Epigenetics and Human Health

Walter Doerfler
Petra Böhm *Editors*

Epigenetics – A Different Way of Looking at Genetics

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Chapter 5
Short Biologically Active Peptides as Epigenetic Modulators of Gene Activity

B.F. Vanyushin and V.Kh. Khavinson

Abstract Short (2–4 aa) peptides investigated increased life span of rats and improved various physiological functions and health status in aged people. Biological activities of peptides are tissue (cell) specific. Peptide bronchogen AlaGluAspLeu (AEDL) regulates synthesis of the Ki67, Mcl-1, p53, CD79, and NOS-3 proteins in cultures of the human bronchoepithelial cells. Bronchogen activates the expression of genes coding for the bronchoepithelial cell differentiation factors Nkx2.1, SCGB1A1, SCGB3A2, FoxA1, FoxA2, as well as MUC4, MUC5AC, and SftpA. Pancragen LysGluAspTrp (KEDW) increases expression of many differentiation factor genes in human pancreatic cell culture. The regulatory peptide actions seem to be due mainly to site-specific peptide–DNA binding. Peptides are able to penetrate into the animal cell, nucleus, and nucleolus; therefore, in principle, they may interact potentially with various components of the cytoplasm and nucleus including DNA and RNA. In vitro peptides interact with various oligonucleotides and DNA; they can discriminate between methylated and unmethylated DNAs. Peptides investigated interact with histone H1 and core histones; this interaction depends on the histone nature and peptide primary structures. Peptides modulate specifically the in vitro action of eukaryotic CNG-site-specific endonucleases. It is suggested that binding of peptides to gene promoters that seems to protect promoter against methylation may be a mechanism of regulation of transcription by peptides. It was shown that in vivo peptides can influence methylation of gene promoters, and, in fact, the modulation of gene activity by peptides is associated often with diminution of CpG methylation in respective gene promoters. Thus, the site-specific (complementary) interactions of short biologically active peptides with DNA may control epigenetically the cell genetic functions, and these interactions seem to play an important role in regulation of gene activity even at the earliest stages of life origin and in the evolution. Besides, the site-specific interactions of peptides with histone tails in chromatin may serve as

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other control epigenetic mechanisms of regulation of gene activity and cell differentiation.

Keywords DNA methylation • Epigenetics • Peptides • Gene promoters • Transcription regulation

5.1 Introduction

Peptides form the extensive and diverse regulatory signal system controlling the physiology, growth, and development of animals and plants. In animals, this is well known for relatively large peptides, most of which are hormones (neuropeptides, growth hormones, and many others). More than 140 peptide therapeutics are already evaluated in clinic as pharmaceuticals: they are highly selective, efficacious, relatively safe, and well tolerated (Fosgerau and Hoffmann 2015). Besides, they are active in the microdoses, and (because they are short) these peptides are quite economical and relatively easy to be synthesized and produced.

The functional role of very small peptides, in particular, consisting of 2–4 amino acid residues was unclear for a long period and underestimated. Nevertheless, short peptides as signal molecules can trigger or inhibit various genetic processes and biochemical reactions in the cell. Under the influence of short biologically active peptides, the mean life span of experimental animals was increased by 30–40 %, and growth of spontaneous, induced, and transplanted tumors was suppressed (Khavinson 2005, 2009; Anisimov and Khavinson 2010). After in vivo introduction of peptides GluTrp, LysGlu, AlaGluAspGly, and AlaGluAspPro in mice, the essential changes in gene expression were observed in the myocardium and brain (Khavinson 2005, 2009). In transgenic mice, these peptides inhibit by two- to fourfold the expression of the breast cancer gene HER-2/neu that correlates with decrease in adenocarcinoma size (Khavinson 2005, 2009). In mice and rats, short peptides increase transcription of IL-2 and c-Fos genes in lymphocytes and various structures of the hypothalamus; this mainly stipulates immunomodulating, oncomodifying, and stress-protecting properties of these peptides (Khavinson 2005, 2009). The geroprotecting action of short peptides is associated with chromatin activation in blood lymphocytes of senile patients (Khavinson 2005, 2009). The treatment of human fibroblasts with peptide AlaGluAspGly induced telomerase activity and extended the length of telomeres by 2.5-fold; this was accompanied with an increase in the cell division number by 42.5 %; thus, Hayflick's limit was surmounted (Khavinson 2005, 2009). In aged and senile patients, the short peptides investigated increase the melatonin level, improve different physiological functions, and decrease mortality by twofold (for 8–12-year period of randomized clinical observation) (Khavinson 2005, 2009).

The essential physiological changes induced by peptides are due mainly to selective modulations of the gene expression pattern in respective tissues or cells. Unfortunately, the molecular mechanisms of the gene expression modulations with

Table 5.1 Peptides used

Formula	Registered (commercial) name
GluAspArg	Pinealon®
LysGluAspTrp	Pancragen®
AlaGluAspArg	Cardiogen®
LysGluAspGly	Testagen®
AlaGluAspLeu	Bronchogen®
AlaGluAspGly	Epitalon®

short peptides remain still essentially unknown, but there is no doubt that these mechanisms are mainly of the epigenetic nature. As far as the cytosine DNA methylation is the epigenetic genome modification playing significant role in stable changes of gene activity, the investigation of the individual gene methylation profiles under the short peptide influence is of a special interest.

More than 40 different short biologically active peptides were synthesized in the Saint Petersburg Institute of Bioregulation and Gerontology (Russia); the biological action and pharmaceutical properties of these peptides were more or less comprehensively investigated, and they are already evaluated in clinic. A short list of some such peptides studied and mentioned here is represented below (Table 5.1).

It has been assumed that tissue- or gene-specific binding of short peptides with DNA is a fundamental principle of their physiological action (Khavinson 2005, 2009). In fact, it can be really spoken on site-specific peptide binding with DNA. This is called the allosteric interaction of peptides with DNA. Some weighty evidences appeared showing that binding of relatively short peptides can be really site specific and depends on the primary structures of peptides and respective DNA sites (sequences) recognized (Laigle et al. 1982; Reddy and Manjula 2009; Khavinson 2009). Binding of short peptides in the large DNA groove is accompanied with essential local changes in the DNA double helix structure and gene expression (Khavinson 2005, 2009). However, detailed mechanisms of such selective binding of short peptides with DNA and of resulting induction or repression of gene expression are still poorly investigated. In spite of many available data on the existence and formation of various specific short peptide–DNA complexes, the site-specific interaction and binding of such peptides and the possible penetration of peptides in the cell nucleus are still often called in question.

5.2 Penetration of Short Peptides in the Cell Nucleus

As far as an idea on the interaction of short peptides with DNA as a possible regulatory mechanism of peptide action was suggested (Khavinson 2005, 2009), it was necessary, at least, to learn that peptides can really penetrate in the nucleus of living cell.

After incubation of HeLa cells in the presence of fluorescein isothiocyanate (FITC)-labeled peptides, the significant fluorescence of many small granules

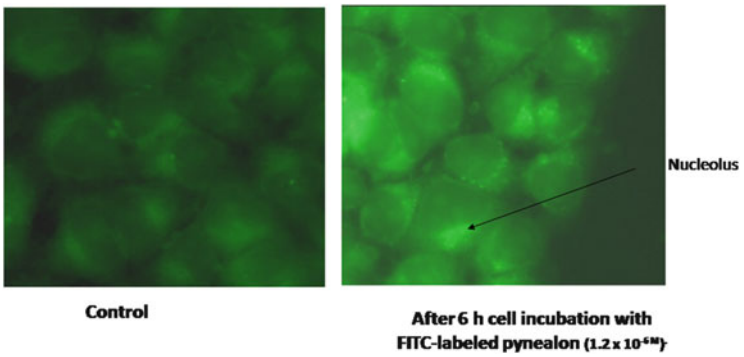

Fig. 5.1 Fluorescence of FITC-labeled peptide GluAspArg in nuclei isolated from HeLa cells (Fedoreyeva et al. 2011)

(probably, endosomes) in the cytoplasm was observed. A marked fluorescence was observed also in isolated nuclei of HeLa cells (Fig. 5.1). The relative intensity of fluorescence of various labeled peptides in nuclei of HeLa cells was different. In particular, the significant fluorescence was markedly expressed in the nuclei of cells incubated with fluorescence-labeled peptides pinealon and epitalon and to lesser degree after cell incubation with peptide testagen. The differences in the fluorescence intensity of nuclei observed can be due to different penetration degree of various fluorescence-labeled peptides into the cell and intracellular structures, different peptide binding degree with these structures and their components in the nucleus, and different quenching degree of fluorescence of labeled peptides used on their interaction with nuclear structures. In fact, we did not expect to observe very intense fluorescence of labeled peptides in the isolated nuclei. The nucleus does not seem to be a depot for such short peptides, and high peptide concentrations are unnecessary for the performance of their signal functions in a possible regulation of gene activity, particularly, on binding with DNA. Unlike the relatively discrete character of the fluorescence distribution in cytoplasm, the fluorescence in nucleoplasm is relatively homogenous, but it is more expressed in the nucleolus (Fig. 5.1). Thus, the fluorescence-labeled peptides used can penetrate into the animal cell and its nucleus and nucleolus. In principle, it is an obligatory event for their possible interaction with nucleic acids of a nucleus and nucleolus. Detection of significant fluorescence in a nucleolus shows that short peptides potentially have possibility to interact with both DNA and RNA (a nucleolus is a place of synthesis of RNA and, in particular, ribosomal RNA).This agrees well with an observation that peptides entered the cells, migrated to the nucleus, bound nucleoli, and poisoned RNA biogenesis, which caused cell death (Kwon et al. 2014).

5.3 The In Vitro Interaction of Peptides with Oligonucleotides and DNA

An investigation of the interaction of fluorescence-labeled deoxyribooligonucleotides with short biologically active peptides showed that peptides having different primary structures bind with one and the same deoxyribooligonucleotide differently. For example, epitalon (AlaGluAspGly) unlike cardiogen (AlaGluAspArg) strongly quenches the fluorescence of FAM-GCG GCG TGA TCA GCG GCG GCG deoxyribooligonucleotide. The Stern–Volmer constants of the fluorescence quenching of labeled deoxyribooligonucleotides by different peptides differ essentially depending on the peptide amino acid sequences (Fedoreyeva et al. 2011). This indicates the site-specific interaction of various short peptides with nucleic acid structures. Cardiogen (AlaGluAspArg) did not quench the fluorescence of single-stranded monotonous oligonucleotides investigated, and it only slightly quenched the fluorescence of some double-stranded oligonucleotides. Therefore, cardiogen seems not to bind with single-stranded oligonucleotides and relatively monotonous nucleotide sequences. Pinealon (GluAspArg) that differs from cardiogen by the absence of one amino acid residue (Ala) induced relatively small quenching of fluorescence of all labeled deoxyribooligonucleotides used. Pinealon predominantly quenches fluorescence of oligo(dT) and least of oligo (dC). Pancragen (LysGluAspTrp) quenches fluorescence of oligo(dA) and oligo (dT) to similar extent, and it does not influence the fluorescence of oligo(dC) and oligo(dGC). Compared to pancragen, the change of one amino acid (Trp) for Ala in testagen results in an increase in the Stern–Volmer constant values by three- to fourfold. Testagen quenches also the fluorescence of oligo(dC) and especially of oligo(dGC). It is interesting that bronchogen (AlaGluAspLeu) does not bind with oligo(dGC) at all.

Interaction of all peptides (except for pinealon) with double-stranded oligo(dA) and oligo(dT) obtained by annealing of single-stranded oligo(dA) and oligo(dT) is characterized by lower (by two- to threefold) Stern–Volmer's constant values compared with that of single-stranded oligos. Thus, short peptides interacting with monotonous oligonucleotides prefer to bind to single-stranded structures.

In addition to monotonous oligonucleotides, we have used also synthesized fluorescence-labeled deoxyribooligonucleotides containing CG and CNG sites that are known to be recognized by eukaryotic cytosine DNA methyltransferases and some specific higher plant endonucleases (Fedoreyeva et al. 2007; Fedoreyeva and Vanyushin 2011): FAM-GCG GCG GAT GCG GCG GCG, FAM-GCG GCG GAT ACG GCG GCG, FAM-GCG GCG GAT CCG GCG GCG GCG, and FAM-GCG GCG TGA TCA GCG GCG. It was established that cardiogen and pancragen do not bind with GC-enriched oligonucleotide FAM-GCG GCG CAT GCG GCG GCG as well as with oligo(dGC), but testagen and epitalon bind with it demonstrating more or less similar constants of the fluorescence quenching. On interaction with pinealon, the fluorescence quenching constant of this oligonucleotide is by almost twofold higher compared to that observed at its binding with

oligo(dGC). Bronchogen binds with FAM-GCG GCG CAT GCG GCG GCG but not with oligo(dGC). Change of one G residue for T after A in an oligonucleotide results in an essential decrease in the Stern–Volmer constant value when this oligonucleotide interacts with all peptides, but change of G for C at the same position is accompanied with an essential increase in this constant value on the oligonucleotide interaction with bronchogen and, particularly, with epitalon. Pancragen was shown to slightly bind with FAM-GCG GCG GAT CCG GCG GCG GCG. Change of adenine residue for cytosine in this oligonucleotide does not affect the fluorescence quenching constant value on its treatment with pinealon or testagen.

Both ionic and hydrophobic interactions play an important role at the interaction of short peptides with oligonucleotides. Hydrophobicity indexes of pinealon (−11.5) and testagen (−11.3) are lower than that of epitalon (−5.6) and bronchogen (−1.4), but the fluorescence quenching constants on oligonucleotide interactions with bronchogen and, particularly, with epitalon are higher than that with testagen and pinealon. Oligonucleotides with TGA and TCA sequences inside of chains bind relatively well with all peptides including cardiogen.

The epitalon (AlaGluAspGly) binding (Fig. 5.2) is sensitive to the share of cytosine or guanine in nucleotide sequences. Judging the fluorescence quenching data, the constant of binding of epitalon with FAM-CGC CGC CAG GCG CCG CCG CGC (12 C residues) is by almost twofold higher than that with FAM-GCG CGG CGG CGC CTG CGC CGC (10 C residues); thus, peptide predominantly binds with oligonucleotide that has more cytosine residues. Introduction of the 5-methylcytosine residue into nucleotide sequence independently on C or G content in it increases the binding of oligonucleotides with epitalon. Thus, the peptide AlaGluAspGly binding is sensitive to the cytosine methylation status of oligonucleotides. Annealing of complementary oligonucleotides both containing and not containing 5-methycytosine resulted in a decrease in the Stern–Volmer constant

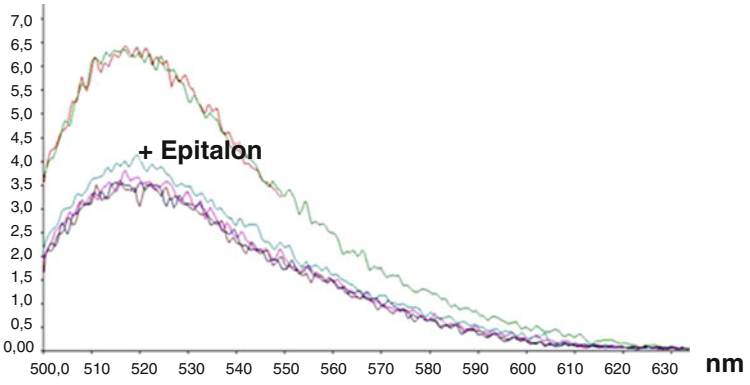

Fig. 5.2 Epitalon (AlaGluAspGly) quenches fluorescence of deoxyribooligonucleotide *(5′)* (FAM)-*cg-ccg-cca-ggc-gcc-gcc-gcg (3′)* containing CNG and CG sites. Ordinate—the fluorescence intensity and relative units (Khavinson et al. 2011)

values compared with that of initial oligos. Epitalon (AlaGluAspGly) prefers to bind with single-stranded oligonucleotide containing methylated cytosine. Testagen binds with oligos containing 5-methylcytosine weaker than with the same but unmethylated oligos. Annealing of complementary oligonucleotides does not decrease their binding with testagen unlike the binding with epitalon. In this particular case on the use of annealed oligonucleotides containing 5-methylcytosine, a small increase in the values of the fluorescence quenching constants was observed. Pancragen (LysGluAspTrp) and pinealon (GluAspArg) interact more or less similarly with single- and double-stranded structures relatively independent on the presence of 5-methylcytosine residues in oligos.

We have investigated the effect of short peptides on the fluorescence of DNA–ethidium bromide (DNA–EB) complexes (Fig. 5.3). In the study, the unmethylated (dam^-, dcm^-) and methylated (dam^+, dcm^+) λ phage DNAs were used. Ethidium bromide itself has very low fluorescence. In the presence of DNA, the fluorescence strongly increased due to ethidium bromide (EB) intercalation between base pairs in the DNA double helix. After addition of epitalon (AlaGluAspGly) to unmethylated phage DNA–EB complex, the fluorescence of this complex was quenched immediately, and maximum of the emission spectrum was shifted (from λ_{max} 591.8 nm to λ_{max} 596.1 nm) (Fig. 5.3). Character of the fluorescence quenching of the methylated phage DNA–EB complexes and unmethylated phage DNA–EB induced by epitalon was different. All other peptides studied except for

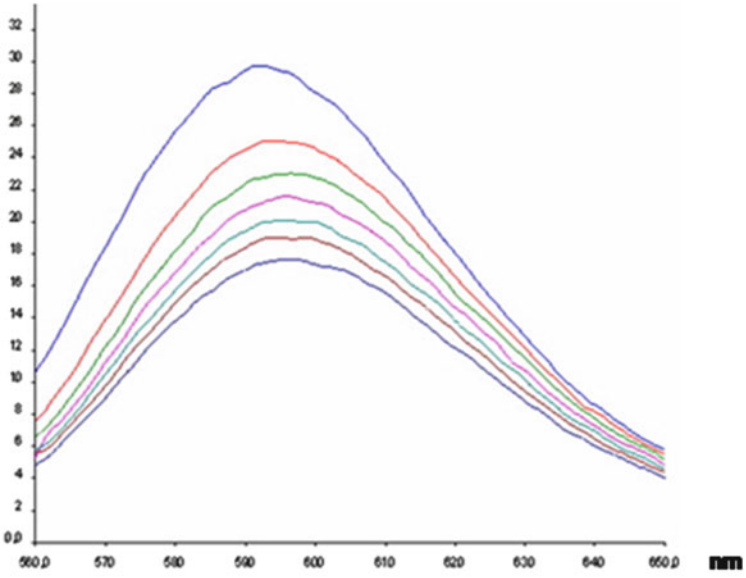

Fig. 5.3 Quenching and shift of fluorescence maximum of DNA–ethidium bromide complex on titration with epitalon AlaGluAspGly. Ordinate—the fluorescence intensity and relative units (Fedoreyeva et al. 2011)

cardiogen have similar character of the fluorescence quenching on titration of the DNA–EB complex.

Along with the investigation of effects of the intact peptides on fluorescence of DNA–EB complexes, we have studied also how these particular complexes may influence the fluorescence of proper fluorescence-labeled peptides. To some extent, it was a sort of reverse task. Titration of fluorescent derivative of epitalon with unmethylated λ phage DNA does not lead to a decrease in peptide fluorescence. But methylated phage DNA slightly quenches the fluorescence of labeled epitalon. As far as unmethylated DNA is more polar than methylated DNA, this seems to essentially influence the interaction of DNA with hydrophobic derivative of epitalon. FITC epitalon interacts preferentially with more hydrophobic methylated DNA compared with more polar unmethylated DNA.

Thus, short biologically active peptides interact specifically with single- and double-stranded deoxyribooligonucleotides and lambda phage DNA. Some of these peptides (e.g., epitalon (AlaGluAspGly)) prefer to bind with single-stranded oligonucleotides (Fig. 5.2). The interaction of peptides with the nucleic acid substrates depends on the nucleotide sequence. In other words, there are specific sites with definite nucleotide sequence in oligonucleotide and the respective amino acid sequences in peptides for mutual biopolymer binding.

In spite of detection of specific binding of short peptides with deoxyribooligonucleotides and DNA dependent on their primary structures, unfortunately we cannot yet name the exact address of binding of any definite peptide in terms of DNA nucleotide sequence.

On investigation of peptide binding with monotonous oligonucleotides, we have observed that pancragen (**Lys**GluAspTrp) was binding only with oligo(dA) and oligo(dT). However, lysin containing testagen (**Lys**GluAspGly) was binding with all monotonous oligonucleotides but preferentially with oligo(dGC). Pinealon (GluAspArg) containing arginine also prefers to bind with oligo(dGC). Though cardiogen (AlaGluAspArg) contains arginine, it does not bind with single-stranded oligonucleotides. Epitalon (AlaGluAspGly) and bronchogen (AlaGluAspLeu) that do not have diaminomonocarbonic amino acids prefer to bind with oligonucleotides containing 5-methylcytosine. Epitalon, testagen, pinealon, and bronchogen bind preferentially with sequences containing CNG sites; the first three peptides prefer to bind with structures containing CAG but bronchogen CTG sequences. It is quite reasonable to recall that CNG sequences are target sites for cytosine DNA methylation in plants and animals.

The existence of specific peptide binding with single-stranded oligonucleotides may have a special significance. Single-stranded stretches are always present in DNA. In particular, they appear on DNA replication, repair, and recombination. The interaction of short peptides with such DNA stretches can specifically control these genetic processes mentioned. Besides, intercalation of short peptides (epitalon) into DNA is accompanied with local unwinding of DNA chains (Khavinson 2009) that leads to origin of single-stranded targets for peptide binding with DNA. It may have a particular sense on the possible conjugative action of different peptides in the cell when one peptide serves as an inducer (modulator) of

origin of the DNA single-stranded structure but the other one may be a proper regulator of biological effect.

Thus, short biologically active peptides are able to in vitro selectively bind with definite oligonucleotides and DNA nucleotide sequences. Besides, some of these peptides can even discriminate between nucleotide sequences with different cytosine methylation status.

We have discovered the tissue, subcellular, and age specificity of DNA methylation (Vanyushin et al. 1970) and were the first to show that the DNA methylation pattern in cancerous cells is different compared with that in normal cells (Romanov and Vanyushin 1981). Taking these data into consideration, we can postulate that one and the same short biologically active peptide can bind with DNA differently depending on the DNA methylation character. Therefore, it may influence genetic processes including transcription and others in a different fashion in different tissues (cells), in the nucleus and mitochondria, in young and aged cells or patients, and in normal and malignant cells. Almost all these postulates are already proved experimentally (Khavinson 2009).

From our point of view, the site-specific peptide binding may or even should modulate the action of many proteins operating with DNA (RNA and DNA polymerases, DNA methyltransferases, endonucleases, DNA repair enzymes, many regulatory protein factors, and others) competing with peptides for the same binding sites in DNA. In particular, we have established that short peptides really modulate the action of eukaryotic CNG-site-specific endonucleases (Fig. 5.4) (Khavinson et al. 2011). Some hexapeptides are strongly selective structural ligands for protein-free Holliday junctions, and they block recombination (Ranjit et al. 2010).

We have suggested one of the most probable mechanisms of gene activation with short peptides (Khavinson et al. 2011): selective peptide binding with CNG or

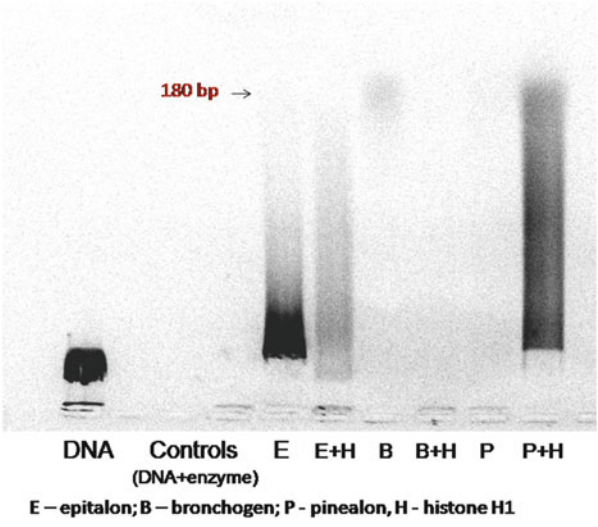

Fig. 5.4 Effects of peptides on hydrolysis of unmethylated lambda phage DNA by eukaryotic CNG-site-specific endonuclease WEN1 (Khavinson et al. 2011)

CG promoter sites may protect them against action of respective DNA methyltransferases, and, as a result, promoter will be unmethylated that is crucial for activation of most genes. The specific (allosteric) peptide–DNA interactions may epigenetically control genetic functions of the cell, and they most probably played a very important role even at the earliest stages of life origin and in subsequent evolution.

5.4 The In Vitro Interaction of Short Peptides with Histones

Judging fluorescence modulation (quenching), the short peptides (AlaGluAspGly, GluAspArg, AlaGluAspLeu, LysGluAspGly, AlaGluAspArg, and LysGluAspTrp) bind with FITC-labeled wheat histones H1, H2в, H3, and H4 (Fedoreyeva et al. 2013). As an example, the fluorescence quenching of FITC-labeled wheat histone H1/1 by the peptide cardiogen is represented in Fig. 5.5. This resulted from the interaction of peptides with N-end histone regions that contain respective and seem to be homologous peptide-binding motifs. Peptide binding with histones and histone–deoxyribooligonucleotide complexes depends on the histone nature and primary structures of peptides; thus, it is site specific. Histones H1 bind preferentially with single-stranded oligonucleotides by homologous sites in the C-end

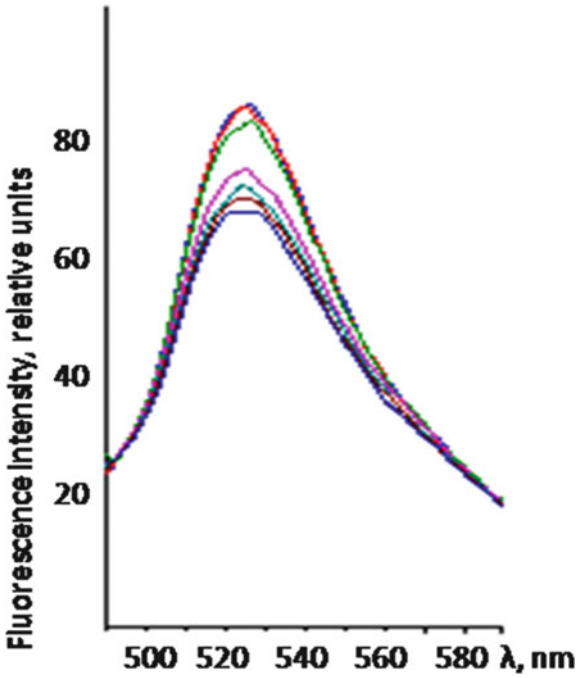

Fig. 5.5 The fluorescence spectrum of FITC-labeled wheat histone H1/1 on titration with peptide cardiogen AlaGluAspArg (Fedoreyeva et al. 2013)

protein region. Unlike histone H1, the core histones bind predominantly with double-stranded methylated oligonucleotides and methylated DNA. DNA or deoxyribooligonucleotides in a complex with histones can enhance or inhibit peptide binding. It is suggested that site-specific interactions of short biologically active peptides with histone tails can serve in chromatin as the control epigenetic mechanisms of regulation of gene activity and cellular differentiation (Fedoreyeva et al. 2013).

5.5 Short Peptides Affect Gene Expression and the Gene Epigenetic Status

Here we shall consider (as examples) the effects of only two peptides—bronchogen and pancragen—on transcription of different genes and CpG methylation status of promoters of these genes.

5.5.1 Bronchogen

Peptide bronchogen (AlaGluAspLeu, AEDL) is a tetrapeptide that restores the lung function in various pathologies. It is efficient in models of acute bacterial lung inflammation, chronicle fibrosis, and sublethal toxic lung damage (Khavinson et al. 2009). These pathologies are characterized by significant changes in lung morphology as well as in cell composition of bronchoalveolar fluid (BAL fluid) with increased neutrophil and lymphocyte quantity, as well as a reduced number of alveolar macrophages. The model of the acute lung inflammation on rats showed that peptide AEDL normalized the BAL fluid composition that evidenced the anti-inflammatory effect of the tetrapeptide. This peptide induced the expression of genes encoding some markers of cell proliferation and activity in bronchial epithelium. However, the molecular mechanisms of these processes have not been yet thoroughly investigated. So it was important to learn how peptide AEDL may influence the gene expression and synthesis of respective proteins (MKI67, MCL1, TP53, CD79A, NOS-3, and others) involved in a normal functioning of bronchoepithelial cells. Ki67 protein is a nonspecific marker of cell proliferation that, for instance, is reduced in chronic obstructive pulmonary (COPD) disease (Chiappara et al. 2013). Mcl-1 protein is an antiapoptotic factor of Bcl-2 family which in bronchial epithelium is reduced in a case of hypoxia. Factor p53 is a proapoptotic protein, the expression of which increases in bronchial epithelium under the influence of adverse environmental factors (such as air pollution) and lung pathology (COPD, cancer) (Porebska et al. 2006).

Membrane-crossing glycoprotein CD79 is a marker of inflammation and local immunity index due to its expression on some epithelial cells as well as on B lymphocytes, when its reduced expression correlated with an autoimmune

pathology. It was also interesting to investigate the NO synthase enzyme (NOS-3), since it is known to act as a catalyst for nitrogen oxide elaboration, which is a cell response modulator in various tissues including bronchial epithelium. Being formed and discharged from endothelium, NO inhibits thrombocyte aggregation, proliferation, and cell migration, regulates apoptosis, and maintains the function of the endothelial cell barrier.

To study the peptide AEDL's influence on the gene expression, we have chosen also the genes (NKX2-1, SCGB1A1, SCGB3A2, FOXA1, and FOXA2) that regulate the differentiation and activity of bronchoepithelial cells. Nkx2.1 protein, encoded by NKX2-1 gene, is known as a differentiation factor of bronchial epithelial burst-forming cells, and the disturbance of NKX2-1 expression is associated with miRNA365 pathology that can lead to lung cancer (Kang et al. 2013). The SCGB1A1 and SCGB3A2 genes encode corresponding secretoglobins produced by lung cells in all mammals. The lack of expression of SCGB1A1 and SCGB3A2 is known to aggravate chronic lung inflammation. Besides, SCGB3A2 protein serves as a marker of burst-forming cells of the respiratory tract. Its expression is regulated by Notch1 and Nkx2.1 proteins (Kurotani et al. 2011). The FOXA1 gene product is a factor of terminal differentiation of alveolar epithelium, and it affects SCGB1A1 secretoglobin expression (Kido et al. 2011). Another gene of this group FOXA2 encodes a factor of embryonic tissue differentiation in the lungs, pancreas, liver, and nervous tissue. It is important to note that the FoxA2 protein regulates gene activity through its interaction with histones (Li et al. 2012). The MUC4, MUC5AC, and SFTPA1 genes take part in a functional activity of bronchial epithelium. Mucin 4 and mucin 5AC are secreted by the respiratory tract epitheliocytes as a protective mucin formation. Disturbance in the proper expression of these genes leads to inflammation. Apart from that, reduced MUC4 expression serves as a potential marker and potential target for treatment of non-small cell lung carcinomas (Kwon et al. 2007). The SPA1 protein (SFTPA1 gene product) stimulates phagocytosis and TNF production and inhibits surfactant secretion. The level of SPA1 secretion correlates with respiratory function indexes in patients with COPD. The transcription aberration of this gene facilitates the development of lung adenocarcinoma.

Peptide AEDL regulated the expression of MKI67, MCL1, TP53, CD79A, and NOS-3 genes and the levels of Mcl-1, p53, CD79, NOS-3, and other markers (Khavinson et al. 2014). For example, peptide AEDL increased the level of Ki67, the inactivation of which leads to an inhibition of cellular proliferation and ribosomal RNA synthesis. The Ki67 level in "young" (3rd passage), "mature" (7th passage), and "old" (14th passage) bronchoepithelial cell cultures increased by 1.5-fold, 2.4-fold, and 3.4-fold, respectively (Khavinson et al. 2014). Both AEDL and KEDW peptides increase the level of antiapoptotic protein Mcl-1; however, AEDL was more efficient. As it was mentioned already, peptide AEDL increased the level of Mcl-1, which inhibits apoptosis and enhances the cell survival. The Mcl-1 level in "young" cultures increased by 40 %, while in "mature" and "old" cells, it increased by 90 % (Khavinson et al. 2014). This may indicate a geroprotective effect of this peptide on bronchial tissue. Besides, peptide AEDL reduced the level

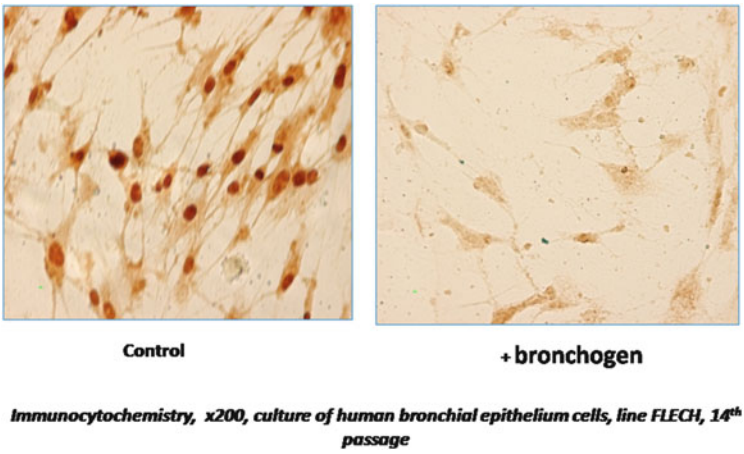

Fig. 5.6 Bronchogen AEDL inhibits expression of proapoptotic protein p53 in cells of human bronchial epithelium (Khavinson et al. 2014)

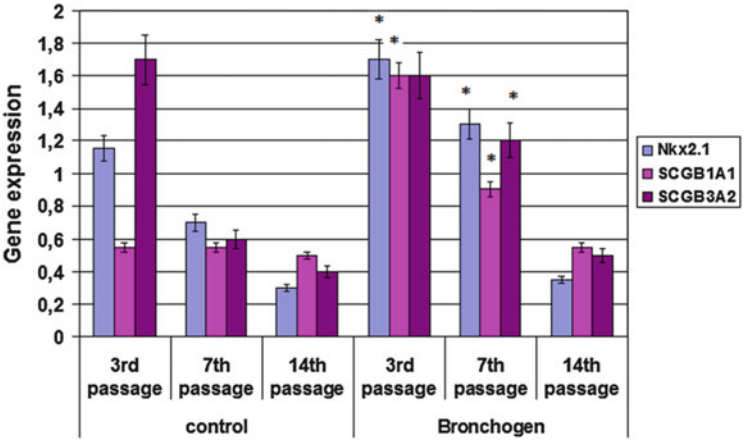

Fig. 5.7 The influence of peptide AEDL on the early differentiation gene expression in the bronchoepithelial cells (Khavinson et al. 2014)

of apoptosis in bronchoepithelial cell culture, through the level of p53 (Fig. 5.6). During cellular senescence, the level of p53 increased in the control cultures. AEDL also increased gene expression and level of membrane glycoprotein CD79 that activates local immunity reactions. Peptide AEDL considerably reduced the level of NOS-3, which generated free radical monoxide (NO), whereas the level of NOS-3 increased in "old" control cell cultures of bronchial epithelium (Khavinson et al. 2014).

Thus, peptide AEDL increased expression of all genes studied including NKX2-1, SCGB1A1, and SCGB3A2 (Fig. 5.7). Hence, the peptide AEDL stimulated

epigenetically the early stages of bronchoepithelial cell differentiation. More evident AEDL stimulating effect on the gene expression is found in "mature" and "old" bronchoepithelial cells. AEDL also activated the expression of genes whose reduced activity correlated with the development of various lung pathologies. Under the influence of AEDL in "young" cell cultures, the expression of MUC5AC and SFTPA1 genes increased by 50 % and 65 %, respectively. AEDL also stimulated the expression of MUC4 and SFTPA1 genes in "adult" cell cultures. In the "old" cell cultures, AEDL has an advantageous effect on expression of genes involved in terminal differentiation and functional activity of bronchoepithelial cells.

Thus, AEDL has an ability to regulate a wide variety of proteins in human bronchial epithelium, and, therefore, it may influence the treatment of acute and chronic lung inflammations. It was found earlier that AEDL increased the expression of the HOXA3 gene transcription factor in cell cultures of human bronchial epithelium during aging (Khavinson et al. 2012). AEDL specifically showed to regulate the cell renewal processes for the bronchial epithelium. AEDL also changed the functional cell state by acting on the content of CD79 and NOS-3 proteins. The increased expression of the glycoprotein CD79, observed in bronchial epithelium by the influence of AEDL, may indicate an increased local immunity of the bronchopulmonary system. AEDL increased cell functional activity and proliferation by enhanced level of proteins, such as proliferation markers Ki67 and antiapoptotic protein Msl-1 (the syntheses of which were reduced in patients with COPD and hypoxia), with a simultaneous reduced p53 apoptotic factor's level (Porebska et al. 2006; Chiappara et al. 2013).

It is most probable that the specific AEDL effects on gene expression observed are mostly due to site-specific binding of the peptide to DNA. Various physical investigation methods showed that the most likely point of binding is the guanine atom N7 in a DNA major groove (Khavinson 2009).

Transcription factor FoxA2 is known to play a significant role at the early embryonic stages of lung development. Together with another transcription factor Nkx2-1, it appears to be an earliest specific molecular marker of lung line cells (Maeda et al. 2007). They are minimal in the young cell cultures and about threefold higher in mature and aged ones. The AEDL peptide addition leads to a more than tenfold increase in FOXA2 expression, whereas in mature and aged cells, only a relatively moderate (two- to threefold) increase in expression is observed. Nevertheless, 5′-end CGI of FOXA2 is totally unmethylated both in control and AEDL-treated cultures at all cell passages studied (Ashapkin et al. 2015). Evidently some other mechanisms, not the DNA methylation, seem to be responsible for age-dependent and peptide-induced modulations of the FOXA2 expression in bronchoepithelial cells.

The NKX2-1 gene codes for a transcription factor involved in early stages of lung bud formation (Maeda et al. 2007). Similarly to the pancreas, the lungs arise from endoderm cells, lung pathway selection being determined by Nkx2-1 and FoxA2 combination, whereas in pancreatic pathway selection—by Pdx1 and FoxA2—the NKX2-1 expression levels are similar in bronchoepithelial cells of

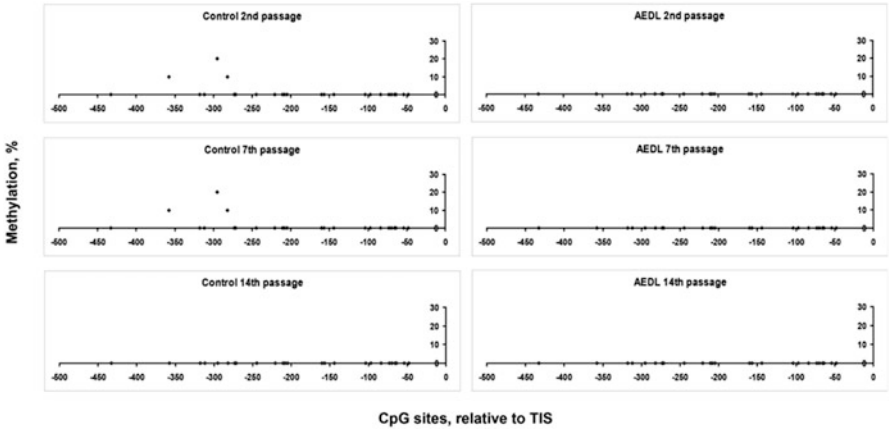

Fig. 5.8 The methylation patterns of the NKX2-1 gene promoter CGI in bronchoepithelial cell cultures (Ashapkin et al. 2015)

various ages. The AEDL addition stimulates NKX2-1 expression in young and mature cell cultures, but it does not affect it in old ones. CGI associated with the promoter region of NKX2-1 is essentially unmethylated in bronchoepithelial cells (Fig. 5.7). The only exception is three CpG sites located between 275 and 370 bp upstream of transcription initiation site (TIS) that are partially (10–20 %) methylated in young and mature cell cultures. The AEDL addition to young and mature cells leads to a full demethylation of these sites (Fig. 5.8). Thus, the NKX2-1 promoter demethylation patterns correlate with gene expression.

The FOXA1 gene codes for a transcription factor controlling terminal differentiation of alveolar cells and the lung secretoglobin and other functionally important protein expression (Maeda et al. 2007). The expression levels of FOXA1 in bronchoepithelial cells are decreased in later passages. The AEDL addition does not affect FOXA1 expression in young cultures and moderately stimulates it in mature and aged cultures. Thus, the peptide slows down age-specific decline in the FOXA1 expression (Fig. 5.9). There is a CGI associated with the FOXA1 promoter. It is fully unmethylated in bronchoepithelial cells in all cell samples investigated. Hence, age-specific and peptide modulation of the FOXA1 expression in bronchoepithelial cells does not depend on its promoter methylation patterns.

Some genes investigated do not have any promoter-associated CGIs (PAX4, SCGB1A1, SCGB3A2, SFTPA1). Few CpG sites in their promoter regions immediately preceding TIS are either methylated (PAX4, SCGB1A1) or unmethylated (SCGB3A2, SFTPA1) (Ashapkin et al. 2015). Most of these CpG sites have similar methylation patterns in all cell samples studied; hence, their connection to age-specific or peptide modulation of gene expression seems to be quite unlikely. The expression levels of SCGB1A1 are similar in the bronchoepithelial cell cultures at all passages but are variably modulated by AEDL. The peptide addition leads to a strong (threefold) stimulation of SCGB1A1 expression in young cell

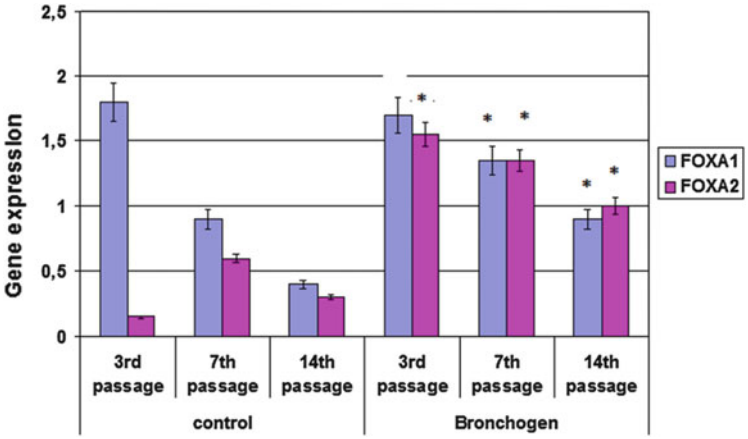

Fig. 5.9 The peptide AEDL influence on expression of genes of the late cellular differentiation in the bronchoepithelial cells (Khavinson et al. 2014)

cultures and moderate (1.5-fold) stimulation in mature ones and practically does not affect it in aged cultures. There are no CGIs in SCGB1A1 promoter region, the methylation level of few CpG sites present being rather high. The methylation levels of these sites are similar in young and mature cell cultures and somewhat lower in the aged ones. The AEDL addition decreases promoter methylation in young and mature cells to a level characteristic of the control aged cells, and it does not affect methylation in aged cells (Ashapkin et al. 2015). These effects correlate with the AEDL modulation of SCGB1A1 expression in aging cell cultures.

5.5.2 Pancragen

Pancragen (LysGluAspTrp, KEDW) is a tetrapeptide with specific biological and therapeutical actions. The intramuscular introduction of pancragen to patients with diabetes increased the insulin level and decreased glucose concentration in blood. The specific peptide modulatory effects on selective induction or stimulation of gene expression were clearly observed during incubation of human pancreatic cell cultures in the presence of pancragen. We investigated particularly how pancragen could influence the gene transcription level and CpG methylation status of promoters of some individual genes responsible for cell differentiation in human pancreatic carcinoma MIA PaCa-2 cell culture.

One of the key cell differentiation genes (PDX1) controlling the early stages of pancreatic tissue development (Arda et al. 2013) is expressed equally in young and mature pancreatic cell cultures, whereas its expression level in aged ones is by about 1.5-fold higher. The peptide KEDW diminishes the level of the PDX1 gene expression in young cell cultures, does not affect its expression in mature ones, and

enhances its expression in aged cell cultures. Most CpG sites of the PDX1 promoter CGI are unmethylated in pancreatic cell cultures. There are about dozen of partially methylated CpG sites in this region. The methylation levels of three sites upstream of TIS are minimally (~10 %) methylated, whereas sites located downstream of TIS have higher methylation levels (~30 %) (Ashapkin et al. 2015). These promoter methylation patterns are identical in young and mature cell cultures, in accordance with similar expression levels of the gene in these cells. The methylation levels of all sites except for two most upstream located ones decreased in aged cells: the third site (−130 bp relative to TIS) is fully unmethylated, the methylation levels of three upper sites of those located downstream of TIS are minimally (~10 %) methylated, and the methylation levels of other sites located downstream of TIS are about 20 %. Thus, a moderate increase in the PDX1 gene expression level in aged cell cultures correlates with a small but appreciable decrease in its methylation level. The addition of the KEDW peptide to young cell cultures does not affect methylation of PDX1. The addition of KEDW leads to a noticeable reduction of the PDX1 methylation level in mature cell cultures and to a significant methylation reduction in aged cell cultures (Ashapkin et al. 2015). This corresponds to significant peptide stimulation of gene expression in aged cell cultures.

Another gene studied, PAX6, codes for a transcription factor involved in β-cell maturation at the late stages of pancreatic development (Arda et al. 2013). The expression levels of this gene are similar in pancreatic cell cultures at all cell passages, but their modulation by KEDW varies. KEDW reduces PAX6 expression level in young cell cultures, increases it in mature ones, and still more increases it in aged cell cultures (Fig. 5.10). The PAX6 promoter region CGI is nearly totally unmethylated in pancreatic cell cultures (Fig. 5.11). The only exceptions are two partially (~30 %) methylated sites located at about 300 bp upstream of TIS. This methylation pattern has been found in control cell cultures of all passages as well as in KEDW-treated young cultures (Ashapkin et al. 2015). In mature cell cultures, the

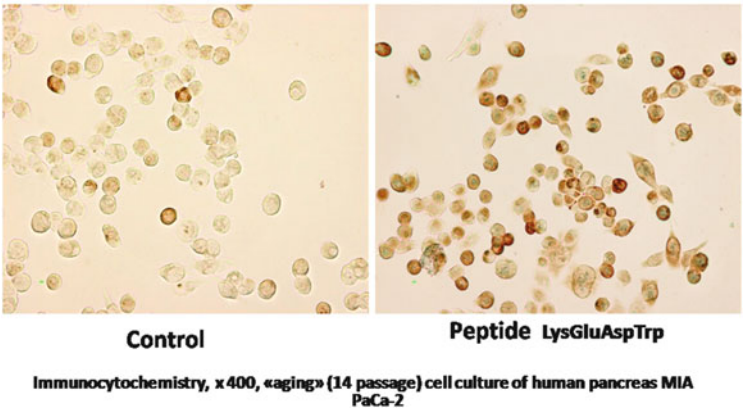

Fig. 5.10 Peptide LysGluAspTrp increases expression of the differentiation factor Pax6 in "aging" cell culture of human pancreas

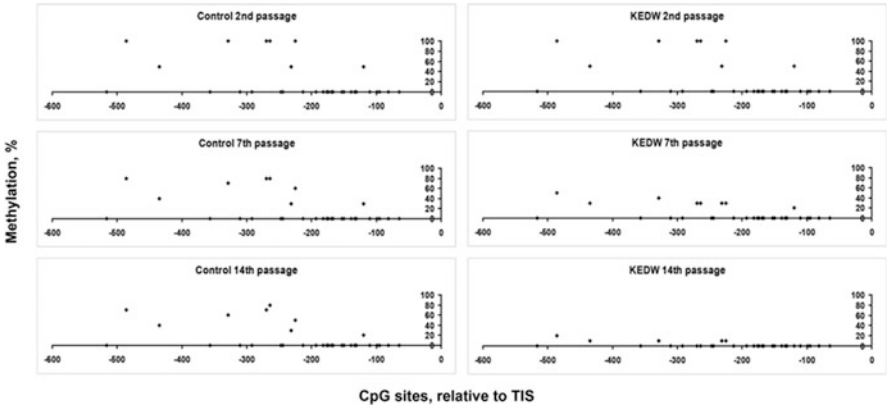

Fig. 5.11 The methylation patterns of the NGN3 gene promoter CGI in pancreatic cell cultures (Ashapkin et al. 2015)

KEDW addition leads to a decrease in the methylation levels of two partially methylated sites, whereas in aged cultures treated with peptide KEDW, these sites become totally unmethylated (Fig. 5.11) (Ashapkin et al. 2015).

Another gene under study, NKX6-1, codes for a transcription factor involved in pancreatic development starting from early embryonic stages (Arda et al. 2013). At the early embryonic stages, this gene is expressed in common multipotent progenitor cells of pancreas, whereas at the late stage, its expression is confined to β-cells. We have found NKX6-1 to be maximally expressed in the mature pancreatic cell cultures, while its expression levels in young and aged cell cultures are somewhat lower and quite similar to each other. KEDW in mature cell cultures does not affect the NKX6-1 expression, whereas in young and aged cultures, it increases expression of NKX6-1 gene to the levels similar to that in mature cultures. A promoter-associated CGI of NKX6-1 is essentially unmethylated in pancreatic cell cultures (Ashapkin et al. 2015). The only exception is a couple of partially methylated sites. The methylation levels of these sites are significantly lower in mature and aged cultures compared to that in young ones. The KEDW addition to young cultures leads to a decrease in promoter methylation down to the methylation levels similar to those in mature and aged cultures. The KEDW addition to mature and aged cell cultures leads to complete demethylation of promoter CGI (Ashapkin et al. 2015). Therefore, no direct correlation between promoter unmethylation and NKX6-1 expression levels has been observed. Probably, minor methylation of few sites in this region does not interfere with the gene transcription.

NGN3 gene codes for a transcription factor involved in the early stages of pancreatic β-cell differentiation (Conrad et al. 2014). The expression levels of NGN3 are similar at all cell culture passages studied. Effects of KEDW on NGN3 expression are age dependent. The peptide addition leads to a small increase in the gene expression levels in young and mature cell cultures, but it very significantly (nearly by threefold) increases the gene expression in aged ones.

The NGN3 promoter CGI is moderately methylated in pancreatic cell cultures (Ashapkin et al. 2015). The KEDW addition does not affect NGN3 gene methylation in young cultures but decreases its methylation level in mature cultures and leads to its nearly full demethylation in aged cultures (Ashapkin et al. 2015). These changes in the promoter methylation may explain the variable effects of KEDW on the NGN3 expression in cell cultures of different ages.

FOXA2 gene codes for a transcription factor involved in pancreatic development at all stages starting from definitive endoderm cells, and it continues to be expressed in differentiated pancreatic cells (Arda et al. 2013); Conrad et al. 2014). One of the specific targets of FOXA2 is the early pancreas development gene PDX1. FOXA2 is actively expressed both in pancreatic and bronchoepithelial cell cultures (Khavinson et al. 2013, 2014). Its expression levels are similar in young and aged cell cultures, whereas the expression level in mature ones is about 1.8-fold higher. The effects of KEDW on FOXA2 expression in cells of the 2nd, 7th, and 14th passages are variable: moderate stimulation in young and aged cells and inhibition in mature cells were observed. Thus, in the presence of KEDW, the expression levels of *FOXA2* become quite similar to that in cultures of all passages studied. There is a CGI in FOXA2 sequence beginning just upstream of TIS (−195 bp) and extending to a better part of the gene-coding region. Most CpG sites in that CGI are unmethylated though there are few partially methylated ones. Their methylation levels vary from 30 to 100 % in young cell cultures, 20 to 60 % in mature, and 10 to 40 % in aged ones (Ashapkin et al. 2015). The KEDW addition to young cultures diminishes the FOXA2 methylation levels to those observed in control mature cultures. The peptide does not change the FOXA2 methylation levels in mature cultures, whereas in aged cultures, it leads to a nearly full demethylation (Ashapkin et al. 2015). Thus, FOXA2 methylation displays only a limited correlation with gene expression.

Thus, the correlation between stimulation of gene expression and promoter undermethylation of some genes (PDX1, PAX6, NGN3, NKX2-1, SCGB1A1) has been found. On the other hand, some genes are similarly methylated irrespective of the cell culture age and peptide action (PAX4 in pancreatic cells and FOXA1, SCGB3A2, and SFTPA1 in bronchial ones). The FOXA2 methylation in pancreatic cells displays some correlation to its expression, whereas in bronchial cells, this gene is completely unmethylated but variously expressed. The peptide modulation of gene expression in the cells studied probably depends on multiple mechanisms, DNA methylation being just one of them.

Anyway, short biologically active peptides are essential tissue-specific modulators of gene activity and in some cases of the DNA methylation status. Thus, short peptides are effective epigenetic regulatory signal molecules affecting gene functioning and cell differentiation.

5.6 Conclusions

Short biologically active peptides represent an efficient natural signaling system of the epigenetic control of cell physiology. Short peptides are able to in vitro interact specifically with single-stranded and double-stranded oligonucleotides, DNA, and histones (H1, core histones). Interaction of peptides with nucleic acids and histones is site specific and depends on the primary structures of both sorts of biopolymers; this may be responsible for gene-specific action of short peptides observed.

Various peptides affect differently the fluorescence of the 5,6-carboxyfluorescein-labeled deoxyribooligonucleotides and the DNA–ethidium bromide complexes. On interaction with nucleic acids, the peptides discriminate between different nucleotide sequences and recognize even their cytosine methylation status. Judging corresponding data on fluorescence quenching, epitalon, pinealon, and bronchogen interact preferentially with CNG-containing deoxyribooligonucleotides (CNG sites are targets for cytosine DNA methylation in eukaryotes). Epitalon, testagen, and pinealon seem to preferentially bind with CAG but bronchogen with CTG-containing sequences.

Peptides investigated modulate specifically the in vitro action of eukaryotic CG- and CNG-site-specific wheat endonucleases (WEN1 and WEN2) on DNA depending on the DNA methylation status. Mostly the peptides strongly inhibit DNA hydrolysis by these enzymes. It is assumed that modulation of the endonuclease action on DNA hydrolysis observed is due to site-specific peptide–DNA binding that seems to protect DNA against enzymatic hydrolysis.

We have been the first to discover the tissue, subcellular, and age specificity of DNA methylation (Vanyushin et al. 1970); besides, it was shown first that the DNA methylation pattern was changed in cancerous cells (Romanov and Vanyushin 1981). Taking these data into consideration, we can predict that one and the same biologically active peptide will bind to DNA and act on the gene functions in a different fashion (1) in various tissues (cells), (2) in the nucleus and mitochondria, (3) in young and old cells or patients, and (4) in normal and malignant cells. The phenomenon of modulation of endonuclease action by peptides of interest discovered may be only a part of the global biological rule: these peptide site-specific or complementary binding with respective DNA sequences recognized (especially regulatory ones) should modulate also functioning of many other proteins operating with DNA (RNA and DNA polymerases, DNA methyltransferases, and very many different regulatory proteins or factors).

As we have observed, short peptides can modulate in vivo DNA methylation, and, therefore, like DNA methylation itself, they may control all genetic functions including DNA transcription, replication, and repair. Short peptides are able to penetrate into the nucleus and nucleolus of the living animal cell and potentially may interact there with various components of the cytoplasm and nucleus including DNA and RNA. The peptide activation of transcription observed in cell cultures is associated often with CpG demethylation of gene promoters. The hypothesis on the most probable mechanism of regulation of transcription by peptides is suggested;

according to this hypothesis, the peptide binding to promoter CpG or CpNpG sites prohibits their methylation that keeps the gene in an activated state.

Judging fluorescence modulation (quenching), the peptides (AlaGluAspGly, GluAspArg, AlaGluAspLeu, LysGluAspGly, AlaGluAspArg, and LysGluAspTrp) bind with FITC-labeled wheat histones H1, H2в, H3, and H4. This is due to the interaction of peptides with N-end histone regions that seem to contain respective peptide-binding motifs. Peptide binding with histones depends on the histone nature and the primary structure of peptides; thus, it is site specific. It is most probable that peptide binding with histones (particularly with histone tails) in chromatin may be another control mechanism of epigenetic regulation of gene functioning.

The further investigation of peptide interactions with DNA and chromatin, in particular, is very important for deciphering of the molecular mechanisms of gene functioning, cell differentiation, and evolution.

The search for and design of new short biologically active peptides is a key promising way to the origin and production of a new generation of drugs that are gene addressed and strongly needed to prevent premature aging and to treat cancer and other diseases.

Acknowledgment I thank very much indeed Professor Walter Doerfler for the invitation to take part in the Fifth Weissenburg Symposium (Epigenetics—A Different Way of Looking at Genetics) and his kind traditional interest to my (B.F.V.) research in the DNA methylation field.

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