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Strains matter: Success of murine *in vitro* spermatogenesis is dependent on genetic background

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ABSTRACT

The current strategy to preserve fertility of male prepubertal cancer patients consists of cryopreservation of a testicular tissue biopsy containing spermatogonial stem cells (SSCs). While in humans, fertility restoration strategies from prepubertal testicular tissues are still under investigation and have not yet resulted in complete germ cell differentiation, in mice various studies have described production of sperm and offspring through testicular organ culture and transplantation of *in vitro* propagated SSCs. Organ culture has shown to be successful in generating mature spermatozoa when using testicular fragments from various mouse strains, including CD1 and C57BL/6 J. Conversely, *in vitro* proliferation of SSCs from C57BL/6 J mice is highly inefficient when compared to other strains such as DBA2 or hybrid mice of C57BL/6 J and DBA2 with 75% C57BL/6 J background (B6D2F2). In this study, we investigated *in vitro* spermatogenesis by organ culture using testicular tissue from C57BL/6 J and B6D2F2 mice. Whereas spermatogenesis was initiated and completed in C57BL/6 J fragments, it could not be effectively supported in B6D2F2 testicular tissue. While maturation of Sertoli cells and Leydig cells functionality appeared to be identical between the two strains, in B6D2F2 tissue spermatogenesis did not proceed past the spermatocyte step, followed by a rapid decline of the number of all germ cells in the fragments. This suggests that the spermatogenic potential *in vitro* is dependent on specialized sites in the genome and therefore the organ culture conditions suboptimal for some strains of mice.

1. Introduction

Spermatogenesis is an intricate and highly regulated process occurring in the seminiferous tubules of the testis through which spermatogonial stem cells (SSCs) develop to mature sperm. The spermatogenic process begins with spermatogonial proliferation, followed by meiotic divisions of spermatocytes giving rise to haploid round spermatids. These spermatids undergo remarkable cellular morphological changes to become functional sperm. The development of germ cells depends on the presence of functioning somatic cell populations, including supporting Sertoli cells and testosterone producing Leydig cells. Correct spermatogenesis underlies male reproductive potential from the moment spermatogenesis is initiated at puberty and continues throughout life.

When cancer treatment is required in prepuberty, boys often face a future of infertility in their adult life (Stukenborg et al., 2018). The spermatogonia present in the prepubertal testis have been identified as very sensitive to chemotherapy and radiation damage (Meistrich, 2013; van der Meer et al., 1992), leading to a medium to high risk of irreversible infertility later in life depending on treatment type and dosage (Schrader et al., 2001; Wallace, 2011). To preserve fertility in these boys, a testicular biopsy containing SSCs can be cryopreserved prior to gonadotoxic treatment (Ginsberg et al., 2010; Picton et al., 2015; Uijldert et al., 2017). A number of methods that allow restoration of fertility from cryopreserved testicular biopsies are being explored in the mouse model including transplantation of *in vitro* propagated SSCs (Brinster and Zimmermann, 1994; Brinster, 2007; Kanatsu-Shinohara and Shinohara, 2007), testicular tissue grafting (Schlatt et al., 2003; Wyns et al., 2007) and *in vitro* spermatogenesis either in cell or organ culture (Stukenborg

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et al., 2009; Sato et al., 2011a). However, none of these methods are approved for clinical application.

Testicular organ culture involves prolonged culture of small fragments from a testicular biopsy at the gas-liquid interphase to allow *in vitro* spermatogenesis (Yokonishi et al., 2013). Successful support of complete spermatogenesis with generation of functional sperm was first achieved by organ culture of neonatal mouse testis fragments (Sato et al., 2011a). Reproducibility and optimization of the murine culture system presented by Sato and colleagues are crucial in order to further assess the potential clinical value of the organ culture method.

For research performed in the field of male fertility, it has been recognized that different mice strains may have differences in physiology. For testicular organ culture, various strains have successfully been used including C57BL/6 J (Chapin et al., 2016; Reda et al., 2017; Yao et al., 2017), CD-1 (Arkoun et al., 2015; Chapin et al., 2016; Dumont et al., 2015, 2016; Rondanino et al., 2017) and also haspin-GFP or acrosin-GFP transgenic mice with a C57BL/6 J and C3H (B6C3F2) hybrid background (Sato et al., 2011b, 2015; Yokonishi et al., 2014). On the other hand, it is known that the choice of mouse strain is crucial for effective *in vitro* proliferation of mouse SSCs in testicular cell culture. Testicular cell cultures originated from C57BL/6 J and 129/SV strains fail to efficiently propagate SSCs *in vitro*, as somatic cells rapidly overgrow the proliferating SSCs and thereby considerably decreasing the percentage of SSCs in culture (Aoshima et al., 2013; Kanatsu-Shinohara et al., 2003a). In contrast, SSCs proliferate massively in testicular cell cultures originated from DBA/2 J, ICR or a hybrid of C57BL/6 J females and DBA/2 J males designated B6D2F1/2 mice (Aoshima et al., 2013; Kanatsu-Shinohara et al., 2003a).

Based on reported data on SSCs proliferation *in vitro* we hypothesized that B6D2F2 hybrid mouse strain with 75% C57BL/6 J background is equally or more efficient in establishing spermatogenesis in neonatal mouse organ cultures compared to C57BL/6 J, due to its greater efficiency in SSCs propagation *in vitro*. Therefore, we aimed to compare the reproducibility and efficiency of spermatogenesis by organ culture with testicular tissue originated from C57BL/6 J and B6D2F2 hybrid mice.

2. Materials and methods

2.1. Ethical approval

All animals were maintained according to the European legislation of animal experimentation and experiments were evaluated and approved by the Animal Ethical Committee (DVF196) of the Academic Medical Center (Amsterdam, The Netherlands).

2.2. Mice

C57BL/6 J (Charles River, France) and B6D2F2 mice were used as testicular tissue donors for organ culture. B6D2F2 animals were generated by crossing of male DBA/2 J (Charles River, France) to female C57BL/6-Tg (CAG-EGFP)131Osb/LeySopJ (Jackson Laboratory, United States of America, stock No: 006567) to create B6D2F1 mice, followed by crossing of male B6D2F1 to C57BL/6 J female (Charles River, France). Hybrid mice (B6D2F2-eGFP) were identified using a hand-held Long-Wave UV lamp (Bio-Rad, United Kingdom).

Donor mice were sedated by 4% isoflurane total body anesthesia prior to euthanization by decapitation and inactivation of the brain. Testicular tissues were harvested and pooled from all male pups from one litter (2–11 animals each) of C57BL/6 J ($n = 3$) and B6D2F2 ($n = 4$) mice at 4–8 days postpartum (dpp), which is within the range of ages demonstrated (Kanatsu-Shinohara et al., 2003b; Sato et al., 2011b) to be more efficient for fertility restoration (Table S1). At these ages the seminiferous epithelium is solely constituted by immature somatic cells and spermatogonia (Drumond et al., 2011).

2.3. Testicular organ culture

Testes were dissected, the tunica albuginea was removed and tissues were further processed into fragments of approximately 1–2 mm^3, in Minimal Essential Medium alpha (α-MEM, Gibco, Thermo Fisher Scientific, USA) on ice. One fragment was directly fixed to serve as pre-culture control and the remaining fragments were used for organ culture. Testicular organ culture was performed according to the gas-liquid interphase method with minor modifications (Sato et al., 2011b; Reda et al., 2017). Briefly, 0.35% (w/v) agarose stands were prepared by mixing equal volumes of 0.7% (w/v) agarose (seaKem LE, 50004, Lonza, Basel, Switzerland) and α-MEM culture medium supplemented with 10% KnockOut serum replacement (KSR; 10828-028, Thermo Fisher Scientific, Massachusetts, USA) and 1% Penicillin-Streptomycin (15140122, Thermo Fisher Scientific, Massachusetts, USA). Up to 3 processed testicular fragments were placed on top of each agarose stand and culture media was added to reach the superior limit of the stands. Testicular fragments were cultured at 34 °C and 5% CO_2 for 1–6 weeks. Weekly, the culture media was refreshed and a minimum of 3 cultured tissue fragments were fixed for further immunohistological analysis.

2.4. Immunohistological examination

Cultured testicular fragments were fixed in diluted Bouin's solution (70% picric acid solution, 25% formaldehyde, 5% glacial acetic acid) for 3 h at room temperature (RT) and embedded in paraffin (ParaPlast Plus - 39602004; Leica Biosystems, Wetzlar, Germany). Serial sections (5 μm) were made from every fragment and sections showing the largest cut surface were selected for morphological assessment of the most advance germ cell developmental step and immunohistological detection of spermatogonial (LIN28), Sertoli cell maturation (androgen receptor - AR) and Leydig cell steroidogenic functionality (CYP17A1) markers. For immunohistochemical staining, after deparaffinization and rehydration, sections were subjected to antigen retrieval in 0.01 M tri-sodium citrate dihydrate (pH 6.0) for 10 min in a microwave. Endogenous peroxidase activity was inactivated with 0.3% H_2O_2/PBS (LIN28 and AR) or 3% H_2O_2/TBS (CYP17A1) for 10 min at RT and non-specific binding sites blocked with Superblock (Klinipath, Olen, Belgium) for 1 h at RT. Sections were subsequently incubated with 1:1000 rabbit polyclonal LIN28 (ab46020, Abcam, UK); 1:100 mouse monoclonal AR (sc-7305, Santa Cruz Biotechnology, USA) or 1:800 rabbit polyclonal CYP17A1 (ab80206, Abcam, UK) primary antibody overnight at 4 °C and, after washing, followed by 1 h incubation with goat-anti Mouse/Rabbit poly-HRP secondary antibody (DPVO110HRP, Immunologic, the Netherlands). The signal was visualized using Bright-DAB (Immunologic, the Netherlands). Sections incubated with isotype IgG instead of the first antibody served as a negative control. Sections were counterstained with Mayer's hematoxylin and entirely scanned using Philips IntelliSite UFS slide scanner (Philips Digital Pathology Solutions, Best, the Netherlands) at 40x magnification.

2.5. Percentage of tubules containing germ cells

To evaluate germ cell development *in vitro*, a total of 7 to 204 tubular cross-sections per fragment were analysed (mean ± SD = 45.08 ± 29.80 for C57BL/6 J and 45.15 ± 27.25 for B6D2F2) (Table S1). Tubules were classified based on the most advanced cell type present as 1) Sertoli cell only (SCO) if no germ cells were found, or containing 2) LIN28-positive spermatogonia, 3) spermatocytes, 4) round spermatids or 5) elongating spermatids. Different germ cell types were identified according to the standard morphology, based on nuclear size, chromatin content and architecture, and location within the seminiferous epithelium (Clermont, 1972).

Testicular fragments from each mouse strain at various time intervals in organ culture period were evaluated and results presented as mean ± SD percentage of seminiferous tubules containing the most advanced germ cell type.

2.6. Statistical analysis

Statistical analysis was performed using SPSS Statistics for Windows, version 22.0 (IBM Corp. Armonk, NY). Mixed-effect linear model was used to evaluate the influence of the variable 'strain' on each outcome (percentage of tubules with Sertoli cell only; spermatogonia; spermatocytes; round or elongating spermatids) during the entire culture period. All fragments for each condition were considered and outliers were not excluded. Statistical significance was considered for p-values lower than 0.05.

2.7. Key Resources Table

Information on the resources used in this study are listed in a Key Resources Table.

Reagent or resource	Source	Identifier
Antibodies		
Rabbit polyclonal anti-LIN28	Abcam	Cat# ab46020; RRID: AB_776033
Mouse monoclonal anti-AR	Santa Cruz Biotechnology	Cat# sc-7305; RRID: AB_626671
Rabbit polyclonal anti-CYP17A1	Abcam	Cat# ab80206; RRID: AB_1603486
Experimental Models: Organisms/Strains		
Mouse: C57BL/6 J	Charles River	Strain code: 027
Mouse: B6D2F2 (wildtype)	Own breeding	N/A
Mouse: B6D2F1 (wildtype)	Own breeding	N/A
Mouse: DBA2/J	Charles River	Strain code: 625
Mouse: C57BL/6-Tg (CAG-EGFP) 131Osb/LeySopJ	Jackson Laboratory	RRID:IMSR_JAX:006567
Software and Algorithms		
SPSS	IBM	RRID:SCR_002865; http://www-01.ibm.com/software/uk/analytics/spss/

3. Results

3.1. Testicular organ culture supports full spermatogenesis for C57BL/6 J, but not B6D2F2

To study the effectiveness of *in vitro* sperm development in mice strains with different genetic backgrounds, testicular fragments from C57BL/6 J and B6D2F2 neonatal testis were cultured in a gas-liquid interphase for 6 weeks (Table S1). In both strains, the seminiferous tubules were constituted by spermatogonia and supportive Sertoli cells (C57BL/6 J: 96 ± 4% tubules; B6D2F2: 99.8 ± 0.4% tubules) or only Sertoli cells (C57BL/6 J: 4 ± 4% tubules; B6D2F2: 0.2 ± 0.4% tubules) indicating that prior to culture there was no differentiation of germ cells in the seminiferous epithelium in either C57BL/6 J or B6D2F2 testicular fragments (Figs. 1 and 2). Additionally, the percentage of tubules with spermatogonia before organ culture was not statistically different between the two strains.

Cultured testicular fragments from C57BL/6 J mice contained LIN28-positive spermatogonia during the entire culture period. The presence of spermatocytes in the prophase of meiosis was first detected from week 1 onwards, irrespective of the mouse age, followed by the appearance of round spermatids from week 2. The percentage of seminiferous tubules containing round spermatids continuously increased *in vitro* from 2 ± 6% at week 2 to 17 ± 10% by the end of the culture period at week 6. Complete spermatogenesis, culminating with the production of elongating spermatids occurred at week 4, being increasingly present in seminiferous tubules and reaching a maximum of 16 ± 18% tubules at week 6 (Figs. 1A and 2A). At the end of the culture period, elongating

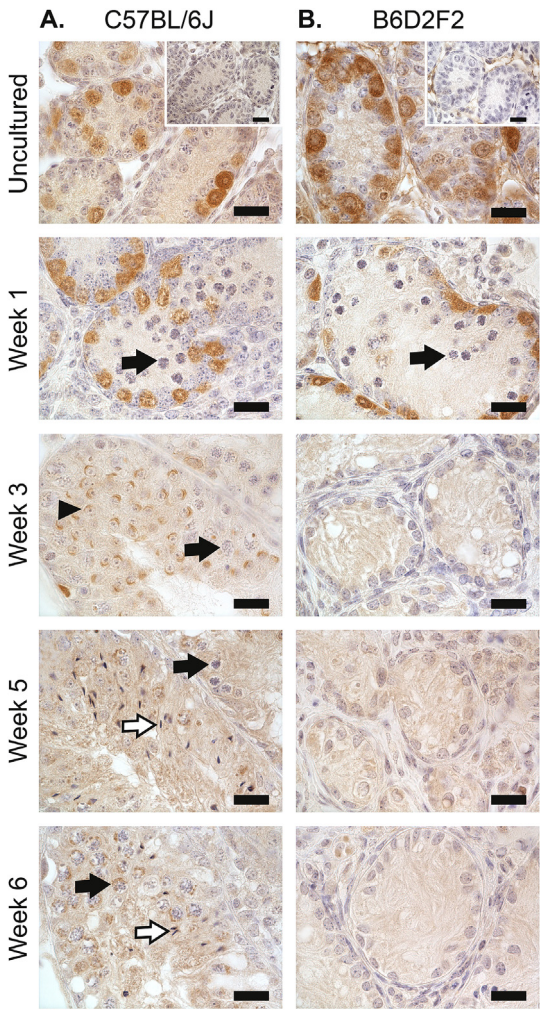

Fig. 1. Immunohistochemical identification of LIN28-positive spermatogonia (brown) in mice testicular fragments sections throughout organ culture. A. C57BL/6 J (n = 3) and **B.** B6D2F2 (n = 4) mouse donors. Insets show isotype IgG negative controls. Complete spermatogenesis was solely observed in C57BL/6 J cultured fragments. Black arrow: spermatocytes; arrowhead: round spermatids; white arrow: elongating spermatids. Scale bars represent 20 μm. Pictures were taken using an Olympus BX41 bright-field microscope with an Olympus DP20 color camera.

spermatids could be observed in 94% of all testicular fragments analysed.

Testicular fragments from B6D2F2 mice showed a different developmental progress *in vitro* when compared to cultured testicular fragments from C57BL/6 J mice. Although cultured testicular fragments from B6D2F2 mice demonstrated progression of spermatogonia to spermatocytes at weeks 1 and 2, no further progression to more advanced germ cell states was observed for this strain in any of the time periods examined. In fact, for B6D2F2 cultured fragments, spermatocytes were absent from week 3 onwards and the seminiferous epithelium rapidly became depleted of all germ cells in 100% of tubular cross-sections at week 6 (Figs. 1B and 2B).

When comparing the organ cultures of both mice strains, the percentage of tubules containing spermatogonia ($p < 0.001$) and spermatocytes ($p < 0.05$) was significantly higher in C57BL/6 J testicular fragments than in B6D2F2 throughout the entire culture period. In fact, the number of spermatogonia on B6D2F2 fragments started to decline immediately after culture (from week 1) and were undetectable in week 6. Consequently, the percentage of tubules without germ cells was significantly higher ($p < 0.001$) compared to that in cultured fragments of C57BL/6 J. The more advanced germ cells, round and elongating

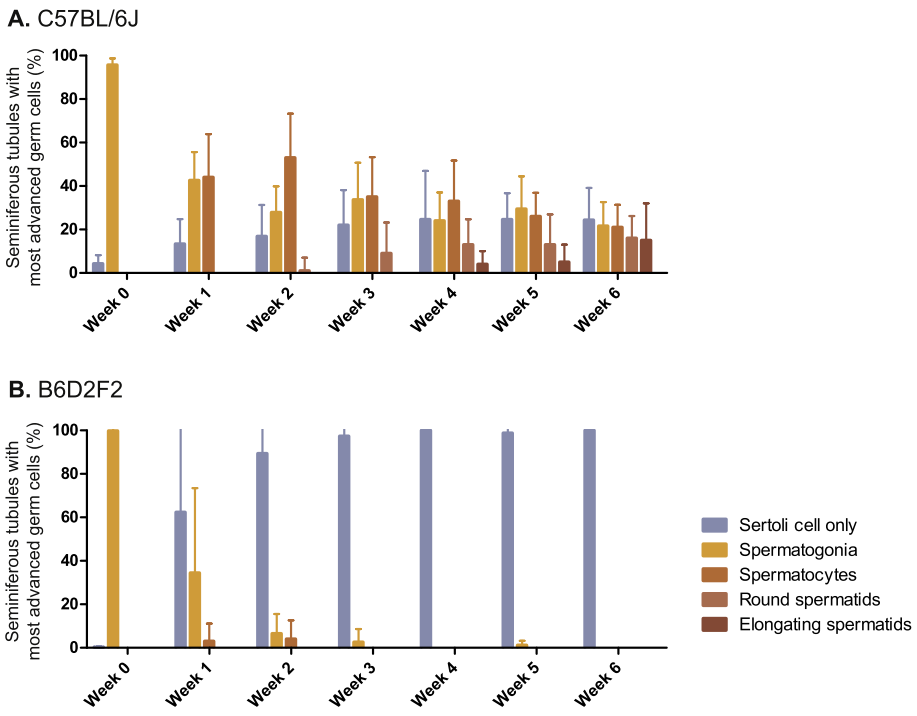

Fig. 2. Percentage (mean ± s.d.) of seminiferous tubules containing the most advanced germ cell type in mice testicular fragments throughout organ culture. A. C57BL/6 J (n = 3) and **B.** B6D2F2 (n = 4) mouse donors. Mixed-effect linear model analysis revealed statistically significant differences in the percentage of tubules without germ cells ($p < 0.001$), containing spermatogonia ($p < 0.001$) and spermatocytes ($p < 0.05$) throughout the entire culture period when comparing the two mice strains.

spermatids, were uniquely detected in C57BL/6 J cultured testicular fragments (Figs. 1 and 2).

Based on the observed differences in initiation of spermatogenesis during testicular organ culture between C57BL/6 J and B6D2F2 testicular fragments, we wondered whether for both strains maturation of the supportive Sertoli cells was occurring *in vitro*. Expression of androgen receptor (AR), a marker of mature Sertoli cells, became detectable from week 1 onwards in cultured testicular fragments of both strains. Additionally, peritubular myoid cells and Leydig cells of C57BL/6 J and B6D2F2 testicular fragments expressed AR before and throughout culture (Fig. 3).

Further functionality of Leydig cells was determined by evaluation of CYP17A1 expression, a key enzyme involved in the steroidogenic pathway. For both strains, expression of CYP17A1 by Leydig cells was detected before initiation of culture and was maintained during the entire culture period, independently of the mouse strain (Fig. 4).

4. Discussion

In this study, we found that organ culture of testicular tissue from B6D2F2 mice could not effectively support spermatogenesis whereas complete spermatogenesis was achieved in C57BL/6 J testicular fragments, in accordance with previous literature. This is to our knowledge the first report of strain-dependence in the research field of *in vitro* spermatogenesis.

It is generally well recognized that the choice of mouse strain differently influences the usefulness of an animal model for each specific research question. Inbred mice strains are most commonly used due to the benefit of the identical genetic make-up in mice within the same strain, thereby reducing differences that may be caused by genetic variation. Nowadays, a plethora of inbred mice strains is commercially available, with C57BL/6 being the most widely used. Even though commonly used mouse strains such as C57BL/6 and DBA2 carry relatively few individual genetic variants (approximately 2%), strain-specific alleles or gene variants can lead to tremendous differences in phenotype (Keane et al., 2011). Our results even implicate a difference between C57BL/6 J and the hybrid derived from the same strain (B6D2F2). Though some degree of genetic segregation takes place at each crossing when creating the F2 hybrid (B6D2F1 male crossed to C57BL/6 J female), B6D2F2 mice share approximately 75% of all genetic material with C57BL/6 J, and therefore the autosomal genetic information should be highly similar. Since hybrid mice are considered to have a greater reproductive potential (e.g. larger litters) than classical inbred mice (Lutz et al., 2012), and as B6D2F2 was shown to be more efficient in propagation of SSCs *in vitro*, we expected to observe higher spermatogenic yields after organ culture of testicular fragments from B6D2F2 compared to C57BL/6 J mice. However, our results were counterintuitive.

Apart from genetic variation in autologous chromosomes between C57BL/6 J and B6D2F2, genetic polymorphisms specifically in the sex chromosomes might explain the differences in spermatogenesis *in vitro*. The Y chromosome of B6D2F2 mice is derived from DBA2 as result of the crossing with a female C57BL/6 J. Genetic polymorphisms in the Y chromosome have been identified in inbred strains (Laman and Palmer, 1984). In fact, mice exhibit single-nucleotide polymorphisms (SNPs) in the Y chromosomal *Usp9y* and *Uty* gene and the number of CAG repeats differ in the *Sry* gene (Satou and Suto, 2015). Particularly, differences in *Usp9y* polymorphisms between DBA/2 J and C57BL/6 J were correlated to different testis weight (an indicator of fertility), with DBA/2 J testis showing higher weights (Satou and Suto, 2015). In contrast, mean sperm counts were reported to be lower in DBA2 mice (9.23 ± 2.16) compared to C57BL/6 J (14.1 ± 7.12) (https://phenome.jax.org/projects/Handel1). The combination of higher testis weight and lower sperm count in DBA2 compared to C57BL/6 might suggest a less efficient testicular somatic environment. Savchuk and colleagues showed strain-related differences in testosterone production by Leydig cells (Savchuk et al., 2013). Additionally, previous reports describe that *in vitro* propagation of SSCs isolated from B6D2F2 testis is more efficient compared to C57BL/6 J, since testicular cell cultures originated from this last strain are rapidly overgrown by somatic cells (Aoshima et al., 2013; Kanatsu-Shinohara et al., 2003a). Hence we reasoned that the hybrid mice B6D2F2 would be more suitable than C57BL/6 J for *in vitro* studies regarding male fertility restoration by organ culture. However, our results show the opposite; spermatogonia to spermatid development *in vitro* occurred in the C57BL/6 J but not in B6D2F2 mice testicular fragments. This contrasting behavior suggests that the *in vitro* balance between proliferation and differentiation of SSCs might be strain-dependent, which possibly reflects

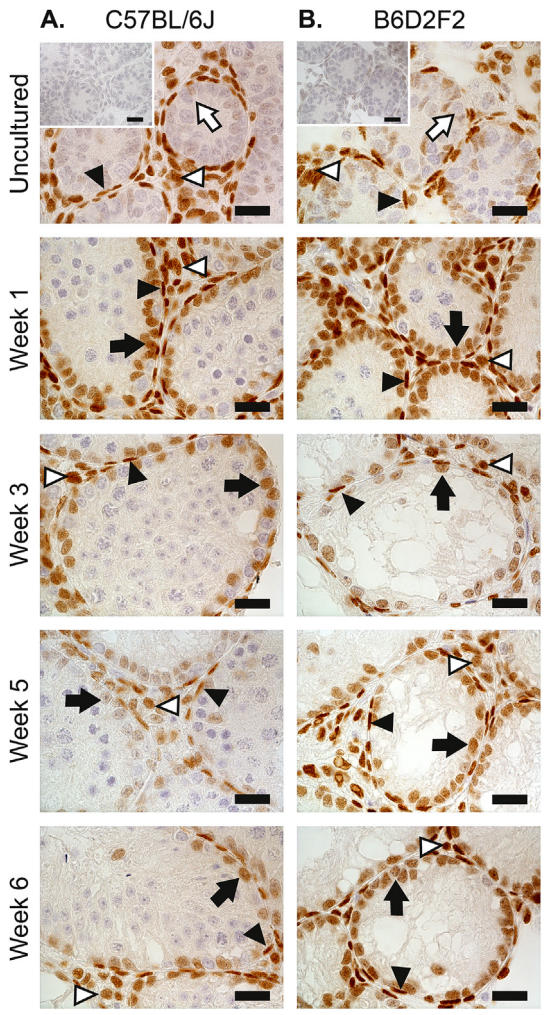

Fig. 3. Immunohistochemical identification of mature Sertoli cells by expression of androgen receptor (AR; brown) in mice testicular fragments sections throughout organ culture. A. C57BL/6 J (n = 3) and **B.** B6D2F2 (n = 4) mouse donors. Insets show isotype IgG negative controls. Before culture, AR was expressed in peritubular myoid cells and Leydig cells but not in Sertoli cells of neither C57BL/6 J nor B6D2F2 testicular fragments. From week 1 onwards in organ culture, Sertoli cells from testicular fragments of both strain presented nuclear expression of AR, indicating a mature phenotype. Black arrow: AR-positive Sertoli cell; white arrow: AR-negative Sertoli cell; black arrowhead: AR-positive peritubular cell; white arrow-head: AR-positive Leydig cell. Scale bars represent 20 μm. Pictures were taken using an Olympus BX41 bright-field microscope with an Olympus DP20 color camera.

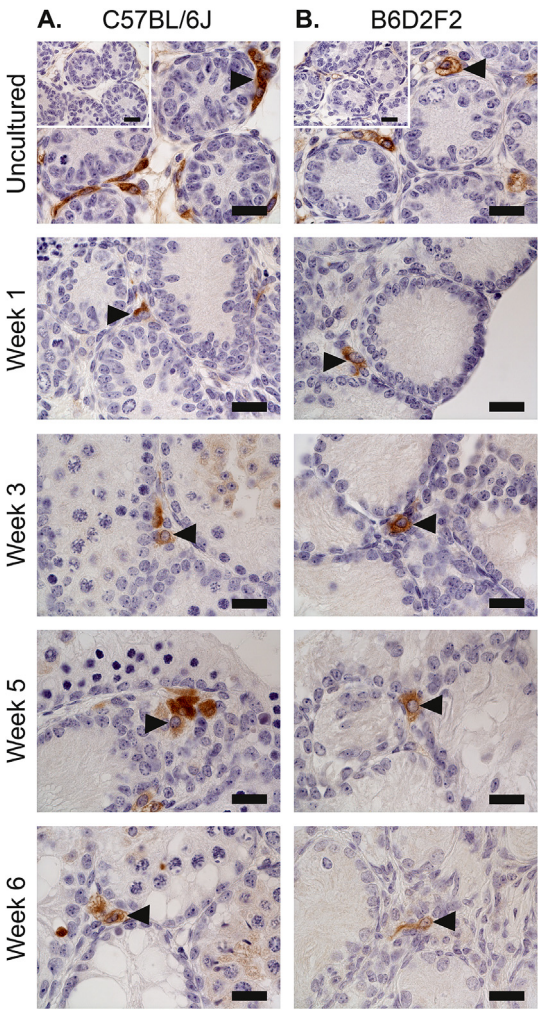

Fig. 4. Immunohistochemical identification of the steroidogenic function of Leydig cells by expression of CYP17A1 enzyme (brown) in mice testicular fragments sections throughout organ culture. A. C57BL/6 J (n = 3) and **B.** B6D2F2 (n = 4) mouse donors. Insets show isotype IgG negative controls. For both strains, CYP17A1 was expressed by Leydig cells before cultured and maintained during the entire culture period. Arrow-head: CYP17A1-positive Leydig cell. Scale bars represent 20 μm. Pictures were taken using an Olympus BX41 bright-field microscope with an Olympus DP20 color camera.

differences between the functional supportive capacity of somatic cells in the testis of C57BL/6 J and B6D2F2 mice. On the other hand, our results indicate that maturation of Sertoli cells in organ culture is successfully initiated in both strains as indicated by AR expression after 1 week. Additionally, steroidogenic functionality of Leydig cells was maintained in culture independently of the strain. Further studies are required to address the differences in molecular control of the somatic cells to initiate full spermatogenesis in these mice strains.

Our data sheds light on a more important problem: the feasibility of testicular organ culture for clinical applications. If the variation in genetic background is an important determinant in the balance between SSCs self-renewal and differentiation *in vitro*, then the vastness of genetic variation amongst human beings should be taken into account. This emphasizes the need for more detailed studies on the effects raised by specific genetic background for the success of spermatogenesis *in vitro* and optimization of testicular organ culture as a method to restore fertility in prepubertal cancer survivors.

In conclusion, in this study we showed that organ culture of testicular tissue collected from C57BL/6 J mice supports complete spermatogenesis *in vitro*, while complete spermatogenesis could not be achieved in cultured testicular fragment from B6D2F2 mice. As the described result for SSCs differentiation is opposite to the efficiency of proliferative activity of SSCs *in vitro* in these mice stains, it strongly suggests that also the balance between proliferation and differentiation of SSCs *in vitro* is strain-dependent or that different genetic backgrounds require different *in vitro* spermatogenesis protocols.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Authors contribution

This study was designed by J.M.D.P., C.L.M., J-B.S., S.R., and A.M.M.P. Neonatal testis were collected by C.L.M. and J.M.D.P. performed testicular organ cultures. Immunohistological stainings were performed by J.M.D.P., S.K.M.D. and C.M.W.K. Histological examinations were done by J.M.D.P. C.L.M. and J.M.D.P. drafted the original manuscript. All authors critically reviewed and revised the manuscript and approved the final version.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.ydbio.2019.08.007.

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