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Reproduction

An *in vitro* prototype of a porcine biomimetic testis-like cell culture system: a novel tool for the study of reassembled Sertoli and Leydig cells

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At present, there is no reliable *in vitro* assembled prepubertal testis-like biomimetic organ culture system designed to assess the functional effects of human gonadotropins on Sertoli and Leydig cells. Spermatogenesis is regulated by endocrine, paracrine, and juxtacrine factors (testicular cross-talk), mainly orchestrated by gonadotropins such as luteinizing hormone (LH) and follicle-stimulating hormone (FSH) that play a pivotal role by stimulating Leydig and Sertoli cells, respectively. The aim of our study was to set up an *in vitro* prepubertal porcine bioengineered construct as a new model for experimental studies on reassembled Sertoli and Leydig cells. We have evaluated Sertoli and Leydig cells obtained from 15- to 20-day-old neonatal pig testes in terms of purity and function. Subsequently, purified Sertoli and enriched Leydig cells were subjected to coincubation to obtain an *in vitro* prepubertal porcine testis-like culture system. We performed enzyme-linked immunosorbent assay (ELISA) for anti-Müllerian hormone (AMH), inhibin B, and testosterone secretion in the medium, and Real-Time PCR analysis of AMH, inhibin B, FSH-r, aromatase, LHr, and 3β-HSD mRNA expression levels. This *in vitro* testis-like system was highly responsive to the effects of human gonadotropins and testosterone. AMH mRNA expression and secretion declined, and inhibin-B increased, while FSH-receptor expression was downregulated upon FSH/LH exposure/treatment. Finally, the production of testosterone was increased selectively upon LH treatment. In summary, our proposed model could help to better determine the action of human gonadotropins on Sertoli and Leydig cells. The potential usefulness of the system for shedding light into male infertility-related issues is evident.
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Keywords: human gonadotropins; Leydig cells; prepubertal biomimetic testis; Sertoli cells

INTRODUCTION

At present, there is no *in vitro* assembled prepubertal testis-like biomimetic organ culture system designed to assess the functional effects of human gonadotropins on Sertoli cells (SC) or Leydig cells (LC). Access to such a model would help develop new approaches for the therapy of male infertility. Infertility is a social problem that involves many couples. In over 30% of cases, it involves a male factor related to altered human sperm function, with special regard to declines in sperm motility and numbers.[1] According to an estimate by the World Health Organization, infertility affects about 15% of couples of childbearing age in industrialized countries. Since a male factor appears in about 30% of the cases, and in 20% both male and female factors involved, cumulatively about 50% of cases of infertility include the male partner. The etiology of impaired sperm production and function may relate to factors acting at pretesticular, posttesticular, or testicular levels. Primary testicular failure accounts for about 50%–70% of all cases of male factor-related infertility. Despite progress, the etiology of male infertility is still unknown in about 50% of the cases, defined as idiopathic infertility.[2] Male infertility is definitely a social disease, with a very high cost of different treatments, including the use of techniques for medically assisted procreation (MAP).

Spermatogenesis is regulated by endocrine and paracrine factors, and juxtacrine testicular cross-talk, mainly orchestrated by gonadotropins such as luteinizing hormone (LH) and follicle-stimulating hormone (FSH), which play pivotal roles by stimulating LC and SC, respectively.[3] SC are the principal actors in spermatogenesis; in fact, they provide for nourishment, structural and functional support to germ cells, and protect them from the host's immune system by means of both the SC-based blood-testis barrier (BTB) and by the production of immunomodulatory factors.[4,5] The contribution of SC to spermatogenesis lies in the production of critical factors necessary for the successful development of spermatogonia, throughout the stage of mature spermatozoa. FSH, whose receptor (FSH-r) is located exclusively on SC, is the principal regulator of SC function

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and modulates testosterone (T) production, through SC factors with the synergistic contribution of LC to attain correct spermatogenesis. In fact, there is clear evidence that both FSH and T are essential for adequate spermatogenesis.[6] In particular, FSH is critical in the early stages (alone and in cooperation with testosterone) and testosterone is essential for the final stages of spermatogenesis, involving the production of elongated spermatids, considered the main limiting factors for *in vitro* spermatogenesis.[7–11] Thus, Dimitriadis *et al.*[6] emphasized this concept in a recent study.

The prepubertal stage in male mammals is associated with the physiological condition of hypogonadotropic hypogonadism, except for the production of anti-Müllerian hormone (AMH) by SC. In fact, at this stage when most of the damage leading to adult male factor infertility begins, SC are the only active testicular cells, as demonstrated by the production of AMH, which remains high for the entire prepubertal period. Therefore, AMH might represent a potential marker of SC function in prepubertal animals, even if its specific role in spermatogenesis remains unclear.[12]

The aim of this study was to establish an *in vitro* prepubertal porcine bioengineered cell culture system as a new model for experimental studies on reassembled SC and LC.

MATERIALS AND METHODS

Ethics statement

This study was conducted in strict compliance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and Perugia University Animal Care.

Sertoli cell isolation, culture, characterization, and function

SC, obtained from neonatal prepubertal Large White pigs, 7–15 days of age, were isolated according to established methods, modified in our laboratory.[13] Briefly, after removing the fibrous capsule, the testes were finely chopped and digested twice enzymatically, with a mixed solution of trypsin and deoxyribonuclease I (DNase I) in Hanks' balanced salt solution (HBSS; Merck KGaA, Darmstadt, Germany) and collagenase P (Roche Diagnostics S.p.A., Monza, Italy). The tissue pellet was centrifuged passed through a 500-µm pore stainless steel mesh, and then resuspended in glycine to eliminate residual LC as well as peritubular cells.[14] The resulting isolate was collected and maintained in HAM's F-12 medium (Euroclone, Milano, Italy), supplemented with 0.166 nmol l^{-1} retinoic acid, (Sigma-Aldrich, Darmstadt, Germany) and 5 ml per 500 ml insulin-transferrin-selenium (ITS, Becton Dickinson cat. no. 354352; Franklin Lakes, NJ, USA) in 95% air/5% CO_2 at 37°C. After 3 days in culture, SC were characterized by immunofluorescence (IF) and flow cytometry for AMH (a prepubertal SC marker), 3β-hydroxysteroid dehydrogenase (3β-HSD, an LC marker), alpha-smooth muscle actin (ASMA, a peritubular cell marker),[13,15] and protein gene product 9.5 (PGP9.5, a gonocyte and spermatogonial marker).[16]

These techniques clearly showed that the purity of our isolated SC preparations was very high, as indicated by the percentage of AMH (97.4% ± 2.4%), with negligible contamination by LC (3β-HSD 1.5% ± 0.2%), peritubular (ASMA 1.0% ± 0.5%), gonocyte, and spermatogonial cells (PGP9.5 0.5% ± 0.1%).[13,15,16]

We also tested for T secretion (a specific LC hormone) to confirm the negligible LC contamination of the final preparation, both basally and after 72 h of stimulation, with LH (Luveris, Merck Serono, Germany) by radioimmunoassay (RIA; testosterone kit IM 1119; Beckman Coulter Webster, TX, USA; intra-assay coefficient of variation, CV ≤8.6%; inter-assay CV ≤11.9%).[15]

Finally, we assessed the functional competence of SC monolayers, in terms of AMH and inhibin B secretion, both basally and after 72 h of stimulation with FSH and/or 0.2 mg ml^{-1} of T (Sigma-Aldrich). AMH and inhibin B were determined by ELISA (inhibin B ELISA kit, Gen II, Beckman Coulter Webster, intra-assay CV = 2.81%, inter-assay CV = 4.33%; and AMH Gen II ELISA kit, Beckman Coulter Webster, intra-assay CV = 3.89%, inter-assay CV = 5.77%, respectively).[15]

Leydig cell isolation, culture, characterization, and function

LC were also obtained from neonatal prepubertal Large White pigs, 15–20 days of age, and isolated according to established methods modified in our laboratory.[17] Briefly, after removal of the fibrous capsule, the testes were finely minced to obtain fragments of 1–2 mm in diameter. Thereafter, these testes underwent stepwise enzymatic digestion in HBSS (Sigma-Aldrich) containing 2 mg ml^{-1} collagenase P (Roche Diagnostics). The dispersed cells were filtered sequentially through 400-, 100-, and 38-µm stainless steel meshes. The filtered cell suspensions were then purified by centrifugation on discontinuous Percoll gradients (5%, 30%, 58%, and 70% Percoll in Ham's/F-12) at 800 ×*g* for 20 min. The cells migrated to form a band between the 30% and 58% Percoll phases. They were collected, washed, and plated in transparent polyethylene terephthalate (PET) membrane inserts for 6-well plates (Corning Incorporate, Corning, NY, USA). The cell preparation was finally cultured in a controlled humidified atmosphere of 95% air/5% CO_2 at 34°C in DMEM/F-12 medium, supplemented with 5 µg ml^{-1} transferrin, 10 µg ml^{-1} Vitamin E, 5 µg ml^{-1} insulin, 15 mmol l^{-1} HEPES, 0.1% fetal bovine serum, 0.1% bovine serum albumin (BSA), and 1% penicillin/streptomycin. Thereafter, LC were characterized by IF and flow cytometry analysis for 3β-HSD, an LC marker, according to reported methods with minor changes.[13,15]

Finally, we assessed the functional competence of LC monolayers, in terms of T production, both basally and upon 72 h of stimulation with LH (Luveris, Merck Serono, Germany) by RIA (testosterone kit; IM 1119; Beckman Coulter Webster, intra-assay CV ≤8.6%, interassay CV ≤11.9%).[15]

Co-culture and treatment

When LC monolayers were confluent after 3 days of culture, the inserts were transferred into multiwell plates containing SC monolayers in HAM's F-12 medium (**Supplementary Figure 1**).

The cell cultures underwent the following treatments: (1) LC alone treated for 72 h with LH (2.5 ng ml^{-1}); (2) SC alone treated for 72 h with: FSH (1000 ng ml^{-1}) and/or T (0.2 mg ml^{-1}) and; (3) SC cocultured with LC treated for 72 h with FSH or LH (as above) and their combination. Co-cultures were maintained in humidified atmosphere of 95% air/5% CO_2 at 34°C.

Real-time PCR analysis

Analyses for AMH, inhibin B, FSH-r, aromatase, 3β-HSD, and LHr employed the primers listed in **Supplementary Table 1**. Briefly, total RNA was extracted from samples obtained in the three experimental groups, using Trizol reagent (Sigma-Aldrich, Milan, Italy) and quantified by reading the optical density at 260 nm. In particular, 2.5 µg of total RNA was subjected to reverse transcription (RT, Thermo Scientific, Waltham, MA, USA) to a final volume of 20 µl. The qPCR was performed using 25 ng of the cDNA prepared by RT and a SYBR Green Master Mix (Stratagene, Amsterdam, The Netherlands). This was performed in an Mx3000P cycler (Stratagene), using FAM for detection and ROX as the reference dye. The mRNA level of each sample was normalized against β-actin mRNA and expressed as fold changes versus the levels in SC + LC cocultures.

Immunofluorescence (IF) staining

To detect the presence of AMH, 3β-HSD, ASMA, and PGP9.5, immunostaining was performed according to reported methods with minor changes.[13,15,16] Briefly, SC monolayers were grown on glass chamber slides (LabTek II, Nunc; Thermo Fisher, Rochester, NY, USA) and fixed in 4% paraformaldehyde in phosphate-buffered saline (PFA-PBS) for 30 min. The fixed cells were then subjected to permeabilization (PBS with 0.2% Triton X-100) for 10 min at room temperature and blocked with 0.5% BSA (Sigma-Aldrich) in PBS for 1 h prior to exposure to polyclonal goat anti-AMH (C-20; Santa Cruz Biotechnology, Dallas, TX, USA; sc6886, 1:100), polyclonal rabbit anti-3β-HSD (Santa Cruz Biotechnology, sc-30821; 1:200), polyclonal rabbit anti-ASMA (Abcam, Cambridge, MA, USA; ab5694; 1:200), or monoclonal rabbit anti-PGP9.5 (Abcam, ab108986; 1:200) antibody overnight at 4°C. The cells were then washed in PBS three times for 5 min and then exposed to a secondary Alexa 488-conjugated donkey anti-goat antibody (Molecular Probes, Grand Island, NY, USA, 1:500) and an Alexa 488-conjugated donkey anti-rabbit antibody (Molecular Probes, 1:500). Thereafter, the cells were treated with RNAse (10 mg ml^{-1}; Sigma-Aldrich) and counterstained for 1 min with DAPI (Sigma-Aldrich). Negative controls omitted the primary antibody treatment. Cells were mounted on slides with ProLong Gold anti-fade reagent (Molecular Probes).

The percentages of AMH-, 3β-HSD-, ASMA-, and PGP9.5-positive cells were determined by IF using an epifluorescence microscope (BX-41; Olympus) equipped with a digital camera (F-viewer, Olympus). IF images were processed with Cell F imaging software (Olympus), and ten different preparations containing at least 500 cells were counted.

Flow cytometry

Flow cytometry analysis was performed according to reported methods.[13,15,16] Briefly SC monolayers were harvested, centrifuged (400 ×*g* for 5 min) to form a cell pellet of approximately 1 × 10^6 cells, and the supernatant was removed. The cells were fixed in 4% PFA-PBS for 30 min, and after washing in flow cytometry buffer (PBS with 3% BSA), the cells were treated with 0.1% Triton X-100 in the same buffer for 10 min. After centrifugation (400 ×*g* for 5 min), the supernatant was removed, and the cells were blocked with 5% BSA in the same buffer for 1 h at room temperature before incubation with primary antibodies (to AMH, 3β-HSD, ASMA, or PGP9.5, 1 μl antibody per 0.5 × 10^6 – 1.0 × 10^6 cells, or buffer alone) for 1 h at room temperature. The cells were washed twice with 2 ml of flow cytometry analysis buffer per tube, pelleted by centrifugation (400 ×*g* for 5 min), and the supernatant was removed. Finally, cells were exposed to a secondary Alexa 488-conjugated donkey anti-goat antibody (1:500) and Alexa 488-conjugated donkey anti-rabbit antibody (1:500) and suspended in 0.5 ml flow cytometry analysis buffer. Then, the cells were centrifuged (400 ×*g* for 5 min), the supernatant was removed, and the pellet was suspended again in 0.5 ml flow cytometry analysis buffer with 1% PFA for analysis.

Data acquisition was performed on 1.0 × 10^4 events per tube based on a total (ungated) count of forward and side light scatter at ~200–300 events per second on a Becton Dickinson flow cytometer and analyzed using Diva software (both from Becton Dickinson Biosciences, Franklin Lakes, NJ, USA).

Statistical analysis

Data were analyzed for statistical significance by one-way analysis of variance (ANOVA) followed by a Tukey's honestly significant difference (HSD) pairwise comparison for at least three replicates. The Benjamini–Hochberg false discovery rate was used to correct for multiple testing, and an asymptotic *P* value computation was used to calculate *P* values. Data were expressed as the mean ± standard deviation (s.d.).

RESULTS

Sertoli cell functional competence

In validating the purity of SC monolayers, T was never detected either basally (0.750 ± 0.022 ng per 10^6 cells) or upon LH stimulation (0.432 ± 0.000 ng per 10^6 cells). In addition, the statistically significant downregulation of AMH (50.170 ± 2.169 ng per 10^6 cells *vs* 105.341 ± 1.607 ng per 10^6 cells after FSH; 49.323 ± 3.756 ng per 10^6 cells *vs* 105.341 ± 1.607 ng per 10^6 cells after FSH and T stimulation) and upregulation of inhibin B (5053.409 ± 421.049 ng per 10^6 cells *vs* 3725.000 ± 81.960 ng per 10^6 cells after FSH; 5630.729 ± 252.643 ng per 10^6 cells *vs* 3725.000 ± 81.960 ng per 10^6 cells after FSH and T stimulation) clearly demonstrated the physiological competence of the isolated SC (**Figure 1a–1c**).

Leydig cell characterization and function

The percentage of 3β-HSD-positive cells in LC monolayers was about 60% ± 3.3%, as determined by IF, with SC at 32% ± 0.7% and in peritubular cells at 8% ± 0.8% (**Figure 2a–2c**). These results were confirmed by flow cytometry with the percentage of 3β-HSD-positive cells at 66% ± 2.2%, of SC at 29% ± 0.5%, and of peritubular cells at 6% ± 1.4% (**Figure 2d–2f**), setting the final values of 3β-HSD-positive cells at 63% ± 2.7%, of SC at 30% ± 0.6%, and of peritubular cells at 7% ± 1.1%, as the mean value between IF and flow cytometry results (**Supplementary Table 2**). Testosterone production upon LH treatment was consistent with prepubertal LC function (**Figure 3**).

Inhibin B and AMH secretion assays

We have demonstrated that, in this *in vitro* construct, composed of functional SC and enriched for functional LC, inhibin B was significantly increased after exposure to FSH alone with no changes under LH stimulation alone, with significant reduction after exposure to FSH and LH, compared with FSH alone (**Figure 4a**). Meanwhile, AMH secretion was significantly downregulated by FSH and LH treatments (alone and in combination; **Figure 4b**).

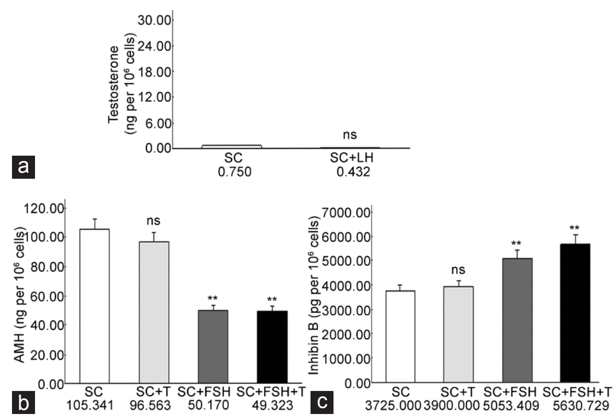

Figure 1: Testosterone secretion and SC functional competence. (**a**) Testosterone levels were measured in culture medium of untreated and treated SC. Functional activity of cultured SC was evaluated by measuring (**b**) AMH and (**c**) inhibin B secretion in culture medium of untreated and treated SC. Data represent the mean of three experiments ± s.d. ***P* < 0.001, treated SC versus untreated SC; ns: not significant, treated SC versus untreated SC. SC: Sertoli cells; s.d.: standard deviation; AMH: anti-Müllerian hormone.

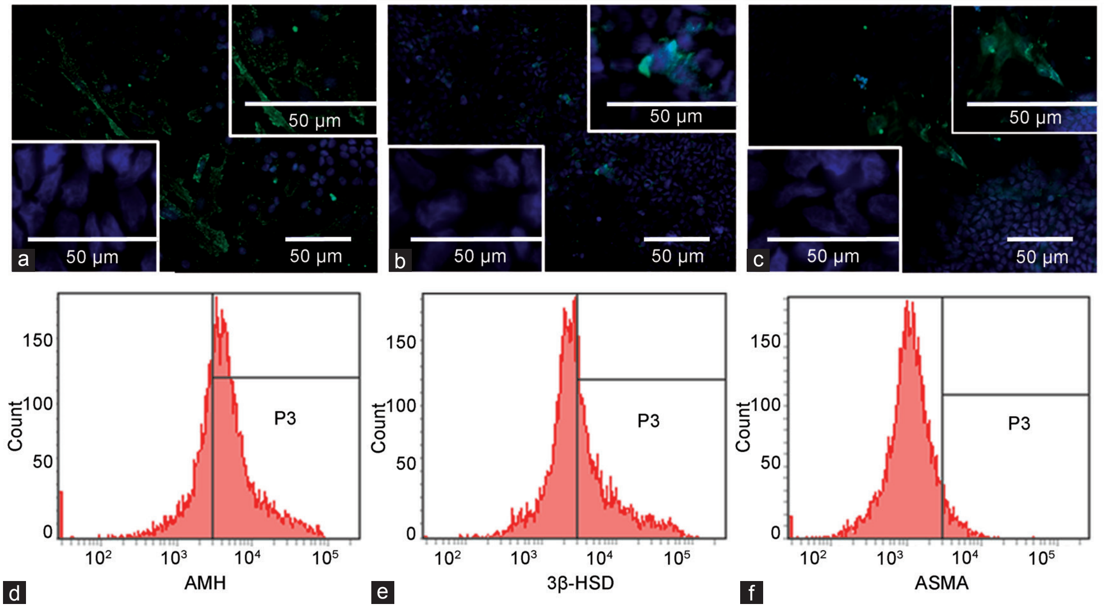

Figure 2: Morphological characterization and flow cytometry analysis. (**a**) Morphological characterization by fluorescence microscopy: LC after immunostaining with AMH antibody and visualized by anti-goat Alexa Fluor 488 (green). (**b**) LC after immunostaining with 3β-HSD antibody and visualized by anti-goat Alexa Fluor 488 (green). (**c**) LC after immunostaining with ASMA antibody and visualized by anti-rabbit Alexa Fluor 488 (green). Nuclei are counterstained with DAPI (blue). In each figure, the magnification insert is showed in the upper right and the negative controls' insert is displayed in the bottom left. Flow cytometry analysis of cultured LC for (**d**) AMH, (**e**) 3β-HSD, and (**f**) ASMA. The images are representative of three separate experiments. LC: Leydig cells; AMH: anti-Müllerian hormone; ASMA: alpha-smooth muscle actin; 3β-HSD: 3β-hydroxysteroid dehydrogenase.

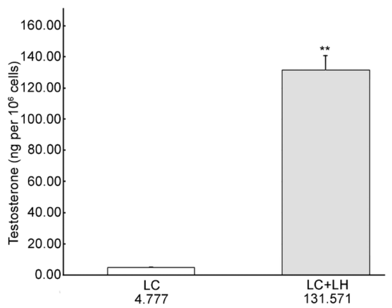

Figure 3: Function of cultured LC. Testosterone secretion measured in the culture medium of untreated and treated LC. Data represent the mean of three experiments ± s.d. $^{**}P < 0.001$, treated LC versus untreated LC. s.d.: standard deviation; LC: Leydig cells.

Real-time PCR

Consistent with the results of the inhibin B secretion assays, expression of inhibin B was significantly increased after FSH treatment (alone and in combination with LH), with a greater increase after FSH alone. AMH expression was significantly downregulated by FSH alone and in combination with LH treatment (**Figure 5a** and **5b**). In addition, we observed a statistically significant reduction of FSH-r upon FSH treatment (alone and in combination with LH). Furthermore, we showed a significant increase of aromatase expression upon FSH and LH treatment (alone and in combination), being stronger when both gonadotropins were present (**Figure 5c** and **5d**). Finally, we found a statistically significant increase of 3β-HSD, upon LH exposure/treatment (alone and in combination with FSH) and a statistically significant reduction of LHr upon LH exposure/treatment (alone and in combination with FSH) (**Figure 5e** and **5f**).

Testosterone secretion assay

We demonstrated that T production by our *in vitro* system was significantly upregulated upon LH treatment (**Figure 6**).

DISCUSSION

Here, we focused on setting up a prepubertal porcine bioengineered *in vitro* cell culture system, as a new model for experimental studies on reassembled SC and LC, both to study paracrine intratesticular interactions and ultimately to generate a bioengineered system for *in vitro* spermatogenesis. In the last few years, the survival rates of children affected by cancer have reached 80%.[18] For this reason, there is an urgent need to preserve fertility in prepubertal boys who do not yet produce spermatozoa because chemo- and radio-therapeutic agents exert significant deleterious effects on the gonads of children treated for cancers.[19,20] Even if some reports claim that an *in vitro* transition of germ cells into haploid cells is possible, the progression throughout meiosis and formation of spermatids to induce spermatogenesis are very difficult to achieve *in vitro*.[21,22] In particular, spermiogenesis *in vitro* - the final differentiation of spermatids into functionally mature gametes, has never been clearly demonstrated with efficiency and reproducibility in humans. In fact, the only available reports include fresh and cryopreserved immature testicular tissue of nonhuman models.[21,22] Obtaining *in vitro* spermatogenesis might depend on partial knowledge of the prepubertal spermatogonial stem cell (SSC) niche, but there have been poor results with different culture systems, such as coculture with Vero cells,[23,24] isolated cell culture with growth factor supplementation,[25] three-dimensional culture,[26] and organotypic cultures.[27] In fact, Vigier *et al.*[28] consider that, without a SC "feeder" system, no *in vitro* system to induce spermatogenesis can function.

SC mainly support spermatogenesis under the regulation of FSH and T. FSH, whose receptor is located exclusively on SC, is the main hormonal regulator of SC function and also modulates androgen production by LC, contributing directly and indirectly to spermatogenesis.

The predominant role of SC in exocrine testicular function is emphasized by three facts: (1) germ cell-only testes have never been reported; (2) SC are required in the vast majority of *in vitro* culture systems

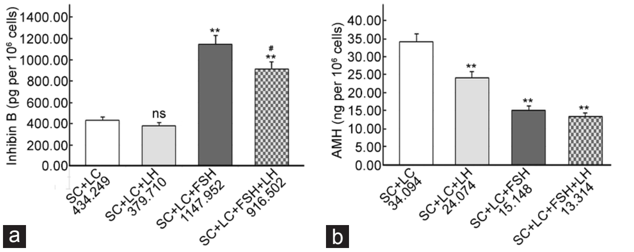

Figure 4: Functional competence of *in vitro* prepubertal porcine system. (**a**) Inhibin B and (**b**) AMH secretion upon 72 h of FSH and LH treatment. Data represent the mean of three experiments ± s.d. $^{**}P < 0.001$, treated SC + LC versus untreated SC + LC; $^{\#}P < 0.05$, SC + LC + FSH + LH versus SC + LC + FSH; ns: not significant, SC + LC + LH versus SC + LC. AMH: anti-Müllerian hormone; FSH: follicle-stimulating hormone; s.d.: standard deviation; LH: luteinizing hormone; SC: Sertoli cells; LC: Leydig cells.

for germ cell differentiation; and (3) the number of SC is directly related to the population of germ cells sustained by the testes.[29] However, increasing evidence indicates that, apart from gonadotropins and other hormones, cell-to-cell interactions are important in the control of testicular function. Increasing numbers of cytokines and growth factors have been implicated in interactions between cells in the testis where they are produced by different cell types at different phases of testicular development.[30] Unfortunately, the physiological roles of many of these factors in the testis are still unknown. In particular, LC produce a number of molecules with putative or demonstrated paracrine activity. Some of them exert inhibitory or stimulatory effects on tubular function. In particular, T is an important paracrine factor in the testis and one of the few hormones clearly demonstrated to act as a local regulator of spermatogenesis in animals and humans. In fact, androgen receptors (ARs), localized in testicular somatic cells such as SC and peritubular cells, play important roles in the regulation of testosterone levels. By contrast, mature germ cells do not seem to require functional ARs.[7] This indicates that androgens also affect spermatogenesis indirectly through SC, as these cells interact directly with developing germ cells.[7] In particular, SC in combination with LC-derived growth factors support the early levels of spermatogenesis (e.g., spermatogonial proliferation). In addition, the high concentrations of intra-testicular T secreted from LC (ranging from 100- to 1000-fold higher than in the systemic circulation) have pivotal roles during spermatogenesis,[3] and in particular during spermiogenesis for the progression from round-to-elongating spermatids.[7,11]

We have successfully isolated pure and functional prepubertal SC preparations (preferred to adult cells because these are very difficult, if not impossible, to isolate) reinforced in an *in vitro* construct by functional-enriched LC. The ability to obtain functional SC and LC cultures was demonstrated by the secretion of AMH and inhibin B after FSH stimulation and T secretion after LH treatment, respectively.

Our results demonstrate that the *in vitro* construction of a biomimetic testis-like organ is feasible, with a system that is very responsive to gonadotropins. This is shown by the increase of inhibin B (mRNA expression and secretion) after FSH exposure, with no changes under LH stimulation alone, supported by the unique contributions of SC. When FSH was used in combination with LH in agreement with previously demonstrated data in individuals with congenital hypogonadotropic hypogonadism, where LH-stimulated testicular androgens outweighed the stimulatory effect of FSH on SC,[31] we found a significant reduction in inhibin B secretion compared with FSH alone. A plausible explanation for the inhibitory action of LH on testicular inhibin B secretion is that this is indirect and mediated by the paracrine action of increased T production from the LC.[32]

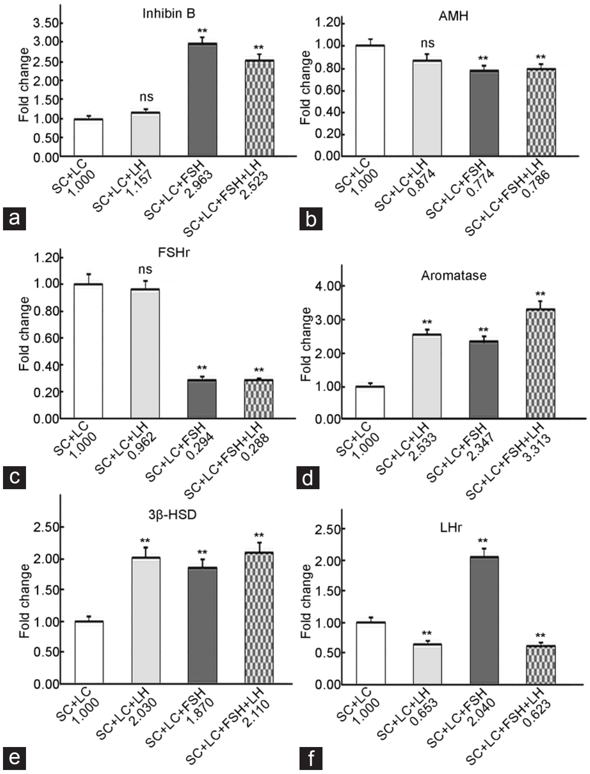

Figure 5: Real-time PCR analysis of (**a**) inhibin B, (**b**) AMH, (**c**) FSH-r, (**d**) aromatase, (**e**) 3β-HSD, and (**f**) LHr gene expression upon 72 h of FSH and LH treatment. Data represent the mean of three experiments ± s.d. $^{**}P < 0.001$, treated SC + LC versus untreated SC + LC; ns: not significant, treated SC + LC versus untreated SC + LC. PCR: polymerase chain reaction; AMH: anti-Müllerian hormone; FSH: follicle-stimulating hormone; s.d.: standard deviation; LH: luteinizing hormone; SC: Sertoli cells; LC: Leydig cells; 3β-HSD: 3β-hydroxysteroid dehydrogenase.

At the onset of puberty in male mammals, AMH serum levels start declining and continue to decrease throughout puberty, thanks to the negative effects of intra-testicular T via ARs. The inhibitory effect of androgens on AMH expression overrides the FSH-dependent stimulation in normal puberty.[33] Accordingly, in our model, we demonstrated reductions in AMH secretion and mRNA expression after FSH stimulation. Furthermore, as expected, we demonstrated a statistically significant reduction in FSH-r expression upon FSH treatment and a significant increase of aromatase expression upon combined FSH and LH treatment. Finally, we demonstrated significant up- and down-regulation of 3β-HSD and LHr or T, respectively, upon LH treatment.[34]

As to whether porcine cells might be able to support human cells *in vitro*, we have demonstrated that a monolayer feeder of our prepubertal porcine SC can preserve human sperm viability for up to 7 days.[35] This demonstrates that porcine SC molecules can cross-react with human cells. In addition, regarding the potential risk of xenozoonotic agents in xenotransplantation, our porcine testicular tissue is totally free from any zoonosis, according to the pathogen list tested on the New Zealand Auckland donor herd, the best colony in the world in terms of monitoring human safety.[36]

In conclusion, our proposed model, by creating an *in vitro* biomimetic testis-like organ, could represent both a new model for experimental studies on paracrine interactions between SC and LC to better understand endocrine issues so far unknown and a bioengineered system to induce spermatogenesis *in vitro*. Investigation of these complex cellular and

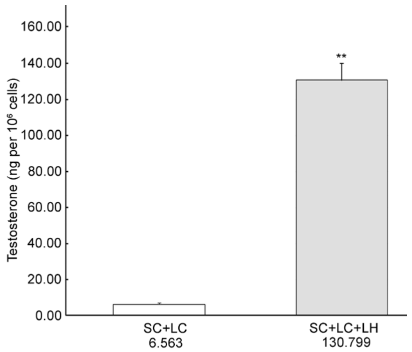

Figure 6: Testosterone secretion. Production of testosterone upon 72 h of LH treatment. Data represent the mean of three experiments ± s.d. $^{**}P < 0.001$, SC + LC + LH versus SC + LC. s.d.: standard deviation; LH: luteinizing hormone; SC: Sertoli cells; LC: Leydig cells.

molecular interactions could be very important to better understand the causes of spermatogenic dysfunction and represent a novel approach for the clinical interpretation and therapy of male infertility.

AUTHOR CONTRIBUTIONS

IA and GL carried out the isolation, coculture, and cell treatments and drafted the manuscript. CB, CL, and MC carried out the RT-qPCR analysis and ELISA assays. BCH, DM, and GG participated in the design of the study and performed the statistical analysis. FM and RC conceived of the study, and participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

COMPETING INTERESTS

All authors declare no competing interests.

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Supplementary information is linked to the online version of the paper on the *Asian Journal of Andrology* website

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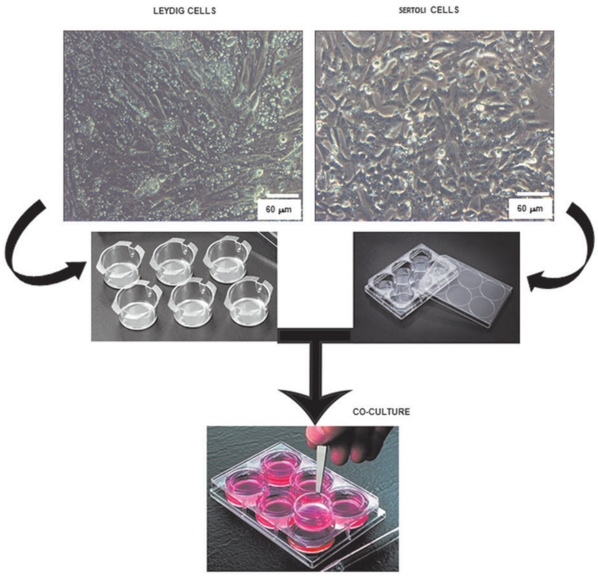

Supplementary Figure 1: Co-culture Leydig and Sertoli cells. Photomicrographs of Leydig and Sertoli culture by Nikon microscopy. After 3 days of "*in vitro*" culture, when LC monolayers were confluent, the inserts were transferred into the 6 multiwell plates containing SC monolayers in HAM's F-12 medium. SC: Sertoli cells; LC: Leydig cells.

Supplementary Table 1: Primer sequences for polymerase chain reaction analyses

Gene	*Forward sequences (5′–3′)*	*Reverse sequences (5′–3′)*
AMH	GCGAACTTAGCGTGGACCTG	CTTGGCAGTTGTTGGCTTGATATG
Inhibin B	CCGTGTGGAAGGATGAGG	TGGCTGGAGTGACTGGAT
Aromatase	CCGTCTGTGCCGATTCCATC	GAAGAGGTTGTTAGAGGTGTCCAG
FSHr	TGAGTATAGCAGCCACAGATGACC	TTTCACAGTCGCCCTCTTTCCC
3β-HSD	GAGAAGGCTGTGCTGGAG	ATGTGGGCAAAGATGAATGG
LHCGr	CGTGACTGTCCTCTTTGTTCTCC	CCAGCAACACTACACCCATTCC
β-actin	ATGGTGGGTATGGGTCAGAA	CTTCTCCATGTCGTCCCAGT

AMH: anti-Müllerian hormone; FSHr: follicle-stimulating hormone receptor; 3β-HSD: 3β-hidroxysteroid dehydrogenase; LHCGr: hormone/choriogonadotropin receptor

Supplementary Table 2: Mean value between immunofluorescence and flow cytometry analysis results of Leydig cells' monolayer

	3β-HSD	*AMH*	*ASMA*
IF	60±3.3	32±0.7	8±0.8
FCA	66±2.2	29±0.5	6±1.4
Average	63±2.7	30±0.6	7±1.1

IF: immunofluorescence; 3β-HSD: 3β-hidroxysteroid dehydrogenase; AMH: anti-Müllerian hormone; ASMA: alpha-smooth muscle actin; FCA: flow cytometry analysis