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The low doses of SWCNTs affect the expression of proliferation and apoptosis related genes in normal human astrocytes

Olha V. Rudnytska [a], Olena O. Khita [a], Dmytro O. Minchenko [a,b], Dariia O. Tsymbal [a], Yuliia V. Yefimova [a], Myroslava Y. Sliusar [a], Oleksandr Minchenko [a,*]

[a] Department of Molecular Biology, Palladin Institute of Biochemistry National Academy of Sciences of Ukraine, Kiev 01030, Ukraine
[b] Department of Pediatrics, National Bohomolets Medical University, Kyiv, Ukraine

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ABSTRACT

The unique properties of single-walled carbon nanotubes (SWCNTs) make them viable candidates for versatile implementation in the biomedical devices. They are able to cross the blood–brain barrier, enter cells and accumulate in cell nuclei. We studied the effect of these carbon nanoparticles on the expression of genes associated with endoplasmic reticulum stress and proliferation, cell viability and cancerogenesis as well as microRNAs in normal human astrocytes. We have shown that treatment of normal human astrocytes by small doses of SWCNTs (2 and 8 ng/ml of medium for 24 hrs) affect the expression of DNAJB9, IGFBP3, IGFBP6, CLU, ZNF395, KRT18, GJA1, HILPDA, and MEST mRNAs as well as several miRNAs, which have binding sites at 3′-UTR of these mRNAs. These changes in the expression profile of individual mRNAs introduced by SWCNTs are dissimilar in magnitude and direction and are the result of both transcriptional and posttranscriptional mechanisms of regulation. It is possible that these changes in gene expressions are mediated by the endoplasmic reticulum stress introduced by carbon nanotubes and reflect the disturbance of the genome stability. In conclusion, the low doses of SWCNTs disrupt the functional integrity of the genome and possibly exhibit a genotoxic effect.

1. Introduction

The unique properties of single-walled carbon nanotubes (SWCNTs) make them viable candidates for multipurpose implementation in various industrial applications as well as in the biomedical devices for targeted delivery of chemotherapeutic agents or cellular-sensing probes. At the same time, the hazardous impact of carbon nanotubes on human health and the environment was also shown, but different variations of these nanoparticles vary in their toxicity (Eldawud et al., 2018; Kobayashi et al., 2017; Møller and Jacobsen, 2017; Tejral et al., 2009; Toyokuni, 2013; Uo et al., 2011). Single-walled carbon nanotubes are able to cross the blood–brain barrier, enter through the cell membrane with accumulation in the cell nuclei. These properties of SWCNTs allow their use in health sciences as imaging probes and carrier of drugs in cancer therapy approaches. At the same time, carbon nanotubes are not biodegradable and their applications in medicine should be limited, raising worries near their chronic toxicity and genotoxicity (Ahmadi et al., 2017; Minchenko et al., 2016b, 2018; Uo et al., 2011). Therefore, these carbon nanoparticles affect an immune response, in particular through strong suppression of the expressions of cell surface glycoproteins HLA-DRA (major histocompatibility complex, class II, DR alpha) and HLA-DRB1 and up-regulate the level of microRNAs, that possibly participate in the suppression these mRNA expression (Minchenko et al., 2018). It is intertesting to note that SWCNTs also disturb alternative splicing, particularly 6-phospho fructo-2-kinase/fructose-2.6-bisphosphatase-3 (PFKFB3) and PFKFB4 as well as dystrobrevin alpha (DTNA) pre-mRNA in human U87 glioma cells (Minchenko et al., 2016b). Furthermore, carbon nanotubes deregulate the expression of cyclin D2, DUSP1, and lamin B1, which participate in the control of cell cycle and genome stability, as well as many other genes indicating genotoxic effect of these nanoparticles (Minchenko et al., 2016b, 2018). However, precise mechanisms of single-walled carbon nanotubes toxicity are still largely unknown and seem to include many pathways including endoplasmic reticulum

* Corresponding author.
E-mail address: ominchenko@yahoo.com (O. Minchenko).

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stress signaling pathways like other nanoparticles (Barberet et al., 2017; Cao et al., 2017; Hu et al., 2018; Simon et al., 2017). Moreover, relief of endoplasmic reticulum stress with 4-phenylbutyric acid inhibited most effects of titanium dioxide nanoparticles (Hu et al., 2018).

In this study we investigate the impact of low quantities of SWCNTs on the expression of DNAJB9 (DnaJ heat shock protein family (Hsp40), member B9), IGFBP3 (Insulin-like growth factor binding protein 3), IGFBP6, CLU (Clusterin), ZNF395 (Zinc finger protein 39), KRT18 (Keratin 18), GJA1 (Gap junction protein alpha 1), HILPDA (hypoxia inducible lipid droplet associated), and MEST (mesoderm specific transcript) mRNAs as well as several miRNAs, which have binding sites at 3′-UTR of these mRNAs in normal human astrocytes line NHA/TS. These genes play an important role in the regulation of multiple metabolic processes and also participate in endoplasmic reticulum stress and cancerogenesis (Amin-Wetzel et al., 2017; Bache et al., 2015; Lee et al., 2015; Liu et al., 2018). Proteins, encoded by *CLU* and *DNAJB9* genes represent cell stress chaperones, have pro-proliferative properties and are increased in variable cancers (Bache et al., 2015; Liu et al., 2018; Lyu et al., 2018). Furthermore, protein DNAJB9 is the endoplasmic reticulum luminal co-chaperone ERdj4, which is a selective ERN1 (endoplasmic reticulum to nucleus signaling 1) repressor. It promotes a complex between the endoplasmic reticulum chaperone BiP (binding immunoglobulin protein), which represent heat shock protein family A (Hsp70) member 5 (HSPA5), and the luminal stress-sensing domain of ERN1 (Amin-Wetzel et al., 2017; Cheng et al., 2019; Lee et al., 2015). It is associated with endoplasmic reticulum stress and plays a role in protecting stressed cells from apoptosis through inhibition of pro-apoptotic function of p53 (López et al., 2017; Lee et al., 2015). Several other proteins (ZNF395 and KRT18) have anti-proliferative properties (Liu et al., 2018; Schroeder et al., 2017; Zhang et al., 2016). Transcription factor ZNF395 is controlled by miR-525-3p and IkB kinase-signaling and activates genes involved in the innate immune response and cancer (Pang et al., 2014; Jordanovski et al., 2013). Furthermore, keratin 18 regulates the transcription and alternative splicing of a number of genes in apoptotic pathways (Cheng et al., 2019). IGF-binding proteins prolong the half-life of the IGFs and have been shown to either inhibit or stimulate the growth promoting effects of the IGFs on cell culture, but IGFBP3 also exhibits IGF-independent antiproliferative and apoptotic effects mediated by its receptor TMEM219/IGFBP-3R (Bai et al., 2019; Canel et al., 2017; Hou et al., 2019; Ingermann et al., 2010; Johnson and Firth, 2014; Wang et al., 2017; Yan et al., 2017; Yang et al., 2019). The mesoderm specific transcript as well as HILPDA has been linked to certain types of cancer, but HILPDA regulates also lipid metabolism and response to microenvironmental stress in tumors (VandeKopple et al., 2019). MEST and HILPDA are responsive to hypoxia and endoplasmic reticulum stress and play an important role in the development through different pathways (Mesman et al., 2017; Minchenko et al., 2016a; VandeKopple et al., 2019).

MicroRNAs (miRNAs) are a class of endogenous small single-stranded non-protein coding small RNA molecules, often phylogenetically conserved, which play a critical role in the control of gene expression preferentially at the posttranscriptional level. Specifically, miRNAs mainly function post-transcriptionally by binding to the 3′ untranslated regions (3′UTR) of target messenger RNAs and induce selected mRNA degradation or translational repression. MicroRNAs play an important role in the regulation all metabolic processes as well as in brain development (Krichevsky et al., 2003; Ma et al., 2019). For this study we selected following microRNAs: miR-7-5p, miR-10a-5p, miR-19a-5p, miR-27-3p, miR-144-5p, miR-145-5p, and miR-150-5p, which have relation to the control of most of studied mRNA and therefore regulate metabolism and numerous processes.

In this study we aim to investigate the effect of SWCNTs on the expression of a subset of genes encoding mRNA and microRNA associated with endoplasmic reticulum stress, cell proliferation, apoptosis, and cancerogenesis in normal human astrocytes.

2. Materials and methods

2.1. Experimental material

The functionalized SWCNTs (diameter tubes – 1–2 nm) were suspended in distilled water and filtered out by means of membrane filter (pore size was 1.2 μm). Characteristics of these SWCNT were described previously (Minchenko et al., 2018).

2.2. Cell culture and treatment

The immortalized normal human astrocytes (NHA/TS cell line) were received from Drs. K. Sasai and S. Tanaka (Japan) and were grown at 37 °C under a humidified atmosphere of 95% air and 5% CO_2 as reported (Sasai et al., 2007). The normal human astrocytes were treated with two doses (2 and 8 ng/ml of medium) of SWCNTs for 24 hrs.

2.3. RNA extraction and cDNA synthesis

Total RNA was extracted from normal human astrocytes using TRIZOL reagent as described previously (Auf et al., 2013). The RNA pellets were washed with 75% ethanol and dissolved in nuclease-free water. For additional purification the RNA samples were re-precipitated with 95% ethanol containing sodium acetate and pellets were dissolved again in nuclease-free water. RNA concentration as well as its spectral characteristics was measured using NanoDrop One (Thermo Scientific). Thermo Scientific Verso cDNA Synthesis Kit was used for reverse transcription. For polyadenylation and reverse transcription of miRNAs we used Mir-X miRNA First-Strand Synthesis Kit (Takara, Japan).

2.4. Real-time quantitative polymerase chain reaction

The expression level of different genes (*CLU, DNAJB9, IGFBP3, IGFBP6, ZNF395, KRT18, GJA1*, and *ACTB*) was measured by real-time quantitative polymerase chain reaction (qPCR) using “QuantStudio 5 Real-Time PCR System” (Applied Biosystems) and Absolute qPCR SYBRGreen mix. Polymerase chain reaction was performed in triplicates. The level of miRNAs was measured by real-time qPCR using Mir-X miRNA qRT-PCR SYBR Kit (Takara, Japan). For analysis of RNA expression four different RNA samples for each experimental group as well as pairs of the specific primers (Sigma-Aldrich, USA; Table 1) were used. For analysis of microRNA expressions an universal qPCR reverse primer and specific for each miRNA forward primers were used (Table 2).

The quality of amplification products was analyzed by melting curves and by electrophoresis using 3% agarose gel. An analysis of quantitative PCR was performed using special computer program “Differential Expression Calculator”. The values of different mRNA expressions were normalized to the level of beta-actin mRNA and represented as percent of control (100%). The values of microRNA expressions were normalized to the level of U6 RNA and represented as percent of control (100%).

2.5. Statistical analysis

All values are expressed as mean ± SEM from triplicate measurements performed in 4 independent experiments. Statistical analysis was performed according to Student's *t*-test using Excel program. A value of $p < 0.05$ was considered statistically significant. All experimental qPCR data from triplicates, performed in 4 different experiments, were analyzed for the normality of distribution using a graphical tool (normal probability plot) and a histogram. A normal distribution was shown for all analyzed data sets. We also used the

Table 1
Characteristics of the primers used for quantitative real-time polymerase chain reaction.

Gene symbol	Gene name	Primer's sequence	Nucleotide numbers in sequence	GenBank accession number
CLU	Clusterin	F: 5′-tcaaaatgctgtcaacgggg	213,232	NM_001831
		R: 5′-tggtctcattgcacactcct	391–372	
DNAJB9	DnaJ heat shock protein family (Hsp40) member B9	F: 5′-gtcggagggtgcaggatatt	346–365	NM_012328
		R: 5′-tcagggtggtacttcatggc	536–517	
IGFBP3	insulin-like growth factor binding protein 3	F: 5′-tctgatcccaagttccaccc	613–632	NM_000598
		R: 5′-tccatttctctacggcaggg	785–766	
ZNF395	Zinc finger protein 395	F: 5′-tctgctcttccaccacctct	1219–1238	NM_018660
		R: 5′-ggtgagactgggatctggaa	1391–1372	
KRT18	Keratin 18, type I	F: 5′-cacagtctgctgaggttgga	966–985	NM_000224
		R: 5′-gagctgctccatctgtaggg	1391–1372	
GJA1	Gap junction protein alpha 1	F: 5′-ggcgtgaggaaagtaccaaa	46–65	NM_000165
		R: 5′-cctccagcagttgagtaggc	268–249	
IGFBP6	insulin-like growth factor binding protein 6	F: 5′-gctgttgcagaggagaatcc	397–416	NM_002178
		R: 5′-ttgggcacgtagagtgtttg	632–613	
HILPDA	hypoxia inducible lipid droplet associated	F: 5′-caagctgagcaccgttgtaa	533–552	NM_013332
		R: 5′-ccaccacacccagctaactt	773–754	
MEST	mesoderm specific transcript	F: 5′-aagatggaggtgtgctgtca	813–832	NM_002402
		R: 5′-gcgccttctgaacttcttcc	1021–1002	
ACTB	beta-actin	F: 5′-gacttcgagcaagagatgg	747–766	NM_001101
		R: 5′-gcactgtgttggcgtacag	980–961	

Table 2
Forward primers used for quantitative real-time polymerase chain reaction of microRNA.

microRNA symbol	microRNA name	Primer's sequence
miR-144	microRNA-144-5p	5′-ggatatcatcatatactgtaag
miR-145	microRNA-145-5p	5′-gtccagttttcccaggaatccct
miR-150	microRNA-150-5p	5′-tctcccaacccttgtaccagtg
miR-10a	microRNA-10a-5p	5′-taccctgtagatccgaatttgtg
miR-19a	microRNA-19a-5p	5′-tgtgcaaatctatgcaaaactga
miR-7	microRNA-7-5p	5′-tggaagactagtgattttgttgt
miR-27a	microRNA-27a-3p	5′-ttcacagtggctaagttccgc

D'Agostino-Pearson test (P was close to 1.00) and the Shapiro–Wilk test ($W > 0.9$; $p > 0.9$) for analysis of some data sets.

3. Results and discussion

3.1. Effect of SWCNTs on the expression of mRNA in normal human astrocytes

The effect of SWCNTs (diameter 1–2 nm) on the expression of genes encoding various important regulatory factors associated with endoplasmic reticulum stress, cell proliferation, apoptosis, and cancerogenesis as well as some other cellular functions in normal human astrocytes line NHA/TS was studied. As shown in Fig. 1, the exposure of normal human astrocytes to SWCNTs (2 and 8 ng/ml of medium) led to significant and dose-dependent up-regulation of the expression of genes encoding three important regulatory factors: clusterin (CLU), DnaJ heat shock protein family (Hsp40) member B9 (DNAJB9), and insulin-like growth factor binding protein 3 (IGFBP3). Thus, the expression level of clusterin mRNA was up-regulated by +45 and +94% and IGFBP3 by +53 and +87% by 2 ng and 8 ng of SWCNTs per 1 ml of medium, correspondingly. Furthermore, the effect of these nanoparticles on the expression of DNAJB9 was significantly stronger (+102 and +160%, correspondingly), as compared to the control (Fig. 1). These results clearly demonstrated that exposure of normal human astrocytes with low amounts of SWCNTs significantly up-regulated the expression of genes encoded the endoplasmic reticulum stress responsible proteins such as CLU, DNAJB9, and IGFBP3, which are increased in variable cancers (Bache et al., 2015; Canel et al., 2017; Hou et al., 2019; Johnson and Firth, 2014; Liu et al., 2018; Lyu et al., 2018). It is possible that SWCNTs introduce the endoplasmic reticulum stress signaling pathways like many other nanoparticles (Barberet et al., 2017; Cao et al., 2017; Hu et al., 2018; Simon et al., 2017). Furthermore, this stress is permanent because these carbon nanoparticles are not biodegradable and can mediate genome reprogramming and genetoxicity by disrupting the functional integrity of the genome. It is possibly that the endoplasmic reticulum stress has a central role in effects of SWCNTs on cell metabolism, surviving and cancerogenesis. Moreover, Hu et al. (2018) shown that relief of endoplasmic reticulum stress with 4-phenylbutyric acid inhibited most effects of titanium dioxide nanoparticles (Hu et al., 2018). There are data that cell stress chaperone DNAJB9 plays a role in protecting stressed cells from apoptosis through inhibition of pro-apoptotic function of p53 (López et al., 2017; Lee et al., 2015). At the same time, a strong increase of IGFBP3 in treated by SWCNTs astrocytes can also mediate antiproliferative and apoptotic effects (Bai et al., 2019; Ingermann et al., 2010; Johnson and Firth, 2014; Wang et al., 2017; Yan et al., 2017).

We also studied the effect of SWCNTs on the expression of keratin 18, type I (*KRT18*) in normal human astrocytes. It was shown that the expression of *KRT18* was less sensitive to toxic action of these carbon nanotubes and its expression level is down-regulated only by −13 and −15% by 2 ng and 8 ng of SWCNTs per 1 ml of medium, correspondingly (Fig. 2). More significant changes were detected for zinc finger protein 395 (ZNF395) in normal human astrocytes treated by SWCNTs (2 and 8 ng/ml of medium) (-44 and −45%, correspondingly) and gap junction protein alpha 1 (GJA1; −72 and −76%, correspondingly). Therefore, the effect of SWCNTs on the expression of all these genes did not significantly depend on quantity of these nanotubes (Fig. 2).

The obtained results demonstrated that low amounts of SWCNTs affect the expression of ZNF395, KRT18, and GJA1 mRNAs in normal human astrocytes and that these changes in their expression can reflect the dysregulation of the functional integrity of the genome, which can contribute to cancerogenesis, because ZNF395 and KRT18 proteins have anti-proliferative properties (Liu et al., 2018; Schroeder et al., 2017; Zhang et al., 2016). Furthermore, protein GJA1 is a component of gap junctions, which are composed of arrays of intercellular channels that provide a route for the diffusion of low molecular weight materials from cell to cell and its dysregulation can also contribute to cancerogenesis (Hills et al., 2018).

We have also studied the effect of SWCNTs on the expression of MEST, HILPDA, and IGFBP6 mRNAs in normal human astrocytes. As shown in Fig. 3, the exposure of normal human astrocytes with SWCNTs (2 and 8 ng/ml of medium) led to significant and dose-dependent down-regulation of the expression of next three genes

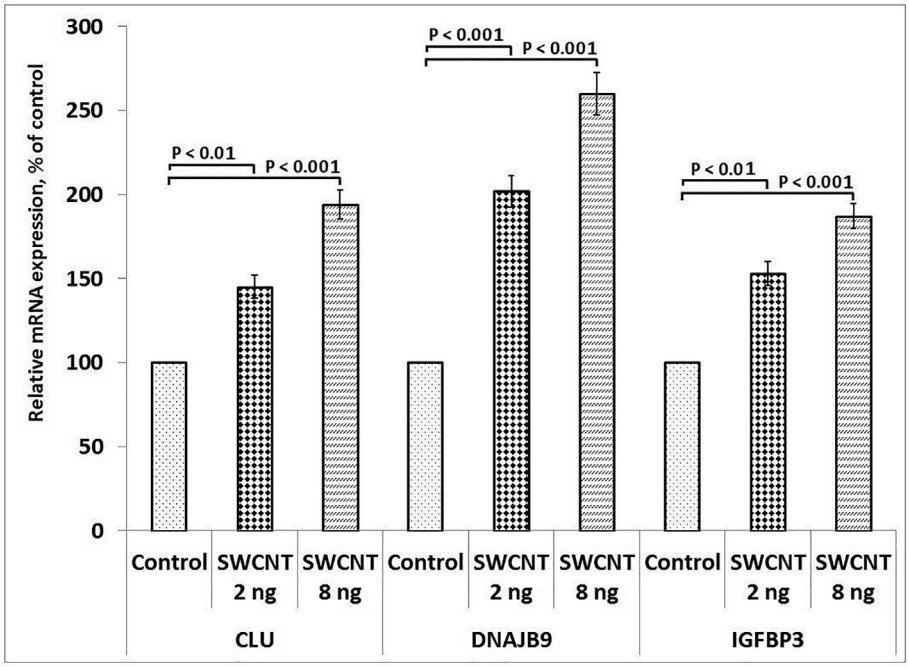

Fig. 1. Effect of single-walled carbon nanotubes (2 and 8 ng/ml for 24 hrs) on the expression of CLU, DNAJB9, and IGFBP3 mRNAs in normal human astrocytes line NHA/TS. Values of studied mRNA expressions were normalized to ACTB mRNA expression; $n = 4$.

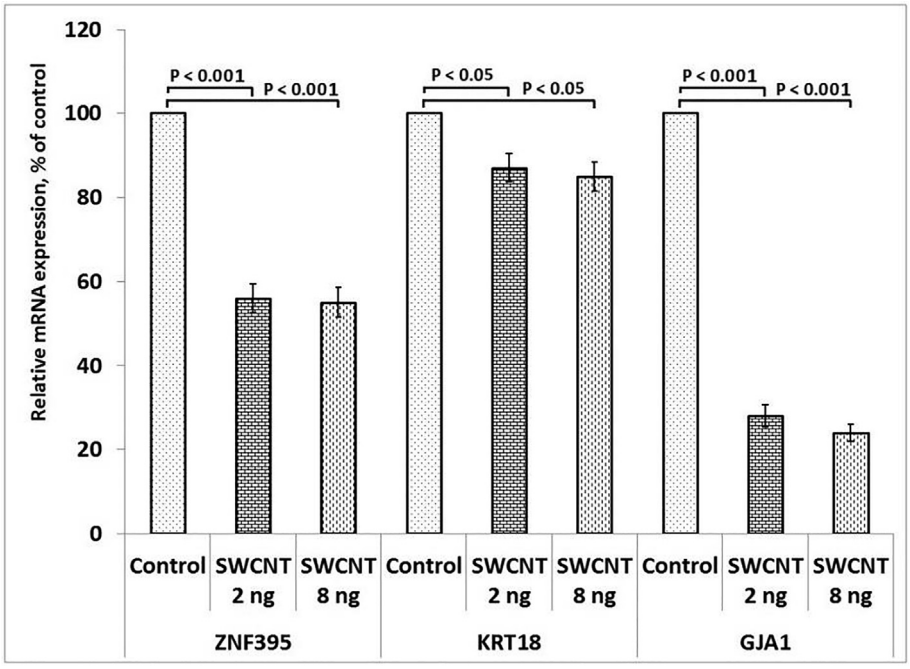

Fig. 2. Effect of single-walled carbon nanotubes (2 and 8 ng/ml for 24 hrs) on the expression of ZNF395, KRT18, and GJA1 mRNAs in normal human astrocytes line NHA/TS. Values of studied mRNA expressions were normalized to ACTB mRNA expression; $n = 4$.

encoding important regulatory proteins such as mesoderm specific transcript, hypoxia inducible lipid droplet associated, and insulin-like growth factor binding protein 6.

Down-regulation of genes encoded MEST and HILPDA proteins, which are responsive to hypoxia and endoplasmic reticulum stress and play an important role in the development, indicate that these carbon nanoparticles destroy important developmental processes (Mesman et al., 2017; Minchenko et al., 2016a; VandeKopple et al., 2019). There are data that mesoderm specific transcript as well as HILPDA has been linked to certain types of cancer and that HILPDA regulates also lipid metabolism and response to micro environmental stress in tumors (VandeKopple et al., 2019).

3.2. Effect of SWCNTs on the expression level of microRNA in normal human astrocytes

We have also studied the effect of SWCNTs on genome function in normal human astrocytes at the level of microRNA expression. As

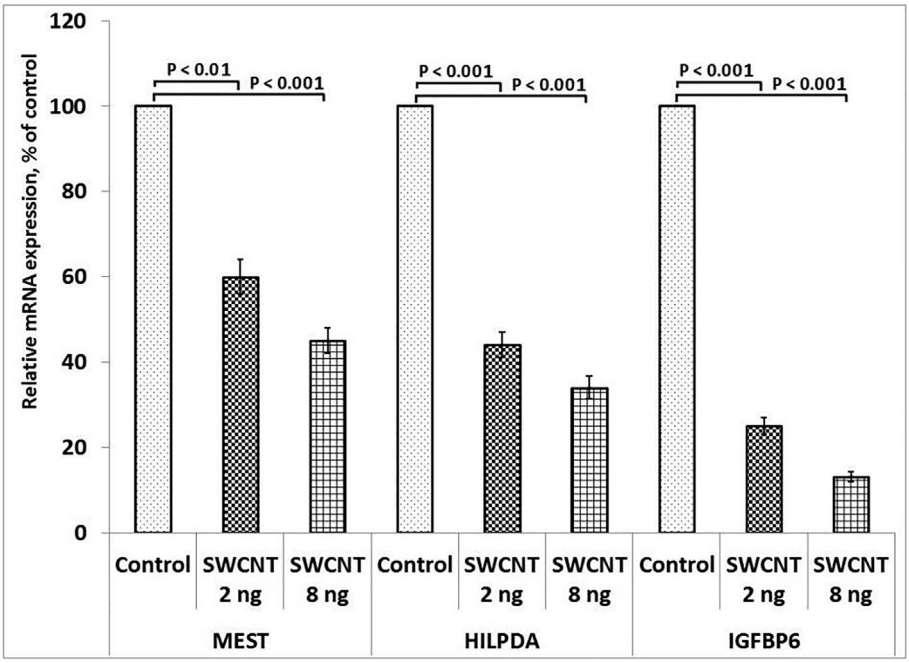

Fig. 3. Effect of single-walled carbon nanotubes (2 and 8 ng/ml for 24 hrs) on the expression of MEST, HILPDA, and IGFBP6 mRNAs in normal human astrocytes line NHA/TS. Values of studied mRNA expressions were normalized to ACTB mRNA expression; $n = 4$.

shown in Figs. 4 and 5, exposure of these cells to SWCNTs (8 ng/ml for 24 hrs) led to variable changes in the expression level of a group of microRNAs, which have binding sites in 3′-UTR of studied mRNAs. It was shown that the expression level of all studied microRNA was up-regulated by SWCNTs except for miR-19a-5p and miR-203-5p. The expression level of miR-19a-5p and miR-27a-3p was reduced in normal human astrocytes treated by SWCNTs: −35 and −55%, correspondingly (Fig. 4). The obtained results agree well with significant up-regulation of CLU and IGFBP3 mRNAs as well as DNAJB9 mRNA, indicating possible participation of these microRNAs in post-transcriptional regulation of the expression of studied mRNAs. Therefore, the level of miR-7-5p, miR-10a-3p, miR-144-5p, miR-145-5p, and miR-150-5p was significantly up-regulated in normal human astrocytes exposure with SWCNTs: +52, +29, +38, +685 and +84%,

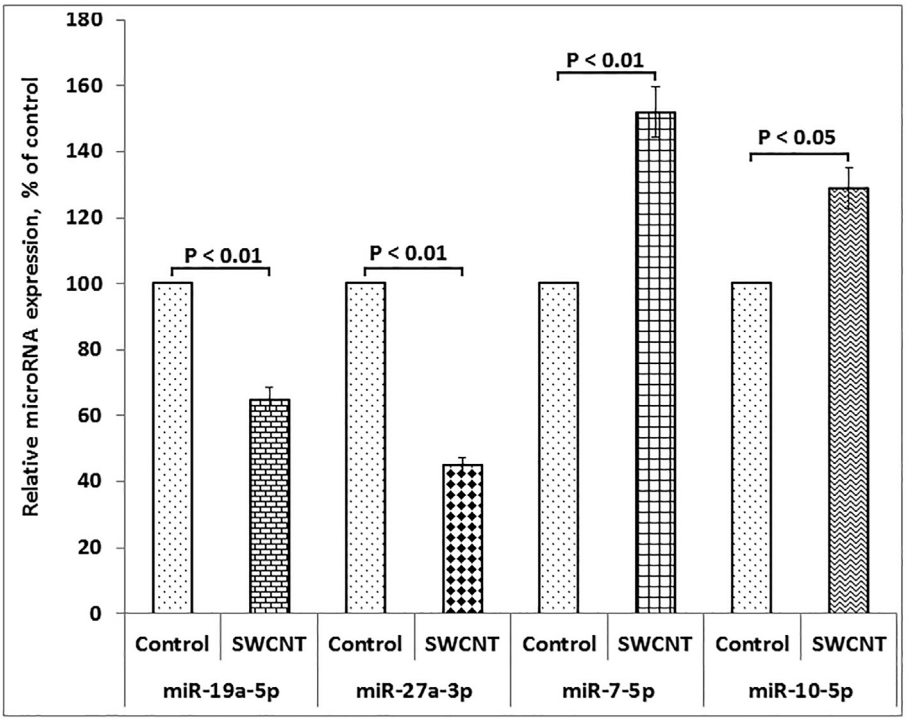

Fig. 4. Effect of SWCNTs (8 ng/ml of medium for 24 hrs) on the expression level of microRNA miR-19a-5p, miR-27a-3p, miR-7-5p, and miR-10-5p in normal human astrocytes line NHA/TS. Values of studied microRNA expressions were normalized to U6 RNA expression; $n = 4$.

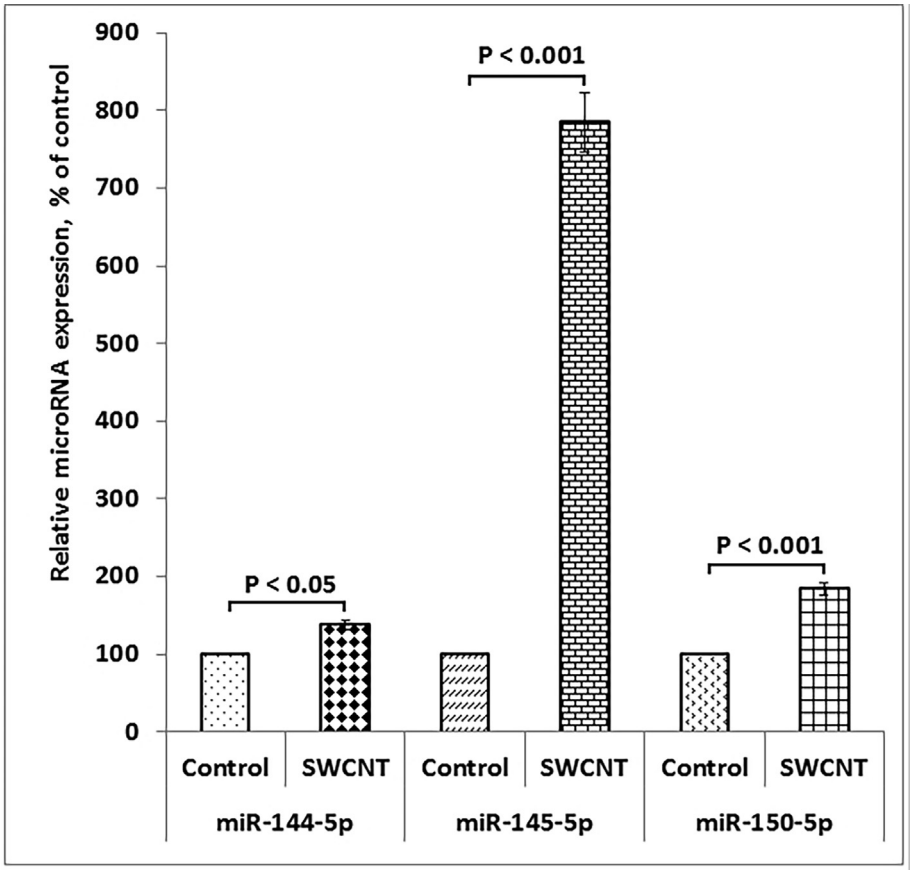

Fig. 5. Effect of single-walled carbon nanotubes on the expression level of microRNA miR-144-5p, miR-145-5p, and miR-150-5p in normal human astrocytes line NHA/TS. Values of studied microRNA expressions were normalized to U6 RNA expression; $n = 4$.

correspondingly (Figs. 4 and 5). An increased level of miR-7-5p, miR-10a-3p, miR-144-5p, miR-150-5p, and miR-145-5p correlate well with down-regulation of the expression of ZNF395, KRT18, GJA1, and HILPDA as well as MEST and IGFBP6 mRNAs. Schematic representation of possible post-transcriptional mechanisms of the regulation of mRNA expressions through changes in microRNA level, which negatively correlated with the level of corresponding mRNA containing specific for selected microRNA sites in their 3′-UTR structure, is shown in (Fig. 6).

Furthermore, SWCNTs at very low amounts dysregulate the expression of numerous mRNAs and microRNAs encoding various important regulatory factors, which related to endoplasmic reticulum stress, cell proliferation, apoptosis, and carcinogenesis, in normal human astrocytes, which possibly reflect the genotoxic and neurotoxic effects of this unique carbon compounds. Our results also agree well with data Krichevsky et al. (2003) and Ma et al. (2019) that microRNAs play also an important role in the regulation all metabolic processes as well as in brain development.

It is possible that most of observed changes in the expression of numerous mRNAs as well as microRNAs in normal human astrocytes treated with SWCNTs are mediated by disturbances in genome function introduced by endoplasmic reticulum stress signaling pathways. Moreover, most of studied mRNAs are endoplasmic reticulum stress responsive supporting an idea that endoplasmic reticulum stress is an important component of SWCNTs action on glial cells similar to other nanoparticles (Barberet et al., 2017; Cao et al., 2017; Hu et al., 2018; Simon et al., 2017). However, precise mechanisms of single-walled carbon nanotubes toxicity are still largely unknown and warrant further investigation.

Thus, results of this investigation clearly demonstrated that SWCNTs at very low amounts dysregulate the expression of mRNAs encoding various important regulatory factors as well as microRNAs related to endoplasmic reticulum stress, cell proliferation, apoptosis, and carcinogenesis in normal human astrocytes and that these changes possibly reflect the genotoxic and neurotoxic effects of this unique carbon nanoparticles.

4. Conclusions

In conclusion, we presented the data that SWCNTs (diameter 1–2 nm), which can potentially be released into environment or used in the biomedical devices, exert dose-dependent effect on genome function in normal human astrocytes, which possibly reflects a genotoxic impact. We speculated that this dysregulation of functional integrity of the genome by SWCNTs may be a result of endoplasmic reticulum stress introduced by these nanoparticles through transcriptional and post-transcriptional mechanisms because changes in the expression of all studied mRNAs negatively correlated with corresponding microRNAs, which recognized specific sites in their 3′-untranslated region. Currently, very little is known regarding the genotoxicity and neurotoxicity of SWCNTs as well as many other nanoparticles and better understanding of detailed molecular mechanisms of carbon nanotubes action is of importance not only in frame of promising therapeutic applications of carbon nanomaterials, but also required for careful assessment of potential exposure-related risks for human health. Furthermore, this data suggest more cautions needed in biomedical applications of carbon nanotubes.

CRediT authorship contribution statement

Olha V. Rudnytska: . **:** Investigation, Data curation. **Olena O. Khita:** Investigation, Visualization. **Dmytro O. Minchenko:** Visualiza-

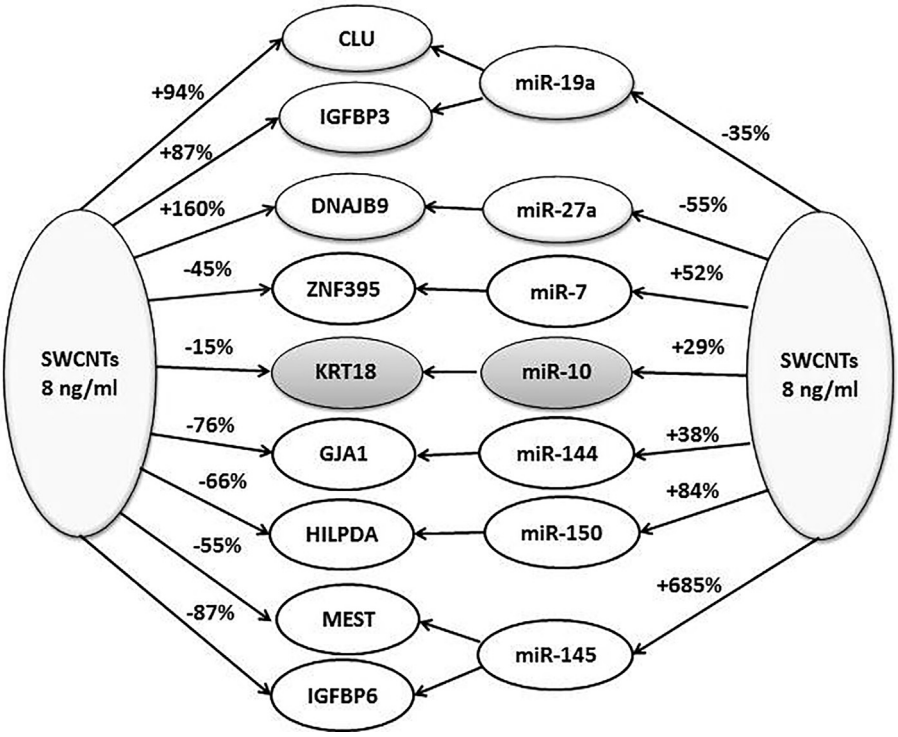

Fig. 6. Schematic representation of transcriptional mechanisms of the regulation of mRNA expressions and possible post-transcriptional mechanisms through changes in microRNA level, which negatively correlated with the level of corresponding mRNAs containing specific for selected microRNA sites in their 3′-UTR sequence.

tion, Writing - original draft. **Dariia O. Tsymbal:** Methodology, Investigation, Writing - review & editing. **Yulia V. Efimova:** Investigation, Visualization. **Myroslava Y. Sliusar:** Investigation, Data curation. **Oleksandr Minchenko:** Conceptualization, Methodology, Investigation, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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