



### Radiation effects on early phase of NT2/D1 neural differentiation in vitro

Journal:	<i>International Journal of Radiation Biology</i>
Manuscript ID	TRAB-2019-IJRB-0083.R1
Manuscript Type:	Original Manuscript
Date Submitted by the Author:	24-Jul-2019
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Keywords:	NT2/D1 cell line, Radiation, neural differentiation, miRNAs, markers of pluripotency

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**Radiation effects on early phase of NT2/D1 neural differentiation *in vitro***

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## Abstract

Purpose: Widespread medical use of radiation in diagnostic, imaging and treatment of different central nervous system malignancies lead to various consequences. Aim of this study was to further elucidate mechanism of cell response to radiation and possible consequence on neural differentiation.

Materials and methods: NT2/D1 cells that resemble neural progenitors were used as a model system. Undifferentiated NT2/D1 cells and NT2/D1 cells in the early phase of neural differentiation were irradiated with low (0.2 Gy) and moderate (2 Gy) doses of  $\gamma$  radiation. The effect was analyzed on apoptosis, cell cycle, senescence, spheroid formation and the expression of genes and miRNAs involved in the regulation of pluripotency or neural differentiation.

Results: 2 Gy of irradiation induced apoptosis, senescence and cell cycle arrest of NT2/D1 cells, accompanied with altered expression of several genes (*SOX2*, *OCT4*, *SOX3*, *PAX6*) and miRNAs (miR-219, miR-21, miR124-a). Presented results show that 2 Gy of radiation significantly affected early phase of neural differentiation *in vitro*.

Conclusions: These results suggest that 2 Gy of radiation significantly affected early phase of neural differentiation and affect the population of neural progenitors. These findings might help in better understanding of side effects of radiotherapy in treatments of central nervous system malignancies.

**Keywords:** NT2/D1 cell line, radiation, neural differentiation, miRNAs, markers of pluripotency

**Biographical note**

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12 **Academician Milena Stevanovic** is full member of Serbian Academy of Sciences and Arts,  
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14 full Professor at Faculty of Biology, University of Belgrade, Head of Human Molecular  
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16 Genetics Laboratory, Institute of Molecular Genetics and Genetic Engineering, University of  
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18 Belgrade. Research interest: human molecular genetics, maintaining pluripotency and neural  
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20 differentiation of stem cells; cytotoxic, antioxidant and anticancer properties of natural and  
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22 synthetic compounds, molecular phylogenetic.  
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## Introduction

Neural progenitor cells (NPCs) comprise undifferentiated population of cells that can differentiate to mature neuronal and glial cells (McKay, 1997; Gage et al., 1998; Temple and Alvarez-Buylla, 1999)). Those cells persist in adult brain of hippocampal dentate gyrus as well as in the sub-ventricular zone (Eriksson et al., 1998; Baptista and Andrade, 2018), where they participate in recovering of neurons after brain injury (Bellenchi et al., 2013). Since NPCs are highly proliferative cells they are radio-sensitive and number of studies documented the side effects of radiation therapy on central nervous system (Kovalchuk and Kolb, 2017). It has been reported that the loss of NPC is caused by DNA damage induced after radiation on rodent brain (Eom et al., 2016; Hladik and Tapio, 2016). Besides, very low dose of radiation also induces DNA double strand breaks and apoptosis in the ventricular and sub-ventricular zones on embryonic day 5.5 to 6.5 in mice (Saha et al., 2014). Just within a few hours of mice exposure to a low dose of radiation some impairment of central nervous system (CNS) that characterized neurodegenerative diseases are observed (Lowe et al., 2009). On the other hand, some studies have reported that radiation have no significant effects in neuronal differentiation of NPC isolated from newborn mouse (Chen et al., 2012) and that NPCs obtained from mouse embryonic stem cells can proliferate and differentiate after exposure to radiation of 1 Gy (Isono et al., 2012). Although radiation can led to delayed effects that are usually associated with neurodegeneration and cognitive decline, there is increasing and widespread medicinal use of radiation in diagnosis, imaging and treatment of different CNS malignancies. It is already hypothesized that radiation can induce the alteration of neuronal differentiation which can cause cognitive impairment, but the mechanism of their response to radiation is still unknown.

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3 It was shown that changes in miRNAs expression level have important roles in  
4 cellular response to ionizing radiation (Moertl et al., 2016). These molecules are recognized  
5 as key players in the regulation of almost every step of radiation response as well as  
6 biomarkers of tissue response to radiation (Moertl et al., 2016). Among others, radiation  
7 induce cellular changes such as DNA damage which further leads to altered expression of  
8 miRNAs (Czochor and Glazer, 2014). Since miRNAs are included in regulation of many  
9 stages of response to DNA damage, these molecules are considered important for further  
10 understanding and improvement of radiation induced response (Czochor and Glazer, 2014).  
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22 Besides important role in response to radiation, miRNAs are important for neural  
23 development (Lau and Hudson, 2010). miRNAs are emerging as regulators of processes such  
24 as transition of pluripotent cells to neural cells, maintenance and self-renewal of neural  
25 progenitor cells and sub-specification of various neural cell types (Stappert et al., 2015). The  
26 level of expression of different miRNAs is important for proper neural development, since  
27 they act as master regulators of cell fate determination (Rajman and Schrott, 2017). Thus,  
28 miR-9 and miR-124 are important for neuronal differentiation and maintaining neuronal  
29 characteristics, miR-21 is involved in gliogenesis, while miR-219 is important for  
30 oligodendrocyte specification (Dugas et al., 2010; Bhalala et al., 2012; Xue et al., 2016; Zhou  
31 et al., 2018) .  
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45 In this study as *in vitro* model system we have used well characterized human  
46 embryonal carcinoma cell line NT2/D1 (Andrews, 1984; Guida et al., 2005; Sanchez et al.,  
47 2009). Phenotype of NT2/D1 cells resembles neuronal progenitor cells (Pleasure and Lee,  
48 1993). In undifferentiated state, NT2/D1 retains some stem cell characteristics (Pleasure and  
49 Lee, 1993), like expression of pluripotency markers OCT4, SOX2 and Nanog (Mojsin et al.,  
50 2014), as well as proliferative capacity. Also, NT2/D1 cells express Nestin and Vimentin,  
51 known to be expressed in neuroepithelial precursor cells (Pleasure and Lee, 1993) and  
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3 members of SOXB1 subgroup that are expressed in neural stem and progenitor cells (Aubert  
4 et al., 2003; Bani-Yaghoub et al., 2006; Wang et al., 2006; Popovic et al., 2014). Early phase  
5 of NT2/D1 neural differentiation is accompanied by up-regulation of *SOX3* gene expression,  
6 one of the earliest neural markers (Stevanovic M 2003 127). High level of PAX6 expression  
7 is important for maintenance of NT2/D1 neural progenitor's population (Coyle D 2011 Plos  
8 one). Differentiation of NT2/D1 cells yields neuronal and glial populations (Coyle et al.,  
9 2011). Neurons obtained following 4 weeks of retinoic acid induction of NT2/D1 cells  
10 represent fully differentiated cells which express neurofilaments, form synapses and release  
11 neurotransmitters (Coyle et al., 2011). Having in mind their molecular characteristics which  
12 resemble neural precursor cells and capacity to differentiate into mature neurons, NT2/D1  
13 cell line represents a good human model system for studying different aspects of the process  
14 of neural differentiation. Thus, this cell line is previously used as a valuable model system for  
15 studying molecular mechanisms of human neural differentiation (Przyborski et al., 2000).

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33 In this study we have analyzed the effects of low (0.2 Gy) and moderate (2 Gy) doses  
34 of  $\gamma$  radiation on undifferentiated NT2/D1 cells and NT2/D1 cells in the early phase of neural  
35 differentiation induced by RA. We have analyzed the effects on cellular processes like  
36 apoptosis, senescence and cell cycle distribution, spheroid formation, as well as the effects on  
37 expression of selected genes and miRNAs involved in regulation of pluripotency and neural  
38 differentiation.

## 39 40 41 42 43 44 45 46 47 48 49 **Material and methods**

### 50 51 *Cell culture and differentiation*

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53 Human NT2/D1 EC stem cells (ATCC® CRL-1973™) were maintained in Dulbecco's  
54 Modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 4500  
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3 mg/L glucose, 2 mmol/L L-glutamine and penicillin/streptomycin (all from Invitrogen™,  
4 NY, USA), at 37°C in 10% CO<sub>2</sub> as previously described (Andrews, 1984).  
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8 Cells were induced to differentiate in culture by addition of 10 µmol/L all-trans retinoic acid  
9 (RA; Sigma-Aldrich, MO, USA) for 1 week. All analyses were performed 8 days after RA  
10 induction (designed as NT2/D1 1W on Figures).  
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14 Cells were monitored and the pictures were taken using microscope (Axiovert, Zeiss,  
15 Germany). Cell density was evaluated using a hemocytometer Z1 Coulter Counter (Beckman  
16 Counter, USA).  
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### 21 ***Irradiation of the cells***

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23 2x10<sup>5</sup> cells were plated in Ti25 flasks. For differentiation, cells were treated with 10 µmol/L  
24 all-trans retinoic acid as previously described (Popovic et al., 2014). Next day cells were  
25 irradiated with a single 0.2 Gy or 2 Gy dose using a Cs<sub>137</sub> irradiator (HWM D-2000,  
26 Wälischmiller, Germany) at a dose rate of 0.95 Gy/min. Doses of 0.2 Gy or 2Gy were  
27 administered at room temperature and control cells were sham irradiated. The exposed and  
28 sham irradiated cells were subsequently incubated at 37°C and harvested after indicated time  
29 points for RNA and protein isolation and for other analysis. The experiment was repeated for  
30 each dose in triplicates (n=3). All analyses were performed 7 days after radiation.  
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### 42 ***RNA isolation***

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44 For isolation of total RNA cells were pelleted by centrifugation at 1,500 rpm for 5 min and  
45 washed with phosphate-buffered saline (PBS). Total RNA, containing small RNAs (<200  
46 nucleotides), was isolated from the cells using the Maxwell®16 miRNA Tissue kit (Promega)  
47 following the protocol provided by the manufacturer. The quantity and quality of the total  
48 RNA was measured with the Nanodrop spectrophotometer (PiqLab Biotechnology,  
49 Germany).  
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### 58 ***TaqMan-miRNA assays and data analysis***

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3 Total RNA from at least three biological replicates was used for specific single TaqMan–  
4 miRNA assay analysis (miR-21, miR-124a, miR-219-5p and miR-941, Applied Biosystems,  
5 Forster City, CA, USA). Reverse transcriptase reactions were performed using  
6 MultiScribe™ reverse transcriptase (Applied Biosystems; Foster City, CA, USA) according  
7 to the manufacturer’s instructions as previously described (Anastasov et al., 2012; Falkenberg  
8 et al., 2013). Quantitative PCR was performed using TaqMan Universal PCR Master Mix on  
9 a StepOne Sequence Detection System (Applied Biosystems, Foster City, CA), according to  
10 the manufacturer’s instructions, as previously described (Kramer, 2011; Anastasov et al.,  
11 2012). The relative expression value of a specific miRNA was calculated by using the  
12  $2^{-\Delta\Delta CT}$  method (Anastasov et al., 2012), normalized to control miRNA (RNU44) and sham  
13 irradiated sample. All experiments were done from three biological replicates with at least  
14 two technical replicates.

### 30 ***Cell cycle analysis***

31  
32 Seven days after radiation, the cells were trypsinized and collected by centrifugation at 300 g  
33 for 5 min, and the supernatant was carefully removed. The cell pellet was gently resuspended  
34 in 500  $\mu$ l of a solution containing 10 mM NaCl, 4 mM Na-citrate, 10  $\mu$ g/ml RNase, 0.3%  
35 Nonidet P-40, and 50  $\mu$ g/ml propidiumiodide (PI). The cell suspensions were incubated for  
36 60 min at room temperature followed by the addition of 500  $\mu$ l of solution containing 70 mM  
37 citric acid, 250 mM sucrose and 50  $\mu$ g/ml PI. The cell suspensions were mixed and stored at  
38 4°C before flow cytometry. Cell cycle distributions were analyzed on a FACScan LSR II  
39 (Becton- Dickinson) (excitation wavelength: 488 nm; emission wavelength: 610 nm, LSR II,  
40 Becton Dickinson/FACS DIVA Software).

### 53 ***Western blot***

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55 Whole cell lysates were isolated as previously described (Popovic et al., 2014). Proteins were  
56 quantified and separated by SDS-PAGE as previously described (Popovic et al., 2014). After  
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3 blocking with 5% non-fat milk at +4°C for 24 h, membranes were incubated for 1 h at RT  
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5 with the following primary antibodies: mouse monoclonal antibody against p53 (DO-1)  
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7 (Santa Cruz Biotechnology, Texas, USA, sc-126X, diluted 1:1000), rabbit monoclonal  
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9 antibody against p21<sup>Waf1/Cip1</sup> (Cell Signaling, Technology, Danvers, MA, USA, 2947, diluted  
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11 1:1000), mouse monoclonal antibody against  $\alpha$  - tubulin (Calbiochem, MA, USA, CP06,  
12  
13 diluted 1:30000). Afterwards, the membranes were incubated for 1 h at RT with the  
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15 following secondary antibodies: horseradish peroxidase-conjugated anti-mouse and anti-  
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17 rabbit IgG (Amersham Biosciences, NJ, USA, diluted 1:10000). Immunoreactive bands were  
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19 detected by chemiluminescence (Immobilion substrate, Millipore, MA, USA).  
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### 24 ***Senescence Analysis***

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26 For assessment of senescence associated  $\beta$ -galactosidase activity 7 days after irradiation, cells  
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28 were washed with PBS, fixed with 4% paraformaldehyde, again washed with PBS, and  
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30 stained for 12 h at 37°C with an X-gal buffer solution (40 mM Na<sub>2</sub>HPO<sub>4</sub>, 150 mM NaCl,  
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32 2 mM MgCl<sub>2</sub>, 5 mM K<sub>3</sub>Fe(CN)<sub>6</sub>, 5 mM K<sub>4</sub>Fe(CN)<sub>6</sub>·3H<sub>2</sub>O, and 1 mg/mL X-gal, pH 6).  
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34 Multicolored microscope images were taken with an Axiovert (Zeiss, Germany) microscope.  
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### 37 ***qRT-PCR***

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39 For quantitative PCR analysis, cDNAs were subjected to real time PCR using Power SYBR  
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41 Green PCR Master Mix (Applied Biosystems®) in 7500 Real Time PCR Systems (Applied  
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43 Biosystems®) as previously described (Popovic et al., 2014). *BAX* was amplified using  
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45 primers 5'-TGGCAGCTGACATGTTTTCTGAC-3' (forward) and 5'-  
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47 TCACCCAACCACCCTGGTCTT-3' (reverse), while for *Bcl2* amplification we used 5'-  
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49 TCGCCCTGTGGATGACTGA-3' (forward) and 5'- CAGAGACAGCCAGGAGAAATC-3'  
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51 (reverse) primers. *SOX3* was amplified using following primer sets 5' -  
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53 GACCTGTTCGAGAGAACTCATCA-3' (forward), 5'-  
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3 CGGGAAGGGTAGGCTTATCAA-3' (reverse). *PAX6* was amplified using following  
4 primer sets 5'-CATATTCGAGCCCCGTGGAA-3' (forward) and 5'-  
5 CCGTTGGACACCTGCAGAAT-3' (reverse). *SOX2* was amplified using following primer  
6 sets 5'-CCCCTGGCATGGCTCTTGGC-3' (forward) and 5'-  
7 TCGGCGCCGGGAGATACAT-3' (reverse). *OCT4* was amplified using following primer  
8 sets 5'-TCTCCAGGTTGCCTTCACT-3' (forward) and 5'-  
9 GCTTTGAGGCTCTGCAGCTT-3' (reverse). *GAPDH* was amplified with 5'-  
10 GGACCTGACCTGCCGTCTAG-3' (forward) and 5'-CCACCACCCTGTTGCTGTAG-3'  
11 (reverse) to control for equivalent amounts of cDNA per reaction. All samples were  
12 measured in triplicate and the mean value was considered. The relative level of *BAX*, *Bcl2*,  
13 *SOX3*, *PAX6*, *SOX2* and *OCT4* expression was determined using a comparative quantification  
14 algorithm where the resulting  $\Delta\Delta C_t$  value was incorporated to determine the fold difference  
15 in expression ( $2^{-\Delta\Delta C_t}$ ). Relative *BAX*, *Bcl-2*, *SOX3*, *PAX6*, *SOX2* and *OCT4* mRNA level  
16 were presented as a percentage of mRNA expression in differentiated NT2/D1 cells sham  
17 irradiated.

### 3D spheroid analysis

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40 Seven days upon radiation with 0.2 Gy and 2 Gy, undifferentiated NT2/D1 and NT2/D1 W1  
41 cells were seeded in 3D hanging drop culture plates (96-well). Cell density was evaluated  
42 using a hemocytometer and 2.000 cells were seeded per well in (96-well) GravityTRAP ULA  
43 Plates (InSphero/Perkin Elmer, USA). The experiment was repeated for each dose in  
44 quadruplicates (n=4). Three days after the seeding the cells formed the 3D-microtissues  
45 (spheroids). They were stained using Live/Dead Cell staining reagents as Hoechst (Thermo  
46 Fisher, USA) for live (blue) cell fraction and Draq7 (Cell Signalling, USA) for dead (red) cell  
47 fraction and analyzed using the Operetta® High Content Imaging System (Perkin Elemer,  
48 USA). Live cells were stained with blue color while red color was specific for dead cells.  
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3 Draq7 is far red fluorescing marker of cell viability. It only labels cells with compromised  
4 plasma membranes indicative of cell death by primary and secondary necrosis. Additionally,  
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6 Hoechst is blue fluorescing marker of living cell fraction. It is only possible to observe the  
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8 cells on outside parts of spheroid structure and two different Z-stack layers were used for  
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10 analysis.  
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## 17 **Results**

### 18 *The effect of radiation on total cell number of undifferentiated NT2/D1 and NT2/D1 cells* 19 20 *in early phase of differentiation* 21 22 23

24 In order to test how populations of undifferentiated NT2/D1 and NT2/D1 cells in  
25 early phase of differentiation (NT2/D1 1W) respond to 0.2 Gy and 2 Gy doses of radiation,  
26 first we analyzed morphology and the total cell number of irradiated cells. Seven days after  
27 irradiation, cells were inspected under the microscope, then trypsinised and subjected to cell  
28 counting and measuring of cell density. As expected, after RA induction NT2/D1 cells  
29 acquired different morphology compared to undifferentiated NT2/D1 cells, indicating that  
30 NT2/D1 cells initiate neural differentiation (Figure 1A: compare panels Ia and IId). Obtained  
31 results showed that 0.2 Gy of radiation did not significantly affect the morphology (Figure  
32 1A: Panel I-compare a with b; Panel II-compare d with e) or cell number (Figure 1B) of both  
33 undifferentiated NT2/D1 and NT2/D1 1W cells. In contrast 2 Gy of irradiation decreased cell  
34 number by approximately 50% in both undifferentiated NT2/D1 cells and NT2/D1 cells in  
35 early phase of differentiation (Figure 1A: Panel I-compare a with c, Panel II-compare d with  
36 f, Figure 1B). Presented results showed that NT2/D1 cells were more sensitive to 2 Gy dose,  
37 compared to lower dose (0.2 Gy) and that 2 Gy irradiation was able to decrease number of  
38 both undifferentiated NT2/D1 cells and cells in early phase of differentiation (Figure 1A and  
39 1B).  
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8 ***The effect of radiation on apoptosis and senescence of undifferentiated NT2/D1 and***  
9 ***NT2/D1 cells in early phase of differentiation***  
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12 Next, we analyzed whether applied doses of radiation induce molecular mechanism of  
13 apoptosis in undifferentiated NT2/D1 and NT2/D1 cells in early phase of differentiation. We  
14 analyzed the initiation of the process of apoptosis by evaluation expression of apoptotic  
15 markers (p53, *BAX* and *Bcl-2*). It is known that p53 protein work as a transcription factor that  
16 regulates apoptosis, mostly through altering the ratio of pro (*BAX*) and anti-apoptotic (*Bcl2*)  
17 genes (Kastan et al., 1995; Sionov and Haupt, 1999; Green and Evan, 2002). Tumor  
18 suppressor p53 leads to changes in *BAX/Bcl-2* ratio that further activates complex network of  
19 caspases, leading cells into the process of apoptosis (Rudel, 1999).  
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31 In undifferentiated NT2/D1 cells lower dose of radiation (0.2 Gy) had no effect on  
32 p53 and *Bcl2* gene expression (Figure 1C and 1F) but decreased *BAX* expression by  
33 approximately 50% (Figure 1D). Dose of 2 Gy increased p53 expression significantly (Figure  
34 1C), decreased the expression of *BAX* also by approximately 50 % (Figure 1D) and had no  
35 effect on *Bcl2* expression in undifferentiated NT2/D1 cells (Figure 1F). In differentiated  
36 NT2/D1 1W cells 0.2 Gy dose had no significant effect on *BAX* expression (Figure 1C and  
37 1E), while this dose of radiation decreased p53 expression (Figure 1C) and *Bcl2* gene  
38 expression, compared to sham irradiated differentiated NT2/D1 cells, by approximately 20%  
39 (Figure 1G). In differentiated NT2/D1 1W cells, 2 Gy of radiation also decreased expression  
40 of p53 protein (Figure 1C) and *Bcl2* gene (by approximately 30%, Figure 1G), while it  
41 increased the expression of *BAX* gene by approximately 30% (compared to sham irradiated  
42 differentiated NT2/D1 1W cells, Figure 1E). Although the level of *BAX* and *Bcl2* genes  
43 expression was differently altered in undifferentiated and differentiated NT2/D1 cells upon  
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3 exposure to radiation, the *BAX/Bcl2* genes ratio was disrupted by radiation in both groups of  
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5 cells (Figure 1D-G).  
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8 When comparing sham radiated differentiated with undifferentiated NT2/D1 cells  
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10 there is an increase in p53 level, indicating that differentiated NT2/D1 1W cells are more  
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12 prone to apoptosis (Figure 1C). Although there is difference between the levels of *BAX* and  
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14 *Bcl2* expression upon radiation (difference between undifferentiated and differentiated  
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16 NT2/D1 cells, Figure 1D-G), the radiation additionally altered *BAX/Bcl-2* ratio. These results  
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18 indicated that both undifferentiated and differentiated NT2/D1 cells enter the process of  
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20 apoptosis.  
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23  
24 Besides apoptosis, it is known that exposure to ionizing radiation induces cell's  
25  
26 senescence (Wang et al., 2016). We analyzed senescence-associated  $\beta$ -galactosidase activity  
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28 in undifferentiated NT2/D1 cells and NT2/D1 cells in early phase of differentiation upon  
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30 exposure to 0.2 Gy and 2 Gy of radiation. No staining was detected in sham irradiated  
31  
32 undifferentiated and differentiated NT2/D1 cells (Figure 2, a and d). Our results showed that  
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34 0.2 Gy of radiation had no effect on senescence induction in NT2/D1 I NT2/D1 1W cells  
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36 (Figure 2-compare a with b and d with e). On the other hand, 2 Gy of radiation induced  
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38 senescence in both undifferentiated NT2/D1 (Figure 2-compare a and c) and NT2/D1 1W  
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40 cells in early phase of differentiation (Figure 2-compare d and f). It could be concluded that 2  
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42 Gy dose of radiation leads both undifferentiated and differentiated NT2/D1 cells to the  
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44 process of senescence.  
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49 Taken together, our results indicated that dose of 2 Gy induced molecular mechanism  
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51 of apoptosis as well as senescence in both undifferentiated and NT2/D1 cells in early phase  
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53 of differentiation.  
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56 **Insert Figure 2 (somewhere near)**  
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6 ***The effect of radiation on cell cycle distribution of undifferentiated NT2/D1 and NT2/D1***  
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8 ***cells in early phase of differentiation***  
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10           Beside apoptosis and senescence, radiation can alter cell cycle distribution. In order to  
11 analyze the effect of radiation on cell cycle, undifferentiated NT2/D1 and NT2/D1 1W cells  
12 seven days after radiation (0.2 Gy and 2 Gy), were subjected to cell cycle distribution  
13 analysis (Figure 3).  
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19           Flow cytometry data analysis show that both undifferentiated NT2/D1 cells and  
20 differentiated NT2/D1 1W cells were more sensitive to 2 Gy of radiation compared to  
21 sensitivity to lower dose of 0.2 Gy radiation (Figure 3A). Results also indicate that, after 2  
22 Gy of radiation, both undifferentiated NT2/D1 cells and differentiated NT2/D1 1W cells have  
23 increased number of cells in sub-G1 phase, while number of cells in G1 and S phase is  
24 reduced (Figure 3A). Cells accumulated in sub-G1 phase represent apoptotic cells with  
25 fractional DNA content which underwent activation-induced apoptosis (Kajstura et al., 2007).  
26 Also, we observed overall increased cell number in G0/G1 phase of NT2/D1 1W,  
27 accompanied with smaller number of cells in S phase compared to undifferentiated NT2/D1  
28 cells (Figure 3A). Thus, NT2/D1 1W cells displayed somewhat altered cells distribution  
29 compared to undifferentiated NT2/D1 cells.  
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45           It is well established that p21<sup>Waf1/Cip</sup> is one of the key factors involved in regulation of  
46 cell cycle distribution and therefore we further analyzed the expression of p21<sup>Waf1/Cip</sup> in  
47 irradiated NT2/D1 and NT2/D1 1W cells. Western blot analysis showed that 2 Gy dose of  
48 radiation increased the level of p21<sup>Waf1/Cip</sup> expression only in NT2/D1 1W cells (Figure 3B).  
49 On the other hand, 0.2 Gy radiation didn't alter the level of p21<sup>Waf1/Cip</sup> expression in both  
50 NT2/D1 and NT2/D1 1W cells (Figure 3B).  
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3 Obtained results confirmed that moderate- dose radiation (2 Gy) induces cell cycle  
4 arrest and stimulates the expression of key regulator of this process- p21<sup>Waf1/Cip</sup>.  
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8 **Insert Figure 3 (somewhere near)**  
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12 ***The effect of radiation on 3D spheroids formation by undifferentiated NT2/D1 and***  
13 ***NT2/D1 cells in early phase of differentiation***  
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17 Since 3D spheroids are shown as advanced *in vitro* neural model which more closely  
18 resemble *in vivo* tissue (Dingle et al., 2015), we used this model in order to further evaluate  
19 radiosensitivity of NT2/D1 cells. Obtained results showed that both NT2/D1 and NT2/D1 1W  
20 cells formed 3D spheroids within a 24 h (Figure 4). The results also indicated a lower growth  
21 efficiency of NT2/D1 1W spheroids compared to spheroids of undifferentiated NT2/D1 cells  
22 (Figure 4). Radiation increased the number of dead cells (red cells in Figure 4) within  
23 spheroids formation of NT2/D1 1W cells. This effect was more prominent with 2 Gy of  
24 irradiation compared to the effect of 0.2 Gy (Figure 4).  
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35 **Insert Figure 4 (somewhere near)**  
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40 ***The effect of irradiation on expression of selected neural markers in undifferentiated***  
41 ***NT2/D1 cells and NT2/D1 in early phase of differentiation***  
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45 In order to analyze the effect of low and moderate- dose radiation on neural  
46 differentiation of NT2/D1 cells we evaluated the expression of selected neural markers upon  
47 irradiation of NT2/D1 and NT2/D1 1W cells. Cellular response to ionizing irradiation affects  
48 expression of different genes encoding proteins and miRNAs involved in regulation of many  
49 signaling pathways. Since miRNAs are often used as markers of neural differentiation, tumor  
50 classification or response to radiation, one of our goals was to analyze expression of selected  
51 miRNAs upon radiation exposure (Cellini et al., 2014).  
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3 Neural marker selected for analysis included genes *SOX3* and *PAX6* as markers of  
4 neural progenitor cells (Wang et al., 2006; Zhang and Jiao, 2015) and the following miRNAs:  
5 miR-21 that is expressed in astrocytes and involved in regulation of astrogliosis (Bhalala et  
6 al., 2012; Rao et al., 2016), miR-124, mostly expressed in neurons and important for  
7 differentiation of adult neural progenitor cells (Cheng et al., 2009; Liu et al., 2011) and miR-  
8 219 that can promote differentiation of embryonic stem cells and neural precursors to neural  
9 cells (Zhao et al., 2010; Hudish et al., 2013; Wu et al., 2017).

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11  
12 The expression of *SOX3* gene was significantly decreased in NT2/D1 1W cells after  
13 exposure to 2 Gy of radiation compared to sham irradiated NT2/D1 1W cells (Figure 5B),  
14 while radiation had no effect on the expression of *SOX3* gene in undifferentiated NT2/D1  
15 cells (Figure 5A). Lower dose of radiation didn't affect the expression of *SOX3* in both  
16 undifferentiated NT2/D1 cells and NT2/D1 1W cells (Figure 5 A).

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19 The expression of *PAX6* was decreased upon 2 Gy radiation in both undifferentiated  
20 NT2/D1 cells (Figure 5C) and in differentiated NT2/D1 1W (Figure 5D), while 0.2 Gy of  
21 radiation didn't significantly alter the expression of *PAX6* gene (Figure 5C and 5D).

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24 Analysis of qRT-PCR results showed that 2 Gy radiation increased the expression of  
25 miR-21 by approximately 2.5 fold in undifferentiated NT2/D1 cells (Figure 5E) and by  
26 approximately 3 fold in NT2/D1 1W cells (Figure 5F), while 0.2 Gy of radiation had no  
27 significant effect (Figure 5E and 5F).

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30 The expression of miR-124a was decreased in both NT2/D1 and NT2/D1 1W cells  
31 after exposure to 2 Gy radiation (Figure 5G and 5H). The expression of miR-124a was not  
32 altered in NT2/D1 cells after exposure to 0.2 Gy of radiation (Figure 5G), while this dose of  
33 radiation decreased the expression of miR-124a in NT2/D1 1W cells in early phase of  
34 differentiation (Figure 5H).

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3 The expression of miR-219 was decreased upon radiation in NT2/D1 cells and  
4 NT2/D1 1W cells (Figure 5I and 5J). Dose of 0.2 Gy of radiation decreased miR-219  
5 expression in undifferentiated NT2/D1 cells only (Figure 5I), while 2 Gy of radiation  
6 decreased miR-219 by approximately 60% and 90% in both NT2/D1 and NT2/D1 1W cells  
7 (Figure 1I and J).  
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14 Here we have presented expression profiles of selected markers associated with neural  
15 differentiation upon radiation. Presented data indicated that radiation, particularly 2 Gy dose,  
16 had effect on expression of analyzed neural markers in undifferentiated, as well as, in  
17 differentiated NT2/D1 cells. Radiation decreased expression of the most of analyzed genes,  
18 except of miR-21 which expression was increased upon radiation as characteristic  
19 antiapoptotic miRNA, indicating that radiation might have effect on regulation of neural  
20 differentiation of NT2/D1 cells.  
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30 **Insert Figure 5 (somewhere near)**  
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35 ***Radiation effect on the expression of pluripotency markers in undifferentiated NT2/D1***  
36 ***and NT2/D1 in early phase of differentiation***  
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40 One of the characteristic of NPC is their ability to maintain stemness and self-renewal  
41 capacities (Shi et al., 2008). Sox2 is known to be regulator of stemness identity in neural  
42 progenitor cells (Bani-Yaghoub et al., 2006; Thiel, 2013). Besides SOX2, OCT4 is  
43 considered as one of the key factors important for maintenance of stemness and regulation of  
44 pluripotency (Kellner and Kikyo, 2010; Shi and Jin, 2010). Among miRNAs, miR-941 is  
45 reported to be highly expressed in pluripotent cells (Hu et al., 2012). In order to see how  
46 radiation affect stemness of NT2/D1 cells, our next goal was to analyze the expression of  
47 SOX2, OCT4 and miR-941 in undifferentiated NT2/D1 and NT2/D1 1W cells. Our results  
48 revealed that the expression of SOX2 wasn't affected upon exposure of undifferentiated  
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3 NT2/D1 cells to radiation (Figure 6A), while it was significantly decreased in differentiated  
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5 NT2/D1 1W cells after exposure to 2 Gy of radiation (Figure 6B). Further, results revealed  
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7 that the expression of *OCT4* wasn't affected upon exposure of undifferentiated NT2/D1 cells  
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9 to radiation, while in the NT2/D1 1W cells the expression of *OCT4* was significantly  
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11 decreased upon exposure to 2 Gy of radiation (Figure 6D). On the other hand, the expression  
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13 of miR-941 wasn't affected upon exposure of undifferentiated NT2/D1 or differentiated  
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15 NT2/D1 cells after exposure to radiation (Figure 6E and 6F). These results indicate that 2 Gy  
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17 of radiation could decrease expression of pluripotency markers in cells in early phase of  
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19 neural differentiation, while it had no effect on stemness of undifferentiated pluripotent  
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21 NT2/D1 cells.  
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26 **Insert Figure 6 (somewhere near)**  
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## 30 **Discussion**

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35 In this study we demonstrated that radiation affects cell number, cell cycle, as well as,  
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37 expression of selected genes and miRNAs in both pluripotent NT2/D1 cells and NT2/D1 cells  
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39 in early phase of neural differentiation. As expected, presented results revealed that cells  
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41 were more affected with moderate-dose radiation (2 Gy), compared to low dose of radiation  
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43 (0.2 Gy). Radiation also affects expression of genes and miRNAs involved in the regulation  
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45 of apoptosis, stemness and neural differentiation. Moreover, it influenced cell cycle  
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47 distribution and senescence. The results presented here show that radiation affects cellular  
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49 processes and genes expression even after seven-days of recovery period. In addition, we  
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51 observed some differences in response to radiation between pluripotent undifferentiated  
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53 NT2/D1 cells and NT2/D1 cells in early phases of differentiation.  
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3 Literature data show differences between undifferentiated NT2/D1 cells and NT2/D1  
4 cells upon RA induction in genes expression and growth rate (Baldassarre et al., 2000;  
5 Houldsworth et al., 2002). Results presented here also show that NT2/D1 and NT2/D1 1W  
6 cells display differences in many aspects. They show difference in the expression of p53, cell  
7 cycle distribution and 3D spheroids formation. Previous study shows that radiation was able  
8 to induce rapid induction of apoptosis in NT2/D1 cells (Guida et al., 2005). Results presented  
9 here show that even after seven-day recovery period, cell death after radiation was still  
10 detectable in both undifferentiated NT2/D1 cells and NT2/D1 1W cells Although 2 Gy is  
11 considered as moderate dose of radiation, we have detected approximately 50% reduction in  
12 cells viability after 7 days. It has been reported that prolonged time between irradiation and  
13 detection leads to increase in detection of cell's death (Fazel et al., 2016). This delayed cell  
14 death is probably the consequence of damage accumulation that occurs upon irradiation of  
15 cells. Upon 2 Gy of radiation, the expression of apoptosis related genes was modulated, total  
16 cell number was decreased and cell cycle distribution was altered. The genome keeper p53 is  
17 considered as one of the important regulator of apoptotic response to radiation (Lee et al.,  
18 2013) and indicator of radiation response (Siles et al., 1996). While lower dose (0.2 Gy) was  
19 not able to induce cell death in undifferentiated pluripotent NT2/D1 cells, decrease of cell  
20 number, as well as, the increased p53 protein expression was observed after exposure to 2  
21 Gy, indicating the induction of cell death. In addition, results obtained by Flow cytometry  
22 indicate that 2 Gy radiation altered cell cycle distribution in both undifferentiated and  
23 differentiated NT2/D1 cells with accumulation of cells in sub-G1 phase. This is indicative for  
24 the process of apoptosis since increase in the sub-G1 phase of the cell cycle is associated with  
25 a lower DNA content, suggesting cell death. Over the years, many investigations supported a  
26 correlation between apoptosis and the increase in the sub-G1 phase of the cell cycle  
27 (Ormerod et al., 1992; Lee et al., 2015; Hurtado-Diaz et al., 2019).

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3 Our results show that RA induced p53 expression which was already reported upon  
4 RA induced differentiation of NT2/D1 cells (Curtin et al., 2001). Interestingly, we observed  
5 that upon radiation, the expression of p53 was decreased in NT2/D1 cells induced with RA.  
6 These results could indicate that NT2/D1 cells in early phases of differentiation are not able  
7 to repair DNA damage induced by radiation. Also, the level of p53 expression in NT2/D1  
8 cells in early phases of differentiation could indicate that radiation can suppress retinoic acid  
9 increased expression of p53. Having in mind that retinoic acid can be used in combination  
10 with radiation therapy (Yan et al., 2016), the possible synergistic effects of radiation and  
11 retinoic acid require further analysis. Although the increased expression of p53 was not  
12 observed, there was decrease in total cell number in irradiated NT2/D1 cells in early phase of  
13 neural differentiation. In addition, radiation induced senescence in NT2/D1 cells in early  
14 phase of differentiation might point to a mechanism responsible for loss of cells upon  
15 exposure to radiation. Having in mind that radiation decreased p53 expression in NT2/D1  
16 cells in early phases of differentiation, these results could indicate that radiation induced  
17 senescence in NT2/D1 cells in early phases of differentiation might be p53 independent.  
18 One of the processes that is altered upon radiation is cell cycle distribution, followed by  
19 delayed progression through the G1, S, and G2 phases of the cell cycle (Bernhard et al.,  
20 1995). Results of flow cytometry revealed that 2 Gy radiation was able to alter cell cycle  
21 distribution, with decreased cell number in G1 phase of pluripotent undifferentiated NT2/D1  
22 cells and increased cell number in G1 phase in NT2/D1 cells in early phases of  
23 differentiation. Further, the expression of cyclin-dependent kinase inhibitor p21<sup>Waf1/Cip1</sup>, the  
24 key factor involved in regulation of cell cycle arrest in response to different stimuli (Dutto et  
25 al., 2015), was highly up-regulated in NT2/D1 cells in early phases of differentiation upon 2  
26 Gy dose of radiation. Moreover, the up-regulation persisted even one week upon radiation  
27 treatment (Fig. 3B). Also, the differences in cell cycle response between pluripotent  
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3 undifferentiated NT2/D1 cells and NT2/D1 cells in early phases of differentiation could be a  
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5 consequence of cell cycle arrest in differentiated NT2/D1 cells, since differentiation process  
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7 is accompanied with cell cycle arrest. Recent data shows that p21<sup>Waf1/Cip1</sup> is involved in  
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9 regulation of hippocampal neural progenitors fate whereas loss of this protein increases  
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11 proliferation of NPC (Li and Wong, 2018). Accordingly, it is possible that radiation can  
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13 affect proliferation of NPC through regulation of p21 expression.  
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17 It is well known that RA induction of neural differentiation of NT2/D1 cells was  
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19 accompanied with decrease expression of pluripotency markers and increased expression of  
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21 neural markers. Accordingly, we analyzed the effects of radiation on the expression of  
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23 markers of pluripotency and neural differentiation. The presented results show that 2 Gy of  
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25 radiation led to changes in the expression of several genes and miRNAs that are known to be  
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27 markers of pluripotency or neural differentiation. Although we could not detect changes in  
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29 morphology or cell number upon 0.2 Gy of radiation, administrated dose had partial effect on  
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31 expression of some genes and miRNAs associated with neural differentiation. Katsura et al.  
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33 published a study regarding the effects of low dose radiation on human neural progenitor  
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35 cells and determined that doses below 0.5 Gy affect only expression profile of various genes  
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37 such as one involved in inflammation, DNA repair or cell adhesion, while dose of  
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39 approximately 0.5 Gy had effect on cellular processes including metabolism, apoptosis and  
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41 neural differentiation (Katsura et al., 2016). Our results suggest that response of NT2/D1  
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43 cells depends on radiation dosage. As expected, these cells are more sensitive to 2 Gy  
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45 radiation, compared to sensitivity to lower dose of 0.2 Gy. Expression analysis of miRNAs  
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47 (particularly miR-21, miR-124a and miR219) was dosage dependent. We also observed  
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49 differences in response to radiation between undifferentiated NT2/D1 cells and NT2/D1 cells  
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51 in early phases of differentiation, with more prominent effects upon RA induction. Our main  
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53 observation is that differentiated NT2/D1 cells are more sensitive to radiation. In  
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3 differentiated NT2/D1 1W cells there is a reduction in expression of pluripotency markers  
4 SOX2 and OCT4 upon 2 Gy of radiation. We have previously shown that SOX2 protein is  
5 gradually upregulated during neural differentiation of NT2/D1 cells (Popovic et al., 2014).  
6 One week upon induction it is expressed at higher level and its expression remained  
7 increased compared to untreated NT2/D1 cells. Reduction of its expression during  
8 differentiation, as a consequence of radiation, could be considered as a sign of impaired  
9 differentiation process.  
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19 It is already known that many genes encoding proteins and miRNAs are involved in  
20 regulation of radiation induced cell damage (Metheetraitut and Slack, 2013; Wahba et al.,  
21 2017). It was shown that radiation can induce transcriptional alteration of human NPCs  
22 (Macaeva et al., 2016). The knowledge of the effect of radiation on genes and miRNAs  
23 expression during neural differentiation could be applied in radiotherapy or for monitoring  
24 the impact of radiation exposure on the brain. Approximately one fourth of miRNAs are  
25 significantly up-regulated after DNA damage, which is one of the essential consequences of  
26 radiation (Halimi et al., 2012). In this study the radiation altered the expression of selected  
27 miRNAs and genes involved in regulation of pluripotency or neural differentiation,  
28 depending on whether NT2/D1 cells were undifferentiated or induced to differentiate with  
29 RA. In NT2/D1 1W cells the expression of *SOX3*, *PAX6*, miR-124 and miR-219 that are  
30 involved in neural differentiation were decreased, while the expression of miR-21 involved in  
31 gliogenesis was increased upon radiation (Figure 5 B, D, F, H and J). In the same cells  
32 radiation also decreased the expression of *SOX2* and *OCT4* that are recognized as markers of  
33 pluripotency (Figure 6 B and D). Obtained results points to differences in radiation response  
34 between un-differentiated NT2/D1 cells and NT2/D1 cells in early phase of neural  
35 differentiation (Figure 5 and Figure 6). Importantly, presented results could indicate that  
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3 expression analysis of selected miRNAs and genes can be used as biomarkers for monitoring  
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5 radiation response of pluripotent and neural progenitor cells.  
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8 miR-21 is involved in regulation of different processes, such as apoptosis, migration,  
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10 invasion and proliferation of tumor cells (Feng and Tsao, 2016) and it was important to  
11  
12 assess the expression level of this miRNA upon radiation exposure. We show that 2 Gy  
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14 radiation was able to induce expression of miR-21 in NT2/D1 cells, which is in line with  
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16 previous results demonstrating that radiation usually induces miR-21 expression in different  
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18 tumor and normal cell lines (Anastasov et al., 2012; Methetraitrut and Slack, 2013). The 2  
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20 Gy radiation was also able to induce the expression of miR-21 in NT2/D1 cells in early  
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22 phases of neural differentiation. Accordingly, increased expression of miR-21 could be  
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24 considered as a biomarker of radiation response (Jiang et al., 2017). Additionally, it was  
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26 reported that increased expression of miR-21 in brain have protective role after traumatic  
27  
28 brain injury (Ge et al., 2014). Further experiments are needed to examine whether miR-21  
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30 have protective role in NT2/D1 cells against radiation induced damage.  
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35 miR-219 has an important role in differentiation of neural precursors (Bruinsma et al.,  
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37 2017). Our results indicate that radiation influences the expression of miR-219 in NT2/D1  
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39 and NT2/D1 1W cells in a dose dependent manner. Our results revealed, for the first time that  
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41 miR-219 could be considered as a neural marker involved in response to radiation.  
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45 Presented data revealed that 2 Gy of radiation can decrease the expression of markers  
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47 of neural precursors and neurons, such as *SOX3*, *PAX6* and miR-124a, in NT2/D1 1W cells  
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49 suggesting that 2 Gy radiation affected early phase of neural differentiation of NT2/D1 cells.  
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51 On the other side, we presented results indicating that 2 Gy dosage decreased expression of  
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53 pluripotency markers, like *SOX2* and *OCT4*, in NT2/D1 1W cells that implies that radiation  
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55 can also alter the pluripotency capacity of NT2/D1 cells in early phase of differentiation.  
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57 Since *SOX2* is considered to be a transcriptional factor with important role in the regulation  
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3 of pluripotency and neural differentiation (Zhang and Cui, 2014), this result also suggest that  
4 radiation of 2 Gy dose induce alterations in expression of important pluripotency factors  
5 which could have a significant impact on capacity of NT2/D1 to enter the program of neural  
6 differentiation. It is also known that Sox2 expression in NPCs is regulated by PAX6, where  
7 loss of PAX6 expression leads to decreased number of SOX2 positive cells (Wen et al.,  
8 2008).

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17 Finally, presented results impose a question about final outcome of radiation on  
18 neural differentiation of NT2/D1 cells, since radiation altered the expression of miRNAs and  
19 genes that are involved in regulation of pluripotency and neural differentiation. Previous  
20 study showed that radiation alters the function of NT2/D1 derived neurons after exposure to 2  
21 Gy of radiation (Sanchez et al., 2009). Also, loss of NPC was observed after exposure to  
22 radiation of rodent brain (Eom et al., 2016). Further analyses of the effect of radiation after  
23 exposure of NT2/D1 cells in early phase of differentiation are needed in order to address this  
24 question.

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In conclusion, these results suggest that 2 Gy of radiation significantly affected early  
phase of neural differentiation and affect the population of neural progenitors. These findings  
might help in better understanding of side effects of radiotherapy in treatments of central  
nervous system malignancies.

### **Acknowledgements**

This work was supported by the Ministry of Education, Science and Technological  
Development, Republic of Serbia (Grant No 173051), the Serbian Academy of Sciences and  
Arts (Grant No: F 24), IBRO/PERC InEurope Short Stay Grants and EURATOM Fission and  
European Commission 7<sup>th</sup> Framework Programme, DarkRisk (contract number 323216).

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3 **Disclosure statement**  
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5 *The authors report no conflicts of interest*  
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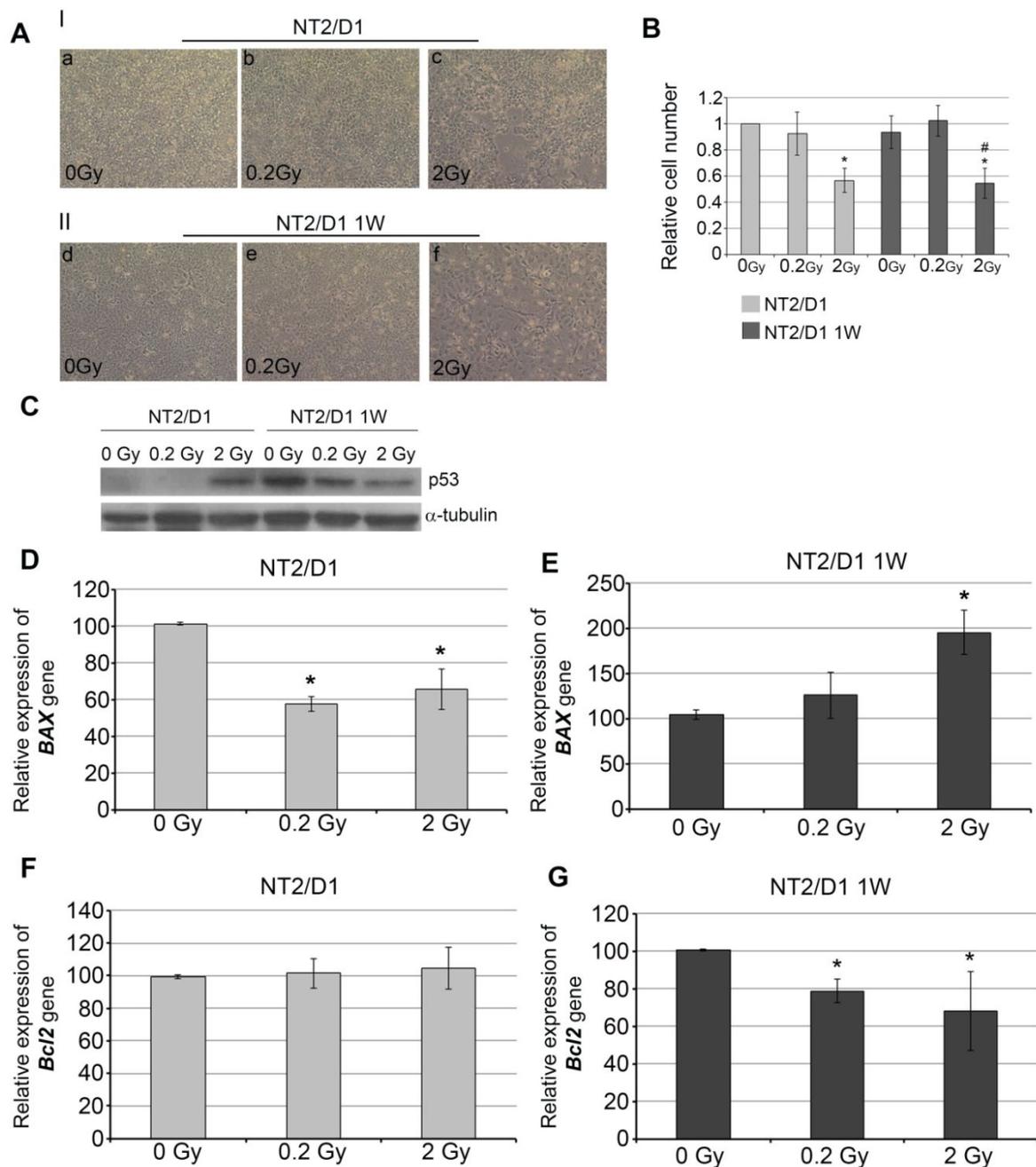
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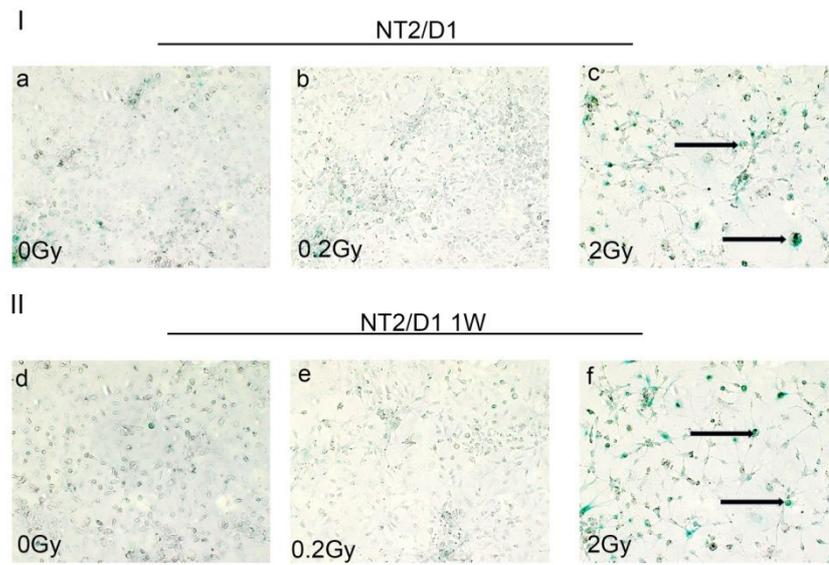
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**Figure 1**



**Figure 2**

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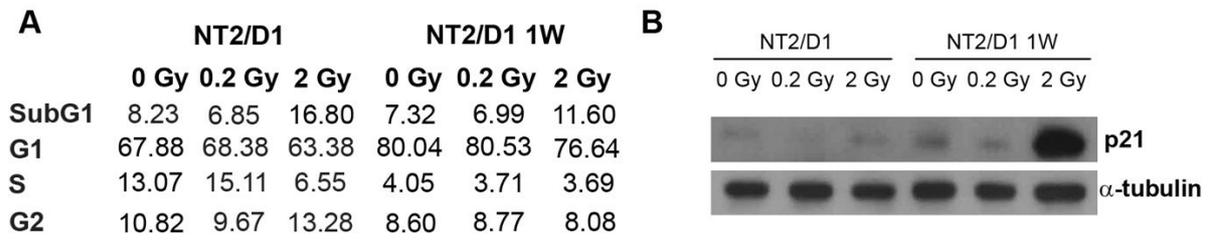
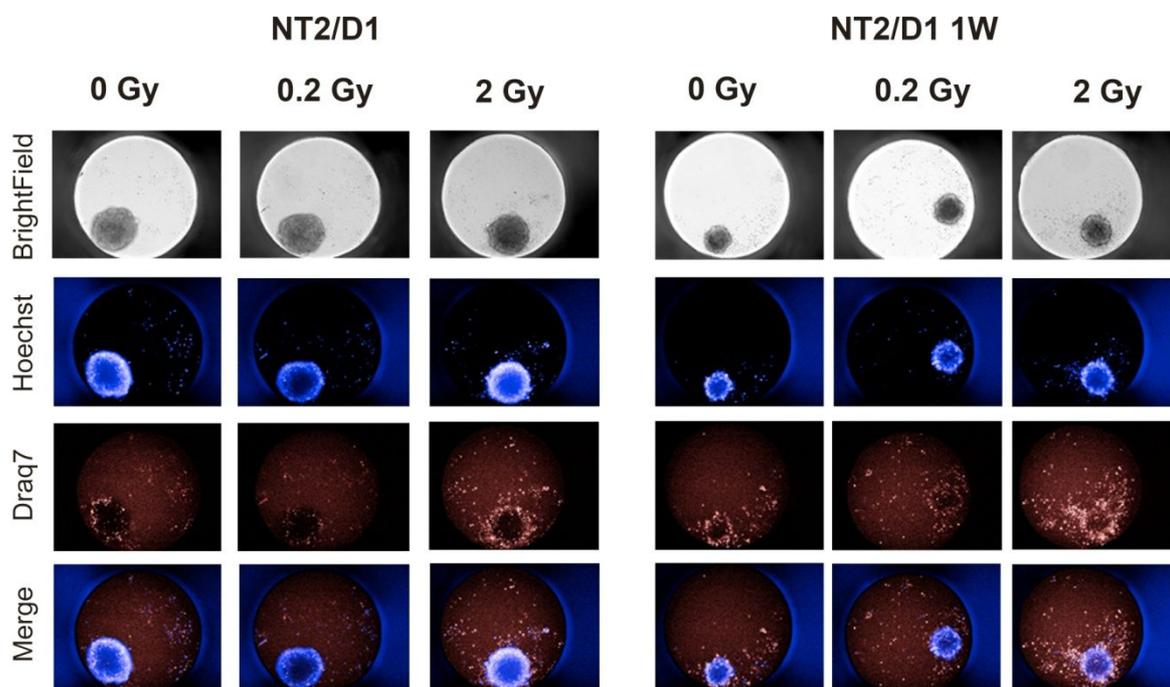
**Figure 3**

Figure 4



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Figure 5

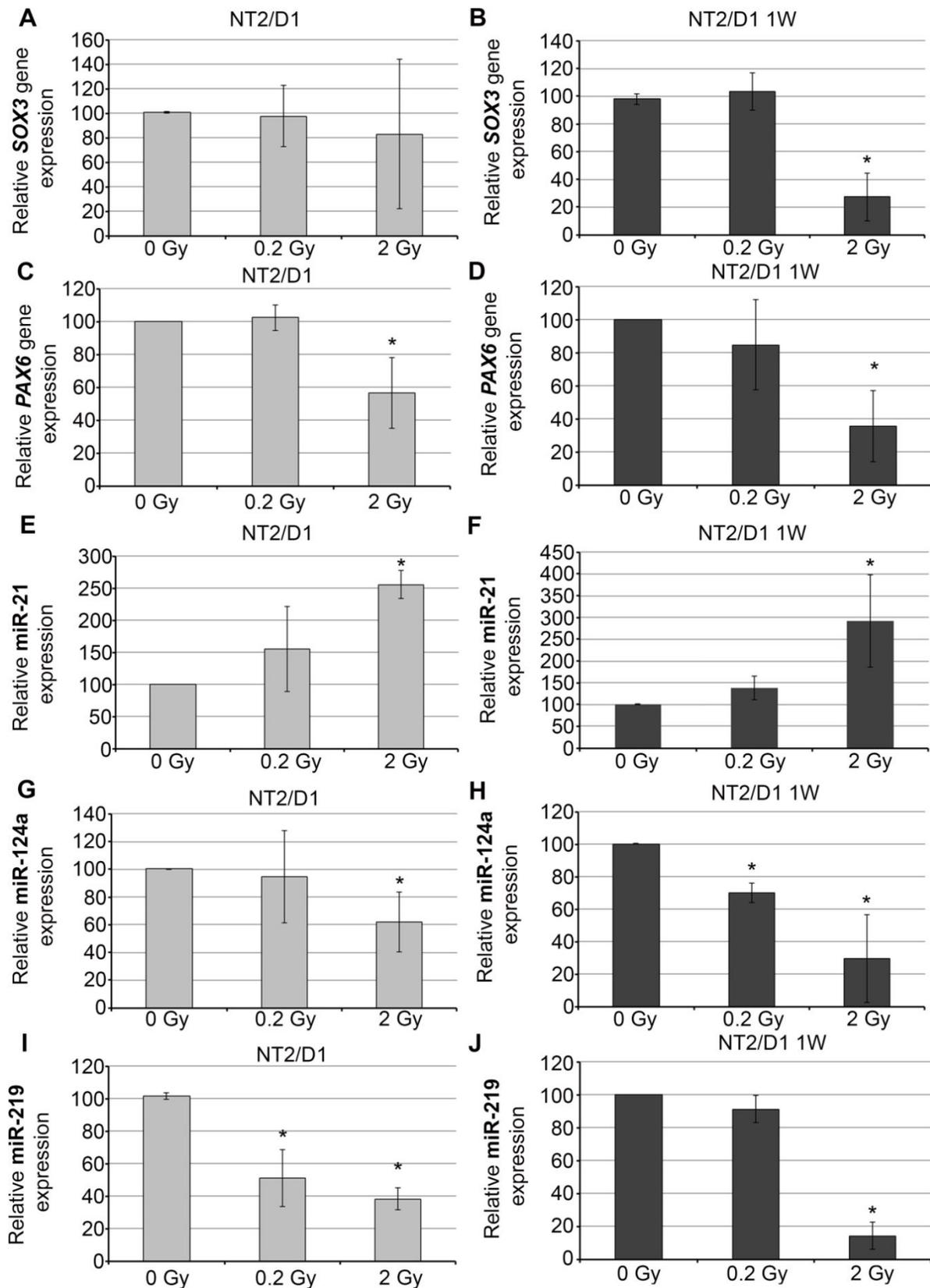
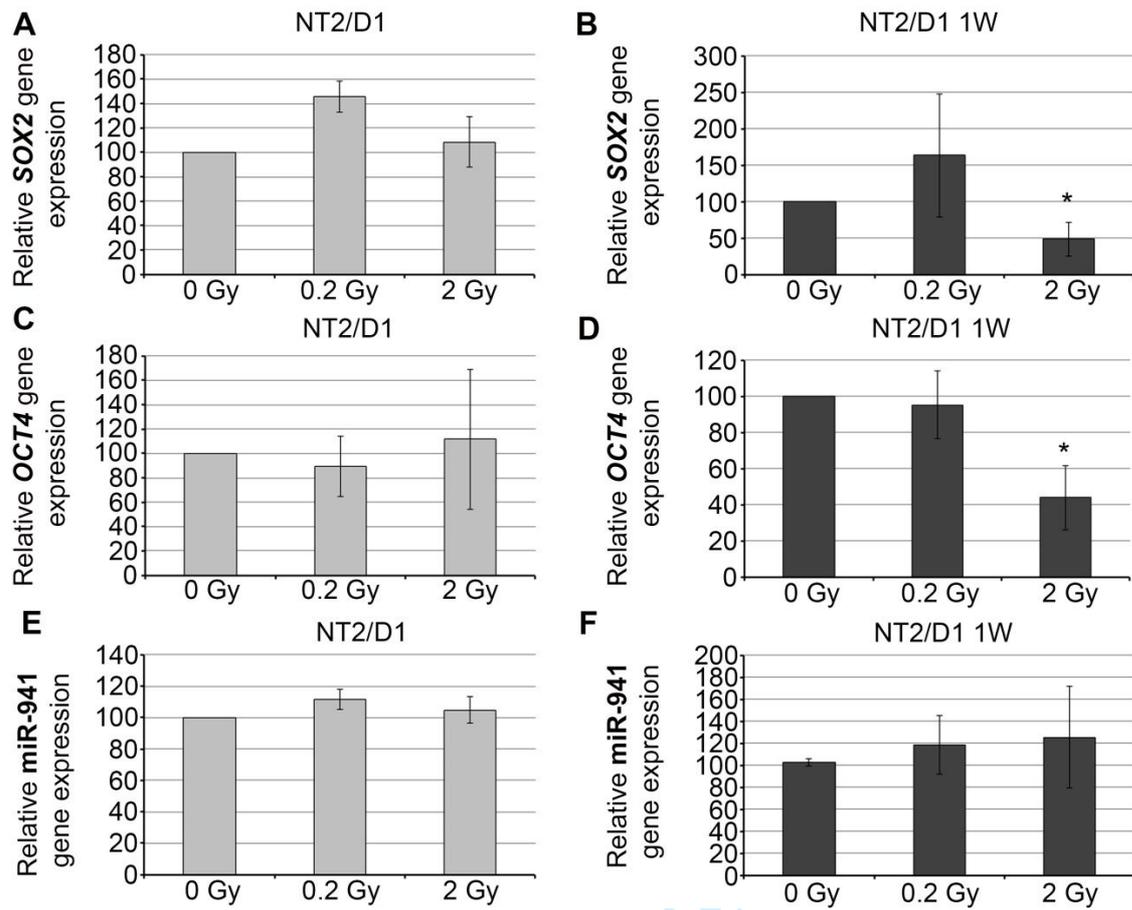


Figure 6



## Figure legends

**Figure 1:** The effect of radiation on morphology and apoptosis of undifferentiated NT2/D1 cells and NT2/D1 cells in early phases of differentiation (NT2/D 1W). **A:** Panel I - undifferentiated NT2/D1 cells, panel II - NT2/D1 cells induced with RA for 1 week (NT2/D1 1W). Cells are irradiated with 0.2 Gy (b and e), and 2 Gy (c and f). Cells were visualized by phase contrast microscope. **B:** Quantification of the relative cell number of undifferentiated NT2/D1 and NT2/D1 1W cells before and after irradiation is presented in histogram. Relative cell number was calculated relative to the sham irradiated undifferentiated NT2/D1 cells, which was set as 1. **C:** Western blot analysis of p53 protein expression in undifferentiated NT2/D1 cells and NT2/D1 1W upon radiation.  $\alpha$  - Tubulin was used as the loading control. Three independent experiments were performed and one representative Western blot is shown. **D** and **F:** qRT-PCR analysis of *BAX* and *Bcl2* gene expression in undifferentiated NT2/D1 cells upon radiation, as indicated. *GAPDH* was used as the loading control. The effect of radiation on *BAX* and *Bcl2* gene expression is shown in histograms. The quantities of *BAX* and *Bcl2* gene expression were calculated as percentages of their respective expression levels in sham irradiated undifferentiated NT2/D1 cells, which were set as 100%. **E** and **G:** qRT-PCR analysis of *BAX* and *Bcl2* gene expression in NT2/D1 1W cells upon radiation, as indicated. *GAPDH* was used as the loading control. The effect of radiation on *BAX* and *Bcl2* gene expression is shown in histograms. The quantities of *BAX* and *Bcl2* gene expression were calculated as percentages of their respective expression levels in sham irradiated differentiated NT2/D1 cells, which were set as 100%. All histograms data are presented as the means  $\pm$  SEM of three independent experiments. Mean values were compared with Student's t-test and P- values were calculated compared to the sham irradiated differentiated NT2/D1 cells were designed with \* $p \leq 0.05$  and P-values calculated compared to the sham irradiated NT2/D1 1W were designed with # $\leq 0.05$ .

**Figure 2:** The effect of radiation on senescence of undifferentiated NT2/D1 and NT2/D1 1W. The undifferentiated NT2/D1 cells (Panel I) and NT2/D1 1W cells (Panel II) seven days upon radiation were stained for  $\beta$ -galactosidase activity and visualized by phase contrast microscope. Green cells represent  $\beta$ -gal positive cells, indicated with arrows. 0 Gy – sham irradiated cells.

**Figure 3:** The effect of radiation on cell cycle distribution of undifferentiated NT2/D1 cells and NT2/D1 cells in early phases of differentiation (**A**) Representative data of G1, S and G2/M cell cycle distribution from three independent experiments (n=3) presented in %. **B** - Western blot analysis of p21<sup>Waf1/Cip</sup> protein expression in undifferentiated NT2/D1 cells and NT2/D1 1W upon radiation.  $\alpha$  - Tubulin was used as the loading control. Three independent experiments were performed and one representative Western blot is shown. 0 Gy – sham irradiated cells.

**Figure 4:** Representative images of 3D-spheroids formation of NT2/D1 and NT2/D1 1W cells upon irradiation with 0.2 Gy and 2 Gy.

**Figure 5:** The effect of radiation on selected neural markers expression in NT2/D1 and NT2/D1 1W cells. qRT-PCR analyses of the effect of radiation on *SOX3* (A and B), *PAX6* (C and D), miR-21 (E and F), miR-124a (G and H) and miR-219 (I and J) gene expression in undifferentiated NT2/D1 cells are presented on graphs. *GAPDH* or RNU44 was used as the

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3 loading control. The quantities of neural marker expression were calculated as percentages of  
4 their respective expression levels in sham irradiated undifferentiated NT2/D1 cells, which  
5 were set as 100% (A, C, E, G and I) or were calculated as percentages of their respective  
6 expression levels in sham irradiated differentiated NT2/D1 cells, which were set as 100% (B,  
7 D, F, H and J). Data are presented as the means  $\pm$  SEM of at least two independent  
8 experiments. Mean values were compared with Student's t-test and P- values were calculated  
9 compared to the sham irradiated undifferentiated NT2/D1 cells were designed with  $*p \leq 0.05$ .  
10 All experiments were done from at least two biological replicates with at least two technical  
11 replicates. 0 Gy: sham irradiated cells, NT2/D1: undifferentiated NT2/D1 cells, NT2/D1-1W:  
12 NT2/D1 in early phase of differentiation.  
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16 **Figure 6:** The effect of radiation on expression of selected pluripotency markers in NT2/D1  
17 and NT2/D1 1W cells. qRT-PCR analyses of *SOX2* (A and B), *OCT4* (C and D) and miR-941  
18 (E and F) expression are presented on graphs. *GAPDH* or RNU44 was used as the loading  
19 control. The quantities of pluripotency markers expression were calculated as percentages of  
20 their respective expression levels in sham irradiated undifferentiated NT2/D1 cells, which  
21 were set as 100% (A, C and E) or were calculated as percentages of their respective  
22 expression levels in sham irradiated differentiated NT2/D1 cells, which were set as 100% (B,  
23 D, F). Data are presented as the means  $\pm$  SEM of three independent experiments. Mean  
24 values were compared with Student's t-test and P- values were calculated compared to the  
25 sham irradiated undifferentiated NT2/D1 cells were designed with  $*p \leq 0.05$ . All experiments  
26 were done from at least three biological replicates with at least two technical replicates. 0 Gy:  
27 sham irradiated cells, NT2/D1: undifferentiated NT2/D1 cells, NT2/D1-1W: NT2/D1 in early  
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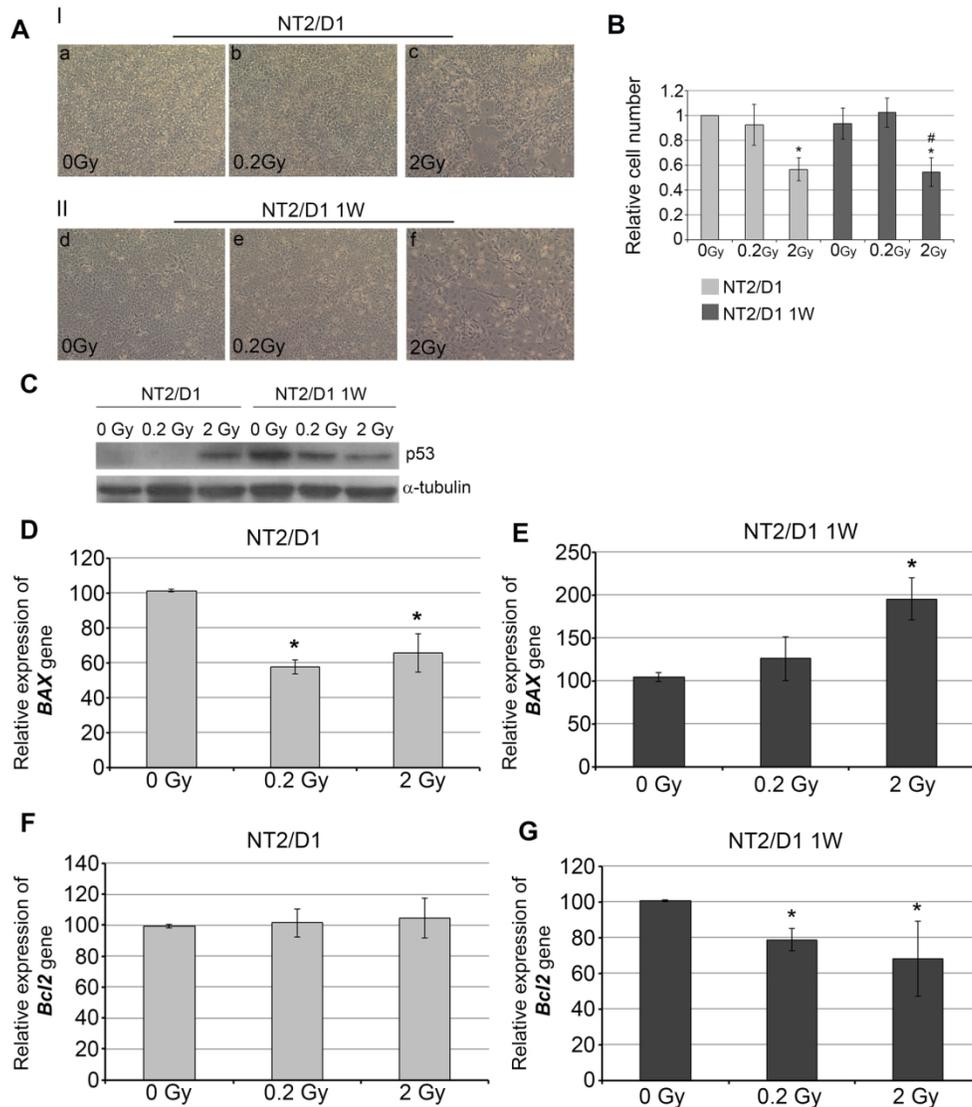


Figure 1: The effect of radiation on morphology and apoptosis of undifferentiated NT2/D1 cells and NT2/D1 cells in early phases of differentiation (NT2/D1 1W). A: Panel I - undifferentiated NT2/D1 cells, panel II - NT2/D1 cells induced with RA for 1 week (NT2/D1 1W). Cells are irradiated with 0.2 Gy (b and e), and 2 Gy (c and f). Cells were visualized by phase contrast microscope. B: Quantification of the relative cell number of undifferentiated NT2/D1 and NT2/D1 1W cells before and after irradiation is presented in histogram. Relative cell number was calculated relative to the sham irradiated undifferentiated NT2/D1 cells, which was set as 1. C: Western blot analysis of p53 protein expression in undifferentiated NT2/D1 cells and NT2/D1 1W upon radiation.  $\alpha$ -Tubulin was used as the loading control. Three independent experiments were performed and one representative Western blot is shown. D and F: qRT-PCR analysis of BAX and Bcl2 gene expression in undifferentiated NT2/D1 cells upon radiation, as indicated. GAPDH was used as the loading control. The effect of radiation on BAX and Bcl2 gene expression is shown in histograms. The quantities of BAX and Bcl2 gene expression were calculated as percentages of their respective expression levels in sham irradiated undifferentiated NT2/D1 cells, which were set as 100%. E and G: qRT-PCR analysis of BAX and Bcl2 gene expression in NT2/D1 1W cells upon radiation, as indicated. GAPDH was used as the loading control. The effect of radiation on BAX and Bcl2 gene expression is shown in histograms. The quantities of BAX and Bcl2 gene expression were calculated as percentages of their respective expression levels in sham irradiated

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3 differentiated NT2/D1 cells, which were set as 100%. All histograms data are presented as the means  $\pm$   
4 SEM of three independent experiments. Mean values were compared with Student's t-test and P- values  
5 were calculated compared to the sham irradiated differentiated NT2/D1 cells were designed with \* $p \leq 0.05$   
6 and P-values calculated compared to the sham irradiated NT2/D1 1W were designed with # $\leq 0.05$ .

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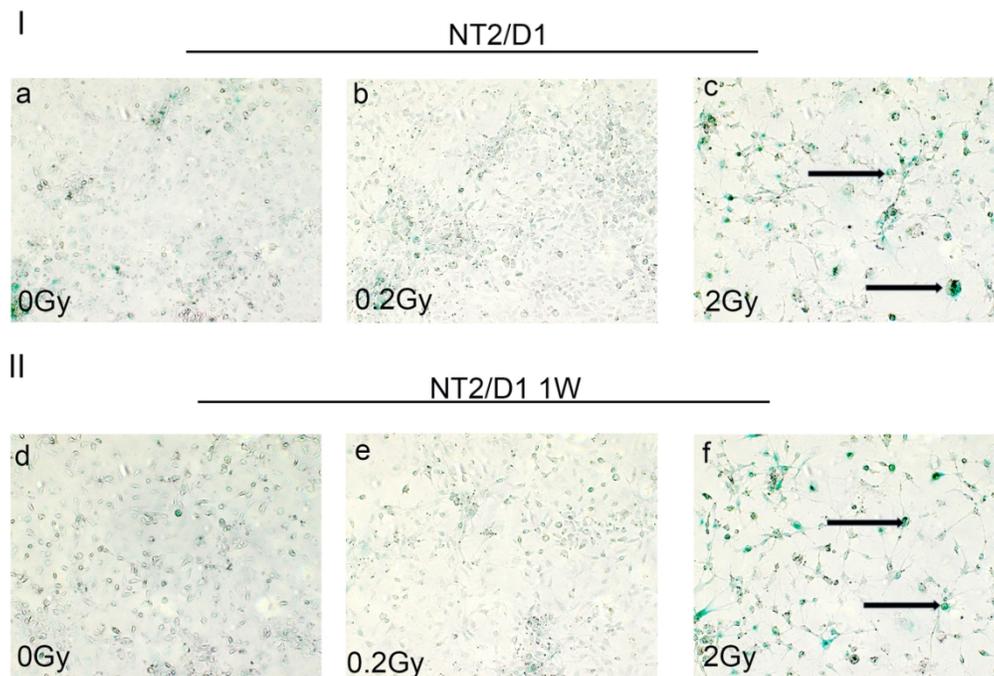


Figure 2: The effect of radiation on senescence of undifferentiated NT2/D1 and NT2/D1 1W. The undifferentiated NT2/D1 cells (Panel I) and NT2/D1 1W cells (Panel II) seven days upon radiation were stained for  $\beta$ -galactosidase activity and visualized by phase contrast microscope. Green cells represent  $\beta$ -gal positive cells, indicated with arrows. 0 Gy – sham irradiated cells.

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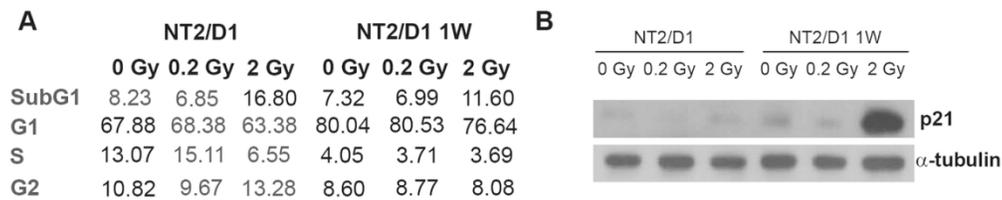


Figure 3: The effect of radiation on cell cycle distribution of undifferentiated NT2/D1 cells and NT2/D1 cells in early phases of differentiation (A) Representative data of G1, S and G2/M cell cycle distribution from three independent experiments (n=3) presented in %. B - Western blot analysis of p21Waf1/Cip protein expression in undifferentiated NT2/D1 cells and NT2/D1 1W upon radiation.  $\alpha$  - Tubulin was used as the loading control. Three independent experiments were performed and one representative Western blot is shown. 0 Gy – sham irradiated cells.

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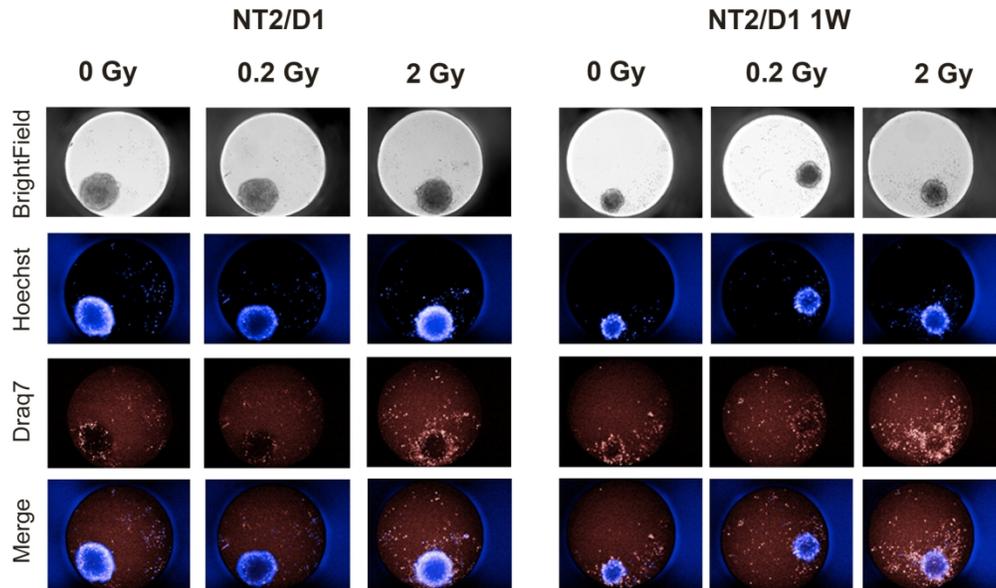


Figure 4: Representative images of 3D-spheroids formation of NT2/D1 and NT2/D1 1W cells upon irradiation with 0.2 Gy and 2 Gy.

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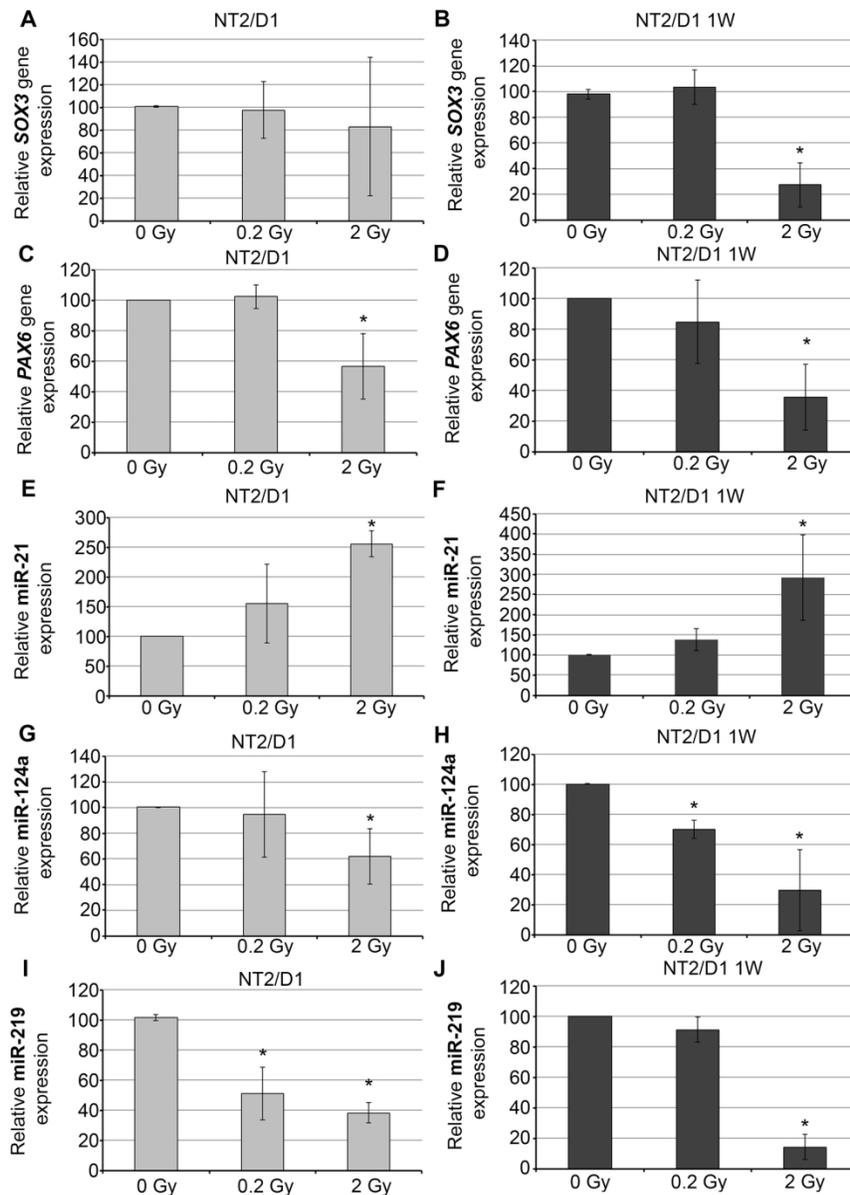


Figure 5: The effect of radiation on selected neural markers expression in NT2/D1 and NT2/D1 1W cells. qRT-PCR analyses of the effect of radiation on SOX3 (A and B), PAX6 (C and D), miR-21 (E and F), miR-124a (G and H) and miR-219 (I and J) gene expression in undifferentiated NT2/D1 cells are presented on graphs. GAPDH or RNU44 was used as the loading control. The quantities of neural marker expression were calculated as percentages of their respective expression levels in sham irradiated undifferentiated NT2/D1 cells, which were set as 100% (A, C, E, G and I) or were calculated as percentages of their respective expression levels in sham irradiated differentiated NT2/D1 cells, which were set as 100% (B, D, F, H and J).

Data are presented as the means  $\pm$  SEM of at least two independent experiments. Mean values were compared with Student's t-test and P-values were calculated compared to the sham irradiated undifferentiated NT2/D1 cells were designed with \* $p \leq 0.05$ . All experiments were done from at least two biological replicates with at least two technical replicates. 0 Gy: sham irradiated cells, NT2/D1: undifferentiated NT2/D1 cells, NT2/D1-1W: NT2/D1 in early phase of differentiation.

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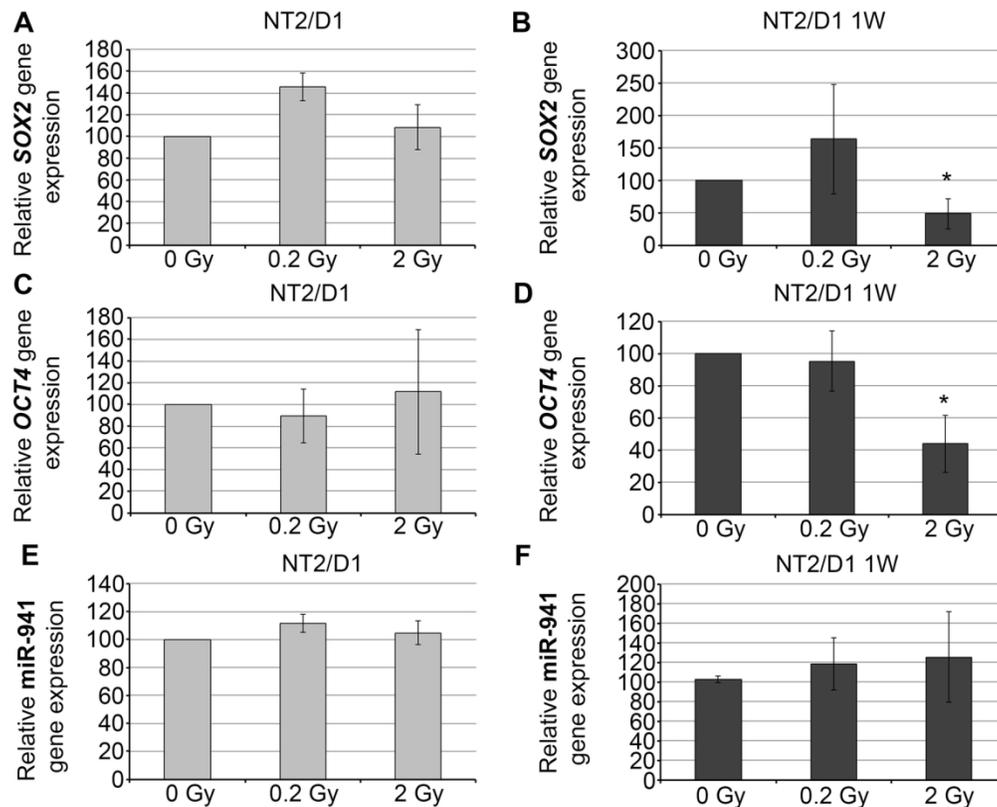


Figure 6: The effect of radiation on expression of selected pluripotency markers in NT2/D1 and NT2/D1 1W cells. qRT-PCR analyses of SOX2 (A and B), OCT4 (C and D) and miR-941 (E and F) expression are presented on graphs. GAPDH or RNU44 was used as the loading control. The quantities of pluripotency markers expression were calculated as percentages of their respective expression levels in sham irradiated undifferentiated NT2/D1 cells, which were set as 100% (A, C and E) or were calculated as percentages of their respective expression levels in sham irradiated differentiated NT2/D1 cells, which were set as 100% (B, D, F). Data are presented as the means  $\pm$  SEM of three independent experiments. Mean values were compared with Student's t-test and P- values were calculated compared to the sham irradiated undifferentiated NT2/D1 cells were designed with \* $p \leq 0.05$ . All experiments were done from at least three biological replicates with at least two technical replicates. 0 Gy: sham irradiated cells, NT2/D1: undifferentiated NT2/D1 cells, NT2/D1-1W: NT2/D1 in early phase of differentiation.

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