

**ORIGINAL ARTICLE**

**INHIBITION OF PARP ACTIVITY DOES NOT AFFECT THE DIFFERENTIATION PROCESSES CAUSED BY RETINOIC ACID IN SH-SY5Y CELLS**

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**Abstract:** Differentiation is a complex process by which cells become specialized in their physiological functions. As this process is followed by a decrease in proliferation ability, the induction of differentiation could be an ideal cancer treatment. We analyzed the effects of a common differentiation agent, retinoic acid, on neuroblastoma SH-SY5Y cells under the conditions of poly(ADP-ribose) polymerase (PARP) inhibition. Namely, PARP1 can be indirectly involved in processes of chromatin remodelling, so the aim was to investigate whether its inhibition can influence the process of differentiation. Cells differentiated after retinoic acid treatment into neural cells, with neurite outgrowth, proliferation arrest and induction of tissue plasminogen activator. PARP inhibition did not influence the process of differentiation. Analysis of gene expression revealed the involvement of several signaling pathways in RA-dependent differentiation. Beside TGF $\beta$  and Notch pathways, master transcription factors directing epithelial-mesenchymal transition were shown to also take part in the differentiation process.

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**INTRODUCTION**

Up to now, one of the most successful tumor therapies applied is the treatment of some forms of promyelocytic leukaemia by derivatives of retinoic acid (RA; all-trans retinoic acid ATRA), inducing B cell differentiation. Other types of tumors and leukemia are mostly refractive to differentiation.<sup>1, 2</sup> This is also true for a group of neuro and glioblastoma tumors: these tumors usually exhibit very aggressive behavior, but a subset of neuroblastoma cell lines can be differentiated by retinoic acid.<sup>3, 4</sup> Retinoic acid is a steroid, binding as a ligand to its receptors (RAR and RAX) in the nucleus, where the complex acts as a transcription factor. RA was also found to be able to interfere with peroxisome proliferator-activated receptor pathways (PPAR), but in dependence on the intracellular milieu. RA has a role in neurogenesis and neural stem cell differentiation. It mediates the proliferation-differentiation switch through a complex network including interactions with the Notch pathway, proneural and neurogenic proteins and genes involved in neural proliferation.<sup>5</sup> Pathways involved in embryonic stem cell differentiation toward neuronal tissue by RA also require the activation of several pathways, including Wnt, MAP kinases, Src kinases, inhibition of GSK $\beta$  and others.<sup>6</sup>

In this article, we analyzed the influence of poly(ADP-ribose)polymerase (PARP) inhibition on the RA-induced differentiation process. PARP1 is an enzyme which adds ADP-ribose chains on proteins, and thus causes changes in the chromatin structure and facilitates the process of DNA damage repair. Its activation in the case of DNA damage can direct cells toward apoptosis or necrosis. Its participation in different processes of chromatin remodeling and cell differentiation is still being uncovering. PARP1 modifies a number of transcription factors and some of

them are involved in differentiation processes. It can influence the processes of DNA methylation and take part in insulator regulation. Also, it was found that it has a role in stem cell establishment and differentiation process.<sup>7</sup>

We analyzed the expression of a set of genes involved in differentiation and epithelial-mesenchymal transition in a neuroblastoma cell line, after treatment with RA and the PARP inhibitor for a prolonged time period. SH-SY5Y cells are model cells for RA-dependent differentiation, a process which requires prolonged treatment and involves numerous changes in cell biology, including signaling pathways and gene expression, cell morphology and proliferation. As PARP1 has the ability to change the chromatin and influence the activity of a number of transcription factors, we wanted to see whether it could influence the process of SH-SY5Y differentiation.

## MATERIAL AND METHODS

### Cell culture and growth assessment

Neuroblastoma SH-SY5Y cells were grown in DMEM/F12 (50:50) medium (Gibco, USA) supplemented with 10% fetal bovine serum (Sigma, USA) at 37 °C and 5% CO<sub>2</sub>. The cell line is commercially available at ATCC (USA). Cells were tested for the presence of mycoplasma with EZ-PCR Mycoplasma Test Kit (Biological Industry, Israel).

Cells were treated with ATRA (all-trans retinoic acid) (Sigma) and PJ-34, PARP inhibitor (Sigma). Control cells were treated with DMSO (Sigma). For prolonged treatment, cells were treated with 10 µM ATRA and 20 µM PJ-34 every second day and reseeded if needed. Cell growth was assessed by crystal violet staining. Cells were seeded on a 96-well plate in triplicates, and every second day one set of cells was fixed with cold methanol. At the end of the assay, the plate was stained with crystal violet and after dissolving in 1% SDS, absorbance was measured on 595 nm by a microplate reader.<sup>8</sup>

### Measurement of neurite length

To measure the length of neurite outgrowth in cells, cells were photographed on the inverted microscope (Zeiss Axiovert 40 CFL) every second day. The neurite length was analysed with NeuroGrowth software in ImageJ program (NIH, USA).

### RNA preparation, cDNA synthesis and quantitative real-time PCR

Total RNA was extracted from cells using TRI Reagent (Sigma), and cDNA was synthesized from 2µg of total RNA by Primescript RTase (Takara, Japan) according

to the manufacturer's instructions. Quantitative real-time PCR (qRT-PCR) was performed using GoTaq® qPCR Master Mix (Promega, USA) in 7500 Fast Real-Time PCR system (Applied Biosystems, USA). Gene expression was validated by comparison with *HPRT* gene expression. Primer sequences, designed by IDT PrimerQuest software package (Integrated DNA Technologies, Inc., USA) used for PCR reactions, are listed in Table 1.

**Table 1. List of primers used for qRT-PCR**

<i>HPRT</i>	F: 5'-CTTTGCTGACCTGCTGGATT-3' R: 5'-TCCCCTGTTGACTGGTCATT-3'
<i>HES1</i>	F: 5'-AGCACAGAAAGTCATCAAAGC-3' R: 5'-CCGCGAGCTATCTTCTTCA-3'
<i>c-MYC</i>	F: 5'-CTGCTTAGACGCTGGATT-3' R: 5'-CTCCTCGTCGCAGTAGAAA-3'
<i>SMAD4</i>	F: 5'-CGCGTCTTTGTACAGAGTTA-3' R: 5'-GATGACACTGACGCAAATCAA-3'
<i>SMAD7</i>	F: 5'-ACCCGATGGATTTTCTCAAACC-3' R: 5'-GCCAGATAATTCGTTCCCCCT-3'
<i>SNAIL1</i>	F: 5'-TCTAGGCCCTGGCTGCTACA-3' R: 5'-CATCTGAGTGGGTCTGGAGGT-3'
<i>SNAIL2</i>	F: 5'-CCTGGTCAAGAAGCATTTC AAC-3' R: 5'-GAGGATCTCTGGTTGTGGTATG-3'
<i>TGFβ</i>	F: 5'-GACACCAACTATTGCTTCAG-3' R: 5'-AGAAGTTGGCATGGTAGCCC-3'
<i>TWIST1</i>	F: 5'-CGGAGACCTAGATGTCATTGTTT-3' R: 5'-ACGCCTGTTTCTTTGAATTTG-3'
<i>ZEB1</i>	F: 5'-GGCAGATGAAGCAGGATGTA-3' R: 5'-GACAGCAGTGCTTGTGTTG-3'
<i>iPA</i>	F: 5'-CTGGGAAGTGCTGTGAAATA-3' R: 5'-TTCTGCAGTAGTTGTGGTTC-3'

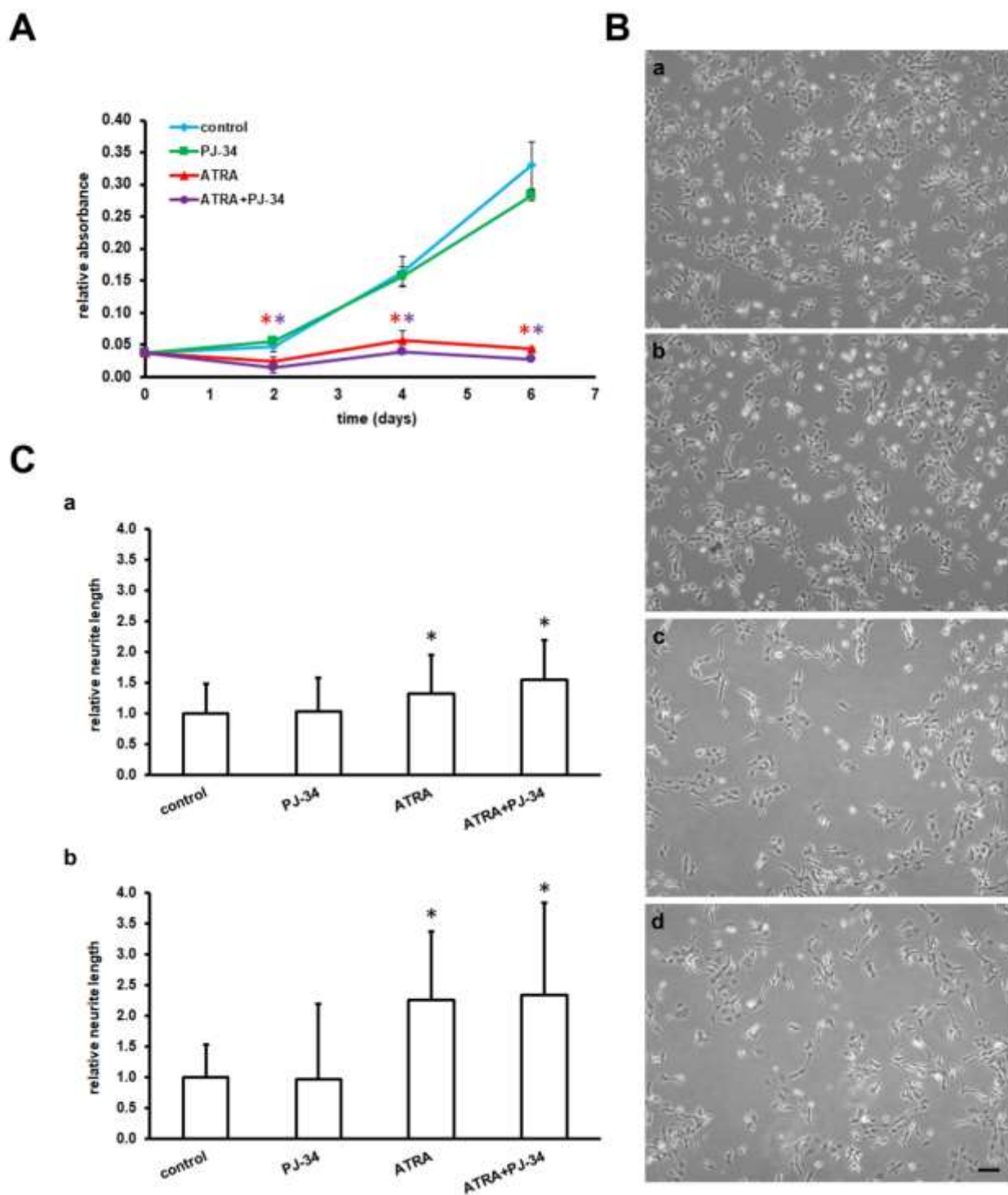
### Statistical analysis

Data were statistically analyzed using the software package Microsoft Office and R Statistical Software (The R Foundation). The parametric test was used for comparison of gene expression between control and treated cells. The significance of independent two-tailed Student's t-test was set at P-value < 0.05. Neurite growth was analyzed using ANOVA and post-hoc Duncan test, and the P-value was set at 0.05.

## RESULTS

### The effect of ATRA treatment and PARP inhibition on growth and differentiation of SH-SY5Y neuroblastoma cells

It was found that RA induced differentiation in a set of neuroblastoma cell lines, such as SH-SY5Y, SK-N-SH, LA-N-5 etc.<sup>9-11</sup> We chose SH-SY5Y cells and treated them for up to 9 days by RA alone and in combination



**Figure 1. A: Growth curve of SH-SY5Y cells treated with ATRA and PARP inhibitor.**

Cells were treated every second day with 10  $\mu$ M ATRA and 20  $\mu$ M PJ-34 and their combination. Cell proliferation was determined by crystal violet staining and absorbance measurement.

**B: Morphology of SH-SY5Y cells after 7 days of treatment with ATRA and PARP inhibitor.**

Microphotographs were taken with the epifluorescent microscope Axiovert 40 CFL (Zeiss). a: control; b: cells treated with 20  $\mu$ M PJ-34; c: cells treated with 10  $\mu$ M ATRA; d: cells treated with 10  $\mu$ M ATRA and 20  $\mu$ M PJ-34.

**C: Neurite length in SH-SY5Y cells after treatment with ATRA and PARP inhibitor.**

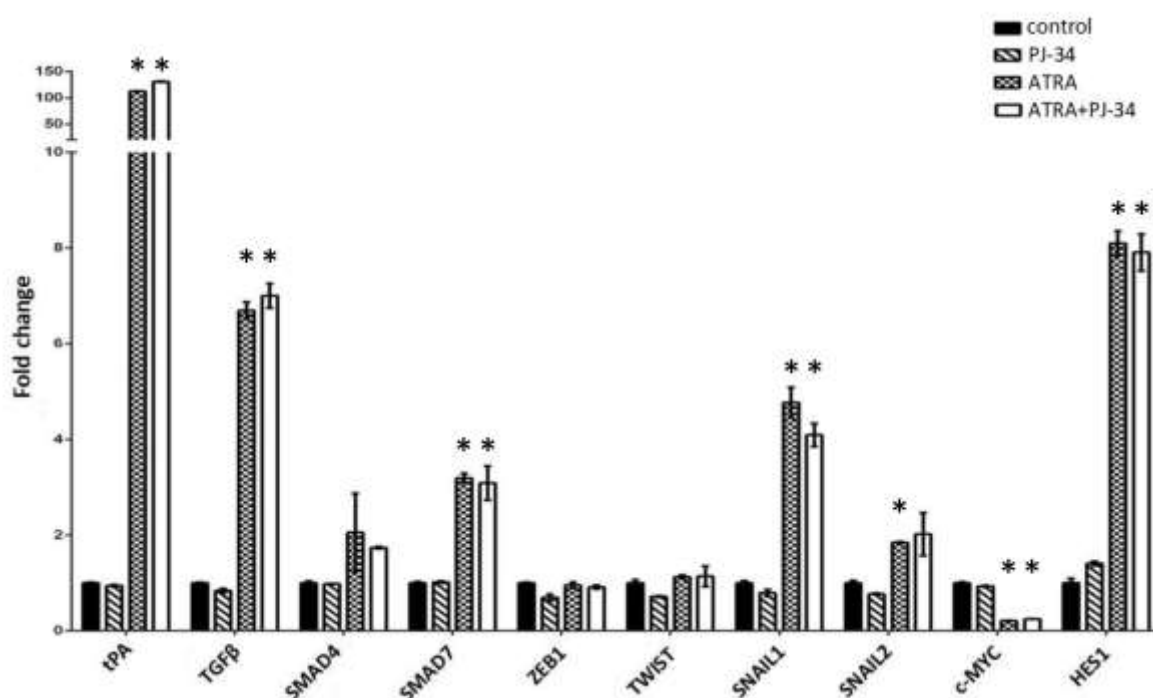
Neurite length was analysed on microphotographs by ImageJ program, after one (a) and seven days (b). \* the mean values were significantly different from control according to Student's t-test ( $p \leq 0.05$ ). Pictures were taken at magnification 100 $\times$ , scale bar = 100  $\mu$ m.

with the PARP inhibitor PJ-34, in order to see whether inhibition of PARP1 could possibly influence chromatin structure and the process of differentiation. To analyze the proliferation of SH-SY5Y cells, the cells were monitored for 6 days while being treated with 10  $\mu$ M ATRA and 20  $\mu$ M PJ-34 PARP inhibitor, alone or in combination every other day. Cell proliferation was assessed by crystal violet staining and absorption measurement. Results (Fig. 1) showed that cells treated with ATRA alone or in combination with the PARP inhibitor stopped proliferating soon after the beginning of the treatment, and their number remained the same throughout the experiment. After 6 days, growth inhibition was around 90% in comparison to untreated cells. Cells treated with the PARP inhibitor alone did not show significant proliferation inhibition (Fig. 1A), nor did PARP inhibition influence the cell arrest observed in RA-treated cells.

Even 24 h after the beginning of the treatment with ATRA, SH-SY5Y cells showed an increase in neurite length as a sign of differentiation. Fig. 1B shows that cell morphology changed on the 7th day of treatment when phenotype differences reached the maximal level. ATRA-treated cells had longer neurites in comparison with untreated cells, and the PARP inhibitor did not influence this process significantly, nor did it prolong the neurites when applied alone (Fig. 1C).

### The effect of ATRA and PARP inhibition on gene expression in SH-SY5Y neuroblastoma cells

Effects of ATRA and PARP inhibitor on SH-SY5Y cells were also explored on the level of mRNA expression after 9 days of treatment (Fig. 2). The most prominent change was the upregulation of a molecule characteristic for differentiated cells, the tissue plasminogen activator (*tPA*).<sup>12</sup> In the cells treated with ATRA, its expression was increased hundredfold and the PARP inhibitor did not influence this induction. Analysis of *c-MYC* expression, as it illustrates the cell's growth and differentiation potential, showed *c-MYC* downregulation by 70% in differentiated cells. A set of genes involved in signaling pathways of differentiation programs was also investigated, such as *TGF $\beta$*  and downstream targets of its signaling. *TGF $\beta$*  was increased seven times in differentiated cells, as well as *SMAD7*, involved in the negative feedback loop and *TGF $\beta$*  downstream target. A downstream target of Notch signaling, involved in neural differentiation, *HES1*, was also upregulated. The third group of genes examined were master transcription factors involved in the epithelial-mesenchymal transition: *TWIST*, *SNAIL1*, *SNAIL2* and *ZEB1*. *SNAIL1* and *SNAIL2* were upregulated in cells differentiated by ATRA.<sup>13</sup>



**Figure 2.** qRT-PCR analysis of expression of genes involved in the differentiation process in SH-SY5Y cells, after 9 days of ATRA and PJ-34 treatment.

Cells were treated with 10  $\mu$ M ATRA and 20  $\mu$ M PJ-34 and their combination every second day. After 9 days, RNA was isolated and cDNA prepared. Relative expression level was normalized with hypoxanthine-guanine phosphoribosyltransferase (*HPRT*) and presented relative to untreated control cell values. Data are expressed as mean  $\pm$  SD. control: untreated control cells; PJ-34: cells treated with PARP inhibitor; ATRA: cells treated with ATRA; PJ-34/ATRA: cells treated with ATRA and PARP inhibitor. tPA: tissue plasminogen activator \* the mean values were significantly different from control according to Student's t-test ( $p \leq 0.05$ ).

## DISCUSSION

Differentiation is an extremely complex process of sequential cellular reprogramming, leading to a cell specialized for performing specific activities, but with low proliferation capacity. Tumor cells, on the other hand, go through the process of dedifferentiation or retain features of undifferentiated cells with high proliferation capacity. Consequently, one of the aims of antitumor therapy is the induction of differentiation. Considering that ATRA is often used as a differentiation agent,<sup>4</sup> we investigated its effects on a neuroblastoma cell line, alone and in combination with the PARP inhibitor, treating cells over a prolonged time period.

Our results showed that ATRA induced differentiation in neuroblastoma SH-SY5Y cells, as was shown previously by other authors.<sup>11, 14</sup> Differentiation by ATRA resulted in inhibition of proliferation and changes in cell morphology. Analysis of gene expression revealed high expression of tissue plasminogen activator, which is characteristic for differentiated cells.<sup>12</sup> Although the primary function of tPA is considered to be plasminogen activation and ECM degradation through plasmin proteolysis, it was found to act as a neuromodulator in neurons and glial cells. It can regulate synaptic plasticity and neuronal survival and influence the release of different growth factors from the ECM, such as guidance molecules F-spondin and VEGF.<sup>15-17</sup> It was also shown that tPA regulated neurite outgrowth in neural progenitor cells through wnt- $\beta$  catenin signaling, and therefore could have an active role in cell differentiation.<sup>18</sup>

We also detected a decrease in *c-MYC* expression that correlated with growth inhibition. Both of these genes could be found in a list of genes upregulated or downregulated by ATRA respectively, in SH-SY5Y cells, obtained by microarray analysis done by Korecka et al.<sup>14</sup> In differentiated cells, an increase in *TGF $\beta$* , as well as *SMAD4* and negative feedback element *SMAD7* expression was detected. *TGF $\beta$*  pathway could participate in a differentiation process, as was recently reported by Duffy et al.<sup>19</sup> PARP inhibition did not influence *TGF $\beta$*  expression in these cells, although PARP was found to modulate Smad transcription ability.<sup>20, 21</sup> We detected high induction of *HES1* which could mirror the activity of the Notch pathway. Notch signaling was found to be involved in embryonic stem cell differentiation toward neural cells,<sup>22, 23</sup> and microarray analysis of SH-SY5Y cells showed an increase in *HES1* and *NOTCH2*.<sup>14</sup> We also detected an increase in *SNAIL1* and *SNAIL2* expression, while *TWIST* and *ZEB1* remained on the control level. Microarray data confirmed the increase in these two genes after ATRA treatment.<sup>14</sup> Ferarri-Toninelli et al.<sup>24</sup> observed increased migration in SH-SY5Y induced by ATRA, which could be a consequence of *SNAIL1* and *SNAIL2* mRNA increase. Joshi et al.<sup>25</sup> found the increase in activity of small Rho GTPases and MMP9, as well as increased invasiveness in SH-SY5Y cells

after 24 h-treatment with ATRA. Transcriptional profiling of ATRA-treated SH-SY5Y cells led to the conclusion that cells enter a pro-differentiation transcriptional state and develop a predominantly mature dopaminergic-like neurotransmitter phenotype.<sup>14</sup>

PARP inhibition did not influence the process of RA-dependent differentiation of SH-SY5Y cells, as could be concluded from the growth curve and expression analysis. PARP influence possibly depends on the intracellular milieu and has a dominant role at certain time points during differentiation. We also cannot exclude its interference with some signaling pathways which were not analyzed and which did not affect the overall cell fate but participate in the fine-tuning of gene expression. Joshi et al.<sup>25</sup> found PARP1 to influence the process of embryo implantation under the control of steroid hormone oestradiol in mice. It was also found to influence the ability of proliferation and self-renewal in neural stem/progenitor cells through p53 pathway modulation.<sup>26</sup> PARP1 was also found to regulate the expression of pluripotency stem cell factors, fine-tune transcription and preserve the capacity for developmental plasticity of embryonal stem cells.<sup>27-30</sup> In neuroblastoma, it was found that their differentiation status depended on PARP1 cooperation with the protein PHF20 which led to increased stemness and aggressiveness.<sup>31</sup> Considering the modification of transcription factors, PARP1 was found to interfere with the *TGF $\beta$*  pathway, influencing the duration of Smad signaling.<sup>20</sup> In SH-SY5Y cells we could not detect such processes, indicating a cell-specific set of signaling pathways influenced by PARP1.

The resistance of RA-induced differentiation on PARP inhibition could also be analyzed in the context of tumor therapy. Namely, some PARP inhibitors, such as olaparib, are used for targeted therapy of tumors with defects in certain DNA damage repair mechanisms.<sup>32</sup> Therefore, it is important to understand the biology of PARP in the process of cell differentiation in different tissues and cell types.

To conclude, in SH-SY5Y neuroblastoma cells RA can trigger the process of differentiation characterized by extensive changes in cell proliferation, morphology and gene expression. This process includes numerous signaling pathways which mutually cooperate and sequentially further induce each other. PARP inhibition did not have an influence on these processes in SH-SY5Y cells.

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