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RNAi mediated silencing of cyclin-dependent kinases of G1 phase slows down the cell-cycle progression and reduces apoptosis

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One of the hypotheses on the origin of Alzheimer's disease (AD) stems from a close relation between a re-activation of a cell-cycle in post-mitotic neurons and a neural cells death observed in pathologically affected parts of AD brains. In the normal, healthy brain almost all neural cells are terminally differentiated and "locked" in the G0 phase of the cell-cycle. For these cells, the consequence of the re-entry to the cell-cycle is targeting them towards cellular divisions and turning on the apoptotic pathway. We used an RNA interference (RNAi) methodology in neural cells to switch-off genes for two cyclin-dependent kinases 4 and 6 (cdk4, cdk6), which control the activation of the initial steps of the cell-cycle. As a result, some evidences are delivered that silencing these genes, which are expressed during cell proliferation but inhibited at mature neurons, prevents the stimulation of apoptotic pathways in the neural cells cultured in a oxidative stress conditions and may have a neuroprotective effect. We demonstrate that down-regulation of genes important in the G1 phase of the cell-cycle may play the protective function on the neuronal cells, and can be considered as the promising approach for the potential gene therapy of neurodegenerative diseases.

Key words: RNAi, siRNA, Alzheimer's disease, cyclin-dependent kinase, cell-cycle re-entry, cell-cycle re-activation

INTRODUCTION

Alzheimer's disease (AD) is a complex and irreversible neurodegenerative disease of brain, clinically characterized by progressive memory loss and severe impairment of cognitive functions. The major hallmark of AD is progressive loss of neurons (with their synaptic connections) and pathological changes in the neural tissue, like senile plaques and neurofibrillary tangles (Ulrich 1990, Braak et al. 2012). The biological mechanisms leading to AD are still poorly understood. More than sixty years ago, Sjögren and coworkers (1952) demonstrated that some patients with AD had an autosomal dominant Mendelian pattern of disease inheritance. Until now, a genetic background of the disease has been identified in about 5% of population of AD patients. Mutations in three genes coding Amyloid Precursor Protein (APP), Presenilin 1 (PSEN1), and Presenilin 2 (PSEN2) were identified in autosomal dominantly inherited form of AD (FAD, Familiar AD) (www.molgen.ua.ac.be/ADMutations). The emergence of mutated proteins with changed structure and activity leads to the intensity of release of longer forms (≥42 amino acids, aa) of beta-amyloid (Aβ) peptide, as a result of sequential cleavage of APP by β- (BACE1, beta side amyloid precursor protein cleavage enzyme) and γ-secretase (with PSEN1 or PSEN2 as the catalytic core of γ-secretase complex). $A\beta_{\geq 42}$ is vulnerable to aggregation and accumulation in the brain as β-amyloid plagues (Parker and Reitz 2000). As to other reasons (>95%), the most frequently occurring sporadic form of disease (SAD, Sporadic AD), has only a partially solved mechanism of the pathogenesis (Braak et al. 2012, Piaceri et al. 2013).

Until now, several hypotheses have been formulated to explain the pathogenesis of disease (Zekanowski and

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Wojda 2009, Nawrot et al. 2010). One of them, the cell-cycle re-entry hypothesis, was proposed by Vincent and other researchers (Vincent et al. 1996, 1997), who revealed an abnormal activation of the cell-cycle components in neurons, in pathologically affected parts of AD brains. The major regulators of the cell-cycle, like cyclin-dependent kinases cdk4, cdk6, and cdc2, cyclins D, E, A and B1, and cdk inhibitors $p27^{Kip1}$, $p16^{INK4a}$, $p18^{INK4a}$, were activated in neurons at very early stages of the disease, prior to any neurofibrillary pathology or accumulation of Aβ (Arendt et al. 1996, Busser et al. 1998, Chow et al. 1998). The consequence of the aberrant activation of these proteins is re-entry of neurons to the cell-cycle (Yang et al. 2003). In healthy brain, in the post-mitotic and terminally differentiated neurons, the cell-cycle proteins activity is arrested by the enrichment of cdk inhibitors and neurons are "locked" in the G0 phase. After the cell cycle re-activation, neurons leave the G0 phase and progress until the S phase and beyond that point. Mosch and colleagues (2007) showed that significant number of hippocampal pyramidal and basal forebrain neurons, in the parts of brain affected by the pathological process, undergo full or partial DNA replication, implying that the neurons had completed the S phase. Progression of neurons into the M phase was never confirmed, so most likely they die at the G2/M transition (Mosch et al. 2007, Arendt 2012). Arendt proposed an explanation for the cell-cycle re-activation suggesting abnormal activation of the Ras-MAPK pathway at very early stages of AD (Gartner et al. 1999, Yang et al. 2006). One of activators of Ras-MAPK pathway could be e.g. the oxidative stress at aging cellular environment (Uttara et al. 2009).

The cell-cycle progression is strongly regulated by coordinated activation of cyclin-dependent kinase (cdk) and cyclin heterodimer complexes (Lim and Kaldis 2013), where cyclins bind and activate the specific cdks. In the G1 phase, a hypo-phosphorylated Retinoblastoma protein (Rb) binds an E2F-DP1 transcription factors forming with a histone deacetylase (HDAC) an inhibitory complex to repress key downstream transcription events. Two cell-cycle related kinase complexes, the cdk4/6-cyclin D1 and cdk2-cyclin E, work to alleviate inhibition of the Rb/E2F transcription complex. After sequential phosphorylation of the Rb by the cdk4/6-cyclin D and cdk2-cyclin E, phospho-Rb dissociates from the repressor complex, permitting transcription of the genes required for DNA replication (Harbour and Dean 2000, Trimarchi and Lees 2002). The cell-cycle protein components can be an attractive goal for therapeutic interventions in the disease-associated cases, especially in cancers and neurodegenerative diseases (Johnson et al. 2010, Comstock et al. 2013, Aarts et al. 2013, Hilgeroth et al. 2014, Kang et al. 2014). Seminal studies focused on the inhibition of cdks activity were performed by Park group (Park et al. 1996, 1998, 2000).

Based on these data, we proposed the strategy of neuroprotection by the cell-cycle inhibition through the repression of the expression of two cyclin-dependent kinases, cdk4 and cdk6. For our studies, we employed RNA interference (RNAi) methodology with several short interfering RNA duplexes, specific for *cdk4*/*cdk6* transcripts and in this paper we demonstrate the RNAi effect on the *cdk4/cdk6* expression and on the cell-cycle progression of neural cells cultured under normal and oxidative stress conditions. After treatment of the cells with appropriate siRNA we noticed significant inhibition of target gene expression and found increased number of cells arrested in the G1 phase. Simultaneously, we observed less cells in the S and G2/M phases, in comparison to the control cells with an untouched *cdk4* and *cdk6* expression. Silencing of cyclin-dependent kinases altered the expression level of other genes important for transfer of cells into the next cell-cycle phases: G1/S and S/G2, like *cyclin E*, *cyclin A*, *PCNA* and surprisingly, some genes involved in Aβ formation: *PSEN1* and *BACE1*, while the level of precursor of Aβ, the APP coding gene, remained unchanged. Additionally, to assess the degree of the induction of apoptosis during the oxidative stress, we determined the level of activity of caspase 3 and 7 in cells. Our results indicate that the silencing of genes of cyclin-dependent kinases, especially cdk4 kinase, protects the cells against negative effects of the oxidative stress. The cells with the silenced *cdk4* gene and exposed to oxidative stress were less vulnerable to activation of apoptotic pathways in comparison to the untreated cells under the same conditions.

METHODS

Synthesis and purification of RNA oligonucleotides

The oligoribonucleotides were synthesized in house, according to the routine phosphoramidite approach (Caruthers 1985, Sierant et al. 2010).

Table I

List of qRT-PCR primers

Gene	Primer name	Sequence	PCR product size
CDK4 human	H CDK4 Fow	5'-GGGCAAAATCTTTGACCTGA-3'	191 bp
	H CDK4 Rev	5'-AGGCAGAGATTCGCTTGTGT-3'	
cdk4 mouse	M cdk4 Fow	5'-CCCGTGGCTGAAATTGGTGT-3'	142 bp
	M cdk4 Rev	5'-GAACTGTGCTGACGGGAAGG-3'	
CDK6 human	H CDK6 Fow	5'-TGCACAGTGTCACGAACAGA-3'	150 bp
	H CDK6 Rev	5'-ACCTCGGAGAAGCTGAAACA-3'	
cdk6 mouse	M cdk6 Fow	5'-GGACGGACAGAGAAACCAAG -3'	140 bp
	M cdk6 Rev	5'-CAGACCTCGGAGAAGCTGAA-3'	
GAPDH Human	H GAPDH Fow	5' GAGTCAACGGATTTGGTCGT 3'	238 bp
	H GAPDH Rev	5' TTGATTTTGGAGGGATCTCG 3'	
gapdh mouse	M gapdh Fow	5'-GTGTGAACGGATTTGGCCGT-3'	238 bp
	M gapdh Rev	5'-TTGATGTTAGTGGGGTCTCG-3'	

Cell culture and transfection

SH-SY5Y (human, bone marrow neuroblastoma) cells were cultured in a F12 Nutrient Mixture (HAM) medium with MEM (Gibco, Life Technologies) (1:1), supplemented with 15% FBS and antibiotics (100 mg/ml streptomycin and 100 U/ml penicillin). Neuro2A (mouse, brain neuroblastoma) cells were cultured in a DMEM medium (Gibco, Life Technologies) with 10% FBS and antibiotics. HeLa (human, cervical carcinoma) cells were cultured in an RPMI 1640 medium (Gibco, Life Technologies) with 10% FBS (Gibco, Life Technologies) and antibiotics. Cells grew at optimal culture conditions, at 37°C and 5% CO_2. Twenty-four hours before transfection, cells were plated on a 6-well plate at density of $3–5\times10^5$ cells per well. Directly before transfection, the medium was replaced with a new one, free of antibiotics. Transfection of HeLa or Neuro2A cells with siRNA was performed using a Lipofectamine 2000 transfection reagent (Invitrogen), at a ratio 2:1. Transfection of SH-SY5Y with siRNA was achieved by nucleofection (Nucleofector II, Lonza) according to the manufacturer's protocol. After 6 to 12 hours incubation, the medium with the transfection mixture was replaced with the fresh medium with antibiotics. After 24–96 hours (depending on the purpose of experiment) incubation at 37°C in 5% CO_2 atmosphere, cells were washed three times with a PBS buffer (free of Ca^{2+} and Mg^{2+}), lysed with TriPure Isolation Reagent (Roche), collected and kept frozen at −70°C until analysis.

Quantitative RT-PCR (qRT-PCR)

Total RNA was extracted from TriPure cellular lysates according to the manufacturer's protocol and analyzed by qRT-PCR as described below. Reverse transcription and PCR amplification reactions were performed in one step using a Light Cycler device (Roche), LightCycler RNA Amplification Kit SYBR Green (Roche). Sequences of all primers used for PCR reactions are showed at Table I. The qRT-PCR reactions were optimized for each studied gene and performed according to the general protocol: a reverse transcription reaction (RT) 30 min at 55°C, and a denaturation step 30 s at 95°C. The PCR quantification reaction comprised of three steps consecutively repeated in 45 cycles: (1) denaturation over 5 s at 95°C, (2) annealing over 10 s at a primer dependent temperature, and (3) product extension over 30 s at 72°C. Subsequent melting experiments were performed by quick denaturation at 95°C, annealing over 10 s at 65°C and slow heating (0.1°C/s) up to 95°C. Obtained amplification curves, in combination with standard curves for con-

trol cytokine RNA with known number of RNA copies (LightCycler Control Kit RNA, Roche) allowed to quantify the number of RNA copies in samples with studied *cdk4* and *cdk6* transcripts, according to CP "crossing point" values characteristic for amplification curves assigned for studied RNAs. Specificity of the amplified PCR products was assessed by performing Melting Curve analysis, which enabled evaluation of the quality of PCR product and discrimination between specific PCR product and potential unspecific primer-dimer product. Full analysis of qRT-PCR results was performed using LightCycler Software 4.05.

Western blot

Studied human or mouse cells were transfected with 100 nM siRNAs targeted against *cdk4 or cdk6* genes. Forty eight hours after transfection, the cells were lysed with TriPure Isolation Reagent (Roche) and the total cellular protein samples were isolated from lysates according to the manufacturer's protocol. Proteins were separated by 10% SDS-PAGE and electro-transferred onto an Immobilon-P membrane (Merck Millipore). The membrane was blocked with 5% non-fat milk and incubated with the primary antibodies: mouse monoclonal anti-cdk4 (1:1000), (Sigma Aldrich), mouse monoclonal anti-cdk6 antibodies (1:500), (Sigma Aldrich) or rabbit polyclonal anti-β-Actin antibodies (1:10 000), (Abcam), overnight at 4°C. Then, the membrane was washed and incubated with secondary antibodies: Alkaline phosphatase (AP) linked anti-mouse or anti-rabbit antibodies (1:5000), (Sigma Aldrich) for 2 hours at room temperature. The protein bands were visualized with the Lumi-Phos WB Chemiluminescent Substrate for AP (Thermo Scientific), according to the manufacturer's instructions. Protein expression analysis was performed at the G-BOX visualization system (Syngene) using GeneTools analysis software.

FACS flow cytometry

Before the flow cytometry analysis, cells were seeded in the 6-well plate (5×10^5 cells per well) and transfected with an appropriate amount of siRNAs (usually at 100 nM) using Lipofectamine 2000, or nucleofection for SH-SY5Y, according to the manufacturer's protocol. For normal culture conditions, a cellular medium with the siRNA transfection mixture was replaced after 6 hours post-transfection and the cells grew for next 24–96 hours at optimal conditions. To develop the oxidative stress conditions, 48 hours after transfection cells were treated with 10–50 μM hydrogen peroxide (H_2O_2) and cultured for 24 hours at 37°C and 5% CO_2. Now, the cells were washed with an ice cold PBS (free of Ca^{2+} and Mg^{2+}), trypsinized, collected and fixed with 70% ethanol at −20°C. Directly before the flow cytometry assay the cells were washed again with the ice cold PBS, treated with RNase A (~50 μg/ml) and stained with Propidium Iodide (PI) (10 μg/ml). Flow cytometry was performed on a BD FACS Calibur Flow Cytometry System (Becton Dickinson) using an Ar-ion laser (488 nm). Fluorescence dot plots and histograms were generated using a Cell Quest software, and the percent of cells at given cell-cycle phases was counted using a ModFit LT software.

MTT assay

HeLa or SH-SY5Y cells were plated in 96-well plate at the density of 7×10^3 cells per well and 24 hours later siRNAs at concentrations: 50, 100, 150, 200 nM were transfected with Lipofectamine 2000 (Invitrogen, Life Technologies) according to manufacturer's protocol. Untreated cells (CC) were used as a control (100% of viability). Cells treated by Lipofectamine 2000 only (CCL) served as control of cytotoxicity of transfection reagent. Assay was performed after 24, 48 and 72 hours of cell culture at 37°C and 5% CO_2, when MTT solution (25 μl of 5 mg/ml solution) in PBS was added to each well and incubated for additional 2 h at 37°C. Finally, 95 μl of lysis buffer (20% SDS, 50% aqueous dimethylformamide, pH 4.5) was added to each well and lysates were incubated overnight at 37°C. Absorbance of a given sample was measured at 570 nm and at the reference wavelength 630 nm (plate reader Synergy HT, BIO-TEK). The percentage of living cells (PLC) was calculated from the equation: $PLC=Abs_{SPL}\times100\%/Abs_{CC}$, where Abs_{SPL} ($Abs_{570}-Abs_{630}$) is the absorbance of a given sample of cells treated with siRNA, Abs_{CC} ($Abs_{570}-Abs_{630}$) is the absorbance of reference cells.

Caspase-3 and 7 activity

Activity of caspases 3 and 7 was determined using Apo-ONE Homogenous Caspase-3/7 Assay (Promega) according to the manufacturer's protocol. Neuro2A cells (2×10^4 cells/well) were cultured at a 96-well plate format

Table II

Sequences of siRNAs specific for human and mouse *cdk2, cdk4, cdk6* genes, used in the studies

Gene	siRNA name	Sequence
CDK4 human NM_000075	Si4-1	5'- GGUAAUCCGGAGUGAGCAATT-3' 3'-TTCCAUUAGGCCUCACUCGUU -5'
	Si4-2	5'- CCUAUGGGACAGUGUACAATT-3' 3'-TTGGAUACCCUGUCACAUGUU -5'
	Si4-3	5'- GGCUGUAUCUUUGCAGAGATT-3' 3'-TTCCGACAUAGAAACGUCUCU -5'
cdk4 mouse NM_009870	Si4-4	5'- CCCUAGUGUUUGAGCAUAUTT-3' 3'-TTGGGAUCACAAACUCGUAUA -5'
	Si4-5	5'- GGAUCUAAUGCGUCAGUUUTT-3' 3'-TTCCUAGAUUACGCAGUCAAA -5'
CDK6 human NM_001259	Si6-9	5'- CUGGAAAGGUGCAAAGAAATT -3' 3'-TTGACCUUUCCACGUUUCUUU -5'
	Si6-10	5'- GGAUAAAGUUCCAGAGCCUTT -3' 3'- TTCCUAUUUCAAGGUCUCGGA -5'
cdk6 human NM_001259 mouse NM_009873	Si6-16	5'- GAUCAAGACUUGACCACUUTT -3' 3'-TTCUAGUUCUGAACUGGUGAA -5'
-	Si-Ctr	5'- AAUCAGAUUGAACCUUCAUTT -3' 3'-TTUUAGUCUAACUUGGAAGUA -5'

(a plate with black walls and a transparent bottom, Perkin-Elmer) and transfected with siRNA specific for the mouse *cdk4* gene only (100 nM), or with siRNAs specific for two mouse genes *cdk4* and *cdk6* (totally 100 nM siRNAs) and Lipofectamine 2000, as mentioned before. After 48 hours of incubation, the cellular medium was changed for a medium with 10 μM H_2O_2 to develop the oxidative stress conditions. The cells were cultured for the next 6 hours at 37°C, 5% CO_2, and after that time 100 μl of Apo-ONE Caspase 3/7 Reagent was added to each well (mixture of Caspase 3/7 buffer and Caspase 3/7 substrate). The cell lysates with the Apo-ONE Caspase Reagent were gently mixed and incubated at room temperature in darkness. Fluorescence of the samples was determined after 2 hours incubation using a Synergy HT reader (BIO-TEK). The excitation wavelength for optimal detection was 499 nm and the emission maximum was present at 521 nm. Quantification of the data was done using a KC4 software).

Statistical analysis

All data are presented as mean ± SE. A Shapiro-Wilk test was used to analyze the data distribution. Statistical analysis of differences between the groups of data was done using a Student *t*-test (for data with normal distribution), or a non-parametric Mann-Whitney *U*-test. Values of $P<0.05$ were regarded as statistically significant.

RESULTS

Design and chemical synthesis of siRNAs targeting *cdk4* and *cdk6* genes: Evaluation of the silencing activity

A set of nine siRNAs, including five duplexes designed for transcripts of human and mouse cdk4: (Si4-1–Si4-5), three duplexes for transcripts of human and mouse cdk6: (Si6-9–Si16), and one duplex with random, non-silencing sequence (control siRNA, Si-Ctr), were synthesized in house (Table II). The sequences of siRNAs were designed using available online software packages: siDesign Center, Dharmacon. Program uses a scoring system based on the state-of-the-art parameters to evaluate the inhibitory potency of proposed siRNAs. The sequence of each RNA duplex was also evaluated for possible

cross-silencing activities by performing BLAST analysis of human and mouse transcriptome (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Target sequences were selected in the coding regions of chosen genes. All siRNAs had a typical structure of 19-base pairs (bp) fully complementary duplex with 2-nt overhangs, two thymidine units (TT) at both 3'-ends. This approach has been recommended to assure proper interaction of an RNA duplex with the proteins of the RNAi machinery (Kim and Rossi 2007).

SiRNA duplexes were tested in human (SH-SY5Y) and mouse (Neuro2A) neural cells and their ability for silencing of target transcripts was determined using quantitative RT-PCR (qRT-PCR) and Western blot techniques (WB). The most active siRNA molecules against human genes inhibited the expression of *cdk4* by 85–90% (mRNA), 60–65% (protein) and *cdk6* by 65–80% (mRNA), 40–55% (protein). In mouse cells, we observed silencing of *cdk4* by 65% (mRNA), 40–55% (protein) and *cdk6* by 70% (mRNA), 60% (protein).

Evaluation of the cell-cycle phases for cells with lowered *cdk4* and *cdk6* expression

To examine the effect of RNAi-induced silencing of *cdk4* gene or simultaneously two genes: *cdk4* and *cdk6* on the cell-cycle phases, we delivered *cdk4* specific siRNA (Si4-1), or *cdk4/cdk6* specific siRNAs (data not shown) and non-silencing control siRNA (Si-Ctr) by nucleofection into SH-SY5Y cells. After transfection, the cells were cultured at optimal conditions for the next 48 hours. The cell-cycle analysis performed by the flow cytometry technique revealed that silencing of target genes caused that number of cells at the G1 phase increased by ~5–7%, while the number of cells at the S phase decreased by ~3–5%, in comparison to cells transfected with non-silencing siRNA (Si-Ctr), where expression of *cdk4/6* genes remained non-changed (Fig. 1). The observed suppression of the cell-cycle progress, induced by the siRNAs, can be interpreted as the "arrest" of a part of cells at the G1 phase. These results confirm that the G1 phase cyclin-dependent kinases are required to push the cells from G1 to the S phase and their silencing results in slowing down the cell-cycle progression. Additionally, we verified the dependence of target genes silencing levels and cell-cycle effects on concentration of the applied siRNAs (50–200 nM siRNAs, data not shown). SiRNAs applied at 100 nM concentration occurred sufficient to develop the biological effects, therefore this concentration was chosen for all next experiments. SiRNA duplexes applied in higher concentrations than 200 nM could elicit unpredictable side-effects, including toxicity to the cells (Jackson et al. 2003, Persengiev et al. 2004).

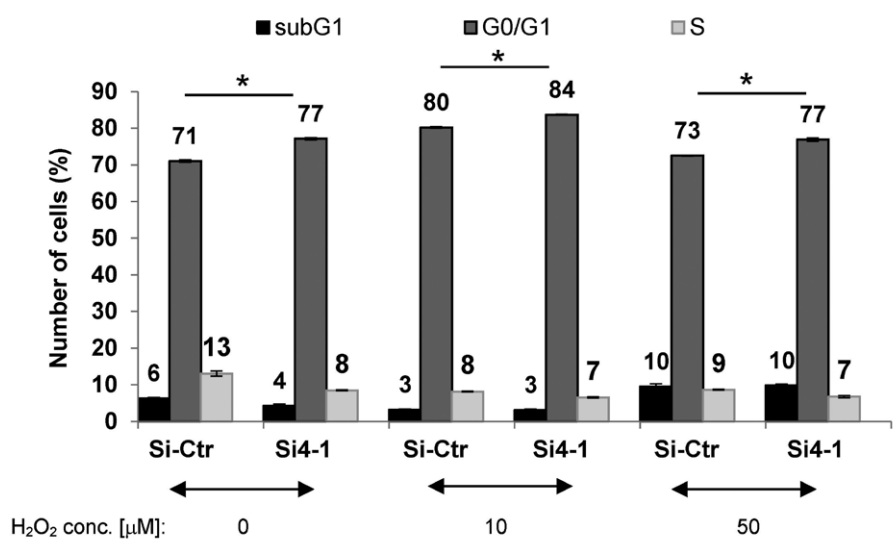

Fig. 1. Evaluation of the cell-cycle phases for SH-SY5Y cells with lowered *cdk4* expression, cultured under normal conditions [H_2O_2 (0 μM)] or under oxidative stress conditions [H_2O_2 (10 or 50 μM)]. SH-SY5Y cells were nucleofected with 100 nM siRNA: control Si-Ctr or *cdk4* gene specific Si4-1. The cell-cycle analysis was performed using flow cytometry on the BD FACS Calibur Flow Cytometry System (Becton Dickinson). **P*<0.05.

Evaluation of the cell-cycle phases for cells with lowered *cdk4* expression, cultured in the oxidative stress conditions

For experiments carried out under oxidative stress conditions, hydrogen peroxide (H_2O_2) was chosen as a source of the reactive oxygen species (ROS). The 10–50 μM H_2O_2 concentration was sufficient for SH-SY5Y cells to stimulate their proliferation and did not cause an immediate cell death. The apoptotic sub-G1 phase of the cell-cycle for cells treated 10–50 μM H_2O_2 was at the tolerable level, 3–10% (Fig. 1). Higher H_2O_2 concentration (100 μM) caused significantly increased amount of apoptotic cells (percent of cells at sub-G1 phase arose up to 45%, data not shown).

The aim of the present study was to examine whether cells with the lowered expression of *cdk4* are similarly susceptible to oxidative stress as those with non-silenced *cdk4* gene. The flow cytometry analysis has shown that in the hydrogen peroxide treated SH-SY5Y cells with silenced *cdk4* the number of cells at the G1 phase was higher by ~4–6%, while the number of cells at the S and G2 phases was lower by ~3%, in comparison to the H_2O_2 treated control cells with normal expression of *cdk4* gene (Fig. 1).

The influence of *cdk4* gene silencing on the expression of genes important for the cell-cycle progression

We intended to decrease the level of cdk4 and cdk6 kinases to block the formation of the cdk4/6-cyclin D complexes, and to inhibit the passage of cells into next stages of the cell-cycle. The expected effect of this inhibition would be the lowered level of phosphorylated Rb protein and reduced expression of genes dependent on the E2F transcription factor (by preventing the dissociation of the Rb/E2F complex). We verified the consequences of *cdk4* gene silencing in the proliferating neural neuroblastoma cells (SH-SY5Y), where the inhibition of the cell-cycle progress was previously proved. The RNAi was exerted with 100 nM control (Si-Ctr) and *cdk4* specific (Si4-1) duplexes, using nucleofection as siRNA delivery method. The level of mRNA of *cdk4*, *Cyclin E*, *Cyclin A* and *PCNA* (Proliferating Cell Nuclear Antigen, which is a processivity factor for DNA polymerase ε), was determined by qRT-PCR (Fig. 2A). We found that high *cdk4* gene silencing (>95%), which was constant until 96 hours in SH-SY5Y cells, strongly affected expression of other studied genes: >70% silencing of *Cyclin E* (50% after 96 h, $P<0.05$), 60% silencing of *Cyclin A* (constant over time, $P<0.05$), and >85% silencing of *PCNA* (80% after 96 h, $P<0.05$).

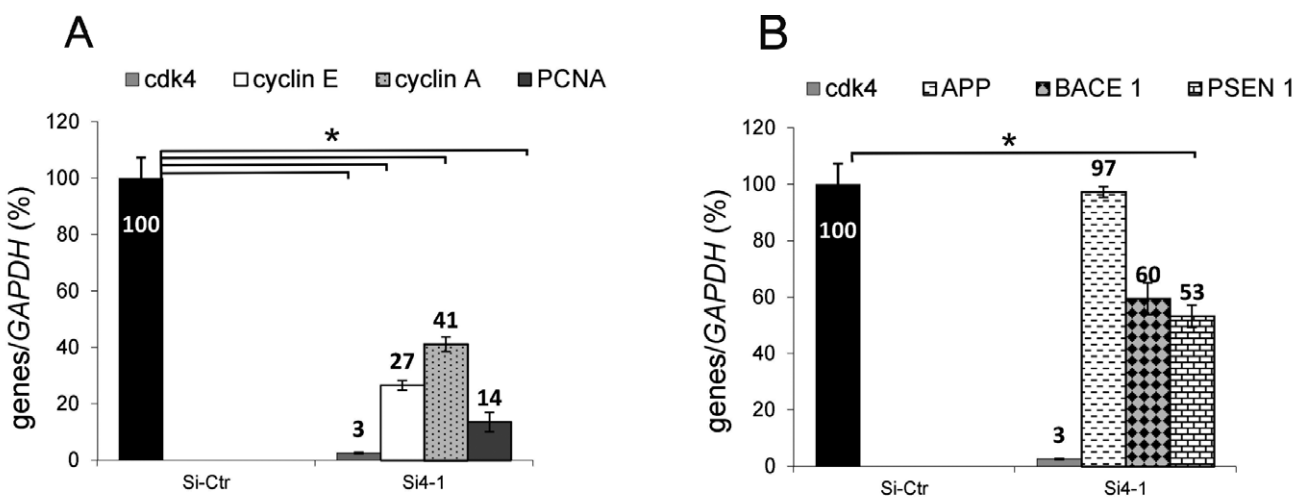

Fig. 2. Evaluation of the influence of *cdk4* gene silencing on the expression of genes important for the cell-cycle progression: *Cyclin E*, *Cyclin A* and *PCNA* (A), and genes involved in an Aβ cascade: *APP*, *BACE1* and *PSEN1* (B) in SH-SY5Y cells. The mRNA level of studied genes in the cells treated with non-silencing Si-Ctr duplex only was assessed as 100% of the expression. Inhibition of *cdk4* gene expression was caused by a gene specific siRNA Si4-1 (100 nM), delivered into cells by nucleofection method. The bars at the graphs represent the levels of expression of respective genes. All results were normalized to the values of *GAPDH* gene expression. *$P<0.05$.

Additionally, we found the influence of lowered *cdk4* expression on the level of genes involved in the Amyloid β (Aβ) cascade. The highest expression of *APP*, *BACE1* and *PSEN1* genes takes place in neuronal cell lines, like SH-SY5Y cells, Fig. 2B. Interestingly, the levels of *BACE1* and *PSEN1* mRNAs were lowered by 50–60% ($P<0.05$), however, there were no changes observed in the *APP* gene expression. This result was difficult to explain and turned our attention to possible unspecific silencing effects elicited by siRNA. Thus, we investigated these unspecific effects using a dual fluorescence assay (DFA) performed in HeLa cells, because this cell line neither expressed endogenous *BACE1* nor *PSEN1* genes (Sierant et al. 2009, 2010, 2011). For transfection of HeLa cells we used plasmids coding fusion genes: pEGFP-BACE (a fusion gene *EGFP-BACE1*) (Sipa et al. 2007, Sierant et al. 2009), or pEGFP-PSEN (a fusion gene *EGFP-PSEN1*) (Sierant et al. 2011), pDsRed-N1 as the reference, and appropriate siRNAs: the control (Si-Ctr) or *cdk4* specific siRNA (Si4-1). We did not observe any significant silencing effects of *cdk4* gene specific siRNA on the *EGFP-BACE1* or *EGFP-PSEN1* genes expression. These results suggest that silencing of BACE1 and PSEN1 proteins occurs by indirect albeit unknown mechanism, which, if further deciphered, may offer a new possibility for protection of neuronal cells by decreasing the level of β- and γ-secretases by *cdk4*-specific siRNA.

Proliferation of cells with lowered *cdk4* and *cdk6* expression

We used MTT assay for determination of the cell number after culturing them in the conditions of lowered expression of *cdk4/cdk6* genes. MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay, based on the conversion of MTT into formazan crystals by dehydrogenases in mitochondria of living cells, is simple and still the most versatile method to monitor the response of cells after treatment with various stimuli. Healthy and active proliferating cells present increase their metabolic activity measured in the assay, while cells, exposed to agents interfering with their ability to proliferation, have decreased activity of dehydrogenase enzymes.

In our case non-neural, proliferating HeLa and neural SH-SY5Y cells were transfected with the control (Si-Ctr), *cdk4* gene specific (Si4-1) and *cdk6* gene specific (Si6-9) siRNAs used at increasing concentrations: 0, 50, 100, 150 and 200 nM, complexed with Lipofectamine 2000. The MTT assay was performed after 24, 48 and 72 hours of incubation of cells at optimal culture conditions (37°C, 5% CO_2) and percent of living cells (PLC) was assessed (Fig. 3). Untreated cells (CC) served as the positive control and represented the 100% cell viability/proliferation. In the case of HeLa cells, for which the RNAi effect on the *cdk4*

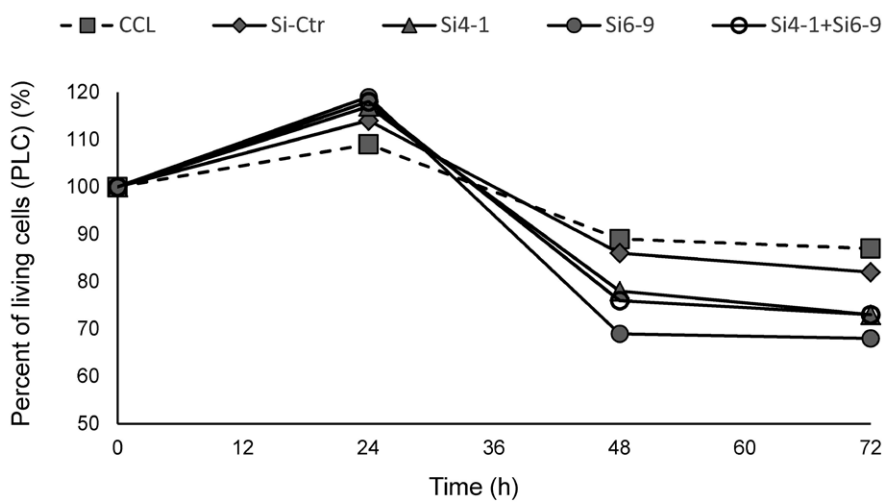

Fig. 3. Results of MTT viability/proliferation assay showing the number of HeLa cells treated with RNAi directed towards *cdk4* and *cdk6* genes and cultured 24, 48 or 72 hours after transfection. (CCL) control cells after treatment by Lipofectamine 2000 only; (Si-Ctr) cells transfected with a control non-silencing siRNA; (Si4-1) cells transfected with siRNA specific for human *cdk4* gene silencing; (Si6) cells transfected with siRNA specific for human *cdk6* gene silencing; (Si4-1+Si6-9) cells transfected with a "cocktail" siRNAs specific for *cdk4* and *cdk6* genes. SiRNAs were used in 100 nM concentration. Presented results are average from four repeats.

gene was very high (~90% of silencing), the cell number was decreased by the siRNAs during the experiment. We found slight proliferation after first 24 hours, ~10% increase of number of cells observed for all samples, but after the next 24 hours the number of cells in control samples (CCL, control cells after treatment by Lipofectamine 2000 only, or Si-Ctr, cells transfected with control non-silencing siRNA) decreased by about 10%, while the number of cells in samples with lowered expression of *cdk4* and *cdk6* genes (Si4-1, Si6-9 or cocktail Si4/Si6) decreased by ca. 20–30% (Fig. 3). We suppose that the first 10% increase the number of cells results from incomplete *cdk4* or *cdk6* genes silencing after 24 hours. Effect observed after 48 hours of cell culture, which maintained until 72 hours and beyond is related to efficient inhibition of target genes expression and stopped proliferation of cells arrested at the G1 phase. An analogous experiment performed in SH-SY5Y cells (transfected with the siRNAs/Lipofectamine 2000 complex) showed almost no influence of the applied siRNA on cell viability/proliferation (data not shown). PLC values were the same (near 100%) for all studied samples. Thus, we suppose that such effect is the result of very low efficiency of transfection of siRNA in neuronal cells.

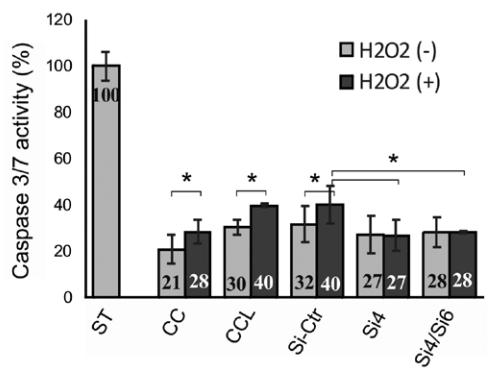

Fig. 4. Determination of caspase 3 and 7 activity in Neuro2A cells with lowered *cdk4* expression, or simultaneously *cdk4* and *cdk6* both genes expression at normal and oxidative stress (10 μM H_2O_2) conditions. (ST) caspases activity in the cells treated with 1 μM staurosporine, the positive control of apoptosis; (CC) caspases activity in the untreated cells; (CCL) caspases activity in the cells treated with transfection reagent, Lipofectamine 2000; (S-Ctr) caspases activity in the cells transfected with control siRNA; (Si4) caspases activity in the cells with lowered *cdk4* gene expression; (Si4/Si6) caspases activity in cells with lowered *cdk4* and *cdk6* genes expression. **P*<0.05.

From our previous studies we know, that neuroblastoma SH-SY5Y cells transfection efficiency with polycationic lipids is much lower than with electroporation/nucleofection approach, which we usually used for siRNA delivery to these cells. For technical reasons the nucleofection method could not be applied in the MTT experiments.

Determination of the level of apoptosis in the neuronal cells during oxidative stress conditions

Apoptosis, defined as a programmed cell death (PCD), is a naturally occurring process, essential for normal development and homeostasis of all multicellular organisms (Schwartzman and Cidkowski 1993). This process is also important for removal of damaged, infected, or potentially neoplastic cells. However, both, decreasing or increasing of the level of apoptotic cells can lead to adverse biological consequences, including the massive loss of neurons in AD (Nagata 1996). There are also evidences that the pathogenesis of AD (and several neurodegenerative diseases), may involve generation (associated with mitochondrial dysfunction) of reactive oxygen species (ROS) and/or reactive nitrogen species (RNS).

In these studies, we aimed to answer the questions whether apoptosis under the mild oxidative stress conditions can be stopped/slowed in neural cells with lowered *cdk4/cdk6* expression, and whether the silencing of *cdk* genes has any neuroprotective effect. The extent of apoptosis was evaluated by measurement of the activity of two apoptotic enzymes caspase 3 and 7. Thus, Apo-One Homogenous Caspase 3/7 Assay (Promega) was used to determine caspase 3 and caspase 7 activity in Neuro2A mouse neuroblastoma cells with lowered expression of *cdk4*, or with lowered expression of both genes *cdk4* and *cdk6*, cultured under normal and oxidative stress conditions (0 and 10 μM H_2O_2, respectively). The cells were transfected with siRNA duplexes directed against mouse *cdk4* gene (Si4-4, at concentration 100 nM) or against two genes simultaneously: *cdk4*, *cdk6* (Si4-4, Si6-16, used at total concentration 100 nM) and cultured for 48 hours (37°C, 5% CO_2). Then, the medium was changed to a medium with 10 μM H_2O_2, to develop a gentle oxidative stress, and cells were incubated for the next 6 hours at 37°C, 5% CO_2. Afterward, the Caspase pro-fluorescent substrate was added with the Caspase bi-functional cell

lysis/activity buffer (in accordance with the manufacturer's protocol), the samples were mixed and a plate with the lysates was kept at darkness for 3 hours. The fluorescence of the samples was measured with a Plate Reader Synergy HT (Bio-Tek). The fluorescence of the medium without the cells was taken as a blank control (FL M), which was subtracted from the fluorescence of studied samples, as well as from the fluorescence of untreated cells (FL CC) and cells with Lipofectamine 2000 (FL CCL), which served as negative control of apoptosis, and from the fluorescence of cells treated with Staurosporine (1 μM) used as the positive control of apoptosis (FL ST, 100% of caspases 3/7 activity).

Differences in caspase 3 and 7 activity were observed between the control samples (CC, the untreated cells, CCL, cells with Lipofectamine 2000, and Si-Ctr, cells after transfection of control siRNA) and studied cells with lowered *cdk4* or simultaneously both *cdk4* and *cdk6* expression (Fig. 4). Activity of caspases at control cells (CCL, Si-Ctr) was about 30%, but in 10 μM H_2O_2 it increased to 40%. The untreated cells (CC) also exhibited some caspase 3/7 activity (21%), which after H_2O_2 treatment increased to 28% in comparison to the staurosporine treated cells. Activity of caspases 3/7 in cells with reduced *cdk*4 and *cdk*6 expression was 27–28%, in comparison to positive control, and was practically unchanged regardless of culture conditions (0 or 10 μM H_2O_2). In this case, the activity of caspases 3 and 7 was also lower comparing the activity in the cells exposed to oxidative stress, but without silencing of cyclin-dependent kinases. These results indicate that the silencing of cyclin-dependent kinases, especially of cdk4 kinase, protects, to some extent, the cells against mild oxidative stress.

CONCLUSIONS

The aim of our studies was to confirm the hypothesis that silencing of cyclin-dependent kinases: cdk4 and cdk6, crucial for the G1 phase progression, might have the neuroprotective effect for neural cells by preventing the induction of apoptosis. We showed that lowering of the expression of *cdk4* gene or simultaneously both genes *cdk4/cdk6* has the impact on the cell-cycle progression, and thus we deliver a hint for development of the RNAi-based therapeutic approach in neurodegeneration.

Firstly, we selected the active siRNA sequences, efficiently inhibiting the expression of human and mouse target *cdk4* and *cdk6* genes (up to 85–90% of mRNA and ~60% of protein), both, in human SH-SY5Y and mouse Neuro2A neural cancer cells. Flow cytometry and MTT based the cell viability/proliferation experiments indicated that inhibition of target genes expression caused the "arrest" of cells in the G1 phase and slowing-down the cell proliferation rate. Additional effect of *cdk4/cdk6* genes silencing was the synchronic down-regulation of the down-stream genes, *Cyclin E*, *Cyclin A* and *PCNA*, as shown by the qRT-PCR analysis. The residual number of cdk4/6-cyclin D complexes present in cells probably was not enough for productive phosphorylation of the Rb protein, therefore preserved dissociation of the Rb/E2F/DP1 complex and stopped the expression of genes dependent on the E2F transcription factor activity. For the same cell models, we found the reduced level of expression of the genes, involved in the secretion of Aβ: *PSEN1* and *BACE1*. The level of the expression of precursor protein *APP* remained unchanged. Finally, we assessed the activity of caspase 3 and 7 indicating the level of apoptosis in the neuronal cells with lowered expression of targets *cdk4*/*cdk6*, where cells were cultured in normal and in oxidative stress conditions. The outcomes of these studies indicate that silencing of cyclin-dependent kinases, 4 and 6, protects cells against oxidative stress. Cells were less vulnerable to activation of the cell-cycle. Activity of caspases 3 and 7 was lower in the cells with vestigial *cdk4/cdk6* expression comparing the activity in cells exposed to oxidative stress, but without silencing of cdks.

In summary, we demonstrate that down-regulation of genes important in the G1 phase of the cell-cycle may play, to some extent, the protective function on the neuronal cells, which are exposed to unfavorable culture conditions

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