either wild type, CAAX-tagged (activated) or cancer-
derived mutants (K545 and R1047) of PIK3CA. In all cases, this led to a strong increase in levels of P-AKT (Figure 1d) and cells efficiently bypassed BRAF<sup>E600</sup>-induced senescence (Figure 1e and f). We reproduced these results in two additional HDF strains, with ectopic expression of PIK3CA or activated AKT1 (AKT<sup>1<sub>MYR</sub></sup>) (Supplementary Figure 1a and b), indicating that these results were not cell strain-specific.

In view of the prominent role of the BRAF<sup>E600</sup> oncogene in nevus and melanoma, we next determined the role of the PI3K pathway in primary human melanocytes that were exposed to oncogenic BRAF. Consistent with previous results (Michaloglou et al., 2005; Denoyelle et al., 2006), BRAF<sup>E600</sup> induced senescence in melanocytes, as judged by a complete loss of proliferative activity (Figure 2a) and a strong increase

**Figure 1 | Activation of the PI3K pathway abrogates BRAF<sup>E600</sup>-induced senescence in HDF.** TIG3 HDF stably expressing one of two non-overlapping PTEN shRNAs (#1 or #2), or PIK3CA (wild type, cancer-associated or artificial mutants), as well as hTERT (to avoid confounding effects due to replicative senescence), were transduced with BRAF<sup>E600</sup>-encoding retrovirus and analyzed. a and d | Samples were analyzed by Western blotting for the indicated proteins 8 days p.i. β-Actin serves as loading control. b and e | Samples were analyzed in a proliferation assay and fixed and stained 8 days p.i. c and f | Samples were analyzed in a BrdU incorporation assay 3 days p.i. Data are represented as mean with SD.
in the number of SA-β-galactosidase-positive cells (Figure 2b). More importantly, as was observed for HDF, PTEN-depleted cells failed to undergo OIS: they continued to proliferate, which correlated with a sharp decline in SA-β-galactosidase-positivity (Figure 2a and b). Of note, similar to primary mammary epithelial cells (Brenner et al., 1998), primary melanocytes are under apparent selective pressure to lose p16INK4A during in vitro propagation (Bennett, 2003). To avoid undefined spontaneous mutations from occurring, these experiments were performed in p16INK4A-knockdown melanocytes (which still undergo BRAFE600-induced senescence), but similar results were obtained in wt melanocytes (data not shown). As expected, PTEN depletion, whether in the absence or presence of BRAFE600, increased the levels of total P-AKT (AKT 1, 2, and 3; Figure 2c). Interestingly, BRAFE600 expression in melanocytes strongly suppressed the accumulation of AKT3, the AKT isoform that is required for survival and transformed properties of melanoma cells (Stahl et al., 2004). This was restored in cells that bypassed BRAFE600-induced senescence as a result of PTEN knockdown. We conclude that activation of the PI3K pathway abrogates BRAFE600-induced senescence, both in HDF and human melanocytes.

**Figure 2** | *Activation of the PI3K pathway abrogates BRAF<sub>E600</sub>-induced senescence in primary human melanocytes.* Primary human melanocytes stably expressing sh-p16INK4A and either control or sh-PTEN were transduced with control or BRAF<sub>E600</sub>-encoding lentivirus and analyzed in a proliferation assay. **a** | BrdU incorporation assay 15 days p.i. A representative experiment out of 8 independent experiments performed is shown. **b** | Samples from (a) were stained for Senescence β-Galactosidase activity and analyzed by Western blotting for protein expression as indicated. β-Actin serves as loading control.
**Significant conservation of BRAF and NRAS mutations during progression from nevus to melanoma**

We next considered the possibility that in human melanocytic neoplasms also, activation of the PI3K pathway represents a rate-limiting step in the outgrowth of BRAF/E600-expressing melanocytes. We investigated a series of 21 human tissue sections with nevi in contiguity with melanomas, which were not separated by normal skin (as determined by an experienced pathologist). We first examined whether the cells of the nevus and the contiguous melanoma in each specimen were clonally related. Genetic evidence has been presented previously in support of a progression model (N-, K-RAS and BRAF mutations Demunter et al., 2001; Yazdi et al., 2003; Dadzie et al., 2009; LOH of 9p21 markers Bogdan et al., 2003), but no statistical analysis was used to rule out random co-occurrence of mutations within these lesions. We therefore set out to identify the conceivable ‘driver’ mutations in the nevus and melanoma compartments, focusing on the likely suspects BRAF and NRAS, as they are most commonly activated in melanomas as well as in nevi. Seventeen specimens were considered suitable for laser capture microdissection, after which the mutational status of BRAF exon 15 and NRAS exon 3 (in which most activating mutations reside; COSMIC database; http://www.sanger.ac.uk/genetics/CGP/cosmic/) were analyzed by genomic DNA PCR amplification and sequencing. Also sections of normal tissue were microdissected in all cases and processed along with the nevi and melanomas, serving as controls for positive identification of the wild type NRAS and BRAF alleles and for excluding possible contamination problems. We found the common BRAF/E600 mutation (encoding BRAF/E600) in eight samples, seven of which showed co-occurrence of the mutation in both the nevi and their flanking melanomas (Figure 3a; Supplementary Table 1). In one sample, we identified the rare BRAF/G1799-A900AA double mutation (also encoding BRAF/E600), in the melanoma as well as the associated nevus (Figure 3c-e; Supplementary Figure 2 for detailed information). In addition, activating mutations in NRAS were detected in four samples, two of which showed co-occurrence in nevi and their contiguous melanomas. The presence of a BRAF or NRAS mutation in three melanomas but not in their contiguous nevi suggests either that the benign and malignant lesions within these specimens are clonally unrelated, or that the lesions harbor driver mutations other than those located in BRAF exon 15 and NRAS exon 3, with mutation in BRAF or NRAS occurring later during melanoma progression. Finally, we identified four specimens with wild-type BRAF exon 15 and NRAS exon 3 sequences in all lesions, although we cannot exclude that some of the wild type alleles are false negatives due to the very small sample size in some cases.

To evaluate the statistical significance of these findings, we collected from the COSMIC database the percentages of each mutation identified in the above-described specimens (Figure 3b) and calculated for all cases the probability
that identical mutations in nevi and melanomas could have occurred by chance (Supplementary Table 1). For each mutation identified in the 17 specimens, we plotted the expected numbers based on random (co-)occurrence in the bars labeled ‘expected’ and the results obtained from the actual mutational analysis in the bars labeled ‘observed’ (Figure 3a). The chance of random co-occurrence based on the percentages from the COSMIC database was then compared to the actual result of the mutational analysis. As is indicated by a p-value <0.05, the co-occurrence of BRAF and NRAS mutations in flanking nevi and melanomas was statistically significant in 10/17 specimens, arguing that at least a considerable proportion of melanomas in this series are clonally related to their contiguous nevi.

![Diagram of mutational analysis](image)

### Supplementary Table 1

<table>
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<th>NRAS C181A</th>
<th>NRAS A182G</th>
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Decrease in PTEN and/or increase in AKT3 and P-AKT expression in melanoma relative to the contiguous nevus

To investigate the role of activation of the PI3K pathway in human melanomagenesis in vivo, we analyzed the relative expression levels of PTEN and AKT3 by immunohistochemical staining of our series of contiguous nevus-melanoma specimens. Five out of 21 samples displayed a reduced PTEN immunostaining in the melanoma compared to the associated nevus (Figure 4a-c and 4j-l; Table 1). Conversely, an increase in AKT3 staining in the melanoma compared to the nevus was detected in nine specimens (Figure 4d-f and 4m-o; Table 1). In two specimens, both PTEN reduction and AKT3 increase were seen. Correspondingly, in the majority of the specimens analyzed we detected induction of P-AKT in the melanoma relative to the nevus (Figure 4g-i; Table 1). These observations reveal a recurrent activation of the PI3K pathway in melanomas relative to their adjacent nevi, with alterations in PTEN and AKT accounting for 12/21 cases.

Figure 3 | Significant conservation of BRAF and NRAS mutations during progression from nevus to melanoma.

a | Bar graph representing expected and observed frequencies of BRAF and NRAS mutations in nevi (N) and contiguous melanomas (M). Seventeen specimens were suitable for laser capture microdissection, after which the mutational status of BRAF exon 15 and NRAS exon 3 (in which most activating mutations reside) were analyzed by genomic DNA PCR amplification and sequencing. The bar labeled ‘expected’ represents the calculated percentages of random (co)-occurrence, and the bar labeled ‘observed’ represents the actual mutational status of BRAF and NRAS in 17 nevi that are contiguous to melanomas. ‘Red’ indicates melanomas and their associated nevi harboring an identical mutation; ‘light grey’ indicates melanomas carrying a mutation, whereas the associated nevi contain corresponding wild-type alleles; ‘grey’ indicates, conversely, melanomas that have wild-type alleles, whereas the associated nevi carry the mutation; ‘dark grey’ indicates that both the melanomas and their associated nevi have wild-type alleles. For statistical analysis, we carried out the ‘one sample test’ for proportions, with the null hypothesis being that the mutations occurred randomly. The ‘two-sided alternative test’ was used to calculate p-values, which were less than 0.05 for the BRAF_{T1799A}, BRAF_{TG1799-1800AA}, and NRAS_{A182G} mutations, indicating that it is highly unlikely that the observed co-occurrence of these mutations (in red) has arisen due to chance. See Supplementary Table 1 for details.

b | To perform the statistical calculations in a, percentages of BRAF_{T1799A}, BRAF_{TG1799-1800AA}, NRAS_{C181A} and NRAS_{A182G} mutations were extracted from the COSMIC database; http://www.sanger.ac.uk/genetics/CGP/cosmic/). Melanomas and benign melanocytic nevi originating from skin were selected, excluding acral, mucosal, ocular and genital origin, and spitz and blue nevi, as these types where not included in our specimen panel.

c-e | A rare BRAF double mutation co-occurring in the nevus and the adjacent melanoma. Chromatograms of BRAF exon 15 PCR fragments amplified from genomic DNA of laser microdissected normal skin, nevus, melanoma, and of a fibroblast cell line as a control. The arrowhead indicates nt Thymine^{1799} in BRAF, which is often found mutated to an Adenine; an asterisk indicates nt Guanine^{1800}, which is rarely found mutated to an Adenine (c). Chromatograms of cloned PCR fragments encompassing BRAF exon 15 show either the wild type allele or the allele with the double mutation (d). Schematic overview of the BRAF_{T1799A} and BRAF_{TG1799-1800AA} mutations, both encoding BRAF^{E600} (e).
In summary, immunostaining of a series of human contiguous nevus/melanoma samples for several proteins within the PI3K pathway (PTEN, AKT3, and P-AKT) revealed that in more than half of the specimens this signaling route is induced in the melanoma relative to the adjacent nevus, either by (partial) loss of PTEN and/or by induction of AKT3. Several of these benign and malignant melanocytic counterparts within single biopsies harbored identical driver mutations in either BRAF (including a rare BRAF double mutation) or NRAS. Correspondingly, both in cultured HDF and human melanocytes, activation of the PI3K pathway, whether by PTEN silencing or ectopic expression of either (wt or mutant) PIK3CA or AKT, abolished BRAF$^{E600}$. 

**Figure 4** | Decrease in PTEN and/or increase in AKT3 and P-AKT expression in melanoma relative to the contiguous nevus. Representative immunohistochemical stainings of contiguous nevus-melanoma specimens with PTEN, AKT3, and P-AKT antibodies. Both nevus and melanoma compartments harbor the $BRAF^{T1799A}$ mutation (a-i and m-o), or the rare $BRAF^{T1799.G1800A}$ double mutation (j-l), as was determined by laser capture microdissection and sequence analysis. The melanoma compartment ('M') exhibits clearly weaker PTEN (a-c and j-l) and/or stronger AKT3 (d-f and m-o) and P-AKT (g-i) immunostaining compared to the nevus compartment ('N'). b, e, h, k and n show higher magnifications of the nevus and c, f, i, l and o of the melanoma. The cell-rich area underneath the melanoma compartment in a-i comprises mainly infiltrating lymphocytes.

**DISCUSSION**

In summary, immunostaining of a series of human contiguous nevus/melanoma samples for several proteins within the PI3K pathway (PTEN, AKT3, P-AKT) revealed that in more than half of the specimens this signaling route is induced in the melanoma relative to the adjacent nevus, either by (partial) loss of PTEN and/or by induction of AKT3. Several of these benign and malignant melanocytic counterparts within single biopsies harbored identical driver mutations in either BRAF (including a rare BRAF double mutation) or NRAS. Correspondingly, both in cultured HDF and human melanocytes, activation of the PI3K pathway, whether by PTEN silencing or ectopic expression of either (wt or mutant) PIK3CA or AKT, abolished $BRAF^{E600}$. 
induced senescence. Taken together, our results support a melanoma progression model, in which oncogene-bearing melanocytic nevi acquire (an) additional mutation(s) activating the PI3K pathway. This is in line with the fact that nevi, during a lifespan that can go up to several decades, show a very low level of proliferative activity (Mooi and Krausz, 2007). We demonstrate that at least one critical function of PI3K pathway activation in this setting corresponds to the abrogation of the OIS program, allowing for malignant progression. Together, our results strongly argue that at least a proportion of human melanomas emerge from nevi.

The observation that identical BRAF and NRAS mutations are present both in the nevi and their flanking melanomas (in at least 10/17 cases) is compatible with a model in which the BRAF/NRAS mutations precede alterations in PTEN and AKT, but leaves open the possibility that mutations in both pathways are acquired within a short time frame. The functional interplay between BRAF<sup>E600</sup> and PI3K pathway

### Table 1 | Activation of the PI3K pathway and mutational status of BRAF and NRAS in a series of contiguous human nevus-melanoma specimens.

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<th>PTEN reduction or loss in melanoma relative to nevus</th>
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V present

? BRAF exon 15 and NRAS exon 3 are wt sequence; remainder of the genes were not sequenced; hence, the driver mutations in these lesions are unknown

ND not determined, because specimen was not suitable for laser capture microdissection

N Nevus

M Melanoma

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activation may explain the frequent co-occurrence of activating mutations in BRAF and the PI3K pathway in melanoma. Clearly, although PI3K induction in melanoma was seen in roughly half of the cases showing diminished PTEN expression or increased AKT3 production, other pathways are likely to contribute to melanoma progression as well. This notwithstanding, our results indicate that alterations at several levels in the PI3K pathway represent a common feature of progression from nevus to melanoma.

Although BRAF\textsuperscript{E600} did not decrease total P-AKT levels in cultured melanocytes, AKT3 expression levels were downregulated. This observation merits further investigation into the relative roles of the three AKT family members in melanocytes. It has been shown previously that AKT3 is the critical isoform in melanoma cell lines (Stahl et al., 2004) and that overexpression of activated AKT3 enhances anchorage-independent growth of melanoma cells (Cheung et al., 2008). Conversely, specific inhibition of AKT3 has been reported to induce apoptosis and inhibit the tumorigenic capacity of melanoma cells in a xenograft model (Stahl et al., 2004). Our in vitro and in vivo data are consistent with, and extend, these observations and support a prominent role of AKT3 in melanomagenesis.

Our results are in agreement with data on two BRAF\textsuperscript{E600} knockin mouse models published recently. Dhomen et al. showed that the induction of BRAF\textsuperscript{E600} in melanocytes results in senescent melanocytes forming (blue) nevi. After a year, half of these mice developed melanoma, presumably by acquiring additional (yet to be defined) mutations. When the BRAF\textsuperscript{E600} nevus-carrying mice were bred onto a p16\textsuperscript{INK4A}-null background, they produced more tumors, which developed earlier, but nonetheless, these mice continued to develop nevi (Dhomen et al., 2009). This is consistent with the mosaic pattern of p16\textsuperscript{INK4A} immunopositivity frequently seen in nevi (Michaloglou et al., 2005) and the observation that p16\textsuperscript{INK4A}-deficient individuals have nevi in addition to an increased propensity to develop melanoma (Pavel et al., 2003). Also Dankort et al. found murine benign melanocytic hyperplasia in the context of an endogenous BRAF\textsuperscript{E600}-encoding allele. These lesions were highly stable and failed to progress to melanoma for at least up to 20 months. However, upon simultaneous activation of BRAF\textsuperscript{E600} and deletion of PTEN, mice quickly developed metastatic melanoma (Dankort et al., 2009). In aggregate, these and our results support a model in which mutant BRAF and activation of the PI3K pathway cooperate in melanomagenesis both in mice and humans, with PTEN loss and PI3K/AKT activation serving, at least in part, to abrogate OIS.

Finally, we show here that by integration of in vitro studies on melanocytes with genetically and immunohistochemically characterized human contiguous nevus/melanoma resection specimens we can identify signaling pathways, deregulation of which contributes to melanoma. The elucidation of the pathobiology of nevi will
improve our understanding of OIS in vivo in general as well as of melanomagenesis in particular, and may well identify diagnostic biomarkers that allow a better distinction from melanoma in problem cases. Furthermore, our results predict that inhibitors of the PI3K pathway may restrain the expansion of tumors with mutations in BRAF or NRAS through restoration of OIS, a concept that is receiving increasing interest (Lin et al., 2010; Alimonti et al., 2010; reviewed in Collado and Serrano, 2010).

MATERIALS AND METHODS

Cell culture, viral transduction
The human diploid fibroblast cell lines TIG3 and HCA2 co-expressing the ecotropic receptor and hTERT, as well as Phoenix and HEK293T cells were maintained in DMEM (Gibco) supplemented with 9% fetal bovine serum (Greiner Bio-One), 2 mM glutamine, 100 units ml⁻¹ penicillin, and 0.1 mg ml⁻¹ streptomycin (all Gibco). The human diploid fibroblast cell line IMR90 expressing the ecotropic receptor, hTERT, and shRNA for p16INK4A was maintained in MEM + Earle’s salts (GIBCO) containing all supplements mentioned above and nonessential amino acids, 0.15% sodium bicarbonate, and 1 mM sodium pyruvate (all GIBCO). Melanocytes were maintained in Medium 254 (Cascade Biologicals) supplemented with melanocyte growth supplement (HMGS; Cascade Biologicals), 100 units ml⁻¹ penicillin and 0.1 mg ml⁻¹ streptomycin (Gibco).

Retroviral transductions were performed using Phoenix cells as producers. Retrovirus production employing Phoenix cells was as described (http://www.stanford.edu/group/nolan/retroviral_systems/phx.html). For production of lentivirus, HEK293T cells were refreshed with complete medium containing 25 μM chloroquine, transfected with 8 μg of lentiviral construct and 3 μg of each of the helper plasmids pMDLgplRRE, pHCMV-G, and pRSVrev, and washed and refreshed in complete medium after 6 hours. Lentivirus was frozen and diluted for transduction. For TIG3, HCA2, and IMR90 cell proliferation assays, cells were transduced with shRNA- or cDNA-encoding retrovirus and checked for successful proviral integration (by Puromycin selection or GFP expression). The cells were plated at equal densities and subsequently transduced with BRAF E600-encoding retrovirus and selected (Blasticidin). The cells were analysed for BrdU incorporation (three hours) three days post-infection (p.i.), and fixed and stained with crystal violet and harvested for Western blotting analysis eight days p.i. For melanocyte proliferation assays, cells were transduced with shRNA- or cDNA-encoding lentivirus, cultured for one week with 254 Medium supplemented with HMGS-2 (-PMA; Cascade Biologicals), and subsequently transduced with BRAF E600-encoding lentivirus. After selection for the successful integration of the oncogene, cells were seeded at equal density and analysed for BrdU incorporation (three hours), SA-β-GAL staining (Senescence β-Galactosidase Staining Kit #9860; Cell Signalling), and harvested for Western blotting analysis 15 days p.i..

Plasmids
pRS₄₀₋sh-PTEN(#1, #2), pMX₄₋GFP-PK3CAwt, K545, K1047, CAAX, and pMSCV₄₋BLAST-BRAF E600 were used for retroviral transduction. KH1₋GFP-sh-p16INK4A, KH1₋GFP-sh-PTEN, and HIV-CSCG₋BLAST-BRAF E600 were used for lentiviral transductions. Sequences for sh-PTEN are described in Supplementary Experimental Procedures.

Antibodies
Antibodies used for Western blotting were against BRAF (sc-5284; Santa Cruz), PTEN (sc-7974; Santa Cruz), AKT3 (#4059; Cell Signaling), phospho-Ser473-AKT (#9271; Cell Signaling), AKT1/2/3 (sc-8312; Santa Cruz), p16INK4A (JC8; Immunologic), p15INK4B (sc-612; Santa Cruz), and β-actin (AC-74; A5316; Sigma).
**Immunohistochemistry**

Twenty-one formalin-fixed paraffin-embedded human nevus in contiguity with melanoma specimens were immunostained for PTEN (clone 6H2.1; DAKO), AKT3 (HPA026441; Sigma-Aldrich), and P-AKT (Ser473) (736E11; Cell Signaling), as previously described (Michaloglou et al., 2005).

**Laser capture microdissection and mutation analysis**

Contiguous nevus-melanoma specimens were sectioned (8 µM) on P.A.L.M. Pen Membrane Slides (Microlaser Technologies), which were pretreated according to the manufacturer’s instructions with UV and Poly-L-Lysine, then deparaffinized and stained with Hematoxyline. Laser capture microdissection was used to isolate normal tissue, nevus cell groups and melanoma cell groups, which were identified by pathologist W.J.M., employing consecutive H&E sections. Genomic DNA was isolated with QIAamp DNA micro kit (Qiagen). PCR conditions (TaqPlus Precision PCR system; Stratagene) and primers for PCRs for BRAF exon 15 and NRAS exon 3 are described in Supplementary Experimental Procedures. PCR products were sequenced using the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems) and an ABI 3730 automated capillary sequencer.

**ACKNOWLEDGEMENTS**

We would like to thank N. Armstrong for statistical analysis; R. Kortlever, M. Voorhoeve and K. Berns for kindly providing constructs; T. Kuilman for critical reading of the manuscript; and all members of the Peeper lab for their valuable input. This work was supported by grants from the Dutch Cancer Society (KWF Kankerbestrijding, including a Queen Wilhelmina Award program) and Vidi and Vici grants from the Netherlands Organization for Scientific Research (NWO) to L.C.W.V., C.M. and D.S.P.
BRAF

E600

and PI3K in OIS and melanoma progression


SUPPLEMENTARY METHODS

Sequences sh-RNAs

sh-p16INK4A GCATGGAGCCTTCGGCTGACT (Michaloglou et al., 2005)

sh-PTEN#1 GTGAAGATGACAATCATGT

sh-PTEN#2 GGCGTATACAGGAACAATA

Primers (underlined are used for sequence PCR)

PCR BRAF exon 15 F 5’ TAATGCTTGCTCTGATAGGA 3’

PCR BRAF exon 15 R 5’ GGCCAAAAATTTAATCAGTG 3’

Nested PCR BRAF exon 15F 5’ TGTITTTTCTTTACTTACCTCA 3’

Nested PCR BRAF exon 15R 5’ CCACAAATGGATCCAGACA 3’

PCR NRAS exon 3F 5’ CACCCCCAGGATCTTACAG 3’

PCR NRAS exon 3R 5’ TGGTAACCTCACTTCCCCATA 3’

Nested PCR NRAS exon 3F 5’ TGGTGAAACCTGTTTTGGG 3’

Nested PCR NRAS exon 3R 5’ TCCGCAAATGACTTGCTATT 3’

PCR TaqPlus Precision (Stratagene)  

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<tr>
<th>PCR TaqPlus Precision (Stratagene)</th>
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<tr>
<td>13.3 µl MQ</td>
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<td>2.5 µl TaqPlus Buffer</td>
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<tr>
<td>0.2 µl dNTP (25 mM each)</td>
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</tr>
<tr>
<td>2.5 µl gDNA/PCR 1</td>
<td>55 °C 1’ 35*</td>
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<td>0.625 µl primer F (10 mM)</td>
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<td>0.25 µl TaqPlus</td>
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<tr>
<td>5 µl Betaine</td>
<td>72 °C 10’</td>
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<td>25 µl total</td>
<td>4 °C ∞</td>
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**Supplementary Figure 1** | Activation of the PI3K pathway allows bypass of BRAF<sup>E600K</sup> induced senescence in HDF. 

a | HCA2 or b | IMR90-sh-p16<sup>INK4A</sup> HDF, stably overexpressing PIK3CA (wildtype, cancer mutants, CAAAX-tagged) or AKT1 (MYR-tagged) as indicated as well as hTERT, were transduced with BRAF<sup>E600K</sup>-encoding retrovirus and used in a proliferation assay. The samples were fixed and stained 8 days p.i.

**Supplementary Figure 2** | Microdissection of a contiguous nevus-melanoma specimen carrying the rare BRAF<sup>TG1799.S1805A</sup> double mutation. 

a | H&E staining of a lesion that was removed from the left upper leg of a 41-year-old male. After a period of many years of clinical stability, the lesion had recently grown to a size of about 7 mm. The lesion was excised with narrow margins. Histologically, the periphery of the lesion of one side showed the features of a melanocytic compound nevus devoid of cytologic or architectural atypia, and devoid of mitotic activity. The center and other side of the lesion, however, consisted of a mitotically active population of enlarged round to oval melanocytes arranged in compact nodules and stands. Mitotic figures were identified near the base of this component. The overlying intact epidermis was slightly flattened and was eccentrically elevated as a result of the subjacent accumulation of these atypical melanocytes. A diagnosis of superficial spreading melanoma, Clark IV, thickness 1.5 mm, devoid of ulceration and in contiguity with melanocytic compound nevus was rendered. Follow-up has so far been unremarkable. Higher magnifications of b | nevus and c | melanoma. d | Image of a section after microdissection stained with hematoxylin. N, M, and NL indicate areas with nevus, melanoma and normal tissue, respectively.
Supplementary Table 1 | Significant conservation of BRAF and NRAS mutations during progression from nevus to melanoma. Percentages of BRAF and NRAS mutations derived from the COSMIC database, calculated percentages of random (co)-occurrence and expected and actual mutational status of 17 contiguous nevus-melanoma specimens. For statistical analysis, the one sample test for proportions was used, with the null hypothesis being that the mutations occurred spontaneously was used. The two-sided alternative was used to calculate p-values, which were less than 0.05 for the **BRAF^T1799A**, **BRAF^TG1799-1800AA**, and **NRAS^A182G** mutations indicating that the co-occurrence of these mutations is unlikely to have arisen due to chance. This table is represented as a bar graph in Figure 3a.

<table>
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<tr>
<th></th>
<th>BRAF T1799A</th>
<th>BRAF TG1799-1800AA</th>
<th>NRAS C181A</th>
<th>NRAS A182G</th>
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<tr>
<td>Nevus</td>
<td>41.7%</td>
<td>0.4%</td>
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<td>Melanoma</td>
<td>38.4%</td>
<td>0.3%</td>
<td>7.2%</td>
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<td>Likelihood of (co-)occurrences</td>
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<td>Mutation in melanoma and nevus</td>
<td>2.7/17 (16.0%)</td>
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<td>0.053/17 (0.31%)</td>
<td>2.9/17 (17.0%)</td>
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<td>Mutation only in melanoma (nevus wildtype)</td>
<td>3.8/17 (22.4%)</td>
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<td>1.4/17 (8.3%)</td>
<td>6.4/17 (37.5%)</td>
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<td>Mutation only in nevus (melanoma wildtype)</td>
<td>4.4/17 (25.7%)</td>
<td>0.068/17 (0.4%)</td>
<td>1.4/17 (8.4%)</td>
<td>0.56/17 (3.3%)</td>
<td>6.4/17 (37.7%)</td>
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<td>Melanoma and nevus wildtype</td>
<td>6.1/17 (35.9%)</td>
<td>16.9/17 (99.3%)</td>
<td>14.4/17 (84.4%)</td>
<td>15 (88.1%)</td>
<td>1.3/17 (7.8%)</td>
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<td>Results of mutational analysis</td>
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<td>Mutation in melanoma and nevus</td>
<td>7/17 (41.2%)</td>
<td>1/17 (5.9%)</td>
<td>2/17 (11.8%)</td>
<td>10/17 (58.8%)</td>
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<td>p = 0.012</td>
<td>p = 0.0002</td>
<td>p = 0.0013</td>
<td>p = 0.00012</td>
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<tr>
<td>Mutation only in melanoma (nevus wildtype)</td>
<td>1/17 (5.9%)</td>
<td>1/17 (5.9%)</td>
<td>1/17 (5.9%)</td>
<td>3/17 (17.6%)</td>
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<tr>
<td>Melanoma and nevus wildtype</td>
<td>10/17 (58.8%)</td>
<td>16/17 (94.1%)</td>
<td>16/17 (94.1%)</td>
<td>4/17 (23.5%)</td>
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Abrogation of oncogene-induced senescence by SV40 small t antigen

Manuscript in preparation
Abrogation of oncogene-induced senescence by SV40 small t antigen

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ABSTRACT

The senescence program corresponds to a largely permanent proliferative arrest that is relayed by the induction of several tumor suppressors, including p53, RB and p16INK4A. Senescence can be triggered by telomere erosion, as a function of cell division. But other types of stress, such as oncogene activation, can induce senescence prematurely, that is, in the absence of telomere shortening. Oncogene-induced senescence (OIS) in vitro has been widely used as a tool to identify and characterize oncogenes and tumor suppressors. Indeed, OIS acts as a major obstacle for a cell to become oncogenically transformed. SV40 Large T antigen (LT) is a viral oncoprotein that simultaneously inactivates p53 and RB. As such, it can be used in conjunction with hTERT to immortalize primary human cells. To acquire a completely transformed phenotype, these cells also need to express SV40 small t antigen (st) as well as an activated oncogene, like RASV12. SV40 st is transcribed from the same viral locus as LT, but is differentially spliced, yielding a unique C-terminus. Previously, we have shown that nevi, long-term arrested melanocytic lesions that often harbor an oncogenic BRAF mutation, display several hallmarks of senescence. To investigate the underlying mechanism of this stable arrest, we have used BRAFV600E-induced senescence in primary cells as a tool to screen for new tumor suppressor pathways. We show that inactivation of both the p53 and the RB/p16INK4A pathways, by LT or specific short-hairpin RNAs, is insufficient to effectively abrogate BRAFV600E-induced senescence. In contrast, we find, unexpectedly, that SV40 st alone resiliently disrupts the BRAFV600E-induced senescence response. Our results therefore point to the existence of a cellular senescence-associated tumor suppressor pathway that is targeted by SV40 st antigen.

INTRODUCTION

Activation of an oncogene in primary cells can lead to programmed cell death (apoptosis) or to premature senescence (reviewed in Lowe et al., 2004). Already in the eighties, several laboratories have noticed that primary rodent cells transfected with
an oncogenic RAS allele failed to undergo oncogenic transformation, but instead underwent cell cycle arrest (Land et al., 1983; Newbold and Overell, 1983; Ruley, 1983; Jenkins et al., 1984; Franz et al., 1986). Not less than a decade later, Serrano et al. described that overexpression of RAS\textsuperscript{V12} in mouse and human primary fibroblasts induces a proliferative arrest that has many of the hallmarks of cells that undergo replicative senescence as a result of telomere erosion (Serrano et al., 1997). Now, it is well established that cells undergoing (oncogene-induced) senescence stop dividing, activate several tumor suppressor pathways. They also commonly display induction of senescence-markers, such as senescence-associated heterochromatin foci (SAHF) (Narita et al., 2003) and increased senescence-associated acidic β-galactosidase (SA-β-GAL) activity (Dimri et al., 1995). Oncogene-induced senescence (OIS) can be elicited by oncogenes like RAS\textsuperscript{V12}, as well as by active forms of its downstream effectors such as BRAF (like BRAF\textsuperscript{E600}; Michaloglou et al., 2005), CRAF (∆RAF-1; Zhu et al., 1998), and MEK (MEK\textsuperscript{P215}b; Lin et al., 1998), in several cell types from different species (Serrano et al., 1997; Lloyd et al., 1997; Olsen et al., 2002; Nicke et al., 2005; Denoyelle et al., 2006).

Unlike replicative senescence, OIS is not caused by telomere attrition. It is often found to depend on the p53 and/or RB/p16\textsuperscript{INK4A} pathway, but this may vary among cell types and oncogenes under study. In senescent cells, p53 is often stabilized and activated, which leads to the induction of transcriptional targets like the cyclin-dependent kinase inhibitor (CKI) p21\textsuperscript{CIP1}. Furthermore, senescent cells often display higher levels of another CKI, p16\textsuperscript{INK4A}. Together with p21\textsuperscript{CIP1}, this will induce hypophosphorylation of RB, which results in repression of several transcription factors, including those comprising the E2F family (reviewed in Ben-Porath and Weinberg, 2005). The individual contributions of the p53 and RB/p16\textsuperscript{INK4A} pathways in OIS vary. For example, RAS\textsuperscript{V12}-induced senescence in human diploid fibroblasts (HDF) is dependent either on the RB/p16\textsuperscript{INK4A} pathway only (Brookes et al., 2002; Huot et al., 2002; Benanti and Galloway, 2004), only on the p53 pathway (Voorhoeve and Agami, 2003), or on both pathways (Serrano et al., 1997; Morales et al., 1999; Hahn et al. 2002; Wei et al., 2003). The differences might be attributed to the use of different HDF cell strains and/or different tools for disrupting the p53 and RB/ p16\textsuperscript{INK4A} pathways. The latter can be accomplished by using cell lines from donors homologous for p16\textsuperscript{INK4A} mutations (Brookes et al., 2002), homologous recombination targeting p53 or p21\textsuperscript{CIP} (Wei et al., 2001; Wei et al., 2003), expression of short hairpin (sh) RNAs (sh-p53, sh-RB, sh-p16\textsuperscript{INK4A}; Voorhoeve and Agami, 2003), overexpression of dominant negative (DN) tumor suppressors (p53\textsuperscript{DN}), or overexpression of (activated) downstream targets (CDK4\textsuperscript{R24C}, CYCLIN D) (Hahn et al., 2002). An alternative to disrupt the cell's major tumor suppressor pathways is to make use of viral oncoproteins like SV40 LT, adenovirus E1A/E1B or HPV E6/E7. Indeed, these factors
have been instrumental in the discovery of tumor suppressors.

SV40 Large T antigen (LT) can bind simultaneously to, and thereby inactivate, both p53 and RB (reviewed in Levine, 2009). It is transcribed from the SV40 early region sequence (ER), which, by means of alternative splicing, encodes two additional proteins, 17KT and small t antigen (st) (Crawford et al., 1978; Zerrahn et al., 1993; and Figure 1). SV40 17KT inhibits RB, whereas SV40 st inhibits the protein phosphatase 2A (PP2A) family of serine-threonine phosphatases, but may inhibit also factors that remain to be identified. SV40 LT and st have been used extensively to study immortalization and oncogenic transformation. Expression of LT in primary cells can induce immortalization through the inhibition of p53 and RB, although human cells also require expression of hTERT to be fully immortalized (Hahn et al., 2002; reviewed in Ahuja et al., 2005). While SV40 st expression causes efficient transformation of LT-immortalized rodent cells, human LT- and hTERT-immortalized cells require the additional presence of both SV40 st and RAS$^{\text{V12}}$ to be fully transformed (reviewed in Arroyo and Hahn, 2005).

**Figure 1 | Schematic overview of Simian Virus 40 Early Region (SV40 ER).** SV40 ER encodes three proteins by alternative splicing; LT (708 amino acid (aa)) is translated from exon 1 and 2; 17KT (135 aa) is similar to the first 131 aa of LT and then splices onto a different reading frame to encode its C-terminal 4 aa; st (174 aa) shares the first 82 aa with LT and 17KT, but then reads into the ‘intron’, yielding a unique C-terminus. All three proteins contain the J domain. LT interacts with p53, both LT and 17KT interact with RB family members, and st binds PP2A; interaction sites are indicated.

BRAF$^{E600}$, the most common BRAF mutation found in human cancer, is a constitutively active mutant kinase (Davies et al., 2002). It is found in several types of cancer, but is most commonly mutated in melanoma and thyroid cancer (reviewed in Michaloglou et al., 2008). Furthermore, BRAF mutations are detected in nevi (moles) (Pollock et al., 2003), which are benign melanocytic lesions that show several hallmarks of OIS (Michaloglou et al., 2005; Gray-Schopfer et al., 2006). While it is now commonly accepted that OIS serves as a bona fide tumor suppressor mechanism...
acting alongside apoptosis, we have an incomplete picture of the pathways by which it is relayed. Therefore, we have used BRAF\textsuperscript{E600}-induced senescence in primary cells as a platform to screen for new tumor suppressor pathways. Here, we dissect the contribution of SV40-encoded viral oncoproteins in this setting.

**RESULTS**

**BRAF\textsuperscript{E600}-induced senescence in human diploid fibroblasts is independent of the p53 and RB/p16\textsuperscript{INK4A} pathways**

Previously, we have reported that BRAF\textsuperscript{E600} induces premature senescence in several HDF strains (Michaloglou et al., 2005). Although this cell cycle arrest is accompanied by the induction of the tumor suppressor p16\textsuperscript{INK4A}, it does not strictly require its presence. Rather, p16\textsuperscript{INK4A} seems to slow down proliferation in general, whereas its depletion enhances the effect of senescence-bypassing events (Michaloglou et al., 2005; and data not shown). Furthermore, although we have observed previously that LT can abrogate senescence induced by BRAF\textsuperscript{E600}, it does not do this robustly (Michaloglou et al., 2005; see also Figure 4). This prompted us to examine the involvement other tumor suppressors in senescence. We first transduced the BJ and TIG3 HDF cell strains with retrovirus expressing hTERT (generating BJ\textsubscript{hTERT} and TIG3\textsubscript{hTERT} cells) to avoid potential confounding effects of replicative senescence. It should be noted that hTERT has been shown previously to be unable to interfere with OIS (Wei and Sedivy, 1999), enabling us to study OIS in this cell system. These immortalized fibroblast lines were transduced with retrovirus encoding BRAF\textsuperscript{E600}. Figure 2a shows that p16\textsuperscript{INK4A} was consistently upregulated by BRAF\textsuperscript{E600} in both HDF strains. For p53 we obtained variable results: while it was downregulated upon overexpression of BRAF\textsuperscript{E600} in BJ\textsubscript{hTERT} cells, it was upregulated in TIG3\textsubscript{hTERT} cells. As shown by western blotting, occasionally we observed a slight downshift in the migration of p53 on SDS polyacrylamide gels, the significance of which is unknown. Both HDF strains showed decreased protein levels of p21\textsuperscript{CIP1}, an established p53 target that is often involved in the induction of senescence. Together, this suggests that neither this CDK inhibitor, nor p53, is involved in bringing about BRAF\textsuperscript{E600}-induced senescence.

To test the validity of this hypothesis, the role of p53 in BRAF\textsuperscript{E600}-induced senescence was addressed directly. To this end, we expressed shRNAs targeting p53 in TIG3\textsubscript{hTERT} cells and subsequently transduced these cells with retrovirus encoding BRAF\textsuperscript{E600}. As shown in a proliferation assay, cells depleted for p53 levels still underwent BRAF\textsuperscript{E600}-induced senescence (Figure 2b and c). Previously, it has been reported that in several settings RAS\textsuperscript{V12}-induced senescence in HDF is dependent on both the p53 and the RB/p16\textsuperscript{INK4A} pathways (see above). This led us to investigate whether simultaneous disruption of these pathways would allow cells to bypass BRAF\textsuperscript{E600}-induced senescence. This was accomplished by combining the overexpression of a p16\textsuperscript{INK4A-}}
insensitive CDK4 mutant (CDK4^{R24C}) and the expression of sh-p53. Consistent with our previous observations (Michaloglou et al., 2005), cells overexpressing CDK4^{R24C} continued to undergo BRAF^{E600}-induced senescence (Figure 2b and c). Remarkably, cells without functional p53 and RB/p16^{INK4A} pathways also underwent BRAF^{E600}-induced cell cycle arrest (Figure 2b and c). Therefore, we conclude that BRAF^{E600}-induced senescence in HDF occurs largely independently of the RB/p16^{INK4A} and p53 pathways. These results are consistent with the observation that simultaneous depletion of p16^{INK4A} and p53, even in conjunction with p15^{INK4B} depletion, fails to abolish BRAF^{E600}-induced senescence (C.M. and D.S.P., unpublished observations). This distinguishes BRAF^{E600} from RAS^{V12}, which triggers cell cycle arrest through the RB and p53 pathways (Campisi, 2005).

Figure 2 | BRAF^{E600} induced senescence in human diploid fibroblasts is independent of the p53 and RB/p16^{INK4A} pathways. a | Regulation of tumor suppressors in HDF upon BRAF^{E600} expression and as a function of p53 expression; western blot analysis of samples analyzed 8 days post infection (p.i.). CDK4 serves as loading control. b | TIG3_{hTERT} HDF stably expressing p53 shRNA and/or CDK4^{R24C} were transduced with BRAF^{E600}-encoding retrovirus and analyzed in a proliferation assay; samples were fixed and stained 15 days p.i. c | Western blot analysis; samples were analyzed for the indicated proteins, β-ACTIN serves as loading control.
**BRAF^{E600} can replace RAS^{V12} in an in vitro oncogenic transformation assay**

This result led us to look for alternative pathways mediating BRAF^{E600}-induced proliferative arrest. As alluded to above, several primary cell types, including HDF, human embryonic kidney cells and human mammary epithelial cells, can be transformed with a defined set of genetic lesions, namely, hTERT, SV40 st and LT, and RAS^{V12} (Yu et al., 2001; Hahn et al., 2002). First, we examined whether BRAF^{E600} can replace RAS^{V12} in this experimental setting. We expressed in BJ_{hTERT} cells either sh-p16^{INK4A} or CDK4^{R24C} together with sh-p53, and subsequently transduced these cell lines with retrovirus encoding SV40 st. These cells served as controls and were unable to form colonies in semi-solid medium (soft agar). But as expected, when RAS^{V12} was introduced, these cells became transformed and grew anchorage-independently (Figure 3). Also expression of BRAF^{E600}, instead of RAS^{V12}, induced the formation of colonies in soft agar, although there were fewer and they were smaller than the RAS^{V12}-transformed colonies (Figure 3). These results obtained in vitro are consistent with our previous in vivo data, showing that RAS^{V12}- and BRAF^{E600}-expressing cell lines are tumorigenic in immunocompromised mice (Michaloglou et al., 2005). Thus, although slightly less efficient than RAS^{V12}, BRAF^{E600} can transform human cells in the context of a set of defined genetic lesions.

**SV40 small t abrogates BRAF^{E600}-induced senescence more effectively than LT**

Thus, BRAF^{E600}-expressing cells that lack functional RB/p16^{INK4A} and p53 pathways continue to undergo senescence, whereas cells that express BRAF^{E600} in the presence of CDK4^{R24C}, sh-p53, and SV40 st not only continue to proliferate, but are also oncogenically transformed. The difference between the two experiments is the expression of SV40 st, which encouraged us to examine what, in fact, the effect of SV40 st expression is in the context of BRAF^{E600}-induced senescence. As shown by a strong increase in the cell number in a proliferation assay (Figure 4a), and a four to five-fold increase in the number of DNA-replicating cells (Figure 4b), SV40 st
expression in TIG3<sub>hTERT</sub> cells abolished BRAF<sub>E600</sub>-induced senescence efficiently. Confirming the results presented in Figure 2, expression of LT was insufficient to cause a robust bypass of BRAF<sub>E600</sub>-induced senescence. Furthermore, the presence of LT failed to enhance the effect of SV40 st in abolishing the senescence response. The DNA expression constructs we used for SV40 st or LT contain only the coding regions of the respective proteins as confirmed by western blotting (Figure 4c) and qRT-PCR analysis (Supplementary Figure 1a). On the contrary, when the entire SV40 ER locus was introduced into TIG3<sub>hTERT</sub> cells, both SV40 st and LT were expressed (Figure 4c; note that we did not detect the 17KT polypeptide). Introduction of ER in TIG3<sub>hTERT</sub> cells resulted in an intermediate bypass of BRAF<sub>E600</sub>-induced senescence, which can be explained by the lower levels of SV40 st expression in this setting than when expressed alone (Figure 4c and Supplementary Figure 1a). The same result was obtained in BJ<sub>hTERT</sub> cells (Supplementary Figure 1b), indicating that the SV40 st-mediated bypass of BRAF<sub>E600</sub>-induced senescence is not cell strain-specific.

**Figure 4 | SV40 small t, but not SV40 LT, allows bypass of BRAF<sub>E600</sub>-induced senescence in human diploid fibroblasts.** TIG3<sub>hTERT</sub> HDF stably overexpressing SV40 st, SV40 LT, both, or SV40 ER were transduced with BRAF<sub>E600</sub>-encoding retrovirus and analyzed. a | Proliferation assay; photomicrographs were taken 10 days p.i.; samples were fixed and stained 13 days p.i. b | BrdU incorporation assay; samples were analyzed 10 days p.i. c | Western blot analysis; samples were analyzed for the indicated proteins 8 days p.i. β-ACTIN serves as loading control.
Previously, it has been reported that AKT, amongst others, is dephosphorylated and inactivated by the PP2A protein phosphatase (Andjelkovic et al., 1996; Van Kanegan et al., 2005). Since SV40 st inhibits PP2A, this can result in an induction of phosphorylated AKT (p-AKT) (Yuan et al., 2002; Zhao et al., 2003). Furthermore, several groups have shown that in a transformation assay, SV40 st can be replaced by factors that activate the PI3K pathway, for example, activated PIK3CA, activated AKT and RACV12 (Zhao et al., 2003), or knockdown of PTEN (Boehm et al., 2005). Consistent with these observations, we have shown that activation of the PI3K pathway can bypass \textit{BRAF}^{E600}-induced senescence (Chapter 3). However, in our experimental system overexpression of SV40 st failed to activate AKT, and also its downstream target \textit{p27}^{KIP1} was not consistently regulated (Figure 4c). Similarly, other known downstream targets of SV40 st, \textit{CYCLIN D1} and \textit{CYCLIN A} (reviewed in Skoczylas et al., 2004), were not regulated by SV40 st in HDF (Figure 3c). Lastly, we investigated another target of PP2A that can also replace SV40 st in a transformation assay, c\textit{MYC} (Yeh et al., 2004). As high expression levels of c\textit{MYC} induce apoptosis, we diluted the c\textit{MYC}-expressing virus and made stable cell lines expressing low levels of c\textit{MYC}. As shown in Supplementary Figure 2, low levels of c\textit{MYC} could bypass \textit{BRAF}^{E600}-induced senescence, albeit less efficiently than SV40 st. Similar results were obtained with a stable mutant of c\textit{MYC} found in Burkitt lymphoma (c\textit{MYC}^{T58A}) (data not shown). We conclude that our results point to the existence of a cellular tumor suppressor pathway that is targeted by SV40 st antigen, but the specific cellular pathway involved remains to be elucidated.

**DISCUSSION**

Previously, we have shown that melanocytic nevi are senescent benign lesions and hypothesized that nevus cells need to circumvent OIS in order to progress to malignant melanoma. Here, we have used \textit{BRAF}^{E600}-induced senescence in primary HDF as a model to study genes involved in this process. We report that \textit{BRAF}^{E600}-induced senescence in HDF is abrogated by a viral oncoprotein, SV40 st. Although we have not yet identified the factor(s) targeted by SV40 st in this context, our results suggest that it acts in a cellular signaling pathway that may be involved in tumor suppression. The ability of SV40 st to efficiently bypass \textit{BRAF}^{E600}-induced senescence is remarkable, because it has been implicated previously only in enhancing cellular transformation. In fact, it has been reported that SV40 st cannot bypass \textit{RAS}^{V12}, or \textit{ΔMEK1}-induced senescence in HDF (Hahn et al., 2002; Collado et al., 2005). However, no actual experimental evidence was included in these reports, which precludes an assessment of the experimental or other differences (e.g. expression levels of SV40 st). To investigate the SV40 st-mediated bypass in more detail, we
previously used a PP2A-gene family knockdown library (Eichhorn et al., 2007) to identify the PP2A subunit(s) involved in BRAF$^{E600}$-induced senescence, but could not validate any of the subunits to be essential for BRAF$^{E600}$-induced senescence. Therefore, it remains to be determined whether a PP2A subunit corresponds to the primary effector of SV40 st in this senescence setting.

To investigate SV40 st-mediated bypass of BRAF$^{E600}$-induced senescence in more detail, we analyzed the expression levels of several known downstream targets of SV40 st. However, p-AKT, CYCLIN D1, and CYCLIN A did not appear to be regulated by SV40 st in our experimental setting, suggesting that they are not involved in SV40 st-mediated bypass of BRAF$^{E600}$-induced senescence. Another downstream target of SV40 st is cMYC (Yeh et al., 2004). Ectopic expression of this effector was able to a bypass BRAF$^{E600}$-induced senescence, albeit less efficiently than SV40 st. This implies that if cMYC is involved in the SV40 st-mediated bypass of BRAF$^{E600}$-induced senescence, it is not the only factor.

Remarkably, unlike what has been reported for RAS$^{V12}$, we find that BRAF$^{E600}$-induced senescence is not dependent on the canonical p53 and RB/p16$^{INK4A}$ pathways. First, in contrast to SV40 st, LT, simultaneously targeting p53 and RB, was unable to interfere effectively with OIS. Second, simultaneous interference, by overexpression and shRNA, of a p16$^{INK4A}$-binding mutant of CDK4 and p53, failed to abrogate BRAF$^{E600}$-induced senescence. These results are in agreement with our observation that even simultaneous silencing of p15$^{INK4B}$, p16$^{INK4A}$ and p53 fails to interfere with this senescence program (C.M. and D.S.P., unpublished observations). Thus, BRAF$^{E600}$ and RAS$^{V12}$ induce senescence through largely non-overlapping pathways. Thus, while several groups found that the type of cell cycle arrest that is triggered by oncogenic RAS is dependent on the p53 and RB/p16$^{INK4A}$ pathways, our results argue that the proliferative standstill that is induced by BRAF$^{E600}$ is relayed through a different cellular signaling pathway, namely one that is inactivated by SV40 st. As we have utilized different HDF cell strains and introduced several perturbations in the p53 and RB/p16$^{INK4A}$ pathways, we deem it unlikely that the type of HDF strain used accounts for the observed differences between senescence induced by BRAF$^{E600}$ and RAS$^{V12}$.

A model in which BRAF$^{E600}$-induced senescence is not primarily dependent on the status of the p16$^{INK4A}$ pathway is consistent with previous observations made by us and others that p16$^{INK4A}$ is not a factor that is uniquely involved in OIS in vitro (Michaloglou et al., 2005; Denoyelle et al., 2006; Haferkamp et al., 2009). Recently, a mouse model, in which endogenous BRAF$^{E600}$ expression and loss of p16$^{INK4A}$ were induced specifically in melanocytes, was generated. Although melanoma formation was accelerated by loss of p16$^{INK4A}$, these mice continued to develop nevi (Dhomen et al., 2009), arguing that also in vivo, p16$^{INK4A}$ does not act primarily in senescence
signaling. This phenomenon has also been reported for humans with homozygous loss of p16\(^{\text{INK4A}}\) (Gruis et al., 1995). Furthermore, human nevi often display increased p16\(^{\text{INK4A}}\) expression, although the expression is often heterogeneous (Michaloglou et al., 2005). Lastly, p53, which is the most frequently mutated tumor suppressor in human cancer, is inactivated in melanoma only at low frequency, suggesting that it does not constitute a major barrier against melanoma formation (reviewed in Hussein et al., 2003).

Important questions remain to be answered. Most importantly: which cellular pathway(s) is involved in the SV40 st-mediated bypass of BRAF\(^{E600}\)-induced senescence? And is this related to research on melanoma or other BRAF\(^{E600}\)-driven cancers? SV40 has not been implicated in melanoma, but a dominant negative mutant of B56\(^{\gamma}1\), a PP2A subunit expressed in the BL6 melanoma cell line, can enhance metastasis (Ito et al., 2000). It is not yet clear if this gene is involved in human melanoma progression, however. To address these issues, our results predict that SV40 st can be used as a probe to identify (new) factors contributing to BRAF\(^{E600}\)-dependent cancers.

**MATERIALS AND METHODS**

**Cell culture, viral transduction**

The human diploid fibroblast cell line TIG3, expressing the ecotropic receptor and hTERT, as well as Phoenix and HEK293T cells, were maintained in DMEM (Gibco) supplemented with 9% fetal bovine serum (Greiner Bio-One), 2 mM glutamine, 100 units ml\(^{-1}\) penicillin, and 0.1 mg ml\(^{-1}\) streptomycin (all Gibco). The human diploid fibroblast cell line BJ expressing the ecotropic receptor and hTERT, was grown in a 4:1 ratio of DMEM to medium 199 (Gibco) supplemented with 15% fetal bovine serum (Greiner Bio-One), 0.1 mM MEM non-essential amino acids, 2 mM glutamine, 100 units ml\(^{-1}\) penicillin, and 0.1 mg ml\(^{-1}\) streptomycin (all Gibco). Retroviral production employing Phoenix cells was carried out as described (http://www.stanford.edu/group/nolan/retroviral_systems/phx.html). For BJ and TIG3 cell proliferation assays, cells were transduced with shRNA- or cDNA-encoding retrovirus and cultured for one week. The cells were plated at equal densities and subsequently transduced with BRAF\(^{E600}\)-encoding retrovirus and selected (Blasticidin). The cells were analysed for BrdU incorporation (3 hours) and fixed and stained with crystal violet 10-15 days post-infection (p.i.) and harvested for protein and mRNA analysis 8 days p.i. For cell transformation assays in semi-solid medium, BJ cells were transduced with shRNA- and cDNA-encoding retrovirus. After selecting for successful proviral integration, 2.5\(\times\)10\(^4\) trypsinized cells were seeded in duplicate in complete DMEM containing 0.4% low-melting-point grade VII agarose (Sigma) on top of a 1% agarose in complete DMEM layer in six-well plates. Pictures were taken 3 weeks after plating.

**Plasmids**

pRS\(_{\text{PURO}}\)-sh-p53 (Brummelkamp et al., 2002), pRS\(_{\text{GFP}}\)-sh-p16\(^{\text{INK4A}}\) (Michaloglou et al., 2005), pLZRS-CDK4\(^{R24C}\)-ires-GFP, pMSCV\(_{\text{BLAST}}\)-BRAF\(^{E600}\), pMSCV\(_{\text{BLAST}}\)-RAS\(^{V12}\), pB\(_{\text{HYGRO}}\)SV40-ER, pB\(_{\text{HYGRO}}\)SV40-LT, pB\(_{\text{HYGRO}}\)SV40-st, and pLZRS-cMYC\(_{\text{ires-GFP}}\) were used for retroviral transduction.

**Antibodies**

Antibodies used for Western blotting were for BRAF (sc-5284; Santa Cruz), p16\(^{\text{INK4A}}\) (JC8; Immunologic), p53 (sc-126; Santa Cruz), p21\(^{\text{CIP1}}\) (sc-397; Santa Cruz), CDK4 (sc-260; Santa Cruz), SV40 LT and st (MS-1833;
Neomarkers), phospho-Ser$^{473}$-AKT (#9271; Cell Signaling), AKT (sc-8312; Santa Cruz), p27$^{kip1}$ (610241; BD Transduction Laboratories), CYCLIN A (sc-751; Santa Cruz), CYCLIN D (sc-753; Santa Cruz), and β-ACTIN (AC-74; A5316; Sigma).

**QRT PCR**

Total RNA was DNase-treated with RQ1 RNase-Free DNase (Promega). Reverse transcription was performed with Superscript II first strand kit (Invitrogen). QRT-PCR was performed with the SYBR Green PCR Master Mix (Applied Biosystems) on an ABI PRISM 7700 Sequence Detection System. Control primers were RPL13. Primer sequences: SV40 st: 5'-AACTGAGGTATTTGCTTCTTCCTTAAAT-3' and 5'-TTGCACACTCAGGCCATTGT-3', SV40 LT: 5'-CACAGTCCCAAGGCTCATTTC-3' and 5'-ACAAATGTGTATGGCTGATTATGAT-3', RPL13: 5'-GAGACAGTTCTGCTGAAGAACTGAA-3' and 5'-TCCGGACGGGCATGAC-3'.

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**REFERENCES**


SUPPLEMENTARY FIGURES

Supplementary Figure 1 | SV40 small t, but not SV40 LT, allows for bypass of BRAF<sup>E600</sup>-induced senescence in human diploid fibroblast. a | Samples described in Figure 4 were analyzed 8 days p.i for SV40 LT (left) and st (right) transcript levels by qRT-PCR. Levels are represented relative to those found in control-infected cells. b | BJ<sub>H</sub>, HDF stably overexpressing SV40 st or both st and LT from the ER were transduced with BRAF<sup>E600</sup>-encoding retrovirus and analyzed in a proliferation assay; samples were fixed and stained 10 days p.i.

Supplementary Figure 2 | cMYC allows bypass of BRAF<sup>E600</sup>-induced senescence in human diploid fibroblast. TIG3<sub>H</sub> HDF stably overexpressing different levels of cMYC (by virus dilution), were transduced with BRAF<sup>E600</sup>-encoding retrovirus and analyzed in a proliferation assay; samples were fixed and stained 12 days p.i.
Functional identification of LRF as an oncogene that bypasses RAS\textsuperscript{V12}-induced senescence via upregulation of CYCLIN E

Functional identification of LRF as an oncogene that bypasses RAS$^{V12}$-induced senescence via upregulation of CYCLIN E

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ABSTRACT

Mutant RAS (RAS$^{V12}$) is known to transform most immortal cells but to induce premature senescence in primary cells. RAS$^{V12}$-induced senescence in murine cells depends on the induction of the ARF/p53 and the retinoblastoma (Rb) family tumor suppressor pathways. We and others have shown previously that oncogene-induced senescence in vitro can be used as a tool to identify new cancer-related genes. In addition, we have shown that oncogene-induced senescence corresponds to an in vivo tumor suppressive mechanism. Therefore, we extended our search for novel genes that bypass of RAS$^{V12}$-induced senescence, with the help of a previously designed unbiased functional screen with cDNA expression libraries. In this screen, we expected to find new mediators feeding into the p53 or Rb pathways or novel signaling factors. We report here the identification of leukemia/lymphoma related factor (Lrf) encoding a transcription factor with a BTB/POZ domain and Krüppel-like zinc fingers. This gene was previously identified as a potential oncogene that is overexpressed in human cancer. We find that LRF enhances E2F-dependent transcription and that it synergizes with RAS$^{V12}$ in activating E2F. Indeed, LRF-mediated bypass of RAS$^{V12}$-induced senescence is accompanied by the induction of several E2F-target genes, including Cyclin E, Cyclin A and p107. Unexpectedly, LRF exerted this activity independent of several critical senescence inducers, such as p19$^{ARF}$, p21$^{CIP}$ and p16$^{INK4A}$. We show that CYCLIN E is necessary for LRF-mediated bypass, suggesting that it corresponds to a critical mediator of LRF-driven oncogenic transformation. Thus, LRF bypasses RAS$^{V12}$-induced senescence in a CYCLIN E-dependent manner, which conceivably contributes to its role in cancer.
INTRODUCTION

It is almost half a century ago that Hayflick showed that primary cells have ‘a limited in vitro lifetime’ (Hayflick, 1965). It took several decades for researchers to uncover the underlying mechanism. Prolonged culturing of primary cells results in the emergence of critically short telomeres, thereby activating tumor suppressor networks (reviewed in Deng et al., 2008). This ultimately leads to replicative senescence, a state in which cells remain metabolically active yet are growth arrested. Similarly, unfavorable tissue culture conditions can induce the p53 and retinoblastoma (Rb) tumor suppressor pathways, leading to cell cycle arrest with several hallmarks of replicative senescence (reviewed in Ben-Porath and Weinberg, 2005). In addition, senescence can be induced prematurely in primary cells by overexpression of activated oncogenes like HRAS^{V12} or BRAF^{E600}. Also in this setting, the p53 and Rb pathways are involved in the induction of the proliferative arrest, now called ‘oncogene-induced senescence’ (Serrano et al., 1997). It is conceivable that the tumor suppressors p53 and Rb owe, at least in part, their important role in cancer suppression by acting as critical mediators of (oncogene induced) senescence.

We and others have recently found evidence indicating that oncogene-induced senescence is not only an in vitro phenomenon but acts as a genuine in vivo tumor suppressive mechanism (Lazzerini Denchi et al., 2005; Michaloglou et al., 2005; Braig et al., 2005; Chen et al., 2005; Collado et al., 2005; reviewed in Prieur and Peeper, 2008). It is for this reason that in vitro senescence bypass screens have been designed to identify new oncogenes and tumor suppressor genes (Prieur and Peeper, 2008; Berns et al., 2004). Here, we performed an unbiased screen with high-complexity retroviral complementary DNA (cDNA) expression libraries and report on the identification of Lrf as a gene that abrogates RAS^{V12}-induced senescence.

RESULTS

A functional genetic screen for genes that bypass RAS^{V12}-induced senescence

A functional RAS^{V12}-induced senescence bypass screen was performed, as described previously (Peeper et al., 2002), in the BTR cell system. Briefly, these cells, BALBc MEFs expressing a temperature-sensitive SV40 Large T (tsT) mutant and RAS^{V12}, allow for a rapid and tight induction of premature senescence at the non-permissive temperature (39.5°C), which is associated with a low background of spontaneous ‘escapees’ (Peeper et al., 2002; Rowland et al., 2005). In this cell system, disruption of either the p53 pathway (Figure 1c) or the Rb pathway (Peeper et al., 2002) results in abrogation of oncogene-induced senescence. This is consistent with previous observations demonstrating that p53^-/-, p19^{ARF/-} or Rb^-/-/p107^-/- MEFs fail to undergo RAS^{V12}-induced senescence (Serrano et al., 1997; Palmero et al., 1998; Peeper et al., 2001). For the library, we used the pEYK3.1 vector system which allows for second
round screening that requires neither wild-type (wt) murine Moloney leukemia virus to mobilize the proviral insert (Peeper et al., 2002), nor subsequent PCR steps (see Koh et al., 2002; and also Materials and Methods and Figure 1a for details).

We introduced by retroviral transduction three high-complexity retroviral cDNA expression libraries into BTR cells. After shifting the cells to the non-permissive temperature, most cells senesced. Indeed, in agreement with our previous observations (Peeper et al., 2002; Rowland et al., 2005), control-transduced BTR cells gave rise to only very few colonies. Amidst these senescent cells, ~60 colonies emerged 2 weeks after the temperature shift. The library-transduced colonies were picked, expanded and the colonies that appeared first were used to perform a second round of screening to discriminate true from false positives. For this, we used a system (pEYK3.1; Figure 1a; Koh et al., 2002), which allows for efficient shuttling of the proviral DNA. Thus, we digested the genomic DNA of the BTR cell clones, performed a religation step and transformed the recombinant shuttle vectors to E.coli. The genomic DNA was digested with either the NotI or Ascl ‘eight-cutters’ to reduce the chance that biologically active inserts would be lost during shuttling. After shuttling, bacterial colonies were pooled and plasmid DNA isolated.

Figure 1 | A functional genetic screen for genes that bypass RAS\textsuperscript{V12}-induced senescence. a | Schematic overview of the pEYK3.1 vector and integrated provirus. b | Outline of the RAS\textsuperscript{V12}-induced senescence bypass screen in the BTR cells with the pEYK3.1 libraries. c | Knockdown of either p53 or p19\textsuperscript{ARF} bypasses RAS\textsuperscript{V12}-induced senescence in BTR cells. Two days after retroviral transduction, BTR cells were shifted to the non-permissive temperature and stained after 2 weeks with Coomassie blue.
Subsequently, this DNA was transfected into Phoenix packaging cells to generate virus for a round of transduction of fresh BTR cells (Figure 1b).

**Identification of LRF**

Upon shuttling of the provirus from several primary clones, only DNA isolated from clone #23A(Ascl) appeared positive in the second round of screening (Figure 2a). Importantly, this result indicated that the biological activity observed in the primary clone was transferable, rather than being caused by an endogenous mutation in the BTR cells. The 23A(Ascl) polyclonal bacterial DNA pool contained three different cellular cDNAs, that is, *Keratin*, *Stearoyl coA desaturase* and *Lrf*. In the third screening round, we tested the individual inserts by retroviral transduction into fresh BTR cells, which revealed *Lrf*, but as expected not either of the remaining cDNAs, as the cDNA that was responsible for efficiently bypassing RAS<sup>V12</sup>-induced senescence (Figure 2c and d). The sequence of *Lrf* lacks an Ascl site but contains a NotI site, which explains the lack of biological activity of the 23A(NotI) DNA pool (Figure 2a). The pEYK3.1-LRF construct contained full-length mouse *Lrf* (NM_010731.3) encoding a 569 amino acid protein. It contains several modules, including a BTB/POZ domain, three Krüppel-like and one unusual Zinc fingers and a nuclear localization signal (Figure 2b). In an independent RAS<sup>V12</sup>-induced senescence bypass screen human *ZBTB7B/hcKROX-α*, the closest homologue of LRF was also identified as gene that can bypass RAS<sup>V12</sup>-induced senescence, although with lower biological activity than LRF (C. Martínez-Muñoz and D.S. Peeper, unpublished data). Upon expression of LRF at the non-permissive temperature, cells continued proliferating despite the presence of RAS<sup>V12</sup> and showed a transformed phenotype (Figure 2c). Proliferation curves confirmed that LRF-expressing BTR cells expanded rapidly, whereas the control cells senesced (Figure 2d).

Next, we determined whether LRF allowed cells to bypass also spontaneous senescence. To this end, we transduced wt and temperature-sensitive Large T expressing MEFs with LRF-encoding retrovirus and shifted the temperature-sensitive Large T expressing MEFs to the non-permissive temperature. Indeed, also this resulted in abrogation of the senescence response (Figure 2e and f). To determine whether LRF can collaborate with more oncogenes than just RAS, we co-expressed it together with cMYC in wt MEFs. LRF strongly enhanced the immortalizing activity of cMYC (Figure 2g). These cells have the ability to form colonies in soft agar with moderate efficiency compared with wt MEFs expressing RAS<sup>V12</sup> and cMYC, whereas wt MEFs immortalized with LRF and RAS<sup>V12</sup> are not oncogenically transformed as measured in this assay (data not shown). These findings confirm previous observations (Maeda et al., 2005) and demonstrate that LRF overexpression can bypass both premature and cellular senescence.
LRF-mediated bypass of RAS\textsuperscript{V12}-induced senescence is accompanied by induction of CYCLINs E and A and occurs independently of critical senescence inducers

To address the mechanism by which LRF rescues RAS\textsuperscript{V12}-induced senescence, we analysed the levels of various proteins known to be involved in senescence induction as well as cell cycle proteins. Inactivation of p19\textsuperscript{ARF} is known to collaborate with RAS\textsuperscript{V12} in oncogenic transformation. However, in a time course experiment, protein levels of p19\textsuperscript{ARF} and p16\textsuperscript{INK4A} showed no difference in control BTR cells compared with their LRF-expressing counterparts, indicating that the LRF-mediated rescue abrogation of
senescence is not mediated by suppression of p19ARF or p16INK4A (Figure 3a). This is in contrast with the findings of Maeda et al. (Maeda et al., 2005), as they observed reported that LRF represses p19ARF transcription. We found that p53 was only slightly downregulated in LRF-expressing BTR cells, even though one of its transcriptional targets p21CIP in fact showed a minor upregulation. Another p53 target, MDM2, was not regulated by LRF. So p53 is downregulated but its targets that have been found to be involved in senescence are not regulated in this experimental setting. Consistent with these observations, knockdown of p53 enhanced the ability of LRF to abrogate senescence-mediated bypass (Supplementary Figure 1a and b). This suggests that p53 is not involved in the LRF-mediated bypass of RASV12-induced senescence.

In contrast, the E2F-targets genes Cyclin E, Cyclin A and p107 showed a clear upregulation in the cells that were rescued from RASV12-induced senescence by LRF, whereas the D-type CYCLINs showed no change in protein levels (Figure 3a). To examine whether the increase in CYCLIN E protein levels is accompanied by an increase in CYCLIN E-associated activity, we performed a CYCLIN E kinase activity assay with Histone H1 as a substrate. As shown in Figure 3b, LRF-expressing BTR cells indeed display increased CYCLIN E kinase activity compared with control cells.

Figure 3 | LRF-mediated bypass of RASV12-induced senescence is accompanied by induction of CYCLINs E and A and occurs independently of critical senescence inducers. a | Western blot analysis of BTR cells transduced with either control or LRF-encoding retrovirus. Two days after transduction, the BTR cells were shifted to the non-permissive temperature. At 8, 12 and 16 days post-infection (p.I.) cells extracts were prepared and equal amounts were used for western blotting with the indicated antibodies. CDK4 serves as a loading control. b | CYCLIN E-associated kinase activity assay. Samples described in a were harvested and lysed 10 days after transduction and immunoprecipitations with control and CYCLIN E antibodies were performed, followed by incubation with Histone H1, as a substrate and [c-32P] adenosine triphosphate for 30 min at 37°C. c | Samples described in a were analyzed 8 and 16 days p.I for Rb transcript levels by quantitative reverse transcription-polymerase chain reaction. Levels are represented relative to those found in control-infected cells, as mean ± SD.
In principle, the activation of E2F-dependent transcription could be indirect, as a result of the downregulation of \( Rb \) by LRF (Jeon et al., 2008), yielding an increase in free E2F competent to activate transcription. However, \( Rb \) transcript levels are not regulated by LRF in the BTR cells (Figure 3c). Thus, LRF-mediated bypass of RAS\(^{V12}\)-induced senescence correlates with induction of several E2F-target genes including CYCLIN E.

**CYCLIN E induction is essential for LRF-mediated bypass of RAS\(^{V12}\)-induced senescence**

To investigate the role of CYCLIN E in the LRF-mediated bypass of RAS\(^{V12}\)-induced senescence, we depleted it using two independent non-overlapping short hairpin RNA (shRNA). Silencing of Cyclin E strongly suppressed the ability of LRF to bypass senescence (Figure 4a and c). Importantly, this was not due to a general cytostatic effect, as the proliferative capacity of both NIH 3T3 mouse fibroblasts (Figure 4a) and BTR cells expressing (wild-type) SV40 LT at the non-permissive temperature (Figure 4c) remained unaffected by silencing of Cyclin E. To determine if CYCLIN E is not only required for LRF to bypass RAS\(^{V12}\)-induced senescence but also sufficient to bypass it, we overexpressed it in the BTR cell system. However, over-expression of CYCLIN E was unable to exert this effect, whether alone or in combination with overexpression of CYCLIN A (Figure 5b and c). Thus, although CYCLIN E is not sufficient to bypass RAS\(^{V12}\)-induced senescence, it is necessary for the LRF-mediated bypass from RAS\(^{V12}\)-induced senescence.

![Image](image_url)

**Figure 4 | CYCLIN E induction is essential for LRF-mediated bypass of RAS\(^{V12}\)-induced senescence.**

a and c | NIH 3T3, BTR-LRF and BTR-SV40LT cells at the non-permissive temperature were transduced with retrovirus expressing short hairpins for CYCLIN E1 (sh-CE). Colony formations was stained 2 weeks after transduction. b and d | Cell extracts were prepared and analysed on western blot for CYCLIN E levels; CDK4 and \( \beta \)-actin serve as loading controls.
LRF and RAS\textsuperscript{V12} synergize in activating E2F-dependent transcription

Since we found three established E2F-targets genes (CYCLIN E, CYCLIN A and p107) being upregulated in BTR cells expressing LRF and found that this was not mediated by changes in Rb mRNA levels, we examined whether LRF enhances E2F-dependent transcriptional activation. We transfected into NIH 3T3 cells a luciferase reporter plasmid, which is driven by six tandem E2F sites (Lukas et al., 1997). This experiment was performed in the presence of E2F and DP1, which we titrated in such amounts that the reporter was only minimally activated (Figure 6a). LRF stimulated the promoter activity in a dose-dependent manner, to up to 60 times. Interestingly, this was even further enhanced in the presence of RAS\textsuperscript{V12}, to up to 190 times. Next, we used the same experimental setting to determine the effect of LRF on the CYCLIN E promoter (Geng et al., 1996), which showed the same pattern: LRF increased E2F/DP1 activity and this was further enhanced by the presence of RAS\textsuperscript{V12} (Figure 6b). The effect of LRF was E2F dependent in the context of RAS\textsuperscript{V12}, as mutation of the corresponding sites in the CYCLIN E promoter greatly impaired activation by LRF. In conclusion, LRF synergizes with RAS\textsuperscript{V12} to strongly activate E2F-dependent transcription, which conceivably underlies its ability to stimulate expression of several E2F targets, thereby contributing to abrogation of the premature senescence program.

DISCUSSION

To identify new regulators of oncogene-induced cellular senescence, we performed a RAS\textsuperscript{V12}-induced senescence bypass screen and found LRF as a potent hit. We used the pEYK3.1 shuttling system to efficiently perform second round screens. One of the advantages of this system is the lack of requirement for PCR steps to isolate hits. Lrf is highly GC-rich and therefore very difficult to amplify by PCR; this might explain why, despite its strong biological effect, it has not been identified in previously performed similar screens that rely on PCR steps (Peeper et al., 2002; Rowland et al., 2005).

Figure 5 | Overexpression of CYCLIN E and A is not sufficient to bypass RAS\textsuperscript{V12}-induced senescence. a and c | BTR cells were transduced with retrovirus encoding human CYCLIN E1 and/or CYCLIN A and shifted to the non-permissive temperature 2 days after transduction. LRF-overexpressing cells were used as positive control. Colony formations were stained 2 weeks after transduction and b | analysed on western blot with a human specific antibody for CYCLIN E and β-actin, serving as loading control.
LRF was originally cloned as a family member of two well-known leukemia/lymphoma related oncogenes, BCL-6 and PLZF (Davies et al., 1999), and the corresponding gene is located on chromosome 19p13.3, a hot spot for chromosomal aberrations in human cancer. The closest homologues of LRF are cKrox-α and AMP-1 (cKrox-γ) (Widom et al., 2001). All the above-mentioned proteins are members of the POK protein family, which have a BTB/POZ domain for homomeric and heterodimeric complex formation and other protein interactions, and Krüppel-like C2H2 zinc fingers, for binding to specific DNA sequences (reviewed in Kelly and Daniel, 2006).

It was recently described that LRF overexpression induces oncogenic transformation in vitro, whereas mice with transgenic expression of LRF in lymphoid cells develop aggressive lymphomas. Furthermore, aberrant overexpression of LRF is found not only in certain types of lymphoma (diffuse large B-cell lymphoma and follicular lymphoma) (Maeda et al., 2005) but also in several other human cancers, including non-small lung cancer (Apostolopoulou et al., 2007; Zhao et al., 2008).

LRF is a transcription factor that is involved in several differentiation processes, like osteoclastogenesis (Kukita et al., 1999), chondrogenesis (Liu et al., 2004), adipogenesis (Laudes et al., 2004) and early lymphoid cell-fate decision (Maeda et al., 2007). Originally, LRF was identified as a factor involved in elongation blocking of HIV-1 (Pessler et al., 1997; Morrison et al., 1999), whereas in the presence of the

Figure 6 | LRF and RAS<sup>V12</sup> synergize in activating E2F-dependent transcription. NIH 3T3 cells were electroporated with a | the 6xE2F-luc reporter or b | the wild-type or 6xE2F mutant human CYCLIN E promoter and as indicated RAS<sup>V12</sup>, E2F, DP1 and (increasing amounts) of LRF. The activation of the reporter is represented relative to the internal control (TK-renilla luciferase) and normalized to the reporter activity in the absence of additional constructs. Representative experiments of three independent experiments are shown. Of note, the mutant construct in b was still slightly responsive to E2F transactivation but contains a full 1.4Kb long promoter that is very GC-rich. Therefore, it conceivably contains one or more cryptic E2F sites that may respond to E2Fs.
HIV-1 transcription factor Tat full-length transcription is induced (Pendergrast et al., 2002). This might involve recruiting other (transcription) factors and/or keeping the promoter in an open and active conformation. Most POK proteins are transcriptional repressors (reviewed in Costoya, 2007) and LRF, too, has been shown to repress transcription of several genes (Widom et al., 2001; Kukita et al., 1999). This occurs via recruitment of HDAC1 and Sin3A repressor complexes to the promoters (Jeon et al., 2008; Liu et al., 2004; Laudes et al., 2008) and/or by inhibiting the transcriptional activity of Sp1 and p53 (Widom et al., 2001; Lee et al., 2002; Choi et al., 2009). However, LRF can also enhance transcription, by increasing the nuclear localization and stabilization of transcription factors (Lee et al., 2005) and by activating Sp1-transcriptional activation (Choi et al., 2008). Whether LRF inhibits or activates transcription probably depends on the different promoter elements, on cofactors and in what ratio they bind to promoters. This is already known for SP1 (reviewed in Wierstra, 2008), another transcription factor that is believed to be involved in most LRF-mediated transcriptional inhibition and activation.

We show that the LRF-mediated bypass of RASV12-induced senescence is accompanied by the induction of the E2F-target genes Cyclin E and A and p107, but occurs independently of several critical senescence inducers, like p19ARF, p21CIP and p16INK4A. Depletion of CYCLIN E abolishes the ability of LRF to bypass senescence. This is not withstanding, CYCLIN E may not be the sole CYCLIN to be involved. However, CYCLIN E is not sufficient to bypass RASV12-induced senescence, not even in the presence of CYCLIN A. Lastly, we find that LRF can enhance, and in synergy with RASV12 super-activate, E2F-dependent transcription. Although we show that it does not involve the downregulation of Rb by LRF, the precise mechanism by which LRF activates E2F-dependent transcription remains to be determined. It may be a direct activation mechanism, as has been shown for LRF-mediated activation of the FASN promoter by Choi et al. (Choi et al., 2008).

Our results are partially in agreement with those obtained by Maeda et al. (Maeda et al., 2005). We confirm here that LRF immortalizes primary murine cells and can collaborate with other oncogenes like RASV12 and cMYC. In contrast, in our experimental system LRF does not consistently downregulate p19ARF. Similarly, in contrast to previous reports on LRF suppressing the levels of p21CIP (Choi et al., 2009), CYCLIN A and p107 (Laudes et al., 2004), we find these genes to be upregulated in our experiments. These differences might be explained by the use of cell lines from different species and/or origins and different experimental setups (e.g. transfection versus retroviral expression). Indeed, how a promoter is regulated by LRF might depend on the presence of different cofactors and in what ratios to LRF they bind to the promoter elements (Choi et al., 2008). Furthermore, we find, like for Rb+/−/p107+/−-MEFs (Peeper et al., 2001), that MEFs expressing LRF are immortalized but not
oncogenically transformed by RAS\textsuperscript{V12}. This finding is in agreement with our data suggesting that LRF acts primarily on the Rb/E2F pathway. Increasing evidence points to an important role of LRF/FBI-1 in cancer. The mouse model directing high levels of LRF to immature T and B lymphoid cells (IckE\(\mu\)-Lrf) develops aggressive tumors (Maeda et al., 2005). Aberrant LRF/FBI-1 expression has been found in several human tumors, however, its relation to expression of the human counterpart of p19\textsuperscript{ARF}, p14\textsuperscript{ARF}, varies. In a subset of B-cell lymphomas (diffuse large B-cell lymphoma), high LRF/FBI-1 levels correlated with low p14\textsuperscript{ARF} (Maeda et al., 2005), whereas an inverse relationship was found in non-small cell lung cancer (Apostolopoulou et al., 2007). The relationship of LRF/FBI-1 expression to clinical outcome also differs; LRF/FBI-1 expression predicts good clinical outcome in diffuse large B-cell lymphoma yet bad clinical outcome in non-small cell lung cancer (Maeda et al., 2005; Zhao et al., 2008). These differences might depend on the cells of origin of the tumors, the collaborating oncogenes, and which other targets are regulated by LRF/FBI-1. In aggregate, the available evidence indicates that LRF/FBI-1 deregulates several important genes associated to cancer, including p14/p19\textsuperscript{ARF}, Rb, p21\textsuperscript{CIP} and, as we show here, Cyclin E and other E2F targets, which conceivably explains its oncogenic activity in different settings.

**MATERIALS AND METHODS**

**Cell culture experiments**

BTR cells, temperature-sensitive Large T expressing MEFs (tsT MEFs) and wild-type mouse embryonic fibroblasts (wt MEFs) were maintained in Dulbecco’s modified Eagle’s medium (Gibco, Zwijndrecht, the Netherlands) supplemented with 10% fetal bovine serum (PAA Laboratories, Cölbe, Germany), 2 mM glutamine, 100 U/ml penicillin, 0.1 mg/ml streptomycin (all Gibco) and 0.1 mM \(\beta\)-mercapto-ethanol. NIH 3T3 cells were maintained in Dulbecco’s modified Eagle’s medium with 10% neonatal bovine serum (Invitrogen, Breda, the Netherlands), 2 mM glutamine, 100 U/ml penicillin and 0.1 mg/ml streptomycin (all Gibco). Phoenix packaging cells (http://www.stanford.edu/group/nolan/retroviral_systems/phx.html) were used to generate ecotropic retroviruses to transduce cells, which were subsequently used to perform colony formations assays and growth curves (all as described previously in Peeper et al., 2002).

**Retroviral cDNA screen**

The retroviral cDNA screen was performed as described previously (Peeper et al., 2002) but with cDNA libraries in the pEYK3.1 vector (Koh et al., 2002). The libraries were prepared from human LMJ216 primary fibroblasts, human MCF-7 breast cancer cells and mouse embryo (day 14) cells. To discriminate between spontaneous colonies and colonies rescued by retroviral encoded by cDNA, we used the pEYK3.1 vector (Koh et al., 2002; Figure 1a) to shuttle the primary clones to Escherichia coli and re-introduced the proviral insert into the BTR cells by retroviral transduction as follows (also Figure 1b): the genomic DNA of the primary clones was isolated and the viral integrations were recovered by digestion with either NotI or Ascl and then religated. The circularized DNA was transformed into E.coli. Subsequently, pEYK3.1 proviral DNA was isolated from multiple bacterial colonies (representing proviral sequences from single primary BTR cell clones) and transfected into Phoenix packaging cells to generate retrovirus. Fresh BTR cells were then transduced with these pools of retrovirus to perform the second round of screening. The polyclonal pool
of DNA from the shuttled proviral inserts that appeared positive in the second round was analyzed by digestion and sequenced. Finally, single inserts were individually expressed by retroviral transduction of BTR cells in the third round of screening.

**Assays**

CYCLIN E-dependent kinase activity assay was performed as in (Dulic et al., 1992), using the M20 (Santa Cruz Biotechnology, Heidelberg, Germany) antibody for CYCLIN E immunoprecipitation and Histone H1 (Roche, Almere, the Netherlands) as a substrate. For the luciferase reporter assays, NIH 3T3 cells were electroporated with 2 μg of luciferase reporter driven by an artificial promoter containing six consensus E2F sites in front of the TATA box (6xE2F; Lukas et al., 1997) or by the human CYCLIN E promoter (2.2 kb DNA fragment 5' of the CYCLIN E gene with or without mutant E2F sites; Geng et al., 1996), as well as increasing amounts (0.5-2 μg) of leukemia/lymphoma related factor (LRF)-expressing plasmid, 0.1 μg of RASV12-expressing plasmid and 0.025 μg of E2F1 and DP1-expressing plasmid. TK-Renilla luciferase was co-introduced as an internal control. After 48 h, the luciferase was measured with the Dual Luciferase Reporter Assay System (Promega, Leiden, the Netherlands).

**Plasmids**

The full-length cDNA of mouse Lrf/Zbtb7a was recloned from pEYK3.1 (pE) in pBABE (pbp) and pCDNA 3.1 expression vectors via EcoRI digestion sites. Sense short hairpin RNA sequences for interference constructs for mouse Cyclin E1: sh-CE#1 5'-TTGATGATGATGAAGGCC-3', sh-CE#2 5'-AGGTTTGGAGGATCATGT-3', and mouse p19ARF: 5'-GTTCGTGCGATCCCGGAGA-3' were cloned into pRS as has previously been described for pRS-mp53 (Dirac and Bernards, 2003).

**Antibodies**

For western blotting, the following antibodies were used from Santa Cruz Biotechnology: M-156 for p16INK4A, C-19 for p21CIP, C-22 for CDK4, H-295 for CYCLIN D1, M20 for CYCLIN D2, M-20 and C-19 (human specific) for CYCLIN E1, C-18 for p107 and C-19 for CYCLIN A; Calbiochem (Beeston Nottingham, UK): Ab-7 for p53; Abcam (Cambridge, UK): R-562 for p19ARF and Sigma-Aldrich Chemie (Zwijndrecht, the Netherlands): AC-74 for β-actin.

**Quantitative reverse transcription–polymerase chain reaction**

Reversetranscription was performed on total RNA with Superscript II first strand kit (Invitrogen). Quantitative reverse transcription-polymerase chain reaction was performed with the SYBR Green PCR Master Mix (Applied Biosystems, Nieuwekerk a/d IJssel, the Netherlands) on an ABI PRISM 7700 Sequence Detection System. All primer pairs span exon-exon barriers. Control primers were HPRT. Primer sequences: Rb: 5’-ATCTACCTCCTTGCCCTGT-3’ and 5’-GAAGCCGTCACAGAGTGA-3’ and HPRT: 5’-CTGGTGAAAAGGACCTCTC-3’ and 5’-TGAAGTACTCATTATAGTCGAGGAC-3’.

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Conflict of Interest Statement
None declared.

REFERENCES


**SUPPLEMENTARY FIGURE**

Supplementary Figure 1 | **Knockdown of p53 enhances the LRF-mediated bypass of RAS<sup>V12</sup>-induced senescence.** BTR cells were first transduced with either control or sh-p53-encoding virus and subsequently with control or LRF-expressing virus. **a** | Two days after infection, the BTR cells were shifted to the non-permissive temperature and stained after 10 days with Coomassie blue. Fewer cells were plated to distinguish between bypass induced by only sh-p53 or LRF, or by the combined expression. **b** | Cell extracts were prepared and analysed by western blotting for p53 levels; β-actin serves as a loading control.
General Discussion: 
Senescence as a tool to find 
factors involved in aging and cancer
Senescence as a tool to find factors involved in aging and cancer

INTRODUCTION

The term senescence (derived from the Latin word *senex*, meaning *old man, old age, or advanced in age*) is used for “growing old”, a process that occurs in almost all species from fungi to plants and from fish to mammals. Senescence (or aging) of an organism involves an intrinsic age-related decline in the functional status of one or more organs, which will eventually lead death of the organism. Both terms, senescence and aging, have been used in the literature to refer to this process. However, throughout this chapter, we will refer to it as aging, and we will use the term senescence (or cellular senescence) specifically for a cellular state that has been originally described for primary human diploid cells in vitro, when they reach the end of their lifespan (Hayflick, 1965). Cellular senescence has been used to describe aging at the cellular level. Aging organisms display increased cellular senescence in some of their tissues. However, it is not always clear whether this contributes to the aging phenotypes and/or mortality.

Many studies have revealed that upon several types of stress, cellular senescence can be induced prematurely, independent of aging (see below). The senescence program induces a proliferation arrest, which, depending on the cell type, can be maintained for several years or is followed by cell death (Wagner et al., 2001). Senescent cells have several characteristics, such as a lack of proliferative capacity, active metabolism and induction of several senescence markers (reviewed in Kuilman et al., 2010). These markers include increased senescence-associated acidic β-galactosidase activity (SA-β-GAL; Dimri et al., 1995), induction of senescence-associated heterochromatic foci (SAHF; Narita et al., 2003), activation of the senescence-mediating secretome (SMS or SASP, for senescence-messaging secretome or senescence-associated secretory phenotype, respectively; Kuilman et al., 2008; Kuilman and Peeper, 2009; Coppe et al., 2008). Furthermore, several biomarkers are induced, including PAI1 (Goldstein et al., 1994), DEC1, DCR2 (Collado et al., 2005), as well as tumour suppressors, like p16<sup>INK4A</sup>, p15<sup>INK4B</sup>, ARF and p53 (reviewed in Ben-Porath and Weinberg, 2005). Although senescence, like apoptosis, can protect an organism from cancer by limiting the ability of cells to divide indefinitely, it might also induce aging by loss of (stem) cells.

In the work described in this thesis, we have used cellular senescence to identify factors involved in cancer. The senescence research field has experienced increasing insight and understanding in recent years. This chapter will summarize the current knowledge on cellular senescence in vitro and in vivo and discuss in detail how we
and others have used this as a tool to identify factors involved in aging and cancer.

**SENESCENCE AND AGING**

Hayflick was the first to report that cells in vitro have a finite lifespan: he showed that prolonged culturing of diploid human fibroblasts leads to ‘an increased generation time, gradual cessation of mitotic activity, accumulation of cellular debris, and ultimately total degeneration of the culture’ (Hayflick, 1965). This phenomenon is now referred to as “replicative senescence”, which can be induced in primary cells by short telomeres and enhanced by inappropriate tissue culture conditions (reviewed in Cristofalo et al., 2004; Ohtani et al., 2009).

Senescence in mammalian cells has been proposed to be involved in aging. Aging humans and primates show increased staining of the senescence markers SA-β-GAL and telomere dysfunction-induced foci (TIFs) in fibroblasts in the skin (Dimri et al., 1995; Herbig et al., 2006; Jeyapalan et al., 2007). In addition, senescence has been reported for human immune cells. Both aging and chronic viral infections are associated with increased numbers of T cells that have shorter telomeres and lack CD28; these cells are unable to proliferate in culture (reviewed in Effros et al., 2003). Furthermore, senescent cells have been found in several human tissues with age-related pathological lesions, such as chondrocytes in osteoarthritis (Price et al., 2002) and endothelial cells in atherosclerosis (Minamino et al., 2002). Lastly, accelerated replicative senescence can be found in human livers affected by chronic hepatitis and liver cirrhosis. These livers suffer from chronic DNA damage induced by, for instance, chronic infection or alcohol; this induces continuous waves of destruction and regeneration, which results in critically short telomeres followed by replicative senescence (Paradis et al., 2001; Wiemann et al., 2002). Senescence is also observed, in activated hepatic stellate cells in response to liver damage in mice, although this cannot be linked to telomere attrition (Krizhanovsky et al., 2008).

**Senescence in vitro**

Replicative senescence in vitro has not been extensively used as a model to find factors involved in aging, but the mechanism of senescence induction and maintenance has been studied widely in vitro. Replicative senescence is induced by telomere attrition, which happens in almost all dividing cells, unless they express telomerase. Critically short telomeres in vitro lead to a DNA damage response (DDR) (reviewed in Deng et al., 2008). Cultured skin fibroblasts from patients with premature aging syndromes display premature senescence and increased DNA damage (Danes, 1971; reviewed in Fossel, 2003). A DNA damage response (DDR) can be induced also by other factors, like oncogenes, pharmacological agents, and oxidative stress (reviewed in Campisi, 2005). Activation of the DNA damage
pathway commonly leads to activation of p53 and subsequent activation of its downstream targets. Which factors influence the cell's decision to subsequently undergo senescence or apoptosis is far from clear. It seems to be dependent on, for instance, the extent of DNA damage and/or the cell type (d'Adda di Fagagna, 2008). Furthermore, p53 can also be activated by ARF, which is induced upon (oncogenic) stress, although this pathway seems more important in mouse cells than human cells. Transcriptional targets of p53 involved in senescence induction include p21\textsuperscript{CIP} and PAI-1 (reviewed in Ben-Porath and Weinberg, 2005; Kortlever and Bernards, 2006). Upregulation of the tumour suppressor p21\textsuperscript{CIP} leads, in a cyclin E-dependent fashion, to activation of RB (and other factors), which in turn inhibits cell-cycle progression. p16\textsuperscript{INK4A} is another important tumour suppressor activating RB and is induced upon several stress signals that induce senescence. Senescence \textit{in vitro} is often, but not always, dependent on p53 and/or RB (reviewed in Ben-Porath and Weinberg, 2005; Campisi, 2005; Nicke \textit{et al.}, 2005; Olsen \textit{et al.}, 2002; and chapter 4). Other factors that have been implicated in senescence are: chromatin remodelling (reviewed in Adams, 2007), secreted factors (reviewed in Kuilman and Peeper, 2009), reactive oxygen species (ROS) (reviewed in Lu and Finkel, 2008), and the PI3K pathway (reviewed in Courtois-Cox \textit{et al.}, 2008).

\textbf{Senescence as a tool to find factors involved in aging}

Several commonly employed model organisms, such as fungi, worms and fruit flies, are used to study the process of aging. Aging in \textit{P. anserine} occurs due to increased ROS that culminates in damaged mtDNA, which in turn leads to mitochondrial dysfunction and eventually aging and death (reviewed in Lorin \textit{et al.}, 2006). This process is also involved in aging of higher organisms, making \textit{P. anserine} a simple, but useful model organism to study this. Furthermore, extensive studies of \textit{C. elegans} longevity mutant strains have revealed insulin-like growth factor signalling and also mitochondrial respiration as important factors that affect aging (Braeckman \textit{et al.}, 2001). Nonetheless, the previously discussed senescence markers have not been used to identify cellular senescence in tissues of these model systems, making it unclear if cellular senescence is involved in their aging process. These model systems however, might be used to test whether genes found \textit{in vitro} cellular senescence screens have an effect on aging and death \textit{in vivo}. For instance, Ha \textit{et al.} assembled a list of proteins that are differentially expressed in human senescent cells \textit{in vitro}, and tested whether depletion of the corresponding factor in \textit{C. elegans} displayed an aging phenotype \textit{in vivo} (Ha \textit{et al.}, 2006). It would be interesting to establish if these useful model organisms display cellular senescence in aging tissues and whether this contributes to the aging phenotype or even to mortality. While most model organisms have not been extensively examined for the presence of senescent cells, zebrafish is one of the few model systems in which senescent
cells have been detected. Whole-body SA-β-GAL staining revealed that this occurs primarily in the skin dermis of aging animals (Kishi et al., 2003). Furthermore, genotoxic stress enhances the SA-β-GAL staining as well as other aging phenotypes, such as a decline in fecundity, increased melatonin levels and impaired fin regeneration, although the body growth of the fish is not affected (Tsai et al., 2007). Retroviral insertional and ENU mutagenesis screens in zebrafish were performed with SA-β-GAL staining as a biomarker. Both screens identified several mutants with increased SA-β-GAL staining, which also display an accelerated aging phenotype (Kishi et al., 2008). At least two homologues of genes identified in these screens have been previously linked to lifespan in flies and telomere protection in human cells. In spite of this progress, however, more careful characterization of senescent cells, establishment of the role of these senescent cells in the aging tissues, and validation of the hits in the senescent tissue is required to really establish a role for cellular senescence in aging zebrafish (Kishi et al., 2008).

Several mouse strains and genetically engineered mice display accelerated aging. For example, the SAM (senescence-accelerated mouse) strains are inbred strains that have several aging phenotypes, such as neurodegeneration, senile osteoporosis, and impaired immune response. However, the occurrence of cellular senescence has not been investigated in these strains (reviewed in Takeda, 2009). The best-known genetically engineered model for studying short telomeres is the TERC-/- mouse model (Blasco et al., 1997). The long-term viability of this mouse strain is severely affected, but the mice are resistant to cancer (reviewed in Blasco, 2007). Although this animal model is widely used to study the impact of short telomeres and aging, only a few studies report on cellular senescence in this mouse model. The lab of Chang showed in several organs of TERC-/- compound mice, which also harbour a mutant p53 that cannot induce apoptosis but still induces cell-cycle arrest, increased SA-β-GAL staining and p21CIP and p53 levels. These double mutant mice have a premature aging phenotype, but are less tumour-prone compared to the TERC-/- mice, which makes it complicated to study aging in this model (Cosme-Blanco et al., 2007). The lab of Rudolph found senescent cells in regenerating livers of TERC-/- mice. Although regeneration is delayed due to a subset of cells that is senescent due to critically short telomeres, cells with sufficient telomeres length are present to fully regenerate the liver (Satyanarayana et al., 2003). Further studies implicate p53 as an important factor for replicative senescence induction in mice (Lechel et al., 2005; Feldser and Greider, 2007). Furthermore, several models have been investigated to implicate the best-known senescence-associated tumour suppressors, p53 and p16INK4A, in aging. Whereas mice expressing constitutively active p53 age prematurely (Tyner et al., 2002), mice with an extra allele of p53 (including endogenous regulatory sequences) do not age prematurely but are resistant to cancer (Garcia-Cao et
This phenotype has also been shown for mice with an extra allele of \( p16^{INK4A} \) (including endogenous regulatory sequences; Matheu et al., 2004). Lastly, telomerase length and \( p16^{INK4A} \) have been implicated in stem cell aging in vivo (Flores et al., 2005; Janzen et al., 2006; Molofsky et al., 2006; Krishnamurthy et al., 2006), suggesting a role for senescence in aging by depleting the stem cell pool. However, these observations have not yet been correlated with induced senescence markers in (stem) cells in vivo. Indeed, it is debatable whether mice are good animal models for replicative senescence, as laboratory mouse strains have long telomeres. Furthermore, ARF seems to have a prominent role in mice, whereas \( p16^{INK4A} \) may be more important in humans. Nonetheless, mice do age and senescent cells are found in aging mice, but one has to be careful with extrapolating results found in lab mice to the human situation.

**SENESCENCE AND CANCER**

Senescence in humans has been proposed to protect against tumour progression. Induction of replicative senescence will stop cells that have exhausted their replicative potential and suffer from critically short telomeres. Furthermore, senescence can be induced prematurely in vitro. For example, oncogene-induced senescence (OIS) is induced upon expression of HRAS\(^{V12}\) or BRAF\(^{E600}\) (Serrano et al., 1997; Michaloglou et al., 2005), while tumour suppressor loss-induced senescence can result from loss of PTEN or NF1 (Chen et al., 2005; Courtois-Cox et al., 2006). In addition, DNA damage can induce senescence. These mechanisms can also cause an arrest in tumours in vivo. Oncogene-induced senescence in vivo in a benign tumour has been described in chapter 2; we and others found that nevi are benign melanocytic neoplasms harbouring several characteristics of senescent cells: they frequently have activating mutations in BRAF or RAS, show cessation of proliferation, induction of SA-\(\beta\)-GAL and \( p16^{INK4A} \), and do not seem to suffer from telomere attrition (Michaloglou et al., 2005; Gray-Schopfer et al., 2006). Furthermore, (premature) senescence has been proposed to inhibit human tumour progression in neurofibromas (Courtois-Cox et al., 2006), non-small cell lung carcinoma (Bartkova et al., 2006), colon adenomas (Kuilman et al., 2008; Fujita et al., 2009), prostate intraepithelial neoplasia (PIN) (Choi et al., 2000; Chen et al., 2005; Acosta et al., 2008; Majumder et al., 2008), and pituitary micro adenomas (Mooi, 2009).

**Senescence in vitro as a tool to find factors involved in cancer**

Already long before the relevance of senescence in vivo was established, it was used as a model system in vitro. Expression of known oncogenes or inactivation of established tumour suppressor genes, such as MYC or p53, respectively, is sufficient to bypass (oncogene-induced) senescence in mouse embryonic fibroblasts. This
suggests that senescence is involved in tumour suppression, indeed, or that at least similar pathways are involved in senescence induction in vitro and tumour suppression. Therefore, senescence in vitro has endlessly been used as a model to test mutant proteins or (regulators of) genes involved in cancer, as well as to find new oncogenes and tumour suppressor genes. Different types of senescence like replicative, oncogene-, stress-, drug-induced senescence in rodent and human cells have been used as model systems for this purpose.

Examination of oncoproteins encoded by DNA tumour viruses has boosted senescence research. SV40 LT, adenovirus E1A and E1B, or papilloma virus E6 and E7 all abrogate cellular senescence (Shay et al., 1991). These proteins inhibit both p53 and RB; further analysis by expression of different combinations and mutant viral proteins suggested a role for both p53 and RB in implementing senescence. This was later confirmed by various other techniques and tools specifically targeting these pathways, like fibroblasts derived from genetically engineered mice or from human donors homologous for mutations, loss of expression by homologous recombination or expressing short hairpin (sh) RNAs, or by overexpression of dominant negative tumour suppressors or activated oncogenes in primary human cells (reviewed in Ben-Porath and Weinberg, 2005). These techniques can also be used to test whether educated guesses are involved in the regulation of senescence. For example, in chapter 3 we have investigated whether loss of a well-known (melanoma) tumour suppressor (PTEN) can contribute to tumour progression by promoting senescence. We found that activation of the PI3K-pathway in primary human cells abrogates BRAF<sup>E600K</sup>-induced senescence. Furthermore, we observed that more than 50 % of human melanoma specimens show decreased PTEN or increased AKT3 and pAKT expression compared to their contiguous nevi. Together, these results imply that at least in a proportion of human melanoma lesions, activation of the PI3K pathway has induced melanoma progression by abrogating OIS. Furthermore, classical experiments with viral oncoproteins can hint to new factors involved in senescence, as we describe in chapter 4. We show that, unexpectedly, expression of SV40 st, which had previously only been implicated as an important factor for cellular transformation, can effectively bypass BRAF<sup>E600K</sup>-induced senescence. Another method is to pick candidate genes or microRNAs identified by differential display or microarray analysis, comparing senescent and non-senescent cells (Qian et al., 2008; Fujita et al., 2009). Designing a mini-library of the top-outliers, for example by Gene Ontology classification, will subsequently allow conducting a more focused approach (Kuilman et al., 2008). Furthermore, mini-libraries of family members or specific pathways could be used to test a group of interesting candidates at once. The use of large-scale screens allows for completely unbiased identification of new mediators of senescence, and this approach has successfully been applied in several cell systems.
For example, we have identified LRF (Leukaemia/Lymphoma Related Factor) in an unbiased functional screen with cDNA expression libraries as a potent gene that can bypass RAS\textsuperscript{V12}-induced senescence (chapter 5). It had previously been shown that LRF is an oncogene that acts on p10\textsuperscript{ARF} (Maeda et al., 2005). We have shown that LRF enhances E2F-dependent transcription of CYCLIN E, which is critical for the abrogation of senescence \textit{in vitro} (Vredeveld et al., 2010). Indeed, the majority of so-called gain of function screens identified potential new oncogenes that regulate the ARF/p53 and or RB/p16\textsuperscript{INK4A} pathways (Jacobs et al., 2000; Shvarts et al., 2002; Peeper et al., 2002; Brummelkamp et al., 2002; Gil et al., 2004; Rowland et al., 2005), but also regulators of other pathways that had not previously been linked to senescence (Kondoh et al., 2005; Kortlever et al., 2008). Also “loss of function screens” have yielded potential new tumour suppressor genes acting on the ARF/p53 and/or RB/p16\textsuperscript{INK4A} pathways (Berns et al., 2004; Wu et al., 2004; Nicke et al., 2005; Castro et al., 2008a; Leal et al., 2008; Castro et al., 2008b; Augert et al., 2009; Humbert et al., 2009; Humbert et al., 2010), and others hits have implicated new pathways in senescence induction and maintenance (Tarunina et al., 2004; Wajapeyee et al., 2008; Acosta et al., 2008).

Although all of these methods have yielded a lot of new clues on the regulation of senescence, we still do not have the full picture. Different cell strains, types, species and methods complicate this matter further. But, improved cell systems and libraries and optimized detection methods, like barcode experiments and deep sequencing, will conceivably provide us with a more complete view. Lastly, it will be crucial to validate genes involved in senescence \textit{in vivo}. Some new senescence markers have been proposed (Collado et al., 2005; Binet et al., 2009). To establish whether the results of these screens can be used to find new drug targets, it has to be determined if the candidate genes are indeed involved in cancer progression and whether the pathway(s) they act on are potentially sensitive for small compounds.

\textbf{Senescence in vivo as a tool to find factors involved in cancer}

Whether senescence occurs \textit{in vivo} has long been debated, but recently several animal models and findings in human lesions have established that it is a relevant tumor-suppressing mechanism \textit{in vivo}. Two groups have studied whether replicative senescence can be involved in tumour suppression \textit{in vivo}, in mouse models with short telomeres and abrogated apoptosis due to expression of mutant p53 or BCL2 (Cosme-Blanco et al., 2007; Feldser and Greider, 2007). Both models show decreased tumour formation and upregulation of several senescence markers, implicating replicative senescence in tumour suppression. Furthermore, oncogene-induced senescence can be induced in different mouse model and several tumours types, such as pituitary gland hyperplasia (Lazzerini Denchi et al., 2005), lung adenoma (Collado et al., 2005; Dankort et al., 2007), urothelial hyperplasia (Mo et al., 2007),
mammary epithelial hyperplasia (Sarkisian et al., 2007), nevi (Dankort et al., 2009; Dhomen et al., 2009; Goel et al., 2009), prostrate intraepithelial neoplasia (Nardella et al., 2008), lymphoma (Ruggero et al., 2004; Campaner et al., 2009), papiloma (Chen et al., 2009), and prelymphomagenic thymocytes (Xu et al., 2008). In addition, senescence induced by other factors is found in several tumour models, like tumour suppressor loss-induced senescence in prostrate intraepithelial neoplasia (Chen et al., 2005; Majumder et al., 2008), pituitary gland hyperplasia (Chesnokova et al., 2007), kidney (Young et al., 2008), and thyroid adenoma (Shamma et al., 2009) and drug-induced senescence in lymphoma (Braig et al., 2005) and papiloma (Collado et al., 2005; Acosta et al., 2008; Yamakoshi et al., 2009). Lastly, a role for senescence has been found in tumour regression upon either re-expression of a tumour suppressor (e.g. p53) or inactivation of the driver oncogene (e.g. c-MYC) in established tumours (Ventura et al., 2007; Xue et al., 2007; Wu et al., 2007). The physiological relevance of some of the models is debatable, however: transgenic overexpression of an oncogene or loss of both alleles of a tumour suppressor is not very commonly found in sporadic tumours at an early stage, except for amplification of HRAS in Spitz nevi (Bastian et al., 2000), or loss of the second allele in patients with an inherited mutation in a tumour suppressor gene (e.g. NF-1 in neurofibromas). In addition, evidence for in vivo senescence in the comparable human tumour types is often not (yet) present, for example an inducible (BRAF^{E600}) thyroid cancer model. By contrast, the BRAF^{E600}-knockin mouse models are nice examples of a model closely resembling the human situation (Dankort et al., 2009; Dhomen et al., 2009). In these models, BRAF^{E600} is expressed from its normal locus, and the mutation can be switched on in a specific tissue type. Upon expression of BRAF^{E600} in melanocytes, the mice develop nevi. This adequately mimics the human situation in which oncogene-induced senescence is established in human nevi that often carry this BRAF^{E600} mutation (Michaloglou et al., 2005; Pollock et al., 2003). These mouse models could therefore be used to further validate our findings that activation of the PI3K pathway abrogates senescence in vitro and that this might be involved in early melanoma progression (chapter 3). We could try to use this model to confirm whether established nevi can progress to melanoma when the PI3K pathway is activated.

Some of these mouse models have been used to test whether factors that are known to be involved in senescence in vitro are also involved in senescence in vivo. Loss of several well-known tumour suppressors, like p53, ARF, p16^{INK4A}, p27^{KIP1} and PTEN, can induce tumour progression, which is accompanied by loss of senescence markers in tumour models for prostate cancer (Chen et al., 2005; Majumder et al., 2008; Nardella et al., 2008), lymphoma (Braig et al., 2005), breast cancer (Sarkisian et al., 2007), lung cancer (Dankort et al., 2007), and melanoma (Dankort et al., 2009;
Dhomen et al., 2009; Goel et al., 2009). Furthermore, a role for heterochromatin remodelling proteins has been found in several senescence models (Braig et al., 2005; Lazzerini Denchi et al., 2005; Collado et al., 2005), while loss of Suv39h1, encoding a histone methyltransferase, can induce tumour progression (Braig et al., 2005). Lastly, DDR markers are often found upregulated in senescent tissue (Bartkova et al., 2006; Di Micco et al., 2008; Xu et al., 2008; Chen et al., 2009; Yamakoshi et al., 2009; Shamma et al., 2009). Although DDR has been proposed to be functionally linked to senescence in vivo in some lesions (Bartkova et al., 2006), this is not found in all cases of OIS in vitro (reviewed in Kuilman et al., 2010). The large overlap in genes that are involved in bypass of senescence in vitro and in vivo indicate that in vitro experiments are indeed valuable. Nevertheless, cell type and tumour environment can influence the involvement of some genes too. For example, loss of the CDKN2A locus (expressing both p16\textsuperscript{INK4A} and ARF) can induce tumour progression of several oncogenic RAS- or BRAF-induced pre-neoplastic lesions (Sarkisian et al., 2007; Dankort et al., 2007; Goel et al., 2009), but not of HRAS\textsuperscript{V12}-induced urothelial hyperplasia (Mo et al., 2007).

Until now only established tumour suppressors have been tested for their capability to bypass senescence in vivo. Some of the above-mentioned mouse models could be used to test ‘new’ senescence-associated genes, too, in particular genes identified by senescence screens in vitro. Furthermore, the reversibility of (oncogene-induced) senescence in vivo could be addressed with these models by manipulating established pre-malignant neoplasms to progress to malignant tumours. Genome-wide screens are technically challenging in mice, but testing family- or mini-libraries might be possible. A model system that is more amenable for unbiased (oncogene-induced) senescence screens in vivo is zebrafish. Transgenic HRAS\textsuperscript{V12} fish display senescent cells with activation of a DDR in heart and brain (Santoriello et al., 2009). Furthermore, transgenic BRAF\textsuperscript{E600} fish develop nevi, but these lesions have not yet been tested for senescence markers. They remain benign and progress to melanoma in cooperation with p53 deficiency (Patton et al., 2005). Retroviral insertional mutagenesis and ENU mutagenesis screens, which have already been successfully used for several phenotypes in zebrafish (see above), can be combined with one of the above-mentioned oncogene-induced senescence fish models.

Another subject that is emerging is the influence of senescent cells on their environment in vivo and the identification of the factors that are involved in this process. Several laboratories have recently reported on the induction of secreted factors in senescent cells (reviewed in Kuilman and Peeper, 2009). For example, when p53 is re-activated in a liver carcinoma mouse model, senescence is induced in the tumour cells. The senescent tumour cells upregulate several inflammatory cytokines, which leads to the activation of the innate immune system that can clear the tumour
(Xue et al., 2007). Along these lines, several reports have shown that senescent fibroblasts promote proliferation and transformation of (pre-)neoplastic epithelial cells (Krtolica et al., 2001; Parrinello et al., 2005; Yang et al., 2006). Several secreted factors with a dual role in senescence and tumour progression have been identified, but it is not yet clear how the switch from anti- to pro-tumourigenic function works (reviewed in Campisi, 2005; Kuilman and Peeper, 2009). This also raises the question whether drug-induced senescence is a good option for cancer therapy. Results from the Jarrard lab suggest that as long as senescence is induced only in the cancer cells and not in the normal cells (fibroblasts, immune cells, endothelial cells, etc.) of the surrounding tissue in vivo, this will not cause a proliferative stimulus, at least in the context of doxorubicin-induced senescence (Ewald et al., 2008). Another concern is the stability of drug-induced senescence: whereas oncogene-induced senescence in nevi in humans is stable for decades, this is not always true for the mouse BRAF/nevus model (Dhomen et al., 2009), although this depends on the setting (Dankort et al., 2009). It remains to be determined how this is for drug-induced senescence in human tumours.

CONCLUDING REMARKS

Senescence has successfully been used as a tool to identify factors in aging and cancer. Since the role of senescence in vivo has now been well established, the previous in vitro results have become even more relevant and applicable. Cells with (at least) some characteristics of senescence have been detected in several human tissues. However, rarely, extensive tests with multiple senescence biomarkers have been performed to prove that they are truly senescent. Furthermore, it would be recommended that also telomere length, mutational status, and extent of DNA damage is determined. Senescence and aging have been linked for a long time, but there is still not a lot of evidence that senescent cells are actually involved in aging-phenotypes or even mortality. Useful animal models for aging research, like yeast, worms and flies, have not yet been analyzed for the presence of senescent cells, but some of these might be suitable for research addressing the contribution of senescence to aging. Furthermore, a lot of mouse models that age prematurely could be used for this line of research. Many factors and pathways have been identified to be important for senescence induction and maintenance, but in spite of this there is still not a very clear picture of how it exactly works. This is further complicated by species- and cell type-dependent differences. The DNA damage-p53 pathway has already been studied extensively and has been found to contribute to some types of senescence. In contrast, although p16INK4A has been implicated in senescence for a long time and has been widely studied, it is still not always clear how certain types of stress induce it. Even less is known
about the identity of factors that are involved in senescence that occurs in a p53- and p16INK4A-independent fashion. Senescence in vitro has mainly been studied in fibroblasts, which is a technically easy system and which has yielded a lot of results that appear to be conserved in other cell types and species. This notwithstanding, also differences have been found with other cell types, so I would recommend to extend experimental systems to other cell types, like epithelial and immune cells. Quite a number of genes that are important for senescence have been identified in screens in vitro; some of these might be acting on pathways that can be targeted with anti-cancer drugs. Recently, several mouse models have been engineered that show senescence in vivo, which can be used for further validating candidate genes. Furthermore, remaining relevant issues, like the effect and role of senescence cells on the tumour environment and the reversibility of senescence in vivo, can be addressed with these models in the near future. In conclusion, senescence has been used extensively and successfully as a model to find factors involved in aging and cancer, but new techniques, new model systems and more senescence-markers will provide us with a more complete overview.

REFERENCES


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ONCOGENE-INDUCED SENESCENCE: FROM IN VITRO TOOL TO IN VIVO TUMOUR SUPPRESSION

In a mature (human) body only several cell types divide, like epithelial stem cells (e.g. skin cells and cells aligning the intestinal tract), blood stem cells, male germ cells and (stem) cells in some tissues that need repair after damage. The division of each cell is very tightly regulated, because uncontrolled cell division might lead to cancer and eventually to death of the organism. Defects in the hereditary material (mutations in DNA) that either over-activate ‘grow-genes’ (oncogenes) or shutdown ‘inhibitor-genes’ (tumour suppressor genes) can lead to uncontrolled cell division. These mutations can be induced by chemicals, radiation, viral infection, or can occur spontaneously. Several mechanisms of the human body and in the cell can prevent the outgrowth of cancer cells. The immune system can recognize cancer cells, which leads to their clearance. Furthermore, tumours require a lot of nutrition, and when they reach a certain size they need their own blood supply to survive. Moreover, build-in protection mechanisms of the cells can induce programmed cell death (apoptosis) or permanent cell cycle arrest (senescence). These processes can be activated by the signals from within the cell or from the cell environment. Therefore, to become a transformed cancer cell these lines of defence have to be evaded; this is a multistep process requiring mutations in several oncogenes and tumour suppressor genes.

Senescence was first described for fibroblast cells that were extensively passaged in a petridish (in vitro). It is induced in response to culture stress and/or severe shortening of the protective ends of chromosomes (telomeres). Later, it was shown that this permanent cell cycle arrest can be induced prematurely also by other types of stress. For example, senescence is triggered when activated oncogenes, such as RAS\textsuperscript{V12} or BRAF\textsuperscript{E600}, are expressed in primary cells. This process is called oncogene-induced senescence. Several markers, like increased senescence-associated acidic β-galactosidase activity (SA-β-GAL), formation of senescence-associated heterochromatic foci (SAHF), and upregulation of tumour suppressor genes are induced alongside and, therefore, can be used to establish whether cells are senescent. Although senescence was thought to be an in vitro artefact for a long time, it was used as a relevant model cell system by several groups nonetheless, because it allowed studying well known tumour suppressor pathways, like those regulated by p53 and RB, which are important for the induction of senescence but also prevent cancer.

In this thesis, we have studied mainly BRAF\textsuperscript{E600}-induced senescence. BRAF is an oncogene that is often mutated in pigment cell cancer (melanoma). In chapter 1, we
describe that mutations in BRAF are found not only in human tumours, but also in benign lesions. We summarize the current knowledge, serving as a model for the role of BRAF in senescence and tumour formation.

In chapter 2, we have shown that cells that form human moles (nevi) are in a senescence-like cell cycle arrest. These nevi are benign tumours of pigment cells (melanocytes) that remain in a growth-arrested state for many years. They often harbour activated mutations in either RAS or BRAF, but show no overt signs of telomere shortening. Furthermore, these lesions display elevated levels of the (melanoma) tumour suppressor protein p16INK4A and SA-β-GAL activity and have a low level of proliferation. This is recapitulated in vitro: expression of BRAF\textsuperscript{E600} in melanocytes and fibroblasts induces oncogene-induced senescence. This is the first report of oncogene-induced senescence in human cells in vivo acting as a tumour-suppressive mechanism.

In chapter 3, we have investigated the role of a well-known melanoma tumour suppressor gene, PTEN, a prime inhibitor of the PI3K pathway, in senescence. This pathway is important for proliferation and survival of cells and is often found to be hyperactive in cancer cells. Its deregulation, by downregulation of PTEN or overexpression of PI3K, allows for prevention of BRAF\textsuperscript{E600}-induced senescence in vitro. This suggests that deregulation of the PI3K pathway might be involved in cancer progression by evading oncogene-induced senescence. To further investigate this, we have linked a number of melanomas to nevi that are in close proximity (within one specimen) by genetic analysis, which led us to propose a human nevus-to-melanoma progression model in vivo. Indeed, we find that in more than half of the cases, the PI3K pathway is activated in the melanoma, but not in the associated nevus. We therefore conclude that abrogation of oncogene-induced senescence by PI3K pathway activation contributes to human nevus-to-melanoma progression in vitro and in vivo.

In chapter 4, we investigated another educated guess in the BRAF\textsuperscript{E600}-induced senescence cell system. Previously, viral oncogenes have been implicated in senescence and cell transformation. SV40 LT can inactivate two very important tumour suppressor genes (i.e., p53 and RB) and has been shown to be able to bypass several types of senescence. Unexpectedly, we found that SV40 LT, or inhibition of the p53 and/or RB pathways cannot effectively bypass BRAF\textsuperscript{E600}-induced senescence. In contrast, SV40 LT’s little brother, SV40 st, can bypass BRAF\textsuperscript{E600}-induced senescence very well. Although it is not yet clear what the mechanism is behind this, it can be used as an effective tool to identify (new) factors contributing to BRAF\textsuperscript{E600}-dependent tumours.

In chapter 5, we have used the RAS\textsuperscript{V12}-induced senescence cell system to screen for potential new oncogenes. Out of a genomic library, we have identified LRF as a
candidate oncogene. The senescence cell system was further used to identify which pathways are involved in the LRF-mediated bypass of senescence. We find that LRF can enhance the activity of E2F, a transcription factor that induces several cell cycle genes. Activation of one of these genes, Cyclin $E$, is critical for the LRF-mediated bypass of oncogene-induced senescence.

Lastly, chapter 6 summarizes the use of the senescence cell system as a tool to identify factors involved in aging and cancer. Senescent cells are found in aging organisms, although it is not yet clear whether these cells also play an actual role in the aging process. In vitro studies have already provided us with a lot of insight into which genes are involved in the induction and maintenance of (oncogene-induced) senescence. Oncogene-induced senescent cells are also found in several benign lesions in humans and model organisms. The model organisms can be used as a tool to recapitulate the in vitro findings in vivo.

In sum, in this thesis we have established a role for oncogene-induced senescence in vivo and have used the oncogene-induced senescence cell system in vitro to identify and study cancer-related genes involved in oncogene-induced senescence, and to link them to cancer progression. Furthermore, we have tried to use the in vitro cell system to unravel what mechanisms are important for senescence induction and maintenance. Better understanding of the pathways involved in this process might eventually lead to development of new anti-cancer medication.
Nederlandse Samenvatting

ONCOGEN-GEÏNDEKUIERDE SENESCENCE: VAN IN VITRO GEREEDSCHAP TOT IN VIVO TUMOR SUPPRESSIE

Slechts een paar soorten cellen in een volwassen (menselijk) lichaam bezitten het vermogen zich voort te planten (te delen), zoals epitheliale stamcellen (bijvoorbeeld huidcellen en cellen die de wand van het maag-darm kanaal bekleden), bloed stamcellen, mannelijke geslachtsstamcellen en (stam)cellen in sommige beschadigde weefsels die gerepareerd moeten worden. De deling van elke cel wordt heel nauwkeurig gereguleerd. Dit is erg belangrijk, want ongecontroleerde celdeling kan leiden tot kanker en dit leidt vaak tot het overlijden van een organisme. Veranderingen in het erfelijke materiaal (mutaties in het DNA) die ervoor zorgen dat ‘groeigenen’ (oncogenen) overactief worden of dat groeiremmers (tumorsuppressor genen) niet meer werken kunnen leiden tot deze ongecontroleerde celdeling. Mutaties in het DNA kunnen worden veroorzaakt door chemicaliën, straling, virussenfecties of kunnen spontaan ontstaan. Gelukkig zijn er in het menselijk lichaam en in de cellen verschillende mechanismes om de groei van deze kankercellen tegen te gaan. Het immuunsysteem kan kankercellen herkennen en deze opruimen. Kankercellen hebben ook veel ‘voeding’ nodig: zodra een tumor van een bepaalde grootte geen eigen bloedvaten heeft zullen de cellen het niet overleven. Verder hebben de cellen ingebouwde beschermingsmechanismen die geprogrammeerde celdood (apoptose) of permanente celdeling stop (senescence) kunnen induceren. Deze processen kunnen worden geactiveerd door signalen vanuit de cel zelf of vanuit de omgeving van de cel. Om een getransformeerde kankercel te worden moeten er dus verschillende processen worden omzeild; hiervoor zijn meerdere stappen nodig die elk één of meerdere specifieke mutaties in oncogenen en tumorsuppressor genen vereisen.

Senescence is oorspronkelijk beschreven voor normale (primaire) cellen die lang in een petrischaaltje (in vitro) in kweek werden gehouden. Senescence kan worden geïnduceerd door de suboptimale kweekcondities of doordat de beschermende eindjes van de chromosomen (telomeren) te kort worden. Andere vormen van stress kunnen ervoor zorgen dat deze permanente celdelingstop vroegtijdig wordt geïnitieerd. Senescence wordt bijvoorbeeld geïnduceerd als bepaalde geactiveerde oncogenen, zoals RAS\textsuperscript{V12} of BRAF\textsuperscript{E600}, tot expressie komen in primaire cellen. Dit proces wordt oncogen-geïnduceerde senescence genoemd. Om te bepalen of een cel senescent is kan er naar verschillende markers worden gekeken, zoals inductie van SA-\beta-GAL activiteit (een enzym), een toename van foci (SAHF) in de celkern, en verhoogde expressie van tumorsuppressor genen. Hoewel er lang werd gedacht dat senescence een in vitro artefact was, is het door een aantal groepen wel gebruikt als
model systeem, omdat verschillende bekende tumorsuppressor genen, zoals p53 en RB, niet alleen belangrijk zijn voor de inductie van senescence maar ook voor het onderdrukken van kanker.

In dit proefschrift hebben we voornamelijk BRAF\textsuperscript{E600}\textsuperscript{-}{-}geïnduceerde senescence bestudeerd. BRAF is een oncogen dat vaak is gemuteerd in kanker van de pigmentcellen (melanoom). In hoofdstuk 1 beschrijven we dat BRAF mutaties niet alleen in kwaadaardige tumoren, maar ook in goedaardige tumoren kunnen worden gevonden. We hebben de actuele kennis samengevat en introduceren een model voor de rol van BRAF in senescence en kanker.

In hoofdstuk 2 hebben we aangetoond dat menselijke moedervlekcellen in een celdelingstop zijn dat veel lijkt op senescence. Moedervlekken (nevi) zijn goedaardige tumoren die zijn ontstaan uit pigmentcellen (melanocyten) en deze blijven vaak jarenlang aanwezig zonder te groeien. De moedervlekcellen hebben meestal een mutatie in BRAF of RAS, maar hun telomeren zijn niet aantoonbaar verkort. Verder vertonen moedervlekcellen een lage celdelingsactiviteit en hebben ze verhoogde expressie van het (melanoom) tumorsuppressoreiwit p16\textsuperscript{INK4A} en een verhoogde SA-β-GAL activiteit. Dit gebeurt ook in vitro: expressie van BRAF\textsuperscript{E600} induceert oncogen-geïnduceerde senescence in normale melanocyten en bindweefselcellen (fibroblasten). Dit hoofdstuk is de eerste publicatie die beschrijft dat oncogen-geïnduceerde senescence in menselijke cellen kan functioneren als een tumorsuppressiemechanisme.

In hoofdstuk 3 hebben we de rol van PTEN in senescence onderzocht. PTEN is een bekend melanoom tumorsuppressorgen, dat gewoonlijk de functie heeft om een groei- en overlevingssignaal dat via de PI3K route gegeven wordt, te remmen. Deze PI3K route is vaak overactief in kankercellen, door verlies van de remmer (PTEN) of door activatie van stimulatoren (bijvoorbeeld PI3K of AKT). Wij hebben gevonden dat activatie van de PI3K route ervoor zorgt dat normale cellen niet meer in senescence gaan. Dit suggereert dat deregulatie van de PI3K route een rol speelt bij het uitgroeien van de kankercellen, doordat de oncogen-geïnduceerde senescence wordt omzeild. Dit hebben we verder onderzocht door naar verschillende melanomen te kijken die vlak naast een moedervlek liggen. Door een genetische analyse uit te voeren op de moedervlekken en de ernaast gelegen melanoomcellen konden we de meeste tumoren genetisch aan elkaar relateren. Op basis hiervan concluderen we dat er sprake is van een moedervlek-naar-melanoom ontwikkelingsmodel. We hebben aangetoond dat de PI3K route geactiveerd is in meer dan de helft van de onderzochte melanomen, maar niet in de aangrenzende moedervlekken. We concluderen dat door activatie van de PI3K route oncogen-geïnduceerde senescence kan worden omzeild en dat dit voor moedervlek-naar-melanoom ontwikkeling zorgt.
In hoofdstuk 4 hebben we de rol van een ander weloverwogen idee in ons oncogen-geïnduceerde senescence celsysteem bestudeerd. Verschillende virale oncogenen kunnen ervoor zorgen dat cellen senescence omzeilen en vervolgens transformeren. De bekendste is SV40 LT, een viraal antigen dat twee belangrijke tumorsuppressorgenen (namelijk p53 en RB) inactiveren en met deze functie senescence omzeilt. We hebben gevonden dat SV40 LT (of remming van de p53 en RB routes op een andere manier) er niet voor kan zorgen dat BRAF$_E^{600}$-geïnduceerde senescence wordt omzeild, maar dat zijn kleine broertje SV40 st dit wel zeer efficiënt kan. Hoewel we er nog niet achter zijn hoe SV40 st dit precies doet, kunnen we dit eiwit wel gebruiken als gereedschap om andere factoren te vinden die betrokken zijn bij BRAF$_E^{600}$-afhankelijke tumoren.

In hoofdstuk 5 hebben we het RAS$_{V12}$-geïnduceerde senescence celsysteem gebruikt om nieuwe oncogenen te screenen. Uit een ‘genoombibliotheek’ (met daarin ongeveer 25.000 genen) hebben we LRF geïdentificeerd als een nieuw potentieel oncogen. Het RAS$_{V12}$-geïnduceerde senescence celsysteem hebben we verder gebruikt om te onderzoeken via welke routes LRF senescence kan omzeilen. We hebben gevonden dat LRF de activiteit kan verhogen van E2F, een transcriptiefactor die ervoor zorgt dat genen die betrokken zijn bij de celcyclus geactiveerd worden. Eén van deze genen is Cycline E, en de activatie van dit gen is essentieel voor omzeiling van oncogen-geïnduceerde senescence door LRF.

In hoofdstuk 6 bespreken we de resultaten die behaald zijn door middel van het gebruik van het senescence celsysteem als gereedschap om genen te vinden die betrokken zijn bij veroudering en de ontwikkeling van kanker. Hoe ouder organismen worden, hoe meer senescent cellen worden gevonden in deze organismen. Maar het is nog niet duidelijk of deze senescent cellen ook een rol spelen bij het verouderingsproces. De talrijke experimenten met het senescence celsysteem in vitro hebben ons al veel inzicht verschaf over de inductie en het onderhoud van senescence. In verschillende goedaardige tumoren in mensen en modelorganismen zijn cellen gevonden die oncogen-geïnduceerde senescence hebben ondergaan. Deze modellen kunnen worden gebruikt om te onderzoeken of de bevindingen die we hebben gedaan met in vitro experimenten ook gelden voor in vivo processen.

Samenvattend, in dit proefschrift hebben we aangetoond dat oncogen-geïnduceerde senescence een belangrijke rol speelt bij het onderdrukken van kanker in het menselijk lichaam. We hebben met het oncogen-geïnduceerde senescence celsysteem laten zien dat kanker-geassocieerde genen een cruciale rol spelen bij oncogen-geïnduceerde senescence. Verder hebben we het oncogen-geïnduceerde senescence celsysteem gebruikt om uit te werken welke mechanismes belangrijk zijn voor de inductie en het onderhoud van senescence. Hopelijk kan een toegenomen inzicht in deze processen uiteindelijk leiden tot de ontwikkeling van medicijnen tegen kanker.
Curriculum vitae

Liesbeth C.W. Vredevelde was born on the 14th of October 1979 in the Openbaar Lichaam Zuidelijke IJsselmeerpoeders (The Netherlands). She received her high school VWO diploma from the Marianum Comprehensive School (Groenlo, The Netherlands) in 1998. The same year she started her studies in Medical Biology at the University of Amsterdam (The Netherlands). As a part of her studies she did an internship in the laboratory of Prof.dr. R. Versteeg at the Department of Human Genetics, Academic Medical Center, University of Amsterdam. Under supervision of Dr. H.A.M. Geerts, she investigated the regulation of nuclear transport by N-myc in neuroblastoma cells. She carried out her second internship at the The Netherlands Cancer Institute in the laboratory of Prof.dr. R. Bernards at the Division of Molecular Carcinogenesis and Center for Biomedical Genetics. Under supervision of Prof.dr. D.S. Peeper, she identified LRF as a putative oncogene in a functional RAS-induced senescence bypass screen. To finalize her studies, she wrote her graduation thesis about RNA Editing in Mammalian Cells, supervised by Dr. R. Benne (Department of Biochemistry, Academic Medical Center, University of Amsterdam). Right after her graduation in 2002, she started in the laboratory of Prof.dr. D.S. Peeper at the division of Molecular Genetics at The Netherlands Cancer Institute, were she continued on the work she started in her second internship and conducted the research described in this thesis. Currently, she is studying at the University of Amsterdam to recieve a Master of Science teaching Biology.
Publications


*These authors contributed equally to this work.
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Liesbeth