Evaluation of apoptotic- and autophagic-related protein expressions before and after IVM of fresh, slow-frozen and vitrified pre-pubertal mouse testicular tissue

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STUDY QUESTION: Do freezing and in vitro culture procedures enhance the expression of proteins involved in apoptotic or autophagic pathways in murine pre-pubertal testicular tissue?

SUMMARY ANSWER: IVM strongly modified apoptosis- and autophagy-related relative protein levels in mice testicular tissue whereas the impact of cryopreservation procedures was minimal at the end of the culture. 

WHAT IS KNOWN ALREADY: In vitro spermatogenesis remains a challenging technical issue as it imposes to find a very close balance between survival and death of germ cell natural precursors (i.e. gonocytes and spermatogonia), which will eventually undergo a complete spermatogenesis close to in vivo conditions. The establishment of efficient culture conditions coupled with suitable cryopreservation procedures (e.g. controlled slow freezing [CSF] and solid surface vitrification [SSV]) of pre-pubertal testicular tissue is a crucial step in the fields of fertility preservation and restoration to improve the spermatic yield obtained in vitro.

STUDY DESIGN, SIZE, DURATION: Here, we study cryopreservation procedures (i.e. CSF or SSV) and the impact of culture media compositions. A first set of 66 mouse pre-pubertal testes were directly cultured during 30, 36, 38 and 60 days (D) from 2.5 to 6.5-day-old CD-1 mice to evaluate the impact of time-aspect of culture and to endorse the reverse phase protein microarrays (RPPM) technique as an adapted experimental tool for the field of in vitro spermatogenesis. Ninety others fresh, slow-frozen and vitrified pre-pubertal testes were cultured during 30 days for the principal study to evaluate the impact of cryopreservation procedures before and after culture. Thirty-four testes dissected from 2.5, 6.5, 36.5, 40.5, 42.5 and 62.5 days postpartum (dpp) mice, corresponding to the time frames of spermatogenesis orchestrated in vitro, were used as in vivo controls.

PARTICIPANTS/MATERIALS, SETTING, METHODS: After in vitro culture, testicular tissue samples originated from 2.5 or 6.5-day-old CD-1 male mice were analyzed using RPPM. This targeted proteomic technique allowed us to assess the expression level of 29 apoptosis- and autophagy-related factors by normalizing blank-corrected signal values. In addition, morphological analyses (e.g. HES, PAS, TRA98 and CREM) and DNA fragmentation in intra-tubular cells (i.e. terminal deoxynucleotidyl transferase dUTP nick end labeling; TUNEL) were assessed for the distinct experimental conditions tested as well as for in vivo control mouse testes.

MAIN RESULTS AND THE ROLE OF CHANCE: A validation of the RPPM procedure in the field of in vitro spermatogenesis was completed with assay and array robustness before a principal study concerning the evaluation of the impact of in vitro culture and cryopreservation procedures. The proportion of elongated spermatids and the total cell number per seminiferous tubule tended to be very different between the in vivo and in vitro conditions (P < 0.05), suggesting the presence of a beneficial regulation on the first spermatogenesis wave by intrinsic
Introduction

Testicular tissue cryopreservation by slow freezing or vitrification has been introduced as an advantageous technique for fertility preservation in boys suffering from cancer (Picton et al., 2015). Because of impairment of human testicular grafting (e.g. inadequate oxygen and nutrient supplies) (Wyss, 2010; Arregui and Dobrinski, 2014), deregulation of angiogenesis-specific signaling (Devi et al., 2017), and risk of contamination by malignant cells (Hou et al., 2007), in vitro tissue culture has been proposed as an alternative procedure to restore fertility (Wyss et al., 2010). However, alterations of testicular tissue after cryopreservation followed by in vitro culture were detected in mice (Yokonishi et al., 2014; Dumont et al., 2015; Arkoun et al., 2016) and human (de Michele et al., 2017). Hence, an understanding of the mechanisms of impairment involved during cryopreservation and IVM seems to be essential for their respective improvements.

Spermatogenesis is a dynamic and synchronized process of spermatogonial stem cell (SSC) differentiation, which takes place in the seminiferous tubules. This process requires the orchestrated regulation of mitotic development of spermatogonia and meiotic process of spermatocytes followed by their differentiation in spermatozoa (Chung and Wolgemuth, 2004). The fate of male germ cells in the testis is determined by a complex network of external and internal signals including the soluble form of stem cell factor/c-kit ligand (SCF) and leukemia inhibitory factor (LIF) (De Felici, 2000) coupled with endocrine factors such as gonadotrophins (i.e. LH and FSH) and testosterone (O’Shaughnessy, 2014). The in vitro self-renewal of mouse SSCs has been reported in the presence of added gial cell line-derived neurotrophic factor (GDNF), epidermal growth factor (EGF), basic fibroblast growth factor (bFGF) and SCF with bovine serum (Kanatsu-Shinohara et al., 2003). Mouse SSCs were also successfully cultured under serum- and feeder-free conditions using StemPro®-34 SFM culture medium supplemented with growth factors (e.g. EGF, bFGF, GDNF and LIF) in vitro. Furthermore, we found in this study that the StemPro®-34 SFM culture medium supplemented with growth factors (e.g. EGF, bFGF, GDNF and LIF) prevented the differentiation of spermatogonial stem cells in favor of a significant proliferation with a better architectural pattern than in vivo culture for 6.5 dpp controls with an increase of seminiferous tubules area for FT (P = 0.0357) and CSF (P = 0.0317).

Limitations Reasons for Caution: Despite our promising results, the evaluation of apoptotic and autophagic-related proteins was studied for a limited amount of proteins and on global testicular tissue.

Wider implications of the findings: The data presented herein will help to improve apoptotic and autophagic understanding during the first spermatogenic wave. Moreover, our findings illustrate for the first time that, using finely-tuned experimental conditions, a testicular in vitro culture combined with proteomic technologies may significantly facilitate the study of cryopreservation procedures and in vitro culture evaluations. This study may also contribute to improve work on testicular tissues from pre-pubertal and adolescent cancer survivors.

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Key words: apoptosis / autophagy / in vitro spermatogenesis / mice / microarray / slow freezing / testsis / vitrification
(Dumont et al., 2016) mouse pre-pubertal testicular tissues. In addition, the regulation of Sertoli cells by FSH is critical for the progression of spermatogenesis (Hogarth and Griswold, 2010). Indeed, the active metabolite of Rol—the all-trans retinoic acid (aTRA)—inhibits FSH transduction pathways in Sertoli cells, by blocking the production of cAMP. In return, FSH suppresses the aTRA-induced nuclear localization, transcriptional transactivation and protein expression of the retinoic acid receptor alpha (RARα) (Braun et al., 2000; Livera et al., 2002). It was recently shown that a synchronous supplementation of both Rol and FSH in the culture medium did not improve the in vitro spermatogenic yield (Arkoun et al., 2015), reflecting an antagonist effect of FSH and aTRA who likely do not act at the same time in Sertoli cells. Moreover, cryopreservation-induced cell death by apoptosis can disrupt the normal biological function of testicular tissues (Onofre et al., 2016). Thus, during cryopreservation and in vitro culture, the increase of free radicals and oxidative stress, which can lead to apoptosis (Santos et al., 2009), result in decrease of antioxidant reagents.

As in several tissues, the cell density in seminiferous tubules is determined by a dynamic balance between cell proliferation and apoptosis (Russell et al., 2002). During testicular development, two peaks of normal physiological germ cell apoptosis are essential for spermatogenesis: the first period is during the migration of primordial germ cells into the gonads, which is controlled by GDNF and aTRA (Culty, 2009); the second is at the beginning of the first round of spermatogenesis (Wang et al., 1998). This period of increased apoptosis occurs during the first 2–3 weeks after birth in rodents as a single wave affecting spermatocytes, suggesting that apoptosis is critical to the normal development and function of the adult testis, possibly by maintaining a specific ratio between the number of germ cells and Sertoli cells (Rodríguez et al., 1997). Indeed, the RARα locates on Sertoli cells and exerts its autocrine effects by reducing GDNF secretion (Pellegrini et al., 2008; Rossi and Dolci, 2013). Sertoli cells rapidly phagocytize apoptotic germ cells and directly instigate the regulation of the apoptotic mechanisms of germ cells through the extrinsic pathway (Rossi et al., 1998; Nakagawa et al., 2005). Even if Fas—receptor of the tumor necrosis factor protein Fas Ligand (Fas-L)—is expressed at low levels in the mouse testis and appears to be restricted to some germ cells, the ‘Fas system’ has been proposed as a key regulator of physiological germ cell apoptosis (Tesarkin et al., 2001). However, Fas, p53 and proteins of the Bcl-2 family provide a part of the signaling apoptotic pathways that appears essential for male germ cells homeostasis. The competitive interactions of pro- and anti-survival Bcl-2 family proteins regulate the maintenance of normal spermatogenesis (Olderied et al., 2001) by the activation of caspase 3, 8 and 9, involved by extrinsic (action of the tumor necrosis factor superfamily of ligands binding to their associated receptors) and intrinsic (through events that result in the release of cytochrome c from the mitochondria) pathways of apoptosis (Moreno et al., 2006). Both pathways result in the subsequent activation of the caspase family that culminates in the characteristic structural, biochemical and morphological changes of apoptosis. Indeed, a dysregulation of apoptotic or autophagic processes in vitro could impair cell survival, proliferation and differentiation.

The aim of our study was to assess the expression of a specific choice of 29 selected proteins from apoptosis, cell proliferation, cell growth, cell survival, stress, differentiation, proto-oncogene and autophagy pathways during IVM of fresh and frozen-thawed mice pre-pubertal testicular tissue. The reverse phase protein microarrays (RPPM) was applied in the current study because this sensitive and high-throughput technique requires small amount of biological material while allowing simultaneous quantification of protein expression levels in large collection of samples. Moreover, this technique displays a good robustness and low signal-to-noise ratio with the use of phospho-specific antibodies. However, to endorse the RPPM technique as an adapted experimental tool for the field of in vitro spermatogenesis, a ‘validation’ study was, firstly, performed on IVM of pre-pubertal mice testes cultured with three different media (Fig. 1), considering our recent published data (Arkoun et al., 2015). Mouse testicular tissues (2.5 or 6.5 days postpartum [dpp]) were cultured for different time points (until D30, D36, D38 or D60) with several culture media (with or without Rol or gonadotropins). Potential effects of testicular age and culture duration on the in vitro expression of apoptotic and autophagic proteins was evaluated and compared to in vivo controls. Then, a principal study focused on the impact of cryopreservation procedures was settled and compared to the fresh testicular tissue. Moreover, we assessed the ability of five culture media (supplemented with Rol; FSH; permutation of Rol and FSH; Rol plus vitamin E [Vit. E]; or StemPro®—34 SFM with growth factors) to enhance the in vitro proliferation of spermatogonia and the differentiation throughout the culture (until D30) of fresh, slow-frozen and vitrified testicular tissue (from 6.5 dpp mice) (Fig. 1). Histological, morphological and DNA damages were coupled with the expression of apoptotic and autophagic proteins and compared with in vivo controls. Finally, the impact of one culture media (StemPro®—34 SFM with growth factors) was assessed on the germ cell kinetics.

**Materials and Methods**

**Ethical approval**

The animal care and the use committee of Rouen University (N/23-11-12/46/11-15) approved all experimental procedures performed in the study.

**Mice testicular tissue collection**

To ensure that the seminiferous tubules contained no germ cells more advanced than gonocytes or spermatogonia, testes were obtained from 2.5– or 6.5-day-old CD-1 male mice, respectively. Indeed, in 2.5 dpp neonatal mice, gonocytes—having a large and easily identified spherical nucleus—are present at the center of the seminiferous cords. From 3.5 to 5.5 dpp, gonocytes migrate from the center to the peripheral region of the seminiferous tubules to become spermatogonia (Yang and Oatley, 2014). Therefore, at 6.5 dpp, testicular tissue is made up of spermatogonia—identified with their localization on the basement membrane of the seminiferous tubules. The tunica albuginea was immediately removed in a phenol red-free minimum essential medium alpha (α-MEM; 41061–029; Gibco® by Thermo Fisher Scientific, Saint-Aubin, France) maintained at 4°C. To evaluate the impact of testicular age at the beginning of the culture (2.5 or 6.5 dpp) and the culture duration (30, 36, 38 and 60 days) on in vitro culture and to confirm that the RPPM technique is an adapted experimental tool in the field of in vitro spermatogenesis evaluation, a ‘validation’ study was undertaken with a total of 66 fresh mice pre-pubertal testicular testes that were cultured directly (for 2.5 dpp) or after being cut into four fragments (for 6.5 dpp). Indeed, histological examination of organotypic cultures previously revealed a complete spermatogenesis able to produce spermatooza corresponding to ages of (i) 40.5 (D38) and 62.5 dpp (D60)
from 2.5 dpp and (i) 36.5 (D30) and 42.5 dpp (D60) from 6.5 dpp (Sato et al., 2011; Arkoun et al., 2015). In the principal study, to determine the impact of cryopreservation procedures and culture media, a total of 90 fresh, slow-frozen and vitrified 6.5 dpp testes were cultured until D30. The in vitro production of spermatids from 2.5 dpp mice testes decreased significantly after long-term culture, notably at 60 days of culture (Sato et al., 2011). In view of these data, the choice of 40.5 (D38) and 62.5 dpp from 2.5 dpp was used to evaluate the apoptotic or autophagic fluctuations. For all these studies, 34 testes composed of a panel of 2.5, 6.5, 36.5, 40.5, 42.5 and 62.5 dpp mice were used as controls.

Validation and principal studies

Cryopreservation and culture

Testicular tissue cryopreservation was performed according to the protocols previously developed by our team for controlled slow freezing (CSF) (Milazzo et al., 2008) and solid surface vitrification (SSV) (Dumont et al., 2015). Testicular tissue was cultured according to the previously published in vitro culture technique (Arkoun et al., 2015).

Because, the robustness of a proteomics analysis will be enhanced if tissue samples are stabilized as soon as possible after excision (Espina et al., 2008), a maximum time interval of 20 min from manipulation (in vivo excision or in vitro removal of the central necrotic part area) to preservation (e.g. flash freezing in liquid nitrogen) has been informally adopted to maintain the integrity of tissue protein. Testicular tissues or the whole explants were analyzed for histological evaluation and the morphological alterations assessment.

Validation study settings

During the first part of the experiment, specific analyses were performed to address the assay and array robustness of the complete RPPM technique as an adapted experimental tool in the field of in vitro spermatogenesis evaluation. Contribution to signal variation was investigated with regard to several aspects. How repetitive sample preparation affect signal reproducibility? Were the majority of protein expression signals stable after archiving chips during a half-year? Did the testicular age of initial fresh testicular tissue (2.5 or 6.5 dpp) and culture duration (D30, D36, D38 or D60) using several culture media (BM, BM Rol, BM FSH + LH) affect the array results? To evaluate the impact of testicular age and culture duration on fresh in vitro culture and to confirm that the RPPM technique is an adapted experimental tool in the field of in vitro spermatogenesis evaluation, a methodology previously described by our laboratory was processed (Arkoun et al., 2015). Briefly, in vitro culture media used for this study were (i) basic medium (BM) alone composed of phenol red-free α-MEM plus 10% (v/v) KnockOut™ serum replacement (KSR) (10828–010; Gibco®) and
5 μg/ml gentamicin (G1397; Sigma-Aldrich®; Saint-Quentin Fallavier), (ii) BM supplemented by Rol (BM + Rol) (all-trans retinol; R7632; Sigma-Aldrich®) at a concentration of 10−6 M (Travers et al., 2013) and (iii) BM supplemented by FSH (Puregon®; Merck & Co. Inc., Courbevoie, France) and LH (Gonadotrophine Chorionique Endo®; Merck & Co. Inc.) at 500 and 50 IU/l (BM FSH + LH), respectively (Arkoun et al., 2015). At D30, D36, D38 and D60, nine testicular explants were pooled to form a sample replicates and 2–3 biological protein RPPM samples replicates per condition were obtained at a concentration of a minimum of 1 mg/ml of proteins—after removal of the central necrotic part of the tissue representing about half of the mass of the explants. Other testicular explants were fixed in Bouin’s solution (HT10132; Sigma-Aldrich®) for further morphological evaluations. Tissues of 2.5, 6.5, 36.5, 40.5, 42.5 and 62.5 dpp were used as in vivo controls.

**Principal study**

To determine which culture media allows proliferation and differentiation of SSCs of fresh, CSF and SSV testicular tissues, six biological replicate samples (composed of four testicular fragments, each) were cultured in 6-well plate ((30184; Thermo Fisher Scientific, Saint-Aubin, France). It was decided to avoid the supplementation of LH in the principal study to focus on the in vitro antagonist effects of FSH and Rol during culture. Indeed, it was shown that the testosterone production induced by LH supplementation suppressed the recovery of spermatogonial differentiation in a dose-dependent manner (Tohda et al., 2001). In addition, an alternate supplementation of Rol (performed every 8 days to mimic the physiologic temporal pattern of entry into differentiation of spermatogonial) and FSH was chosen considering their well-known antagonist effects, recently highlighted on comparable in vitro culture systems (Arkoun et al., 2015). In vitro culture media were (i) BM supplemented by Rol (BM_{Rol+}), (ii) BM supplemented by FSH (BM_{FSH}), (iii) BM alternatively supplemented by Rol and FSH (BM_{Rol+FSH}), (iv) BM supplemented by Rol plus Vit. E (T3251; Sigma-Aldrich®) at 3.4 μM (BM_{Rol+Vit. E}) and (v) StemPro®–34 SFM medium with growth factors (StemPro®–34 SFM). The StemPro®–34 SFM medium was adapted from previous study of Shinoara (Kanatsu-Shinohara et al., 2011): customized phenol red-free StemPro®–34 SFM medium (1001229; Gibco®) supplemented with StemPro®–34 Nutrient supplement (10641–025, Gibco®), insulin-transferrin-selenium (10.5, 5.5 and 6.7 mg/ml, 41400–045, Gibco®), 6 mg/ml D(-)-glucose (G6152; Sigma-Aldrich®), 2 mM L-glutamine (25030–024; Gibco®), 1% Non-Essential Amino Acids solution (11140–035; Gibco®), 1% vitamin solution (11120–037; Gibco®), 50 μg/ml gentamycin (G1397; Sigma-Aldrich®), 1 mM sodium pyruvate (11360–039; Gibco®), 0.1 mM vitamin C (A4403; Sigma-Aldrich®), 1 μg/ml L-(-)-lactic acid (L1750; Sigma-Aldrich®), 30 ng/ml β-estradiol (E2758; Sigma-Aldrich®), 60 ng/ml progesterone (P0130; Sigma-Aldrich®), 0.2% bovine serum albumin (BSA; A7906; Sigma-Aldrich®), 1% KSR, 20 ng/ml mouse EGF (L1750; Sigma-Aldrich®), 10 ng/ml bFGF (PMG0034; Gibco®), 10 ng/ml GDNF (G1401; Sigma-Aldrich®) and 103 U/ml LIF (L5158; Sigma-Aldrich®). At D30, for each culture plate, a total of 18 testicular explants were pooled to form two biological protein RPPM samples and six testicular explants were fixed in Bouin’s solution for further morphological evaluations. Tissues of 6.5 and 36.5 dpp were used as in vivo controls to evaluate the impact of cryopreservation procedures and culture, respectively.

**Preparation of tissue lysates**

Testicular tissue fragments were collected after culture, thoroughly washed with phenol red-free α-MEM medium and further processed to generate lysate extracts used for protein array analysis. In vivo and in vitro testes were incubated under denaturing conditions in lysis buffer composed of 20 mM of Heps (pH = 7.9; H4034; Sigma-Aldrich®), 1 mM of MgCl₂ (M8266; Sigma-Aldrich®), 1% of Nonidet™ P 40 Substitute solution (98379; Sigma-Aldrich®), 0.5% of sodium cholate (C6445; Sigma-Aldrich®), 0.25% of n-Dodecyl β-D-Maltoside (324355; Merck Millipore, Billenica, MA, USA), 1 mM of sodium orthovanadate (450243; Sigma-Aldrich®) and 50 mM of sodium fluoride (201154; Sigma-Aldrich®) with freshly added protease and phosphatase inhibitors (87785; Thermo Fisher Scientific Inc., Waltham, MA, USA). Tissues were homogenized for 20 s with a mechanical pellet pestle (2359971–1EA; Sigma-Aldrich®) and lysed on ice for 20 min. After centrifugation at 14 000 g during 20 min, crude extract supernatants were stored at −80°C. Total protein concentrations were determined for all lysates according to bichoninic acid assay colorimetric test (Pierce™ BCA protein assay kit; E9981L; Thermo Fisher Scientific Inc).

**Reverse phase protein microarray**

Protein expression profiling was performed according to the recommendation given by the ‘preparation and use of reverse protein microarrays’ (Pin et al., 2014) and by the protocol developed for the validation of the RPM technique (Peet et al., 2016).

**Samples and reagents**

National and international efforts are underway to develop, catalog and validate well-characterized libraries of high-quality affinity reagents that can be used by the community:

- Antibodypedia at https://www.antibodypedia.com/
- Antibodyresource at http://www.antibodyresource.com/home
- Institut Curie at http://parys.cure.fr/ (inside Institut Curie only)
- MD Anderson Cancer Center at https://www.mdanderson.org/research/research-resources/core-facilities/functional-proteomics-rppa-core/antibody-information-and-protocols.html

Validation methods involving various forms of manipulation such as immunohistochemistry, treatment of phosphoproteins with phosphate, knockdown of the gene of interest using siRNA and studies of knockout material were assessed for the majority of the antibodies used in this study. Moreover, all antibodies were subjected to a stringent validation procedure by western blot (a single or explainable multiple bands to be observed) on a panel of standard cell lines (i.e. Nalm-6, glioblastoma CB74) and tissue samples (i.e. bovine, human xenografting) followed by a ‘reverse’ validation by RPM (Peet et al., 2016). An overview of the experimental set-up of the principal study is available in Fig. 2 and the list of antibodies against the 29 antigens, purchased from Cell Signaling Technology (Ozyme®, Saint-Quentin en Yvelines, France) and from Dako (Dako®, Les Ulis, France), is detailed in Supplementary Information Table S1. An overview of protein–protein interactions pathways of the 29 antigens assessed in the current study is available in Supplementary Information Fig. S1. Secondary antibodies were labeled with a fluorochrome emitting in near infrared (680 nm) (LI-COR Biosciences®, NE, USA).

**Array production**

After protein quantification, the lysates were placed into 384-well plates in printing buffer composed of 250 mM Tris(hydroxymethyl)aminomethane (252859; Sigma-Aldrich®), 50% (v/v) glycerol (G8773; Sigma-Aldrich®), 4% (v/v) sodium dodecyl sulfate (L3771; Sigma-Aldrich®), 10% (v/v) β-mercaptoethanol (M7154; Sigma-Aldrich®) and 0.1% (v/v) Tween® 20 (P1379; Sigma-Aldrich®) in ddH₂O water (34877; Sigma-Aldrich®) to obtain a uniform protein concentration. Each pooled biological repetition lysate was spotted at 1:1 and 1:2 starting at an ideal total protein...
concentration of 1 mg/ml; each dilution was spotted in technical duplicate. Sixteen arrays of 12 × 14 (168) and 12 × 13 (156) spots were generated on each chip for the two studies (see chip and array layout of the principal study in Supplementary Information Fig. S2A). Chips processed in the principal study were scanned 72 h after experiments to limit any variation. A control slide corresponding to total protein concentrations was determined by the Fast Green FCF method (Luo et al., 2006) commonly used for staining connective tissues in histological samples (Loebke et al., 2007).

Protein samples were printed onto nitrocellulose-coated glass slides (2UNY2GW00600616G; Sartorius®, Aubagne, Germany) with an automated robotic SpotBot® 3 microarrayer (SPA3PRO; Arrayit Corporation®, CA, USA) under a temperature of 25°C with a relative humidity of 60–70%. This contact method allows ~500 pl of protein lysate to be transferred to the nitrocellulose glass slide per array pin touch (65.5 μm microelectrode). After the printing process, slides were stored overnight at 4°C for a complete binding of proteins to nitrocellulose. All the arrays were produced under cleanroom conditions with a lateral distance of 1.8 mm, a vertical distance of 1.6 mm and spot-to-spot distances of 380/380 μm (x/y direction). After printing, the microarrays were blocked with 50% of Odyssey blocking buffer (927–40000; Eurobio®) in PBS for 1 h at room temperature (RT).

Assay procedure
For each protein of interest, primary antibodies were incubated on a dry RPPM for 2 h at 4°C. After the removal of excess antibody and subsequent washing with array buffer (PBS with 0.1% Tween 20), the microarrays were incubated with IRDye®-labeled goat anti-rabbit (1:2000; 926–68021; LI-COR Biosciences®) or anti-mouse (1:2000; 926–68020; LI-COR Biosciences®) IgG (H + L) for 1 h at RT in the dark. Subsequently, the arrays were washed and scanned with an InnoScan 710-IR scanner (Innopsys®; Carbonne, France). Read-out was performed at excitation wavelengths of 670 and 785 nm while avoiding support background intensity signals. The Fast Green FCF maximum dye absorption at 625 nm was turned out to be detectable in the near-infrared range at 700 nm (Loebke et al., 2007).

For each RPPM, four fluorescence images per slide (3 slides per study) were recorded at several gain powers between 10 and 70 and stored in 16-bit in tiff format for further data analysis. One entire slide was stained for each study to measure the relative amount of immobilized protein per spot. Chips were blocked and then incubated with Fast Green FCF (F7258; Sigma-Aldrich®) according to the manufacturer’s instructions.

Microarray analysis and data processing
Microarray images, selected at the highest exposure time without saturation, were generated with the Arrayit® software (Arrayit Corporation—ARYC) and analyzed using the Mapix® microarray controller, qualification and analysis software (Innopsys). Background-corrected relative fluorescent unit intensities of the technical duplicate spots were averaged for each lysate sample. To correct the assay signals for non-specific binding contribution of secondary antibody
(blank signals, β), images of the blank assay experiment (α) were analyzed and the blank images subtracted from the assay images (α – β), resulting in blank-corrected signals (Supplementary Information Fig. S2B). Then, this blank-corrected signal was normalized to the total protein concentration by making a ratio between the blank-corrected signals of the assay images with the blank-corrected signals of the Fast Green FCF slide. The final signals resulted in normalized blank-corrected signal values. Pooled biological replicate intensities were averaged and Log2 transformed for further analysis.

### Immunohistochemistry, kinetic and DNA fragmentation assessment

**Immunohistochemistry**

Testicular sections and explants used for the evaluation of morphological alterations were fixed in Bouin’s solution for 2 h at RT then dehydrated in a graded series of ethanol washes and embedded in paraffin. Sections (3 μm thick) were cut using a microtome (JungRM 2035; Leica Microsystems© GmbH). Five serial tissue sections were mounted on each Polyvinyl alcohol (PVA) slide (28000ANZ; Thermo Fisher Scientific Inc., Waltham, MA, USA). For each repetition of each experiment, three slides, representing the two side-ends and the middle of the sample, were examined to obtain a more accurate and global assessment of the tissue (Dumont et al., 2015; 2016). Hemalun eosin saffron (HES) and periodic acid-Schiff (PAS)-hemalun (RAL diagnostic, Martillac, France) stainings were chosen for the analysis of (i) in vivo pre-pubertal testicular tissue and (ii) in vivo pubertal testes and in vitro testicular tissue fragments, respectively. To estimate clearly the different stages of germ cells, testicular tissue sections were immunostained using antibodies directed against (i) TRA98 (JLP Antibody; 1:50; ab25227; Abcam, Paris, France), specific for spermatogonia as well as leptotene/zygotene and early pachytene spermatocytes I or (ii) CAMP responsive element modulator (CREM, X-12; 1:50; sc-440; Santa Cruz Biotechnology Inc., Heidelberg, Germany), to facilitate the detection of post-meiotic germ cells. Diaminobenzidine substrate (TA-060-HDX; Microm Microtech) was used as a chromogen. Tissue sections were counterstained with haematoxylin (S2020; Dako) and then observed under a light microscope (DM4000B©; Leica Microsystems© GmbH) equipped with Leica Application Suite© software (LAS©; Leica Microsystems© GmbH).

**Kinetic evaluation of the StemPro®—34 SFM medium with growth factors**

To evaluate the impact of the StemPro®—34 SFM medium (with growth factors) on the germ cell kinetic of proliferation and differentiation during the first wave of spermatogenesis, morphological evaluations were independently performed after the examination of at least 30 seminiferous tubules at D0, D5, D8, D15, D22 and D30, corresponding to 6.5 (before meiosis initiation), 11.5 (leptotene/zygotene spermatocyte I), 14.5 (pachytene spermatocyte I), 21.5 (round spermatid), 28.5 (elongating and elongated spermatid) and 36.5 dpp (spermatozoon), respectively. Serial digital images were obtained with a light microscope equipped with LAS®.

**DNA damages evaluations**

TUNEL procedure was performed on explants using an In Situ Cell Death Detection Kit (1168417910; F. Hoffmann-La Roche SA, Penzberg, Germany) according to the manufacturer’s recommendations and specific evaluations (Labat-Moleur et al., 1998). Seminiferous tubules present on the peripheral and intermediate area were counted and tubules located on the central necrotic—receiving insufficient nutrients and oxygen—area were excluded for analysis to avoid ‘false TUNEL-positive cells’ linked to the organotypic technology itself and not to the culture media nor the cryopreservation procedure. However, depending on the size of the explant or its necrotic area, the surface could differ. The central necrotic area was identified under a stereomicroscope (Leica S8 APO; Leica Microsystems© GmbH) with the use of a microscopy halogen light source (KL 1500 LCD; Leica Microsystems© GmbH).

### Bioinformatics and statistical analyses

**RPPM data analysis** was carried out using the AMEN suite of tools (Chalmel and Primig, 2008) and the FactoMineR (Lê et al., 2008), missMDA (Josse and Husson, 2013) and LIMMA (Smyth, 2004) packages implemented in the R statistical environment (2011—R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/).

**Imputation of the missing values**

We first used the impute PCA function from the R package missMDA to impute the missing values of the RPPM dataset with a principal component analysis (PCA) model performed with the R package FactoMineR. The number of dimensions for the PCA analysis was estimated by using the estim_ncpPCA function (FactoMineR).

**Statistical filtration**

The linear models for microarray data package (LIMMA) implemented in AMEN were next used to define the set of protein markers displaying significant statistical changes across comparisons (F-value ≤ 0.05).

**PCA and data clustering**

RPPM data of differentially expressed proteins were sequentially averaged, scaled to unit variance and then projected on orthogonal components in which differentially expressed protein markers were considered as variables and experimental culture conditions as individuals (FactoMineR). Next, the estim_ncpPCA function was applied to find the best number of components. Finally, we performed an agglomerative hierarchical clustering using the HCPC function on the coordinates of the individuals on the selected principal components. Individuals were automatically partitioned in clusters of experimental culture conditions showing close similarities in their protein expression patterns.

**Correlation matrix**

The correlation matrix of final protein-normalized signals and morphological evaluations was based on Tiyun Wei, 2013—Corrplot: visualization of a correlation matrix—https://cran.r-project.org/web/packages/corrplot/ with a Spearman’s correlation coefficient (with $r > 0.7$ considered as correct for a correlation).

**Statistics**

Other statistical analyses were performed with GraphPad Prism version 6.00 (GraphPad Software, La Jolla, CA, USA). The Mann—Whitney test was used for unpaired rank comparisons and the nonparametric Wilcoxon test was used for paired rank comparisons determined by a unilateral one-tailed. The one-way ANOVA test was used for the evaluation of the assays reproducibility, followed by a Tukey’s multiple comparison post hoc test. Data are presented as the means ± SEM and $P < 0.05$ was considered to be significant.

### Results

**Testicular age and culture duration of fresh testicular tissue**

**Validation of the RPPM assay and array robustness**

Inter- and intra-array variations—which contributed to the overall reproducibility—were determined individually according to the coefficient of variance. All variations of this study were in the approval
limits of the US Food and Drug Administration and the European Medicines Agency for spot-to-spot signal (~3 and 3–5%, respectively), intra-array signal (~10 and 6–13%, respectively) and inter-array signal (~20 and 7–14%, respectively), suggesting that repetitive sample preparation did not affect the signal reproducibility. Among the chips scanned after more than 24 weeks, 21 of the 29 selected proteins were considered as having a similar fluorescence intensity (Supplementary Information Fig. S3), confirming the reproducibility of the RPPM data over a long-term and showing that assays correctly reproduced their protein expression signals after a half-year.

**Cultures of 6.5 dpp tests close to in vivo controls**

All in vivo testicular tissues (36.5, 40.5, 42.5 and 62.5 dpp) have a similar protein profile, allowing them to cluster into a single entity, excluding at the same time in vitro testicular tissues (Supplementary Information Fig. S4A and C). In vitro cultures performed with testicular tissues from 2.5 dpp mice and cultured until D38 and D60 presented a very distant protein profile compared to their corresponding in vivo controls (40.5 and 62.5 dpp, respectively). Interestingly, tissue cultured from 2.5 dpp were highly affected by apoptosis mechanisms (i.e. p53, Puma, Bax, pFADD, Fas, Akt) and cell-growth survival (i.e. mTOR) whereas those cultured from 6.5 dpp were related to autophagy (i.e. Beclin-1, Atg12), stress-differentiation (i.e. C-abl) and proto-oncogene (i.e. C-kit, C-myc, C-fos) (Supplementary Information Fig. S4D). Caspase_9—corresponding to the ratio of cleaved Caspase 9 on total Caspase 9 over a long-term and showing that assays correctly reproduced their protein pro.

**Cryopreservation procedures, in vitro culture and media**

**Principal study settings**

During the study performed with fresh testicular tissue only, the concentration of proteins was low (0.8–1.2 mg/ml for explants from 2.5 dpp and 0.4–0.9 mg/ml for explants from 6.5 dpp) (Supplementary Information Table S2). Typical protein concentrations of tissue lysates in the principal study were 10.5 ± 4.10 μg/ml for in vivo 36.5 dpp, 6.5 dpp and D30 in vitro cultures, respectively (Supplementary Information Table S3).

**Proteomic evaluation of cryopreservation procedures, in vitro culture and media**

Compared to 6.5 dpp fresh tissue (FT), testicular tissues of 36.5 dpp mice were similar to in vivo controls exhibited a basal protein expression with low levels of Akt—corresponding to the ratio of phosphorylated Akt on total Akt (4–fold) and Atg12 (8-fold) (Fig. 3A). The impact of the cryopreservation procedures on 6.5 dpp in vitro testicular tissues was assessed for CSF (Fig. 3B1) and SSV (Fig. 3B2) in comparison to FT. There was no significant variation of apoptotic or autophagic protein levels in testicular tissues after CSF. However, the expression of Bcl-2 (4-fold) and pFADD.Fas—corresponding to the ratio of phosphorylated FADD on Fas—(4-fold) increased after SSV, which could be linked to a harmful impact of this cryopreservation procedure on pre-pubertal testicular tissue.

The impact of in vitro culture system on 6.5 dpp testicular explants cultured until D30 (with several media) was assessed for FT (Fig. 3C1, S3). CSF (Fig. 3D1, S5) and SSV (Fig. 3E1, S5) compared with corresponding 6.5 dpp in vivo controls. For FT, the expression of Caspase_9—corresponding to the ratio of cleaved Caspase 9 on total Caspase 9 was reduced for BM/FSH (4-fold), BMRol/FSH (4-fold) and BMRol/Vit.E (4-fold) whereas expressions of Bcl-2 (8-fold for BMRol/Vit.E) and Beclin-1 (4-fold for BMRol/FSH and BMRol/Vit.E) were increased. For CSF, expressions of pFADD.Fas (4-fold for BMRol/Vit.E and StemPro®–34 SFM) and SAPK—corresponding to the ratio of phosphorylated SAPK on total SAPK—(8-fold for BMRol/−) were reduced whereas expressions of Bcl-2 (4-fold for BMRol/FSH, BMRol/Vit.E and StemPro®–34 SFM), Beclin-1 (4-fold for BMRol/FSH and BMRol/Vit.E) and C-kit (4-fold for BMRol/FSH) were increased. For SSV, expressions of pFADD.Fas (4-fold for BMRol/− and BM/FSH; 4-fold for BMRol/FSH and BMRol/Vit.E) and SAKP (16-fold for BMRol/−) were increased whereas the expression of Bcl-2 (4-fold for BMRol/FSH) was increased.

Conditions allowing only small protein expression fluctuations compared to their respective controls were BMRol/− for FT, StemPro®–34 SFM for FT and StemPro®–34 SFM for testes cryopreserved by SSV.

**Histological and morphological evaluations of cryopreservation procedures, in vitro culture and media**

The in vivo 6.5 dpp testicular tissue architecture was slightly affected by cryopreservation procedures. Indeed, SSV did not have any impact on the morphological parameters evaluated whereas only the cell density per seminiferous tubule was higher after CSF (8.9 ± 2 cells/1000 μm²) than FT (7.6 ± 0.2 cells/1000 μm², P = 0.0079) (Fig. 4C). A harmful impact of CSF on testicular tissues after D30 was observed depending on the culture medium tested. Indeed, with BMRol/Vit.E, the seminiferous tubules area was higher for FT (9711 ± 855 μm²) compared to CSF (4125 ± 1013 μm², P = 0.0286) (Fig. 4A) whereas the cell density was lower for FT (3.6 ± 0.3 cells/1000 μm²) than CSF (6.0 ± 0.5 cells/1000 μm², P = 0.0286) (Fig. 4C). Moreover, with BMRol/FSH, the proportion of tubules with round and elongated spermatids was higher for FT (8.9 ± 1.5% and 11 ± 4.7%, respectively) than CSF (2.4 ± 1.3%, P = 0.0173 and 1.8 ± 1.3%, P = 0.0216, respectively) (Fig. 4D). The harmful impact of the SSV procedure on the testicular tissue after D30 was assessed. Indeed, even if the seminiferous area of tubules observed at D30 was higher for FT than SSV with BMRol/− (7778 ± 1077 μm² vs. 4048 ± 701 μm², P = 0.0173) and BMRol/Vit.E (9711 ± 855 μm² vs. 4717 ± 524 μm², P = 0.0159) (Fig. 4A), the cell density was higher with BMRol/Vit.E for SSV (3.6 ± 0.3 cells/1000 μm²) in comparison to FT (4.9 ± 0.3 cells/1000 μm², P = 0.0317) (Fig. 4C). Finally, the effect of CSF and SSV on the testicular tissue after D30 was compared. The seminiferous tubule area observed at D30 was higher for CSF than SSV with BMRol/− (6810 ± 796 μm² vs. 4048 ± 701 μm², P = 0.0303) but lower with BMRol/FSH (7736 ± 1395 μm² vs. 15834 ± 2017 μm², P = 0.0087) (Fig. 4A). Moreover, the total number of cells was higher for SSV (35 ± 2.8 cells) in comparison to CSF (25 ± 4.1 cells, P = 0.0152) with BMRol/− (Fig. 4B). However, with BMRol/FSH and BMRol/Vit.E, the proportion of tubules with round spermatids was higher for SSV (14 ± 3.0% and 19 ± 0.0% of the testicular tissue area) in comparison to CSF (10 ± 1.2% and 13 ± 0.8% of the testicular tissue area) for BMRol/−.
For all the culture protocols, except with StemPro®-34 SFM, an achievement of the spermatogenesis was obtained with the production of round and elongated spermatids (Fig. 4D). However, testicular fragments cultured until D30 with StemPro®-34 SFM exhibited better architectural patterns than CSF (6788 ± 1435 μm² vs. 3652 ± 163 μm², P = 0.0357) and CSF (4039 ± 306 μm² vs. 3075 ± 98, P = 0.0317) (Fig. 4A).

DNA damages evaluations of cryopreservation procedures, in vitro culture and media

Regarding cell DNA damages, differences were observed between cryopreservation procedures performed with the same culture medium (Fig. 5). Indeed, there is more DNA damages for BMRol + Vit. E after SSV (4.12 ± 0.26%) than CSF (1.86 ± 0.12%, P = 0.0022) or FT (2.69 ± 0.33%, P = 0.0108). However, we notice that the proportion of TUNEL-positive cells compared to their respective 6.5 dpp controls was similar for FT (2.54 ± 0.22 vs. 2.69 ± 0.33%) and SSV (4.00 ± 0.68 vs. 4.12 ± 0.26%).
Figure 4 Morphological evaluations of 36.5 dpp in vivo testicular tissues and of testicular explants obtained after an in vitro 30-day-organ culture of fresh, slow-frozen and vitrified testes. The effects of in vitro culture conditions (BM_Rol-, BM_FSH, BM_Rol+FSH, BM_Rol + Vit. E and StemPro®-34 SFM) on culture explants were evaluated via the area of seminiferous tubules (A), the total cell number per seminiferous tubule (B) and the cell density per tubule (C). (D) Percentage of seminiferous tubules containing differentiated germ cells at the most advanced stage, obtained after an in vitro 30-day-organ culture of fresh, slow-frozen and vitrified testes. Columns are presented as the mean ± SEM with n = 6. Letters a, b and c represent a statistically significant difference for FT vs. CSF, FT vs. SSV and CSF vs. SSV, respectively. *P < 0.05, **P < 0.01 in comparison to the corresponding 6.5 dpp control.

BM, basic medium; CSF, controlled slow freezing; dpp, days postpartum; FT, fresh tissue; FSH, follicle-stimulating hormone; ns, non-significant; Rol, retinol; SSV, solid surface vitrification; Vit. E, vitamin E.
Kinetic evaluation of the StemPro®–34 SFM medium with growth factors

A specific study of the action of the StemPro®–34 SFM medium (with growth factors) on the kinetics of the first spermatogenesis (Supplementary Information Fig. S5) revealed that the cell density increased from D15 (8.8 ± 0.4 cells/1000 μm²) to D22 (18 ± 1.7

Figure 5  Representation of DNA fragmentation in 36.5 dpp in vivo testicular tissues and in testicular explants obtained after an in vitro 30-day-organ culture of fresh, slow-frozen and vitrified testes. (A) TUNEL-positive nuclei, corresponding to DNA fragmentation, are shown in insets at a higher magnification for each photomicrograph. Representation of the effects of the cryopreservation procedure on 6.5 dpp in vivo testicular tissues for CSF (b2) and SSV (b3). Representation of the effects of in vitro culture conditions—BM_{Rol}, (c1–c3), BM_{FSH} (d1–d3), BM_{Rol/FSH} (e1–e3), BM_{Rol} + Vit. E (f1–f3) and StemPro®–34 SFM (g1–g3)—on culture explants. Scale bar = 20 μm (magnification ×500). (B) Representation of the DNA damages per seminiferous tubules obtained after in vitro 30-day-organ cultures of fresh, slow-frozen and vitrified testes. Whiskers on each plot extend from minimum to maximum values of DNA damages within a given group. The middle line in each box represents the median and the bottom and top of each box represent the 25th and 75th percentile, respectively, with n = 6. The red line represents the median of the 36.5 dpp control. *P < 0.05, **P < 0.01 in comparison to the corresponding 6.5 dpp control or between cryopreservation procedures. BM, basic medium; CSF, controlled slow freezing; dpp, days post-partum; FT, fresh tissue; FSH, follicle-stimulating hormone; ns, non-significant; Rol, retinol; SSV, solid surface vitrification; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling; Vit. E, vitamin E.

vs. 4.12 ± 0.26%) or even smaller for CSF (1.86 ± 0.12 vs. 2.62 ± 0.25%, P = 0.0390). Moreover, there is more DNA damages in cultures carried out with StemPro®–34 SFM after CSF (3.25 ± 0.17%) than SSV (2.42 ± 0.25%, P = 0.0108) or FT (1.83 ± 0.12%, P = 0.0022). However, we notice that their proportion of TUNEL-positive cells is smaller than their respective 6.5 dpp controls.
cells/1000 μm², P < 0.0001) and then decreased at D30 (7.4 ± 0.5 cells/1000 μm², P < 0.0001). Moreover, the germ to Sertoli cell ratio decreased from D22 (3.56 ± 0.40) to D30 (0.67 ± 0.11, P < 0.0001). At D22 and D30, numerous gap formations were observed, suggesting that the StemPro®−34 SFM medium (with growth factors) has a harmful impact after 15 or 22 days of culture.

Main apoptotic and autophagic pathways involved at the first spermatogenic wave—RPPM profiling results coupled with tissue analyses and morphological evaluations

All samples were subjected to a hierarchical clustering using final protein-normalized signals, DNA damages, histological and morphological evaluations to reveal further interesting aspects in terms of...
protein-expression profiling. The pubertal 36.5 dpp in vivo testicular tissues, having a very distant protein profile in comparison to the 6.5 dpp in vivo tissues and the in vitro explants, segregated alone (Fig. 6A and C). In vivo 6.5 dpp testicular tissues clustered into a single entity, excluding at the same time the in vivo 36.5dpp control and in vitro cultured tissues (Fig. 6A and C). The three protein profiles corresponding to the explants cultured with StemPro®−34 SFM were similar and clustered into a single entity, near to the 6.5 dpp in vivo controls (Fig. 6A and C). Not surprisingly, many of the protein signals expressed in the explants cultured with StemPro®−34 SFM were very low, suggesting a dysregulation in apoptotic and autophagic pathways. The other in vitro protein profiles aggregated in one large cluster with the exclusion of the explants vitrified and cultured with BM-/FSH (Fig. 6A and C). Indeed, 17 of 19 proteins were highly expressed (in red) on the expression profile of vitrified tissues cultured with BM-/FSH (Fig. 6A). In particular, the expression of proteins related to apoptosis (i.e. Akt, total Bad), autophagy (i.e. Atg3), stress - differentiation (i.e. C- abl) and proto-oncogene (i.e. C-kit, C-fos, C-myc) was markedly different from all other conditions. These observations are reflected by distances between the samples in the hierarchical clustering heatmap representation (Fig. 6A).

All morphological evaluations assessed for this study were reported in the variable factor map (Fig. 6D). Protein expressions of Caspase_9 and Atg5 associated with the proportion of elongated spermatids and the total cell number tended to be very different between the in vivo and in vitro conditions. Despite the integration of TUNEL experiments, no association between DNA damages and a specific apoptosis protein was highlighted in the in vitro system used in this study.

Concerning apoptosis, correlations are mainly observed between Caspase_9 and pro-apoptotic proteins involved in the regulation of the intrinsic pathway via the inhibition of pro-survival proteins (Fig. 7). Indeed, (i) Bad (P < 0.0001 and $r^2 = 0.77$) inhibits Bcl-xL, (ii) Puma (P < 0.0002 and $r^2 = 0.76$), transcriptionally activated by p53, inhibits Bcl-2 and (iii) Bim (P < 0.0004 and $r^2 = 0.73$) inhibits Bcl-xL and Bcl-2. All these proteins lead to the regulation of apoptotic pathways related to the secretion of cytochrome c, the formation of the apoptosome and the generation of Caspase_9 (Hengartner, 2000). Concerning autophagy, a positive correlation between Atg12 and Atg3 ($P < 0.0005$ and $r^2 = 0.72$) was observed. Moreover, the correlation between Caspase_9 and the autophagic actor Atg5 (P < 0.0003 and $r^2 = 0.74$) was observed. Surprisingly, a positive correlation between C-kit and Caspase_3 (P < 0.0002 and $r^2 = 0.76$) and Beclin-1 (P < 0.0003 and $r^2 = 0.75$) was observed.

![Figure 7](https://academic.oup.com/molehr/article-abstract/23/11/738/4430957)
Discussion

In this study, we have evaluated the complex impact of cryopreservation procedures and in vitro culture for apoptosis, autophagy, differentiation and proliferation on the seminiferous gonial cell fate at different developmental stages during the first spermatogenic wave. Indeed, a disturbance in the balance between pro- and anti-apoptotic proteins expression during in vitro culture could be one of the reasons for altered apoptosis in less effective (or impaired) spermatogenesis. Unfortunately, apoptotic and autophagic mechanisms underlying physiologic or abnormal spermatogenesis during spermatogenic wave are poorly understood.

We showed that the use of germ cells at spermatogonia stage (at 6.5 dpp) instead of gonocytes (at 2.5 dpp) show protein expression closer to corresponding in vivo profiles at the end of the culture. These findings observed with the targeted proteomic analysis are consistent with those described on morphological analyses performed previously in the laboratory (Arkoun et al., 2015), confirming that the RPPM technique could be an adapted experimental tool in the field of in vitro spermatogenesis evaluation. In vitro cultures performed with testicular tissues from 6.5 dpp mice cultured until D30 were closer to in vivo controls.

Immunohistochemistry and morphological evaluations are not able to assess protein expression levels and could suffer from intra- and inter-laboratory variability. We have established a new functional protein expression profiling approach combining the opportunities presented by RPPM technology—requiring small amount of biological material while allowing the analysis of a given protein in a large collection of samples using phosphospecific antibodies (Pawelcz et al., 2001)—and classical in vitro tissue culture evaluations. In the present study, near-infrared detection systems indicate that RPPM has adequate sensitivity to detect antigens from a very limited total protein content input, as previously described (Dupuy et al., 2009). Indeed, RPPM represents a high-throughput technique suited for quantitative or semi-quantitative detection of signaling proteins present in low abundance with high sensitivity (picomole-femtomole range) and precision (coefficient of variance <1.5%) (Liotta et al., 2003).

Moreover, experiments performed during this study provided valuable information about the robustness of the entire analysis system for a broad panel of molecular analyses. These features allow functional studies to be performed on clinical testicular human tissue analyses in the future. However, before this new approach can be routinely used in biomedical research, assay sensitivity and robustness needs to be proven in human testicular tissue and proof-of-concept experiments must be performed.

Concerning cryopreservation procedures, long-term impact of cryopreservation procedures is very limited since the architecture of seminiferous tubules is well-preserved, as previously observed in other culture conditions (Dumont et al., 2015). The present study showed that the 29 proteins involved in apoptotic or autophagic pathways were unchanged for slow-frozen 6.5 dpp testicular tissues. Moreover, a prolonged exposure with high concentrations of low molecular weight cryoprotectants during the vitrification technique can cause severe toxicity (Yavin and Arav, 2007; Dumont et al., 2015) or apoptosis initiation (Curaba et al., 2011) compared with the controlled slow freezing procedure. Vitrification, requiring the use of cryoprotectants in high quantities, leads to the activation of some precursors involved in the extrinsic apoptotic pathway (Orrenius et al., 2011), which could explain the high expression of apoptotic proteins after SSV. The overexpressed pFADD.Fas ratio level observed after SSV procedure on 6.5 dpp in vivo testicular tissues suggest a harmful activation through the death-inducing signaling complex. Indeed, it was recently shown that the use of DMSO during vitrification activate early apoptotic pathways in testicular tissue during the first 3 h of in vitro culture with a high expression of Fas-L and Fas (Hajaghahelou et al., 2016), suggesting a rapid phosphorylation of FADD. Nevertheless, the tendency of the pFADD.Fas ratio to be intensely down-regulated at D30 in vitrified tissues suggests a beneficial up-regulation of Fas through the culture. Even, if Fas is expressed at low levels in the mouse testis, the ‘Fas system’ has been proposed as a key regulator of physiological germ cell apoptosis (Tesarik et al., 2001). Indeed, high levels of Fas in rat were shown to be associated with a regulation of germ cell and a spermatocyte apoptosis during the first spermatogenesis wave (Lizama et al., 2007).

In the current study, a relative low number of TUNEL-positive cells per seminiferous tubule were observed on in vivo controls and after in vitro cultures; as observed in a recent study showing the preservation of seminiferous tubule integrity after long-term culture of pre-pubertal human testicular tissue (de Michele et al., 2017). These observations could be explained by the well-known phagocytosis activity orchestrated by Sertoli cells (Hai et al., 2014; Xiao et al., 2014)—who can directly instigate the regulation of the apoptotic mechanisms of germ cells through the extrinsic pathway involving Fas-L (Orrenius et al., 2011)—suggesting that the phagocytosis activity of Sertoli cells was preserved during in vitro culture, even after vitrification. Moreover, the maturity of Sertoli cells (who compartmentalizes the seminiferous epithelium into basal and apical domains) was validated in recent work coupled with an establishment and a maintenance of the blood-testis barrier coupled throughout in vitro culture of fresh, slow-frozen and vitrified pre-pubertal testicular tissue from mice (Rondanino et al., 2017).

The fact that Caspase_9 and Bcl-2 tended to be highly expressed in in vitro conditions compared to in vivo could reflect the establishment of an intrinsic apoptosis regulation (mitochondrial) within the testicular tissues during culture with the various media used in this study. Moreover, the protein expressions of Caspase_9 and Atg5 associated with the proportion of elongated spermatids and the total cell number tended to be very different between the in vivo and in vitro conditions, suggesting the presence of an in vivo beneficial regulation during the first spermatogenesis wave by intrinsic apoptosis and autophagy factors. The p53 levels of cryopreserved fragments or in vitro explants were not over- or under-expressed compared to respective controls. These data suggest that cryopreservation procedures and in vitro spermatogenesis did not impair testicular tissue organization and viability by a p53 intrinsic-dependent apoptotic pathway. Indeed, a stabilization of p53 levels occurred in the first round of spermatogenesis in rats (Lizama et al., 2007) and a p53-independent apoptosis incidence after vitrification was recently observed in pre-pubertal testicular tissue from mice (Hajaghahelou et al., 2016).

The presence of C.kit significant correlations with Caspase_3 and Beclin-1 could reflect the comeback of a study performed on tissue materials and not on individual cell types, making analysis of the results even more complex. Moreover, Caspase_3 represents the final and irreversible executor of apoptosis that brings signals together from
both the extrinsic and intrinsic pathways, leading to a difficult interpretation of regulations pathways implicated. Primordial germ cells begin to divide mitotically around 3.5 dpp during which the expression of C.KIT is dramatically reduced (Orr-Urtreger et al., 1990) and the reactivation of C.KIT in postnatal testis is detected in differentiating SSCs, but not in undifferentiated SSCs (Yoshinaga et al., 1991; Schrans-Stassen et al., 1999), and KIT ligand (also called stem cell factor) is necessary for the proliferation of putative stem Leydig cells (Liu et al., 2017). Taken together, these observations leave an open question of whether other C.KIT+ cells exist and play important roles in postnatal development of testis (Zhang et al., 2014).

An overall evaluation of the BMRol/FSH condition with BMRol+ and BMRol− does not promote a media against one other. Independently of the supplementation of Rol, the addition of FSH or testosterone in vitro remains difficult to estimate due to the testicular tissue spatial-, dose- and time-dependent machinery. Indeed, on identical in vitro culture system, it has been reported that the addition of FSH did not change the spermatic yield (Sato et al., 2011); however, the concentration used (i.e. 200 ng/ml) was very low. Furthermore, the addition of testosterone (alone or in combination with FSH) could even lead to a sharp decrease of the spermatic performance (Sato et al., 2011).

A dysregulation of vitrified testicular tissues cultured with BMRol− was observed for the expression of protein related to stress - differentiation (i.e. C-abl) and proto-oncogene (i.e. C-kit, C-fos and C-myc) pathways, excluding this procedure in hierarchical clustering and PCA representation. These data were concordant with the presence of a large amount of abnormal and multinucleated round spermatids in testicular tissues cultured after vitriification with a medium exempt of Rol (Dumont et al., 2016). Among all various culture media tested, BMRol+VIL E is the medium showing the most beneficial effect on architecture and spermatic yield on fresh testicular tissue.

Additionally, our work showed that the StemPro®–34 SFM medium supplemented with growth factors such as GDNF, bFGF, EGF and LIF prevented the differentiation of SSCs in favor of a significant proliferation. Interestingly, comparative results were observed in human SSCs originated from testicular tissue of patients with obstructive azoospermia and cultured using StemPro®–34 SFM medium supplemented with the same growth factors followed by an in vitro survival rate maintained for a period of two months without any differentiation (Piravar et al., 2013). Previously, it was demonstrated that an atRA treatment in GDNF-overexpressing mice resulted in apoptosis of undifferentiated spermatogonial cells with an inhibition of spermatogonial differentiation (Meng et al., 2000). Indeed, GDNF and atRA signaling represents two independent means for regulating germ cell maturation and abnormal expression of GDNF suppresses spermatogonial differentiation. Thus, GDNF and Rol should therefore be added sequentially throughout in vitro cultures. Moreover, bFGF has been shown to regulate mouse SSCs proliferation via an autocrine pathway (Zhang et al., 2012) and to play a role in their survival in vitro (Kubota et al., 2004; Hofmann et al., 2005). It would therefore seems to be beneficial to perform a SSV at 6.5 dpp followed by a proliferation of SSCs during 2–3 weeks with the use of StemPro®–34 SFM medium supplemented with appropriate growth factors prior to the differentiation step with the use of Rol and antioxidant compounds may help to greatly increase the final spermatic performance.

The present study was performed on tissue materials and not on individual cell types, making analysis and interpretation of the results complex. Indeed, the niche is critical to keep the testicular environment, regulated precisely by the microenvironment of the testis (Hofmann, 2008). Therefore, it is necessary to make a choice within the key proteins to select for RPPM analysis (compared to global proteomic analysis), requiring a great understanding of mechanisms and pathways which need to be analyzed. Thus, regulation systems implicated in the proliferation of germ cells via the action of Sertoli cells (Murphy and Richburg, 2014) resulting in an increase of ‘beneficial apoptotic pathways’ (Xu et al., 2016) in the testis have to be analyzed carefully in the future.

**Conclusion**

In this study, we validated an RPPM-based readout platform, providing a very robust and sensitive analysis, allowing the screening of a large number of biological materials in the field of biological reproduction. From our findings, we can conclude that suitable cryopreservation procedures of pre-pubertal testicular tissue associated with efficient culture conditions are crucial in the fields of fertility preservation and restoration. Indeed, harmful impact of cryopreservation procedures observed after freezing is minimal at the end of the culture. Moreover, the in vitro spermatogenesis remains challenging as it strongly modifies apoptotic- and autophagy-related relative protein levels in mice testicular tissue compared to physiological in vivo process. A disturbance in the balance between pro- and anti-apoptotic proteins expression during in vitro culture could be one of the reasons for altered apoptosis in less effective (or impaired) spermatogenesis.

### Supplementary data

Supplementary data are available at Molecular Human Reproduction online.

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### Authors’ roles

L.D.: conception and design of experiments, acquisition, analysis and interpretation of data, statistics, writing manuscript and final approval of the version to be published. F.C.: RPPM bioinformatics data filtration, hierarchical clustering and PCA representations. A.O., B.B. and A.R.: provided advices during the interpretation of data. V.D.: assisted in the immunohistochemical and TUNEL experiments. C.R.: provided advices during the conception of experiments, discussion of the results and revision of the manuscript. N.R.: conception of the research, responsible for revision and critical review of the manuscript.

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Conflict of interest

The authors declare that there is no conflict of interest.

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