Developmental Biology

Steroidogenesis and androgen/estrogen signaling pathways are altered in *in vitro* matured testicular tissues of prepubertal mice

Reviewed Preprint

Revised by authors after peer review.

About eLife's process

Reviewed preprint version 3
November 24, 2023

Reviewed preprint version 2
August 25, 2023 (this version)

Reviewed preprint version 1
May 2, 2023

Sent for peer review
January 13, 2023

Posted to preprint server
November 18, 2022

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Abstract

Children undergoing cancer treatments are at risk for impaired fertility. Cryopreserved prepubertal testicular biopsies could theoretically be later matured *in vitro* to produce spermatozoa for assisted reproductive technology. A complete *in vitro* spermatogenesis has been obtained from mouse prepubertal testicular tissue, although with low efficiency. Steroid hormones being essential for the progression of spermatogenesis, the aim of this study was to investigate steroidogenesis and steroid signaling in organotypic cultures. Histological, RT-qPCR, western blot analyses and steroid hormone measurements were performed on *in vitro* cultured mouse prepubertal testicular tissues and age-matched *in vivo* controls. Despite a conserved density of Leydig cells after 30 days of culture (D30), transcript levels of adult Leydig cell and steroidogenic markers were decreased. Increased amounts of progesterone and estradiol and reduced androstenedione levels were observed at D30, together with decreased transcript levels of steroid metabolizing genes and steroid target genes. hCG was insufficient to facilitate Leydig cell differentiation, restore steroidogenesis and improve sperm yield. In conclusion, this study reports the failure of adult Leydig cell development and altered steroid production and signaling in tissue cultures. The organotypic culture system will need to be further improved before it can be translated in clinics for childhood cancer survivors.

eLife assessment

This study reports **useful** information on the limits of the organotypic culture of neonatal mouse testes, which has been regarded as an experimental strategy that can be extended to humans in the clinical setting for the conservation and subsequent re-use of testicular tissue. The evidence that the culture of testicular fragments of 6.5-day-old mouse testes does not allow optimal differentiation of steroidogenic cells is **compelling** and would be **useful** to the scientific community in the field for further optimizations.

Introduction

Pediatric cancer treatments such as chemotherapy have recognized toxicity on germline stem cells, which could lead to infertility at adulthood (1,2). Freezing of prepubertal testicular tissue containing spermatogonia is a fertility preservation option proposed to prepubertal boys with cancer prior to highly gonadotoxic treatments. Several fertility restoration approaches, whose aim is to mature cryopreserved tissues *in vivo* or *in vitro* in order to produce spermatozoa, are being developed. So far, these approaches have not been clinically validated. *In vitro* maturation strategies are currently being optimized in animal models. *In vitro* spermatogenesis could indeed be proposed to patients with testicular localization of residual tumor cells, for whom testicular tissue autografting is not indicated (about 30% of patients with acute leukemia). The organotypic culture procedure, which preserves testicular tissue architecture, microenvironment and cell interactions, has been used successfully to obtain spermatozoa from fresh or frozen/thawed mouse prepubertal testicular tissues (3–6). In addition, viable and fertile mouse offspring have been obtained from *in vitro* produced spermatozoa by oocyte microinjection (3,4).

It has been previously shown that supplementing organotypic culture media with retinol improves the *in vitro* production of spermatids and spermatozoa in prepubertal mouse testicular tissues cultured at a gas-liquid interphase (5,7). However, *in vitro* sperm production still remains a rare event. Transcript levels of the androgen receptor (AR)-regulated gene *Rhox5* were decreased at the end of the culture period, suggesting that testosterone production by Leydig cells and/or AR transcriptional activity could be impaired in organotypic cultures (8). A decline in intratesticular testosterone levels has been highlighted *in vitro* in 35- to 49-day cultures of 5 d*pp* mouse testes, i.e. after the end of the first wave of spermatogenesis (9). Moreover, our recent transcriptomic analysis reveals a decline in mRNA levels of *Cyp17a1*, encoding a steroidogenic enzyme, in 4- to 30-day cultures of 6 d*pp* mouse testes (10). Based on these results, Leydig cell maturation and functionality need to be thoroughly explored in order to identify the molecular mechanism that are deregulated *in vitro*.

Two distinct populations of Leydig cells appear during mouse testicular development: (i) fetal Leydig cells arising during embryonic development and regressing over the first two weeks of postnatal life and (ii) adult Leydig cells appearing around one week after birth. Although they both express StAR (steroidogenic acute regulatory protein), CYP11A1 (P450 side chain cleavage), 3β-HSD1 (3β-hydroxysteroid dehydrogenase type 1) and CYP17A1 (P450 17α-hydroxylase/C17-20 lyase), which are steroidogenic proteins required for androgen production, 17β-HSD3 (17β-hydroxysteroid dehydrogenase type 3), which converts androstenedione to testosterone, is expressed only by adult Leydig cells (11,12). Consequently, the major androgens produced in fetal Leydig cells and adult Leydig cells are androstenedione and testosterone, respectively (13). In rodents, adult Leydig cells develop from undifferentiated stem Leydig cells through two

intermediate cells, progenitor Leydig cells and immature Leydig cells (14). All these cells, except stem Leydig cells, express CYP11A1, 3β-HSD1 and CYP17A1 (15,16). 17β-HSD3 begins to be expressed in immature Leydig cells (17). The expression of the androgen metabolizing enzyme SRD5A1 (5α-reductase 1), which is high in progenitor and immature Leydig cells, is being silenced in adult Leydig cells (17). Adult Leydig cells express INSL3 (Insulin-like peptide 3) (18,19) as well as SULT1E1 (estrogen sulfotransferase) (12), which protects Leydig cells and seminiferous tubules against estrogen overstimulation by catalyzing the sulfoconjugation and inactivation of estrogens. Estrogens and androgens can also be inactivated in the testis by 17β-HSD2 (17β-hydroxysteroid dehydrogenase type 2), which converts estradiol to estrone and testosterone to androstenedione (20). Different factors, among which DHH (Desert hedgehog), IGF1 (Insulin-like Growth Factor 1) and LH (Luteinizing hormone) regulate the proliferation and differentiation of the Leydig cell lineage: DHH is required for both proliferation and differentiation of stem Leydig cells (21), IGF1 stimulates the proliferation of progenitor Leydig cells and immature Leydig cells (22) and promotes the maturation of immature Leydig cells into adult Leydig cells (23), and LH is critical to both adult Leydig cell differentiation and their precursor proliferation (24).

The steroid hormones produced by Leydig cells are essential for the progression of spermatogenesis. Testosterone, synthesized under the control of LH, is essential for many aspects of spermatogenesis, including meiotic progression, spermiogenesis and germ cell survival (25–28). Androgen-binding protein (ABP), produced by Sertoli cells under the regulation of FSH, increases the accumulation of androgens in the seminiferous epithelium and makes them available for binding to intracellular AR (29). Estrogens, derived from the aromatization of androgens by CYP19A1 (aromatase) in somatic and germ cells, have been shown to be important for the long-term maintenance of spermatogenesis in the ArKO mouse (lacking aromatase) and for the progression of normal germ cell development in the ENERKI mouse (estrogen nonresponsive ERα knock-in) (30,31). The expression of aromatase is age-dependent, being mostly in Sertoli cells in immature rat testes and then in Leydig cells during adulthood (32,33). In mouse, aromatase is also present within seminiferous tubules, mainly in spermatids (34).

Androgens act directly on somatic cells (Sertoli cells and peritubular myoid cells) through AR to support germ cell development by regulating the expression of target genes. *Rhox5*, encoding a transcription factor that promotes germ cell survival (35), and *Eppin*, encoding a serine protease inhibitor, are target genes of testosterone in Sertoli cells, with androgen response elements (ARE) in their promoters (36). Several estrogen receptors mediate the effects of estrogens in the testis. In rodents, ERα was found in Leydig cells and peritubular myoid cells, ERβ was found in Sertoli cells and some germ cells (spermatogonia, spermatocytes) and GPER was detected in Leydig cells, Sertoli cells and germ cells (spermatocytes, spermatids) (37–41). *Faah*, encoding a hydrolase that promotes Sertoli cell survival by degrading anandamide, is a direct target gene of estrogens in mature Sertoli cells (42,43). *Septin12*, containing 1 AR and 2 ERα binding sites in its promoter, has been identified as a potential novel target of the androgen and estrogen receptors in human testicular cells (44).

Since steroid hormones play an essential role in the progression of spermatogenesis, it appears necessary to ensure that their syntheses and mechanisms of action are not altered in *in vitro* cultured testicular tissues. The aim of the present work was therefore to study Leydig cell maturation, steroidogenesis and androgen/estrogen signaling in a comprehensive manner during the *in vitro* maturation of mouse prepubertal testicular tissues. The expression of Leydig cell markers, Leydig cell differentiation factors, steroidogenic enzymes, steroid receptors, steroid target genes and steroid contents were quantified in cultured tissues and in their *in vivo* counterparts. Although our organotypic culture conditions support a complete spermatogenesis, a failure of adult Leydig cell development with impaired steroidogenesis and altered steroid hormone signaling was demonstrated in this study.

Results

Leydig cells are partially mature after 30 days of organotypic culture

We first wondered whether Leydig cell number, survival, proliferation and differentiation could be altered under *in vitro* conditions. 3β-HSD immunofluorescence staining was performed to detect and quantify Leydig cells during mouse postnatal development and in *in vitro* cultured fresh testicular tissues (FT). The percentage of Leydig cells in apoptosis or in proliferation was measured after cleaved caspase 3 or Ki67 immunofluorescence staining respectively, and the percentage of Leydig cells expressing AR, which is required for their proliferation and maturation (51), was also determined. The transcript levels of genes necessary for Leydig cell differentiation (*Igf1*, *Dhh*) and markers of fetal Leydig cells (*Mc2r*), progenitor/immature Leydig cells (*Srd5a1*) or adult Leydig cells (*Sult1e1*, *Insl3*) were then assessed by RT-qPCR. Finally, the concentration of INSL3, a marker of Leydig cell maturity, was measured by RIA in organotypic culture media and in testicular homogenates.

After 16 days of culture, the number of Leydig cells per cm² of testicular tissue was not significantly different from 22 d*pp*, the age-matched *in vivo* control (**Figure 1A-B**). However, at this time point, the percentage of apoptotic Leydig cells was increased compared to the *in vivo* control while the percentage of proliferating Leydig cells was unchanged (**Figure 1C-D**). In addition, the percentage of Leydig cells expressing AR was similar at D16 and 22 d*pp* (**Figure 1E**). Whereas no difference was observed in the mRNA levels of *Dhh*, *Srd5a1* and *Sult1e1* between D16 and 22 d*pp*, *Igf1* and *Insl3* transcripts levels were respectively higher and lower in 16-day organotypic cultures than *in vivo* (**Figure 1F-J**). *Mc2r* transcripts were barely detected at D16 and 22 d*pp* as well as at later time points, thereby reflecting the low amount of fetal Leydig cells in prepubertal testicular tissues (data not shown).

After 30 days of culture, no significant difference in the number of Leydig cells per cm² of testicular tissue and in the percentages of apoptotic and proliferating Leydig cells was observed compared to 36 d*pp*, the corresponding *in vivo* time point (**Figure 1A-D**). Furthermore, the percentage of Leydig cells expressing AR was comparable after *in vitro* or *in vivo* maturation (**Figure 1E**). The transcript levels of *Dhh* were also similar in *in vitro* and *in vivo* matured tissues at the end of the first spermatogenic wave (**Figure 1G**). However, a reduction in the mRNA levels of *Igf1*, *Srd5a1* and the two adult Leydig cell markers (*Sult1e1*, *Insl3*) was found at D30 in comparison to 36 d*pp in vivo* controls (**Figure 1F, H-J**). Moreover, intratesticular INSL3 was below the detection limit (<10 pg/mL) in *in vitro* matured tissues (**Figure 1K**) and a significant elevation in the concentration of INSL3 was observed in culture medium from D22 to D30 for FT tissues (**Figure 1L**).

In summary, while the number and proliferation of Leydig cells are not affected by organotypic culture conditions, their differentiation into mature adult Leydig cells is impaired *in vitro*.

Controlled slow freezing has no impact on Leydig cell density or state of differentiation before or after organotypic culture

In the clinics, testicular biopsies from prepubertal boys are frozen and stored in liquid nitrogen for later use. In order to assess the impact of freezing/thawing procedures (CSF) on Leydig cell number, survival, proliferation and differentiation in organotypic cultures, we conducted the same analyses as above on 6 d*pp* CSF mouse testicular tissues and on *in vitro* matured 6 d*pp* CSF tissues (**Figure 1** -figure supplement 1).

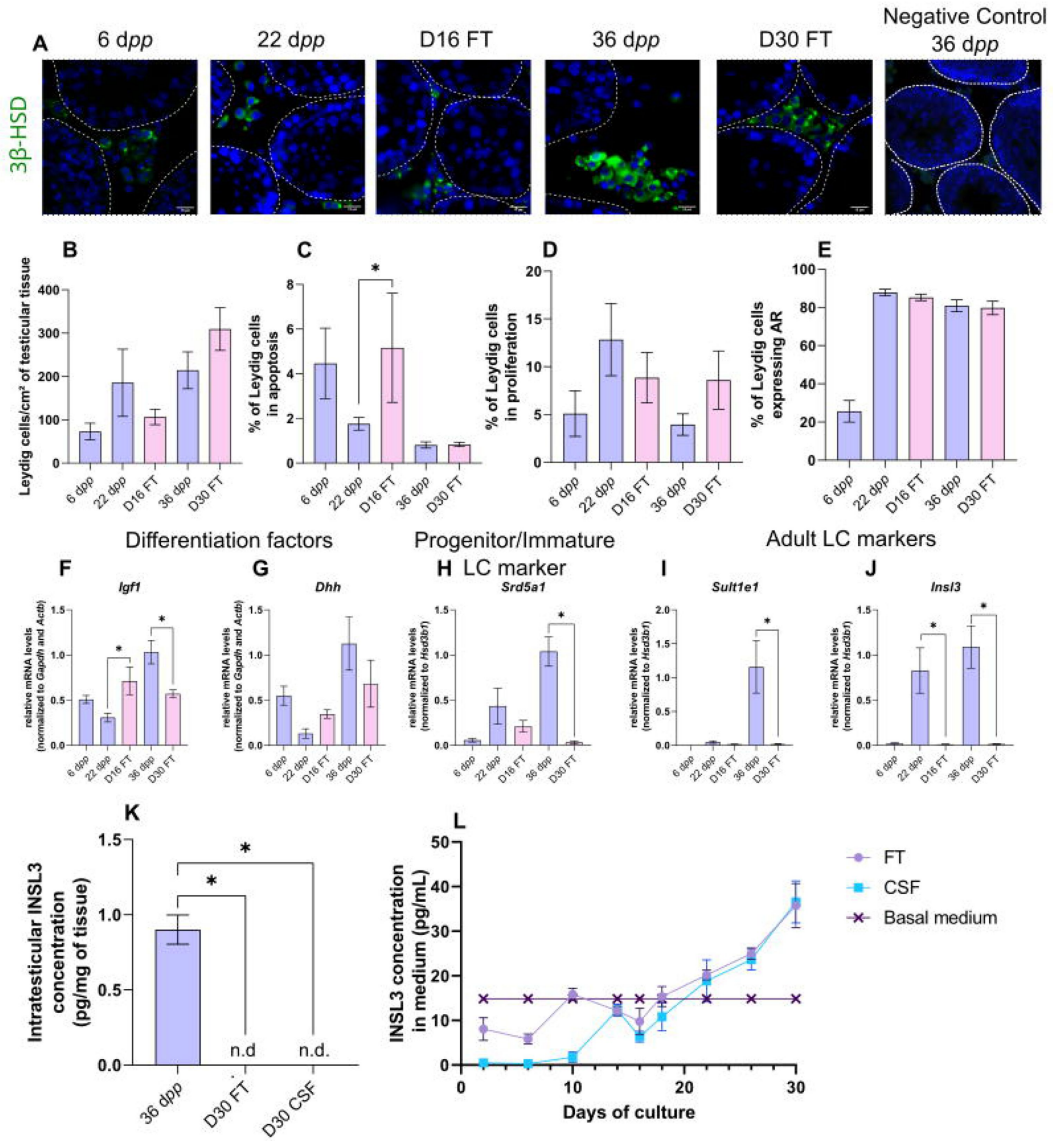

Figure 1.

Leydig cells are partially mature after 30 days of organotypic culture.

(A) Representative images of 3β-HSD expression by Leydig cells during mouse postnatal development (6 d*pp*, 22 d*pp* and 36 d*pp*) and in *in vitro* cultured tissues after 16 days of culture (D16) or 30 days (D30). A representative image of a negative control, carried out by omitting the primary antibody, is also shown. Testicular tissue sections were counterstained with Hoechst (blue). Dotted lines delineate seminiferous tubules. Scale: 15 µm. (B) Number of 3β-HSD+ Leydig cells per cm² of testicular tissue during mouse postnatal development (6 d*pp*, 22 d*pp* and 36 d*pp*) and in *in vitro* cultured tissues (D16 and D30). (C-D) Percentage of Leydig cells (C) in apoptosis (3β-HSD and cleaved caspase 3 positive) or (D) in proliferation (3β-HSD and Ki67 positive) in *in vivo* and *in vitro* matured tissues. (E) Percentage of 3β-HSD positive Leydig cells expressing AR in *in vivo* and *in vitro* matured tissues. (F-J) Relative mRNA levels of Leydig cell differentiation factors (*Igf1*, *Dhh*), progenitor/immature Leydig cell (*Srd5a1*) and adult Leydig cell markers (*Sult1e1*, *Insl3*) (normalized to *Gapdh* and *Actb* or to *Hsd3b1*). (K) Intratesticular concentration of INSL3 (pg/mg of tissue) or in the culture medium (pg/mL).
Data are presented as means ± SEM with n = 4 biological replicates for each group. A value of $*P < 0.05$ was considered statistically significant. n.d.: not determined (under the detection limit)
FT: Fresh Tissue; CSF: Controlled Slow Freezing.

CSF had no impact on the number of Leydig cells per cm² of tissue at 6 d*pp* as well as after 16 or 30 days of culture (**Figure 1** -figure supplement 1**A-B**). Although the percentage of apoptotic Leydig cells was lower in CSF than in FT tissues at 6 d*pp*, it was similar in CSF and FT tissues at D16 and D30 (**Figure 1** -figure supplement 1**C**). Furthermore, no change in the percentage of Leydig cells in proliferation or expressing AR was found after CSF (**Figure 1** - figure supplement 1**D-E**). Moreover, *Dhh* and *Srd5a1* mRNA levels were similar in CSF and FT tissues (**Figure 1** -figure supplement 1**G-H**). *Igf1* transcript levels were decreased in CSF tissues at D16 (**Figure 1** -figure supplement 1**F**). Furthermore, *Sult1e1* mRNA levels were higher in CSF tissues at D30 (**Figure 1** -figure supplement 1**I**) and *Insl3* transcript levels were higher in CSF than in FT tissues at D16 and D30 (**Figure 1** - figure supplement 1**J**).

Since the number and the state of differentiation of Leydig cells are rather similar in FT and CSF tissues, it can be concluded that the freezing/thawing procedures are not harmful to these cells.

The expression of several actors of steroidogenesis is affected in organotypic cultures

As the differentiation of Leydig cells is not fully completed in organotypic cultures, we next wanted to know if actors of the steroidogenic pathway show deregulated expression *in vitro* in comparison to physiological conditions, and thus which steps of the steroid hormone biosynthesis pathway may be impaired. The transcript levels of several genes involved in steroidogenesis were measured by RT-qPCR to highlight a potential deregulation of their expression in cultured tissues (**Figure 2A-D, F, H-I**). The protein levels of two steroidogenic enzymes, 3β-HSD and CYP17A1, were also quantified by western blot (**Figure 2E, G**).

Controlled slow freezing had no impact on the mRNA levels of all the genes examined at 6 d*pp*, *i.e.* before culture (**Figure 2A-I**). At D16, the mRNA levels of *Star, Cyp17a1* and *Hsd17b3* were decreased in FT and CSF testicular tissues compared to 22 d*pp* testes (**Figure 2B, F, H**). *Cyp11a1* transcript levels were also lower in FT tissues at D16 than at 22 d*pp* (**Figure 2C**). In contrast, *Lhcgr*, *Hsd3b1* and *Hsd17b2* transcript levels remained unchanged at this time point (**Figure 2A, D, I**). 3β-HSD and CYP17A1 protein levels were not different between D16 and 22 d*pp* (**Figure 2E, G**).

The mRNA levels of *Cyp11a1*, *Hsd3b1*, *Cyp17a1* and *Hsd17b2* were lower at D30 in both FT and CSF tissues than in the physiological situation (**Figure 2C-D, F, I**). Despite a significant decrease in *Hsd3b1* mRNA levels at D30, the protein levels of 3β-HSD were not significantly different after 30 days of culture and at 36 d*pp* (**Figure 2E**). Western blot experiments however showed that the expression of CYP17A1 was also reduced at the protein level in both FT and CSF tissues at D30 compared to 36 d*pp* (**Figure 2G**). This steroidogenic enzyme was still detectable by immunofluorescence in Leydig cells in cultured tissues (**Figure 2J**). Finally, *Lhcgr* mRNA levels were found significantly reduced at D30 in FT tissues (**Figure 2A**), while *Star* and *Hsd17b3* mRNA levels were reduced at D30 in CSF tissues (**Figure 2B, H**).

Thus, the expression of several genes encoding steroidogenic enzymes is decreased *in vitro*, notably that of *Cyp17a1*, necessary for the conversion of progesterone to androstenedione.

Increased intratesticular concentrations of progesterone and estradiol combined with decreased intratesticular concentration of androstenedione in organotypic cultures

We then analyzed the steroid hormone content in *in vitro* cultured testicular tissues. The concentrations of androstenedione, DHEA and testosterone were measured by LC-MS/MS and the concentrations of progesterone and estradiol were assessed by ELISA in both testicular samples and the culture media (**Figure 3**). DHEA was below the detection limit (<1 ng/mL) in all the

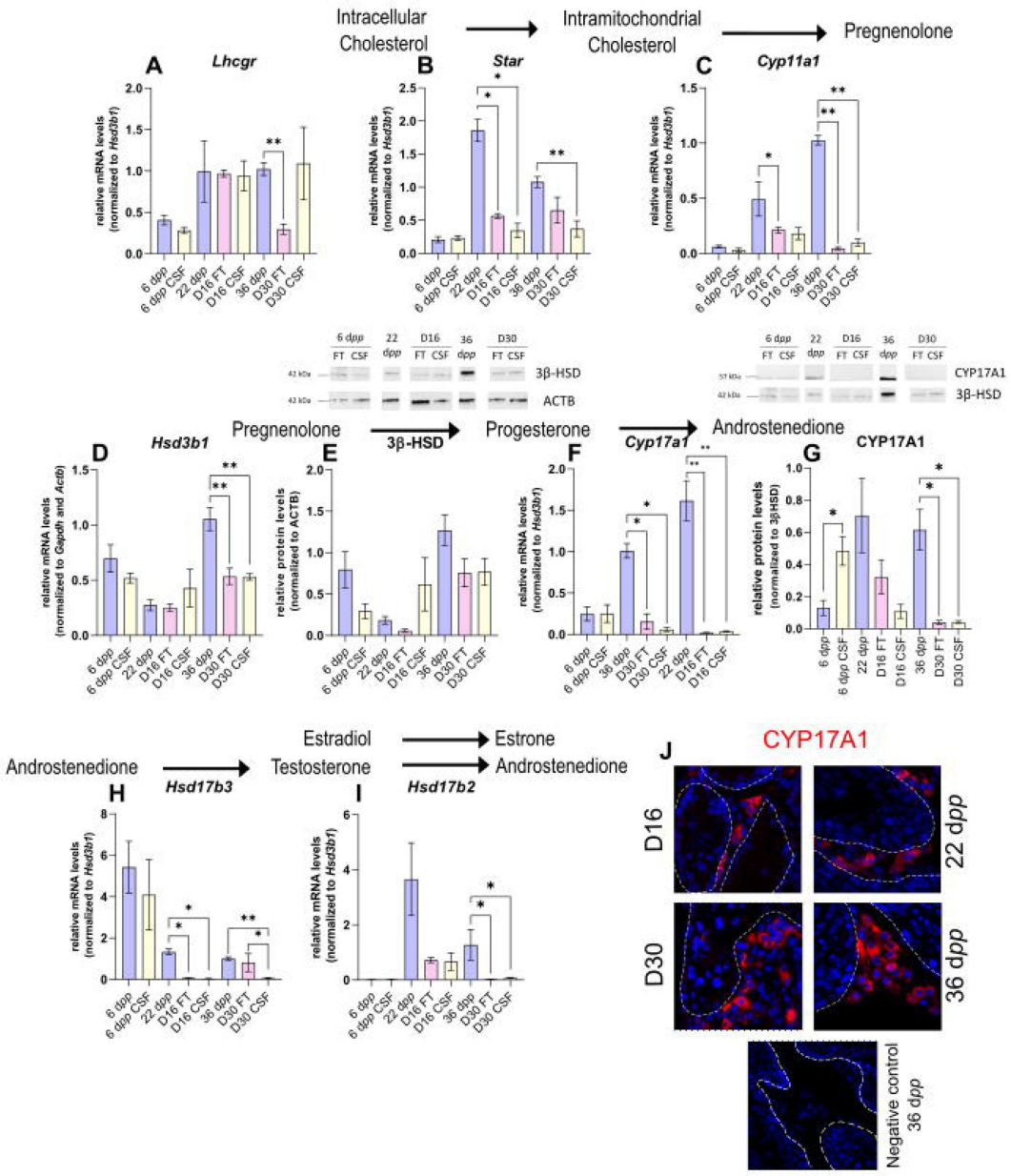

Figure 2.

The expression of several actors of steroidogenesis is downregulated in 30-day organotypic cultures.

(A-I) Relative mRNA levels of *Lhcgr*, *Star*, *Cyp11a1*, *Hsd3b1*, *Cyp17a1*, *Hsd17b3* and *Hsd17b2* (normalized to *Gapdh* and *Actb* or to *Hsd3b1*) and relative protein levels of 3β-HSD (normalized to ACTB) and CYP17A1 (normalized to 3β-HSD) during mouse postnatal development (6 d*pp*, 22 d*pp* and 36 d*pp*) and in *in vitro* cultured FT or CSF tissues (D16 and D30). (J) Representative images of CYP17A1 expression during mouse postnatal development (22 d*pp* and 36 d*pp*) and in *in vitro* cultured tissues (D16 and D30). A representative image of a negative control, carried out by omitting the primary antibody, is also shown. Testicular tissue sections were counterstained with Hoechst (blue). Dotted lines delineate seminiferous tubules. Scale: 15 µm.
Data are presented as means ± SEM with n = 4 biological replicates for each group. A value of $*P < 0.05$ was considered statistically significant.

samples examined (data not shown). The intratesticular concentrations of progesterone were significantly increased at D16 and D30 in both FT and CSF tissues compared to their respective *in vivo* controls (**Figure 3A**). In contrast, the intratesticular concentrations of androstenedione were significantly decreased at D16 in both FT and CSF tissues and at D30 in FT tissues compared to *in vivo* controls (**Figure 3B**). The intratesticular concentrations of testosterone were however not different in *in vitro* and *in vivo* matured tissues at both time points (**Figure 3C**). In addition, the intratesticular concentrations of estradiol were significantly higher at D16 and D30 in FT and CSF tissues than in their respective *in vivo* controls (**Figure 3D**).

In the organotypic culture medium, a significant increase in the concentrations of progesterone was observed at D26 only (**Figure 3E**). Furthermore, a significant elevation in the concentrations of androstenedione was observed from D16 to D30 (**Figure 3E**). Regarding testosterone, following a decrease in the levels of this androgen in the culture media between D2 and D14, an augmentation was then detected between D26 and D30 (**Figure 3E**). At D30, the concentrations of both androstenedione and testosterone were higher in the culture media of FT tissues than of CSF tissues (**Figure 3** -figure supplement 1). Regarding estradiol, no significant change in its concentrations was observed in the media between D6 and D30 (**Figure 3F**).

Since steroids are derived from cholesterol, intratesticular levels of total, free and esterified cholesterol were also analyzed (**Figure 3G**). The intratesticular levels of esterified cholesterol were comparable between the different experimental conditions (**Figure 3G**). However, total cholesterol levels were higher in FT and CSF cultured tissues at D16 and D30 compared to their corresponding *in vivo* time points (**Figure 3G**). Moreover, free cholesterol levels were increased in FT and CSF cultured tissues at D30 compared to 36 d*pp* and in FT tissues at D16 (**Figure 3G**).

Overall, our data show that the steroid content is altered in cultured testicular tissues, with an excess of free cholesterol, progesterone and estradiol and a deficiency of androstenedione.

Androgen and estrogen signaling are altered in 30-day organotypic cultures

The impact of imbalanced steroid hormone levels on downstream target gene expression was then investigated. The mRNA levels of actors of androgen and estrogen signaling were measured by RT-qPCR (**Figures 4** and **5**). The protein expression of the androgen receptor (AR), aromatase (CYP19) and fatty acid amide hydrolase (FAAH whose expression is regulated by estrogen) was also assessed by western blot (**Figures 4** and **5**).

The transcript levels of *Ar*, encoding the androgen receptor, were higher at D16 in FT tissues than at 22 d*pp* but were not significantly different between D30 and 36 d*pp* (**Figure 4A**). Conversely, AR protein levels were comparable at D16 in FT tissues and 22 d*pp* but were more elevated at D30 in both FT and CSF tissues than at 36 d*pp* (**Figure 4B**). Our immunofluorescence data showed that AR was expressed in Sertoli cells, peritubular myoid cells and Leydig cells in 16-day and 30-day cultured tissues as well as in the corresponding *in vivo* controls (**Figure 4C**). The transcript levels of two AR-regulated genes (*Rhox5, Eppin*) and the AR/Erα-regulated gene *Septin12* were decreased in FT and CSF tissues cultured for 16 days compared to 22 d*pp* (**Figure 4D-F**). At D30, *Rhox5* and *Septin12* mRNA levels were still lower than *in vivo* while *Eppin* mRNA levels were higher than at 36 d*pp* (**Figure 4D-F**). In addition, a significant increase in *Abp* mRNA levels was found at D16 and D30 in CSF but not in FT tissues (**Figure 4G**).

Intriguingly, the expression of *Cyp19a1*, encoding aromatase, was drastically diminished after 30 days of culture in FT and CSF tissues compared to 36 d*pp* (**Figure 5A**). The protein levels of CYP19 were found reduced in both FT and CSF tissues at D16 and D30 (**Figure 5B**). CYP19 was expressed in Leydig cells at 6 d*pp* and was then found in elongated spermatids and spermatozoa at 36 d*pp*, whereas no expression could be detected in cultured testes (**Figure 5C**). CYP19

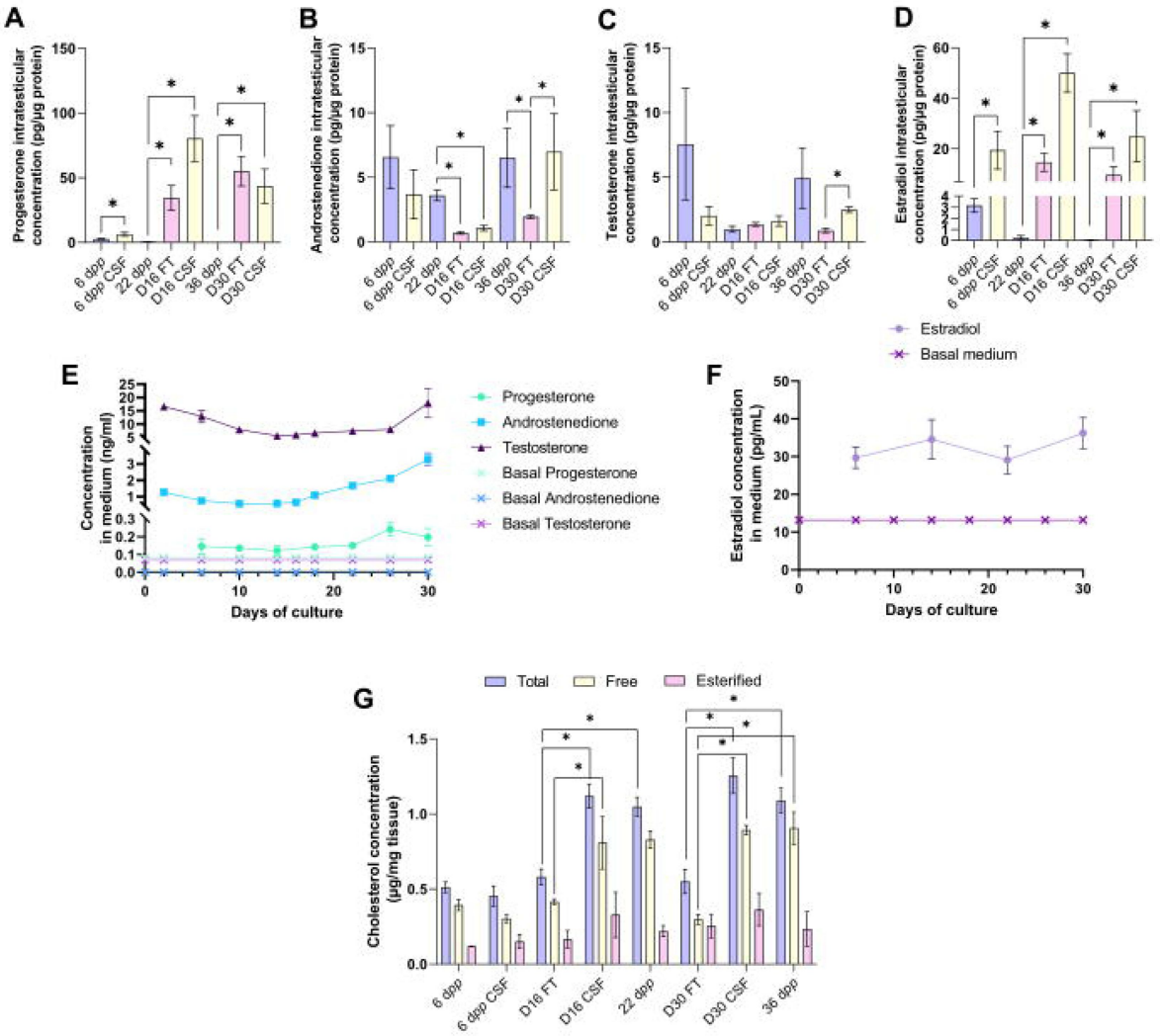

Figure. 3

An increased production of progesterone and estradiol and a decreased production of androstenedione are observed after 16 and 30 days of culture of prepubertal mouse testicular tissues.

Intratesticular concentrations of (A) progesterone, (B) androstenedione, (C) testosterone and (D) estradiol during mouse postnatal development (6 d*pp*, 22 d*pp* and 36 d*pp*) and in *in vitro* cultured fresh (FT) or frozen/thawed (CSF) tissues (D16 and D30). Steroid concentrations were normalized to protein levels. (E-F) Concentrations of (E) progesterone, androstenedione, testosterone and (F) estradiol in the culture medium of FT tissues. (G) Intratesticular concentrations of total, free and esterified cholesterol normalized to tissue mass.

Data are presented as means ± SEM with n = 4 biological replicates for each group. A value of $*P < 0.05$ was considered statistically significant.

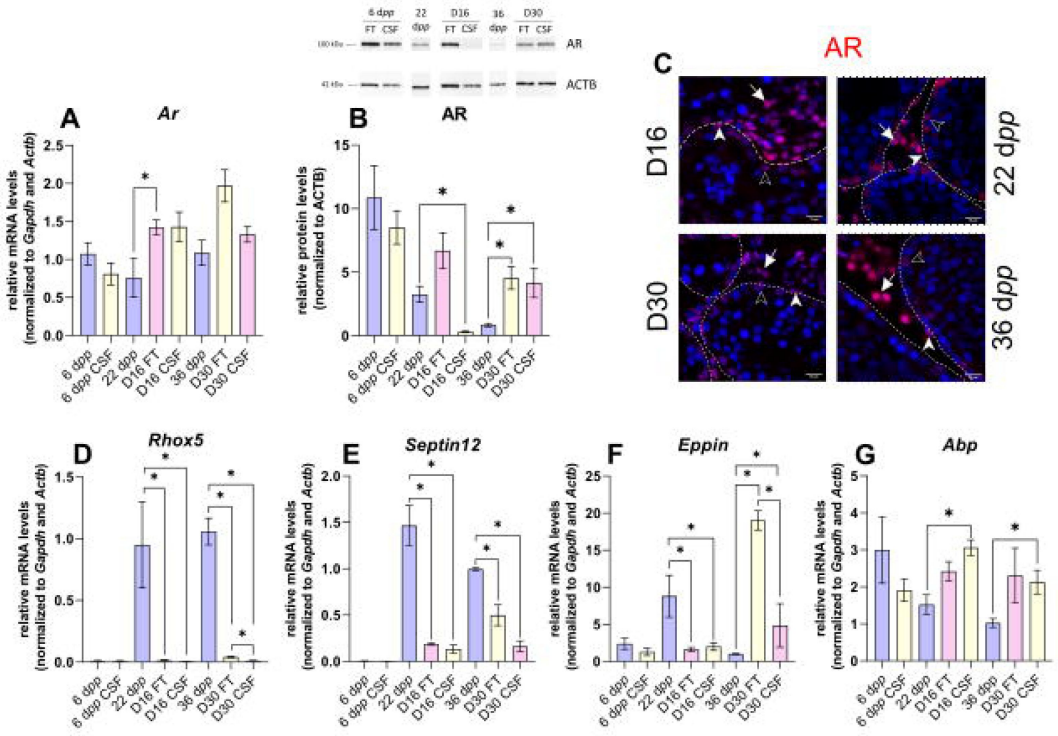

Figure. 4

Androgen signaling is altered in 30-day organotypic cultures.

(A) Relative mRNA levels of *Ar* (normalized to *Gapdh* and *Actb*) and (B) relative protein levels of AR (normalized to ACTB) during mouse postnatal development (6 d*pp*, 22 d*pp* and 36 d*pp*) and in *in vitro* cultured FT or CSF tissues (D16 and D30). (C) Representative images of AR expression at 22 d*pp* and 36 d*pp* and at corresponding *in vitro* time points (D16 and D30). A representative image of a negative control is shown in **Figure 2J** (same secondary antibody as for CYP17A1). Testicular tissue sections were counterstained with Hoechst (blue). Solid arrowheads: peritubular myoid cells. Open arrowheads: Sertoli cells. Arrows: Leydig cells. Dotted lines delineate seminiferous tubules (ST). Scale: 15 µm. (D-G) Relative mRNA levels of *Rhox5*, *Septin12*, *Eppin* and *Abp* (normalized to *Gapdh* and *Actb*).
Data are presented as means ± SEM with n = 4 biological replicates for each group. A value of $*P < 0.05$ was considered statistically significant.

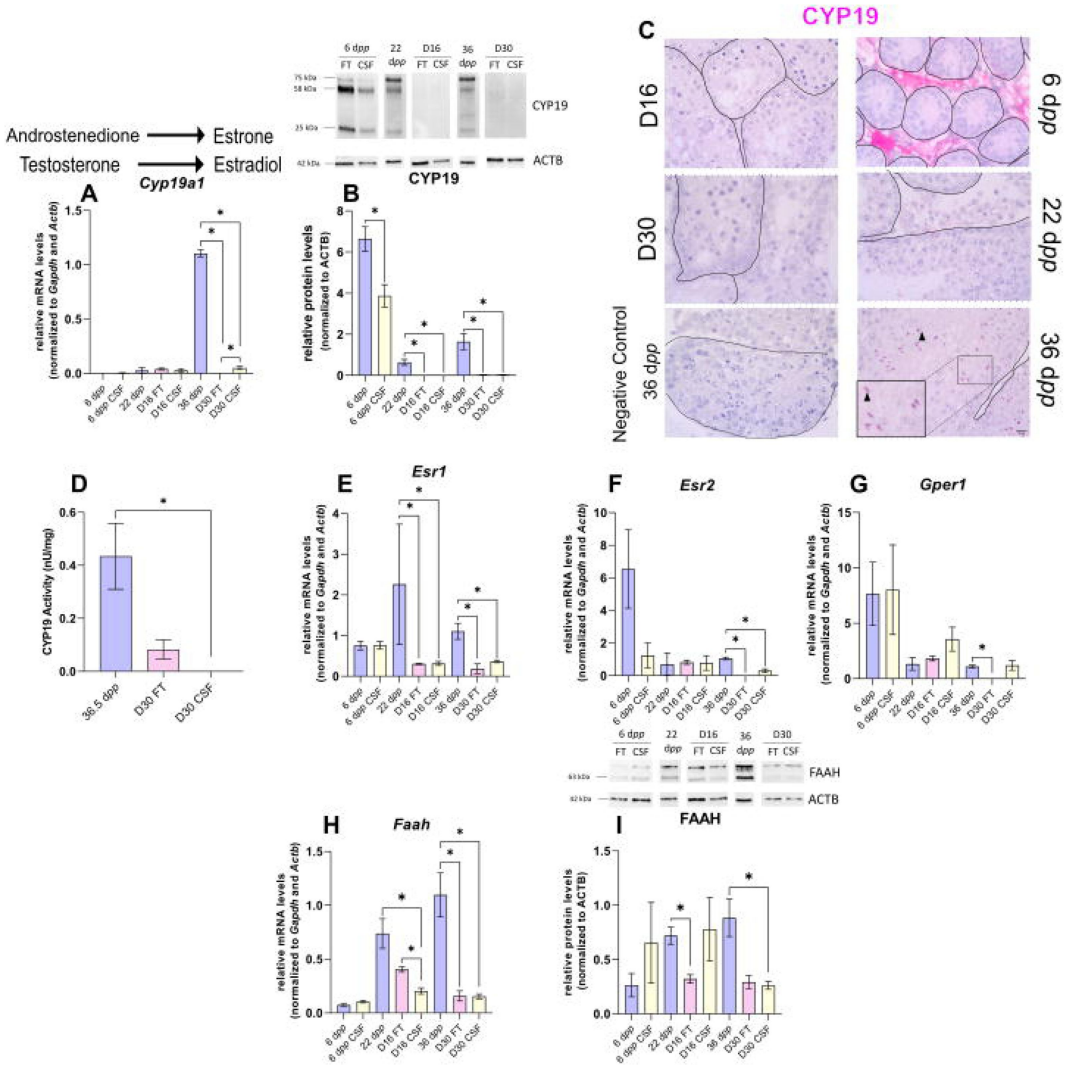

Figure. 5

The expression of aromatase and estrogen signaling are impaired after 30 days of organotypic culture.

(A) Relative mRNA levels of *Cyp19a1* (normalized to *Gapdh* and *Actb*) and (B) relative protein levels of CYP19A1 (normalized to ACTB) during mouse postnatal development (6 d*pp*, 22 d*pp* and 36 d*pp*) and in *in vitro* cultured FT or CSF tissues (D16 and D30). The bands on the western blot correspond to different isoforms of CYP19. (C) Representative images of CYP19A1 expression at 6 d*pp*, 22 d*pp* and 36 d*pp* and at corresponding *in vitro* time points (D16 and D30). A representative image of a negative control, carried out by omitting the primary antibody, is also shown. Testicular tissue sections were counterstained with Hematoxylin. Scale: 15 µm. Arrow: Leydig cells. Arrowheads: elongated spermatids. (D) Aromatase activity (normalized to tissue weight). (E-G) Relative mRNA levels of *Esr1*, *Esr2* and *Gper1* (normalized to *Gapdh* and *Actb*). (H) Relative mRNA levels of *Faah* (normalized to *Gapdh* and *Actb*) and (I) relative protein levels of FAAH (normalized to ACTB). The second band at 80 kDa is an isoform of FAAH (Q8BRM1, UniProtKB).

Data are presented as means ± SEM with n = 4 biological replicates for each group. A value of $*P < 0.05$ was considered statistically significant.

enzymatic activity was also significantly decreased in CSF cultures at D30 (**Figure 5D**). In addition, the transcript levels of the three genes encoding estrogen receptors (*Esr1*, *Esr2*, *Gper1*) and of the estrogen target gene *Faah* were significantly lower at D30 than at 36 d*pp* (**Figure 5E-H**). *Esr1* mRNA levels were also decreased at D16 (**Figure 5E**). FAAH protein levels were decreased in 16-day FT and 30-day CSF organotypic cultures (**Figure 5I**).

Thus, the expression of many genes in the androgen and estrogen signaling pathways are deregulated under organotypic culture conditions.

hCG is insufficient to stimulate Leydig cell differentiation and to restore steroidogenesis and steroid hormone signaling

As LH is known to stimulate the differentiation of the adult Leydig cell lineage and steroidogenesis, we wondered whether supplementation of organotypic culture media with hCG (LH homolog) could restore steroidogenesis to control levels. In order to determine the dose to be used, we examined the impact of different hCG concentrations (5 pM, 50 pM, 1 nM, 5 nM or 50 nM) on the intratesticular concentrations of testosterone and the secretion of this hormone in the organotypic culture medium. Since the concentration of hCG leading to the best response (*i.e.* plateau of the dose-response curves for intratesticular and secreted testosterone) was 1 nM, we used this dose for the rest of the analyses (**Figure 6** -figure supplement 1).

Upon supplementation with 1 nM hCG, intratesticular androstenedione levels were significantly increased at D30 in FT but not CSF tissues (**Figure 6A**). The intratesticular testosterone levels were higher at D30 in both FT and CSF tissues following hCG supplementation (**Figure 6B**). Moreover, a significant increase in androstenedione and testosterone concentrations was observed in the culture media of both FT and CSF tissues following hCG supplementation (**Figure 6C-D**). The amounts of androgens released into the culture media were however lower for CSF tissues than for FT tissues (**Figure 6C-D**).

The addition of 1 nM hCG had no impact on the transcript levels of *Star*, *Hsd17b3*, *Hsd3b1*, *Ar* and *Esr1* in both FT and CSF cultures (**Figure 6F, I, J, L, P**). *Hsd17b3* and *Esr1* mRNA levels still remained lower upon hCG supplementation than *in vivo* (**Figure 6I, P**). No effect of hCG was also observed in FT cultures for *Cyp17a1*, *Faah*, *Srd5a1*, *Sult1e1*, *Insl3*, *Igf1*, *Dhh*, *Abp* and *Hsd17b2* mRNA levels (**Figure 6I, J, S**, **Figure 6** -figure supplement 2). *Lhcgr*, *Cyp11a1*, *Cyp19a1*, *Esr2* and *Gper1* mRNA levels were increased after hCG supplementation in FT cultures (**Figure 6E, G-H, K, Q, R**). Whereas *Cyp11a1* and *Cyp19a1* mRNA levels were still lower after hCG supplementation than *in vivo* **(Figure 6G, K)**, *Esr2* and *Gper1* transcripts were restored to their physiological levels (**Figure 6Q-R**). In contrast, mRNA levels of *Rhox5*, *Eppin*, *Septin12* and *Igf1* were decreased after hCG supplementation in FT cultures, with *Rhox5* and *Septin12* transcript levels lower than those observed *in vivo* (**Figure 6M-O**).

Finally, the mean proportions of seminiferous tubules reaching the round spermatid and elongated spermatid stages were lower in tissues cultured with than without hCG (**Figure 6T**). The number of Sertoli cells inside seminiferous tubules was unchanged following hCG supplementation, but was higher than in *in vivo* controls (**Figure 6U**).

Although the addition of hCG resulted in increased androgen levels, the expression of adult Leydig cell markers, of several steroidogenic genes and of steroid hormone-regulated genes remained low, thus not promoting the progression of *in vitro* spermatogenesis.

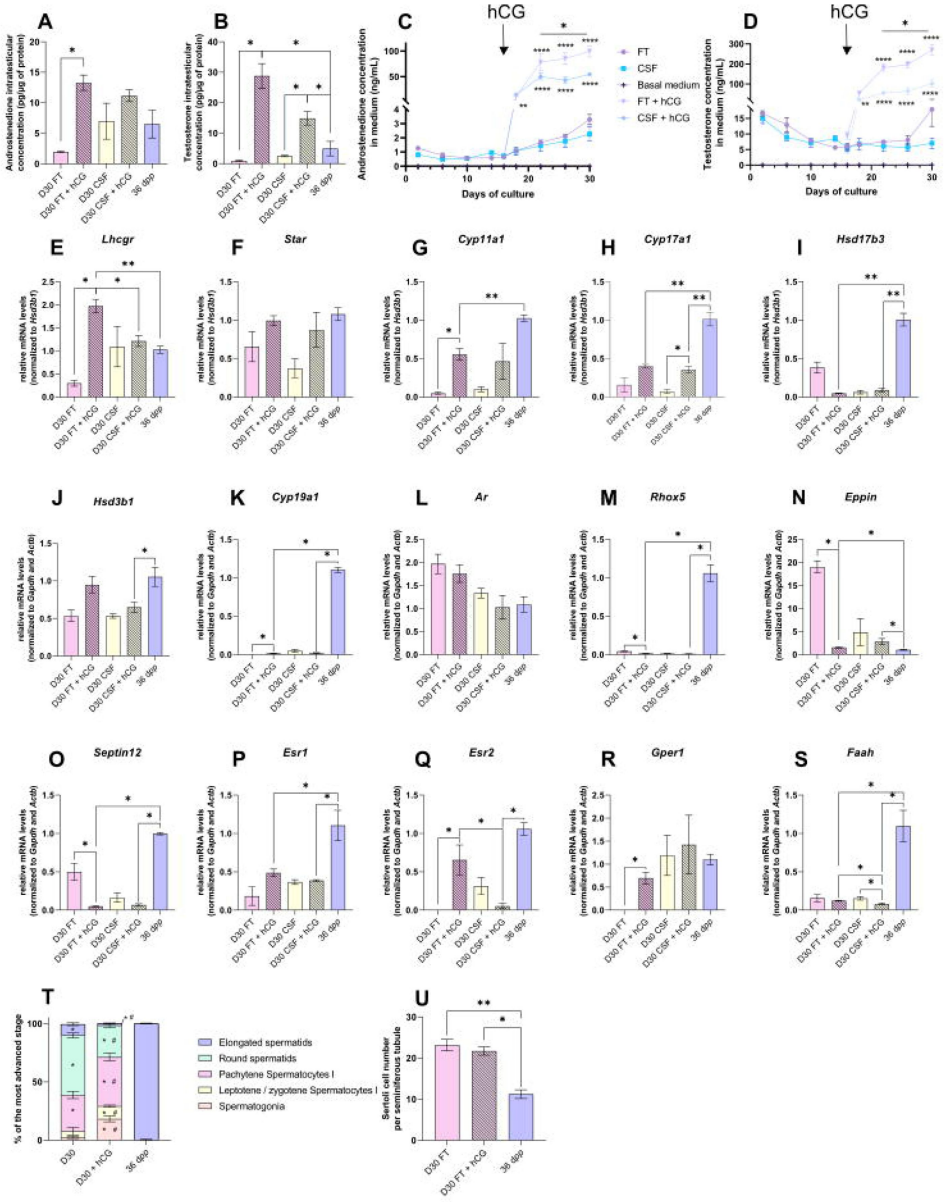

Figure. 6

hCG is insufficient to stimulate Leydig cell differentiation and restore steroidogenesis and steroid hormone signaling.

(A-D) Levels of androgens (androstenedione and testosterone) (A-B) in testicular tissues and (C-D) in culture medium with 1 nM hCG or without supplementation. (E-S) Relative mRNA levels of genes involved in steroidogenesis and androgen/estrogen signaling pathways (normalized to *Gapdh* and *Actb* or to *Hsd3b1*) with or without hCG supplementation. (T) Proportion of seminiferous tubules containing the most advanced type of germ cells after *in vitro* culture with or without hCG. $P < 0.05$: * *vs* 36 d*pp* and # *vs* D30 FT. (U) Sertoli cell number in seminiferous tubules after *in vitro* culture with or without hCG.
Data are presented as means ± SEM with n = 4 biological replicates for each group. A value of $*P < 0.05$ was considered statistically significant.
FT: Fresh Tissue; CSF: Controlled Slow Freezing.

Discussion

The present study shows for the first time and in a comprehensive manner that Leydig cell maturation and functionality as well as steroid hormone signaling are impaired in cultures of fresh and frozen/thawed immature mouse testes. Only few differences were found between fresh and frozen/thawed *in vitro* matured tissues.

A similar density of Leydig cells was found in organotypic cultures and in corresponding *in vivo* controls. However, Leydig cells only partially matured *in vitro*. At D16, the expression of the Leydig cell differentiation factors *Igf1* and *Dhh* was unaffected. At D30, the proliferation/apoptosis balance was undisturbed in Leydig cells while the expression of *Insl3* was faint, as well as that of *Sult1e1*, another Leydig cell maturity marker. The deficient Leydig cell maturation observed at D30 could be related to the decreased levels of *Igf1*, which encodes insulin-like growth factor 1 known to promote the maturation of immature Leydig cells into adult Leydig cells (23).

Leydig cell functionality was also altered in organotypic cultures. Indeed, the expression of several genes encoding steroidogenic enzymes (*Cyp11a1*, *Cyp17a1*, *Hsd17b3*, *Hsd17b2*) was downregulated in these cells (**Figure 7**). Reduced *Cyp17a1* mRNA levels in organotypic cultures were also recently highlighted using a bulk RNA-seq approach (10). Decreased *Cyp11a1* and *Cyp17a1* transcript levels were previously reported in *Igf1*$^{-/-}$ mice, together with decreased intratesticular testosterone levels (22). Furthermore, deficiency in insulin-like growth factors signaling in *Insr*$^{-/-}$/*Igf1r*$^{-/-}$ mouse Leydig cells was shown to impair testicular steroidogenesis (decreased *Lhcgr*, *Star*, *Cyp11a1*, *Cyp17a1*, *Hsd17b3*, *Srd5a1* and *Insl3* mRNA levels) and increase estradiol production (52). Here, we report for the first time an accumulation of estradiol in *in vitro* cultured tissues, which could be deleterious for sperm production. To promote Leydig cell maturation and lower intratesticular estradiol levels *in vitro*, supplementation of culture medium with IGF1 could thus be later envisaged, and even more so since this factor has been shown to increase, although modestly, the percentages of round and elongated spermatids in cultured mouse testicular fragments (53). To reduce estrogen levels and increase sperm production, supplementation of the organotypic culture medium with selective estrogen receptor modulators such as tamoxifen or with aromatase inhibitors such as letrozole could also be considered in future studies. These molecules have indeed proven to be effective in increasing sperm concentration in infertile men (54–56).

Our work also revealed an elevation in progesterone and a reduction in androstenedione in *in vitro* matured tissues (**Figure 7**), which could arise from the reduced expression of *Cyp17a1*. A greater sperm production has been highlighted in PRKO mice (lacking the progesterone receptor), thereby showing the inhibitory action of progesterone on spermatogenesis (57). The cumulative excess of progesterone and estradiol in cultured testicular tissues may therefore slow the progression of *in vitro* spermatogenesis and lead to a poor sperm yield.

Despite the disturbed steroidogenic activity of Leydig cells *in vitro*, the accumulation of estradiol and progesterone, the decrease in androstenedione, and in contradiction with a previous study (9), intratesticular testosterone levels in cultured tissues were not significantly different from *in vivo*. Moreover, a complete *in vitro* spermatogenesis was achieved, albeit with a low efficiency as previously described (5,6). We also found that the percentage of Leydig cells expressing AR was unchanged under *in vitro* conditions. The increased AR protein levels at D30 could thus be the result of the higher number of Sertoli cells within seminiferous tubules. Intriguingly, common points can be observed in our organotypic cultures and in LCARKO (Leydig cell-specific *Ar*$^{-/-}$) mice (58): decreased *Insl3*, *Cyp17a1* and *Hsd17b3* mRNA levels, increased intratesticular progesterone levels and unchanged intratesticular testosterone levels compared to controls. This could suggest that AR signaling is dysfunctional in Leydig cells *in vitro*. The downregulation of *Rhox5* expression (**Figure 7**), which confirms our previous findings (8), further suggests that AR signaling may also be impaired in Sertoli cells. Recently, in a mouse model with disrupted dimerization of the AR

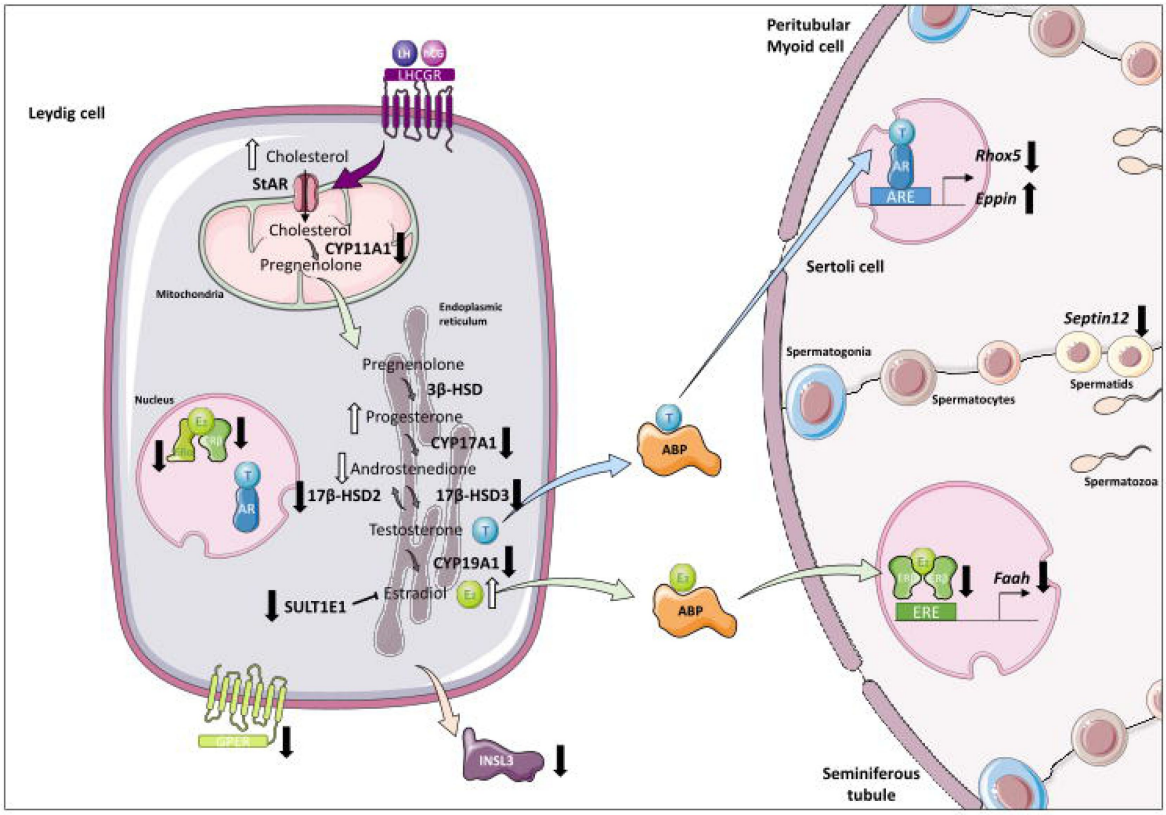

Figure. 7

Altered steroidogenesis and androgen/estrogen signaling pathways in *in vitro* matured testicular tissues of prepubertal mice

A similar density of Leydig cells is found after 30 days of organotypic culture (D30) and at 36 days *postpartum*, the corresponding *in vivo* time point. However, Leydig cells are partially mature *in vitro* with a decrease in *Sult1e1* and *Insl3* mRNA levels (adult Leydig cell markers). The mRNA levels of *Cyp11a1*, *Cyp17a1* and *Hsd17b3* encoding steroidogenic enzymes and the protein levels of CYP17A1 are decreased *in vitro*. Increased amounts of cholesterol, progesterone and estradiol and decreased androstenedione intratesticular levels are observed at D30. Furthermore, despite testosterone levels similar to *in vivo*, the expression of the androgen receptor (AR) and of the androgen binding protein (*Abp*), androgen signaling is altered at D30, with decreased transcript levels of the androgen target gene *Rhox5* and of *Septin12.* Moreover, with decreased expression and activity of aromatase and decreased estrogen receptor expression, estrogen signaling is impaired at D30, leading to decreased transcript and protein levels of the estrogen target gene *Faah*.

ligand-binding-domain, a decreased expression of *Hsd17b3* and of AR-regulated genes, such as *Rhox5* and *Insl3*, a higher percentage of Sertoli cells and a decreased production of round and elongated spermatids were found (59), which further supports our hypothesis that AR signaling may be defective in organotypic cultures. In the SPARKI (SPecificity-affecting AR KnockIn)-AR mouse model, which has a defect in binding to AR-specific DNA motifs while displaying normal interaction with the classical AREs, it has been shown that *Rhox5* expression is regulated by an AR-selective ARE, while *Eppin* expression is regulated by a classical, nonselective ARE (60). The differential regulation of these two androgen-responsive genes could explain why *Rhox5* mRNA levels were downregulated while *Eppin* mRNA levels were upregulated in our cultures. We also report here that the transcript levels of *Septin12*, a potential target of the androgen and estrogen receptors, was reduced *in vitro* (**Figure 7**).

Additionally, estrogen signaling was impaired in organotypic cultures, with a low expression of the estrogen-synthesizing enzyme aromatase, of estrogen receptors and of the estrogen target gene *Faah* (**Figure 7**). Since the expression of *Cyp19a1*, *Esr1* and *Esr2* (encoding aromatase, ERα and ERβ, respectively) can be downregulated by estradiol in the rat testis (61,62), we hypothesize that the transcription of these genes may be negatively controlled by elevated estradiol levels in our tissue cultures. *Faah*, whose promoter activity engages ERβ and the histone demethylase LSD1, is a direct target gene of estrogens in Sertoli cells (42). The *Faah* proximal promoter is not regulated by estrogen in immature Sertoli cells, as they express ERβ at the same level as mature cells but do not express LSD1 (42). Thus, the decreased *Faah* transcript levels in organotypic cultures could be the consequence of the downregulated ERβ expression and/or of the immaturity of Sertoli cells. Whether Sertoli cells in organotypic cultures are as mature as *in vivo* is unknown and will be the focus of future studies. The transcription of *Insl3*, which is repressed by estradiol in a MA-10 Leydig cell line model (63), could also be inhibited by the excess of estradiol in our tissue cultures. Interestingly, our *in vitro* cultured testes exhibit common features with the testes of *Sult1e1*$^{-/-}$ mice lacking estrogen sulfotransferase, an enzyme that catalyzes the sulfoconjugation and inactivation of estrogens (64): decreased *Cyp17a1* expression as well as increased progesterone levels and local estrogen activity (65,66). In organotypic cultures, estrogen excess could be explained by low *Sult1e1* expression. In addition, reduced transcript levels of *Hsd17b2*, encoding an enzyme that converts estradiol to estrone and testosterone to androstenedione, may also explain why estradiol levels remain elevated in cultures while testosterone levels are similar to controls and androstenedione levels are low.

Finally, as previously reported (5,6), the presence of LH or its homolog hCG was not necessary to reconstitute a full first spermatogenic wave *in vitro*. However, plasma LH concentration is known to rise *in vivo* from 21-28 d*pp* in the mouse model (67). We previously showed that the addition of 0.075 nM (50 IU/L) hCG together with FSH from D7 onwards modestly increased the number of spermatozoa produced in organotypic cultures (5). To mimic as closely as possible the physiological conditions, 1 nM hCG was added in the culture medium from D16 onwards. Even though Leydig cells responded to this stimulation by synthesizing and secreting more androgens, supplementation of the culture medium with 1 nM hCG alone was not sufficient to facilitate Leydig cell differentiation, restore steroidogenesis and steroid hormone signaling and increase the yield of *in vitro* spermatogenesis.

In conclusion, the present study shows the partial maturation and the disturbed steroidogenic activity of Leydig cells, the abnormal steroid hormone content as well as the altered androgen and estrogen signaling in organotypic cultures of fresh and frozen/thawed prepubertal mouse testicular tissues. Altogether, these defects could contribute to the low efficiency of *in vitro* spermatogenesis. It will therefore be necessary to optimize the culture medium by adding factors that promote proper Leydig cell differentiation as well as the culture design to prevent central necrosis from affecting the survival and/or the growth and/or the differentiation of the testis in culture. The development of an optimal model of *in vitro* spermatogenesis could be useful in

advancing the field of fertility restoration, but could also have wider applications, as it could be useful for studying the initiation of puberty as well as the impact of cancer therapies, drugs, chemicals and environmental agents (e.g. endocrine disruptors) on the developing testis.

Materials and methods

Key resources table

Key Resources Table				
Reagent type (species) or resource	**Designation**	**Source or reference**	**Identifiers**	**Additional information**
biological sample (*Mus musculus*, male)	Testis	CD-1 Mice from Charles River		Freshly isolated from male *Mus musculus* (CD-1)

antibody	Anti-3β-HSD (mouse monoclonal)	Santa Cruz Biot.	sc-515120 (AF488)	IF (1:100), WB (1:1000)
antibody	Anti-β-Actin (mouse monoclonal)	Abcam	ab8226	WB (1:5000)
antibody	Anti-AR (rabbit monoclonal)	Abcam	ab133273	IF (1:100), WB (1:5000)
antibody	Anti-CC3 (rabbit polyclonal)	Abcam	ab49822	IF (1:200)
antibody	Anti-CYP17A1 (rabbit polyclonal)	Abcam	ab231794	IF (1:200), WB (1:10000)
antibody	Anti-CYP19A1 (mouse monoclonal)	BioRad	MCA2077 S	IHC (1:50), WB (1:250)
antibody	Anti-FAAH (rabbit polyclonal)	Proteintech	17909-1-AP	WB (1:1000)
antibody	Anti-Ki67 (rabbit monoclonal)	Abcam	ab16667	IF (1:100)
antibody	Anti-mouse Alexa 488 (goat)	Abcam	ab150113	IF (1:200)
antibody	Anti-rabbit Alexa 488 (goat)	Abcam	ab150077	IF (1:200)
antibody	Anti-rabbit Alexa 594 (goat)	Abcam	ab150080	IF (1:200)
antibody	Anti-mouse HRP (goat)	Invitrogen	31430	WB (1:5000)
antibody	Anti-rabbit HRP (goat)	Invitrogen	A16110	WB (1:5000)
commercial assay or kit	RNeasy Micro kit	Qiagen	74004	

commercial assay or kit	qScript cDNA SuperMix	QuantaBio	95048	
commercial assay or kit	Lipid Extraction kit	Abcam	ab211044	
commercial assay or kit	Cholesterol/Cholesteryl Ester assay kit	Abcam	ab65359	
commercial assay or kit	Progesterone ELISA kit	Cayman Chemical Company	582601	
commercial assay or kit	Estradiol ELISA kit	Cayman Chemical Company	501890	
commercial assay or kit	Aromatase (CYP19A) Activity assay kit	Abcam	ab273306	
commercial assay or kit	INSL3 RIA kit	Phoenix Pharmaceuticals	RK-035-27	
chemical compound, drug	KSR	Gibco by Life Technologies	10828010	(10%)
chemical compound, drug	Retinol	Sigma-Aldrich	R7632	1 µM
chemical compound, drug	hCG	MSD France	Ovitrelle	1 nM
chemical compound, drug	SYBR Green	Thermo Fisher Scientific	4385616	

Ethical approval

All the experimental procedures were approved by the Institutional Animal Care and Use Committee of Rouen Normandy University under the licence/protocol number APAFiS #38239.

Mice and testis collection

CD-1 mice (Charles River Laboratories, L'Arbresle, France) were housed in a temperature-controlled room (22–23°C) under a 12-h light/dark cycle. Prepubertal 6-day *postpartum* (d*pp*) male mice were euthanized by decapitation and underwent a bilateral orchiectomy. Testes were transferred to Petri dishes containing α-MEM without phenol red (Gibco by Life Technologies, Saint-Aubin, France) and the complete removal of the tunica albuginea was performed with two needles under a binocular magnifier (S8AP0, Leica Microsystems GmbH, Wetzlar, Germany). Testes were then either cultured immediately (culture from fresh tissues), or after a

freezing/thawing cycle (**Figure 8**). Moreover, mice aged 22 and 36 d*pp* were euthanized by CO_2 asphyxiation and their testes were used as *in vivo* controls for 16 and 30 days of culture, respectively (**Figure 8**).

Controlled slow freezing (CSF) and thawing of testicular tissues

Freezing procedure

Testes were placed into cryovials (Dominique Dutscher, Brumath, France) containing 1.5 mL of the following cryoprotective medium: Leibovitz L15 medium (Eurobio, Courtabœuf, France) supplemented with 1.5 M dimethylsulfoxyde (DMSO, Sigma-Aldrich, Saint-Quentin Fallavier, France), 0.05 M sucrose (Sigma-Aldrich), 10% (v/v) fetal calf serum (FCS, Life Technologies) and 3.4 mM vitamin E (Sigma-Aldrich) (45). After a 30-min equilibration at 4°C, samples were frozen in a programmable freezer (Nano Digitcool, CryoBioSystem, L'Aigle, France) with a CSF protocol: start at 5°C, then −2°C/min until reaching −9°C, stabilization at −9°C for 7 min, then −0.3°C/min until −40°C and −10°C/min down to −140°C. Testicular tissues were then plunged and stored in liquid nitrogen.

Thawing procedure

Cryotubes were warmed for 1 min at room temperature (RT) and then for 3 min in a water bath at 30°C. They were then successively incubated at 4°C in solutions containing decreasing concentrations of cryoprotectants for 5 min each [solution 1: 1 M DMSO, 0.05 M sucrose, 10% FCS, 3.4 mM vitamin E, Leibovitz L15; solution 2: 0.5 M DMSO, 0.05 M sucrose, 10% FCS, 3.4 mM vitamin E, Leibovitz L15; solution 3: 0.05 M sucrose, 10% FCS, 3.4 mM vitamin E, Leibovitz L15; solution 4: 10% FCS, 3.4 mM vitamin E, Leibovitz L15].

Organotypic cultures at a gas–liquid interphase

In vitro tissue cultures were performed as previously described (3,5). Briefly, prepubertal 6-day old mouse testes, which contain spermatogonia as the most advanced type of germ cells, were first cut into four fragments (approximately 0.75 mm^3 each, which was previously determined to be the most appropriate size for mouse *in vitro* spermatogenesis) (7). They were placed on top of two 1.5% (w/v) agarose Type I gels (Sigma-Aldrich) half-soaked in medium. Testicular tissues were then cultured under 5% CO_2 at 34°C for 16 days (D16), which corresponds to the end of meiosis and the appearance of the first round spermatids, or 30 days (D30) to explore the end of the first spermatogenic wave. No growth of the fragments was observed during the organotypic culture period (**Figure 8**-figure supplement 1A). The basal medium (BM) contained α-MEM without phenol red, 10% KSR (KnockOut Serum Replacement, Gibco by Life Technologies), 0.1 mg/mL streptomycin and 100 UI/mL penicillin (Sigma-Aldrich). The medium was chosen without phenol red because of its estrogen activity (46). Retinol (10^{-6} M, Sigma-Aldrich) was added in all organotypic cultures from D2 and then every 8 days in order to respect the meiosis entry cycle of spermatogonia (5). Furthermore, the basal medium was supplemented or not with 1 nM hCG (human Chorionic Gonadotropin, MSD France, Courbevoie, France) from D16 in order to stimulate Leydig cell differentiation and assess Leydig cell functionality. Media were prepared just before use and were replaced twice a week.

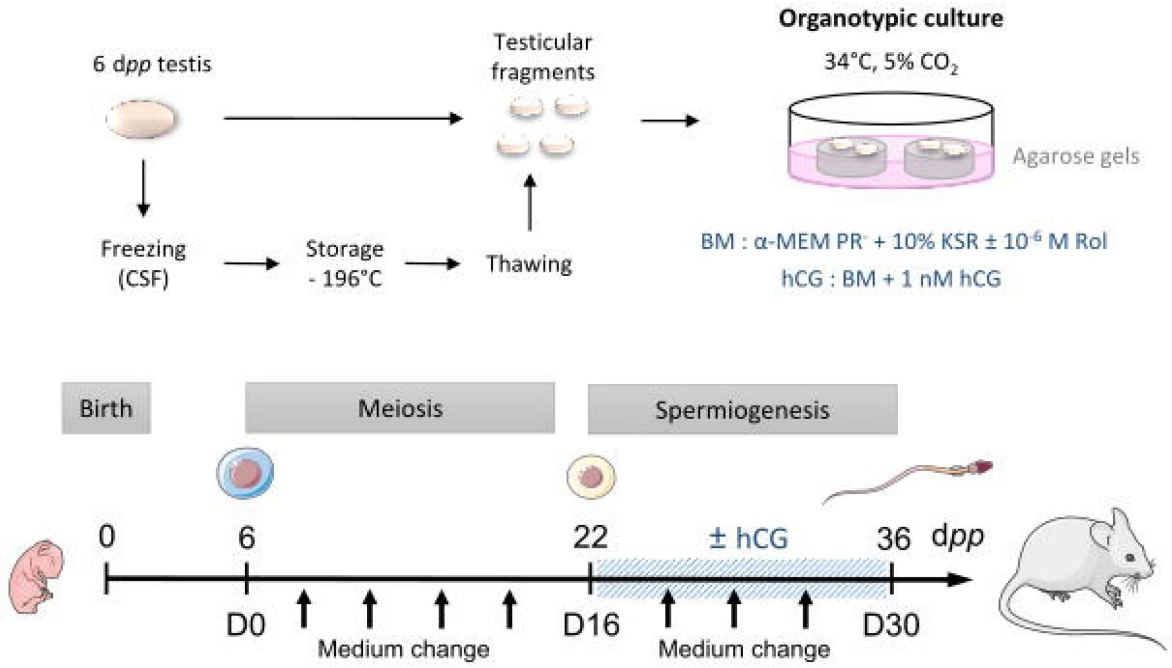

Figure 8.

Scheme of the study design.

At 6 d*pp* (D0), the testis contains only spermatogonia and the initiation of meiosis occurs between 7 and 9 d*pp*. At 22 d*pp* (D16), meiosis ends and the first round spermatids appear. At 36 d*pp* (D30), the first spermatozoa appear and this is the end of the first spermatogenic wave. Arrows represent times at which the medium was collected (D0, D2, D6, D10, D14, D18, D22, D26 and D30).

BM: Basal Medium; CSF: Controlled Slow Freezing; d*pp*: days *postpartum*; hCG: human Chorionic Gonadotropin; KSR: KnockOut Serum replacement; PR-: without phenol red; Rol: retinol

Histological analyses

Tissue fixation, processing and sectioning

Testicular tissues were fixed with Bouin's solution (Sigma-Aldrich) or 4% paraformaldehyde (PFA, Sigma-Aldrich) for 2 h at room temperature. They were then dehydrated in ethanol in the Citadel 2000 tissue processor (Shandon, Cheshire, UK) and embedded in paraffin. Tissue sections (3 µm thick) were prepared with the RM2125 RTS microtome (Leica) and were mounted on Polysine slides (Thermo Fisher Scientific, Waltham, MA, USA).

Periodic Acid Schiff (PAS) reaction

A PAS reaction was then performed on *in vitro* matured tissues. Tissue sections were deparaffinized in xylene and rehydrated in decreasing concentrations of ethanol. Slides were then immersed in 0.5% periodic acid (Thermo Fisher Scientific) for 10 min, rinsed for 5 min in water and then placed for 30 min in the Schiff reagent (Merck, Darmstadt, Germany). After three 2-min washes in water, sections were counterstained with Mayer's hematoxylin and mounted with Eukitt (CML, Nemours, France). Images were acquired on a DM4000B microscope (Leica) at a ×400 magnification. The most advanced type of germ cells present in the testicular fragments was analyzed in at least 30 cross-sectioned seminiferous tubules (located at the periphery of the cultured fragments, i.e. outside of the necrotic region) (**Figure 8**-figure supplement 1E-F) from 2 sections with the Application Suite Core v2.4 software (Leica).

Immunofluorescence staining

After deparaffinization, rehydration and a 3-min wash in phosphate buffered saline (PBS, Sigma-Aldrich), tissue sections were boiled in 10 mM citrate buffer pH 6.0 (Diapath, Martinengo, Italy) for 40 min at 96°C. They were cooled for 20 min at RT and rinsed in distilled water for 5 min. A permeabilization step with 0.1% (v/v) Triton X-100 (Sigma-Aldrich) was performed at RT for 15 min for Ki67/3β-HSD immunostaining. Non-specific sites were blocked with 5% (w/v) bovine serum albumin (BSA, Sigma-Aldrich) and 5% (v/v) horse serum (Sigma-Aldrich). Slides were then incubated in humidified environment with primary antibodies (**Supplementary File 1a**), rinsed 3 times in PBST (PBS with 0.05% Tween-20) and incubated with appropriate secondary antibodies (**Supplementary File 1a**). For Ki67/3β-HSD, a sequential protocol was performed as follows: incubation with anti-Ki67 antibodies overnight at 4°C, incubation with secondary antibodies coupled to Alexa 594 for 60 min at RT, fixation with 4% PFA for 15 min at RT and incubation with anti-3β-HSD antibodies directly coupled to Alexa 488 for 90 min at RT. Negative controls were carried out by omitting the primary antibodies. Sections were washed, dehydrated with ethanol and mounted in Vectashield with Hoechst. Images were acquired on a THUNDER Imager 3D Tissue microscope (Leica) at a ×400 magnification. Leydig cell number ($3\beta\text{-HSD}^+$) was normalized to tissue area (cm^2).

Immunohistochemical staining

After deparaffinization and rehydration, endogenous peroxidases were blocked with HP Block (Dako, Les Ulis, France) for 30 min and non-specific binding sites were blocked with Ultra-V Block solution (Thermo Fisher Scientific) for 10 min at RT. Tissue sections were then incubated overnight at 4°C with anti-CYP19A1 antibodies (**Supplementary File 1a**). After three 5-min washes in PBS, they were incubated for 10 min at RT with biotinylated polyvalent secondary antibodies (UltraVision Detection System HRP kit, Thermo Fisher Scientific). After three 5-min washes in PBS, a 10-min incubation at RT with streptavidin associated with peroxidase (UltraVision Detection System HRP kit, Thermo Fisher Scientific) was performed. The labeling was revealed after application of a chromogenic substrate (EnVision FLEX HRP Magenta Chromogen, Dako) for 10 min at RT. A negative control was carried out by omitting the primary antibody.

RNA extraction and RT-qPCR

RNA extraction

Total RNA was extracted from testicular samples using RNeasy Micro kit (Qiagen, Courtabœuf, France) according to the manufacturer's instructions. For *in vitro* cultured tissues, the central necrotic area (16-27% of the explants, **Figure 8**-figure supplement 1B-F) was carefully removed before RNA extraction, so that transcript levels in the healthy part of the samples (i.e. where *in vitro* spermatogenesis occurs) could be measured and compared with *in vivo* controls. To avoid contamination with genomic DNA, extracted RNA was incubated with two units of TURBO DNase (Life Technologies) for 45 min at 37°C. The amount of the RNA samples was measured with a NanoDrop Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and purity was determined by calculating the ratio of optical densities at 260 nm and 280 nm.

Reverse transcription

The reverse transcription reaction was performed in a 96-well plate from 1 µg total RNA with 4 µL of qScript cDNA SuperMix 5X (QuantaBio, Gaithersburg, MD, USA) and ribonuclease-free water to adjust the volume to 20 µL. Reverse transcription was performed according to the following program: 5 min at 25°C, 30 min at 42°C and 5 min at 85°C. The complementary DNA (cDNA) obtained were diluted 1:10.

Polymerase chain reaction

cDNA amplifications were carried out in a total volume of 13 µL containing 6 ng of cDNA templates, 6.5 µL of SYBR Green (Thermo Fisher Scientific) and 300 nM of each primer. Specific primers are listed in **Supplementary File 1b**. Samples were dispensed using the Bravo pipetting robot (Agilent Technologies, Santa Clara, CA, USA). Reactions were performed in 384-well plates (Life Technologies) in a Quant Studio 12K Flex system. The amplification condition was 20 s at 95°C followed by 40 cycles (1 s at 95°C, 20 s at 60°C) and a final step of denaturation of 15 s at 95°C, 1 min at 60°C and 15 s at 95°C. Melting curves were obtained to ensure the specificity of PCR amplifications. The size of the amplicons was verified by agarose gel electrophoresis (E-gel 4%, Life Technologies). The relative expression level of each gene was normalized to two housekeeping genes as recommended by the MIQE guidelines (47): *Gapdh* and *Actb*, which were identified and validated as the most stable and suitable genes for RT-qPCR analysis in mouse testis development (48). 3β-hydroxysteroid dehydrogenase (*Hsd3b1*), a selective Leydig cell marker, has been used as a normalization factor for the analysis of genes expressed in Leydig cells (49). Data were analyzed using the $2^{-\Delta\Delta Ct}$ method (50).

Western blot

Protein extraction

Testes were homogenized in ice-cold RIPA buffer (50 mM Tris HCl, 0.5% cholic acid, 0.1% SDS, 150 mM NaCl) containing a protease inhibitor cocktail (Sigma-Aldrich). For *in vitro* cultured tissues, the central necrotic area was carefully removed before protein extraction.

Determination of protein concentration by the Bradford method

Total protein concentration was measured in the homogenates. The assay was performed in a 96-well plate in a final volume of 200 µL containing the sample and Bradford's solution (BioRad, Marnes-la-Coquette, France). After a 5-min incubation at RT, the optical density at 595 nm was measured using a Spark spectrophotometer (Tecan, Lyon, France).

Western blot

Protein extracts (20 µg) were separated on 12% resolving polyacrylamide gels (BioRad) and transferred onto nitrocellulose membranes (BioRad). The membranes were blocked with 5% milk or 5% BSA in TBST (0.2% Tween-20 in Tris Buffered Saline) for 30 min at 37°C before incubation overnight at 4°C with the appropriate primary antibody (**S1 Table**). After washes, membranes were incubated with the appropriate HRP-conjugated secondary antibodies (**S1 Table**). ECL reagents (BioRad) were used to detect the immunoreactivity. Images were captured with the ChemiDoc XRS+ Imaging System (BioRad). Densitometric analyses were performed using Image Lab™ v5.0 software (BioRad) and protein levels were normalized to β-actin or to the Leydig cell-specific marker 3β-HSD.

Intratesticular cholesterol levels

Total testicular lipids were extracted from ~10 mg tissue with a Lipid Extraction kit (ab211044; Abcam, Paris, France) according to the manufacturer's instructions. Dried lipid extracts were reconstituted in 200 µL of assay buffer. Intratesticular levels of total, free, and esterified cholesterol were measured with a colorimetric Cholesterol/Cholesteryl Ester assay kit (ab65359; Abcam) according to the manufacturer's instructions.

Hormonal assays

Sample preparation for liquid chromatography coupled to mass spectrometry (LC-MS/MS)

Testicular fragments were homogenized in 200 µL (6 d*pp* tissues and *in vitro* matured tissues) or 400 µL (22 d*pp* and 36 d*pp* tissues) of 0.1 M phosphate buffer pH 7.4 with a cocktail of protease inhibitors (Sigma-Aldrich). Total protein concentration was measured as described above.

Lc ms ms

The standards for androstenedione, testosterone, dehydroepiandrosterone (DHEA) and their stable labeled isotopes were obtained from Merck. Working solutions were prepared in methanol. Serial dilutions from working solutions were used to prepare seven-point calibration curves and 3 quality control levels for all analytes. For the final calibration and quality control solutions, PBS was used for testicular fragments and α-MEM for medium. The linearity ranges were from 0.05 to 10 ng/mL for androstenedione and testosterone, and from 1 to 200 ng/mL for DHEA. A simple deproteinization was carried out by automated sample preparation system, the CLAM-2030 (Shimadzu Corporation, Marne-la-Vallée, France) coupled to a 2D-UHPLC-MS/MS system. Once the sample on board, 30 µL was automatically pipetted in a pre-conditioned tube containing a filter, in which reagents were added, then mixed and filtered. Briefly, the polytetrafluoroethylene (PTFE) filter vial (0.45 µm pore size) was previously conditioned with 20 µL of methanol (Carlo Erba, Val-de-Reuil, France). Successively, 30 µL of sample and 60 µL of a mixture of isotopically labeled internal standards in acetonitrile were added. The mixture was agitated for 120 s (1900 rpm), then filtered by application of vacuum pressure (−60 to −65 kPa) for 120 s into a collection vial. Finally, 30 µL of the extract was injected in the 2D-UHPLC-MS/MS system.

Analysis was performed on a two dimensions ultra-performance liquid chromatograph-tandem mass spectrometer (2D-UHPLC-MS/MS) consisting of the following Shimadzu® modules (Shimadzu Corporation): an isocratic pump LC20AD SP, for pre-treatment mode, a binary pump consisting of coupling two isocratic pumps Nexera LC30AD for the analytical mode, an automated sampler SIL-30AC, a column oven CTO-20AC and a triple-quadrupole mass spectrometer LCMS-8060. The assay was broken down into two stages. The first was the pre-treatment where the sample was loaded on the perfusion column. The second step was the elution of the compounds of interest to the

analytical column. The deproteinized extract performed by the CLAM-2030 was automatically transferred to the automated sampler, where 30 µL was directly analyzed into the chromatographic system. The LC-integrated online sample clean-up was performed using a perfusion column Shimadzu® MAYI-ODS (5 mm L × 2 mm I.D.). The first step consisted of loading the extract on the perfusion column with a mobile phase composed of 10 mM ammonium formate in water (Carlo Erba) at a flow rate of 0.5 mL/min during 2 min. Then the system switched to the analytical step to elute the analytes from the perfusion column to the analytical column to achieve chromatographic separation. During this step, the loading line was washed with propan-2-ol (Carlo Erba) during 3 min. Chromatographic separation was achieved on a Restek® Raptor Biphenyl (50 mm L × 3 mm I.D., 2.7 µm) maintained at 40 °C and a gradient of (A) 1 mM ammonium fluoride buffer in water (Carlo Erba) and (B) methanol (Carlo Erba) at a flow rate of 0.675 mL/min as follows: 0.0–2.10 min, 5% (B); 2.1–3.0 min, 5 to 65% (B); 3.0–4.75 min, 65% (B); 4.75–5.0 min, 65 to 70% (B); 5.0–6.6 min, 70% (B); 6.6–8.0, 70 to 75% (B); 8.0–8.5 min, 75 to 100% (B); 8.50–9.5 min, 100% (B); 9.5–9.6 min, 100% to 5% (B); 9.6–12.0 min, 5% (B). Detection and quantification were performed by scheduled-Multiple Reaction Monitoring (MRM) using 1 ms pause time and 50 ms dwell times to achieve sufficient points per peak. The interface parameters and common settings were as follows: interface voltage: 1 kV; nebulizing gas flow: 3 L/min; heating gas flow: 10 L/min; drying gas flow: 10 L/min; interface temperature: 400 °C; desolvation line (DL) temperature: 150 °C; heat block temperature: 500 °C; collision gas pressure 300 kPa. Compound-specific MRM parameters are shown in **Supplementary File 1c**.

Enzyme-linked immunosorbent assay (ELISA)

Steroids were extracted from testicular homogenates and from organotypic culture media with 5 volumes of diethyl ether (Carlo Erba). The organic phase was then recovered and evaporated in a water bath at 37°C. The tubes were stored at −20°C until use. Progesterone and estradiol levels were measured in 50 µL of testicular homogenates and in 50 µL of culture media using Cayman ELISA kit (582601 for progesterone and 501890 for estradiol, Cayman Chemical Company, Ann Arbor, MI, USA), according to the supplier's recommendations. The sensitivity limit for the progesterone assay was 10 pg/mL with average intra- and inter-trial variations of 13.9% and 9.6%, respectively. The sensitivity limit for the estradiol assay was 20 pg/mL and the lower limit of detection was 6 pg/mL with average intra- and inter-trial variations of 20.25% and 14.975%, respectively.

Aromatase activity

After homogenization of ~10-50 mg of tissues in 500 µL of Aromatase Assay Buffer, the aromatase activity was measured with the fluorometric Aromatase (CYP19A) Activity assay kit (ab273306; Abcam) according to the manufacturer's instructions.

Radioimmunoassay (RIA)

INSL3 was assayed by using RIA kit with ^{125}I as tracer (RK-035-27, Phoenix Pharmaceuticals, Strasbourg, France). According to the manufacturer's instructions, analyses were performed on 100 µL of culture media or tissue homogenates. Assay validation was assessed by determining the recovery of expected amounts of INSL3 in samples to which exogenous INSL3 was added. The sensitivity of the INSL3 RIA kit was 60.4 pg/mL and the range of the assay was 10-1280 pg/mL.

Statistical analyses

Statistical analyses were carried out with GraphPad Prism 8 software (GraphPad Software Inc., La Jolla, CA, USA). Data are presented as means ± SEM. The non-parametric Mann-Whitney test was used to compare *in vitro* cultures and *in vivo* controls (D16 FT *vs* 22 d*pp*, D16 CSF *vs* 22 d*pp*, D30 FT *vs* 36 d*pp*, D30 CSF *vs* 36 d*pp*, D30 FT + hCG *vs* 36 d*pp*, D30 CSF + hCG *vs* 36 d*pp*); cultures of fresh

and CSF tissues (6 d*pp* *vs* 6 d*pp* CSF, D16 FT *vs* D16 CSF, D30 FT *vs* D30 CSF, D30 FT + hCG *vs* D30 CSF + hCG); cultures with or without hCG (D30 FT + hCG *vs* D30 FT, D30 CSF + hCG *vs* D30 CSF). A value of $P < 0.05$ was considered statistically significant.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Funding

This work was supported by a PhD grant from Région Normandie (awarded to LM) and by fundings from Ligue contre le Cancer Comité de Seine-Maritime (awarded to CR), France Lymphome Espoir (Bourse Sacha awarded to CR), Région Normandie and Europe (RIN Steroids awarded to HL) and ANR (ANR-21-CE14-0068, sc-SpermInVitro awarded to NR). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Acknowledgements

Data from RT-qPCR and images were obtained on PRIMACEN (*http://www.primacen.fr*), the Cell Imaging Platform of Normandy, IRIB, Faculty of Sciences, University of Rouen, 76821 Mont-Saint-Aignan. The authors also thank the U1096 team for the access to their western blot devices.

Figure 1 – figure supplement 1. Impact of controlled slow freezing on Leydig cells in organotypic cultures. (A) Representative images of 3β-HSD expression in Leydig cells at 6 d*pp* and in *in vitro* cultured fresh (FT) or frozen/thawed (CSF) testicular tissues (D16 and D30). Testicular tissue sections were counterstained with Hoechst (blue). Dotted lines delineate seminiferous tubules. Scale: 15 µm. (B) Number of 3β-HSD positive Leydig cells per cm² of FT or CSF testicular tissue. (C-D) Percentage of Leydig cells (C) in apoptosis (3β- HSD and cleaved caspase 3 positive) or (D) in proliferation (3β-HSD and Ki67 positive) in *in vivo* and *in vitro* matured FT or CSF tissues. (E) Percentage of 3β-HSD positive Leydig cells expressing AR in *in vivo* and *in vitro* matured FT or CSF tissues. (F-J) Relative mRNA levels of Leydig cell differentiation factors (*Igf1*, *Dhh*), progenitor/immature Leydig cell (*Srd5a1*) and of adult Leydig cell markers (*Sult1e1*, *Insl3*) (normalized to *Gapdh* and *Actb* or to *Hsd3b1*).

Data are presented as means ± SEM with n = 4 biological replicates for each group. A value of $*P < 0.05$ was considered statistically significant.

FT: Fresh Tissue, CSF: Controlled Slow Freezing

Figure 3 – figure supplement 1. Impact of controlled slow freezing on steroids production by Leydig cells in organotypic cultures. Concentrations of androgens (androstenedione and testosterone) in the culture medium of fresh (FT) or frozen/thawed (CSF) testicular tissues.

Data are presented as means ± SEM with n = 4 biological replicates for each group. A value of $*P < 0.05$ was considered statistically significant.

Figure 6 – figure supplement 1. Impact of different hCG concentrations on testosterone production in organotypic cultures. (A) Concentrations of testosterone in the culture medium after supplementation with different hCG concentrations (5 pM, 50 pM, 1 nM, 5 nM, 50 nM) from

D16 to D30. (B) Concentrations of testosterone in FT testicular tissues (D30) normalized to *in vivo* control (36 d*pp*) and in culture medium at D30 normalized to *in vitro* control without hCG, after supplementation with different hCG concentrations (5 pM, 50 pM, 1 nM, 5 nM, 50 nM).

Data are presented as means ± SEM with n = 4 biological replicates for each group. A value of $*P < 0.05$ was considered statistically significant.

Figure 6 – figure supplement 2. Impact of hCG supplementation on mRNA levels of genes involved in steroidogenesis and androgen signaling in organotypic cultures. Relative mRNA levels of Leydig cell markers, actors of steroidogenesis and androgen signaling (normalized to *Gapdh* and *Actb* or to *Hsd3b1*) after supplementation with 1 nM hCG.

Data are presented as means ± SEM with n = 4 biological replicates for each group. A value of $*P < 0.05$ was considered statistically significant.

Figure 8 – figure supplement 1. (A) Testicular tissue area (in mm²) measured on tissue sections from mice of different ages (6 d*pp*, 22 d*pp* and 36 d*pp*) and after 16 days (D16) or 30 days (D30) of organotypic cultures. (B) Necrotic region (percentage of the tissue section area) at D16 and D30. (C-D) Representative images of cleaved caspase 3 (CC3) immunostaining, mostly detected in the central necrotic region (dotted lines). Testicular tissue sections were counterstained with Hoechst (blue). Scale: 100 µm. (E-F) Representative images of PAS staining, with the necrotic area delineated by dotted lines. Testicular tissue sections were counterstained with hematoxylin. Scale: 200 µm.

Data are presented as means ± SEM with n = 4 biological replicates for each group. A value of $*P < 0.05$ was considered statistically significant.

FT: Fresh Tissue; CSF: Controlled Slow Freezing.

Supporting information

Supplementary File 1a. Detailed list of antibodies used in this study HRP: horseradish peroxidase; IF: immunofluorescence; IHC: immunohistochemistry; O/N: overnight; RT: room temperature; WB: western blot

Supplementary File 1b. List of PCR primers used in this study

Supplementary File 1c. Compound-specific MRM parameters for LC-MS/MS CE: collision energy; DHEA: Dehydroepiandrosterone; MRM: multiple reaction monitoring

Supplementary File 2. Western Blot for 3β-HSD, CYP17A1, AR, CYP19 and FAAH.

Figure 1-source data 1: Source data of figure 1

Figure 1-figure supplement 1-source data 1: Source data of figure 1 – figure supplement 1

Figure 2-source data 1: Source data of figure 2

Figure 3-source data 1: Source data of figure 3

Figure 3-figure supplement 1-source data 1: Source data of figure 3 – figure supplement 1

Figure 4-source data 1: Source data of figure 4

Figure 5-source data 1: Source data of figure 5

Figure 6-source data 1: Source data of figure 6

Figure 6-figure supplement 1-source data 1: Source data of figure 6 – figure supplement 1

Figure 6-figure supplement 1-source data 2: Source data of figure 6 – figure supplement 2

Figure 8-figure supplement 1-source data 1: Source data of figure 8 – figure supplement 1

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Reviewer #1 (Public Review):

In this manuscript, the authors aimed to compare, from testis tissues at different ages from mice in vivo and after culture, multiple aspects of Leydig cells. These aspects included mRNA levels, proliferation, apoptosis, steroid levels, protein levels, etc. A lot of work was put into this manuscript in terms of experiments, systems, and approaches. The technical aspects of this work may be of interest to labs working on the specific topics of in vitro spermatogenesis for fertility preservation.

Second review:

The authors should be commended for substantial improvement in their manuscript for resubmission.

- https://doi.org/10.7554/eLife.85562.2.sa1

Reviewer #3 (Public Review):

Moutard, Laura, et al. investigated the gene expression and functional aspects of Leydig cells in a cryopreservation/long-term culture system. The authors found that critical genetic markers for Leydig cells were diminished when compared to the in-vivo testis. The testis also showed less androgen production and androgen responsiveness. Although they did not produce normal testosterone concentrations in basal media conditions, the cultured testis still remained highly responsive to gonadotrophin exposure, exhibiting a large increase in androgen production. Even after the hCG-dependent increase in testosterone, genetic markers of Leydig cells remained low, which means there is still a missing factor in the culture media that facilitates proper Leydig cell differentiation. Optimizing this testis culture protocol to help maintain proper Leydig cell differentiation could be useful for future human testis biopsy cultures, which will help preserve fertility and child cancer patients.

Overall, the authors addressed most comments and questions from the previous review. The additional data regarding the necrotic area is helpful for interpreting the quality of the cultures.

The authors did not conduct a multiple comparison tests although there are multiple comparisons conducted on for a single dependent variable (Fig 2J, Fig 3F, among many others), however, the addition of this multiple comparison is unlikely to change the conclusions of the paper or the figure and, thus is a minor technical detail in this case.

- https://doi.org/10.7554/eLife.85562.2.sa0

Author Response

The following is the authors' response to the original reviews.

We would like to thank you for your thorough review of the manuscript. We have taken all comments into account in the revised version of the manuscript. Please find below our detailed responses to your comments.

> ***eLife assessment***
>
> *This study reports useful information on the limits of the organotypic culture of neonatal mouse testes, which has been regarded as an experimental strategy that can be extended to humans in the clinical setting for the conservation and subsequent re-use of testicular tissue. The evidence that the culture of testicular fragments of 6.5-day-old mouse testes does not allow optimal differentiation of steroidogenic cells is compelling and would be useful to the scientific community in the field for further optimizations.*

Thank you for this assessment. We have carefully considered all comments and made the requested revisions to improve the manuscript.

> ***Reviewer #1 (Public Review):***
>
> *In this manuscript, the authors aimed to compare, from testis tissues at different ages from mice in vivo and after culture, multiple aspects of Leydig cells. These aspects included mRNA levels, proliferation, apoptosis, steroid levels, protein levels, etc. A lot of work was put into this manuscript in terms of experiments, systems, and approaches. However, as written the manuscript is incredibly difficult to follow. The Introduction and Results sections contain rather loosely organized lists of information that were altogether*

> *confusing. At the end of reading these sections, it was unclear what advance was provided by this work. The technical aspects of this work may be of interest to labs working on the specific topics of in vitro spermatogenesis for fertility preservation but fail to appeal to a broader readership. This may be best exemplified by the statements at the end of both the Abstract and Discussion which state that more work needs to be done to improve this system.*

As suggested, we have reworked the manuscript to make it clearer, more meaningful and more precise. We believe that this work may be of interest to a broader readership. Indeed, the development of a model of in vitro spermatogenesis could be of interest for labs working on the specific period of puberty initiation, on germ and somatic cell maturation and on steroidogenesis under physiological and pathological conditions, and could also be useful for testing the toxicity of cancer therapies, drugs, chemicals and environmental agents (e.g. endocrine disruptors) on the developing testis.

There is a crucial unmet need to optimize the culture conditions for in vitro spermatogenesis. It is important to identify the deregulated molecular mechanisms leading to a decreased in vitro spermatogenic yield. Such results will be of great help to improve organotypic culture conditions. In the present study, we not only uncovered for the first time a failure in adult Leydig cell development, but also an alteration in the expression of several steroidogenic and steroid-metabolizing genes, which could explain the accumulation of progesterone and estradiol and the deficiency of androstenedione in cultured tissues. This hyperestrogenic and hypoandrogenic environment could explain, at least in part, the low efficiency of in vitro spermatogenesis. Furthermore, we show that the addition of hCG (LH homolog) is not sufficient to facilitate Leydig cell differentiation, restore steroidogenesis and improve sperm yield. These data provide valuable information for improving culture conditions. More fundamentally, this culture system could be a useful tool for identifying factors that are essential for the differentiation and functionality of adult Leydig cells during puberty initiation.

> ***Recommendations For The Authors:***
>
> *This reviewer appreciates that a lot of work was put into this manuscript in terms of experiments, systems, and approaches. However, the manuscript needs significant revision, and in this reviewer's opinion is not appropriate for a broader readership journal. The results seem rather incremental, and the topic is too specialized in its current format.*

The manuscript was significantly revised taking into account the reviewer's comments. In addition, as mentioned above, the development of a model of in vitro spermatogenesis could have wider applications and be of interest to a broader audience.

> *Comments for improvement, roughly in order of appearance:*
>
> *1. Abstract - would recommend condensing to hit the main points of the manuscript.*

The abstract has been condensed as suggested.

> *1. Introduction, overall - this is a rather loosely organized list of information that is not synthesized or communicated in a meaningful way. It contains overstatements and lumps together findings from both mice and primates and thus several statements for the actions of these steroid hormones are inaccurate. The authors rely much too heavily upon reviews and need to replace those with a more scholarly approach of carefully reading and citing primary literature.*

The Introduction has been reorganized to make it clearer, more synthetic, more meaningful and more accurate. Only findings from rodents are presented. We carefully read the literature and replaced most of reviews by primary literature.

> 1. *Results - this section was extremely difficult to read and comprehend, as it's essentially a laundry list of measurements of mRNAs, steroids, cholesterols, and proteins that go up or down or don't change at multiple ages, both in vitro and in vivo. The section would be improved greatly by an organization with rationale and concluding statements to prepare the reader for the factoid-style data that are presented.*

As suggested, the Results section has been improved by an organization with rationale and concluding statements to make it easier to read and comprehend.

> 1. *47 - is this approach going to both "preserve and restore"? Sounds more like it will allow for the production of offspring, but the other goals are not going to happen from the approach listed in the latter part of that sentence - so not really "fertility restoration" but more of an insurance program that sperm can be produced for ART*

Freezing of prepubertal testicular tissue, which contains spermatogonia, is a fertility preservation option proposed to prepubertal boys with cancer prior to highly gonadotoxic treatments. Several fertility restoration strategies, which aim to allow the production of spermatozoa from cryopreserved spermatogonia, are being developed, including in vitro spermatogenesis. This sentence has been rewritten.

> 1. *62 - specify whether this "decreased expression" is mRNA or protein, and is this because of a loss of Sertoli cells?*

“Decreased expression” was replaced by “decreased mRNA levels”. The results we obtained in the cited study (Rondanino et al., 2017) suggest that the decrease in Rhox5 mRNA levels is not the consequence of a change in the proportion of Sertoli cells but reflects an alteration in Rhox5 gene expression. In Figure 6U of the present study, we show indeed that there is no loss of Sertoli cells in organotypic cultures.

> 1. *66 - what is "the first wave of mouse in vitro spermatogenesis"? Are these cultures from the first wave of mouse in vivo spermatogenesis, or is there a second wave of in vitro spermatogenesis? Please specify*

In the mouse, the first entry into meiosis occurs around 8-10 dpp and the first spermatozoa are produced at around 35 dpp: this is the first wave of spermatogenesis which takes place at the onset of puberty. By culturing 6 dpp-old testes for 30 days, our aim is to reproduce in vitro all the stages of this first wave of spermatogenesis, i.e. entry into meiosis, completion of meiosis and spermiogenesis.

In the cited study (Pence et al., 2019), the authors cultured 5 dpp testes for 35 to 49 days and observed a decline in intratesticular testosterone levels in the cultured tissues, i.e. after the end of the first spermatogenic wave, compared to in vivo controls. Our sentence has been rewritten to make it clearer.

> 1. *78 - is there a difference in T production by Fetal vs Adult LCs? It is this reviewer's understanding that the levels of T around birth in mice (and then a few months after birth in humans) are quite high, similar to adults. So, what are the authors suggesting here by providing the list of expressed genes in these two LC populations?*

As mentioned in the Introduction section, 17β-HSD3 – the enzyme responsible for the conversion of androstenedione to T – is not expressed in fetal Leydig cells but is expressed in adult Leydig cells. Therefore, unlike adult Leydig cells, fetal Leydig cells are not capable of synthesizing T.

In the present study, we investigated steroidogenesis but also wondered which types of Leydig cells could be detected under in vitro conditions. It is therefore important to explain to the reader which steroidogenic proteins are expressed by the different Leydig cell populations.

As described in O'Shaughnessy et al., 2002, levels of intratesticular T decline after birth, being very low between 10 and 20 dpp. Then, T levels increase. At 25 dpp, T levels are close to those observed at 1 dpp. T levels increase more than 16-fold between 25 and 30 dpp and then double between 30 dpp and adulthood. Therefore, intratesticular T levels around birth in mice are not as high as in adults, but are about 36-fold lower after birth than in adulthood. It has been shown that in the fetal testis, the conversion of androstenedione produced by fetal Leydig cells is achieved by the adjacent fetal Sertoli cells that express 17β-HSD3 (O'Shaughnessy et al., 2000; Shima et al., 2013). During postnatal development however, Sertoli cells lose the expression of 17β-HSD3 (O'Shaughnessy et al., 2000).

> *1. 79 -99 - can the authors revise this long list of information to provide a summary of what they are trying to communicate to the reader? What is the intention of this information?*

This paragraph has been modified to make it clearer and more synthetic. As different Leydig cell markers are presented in the Results section, it is important to introduce the reader to the different types of Leydig cells, the proteins expressed by these cells and the factors involved in their proliferation and differentiation.

> *1. 101-2 - replace "involved in" with a more meaningful word - and it is this reviewer's understanding that T has not been shown convincingly to have much of a role in spermatogonial development, at least in mice - that statement is likely true in primates, but not mice; provide primary literature citations to be more precise, rather than a broad review that covers multiple species*

"involved in" was replaced by "is essential for many aspects of spermatogenesis, including". Moreover, we removed "spermatogonial proliferation and differentiation" and provide primary literature citations to be more precise.

> *1. 105-7 - similar concern for E as for T, above - KO mouse models for ERalpha and beta did not show defects in spermatogenesis as described - not sure what evidence the authors are specifically referring to here - cite primary literature rather than a review on Vitamin D + estrogen*

We agree that the question of whether estrogens play a direct role in spermatogenesis was unanswered by the ER null mice. However, estrogens have been shown to be important for the long-term maintenance of spermatogenesis in the ArKO mouse (Robertson et al., 1999) and for the progression of normal germ cell development in the ENERKI mouse (Sinkevicius et al., 2009). This sentence has been reworded and primary literature is cited to be more precise.

> *1. 113-4 - there is no convincing evidence this reviewer is aware of that the AR is expressed in male germ cells, and therefore T actions on germ cells are indirect, through Sertoli cells and perhaps PTMs; if there is some, this sentence needs a citation showing that*

We agree that there is no evidence that AR is expressed in male germ cells and that T acts indirectly on germ cells. This sentence has been rewritten.

> *1. 114-6 - this is untrue - nowhere in that paper was testosterone or androgen even mentioned!*

This reference has been removed. We apologize for this mistake.

> *1. 116-7 - again, E actions through the ERs are thought to be indirect in the testis, not acting on germ cells; if this is incorrect, please add supportive citations and explain; replace "involved" with a more meaningful word; Rhox5 has a very minor role in spermatogenesis*

In contrast to androgen receptors, which are localized in somatic cells, estrogen receptors have been found in most testicular cells, including germ cells. The studies reporting the expression of estrogen receptors in germ cells are cited in the Introduction section. The word “involved” was replaced by “promotes”.

Rhox5 (also known as Pem) has not a very minor role in spermatogenesis. On the contrary, its expression is crucial for normal spermatogenesis and sperm maturation, as loss of Rhox5 in male mice leads to reduced fertility, increased germ cell apoptosis, decreased sperm count and decreased sperm motility (MacLean et al., 2005).

> *1. 117 - Ref 29 does not support the statement about Rhox5's role in spermatogenesis*

The reference (MacLean et al., 2005), supporting the statement about Rhox5’s role in spermatogenesis, was added in the manuscript.

> *1. 120 - Does FAAH have a protective role in that it is anti-apoptotic? Or just required for some other Sertoli cell function? Should re-word to be more specific.*

FAAH (fatty acid amide hydrolase), whose expression is stimulated by estrogens, has been shown to have a crucial role in promoting survival of Sertoli cells by degrading anandamide (N-arachidonoylethanolamine), an endocannabinoid which has a pro-apoptotic activity (Rossi et al., 2007).

The sentence has been reworded to be more specific.

> *1. 127 - should complete the Introduction with a sentence summarizing what was done and found, for reader clarity*

The Introduction has been completed for reader clarity.

> *1. 136 - misspelled the procedure*

Orchidectomy was replaced by orchiectomy.

> *1. Mice - why use half-day nomenclature for postpartum mice? This is not standard in the literature.*

Half-day nomenclature was used due to the uncertainty of the time of birth, which mostly takes place during the night. Since this is not standard in the literature, half-day nomenclature was removed in the entire manuscript.

> *1. 172-3 - the half-life of RA is very short (<1 hr), and it is light-sensitive. This addition every 8 days means that retinoids are present for a very minimal window of time - are the authors sure retinoids have no requirement elsewhere during spermatogenesis? And in the literature, the measured pulse of RA in the mouse lasts >40 hours (stages VII-IX)...*

RA is mandatory for proper spermatogenesis and is needed many times during spermatogenesis (for review, see Schleif et al., 2022): RA is involved in spermatogonial differentiation, pre-meiotic activation and meiotic completion, establishment of the blood-testis barrier and spermiation. In our study, we did not add RA in the culture medium but retinol, the precursor of RA. Indeed, our previous studies have shown beneficial effects of retinol on in vitro spermatogenesis, including an increased production of spermatids with less nuclear alterations and DNA damage (Arkoun et al., 2015; Dumont et al., 2016).

The reason we added retinol (and not RA, which has a very short half-life) in this study and in our previous studies is that it can be oxidized into RA but also be stored in Sertoli cells in the form of retinyl esters for later use. As retinol is photosensitive, handling and storage were performed in tubes covered with aluminum foil, which protects from direct light exposure.

> *1. 362 - Start the Results section with a broader statement(s) that prepares the readers rather than jumping into specific experiments; it would be helpful for readers to have concluding sentences included as well for readers to navigate the Results section.*

As suggested, the Results section has been improved by an organization with rationale and concluding sentences to facilitate reading.

> *1. 364 - KI67 is a marker of.*

Ki67 is widely used as a cell proliferation marker.

> *1. 367 - replace "involved".*

“involved” was replaced by “necessary for”.

> *1. What intensity thresholds were used to define a cell as positive or negative for a given marker? And there seemed to be no mention of controls - especially no primary antibody controls. This is a significant oversight if these were not done in parallel with every single immunostaining experiment.*

We did not apply intensity thresholds. Cells presenting detectable labeling were defined as positive, while unlabeled cells were defined as negative.

Negative controls, performed by omitting the primary antibodies, were of course done in parallel to each immunostaining and are presented in Figure 1A, Figure 2J and Figure 5C. The mention of negative controls has been added in the Materials and methods section.

> *1. 388 - INSL3 - is this referring to mRNA or protein? Protein nomenclature is used...*

INSL3 is here referring to the protein, whose concentrations were measured by radioimmunoassay.

> *1. 402 - typo.*

“expect” was replaced by “except”.

> *1. 409 - do mRNA levels really "determine the testicular steroidogenic potential"??*

This sentence has been reworded: “determine the testicular steroidogenic potential” was replaced by “highlight a potential deregulation of their expression”.

> *1. 410 - western should not be capitalized.*

Western Blot was replaced by western blot in the entire manuscript.

> *1. 405-28 - this reviewer is underwhelmed by qRT-PCR results for a handful of markers - what is the purpose? The results do not prove anything about the function of the system.*

As the differentiation of Leydig cells is not fully completed in organotypic cultures, we wanted to know which actors of the steroidogenic pathway show deregulated expression in vitro in comparison to physiological conditions, and thus which steps of the steroid hormone biosynthesis pathway may be impaired. We found that the expression of several genes encoding steroidogenic enzymes was decreased in vitro, notably that of Cyp17a1, necessary for the conversion of progesterone to androstenedione. Transcript levels of Hsd17b2, encoding an enzyme that converts estradiol to estrone and testosterone to androstenedione, were also decreased at D30.

Our data therefore show that the expression of several steroidogenic genes and steroid metabolizing genes is deregulated in organotypic cultures but we agree that these results do not prove anything about the function of the system.

We then found an accumulation of estradiol and progesterone, a decrease in androstenedione and unchanged testosterone levels in cultured tissues. The elevation in progesterone and the reduction in androstenedione in in vitro matured tissues could arise from the reduced expression of Cyp17a1. In addition, reduced Hsd17b2 transcript levels may explain why estradiol levels remain elevated in cultures while testosterone levels are similar to controls and androstenedione levels are low.

> *1. How do the authors interpret data gleaned from tissues containing a variably-sized necrotic core?*

In the present study, the central necrotic area was consistent between all samples and variables: it represents on average 16-27% of the explants.

As in our previous publications and recent RNA-seq analyses (Rondanino et al., 2017; Oblette et al., 2019; Dumont et al., 2023), the central necrotic area was removed so that transcript and protein levels in the healthy part of the samples (i.e. where in vitro spermatogenesis occurs) could be measured and compared with in vivo controls. In order to be able to compare the healthy part of the in vitro matured tissues with in vivo controls, transcript levels were

normalized to housekeeping genes (Gapdh and Actb) or to the Leydig cell-specific gene Hsd3b1 while protein levels were normalized to ACTB or to 3β-HSD.

> 1. *520 - after reading to this point, this reviewer was left confused and wondering why any of this is important to the reader unless that reader specifically works on this topic. The way the data were presented makes it nearly impossible for the reader to keep any of the data in their mind as they read. It's a seemingly endless list of ups and downs of many things under many conditions. What is the point of all of this? How will it advance our understanding of spermatogenesis? Or improve in vitro culture? Or help prepubertal cancer patients? Presumably, that will be explained in the Discussion, but at this point, this reviewer honestly has no idea what this all means. Why is this important??*

We have modified the Results section by including rationale and concluding statements to make it easier to read and follow for all readers, not necessarily for those working on this topic.

As mentioned above, the identification of the molecular mechanisms that are deregulated in vitro will give us important insights for the optimization of the culture system. The development of an optimized model of in vitro spermatogenesis could lead to several applications, including improving our knowledge of the regulation of spermatogenesis during pubertal development.

In this study, our main findings are that the differentiation of the adult Leydig cell lineage, steroid biosynthesis, metabolism and signaling are altered in organotypic cultures, leading to a hyperestrogenic and hypoandrogenic environment. In addition, we show that the presence of an LH homolog, known to be critical to adult Leydig cell differentiation and to stimulate steroidogenesis, does not rescue the expression of adult Leydig cell markers and of several steroidogenic genes, steroid metabolizing genes and steroid target genes. Other factors required for Leydig cell maturation and functionality will have to be tested in the future on cultured testicular tissues. Improvements to this in vitro maturation procedure in animal models may be useful for future cultures of human testicular biopsies, although we are aware that more work needs to be done before prepubertal cancer patients can benefit from this in vitro maturation approach.

> 1. *619-20 - this sort of summarizes this reviewer's overall opinion of the manuscript. Not much seems to have been learned here that would justify publication in a broad readership journal like eLife. More work needs to be done to provide that sort of meaningful advance. The current work, with considerable re-writing to improve accuracy and clarity, is much better suited to a specialty journal where others who are working on this specific topic will appreciate its value.*

We have carefully considered the reviewer’s comments and modified the manuscript to improve accuracy and clarity. We understand the reviewer’s point of view, but we believe that this work may be of interest not only to labs working on fertility preservation and restoration, but also to those working on puberty initiation, germ and somatic cell maturation, steroidogenesis under physiological and pathological conditions, and on the effect of cancer therapies, drugs, chemicals and environmental agents (e.g. endocrine disruptors) on the developing testis.

As mentioned above, we not only uncovered for the first time a failure in adult Leydig cell development, but also an alteration in the expression of several steroidogenic and steroid-metabolizing genes, which could explain the accumulation of progesterone and estradiol and the deficiency of androstenedione in cultured tissues. This hyperestrogenic and hypoandrogenic environment could explain, at least in part, the low efficiency of in vitro

spermatogenesis. Furthermore, we show that the addition of hCG (LH homolog) is not sufficient to facilitate Leydig cell differentiation, restore steroidogenesis and improve sperm yield. These data provide valuable information for improving culture conditions. More fundamentally, this culture system could be a useful tool for identifying factors that are essential for the differentiation and functionality of adult Leydig cells during puberty initiation.

> *1. Why are the figures repeated at the end of the manuscript?*

During the submission process, our bioRxiv preprint (which contains the figures) was merged with the same but higher quality figures.

> ***Reviewer #2 (Public Review):***
>
> *Preserving and restoring the fertility of prepubertal patients undergoing gonadotoxic treatments involves freezing testicular fragments and waking them up in a culture in the context of medically assisted procreation. This implies that spermatogenesis must be fully reproduced ex vivo. The parameters of this type of culture must be validated using non-human models. In this article, the authors make an extensive study of the quality of the organotypic culture of neonatal mouse testes, paying particular attention to the differentiation and endocrine function of Leydig cells. They show that fetal Leydig cells present at the start of culture fail to complete the differentiation process into adult Leydig cells, which has an impact on the nature of the steroids produced and even on the signaling of these hormones.*
>
> *The authors make an extensive study of the different populations of Leydig cells which are supposed to succeed each other during the first month of life of the mouse to end up with a population of adult and fully functional cells. The authors combine quantitative in situ studies with more global analyzes (RT-QtPCR Western blot, hormonal assays), which range from gene to hormone. This study is well written and illustrated, the description of the methods is honest, the analyses systematic, and are accompanied by multiple relevant control conditions.*
>
> *Since the aim of the study was to study Leydig cell differentiation in neonatal mouse testis cultures, the study is well conceived, the results answer the initial question and are not over-interpreted.*
>
> *My main concern is to understand why the authors have undertaken so much work when they mention RNA extractions and western blot, that the necrotic central part had to be carefully removed. There is no information on how this parameter was considered for immunohistochemistry and steroid measurements. The authors describe the initial material as a quarter testis, but they don't mention the resulting size of the fragment. A brief review of the literature shows that if often the culture medium is crucial for the quality of the culture (and in particular the supplementations as discussed by the authors here), the size of the fragments is also a determining factor, especially for long cultures. The main limitation of the study is therefore that the authors cannot exclude that central necrosis can have harmful effects on the survival and/or the growth and/or the differentiation of the testis in culture. In this sense, the general interpretation that the authors make of their work is correct, the culture conditions are not optimized.*

When using the organotypic culture system at a gas-liquid interphase, the central part of the testicular tissue becomes necrotic. As previously reported (Komeya et al., 2016), the central region receives insufficient nutrients and oxygen. In vitro spermatogenesis therefore only occurs in the seminiferous tubules present in the peripheral region. As in our previous publications and recent RNA-seq analyses (Rondanino et al., 2017; Oblette et al., 2019;

Dumont et al., 2023), the central necrotic area was removed so that transcript and protein levels in the healthy part of the samples (i.e. where in vitro spermatogenesis occurs) could be measured and compared with in vivo controls. For histological and immunohistochemical analyses, only seminiferous tubules located at the periphery of the cultured fragments (outside of the necrotic region) were analyzed. Steroid measurements were performed on the entire fragments.

The initial material was indeed a quarter testis, which represents approximately 0.75 mm3. No growth of the fragments was observed during the organotypic culture period (Figure 8-figure supplement 1). We agree with the reviewer that the composition of the culture medium is not the only parameter to be considered for the quality of the culture and that the size of the fragments is also a determining factor. We previously determined that 0.75 mm3 was the most appropriate size for mouse in vitro spermatogenesis (Dumont et al., 2016). We do not exclude at all that central necrosis can have harmful effects on the survival and/or the growth and/or the differentiation of the testis in culture. Optimization of the culture medium and culture design (so that the tissue center receives sufficient nutrients and oxygen) will be necessary to increase the yield of in vitro spermatogenesis.

> *Organotypic culture is currently trying to cross the doors of academic research laboratories to become a clinical tool, but it requires many adjustments and many quality controls. This study shows a perfect example of the pitfall often associated with this approach. The road is still long, but every piece of information is useful.*
>
> ***Reviewer #3 (Public Review):***
>
> *Moutard, Laura, et al. investigated the gene expression and functional aspects of Leydig cells in a cryopreservation/long-term culture system. The authors found that critical genetic markers for Leydig cells were diminished when compared to the in-vivo testis. The testis also showed less androgen production and androgen responsiveness. Although they did not produce normal testosterone concentrations in basal media conditions, the cultured testis still remained highly responsive to gonadotrophin exposure, exhibiting a large increase in androgen production. Even after the hCG-dependent increase in testosterone, genetic markers of Leydig cells remained low, which means there is still a missing factor in the culture media that facilitates proper Leydig cell differentiation. Optimizing this testis culture protocol to help maintain proper Leydig cell differentiation could be useful for future human testis biopsy cultures, which will help preserve fertility and child cancer patients.*
>
> *Methods: In line 226, there is mention that the central necrotic area was carefully removed before RNA extraction. This is particularly problematic for the inference of these results, especially for the RT-qPCR data. Was the central necrotic area consistent between all samples and variables (16 and 30FT)? How big was the area? This makes the in-vivo testis not a proper control for all comparisons. Leydig cells are not evenly distributed throughout the testis. A lot of Leydig cells can be found toward the center of the gonad, so the results might be driven by the loss of this region of the testis.*

When using the organotypic culture system at a gas-liquid interphase, the central part of the testicular tissue becomes necrotic. As previously reported (Komeya et al., 2016), the central region receives insufficient nutrients and oxygen. In vitro spermatogenesis therefore only occurs in the seminiferous tubules present in the peripheral region. As in our previous publications and recent RNA-seq analyses (Rondanino et al., 2017; Oblette et al., 2019; Dumont et al., 2023), the central necrotic area was removed so that transcript levels in the healthy part of the samples (i.e. where in vitro spermatogenesis occurs) could be measured and compared with in vivo controls. In order to be able to compare the healthy part of the in vitro matured tissues with in vivo controls, transcript levels of the selected genes were

normalized to housekeeping genes (Gapdh and Actb) or to the Leydig cell-specific gene Hsd3b1.

The central necrotic area was consistent between all samples and variables: it represents on average 16-27% of the explants.

Moreover, we would like to point out that the gonads were cut into four fragments before in vitro cultures. It is therefore the central part of the cultured explants that was removed and not the central part of the gonads. The central part of the gonads was thus included in our analyses.

> *What did the morphology of the testis look like after culturing for 16 and 30 days? These images will help confirm that the culturing method is like the Nature paper Sato et al. 2011 and also give a sense of how big the necrotic region was and how it varied with culturing time.*

Images showing mouse testicular tissues cultured for 16 and 30 days are presented in Figure 8-figure supplement 1. The cultured tissues resemble those shown by Sato et al., 2011. As mentioned above, the central necrotic area represents on average 16-27% of the explants. No significant difference in the area of the necrotic region was found between the two culture time points.

> *There are multiple comparisons being made. Bonferroni corrections on p-value should be done.*

Bonferroni corrections are used when multiple comparisons are conducted. As mentioned in the Materials and methods section, multiple comparisons were not made in this study. Indeed, the non-parametric Mann-Whitney test was used to compare two conditions: in vitro vs in vivo (D16 FT vs 22 dpp, D16 CSF vs 22 dpp, D30 FT vs 36 dpp, D30 CSF vs 36 dpp, D30 FT + hCG vs 36 dpp, D30 CSF + hCG vs 36 dpp), cultures of fresh vs frozen tissues (6 dpp vs 6 dpp CSF, D16 FT vs D16 CSF, D30 FT vs D30 CSF, D30 FT + hCG vs D30 CSF + hCG) and cultures with vs without hCG (D30 FT + hCG vs D30 FT, D30 CSF + hCG vs D30 CSF). These comparisons were added in the Materials and methods section.

> *Results: In the discussion, it is mentioned that IGF1 may be a missing factor in the media that could help Leydig cell differentiation. Have the authors tried this experiment? Improving this existing culturing method will be highly valuable.*

The decreased Igf1 mRNA levels found in the present study are in line with the RNA-seq data of Yao et al., 2017. As mentioned in the Discussion section, the addition of IGF1 in the culture medium led to a modest increase in the percentages of round and elongated spermatids in cultured mouse testicular fragments (Yao et al., 2017). However, the effect of IGF1 supplementation on Leydig cell differentiation was not investigated. The supplementation of organotypic culture medium with IGF1 is currently being tested in our research team.

> *Add p-values and SEM for qPCR data. This was done for hormones, should be the same way for other results.*

p-values and SEM are shown for both qPCR and hormone data.

> *Regarding all RT-qPCR data-There is a switch between 3bHSD and Actb/Gapdh as housekeeping genes. There does not seem to be as some have 3bHSD and others do not. Why do Igf1 and Dhh not use 3bHSD for housekeeping? If this is the method to be used, then 3bHSD should be used as housekeeping for the protein data, instead of ACTB. Also, based on Figure 1B and Figure 2A (Hsd3b1) there does not seem to be a strong*

> *correlation between Leydig cell # and the gene expression of Hsd3b1. If Hsd3b1 is to be used as a housekeeper and a proxy for Leydig cell number a correlation between these two measurements is necessary. If there is no correlation a housekeeping gene that is stable among all samples should be used. Sorting Leydig cells and then conducting qPCR would be optimal for these experiments.*

Hsd3b1 was used as a housekeeping gene only to normalize the mRNA levels of Leydig cell-specific genes. Therefore, Igf1 and Dhh transcript levels were not normalized with Hsd3b1 since Igf1 is expressed by several cell types in the testis (Leydig cells, Sertoli cells, peritubular myoid cells) and Dhh is expressed by Sertoli cells.

Regarding western blots, the expression of AR, CYP19 and FAAH could not be normalized with 3□-HSD since AR is expressed by Leydig cells, Sertoli cells and peritubular myoid cells, CYP19 is expressed by Leydig cells and germ cells and FAAH is expressed by Sertoli cells. For CYP17A1 however, 3B-HSD was used as housekeeping instead of ACTB (Figure 2G).

No correlation was found between the number of Leydig cells per cm2 of testicular tissue shown in Figure 1 and Hsd3b1 mRNA levels presented in Figure 2. However, this result was expected since on the one hand the number of Leydig cells per cm2 was determined in the peripheral region of one tissue section whereas on the other hand Hsd3b1 transcript levels were measured in the entire peripheral region of the cultured fragments. The correction factor used for the analysis of genes expressed in Leydig cells present in the healthy part of the cultured tissues was therefore the Leydig cell selective marker Hsd3b1, as previously described (Cacciola et al., 2013).

> *Figure 2A (CYP17a1): It is surprising that the CYP17a1 gene and protein expression is very different between D30FT and 36.5dpp, however, the immunostaining looks identical between all groups. Why is this? A lower magnification image of the testis might make it easier to see the differences in Cyp17a1 expression. Leydig cells commonly have autofluorescence and need a background quencher (TrueBlack) to visualize the true signal in Leydig cells. This might reveal the true differences in Cyp17a1.*

RT-qPCR and western blot analyses show that both Cyp17a1 mRNA levels and CYP17A1 protein levels are decreased in organotypic cultures at D30. However, we agree that such a decrease is not visible in immunostaining. No autofluorescence of Leydig cells could be observed in the negative controls (Figure 2J).

> *Figure 3D: there are large differences in estradiol concentration in the testis. Could it be that the testis is becoming more female-like? Leydig and Sertoli cells with more granulosa and theca cell features? Were any female markers investigated?*

We show in the present study that the expression levels of the Sertoli cell-specific gene Dhh are not reduced in organotypic cultures. We also previously found that the expression levels of the Sertoli cell-specific gene Amh were not reduced in in vitro matured testicular tissues (Rondanino et al., 2017). Moreover, we have recently shown that Sox9, encoding a testis-specific transcription factor, is expressed in organotypic cultures (Dumont et al., 2023). Our recent transcriptomic analysis also revealed that the transcript levels of the pro-male sexual differentiation marker Sry and of the Sertoli cell-specific gene Dmrt1 remained unchanged in organotypic cultures compared to in vivo controls (Dumont et al., 2023). In addition, no increase in the mRNA levels of the female sex-determining genes Foxl2 and Rspo1 was found in vitro (Dumont et al., 2023). However, we cannot rule out that in vitro cultured testes are becoming more female-like as the expression of Hsd17b3, encoding an androgenic enzyme, is reduced (this study) while the expression of the feminizing gene Wnt4 is upregulated (Dumont et al., 2023).

> *Figure 3D and Figure 5A: It is hard to imagine that intratesticular estradiol is maintained for 16-30 days without sufficient CYP19 activity or substrate (testosterone). 6.5 dpp was the last day with abundant CYP19 expression, so is most of the estrogen synthesized on this first day and it sticks around? Are there differences in estradiol metabolizing enzymes? Is there an alternative mechanism for E production?*

In the present study, abundant CYP19 expression was indeed found at 6 dpp. However, the expression of this enzyme was not measured between 6 dpp and D16. Therefore, we cannot be sure that 6 dpp is the last day with abundant CYP19 expression. We assume that the estradiol synthesized before D16 may then accumulate within the cultured tissues. In our study, we quantified the transcript levels of Sult1e1, encoding an estradiol metabolizing enzyme. SULT1E1 is thought to play a physiological role in protecting Leydig cells from estrogen-induced biochemical lesions (Tong et al., 2004). A reduction in Sult1e1 mRNA levels was found at D30 in comparison to in vivo controls, but this may occur earlier during organotypic culture. In addition, decreased transcript levels of Hsd17b2, which encodes an estrogen metabolizing enzyme that converts estradiol to estrone, were found at D30 in this study. We suggest in the Discussion section that elevated estradiol levels in cultured tissues could be a consequence of low Sult1e1 and Hsd17b2 expression. Our recent transcriptomic analyses show that the levels of Cyp1a1, Cyp1b1 and Comt, encoding other estrogen metabolizing enzymes, are unchanged in organotypic cultures (Dumont et al., 2023). To our knowledge, there is no alternative mechanism for estradiol production.

> ***Recommendations For The Authors:***
>
> *1. The acronyms, PLC, SLC, ILC, ALC, and FLC, become hard to follow. It is recommended to spell out the names.*

PLC was replaced by progenitor Leydig cells, SLC by stem Leydig cells, ILC by immature Leydig cells, ALC by adult Leydig cells and FLC by fetal Leydig cells in the entire manuscript.

> *1. All Figures: Use letters for each bar graph. Difficult to make a connection from text to figure.*

A letter was added to each bar graph.

> *1. Supplemental figure 1: Change "Changement du milieu" to English.*

These words were replaced by “Medium change”.

> *1. Catalog numbers for antibodies are necessary.*

The catalog numbers of the antibodies used in this study are presented in Supplementary Table 1.