Erschienen in: Archives of toxicology ; 93 (2019), 10. - S. 2879-2893
https://dx.doi.org/10.1007/s00204-019-02553-z

Toxicity of ionizing radiation (IR) in a human induced pluripotent stem cell (hiPSC)-derived 3D early neurodevelopmental model

Annemarie Klatt[1] · Eugenia Salzmann[1] · Lisanne-Josephin Schneider[1] · Alexander Reifschneider[1] · Milena Korneck[1] · Patrick Hermle[1] · Alexander Bürkle[2] · Dieter Stoll[1] · Suzanne Kadereit[1]

Abstract
Prenatal brain development is a complex and sensitive process, highly susceptible to environmental influences such as pollutants, stress, malnutrition, drugs, tobacco exposure, or ionizing radiation (IR). Disturbances in development may cause life-long disabilities and diseases, such as ADHD, childhood cancers, cognitive problems, depression, anxiety and more severe developmental disabilities. Due to increasing medical imaging, radiation therapy, natural terrestrial radiation, radioactive pollution and long-distance flights, humans are increasingly exposed to IR. However, data on impact of IR on very early human brain development are scarce, particularly in the very first weeks of gestation. Here we investigated the effects of low-dose X-ray IR (1 Gy) in a 3D early brain developmental model derived from human pluripotent stem cells. In this model very early neural stem cells, neuroectodermal progenitor cells (NEP), were exposed to low-dose IR and direct as well as delayed effects were investigated. Expression of 20 different marker genes crucial for normal neural development was determined 48 h and 9 days post IR (pIR). All but one of the analyzed marker genes were reduced 48 h after IR, and all but seven genes normalized their expression by day 9 pIR. Among the seven markers were genes involved in neurodevelopmental and growth abnormalities. Moreover, we could show that stemness of the NEP was reduced after IR. We were thus able to identify a significant impact of radiation in cells surviving low-dose IR, suggesting that low-dose IR could have a negative impact on the early developing human brain, with potential later detrimental effects.

Keywords Ionizing irradiation · Developmental neurotoxicity · Pluripotent stem cells · 3D in vitro model

Introduction

Despite the fact that exposure to ionizing radiation is increasing in humans, the impact on the early developing human brain is only scarcely characterized.

The sources of ionizing radiation (IR) can be divided into two clusters: man-made and natural (UNSCEAR Report 2008). While artificial IR sources such as nuclear reactors and waste, industrial, military and medical IR are often sources of higher levels of exposure, natural occurring IR such as cosmic and terrestrial radiation is seen as a stable low level, long-term exposure (UNSCEAR Report 2008, 2013). Underlining the importance of particularly low-dose radiation research is the observation that CNS tumor incident rates were found to be associated with naturals background radiation levels in Germany, varying from 0.5 to 1.5 mSv (Spix et al. 2017).

IR is extensively used in varying dosages in medical imaging and treatment reaching from very low-dose exposure in X-ray imaging (0.005–1.5 mGy depending on imaged body part) to doses in CT scans of 2–65 mGy per scan (Brenner and Hall 2007; Gregory et al. 2008; Pearce et al. 2012; Smith-Bindman et al. 2009). In therapy of non-malignant disorders (e.g., inflammatory or degenerative or diseases), higher doses of 3–30 Gy are used, typically delivered in fractions between 1 and 3 Gy (Seegenschmiedt et al. 2015). The overall doses used in RT for cancer treatment

✉ Suzanne Kadereit
Kadereit@hs-albsig.de

[1] Department of Life Sciences, Albstadt-Sigmaringen University of Applied Sciences, Anton Guenther-Strasse 51, 72488 Sigmaringen, Germany

[2] Department of Biology, University of Konstanz, Universitaetstrasse 10, 78467 Konstanz, Germany

Konstanzer Online-Publikations-System (KOPS)
URL: http://nbn-resolving.de/urn:nbn:de:bsz:352-2-hmil7ruxb0k73

vary between about 16 and 75 Gy, depending on position, size and type of the tumor (Pinnix et al. 2018; UNSCEAR Report 2008; Yamoah et al. 2015).

Unlike in adults, brain and CNS tumors are the most common cancer types in children, followed by leukemia (Ostrom et al. 2015). One of the major problems of RT in general and particularly in brain- and CNS-associated cancer is the damage to the surrounding normal tissue. This has particularly pronounced effects in the still-developing brain of pediatric patients. Severe consequences such as memory and intellectual impairment, hearing and visual memory loss, appear directly after treatment, and progress further as the patients age (Gan et al. 2011; Hall et al. 2004; Khan et al. 2018; Soussain et al. 2009; Spiegler et al. 2004). As the survival rate has increased over the years, these children, now adults, show rapidly progressing mental impairment, similar to Alzheimer's disease. These side effects are more commonly seen in patients that were less than 3 years of age at the time of treatment, suggesting an impact of RT on neurogenesis, which is still ongoing in humans after birth (Chin and Maruyama 1984; Packer et al. 1987; Sorrells et al. 2018). This is supported by the finding that in contrast to adult neurons and astrocytes which demonstrate considerable radio resistance, neural stem cells (NSC) are very sensitive to radiation (Barazzuol and Jeggo 2016; Etienne et al. 2012; Ivanov and Hei 2014; Katsura et al. 2016; Shimura et al. 2017). The high sensitivity of NSCs to irradiation could be confirmed not only for conventional irradiation methods but also for carbon ion irradiation (Morini et al. 2018). However, there is only a limited number of studies investigating molecular effects of IR specifically in human stem cells and particularly in NSC. Low doses of radiation (0.05–0.2 Gy) were found to possibly enhance cell viability, increase ATP levels and decrease ROS levels (Betlazar et al. 2016; Sokolov and Neumann 2013). However, other studies found changes in expression of genes involved in cell death, p53 signaling and embryonic development, and an altered protein expression profile similar to stem cells treated with doses ≥ 0.5 Gy of IR (Bajinskis et al. 2011; Wilson et al. 2010). In NSC tissue culture models the response to IR was found to involve neuroinflammatory processes and mitochondrial dysfunction leading to cell death and cellular senescence (Betlazar et al. 2016; Manda et al. 2014; Yamaguchi and Kashiwakura 2013).

In mice, IR reduces significantly the production of new neurons in the brains of young mice, leading to changes in behavior in 3-month old mice and to spatial memory retention deficits (Fishman et al. 2009; Rola et al. 2004). In utero irradiated mouse embryos (1 Gy) developed a severe microcephaly due to a depletion of neural progenitor cells in the cortical layers (Shimada et al. 2016). In addition to reduced neurogenesis, the number of astrocytes increased significantly in the brain of mice, presumably due to processes of neuroinflammation and activation of microglial cells. Additionally, a loss of microglia in the brains of young mice and apoptosis of cells in the human microglia cell line CHME5 was determined (Chen et al. 2016; Kalm et al. 2009).

In human NSC treated with cell culture supernatants from irradiated glioblastoma cells, the differentiation to neurons, but not to astrocytes, was selectively suppressed, suggesting bystander effects on neighboring cells during therapeutic IR (Ivanov and Hei 2014). Secreted factors from irradiated medulloblastoma cells were also found to increase oxidative stress levels in bystander NSCs (Sharma et al. 2016). Those bystander effects were not only limited to directly neighboring cells but also other organ parts. When one lung was irradiated in mice, bystander effects were found in the protected other lung (Kovalchuk and Kolb 2017). Furthermore, even low-dose irradiation was found to cause inhibition of cell-cycle progression and lead to elevated levels of apoptosis in human NSC (Acharya et al. 2010). Irradiated NSC were found to be more likely to differentiate to astrocytes showing features of cell senescence, demonstrating a senescence-associated secretory phenotype by secretion of interleukin 6, 8 and 1α (Zou et al. 2012). These results point towards sensitivity of NSC and a significant impact of ionizing radiation on NSC, not only through direct effects but also through bystander effects.

Most studies have focused on in vivo or cell cultures, mostly adherent cells, to investigate radiation and its direct and indirect effects on NSC. In this study, we focused on the effect of IR on human neuroectodermal progenitor cells (NEP), very early NSC of the human brain (Chambers et al. 2009). In a 3D in vitro model we developed previously, we generated NEP from pluripotent stem cells which we then differentiated further within neurospheres (NS), three-dimensional round aggregates that are formed by neural stem and progenitor cells, in which the NSC self-renew, but also continue to differentiate to neural cells. In this model, gene expression profiling had revealed robust differentiation toward neuronal precursor cells and neurons, and indicated a predominantly forebrain-like development (Hoelting et al. 2013). We could show that the model could detect developmental neurotoxicity (DNT). Here we adapted this model to human induced pluripotent stem cells (hiPSC) and investigated the impact of ionizing irradiation on very early neural cell development.

Materials and methods

Cell culture

hIPSC and hESC (WA09, hESC work is approved under the license AZ 3.04.02-0075) were cultured in Dulbecco's Modified Eagle's Medium (DMEM)/F12 medium supplemented

with 20% serum replacement, 1% nonessential amino acids, 1% glutamax, 0.1 mM β-mercaptoethanol, and 10 ng/ml basic fibroblast growth factor (bFGF), all from Life Technologies/Thermo Fisher, on mitomycin C (MMC)-treated mouse embryonic fibroblasts (MEFs) feeder cells (Thomson et al. 1998). For feeder-free cultures, hiPSC were cultured on Matrigel (Corning) using mTeSR1 (Stemcell Technologies). Stem cells were passaged using Dispase (Worthington) and used at passages 8–20.

Differentiation to NEP and neurospheres

Cells were differentiated in adherent culture to PAX6$^+$ neuroectodermal progenitor cells (NEP) as described previously (Hoelting et al. 2013). Briefly, hiPSC, and where indicated also hESC, were plated onto Matrigel-coated flasks as single-cell suspension (differentiation day 0, dd0) and cultured for 3 days in MEF-conditioned medium (Xu et al. 2001). Three days later, on dd3, directed neural differentiation was initiated by adding neural differentiation medium (Hoelting et al. 2013). On differentiation day 11 (dd11) when cultures were predominantly PAX6-positive, cells were either digested with Dispase to clumps or to single cells with Accutase (Life Technologies), washed twice, thus ensuring removal of dead cells, and counted using trypan blue exclusion. Cells were transferred into 6-well or 96-well ultra-low adhesion plates (Corning) in DMEM/F12 medium supplemented with B27 (Life Technologies), 600 nM dorsomorphin (Torcis), 42 ng/ml noggin (Peprotech), 20 ng/ml FGF2 (Peprotech) and 10 µM Rock inhibitor (Tocris), to form neurospheres as previously described (Hoelting et al. 2013). Neurospheres were fed every 3–4 days and extracted after 1 week in culture, on dd18, for gene expression analysis.

Irradiation of NEP

On differentiation day 9 (dd9), cells were irradiated for 100 s at a dose rate of 0.6 Gy/min, resulting in a total dose of 1 Gy of gamma radiation (X-ray) in an X-rad 225 iX (Precision X-ray, USA) with a 1.25 mm Al filter. Medium was changed after 1–2 h, to reduce stress factors in the medium of irradiated cells. On dd11, i.e., 48 h pIR, cells were extracted for expression analyses or transferred into neurosphere cultures. 7 days later, on dd18, NS were extracted for expression analyses.

Stemness assay

On differentiation day 11 (48 h pIR), single-cell suspensions were generated as described above and plated into 96-well ultra-low adhesion plates as above, at 500, 200, 100, 50 and 25 cells/well, with 10 wells for each cell concentration, thus diluting down the stem cells contained in the cell suspension. After 4 days, each well was assessed visually for content of a neurosphere. Only round spheres with sharp borders were counted and wells containing a neurosphere were expressed as proportions of seeded wells (Suppl. Fig. 3).

RNA extraction and qRT-PCR

Cells were differentiated as described in Fig. 1, where indicated irradiated on dd9 and extracted 48 h later (on dd11) or 9 days later, on dd18. Total RNA was isolated using TRIzol (Invitrogen). cDNA was prepared with the iScript Advanced cDNA Synthesis Kit (Bio-Rad) using 1 µg total RNA. Quantitative RT-PCR was performed in 3 technical replicates with fluorescent-labeled probes using SsoAdvanced Universal Probes Supermix (Bio-Rad). Primer tests were performed using the iQ SYBR green super mix (Bio-Rad). For primer and probe sequences see Suppl. Table 1. For quantification of expression, the $2^{-\Delta\Delta Ct}$ method was used to determine relative expression level, normalized to beta-2 microglobulin expression levels. For hiPSC experiments we performed ten biological replicates from three different thaw-batches of the hiPSC line.

Western blotting

Cells were differentiated and treated as above and samples were taken at indicated time points and were denatured in NuPage® LDS Sample buffer 4× (Invitrogen, UK). A total of 5 µg protein was loaded for gel electrophoresis and blotting, which was performed using the NuPAGE SDS-PAGE gel system (Life Technologies). For protein separation 4–12% Bis–Tris gels in NuPAGE MES running buffer were used according to the manufacturer's instructions. Proteins were blotted onto nitrocellulose membranes (GE Healthcare, Freiburg, Germany). Blocking was performed using 3% milk powder in TBST. Antibodies (Suppl. Table 2) were incubated overnight at 4 °C in the recommended dilutions in 1% bovine serum albumin in TBST. Before and after the fluorescent-labeled secondary antibody was added and incubated for 1 h at room temperature, the membrane was washed 5 times for 5 min with TBST. For detection, a sensitive near-infrared fluorescence scanner (Li-COR Odyssey, Li-COR Instruments, Bad Homburg, Germany) was used. Quantification was performed with ImageJ 1.51n.

Neurosphere diameter measurement

Cells were differentiated and treated as described in Fig. 1c and irradiated on dd9. 48 h pIR, irradiated and non-irradiated cells were detached, washed thoroughly and 500 cells/well were plated into 96-well ultra-low adhesion plates (Corning) to form spheres. At those cell numbers, the cells formed one single neurosphere/well. Images of spheres were

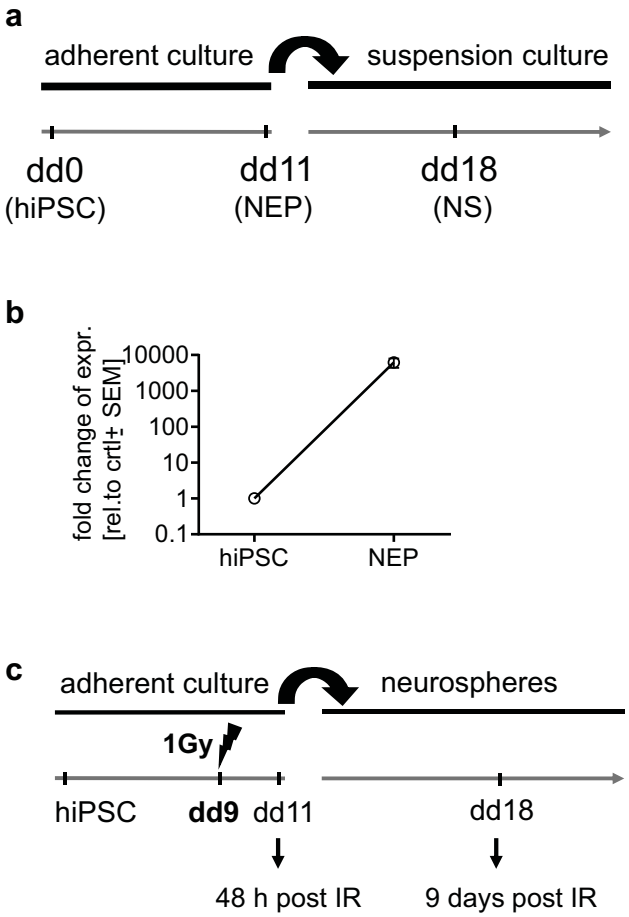

Fig. 1 Strong upregulation of PAX6 expression during differentiation from hiPSC to NEP. hiPSC were differentiated as described in Hoelting et al. (2013) and PAX6 expression assessed as a marker for successful differentiation to neuroectodermal progenitor cells (NEP). **a** Graphic of the differentiation scheme used throughout the experiments. Pluripotent stem cells were seeded and differentiated for 11 days to NEP in adherent cultures. On differentiation day 11 (dd11) cells were extracted for analyses or replated as clumps into suspension cultures and matured for additional 7 days within neurospheres (NS) and extracted on dd18. **b** On dd11, PAX6 gene expression was measured in NEP by quantitative RT-PCR and expressed as fold-increase over expression in undifferentiated hiPSC, arbitrarily set to 1. $n = 10$. Data are shown as the mean percentage ± SEM. **c** On dd9, NEP were irradiated with one dose of 1 Gy of X-rays and extracted 48 h later, i.e., on dd11 (for analysis of expression in NEP), or transferred as clumps or single cells to suspension cultures to form NS. For gene and protein expression analyses NS cells were extracted 7 days later, on dd18

acquired over time, starting from day 5 pIR and sphere diameters were measured using ImageJ 1.51n.

Statistical analyses

The number of biological repeat experiments is indicated in each figure. All statistical analyses were performed using GraphPad Prism 8. Data were tested for normal distribution using D'Agostino & Pearson normality test. A two-tailed one sample *t* test was used to test for statistically significant differences.

Results

To determine the impact of low-dose ionizing radiation (IR) on early brain development, neuroectodermal progenitor cells (NEP) were generated from human embryonic stem cells (hESC) and human induced pluripotent stem cells (hiPSC), as previously described, according to the scheme depicted in Fig. 1a. Characterization of differentiation to NEP from hESC has been described and characterized previously (Hoelting et al. 2013).

Differentiation of hiPSC to NEP and NS

Efficient differentiation of hiPSC to NEPs and neurospheres was verified by quantitative RT-PCR analysis.

First, we identified appropriate house-keeping genes. We had previously observed that classical house-keeping genes such as GAPDH or actin changed in expression over the course of differentiation from pluripotent stem cells to neural cells (Hoelting et al. 2013). Moreover, treatment with certain compounds also affected the expression of classical house-keeping genes, as was also shown to be the case with radiation (Banda et al. 2008). The best results after IR were obtained using beta 2 microglobulin (B2M) and ribosomal protein L13 (RPL13), both remaining stable throughout the course of differentiation, and also after irradiation (suppl. Fig. 1). As B2M displayed CT values closer to our genes of interest, we chose B2M as the house-keeping gene for all the following gene expression analyses.

We next examined upregulation of PAX6 in differentiating hiPSC. PAX6 is a marker for very early human brain progenitor cells, the neuroectodermal progenitor cells (NEP) (Zhang et al. 2010). On day 11 of differentiation (dd11, NEP) from hiPSC, upregulation of PAX6 was strong, robust and reproducible (Fig. 1b).

To ascertain that hiPSC performed similar to hESC in the 3D model, we next analyzed the expression of key genes involved in neurogenesis (Table 1). hiPSC were differentiated to NEP and subsequently to 1 week-old (dd18) NS, according to the differentiation scheme depicted in Fig. 1a.

Gene expression analyses revealed that expression of the chosen genes followed expected expression patterns, similar to those observed when differentiating from hESC (Hoelting et al. 2013). Neural stem cell markers such as PAX6, EPHA4 and NES all increased significantly over differentiation time (Fig. 2a). The same could be observed for the neuronal precursor markers HES5, DLL1 and ASCL1 (Fig. 2b). All three markers increased significantly during differentiation

Table 1 Function and human pathologies of analyzed markers

Gene	Protein	Function	Human pathologies	References
ARX	Aristaless related homeobox	Neuron migration/communication	Early infantile epileptic encephalopathy 1, Partington syndrome, X-linked lissencephaly	Cho et al. (2017) and Kato and Dobyns (2003)
ASCL1	Achaete-scute family bHLH transcription factor 1	Neuronal commitment and differentiation, generation of olfactory and autonomic neurons	Congenital central hypoventilation syndrome (CCHS)	Memic et al. (2016) and Park et al. (2017)
DCX	Doublecortin	Neuronal migration, regulating the organization and stability of microtubule	Subcortical band heterotopia in females, lissencephaly in males	Ayanlaja et al. (2017) and Shahsavani et al. (2018)
DLL1	Delta Like Canonical Notch Ligand 1	Neocortex development, neuronal differentiation, cerebellar development, fate determination of neural stem cells, notch pathway	Alobar holoprosencephaly, septopreoptic holoprosencephaly	Dupe et al. (2011) and Kageyama et al. (2018)
EMX2	Empty spiracles homeobox 2	Patterning neocortex, generates boundary between roof and archipallium	Associated with schizencephaly (SCHZC)	Bishop et al. (2000) and Dies et al. (2013)
EPHA4	Ephrin type-A receptor 4	Controls axonal guidance, synaptic plasticity, development of thymic epithelium	Amyotrophic lateral sclerosis (ALS)	Van Hoecke et al. (2012) and Zhao et al. (2018)
FOXG1	Forkhead box G1	Patterning of the fetal brain, development of telencephalon	FOXG1 syndrome, Lennox–Gastaut syndrome	Florian et al. (2012) and Tohyama et al. (2011)
HDAC6	Histone deacetylase 6	Formation of stress granules, involved in cell motility, cell survival, cell-cycle progression, developmental events, protein degradation, radiation stress response	Chondrodysplasia with platyspondyly, distinctive brachydactyly, hydrocephaly and microphthalmia (CDP-PBHM), Alzheimer's disease	Hubbert et al. (2002) and Yang et al. (2018)
HES5	Hairy and enhancer of split 5	Regulates cell differentiation and timing of neurogenesis and gliogenesis	Various cancer types	Bansod et al. (2017), Massie et al. (2015) and Panaliappan et al. (2018)
LHX2	LIM/homeobox protein Lhx2	Forebrain development, regulation of neuron-glia cell fate	Putative involvement in some cancer types	Hou et al. (2013), Morel et al. (2017) and Zhou et al. (2014)
MAP2	Microtubule-associated protein 2	Stabilizes microtubules growth, putative role during neuron development	Age-related macular degeneration, MAP2 Splicing altered in Huntington's Disease	Cabrera and Lucas (2017) and Gumy et al. (2017)
MAPT	Microtubule-associated protein tau	Promotes microtubule assembly and stability, might be involved in neuronal polarity	Frontotemporal dementia (FTD), Alzheimer's disease, Parkinsonism	Dehmelt and Halpain (2005) and Lee et al. (2001)
MCPH1	Microcephalin 1	Regulator of telomere integrity and of cell-cycle control, controls neurogenesis, DNA damage response	Primary autosomal recessive microcephaly 1, autosomal recessive primary microcephaly	Arroyo et al. (2017) and Liu et al. (2016)
NES	Nestin	Neural stem cell marker, assembly/disassembly of intermediate filaments	Various cancer types	Neradil and Veselska (2015) and Yan et al. (2016)
PAX6	Paired box protein Pax-6	Formation of the eyes, nose, central nervous system and pancreas, patterning	Aniridia, Gillespie syndrome, Peters anomaly, Coloboma, microphthalmia, WAGR syndrome	Bishop et al. (2000), O'Leary and Sahara (2008) and Tzoulaki et al. (2005)

Table 1 (continued)

Gene	Protein	Function	Human pathologies	References
RSPO1	R-Spondin-1	Promotes canonical WNT/β catenin signaling, activates TGFβ signaling, enhancing stress-induced apoptosis, radiation stress response	Keratoderma, palmoplantar, with squamous cell carcinoma of skin and sex reversal (PKKSCC)	Chen et al. (2017) and Tallapaka et al. (2018)
TP53	Tumor protein p53	Regulates cell division, induces growth arrest/apoptosis, DNA damage response, radiation stress response	Various cancer types	Broustas and Lieberman (2014) and Guo et al. (2000)
TRH	Thyrotropin releasing hormone	Controls secretion of TSH, modulator of hair growth, neurotransmitter	Hypothalamic hypothyroidism	Joseph-Bravo et al. (2015) and Waniek et al. (2015)
TUBB	Tubulin beta chain	Major constituent of microtubules, cortical development	Cortical dysplasia complex with other brain malformations 6 (CDCBM6), skin creases, congenital symmetric circumferential 1 (CSCSC1)	Parrini et al. (2016) and Romaniello et al. (2018)
ZEB2	Zinc finger E-box binding homeobox 2	Regulates induction of neuroectoderm and neural crest, Schwann cell differentiation	Mowat–Wilson syndrome, Coloboma	Quintes et al. (2016) and Zweier et al. (2005)

to NEP and plateaued (DLL1) or increased further (HES5 and ASCL1) in NS (dd18), with a stronger increase in HES5, as also observed in differentiation from hESC. The three patterning markers EMX2, LHX2, and FOXG1 were all strongly upregulated in NEP differentiated from hiPSC and increased further in expression in NS, similar to what we observed in hESC-derived NEP and NS (Fig. 2c). Neuronal genes such as MAP2 and MAPT (tau protein) also increased during differentiation, with MAP2 increasing strongly already in NEP whereas MAPT increased only in the NS stage (Fig. 2d). TUBB, beta-5 tubulin, although expressed strongly, did not show an increase during differentiation. A similar pattern was also observed in differentiation from hESC (Hoelting et al. 2013).

Impact of IR on NEP

To investigate the impact of ionizing radiation (IR) on very early human forebrain development, we assessed the impact of a one-time, low-dose, ionizing irradiation on still differentiating NEP. To this aim, we irradiated NEP on dd9 (Fig. 1c) with a single dose of 1 Gy and cultured the NEP for additional 48 h to reduce the number of apoptotic cells (Acharya et al. 2010). Subsequently, NEP were detached from the culture surface, washed twice to eliminate dead cells and counted. Decrease in cell numbers was dose-dependent, with increasing cell loss at increasing doses (data not shown). As at lower irradiation doses (0.25 Gy and 0.5 Gy) no significant differences could be observed in gene expression analyses we used a dose of 1 Gy in the following experiments. At 1 Gy both in hESC and hiPSC-derived NEP, a large amount of the irradiated cells had died, with on average 40% and 50% surviving cells, respectively (Fig. 3).

Next, cells were washed thoroughly, counted by trypan blue exclusion and viable cells were analyzed for expression of genes crucial for neurodevelopment. Interestingly, all but one of 20 analyzed genes were downregulated 48 h pIR, to varying extent. Only NES (nestin), a type VI intermediate filament protein, and marker for neuroepithelial stem cells was not affected by 1 Gy of X-rays (Fig. 4a). In contrast, two neural stem cell markers were downregulated over twofold, with a downregulation of PAX6 of threefold and EPHA4 of 2.6-fold (Fig. 4a). Neuronal precursor markers were also expressed at reduced levels with DLL1 expressed 1.9-fold less, ASCL1 threefold less and HES5 expressed 4.6-fold less after irradiation (Fig. 4b). Similar reductions in expression were also observed with the patterning genes EMX2 (1.8-fold less), FOXG1 (2.6-fold less) and LHX2 (3.9-fold less) (Fig. 4c). The neuronal genes MAP2 and MAPT were also both expressed at reduced levels with a 2.5- and 2.3-fold decrease, respectively (Fig. 4d). TUBB, which unlike MAP2 and MAPT did not increase during differentiation, albeit being expressed at

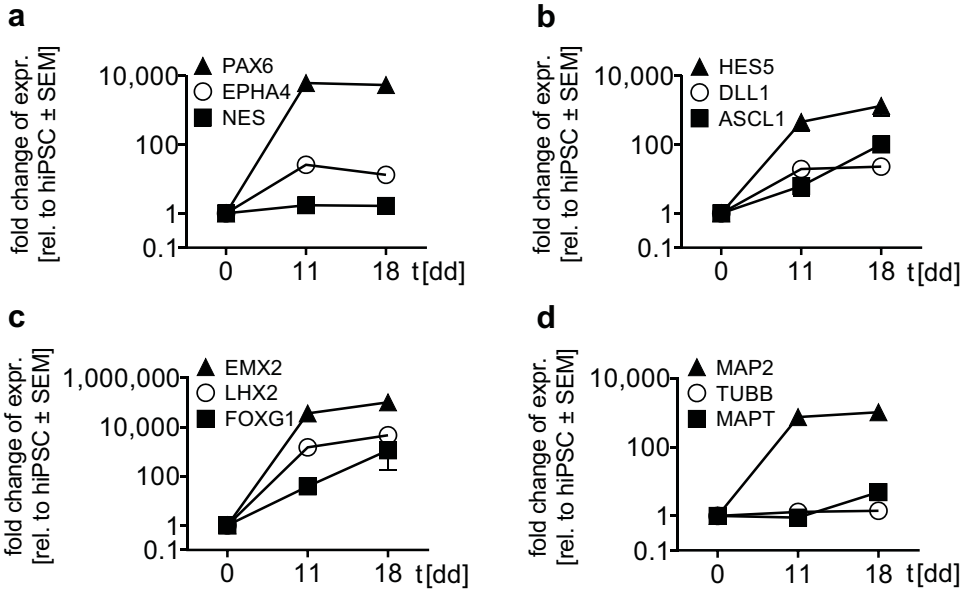

Fig. 2 Marker expression during the differentiation from hiPSC. hiPSC were differentiated as in Fig. 1 and NEP were extracted on dd11, neurospheres on dd18. Gene expression was analyzed by qRT-PCR and graphed as fold-increase over undifferentiated hiPSC, arbitrarily set to 1. **a** Neural stem cell genes, **b** neuronal precursor genes, **c** patterning genes, **d** neuronal genes. $n \geq 8 \pm$ SEM

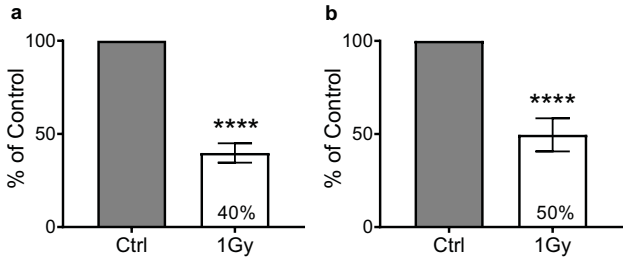

Fig. 3 Decreases in cell numbers after IR. hiPSC were differentiated to NEP over 11 days as in Fig. 1. On day 9 of differentiation (dd9) cells were irradiated with 1 Gy of ionizing radiation (X-ray). 48 h pIR, cells were detached and washed twice and living cells counted using trypan blue exclusion. Cell numbers of non-irradiated cells (Ctrl) were set arbitrarily as 100% and cell numbers of the irradiated cells expressed as proportions. **a** NEP differentiated from hESCs ($n=4$); **b** NEP differentiated from hiPSC ($n=11$). All data are shown as the mean ± SEM ****$p<0.0001$)

strong levels (data not shown), was found reduced twofold after irradiation (Fig. 4d). We also analyzed expression of neurodevelopmental disease-associated genes (Fig. 4 e). Out of this group, DCX (doublecortin, lisencephaly) was reduced the most with a reduction of 4.1-fold after irradiation. ZEB2 (Hirschsprung disease/Mowat–Wilson syndrome) was reduced threefold, whereas ARX (lisencephaly) was reduced 2.8-fold, and MCPH1 (microcephalin 1, microcephaly) was found reduced 1.8-fold in irradiated cells (Kato and Dobyns 2003). Expression of stress-related genes such as TP53 (p53), HDAC6, RSPO1 and TRH was also analyzed. All 4 stress genes were downregulated, with P53 1.8 fold, TRH 2.5-fold, HDAC6 2.7-fold and RSPO1 3.6-fold (Fig. 4f).

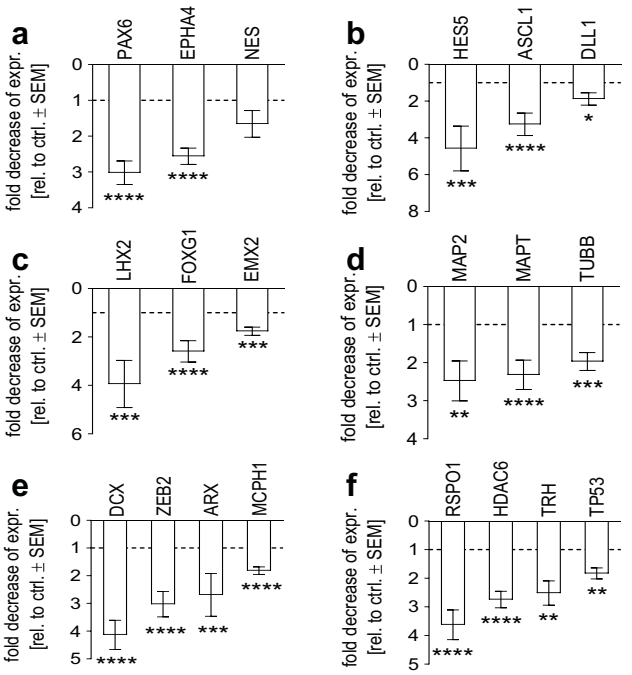

Fig. 4 Significant downregulation of all but one marker genes 48 h pIR. hiPSC were differentiated and irradiated as described in Fig. 1c. At dd11 (48 h pIR) marker gene expression was assessed by quantitative RT-PCR. Expression of **a** neural stem cell genes **b** neuronal precursor genes **c** patterning genes **d** neuronal genes **e** neuronal development genes and **f** stress response genes. Dotted lines indicate control levels. All data are shown as the mean fold change ± SEM relative to non-irradiated cells, set arbitrarily to 1 ($n \geq 8$; *$p<0.05$; **,****$p<0.01$)

Long-term effects of IR

To assess potential late effects of IR in cells differentiating from the surviving cells, NEP were irradiated as above. At 48 h pIR, neurospheres were generated from irradiated and non-irradiated NEP. The NS were matured for 1 week, until dd18, and gene expression analyzed. In this time frame, no significant difference in growth of the NS had been observed between irradiated and non-irradiated cells (Suppl. Fig. 2). Most of the analyzed genes, 19 of which were found downregulated in NEP at 48 h pIR, were expressed at control levels after 1 week of maturation in NS, suggesting that surviving cells mostly compensated irradiation effects (Fig. 5). Seven genes, however, were not expressed at control levels. The neural precursor marker HES5 was still expressed at a 1.9-fold reduced level when compared to non-irradiated cells (Fig. 5b). The same was observed for the expression of the neuronal microtubule protein tau MAPT, with a 1.6-fold reduction (Fig. 5d). ZEB2 and MCPH1, two genes involved in neurodevelopmental defects such as the Mowat–Wilson syndrome and microcephaly were also not expressed at normal levels, with a 1.5-fold and 1.2-fold reduction, respectively (Fig. 5e). The gene encoding the crucial neurotransmitter thyrotropin releasing hormone (TRH) had also a significantly reduced (2.5-fold) expression level. The stress-associated gene HDAC6 was also still reduced after 1 week of neurosphere culture, with a 1.3-fold reduction when compared to control (Fig. 5f). Interestingly, we found that the stress-associated gene RSPO1 did not only not normalize its reduced expression at 48 h pIR, but rather was overexpressed 1.7-fold in NS from irradiated NEP, compared to NS from non-irradiated NEP (Fig. 5f).

We next verified gene expression at the protein level. For this, we chose genes that were not back to normal mRNA expression levels on dd18, which were not secreted (TRH and RSPO1), and for which antibodies of acceptable quality were available. NEP were generated as above, irradiated on dd9 and proteins extracted 48 h pIR (dd11) and on dd18. We analyzed expression of HES5, HDAC6, and ZEB2 (Fig. 6). Of these three genes, only HES5 protein expression mirrored mRNA expression, with protein levels similarly reduced at 48 h after irradiation (dd11) and on dd18 (Fig. 6a). After irradiation, HES5 protein was expressed at 64% and 66% of control levels, on dd11 and on dd18, respectively (Fig. 6b). Protein levels of HDAC6 and ZEB2, however, were only reduced 48 h after irradiation and returned to control levels by dd18 (Fig. 6c, d). On dd11, HDAC6 was expressed at 48% of control levels, and returned to slightly, but not significantly, reduced levels of

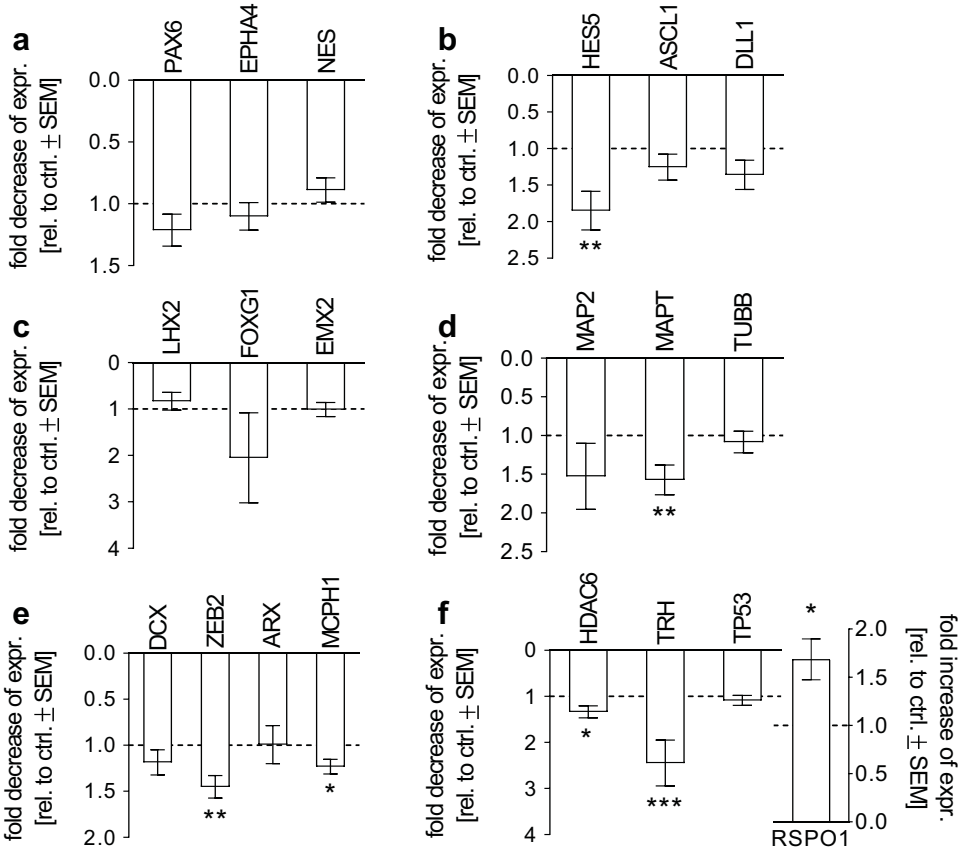

Fig. 5 Gene expression was still altered 9 days pIR. hiPSC were differentiated and irradiated as described in Fig. 1c. On d11 (48 h pIR) the irradiated cells were detached and neurospheres generated. On dd18, marker gene expression was assessed by quantitative RT-PCR. **a** Neural stem cell genes **b** neuronal precursor genes **c** patterning genes **d** neuronal genes **e** neuronal development genes and **f** stress response genes. Dotted lines indicate control levels. All data are shown as the mean fold change ± SEM relative to non-irradiated cells ($n \geq 8$; $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$)

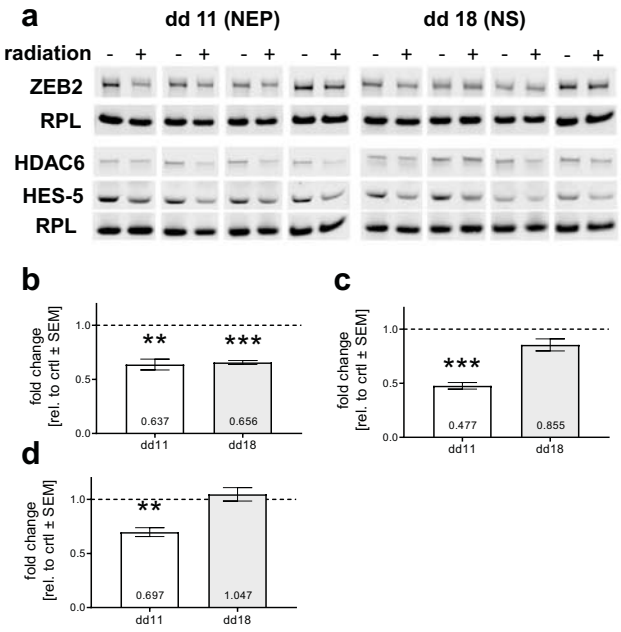

Fig. 6 Protein expression in irradiated NEP and NS. hiPSC were differentiated and irradiated as before, protein extracted from NEP on dd11 (48 h pIR) and from NS on dd18 (9 d pIR) and analyzed by Western blot for indicated proteins. **a** Western blots on dd11 and on dd18; **b**–**d** protein levels were quantified and compared to control expression, arbitrarily set to 1 (dotted lines). **b** HES-5; **c** HDAC6 and **d** ZEB2. Data are shown as mean ± SEM, $n = 4$; $^{**}p < 0.01$; $^{***}p < 0.001$

86%. ZEB2 was reduced to 70% of control levels on dd11 and returned to control levels on dd18.

Stemness after IR

PAX6$^+$ NEP are very early neural stem cell of the human brain (Zhang et al. 2010). To evaluate whether the observed radiation-induced reduction in mRNA and protein expression could have a functional impact on stemness of these early central nervous system progenitor cells, single cell suspensions were prepared 48 h pIR from NEP generated from hESC and hiPSC, titrated to decreasing numbers and plated into 96-well low adhesion plates. When prevented to adhere, neural stem cells (including NEP) form neurospheres in which the cells continue to proliferate and differentiate (Fasano et al. 2007; Hoelting et al. 2013; Ohtsuka et al. 2001). In limiting dilution assays, the stronger the stemness of the NEP is, the less cells are needed to form a neurosphere. We hypothesized that if stemness of the NEP was impaired by irradiation, as suggested by the reduced expression of developmentally crucial genes, neurosphere formation capacity should be reduced, particularly at lower cell numbers. As expected, 4 days after plating, we found neurosphere formation reduced in NEP that were irradiated with a single dose of X-rays 6 days earlier. When titrating non-irradiated NEP derived from hESC, no impact on NS formation could be observed at 1000 and 500 cells per well (data not shown). However, starting at 200 cells/well, a reduction in NS formation capacity was observed in non-irradiated cells, as not all wells received sufficient self-renewing NEP to form a NS. Neurosphere-forming capacity was reduced to neurosphere formation in 92% of the wells (Fig. 7a, ctrl. bar). At 100 cells/well this was further reduced to 75% of the seeded wells, and with 50 cells/well, only in 43% of the wells a NS had formed. After irradiation of the NEP, NS-formation capacity was even further reduced, to 63% of the wells containing a NS when seeding 200 cells/well, 38% of the wells when seeding 100 cells and 15% of the wells when seeding 50 cells/well. This equaled an IR-induced reduction of 68% at 200 cells, 51% at 100 cells and 35% at 50 cells/well, compared to the NS formation found in non-irradiated cells. When testing NEP derived from hiPSC, an impact of IR on stemness was only apparent at lower cell concentrations. At 50 cells/well, both in the non-irradiated

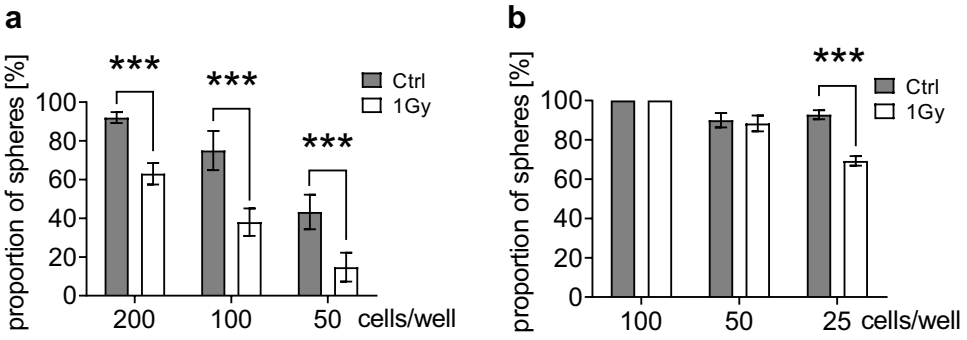

Fig. 7 Impaired neurosphere generation from irradiated cells. hiPSC were differentiated and irradiated as above, cells detached 48 h later, washed twice and live cells counted using trypan blue exclusion. Single-cell suspensions were plated into 96-well ultra-low adhesion round-bottom plates, at indicated cell numbers. After 4 days, wells containing an intact spheroid were counted and expressed as proportion of seeded wells. **a** Spheres generated from hESC (H9; $n = 4$); **b** spheres generated from hiPSC ($n = 6$). Data are shown as the mean percentage ± SEM; $^{***}p < 0.001$)

and irradiated cells NS formation was still observed in 90% of the wells, with no significant difference between the two (Fig. 7b). Only at 25 cells/well did we observe a significant IR-induced reduction of NS forming capacity. In control cells NS were formed in 93% of the wells, whereas in the irradiated cells NS were generated only in 69% of the wells, i.e., 74% of control.

Further titration to 12.5 cells/well was not conclusive, as with such low cell numbers distinguishing between cell aggregates and real spheres was difficult (Suppl. Fig. 3).

Taken together, these results strongly suggested impaired functional properties of NEP after irradiation, with potential late consequences.

Discussion

Prenatal development is a complex and sensitive process that is highly susceptible to environmental influences. Particularly development of the nervous system is very sensitive to environmental impact, resulting in so-called neurodevelopmental disabilities. These include learning and intellectual disabilities, difficulties with language and speech, autism, attention-deficit/hyperactivity disorder (ADHD), conduct disorders, cerebral palsy, spina bifida, impairments in vision and hearing, and other neurological dysfunctions. Millions of children are affected worldwide, with every 6th child affected in the USA (Boulet et al. 2009; Grandjean and Landrigan 2006).

Environmental chemicals have in certain instances been shown to play a role in neurodevelopmental disabilities (Grandjean and Landrigan 2014). However, despite increasing exposure to ionizing radiation due to medical diagnostics, radiotherapy for cancer and non-malignant disease, long-distance flights, transport of nuclear waste, and fallout pollution after nuclear reactor accidents, not much is known about its impact on the very early developing human brain. Importantly, however, human epidemiological data on environmental chemicals have clearly shown that neurodevelopmental disorders are caused at concentrations far below toxicity thresholds in adults, suggesting that likewise, low doses of radiation could cause damage to the developing brain (Grandjean and Landrigan 2006). This is supported by findings that neural stem cells are particularly sensitive to IR (Etienne et al. 2012; Fike et al. 2009). Studies of prenatally exposed survivors of Hiroshima and Nagasaki have shown harmful effects on the developing human brain, with severe mental retardation, reduced IQ and lower school performance. This was particularly pronounced when exposure took place 8–15 weeks after ovulation, when cells are highly proliferative and migratory (Otake and Schull 1998). Moreover, interference with normal brain development, which is still occurring after birth and is fueled by neural stem cells, is suggested by the observation that adults that had received radiotherapy in their childhood, developed cognitive impairment and rapidly progressing Alzheimer-like symptoms (Hall et al. 2004; Spiegler et al. 2004).

In humans, development of the central nervous system (CNS) begins with the formation of the neural tube. In rodents this event occurs approximately mid-gestation, on gestational day 10.5–11 in rats, and on day 9–9.5 in mice (DeSesso et al. 1999). Comparatively, the formation of the neural tube occurs much earlier during human fetal development (gestational day 24–28), in a gestation period of 266–280 days (Rice and Barone 2000). At this time, a pregnancy is often still unknown and protection of the fetus is not factored in during radiation-based diagnostics or therapy of the mother (Hill 2019). Particularly during the very early developmental time frame, environmental influences can have a severe impact on the developing brain, particularly as early as during neural tube formation. Another major difference between human and rodent brain development is that, in humans, brain development continues after birth, into early adulthood, resulting in continued sensitivity to insults (Gogtay et al. 2004). Both the very early time points of neurogenesis and the impact on postnatal human brain development can only be poorly modeled in rodent models. Here, we modelled the very early phase of human brain development with a pluripotent stem cell (PSC)-derived in vitro model in which we differentiated PSC into $PAX6^+$-progenitor cells. In human fetuses the very first CNS, $PAX6^+$, progenitor cells appear in the third week of gestation, when the neural tube forms. In week 4, at E26, when forebrain and midbrain have already been clearly demarcated, PAX6 expression is restricted to the forebrain and part of the spinal cord (Zhang et al. 2010). Here, we investigated the effects of IR on these very early $PAX6^+$ progenitor cells (NEP), in a 3D differentiation model favoring forebrain development (Hoelting et al. 2013). We chose to irradiate at a dose of 1 Gy, as several developmental studies in rats had previously detected effects on the nervous system at doses from 1 to 2 Gy (Schull et al. 1990; Verheyde and Benotmane 2007). In addition, in human studies, cognitive defects after cranial radiation exposure for ringworm treatment were detected for doses in the range of 1–1.5 Gy (Ron et al. 1982; Verheyde and Benotmane 2007). Furthermore, in RT for cancer and non-malignant disease, the overall doses vary between 3 and 75 Gy, but are typically delivered in fractions of 1–5 Gy (Seegenschmiedt et al. 2015; UNSCEAR Report 2008). At 1 Gy, when analyzing gene expression of genes crucial for neurodevelopment, we observed mild to strong reduction in expression in all but 1 gene (NES) of the 20 genes analyzed 48 h pIR. The genes analyzed included markers for NSC, neuronal precursor cells, patterning, neuronal cells, and genes associated with disease phenotypes, suggesting that neural development may be severely affected. Proliferation

of the cells was also reduced at 48 h (data not shown). It is, however, a possibility, that gene expression at that time point (48 h pIR) may still have been generally depressed in the surviving cells, so shortly after irradiation-induced-apoptosis. Depending on the cell type analyzed, global mRNA decay has been described after 2.5–6 h of induction of apoptosis (Thomas et al. 2015). In contrast to this, in our hands, 48 h after irradiation, the four tested house-keeping genes and the neural stem cell marker NES were not affected at 48 h pIR. Nevertheless, a reduction in gene expression at that early time point after irradiation should not be over-interpreted.

As the herein used stem cell-derived neurodevelopmental model enables long-term exploration of gene expression, we cultured the irradiated cells for additional 9 days and analyzed gene expression on dd18. At this stage of in vitro development, expression of most genes in the irradiated cells was back to normal expression levels, suggesting a recovery of most radiation damage. However, six genes were still reduced in their mRNA expression on dd18. MCPH1 associated with primary autosomal recessive microcephaly, was still slightly, but significantly, reduced in mRNA expression on dd18 (Fig. 5) (Arroyo et al. 2017). Expression of the gene encoding the crucial neurotransmitter TRH was also still significantly reduced in expression. TRH is best known for its function in regulation and release of TSH (thyroid-stimulating hormone) (Joseph-Bravo et al. 2015). Furthermore, it leads to stress-induced depression-like behavior in mice when injected in the basolateral amygdala and is, together with TSH involved in Alzheimer's disease (Choi et al. 2015; Kelly et al. 2015; Molchan et al. 1991; Yates et al. 1983). Interestingly, when differentiating from hESC, TRH was the second most upregulated gene on dd18, with a 122-fold upregulation in gene arrays (Hoelting et al. 2013). MAPT, coding for the Tau protein associated with neurofibrillary tangle formation in Alzheimer's disease, was still reduced in mRNA expression 9 days pIR. ZEB2, a DNA-binding transcriptional repressor, interacts with activated SMADs, represses transcription of E-cadherin and has a crucial role in nervous system development and Schwann cell differentiation (Bar Yaacov et al. 2019; Comijn et al. 2001; Quintes et al. 2016). It is also mutated in Hirschsprung disease/Mowat–Wilson syndrome, including intellectual and delayed development. Its expression was still reduced on the mRNA level on dd18 (Fig. 5). Reduced expression was confirmed on the protein level by Western blot at 48 h pIR. By dd18 protein levels of ZEB2 were, however, back to control levels in the irradiated cells, while mRNA level recovered from threefold decrease to a 1.5-fold decrease, but still remained significantly reduced compared to the control. Posttranscriptional and posttranslational regulation of ZEB2 are not well characterized. ZEB2 can be regulated by microRNA 141 and Snail1 on the posttranscriptional level, but it can also be stabilized on the posttranslational level by FAT10 by decreasing its ubiquitination (Beltran et al. 2008; Hu et al. 2010; Zou et al. 2018). It is, however, likely, that no significant differences in protein levels were observed due to a lesser sensitivity of this method compared to quantitative PCR. Similarly, expression of the stress-associated gene HDAC6 was still reduced at the mRNA level on dd18, but only slightly, although not significantly, reduced in its protein expression, compared to control cells (Fig. 6c) (Matthias et al. 2008).

Interestingly, HES5, a basic helix–loop–helix transcriptional repressor in neural stem cells and target of NOTCH signaling, was still reduced at day 9 pIR. This could be confirmed at the protein level, with reduced expression of HES5 protein both at 48 h and 9 days pIR. In hESC-derived neurospheres, HES5 was the most upregulated gene from hESC to dd18, with a 134-fold upregulation of gene expression in gene arrays, indicating that this model is suitable to investigate the role of HES5 in human brain development in more detail (Hoelting et al. 2013). In the murine embryonic brain, HES5 plays a crucial role in the maintenance of neural stem cells. Overexpressing HES5 inhibits both neurogenesis and gliogenesis by keeping the cells undifferentiated, whereas in the absence of HES5 neural stem cells are not properly maintained, generating fewer and smaller NS (Ohtsuka et al. 1999, 2001). In our model, reduced expression of HES5 could suggest altered neurogenesis after IR. Supporting this hypothesis, we could also observe a significantly smaller NS diameter at a later time point (dd33) in NS generated from irradiated cells (Suppl. Fig. 2). We could likewise observe a decrease in NS formation capacity after irradiation (Fig. 7). A reduced expression of HES5 after irradiation thus suggests potential developmental problems and could be the underlying mechanism for the observed mental retardation, reduced IQ and lower school performance after in utero exposure to IR. Whether reduced stemness of the NEP after irradiation in our model is due to reduced HES5 expression remains to be investigated in more detail with experiments restoring HES5 expression after irradiation.

Most interestingly, however, mRNA expression of RSPO1, a canonical Wnt signaling agonist, which was reduced 3.6-fold 48 h pIR was not only not reduced anymore on dd18, but exceeded control levels by 1.7-fold. In mice, it has been shown to ameliorate lethal radiation-induced injury of intestinal crypts when delivered with mesenchymal stem cells, and in vitro to be crucial for survival and proliferation of intestinal crypts (Chen et al. 2017). RSPO1 plays also a role in cancer and in sex determination (Yoon and Lee 2012). In early development, RSPO1 expression is found on both sides of the roof plate, in the fore- hind- and midbrain as well as in the spinal cord (Kamata et al. 2004). In rats, RSPO1 was found to be colocalized with the neuronal marker HuD, indicating an expression by neurons (Li et al. 2014). While Wnt signaling is known to perform many

crucial tasks during development such as cell fate determination and early patterning events, the role of RSPO1 in human brain development is less well characterized (Noelanders and Vleminckx 2017). In our 3D model RSPO1 was the fifth most regulated gene in cells differentiating from hESC, with a 44-fold increase in gene arrays on dd18, underscoring a crucial role in neural development (Hoelting et al. 2013).

Thus, the herein described 3D model will enable the molecular exploration of the role of RSPO1 and other genes in human brain development in more detail and to investigate the molecular mechanisms underlying observed detrimental effects of IR on human brain development. It could also be used to identify mitigating strategies against radiation-induced damage to improve treatment outcome.

Acknowledgements This work was supported by Grant 02NUK025B from the German Ministry for Education and Research (BMBF), AK and ES were supported by the InViTe PhD program from the Baden-Wuerttemberg Ministry for Science, Research and Art (MWK Baden-Württemberg). We also thank George Daley, Harvard Medical School/Children's Hospital Boston, for providing the human iPSC, and Maria Moreno-Villanueva and Thilo Sindlinger, University of Konstanz, for very helpful input with the irradiation experiments.

Compliance with ethical standards

Conflict of interest The manuscript does not contain clinical studies or patient data and the authors declare no conflict of interest.

References

Acharya MM, Lan ML, Kan VH et al (2010) Consequences of ionizing radiation-induced damage in human neural stem cells. Free Radic Biol Med 49:1846–1855. https://doi.org/10.1016/j.freeradbiomed.2010.08.021

Arroyo M, Kuriyama R, Trimborn M et al (2017) MCPH1, mutated in primary microcephaly, is required for efficient chromosome alignment during mitosis. Sci Rep 7:13019. https://doi.org/10.1038/s41598-017-12793-7

Ayanlaja AA, Xiong Y, Gao Y et al (2017) Distinct features of doublecortin as a marker of neuronal migration and its implications in cancer cell mobility. Front Mol Neurosci 10:199. https://doi.org/10.3389/fnmol.2017.00199

Bajinskis A, Lindegren H, Johansson L, Harms-Ringdahl M, Forsby A (2011) Low-dose/dose-rate γ radiation depresses neural differentiation and alters protein expression profiles in neuroblastoma SH-SY5Y cells and C17.2 neural stem cells. Radiat Res 175:185–192

Banda M, Bommineni A, Thomas RA, Luckinbill LS, Tucker JD (2008) Evaluation and validation of housekeeping genes in response to ionizing radiation and chemical exposure for normalizing RNA expression in real-time PCR. Mutat Res 649:126–134. https://doi.org/10.1016/J.MRGENTOX.2007.08.005

Bansod S, Kageyama R, Ohtsuka T (2017) Hes5 regulates the transition timing of neurogenesis and gliogenesis in mammalian neocortical development. Development 144:3156–3167. https://doi.org/10.1242/dev.147256

Bar Yaacov R, Eshel R, Farhi E, Shemuluvich F, Kaplan T, Birnbaum RY (2019) Functional characterization of the ZEB2 regulatory landscape. Hum Mol Genet 28:1487–1497. https://doi.org/10.1093/hmg/ddy440

Barazzuol L, Jeggo PA (2016) In vivo sensitivity of the embryonic and adult neural stem cell compartments to low-dose radiation. J Radiat Res 57:i2–i10. https://doi.org/10.1093/jrr/rrw013

Beltran M, Puig I, Pena C et al (2008) A natural antisense transcript regulates Zeb2/Sip1 gene expression during Snail1-induced epithelial–mesenchymal transition. Genes Dev 22:756–769. https://doi.org/10.1101/gad.455708

Betlazar C, Middleton RJ, Banati RB, Liu G-J (2016) The impact of high and low dose ionising radiation on the central nervous system. Redox Biol 9:144–156. https://doi.org/10.1016/j.redox.2016.08.002

Bishop KM, Goudreau G, O'Leary DD (2000) Regulation of area identity in the mammalian neocortex by Emx2 and Pax6. Science 288:344–349

Boulet SL, Boyle CA, Schieve LA (2009) Health care use and health and functional impact of developmental disabilities among US children, 1997–2005. Arch Pediatr Adolesc Med 163:19. https://doi.org/10.1001/archpediatrics.2008.506

Brenner DJ, Hall EJ (2007) Computed tomography—an increasing source of radiation exposure. N Engl J Med 357:2277–2284. https://doi.org/10.1056/NEJMra072149

Broustas CG, Lieberman HB (2014) DNA damage response genes and the development of cancer metastasis. Radiat Res 181:111–130. https://doi.org/10.1667/RR13515.1

Cabrera JR, Lucas JJ (2017) MAP2 splicing is altered in Huntington's disease. Brain Pathol 27:181–189. https://doi.org/10.1111/bpa.12387

Chambers SM, Fasano CA, Papapetrou EP, Tomishima M, Sadelain M, Studer L (2009) Highly efficient neural conversion of human ES and iPS cells by dual inhibition of SMAD signaling. Nat Biotechnol 27:275–280. https://doi.org/10.1038/nbt.1529

Chen H, Chong ZZ, De Toledo SM, Azzam EI, Elkabes S, Souayah N (2016) Delayed activation of human microglial cells by high dose ionizing radiation. Brain Res 1646:193–198. https://doi.org/10.1016/j.brainres.2016.06.002

Chen W, Ju SS, Lu T et al (2017) Directional delivery of RSPO1 by mesenchymal stem cells ameliorates radiation-induced intestinal injury. Cytokine 95:27–34. https://doi.org/10.1016/J.CYTO.2017.02.004

Chin HW, Maruyama Y (1984) Age at treatment and long-term performance results in medulloblastoma. Cancer 53:1952–1958

Cho I-T, Lim Y, Golden JA, Cho G (2017) Aristaless related homeobox (ARX) interacts with β-catenin, BCL9, and P300 to regulate canonical Wnt signaling. PLoS One 12:e0170282. https://doi.org/10.1371/journal.pone.0170282

Choi J, Kim JE, Kim TK et al (2015) TRH and TRH receptor system in the basolateral amygdala mediate stress-induced depression-like behaviors. Neuropharmacology 97:346–356. https://doi.org/10.1016/j.neuropharm.2015.03.030

Comijn J, Berx G, Vermassen P et al (2001) The two-handed E box binding zinc finger protein SIP1 downregulates E-cadherin and induces invasion. Mol Cell 7:1267–1278. https://doi.org/10.1016/S1097-2765(01)00260-X

Dehmelt L, Halpain S (2005) The MAP2/Tau family of microtubule-associated proteins. Genome Biol 6:204. https://doi.org/10.1186/gb-2004-6-1-204

DeSesso JM, Scialli AR, Holson JF (1999) Apparent lability of neural tube closure in laboratory animals and humans. Am J Med Genet 87:143–162

Dies KA, Bodell A, Hisama FM et al (2013) Schizencephaly: association with young maternal age, alcohol use, and lack of prenatal care. J Child Neurol 28:198–203. https://doi.org/10.1177/0883073812467850

Dupe V, Rochard L, Mercier S et al (2011) NOTCH, a new signaling pathway implicated in holoprosencephaly. Hum Mol Genet 20:1122–1131. https://doi.org/10.1093/hmg/ddq556

Etienne O, Roque T, Haton C, Boussin D, ç ois Boussin FD (2012) Variation of radiation-sensitivity of neural stem and progenitor cell populations within the developing mouse brain. Int J Radiat Biol 88:694–702. https://doi.org/10.3109/09553002.2012.710927

Fasano CA, Dimos JT, Ivanova NB, Lowry N, Lemischka IR, Temple S (2007) shRNA knockdown of Bmi-1 reveals a critical role for p21-Rb pathway in NSC self-renewal during development. Cell Stem Cell 1:87–99. https://doi.org/10.1016/J.STEM.2007.04.001

Fike JR, Rosi S, Limoli CL (2009) Neural precursor cells and central nervous system radiation sensitivity. Semin Radiat Oncol 19:122–132. https://doi.org/10.1016/j.semradonc.2008.12.003

Fishman K, Baure J, Zou Y et al (2009) Radiation-induced reductions in neurogenesis are ameliorated in mice deficient in CuZnSOD or MnSOD. Free Radic Biol Med 47:1459–1467. https://doi.org/10.1016/j.freeradbiomed.2009.08.016

Florian C, Bahi-Buisson N, Bienvenu T (2012) FOXG1-related disorders: from clinical description to molecular genetics. Mol Syndromol 2:153–163 **(000327329)**

Gan HK, Bernstein LJ, Brown J et al (2011) Cognitive functioning after radiotherapy or chemoradiotherapy for head-and-neck cancer. Int J Radiat Oncol Biol Phys 81:126–134. https://doi.org/10.1016/j.ijrobp.2010.05.004

Gogtay N, Giedd JN, Lusk L, Hayashi KM, Greenstein D, Vaituzis AC, Nugent TF 3rd, Herman DH, Clasen LS, Toga AW, Rapoport JL, Thompson PM (2004) Dynamic mapping of human cortical development during childhood through early adulthood. Proc Natl Acad Sci USA 101:8174–8179. https://doi.org/10.1073/pnas.0402680101

Grandjean P, Landrigan PJ (2006) Developmental neurotoxicity of industrial chemicals. Lancet 368:2167–2178. https://doi.org/10.1016/S0140-6736(06)69665-7

Grandjean P, Landrigan PJ (2014) Neurobehavioural effects of developmental toxicity. Lancet Neural 13:330–338. https://doi.org/10.1016/S1474-4422(13)70278-3

Gregory KJ, Bibbo G, Pattison JE (2008) On the uncertainties in effective dose estimates of adult CT head scans. Med Phys 35:3501–3510. https://doi.org/10.1118/1.2952359

Gumy LF, Katrukha EA, Grigoriev I et al (2017) MAP2 defines a preaxonal filtering zone to regulate KIF1- versus KIF5-dependent cargo transport in sensory neurons. Neuron 94:347.e7–362.e7. https://doi.org/10.1016/J.NEURON.2017.03.046

Guo A, Salomoni P, Luo J et al (2000) The function of PML in p53-dependent apoptosis. Nat Cell Biol 2:730–736. https://doi.org/10.1038/35036365

Hall P, Adami H-O, Trichopoulos D et al (2004) Effect of low doses of ionising radiation in infancy on cognitive function in adulthood: swedish population based cohort study. BMJ (Clin Res ed) 328:19. https://doi.org/10.1136/bmj.328.7430.19

Hill MA (2019) Embryology Carnegie stage table. https://embryology.med.unsw.edu.au/embryology/index.php/Carnegie_stage_table Accessed 27 May 2019

Hoelting L, Scheinhardt B, Bondarenko O et al (2013) A 3-dimensional human embryonic stem cell (hESC)-derived model to detect developmental neurotoxicity of nanoparticles. Arch Toxicol 87:721–733. https://doi.org/10.1007/s00204-012-0984-2

Hou P-S, Chuang C-Y, Kao C-F et al (2013) LHX2 regulates the neural differentiation of human embryonic stem cells via transcriptional modulation of PAX6 and CER1. Nucleic Acids Res 41:7753–7770. https://doi.org/10.1093/nar/gkt567

Hu M, Xia M, Chen X et al (2010) MicroRNA-141 regulates Smad interacting protein 1 (SIP1) and inhibits migration and invasion of colorectal cancer cells. Dig Dis Sci 55:2365–2372. https://doi.org/10.1007/s10620-009-1008-9

Hubbert C, Guardiola A, Shao R et al (2002) HDAC6 is a microtubule-associated deacetylase. Nature 417:455–458. https://doi.org/10.1038/417455a

Ivanov VN, Hei TK (2014) Radiation-induced glioblastoma signaling cascade regulates viability, apoptosis and differentiation of neural stem cells (NSC). Apoptosis 19:1736–1754. https://doi.org/10.1007/s10495-014-1040-x

Joseph-Bravo P, Jaimes-Hoy L, Uribe R-M, Charli J-L (2015) 60 years of neuroendocrinology: TRH, the first hypophysiotropic releasing hormone isolated: control of the pituitary–thyroid axis. J Endocrinol 226:T85–T100. https://doi.org/10.1530/JOE-15-0124

Kageyama R, Shimojo H, Ohtsuka T (2018) Dynamic control of neural stem cells by bHLH factors. Neurosci Res 138:12–18. https://doi.org/10.1016/J.NEURES.2018.09.005

Kalm M, Lannering B, Björk-Eriksson T, Blomgren K (2009) Irradiation-induced loss of microglia in the young brain. J Neuroimmunol 206:70–75. https://doi.org/10.1016/j.jneuroim.2008.11.002

Kamata T, Katsube K-i, Michikawa M, Yamada M, Takada S, Mizusawa H (2004) R-spondin, a novel gene with thrombospondin type 1 domain, was expressed in the dorsal neural tube and affected in Wnts mutants. Biochim Biophys Acta 1676:51–62. https://doi.org/10.1016/J.BBAEXP.2003.10.009

Kato M, Dobyns WB (2003) Lissencephaly and the molecular basis of neuronal migration. Hum Mol Genet 12:89R–96R. https://doi.org/10.1093/hmg/ddg086

Katsura M, Cyou-Nakamine H, Zen Q et al (2016) Effects of chronic low-dose radiation on human neural progenitor cells. Sci Rep 6:20027. https://doi.org/10.1038/srep20027

Kelly JA, Boyle NT, Cole N et al (2015) First-in-class thyrotropin-releasing hormone (TRH)-based compound binds to a pharmacologically distinct TRH receptor subtype in human brain and is effective in neurodegenerative models. Neuropharmacology 89:193–203. https://doi.org/10.1016/j.neuropharm.2014.09.024

Khan A, Budnick A, Barnea D, Feldman DR, Tonorezos ES (2018) Hearing loss in adult survivors of childhood cancer treated with radiotherapy. Children 5:1–9. https://doi.org/10.3390/children5050059

Kovalchuk A, Kolb B (2017) Low dose radiation effects on the brain—from mechanisms and behavioral outcomes to mitigation strategies. Cell Cycle 16:1266. https://doi.org/10.1080/15384101.2017.1320003

Lee VMY, Goedert M, Trojanowski JQ (2001) Neurodegenerative tauopathies. Annu Rev Neurosci 24:1121–1159. https://doi.org/10.1146/annurev.neuro.24.1.1121

Li J-Y, Chai B, Zhang W, Fritze DM, Zhang C, Mulholland MW (2014) LGR4 and its ligands, R-spondin 1 and R-spondin 3, regulate food intake in the hypothalamus of male rats. Endocrinology 155:429–440. https://doi.org/10.1210/en.2013-1550

Liu X, Zhou Z-W, Wang Z-Q (2016) The DNA damage response molecule MCPH1 in brain development and beyond. Acta Biochim Biophys Sin (Shanghai) 48:678–685. https://doi.org/10.1093/abbs/gmw048

Manda K, Kavanagh JN, Buttler D, Prise KM, Hildebrandt G (2014) Low dose effects of ionizing radiation on normal tissue stem cells. Mutat Res Rev Mutat Res 761:1–9. https://doi.org/10.1016/j.mrrev.2014.02.003

Massie CE, Spiteri I, Ross-Adams H et al (2015) HES5 silencing is an early and recurrent change in prostate tumourigenesis. Endocr Relat Cancer 22:131–144. https://doi.org/10.1530/ERC-14-0454

Matthias P, Yoshida M, Khochbin S (2008) HDAC6 a new cellular stress surveillance factor. Cell Cycle 7:7–10. https://doi.org/10.4161/cc.7.1.5186

Memic F, Knoflach V, Sadler R et al (2016) Ascl1 is required for the development of specific neuronal subtypes in the enteric nervous system. J Neurosci 36:4339–4350. https://doi.org/10.1523/JNEUROSCI.0202-16.2016

Molchan SE, Lawlor BA, Hill JL et al (1991) The TRH stimulation test in Alzheimer's disease and major depression: relationship to clinical and CSF measures. Biol Psychiatry 30:567–576. https://doi.org/10.1016/0006-3223(91)90026-I

Morel L, Chiang MSR, Higashimori H et al (2017) Molecular and functional properties of regional astrocytes in the adult brain. J Neurosci 37:8706–8717. https://doi.org/10.1523/JNEUROSCI.3956-16.2017

Morini J, Babini G, Barbieri S et al (2018) A comparison between X-ray and carbon ion irradiation in human neural stem cells. Radiat Prot Dosim 183:1–5. https://doi.org/10.1093/rpd/ncy231

Neradil J, Veselska R (2015) Nestin as a marker of cancer stem cells. Cancer Sci 106:803–811. https://doi.org/10.1111/cas.12691

Noelanders R, Vleminckx K (2017) How Wnt signaling builds the brain: bridging development and disease. Neuroscientist 23:314–329. https://doi.org/10.1177/1073858416667270

Ohtsuka T, Ishibashi M, Gradwohl G, Nakanishi S, Guillemot F, Kageyama R (1999) Hes1 and Hes5 as notch effectors in mammalian neuronal differentiation. EMBO J 18:2196–2207. https://doi.org/10.1093/emboj/18.8.2196

Ohtsuka T, Sakamoto M, Guillemot F, Kageyama R (2001) Roles of the basic helix-loop-helix genes Hes1 and Hes5 in expansion of neural stem cells of the developing brain. J Biol Chem 276:30467–30474. https://doi.org/10.1074/jbc.M102420200

O'Leary DD, Sahara S (2008) Genetic regulation of arealization of the neocortex. Curr Opin Neurobiol 18:90–100. https://doi.org/10.1016/j.conb.2008.05.011

Ostrom QT, Gittleman H, Fulop J et al (2015) CBTRUS statistical report: primary brain and central nervous system tumors diagnosed in the United States in 2008–2012. Neuro Oncol 17:1–62. https://doi.org/10.1093/neuonc/nov189

Otake M, Schull WJ (1998) Radiation-related brain damage and growth retardation among the prenatally exposed atomic bomb survivors. Int J Radiat Biol 74:159–171. https://doi.org/10.1080/095530098141555

Packer RJ, Meadows AT, Rorke LB, Goldwein JL, D'Angio G (1987) Long-term sequelae of cancer treatment on the central nervous system in childhood. Med Pediatr Oncol 15:241–253

Panaliappan TK, Wittmann W, Jidigam VK et al (2018) Sox2 is required for olfactory pit formation and olfactory neurogenesis through BMP restriction and Hes5 upregulation. Development. https://doi.org/10.1242/dev.153791

Park NI, Guilhamon P, Desai K et al (2017) ASCL1 reorganizes chromatin to direct neuronal fate and suppress tumorigenicity of glioblastoma stem cells. Cell Stem Cell 21:209.e7–224.e7. https://doi.org/10.1016/J.STEM.2017.06.004

Parrini E, Conti V, Dobyns WB, Guerrini R (2016) Genetic basis of brain malformations. Mol Syndromol 7:220–233. https://doi.org/10.1159/000448639

Pearce MS, Salotti JA, Little MP et al (2012) Radiation exposure from CT scans in childhood and subsequent risk of leukaemia and brain tumours: a retrospective cohort study. Lancet 380:499–505. https://doi.org/10.1016/S0140-6736(12)60815-0

Pinnix CC, Yahalom J, Specht L, Dabaja BS (2018) Radiation in central nervous system leukemia: guidelines from the international lymphoma radiation oncology group. Int J Radiat Oncol Biol Phys 102:53–58. https://doi.org/10.1016/j.ijrobp.2018.05.067

Quintes S, Brinkmann BG, Ebert M et al (2016) Zeb2 is essential for Schwann cell differentiation, myelination and nerve repair. Nat Neurosci 19:1050–1059. https://doi.org/10.1038/nn.4321

Rice D, Barone S Jr (2000) Critical periods of vulnerability for the developing nervous system: evidence from humans and animal models. Environ Health Perspect 108(Suppl 3):511–533. https://doi.org/10.1289/ehp.00108s3511

Rola R, Raber J, Rizk A et al (2004) Radiation-induced impairment of hippocampal neurogenesis is associated with cognitive deficits in young mice. Exp Neurol 188:316–330. https://doi.org/10.1016/j.expneurol.2004.05.005

Romaniello R, Arrigoni F, Fry AE et al (2018) Tubulin genes and malformations of cortical development. Eur J Med Genet 61:744–754. https://doi.org/10.1016/J.EJMG.2018.07.012

Ron E, Modan B, Floro S, Harkedar I, Gurewitz R (1982) Mental function following scalp irradiation during childhood. Am J Epidemiol 116:149–160. https://doi.org/10.1093/oxfordjournals.aje.a113389

Schull WJ, Norton S, Jensh RP (1990) Ionizing radiation and the developing brain. Neurotoxicol Teratol 12:249–260. https://doi.org/10.1016/0892-0362(90)90096-U

Seegenschmiedt MH, Micke O, Muecke R, German Cooperative Group on Radiotherapy for Non-malignant Diseases (BSG-BD) (2015) Radiotherapy for non-malignant disorders: state of the art and update of the evidence-based practice guidelines. Br J Radiol 88:20150080. https://doi.org/10.1259/bjr.20150080

Shahsavani M, Pronk RJ, Falk R et al (2018) An in vitro model of lissencephaly: expanding the role of DCX during neurogenesis. Mol Psychiatry 23:1674–1684. https://doi.org/10.1038/mp.2017.175

Sharma N, Colangelo NW, de Toledo SM, Azzam EI (2016) Diffusible factors secreted by glioblastoma and medulloblastoma cells induce oxidative stress in bystander neural stem progenitors. ASN Neuro. https://doi.org/10.1177/1759091416662808

Shimada M, Matsuzaki F, Kato A, Kobayashi J, Matsumoto T, Komatsu K (2016) Induction of excess centrosomes in neural progenitor cells during the development of radiation-induced microcephaly. PLoS One 11:e0158236. https://doi.org/10.1371/journal.pone.0158236

Shimura T, Sasatani M, Kawai H et al (2017) A comparison of radiation-induced mitochondrial damage between neural progenitor stem cells and differentiated cells. Cell Cycle 16:565–573. https://doi.org/10.1080/15384101.2017.1284716

Smith-Bindman R, Lipson J, Marcus R et al (2009) Radiation dose associated with common computed tomography examinations and the associated lifetime attributable risk of cancer. Arch Intern Med 169:2078–2086. https://doi.org/10.1001/archinternmed.2009.427

Sokolov M, Neumann R (2013) Lessons learned about human stem cell responses to ionizing radiation exposures: a long road still ahead of us. Int J Mol Sci 14:15695–15723. https://doi.org/10.3390/ijms140815695

Sorrells SF, Paredes MF, Cebrian-Silla A et al (2018) Human hippocampal neurogenesis drops sharply in children to undetectable levels in adults. Nature 555:377–381. https://doi.org/10.1038/nature25975

Soussain C, Ricard D, Fike JR, Mazeron JJ, Psimaras D, Delattre JY (2009) CNS complications of radiotherapy and chemotherapy. Lancet 374:1639–1651. https://doi.org/10.1016/S0140-6736(09)61299-X

Spiegler BJ, Bouffet E, Greenberg ML, Rutka JT, Mabbott DJ (2004) Change in neurocognitive functioning after treatment with cranial radiation in childhood. J Clin Oncol 22:706–713. https://doi.org/10.1200/JCO.2004.05.186

Spix C, Grosche B, Bleher M, Kaatsch P, Scholz-Kreisel P, Blettner M (2017) Background gamma radiation and childhood cancer in Germany: an ecological study. Radiat Environ Biophys 56:127–138. https://doi.org/10.1007/s00411-017-0689-2

Tallapaka K, Venugopal V, Dalal A, Aggarwal S (2018) Novel RSPO1 mutation causing 46, XX testicular disorder of sex development with palmoplantar keratoderma: a review of literature and expansion of clinical phenotype. Am J Med Genet A 176:1006–1010. https://doi.org/10.1002/ajmg.a.38646

Thomas MP, Liu X, Whangbo J et al (2015) Apoptosis triggers specific, rapid, and global mRNA decay with 3′ uridylated

intermediates degraded by DIS3L2. Cell Rep 11:1079. https://doi.org/10.1016/J.CELREP.2015.04.026

Thomson JA, Itskovitz-Eldor J, Shapiro SS et al (1998) Embryonic stem cell lines derived from human blastocysts. Science 282:1145–1147

Tohyama J, Yamamoto T, Hosoki K et al (2011) West syndrome associated with mosaic duplication of FOXG1 in a patient with maternal uniparental disomy of chromosome 14. Am J Med Genet A 155:2584–2588. https://doi.org/10.1002/ajmg.a.34224

Tzoulaki I, White IMS, Hanson IM (2005) PAX6 mutations: genotype–phenotype correlations. BMC Genet 6:27. https://doi.org/10.1186/1471-2156-6-27

United Nations Scientific Committee on the Effects of Atomic Radiation UNSCEAR 2013 Report to the General Assembly, Vol II. Scientific Annex B https://www.unscear.org/docs/reports/2013/UNSCEAR2013Report_AnnexB_Children_13-87320_Ebook_web.pdf. Accessed 03 Mar 2019

United Nations Scientific Committee on the Effects of Atomic Radiation UNSCEAR 2008 Report to the General Assembly, Scientific Annexes A and B. https://www.unscear.org/docs/publications/2008/UNSCEAR_2008_Report_Vol.I.pdf. Accessed 20 Feb 2019

Van Hoecke A, Schoonaert L, Lemmens R et al (2012) EPHA4 is a disease modifier of amyotrophic lateral sclerosis in animal models and in humans. Nat Med 18:1418–1422. https://doi.org/10.1038/nm.2901

Verheyde J, Benotmane MA (2007) Unraveling the fundamental molecular mechanisms of morphological and cognitive defects in the irradiated brain. Brain Res Rev 53:312–320. https://doi.org/10.1016/J.BRAINRESREV.2006.09.004

Waniek A, Hartlage-Rübsamen M, Höfling C et al (2015) Identification of thyrotropin-releasing hormone as hippocampal glutaminyl cyclase substrate in neurons and reactive astrocytes. Biochim Biophys Acta 1852:146–155. https://doi.org/10.1016/J.BBADIS.2014.11.011

Wilson KD, Sun N, Huang M et al (2010) Effects of ionizing radiation on self-renewal and pluripotency of human embryonic stem cells. Cancer Res 70:5539–5548. https://doi.org/10.1158/0008-5472.CAN-09-4238

Xu C, Inokuma MS, Denham J et al (2001) Feeder-free growth of undifferentiated human embryonic stem cells. Nat Biotechnol 19:971–974. https://doi.org/10.1038/nbt1001-971

Yamaguchi M, Kashiwakura I (2013) Role of reactive oxygen species in the radiation response of human hematopoietic stem/progenitor cells. PLoS One 8:1–7. https://doi.org/10.1371/journal.pone.0070503

Yamoah K, Showalter TN, Ohri N (2015) Radiation therapy intensification for solid tumors: a systematic review of randomized trials. Int J Radiat Oncol Biol Phys 93:737–745. https://doi.org/10.1016/j.ijrobp.2015.07.2284

Yan S, Li P, Wang Y et al (2016) Nestin regulates neural stem cell migration via controlling the cell contractility. Int J Biochem Cell Biol 78:349–360. https://doi.org/10.1016/J.BIOCEL.2016.07.034

Yang W, Liu Y, Gao R, Yu H, Sun T (2018) HDAC6 inhibition induces glioma stem cells differentiation and enhances cellular radiation sensitivity through the SHH/Gli1 signaling pathway. Cancer Lett 415:164–176. https://doi.org/10.1016/J.CANLET.2017.12.005

Yates CM, Harmar AJ, Rosie R et al (1983) Thyrotropin-releasing hormone, luteinizing hormone-releasing hormone and substance P immuno-reactivity in post-mortem brain from cases of Alzheimer-type dementia and Down's syndrome. Brain Res 258:45–52. https://doi.org/10.1016/0006-8993(83)91224-6

Yoon JK, Lee JS (2012) Cellular signaling and biological functions of R-spondins. Cell Signal 24:369–377. https://doi.org/10.1016/j.cellsig.2011.09.023

Zhang X, Huang CT, Chen J et al (2010) Pax6 is a human neuroectoderm cell fate determinant. Cell Stem Cell 7:90–100. https://doi.org/10.1016/j.stem.2010.04.017

Zhao J, Cooper LT, Boyd AW, Bartlett PF (2018) Decreased signalling of EphA4 improves functional performance and motor neuron survival in the SOD1G93A ALS mouse model. Sci Rep 8:11393. https://doi.org/10.1038/s41598-018-29845-1

Zhou F, Gou S, Xiong J, Wu H, Wang C, Liu T (2014) Oncogenicity of LHX2 in pancreatic ductal adenocarcinoma. Mol Biol Rep 41:8163–8167. https://doi.org/10.1007/s11033-014-3716-2

Zou Y, Zhang N, Ellerby LM et al (2012) Responses of human embryonic stem cells and their differentiated progeny to ionizing radiation. Biochem Biophys Res Commun 426:100–105. https://doi.org/10.1016/j.bbrc.2012.08.043

Zou Y, Ouyang Q, Wei W, Yang S, Zhang Y, Yang W (2018) FAT10 promotes the invasion and migration of breast cancer cell through stabilization of ZEB2. Biochem Biophys Res Commun 506:563–570. https://doi.org/10.1016/j.bbrc.2018.10.109

Zweier C, Thiel CT, Dufke A et al (2005) Clinical and mutational spectrum of Mowat–Wilson syndrome. Eur J Med Genet 48:97–111. https://doi.org/10.1016/J.EJMG.2005.01.003