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p16$^{\text{INK4A}}$ and p15$^{\text{INK4B}}$

in senescence, immortalization

and cancer

Gene transfer by adenovirus vectors

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ABSTRACT

The astrocytic gliomas are the most common form of brain tumors in humans. The malignant progression from low grade (astrocytoma, grade II) to high-grade (glioblastoma multiforme, grade, IV) astrocytomas is well documented and is accompanied by an increasing number of genetic aberrations. Many of these genetic alterations center round genes that regulate the G1/S transition of the cell cycle. This is particularly the case with the genes encoding the cyclin dependent kinase inhibitors (CDKIs), p16^{INK4A} (p16) and p15^{INK4B} (p15). They are homozygously deleted in 40–50% of primary gliomas and in an even higher percentage in glioma cell lines. In addition, the p16 gene is frequently inactivated by point mutations and by DNA methylation, and mice that are deficient for the p16 gene spontaneously develop tumors within their first year of life. While the role of p16 in senescence and tumor suppression has been amply documented, the role of p15 remained less clear.

Recombinant adenoviruses (Ad) expressing p15 (Adp15) or p16 (Adp16) were constructed and used to infect human glioma cells with different status of the p15, p16, and pRB genes. The results indicated that p15 is as potent as p16 in inducing cell cycle arrest and senescence-like changes in human glioma cells with an intact pRB. In addition, both p15 and p16 strongly inhibited telomerase activity in these cells. This shows the potential of p15 to function as a tumor suppressor and mediator of senescence. Together with the fact that p15 accumulates in T lymphocytes as they approach senescence and that p15 deficient mice display tumors of the hematopoietic cell system, these results suggest that p15 plays a role in regulating homeostasis in these (and perhaps other) cell systems.

The mechanism behind the synthesis of an alternative isoform of p15 was also elucidated. This protein, termed p15.5, was found to be an N-terminally extended variant of p15, initiated from an upstream GUG codon. It was shown to separately, or in combination with p15, interact with both CDK4 and CDK6, and to induce cell cycle arrest and a senescent-like phenotype when transfected into human glioma cells.

The involvement of the p16/pRB pathway components in telomerase-mediated immortalization of bovine capillary endothelial (BCE) cells was determined. In these studies, transfection of the catalytic subunit of human telomerase (hTERT) alone was sufficient to immortalize BCE cells. Surprisingly, the telomere lengths in the hTERT-BCE cells were consistently shorter than in the senescent parental cells. pRB was hyperphosphorylated, and the expression of p16 (and p21^{CIP1}) was repressed by promoter methylation. Reactivation of p16 by either Ad-mediated expression or by demethylation reversed the immortalized phenotype and induced senescence-like changes. These results suggest that the immortalization of BCE cells by hTERT is mediated through inactivation of crucial cellular senescence machineries including p16/pRB and p21^{CIP1}, proteins that also by others have been implicated in inducing and maintaining a senescent phenotype in mammalian cells.

The ability of type C Ad to infect human cells is dependent on the expression of the coxsackie- and adenovirus receptor, hCAR. Since very little is known about the expression of hCAR in primary human gliomas and since hCAR has been implicated to function as a tumor suppressor, such expression studies were performed. A great variation in hCAR expression was detected, both in glioma cell lines and in the primary tumors, and the expression correlated well with Ad infectability. A significant decrease in the mean CAR expression levels was detected in the grade IV tumors as compared to the values for the grade II and grade III tumors. Moreover, a mean 12-fold higher expression of hCAR was detected in xenografts derived from glioblastomas, compared to the parental tumors. Interestingly, the two xenografts that did not show any upregulation of hCAR expression grew much faster than the hCAR-expressing cells. This points to an inverse correlation between growth rate and hCAR expression, and suggests that hCAR may contribute to suppression of tumor growth.

ABBREVIATIONS

aa	amino acid
Ad	adenovirus
ALT	alternative lengthening of telomeres
bp	base pair
CAK	CDK-activating kinase
CAR	coxsackie- and adenovirus receptor
CDK	cyclin-dependent kinase
CDKI	cyclin-dependent kinase inhibitor
CDK4	CDK4 gene
CDKN2A	cyclin-dependent kinase inhibitor 2A (= *INK4A*) gene
CDKN2B	cyclin-dependent kinase inhibitor 2B (= *INK4B*) gene
cDNA	complementary deoxyribonucleic acid
CIP	cyclin dependent kinase inhibitor protein
CpG	DNA methylation site
Dnmt	DNA methyltransferase
EGFR	epidermal growth factor receptor
FGF	fibroblast growth factor
HDAC	histone deacetylase
INK4	inhibitor of cyclin dependent kinase 4 and 6
IFNα	interferon alpha
IFNγ	interferon alpha
kb	kilobases
MDM2	mouse double minutes 2
MEFs	mouse embryonic fibroblasts
mTR	mouse telomerase RNA component
N-CAM	neural – cell adhesion molecule
p14^{ARF}	human alternative reading frame (ARF) protein
p19^{ARF}	mouse alternative reading frame (ARF) protein
p15	p15^{INK4B}
p16	p16^{INK4A}
PCNA	proliferating cell nuclear antigen
PCR	polymerase chain reaction
PDGFRα	platelet-derived growth factor receptor α gene
PDLs	population doublings
pRB	retinoblastoma protein
PTEN	phosphatase and tensin homologue deleted on chromosome 10 gene
Rb	retinoblastoma gene
RT-PCR	reverse transcriptase – polymerase chain reaction
SA-β-gal	senescence associated beta galactosidase
TERT	catalytic subunit of telomerase
TGFβ	transforming growth factor beta
TIMP	tissue inhibitor of metalloproteinase
TSP-1	thrombospondin-1
TP53	p53 gene
VEGF	vascular endothelial growth factor
WHO	world health organization

TABLE OF CONTENTS

LIST OF ARTICLES

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals:

I.   **Jonas Fuxe**, Göran Akusjärvi, Helena M. Goike, Göran Roos, V. Peter Collins, and Ralf F. Pettersson. Adenovirus-mediated overexpression of p15^{INK4B} inhibits human glioma cell growth, induces replicative senescence, and inhibits telomerase activity similarly to p16^{INK4A}. (2000) *Cell Growth & Diff.*, **11**, 373-384

II.   **Jonas Fuxe**, Elisabeth Raschperger and Ralf F. Pettersson. Translation of p15.5^{INK4B}, an N-terminally extended and fully active form of p15^{INK4B}, is initiated from an upstream GUG codon. (2000) *Oncogene*, **19**, 1724-1728

III.   Niina Veitonmäki*, **Jonas Fuxe***, Magnus Hultdin, Göran Roos, Ralf F. Pettersson and Yihai Cao. Immortalization of bovine capillary endothelial cells by telomerase is mediated by a telomere length independent mechanism. (2001) *Submitted Manuscript*.

IV.   **Jonas Fuxe***, Lu Liu*, Stephen Malin, Lennart Philipson, V. Peter Collins and Ralf F. Pettersson. Expression of the human coxsackie- and adenovirus receptor in astrocytic tumors and xenografts. (2001) *Submitted manuscript*

* Equal contributions

INTRODUCTION

Human cancer

General aspects

Cancer is a genetic disease with an overall incidence of one in three life times. More than a hundred subtypes of malignancies exist, arising from different cell types in different organs. In accordance with this, the symptoms of cancer vary, depending on the location and the growth characteristics of the tumor. A common feature of all cancers is uncontrolled cell growth – the appearance of disorganised tissues that expand without limit, compromising the function of the organs and threatening the life of the organism (Varmus, 1989). The origin of all malignant tumors is thought to be clonal (Garcia et al., 2000; Kern, 1993; Vogelstein and Kinzler, 1998), meaning that all tumor cells in a specific cancer are derived from a single normal cell that has been transformed. However, although all tumor cells that grow in a single tumor share common signitures, e.g. a specific mutation, a chromosomal aberration or an X-chromosome inactivation, different regions within the tumor show a more complex picture. In fact, as a tumor develops, the initial monoclonal population of cells may become more and more heterogeneous as some of the cells acquire new genetic aberrations. This leads to sub-populations within the tumor (Cahill et al., 1999; Hanahan and Weinberg, 2000; Nowell, 1976).

Cancer cells are genetically less stable than the normal counterparts and are therefore more prone to develop additional genetic aberrations as they grow beyond their normal life span (Cahill et al., 1999). Additional mutations as well as rearrangements, duplications and deletions of portions of their chromosomes during cell division are new traits that may be acquired during tumorigenesis. This genomic instability is thought to be due to disturbed "checkpoint" controls operating at several points in the cell cycle (Hartwell and Weinert, 1989). Cells that accumulate genetic changes that favor a more rapid and aggressive growth compete more efficiently with other cells in the surrounding tissue (Varmus, 1989). Tumor development may

therefore be regarded as a "Darwinian" evolution process, where a natural selection favours growth of cells with the most dominant pheno- and genotype (Cahill et al., 1999).

Genes involved in cancer

Traditionally, cancer genes are classified as oncogenes or tumor suppressor genes. Oncogenes are mutated dominant acting variants of normal genes called proto-oncogenes (Hanahan and Weinberg, 2000; Weinberg, 1995). They act by overexpressing proteins that stimulate cell proliferation in an aberrant fashion. Some oncogenes code for growth factors, like platelet-derived growth factor (PDGF) or transforming growth factor alpha (TGFα). Others encode oncogenic versions of growth factor receptors such as the epidermal growth factor receptor (EGFR), which is being overexpressed in e.g. high-grade gliomas and ErbB2, which is over-expressed in breast carcinomas.

The tumor suppressor genes, e.g. *TP53, Rb, INK4A*, normally act as negative regulators of cell growth, and/or are involved in cellular processes like apoptosis, differentiation and senescence (Brown, 1997; DiCiommo et al., 2000; Fisher, 2001; Grander, 1998; Macleod, 2000; Weinberg, 1995). In tumor cells, the damaged or altered function of such genes makes the cells divide in a fast and uncontrolled manner. Already in 1971, Knudson proposed the "two-hit" hypothesis for the involvement of tumor suppressor genes in tumorigenesis. He suggested that tumor suppressor genes function in a recessive manner, i.e. that both alleles must be mutated for a phenotype to occur (Knudson, 1971). This hypothesis still holds true for tumor suppressor genes in general. *TP53,* the most commonly mutated or deleted gene in human cancer, represents an exception from that rule. Disruption of only one allele gives profound consequences, either in the germline of patients with Li-Fraumeni Syndrome, or in mice with targeted gene knockouts (Fisher, 2001). p53 has a dual role in regulating cell growth because it can both induce cell cycle arrest as well as apoptosis. A third class of cancer genes that have been identified is the DNA repair genes. They normally take part in the repair of DNA damage, and loss of their function leads to increased genetic instability (Cleaver, 1994).

Taken together, the number of different genes involved in human cancer is continuously growing. One can therefore argue that the search for the origin and complexity of the disease will continue for quite some time. However, others are now beginning to foresee cancer research develop into a logical science, where the complexities of the disease will become understandable in terms of a small number of underlying principles (Hanahan and Weinberg, 2000). Hanahan and Weinberg suggest that research over the past decade has revealed a small number of molecular, biochemical, and cellular traits – acquired capabilities – shared by most and perhaps all types of human cancer. By classifying cancer-associated genes by their normal function and role in different cellular processes they suggest that in total, there are six essential alterations in cell physiology that manifest malignant growth (Hanahan and Weinberg, 2000). These six acquired capabilities are:

1. Self-sufficiency in growth signals – e.g. oncogene activation; PDGF, TGFβ
2. Insensitivity to antigrowth signals – e.g. loss of pRB, p15^{INK4B}, TGFβ receptors.
3. Resistance to apoptosis – e.g. loss of p53, upregulation of anti-apoptotic *bcl-2*.
4. Limitless replicative potential – e.g. activation of telomerase or ALT, loss of p16^{INK4A}.
5. Sustained angiogenesis – e.g. upregulation of VEGF, FGF, loss of IFNα, TSP-1.
6. Tissue invasion and metastasis – e.g. loss of E-cadherin, N-CAM.

Some of these acquired capabilities operate on a cell-autonomous basis, and others are coupled to signals that cells receive from their surrounding microenvironment.

Human astrocytomas

General features

The astrocytic gliomas are the most common form of brain tumors in humans. They are sub-classified, according to the World Health Organization (WHO) (Kleihues and Cavenee, 1997), into four different malignancy grades: pilocytic astrocytoma (grade I), astrocytoma (grade II), anaplastic astrocytoma (grade III) and glioblastoma multiforme (grade IV). The highest malignancy grade, glioblastoma, is also the most common grade at clinical presentation (Kleihues and Cavenee, 1997; Russell and Rubinstein, 1989), and is associated with a mean survival time of less than one year (Barker et al., 1996; Furnari et al., 1995). Glioblastomas grow invasively into the surrounding tissue, but does not metastasize outside the CNS. Other characteristics of this tumor type are areas of necrosis and pronounced proliferation of endothelial cells. Surgery, when possible, is the primary treatment, supplemented with radiotherapy and/or chemotherapy. The benefit of the two latter modes of treatment for survival is marginal. The prognosis for glioblastomas has remained nearly unaltered for the last 30-40 years. Thus, there is a great need for new and more efficient treatment modalities for glioblastomas.

Genetic alterations in gliomas – a model for malignant progression

The genetic aberrations found in astrocytic gliomas follow distinct patterns. Generally, a higher malignancy grade is associated with a higher frequency of genetic abnormalities. This suggests malignant progression and indeed, progression from low grade astrocytomas to high grade glioblastomas is today a well documented phenomenon which is accompanied by an increasing number of genetic abnormalities (Collins, 1999) (Fig. 1). Many of the genetic lesions found in gliomas center around genes regulating the progression through the G1 phase of the cell cycle. For example, mutations in different tumor suppressor genes such as *TP53*, *PTEN* and *Rb* are often detected. Oncogenes such as CDK4 and MDM2, as well as growth factor receptors such as EGFR, PDGFR-α, are amplified in a number of cases.

Astrocytoma (A) *grade II*	Anaplastic Astrocytoma (AA) *grade III*	Glioblastoma (GB) *grade IV*
10p	10p/q	10 (*PTEN*)
13q (*Rb*)	13q (*Rb*)	13q (*Rb*)
17p (*TP53*)	17p (*TP53*)	17p (*TP53*)
22q	22q	22q
	6q	6q
	9p (*INK4A,INK4B*)	9p (*INK4A,INK4B*)
	11p	11p
	12q amp (*CDK4*)	12q amp (*CDK4*)
	19q	19q
		4q (*PDGFRα*)
		7p (*EGFR*)

Figure 1. Genetic abnormalities in human astrocytic gliomas of malignancy grade II-IV. An increasing number of genetic lesions is detected with higher malignacy grades. Many of these genetic abnormalities center around genes that are involved in the G1/S transition of the cell cycle. *The experimental findings that support this figure are reviewed in Collins, V.P., 1999.*

A locus on the short arm of chromosome 9 (9p21) which is deleted in 40% of human glioblastomas harbor the two genes *CDKN2A* (or *INK4A*) and *CDKN2B* (or *INK4B*) (Ichimura et al., 1996; Schmidt et al., 1994). They code for the three tumor suppressors p16^{INK4A}, ARF and p15^{INK4B}. In total, the majority of glioblastomas have abnormalities in the pRB- and p53-pathway genes that normally are involved in the regulation of the G1/S transition of the cell cycle. Interestingly, genes inactivated in the pRB pathway are mutually exclusive in glioblastomas. Individual tumors have either loss of the *INK4A*/*INK4B* loci or pRB, or they overexpress CDK4 (Ichimura et al., 1996). Only 3% of glioblastomas show two of these abnormalities. If tumors with loss of p16^{INK4A} expression without any detectable genetic lesions, but with

promoter hypermethylation (discussed below) are included then 90% of glioblastomas show these abnormalities (Collins, 1999).

The cell cycle

Overview

The cell cycle is the process by which cells multiply by cell division. It plays an essential role in the normal development of multicellular organisms and in tissue homeostasis. Since loss of cell cycle control ultimately leads to cancer, it is a process that needs to be tightly regulated. This is achieved by internal regulatory machineries, which ensure that the cell only goes through the cell cycle and divides if the genome is intact. One might argue that the cell cycle represents an intrinsic event of the cell. However, the cell cycle is regulated at many different levels. Only a few percentage of the cells in a specific tissue at a specific time point, are dividing. In the brain for example, most neurons are terminally differentiated and do not divide at all. In the epithelial cell layers of the skin and of the gastrointestinal tract, the basal cell layer contains actively dividing stem cells, while the upper cell layers harbor more differentiated cells that have stopped dividing. In cellular processes like senescence cells exit the cell cycle permanently. Wound healing, on the other hand, is a process in which growth stimulatory signals from the surroundings make cells divide more rapidly. In cell culture, different growth conditions can force cells in and out of the cell cycle. In general, a cell in a multicellular organism can not simply be regarded as an isolated functional unit. Instead, not just the whole organism, as such, but also the single cell is dependent on interactions with its neighbors. It is therefore more correct to say that the cell cycle is an intrinsic event that is regulated by extrinsic signals from its surroundings.

The regulation of the cell cycle is mainly focused on two critical events: the initiation of nuclear DNA synthesis (S phase) and the initiation of mitosis (M phase)(Fig. 2). In early embryonic cell cycles, the S phase and the M phase are the only two phases present, and the cycle alternates rapidly between them. In somatic cells, two gap phases exist, separating S- and M-phase in a temporal manner. These are termed the G1 phase (the first gap), and the G2 phase (the second gap). In G1, the cell prepares for DNA synthesis and checks the integrity of the

DNA before it enters the S phase. In the G2 phase, the duplication of the genome has been completed, and the cell prepares for mitosis. Results from pioneering studies on the cell cycle in yeast and other organisms have established the need for a temporal order of the S and M phase, to ensure that they alternate in a correct manner (Hartwell and Weinert, 1989; Nurse, 1994). To ensure that the onset of one cell cycle phase is not initiated before the previous one is completed in a correct way a number of checkpoints, at which cell cycle progression will stop unless certain criteria are met, have evolved (Hartwell and Weinert, 1989; Nurse, 1975; Pardee, 1974). Two of the most critical checkpoints occur at the G1/S and the G2/M transitions.
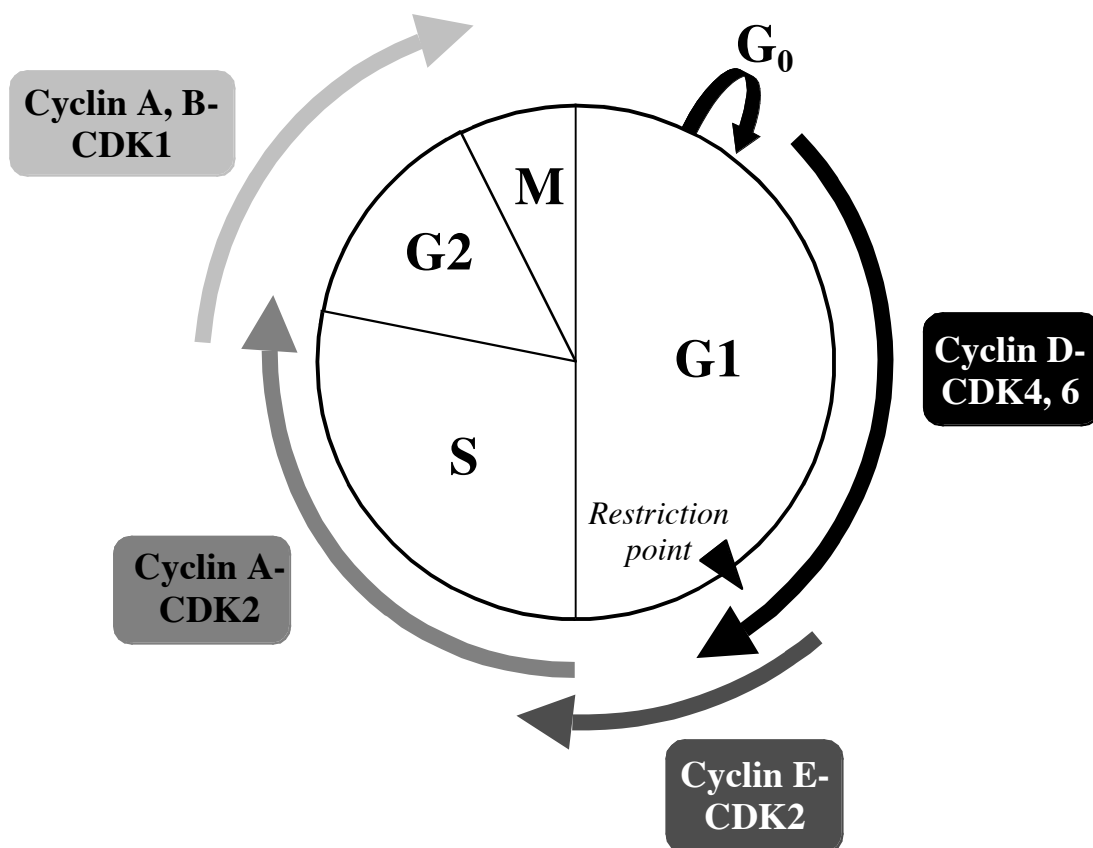

Figure 2. The mammalian cell cycle. Somatic cell cycles consist of alternating DNA synthetic (S) and mitotic (M) phases, which are separated by two gaps (G1 and G2). Specific combinations of cyclins and CDKs are active in different phases of the cell cycle.

The cell cycle engine

The cell cycle is driven by the cyclin dependent kinases (CDKs) (Murray, 1994; Pardee, 1989; Pines, 1994; Pines, 1995). The first member of this family was identified in yeast (Hartwell et al., 1974), but homologues have been found in humans (Lee and Nurse, 1987), as well as in all other examined organisms (Pines, 1995). The CDKs are proteins with molecular weights of approximately 34 kDa that are highly homologous to each other. They are expressed at similar levels throughout the cell cycle, but cannot function as active serine/threonine kinases on their own. Instead they become activated by binding to regulatory cyclins, which were originally discovered as proteins that are expressed at different levels in different phases of the cell cycle (Evans et al., 1983; Rosenthal et al., 1980). Because of their cyclic expression pattern, they were named cyclins. The cyclins consist of a family of proteins that share a 150 amino acid region of structural homology, called the cyclin box. This is the region responsible for binding to the CDK subunit. The active cyclin-CDK complexes drive the cell cycle by phosphorylating phase-specific substrates, which induce the transcription of critical enzymes for DNA replication, nuclear envelope breakdown, chromosome segregation etc. Each combination of cyclin and CDK is formed and activated in a specific phase of the cell cycle (Evans et al., 1983; King et al., 1996; Koch and Nasmyth, 1994; Motokura and Arnold, 1993; Pines, 1995; Sherr, 1994). When their role has been completed, they are subsequently inactivated by rapid ubiquitination and cyclin degradation.

Regulation of the G1 checkpoint

The G1 phase is the period of the cell cycle in which the cell responds to external signals. If such signals, received by receptors on the cell surface and transmitted to the nucleus via intracellular signaling pathways, are of a stimulatory kind, the cell completes the G1 phase and eventually enters S phase. In the absence of such signals, or, if growth inhibitory signals are present, the cell may exit the cell cycle into a quiescent state, termed G0. In late G1 phase, a checkpoint originally identified as START (S) was identified in yeast by a mutation in the *cdc28* gene (Hartwell et al., 1974).
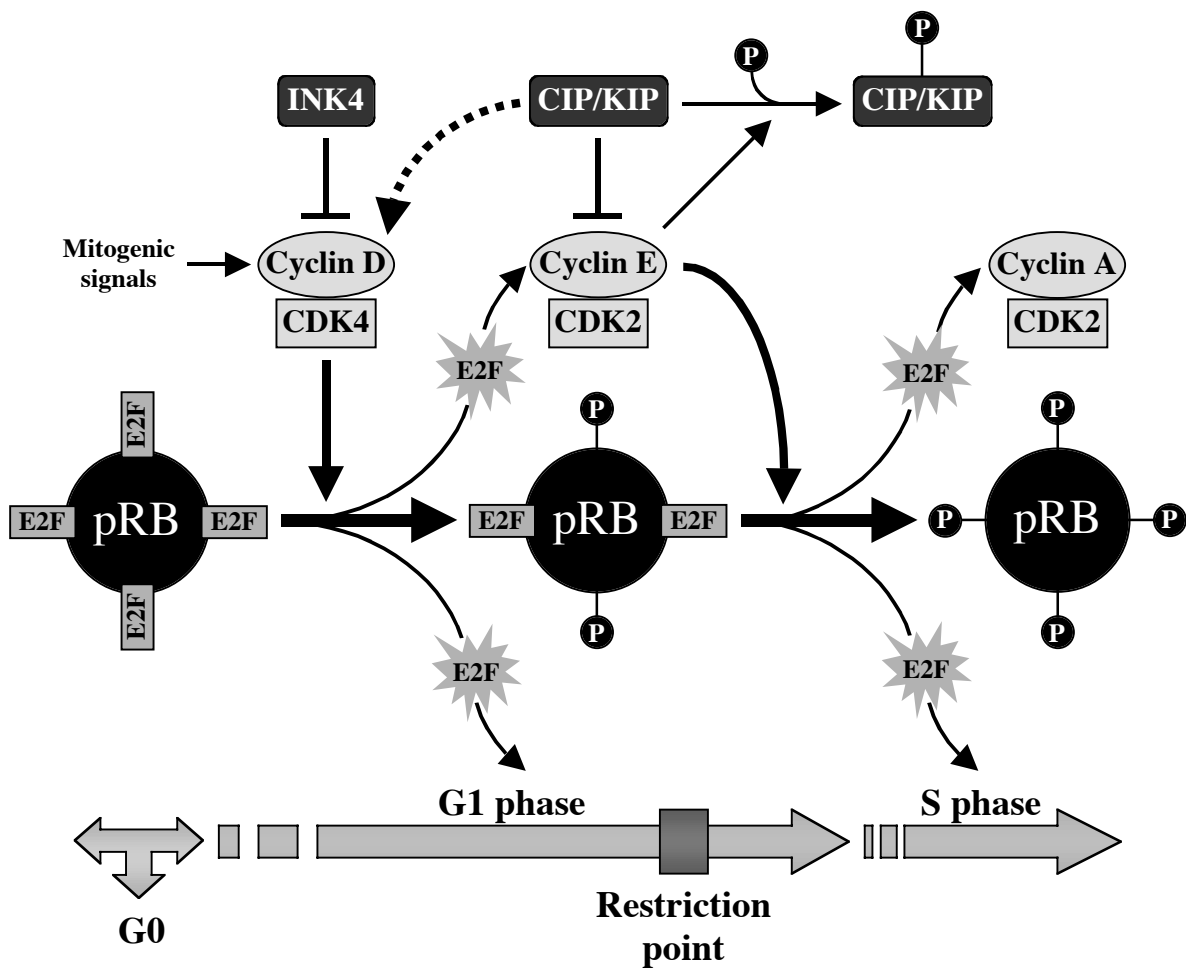
Figure 3. Regulation of the G1 phase of the the cell cycle. Mitogenic stimuli activate Ras-signaling and subsequently the expression of the D-type cyclins. They form active kinase complexes together with CDK4 or CDK6, and members from the CIP/KIP family. These complexes function by phosporylating pRB, which then releases E2F transcription factors. The E2Fs initiate transcription of cyclin E, plus a variety of genes that are needed for S phase. Cyclin E/CDK2 complexes then phosphorylates both pRB (on additional sites) and the CIP/KIP inhibitors, which leads to their degradation. These phosphorylation steps contributes to the irreversability of the cell cycle progression that is achieved in the G1 restriction point. The INK4 inhibitors specifically bind and inhibit CDK4 and CDK6. While the CIP/KIP proteins bind to and inactivate cyclin E/CDK2, their binding to cyclin D/CDK4/6 is essential for the function of these complexes. *Modified from Malumbres et al., 2000*

This checkpoint was subsequently identified as the restriction point (R) in mammalian cells (Nurse, 1994; Pardee, 1974; Zetterberg et al., 1995) (Figures 2 and 3) exists. This is a highly regulated event that governs the integrity of the chromosomal DNA before the cells enter S phase. When cells have passed through the G1 restriction point, they complete the whole cell cycle and divide into two daughter cells.

If quiescent cells are stimulated with mitogenic growth factors a continuous supply of these factors is generally needed until the cells pass the restriction point. At that moment, cells become independent of such mitogenic stimuli (committed) and enter the S phase even in their absence.

Conversely, cells progressing through the G1 phase are sensitive to anti-proliferative growth factors, such as transforming growth factor beta (TGF-β), before they reach the restriction point.

G1 cyclins and cyclin-dependent kinases

Two classes of CDKs function and drive the cell cycle through the G1-S transition (Draetta, 1994; Reed, 1997; Sherr, 1994); the cyclin D- and the cyclin E-dependent kinases. The D-type cyclins (cyclin D1, D2, and D3) are expressed in a tissue-specific manner (Sherr, 1993), and they exclusively interact with CDK4 and CDK6 (Matsushime et al., 1992; Matsushime et al., 1991; Meyerson and Harlow, 1994; Motokura et al., 1991; Xiong et al., 1991). Expression of the D-type cyclins is induced in response to mitogenic signals, and their synthesis is highly dependent on those. If growth factors are withdrawn, cyclin D synthesis ceases immediately (Matsushime et al., 1991). Both the transcriptional activation of the cyclin D1 gene and the formation of the cyclin D1/CDK4 complexes is mediated by Ras, which signals via a kinase cascade that involves Raf-1, mitogen-activated protein kinase kinase (MEK1 and MEK2), and extracellular signal-regulated protein kinases (ERKs) (Filmus et al. 1994; Albanese et al. 1995; Lavoie et al. 1996; Winston et al. 1996; Aktas et al. 1997; Kerkhoff and Rapp 1997; Weber et al. 1997). When the cyclin D/CDK 4/6 complexes are formed, they enter the cell nu-

cleus where they become activated by phosphorylation. This is crucial for their function as kinases and is mediated by a cdk-activating kinase (CAK).

The active cyclin D/CDK 4/6 kinases act by phosphorylating the retinoblastoma protein (pRB), which binds to various members of the E2F family of transcription factors (Fig. 3). When pRB becomes phosphorylated its association with the E2F:s is disrupted (Sherr, 1996; Taya, 1997; Weinberg, 1995). Free E2F molecules then bind to and activate the transcription of multiple target genes, including cyclin E and cyclin A, which are essential for S phase entry (Dyson, 1998; Nevins, 1998). Accumulation of cyclin E subsequently leads to activation of cyclin E/CDK2 complexes, which complete the G1-S transition by phosphorylating pRB on additional sites. This shift in pRB phosphorylation from the mitogen-dependent cyclin D-CDK4/6 to the mitogen-independent cyclin E-CDK2, coincides with cells passing through the G1 restriction point (Fang and Newport, 1991; Krude et al., 1997) (Fig. 3). Beyond the G1-S transition, pRB is kept in a hyperphosphorylated state by the action of the cyclin A- and B-dependent CDKs. It is not until the cell exits mitosis and enters the next G1 phase that pRB returns to its hypophosphorylated state (Ludlow et al., 1993; Ludlow et al., 1990).

pRB pocket proteins

Rb was originally identified as a gene that when it was mutated in the germ line resulted in the development of retinoblastoma (Friend et al., 1986; Lee et al., 1987). It has since then been found to be mutated in a large variety of tumors and is regarded as the prototype tumor suppressor gene. Individuals heterozygous for *Rb* have a 95% risk of developing retinoblastoma (Gallie et al., 1990) and *Rb* heterozygous mice (*Rb-/+*), develop tumors of the pituitary and thyroid (Clarke et al., 1992; Jacks et al., 1992; Lee et al., 1992). Mice with a targeted deletion of both *Rb* alleles (*Rb-/-*) die during embryonic development. Additional evidence that pRB plays a key role in regulating cell proliferation is that many DNA tumor viruses, with the capacity to transform cells, act by targeting pRB. The adenovirus E1A protein (Egan et al., 1989; Whyte et al., 1988), the simian virus 40 (SV40) large T antigen (DeCaprio et al., 1988; Dyson

et al., 1989), the human papilloma virus (HPV-16) E7 protein (Munger et al., 1989), and the large T antigen of polyomaviruses (Dyson et al., 1990), all directly bind and inactivate pRB. This undermines pRB's ability to bind to E2F and thereby also its role in growth suppression. The consequence is an excess of free E2F, which acts by pushing cells into a hyperproliferative state.

Two other proteins, p107 and p130, belongs to the same family as pRB (Classon and Dyson, 2001). They have the similar over all structure as pRB and also contain the highly conserved "pocket" domains, which are specific for proteins within this family. These pocket domains are used for interactions with both viral oncoproteins and E2F transcription factors (Ewen et al., 1992; Ewen et al., 1991; Hannon et al., 1993). pRB is expressed at a constant, moderate, level throughout the cell cycle (Buchkovich et al., 1989; Chen et al., 1989; DeCaprio et al., 1989; Mihara et al., 1989). In contrast, p130 is highly expressed in quiescent and differentiated cells, but is downregulated in cells that are pushed into the G1 phase by mitogenic signals (Cobrinik et al., 1993; Smith et al., 1996). The expression of p130 then stays at quite low levels in dividing cells. p107 is expressed in an opposite pattern, being not very abundant in terminally differentiated cells (Kiess et al., 1995; Raschella et al., 1997; Shin et al., 1995), but increasing in cells entering the cell cycle (Beijersbergen et al., 1995; Smith et al., 1996). In sharp contrast to pRB, neither p107 nor p130 seem to have any tumor suppressive properties. None of these genes are mutated or deleted in human cancer and in contrast to *Rb-/-* mice, mice lacking p107 (p107-/-) or p130 (p130-/-) are born healthy and do not develop tumors (Cobrinik et al., 1996; Lipinski and Jacks, 1999).

Apart from its role in modulating transcriptional activity by sequestering the E2Fs, pRB also actively, in complex with E2Fs, takes part in repressing transcription (Bremner et al., 1995; Sellers et al., 1995; Weintraub et al., 1995). The transcription of several cell cycle genes (e.g. cyclin A, cyclin E, CDK2, cdc2) which contain E2F sites in their promoters is repressed by pRB/E2F complexes (Dyson, 1998; Weinberg, 1995). In fact, active repression of transcrip-

tion of these genes is required for pRB-mediated growth suppression (Zhang et al., 1999). Studies to find out the mechanism behind the pRB/E2F mediated repression have revealed a direct link between cell cycle regulation and chromatin remodelling (Zhang and Dean, 2001). pRB binds to histone deacetylases (HDACs), which function to reverse histone acetylation, a process in which nucleosomes in the chromatin structure are "unwrapped" to give transcription factors free access to promoters in the DNA. The recruitment and binding of HDACs by pRB/E2F to certain promoters work in the opposite direction by promoting nucleosome formation and repression of transcription. The HDACs seem to be essential for pRB-mediated G1 arrest (Zhang et al., 2000). In addition, pRB has recently been shown to participate in chromatin remodelling by interacting with ATP-dependent nucleosome remodelling complexes (Kingston and Narlikar, 1999). The yeast SW1/SNF complex was the first member of this family of chromatin modulators that was isolated, but homologues have now been found in human and other mammalian cells as well. p107 and p130 also seem to be involved in these two different mechanisms of chromatin remodelling because they also interact with both HDAC and the human homologues of the SW1/SNF complexes, BRG1 and hBRM (Dunaief et al., 1994; Ferreira et al., 1998; Strober et al., 1996).

The CDK inhibitors (CDKIs)

The CDKs that drive the cell cycle through its different phases are also negatively regulated by a number of small proteins called the CDKIs. These are grouped into two families; the CIP/KIP family consist of the p21^{CIP1} (Gu et al., 1993; Harper et al., 1993), p27^{KIP1} (Polyak et al., 1994; Toyoshima and Hunter, 1994), and p57^{KIP2} (Lee et al., 1995; Matsuoka et al., 1995) proteins, and the INK4 family includes p16^{INK4A} (Serrano et al., 1993), p15^{INK4B} (Hannon and Beach, 1994), p18^{INK4C} (Guan et al., 1994) and p19^{INK4D} (Chan et al., 1995; Hirai et al., 1995). The CDKIs act by binding and inhibiting the function of different cyclins/CDKs throughout the cell cycle. Members of the CIP/KIP family are found in complex with the cyclin D-, cyclin E-, and cyclin A-dependent kinases. They bind to both the cyclin and the CDK subunit of the complex and by having a broad specificity for different cyclins and CDKs they act in different

phases of the cell cycle (Sherr, 1996). In contrast, the members of the INK4 family, which are composed of ankyrin repeats, specifically bind to and inhibit the function of the cyclin D-dependent kinases, CDK4 and CDK6 (Hall et al., 1995).

The CIP/KIP family

p21^{CIP1} (p21) was the first member within this family that was cloned, more or less simultaneously from different sources. One group isolated the cDNA from a cDNA library that was induced by the tumor suppressor p53 (el-Deiry et al., 1993). At the same time, others cloned the cDNA from a library that was constructed from senescent cells (Noda et al., 1994), or identified p21 as a CDK-interacting protein with a CDK inhibitory activity (Gu et al., 1993; Harper et al., 1993; Xiong et al., 1993). In addition, p21 was also found as a p53-dependent CDK inhibitor in cells subjected to ionizing radiation (Dulic et al., 1994). p21 directly interacts with the proliferating cell nuclear antigen (PCNA), a subunit of DNA polymerase-δ. When PCNA is associated together with p21 in cyclin/CDK complexes, its ability to activate DNA polymerase-δ is blocked (Xiong et al., 1992; Zhang et al., 1993). This disrupts the function of DNA polymerase-δ in DNA replication (Flores-Rozas et al., 1994; Waga et al., 1994), but not in DNA repair (Li et al., 1994; Shivji et al., 1994). The interaction between PCNA and p21 suggests a mechanism for balancing the action of the DNA replication and repair machinery's throughout the cell cycle. p27^{KIP1} (p27), is highly expressed in quiescent cells, but its levels decline when cells enter the cell cycle. p27 functions as a mediator of cell cycle arrest in response to various anti-mitogenic signals such as TGF-β, serum starvation, contact inhibition (Hengst and Reed, 1998), and IFNα and IFNγ (Grander et al., 1997).

Members within this family of CDKIs were initially thought to interfere with the function of the cyclin D-, cyclin E-, and cyclin A-CDKs. However, later experiments have shown that while the CIP/KIP proteins truly interact and inhibit the function of both cyclin E/CDK2 and cyclin A/CDK2 complexes, the binding of these proteins do not inhibit the activity of the cy-

clin D-dependent kinases (Blain et al., 1997; LaBaer et al., 1997; Soos et al., 1996) (Fig. 3). Instead, binding of CIP/KIP proteins to the cyclin D/CDK4/6 complexes is necessary for their function as active serine/threonine kinases (Cheng et al., 1999). The CIP/KIP proteins therefore have dual roles in cell cycle regulation, serving as negative regulators of the cyclin E- and cyclin-A dependent kinases but positive regulators of cyclin D/CDK4/6. The binding and sequestration of the CIP/KIP proteins show a more complex role for the cyclin D/CDK4/6 complexes during the cell cycle. In addition to phosphorylating and inactivating pRB, active cyclin D/CDK4/6 complexes also compete with cyclin E/CDK2 complexes for p21 and p27 molecules (Sherr and Roberts, 1999). Cyclin E/CDK2 not only neutralizes pRB by phosphorylation, but also inactivates p27 by the same mechanism. This leads to degradation of p27 (Montagnoli et al., 1999; Nguyen et al., 1999; Pagano et al., 1995; Sheaff et al., 1997; Vlach et al., 1997), and contributes to the irreversibility of the reaction that is achieved when cells pass through the G1 restriction point.

The INK4-family

The INK4 inhibitors, named for their ability to inhibit CDK4, induce growth arrest by specific targeting and inactivation of CDK4 and CDK6 (Chan et al., 1995; Guan et al., 1994; Guan et al., 1996; Hannon and Beach, 1994; Hirai et al., 1995; Quelle et al., 1995; Serrano et al., 1993). They form binary inactive complexes with the CDKs and block the ability of these to interact with both the D-type cyclins and the CIP/KIP proteins. Thus, activation of the INK4 inhibitors mediates transfer of CIP/KIP proteins from the cyclin D-CDK4/6 to the cyclin E-CDK2 complexes. This inactivates the latter complexes, which shows that INK4 proteins apart from being direct inhibitors of CDK4/6 also indirectly inhibit the function of CDK2.

Among the members of the INK4 family, p16^{INK4A} (p16) was the first one to be identified. It was originally discovered as a protein that interacts with CDK4 in cells transformed with SV40 virus (Xiong et al., 1993). The cDNA was cloned in a yeast two-hybrid screen with CDK4 as a bait, and it became clear that this protein acts as a strong inhibitor of CDK4 (Serrano et al., 1993). In addition, two other groups, independently, used a positional cloning

approach to map a melanoma susceptibility gene and suspected tumor suppressor gene located on chromosome 9p21 (Fig. 4a). In both cases, the gene encoding p16 was identified as the gene of interest (Kamb et al., 1994; Nobori et al., 1994). From these studies alternative names for the p16 gene were proposed; *INK4A*, *CDKN2A* and *MTS1* are all different names of the same gene. The gene encoding the second member of the family, p15^{INK4B} (p15), is located in the same genomic region as *INK4A*, only 30 kb away (Fig. 4a). The p15 gene has been termed *CDKN2B* or *INK4B,* and is transcribed in the same direction as *INK4A* (Jen et al., 1994; Stone et al., 1995). In contrast to p16, which is encoded by three exons, p15 only has two. The p16 and p15 proteins are highly homologous, displaying a 82% identity in the region of overlap, and 95% identity in exon 2. In addition, a second transcript is produced from the *INK4A* locus. This mRNA is initiated from an alternative exon 1 (exon 1β) located around 20 kb upstream of exon 1α (Fig. 4b). Both the α- and the β-transcripts are spliced into the common exon 2. However, this exon is read in two alternative reading frames, which results in two completely different protein products from the same gene (Duro et al., 1995; Mao et al., 1995; Quelle et al., 1995; Stone et al., 1995). The protein translated from the β-transcript has been named ARF (p14^{ARF} in humans and p19^{ARF} in mouse).

An alternative transcript derived from the p15^{INK4B} gene, and encoding a p10 protein has also been described (Tsubari et al., 1997). Although this protein has been shown to inhibit CDK4/6 activity in tissue cultures, it is unclear whether it is expressed and has a role *in vivo*. The genes encoding the two additional members of the INK4 family, p18^{INK4C} (p18) and p19^{INK4D} (p19) have been mapped to chromosome 1p32 (Guan et al., 1994) and 19p13 (Chan et al., 1995; Guan et al., 1996; Okuda et al., 1995), respectively. The primary sequences of p18 and p19 show about 45% identity to p16 and p15. However, the exon/intron structure, the presence of ankyrin-like motifs in the sequences, and the fact that they also function as specific inhibitors of CDK4 and CDK6, clearly reinforces them as members of the same family.

a

b

p16^{INK4a} transcript
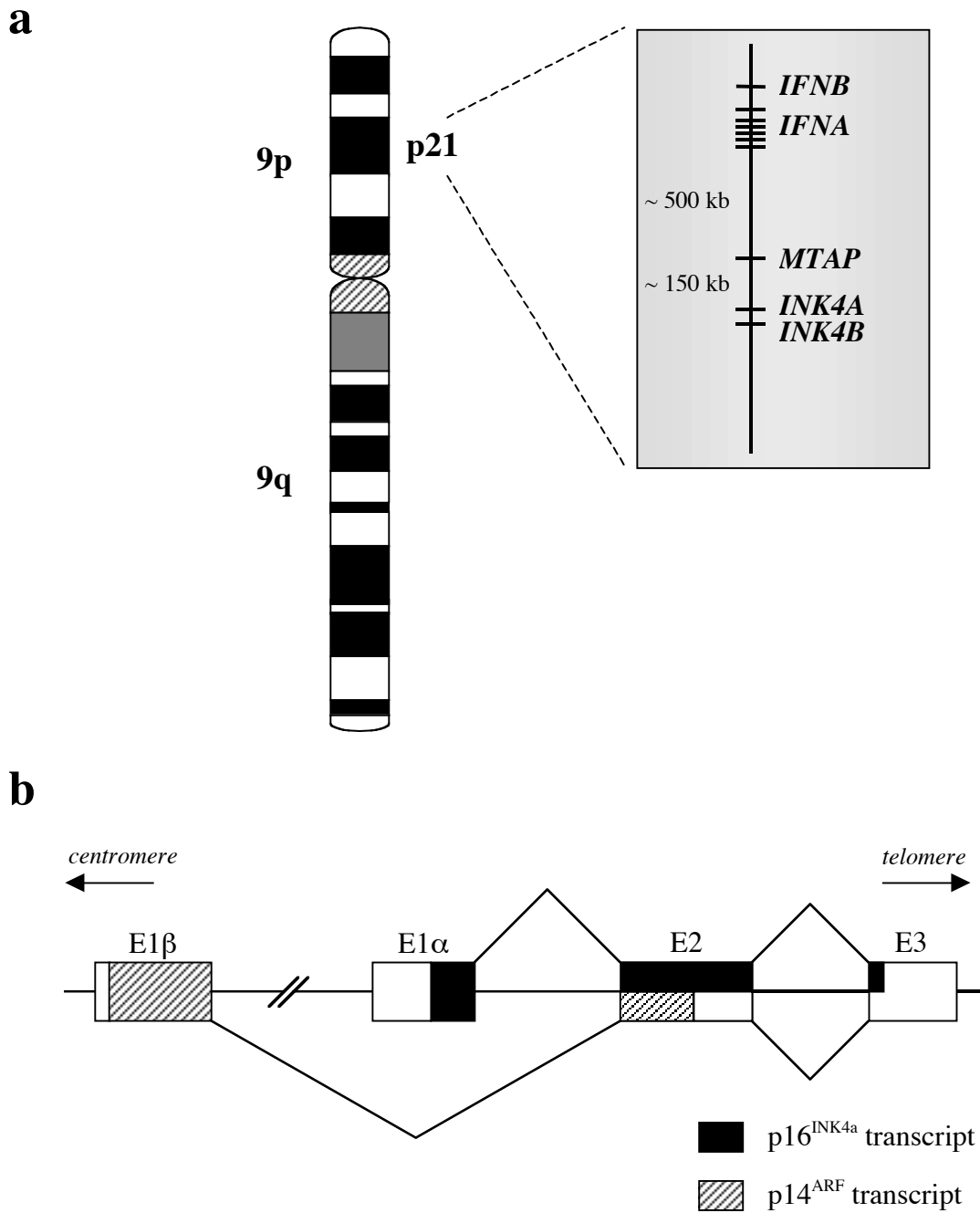
p14^{ARF} transcript

Figure 4. Chromosome 9p21 and the genomic organization of *INK4A*. **a**: The ideogram of chromosome 9 identifies band p21 and the linear map on the right shows the relative locations of several known genes. **b**: An expanded map of the INK4A locus shows the positions of the exons that encode p16^{INK4A} and ARF. *Modified from Ruas et al., 1998*

- 28 -

On the primary protein sequence level, the members of the INK4 family share the presence of ankyrin-like motifs (Guan et al., 1994; Serrano et al., 1993). Since this consensus motif is found in a large number of different proteins (Bork, 1993) with diverse functions, it has been suggested to be important from a structural point of view, facilitating protein-protein interactions. By using crystallographic and nuclear magnetic resonance (NMR) imaging to determine the three-dimensional structure of p16, p18 and p19 (Byeon et al., 1998; Luh et al., 1997; Venkataramani et al., 1998), this view has been supported.

The expression pattern of the four INK4 inhibitors in normal tissues varies. During mouse embryonic development, neither p16 nor p15 (or ARF) seem to be expressed at all or at very low levels (Zindy et al., 1997). In contrast, the expression of both p18 and p19 is detected early in mouse embryogenesis and increases during development. Four weeks after birth, the expression of p16 remains at low levels, but both p15 and ARF is detectable in many tissues. In older mice, p16 expression is elevated in several organs and cell types. When cells from mouse embryos are isolated and cultured as mouse embryonic fibroblasts (MEFs), both p16 and p15 expression is induced. These cells continue to proliferate for a finite number of passages in culture before they stop dividing and enter a state called replicative senescence. As MEFs approach senescence, the levels of p16 and ARF (as well as p21 and p53), but not p15 or p19, are increasing (Zindy et al., 1997). In normal human tissues, p16 expression is also low. In infants, the expression is restricted to the thymus (Hassall's corpuscles), and to rare thymic lymphocytes (Nielsen 1999). In adults, the expression levels are generally higher, but still only limited to certain tissues and cell types including proliferative endometrium, breast ductal epithelium, esophageal squamous epithelium and salivary glands. Endocrine glands, including Langerhans cells in the pancreas, anterior pituicytes and Leydig and Sertoli cells in testis, are also positive.

The regulation of the expression of the INK4 proteins is not well understood. The transcription of p15 is induced in response to the growth inhibitory factor, transforming growth factor beta

(TGFβ) (Florenes et al., 1996; Hannon and Beach, 1994; Reynisdottir et al., 1995; Sandhu et al., 1997) and TGFβ-responsive sequences have been identified in the p15 promoter region (Li et al., 1995). In addition, interferon-α can induce p15 expression in some hematopoietic cell lines (Sangfelt et al., 1997). As mentioned earlier, p16 is expressed at low levels in young cells, but its expression increases with age. This correlates with the fact that the human p16 promoter is repressed by methylation in young cells, but becomes gradually more and more demethylated and activated as cells get older (Brenner et al., 1998; Foster et al., 1998; Wong et al., 1999). Furthermore, the p16 promoter contains AP-1 binding sites that are responsive to JunB-containing complexes (Passegue and Wagner, 2000). Upon UV irradiation, such AP1/JunB complexes are formed and induce p16 expression, which suggests that p16 might play an important role in the response to UV damage (Isoherranen et al., 1998; Pavey et al., 1999). P18 and p19 are both widely expressed in primary tissues at variable levels. In one study, the expression in the developing nervous system pointed to a role for these proteins in differentiation (Zindy et al., 1997). More such evidence comes from studies showing that a 50-fold increase of p18 expression levels is detected upon terminal differentiation of mouse myoblasts (Franklin and Xiong, 1996). In addition, alterations of p15, p18, and p19 levels have been noticed in monocytic and granulocytic differentiation models (Schwaller et al., 1997), and ectopically expressed p19 can induce features of macrophage differentiation in 32D myeloid cells (Adachi et al., 1997).

ARF

The alternative product from the INK4a locus, ARF, is a highly basic protein that localizes to the nucleolus in cells (Pomerantz et al., 1998; Quelle et al., 1995; Weber et al., 1999). Although it does not interact with any of the CDKs (An et al., 1996; Quelle et al., 1995), it has the capacity, when induced or overexpressed, to induce growth arrest. Compared to the INK4 proteins, though, which induce G1 arrest in a pRB-dependent manner, ARF causes cell cycle arrest in both G1 and G2, and does not need an intact pRB for its action (Arap et al., 1997; Liggett et al., 1996; Quelle et al., 1997; Quelle et al., 1995; Stott et al., 1998). Instead, ARF-

induced growth arrest is dependent on p53 (Kamijo et al., 1997; Pomerantz et al., 1998; Stott et al., 1998). ARF binds to Mdm2, a protein which normally targets p53 and activates its degradation. When bound to ARF, Mdm2 is imported and captured in the nucleolus, allowing p53 to accumulate in the nucleoplasm (Lohrum et al., 2000; Weber et al., 2000; Weber et al., 1999).
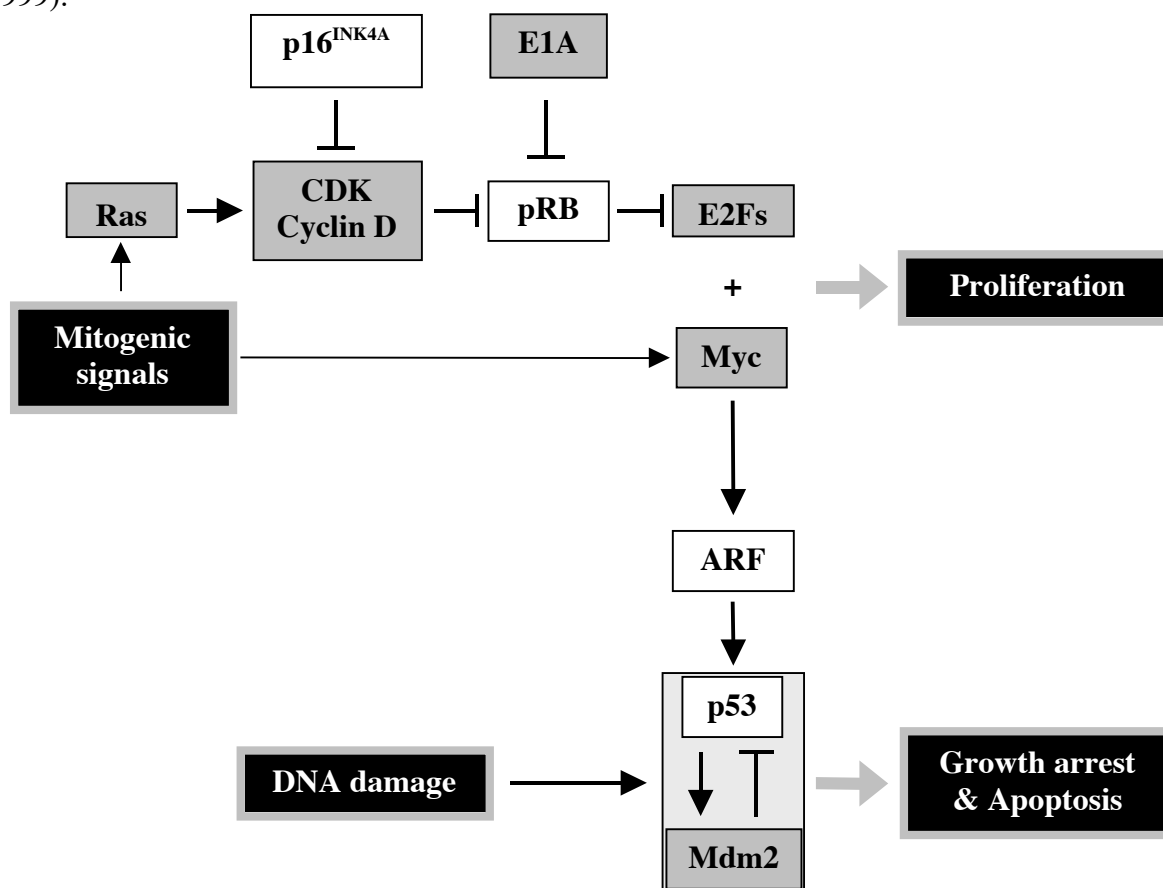
Figure 5. ARF connects the pRB and the p53 pathways. Mitogenic signals acting through Ras stimulates the formation of cyclin D/CDK4 complexes, which phosphorylate pRB. This leads to the release of E2F transcription factors that promote S phase entry. Myc acts in the same way as the E2Fs in the sense that they both promote the entry into S phase. p16^{INK4A} inhibits CDK4-activity and the adenovirus E1A oncogene interferes with pRB function. Aberrantly increased expression of E2F or Myc, triggers ARF expression, which activates p53 to induce either growth arrest or apoptosis. DNA damage triggers p53 in an ARF independent way. However, loss of ARF expression enables MDM2 to work more efficiently to counteract the function of p53 in response to DNA damage.
Modified from Sherr and Weber, 2000.

Overexpression of oncoproteins like Myc (Zindy et al., 1998), E1A (Zindy et al., 1998), E2F-1 (Bates et al., 1998), Ras (Palmero et al., 1998), and v-Abl (Radfar et al., 1998), upregulates ARF expression. Thus, ARF functions as a fuse, which is activated by abnormal mitogenic signalling, putting brakes on the cell cycle machinery by activating p53. Depending on the biological setting of the cell, p53 induces either growth arrest or apoptosis (Ko and Prives, 1996; Levine, 1997), preventing transformed cells to evolve into cancer. ARF therefore plays an important role by connecting the pRB and the p53 pathways (Fig. 5) (Sherr, 2000; Sherr, 1998).

Cell cycle inhibitors and cancer

The role of the CDK inhibitors and ARF in normal proliferating cells is not completely understood. Differences in temporal and spatial expression patterns of the members within the sub-families suggest that although they share similar, conserved, features and functions they might act in different cell types at different time points. Considering the role of the CDKIs as negative regulators of the cell cycle, one might expect that loss of their function would facilitate tumorigenesis. While this seems to be true for some of the proteins within these families, the role of the other members in human cancer is less clear.

Inactivation of CDKIs by genetic alterations

The chromosomal region 9p21, where *INK4A* and *INK4B* are localized in tandem, is frequently homozygously deleted in a broad range of primary tumors and tumor cell lines (Cairns et al., 1995; Kamb et al., 1994; Nobori et al., 1994; Spruck et al., 1994). Since the three distinct proteins p16, p15 and ARF are encoded by these two genes an obvious question is which of these proteins, or some combination thereof, confers the tumor-suppressor activity of the 9p21 locus. A growing body of evidence suggests that both p16 and ARF are bona fide tumor suppressors of perhaps relative importance in different species and cell types. Although deletions are the most common way of inactivating these genes/proteins, mutations affecting the

function of the proteins have been detected in both primary tumors and tumor cell lines. Germline mutations in kindreds with familial melanoma and pancreatic adenocarcinoma have been found (FitzGerald et al., 1996; Flores et al., 1997; Goldstein et al., 1995; Holland et al., 1995; Hussussian et al., 1994; Kamb et al., 1994), and a majority of these mutations affect both p16 and ARF. Mutations that specifically target p16, but not ARF have also been detected, but not vice versa (Ruas and Peters, 1998; Sherr and Weber, 2000). An alternative, indirect way of inactivating p16 would be to inhibit its binding to CDK4. Indeed, mutations in *CDK4* that abrogate p16 binding has been found in melanoma-prone families (Wolfel et al., 1995; Zuo et al., 1996). The role of p15 in tumorigenesis is less clear. The general opinion is that although the *INK4B* locus is deleted in many human tumors, this is secondary to *INK4A* targeting. However, there have been cases reported in which the *INK4B* locus is lost with retention and expression of p16INK4A (Glendening et al., 1995). Others have detected point mutations in *INK4B* in some human malignancies as well (Drexler, 1998; Flores et al., 1996; Gardie et al., 1998; Hangaishi et al., 1996). Thus, the *INK4A/INK4B* locus on the short arm of chromosome 9 harbors two verified (p16 and ARF), and one potential (p15) tumor suppressor. Even though tumor analyses favor p16 as the primary target of inactivation, it is not difficult to understand that deletion of the whole locus could represent a preferential target in human cancer. Both ARF and p15 are also two potent inhibitors of cell proliferation and one hit therefore results in the inactivation of three negative regulators of cell growth.

Thorough analyses have failed to detect genetic alterations in any of the genes encoding the other CDK inhibitors, including p18, p19, p21, p27, and p57. However, despite the absence of genetic alterations, there are examples of specific tumors with decreased expression of certain CDK inhibitors. Loss of p27 expression is a frequently detected phenomenon that has been observed in breast, prostate, colon, and gastric carcinomas. This is mainly mediated by protein degradation by the ubiquitin-proteasome pathway, but in some studies DNA methylation has been suggested as an alternative mechanism for repression of p27 expression (Lloyd et al., 1999; Qian et al., 1998). It has been suggested that deregulation of p27 might correlate with

the progression from low-grade to high-grade malignancies, onset of tumor invasion and subsequent metastasis (Catzavelos et al., 1997; Thomas et al., 1998; Tsihlias et al., 1998). This has led to studies in which loss of p27 expression is used as a prognostic marker for some human cancers (Catzavelos et al., 1997; Lloyd et al., 1999; Porter et al., 1997; Tan et al., 1997).

Inactivation of p16, p15 and ARF by DNA methylation

DNA methylation is, in addition to histone acetylation/deacetylation, a type of epigenetic event that regulates gene expression in mammalian cells (Bird, 1992; Eden and Cedar, 1994). It is a process that involves at least three different DNA methyltransferases (Dnmt1 (Bestor et al., 1988), Dnmt3a and Dnmt3b (Okano et al., 1998)), enzymes that catalyze the transfer of a methyl group to cytosine residues at CpG sites in the genome. The 5-methylcytosine residues then block transcription by interfering with the binding of transcription factors, or other DNA-binding proteins to promoter regions within different genes. In normal mammalian cells, approximately 3-5% of the cytosine residues in the genome is present as 5-methylcytosine (Ehrlich et al., 1982). Since certain genes contain methylated CpG islands in some tissues but not in others, DNA methylation has been suggested as an important mechanism for tissue-specific gene expression. This is supported by the fact that house-keeping genes, which are more or less equally expressed in every tissue, have unmethylated CpG islands (Yeivin and Razin, 1993). DNA methylation has also been suggested to be important in embryonic development, a process which demands sequential activation and deactivation of different classes of genes (Kafri et al., 1992). Methylation is also involved in other cellular processes such as inactivation of X-chromosome specific genes (Riggs and Pfeifer, 1992), senescence and aging (Cooney, 1993; Issa, 2000).

In tumor cells, there are now extensive evidence for aberrant DNA methylation as an alternative way to silence gene expression of p16 (Rocco and Sidransky, 2001; Ruas and Peters, 1998), p15 (Rocco and Sidransky, 2001; Ruas and Peters, 1998), and ARF (Robertson and Jones, 1998). Characteristic CpG islands are found within the promoter regions and the first

exon (exon 1α) of both *INK4A* and *INK4B*. In both genes, an approximately 500-bp region which shows a G+C content of 67%, and a CpG frequency of 7%, exist. These numbers are much higher than the average frequency detected in genomic DNA (Herman et al., 1996; Merlo et al., 1995). Today, hypermethylation of the p16 promoter region has been detected in a variety of human cancers (Herman et al., 1995; Rocco and Sidransky, 2001; Ruas and Peters, 1998). Since mutational events affecting the 9p21 locus in human cancer mainly target p16 and more rarely p15, it was not surprising that while the p16 promoter often is hypermethylated in epithelial tumors, p15 is not (Ruas and Peters, 1998). However, in some gliomas and hematological malignancies, p15 and not p16 appears to be the specific target of methylation (Herman et al., 1997; Herman et al., 1996). In acute myeloid leukemia (AML), 82% of analyzed cases showed selective methylation of p15. The frequency in acute lymphatic leukemia (ALL) was 57%. p16 methylation was detected at very low frequencies in both these tumor variants. Treatment of methylated tumor cell lines with the demethylating agent, 5-aza-2´-deoxycystine, leads to a significant decrease in p16 promoter methylation, reexpression of p16 and consequently G1 arrest (Merlo et al., 1995).

Figure 6. A model for biallelic inactivation of tumour suppressor genes by DNA methylation alone, or in combination with mutation or deletion. MUT, mutation, CH_3-CpG, indicates methylation. *Modified from Momparler and Bovenzi, 2000.*

Mutation + Methylation   **Deletion (LOH) + Methylation**   **Biallelic Methylation**
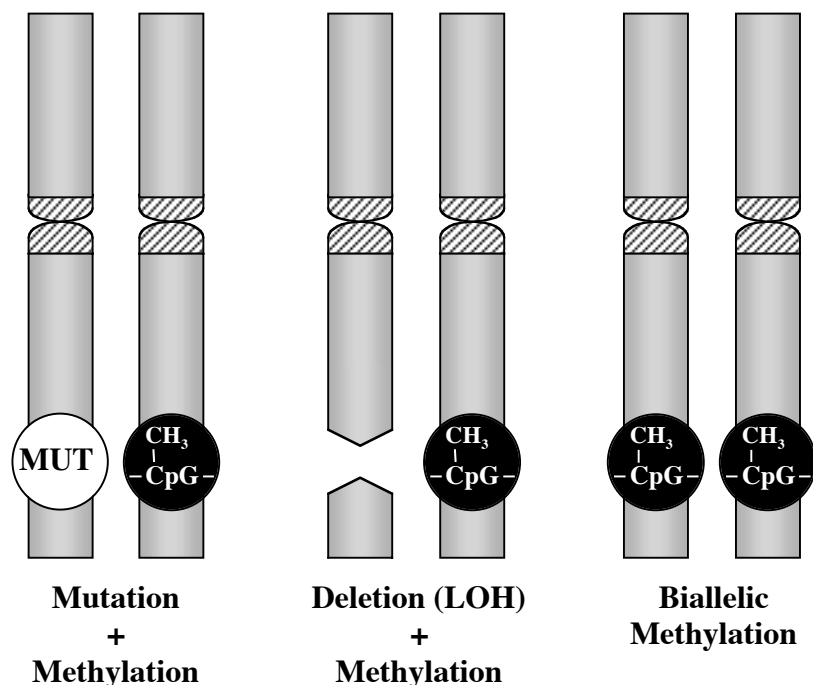
CpG islands within promoters of other tumor suppressor genes such as *Rb*, Wilms tumor, von Hippel-Lindau's syndrome and the estrogen receptor have also been found to be hypermethylated (Momparler and Bovenzi, 2000). This is true also for some suppressors of invasion and metastasis, such as E-cadherin and tissue inhibitor of metalloproteinase-3 (TIMP-3), as well as for inhibitors of angiogenesis, e.g. thrombospondin-1 (TSP-1) (Momparler and Bovenzi, 2000). In total, this has led to a new formulation the Knudsen two-hit model (1971), used for definition of tumor suppressors. In this new model, methylation alone or in combination with mutations or deletions is regarded as an alternative way for biallelic inactivation of tumor suppressor genes (Fig. 6).

Mouse models

Genetic approaches to study the involvement of the CDK inhibitors and ARF in the regulation of the normal cell cycle, and the importance of their inactivation in the cancer cell cycle have also been used. In these studies, inactivating mutations or deletions in the genes encoding the different members have been introduced in mouse ES cells to create knockout mice. Today, all the different members of the INK4 and the CIP/KIP families, as well as ARF, have been separately ablated from the mouse genome. The results from these studies are summarized in Table 1.

Briefly, mice lacking p21 are born healthy and are fertile (Brugarolas et al., 1995; Deng et al., 1995). No increase in spontaneous tumor formation is seen, which correlates with the fact that genetic alterations in the p21 gene are seldomly detected in cancer. In agreement with its role as a mediator of p53-dependent growth arrest in response to γ-irradiation, cells from the p21-/- mice are impaired in their capacity to do this. More detailed studies have revealed that although the p21-null mice are grossly normal, several defects in specific cellular lineages, such as lymphocytes and renal cells, have been observed. T lymphocytes from these animals are hyperproliferative, which leads to the accumulation of abnormal amounts of CD4^+ cells and a loss of tolerance against nuclear antigens. Subsequently, a severe lupus-like syndrome develops, characterized by lymphadenopathy, glomerulonephritis and glomerular autoantibody de-

posits against dsDNA, resulting in renal failure and decreased viability (Balomenos et al., 2000). Furthermore, p21 has been shown to function as a molecular switch, governing the entrance of quiescent hematopoietic stem cells into the cell cycle. In p21-/- animals, hematopoietic stem cells divide continously, which leads to impaired self-renewal, stem cell exhaustion and hematopoietic failure (Cheng et al., 2000).

p27 is under normal conditions strongly expressed in non-proliferating quiescent cells. It has been suggested that it plays an important role in cell differentiation and in accordance with this, cells from the p27-/- mice show aberrant differentiation programs (Fero et al., 1996; Kiyokawa et al., 1996; Nakayama et al., 1996). These mice have increased body size and overgrowth of several organs, confirming the role for p27 in quiescence and tissue maintenance. Although no genetic alterations in the p27 gene has been detected in human cancer the p27 knockout mice develop both pituitary and parathyroid tumors. Furthermore, mice treated with γ-irradiation display an increased susceptibility to a broad range of tumors including adenomas of the small intestine and colon, angiosarcomas, endometrial carcinomas, adrenal tumors, lung tumors and fibromas (Fero et al., 1998; Nakayama et al., 1996). p27 therefore is a multiple-tissue tumor suppressor in mice. However, molecular analyses of tumors in p27 heterozygous mice show that the remaining wild-type allele is neither mutated nor silenced, Thus p27 does not fulfill Knudson's "two-hit" criterion for a tumor-suppressor gene, but might instead be regarded as haplo-insufficient for tumor suppression. The p57 gene has been reported to be mutated in patients with the Beckwith-Wiedemann syndrome and it has also been found to be deleted in lung carcinomas and Wilms' tumors. p57-/- mice display severe developmental defects and die soon after birth (Yan et al., 1997; Zhang et al., 1997). Because of this, the role of p57 in the development of tumors in these mice is unclear.

In 1996 one group, with the intention to knockout p16, deleted both exons 2 and 3 of the *INK4A* gene (Serrano et al., 1996). However, during the generation of these mice, ARF was discovered as an additional protein produced from the *INK4A* locus. The knockout mice gen-

erated in these experiments therefore show loss of expression of both p16 and ARF. These INK4a^{Δ2,3}-deficient mice are born healthy and are fertile, but spontaneously develop tumors early in life, especially sarcomas and lymphomas (Serrano et al., 1996). MEFs derived from these animals proliferate more rapidly than their normal counterparts, bypass replicative senescence and can be directly transformed with Ras. In total, these MEFs remind more of established immortalized cell lines than normal primary fibroblasts. In 1997, disruption of exon 1β was used to generate mice lacking only p19^{ARF} (Kamijo et al., 1997). The results from these studies were unexpected. All the cardinal features of the INK4/ARF knockout mice, including both tumor development *in vivo* and growth properties of MEFs *in vitro*, were also seen in the ARF null mice. This suggested that the phenotype of the INK4/ARF mice, initially thought to be due to the lack of p16 expression, instead reflected loss of ARF. The obvious question since then has been; what is the phenotype of a clean p16 knockout? Very recently, the generation of such animals (INK4A-/-) was reported (Krimpenfort et al., 2001; Sharpless et al., 2001). The results from these studies show that in contrast to MEFs from the ARF null mice, MEFs from INK4A-/- animals proliferate normally in culture and enter senescence when grown on a 3T3 or 3T9 protocol. Moreover, these cells cannot be transformed with oncogenic Ras alone. However, some spontaneously immortalized clones, with retained ARF and p53 function, has been established as permanent cell lines. Also, compared to wild-type primary MEFs, *INK4A* null MEFs are more easily transformed by the combination of Myc and Ras. About 25% of the *INK4A* null mice spontaneously develop tumors, including sarcomas, lymphomas and a single detected melanoma, within the first year of life (Sharpless et al., 2001). Many animals also develop skin and lung tumors after carcinogen treatment. Interestingly, no p16 expression was detected in the lung tumors that arose in the INK4+/- heterozygots, after treatment with the carcinogen dimethylbenzanthracene (DMBA). The wild-type *INK4A* allele was intact, but epigenetically shut down by DNA methylation. p16 therefore seems to play an important role in tumor suppression after carcinogen treatment.

Mice deficient for p15 have also been generated (Latres et al., 2000). These animals develop normally and are fertile. However, loss of p15 results in a high incidence of lymphoproliferative disorders such as increased extramedullar hematopoiesis in spleen and frequent formation of secondary follicles in lymph nodes, a process characteristic of lymphocyte activation after infections or inflammations. p15 null mice also display a modest, but reproducible increased incidence of tumor development (about 8% incidence). Interestingly, most tumors are angiosarcomas (Latres et al., 2000), which correlates with the fact that p15 is strongly expressed in endothelial cells (Thullberg et al., 2000). It has been proposed that p15 is the mediator of TGF-β-mediated growth inhibition, but cells from p15 null mice, still respond to TGF-β in the same manner as wild-type cells.

Table 1 Summary of the major phenotypes of mice lacking each of the CDK inhibitors.

Gene targeted	Major phenotype	Reference
p21^{CIP1}-/-	Mice develop normally but cultured cells arrest inefficiently upon DNA damage.	Deng *et al.*, 1995; Brugarolas *et al.*, 1995
p27^{KIP1}-/-	Increased body size and overgrowth of organs, female sterility, pituitary tumors. Haplo-insufficient for tumor suppression.	Kiyokawa *et al.*, 1996; Nakayama *et al.*, 1996; Fero *et al.*, 1996
p57^{KIP2}-/-	Mice die soon after birth from severe developmental defects.	Yan *et al.*, 1997; Zhang *et al.*, 1997
INK4A^{Δ2,3}	No expression of p16 or ARF. Lymphomas and sarcomas similarly to p19ARF-/- and p53-/- mice.	Serrano *et al.*, 1996
ARF-/-	Similar phenotype to the INK4A^{Δ2,3} mice, including tumor formation.	Kamijo *et al.*, 1997
p16^{INK4A}-/-	Sarcomas and lymphomas. Skin and lung tumors after carcinogen treatment.	Sharpless *et al.*, 2001; Krimpenfort *et al.*, 2001
p15^{INK4B}-/-	Lymphoproliferative disorders and angiosarcomas.	Latres *et al.*, 2000
p18^{INK4C}-/-	Increased body size and overgrowth of some organs, lymphoproliferative disorders, pituitary tumors and other malignancies.	Franklin et al., 1998; Latres *et al.*, 2000
p19^{INK4D}-/-	Except for testicular atrophy, these mice are normal.	Zindy *et al.*, 2000

Modified from Malumbres et al., 2000

This suggests that TGF-β induced growth arrest can be mediated by some other pathways as well (Latres et al., 2000). MEFs from these mice are hyperproliferative and more easily transformed by either Ras alone or in combination with Myc. In short, these data indicate that p15 indeed is a tumor suppressor, at least in some cellular lineages. Mice lacking p18 are larger than their normal littermates and display features of organomegaly, pituitary hyperplasia and adenoma (Franklin et al., 1998; Latres et al., 2000). They also develop testicular tumors, pheochromocytomas and more rarely, angiosarcomas, findings that support the role of p18 as a tumor suppressor. In addition, hyperproliferative hematopoietic defects are commonly detected in these animals. Mice lacking the fourth member of this family, p19, were also described recently (Zindy et al., 2000). These mice are viable and fertile, and do not develop tumors even after treatment with carcinogens.

Cell cycle inhibitors in senescence and immortalization

Replicative senescence

It was established already more than 30 years ago that human diploid fibroblasts, explanted from living tissue and grown as cell cultures, have a finite life span (Hayflick and Moorhead, 1961). After a critical number of population doublings (PDLs), cells stop dividing and enter a state known as replicative senescence. Later, it has become clear that a variety of normal mammalian cells behave in the similar way when they are grown in culture. Although controversial for a long time, there is now widespread (Campisi, 1997; Kipling et al., 1999), although not universal (Rubin, 1998), agreement that normal human somatic cells have an intrinsically limited proliferative life span even in ideal growth conditions. In contrast, tumor cells bypass senescence and continue to divide indefinitely.

The senescent phenotype

Senescent cells differ from presenescent cells in three fundamental ways (Campisi, 1997). First, they are irreversibly growth arrested in the G1 phase of the cell cycle, and once this growth arrest occurs, physiological mitogens cannot stimulate them to enter the S phase of the cell cycle (Cristofalo and Pignolo, 1993; Goldstein, 1990; McCormick and Campisi, 1991; Shay et al., 1991). The failure of senescent cells to initiate DNA replication is not due to a general breakdown in growth factor signal transduction (Campisi, 1997), but instead a result of a change in expression of several growth inhibitory proteins (see below). Secondly, senescent cells acquire resistance to apoptotic stimuli (Wang et al., 1994). Little is known about this phenomenon, but it may explain why senescent cells seem to accumulate *in vivo* (Dimri et al., 1995; Mishima et al., 1999; Pendergrass et al., 1999). Thirdly, senescent cells are both morphologically and functionally altered. These changes include a larger and more flattened cell size, an increased lysosomal biogenesis and decreased rates of protein synthesis and degradation (Campisi et al., 1996; Stanulis-Praeger, 1987). A marker for senescence, senescence-associated β-galactosidase (SA-β-gal), was originally discovered as an endogenous β-galactosidase activity that is detectable in senescent, but not in presenescent cells, at pH 6.0 (Dimri et al., 1995). Recently, it was shown that this reflects a residual lysosomal activity at a suboptimal pH, which becomes detectable due to the increased lysosomal content in senescent cells (Kurz et al., 2000). Senescent changes in gene expression are also cell-type specific. For example, senescent human endothelial cells overexpress interleukin-1α and the cell adhesion molecule I-CAM and senescent adrenocortical epithelial cells loose their ability to induce 17α-hydroxylase, a key enzyme in cortisol biosynthesis. Moreover, senescent dermal fibroblasts display increased expression of collagenase and stromelysin (proteases that degrade extracellular matrix proteins) and reduced expression of TIMP 1 and 3 (tissue inhibitor of metalloproteinase 1 and 3) (Campisi, 2000).

Inducers of senescence

Different physiological stimuli can provoke a cell to enter senescence (Fig. 7). As mentioned, extensive passaging in culture eventually leads to replicative senescence. This has been proposed to be due to a progressive shortening of telomere length (Allsopp et al., 1992; Harley et al., 1990; Vaziri et al., 1993). Telomeres are located at the end of mammalian chromosomes and consist of a repetitive DNA sequence (TTAGGG in human and other vertebrates) and specialized proteins. Their role is to protect the chromosomal ends from end-to-end fusions (Greider and Blackburn, 1996). Because of the fact that mammalian DNA polymerases only can move unidirectionally and require a primer for initiation, each round of DNA replication leaves 50-200 3' bases at the telomeres unreplicated (Olovnikov, 1973). After a number of PDLs, the telomeres reach a critical length where they no longer can protect the ends of the chromosomes. This has been suggested to be the time point when chromosomal ends may activate the senescent machinery in the cell. The fact that cells in culture display progressively shorter telomeres as they approach senescence, and that cells collected from older organisms have shorter replicative life spans in culture, as well as shorter telomeric DNA (Harley et al., 1990; Hastie et al., 1990), support this theory. Furthermore, ectopic expression of telomerase, an enzyme that protects telomeres from getting shorter by adding nucleotides to telomeric ends, in fibroblasts and other cell types is sufficient to make cells bypass senescence and become immortalized (Bodnar et al., 1998; Vaziri and Benchimol, 1998). However, other results suggest alternative mechanisms for initiation of senescence. Firstly, it has become clear that telomere shortening is an inconsistent measure of the onset of senescence as the rate of telomere erosion varies between cells during in vitro culture (Counter et al., 1995; Harley et al., 1990; Hastie et al., 1990; Klingelhutz et al., 1994; Lansdorp et al., 1996; Martens et al., 2000). Secondly, no consistent telomere length at which senescence takes place has been detected (Ouellette et al., 2000) and finally, the ectopic expression of telomerase in keratinocytes and mammary epithelial cells does not allow these cells to bypass senescence (Dickson et al., 2000; Kiyono et al., 1998). Taken together, results from various studies indicate that addi
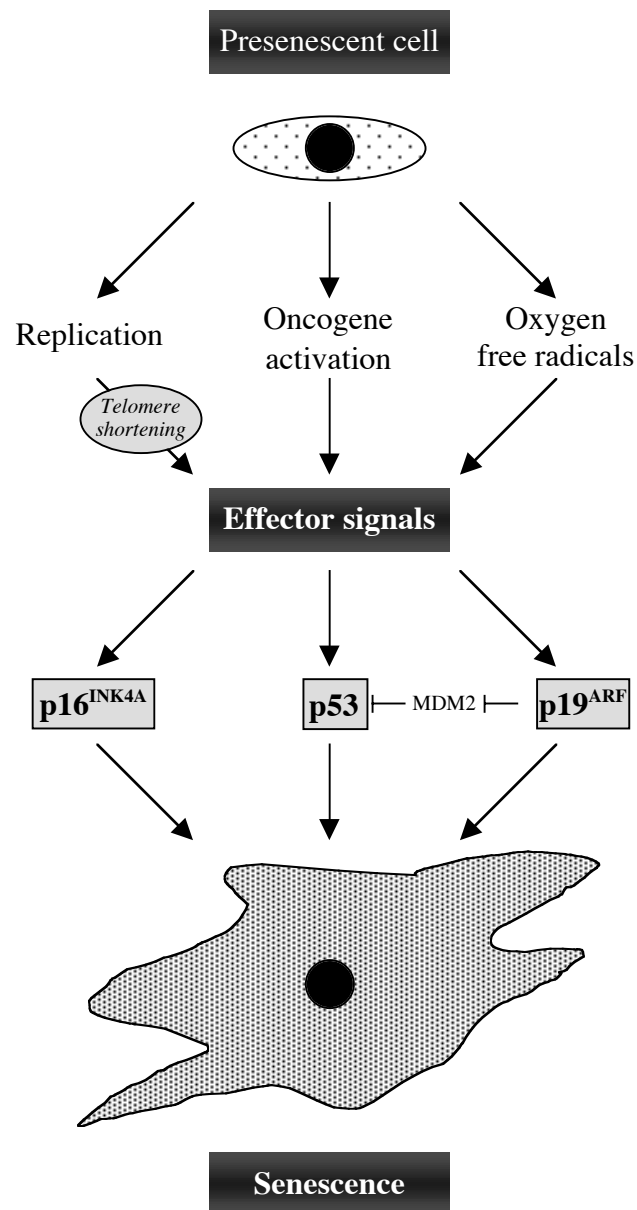
Figure 7. Signals that activate the senescence program. Following extensive passage in culture (replication), oncogene activation or exposure to oxidative damage, primary cultures of mammalian cells enter an irreversible growth arrest and display hallmarks of senescence.

(Modified from Lundberg et al., 2000)

tional signals apart from telomere shortening are important for induction of replicative senescence, at least in some cell types.

Apart from the induction of senescence as a consequence of replication, oncogene activation can also induce senescence (Fig. 7). In primary cells, overexpression of Ras can induce senescence (Serrano et al., 1997), and downstream effectors such as Raf or MEK, can do the same (Lin et al., 1998; Zhu et al., 1998). Ras-mediated senescence is phenotypically equivalent to replicative senescence, including a flattened morphology, SA-β-gal activity, and induction of cell cycle inhibitors (Serrano et al., 1997). One difference, however, is that ectopically expressed hTERT can make fibroblasts escape from replicative, but not Ras-induced senescence (Wei and Sedivy, 1999). In addition, oxidative stress is a third type of condition that may induce a senescent-like phenotype (Fig. 7). While mild hyperoxia shortens the replicative life span of cells in culture (von Zglinicki et al., 1995), low oxygen pressure have the opposite effect (Chen et al., 1995; Saito et al., 1995). Reactive oxygen species (ROS), such as hydrogen peroxide, has also been strongly implicated in both cellular senescence and organismal aging (Beckman and Ames, 1998).

Mediators of senescence

Some cell cycle inhibitors have been implicated as being mediators of senescence (Fig. 7). The most studied one is perhaps p16, which accumulates in cultured fibroblasts as they approach senescence (Alcorta et al., 1996; Hara et al., 1996). Spontaneously immortalized clones of mouse fibroblasts that escape senescence often display loss of p16 expression. In human cell types which are resistant to immortalization by telomerase alone (e.g. keratinocytes and mammary epithelial cells), co-inactivation of p16 by genetic or epigenetic mechanisms is required for immortalization (Dickson et al., 2000; Kiyono et al., 1998). ARF has also been implicated as an important mediator of senescence (Fig. 7). Its binding to MDM2 indirectly results in p53 activation and subsequent growth arrest (Hara et al., 1996), but ARF may also act through p53-independent mechanisms (Carnero et al., 2000). Similar to p16, ARF also accu-

mulates in senescent cells. (Zindy et al., 1998). The activation of the p16/pRB pathway or the ARF/p53 pathway for mediating senescence seems to be a species- and cell-type specific matter (Kiyono et al., 1998; Munro et al., 1999)

The same molecules seem to be important as mediators of senescence following oncogene activation as well. In rodent cells, overexpression of oncogenes such as E1A, Myc or Ras induces upregulation of p15, p16 and ARF. Both the p16/pRB and the ARF/p53 pathways seem to be essential for oncogene-mediated senescence (Palmero et al., 1998; Serrano et al., 1997). Thus, both Myc and E1A inactivates pRB, which thereby releases E2F transcription factors that promote transcription of ARF (de Stanchina et al., 1998; Zindy et al., 1998). p15 is involved in v-*ras*-mediated senescence in mouse keratinocytes, a process that is dependent on a TGF-β (Tremain et al., 2000). Also p21 has been implicated to play a role in senescence (Noda et al., 1994). It accumulates in cultured human fibroblasts but in contrast to p16, which is continuously expressed at high levels in senescent cells, the expression of p21 decreases as cells enter senescence (Stein et al., 1999) (Fig. 8). It has therefore been suggested that p16 and p21 have different roles during the senescence of human fibroblasts. p21, which is expressed at its maximum levels at the time point when cells stop proliferating, is suggested to be the driving force for pushing cells into early senescence by inactivating both the cyclin D- and the cyclin E-dependent kinases. p16, on the other hand, which peaks at a time point when the fibroblasts already have stopped growing and reached late senescence, is suggested to be essential for maintenance of the senescent-associated cell cycle arrest.
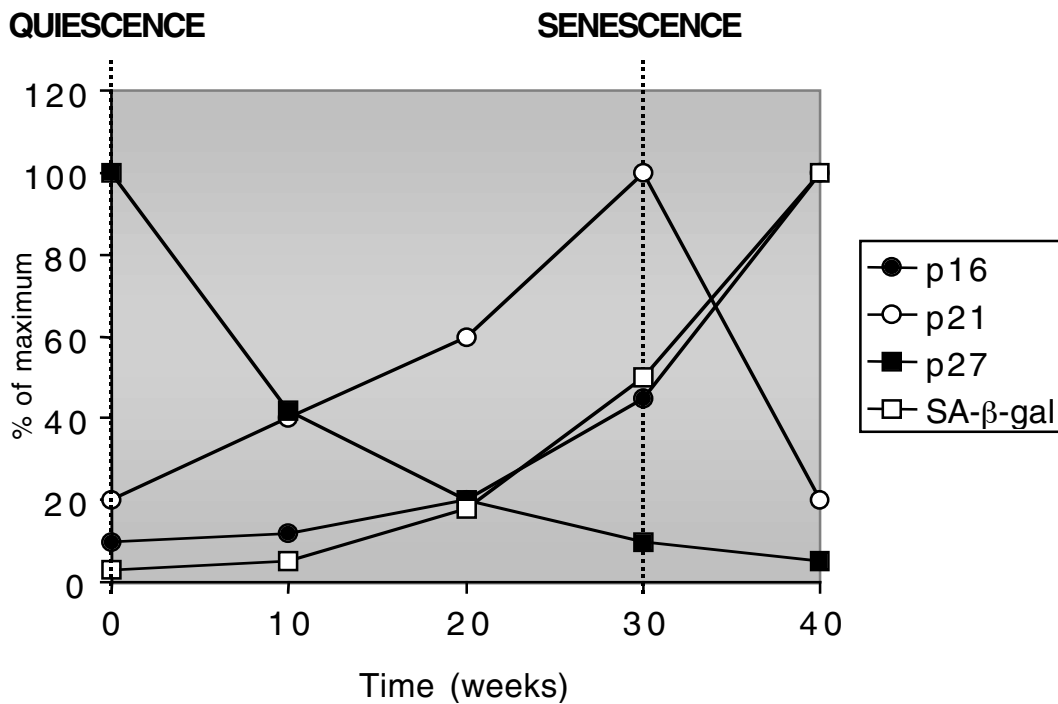
Figure 8. Changes in CDKI expression levels during the life span of primary human IMR90 fibroblasts in culture. The fibroblasts were seeded at a constant cell density, fed after 1 week, and harvested after 2 weeks. Regardless of their age, the cells were arrested at the end of the 2-week period. Initially, the cells were reversibly arrested at high cell density with a high amount of the p27Kip1 inhibitor of CKIs, as expected from the known association of p27 with high-cell-density arrest. As the cells aged, their density at quiescence declined and consequently so did their amount of p27, In contrast, p21 and p16 expression increased with age. *(Modified from Stein et al., 1999)*

Telomerase

The maintenace of telomeric repeats throughout many cycles of cell division requires telomerase, a telomere-specific ribonucleoprotein (RNP) reverse transcriptase. Telomerase functions by copying a template sequence carried within its integral RNA and adds single-stranded telomeric repeats to the chromosome 3' ends. This prevents telomere erosion and chromosome instability.

The telomerase enzyme complex consist of an RNA component, telomerase-associated protein 1 (TEP1) and telomerase reverse transcriptase (TERT) (Nugent and Lundblad, 1998). The enzyme activity was first identified in Tetrahymena and later in other species, including ciliates, yeast, and human (Cohn and Blackburn, 1995; Greider and Blackburn, 1985; Lin and Zakian, 1995; Morin, 1989; Shippen-Lentz and Blackburn, 1990). It was initially thought that since most cells produce both the RNA component of telomerase and TEP1, but not TERT, telomerase activity is regulated by the transcriptional regulation of TERT. However, the situation seems more complex. Additional, alternatively spliced isoforms of TERT have been discovered (Kilian et al., 1997), and since some of these are catalytically inactive, cells with TERT can lack telomerase activity (Ulaner et al., 1998). Also post-translational regulation of telomerase activity have been suggested (Kang et al., 1999; Li et al., 1998; Liu et al., 1999).

Telomerase is active in human embryogenesis, but in adults the expression is limited to germ cells, stem cells and stimulated cycling lymphocytes. In most human somatic cells, telomerase is not expressed and these cells therefore exhibit telomere shortening as a function of cell division *in vitro* (Harley et al., 1990), or advancing age *in vivo* (Allsopp et al., 1992). In contrast, most primary tumors and tumor cell lines have telomerase activity (Kim et al., 1994; Shay, 1995; Shay and Wright, 1996). In fact, telomerase activity seems to be essential for maintaining cell immortality, since mutations introduced into the telomerase RNA component leads to telomere instability and a senescent phenotype (McEachern and Blackburn, 1995; Singer and Gottschling, 1994; Yu et al., 1990).

The RNA component of telomerase has been deleted from the mouse germline (mTR-/- mice) (Blasco et al., 1997). However, since mouse telomeres are much longer than the human counterparts, no phenotype was present until the sixth generation of littermates. Telomere shortening associated with aneuploidy and chromosomal end fusions were frequent in cultures from mTR-/- mice in generation G2 to G6. Telomerase-deficient cells could be immortalized in

culture, transformed by viral oncogenes, and they generated tumors in nude mice following transformation.

Ectopic expression of TERT in human fibroblasts halts telomere shortening, stabilizes telomere length and leads to immortalization (Bodnar et al., 1998; Vaziri and Benchimol, 1998). However, this phenomenon seems to be cell type specific because expression of TERT alone in human foreskin keratinocytes (HFKs) and human mammary epithelial cells (HMECs) is not sufficient for immortalization (Counter et al., 1998; Hahn et al., 1999; Kiyono et al., 1998). In one study it was suggested that inactivation of the pRB/p16^{INK4A} pathway was required in addition to telomerase activity to immortalize these cell types (Kiyono et al., 1998).

Differences between species and cell types

As already touched upon, the molecular machinery governing the processes of cellular senescence and immortalization varies in different species and cell types. Compared to MEFs, human fibroblasts have a proliferative potential in culture, which is three times longer. Also, they hardly ever, in contrast to mouse fibroblasts, escape senescence and become immortalized. The human counterparts are also more resistant to oncogene-mediated transformation, and they are more chromosomally stable. Recently, it has been argued that replicative senescence of cultured cells depends on two different sources of signals (Sherr and DePinho, 2000). Apart from the "mitotic clock" that monitors the integrity of the chromosomes with increasing number of PDLs, a second source is the extrinsic signals that are associated with the non-physiological growth conditions associated with cell culturing. These signals have been gathered under the term "culture shock", and includes disruption of cell-cell contacts, lack of heterotypic interactions between different cell types, the medium-to-cell ratio, persistent Ras activation by mitogens, absence of appropriate survival factors, hyperoxia, and plating on plastic culture dishes. The authors claim that replicative senescence in human and mouse cells depend in part on a differential response to the tissue culture environment (Fig. 9).

Mouse cells may be relatively more sensitive to culture shock, because shorter-lived rodents may not have evolved the putatively more effective damage repair machinery that is present in longer-lived humans. Many somatic mouse tissues and cultured cells express telomerase and in these cells, disruption of the p53 pathway is sufficient for immortalization. In human cells, disruption of both the pRB and the p53 pathways is insufficient for immortalization, but leads to prolongation of the life span. Such human cells eventually reach "crisis", a stage at which many chromosomes have undergone telomere erosion. Rare clones, which additionally acquire the capacity to solve the problems with telomere instability, either by the activation of telomerase or an alternative recombinational mechanism (ALT), may escape this stage and become immortalized (Fig. 9).
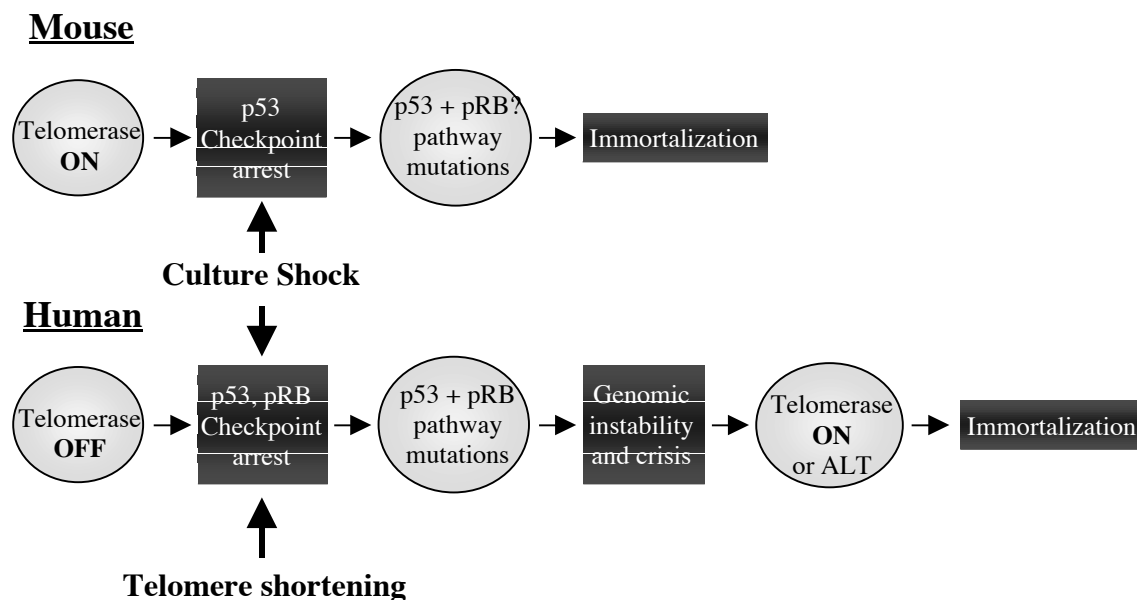
Mouse

Figure 9. Pathways to senescence in mouse and human cells. In mouse cells, which have telomerase activity, culture conditions is suggested to play an important role in the induction of senescence. Mutations in the p53 pathway are sufficient for immortalization. Human cells are capable of many more cell divisions in culture (60-80 doublings) before they undergo senescence. Telomerase is not expressed in human cells and the telomeres are much shorter than those in laboratory mice. Over the course of many cell divisions, shortened telomeres may therefore initiate DNA damage responses, which might activate both p53- and pRB-dependent checkpoints that may contribute to senescence. If these pathways are inactivated by viral oncoproteins or mutations, cells may continue to proliferate until they reach "crisis", a state which is characterized by telomere erosion, chromosomal instability and cell death. Rare survivors from "crisis" have activated telomerase, or an alternative recombinational mechanism (ALT) to maintain telomeres and chromosomal integrity. *(Modified from Sherr & DePinho, 2000)*

Senescence as a barrier to human cancer

During recent years the amount of supporting evidence that senescence indeed functions as a tumor suppressor mechanism, have arose (Campisi, 2000; Lundberg et al., 2000; Parkinson et al., 2000; Wright and Shay, 2001; Wynford-Thomas, 1999). Tumor cells are immortal, and cells that grow beyond their normal proliferative life span are at a greatly increased risk of being transformed, since this allows them to acquire multiple mutations. Many oncogenes specifically target the senescent machinery, rendering cells insensitive to such stimuli. The two tumor suppressor genes that are most frequently targeted in human cancer, p53 and pRB, are also the most central players in cellular senescence. The *INK4A/INK4B* locus, which harbors the cell cycle inhibitors p16, ARF and p15, is also frequently inactivated in tumors. These tumor suppressor proteins have all three been shown to be mediators of senescence in different species and cell types. Interestingly, the whole *INK4A/INK4B* locus is often inactivated by a single deletion event. By one hit, the function of three strong tumor suppressors and mediators of senescence is lost. Thus, the genomic organization of the *INK4A/INK4B* locus makes it an attractive target in tumor development. In fact, next to p53 mutations, the inactivation of the *INK4A/INK4B* locus by deletions, mutations or DNA methylations, is the most frequent aberration in human cancer.

Gene transfer by adenovirus vectors

Adenovirus vectors

Adenoviruses (Ads) consist of a large family of non-enveloped viruses that contain a double-stranded DNA genome of approximately 36 kb (Horwitz, 1996; Philipson et al., 1995). The capsid is composed of pentons (penton base and fiber) and hexons. About 50 different human Ad serotypes exist and these are associated with respiratory, ocular, and gastrointestinal diseases. Accumulation of information on the structure, molecular, and cell biology of Ad over the last decades have allowed the development of Ad vectors for both *in vivo* and *in vitro* gene transfer (Falck-Pedersen, 1998). Compared to other vector systems, the advantages of using Ads are that they infect a broad range of both dividing and non-dividing cells, and can be produced in large quantities and at high titers (Benihoud et al., 1999). Compared to various transfection methods, Ad vectors are often superior in delivering genes into various cells. This has made them popular tools for gene expression analyses.

The first generation recombinant Ad vectors are based on Ad type 5 (or type 2), which causes mild respiratory infection in humans (Horwitz, 1996). The Ad genome comprises early (E1-E4) and late (L1-L5) genes. In the first generation vectors, the E1 genes are deleted, rendering the virus unable to replicate in normal cells. In Ad vectors used for gene delivery, the E3 region is often deleted as well to make more space for the expression cassette. The expression cassette, containing a promoter, the gene of interest, a splicing signal and a poly-adenylation signal, is usually inserted into the E1 region of the viral DNA (Fig. 10). To produce recombinant adenoviruses, vector DNA containing the expression cassette and some flanking Ad sequences is co-transfected together with viral DNA (often circularized in a way that makes it too large to be packaged into viral particles) into 293 human embryonic kidney cells. The expression cassette is inserted into the viral genome by homologous recombination. The 293 cells express the missing E1 gene products, which permits the formation of recombinant viral particles that can be isolated and purified and used for infections.
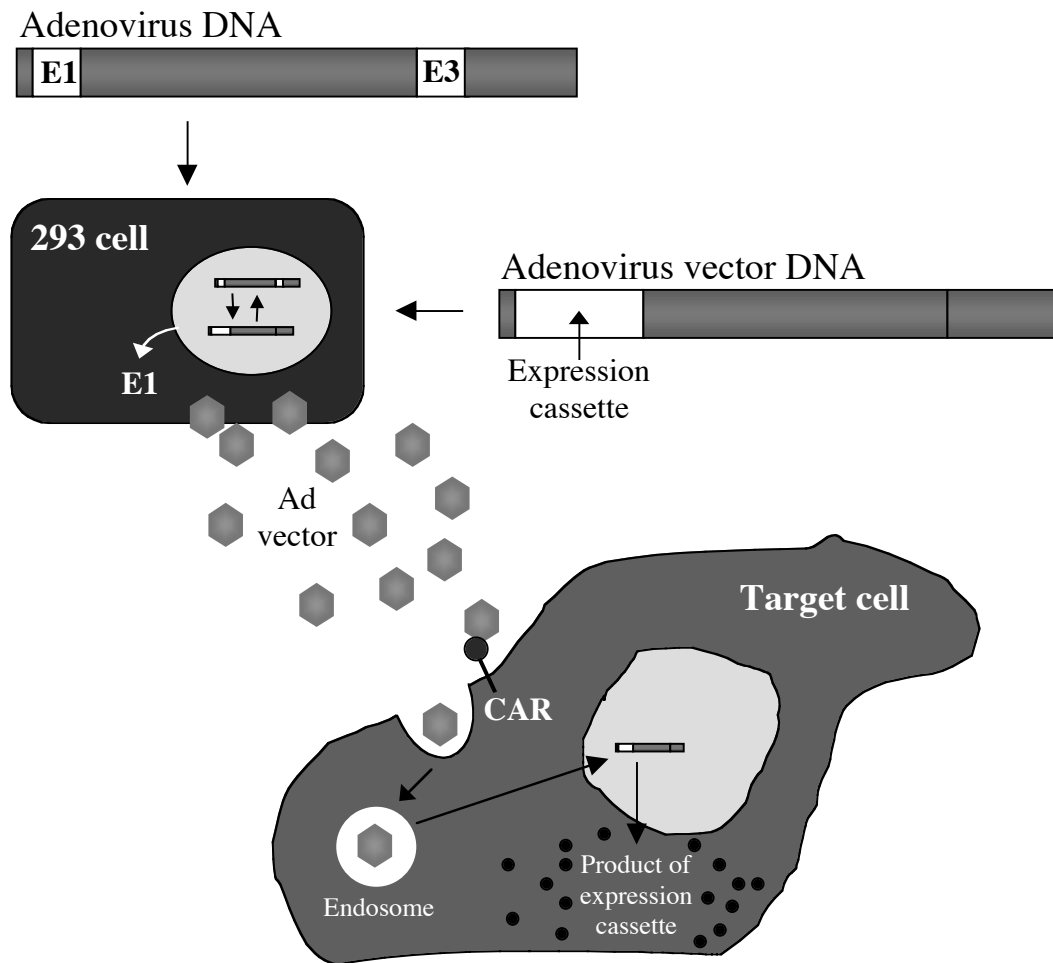
Figure 10. Design, production and gene transfer of the first generation replication-deficient Ad vectors. The E1 and E3 regions are deleted from the viral DNA, which then is co-transfected into 293 cells together with an Ad vector DNA that contains an expression cassette (EC) with the promoter and the transgene. In 293 cells, homologous recombination occurs, leading to the formation of a recombinant viral DNA in which the EC has replaced the E1 region. The 293 cells provide the E1 proteins, which permits the virus to replicate in these cells, but not in others. The recombinant virus infects the target cell by attachment to CAR. The uptake is then mediated by endocytosis, and subsequently the virus delivers the dsDNA into the nucleus where it functions in an epichromosomal fashion to express its product.

Modified from Crystal R.G., 1999

The coxsackie- and adenovirus receptor, CAR

The virus utilizes its penton fiber to attach to a cellular receptor named CAR (Coxsackie- and Adenovirus Receptor) (Bergelson et al., 1997; Tomko et al., 1997) (Fig. 11). The ability of Ads to infect different cells is dependent on the cellular expression of CAR, which is a 46 kDa glycoprotein composed of an extracellular part containing two Ig-like domains, a transmembrane region and a cytoplasmic tail (Bergelson et al., 1997; Tomko et al., 1997). Structural analysis has revealed that the Ad fiber knob interacts with the N-terminal Ig domain of the receptor (Bewley et al., 1999; Roelvink et al., 1999; Tomko et al., 2000).
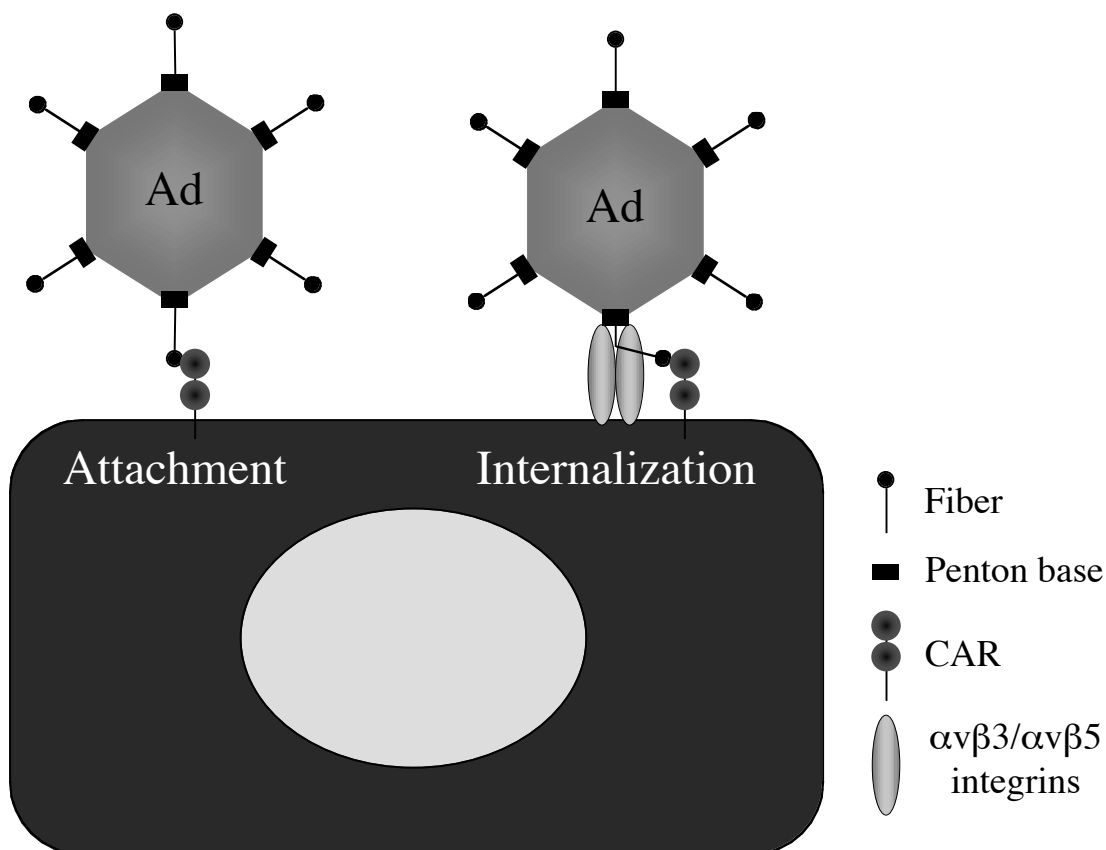

Figure 11. The involvement of different cell surface receptors in the attachment and internalization of adenovirus. (*Modified from Nemerow G.R., 2000)*

CAR belongs to the CTX-group within the immunglobulin superfamily (Chretien et al., 1998) and seems, although some conflicting results exist, to be expressed in many different tissues (Bergelson et al., 1998; Fechner et al., 1999; Tomko et al., 2000; Tomko et al., 1997). In polarized cells, such as airway epithelia, it localizes to the basolateral membrane (Walters et al., 1999). This limits adenovirus infection from the apical side, and is most likely the reason for why adenovirus-mediated gene transfer to the airway epithelium of patients with cystic fibrosis not has been as efficient as expected (Goldman and Wilson, 1995; Pickles et al., 1998). While the fiber interaction with CAR clearly is required for virus attachment, virus internalization is facilitated by the two integrins $\alpha v\beta3$ and $\alpha v\beta5$ (Wickham et al., 1993). The αv integrins recognize a highly conserved RGD motif that is present in the penton base of many but not all Ad serotypes. It is not yet known whether Ad interacts with both CAR and the integrins simultaneously, or sequentially. In one model it is suggested (Nemerow, 2000) that the fiber upon CAR binding changes its conformation, allowing the virus capsid to get closer to the cell surface, which facilitates interaction between the penton base and the αv integrins (Fig. 12).

The internalization is mediated by endocytosis of the virus-receptor complex into clathrin-coated vesicles (Fig. 12). The viral particles are uncoated in the endosomes and subsequentially released into the cytoplasm by rupture the endosomal membrane (Greber et al., 1993). There are several lines of evidence suggesting that the integrins play an important role in this process (Wang et al., 2000). The Ad particles then associate with microtubuli for their transport to the nucleus (Nakano and Greber, 2000; Suomalainen et al., 1999).
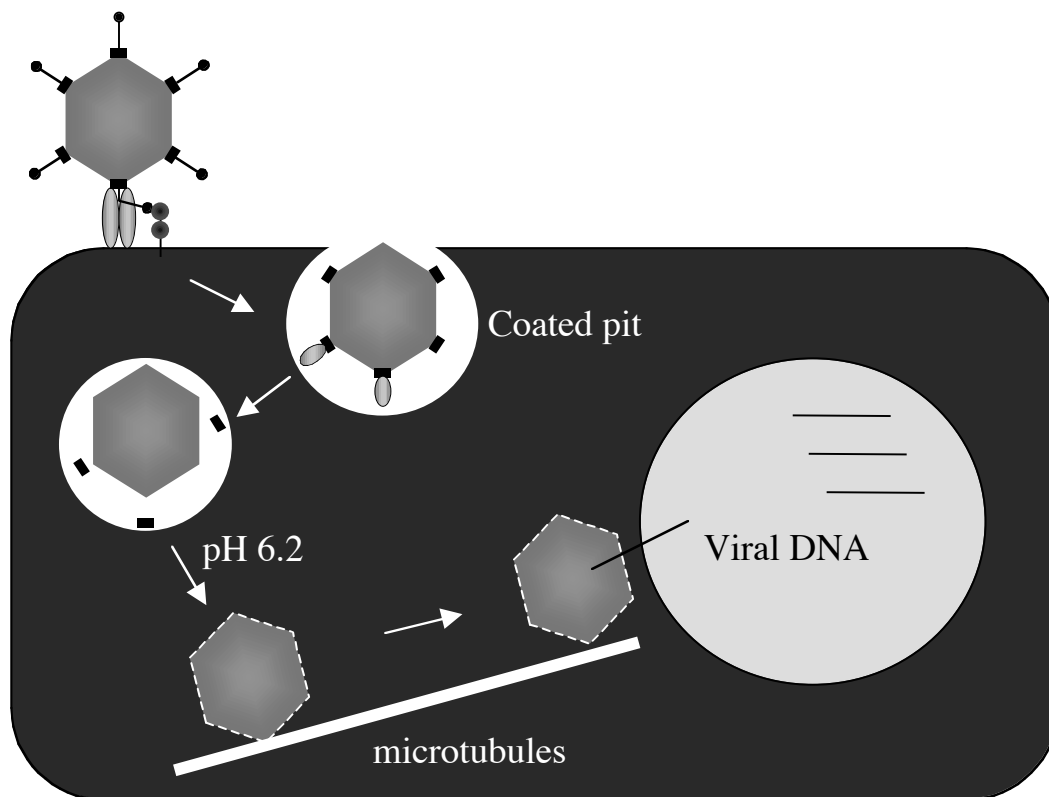
Figure 12. Adenovirus cell entry pathway. The virus and the cellular integrins are internalized into coated pits. Acidic conditions in the early endosomes triggers partial disassembly of the viral capsid allowing the virus to be released into the cytoplasm. It is then associated with microtubuli and transported to the nucleus, where the virus delivers its DNA. (*Modified from Nemerow G.R., 2000*)

Adenovirus vectors in cancer research

For *in vivo* cancer gene therapy applications, replication-defective recombinant adenoviruses are the most widely used vectors. They have been used in cancer trials to deliver therapeutic genes that can act to directly, or indirectly kill or block the growth of the tumor cells, inhibit angiogenesis, stimulate immune responses to tumor antigens and block tumor cell invasion (Anklesaria, 2000; Crystal, 1999; Zhang, 1999). In addition, replicating, tumor cell-selective, oncolytic adenoviruses have been explored as toxic agents (Alemany et al., 2000). In different

human tumors and tumor cell lines examined so far, CAR is expressed at very variable levels (Cripe et al., 2001; Dmitriev et al., 1998; Fechner et al., 2000; Hemmi et al., 1998; Li et al., 1999; Miller et al., 1998; Okegawa et al., 2000). Not much is known about the expression of CAR in primary human gliomas. Miller et al. (1998) found that the level of CAR expression varied a lot between and within different astrocytoma grades and that the histological type of the tumor did not appear to influence the susceptibility of the cells to Ad infection. Tumor cells lacking or expressing low levels of CAR are resistant to adenovirus infection and to efficient oncolysis by replicating Ad (Douglas et al., 2001). This has led to new approaches such as tumor targeting, where modifications of the fiber protein have been used to direct the attachment of Ad to other cell surface molecules than CAR (Hidaka et al., 1999; Krasnykh et al., 2000; Reynolds et al., 1999; Vanderkwaak et al., 1999; Wickham, 2000; Wickham et al., 1997).

AIMS OF THE STUDY

Based on the findings that the genes encoding the cyclin dependent kinase inhibitors p16^{INK4A} and p15^{INK4B} are frequently inactivated in human gliomas (and in a large number of other tumors), the major aim of this thesis was to study the role of these proteins in cell cycle regulation, senescence and immortalization. To introduce p16^{INK4A} and p15^{INK4B} in various cells, the aim was also to construct recombinant adenoviruses expressing these proteins, and to establish the adenovirus expression system in the lab. This subsequently led to studies on the expression of the human coxsackie- and adenovirus receptor, hCAR, in human gliomas.

More specifically, the aims were:

(i)   To compare the phenotypic and molecular consequences of using recombinant adenoviruses to express either p16^{INK4A} or p15^{INK4B} in human glioma cells, in which both these genes are co-inactivated, and in bovine capillary endothelial cells (BCE), immortalized with human telomerase (hTERT) (Papers I and III)

(ii)   To find out the molecular and possible functional differences between the two isoforms of p15^{INK4B} (p15^{INK4B} and p15.5^{INK4B}) (Paper II)

(iii)   To analyze changes in expression levels of p16^{INK4A} and p15^{INK4B}, as well as other cell cycle genes regulating the G1 phase of the cell cycle, in BCE cells immortalized with hTERT (Paper III)

(iv)   To study the expression of the coxsackie- and adenovirus receptor, hCAR, in human gliomas and to find out if any correlation between hCAR expression levels and tumor malignancy grade exist (Paper IV)

METHODS

Apart from the standard molecular biology and cell culture techniques used in this thesis, the more specific methods are summarized in the table below. For detailed information, see the materials and methods sections in the referred papers and manuscripts.

Methods	Paper
Construction of recombinant adenoviruses	I
Adenovirus infections	I, II, III, IV
Immunoblotting	I, II, III, IV
Immunofluorescence	I, II, III, IV
Southern blotting	I, III
Polymerase chain reaction (PCR)	I, IV
Reverse transcriptase (RT) – PCR	I
Real-time quantitative PCR	IV
Immunoprecipitation	I
Senescence-associated beta gal staining (SA-β-gal)	I, II, III
Telomeric repeat amplification protocol (TRAP) assay	I, III
Single stranded mutagenesis	II
FACS analysis	III, IV
Subcutaneous transplantation of tumor tissue to nude mice	IV

RESULTS AND DISCUSSION

Overexpression of p15[INK4B] in human glioma cells leads to cell cycle arrest, a senescent-like phenotype and repression of telomerase (paper I).

The CDKIs have attracted much attention because of their potential as tumor suppressors. This is particularly the case with the genes encoding p16[INK4A] (p16) and p15[INK4B] (p15), since they are frequently homozygously deleted in a broad range of tumor cell lines and primary tumors. In human glioblastomas, they are homozygously deleted in 40–50% of primary tumors and in an even higher percentage in glioma cell lines. Inactivation of p16 by point mutations or promoter methylation is also frequently detected in human cancer. Mice with a targeted deletion of the p16 gene spontaneously develop tumors within the first year of life. Ectopic expression of p16 induces G1 growth arrest in cells with an intact pRB (Craig et al., 1998; Koh et al., 1995; Lukas et al., 1995; Medema et al., 1995) and marked morphological changes reminiscent of replicative senescence (Uhrbom et al., 1997). A role for p16 in replicative senescence is also supported by the fact that it accumulates in various cell types as they approach senescence. Thus, while the role of p16 in senescence and tumor suppression has been amply documented, the role of p15 has been less clear.

To compare the capability of p15 to induce growth arrest and senescence with that of p16, recombinant adenoviruses expressing p15 (Adp15) or p16 (Adp16) were constructed and used to infect two human glioma cell lines with different status of the p16, p15 and pRB genes. In U251MG cells both the p16 and the p15 genes are homozygously deleted, but the pRB gene remains intact. In contrast, U373MG cells retain wild-type p16 and p15 alleles, but have a loss of pRB function. By infecting U251MG and U373MG cells with Adp16 or Adp15 and analyzing the cell lysates from these cells we observed that both p16 and p15 were found in complexes with CDK4 and CDK6, indicating that the adenovirus expressed proteins were active. It turned out that p15 was equally effective as p16 in causing growth arrest in U251MG cells. In

contrast, U373MG cells, in which pRB function is lost, were not growth arrested by neither p16 nor p15. A control virus expressing β-galactosidase (AdlacZ) did not induce growth arrest in any of the cell lines, indicating that the growth inhibitory effect of U251MG cells were specifically induced by the CDKIs, and not due to virus toxicity. These results are in agreement with previous studies showing that p16-mediated growth arrest is dependent upon an intact pRB (Craig et al., 1998; Koh et al., 1995; Lukas et al., 1995; Medema et al., 1995). The results were fully reproducible in another pair of glioma cell lines, Tp483MG and SW1723, which have the same status of the *RB*, *CDKN2A/2B* genes as the U251MG and U373MG, respectively.

Immunofluorescent analysis of infected U251MG cells expressing p16 or p15 showed strong predominantly nuclear but also diffuse cytoplasmic staining. Already at 48 h post infection (p.i.), both p15- and p16-expressing cells displayed clear morphological changes as compared with mock-infected or AdlacZ control virus-infected cells. Transduced cells were enlarged and flattened, an effect that was even more pronounced at 7 days p.i. In contrast, these morphological changes were absent in infected U373MG cells, even 7 days after infection. Since the morphological changes were reminiscent of those seen in cells entering replicative senescence, we stained infected U251MG and U373MG cells with the senescence marker, SA-ß-gal. We found that cells that displayed a large and flattened morphology also became positive for SA-β-gal, which shows that p15, similarly to p16, has the capacity to induce senescent-like changes in glioma cells with an intact pRB. Furthermore, telomerase activity has been shown to be a prerequisite for the immortalization of mammalian cells. Since the growth arrest and senescent-like changes induced by p15 and p16 in U251MG cells indicated an escape from an immortalized phenotype, we also analyzed if telomerase activity was affected by the expression of p15 or p16. The results clearly showed that telomerase activity was almost completely inhibited in these cells by day 6 after infection with either Adp15 or Adp16. This was not seen in U373MG cells.

Whether p15 and p16 are equally efficient, on a molar basis, in exerting these biological effects could not be fully assessed using the adenovirus expression system. The mRNAs encoding p15 and p16 were both expressed from the cytomegalovirus (CMV) early promoter, and the same titer of recombinant adenoviruses was used in each experiment comparing the biological effects. In addition, we carried out extensive control experiments to ascertain that the level of expression of p15 and p16 was the same in each experiment. These included careful titration of the viruses, the use of different multiplicities of infection (MOI) and titration of the antisera used. All of the results from these experiments suggested that p15 and p16 both qualitatively and quantitatively have the same biological effects. So, what are the differences between p15 and p16, and does p15 play any role in tumor suppression?

As discussed earlier, the expression pattern of p15 and p16 in tissues is different. In addition, the regulation of expression of these CDKIs differs from one another. p15, but not p16, is upregulated by both TGF-β and IFN-α, growth inhibitory factors that potently inhibit the proliferation of a variety of cells of different origin by inducing G1 growth arrest (Massague et al., 2000; Sangfelt et al., 1997) p15 is rapidly upregulated by these factors and induces cell cycle arrest by directly inhibiting CDK4/6, and by indirectly inactivating cyclin E-CDK2, via the displacement and shuttling of p27 from CDK4/6 to cyclin E-CDK2. In many cases TGF-β-mediated growth arrest is reversible, but sometimes it is associated with terminal differentiation, or apoptosis. In some reports, TGF-β has also been suggested to be involved in age-related changes and senescence both *in vivo* (Ding et al., 2001; Kordon et al., 1995; Ruiz-Torres et al., 1998) and *in vitro* ((Frippiat et al., 2001; Katakura et al., 1999), and our own unpublished results)). Interestingly, TGF-β and IFN-α have been shown to down-regulate telomerase activity in both malignant and non-malignant cells (Xu et al., 2000; Yang et al., 2001). Moreover, the expression of p15 and p16 accumulates in T-lymphocytes as they approach senescence *in vitro* (Erickson et al., 1998). Together with the fact that we found that p15 caused a replicative senescence phenotype indistinguishable from that caused by p16 suggests that p15 in some situations/cell types plays a role in senescence, and that the two cell cycle inhibi-

tors may work through a similar mechanism. Future studies will show if p15, and p16, in agreement with our results, are involved in senescence-related processes *in vivo*.

The role of p15 in tumor suppression has been debated. Our results indicate that p15 is as potent as p16 in inducing cell cycle arrest and senescence, as well as in repressing telomerase activity in human glioma cells. Apart from frequent inactivation of p15 by co-deletions together with the p16 gene, both point mutations and promoter methylation have recently been reported to be additional ways of inactivating p15 in human cancer. These results, plus the fact that p15 deficient mice develop tumors, clearly indicate that p15, in addition to p16, has tumor suppressor properties at least in some cell types.

p15.5^INK4B is an N-terminally extended and fully active form of p15^INK4B (paper II).

During the course of our studies on p15, we consistently observed that human p15 migrated as a doublet on SDS-polyacrylamide gels. These two species, called p15.5 and p15, were already noted in the original report by Hannon and Beach (1994) describing p15, and has been observed subsequently also by others (Reynisdottir et al., 1995). Since isoforms of various proteins may possess different functions, have different stability, or display different subcellular distribution, we set out to analyze the molecular and possible functional differences between the two p15 isoforms.

One mechanism of generating isoforms of proteins is initiation of translation from codons other than the classical AUG (e.g. ACG, CUG, or GUG) (Kozak, 1991). We therefore looked for alternative initiation sites in the region upstream from and in frame with the first AUG within the p15 gene. Constructs, in which the coding region of the human p15 cDNA plus different numbers of nucleotides upstream of the first AUG were included, were analyzed by *in vitro* translation. From these studies we identified a region located around 35-45 nucleotides upstream from the first AUG that was crucial for p15.5 synthesis. In this region, two codons with the potential to function as alternative initiation codons for translation are located at po-

sitions 286 (ACG) and 289 (GUG). Additional experiments with constructs in which these two candidate initiation codons were mutated into GCA suggested that p15.5 is initiated at the GUG codon at position 289. Subsequently, these results were confirmed in transfection experiments that also showed that both the p15.5 and p15 isoforms, when expressed in combination or separately, associate with both CDK4 and CDK6, block cell proliferation, and induce a senescent-like phenotype in human U251MG glioma cells.

The context around the GUG codon initiating p15.5 synthesis is more close to the optimal Kozak consensus sequence than the sequence context around the first AUG. In addition, the 5' noncoding region of the p15 mRNA is unusually long (327 nucleotides) and GC rich (65%), characteristics that have shown to slow down the transit of the 40S ribosomal subunit down the mRNA. Thus, these are factors that could contribute to the initiation at the GUG codon. An N-terminal extension of proteins has in some cases been shown to affect the function and/or the fate of the protein. For example, four versions of the fibroblast growth factor-2 are synthesized due to initiation at three upstream CUG codons, in addition to initiation at an AUG codon (Florkiewicz and Sommer, 1989). Translation at the CUG codons is activated in transformed and stressed cells (Vagner et al., 1996). The N-terminally extended forms are translocated to the nucleus, while the AUG-initiated form remains cytoplasmic (Bugler et al., 1991; Cao and Pettersson, 1993). In light of these and other examples, it seemed plausible that p15.5 and p15 also could have different functions or cellular distributions. Our analyses did not, though, show any differences in the capacity of p15 and p15.5 to bind to CDK4 and CDK6, to inhibit DNA synthesis, nor to induce a replicative senescent phenotype. In addition, we also found that the stability and intracellular distribution of the two isoforms of p15 were identical (unpublished results). However, since we only used a limited set of assays, it is still possible that the two isoforms may display functional differences in some other assays. Finally, we compared the expression of human p15 with p15 in other species and noticed that p15.5 is absent in mouse cell lysates (Latres et al., 2000), but present in lysates from lung epithelial cells from mink (Mv1Lu cells) (Reynisdottir and Massague, 1997; Zhang et al.,

2001). The GUG codon is not present in the mouse p15 cDNA sequence, which probably explains the absence of p15.5 in mouse cell lysates. If the p15.5 isoform in mink cells is initiated from the same GUG codon as in humans is not clear, since the p15 cDNA from mink has not been sequenced.

Immortalization of bovine capillary endothelial cells by telomerase is mediated by a telomere length independent mechanism (paper III).

Primary BCE cells derived from the adrenal gland cortex are widely used in angiogenesis and antiangiogenesis studies (Folkman and Haudenschild, 1980; Folkman et al., 1979). Like human primary cells, they undergo a limited number of divisions in culture before they enter a non-dividing state *i.e.*, replicative senescence. Shortening of telomere repeats during cell divisions has been considered as the mechanism of replicative senescence. In contrast to primary human and bovine cells, rodent cells do not seem to have a cell division counting mechanism. Introduction of hTERT alone into primary human skin fibroblasts (Bodnar et al., 1998; Vaziri and Benchimol, 1998), retinal pigment epithelial cells (Bodnar et al., 1998) or endothelial cells (Yang et al., 1999) is sufficient for immortalization. However, others have reported that both inactivation of the p16/pRB pathway and simultaneous expression of hTERT are required to immortalize primary human skin keratinocytes and mammary epithelial cells (Dickson et al., 2000; Farwell et al., 2000; Kiyono et al., 1998), suggesting a cell type specificity of hTERT-mediated immortalization.

In this study, we transfected primary BCE cells with a vector encoding hTERT alone, which turned out to be sufficient for immortalization. Cells expressing telomerase were isolated and, in contrast to normal BCE cell cultures, where more than 90% of the BCE cells entered replicative senescence after 45 population doublings (PDLs), the telomerase positive cells escaped senescence. Compared to normal BCE cells, which stained positive for the SA-β-gal marker and appeared large and flattened, only a few of the hTERT-expressing cells at higher PDLs showed SA-β-gal staining and their morphology resembled young primary BCE cells. Expres-

sion of hTERT in BCE cells did not alter their ability to take up acetylated-LDL, an endothelial cell marker. Telomerase activity was detected in hTERT[+]-BCE cells that have been grown for over 200 PDLs and these cells continue to proliferate as primary young BCE cells, without evidence of obvious altered morphology or growth rate. Like telomerase-immortalized human cells, hTERT[+]-BCE cells were not able to form tumors when implanted subcutaneously into immunodeficient SCID mice. Surprisingly, the peak telomere lengths in hTERT[+]-BCE cells were progressively shortened during cell doublings. At the early PDL of 50, the peak length of hTERT[+]-BCE cells was similar to that of senescent primary BCE (PDL 45), with telomere sizes of approximately 11-12 kb. However, telomere lengths were found to be significantly shorter in later population doublings, ranging from 8.6 to 7.8 in PDL 150 and PDL 200, respectively.

As in cultured primary human cells, expression of p16 and p21 accumulated in normal BCE cells as they approached senescence. In hTERT[+]-BCE cells at both early and late number of PDLs, both p16 and p21 expression were repressed. Still after serum starvation for 24h, a substantial amount of pRB protein remained in a hyperphosphorylated state. By 4h after addition of serum, virtually all pRB molecules became hyperphosphorylated. Cell cycle analysis revealed that although serum was depleted from the growth media, a substantial portion of hTERT[+]-BCE cells was found in the G2/M phase. In comparison, presenescent BCE cells accumulated in the G0/G1 phase of the cell cycle.

We reasoned that if p16 inactivation was important for the hTERT[+]-BCE cells to bypass replicative senescence, overexpression of p16 in these cells should reverse the replicating phenotype. To test this hypothesis, we infected hTERT[+]-BCE cells at 150 PDLs with adenoviruses encoding either p15 (Adp15), or p16 (Adp16). Both p15 and p16 induced a senescent-like phenotype in the telomerase positive cells, as determined by SA-β-gal activity and morphological changes like enlargement of cellular sizes and flattened cell shapes. In addition, ectopic expression of both p15 and p16 resulted in a complete growth arrest.

In these studies, ectopic expression of the hTERT cDNA alone was sufficient to immortalize primary BCE cells. This was also accompanied by repression of both p16 and p21 expression. Since we found it unlikely that both these genes underwent simultaneous mutations and/or deletions in the hTERT$^+$-BCE cells, we hypothezised that methylation could be involved in the inactivation of these genes. To determine the methylation state of these genes, hTERT$^+$-BCE cells were treated with the demethylating agent, 5-azadeoxycytidine. This increased the expression of both p16 and p21 in the hTERT$^+$-BCE cells, and induced a senescent-like phenotype. Thus, we conclude that promoter methylation is likely to be the mechanism of inactivation of these genes in the hTERT immortalized cells.

All together, our data indicate that human TERT alone is sufficient to immortalize bovine primary endothelial cells and that immortalization is mediated through inactivation of crucial cellular senescence machineries including p16/pRB and p21. Despite the fact that stable and high levels of telomerase activity are detected in various PDLs, telomere lengths are significantly shorter in hTERT-immortalized BCE cells as compared with senescent BCE cells. This finding is unexpected and surprising because the key function of TERT is thought to be to extend and maintain the critical length of telomeres that are essential for genomic stability. If telomere lengths in senescent cells are shorter than the minimal and critical length that is required to stabilize chromosomes, introduction of TERT into subsenescent cells should increase or prevent further shortening of telomeres and thus protect cells from senescence. In some primary human cells including fibroblasts and retinal pigment epithelial cells, this seems to be the case. However, others have also reported that telomeres become progressively shorter in hTERT-immortalized human fibroblasts and endothelial cells compared to their senescent parental cells (Farwell et al., 2000; Ouellette et al., 1999; Yang et al., 1999). This might reflect that telomere shortening is not the only factor that determines the entry into replicative senescence, an issue that also has been addressed by others (Sherr and DePinho, 2000). Our results suggest that immortalization of bovine endothelial cells by human TERT alone may not pri-

marly be due to telomere stabilization, but instead of inactivation of p16 and p21, proteins that are known to induce and maintain a senescent phenotype in mammalian cells. The fact that reactivation of p16 in hTERT[+]-BCE cells by either adenovirus-mediated expression or by de-methylation induced a senescent-like phenotype, further supports this theory.

Heterogeneous expression of hCAR in primary human gliomas (paper IV).

Replication-defective recombinant adenoviruses are currently the most widely used vectors for *in vivo* cancer gene therapy applications. They have been used to deliver therapeutic genes that can act to directly, or indirectly, kill or block the growth of the tumor cells, inhibit angiogenesis, stimulate immune responses to tumor antigens, or block tumor cell invasion. The infectability of human tissues and tumors with type C adenoviruses correlates with the expression of the human coxsackie B- and adenovirus receptor, hCAR, which is heterogeneously expressed in various types of human cancer cells. In primary gliomas, not much is known about CAR expression levels. Miller et al. (1998), studied CAR expression in short term cultures of glioblastomas and noticed that CAR expression levels varied a lot between and within different astrocytoma grades. Ectopic expression of hCAR in a prostate cancer cell line have been shown to result in a lowered proliferative potential of these cells, both *in vitro* and *in vivo*, indicating a tumor suppressive role for CAR. A cell type specific regulation of endogenous hCAR expression in response to cell density has also been reported. These results, and the fact that the expression levels of hCAR in primary tumors has implications for the use of adenoviruses as vectors in cancer gene therapy, prompted us to investigate if there could be a correlation between tumor malignancy grade and expression of hCAR in primary astrocytomas.

By using immunoblotting, real-time PCR, FACS-analysis, and sensitivity to infection with adenovirus-lacZ, we analyzed the expression levels of hCAR in glioma grade IV cell lines. Analysis of a set of 10 cell lines revealed great variation in hCAR expression, which correlated well with the infectability with Ad-lacZ. No such correlation was observed with the expression of $\alpha v\beta 3/\alpha v\beta 5$ integrins, which are proposed to function as co-receptors for adenovi-

ruses. The mean values of CAR expression was significantly lower in 22 grade IV tumors as compared to the values for 6 grade II ($P = 0.01$) and 6 grade III ($P = 0.01$) tumors. This suggests a possible growth advantage of cells with lower levels of hCAR expression. When hCAR expression in 11 xenografts derived from glioblastomas were compared to the level detected in the original parental tumors, a mean 12-fold higher expression was seen in the xenografts ($P = 0.01$). Interestingly, the two xenografts with no apparent upregulation of hCAR expression grew much faster than the hCAR-expressing cells. This points to an inverse correlation between growth rate and hCAR expression, and suggests that hCAR may contribute to suppression of tumor growth.

In general, our results on hCAR expression in the cell lines are in good agreement with those now obtained for a range of tumor cell lines. A variability of CAR expression levels has been found in e.g. bladder (Li et al., 1999; Okegawa et al., 2000), prostate (Okegawa et al., 2000), melanoma (Hemmi et al., 1998) and in glioma cell lines (Asaoka et al., 2000; Miller et al., 1998; Mori et al., 1999). In some tumor cell lines, such as rhabdomyosarcoma (Cripe et al., 2001) and gastrointestinal cancers (Fechner et al., 2000), CAR expression has been found to be low or undetectable.

To date there is almost no information in the literature on the expression of CAR protein, or mRNA, in primary astrocytic tumors. This is partly due to the inherent problems of reliably quantifying CAR protein expression from tumor tissue. Miller et al. (1998) demonstrated highly variable adenovirus infectability of primary glioma cultures after short-term *in vitro* culturing. Interestingly, they found that the susceptibility did not correlate with the original histological type of the tumor. However, even a few *in vitro* passages of tumor specimens may select subsets of cells, and result in altered gene expression, such as CAR. Combined with our results this information should be of importance in regard to clinical trials in which adenovirus vectors are being used for the treatment of glioblastomas (Puumalainen et al., 1998). From the point of view of practical gene therapy, the expression levels of CAR in the primary tumors,

rather than in established cell lines, is of crucial relevance. Future work should thus focus on determining CAR expression in primary tumors and analyzing their susceptibility to adenovirus infection.

The observation that grade IV tumors express lower levels of CAR than the grade II and III tumors may point to a possible growth advantage for tumors displaying low CAR expression levels. Alternatively, CAR may be secondarily down-regulated in fast growing cells. The normal cellular function of CAR is not known. However, some recent results suggest that CAR may serve as a cell-cell adhesion molecule (Honda et al., 2000). Furthermore, other results suggest that CAR expression may inversely correlate with cell growth. Bladder carcinoma cell lines expressing elevated levels of CAR showed increased cell-cell aggregation, displayed a slower growth rate, and antibodies to the CAR ectodomain prevented the inhibitory effect of CAR on growth (Okegawa et al., 2001). Our results also show that loss of CAR expression correlates with increased growth rate of glioma cells. The two xenografts that did not show any CAR expression grew considerably faster in nude mice than the 9 CAR-positive tumors. However, although CAR may thus find a role as a tumor suppressor, in addition to being the entry ticket for adenoviruses into cells, we wish to stress that these observations need to be corroborated experimentally.

Finally, the observation that CAR expression was upregulated in 9 out of 11 glioblastoma xenografts as compared to their parental, primary tumors, is interesting. The reason for this is unclear, but may suggest that CAR gene expression is induced by factor(s) produced in the mice. So far little is known regarding signaling that leads to regulation of the CAR promoter. Analysis of CAR regulation will have to await a detailed definition of the CAR promoter region and the development of a reporter system for analyzing factors regulating CAR expression.

CONCLUSIONS

p15^{INK4B} is as potent as p16^{INK4A} in inducing cell cycle arrest and a senescence-like phenotype in glioma cells with an intact pRB. This shows the potential of p15 to function as a tumor suppressor and mediator of senescence. Together with the fact that p15 accumulates in T lymphocytes as they approach senescence and that p15 deficient mice display tumors of the hematopoietic cell system, this suggests that p15 plays a role in regulating homeostasis in these (and perhaps other) cell systems. We also conclude that p15 and p16 act through similar mechanisms.

One of the original aims was to study whether adenovirus-mediated transfer of wild-type p16 could inhibit the growth of gliomas with inactivated p16 expression *in vivo*. Our preliminary and unpublished results were, however, disappointing, since only a small fraction of the glioma cells that were grown subcutaneously in nude mice were infected by Adp16. Thus we were discouraged to continue these studies. The prospects of being able to correct genetic lesions by gene therapy in cases where the gene product remains within the cell, and when no bystander effects are obtained, seem at present unrealistic.

Adenovirus-mediated overexpression of either p15 or p16 strongly inhibits telomerase activity in the glioma cells and is sufficient to reverse the immortalized phenotype of both these cells, and of BCE cells expressing an ectopically introduced telomerase (expressed from a constitutively active promoter). Since p16 expression (and p21) is repressed in the immortalized BCE cells by DNA methylation, we conclude that p16 is one of the important players that are involved in maintaining homeostasis. In fact, others have also suggested that the p16/pRB pathway needs to be inactivated for the immortalization of at least certain cell types. This is also supported by the fact that the pRB pathway is disturbed in many, if not all human cancers. The importance of replicative senescence *in vivo* and its role as a barrier to immortalization and

cancer needs to be more thoroughly investigated. Studies that address these issues will also provide further information about the involvement of p15 and p16 in such cellular processes, and why they are so frequently inactivated in human cancers.

An alternative isoform of p15, p15.5, is initiated from an alternative GUG initiation codon located upstream of the first AUG in the p15 gene. Both these isoforms target CDK4 and CDK6, and have similar capacities to induce cell cycle arrest and a senescent-like phenotype in human glioma cells. The importance of the existence of two different p15^{INK4B} isoforms remains to be solved. Even though we could not find any functional differences between p15^{INK4B} and p15.5^{INK4B} in our assays, one cannot exclude the possibility that differences do exist under certain conditions.

Finally, the expression of CAR, the adenovirus receptor, seems to decrease in astrocytic tumors as they progress to the most malignant form, suggesting a role for CAR in tumor suppression. In contrast, CAR expression levels are significantly upregulated in xenografts derived from high-grade glioblastomas, and grown subcutaneously in nude mice. The mechanism behind the upregulation remains to be determined in future studies. Potentially, if specific factors with the ability to regulate CAR expression *in vivo* could be identified, these may be used to increase the efficiency of infection with adenovirus vectors in cancer research, or to upregulate CAR with the aim of slowing down tumor cell growth.

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