ISSN 0006-2979, Biochemistry (Moscow), 2015, Vol. 80, No. 3, pp. 310-322. © Pleiades Publishing, Ltd., 2015.
Original Russian Text © V. V. Ashapkin, N. S. Linkova, V. Kh. Khavinson, B. F. Vanyushin, 2015, published in Biokhimiya, 2015, Vol. 80, No. 3, pp. 374-388.

Epigenetic Mechanisms of Peptidergic Regulation of Gene Expression during Aging of Human Cells

V. V. Ashapkin[1]*, N. S. Linkova[2,3], V. Kh. Khavinson[2,4], and B. F. Vanyushin[1]

[1]*Belozersky Institute of Physico-Chemical Biology, Lomonosov Moscow State University, 119992 Moscow, Russia; fax: (495) 939-3181; E-mail: ashapkin@genebee.msu.ru*
[2]*St. Petersburg Institute of Bioregulation and Gerontology, 197110 St. Petersburg, Russia; fax: (812) 230-0049; E-mail: linkova@gerontology.ru*
[3]*St. Petersburg State Polytechnical University, 195251 St. Petersburg, Russia; fax: (812) 552-6080; E-mail: linkova@medfiz.ru*
[4]*Mechnikov North-West State Medical University, 191015 St. Petersburg, Russia; fax: (812) 303-5035; E-mail: khavinson@gerontology.ru*

Received July 29, 2014
Revision received September 13, 2014

Abstract—Expression levels of genes encoding specific transcription factors and other functionally important proteins vary upon aging of pancreatic and bronchial epithelium cell cultures. The peptides KEDW and AEDL tissue-specifically affect gene expression in pancreatic and bronchial cell cultures, respectively. It is established in this work that the DNA methylation patterns of the *PDX1*, *PAX6*, *NGN3*, *NKX2-1*, and *SCGB1A1* genes promoter regions change upon aging in pancreatic and bronchial cell cultures in correlation with variations in their expression levels. Thus, stable changes in gene expression upon aging of cell cultures could be caused by changes in their promoter methylation patterns. The methylation patterns of the *PAX4* gene in pancreatic cells as well as those of the *FOXA1*, *SCGB3A2*, and *SFTPA1* genes in bronchial cells do not change upon aging and are unaffected by peptides, whereas their expression levels change in both cases. The promoter region of the *FOXA2* gene in pancreatic cells contain a small number of methylated CpG sites, their methylation levels being affected by cell culture aging and KEDW, though without any correlation with gene expression levels. The promoter region of the *FOXA2* gene is completely unmethylated in bronchial cells irrespective of cell culture age and AEDL action. Changes in promoter methylation might be the cause of age- and peptide-induced variations in expression levels of the *PDX1*, *PAX6*, and *NGN3* genes in pancreatic cells and *NKX2-1* and *SCGB1A1* genes in bronchial cells. Expression levels of the *PAX4* and *FOXA2* genes in pancreatic cells and *FOXA1*, *FOXA2*, *SCGB3A2*, and *SFTPA1* genes in bronchial cells seem to be controlled by some other mechanisms.

DOI: 10.1134/S0006297915030062

Key words: short peptides, DNA methylation, transcription, cell differentiation, pancreatic cell culture, bronchial cell culture, differentiation factors

We established earlier that pancreatic cell aging *in vitro* is accompanied by changes in expression of a number of genes. Most of these genes code for proteins important for pancreatic cell differentiation. Addition of the tetrapeptide KEDW changes expression of these genes and synthesis of their respective encoded proteins in young, mature, and aged cell cultures [1]. Besides, the KEDW peptide has been shown to reduce blood glucose levels in rats with streptozotocin- and alloxan-induced diabetes mellitus [2]. The KEDW peptide also lowers blood glucose levels on empty stomach and at standard glucose tolerance test, as well as decreases the insulin sensitivity index, in patients of advanced age with diabetes mellitus type 1 and 2 [3, 4]. Upon aging *in vitro* of human bronchoepithelial cells, a decrease in expression levels of genes *NKX2-1* and *FOXA1*, coding for respective transcription factors, and *SCGB3A2*, coding for the second secretoglobin of the 3A family, occurs, whereas the expression levels of *FOXA2* and *SCGB1A1* genes are unchanged [5]. Addition of a tetrapeptide AEDL increases the expression levels of the *NKX2-1* and *SCGB1A1* genes in young and mature bronchoepithelial cell cultures but does not affect them in aged cultures. The *SCGB3A2* gene expression is stimulated by the peptide in mature cultures only, the *FOXA1* gene expression in mature and

Abbreviations: AEDL, peptide H-Ala-Glu-Asp-Leu-OH; bp, base pair; CGI, CpG-island; KEDW, peptide H-Lys-Glu-Asp-Trp-NH_2; TIS, transcription initiation site.

* To whom correspondence should be addressed.

aged cultures, and the *FOXA2* gene expression in all cultures. The expression levels of genes *MUC4*, *MUC5AC*, and *SFTPA1*, coding for functionally active proteins of bronchial epithelium, are progressively diminished upon aging of the cell cultures. The addition of peptide AEDL stimulates *SFTPA1* expression in cultures at any passage, *MUC4* expression in mature and aged cultures, and *MUC5AC* expression in young cultures only. Besides, the peptide AEDL enhances the synthesis of proteins controlling cell differentiation, proliferation, and apoptosis in bronchial epithelium upon aging. Bronchial protective activity of AEDL has been observed in bacterial acute lung inflammation animal models, chronic fibrosis, and sublethal hypertoxic lung damage [6]. The actions of peptides KEDW and AEDL appeared to be not only selective with respect to different genes, but also tissue-specific, KEDW preferentially affecting pancreatic cells but not the bronchial ones, and AEDL *vice versa* [6].

The molecular mechanisms of gene expression modulation with tetrapeptides remain essentially unknown, but the main features of this modulation (age- and tissue-specificity, gene-selective action, timescale of multiple cell passages) suggest an epigenetic mode of action. Cytosine DNA methylation is the most extensively studied epigenetic genome modification, playing significant role in stable changes of gene activity upon cell differentiation and aging in mammals [7-12].

We describe in this work investigation of the cytosine methylation patterns of genes modulated by the KEDW and AEDL peptides in pancreatic and broncoepithelial cell cultures upon aging, as well as possible effects of these peptides on these methylation patterns.

MATERIALS AND METHODS

Human epithelial cell cultures of pancreatic carcinoma MIA PaCa-2 (Institute of Cytology, Russian Academy of Sciences) and human bronchoepithelial embryonic cell line FLECH (Research Institute of Influenza, Ministry of Healthcare of the Russian Federation) were used.

The cells of 2nd, 7th, and 14th culture passages were regarded as young, mature, and aged, respectively, in compliance with recommendations of the International Association for Cultural Studies (USA, San Francisco, 2007). The cell cultures were divided into control and experimental groups. The respective tetrapeptide (KEDW for pancreatic cells, AEDL for bronchial cells) was added at each passage to cells of experimental groups at 20 ng/ml final concentration, and an equivalent volume of physiological solution was added to the control group cells. The pancreatic cells were cultured in vials with 25 cm^2 surface (JetBiofil, Japan) in 5 ml of growth medium consisting of DMEM with addition of L-glutamine (Biolot, Russia), 15% of cows fetal serum SC-BIOL (Biolot), and 1% penicillin–streptomycin solution in a CO_2 incubator under standard conditions (5% CO_2, 37°C). Bronchial cells were cultured in Petri dishes (3.5 cm diameter) treated with gelatin solution (Biolot), 15% fetal bovine serum, 82.5% iMEM, 1.5% of HEPES buffer with L-glutamine, and 1% penicillin–streptomycin solution in a CO_2 incubator under standard conditions (5% CO_2, 37°C). We used trypsin–Versene solution for cell subcultivation at ratio 3 : 1.

DNA isolation from cultured cells and its treatment with sodium bisulfite were carried out with the QIAamp DNA Mini Kit and EpiTect Bisulfite Kit (Qiagen, Germany) by protocols recommended by the supplier. The oligonucleotide primers for PCR amplification of bisulfite-modified genomic DNA were constructed with the BiSearch web-based service [13, 14]. The target sites were chosen located in TIS proximal CpG-island (CGI) sequences, or immediately preceding the TIS sequences for genes devoid of CGIs. PCR amplification was carried out in two consecutive steps, changing one of the primers for a more internal one and using 2 μl aliquot of the first step PCR mix as a matrix on the second step. A DT-322 real-time PCR detection system (DNA-Technology, Russia) and qPCRmix-HS SYBR+ROX kit (Evrogen, Russia) were used. Typical amplification conditions used were 95°C – 5 min matrix denaturation/polymerase activation; 40 cycles of (95°C – 30 sec denaturation; 52-56°C (minimal melting temperature of respective primer pair minus 3°C) – 30 sec primer annealing; 72°C – 45 sec elongation); 72°C – 2 min final elongation. The final PCR products were fractionated by 2% agarose gel electrophoresis, amplified target DNA fragments were quickly cut out under long wavelength (310 nm) UV light, and extracted with a QIAquick Gel Extraction Kit (Qiagen). The target DNA fragments were sequenced at the Genome service center (http://www.genome-centre.ru/) with an ABI PRISM® BigDye™ Terminator v. 3.1 kit on an Applied Biosystems 3730 DNA Analyzer (Applied Biosystems, USA). Free Sequence Scanner v. 1 soft (Applied Biosystems) was used for visualization of results and their export into fasta-format files. The cytosine methylation patterns were obtained from sequence data with the Meth Tools 2.0 web-based service [15]. The methylation levels of partially methylated sites were calculated from the areas of respective peaks of cytosine vs. thymine in four-dye electrophoregram data produced by the sequencing machine [16, 17]. Arithmetic means of three independent determinations (biological parallels) were rounded to the next multiple of 10%.

RESULTS

The methylation patterns of a promoter proximal CpG island of the *PDX1* gene in pancreatic cells are shown in Fig. 1. This gene codes for a transcription factor

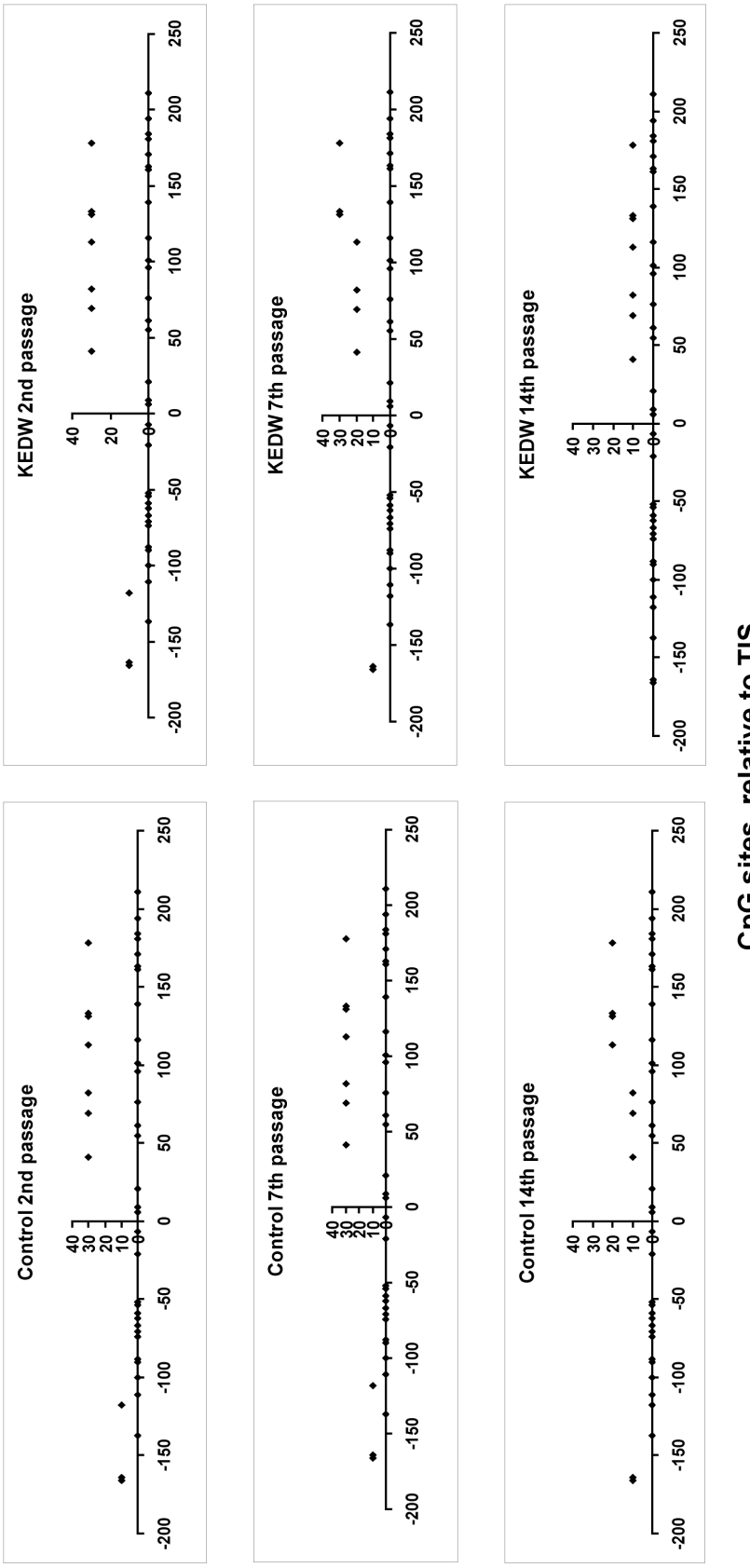

Fig. 1. Methylation patterns of the *PDX1* gene promoter CGI in pancreatic cell cultures on aging.

protein controlling the early stages of pancreatic development [18]. We found previously that this gene is equally expressed in young and mature pancreatic cell cultures, whereas its expression level in aged ones is about 1.5-fold higher. The peptide KEDW diminishes the level of *PDX1* gene expression in young cell cultures, does not affects its expression in mature ones, and enhances its expression in aged cell cultures. As can be seen in Fig. 1, most CpG sites of the *PDX1* promoter CGI are unmethylated in pancreatic cell cultures. There are about a dozen partially methylated CpG sites in this region. It must be noted that the methylation levels of three sites upstream of TIS (transcription initiation site) are minimal (~10%), whereas seven sites located downstream of TIS have higher methylation levels (~30%). These 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 the two most upstream ones are decreased in aged cells: the third site (−130 bp relative to TIS) is fully unmethylated, methylation levels of the three upper sites of those located downstream of TIS are minimal (10%), and methylation levels of other sites downstream of TIS are about 20%. Thus, a moderate increase in *PDX1* gene expression level in aged cell cultures correlated 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 in the *PDX1* methylation level in mature cell cultures and to a significant reduction in aged cell cultures (all the sites upstream of TIS become completely unmethylated, whereas the methylation levels of downstream sites diminish to a minimum). These data are in accord with significant stimulation of the gene expression in aged cell cultures.

Another studied gene, *PAX6*, codes for a transcription factor involved in β-cell maturation during the late stages of pancreatic development [19]. The expression levels of this gene are similar in pancreatic cell cultures at all passages, but their modulation by KEDW varies. Addition of KEDW reduces *PAX6* expression level in young cell cultures, increases it in mature ones, and increases it still more in aged ones. The *PAX6* promoter region CGI is nearly completely unmethylated in pancreatic cell cultures (Fig. 2). The only exceptions are two partially (~30%) methylated CpG sites located about 300 bp upstream of TIS. This methylation pattern was found in control cultures of all passages as well as in KEDW-treated young cultures. In mature cultures, KEDW addition leads to a decrease in methylation levels of two partially methylated sites, whereas in KEDW-treated aged cultures these sites become completely unmethylated. Whether these small differences in methylation are connected with variable *PAX6* gene expression is unclear.

Still another studied gene, *NKX6-1*, codes for a transcription factor involved in pancreatic development starting from early embryonic stages [19]. At early embryonic stages, this gene is expressed in common multipotent progenitor cells of pancreas, whereas at late stages its expression is confined to β-cells. We found *NKX6-1* to be maximally expressed in mature pancreatic cell cultures, whereas its expression levels in young and aged cell cultures are somewhat lower and quite similar to each other. The addition of KEDW to mature cell cultures does not affect the *NKX6-1* expression, whereas in young and aged cultures it increases expression of the *NKX6-1* gene to levels similar to that in mature cultures. A promoter-associated CGI of *NKX6-1* is essentially unmethylated in pancreatic cell cultures (Fig. 3). The only exceptions are a couple of partially methylated sites. Methylation levels of these sites are significantly lower in mature and aged cultures as compared to the young ones. Addition of KEDW to young cultures leads to a decrease in methylation to levels similar to those in mature and aged cultures. Addition of KEDW to mature and aged cultures leads to complete demethylation of promoter CGI. Thus, no direct correlation between promoter methylation and *NKX6-1* expression levels was observed. Probably, minor methylation of a few sites in this region does not interfere with the gene transcription.

The *NGN3* gene codes for a transcription factor involved in early stages of pancreatic β-cell differentiation [19]. The expression levels of *NGN3* are similar at all cell culture passages studied. Effects of KEDW on *NGN3* expression are age-dependent. Its addition leads to a small increase in expression levels in young and mature cell cultures and to a very significant increase (nearly 3-fold) in aged ones. The *NGN3* promoter CGI is moderately methylated in pancreatic cell cultures (Fig. 4). Of three dozens of CpG sites analyzed, most are completely unmethylated. There are eight sparse CpG sites in this region with highly variable (20-100%) methylation levels. Methylation levels of these sites tend to be lower in mature cells as compared to young ones, and in aged cells as compared to mature ones. Addition of KEDW does not affect *NGN3* gene methylation in young cultures, decreases its methylation level in mature cultures, and leads to its nearly full demethylation in aged cultures. These changes in promoter methylation might explain variable effects of KEDW on *NGN3* expression in cell cultures of different ages.

The *FOXA2* gene codes for a transcription factor involved in pancreatic development at all stages starting from definitive endoderm cells and continues to express in differentiated pancreatic cells [18, 19]. One of the specific targets of *FOXA2* is the early pancreas development gene *PDX1*. *FOXA2* is actively expressed in both pancreatic and bronchoepithelial cell cultures [1, 5]. Its expression levels are similar in young and aged cell cultures, whereas expression level in mature ones is about 1.8-fold higher. The effects of KEDW on *FOXA2* expression in 2nd, 7th, and 14th passage cells are variable: moderate stimulation

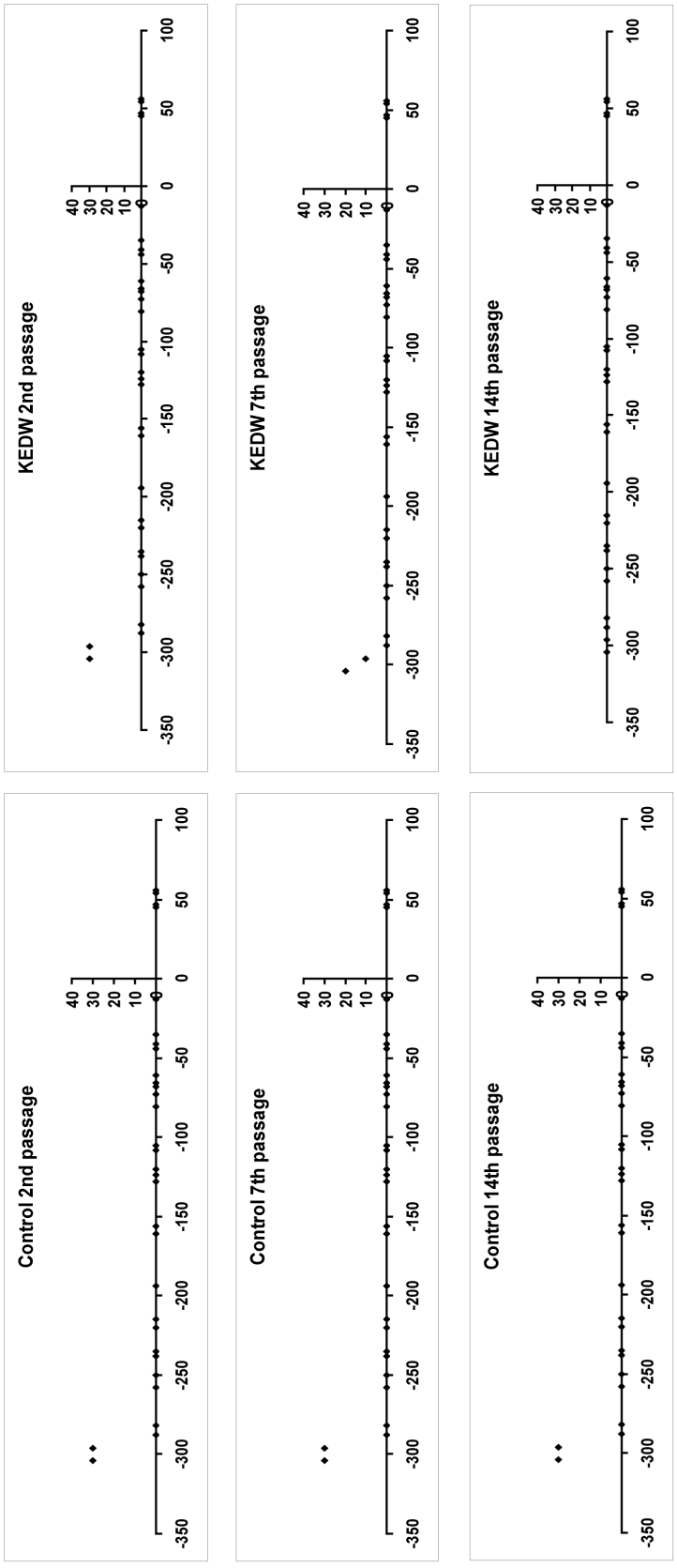

Fig. 2. Methylation patterns of the *PAX6* gene promoter CGI in pancreatic cell cultures on aging.

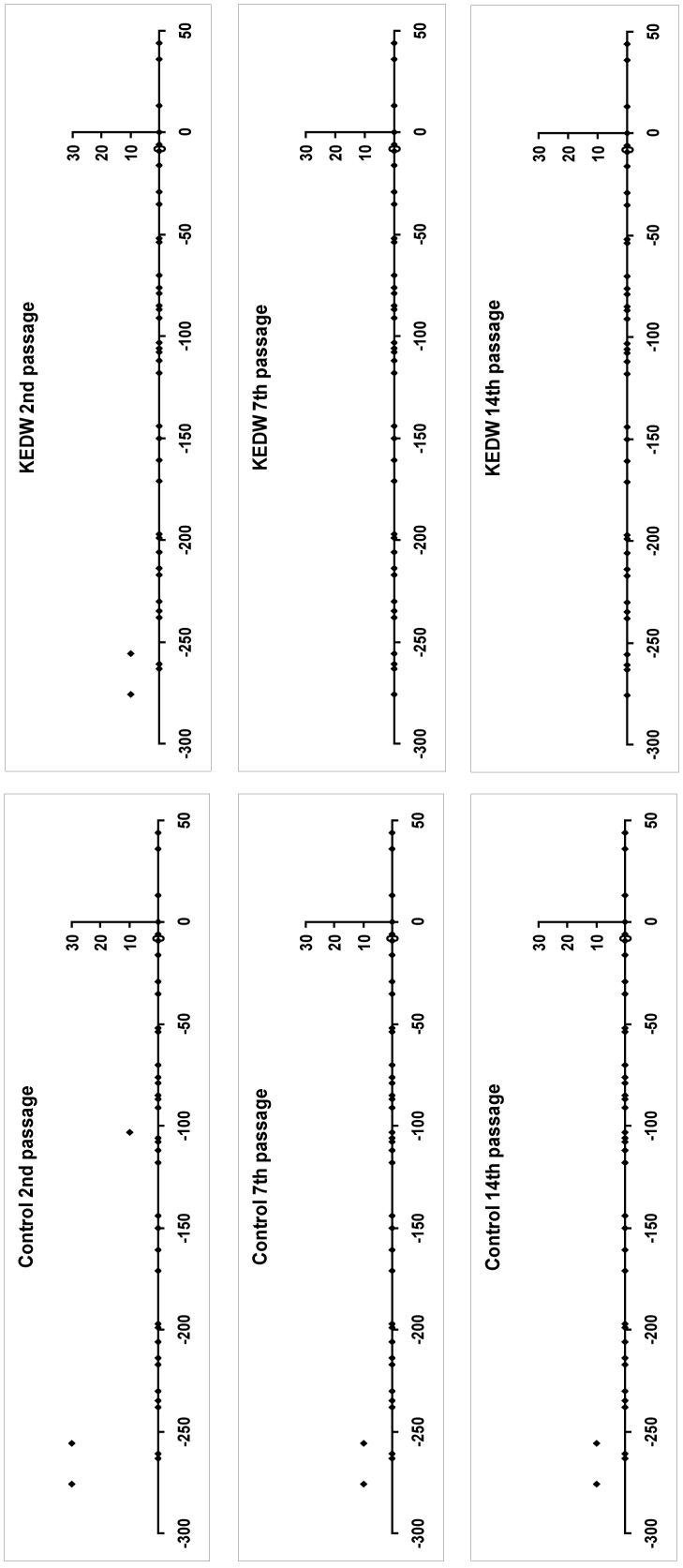

Fig. 3. Methylation patterns of the *NKX6-1* gene promoter CGI in pancreatic cell cultures on aging.

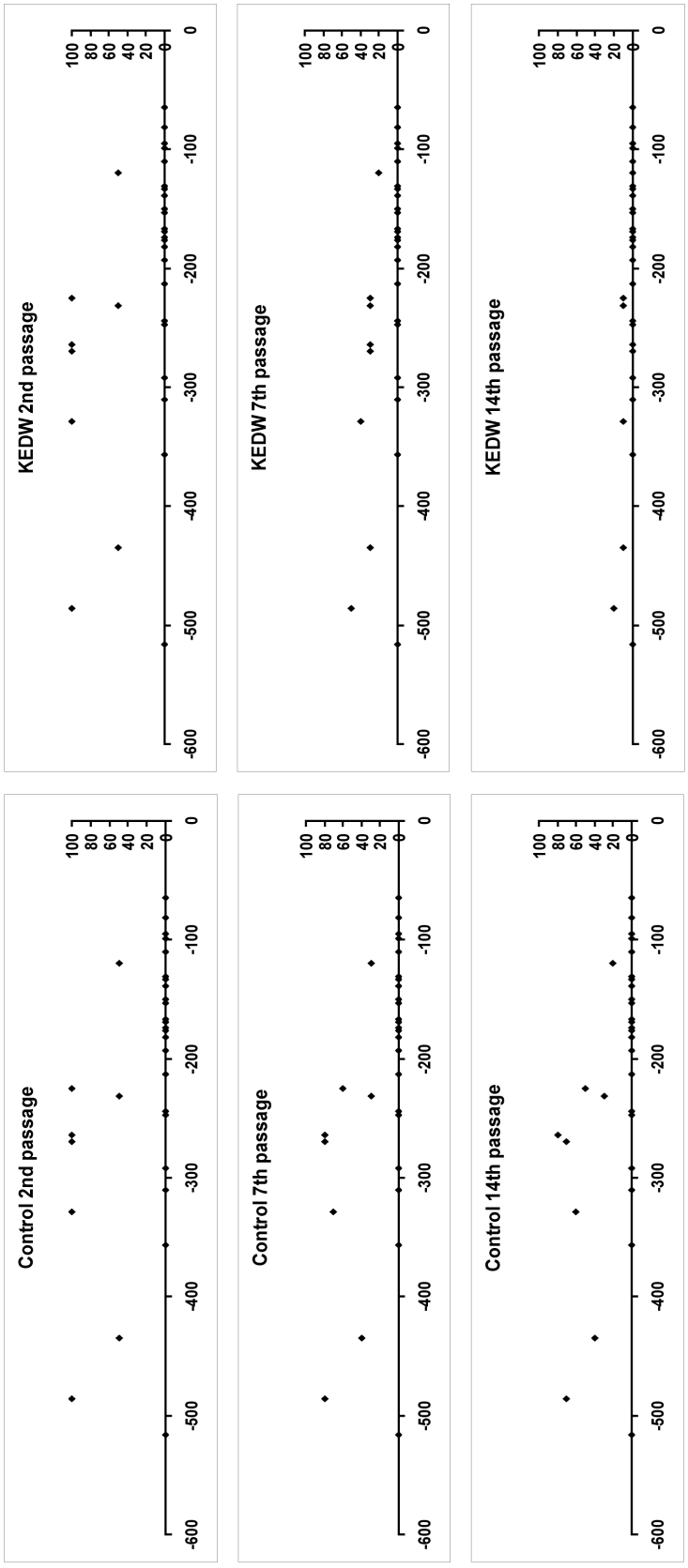

Fig. 4. Methylation patterns of the *NGN3* gene promoter CGI in pancreatic cell cultures on aging.

in young and aged cells, and inhibition in mature cells. Thus in the presence of KEDW the expression levels of *FOXA2* become quite similar in cultures of all passages studied. There is a CGI in the *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 a few partially methylated ones (Fig. 5). Their methylation levels vary from 30 to 100% in young cell cultures, 20 to 60% in mature, and 10 to 40% in aged ones. Addition of KEDW to young cultures diminishes *FOXA2* methylation levels to those observed in control mature cultures. The peptide does not change *FOXA2* methylation levels in mature cultures, whereas in aged cultures it leads to nearly full demethylation. Thus, *FOXA2* methylation displays only a limited correlation with gene expression. We suggest that 5′-end CGI methylation does not affect *FOXA2* expression or is only one of multiple factors controlling the expression.

Transcription factor FoxA2 is known to play a significant role in early embryonal stages of lung development. Together with another transcription factor, Nkx2-1, it appears to be an earliest specific molecular marker of lung line cells [20]. Expression levels of the *FOXA2* gene in bronchoepithelial cell cultures are variable upon aging. They are minimal in young cell cultures and about 3-fold higher in mature and aged ones. Addition of the AEDL peptide leads to a more than 10-fold increase in *FOXA2* expression in young cells, whereas in mature and aged cells only a moderate (2-3-fold) increase in expression is observed. Nevertheless, the 5′-end CGI of *FOXA2* is completely unmethylated in both control and AEDL-treated cultures at all studied passages. Evidently, some other mechanisms, not DNA methylation, are responsible for the age-dependent and peptide-induced modulation of *FOXA2* expression in bronchoepithelial cells.

The *NKX2-1* gene codes for a transcription factor involved in early stages of lung buds formation [20]. Similar to pancreas, lungs arise from foregut endoderm cells, lung pathway selection being determined by Nkx2-1 and FoxA2 combination, whereas pancreatic pathway selection by Pdx1 and FoxA2. The *NKX2-1* expression levels are similar in bronchoepithelial cells of various ages. Addition of AEDL stimulates *NKX2-1* expression in young and mature cell cultures, but does not affect it in old ones. There is a CGI associated with the promoter region of *NKX2-1*, essentially unmethylated in bronchoepithelial cells (Fig. 6). The only exception are three CpG sites located between 275 and 370 bp upstream of TIS that are partially (10-20%) methylated in young and mature cell cultures. The addition of AEDL to young and mature cells leads to full demethylation of these sites. Thus, the *NKX2-1* promoter methylation patterns correlates with expression.

The *FOXA1* gene codes for a transcription factor controlling terminal differentiation of alveolar cells and the lung secretoglobin and other functionally important proteins expression [20]. The expression levels of *FOXA1* in bronchoepithelial cells are decreased in later passages. Addition of AEDL does not affect *FOXA1* expression in young cultures and moderately stimulates it in mature and aged cultures. Thus, the peptide slows age-specific decline in *FOXA1* expression. There is a CGI associated with the *FOXA1* promoter. It is fully unmethylated in bronchoepithelial cells in all samples investigated. Hence, age-specific and peptide modulation of *FOXA1* expression in bronchoepithelial cells does not depend on its methylation patterns.

Some of the genes investigated do not have any promoter-associated CGIs (*PAX4*, *SCGB1A1*, *SCGB3A2*, and *SFTPA1*). Few CpG sites in their promoter regions immediately preceding TIS could be either methylated (*PAX4*, *SCGB1A1*) or unmethylated (*SCGB3A2*, *SFTPA1*). Most of these CpG sites have similar methylation patterns in all cell samples studied, and hence their connection to age-specific or peptide modulation of gene expression seems to be quite unlikely. The only notable exception could be *SCGB1A1*. It has similar expression levels in bronchoepithelial cell cultures at all passages but is variably modulated by AEDL. Addition of the peptide leads to a strong (3-fold) stimulation of *SCGB1A1* expression in young cell cultures, moderate (1.5-fold) stimulation in mature ones, and practically does not affect it in aged cultures. There are no CGIs in the *SCGB1A1* promoter region, the methylation level of few CpG sites present being rather high (Fig. 7). The methylation levels of these sites are similar in young and mature cell cultures and somewhat lower in the aged ones. Addition of AEDL decreases promoter methylation in young and mature cells to a level characteristic of the control aged cells and does not affect methylation in aged cells. These effects correlate with the AEDL modulation of *SCGB1A1* expression in aging cell cultures.

DISCUSSION

Most of the genes investigated in this work have promoter-associated or TIS-proximal CGIs. Such promoter CGIs are associated in the first place with the genes coding for housekeeping proteins and transcription factors involved in organ and tissue development [21-24]. Most of these CGIs are fully unmethylated, their methylation serving as a fixation mechanism of stable gene repression. This mechanism is used for repression of pluripotency genes in somatic cells, inactivation of X chromosome genes, and allele-specific repression of imprinted genes. All the genes studied here are actively expressed in the respective cell cultures. Thus, low methylation levels of their promoter CGIs are quite anticipated. Partial methylation of a few CpG sites in these CGIs appears to be an indication of epigenetic heterogeneity of the studied cell

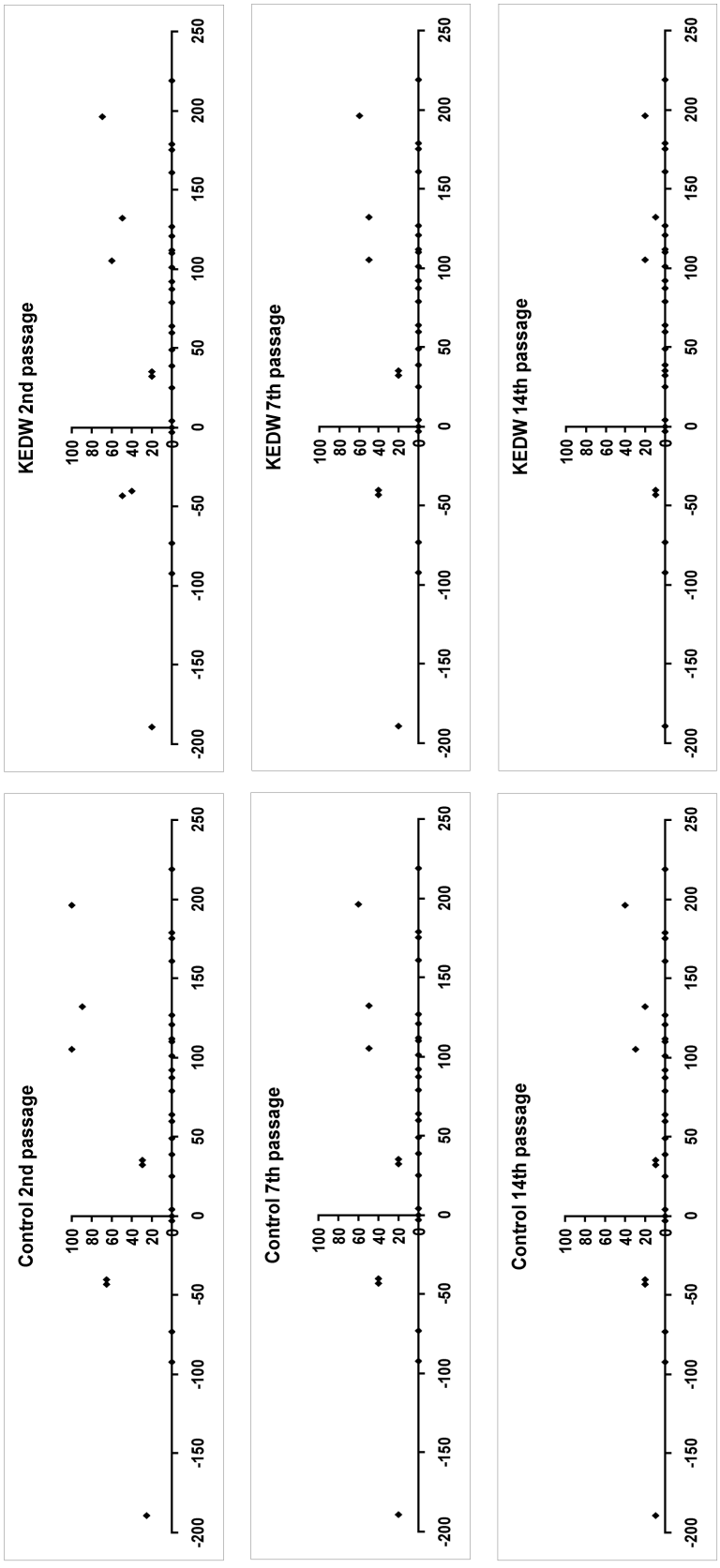

Fig. 5. Methylation patterns of the *FOXA2* gene 5′-end proximal CGI in pancreatic cell cultures on aging.

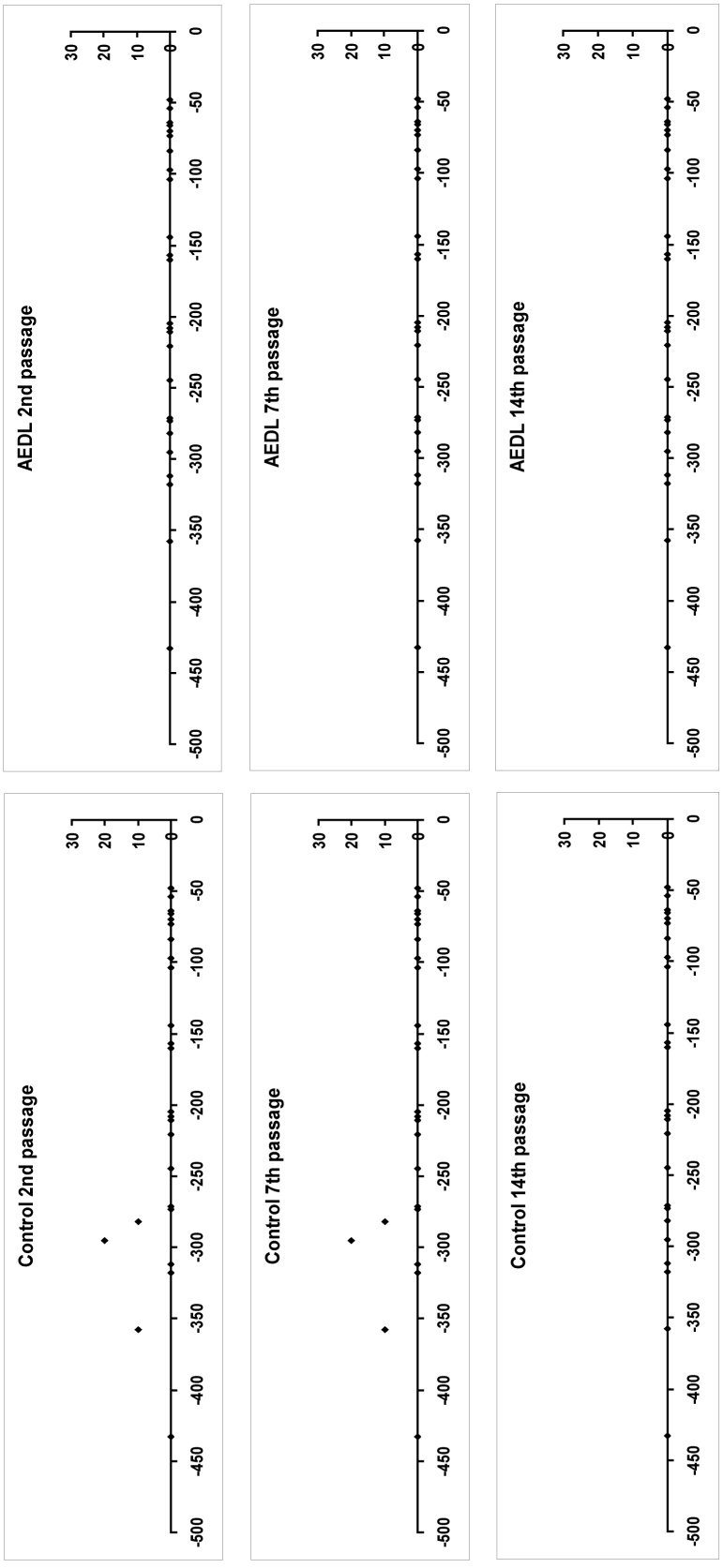

Fig. 6. Methylation patterns of the *NKX2-1* gene promoter CGI in bronchoepithelial cell cultures on aging.

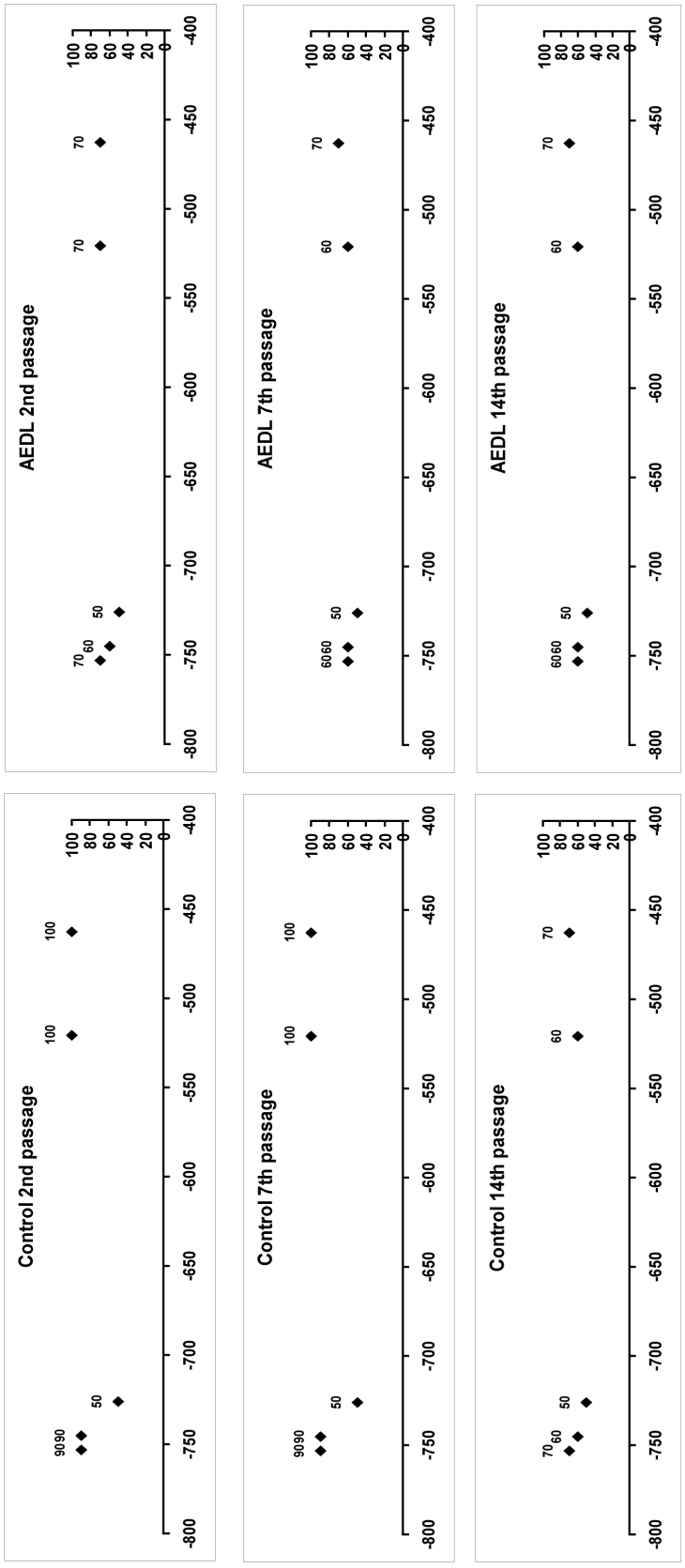

Fig. 7. Methylation patterns of the *SCGB1A1* gene promoter region in bronchoepithelial cell cultures on aging. Methylation levels of specific CpG sites are shown with nearby numbers.

cultures. We suppose the cell aging in culture to be accompanied by asynchronous epigenetic changes. Thus, at any moment there are epigenetically different cells representing various aging stages. The epigenetic changes accompanying cell aging in culture are probably not strictly time-programmed, time and order of their occurrence being variable. The epigenetic heterogeneity of cell cultures could account for both partial DNA site methylation and differences in gene expression levels. The complete methylation of all CpG sites in CGIs is known to invoke a "closed" chromatin structure inaccessible to transcription factors [21-24]. Such changes do not occur in genes studied in this work. First, most of their CpG sites remain unmethylated. Second, expression levels of these genes are variable but high in all cells. Could relatively small changes in methylation levels of a few CpG sites be responsible for quantitative variations in gene expression? We suppose this to be quite possible for some of the genes investigated. It has been shown that methylation of even single CpG sites in promoter region, both CGI containing and devoid of CGIs, affects their affinity to specific transcription factors [25-27]. It is worth a special note that the connection between promoter methylation and inhibition of its activity is not obligatory. In most cases, DNA methylation of promoter region does interfere with transcription by inhibiting binding of respective protein factors. However, there are some examples of positive effects of promoter methylation on transcription [25, 27]. DNA methylation can either inhibit or stimulate binding of transcription regulatory proteins, the effect of this binding being dependent on protein function. Thus, DNA methylation can be a part of either negative or positive gene expression control. Variable gene expression levels in pancreatic and bronchoepithelial cell cultures found in this study are probably caused by local epigenetic changes.

Whether the peptide effects found are of epigenetic nature is less clear. On one hand, an evident correlation between expression stimulation and promoter undermethylation has been found (*PDX1*, *PAX6*, *NGN3*, *NKX2-1*, and *SCGB1A1*). On the other hand, such correlation has not been found for other genes. Some genes are similarly methylated irrespective of age and peptide action (*PAX4* in pancreatic cells, *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 studied cells probably depends on multiple mechanisms, DNA methylation being just one of them. It must be noted that some of the studied genes are targets of protein factors encoded in other genes (for example, *PDX1* is a specific target of FoxA2, *NGN3* is target of Pdx1, etc.). Thus, some of the effects found in this study could be secondary ones.

Short biologically active peptides are essential tissue-specific gene expression modulators and, in some cases, DNA methylation modulators. Thus, such short peptides could be effective epigenetic regulatory signal molecules affecting gene function and cell differentiation. Molecular mechanisms of their action are generally unstudied. However, there are some data concerning methylation-dependent interaction between peptides and DNA [28]. A hypothesis has been proposed that peptides can interfere with DNA methylation, thus affecting gene expression. Such interference could be one of the mechanisms of transcription modulation by peptides [29]. The data concerning effects of peptides on promoter methylation obtained in this study are in general accordance with this hypothesis.

REFERENCES

1. Khavinson, V. Kh., Durnova, A. O., Polyakova, V. O., Tolibova, G. H., Linkova, N. S., Kvetnoy, I. M., and Tarnovskaya, S. I. (2013) Effects of pancragen on the differentiation of pancreatic cells during their ageing, *Bull. Exp. Biol. Med.*, **154**, 501-504.
2. Khavinson, V. Kh., Gapparov, M. M.-G., Sharanova, N. E., Vasilyev, A. V., and Ryzhak, G. A. (2010) Study of biological activity of Lys-Glu-Asp-Trp-NH_2 endogenous tetrapeptide, *Bull. Exp. Biol. Med.*, **149**, 351-353.
3. Korkushko, O. V., Khavinson, V. Kh., Shatilo, V. B., and Antonyk-Sheglova, I. A. (2011) Peptide geroprotector from the pituitary gland inhibits rapid aging of elderly people: results of 15-year follow-up, *Bull. Exp. Biol. Med.*, **151**, 366-369.
4. Korkushko, O. V., Khavinson, V. Kh., Shatilo, V. B., Antonyk-Sheglova, I. A., and Bondarenko, E. V. (2011) Prospects of using pancragen for correction of metabolic disorders in elderly people, *Bull. Exp. Biol. Med.*, **151**, 454-456.
5. Khavinson, V. Kh., Tendler, S. M., Vanyushin, B. F., Kasyanenko, N. A., Kvetnoy, I. M., Linkova, N. S., Ashapkin, V. V., Polyakova, V. O., Basharina, V. S., and Bernadotte, A. (2014) Peptide regulation of gene expression and protein synthesis in bronchial epithelium, *Lung*, **192**, 781-791.
6. Khavinson, V. Kh., Linkova, N. S., Polyakova, V. O., Kheifets, O. V., Tarnovskaya, S. I., and Kvetnoy, I. M. (2012) Peptides tissue-specifically stimulate cell differentiation during their aging, *Bull. Exp. Biol. Med.*, **153**, 148-151.
7. Pogribny, I. P., and Vanyushin, B. F. (2010) Age-related genomic hypomethylation, in *Epigenetics of Aging* (Tollefsbol, T. O., ed.) Springer Science+Business Media, N. Y., pp. 11-27.
8. Schumacher, A. (2011) Aging epigenetics, in *Handbook of Epigenetics. The New Molecular and Medical Genetics* (Tollefsbol, T. O., ed.) Elsevier Inc., Amsterdam, pp. 405-422.
9. Bell, J. T., Tsai, P.-C., Yang, T.-P., Pidsley, R., Nisbet, J., Glass, D., Mangino, M., Zhai, G., Zhang, F., Valdes, A., Shin, S. Y., Dempster, E. L., Murray, R. M., Grundberg, E., Hedman, A. K., Nica, A., Small, K. S., Dermitzakis, E. T., McCarthy, M. I., Mill, J., Spector, T. D., and Deloukas,

P. (2012) Epigenome-wide scans identify differentially methylated regions for age and age-related phenotypes in a healthy ageing population, *PLoS Genet.*, **8**, e1002629; DOI: 10.1371/journal.pgen.1002629.

10. Day, K., Waite, L. L., Thalacker-Mercer, A., West, A., Bamman, M. M., Brooks, J. D., Myers, R. M., and Absher, D. (2013) Differential DNA methylation with age displays both common and dynamic features across human tissues that are influenced by CpG landscape, *Genome Biol.*, **14**, R102.
11. Hannum, G., Guinney, J., Zhao, L., Zhang, L., Hughes, G., Sadda, S., Klotzle, B., Bibikova, M., Fan, J. B., Gao, Y., Deconde, R., Chen, M., Rajapakse, I., Friend, S., Ideker, T., and Zhang, K. (2013) Genome-wide methylation profiles reveal quantitative views of human aging rates, *Mol. Cell*, **49**, 359-367.
12. Florath, I., Butterbach, K., Mueller, H., Bewerunge-Hudler, M., and Brenner, H. (2014) Cross-sectional and longitudinal changes in DNA methylation with age: an epigenome-wide analysis revealing over 60 novel age-associated CpG sites, *Hum. Mol. Genet.*, **23**, 1186-1201.
13. Tusnady, G. E., Simon, I., Varadi, A., and Aranyi, T. (2005) BiSearch: primer-design and search tool for PCR on bisulfite treated genomes, *Nucleic Acids Res.*, **33**, e9.
14. Aranyi, T., Varadi, A., Simon, I., and Tusnady, G. E. (2006) The BiSearch web server, *BMC Bioinformatics*, **7**, 431.
15. Grunau, C., Schattevoy, R., Mache, N., and Rosenthal, A. (2000) MethTools – a toolbox to visualize and analyze DNA methylation data, *Nucleic Acids Res.*, **28**, 1053-1058.
16. Lewin, J., Schmitt, A. O., Adorjan, P., Hildmann, T., and Piepenbrock, C. (2004) Quantitative DNA methylation analysis based on four-dye trace data from direct sequencing of PCR amplificates, *Bioinformatics*, **20**, 3005-3012.
17. Rakyan, V. K., Hildmann, T., Novik, K. L., Lewin, J., Tost, J., Cox, A. V., Andrews, T. D., Howe, K. L., Otto, T., Olek, A., Fischer, J., Gut, I. G., Berlin, K., and Beck, S. (2004) DNA methylation profiling of the human major histocompatibility complex: a pilot study for the human epigenome project, *PLoS Biol.*, **2**, e405.
18. Arda, H. E., Benitez, C. M., and Kim, S. K. (2013) Gene regulatory networks governing pancreas development, *Devel. Cell*, **25**, 5-13.
19. Conrad, E., Stein, R., and Hunter, C. S. (2014) Revealing transcription factors during human pancreatic β cell development, *Trends Endocrinol. Metab.*, **25**, 407-414.
20. Maeda, Y., Dave, V., and Whitsett, J. A. (2007) Transcriptional control of lung morphogenesis, *Physiol. Rev.*, **87**, 219-244.
21. Illingworth, R. S., and Bird, A. P. (2009) CpG islands – "a rough guide", *FEBS Lett.*, **583**, 1713-1720.
22. Deaton, A. M., and Bird, A. (2011) CpG islands and the regulation of transcription, *Genes Devel.*, **25**, 1010-1022.
23. Jones, P. A. (2012) Functions of DNA methylation: islands, start sites, gene bodies and beyond, *Nat. Rev. Genet.*, **13**, 484-492.
24. Smith, Z. D., and Meissner, A. (2013) DNA methylation: roles in mammalian development, *Nat. Rev. Genet.*, **14**, 204-220.
25. Huntriss, J., Lorenzi, R., Purewal, A., and Monk, M. (1997) A methylation-dependent DNA-binding activity recognizing the methylated promoter region of the mouse *Xist* gene, *Biochem. Biophys. Res. Commun.*, **235**, 730-738.
26. Han, H., Cortez, C. C., Yang, X., Nichols, P. W., Jones, P. A., and Liang, G. (2011) DNA methylation directly silences genes with non-CpG island promoters and establishes a nucleosome occupied promoter, *Hum. Mol. Genet.*, **20**, 4299-4310.
27. Gustems, M., Woellmer, A., Rothbauer, U., Eck, S. H., Wieland, T., Lutter, D., and Hammerschmidt, W. (2014) c-Jun/c-Fos heterodimers regulate cellular genes via a newly identified class of methylated DNA sequence motifs, *Nucleic Acids Res.*, **42**, 3059-3072.
28. Fedoreeva, L. I., Kireev, I. I., Khavinson, V. Kh., and Vanyushin, B. F. (2011) Penetration of short fluorescence-labeled peptides into the nucleus in HeLa cells and *in vitro* specific interaction of the peptides with deoxyribooligonucleotides and DNA, *Biochemistry (Moscow)*, **76**, 1210-1219.
29. Khavinson, V. Kh., Fedoreeva, L. I., and Vanyushin, B. F. (2011) Short peptides modulate the effect of endonucleases of wheat seedling, *Doklady Biochem. Biophys.*, **437**, 64-67.