SECTION I

EPIGENETICS AND CELL CYCLE

1

EPIGENETIC MODULATION OF CELL CYCLE: AN OVERVIEW

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1.1 INTRODUCTION

The progression of the cell cycle is a very finely tuned process that responds to the specific needs of any specific tissue or cell, and is strictly controlled by intrinsic and extrinsic surveillance mechanisms (Giacinti and Giordano, 2006; Montanari et al., 2006; Satyanarayana and Kaldis, 2009). The intrinsic mechanisms appear at every cycle whereas the extrinsic mechanisms only act when defects are detected (Macaluso and Giordano, 2004; Johnson, 2009). The loss of these control mechanisms by genetic and epigenetic alterations leads to genomic instability, accumulation of DNA damage, uncontrolled cell proliferation, and eventually,

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tumor development. While genetic abnormalities are associated with changes in DNA sequence, epigenetic events alter the heritable state of gene expression and chromatin organization without change in DNA sequence. The most studied epigenetic modifications of DNA in mammals are methylation of cytosine in CpG dinucleotides (DNA methylation), imprinting, posttranslational modification of histones (principally changes in phosphorylation, acetylation, and ubiquitination status), and small RNA-mediated control, specifically miRNAs (Garzon et al., 2009; Kampranis and Tsichlis, 2009; Mendez, 2009; Simon and Kingston, 2009). Important biological processes are regulated by epigenetic mechanisms, including gene reprogramming during early embryogenesis and gametogenesis, cellular differentiation, and maintenance of a committed lineage. Epigenetic marks are established early during development and differentiation; however, modifications occur all through the life in response to a variety of intrinsic and environmental stimuli, which may lead to disease and cancer (Delcuve et al., 2009; Maccani and Marsit, 2009). Although the importance of genetic alterations in cancer has been long recognized, the appreciation of epigenetic changes is more recent. Numerous studies have provided evidence that aberrant epigenetic mechanisms affect the transcription of genes involved in cell proliferation, differentiation, survival, apoptosis, and genome integrity, and play an important role in cancer formation and progression (Humeniuk et al., 2009; Lopez et al., 2009; Toyota et al., 2009).

1.2 EPIGENETIC AND GENETIC ALTERATIONS OF pRb AND p53 PATHWAYS

The progression of the cell cycle is tightly monitored by surveillance mechanisms, or cell cycle checkpoints, which ensure that the initiation of a later event is coupled with the completion of an early cell cycle event. The pRb (pRb/$p16^{INK4}$/Cyclin D1) and p53 ($p14^{ARF}$/mdm2/p53) pathways are the two main cell cycle control pathways (Fig. 1.1). The importance of these pathways in controlling cellular growth and apoptosis is underscored by many studies, indicating that mutations of the components of these pathways in all human cancers. Almost all human cancers show deregulation of either the pRb or p53 pathway, and often both pathways simultaneously (Macaluso et al., 2006; Yamasaki, 2006; Polager and Ginsberg, 2009).

A combinatorial signaling network between pRb and p53 pathways controls cell cycle progression through an array of autoregulatory feedback loops where pRb and p53 signals exhibit very intricate interactions with other proteins involved in the determination of cell fate (Hallstrom and Nevins, 2009; Polager and Ginsberg, 2009).

Alterations in the pRb and/or p53 pathway converge to reach a common goal: uncontrolled cell cycle progression, cell growth, and proliferation. Then, loss of cell cycle control may lead to hyperplasia and eventually to tumor formation and progression (Sun et al., 2007; Lapenna and Giordano, 2009; Polager and Ginsberg, 2009).

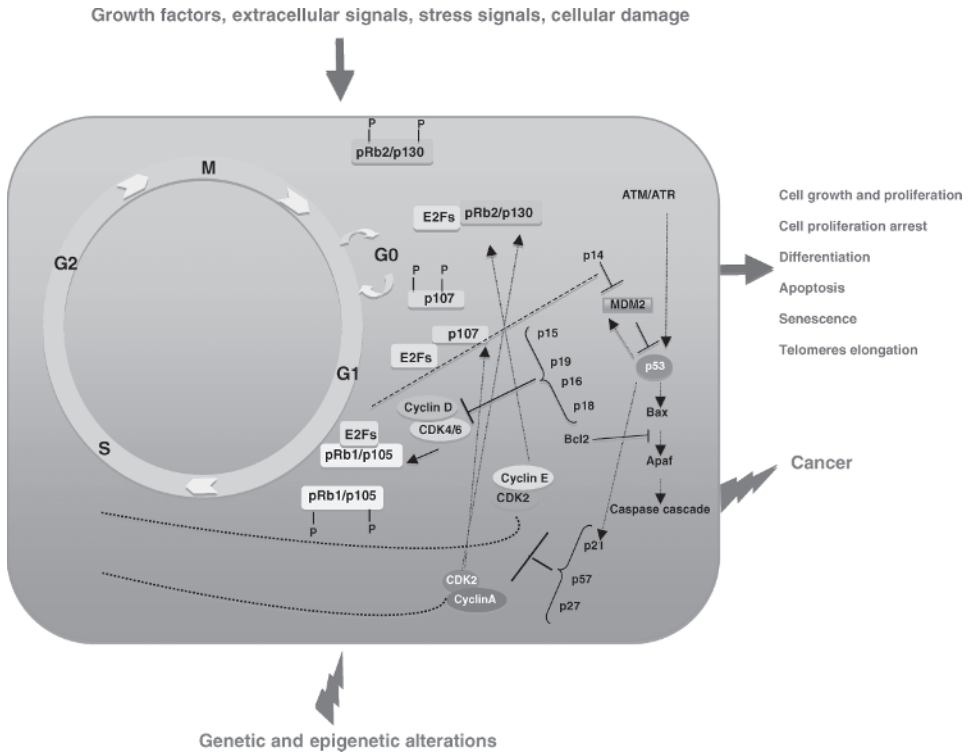

FIGURE 1.1 A combinatorial signaling network between pRb (pRb/p16^{INK4}/cyclin D1) and p53 (p14^{ARF}/mdm2/p53) pathways control cell cycle progression through an array of autoregulatory feedback loops where pRb and p53 signals exhibit very intricate interactions with other proteins involved in the determination of cell fate. Loss of cell cycle control by genetic and epigenetic alterations leads to genomic instability, accumulation of DNA damage, uncontrolled cell proliferation, and eventually tumor development. (*See insert for color representation of the figure.*)

1.2.1 pRb (pRb/p16^{INK4}/Cyclin D1) Pathway

Several studies have documented the role of the pRb pathway, and its family members pRb2/p130 and p107, in regulating the progression through the G1 phase of the mammalian cell cycle (Giacinti and Giordano, 2006; Johnson, 2009; Poznic, 2009). In addition to pRb family proteins, key components of this pathway include the G1 cyclins, the cyclin-dependent kinases (CDKs), and the CDK inhibitors (Lapenna and Giordano, 2009; Poznic, 2009).

Alterations in the signaling network in which pRb, p107, and pRb2/p130 act have been reported in most human cancers. Genetic changes, such as mutations, insertions, and deletions, and also epigenetic alterations, such as promoter hypermethylation, are the most common molecular alterations affecting the function of pRb family proteins. Moreover, it has been reported that inherited allelic loss of pRb confers increased susceptibility to cancer formation (Mastrangelo et al., 2008; Sabado Alvarez, 2008; Poznic, 2009).

Numerous observations have indicated that pRb family proteins interact with a variety of transcription factors and chromatin-modifying enzymes (Brehm et al., 1998; Harbour et al., 1999; Macaluso et al., 2007). Nevertheless, the binding of pRb family proteins with the E2F family of transcription factors appears to be crucial in governing the progression of the cell cycle and the DNA replication by controlling the expression of cell cycle E2F-dependent genes. These genes include *CCNE1* (cyclin E1), *CCNA2* (cyclin A2), and *CDC25A*, which are all essential for the entry into the S phase of the cell cycle, and genes that are involved in the regulation of DNA replication, such as *CDC6, DHFR*, and *TK1* (thymidine kinase) (Attwooll et al., 2004; Polager and Ginsberg, 2009). The *INK4a/ARF* locus (*9p21*) encodes two unique and unrelated proteins, p16INK4a and p14^{ARF}, which act as tumor suppressors by modulating the responses to hyperproliferative signals (Quelle et al., 1995). One of the most frequent alterations affecting the pRb pathway regulation in cancer involves p16^{INK4a}. Loss of p16^{INK4a} occurs more frequently than loss of pRb, suggesting that p16^{INK4a} suppresses cancer by regulating pRb as well as p107 and pRb2/p130. Loss of function of p16^{INK4a} by gene deletion, promoter methylation, and mutation within the reading frame has frequently been found in human cancers (Sherr and McCormic, 2002). Different studies have indicated that p16^{INK4A} can modulate the activity of pRb and it also seems to be under pRb regulatory control itself (Semczuk and Jacowicki, 2004). p16^{INK4a} blocks cell cycle progression by binding Cdk4/6 and inhibiting the action of D-type cyclins. Moreover, p16^{INK4a} controls cell proliferation through inhibition of pRb phosphorylation, then promotes the formation of pRb-E2Fs repressing complexes, which blocks the G1–S-phase progression of the cell cycle (Zhang et al., 1999). It has been reported that pRb forms a repressor containing histone deacetylase (HDAC) and the hSWI/SNF nucleosome remodeling complex, which inhibits transcription of genes for cyclins E and A, and arrests cells in the G1 phase of the cell cycle (Zhang et al., 2000). Both cyclin D1 overexpression and p16^{INK4a} protein alteration produce persistent hyperphosphorylation of pRb, resulting in evasion of cell cycle arrest. Phosphorylation of pRb by cyclin D/cdk4 disrupts the association of the HDAC-Rb-hSWI/SNF

complex, relieving repression of the *cyclin E* gene and G1 arrest. However, the persistence of Rb-hSWI/SNF complex appears to be sufficient to maintain the repression of the *cyclin A* and *cdc2* genes, inhibiting exit from S phase (Zhang et al., 2000; Beasley et al., 2003). Interestingly, there is evidence that suppression of pRb2/p130, perhaps due to epigenetic alterations, abolishes the G1–S phase block, leads to cyclin A expression, and extends S-phase activity. In addition, it has also been reported that overexpression of $p16^{INK4a}$ or p21 causes accumulation of pRb2/p130 and senescence (Helmbold et al., 2009; Fiorentino et al., 2011). While p16INK4a mutations are not commonly reported, small homozygous deletions are the major mechanism of $p16^{INK4a}$ inactivation in different primary tumors such as glial tumors and mesotheliomas. The INK4a/ARF locus on 9p21 is deleted or rearranged in a large number of human cancers, and germline mutations in the gene have been shown to confer an inherited susceptibility to malignant melanoma and pancreatic carcinoma (Meyle and Guldberg, 2009; Scaini et al., 2009). Interestingly, it has been reported an increased risk of breast cancer in melanoma prone kindreds, owing to the inactivation of p16INK4a, p14ARF or both genes (Prowse et al., 2003). Aberrant methylation of p16INK4a has been reported in a wide variety of human tumors including tumors of the head and neck, colon, lung, breast, bladder, and esophagus (Blanco et al., 2007; Gold and Kim, 2009; Goto et al., 2009; Phe et al., 2009; Xu et al., 2010). Inactivation of the p16INK4a gene by promoter hypermethylation has been frequently reported in approximately 50% of human, non-small-cell lung cancer (NSCLC) (Zhu et al., 2006). Moreover, p16INK4a loss in preneoplastic lesions occurred exclusively in patients who also showed loss of $p16^{INK4a}$ expression in their related invasive carcinoma, indicating that $p16^{INK4a}$ may constitute a new biomarker for early diagnosis of this disease (Brambilla et al., 1999; Beasley et al., 2003).

Deregulated tumor expression of p16INK4a has been described in association with clinical progression in sporadic colorectal cancer (CRC) patients (McCloud et al., 2004). p16INK4a hypermethylation has been shown to occur in advanced colorectal tumors and has been associated with patient survival (Cui et al., 2004). Significant correlation has also been reported between aberrant p16INK4a methylation and Dukes' stage and lymphatic invasion in colorectal carcinoma (Goto et al., 2009). Although the inactivation of *p16INK4a* seems to be a crucial event in the development of several human tumors, the relevance of this alteration in mammary carcinogenesis remains unclear. For example, p16INK4a homozygous deletions have been reported in 40–60% of breast cancer cell lines, while both homozygous deletions and point mutations are not frequently observed in primary breast carcinoma, suggesting that these alterations might have been acquired in culture (Silva et al., 2003). In addition, p16INK4a hypermethylation has been reported in breast carcinoma, although the relevance of this $p16^{INK4a}$ alteration is discordant among different studies (Lehmann et al., 2002; Tlsty et al., 2004). Interestingly, although methylation of p16INK4a promoter is common in cancer cells, it has been reported that epithelial cells from histologically normal-appearing mammary tissue of a significant fraction of healthy women show *p*16

promoter methylation as well (Holst et al., 2003; Bean et al., 2007). However, a recent study indicates a strong association between aberrant p16INK4a methylation and breast-cancer-specific mortality (Xu et al., 2010). Cyclin alteration represents one of the major factors leading to cancer formation and progression. Evidence indicates that a combination of cyclin/cdks, rather than a single kinase, executes pRb phosphorylation and at specific pRb-phosphorylation sites (Mittnacht, 2005). Moreover, it has been reported that the activation of the mitogen-activated protein kinase (MAPK) leads to pRb inactivation by sustaining cyclin levels and consequently activating CDKs (Hansen et al., 2009). Constitutive cell surface kinase receptors and persistent phosphorylation/inactivation of pRb, p107, and pRb2/p130 proteins have been implicated in conferring uncontrolled growth to melanoma cells (von Willbrand et al., 2003). A statistically significant difference has been reported in the expression profiles of p16, cyclin D1, and pRb between naevi and melanomas, with decreased, increased, and increased expression in the melanomas, respectively, supporting the hypothesis that cell cycle checkpoint proteins of G1/S transition are critical in the pathogenesis of melanoma (Karim et al., 2009). Moreover, overexpression of cyclin D1 has been found in a wide variety of cancers, including breast carcinoma, endocrine pancreatic tumors, multiple myeloma, mantle cell lymphoma, colon cancer, and various sarcomas (Kim and Diehl, 2009). The mechanisms altering the pRb pathway converge to reach a common goal: uncontrolled expression of key regulators that trigger, even in the absence of growth signals, an irreversible transition into the S phase and cell cycle progression. It is important to underscore that alterations affecting the components of pRb pathway often occur in a mutually exclusive manner, in that one alteration is unaccompanied by others. Moreover, the frequency of particular genetic and epigenetic events varies among tumor types.

1.2.2 p53 ($p14^{ARF}$/mdm2/p53) Pathway

The tumor suppressor gene *p53* is a key regulator of cell cycle checkpoints, which is activated in response to virtually all cancer-associated stress signals, including DNA damage and oncogene activation. Once activated, p53 can trigger several cellular responses, including growth arrest, apoptosis, and senescence. (Junttila and Evan, 2009; Menendez et al., 2009). The key role of p53 in tumor suppression is demonstrated by the prevalence of *TP53* gene mutations in cancer: mutations of this gene occur in more than 50% of all human cancers (Vousden and Prives, 2009). Moreover, because *p*53 is the most frequently mutated gene in human cancer, it appears to be a crucial target for therapy with respect to tumor formation and elimination of the tumor cell (Portugal et al., 2009).

The p53 ($p14^{ARF}$/mdm2/p53) pathway appears to play a major role in mediating oncogene-induced apoptosis; therefore, the suppression of apoptosis by inactivation of this pathway has an important role in tumor development (Menendez et al., 2009). The check and balance existing between the pRb (pRb/$p16^{INK4}$/Cyclin D1) and p53 ($p14^{ARF}$/mdm2/p53) pathways involves the regulation of the G1 to S transition and its checkpoints. This network consists of, but is not limited to, an array of autoregulatory feedback loops, where pRb and

p53 signals exhibit very intricate interactions with other proteins known to exert important roles in the determination of cell fate (Junttila and Evan, 2009; Polager and Ginsberg, 2009). p53 is activated in response to DNA damage, cellular stress, and ultraviolet irradiation, and the turnover of this protein is regulated by ubiquitination through mdm2 binding, which leads to p53 degradation by proteosomes. Moreover, p53 activates E3 ubiquitin ligase mdm2 transcription, ensuring a negative feedback regulation (Xin, 2005). Furthermore, it has been recently reported that mdm2 inhibits TP53 mRNA translation (Ofir-Rosenfeld et al., 2008). In tumors lacking p53 gene mutations, p53 function is often abrogated indirectly through the overexpression of mdm2 or the inactivation of the cell cycle inhibitor $p14^{ARF}$ (also known as p19 in rodents). $p14^{ARF}$ interferes with all the known functions of mdm2 and it has been shown that $p14^{ARF}$ binds to the mdm2−p53 complex, resulting in a stabilization of both proteins (Moule et al., 2004).

Significantly, $p14^{ARF}$ expression is positively regulated by members of the E2F family of transcription factors. This observation provides a link between the pRb (pRb/$p16^{INK4}$/Cyclin D1) and p53 ($p14^{ARF}$/mdm2/p53) pathways, suggesting a mechanism whereby the loss of function of pRb proteins leads to deregulation or hyperactivation of E2Fs, resulting in the functional inactivation of p53. These concurrent alterations have been observed in a wide range of human tumors, highlighting the crucial role of pRb (pRb/$p16^{INK4}$/Cyclin D1) and p53 ($p14^{ARF}$/mdm2/p53) pathways in oncogenesis in general (Polager and Ginsberg, 2009). p53 also activates the transcription of $p21^{Cip/Kip}$, which is largely responsible for the p53-dependent G1 arrest in response to different cellular stress and DNA damage (Sherr, 2004). p21Cip/Kip regulates cyclin E/Cdk2 and cyclin A/Cdk2 complexes, both of which phosphorylate pRb, contributing to an irreversible transition into the S phase and cell cycle progression even in the absence of growth signals. Deletion inactivation of p14ARF has been reported in human cancers, but in these studies *p16INK4a* was always codeleted (Fulci et al., 2000; Newcomb et al., 2000; Sarkar et al., 2000). Only germline deletion of p14ARF-specific exon 1b in a family characterized by multiple melanoma and neural cell tumors has been reported (Randerson-Moor et al., 2001). Different studies have reported that epigenetic alterations such as CpG hypermethylation may be the first cause of *p14ARF* gene silencing, followed by *p14ARF* loss of heterozygosity (LOH) and homozygous deletions. *p14ARF* hypermethylation has been detected in several tumors including primary colorectal, breast, gastric, and lung tumors (Furonaka et al., 2004; Sharma et al., 2007; Zhao et al., 2007; Kominami et al., 2009).

1.3 CONCLUSION

The intricate crosstalk of signals connecting pRb (pRb/$p16^{INK4}$/cyclin D1) and p53 ($p14^{ARF}$/mdm2/p53) pathways is crucial in regulating cell cycle progression and viability. Genetic and epigenetic alterations disturbing this crosstalk appear to be a common part of the life history of human cancers, independent of age or tumor type. Data accumulated over the past years clearly indicate that although

pRb and p53 pathways are each typically deregulated in human cancer, they do not function independently but through a complex network of communicating signals. Understanding the complex molecular mechanisms that regulate cell cycle progression and are involved in tumor formation and progression still remains the most important goal in cancer research. Indeed, an increased knowledge of the alterations in pRb and p53 pathways will be useful in improving anticancer treatments. Importantly, progress over the past years has greatly enhanced our understanding of the epigenetic mechanisms affecting the action of cell cycle key regulators and leading to cancer formation and progression, thus offering important tools for the diagnosis and prevention of this disease.

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