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EPIGENETIC CHANGES PROMOTING HELA CELL APOPTOSIS ARE LINKED TO VALPROIC ACID-INDUCED DOWN-REGULATION OF REST AND ITS COREPRESSOR CoREST

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ABSTRACT

REST (RE-1 silencing transcription factor, or NRSF –neuron-restrictive silencing factor) binds to a conserved RE-1 motif present in the promoter region of regulated genes and represses their transcription in neuronal and non-neuronal cells (Bruce et al., 2004). REST recruits corepressors (CoREST, mSin3a) and multiple chromatin modifying enzymes (HDAC1/2, demethylase LSD1 and methyltransferase G9a), causing chromatin compaction and altering gene expression by changing epigenetic tagets (Ballas et al., 2005). REST contributes to orchestrate the epigenetic regulation of target genes through several miRNAs including miR-9/9*, miR-29a, miR-124a, miR-218 and others (Wu and Xie, 2006).

My thesis has ascertained an anti-tumor properties of transcription factor REST on a model of cervical adenocarcinoma where class I histone deacetylase (HDAC) inhibitor: valproic acid (VPA) down-regulates REST and its corepressors CoREST and HDAC1 at mRNA and protein level. These effects are related to a potent effect on cell apoptosis, possibly mediated by miR-9 overexpression as consequence of REST and CoREST down-regulation. I report the presence of a double-negative feedback loop between REST and miR-9 in HeLa cell line: in absence of REST, miR-9 levels substantially increase while miR-9 overexpression promotes REST down-regulation. Interestingly, I have observed that REST is sufficient to induce a noteworthy chromatin remodeling in HeLa cells.

HeLa cell apoptosis induced by these events, involves mitochondrial control of apoptosis signaling pathways, particularly Bcl-2 family gene BAX.

In conclusion, the present study aims to contribute to a more accurate comprehension of the processes responsible for REST activity in a model of epithelial cervical adenocarcinoma, and relevant for a detailed knowledge of important events causing oncogenesis. Moreover, considering the crucial role of epigenetic regulation of gene transcription in the etiology of many pathological conditions, any further knowledge in this field could find important and innovative pharmacological applications.

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1. The transcription factor REST

A complex multicellular eukaryotic organism contains the same genomic DNA in each cell; however, it is composed of many different types of tissues. The particular combination of genes that are expressed or repressed determines cellular morphology and function. For example, only the lymphocytes of the human immune system make antibodies, while developing red blood cells are the only cells that make the oxygen-transport protein hemoglobin. Thus, regulation of gene expression is essential process in multicellular organisms that drives cellular differentiation and morphogenesis, forming different cell types that possess certain gene expression profiles, and hence produce different proteins according to their functions.

Eukaryotes often have a promoter region upstream from the gene, or enhancer regions up or downstream from the gene, with certain specific motifs that are recognized by the various types of transcription factors (TFs). TFs are molecules involved in regulation of gene expression. They are usually proteins, although they can also consist of short, non-coding RNAs. TFs are also usually found working in groups or complexes, forming multiple interactions that allow for varying degrees of transcription control. In addition, the packaging of DNA into chromatin and its modification by methylation impart further levels of complexity to the control of eukaryotic gene expression.

Deregulation of a single transcription factor can be sufficient to alter cell morphology and eventually lead to many forms of human cancer (Lee and Young, 2013). In tumor cells, genes encoding TFs could be deleted, amplified, translocated, or subjected to point mutations that result in a gain- or loss-of-function (Darnell, 2002).

REST (RE-1-silencing transcription factor, also known as neuron-restrictive silencing factor NRSF) is a transcriptional repressor with a role in regulation of gene expression throughout the body. REST was independently identified in 1995 by two research groups as a protein that binds to repressor element 1 (RE1, also known as NRSE – neuron restrictive silencer element) sequences that are present in the rat Scn2a2 and Stmn2 genes (Chong et al., 1995;

Schoenherr et al., 1996). Because RE1 sites had been identified in several neuron-specific genes (Kraner et al., 1992), REST was initially suggested to function as a master regulator of the neuronal phenotype that blocks transcription of its target genes by binding to a specific consensus sequence present in the target genes' regulatory region (Schoenherr et al., 1996). Since REST discovery as a negative regulator of neuronal differentiation, this transcription factor has been implicated in several processes such as maintenance of embryonic stem cell pluripotency, self-renewal and regulation of mitosis in non-neuronal cells.

By fluorescence in situ hybridization analysis of the REST gene (3365 base pairs) was allocated to the chromosomic region 4q12 [GeneBank NM_005612] (Cowan et al., 1996). The gene encodes for a protein of 1097 aminoacids: it is a 116-kDa Krüppel family zinc finger protein that contains a DNA-binding domain, composed of eight zinc-fingers, and have the ability to bind both RNA and DNA. The DNA-binding domain of REST binds to a 21-bp consensus DNA sequence, the RE-1 binding site/neuron-restrictive silencer element (RE-1/NRSE), present in the target gene's regulatory regions. Interestingly, REST has an N-terminal repression domain and a C-terminal repression domain, which contains a single zinc finger motif (Ballas et al., 2005) (Figure 1.1). REST protein is expressed at higher levels in the nuclei of pluripotent embryonic stem (ES) cells; since ES cells differentiate to neural progenitors REST mRNA levels stay relatively constant but REST protein is downregulated post-translationally to minimal levels. The release of REST from neuronal gene chromatin as progenitor cells differentiate into neurons is concomitant with its own transcriptional repression. By recruitment of several cofactors, REST promotes dynamic modifications of DNA, histones and nucleosomes to maintain genomic stability.

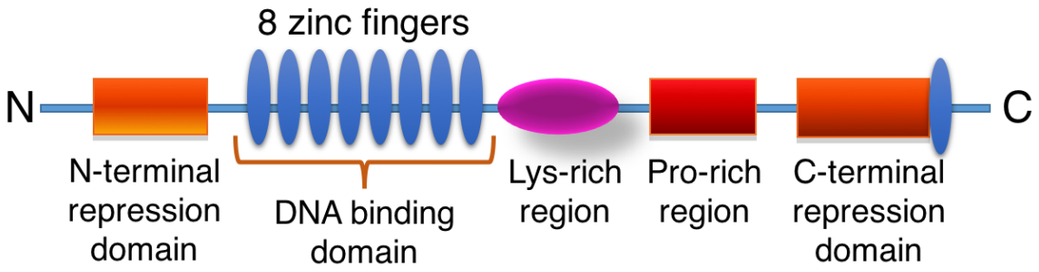

Figure 1.1 Schematic representation of REST protein domains.

REST is necessary for the correct development of vertebrates, since perturbations of its expression or function in the developing embryo results in the ectopic expression of neuronal genes in non-neuronal tissues and early embryonic lethality: mice homozygous null for REST showed brain malformation at embryonic day E9.5, followed by extensive apoptosis and lethality by E11.5. These results suggested a highly important role for REST in brain development (Chen et al., 1998).

The molecular weight calculated on the basis of REST sequence is around 116 kDa, however, this transcription factor appears to have several molecular weights detected by western blot technique. This difference could be explained by post-translational modifications of the proteins and existence of REST splice variants. It was found that splice variants of REST encode proteins with four or five zinc finger motifs (Palm et al., 1998). Two variants, termed REST4 and REST5, were detected only in neuronal tissues and are conserved in human, mouse and rat (Palm et al., 1998). These transcripts are generated by alternative splicing of a neuron-specific exon (exon N) located between exons V and VI. REST4 in particular have an insertion of 16 nucleotides followed by an in-frame stop codon, so it retains the N-terminal repression domain and five of the eight zinc fingers (Figure 1.2).

REST protein was found to migrate on SDS-PAGE as a protein of ~200 kDa, while the predicted molecular weight, based on amino acid sequence, is 116 kDa; the isoforms REST4

and REST5 migrated on SDS-PAGE as 53 kDa and 50 kDa proteins, respectively, while the predicted molecular weights are 37 kDa and 33 kDa, consistent with glycosylation of the protein backbone. REST4, and presumably REST, was found to be O-glycosylated between residues 87 and 152: however full length REST could contain additional glycosylation sites (Lee et al., 2000).

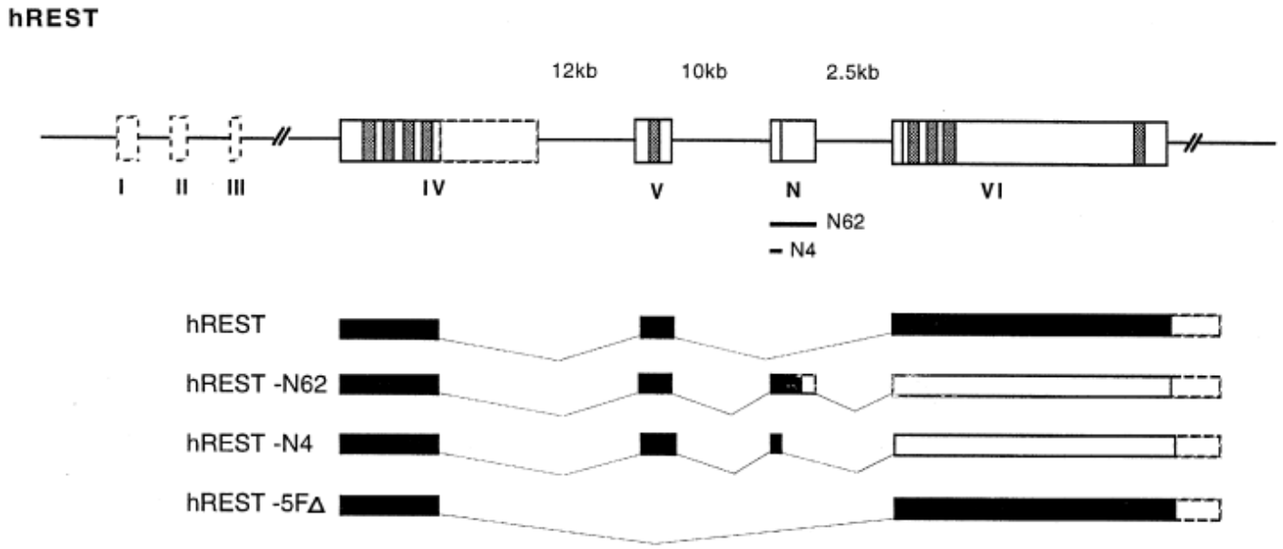

Figure 1.2 Structure of the human REST gene and alternative transcripts. Exons are shown as boxes, and introns as lines. The numbers above the introns indicate their respective sizes. Exon numbers in bold Roman characters from I to VI are shown below the respective exons. The neuron-specific exon located between exons V and VI is indicated as N. Undefined 5' exons I, II and III, and the putative extension of exon IV which have been shown to be present in rat REST gene, are indicated with the dashed stroke. The vertical grey bars denote zinc finger motifs. The schematic representation of human REST transcripts in relation to the human REST gene, is shown below the corresponding gene structure. The ORF of each transcript is shown as a filled box, and 3'-UTRs as open boxes with dashed stroke. Abbreviations: hREST – human REST transcript with the longest ORF encoding the protein isoform with nine zinc fingers; hREST-N62 – human REST transcript with the inclusion of exon N62; hREST-N4 – human REST transcript with the inclusion exon N4; hREST-5FΔ – human REST transcript with skipped exon V that encodes protein isoform with eight zinc finger motifs.

1.1 REST Binding site

REST binds to a 21 base-pair sequence termed RE-1 (Repressor Element-1)/NRSE (Neuron Restrictive Silencer Element) and found in the regulatory regions of its target genes. Canonical RE-1 element motifs (Figure 1.3) that were defined by bioinformatic approaches or genome-wide binding analysis facilitate strong REST binding and control functional classes of its target genes. Although it was shown that the canonical REST-binding motif of 21 bp could be naturally subdivided into two nonidentical, nonpalindromic half sites separated by a "spacer" sequence that increases the center-to-center distance from the canonical 11 bp to 16 to 19 bp, or decreases it by 1 bp to 10 bp (Johnson et al., 2007). Although REST could bind to half-sites of the RE-1 element, neither the left nor the right half-site by itself is an effective repressor, indicating that half-site binding is not as strong and functional as complete binding to canonical RE-1 element (Jothi et al., 2008). 1892 putative RE-1s in the human genome have been identified.

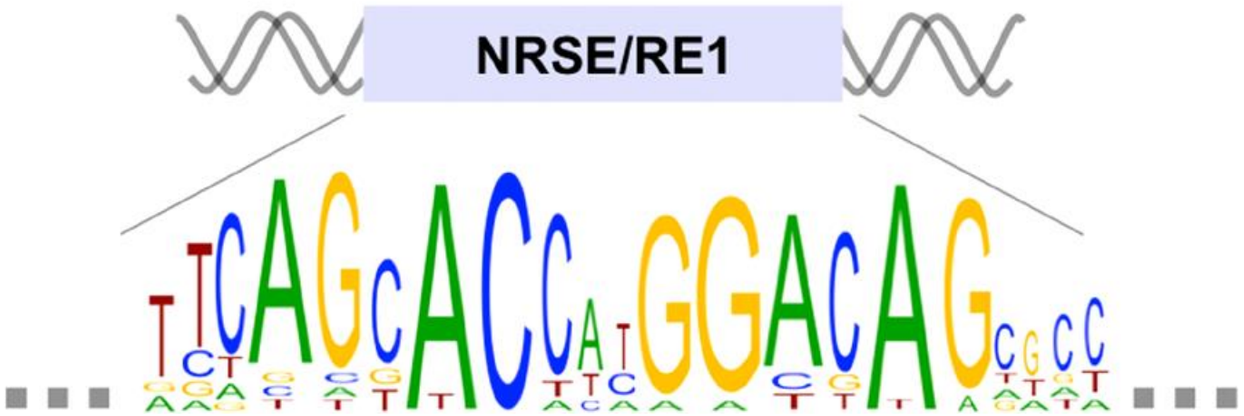

Figure 1.3 Canonical REST binding motif. The relative height of each letter in the RE1 sequence reveals its degree of evolutionary conservation in different RE1 sequences, reflecting its relative importance to REST binding.

A 21-bp Repressor Element-1 has been found in the regulatory regions of many neuron-specific genes. These genes include those encoding ion channels (e.g., NaV1.3, Kv3.4, and

Cavl.3), neurotransmitter synthesizing enzymes and receptors (e.g., M4 muscarinic, D3 dopamine, and g-aminobutyric acid type b3 receptors), transporters (e.g., g-aminobutyric acid transporter 4), neurotrophic receptors and proteins involved in vesicular trafficking (e.g., synaptosomal associated protein SNAP25; synaptotagmins IV, V and VII; syntaxin 8; Rab3), neuronal cytoskeleton (tubulin b3) and axonal guidance (e.g., SCG10, stathmin 3, netrin-2, roundabout, semaphorin 5A, L1 cell-adhesion molecule L1CAM). Among REST targets many genes not related to neuron-specific functions were identified, such as genes involved in cellular metabolic processes (peroxisome and proteasome components) or in immune and inflammatory responses. Additionally, there are genes which perform neuronal functions but are also required in non-neuronal tissues, including those involved in the regulation of cardiovascular tone (endothelial nitric oxide synthase, vasoactive intestinal peptide, atrial natriuretic peptide, brain natriuretic peptide, and KCNH2) (Bruce et al., 2004) (Figure 1.4).

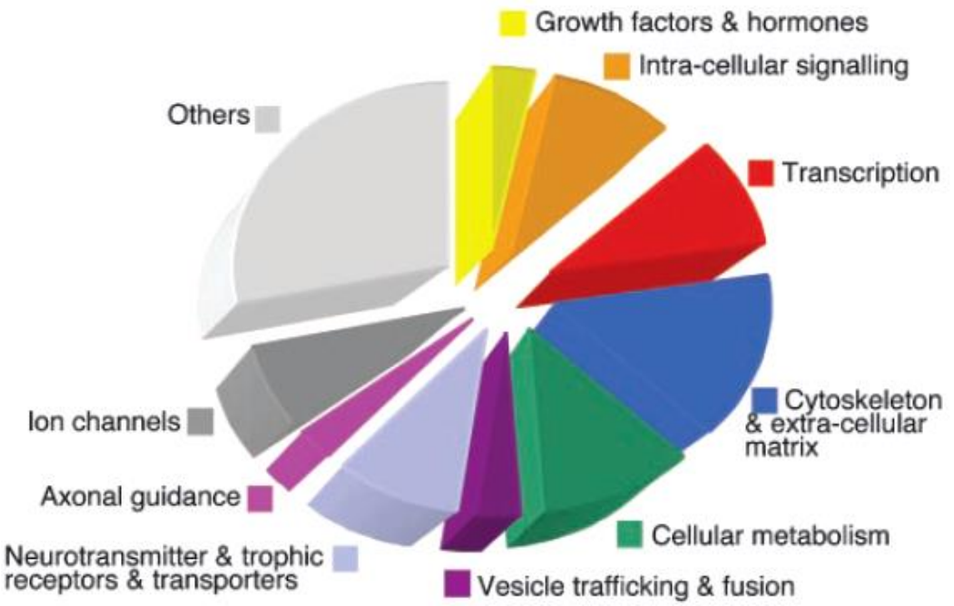

Figure 1.4 Assignment of putative REST target genes.

1.2 REST repression complex

REST-mediated gene repression is achieved by the recruitment of two separate corepressor complexes, mSin3 and CoREST. The N-terminal repression domain of REST interacts with mSin3, that recruits two class I histone deacetylases (HDAC1 and HDAC2) and the retinoblastoma-associated proteins RbAp48 and RbAp46, that are thought to interact with histones (Grimes et al., 2000; Naruse et al., 1999; Roopra et al., 2000). The mSin3–HDAC complex, however, is associated primarily with a dynamic mode of repression that can alternate between repression and activation and therefore by itself is not sufficient for long-term silencing of neuronal genes. In turn, the C-terminal repression domain of REST interacts with the corepressor termed CoREST (Ballas et al., 2005) which forms a complex containing HDAC1 and HDAC2, the histone H3 lysine 4 (H3-K4) demethylase LSD1, the chromatin-remodeling enzyme BRG1, the histone H3– K9 methyltransferase G9a and the methyl-CpG-binding protein MeCP2 (Figure 1.5).

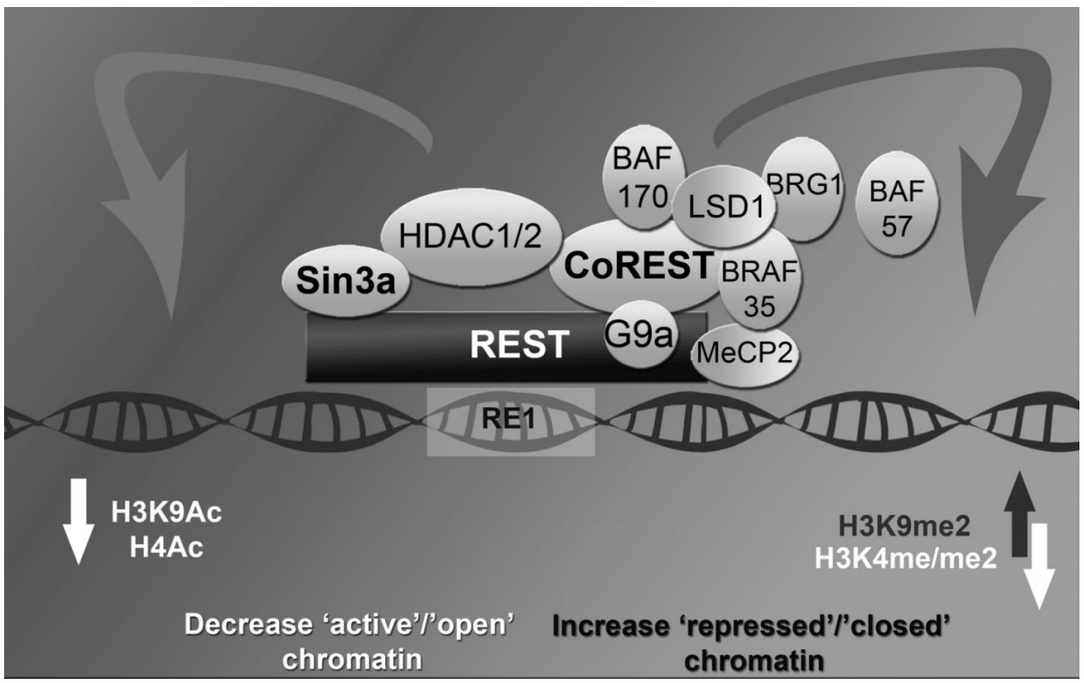

Figure 1.5 Schematic representation of target genes silencing mediated by REST repression complex.

Outside the nervous system, REST recruits chromatin modifiers necessary for a long-term silencing of selective neuronal genes. BRG1 is recruited by the C-terminal repression domain of REST, as part of the CoREST complex: it stabilizes REST binding on the RE-1 and allows REST to gain better access to its chromatin targets (Ooi and Wood, 2007). Once associated with the chromatin, REST mediates repression through histone deacetylase, histone demethylase and histone methylase activities. HDACs deacetylate H3-K9 and H3-K14, a process that is required for subsequent demethylation of H3-K4 by LSD1 and demethylation of H3-K9 by G9a: the addition of methyl groups to H3-K9 prevents the re-acetylation and is necessary for the subsequent recruitment of the Heterochromatin protein 1(HP1), which mediates chromatin condensation. The overall effect of these modifications results in the silencing of several nuclear proteins that are associated with transcriptional activation, followed by the replacement of chromatin compaction that is associated with transcriptional repression.

In general, an increase of histone acetylation by HATs (Histone Acetyl Transferases) induces the remodelling of chromatin from a tightly to a loosely packed configuration, which facilitates transcriptional activation; conversely, a decrease of histone acetylation by HDACs (Histone DeACetylases) yields a condensed chromatin structure and thus transcriptional silencing. Histone H3 lysine 4 (H3-K4) methylation is a well-known marker of transcriptionally active chromatin, while methylated H3-K9 and H3-K27 mark transcriptionally inactive chromatin (Juliandi et al., 2010).

1.3 REST and ncRNAs

Non-coding (nc)RNAs have generated tremendous interest over the past 10 years and have been shown to encompass regulatory activities to control the genome expression and stability. The term non-coding RNA (ncRNA) is commonly employed for RNA that does not encode a protein, but function directly as structural, catalytic, or regulatory molecules. Examples include the small nucleolar RNAs (snoRNAs), which guide site- specific RNA modifications,

the micro-RNAs (miRNAs), which post-transcriptionally repress gene expression, and the rasiRNAs (repeat associated small interfering RNAs), which direct heterochromatin formation at centromeres.

MicroRNAs (miRNAs) are small non-coding single-stranded RNA molecules (containing about 21 nucleotides) that function in mRNA silencing and post-transcriptional regulation of gene expression (Ambros, 2004; Bartel, 2004). The first miRNA was discovered in 1993 during studies of the lin-4 gene known to be essential for the timing of C. elegans larval development by repressing the lin-14 gene. It was found that instead of producing mRNA that encodes a protein, lin-4 produced short noncoding RNAs (Lee et al., 1993). One of these noncoding RNAs was a ~22-nucleotide RNA that contained sequences partially complementary to multiple sequences in the 3' UTR of the lin-14 mRNA. This complementarity was proposed to inhibit the translation of the lin-14 mRNA into the LIN-14 protein. Since then, many other miRNAs were discovered in the human genome which may encode over 1000 miRNAs (Bentwich et al., 2005). Micro RNAs are abundant in many mammalian cell types and appear to target about 60% of the genes of humans and other mammals (Friedman et al., 2009).

MicroRNAs are produced from either their own genes or from intron sequences of protein coding genes. The majority of the characterized miRNA genes are intergenic or oriented in an antisense pattern to neighboring genes. However, some microRNA genes are transcribed together with their host genes (Rodriguez et al., 2004). MicroRNAs are usually transcribed by RNA polymerase II (Pol II) producing a several hundred nucleotide-long miRNA precursors termed primary miRNAs (pri-miRNAs); then, these transcripts are processed by the Drosha/DGCR8 complex resulting in the formation of ~70 nucleotide pre-miRNAs. The Ran-GTP-dependent-factor Exportin 5 then transports the pre-miRNAs from the nucleus into the cytoplasm, where Dicer processes them into ~21 nucleotide duplexes that contain the mature miRNA product (Kim and Kim, 2007; Lee et al., 2003). One strand of the miRNA duplex incorporates with the RNA-induced silencing complex (RISC), which directs the miRNA to target mRNAs (Figure 1.6) (Winter et al., 2009).

miRNAs function as negative regulators of gene expression by base-pairing with the 3'-

untranslated region (3'-UTR) of target mRNAs in the form of ribonucleoprotein complexes, known as RNA-induced silencing complexes (RISCs). Because the interaction between a miRNA and its target requires only a partial complementarity, one miRNA could regulate hundreds of mRNAs (Cao et al., 2006). MiRNAs expression contributes to regulate, in a temporal and/or tissue-specific manner, expression of many mammalian genes. Genome-wide analysis of RE-1 sites has shown that some REST-binding sites are adjacent to non-coding RNA genes that encode miRNAs.

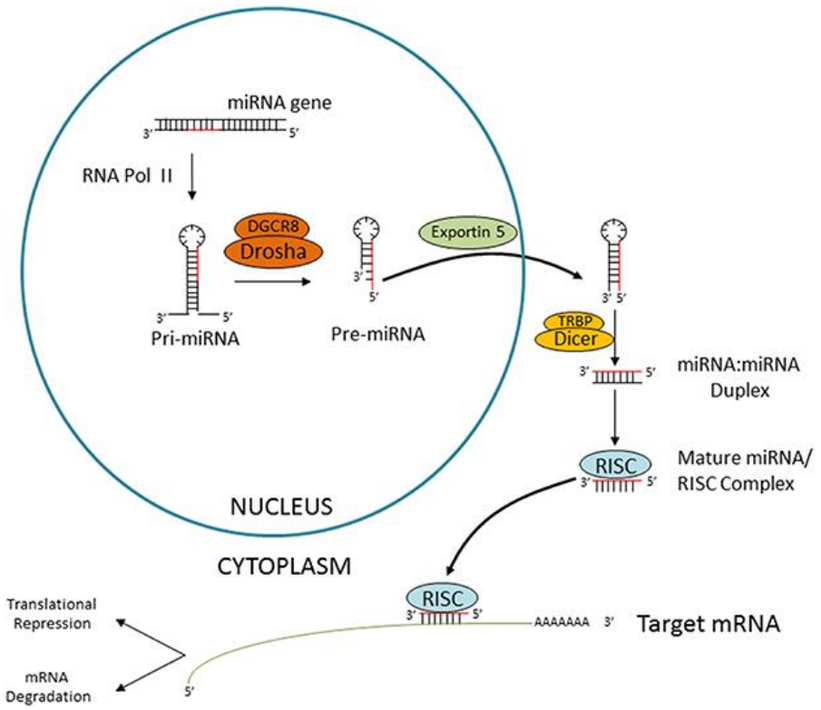

Figure 1.6 miRNA processing and mechanism of action. Partial sequence complementarity between the miRNA and the target results in translational repression whereas complete sequence complementarity results in the degradation of the target mRNA.

One of the first miRNAs identified as a direct target of REST by binding studies and expression analysis was miR-124, the most abundant miRNA in the adult mammalian brain,

accounting for 25%–48% of all brain miRNAs; more than 1100 genes were predicted to be putative targets of miR-124. During neurogenesis miR-124 expression is undetectable or expressed at low levels in progenitor cells and is up-regulated in differentiating and mature neurons, and blocking miR-124 activity in mature neurons leads to increased levels on non-neuronal transcripts (Lim et al., 2005). Thus, the establishment and maintenance of neuronal identity requires both derepression of REST-regulated neuronal genes as well as post-transcriptional down-regulation of non-neuronal transcripts by proneural miRNAs (Conaco et al., 2006). Moreover, miR-124 may act in turn by silencing REST, allowing the transition form a progenitor state to a differentiated neuronal phenotype (Figure 1.7).

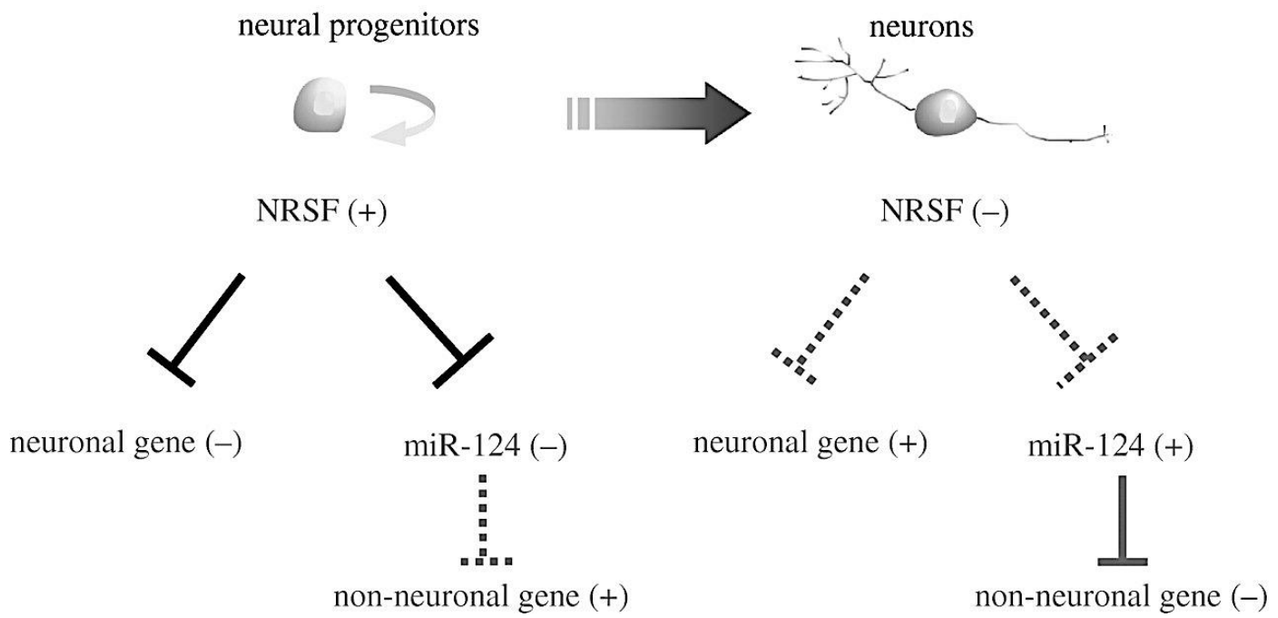

Figure 1.7 Reciprocal function of REST/NRSF and miR-124 in modulating neuronal identity. In neural progenitors, REST/NRSF is expressed and inhibits miR-124a expression, allowing the persistence of non-neuronal transcripts. When the progenitors differentiate into neurons, REST/NRSF is down-regulated and its dissociation from the miR-124a gene loci induces derepression of the gene coding for MiR-124, resulting in the selective degradation of non-neuronal gene transcripts.

Since these initial reports, the list of putative miRNAs targeting REST has grown: computational analyses, genome-wide binding studies and ChIP analyses have identified several neuron-specific miRNAs, including miR9, miR-29a/miR-29b, miR-95, miR-132, miR-133, miR-135b, miR-139, miR-153, miR-218- 212, miR-346, miR-375 and miR-455, that display the potential to be regulated by REST. And in turn, some miRNAs were found to target 3'UTRs and negatively regulate REST and other the members of the REST repression complex, e.g. MeCP2 and CoREST (Figure 1.8) (Wu and Xie, 2006).

miR-9 has been proposed as one of the crucial regulators of neuronal development as well as of other physiological or pathological events in several organisms. As regards mammals, REST is induced upon embryonic stem cell neuronal differentiation and blocking it during this process decreases neuronal differentiation at the expense of astrocytes. In addition, it has onco-suppressor properties, with a crucial role in repressing cell proliferation of human neuroblastoma and medulloblastoma cells. Moreover, recently miR-9 was shown to be potential marker for cervical cancer prognosis (Cheung et al., 2012). In proliferating cells, where it is highly expressed, REST binds to the miR-9 promoter preventing its transcriptional activity; upon neuronal differentiation, REST is down-regulated and the concomitant phosphorylation of the neuronal activator CREB, already bound to miR-9 promoter activates gene transcription. It has been reported that miR-9 targets 3'UTR of REST and significantly reduces endogenous REST whereas a reduction of REST enhances miR-9 expression (Laneve et al., 2010).

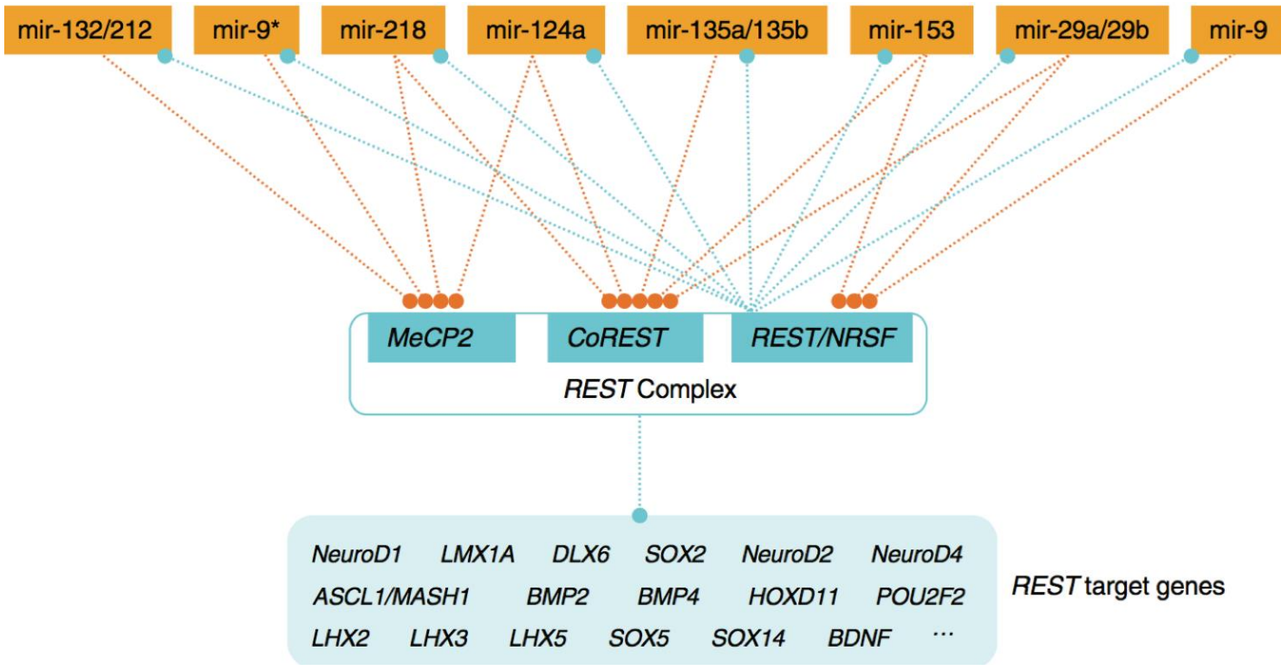

Figure 1.8 Schematic diagram of the interactions among REST repression complex and miRNAs. REST complex shown in blue, miRNAs shown in orange, a list of REST target genes is shown in light blue. Negative interactions are denoted with dotted lines with filled circles (Wu and Xie, 2006, with modifications).

Perturbations of REST activity and related deregulation of ncRNAs have been implicated in the molecular pathophysiology of diverse disorders that range from cancer to neurodegenerative and neurodevelopmental diseases (Qureshi and Mehler, 2009; Rossbach, 2011).

1.4 Regulation of REST transcription

In contrast to regulation of REST target genes, relatively little is known about regulation of REST expression. Analysis of the human, mouse and rat gene sequences has shown 68% homology between them and a conserved exon–intron structure. REST gene transcription starts with one of three alternatively used 5' exons (1a, 1b and 1c) with the most abundant transcription start site at the exon 1a (Figure 1.9) (Koenigsberger et al., 2000).

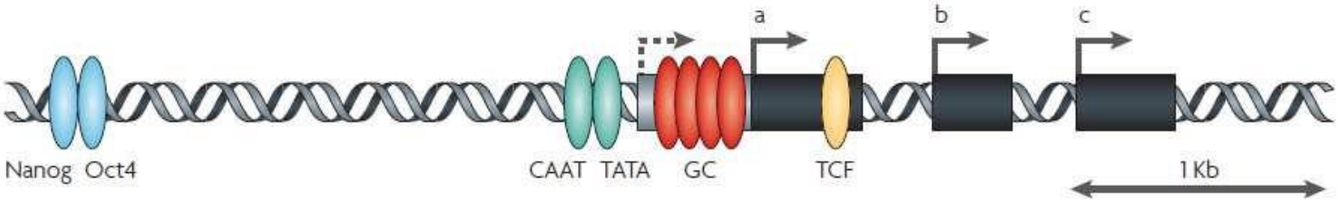

Figure 1.9 Schematic representation of REST promoter region.

The presence of three alternatively available 5' exons suggests that REST contains at least three different promoters: promoter A has been shown to be the most active in non-neuronal cells, while promoter B is active in neuronal cells. Promoter C, on the contrary, did not show any activity in neuronal cells and only a weak activity in non-neuronal and neural progenitors. In the mouse, the exon 1a promoter was found to contain TATA and CAAT boxes. For the human REST gene regulation, the same functionality could not be suggested due to low interspecies conservation (Kojima et al., 2001).

However, both human and mouse REST promoters contain GC boxes that recruit the Sp1 family of transcription factors; other transcription factors involved in the regulation of REST expression are PBX, AP-1, CREB and Sox (Koenigsberger et al., 2000). REST was also identified as a target for the transcription factors Nanog and Oct4 in mouse and human embryonic stem cells and both Nanog and Oct4 have been shown to be bound to a region about 2 kb from exon 1a.

Chromatin immunoprecipitation analysis of the REST promoter revealed also the presence of an HDAC complex that contains mSin3, CoREST and MeCP2. Intriguingly the retinoic acid Receptor (RAR) and its corepressor, N-CoR, were part of this repressor complex, bound to a region containing a Retinoic Acid Response Element (RARE), located 400 bp upstream of REST transcriptional start site (Ballas et al., 2005). During terminal differentiation an unliganded RAR repressor complex is recruited to a RARE site located upstream from the transcriptional start site, causing the repression of REST transcription and exit from the cell cycle and the onset of terminal differentiation.

1.5 REST protein degradation

REST protein levels appear to be regulated by transcriptional and post-transcriptional mechanisms depending on the cell type: during neuronal differentiation, post-translational degradation of REST protein occurs, which leads to both REST release from RE-1sites and transcriptional inactivation of the REST gene itself.

It has been revealed that REST protein levels are regulated through the proteasomal degradation mediated by F-box protein β-TRCP (SCFβ-TRCP), an E3 ubiquitin ligase. During neural differentiation β-TRCP binds and ubiquitinates REST through its conserved phospho-degron with phosphorylated serine residues. Interestingly, REST has two adjacent similar motifs at its C-terminus and degron-mutant REST was shown to be substantially more stable than wild-type REST, thus attenuating neural differentiation (Westbrook et al., 2008). In non-neuronal cells, REST β-TRCP-dependent regulation contributes to genetic instability at any disruption of the cell-cycle: REST degradation in the G2 phase of the cell cycle might be necessary to de-repress genes involved in mitosis. Particularly, SCFβ-TRCP-dependent degradation of REST during G2, allows transcriptional de-repression of Mad2, an essential component of the spindle assembly checkpoint (Guardavaccaro et al., 2008). Mutant REST is unable to bind β-TRCP, and inhibits Mad2 expression causing specific defects of mitosis such as premature sister-chromatid separation, chromosome bridges and mis-segregation in anaphase.

Furthermore, it had been reported that β-TRCP over-expression is a common event in human epithelial cancers and causes oncogenic transformation of human mammary epithelial cells, and that this function of β-TRCP requires REST degradation (Kudo et al., 2004; Saitoh and Katoh, 2001). Thus, REST proteolysis must be accurately controlled to avoid subjecting neuronal tissues to cancer formation, as REST is a key target in β-TRCP-driven transformation.

Reciprocal mechanisms that stabilize REST are discovered too. The deubiquitylase HAUSP (Herpesvirus-Associated-Ubiquitin-Specific Protease 7) antagonizes β-TrCP-mediated ubiquitylation of REST and prevents NSCs differentiation: the balance between ubiquitylation and deubiquitylation may determine the net REST protein levels, defining the maintenance of stemness or initiation of neuronal differentiation (Figure 1.10). When the HAUSP-mediated deubiquitylation overrides β-TRCP-mediated ubiquitylation, REST is stabilized to suppress differentiation and promote NSCs maintenance. In contrast, when β-TRCP-mediated ubiquitylation exceeds HAUSP-mediated deubiquitylation, REST is targeted for degradation, which promotes cell differentiation by releasing repression of differentiation- associated genes (Huang et al., 2011).

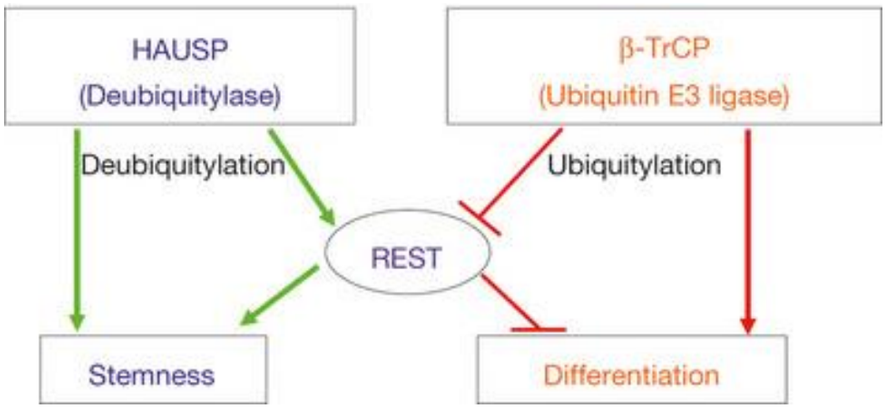

Figure 1.10 Control model of REST protein at the post-translational level. Both HAUSP-mediated deubiquitylation and β-TRCP-mediated ubiquitylation regulate REST protein levels in NPCs. HAUSP deubiquitylase stabilizes REST protein to promote NPC maintenance. In contrast, the β-TRCP E3 ubiquitin ligase drives REST proteasomal degradation and neural differentiation.

1.6 Involvement of REST in cancer

REST was reported to be a regulator of several protein-coding and miRNA genes that may affect important biological processes. Increased levels of REST were found in rat hippocampal and cortical neurons in response to epileptic seizures and ischaemia: these insults de-repress REST mRNA and protein in dying neurons, and this was suggested to be a critical mechanism

of insult-induced neuronal-death (Calderone et al., 2003). REST was also found to repress the m-opioid receptor in neuronal cells, and thus may have a neuroprotective role in opium addiction (Bedini et al., 2008). Similarly, REST was found to repress the serotonin 1A receptor, which is implicated in depression and anxiety (Lemonde et al., 2004). In patients with Huntington's disease, REST could not be recruited by mutated huntingtin and translocated from the cytoplasm to the nucleus, which causes blockade of neuronal gene expression (Zuccato et al., 2003). Other neurological diseases that REST is associated with, include Schizophrenia (Warburton et al., 2014), Alzheimer's disease (Lu et al., 2014b), Parkinson's disease (Yu et al., 2013), Down's Syndrome (Bahn et al., 2002) and others.

REST seems to act as an oncogene or a tumor suppressor depending on the cell type and tumor progression. Thus, it contributes to promote the development of brain tumors such as neuroblastomas, medulloblastomas and glioblastomas (Conti et al., 2012; Liang et al., 2014; Taylor et al., 2012).

Medulloblastoma is one of the most malignant brain tumors in children and is believed to arise from undifferentiated neuroectodermal stem cells in the cerebellum: these cells have a primitive embryonal phenotype and show the capacity for divergent differentiation. It was reported that over 50% of human medulloblastomas express REST at high levels and fail to differentiate (Fuller et al., 2005). Recently it has been shown that high REST in medulloblastomas represses USP37, a protein deubiquitinase, thus accelerating turnover of p27 (kip1), a cyclin- dependent kinase inhibitor that normally stops cell proliferation (Figure 1.11-1).

In glioblastoma multiforme, the most common malignant brain tumor in adults, over 30% cases depend on high REST (Kamal et al., 2012). It has been shown that in these tumors REST represses its target miR-124a expression, which in turn causes overexpression of miR-124a targets such as SNAI2, Scp1 and PTPN12 (Figure 1.11-2) (Fowler et al., 2011). SNAI2 is a transcription factor known to be responsible for metastases formation and cell invasion; Scp1 and PTPN12 are small phosphatases (Conti et al., 2012).

Pheochromocytomas are differentiated tumors derived from the adrenal medullary tissue with around 10% malignancy rate. In a recent study it has been reported that spontaneous high-REST PC12 clones (a cell line derived from rat pheochromocytoma) are characterized by low levels of tuberous sclerosis complex 2 (TSC2) and by increased levels of nuclear b-catenin (Figure 1.11-3). TSC2 controls cell growth and size and is known for its tumor suppressor function; low TSC2 decreases turnover and increases transfer to the nucleus of b-catenin, which governs the expression of oncogenes such as cMyc and cyclin D1 (Tomasoni et al., 2011).

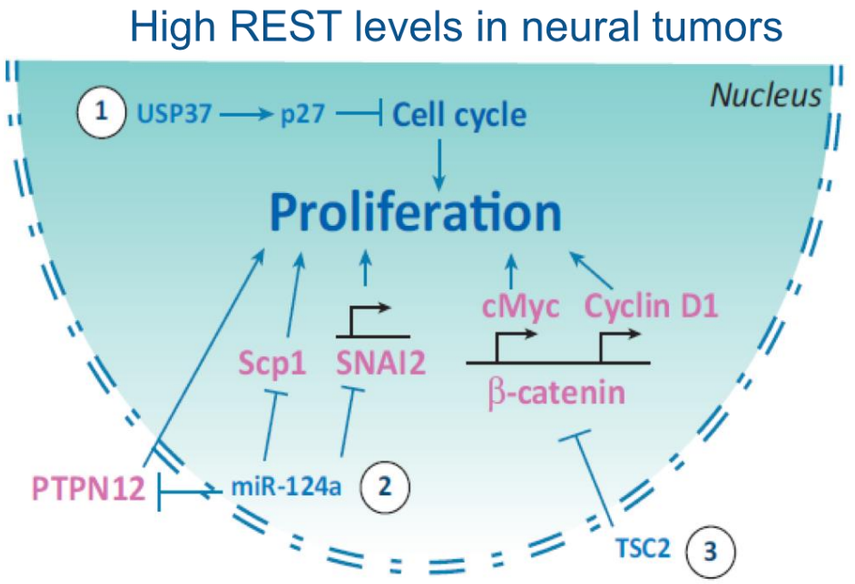

Figure 1.11 Mechanisms of stimulated proliferation in three types of high-REST neural tumors: 1 – in medulloblastomas, 2 – in glioblastomas, 3 – in pheochromocytoma cells.

On the other hand, a variety of tumors, including those arising in breast, ovary, lung and colon, activates expression of neuron-specific genes: the expression of neuronal genes out of their normal context causes the permanence of malignant cells in the cell cycle. Abnormal expression of neuronal genes results from the loss of REST activity, which normally represses their expression and leads to oncogenic conversion of these cells, perhaps through the

aberrant expression of REST target genes, resulting in an abnormal neuroendocrine phenotype. Moreover, inactivating mutations of the REST gene were identified in colorectal cancer: deletions of human chromosome 4q12, on which REST is located, are frequent events in this type of cancer. A frame shift mutation in the REST coding region, resulting in a premature termination codon in the center of the coding sequence and yielding a truncated protein that lacks the C-terminal repression domain, was also identified in primary colon adenocarcinoma: this dominantly acting truncation is capable of transforming epithelial cells through an increase of PI(3)K-dependent signaling, in both intensity and duration (Westbrook et al., 2005). Finally, a reduced REST function caused by an increased expression of a splice variant that lacks some of the DNA binding domain and the C-terminal repression domain has been associated with small-cell lung carcinomas (SCLCs): this event leads to the expression of neuronal markers, like L1-cell adhesion molecule (L1-CAM) and neural cell adhesion molecule (NCAM), enhanced cell proliferation and anchorage independent cell growth (Kreisler et al., 2010). In some instances, the inappropriate expression of neural genes elicits an autoimmune response that culminates in neurological disorders, collectively known as Paraneoplastic Neurologic Degenerations (PNDs).

In summary, depending on the cell type, REST may possess tumorigenic or tumor suppressor effects. This complex role would indicate that REST is a major biological regulator of normal and abnormal development: in epithelial cells REST functions as a tumor suppressor whereas in differentiating or differentiated neuronal cells REST repressor activity has an oncogenic function. Thus, it is important to investigate roles of REST in different types of cancer, both neural and non-neural to develop new REST-based therapies, since REST-dependent tumors appear to be more aggressive than REST-independent ones (Lv et al., 2010; Wagoner and Roopra, 2012).

2. Epigenetic regulation of gene expression

The term 'epigenetics' was first used by Conrad Waddington in the early 1940s which literally means 'besides genetics' and refers to heritable changes in gene expression without changing the DNA sequence (Waddington, 2012). Waddington defined epigenetics as "the branch of biology which studies the causal interactions between genes and their products which bring the phenotype into being". But during the following 50 years, the knowledge of the molecular mechanisms of regulation of gene expression in eukaryotes increased dramatically. Thus, modern usage of "epigenetics" has a stricter definition and was developed by Arthur Riggs and colleagues as "the study of mitotically and/or meiotically heritable changes in gene function that cannot be explained by changes in DNA sequence" (Fincham, 1997).

Nowadays, it is postulated that epigenetic changes modify the activation of genes, but do not modify the sequence of DNA, and that these changes may be preserved through cell division. Such a mechanism enables differentiated cells in a multicellular organism to express only the genes that are necessary for their function. At least three systems are considered as parts of epigenetic regulation of gene expression: DNA methylation, histone modifications and gene silencing by non-coding RNAs (ncRNAs).

DNA methylation is an epigenetic mechanism by which methyl (-CH3) groups are covalently added to DNA. DNA methylation occurs at the cytosine bases of eukaryotic DNA, which are thereby converted to 5-methylcytosine by DNA methyltransferase (DNMT) enzymes (Figure 2.1-a). The methylated cytosine residues are usually adjacent to guanine nucleotides (CpG sites), resulting in two methylated cytosine residues placed diagonally to each other on opposing DNA strands. Different members of the DNMT family act either as de novo DNMTs, putting the initial pattern of methyl groups on a DNA sequence, or as maintenance DNMTs, copying the methylation from an existing DNA strand to its new strand after replication (Figure 2.1-b) (Jeltsch, 2006). It was shown that in human DNA, 5-methyl-cytosine is found in approximately 1.5% of genomic DNA with methylated 60-90% of all CpGs (Siegfried and

Cedar, 1997). Unmethylated CpG sites are often grouped in clusters called CpG islands, which are present in the 5' regulatory regions of many genes, DNA methylation in such regulatory regions results in transcriptional gene silencing (Kass et al., 1997). It has been proposed that DNA methylation may affect the silencing of genes in two ways: transcriptional repression by direct interference with the binding of transcription factors to DNA (Eden and Cedar, 1994) and specific bindig of methylated DNA by methylated-DNA binding domain (MBD) proteins that may recruit additional chromatin remodeling proteins and form compact inactive chromatin (Lewis et al., 1992).

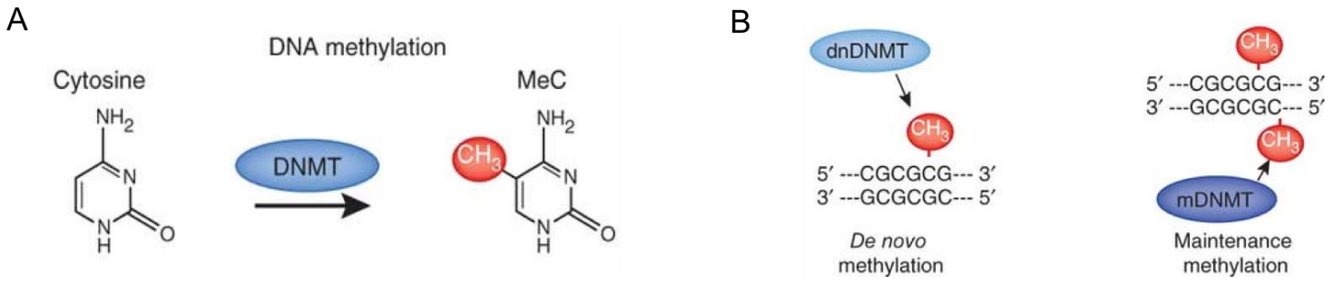

Figure 2.1 Schematic representation of DNA methylation: A – DNA methyltransferase (DNMT) converts cytosine to 5'methyl-cytosine, B – scheme of *de novo* and maintenance DNA methylation.

Histones are the proteins that pack and order DNA into nucleosomes: each nucleosome contains two subunits of histones H2A, H2B, H3 and H4, known as the core histones (Kornberg, 1974). The linker histone H1 does not form part of the nucleosome but acts as a stabilizer of the internucleosome DNA. Histone post-translational modifications are heritable and reversible modifications of histone tails that include phosphorylation, methylation, acetylation, ubiquitynation and sumoylation (Bannister and Kouzarides, 2011; Cosgrove and Wolberger, 2005).

Although histones do not interact with polymerase enzymes directly, their modifications can affect the way DNA wraps around them and thereby influence which genes are expressed and which are silenced. Histone modifications are necessary for recruiting cofactors,

polymerase binding, and for maintaining chromatin stability; most modifications of histones occur at their alkaline N-terminal tails.

Among modifications on histone tails, histone acetylation of lysine residues and methylation of lysine and arginine residues are the most extensively studied (Eberharter and Becker, 2002; Zhang and Reinberg, 2001). Histone acetyl transferase (HATs) enzymes remove the positive charge from the histones H3 and H4 by covalent adding of acetyl groups to the NH3+ group on their lysine residues, thereby decreasing the interaction of histones with the negatively charged phosphate groups of DNA (Figure 2.2-a) (Roth et al., 2001). As a consequence, the chromatin is transformed into a more relaxed structure that is associated with higher levels of gene transcription (Verdone et al., 2006). Lysine acetylation is reversible: deacetylation, catalyzed by histone deacetylases (HDACs), remove acetyl groups from histone tails inducing histone hypoacetylation that is associated with gene silencing (de Ruijter et al., 2003). There were identified 18 human HDACs with some differences in their structure, enzymatic function, subcellular localization and expression patterns. Basing on these differences HDACs were divided into four classes: I (HDAC1, -2, -3, and -8), II (HDAC4, -5, -6, -7, -9, and -10), III (Sirt1, -2, -3, -4, -5, -6, and -7) and IV (HDAC11) (Shahbazian and Grunstein, 2007). Class I HDACs is partially homologous to members of classes II and IV but not to members of class III. Accordingly, similar in structure HDACs catalyze the deacetylation of acetyl lysine via similar mechanisms: class I, II, and IV HDACs are zinc-dependent enzymes, whereas the deacetylase activity of class III members is NAD+ dependent.

The regulatory role of lysine methylation is complex: methylation of some lysine residues is associated with transcription activation, while methylation of other lysines is associated with repression of transcription (Wood and Shilatifard, 2004). For example, the methylation of lysine 9 on histone H3 (H3K9me3) in the promoter region of genes prevents their expression (Chen et al., 2011). In total, four N-terminal lysine residues (K4, K9, K27, K36) and two structural residues (K56, K79) are able to be methylated on H3 histone tail (Lachner et al., 2003). Interestingly, methylation of H3K4, H3K36 and H3K79 is associated with gene

activation, while methylation of H3K9, H3K27, H3K56, H4K20 and H1.4K26 is linked to gene silencing (Jack et al., 2013). It was shown that histone lysine methyltransferases are able to catalyze mono-, di- and trimethylation of lysine residues (Figure 2.2-b). S-adenosyl-l-methionine (AdoMet) serve as the source of the methyl groups, which is converted to S-adenosyl-l-homocysteine (AdoHcy) during the reaction.

Methylated lysines on histone tails could be demethylated: lysine demethylation is catalyzed by lysine demethylases (KDMs). Structurally, histone lysine demethylases can be divided into two functional families. The first family, lysine specific demethylases (LSD1/KDM), includes lysine specific demethylases LSD1 (also known as KDM1A) and LSD2 (KDM1B), the second family, jumonji histone demethylases (JHDMs), consists of the Jumonji C (JmjC)-domain containing proteins. LSD1 and LSD2 can remove mono- and dimethyl histone lysine marks and are unable to demethylate trimethyl lysine residues (Shi et al., 2004). On the contrary, JHDMs are able to demethylate specific histone mono-, di- and tri-methyllysine residues via an oxygenase mechanism (Tu et al., 2007).

Moreover, histones can also be methylated at arginine residues: as with lysine methylation, the regulatory role of arginine methylation is complex and reversible. The mammalian family of arginine methyltransferases (PRMTs) consists of nine members that transfer methyl groups from S-adenosylmethionine (AdoMet) to a guanidino nitrogen of arginine residues of histones resulting in S-adenosylhomocysteine (AdoHcy) and methylarginine. It was reported that there are three main forms of methylated arginine identified in mammalian cells: monomethylarginines (MMA), asymmetric dimethylarginines (ADMA) and symmetric dimethylarginines (SDMA) (Figure 2.2-c) (Scott et al., 1998). There is only one enzyme known to demethylase histone arginine residues - Jumonji domain-containing protein 6 (JMJD6). JMJD6 catalyses demethylation of H3R2me2, H4R3me2 and H4R3me1 (Chang et al., 2007).

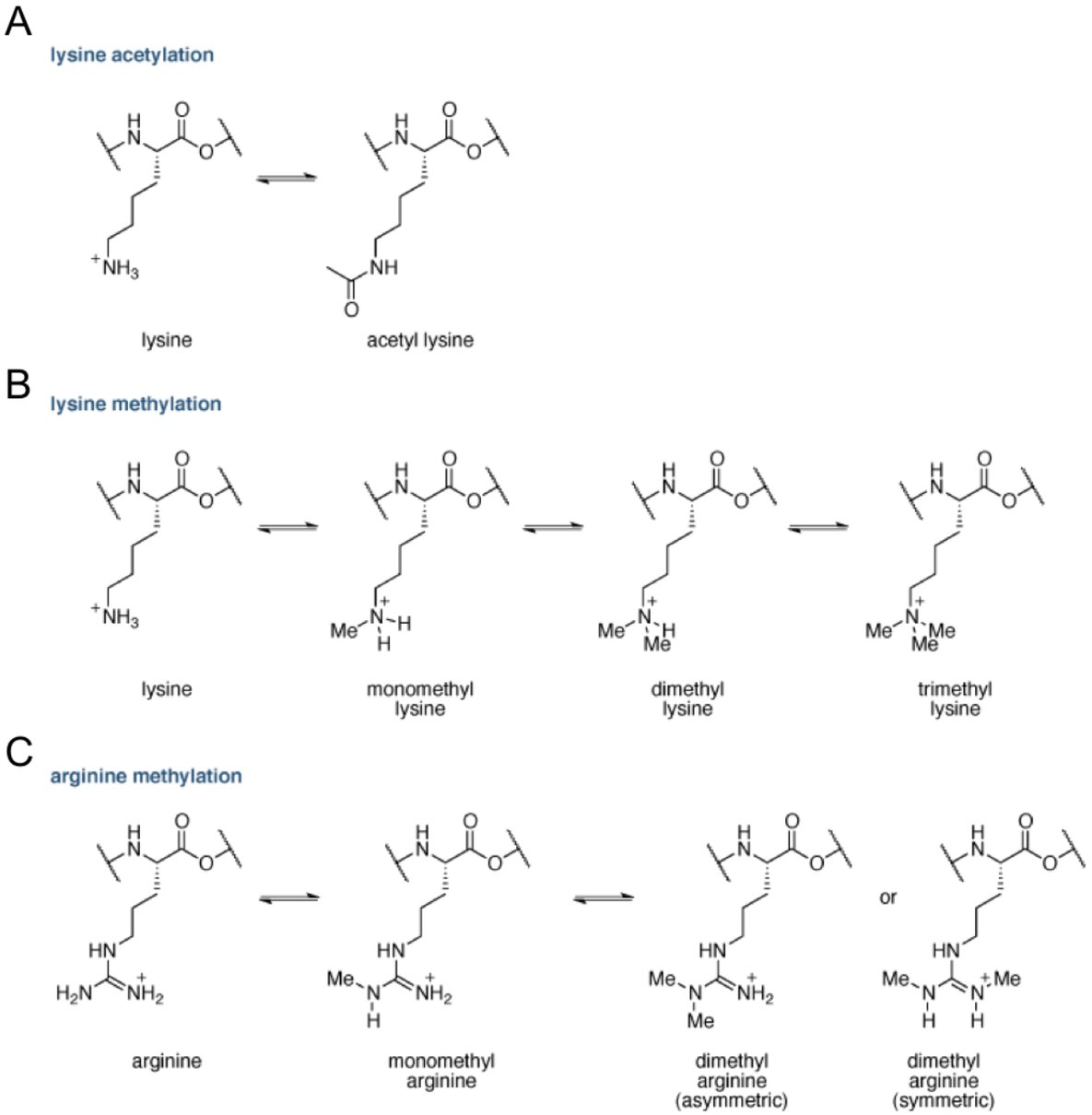

Figure 2.2 Structures of important epigenetic histone modifications: A – lysine acetylation, B – lysine methylation, C – arginine methylation.

In summary, DNA methylation, histone modifications and small noncoding RNA regulation (discussed in chapter 1.3) are different epigenetic mechanisms directly or indirectly linked to transcriptional activity of genes and post-translational modifications of proteins (Figure 2.3). The disruption of normal transcriptional patterns through altered epigenetic marks may trigger signaling pathways that are linked with a number of pathological states including cancer.

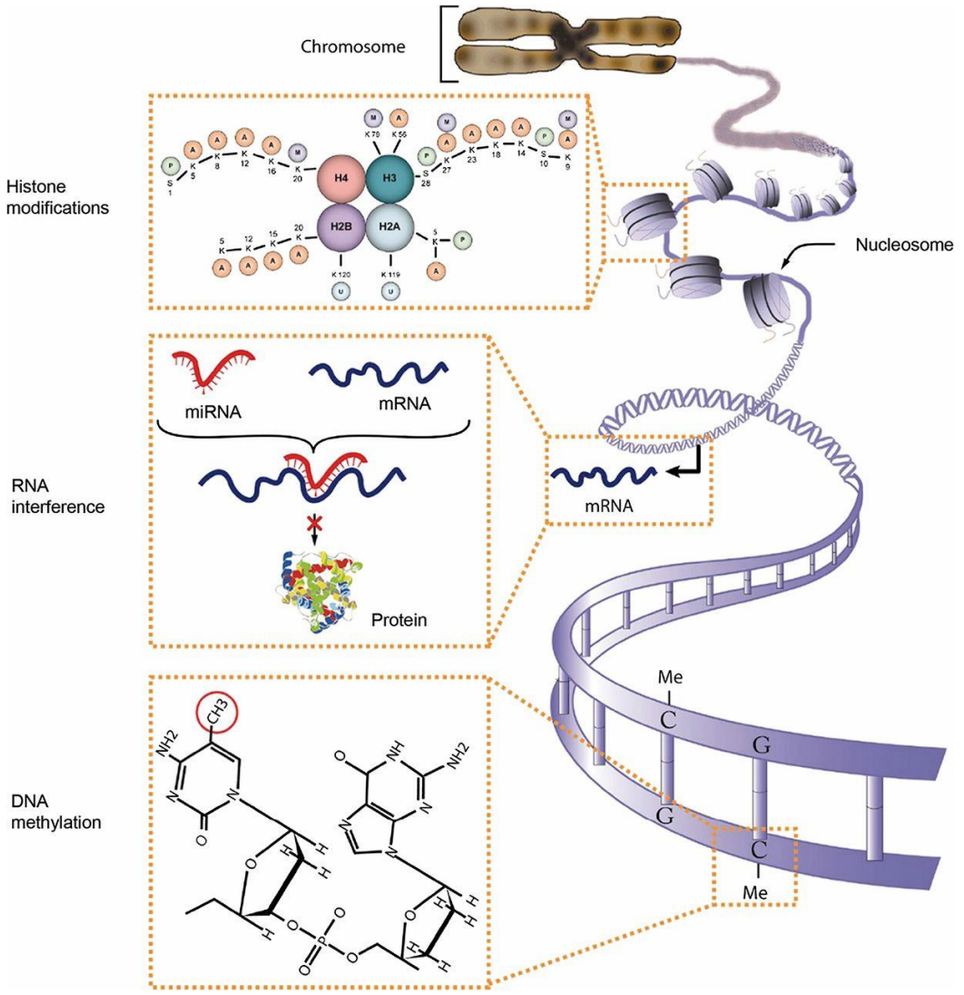

Figure 2.3 Schematic representation of 3 fundamental epigenetic mechanisms of gene transcription regulation: histone modifications, RNA-based mechanisms (i.e., ncRNAs) and DNA methylation.

2.1. Epigenetics in Cancer

Nowadays it is known that multiple changes in cancer cells, including chromosomal instability, oncogene activation and/or silencing of tumor-suppressors and inactivation of DNA repair systems, are due not only to genetic or genomic abnormalities but also to epigenetic alterations (Kulis and Esteller, 2010). During carcinogenesis the genome is subjected to a massive hypomethylation and regional hypermethylation of CpG islans, while in a normal cell DNA methylation occurs at CpG sites, but CpG islands remain unmethylated (Esteller and Herman, 2002). Thus, it was first found in 1980-s in studies of colon cancer that DNA methylation patterns in tumor cells are different from those observed in normal cells (Feinberg and Vogelstein, 1983). During the last decade, the list of genes that could be inactivated by DNA hypermethylation has grown and included most of the pathways that are important for tumor formation and progression, such as control of cell cycle, apoptosis, cell signaling, motility and adhesion, angiogenesis, cancer cell invasion and metastasis (Sigalotti et al., 2007). Interestingly, changes in DNA methylation are also linked to anticancer chemotherapy resistance: methylation of the mutL homolog 1 (MLH1) gene in colorectal cancer is associated with increased resistance to a widely used chemotherapeutic drug cisplatin (Teodoridis et al., 2004).

Specific histone modifications are also associated with tumor formation. For example, many types of tumors exhibit decreased levels of monoacetylated and trimethylated forms of histone H4 (H4ac and H4me3) (Fraga and Esteller, 2002). Other histone modifcations associated with tumorigenesis include: increased deacetylation (or decreased acetylation) of histones H3 and H4, decreased trimethylation of histone H3 Lysine 4 (H3K4me3), increased monomethylation of histone H3 Lysine 9 (H3K9me) and trimethylation of histone H3 Lysine 27 (H3K27me3). Mutations in the epigenetic machinery itself are potentially responsible for the modified epigenetic profile of cancerous cells (Table 1). Thus, altered expression and mutations both in histone acetyltransferase (HAT) and histone deacetylase (HDAC) genes are linked to tumor development since they both are able to change the transcription of key genes that regulate important cellular functions such as cell proliferation (Kim et al., 2001), cell-

cycle regulation (Wang et al., 2001) and apoptosis (Hanigan et al., 2008). An inactivating frameshift mutation in HDAC2 has been associated with cancers showing altered histone acetylation patterns (Ropero et al., 2006).

Table 1. Epigenetic regulators which are altered in cancer

Epigenetic regulator	Function	Histone modification	Associated cancer	Alteration in cancer
DNMT 1, 3a, 3b	DNMT	Methyl CpG	Various types	Overexpression
P300	HAT	Multiple lysines	Leukemia, myeloplasia	Translocation/ inactivating mutation
CBP	HAT	Multiple lysines	Leukemia, myeloplasia	Translocation/ inactivating mutation
MOZ	HAT	Multiple lysines	Leukemia	Translocation
MORF	HAT	Multiple lysines	Leukemia	Translocation
HDAC1-3, 6	HDAC	General	Various types	Overexpression
RIZ1	HMT	H3K9	Various types	Down-regulation/ mutation
MLL1	HMT	H3K4	Leukemia, lymphoma	Translocation
NSD1	HMT	H3K36	Leukemia, hepatocellular carcinoma	Translocation/ inactivating mutation
NSD3	HMT	H3K36	Breast cancer	Translocation/ overexpression
JMJD2C	Histone demethylase	H3K9	Various types	Overexpression
MeCP2	Methyl-CpG-binding protein	Methyl CpG	Various types	Overexpression

miRNAs have emerged as another important part of epigenetic regulation of gene expression that was shown to be implicated in cancer development. Despite the relatively small number of discovered miRNAs, by computational and experimental studies it was found that thousands of human protein-coding genes are regulated by miRNAs (Lewis et al., 2005). Recent studies have indicated that numerous miRNA genes are located at fragile genomic regions and are frequently dysregulated in various cancers (Table 2) (Zhang et al., 2006). Thus, first miRNAs found to be dysregulated in cancer were miR-15 and miR-16: their cluster was allocated to a frequently deleted in chronic lymphocytic leukemia (CLL) chromosomal region 13q14.3 (Calin et al., 2002). Deletion of these two microRNA genes was shown to allow higher expression of their anti-apoptotic target gene B-cell lymphoma 2 (BCL2), keeping such cells from apoptosis.

Table 2. miRNAs which are up- or down-regulated in various tumors relatively to normal tissues

miRNA	**Up/down**	**Type of cancer**	**References**
let-7	Up	Colon cancer	(Nakajima et al., 2006)
	Down	Lung cancer	(Johnson et al., 2005)
	Up	Pancreatic cancer	(Lee et al., 2007)
miR-9	Down	Breast cancer	(Lehmann et al., 2008)
	Up	Breast cancer	(Ma et al., 2007)
	Down	Neuroblastoma	(Laneve et al., 2007)
miR-21	Down	Pituitary adenoma	(Bottoni et al., 2007)
	Up	Cervical cancer	(Lui et al., 2007)
miR-30a	Down	Thyroid cancer	(Visone et al., 2007)
miR-124a	Down	Colon, lung cancer	(Lujambio et al., 2007)
miR-135b	Up	Colorectal cancer	(Bandrés et al., 2006)
miR-212	Up	Pancreatic cancer	(Lee et al., 2007)

Moreover, signatures of miRNA expression are thought to be promising biomarkers for the classification and even outcome prediction in a wide number of human cancers (Jay et al., 2007). For example, a five-miRNA signature (hsa-let-7a, hsa-miR-221, hsa-miR-137, hsa-miR-372, and hsa-miR-182*) has been identified by expression profiling for the prediction of lung cancer outcome (Yu et al., 2008); miR-200a and miR-9 were identified as two miRNAs that could predict patient survival with cervical cancer (Hu et al., 2010).

In contrast to genetic mutations, which are irreversible, epigenetic modifications may be, to varying degrees, reversible and preventable. The goal of epigenetic pharmacological therapy in cancer treatment is to restore epigenome to a 'normal' state. Pharmacological targeting of DNA methyltransferases and histone acetylases and methylases is now possible with some agents already approved by FDA (e.g. azacytidine, decitabine and vorinostat) and some others under investigation (Figure 2.4, Table 3) (Ellis et al., 2009; Nervi et al., 2015).

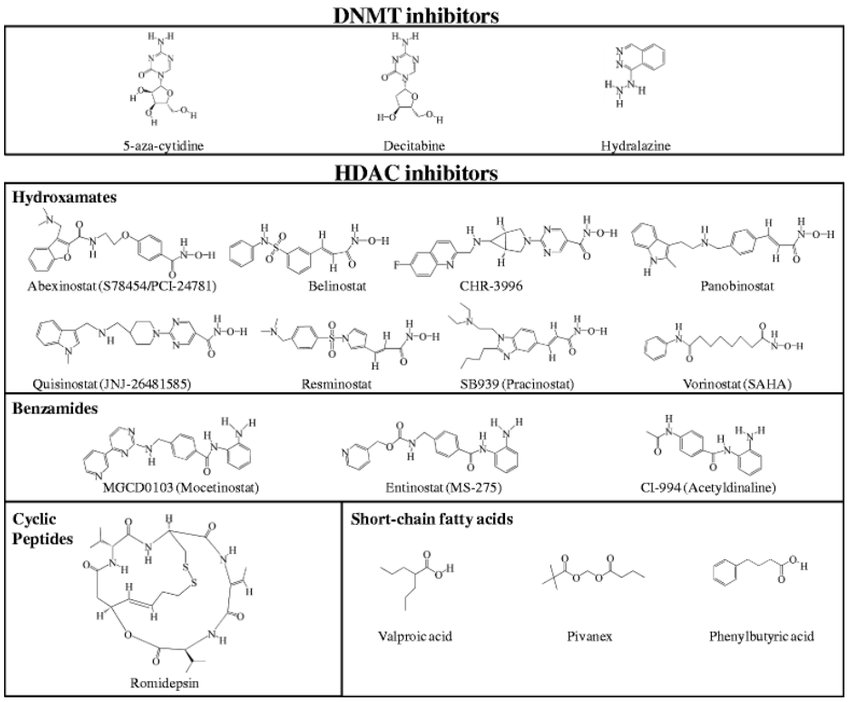

Figure 2.4. Molecular structures of the most studied epigenetic drugs among DNA methyltransferase (DNMT) and histone deacetylase (HDAC) inhibitors

Table 3. Selected epigenetic drugs.

Drug	**Compound**	**Study phase**
DNMT inhibitors	Azacitidine (Vidaza)	US FDA-approved in MDC
	Decitabine (Dacogen)	US FDA-approved in MDC
	S110	Phase I
	CP-4200 (elaidic azacytidine)	Preclinical
	Nanaomycin A	Preclinical
HDAC inhibitors	Vorinostat (Zolinza)	US FDA-approved in CTCL
	Romidepsin (Istodax)	US FDA-approved in CTCL
	Panobinostat	Phase II
	Belinostat	Phase I-III
	Valproic acid	Phase III
HMT inhibitors	Deazaneoplanocin A (DZNep)	Preclinical
	Quinazoline derivatives	Preclinical
	Ellagic acid	Preclinical
Histone demethylase inhibitors	Polyamine analogues	Preclinical
	Hydroxamate analogues	Preclinical
HAT inhibitors	Spermidinyl-CoA derivatives	Preclinical
	Hydrazinocurcumin	Preclinical
	Pyrazolone-containing small molecules	Preclinical

CoA = coenzyme A; CTCL = cutaneous T-cell lymphoma; DNMT = DNA methyltransferase; HAT = histone acetyltransferase; HDAC = histone deacetylase; HMT = histone methyltransferase; MDS = myelodysplastic syndrome.

2.2. Valproic Acid: a potent epigenetic-acting drug

Valproic acid (VPA; valproate; di-n-propylacetic acid, DPA; 2-propylpentanoic acid, or 2-propylvaleric acid) (Figure 2.5) is a synthetic substance that was first synthesized in 1882 by Burton as an analogue of valeric acid, found naturally in valerian (Burton, 1882).

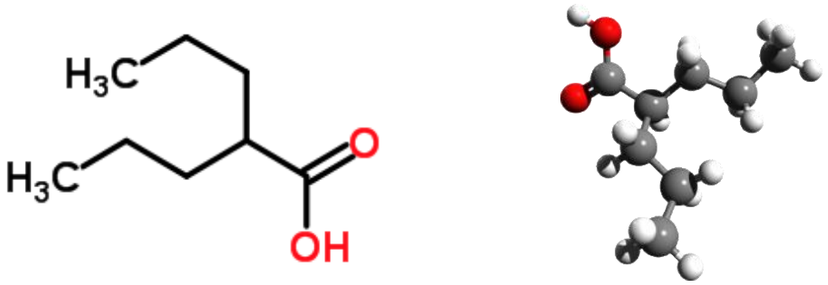

Figure 2.5 Chemical structure of Valproic acid

Nearly for the first 90 years of its history, valproic acid was used as a lipophilic vehicle to dissolve water-insoluble compounds during preclinical drug testing. In 1962, Pierre Eymard sinthesized kheline derivatives and tested them together with Meunier in the pentylenetetrazole (PTZ) seizure test using VPA as a vehicle. Researchers found that this vehicle alone exerted an anticonvulsant effect in rodents (Meunier et al., 1963). In 1967 VPA was approved as an antiepileptic drug in France and became among the most widely prescribed antiepileptic drug worldwide. Later, VPA started to be used not only as an anticonvulsant and mood-stabilizing drug (McElroy et al., 1989), but also in clinical depression (Calabrese and Delucchi, 1989) absence seizures (Erenberg et al., 1982), tonic-clonic seizures, complex partial seizures (Dean and Penry, 1988), juvenile myoclonic epilepsy (Calleja et al., 2001), seizures associated with Lennox-Gastaut syndrome (Friis, 1998), migraine headaches, and schizophrenia (Schwarz et al., 2008).

It was shown that in the human brain, valproic acid affects the function of the inhibitory neurotransmitter in the central nervous system gamma-aminobutyric acid (GABA) by increasing the inhibitory activity of GABA through several mechanisms such as inhibition of GABA protein degradation, its increased synthesis, and decreased turnover (Chateauvieux et al., 2010). Moreover, its

anticonvulsant activity may be related to VPA-induced blockade of voltage-gated sodium, potassium and calcium channels and modulation of dopaminergic and serotoninergic transmission (VanDongen et al., 1986).

Valproic acid is rapidly absorbed after oral administration, with peak serum levels 1–4 h after a single dose. The half-life of VPA in serum is in the range of 7–16 h; the drug is ~95% bound to human plasma proteins. VPA is primarily metabolized to the glucuronide conjugate in the liver and eliminated with the urine. VPA is generally well tolerated by patients, the most serious side effects are liver failure and teratogenicity, however, fatal hepatotoxicity is rare (~1:15,000) and mainly occurs in children younger than 2 years (DeVane, 2003). VPA may cause birth defects such as defects of neural tube closure and other malformations if administered during early pregnancy (DiLiberti et al., 1984).

Besides its anticonvulsant activity, recently VPA has been recognized as a promising HDAC inhibitor showing anti-tumor activity that affects cell growth in different types of cancer in vitro and in vivo (Nagai, 2013; Wang et al., 2013). This finding was described by Gottlicher and colleagues who found that VPA causes hyperacetylation of the N-terminal tails of histones H3 and H4 and inhibition of HDAC enzymatic activity at VPA concentration of 0.5 mM (Göttlicher et al., 2001). From these previous studies as well as from other works, now it is acknowledged that VPA specifically targets 2 of the 4 classes of HDACs: class I, subclasses Ia and Ib, and class II, subclass IIa (Gurvich et al., 2004; Phiel et al., 2001). VPA, as well as other HDAC inhibitors (HDACi), are able to alter expression of genes with important roles in cell differentiation, apoptosis and anti-tumor action.

Anti-cancer effect of VPA was revealed by several studies which found that VPA inhibits growth of several types of cancer cells and promotes their differentiation (Blaheta and Cinatl, 2002; Kuendgen and Gattermann, 2007), inhibits angiogenesis in tumor tissue (Michaelis et al., 2004a) and induces cell apoptosis (Kawagoe et al., 2002). Moreover, VPA is able to enhance the effect of other anti-tumor treatments such as retinoic acid and interferon alpha (Bug et al., 2005; Michaelis et al., 2004b) and to strengthen sensitivity of human cancer cells to ionizing radiation (Tom C Karagiannis, 2006).

The restoration of histone acetylation patterns has been shown to correlate with antitumour activity, and HDAi including valproic acid have been intensively investigated. Many clinical trials where patients

affected by various forms of cancer are treated with VPA (both mono- or multi-therapeutic approaches) are ongoing (Table 4).

Table 4. Ongoing studies of valproic acid alone or in combination with other antitumor therapies

Tumor	Phase	VPA schedule
Thyroid advanced	II	VPA daily for 10-weeks, if increased radioiodine uptake, then continue VPA up to 16 weeks
Melanoma	I/II	Cycle 1: karenitecin day1–5 Cycle 2: karenicetin days 1–7 & days 10–12 VPA d1–12
Advanced solid tumors	I	VPA day1–5 every 21 days, 5-FU/cyclophosphamide
Advanced solid tumors	I	VPA day1–7 every 21 or 28 days + either: dasatinib, erlotinib, lapatinib, lenalidomide, sorafenib or sunitinib
Locally advanced breast cancer	II	VPA six doses every 12 h+ FEC100 at day 3 every 21-days.
Ovarian cancer	I/II	VPA d1–5, Azactidine day1–5. Carboplatin days 3 and 10, every 21 days.
Nasopharyngeal	I	VPA day1–14 (primary outcome: expression of EBV lytic cycle antigens in tumor tissues)
Advanced solid tumors	I	VPA d1–28. Bevacizumab every 2 weeks, cycles of 28-days
Neuroectodermal tumors & brain metastases	I	VPA daily oral etoposide daily. For up to two years.
Gliobastoma multiforme	II	VPA d7-49. Temozolomide 1–5 and 29–33 External radiation for 6.5 weeks.
Brain metastases	I	VPA day 1–21, Temozolomide 1–21. External radiation for 3 weeks.
Non-Hodgkin lymphoma	I	Stage 1: decitabine days 1–5 or 1–10 Stage 2: VPA days 5–25 + decitabine as stage 1
Cervical cancer recurrent/metastatic	III	Randomized to either: Cisplatin-topotecan + daily VPA & hydralazine Cisplatin-topotecan + placebo. 6 cycles of 21 days.
Ovarian cancer cisplatin-resistant	III	Randomized to either: Topotecan + daily VPA & hydralazine Topotecan + placebo. 6 cycles of 28 days.

3. Aim of the study

Genetic and genomic alterations may result in activation of oncogenes and/or inactivation of tumor-suppressors contributing to cancer development. Furthermore, epigenetic events such as histone post-translational modifications, DNA methylation, mRNA and/or protein degradation by non-coding RNAs (siRNA, miRNA), have emerged as another key mechanism in the development of human cancer (Herceg and Hainaut, 2007).

Histone post-translational modifications are heritable and reversible modifications of histones that include phosphorylation, methylation, acetylation, ubiquitylation and sumoylation (Bannister and Kouzarides, 2011). Among modifications on histone tails, histone acetylation and methylation of specific lysine residues on histones H3 and H4 are the most extensively studied. Histone acetyl transferases (HATs) remove the positive charge from the histones H3 and H4 by covalent adding of acetyl groups to the NH3+ group on lysine, thereby decreasing the interaction of histones with the negatively charged phosphate groups of DNA (Roth et al., 2001). As a consequence, the chromatin is transformed into a more relaxed structure that is associated with higher levels of gene transcription. On the contrary, histone deacetylases (HDACs) remove acetyl groups from histone tails inducing histone hypoacetylation that is associated with gene silencing. Altered expression and mutations in HATs and HDACs genes are linked to tumor development since they both are able to change the transcription of key genes that regulate important cellular functions such as cell proliferation, cell-cycle regulation and apoptosis (Hanigan et al., 2008; Wang et al., 2001). Accordingly, several drugs such as valproic acid (VPA) have been proposed to inhibit HDACs and are in clinical trials for the treatment of several types of cancer (Marson, 2009).

Valproic Acid (VPA, 2-propylpentanoic acid) is a drug that initially was used for the treatment of epilepsy, bipolar disorder and as a mood stabilizer in psychiatry (Bowden, 2004; Trinka et al., 2014). VPA-induced anticonvulsant activity may be related to increased brain concentrations of gamma-aminobutyric acid (GABA), an inhibitory neurotransmitter in the

central nervous system. VPA also may have an anti-epileptic activity by blocking voltage-gated sodium, potassium and calcium channels.

Recently VPA has been recognized as a promising HDAC inhibitor showing anti-tumor activity that affects cell growth in different types of cancer in vitro and in vivo (Wang et al., 2013). For instance, it has been reported that intraperitoneal injection of VPA inhibited tumor growth and angiogenesis in mice transplanted with Kasumi-1 cells, human acute myeloid leukemia cells, through an anti-angiogenic mechanism (ZHANG et al., 2014). Several studies have reported that VPA selectively inhibits histone deacetylase 1 (HDAC1), which potentially increases the expression of genes involved in differentiation, apoptosis and anti-tumor action (Göttlicher et al., 2001; Phiel et al., 2001). Epigenetic mechanisms contributing to VPA-mediated chromatin remodeling have been poorly investigated. It has been shown that VPA affects the activities of several enzymes and signal transduction pathways, but the mechanisms underlying the anti-cancer properties of VPA are not well characterized.

In this study we have focused on the transcriptional repressor REST (RE-1 silencing transcription factor, or NRSF –neuron-restrictive silencing factor) that binds to a conserved RE-1 motif present in the promoter region of regulated genes and represses their transcription in neuronal and non-neuronal cells (Bruce et al., 2004). REST recruits corepressors (CoREST, mSin3a) and multiple chromatin modifying enzymes (HDAC1/2, demethylase LSD1 and methyltransferase G9a), causing chromatin compaction and altering gene expression by changing epigenetic marks (Ballas et al., 2005).

REST seems to act as an oncogene or a tumor suppressor depending on the cell type and tumor progression (Negrini et al., 2013). Thus, it contributes to promote the development of brain tumors such as neuroblastomas, medulloblastomas and glioblastomas (Conti et al., 2012; Taylor et al., 2012). Whereas, REST acts as a tumor suppressor inhibiting tumorigenesis in epithelial cells like carcinomas of the lung, breast, and colon (Kreisler et al., 2010; Wagoner et al., 2010). Furthermore, REST contributes to orchestrate the epigenetic regulation of its target genes through several miRNAs including miR-9/9*, miR-29a, miR-124a, miR-218 and others (Wu and Xie, 2006). It has been reported that miR-9 targets 3'UTR

of REST and significantly reduces endogenous REST, whereas a reduction of REST results in enhanced miR-9 expression; interestingly, miR-9 could be used as a marker for cervical cancer prognosis (Conaco et al., 2006; Packer et al., 2008). In a previous study, we have reported that REST-mediated chromatin compaction through HDAC recruitment reduces apoptosis in HeLa cells caused by anti-Fas antibody plus PD 98059; thus, suggesting that REST has an antiapoptotic effect in these cells (Baiula et al., 2012).

Moving from the observations that REST repression complex is able to change its target gene and miRNA transcription via recruitment of multiple chromatin modifying enzymes, including HDAC1, and it may act as an oncogene or a tumor-suppressor in different human cancers, we hypothesized that REST also may have an important role in cervical cancer. Worldwide, cervical cancer is the fourth-most common cause of death from cancer in women, about 8% of the total cases and total deaths from cancer. In biomedical research, the most common cell line used as a model of cervical adenocarcinoma, is HeLa cell line (Masters, 2002). Recently it was shown that valproic acid inhibits the growth of HeLa cells via caspase-dependent apoptosis and now is in phase III clinical trials for the treatment of human cervical cancer (Coronel et al., 2011; Han et al., 2013).

Thus, the aim of this study was to assess whether in HeLa cells VPA-mediated histone acetylation involves changes of REST, its corepressors (mSin3a, CoREST, HDAC1) and/or any of its target microRNAs (miR-9, miR-124a) and if these peculiar effects may influence apoptosis of the cervical cancer cell line HeLa.

4. Materials and methods

4.1 Materials

Eagle's Minimum Essential Medium, (EMEM), Phosphate Buffered Saline (PBS) and L-Glutamine (200mM) were from Lonza (Verviers, Belgium), fetal bovine serum (FBS) was from EuroClone S.p.A. (Milan, Italy). Antibiotic-Antimycotic solution (100x), Trypsin-EDTA (0.25%, 1x), MEM Non-Essential Amino Acids Solution (MEM NEAA, 100x) and Opti-MEM® I Reduced Serum Medium (no phenol red) were from Gibco®, Life Technologies Corporation (Grand Island, NY, USA). Lipofectamine® 2000 Transfection Reagent and Lipofectamine® RNAiMAX Transfection Reagent were from Invitrogen™, Life Technologies Corporation (Carlsbad, CA, USA).

Goat anti-rabbit IgG-HRP (sc-2030), goat anti-mouse IgG-HRP (sc-2031), rabbit anti-mSin3A (sc-994) antibodies were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Rabbit anti-REST (07-579), rabbit anti-CoREST (07-455), rabbit anti-HDAC1 (06-720), mouse anti-histone H1 (05-457), rabbit anti-histone H3 (05-928), rabbit anti-acetyl-Histone H3 (06-599) antibodies and Immobilon™ Western Chemiluminescent HRP Substrate were purchased from Millipore (Billerica, MA, USA). Monoclonal anti-β-actin antibodies, oligonucleotide primers, polyacrylamide, N,N,N′,N′-tetramethylethylenediamine and sodium dodecyl sulfate were purchased from Sigma-Aldrich (St. Louis, MO, USA). Restriction enzymes were obtained from Roche (Mannheim, Germany) or Fermentas (Hanover, MD, USA).

NE-PER® Nuclear and Cytoplasmic Extraction Kit was obtained from Thermo Fisher Scientific Inc. (Rockford, IL, USA). MG132 (Z-Leu-Leu-Leu-al), polyacrylamide gel, N,N,N′,N′-tetramethylethylenediamine and sodium dodecyl sulfate were purchased from Sigma-Aldrich®. All other reagents utilized were of analytical grade or of the highest purity available and purchased from Sigma-Aldrich® or Roche. All plastic disposables were from Sarstedt AG & Co (Nümbrecht, Germany).3′UTR Reporter Vector pLightSwitch_3UTR (S890005) and

LightSwitch Luciferase Assay Kit (LS010) were purchased from © SwitchGear Genomics. All other reagents were of analytical grade or of the highest purity available, purchased from Sigma-Aldrich (St. Louis, MO, USA).

4.2 Cell culture

HeLa cells, a human cervical adenocarcinoma cell line obtained from the American Type Culture Collection (Rockville, MD, USA), were cultured as monolayers in Eagle's minimum essential medium containing 1x nonessential amino acids, 10% (vol/vol) FBS, 2mM L-Glutamine and 1x antibiotics-antimycotics at 37 °C in a humidified environment containing 5% CO2 and 95% air.

4.3 Plasmid and siRNA transfection

HeLa cells were plated in 100mm dishes and at 80%–85% confluence were transiently transfected with 10 µg/dish of the mammalian expression vector pCMV-Taq2B+REST (©Agilent Technologies, Inc., Santa Clara, CA, USA) containing the cDNA coding sequence of human REST; alternatively, the mock plasmid pCMV-Taq2B was used as a control instead of the REST expressing plasmid. Cell transfection was carried out using the Lipofectamine® 2000 transfection reagent.

For siRNA transfection, HeLa cells were plated in 100mm dishes or 6-well plates and at 80%–85% confluence were transiently transfected with 20nM of Silencer® Select Pre-Designed & Validated siRNA (Ambion®) against human REST (ID s11932) and human CoREST (ID 136795). Silencer® Select Negative Control No.1 siRNA (Ambion®) was used as a control. Cell transfection was carried out using the Lipofectamine® RNAiMAX Transfection Reagent.

4.4 Protein extraction and Western Blot analysis

HeLa cells were detached with trypsin-EDTA, centrifuged (2000g for 3 min) and the pellet was used for protein extraction with NE-PER® Nuclear and Cytoplasmic Extraction Kit according to the manufacturer's instructions. The protein content was quantified by Lowry assay. Then proteins were denatured at 95 °C for 3 minutes, loaded and separated by SDS-PAGE. MagicMark™ XP Western Protein Standard (Invitrogen) served as a molecular weight standard.

Proteins were electrophoretically transferred to Amersham™ Protran™ 0.45µm nitrocellulose membranes, which were probed overnight with primary antibody of interest: anti-REST, anti-CoREST, anti-HDAC1, anti-AcH3 and anti-H1 (1:2000, Millipore); anti-mSIN3a (1:200, Santa Cruz Biotechnology), anti-β-actin (1:5000, Sigma). After washing with TBST, the membranes were incubated in corresponding horseradish peroxidase (HRP) conjugated secondary antibody and developed with Immobilon™ Western Chemiluminescent HRP Substrate for 3 minutes. Chemiluminescence was detected using a luminescent image analyzer LAS-3000 (Fujifilm, Tokyo, Japan). The experiments were carried out in triplicate and statistical analysis was performed using GraphPad Prism 5 software.

To reprobe membranes with another primary antibody, they were incubated with a stripping solution (NaOH 0,2 N, NaCl 0,5 M) for 10 minutes in agitation at room temperature. Then, membranes were washed 3 times with water and twice with TBS-0,1% Tween 20 for 10-15 minutes.

4.5 Histone extraction

Cells were detached with trypsin-EDTA, pelleted and resuspended in 750 µl of lysis buffer: NaH_2PO_4/Na_2HPO_4 buffer - 10 mM, NaCl - 10 mM, $MgCl_2$ - 5 mM, NP-40 0,1%, sodium butyrate - 10 mM, protease inhibitors (aprotinin 10 µg/ml, leupeptin 10 µg/ml, pepstatin 10 µg/ml and PMSF 5 µg/ml). After 15 minutes of incubation on ice, 250 µl of paraformaldehyde

3% were added to each sample and incubated for 30 minutes on ice. Samples were centrifuged at 2000g for 5 minutes at 4°C and the pellet containing nuclei was washed twice in PBS supplemented of sodium butyrate 10 mM (Sigma-Aldrich). Histones were extracted by incubating the nuclei in 0.2 ml H2SO4 0.2 M for 4 hours at 4°C, then samples were centrifuged at 15000g for 5 minutes at 4°C. The supernatants were mixed with 1 ml of acetone (Sigma-Aldrich) and incubated at -20°C overnight to precipitate histones. The following day, samples were centrifuged at 15000g for 5 min at 4°C and the pellets of histones were solubilized in 20-30 µl of nuclease-free water (Shechter et al., 2007).

4.6 Total RNA preparation and Real-Time Polymerase Chain Reaction (RT-PCR) analysis

For RT-PCR experiments, HeLa cells were treated as indicated in various experiments, then detached with trypsin, centrifuged (2000g for 3 min) and rinsed with phosphate-buffered saline (PBS). Total cellular RNA was extracted with Trizol® reagent (Sigma-Aldrich) and 2-µg sample was reverse-transcribed using the High-Capacity cDNA Reverse Transcription Kit™ (Applied Biosystems) according to the manufacturer's instructions using Applied Biosystems 2720 Thermal Cycler® (Applied Biosystems) instrument.

A 0.05 µg of cDNA was employed for relative quantification of mRNA transcripts using SYBR® Select Master Mix and the StepOne™ RT-PCR System (Applied Biosystems) in accordance with the manufacturer's instructions. Primer pairs which were used are indicated in the Table 5.

To validate and quantify mature microRNA sequences, the following TaqMan® Small RNA Assays (Applied Biosystems) were used: hsa-miR-9 (assay ID 000583), mmu-miR-124a (assay ID 001182) and U6 snRNA (assay ID 001973). A 0.5-µg samples of total cellular RNA were reverse-transcribed with TaqMan® MicroRNA Reverse Transcription Kit, 0.044 µg of cDNA

were amplified using the TaqMan® Universal PCR Master Mix II (No UNG) and the StepOne™ RT-PCR System (Applied Biosystems) according to the manufacturer's instructions.

Relative expression levels of RT-PCR products were determined using the $2^{-\Delta\Delta Ct}$ method, each sample was tested in triplicate and the mean Ct was used in the $2^{-\Delta\Delta Ct}$ equation.

Table 5. Primer sequences used for RT-PCR

Gene	Primer sequence		Amplicon length, bp
REST	Sense	5′-TCGAAGACCAAACCCTTTCG-3′	226
	Antisense	5′-GATTAGTATTGTAGCCGCAGCG-3′	
CoREST	Sense	5′-AACAATCCCATTGACATTGAGGT-3′	244
	Antisense	5′-GTCGTTTGACTGAAACCAATTCC-3′	
HDAC1	Sense	5′-CGCCCTCACAAAGCCAATG-3′	108
	Antisense	5′-CTGCTTGCTGTACTCCGACA-3′	
mSin3a	Sense	5′-CCTGTGGAGTTTAATCATGCCA-3′	248
	Antisense	5′-GCATCTGGTAGGAATTGTCCAAA-3′	
L19	Sense	5′-CTAGTGTCCTCCGCTGTGG-3′	169
	Antisense	5′-AAGGTGTTTTTCCGGCATC-3′	
BAX	Sense	5′- CCCGAGAGGTCTTTTTCCGAG-3′	155
	Antisense	5′- CCAGCCCATGATGGTTCTGAT-3′	

4.7 Flow Cytometry analysis

Guava Nexin® Reagent (Millipore) was used for labeling of early apoptotic cells and for discriminating between early apoptotic and late apoptotic/dead cells in accordance with the manufacturer's instructions. The Guava Nexin® assay utilizes Annexin-V conjugated with the fluorochrome phycoerythrin (PE), to detect phosphatidylserine (PS) on the external membrane of apoptotic cells, and a cell impermeant dye, 7-AAD, as an indicator of cell membrane structural integrity. 7-AAD is an analogue of propidium iodide, which is excluded from live, healthy cells as well as early apoptotic cells. Three populations of cells can be distinguished

in this assay: viable cells are Annexin-V and 7-AAD negative, apoptotic cells are Annexin-V positive and 7-AAD negative, necrotic cells are Annexin-V and 7-AAD positive.

In brief, HeLa cells were treated as indicated in various experiments, then harvested by trypsinization and cell pellet was resuspended in a complete medium to achieve the concentration $2x10^5$ – $1x10^6$ cells/ml. A 100-µl samples were transferred into an eppendorf tube and 100 µl of pre-warmed Guava Nexin® Reagent was added. After 20 minutes of incubation in the dark, samples were acquired on Guava EasyCyte™ System and analyzed with GuavaSoft 2.2.3 software (Millipore).

Thereafter, 100 µl of each cell suspension sample were mixed with Nexin® reagent (Millipore), previously warmed at room temperature, according 1:1 ratio; then samples were incubated for 20 minutes at room temperature in the dark, to allow cell staining. Thereafter, the suspension was resuspended accurately and read at the flow cytometer instrument (Guava EasyCyte™ System, Millipore). Percentage of apoptotic cells was counted in every sample; the same experiment was performed in triplicate.

4.8 MTT assay

The effect of VPA on cell growth was determined by measuring 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich) absorbance in living cells. This assay requires cells that are able to take up and metabolize the soluble 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) salt to an insoluble formazan precipitate. The salts initially formed can be reduced within the cell to blue-colored formazan crystals that can be visualized on a spectrophotometer. The assay is used as an indicator of mitochondrial function in live cells; however, loss of activity cannot be used to distinguish compromised from dead cells.

Cells were seeded in a 96-well plate (5000 cells/well) and grown in complete medium for 24 hours. Then cells were treated as reported in the results, each treatment was performed in quadruplicate. Thereafter, 10 µl of MTT 5 mg/ml (Sigma), dissolved in PBS and filtered, were added to each well, and the plate was incubated for 4 hours at 37 °C in the dark. Thereafter, the MTT solution was withdrawn from the plates by pipetting surnatants were discarded and 200 µl of isopropanol-HCl 0,04N solution were added to each well to solubilize the formazan crystals. The optical density was measured at 540 nm using a plate-reader spectrophotometer (Victor2, PerkinElmer).

4.9 REST 3'UTR cloning and Gene reporter assay

The fragment of REST 3'UTR which contains 2 miR-9 recognition elements, was amplified from human genomic DNA by PCR using the following primers: 5'-CACTCCTCTAGAAAAGGTAGTATGAGTGCTGGTAGAC-3' (forward) and 5'-ACTGTACTCGAGAGGCTAAATTCTATCCAGTGTGGG-3 (reverse). Primers were designed to contain restriction sites for further manipulations: the forward primer contains XbaI restriction site, the reverse primer – XhoI restriction site.

The PCR product with length of 1319bp, was inserted into an intermediate vector pCR-Blunt II–TOPO® plasmid (Invitrogen™, Carlsbad, CA, USA) according to manufacturer's instructions and the restriction analysis of the pCR-BluntII–TOPO+REST-3'UTR was done to confirm the correct insert. Using XbaI and XhoI restriction enzymes, the fragment of REST 3'UTR was transferred from an intermediate to a final vector pLightSwitch_3UTR which contains XbaI and XhoI restriction sites as well. Finally, the novel plasmid pLightSwitch_3UTR+REST-3'UTR was sequenced (BMR Genomics, Padua, Italy); this analysis confirmed the correct insert of REST 3'UTR and both miR-9 recognition elements were correctly placed. Thus, this novel genetic vector for employed to study regulation of REST transcription by miR-9.

For Gene reporter assay, HeLa cells were plated in 96 well-plates and co-transfected with 0.4 µg/well of pLightSwitch_3UTR+REST-3'UTR plasmid and 20nM of human mirVana® miR-9-5p mimic. miRNA mimics are small, chemically modified double-stranded RNAs that mimic endogenous miRNAs and enable miRNA functional analysis by up-regulation of miRNA activity. After 24 hours of transfection, cells were treated with 0.5 or 1 mM of VPA for another 24 hours. After treatment the medum was replaced with LightSwitch Luciferase Assay Reagent (©SwitchGear Genomics) according to manufacturer's instructions (http://switchgeargenomics.com/resources/protocols). Cells were incubated for 10 minutes and the signal was detected on a plate-reading luminometer (Victor2; PerkinElmer). Calculations of knockdown were made by calculating luminescence signal ratio for each treatment over the non-targeting control.

4.10 Data analysis

All data were expressed as mean ± standard error of the mean (SEM) for the number of experiments indicated. Statistical comparisons were made by analysis of variance (ANOVA), and post-hoc Newman–Keuls; differences of $P<0.05$ were considered significant (*), $p< 0,01$ very significant (**) and $p< 0,001$ extremely significant (***). (Prism version 5.0, GraphPad Prism Software, Inc., San Diego, CA).

5. Results

5.1 Analysis of native REST protein in HeLa cells

Although the molecular weight calculated on the basis of REST aminoacidic sequence is about 116 kDa, western blotting analysis shows that this transcription factor has higher apparent molecular weight; the difference could be explained by post-translational modifications of the protein, such as glycosylation, as previously demonstrated in Prof. Spampinato's laboratory.

To study the endogenous form of REST we evaluated the expression of this transcription factor in the cervical adenocarcinoma cells HeLa: to recognize REST protein, analysis was carried out using commercial antibodies, raised against the C-terminal region of REST (07-579, Millipore).

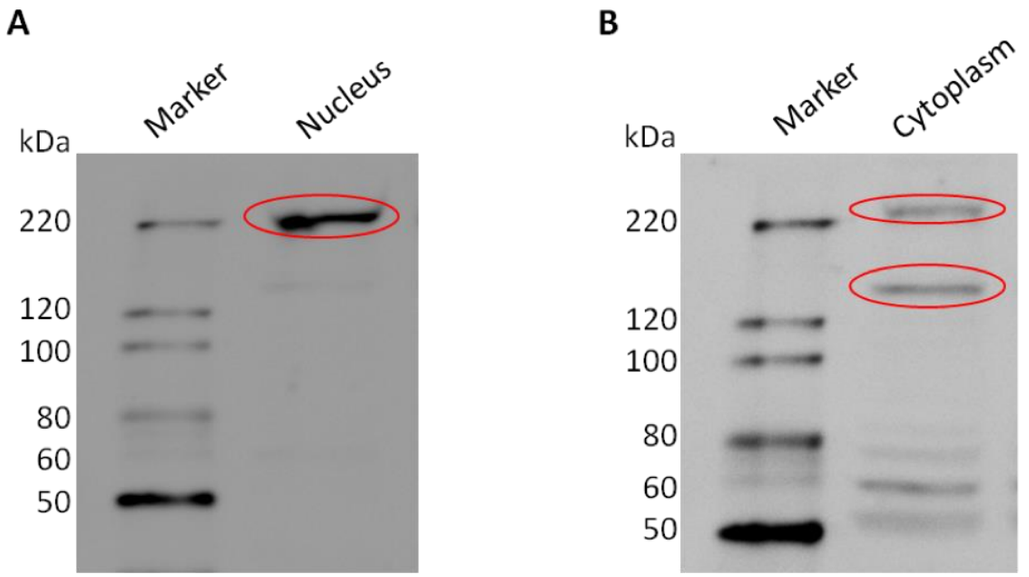

Figure 5.1.1 Western blot analysis of REST expression in cervical adenocarcinoma cells HeLa. Panel A, nuclear extract; panel B, cytoplasmic extract. Detection was carried out using a commercial antibody, raised against the C-terminal region of REST.

In nuclear protein extracts of HeLa cells, we found one specific band corresponding to REST with an apparent molecular weight of ~220 kDa (Figure 5.1.1-A). Cytoplasmic REST migrated as two bands of apparent molecular weights around 150 and 220 kDa (Figure 5.1.1-B).

5.2 Analysis of histone H3 acetylation in HeLa cells

The N-terminal repression domain of REST interacts with mSin3, which recruits two class I histone deacetylases (HDAC1 and HDAC2); REST C-terminal repression domain interacts, as previously described, with CoREST and forms a complex containing HDAC1 and HDAC2, LSD1, BRG1, G9a and MeCP2. Thus, once recruited by REST, the complex HDAC1 and HDAC2 deacetylate histone H3 lysine 9 (H3-K9) and histone H3 lysine 14 (H3-K14), causing a condensed chromatin structure that contributes to silence target genes transcriptional activity.

In order to evaluate a degree on which REST influences chromatin structure and, subsequently, transcription activity in HeLa cells, we evaluated the histone H3 acetylation levels which may be related to the amount nuclear REST protein content (Figure 5.2.1).

H3 histone acetylation was decreased by 47% in cells overexpressing REST as these were transfected with 10 μg of the plasmid pCMV-Taq2B containing REST coding sequence; conversely, it was increased by 89% after REST down-regulation using a specific siRNA (Figure 5.2.1). Thus, I found that the levels of nuclear REST and of acetylated form of histone are in an inverse relationship; suggesting that REST is sufficient to induce a notheworthy chromatin remodeling in HeLa cells.

Furthermore, I assayed the capability of valproic acid (VPA) to change histone H3 acetylation levels in HeLa cells. VPA is a powerful class I HDAC inhibitor that causes hyperacetylation of the N-terminal tails of histones H3 and H4 in cultured cells and in vivo. Valproic acid relieves HDAC-dependent transcriptional repression and possess antitumor activity.

Western Blot analysis carried out on nuclear fraction of protein extracts was performed after VPA treatment at four concentrations (0.25 mM, 0.5 mM, 0.75 mM and 1 mM) and two time point-cell exposure (24 and 48 hours). I found a significant increase of H3 histone acetylation levels in VPA-treated cells (Figure 5.2.2). Histone H3 acetylation changes are related to VPA concentration and to cell exposure time of this HDAC inhibitor. In fact, after 24 hours of treatment, 0.25 mM valproic acid caused a 75% increase of histone H3 acetylation in comparison to untreated cells (control) whereas 1 mM VPA, a 180% increase was observed. After 48 hours of VPA exposure, histone H3 acetylation levels increased by approx. 40% in cells exposed to 0.25 mM VPA and up to 700% after 1 mM VPA, in comparison to control cells (Figure 5.2.2).

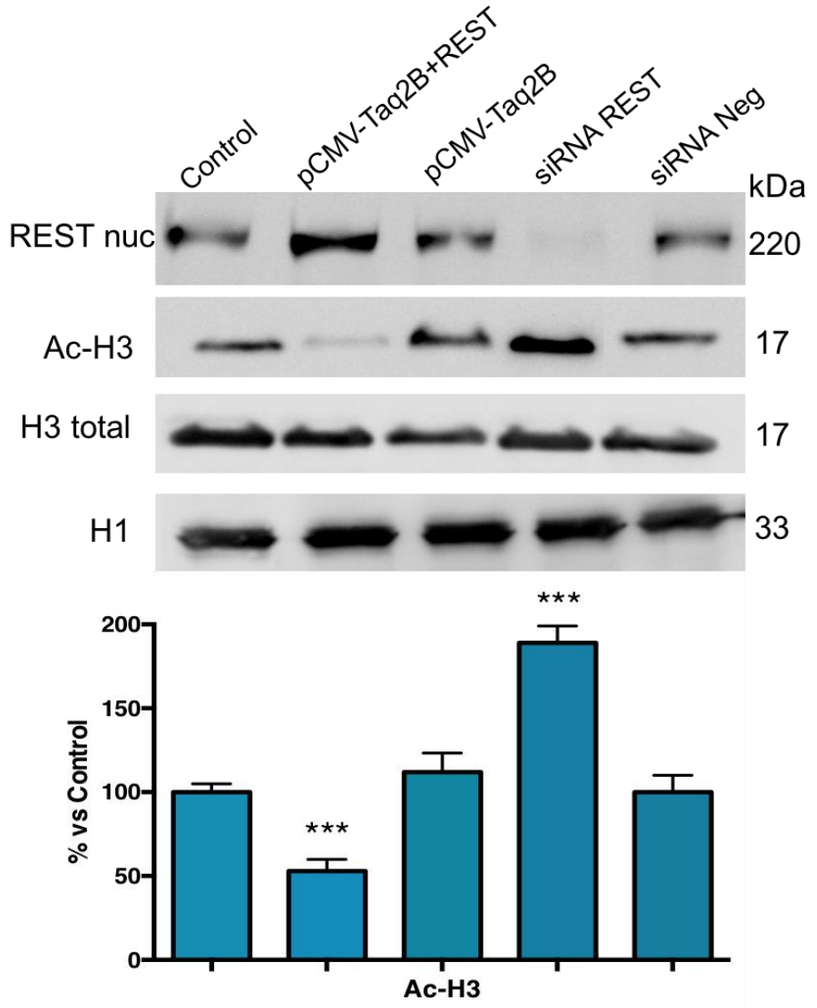

Figure 5.2.1 Representative western blot analysis of nuclear REST (REST nuc) protein expression and acetylated form of H3 histone (Ac-H3) from HeLa cells, transfected with REST-expressing plasmid (pCMV-Tag2B+REST), mock plasmid (pCMV-Tag2B), REST-specific siRNA (siRNA REST) and Silencer® Select Negative Control No.1 (siRNA Neg). Cells were collected 48 hours after transfection or treatment. The blot was reprobed for total H3 histone and histone H1 as loading controls. Below: densitometric analysis of the Ac-H3 bands, values are the mean ±SEM, n=3 of % changes vs. Ctrl. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ vs Ctrl (Newman-Keuls test after ANOVA).

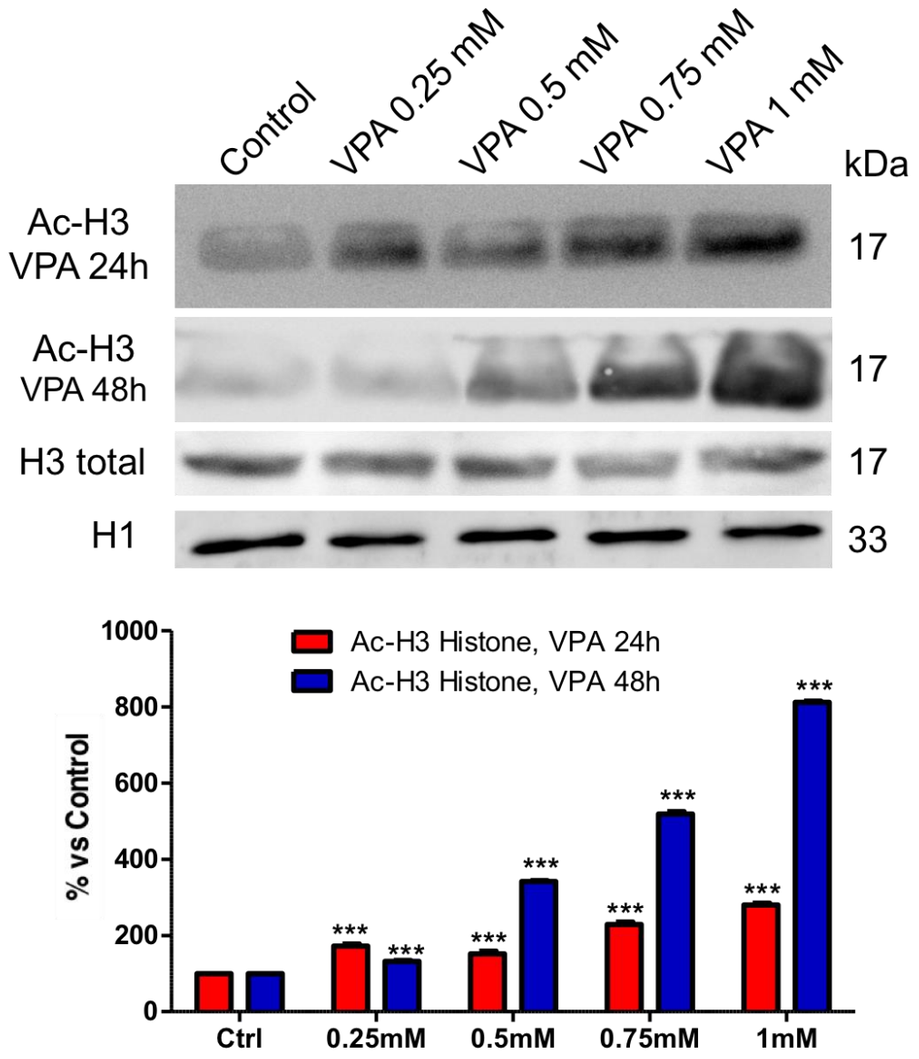

Figure 5.2.2 Representative western blot analysis of acetylated form of H3 histone carried out on histone extracts of HeLa cells treated with VPA of different concentrations (0.25-1mM) for 24 and 48 hours (Ac-H3, VPA 24h and Ac-H3, VPA 48h respectively). The blots were reprobed for histone H1 (H1) as loading control. Below panel reports densitometric analysis of the bands, values are presented as % changes (mean ±SEM; n=3 vs Ctrl) *** $p < 0.001$ vs Ctrl (Newman-Keuls test after ANOVA).

5.3 Evaluation of VPA cytotoxicity in HeLa cells

To investigate whether valproic acid has any cytotoxic effect on HeLa cells, we performed the MTT assay after VPA treatment. The MTT assay is a based on a colorimetric evaluation for assessing cell metabolic activity: NAD(P)H-dependent cellular oxidoreductase enzymes may reflect the number of viable cells. These enzymes are capable of reducing the tetrazolium dye MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to its insoluble formazan, which has a purple color. Thus, MTT assay is employed to measure cytotoxicity (i.e. loss of viable cells) or cytostatic activity (shift from proliferation to quiescence) of certain agents.

HeLa cells were first seeded in a 96-well plate and grown in complete medium for 24 hours; thereafter, cells were treated with an extended range of concentrations of VPA (0.25 – 1.75 mM) for 48 hours and the MTT assay was performed as reported in the materials and methods chapter. We found that valproic acid treatment slightly increased HeLa cell death (cytotoxicity) to a maximum 1.25-fold change with 0.75 mM VPA for 48 hours comparing to untreated cells (Ctrl), however, this increase is not statistically significant (Figure 5.3.1). These data, thus suggest that VPA contribution to HeLa cell death was not considered in the following experiments.

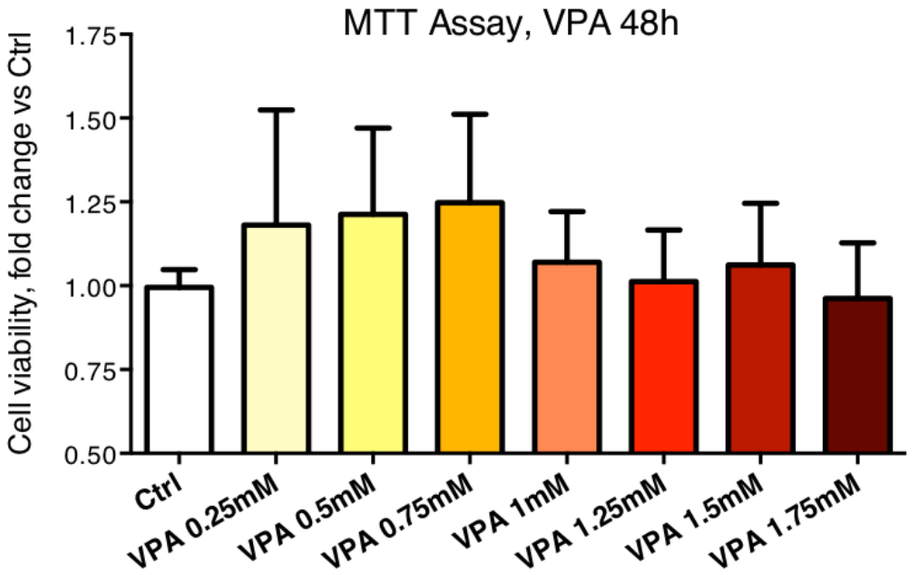

Figure 5.3.1 Effects of VPA on HeLa cell cytotoxicity evaluated by the MTT assay. Cell cytotoxicity determined by MTT assay is expressed as fold change of dead cells and is presented as mean of three experiments made in quadruplicate. No any significant different mean value *vs* control was found.

5.4 VPA influences expression of the REST complex with corepressors and HDAC1

5.4.1 REST, CoREST and HDAC1 are influenced by VPA

In order to assess whether in HeLa cells VPA-mediated histone H3 acetylation (described in chapter 5.2) involves changes of REST and/or its corepressors mSin3a, CoREST and HDAC1, we performed a series of experiments in which HeLa cells were treated with 0.25 – 1 mM VPA for 24 or 48 hour; thereafter, nuclear and cytoplasmic proteins were extracted and reprobed with antibodies selective for the mentioned above proteins.

Western Blot analysis of HeLa nuclear extracts after 24 hours of exposure to different concentrations of VPA showed that REST levels were not affected by the treatment, mSin3a and CoREST show a trend to increase and HDAC1 displays a bell-shape profile: it increases

rapidly after treatment with 0.25mM VPA but drop in cells exposed to higher concentrations (Figure 5.4.1-A). Western blot of cytoplasmic extracts shows that the two forms of REST in the cytoplasm (approximately 220kDa and 150kDa) do not change significantly after VPA treatment. CoREST, as well as mSin3a, in the cytoplasm are also present in two forms, that could be ascribed to post-translational modifications of these proteins. The levels of CoREST and mSin3a do not seem to be affected by VPA treatment whereas HDAC1 displays a tendency to decrease at higher VPA concentrations (Figure 5.4.1-B).

Analysis of western blot on HeLa nuclear extracts after 48 hours of exposure to VPA indicates that REST protein levels decrease in a concentration-dependent manner from 77% (0.25mM VPA) to 61% (1mM VPA) in comparison to control cells. mSin3a levels have a tendency to increase, CoREST and HDAC1 – to decrease (to 68% and 70% respectively at 1mM VPA) (Figure 5.4.2-A). Western blots of HeLa cytoplasmic extracts, 48 hours after VPA treatment, showed that VPA produces a concentration-dependent decrease of the assayed proteins: both forms of REST decrease from 66% (0.25mM VPA) to 29% (1mM VPA); CoREST levels decrease from 70% (0.25mM VPA) to 30% (1mM VPA) and HDAC1 levels decrease from 77% (0.25mM VPA) to 42% (1mM VPA) (Figure 5.4.2-B).

According to the above presented data, I found that both nuclear and cytoplasmic REST protein levels are decreased in HeLa cells exposed to VPA for 48 hours but not after 24 hours in a concentration-dependent manner. Moreover, a similar pattern is observed for REST corepressors: CoREST and HDAC1, but not for mSin3a. Present data, thus, suggest that VPA is capable not only to inhibit HDAC1 in HeLa cells, but also to down-regulate REST repression complex acting at transcriptional level and/or through processes that degrade these proteins.

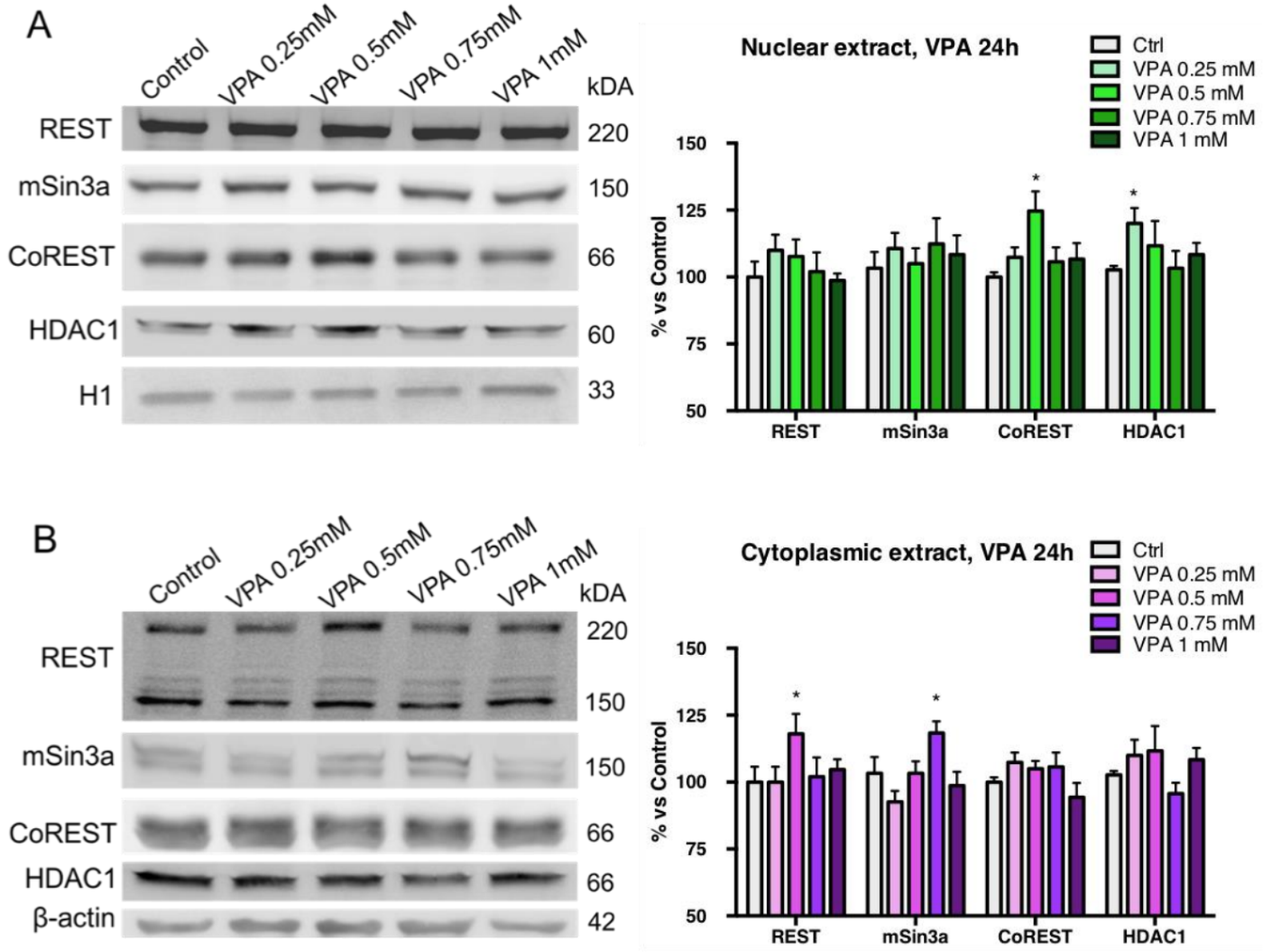

Figure 5.4.1 Expression of REST complex with corepressors and HDAC1 in HeLa cells after 24 hours of treatment with valproic acid (0.25, 0.5,0.75,1mM). Panel A, western blots carried out on nuclear extracts. Panel B, western blots carried out on cytoplasmic extracts. Histone H1 and beta-actin served as loading controls. On the right: densitometric analysis of bands, values are the mean ±SEM; n=3, expressed as % vs. Ctrl. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ *vs* Ctrl (Newman-Keuls test after ANOVA).

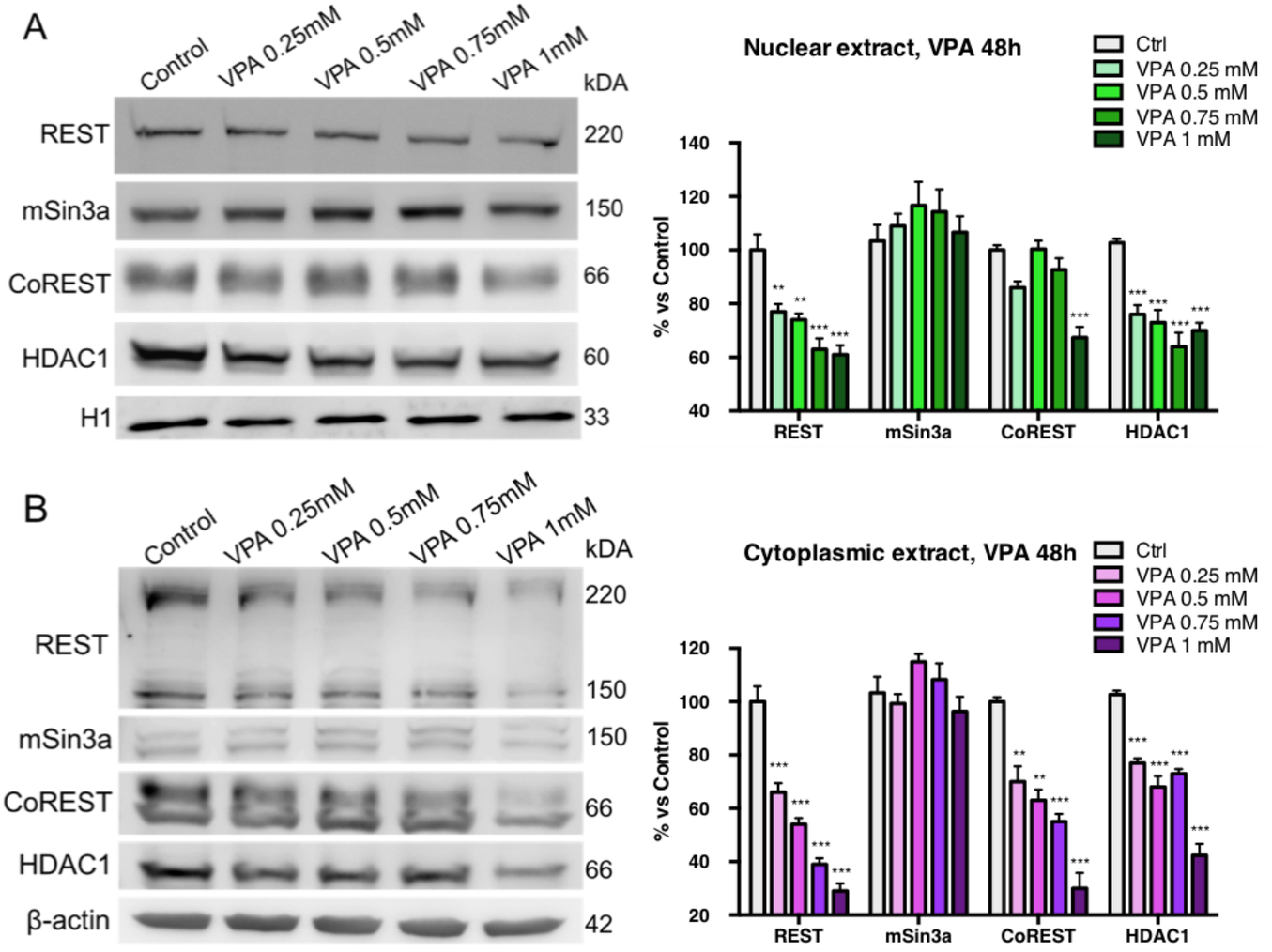

Figure 5.4.2 Expression of REST complex with corepressors and HDAC1 in HeLa cells after 48 hours of treatment with valproic acid (0.25,0.5,0.75,1mM). Panel A, western blots on nuclear extracts. Panel B, western blots carried out on cytoplasmic extracts. Histone H1 and beta-actin served as loading controls. On the right: densitometric analysis of bands, values are the mean ±SEM; n=3, expressed as % vs. Ctrl. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ *vs* Ctrl (Newman-Keuls test after ANOVA).

5.4.2 VPA modulates REST degradation through ubiquitin-proteasome system

To investigate if VPA-caused REST down-regulation involves post-translational mechanisms, I treated HeLa cells with VPA (0.5 – 1 mM) and during the last 6 hours of treatment, I added to the cells the proteasome inhibitor MG132 to the final concentration of 10μM. MG132 (amino acid sequence Z-Leu-Leu-Leu-al) is a specific, potent and cell-permeable proteasome inhibitor (Ki = 4 nM). It reduces the degradation of ubiquitin-conjugated proteins in mammalian cells by the 26S proteasome complex (Delcros et al., 2003).

Western blot analysis of HeLa nuclear and cytoplasmic extracts shows that MG132 is able to prevent VPA-mediated REST decline in the nuclear and in the cytoplasmic cell compartment (Figure 5.4.3-A, B). Thus, VPA modulates REST down-regulation in HeLa cells, at least partially, through its degradation by Ubiquitin-proteasome system, accelerating this process.

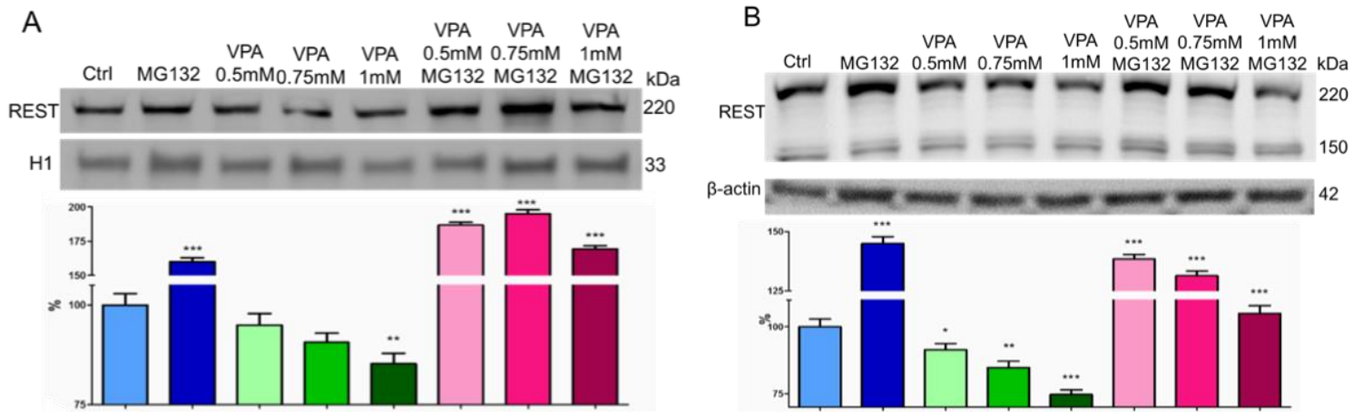

Figure 5.4.3 Representative immunoblots of REST protein levels in HeLa cells treated with VPA and the proteasome inhibitor MG132. Panel A, western blots carried out on nuclear extracts. Panel B, western blots carried out on cytoplasmic extracts. Histone H1 and beta-actin served as loading controls. Below: densitometric analysis of REST bands, values are the mean ±SEM; n=3, expressed as % vs. Ctrl. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ *vs* Ctrl (Newman-Keuls test after ANOVA).

5.5 Evaluation of VPA influence on REST complex with corepressors/HDAC1 and micro-RNA target

As previously described, REST is part of a repression complex with two cofactors (mSin3a at the N terminus and CoREST at the C terminus) to bind to genomic DNA and to silence target genes and micro-RNAs. Moving from the previous observations on VPA-caused decrease in REST, CoREST and HDAC1 protein levels, we hypothesized that this effect of VPA may occur at transcriptional level.

Thus, to ascertain any change of REST repression complex (REST, CoREST, HDAC1 and mSin3a) in HeLa cells, we treated them with VPA (0.25-1mM) for 24 hours; according to previous studies, this time frame is sufficient for switching VPA-mediated transcription profile in vitro. Thereafter, total cellular RNA was extracted and mRNA transcripts were quantified by RT-PCR analysis using specific primers indicated under materials and methods (Table 5).

In the result section, I have reported that VPA treatment for 24 hours did not cause any significant change in HDAC1 and mSin3a mRNA levels (Figure 5.5.1). On the contrary, REST and CoREST mRNA levels were significantly down-regulated in HeLa cells exposed to the heist concentration of VPA (0.75mM). In fact, REST mRNA level reached 0.232-fold change comparing to the controls, CoREST mRNA level reached 0.14-fold change in comparison to control cells.

One of the first miRNAs identified as a direct target of REST by binding studies and expression analysis was miR-124a, the most abundant miRNA in the adult mammalian brain. It was found that miR-124a may act in turn by silencing REST, allowing differentiation of neuronal phenotype (Lim et al., 2005). Nowadays the list of putative REST-target miRNAs has grown substantially: computational analyses, genome-wide binding studies and ChIP analyses have identified several neuron-specific miRNAs, including miR-9, miR-29a/miR-29b, miR-95, miR-132, miR-133, miR-135b, miR-139, miR-153, miR-218-212, miR-346, miR-375 and miR-455, which are regulated by REST (Otto et al., 2007). And

in turn, some miRNAs were found to target 3'UTRs to negatively regulate the members of REST repression complex, e.g. MeCP2, CoREST and REST itself. For instance, miR-9 targets 3'UTR of REST and significantly reduces endogenous REST, whereas a reduction of REST results in enhanced miR-9 expression (Laneve et al., 2010).

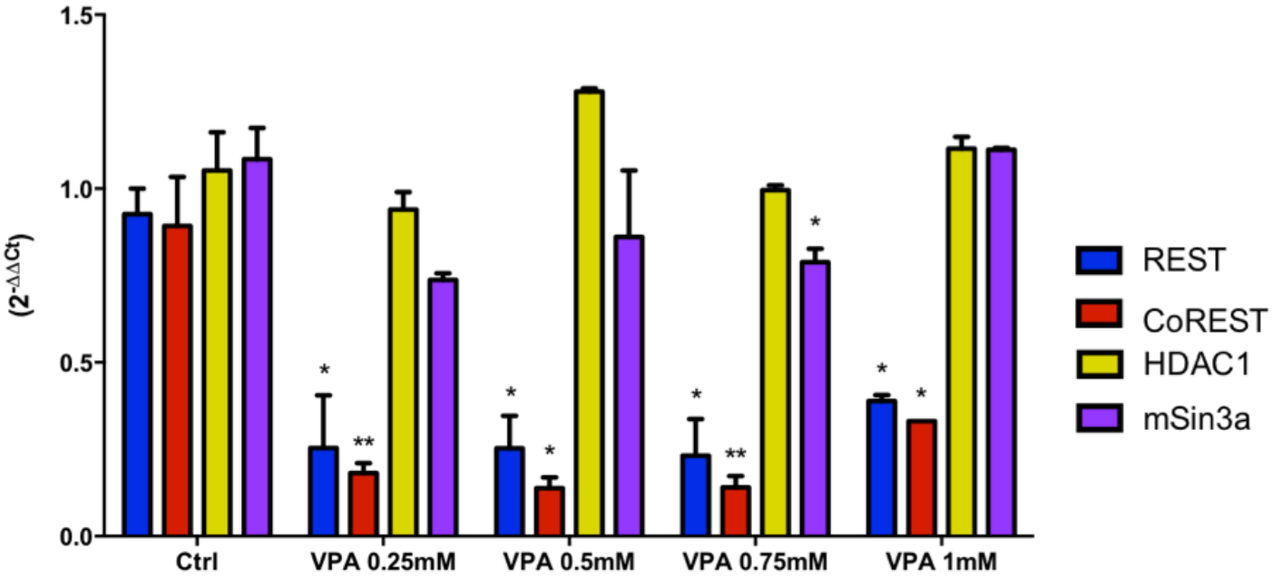

Figure 5.5.1 REST and corepressors mRNA levels in HeLa cells after 24 hours of treatment with VPA. Values were calculated using the $2^{-\Delta\Delta Ct}$ method and are the mean of experiments carried out in triplicates. *p<0.05; **p<0.01; ***p<0.001 *vs* Ctrl (Newman-Keuls test after ANOVA).

To test the possible outcome of VPA-mediated decrease of REST, its corepressors CoREST and HDAC1 on REST-regulated micro-RNA levels, we treated HeLa cells as reported previously, for 24 and 48 hours. After VPA treatment, total cellular RNA was extracted, and REST target miRNAs miR-9 and miR-124a were quantified by RT-PCR analysis using specific TaqMan probes reported under materials and methods. VPA treatment caused up-regulation of both miRNAs: miR-9 and miR-124a following a time- and concentration-dependent manner (Figure 5.5.2 – A, B). Levels of mir-9 after 24 hours of VPA increased up to 8.2-fold change in cells exposed to the highest concentrations of this agent (0.75 mM and 1 mM) in

comparison to untreated cells. A more prolonged exposure to VPA for 48 h induced a maximum increase of miR-9 levels in cells treated with 1mM VPA as it reached a 35-fold change (Figure 5.2.2 - A). RT-PCR analysis of another REST target microRNA, miR-124a, revealed a less significant and evident relationship between VPA and this miRNA. Thus, after 24 hours exposure to VPA, no any statistically significant change in miR-124a expression was detected in HeLa cells. Conversely, after 48 hours, of VPA (1 mM) induced a significant up-regulation of miR-124a levels to 4.9-fold change (Figure 5.2.2 - B).

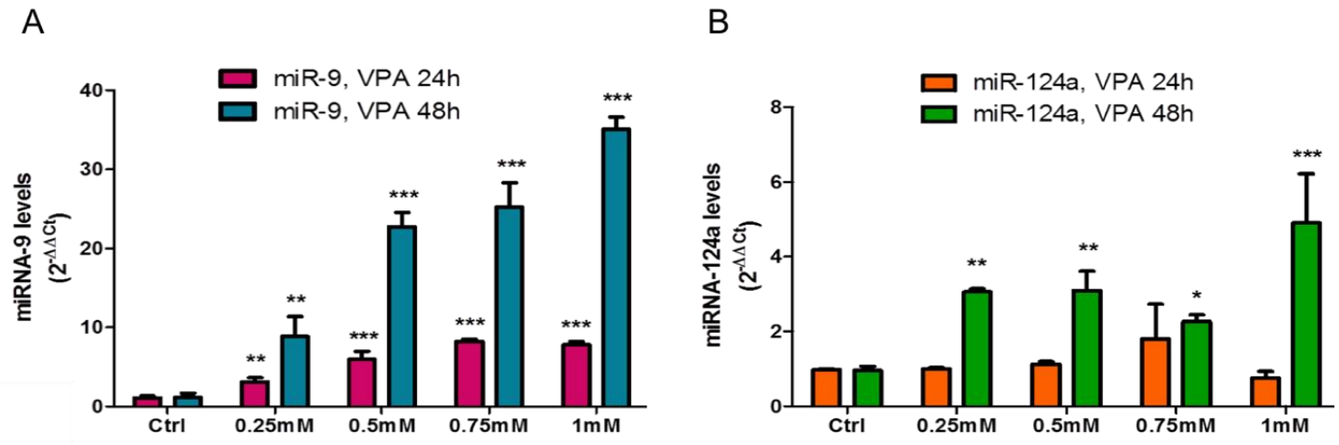

Figure 5.5.2 VPA effects the expression of REST target micro-RNAs. A – miR-9 levels and B – miR-124a levels in HeLa cells after 24 and 48 hours of treatment with VPA. Identical experiments were performed three times. Values were calculated using the $2^{-\Delta\Delta Ct}$ method and are the mean of experiments carried out in triplicates. *p<0.05; **p<0.01; ***p<0.001 *vs* Ctrl (Newman-Keuls test after ANOVA).

These data seem to indicate that VPA down-regulates REST and its corespressor CoREST at the transcriptional level and, possibly, this effect could be related to an up-regulation of REST target microRNAs miR-9 and miR-124a. A more significant effect was observed for miR-9 levels.

5.6 Cloning of REST 3' untranslated region (3'UTR)

To further investigate the role of REST target miRNAs in cervical cancer cells HeLa, I have focused on miR-9, in agreement with the results above shown. Moreover, it should be mentioned that miR-9 may represent a marker of cervical cancer prognosis (Conaco et al., 2006; Packer et al., 2008). miR-9 and miR-9* are, in fact, processed from the same primary transcript from 3 genomic loci (miR-9-1, 9-2 and 9-3); miR-9-1 and miR-9-3 both have upstream RE-1 sequences that could be occupied by REST. Moreover, the 3'UTRs of REST and CoREST contain miRNA recognition elements (MRE) for miR-9 and miR-9*, respectively. The full length 3'UTR of REST contains two conserved MRE sites for miR-9 close to each other in the middle of 3'UTR, one miR-9* MRE and the third miR-9 MRE are located at the end of REST 3'UTR.

To further explore any relationship between miR-9 and REST in HeLa cells, I have cloned the portion of REST 3'UTR containing two miR-9 recognition elements downstream to the sequence encoding Renilla luciferase to construct, by this approach, a gene reporter system capable to evaluate any effect of mirR-9 on transcription activity of REST 3'UTR (Figure 5.6.1).

This fragment of REST 3'UTR, length 1319bp, was amplified by PCR (Figure 5.6.2; panel A) and inserted into an intermediate vector pCR-Blunt II–TOPO® plasmid (Invitrogen™, Carlsbad, CA, USA) according to manufacturer's instructions. An example of restriction analysis of the pCR-BluntII–TOPO+REST-3'UTR is shown in Figure 5.6.2 (panel B). The restriction enzyme HindIII cuts only once the inserted DNA fragment and once the DNA sequence of the pCR-BluntII–TOPO plasmid. The agarose gel electrophoresis shown in Figure 5.6.2 (panel B), two bands of different size: 3976 and 862 base pairs can be observed.

Furthermore, using XbaI and XhoI restriction enzyme, the fragment of REST 3'UTR was transferred into the final vector pLightSwitch_3UTR which contains XbaI and XhoI restriction sites as well. Finally, the novel plasmid pLightSwitch_3UTR+REST-3'UTR was sequenced (BMR Genomics, Padua, Italy); this analysis confirmed the correct insert of REST

3'UTR and both miR-9 recognition elements were correctly placed. Thus, this novel genetic vector for employed to study regulation of REST transcription by miR-9.

A

5′ctcatagatggttggtgttcatatggctactttacaataaagagaactgtaagtgatatttggaaactaca
aacctggaattaggagatataattattccttcaagttttatagaatatcacttgggagattccaaagccatag
ctattacgcggcaaacctaggataag**(FOR)**cacctccctctagaaaaggtagtatgagtgctggtagaccag
ctgcaactttcctatacagtgaaaaaggctggtgaaacaagtacagtccagatttttaaaatcatactttct
cagggatctccacaaactggtgggtgtcctggctgtctgtgtgatagcctctttctataggtgaggcctcaaa
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tgaggagtaagttgtgtgataatggtatgtgttttgtgtgcatgaatggacattgtaaatgttgaattctagg
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ggaagtttttccatttgaaaaatgtgatgtttgcatggaactgtttgaaacttttttatttttctagtccccct
cccccacactggatagaatttagcctctcgagtacagt**(REV)**agaattttccctttggataaaagaacaaaa
attgaacatgttatttgtaaattgatgtttagtaattagtgataaacttgaaatactagcatatattataagc
cttaatcttaggtagtcttatgaaaatgaatctcttaactatcttttgaacctgtattcacatt**3′**

B

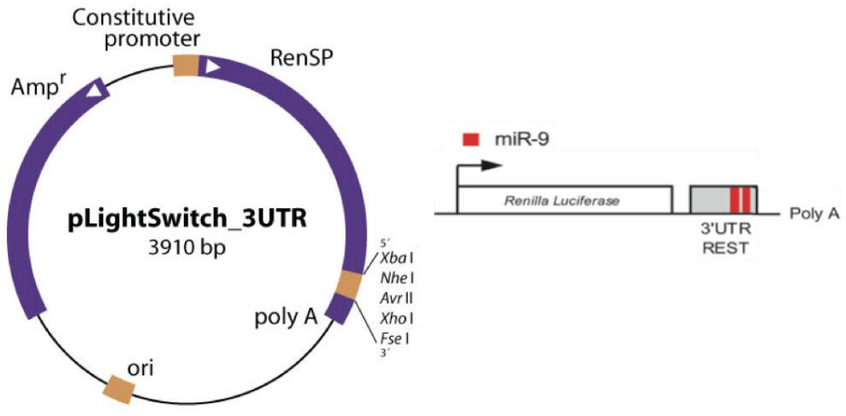

Figure 5.6.1 REST 3'UTR cloning scheme. Panel A, portion of REST 3'UTR which contains two miR-9 binding sites (marked in re), HindIII (green) and SpeI (yellow) are the choosen restriction enzyme sites. Primers (marked in blue) were designed to amplify a 1319 bp fragment of REST 3'UTR. Panel B, Map of pLightSwitch_3UTR plasmid that was used as a final vector for REST 3'UTR cloning.

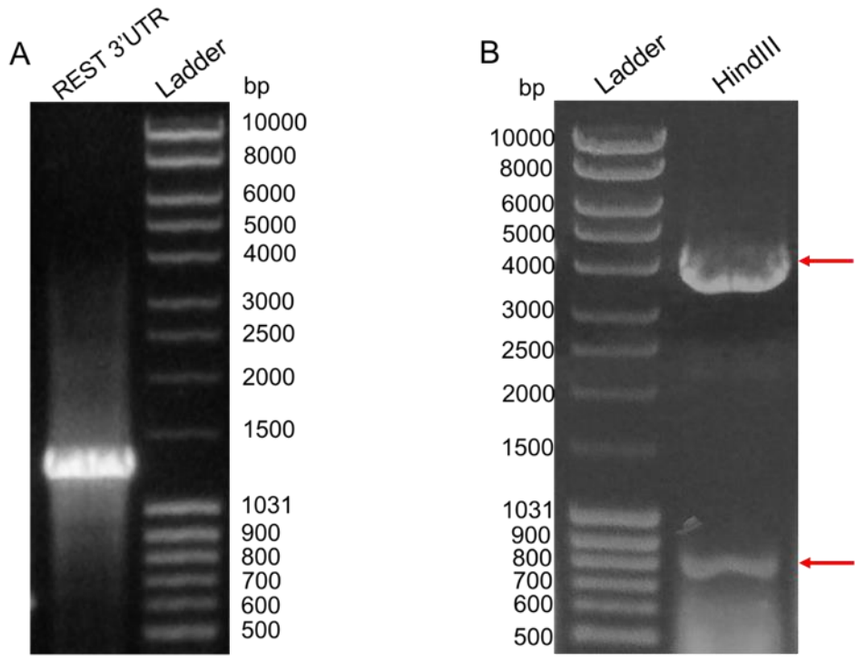

Figure 5.6.2 Agarose gel electrophoresis: A – amplified REST 3'UTR fragment with length of 1319 bp; B – restriction analysis of pCR-BluntII–TOPO+REST-3'UTR with HindIII restriction enzyme.

5.7 miR-9 regulates REST transcription at 3'UTR

To further investigate the contribution of miR-9 on regulation of REST transcription in HeLa cells exposed to VPA, we performed Gene Reporter Assay with the newly constructed vector pLightSwitch_3UTR+REST-3'-UTR.

Gene Reporter Assay showed that VPA treatment significantly reduces Renilla luciferase expression acting on REST 3'UTR which contains mir-9 binding sites (Figure 5.7.1). Thus, 0.5 mM VPA decreased R. luciferase expression to 56.5%, 1 mM VPA – to 44.3% *vs.* control cells. Moreover, transfection of HeLa cells with miR-9 mimic reduced R. luciferase expression to 36.6% compared to the control. VPA exposure along with miR-9 mimic transfection further reduced REST transcription in an additive mannerto cells exposed to

VPA alone. These results indicate that previously detected down-regulation of REST after VPA treatment along with miR-9 up-regulation may be due to REST mRNA degradation after miR-9 binding to its 3'UTR (Conaco et al., 2006).

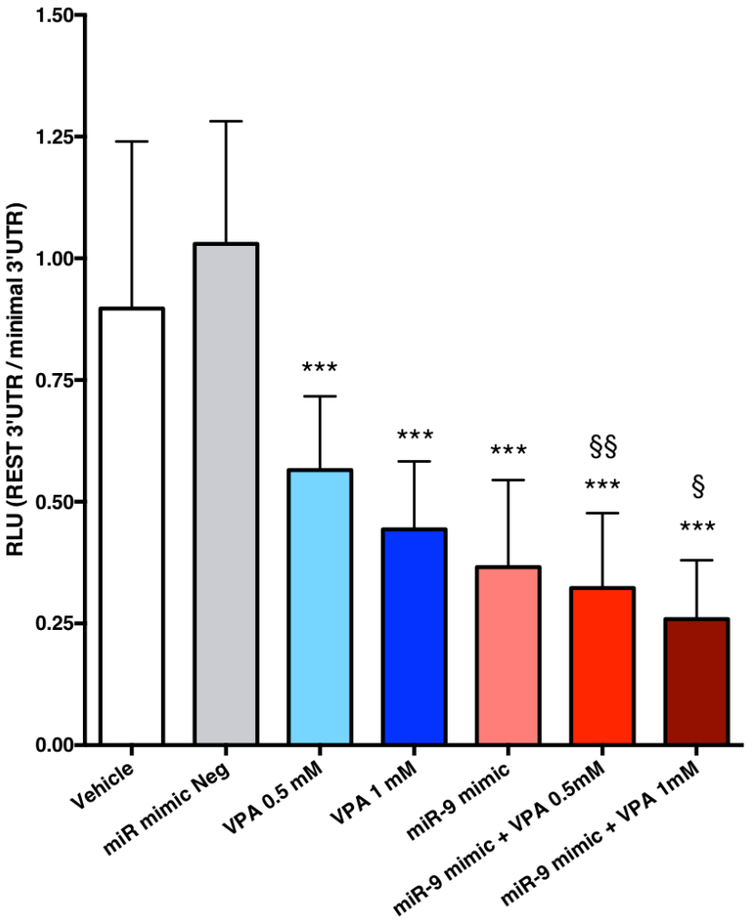

Figure 5.7.1 Gene Reporter Assay. Relative Luminescence Units of Hela cells co-transfected with pLightSwitch_3UTR+REST-3'UTR and human miR-9 mimic and treated with VPA for 24 hours over minimal 3'UTR. Values are the mean of at least 3 different experiments carried out in triplicates. *p<0.05; **p<0.01; ***p<0.001 vs Vehicle, §p<0.05; §§p<0.01; §§§p<0.001 *vs* corresponding cells treated with VPA alone (Newman Keuls test after ANOVA).

5.8 REST and CoREST down-regulation promote HeLa cell apoptosis

It has been reported that REST-mediated chromatin compaction through HDAC recruitment reduces apoptosis in HeLa cells caused by anti-Fas antibody plus PD 98059; thus, suggesting that REST has an antiapoptotic effect on these cell line (Baiula et al., 2012). Moving from the previous results on VPA-caused REST and CoREST down-regulation (Paragraphs 5.4 and 5.5), we hypothesized that this effect of VPA on REST complex and its target miR-9 may contribute to induce cell apoptosis. To confirm this hypothesis, we performed a series of experiments using Guava Nexin® Assay to recognize apoptotic cells and to discriminate between apoptotic and necrotic/dead cells in accordance with the manufacturer's instructions.

Thus, to investigate if any REST or CoREST down-regulation may contribute to apoptosis induction, HeLa cells were transfected with 20 nM of specific siRNA for REST or CoREST and 24 hours later were treated with VPA (0.25 – 1 mM) for 48 hours. Similarly, I analyzed the effect of miR-9, that reduces REST levels, on cell apoptosis. HeLa cells were transfected with 20nM of human mirVana® miR-9-5p mimic and treated with VPA as previously described. An additional set of cells were transfected with siRNA Negative (siRNA Neg) or miR mimic negative (miR mimic Neg) and treated with VPA; alternatively, HeLa cells were exposed to VPA alone for 48 hours.

I observed that HeLa cells undergo to apoptosis after 48 hours of VPA treatment, an effect produced in a concentration-dependent manner: the maximum effect of 10% of apoptotic cells was achieved at 1mM VPA (Figure 5.8.1). Following siRNA-mediated REST down-regulation, a significant increase of VPA-mediated cell apoptosis was noted, in fact, apoptosis reached 25.8% in cell exposed to 1 mM VPA and treated with siRNA REST (Figure 5.8.1). Thus, indicating that REST down-regulation contributes to apoptosis in HeLa cells, an event that pontentiate VPA-mediated apopotosis. A similar, albeit less pronounced effect, was observed following CoREST down-regulation with specific siRNA and VPA treatment: a maximum level of 19.5% apoptotic cells, following 1mM VPA (Figure 5.8.1) was found. The up-regulation of miR-9 by transfection of miR-9 mimic had the most distinct effect on

apoptosis induction. miR-9 mimic alone caused 24.3% of cell death which is the same level of apoptotic cells as with REST siRNA + VPA 0.75mM (Figure 5.8.1). The maximum effect on HeLa cell apoptosis induction was achieved in cells transfected with miR-9 mimic and treated with 1 mM VPA.

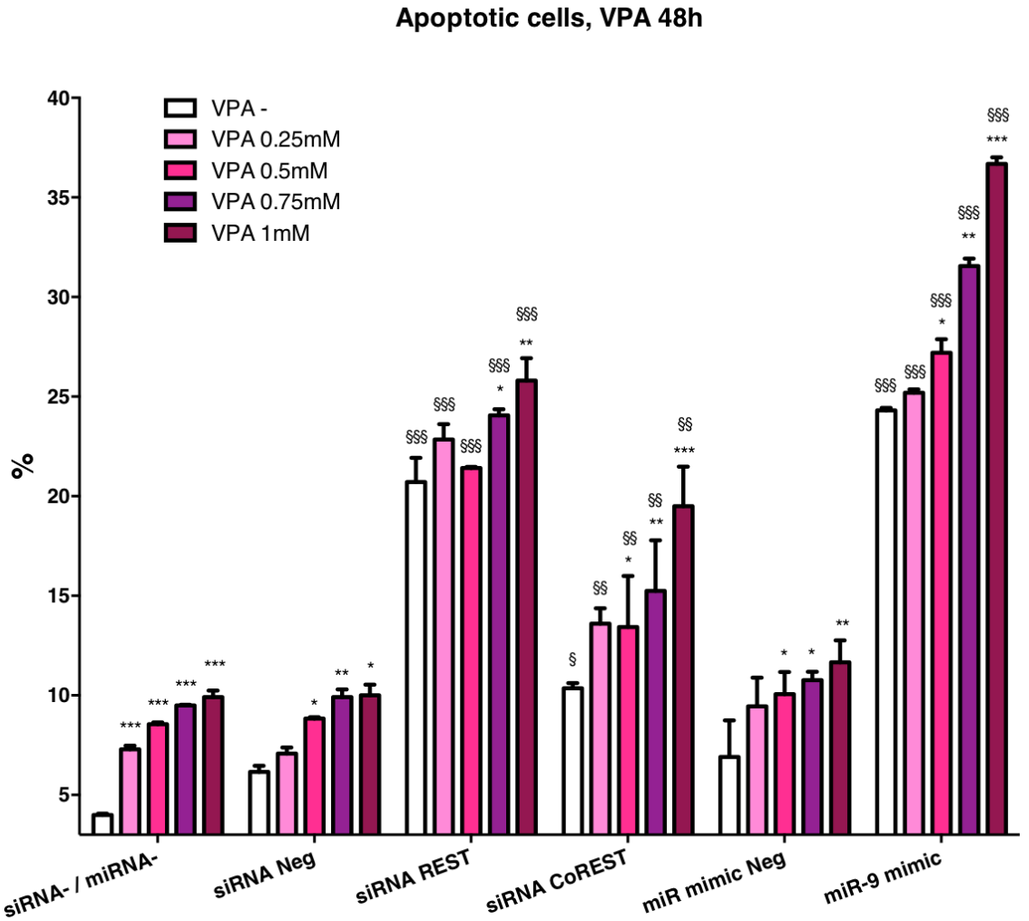

Figure 5.8.1 Detection of apoptotic HeLa cells by the Guava Nexin® Assay. Statistical plot of flow cytometry analysis of apoptotic HeLa cells, transfected with REST or CoREST siRNA, or with miR-9 mimic and treated with VPA for 48 hours. Values are the mean ± SEM of at least three experiments carried out in triplicates. *p<0.05; **p<0.01; ***p<0.001 vs not treated cells (VPA-) of each set of columns, §p<0.05; §§p<0.01; §§§p<0.001 vs corresponding cells treated with VPA alone (Newman-Keuls test after ANOVA).

These data confirm the hypothesis on any anti-apoptotic role of REST in HeLa cells, and show a partial involvement of REST corepressor CoREST in apoptosis induction and, furthermore, uncover a pro-apoptotic role of REST target miR-9.

5.9 REST down-regulation is related to BAX upregulation

To clarify any cell pathway through which REST down-regulation contributes to HeLa apoptosis, I performed a series of RT-PCR experiments in which I searched for transcriptional changes of known pro-apoptotic markers such as BAX and BAK and/or down-regulation of anti-apoptotic markers such as Bcl-2 and Bcl-XL. The BCL-2 protein family determines the commitment of cells to apoptosis through mitochondrial dysfunction (Donovan and Cotter, 2004). Mitochondrial dysfunction includes perturbations in the mitochondrial membrane potential, production of reactive oxygen species (ROS) and the release of the cytochrome C (Cyt C). When released, cytochrome C activates a downstream caspase program, which leads to death substrates cleavage (e.g., PARP) and ultimately to cell apoptosis.

In these experiments, I found that VPA alone, as well as its association to REST siRNA, promotes up-regulation of BAX (Bcl-2-associated X protein) gene transcription in a concentration-dependent manner. In cells treated with VPA alone, up to 1.91-fold change was observed, and up to to 2.37- fold change in cells treated with siRNA REST and VPA (Figure 5.9.1). BAX is a member of Bcl-2 family that binds to and antagonizes the Bcl-2 protein, therefore acting as an apoptotic activator. Interestingly, transfection with REST siRNA caused a higher and statistically different BAX mRNA levels in comparison to corresponding VPA treatments without REST siRNA. This finding is consistent with previous results on higher percentage of apoptotic cells after REST down-regulation along with VPA treatment in comparison to VPA alone (see Paragraph 5.8).

Thus, VPA treatment along with REST down-regulation promotes a marked apoptosis of HeLa cells possibly via Mitochondrial Control of Apoptosis Signaling Pathway controlled by Bcl-2 family

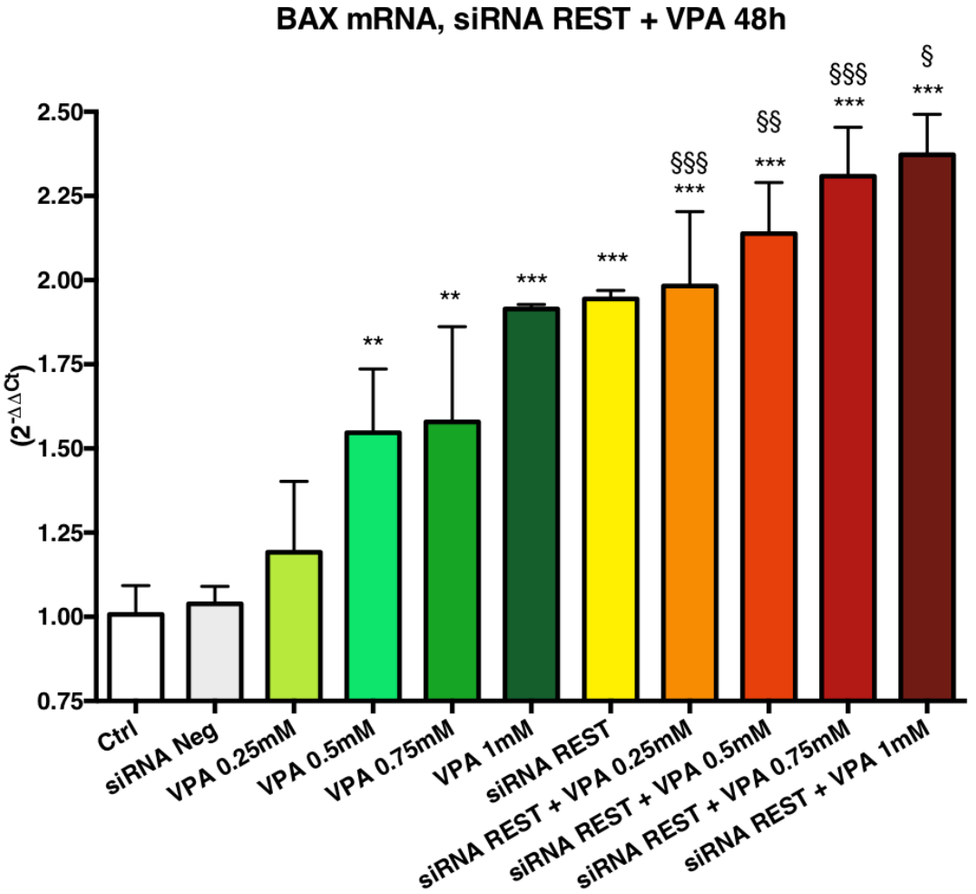

Figure 5.9.1 BAX mRNA levels in HeLa cells after transfection with REST siRNA and VPA treatment (0.25 – 1 mM) for 48. Values were calculated using the $2^{-\Delta\Delta Ct}$ method and are the mean of experiments carried out in triplicates. *p<0.05; **p<0.01; ***p<0.001 vs Ctrl; §p<0.05; §§p<0.01; §§§p<0.001 *vs* corresponding type of VPA treatment without transfection (e.g. siRNA REST + VPA 0.25mM *vs* VPA 0.25mM) (Newman-Keuls test after ANOVA).

6. Discussion

REST (RE-1 silencing transcription factor, or NRSF –neuron-restrictive silencing factor) that binds to a conserved RE-1 motif present in the promoter region of regulated genes and represses their transcription in neuronal and non-neuronal cells (Bruce et al., 2004). REST recruits corepressors (CoREST, mSin3a) and multiple chromatin modifying enzymes (HDAC1/2, demethylase LSD1 and methyltransferase G9a), causing chromatin compaction and altering gene expression by changing epigenetic marks (Ballas et al., 2005).

Furthermore, REST contributes to orchestrate the epigenetic regulation of its target genes through several miRNAs including miR-9/9*, miR-29a, miR-124a, miR-218 and others (Wu and Xie, 2006). It has been reported that miR-9 targets 3'UTR of REST and significantly reduces endogenous REST, whereas a reduction of REST results in enhanced miR-9 expression; interestingly, miR-9 could be used as a marker for cervical cancer prognosis (Conaco et al., 2006; Packer et al., 2008). In a previous study, we have reported that REST-mediated chromatin compaction through HDAC recruitment and following histone H3 deacetylation reduces apoptosis in HeLa cells caused by anti-Fas antibody plus PD 98059; thus, suggesting that REST has an antiapoptotic effect in these cells (Baiula et al., 2012).

REST seems to act as an oncogene or a tumor suppressor depending on the cell type and tumor progression (Negrini et al., 2013). Thus, it contributes to promote the development of brain tumors such as neuroblastomas, medulloblastomas and glioblastomas (Conti et al., 2012; Taylor et al., 2012). Conversely, REST acts as a tumor suppressor inhibiting tumorigenesis in epithelial cells like carcinomas of the lung, breast, and colon (Kreisler et al., 2010; Wagoner et al., 2010). To date, the role of REST in another type of epithelial cancer, cervical adenocarcinoma, has not been reviewed. Worldwide, cervical cancer is the fourth-most common cause of death from cancer in women, about 8% of the total cases and total deaths from cancer. Cervical cancer develops either from squamous cells and called squamous cell carcinomas, either from the mucus-producing gland cells of the endocervix

and called cervical adenocarcinomas. One of the main risk factors in developing cervical cancer is infection by high-risk types of the human papilloma virus (HPV), HPV 16 and 18 (Castle et al., 2012). In medical research, the most common cell line used as a model of cervical adenocarcinoma, is HeLa cell line (Masters, 2002).

Moving from the observations that REST repression complex is able to change its target gene and miRNA transcription via recruitment of multiple chromatin modifying enzymes, including HDAC1, and it may act as an oncogene or a tumor-suppressor in different human cancers, we hypothesized that REST also may have an important role in cervical cancer. Recently it was shown that valproic acid, which was found to a potent class I HDAC inhibitor, reduces the growth of HeLa cells via caspase-dependent apoptosis, and now VPA is in phase III clinical trials for the treatment of human cervical cancer (Coronel et al., 2011; Han et al., 2013).

Valproic Acid (VPA, 2-propylpentanoic acid) is a drug that initially was used for the treatment of epilepsy, bipolar disorder and as a mood stabilizer in psychiatry (Bowden, 2004; Trinka et al., 2014). Recently VPA has been recognized as a promising HDAC inhibitor showing anti-tumor activity that affects cell growth in different types of cancer in vitro and in vivo (Wang et al., 2013). However, epigenetic mechanisms contributing to VPA-mediated chromatin remodeling have been poorly investigated: it has been shown that VPA affects the activities of several enzymes and signal transduction pathways, but the mechanisms underlying the anti-cancer properties of VPA are not well characterized.

In this thesis, I have demonstrated that in the HeLa cell line, a model of cervical adenocarcinoma, nuclear REST accumulates in the nucleus as a single form with an apparent molecular weight of ~220 kDa whereas cytoplasmic REST is present in two isoforms with molecular weights around 150 and 220 kDa (Paragraph 5.1). In other reports, REST was shown to have different molecular weights from 116-120 kDA in PC12 cell line and murine endothelial cells (Chong et al., 1995), 150 kDa in mouse cortical neurons (Qiang et al., 2005), 200 kDa and 160-180 kDa in neuronal murine cells (Zuccato et al., 2003) and 200 kDa in HEK293, SH-SY5Y, PC12 and colorectal cancer cell lines (Grimes et al., 2000; Westbrook et

al., 2005). This variability was recently ascertained to post-translational modifications of REST protein by complex O- and N-linked glycosylation (http://amsdottorato.unibo.it/4288/).

Then, I ascertained the influence of REST on the chromatin structure and, subsequently, transcription activity in HeLa cells, by evaluation of histone H3 acetylation levels. I found that the levels of nuclear REST and of acetylated form of histone H3 are in an inverse relationship: H3 histone acetylation is decreased in cells overexpressing REST and conversely, it is increased after REST down-regulation. Furthermore, I assayed the capability of valproic acid (VPA) to change histone H3 acetylation levels in HeLa cells and I observed a significant increase in H3 histone acetylation levels in VPA-treated cells, in time- and concentration dependent manner (Paragraph 5.2).

Cellular levels of histone acetylation depend on the opposing roles of histone acetyltransferases (HATs) and histone deacetylases (HDACs). Histone acetyl transferase (HATs) enzymes remove the positive charge from the histones H3 and H4 by covalent adding of acetyl groups to the NH3+ group on their lysine residues, thereby decreasing the interaction of histones with the negatively charged phosphate groups of DNA (Roth et al., 2001). As a consequence, the chromatin is transformed into a more relaxed structure that is associated with higher levels of gene transcription (Verdone et al., 2006). Lysine acetylation is reversible: deacetylation, catalyzed by histone deacetylases (HDACs), remove acetyl groups from histone tails inducing histone hypoacetylation that is associated with gene silencing (de Ruijter et al., 2003).

Thus, REST absence compromises the formation of an effective repression complex with its co-repressors to inhibit histone acetylation. As a consequence, lacking REST, active HATs unfold chromatin and activate gene transcription. Conversely, REST over-expression promotes histone H3 deacetylation by HDACs, which counteract gene transcription. These observations are consistent with previously reported data on evaluation of REST regulation by epidermal growth factor in HeLa cells (Baiula et al., 2012).

Furthermore, I have studied the influence of VPA-mediated histone H3 acetylation on changes of REST, its corepressors mSin3a, CoREST and HDAC1 at mRNA and protein levels. I showed that in VPA treated HeLa cells, REST, CoREST and HDAC1, but not mSin3a mRNA levels were decreased depending on VPA concentration (Paragraph 5.5). Protein levels did not change after 24 hours of VPA exposure, but after 48 hours of treatment both nuclear and cytoplasmic REST, CoREST and HDAC1 protein levels decrease significantly and in relationship with VPA concentration (Paragraph 5.4). Moreover, proteasome inhibitor MG-132 restores VPA-caused decline in REST protein, suggesting that this drug may modulate REST degradation in HeLa cells through the ubiquitin-proteasome system.

Indeed, REST protein levels are regulated through the proteasomal degradation mediated by F-box protein β-TRCP (SCFβ-TRCP), an E3 ubiquitin ligase. During neural differentiation β-TRCP binds and ubiquitinates REST through its conserved phospho-degron with phosphorylated serine residues (Westbrook et al., 2008). In non-neuronal cells, TrCP-dependent degradation of REST allows transcriptional de-repression of Mad2, an essential component of the spindle assembly checkpoint (Guardavaccaro et al., 2008). Furthermore, β-TRCP over-expression is a common event in human epithelial cancers and causes oncogenic transformation of human mammary epithelial cells; interestingly, this action requires REST degradation (Kudo et al., 2004; Saitoh and Katoh, 2001). On the contrary, the deubiquitylase HAUSP (Herpesvirus-Associated-Ubiquitin-Specific Protease 7) antagonizes β-TRCP-mediated ubiquitination of REST: the balance between ubiquitination and deubiquitination may determine the net REST protein levels. When the HAUSP-mediated deubiquitination overrides β-TRCP-mediated ubiquitination, REST is stabilized. In contrast, when β-TRCP-mediated ubiquitination exceeds HAUSP-mediated deubiquitination, REST is targeted for degradation, which promotes cell differentiation by releasing repression of differentiation- associated genes (Huang et al., 2011).

I have evaluated any possible outcome of VPA-mediated decrease of REST, its corepressors and HDAC1 on REST-regulated micro-RNA levels by RT-PCR analysis and I found that VPA treatment causes significant up-regulation of both REST target miRNAs: miR-9 and miR-124a

in a time- and concentration-dependent manner (Paragraph 5.5). These data suggest that VPA influences REST and its corespressors CoREST expression at the transcriptional level and, probably as a consequence, causes an up-regulation of REST target microRNAs miR-9 and miR-124a with a more evident effect on miR-9 levels.

miR-9 has been proposed as one of the crucial regulators of neuronal development as well as of other physiological or pathological events and shown to possess onco-suppressor properties, with a crucial role in repressing cell proliferation of human neuroblastoma and medulloblastoma cells (Fiaschetti et al., 2014). Moreover, recently miR-9 was shown to be potential marker for cervical cancer prognosis (Cheung et al., 2012). Thus, in vitro cell profiling and cervical tumor tissue profiling showed that HPV oncogene activity resulted in upregulation of miR-9 expression and suppression of its targets, which may result in increased mobility of HPV infected cells (Liu et al., 2014). Moreover, miR-9 signature had been proposed to be important in nasopharyngeal carcinoma (Lu et al., 2014a), hepatocellular carcinoma (Cai and Cai, 2014) and papillary thyroid cancer (Sondermann et al., 2015).

3'UTRs of REST and CoREST contain miRNA recognition elements (MRE) for miR-9 and miR-9* respectively. The full length 3'UTR of REST contains two conserved MRE sites for miR-9 close to each other in the middle of 3'UTR, one miR-9* MRE and the third miR-9 MRE are located at the end of REST 3'UTR. To test miR-9 and REST interaction in HeLa cells, I cloned the part of REST 3'UTR containing two miR-9 recognition elements downstream from the gene encoding Renilla luciferase (Paragraph 5.6). Finally, the novel genetic vector for studying regulation of REST expression, plasmid pLightSwitch_3UTR+REST-3'UTR, was obtained.

To further investigate any contribution of miR-9 to REST transcription in HeLa cells exposed to VPA, I performed Gene Reporter Assay experiments which demonstrated that VPA significantly reduces Renilla luciferase expression acting on REST 3'UTR which contains mir-9 binding sites and transfection of HeLa cells with miR-9 mimic reduced R. luciferase expression, following a VPA concentration-dependent profile (Paragraph 5.7). These results indicate that previously detected down-regulation of REST following VPA treatment along with miR-9 up-regulation is due to REST mRNA degradation after miR-9 binding to its 3'UTR.

Interestingly, in Huntington Disease several microRNA levels with upstream RE-1 sites found to be decreased in HD cortices samples in comparison to healthy subjects. One of these deregulated miRNAs, bi-functional miR-9/9* refers to two components of the REST complex: miR-9 targeted REST and miR-9* targeted CoREST (Packer et al., 2008).

Moving from the previous results on VPA-mediated REST and CoREST down-regulation (Paragraphs 5.4 and 5.5), I have hypothesized that this effect of VPA on REST complex and its target miR-9 may contribute to the induction of HeLa cell apoptosis. Using Guava Nexin Assay, I found that HeLa cells undergo apoptosis after 48 hours of VPA treatment in a concentration-dependent manner: REST down-regulation with a specific siRNA significantly increases apoptosis. A similar but less pronounced effect was observed in HeLa cells treted with VPA following CoREST down-regulateion with a specific siRNA. In the expesriments, I have introduced an internal control group: up-regulation of miR-9 by transfection of miR-9 mimic, that induced cell apoptosis. These data have confirmed my hypothesis on anti-apoptotic role of REST in HeLa cells and suggest a contribution of the REST corepressor CoREST in apoptosis; furthermore, I have uncovered a pro-apoptotic role of REST target miR-9.

Finally, I found that VPA alone, as well as its association with REST siRNA, promotes up-regulation of BAX gene transcription promoted by VPA in a concentration-dependent manner. BAX (Bcl-2-associated X protein) is a member of Bcl-2 family that binds to and antagonizes Bcl-2 protein expression; therefore, acting as an apoptotic activator. Taken together, these data support the idea that VPA treatment along with REST down-regulation promotes apoptosis of HeLa cells, possibly via mitochondrial control of apoptosis signaling pathways controlled by Bcl-2 family.

Deregulation of a single transcription factor can be sufficient to alter cell morphology and eventually lead to many forms of human cancer (Lee and Young, 2013). In tumor cells, genes encoding transcription factors could be deleted, amplified, translocated or subjected to point mutations that result in a gain- or loss-of-function (Darnell, 2002). MicroRNAs (miRNAs), a class of small noncoding RNA molecules, have emerged as a novel class of regulatory

molecules that are essential to influence various cellular processes, including cell signaling, cell development, cell death, cell proliferation and differentiation.

In conclusion, the present study aims to contribute to a more accurate comprehension of the processes responsible for REST activity in a model of epithelial cervical adenocarcinoma, and relevant for a detailed knowledge of important events causing oncogenesis. Moreover, considering the crucial role of epigenetic regulation of gene transcription in the etiology of many pathological conditions, any further knowledge in this field could find important and innovative pharmacological applications.

7. References

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