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An engineered 3D blood-testis barrier model for the assessment of reproductive toxicity potential

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ABSTRACT

We have developed an *in vitro* model that replicates the composition, organization, and barrier and spermatogenesis functions of the *in vivo* rat blood-testis barrier. This engineered blood-testis barrier (eBTB) is based on a three-dimensional (3-D) culture in a bicameral chamber of testicular cells isolated from 18-day-old rats. Peritubular cells were cultured on the bottom of the insert. On the top of the insert, a mixture of Sertoli and germ cells were coated within an artificial extracellular matrix, thereby mimicking the basement membrane. The matrix composition was defined to obtain a cord-like organization. This structure was revealed depending on morphogenetic gradients, and was made of polarized Sertoli cells and germ cells in the center of the structure. The *in vivo* functionality of the BTB was characterized by tight junctions between Sertoli cells. Claudin-11 protein immunodetection suggests that these junctions *in vitro* in the cord-like structure, suggesting the presence of a physical compartment with apical and basal spaces. Measurement of the trans-epithelial electrical resistance characterized the relationship between the Sertoli cells, peritubular cells, and matrix/cells that influenced the tightness of their junctions during the course of the culture. *In vitro* germ cell differentiation was confirmed with the detection of haploid cells. The development of the eBTB under optimum conditions addresses the involvement of new models, testing the barrier and spermatogenesis functions that are sensitive to chemical compounds from the environment. In this way, the eBTB could be used as an alternative method to animal reprotoxicity studies, and would be of high interest in the scope of regulatory requests for chemical risk assessment.

1. Introduction

In vivo, the blood-testis barrier (BTB) is a key part of the possible alteration of the male reproduction function by xenobiotics. In the testis, BTB formation occurs in 16–19-day-old rats [1] to obtain a physical and physiological barrier that ensures functions in hormonal regulation and spermatogenesis [2] with BTB formation occurring in 16–19 day-old rats [1]. BTB is a testis-specific structure composed of tight junctions, basal ectoplasmic specializations, basal tubulobulbar complexes, desmosome-like junctions, and gap junctions [3,4].

The delineation between the basal and apical spaces is defined according to the presence of tight junctions between Sertoli cells all around the seminiferous tubules. Claudin-11 is a specific protein of

these tight junctions, and its presence contributes to BTB functionality that is essential for the regulation function of the barrier by clearing substances through the germinal epithelium [5–8]. The BTB is composed of three parts. The first part is an interstitial compartment containing interstitial cells (Leydig cells and testicular macrophages) and endothelial cells. This compartment is the first actor in defending the testis against exogenous pathogens and environmental xenobiotics after these toxic substances are transported through the capillary endothelium. Then, in the seminiferous epithelium, the basal and the apical spaces are well delimited physically and form the other two compartments. The basal compartment is consistent with a tunica propria (peritubular cells, basement membrane), Sertoli cells, and two types of germ cells -spermatogonia and preleptotene spermatocytes- represent the first step in spermatogenesis. In a rat, the monolayer of peritubular cells surrounding the tubules forms a partial barrier because tight junctions between peritubular cells could be insufficient to prevent the passage of molecules as a lanthanum tracer [2]. After passage,

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meiotic and post-meiotic germ cells present in the apical compartment could be accessible and targeted by reprotoxic compounds.

Since 1960, several research efforts to create an artificial testis have been attempted based on culture systems of male germ cells under biochemical conditions close to the seminiferous tubular biochemical environment [9]. Recent studies have been devoted to the establishment of an ideal culture system in which spermatogonia stem cells could be maintained, and directed to proliferate and undergo meiosis and complete spermiogenesis. Many systems have been tested as organ cultures, cell cultures, or cocultures of Sertoli cells and germ cells. Two types of support could be used: impermeable (e.g. Petri dishes or flasks) or permeable support such as a bicameral chamber and/or gel matrix (e.g. collagen or Matrigel). Several works using bicameral chambers successfully demonstrated *in vitro* differentiation of germ cells into haploid cells [10–14]. Recent research has shown that a three-dimensional (3-D) *in vitro* culture system has good potential for *in vitro* differentiation of spermatogenic cells, and provides a 3-D environment for the testicular cells and their interactions [11]. Consistent with the works of Hadley et al. and Ito et al. [15,16], an extracellular matrix allows improvements in the architecture of Sertoli cells, in their proliferation, and in germ cell differentiation in the culture. These types of cultures allow us to understand the regulation of *in vitro* spermatogenesis and offer new possibilities for the treatment of male sterility; however, they are not useful for studying the function of physical-chemical BTB.

Few models are presented as an *in vitro* BTB model for toxic studies. Indeed, primary Sertoli cell cultures are used to study the effects of phorbol ester or cadmium on tightness junctions [17,18]. Cultures of Sertoli cell lines on impermeable supports have also improved our understanding of the effects of toxicants on junctional membrane proteins of seminiferous epithelium [19,20]. Another study was performed combining a bicameral chamber and 3-D culture with only Sertoli cells; the authors presented a dynamic model of BTB to study its integrity after exposure to bisphenol A [21]. However these models do not reflect exactly *in vivo* BTB which is composed of a 3-D structure with three compartments (interstitial, basal, and apical) and defined by many cell junctions, all contributing to barrier function and germ cell differentiation.

To date, no *in vitro* reprotoxicity test on the male reproduction function has allowed the demonstration of the toxic effects on both BTB and spermatogenesis. Only one type of testicular cell (Sertoli or Leydig cells) was tested, thereby limiting the interpretation of the observed toxic effects to *in vivo* extrapolation. Development of an *in vitro* engineered blood-testis barrier (eBTB) model appears to be the most effective alternative for reprotoxicity tests in males. Indeed, the major steps link to fertility adverse effects are the clearing of toxicants through BTB and effects on germ cell differentiation. Using this type of model, we could detect the effect of chemical compounds on both the barrier and on the spermatogenesis functions. The aim of this study is to reproduce the *in vivo* organization of rat testis seminiferous epithelium with a two-layered epithelium (i.e., two compartments). By using a model with a 3-D culture in a bicameral chamber, the apical and basal compartments have been set up with three types of testicular cells isolated from 18-day-old rat testis (peritubular cells, Sertoli cells, and germ cells). Different culture models were tested and compared for their morphology features. The most robust method was selected on the basis of its ability to demonstrate both the barrier and spermatogenesis functions. Thus, we set up the *in vitro* eBTB as closely as possible to the *in vivo* BTB.

2. Material and methods

2.1. Animals

Animals were provided by the Charles River Animal Center (L'Abresle, France) and kept in a controlled environment with free access to food and water. They were housed at the National Institute of Industrial Environment and Risk with a 12 h-to-12 h light-to-dark cycle at 22 °C with food and water *ad libitum*. All experiments were approved by the Animal Experimental Committee of the National Institute of Environment and Industrial Risks.

2.2. Cell preparation

Testicular cells were isolated from 18-day-old Sprague–Dawley male rats. At this age, the spermatogenesis cycle is not achieved; thus, the zygotene spermatocytes are the most differentiated germ cells that are present in tubules. However some early pachytene spermatocytes are likely to be present [23]. Animals were euthanized with a lethal dose of sodium pentobarbital (Ceva Santé Animale, France). After exsanguination, testes without albuginea were washed in phosphate buffer saline (PBS) (pH 7.2, Invitrogen, Cergy Pontoise, France) and disposed in Dulbecco's Modified Eagle Medium (DMEM)/F-12 culture medium (Invitrogen) with 1X antimycotic antibiotic (Invitrogen). This supplemented medium was used in all experiments. Cell preparation was adapted from Lee et al. [11]. Briefly, testes were decapsulated and minced into small pieces with a scalpel. Under sterile conditions, three washes in a culture medium were carried out, allowing sedimentation for 10 min. After each wash, the supernatant containing interstitial cells was discarded. The dissociation medium was composed of collagenase (2 mg/mL), soybean trypsin inhibitor (1 µg/mL), DNase (10 µg/mL), and hyaluronidase (1 mg/mL) in culture medium (Sigma–Aldrich, Saint–Quentin Fallavier, France). The small pieces of seminiferous tubules were digested for 30 min at 32 °C with rotating agitation (50 rpm/min) in a dissociation medium. After dissociation, tubules were transferred in 50 mL tubes for sedimentation for 5 min, and the supernatant was removed. After first centrifugation at 110 g (20 °C, 10 min), the pellet was constituted by Sertoli cells and germ cells, and the supernatant containing peritubular cells was isolated. The isolation experiments were repeated at least eight times, and we used an average of 20 testes (10 rats, 18 day post-natal) per experiment.

The purity of Sertoli and the germ cell population was assessed by staining an aliquot for the enzyme alkaline phosphatase and for the 3 beta-hydroxysteroid dehydrogenase, according to the Sigma–Aldrich procedures, because these enzyme were specifically expressed by peritubular cells or Leydig cells [22–24]. For the preparation of 3-D culture, the mix of cells was washed three times with culture medium and centrifuged at 400 g (20 °C, 10 min). The supernatant containing peritubular cells was centrifuged at 250 g (20 °C, 5 min). The cells were counted on a Z2 Coulter® particle counter and size analyzer (Beckman Coulter, Roissy, France), and the viability was determined with trypan blue dye exclusion before the cells were seeded in bicameral chambers (Falcon, Becton Dickinson, Le Pont de Claix, France).

2.3. Peritubular cell cultures

Purity of the peritubular cells was confirmed by phosphatase alkaline staining as previously described. First, peritubular cells were cultured in culture medium supplemented with 10% fetal bovine serum (Invitrogen) as reported in Tung et al. [25]. A culture flask was placed at 32 °C in a humidified chamber supplied with 5% CO₂. A minimum of one passage was carried out to purify the culture and obtain a sufficient quantity of cells.

Three days before the animal sacrifice for Sertoli and germ cell isolation, peritubular cells were ensemenced at a 1.10⁵ cell density/insert on the underside of the bicameral chamber as in the methodology of Ailenberg et al. [26]. The inserts were turned over for 5 h to permit total cell adhesion. Next, the inserts were reverted in the well and the culture medium containing 10% FBS was added to the apical and basal sides. The confluence of cell culture on the underside of the insert was always controlled before the preparation of the 3-D culture.

2.4. Preparation of 3-D culture

The Sertoli and germ cells were ensemenced at a high cell density of 1.10⁶ cells/insert in a culture medium supplemented with a 6.25 µg/mL insulin-transferrin-selenium solution (Becton Dickinson, Biosciences), 10⁻⁴ M vitamin C, 10 µg/mL vitamin E, 3.3.10⁻⁷ M retinol, 1 mM pyruvate, 100 mIU recombinant follicle stimulating hormone, and 10⁻⁷ M testosterone, all purchased from Sigma–Aldrich, and 5% fetal bovine serum. This culture medium allows germ cell differentiation [11]. The *in vitro* culture was kept for 22 days at 32 °C in a humidified chamber supplied with 5% CO₂ with slight agitation (45 rpm/min).

To generate a 3-D culture of testicular cells, SGC was grown within an artificial extracellular matrix (EM) made of a Matrigel matrix, a solubilized basement membrane preparation extracted from Engelbreth–Holm–Swarm mouse sarcoma (BD, Biosciences). The matrix preparations tested were composed of different final concentrations of Matrigel with or without a high concentration of collagen type I

(BD, Biosciences). The tested compositions and concentrations are shown in Table 1. Each type of EM was tested in triplicate.

Sertoli and germ cells were mixed with the extracellular matrix and plated on the top of the insert. The plates were incubated for 60 min at 32 °C to allow for gelification in the static state in a humidified chamber supplied with 5% CO₂. The culture media of the apical and basal compartments were replaced on the first day of the culture; then, at each 48 h interval, only the apical culture medium was replaced with supplemented medium culture, as previously described.

2.5. Live imaging and quantitative analysis

For live imaging of cultures in the EM, pictures were captured with a Leica DMIL phase contrast microscope (Leica, Melville, NY, USA) and a Qicam Fast 1394 camera (Qimaging, Canada). The spatial arrangement of the spherical cell aggregates was analyzed from day 3 of the culture as described below, and bright field micrographs were obtained with a Leica MZ12.5 stereomicroscope (Leica) or binocular magnifying glass (BMG). Five micrographs, each covering a culture area of 4 mm², were taken from each of the three culture wells (a total of 300 clusters) for every day studied (day 3, day 10, and day 22), and for each experiment.

2.6. Histology

The tested eBTBs (different matrix compositions) were fixed in tamponned formol 4% with 0.1 % Light Green (Sigma–Aldrich) for 4 h and preembedded in agarose gelling low temperature (Sigma–Aldrich). *In vivo* testis from an 18-day-old rat was fixed in Bouin's liquid for 24 h and then decapsulated. *In vitro* and *in vivo* tissues were dehydrated, embedded in paraffin, and cut into 5 μm-thick sections. The cut sections were disposed on Superfrost plus glass slides (Labonord, Templemars, France) and were immunostained by fluorescence.

Cut sections disposed on Superfrost glass slides (Labonord) were stained with periodic acid Schiff–Hematoxylin (PAS–H). A Sigma–Aldrich staining procedure was performed according to the manufacturer's instructions. The type IV collagen of the EM and the acrosome of the spermatid were stained in pink.

2.7. Transmission electron microscopy (TEM)

eBTBs were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer for 1 h 30 min at 4 °C. After washing, the samples were post-fixed with 1% osmium tetroxide in 0.1 M cacodylate for 1 h, serially dehydrated with different ethanol degrees, and placed in propylene oxide. Samples were included in Araldite (Sigma–Aldrich) and sectioned into 1 μm semi-thin sections. The sections were stained with 1% toluidine blue and examined under a light microscope. The areas of interest were finely sectioned with an ultramicrotome into 60 nm ultra-thin sections and attached to a copper grid (200 mesh). The samples were doubly-stained with 4% uranyl acetate and plumb citrate, and observed with a Philips CM10 electron microscope at 120 kV.

2.8. Trans-epithelial electrical resistance

A measurement of trans-epithelial electrical resistance (TEER) was performed with an Endohm 12 chamber (EVOM, World Precision Instruments, Florida, USA). The culture was equilibrated at room temperature 20 min before the measurement as described by Janecki et al. [27]. The blank was performed with Hank's buffered salt solution (Invitrogen), a different medium of germ cell differentiation culture medium. The other controls were: insert alone, insert with peritubular cells, insert with EM without cells, insert with EM and peritubular cells, and insert with EM, Sertoli, and germ cells but without peritubular cells. The TEER was calculated according to the following equation: $TEER = (R_{total} - R_{control})/A$ (Ohm/cm²), where R_{total} is the resistance measured, $R_{control}$ is the resistance of the considered control insert, and A is the surface area of the insert (i.e., 1.13 cm²). According to the control, the resistance difference was subtracted, and was considered to reflect the effect of one of the considered subtracted compounds (EM, peritubular cells, Sertoli cells, and germ cells). Functional TJs have been detected when TEER oversteps the threshold of 25 Ohm/cm² [27].

2.9. Cell staining

Lyses of matrix and cell dissociations were performed with dispase (10 U/cm², BD Bioscience) and collagenase (2.5 mg/mL, Sigma–Aldrich). After 2 h of incubation at 32 °C, the cells were centrifuged at 250 g for 5 min at 4 °C. The cells were

cytospined and were then ready for further analysis using May-Grünwald staining and immunodetection by fluorescence.

2.10. May-Grünwald staining

According to interne procedures, the cells were stained with May-Grünwald Giemsa (MGG) in order to identify testicular cells. On every day studied, sections were randomly selected and at least 500 cells were counted. Because of their identification with their cytoplasm characterized by basophily/acidophily and DNA condensation [28,29], a percentage of Sertoli cells, spermatogonia, meiosis cells (spermatocytes and metaphasis cells–2N cells), haploid cells (spermatids–1N cells), and unidentifed cells were evaluated for every replicate. A ratio of the number of Sertoli cell per germ cell was also determinate.

2.11. Immunofluorescence

The following primary antibodies were used in immunofluorescence staining: vimentin as usual Sertoli cell marker; claudin-11 as the tight junction of Sertoli cell marker [7,30]; and alpha smooth muscle actin (α SMA) as the peritubular cell marker [31]. To identify germ cells, the antibody against Chk2 as a spermatogonia marker [32] and against γ -H2AX as leptotene, zygotene, and pachytene spermatocytes marker were used [33–36]. A summary of primary antibodies is shown in Table 2. After incubation overnight at 4 °C, the testis sections, or cells fixed on a slide after cytospin, were incubated with the corresponding alexa fluor secondary antibody: Alexa Fluor[®] 488 goat anti-mouse antibody (A31619), Alexa Fluor[®] 594 goat anti-mouse antibody (A31623), Alexa Fluor[®] 488 goat anti-rabbit antibody (A31627), and Alexa Fluor[®] 594 goat anti-rabbit antibody (A31631). All secondary antibodies were purchased from Invitrogen. Controls were performed by omitting primary antibodies. Sections of testis from pubertal and adult rats stained with the same antibodies were used as a positive control. Photomicrographs were acquired using a ZeissAxioImager.Z1 fluorescence microscope with an ApoTome (Carl Zeiss, Le Pecq, France).

2.12. Reverse transcriptase polymerase chain reaction

Cells from *in vitro* eBTB were dissociated with BD cell recovery (BD, Biosciences) according to the manufacturer's procedures. After incubation for 1 h, the cells were centrifuged at 250 g for 5 min at 4 °C and counted. Total RNA from *in vitro* eBTBs and m 18-, 21-, 40-, and 60-day-old rat testis (20 mg of tissue) was extracted according to Chomczynski et al. [37] using Trizol reagent (Invitrogen). The purity and the integrity of the RNA were checked using a Nanodrop 8000 UV–V is spectrophotometer (Thermo Scientific) and by gel electrophoresis, respectively. Reverse transcription (RT) was performed with an Oligo dT primer according to the manufacturer's instructions (Omniscript RT kit, Qiagen, France) to prepare cDNA with 1 μg of RNA. 1 μL of cDNA was used as a template to amplify the considered gene. The primer sequences, size of the amplified products, Genbank accession number, and cell marker are shown in Table 3. The *S16* gene was amplified as a housekeeping gene for normalization. Controls were performed with ARN (without RT) and with sterile water (without cDNA, PCR). During the polymerase chain reaction (PCR), amplification was carried out with a Hot Star Taq Master mix kit (Qiagen) as follows: 94 °C for 5 min, 40 cycles consisting of 94 °C for 1 min, 57 °C for 1 min and 72 °C for 1 min, followed by a complementary final Taq inactivation step at 72 °C for 10 min. For the *TH2B* gene only, RT-PCR was performed for 50 cycles. The amplified products were electrophorised in parallel with size markers on 3% agarose gels.

2.13. Analysis of the data

Each experiment was repeated at least three times except for cell isolation. The selected data are presented herein. The numerical data are presented as the mean ± standard error of the mean (SEM) except for Fig. 7 in which individual values are shown for three different cultures. Statistical analyses were performed by a one-way analysis of variance (ANOVA) test with a Bonferroni's post-test using GraphPad statistical analysis (Prism 5, version 5.02).

3. Results

3.1. Morphology of 3-D testicular cell culture

During the first day, cells migrated inside the EM and started to form clusters of cells. After the second day of culture, the engineered *in vitro* tissue presented spherical clusters with different degrees of organization and different diameters depending on the EM composition (i.e., (un)diluted BD Matrigel, with or without Type I Collagen) (Figs. 1 and 2). As early as the ninth day of culture, the collagen presence promoted a visible matrix retraction with a final reduction of 50% of the surface of the EM at 22 days of culture

Table 1
Final concentrations and composition of tested extracellular matrix (EM).

Name of EM	Matrigel [®]	Type I Collagen
Type 1 EM 2.5: 4.3	4.3 mg/mL	2.5 mg/mL
Type 2 EM 4.3	4.3 mg/mL	No
Type 3 EM 2.5	2.5 mg/mL	No

Table 2

Summary of primary antibodies used in this study for immunofluorescence on tissue and cell application. Ab: antibody.

Antigen	[Catalog#]	Immunogen	Type (clone)	Vendor	Working dilution
Vimentin	M 0725	Purified vimentin from porcine eye lens	Monoclonal Mouse Ab (Clone V9)	Dakocytomation	1:75
Claudin-11	Sc-25711	Amino acids 101–207 mapping at the C-terminus of Claudin-11 of human origin	Polyclonal Rabbit Ab (H-107)	Santa Cruz Biotechnology	1:50
Alpha smooth muscle actin	Sc-53142	Chicken gizzard Actin	Monoclonal Mouse Ab (Clone B4)	Santa Cruz Biotechnology	1:50
Chk2	Sc-9064	Amino acids 1–300 mapping at the N-terminus of Chk2 of human origin	Polyclonal Rabbit Ab (H-300)	Santa Cruz Biotechnology	1:150
γ Histone H2A.X	05–636	Peptide (C-KATQA[ρ S]QEY) corresponding to amino acids 134–142 of human histone H2A.X	Monoclonal Mouse Ab (JBW301)	Millipore Home	1:200

(Fig. 1A–E). A matrix retraction is totally absent along the culture with the EM made only of Matrigel (Fig. 1F–O). Interestingly, a cluster organization was observed with diluted Matrigel at a 2.5 mg/mL final concentration (Fig. 2A). At this concentration, the regularity of spaces between clusters is consistent with the morphogenetic gradient hypothesis as observed by Gassei et al. [38]. A number of 6.3 ± 0.1 neighboring aggregates were separated by approximately $988 \pm 95 \mu\text{m}$. The clusters were circular with a diameter of $158 \pm 27 \mu\text{m}$ versus a diameter of less than $104 \pm 39 \mu\text{m}$ with a 4.3 mg/mL final concentration of Matrigel, and are similar to *in vivo* seminiferous tubules ($150 \pm 46 \mu\text{m}$) from 18-day-old rats (Fig. 1F–J and 2C). Moreover, the proliferation of primary cells depends on the concentration of fetal bovine serum: 10% FBS-culture medium over-stimulates the proliferation of peritubular cells that are under the insert and that are above the insert, and contaminate the Sertoli and germ cells mix, thereby accentuating the matrix retraction. Additionally, the prehydration of the insert due to seeding of peritubular cells under the insert demonstrated a negative effect on matrix retraction (data not shown). Repeatability of the experiments described above was obtained with diluted Matrigel at 2.5 mg/mL final concentration without collagen in 5% FBS-supplemented medium.

3.2. Identification and structural organization of different actors in the eBTB

The total cell number decreased slightly after the first day of culture because all cells were not embedded in the EM and approximately 0.5% of the cells were in suspension in a culture medium. At the end of the experiments, the cell number had decreased with 26% cell loss. The cell viability was between 87% and 91% at day 11 (the half-time of the culture). Until the end of the culture, the maximum of cell viability was around 85% (data not shown).

After seeding under the insert, the presence of peritubular cells was confirmed by their positive alkaline phosphatase activity. Cells were also detected by showing expression of the specific protein alpha smooth muscle actin (αSMA) by RT-PCR [31] (Fig. 3A) and immunofluorescence (Fig. 3B and C). Sertoli cell presence in *in vitro* eBTB has been confirmed through molecular analysis of a specific *Gata4* gene (Fig. 3A) [39] and immunostaining of a vimentin protein (Fig. 3D). In the same way, present in 18-day-old rat testis, spermatogonia have been specifically detected in *in vitro* eBTB by mRNA detection of the *c-kit* gene (Fig. 3A) [40,41] and by Chk2 protein immunodetection, which is a specific check point protein of the cell cycle (Fig. 3E). At day 4, MGG staining allowed the determination of the Sertoli cell/germ cell ratio, which is 1.2 ± 0.2 . Excluding peritubular cells, the Sertoli cell number represented $49.7 \pm 3.2\%$ of the total testicular cells in eBTB (Fig. 4B). Quantitative analysis of the Sertoli cell/germ cell ratio and the Sertoli cell numbers presented no significant difference during the 22 days of culture.

The PAS staining identified the type IV collagen, which was colored in pink, from Matrigel (Fig. 5A) and from the basement membrane of the tubules (Fig. 5B). This staining showed that cell clusters are embedded in EM in the same way that the seminiferous epithelium surrounded by the basement membrane. The cord-like structure possessed no defined lumen in the center, but polarization of the Sertoli cells and the presence of germ cells was identified by their nucleus size (Fig. 5A, C, and E). Spherical structure was confirmed with 3-D immunofluorescence (data not shown). Epithelial and polarized organization was confirmed by semi- and ultra-thin sections obtained for TEM analysis, which showed the characteristic invaginated nucleus and enlarged nucleolus from Sertoli cells surround with germ cells in eBTB (Fig. 5C and E) and in 18-day-old rat testis (Fig. 5D and F). These observations suggest a similar organization for *in vitro* eBTB to *in vivo* seminiferous tubules.

Table 3

Sequence of specific primers used for amplification of targeted genes. F represents forward, R represents reverse, and Tm is the melting temperature.

Gene	Genbank accession no.	Cell marker	Primer sequence (5'–3')	Tm (°C)	Length (bp)
<i>c-kit</i>	NM_021099	Spermatogonia	F: gcatccacatcaaaaacgtg R: gatagtcagcgtctctggc	57 57	332
<i>TH2B</i>	M18045	Pachytene spermatocytes	F: tgagacgttggagtgacaa R: gtaactctctcgcggcatc	57 57	152
<i>TP2</i>	U52958	Spermatids	F: ggctcaaagtcacaccaat R: ttccctccaaggtcttct	57 57	205
<i>Gata4</i>	NM_144730	Sertoli cells	F: gaaaggacaagggtgatgga F: ctctgctacggcagtaagg	57 57	225
<i>Alpha SMA</i>	NM_031004	Peritubular cells	F: catcaggaaactcgagaagc R: tcggatactcagggtcagg	57 57	247
16S	XM_341815	Housekeeping gene	F: gaccctgctgtagctgacc R: cagtcaccacagccacttat	57 57	224

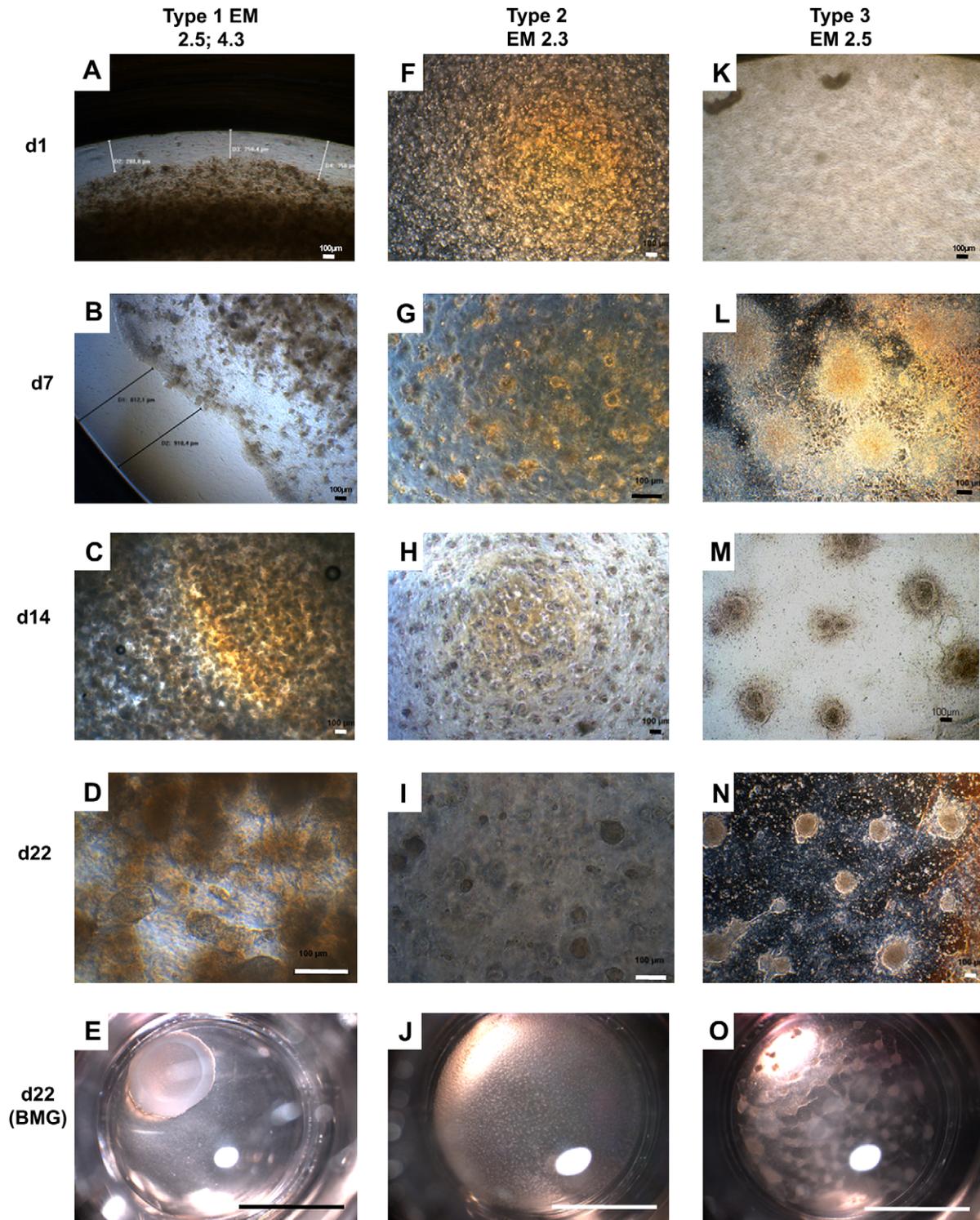


Fig. 1. Comparison of extracellular matrix (EM) composition effect on morphology of 3-D testicular cell culture. Evolution of cell organization (cluster formation, diameter of spherical structure) and matrix retraction along the 22 day culture (d1-7-14-22) for three types of EM composition. (A to D): The EM is composed of Matrigel 4.3 mg/mL + high concentration of collagen Type I, 2.5 mg/mL (Type 1 EM 2.5; 4.3). (F–I): The EM is composed of Matrigel only at 4.3 mg/mL (Type 2 EM 4.3). (K–N): EM is composed of 2.5 mg/mL (Type 3 EM 2.5). Corresponding bar types 1, 2, and 3 are indicated in μm in all views. (E, J, O): Pictures are representative views of inserts (diameter = 12 mm) observed at the 22nd day of culture with a binocular magnifying glass (BMG) for the three types of EM. Corresponding bar is 60 mm. Each type of EM was tested in triplicate.

3.3. The barrier function: presence of tight junctions

The presence of tight junctions was detected *in vitro* between Sertoli cells by immunofluorescence using primary antibodies raised against rabbit claudin-11 (Fig. 6). A colocalization of

vimentin (Fig. 6A) and claudin-11 (Fig. 6B) was performed and an overlay confirmed the presence of Sertoli cells with tight junctions consisting of claudin-11 (Fig. 6D). In the adult testis, this protein is specific to tight junctions between Sertoli cells and is characteristic of the compartmentalization of BTB. In 18-day-old rat testis, the

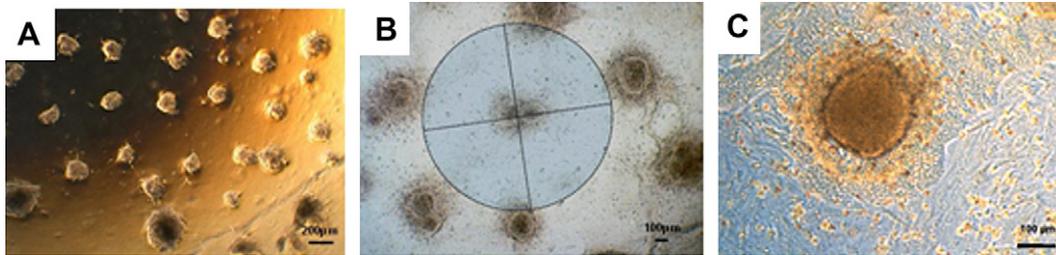


Fig. 2. Morphology details of 3-D testicular cell culture with type 3 EM composition (Matrigel 2.5 mg/mL). (A) Clusters of cells with spherical structure included in EM. (B) The space between the clusters is identified by blue circle and shows morphogenetic gradients with a distance around $988 \pm 95 \mu\text{m}$. (C) Cluster diameter is around $158 \pm 27 \mu\text{m}$. Corresponding bars are indicated in μm in all views. The isolation experiments with this EM composition were repeated at least eight times. Numerical data is representative of the mean of three replicates on three different days (Day 3, Day 10, Day 22) in each experiment.

localization of claudin-11 is diffuse along the seminiferous epithelium because the BTB is undergoing formation (Fig. 6E), and correlates with our observation in *in vitro* eBTB. The functionality of tight junctions has also been studied with the measurement of electrical resistance in *in vitro* eBTB. We demonstrated that functional tight junctions were present between Sertoli cells and peritubular cells because TEER overstepped the threshold of $25 \Omega/\text{cm}^2$ (Fig. 7). The TEER evolutions present a maximum level from the 5th to the 13th days with values of $83 \Omega/\text{cm}^2$ and $32 \Omega/\text{cm}^2$ for tight junctions of Sertoli cells and peritubular cells, respectively. Indeed, controls allowed us to deduct the TEER from tight junctions of the two cell types and show a difference in their tightness function. The TEER total depends on 70–80% tight junctions between Sertoli cells. Moreover, the measurement of TEER in controls (insert with peritubular cells alone) permit us to observe no significant and evaluative values during the time of culture. These observations proved that the concomitant presence of EM and Sertoli and germ cells is the strongest link to the development of tight junctions between peritubular cells. The results prove that the *in vitro* eBTB presents a feature of functional physico-chemical barrier with the presence of tight junctions.

3.4. The spermatogenesis function: the *in vitro* germ cell differentiation

Immunofluorescence with anti-phosphorylated serine 139 of histone H2AX (γ H2AX) is detectable in three types of spermatocytes: in control, in 60-day-old rat testis (Fig. 8A), and at day 22 of the eBTB culture (Fig. 8B). In leptotene and zygotene spermatocytes, H2AX is phosphorylated (in γ H2AX) throughout the chromatin to activate the DNA double strand break repair, and in pachytene spermatocytes the reactivity takes place in the XY body. The presence of pachytene spermatocytes was also confirmed by detection of the *TH2B* gene (testis-specific histone *TH2B*) (Fig. 9A). A low level expression was detected at the start of the culture corresponding to preleptotene spermatocytes and early pachytene spermatocytes presence in 18-day-old Sprague–Dawley rat testis, as already shown [42,43]. RT-PCR was performed on post-meiotic gene, transition protein 2 (*TP2*) [44,45], on cultivated cells in comparison with pubertal (18–21 dpn) and adulthood (40–60 dpn) testis, and confirmed *in vitro* germ cell differentiation in 1N spermatids (Fig. 9A). Consistent with Marret et al. and Lee et al. [11,42], a very low expression of *TP2* mRNAs could be amplified in 18-day-old Sprague–Dawley rat testis when no post-meiotic germ cells were present and no *TP2*-positive germ cells were immunodetected. According to the timing of *in vivo* differentiation of germ cells [10,46], the detection of a high level of *TP2* mRNAs at 18 days of culture confirms the differentiation of germ cells into round spermatids. An ultra-thin section performed for TEM observation

revealed around spermatid fused into the same giant cells in 22 day-old culture (Fig. 9B). At culture day 22 of the eBTB, different DNA condensation stages were observed by MGG staining: Sertoli cells, meiosis cells (spermatocyte and metaphase cells—2N cells) and haploid cells (spermatids—1N cells) (Fig. 10A). Furthermore, MGG staining could provide an approximation of the quantitative evolution of a percentage of the meiosis and haploid cells during the 22 days of culture. A significant difference ($P < 0.1$) was detected between day 4 and day 22 for meiosis cells, proving a decrease in the cell number in eBTB (Fig. 10B). As expected, a significant increase in the number of haploid cells was observed ($P < 0.001$) on days 9 and 22 versus day 4 (Fig. 10C), thereby confirming *in vitro* spermatogenesis.

4. Discussion

Our model attempts to reproduce a 3-D eBTB that is highly representative of the morphology and physiology of rat *in vivo* seminiferous epithelium. The eBTB keeps the interactions between somatic and germ cells that are very important for advancement to subsequent steps of spermatogenesis, and for the creation of cell junctions. Many studies have shown strong relationships between the regulation of cell junctions such as ectoplasmic specializations [47]; gap junctions [48] or tight junctions [49]; and spermatogenesis (for a review, see [3]). In mammalian species, the BTB acts as a physico-chemical barrier, an efflux pump, and an immunological barrier, and protects germ cells from harmful agents and immunological influences. Thus, it also impedes the delivery of chemotherapeutic drugs to the testis, and could regulate the passage of most of the molecular compounds that can disturb the delicate process of meiotic cell division.

The need for the development of *in vitro* BTB is linked to the need for alternative methods in animal experimentation in the field of reproductive toxicology. Such an *in vitro* BTB could significantly contribute to study of the effects of environmental toxicants on testicular function disruption. Our research contributes to this scientific effort, and we presented here an original model of BTB which could be used to test the effects of toxic substances on both the barrier and spermatogenesis functions. We used a coated method using a mix of Matrigel, Sertoli, and germ cells cultivated inside a bicameral chamber, and a peritubular cell layer under the insert. Cell organization is more appreciable in this coating method whereas in the precoated methods, the organization of cell clusters was more irregular (data not shown). With the precoated method, Sertoli cells maintained a columnar cell shape with normal polarity and developed tight junctional complexes in the basolateral region [26,50–52]; however, the organization was not a cord-like structure similar to those present in *in vitro* eBTB and *in vivo* (Fig. 4) rat testis seminiferous epithelium. Our engineered *in vitro* tissue

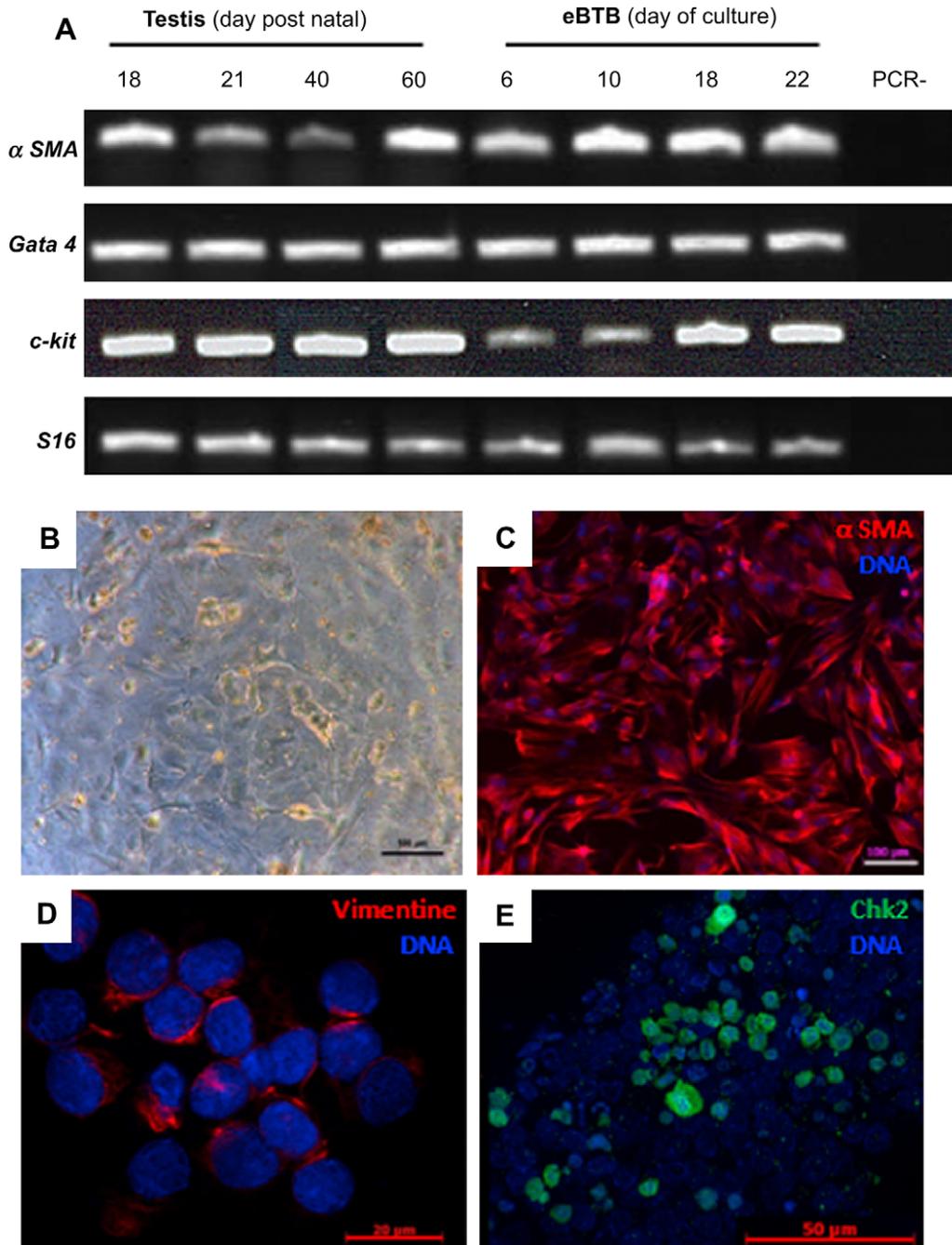


Fig. 3. Identification of different cell types present in *in vitro* eBTB in comparison within rat testis. (A) RT-PCR analysis was used to detect mRNA expression detection of α SMA gene (specific to peritubular cells), *Gata4* gene (specific of Sertoli cells), *c-kit* gene (specific of spermatogonia) and S16 gene, (housekeeping gene). The molecular analysis was performed on 1–21–40–60 dpn rat testis or on 6–10–18–22 days of eBTB culture. Controls omitting cDNA were indicated in column PCR-. Gel is representative of four separate experiments. (B–C) Peritubular cells on the underside of the insert at Day 4 were identified by immunodetection of the specific marker α Smooth muscle actin (α SMA) (red fluorescence). (D–E) Germ cells and Sertoli cells were identified in cluster after digestion of extracellular matrix and cell dissociation. (D) Expression of vimentin, a Sertoli cell specific marker, was detected (red fluorescence), and (E) the presence of spermatogonia was confirmed by expression of Chk2 (green fluorescence) at Day 4. Dpn: day post-natal. (A–D): Corresponding bars were indicated in μ m in all views.

presented, after the 2nd day of culture, a cord-like structure embedded into the EM with Sertoli cells in the periphery, and germ cells in the center of the structure, as reported in Hadley et al. [15]. The delay and the steps for the formation of these structure were the same observed by others [53]. Their diameter was close to what we observed in *in vivo* seminiferous tubules (Fig. 2). Other works have recently described an improved 3-D culture system consisting of a culture of Sertoli cells on Matrigel for the *de novo* generation of testicular cord like structures which were formed after 3 days in

culture [38,54]. Our clusters have different behaviors with a higher diameter and larger spaces between neighboring aggregates (Fig. 2), confirming the difference between these two types of cultures depending on the coating and precoating methods, and on the different types of cultured testicular cells.

Matrigel allows us to obtain an artificial extracellular matrix resembling the mammalian cellular basement membrane, and participates in the organization and functions of the eBTB. Indeed, in seminiferous epithelium, basement membrane components

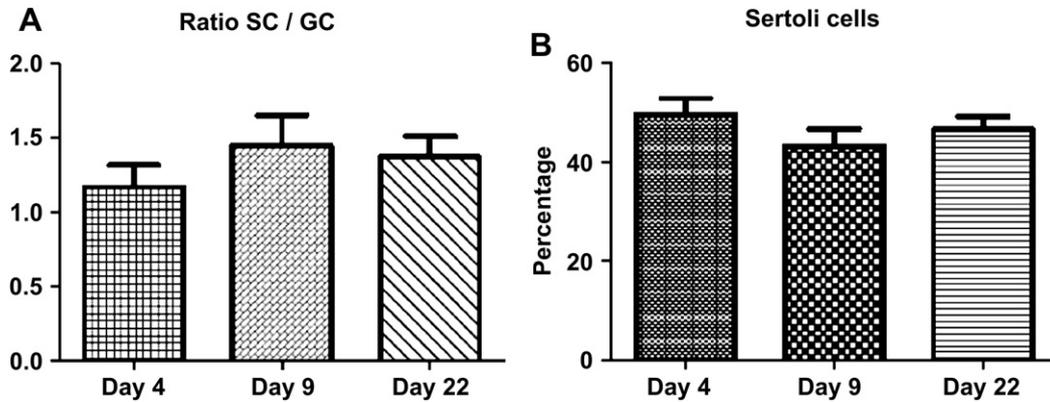


Fig. 4. Quantitative evolution of cell population during 22 days of culture by MGG staining. (A) Percentage of Sertoli cells was identified in cluster of spermatogenic cells at culture Days 4, 9, and 22. (B) A ratio of Sertoli cell/germ cell was evaluated at culture Days 4, 9, and 22. The percentage of different types of cells were evaluated for every replicate. At least 500 cells were counted for each day in three different experiments. Results are the mean \pm SEM of determinations.

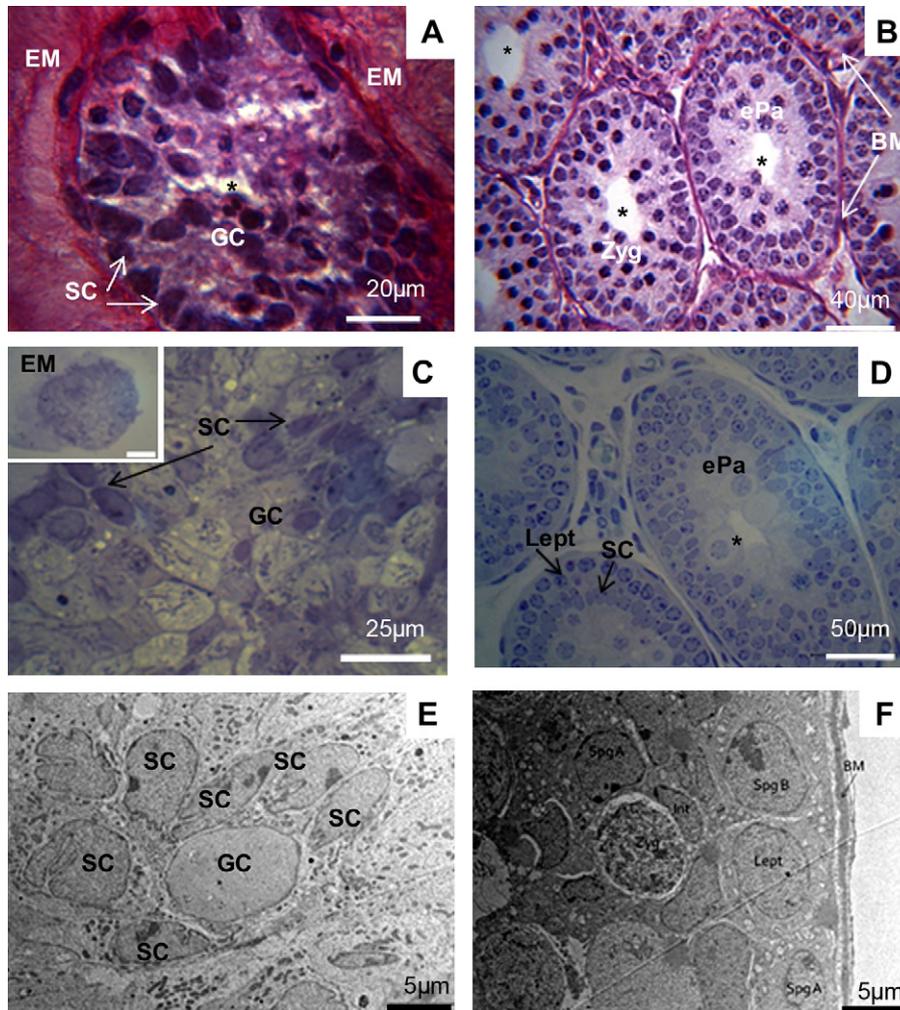


Fig. 5. Microscopic observations of eBTB structural organization in comparison with *in vivo* rat seminiferous tubules. (A–E) Representative picture of the eBTB at 10 days old culture. (B–D–F) Seminiferous tubules in rat testis at 18-days-old. We note that early pachytene (ePa) as the last step in germ cell differentiation can be identified (D). The presence of EM with Type IV Collagen detection (in pink with periodic acid Schiff staining) was confirmed (A) and its composition correlates with the presence of basement membrane (BM) in rat testis (B). (A–B) The organization is very similar to *in vivo* observation as proven with periodic acid Schiff-hematoxylin staining and (C–D) with toluidine staining, both showing spherical structures. (C) In eBTB, Sertoli cells (SC) are polarized and (D) the localization of germ cells (GC) on the center are identified in *in vivo* seminiferous tubules. In (C), the view represents the magnification of the global cord-like structure present in the inset (bars corresponding 50 μ m). (E) In the TEM results, the presence of Sertoli cells with characteristic invaginated nuclei was observed in eBTB. (F) In 18-day-old rat *in vivo* seminiferous tubules, many types of germ cells were identified: Type A & B of spermatogonia (Spg A, Spg B), intermediary spermatogonia (Int), Leptotene spermatocyte (Lept), zygotene spermatocyte (Zyg), and early pachytene (ePa). * represents lumen of structure. Corresponding bars are indicated in μ m in all views.

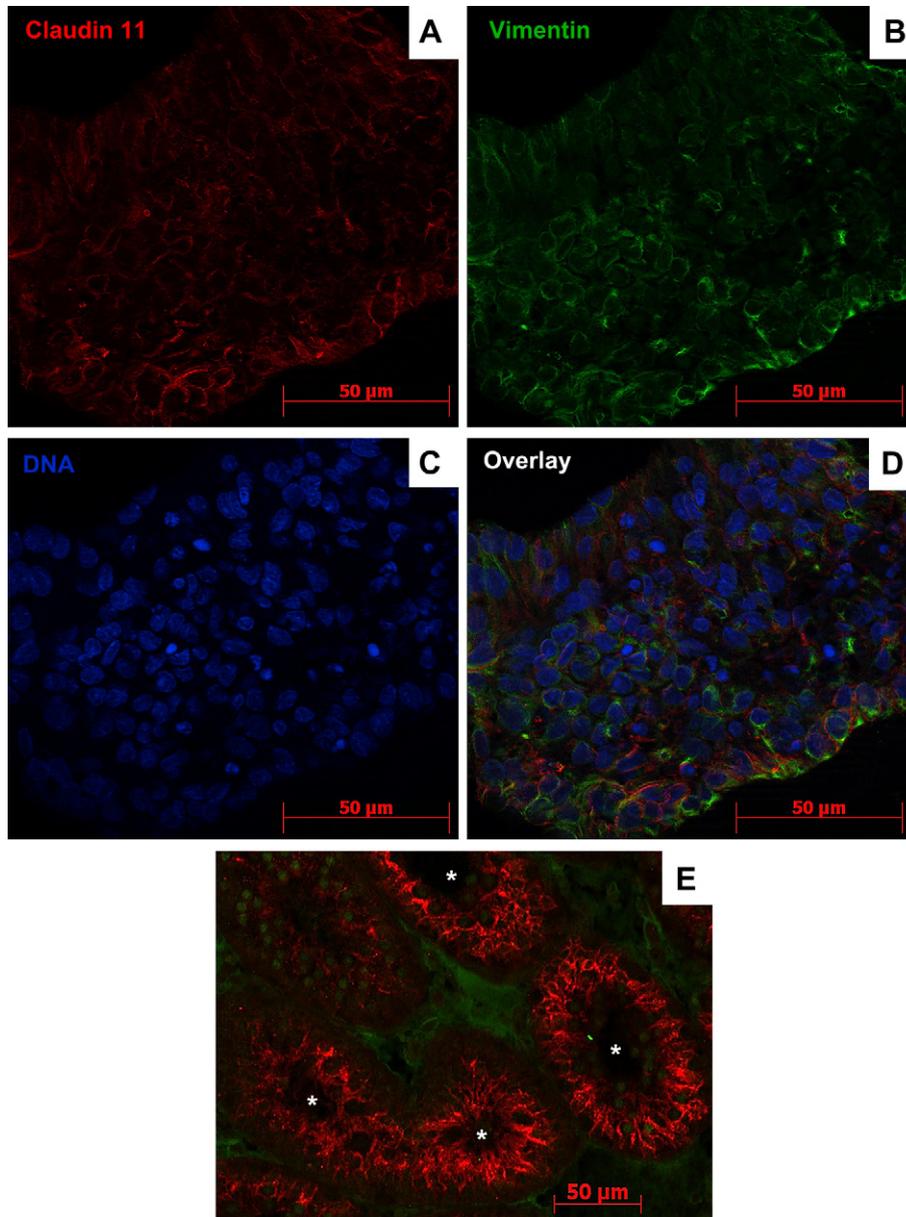


Fig. 6. Detection of claudin-11 by immunofluorescence. (A) Claudin-11, a protein involved in tight junctions and establishment of BTB, is expressed at culture Day 10 in *in vitro* eBTB model (red fluorescence). (B) vimentin was immunolocalized in Sertoli cells (green fluorescence). (C) DNA was stained by Hoechst 33342. (D) Overlay of claudin-11, vimentin, and DNA immunodetection. (E) Immunolocalization of claudin-11 and vimentin in 18-day-old rat testis. In comparison with *in vitro* eBTB, the tight junctions in 18-day-old rat testis were present between Sertoli cells, and the delimitation between the basal and apical compartments can be identified. * represents lumen of the structure on the apical pole. Corresponding bars are 50 μm in all views.

helped maintain epithelium, Sertoli cell function, and germ cell differentiation [55]. In addition, *in vitro* biological activity of Matrigel ensures cell morphogenesis, cell differentiation, and tumor growth [56]. The regular spaces observed between aggregates in eBTB respect morphogenetic gradients (Fig. 2) which are responsible *in vivo* for the control of seminiferous tubule formation during testis development [53]. This confirms the relationships between cells to obtain an organized structure. Consistent with Hadley et al. [57], our results confirmed that laminin and collagen IV present in BD Matrigel are essential for the formation of testicular cords. Indeed, neither cell type present in eBTBs are capable of producing detectable quantities of soluble laminin [58]. These observations confirm that EM components are essential for 3-D structure and spermatogenesis as reported with infertile patients with aspermatogenesis, which exhibits abnormal basement

membrane structures [59]. *In vivo*, the cord formation is surrounded by peritubular myoid cells which cooperate with Sertoli cells to form the basement membrane localized between the two cell types [58,60].

In rat testis, the peritubular cell layers surrounding the seminiferous tubule are composed of both myoid and non-myoid cell populations [58,61]. The first type consists of contractile elements similar to smooth muscle and secretes many compounds in the presence of serum such as fibronectin, type I collagen, and type IV collagen, which are important basement membrane components [62,63]. Myoid peritubular cells express alpha smooth muscle action, a classic marker for terminally differentiated myoid peritubular cells, and phosphatase alkaline, another specific marker. Both depend on cell density and time culture [31,63–65]. In our model, peritubular cells were isolated from 18-day-old rat testis,

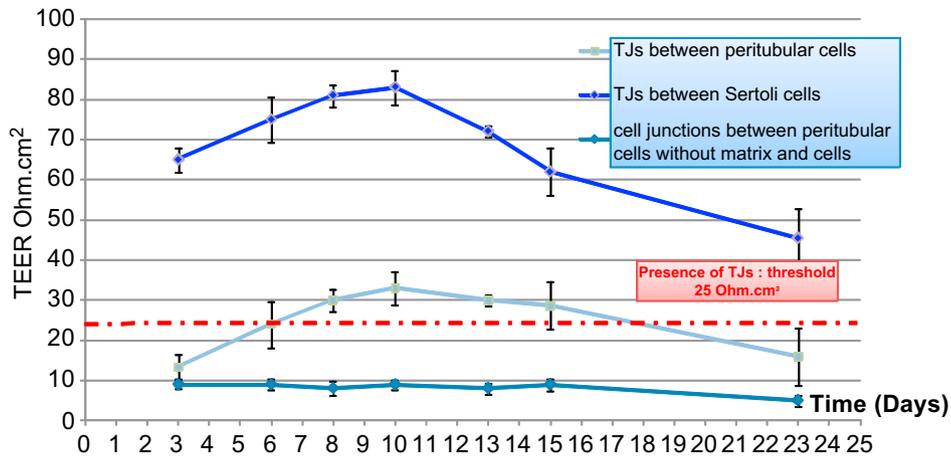


Fig. 7. Evolution of tight junction functionality between Sertoli cells and peritubular cells detected with TEER measurement in *in vitro* eBTB model. Functional tight junctions were detected with maximum levels of TEER between the 8th to the 13th day of culture. The tightness is more appreciable between Sertoli cells, but decreases together in the middle of the culture. Measurement between peritubular cells (seeding without matrix and Sertoli and germ cells) presented no evolution of electrical resistance during the time confirming the role of relationships between this 3-D culture. The threshold of 25 Ω/cm^2 was determined according to information in the literature [1]. Results are the mean \pm SD of triplicate determinations in one representative experiment.

and these markers were detected at the first day of culture. (Fig. 3B and C). This suggests the presence of a myoid cell population. Moreover, peritubular cells expressed vimentin protein (data not shown), as reported by Tung et al. [31], relative to the mesenchymal origin of peritubular cells. The experiments with the EM composed or not composed of high concentration collagen type I demonstrated the relationships between the presence of peritubular cells and type I collagen secretion. Indeed, type I collagen, which is essential for basement membrane functions, could also be secreted in the culture medium by peritubular cells [31]. Moreover, the presence of type I collagen did not improve the eBTB behavior, and induced more retraction of the EM during the time of the culture (Fig. 1A–E). Lee et al. [11] compared the behavior of SGC seeded on the EM composed of high concentration collagen type I with or without Matrigel, thereby proving the importance of collagen. Despite the retraction, which was approximately a 75% reduction in size after 12 days in culture, Lee et al. promoted the mix with collagen and Matrigel [66]. In our study, we show the best results, without the retraction of the matrix (Fig. 1K–O), with an extracellular matrix composed of Matrigel alone against the Matrigel and Collagen mixture.

Relationships between different types of cells could be investigated in eBTB. In the literature, cocultures of peritubular and Sertoli cells, or cultures of Sertoli cells on laminin composing the EM, showed increasing Sertoli cell survival. They also stimulated secretion of Sertoli cell products such as transferrin and androgen-binding protein as well as lactate, which is an energy substrate required for the survival of post-meiotic germ cells [15,67]. These Sertoli cell products are glycoproteins that facilitate the transport of ions and hormones, or provide bioprotective functions [9]. Additionally, Ailenberg et al. [26] reported that peritubular cells assume a more important role than Matrigel in the modulation of transferrin secretion. In this way, a characterization of the intracellular cell signalizations could be performed inside eBTB as a transferrin secretion by Sertoli cells. Furthermore, Sertoli cells interact directly with germ cells in testis and perform a number of functions critical for spermatogenesis, including compartmentalization of the seminiferous tubule, physical and metabolic support of germ cells. Secretion of numerous factors equally promotes germ cell viability and differentiation [68], thereby confirming our observations.

Sertoli cells from early pubertal rat testis were isolated because they exhibited a synergistic responsiveness to FSH and testosterone

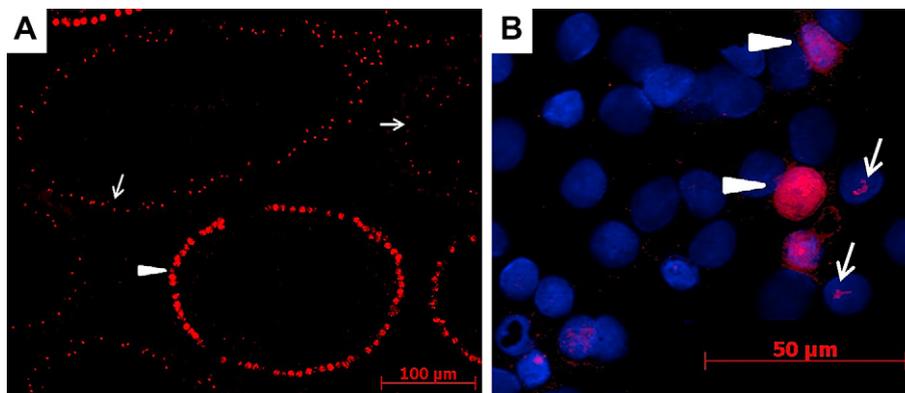


Fig. 8. Immunodetection of γ H2AX, a specific histone of the leptotene, zygotene, and pachytene germ cell development steps. (A) The immunofluorescence staining in 60-day-old rat testis confirmed γ H2AX expression (red fluorescence) in leptotene, zygotene, and pachytene spermatocytes. As detected in 22-day-old eBTB (B), immunoreactivity in leptotene and zygotene spermatocytes correspond with phosphorylation of H2AX (in γ H2AX) throughout the chromatin to activate DNA double strand break repair (arrowheads). Expression of pachytene spermatocytes on a characteristic XY body was detected, confirming its role in processing the meiotic sex chromosome inactivation reactivity (arrow).

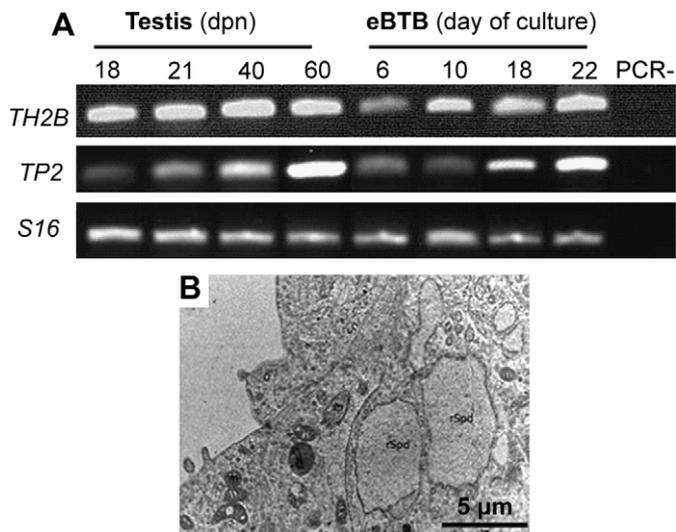


Fig. 9. Detection of germ cell differentiation in eBTB models. (A) RT-PCR allows mRNA expression detection of the *TH2B* gene, specific to preleptotene and pachytene spermatocytes; and the *TP2* gene, specific to round spermatids (1N germ cells) and the housekeeping gene (*S16* gene). Molecular analysis was performed on 18–21–40–60 dpn rat testis or on 6–10–18–22 days of eBTB culture. The testis 18 dpn was designated as negative control for *TP2* mRNA expression despite low expression of *TP2* mRNA. Controls omitting cDNA were indicated in column PCR. Gel is representative of four separate experiments. Dpn represents day post-natal. (B) TEM analysis permitted detection of fused round spermatids. Note the absence of a cytoplasmic bridge between the two nuclei, mitochondria (m), and lysosome (l). The corresponding bar is indicated in μm.

that benefited the maturation of Sertoli and germ cells and the process of *in vitro* spermatogenesis [69–71]. In our eBTB, viability seemed increase during the first ten days of the culture, but more investigations should be performed. Besides gentle shaking, cultures have better viability because the penetration of oxygen in the air is easier in the eBTB cultures. The number of Sertoli cells supporting germ cells is determinant in spermatogenesis [72]. Indeed, morphometric analysis of the adult rat testis has shown that each Sertoli cell is associated with ~30–50 germ cells at each stage of the spermatogenic cycle in the epithelium [3,73]. However, in our study, at day 4, the Sertoli cell/germ cell ratio results in 0.8 germ cells associated with one Sertoli cell (Fig. 4A). The results show that the proportion of these two population cells is approximately equal in eBTB (Fig. 4B). This observation suggests that the Sertoli cell number does not to be a limiting factor for *in vitro* spermatogenesis.

In a Sprague–Dawley rat, spermatogenesis is a long-lasting process that takes about 53.2 days from A stem spermatogonia to spermatozoa, and more than 7–12 days are required for a leptotene primary spermatocytes to complete meiosis and produce spermatids [46,74]. As reported in the literature, at post-natal days 17–18, zygotene spermatocytes are present and haploid round spermatids first appear at days 24–25 [75]. In our work, early pachytenes have been identified in 18-day-old rat Sprague–Dawley testes (Fig. 5B) correlating with a very low level of *TH2B* gene detection in these testes (Fig. 9B), as expected [42,43]. Spermatogonia were immunodetected with their cell cycle specific check point protein, Chk2 in eBTB, confirming this expression in rat testes as observed in human testes [32,76]. H2AX has a synergistic function with ataxia-telangiectasia mutated (ATM) in the development and maintenance of a genomic instability in a mouse germ line [77,78]. The immunodetection of γ H2AX confirmed the presence of leptotene and zygotene spermatocytes, and pachytene spermatocytes (Fig. 8) where γ H2AX is localized to the XY body to process the meiotic sex chromosome inactivation reactivity [33–36]. The results show the

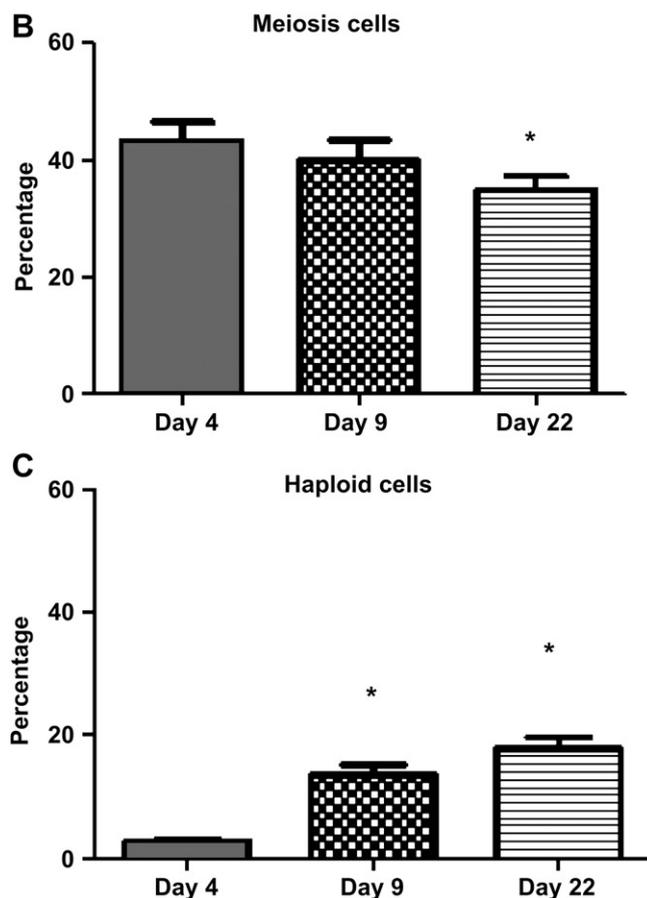
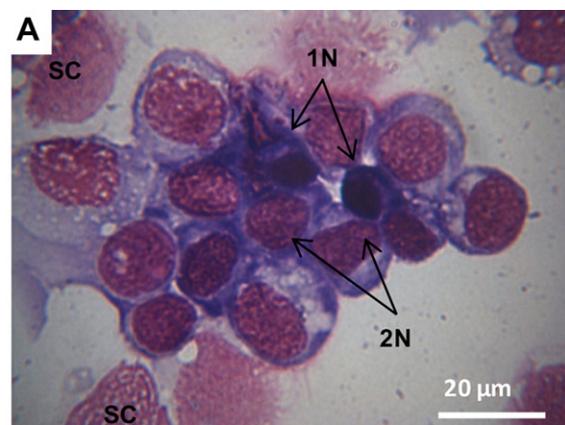


Fig. 10. Quantitative evolution of cell population during 22 days of culture by MGG staining. (A) Representative view allows identification of cluster of spermatogenic cells at culture day 22 in *in vitro* eBTB as Sertoli cells (SC), meiosis cells (spermatocytes and metaphase cells–2N), and haploid cells (spermatids–1N). * $P < 0.1$ significantly different versus Day 4. (B) Percentage of meiosis cells evaluated for every replicate at culture days 4, 9, and 22. (C) Percentage of haploid cells evaluated for every replicate at culture days 4, 9, and 22. * $P < 0.001$ significantly different versus Day 4. At least 500 cells were counted for each day in three different experiments. Results are the mean ± SEM of determinations.

presence, at the start of the culture, of the last development step in 18-day-old rat testicular cells. Further analysis of protein and mRNA expression that we developed confirmed the presence of others testicular cells as peritubular cells and Sertoli cells isolated from 18-day-old rat testis (Fig. 3).

Sertoli cells and germ cells develop many cell junctions during BTB formation, and modulate these during the spermatogenic cycle.

These associations create an intimate interaction that promotes germ cell survival differentiation. In our work, immunohistochemical examination revealed claudin-11 positive and vimentin positive cells in cell clusters. Because these two proteins are specific to Sertoli cells and their tight junctions [7,8,30], these observations suggest that *in vitro* Sertoli cells have the same phenotype as *in vivo*, and give a compartmentalization of spherical structure in the two compartments as we observed in *in vivo* rat testis BTB (Fig. 6). Moreover, tight junction presence is very important because it prevents *in vivo* molecules from crossing through the barrier. The tight junctions are essential to protect the testis against environmental toxicants to maintain an efficient BTB against testicular function disruption. In this way, the presence of this closed organization could be a marker to study *in vitro* eBTB with respect to the crossing and the effect of environmental toxicants [5,79].

In addition, we studied the functions of barrier and *in vitro* spermatogenesis and improved on the originality of the concept. The TEER measurement confirmed that eBTB creates permeability weakness due to the tight junctions of Sertoli cells and peritubular cells overstepping the threshold of 25 Ω/cm^2 . The tightness of these junctions has a distinct evolution and the different controls confirmed the importance of the EM that promotes the formation of tight junctions between Sertoli cells and between peritubular cells. When the data from all experiments were pooled, we concluded that (i) Matrigel is more important than peritubular cells in increasing the tightness of the permeability barrier generated by Sertoli cells in cord-like structures, and (ii) in the absence of Sertoli cells, neither EM nor peritubular cells can generate an efficient barrier, as was reported by Ailenberg et al. [26]. Moreover, the tight junctions between peritubular cells show less tightness than between Sertoli cells (Fig. 7). According to Dym et al. [80], a majority of closed junctions and few open junctions in one peritubular cell layer exists in rat testis which could explain our *in vitro* results. The tightness of Sertoli cells is more representative of exclusive barrier presence even though the tightness between peritubular cells corresponds to a partial barrier presence. The threshold is overstepped more rapidly between Sertoli cells than between peritubular cells (the 6th day-old culture). The establishment of this interaction correlates with the role of peritubular cells [81,82] and Sertoli cells to act indirectly or directly on germ cell differentiation. Indeed, 1N cells have been detected in eBTB (Fig. 9) using mRNA expression analysis of a haploid stage-specific *TP2* gene [44,45]. However, the transcription of *TP2* mRNA has very low level in seminiferous tubules devoid of round spermatids, proving that these genes are transcribed before completion of meiosis (Fig. 9B). This expression increases for mature round spermatids; i. e., only between steps 7 and 13 [42], suggesting very early steps of spermiogenesis during *in vitro* processes in eBTB. However, we did not clearly observe the characteristic morphology of these spermatid steps [46,74] in *in vitro* eBTB, and they must be confirmed. According to Hue et al. and Staub et al. [12,13], the differentiation of spermatocytes into round spermatids required 10 days, in contrast to 18 days of culture in our study (Fig. 9). More investigations will be performed to detect *TP2* mRNA between the 10th and the 18th day of culture because quantitative analysis by MGG staining showed a significant difference between the percentage of haploid cells at day 9 versus day 22 (Fig. 10C). As demonstrated by the time required for *in vitro* germ cell differentiation, more investigations will be performed to study the correlation between our eBTB and *in vivo* events.

5. Conclusion

Our model of eBTB has various similarities with the organization and function of seminiferous epithelium in rat testis, and presents

the many advantages of *in vitro* testicular cell cultures. The aim of our method was to reproduce *in vitro* the *in vivo* organization of rat testis seminiferous epithelium with barrier and germ cell differentiation functions. *In vitro* BTB models are urgently needed as alternatives to animal testing for compliance with regulatory chemical risk assessment. The passage of pollutants through the BTB presents the last step for adverse effects on reproductive functions. Therefore, our eBTB could be a suitable model for studying BTB toxicity and thus could contribute to the prediction of toxic effects from chemical compounds on the male reproductive function, which may affect human fertility.

Acknowledgement

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Appendix

Figure with essential color discrimination. Figs. 1–3,5–8 and 10 in this article have parts that are difficult to interpret in black and white. The full colour images can be found in the on-line version, at [doi:10.1016/j.biomaterials.2010.02.029](https://doi.org/10.1016/j.biomaterials.2010.02.029).

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