1 Introduction

1.1 Epigenetics

The malignant program of the cancer cells is associated with altered function of genes or its inactivation. This can be mediated by disruption of coding sequences and epigenetical alterations. Two epigenetical modifications are critical for transcription regulation: DNA methylation and chromatin modification. Recent studies showed that epigenetical changes have a central role in neoplastic progression.

1.1.1 DNA methylation

Mammalian genomic DNA contains four bases. In addition to these, a fifth base, methylated cytosine is found (reviewed by Laird, 1999; Herman and Baylin, 2003). The cytosine methylation is a post-replicative event occurring symmetrically on both DNA strands at CpG sites and provided by DNA methyltransferases (*DNMT*). The pattern and content of CpG methylation is cell specific. In the genome, CpGs are mostly clustered in CpG islands (reviewed by Herman and Baylin, 2003). As supposed, methylated cytosine can be deaminated with subsequent replacement by thymidine (reviewed by Laird, 1999). Hence, most of the CpGs without any regulatory role were eliminated in evolution to prevent these mutations. The localization of CpG islands is often associated with the promoter regions (reviewed by Jones and Laird, 1999; Esteller and Herman, 2002). In the transcribed genes, upstream regions of the CpG islands are usually unmethylated. The DNA methylation in the 5' end of the promoter CpG island mostly leads to inactivation of transcription. Imprinted genes, germ-specific genes, tissue-specific genes and X chromosome are examples of the transcriptional inactivation by DNA methylation (reviewed by Rountree *et al.*, 2001; Esteller and Herman, 2002; Herman and Baylin, 2003). The control of the mechanism, which protects promoter from DNA methylation, can be lost during aging, since gradual increase of the *de novo* DNA methylation takes place in non-imprinted genes during senescence (reviewed by Jones and Laird, 1999). In cancer cells, hyper- and hypomethylation of DNA are found (reviewed by Herman and Baylin, 2003). Aberrant DNA hypomethylation is identified in normally imprinted genes and chromosome pericentromeric regions of malignant cells. DNA hypomethylation of pericentromeric

regions leads to chromosome instabilities and mistakes in replication. *De novo* DNA methylation of the promoters and the following gene silencing were observed in several tumor suppressor genes in cancer cells. In some cases, the DNA methylation can mediates gene inactivation even when CpG island is located outside the promoter (reviewed by Jones and Laird, 1999). However, aberrant DNA methylation of the CpG islands does not always lead to transcriptional inactivation and also the genes inactivated by DNA methylation in cancer cells are not always tumor suppressor genes.

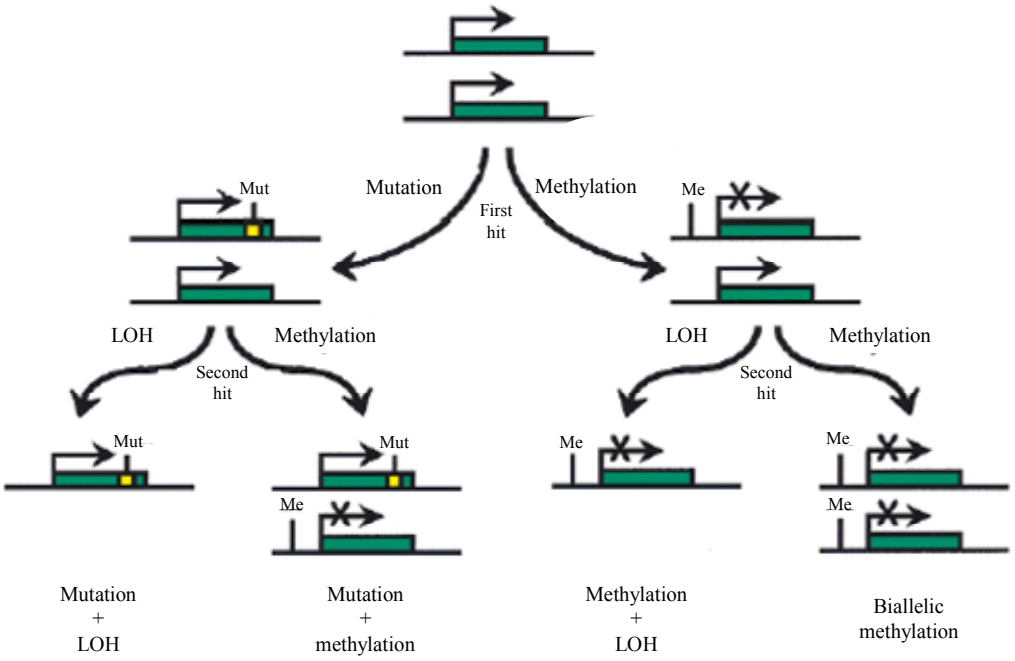

Figure 1-1. Knudson's two-hit hypothesis revised. Two active alleles of a tumor suppressor gene are indicated by the two green boxes shown at the top. The first step of gene inactivation is shown as a localized mutation on the left or by transcriptional repression by DNA methylation on the right. The second hit is shown by either LOH or transcriptional silencing (Adopted from Jones and Laird, 1999).

The inactivation mechanism of the tumor suppressor gene by DNA methylation was proposed by Jones and Laird (Figure 1-1) using Knudson's two-hits model (Jones and Laird, 1999). Knudson's model defines that the inactivation of both alleles is necessary for the loss of gene function (Figure 1-1). According to Jones and Laird, the first and second inactivation hits can be mediated by aberrant DNA methylation in promoter region of the tumor suppressor gene (Figure 1-1). Using DNA demethylating drug 5-aza-2'-deoxycytidine (5-Aza-CdR), the importance of promoter DNA hypermethylation in gene expression was illustrated (reviewed by Herman and Baylin,

2003). The mechanism of the DNA methyltransferase (*DNMT*) inactivation by 5-Aza-CdR can be explained as an analog of cytosine, 5-Aza-CdR integrating in the DNA sequence by replication. In contrast to cytosine, 5-Aza-CdR can not be methylated by *DNMT*, while it contains nitrogen in place of carbon at fifth position of cytosine. DNA methylation is not only involved in transcription control and chromosome stability, but also in the replication time of DNA (reviewed by Herman and Baylin, 2003). The level of the DNA methylation is correlated with time of replication i.e., heavy methylated DNA replicates late as compared to unmethylated DNA regions containing active genes. DNA methylation alone does not repress transcription, since only addition of proteins to the methylated DNA and following organization of chromatin lead to transcriptional inactivation (reviewed by Rountree *et al.*, 2001; Herman and Baylin, 2003). Thus, both DNA methylation and chromatin structure are involved in regulation of transcriptional activity.

1.1.2 Chromatin

In the eukaryotic nuclei, the genomic DNA is highly folded and compacted by proteins in a dynamic structure termed chromatin. The unit of chromatin is termed nucleosome and contains 146 bp of DNA wrapped around nucleosome core (reviewed by Jenuwein and Allis, 2001; Rountree *et al.*, 2001; Ehrenhofer-Murray, 2004). The nucleosome core is formed by octamer of four core histone proteins: H2A, H2B, H3 and H4. Histone H1 is located between nucleosomes and responsible for the DNA folding in high-order chromatin structure, 30 nm fiber. Chromatin is a dynamic structure, which controls access of transcription regulators to DNA (reviewed by Herman and Baylin, 2003). When DNA is heavy methylated, nucleosomes are closely compacted. At such state, chromatin is inaccessible (heterochromatic) for the transcription regulators. In contrast, nucleosomes are spaced with wide and irregular intervals at the sites of transcribing genes. At this state, chromatin is accessible (euchromatic) for other proteins. Euchromatic and heterochromatic states have own chromatin modifications. These modifications are post-translation modifications of the histones such as histone acetylation, methylation, phosphorylation and ubiquitylation (reviewed by Ehrenhofer-Murray, 2004). Except ubiquitylation, most of the histone modifications are observed in N-terminal tails of histones. Recent studies demonstrate the importance of histone modifications in the gene regulation. Transcriptionally active chromatin is marked by

histone H3 with acetylated lysine 9, 14 and histone H3 with methylated lysine 4 (Figure 1-2) (reviewed by Jenuwein and Allis, 2001; Sarraf and Stancheva, 2004). Methylation at lysine 9 of histone H3 is associated with transcriptionally silenced gene promoters and inactive chromatin (Figure 1-2). This histone modification is found in the inactivated X chromosome and pericentromeric chromosome regions (reviewed by Nguyen *et al.*, 2002; Santoro *et al.*, 2002).

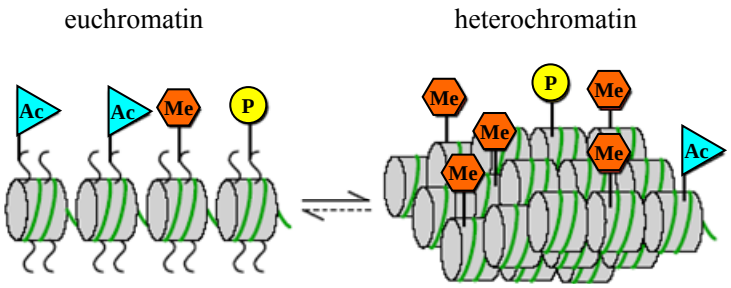

Figure 1-2. Euchromatin and heterochromatin. Schematic representation of euchromatin and heterochromatin as accessible or condensed nucleosome fibers containing acetylated (AC), phosphorylated (P) and methylated (Me) histone NH_2-termini (Adopted from Jenuwein and Allis, 2001).

Histone acetylation is carried out by histone acetyltransferases (*HATs*) (reviewed by Ehrenhofer-Murray, 2004). *HATs* as *p300* and *CREB-binding protein* play a role in the initiation of transcription. Removal of the acetyl groups from histone H3 by histone deacetylases (*HDACs*) leads to transcriptional inactivation, which is mediated by increasing of the chromatin compactization (reviewed by Rountree *et al.*, 2001; Herman and Baylin, 2003). Formation of inaccessible chromatin is also mediated by interaction partner of *HDAC*, histone H3 lysine 9 methyltransferase (*SU(VAR)3-9*) (Czermin *et al.*, 2001). *DNMTs* can repress gene transcription by binding of *HDACs* and transporting it to the gene promoters (Fuks *et al.*, 2000; Robertson *et al.*, 2000; Rountree *et al.*, 2000). Moreover, *HDACs* can be recruited by proteins, which specifically bind to methylated CpGs (Fuks *et al.*, 2000; Suzuki *et al.*, 2003; reviewed by Rountree *et al.*, 2001; Esteller and Herman, 2002; Herman and Baylin, 2003). These proteins are termed Methyl-CpG binding domain proteins (*MBDs*). *MBDs* have the ability to repress transcription by itself (reviewed by Herman and Baylin, 2003) and interact with histone H3 lysine 9 methyltransferase (Fuks *et al.*, 2003; Sarraf and Stancheva, 2004). Moreover, *DNMT1* is identified in complexes with *MBDs* (*MBD2*, *MBD3 and MeCP2*) (Tatematsu *et al.*, 2000; Kimura and Shiota, 2003). Out of six

mammalian *DNMTs* (*DNMT1*, *DNMT1o*, *DNMT2*, *DNMT3a*, *DNMT3b* and *DNMT3L*), *DNMT1* methylates DNA with hemi-methylated CpGs and is responsible for maintaining of DNA methylation after each round of replication; whereas other *DNMTs* are only specific for the development or methylate *de novo* DNA or have no catalytic activity (reviewed by Robertson, 2002; Jaenisch and Bird, 2003). In addition, absence of *DNMT1* in cancer cells leads to disorganization of nuclear structure, increasing in acetylation and decreasing in methylation at lysine 9 of histone H3 (Espada *et al.*, 2004). In concordance with this observation, Fuks and colleagues identified *SUV39H1* histone H3 lysine 9 methyltransferase in a complex with *DNMT1* and *DNMT3a* (Fuks *et al.*, 2003).

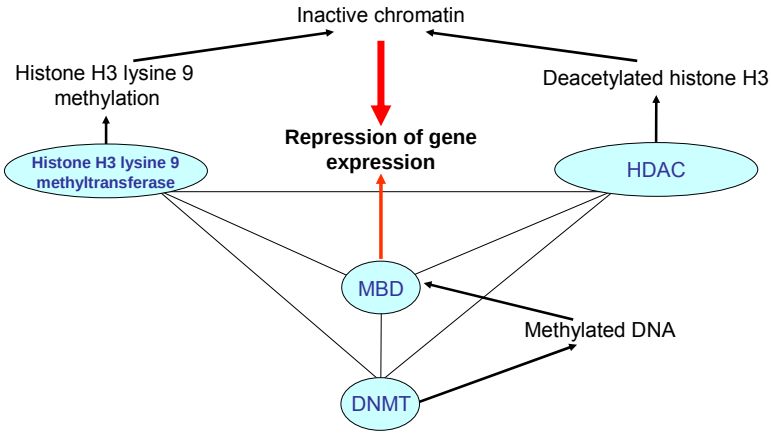

Figure 1-3. Maintaining of inactive chromatin. Connecting lines indicate interaction partners. Arrows indicate functions or following effect.

Furthermore, after 5-Aza-CdR treatment of cancer cells with the epigenetically inactivated $p16^{INK4}$ promoter, the following changes were observed: the upregulation of $p16^{INK4}$ expression, reduction of levels of methylated histone H3 lysine 9 and *MBD* (*MeCP2*), increasing levels of acetylated histone H3 and methylated histone H3 lysine 4 (Nguyen *et al.*, 2002). Thus, histone modifications and DNA methylation are related events, which play a role in the promoter inactivation (Figure 1-3).

1.1.3 Specificity protein 1 (*Sp1*)

Many housekeeping, tissue-specific and viral genes contain functionally important GC- and related GT/CACC-boxes. The proteins from *Sp* family recognize and bind to

these motifs. The *Sp* family includes four isoforms: *Sp1*, *Sp2, Sp3* and *Sp4* (Hagen *et al.*, 1992; reviewed by Suske, 1999; Samson and Wong, 2002). Three of them: *Sp1*, *Sp3* and *Sp4* recognize and bind to GC boxes as well as to GT/A-rich motifs with similar affinity (Hagen *et al.*, 1992; reviewed by Suske, 1999; Samson and Wong, 2002). *Sp1* and Sp3 are expressed in a wide variety of mammalian cells. *Sp2* binds preferentially to GT/A-rich sequences and is detected predominantly in neuronal tissues. *Sp* proteins belong to a family of transcription regulators known as the mammalian *Sp/XKLF* or "Krüppel-like" factors. *Sp/XKLF* proteins are characterized by a highly-conserved DNA-binding domain containing three Krüppel-like C_2H_2 zinc fingers. Homological proteins to the *Sp/XKLF* were identified in *Drosophila melanogaster*, *Caenorhabdidtis elegans* and yeast (reviewed by Philipsen and Suske, 1999; Suske, 1999; Turner and Crossley, 1999; Samson and Wong, 2002). *Sp1* was one of the first eukaryotic transactivators to be isolated. In 1983, using *Sp1* isolated from HeLa cell extracts, Dynan and Tjian performed *in vitro* transcription from the *SV40* viral early promoter (Dynan and Tjian, 1983). *Sp1* may form tetramers by interaction of glutamine-rich domains (Figure 1-4) (reviewed by Samson and Wong, 2002). Formation of these tetramers can be involved in a DNA loop formation. The carboxyl-terminal domain of *Sp1* may play an important role in the *Sp1* synergistic activation by the stacking of tetramers (Figure 1-4) (Matsushita *et al.*, 1998). By direct interactions with DNA or via interactions with other transcription regulators, *Sp1* mediates activation and repression of different promoters (reviewed by Kavurma and Khachigian, 2004).

Figure 1-4. Structural features of the *Sp1* protein. The *Sp1* length is indicated on the right. Red boxes indicate 2 glutamine rich regions and blue box represents a carboxyl-terminal domain. The black boxes label the zinc fingers.

Sp1 and its isoform *Sp3* bind to the same motif. *Sp3* protein was originally found as a repressor of the *Sp1*-mediated activation by binding to the same site (Hagen *et al.*, 1994). Analysis of different promoters showed that *Sp1* and *Sp3* may display parallel or opposing transcription activities (reviewed by Samson and Wong, 2002). *Sp1*

interacts with *TAFII130*, which is a subunit of the general transcription factor *TFIID* (Pugh and Tjian, 1990; Tanese *et al.*, 1996). There are speculations that *Sp1* may play a role as TATA box in the TATA less promoters, since *Sp1* can recruit *TFIID* (Kaufmann and Smale, 1994; Tanese *et al.*, 1996). Also, *Sp1* can interact with other proteins such as the early growth response factor *Egr-1*, octamer transcription factor *Oct-1* and nuclear receptors for estrogens or androgens (reviewed by Samson and Wong, 2002). Furthermore, *Sp1* interacts with *E2F1*, *c-Myc*, *p53* and *HDAC1* (Karlseder *et al.*, 1996; Doetzlhofer *et al.*, 1999; Gartel *et al.*, 2001; Lagger *et al.*, 2003). Importance of *Sp1* was shown by Marin and colleagues. They demonstrated that *Sp1 null* embryos are severely retarded in growth and die after day 10 of embryonic development (Marin *et al.*, 1997). The *Sp1* sites appear to play a critical role in the maintenance of the methylation-free CpG islands, since the removal or mutation of the *Sp1* motif exposes DNA methylation of the CpG-rich regulator regions (Macleod *et al.*, 1994; Brandeis *et al.*, 1994; Gazzoli and Kolodner, 2003). The open question is still: is the *Sp1* binding sensitive to the DNA methylation at the *Sp1* site or not? Several reports demonstrated that *Sp1* is insensitive to the DNA methylation (Holler *et al.*, 1988; Harrington *et al.*, 1988; Mancini *et al.*, 1999). Whereas in other studies, methylated CpGs variably reduce the *Sp1* binding (Gazzoli and Kolodner, 2003; Chang *et al.*, 2004; Butcher *et al.*, 2004). However, Wei-Guo Zhu and colleagues demonstrated that methylation at CG sites outside of the consensus *Sp1*-binding site may directly reduce the ability of *Sp1/Sp3* to bind (Zhu *et al.*, 2003). In summary, *Sp1* plays an important role in transcription regulation by recruiting *RNA polymerase II* via *TFIID*, by interacting with DNA, transcriptional factors and *HDAC1* and also by protecting the promoters from aberrant DNA methylation.

1.2 RASSF1A

In 2000, Dammann and colleagues discovered and cloned a new gene, *RASSF1* from common homozygous deletion area at 3p21.3 (Dammann *et al.*, 2000). This gene is termed *Ras- association domain family 1* (*RASSF1*) gene because of the predicted *Ras*-association domain and homology to the murine *Ras*-effector *NORE1*. Homology search and cDNA screening identified 7 alternatively spliced transcripts: *RASSF1A*, *RASSF1B* (minor form), *RASSF1C*, *RASSF1D* (cardiac-specific), *RASSF1E* (pancreas-specific), *RASSF1F* and *RASSF1G* (reviewed by Dammann *et al.*, 2003). *RASSF1A*

and *RASSF1C* are major transcripts, which are expressed in normal tissues (Dammann *et al.*, 2000). Both isoforms have four common exons, which encode the *RAS*-association domain (Figure 1-5). The transcription of *RASSF1A* and *RASSF1C* starts from two different CpG islands, which are approximately 3.5 kb apart (Figure 1-5). The *RASSF1A* transcript is frequently missing in human cancer cells in contrast to *RASSF1C*, which is identified in all analyzed malignant cells except cells containing homozygous deletion of this region (reviewed by Dammann *et al.*, 2003). To identify mutations of *RASSF1A*, sequences of its exons were analyzed in cancer cells. Only two confirmed somatic mutations were identified in more than 200 different carcinoma samples. Thus, mechanism of the *RASSF1A* inactivation is other than mutagenesis. Analysis of the *RASSF1A* CpG island identified frequent methylation of the *RASSF1A* promoter in cancer cells. Further research showed that DNA hypermethylation of the *RASSF1A* CpG island is the most frequent event in primary human cancer. In lung tumor, DNA hypermethylation of the *RASSF1A* promoter is correlated with advanced tumor stages and impaired survival of patients. Methylation of the *RASSF1A* promoter corresponds also with LOH frequency in several types of cancer. Furthermore, the *RASSF1A* inactivation is associated with viral infections of *SV40* and *EBV*. Expression of exogenous *RASSF1A* inhibits tumor growth *in vitro* and *in vivo* (Dammann *et al.*, 2000; Li *et al.*, 2004; Song *et al.*, 2004; reviewed by Dammann *et al.*, 2003). Moreover, the *RASSF1A* knockout mice are prone to spontaneous and induced carcinogenesis (Tommasi *et al.*, 2005). Thus, *RASSF1A* plays a role as tumor suppressor gene.

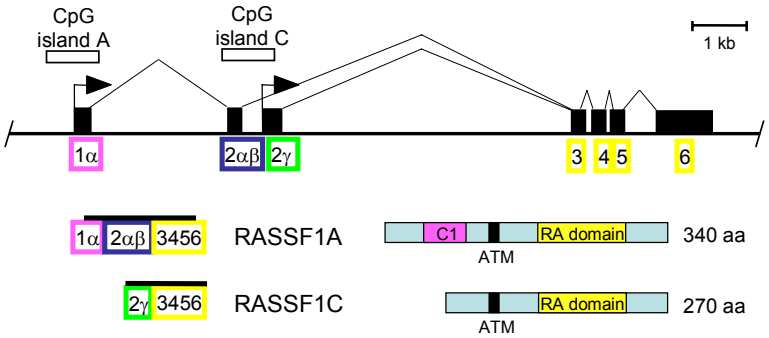

Figure 1-5. Map of the *RASSF1* gene with two main isoforms. Two promoters of *RASSF1* (arrows) are located in the CpG islands (open squares). *RASSF1A* and *RASSF1C* are made by alternative promoter usage und RNA splicing of exons (black boxes). The encoded protein length is indicated in amino acid (aa) and domains are marked as: C1 - diacylglycerol/phorbol ester binding domain; RA - RAlGDS/AF6 *Ras*-association domain; ATM - putative ATM phosphorylation site consensus sequence (Adopted from Dammann *et al.*, 2003).

Several different groups reported that *RASSF1A* is a microtubule-binding protein (Figure 1-6) (Liu *et al.*, 2003; Dallol *et al.*, 2004; Rong *et al.*, 2004; Vos *et al.*, 2004), which can directly interact with tubulins and microtubule-associated proteins (Dallol *et al.*, 2004; Rong *et al.*, 2004). *RASSF1A* stabilizes microtubules and induces growth arrest in G2/M and G1/S phases (Figure 1-6) (Shivakumar *et al.*, 2002; Liu *et al.*, 2003; Rong *et al.*, 2004). Its association with microtubules was observed at interphase; whereas in mitosis, *RASSF1A* colocalizes with spindles and centrosomes (Figure 1-6) (Liu *et al.*, 2003). Additionally, control of the cell cycle can be mediated by the *RASSF1A* interaction with *Cdc20*, an activator of anaphase-promoting complex (*APC*) (Song *et al.*, 2004). After interaction with *RASSF1A, Cdc20* will not activate *APC* and the cell cycle is blocked at prometaphase. The other interaction partner of *RASSF1A* is $p120^{EAF}$ (Fenton *et al.*, 2004). $p120^{EAF}$ interacts with *retinoblastoma* (*RB*) and *p53* and is involved in the control of entering the S-phase (Fenton *et al.*, 2004). Furthermore, *RASSF1A* negatively regulates cyclin *D1* (Shivakumar *et al.*, 2002), which mediates phosphorylation of *RB* and controls the exit from G1 phase (reviewed by Sherr, 1996).

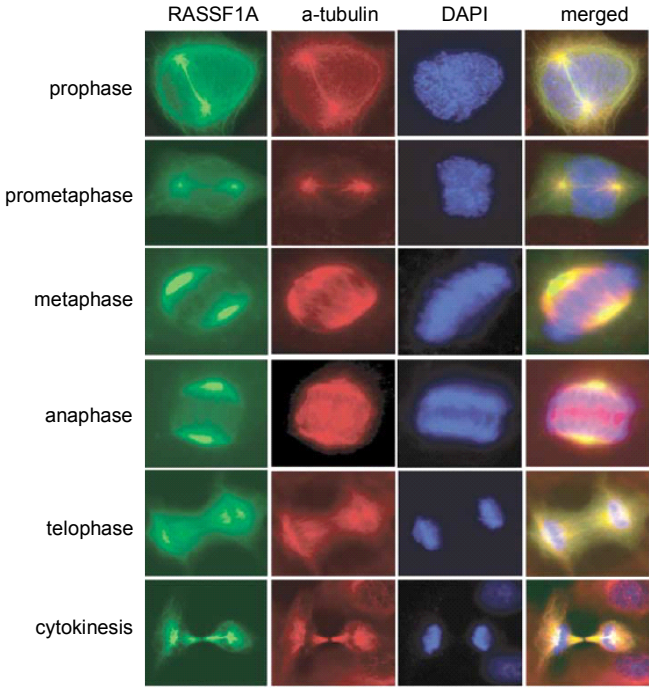

Figure 1-6. *RASSF1A* localizes to the mitotic apparatus during mitosis. COS-7 cells transfected with *GFP-RASSF1A* (green) were fixed, permeabilized and co-stained with an anti-a-tubulin antibody (red) and DAPI (blue). Cells at each mitotic stage are as indicated (Adopted from Liu *et al.*, 2003).

RASSF1 as *NORE1* can bind the serine/threonine kinase *MST1* (*mammalian sterile twenty-like*), which mediates the *Ras*-apoptotic effect (Khokhlatchev *et al.*, 2002). *RASSF1* and *NORE1* can form heterodimers, therefore, as supposed, *RASSF1* is also involved in the *Ras*-signaling (Ortiz-Vega *et al.*, 2002). However, the *RASSF1C-NORE1* interaction is weaker compared to the *RASSF1A-NORE1* binding. Furthermore, *RASSF1C* can stabilize microtubules but not as effective as *RASSF1A* (Rong *et al.*, 2004; Vos *et al.*, 2004). *RASSF1C* induces cell cycle arrest (Rong *et al.*, 2004). The inactivation of *RASSF1C* and *RASSF1A* leads to the *Ras*-induced genomic instability (Vos *et al.*, 2004). Moreover, Li and colleagues identified that *RASSF1C* may play a role as a tumor suppressor gene, since *RASSF1C* can repress the growth of cancer cells *in vitro* and *in vivo* and the mutations or loss of expression of *RASSF1C* were observed in tumors (Li *et al.*, 2004). The connector enhancer of *KSR* (*CNK*) is a *c-Raf* binding protein mediating *Ras*-induced *Raf* activation (Rabizadeh *et al.*, 2004). *CNK1* is an interaction partner of *RASSF1*. *CNK* can repress division of cancer cells and initiate apoptosis via complex *RASSF1A-MST1* (*or MST2*). Hence, *RASSF1A* supports the *CNK1* apoptotic effect in contrast to *RASSF1C*, which does not influence the *CNK1* induced apoptosis. In addition, both isoforms contain a consensus of phosphorylation site for ataxia telangiectasia-mutated kinase (*ATM*) (Dammann *et al.*, 2000), which plays a role in cycle arrest, apoptosis and maintaining of the genomic instability (reviewed by Shiloh and Kastan, 2001). Mutation of this site in *RASSF1* leads to significant reduction in the protein phosphorylation level compared to wildtype (Shivakumar *et al.*, 2002). Moreover, cancer cell transfected with mutated *RASSF1A* at position for *ATM* phosphorylation could enter the synthesis phase in contrast to cells transfected with the *RASSF1A* construct without mutation. Additionally, mutations at this site were observed in cancer cells. The binding of *RASSF1* to *Ras* is still an open question. Ortitz-Vega and colleagues described this binding as weak (Ortiz-Vega *et al.*, 2002). From other side, Vos and colleagues demonstrated the *RASSF1* binding to a *Ras* in GTP-dependent manner *in vivo* and *in vitro* and the *RASSF1A* induced apoptosis (Vos *et al.*, 2000). Taken together, both *RASSF1* isoforms are associated with microtubules and can mediate cell cycle arrest and prevent *Ras*-induced genomic instability. In contrast to *RASSF1A*, *RASSF1C* shows weak or no characteristic related with cell cycle control. This could be revealed to the absence of diacylglycerol/phorbol ester binding domain in *RASSF1C*. The

frequent epigenetical inactivation of *RASSF1A* in cancer cells is associated with the function of *RASSF1A* as a tumor suppressor in cell proliferation.

1.3 Human mammary epithelial cells (HMECs)

Detection of breast cancer at early stage may save lives of patients. Hence, to study the mechanism of malignant transformation of breast cells is very important. There are evidences that inactivation of $p16^{INK4}$ tumor suppressor by its promoter methylation occurs in histological normal breast tissues (Holst *et al.*, 2003) and it may promote a premalignant cell program (Crawford *et al.*, 2004). In addition to the $p16^{INK4}$ promoter methylation in breast carcinomas, an epigenetical inactivation of $p16^{INK4}$ was identified during HMEC senescence (Brenner *et al.*, 1998; Foster *et al.*, 1998; Esteller *et al.*, 2001; Dominguez *et al.*, 2003). Analogous to $p16^{INK}$, high rate of the *RASSF1A* promoter methylation was observed in breast carcinomas (Dammann *et al.*, 2001; Dulaimi *et al.*, 2004). Moreover, histological normal breast tissues contain cells with aberrant methylation of the *RASSF1A* CpG island (Yan *et al.*, 2003; Lewis *et al.*, 2005). Thus, *RASSF1A* may be epigenetically inactivated in HMECs analogous to $p16^{INK4}$.

In tissue culture, human mammary fibroblasts (HMF) proliferate for a limited number of population doublings (PD) and then enter a plateau termed replicative senescence or Hayflick limit (reviewed by Figueroa *et al.*, 2000). It is believed that the reason for the Hayflick limit is the telomeres shortening (reviewed by Sandhu *et al.*, 2000). The shortening of telomeres is mediated due to the instability of DNA polymerase in the replication of the outermost ends of the lagging strand DNA (reviewed by Figueroa *et al.*, 2000). This instability results in a lost of approximately 50-200 bp of telomeres in each round of replication. In stem cells and some cancer cell lines, the telomere shortening is overcome by telomerase, a ribonucleoprotein complex that adds *de novo* telomeric sequences. At Hayflick limit, HMF have a mean telomere restriction fragment (TRF) of approximately 6-8 kb (reviewed by Romanov *et al.*, 2001). HMF, which reached Hayflich limit, are large vacuolated cells with a flat form and expressing senescence-associated β-galactosidase (SA-β-gal) (reviewed by Tlsty *et al.*, 2001). These cells persist to stay at this stage. After 15-30 PD, HMECs attain senescence morphology i.e., large, flat and becomes vacuolated (Figure 1-7) (reviewed by Romanov *et al.*, 2001). Similar to HMF, HMECs express SA-β-gal and have a

mean TRF of approximately 6-8 kb. At this phase, HMECs and HMF have elevated levels of the $p16^{INK4}$ expression (Alcorta *et al.*, 1996; Brenner *et al.*, 1998; Foster *et al.*, 1998). In contrast to HMF obtained from the same mammary tissue, HMECs escape from this proliferation block with a high frequency of spontaneous emergence (Romanov *et al.*, 2001). After senescence selection, HMECs enter a second period of exponential growth (Brenner *et al.*, 1998; Foster *et al.*, 1998). At the post-selection stage, HMECs are characterized by heavy DNA methylation of the $p16^{INK4}$ promoter followed by the absence of the $p16^{INK4}$ expression (Brenner *et al.*, 1998; Foster *et al.*, 1998). After 25-45 PD, HMECs go back to senescence phenotype associated with SA-β-gal expression and enter a new plateau termed agonescence (Romanov *et al.*, 2001). Cells at agonescence are characterized by high levels of chromosome abnormalities compared to cells at senescence proliferation state. At five PD before agonescence, these abnormalities are detected in 66-100% metaphases and are mediated by critically shortened telomeres with a mean TRF of approximately 5 kb (Tlsty *et al.*, 2001; Romanov *et al.*, 2001). As proposed, the proliferative barrier at agonescence is a telomere-dependent proliferative barrier (reviewed by Stampfer and Yaswen, 2003).

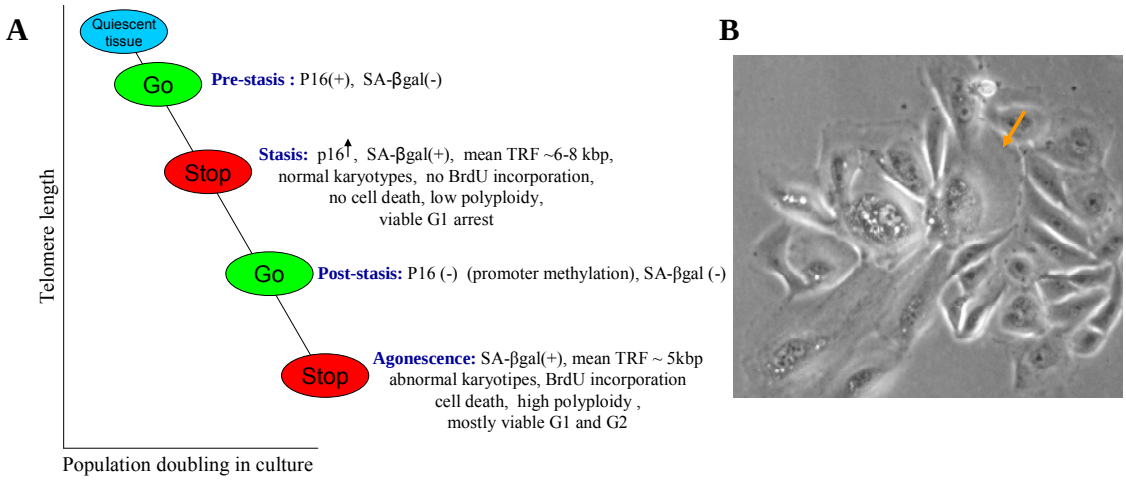

Figure 1-7. HMECs in culture. A. A model of HMEC proliferation in cell culture. **B.** HMECs in post-stasis proliferation phase (100x). Yellow arrow indicates cell in agonescence proliferation phase.

Yaswen and Stampfer suggest that the generation of chromosomal abnormalities during post-stasis may lead to telomerase reactivation (Yaswen and Stampfer, 2002). This reactivation can be a preferential way of tumor progressing of epithelial cells.

When escaping from stasis by HMECs was observed, researchers speculated that first proliferation barrier is associated with short length of telomeres (Hayflick limit)

(Romanov *et al.*, 2001). However, studies of the last years show that the elevated level of $p16^{INK4}$ is responsible for this proliferation plateau via *RB* using following mechanism: $p16^{INK4}$ interacts and inhibits cyclin *D1* dependent protein kinases; as a result of this interaction, *RB* can not be phosphorylated by these kinases; therefore, cells go to G1 arrest (reviewed by Sherr, 1996; Stampfer and Yaswen, 2003). Recent studies showed that the upregulation of $p16^{INK4}$ is stress associated. Ramirez and colleagues found that HMECs, which were grown on feed layers, do not enter the proliferation plateau and do not show any change in the $p16^{INK4}$ expression (Ramirez *et al.*, 2001; Herbert *et al.*, 2002). Furthermore, evaluation of the $p16^{INK4}$ expression can be mediated by other stress types, such as high level of O_2 and inadequate medium; therefore, changing of incubation conditions can delay senescence (reviewed by Drayton and Peters, 2002). Thus, the $p16^{INK4}$ inactivation in an inadequate culture environment is an event, which is necessary for proliferation. Interestingly, that researcher could not identify gross chromosome abnormalities in mouse embryo cells, which avoid stasis (Loo *et al.*, 1987). Basis on new knowledge's, the new term, stasis (stress or aberrant signaling induced senescence) had been introduced to refer to stress-associated senescence (Drayton and Peters, 2002). Stasis was identified in experiments with culture of mouse embryo cells, keratinocytes, skin fibroblast, oligodendrocyte precursor cells and normal rodent glia (Loo *et al.*, 1987; Mathon *et al.*, 2001; Tang *et al.*, 2001; Ramirez *et al.*, 2001). Shortly, HMECs in culture enter two proliferation plateaus; first is mediated by $p16^{INK4}$ via *RB* and second is telomere-dependent proliferative barrier. The post-stasis cells are characterized by the epigenetically inactivated $p16^{INK4}$ promoter.

1.4 Aim of study

Aim of the present study was localization and analysis of the regulatory elements in the *RASSF1A* promoter. Moreover, mechanism of epigenetical inactivation of the *RASSF1A* promoter should be elucidated in present research. In concordance with this aim, mechanism of methylation of the *RASSF1A* promoter should be analyzed. Furthermore, roles of chromatin state and transcription regulatory elements in epigenetical inactivation of the *RASSF1A* promoter should be investigated. Additionally, hypothesis about epigenetical inactivation of the *RASSF1A* promoter during senescence of HMECs should be verified.