4 Discussion

4.1 Regulation of *RASSF1A* transcription

RASSF1A is a tumor suppressor gene, which is frequently inactivated in human cancers (Dammann *et al.*, 2003). This silencing is associated with a DNA hypermethylation of the *RASSF1A* CpG island. However, the epigenetic mechanisms responsible for the *RASSF1A* inactivation are unknown. Since a protectional role of the *Sp1* sites from DNA methylation was demonstrated in several studies (Frank *et al.*, 1991; Brandeis *et al.*, 1994; Macleod *et al.*, 1994; Matsuo *et al.*, 1998; Qu and Ehrlich, 1999; Gazzoli and Kolodner, 2003); the four putative *Sp1* sites, which were identified in the *RASSF1A* promoter by *in silico* analysis, were intensively analyzed in present research. Three of these *Sp1* sites are located in the *RASSF1A* CpG island, whereas the fourth *Sp1* site is detected 478 bp upstream from the putative translation start site, in the last exon of the *BLU* gene. Luciferase assay analysis identified that mutation of the upstream *Sp1* site leads to a decrease of the *RASSF1A* promoter transcription activity. In this site, a hypersensitive G was detected in the *in vivo* footprinting experiments. Additionally, EMSA showed that proteins, which form complex with *Sp1* antibodies, bind to a 22 bp fragment of the *RASSF1A* promoter containing this *Sp1* site. Analogously, the binding of *Sp1* to this site was detected by ChIp. Thus, present data suggest that the upstream *Sp1* site in the *RASSF1A* promoter is functional. Surprisingly, the deletion of a 63 bp fragment containing this binding site leads to a three fold increase of the promoter activity. Analysis of footprinting *in vivo* identified additional hypersensitive Gs located in the 63 bp fragment. This indicates that this fragment is bound also by other regulatory elements, which may be involved in repression of the *RASSF1A* transcription.

Binding of *Sp1* at the *RASSF1A* CpG island was detected by EMSA *in vitro* and by ChIp *in vivo*. Luciferase assay of the *RASSF1A* promoter demonstrated that the *RASSF1A* CpG island fragment, which is located between -137 and +17 positions and containing the three *Sp1* sites, has 3% of promoter activity compared to the construct containing four *Sp1* sites. Deletion of this CpG island fragment resulted in a 45% decrease of transcription activity compared to the construct with all four *Sp1* sites. Thus, elements located in this CpG island fragment can mediate a very weak transcription activity (3%) by itself, but its presence supports the *RASSF1A* promoter

activity (45%). Hence, three *Sp1* sites located in the *RASSF1A* CpG island may be involved in induction of the *RASSF1A* promoter transcription. Further, promoter analysis by luciferase assay showed that the fragment located between -205 and +17 positions has 40% of promoter activity. Thus, adding of the 68 bp fragment located upstream from three *Sp1* sites leads to an increase of promoter activity from 3% up to 40%. Elements located in this 68 bp DNA region may be essential for expression of the *RASSF1A* promoter. Moreover, this region may be bound by the general transcription factor *TFIID*. Studies of *Sp1* from the last 25 years suggest that *Sp1* is involved in recruitment of the general transcription factor *TFIID* to the DNA through direct interaction with *TAFII130* (Pugh and Tjian, 1990; Tanese *et al.*, 1996). It is commonly believed, that *Sp1* can recruit *TFIID* to TATA-less promoter similar to the TATA box (Pugh and Tjian, 1990; Kaufmann and Smale, 1994; Tanese *et al.*, 1996). In the *RASSF1A* TATA-less promoter, three *Sp1* sites located in the *RASSF1A* CpG island flank a region, which may be bound by general transcription factor *TFIID*. Thus, the *TFIID* recruitment to the *RASSF1A* promoter may be supported by the *Sp1* binding to the CpG island.

In the *RASSF1A* promoter of pre-stasis HMECs, the *Sp1* binding pattern is similar to binding in HeLa cells. However, in pre-stasis HMECs, it is associated with the eight times decreased level of the *RASSF1A* transcription activity compared to HeLa. Chromatin state in the *RASSF1A* promoter in these cells is characterized by decrease of frequency of acetylated histone H3 and increase of level of trimethylated histone H3 lysine 9 compared to HeLa. Methylated histone H3 at lysine 9 is a histone modification, which is associated with inactive X chromosome and pericentric heterochromatin (Mermoud *et al.*, 2002; Peters *et al.*, 2002; Lehnertz *et al.*, 2003; Rougeulle *et al.*, 2004). Moreover, an elevated frequency of methylated histone H3 lysine 9 is an important characteristic of inactive genes (Litt *et al.*, 2001; Nielsen *et al.*, 2001; Fahrner *et al.*, 2002; Nguyen *et al.*, 2002; Kondo *et al.*, 2003; Mutskov and Felsenfeld, 2004; Su *et al.*, 2004). Thus, methylation at lysine 9 of histone H3 is a feature of repressed chromatin, which is not accessible for transcription factors. In contrast, absence or low level of acetylated histone H3 is correlated with inactive chromatin (Litt *et al.*, 2001; Fahrner *et al.*, 2002; Nguyen *et al.*, 2002; Kondo *et al.*, 2003; Mutskov and Felsenfeld, 2004; Su *et al.*, 2004). Thus, the reduced *RASSF1A* transcription in pre-stasis HMECs is associated with the active *Sp1* binding and repressed chromatin. Analogously, a number of studies demonstrated that

transcription, which is mediated by *Sp1*, can be repressed; and this is associated with inactivation of chromatin. Lagger and colleagues reported that the regulation of the $p21^{CIP1}$ expression is under control of *HDAC* and *p53* through interaction with C terminus of *Sp1* (Lagger *et al.*, 2003). In a suppressed state, *HDAC* binds *Sp1* and deacetylate histones; whereas active state of the $p21^{CIP1}$ promoter is characterized by the interaction of *Sp1* with *p53*, which recruits *HAT* and *p300* and this interaction is crucial for transcription activation. Analogous to the $p21^{CIP1}$ promoter, repression of transcription activity by *Sp1-HDAC* complex was demonstrated for *S-phase specific mouse thymidine kinase*, *human telomerase reverse transcriptase*, *manganese superoxide dismutase*, *ORF50* core promoter of Kaposi's sarcoma-associated herpes virus, $p19^{INKd}$, *transforming growth factor-β receptor type I*, *human luteinizing hormone receptor*, *dihydrofolate reductase* and *reversion-inducing-cysteine-rich protein* (*RECK*) (Doetzlhofer *et al.*, 1999; Chang *et al.*, 2001; Hou *et al.*, 2002; Maehara *et al.*, 2002; Won *et al.*, 2002; Zhang and Dufau, 2002; Ammanamanchi and Brattain, 2004; Chang *et al.*, 2004; Yokota *et al.*, 2004). Thus, changing of chromatin state around the *Sp1* binding site may result in changing of transcription activity, which is regulated by *Sp1*. Therefore, in pre-stasis HMECs, the inactivation of chromatin in the *RASSF1A* promoter may result in repression of transcription.

From the other side, *Sp1* interacts with *HAT* and this results in acetylation of the *Sp1* zinc fingers (Suzuki *et al.*, 2000; Torigoe *et al.*, 2004). Torigoe and colleagues demonstrated that the *Sp1* acetylation is associated with reactivation of the *SV40* promoter (Torigoe *et al.*, 2004). Thus, additionally to the chromatin state, acetylation and deacetylation of *Sp1* can regulate changes in transcription. The *RASSF1A* promoter in pre-stasis HMECs is characterized by decreased level of acetylated histone H3 compared to HeLa. This indicates a decrease of level of *HATs* and an increase of level of *HDAC*. These changes may result in deacetylation of the *Sp1* in the *RASSF1A* promoter with a following repression of the *RASSF1A* promoter activity in pre-stasis HMECs.

Additionally, transcription regulated by *Sp1* can be repressed by *MBD*. In 1998, Kudo demonstrated that the human leukosialin transcription activated by *Sp1* is repressed by DNA methylation through methyl-CpG binding protein *MeCP2* (Kudo, 1998). Moreover, an other methyl-CpG binding protein, *MBD1* in a complex with MBD1-containing chromatin-associated factor (*MCAF*) can interact with *Sp1* through *MCAF* and repress transcription of the methylated *p16* and *small nuclear ribonucleoprotein*

polypeptide N (*SPRPN*) promoters (Fujita *et al.*, 2003). In unmethylated promoters, *MCAF* facilitates the *Sp1*–mediated transcription. In pre-stasis HMECs, the *RASSF1A* promoter is characterized by heavy methylation of the upstream *Sp1* site. Thus, presence of *MBD* in the upstream *Sp1* site of the *RASSF1A* promoter in pre-stasis HMECs may result in a repression of the *RASSF1A* transcription. Hence, repression of the *RASSF1A* transcription in pre-stasis HMECs may be regulated by deacetylation of *Sp1*, repression of chromatin and by *MBD*, which are directed by elevated level of DNA methylation in the *RASSF1A* promoter.

4.2 DNA methylation and the *RASSF1A* promoter inactivation

The role of DNA methylation in the inactivation of the *RASSF1A* transcription was revealed by treatment of post-stasis HMECs and no expressing *RASSF1A* cancer cells with 5-Aza-CdR, since after this treatment, transcriptional reactivation of the *RASSF1A* promoter was observed. Recent studies of the hypermethylated $p16^{INKa}$, *mutL homologue 1* (*MLH1*) and O^6*-methylguanine-DNA methyltransferase* promoters showed that the inhibition of *DNMT* with 5-Aza-CdR results in transcriptional reactivation, decrease of DNA methylation and reactivation of chromatin in these promoters (Nguyen *et al.*, 2002; Kondo *et al.*, 2003). Moreover, open chromatin structure occurs in whole genome of cancer cells after 5-Aza-CdR treatment (Espada *et al.*, 2004). Thus, in cancer cells, inactivation of *DNA* methyltransferases leads to opening of chromatin and transcriptional reactivation of silenced genes. Interaction partners of *MBD* are *HDAC* and histone H3 lysine 9 methyltransferase (Jones *et al.*, 1998; Ng *et al.*, 1999; Fuks *et al.*, 2000; Tatematsu *et al.*, 2000). Moreover, *MBDs* have the ability to repress transcription by itself (reviewed by Herman and Baylin, 2003). Thus, the *RASSF1A* transcription may be repressed by *MBD* directly and by *HDAC* and histone H3 lysine 9 methyltransferase through compactization of chromatin. Therefore, decrease of the *MBD* level in the *RASSF1A* promoter after 5-Aza-CdR treatment may result in a reactivation of the transcription through decrease of the *MBDs* negative control of transcription and through opening of chromatin structure for transcriptional factors. This suggestion is supported by observation of Sarraf and Stancheva. They demonstrated that the absence of *MBD1* resulted in loss of methylated histone H3 lysine 9 at multiple genomic loci and the *p53BP2* transcriptional reactivation (Sarraf and Stancheva, 2004).

Analysis of the DNA methylation pattern in the *RASSF1A* promoter identified an methylation free region around the unmethylated CpG island in the *RASSF1A* transcribing cells such as HeLa, HF and blood, whereas this DNA area is frequently methylated in the no expressing *RASSF1A* cancer cells. The 3'-end of this unmethylated DNA fragment in the *RASSF1A* expressing cells flanks a partly methylated (50%) *Alu* repeats; whereas the 5'-end of this region flanks a lightly methylated or completely unmethylated DNA region containing the upstream *Sp1* site. The region containing the upstream *Sp1* site is close to a DNA area, which is strongly methylated in the *RASSF1A* expressing and not expressing cells. Thus, in the *RASSF1A* expressing cells, the DNA methylation level is gradually increased outside from the methylation free area of the *RASSF1A* promoter. In pre-stasis HMECs, the DNA methylation free region in the *RASSF1A* promoter is smaller compared to the *RASSF1A* expressing cells as HeLa, HF and blood. Pre-stasis HMECs are characterized by methylation of the *RASSF1A* exon 1α and region containing the upstream *Sp1* site; whereas the methylation free area was detected only in the *RASSF1A* CpG island fragment located upstream from translation start site. In post-stasis HMECs, this CpG island fragment was partially methylated and an increase of DNA methylation was observed in the *RASSF1A* exon 1α and the region containing the upstream *Sp1* site. Thus, DNA methylation spreads from upstream and downstream into the *RASSF1A* promoter during senescence of HMECs. Similar results were reported by Yan and colleagues (Yan *et al.*, 2003). They identified DNA methylation in the *RASSF1A* exon 1α in all normal breast tissues, primary tumors and cancer cell lines. They detected DNA methylation in the *RASSF1A* CpG island fragment located upstream from translation start site in 90% of normal breast tissue and 90% of primary breast tumors. However, the methylation level in this CpG island fragment in normal breast tissue and primary breast tumors was lower compared to breast cancer cell lines. Thus, DNA methylation pattern of the *RASSF1A* CpG island, which was observed by Yan and colleagues in normal breast tissues and primary breast tumors, is similar as we observed in pre-stasis and post-stasis HMECs. Further, Yan and colleagues showed that level of DNA methylation is strongly enhanced in the exon 1α in normal breast tissues and primary breast tumors compared to the CpG island fragment located upstream from translation start site. Moreover, they detected the methylation pattern of the *RASSF1A* promoter in breast tissues where exon 1α was methylated and the CpG island fragment located upstream from translation start site was unmethylated. Thus,

the observed spreading of DNA methylation from downstream into the *RASSF1A* promoter in our study is also supported by methylation profiling detected by Yan and colleagues (Yan *et al.*, 2003). Analogously, Millar and colleagues identified a spreading of the DNA methylation from upstream and downstream into the *GSTP1* promoter in prostate cancer cells (Millar *et al.*, 2000). Inactivation of the *E-cadherin* gene promoter in human fibroblasts, which overexpress *DNMT1*, is associated with a spreading of DNA methylation from upstream and downstream into promoter (Graff *et al.*, 1997). Thus, spreading of DNA methylation from upstream and downstream into CpG islands can be a common mechanism of epigenetical inactivation of genes.

Further, analysis of the DNA methylation pattern of the region containing upstream *Sp1* site of the *RASSF1A* promoter by bisulfite sequencing revealed only two methylated CpGs in the *RASSF1A* expressing cells, HeLa and PBMC. One methylated cytosine belongs to the 5'GGG**C**GG sequence of the upstream *Sp1* site in the *RASSF1A* promoter, whereas the second methylated cytosine is an upstream nucleotide (5'**C**GGGCGG). An *in vivo* footprinting analysis of the upstream *Sp1* site detected hypersensitive G next to this cytosine (5'C**G**GGCGG). Moreover, two research groups defined the *Sp1* consensus sequence as 5'**C**GGGCGG (Seguin and Hamer, 1987; Boyer and Maquat, 1990). Thus, this cytosine can be involved in the *Sp1* binding. Additionally, the DNA methylation pattern of this region was analyzed in cell lines and human mammary epithelium. Analysis of clones containing this fragment of the *RASSF1*A promoter from different cell types identified similarity in the methylation pattern of partly methylated sequences. All partly methylated sequences contained at least one methylated CpG in the upstream *Sp1* site. In these clones, the frequency of methylation of both CpGs in the upstream *Sp1* site was 5.5 times higher compared to the event, when only one from these CpGs was methylated. This indicates that DNA methylation occurs preferently in the upstream *Sp1* site of the *RASSF1A* promoter compared to flanking CpGs (Figure 4-1).

Since the upstream *Sp1* site is frequently methylated, *Sp1* at this site can be associated with *DNMT1*. *DNMT1* in complex with *DNMT3* controls about 95% DNA methylation in cells and is involved in aberrant DNA methylation in cancer cells (Rhee *et al.*, 2002). Overexpression of *DNMT1* in cells leads to an increase of DNA methylation, which is accompanied by malignant transformation (Wu *et al.*, 1993; Graff *et al.*, 1997). *In vitro* experiments showed that *DNMT1* methylates unmethylated CpGs with very low efficiency *de novo* (Fatemi *et al.*, 2002; Hermann *et al.*, 2004). The

association of *Sp1* with *DNMT1* was demonstrated by Milutinovic and colleagues (Milutinovic *et al.*, 2004). In the *DNMT1* antisense knock down cells, they identified expressional induction of the unmethylated *p21* promoter in a *Sp1*-dependent manner. Moreover, it is well known that aberrant DNA methylation occurs during aging of cells (reviewed by Issa, 1999). Thus, *DNMT1* may be associated with the upstream *Sp1* site of the *RASSF1A* promoter; and this may result in preferential aberrant DNA methylation of this site during aging (Figure 4-1). Additionally, the association of *Sp1* with *DNMT1* may be performed through *HDAC* in the *RASSF1A* promoter. Since, *HDAC* is a interaction partner of *Sp1* and *DNMT1* (Doetzlhofer *et al.*, 1999; Fuks *et al.*, 2000; Robertson *et al.*, 2000; Rountree *et al.*, 2000; Chang *et al.*, 2001; Hou *et al.*, 2002; Won *et al.*, 2002; Zhang and Dufau, 2002; Lagger *et al.*, 2003; Ammanamanchi and Brattain, 2004). *In vitro* studies of the activity of *DNMT1* showed that *de novo* DNA methylation is stimulated by the presence of methylated DNA in solution (Hermann *et al.*, 2004; Fatemi *et al.*, 2002; Fatemi *et al.*, 2001). Therefore, DNA methylation at the upstream *Sp1* site in the *RASSF1A* promoter may initiate the methylation of the surrounding CpGs (Figure 4-1). This is supported by methylation profiling of the fragment containing the upstream *Sp1* site. Analysis of this region in mammary epithelium demonstrated all steps of possible progression of the DNA methylation from unmethylated state and gradual increase of the DNA methylation around the *Sp1* site. Thus, the DNA methylation may spread from the upstream *Sp1* site (Figure 4-1). Moreover, *de novo* DNA methylation of this region may be initiated by the heavy methylated DNA fragment located upstream from this region (Figure 4-1).

Methylated DNA is a target for *MBDs*, which interact with *DNMT1*, *HDAC* and histone H3 lysine 9 methyltransferase (Jones *et al.*, 1998; Ng *et al.*, 1999; Tatematsu *et al.*, 2000; Fuks *et al.*, 2003; Kimura and Shiota, 2003; Sarraf and Stancheva, 2004). Recruitment of *HDAC* and histone H3 lysine 9 methyltransferase by *MBD* can lead to chromatin inactivation, which can directs DNA methylation (Tamaru and Selker, 2001; Johnson *et al.*, 2002; Lehnertz *et al.*, 2003; Jackson *et al.*, 2004). Thus, the *RASSF1A* transcription can be inactivated using the following mechanism. The association of *Sp1* with *DNMT1* at upstream *Sp1* site of the *RASSF1A* promoter may result in aberrant DNA methylation at this site during aging and in the subsequent *MBDs* binding (Figure 4-1, Figure 4-3). *MBDs* repress the *RASSF1A* transcription through *Sp1* and recruit the *HDAC* and histone H3 lysine 9 methyltransferase to the *RASSF1A* promoter

(Figure 4-3). These enzymes inactivate chromatin and recruit *DNMT1*, which actively methylate *de novo* the *RASSF1A* promoter (Figure 4-3). Thus, epigenetical inactivation of the *RASSF1A* promoter may be mediated by DNA methylation at the upstream *Sp1* site (Figure 4-3).

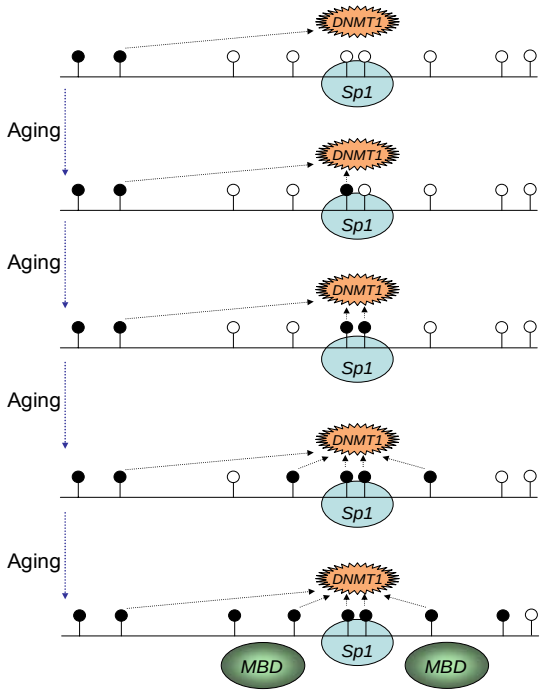

Figure 4-1. Model of mechanism of aberrant DNA methylation of the upstream *Sp1* site in the *RASSF1A* promoter during aging. The association of *Sp1* with *DNMT* at the upstream *Sp1* site results in *de novo* aberrant DNA methylation of this site during aging. Presence of the methylated CpGs stimulates *de novo* methylation of the other CpGs by *DNMT*. Subsequently, aberrant DNA methylation is resulted in the recruitment of *MBDs* to promoter. Black and white lollipops represent methylated and unmethylated CpGs, respectively. Arrows indicate an effect of the methylated CpGs to *DNMT1*.

In pre-stasis HMECs, the *RASSF1A* promoter is characterized by the active binding of *Sp1* and elevated frequency of methylated CpGs compared to HeLa. This indicates that the binding of *Sp1* does not prevent the epigenetical inactivation of *RASSF1A* in HMECs. This observation contradicts a number of studies. In 1994, two independent groups reported that mutation of the *Sp1* site located in the *adenine phosphoribosyltransferase* (*Aprt*) promoter results in spreading of *de novo* DNA methylation in transgenic mice and transfected embryonic cells (Brandeis *et al.*, 1994; Macleod *et al.*, 1994). Moreover, in mouse embryonic stem cells, the presence of *Sp1* site in an *in vitro* methylated construct leads to demethylation of a CpG island upstream from the hamster *Aprt* gene (Brandeis *et al.*, 1994). Matsuo and colleagues

demonstrated, that the binding of *Sp1* induces demethylation of the *in vitro* methylated *β-globin* promoter in *Xenopus* fertilized eggs after midblastula transduction (Matsuo *et al.*, 1998). Qu and Ehrlich identified demethylation of DNA induced by *Sp1* in non-embryonic cells (Qu and Ehrlich, 1999). Thus, *Sp1* is involved in demethylation of promoters in embryonic and non-embryonic cells. Recently, this hypothesis was supported by observation of Gazzoli and Kolodner. They identified that polymorphisms at the *Sp1* site in the *MSH6* promoter are associated with the epigenetical inactivation of *MSH6* (Gazzoli and Kolodner, 2003). Moreover, the localizations of the *Sp1* sites between methylated flanking DNA and unmethylated promoter regions in *E-cadherin* (E-cad) gene, von *Hippel-Lindau* gene (*VHL*), *BRCA1* and *iduronate-2-sulfatase* gene (*IDS*) may be additional proofs of the protectional role of *Sp1* from spreading *de novo* DNA methylation into promoter (Graff *et al.*, 1997; Butcher *et al.*, 2004; Tomatsu *et al.*, 2004). In contrast to this, epigenetical inactivation of the *RASSF1A* promoter is not sensitive to the binding of *Sp1*, since the *Sp1* binding to the *RASSF1A* promoter in pre-stasis HMEC is associated with repressed chromatin and an increase of DNA methylation level in promoter. There are several explanations for these observations. First, the control of the DNA methylation free promoter can be performed by other mechanism as *Sp1*. The presence of such mechanism is supported by a study of Marin and colleagues, which identified DNA methylation free CpG islands in the genome of *Sp1-null* mice (Marin *et al.*, 1997). Moreover, Mummaneni and colleagues demonstrated that only one from four functional *Sp1* sites in the *Aprt* promoter has the ability to inhibit the epigenetical inactivation of *Aprt* and is not required for transcriptional activity in contrast to the other three *Sp1* sites (Mummaneni *et al.*, 1998). Analysis of the *glutathione-S-transferase* gene (*GSTP1*) showed that a *Sp1* site located in the promoter is not involved in its epigenetical protection, since mutation and deletion of this site does not lead to DNA methylation (Song *et al.*, 2002). Thus, different *Sp1* sites may realize different functions. One of several *Sp1* sites regulates transcriptions, whereas others are involved in the protection from epigenetical inactivation of the promoters. Hence, the *Sp1* sites in the *RASSF1A* promoter may be only involved in transcriptional regulation and do not mediate protection from epigenetical inactivation. Second explanation is that the *Sp1* binding may protect the *RASSF1A* promoter from *de novo* DNA methylation only during embryonic reprogramming; and the control of the methylation free *RASSF1A* promoter may be mediated by mechanisms other as *Sp1* in non-embryonic cells.

4.3 Mechanism of epigenetical inactivation of the *RASSF1A* promoter

In pre-stasis HMECs, the chromatin state in the *RASSF1A* promoter is repressed compared to HeLa cells, which actively transcribe *RASSF1A*. The chromatin state of the methylated region containing the upstream *Sp1* sites is similar to the unmethylated CpG island fragment containing three *Sp1* sites. Thus, a repressed state of chromatin is detected in the unmethylated and methylated *RASSF1A* promoter fragments. Further, analysis of post-stasis HMECs identified a *de novo* DNA methylation in the *RASSF1A* CpG island fragment and enhanced repression of chromatin compared to pre-stasis HMECs. Thus, an inactive chromatin state observed in the *RASSF1A* CpG island fragment at pre-stasis proliferation state precedes the *de novo* DNA methylation of this region detected in post-stasis HMECs. This observation is supported by several studies, which demonstrated that repression of chromatin occurs before DNA methylation during epigenetical inactivation. The best example of epigenetical inactivation is methylation of X chromosome. During X chromosome inactivation, repression of chromatin and following inactivation of *X* linked genes occur before DNA methylation (Heard *et al.*, 2001). Similar data were obtained by Bachman and colleagues when they analyzed the promoter silencing of $p16^{INK4a}$ in colorectal cancer cells with inactivated *DNMT1* and *DNMT3* (Bachman *et al.*, 2003). They observed that changing of chromatin precedes inactivation of the $p16^{INK4}$ transcription and DNA methylation of the promoter is the last step of this inactivation process. Furthermore, study of thymocytes maturation showed that chromatin repression in the *terminal deoxynucleotidyltransferase* (*Dntt*) promoter occurs before transcription inactivation and an increase of DNA methylation is only observed in mature T-lymphocytes (Su *et al.*, 2004). Analogously, repression of chromatin takes place before DNA methylation during inactivation of transgene (Mutskov and Felsenfeld, 2004). However, Stirzaker and colleagues identified that chromatin inactivation is directed by DNA methylation when prostate cancer cells were transfected with a plasmid containing the *GSTP1* promoter and exon 1 (Stirzaker *et al.*, 2004). Histone deacetylation of this construct was directed by seeds of introduced DNA methylation and occurs before histone methylation. However, this study was performed in an artificial situation. Whereas inactivation of the X chromosome during embryonic development, inactivation of *Dntt* during thymocytes maturation and the *RASSF1A* inactivation in HMECs take place *in vivo*. Moreover, the inactivation of $p16^{INKa}$ in DKO cells and transgene inactivation are models, which represent *in vivo* situation.

In pre-stasis HMECs, the *RASSF1A* transcription was reduced compared to the strongly *RASSF1A* expressing cells. As discussed above, chromatin modifications and DNA methylation may be responsible for the decreased level of the *Sp1*-mediated *RASSF1A* transcription in pre-stasis HMECs. Moreover, transcription, which is not mediated by *Sp1*, can be also repressed by chromatin, since during epigenetical inactivation of the $p16^{INK4}$ promoter, X chromosome, *Dntt* and transgene, changing of chromatin was associated with repression of transcription and DNA methylation was observed after this repression (Heard *et al.*, 2001; Bachman *et al.*, 2003; Su *et al.*, 2004; Mutskov and Felsenfeld, 2004). Moreover, *Caenorhabdidtis elegans* and *Saccharomyces cerevisiae* lack DNA methylation (Reuben and Lin, 2002; Osley, 2004; reviewed by Rountree *et al.*, 2001; Martin *et al.*, 2004). Thus, repression of gene activity can be performed by chromatin in absence of DNA methylation. A similar mechanism was observed in mammals by Lewis and colleagues. They found in placenta that imprinted genes flanking the imprinting center 2 (*IC2*) on mouse distal chromosome 7, are inactivated by repressive chromatin modifications in the absence of DNA methylation (Lewis *et al.*, 2004). Thus, repression of transcription by chromatin is a common mechanism, and the *RASSF1A* expression may be repressed by chromatin state independent from *Sp1* in pre-stasis HMECs. In post-stasis HMECs, the DNA methylation pattern of the *RASSF1A* CpG island is completely different from breast cancer cell lines; however, the transcriptional rate is only slightly higher. Thus, in the proliferating post-stasis HMECs a new transcriptional pattern is established by a repressed chromatin state and then this aberrant expression profile is maintained by DNA hypermethylation in tumor cells.

In post-stasis HMECs, *de novo* DNA methylation in the *RASSF1A* promoter is associated with elevated frequency of trimethylated histone H3 lysine 9. This observation supported by interaction of *DNMT1* with *SUV39H1* histone H3 lysine 9 methyltransferase (Fuks *et al.*, 2003). Moreover, recent studies showed that DNA methylation can be directed by methylation of histone H3 at lysine 9 position (Tamaru and Selker, 2001; Johnson *et al.*, 2002, Lehnertz *et al.*, 2003). In experiments with fungus *Neurospora crassa,* the control of DNA methylation by histone methylation was demonstrated, since absences of histone methyltransferase or DNA methyltransferase result in the loss of DNA methylation (Tamaru and Selker, 2001). Analogously, in *Arabidopsis thaliana*, mutation of the methyltransferase gene, which is specific for histone H3 lysine 9 (homolog of *SU(VAR)3-9*), and mutation of the

DNA methyltransferase lead to loss of cytosine methylation at CpNpG trinucleotides (where N is A, C, G or T) (Johnson *et al.*, 2002; Jackson *et al.*, 2004). Moreover, Lehnertz and colleagues identified that presence of trimethylated histone H3 lysine 9 is necessary for DNA methylation of major satellite repeats at pericentric heterochromatin in mice (Lehnertz *et al.*, 2003). However, absence of *DNMT1* or *DNMT3a/DNMT3b* does not result in changing of chromatin state at these sites (Lehnertz *et al.*, 2003). Thus, histone methylation can direct DNA methylation in different organisms. Additional proof of the control of DNA methylation by chromatin is a *de novo* DNA methylation in early development, since DNA should be marked for this process to direct the work of *DNMT*. Proteins like *Sp1*, which interact with DNA, may play the role of these marks. There is no evidence, that every unmethylated DNA area is marked by *Sp1*. Histones are the best candidates to direct *de novo* DNA methylation in development. Thus, DNA methylation can be directed by chromatin. Hence, d*e novo* DNA methylation in the *RASSF1A* CpG island may be mediated by elevated level of trimethylated histone H3 lysine 9. Similar explanation may be used for observations, which were done by two research groups. Mutskov and Felsenfeld, 2004 showed that *de novo* DNA methylation of transgene occurs in the same window of time as histone H3 lysine 9 methylation (Mutskov and Felsenfeld, 2004). Moreover, analysis of prostate cancer cells transfected with a partly methylated plasmid containing the *GSTP1* promoter and exon 1 showed that extensive DNA methylation of the *GSTP1* CpG island is associated with histone H3 lysine 9 methylation (Stirzaker *et al.*, 2004). Thus, direction of *de novo* DNA methylation in the *RASSF1A* CpG island by chromatin may be a common mechanism during epigenetical inactivation.

4.4 The modulation of the binding of *Sp1* to the *RASSF1A* promoter

Using ChIp, the *Sp1* binding to the upstream *Sp1* site of the *RASSF1A* promoter was observed, when this site is unmethylated in HeLa and methylated in pre-stasis HMECs. Similar results were obtained by EMSA, which indicated binding of *Sp1* to the methylated and unmethylated oligos containing *Sp1* sites. Thus, the binding of *Sp1* to the upstream consensus of *RASSF1A* is unaffected by DNA methylation. Concordantly, Holler and colleagues showed that DNA methylation of the *Sp1* site does not influence the binding of *Sp1 in vitro* (Holler *et al.*, 1988). Moreover, an *in vitro* analysis by EMSA identified that *Sp1* binds with same efficiency methylated and

unmethylated oligos containing the *human metallothionein IIA* promoter fragment and *neurofibromatosis* gene (*NF1*) promoter fragment (Harrington *et al.*, 1988; Mancini *et al.*, 1999). Interestingly, analysis of the *Sp1* binding to the $p21^{Cip1}$ promoter by EMSA showed that *Sp1* is insensitive to DNA methylation at binding consensus, however sensitive to DNA methylation of the CpGs in the surrounding sequences (Zhu *et al.*, 2003). Analysis of *Sp1* sites in the *MSH6* (mismatch repair protein) and *insulin-like growth factor-binding protein-3* (*IGFBP-3*) promoters by EMSA showed, that *Sp1* binds more effectively to unmethylated oligos compared to methylated (Gazzoli and Kolodner, 2003; Chang *et al.*, 2004). Furthermore, an increase of the DNA methylation around the methylated *Sp1* site of the *IGFBP-3* promoter resulted in completely inactivation of the *Sp1* binding in EMSA experiments (Chang *et al.*, 2004). In our experiments, the binding of *Sp1* to the methylated and unmethylated upstream *Sp1* site of the *RASSF1A* promoter was analyzed *in vitro* and *in vivo*, however the binding of *Sp1* was investigated in the discussed studies *in vitro* (Harrington *et al.*, 1988; Zhu *et al.*, 2003; Gazzoli and Kolodner, 2003; Chang *et al.*, 2004). Butcher and colleagues studied the *Sp1* binding to the *BRCA1* promoter by *in vitro* and *in vivo*. They showed that the binding of *Sp1* to the methylated *BRCA1* promoter fragment is inhibited in EMSA experiments; however, active binding of *Sp1* to the methylated site in transcribing promoter was observed by ChIp (Butcher *et al.*, 2004). Thus, *Sp1* may bind methylated DNA *in vivo* even if *in vitro* experiments demonstrated inhibition of the *Sp1* binding by DNA methylation. Additionally, the differences in the *Sp1* binding to methylated DNA *in vitro* may be attributed to differences in DNA sequences surrounding the *Sp1* sites. Thus, present data suggest, that the *Sp1* binding to the upstream *Sp1* site is insensitive to DNA methylation in the *RASSF1A* promoter.

Since the binding of *Sp1* to the upstream site of *RASSF1A* is independent from the DNA methylation, this binding may be mediated by chromatin state. In accordance, a decrease of acetylated histone H3 level at this site in post-stasis HMECs compared to pre-stasis HMECs was associated with occlusion of the *Sp1* binding. It is important to note that chromatin at the upstream *Sp1* site of the *RASSF1A* promoter was repressed in pre-stasis HMECs compared to HeLa; however, this chromatin changing does not inhibit the binding of *Sp1* to this site. Recently, a factor was revealed, which interacts with *Sp1* and may mediate the binding of *Sp1*. Suzuki and colleagues reported that the DNA binding domain of *Sp1* interacts with acetyltransferase region of *p300* and the *Sp1-p300* interaction stimulates the binding of *Sp1* to DNA (Suzuki *et al.*, 2000). Thus,

a decrease of *p300* at the upstream *Sp1* site of *RASSF1A* may be associated with reduction of the *Sp1* binding to DNA. Additionally, deacetylation of histone H3 could be involved in the formation of inaccessible chromatin for transcription factors in post-stasis HMECs. For instance, inactive chromatin is detected in the *RASSF1A* promoter of ZR75-1 cells and associated with inhibition of the *Sp1* binding. Moreover, the *RASSF1C* promoter in post-stasis HMECs is characterized by an increased level of acetylated histone H3 and an decreased frequency of trimethylated histone H3 lysine 9 and this is associated with increasing of *Sp1* binding. Thus, two factors may be involved in inhibition of *Sp1* binding to the upstream *Sp1* site of the *RASSF1A* promoter in post-stasis HMECs: a repressive chromatin state and the absence of *p300*.

Analogous to the upstream *Sp1* site region of the *RASSF1A* promoter, the *Sp1* binding in the *RASSF1A* CpG island may be inhibited by additional chromatin modifications and a decrease of the *p300* level in post-stasis HMECs. Additionally, *de novo* DNA methylation may be reason for the *Sp1* binding inactivation in the *RASSF1A* CpG island of post-stasis HMECs. As was shown previously, *Sp1* binding is sensitive to DNA methylation (Zhu *et al.*, 2003; Butcher *et al.*, 2004; Gazzoli and Kolodner, 2003; Chang *et al.*, 2004). In the *RASSF1A* CpG island of post-stasis HMECs, seeds of DNA methylation were detected in *Sp1* sites. However, *Sp1 in vitro* binds oligos containing the *Sp1* sites of the *RASSF1A* promoter in unmethylated as completely methylated state. There are no evidences that the *Sp1* binding is dependent from the DNA methylation in the *RASSF1A* CpG island. Thus, the binding of *Sp1* to the *RASSF1A* promoter may be mediated by chromatin state.

4.5 Comparing the *RASSF1A* promoter to the *RASSF1C* promoter

RASSF1A and *RASSF1C* are the two major transcripts of *RASSF1*, which are transcribed from two different CpG islands located approximately 3.5 kb apart and expressed in normal cells (Dammann *et al.*, 2000). *RASSF1A* is frequently epigenetically inactivated in cancer cells in contrast to the unaffected *RASSF1C* transcript. Here, the epigenetic states of *RASSF1C* and *RASSF1A* promoter were analyzed and compared between each other.

DNA methylation analysis identified that the *RASSF1C* promoter is completely unmethylated in all analyzed cells. The transcription analysis of *RASSF1C* revealed no inactivation in HMECs, cancer cells and non malignant cells. Thus, the transcription

and DNA methylation status of *RASSF1C* are not associated with the *RASSF1A* epigenetical inactivation. Analysis of chromatin state in the *RASSF1C* promoter identified the increased level of trimethylated histone H3 lysine 9 in pre-stasis HMECs compared to HeLa, whereas the frequency of trimethylated histone H3 lysine 9 in post-stasis HMECs was similar to HeLa. Thus, establishment of inactive chromatin in the *RASSF1A* promoter in pre-stasis HMECs may influence to chromatin state in the *RASSF1C* promoter. However, these chromatin changes do not result in an inhibition of *Sp1* binding to the *RASSF1C* promoter and inactivation of the *RASSF1C* transcription. In post-stasis HMECs, chromatin is repressed in the *RASSF1A* promoter, whereas the *RASSF1C* promoter is characterized by increase of level of active chromatin compared to HeLa. Moreover, this chromatin remodeling of the *RASSF1* locus in post-stasis cells is associated with loss of *Sp1* in the *RASSF1A* promoter and an increase of the *Sp1* binding in the *RASSF1C* promoter. Analogously, the elevated level of the *Sp1* binding to the *RASSF1C* promoter was observed in ZR75-1 compared to HeLa and pre-stasis HMECs. Thus, chromatin inactivation in the *RASSF1A* promoter may result in an increase of active chromatin state in the *RASSF1C* promoter.

These observations suggest that there is a mechanism, which prevents spreading of inactive chromatin and DNA methylation into the *RASSF1C* promoter. *RASSF1C* promoter contains five *Sp1* sites upstream from translation start site, whereas in the *RASSF1A* promoter, only four *Sp1* sites are found. Mutations in two *Sp1* sites in the *RASSF1C* promoter result in abolishment of promoter activity. Thus, *Sp1* may play a critical role in transcription regulation of the *RASSF1C* promoter. As discussed above, *Sp1* is involved in promoter protection from epigenetical inactivation (Brandeis *et al.*, 1994; Matsuo *et al.*, 1998; Qu and Ehrlich, 1999; Gazzoli and Kolodner, 2003). Thus, DNA methylation free state of the *RASSF1C* promoter may be promoted by one from five *Sp1* sites in the promoter. From other side, the *RASSF1C* promoter is more transcriptional active compared to the *RASSF1A* promoter. Thus, active transcription of the *RASSF1C* may be involved in the protection of the promoter from its inactivation. Interestingly, that *RASSF1A* and *RASSF1C* are expressed in similar pattern in different human tissues. This suggests that both proteins are involved in the same pathways. This hypothesis may be supported by recent studies of the *RASSF1A* and *RASSF1C* functions. As was found, both proteins have the following common characteristics: an association with microtubules, induction of cell cycle arrest, binding of proapoptotic kinase *MST1*, repression of *Ras*-genomic instability and growth

inhibition of the cancer cells *in vivo* and *in vitro* (Dammann *et al.*, 2000; Khokhlatchev *et al.*, 2002; Ortiz-Vega *et al.*, 2002; Liu *et al.*, 2003; Li *et al.*, 2004; Rong *et al.*, 2004; Song *et al.*, 2004; Vos *et al.*, 2004). However, the *RASSF1A* promoter is frequently inactivated in cancer cells in contrast to *RASSF1C*.

4.6 The role of the *RASSF1A* transcription in HMECs

The *RASSF1A* promoter is methylated in 49% up to 65% of primary breast carcinomas (reviewed by Dammann *et al.*, 2003). Its methylation in serum of breast cancer patients is associated with poor prognosis (Muller *et al.*, 2003). Moreover, a new study of Lewis and colleagues showed that methylation of the *RASSF1A* promoter is associated with an increase of breast cancer risk (Lewis *et al.*, 2005). Thus, analysis of methylation of the *RASSF1A* promoter in HMECs may elucidate the mechanism of malignant reprogramming of the mammary epithelium. This is the first study, which analyzes the inactivation of *RASSF1A* in the proliferation of HMECs before and after overcoming stasis. Our results show that *RASSF1A* is epigenetically inactivated during senescence of HMECs. Transcriptional inactivation of *RASSF1A* during proliferation of HMECs is associated with spreading of DNA methylation into promoter and repression of chromatin. As discussed above, healthy breast tissues have the methylation pattern of the *RASSF1A* promoter similar as pre-stasis and post-stasis HMECs (Yan *et al.*, 2003). Thus, initiation of the epigenetical inactivation of *RASSF1A* already occurs in normal breast tissues and continues in dividing HMECs. Moreover, this demonstrates that epigenetical inactivation, which we observed in HMECs, takes place in tissues during aging. This is also supported by observation of Meeker and colleagues. They identified that cells from histological-normal terminal duct lobular units are characterized by a high level of the chromosome shortenings, which were detected in post-stasis HMECs (Meeker *et al.*, 2004). Thus, analogous to *de novo* DNA methylation of the *RASSF1A* promoter, shortenings of chromosomes take place in HMECs in culture and in healthy breast tissues. These data suggest that the processes, which are observed during proliferation of HMECs in culture, may be equal to processes in HMECs in tissue.

Bisulfite sequencing identified that the level of DNA methylation in the region containing the upstream *Sp1* site is increased in pre-stasis HMECs compared to normal mammary epithelium. Thus, cells proliferating in culture have a higher level of DNA

methylation compared to breast tissue. Increased level of DNA methylation may be associated with repression of the *RASSF1A* transcription activity. In concordance, in pre-stasis HMECs, the *RASSF1A* transcription level was decreased compared to HeLa, HF and PBMC. Repression of this transcription may be associated with selection, which may occur during cultivation of HMECs. This transcriptional inactivation may be necessary for further proliferation of HMECs in culture. From other side, normal mammary epithelium has a low level of the *RASSF1A* transcription. However, breast tissue contains various types of cells, which may have differences in transcription patterns. Thus, the *RASSF1A* transcription in normal breast tissue may be distinct from isolates of mammary epithelium. This is supported by the *RASSF1C* transcription, which is strongly decreased in normal mammary epithelium compared to proliferating HMECs. Thus, repression of the *RASSF1A* transcription may be associated with selection of HMECs in culture.

Additionally to the *de novo* methylation of the *RASSF1A* CpG island fragment, the post-stasis HMECs are characterized by DNA hypermethylation of the $p16^{INK4}$ promoter and absence of the $p16^{INK4}$ transcription (Brenner *et al.*, 1998; Foster *et al.*, 1998). In contrast to *RASSF1A*, $p16^{INK4}$ is induced in pre-stasis and highest transcription activity of $p16^{INK4}$ is observed in stasis proliferation phase Brenner *et al.*, 1998, Foster *et al.*, 1998). In stasis, HMECs with the elevated $p16^{INK}$ transcription went under G1 arrest; whereas the $p16^{INK}$ negative cells overcome this proliferation barrier. The *RASSF1A* inactivation is not associated with stasis of HMECs, since its epigenetical inactivation occurs during pre-stasis and post-stasis proliferation phases. Thus, epigenetical inactivation of the two tumor suppressor genes, *RASSF1A* and $p16^{INK}$ during senescence of HMECs may be associated with different processes in cells. Aberrant methylation was observed in the *RASSF1A* and $p16^{INK}$ promoters in normal breast tissues (Dammann *et al.*, 2001; Holst *et al.*, 2003; Yan *et al.*, 2003; Crawford *et al.*, 2004). However, pre-stasis cells are characterized by active $p16^{INK}$ and repressed *RASSF1A* transcriptions. This indicates that transcription of $p16^{INK}$ does not inhibit proliferation of HMECs and all cells with active and the not active $p16^{INK}$ promoter dividing in culture, whereas the repressed transcription of *RASSF1A* may be a necessary condition for proliferation of HMECs. This may be an additional proof of selection, which can take place during cultivation of HMECs. Moreover, this indicates a function of *RASSF1A* as tumor suppressor gene.

The inactivation of *RASSF1A* plays an important role in the breast cancer. This was demonstrated by a study of Lewis and colleagues, who observed DNA methylation of the *RASSF1A* promoter in 70% of healthy women with high risk of breast cancer (Lewis *et al.*, 2005). In concordance, epigenetical inactivation of the *RASSF1A* promoter is frequently detected in primary breast tumors (reviewed by Dammann *et al.*, 2003). Recent study of Shivakumar and colleagues demonstrated the possible function of *RASSF1A*. They identified that the accumulation of cyclin *D1* in cells is negatively regulated by *RASSF1A* (Shivakumar *et al.*, 2002). Cyclin *D1* plays a critical and uncompensated role in the breast tissue development (reviewed by Sherr, 1996). Overexpression of cyclin D1 in transgenic mice results in abnormal mammary cell proliferation including the development of mammary adenocarcinomas (Wang *et al.*, 1994). Moreover, cyclin *D1* gene is amplified in 15% and overexpressed in 30–50% of primary human breast tumors (reviewed by Fu *et al.*, 2004). Thus, inactivation of the *RASSF1A* may be necessary for hyperproliferation of mammary epithelial cells, which can be mediated by an increased level of cyclin *D1*. Moreover, the repression of *RASSF1A* transcription in HMEC and not in other nonmalignant cells can be explained by role of cyclin D1 in HMEC.

$p16^{INK4}$ negatively mediates cyclin *D1* dependent kinase activity (reviewed by Sherr, 1996). Analogous to $p16^{INK4}$, *RASSF1A* may negatively regulate cyclin *D1* dependent kinase activity, since control of this activity can be performed by different mechanism. The mechanism used by $p16^{INK4}$ is direct blocking of cyclin *D1* dependent kinase activity (reviewed by Sherr, 1996). Analogously, cyclin *D1* dependent kinase activity is decreased when level of cyclin *D1*, which is controlled by *RASSF1A*, is reduced. Thus, post-stasis HMECs are characterized by repression of two negative regulators of cyclin *D1* dependent kinase activity and this may be involved in the premalignant program of HMECs. Sandhu and colleagues identified reduction of cyclin *D1* dependent kinase activity during proliferation of post-stasis HMECs; and HMECs at agonescence are characterized by the lowest level of this activity (Sandhu *et al.*, 2000). This indicates that activation of other such as $p16^{INK4}$ and *RASSF1A* negative regulators of cyclin *D1* dependent kinase activity takes place in post-stasis HMECs.

In post-stasis proliferation phase, HMECs are characterized by chromosome abnormalities associated with critically shortened telomeres (Romanov *et al.*, 2001; Tlsty *et al.*, 2001). Vos and colleagues demonstrated that the *RASSF1A* inactivation results in genomic instability (Vos *et al.*, 2004). Thus, inactivation of the *RASSF1A*

promoter may promote the dividing of HMECs with chromosome abnormalities. Moreover, recent studies demonstrated that *RASSF1A* is associated with the control cell of cycle and apoptosis. Since chromosome abnormalities can mediate malignant transformation of the cells and the *RASSF1A* negatively regulates this process, silencing of *RASSF1A* may result in malignant transformation of HMECs.

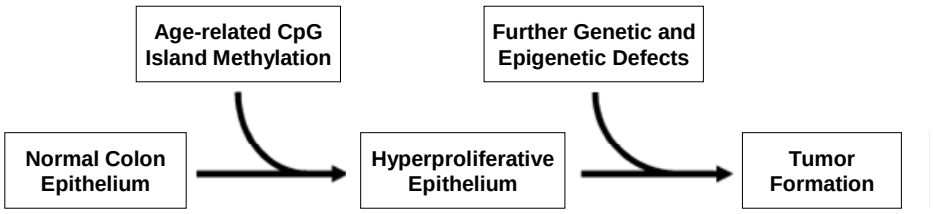

Figure 4-2. A model of the interactions between aging, CpG island methylation and cancer (Adopted from Issa, 1999).

In 1999, Issa suggested a model of neoplastic transformation of normal colon epithelium to tumor (Figure 4-2) (Issa, 1999). A similar mechanism may be involved in the malignant transformation of mammary epithelium (Figure 4-3). The epigenetical inactivation of *RASSF1A* during malignant transformation of mammary epithelium may have following steps. During aging, aberrant DNA methylation in the *RASSF1A* promoter accumulates in normal mammary epithelium and may induce the epigenetical inactivation of the *RASSF1A* promoter (Figure 4-3). This inactivation may mediate repression of the *RASSF1A* transcription and subsequent increase of cyclin *D1* level, which may allow a hyperproliferation of HMECs. Further, the repressed *RASSF1A* transcription permits a proliferation of cells with chromosome abnormalities, as was detected in post-stasis HMECs and histological-normal terminal duct lobular units. Accumulation of chromosome abnormalities may result in new mutations, which may support malignant transformation of HMECs. The epigenetical inactivation of *RASSF1A* started in normal mammary cells leads to completely inactivation of the *RASSF1A* transcription and to hypermethylation of the *RASSF1A* promoter in cancer breast cells (Figure 4-3). Thus, aberrant methylation of the *RASSF1A* promoter may mediate malignant transformation of HMECs.

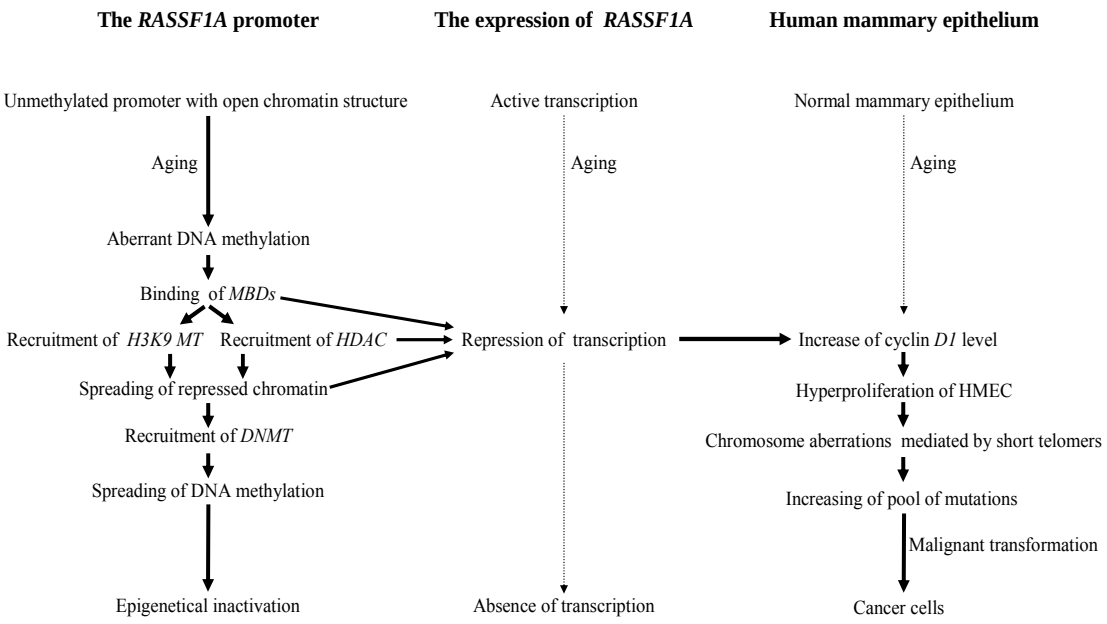

Figure 4-3. Epigenetical inactivation of the *RASSF1A* promoter during malignant transformation of human mammary epithelium. In normal mammary epithelium, the *RASSF1A* promoter may be characterized by active expression, unmethylated promoter area and open chromatin structure. During aging, the *SP1-DNMT* association at the upstream *Sp1* site may result in aberrant DNA methylation with following binding of *MBDs*. *MBDs* may repress the *RASSF1A* transcription through *Sp1* and recruit histone H3 lysine 9 methyltransferase (*H3K9 MT*) and *HDAC*. Both of these enzymes repress chromatin. This repression may result in additional inactivation of *RASSF1A* expression. Moreover, an increased level of *HDAC* may mediate deacetylation of *Sp1* in the *RASSF1A* promoter and following repression of transcription. The increase of histone H3 lysine 9 methyltransferase level may direct a *de novo* DNA methylation in the *RASSF1A* promoter. Repressed *RASSF1A* transcription may result in increase of level of cyclin *D1* and subsequent hyperproliferation of mammary epithelium. This proliferation and the extremely low expression level of *RASSF1A* may lead to occurrence of the cells with chromosome abnormalities. Accumulation of the mutations may mediate malignant transformation of mammary epithelium, which is characterized by epigenetically inactivated *RASSF1A* promoter. Arrows indicate directed processes. Dot arrows represent processes, which are directed by other mechanisms.

4.7 Outlook of project

To understand the mechanism of epigenetical inactivation of the *RASSF1A* promoter in HMECs, it is very important to analyze the chromatin state in the *RASSF1A* promoter by ChIp using antibodies to *MBD*, monomethylated and dimethylated histone H3 lysine 9 and other chromatin modifications during HMEC senescence. *In vivo* footprinting of the *RASSF1A* promoter fragments located from -429 up to -315 and from -205 up to -137 should be performed to identify transcriptional regulators in these regions. The *Sp1* interaction with putative partners and modifications of *Sp1* should be analyzed in the *RASSF1A* promoter. Analysis of nucleosome positions in the *RASSF1A* promoter during HMEC proliferation could help to reveal the mechanism of the transcriptional inactivation of *RASSF1A*. It would be very interesting to study the *RASSF1A* expression in HMECs grown on feed layers; this could help to understand the role of *RASSF1A* in HMECs. The inactivation of *Sp1* by siRNA in embryonal cells can elucidate the involvement of *Sp1* in the epigenetical protection of *RASSF1A* from *de novo* DNA methylation. Taken together, analysis of *RASSF1A* promoter in HMECs and in embryonal cells could help to understand mechanism of epigenetical control of genes.