The Regulation and Function of CDK5 in Cellular Senescence and Tumorigenesis

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Abstract

The cellular senescence program evolved as a response to diverse forms of damage and stress. Importantly, although senescence was considered a tissue culture phenomenon for many years, recent *in vivo* studies demonstrated that cellular senescence represents a potent failsafe mechanism against tumorigenesis, by halting aberrant proliferation of cells harboring cancer-promoting mutations in benign lesions. Moreover, senescence can be restored in a number of cancer cells. It is clear now, as with apoptosis, the senescence response also contributes to the efficacy of certain anticancer agents.

The atypical cyclin-dependent kinase family member CDK5 is a postmitotic kinase whose activity is mainly associated with proper development of the central nervous system (CNS), but accumulating evidence suggests that CDK5 is also involved in other non-neuronal settings and functions in a diverse range of processes, such as wound healing, migration, apoptosis and senescence.

Recent studies in our lab have shown that CDK5 is activated during cellular senescence in both primary and tumor cells, and its activity is required for the senescent morphology change. In the present study, we show that p35, one of the activators of CDK5 in neurons, is required for CDK5 activation during senescence and the expression of p35 itself is upregulated in senescing cells. In addition to the shape change, CDK5/p35 is also required for the altered expression of senescent secretome. We further investigated whether such changes would confer any biological or physiological consequences, for example, on cell proliferation and tumorigenesis. Here we show that CDK5 is required for the expression of senescence markers in murine embryonic fibroblasts, as well as fibrosarcomas. Although acute loss of CDK5 does not affect the

proliferation of pre-senescent cells, CDK5 loss cooperates with Ras and dominant-negative p53 (DNp53) in the transformation of mouse embryonic fibroblasts (MEFs), as shown by an increased anchorage-independent growth of *Cdk5*-null cells in *in vitro* soft agar assays. *In vitro* migration/invasion assays performed with these transformed cells also show an increased motility and invasiveness in the *Cdk5*-null MEFs. However, CDK5 loss does not accelerate tumor formation in a xenograft model using transformed MEFs. Put together, these results suggest that although CDK5 is not directly involved in the regulation of cell proliferation and tumor growth once the senescence barrier is overcome, it may control cell motility and the metastatic potential of cancerous cells. In addition, CDK5 could have profound effects on the microenvironment and neighboring cells through its regulation of the secretome in senescent cells.

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Abbreviations

RB1, retinoblastoma gene 1

pRB, retinoblastoma protein

CDK5, cyclin-dependent kinase 5

SA-β-gal, senescence-associated β-galactosidase

HDF, human diploid fibroblast

MEF, murine embryonic fibroblast

OIS, oncogene-induced senescence

SAHFs, senescence-associated heterochromatin foci

SDFs, senescence-associated DNA-damage foci

DDR, DNA damage response

DSB, double-strand break

ROS, reactive oxygen species

HDAC, histone deacetylase

H3K9, histone H3 lysine 9

PAI1, plasminogen activator inhibitor 1

MMP3, matrix metalloproteinase 3

IL6, interleukin 6

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Dedication

I dedicate this work to my late grandfather Lin Wenhai, and my dearest parents.

Chapter I

Introduction

I. Cellular Senescence

1.1 Overview

The process of cellular senescence was described almost five decades ago when Hayflick and colleagues first observed that normal somatic cells had a limited ability to proliferate in culture (Hayflick, 1965). After a finite number of cell divisions, the human fibroblasts eventually entered a permanent cell cycle arrest. The non-dividing cells remained viable for many weeks, but failed to proliferate despite adequate growth conditions (i.e. the presence of ample space, nutrients and growth factors in the medium). This phenomenon is thus also called 'replicative senescence', which has been shown to be due to telomere attrition after repeated DNA replication in the absence of telomerase activity (Shay and Wright, 2000).

In parallel, other cellular stresses, such as non-telomeric DNA damage, oxidative stress and oncogene activation can acutely produce a phenotypically indistinguishable process, termed 'premature senescence', that seems to be independent of progressive telomere shortening (Campisi, 2005).

Thus, the senescence response is a fundamental cellular program that is activated in various situations of physiologic stress and ensures that damaged cells withdraw from the cell cycle without further proliferation. This function places senescence as a cell intrinsic mechanism that is analogous to the well-studied phenomenon of programmed cell death – apoptosis. In some instances, the same stimulus might induce either senescence or apoptosis, but the mechanisms that govern the decision to engage one or the other are not known.

Characteristics of senescent cells

When mitotic cells undergo senescence in response to various stresses, which in essence is irreversible, they become unresponsive to mitogenic stimuli and acquire widespread changes in gene expression. Senescent cells in culture often exhibit a unique enlarged and flattened morphology and accumulate a senescence-associated β-galactosidase (SA-β-gal) activity that distinguishes them from most quiescent cells (Fig.1.1).

The hallmark of cellular senescence is an inability to progress through the cell cycle. Senescent cells usually arrest at G1 phase, yet they remain viable and metabolically active for weeks. In contrast to quiescence, the senescence growth arrest cannot be stimulated to proliferate by known physiological stimuli. The features and stringency of the senescence growth arrest vary depending on the species and the genetic background of the cell. For example, although murine cells also have a finite proliferative capacity in culture, they frequently acquire spontaneous variants that can bypass senescence. Such variants are exceedingly rare in human cell cultures (Campisi and d'Adda di Fagagna, 2007).

Senescent cells show striking changes in gene expression, including changes in known cell-cycle inhibitors or activators. Two cell-cycle inhibitors that are often expressed by senescent cells are the cyclin-dependent kinase inhibitors (CDKIs) p21 (also termed CDKN1a, p21Cip1, Waf1 or SDI1) and p16 (also termed CDKN2a or p16INK4a), components of tumor-suppressor pathways that are governed by the p53 and retinoblastoma (pRB) proteins, respectively. Both pathways can establish and maintain the growth arrest that is typical of senescence. Senescent cells also repress genes that encode proteins that stimulate or facilitate cell-cycle progression (e.g.

replication-dependent histones, c-FOS, cyclin A, cyclin B and PCNA) (Campisi and d'Adda di Fagagna, 2007).

Remarkably, it has been known for some time that senescence is not a passive proliferation arrest that impacts only the senescent cell itself. Rather senescent cells influence their environment and neighboring cells through an active secretory program. Many senescent cells show an altered production of a mix of secreted proteins into their environment (Campisi and d'Adda di Fagagna, 2007). The senescence secretome (Adams, 2009) (also referred to as the senescence-associated secretory phenotype (SASP) (Coppe et al., 2008) and the senescence-messaging secretome (SMS) (Kuilman and Peeper, 2009)) is complex and includes a plethora of factors that reinforce senescence-associated proliferation arrest (e.g. reduced levels of WNT2, IGF1 and increased levels of PAI-1, IGFBP proteins, and TGFβ), factors that regulate immune response (e.g. IL6, IL8, CXCL1, CXCL5, CXCL7), and factors that remodel the extracellular matrix (e.g. MMP1, MMP3). This altered secretome is emerging as one of the most exciting aspects of the senescence program, because of its potential wide-ranging impact on a tissue's function, response to damage, and tissue degeneration.

Senescence markers

The study of senescence has yielded several markers that are useful for the detection of this response not only *in vitro* but also *in vivo* (summarized in Collado's review (2006), Table 1)(Collado and Serrano, 2006).

The first and most widely used marker for identifying senescent cells was the cytochemical detection of senescence-associated β-galactosidase (SA-β-gal) activity at a suboptimal pH 6.0 (Dimri et al., 1995). SA-β-gal is generally accepted as a marker of senescence both *in vitro* and *in vivo*. Lee *et al*. (2006) showed that SA-β-gal induction

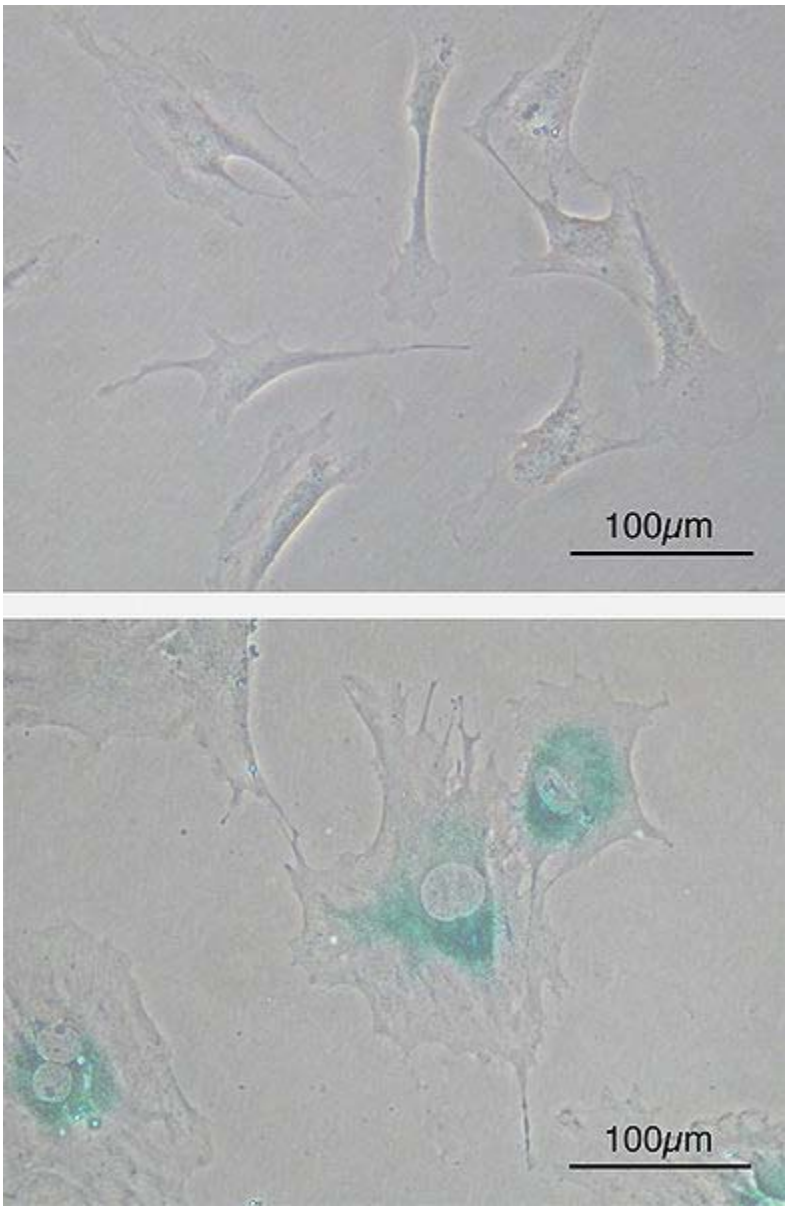
during senescence was due at least in part to increased expression of the lysosomal beta-galactosidase protein, encoded by GLB1, and the expression of SA-β-gal was not required for senescence (Lee et al., 2006). There is also evidence for a positive SA-β-gal reaction in settings of cellular stress that are unrelated to senescence, such as serum withdrawal or high confluency in cell culture (Severino et al., 2000), although this is not commonly observed.

Some secreted factors that are upregulated during senescence are also used as markers, such as PAI1 (plasminogen activator inhibitor 1) (Mu and Higgins, 1995) and MMP3 (matrix metalloproteinase 3) (Parrinello et al., 2005). Recently, several new markers were identified in a screen for genes that were expressed following oncogene-induced senescence, and could help to identify senescent cells *in vivo*: p15INK4b, DEC1 (differentiated embryo-chondrocyte expressed-1), and DCR2 (decoy death receptor-2) (Collado et al., 2005). The specificity and significance of these proteins for senescent cells are not yet clear, but they are promising additional markers.

Some senescent cells can also be identified by the cytological markers of senescence-associated heterochromatin foci (SAHFs) and senescence-associated DNA-damage foci (SDFs). SAHFs bear the hallmarks of heterochromatin, such as the trimethylation of lysine 9 in histone 3 (H3K9me3), the recruitment of heterochromatin protein 1-γ (HP1γ) (Narita et al., 2003) and macroH2A histone (Narita et al., 2006; Zhang et al., 2005). This genomic alteration can be microscopically visualized by the appearance of clusters of DAPI-stained heterochromatic regions. Senescence-associated DNA-damage foci (SDFs) are also often detected in senescent cells. Many proteins participate in the DNA-damage response (DDR), particularly those activated by double-strand breaks (DSBs) such as ataxia telangiectasia mutated (ATM), checkpoint-

2(CHK2), 53BP 1 and γ-H2AX, are found to localize to the DNA-damage foci in senescent cells (Bartkova et al., 2006; Di Micco et al., 2006; Mallette et al., 2007).

However, it is important to note that, no markers are exclusive to the senescent state. Aside from the decline in DNA replication, all of them require several days to develop.



(http://en.wikipedia.org/wiki/File:SABG_MEFs.jpg)

Fig.1.1. Proliferating cells *vs.* senescent cells. Primary mouse embryonic fibroblast cells (MEFs) before senescence (upper). Cells are typically spindle-shaped. MEFs became senescent after serial passages (lower). Cells become enlarged, flattened and express senescence-associated β-galactosidase (SA-β-gal, blue areas), a marker of cellular senescence.

1.2 Inducing pathways

Senescence is a cellular stress response

The concept of senescence was first applied to the permanent cell cycle exit of cells after prolonged proliferation under *in vitro*, non-physiological conditions. Now this concept is applied in general to the irreversible growth arrest of cells caused by various stresses, including telomere dysfunction, DNA damage, oxidative stress, and oncogene activation. Senescence thus functions as a self-defense mechanism to prevent the proliferation of potentially damaged cells.

A population of growing cells may suffer from a combination of different physiologic stresses acting simultaneously. Recent studies have provided important insights regarding the manner by which different stresses and stimuli activate the signaling pathways leading to senescence. The signaling pathways activated by these stresses are funneled to the p53 and pRb proteins, and the combined levels of stresses determine whether and how rapidly cells enter senescence.

Telomere dysfunction

Telomeres, the structures that cap the ends of linear chromosomes and protect them from degradation or fusion by DNA-repair processes, have received much attention as a trigger for replicative senescence.

A telomere consists of a stretch of repetitive double-strand DNA (5'-TTAGGG-3' in vertebrates), a 3'single-strand G-rich overhang and associated proteins. The precise telomeric structure is not known, but mammalian telomeres are thought to end in a large circular structure, termed a T-loop. During the proliferation of human cells, a gradual shortening of the average length of telomeres is observed and thought to function as a 'molecular clock' that limits replicative lifespan. The molecular cause of this shortening

stems from the “end-replication problem” -- the inability of standard DNA polymerases to complete DNA synthesis at the very end of the replicated lagging strand, therefore cells lose 50–200 base pairs of telomeric DNA during each S phase (Harley et al., 1990). Human telomeres range from a few kilobases to 10–15 kb in length, so many cell divisions are possible before the end-replication problem renders telomeres critically short and dysfunctional. The end-replication problem is a major (but not the sole) reason why normal cells do not proliferate indefinitely. Disruption of the proper structure of the protective cap at the end of the telomere, for example the loss of the telomeric overhang or inhibition of the TRF2 telomere binding protein, causes the collapse of T-loop and the exposure or ‘uncapping’ of the telomere end, and triggers senescence even when telomeres are still quite long (Stewart et al., 2003; van Steensel et al., 1998).

Dysfunctional telomeres resemble double-strand breaks (DSBs) and trigger a classical DNA damage response (DDR) -- most prominently through ATM and ATR and their targets Chk2 and Chk1 – to activate p53, which leads to the arrest of cell-cycle progression. Only one or a few such telomeres are sufficient to trigger senescence (Hemann et al., 2001; Martens et al., 2000). Ectopic expression of the catalytic subunit of telomerase (TERT), which is not expressed or is expressed at very low levels in most normal cells, prevents senescence in many cell types (Bodnar et al., 1998; Vaziri and Benchimol, 1998). By adding repeat sequences to the 3’ telomeric end, telomerase extends not only overall telomere length but also generates longer single-strand overhangs, and thus prevents entry to senescence by maintaining a proper telomere structure.

Non-telomeric DNA-damage

The direct damaging of DNA, especially DSBs, achieved either by irradiation of cells or by treatment with DNA damaging agents, can induce cells to undergo senescence (Wahl and Carr, 2001). The presence of the senescence-associated DNA-damage foci (SDFs) in senescent cells may provide constitutive signals to p53 to maintain the senescence growth arrest.

Genomic damage initiates a cascade of phosphorylation events in the nucleus, which in turn orchestrate DNA repair or – if the damage is severe or irreparable – cell death or senescence. The pivotal integrator and effector of these damage signals is p53. It is not clear how cells choose between senescence and apoptosis upon DNA damage-induced p53 activation, although it has been suggested that this is determined by differences in the post-translational modifications that p53 undergoes in response to different stimuli (Webley et al., 2000), by binding of different proteins to p53, or through the activation of different sets of transcriptional targets (Wahl and Carr, 2001). It is known that normal cells, in which all signaling pathways are intact, have a preference toward senescence when compared to transformed cells, and that senescence is usually induced by lower levels of damage than those leading to apoptosis (Ben-Porath and Weinberg, 2005).

Oxidative stress

Oxidative stress and the accumulation of intracellular reactive oxygen species (ROS) play an important role in the induction of senescence. In contrast to most human cells, cells from laboratory mice have long telomeres (>20 kb) and many express telomerase. Nonetheless, many mouse cells senesce after only a few doublings under standard culture conditions. This arrest is due to the supraphysiological oxygen level

(20%), to which mouse cells are much more sensitive than human cells. When these cells are grown at a lower oxygen level (3%), senescence is greatly delayed or avoided (Parrinello et al., 2003). Similarly, increase of intracellular ROS levels through hydrogen peroxide treatment or through the inhibition of ROS scavenging enzymes, such as superoxide dismutase Sod1, causes premature senescence (Blander et al., 2003; Chen et al., 1998; Frippiat et al., 2000).

Internal ROS can damage cellular components through the oxidation of DNA, proteins and lipids (Chen et al., 1998; Sitte et al., 2000), and can also act directly as second messengers to regulate specific signaling pathways (Saitoh et al., 1998).

Oncogene activation

Normal cells respond to many oncogenes by undergoing senescence. This phenomenon was first observed when an oncogenic form of H-Ras, a cytoplasmic transducer of mitogenic signals, was expressed in normal human and mouse fibroblasts (Serrano et al., 1997). Subsequently, other members of the Ras signaling pathway (for example, Raf, MEK, MOS and BRAF), as well as pro-proliferative nuclear proteins (for example, E2F1), were shown to cause senescence when overexpressed or expressed as oncogenic versions (Dimri et al., 2000; Lin et al., 1998; Michaloglou et al., 2005; Zhu et al., 1998). In addition, the loss of anti-mitogenic tumor-suppressor genes can also induce senescence in normal cells (for example, PTEN, NF1, pRB) (Chen et al., 2005; Courtois-Cox et al., 2006; Shamma et al., 2009). Oncogene-induced senescence (OIS) has recently been recognized as a tumor-suppressive mechanism *in vivo*, in human lesions and in several mouse tumor models. These findings show that oncogenes elicit a senescence response *in vivo* that curtails the development of cancer (Braig et al., 2005; Chen et al., 2005; Collado et al., 2005; Lazzerini Denchi et al., 2005; Michaloglou et al.,

2005). Genes that have been shown to trigger oncogene induced senescence, including a subset that has been confirmed to do so *in vivo*, are listed in Table 1 (adapted from (Courtois-Cox et al., 2008),Table 1).

Important advances have been made toward elucidating the mechanisms that regulate this response; however, there is presently no unified model that integrates all current findings. DNA damage, replicative stress, reactive oxygen species, heterochromatin formation and negative feedback signaling networks have all been proposed to play an integral role in promoting senescence in response to various oncogenic insults. In all cases, these signals have been shown to function through pRb and p53, but utilize different intermediaries (Courtois-Cox et al., 2008).

Cumulative effect of multiple stresses

It appears that senescence is not triggered by a single, linear series of events. Different triggers are not independent of one another or mutually exclusive, but instead are regulated by a complex signaling network. Accordingly, multiple mechanisms may cooperate to establish a senescence response.

Oxidative stress can cause DNA damage and also accelerate telomere shortening rates (Parrinello et al., 2003; von Zglinicki et al., 1995). While ROS can induce DNA damage, ROS may trigger senescence via a kinase cascade involving p38 MAPK and one of its effector kinases known as PRAK, which can phosphorylate and activate p53 (Sun et al., 2007).

The induction of senescence by the Ras oncogene has been suggested to occur through the induction of ROS (Lee et al., 1999). Consistent with this hypothesis, oncogene induced senescence can be bypassed in murine cells by growing cells in low oxygen (Lee et al., 1999; MacLaren et al., 2004; Parrinello et al., 2003). Similarly, ROS

scavengers can suppress senescence in some systems, and inactivation of the seladin-1 gene, which has been proposed to be a sensor of ROS, is also sufficient to bypass Ras-induced senescence in BJ fibroblasts (Lee et al., 1999; Wu et al., 2004). Despite these findings, not all oncogenes increase ROS (for example, Raf, Myc) (Dolado et al., 2007; Ray et al., 2006).

Many oncogenes induce a robust DDR owing to the DNA damage that is caused by aberrant DNA replication. These senescent cells stall in S phase, have an augmented number of active replicons and exhibit defects in DNA replication fork progression, resulting in an activation of ATR and ultimately ATM. This DDR has a causal role in both the initiation and maintenance of oncogene-induced senescence because its experimental down-regulation prevents senescence, allows cell proliferation and predisposes cells to oncogenic transformation (Bartkova et al., 2006; Di Micco et al., 2006). However, while DNA damage appears to be one signal that can cause senescence, not all oncogenes induce DNA damage (Hemann and Narita, 2007). Moreover, while inactivation of ATM is sufficient to bypass senescence triggered by some oncogenes in some cell types, other studies demonstrate that additional senescence regulators must also be inactivated, indicating that multiple pathways can be simultaneously engaged (Mallette et al., 2007).

These observations and others demonstrate the complexity of the cellular response to various stresses. It is of great interest to dissect the effects of these different stresses and to study the manner by which each activates the senescence program.

Gene	Cell type showing senescence response	Tumor/tissue showing senescense response	References
Oncogene activation			
Ras	HDF; MEF*	Lung adenomas;	Serrano et al., 1997;
		Atherosclerotic lesions;	Collado et al., 2005;
		Mammary epithelial hyperplasia	Sarkisian et al., 2007
Raf	HDF; MEF*; melanocytes	Lung adenomas	Dankort et al., 2007;
			Michaloglou et al., 2005
Akt	Endothelial cells; MEF	–	Chen et al., 2005;
E2F1/3	HDF	Pituitary gland hyperplasia	Dimiri et al., 2000; Lazzerini Denchi et al., 2005
Cyclin E	U2OS	–	Barkova et al., 2006
mos	HDF	–	Barkova et al., 2006
cdc6	HDF	–	Barkova et al., 2006
Tumor suppressor inactivation			
PTEN	MEF; IMR90	HG-PIN	Chen et al., 2005; Courtois-Cox et al., 2006
NF1	HDF	Dermal benign neurofibroma	Courtois-Cox et al., 2006

Table 1. Genes that trigger senescence *in vitro* and *in vivo*. Abbreviations: HDF, human diploid fibroblas; MEF, murine embryonic fibroblast; HG-PIN, high-grade prostatic intrapithelial neoplasia. MEF*: requires overexpression to induce senescence. (Adapted from (Courtois-Cox et al., 2008), Table 1).

1.3 p53 and pRB

Although diverse stimuli can induce a senescence response, they appear to converge on either or both of two pathways that are governed by the p53 and pRB tumor suppressor proteins (Bringold and Serrano, 2000; Campisi, 2001; Lundberg et al., 2000). Both p53 and pRB are activated upon the entry into senescence. The p53 protein is stabilized and proceeds to activate its transcriptional targets, such as p21CIP1, which leads to proliferation inhibition (Kulju and Lehman, 1995). pRB is found at senescence in its active, hypophosphorylated form, in which it binds to the E2F protein family members to repress their transcriptional targets that are required for G1 phase progression (Narita et al., 2003).

How do such diverse stimuli engage the p53 and pRB pathways? Are both pathways required to initiate the senescence response? And do both pathways maintain the senescent state? Answers to these questions are still fragmented and incomplete. Nonetheless, a consolidated, if not yet comprehensive, picture is emerging (Campisi, 2005; Campisi and d'Adda di Fagagna, 2007).

<u>The p53 pathway</u>

The p53 pathway is composed of a network of genes and their products that are targeted to respond to a variety of intrinsic and extrinsic stress signals that impact upon cellular homeostatic mechanisms that monitor DNA replication, chromosome segregation and cell division (Vogelstein et al., 2000). Different types of stress signals stabilize and activate the p53 protein in a specific manner by a code of post-translational modifications including phosphorylation, acetylation, methylation, ubiquitination or sumoylation of the p53 protein (Appella and Anderson, 2001). p53 is a crucial mediator of senescence responses to DNA damage (Wahl and Carr, 2001), and p21CIP1, a major

target for p53 transactivation and inhibitor of cell cycle progression, is often upregulated upon entry into senescence (Di Leonardo et al., 1994; Kulju and Lehman, 1995). Dysfunctional telomeres resemble DSBs and thus trigger a p53-dependent DNA damage response, via ATM kinase (d'Adda di Fagagna et al., 2003; Herbig et al., 2004). The p53 pathway is also important for the senescence response to overexpressed oncogenes such as activated Ras (Ferbeyre et al., 2000; Pearson et al., 2000; Serrano et al., 1997), which may also trigger a p53-dependent damage response, in this case by producing high levels of DNA-damaging ROS. The activation of p53 by a number of different oncogenes (E2F1, β-catenin, Myc, Ras and adenovirus E1A) is mediated in part by a positive feedback loop that results in the transcription of p19 or p14 ARF (Bates et al., 1998; Damalas et al., 1999; de Stanchina et al., 1998; Palmero et al., 1998; Zindy et al., 1998), which in turn inhibits the MDM-2 or HDM-2 ubiquitin ligase that is responsible for inactivating p53 (Honda and Yasuda, 1999), resulting in an increased level of p53 in the cell (Harris and Levine, 2005). Thus, at least in some cells, the induction of senescence by DNA damage, telomere dysfunction and possibly oncogene overexpression converge on the p53 pathway, which is both necessary and sufficient to establish and maintain the senescent growth arrest.

Consequently, although the senescence growth arrest cannot be reversed by physiological signals, in such cells it can be reversed by loss of p53 function (Campisi, 2005). It is well established that loss of p53 function delays or abrogates the senescence of human cells. Inactivation of p53 in at least some replicatively senescent human cells completely reverses the senescent growth arrest (Beausejour et al., 2003; Brown et al., 1997; Gire and Wynford-Thomas, 1998). In these cases, experimental manipulations that abolish p53 function or its target p21CIP1, cause postmitotic senescent cells to resume growth for many doublings, despite short telomeres, until

widespread severe telomere dysfunction drives them into crisis, a state of acute genomic instability.

The p16INK4a-pRB pathway

The retinoblastoma family proteins pRB, p107 and p130 physically interact with many proteins, but their binding to members of the E2F family of transcription factors appears to be central to their role in governing cell-cycle progression (Dyson, 1998; Nevins, 2001; Trimarchi and Lees, 2002). Complexes between the active hypophosphorylated forms of pRB and various E2Fs actively repress gene expression by recruiting histone deacetylases (HDACs) and other chromatin remodeling factors to E2F-responsive promoters, thus blocking S phase entry. Apart from E2Fs, pRB also interacts with other transcription factors that govern cell differentiation (Sherr and McCormick, 2002). Although the pRB protein family members have overlapping and compensatory functions in cell-cycle control, several lines of evidence have shown a nonredundant role for pRB in senescence. In mouse embryonic fibroblasts, acute loss of pRb in senescent cells leads to reversal of the cellular senescence program (Sage et al., 2003). Recent studies have shown that the pRB pathway is essential for the transcriptional repression of loci in senescent cells, termed senescence-associated heterochromatin foci (SAHF), through a reorganization of chromatin (Bandyopadhyay et al., 2007; Narita et al., 2006; Narita et al., 2003; Ye et al., 2007b), although its role in the induction of gene expression at senescence is largely unknown. Further, in a recent study, Chicas *et al.* (2010) demonstrate that pRB, but not p107 or p130, preferentially associates with E2F target genes involved in DNA replication and is uniquely required to repress these genes during senescence but not other growth states (Chicas et al., 2010). Notably, inactivation of the pRB tumor suppressor can also trigger senescence, and this depends on enhanced isoprenylation and activation of N-Ras (Shamma et al., 2009).

Four INK4 proteins (p16INK4a, p15INK4b, p18INK4c, and p19INK4d) specifically inhibit the activity of cyclin D-dependent kinases to prevent phosphorylation and therefore inactivation of pRB family proteins (Roussel, 1999; Ruas and Peters, 1998; Sherr and McCormick, 2002; Sherr and Roberts, 1999). A progressive increase in p16INK4a levels as cells are serially passaged correlates with their diminishing proliferative capacity, arguing for a role for p16INK4a in cellular senescence (Alcorta et al., 1996; Hara et al., 1996; Zindy et al., 1997). p16INK4a is induced by a variety of stressful stimuli, including overexpression of oncogenes such as Ras and suboptimal culture conditions loosely defined as 'culture shock' (Benanti and Galloway, 2004; Brookes et al., 2002; Ramirez et al., 2001). In some cells, oxidative stress induces p16INK4a (Forsyth et al., 2003), mostly through stress-activated p38 MAPK (Bulavin et al., 2004; Deng et al., 2004; Ito et al., 2006), but this is not always the case (Beausejour et al., 2003). In many cells, DNA damage and dysfunctional telomeres also induce p16INK4a, albeit with delayed kinetics. In addition, forced telomere 'uncapping' by TRF2 inhibition causes an induction of p16INK4a and senescence, even in the absence of p53 (Jacobs and de Lange, 2004). p16INK4a, then, plays a specialized role within the INK4 family in countering certain signals that abnormally drive cell proliferation, a checkpoint function that leads to senescence and is eroded in tumor cells.

Cell culture studies of human cells indicate that p16INK4a prevents the reversal of senescence by p53 inactivation. Although p53 inactivation reverses the senescence arrest in some cells, it fails to do so in others (Beausejour et al., 2003; Herbig et al., 2004; Sakamoto et al., 1993). The difference lies in the ability of cells to express p16INK4a. The reversal of senescence by p53 inhibition is achievable only in cultures with low levels of p16INK4a and is blocked at high p16INK4a levels. Of special interest, once the pRB pathway is engaged, the senescence growth arrest cannot be reversed by

subsequent inactivation of p53, silencing of p16INK4a, or inactivation of pRB (Beausejour et al., 2003).

Interconnections between senescence pathways

Molecular interactions link the p53 and pRB pathways in regulating cellular senescence (Sherr and McCormick, 2002). An important example is the induction of p21CIP1 expression by p53. p21CIP1 is a more global inhibitor of cyclin-dependent kinases than p16INK4a and thus also causes hypophosphorylation and activation of pRB (Fig.1.2) (adapted from (Ben-Porath and Weinberg, 2005), Figure 2). Engagement of the p53 pathway should, therefore, engage the pRB pathway in a linear fashion. Nonetheless, the consequences of pRB activation by p21CIP1 differ from that of activation by p16INK4a, at least in some respects. When ectopically overexpressed, p16INK4a is more effective than p21CIP1 at inducing human fibroblasts to arrest with features of senescence (McConnell et al., 1998). In some human fibroblasts, replicative senescence is marked by the sequential rise in p21CIP1 expression, followed by a decline in p21CIP1 and concomitant rise in p16INK4a (Stein et al., 1999). Thus, signaling from the p53 to p16INK4a-pRB pathway might occur in some cells, with the p53-generated signal being transient.

p53 and p16INK4a–pRB pathways interact but can independently halt cell-cycle progression. To some extent, they also respond to different stimuli. In addition, there are both cell-type-specific and species-specific differences in the propensity with which cells engage one or the other pathway, and in the ability of each pathway to induce senescence.

Cellular senescence has been best characterized in cultures of human fibroblasts and mouse embryo fibroblasts (MEFs), and a complex picture emerges from these

studies. In MEFs, inactivation of p53 is sufficient to prevent senescence, allowing these cells to divide indefinitely (Dirac and Bernards, 2003). Cells carrying a null mutation in the *RB1* gene senesce normally, yet inactivation of additional pRB family members – p107 and p130 – is sufficient to prevent senescence (Dannenberg et al., 2000; Sage et al., 2000). This indicates that both p53 and pRB are necessary for the initiation of senescence, but also that the other pRB family members are capable of providing redundancy for this function of pRB. Once MEFs have undergone senescence, the continued activity of both p53 and pRB is required to maintain this state—inactivation of either of these genes in senescent MEFs allows these cell populations to resume a proliferating state (Dirac and Bernards, 2003; Sage et al., 2003).

Various experiments with human fibroblasts indicate that, unlike the behavior of mouse cells, the inactivation of both p53 and pRB is required to prevent the onset of senescence (Smogorzewska and de Lange, 2002). Accordingly, the inactivation of either protein alone generally only delays the onset of senescence. This suggests that in human cells, p53 and pRB are activated in parallel and perform partially redundant roles. In some human fibroblast cell strains, pRB growth-suppressive activity is apparently maintained independently of p53 (Beausejour et al., 2003). Moreover, as a population of cells is propagated in culture, cells are exposed to various extrinsic and intrinsic stresses, which lead to the activation of the p53 pathway in some cells and of the p16INK4a pathway in others. It has been shown that upon replicative senescence, some human fibroblasts express either p21CIP1 or p16INK4a, but rarely both (Herbig et al., 2004). As the population continues to be propagated in culture, more and more cells activate one or both of these pathways, and the population as a whole eventually undergoes senescence. A population of human cells in culture is thus a mosaic of distinct subpopulations responding to different stresses. Such a mechanism could provide an

extra layer of protection against the bypass of senescence and thus against tumor development.

Activation of the p53 and pRB pathways by different stimuli is summarized in Figure 1.3 (adapted from (Ben-Porath and Weinberg, 2005), Figure 3).

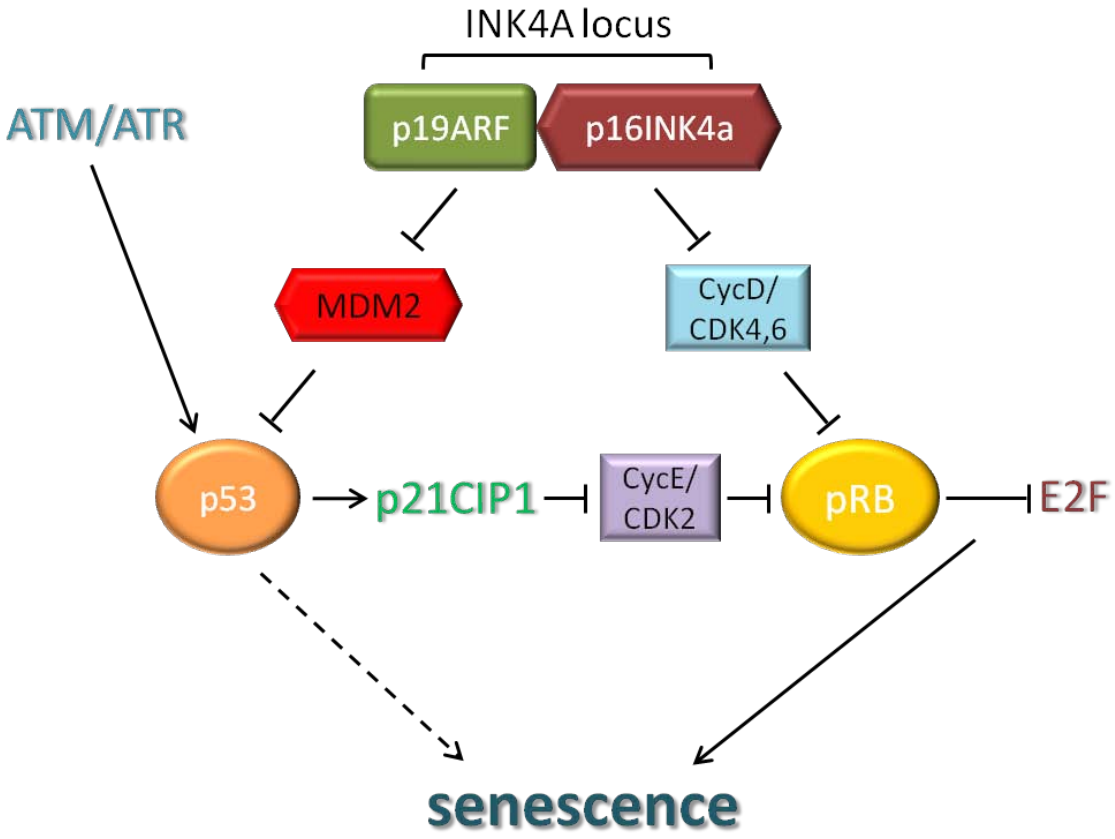
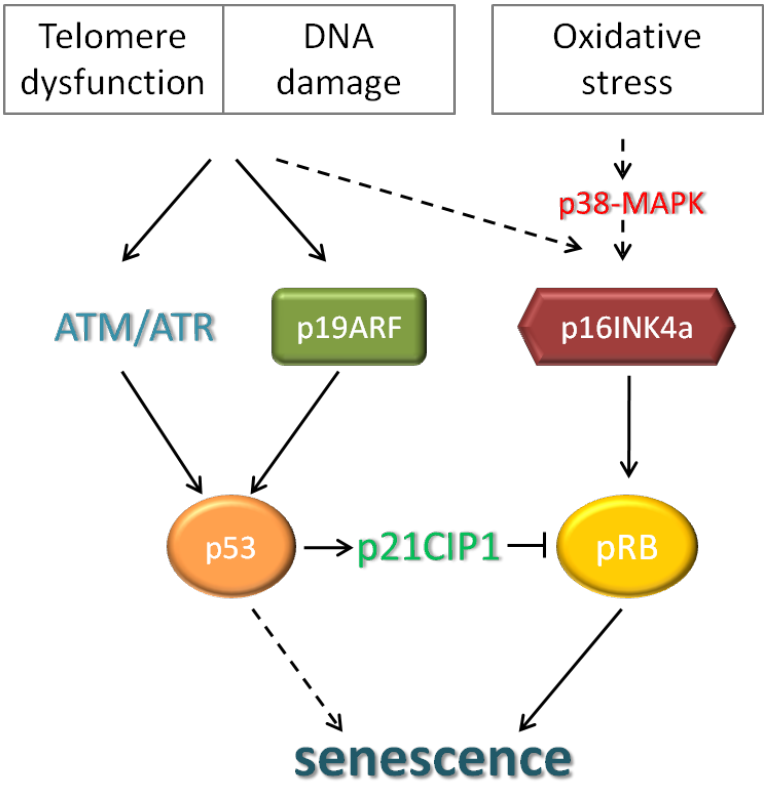


Fig.1.2. A model of a linear activation of pRb by p53. p53 and pRB are the main activators of senescence. p53 can activate senescence by activating pRb through p21CIP1, an inhibitor of cyclin E/Cdk2 complexes, and also, in human cells, can activate senescence independently of pRB. pRB is activated either by p21CIP1, or by the *p16INK4a* product. p53 is activated either by the checkpoint proteins ATM/ATR and Chk1/Chk2, or by the *p19ARF* product of the *INK4a* locus, which sequesters Mdm2 in the nucleolus. (Adapted from (Ben-Porath and Weinberg, 2005), Figure 2).

(A)


Telomere dysfunction
DNA damage
Oxidative stress
p38-MAPK
ATM/ATR
p19ARF
p16INK4a
p53
p21CIP1
pRB
senescence

(B)

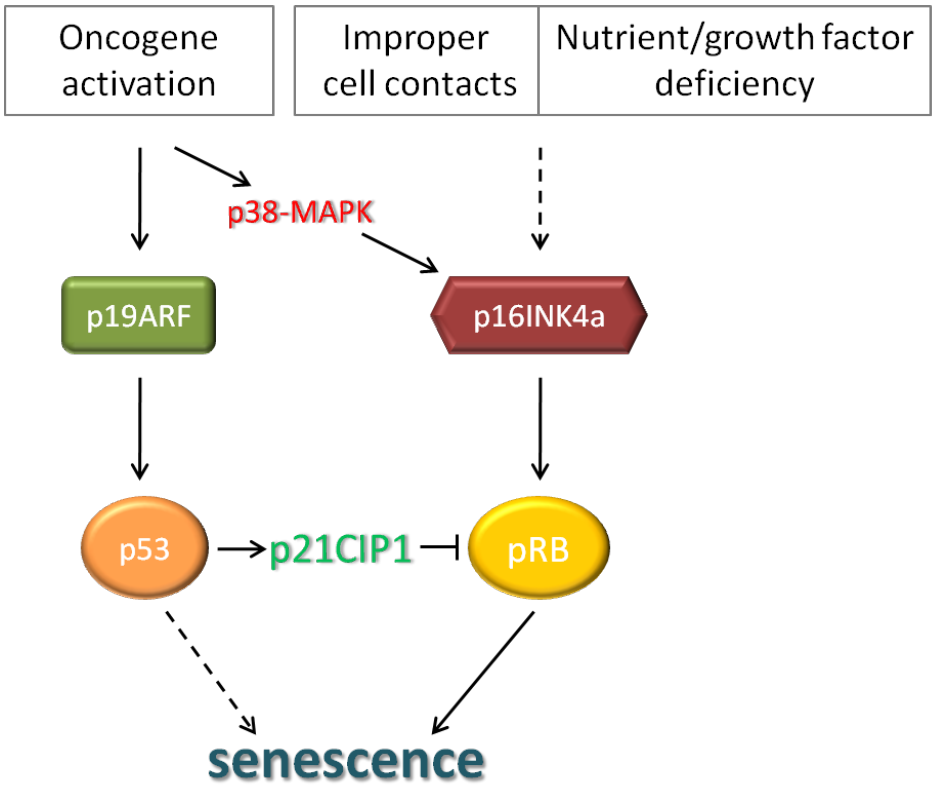

Oncogene activation
Improper cell contacts
Nutrient/growth factor deficiency
p38-MAPK
p19ARF
p16INK4a
p53
p21CIP1
pRB
senescence

Fig.1. 3. Activation of the p53 and p16INK4a-pRb pathways by different stimuli. (A) Telomere dysfunction induces senescence mainly through the DNA damage response, activating ATM/ATR and Chk1/Chk2 proteins to stabilize p53. In mouse this response is dependent on p19ARF, while in humans the role of ARF in this response is not known. In human cells p16INK4a is activated in certain settings in response to telomere dysfunction, through unknown pathways. Direct DNA damage activates the senescence program mainly through p53, in essentially the same manner as telomere dysfunction. Oxidative stress induces DNA damage, and also accelerates telomere shortening. The downstream response is mediated through the DNA damage pathway and through p19ARF. Activation of p16INK4a by oxidative stress is seen in certain conditions mainly in human cells, and may be mediated through p38-MAPK. (B) Ras oncogene activation induces senescence through p19ARF in the mouse, and mainly through p16INK4a in human cells. p38-MAPK plays a role in mediating Ras-induced senescence. p16INK4a is activated by additional physiologic stresses to induce senescence, such as nutrient and growth factor deficiency, and improper cell–cell and cell–matrix contacts. The exact stimuli for this activation and the pathways mediating it are unknown. (Adapted from (Ben-Porath and Weinberg, 2005), Figure 3).

1.4 Senescence in tumorigenesis and cancer therapy

Senescence as anti-cancer mechanism

In complex animals such as mammals, many somatic tissues contain mitotic cells and thus are capable of renewal, repair, and, in some cases, regeneration. Unfortunately, renewable tissues are also susceptible to hyperproliferative disease, the most deadly of which is cancer.

Soon after its discovery, cellular senescence was proposed to be an anti-cancer mechanism. In oncogene-induced senescence (OIS), cells expressing activated oncogenes typically undergo a brief burst of proliferation ("the mitotic phase"), and then senescence. Serrano *et al.* (1997) first observed that expression of oncogenic Ras in primary human or rodent cells resulted in a permanent G1 arrest, accompanied by accumulation of p53 and p16INK4a. This arrest was phenotypically indistinguishable from cellular senescence. Inactivation of either p53 or p16INK4a prevented Ras-induced arrest in rodent cells, and E1A achieved a similar effect in human cells. Thus, this study suggested that the onset of cellular senescence does not simply reflect the accumulation of cell divisions, but can be prematurely activated in response to an oncogenic stimulus. Negation of Ras-induced senescence may be relevant during multistep tumorigenesis. (Serrano et al., 1997).

Ever since its original description, the possibility that oncogene-induced senescence functioned as a potential fail-safe mechanism seemed logical, but the physiological relevance of oncogene-induced senescence remained an issue of debate for years. Opponents of the concept speculated that it might be an *in vitro* phenomenon caused by supraphysiological levels of activated Ras. This hypothesis was fueled by the development and characterization of mouse models that were designed to express a

single activated *K-ras* allele driven by its endogenous promoter (Guerra et al., 2003; Tuveson et al., 2004). Initial reports demonstrated that this allele promoted hyperplasia and tumor development in a subset of tissues. Moreover, this allele did not induce the cellular senescence of mouse embryonic fibroblasts (MEFs), but rather resulted in their immortalization, supporting the possibility that at least in this cell type, senescence might be an artifact of Ras overexpression (Courtois-Cox et al., 2008).

However, there is now substantial evidence that cellular senescence does indeed occur *in vivo* and function as a potent barrier to tumorigenesis, thus this phenomenon is no longer interpreted as a mere culture artifact. A series of studies recently published establish that OIS occurs during the early stages of tumor development both in mouse models and in humans (Braig et al., 2005; Chen et al., 2005; Collado et al., 2005; Courtois-Cox et al., 2006; Dankort et al., 2007; Lazzerini Denchi et al., 2005; Michaloglou et al., 2005; Sarkisian et al., 2007). These observations strongly indicate that OIS restricts the growth of oncogenically stressed cells, therefore maintaining the tumor in a non-aggressive, premalignant state; by contrast, the absence of OIS, which is caused by the defects in the senescence response, leaves the road to oncogene-driven malignant progression unimpeded. These articles underscore the significant role of oncogene-induced senescence in preventing or interrupting the progression to full-blown cancer formation, in a wide spectrum of premalignant scenarios of both solid tumors and hematologic malignancies, mesenchymal and epithelial cancer types. Moreover, the data clearly rebut the view that oncogene-induced senescence may occur only when the driving mitogenic oncogene is expressed at supraphysiologic levels. In fact, mutant BRAF activities in patient-derived samples, titrated expression levels of exogenous $BRAF^{V600E}$, transgenic $N\text{-}Ras^{G12D}$ expression, and activation of endogenous $K\text{-}ras^{G12V}$ alleles equally produced cellular senescence in their respective contexts *in vitro* and in

vivo, and cellular senescence was also observed in PTEN$^{-/-}$ prostate glands with intraepithelial neoplastic lesions without any explicit manipulation of the Ras/Raf oncogene family (Braig and Schmitt, 2006). Recent work in mouse models also implicated oncogene-induced senescence as part of the barrier imposed by DNA damage checkpoints to progression of preneoplastic lesions to neoplasia (Bartek et al., 2007; Bartkova et al., 2006), hereby highlighting the universal nature of this program as an antioncogenic safeguard mechanism that may prevent the proliferation of damaged or stressed cells that are at risk for malignant transformation.

The observation that senescent cells accumulate in benign human tumors strongly supports the hypothesis that senescence restricts tumor progression. However, there are still questions about how senescence suppresses tumor development. Several studies have now established a more causal role for senescence in this process (Sarkisian et al., 2007; Ventura et al., 2007; Xue et al., 2007). First, while senescence has been historically defined as 'irreversible', until recently, evidence of its irreversibility *in vivo* has been lacking. By using a transgenic mouse model designed to express a doxycycline regulated mutant *H-ras* allele, Sarkisian *et al*. (2007) have shown that once senescence is triggered, cells are not capable of re-entering the cell cycle or developing into tumors (Sarkisian et al., 2007). Using a different genetic approach, two other studies have demonstrated that the acute re-expression of endogenous p53 rapidly induces senescence and halts tumorigenesis in genetically engineered mouse models of hepatocellular carcinoma and sarcoma (Ventura et al., 2007; Xue et al., 2007). Xue *et al*. (2007) further showed that even a brief reactivation of p53 is sufficient to trigger this irreversible response. Moreover, these lesions ultimately regressed, at least in part due to clearance mediated by an innate immune response (Xue et al., 2007). However, it remains to be determined whether other mechanisms also participate in tumor

regression. In addition, it will be important to establish how some senescent cells (for example, cells within benign lesions) may evade mechanisms leading to their elimination. Nevertheless, all three reports have provided important evidence that senescence does suppress tumorigenesis *in vivo*.

Do senescent fibroblasts prevent or promote cancer?

While there have been many theoretical and some experimentally derived lines of evidence to support the concept that senescent cells may represent a substantial barrier to cancer progression, recent results have also suggested that senescent stromal cells may actually promote the proliferation and tumorigenesis of mutant epithelial cells (Campisi, 2001; Krtolica et al., 2001). Malignant tumors require a permissive tissue environment in order to survive, proliferate, and migrate. Most age-related cancers arise from the epithelial cells of renewable tissues. An often overlooked but essential component of epithelial tissue is the stroma, the subepithelial layer composed of extracellular matrix and several cell types (but mostly fibroblasts). While it is clear that cell autonomous changes are crucial to the tumorigenic process, concomitant changes of in the surrounding stromal tissue, also play an instrumental role in the transformation process (Campisi, 2005).

There is increasing evidence that one change that occurs in tissues during aging is the accumulation of epithelial cells and fibroblasts that have undergone cellular senescence. Cellular senescence arrests proliferation in response to damage or stimuli that put cells at risk for neoplastic transformation. However, senescent cells also secrete growth factors as well as extracellular matrix components, matrix-degrading enzymes, and inflammatory cytokines that can disrupt tissue integrity and/or stimulate nearby cells to proliferate (Campisi, 2001). As a result, senescent fibroblasts admixed with

preneoplastic epithelial cells stimulate the growth of the latter in culture as well as in tumor models (Krtolica et al., 2001). Campisi's original report led several groups to examine whether senescent stromal cells might be responsible for altering tissue architecture, leading to growth of preneoplastic cells. Senescent cells often secrete large amounts of MMP3, a metalloprotease that acts on the extracellular matrix. Parrinello *et al*. (2005), using a three dimensional (3D) breast model, showed that stromal-derived MMP3 altered branching morphology, suggesting that senescent stromal cells could alter breast architecture, which in turn might contribute to preneoplastic cell growth (Parrinello et al., 2005). In another study, Coppe *et al*. (2006) demonstrated that senescent cells express VEGF and stimulate angiogenesis in mice (Coppe et al., 2006), suggesting senescent cells stimulate tumor growth through enhanced angiogenesis. Finally, analysis of senescent prostate fibroblasts indicated numerous genes were upregulated, including fibroblast growth factor 7 (FGF7), amphiregulin (AREG), and hepatocyte growth factor (HGF), all previously linked to the growth of neoplastic cells *in vivo* (Bavik et al., 2006). RNAi-directed loss of AREG resulted in reduced preneoplastic prostate cell growth, highlighting the importance of AREG in this model. Together, these studies reveal the complex nature of the senescent secretory profile and indicate that senescent cells stimulate preneoplastic cell growth *in vitro* and *in vivo* (Pazolli and Stewart, 2008). In addition, another report has suggested that initiated epithelial cells may be capable of inducing senescence in the surrounding stromal cells (Yang et al., 2006). Yang *et al*. (2006) showed that Ras expression leads to growth-related oncogene (GRO1) expression in ovarian epithelial cells. Epithelial-derived GRO1 is then able to drive fibroblasts into senescence, which could further support the growth of the initiated epithelium. This study therefore leads to the tempting possibility that the initiated (i.e. preneoplastic) epithelium actively recruits a supportive stromal environment by activating the senescent phenotype.

These observations allow us to envision a model in which both senescent stromal cells and preneoplastic epithelial cells arise within the vicinity of one another (Fig.1.4) (adapted from (Pazolli and Stewart, 2008), Figure 1). While senescence may have evolved to initially protect humans against the formation of early onset cancers, senescent stromal cells may in certain contexts be the culprit by creating a pro-oncogenic tissue environment, which leads to decline in tissue function that may in some cases synergize with oncogenic mutations to drive the rise in cancer incidence with age.

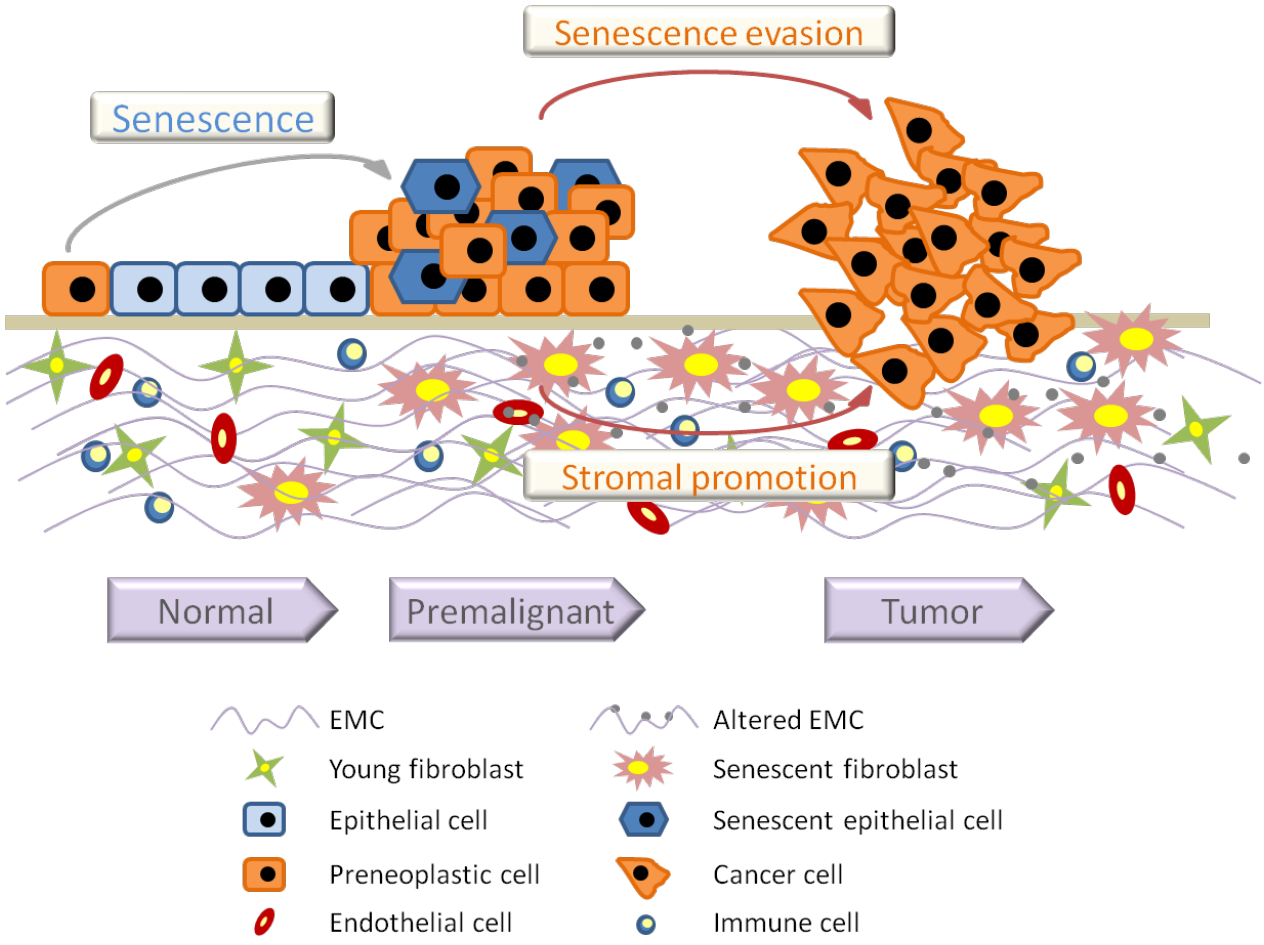


Fig.1.4. Senescent stromal cells might contribute to tumorigenesis. In a tissue setting, an oncogenic assault triggers senescence in the epithelial cells. Such genetic assaults accumulate over time, leading to an increase in preneoplastic cells. Meanwhile, senescence triggered in the stroma results in alterations in the surrounding microenvironment. These alterations occur through the secretion of matrix remodeling proteins and growth factors and possess the capacity to contribute to the transformation process. Evasion of senescence by the preneoplastic epithelial cell in combination with a promoting stroma leads to tumorigenesis. (Adapted from (Pazolli and Stewart, 2008), Figure 1).

Senescence and cancer therapy

Analyses using genetically tractable mouse models and human tumor biopsies have shown that cellular senescence is a physiological mechanism for thwarting the proliferation of tumor cells. Encouraging cancer-prone cells to senesce might therefore be recruited as an ultimate fail-safe mechanism to blunt oncogenic activity *in vivo* and constitute an important block to tumor progression at a very early stage. So, from a therapeutic point of view, restoration of senescence can be an option for cancer treatments.

The process of tumorigenesis involves a series of changes that allow tumor cells to bypass the senescence program. Nevertheless, tumor cells retain the ability to undergo a senescence-like arrest, especially in response to certain anti-cancer therapies (Shay and Roninson, 2004). Many recent studies have demonstrated that a variety of stimuli, as described below, can shift this equilibrium in favor of senescence, thereby stopping the growth of tumor cells.

(1) Although telomerase induction is the best-known senescence-restraining mechanism, only a few studies have shown that telomerase inhibitors induce senescence in tumor cells (Kim et al., 2003). Instead, most observations suggest that telomerase inhibition by small molecules (Herbert et al., 1999) or by dominant-negative mutants (Hahn et al., 1999) produces cell death rather than senescence in tumor cell lines.

(2) Most conventional chemotherapeutic drugs and ionizing radiation cause severe DNA damage and induce apoptosis in cells, but in many instances cells do not die but rather undergo a senescence-like terminal growth arrest, which has been observed in both mouse and human *in vitro* and *in vivo* (Chang et al., 1999a; Christov et

al., 2003; Elmore et al., 2002; Han et al., 2002; Haq et al., 2002; Michishita et al., 1999; Park et al., 2000; Roninson, 2002; Suzuki et al., 2001; te Poele et al., 2002; Wang et al., 1998). Of practical importance, the genetic makeup of the tumor cells determines the outcome of chemotherapy. In this regard, p53, p21CIP1, and p16INK4a, which play a central role in normal cell senescence, are also implicated in the senescence of tumor cells. Drug-induced *in vitro* senescence in HCT116 colon carcinoma cell line is decreased several-fold upon the knockout of either p53 or p21CIP1, and the same effect is observed in HT1080 fibrosarcoma cells upon the inhibition of p53 expression (Chang et al., 1999b). In murine Eμ-*myc* lymphoma, treatment-induced *in vivo* senescence becomes undetectable upon the knockout of either p53 or p16INK4a, and these mice died earlier than those in which senescence response could be triggered (Schmitt et al., 2002). In the case of human tumors, senescence induced by chemotherapy has been shown to be a relevant factor in determining treatment outcome. A role for p53 and p16INK4a is also suggested by the analysis of chemotherapy-induced senescence in clinical breast cancer, where SA-β-gal staining was significantly associated with low p53 staining (indicative of the lack of p53 mutations), and with strong staining for p16INK4a (te Poele et al., 2002). Similarly, correlation between p21CIP1 and chemotherapy-induced senescence was shown in lung carcinoma (Roninson, 2003). Therefore, DNA-damaging therapies are more likely to be efficacious in tumors that senesce, compared with those that do not (Roberson et al., 2005; Roninson, 2003; Schmitt et al., 2002; te Poele et al., 2002).

Among different classes of anticancer agents, the senescent phenotype is induced most strongly by agents that affect DNA structure, such as doxorubicin, aphidicolin, cisplatin, ionizing radiation, cytarabine, and etoposide (Chang et al., 1999a). Interestingly, chemical histone deacetylase inhibition (HDACi), which promotes

heterochromatin disruption, also induces senescence (Munro et al., 2004; Ogryzko et al., 1996), probably by initiating a p53-dependent DDR.

Therefore, senescence inducing drugs could represent an attractive alternative approach to treat tumors that are resistant to apoptosis-based therapies.

(3) However, to avoid the genotoxicity unselectively delivered by conventional chemotherapeutics, it is appealing to identify drug targets that act as signaling mediators in the senescence program. These targets could include pRB, p53, and two p53-related proteins (p63 and p73), and several CDK inhibitors (p21CIP1, p16INK4a, p57Kip2, and p15INK4b) (Roninson, 2003). The most striking example is that the inhibition of papillomavirus oncoproteins E6 and E7, inhibitors of p53 and pRb tumor suppressors, respectively, rapidly induces stable growth arrest and the senescent phenotype in HeLa cells and human cervical carcinoma cell lines (DeFilippis et al., 2003; Goodwin et al., 2000; Hall and Alexander, 2003; Wells et al., 2000).

Recent novel findings have revealed the involvement of additional signaling players in senescence, including PML, CK2, Bcl-2, PI3K effectors such as Rheb, Rho small GTPases, and cytokines (reviewed in (Caino et al., 2009)), all of which could be potential drug targets. Moreover, inducible antagonism of the senescence-suppressing protein Tbx2, a T-box gene family member that represses the expression of ARF and p21CIP1 in melanoma cells, was recently shown to directly restore cellular senescence in these fully malignant cells (Jacobs et al., 2000; Vance et al., 2005). Pharmacologic stabilization of the p53 tumor suppressor in PTEN-deficient prostate cancer has also been proposed as a novel therapeutic approach to induce senescence (Chen et al., 2005). Recent studies have implicated protein kinase C (PKC) isozymes as modulators of senescence phenotypes and showed that phorbol esters, widely used PKC activators,

can induce senescence in a number of cancer cells (Caino et al., 2009). Tumor cell senescence is also induced by TGF-β (Katakura et al., 1999), antiestrogens (Christov et al., 2003), and by so-called 'differentiating agents', including sodium butyrate (Terao et al., 2001) and retinoids (Chang et al., 1999a; Christov et al., 2003; Dokmanovic et al., 2002).

Thus, the emerging knowledge about the pathways that lead to senescence and determine the pattern of gene expression in senescent cells may therefore lead to more effective treatments for cancer.

(4) In addition to the fundamental area of investigating the diverse signaling pathways that cause cells to undergo senescence, another novel area is to analyze the roles of senescent cells and their secreted factors in tumor growth and treatment response.

The clearance of oncogenically primed cells by induced senescence is gaining increasing attention as accumulating evidence points to its role in tumor suppression (Collado and Serrano, 2010). One possibility is that senescent cells are removed by the immune system. Senescent cells secrete cytokines to attract immune cells to their location (for their removal), and secrete matrix degrading proteins to allow the easy access by these immune cells. Indeed, in experimental mouse models, senescent cells can be engulfed by phagocytic cells in a process that is essentially similar to the removal of apoptotic cells (Krizhanovsky et al., 2008; Xue et al., 2007). These results indicate that enhancement of the program of senescence in tumor cells provides a biologically justified approach to cancer therapy.

However, as discussed above, an important consideration is that senescent cells may also favor the growth of adjacent or neighboring tumor cells. If this is proved to be

the case then the induction of senescence in tumors could be a double-edged sword, both limiting the proliferation of senescent-competent tumor cells, but at the same time promoting the proliferation of a subset of tumor cells that are no longer able to execute the senescence program.

1.5 Senescence and aging

Senescence appears to have specific physiological roles and pathological consequences. At least two of these, tumor suppression and aging, were predicted by Hayflick (Hayflick, 1965). It is debated if the so-called ‘‘Hayflick limit’’ reflects the aging process of the whole organism *in vivo*. Although a causal relationship has yet to be proved, growing evidence indicates that, at least in complex animals such as mammals, there is correlation between cellular senescence and organismal aging. Senescent cells accumulate *in vivo* in mammals with increasing age and at sites of pathology, and many mouse and human models of premature aging (i.e. mutations in DNA repair genes) are accompanied by premature cellular senescence *in vitro* (Itahana et al., 2004).

Senescent stromal cells have been observed in human tissue. The number of senescent cells present in the skin increases in older individuals (Dimri et al., 1995), as well as in other primates (Herbig et al., 2006; Jeyapalan et al., 2007), demonstrating an increase in senescent cell numbers within the same individual as a function of age. Senescent cells have also been detected in the vasculature and various epithelial tissues as well as in atherosclerotic lesions (Vasile et al., 2001), suggesting that the presence of senescent cells correlates with age-related pathologies. Together, these studies suggest that cellular senescence is a hallmark of organismal aging.

If senescence truly reflects the aging process at a cellular level, it would represent an ideal model system to study the molecular basis of aging. Two general

models have been proposed to explain how cellular senescence may contribute to organismal aging and age related pathology. First, senescence could lead to the reduction of the regenerative potential of the stem or progenitor cell pool, thus impairing tissue renewal. In this regard, the Polycomb group repressor Bmi appears to control levels of stem cells via negatively regulating the induction of senescence in these cells. Correlatively, expression of p16INK4a increases with age in many murine tissues (Krishnamurthy et al., 2004; Zindy et al., 1997) and contributes to the decline in the function of haematopoietic, neuronal and pancreatic stem cells or progenitors *in vivo* (Janzen et al., 2006; Krishnamurthy et al., 2006; Molofsky et al., 2006). So far, a few studies have also analyzed global gene expression patterns in somatic stem cells upon aging and these were performed in murine hematopoietic stem and progenitor cells (Chambers et al., 2007; Prall et al., 2007; Rossi et al., 2005). Second, aging may be due in part to cellular responses to damage, not damage per se, and may be a side effect of the natural safeguards that protect us from cancer. As mentioned in the previous section, senescent cells are dysfunctional and secrete proteases and other factors that may actively disrupt normal tissues function as they accumulate and may contribute to age-related diseases, even, paradoxically, late-life cancer. In this regard, although the senescence response constitutes a potent anticancer mechanism, it has a complex relationship with neoplasia (Campisi, 2005; Krtolica and Campisi, 2002; Pelicci, 2004).

Therefore, treatments that inhibit senescence in healthy individuals might slow aspects of aging. A better understanding of the age-specific changes in signal transduction networks is critical to understanding aging in molecular terms and to development of novel therapies for age-specific disorders. However, given the fact that cancer and aging are nonexclusive and possibly concomitant, future research will also need to clarify whether therapies that induce senescence are useful for cancer treatment,

and determine their effect on aging. Conversely, the risk of cancer should also be evaluated when considering regenerative medicine-based approaches for aging therapy.

1.6 New effectors

Understanding how senescence is established and maintained is an important area of study both for normal cell physiology and in tumorigenesis. An emerging field of investigation is the search for new senescence effectors.

Epigenetic regulation

Emerging evidence has pointed to the modulation of epigenetic modifications as a potential driving force of cellular senescence in mammals. Epigenetic mechanisms such as DNA methylation of specific gene promoters and modification of histone proteins, which leads to chromatin remodelling, have been shown to be sufficient to alter gene expression profile in senescent cells. For example, Wilson *et al*. (1987) showed the gradual loss of DNA methylation with age in various mouse tissues and in human bronchial epithelial cells (Wilson et al., 1987). Similarly, Fuke *et al*. (2004) recently found an age-dependent decrease in global methylation levels in human leukocytes (Fuke et al., 2004). A direct and well-established function for a histone modifier in senescence is that of the PcG complexes, whose key subunits include EZH2 and BMI1, a histone H3 lysine 27 (H3K27) methyltransferase and a RING finger protein, respectively. Early studies showed that, in both mouse and human cells, BMI1 antagonizes cell senescence through repression of the *p16INK4a/CDKN2a* gene (Jacobs et al., 1999). Recent studies have also implicated the EZH2-containing complex (known as PRC2) in the transcriptional repression of the INK4A locus in proliferating cells. Upon signals triggering senescence, EZH2 levels decrease, concomitant with the loss of the H3K27me3 mark at the INK4A locus (Bracken et al., 2007). Similarly, the histone

demethylases KDM2a and KDM2b, which target methylated H3K36, regulate the p15INK4B locus (He et al., 2008; Pfau et al., 2008), and therefore prevent senescence by modulating the p53 and pRb pathways. Together, these data demonstrate that histone modifiers are able to contribute in a gene-specific manner to the induction or prevention of cellular senescence. By contrast, the contribution of chromatin modifiers to cellular senescence and aging as global regulators of chromatin structure remains elusive and awaits genome-wide approaches to definitively ascertain these functions. Similar to what was reported for DNA methylation, the total levels of histone acetylation are likely to change as the organism ages. Consistent with this observation, the levels of the histone deacetylase HDAC-1 decrease upon serial passaging of primary human fibroblasts (Wagner et al., 2001). Importantly, treatment of primary human fibroblasts with the HDAC inhibitors Trichostatin A (TSA) or sodium butyrate induces a senescence-like state, suggesting that modulation of histone acetylation through class I and II HDACs is an essential step in the establishment of senescence (Ogryzko et al., 1996). In addition, trimethylation of histone H4 at lysine 20 (H4K20me3), a hallmark of constitutive heterochromatin, increases in rats livers with age, and is found upregulated in a cellular model of progeria (Sarg et al., 2002; Shumaker et al., 2006).

More recently, pRB protein-mediated senescence-associated heterochromatin foci (SAHFs) have been identified as sites of local transcriptional repression, where heterochromatin proteins are recruited to E2F-responsive promoters, for example the promoter of cyclin A2 (Narita et al., 2003). This observation fueled the view that cellular senescence might be an epigenetically controlled process in which S-phase-related gene activities are selectively shut down in the vicinity of methylated lysine 9 residues at histone H3 (H3K9). Later, using Eμ-*N-ras*^{G12D} transgenic mice, Braig *et al*. (2005) further showed that oncogenic Ras-induced senescence in primary lymphocytes strictly

depended on Suv39h1 (Braig et al., 2005). Hence, a histone modification signature (i.e., Suv39h1-mediated methylation of H3K9) acts as an early safeguard against imminent lymphoma development initiated by oncogenic Ras *in vivo*. Moreover, the high-mobility group A proteins have been shown to be essential components of SAHFs and when inactivated, along with p16INK4a, result in a bypass of Ras-induced senescence in some cell types (Narita et al., 2006). Bandyopadhyay *et al*. (2007) have shown that in human melanocytic nevi, HDAC1 dynamically forms complexes with pRB during senescence and drives a sequential and cooperative activity of chromatin remodeling effectors, including transient recruitment of Brahma (Brm1) into pRB/HDAC1 mega-complexes, formation of HP1β/SUV39H1 foci, methylation of H3-K9, stable association of pRB with chromatin and significant global heterochromatinization (Bandyopadhyay et al., 2007). Thus, the identification of a common epigenetic signature of histone H3K9 methylation in different mouse model settings as well as in human tumor material strongly supports the concept of cellular senescence as an inherited principle that irreversibly reprograms cellular capabilities via chromatin remodeling. In addition, Narita *et al*. (2003) showed that SAHF contain chromatin marks that are reminiscent of those found in constitutive heterochromatin, including hypoacetylated histones, methylation of lysine 9 of histone H3, and Heterochromatin Protein 1 (HP1) (Narita et al., 2003). However, SAHF also contain specific marks, absent from constitutive heterochromatin such as enrichment of macroH2A and HMGA proteins and depletion of linker histone H1 (Funayama et al., 2006; Narita et al., 2006; Zhang et al., 2007). A complex of histone chaperones, including HIRA, ASF1a, and UBN1, also contributes to chromosome condensation during SAHF assembly in human cells (Banumathy et al., 2009; Zhang et al., 2005). In addition, the pRB pathway appears to converge with the HIRA/ASF1a/UBN1 pathway at the level of chromatin (Ye et al., 2007b), again,

suggesting a role for pRB in providing a nucleation site for chromosome condensation and formation of SAHF.

It has also been suggested that DNA damage may precede and trigger SAHF formation (Hemann and Narita, 2007), thereby linking these two processes. However, not all senescence-inducing signals trigger SAHF formation (Hemann and Narita, 2007), and some cell types appear to be incapable of forming SAHFs (Mallette et al., 2007). Nevertheless, more subtle changes in chromatin and transcriptional repression may still be important in mediating the senescence response. Understanding how these processes are inter-related will be an important question for the future.

Overall, these findings suggest that epigenetic mechanisms are important in senescence and further suggest epigenetic deregulation could play an important role in the bypass of senescence and the acquisition of a tumorigenic phenotype. In the near future, technically more challenging screens for genetic defects at the level of repressive histone signatures will help to uncover potentially deregulated downstream components of the senescence machinery.

Autophagy

Autophagy was originally characterized as a ‘‘recycling’’ process that promotes cell survival during periods of nutrient deprivation. Recently, autophagy has been identified as an important component of the senescence program required for efficient establishment and quality of the senescence phenotype (Gamerdinger et al., 2009; Young et al., 2009). It has been reported that the stress of oncogene activation also triggers autophagy that is required for efficient establishment of the senescence phenotype (Young et al., 2009). A subset of autophagy-related genes (ATGs) are up-regulated in senescence, including ULK3, LC3B, BNIP3, and BNIP3L, which correlates

with down-regulation of mammalian target of rapamycin (mTOR) and induction of FoxO3a that induces ATG gene expression. Inhibition of autophagy leads to delayed senescence and affects the accumulation of senescence-associated secreted proteins, such as IL6 and IL8. Thus, senescence-associated autophagy might have a dual role in the physical breakdown of cell constituents during remodeling and in providing the building blocks for restructuring and secretion.

Recent evidence has indicated a role for autophagy in tumor suppression (Mathew et al., 2007). The impairment of autophagy might facilitate escape from senescence, and therefore contribute to increased cancer incidence. The potential role for autophagy in preventing senescence bypass can begin to be assessed by comparing cancer incidence following oncogene activation and induction of OIS in mice with an autophagy wild-type and defective (*Beclin1*$^{+/-}$) genetic background. Defining which aspects of autophagy are required for senescence and if or how this impacts tumor suppression through senescence are the next important issues.

miRNA

MicroRNAs (miRNAs) are small, 19-23 nucleotide, non-coding RNAs that repress the expression of target genes by either preventing translation of the target mRNA or causing its degradation. Accumulating evidence points to a role for a miRNA network in orchestrating the senescence program. Expression profiling has revealed expression of miRNAs to be changed in senescence (Lafferty-Whyte et al., 2009). For example, an abundance of evidence has linked members of the miR-34 family of miRNAs (miR-34a-c) to senescence. miR-34 miRNAs are shown to be directly activated by p53 and to drive proliferation arrest and cell senescence (He et al., 2007), in part, by posttranscriptional downregulation of cell-cycle progression genes, including CDK4 and cyclin E. miR-34a is

also shown to regulate endothelial senescence in both primary and progenitor cells (Ito et al., 2010; Zhao et al., 2010). In addition, recent work by Maes *et al*. (2009) describes the miRNA profile of replicative senescence in comparison to premature senescence and serum-starved cells using WI-38 fibroblasts (Maes et al., 2009). In a similar study, Bonifacio *et al*. (2010) presents the miRNA profile of replicative senescence in human BJ fibroblasts, and compares this to the miRNA expression profile of BJ fibroblasts immortalized by the catalytic subunit of human telomerase (hTERT). In addition, a p16INK4a-independent senescence association of several miRNAs is shown by comparing the miRNA profile of senescent BJ cells (p16INK4a deficient) to that in WI-38 cells (p16INK4a positive) (Bonifacio and Jarstfer, 2010). Moreover, miRNAs might also regulate some of the factors that are secreted by senescent cells. In a recent study, Bhaumik *et al*. (2009) have shown that miR-146a/b is expressed in response to rising inflammatory cytokine levels as part of a negative feedback loop that restrains excessive secretion of IL-6 and IL-8 (Bhaumik et al., 2009) in senescent human fibroblasts, suggesting that the miRNA regulatory network may be broadly interwoven throughout the senescence program.

Deregulation of microRNAs has roles in human carcinogenesis (Croce, 2009). Among the miRNAs up-regulated in senescent BJ cells, miR-143 and miR-145 have been reported to be down-regulated in several cancer cells, suggesting that miR-143 and miR-145 have a general role of in regulating cellular proliferation and may function as tumor suppressor miRNAs (Bonifacio and Jarstfer, 2010). In addition, miR-34a induces senescence in human colon cancer cells, through modulation of the E2F pathway, and is shown to be down-regulated in human colon cancers (Tazawa et al., 2007). By contrast, the miR-17-92 cluster, which is frequently overexpressed in human cancers, confers tumorigenicity by inhibiting oncogenic Ras-induced senescence (Hong

et al., 2010). Moreover, the miR-17/20a seed family and other miRNAs confer resistance to Ras-induced senescence by directly targeting p21CIP1 (Borgdorff et al., 2010; Hong et al., 2010). No doubt, the investigation of the interface between miRNAs and senescence will help to reveal cancer biomarkers of diagnosis, prognosis, and therapeutic outcome.

Senescence secretome

Senescent cells are metabolically and biosynthetically active and secrete an altered mix of mediators essential for entry and maintenance of senescence (Acosta et al., 2008; Kuilman et al., 2008; Wajapeyee et al., 2008) and immune surveillance (Krizhanovsky et al., 2008; Xue et al., 2007). As the physiological functions and pathological impact of the senescence program become clearer in a whole tissue context, this altered secretome is emerging as one of the most exciting aspects of the senescence program, because of its potential wide-ranging impact on a tissue's function, response to damage, and tissue degeneration (Adams, 2009; Campisi and d'Adda di Fagagna, 2007).

One category of the secreted factors consists of those that tend to reinforce senescence-associated proliferation arrest. Altered expression the secreted gene products includes reduced levels of IGFI and WNT2 (Ferber et al., 1993; Ye et al., 2007a), and increased production of PAI-1, IGFBP proteins, and TGFβ1 (Kortlever et al., 2006; Tremain et al., 2000; Wajapeyee et al., 2008).

Senescent cells also express increased amounts of factors best known for their immune-regulatory functions, namely, cytokines, chemokines, and their receptors. Some of these factors also contribute to proliferation arrest. For example, IL6 reinforces senescence-associated proliferation arrest of untransformed primary cells in a cell-

autonomous autocrine manner (Kuilman et al., 2008). Likewise, increased CXCR2 and IL8 reinforce the senescence program, including proliferation arrest (Acosta et al., 2008). More importantly, these immune regulators produced by senescent cells appear to impact the immune system. Senescent cells are shown to activate NKG2D signaling, a receptor expressed on NK cells and T cells, by upregulating the NKG2D ligands, MICA and ULBP2, and IL15 (Krizhanovsky et al., 2008; Xue et al., 2007). Senescent cells thereby contribute to tumor clearance by triggering an innate immune response that targets the tumor cells *in vivo*.

A third group of secreted factors whose expression is often altered in senescence includes factors that remodel the extracellular stroma or matrix (ECM), such as increased production of Matrix Metalloproteinase1 (MMP1) and MMP3, and decreased expression of fibronectin and collagen (Krizhanovsky et al., 2008; West et al., 1989). These changes appear to promote breakdown of the ECM, therefore affecting tissue architecture and facilitating cell migration. Secreted MMPs also regulate the activity of cytokines, suggesting that these two components of the senescence secretome are functionally linked. MMP3 has also been linked to the inflammatory response in skin (Wang et al., 1999). Therefore, the ECM remodeling activity of senescent cells may play an important role in tissue wound healing response and have profound impacts on tissue aging, as well as tumor formation, by changing the tissue microenvironment.

Analysis of regulation of the senescence secretome has identified the transcription factors C/EBPβ and NFκB as two key activators. Several CXRC2 ligands, namely IL8, CXCL1, CXCL5, and CXCL7, are activated in senescent cells in an NFkB-dependent manner (Acosta et al., 2008). Similarly, a central role for C/EBPβ in expression of several cytokines, such as IL6, IL8, CXCL1, and CXCL7, has been shown

in senescent cells (Acosta et al., 2008; Kuilman et al., 2008). Recent reports have also indicated roles for DNA damage signaling, autophagy and microRNAs in activation of the senescence secretome (Bhaumik et al., 2009; Rodier et al., 2009; Young et al., 2009).

Taken together, the senescence secretome may be employed to communicate signals to both senescent cells themselves and surrounding cells, informing them of a stress response, altering the extracellular matrix and even regulating the immune response.

1.7 Control mechanisms

Signal intensity and cell sensitivity

As noted in section 1.4, one of the initial concerns in accepting senescence as a mechanism of tumor suppression was that perhaps only supraphysiological levels of activated Ras could trigger this response. This skepticism was fueled by the observation that while MEFs senesce in response to overexpressed oncogenic H-Ras, MEFs expressing a single activated *K-ras* allele are immortal (Guerra et al., 2003; Tuveson et al., 2004). However, it has been shown now that a subset of adenomas from these same mice do undergo senescence *in vivo* (Collado et al., 2005), raising the possibility that different cell types may be differentially sensitive to the same signal. This hypothesis has been formally proven by analyzing the consequences of inactivating the NF1 (neurofibromatosis type I) tumor suppressor, the gene encoding a RasGAP, and the subsequent acute activation of endogenous Ras and Ras effector (Courtois-Cox et al., 2006). Similar to MEFs expressing a single activated *K-ras* allele, inactivation of NF1 results in the immortalization of this cell type. In contrast, NF1 ablation rapidly triggers cellular senescence of normal human diploid fibroblasts. Importantly, NF1 deficiency also induces senescence *in vivo*, in cells within benign human tumors from patients with

NF1. Therefore, it can be concluded that (1) oncogene-induced senescence is not an artifact of Ras overexpression and (2) different cell types exhibit different sensitivities to oncogenic stress. The challenge of the future will be to elucidate the mechanisms that dictate the sensitivity or resistance to a specific oncogenic signal (Courtois-Cox et al., 2008).

A direct example is shown by Sarkisian *et al*. (2007) that signal intensity may play an important biological role in mediating a senescence response in some instances. It has been shown that high but not low levels of activated H-Ras trigger senescence in mammary epithelial cells *in vivo*. Moreover, in this system, low levels of Ras ultimately triggered tumor formation, but only after active Ras was spontaneously upregulated, and senescence checkpoints were inactivated (Sarkisian et al., 2007). These observations suggest that some cell types, at least in the mouse, are not sensitive to low levels of oncogenic Ras activity. Therefore, it is possible that in some early lesions, a single copy of an activated *ras* allele might be initially selected for, and that after additional genetic alterations occur (for example, p53 loss), Ras might be amplified or its activity upregulated, further promoting tumorigenesis. This leaves us with several questions that remain to be answered. For example, how much oncogenic activity is necessary to induce senescence? How much oncogenic activity is needed for neoplastic transformation?(Courtois-Cox et al., 2008)

In order to find a way to trigger the defensive senescence response more efficiently at an early stage of tumor development, two groups have independently attempted to lower the bar of senescence and look at the effects on cancer (Campaner et al., 2010; Lin et al., 2010). Using different mouse models, both studies focus on whether decreasing the activity of G1 CDKs (i.e. CDK2, CDK4, CDK6), previously shown to be crucial for establishing senescence, might lower the 'critical point' for triggering

senescence in oncogenically primed cells. Lin *et al*. (2010) genetically eliminated the Skp2 protein, which normally mediates degradation of CIP/KIP family cyclin-dependent kinases inhibitors p21CIP1and p27KIP1, and resulted in a reduced G1 CDK activity in mice. In the absence of Skp2, cancer-prone cells expressing the Ras oncoprotein or partially lacking the tumor suppressor protein Pten become more sensitized to senescence. Intriguingly, mice deficient in both Pten and Skp2 (*Pten*$^{+/-}$; *Skp2*$^{-/-}$) are strongly protected from cancer, compared to mice carrying only one copy of the *Pten* gene (*Pten*$^{+/-}$ mice). More importantly, analysis of the tissues protected from cancer, particularly the lymph nodes and prostate, showed abundant senescent cells and a low proliferation rate within the pre-tumoral tissues (Lin et al., 2010). Campaner *et al*. (2010) focused directly on CDK2-deficient mice. They found that oncogenic stress, in this case exerted by the Myc oncoprotein, caused the cells of mice lacking CDK2 to become sensitized to senescence. Overexpressing oncogenic Myc in B lymphocytes normally led to a strong apoptotic response, which, despite its protective potential, was not sufficient to prevent the development of lymphoma. By contrast, Myc expression in CDK2-deficient mice resulted not only in apoptosis but also in increased levels of senescent B lymphocytes. This double anti-oncogenic response was accompanied by a lower number of proliferative cells and delayed development of lymphoma. The same effect was also observed in other tissues, for example, in pancreas (Campaner et al., 2010). Both studies demonstrate that it is feasible to make oncogenically primed mouse cells more sensitive to senescence induction and therefore make the organism better protected from cancer, and again, point out the importance of signal intensity (Serrano, 2010).

Furthermore, oncogenic signaling is transmitted through complex cascades of mediators that have ample opportunities for self attenuation through negative-feedback regulation, to be discussed below. Consequently, the activation of an endogenous

oncogene does not necessarily translate into the full activation of its downstream effectors. A more realistic scenario is one in which the influence of a particular oncogene on its downstream effectors increases progressively during tumor development, which reflects the cumulative loss of negative-feedback regulators (Collado and Serrano, 2006).

Negative feedback loops

Recent studies have shown that, in addition to activating p53 and pRB, oncogene activation triggers an elaborate process of autoregulatory negative feedback loops that initiate a coordinated shutdown of intracellular signaling pathways, presumably designed to eliminate the aberrant signal, and actively promote the senescence response to the persistent oncogenic stimulus.

This discovery was first made in the course of studying the NF1 tumor suppressor, ablation of which results in an activation of endogenous levels of Ras. Courtois-Cox *et al*. (2006) showed that in MEFs, which became immortalized in response to NF1 deficiency, NF1 ablation triggered a sustained activation of Ras and AKT, as expected. However, in normal human diploid fibroblasts, Ras and its effectors were transiently activated following NF1 inactivation but were potently suppressed shortly thereafter. Notably, an oncogenic Raf allele triggered an even more potent and rapid suppression of Ras and AKT activity, indicating that this was not a unique feature of senescence triggered by NF1 deficiency. These data suggest that in sensitive cells, this oncogenic signal was triggering a potent negative feedback response. Furthermore, these same 'feedback signals' were shown to be actively involved in triggering the senescence response, by meeting the following criteria: (1) suppression of Ras signaling precedes senescence, (2) suppression of Ras or Ras effector pathways alone is sufficient to trigger senescence, (3) Ras suppression activates known regulators of the

senescence pathway (pRb and p53), (4) suppression of Ras signaling occurs in response to multiple genetic insults and (5) evidence of 'negative feedback' is observed in benign tumors *in vivo* (Courtois-Cox et al., 2006).

Using proteomic and genomic approaches, this study further discovered that cells exposed to an oncogenic insult suppressed Ras signaling in two waves (Courtois-Cox et al., 2006). By using an inducible activated Raf allele, which allows for a more precise kinetic analysis, Ras-GTP levels were found to be dramatically reduced within 30 min of Raf induction. This suppression appeared to be primarily regulated by post-translational events, such as phosphorylation and inactivation of the RasGEF protein SOS, among others. This initial negative feedback response resulted in a significant, but incomplete attenuation of Ras and Ras effector pathways, suggesting that cells might be attempting to neutralize the aberrant signal. However, within 42 h, a global negative feedback signaling network was transcriptionally induced. At least 14 proteins known to directly regulate Ras and Ras effectors were affected, including several RasGAPs, RasGEFs, Sproutys, SPREDs, MAP kinase phosphatases, and the AKT target FOXO. Importantly, a robust suppression of Ras and AKT activity accompanied this transcriptional program (Courtois-Cox et al., 2008).

A second example is from the studies of the PI3K pathway and cellular senescence. In normal human diploid fibroblasts, suppression of the PI3K pathway is sufficient to induce senescence (Courtois-Cox et al., 2006). This finding is consistent with the long-standing observation that PI3K-deficient MEFs cannot be established, as they rapidly become senescent in culture (Brachmann et al., 2005). On the surface, these models appear to contradict findings indicating that activation of PI3K, through loss of PTEN, also triggers senescence (Chen et al., 2005). Although PTEN inactivation does trigger a sustained activation of AKT *in vitro*, several observations suggest the

possibility that a negative feedback event may occur downstream of cytoplasmic AKT activation. Inactivation of PTEN in the mouse prostate results in neoplastic (PIN) lesions, where phosphorylated AKT is exclusively cytoplasmic (Trotman et al., 2003). Whereas, in more advanced lesions caused by compound mutations in PTEN and PML, active AKT is found in the nucleus. PML has been reported to promote the dephosphorylation of nuclear AKT via recruitment of PP2A. Accordingly, loss of PML results in the nuclear accumulation of activated AKT and inactivation of FOXO-3a, a nuclear target of AKT, along with promoting the development of more advanced tumors in the PTEN model (Trotman et al., 2006). This finding raises the intriguing possibility that the key negative feedback signals in response to PTEN loss may function to suppress nuclear AKT activity (or localization). Consistent with this possibility, a constitutively active FOXO mutant, deficient in its ability to be phosphorylated by AKT, has been shown to be a potent activator of senescence in sensitive cell types. Moreover, FOXO has been shown to be rapidly activated by Raf, before senescence (Courtois-Cox et al., 2006). Interestingly, Paik *et al*. (2007) have found that Sprouty 2 is a direct target of FOXO, indicating that a positive feedback loop may function within this negative feedback pathway (Paik et al., 2007). Taken together, these data suggest that once a threshold inhibitory response is achieved, a negative-feedback loop designed to terminate intracellular PI3K signaling is engaged and amplified to promote a senescence response (Courtois-Cox et al., 2008).

Another intriguing observation is that the suppression of canonical WNT signaling may also participate in the senescence response. Ye *et al*. (2007a) reported repression of Wnt2 occurs early in senescence and independently of the pRB and p53 tumor suppressor proteins and drives relocalization of HIRA to PML bodies, formation of SAHF

and senescence (Ye et al., 2007a), further suggesting a role for negative feedback signaling in tumor suppression.

It is tempting to speculate that some of these genes functioning in the negative-feedback network might be mutational targets in human cancer. Indeed, a number of genes that negatively regulate the Ras pathway have already been implicated in tumorigenesis (Courtois-Cox et al., 2006). In addition, it is now becoming more generally apparent that many mouse tissues designed to express a single, activated *K-ras* allele do not exhibit much if any ERK and AKT activation, and the negative feedback pathways are responsible for attenuating the Ras signal (Kim et al., 2005; Sansom et al., 2006). In contrast, when these mice are crossed to other tumor-prone strains to generate animals with compound mutations, the resulting tumors do exhibit activation of Ras effectors, implying that this suppression is somehow alleviated in the course of tumor development and/or progression (Dinulescu et al., 2005; Hingorani et al., 2005). A more direct example is, in a mouse model that expressed a single oncogenic *K-ras* allele in the lung, Sprouty 2 was dramatically upregulated. Strikingly, genetic inactivation of Sprouty 2 potently enhanced Ras-mediated lung tumorigenesis, affecting tumor number, latency, size and grade, and ERK activation (Shaw et al., 2007). These findings show that the senescence network is quite complicated and that much remains to be learned about these negative regulatory signals, both in the context of normal growth control and tumor suppression (Courtois-Cox et al., 2008).

II. CDK5

Cyclin-dependent kinases (CDKs) are a family of proline-directed Ser/Thr kinases known for their role in the control of cell cycle progression. In 1992, an atypical member called CDK5 (also known as Neuronal CDC2-Like Kinase, NCLK) was identified. Unlike other mitotic CDKs, it is regulated by its own activators rather than by cyclins and does not act as a checkpoint kinase to regulate cell cycle progression (Lew et al., 1994; Tang et al., 1995).

CDK5 was recovered from bovine brain and from brains of Alzheimer patients as a cyclin-free 33 kD/25 kD kinase complex with the ability to phosphorylate synthetic Ser/Thr-Pro-containing peptides (Ishiguro et al., 1992; Lew et al., 1992). Meanwhile, low-stringency screenings for CDK1 kinases resulted in the identification of CDK5 (Hellmich et al., 1992; Meyerson et al., 1992).

The functional counterpart of CDK5 in yeast is the multifunctional Pho85 kinase (Andrews and Measday, 1998; Huang et al., 1999). The best known role for CDK5 is in regulating cytoarchitecture during the development of the central nervous system (CNS), as well as other post-mitotic neuronal activities, such as neurite outgrowth, axon guidance, membrane transport, synaptic function, dopamine signaling and drug addiction. Deregulation of CDK5 might contribute to the pathology of neurodegenerative diseases, such as Alzheimer's disease and amyotrophic lateral sclerosis (ALS) (Dhavan and Tsai, 2001).

2.1 CDK5 structure

Overall, CDK5 retains the main structural features characteristic of CDKs (Tarricone et al., 2001). Mitotic cyclin-dependent CDKs have a bilobate structure that is divided into a small N-lobe and a large C-lobe (Jeffrey et al., 1995). In the cleft between

the N- and C-lobes lay the ATP and substrate binding sites. All CDKs have a unique activation segment (T-loop) that sterically blocks the access of ATP and protein substrates to their respective binding sites in the inactive conformation (Lalioti et al., 2010).

Mitotic CDKs and CDK5 display identical peptide-substrate specificity, however, the structural rearrangements of CDK5 in the activation process are substantially different from those of other CDKs (Mapelli and Musacchio, 2003). Activation of CDK5 is currently known to occur only in postmitotic cells and involve two specific activators, p35 (CDKR1) and p39 (CDKR2). The structurally related activators do not share sequence similarity with the cyclins (57% amino-acid identity), but remarkably adopt a conformation similar to the characteristic cyclin box fold through which they interact with CDK5 (Tang et al., 1997; Tarricone et al., 2001). CDK5 also binds cyclins D and E (Miyajima et al., 1995; Xiong et al., 1992), but its kinase activity is not promoted by them. Conversely, p35 and p39 are selective and do not bind strongly to other CDKs. Moreover, the CDK inhibitors (CKIs) p21CIP1 and p27KIP1 effectively inhibit CDK2, but have minimal effect on CDK5-p35, supporting the idea that interaction with a non-cyclin activator might allow CDK5 to escape from the CKIs for mitotic CDKs (Lee et al., 1996). In addition, it is noteworthy that the PSTAIRE sequence, the central motif in the interaction of the mitotic CDK with its regulatory cyclin subunit, is modified to PSSALRE in Cdk5 (Meyerson et al., 1992).

Structural analyses show that the selectivity of p25, an active fragment of p35, for CDK5 rests in the extensive involvement of the T-loop and of Ser159 in the binding of the activator. In contrast to the effect of cyclin A binding to CDK2, the binding of p25 to CDK5 does not change the position of the C-helix in the N-lobe, but rather extends the T-loop into a stretched active conformation, which is shown to be important in facilitating

the CDK5 recognition of substrates with a +1 Pro (Tarricone et al., 2001) (Fig.1.5)(adapted from (Lalioti et al., 2010), Figure 1). Moreover, the p35 activator participates in binding of the basic residue in the +3 position of the substrate, resulting in the preference of CDK5 for peptides that contain the S/TPXH/R/K substrates (Songyang et al., 1996).

The activation of CDK5 by the p35/p39 activators is independent of CDKs-activating kinase (CAK), which phosphorylates Thr160 in the T-loop of CDKs (Qi et al., 1995). More differences between the mechanisms of mitotic CDKs and CDK5 activation involve phosphorylation of Thr14 and Tyr15 in the G loop. As for mitotic CDKs, phosphorylation of Thr14 in CDK5 inhibits kinase activity, but surprisingly, phosphorylation of Tyr15 is stimulatory (Zukerberg et al., 2000). Furthermore, Tyr15 of CDK5 is not phosphorylated by Wee1 *in vitro* (Poon et al., 1997) as in mitotic CDKs, but instead is phosphorylated by c-Abl, through an Abl-binding adaptor protein called Cables (Zukerberg et al., 2000) and the Src kinase Fyn (Sasaki et al., 2002). It is reasonable to speculate that there might be independently regulated phosphatases with separate specificity for Thr14 and Tyr15, activation of which would have opposite effects on the state of CDK5 activation, in contrast to the dephosphorylation of Thr14 and Tyr15 in mitotic CDKs by the dual-specificity phosphatase Cdc25 (Lalioti et al., 2010).

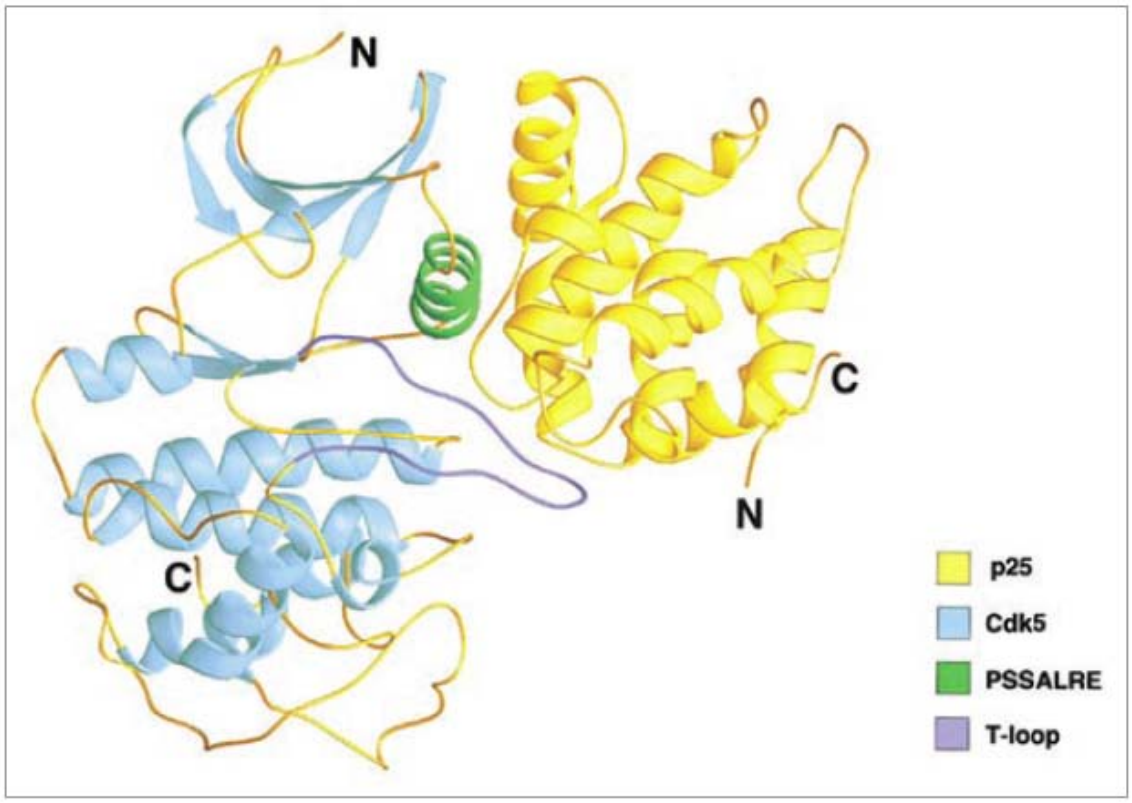


Fig.1.5. Ribbon diagram of the p25-Cdk5 structure. p25 (yellow) binds Cdk5 (light blue) in the region of the PSSALRE helix (green) in the kinase's small N-lobe. The contact between p25 and the activated T-loop (dark blue) is extensive. The N- and C-termini are shown. (Adapted from (Lalioti et al., 2010), Figure 1).

2.2 Regulation of CDK5 by its activators

Activation of CDK5 requires its association with either of its two known regulatory subunits, p35 and p39. Inactivation of CDK5 involves ubiquitylation and degradation of these activating subunits by the proteasome (Hisanaga and Saito, 2003; Patrick et al., 1998). p35 is highly unstable and exhibits a half life of only 20–30 min. Phosphorylation of p35 by CDK5 targets it for ubiquitin-mediated proteolysis. Proteolytic cleavage by Calpain also directly truncates p35 into a much more stable fragment of 25KD, p25, extending the half-life by 3–5-fold (Kusakawa et al., 2000; Lee et al., 2000; Patrick et al., 1998; Patrick et al., 1999). Furthermore, cleavage of p35 to p25 removes the N-terminal myristoylation sequence from p35, resulting in a re-distribution of the CDK5 activator from membrane-associated to cytosolic form. Thus, the generation of p25 releases CDK5 activity into the cytosol and results in prolonged activation of CDK5, which has been proposed to mediate neuronal death in various models of neurodegenerative diseases. Recent evidence indicates that p39 is also cleaved into p29 by calpain. Similar to p35, p29 is more stable than p39, and redistribution of CDK5 activity into the cytosol was observed following p39 cleavage (Patzke and Tsai, 2002).

Studies on age and regional distribution of p35 and p39 protein levels in rat brain show that the pattern of expression of p39 is inverse to that of p35. p35 mRNA expression is highest in the newborn brain (Tomizawa et al., 1996) while p39 mRNA expression peaks postnatally at 3 weeks and levels remain particularly high in the cerebellum of the adult (Cai et al., 1997). At the protein level, p35 and p39 are localized in both the cerebral and cerebellar cortices (Honjyo et al., 1999; Matsushita et al., 1996) but it appears that p35 is the major CDK5 activator in the cerebral cortex. In contrast, the higher level of p39 expression during cerebellar development (Jeong et al., 2003) may reflect its important role in the functional compartmentalization of the cerebellum. Taken

together, these findings suggest that they might have developmental stage- and region-specific complementary functions in brain (Lalioti et al., 2010; Wu et al., 2000).

2.3 CDK5 function in CNS development

Initial findings implicated CDK5 activity in critical postmitotic neuronal activities such as neurite outgrowth, axon guidance, neuronal migration, secretion, dopamine signaling and synaptic transmission (Dhavan and Tsai, 2001). Despite the ubiquitous expression of CDK5, kinase activity of CDK5 is largely detected only in the nervous system (Tsai et al., 1993). This puzzle was later resolved by the isolation of its activators, p35 and p39, whose expression is found almost exclusively in the nervous system (both CNS and PNS) (Tsai et al., 1994; Zheng et al., 1998).

Cdk5 knockout mice display severe cortical lamination defects and perinatal death. While newly born neurons usually migrate to the outer cortical layers in an 'inside-out manner', this stratification of neocortex is absent in *Cdk5*$^{-/-}$ mice (Ohshima et al., 1996). Remarkably, deletion of *p35* results in similar abnormality in the formation of cortical lamina, although mortality of *p35*$^{-/-}$ mice is reduced to 15% and they generally live until 3 months of age (Chae et al., 1997). On the other hand, *p39*$^{-/-}$ mice, display no overt phenotypic abnormality compared to wild-type. The disparity in the phenotypes between the *p35*$^{-/-}$ and *p39*$^{-/-}$ mice underscores the importance of p35 during development. Importantly, double deletion of p35 and p39 results in phenotypes that are indistinguishable from CDK5 null mice, strongly suggesting that p35/p39 are necessary and sufficient to activate CDK5 in the nervous system. Further analysis of p35 and p39 indicates that these proteins have distinct and redundant functions and that both are essential for CDK5 function during neurodevelopment (Ko et al., 2001; Smith et al., 2001).

Recently, the potential involvement of CDK5 as a regulator of neuronal death and survival has attracted considerable interests. CDK5 activity is indispensable for neuronal survival during development. Swelling of cell soma and nuclear margination was observed in the brainstem and spinal cord neurons of $CDK5^{-/-}$ mice (Ohshima et al., 1996). Reconstitution of CDK5 function in $Cdk5^{-/-}$ mice abolishes the perinatal lethality of *Cdk5* null mice, and reverses the chromatolytic morphology of $Cdk5^{-/-}$ neurons (Tanaka et al., 2001). Furthermore, CDK5 has been shown to protect neuronal cells from apoptosis by inhibiting c-Jun N-terminal kinase 3 (JNK3) following UV irradiation, and is indispensable for the maintenance of survival signals by regulating PI3K/Akt activity in neurons (Li et al., 2003; Li et al., 2002).

In contrast to the observed involvement of CDK5 in neuronal survival, CDK5 has also been shown to act as a death inducing agent in neurons. Increasing evidence has linked CDK5 to the etiopathology of neurodegenerative diseases, such as Alzheimer's disease, Parkinson's disease and amyotrophic lateral sclerosis (ALS). p25, which is a cleaved fragment of CDK5 activator p35, plays a central role in the function of CDK5 under these pathological conditions. Generation of the p25 fragment often accompanies apoptosis upon challenge by a variety of apoptotic stimuli, ranging from neurotoxicity, ischemia, oxidative stress, incubation with amyloid beta-peptide to nerve injury. Deregulation of CDK5 is therefore principal in the development of serious neuronal diseases through its contribution to cell death (Cheung and Ip, 2004; Dhavan and Tsai, 2001).

2.4 CDK5 function in non-neuronal settings

Although CDK5 was first recognized as an essential molecule in the brain, later, the demonstration that CDK5 was active in virtually every tissue extended the search to

non-neuronal tissues and a broad variety of cellular functions. An increasing body of evidence has established CDK5 kinase activity, the presence of the CDK5 activators, p35 and p39, and CDK5 functions in non-neuronal cells, including lens epithelial cells, myocytes, monocytic and neutrophilic leucocytes, pancreatic β-cells, glial cells and germ cells (Chen and Studzinski, 1999; Gao et al., 1997; Lazaro et al., 1997; Lilja et al., 2001; Philpott et al., 1997; Rosales et al., 2004a; Rosales et al., 2004b; Tang et al., 1998). The functional significance is highlighted by the numerous and varied targets of CDK5 within pathways that control the organization of the cytoskeleton and focal adhesions, signaling cascades, membrane dynamics and function, cell metabolism, cell cycle arrest in postmitotic cells, gene transcription and cell survival (summarized in Table 2, adapted from (Rosales and Lee, 2006), Table 2).

Gene expression: Although it is not clear whether CDK5/p35 acts directly on the nuclear machinery to regulate gene transcription, accumulating evidence indicates that CDK5 can regulate the activity of a number of transcription factors; moreover, this regulation does not always require its presence in the nucleus. Thus, CDK5/p35-directed phosphorylation plays a pivotal role in regulating the activity of numerous signaling and transcriptional molecules governing diverse cellular processes. For example, CDK5-directed phosphorylation of the retinoblastoma and p53 tumor suppressors can modulate their activities and influence apoptotic processes (Lee et al., 1997; Zhang et al., 2002). CDK5-dependent phosphorylation of the transcription factor myocyte enhancer factor-2 (MEF2) leads to the inhibition of its prosurvival functions and also engenders apoptosis (Gong et al., 2003). The CDK5 complex can also phosphorylate STAT3 and modulate its transcriptional activity (Fu et al., 2004). In pancreatic β-cells, elevated glucose level results in increased CDK5/p35 activity, which in turn, upregulates the transcriptional activation of the insulin gene (Ubeda et al., 2004). CDK5/p35 activity is also a key

regulator of non-specific esterase expression in monocytes (Studzinski and Harrison, 2003). Finally, SET protein, a chromatin remodeling factor, serves as a substrate for CDK5 and binds the core histones, protecting it from acetylation by different histone acetyltransferases (Qu et al., 2002).

Differentiation: Although CDK5 activity has mainly been associated with differentiation of neurons, CDK5 activity has also been observed in other non-neuronal cells during differentiation. CDK5/p35 is expressed in lens epithelial cells and in differentiating lens fibers (Gao et al., 1997; Negash et al., 2002). Differentiation of primary oligodendrocyte precursor cells is accompanied by increased level of CDK5 activity (Tang et al., 1998). CDK5 is required for the expression of the muscle differentiation markers, such as MyoD and MRF4, and has been shown to be a positive regulator of early myogenesis (Lazaro et al., 1997; Philpott et al., 1997; Sahlgren et al., 2003). CDK5 is expressed in Leydig cells, Sertoli cells, spermatogonia and peritubular cells, indicating a role in spermatogenesis (Rosales et al., 2004b). In the human myeloid leukemia HL60 cells, induction of differentiation into monocytic cells using 1α,25-dihydroxyvitaminD_3 (1,25D_3) results in increased CDK5 level and activity (Chen and Studzinski, 1999, 2001; Chen et al., 2004). CDK5 activity is also present in podocytes during glomerulogenensis (Griffin et al., 2004).

Migration/wound healing: CDK5 plays an essential role in neuronal cell–cell, cell–matrix adhesion and migration. Similarly, CDK5 also regulates adhesion and migration in lens epithelial cells, corneal epithelial cells and keratinocyte-derived HaCaT cells (Gao et al., 2002; Kwon et al., 2000; Nakano et al., 2005; Negash et al., 2002). In addition, CDK5 is further involved in corneal wound healing. CDK5 overexpression in mice causes retarded corneal wound closure as well as suppressed actin remodeling. In contrast, inhibition of CDK5 activity by olomoucine treatment hastens wound healing,

along with increased level of activated Src, dissolution of focal adhesions, formation of lamellipodia and actin remodeling (Gao et al., 2004).

Exocytosis/hormonal regulation: In addition to the regulation of exocytosis in neurons, CDK5 is also involved in secretory exocytosis in other cell types. In pancreatic β-cells, inhibition of CDK5 activity reduces insulin secretion, indicating that CDK5 might be a positive regulator of insulin exocytosis (Lilja et al., 2001). p39 appears to be the major CDK5 activator involved in secretion by β-cells (Lilja et al., 2004). A role for CDK5 in secretory exocytosis has further been implicated in the anterior pituitary gland and neutrophils (Rosales et al., 2004a; Xin et al., 2004). Moreover, CDK5 activity is involved in hormone regulation. Upregulated CDK5 level and activity are observed in Leydig TM3 cells in the presence of epidermal growth factor or luteinizing hormone, and are associated with increased testosterone production (Musa et al., 2000). CDK5 is activated in the uterus following 17 beta-estradiol administration to adult ovariectomized rats, suggesting its involvement in estrogen activity in the adult rat uterus (Altucci et al., 1997).

Senescence: CDK5 has been shown to play a key role in the morphological alteration in senescent cells (Alexander et al., 2004). Abrogation of CDK5 activity blocks flat cell formation in different types of senescent cells. In senescent cells, both CDK5 level and activity are elevated. Suppression of Rac1 activity through phosphorylation of Pak1 at Thr212 by CDK5 is essential for increased actin polymerization that accompanies senescent morphology. Ezrin, an actin-binding protein involved in membrane–cytoskeletal signaling, has also been implicated in the CDK5–Rho senescence pathway (Yang and Hinds, 2003, 2006). Senescence of SAOS-2 cells is accompanied by increased expression and altered subcellular localization of ezrin. Phosphorylation of Ezrin at T235 by CDK5 in response to pRB expression stimulates

further membrane association as well as changes in cell shape. CDK5 phosphorylation of Ezrin also causes dissociation of Rho GDP dissociation inhibitor (Rho-GDI) from the Ezrin–Rho-GDI complex. The released Rho-GDI interacts with and inhibits Rac1 GTPase activity, which is associated with the formation of senescent morphology (Yang and Hinds, 2006).

Function	Cell type/tissue	References
Senescence	Osteosarcoma cells	Alexander et al., 2004; Yang et al., 2003; 2006
	Breast carcinoma cells	Alexander et al., 2004
Differentiation	Murine C2 cells	Lazaro et al., 1997
	Myoblasts	Sahlgren et al., 2003
	HL60	Chen et al., 1999; 2001; 2004
	Elongating spermatids	Rosales et al., 2004b
	Lens fiber	Negash et al., 2002
	Podocytes	Griffin et al., 2004
Gene expression	Myotube	Fu et al., 2001
	Muscle	Fu et al., 2004
	Pancreatic β-cells	Ubeda et al., 2004
Wound healing	Corneal epithelial cells	Gao et al., 2004
Adhesion/migration	Lens epithelial cells	Negash et al., 2002
	Corneal epithelium	Gao et al., 2002
	Keratinocytes	Nakano et al., 2005
Apoptosis	Astrocytoma cells	Gao et al., 2001
	Prostate cancer cells	Lin et al., 2004
	Promyelocytic cells	Sandal et al., 2002
Exocytosis	Pancreatic β-cells	Lilja et al., 2004
	Anterior pituitary cells	Xin et al., 2004
	Neutrophils	Rosales et al., 2004a
Hormone regulation	Leydig/Sertoli cells	Musa et al., 2000
	Uterus	Altucci et al., 1997

Table 2. Regulation of non-neuronal cell functions by CDK5. (Adapted from (Rosales and Lee, 2006), Table 2).

Chapter II

p35 Is Required for CDK5 Activation in Cellular Senescence

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Abstract

The retinoblastoma tumor suppressor gene (*RB-1*) is a key regulator of cellular senescence. Expression of the retinoblastoma protein (pRB) in human tumor cells that lack it results in senescence-like changes. The induction of the senescent phenotype by pRB requires the postmitotic kinase CDK5, the best known function of which is in neuronal development and postmitotic neuronal activities. Activation of CDK5 in neurons depends on its activators p35 and p39; however, little is known about how CDK5 is activated in non-neuronal senescent cells. Here we report that p35 is required for the activation of CDK5 in the process of cellular senescence. We demonstrate that: (i) p35 is expressed in osteosarcoma cells, (ii) p35 is required for CDK5 activation induced by pRB during senescence, (iii) p35 is required for the senescent morphological changes in which CDK5 is known to be involved as well as for expression of the senescence secretome, and (iv) p35 is up-regulated in senescing cells. Taken together, these results suggest that p35 is at least one of the activators of CDK5 that is mobilized in the process of cellular senescence, which may provide insight into cancer cell proliferation and future cancer therapeutics.

Introduction

Cellular senescence was originally described as the process of cell cycle arrest that accompanies the exhaustion of replicative potential in cultured somatic cells (Hayflick, 1965). Senescent cells display characteristic changes in cell morphology, physiology, gene expression, and typically express a senescent-associated β-galactosidase (SA-β-gal) activity (Dimri et al., 1995; Shelton et al., 1999). Although the term 'replicative senescence' indicates the widely accepted model of a terminal growth arrest due to telomere attrition, an apparently identical process called 'premature senescence' can be acutely produced in response to activated oncogenes, DNA damage, oxidative stress, and suboptimal cell culture conditions (Campisi and d'Adda di Fagagna, 2007). These observations imply that senescence is a cellular response to stress that limits the proliferation of damaged cells. Based on such antiproliferative effects, cellular senescence was proposed to be a tumor-suppressive, fail-safe mechanism that shares conceptual and possibly therapeutic similarities with the apoptosis machinery (Braig and Schmitt, 2006; Campisi, 2001; Mathon and Lloyd, 2001). There is now substantial evidence that cellular senescence is a bona fide barrier to tumorigenesis and cells must overcome it to progress to full-blown malignancy. For example, recent studies suggest that oncogene-induced senescence occurs and suppresses tumorigenesis *in vivo*. Together, the findings identify senescent cells in premalignant hyperplastic lesions but not in malignant ones, and show that oncogene-induced senescence potently restricts tumor progression at an early stage. Mutations in certain tumor suppressor genes compromise senescence, thereby contributing to cell immortalization and cancer (Bartkova et al., 2006; Braig et al., 2005; Chen et al., 2005; Collado et al., 2005; Lazzerini Denchi et al., 2005; Michaloglou et al., 2005). Furthermore, cytotoxic agents used in cancer chemotherapy can induce cellular

senescence, and defects in this process contribute to drug resistance *in vivo* (Chang et al., 1999a; Roberson et al., 2005; Schmitt et al., 2002; te Poele et al., 2002).

The *RB-1* and *p53* tumor suppressors are important senescence regulators. p16INK4a/pRB and p14ARF/p53 pathways are typically activated during senescence, and enforced expression of components of either signaling pathway induces senescence in some cell types (Dimri et al., 2000; Ferbeyre et al., 2002; Kelly-Spratt et al., 2004; Lin et al., 1998; Lin and Lowe, 2001; Stein et al., 1990; Stein et al., 1999). Oncogenic lesions that disable these tumor suppressor systems bypass senescence (Beausejour et al., 2003; Brookes et al., 2002; Brown et al., 1997; Rheinwald et al., 2002; Serrano et al., 1997; Shay et al., 1991). Significantly, the role of p16INK4a/pRB in the senescence of primary cells can be recapitulated in tumor cells. The reintroduction of pRB or p16INK4a into tumor cells that lack either protein induces a premature senescence requiring p21CIP1 or, in the absence of an intact p53 pathway, p27KIP1 (Alexander and Hinds, 2001; Dai and Enders, 2000; Xu et al., 1997). Intriguingly, cyclin-dependent kinase inhibitors like p14ARF, p21CIP1, and p27KIP1, which are required for senescence, can induce markers of senescence on their own. However, they cannot mediate the senescent shape change, demonstrating that these two processes in senescence are separable (Alexander and Hinds, 2001; Collado et al., 2000; Dulic et al., 2000).

Using several model systems of senescence, including long-term passage and acute expression of Ras or pRB, work in our lab has shown that cyclin-dependent kinase 5 (CDK5), a serine/threonine kinase that displays kinase activity predominantly in post-mitotic neurons, plays a central role in the morphology change of senescent cells (Alexander et al., 2004; Yang and Hinds, 2003, 2006). Expression of pRB in pRB-deficient SAOS-2 cells activates CDK5 during the course of senescence. Induction of CDK5 activity leads to the phosphorylation and activation of the ERM family member,

Ezrin, as well as the repression of Rac GTPase activation, which are coincident with acquisition of the pRB-induced senescent phenotypes. However, little is known about how CDK5 is activated in senescent cells induced by pRB.

In this study, we show that p35, one of the known activators of CDK5 in neurons, is required for CDK5 activation and the cell morphology change in pRB-induced SAOS-2 senescence. An increase of p35 at the mRNA level was also detected upon pRB expression in SAOS-2 cells, as well as in senescing IMR90 human diploid fibroblasts after long-term passage. These results further support a role for the CDK5/p35 pathway in regulating cellular senescence, which may provide insight into the regulatory mechanism underlying the induction of the senescent phenotype and its impact on cell proliferation and tumorigenesis.

Results

Expression of p35 in SAOS-2 and U2OS cells.

By using several model systems of senescence, we previously showed that cyclin-dependent kinase 5 (CDK5) activation is upregulated in senescing cells (Alexander et al., 2004). CDK5 kinase activity is stimulated by either one of two non-cyclin regulatory proteins, p35 and p39. Although CDK5 expression is ubiquitous in mammalian tissues, the expression of its activators is largely restricted to post-mitotic neurons (Dhavan and Tsai, 2001). No other activators are known. Thus, we asked if p35 and p39 are also expressed in the osteosarcoma cell lines used as senescence models in our study. The expression of mRNA encoding p35 and p39 in SAOS-2 (pRB deficient) and U2OS (wtpRB) cells was detected by RT-PCR (Fig.2.1A). Immunoblotting for p35 was then performed with α-p35 (C19) polyclonal antibody. Due to a short half-life of less than 30 minutes (Patrick et al., 1998), p35 only became detectable when U2OS and SAOS-2 cells were treated with the proteasome inhibitor MG132 (20 μM) for 16hrs (Fig.2.1B). These experiments indicate that both p35 and p39 are candidates for CDK5 activation in senescing cells. Due to a lack of appropriate reagents for analyzing p39, the following experiments focused on p35.

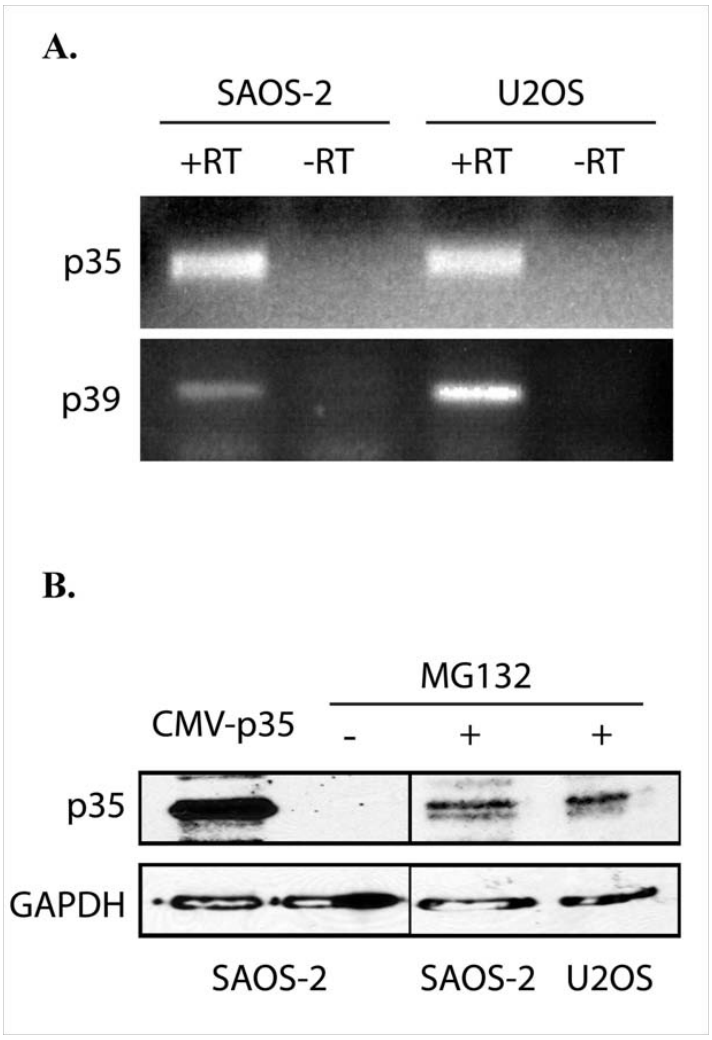


Fig.2.1. Expression of p35 and p39 in SAOS-2 and U2OS cells. *A.* RT-PCR for p35 and p39 from the total RNA of SAOS-2 and U2OS cells. Lane '-RT ', negative control using RNA without reverse transcription. *B.* SAOS-2 and U2OS cells were treated with MG132 (20 μM) for 16hr before harvest, whole cell extracts (30 μg of protein per lane) were analyzed by immunoblotting with anti-p35 antibody, SAOS-2 cells were also transfected with CMV-p35 or control empty vector, the ectopically expressed p35 was used as positive control.

Requirement of p35 for pRB-induced CDK5 activation in SAOS-2.

Both the protein levels and activity of CDK5 were shown to be upregulated in pRB-transfected SAOS-2 cells (Alexander et al., 2004). We therefore investigated the involvement of p35 in CDK5 activation. Using both retrovirus (data not shown) and lentivirus-based p35 shRNA constructs (Fig.2.2A), the ectopically expressed p35 was shown to be efficiently knocked-down in SAOS-2 cells. SAOS-2 cells were then cotransfected with a pRB expression vector and a p35 shRNA construct or control empty vectors. Cells were harvested for cell lysates 5 days after pRB reintroduction.

Performance of a CDK5 immunoprecipitation and a subsequent *in vitro* kinase assay using histone H1 as a substrate showed that the activity of CDK5 decreased upon knocking down p35, despite the continued presence of pRB (Fig.2.2B). The data suggest that p35 is an activator of CDK5 in pRB-induced senescence in SAOS-2 cells.

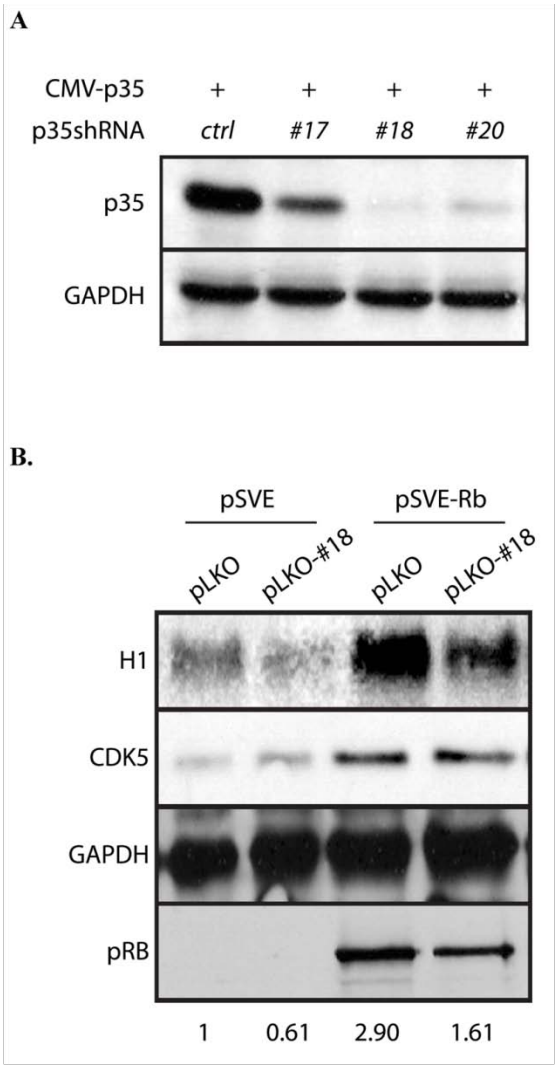
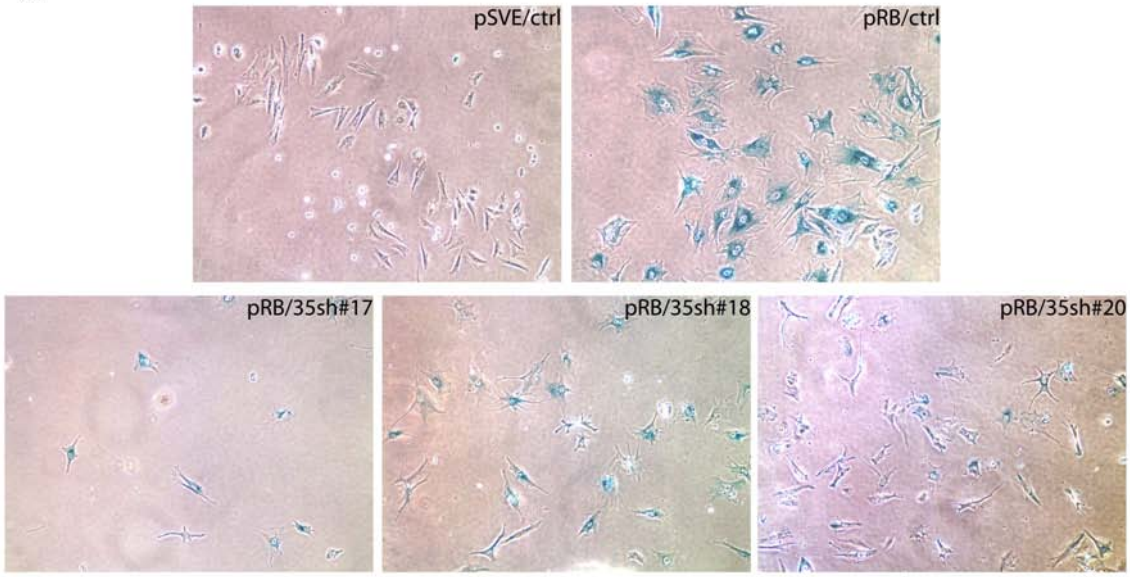


Fig.2.2. Knockdown of p35 decreases CDK5 activity in SAOS-2 Cells. *A.* The efficacy of lentivirus-based shRNAs for p35 was tested by cotransfecting pCMV-p35 and pLKO-p35shRNA-#17, #18, #20 or control empty vector into SAOS-2 cells. Cell lysates were collected 2 days after transfection and were immunoblotted for p35. *B.* pSVE or pSVE-Rb was cotransfected with pLKO-shp35-#18 or pLKO vector into SAOS-2 cells. Cells were selected with puromycin (0.5 μg/ml) 2 days after transfection, cell lysates were collected on day 5 and immunoprecipitated with anti-Cdk5 antibody to perform an *in vitro* kinase assay using histone H1 as the substrate. Cell lysates were also analyzed for CDK5 and pRB expression by immunoblotting. Numbers at bottom of panels show ratio of kinase activity in each lysate to control lysate.

Impact of p35 on senescent morphology change in SAOS-2 and U2OS cells.

Ectopic expression of pRB in the SAOS-2 osteosarcoma cell line produces a response that displays many characteristics of senescence in primary diploid cells, including the distinct morphological change termed 'flat cell' formation, typified by an increased cell area and a flattened appearance. This phenotype appears identical to that observed during classical senescence, and is taken as an indicator of the senescent state (Alexander and Hinds, 2001; Hinds et al., 1992; Xu et al., 1997). Because we previously found that CDK5 was required for this aspect of senescent cell morphology (Alexander et al., 2004), we next investigated the role of p35 in the senescence phenotype. SAOS-2 cells cotransfected with a pRB expression vector and p35 shRNA constructs were examined for senescence by SA-β-gal assay 10 days post-transfection. Cells transfected with empty vectors were used as a control (Fig.2.3A). Knockdown of p35 antagonized the formation of pRB-induced senescent flat cells, and cells in the knockdown groups were generally smaller. In order to better analyze the shape change, cell area was measured in each group and subjected to statistical analysis (Fig.2.3B). The result showed that by knocking down p35, the average area of pRB-induced senescent cells was reduced, and this difference was statistically significant, supporting a role for p35 as a regulator of the senescent phenotype. To further confirm the specificity of the p35 knockdowns, shape changes were analyzed in pRB-transfected cells in which CDK5, p35, or both were subjected to knockdown (Fig.2.3C). By measuring cell area, cells in each knockdown group were shown to have significantly reduced flat cell formation compared to pRB control cells, but there were no significant differences among all knockdown groups. In the absence of CDK5, changes in p35 levels did not confer further reduction in cell area, suggesting that the effects of p35 on senescence are mediated through CDK5.

A.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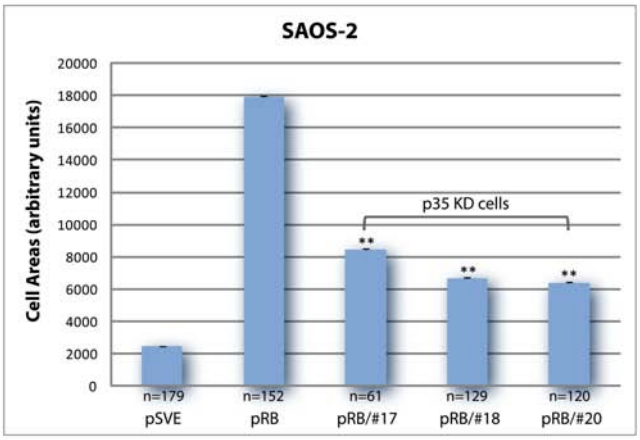
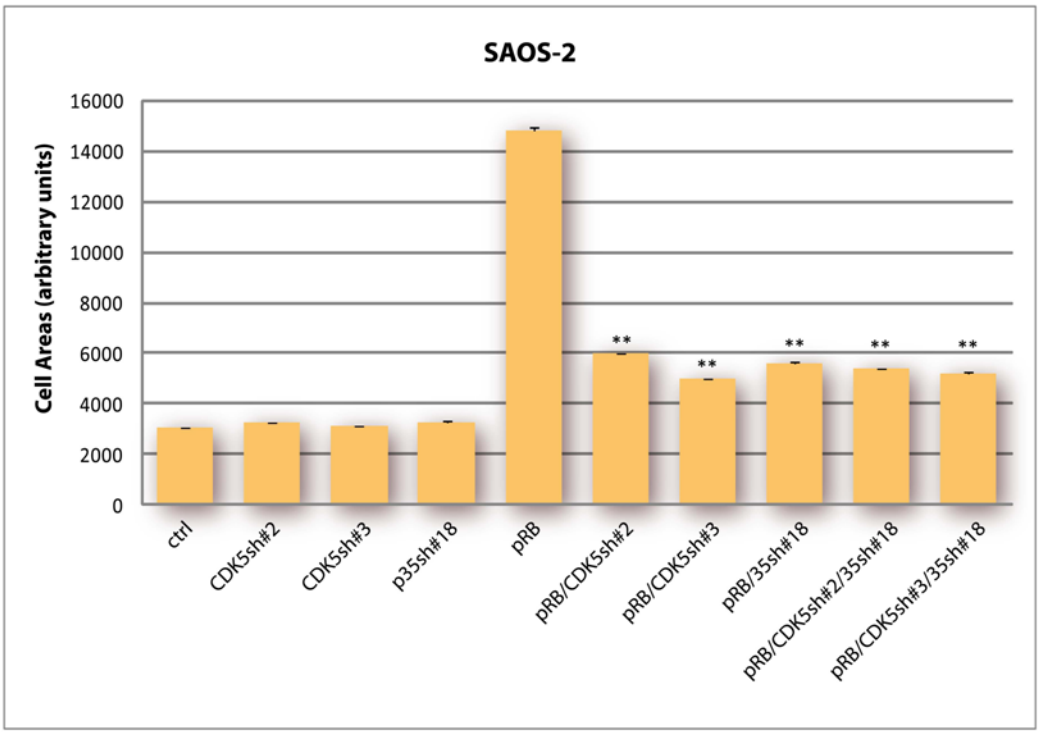
pRB/ctrl
pRB/35sh#17
pRB/35sh#18
pRB/35sh#20

B.


SAOS-2
Cell Areas (arbitrary units)
20000
18000
16000
14000
12000
10000
8000
6000
4000
2000
0
p35 KD cells
**
**
**
n=179
pSVE
n=152
pRB
n=61
pRB/#17
n=129
pRB/#18
n=120
pRB/#20

C.


SAOS-2
Cell Areas (arbitrary units)
16000
14000
12000
10000
8000
6000
4000
2000
0
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ctrl
CDK5sh#2
CDK5sh#3
p35sh#18
pRB
pRB/CDK5sh#2
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pRB/CDK5sh#3/35sh#18

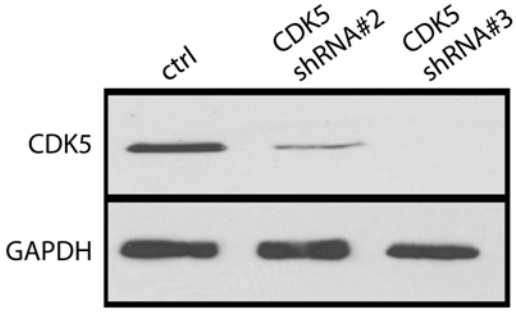

ctrl
CDK5 shRNA#2
CDK5 shRNA#3
CDK5
GAPDH

Fig.2.3. Knockdown of p35 reduces pRB-induced flat cell formation in SAOS-2 cells. *A.* SAOS-2 cells were cotransfected with pSVE-Rb and pLKO-p35shRNA-#17, #18, #20 or control empty vector. Cells transfected with empty vectors were used as control. 16hrs after transfection, cells were selected with puromycin at a final concentration of 0.5ug/ml. 10 days post-transfection, cells were assayed for SA-ß-gal expression and the morphology change in these cells is shown. All images were obtained by using phase contrast microscopy at a magnification of x10. *B.* Cell area in pRB-induced senescent SAOS-2 cells was measured using ImageJ. Means and standard errors of data collected in each group are presented in the column chart. To compare the difference in size between pRB-transfectant cells and p35-knockdown cells, P-values were determined using both unpaired two-tailed Student's t-test and one-way ANOVA. A P-value < 0.001 is indicated by **. The experiment was repeated three times. *C.* pSVE or pSVE-Rb was transfected into SAOS-2 cells with MKO-CDK5 shRNA#2, #3, and/or pLKO-p35shRNA-#18. Cell area in each group was measured using ImageJ. Means and standard errors of three experiments are presented in the column chart. A P-value < 0.001 versus pRB control is indicated by **. Bottom panels, the efficacy of shRNAs for CDK5 was tested by transfecting MKO-CDK5shRNA-#2, #3 or control empty vector into SAOS-2 cells. Cell lysates were collected 2 days after transfection and were immunoblotted for CDK5.

To demonstrate that similar results can be observed in other cells that lack a functional pRB pathway, we used the human osteosarcoma cell line U2OS, which does not express p16INK4a. By reintroducing p16INK4a into these cells, a premature senescence response can also be induced, as in the case of adding pRB to SAOS-2 (Dai and Enders, 2000). Firstly, U2OS cells cotransfected with a p16INK4a expression vector and p35 shRNA constructs were examined for senescence by SA-β-gal assay 10 days post-transfection, using cells transfected with empty vectors as a control (Fig.2.4A). Secondly, shape changes were quantified by measuring cell area in each group (Fig.2.4B). The results showed that knockdown of p35 in U2OS significantly suppressed the senescent morphology change induced by p16INK4a overexpression.

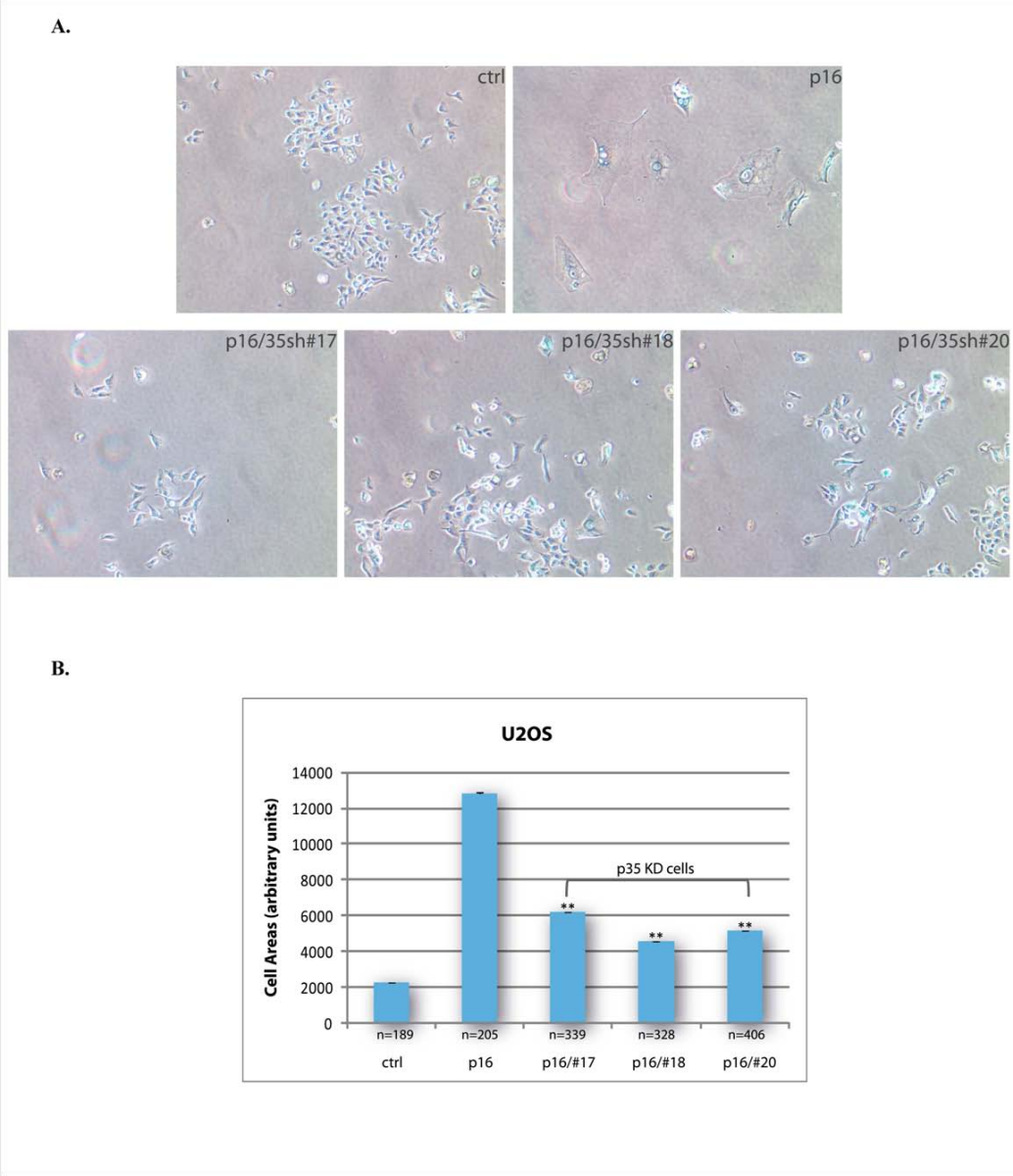


Fig.2.4. Knockdown of p35 reduces p16INK4a-induced flat cell formation in U2OS cells. A. U2OS cells were cotransfected with CMV-p16INK4a and pLKO-p35shRNA-#17, #18, #20 or control empty vector. Cells transfected with empty vectors were used as control. 16hrs after transfection, cells were selected with puromycin at a final concentration of 2ug/ml. 10 days post-transfection, cells were assayed for SA-ß-gal expression and the morphology change in these cells is shown. All images were obtained by using phase contrast microscopy at a magnification of x10. B. Cell area in each group of cells was measured using ImageJ. Means and standard errors of three experiments are presented in the column chart. A P-value < 0.001 versus p16INK4a control is indicated by **.

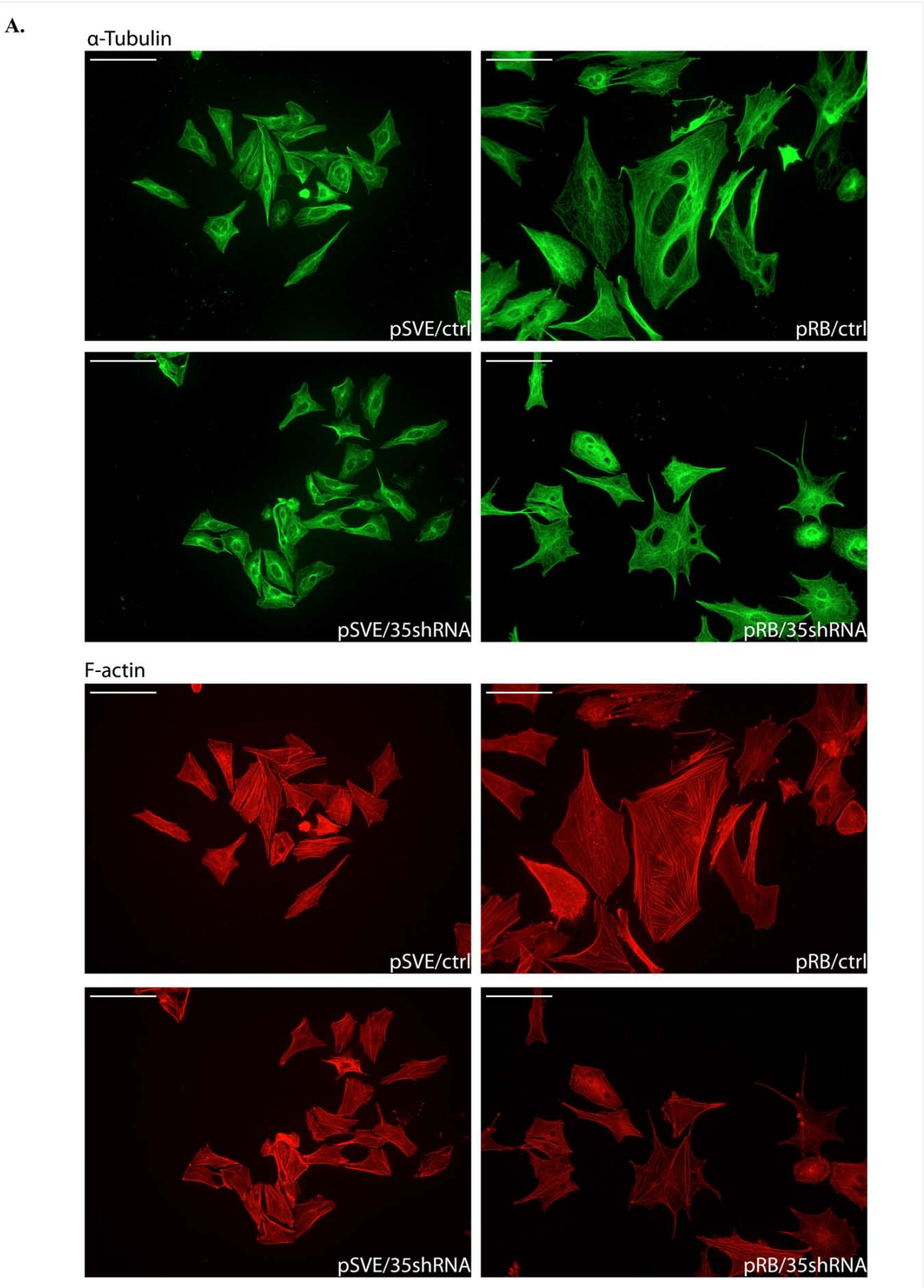
p35 regulates cytoskeletal organization in senescent SAOS-2 and U2OS cells.

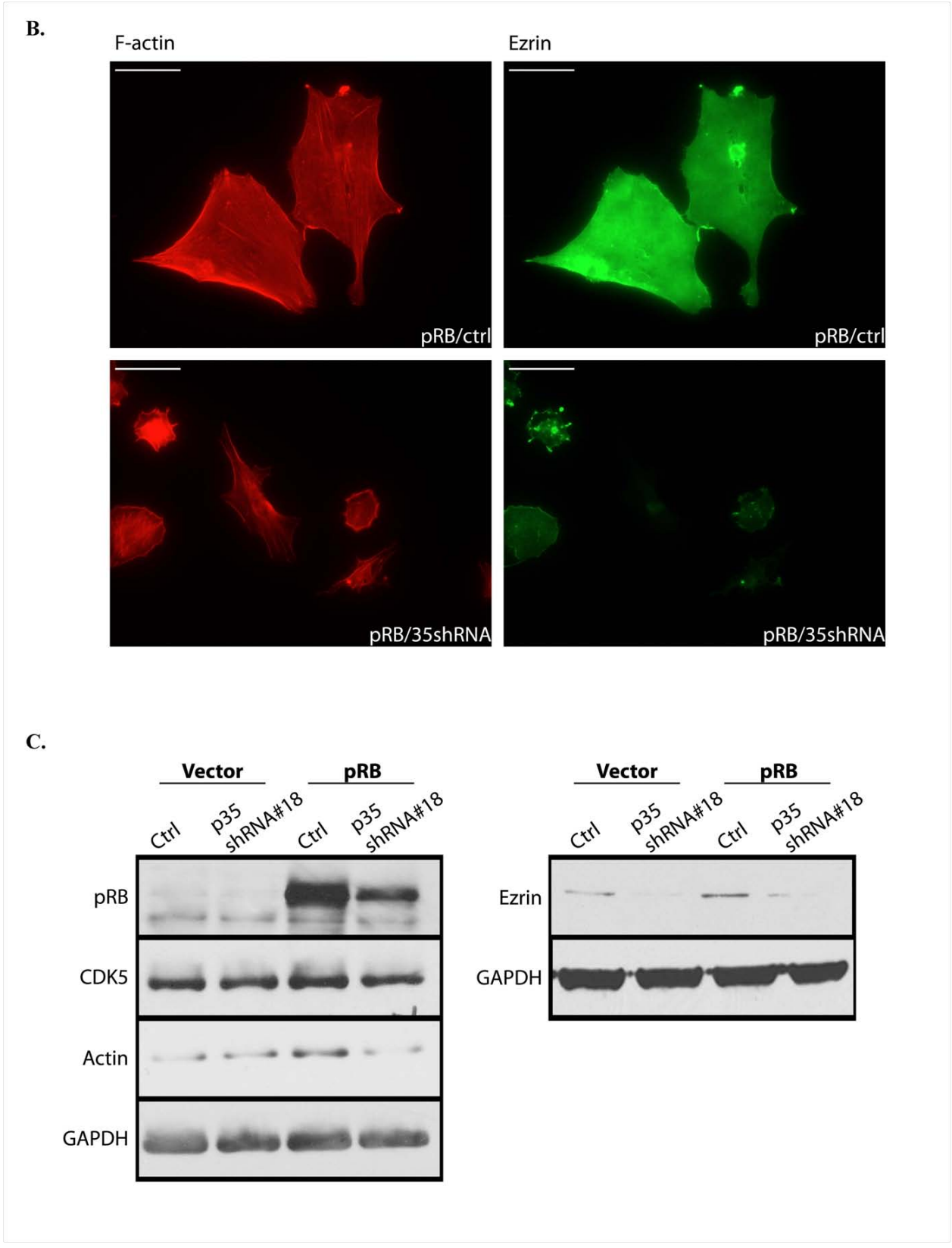
CDK5-dependent flattening of senescent cells suggests that extensive changes must occur to the cellular cytoskeletons as cells undergo senescence. CDK5 has known roles in regulating cell shape through actin reorganization. With this in mind, we investigated the effect of p35 on the changes in cytoskeleton structure as cells underwent senescence. SAOS-2 cells cotransfected with a pRB expression vector and a p35 shRNA construct or control vectors were fixed with 4 % formaldehyde 10 days post-transfection and immunofluorescence staining for cytoskeleton organization in the samples was performed by using rhodamine phalloidin or α-tubulin antibody. No significant change in microtubule arrangement was observed (Fig.2.5A upper panels), whereas knockdown of p35 interfered with actin polymerization. In the knock-down group, cells exhibited less intense F-actin fiber staining, along with a reduction in size (Fig.2.5A lower panels). Further, immunoblotting showed that the level of actin was reduced by p35 knockdown (Fig.2.5C).

Previous studies in our lab have shown that the expression of the ERM family member, Ezrin, an actin binding protein involved in membrane-cytoskeletal signaling, is increased upon pRB-induced senescence. The activation of Ezrin appears to be the consequence of direct phosphorylation of T235 of the protein by CDK5, which is activated in response to pRB expression (Yang and Hinds, 2003). We thus also checked the status of Ezrin in SAOS-2 cells transfected with pRB and p35 shRNA constructs or control vectors. p35 knock-down cells showed reduced immunofluorescence staining for both F-actin and Ezrin (Fig.2.5B), compared to control senescent cells induced by pRB, that was accompanied by an overall decrease in Ezrin levels (Fig.2.5C). Furthermore, similar results were observed in U2OS cells (Fig.2.5D). p35 knock-down cells showed less intense staining for both F-actin and Ezrin when compared to p16INK4a-induced

senescent cells. The localization of Ezrin in p35 knock-down cells also remained mostly cytosolic as in control cells, whereas in senescent cells Ezrin was relocated to the membrane periphery.

Taken together, these data suggest that p35 is required for the acquisition of the senescent morphology in pRB-induced senescence, which is consistent with our previous finding that CDK5 activity is required in such changes.


A.
α-Tubulin
pSVE/ctrl
pRB/ctrl
pSVE/35shRNA
pRB/35shRNA
F-actin
pSVE/ctrl
pRB/ctrl
pSVE/35shRNA
pRB/35shRNA


B.
F-actin
Ezrin
pRB/ctrl
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pRB/35shRNA
pRB/35shRNA
C.
Vector
pRB
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p35
shRNA#18
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p35
shRNA#18
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Ezrin
GAPDH

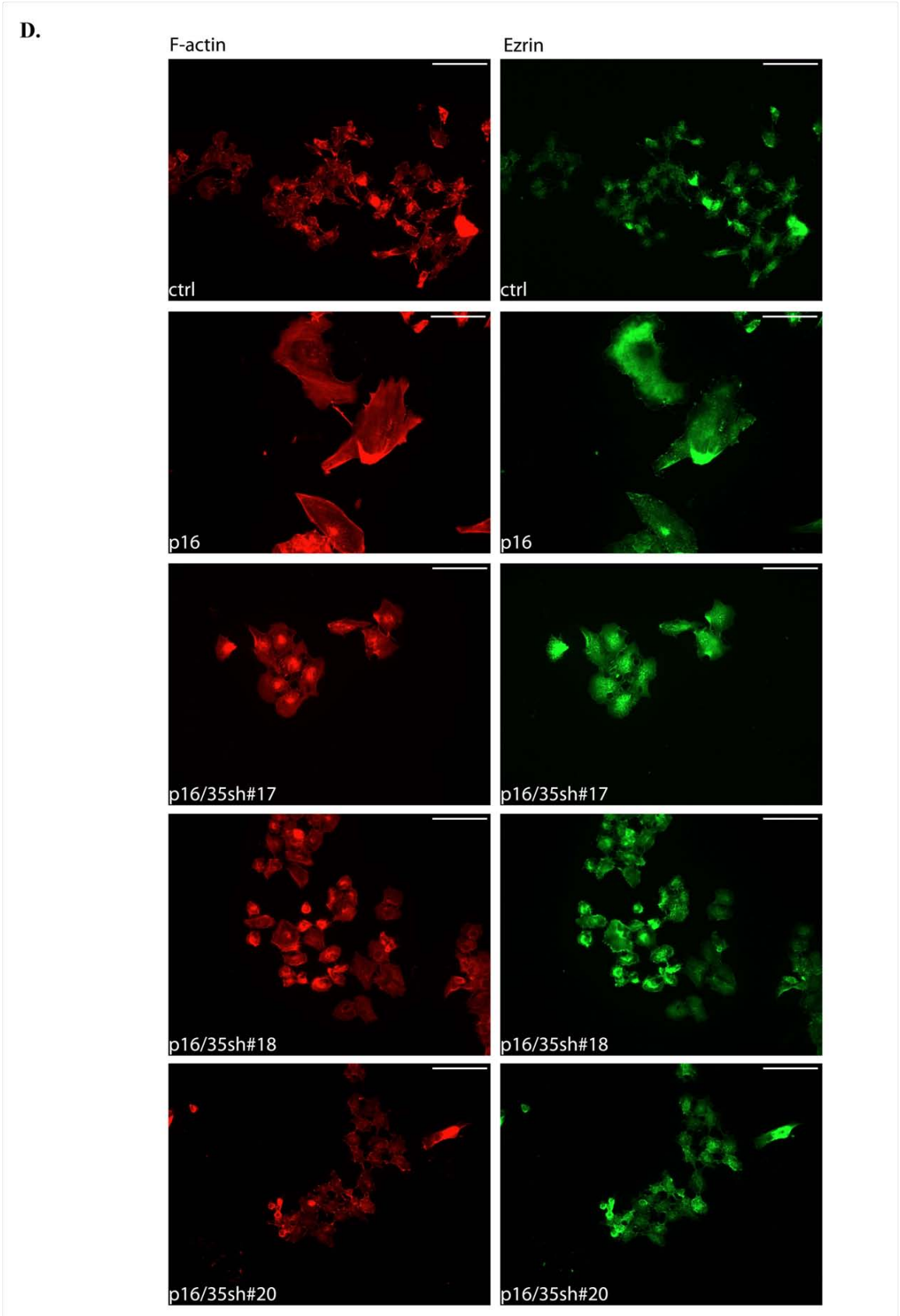

D.
F-actin
Ezrin
ctrl
ctrl
p16
p16
p16/35sh#17
p16/35sh#17
p16/35sh#18
p16/35sh#18
p16/35sh#20
p16/35sh#20

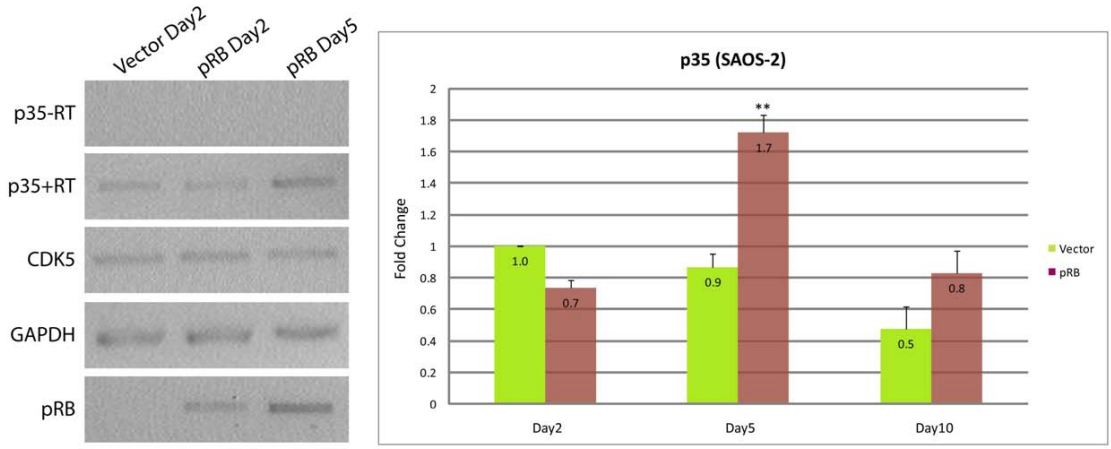
Fig.2.5. Knockdown of p35 affects cytoskeletal organization in senescent SAOS-2 and U2OS cells. *A.* SAOS-2 cells were seeded on coverslips and cotransfected with pSVE or pSVE-Rb and pLKO-p35shRNA-#18 or control pLKO vector. 16hrs after transfection, cells were selected with puromycin at a final concentration of 0.5ug/ml. Samples were collected 10 days posttransfection and fixed with 4% formaldehyde. Immunofluorescence staining was performed using anti-α-tubulin antibody (DM1A) (upper panels) and rhodamine-phalloidin (lower panels), to show the structure of microtubules and F-actin filaments, respectively. (Scale bars: 100 μm). *B.* Immunofluorescence staining for F-actin (red) and Ezrin (green) in SAOS-2 cells cotransfected with pSVE-Rb and pLKO-p35shRNA-#18 or control vectors following 8 days of selection. (Scale bars: 100 μm). *C.* Immunoblotting for actin and Ezrin in SAOS-2 cells transfected with pSVE-Rb and pLKO-p35shRNA-#18 or control vectors following 5 days of selection. *D.* Immunofluorescence staining for F-actin (red) and Ezrin (green) in U2OS cells cotransfected with CMV-p16INK4a and pLKO-p35shRNA-#17, #18, #20 or control vectors following 8 days of puromycin selection (2ug/ml). (Scale bars: 100 μm).

Expression of p35 is increased during cellular senescence.

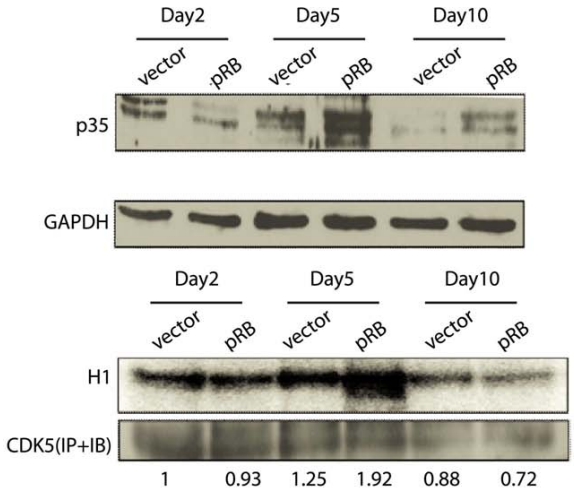
To understand if the regulation of p35 mRNA or protein levels is a significant determinant of CDK5 activity during pRB-induced senescence, SAOS-2 cells infected with pRB lentivirus or control GFP virus were collected at 2, 5 and 10 days post-infection. Both semi-quantitative RT-PCR and quantitative real-time PCR were performed to examine p35 mRNA levels in these samples. Compared to control, pRB infectants showed an approximate 2-fold increase in p35 levels at day 5 and a slight increase at day 10 (Fig.2.6A). Concomitantly, both endogenous p35 protein levels and CDK5 activity increased 5 days after pRB-infection (Fig.2.6B).

We have also shown that CDK5 is activated in different model systems of senescence, including long-term passage and acute expression of Ras or pRB (Alexander et al., 2004). In order to see if the increase of p35 expression is a general phenomenon in cells undergoing senescence, we induced senescence in IMR90 HDFs by long-term passage. The p35 mRNA levels were examined by real-time PCR in early and mid passages of IMR90 human diploid fibroblasts (Fig.2.6C left panel). This showed that expression of p35 mRNA was also upregulated in senescing IMR90 HDFs. Concomitantly, SA-β-gal staining showed more senescent cells at later passage (Fig.2.6C right panel). These findings strongly suggest that p35 regulates CDK5 activity during the process of senescence.

A.


Vector Day2
pRB Day2
pRB Day5
p35-RT
p35+RT
CDK5
GAPDH
pRB
p35 (SAOS-2)
Fold Change
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
**
1.0
0.7
0.9
1.7
0.5
0.8
Day2
Day5
Day10
Vector
pRB

B.


Day2
Day5
Day10
vector
pRB
p35
GAPDH
H1
CDK5(IP+IB)
1
0.93
1.25
1.92
0.88
0.72

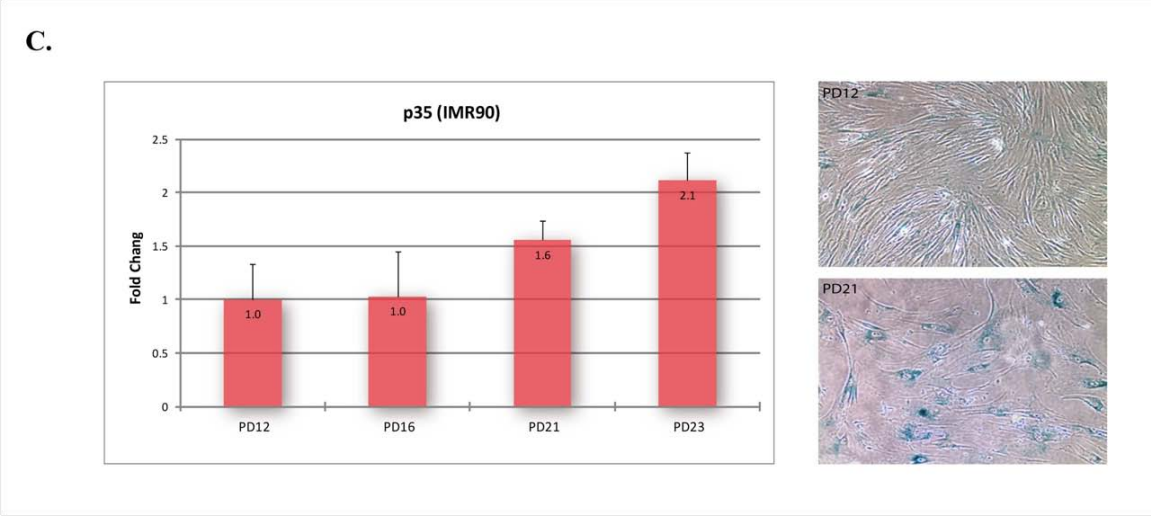


Fig.2.6. p35 is increased in senescing SAOS-2 and IMR90 cells. *A.* SAOS-2 cells were infected with pRB or control GFP lentiviruses. 16hrs after infection, cells were selected with puromycin at a final concentration of 0.5ug/ml. p35 mRNA levels at 2, 5 and 10 days posttransfection were examined by semi-quantitative RT-PCR (left), '-RT', negative control using RNA samples not subjected to reverse transcription, or quantitative real-time PCR in triplicate (right), error bars show standard deviation. **, P <0.001 versus day 2 empty vector control. *B.* Cell lysates were prepared from cells infected with pRB or control GFP lentiviruses, followed by MG132 (20 μM) treatment for 16hr before harvest. Immunoblotting was performed using anti-p35 antibody (upper panels), and immunoprecipitation with anti-Cdk5 antibody was performed followed by an *in vitro* kinase assay using histone H1 as the substrate (lower panels). Numbers at bottom of panels show ratio of kinase activity in each lysate to control lysate of day2. *C.* Real-time PCR (in triplicate) for p35 (left) was performed with total mRNA prepared from early and middle passage IMR90 cells, error bars show standard deviation. SA-ß-gal assay (right) was performed on IMR90 cells at early and middle passage.

p35 regulates senescence secretome in SAOS-2 cells.

Our previous work and that shown above clearly implicate p35/CDK5 in the senescent cell shape change. However, the physiological significance of this shape change and its impact on the microenvironment of the senescent cell is unclear. To determine if p35/CDK5 could mediate senescent cell processes that may directly affect tissue homeostasis and/or tumor formation, we chose to analyze the impact of p35 knockdown on the expression of genes encoding secreted factors. This analysis was based on increasing evidence that senescent cells show altered expression of many secreted gene products that regulate proliferation (i.e. increased production of PAI-1), impact the immune response (i.e. increased amounts of IL-6, IL-8), and influence remodeling of the stroma or extracellular matrix (ECM), (i.e. increased production of Matrix Metalloproteinase 1(MMP1) and MMP3) (Adams, 2009). To understand if the regulation of p35 mRNA affects the senescence secretome in pRB-induced senescent SAOS-2 cells, cells cotransfected with a pRB expression vector and a p35 shRNA construct or control vectors were subjected to quantitative real-time PCR for examining changes in a variety of secreted factors in these samples (Fig.2.7). Compared to control, pRB transfectants showed a significant increase in all the factors analyzed except MMP3, whereas upon p35 knockdown, the production of these factors was suppressed. This suggests that p35 not only participates in the regulation of cytoskeletal structures but affects other aspects of senescence as well.

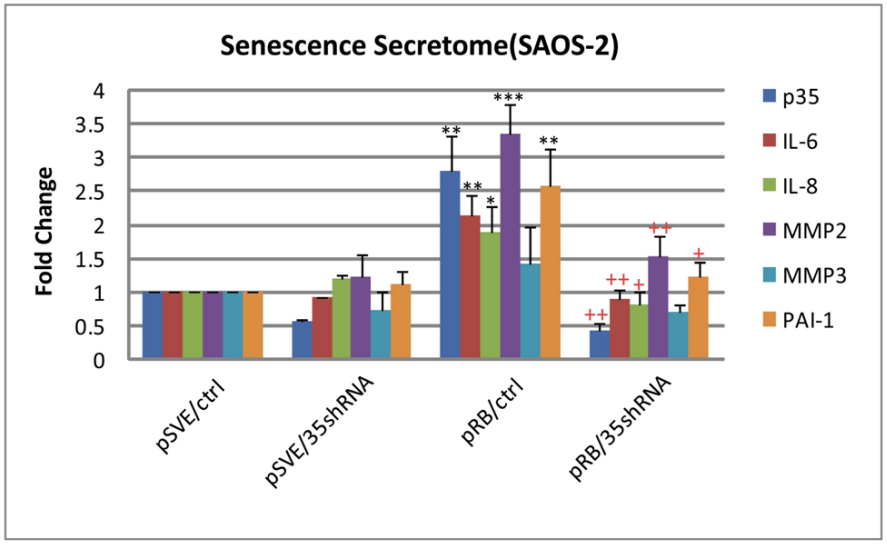


Fig.2.7. Knockdown of p35 affects senescence secretome in SAOS-2 cells. A. SAOS-2 cells were cotransfected with pSVE or pSVE-Rb and pLKO-p35shRNA-#18 or control pLKO vector. 16hrs after transfection, cells were selected with puromycin at a final concentration of 0.5ug/ml. Samples were collected 5 days posttransfection and mRNA levels for p35, IL-6, IL-8, MMP-2, MMP3 and PAI-1 were examined by quantitative real-time PCR (in triplicate). Error bars show standard deviation. *, $P < 0.05$ versus control; **, $P < 0.01$ versus control; ***, $P < 0.001$ versus control; +, $P < 0.05$ versus pRB; ++, $P < 0.01$ versus pRB.

Discussion

We have previously reported the activation of CDK5 in human primary and tumor cells induced to senesce by a variety of stimuli, and the activity of CDK5 is necessary for proper acquisition of the cytoskeletal changes accompanying senescence. Discovery of this role for CDK5 was unexpected, since its activity has primarily been associated with post-mitotic neurons. However, given that CDK5 is ubiquitously expressed in mammalian tissues, an increasing body of evidence has established CDK5 kinase activity and functions in non-neuronal cells. In this report we show the presence of the CDK5 activators, p35 and p39, in our model systems of senescence. Upregulation of the expression of p35 is concomitant with CDK5 activation in senescing SAOS2 and IMR90 cells. Knockdown of p35 by shRNA markedly suppresses the morphologic changes of senescent SAOS-2 cells induced by pRB, which coincides with a decrease in CDK5 activity. The polymerization of actin filaments is inhibited in the p35 knockdown cells, and the levels of actin and the F-actin-associated Ezrin are reduced as well. These findings underscore a role for CDK5/p35 activity in mediating the cytoskeletal reorganization in the non-neuronal senescent cells. Furthermore, the use of model systems in which senescence is induced by pRB reintroduction in p16INK4a/pRB-deficient tumor cells strongly implicates the pRB pathway in CDK5/p35 upregulation at least in part through transcriptional upregulation of p35. Although the pRB pathway is essential for the transcriptional repression of loci in senescent cells (Narita et al., 2003), its role in the induction of gene expression in senescence is poorly understood. Because pRB/E2F complexes are usually repressive, they most likely do not directly regulate the genes that are highly expressed by senescent cells, although they could indirectly control such genes, for example, by silencing a repressor. At present, we have been unable to test for a direct role for pRB in regulating p35 mRNA production, as reporter

constructs containing the p35 promoter do not respond to pRB (data not shown). It is possible that pRB effects on the p35 promoter, whether direct or indirect, require an appropriate chromatin context as observed for differentiation-specific promoters(Thomas et al., 2001).

Senescent cells show striking changes in gene expression. Interestingly, many changes in gene expression appear not to be directly related to growth arrest. Despite the universality of morphological changes observed in a wide variety of senescent cells, little is known about the potential contribution of this phenotype to the establishment or maintenance of the irreversible growth arrest that accompanies senescence. Significant additional studies of the role of cytoskeletal rearrangement in the biochemical and proliferative aspects typical of senescence are needed to fully appreciate the role of CDK5/p35 in this process. The role of CDK5/p35 activity in regulating the differentiation of monocytes might provide some clues. In promyelocytic HL60 cells, treatment with 1,25-dihydroxyvitamin D3 (1,25D3) results in the upregulation of the Egr1 gene, which in turn activates p35 transcription and expression, and subsequently enhances CDK5 activity. p35-associated CDK5 phosphorylates MEK1 on T286, preventing MAPK/ERK phosphorylation and cell proliferation. However, MEK1 phosphorylation at T286 requires prior S218 and S222 phosphorylation and activation by Raf1 induced by growth factors or cytokines. These events are suggested to result in the upregulation of p27KIP1 and eventually, monocytic differentiation (Chen et al., 2004; Rosales and Lee, 2006). Given the established role of pRB in osteoblast differentiation (Thomas et al., 2001), it is possible that complex mechanisms such as these are stimulated by pRB in senescing mesenchymal cells that are unable to properly differentiate.

There is now substantial evidence that induction of senescence constitutes an important block to tumor progression. It has also become clear that senescent cells have

characteristic alterations in secreted growth factors, inflammatory cytokines, extracellular-matrix components and matrix-degrading enzymes, which could influence the growth of adjacent neighboring tumor cells by altering the tissue microenvironment (Adams, 2009; Campisi and d'Adda di Fagagna, 2007). We find that p35/CDK5 can influence this senescence secretome, implicating CDK5 in the physiological response to this aspect of senescence. Further, recent studies suggest that, as with CDK5 in neurons, the CDK5 activity in non-neuronal cells may influence phenotypic changes mostly through its direct or indirect effect on the organization of cytoskeletal structures. CDK5 regulates cellular processes such as cell-cell and cell-matrix adhesion, cell migration and wound healing in a variety of tissues (Rosales and Lee, 2006). Thus the induction of senescent shape change may also impact the neighboring tumor cells and their microenvironment.

Tumor cells are exposed to many sources of stress, especially those derived from the aberrant proliferative signals of oncogenes. As one of the cellular responses to these stresses, senescence has emerged as a compelling target for future cancer therapeutics and may determine the response of tumor cells to chemotherapeutic drugs. Because senescence effector mechanisms are still largely unknown, understanding the molecular mechanisms through which CDK5 affects cellular senescence may give clues to potential therapeutic approaches for cancer and age-related diseases. Finally, the intriguing relationship of this aspect of senescence to neuronal and non-neuronal differentiation (Chen et al., 2004; Lazaro et al., 1997; Sarker and Lee, 2004) may help to unravel the role of senescence effectors in the physiology of tissues that accumulate a senescent cell burden following acute or chronic stress.

Materials and Methods

Cell Culture and Recombinant Vector

The human osteosarcoma cell line SAOS-2 subclone 2.4 (39) was maintained in Dulbecco's modified Eagle's medium (DMEM) (Gibco) supplemented with 15% fetal bovine serum (FBS) and 1% penicillin-streptomycin. Human U2OS osteosarcoma cells and IMR90 HDFs were maintained in DMEM supplemented with 10% FBS. Cells were cultured in a 5% CO2 incubator at 37 °C. The pSVE and pSVE-Rb expression plasmids have been previously described (39, 40). The lentivirus expression plasmids pZsG and pZsG-Rb were constructed in our laboratory. The pLKO-p35shRNA-17, -18, and -20 constructs were purchased from Open Biosystems (Clone IDs: TRCN0000006217, TRCN0000006218, TRCN0000006220). SAOS-2 cells were transfected at 80% confluency with the indicated plasmids by using Fugene6 (Roche). SAOS-2 transfectants were selected with puromycin (0.5μg/ml) 24-h post-transfection or infection and maintained under selection for the duration of the experiment.

Immunoblotting

Cells were lysed in 100–200 μl of lysis buffer (50mM HEPES pH 8.0, 150mM NaCl, 1mM EDTA, 0.1% Nonidet P-40) plus protease, and phosphatase inhibitors (1 mg of aprotinin/ml, 1μg of leupeptin/ml, 100μg of phenylmethylsulfonyl fluoride/ml, 4 mM sodium orthovanadate, 2 mM sodium PPi) per 10-cm plate. Protein concentrations of the cell lysates were determined by the Bradford assay (Bio-Rad). For immunoblotting, 30μg of protein was separated by SDS-PAGE and transferred to nitrocellulose membrane in a trans-blotting buffer (25 mM Tris, 192 mM glycine, 20% (v/v) methanol).Immunoblot analysis was performed as described previously (36, 39). Antibodies used for immunoblotting include: anti-Cdk5 monoclonal J-3, polyclonal C-8, and anti-p35 polyclonal C-19 antibodies (Santa Cruz Biotechnology), anti-pRB monoclonal 245

(Pharmingen), anti-Ezrin monoclonal 3C12 (NeoMarkers), anti-GAPDH monoclonal MAB374 (Chemicon), anti-actin monoclonal C-2 (Santa Cruz Biotechnology), and anti-α-tubulin monoclonal DM1A (Calbiochem). Horseradish peroxidase-conjugated donkey anti-mouse or anti-rabbit secondary antibodies (Jackson Immunosciences) were used, and signal was detected by ECL (PerkinElmer).

Immunoprecipitation and in Vitro Kinase Assays

An *in vitro* CDK5-associated histone H1 kinase activity (CDK5 kinase activity) assay was carried out as described by Zheng *et al.*, with slight modifications (37, 41). Immunoprecipitation for CDK5 was performed by incubating 100 µg of cell lysate with 1 µg of anti-Cdk5 C-8 antibody (Santa Cruz Biotechnology) and 30 µl of protein A-Sepharose CL-4B beads (Amersham Biosciences) overnight at 4 °C. The beads were washed with lysis buffer, and the immunocomplexes were used to determine the kinase activity at 37 °C for 30 min, using 1 µg of histone H1 as a substrate.

Immunofluorescence

SAOS-2 cells seeded on coverslips were transfected with the indicated plasmids using Fugene6 (Roche). Cells fixed with 4% formaldehyde were incubated with PBS containing 0.1% Triton X-100 and 2% BSA for 1hr at room temperature, followed by primary antibody or rhodamine phalloidin (Cytoskeleton) incubation overnight at 4°C. Cells were then washed with PBS plus 0.1% Triton X-100 and incubated with fluorophore-conjugated secondary antibody. After staining, coverslips were mounted using Fluoromount G and visualized using a Nikon Eclipse 80i fluorescence microscope.

Quantitative Real-Time PCR

Total RNA was extracted from cells transfected with pRB using TRIzol (Invitrogen). 100 nanograms of DNase I treated RNA was used for first-strand cDNA

synthesis using the iScript cDNA synthesis kit (Bio-Rad) according to the manufacturer's instructions. Quantitative PCR was carried out by employing the QuantiTect SYBR green PCR kit (Qiagen) and using 1 µl of the cDNA per reaction. The primer sequences are as follows,

p35: 5'-AAGAACGCCAAGGACAAGAA-3' and 5'-TCATTGTTGAGGTGCGTGAT-3';

GAPDH: 5'-GAAGGTGAAGGTCGGAGTC-3' and 5'-GTGCGGCTGCTTCCATAA-3';

IL-6: 5'-AACCTGAACCTTCCAAAGATGG-3'and 5'-TCTGGCTTGTTCCTCACTACT-3';

IL-8: 5'-ACTGAGAGTGATTGAGAGTGGAC-3' and

5'-AACCCTCTGCACCCAGTTTTC-3';

MMP2: 5'-CCGTCGCCCATCATCAAGTT-3' and 5'- CTGTCTGGGGCAGTCCAAAG-3';

MMP3: 5'-ATGGACAAAGGATACAACAGGGA-3' and

5'-TGTGAGTGAGTGATAGAGTGGG-3';

PAI-1: 5'-GCTTGTCCAAGAGTGCATGGT-3' and 5'-AGGGCTGGTTCTCGATGGT-3'.

Relative quantification of gene expression was carried out by the comparative C(T) method (Livak and Schmittgen, 2001).

Senescence-associated β-galactosidase staining assay

SAOS-2 cells were cotransfected with the indicated plasmids. Transfectants were selected with puromycin (0.5µg/mL) 24-h posttransfection for the duration of the experiment. The staining for perinuclear senescence associated -β-galactosidase (SA-β-gal) activity was performed as described (Dimri et al., 1995). Briefly, cells were washed in PBS and fixed in 2% formaldehyde/0.2% glutaraldehyde. Cells were then washed and

incubated at 37°C overnight with fresh senescence associated -β-galactosidase staining solution (1 mg of 5-bromo-4-chloro-3-indolyl-ß-D-galactoside per ml, 40 mM citric acid/sodium phosphate (pH 6), 150 mM NaCl, 2 mM $MgCl_2$, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide).

Chapter III

Role for CDK5 in Cell Proliferation and Tumorigenesis

Abstract

Cyclin-dependent kinase 5 (CDK5), a known regulator of postmitotic neuronal activities, has been shown to play an important role in morphological change and secreted protein expression during cellular senescence in non-neuronal cells. Senescence has been established to be an important fail-safe mechanism to impede tumorigenesis *in vivo*, but the role of these CDK5-mediated senescence phenotypes in tumor suppression remains unclear. To address this, we studied the effect of CDK5 loss on transformation and tumorigenesis using murine embryo fibroblasts (MEFs). Acute loss of CDK5 results in bypass of senescence in MEFs, but does not affect the proliferation of pre-senescent cells. Loss of CDK5 cooperates with Ras and DNp53 in the transformation of MEFs, resulting in increased colony numbers in *in vitro* soft agar assays. However, CDK5 loss does not accelerate tumor formation in a xenograft model using transformed MEFs. Migration assays performed with these transformed cells show an increased motility and invasiveness in the *Cdk5*-null MEFs. These results suggest that although CDK5 is not directly involved in the regulation of cell proliferation and tumor growth once the senescence barrier is overcome, it may control cell motility and the metastatic potential of cancerous cells.

Introduction

Cellular senescence can be induced upon the passage of primary cells in culture or prematurely upon oncogenic stimulation or reintroduction of tumor suppressors (e.g. p16INK4A, pRB or p53) into tumor cell lines that lack them (Dimri et al., 2000; Ferbeyre et al., 2002; Hayflick, 1965; Kelly-Spratt et al., 2004; Lin et al., 1998; Lin and Lowe, 2001; Serrano et al., 1997; Stein et al., 1990; Stein et al., 1999). The mechanisms by which senescence is triggered suggest that the process is tumor suppressive by preventing the proliferation of cells at risk of neoplastic transformation (Braig and Schmitt, 2006; Campisi, 2001; Mathon and Lloyd, 2001). Recent studies have shown that senescence indeed functions as a potent barrier to tumorigenesis *in vivo* (Bartkova et al., 2006; Braig et al., 2005; Chen et al., 2005; Collado et al., 2005; Lazzerini Denchi et al., 2005; Michaloglou et al., 2005). Besides an irreversible G1 growth arrest, senescent cells undergo characteristic changes in cell morphology, the production of a plethora of secreted proteins, and typically express a senescent-associated β-galactosidase (SA-β-gal) activity (Dimri et al., 1995; Shelton et al., 1999). Using several model systems of senescence including pRB-induced senescence in the osteosarcoma line SAOS-2, work in our lab has shown that cyclin-dependent kinase 5 (CDK5), a serine/threonine kinase that has previously been found to display kinase activity predominantly in post-mitotic neurons, plays a key role in the morphology change of senescent cells (Alexander et al., 2004; Yang and Hinds, 2003, 2006). Moreover, in chapter two, we have shown that p35, one of the known activators of CDK5, is required for CDK5 activation and the cell morphology change in pRB-induced SAOS-2 senescence. Interestingly, the induction of the senescent secretome also depends on p35, and hence CDK5 activity. These results further support a role for the CDK5/p35 in regulating cellular senescence phenotypes.

However, the physiological relevance of these *in vitro* manifestations has yet to be elucidated.

The best-known physiological roles for CDK5 are in regulating postmitotic neuronal activities such as neurite outgrowth, axon guidance, membrane transport, synaptic function, dopamine signaling, and drug addiction (Dhavan and Tsai, 2001). CDK5 also has connections to neuronal apoptosis and degeneration (Tang et al., 1996). Recent studies indicate that in addition to the role of CDK5/p35 in the central nervous system, it has some extra-neural functions particularly in cancer cells. Activation of CDK5/p35 has been reported to be involved in cell death in several tumor cell lines, including astrocytoma (Gao et al., 2001), leukemia (Sandal et al., 2002), (Lin et al., 2004), breast (Goodyear and Sharma, 2007; Upadhyay et al., 2008) and medullary thyroid carcinoma cells (Lin et al., 2007). Yet little is known about its role in the senescence response *in vivo* and the functional correlation with cancer. Moreover, CDK5/p35 has also been implicated in the regulation of the motility of prostate, glioblastoma and pancreatic cancer cells (Feldmann et al., 2010; Liu et al., 2008; Strock et al., 2006).

In this study, we investigated the biological consequences of acute CDK5 loss on cell proliferation and tumorigenesis in murine embryonic fibroblasts (MEFs). We generated *Cdk5*-null MEFs using cre-lox recombinase technology *in vitro* and showed that acute depletion of CDK5 didn't affect the proliferation of primary cultured MEFs, whereas the induction of senescence markers were much reduced in these cells. CDK5 loss cooperated with oncogenic Ras and dominant-negative p53, resulting in an enhanced anchorage-independent growth. However, when these transformed cells were subcutaneously injected into nude mice, loss of CDK5 didn't confer an advantage to tumor growth. Interestingly, we found that the motility and invasion of the transformed

cells were significantly increased in the *Cdk5*-null background, suggesting a potential role of CDK5 in metastasis. Overall, the data presented here not only emphasize the significance of CDK5 in induction of the senescence response both *in vitro* and *in vivo*, but also begin to illuminate the biological significance of CDK5 in tumor incidence and progression.

Results

Acute loss of CDK5 does not affect proliferation of presenescent MEFs.

In order to determine whether CDK5 affects the proliferative capacity of murine embryo fibroblasts (MEFs), we derived MEFs from a conditional LoxP *Cdk5* mouse (*Cdk5*$^{flox/flox}$ MEFs), and then used the cre/loxP recombination technique to achieve acute inactivation of CDK5 in the MEFs *in vitro*. When exponentially growing *Cdk5*$^{flox/flox}$ MEFs were infected with Cre- or control GFP- expressing adenoviruses (Ad-Cre or Ad-GFP, respectively), CDK5 was no longer detectable 6 days after infection with Ad-Cre (Fig.3. 1A). The proliferation rate of *Cdk5*$^{flox/flox}$ MEFs infected with Ad-Cre (*f/f*-Cre) at passage 3 was indistinguishable from that of GFP controls (*f/f*-GFP). In addition, when *Cdk5*$^{+/+}$ MEFs were also infected with Ad-Cre or Ad-GFP (+/+-Cre or +/+-GFP, respectively), no inhibitory or growth-promoting effect of Ad-Cre was observed, indicating that at the dose used, Ad-Cre has no detrimental effect in this system (Fig.3.1B). *f/f*-Cre and *f/f*-GFP MEFs were indistinguishable in several other assays as well, including response to contact inhibition and proliferation at low density (data not shown). In summary, we have developed a system to eliminate CDK5 function acutely in primary MEFs. This allows us to explore the biological consequences of CDK5 loss in this system.

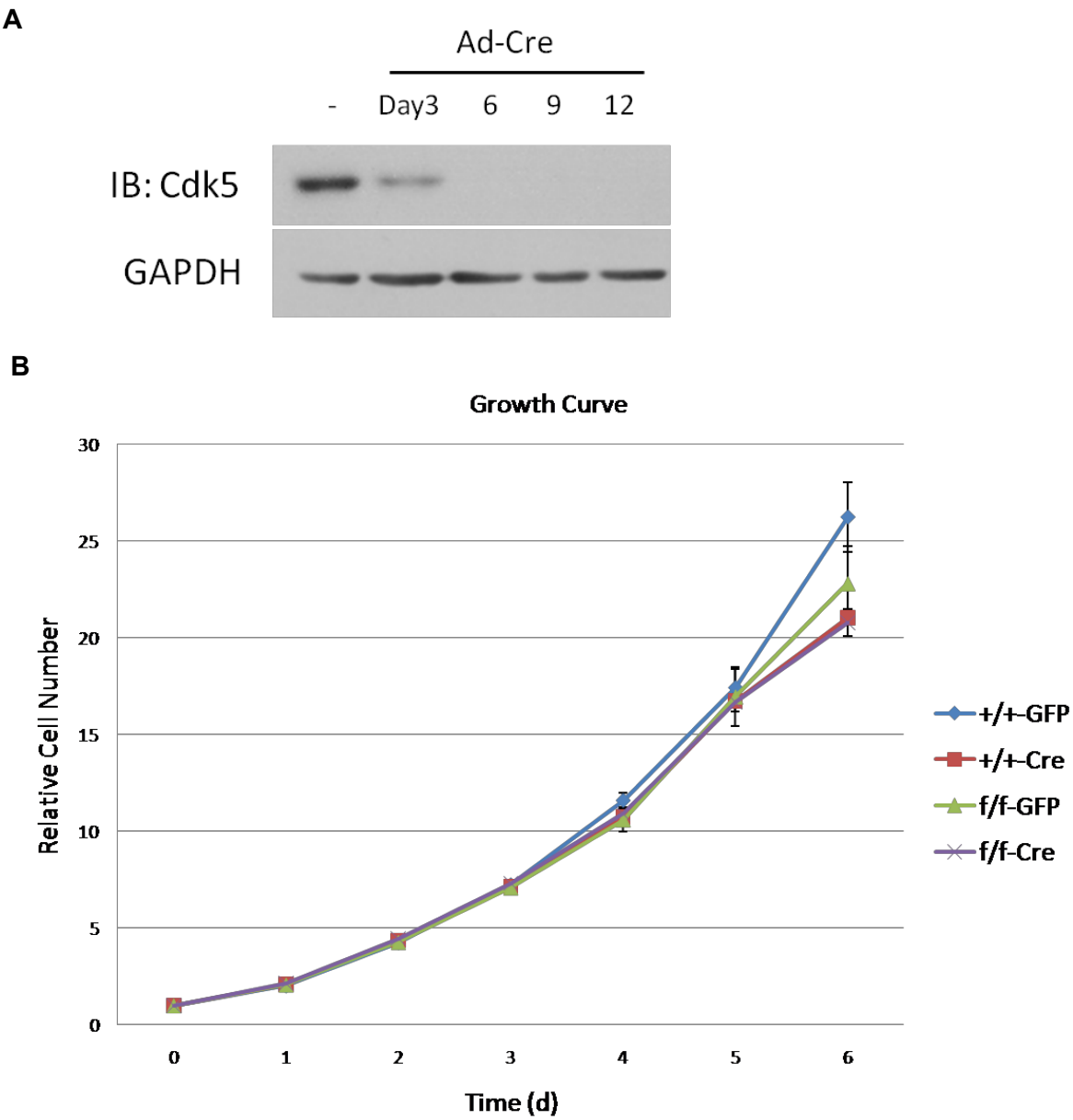
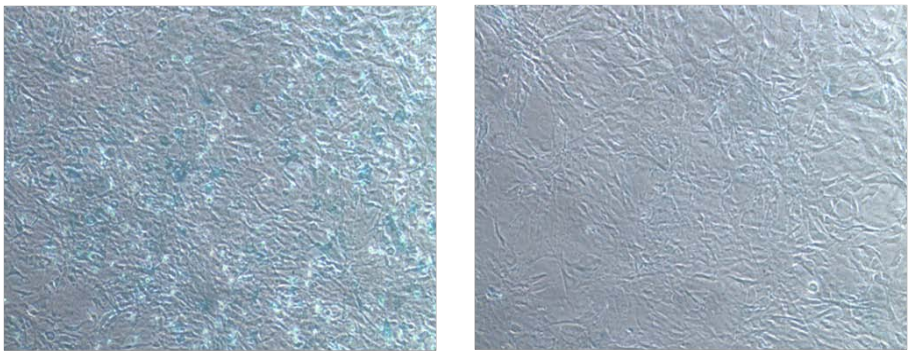


Fig.3.1. Acute loss of CDK5 does not affect the proliferation of mouse embryo fibroblasts. A, Immunoblot analysis for CDK5 at various times after infection as indicated. *Cdk5*$^{flox/flox}$ MEFs were infected with Ad-Cre at passage 3. *B,* Growth curves of *Cdk5*$^{+/+}$ and *Cdk5*$^{flox/flox}$ MEFs. Cells infected with Ad-Cre or control Ad-GFP were followed over a 6-day period. Error bars, ±s.d.; +/+, *Cdk5*$^{+/+}$; f/f, *Cdk5*$^{flox/flox}$. The curves represent the average of two *Cdk5*$^{+/+}$ and five *Cdk5*$^{flox/flox}$ independent MEF cultures.

Diminished senescence markers in Cdk5-null MEFs.

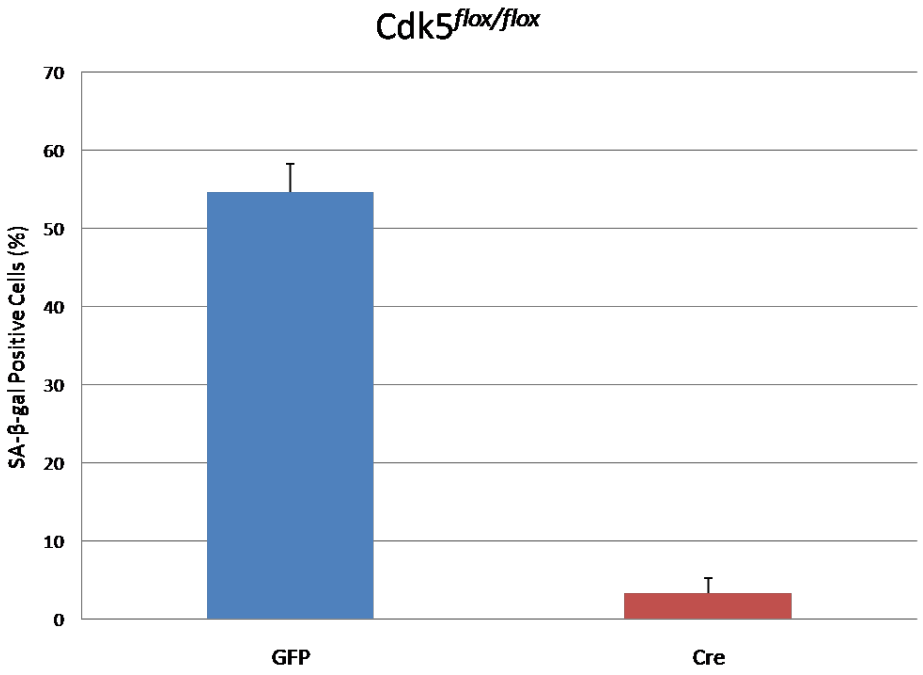
As CDK5 is required for the acquisition of senescent phenotypes in human primary and tumor cells, we investigated whether *Cdk5*-null MEFs are able to undergo senescence. *Cdk5*$^{flox/flox}$ MEFs infected with Ad-Cre or Ad-GFP at passage 3 were serially passaged and monitored for senescence. Under the culture conditions used in our lab, wild-type MEFs stopped dividing after about 6 passages and appeared to enter senescence. The senescence levels of wild-type (Ad-GFP) and *Cdk5*$^{-/-}$ (Ad-Cre) MEFs were examined through SA-β-galactosidase staining, which is widely used as a biomarker of senescence. *Cdk5*$^{-/-}$ (Ad-Cre) MEFs showed very few SA-β-gal positive cells, in contrast to the substantial number of senescent cells observed in the wild-type (Ad-GFP) MEFs. Determination of the percentage of SA-β-gal positive cells indicated that *Cdk5*$^{-/-}$ (Ad-Cre) MEFs displayed greatly and significantly diminished levels of this senescence marker compared to their wild-type counterparts (Fig.3.2A). Interestingly, when cell lysates from different passages were subjected to immunoblot analysis, the accumulation of cell cycle inhibitors p16INK4A and p27KIP1 were found to be markedly delayed in *Cdk5*$^{-/-}$ (Ad-Cre) MEFs (Fig.3.2B), indicating that these cells exhibit disruption of pathways leading to senescence. Taken together, these findings suggest that acute loss of CDK5 leads to bypass of the senescence program in MEFs.

A



Cdk5$^{flox/flox}$, Ad-GFP

Cdk5$^{flox/flox}$, Ad-Cre



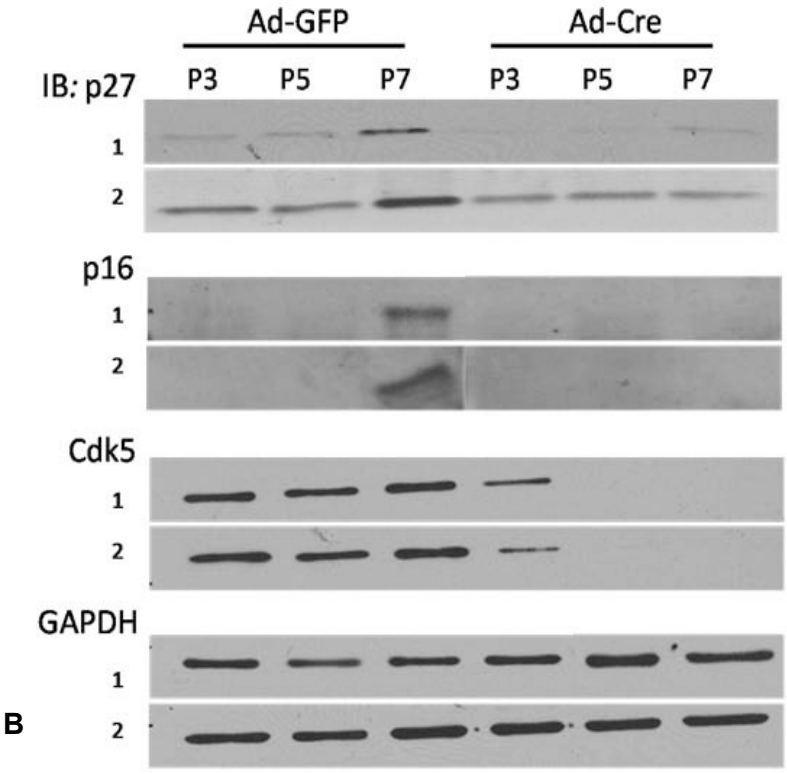


Fig.3.2. Acute Loss of CDK5 triggers bypass of senescence. A, Senescence assay. Wild-type (*Ad-GFP*) and *Cdk5*$^{-/-}$ (Ad-Cre) MEFs undergoing senescence were stained for SA-β-gal (top). Percentages of SA-β-gal positive cells were determined by counting cells from three different fields for each sample (bottom). The average of nine independent cultures is shown. Error bars, s.d., ** $p < 0.001$, using two-tailed Student's t-test, n=9. B, Immunoblot analysis for p16INK4A and p27KIP1 cell cycle inhibitors and for CDK5 in wild-type (Ad-GFP) and *Cdk5*$^{-/-}$ (Ad-Cre) MEFs undergoing senescence. Cell lysates were prepared at different passages, as indicated. Immunoblot for GAPDH protein was used as a loading control. Two representative samples are shown.

Acute loss of CDK5 cooperates with H-Ras and DNp53 in the neoplastic transformation of MEFs.

Most normal cells in culture cease proliferation when confluent and require attachment to a substratum for proliferation, while neoplastically transformed cells are much less contact-inhibited, exhibiting anchorage-independent growth. In addition to these limitations on proliferation, many normal cell types respond to oncogenic signaling with cellular senescence. In order for an oncogene such as Ras to be transforming, additional genetic changes provided by so-called cooperating genes are required, for example, loss of p53 is sufficient to cooperate with Ras to transform MEFs. To test if CDK5's role in senescence might be limiting for transformation, *Cdk5*$^{+/+}$ or *Cdk5*$^{flox/flox}$ MEFs infected with Ad-GFP or Ad-Cre (+/+-GFP, +/+-Cre, f/f-GFP, f/f-Cre) were transduced with retroviral H-RasV12 and a dominant negative p53 (DNp53). The anchorage-independent growth of these cells was examined by *in vitro* soft agar assay. Wild-type (+/+-GFP, +/+-Cre, f/f-GFP) MEFs expressing oncogenic Ras and DNp53 formed colonies in soft agar efficiently in a period of 3 weeks. However, in the setting of CDK5 loss (f/f-Cre), the number of colonies was significantly increased ($p < 0.001$) (Fig.3.3A). Expression of CDK5 in f/f-GFP and f/f-Cre transformed MEFs were examined by immunoblot analysis, showing a stable loss of CDK5 in the Cre-treated cells (Fig.3.3B). Neither wild-type nor *Cdk5*–null cells expressing Ras or DNp53 alone were able to proliferate independently of anchorage and remained as single cells (data not shown). Moreover, loss of CDK5 function is not sufficient for Ras to overcome the growth arrest in MEFs, or to facilitate the immortalization of cells by DNp53 (data not shown). Taken together, our data suggest that although loss of CDK5 alone cannot cooperate with Ras to transform MEFs, it synergizes with H-RasV12 and DNp53 to enhance the anchorage-independent growth of MEFs.

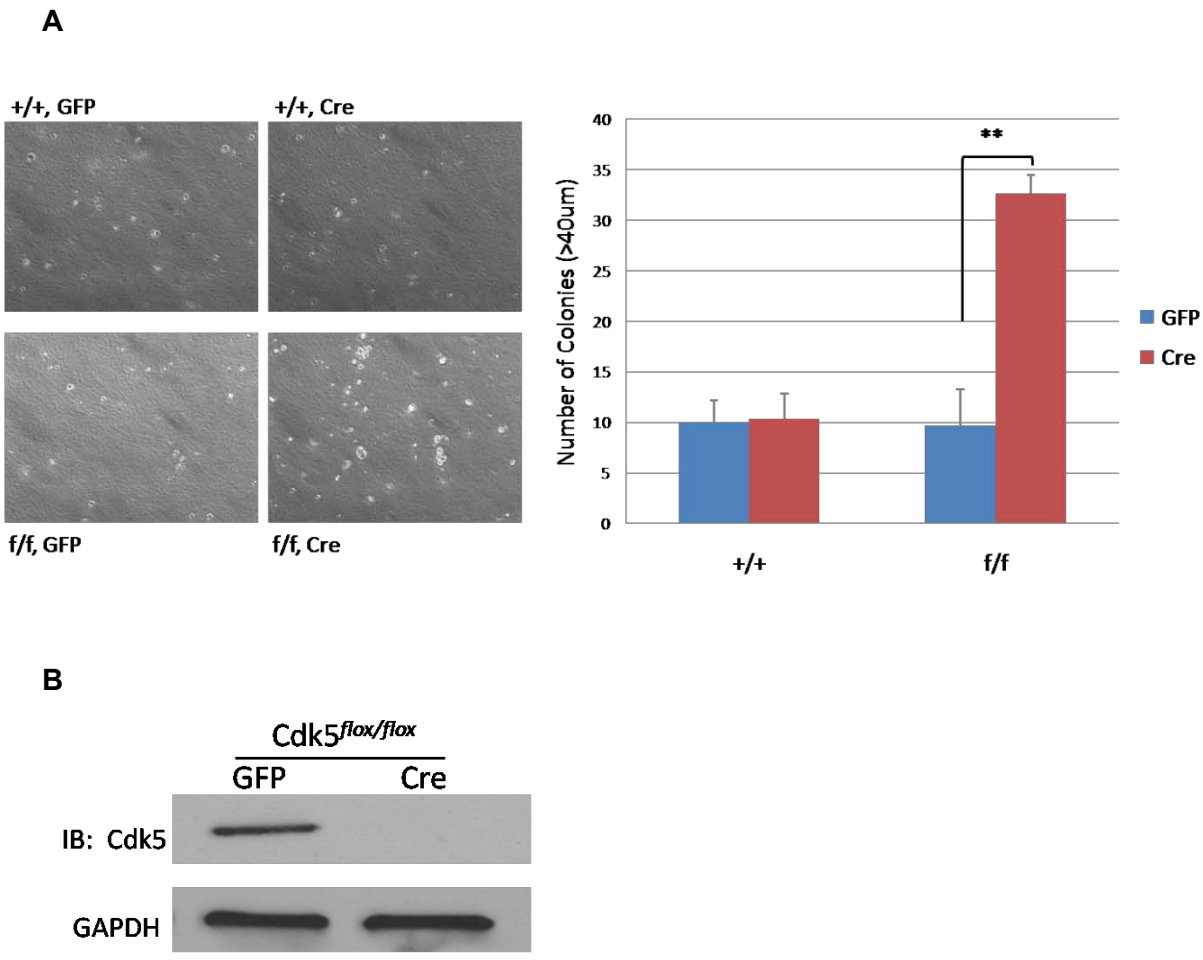
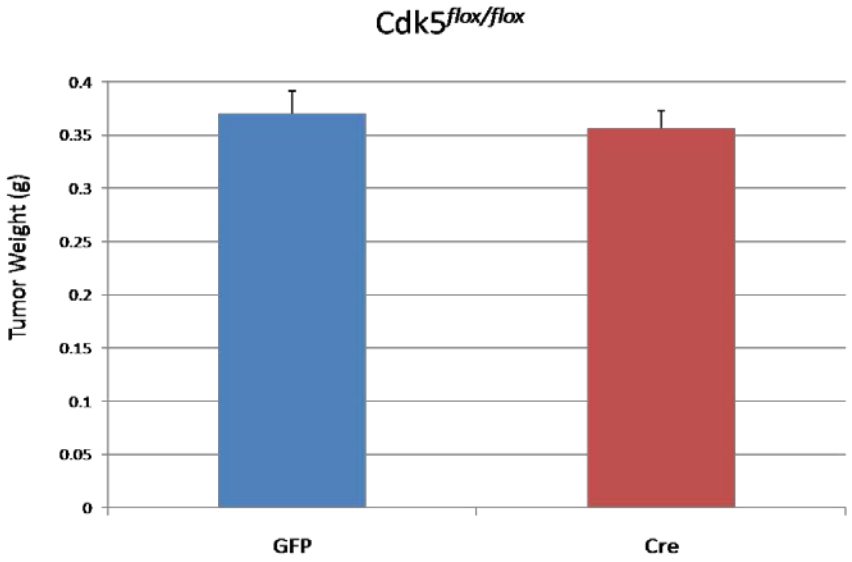


Fig.3.3. Loss of CDK5 cooperates with H-RasV12 and DNp53 in anchorage-independent growth of MEFs. A, Soft agar assay of *Cdk5*$^{+/+}$ and *Cdk5*$^{flox/flox}$ MEFs. Cells infected with Ad-Cre or control Ad-GFP were transduced with retroviral H-RasV12 and DNp53 and grown in soft agar for a 3-week period. Representative microscopic images are shown (left), and numbers of colonies were counted in three random fields from each sample (right). Data represent the average of three independent cultures. Error bars, s.d.; ** $p < 0.001$; +/+, *Cdk5*$^{+/+}$; f/f, *Cdk5*$^{flox/flox}$. B, Immunoblot analysis for CDK5 in wild-type (Ad-GFP) and *Cdk5*$^{-/-}$ (Ad-Cre) MEFs following transformation.

Acute loss of CDK5 does not contribute to tumor formation of H-Ras- and DNp53-transformed MEFs.

Cells that have acquired the ability to proliferate independently of anchorage *in vitro* usually have the ability to form tumors *in vivo*. To test for the tumorigenicity of the transformed cells, wild-type (Ad-GFP) or *Cdk5*$^{-/-}$ (Ad-Cre) MEFs transduced with retroviral H-RasV12 and DNp53 were injected subcutaneously into the flanks of NOD/SCID/γnull mice. Palpable tumors derived from both genotypes were detected in all of the animals inoculated within a short time (2-3 weeks). On day 24, tumors were removed, weighed, dissected and processed for histological analysis. Average tumor weights were not statistically different in these two genotypes (Fig.3.4A), indicating that CDK5 loss has no direct effect on tumor growth following subcutaneous injection of fully transformed cells. Interestingly, tumors derived from wild-type (Ad-GFP) transformed MEFs showed prominent SA-β-gal staining, whereas in the *Cdk5*$^{-/-}$ (Ad-Cre) counterparts, this senescence response was essentially absent (Fig.3.4B top), consistent with the observation made in the primary MEFs (Fig.3.2A). However, when paraffin sections from both wild-type (Ad-GFP) and *Cdk5*$^{-/-}$ (Ad-Cre) tumors were subjected to Hematoxylin and Eosin (H & E) staining, there were no demonstrable histological differences between the two genotypes (Fig.3.4B bottom). Thus, loss of CDK5 did not directly influence tumor formation by fully transformed, H-Ras- and DNp53- expressing MEFs in nude mice, and the senescence response observed in tumors derived from CDK5-expressing transformed cells does not appear to be rate limiting in this assay.

A


Cdk5flox/flox
Tumor Weight (g)
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0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
GFP
Cre

B

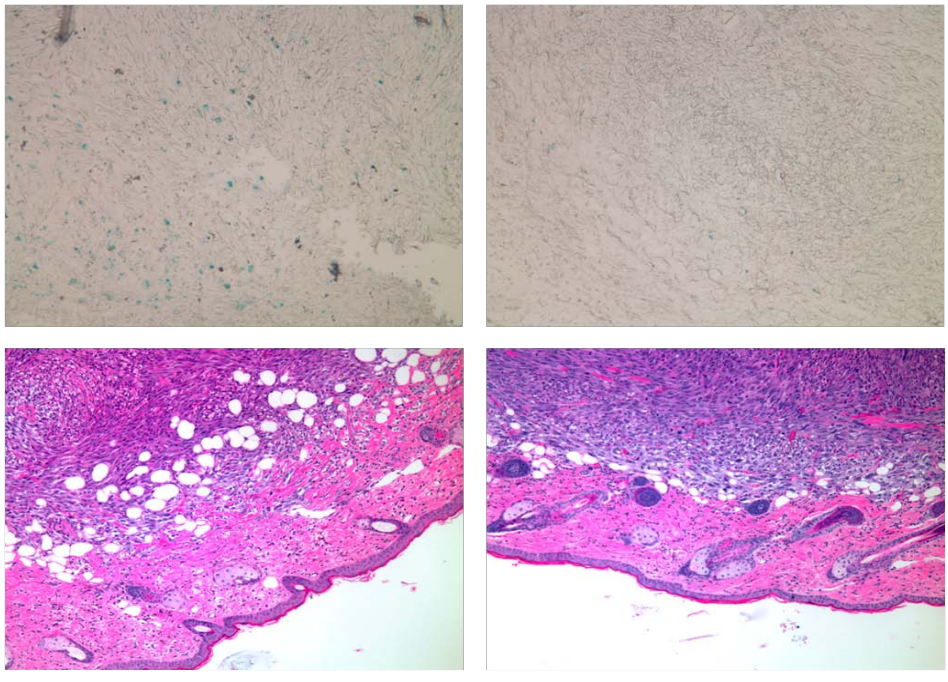
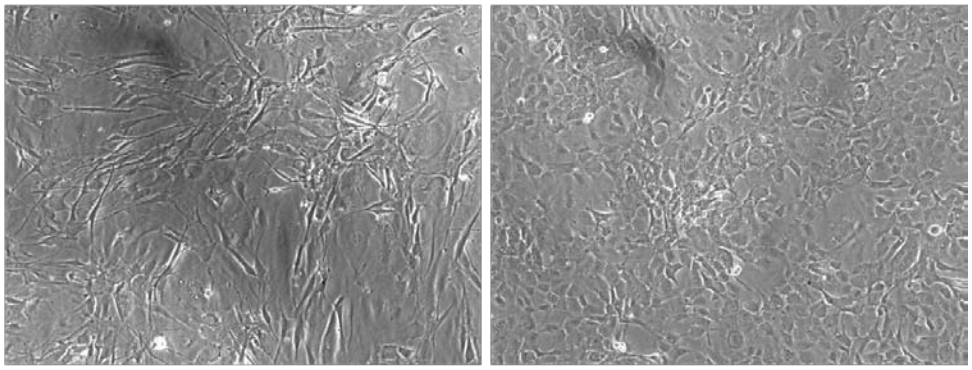

Cdk5flox/flox, Ad-GFP
Cdk5flox/flox, Ad-Cre

Fig.3.4. Loss of CDK5 leads to bypass of senescence response *in vivo* but does not affect tumor growth and histology. Wild-type (Ad-GFP) and *Cdk5*$^{-/-}$ (Ad-Cre) MEFs transduced with retroviral H-RasV12 and DNp53 were injected s.c. into both flanks of NOD/SCID mice and tumors were isolated after 3 ½ weeks. A, Weights of tumors after biopsy. The bar graph represents average weight of tumors derived from 12 injected mice for each cell type in four experiments. Error bars, s.d. B, Representative images of senescence analysis (top) and histological analysis (haematoxylin/eosin staining, bottom) of frozen and paraffin tumor sections, respectively.

Increased motility and invasiveness in Cdk5-null transformed MEFs.

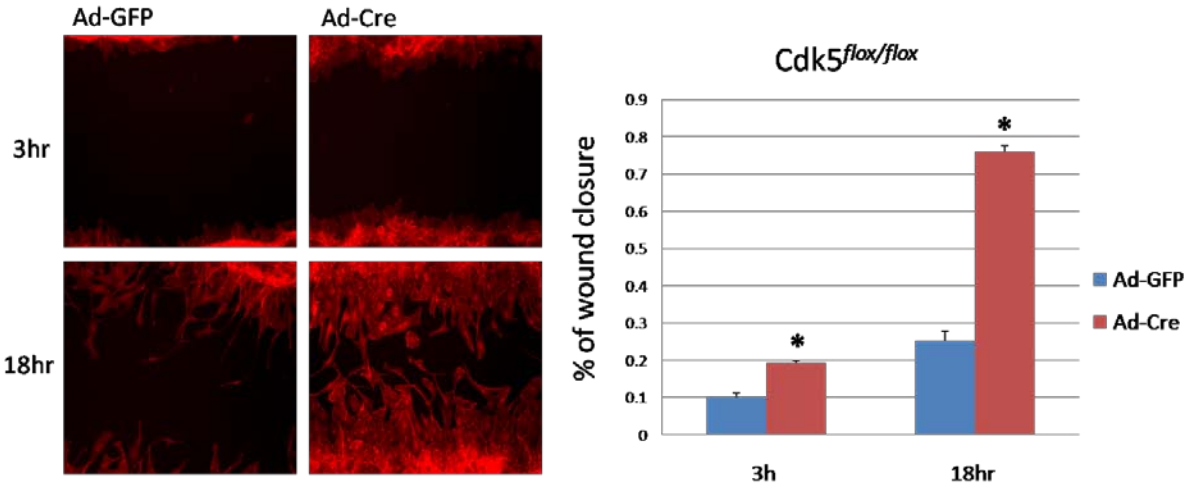
After being transformed by H-Ras and DNp53, *Cdk5*$^{-/-}$ (Ad-Cre) MEFs showed remarkable changes in morphology, compared to the wild-type controls (Fig.3.5A). To understand if such morphological changes are accompanied by other phenotypic changes, we proceeded to examine motility and invasiveness in these transformed MEFs. Surprisingly, in the wound closure assay, transformed *Cdk5*$^{-/-}$ (Ad-Cre) MEFs showed a significant increase in cell motility, compared to wild-type controls ($p<0.01$) (Fig.3.5B). To eliminate any contribution of cell proliferation to this process, cells were starved in serum-free medium for at least 24 hr before scratching, and were further maintained in serum-free medium in the presence of mitomycin C. BrdU incorporation assays were also performed to confirm that cell division was blocked during the 'wound closure' process (data not shown). Next, using transwell migration assays, we obtained comparable results (Fig.3.5C). In addition, using Matrigel-coated Transwell inserts in the same transwell assays, transformed *Cdk5*$^{-/-}$ (Ad-Cre) MEFs also exhibited significantly increased invasiveness (Fig.3.5D). Thus, H-RasV12 and DNp53 cooperated to induce cell migration and invasion in *Cdk5*-null MEFs, phenotypes that have strong implications for the metastatic potential of cancer cells.

A

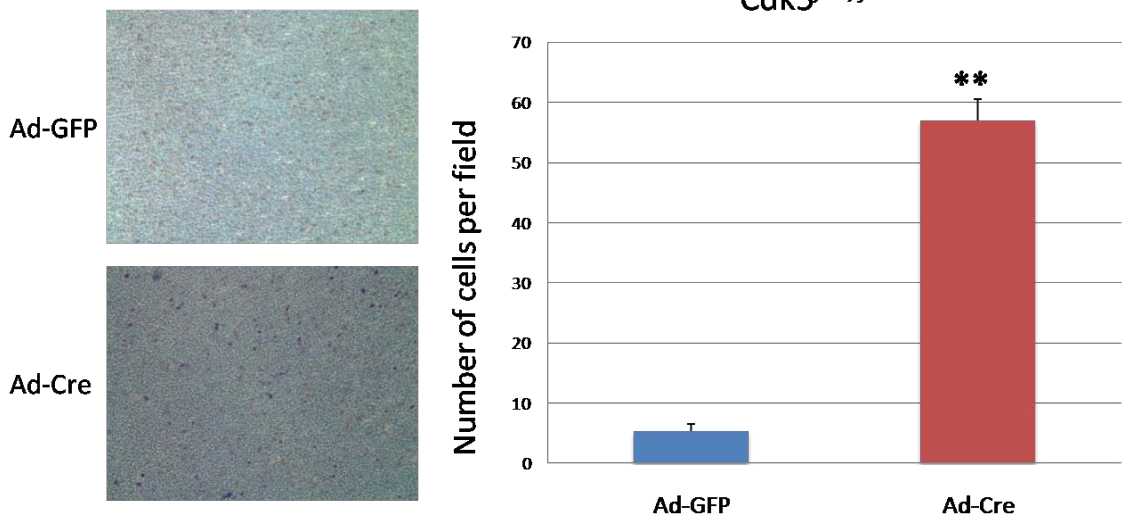


Cdk5$^{flox/flox}$, Ad-GFP Cdk5$^{flox/flox}$, Ad-Cre

B



C


Ad-GFP
Ad-Cre
Cdk5flox/flox
Number of cells per field
70
60
50
40
30
20
10
0
**
Ad-GFP
Ad-Cre

D

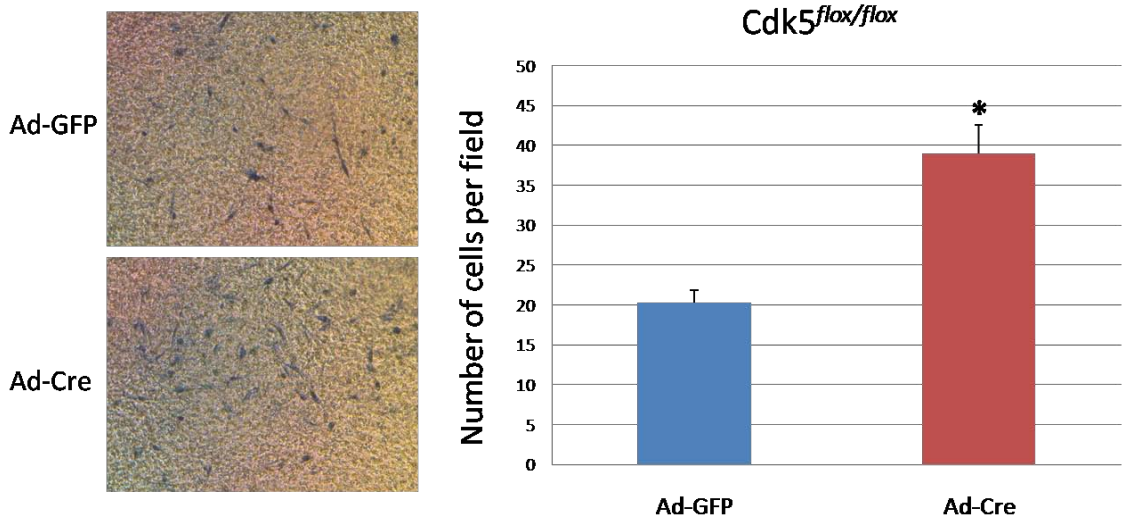

Ad-GFP
Ad-Cre
Cdk5flox/flox
Number of cells per field
50
45
40
35
30
25
20
15
10
5
0
*
Ad-GFP
Ad-Cre

Fig.3.5. Loss of CDK5 enhances migration and invasion in transformed MEF. Wild-type (Ad-GFP) and *Cdk5*$^{-/-}$ (Ad-Cre) MEFs expressing H-RasV12 and DNp53 were examined by A, Microscopic morphology. B, *In vitro* wound-closure assay. *left,* representative images of cell migration 3 h after scratching the cultures and 18 h later; *right,* to quantify cell migration, phase-contrast images of identical locations in each wound were taken at 0 h, 3 h and 18 h after wounding. The rate of cell migration was then calculated as the average percentage of wound closure from at least three independent experiments. Error bars, s.d.; *, $p<0.01$. C-D, Transwell migration and invasion assays. *left,* representative images of cell populations after migration (C) and invasion (D) through transwell membrane. *right,* quantifications of migrated (C) and invaded (D) cells in transwell assays. Experiments were performed at least three times. Error bars, s.d.; **, $p<0.001$; *, $p<0.01$. For invasion assay, the membrane filters of the Transwell inserts were coated with Matrigel as described under Materials and Methods.

Discussion

Cellular senescence has emerged as a programmed cellular stress response that is induced due to the accumulation of damage to a cell. Increasing evidence shows that senescence plays a critical role as a tumor suppressive process *in vivo* (Braig et al. 2005; Chen et al. 2005; Collado et al. 2005; Michaloglou et al. 2005). The effect of CDK5 on senescent morphological changes has been well documented in the context of human primary and tumor cells induced to senesce by a variety of stimuli. However, the biological consequences of these changes in regulating cell proliferation and tumorigenesis have remained largely unexplored. Mouse embryonic fibroblasts (MEFs) have been widely used as a model for the study of cell growth, immortalization and neoplastic transformation, and have provided important clues about genetic control of cellular senescence. In this study, we took advantage of the *Cdk5*$^{flox/flox}$ conditional mouse to investigate the biological functions of CDK5 in these primary cells.

Our results show the bypass of cellular senescence in MEFs upon ablation of CDK5 function. Concomitantly, CDK5 deficiency impaired the accumulation of key cell-cycle attenuators and checkpoint regulators, including p16INK4a and p27KIP1, in cells undergoing senescence. Unlike normal human primary cells in culture, which undergo replicative senescence due to telomere shortening, the senescence response in MEFs is mainly caused by suboptimal culture conditions. Reactive oxygen species (ROS) resulting from supraphysiological levels of oxygen have been identified as a key trigger of the senescence response in MEF culture in standard (18% oxygen) tissue culture incubators (Busuttil et al., 2003; Parrinello et al., 2003). Thus, the diminished expression of senescence markers in *Cdk5*-null MEFs, as well as the delayed accumulation of the cell cycle inhibitor p16INK4a, a general indicator of stress, suggests that CDK5 might play a role in sensitizing these cells to oxidative stress. Oncogenic Ras triggers

senescence by increasing intracellular levels of ROS in primary cells (Lee et al., 1999; Nicke et al., 2005). The stress-responsive p38MAPK (Tobiume et al., 2001) is required for Ras-induced senescence (Haq et al., 2002; Iwasa et al., 2003; Wang et al., 2002), and has recently been reported to negatively regulate the onset of cancer by sensing oxidative stress induced by oncogenes that produce ROS (Dolado et al., 2007). CDK5 has been shown to be a major regulator of p38 cascade in neurons (Chang et al., 2010). In addition, CDK5 dysregulation upon neurotoxic insults results in ROS accumulation in neuronal cells. The elevated ROS then activates CDK5 in a positive feedback loop (Sun et al., 2008). Therefore, we speculate that CDK5 plays a key role in mediating p38MAPK activation in response to the increased intracellular levels of ROS, which in turn leads to the cellular senescence of MEFs.

It is well known that although oncogenic Ras efficiently transforms most immortal rodent cell lines, it fails to do so in primary cells. Overexpression of oncogenic H-Ras results in senescence in both primary human and rodent cells, a phenomenon later described as oncogene-induced senescence (Serrano et al., 1997). However, Ras cooperates with certain other alterations, which disrupt p53 or p16INK4A/pRB pathways, to transform primary rodent cells. For example, SV40 T-antigen or adenovirus E1A inactivates both p53 and pRB; cyclin D1, Cdc25A and B phosphatases lead to the inactivation of pRB. In our report, we show that although loss of CDK5 is not sufficient to cooperate with Ras to transform MEFs, it enhances the ability of MEFs transduced with oncogenic H-RasV12 and dominant negative p53 to grow anchorage-independently in soft agar. However, when these cells were injected s.c into SCID mice, we did not observe any effect of CDK5 loss on either an enhancement of tumor growth or the overall histopathology of the tumors. Surprisingly, a senescence response was still detectable in tumors derived from wild-type but not *Cdk5*-null transformed MEFs, which

is consistent with what we found in primary MEFs. Possible explanations for these results may include: (1) The transformed wild-type MEFs are already very refractory to further tumor suppressive stimuli, having already bypassed the senescence response *in vitro*, and rapidly form large tumors in two to three weeks after inoculation. In this case, the additional effect of loss of CDK5 is marginal and hard to evaluate. The fact that a small proportion of cells were able to undergo senescence in the tumors derived from transformed wild-type but not *Cdk5*-null MEFs suggests that CDK5 can still modulate a senescence response even in these highly tumorigenic cells, and this response is likely to be p53 independent. If cells are put under more stringent conditions, CDK5 may help to trigger a more robust senescence response and thus limit tumor growth. In line with this idea, CDK5 may thereby contribute to the outcome of chemotherapeutic treatment; (2) CDK5 is not required for tumor initiation, but rather for tumor progression. This is supported by our observations that acute loss of CDK5 did not promote proliferation of pre-senescent primary or transformed MEFs (data not shown), and loss of CDK5 could not immortalize MEFs or enhance the ability of foci formation in the DNp53-immotalized cells (data not shown). However, in addition to the enhanced anchorage-independent growth in soft agar, CDK5 loss also promoted the migration and invasion of the transformed MEFs, as shown in our *in vitro* assays, suggesting that these cells might attain increased metastatic potential. To explore this possibility, appropriate metastastic tumor models will be of great help.

Taken together, our results provide strong evidence that CDK5 functions as a key player in cellular senescence in primary mouse embryonic fibroblasts, and might regulate tumor development by modulating metastatic potential of cancer cells.

Materials and Methods

Cell Culture.

Mouse embryo fibroblasts (MEFs) were generated from embryos at E13.5 to E15.5 from $Cdk5^{flox/flox}$ mice. Cells were cultured in a 5% CO2 incubator at 37°C and maintained in Dulbecco modified Eagle medium (DMEM; Gibco) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. Adenoviral stocks were purchase from the MCRI Adenovirus Core Facility of Tufts University School of Medicine.

Growth curves.

Wild-type or $Cdk5^{flox/flox}$ MEFs were infected with Ad-Cre or control Ad-GFP virus at passage 3 (P3). At passage 4 (P4) $3X10^4$ MEF cells were seeded per well in triplicate in 24-well plates. Six hours after seeding, one plate of cells was washed with PBS and fixed with 100% ethanol for 15 min, after three washes with water, cells were stained with 0.1% crystal violet for 20 min, followed by another three washed and then were air dried. One plate of cells was fixed and stained each day for six days. At the end point, 500µl of 10% acetic acid was added to each well and incubated 20 min with shaking. The absorbance of each well was determined at wavelengths of 590 nm using a microplate reader (BioTek).

Cell transfor bmation, anchorage-independence assay and tumour formation assay.

To test for tumorigenicity, wild-type (Ad-GFP) and $Cdk5^{-/-}$ (Ad-Cre) MEFs were transduced with retrovirus-based human oncogenic H-RasV12 and the dominant-negative p53 (DNp53) at passage 4. Retroviral supernatants were produced by transfection of 293T packaging cells. Viral supernatants were filtered through a 45 µM Millex HA filter (Millipore) and infections were performed in the presence of 10 µg/ml

polybrene (Sigma). Transformed MEF cell lines were selected with 2 μg/ml puromycin and 50 μg/ml hygromycin in DMEM containing 10% fetal bovine serum and 1% penicillin-streptomycin.

For anchorage-independent growth, $5X10^3$ tranformed cells at passage 8 were resuspended in a medium containing 0.4% low-melting-point agarose and plated onto a solidified bottom layer medium containing 0.8% agarose in 6-well plates. Colonies were photographed after 3 weeks and counted in three randomly chosen fields. Values are expressed as means ± standard deviations for three independent experiments.

For tumor formation assays, 1×10^5 cells were injected subcutaneously into the flanks of 6-8 weeks old male NOD/SCID/γnull mice in 200 μl of serum-free DMEM. Tumor growth was monitored weekly for 3 and half weeks. At the end point, mice were sacrificed, each tumor were dissected, measured for the weight, and processed for histological analysis.

In vitro wound-closure assay.

For the wound-closure assay, $3X10^4$ cells were seeded in 24-well plates and incubated at 37°C overnight to generate confluent culture. Cells were then serum-starved for 24 h and treated with Mitomycin C (10 mg/ml) for 2 h before wounding. The cell layers were scraped with a plastic pipette tip and washed three times with serum-free medium. The remaining cell culture was incubated 3 h or 18h to allow cells to migrate into the cleared space. To quantify cell migration, phase-contrast images of identical locations in each wound were taken at 0 h, 3 h and 18 h after wounding. The rate of cell migration was then calculated as the average percentage of wound closure from at least three independent experiments. To visualize cell migration, 3 h and 18 h after wounding, the plate was washed with PBS and cells remaining attached were fixed

for 20 min with 4% paraformaldehyde per well. Fixed cells were washed once with PBS and then stained for F-actin with rhodamine phalloidin as described previously in Chapter 2. Stained cells were mounted using Fluoromount G and images were taken using a Nikon Eclipse Ti-U inverted microscope.

Transwell migration and invasion assay.

Transwell migration and invasion assays were performed using 24-well Transwell inserts (8.0 μm Polycarbonate membrane, Corning). $5X10^4$ cells were added to the top well resuspended in serum-free medium, and the lower well was filled with medium containing 10% FBS. After a 3-h or 22-h incubation for migration assay or invasion assay, respectively, cells remaining on the upper side of the filter were removed with cotton swabs, and the cells that had migrated to the bottom surface of the filter were fixed in 4% paraformaldehyde and stained with 0.1% crystal violet. Migrated cells were photographed and quantified by counting the total number of cells in at least three randomly selected fields. Data were obtained from three independent experiments. For invasion assay, the filters were coated with 70μl of 0.125 mg/ ml Matrigel (basement membrane matrix, BD Biosciences), then dried overnight in a laminar flow hood.

Immunoblotting

Cells were lysed in 100–200 μl of lysis buffer (50mM HEPES pH 8.0, 150mM NaCl, 1mM EDTA, 0.1% Nonidet P-40) plus protease, and phosphatase inhibitors (1 mg of aprotinin/ml, 1μg of leupeptin/ml, 100μg of phenylmethylsulfonyl fluoride/ml, 4 mM sodium orthovanadate, 2 mM sodium PPi) per 10-cm plate. Protein concentrations of the cell lysates were determined by the Bradford assay (Bio-Rad). For immunoblotting, 30μg of protein was separated by SDS-PAGE and transferred to nitrocellulose membrane in a trans-blotting buffer (25 mM Tris, 192 mM glycine, 20% (v/v) methanol).Immunoblot

analysis was performed as described previously (36, 39). Antibodies used for immunoblotting include: anti-Cdk5 monoclonal J-3 and polyclonal C-8, anti-p16 polyclonal M-156, anti-p27 monoclonal F-8 (Santa Cruz Biotechnology), anti-GAPDH monoclonal MAB374 (Chemicon). Horseradish peroxidase-conjugated donkey anti-mouse or anti-rabbit secondary antibodies (Jackson Immunosciences) were used, and signal was detected by ECL (PerkinElmer).

Tumorigenicity assays and histological analysis.

NOD/SCID/γnull mice were generously provided by Kuperwasser lab (MORI, Tufts Medical Center). For tumorigenicity assay, 1 $X10^5$ cells in 200 µl of serum-free medium were injected s.c. into both flanks of nude mice. After 3 and half weeks, the animals were sacrificed and tumors were removed, weighed, dissected and processed for histological analysis. Tumor samples were fixed in 10% formalin and processed for hematoxylin and eosin (H&E) staining.

Senescence-associated β-galactosidase staining assay

MEF cells or frozen sections of tumors were stained for perinuclear senescence associated -β-galactosidase (SA-β-gal) activity as described (Dimri et al., 1995). Briefly, samples were washed in PBS and fixed in 2% formaldehyde/0.2% glutaraldehyde, and then washed and incubated at 37°C overnight with fresh senescence associated -β-galactosidase staining solution (1 mg of 5-bromo-4-chloro-3-indolyl-ß-D-galactoside per ml, 40 mM citric acid/sodium phosphate (pH 6), 150 mM NaCl, 2 mM MgCl2, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide).

Chapter IV

Discussion

I. How might CDK5 contribute to senescence program?

(1) CDK5-p27KIP1 relationship.

Reintroduction of pRB or its upstream regulator p16INK4a into tumor cells that lack them triggers premature senescence. This senescence response triggered by pRB can occur in the absence of a functional p53/p21CIP1 pathway and instead induces an accumulation of p27KIP1 for G1 arrest. Intriguingly, while overexpression of p27KIP1 or p21CIP1 can induce growth arrest and SA-β-gal expression even in the absence of functional pRB, they cannot mediate the senescent shape change, demonstrating that this is a function unique to pRB and further investigation has established a role of CDK5/p35 activity in this senescent phenotype. These findings suggest that the processes of cell cycle arrest and morphology change are two distinct aspects of cellular senescence and can be separately regulated.

However, there is also considerable evidence for 'cross-talk' between the two pathways. Studies in neurons have recently indicated that CDK5/p35 directly phosphorylates p27KIP1 at Ser10 and Thr187 and stabilizes it (Kawauchi et al., 2006). p27KIP1 is localized not only in the nucleus but also in the cytoplasm. Phosphorylation of p27KIP1 at Ser 10 participates in its cytoplasmic localization, contributing to actin organization and cortical neuronal migration (Boehm et al., 2002; Kawauchi et al., 2006; Rodier et al., 2001), whereas CDK5 phosphorylation of p27KIP1 at Thr187 is crucial to neural differentiation (Zheng et al., 2010). Analogously, CDK5 phosphorylation of p27KIP1 may regulate the progress of senescence from cell cycle arrest through cytoskeletal regulation and morphology change in non-neuronal post-mitotic cells.

(2) Gene expression regulation.

Senescence is accompanied by large-scale gene expression changes. Accumulating evidence suggests a direct role for CDK5 in the regulation of gene transcription. For example, in pancreatic β-cells, elevated glucose level in the culture medium induces p35 expression and therefore, CDK5/p35 activity, which in turn upregulates the transcriptional activation of the insulin gene (Ubeda et al., 2004). CDK5/p35 activity is a key regulator of non-specific esterase expression and therefore influences differentiation of mouse monocytes (Studzinski and Harrison, 2003). In skeletal muscle, the neuregulin-1(NRG)-induced expression of acetylcholine receptors (AchR) is regulated by CDK5, thus involving CDK5 in the NRG signaling pathway and synaptic transmission (Fu et al., 2001).

pRB and p53 are the key regulators of cellular senescence. Studies in our lab have shown that CDK5 is activated in several human tumor cell lines induced into senescence by p16INK4a/pRB, irrespective of p53 status. Activation of CDK5 may in turn regulate these checkpoint proteins through direct phosphorylation modifications. In neurons, both pRB and p53 are CDK5 targets. p25/CDK5 has been shown to directly mediate phosphorylation of pRB, and the deregulation of CDK5 was linked to neuronal cell death (Hamdane et al., 2005; Lee et al., 1997). Direct evidence to demonstrate the regulation of pRB phosphorylation and its cellular function by CDK5 in cells is still lacking. Nevertheless, it raises the possibility that such a functional relationship between CDK5 and pRB might exist. Stabilization and activation of p53, via its phosphorylation on Ser15, Ser33 and Ser46 by CDK5, also contribute to neuronal cell death. CDK5 mediates disruption of the interaction between p53 and HDM2 (also known as MDM2), thus preventing HDM2-induced p53 ubiquitylation and downregulation. In addition, CDK5 enhances phosphorylation-dependent binding of the p300 coactivator, inducing

acetylation of p53. CDK5-stabilized p53 protein is transcriptionally active, resulting in induction of p53 target genes, such as p21CIP1 and Bax (Lee and Kim, 2007; Zhang et al., 2002). The ability of p53 to promote apoptosis or senescence is context dependent. Likewise, although CDK5 activation is implicated in the pro-apoptotic effects of many drugs (Kuo et al., 2009; Lee et al., 2007; Lin et al., 2004; Upadhyay et al., 2008), its activity is also involved in the terminal differentiation of different cell types and exhibits an anti-apoptotic function, for example, through regulating the expression of Bcl-2 and Bcl-XL (Brinkkoetter et al., 2009; Lee and Kim, 2004; Sarker and Lee, 2004; Zheng et al., 2010). Thus, CDK5 might enforce the senescence response via modulation of p53 stability and activity.

In oncogene-induced senescence, hyperproliferative signaling in response to certain mitogenic oncogenes, such as the small GTPase Ras and its effector Raf, ultimately triggers premature senescence via p53 and pRB in a variety of human and mouse primary cells. Interestingly, the aberrant activation of Raf and Ras trigger a global negative feedback signaling program, leading to the rapid inactivation of Ras effector pathways, as assessed by the levels of phospho-ERK and phospho-AKT (Courtois-Cox et al., 2006; Menges and McCance, 2008). One target of CDK5 is MEK1 (MAPK/ERK kinase-1). Phosphorylation of MEK1 by CDK5 represses MEK1 activity and blocks downstream cellular responses (Chen et al., 2004; Zheng et al., 2007). The activation of p35 by MAPK (Mitogen-Activated Protein Kinase) pathways followed by deactivation of MAPK signaling by the CDK5/p35 complex, through the inhibition of MEK1, completes the loop of a negative feedback circuit to terminate MAPK signaling, and therefore implicates CDK5/p35 as a participant in Ras/Raf-induced senescence.

In addition, in Ras-provoked senescent cells, pRB protein-mediated senescence-associated heterochromatin foci (SAHFs) have been identified as sites of local

transcriptional repression, where heterochromatin proteins are recruited to E2F-responsive promoters (Narita et al., 2003), highlighting the significance of a pRB pathway- and histone modification-governed senescence program *in vivo*. CDK5 has also been shown to interact with chromatin-remodeling factors such as SET and histone deacetylases, which contribute to the formation of repressive chromatin. SET is present in the nucleus within a complex that binds to chromatin and blocks histone from acetylation by p300/CBP and PCAF. Nuclear SET protein specifically binds to the N-terminal region of p35 or p39. This interaction stimulated CDK5/p35 kinase activity (Qu et al., 2002). A physical and functional interaction has also been established between the CDK5/p35 complex and mouse Sds3 (mSds3), an essential component of mSin3-histone deacetylase (HDAC) co-repressor complex. mSds3 binds to p35 both *in vitro* and *in vivo*, enabling active CDK5 to phosphorylate mSds3 at serine 228. p35 overexpression is shown to augment mSds3-mediated transcriptional repression *in vitro* (Li et al., 2004). Moreover, pRB family proteins were previously shown to associate via the pocket with mSIN3-SAP30-HDAC complexes containing exclusively class I HDACs (Lai et al., 2001). Thus, CDK5/p35 may contribute to altered gene expression during senescence by participating in pRB-mediated SAHF formation.

Moreover, CDK5 also regulates gene expression in various cell types by directly phosphorylating transcription factors, such as Myc, STAT3 and MEF2 (myocyte enhancing factor-2) (Fu et al., 2004; Gong et al., 2003; Seo et al., 2008). It would be interesting to identify transcription factors as substrates of CDK5 that are specifically modulated during cell senescence.

(3) Response to diverse senescence-inducing signals.

Numerous studies in both neuronal and non-neuronal contexts have shown that CDK5 activity can be modulated by a variety of signaling cascades, many of which are also identified as senescence-inducing signals in different cell types.

For example, the two most important Ras effector pathways, Raf–MEK–ERK and PI3K-Akt signaling, are both able to activate CDK5 during differentiation in various cell types. During retinoic acid-induced neuronal differentiation, retinoic acid causes activation of ERK (via MEK1) and PKA. ERK activation results in increased expression of Egr1, c-fos and c-jun. Egr1 transcriptionally activates p35 expression by binding to the p35 promoter region. In addition, PKA increases the phosphorylation of CREB, which together with c-fos and c-jun, increase CDK5 expression. The formation of the CDK5/p35 complex then induces neuronal differentiation (Lee and Kim, 2004). Differentiation induced by nerve growth factor (NGF) and IFN-gamma-induced neurite outgrowth also utilizes the MEK1–ERK–Egr1 pathway to enhance p35 expression (Harada et al., 2001; Song et al., 2005). In promyelocytic HL60 cells, treatment with 1,25-dihydroxyvitamin D3 (1,25D3) also results in the upregulation of the Egr1 gene and increased p35 transcription and expression. p35-associated CDK5 activation phosphorylates MEK1 on T286, preventing ERK phosphorylation and cell proliferation. It is noted, however, that MEK1 phosphorylation at T286 requires prior S218 and S222 phosphorylation and activation by Raf1 inducible by growth factors or cytokines. These events were suggested to result in the upregulation of p27KIP1 and eventually, monocytic differentiation (Chen et al., 2004).

Activation of the PI3K–Akt–p70S6K–Egr1 pathway is identified as the upstream modulator of CDK5 activity during myoblast differentiation. In myocytes, serum reduction

causes activation of PI3K that results in Akt and p70S6K activation via PDK1 and FRAP/mTOR, respectively. Subsequently, Egr1 is upregulated causing increased p35 expression, CDK5/p35 activation and myocyte differentiation (Sarker and Lee, 2004).

Senescence can be view as a special form of terminal differentiation. Both processes exhibit strikingly similar characteristics, because both can lead to irreversible growth arrest. More specifically, both differentiated and senescent cells are growth-arrested with a G1 DNA content and neither can be stimulated to enter the S phase of the cell cycle by any known combination of physiological mitogens, even though such cells can be maintained in this viable, albeit nonreplicating state for very long periods of time. Therefore, it is not surprising that related and overlapping regulatory pathways mediate both of these phenomena.

Recently, CDK5 has also been shown to serve as a critical signal to initiate the ATM response to DNA damage in neuronal death. Calpain-mediated CDK5 activation, in response to DNA damage, directly phosphorylates ATM at Ser 794 in post-mitotic neurons. Phosphorylation at Ser 794 precedes, and is required for, ATM autophosphorylation at Ser 1981, and activates ATM kinase activity. The CDK5-ATM signal regulates phosphorylation and function of the ATM targets p53 and H2AX, and is required for the expression of p53 target genes including *PUMA* and *BAX* (Tian et al., 2009). Since the result of the activation of a certain signaling pathway can be cell-type specific, it is reasonable to speculate that CDK5-mediated regulation of ATM may represent a mode of DNA damage response that leads to senescence in certain circumstances.

Another important control point of CDK5 lies in the activation of p38 MAPK pathway, a well-studied stress modulator, thus linking CDK5 function to stress

responses, especially oxidative stress. CDK5 has been shown to be a major regulator of p38 MAPK cascade in neurons. CDK5 deregulation promotes oxidative stress by inactivating the peroxidase activities of Prx-I and Prx-II. Subsequently, elevated levels of reactive oxygen species (ROS) induce p38 MAPK activation, and activate CDK5 in a positive feedback loop as well, thus establishing a mechanistic link between CDK5 and p38 MAPK (Chang et al., 2010; Otth et al., 2003; Sun et al., 2008). Reactive oxygen species (ROS) is known to induce senescence in a number of systems. p38 MAPK activation is required for Ras-induced senescence (Haq et al., 2002; Iwasa et al., 2003; Wang et al., 2002), and has recently been reported to negatively regulate the onset of cancer by sensing oxidative stress induced by oncogenes that produce ROS (Dolado et al., 2007). These data, taken together, strongly point to a role for CDK5 in the senescence response triggered by oxidative stress. Moreover, CDK5 also cooperates with c-Abl in regulating activation of p53, resulting in neuronal death in response to oxidative stress (Lee et al., 2008).

By investigating the molecular mechanism underlying the irreversibility of senescence cell cycle arrest, a recent study has defined another function for ROS — enforcer of senescence. The study showed that p16INK4a/pRB cooperates with mitogenic signals to induce ROS, thereby activating protein kinase Cδ (PKCδ), which, in turn, leads to the further production of ROS, thus establishing a positive feedback loop to sustain ROS–PKCδ signaling. Sustained activation of ROS–PKCδ signaling irreversibly blocks cytokinesis, at least partly through reducing the level of WARTS (also known as LATS1), and therefore is likely to act as a second barrier to cellular immortalization by ensuring stable cell-cycle arrest in human senescent cells (Takahashi et al., 2006). Although a direct link between CDK5 and cytokinesis inhibition is still lacking, a functional link between CDK5 and PKCδ has recently been established. Zhao *et al*.

(2009) showed that PKC can phosphorylate and thus stabilize p35 in cultured cortical neurons. PKCδ is required for the activation of CDK5 by BDNF and regulates cortical radial migration through maintaining the proper level of p35 in newborn neurons (Zhao et al., 2009). Thus, it would be interesting to know in non-neuronal senescent cells, whether CDK5 can be activated via ROS–PKCδ signaling and how it contributes to the irreversibility of the senescent state.

Conclusion: Since CDK5 and its activator p35 are widely expressed by a variety of cell types including cancers, it raises the possibility that CDK5-p35 activity may be engaged in broad tissue types and modulate regulation of cellular senescence in response to a wide spectrum of signals. CDK5-p35 is detected in both cytoplasmic and nuclear compartments in cells. This may allow CDK5 to execute multiple tasks and critically distinguish diverse signals.

II. Beyond the shape change.

One of the most visible changes that occur when cells undergo senescence is a change in morphology. Cells become dramatically enlarged and flattened and more irregularly shaped, showing more prominent intracellular actin fibers. In addition, the cells accumulate intracellular vesicles, many of which are lysosomes. For most cell types, the senescent morphology is quite distinct from that of proliferating, quiescent, or terminally differentiated cells from the same lineage.

Studies in our lab have established a clear link between CDK5/p35 activity and the senescent morphology change. However, the *in vivo* significance of such change is still elusive and the mechanism is not well understood. As with CDK5 in neurons, CDK5 activity in non-neuronal cells appears to influence phenotypic changes mostly through its direct or indirect effect on the organization of cytoskeletal structures such as actin and

microtubules. Alterations in the cytoskeletal organization could, in turn, lead to alterations in cell adhesion, cell-cell contact, motility, and many other cellular processes, which may provides new insights into our understanding of the physiological consequences of cellular senescence.

(1) Cell adhesion, cell-cell contact, motility

The mechanism by which CDK5 mediates the senescence morphological alteration has yet to be investigated, but it at least in part involves the Rho family of small GTPases and ERM family member Ezrin (Alexander et al., 2004; Yang and Hinds, 2003).

The Rho GTPases, including Rac1, Cdc42, and RhoA, have been implicated in the establishment of cell– cell contacts and of cell–matrix interactions and they are known for their regulation of the actin cytoskeleton and cell morphology (Hall, 1998). Under aberrant conditions, however, they have been implicated in motility, invasion, and some aspects of metastasis (Schmitz et al., 2000). Studies investigating the relationship between Ras and Rho proteins have shown that Ras can activate Rac1 (Ridley et al., 1992). However, in Ras-transformed fibroblasts, Rac1-GTP levels are reduced and RhoA-GTP levels are increased (Sahai et al., 2001). Our previous findings showed that repression of the activity of Rac1 by CDK5 is required for actin polymerization accompanying senescent morphology. In addition, CDK5 directly phosphorylates PAK1 (p21-Activated Kinase), a Rac1 effector, and results in downregulation of PAK1 kinase activity (Alexander et al., 2004). In chapter 3, we reported that Ras/Dnp53-transformed mouse embryonic fibroblasts (MEFs) exhibited increased motility in a *Cdk5*-null background, which we speculated was due to an increased Rac1 activity in the absence of CDK5. In fibroblasts, the formation of membrane ruffles, the actin-rich structures at

the leading edge of the cell, is Rac-dependent (Ridley et al., 1992), and is a driving force of cell movement. In contrast, RhoA inhibits migration by regulating the formation of actin stress fibers and focal adhesions (Amano et al., 1997; Arthur and Burridge, 2001; Nobes and Hall, 1995), which are also prominently increased in senescent cells. Consistently, unpublished data in our lab showed that while normal SAOS-2 cells were able to migrate, senescent SAOS-2 cells didn't exhibit any detectable motility. Therefore, by suppressing Rac1 activity, and thereby elevating relative signaling by RhoA, CDK5 might help to make cells less motile.

In addition to the shape change, senescent cells typically acquire increased adhesion to the extracellular matrix while losing cell–cell contacts. Similarly, in lens epithelial cells and keratinocytes, CDK5 overexpression increases cell-matrix interactions, whereas it decreases cell-cell adhesion (Nakano et al., 2005; Negash et al., 2002). Moreover, in corneal epithelial cells, CDK5/p35 activity correlates with increased cell adhesion and decreased migration, and inhibition of CDK5 promotes corneal wound closure *in vivo* (Gao et al., 2002; Tripathi et al., 2008). A plethora of functionally diverse proteins involved in cytoskeleton dynamics, cell adhesion and migration have been identified as CDK5 substrates both in the CNS and in other non-neuronal settings. For example, β-catenin is a substrate of CDK5, and phosphorylation by CDK5 regulates its association with the Cadherins (Kesavapany et al., 2001; Kwon et al., 2000; Nakano et al., 2005). CDK5 can phosphorylate nonreceptor tyrosine kinases Src and FAK, which are associated with the integrins (Kato and Maeda, 1999; Xie et al., 2003).

(2) Invasion and metastatic potential

Acquisition of cell motility is a prerequisite for progression of neoplastic cells to the malignant phenotype. Oncogenic deregulation, apart from a proliferative

hyperactivity, can induce a switch in cells from a stable adherent phenotype to a motile and invasive one. For example, while an untransformed adherent cell undergoes a particular form of apoptosis known as anoikis after a loss of adhesion, the activity of certain oncogenes appears to be able to overcome this effect. In chapter 3, we reported an enhanced anchorage-independent growth of *Cdk5*-null MEFs when transformed by oncogenic Ras and dominant negative p53. Moreover, in addition to increased motility, these transformed cells also showed increased invasion by *in vitro* assays. However, it is difficult, at present, to extrapolate these findings to the role of CDK5 in tumor invasiveness and metastasis. Several lines of evidence actually indicate that CDK5 might promote these processes. CDK5/p35 activity has been shown to be important for invasion and metastasis in various types of carcinomas. And a poorer prognosis has been recently linked to CDK5/p35 in patients with non-small cell lung cancer (NSCLC) (Feldmann et al., 2010; Liu et al., 2010; Liu et al., 2008; Strock et al., 2006). Moreover, the ERM family protein Ezrin, which was previously identified as a direct target of CDK5, has also been implicated in promoting metastasis (Khanna et al., 2004; Yu et al., 2004).

Effects of CDK5 activation can be varied due to cell type, timing, protein localization, the nature of the activating signals and differences between the *in vitro* and *in vivo* microenvironment. It is to be hoped that further work, using *in vivo* models of tumor progression and more detailed analysis of the expression of CDK5 and its regulators in human tumors, might resolve these issues.

(3) Protein secretion/trafficking

The striking change in cell morphology observed during cellular senescence very likely reflects, at least in part, the functional changes that accompany replicative senescence. In the case of fibroblasts, upon senescence, cells switch from a matrix-

producing phenotype to a matrix-degrading phenotype. Senescent fibroblasts produce less collagen and elastin, compared to presenescent cells. Meanwhile, senescent cells overexpress several matrix metalloproteinases. These enzymes degrade the stromal fibers and extracellular matrix. Senescent fibroblasts also secrete pro-inflammatory cytokines and a variety of epithelial cell growth factors, which, combined, is termed the 'senescence secretome'. Thus, senescent fibroblasts, despite their inability to divide, adopt a phenotype that at least partially resembles a wounding response. As discussed in the introduction, this senescence-associated secretory phenotype may have a profound impact on tumor development and organismal aging by changing the local tissue environment. In Chapter 2, we reported that CDK5/p35 is required for the induction of the secretory phenotype in senescent SAOS-2 cells. This may reflect the function of CDK5 in modulating protein trafficking and secretion in other systems such as synaptic activity in neurons and insulin secretion in pancreatic β-cells

(4) A possible cytoskeletal checkpoint

Why would CDK5/p35, the primary function of which is to regulate the cytoskeleton, be coupled to cellular senescence, one of the key mechanisms to inhibit proliferation? Among the substrates of CDK5, many have both structural and signaling functions, for example, Rho proteins, integrin, β-catenin, Nestin, FAK, Src, Ezrin, etc. Therefore, CDK5 might be a good candidate for a checkpoint protein that relays physical cues to the cell-cycle machinery, ensuring cells proliferate only in the correct environment. Activation of CDK5 in senescent cells in response to a variety of stresses may thus help to reinforce growth arrest and lock the cells in a 'non-proliferating' state.

Conclusion: The CDK5/p35-mediated morphological change in senescent cells is presumed to reflect some important alterations in their biological functions and

physiological traits. Although these have not been fully elucidated in the present study, they may well be better understood following analyses such as those suggested above, based on known and suspected relationships between CDK5 and important cellular signaling networks.

III. Cdk5 and cancer.

The cellular senescence program has evolved to curtail proliferation of cells bearing oncogenic mutations in benign lesions, and therefore acts as a natural barrier against tumorigenesis. During the last decades it has become apparent that not only apoptosis, but also senescence determines the outcome of cancer therapy.

In the present study, CDK5/p35 is shown to be required for the acquisition of changes in senescent morphology, as well as expression of the senescent secretome in osteosarcoma cell lines. Expression of Cdk5 is also required for the induction of senescent markers in the murine fibrosarcoma. As discussed in part I, CDK5 can be activated in response to various stresses, in particular oxidative stress, in both neuronal and non-neuronal settings. In addition, CDK5/p35 has been implicated in apoptosis in a variety of tumor cell lines (Lin et al., 2004; Sandal et al., 2002; Upadhyay et al., 2008). Therefore, it is very likely that CDK5 can be induced in various situations of stress and result in senescence in some types of cancers, and thus contributes to the outcome of drug treatment. Although our data show that CDK5 deficiency in MEFs doesn't strongly affect cell proliferation properties, or tumor formation of H-Ras/DNp53–transformed cells when injected subcutaneously into nude mice, loss of CDK5 does seem to facilitate anchorage-independent growth in the transformed MEFs, suggesting that even though CDK5 loss per se is not sufficient to induce tumorigenesis, it might cooperate with other mutations to facilitate malignant transformation. To further investigate if CDK5 functions

as an extra layer of safety, it will be interesting to see if conditional loss of Cdk5 accelerates progression to malignancies when combined with other oncogenic events in particular tissue types. To this end, two tumor models in our lab, namely, MMTV-*neu* and Tyr-*BRAF*^{V600E} transgenic mice, can be used to examine the effect of CDK5 deficiency on the formation and progression of mammary tumors and melanoma, respectively. Interestingly, a re-analysis of previously published data from breast cancers (Chin et al., 2006) revealed that genomic loss of CDK5 occurred in 5.5% (8/145) of breast cancers, with evidence of homozygous loss in one cancer (Turner et al., 2008). Moreover, a putative site, which lies in close proximity to the Ras binding domain, has been reported for CDK5-mediated phosphorylation in BRAF (Wissing et al., 2007).

The present study also shows that CDK5 loss promotes migration and invasion of transformed MEFs *in vitro*, suggesting that CDK5 might control tumor cell motility and metastatic potential. Given the fact that CDK5 is crucial to neuronal migration during embryogenesis, an implication of CDK5 in metastasis might be envisioned. Notably, contrary to our findings, studies by the Nelkin group have shown that ablation of CDK5 inhibits migration and invasion of prostate and pancreatic cancer cells *in vitro*. Moreover, CDK5 activity is important for lung metastasis of subcutaneously injected prostate cancer cells and systemic metastasis of pancreatic cancer cells in an orthotopic xenograft model (Feldmann et al., 2010; Strock et al., 2006). However, the seemingly contradictory observations might reflect effects of CDK5 on cell migration that depend on cell type, supporting substrate, and the particular genetic makeup of a cell.

Finally, CDK5 might also influence tumorigenesis through its regulation of the secretome in senescent cells. Senescent fibroblasts stimulate the growth of preneoplastic cells in several relevant cancer models (Campisi, 2001; Krtolica et al., 2001), but it has also been shown that senescence triggers tumor regression, at least in

part due to clearance mediated by an innate immune response (Xue et al., 2007). This, again, highlights the importance of assessing CDK5 function at the tissue and organismal levels in future studies.

Conclusion:

The use of mouse tumorigenesis models, together with studies on human tumors, should help to identify the important connections that link CDK5 functions to cancer *in vivo*. Such studies will surely provide novel insights into our understanding of both CDK5 and senescence in tissue homeostasis and cancer progression, and hopefully, will be useful leads in the design of drugs and development of therapies that can be taken into the clinic.

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