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Tetrapeptide KEDW Interacts with DNA and Regulates Gene Expression

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Abstract

Peptide KEDW (Lys-Glu-Asp-Trp-NH2) is known to reduce the blood glucose level in rats with streptozotocin- and alloxan-induced diabetes mellitus. Here, we examine the influence of KEDW peptide on cell differentiation and DNA structure. KEDW peptide increased the expression of PDX1, NGN3, PAX6, FOXA2, NKX2-2, NKX6.1, and PAX4 genes but decreased MNX1 and HOXA3 gene expression when added to pancreatic cell culture. Moreover, KEDW peptide caused an increase in expression of PDX1, NGN3, PAX6, FOXA2, NKX2-2, NKX6.1, and PAX4 proteins without affecting synthesis of MNX1 and HOXA3 when added to pancreatic cell culture. Results obtained through physical methods (UV–visible absorption, circular dichroism) and molecular modelling methods suggest that the peptide binds to DNA along the major groove. Experimental and theoretical data provided a 3D model of the stable DNA-peptide complex. We propose that regulation of differentiation factor expression in pancreatic (endocrine) cells by KEDW peptide occurs through specific binding of the peptide to regulatory elements of corresponding genes.

Keywords: KEDW peptide, diabetes mellitus, gene expression, protein synthesis, molecular modelling, DNA binding

1. Background

Occurrences of type 2 diabetes mellitus (DM) and chronic pancreatitis have steadily increased worldwide in recent decades [1][2][3][4]. These pancreatic pathologies occur when the expression of transcription factors, which regulate pancreatic cells differentiation, is affected. Significant transcription factors of pancreatic cell differentiation include: Pdx1, Ngn3, Mnx1, Pax6, Foxa2, Nkx2.2, Nkx6.1, Hoxa3, Cxcl12 and Pax4 [5][6]. Mutations in these differentiation and expression genes can disturb insulin synthesis in β-cells and lead to DM [7][8][9][10][11][12]. Expression of differentiation and proliferation factors, which regulate the maturation of endocrine insular cells and exocrine acinar, gradually decreases with continuous propagation of pancreatic cell cultures [13][14][15].

KEDW peptide reduces blood glucose levels in rats with streptozocin- and alloxan-induced DM [16]. KEDW peptide is also shown to lower blood glucose after fasting and during standard glucose tolerance tests; additionally, KEDW peptide can lower the concentration of plasma insulin and the insulin sensitivity index in patients of advanced age with DM types 1 and 2 [17]. Furthermore, KEDW peptide increases synthesis of Pax6, Foxa2, Nkx2.2, Nkx6.1, Hoxa3, and Pax4 factors in cell cultures at the 14^{th} passage [13][14][15].

Herein, the project aim is to study the mechanism of KEDW peptide influence on gene expression of aforementioned transcription factors in human pancreatic cell cultures.

2. Results and discussion

2.1 Gene expression and protein synthesis in pancreatic cells

Addition of KEDW peptide to pancreatic cells (14^{th} passage) increased mRNA expression of all investigated genes except for that of MNX1 and HOXA3 (Table 1).

The most pronounced effects observed were associated with PDX1, NGN3, and PAX6 genes: the peptide increased expression by 1.6, 2.7, and 1.8 times, respectively (Table 1). On the contrary, expression of MNX1 and HOXA3 genes decreased by 1.5 and 2.4 times, respectively. Thus, differentiation factors of mRNA expression increased in cell cultures under the influence of KEDW peptide; therefore, it is appropriate to conclude that the peptide activates transcription of specific genes involved in differentiation of pancreatic cells.

Table 1. Influence of KEDW peptide on expression of genes coding for differentiation factors in pancreatic cells.

mRNA	Square of expression, %	
	Control	KEDW peptide
PDX1	0.85±0.07	1.35±0.06*
NGN3	0.50±0.02	1.35±0.06*
MNX1	1.10±0.20	0.75±0.05*
PAX6	0.75±0.06	1.37±0.08*
FOXA2	0.95±0.09	1.25±0.06*
NKX2.2	0.50±0.04	0.80±0.06*
NKX6.1	0.80±0.05	1.20±0.07*
HOXA3	1.10±0.20	0.45±0.03*
PAX4	0.75±0.06	1.00±0.10*

*$p < 0.05$ relative to the control; the relative intensity of mRNA was determined by comparing intensities of specific cDNA from our experiment with that of GAPDH, which is the standard gene of a normal cell population.

Results obtained by immunofluorescence confocal microscopy suggest that the peptide increased expression of PDX1, NGN3, PAX6, FOXA2, NKX2.2, NKX6.1, PAX4 by 1.5, 3.2, 2.4, 2.1, 2.4, 3.5, and 1.9 times, respectively. However, the peptide did not affect MNX1 and HOXA3 synthesis (table 2, Fig 1), whichcorrelates with the decreased expression of corresponding genes (table 1).

Table 2. Influence of KEDW peptide on expression of proteins coding for differentiation factors in pancreatic cells

Protein	Square of expression, %	
	Control	KEDW peptide
PDX1	2.58±0.17	3.97±0.18*
NGN3	0.95±0.10	3.04±0.22*
MNX1	2.11±0.23	2.02±0.19
PAX6	1.55±0.10	3.78±0.26*
FOXA2	1.14±0.13	2.45±0.15*
NKX2.2	0.79±0.06	1.93±0.17*
NKX6.1	0.97±0.12	3.36±0.20*
HOXA3	1.40±0.20	1.50±0.24
PAX4	2.23±0.16	4.13±0.12*

*p < 0.05 relative to the control

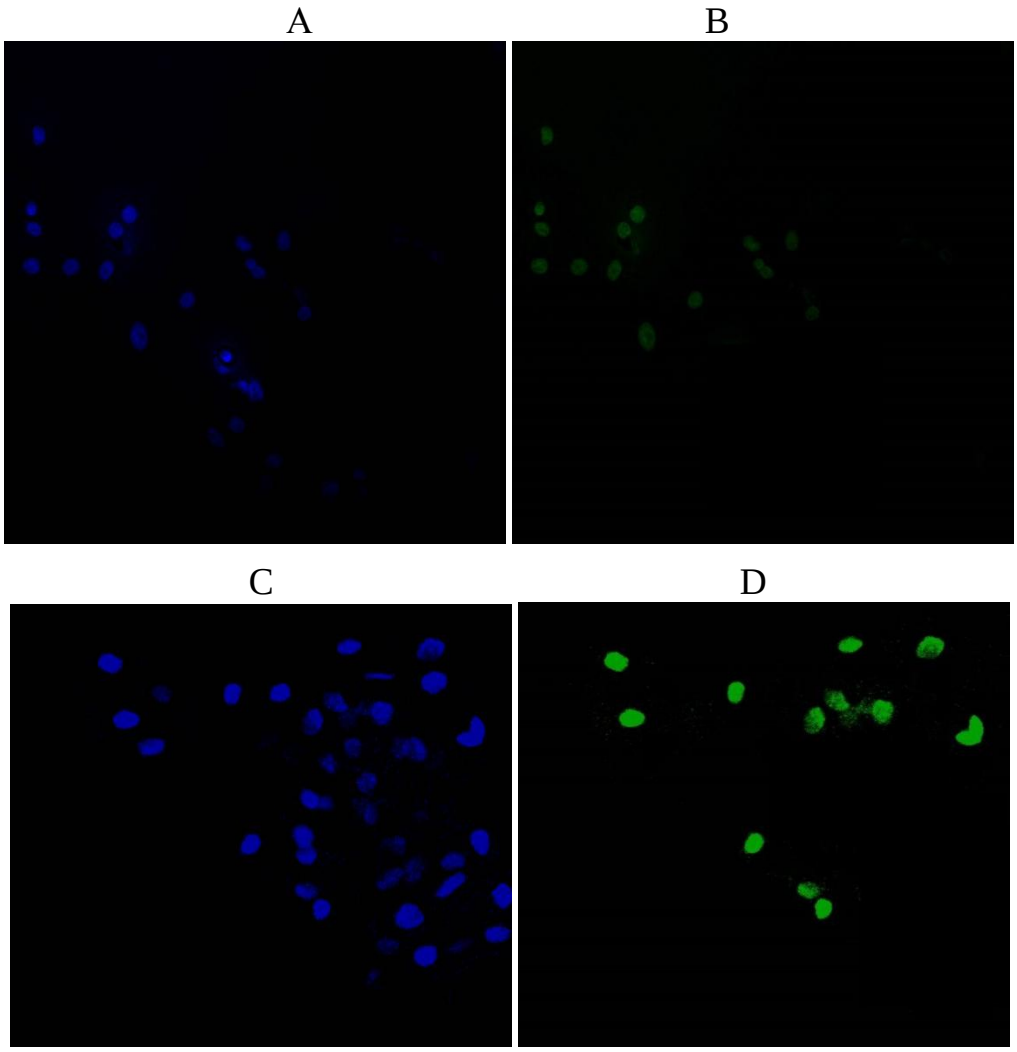


Figure. 1. Influence of peptide KEDW on expression of PAX4 in pancreatic cells culture. Confocal microscopy at magnification 400x; (A) control, blue color – immunofluorescence, Hoechst – 33258; (B) control, green – antibody against PAX4+Alexa 488; (C) peptide KEDW, blue color – immunofluorescence, Hoechst – 33258; (D) peptide KEDW, green – antibody against PAX4+Alexa 488.

2.2 Spectral characteristics of DNA-peptide complexes in solution

Figure 2A shows the absorption spectra of DNA, KEDW peptide, and associated complexes at two concentrations of NaCl (0.005 M and 1 M). KEDW peptide includes a tryptophan amino acid residue, which has 3 or more electron transitions visible between 240 and 290 nm [10]. Corresponding bands for KEDW peptide have local maxima at 271,

278 (global maximum), and 286 nm. Absorption spectra obtained immediately upon mixing of equal volumes of DNA and peptide solution in 0.005 M NaCl correspond with the sum of spectra observed for free DNA and for free peptide at equivalent concentrations. This indicates an absence of complex formation during the initial moments of the test. After 24 hours of storage at 4°C, associated spectra show a higher intensity than that associated with the sum of spectral components (Fig. 2, curves 4 and 3, respectively).

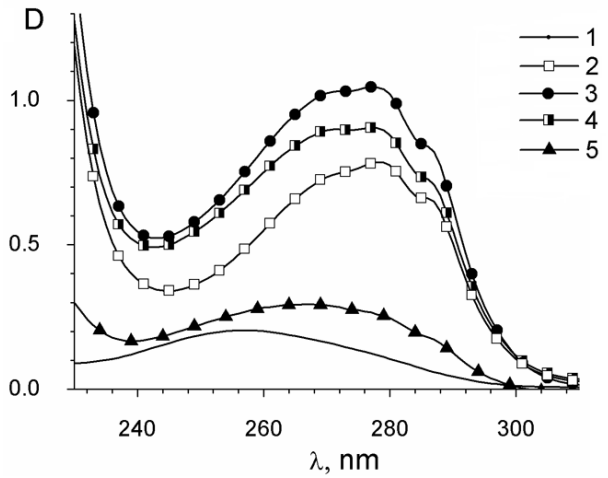


Figure 2. Absorption spectra of DNA-peptide complexes in 0.005 M NaCl. D denotes molar absorptivity ($x10^{-3}$); λ denotes wavelength (nm). 1 - absorption spectrum of DNA; 2 - absorption spectrum of KEDW peptide; 3 - absorption spectrum of DNA-peptide complex; 4 - calculated sum of peptide and DNA spectra; 5 - calculated DNA spectrum as part of DNA-peptide complexes.

Thus, it is clear that an interaction occurs between the components. In order to evaluate the change in the absorption spectrum of DNA that occurs upon binding to the peptide, we subtracted peptide spectrum from the spectrum of DNA-peptide complex. DNA spectrum calculated from that of the complex (Fig. 2. curve 5) was substantially different from the absorption spectrum of free DNA (Fig. 2. curve 1).

In addition to the hyperchromatic effect, which may indicate some destabilization of the DNA secondary structure upon binding, a bathochromic shift of absorption maximum occurred from 260 to 267 nm (Fig. 2, curve 3). Similar results we obtained using DNA and peptide solutions in 1 M NaCl (Fig. 2, curve 4). Since the overall DNA charge is completely hidden in 1 M NaCl solution, this finding indicates that electrostatic interactions do not influence the complex formation

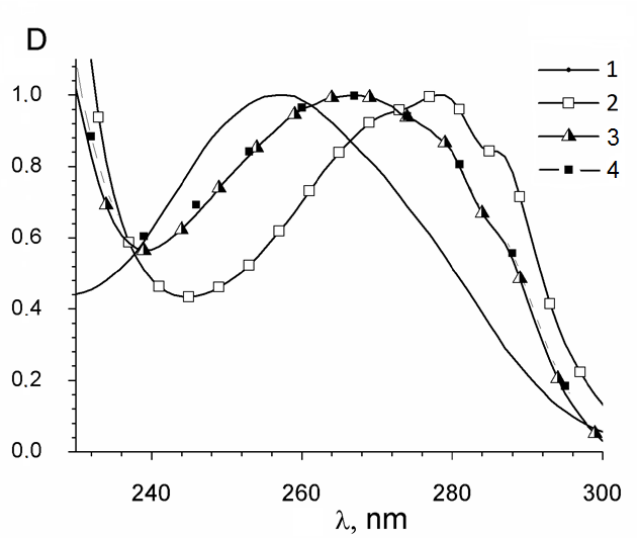


Figure 3. Relationship between DNA absorbance and ionic strength. 1 - absorption spectrum of DNA in 0.005 M NaCl; 2 - absorption spectrum of KEDW peptide in 0.005 M NaCl; 3 - calculated DNA spectrum as part of DNA-peptide complex in 0.005 M NaCl; 4 - calculated DNA spectrum as part of DNA-peptide complex in 1 M NaCl. D denotes molar absorptivity ($x10^{-3}$); λ - wavelength (nm).

When normalized at their maximal values (Fig. 3), the calculated absorption spectra of DNA-peptide complexes in 1 M and 0.005 M NaCl completely coincide. The calculated DNA absorption spectra include distinguishable additional peaks at 278 and 286 nm (Fig. 3), indicative of the change in peptide absorption spectrum at complex formation.

Dependence of the KEDW peptide absorption spectrum on DNA and peptide concentration ratio (r) is shown in Figure 4. Spectral changes associated with the interaction of KEDW with DNA are more intense at low values of r; the extra band at 286 nm becomes less intensive, whereas the intensity of the band observed as 271 nm increases in intensity.

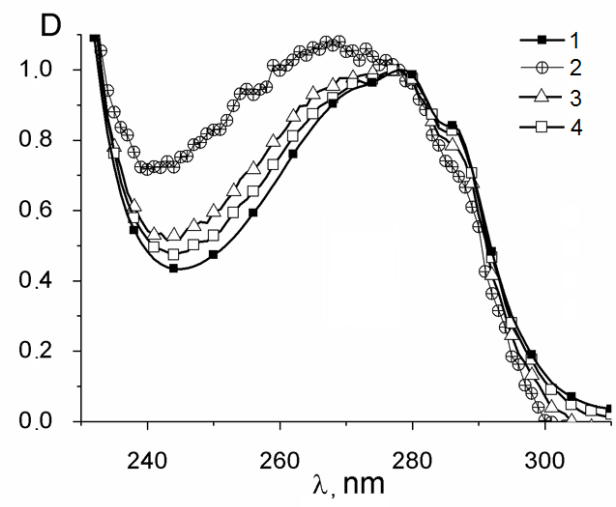


Figure 4. Relationship between the absorption spectrum of KEDW peptide in complex with DNA and DNA-peptide concentration ratio (r). 1 - absorption spectrum of free peptide (r = 0); 2 - r = 0.53; 3 - r = 1.7; 4 - r = 2.8. D - molar absorptivity (x10^{-3}); λ - wavelength (nm).

However, the increase in intensity at 271 nm may be due to an unconsidered contribution of the DNA absorption change in the complex. At higher r-values, the spectrum depends on free peptide unbound to DNA. Calculations of r-values are based on total molar concentration ratios of peptide to DNA phosphate groups. For example, r = 0.5 denoted a specific ratio of one base pair of DNA present in the solution for each peptide molecule. This ratio corresponds to the maximum change of the peptide absorption spectrum at DNA binding observed in our experiment. At high r-values, the peptide spectrum in the solution increases and approaches that of typical free peptide. The peptide binds to DNA along the major groove; when all possible binding sites in DNA groove are occupied, excess peptide molecules remain in the solution, unable to bind DNA.

Characteristic circular dichroism (CD) spectrum of KEDW peptide, of free DNA, and of peptide-DNA complexes in 0.005 M and 1 M NaCl solutions are shown in Figures 5 and 6.

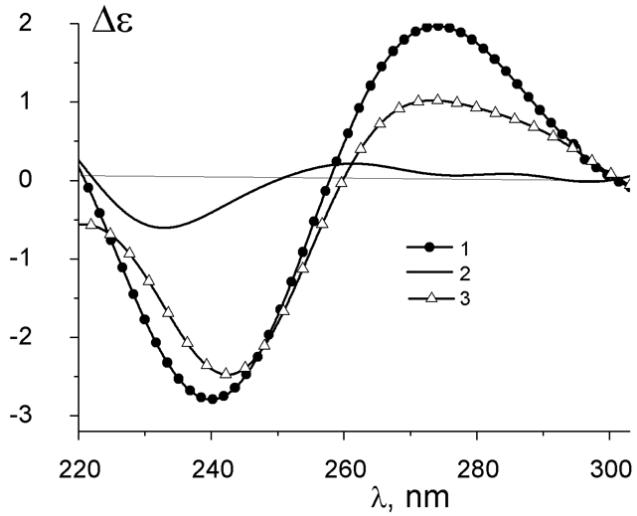


Figure 5. Circular dichroism spectra of DNA (1), peptide (2), and associated complexes (3) in 0.005 M NaCl. Concentration of peptide was 1 mg/mL; DNA concentration was 0.05 mg/mL. λ - wavelength (nm); Δε - difference in absorption intensities for left and right circularly polarized light.

CD spectra of the peptide-DNA complex and the peptide at a high salt concentration (1 M NaCl) differ from those at low salt concentration (0.005 M NaCl). Evidently, conformation of the peptide depends on masking the ionogenic groups. However, peptide binding to DNA occurs irrespective of such masking. The complex spectrum differs from that of the sum of individual components. On the basis of difference in CD spectra of the peptide in solutions with high and low ionic strength, we suggest that changes of the peptide conformation upon binding to DNA determine the results obtained. Peptide configuration changes in

NaCl solutions affect the formation of peptide-DNA complex. On the other hand, similar changes to the complex CD spectra in 1 M and 0.005 M NaCl do not provide adequate explanation for the differences in complex formation under these conditions.

However, UV-vis spectroscopy studies show that ionic strength does not affect peptide binding to DNA. The complex formation between peptide and DNA depends on the concentration of these components, as suggested by observed changes of CD spectra of the complex (Fig. 7 and 8). This phenomenon is likely related to the destabilization of secondary DNA structure accompanied by insignificant reduction of bending stiffness of the macromolecule.

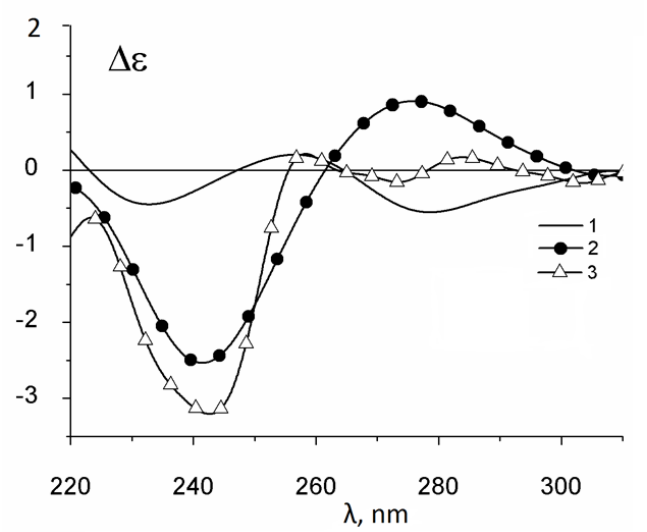


Figure 6. Circular dichroism spectra of DNA (1), peptide (2), and associated complexes (3) in 1 M NaCl. Concentration of peptide – 1 mg/mL; DNA – 0.05 mg/mL. λ - wavelenght (nm); Δε - difference in absorption intensities for left and right circularly polarized light.

There were no observed changes of polyelectrolytic DNA swelling in the systems under study because binding is not dependent on ionic strength and, hence, did not result in any change of electrostatic interactions in the solution. An alternative explanation may be spiral deformation upon binding the peptide without disturbance of hydrogen bonds – but weakening of π-stacking interactions. It is possible that the peptide binds specific sites located in gene promoters, causing activation of their expression.

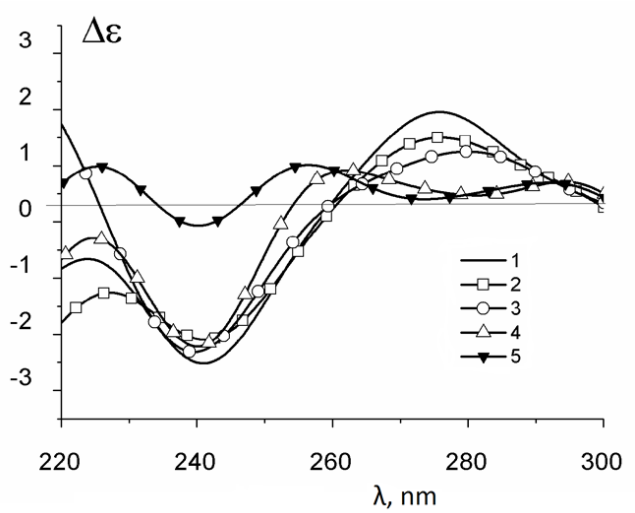


Figure 7. Circular dichroism spectra of DNA (1) and its complexes with the peptide in 0.005 M NaCl at C(pep) = 0.15 (2), 0.3 (3), 0.75 (4) and 1.5 mM (5). C(DNA) = 0.05 mg/mL. λ - wavelenght (nm); Δε - difference in absorption intensities for left and right circularly polarized light.

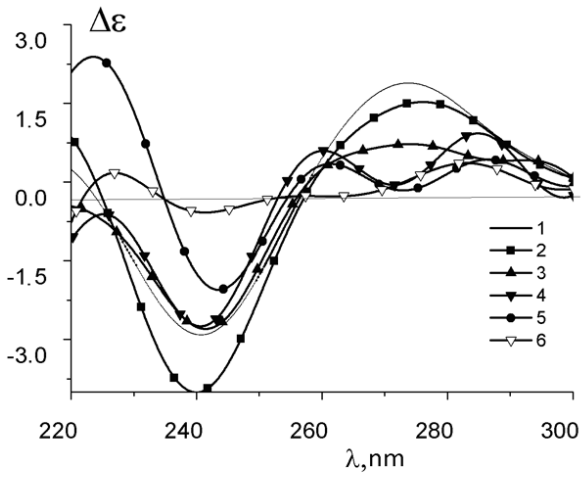


Figure 8. Circular dichroism spectra of DNA (1) and its complexes with the peptide in 1 M NaCl at C(pep) = 0.08 mM (2), 0.26 mM (4), 0.43 mM (5) and 1.3 mM (6). C(DNA) = 0.05 mg/mL. λ - wavelenght (nm); Δε - difference in absorption intensities for left and right circularly polarized light.

2.3 Docking

Docking experiments were performed using default parameters with MOE. From analysis docked structures, we concluded that the KEDW peptide is stabilized by one or more H-bonds with the DNA bases (Table 3). The peptide showed the lowest dG energy pose -5.9 kcal/mol in compound with the DNA sequence ACCT (Fig 9). Figure 9 shows results of KEDW peptide docking in the major DNA groove formed by sequences, corresponding to those found in the differentiation factors genes (Table 4).

In the complex relationship between the KEDW peptide and site ACCT, there is a network of five hydrogen bonds and two ion-ion interactions (Table 3). The peptide simultaneously binds to nucleotides of both DNA strands, acting as an additional bond between the strands. Calculations obtained by molecular docking method confirm the experimental data. The peptide binds in the DNA major groove via non-covalent bonds to nitrogenous bases. Peptide binding to DNA is site-specific due to primary DNA structure.

Table 4 shows promoter sites of the differentiation factor genes of pancreatic cells in the range of 499 to 100 nucleotide pairs, relative to the transcription initiation site [18]. The molecular docking method was used to find sequence ACCT, which appears as an energetically favourable sequence for peptide-DNA complex formation.

Table 3. Peptide docking in DNA sites

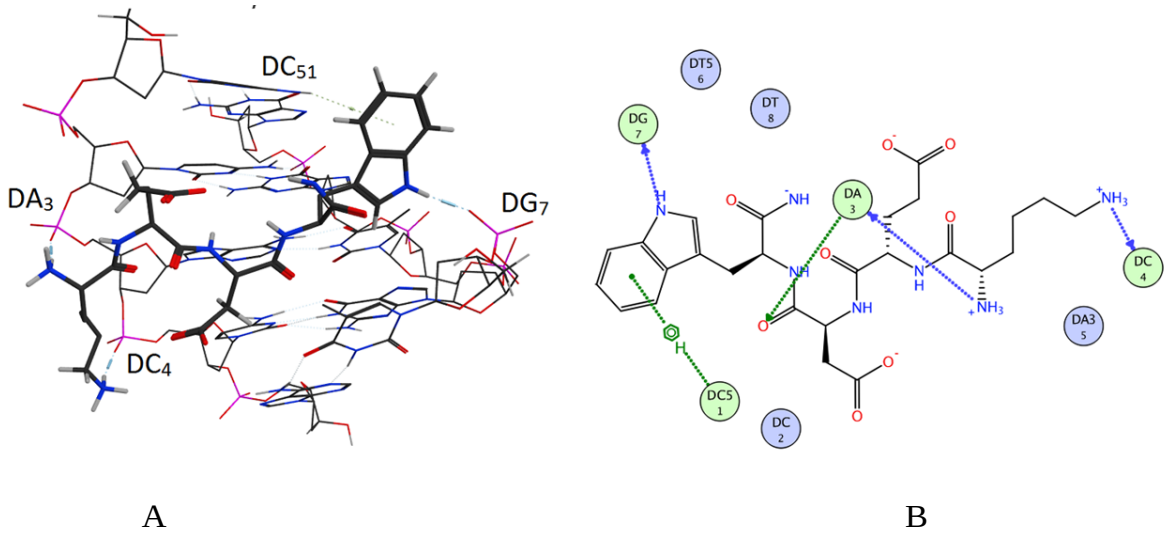
Peptide KEDW	DNA sequence ACCT	Interaction	Distance, Å
N1	OP2 (DA1)	H-donor	2.83
N1	N7 (DA1)	H-donor	3.16
NZ21	OP1 (DC2)	H-donor	3.03
OE39	N4 (DC3)	H-acceptor	3.04
OD51	N6 (DA1)	H-acceptor	3.13
N1	OP2 (DA1)	ionic	2.83
NZ21	OP1 (DC2)	ionic	3.03

Letters in the peptide column: N – the N-terminal nitrogen atom of lysine, NZ - the nitrogen atom in the side chain of lysine, OE – oxygen atom of carboxyl group of glutamic acid, OD2 - oxygen atom of carboxyl group of aspartic acid. Letters in the DNA column: DG - guanine, DC - cytosine, DA - adenine, DT - thymine. The serial number corresponds to atom number in peptide or DNA models.

Table 4. Promoter sequences of *Homo sapiens* genes

Gene	Regulatory regions of the genes within the range from 499 to 100 base pairs relative to transcription initiation point	GenBank/ ENSEMBL Gene ID
PDX1	ATCAAATGCTTCTG**ACCT**AGAGAGCTGGGTCTGCAAACTTTTTTTTTATCGTATTCCGCAACAGTT AAATAAAAAATTAAAAACTCAACATGTCTCCTTGTAAACTACATCAATTAACAAACACACTATG**T CCA**TTATCAAATATAATAGAAAAAATATAGGAAAATAGAAAATAGAAAAATATAGGAAAATAGA AACTTTTAAGCCACGGTGAAAATGTTTCTATAAATGAGTGGTTCTAATGTTTTCGTGAGCGCCCAT TTTGGGGAGCACCGCCAGCTGCCCGTTCAGGAGTGTGCAGCAAACTCAGCTGAGAGAGAAAAT**T GGA**ACAAAAGC**AGGT**GCTCGCGGGT**ACCT**GGGCCTAGCCTCTTAGTGCGGCCAGCCAGGCCAAT CACGGCCCCCGGCTGAACCACGTGGGGCCCCGCGGAGCCTATGGTGCGGCGGCCGGCCCGCCGG TCCGCGCTGGCTGTGGGTTCCCTCTGAGATCAGTGCGGAGCTGTCAAAGCGAGCAGGGGTGGCGC CGGGAGTGGGAACGCCACACAGTGCCAAATCCCCGGC**TCCA**GCTCCCGACTCCCGGCTCCCGGCT CCCGGCTCCCGGTGCCC	ENSG00000139515
NGN3	GGCTAGGAGCAAAGCCGTCTG**AGGT**GGCCTGACCAGAGCCACACGAGGCTCTTCTCACTGGGCG AGGCTCTTTGAGGAACCGAGAGTTGCTGGGACCCAGCCCGCCCTCGAGAGAGCAAACAGAGCGG CGCTCCCCTCCCCCGACCCCGGCCCTTTGTCCGGAA**TCCA**GCTGTGCCCTGCGGGGGAGGAGCGG GCTCGCGTGGCGCGGCCCCAGGGCCCCGGCGCTGATTGGCCGGTGGCGCGGGCAGCAGCCGGGC AGGCACGCTCCTGGCCCGGGCGAAGCAGATAAAGCGTGCCAAGGGGCACACGACTTGCTGCTCA GGAAATCCCTGCGGTCTCACCGCCGCGCCTCGAGAGAGAGCGTGACAGAGGCCTCGGACCCCATT CTCTCTTCTTTTCTCCTTTGGGGCTGGGGCAACTCCCAGGCGGGGGCGCCTGCAGCTCAGCTGAAC TTGGCGACCAGAAGCCCGCTGAGCTCCCCACGGCCCTCGCTGCTCATCGCTCTCTATTCTTTTGCG	ENSG00000122859

	CCGGTAGAA**AGGT**AATATT**TGGA**GGCCTCCGAGGGACGGGCAGGGGAAAGAGGGATCCTCTGA CCCAGCGGGGGCTGGGAGG	
MNX1	AGTGCGCTGCCGTCGCCTCGCAGAGCGGCC**AGGT**CTGCGCAGCGTGGGCGCTCAGGAGCG**TGGA** ACCACCGCGCTCCGCAAGGCAGACG**TGGA**CCCAC**TCCA**GCGCCGACAAGGAGGCCGGAGAGGA CGCGCGCAGCTGGC**TCCA**GGGACCAACCAAGTCCGGAGGGGCCCACGTGTCTCGGGAGCGGGTC TTGGGCCGCCGCGGGGCTC**TCCA**GGGGCCCGGGGGTTGCCAGTGCCCGCCGTCCGGCTCCGCACC CAGGGGAGGGCGGGCAGAGGCCCC**AGGT**TGAGCCTCGGAGTA**TCCA**CTCCCCGTTGGTGTCCCC GCCCGCCAGCGCCGCGCAACAGCCCGGGCTGCTTAGG**ACCT**CGCGGCGCGGGGGCGGGGCGGCG GGCGGCCGGGACGGTGAC**AGGT**GCGCCCCTCGGCCAATGGCGGAGCCCGCGGCCCTCCCCGGGC CCCCGCTGCGCGCGCCAATCGGAAGCCGCGCTGAGCTTCAAGTGCGCGGAGGGGCGCGGGTCCC CACCACGGCGGCAGCGGCCTCCGCGCGGTCGCCTGAGGGCTGCAGCGCAAAGAACCGGGCTCCC GGGCCCGGACGCGGGGAAGCACTC	NM005515.3
PAX6	GGCCCGAAGCCGCCGAGAGAGCTCGGGACAGCGCAGGACCAGGCAGCCGCTCGCTCTCCTGTC**A** **CCT**TAACTGCAGGCTCCGAGGGGCGCCTT**TGGA**GTGTACTG**AGGT**GTGTCCTAATCGTGCGGCAT TCAACAAA**TGGA**CTTCTGGTGTGTGGTCAGAAGAGAAAAGCCATTTACTTACTTTCCTCCCCGGT TTTCTGGCAACAGCTGAAGGGGAGCTGCCTCCG**TGGA**CTGAGCAGACCCAGGAGAGGGAGTCGT GGTGCGGAGACACACGCACCACACACAGATGACCGGTGGCACACACGACACACGCTGACATACC GACATCGCCAGTGGGACACACACACACACACACACACACACACACACAGAGAGAGAG AGAGAATCCCTCCCAGCATTGGTCATCCGCCCCCCCACCCAGGCT**TCCA**CTCCCCCTCCCCTCTTA TCTCCCCTGGCTTCCCCTCCTCTCGGGCGCTGCGAAAAGCAGCCGCACTTAGTCAACAAATGGCA CGTGGGAGAAGTTGGTGAGTGTTTGGTGAGGACTCTTCAGGGCTTTTCACAAGAACCCTCTGTAC ACAAAGTAAGTGGCGTGTT	ENSG00000007 372
FOXA2	CGAAGCTCCGTGTCTGCCATCTCGCCTGTCTTCTGCCACCATCGCCCCCAATTT**TGGA**C**AGGT**GG GC**TGGA**TGCCCACTAGTTCCTATGCATTCTCTGTGTCTGAGGGGGTGGGTACAGGGC**TGGA**TCCC CA**AGGTCCA**GCC**AGGT**TTTCAGAACCAAGAAAGAGCC**TCCA**CA**TCCA**AAC**ACCT**GCAATATCC CCCCAC**TCCA**AATCTGGGCTCACAGGCTAACCCAGAACAGAAGACAATTTTTGAACCCAAGAGCT GCTGGGGAAATAAAAGTATACGATTGC**TGGA**GTTTCTAATTTCTATTAAGCAGTCCCTC**TGGA**AG ACAGAGAGGACAGAGACGCTCTTGAAGTCAAC**TCCA**TATGCCCCATCATTGATTCC**TGGA**TTCTT CTCTCCTCACCCCTCCCTCCCC**ACCT**CCTGCCCTGTTTGTTTTAGTTACGAAATGCTGTGGGC**ACC** **T**CGGTTGTGACTGAAAAGTA**ACCT**TGAAACACGCCGGCCTGAATATCAGAGACAAATCTCAGCCT CCCAACCGTCGGCCGCTGCTAGAGGGGCTGCTTGCGCCAGGCGCCGGCCGCCCCACTGCGGGTCC CTGGCGGCCGGTGTCT	NM153675.2
NKX2-2	TCCCCTCCTCCTCCCCACCCCC**ACCT**TTTTTAAGATGCAATTTGTTAAAACGGCCCTTTCAAGTGT G**TGGA**CTCGCGAGCGACGCGGTGGCCCTTTGTATGTAAATACTGGGTTTAAAAAAAAAAAGGCT CGCCCCGTCTTTGCAATTAATTGACACGTTAC**ACCT**CTCATCTTGCTCTAGAGGGCCGTTGGCTGG GAGCGCGGAGCTCCCCAAAACCCACAATTTCACATCTGCAAATACTGTCTTCA**TCCA**CTTGACTC CCAAGACCCGCCCACACGTGGCCA**ACCT**TTGCGTTTTTAATGTCTCTTCCCCCTTTTT**TCCA**CCCT TCTCCCGCTCCCTCTCTCGCTCCCCCTCCCTCCCTCTTTCTTTCCCTCCCTCCCTCTCTTTCTCCCCC TCTCCCCGCCTCCCC**AGGT**TCGTGAG**TGGA**GCCCAGCCTTATA**TGGA**CTGATCGCTCGGGCAATG GCCCATTTTTTCCTCGCCACCAGCCGCCACCGCGCGCCGAGCGGCCGCCGGAGCCCGAGCTGACG CCGCCTTGGCACCCCTCC**TGGA**GTTAGAAACTAAGGCCGGGGCCCGCGGCGCTCGGCGCGCAGG CCGCCCGGCTT	ENSG00000125 820
NKX6.1	TGGGCCAGAGGGCAAGGCGGG**TGGAGGT**GAGG**ACCT**GGGAGCCGCGGGGATCCGTGGCACTGC CCTTTCTGGCGCAGCAGCCCGGGGCAGCGTGGGCGGAGGAAGCCCGCACAGAGGCTAGATCTCC CGCGGGC**TGGA**TGCGCTTTCTCCCCGGGCACAGTGAGCGTCGAATGCGAATCAGCCGCGCGACC GAAAGAGCAGAGCATCCCAGTAAGATCAGAGGAGCGCCACGGGCTGCACAAGGCGTCCTTTGA**A** **CCT**CCCCAAAGAAAGCAAGCCACCCCCACCC**TCCA**ACTTCAAAG**TGGA**GATTCGGCAACTAACT TTGCTACAAACTCTCCGGAGCCAGCCTGGGTTTTGTTTTGCTTATTTCCCGGGGGCAGAAGATGAG AAGTAGCGCACTTTGAACAGCTAGGAAAAGTGAGGAAGAGAGAATAGCCAGGGATCGAATCTAG GACTCGCGGAACGAAAGGACTGCCTAGCCCGCCGGGACGCCTGCTTTTCTCGGCGAGCTGCCGCC TCCCGCG**TGGA**GGGTT**TGGA**CATCTCTGCTGCGCAGCTAGGCGAGCAACTCCCGGCAGCGGCAT TTTTGGTTCAGTTGGCAGCTCG	NM006168.2
HOXA3	**TCCA**GGGCAGAGGAATACGAAGTTTCTCAAACGAATTCCTAAGAAATAGAGCCCAGCAAGAGAG CCCCTTTGTGAGAGCCGTTTGTCCTCATTAGCATAATTTTCC**TGGA**CTTTCGTGACGCCTGGGAGC AGGAGAGGGAGGGCGTTGGGGGGTGGGGGGAGGCTGCCCTCCTCCCGCCACCGCCGAGCTCCCC TTCTCCCTCCGGTCCCTCCCCTCGCCTCAGCCCCATCCCCCACACCAGCCCGCGCTTCCCCAGCCG CCTTGGTCTCCCCTCCCGCCCGCCCCAACACCCCCCACCGCACCCCCGGACCAGACGCTGCTCCGC GTGTAATATTCATAAGAAACATACCCAAGTCGGTGCCACTAGCCCAGGCAGAGCCCGGCGCCGCA CTAGCGCTTATCTCCGGGCCGCGGCTGCTGCCCTCGGGAAGGGCAGGGGGCGGGGGTGTGGGGG AGGGGTGGGTGGGTGGGGTGGGTGGGAGGGGGG**TCCA**AGCCGCCCCAGTCGGCCCTGGGCGAGC CGTGC**TGGA**GCAGCGAGGCGGCCACGCTCTGCGCGGCGGTGACCGGGCCTCGGGGTC**TCCA**GCC CGCCTGCCATCTCGGCT	NM030661.4
PAX4	GCCAGCTCTCAAAGAAAGCAGCTTGCGTTGACAGCCTGGGGGCAGCAAGGATGCAGTCTCCCAG GAGAGGATGCACTCGGTGGTGGGAAGCCAGGC**TGGA**GGGGCCTGAGTGACCCTC**TCCA**CAGGCG GGCAGGGCAGTGGGAG**AGGT**GGTGTG**TGGA**T**ACCT**CTGTCTCACGCCCAGGGATCAGCAGCATG AACCAGCTTGGGGGGCTCTTTGTGAATGGCCGGCCCCTGCCTC**TGGA**TACCCGGCAGCAGATTGT GCGGCTAGCAGTCAG**TGGA**ATGCGGCCCTGTGACATCTCACGGATCCTTA**AGGT**AATGGGCCAG C**ACCT**TTACCCAGTGATGGGGACAGGAAGCAGGGAGAAAGGGCTCCTCTGAAGGCAAGAGCCTG GGGCTGTTGCAGGCTCTGAGGGCTTCTGGGACTTGGGTCACTTCCTGGGAGATCCTCTCGG**AGGT** TGAAAAGGGGAGCCTCAGGCCCTCAA**AGGT**GAGGC**TGGA**CTCCCGACTTCATGGCCTGG**TCCA**G TAAGTCTTGGCTTTGTCTTATAGCCTCCTCCTGTCCCAGGGACACTCTCCTTCCTTCTGCCCATCAT GCCTC**ACCT**GTCCCTGCTT	ENSG00000106 331



A B

Figure 9. Interaction of KEDW peptide with DNA sequence CCACA. A: The peptide located in the major DNA groove. B: plot of the active site of DNA-peptide complex. Grey color represents carbon atoms, light grey: polar hydrogen atoms. Atoms of non-polar hydrogens are not shown. The dotted lines represent hydrogen bonds between atoms of the peptide and the nitrogenous bases. π-cation interactions are dotted lines with the image of benzene ring and hydrogen atom from the corresponding sides. Letters representsnucleotides: DG - guanine, DC - cytosine, DA - adenine, DT - thymine. The serial number corresponds to nucleotide number in DNA model.

The PDX1 gene promoter has 6 binding sites; NGN3 contains 4 sites; MNX1 promoters have 10 sites; HOXA3 – 5 sites, PAX4 – 14 sites, PAX6 – 6 sites, FOXA2 – 18, NKX2-2 promoters – 10, and NKX6.1 contains 9 sites. Though promoters of PAX4 and FOXA2 genes appear to have a maximal number of ACCT sites, it does not correlate with the increase in expression observed at KEDW addition. The peptide activity seems to be unproportional to the number of its target sites in the promoter.

An increase in cell culture passages number results in decreased gene expression levels of differentiation factors; this is necessary in order to induce maturation of islet pancreatic cells [15]. It is understood that absence/depression of synthesis of these proteins results in a disturbed development of pancreatic cells from pluripotent cells [6].

The KEDW peptide increases expression of differentiation factors genes PDX1, NGN3, PAX6, FOXA2, NKX2-2, NKX6.1, and PAX4 at 14th passage of cell cultures. The most pronounced effect is observed for the PDX1, NGN3, and PAX6 genes. Hence, the peptide stimulates differentiation of various endocrine pancreatic cells. This data correlates with the results of the KEDW effect on corresponding peptides synthesis. Indeed, the increased gene expression of 2-2.7 could be esteemed of small account. However, other studies concerning peptide regulation of gene expression obtained similar data: maximum regulatory effects were demonstrated on Semaks peptide (MEHFPGP), which increased NGF and BDNF gene expression by 5-8 times, respectively [7]; effects were also demonstrated on Epithalon peptide (AEDG), which changed expression of 98 genes by 2.7-6.6 times [1]. However, we assume that peptide regulation of gene expression existing under physiological conditions should not lead to overexpression of genes. Even an increase of genes expression 2-3 times by peptide KEDW promotes the synthesis of pancreatic differentiation factor regulators by

2-3 times, which leads to the restoration of pancreatic function, as shown in animal studies [13][14][15]. We could assume that if the expression of genes and the synthesis of the corresponding proteins increased tenfold it would lead to short-term improvement of pancreatic function and rapid resource depletion with long-term negative consequences.

KEDW is also able to restore synthesis of insulin, glucagon, somatostatin, and pancreatic polypeptide. Based on data obtained by UV spectroscopy and circular dichroism studies, we may conclude that the peptide binds to DNA. This process takes several hours and occurs nearly without any participation of electrostatic forces. The complex formation takes place in the major DNA groove and involves the formation of bonds between DNA nitrogenous bases and the peptide. Peptide-DNA complex formation does not depend on ionic strength of the solutions; spectrum type corresponds to those obtained at binding of various ligands (divalent ions of alkaline-earth metals, protons, cis-DDP) to N7 of guanine in the DNA major groove [19].

These bonds lead to a decrease in DNA bending stiffness and destabilization of secondary structure. Interaction of the peptide with DNA involves van der Waals, π-stacking, and hydrogen bonding between functional groups of both molecules. The results of molecular modelling as well as identified features from the peptide-DNA interaction confirms earlier assumptions about the possibility and principle of the peptide complex formation with nucleic acids [14][15][20].

3. Conclusion

In summary, we found that KEDW peptide increases the expression of genes important for maintaining functional activity of endocrine pancreatic cells. The mechanism of peptide action is likely due to interaction with DNA and subsequent activation of corresponding gene expression. The peptide-DNA binding process is relatively slow and associated maximal effects are achieved late. Due to the induction influence of the peptide on differentiation and functional activities on various types of endocrine pancreatic cells, it can be used as a perspective marker for correction of functional activity of pancreatic cells. This is significant for the development of new, efficient therapy methods for treating diabetes mellitus and other pancreatic diseases.

Ultimately, knowledge acquired from these projects can be used to develop new preclinical/clinical trials that can improve the life expectancy of patients with diabetes mellitus and pancreatic diseases.

4. Materials and methods

4.1 Human pancreatic cell cultures

Human pancreas cells cultures of the 14th passage from epithelial cell of pancreatic carcinoma called MIA PaCa-2 received from the Institute of Cytology of the Russian Academy of Sciences were used in this study. To investigate cell functional morphology in the 14th passage, we measured optimal cell mass in this culture. It has a stabilize cell population with the most distinct biological activity of the peptide. Cell cultures were divided into 2 equal groups: to the first group, we added 165 mL of normal saline solution and to the second group, the same 165 mL solution and additional KEDW peptide (20 ng/mL). This concentration was used as it seemed the most effective in previous studies [15][14]. Cells were grown in vials with 25 cm surface (JetBiofil, Japan) in 5 mL of growth DMEM medium at 37ºC with addition of L-glutamine (Biolot), 15% of fetal bovine serum SC-BIOL (Biolot), and 1% Penicillin-Streptomycin solution.

4.2 Quantitative RT-PCR

To measure the influence of KEDW peptide on expression of several genes (PDX1, NGN3, MNX1, PAX6, FOXA2, NKX2-2, NKX6.1, HOXA3, PAX4) in pancreatic cell cultures, we used quantitative

polymerase chain reaction (PCR). Cells were treated with RNAprotect Cell Reagent (Qiagen, Germany) and total RNA was isolated using the RNeasy Mini Kit, as suggested by the manufacturer (Qiagen, Germany). The synthesis of the first cDNA strand from RNA samples was carried out with oligo (dT)18 primer (Synthol, Russia) and Omniscript RT Kit reverse transcription kit (Qiagen, Germany). Specific cDNA for differentiation factors was amplified by a real-time PCR detection system CFX96 (BioRad Laboratories, Inc., USA) using green fluorescence QuantiFast SYBR Green PCR kit (Qiagen, Germany). The relative intensity was determined by comparing specific cDNA from our experiment with the GAPDH (Glyceraldehyde 3-phosphate dehydrogenase), which is the standard gene of a normal cell population. Statistical analyses were carried out in automatic mode using CFX manager software. We used three independently grown cell cultures in each group (biological parallels) and three or more identical parallel PCR assays for each cDNA sample (technical parallels). Oligonucleotide primer design was carried out with the online database NCBI Primer-Blast service. Corresponding oligonucleotides were synthesized and supplied by Synthol (Russia).

4.3 Immunofluorescence staining

Pancreatic cells were incubated with 20 ng/mL MitoTracker (M-7510; Invitrogen) for 15 min, washed with PBS, fixed with 3.7% formaldehyde in PBS, and premobilized with 0.1% Triton X-100 in PBS for 5 min. After washing, the samples were blocked with 2% (wt/vol) BSA in PBS for 30 min and incubated with mouse monoclonal anti tau-protein (1:200, Abcam, UK) or mouse monoclonal anti PDX1, NGN3, PAX6, FOXA2, NKX2.2, NKX6.1, PAX4, MNX1, HOXA3 (all 1:100, Novocastra, UK) and diluted in PBS with 2% (wt/vol) BSA for 1 h. Samples were rinsed three times with PBS and incubated with Alexa Fluor 488-conjugated goat anti-rabbit secondary antibodies diluted in PBS with 1% (wt/vol) BSA for 1.5 h. DNA was stained with Hoechst 33258 (Sigma, USA). Data acquisition was performed on an Olympus FluoView 1000 microscope (Japan). In each case, 10 fields of vision were analyzed at a magnification of 400x. Expression surface was calculated in Vidiotest-Morphology 5.2 programm. The square of expression was estimated in % by Vidiotest-Morphology 5.2. The square of expression was estimated as the ratio of immune positive cell area to the total area of cells viewed, and was expressed in percentage. The index characterized the intensity of studied tau-protein level in cells. Vidiotest-Morphology 5.2 is a firmware complex (license number FS-99-03-003672, registration certificate Russian Federation N FSR 2012/13356) used for extended analysis goals in cytology, histology, hematology, immunocytochemistry and other scientific areas.

4.4 UV-spectroscopy and circular dichroism

Studies of peptide interactions with natural DNA (Sigma-Aldrich) were carried out using water-salt solutions of high-molecular weight double-stranded DNA ($MM=9\times10^6$). We used NaCl as a supporting low-molecular electrolyte in DNA solutions. Measurements have been done at two salt concentrations: 0.005 M (low ionic strength with strongly pronounced polyelectrolyte effects) and 1 M NaCl (suppression of electrostatic interactions in polyelectrolyte solutions).

It is known that the concentration of salt solution (NaCl) in the cytoplasm is 0.009 M; however, it is about 0.005 M in the nucleus. [21][22]. We assumed that the tetrapeptide KEDW penetrates into the nucleus and interacts there with DNA. Thus, we used saline concentrations of > 0.0005 M in our experiments. In order to mimic conditions of the nucleus, some concentration variations allowed us to consider electrostatic interactions between the peptide and DNA. This saline condition has been previously tested and successfully used for the study of biologically active molecules interaction in solution [23].

DNA-peptide complexes were prepared by mixing similar volumes of solutions of the

DNA and peptide with known ionic strengths. We determined the DNA concentrations by optical absorption differences at 270 and 290 nm after acid hydrolysis in 6% $HClO_4$ for 15 min at 100°C. Nativity of DNA molecules was controlled by values of hyper-chromatic effects at 260 nm at thermal denaturation. After mixing and storing DNA and KEDW peptide solutions together at 4°C for 24 h, we studied spectral characteristics using spectrophotometer «SF-56» (Russia) and dichrograph «Autodichrograph Mark IV» (France).

4.5 Statistical methods

Statistical analyses included finding the arithmetic mean as well as the standard deviation for each sample and was performed using the Statistica 7.0 software. To test a null hypothesis of normal distribution, we used Shapiro-Wilk test. To assess statistical homogeneity among multiple samples, we used the non-parametric ANalysis Of VAriance (ANOVA) (Kruskal–Wallis test). Differences between groups were considered statistically significant at $p < 0.05$. Graphics and tables were performed by «EXEL».

4.6 Molecular modelling

All molecular modelling and calculations were conducted using Molecular Operating Environment 2013.10 software (Chemical Computing Group, Canada). Solvent influence was considered with introduction of internal dielectric constant equal to 1 and the external equal to 80 (generalized Born model). Peptide KEDW was built in a left-handed stereo configuration. We modelled the 3-D DNA structures in the common B-form conformation, choosing sequences of all combinations of four nucleic acids excepting repeats. After construction, all studied molecules were protonated under conditions of pH = 7 and T = 300 Kelvin with optimization of their geometries in Amber12EHT force field. Modelling of the complexes of double-stranded DNA with KEDW peptide was carried out using molecular docking method. This includes the measurement of bond energy and energetically favourable location of peptide in DNA. For docking, DNA was chosen as rigid body, peptide was chosen as flexible body. The docking solutions were represented by values of an enthalpic contribution to the free binding energy (Affinity dG scoring function). Lower scores indicate more favourable poses, the unit for scoring function is kcal/mol. Promoter region of differentiation factors were found in the Eukaryotic Promoter Database [18].

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List of abbreviations

cDNA – complementary DNA
Cxcl12 – chemokine 12
FOXA2 – gene of forkhead box protein A2 (Foxa2)
GAPDH – glyceraldehyde 3-phosphate dehydrogenase
HOXA3 – gene of homeobox A3 (Hoxa3)
KEDW – Lys-Glu-Asp-Trp-NH2 peptide
MD – diabetes mellitus
MNX1 – motor neuron and pancreas homeobox 1 (Mnx1)
NGN3 – gene of neurogenin 3
NKX2.2 – gene of homeobox protein Nkx2.2
NKX6.1 – gene of Nkx6.1 transcription factor homolog A
PAX6 – gene of paired box protein 6 or oculorhombin (Pax6)
PAX4 – gene of paired box gene 4 (Pax4)
PDX1 – gene of pancreatic and duodenal homeobox-1
RT-PCR – real-time polymerase chain reaction

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