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The influence of retinoic acid-induced differentiation on the radiation response of male germline stem cells

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Abstract

Lifelong mammalian male fertility is maintained through an intricate balance between spermatogonial proliferation and differentiation. DNA damage in spermatogonia, for instance caused by chemo- or radiotherapy, can induce cell cycle arrest or germ cell apoptosis, possibly resulting in male infertility. Spermatogonia are generally more radiosensitive and prone to undergo apoptosis than somatic cells. Among spermatogonial sub-types the response to DNA damage is differentially modulated; undifferentiated spermatogonia, including the spermatogonial stem cells (SSCs), are relatively radio-resistant, whereas differentiating spermatogonia are very radiosensitive. To investigate the molecular mechanisms underlying this difference, we used an *in vitro* system consisting of mouse male germline stem (GS) cells that can be induced to differentiate. Using RNA-sequencing analysis, we analyzed the response of undifferentiated and differentiating GS cells to ionizing radiation (IR). At the RNA expression level, both undifferentiated and differentiating GS cells showed a very similar response to IR. Protein localization of several genes found to be involved in either spermatogonial differentiation or radiation response was investigated using mouse testis sections. For instance, we found that the transcription factor PDX1 was specifically expressed in undifferentiated spermatogonia and thus may be a novel marker for these cells. Interestingly, also at the protein level, undifferentiated GS cells showed a more pronounced upregulation of p53 in response to IR than differentiating GS cells. The higher p53 protein level in undifferentiated spermatogonia may preferentially induce

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Conflict of interest

The authors declare that there are no conflicts of interest.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <https://doi.org/10.1016/j.dnarep.2018.08.027>.

cell cycle arrest, thereby giving these cells more time to repair inflicted DNA damage and increase their radio-resistance.

Keywords

Spermatogonia; Differentiation; Spermatogonial markers; DNA damage response; p53; Transcriptome

1. Introduction

Spermatogenesis is an intricate process that takes place in the seminiferous tubules within the testis. In mammals, the entire process of spermatogenesis is comprised of three consecutive phases: a mitotic phase (spermatogonial proliferation and differentiation), a meiotic phase (spermatocyte meiotic divisions to generate haploid spermatids) and spermiogenesis (elongation and maturation of spermatids) [1]. For continuous spermatogenesis spermatogonial stem cells (SSCs) are essential. SSCs can be defined as a subpopulation of undifferentiated spermatogonia able to generate and maintain donor-derived spermatogenesis when transplanted into infertile recipient testes [2,3]. Continuous spermatogenesis requires a constant balance between SSC self-renewal, proliferation and differentiation [1]. Within the seminiferous tubules, spermatogenesis occurs in an orchestrated spatiotemporal fashion in which specific germ cell types are grouped in specific stages of the seminiferous epithelium. The undifferentiated spermatogonia may divide freely during all of these epithelial stages. In contrast, differentiating spermatogonia are irreversibly committed towards meiosis and their subsequent divisions are strictly dictated by the epithelial stage in which they are present [4].

Because DNA damage, for instance caused by gonadotoxic chemicals or ionizing radiation (IR), can result in gene mutations or chromosomal aberrations, DNA damage often causes spermatogonial apoptosis or activates a male-specific meiotic arrest checkpoint [5–9]. These forms of spermatogenic arrest then prevent genomic aberrations being transmitted via the sperm. Indeed, in the human, treatment with chemo- or radiotherapy in adult males often results in impaired fertility [10]. Spermatogenic cells generally exhibit a slow rate of DNA repair together with a high incidence of unrepaired DNA damage, which renders them more radiosensitive than somatic cells [11,12].

Because genetic aberrations in SSCs have the potential to result in lifelong generation of mutated sperm, one might expect that SSCs, when compared to all other spermatogonia, are most prone to undergo apoptosis in response to IR. However, this appears not to be the case. It turns out that differentiating spermatogonia are actually much more radiosensitive and show a stronger apoptotic response [13–15]. Among undifferentiated spermatogonia, the self-renewing SSCs are most resistant to DNA damage induced by either the alkylating agent busulfan or IR [16–18]. Evidently, while the damaged differentiating spermatogonia are more easily sacrificed, preservation of SSCs, and thus the long-term fertility, seem to outweigh a certain risk of mutated offspring.

What determines the differences in radio-sensitivity among spermatogonial subtypes is currently unknown. Several DNA damage response proteins have been reported to be differentially regulated during spermatogonial differentiation. For instance, phosphorylated histone H2 AX (γ -H2 AX), usually marking DSBs, has been described to increase with spermatogonial differentiation [12,19] and is highly expressed in intermediate and B spermatogonia [20]. The DNA damage response protein p53 has been found to be induced in all spermatogonia by irradiation, but knockout of p53 seems to predominantly affect the apoptotic response of undifferentiated spermatogonia [5,21,22]. Nevertheless, transplantation assays of mutated SSCs revealed that deficiency in a specific p53 pathway (Trp53-Trp53inp1-Tnfrsf10b) actually increased survival of SSCs after irradiation [23]. The same study also reported that the apoptosis-inducing protein BBC3 was specifically active in differentiating spermatogonia after irradiation [23].

To investigate the relation between the IR-induced DNA damage response and spermatogonial differentiation, we used an established culture system for undifferentiated mouse spermatogonia [24,25]. In this culture system, primary isolated mouse SSCs, then referred to as male germline stem (GS) cells, can propagate *in vitro* for years without losing SSC properties [25]. GS cells can also be induced to differentiate by adding retinoic acid (RA) to the culture medium [26,27]. Moreover, by way of RNA-sequencing (RNA-seq), the transcriptome of RA-induced differentiating GS cells was reported recently [27]. To gain insights into the differential DNA damage responses of undifferentiated and differentiating spermatogonia, we investigated the transcriptomes of irradiated and non-irradiated GS cells with or without RA treatment.

2. Materials and methods

2.1. Animals

Neonatal (4–5 d.p.p) DBA/2 J male mice were used for GS cell isolation, and adult (~8 weeks) C57BL/6 J male mice were used for irradiation and immunohistochemical analysis. For histological analysis on neonatal testis sections, 8 d.p.p old C57BL/6 J male mice were used. All animal procedures were in accordance with and approved by the animal ethical committee of the Academic Medical Center, University of Amsterdam or in accordance with the National Institutes of Health and US Department of Agriculture criteria approved by the Institutional Animal Care and Use Committees of Johns Hopkins University.

2.2. GS cell culture

A mouse GS cell line was established as previously reported [24,28]. Briefly, testes were harvested from neonatal DBA/2 J male mice, and after removing the tunica albuginea, testicular tissues were mechanically dissociated and subjected to a collagenase-trypsin dissociation to obtain a single-cell suspension. Germ cells were enriched by an overnight differential plating and cultured in a medium mainly composed of StemPro-34 SFM medium (Thermo Fisher Scientific), StemPro-34 Supplement (Thermo Fisher Scientific), 1% fetal bovine serum (FBS), recombinant human GDNF (10 ng/ml, Peprotech), recombinant human bFGF (10 ng/ml, Peprotech), as well as other components as previously reported [24]. The cells were cultured on mitotically inactivated mouse embryonic fibroblasts (MEFs) since the

third passage and were refreshed every 2–3 days and passaged every 5–7 days at a ratio of 1:4–6. The cells were maintained at 37 °C in an atmosphere of 5% CO₂ in air.

2.3. RA treatment

Before RA treatment, GS cells cultured on MEFs were transferred to laminin (20 µg/ml, Sigma-Aldrich)-coated wells. On the next day, GS cells were treated with 2µM all-trans-RA (Sigma-Aldrich) in culture medium for 48–72 hours. In control groups, vehicle (0.1% ethanol in medium) was applied to the cells.

2.4. Ionizing irradiation (IR)

Before IR treatment, GS cells cultured on MEFs were transferred to laminin (20 µg/ml, Sigma-Aldrich)-coated wells. On the next day, GS cells were subjected to 1 Gy of IR emitted by a ¹³⁷Cs source, a dose that causes substantial DNA damage but does not necessarily kill spermatogonia *in vivo* [15]. Because spermatogonial p53 is significantly induced in response to IR after 3 h [5], cells were used 3 h after IR or sham irradiation (same treatment but leaving out the actual exposure to IR). To prepare irradiated mice, adult C57BL/6 J male mice were exposed to a whole-body IR (1 Gy) and killed 3 h post IR (or sham IR), after which the testes were fixed in 4% paraformaldehyde (PFA).

2.5. Quantitative-real time PCR (Q-PCR)

Total RNA was extracted from GS cells using ISOLATE II RNA Mini Kit (Bioline) and following the protocol provided by the manufacturer. After treatment with DNase (Qiagen) and tests for genomic DNA-free, RNA samples were reversely transcribed, using SensiFAST cDNA Synthesis Kit (Bioline). The synthesized cDNA was then used for Q-PCR reactions, using the Roche LightCycler 480 platform (the 384-well plate format). The Q-PCR reaction was performed in a 10 µl volume system including 2× LightCycler 480 SYBR Green I Master (Roche). *Ppt2* and *Mtg1* were used as reference genes, and the data were analyzed using the – C_t method. Data were presented as the mean ± standard error of mean (SEM) of 3 independent experiments (n = 3). Differences between groups were assessed using the Student's *t*-test. *P* < 0.05 was considered statistically significant and *P* < 0.01 was considered extremely significant. The primers for Q-PCR analysis are listed in Table 1.

2.6. Western blot

Proteins were extracted from the cells and quantified with Qubit Protein Assay Kit (Thermo Fisher Scientific). Then Western blot analysis was performed as reported previously [28–30], using the LI-COR Odyssey imaging system (LI-COR Biosciences). The primary antibodies used were mouse anti-PLZF (1:100; D-9, Santa Cruz Biotechnology), mouse anti-OCT4 (1:200; C-10, Santa Cruz Biotechnology), rabbit anti-STRA8 (1:500; ab49602, Abcam), rabbit anti-p53 (1:100; FL-393, Santa Cruz Biotechnology), rabbit anti-GAPDH (1:400; FL-335, Santa Cruz Biotechnology) and mouse anti-β-actin (1:5000; A1978, Sigma-Aldrich). For quantification of the relative p53 protein expression, p53 band intensities were divided by those of GAPDH. Data were presented as the mean ± SEM of 4 independent experiments (n = 4). Differences among groups were assessed using the one-way ANOVA

followed by the LSD test. $P < 0.05$ was considered statistically significant and $P < 0.01$ was considered extremely significant.

2.7. Immunocytochemistry (ICC) and immunohistochemistry (IHC)

For ICC, GS cells were grown on laminin (20 $\mu\text{g}/\text{ml}$, Sigma-Aldrich)-coated glass coverslips in 24-well plates or multi-chambered cover-glass slides (Labtek II, Nunc). Cells were fixed, permeabilized and blocked as previously described [28,30], followed by 4 °C overnight incubation with the following primary antibodies: mouse anti-PLZF (1:50; D-9, Santa Cruz Biotechnology), mouse anti-OCT4 (1:50; C-10, Santa Cruz Biotechnology), rabbit anti-LIN28 A (1:1000; ab46020, Abcam), rabbit anti-ID4 (1:100; M106, CalBioReagents), mouse anti- γ -H2AX (1:20,000; 05-636, Merck Millipore), rabbit anti-WNT16 (1:100; H-96, Santa Cruz Biotechnology), rabbit anti-SLIT2 (1:80; ab7665, Abcam). Replacing primary antibodies with phosphate buffered saline (PBS) containing 0.5% bovine serum albumin (BSA) was used as negative controls. On the next day, the cells were washed and incubated with the corresponding host-specific secondary antibodies (Alexa Fluor 488 or 555, 1:1000; Thermo Fisher Scientific), and counterstained with DAPI. The cells were mounted on glass slides with the Prolong Gold anti-fade mountant (Thermo Fisher Scientific) and later visualized under the microscope. Microscopy was performed as previously described [28]. For quantification of γ -H2AX⁺ cells, at least 200 cells were analyzed in each group. Data were presented as the mean $\pm$ SEM of 4 independent experiments ($n = 4$). Differences among groups were assessed using the one-way ANOVA followed by the LSD test. $P < 0.05$ was considered statistically significant and $P < 0.01$ was considered extremely significant.

For IHC, testes were collected from normal adult and neonatal mice, or the experimental and control mice at 3 h post (sham) IR, fixed in diluted Bouin's solution or 4% PFA, and embedded in paraffin. Testis sections were sliced at a thickness of 5 μm . After deparaffinization and rehydration, testis sections were alternatively subjected to microwave-mediated antigen retrieval in sodium citrate buffer (0.01 M, pH 6.0), followed by blocking in Super Block (ScyTek Laboratories) or PBS with 1% BSA and 0.1% Tween-20, for 1 h at room temperature (RT). Then the sections were incubated with primary antibodies diluted in Normal antibody Diluent (ImmunoLogic) or PBS containing 0.5% BSA and 0.1% Tween-20, at 4 °C overnight. The primary antibodies used were rabbit anti-WNT16 (1:100; H-96, Santa Cruz Biotechnology), rabbit anti-SLIT2 (1:80; ab7665, Abcam), rabbit anti-PDX1 (1:1000; ab47267, Abcam), rabbit anti-ADORA1 (1:600; ab82477, Abcam), rabbit anti-p53 (1:100; FL-393, Santa Cruz Biotechnology), rabbit anti-BBC3 (1:250; ab9643, Abcam), rabbit anti-PLK2 (1:100; H-90, Santa Cruz Biotechnology), rabbit anti-PDRG1 (1:100; 16968-1-AP, Proteintech) and rabbit anti-SESN2 (1:100; 10795-1-AP, Proteintech). Isotype rabbit IgGs replaced primary antibodies in negative controls (Fig. S1). On the next day, the sections were washed and incubated with PowerVision Poly-HRP-Anti-mouse/rabbit/rat secondary antibody (ImmunoLogic) for 1 h at RT. After washing, the sections were stained with diaminobenzidine (DAB) and counterstained with hematoxylin. Then the slides were dehydrated and embedded in Entellan (Merck Millipore). Microscopy was performed as previously described [29]. For IHC, all experiments were repeated 3 times using different mice ($n = 3$).

2.8. EdU assay

GS cells were grown on laminin-coated multi-chambered cover-glass slides (Labtek II, Nunc) and subjected to IR treatment and/or RA exposure as described. The EdU assay was performed as previously described [28]. For quantification of EdU⁺ cells, at least 300 cells were analyzed in each group. Data were presented as the mean $\pm$ SEM of 3 independent experiments ($n = 3$). Differences among groups were assessed using the one-way ANOVA followed by the LSD test. $P < 0.05$ was considered statistically significant and $P < 0.01$ was considered extremely significant.

2.9. RNA-sequencing (RNA-seq) analysis

Total RNA was extracted from GS cells which had been subjected to RA treatment for 48 h and/or IR 3 h before, using ISOLATE II RNA Mini Kit (Bioline) and following the protocol provided by the manufacturer. After treatment with DNase (Qiagen) and tests for genomic DNA-free, RNA samples from 3 independent experiments (biological triplicates) were sent to BGI Tech Solutions (HongKong) where libraries were constructed (TruSeq, 160bp) and paired-end sequencing was performed (101bp, Illumina HiSeq 4000). RNA-seq analysis was conducted as described previously [28]. In brief, clean reads were subjected to quality control, and then aligned to UCSC mm10 GRCm38.p4 GTF using HISAT2 (v2.0.4) [31]. Counts were obtained using HTSeq (v0.6.1) [32]. Count tables were made and all genes without counts in any of the samples were removed, whilst genes with more than 1 count-per-million reads (CPM) in 2 of the samples were kept. Genes were re-annotated using biomaRt and mm10 of Ensembl. Count data were transformed to log₂-counts per million (logCPM) using voom, estimating the mean-variance relationship. Differential expression was assessed using a moderated *t*-test and the linear model framework from the limma package. Benjamini-Hochberg false discovery rate (FDR) was used to correct for multiple testing of the resulting *p*-values. The entire analysis was performed using R v3.2.2 and Bioconductor v3.0 [33,34].

Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses were conducted using DAVID bioinformatics resources 6.8 [35,36], and Gene Set Enrichment Analysis (GSEA) [37,38] was used to analyze differentially expressed gene sets.

2.10. Accession numbers

All sequencing data have been submitted to NCBI (SRA) and will be available under the accession number: SUB2838515 (BioProject ID PRJNA392875).

3. Results

3.1. Culture and differentiation of GS cells

First, we established a GS cell line according to a previously published and well-demonstrated protocol [24]. GS cells can be maintained on either a feeder layer of MEFs or in laminin-coated wells [39]. Like previously described [39], GS cells cultured on mouse embryonic fibroblasts (MEFs) formed typical grape/morula-like colonies, whereas they started to form chain-like structures when seeded in laminin-coated wells (Fig. 1A). The

cultured GS cells were positive for putative SSC/progenitor markers PLZF, LIN28 A, OCT4 and ID4 (Fig. 1B), indicative of their undifferentiated spermatogonial properties.

To induce GS cell differentiation, we transferred them to laminin-coated wells and added RA to the culture medium. Multiple studies have shown that RA can drive spermatogonial differentiation both *in vivo* and *in vitro* [26,27,40,41]. Consistent with these reports, exposure to RA for 3 days made the GS cells gradually lose the structure characteristic for undifferentiated spermatogonia and display an enlarged cell size and round morphology with clear cellular boundaries (Fig. 1C), indicative of spermatogonial differentiation. Q-PCR and Western blot analyses showed substantial downregulation of the SSC self-renewal genes *Plzf* and *Oct4*, while RNA and protein levels of the differentiation marker *Stra8* markedly increased after 3 days of RA exposure (Fig. 1D, E). These results demonstrate that *in vitro* spermatogonial differentiation was successfully induced in our culture system.

3.2. GS cell differentiation induces a high transcriptomic change

Differentiating spermatogonia are known to be much more radio-sensitive than undifferentiated spermatogonia [13–15]. To study the underlying molecular mechanism causing this increased radio-sensitivity, we performed a whole transcriptomic RNA-seq analysis. First, we probed the transcriptomic variation of GS cells in response to RA-induced differentiation. As expected, many genes encoding known markers for differentiating spermatogonia and (pre)meiotic germ cells, such as *c-Kit*, *Stra8*, *Rec8*, *Prdm9*, were upregulated. Likewise, well-known marker genes for SSCs/progenitors, e.g. *Plzf*, *Oct4*, *Gfra1*, *c-Ret*, *Nanos2*, *Nanos3*, *Lin28A*, *Id4* and *Pax7*, as well as self-renewal genes such as *Bcl6b* and *Etv5*, showed a significant downregulation (Fig. 1F, Table S1). Together with other results presented in Fig. 1, this confirms that GS cells were indeed undifferentiated before the addition of RA and that administration of RA indeed induced spermatogonial differentiation as expected.

To compare the transcriptomes of GS cells with/without RA and/or IR treatment, 4 treatment groups were compared with each other (Fig. 2A). A multidimensional scaling (MDS) plot and heat map showed that RA treatment triggered a much larger transcriptomic change than IR (Fig. 2B). 1748 upregulated and 966 downregulated differentially expressed genes (DEGs, adj.P < 0.05 and fold change > 2) resulted from RA treatment (Table S1). Gene Ontology (GO) analysis demonstrated that RA-induced upregulated genes were involved in cell adhesion, differentiation and the response to retinoic acid. Downregulated genes were mainly related to transcription, proliferation, regulation of cell death and apoptosis (Fig. 2C, Table S2). Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis, which can reveal altered pathways by DEGs, showed that differentiation-upregulated genes were enriched with pathways mediating extracellular matrix (ECM)-receptor interaction, PI3K-Akt and Rap1 signaling, whereas downregulated genes were primarily related to stem cell pluripotency regulation and cancer pathways (Fig. 2D, Table S3). We further conducted a Q-PCR analysis for 16 RA-induced DEGs representative of the aforementioned cellular processes (Fig. 2E). For most genes the Q-PCR results were in line with the RNA-seq data, confirming the validity of the RNA-seq analysis.

3.3. Undifferentiated and differentiating GS cells display similar transcriptomic changes in response to IR

Also IR yielded transcriptomic variations in both undifferentiated and differentiating GS cells (Fig. 2B). Between the -RA-IR and -RA + IR cell populations, 31 and 8 DEGs (adj.P < 0.05 and fold change > 2) were up- and downregulated, respectively (Table S1). KEGG analysis disclosed that IR-upregulated genes, including *Bbc3*, *Gtse1*, *Ccng1*, *Cdkn1a* and *Sesn2*, were predominantly related to the p53 signaling pathway. To validate the RNA-seq data, we used Q-PCR to quantify the expression of 12 IR-induced DEGs that are implicated in the p53 signaling pathway, cell cycle arrest or apoptosis. The Q-PCR results were in line with the RNA-seq data for most genes (Fig. 2F).

Next, we investigated IR-induced DEGs in differentiating GS cells. Between the + RA-IR and + RA + IR cell populations, there were 20 DEGs (adj.P < 0.05 and fold change > 2) that all showed upregulation (Table S1). As the expression of DEGs can be influenced by RA-induced differentiation independent of IR, we directly compared the -RA + IR and + RA + IR cell populations after correction for their respective baseline levels of gene expression without exposure to IR (i.e. -RA-IR and + RA-IR, respectively). Apart from the genes affected by RA treatment independent of IR, no significant DEGs (adj.P < 0.05 and fold change > 2) were detected between the -RA + IR and + RA + IR cell populations. Hence, undifferentiated and differentiating GS cells did not generate significantly differential transcripts in response to IR.

In order to find significant up- or downregulation of specific pathways or gene sets, we performed a Gene Set Enrichment Analysis (GSEA). In contrast to GO or KEGG analysis, GSEA is not based on observed DEGs but instead takes into account all minor variations at the whole transcriptome level. GSEA showed that mesenchymal transition pathways went up in + RA + IR cell populations compared to their -RA + IR counterparts (FDR = 4.37×10^{-5} , Table S4).

3.4. Undifferentiated GS cells display a more robust p53 induction in response to IR

Despite the similar transcriptomic changes, we determined to gain more knowledge on the effects of RA-induced differentiation on the spermatogonial response to IR. First, to quantify the amount of DNA damage 3 h after IR, we stained the 4 groups of cells with γ -H2AX, a widely used DSB marker. A small fraction of GS cells displayed γ -H2AX staining, independent of either differentiation or IR (-IR-RA). Although statistically significant, the number of γ -H2AX⁺ cells increased only slightly after RA-induced differentiation (-IR + RA). Nonetheless, in line with previous reports [13–15], differentiating GS cells showed a clear increase of γ -H2AX⁺ cells in response to IR (+IR + RA, Fig. 3A, B). Next, we conducted an EdU assay to investigate the proliferation activity of GS cells. RA-induced differentiation resulted in a significant decrease in GS cell proliferation (-IR + RA vs -IR -RA). Also, IR alone resulted in a significant decrease in GS cell proliferation (-IR-RA vs + IR-RA). RA-induced differentiation further aggravated the decrease of EdU⁺ GS cells in response to IR (+IR + RA vs + IR-RA, Fig. 3C, D). Overall, these results indicate that RA-induced differentiation intensifies the effects of IR on GS cells.

Because IR-upregulated genes were predominantly related to the p53 signaling pathway and p53 is known to be upregulated in spermatogonia in response to IR [5], we performed a Western blot analysis to investigate the IR-induced p53 in both undifferentiated and differentiating GS cells. Interestingly, we found that undifferentiated GS cells displayed a more pronounced induction of p53 than the differentiating population at 3 h post IR (Fig. 3E).

3.5. Protein localization of differentiation-regulated genes in the testis

To study the protein localization of differentiation-regulated DEGs, we performed IHC on testis sections. We examined the protein localization patterns of 4 RA-regulated DEGs (*Wnt16*, *Slit2*, *Pdx1*, *Adora1*) for which working antibodies were available in adult mouse testes (Fig. 4A–D).

WNT family members have been reported to regulate the growth and differentiation of stem cells including SSCs [42,43]. RNA-seq and Q-PCR analyses revealed the *Wnt16* upregulation by RA-induced differentiation. While both undifferentiated and differentiating GS cells were negative for WNT16 staining *in vitro* (Fig. S2), its staining was observed in the cytoplasm of pachytene spermatocytes, round and elongating spermatids in testis sections (Fig. 4A).

SLIT2, a member of the SLIT family, has been reported to inhibit endothelial cell proliferation and migration [44]. We observed SLIT2 staining in the nuclei of round spermatids. In addition, some staining was observed in chromatid bodies in pachytene spermatocytes (Fig. 4B). Again, despite the RNA-seq and Q-PCR data which showed differentiation-induced upregulation of *Slit2* gene expression, neither undifferentiated nor differentiating GS cells were positive for SLIT2 staining (Fig. S2).

PDX1 is a transcription factor that acts as a regulator in pancreatic development and β cell function [45]. RNA-seq and Q-PCR analyses showed that *Pdx1* was significantly downregulated by RA-induced differentiation. In testes, PDX1 staining was exclusively found in the nuclei of a subpopulation of type A spermatogonia (Fig. 4C). Spermatogonia with clear heterochromatin patches at the rim of their nuclei, indicative of spermatogonial differentiation, and intermediate and type B spermatogonia did not stain for PDX1. To pinpoint whether it is the undifferentiated spermatogonia that are positive for PDX1 staining, we performed IHC on testis sections from neonatal (8 d.p.p) mice. Indeed, PDX1 staining was observed in undifferentiated spermatogonia but not in Sertoli cells (Fig. 5A), consistent with its staining pattern in adult testis sections.

ADORA1 belongs to the G protein-coupled receptor 1 family and modulates respiration and metabolism [46,47]. While RA-induced differentiation significantly lowered the number of *Adora1* transcripts, ADORA1 protein localization in testes appeared more dynamic. Like PDX1, ADORA1 staining was observed in the nuclei of some but not all type A spermatogonia. Also, pachytene spermatocytes and round spermatids up to stage VII of the seminiferous epithelium were stained (Fig. 4D). To investigate whether ADORA1 positive spermatogonia represent the undifferentiated spermatogonial population, we again performed IHC on neonatal testis sections. However, unlike PDX1, all neonatal testicular

cells, consisting of undifferentiated spermatogonia and Sertoli cells, were negative for ADORA1 staining (Fig. 5B).

3.6. Protein localization of irradiation-induced genes in the testis

To study the protein localization of irradiation-induced genes *in vivo*, adult mice were subjected to a whole-body IR (1 Gy), after which the protein localization patterns of 4 IR-induced DEGs (*Bbc3*, *Plk2*, *Pdrg1*, *Sesn2*) for which working antibodies were available were studied. IHC was performed on testis sections from experimental (3 h post IR) and control (sham IR treatment) mice. p53 was used as a positive control for the testicular radiation response [5]. As shown in Fig. 6A, nuclear p53 staining was clearly induced in spermatogonia after IR.

BBC3 is a key determinant of the intrinsic p53-dependent apoptotic pathway [48]. In line with our RNA-seq and Q-PCR data and a previous report [23], IR induced a strong increase of BBC3 staining in all testicular cells (Fig. 6B).

PLK2 is a serum-inducible kinase that functions in cell proliferation [49]. Although significantly induced at the RNA level in GS cells, PLK2 showed no significant difference in response to IR with regard to its localization *in vivo*. PLK2 staining was observed in pachytene spermatocytes and all subsequent germ cells but not in spermatogonia, (pre-) leptotene or zygotene spermatocytes (Fig. 6C).

PDRG1 is a DNA damage protein modulated by p53 and ultraviolet (UV) radiation [50]. Like PLK2, also PDRG1 staining in testes was not influenced by IR treatment. While all germ cells displayed a vague cytoplasmic staining, clear nuclear PDRG1 staining was observed in spermatocytes and round spermatids (Fig. 6D).

SESN2, a tumor suppressor, is able to induce autophagy [51] and implicated in the p53 signaling pathway. SESN2 staining was present in Sertoli cells before IR. After IR, SESN2 staining appeared more intense and was clearly induced in spermatogonia and, albeit less intense and consistent, in spermatocytes (Fig. 6E).

4. Discussion

Several factors, such as bone morphogenetic protein 4 (BMP4), activin A [52,53] and RA [54], have been reported to be involved in the regulation of spermatogonial differentiation. Of these, RA, the active metabolite of vitamin A, plays pivotal roles in the transition of undifferentiated spermatogonia into differentiating spermatogonia, as well as in the initiation and progression of meiosis [54]. A recent article reported that RA alone was able to induce GS cell differentiation into zygotene spermatocytes [27]. In this present study, we first used RA to induce GS cell differentiation as described previously [26,27] and compared the transcriptomes of undifferentiated and differentiating GS cells. Our differentiation protocol induced the transcriptomic alteration characteristic for spermatogonial differentiation. Markers for differentiating spermatogonia and (pre-)meiotic germ cells were significantly upregulated, whereas well-known markers for SSCs/progenitors and genes essential for SSC self-renewal showed a substantial down-regulation. GO and KEGG analyses further

uncovered that, apart from the expected response to RA, spermatogonial differentiation orchestrated the expression of genes involved in diverse biological processes. Collectively, in terms of transcriptomic variation, RA-induced differentiation of GS cells for a large part mimics *in vivo* spermatogonial differentiation, supporting the use of this *in vitro* model in our study.

We further examined the protein localization patterns of several RA-regulated DEGs *in vivo*. *Wnt16* and *Slit2* were upregulated by RA treatment, as shown by our RNA-seq and Q-PCR data. As a member of the WNT family, WNT16 has been found to be associated with estrogen withdrawal and bone loss during aging [55], and it regulates periosteal bone formation via activation of the canonical WNT signaling pathway [56]. We observed WNT16 staining mainly in the cytoplasm of pachytene spermatocytes and spermatids. Similar to WNT16, SLIT2 staining was observed in advanced germ cells, in this case the nuclei of round spermatids. Both protein localization patterns support the idea that genes not required until later stages of spermatogenesis are often already expressed upon spermatogonial differentiation [57–60].

Pdx1 and *Adora1* showed a significant downregulation upon RA treatment. Intriguingly, we found PDX1 staining exclusively in a sub-population of type A spermatogonia. Further IHC analysis on neonatal testis sections demonstrated that the spermatogonia positive for PDX1 staining were indeed undifferentiated spermatogonia. PDX1 may thus be a suitable novel marker for these cells. Because *Pdx1* is down-regulated upon spermatogonial differentiation, it may be involved in SSC self-renewal or proliferation. Like PDX1, ADORA1 staining was observed in some but not all type A spermatogonia in adult testes. However, ADORA1 staining was absent from spermatogonia in neonatal testes. Also, pachytene spermatocytes and a fraction of round spermatids were stained, suggestive of a more dynamic role for ADORA1 during spermatogenesis. Interestingly, ADORA1 staining disappeared from round spermatids after stage VII of the seminiferous epithelium. After stage VII, spermatid nuclei start to elongate while the cytoplasm and a more pronounced flagellum move to the luminal side of the seminiferous tubule [61,62]. This is also exactly the stage at which spermatogonial differentiation and meiotic initiation occur in response to RA signaling [61–63]. Because *Adora1* showed a significant down-regulation upon RA treatment in our experiments, it may thus be the case that, at stage VII of the seminiferous epithelium, also down-regulation of ADORA1 during spermiogenesis is regulated by RA signaling, which, however, requires further investigation.

To unravel why differentiating spermatogonia are more radio-sensitive than their undifferentiated counterparts, we compared the molecular responses of both undifferentiated and differentiating GS cells to IR by way of RNA-seq. We found no significant DEGs between –RA + IR and + RA + IR GS cells after correction for their respective baselines. Despite that, GSEA did reveal upregulation of the epithelialmesenchymal transition (EMT) pathway in irradiated differentiating GS cells. EMT is a dynamic developmental process by which epithelial cells that normally interact with basal membranes convert to migratory cells with fibroblast-like morphology and mesenchymal secretory characteristics [64]. Developing SSCs, since they express markers for both epithelial (*e.g.* CDH1 [65]) and mesenchymal cells (*e.g.* THY1 [66]), are typically regarded as a heterogeneous cell population containing

spermatogonia that are more epithelial-like or more mesenchymal-like, with the latter exhibiting distinctive DNA methylation dynamics [67]. Hence, supported by our RNA-seq data showing downregulation of *CDH1* and upregulation of *THY1* by RA treatment, spermatogonial differentiation, characterized by movement from the basal membrane towards the lumen, can be associated with enhanced EMT. GS cells can, albeit rarely, spontaneously reprogram to pluripotency, becoming multipotent GS (mGS) cells. Interestingly, EMT has recently been found to block this reprogramming of GS cells to pluripotency [64]. Moreover, mGS cells have been shown to be more resistant to IR than GS cells [23]. The other way around, it could thus make sense that differentiating GS cells, which are associated with increased EMT and the decreased level of pluripotency, are less resistant to IR.

In addition to the upregulation of the EMT pathway revealed by GSEA, differentiating GS cells displayed a less robust increase of the p53 protein level in response to IR. p53 is a well-known tumor repressing factor. Activated by post-translational modifications, it regulates the transcription of genes involved in cell cycle arrest and apoptosis [68,69]. p53 can initiate apoptosis to eliminate damaged cells or, alternatively, arrest the cell cycle. The latter will give the cells an opportunity to repair the damaged DNA before resuming the cell cycle [68,69]. Yet, how p53 signaling triggers one of these cascades remains unknown for most cell types. Our Western blot data showed a more robust p53 induction in undifferentiated GS cells in response to IR. As undifferentiated spermatogonia are relatively radio-resistant, it seems plausible that spermatogonial p53 preferentially induces cell cycle arrest. Differentiating spermatogonia, which induce less p53 in response to IR, would then be less likely to undergo cell cycle arrest and thus have fewer opportunities to repair the inflicted DNA damage. This would then render differentiating spermatogonia more radiosensitive.

Another process that characterizes spermatogonial differentiation is the condensation of repetitive sequences surrounding centromeres in pericentromeric heterochromatin domains. From spermatogonial differentiation and onwards through meiosis, these heterochromatic regions are marked by the presence of SMC6, a major subunit of the SMC5/6 complex known to be involved in the repair of DNA double-strand breaks (DSBs) [29,70]. Within these regions the SMC5/6 complex has been postulated to inhibit aberrant homology-driven recombinational repair of DSBs that would otherwise easily occur between repetitive sequences [29]. Hence, this changed chromatin architecture in differentiating spermatogonia may profoundly influence their response to DNA damage.

We also examined the protein localization patterns of several IR-induced DEGs *in vivo*. Our RNA-seq and Q-PCR data demonstrated a clear upregulation of *Bbc3*, *Plk2*, *Pdrg1* and *Sesn2* in irradiated GS cells. As a positive control for the IR-induced DNA damage response, we stained testis sections from irradiated and non-irradiated mice with an anti-p53 antibody. Consistent with a previous literature [5], p53 staining was clearly discerned in spermatogonia after IR treatment. A previous study showed that spermatogonial BBC3 was specifically up-regulated in differentiating spermatogonia by IR, whereas its knockdown attenuated apoptosis and increased spermatogonial survival [23]. We found an increase of BBC3 staining in all testicular cells after IR treatment, suggesting a general role in the testicular response to DNA damage. PLK2 initiates its expression at the G₁ phase, and it was

recently found to modulate mitotic spindle orientation as well as mammary gland development [71]. Our protein staining showed that IR treatment did not change the expression pattern of PLK2 in testes. Interestingly, PLK2 staining was not observed in spermatogonia, (pre-) leptotene or zygotene spermatocytes but in more advanced germ cells, in line with a previous article [72] and suggesting a role in meiosis as well as spermiogenesis. PDRG1 was recently identified as a novel tumor marker potentially functioning in cancer development [73]. In addition, *Pdrg1* transcripts have been shown to be highly expressed in human testes [50]. Like PLK2, we found PDRG1 staining, irrespective of IR treatment, in pachytene spermatocytes and round spermatids. Both staining patterns again support the notion that many mRNAs that are induced upon spermatogonial differentiation are actually required later during spermatogenesis. These are then stored in ribonucleoprotein particles (RNPs) and translated later during spermatogenesis [57–60]. Before IR, we observed SESN2 staining in Sertoli cells. In the IR group, this staining appeared more intense. Furthermore, after IR, also spermatogonia were clearly stained, suggesting a role for SESN2 in the spermatogonial response to IR.

Male cancer patients subjected to chemo- or radiotherapy are often confronted with sub-fertility caused by spermatogonial apoptosis. Nevertheless, the specific DNA damage-induced gene expression profiles of undifferentiated and differentiating spermatogonia have not been studied so far. We took advantage of a well-established *in vitro* model of spermatogonial differentiation to study the increasing radio-sensitivity of differentiating spermatogonia, and found no DEGs that were induced specifically in undifferentiated or differentiating GS cells in response to IR. Nevertheless, at the protein level, undifferentiated GS cells showed a stronger upregulation of p53 in response to IR than the differentiating population. Spermatogonial p53 may preferentially induce cell cycle arrest so as to repair the inflicted DNA damage, which would then endow undifferentiated spermatogonia with increased radio-resistance. Aside from protein content, other properties such as chromatin architecture or the proliferation activity, which are already affected by differentiation, might also be potential contributors to the increased radio-sensitivity of differentiating spermatogonia.

The difference in radio-sensitivity may reflect the divergent roles of undifferentiated and differentiating spermatogonia in maintaining genome integrity and male fertility. When damaged, differentiating spermatogonia can easily be sacrificed, thereby preventing the transmission of mutations to the offspring. When differentiating spermatogonia are lost, SSCs are still there to reinitiate spermatogenesis and preserve the long-term fertility. In contrast, elimination of undifferentiated spermatogonia could potentially remove the SSC pool and lead to permanent infertility. No longer being able to reproduce is an evolutionary dead end. Undifferentiated spermatogonia may have therefore developed a relatively high resistance to DNA damage. From an evolutionary point of view, mutated offspring is better than no off-spring.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

In vitro culture and differentiation of GS cells. (A) Representative images of GS cells cultured on MEFs or laminin. Bar = 100 μ m. (B) PLZF, LIN28A, OCT4 and ID4 staining of GS cells on laminin. NC: omitting primary antibodies. Bar = 10 μ m. (C) Representative images of GS cells (on laminin) with/without exposure to RA. Asterisks (*) refer to the long extensions typical for undifferentiated spermatogonia. Bar = 25 μ m. (D) Q-PCR analysis of *Plzf*, *Oct4* and *Stra8* expression in GS cells with/without exposure to RA. Data are presented as the mean $\pm$ standard error of mean (SEM), n = 3. *: P < 0.05; **: P < 0.01. (E) Western blot analysis of PLZF, OCT4 and STRA8 expression in GS cells with/without exposure to RA. β -actin or GAPDH is used as the loading control. (F) A heat map showing up- and down-regulation of markers for spermatogonial differentiation and progenitors/SSC self-renewal respectively in response to RA.

Fig. 2.

Transcriptomic profiles of GS cells in response to RA and IR treatment. (A) A schematic overview of the experimental design. (B) The MDS plot (left panel) and heat map (right panel) showing the expression profiles of 4 experimental groups of cells (-RA-IR, +RA-IR, -RA + IR, +RA + IR). (C) GO term enrichment analysis of RA-induced up- and downregulated genes. (D) The representative enriched pathways shown by KEGG analysis. (E) Q-PCR analysis of a panel of RA-induced DEGs. Data are presented as the mean $\pm$ SEM, n = 3. *: P < 0.05; **: P < 0.01. (F) Q-PCR analysis of a panel of IR-induced DEGs. Data are presented as the mean $\pm$ SEM, n = 3. *: P < 0.05.

Fig. 3. γ -H2 AX and EdU staining, as well as Western blot analysis of p53 protein levels, in -IR -RA, -IR + RA, +IR-RA and + IR + RA cell groups. (A) γ -H2 AX staining in 4 cell groups. NC: negative control. Bar = 100 μ m. (B) Percentages of γ -H2 AX⁺ cells in 4 groups. Data are presented as the mean $\pm$ SEM, n = 4. (C) EdU staining in 4 cell groups. Bar = 100 μ m. (D) Percentages of EdU⁺ cells in 4 groups. Data are presented as the mean $\pm$ SEM, n = 3. (E) p53 immunoblotting and the p53 band intensities relative to GAPDH. GAPDH is used as a loading control. Data are presented as the mean $\pm$ SEM, n = 4. *: P < 0.05; **: P < 0.01.

Fig. 4.

The protein localization of several RA-regulated DEGs in adult testes. Shown is the localization of (A) WNT16, (B) SLIT2, (C) PDX1 and (D) ADORA1 in adult testis sections. Examples of several cell types are marked: A, type A spermatogonia; Int, intermediate spermatogonia; B, type B spermatogonia; pL, pre-leptotene spermatocytes; L, leptotene spermatocytes; Z, zygotene spermatocytes; P, pachytene spermatocytes; D, diplotene spermatocytes; R, round spermatids; E, elongating spermatids; Ser, Sertoli cells; Ley, Leydig cells. Stages of the seminiferous epithelium are indicated with Roman numerals. Bar = 20 μm. Negative controls, using the isotype rabbit IgG, do not show any staining apart from interstitial cells.

Fig. 5. The protein localization of PDX1 (A) and ADORA1 (B) in neonatal testes. Asterisks (*) indicate the positive undifferentiated spermatogonia. Bar = 20 μm. Negative controls, using the isotype rabbit IgG, do not show any staining apart from interstitial cells.

Fig. 6. The protein localization of several IR-induced DEGs in adult testes. Shown is the localization of (A) p53, (B) BBC3, (C) PLK2, (D) PDRG1 and (E) SESN2 in adult testis sections with/without IR treatment. Examples of several cell types are marked: A, type A spermatogonia; Int, intermediate spermatogonia; B, type B spermatogonia; pL, pre-leptotene spermatocytes; L, leptotene spermatocytes; Z, zygotene spermatocytes; P, pachytene spermatocytes; D, diplotene spermatocytes; M, metaphase I spermatocytes; R, round spermatids; E, elongating spermatids; Ser, Sertoli cells; Ley, Leydig cells. Stages of the seminiferous epithelium are indicated with Roman numerals. Bar = 20 μ m. Negative controls, using the isotype rabbit IgG, do not show any staining apart from interstitial cells.

Table 1

Primer sequences for Q-PCR analysis.

Gene	Forward primer	Reverse primer	Product size (bp)
<i>Ptn2</i>	CCTGCTGGACTATATCAATGAGAC	TCTCGGAACCCCTTGACCTG	114
<i>Mtg1</i>	CACGATGTAGCACGCTGGTT	GGGTTTCGACCTGAAAATGGG	128
<i>Phsf</i>	TCTCGGAACCCCTTGACCTG	ACCGAAAGAGGTGGAGACTGA	132
<i>Oct4</i>	CACGAGTGGAAAGCAACTCA	CTTCTGCAGGGCTTTCATGT	125
<i>Strat8</i>	GGAAAGGCAGTTTACTCCCCAGTC	GATTCCTCATCTTCGACAGTTGA	144
<i>Clu</i>	CAGTTCCAGACGTGGATT	GGGCAGGATTGTTGGTTG	157
<i>Nr1k3</i>	TTTGGGGTGTCCATAGCAG	AGCCACAGGACCCCTTCATT	120
<i>Wnt16</i>	CTTCCCATCAGAAACACCACA	GCGGCAGTCCACAGACATTA	114
<i>Agn2</i>	GAAGAAACAGAAATTACCCGTGAC	AGGGAAGCCAGCAAAATGA	80
<i>Bmp2</i>	ATCTGTACCGCAGGCATC	ACGGCTTCTTCGTGATGG	112
<i>Surm1</i>	CAAGGAGATTGTGACTGCTTTA	GGTACTCATGGGACCAATTTGA	143
<i>Ins1</i>	GGTGTTCCTCTGCAAGTACT	CTATTCTCAGACGGGTGGC	90
<i>Slit2</i>	ATGGAGAACAGAAATCAGCACC	TCGCAGTCCCGAGAAACA	124
<i>Adgrg1</i>	AGCCAAGTCTGGGTGAGA	TTGATGCCGGGTCTTCAA	154
<i>Myog</i>	GTCCCAACCCAGGAGATCA	AACAGACATATCCTCCACCGT	107
<i>Pax6</i>	AACAGACATATCCTCCACCGT	TATCATAAATCCTCCGCCATTTC	135
<i>Vsx2</i>	CACCTACCCAGATGTCTACGCC	CACCTTCTCCCTCTTCTCTCCAC	116
<i>Adora1</i>	AACCCAGCATCCTCATCTACA	GTGGTCGTTCCTCCAAATCTCA	125
<i>Sfrp2</i>	GTGGTCGTTCCTCCAAATCTCA	GCTCTTTGTCTCCAGGATGAT	130
<i>Irf1</i>	AATGGGGATGAGACCCTG	ATGTCCAGCCGTGCTTA	129
<i>Pdx1</i>	AATCCACCAAGCTCACGC	CGGGTCTCTTGTTTTCCTC	82
<i>Bbc3</i>	GAGCGCGGAGACAAAGAAGA	ATCCCTGGGTAAGGGGAGGA	96
<i>Plk2</i>	ATGTGGAACCCCAAAATATCTC	GGTCTTCTTAGCAGCATCGTAT	114
<i>Trp53inp1</i>	GACACCAAGTGATTCCTGCTTC	GGACTTGTTCACCTTGATAG	122
<i>Ddias</i>	TGTCTTTGAAAGTGGCAGAA	GTGTAAACCAAGTGGCCGTAA	98
<i>Ceng1</i>	ATAATGGCTCAGAAATGACTGC	CCAAGATGCTTCGCCCTGTAC	160
<i>Kh42</i>	CAATCCCATCACCAACGAG	TAGAAACAGCCTGCCACC	116
<i>Psrc1</i>	TGCCCAACCGTGAGTTCTT	GTGGGTGATTCTCTTTATGC	139

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Gene	Forward primer	Reverse primer	Product size (bp)
<i>Eda2r</i>	ACTTGTGCTGTCAATCGG	CGTGTCTTTTCGGTAGAACCTG	98
<i>Pdgl</i>	GGAAGGAGCCAAAGGTGAAAGT	CCTCGGCCCAACTCCTCTAC	117
<i>Sesn2</i>	AACACATCCACTGCGTCTTTG	CATCCTACGGGTCTCTCT	135
<i>Slc2a10</i>	TACTTGTTCCTGTGAACCCAAAGG	TCCAGGGGATGGTACTGAA	121
<i>Far2</i>	TTATTGGAAACACCGTCAGCC	CAGCATTTCTGGGTTTCCTTC	85