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# Tumor Suppressor Gene p16/INK4A/CDKN2A and Its Role in Cell Cycle Exit, Differentiation, and Determination of Cell Fate

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## 1. Introduction

Tumor suppressor genes and oncogenes are important regulatory genes which encode proteins regulating transitions in and out of the cell cycle and which also have a role in the gateway to terminal differentiation (Tripathy & Benz, 1992). Defects in tumor suppressor genes and oncogenes result in uncontrolled cell division, which leads to cancer (Tripathy & Benz, 1992). Oncogenes are mutated proto-oncogenes that have a role in malignancy of tumors and most frequently regulate cell cycle re-entry. Gain-of-function mutations result in transformation of proto-oncogenes into dominant oncogenes. Tumor suppressor genes encode proteins that suppress cell growth and most frequently result in exit from the cell cycle. Loss-of-function mutations in tumor suppressor genes result in tumor malignancy and can account for hereditary cancers. Every gene has two alleles present in the genome (with a few exceptions in the hemizygous regions of the sex chromosomes). For tumor suppressor genes to be inactivated either deletion of one allele and somatic mutation of the other allele is required resulting in a loss of heterozygosity (Swellam et al., 2004), or somatic deletion of both of the alleles is required resulting in a complete loss of homozygosity (Quelle et al., 1997). Tumor suppressor genes can also be inactivated by hypermethylation of the gene resulting in promoter suppression so that genes can not be transcribed further (Herman et al., 1997). Telomere shortening and tumor suppressor gene promoter hyper-methylation can be used as potential breast cancer biomarkers (Radpour et al., 2010).

Regulation of cell proliferation and differentiation is important in due course of growth and development of an organism. Cell proliferation is not an infinitely continuous process as cells undergo a finite number of cumulative population doublings (CPDs) in culture before entering replicative senescence (RS) (Hayflick, 1965). Cell replication or growth is controlled by a complex network of signals that control the cell cycle, the orderly sequence of events that all cells pass through as they grow to approximately twice their size, copy their chromosomes, and divide into two new cells. The cell cycle consists of 4 phases; G1, S, G2, and M phase (Enoch & Nurse, 1991). DNA duplication takes place in S phase and cytokinesis in M phase. G1 and G2 are gap phases, which provide the time for cells to

ensure suitability of the external and internal environment and preparation for DNA duplication and division. Cell cycle progression from one phase to another is controlled principally by cell cycle proteins; cyclins, the cofactors of cyclin dependent kinases (CDKs), a family of serine/threonine kinases (Afshari & Barrett, 1993). Cyclins are the cell cycle proteins, which bind to CDKs and activate them to function and enhance cell cycle progression (Pines & Hunter, 1991). Cyclin/CDK complexes are specific for each phase transition. In complex eukaryotic cells there are approximately 20 CDK related proteins. Complex combination of all these different CDKs and cyclins in different phases of the cell cycle provide tightly regulated control of cell cycle progression (Satyanarayana & Kaldis, 2009). Levels of CDKs in cells vary little throughout the cell cycle, but cyclins, in contrast are periodically synthesized and destroyed in a timely manner to regulate the CDK's activity during cell cycle (Malumbres & Barbacid, 2009).

Early G1 phase progression is facilitated by CDK4/6 binding with cyclin D family proteins. These complexes phosphorylate members of the retinoblastoma protein (Rb) family (Rb, p130, and p107) (Sherr & Roberts, 1999). Phosphorylation of Rb results in release of E2F protein, which otherwise binds to Rb. E2F is a transcription factor, which activates E2F responsive genes, which are required for further cell-cycle progression in S phase (Weinberg, 1995). CyclinE/CDK2 complexes complete Rb phosphorylation and promote further progression of the cell cycle through late G1 phase. These complexes further activate E2F-mediated transcription and passage through the restriction point to complete G1/S phase transition (Sherr & Roberts, 1999). At the onset of S phase, cyclin A is synthesized, forms a complex with CDK2 and phosphorylates proteins involved in DNA replication (Petersen et al., 1999).

During replication of DNA in S phase of the cell cycle, CDC6 and Cdt1 are recruited to recognition complexes. These factors help in the recruitment of mini-chromosome maintenance (MCM) proteins to replication origins which are known as pre-replicative complexes (preRC). In early S phase, preRC recruits the functional replication complex including DNA polymerase and associated processivity factors such as proliferating cell nuclear antigen (PCNA). Subsequent cell cycle transition takes place through the activity of the CDK1/cyclinA complex initiating prophase of mitosis (Furuno et al., 1999). Finally, activation of CDK1/cyclin B complex activity completes entry into mitosis (Riabowol et al., 1989).

Along with the cyclins and CDKs, other proteins such as the tumor suppressor genes, the retinoblastoma protein (Rb), p53 and transcription factors such as the E2F proteins, play important roles in regulating cell cycle progression. The cell cycle has two important check points that occur at the G1/S and G2/M phase transitions (Hartwell & Weinert, 1989). These check points control cell cycle progression during normal proliferation and during stress, DNA damage, and other types of cellular dysfunction. At these cell cycle check points, cellular CDKs can be inhibited by cyclin-dependent kinase inhibitors (CKIs); thus, inhibiting and regulating cell cycle progression (Morgan, 1997). Rb can remain active suppressing downstream transcription factors if cyclin/CDKs are suppressed and p53 can directly activate CKI gene expression (Udayakumar et al., 2010).

All of the CKIs are proven tumor suppressor genes or suspected of having this potential. Two CKI families which play important roles in regulating cell division are; the INK4 family and the KIP/CIP family (Vidal & Koff, 2000). INK4 family inhibitors inhibit CDK4 and CDK6 in association with cyclin D, while KIPs inhibit CDK1, CDK2 and CDK4 associations with cyclin A, cyclin B, and cyclin E. The INK4 family consists of p16 (INK4A), p15 (INK4B), p18 (INK4C), and p19 (INK4D). The KIP family consists of p21 (CIP1), p27 (KIP1), and p57 (KIP2).

### 1.1 INK4A/CDKN2A/p16

p16 is an important CKI and a tumor suppressor gene encoded on the 9p21 region of the human genome, chromosome number 4 in mouse, and chromosome 11 in dogs (Serrano et al., 1993; Kamb et al., 1994; Asamoto et al., 1998; Fosmire et al., 2007) at the INK4A/ARF/INK4B locus. This gene locus is a 35kb multigene region which encodes three distinct major tumor suppressor genes, p15, p14ARF, and p16 (Sherr & Weber, 2000). INK4A/ARF/INK4B gene locus is repressed in young and normal cells by polycomb proteins and histone H3 lysine27 (H3K27) trimethylation (Kotake et al., 2007; Kia et al., 2008; Agger et al., 2009) and is induced during aging or by hyperproliferative oncogenic stimuli or stress. The INK4A/ARF locus has been speculated to have a global anti-aging effect by favoring cell quiescence and limiting cell proliferation (Matheu et al., 2009).

The classic role of p16/INK4A/CDKN2A is to check the cell cycle in early G1 phase and inhibit further transition of the cell cycle from G1 to S phase as a component of a multi-protein regulatory complex. During G1 phase, CDK4 and CDK6 form complexes with cyclin D1 which in turn phosphorylate the Rb protein family resulting in additional phosphorylation by cyclin E/CDK complexes. These inhibitory phosphorylations of Rb cause release of the E2F transcription factor from Rb/E2F complexes. Rb otherwise inhibits transcription factor E2F (Weinberg, 1995). E2F is a transcription factor which initiates transcription of genes required for S phase such as DNA polymerase, thymidine kinase, dihydrofolate reductase, replication origin binding protein HsOrc1 and MCM (Lukas et al., 1996). Action of p16 inhibits binding of CDK4/6 with cyclin D1 which leaves Rb, and Rb-related proteins like p107 and p130, un-phosphorylated and E2F bound and inactive (Serrano et al., 1993; Walkley & Orkin, 2006). INK4 proteins cause both inhibitory structural changes and block activating structural changes to bound CDKs. p16 binds next to the ATP binding site of the catalytic cleft, opposite to the cyclin binding site, which results in a structural change in the cyclin binding site (Russo et al., 1998). p16/INK4A targets CDK4 and CDK6, rather than the cyclin subunit, and actually competes with cyclin D1 for CDK binding. Binding of p16 results in changes in conformation of CDK proteins so that they can no longer bind cyclin D1 (Russo et al., 1998). p16 distorts the kinase catalytic cleft, interferes with ATP binding, and thus may also deactivate pre-assembled CDK4/6-cyclin D1 complexes blocking their function (Russo et al., 1998). Binding sites for p16 and cyclin D1 on CDK4 are overlapping in some cases and are present near the amino terminus where a majority of the mutations in CDK4 are found. Mutations in the p16 binding site result in diminished capability of p16 binding to CDK4 and also compromise the binding of cyclin D1 to CDK4, which can also lead to melanoma (Coleman et al., 1997; Tsao et al., 1998). Other than inhibiting the pRb/E2F pathway, the very recently reported function of p16 is to downregulate CDK1 expression by upregulating miR-410 and miR-650 (Chien et al., 2011). CDK1 is an indispensable kinase which is most important for cell cycle regulation during G2/M phase (Santamaria et al., 2007). The regulation of CDK1 by p16 is post-transcriptional. Thus, p16 is an important tumor suppressor gene which regulates gene expression at different levels by modifying functional equilibrium of transcription factors, and consequently of miRNAs, and also by binding to post-transcriptional regulators (hnRNP C1/C2 and hnRNP A2/B1) (Souza-Rodrigues et al., 2007). The role of p16 in cell growth can also be attributed by irreversible repression of the hTERT (human telomerase) gene by increasing the amount of histone H3, trimethylated on lysine 27 (H3K27), bound to the

hTERT promoter (Bazarov et al., 2010). hTERT encodes the catalytic subunit of telomerase; therefore, p16 induction results in repression of telomerase and thus telomere shortening. Another binding partner important for cell growth inhibition by p16 is GRIM-19 (Gene associated with Retinoid-IFN-induced Mortality-19). GRIM-19 is a tumor suppressor gene mutations of which have been found in primary human tumors. GRIM-19 and p16 synergistically inhibit cell cycle progression via the E2F pathway (Sun et al., 2010).

## 2. Gene location and mapping of the p16 gene

The region of the human chromosome, 9p21 encompassing the INK4A gene locus, corresponds to regions of dog chromosome 11, mouse chromosome 4, and rat chromosome 5. These regions have been demonstrated to be frequently mutated in various types of cancer (Ruas & Peters, 1998; Sharpless, 2005). The INK4A gene locus also alternatively named the CDKN2B/CDKN2A or INK4A/ARF/INK4B locus, encodes three members of the INK4 family of cyclin dependent kinase inhibitors (CKIs), including p15, p16, and the MDM2 ubiquitin ligase inhibitor p14ARF (Gil & Peters, 2006). p15 has its own reading frame and is physically distinct, but p14ARF and p16 share a common second and third exon but each has a different and unique first exon (Kim & Sharpless, 2006). It has been reported that tandem gene duplication and rearrangement occurred during the evolution of INK4A (p16) and INK4B (p15) that are located 30 kbp apart on the same chromosome (Fig.1) (Sharpless, 2005).

The INK4A gene was initially discovered to have three exons. Subsequent evidence identified an additional exon between the INK4B and INK4A genes, designated as exon1 $\beta$ , that was alternatively spliced from INK4A exon 1 $\alpha$  (Mao et al., 1995; Quelle et al., 1995; Stone et al., 1995a). This alternative exon 1 $\beta$  was transcribed from a promoter different from the p16INK4A first exon (exon 1 $\alpha$ ) and then spliced to the same second and third exons of INK4A to form a transcript, usually shorter than that encoding p16INK4A (Stone et al., 1995b). The 1 $\beta$  transcript encodes a completely different protein from p16 because splicing of exon 1 $\beta$  to exon 2 allows translation from an alternative reading frame resulting in the different protein sequence (Fig. 2) (Quelle et al., 1995; Stone et al., 1995a).

In most mammals this later protein is referred to as p14ARF ('14' indicates molecular weight of the protein and ARF for alternative reading frame). An ortholog of exon1 $\beta$  in mouse and rat is longer than those from other mammals resulting in a larger protein and is designated p19ARF (Quelle et al., 1995). Thus, these two alternative INK4A transcripts (p16 and p14ARF/p19ARF) share a large overlapping nucleotide sequence for the common exons 2 and 3 but result in structurally unrelated proteins due to presence of unique alternative first exons. Both have become important candidates for the study of novel cancer mechanisms.

In dogs, the p16 and p14ARF transcripts derived from INK4A locus have not been fully elucidated. There are no full-length mRNAs or expressed sequence tags (ESTs) available that would completely define these transcripts. In addition this region of the chromosome is extremely GC-rich making it difficult to clone and sequence and causing a gap in the CanFam 2.0 genome assembly (Lindblad-Toh et al., 2005). The biological functions of these two proteins are fairly well understood compared to their genomic structure. Several lines of evidence suggest that both p16 and p14ARF act as potent tumor suppressors apart from their roles as cell cycle regulators during the G1 to S phase transition and p53 mediated cell cycle arrest, respectively.

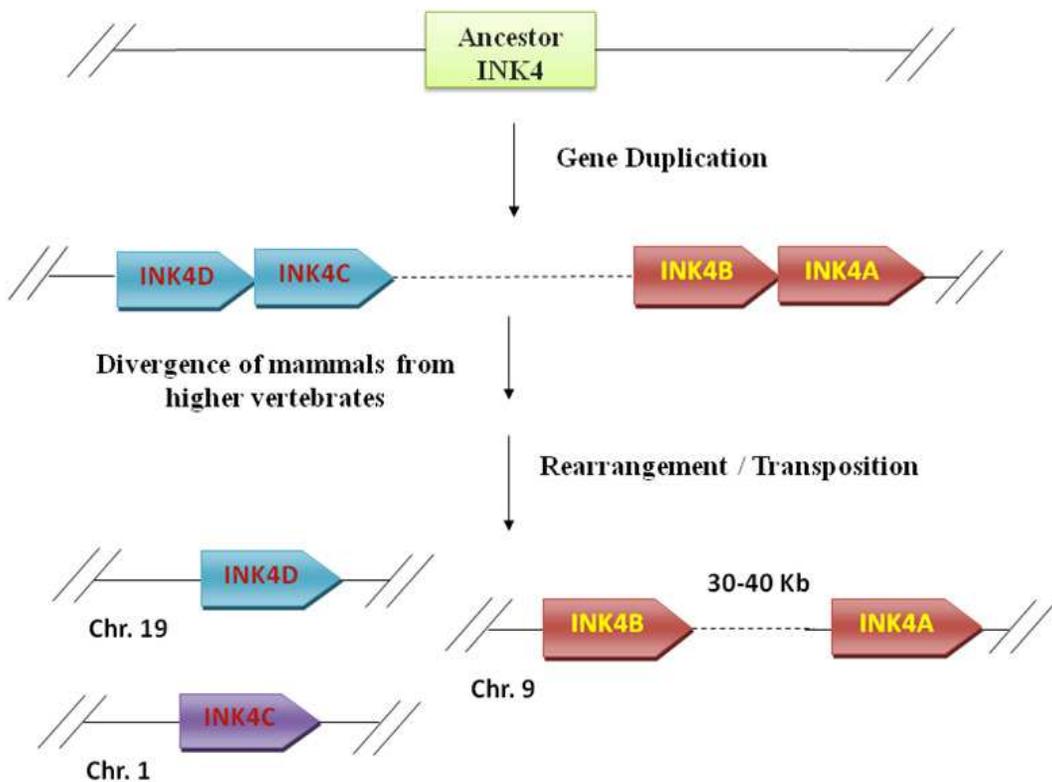


Fig. 1. Evolution of mammalian CKIs. Schematic representation of gene duplication and the evolution of CKIs (p16INK4A, p15INK4B, p18INK4C and p19INK4D) from a single ancestor INK4 gene. The chromosomal localization of INK4 genes are widely conserved across mammals. During the course of evolution, INK4C and INK4D were integrated into different chromosomes while INK4A and INK4B remained located on the same chromosome. Here human chromosomes and corresponding INK4 genes are shown.

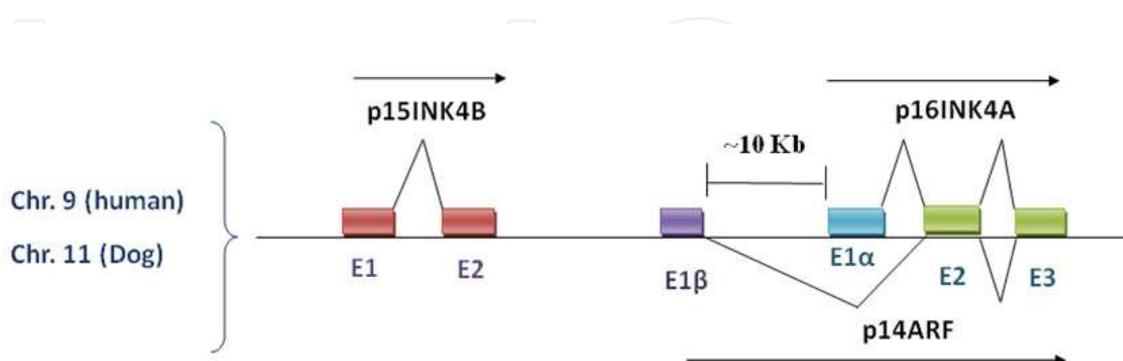


Fig. 2. Alternative splicing of p16INK4A and p14ARF. Exon E1α is spliced to INK4A exons - E2 and E3 forming the p16 mature transcript whereas E1β is alternatively spliced to the same E2 and E3 exons generating the mature p14ARF transcript. The latter produces a different protein from p16 because translation occurs from an alternative reading frame.

Primary melanomas, osteosarcoma and mammary tumor cell lines from dogs have been shown to harbor frequent loss of p16 (Levine & Fleischli, 2000; Koenig et al., 2002; DeInnocentes et al., 2009). Opposing roles of p16 and p14ARF have also been documented, where p16 inactivation attenuates senescence and ageing while p14ARF inactivation induces senescence and aging in skeletal muscle of BubR1 mice (Baker et al., 2008). p16 and p14ARF contribute to reduced growth and survival of B lymphopoiesis and inhibit malignant transformation (Signer et al., 2008). This contrasting behavior could be due to a level of tissue-specific activity of these CKIs.

### **2.1 Cellular location of p16**

The subcellular localization of p16 has been even more cryptic than its genetic behavior and expression. Most studies have supported the localization of this protein both in the nucleus and the cytoplasm. But there are some debates on its specific roles, being in both cellular fractions, and in the context of normal and tumor cell lines. It is generally assumed that p16 is transported to the nucleus and acts as a CKI to regulate the G1 phase cell cycle checkpoint. This phenomenon has been reported in normal cells where the protein was mainly found in the nucleus but not in the cytoplasm (Bartkova et al., 1996). However many tumor cell lines have been shown to harbor p16 in the cytoplasm as well as in the nucleus (Geradts et al., 2000; Nilsson & Landberg, 2006). Two major populations of p16 have been identified in subcellular fractions – one is unphosphorylated or basic in form and the other is phosphorylated or acidic in form and both are generally derived from post-translational modification. The phosphorylated form was found to be associated with CDK4 in normal human fibroblasts (Gump et al., 2003). It has been reported that the localization of the two forms of p16 in both cellular compartments mostly depends on cancer types. In breast cancer cell lines, both forms of p16 were observed in the cytoplasm while the phosphorylated form was predominant in the nucleus (Nilsson & Landberg, 2006). In addition, strong cytoplasmic expression of p16 was observed in many tumor cell lines including primary breast carcinoma associated with a malignant phenotype (Emig et al., 1998; Evangelou et al., 2004) suggesting that the protein might have specific roles for its cytoplasmic localization in certain malignancies. But so far there is no direct evidence for the function of this tumor suppressor in the cytoplasm. One possible mechanism is that p16 can bind to CDK4/6 in the nucleus and the complex is transported to the cytoplasm, inhibiting the association of CDK4/6 with cyclinD in the nucleus and thereby blocking the G1/S phase transition of the cell cycle. In normal cells and epithelial-derived breast carcinoma, a novel substrate for CDK4/6 has been identified which is more prevalent in the cytoplasm than in the nucleus (Kwon et al., 1995). This might cause p16 localization bound to CDK4/6 to the cytoplasm and thus prevent CDK4/6 from acting on the substrate cyclinD1. Another mechanism may be hinted that p16 is mutated in some tumors and resulting in the defective protein being localized in the cytoplasm. However, this speculation is not supported by the fact that p16 is expressed in both the nucleus and the cytoplasm in cell lines with wild-type p16 protein (Craig et al., 1998). Other studies have suggested that the cytoplasmic localization might represent a mechanism for p16 inactivation in various tumors (Evangelou et al., 2004; Nilsson & Landberg, 2006).

### **2.2 Other INK4 family CKIs – p15, p18, p19**

There are two classes of CKIs that interact with cyclin-dependent kinases (CDKs) and reversibly block their enzymatic activities. The first group consists of p21, p27, and p57 and the second group is comprised of p16/INK4A, p15/INK4B, p18/INK4C and p19/INK4D.

The INK4 family CKIs (the second group) generally inhibit the assembly of CDKs by binding to CDK4 or CDK6 (Sherr & Roberts, 1995). Like p15/INK4B and p16/INK4A genes, p18/INK4C and p19/INK4D have been demonstrated to have evolved through tandem gene duplication and rearrangement during the course of evolution. Cross-species observations have suggested that a common vertebrate ancestor containing a single INK4 gene that was duplicated and gave rise to the INK4B-INK4A and INK4C-INK4D gene clusters. After the divergence of mammals and other higher animals from lower vertebrates (~350 million years ago), further gene duplication and rearrangement resulted in the four different INK4 genes (Fig.1) (Gilley & Fried, 2001; Sharpless, 2005). It has been reported that p15 and p16 arose from a common ancestor or single gene locus placed on the same chromosome whereas, p18 and p19 mapped to different chromosomes in humans and other mammals (Guan et al., 1994; Hirai et al., 1995).

Both p18 and p19 proteins share basic structural and biochemical properties with p15 and p16 proteins. All of them consist of repeated ankyrin motifs that play important roles in folding of proteins and in molecular interactions with other proteins such as CDK4/6 (Hirai et al., 1995). The p18 and p19 have not been studied as extensively as p16 and there is also some debate about their roles as independent tumor suppressors (Hirai et al., 1995). Unlike p16 and p15, which are deleted in a number of established tumor cell lines (both human and canine), the expression of p18 and p19 can be readily detected in many cell lines including many different primary tissues (Hirai et al., 1995). For example, both p18 and p19 are uniformly expressed in canine mammary tumor (CMT) cell lines and normal canine fibroblasts (NCF) (Bird et al., unpublished data). Although p15 and p16 differ significantly from each other as one is encoded by two exons and the other by three exons with alternative splicing of the first exon, respectively (Stone et al., 1995a), the two proteins are closely related in their structures and functions. Both have four ankyrin repeats, are involved in similar mechanisms of cell cycle regulation and in some instances may be interchangeable as tumor suppressors (Krimpenfort et al., 2007).

Expression of p18 and p19, have been shown to predominate during early to mid-gestation in mouse development (Zindy et al., 1997) while expression of p15 has been found in later stages of gestation (Zindy et al., 1997). Circumstantially, it appears that different INK4 proteins are not functionally redundant as they appear to be expressed during different periods of development and may also be expressed in distinct tissue-specific profiles. Expression of p15 is down-regulated during human lymphocyte mitogenesis with a marked increase in Rb kinase activity providing a potential role for p15 in cell cycle arrest. p15 mediated growth suppression is induced by TGF $\beta$  mediated by SP1 and SP3 transcription factors (Li et al., 1995). p15 and p27 levels were decreased during lymphocyte activation and appear important in maintaining cell quiescence (Lois et al., 1995). Although p15 acts as a tumor suppressor, the frequency of mutations and defects in p15 in tumor cells is lower than p16 (Stone et al., 1995a). Overexpression of p15 can induce cell cycle arrest in cancer cells (Thullberg et al., 2000), TGF $\beta$ -mediated cell cycle arrest in human keratinocytes (HaCaT) (Hannon & Beach, 1994), and cell cycle arrest by the pyrido-pyrimidine derivative JTP-70902 in the human colon cancer cell line HT-29 (Yamaguchi et al., 2007). p18 inhibits the CDK-cyclin binding site by distorting the ATP binding site and by misaligning catalytic residues. p18 can also distort the cyclin-binding site of CDKs by reducing the size of the interface of bound cyclin (Jeffrey et al., 2000). A lack of mutations in p18 and p19 has been reported in tumor-derived cell lines and primary tumors, which were mutated for p16 and p15 expression, which shows distinct biological function of evolutionary related INK4 proteins (Zariwala & Xiong, 1996).

The INK4 and CIP cyclin dependent kinase inhibitor families have overlapping roles of cell cycle arrest in mouse embryo fibroblasts. Loss of both INK4 (p15, p16, and p18) and CIP (p21) promotes pRB inactivation, cell immortalization, and H-rasV12/c-myc-induced loss of contact inhibition. However, loss of both families of CKIs is still only weakly able to cause cell immortalization largely due to active apoptosis induction (Carbone et al., 2007). This data strongly supports the concept that both CKI inactivation and apoptosis failure are required to promote a neoplastic phenotype.

### 2.3 Structure of p16

p16 encodes four or five ankyrin repeats (Russo et al., 1998). Ankyrin repeats are 30 amino acid structural motifs that resemble the letter 'L' with a stem made of a pair of anti-parallel helices with a beta-hairpin region forming the base (Russo et al., 1998). The functional domain of p16 involved in interaction with CDK4/6, is located in the C-terminal half including the III and IV ankyrin repeats and the C-terminal flanking region accompanied by loops 2 and 3 (Fahham et al., 2010). p16 interacts with the N and C lobes of CDK6 and binds to one side of the catalytic cleft opposite to the cyclin binding site. CDK6 bound to p16 is inactive because it can not bind to cyclin and is not phosphorylated; thus, proliferation is suppressed (Russo et al., 1998). p16/INK4A also exerts transcriptional control over cyclin D1. Activating transcription factor-2 (ATF-2) and cAMP-responsive element-binding protein (CREB) induce the cyclinD1 expression by binding to cAMP-response element/activating transcription factor-2 (CRE/ATF-2) binding site at cyclinD1 promoter side, p16 represses the ATF-2 and CREB expression by 40-50%, thus inactivates cyclinD1 independent of its CDK4 inactivating properties (D'Amico et al., 2004).

### 2.4 p14ARF/p16gamma/p12

As has been noted, the INK4A locus encodes two distinct p16 and p14ARF proteins. However, it has also been reported that besides these two proteins, this gene locus also encodes two additional proteins; p16gamma and p12 (Fig.3).

p14ARF inhibits MDM2, which results in stabilization of the important tumor suppressor p53. p53 is a transcription factor, which activates expression of proteins required for cell-cycle inhibition and apoptosis (Boehme & Blattner, 2009). One of the downstream regulatory protein activations mediated by p53 is p21 up-regulation which checks the cell cycle late in the G1/S phase transition. p53 also acts as a transcription repressor of other genes (Gomez-Lazaro et al., 2004). p53 is more stable in mammary epithelial cells in comparison to fibroblasts in humans, which indicates the importance of p53 in mammary epithelial cell growth (Delmolino et al., 1993). Under normal conditions, p53 is rapidly degraded to keep its protein level low, mediated through the E3 ubiquitin ligase MDM2. Under conditions of stress or other dysfunction, p14ARF binds to MDM2, thus releasing and stabilizing p53 by blocking MDM2. Wild type p53-induced phosphatase 1 (Wip1/Ppm1d) stabilizes MDM2 and downregulates p53, p38MAPK, and p16 expression (Lin et al., 2007; Yu et al., 2007). Disruption of Wip1 activates p53, p16, and p14ARF pathways, through p38MAPK signaling, and suppresses mouse embryo fibroblast transformation by oncogenes *in vivo* (Bulavin et al., 2004). Another mechanism of p14ARF induction and p53 stabilization is stimulation of the DMP1 promoter by HER2/neu growth factor receptor overexpression (Mallakin et al., 2010). HER2/neu activates the DMP1 promoter through the phosphatidylinositol-3'-kinase-Akt-NF- $\kappa$ B pathway, which in turn activates p14ARF transcription (Taneja et al., 2010).

p14ARF expression is not directly involved in the response to DNA damage although p53 negatively regulates p14ARF expression and both of them have an inverse correlation with each other with respect to activity (Stott et al., 1998). Function of p14ARF is not limited to p53 as p14ARF also has other independent roles in cellular systems such as vascular regression in the developing eye (McKeller et al., 2002) and arrest of cell cycle in murine embryo fibroblasts in the absence of p53 (Weber et al., 2000). Loss of p14ARF, results in tumorigenesis by facilitating angiogenesis, which is independent of the p53 pathway (Ulanet & Hanahan, 2010). Other than MDM2, p14ARF also binds to E2F-1, MDMX, HIF1- $\alpha$ , topoisomerase I, c-myc, and nucleophosmine (NPM) (Boehme & Blattner, 2009). p19ARF (the mouse homolog of human p14ARF) is able to induce cell cycle arrest in mammalian fibroblasts analogous to p16 (Quelle et al., 1995).

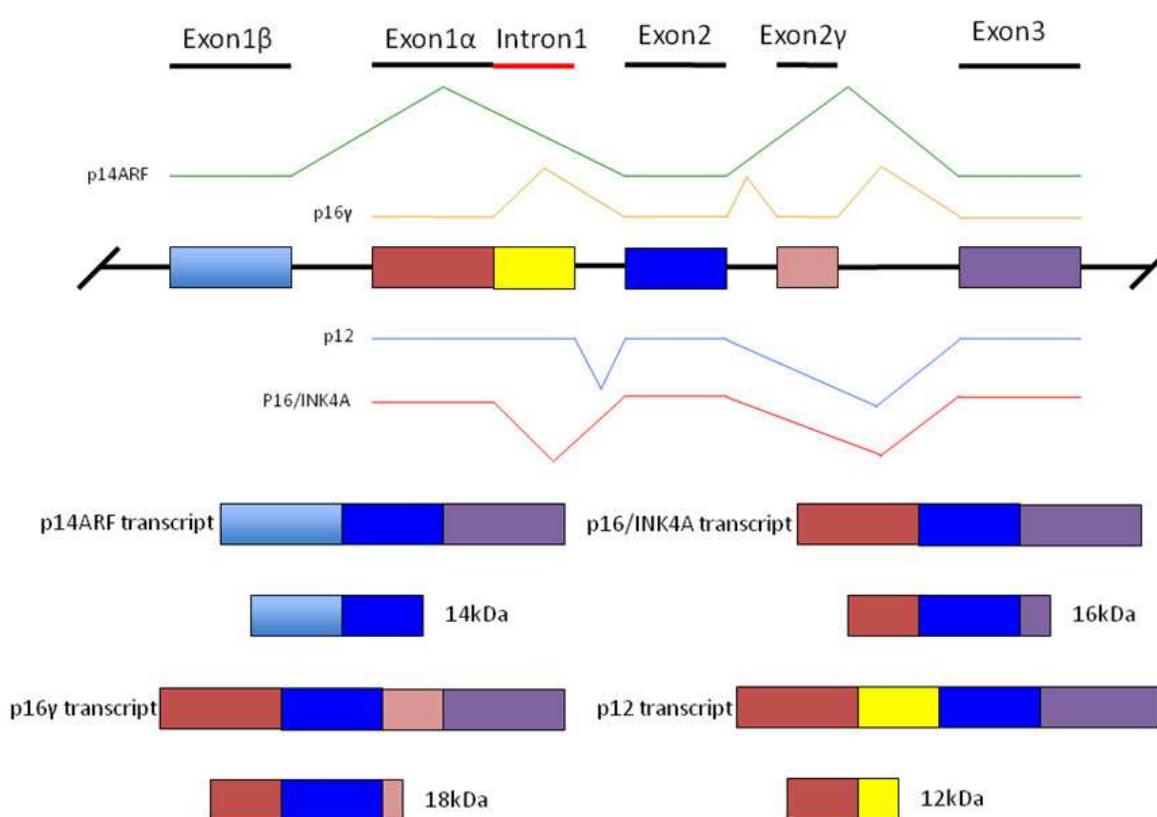


Fig. 3. Transcription from the INK4A/ARF locus. Schematic representation of all the four different transcripts, transcribed from the common INK4A/ARF gene locus. p14ARF and p16/INK4A share exon 2 and exon 3, but differ in their first exons. p14ARF includes exon 1 $\beta$  while p16 includes exon 1 $\alpha$ . The stop codon for p14ARF reading frame is located in exon2. The stop codon for the p16/INK4A is located in exon 3. Another transcript transcribed from this locus is p16 $\gamma$ , which has an extra exon (exon2 $\gamma$ ) along with all three exons of the p16/INK4A transcript. Exon2 $\gamma$  (197bp) is located in intron 2 between exon 2 and exon 3. p16 $\gamma$  encodes a 18kDa protein. The smallest 12kDa protein encoded from INK4A locus is p12. p12 shares the first exon, exon1 $\alpha$  with p16, but first exon of p12 transcribes little longer in intron 1 to give an additional 274bp sequence. Stop codon for p12 is located in the additional intron 1 $\alpha$  sequence and introduces an earlier stop codon and encoding a 12kDa protein spite having a longer transcript than p16/INK4A.

E2F induces cell proliferation by activating S phase regulatory proteins but, according to one report, E2F can induce senescence in human diploid fibroblasts by inducing p14ARF expression, which is required for p53 stabilization (Dimri et al., 2000). Id1 encodes a helix-loop-helix transcription factor that is overexpressed in high grade breast tumors and estrogen receptor-negative diseases (Gupta et al., 2007). Overexpression of Id1 or inactivation of the p14ARF-p53-p21 pathway can also revert senescence induced by ras signaling in mouse mammary carcinoma (Swarbrick et al., 2008). Other than facilitating the DNA-damage-induction response of p53, p14ARF also has a role in nucleotide excision repair. p14ARF induces expression of the damaged-DNA recognition protein xeroderma pigmentosum, complementation group C (XPC), by disrupting the interaction of E2F-4 and DRTF polypeptide 1 (DP1). p14ARF also reduces the interaction of the E2F-4-p130 repressor complex with the XPC promoter (Dominguez-Brauer et al., 2009). TGF- $\beta$  activity controls the expression of p14ARF during mouse embryonic development (Freeman-Anderson et al., 2009).

Other than p16 and p14ARF transcription from the INK4A locus, there is one more alternative transcript that has been reported derived from this gene in human lymphoblastic leukemia which is termed as p16 gamma (p16 $\gamma$ ) (Fig.3) (Lin et al., 2007). p16 $\gamma$  has been demonstrated to be expressed at both transcriptional and translational levels confirming its functional potential (Lin et al., 2007). p16 $\gamma$  shares the same exon 1 $\alpha$ , exon 2, and exon 3 as p16 but with a 197 bp insertion between exon 2 and 3 due to an alternative splicing event that extends exon 2 and concedes a stop codon. p16 $\gamma$  is also an ankyrin-repeat protein and interacts with CDK4. p16 $\gamma$  suppresses E2F activity and induces cell cycle arrest like p16/INK4A. It is not known what functionally distinguishes these 2 transcripts or their encoded proteins.

There is an alternative splice variant of p16 present in human pancreas as well, known as p12 (Fig.3) (Robertson & Jones, 1999). p12 is a 12kd size protein, which is encoded from the same INK4/ARF locus. The p12 gene shares the p16 promoter, 5'UTR, ATG-start codon and exon 1 $\alpha$ , and uses the alternative splice donor site to splice to exon 2. The extra sequence encodes a premature stop codon that results in a smaller protein. p12 shares the first ankyrin repeat with p16 but is not predicted to bind to CDK4 or CDK6 based on crystal structure studies. p12 is reported to suppress cell growth but in a pRb-independent mechanism (Sharpless, 2005). When the effect of ectopic expression of all the three transcripts, p16, p14ARF, and p12 was compared, p16 had the most inhibitory effects on cell growth of the human lung cancer cell line A549 (Zhang et al., 2010b).

### 3. The role of p16

#### 3.1 p16 as a tumor suppressor gene

CKI p16 is an important tumor suppressor gene, defects in which are associated with cancer (Koh et al., 1995). p16 is functional as a growth suppressor gene as introduction of full length p16 cDNA caused marked growth suppression in p16-null human glioma cells (Arap et al., 1995), lung cancer *in vitro* and *in vivo* (Jin et al., 1995), carcinoma cell lines *in vitro* and *in vivo* (Spillare et al., 1996), esophageal cancer cells (Schrumpp et al., 1996), and human and canine breast cancer cells (Campbell et al., 2000; DeInnocentes et al., 2009). p16 defects are second in frequency only to those in p53 for human malignancies (Baylin et al., 1998). p53 and p16 are thought to work via two independent pathways of growth suppression, but both of them are important in suppressing malignant transformation (Gruis et al., 1995). p16

gene deletions are associated with, and appear permissive for, late-stage, high-grade cancers such as; melanoma, bladder cancer, schistosomal bladder cancer, esophageal cancer, breast cancer, and glioblastoma (Gruis et al., 1995; Izumoto et al., 1995; Zhou et al., 1995; Swellam et al., 2004; DeInnocentes et al., 2009). Deletion of the 9p21 region encoding the p16-INK4A/p14ARF/p15 tumor suppressor loci in humans results in tumor formation in a wide range of tissues (Kamb et al., 1994; Kleihues et al., 1994; Packenham et al., 1995). Loss of heterozygosity (Swellam et al., 2004), loss of homozygosity (Ranade et al., 1995; Quelle et al., 1997), and hypermethylation of the promoter (Herman et al., 1997) in the 9p21 region are all important mechanisms which have been shown to result in loss of p16 expression and promote p16-related neoplasms. Hyper-methylation of the p16 promoter region appears to occur early in neoplastic transformation before development of tumorigenicity in rat respiratory epithelium (Yamada et al., 2010). Loss of p16 is associated with extended life span but is not sufficient for immortality (Loughran et al., 1996; Noble et al., 1996). Frequency of loss of p16 is high in pre-malignant lesions suggesting the importance of loss of p16 activity as an early event in cancer progression (Liggett & Sidransky, 1998) and evaluation of p16 expression could have value as an early prognostic indicator for predicting cancer recurrence (Bartoletti et al., 2007). Hypermethylation of CpG islands in the p16 promoter results in enhanced cell proliferation in human colorectal cancer and can activate DNA demethylation in the invasive region suppressing proliferation but enhancing tumor invasion (Jie et al., 2007). Additionally INK4A/ARF hypermethylation occurs frequently in mammary epithelial cells in high risk women with sporadic breast cancer (Bean et al., 2007; Jing et al., 2007; Sharma et al., 2007).

K-cyclin (ORF72) is a human homolog of cyclinD1 in Kaposi's sarcoma-associated herpesvirus (KSHV/HHV-8) which is oncogenic in immune suppressed individuals. p16 inhibits the unphosphorylated CDK6-K-cyclin complex and functional availability of K-cyclin for tumorigenesis is largely dependent upon the balance of expression of p16 and CDK6 (Yoshioka et al., 2010). This complex is resistant to CKI p21 and p27 and can phosphorylate both of them explaining the important role of p16 as a tumor suppressor gene in malignancies induced by KSHV and their resistance to multiple CKI activities.

Mutations in the p16 encoding gene have also been reported in other cancer types such as glioblastomas, pancreatic adenocarcinomas, and melanoma-prone pedigrees. Allelic variants of p16 in melanoma-prone pedigrees have been found, which are deficient in interaction with CDK4 and CDK6. p16 allelic variants with decreased CDK interaction capability predisposes these individuals to increased risk of cancer which reinforces the important role of p16 as a tumor suppressor gene (Reymond & Brent, 1995). Mutations in CDK4 prevent p16 binding to CDK4 and have been identified for several noncontiguous amino acid sequences. This suggests there may be multiple binding sites for p16. Such mutated CDK4s have oncogenic potential and occur spontaneously in melanomas and other neoplasms (Ceha et al., 1998).

Matrix metalloproteinases (MMPs) are the zinc-dependent endopeptidases, which are capable of degrading components of the extracellular matrix. The MMP family is composed of at least 20 enzymes. One of the MMP family enzymes is MMP-2, which has been reported to be strongly linked with various types of human cancers such as glioma (Uhm et al., 1996) and astroglomas (Qin et al., 1998). p16 represses expression of MMP-2 and invasiveness of gliomas (Chintala et al., 1997) by blocking Sp1 to mediate gene transcription of MMP-2 (Wang et al., 2006). Thus, p16 can inhibit the cyclin-CDK complex to suppress the cell cycle can also suppress tumor invasion through other cell regulatory functions.

Rab proteins are members of Ras-related small GTPase family. Rab27A is also linked with human genetic diseases (Seabra et al., 2002). Rab27A is associated with invasive and metastatic breast cancer, which is facilitated by down-regulation of p16 and up-regulation of cyclinD1 (Wang et al., 2008). Concomitant overexpression of p16 and p73 has oncogenic potential and affects the development and growth of breast carcinomas (Garcia et al., 2004).

Alteration of p15 and p16 expression and overexpression of TGF- $\alpha$  are found frequently in schistomal bladder cancers and squamous cell carcinomas (Swellam et al., 2004). Loss of p16 expression has prognostic value in predicting recurrence-free probability in patients affected by low-grade urothelial bladder cancer (Bartoletti et al., 2007). p16 has been reported to be inactive in human colorectal cancer but p16 expression is elevated by the demethylation of the p16 promoter in invasive cancer cells, as these cells cease proliferation at the invasive front (Jie et al., 2007).

$\beta$ -catenin is the key downstream effector of Wnt signaling and is also a potent oncogene.  $\beta$ -catenin can also inhibit cell proliferation by activating the p14ARF-p53-p21 pathway during trans-differentiation of squamous cell differentiation associated with endometrial carcinoma (Saegusa et al., 2005). p16 is also induced along with loss of pRb expression in trans-differentiation of endometrial carcinoma cells, mediated by  $\beta$ -catenin and p21 (Saegusa et al., 2006). Deletion of p16 has been reported in high-grade B-cell non-Hodgkin's lymphoma (Fosmire et al., 2007) along with increases in Rb phosphorylation at CDK4 phosphorylated sites. Inactivation of p16 has also been reported in high grade non-Hodgkin's lymphoma and is less prevalent in low-grade tumors (Modiano et al., 2007). p16 inactivation is more frequent in blastoid mantle cell lymphoma (Dreyling et al., 1997).

p16 forms a complex with HIF-1 $\alpha$ , the transcription factor for the VEGF gene promoter, thus represses the transactivation of VEGF. p16 inhibits VEGF gene expression and inhibits cancer cell induced angiogenesis in breast cancer cells and the loss of p16 is a significant transition in neoplastic development (Zhang et al., 2010a).

### 3.2 Role of p16 in cell quiescence

p16 checks the cell cycle at the G1/S phase transition and thus has an important role in cell cycle exit and quiescence in a variety of cell systems. Growth suppression by p16 depends upon the presence of functional Rb. Growth in Rb null fibroblasts failed to be suppressed by p16 (Medema et al., 1995). Ectopic p16 expression prevents re-entry into the cell cycle (Lea et al., 2003) and p16 expression can induce a G0-like state in hematopoietic cells (Furukawa et al., 2000). p16 expression is up-regulated by exposure to cellular stressors such as oxidative stress, aging, UV exposure, ionizing radiation, chemotherapeutic agents, telomere dysfunction, and wound healing (Kim & Sharpless, 2006; Natarajan et al., 2006). In response to non-lethal UVC irradiation, only p16 positive cell lines can induce cell cycle delay in comparison to p16 null cell lines (Wang et al., 1996). p16 is induced by MAPK activation, in response to stimulation of the ERK/MAPK pathway through RAS/RAF signaling (Ohtani et al., 2001). RAS activation induces p16 expression through ERK mediated activation of Ets1/2 (Ohtani et al., 2001) and p14ARF through Jun-mediated activation of DMP1 (Sreeramaneni et al., 2005). Histone acetyltransferases (HATs), such as p300/CBP are important transcriptional up-regulators. The GC-rich region in the p16 promoter is the putative binding site for transcription factor Sp1 (Gizard et al., 2005). p300 in-cooperation with Sp1 transcriptionally upregulates p16 expression and induces cell cycle arrest in HeLa

cells (Kivinen et al., 1999; Wang et al., 2008). Smooth muscle cells in mature arteries in rat have low rates of proliferation. Suppression of proliferation is dependent on the up-regulated levels of p16 and p27, which makes these cells unable to activate cyclinD1 and cyclinE and their associated kinase activities (Izzard et al., 2002).

p16 has also been associated with a variety of additional cell proliferation control proteins that either bind and suppress its function or compete for p16 targets. SEI-1/p34/TRIP-Br1 protein induces CDK4-mediated Rb phosphorylation through physical binding, independent of p16 (Li et al., 2005). SEI-1 facilitates CDK4 function making it resistant to p16 inhibition. ISOC2 protein binds and co-localizes with p16 inhibiting the function of p16 (Huang et al., 2007). Other than ISOC2, p16 protein has also been found to bind to proliferating cell nuclear antigen (PCNA) and minichromosome maintenance protein 6 (MCM6) (Souza-Rodrigues et al., 2007). The same authors have reported that p16 interacts with DNA polymerase $\delta$  accessory protein PCNA, thus inhibiting the function of DNA polymerase.

p16 is normally localized in the nucleus where it functions as an inhibitor of CDK/cyclin complexes but it has also been reported that p16 can be co-localized in the cytoplasm (Nilsson & Landberg, 2006). Both cytoplasmic and nuclear p16 bind CDK6 and have a role in cell cycle arrest. Human melanocytes initiate differentiation by activation of the cAMP synthesis pathway. This results in increased association of p16 and p27 with CDK4 and CDK2, respectively, Rb phosphorylation failure and decreased expression of E2F proteins with decreased DNA-binding activity (Haddad et al., 1999). Senescence induced by the cAMP pathway in these cells can be attributed to the complex formation of CKI/CDK complexes, which cause cell cycle exit (Haddad et al., 1999).

It is known that p16 induces cell cycle arrest via Rb, but there is one more mechanism by which p16 can arrest cell cycle independent of Rb. I $\kappa$ B $\alpha$  is a specific inhibitor of NF $\kappa$ B, which competes with p16 for binding to CDK4 and inhibits its activity (Li et al., 2003). This observation has led to speculation that I $\kappa$ B $\alpha$  could substitute for p16 in CDK4 inhibition in malignant cells. Other than that, in G1 phase, activity of CDKs is required for proper recruitment of mini-chromosome maintenance (MCM) protein to the origin recognition complex. p16 influences CDKs and thus influences prereplicative complex (preRC) at the MCM level resulting in arrest of cell cycle (Braden et al., 2006).

c-myc is a transcription factor that plays an important role in cell proliferation. c-myc can induce cell cycle progression from G1 phase to S phase in quiescent cells (Eilers et al., 1991). Oncogenic activity of altered CDK4 is due to its inability to bind p16 and inhibiting its enzymic activity. The oncogenic activity of c-myc and the CDK4/cyclin D1 complex require each other to effectively transform cells. CDK4 requires Myc protein for proper function and, similarly, Myc requires the CDK4 cyclinD complex kinase activity to effect tumor transformation. p16 inhibits the transcription regulatory activity of c-myc by blocking cyclin D1/CDK4 complex formation (Haas et al., 1997).

Introduction of adenovirus expressing p16 in human cancer cell lines result in p16-mediated cytotoxicity and results in apoptosis (Kim et al., 2000). p16 expression and estrogen receptor (ER) gene expression are inversely related (Hui et al., 2000). p16 expressing adenovirus vector resulted in delay in tumor growth in a polyomavirus middle-T antigen model of murine breast carcinoma, in comparison with, p19, p27, p18, and p21 which were ineffective (Schreiber et al., 1999).

In dogs, the p16 and p14ARF transcripts derived from INK4A locus have not been fully elucidated. There are no full-length mRNAs or expressed sequence tags (ESTs) available that would completely define these transcripts. In addition this region of the chromosome is extremely GC-rich making it difficult to clone and sequence and causing a gap in the CanFam 2.0 genome assembly (Lindblad-Toh et al., 2005). The biological functions of these two proteins are fairly well understood compared to their genomic structure. Several lines of evidence suggest that both p16 and p14ARF act as potent tumor suppressors apart from their roles as cell cycle regulators during the G1 to S phase transition and p53 mediated cell cycle arrest, respectively.

### 3.3 Role of p16 in cell differentiation

Other than senescence and quiescence, p16 also has a role in cell differentiation like other CKIs. Expression of p16 increases by several fold in terminally differentiated human adult brain tissue and p16 is thought to play role in human brain development (Lois et al., 1995). During differentiation of human embryonic teratocarcinoma cells (NT2) into post-mitotic neurons, expression of p16 and p15 protein levels become elevated (Lois et al., 1995). The role of p16 in melanocyte differentiation has also been investigated. Microphthalmia transcription factor (MITF) is able to induce cell cycle arrest prior to cell differentiation by activation of p16 protein (Loercher et al., 2005).

A-type lamins are intermediate filaments which affect gene expression in differentiation and are thought to function through an Rb-dependent mechanism. Rb associates with a number of tissue specific transcription factors in an E2F-independent manner and induces differentiation in those tissues. Rb associates with MyoD and Mef2 in skeletal muscle cells (Sellers et al., 1998; Novitch et al., 1999), CBFA1 and Runx2 in osteocytes (Thomas et al., 2001; Thomas et al., 2004), and C/EBP in adipocytes and during macrophage differentiation (Chen et al., 1996). pRb is essential for muscle and fat cell differentiation (Korenjak & Brehm, 2005) and cellular senescence (Ohtani et al., 2001). Cells lacking A-type lamins do not arrest in the presence of p16 because destabilization of pRb (Nitta et al., 2006). This report suggests a dependence of p16-induced cell cycle arrest on Rb and posits a role for A-type lamins in Rb-dependent cell cycle arrest.

Cyclin, CDKs, and CKIs have been associated with proliferation and differentiation in a variety of cell and tissue systems. CyclinD1, the principle cofactor of p16, targets CDK4/6 and may participate in myoblast differentiation (Rao & Kohtz, 1995). Thus, p16 appears to play a key regulatory role in cell differentiation and senescence through management of cell cycle exit. CDK4/6 also regulates cell division at different stages of erythroid maturation (Malumbres et al., 2004). CDK4 knock-out mice lack postnatal homeostasis of pituitary somato/lactotrophs and pancreatic B cells (Jirawatnotai et al., 2004). CDK6 knock-out mice have mild defects in hematopoietic cell differentiation. Double deficiency of CDK4/6 in embryos appears to have no effect on organogenesis and associated cell proliferation although they are lethal due to defects in the erythroid lineage (Malumbres et al., 2004). Thus, CDK4/6 are required for many specific tissue differentiation events along with cell cycle progression. For example CDK4 activity is required for pancreatic  $\beta$ -cell proliferation and increased expression of p16, limits the regenerative capacity of  $\beta$ -cells with aging (Krishnamurthy et al., 2006).

Cell proliferation inhibits cell differentiation while, conversely, factors inducing cell cycle exit often lead to differentiation. Cell cycle regulatory proteins are multifunctional and can

also affect cell differentiation independent of their role in cell cycle. p21 deficient cells are defective in differentiation although differentiation can resume by transducing cells with p16 to complement the mutation (Gius et al., 1999). Cyclin-CDK complexes can inhibit differentiation in a kinase-dependent manner. The cyclin D1-CDK4 complex can phosphorylate and inhibit DMP1 or Mef2c transcription factors that are essential for differentiation of skeletal muscle and pre-hypertrophic chondrocytes (Hirai & Sherr, 1996; Lazaro et al., 2002; Arnold et al., 2007). CDK2 and CDK4 can inhibit TGF- $\beta$  induced growth arrest by phosphorylating Smad3 (Matsuura et al., 2004). p16, as a direct inhibitor of cyclin D1 and CDK4 complexes may thus have an indirect role in cell differentiation.

### 3.4 Role of p16 in cell senescence

Cell senescence is a permanent cell resting phase and is related to cell aging (Smith & Pereira-Smith, 1996). Senescence can be either induced by DNA replication stress or by oncogene expression but is most often the result of replicative senescence. Accumulation of p16 is associated with replicative senescence. Increased p16 expression has been found in lymphocytes only a few cell doublings before replicative senescence (Chebel et al., 2007). Oncogene induced senescence is linked with elevated p16 and p14ARF expression (Serrano, 1997; Markowski et al., 2010). Deletion of the INK4/ARF gene locus, in K-ras constitutively expressing mice, results in loss of senescence and invasive, metastasizing tumors (Bennecke et al., 2010).

p16 expression has been shown to promote premature cell senescence (Zindy et al., 1997). Level of p16 expression increases as mouse embryonic fibroblasts reaches senescence (Zindy et al., 1997). Immortal fibroblast (NIH3T3) and tumor cell lines frequently lack p16 expression suggesting the removal of p16 as a potential pathway to bypass senescence and also points towards the importance of p16 as a tumor suppressor gene (Kamb et al., 1994; Nobori et al., 1994; Zindy et al., 1997). T box proteins (Tbx2) and polycomb proteins (BMI1, Cbx7, Mel18) have been reported to be repressors of all three genes of the INK4 locus (p16, p14ARF, and p15) (Jacobs et al., 1999; Gil et al., 2004). Repression of the INK4B/INK4A/ARF locus is controlled by methylation of histone 3 at lysine 27, by binding of chromobox 7 (CBX7) within the polycomb repressive complex 1 to ANRIL (antisense non-coding RNA of INK4B/INK4A/ARF locus) (Yap et al., 2010). Bmi-1 encodes the polycomb protein, which represses both p16 and p14ARF and is linked with regulation of the replicative life span of human fibroblasts (Itahana et al., 2003). BMI1 protein represses p16 expression by binding directly to the Bmi-1 response element (BRE), within the p16 promoter (Meng et al., 2010), and is dependent on the continued presence of EZH2-containing Polycomb-Repressive Complex 2 (PRC2) complex (Bracken et al., 2007). Enhancer of zeste homolog 2 (EZH2) is a histone methyltransferase and a component of the polycomb group protein complex which represses INK4a/ARF gene expression in pancreatic islet beta cells (Chen et al., 2009).

Under a stress and senescence stimulus EZH2 levels decrease coinciding with up-regulated p16. Coincidentally PRC2 and PRC1 complexes, localized at the regulatory domain of p16, are lost when cells enter senescence which in turn results in decreased levels of histone H3K27 trimethylation (H3K27me3) and increased levels of the histone demethylase Jmjd3 with the recruitment of the MLL1 protein (Agherbi et al., 2009). Polycomb proteins are recruited to the INK4/ARF locus through CDC6 and, upon senescence and with an increase in Jmjd3 levels, MLL1 protein is recruited to the locus provoking dissociation of polycomb protein from the INK4/ARF locus. This leads to transcription and replication of the INK4/ARF locus

in early S phase prior to reaching senescence (Agherbi et al., 2009). CDC6 is an essential DNA replication regulator. CDC6 overexpression induces increased INK4/ARF tumor suppressor gene expression through epigenetic modification of chromatin at the INK4/ARF locus (Borlado & Mendez, 2008). COOH-terminal-binding protein (CtBP), a physiologically regulated co-repressor, has also been reported to have a significant role in p16 repression. Several of the pathways noted above repress p16 via CtBP-mediated repression as CtBP forms bridges between proteins having PxDLS amino acid motifs including several transcription factors and other proteins involved in transcription (Mroz et al., 2008).

The levels of p27 and p16 proteins are significantly increased in contact-inhibited human fibroblasts while in contrast, levels were low in serum-deprived human fibroblasts but in both cases, even through the mechanisms of growth arrest are different, they both affect the same pathway involving CDK4, cyclin D1, and Rb (Dietrich et al., 1997). Maintenance of p16 and p27 levels have also been shown to contribute to the low levels of proliferation in normal blood vessels (Izzard et al., 2002) and p16 mRNA and protein accumulate in human fibroblasts as they become senescent (Hara et al., 1996).

#### **4. Potential of p16 as a therapeutic or gene therapy target**

p16 is an important tumor suppressor gene, deletion of which causes various types of tumors making it an important potential target for cancer gene therapy. It has been reported that p16 positive oropharyngeal squamous cell carcinoma (OPSCC) patients respond more favorably to intensity-modulated radiotherapy treatment in comparison to similar p16 negative tumors (Shoushtari et al., 2010). Infectious delivery of the whole p15/p16/p14ARF locus, in infectious bacterial artificial chromosomes, results in growth suppression in human glioma cells (Inoue et al., 2004). Induction of p16 using the DNA methyltransferase inhibitor zebularine combined with the histone deacetylation (HDAC) inhibitors depsipeptide led to inhibition of cell growth in lung tumor cell lines (Chen et al., 2010). Ectopic p16 introduction in cancer cells alone or with other tumor suppressor genes inhibits cell growth and induces apoptosis and senescence, while p16 gene silencing reduced the p53-mediated response to chemotherapeutic agents in cancers (Derenzini et al., 2009). Histone methyltransferase EZH2 inhibitor 3-deazaneplanocin A and the histone deacetylase inhibitor panobinostat together induce p16, p21, p27, and FBX032 and down-regulates cyclin E and HOXA9 levels, which induces apoptosis in cultured and primary human acute myeloid leukemia (AML) cell line cells (Fiskus et al., 2009). p16 along with the murine granulocyte-macrophage colony-stimulating factor gene (AdGM-CSF) can induce effective anti-tumor immunity (Wang et al., 2002). Exogenous expression of p16 and p53 induce apoptosis in lung carcinoma cells (Bai et al., 2000), leukemia cell line K562 (Rui & Su, 2002), non-small lung cancer (Wu et al., 2000), and pancreatic cancer (Ghaneh et al., 2001). p16 along with p27 inhibits angioplasty-induced neointimal hyperplasia and coronary artery occlusion (Tsui et al., 2001), inhibits proliferation in neointimal hyperplasia (McArthur et al., 2001), and a wide range of other tumor types (Patel et al., 2000). Ectopic expression of p16 by replication-competent adenovirus leads to potent anti-tumor effects in gastric cancer xenografts in nude mice (Ma et al., 2009) while p16 transfection along with cisplatin treatment increased senescence and growth inhibition in non-small cell lung cancer xenografts in mice (Fang et al., 2007). Ectopic p16 expression was able to induce growth arrest in pancreatic carcinoma JF305 cell lines (Ma et al., 2007), human laryngeal squamous cell carcinoma (Liu et al., 2003; Fu et al., 2004), and inhibit experimental lung metastasis in Balb/c nude mice (Kim et al., 2003), inhibit cell

growth in nasopharyngeal carcinoma (Lee et al., 2003), and a murine model of head and neck cancer (Rhee et al., 2003), human mesothelioma (Yang et al., 2003), and suppress tumor growth by glioblastoma cells *in vivo* and *in vitro* (Adachi et al., 2002). p16 transfection suppressed growth of Bcap-37 breast cancer cells (Bai et al., 2001), the human melanoma cell line WM-983A (Cheng et al., 1999), human lung adenocarcinomas (Fu et al., 1999), pancreatic cancer (Calbo et al., 2001), and inhibits cardiac hypertrophy *in vitro* and *in vivo* (Nozato et al., 2001). p16 also inhibited cell growth in a human ovarian cancer cell line (Wang et al., 1999), a human gastric cell line (Sun & Lu, 1997), and a small cell lung carcinoma (Sumitomo et al., 1999). All of these diverse examples demonstrate the potent and broadly efficient effects of exogenous p16 expression on cell proliferation in p16 negative cancer cells *in vitro* and *in vivo* and reflects the importance of p16 as regulatory factor and potential target for gene therapy and cancer therapeutics.

## 5. Conclusions

p16/INK4A/CDKN2A is an important tumor suppressor gene, which is required for the control of unregulated cell growth in many and perhaps most cell types. The INK4A locus is also unique in eukaryotes, where 3 and perhaps 4 transcripts are derived which have similar functions of suppressing cell growth but that work via very different pathways. Most surprising is the utilization of alternative open reading frames from a single gene complex mandating co-evolution of the unrelated protein sequences. Despite these constraints, mutation of p16 is second only to p53 in mutation frequency in a wide range of tumors. This strongly suggests that p16 may have real potential as an important new target for cancer gene therapy. p16 is not just an important cell cycle regulatory protein that helps suppress the cell growth and tumor formation, p16 also has a role in other cell cycle phases. p16 has been reported to play an important role in cell differentiation, cell quiescence, and cell senescence, which makes it not just a tumor suppressor protein but a cell regulatory protein that plays a critical role in regulating terminal differentiation and the aging process. There is a great need to investigate all the subtleties surrounding p16 function and to unravel all the pathways and binding partners of p16. p16 is a promising gene located within a complex gene locus with many roles in cell metabolism.

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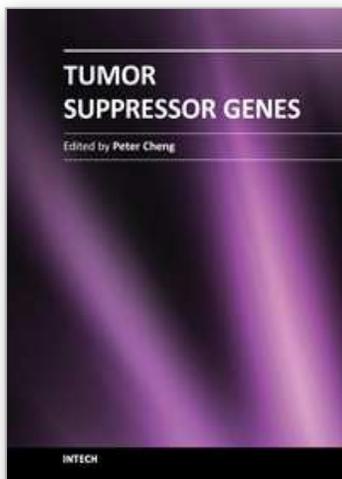
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## **Tumor Suppressor Genes**

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Functional evidence obtained from somatic cell fusion studies indicated that a group of genes from normal cells might replace or correct a defective function of cancer cells. Tumorigenesis that could be initiated by two mutations was established by the analysis of hereditary retinoblastoma, which led to the eventual cloning of RB1 gene. The two-hit hypothesis helped isolate many tumor suppressor genes (TSG) since then. More recently, the roles of haploinsufficiency, epigenetic control, and gene dosage effects in some TSGs, such as P53, P16 and PTEN, have been studied extensively. It is now widely recognized that deregulation of growth control is one of the major hallmarks of cancer biological capabilities, and TSGs play critical roles in many cellular activities through signaling transduction networks. This book is an excellent review of current understanding of TSGs, and indicates that the accumulated TSG knowledge has opened a new frontier for cancer therapies.

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