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Oncogene. Author manuscript; available in PMC 2013 November 29.

Published in final edited form as:
Oncogene. 2008 October 9; 27(46): . doi:10.1038/onc.2008.213.

Critical pathways in cellular senescence and immortalization revealed by gene expression profiling

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Abstract

Bypassing cellular senescence and becoming immortal is a prerequisite step in the tumorigenic transformation of a cell. It has long been known that loss of a key tumor suppressor gene, such as p53, is necessary, but not sufficient, for spontaneous cellular immortalization. Therefore, there must be additional mutations and/or epigenetic alterations required for immortalization to occur. Early work on these processes included somatic cell genetic studies to estimate the number of senescence genes, and microcell-mediated transfer of chromosomes into immortalized cells to identify putative senescence-inducing genetic loci. These principal studies laid the foundation for the field of senescence/immortalization, but were labor intensive and the results were somewhat limited. The advent of gene expression profiling and bioinformatics analysis greatly facilitated the identification of genes and pathways that regulate cellular senescence/immortalization. In this review, we present the findings of several gene expression profiling studies and supporting functional data, where available. We identified universal genes regulating senescence/immortalization and found that the key regulator genes represented six pathways: the cell cycle pRB/p53, cytoskeletal, interferon-related, insulin growth factor-related, MAP kinase and oxidative stress pathway. The identification of the genes and pathways regulating senescence/immortalization could provide novel molecular targets for the treatment and/or prevention of cancer.

Keywords

immortalization; senescence; cell cycle; interferon; cytoskeletal; IGF-related genes

Introduction

One of the critical steps in human carcinogenesis is cellular immortalization, a process in which cells must escape senescence and acquire an infinite lifespan. In the absence of immortalization although a cell might undergo malignant transformation it could not proliferate indefinitely. Frequently, the loss of tumor-suppressive genes contributes to cellular immortalization. One approach to understanding senescence and immortalization is comprehensive gene expression profiling of cells prior to and after their acquisition of an infinite lifespan. Although there are several studies that employ rodent cells as models for identifying candidate genes in replicative senescence (Benvenuti *et al*., 2002a, b), we have chosen not to include these studies in this review because of the differences between rodent

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and human cell immortalization. The differences between the mechanisms of human and mouse cell immortalization have been reviewed in Itahana *et al.* (2004).

A comparison of gene expression profiling studies is limited by the lack of overlap among the available genes on the various experimental platforms. Large profiling studies are only a tool for identifying potential genes that regulate a particular process, in the case of this review, immortalization and senescence. Researchers conducting these large-scale studies must be cognizant of such pitfalls including the potential lack of universality of the results, whether the gene expression change is primary or a consequence of other gene changes, and the potential for the identification of unrelated genes that coincidently respond to the same regulatory effectors (Chuaqui *et al.*, 2002; Quackenbush, 2003). Our goal was not only to present data from several immortalization and senescence studies but also to determine whether there were gene(s) and/or pathway(s) that are regulated across cell models of senescence and immortalization. Furthermore, where available, we show supporting functional studies for genes identified as effectors of senescence and/or immortalization.

Replicative senescence

Normal somatic cells grown in culture cease to proliferate, senesce, after a finite number of divisions. This phenomenon, first described by Hayflick (1965), is referred to as replicative senescence or mortality stage 1 (M1) (Dimri *et al.*, 1995; Coates, 2002; Cong *et al.*, 2002). Apparent senescence can also be induced with chemicals, by the overexpression of tumor suppressor genes or with oncogenes, and is referred to as premature or induced senescence (Drayton and Peters, 2002). The validation for the involvement of a gene in cellular senescence is whether it is regulated during naturally occurring senescence of cells aging in culture. Senescent cells generally have a large, flattened morphology (Berube *et al.*, 1998). These cells are growth arrested in G1 phase of the cell cycle, are incapable of synthesizing DNA and are unresponsive to stimulation with growth factors; yet interestingly, are still metabolically active (Berube *et al.*, 1998; Lundberg *et al.*, 2000). Senescent cells can be distinguished from pre-senescent, immortal, quiescent or terminally differentiated cells by histochemical detection of the biomarker β-galactosidase (SA β-gal) at pH 6 (Dimri *et al.*, 1995). Other markers of cellular senescence include p16^{INK4a}, p 21$^{CIP1/WAF1}$, PAI-1, phosphorylated H2AX, activated CHK1 and CHK2 (Suzuki *et al.*, 2001; d'Adda di Fagagna *et al.*, 2003).

Cell types that are capable of entering replicative senescence include fibroblasts, epidermal keratinocytes, vascular smooth muscle cells, lens epithelial cells, glial cells, endothelial cells, melanocytes, T lymphocytes and adrenocortical cells (Cristofalo and Pignolo, 1993; Berube *et al.*, 1998). Although there are similarities among the cell types in the senescent phenotype, the process by which a cell senesces is likely to have features that are cell-type specific.

Replicative senescence: fibroblast cells

In normal human fibroblast cells, telomeres become progressively shorter with each population doubling until they reach a critically short length (Cristofalo and Pignolo, 1993; Cong *et al.*, 2002). The progressive shortening of telomeres in normal somatic cells is more prominent than in germ cells, and to a lesser extent stem cells, where the telomeres are maintained by telomerase (Cong *et al.*, 2002). Critically, short telomeres may be one of the signals that can induce senescence or M1. Inactivation of p53, pRB/p16^{INK4a} or another key proliferative checkpoint gene can extend the lifespan of a cell beyond M1. The telomeres of these checkpoint-defective cells continue to shorten and eventually the cells enter a stage called crisis, or M2. Only a rare cell in such populations is able to survive the widespread cell death characterized by M2. This rare cell escapes crisis and becomes immortalized

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through telomere stabilization either by telomerase activation or alternate lengthening of telomeres (Bischoff *et al.*, 1990; Kim *et al.*, 1994; Bodnar *et al.*, 1998; Gollahon *et al.*, 1998; Cong *et al.*, 2002). Ectopic expression of the catalytic unit of telomerase, hTERT, can stabilize telomere length allowing cells to grow indefinitely (Bodnar *et al.*, 1998).

Replicative senescence: epithelial cells

Epithelial cells have two stages of senescence. The first stage, termed M0, is a transient growth plateau that occurs after only a few cell divisions (Romanov *et al.*, 2001; Yaswen and Stampfer, 2002; Stampfer and Yaswen, 2003; Zhang *et al.*, 2003). Arrest of epithelial cells in M0 is associated with an increase in $p16^{INK4a}$ protein expression, but it is not a consequence of telomere shortening (Romanov *et al.*, 2001; Yaswen and Stampfer, 2002; Stampfer and Yaswen, 2003; Zhang *et al.*, 2003). In spite of there being no association of telomere shortening with M0-arrested epithelial cells, some consider this stage the most analogous to M1-senesced fibroblasts (Romanov *et al.*, 2001). Following inactivation of $p16^{INK4a}$, and in some cell types the inactivation of p53, epithelial cells are able to emerge from M0 and continue to proliferate for approximately another 20–70 population doublings before the second stage of growth arrest, termed M1 or agonoescence (Stampfer and Yaswen, 2003). Epithelial cell M1 growth arrest is associated with telomere shortening (Yaswen and Stampfer, 2002; Stampfer and Yaswen, 2003; Zhang *et al.*, 2003).

Comparison of senescence in fibroblast and epithelial cells

There are several differences between M1 senescent fibroblasts, M0-arrested epithelial cells and M1-arrested epithelial cells (Romanov *et al.*, 2001). M1-arrested epithelial cells do not express $p16^{INK4a}$, whereas $p16^{INK4a}$ is expressed in M0 epithelial cells and M1 fibroblasts (Yaswen and Stampfer, 2002; Stampfer and Yaswen, 2003). There are a higher percentage of M1 epithelial cells stained for Annexin-V, a marker of apoptosis, than in M0-arrested epithelial cells or M1-arrested fibroblasts (Romanov *et al.*, 2001). For more in depth information on senescence of fibroblast and epithelial cells, we refer readers to reviews by Cristofalo and Pignolo (1993) and Yaswen and Stampfer (2002), and to a study that compares senescence of isogenic sets of human mammary fibroblasts and epithelial cells (Romanov *et al.*, 2001).

Correlation of senescence with age of a cell and with lifespan of an organism

It has been proposed that senescence reflects aging of cells, but this is controversial (Rubin, 2002). This theory is supported by evidence that shows a percentage of all cell cultures contain senescent cells and this percentage increases as the cell cultures age (Smith and Pereira-Smith, 1996; Campisi *et al.*, 2001). For human fibroblasts, there is evidence from several studies of an inverse relationship between replicative potential and the age of the cell donor (Rohme, 1981; Hayflick, 1985; Smith and Pereira-Smith, 1996). There is also evidence suggesting that there is a correlation between the average life expectancy of a species and the number of doublings that cells from the organism undergo before senescing in culture (Rohme, 1981; Hayflick, 1985; Campisi *et al.*, 2001). For example, cultured fibroblasts from a mouse, which has a maximal lifespan of 2 years, reach growth crisis after an average of nine population doublings, whereas fibroblasts from a human reach crisis after an average of 61 population doublings (Rohme, 1981). However, there are many who are skeptical of the correlation between lifespan of an organism and senescence. In part, this is because there are many exceptions to this correlation (Rubin, 2002).

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Mechanisms by which cells escape senescence

Because cellular senescence is growth suppressive, it would seem that the accumulation of senescent cells during aging would result in a decrease in the incidence of cancer (Krtolica *et al.*, 2001), yet we know that there is an increased incidence of cancer with age. This might be reconciled by the fact that there is also an accumulation of mutations with aging that can confer selective growth advantages to the cell and therefore mutations in key regulatory genes can result in bypassing senescence. Consequently, senescence is thought to be a tumor-suppressive mechanism. However, there is also evidence from a mouse model that suggests senescent fibroblasts can actually stimulate growth of immortalized and malignant epithelial cells (Krtolica *et al.*, 2001). Tumors, whether naturally occurring or experimentally induced, have at the very least, an extended lifespan and are usually replicatively immortal.

Cancer cells that have escaped replicative senescence are immortal, capable of growing indefinitely. There are several mechanisms that contribute to a cell being able to escape senescence and become immortal, including genomic instability, telomere length stabilization, epigenetic gene silencing by selective promoter methylation, oxidative DNA damage, inactivation of cell cycle regulatory genes such as p16^{INK4a}, p53, pRB or p21$^{CIP1/WAF1}$, overexpression of a cellular oncogenic protein such as c-MYC or Bmi-1, or through expression of viral oncogenes (Berube *et al.*, 1998; Bringold and Serrano, 2000; Lundberg *et al.*, 2000; Neumeister *et al.*, 2002; Itahana *et al.*, 2003). Shortening of telomeres is another mechanism associated with replicative senescence. Telomeres are the tandem GT-rich repeats (TTAGGG) at the ends of chromosomes. Among their many roles telomeres act as ‘molecular clocks,’ determining the lifespan of cells (Cong *et al.*, 2002; Harley, 2002). Telomeres become successively shorter, by 50–200 bp, with each round of replication. In most cases, maintenance of telomere length is required for immortalization. Telomere length is maintained through reactivation of telomerase or by the alternate lengthening of telomere pathway. Cellular senescence, apoptosis and genomic instability are the consequences resulting from dysfunctional telomeres (Campisi *et al.*, 2001).

Methylation of CpG islands in gene promoters

There are several normal cellular processes that are regulated in part by epigenetic modification through DNA methylation, including developmental imprinting, X-chromosome inactivation and tissue-specific gene expression. In addition, aberrant promoter methylation has been found in growth-suppressive genes in human tumorigenesis (Baylin and Herman, 2000; Baylin *et al.*, 2001; Esteller *et al.*, 2001; Feinberg *et al.*, 2002). CpG dinucleotides are typically methylated. In contrast CpG islands, which are CpG rich stretches of DNA ranging from 200 to 2000 bp in length in the regulatory regions of genes, are generally unmethylated. The transfer of a methyl group from *S*-adenosyl-L-methionine to the cytosines in CpG sites is catalysed by DNA-methyltransferases (DNMT) (Lopatina *et al.*, 2002). There are three well-characterized DNMTs, DNMT1, DNMT3A and DNMT3B. DNMT1, the most abundant DNMT, is mainly responsible for maintenance methylation, whereas DNMT3a and DNMT3b are responsible for *de novo* methylation (Lopatina *et al.*, 2002).

In human cancers, the silencing of tumor suppressor genes through aberrant DNA methylation of a CpG island(s) in the promoters in these genes is a common epigenetic change (Baylin and Herman, 2000). There are an assortment of pathways from which genes have been shown to be hypermethylated in cancer cells, including DNA repair, cell cycle control, invasion and metastasis. The tumor suppressor genes *BRCA1*, *p16*^{INK4a}, *p15*^{INK4b},

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p14^{ARF}, *p73* and *APC* are among those that are silenced by hypermethylation, although the frequency of aberrant methylation is somewhat tumor-type specific (Esteller *et al.*, 2001).

Aberrant hypermethylation of DNA can be reversed with chemical agents that inhibit DNMTs, which in effect 'demethylate' DNA. A commonly used inhibitor of DNMT is 5-aza-deoxycytidine (5-aza-dC), a cytosine analog. 5-aza-dC and related drugs work by substituting for cytosine during replication. DNMTs recognize and covalently bind 5-aza-dC in DNA. The covalently bound DNMT1 is unable to catalyse the transfer of methyl groups to the cytosine analog because the substituted nitrogen base cannot be methylated. Consequently, DNMT1 is depleted following several rounds of replication. This in turn results in DNA hypomethylation and the expression of genes that were silenced by methylation (Haaf, 1995; Kanai *et al.*, 2001; Takebayashi *et al.*, 2001).

Methylation of CpG Islands, a preneoplastic event

Vogt *et al.* (1998) found that when spontaneously immortalized fibroblasts with germline p53 mutations (from Li-Fraumeni patients (LFS)) were treated with 5-aza-dC, they growth arrested and senesced. They concluded that methylation of CpG islands may contribute to the spontaneous immortalization of LFS cells. Treatment with 5-aza-dC was found to restore the expression of genes such as p16^{INK4a} and p21$^{CIP1/WAF1}$ that were presumably silenced by aberrant DNA methylation (Vogt *et al.*, 1998). We used 5-aza-dC as a tool to identify genes epigenetically silenced during immortalization. Our findings were the first to demonstrate pathway-specific epigenetic changes as a preneoplastic event (Kulaeva *et al.*, 2003; Fridman *et al.*, 2006).

Complementation groups of immortal cells

It is generally believed that many genes and pathways are involved in senescence. Losing the wild-type p53 allele in LFS human fibroblasts is necessary, but not sufficient, for spontaneous cellular immortalization as p53-deficient cells do senese; however, the absence of p53 does extend their lifespan (Bischoff *et al.*, 1990, 1991; Harvey *et al.*, 1993; Tsutsui *et al.*, 1997). The loss of p53 and the acquisition of the immortal phenotype can be accelerated by the treatment of p53$^{+/-}$ LFS fibroblasts with the chemical carcinogen aflatoxin B1 (Tsutsui *et al.*, 1995). Because the loss of the wild-type p53 allele is insufficient to cause immortalization (Harvey *et al.*, 1993), there must be additional mutations and/or epigenetic alterations necessary for immortalization to occur. Somatic cell genetics complementation studies were used to estimate the number of senescence genes. In somatic cell hybrids of mortal and immortal cells, the replicative senescence phenotype is dominant over the immortal phenotype (Smith and Pereira-Smith, 1996; Berube *et al.*, 1998). Consequently, when two unrelated immortal cell lines with defects in different genes are fused together, the hybrid cells senesce and it can be concluded that they belong to different complementation groups (Smith and Pereira-Smith, 1996). To date four immortalization complementation groups have been identified to which 40 immortal human cell lines were assigned (Smith and Pereira-Smith, 1996; Berube *et al.*, 1998). This suggests that there are at least four senescence genes or gene pathways that must be abrogated to achieve cellular immortalization (Smith and Pereira-Smith, 1996; Berube *et al.*, 1998).

Chromosomes associated with a senescence-like phenotype

Microcell-mediated transfer of chromosomes into immortalized cells was used to identify putative senescence genes associated with the complementation groups (Smith and Pereira-Smith, 1996; Berube *et al.*, 1998). Using this method, several chromosomes were identified as potentially encoding senescent genes, including chromosomes 1, 2, 3, 4, 6, 7, 10, 11, 16, 17, 18 and X (Smith and Pereira-Smith, 1996; Berube *et al.*, 1998; Tominaga *et al.*, 2002).

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Of these, only three chromosomes have been assigned to a complementation group: chromosome 4 to complementation group B, chromosome 1 to complementation group C, and chromosome 7 to complementation group D (Smith and Pereira-Smith, 1996). Complementation group A, thus far, does not have a chromosome associated with it, although some studies indicate that it may be chromosome 6 (Sandhu *et al*., 1994; Berube *et al*., 1998). A chromosome was assigned to a complementation group if it induced senescence in several cell lines within a complementation group, but did not induce senescence in cell lines from the other complementation groups (Smith and Pereira-Smith, 1996). Mapping known key senescence and immortalization regulatory genes to chromosomes does not reveal any gene clustering or pattern (MA Tainsky lab, unpublished data). However, we identified several clusters, defined as three or more genes within a distance of 2.5Mb, of coregulated genes on chromosome 1q that were commonly regulated in four independent immortal LFS cell lines (AL Fridman and MA Tainsky, unpublished data).

Senescence genes and pathways

Cellular senescence pathways are believed to have multiple layers of regulation with additional redundancy built into these layers (Smith and Pereira-Smith, 1996). On the basis of the complementation studies there are at least four senescence genes or pathways. There are, however, many more chromosomes that can induce senescence than there are senescence complementation groups. Furthermore, there are some immortal cell lines that have been assigned to multiple complementation groups (Duncan *et al*., 1993; Berry *et al*., 1994). This indicates that in any one immortal cell line there are probably multiple senescence genes/pathways that are abrogated (Sasaki *et al*., 1994; Vojta *et al*., 1996). Many of the functional studies, where a putative senescence gene is overexpressed in cells, indicate that although multiple genes/pathways may be abrogated in a particular cell line, as little as one gene/pathway is required for repair and subsequent reversion to senescence.

Two candidate regions on chromosome 1, 1q25 and 1q41–42 were identified as potentially encoding senescence genes (Berube *et al*., 1998). To date, the specific senescence gene on chromosome 1 has not been identified. On chromosome 6 the region 6q13–6q21 is believed to contain potential senescence genes as transfection of this region into ovarian tumor cells induced senescence (Morelli *et al*., 2000). The region containing the senescence gene was further narrowed down to fragile site FRA6F located at 6q21, but the gene in this region responsible for senescence has not yet been identified (Morelli *et al*., 2002). MORF4 is a senescence gene identified on chromosome 4 (Berube *et al*., 1998; Bertram *et al*., 1999) and complements cell lines in complementation group B.

Genes that have been shown to induce a senescence-like phenotype, or at the very least inhibit cell growth in tumor cells, include *p14^{ARF}* (Dimri *et al*., 2000; Sekaric *et al*., 2007), *E2F-1* (Dimri *et al*., 2000), *IGFBP3* (Fridman *et al*., 2007), *IGFBPrP1* (Wilson *et al*., 2002; Fridman *et al*., 2007), *PAI-1* (Kortlever *et al*., 2006), *MKK3* (Wang *et al*., 2002), *MKK6* (Haq *et al*., 2002; Wang *et al*., 2002), *Smurf2* (Zhang and Cohen, 2004) and *HIC-5* (Shibanuma *et al*., 1997). Other genes that can induce a senescence-like phenotype are reviewed in Bringold and Serrano (2000), Lundberg *et al*. (2000) and Roninson (2003) and include *p53*, *p63*, *p73*, *pRB*, *p16^{INK4a}*, *p21$^{CIP1/WAF1}$*, *p15^{INK4b}*, *p57^{KIP2}*, *RAF-1*, *E2 papillomavirus protein* (inhibitor of E6 and E7), oncogenic ras and hTERT. Pathways known to regulate cellular senescence/immortalization, including the p16^{INK4a}/pRB pathway, the p19^{ARF}/p53/p21$^{CIP1/WAF1}$ pathway, and the PTEN/p27^{KIP1} pathway are reviewed in Berube *et al*. (1998), Bringold and Serrano (2000), Lundberg *et al*. (2000) and Campisi (2001).

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Genomic approaches to identify senescence/immortalization genes and pathways

There are a variety of model systems that have been used to identify and study senescence/immortalization genes and pathways. Normal cells grown *in vitro* senesce after a finite number of divisions (Hayflick, 1965) and such model systems are highly suitable for studying genes that are regulated during senescence. Cells that are derived from patients with Werner syndrome, a disease typified by premature aging and shortened lifespan, provide useful genetic variant systems for the study senescence as well as aging. Alternatively, senescence model systems have also employed immortalized cells that are chemically induced to senesce. A cellular senescence-like phenotype can be induced in immortalized cells that are treated with reagents such as 5-aza-dC, H_2O_2 or 5-bromodeoxyuridine (BrdU) (Chen *et al*., 1995; Vogt *et al*., 1998; Suzuki *et al*., 2001; Kulaeva *et al*., 2003; Fridman *et al*., 2006). Although not the focus of this review there are *in vivo* mouse models for studying senescence and aging (Sommer *et al*., 2006).

In vitro immortalization models have been developed using cells immortalized chemically, virally or with a biological agent. Mortal cells can be immortalized artificially by stabilizing the telomeres by overexpression of *hTERT* (Bodnar *et al*., 1998), through the addition of a chemical mutagen such as aflatoxin B1 (Tsutsui *et al*., 1995), or by transduction with viral oncogenes such as SV40 T-antigen, adenovirus E1a/E1B and HPV16-E6 or -E7 (Jha *et al*., 1998; Garbe *et al*., 1999; Schwarze *et al*., 2002; Boulet *et al*., 2007). These viral oncogenes act by inhibiting key genes such as the tumor suppressor gene proteins p53 and pRB, thus allowing the cell to bypass senescence and become immortal (Jha *et al*., 1998; Boulet *et al*., 2007). Alternatively, at a low but finite frequency spontaneous immortalization occurs in cells with a germline mutation such as *p53*$^{+/-}$ fibroblasts or epithelial cells derived from a patient with LFS (Bischoff *et al*., 1990; Rogan *et al*., 1995; Shay *et al*., 1995; Gollahon *et al*., 1998), or as in the case of *APC*$^{+/-}$ fibroblasts from a patient with familial adenomatous polyposis (Forsyth *et al*., 2004). Using fibroblasts from a patient with LFS, our lab was the first to spontaneously immortalize a human fibroblast cell line *in vitro* (Bischoff *et al*., 1990). As is typical for LFS patients, the fibroblasts had a germline *p53* tumor suppressor gene mutation (Varley *et al*., 1997; Bachinski *et al*., 2005) that results in significant genomic instability. Consequently, low-passage precrisis LFS cells with genomic instability immortalize without intervention, thereby providing an ideal model of spontaneous cellular immortalization. The disadvantage of inducing immortalization with viral or chemical agents is that gene expression changes may be altered beyond those relevant to immortalization. However, because human cells have only been shown to spontaneously immortalize in culture using strains with certain pre-existing germline mutations (*p53* and *APC*), all other human cell systems require pretreating cells with a chemical or by transducing them with a viral oncogenes to achieve cellular immortalization.

Immortalization is an essential step in the tumorigenic transformation of a cell, yet it can be reversed hence halting the tumorigenic transformation of a cell. Immortalization can be reversed and cellular senescence induced by reexpression of a key senescence gene that was abrogated as a consequence of mutation, infection with a viral oncogene or suppressed by epigenetic silencing. Furthermore, a cellular senescence-like phenotype can be induced in immortalized LFS cells as well as many tumor cell lines when they are treated with the DNMT inhibitor 5-aza-dC, further indicating that the immortal phenotype of cells is reversible (Vogt *et al*., 1998; Karpf and Jones, 2002; Kulaeva *et al*., 2003; Paz *et al*., 2003; Fridman *et al*., 2006). Therefore, understanding the process by which cells bypass senescence and become immortal could provide novel molecular targets for the treatment of preneoplastic lesions and/or prevention of cancer.

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Senescence and immortalization model systems are essential for elucidating the genes and pathways involved in cellular senescence/immortalization. Recently model systems have been studied using functional genomics approaches, including differential proteomics, serial analysis of gene expression (SAGE) and microarrays, thus allowing researchers to identify relevant genes and pathways involved in senescence/immortalization. The findings of these studies, which are summarized in Supplemental Tables 1 and 2, are the focus of this review. The genomic approaches in combination with traditional methods including overexpression of putative senescence or immortalization genes has led to the discovery of six key distinct, yet interacting, senescence/immortalization pathways, including the cell cycle pathway, cytoskeletal genes, interferon (IFN) pathway, insulin growth factor (IGF)-related pathway genes, MAP kinase pathway and oxidative stress pathway (Figure 1).

SAGE was used to characterize genes differentially expressed during the senescence of prostate epithelial cells (Untergasser *et al.*, 2002). Untergasser *et al.* (2002) found 157 mRNA (70 known genes) that were upregulated and 116 mRNA (65 known genes) that were repressed during senescence. Among these genes, 70 upregulated and 65 repressed genes were classified as functioning in the cell cycle, IFN-related, extracellular matrix or cytoskeletal pathways.

cDNA microarrays were used to identify genes in human prostate epithelial cells (HPECs) upregulated in senescence and repressed during the immortalization of the cells. Three genes, *DOC1*, *BRAK* and *IGFBP3*, were identified that were decreased in immortalized cells and upregulated in senescent cells (Schwarze *et al.*, 2002). The identification of these genes is consistent with the involvement of the IFN, cytoskeletal, cell cycle and IGF pathway in immortalization; however, these genes did not overlap with those identified with expression changes in the study of prostate epithelial cells (Untergasser *et al.*, 2002).

We used precrisis fibroblast LFS cell lines and spontaneously immortalized LFS fibroblast cell lines to investigate the nature of the genetic changes that might have given these cell lines the prerequisite growth advantage to become immortal (Kulaeva *et al.*, 2003; Fridman *et al.*, 2006). A cellular senescence-like phenotype can be induced in immortalized LFS cells that are treated with the DNMT inhibitor 5-aza-dC, thus reversing the immortal phenotype of these cells. On the basis of this observation, we hypothesized that genes epigenetically silenced by methylation are putative key regulatory cellular senescence genes. Consistent with previous studies, we found that the loss of checkpoint proteins such as $p16^{INK4a}$, $p21^{CIP1/WAF1}$ and p53 led to immortalization-initiating events including genomic instability and telomere stabilization (Fridman *et al.*, 2006). Using four independently derived immortal LFS cell lines, our analysis identified 149 upregulated genes and 187 downregulated genes after immortalization, 14 of which were epigenetically downregulated. Pathway analysis of the expression data with gene ontology bioinformatics software revealed a statistically significant contribution of IFN-related, cell cycle and cytoskeletal genes in the process of immortalization (Kulaeva *et al.*, 2003; Fridman *et al.*, 2006). In total, 12 of the 14 genes that are epigenetically regulated in all four immortal LFS cell lines have a known function and 8 of these genes could be categorized in one of the six senescence/immortalization pathways.

Cell Cycle

The cell cycle has long been known as one of the pathways that regulates senescence, in particular through pRB and p53. In our study of the spontaneously immortalized LFS cells, in addition to the genes known to be involved in the regulation of cellular senescence/immortalization ($p16^{INK4a}$, $p21^{CIP1/WAF1}$ and *p53*), we identified other cell cycle regulators among the 14 epigenetically regulated genes that were commonly regulated in all four immortal LFS cell lines. One of the 14 genes, *HTATIP2*, is categorized in the gene ontology

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cell cycle category. Two genes, *IGFBPrP1* and *ALDH1A3*, are regulated by the cell cycle-regulating gene *p53*, and two genes, *CREG* and *SERPINB2*, have been shown to physically associate with the cell cycle regulator pRB. Our identification of these genes as epigenetically regulated in senescence/immortalization is supported by research from other laboratories. *HTATIP2* is upregulated in senescent human mammary epithelial cells (HMECs) (Zhang *et al.*, 2003). Overexpression of *CREG* in human teratocarcinoma NTERA-2 cells delays G1/S transition and inhibits growth (Di Bacco and Gill, 2003). SERPINB2 is one of only three genes that was upregulated in human HMEC during senescence on feeder layers, in HMEC during senescence on plastic and in senescent human fibroblasts (Zhang *et al.*, 2003, 2004). Expression of SERPINB2 is also upregulated in the bladder tumor cell line T24 following 5-aza-dC treatment (Liang *et al.*, 2002), as well as upregulated in senescent BJ fibroblast cells (a human dermal fibroblast cell line) (Linskens *et al.*, 1995). In addition, expression of the p53-downregulated gene *CDC25B* is induced after immortalization and then following 5-aza-dC treatment its expression is repressed (MA Tainsky lab, unpublished data). CDC25B was also increased in immortalized HMEC, but not prestasis or postselection HMEC (Li *et al.*, 2007).

The cell cycle protein inhibitor of growth 1 (p33ING1), located on chromosome 13q34, was identified as a senescence-inducing gene (Garkavtsev and Riabowol, 1997; Berube *et al.*, 1998; Goeman *et al.*, 2005). Expression of this tumor suppressor gene is increased in senescent human diploid fibroblasts, and when expression of it is blocked in pre-senescent cells the life span of the cells increases (Garkavtsev and Riabowol, 1997). Furthermore, ectopic expression of p33ING1 in IMR90 fibroblasts induces senescence (Goeman *et al.*, 2005). There is a functional connection of p33ING1 with oncogenic ras (Goeman *et al.*, 2005) and $p14^{ARF}$ (Gonzalez *et al.*, 2006), which are both capable of inducing senescence. p33ING1 has also been shown to bind p53 (Garkavtsev *et al.*, 1998; Leung *et al.*, 2002). p33ING1 protein leads to the stabilization of p53 through disruption of the p53–MDM2 interaction (Leung *et al.*, 2002). Additionally, the interaction of p33ING1 with p53 induces transcription of $p21^{CIP1/WAF1}$ (Garkavtsev *et al.*, 1998).

PAI-1, a downstream target of p53 (Kortlever *et al.*, 2006), is among the genes that were overexpressed in senescent Werner syndrome fibroblasts (Murano *et al.*, 1991; Lecka-Czernik *et al.*, 1996). PAI-1 was also upregulated in senescent BJ fibroblasts (Shelton *et al.*, 1999), senescent TIG-7 (a human embryonic lung fibroblast cell line) (Suzuki *et al.*, 2001), in HeLa cells senesced with BrdU (Suzuki *et al.*, 2001), and in senescent prostate epithelial cells (Untergasser *et al.*, 2002). *PAI-1* decreases in immortalized HMEC when compared with prestasis HMEC (Li *et al.*, 2007). Functional studies of PAI-1 in mouse embryonic fibroblasts and human BJ fibroblasts show that PAI-1 is necessary and sufficient for senescence of these fibroblasts (Kortlever *et al.*, 2006). PAI-1 and its connection to cytoskeletal genes are discussed below. The role of PAI-1 in senescence has been recently reviewed in Kortlever and Bernards (2006).

Cytoskeletal genes

Given the distinct morphological transition as cells transition from proliferation to senescence, it was no surprise that genes associated with the cytoskeleton have been found to be altered as cells senesce. Supporting evidence for the involvement of cytoskeletal genes in senescence comes from several laboratories that have shown an increase in expression of cytoskeletal genes, such as vimentin and fibronectin, in senescent human fibroblasts (Murano *et al.*, 1991; Satoh *et al.*, 1994; Kaneko *et al.*, 1995). Among the genes that were overexpressed in senescent Werner syndrome fibroblasts, several are cytoskeletal related including fibronectin, PAI-1 and thrombospondin (Murano *et al.*, 1991; Lecka-Czernik *et al.*, 1996). Fibronectin was also upregulated in senescent human vascular endothelial cells (Shelton *et al.*, 1999), senescent TIG-7 (Suzuki *et al.*, 2001), HeLa cells senesced with BrdU

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(Suzuki *et al*., 2001), senescent prostate epithelial cells (Untergasser *et al*., 2002), senescent human oral keratinocytes (Kang *et al*., 2003) and senescent HMECs (Zhang *et al*., 2003). *PAI-1* expression is induced in anchorage-dependent cells, but was not detectable in an anchorage-independent cell line (Lee *et al*., 2002) Fibronectin- and vitronectin-induced expression of *PAI-1* (Lee *et al*., 2002). PAI-1 inhibits cleavage of focal adhesions, thus further connecting PAI-1 to senescence (Sorrell *et al*., 2006). *DOC1* was identified by Schwarze *et al.* (2002) as upregulated in senescence and repressed during the immortalization of HPECs, and identified by Zhang *et al.* (2003) as upregulated in senescent fibroblasts. *DOC1* is homologous to a mouse cytoskeleton-associated protein (Tandle *et al*., 2005). In our LFS study, there were a statistically significant number of cytoskeletal genes that were silenced during immortalization, including 2 of the 14 genes that were regulated across four LFS cell lines, *HPS5* and *MAP1LC3B*. In their proteomic analysis of senescent rat fibroblasts, Benvenuti *et al.* (2002b) identified several cytoskeletal proteins upregulated during senescence.

IFN pathway

Probably the most unanticipated pathway found to be differentially regulated in senescence and immortalization was the IFN pathway. In our initial gene expression profiling on oligonucleotide microarrays where we compared gene expression profiles of precrisis with immortal LFS cells, and untreated with 5-aza-dC-treated LFS immortal cells, we demonstrated treatment of MDAH041 cells with 5-aza-dC induced senescence and resulted in the upregulation of 85 genes that appeared to be epigenetically silenced after immortalization (Kulaeva *et al*., 2003). Of these genes, there was a statistically significant portion that was linked with the IFN signaling pathway (39 out of 85) (Kulaeva *et al*., 2003). In an expanded study using four independent immortal LFS cell lines (Fridman *et al*., 2006), we confirmed our initial findings that the IFN pathway plays a significant role in immortalization. Furthermore, of the 14 genes that were epigenetically regulated in four LFS cell lines we found that 3 were regulated by IFN: *ALDH1A3*, *OPTN* and *SERPINB2* (Fridman *et al*., 2006). Likewise, IFN-regulated genes were also found to be downregulated in tumorigenic benign prostatic hyperplasia cells (Shou *et al*., 2002), upregulated in senescent prostate epithelial cells (Untergasser *et al*., 2002) and upregulated in senescent human diploid fibroblasts (Yoon *et al*., 2004). In addition, IFN-regulated genes were sensitive to demethylation treatment in the bladder tumor cell line T24 following 5-aza-dC treatment (54% of upregulated genes) (Liang *et al*., 2002). *BRAK*, a chemokine that when over-expressed suppresses tumor growth (Ozawa *et al*., 2006), was identified as upregulated in senescence and repressed during the immortalization of HPEC (Schwarze *et al*., 2002). Treatment with IFN-γ reduced the tumorigenic potential of the benign prostatic hyperplasia cells (Shou *et al*., 2002). Expression of IFN-γ is increased in senescent BJ fibroblasts (Linskens *et al*., 1995). Furthermore, young and senescent cells respond differently to treatment with IFN-γ (Stratford *et al*., 2006), yet further proof that the IFN pathway plays a significant role in cellular senescence. We also find overexpression of either IRF-5 or IRF-7 is sufficient for inducing cellular senescence in LFS cells (Li *et al*., 2008). These data strongly corroborate the involvement of the IFN pathway in senescence/immortalization.

IGF pathway genes

The insulin-like growth factors, their receptors and their binding proteins have frequently been found to be altered in their expression during immortalization and complete carcinogenesis. *IGFBP3* was overexpressed in senescent fibroblasts from a patient with Werner syndrome (Goldstein *et al*., 1991; Murano *et al*., 1991), in BrdU-senesced HeLa cells (Suzuki *et al*., 2001), in senescent TIG-7 fibroblasts (Suzuki *et al*., 2001), in senescent human oral keratinocytes (Kang *et al*., 2003), in senescent human diploid fibroblasts (Yoon *et al*., 2004) and in prestasis HMEC when compared with fully immortalized HMEC (Li *et*

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al., 2007). Consistent with these studies, *IGFPB3* was identified by Schwarze *et al.* (2002) as upregulated in senescence and repressed during the immortalization of HPEC. We found that expression of IGFBP3 transcript and protein expression decreased during immortalization of LFS fibroblasts (Fridman *et al*., 2006), which further supported the involvement of IGFBP3 in this process. Functional studies have demonstrated that overexpression of this gene in immortalized LFS cell lines suppressed cell growth and inhibited colony formation (Fridman *et al*., 2007).

IGFBPrP1 was upregulated in senescent epithelial cells and senescent fibroblasts (Zhang *et al*., 2003), in HMECs (Swisshelm *et al*., 1995) and in HPECs (Lopez-Bermejo *et al*., 2000). Immortalization of LFS cells results in the repression of *IGFBPrP1*, and the epigenetic regulation of this gene was demonstrated by its upregulation by 5-aza-dC treatment (Fridman *et al*., 2006). IGFBPrP1 is upregulated in senescing normal fibroblasts and senescing precrisis LFS fibroblasts (Fridman *et al*., 2007). When IGFBPrP1 is expressed in LFS cells it inhibits both growth and colony formation (Fridman *et al*., 2007). Expression of IGFBPrP1 in MCF-7 cells induces a senescence-like state (Swisshelm *et al*., 1995; Wilson *et al*., 2002).

Other IGFBP genes, including *IGFBP2*, *IGFBP4*, *IGFBP5*, *IGFBP6* and *IGFBPrP2*, have also been shown to be regulated during senescence/immortalization. *IGFPB2* was upregulated in senescent cells, including BJ fibroblasts (Shelton *et al*., 1999), human retinal pigment epithelial cells (Shelton *et al*., 1999) and HMECs (Zhang *et al*., 2003). *IGFBP6* mRNA expression increased in the senescent colon carcinoma cell line HCT116 (Chang *et al*., 2002). IGFBPrP2 expression increased in senescent HPECs (Lopez-Bermejo *et al*., 2000). *IGFBP4* and *IGFBP5* decrease during immortalization of LFS cells (Fridman *et al*., 2006, 2007). BrdU-induced senescence of HeLa cells results in the upregulation of *IGFBP4* (Suzuki *et al*., 2001). *IGFBP5* is upregulated in senescent cells, including IMR90 (Linskens *et al*., 1995), BJ fibroblasts (Linskens *et al*., 1995; Shelton *et al*., 1999), human retinal pigment epithelial cells (Shelton *et al*., 1999), human vascular endothelial cells (Shelton *et al*., 1999), TIG-7 fibroblasts (Suzuki *et al*., 2001) and HDF isolated from foreskin (Yoon *et al*., 2004). *IGFBP5* was shown to decrease in E7-immortalized HPEC (Schwarze *et al*., 2002) and in fully immortalized HMEC when compared with prestasis HMEC (Li *et al*., 2007). Interestingly, the senescence cell cycle gene PAI-1 has been shown to bind IGFPB5 and partially protects it from proteolysis (Nam *et al*., 1997). However, there is some evidence that IGFBP5 may be inhibiting PAI-1 (Sorrell *et al*., 2006). Significantly, premature senescence was induced when IGFBP5 was overexpressed in human umbilical vein endothelial cells (Seok Kim *et al*., 2007). Induction of senescence by IGFBP5 in the human umbilical vein endothelial cells was p53 dependent (Seok Kim *et al*., 2007). The expression data and the functional studies of genes in the IGFBP family all support these genes as at the very least involved and potentially as key regulators of senescence/immortalization.

MAP kinase pathway

The upregulation of the MAP kinase pathway in cells with normal checkpoint control often results in growth arrest, apoptosis and/or senescence The decrease in MKK3 expression during immortalization of LFS fibroblasts (Fridman *et al*., 2006) is supported by functional studies of this pathway. Activation of p38HOG by constitutively active MKK3 or MKK6 was found to induce a senescence-like phenotype in BJ fibroblasts (Wang *et al*., 2002) and U2OS, a human osteogenic sarcoma cell line (Haq *et al*., 2002). Experimental evidence from Wang *et al.* (2002) shows oncogenic ras, which was previously shown to induce senescence-like growth arrest in human fibroblasts (Serrano *et al*., 1997), induces activation of p38HOG via the MEK-ERK pathway. Induction of premature senescence through the oncogenic ras-Raf/MEK/ERK-p38HOG pathway results in an increase in p53 and $p16^{INK4a}$ protein

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expression. Although Wang *et al.*, found activation of p38HOG was sufficient to cause an increase in p53 and $p16^{INK4a}$ protein expression in BJ fibroblasts, Haq *et al.* (2002) did not find p53 protein levels increased following activation of p38HOG, but there was an increase in $p21^{CIP1/WAF1}$ protein expression in U2OS cells; U2OS cells do not express $p16^{INK4a}$. That there are multiple known senescence genes that can be activated by p38HOG indicates that even within a senescence pathway there is redundancy.

Oxidative stress pathway

Oxidative DNA damage has long been known as a contributing factor in the senescence of human diploid fibroblasts grown in culture (Chen *et al.*, 1995). An increase in reactive oxygen species (ROS) has been demonstrated in cellular senescence (Chen *et al.*, 1995) with senescing and aged cells having a higher level of ROS than normal cells (Hagen *et al.*, 1997). Treatment of human fibroblasts with H_2O_2 induces a replicative senescence-like phenotype (Chen *et al.*, 1998). In normal diploid cells, ras oncogenes (Lee *et al.*, 1999) and $p21^{CIP/WAF1}$ (Macip *et al.*, 2002) can induce senescence with increased intracellular ROS. Treatment of cells with H_2O_2 (Chen *et al.*, 1998) or the induction of hyperoxia (von Zglinicki *et al.*, 1995; Chen *et al.*, 1998) induces senescence through telomere shortening through a mechanism relying on proper cell cycle control (Chen and Ames, 1994). H_2O_2-treated cells have elevated levels of p53 and $p21^{CIP/WAF1}$, and hypophosphorylated pRB relative to untreated cells (Chen *et al.*, 1998). Interestingly however, oxidative stress pathway genes were also found to be increased in *hTERT* and spontaneously immortalized primary breast tumors (Dairkee *et al.*, 2007). Because ROS is involved in senescence and p53 can enhance ROS levels, the ROS/oxidative stress pathway likely plays a role in senescence. p53-regulated genes such as PIG3 and FDXR genes are involved in the response to ROS (Polyak *et al.*, 1997). In addition, ROS alone can induce $p21^{CIP/WAF1}$ expression even in the absence of p53, but to a lesser extent (Russo *et al.*, 1995). Treatment of three p53-deficient human fibroblast strains with five different antioxidants failed to inhibit their progression toward immortalization using intermediate markers such as anchorage-independent growth or cytogenetic abnormalities, although one antioxidant, oltipraz, was significantly effective in transiently delaying a shift to hyperdiploidy in all three cell strains (Kraniak *et al.*, 2006). Therefore, ROS inhibition alone is not sufficient to block the induction of senescence. p53 provides a critical switch in cell fate between entering apoptosis or reversible cell cycle arrest events such as G1, G0 or senescence. It is therefore no surprise that cells deficient in one or both copies of p53 are prone to spontaneous immortalization (Bischoff *et al.*, 1990), but resistance to ROS-induced growth arrest is not sufficient to escape senescence.

Common senescence/immortalization pathways

Shelton *et al.* (1999) using cDNA microarrays studied replicative senescence in three cell types: dermal fibroblasts, retinal pigment epithelial cells and vascular endothelial cells. They came to the conclusion that although these cell types have similar senescence phenotypes, the genes and pathways involved vary among them. In our analysis of the spontaneously immortalized cell lines, we were able to identify common pathways that were dysregulated during immortalization despite somatic cell hybrid studies showing the LFS cell lines used were in different complementation groups. Somatic cell hybrids were made between MDAH041 (tel+, p $53^{-/-}$, N-ras-transformed) or MDAH087 (tel-,$p53^{-/-}$, N-ras-transformed) and HT1080 (tel+, p 53^{wt}, N-ras) (Gollahon *et al.*, 1998), a fibrosarcoma cell line that had been assigned to senescence complementation groupA (Pereira-Smith and Smith, 1988). Both the MDAH041–HT1080 and the MDAH087–HT1080 hybrids were telomerase positive (Gollahon *et al.*, 1998). The MDAH041–HT1080 hybrids senesced rapidly whereas the MDAH087–HT1080 grew indefinitely (Gollahon *et al.*, 1998). It can be concluded that MDAH041 and HT1080 are in different complementation groups. MDAH087 is probably in

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the same complementation group as HT1080, complementation group A. That we were able to identify both genes and pathways that were dysregulated during immortalization that were in common to cell lines in different complementation groups suggests that it is likely there are certain senescence/immortalization pathways that are consistently involved in this process. In our review of senescence and immortalization studies, we find that across cell types and model systems consistently genes in the cell cycle pathway, cytoskeletal genes, IFN pathway, IGF pathway genes, MAP kinase pathway and oxidative stress pathway were identified as key regulators of senescence/immortalization.

Conclusion

Carcinogenesis is widely accepted to be a multistep process resulting from the accrual of mutations in tumor suppressor genes and oncogenes that confers growth advantages and/or genomic instability to the cell. One of the critical steps in this process is immortalization. Senescence is a mechanism by which cells can suppress unregulated growth by arresting cell proliferation. In contrast, cancer cells have the ability to grow indefinitely because they bypassed replicative senescence and become immortal. In the absence of immortalization, a cell is unable to undergo malignant transformation. Therefore, identification of the genes and pathways regulating the process by which cells bypass senescence and become immortal could provide novel early molecular targets for the treatment and/or prevention of cancer.

In this review, we have presented what we believe are the critical pathways in cellular senescence and immortalization based on information gathered from several gene expression profiling studies and some of these genes have functionally implicated certain cellular processes in senescence and immortalization (Figure 1 and Supplemental Table 1). There are a variety of cellular factors and mechanisms involved in bypassing senescence leading to the cellular immortalization including telomere length, genomic instability and epigenetic gene silencing due to gene methylation. The loss of growth-suppressive genes, and the ensuing cellular immortalization, can result from multiple mechanisms including chromosomal recombination, numerical changes in chromosomes, point mutations and epigenetic silencing. These changes have been considered cell origin dependent as well as dependent on their cell culture conditions (Zhang *et al.*, 2004). However, there is considerable overlap of the genes and pathways that regulate senescence/immortalization, even across cell types, indicating at least some commonality among these regulatory pathways (Supplemental Tables 1 and 2).

In our analysis, there are several common genes regulating these processes and many of these genes that were identified as regulators of senescence/immortalization fall into six main pathways: the cell cycle pRB/p53 pathway, cytoskeletal genes, IFN pathway, IGF pathway genes, MAP kinase pathway and oxidative stress pathway. In reality, these pathways are so interwoven that it is difficult to describe them as individually involved in the transition from senescence to immortalization (Figure 1).

Our conclusion is that a comprehensive, well-controlled gene-profiling study is necessary to fully understand senescence/immortalization. Ideally, this mega study would be performed using different cell systems on a single experimental gene-profiling platform. It would necessarily require functional data on the most common senescence/immortalization regulatory genes identified to produce a universal picture of the genes and pathways that regulate senescence/immortalization. A study of this proportion may be difficult to fund, thus we propose the next step should be to extend the work in this review and compile data from gene expression profiling experiments and functional data of senescence/immortalization regulatory genes into a large database. An in-depth analysis of this database could potentially help define and identify novel senescence/immortalization genes.

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Supplementary Material

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Acknowledgments

This study was supported by the Barbara and Fred Erb Endowed Chair in Cancer Genetics (to MAT), and by funds from the Barbara Ann Karmanos Cancer Institute, the State of Michigan Life Sciences Corridor, Wayne State University and Michigan Center for Genomic Technologies Applied Genomics Technology Center (MEDC 085P10009816), and the Genomics and Biostatistics Cores of the Barbara Ann Karmanos Cancer Institute (P30CA022453). We thank the numerous members of the Tainsky lab who over the past 20 years have contributed to the studies of the immortalization of Li-Fraumeni cells, including Sun Yim, Farideh Bischoff, Tian-Ai Wu, Eliayhu Kraus, Sylvia Dryden, Olga Kulaeva, Lin Tang, Rita Rosati, Janice Kraniak and Qunfang Li. We acknowledge Louise C Strong whose insight into human cancer genetics led to the establishment of cell lines from Li-Fraumeni patients. We thank Dr Janice Kraniak for critical reading of the manuscript.

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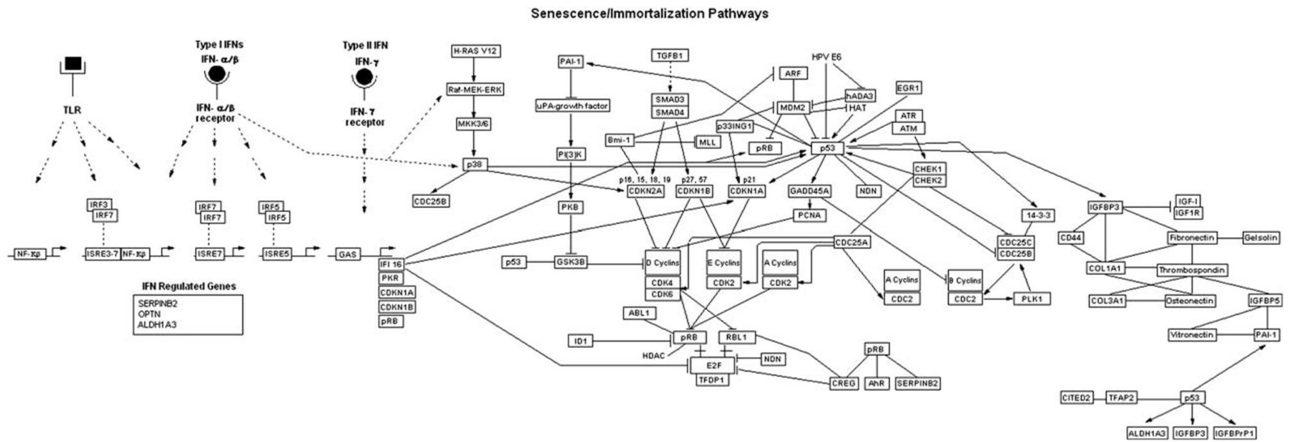

Figure 1.

Senescence/immortalization pathways. This figure was drawn using GenMAPP version 2 (http://www.genmapp.org/; Salomonis *et al.* (2007)). Pathway and gene interactions were derived from literature referenced in this paper and the supplemental section of this paper, GenMAPP, KEGG Pathway (Kanehisa and Goto, 2000), NCBI Entrez Gene (http://www.ncbi.nlm.nih.gov/sites/entrez), InvivoGen (http://www.invivogen.com/docs/Insight200509.pdf), Schroder *et al.* (2004), and Takaoka and Yanai (2006).

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