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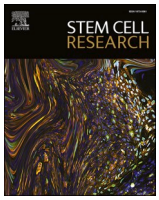

Differentiation of mouse embryonic stem cells into cells with spermatogonia-like morphology with chemical intervention-dependent increased gene expression of LIM homeobox 1 (*Lhx1*)

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ABSTRACT

Spermatogonial stem cells (SSCs) originate from gonocytes that differentiate from primordial germ cells (PGCs). In the developing mouse testis, expression of the gene LIM homeobox 1 (*Lhx1*) marks the most undifferentiated SSCs, which has not yet been reported for spermatogonia-like cells generated *in vitro*. Previously, it was shown that a chemical intervention in male mouse embryonic stem (ES) cells in serum culture, including Sirtuin 1 (SIRT1) inhibitor Ex-527, DNA methyltransferase (DNMT) inhibitor RG-108 and electrophilic redox cycling compound *tert*-butylhydroquinone (tBHQ), was associated with molecular markers of PGC to gonocyte differentiation. Here, we report the *in vitro* differentiation of male mouse ES cells, cultured under dual chemical inhibition of GSK3β and MEK (2i) with leukemia inhibitory factor (LIF) (2iL) and serum, into cells with spermatogonia-like morphology (CSMs) and population-averaged expression of spermatogonia-specific genes by removal of 2iL and a specific schedule of twice daily partial medium replacement. Combination of this new protocol with the previously reported chemical intervention increased population-averaged gene expression of *Lhx1* in the resulting CSMs. Furthermore, we detected single CSMs with strong nuclear LHX1/5 protein signal only in the chemical intervention group. We propose that further investigation of CSMs may provide new insights into male germline development.

1. Introduction

Embryonic stem (ES) cells are pluripotent stem (PS) cells determined by their potential to differentiate into the germline and all somatic lineages (Nichols and Smith, 2012). They can function as a cell model to investigate developmental processes *in vitro*, such as the development of the male germline (Kurimoto and Saitou, 2015). Primordial germ cells (PGCs), the developmentally first cells of the germline, are induced from epiblast cells on around embryonic day (E)6.25 by external signals, consisting of bone morphogenetic protein 4 (BMP4) signaling (Kurimoto and Saitou, 2015). Gonocytes are induced from PGCs on around E12.5-E13.5 (Kurimoto and Saitou, 2015). The stem cells of the male germline, defined as spermatogonial stem cells (SSCs), originate on around postnatal day (P)3-P6 from gonocytes (Yoshida et al., 2006). Previous efforts to reconstitute these developmental stages *in vitro* first induced ES cells into epiblast-like cells, which in turn were induced into PGC-like cells (Hayashi et al., 2011), and then further differentiated into spermatogonia-like cells (Ishikura et al., 2016; Ishikura et al., 2021). PGC-like cells and spermatogonia-like cells both had the capacity to generate offspring after transplantation into testes of germ cell-deficient mice (Hayashi et al., 2011; Ishikura et al., 2016; Ishikura et al., 2021). However, spermatogonia-like cells lacked enriched expression of important SSC marker genes such as LIM homeobox 1 (*Lhx1*) (Ishikura et al., 2016; Ishikura et al., 2021), which is a marker gene for the most undifferentiated SSCs in the developing and regenerating mouse testis (La et al., 2018; Tan et al., 2020). This observation suggests that some important aspects of spermatogonia development have not yet been reconstituted *in vitro*. Dual chemical inhibition of GSK3β and MEK (termed 2i) with leukemia inhibitory factor (LIF) (termed 2iL), is routinely used *in vitro* to stabilize the so-called ground state of

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pluripotency in ES cells, corresponding to cells of the inner cell mass (ICM) of the blastocyst (Nichols and Smith, 2012). However, it was reported that mouse ES cells *in vitro*, depending on culture conditions, exist within a spectrum of pluripotent states that influences their commitment towards the germline or somatic lineages (Hackett et al., 2017). 2i induced a PGC-like state in ES cells, while LIF inhibited this PGC-like state (Hackett et al., 2017); and serum increased heterogeneity of pluripotent states (Welling et al., 2015). Furthermore, it was shown for male mouse ES cells cultured in serum that a chemical intervention, including a timed combination of the Sirtuin 1 (SIRT1) inhibitor Ex-527, the DNA methyltransferase (DNMT) inhibitor RG-108 and the electrophilic redox cycling compound *tert*-butylhydroquinone (tBHQ), was associated with molecular markers of the PGC to gonocyte differentiation process (Moshfegh et al., 2016). The chemical intervention was originally designed to rescue cell viability of mouse ES cells cultured in the absence of βME, based on the induction of nuclear factor erythroid 2-related factor 2 (NRF2) signaling by tBHQ (Moshfegh et al., 2016). tBHQ also increases intracellular ROS by undergoing redox cycling reactions (Lee et al., 2001). βME is an external antioxidant essential for the viability of ES cells in culture but was removed to avoid potential interference with differential reactive oxygen species (ROS) signaling (Chae and Broxmeyer, 2011). The timed use of RG-108 was necessary for the rescue of cell viability by tBHQ and the timed combination of Ex-527 and RG-108 further improved this (Moshfegh et al., 2016). As RG-108 inhibits DNMTs its necessity was likely based on the demethylation of NRF2-target genes although DNA methylation was not measured. Ex-527 inhibits SIRT1 deacetylase activity and it was previously shown that inhibition of SIRT1-mediated deacetylation of NRF2 increased its nuclear localization upon induction by tBHQ (Kawai et al., 2011). The aim of this study was to investigate whether the combination of a new cell culture protocol based on 2iL and serum with the previously reported chemical intervention (Moshfegh et al., 2016) can induce aspects of spermatogonia development in male mouse ES cells *in vitro*. We found that male mouse ES cells cultured in 2iL and serum could be differentiated into cells with spermatogonia-like morphology (CSMs) by removal of 2iL and a specific schedule of partial medium replacements, and that a combination of this new cell culture protocol with the previously reported chemical intervention resulted in the first observation of CSMs *in vitro* with enriched *Lhx1* expression. Further investigation of these cells may provide new insights into male germline and stem cell development. Fig. 3.

2. Results and discussion

To investigate aspects of spermatogonia development in male mouse ES cells, using 2iL and serum as the basis for the culture, we designed a combined cell culture protocol without (control) and with chemical intervention (intervention) (Moshfegh et al., 2016). The intervention is described in Materials and Methods and Fig. 1.

We purchased male mouse C57BL/6N ES cells from Millipore and confirmed their male genetic sex (xy) by fluorescence in situ hybridization (FISH) (S1 Fig). We also found aneuploidy in these ES cells with a duplication of chromosome 8 in all three analyzed metaphase spreads (S1 Fig). Trisomy 8 is a common chromosomal abnormality in ES cells cultured long-term *in vitro*, as it confers a selective proliferation advantage (Liu et al., 1997). Although ES cells with trisomy 8 have a diminished efficiency to contribute to the germline of chimeric mice (Liu et al., 1997), a diminished *in vitro* germline developmental potential for ES cells with duplication of chromosome 8 has not been reported. Therefore, we continued to use these ES cells.

Typical for ES cells (Hayashi et al., 2011), on day 0, the culture consisted of compact multicellular colonies, while on day 7 it had grown to confluent multilayered cell sheets of variable thickness with some visible debris from dead cells in both groups (Fig. 2). Thicker cell sheets with holes in some areas and reduced debris were observable on day 19 in both groups. Starting on day 23 in both groups, these cell sheets progressively dissociated into round mostly non-adherent cells, described here as CSMs due to their morphological similarity to previously reported isolated SSCs from P4.5-P7.5 mouse testis (Kubota et al., 2004). By day 32 a large portion of the cell sheets had dissociated into CSMs in both groups (Fig. 2). This process progressed faster and more completely in the intervention group than in the control group (S2 Fig). This may suggest a faster or changed differentiation process in the intervention group compared to the control group. CSMs were morphologically indistinguishable between both groups. However, we noticed that the twice daily partial medium replacement at hour 8 and hour 16 with a third of the volume from day 19 onwards was necessary for the appearance of CSMs. When we replaced the medium twice daily every 12 h with half of the volume, the cell sheets remained intact and CSMs did not appear in either group. This observation suggests that endogenously produced soluble factors may be necessary for the transition of cell sheets into CSMs. Interestingly, it was previously reported that the transition of gonocytes into spermatogonia in the mouse required FGF signaling (Pui and Saga, 2017) and that accumulation of endogenously produced FGF2 in the culture medium was essential for the survival and proliferation of cultured mouse SSCs (Zhang et al., 2012). We then measured the population-averaged expression of genes specific to spermatogonia development on day 0, day 7, day 19 and day 32 by real-time PCR analysis to detect mRNA levels. From day 0 to day 19, all cells were adherent and collected. On day 32, because our focus

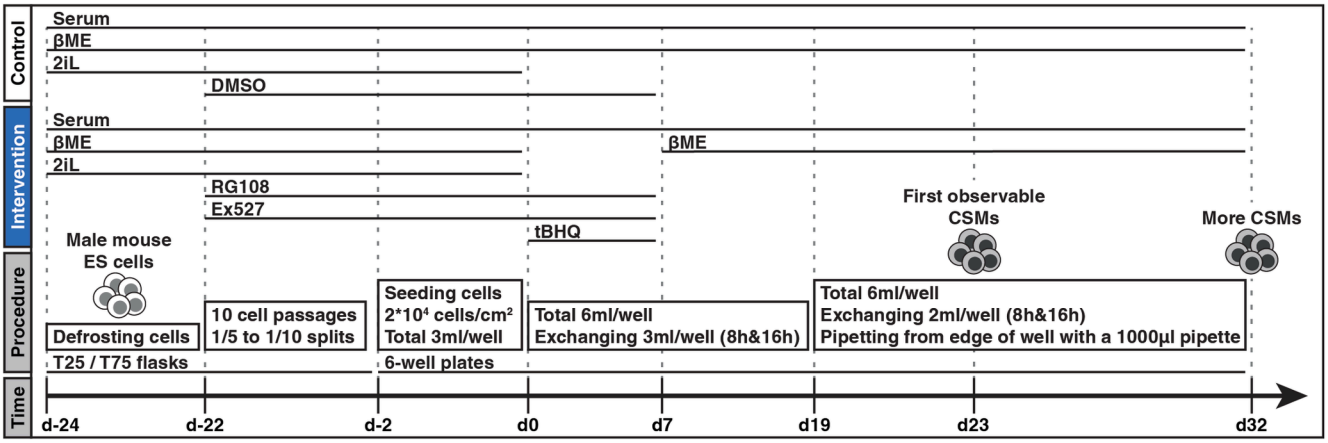

Fig. 1. Schematic procedure for the differentiation of male mouse ES cells into CSMs. A schematic description of the procedure in the control (white) and intervention group (blue) for the differentiation of male mouse ES cells (day −24) into CSMs (day 32, first observable CSMs on day 23). See Materials and Methods for a detailed description.

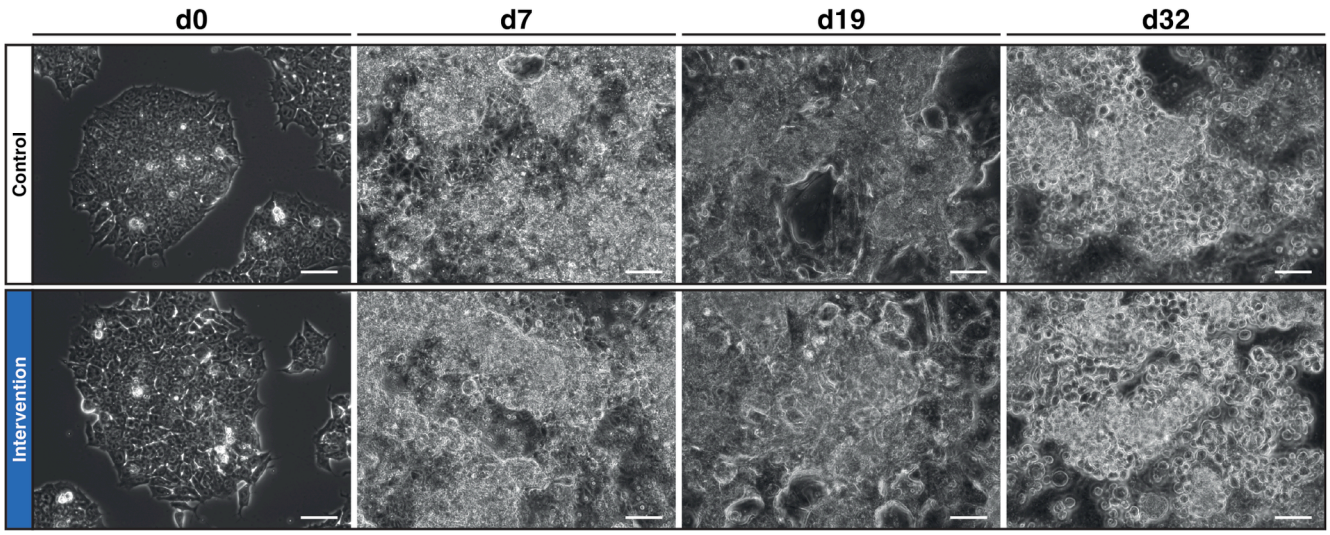

Fig. 2. Phase contrast images during the differentiation process. Phase contrast images during the differentiation process of ES cells to CSMs in the control (white) and intervention group (blue) on day 0, day 7, day 19 and day 32 (S2 Fig). Scale bar = 50 μm.

was on the CSMs, we only collected non-adherent cells by gentle rinsing, capturing most of the CSMs.

We found that expression of *Zbtb16*, *Gfra1*, *Bmi1*, *Kit* and *Klf4* was most upregulated on day 32 (containing CSMs) relative to all other analyzed time points in both groups, while *Klf4* expression was more upregulated in the control group compared to the intervention group on day 32 (Fig. 3). *Zbtb16* and *Gfra1* are marker genes of SSCs but are already expressed in some gonocytes from E18.5-P1.5 onwards (Pui and Saga, 2017). *Bmi1* is a marker gene of SSCs but is also expressed in gonocytes from E16.5 onwards (Hammoud et al., 2014). *Kit* is a marker gene of differentiating spermatogonia from P2-P6 onwards (Manova et al., 1990), including the first round of spermatogenesis that begins directly from gonocytes and omits the SSC-stage (Yoshida et al., 2006). *Klf4* is a pluripotency-associated gene but is also expressed in SSCs (Corbineau et al., 2017), differentiating spermatogonia and spermatids (Hammoud et al., 2014). Therefore, these results suggest that aspects of spermatogonia development and differentiation were present on day 32 in both groups.

Furthermore, *Lin28a* expression was upregulated on day 32 relative to day 0 but showed the highest upregulation on day 7 in both groups. *Lin28a* is a marker gene of SSCs (Zheng et al., 2009) but is also expressed in PGCs on E13.5 (Shinoda et al., 2013). Expression of *Csf1r*; *Ret*; *Stra8* and *Dazl* showed the highest upregulation on day 19 in both groups, while *Csf1r* expression was also upregulated on day 32 relative to day 0 in both groups. *Ret* expression was more strongly downregulated in the control group compared to the intervention group on day 32 (Fig. 3). *Csf1r* is expressed in SSCs on P6 (Kokkinaki et al., 2009). *Ret* is expressed together with *Gfra1* and *Zbtb16* in SSCs as well as in some gonocytes from E18.5-P1.5 onwards, while *Stra8* is expressed in a subset of these gonocytes (Pui and Saga, 2017). DAZL mediates a broad translational program to regulate proliferation and differentiation in the germline and is robustly expressed in male gonocytes on P0 and P4 (Mikedis et al., 2020). Therefore, these results further suggest that aspects of gonocyte development preceded the transition of cell sheets into CSMs.

We then found differences in the expression of *Dppa3* and *Tex101* in the intervention group compared to the control group (Fig. 3). *Dppa3* is expressed in PGCs and in male gonocytes until E15.5 (Sato et al., 2002), and then its expression gradually decreases until it is no longer detectable on P1 or later (Sato et al., 2002). *Tex101* is expressed in early gonocytes from E14-E16 onwards until early spermatogonia on P8-P10, in spermatocytes and spermatids, but not in PGCs, during gonocyte induction on E12.5-E13.5 or in spermatogonia from P12 onwards (Takayama et al., 2005). *Dppa3* expression was upregulated on day 7 relative to day 0 in both groups. In the intervention group, *Dppa3* expression remained at this level on day 19 and was downregulated to a level like day 0 on day 32. In the control group, *Dppa3* expression was downregulated already on day 19 to a level like day 0 and was further downregulated around 160-fold on day 32. In the intervention group, *Tex101* expression was upregulated on day 19 relative to day 0 but not earlier and remained at that level on day 32. This means that *Tex101* expression was not upregulated during the chemical intervention. In the control group, *Tex101* expression was upregulated already on day 7 relative to day 0, remained at that level on day 19 and was downregulated on day 32. This suggests that some aspects of spermatogonia development up to P1 and P8 progressed later in the intervention group compared to the control group.

We found further differences in the expression of *Neurog3* and *Lhx1* in the intervention group compared to the control group that suggested a changed differentiation process. *Neurog3* is expressed in undifferentiated spermatogonia from P3-P6 onwards but not in gonocytes, differentiating spermatogonia or other germ cells (Yoshida et al., 2006). Although not all adult SSCs express *Neurog3* (Zheng et al., 2009), all adult spermatogenic cells originate from these *Neurog3*-expressing spermatogonia established after birth (Yoshida et al., 2006). Therefore, upregulation of *Neurog3* expression was expected only when CSMs were present (day 32) but not otherwise. *Lhx1* is a marker gene of the most undifferentiated SSCs in the developing (P7-P10, potentially earlier) and regenerating mouse testis (La et al., 2018; Tan et al., 2020). In the intervention group, *Neurog3* expression was only upregulated on day 32 relative to day 0 (Fig. 3). In the control group, *Neurog3* expression was upregulated only on day 7 and day 32 relative to day 0 (Fig. 3). The expected upregulation of *Neurog3* expression only when CSMs were present (day 32) was confirmed in the intervention group but not in the control group. The upregulated expression of *Neurog3* on day 7 in the control group but not in the intervention group may indicate a premature activation of early spermatogonia markers in the control group, which was prevented by the chemical intervention. *Lhx1* expression was upregulated on day 32 relative to day 0 in both groups. However, the expression of *Lhx1* on day 32 was around 10-fold higher in the intervention group compared to the control group. Among the analyzed genes with an upregulated expression on day 32 relative to the other analyzed time points *Lhx1* showed the largest difference between the two groups. This suggests that the differentiation process in the intervention group compared to the control group was fundamentally different, leading to an increased population-averaged expression of a marker gene of the most undifferentiated SSCs (*Lhx1*) in the resulting

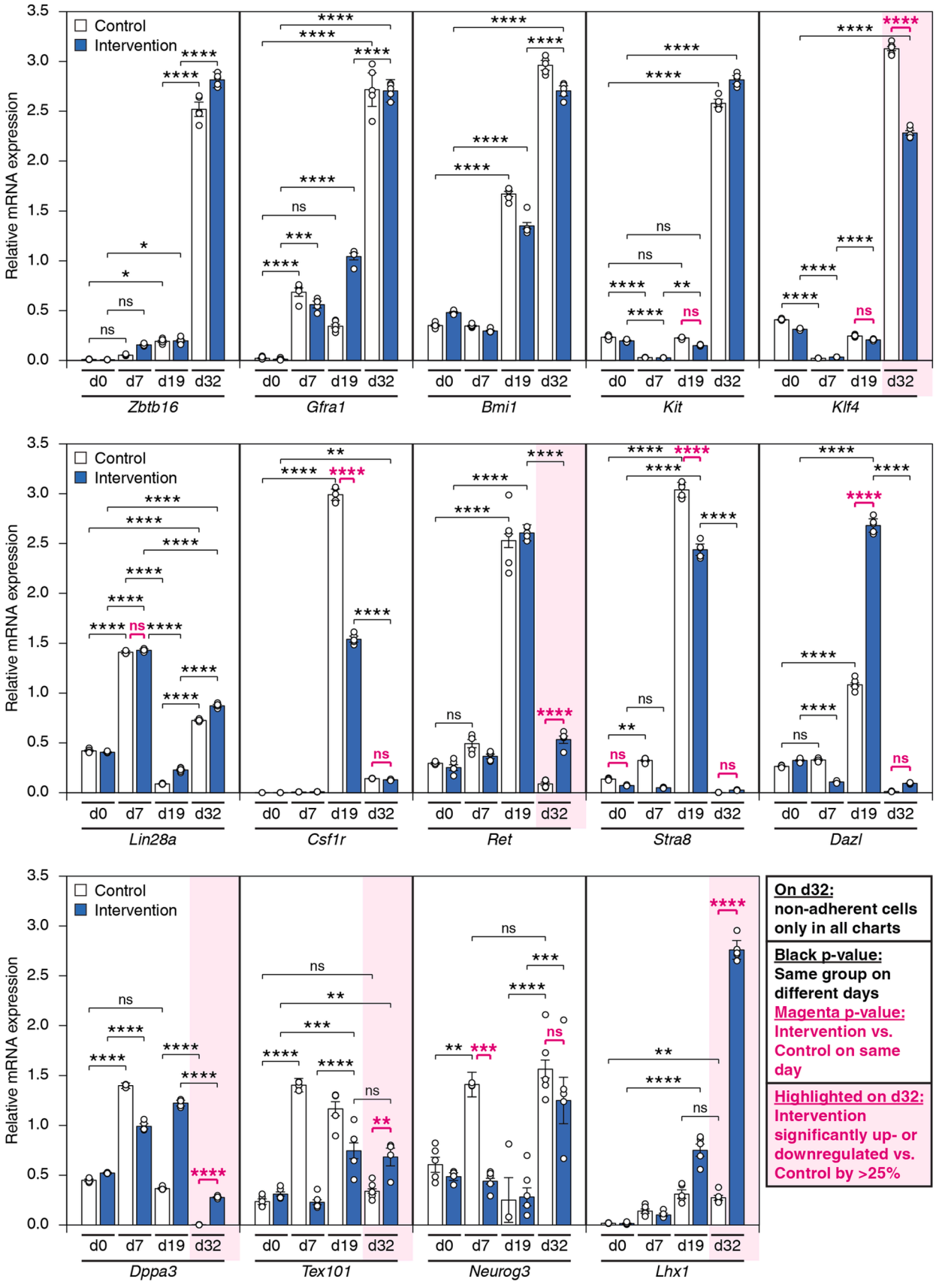

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Fig. 3. Expression of genes specific to spermatogonia development during the differentiation process. Real-time PCR analysis of genes specific to spermatogonia development during the differentiation process of ES cells to CSMs in the control (white) and intervention group (blue) on day 0, day 7, day 19 and day 32. From day 0 to day 19, all cells were adherent and collected, while on day 32, only non-adherent cells were collected. Black p-values indicate comparison of expression between different days within the same group, while magenta p-values indicate comparison of expression between the intervention group and the control group on the same day. Magenta highlighted areas on day 32 indicate a significant up- or downregulation of expression in the intervention group compared to the control group by greater than 25%. Relative gene expression levels were calculated based on the ΔΔCt method with normalization using *Gapdh*. White circles indicate individual expression data of all replicates before inter-run calibration. Error bars indicate standard error of the mean (SEM). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, ns non-significant. PCR reactions were performed with technical replicates of 5 ($n = 5$) from pooled samples.

CSMs in the intervention group.

On day 32, a significantly up- or downregulated expression in the intervention group compared to the control group by more than 25% implied that the chemical intervention influenced the expression of the respective genes in the resulting CSMs, and this was only detected for *Dppa3*, *Tex101*, *Lhx1*, *Ret* and *Klf4* but not for *Neurog3* (Fig. 3).

We then tested for LHX1 protein in single cells on day 32 compared to mouse embryonic fibroblasts (MEFs) and ES cells by immunofluorescence using an antibody that detects LHX1/5 (Developmental Studies Hybridoma Bank, 4F2). Congruence of the LHX1/5 and DAPI signal in the images and z-axis profiles defined a nuclear signal. We found that LHX1/5 signal was detectable in the cytoplasm and nucleus of some cells in all analyzed samples. A strong nuclear LHX1/5 signal was detected in 3 out of 187 cells in CSMs in the intervention group but not in the other analyzed cells (MEFs: 160 cells, ES cells: 226 cells, CSMs in control group: 144 cells) (Fig. 4; S3 and S4 Fig). As LHX1 is a transcription factor and shows a strong nuclear signal in SSCs from P7 mouse testis (Tan et al., 2020), a strong nuclear LHX1/5 signal supports this function. The small fraction of CSMs with a strong nuclear LHX1/5 signal may result from a combination of increased *Lhx1* expression in only a small fraction of CSMs, post-transcriptional, and post-translational regulation. These results support the population-averaged gene expression analysis of *Lhx1*. This represents the first experimental evidence for the *in vitro* generation of CSMs with an enrichment of *Lhx1* expression. Our results identify genes involved in spermatogonia development, including *Dppa3*, *Tex101*, *Neurog3*, *Lhx1*, *Ret* and *Klf4*, as differentially expressed during early and late stages of the *in vitro* differentiation process, depending on a chemical intervention. How the chemical intervention achieves this was not studied here and may be a subject of future experiments. Further studies based on this cellular *in vitro* system may provide insights into new aspects of male germline and stem cell development.

3. Conclusions

Here, we demonstrate a new *in vitro* culture system using male mouse ES cells to study and manipulate aspects of spermatogonia development, including changes in gene expression and the generation of CSMs. We show that a transient chemical intervention at a time before upregulation of *Tex101* expression changes the differentiation process and leads to an enrichment of *Lhx1* expression in the resulting CSMs.

4. Limitations

This is a cell culture-based *in vitro* differentiation system, where we used commercially available male mouse C57BL/6N ES cells (Millipore) that had a duplication of chromosome 8. Further studies using euploid ES cells from different genetic backgrounds are necessary to prove that the observed results are not specific to the ES cells used here. Future studies should analyze the protein expression of additional germ cell-specific genes such as DDX4, DAZL or KIT by methods including but not limited to Western blot analysis and immunofluorescence. Mouse testis tissue or isolated SSCs should be used as a comparison to CSMs in the analysis in future studies, including gene expression analysis, identification of spermatogonia types, and studies of biological functionality in testis transplantation experiments with the test for spermatogenic colonies in the host testis and the generation of healthy offspring.

5. Materials and methods

5.1. Maintenance cell culture

All cells were cultured in an incubator with a humidified atmosphere at 37 °C and 5% (v/v) CO_2. Male (xy) mouse C57BL/6N ES cells (Millipore, SCC050) (S1 Fig) were cultured in T25 or T75 tissue culture flasks (TPP, Switzerland) that were coated with 0.1% gelatin solution (Millipore, ES-006-B). ES cells were passaged every 2 days with accutase (Thermo Fisher Scientific, A1110501). Basic medium (BM) consisted of Embryomax DMEM (Millipore, SLM-220-B), supplemented with 15% (v/v) Embryomax fetal bovine serum (FBS) (Millipore, ES-009-B), 1 mM sodium pyruvate (Millipore, TMS-005-C), 0.1 mM non-essential amino acids (Thermo Fisher Scientific, 11140050), and 2 mM Glutamax-I supplement (Thermo Fisher Scientific, 35050061). ES cells were cultured in 2iL medium, consisting of BM supplemented with 0.1 mM βME (Thermo Fisher Scientific, 31350010), 1000 units/ml LIF (Millipore, ESG1107), 1 μM CHIR99021 (Cayman Chemical, 13122) and 1 μM PD 0,325,901 (Cayman Chemical, 13034). Immortalized MEFs were kindly provided by Reinhard Fässler (Max Planck Institute of Biochemistry, Germany). MEFs were cultured in DMEM (Thermo Fisher Scientific, 21885) supplemented with 10% (v/v) FBS (Biowest, S181H).

5.2. Intervention & control culture

For the intervention and control group the procedure illustrated in Fig. 1 was followed. In the control group βME was always supplemented, while Ex-527 (Cayman Chemical, 10009798), RG-108 (Cayman Chemical, 13302) and tBHQ (Sigma-Aldrich, 112941) were replaced with dimethyl sulfoxide (DMSO) with the same final concentration as in the intervention group. Ex-527, RG-108 and tBHQ were added from stock solutions in DMSO. The intervention and control group were identical in all other aspects of the procedure. On day −24, ES cells were defrosted and cultured for 2 days in 2iL medium. On day –22, the medium of the intervention group was changed to 2iL medium supplemented with 5 μM Ex-527 and 100 μM RG-108 and the cells were passaged 1:5 to 1:10 every 2 days for 10 cell passages (20 days) in T25 or T75 tissue culture flasks. On day −2, cells were seeded into gelatin-coated 6-well plates (9.5 cm^2 growth area per well, TPP, Switzerland) at 2×10^4 cells/cm^2 (3 ml/well) and cultured for 2 days in the same medium. No further cell passaging was performed from this point onwards. The total DMSO concentration was 0.03% (v/v). On day 0, the medium of the intervention group was changed to BM supplemented with 5 μM Ex-527, 100 μM RG-108 and 10 μM tBHQ and the total volume was increased to 6 ml/well. βME and 2iL were not present. The cells were cultured in this medium for 7 days with partial medium replacement of 3 ml/well (half of well volume) two times per day at hour 8 and hour 16. The total DMSO concentration was 0.04% (v/v). On day 7, the medium of the intervention group was changed to BM supplemented with βME, while keeping the same replacement frequency, volume and total volume. Until day 19, there was no noticeable cell loss during medium replacements because all cells were strongly adherent. From day 19 until day 32, the partial medium replacements were changed to 2 ml/well (one third of well volume) pipetting slowly from the edge of the well with a 1000 μl pipette to avoid the loss of non-adherent CSMs, while keeping the same replacement frequency, medium composition and total volume.

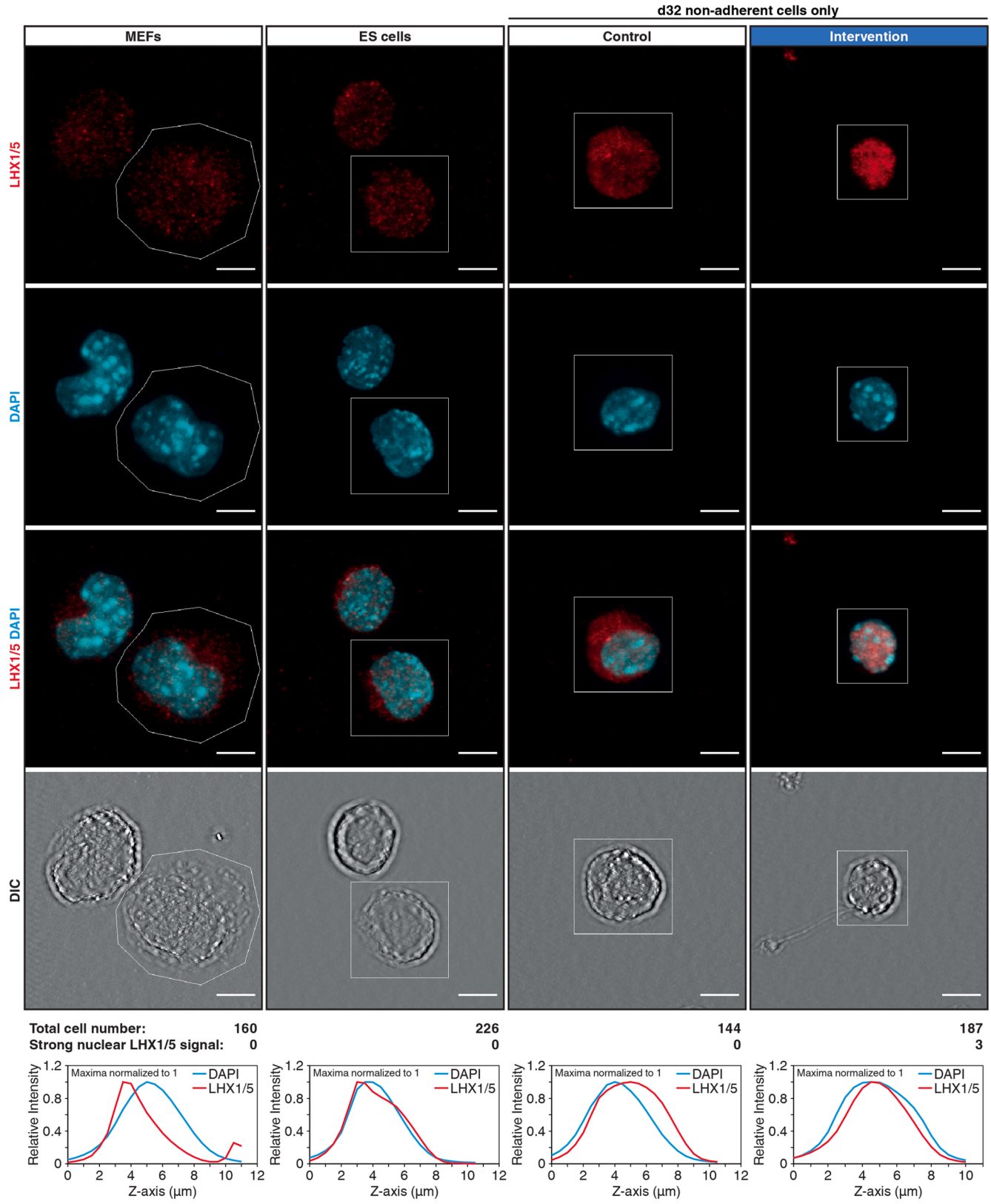

Fig. 4. Zoom-in immunofluorescence images of LHX1/5. Zoom-in immunofluorescence images of LHX1/5 (red) in MEFs, ES cells, and CSMs in the control and intervention group, shown as maximum z-projections. DNA was counterstained with DAPI (blue). MEFs and ES cells were dissociated with accutase, while CSMs were obtained by collecting the non-adherent cell fraction of day 32 samples without enzymatic dissociation. After collection, all cells were stored alive for more than 6 months in liquid nitrogen before processing. DIC images at the bottom show the membrane boundaries of cells. The z-axis profiles (maximum intensities normalized to 1) of LHX1/5 and DAPI are plotted for regions marked by a white line in the respective images. Congruence of the LHX1/5 and DAPI signal in the images and z-axis profiles define a nuclear signal (S3 and S4 Fig). Scale bar = 5 µm.

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CRediT authorship contribution statement

Cameron Moshfegh: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Visualization, Validation, Software, Project administration, Supervision, Funding acquisition, Writing – original draft, Writing – review & editing. **Sebastian G. Rambow:** Investigation, Methodology, Writing – review & editing. **Seraina A. Domenig:** Investigation, Methodology, Writing – review & editing. **Aldona Pieńkowska-Schelling:** Investigation, Data curation, Writing – review & editing. **Ulrich Bleul:** Supervision, Writing – review & editing. **Viola Vogel:** Resources, Supervision, Writing – review & editing.

Declaration of Competing Interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scr.2022.102780.

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