Antioxidant vitamins and lysophospholipids are critical for inducing mouse spermatogenesis under organ culture conditions

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Abstract
In vitro mouse spermatogenesis using a classical organ culture method became possible by supplementing basal culture medium with only the product of bovine serum albumin purified by chromatography (AlbuMAX), which indicated that AlbuMAX contained every chemical factor necessary for mouse spermatogenesis. However, since the identity of these factors was unclear, improvements in culture media and our understanding of the nutritional and signal substances required for spermatogenesis were hindered. In the present study, chemically defined media (CDM) without AlbuMAX was used to evaluate each supplementary factor and their combinations for the induction of spermatogenesis. Similar to in vivo conditions, retinoic acid, triiodothyronine (T3), and testosterone (T) were needed. Based on differences in spermatogenic competence between AlbuMAX, fetal bovine serum, and adult bovine serum, we identified α-tocopherol, which strongly promoted spermatogenesis when combined with ascorbic acid and glutathione. Differences were also observed in the abilities of lipids extracted from AlbuMAX using two different methods to induce spermatogenesis. This led to the identification of lysophospholipids, particularly lysophosphatidylcholine, lysophosphatidic acid, and lysophosphatidylserine, as important molecules for spermatogenesis. New CDM formulated based on these results induced and promoted spermatogenesis as efficiently as AlbuMAX-containing medium. In vitro spermatogenesis with CDM may provide a unique experimental system for research on spermatogenesis that cannot be performed in in vivo experiments.
INTRODUCTION

Spermatogenesis is a long and complex process, and reproducing spermatogenesis in vitro has been an elusive target.1 If this becomes possible in a number of animals, the common principal and species-specific mechanisms underlying spermatogenesis may be elucidated in more detail. This will not only expand our understanding of spermatogenesis, but also create opportunities for practical applications. In the case of humans, in vitro spermatogenesis may be an invaluable approach for fertility preservation for male juvenile patients who undergo cytoablative treatments, such as chemo- or radiotherapy for malignant diseases. By banking testis tissues, sperm may be obtained later in life through in vitro spermatogenesis.2

Attempts to achieve in vitro spermatogenesis dates back to a century ago.1 One of the successful results reported in such early period described that newborn mouse testis tissues were cultured on clot of fowl embryo extract and meiotic germ cells appeared.3 After this, however, the organ culture method did not show prominent progress in advancing the spermatogenesis. Then, cell culture method became popular and dominant since around 1980s. Although cell culture had many advantages over the organ culture method, germ cells isolated from the original seminiferous tubules hardly differentiated toward sperm formation.1 With this history in mind, we re-evaluated the organ culture method and found its potential.4 In 2011, we reported for the first time that in vitro mouse spermatogenesis, from spermatogonial stem cells to sperm, was successful using an organ culture method, which led to the production of healthy offspring by micro-insemination.5,6 This achievement was possible by using a serum replacement agent called Knockout Serum Replacement (KSR). Several independent research groups repeated this study and validated the effects of KSR in in vitro spermatogenesis.7-10 We identified AlbuMAX, which is a lipid-rich BSA produced by chromatography, as an essential component of KSR. In vitro spermatogenesis was equally supported with KSR and AlbuMAX. Of note, culture medium composed of only AlbuMAX and basic medium, α-Minimum Essential Medium (α-MEM), maintained complete spermatogenesis for more than 6 months.11 Hence, AlbuMAX contains every substance and factor needed for the induction, progression, and maintenance of mouse spermatogenesis.

In our previous study, we formulated chemically defined medium (CDM) using α-MEM as basal medium and BSA extracted by an ethanol-based method (Et-BSA), which did not exhibit spermatogenic activity by itself, as a carrier of lipid-soluble ingredients. By supplementing other ingredients, including retinoic acid (RA), retinol (Re), luteinizing hormone (LH), follicle-stimulating hormone (FSH), testosterone (T), triiodothyronine (T3), fatty acids (FAs), cholesterol (Chol), phosphatidylcholine (PC), and sphingomyelin (SM), the CDM induced mouse spermatogenesis.12 However, spermatogenesis with CDM was not successful when the testis tissues of neonatal mice, younger than 2 days old, were used. Furthermore, haploid cell production was rare with CDM, but was frequently observed with AlbuMAX medium, indicating that CDM was missing some important factors.12

In the present study, we focused on the factors needed for the induction of spermatogenesis from neonatal mouse testes. To make each culture experiment sensitive and reliable, we used testes of Acr-GFP transgenic mouse whose testicular germ cells express strong GFP in the cytoplasm of spermatocytes at mid-pachytene stage of meiosis onward.13,14 That GFP then accumulates into the acrosome which is a unique organelle appears in haploid spermatids, by which haploid cell formation could be faithfully evaluated without any additional marker staining or gene-expression studies.13 When applying Acr-GFP expression in the organ culture experiments, area ratio of Acr-GFP expression in each tissue, GFP-expression grade, functioned as a reliable measure for evaluating the initiation and progression of mouse spermatogenesis.11,12

For CDM preparation, we started with treating Et-BSA with charcoal and de-ionization procedures, which cleared the batch difference in BSA quality and significantly improved culture results. We also re-evaluated the effects of four hormones, LH, FSH, T, and T3, and demonstrated that T3 was...
the most important factor for spermatogenic induction in our system. We then showed that the combination of α-tocopherol (αT), ascorbic acid (AA), and glutathione (GSH) significantly promoted spermatogenesis. We also examined lipids in AlbuMAX and found that lysophospholipids (LPLs), specifically LPC, LPA, and LysoPS, promoted spermatogenesis. An improved version of CDM induced spermatogenesis as efficiently as AlbuMAX medium even from neonatal mouse testis tissues. These novel results expand our understanding of spermatogenesis and are the basis for further improvements in culture medium.

2 | MATERIALS AND METHODS

2.1 | Mice and treatments

Acr-GFP transgenic mice (C57BL/6 strain) were provided by RIKEN BRC through the National Bio-Resource Project of MEXT, Japan. Female ICR, C57BL/6 (CLEA Japan), or ICR × C57BL/6 F1 mice were used for breeding, resulting in Acr-GFP mice with a strain background that was a mixture of ICR and C57BL/6. Spermatogenic cells in Acr-GFP mice express GFP from the mid-pachytene stage and onward. GFP accumulates and concentrates in the acrosomes of spermatids. All animal experiments conformed to the Guide for the Care and Use of Laboratory Animals, and were approved by the Institutional Committee of Laboratory Animal Experimentation (Animal Research Center of Yokohama City University, Yokohama, Japan).

2.2 | Culture method

The testes of male mice, postnatal days 0-4 (P0-P4) and Acr-GFP (+/+ or (+/-), were decapsulated and gently separated by forceps into three to eight pieces with diameters of 1-3 mm. Tissue fragments were then placed on blocks of agarose gel in the wells of a culture plate. To make the agarose gel block, agarose powder (Dojindo Molecular Technologies) was dissolved in water purified with Milli-Q (Millipore, Integral3) at 1.5% (w/v) and autoclaved. During cooling, 33 mL of agarose solution was poured into 10-cm dishes to form a 5-mm-thick gel. The gel was cut into approximately 10-mm square pieces, which were used as stands for testis tissue placement. Gels were submerged in culture medium in 12-well culture plates for more than 6 hours, twice, with a medium change in between. After medium removal, 0.5 mL of new medium was added to each well to half-soak gels, upon which testis tissues were placed. Each gel stand was loaded with 3 to 4 tissues. Medium was changed once a week. The culture incubator was supplied with 5% of carbon dioxide in air and maintained at 34°C. The protocol was previously described in detail.

2.3 | GFP expression observation

Cultured tissues were observed once a week under a stereomicroscope equipped with an excitation light for GFP (Leica M205 FA; Leica, Germany). The area ratio of the GFP expression region was visually assessed and classified into 7 grades: 0, 0.5, 1, 2, 3, 4, and 5, corresponding to GFP-expressing area ratios of 0, -10, -20, -40, -60, -80, and -100%, respectively (Figure 1A). To identify haploid cells with an acrosome cap structure, cultured tissues were transferred to a slide glass, covered with a cover glass, and moistened with PBS. They were observed under an inverted microscope, applying the GFP-excitation light (Olympus IX 73; Olympus, Tokyo, Japan).

2.4 | Histological and immunohistochemical examinations

In histological examinations, specimens were fixed with Bouin’s fixative and embedded in paraffin. One section showing the largest cut surface was made for each specimen, and stained with hematoxylin and eosin (H&E) or periodic acid-Schiff (PAS). In immunofluorescence staining, tissues were fixed with 4% of paraformaldehyde in PBS at 4°C overnight. Tissues were then soaked in solutions of 10, 15, and 20% (w/v) sucrose in PBS for 1 hour each in succession for cryoprotection. They were cryo-embedded in OCT compound (Sakura Finetek Japan) and cut into 7-μm-thick sections. Antibodies used as primary antibody were rabbit anti-AR (1:100, ab133273, abcam, Cambridge, EU), anti-GFP (1:1000, ab13970, abcam), anti-synaptonemal complex protein 3 (SCP3) (1:100, ab97672, abcam), anti-GFRα1 (1:200, AF560, Bio-Techne, MN, USA), and anti-TRA98 (1:500, 73-003, BioAcademia, Osaka, Japan). Lectin PNA From Arachis hypogea (peanut), Alexa Fluor 568 Conjugate (1:1000, L32458, Thermo Fischer Scientific, MA, USA) was used to identify acrosome. Antibodies used for secondary antibody were Alexa Fluor 488-conjugated goat anti-chicken antibody (A-11039, 1:200, Thermo Fischer Scientific), Alexa Fluor 555-conjugated goat anti-mouse antibody (A-21424, 1:200, Thermo Fischer Scientific), Alexa Fluor 555-conjugated goat anti-rabbit antibody (A-21428, 1:200, Thermo Fischer Scientific), Alexa Fluor 647-conjugated goat anti-rat antibody (A-21247, 1:200, Thermo Fischer Scientific), and Alexa Fluor 647-conjugated donkey anti-goat antibody (A-21447, 1:200, Thermo Fischer Scientific). Nuclei were counterstained with Hoechst33342 dye. Observation of immunostained samples were performed with a confocal laser microscope (FV1000-MPE; Olympus).
FIGURE 1  GFP grading scale and effects of BSA purification. A, Acr-GFP grading scale. Based on the percentage of the GFP-expressing area observed under a stereomicroscope, the extent of GFP expression was classified into 7 grades; 0, 0.5, 1, 2, 3, 4, and 5. B, The concentrations of fatty acids in a batch of Et-BSA (F5061) were measured before and after the purification procedure. Purification removed most fatty acids from Et-BSA. C, When non-treated Et-BSA was used in CDM, cultured tissue showed degenerative changes, appearing as yellowish-white auto-fluorescence. The purification of Et-BSA reduced tissue deterioration and improved Acr-GFP expression. Photos were taken at culture day 27. Scale bars: 0.5 mm. D, A histological examination on culture day 34 confirmed that CDM containing purified Et-BSA supported germ cell survival and differentiation in cultured tissue. Meiotic cells were observed in media containing AlbuMAX or CDM with purified Et-BSA. In tissues cultured with non-treated Et-BSA showed degenerative or necrotic foci at its periphery (white arrow heads). A Scale bars: 0.5 mm (upper raw) & 50 μm (bottom raw). E, Testis tissues of different ages of mice, P1-P4, were cultured with medium using AlbuMAX, non-treated Et-BSA, and purified Et-BSA. The purification procedure increased the Acr-GFP expression grade in mouse testis tissues of P2-P4. *P < .05, **P < .01, n.s., not significant.
2.5 | Purification of BSA

To make dextran-coated charcoal, 2 g activated charcoal (Wako, Japan) was initially mixed with 0.2 g dextran (D299100, TRC Canada) in 200 mL of Milli-Q water and kept at room temperature for 30 minutes with occasional agitation. After centrifugation at 3000 g for 15 minutes, the supernatant was discarded and rinsed with Milli-Q water once. Ten grams of BSA (A9418, Sigma-Aldrich) was dissolved in charcoal solution, followed by a pH adjustment to 3.0 by the dropwise addition of concentrated HCl with stirring. The mixture was heated to 56°C for 60 minutes with frequent agitation. Charcoal was then mostly removed by centrifugation at 3000 g for 30 minutes. pH was adjusted to 5.5 with NaOH (1 N), and the remaining charcoal was removed using a Millipore filter with a pore size of 0.45 µm (MILLIPORE Express PLUS). The solution was deionized at 4°C overnight using 30 g of mixed bed resin (Bio-Rex MSZ 501 (D), Resin Cat. #142-7245, BIO-RAD), and concentrated using an ultracentrifugation filter (VIVASPIN TURBO 15 sartorius) at 3000 g for 40 minutes. After a pH adjustment to 7.0 with HCl (1 N), the concentration of BSA was measured with the Quick Start Bradford kit (BIO-RAD, 500-0205, Lot.210007090), which generally settled at approximately 200 mg/mL. The solution was sterilized using a filter with a pore size of 0.22 µm (MILLIPORE Express™ PLUS) and stored at 4°C.

2.6 | Culture medium preparation

To prepare AlbuMAX medium, AlbuMAX I (11020-021, ThermoFisher Scientific) was dissolved in α-MEM (12000-022, Gibco) solution (40 mg/mL final concentration), stirred until complete dissolution, and 7% of NaHCO₃ solution was then added (0.026 ml/L) to achieve a final concentration of 1.82 g/L (0.0182 g for 10 ml of medium). Antibiotic-Antimycotic (15240062, ThermoFisher) was added at a concentration of 1.82 g/L (0.0182 g for 10 ml of medium). Antibiotic-Antimycotic was added, and Millipore filtration was performed according to the original study with some modifications.18 Briefly, AlbuMAX dissolved in DDW at 5% (w/v), 35 mL, was added and mixed by stirring sequentially with 0.7 mL of αT acetate (47786, Supelco, 1 M stock sol. in ethanol), linoleic acid (L1012, Sigma, 1 M of stock sol. in ethanol), linolenic acid (L2376, Sigma, 1 M of stock sol. in ethanol), and stearic acid (S4751, Sigma, 300 mM of stock sol. in ethanol). Chol (C3045, Sigma, 3.5 mg/mL of stock sol. in ether or 47127-U, Supelco, chloroform), PC (P3556, Sigma, 20 mg/mL of stock sol. in ethanol), and SM (S0756, Sigma, 3.2 mg/mL of stock sol. in ethanol) were also stored in the freezer until used. When preparing media, these stock solutions of lipids were poured into an empty beaker to evaporate the solvent. Following evaporation, purified Et-BSA solution was added and stirred at 37°C for at least 2 hours to achieve the complete dissolution of lipids. Double-concentrated α-MEM, at half the volume of the final solution and pre-warmed to 37°C, was mixed with Et-BSA-lipid solution, followed by the addition of factors including all-trans RA (R2625, Sigma, 10 mM of stock sol. in DMSO), Re (R7632, Sigma, 10 mM of stock sol. in DMSO), T (20808341, Wako, 10 mM of stock sol. in ethanol), T3 (T6397, Sigma, 2.0 µg/mL of stock sol. in ethanol), LH (L5259, Sigma, 50 µg/mL of stock sol. in Milli-Q water), and FSH (F4021, Sigma, 50 µg/mL of stock sol. in Milli-Q water) at the concentrations indicated in Table 1. NaHCO₃ solution and Antibiotic-Antimycotic were then added followed by Milli-Q water up to the final volume. L-Ascorbic acid 2-glucoside (G0394, TCI, 100 mM of stock sol. in Milli-Q water), αT acetate (47786, Supelco, 1 M stock sol. in ethanol), and L-glutathione (G6013, Sigma, 100 mg/mL of stock sol in Milli-Q water) were added to medium to the final concentration indicated in the text and shown in Table 1. Oleoyl-L-a-lysophosphatidic acid (LPA) (L7260, Sigma, 10 mM of stock sol. in 10% BSA), L-a-lysophosphatidylcholine (LPC) (L4129, Sigma, 50 mg/mL of stock sol. in ethanol), and 18:1 lysophosphatidylserine (LysyOS) (858143P, Avanti, 2 mM of stock sol. in ethanol) were added to medium as indicated in Table 1. After sterilization with Millipore filters, media were stored at 4°C.

2.7 | Lipid extraction from AlbuMAX

The Bligh-Dyer method of lipid extraction was performed according to the original study with some modifications. The Bligh-Dyer method of lipid extraction was performed according to the original study with some modifications.18 Briefly, AlbuMAX dissolved in DDW at 5% (w/v), 35 mL, was added and mixed by stirring sequentially with 0.7 mL of glacial acetic acid, 87.5 mL of methanol, 43.75 mL of chloroform, 43.75 mL of DDW, and 43.75 mL of chloroform. The mixture was centrifuged at 4000 g for 1 hour. The bottom organic layer was transferred to an eggplant-shaped flask for evaporation of the solvent at 30°C on a rotary evaporator. The remaining water layer was added with 87.5 mL of chloroform to repeat extraction, followed by centrifugation and transferal to the flask, which was repeated twice. After evaporation was completed, the mass of lipids was dried under a stream of N₂ gas. The flask was then filled with N₂ gas and stored in a freezer at −30°C until used. The butanol
extraction method was performed as described previously.19 Briefly, AlbuMAX solution in DDW at 10% (w/v), 14 mL, was added and mixed sequentially with 47 mL of buffered Na2HPO4-citric acid solution (pH 4), 124 mL of 1-butanol, and 62 mL of water-saturated 1-butanol. The mixture was centrifuged at 4000 g for 10 min. The upper organic layer was transferred to an eggplant-shaped flask. The remaining water layer was added with 124 mL of 1-butanol and 62 mL of water-saturated 1-butanol again for re-extraction, followed by centrifugation and evaporation. The upper layer was combined for evaporation of the solvent at 60°C. After evaporation, lipids were further dried under a stream of N2 gas. The flask was then filled with N2 gas and kept in a freezer at −30°C until used.

### 2.8 Measurement of FAs

FAs were extracted from albumin solutions using the Bligh-Dyer method18 with margaric acid and cyclohexylbutyric acid as the internal standard. FAs were derivatized to their trimethylsilyl esters using BSTFA + TMCS solution (33148, Supelco) and analyzed on a GC-MS system (7890A GC & 5975C GC/MSD, Agilent). An Agilent J&W GC capillary column (0.25 mm ID × 30 m, film thickness 0.25 μm) was used in GC. The column temperature was held at 100°C for 1 minute immediately after the injection, elevated from 100 to 320°C at 5°C/min, and then, held at the final temperature for 5 minutes. Helium was used as a carrier gas at a constant flow rate of 1 ml/min. The temperatures of the ion source, quadrupole, and auxiliary were 230, 150, and 280°C, respectively. A 1-μL sample was injected, with the split ratio of the injector being 1:10. The total run time was 50 minutes. Mass spectra were obtained by the electron ionization method (70 eV). Protein concentrations were measured to calculate the amount of FAs per protein with the Bradford method using the Quick Start Bradford kit (BIO-RAD, 500-0205, Lot. 210007090) according to the manufacturer’s instructions.

### 2.9 Untargeted lipidomics for extracts from AlbuMAX

Untargeted lipidomics was performed, as described previously,20 using an ACQUITY UPLC system (Waters, Milford, MA) coupled with quadrupole time-of-flight...
LC separation was performed using a reverse-phase column [ACQUITY UPLC BEH peptide C18 (2.1 × 50 mm, particle size of 1.7 µm; Waters)] with a gradient elution of mobile phase A [methanol/acetonitrile/water (1:1:3, v/v/v) containing 5 mM of ammonium acetate (Wako Chemicals, Osaka, Japan) and 10 mM EDTA (Dojindo, Kumamoto, Japan)] and mobile phase B (100% of isopropanol containing 5 mM of ammonium acetate and 10 mM EDTA); the composition was produced by mixing these solvents. The LC gradient consisted of holding solvent (A/B:100/0) for 1 minute, then, linearly converting to solvent (A/B:60/40) for 4 minutes, linearly converting to solvent (A/B:36/64) for 2.5 minutes and holding for 4.5 minutes, then, linearly converting to solvent (A/B:17.5/82.5) for 0.5 minutes and linearly converting to solvent (A/B:5/95) for 1 minute, followed by returning to solvent (A/B:100/0) and holding for 5 minutes for re-equilibration. The injection volume was 2 µL, the flow rate was 0.300 mL/min, and column temperature was 45°C. The information-dependent acquisition (IDA) mode was applied to confirm each of the lipid structures in the negative-ion mode. Acquisition conditions were as follows: the accumulation time for a full scan was 100 ms for scanning a mass range between m/z 75 and m/z 1,250. The accumulation time for each IDA experiment was 50 ms, and collision energies (CEs) were set to −35 eV with a CE spread of 15 eV in the high-resolution mode.

### 2.10 Lipidomics specific for LPLs

Medium containing AlbuMAX, Ch-BSA (A2058, Sigma), FBS, or ABS was mixed with methanol (including 17:0-LPA as the internal standard; final concentration 100 nM), sonicated, and centrifuged as described previously. The resulting supernatant was stored at −80°C. A LC-MS/MS analysis was performed according to a previously described method with minor modifications. In the present study, the LC-MS/MS system that included an Ultimate 3000 HPLC and TSQ Quantiva triple quadruple mass spectrometer (Thermo Fisher Scientific) was used. LPA analyses were performed in multiple reactive monitoring (MRM) in the negative mode. LC was performed using a reverse phase column (CAPCELL PAK C18 (1.5 mm ID × 250 mm, particle size of 3 µm)) with a gradient elution of solvent A (5 mM of ammonium formate in 95% (v/v) water, pH 4.0), and solvent B (5 mM of ammonium formate in 95% (v/v) acetonitrile, pH 4.0) at 200 µL/min. Gradient conditions were as follows: hold 50% B for 0.2 minutes, followed by a linear gradient to 100% B over 11.8 minutes, hold 100% B for 5 minutes, return to the initial condition over 0.5 minutes, and maintain for 2.5 minutes until the end of the run (total run time 20 minutes).

### 2.11 Metabolome analyses

Metabolite extraction and a metabolome analysis were conducted at Human Metabolome Technologies (Tsuruoka, Yamagata, Japan). Samples were prepared and analyzed by two modes (for the detection of both cationic and anionic metabolites): capillary electrophoresis time-of-flight mass spectrometry (CE-TOFMS) and liquid chromatography time-of-flight mass spectrometry (LC-TOFMS). The identification of metabolites from the peaks was based on the annotated tables of m/z values and normalized migration times based on the data of the HMT standard library. Relative peak areas under the curves were quantified. Data from four samples were normalized by albumin concentrations.

### 2.12 Statistical analysis

Data on Acr-GFP expression grades were shown as averages and SD in graphs. Non-parametric multiple comparison tests, the Kruskal-Wallis test, followed by the Steel-Dwass test, were performed to assess the significance of differences in GFP grades.

### 3 RESULTS

#### 3.1 Batch difference in BSA quality was cleared by charcoal and deionization treatments

CDM formulated in our previous study contained Et-BSA in addition to RA, Re, LH, FSH, T, T3, Chol, PC, SM, and FAs (Table 1, original CDM). Et-BSA was necessary for dissolving hydrophobic ingredients, such as lipids, into media. Importantly, we noticed that the batch of Et-BSA had a significant effect on the efficiency of spermatogenesis. Different batches produced different results. We also found that the composition of FAs contained in Et-BSA markedly differed between batches. We then introduced the “BSA purification” procedure, which consisted of charcoal and deionization treatments, to remove most FAs from Et-BSA (Figure 1B). This purification effectively improved culture results by avoiding the degeneration of tissues as well as more efficiently inducing Acr-GFP expression (Figure 1C-E). Thus, we routinely purified Et-BSA in the preparation of CDM.

#### 3.2 T3 was critical in inducing spermatogenesis

Although the effects of hormones on spermatogenesis in mice have been extensively examined, few in vitro studies...
have been conducted to date. We investigated each of the four hormones, LH/FSH/T/T3, which exerted significant effects on the induction of spermatogenesis when supplemented together.\textsuperscript{12} We used original CDM (Table 1) with purified Et-BSA and the testis tissues of P2-P3 mice. We added each hormone separately to CDM and found that T3 alone induced Acr-GFP expression (Figure 2A). We then omitted each one from LH/FSH/T/T3. The results showed that Acr-GFP was not expressed with medium devoid of T3 (Figure 2B). Therefore, T3 is a requisite factor for the initiation of in vitro mouse spermatogenesis. A histological and immunohistochemical examination on cultured tissue on day 20 demonstrated that seminiferous tubules contained meiotic germ cells in the pachytene or later stages when AlbuMAX or CDM containing T3 was used. On the contrary, the tubules were thin with sparse germ cells of spermatogonia and some less progressed meiotic cells, SCP3 (+) and Acr-GFP (−), in groups of CDM lacking T3 (Figure 2C). T3 is known to promote the differentiation of Sertoli cells.\textsuperscript{23} An immunohistochemical examination with an antibody to androgen receptors (AR), which is a marker of matured Sertoli cells, demonstrated that Sertoli cells cultured with T3 were positive for AR, but negative when cultured without T3. Cells in the interstitium and peritubular cells were AR-positive regardless of the presence of T3 (Figure 2D). These results corresponded with the function of T3 in vivo to stimulate the maturation of Sertoli cells. By contrast, the effects of the 3 other hormones were not obvious. However, when older P3-4 mouse testis tissues were cultured, T in combination with T3 enhanced Acr-GFP expression significantly (Figure 2E). Although these results did not show any advantages of supplementing LH and FSH, we continued to use them in subsequent studies considering their potential benefits on spermatogenesis.

### Metabolomic analysis identified α-tocopherol as an essential component in AlbuMAX

FBS had limited ability to promote in vitro spermatogenesis,\textsuperscript{5} whereas AlbuMAX induced complete spermatogenesis even though AlbuMAX was entirely derived from bovine serum. When we used ABS instead of FBS for testis tissue cultivation, ABS induced Acr-GFP expression to a similar level as AlbuMAX, even in neonatal testis tissues (Figure 3A). This result indicated that some important factors for the induction of spermatogenesis were contained in ABS as well as in AlbuMAX, but not in FBS. We then performed a metabolome analysis and focused on differences among AlbuMAX, FBS, and ABS. Another BSA produced by chromatography (Ch-BSA) was examined for comparison. Ch-BSA was not as efficient as AlbuMAX, but induced Acr-GFP expression when supplemented in α-MEM, suggesting that Ch-BSA also contains most of the spermatogenesis-inducing factors, similar to AlbuMAX.\textsuperscript{12} The metabolome analysis identified 384 substances in four samples, with 182 out of 384 substances being detected in AlbuMAX. A hierarchical clustering analysis demonstrated that AlbuMAX and Ch-BSA were similar and also markedly differed from FBS and ABS (Figure 3B). We predicted that the concentrations of candidate factors were the highest in AlbuMAX, followed by ABS, Ch-BSA, and FBS. Several substances followed this concentration pattern; however, most did not appear to be relevant to the activation of spermatogenesis (Data S1). The only substance identified for further testing was vitamin E, namely, αT or αT acetate, which was detected in AlbuMAX, Ch-BSA, and ABS, but not in FBS (Figure 3C). αT mainly functions as an antioxidant,\textsuperscript{24-26} and previous studies reported that vitamin E was essential for spermatogenesis.\textsuperscript{27,28} Thus, we considered it to be a candidate critical factor.

### Combination of αT, AA, and GSH was effective in promoting spermatogenesis

αT supplemented in CDM did not initially exert any effect on Acr-GFP expression levels. However, when combined with AA to enhance its effects\textsuperscript{29} and with GSH to enhance the effects of AA,\textsuperscript{30} Acr-GFP expression levels significantly increased (Figure 4A). This triple combination (αT/AA/GSH) increased the Acr-GFP expression in a dose-dependent manner (Figure 4B). Testis tissues cultured with CDM sometimes showed a degenerative change that appeared as yellowish-white auto-fluorescence emission. This degenerative change was ameliorated by supplementing αT/AA/GSH into CDM in a dose-dependent manner, suggesting that αT/AA/GSH functioned to prevent tissue degeneration by suppressing the peroxidation of cellular lipids (Figure 4C). When αT/AA/GSH was added to FBS along with RA/Re and LH/FSH/T/T3, Acr-GFP expression levels significantly increased, thereby confirming the effects of αT/AA/GSH to promote spermatogenesis (Figure 4D).

### LPLs emerged as factors promoting spermatogenesis

The original CDM contained lipids of FFA, Chol, PC, and SM, and their promoting effects on spermatogenesis were previously demonstrated (Table 1).\textsuperscript{12} In order to investigate lipids in a broader category that may affect spermatogenesis, we extracted lipids from AlbuMAX. Two methods, Bligh-Dyer (BD)\textsuperscript{18} and butanol extraction,\textsuperscript{19} were employed. These extracts were dissolved in purified Et-BSA solution.
and added to α-MEM with no other supplements. Medium containing BD extract did not induce Acr-GFP expression, whereas butanol extract medium induced Acr-GFP expression to a level similar to AlbuMAX medium, even with neonatal testis tissues (Figure 5A). This result indicated that the majority of factors necessary for spermatogenic induction or promotion were extracted from AlbuMAX into butanol, while the BD extract was missing some of them. Untargeted lipidomics on these two extracts showed that both extracts contained similar amounts of FAs, LPCs, PCs, and SMs.
(Data S2). On the contrary, LysoPS and LPAs were exclusively detected in the butanol extract (Figure 5B). Thus, we focused on LPLs and performed lipidomics to analyze LPLs in more detail using samples of culture media containing AlbuMAX, butanol extract, BD extract, FBS, and ABS. Six species of LPLs, namely, LPC, LPA, LysoPS, LPE, LPI, and lysophosphatidylglycerol (LPG), were identified in this order of concentrations (Figure 5C, Figure S1). No single lipid species alone was responsible for the different ability of each of the five samples for the induction/promotion of spermatogenesis. However, when butanol and BD extracts were compared, LPA and LysoPS were significantly higher in butanol, which is consistent with the results of lipidomics described above (Figure 5B). LPC appeared to be important based on its levels being the highest in AlbuMAX and more prominent than in FBS. Thus, we considered LPA, LysoPS, and LPC to be the main LPLs that appear to play important roles in enhancing spermatogenesis.

3.6 | CDM containing LPLs effectively induced spermatogenesis in neonatal testes

We examined the effects of LPLs, including LPA, LysoPS, and LPC as well as PEA and PI, by supplementing each of them into CDM that already contained the FFAs, Chol/PC/SM, RA/Re, LH/FSH/T/T3, and αT/AA/GSH. The concentrations of each LPL were set proportional to their amounts in AlbuMAX. Each LPL exerted a positive effect on the induction of Acr-GFP expression, with LPC showing the strongest and most stable effect (Figure 6A). We then investigated the combination of LPC, LPA, and LysoPS, and found that all of them induced Acr-GFP expression as efficiently as AlbuMAX medium (Figure 6B). We explored the haploid cell formation by observing each tissue on a slide glass under high magnification using the inverted microscope. Round spermatids were observed as cap- or crescent-like shapes with condensed GFP, but were very few. Instead,
FIGURE 4  Combination of three antioxidants promoted spermatogenesis. A, Original CDM was supplemented additively with α-tocopherol (αT), ascorbic acid (AA), and glutathione (GSH), showing that the combination of these three was effective for inducing Acr-GFP expression in neonate testis tissues. Photos were taken on culture day 27. Scale bars: 0.5 mm. B, The effect of αT/AA/GSH was dose-dependent, from 100 to 1000 µM. C, A degenerative change observed as yellowish-white auto-fluorescence was reduced by αT/AA/GSH supplementation. P1 mouse testis tissues were cultured and photos were taken on culture day 14. D, Although FBS alone did not induce Acr-GFP expression and the supplementation of RA/Re and LH/FSH/T/T3, exerted limited effects, the addition of αT/AA/GSH significantly up-regulated Acr-GFP expression. Scale bar: 1 mm. *P < .05, **P < .01, n.s., not significant
there were many irregularly formed rod-shaped GFP condensates or unevenly sized GFP dots. Several of them were assembled in a single cyst, appearing like a multinuclear cell, which were most likely reflecting the opening of intercellular bridges between round spermatids (Figure 6C). A histological examination revealed that spermatids in tissues cultured with CDM were degenerative, forming atypical multi-nuclear cells with cytoplasm that was densely stained by PAS (Figure 6D). These cystic degenerative spermatids were also observed, along with regular spermatids, in tissues

**FIGURE 5** Lipids extracted from AlbuMAX maintained spermatogenesis induction/promotion activity. A, Lipids extracted from AlbuMAX with the butanol method, but not the Bligh-Dyer method, strongly induced Acr-GFP expression with neonatal mouse testis tissue. Scale bars: 0.5 mm. * P < .05, n.s., not significant. B, Thirty-five molecules, including LPEs, a LysoPS, LPAs, PCs, and SMs, detected in BD and/or butanol extracts were selected from Data S2 and their relative amounts were shown in the bar graph. C, A lipidomic analysis focusing on LPLs detected 6 species of LPLs, including LPC, LPA, LysoPS, LPE, LPI, and LPG, in five samples of culture media; AlbuMAX, butanol extract, BD extract, 10% ABS, and 10% FBS.
FIGURE 6  LPLs effectively promoted spermatogenesis from the neonatal testis. A, Each lysophospholipid of LPA, LPC, and LysoPS and phospholipid of PEA and PI was supplemented into CDM containing αT/AA/GSH (300 µM). They all increased the expression of Acr-GFP, with LPC exerting the strongest effect. Scale bars: 0.5 mm. *P < .05. B, CDM supplemented with αT/AA/GSH and combinations of LPLs induced Acr-GFP expression in the neonatal testis to a similar level as that with AlbuMAX medium. C, Neonatal testis tissue cultured with CDM containing αT/AA/GSH and LPC/LysoPS. Observations were performed under an inverted microscope on culture day 41. Round spermatids (arrow) along with irregularly formed GFP assembled in cysts (arrowhead) were recognized. Dashed rectangular areas were enlarged sequentially in the panel with corresponding marks; a-h. Scale bars: 1 mm (a), 200 µm (b), 100 µm (c), 50 µm (d), and 10 µm (e-h). D, Histological view of tissues cultured with AlbuMAX medium and CDM with αT/AA/GSH, LPC/LysoPS for 45 days. Cells densely stained with PAS, mostly multi-nucleated, were observed in CDM samples, indicating degenerative changes corresponding to irregular forms of GFP observed in C. Scale bars: 100 µm (upper raw), and 50 µm (bottom raw)
cultured with AlbuMAX medium, but were predominant in those cultured with CDM (Figure S2). This suggested that there are still other factors missing in CDM, which could be other than lipids, for proper haploid cell formation. Alternatively, the lipid composition of CDM may need further adjustments.

3.7 | Deficient in BD extract of AlbuMAX were RA, T, T₃, αT, and LPLs

Although LPA and LysoPS were suggested to be the missing factors in the BD extract, the addition of LPA and LysoPS alone and even extra LPC into BD extract medium did not
induce Acr-GFP expression. We then supplemented the BD extract with RA/Re/LH/FSH/T/T₃, αT/AA/GSH, and then, LPA (Figure 7A). The addition of RA/Re and LH/FSH/T/T₃ induced Acr-GFP expression. This result suggested that RA/Re, T, and T₃, which are all lipophilic, were missing in the BD extract, but included in the butanol extract. The further addition of αT/AA/GSH to BD extract medium increased the GFP grade, and addition of LPA further increased the grade. Observations with the inverted microscope revealed many round spermatids in seminiferous tubules that were cultured with CDM containing αT/AA/GSH, and CDM containing αT/AA/GSH and LPA, as well as with AlbuMAX medium (Figure 7B). Immunohistochemical staining with PNA, which reacts with acrosome of spermatids, demonstrated round spermatids not only in AlbuMAX samples, but also in the supplemented BD-extract samples (Figure 7C). Based on these results, we concluded that the difference in spermatogenesis induction/promotion activity between BD and butanol extracts was mostly attributable to RA, T, T₃, αT, and LPLs, such as LPA. Collectively, lipids extracted from AlbuMAX were capable to promote mouse spermatogenesis up to spermatid formation, indicating that the potency of AlbuMAX resided almost exclusively in the lipid-soluble fraction.

4 | DISCUSSION

In the present study, we employed the testis organ culture method to assess the effects of each component in CDM. We initially succeeded in refining medium by purifying Et-BSA, which showed variable results in different batches. The BSA-purification procedure not only gave stable culture results, but also improved spermatogenic efficiency. We then examined the effects of each of the four hormones, LH, FSH, T, and T₃, and found that T₃ was the most critical for the induction of spermatogenesis. T₃ was previously shown to induce the maturation of Sertoli cells. The present results were consistent with previous findings showing the significant effects of T₃ on Sertoli cell maturation and the initiation of spermatogenesis. T was not responsible for the induction of spermatogenesis in the present study. This was also compatible with results in previous reports performed with animal experiments. Studies depriving T in rodents demonstrated that T was required for germ cells to progress beyond meioisis and for the release of mature spermatids. In addition, knockout of AR specific to Sertoli cells resulted in the arrest of spermatogenesis at the diplotene stage, while AR-KO in specific cell types, including Leydig, peritubular myoid, and germ cells, exerted milder or no adverse effects on spermatogenesis. Therefore, spermatogenesis can proceed to the diplotene stage without T, and, as such, our Acr-GFP expression assay system did not detect the effects of T because GFP appeared in pachytene spermatocytes. Regarding FSH, its effects on spermatogenesis were mediated by Sertoli cells, which express the FSH receptor. However, the role of FSH in spermatogenesis markedly varies among species. In the case of mice, its effects on spermatogenesis were reported to be negligible. The gene knockout of FSH-β or FSH receptors did not result in evident abnormalities in spermatogenesis itself; however, testes were smaller and fertility was reduced in male mice. Thus, our culture results were consistent with these findings. The effects of LH on spermatogenesis were mainly mediated by T produced by Leydig cells. Thus, LH and T in our culture medium may be redundant. Collectively, our testis organ culture with CDM reproduced processes and reactions occurring in an in vivo testis, thereby providing an experimental system with the capacity to evaluate the effects of individual factors in medium.

We employed metabolomics technology to investigate factors that are critical for spermatogenic induction in AlbuMAX, and identified αT. Although αT alone was not sufficiently effective in promoting spermatogenesis, the addition of AA and GSH significantly improved it. αT, AA, and GSH have mutual interactions. Specifically, GSH converts oxidized AA back to its reduced form. Reduced AA converts oxidized αT to its reduced form. Thus, GSH and AA are both expected to reinforce and sustain the effects of αT, while functioning as general reductants for various molecules. However, since AA and GSH were not detected in AlbuMAX by the metabolome analysis, the combination of αT/AA/GSH may be unique in CDM. Nevertheless, the addition of αT/AA/GSH to CDM exerted significant effects on spermatogenic induction and promotion, which led to the discovery of LPLs and their effects on spermatogenesis.

Historically, the importance of lipids in culture medium was recognized in pursuits to formulate synthetic medium from which serum is omitted. Ham's group for the first time reported that lipid-rich synthetic medium facilitated the growth of human fibroblasts. FFAs were found to be essential for the proliferation of liver epithelial cells. In the long-term proliferation of hepatic progenitors or stem cells, not only FFA, but also other lipids were required as high-density lipoproteins. This is also the case for spermatogonial stem cells (SCs), and lipids were required for their proliferation. These findings on SSC culture conditions were consistent with the present results showing that lipids are critical ingredients in CDM to induce spermatogenesis, suggesting that the induction of spermatogenesis and proliferation of SSCs are closely linked.

The difference between BD and butanol extracts demonstrated that some LPLs were only extracted by butanol. The addition of LPLs, specifically LPC along with LPA and/or LysoPS, to CDM promoted spermatogenesis in culture. Although the roles of LPLs in the body have not yet been examined in detail, their function as extracellular signaling molecules is being established. Their receptors, members of the
G protein-coupled receptor family (GPCRs), and/or specific enzymes for their production have been modified through knockout technology, which revealed the function of LPLs, particularly LPA, by demonstrating a variety of phenotypes relating to hair follicle development, vascular formation, fibrosis in the lungs and kidneys, and embryo implantation. Regarding spermatogenesis, a previous study reported that mice having the null-mutation of LPA receptors, specifically LPAR1, LPAR2, and LPAR3, had elevated levels of germ cell apoptosis and smaller sperm numbers, particularly as animals aged. Recent studies identified several GPCRs for other LPLs, such as LysoPS. Thus, activity of LPLs to promote spermatogenesis in the present study could be dependent on the activation of their receptors. However, the effects of LPLs were not specific to LPA or LysoPS, they were also observed with LPC. Notably, LPLs may be selectively moved through the blood-brain barrier by the transporter protein, Mfsd2a, which is specifically expressed in the endothelium and resides in the plasma membrane. If a similar mechanism exists in the testis, LPLs, rather than unesterified FAs and phospholipids, may be preferentially incorporated in cells to be used as substrates for lipid metabolism, which may explain the effects of LPLs observed in the present study. However, the mechanisms responsible for the effects of LPL on spermatogenesis currently remain unclear. This is partly due to the innate nature of our culture method, which takes several weeks to evaluate spermatogenesis. Since lipids in cellular structures are under rapid turnover, particular lipids initially supplemented may be converted to other forms within this time span. For example, LPLs and phospholipids are mutually convertible through the addition and removal of acyl moieties, which are mediated by acyltransferase and phospholipase, respectively. This hinders our mechanistic understanding of the role of lipids in the progression of spermatogenesis. Thus, an experimental system with the capacity to evaluate the progression of spermatogenesis at each step in a more precise manner is needed. Nevertheless, the present study demonstrated for the first time the importance of lipids, particularly LPLs, for the induction of spermatogenesis up to the completion of meiosis.

The final CDM was defective at promoting spermiogenesis, namely, the maturation of spermatids to spermatozoa was severely hindered. It appears, therefore, that some lipids, or lipid-soluble substances, that adequately support spermiogenesis have yet to be identified. The mechanisms by which lipids affect spermatogenesis are an important issue for future study, which may contribute to the development of therapeutic protocols, whether pharmaceutical or nutritional, for males with fertility issues.

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CONFLICT OF INTEREST
The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS
H. Sanjo, T. Yao, and T. Ogawa designed the research; H. Sanjo, K. Katagiri, T. Sato, M. Komeya, H. Yamanaka, and T. Matsumura performed culture experiments; T. Matsumura performed immunohistochemistry. K. Ikeda, K. Kano, J. Aoki, and M. Arita performed lipidomic analyses; T. Yao, and Y. Asayama performed lipid extraction; H. Sanjo, T. Yao, T. Matsumura, and T. Ogawa analyzed data; M. Yao and A. Matsuhisa supervised the research; and H. Sanjo, T. Yao, and T. Ogawa wrote the manuscript.

REFERENCES
1. Komeya M, Sato T, Ogawa T. In vitro spermatogenesis: a century-long research journey, still half way around. Reprod Med Biol. 2018;17:407-420.
2. Yokonishi T, Ogawa T. Cryopreservation of testis tissues and in vitro spermatogenesis. Reprod Med Biol. 2016;15:21-28.
3. Martinovitch PN. Development in vitro of the mammalian gonad. Nature. 1937;139:413.
4. Gohbara A, Katagiri K, Sato T, et al. In vitro murine spermatogenesis in an organ culture system. Biol Reprod. 2010;83:261-267.
5. Sato T, Katagiri K, Gohbara A, et al. In vitro production of functional sperm in cultured neonatal mouse testes. Nature. 2011;471:504-507.
6. Sato T, Katagiri K, Yokonishi T, et al. In vitro production of fertile sperm from murine spermatogonial stem cell lines. Nat Commun. 2011;2:472.
7. Arkoun B, Dumont L, Milazzo J-P, et al. Retinol improves in vitro differentiation of pre-pubertal mouse spermatogonial stem cells into sperm during the first wave of spermatogenesis. PLoS One. 2015;10:e0116660.
8. Dumont L, Oblette A, Rondanino C, et al. Vitamin A prevents round spermatid nuclear damage and promotes the production of motile sperm during in vitro maturation of vitrified pre-pubertal mouse testicular tissue. Mol Hum Reprod. 2016;22:819-832.
9. Isoler-Alcaraz J, Fernández-Pérez D, Larriba E, Del Mazo J. Cellular and molecular characterization of gametogenic progression in ex vivo cultured prepubertal mouse testes. Reprod Biol Endocrinol. 2017;15:85.
10. Nakamura N, Merry GE, Inselman AL, et al. Evaluation of culture time and media in an in vitro testis organ culture system. Birth Defects Res. 2017;109:465-474.
11. Komeya M, Kimura H, Nakamura H, et al. Long-term ex vivo maintenance of testis tissues producing fertile sperm in a microfluidic device. Sci Rep. 2016;6:21472.

12. Sanjo H, Komeya M, Sato T, et al. In vitro mouse spermatogenesis with an organ culture method in chemically defined medium. PLoS One. 2018;13:e0192884.

13. Nakanishi T, Ikawa M, Yamada S, et al. Real-time observation of acrosomal dispersal from mouse sperm using GFP as a marker protein. FEBS Lett. 1999;449:277-283.

14. Nakanishi M, Ikawa M, Yamada S, Toshimori K, Okabe M. Alkalization of acrosome measured by GFP as a pH indicator and its relation to sperm capacitation. Dev Biol. 2001;237:222-231.

15. Ohta H, Sakaide Y, Wakayama T. Functional analysis of male mouse haploid germ cells of various differentiation stages: early and late round spermatids are functionally equivalent in producing progeny. Biol Reprod. 2009;80:511-517.

16. Yokonishi T, Sato T, Katagiri K, Ogawa T. In vitro spermatogenesis using an organ culture technique. Methods Mol Biol. 2013:927:479-488.

17. Sato T, Katagiri K, Kubota Y, Ogawa T. In vitro sperm production from mouse spermatogonial stem cell lines using an organ culture method. Nat Protoc. 2013;8:2098-2104.

18. Bligh EG, Dyer WJ. A rapid method of total lipid extraction and purification. Can J Biochem Physiol. 1959;37:911-917.

19. Baker DL, Desiderio DM, Miller DD, Tolley B, Tigyi GJ. Direct quantitative analysis of lysophosphatidic acid molecular species by stable isotope dilution electrospray ionization liquid chromatography-mass spectrometry. Anal Biochem. 2001;292:287-295.

20. Aoyagi R, Ikeda K, Isobe Y, Arita M. Comprehensive analyses of oxidized phospholipids using a measured MS/MS spectra library. J Lipid Res. 2017;58:2229-2237.

21. Okudaira M, Inoue A, Shuto A, et al. Separation and quantification of 2-acetyl-1-lysophosphatidic acid and 1-acetyl-2-lysophosphatidic acid in biological samples by LC-MS/MS. J Lipid Res. 2014;55: 2178-2192.

22. Iscove NN, Guilbert LJ, Weyman C. Complete replacement of serum in primary cultures of erythropoietin-dependent red cell precursors (CFU-E) by albumin, transferrin, iron, unsaturated fatty acid, lecithin and cholesterol. Exp Cell Res. 1980;126:121-126.

23. Holsberger DR, Kiesewetter SE, Cooke PS. Regulation of neontal sertoli cell development by thyroid hormone receptor α1. Biol Reprod. 2005;73:396-403.

24. Olcott HS, Emerson OH. Antioxidants and the autooxidation of fats. IX. The antioxidant properties of the tocopherols. J Am Chem Soc. 1937;59:1008-1009.

25. Burton GW, Joyce A, Ingold KU. First proof that vitamin E is major lipid-soluble, chain-breaking antioxidant in human blood plasma. Lancet. 1982;2:327.

26. Niki E. Antioxidants in relation to lipid peroxidation. Chem Phys Lipids. 1987;44:227-253.

27. Mason KE. Differences in testis injury and repair after vitamin A-deficiency, vitamin E-deficiency, and inanition. American J Anatomy. 1933;52:153-239.

28. Bensoussan K, Morales CR, Hermo L. Vitamin E deficiency causes incomplete spermatogenesis and affects the structural differentiation of epithelial cells of the epididymis in the rat. J Androl. 1998;19:266-288.

29. Packer JE, Slater TF, Willson RL. Direct observation of a free radical interaction between vitamin E and vitamin C. Nature. 1979;278:737-738.

30. Winkler BS, Orselli SM, Rex TS. The redox couple between glutathione and ascorbic acid: a chemical and physiological perspective. Free Radic Biol Med. 1994;17:333-349.

31. Smith LB, Walker WH. Hormone signaling in the testis. In: Plant TM, Zelznik AJ, eds. Knobil and Neill’s Physiology of Reproduction. Vol. 1. San Diego: Academic Press. 2015: 637-690.

32. Sharpe RM. Regulation of spermatogenesis. In: Knobil E, Neill JD, eds. The Physiology of Reproduction. New York: Raven Press; 1994:1363-1434.

33. Wang RS, Yeh S, Tseng CR, Chang C. Androgen receptor roles in spermatogenesis and fertility: lessons from testicular cell-specific androgen receptor knockout mice. Endocr Rev. 2009;30:119-132.

34. Moudgal NR, Sairam MR. Is there a true requirement for follicle stimulating hormone in promoting spermatogenesis and fertility in primates? Hum Reprod. 1998;13:916-919.

35. Kumar TR, Wang Y, Lu N, Matzuk MM. Follicle stimulating hormone is required for ovarian follicle maturation but not male fertility. Nat Genet. 1997;15:201-204.

36. Dierich A, Sairam MR, Monaco L, et al. Impairing follicle-stimulating hormone (FSH) signaling in vivo: targeted disruption of the FSH receptor leads to aberrant gametogenesis and hormonal imbalance. Proc Natl Acad Sci U S A. 1998;95:13612-13617.

37. Singh J, O’Neill C, Handelsman DJ. Induction of spermatogenesis by androgens in gonadotropin-deficient (hpg) mice. Endocrinology. 1995;136:5131-5321.

38. Niki E, Noguchi N. Dynamics of antioxidant action of vitamin E. Acc Chem Res. 2004;37:45-51.

39. Packer L. Vitamin C and redox cycling antioxidants. In: Packer L, Fuchs J, eds. Vitamin C in Health and Disease. New York: Marcel Dekker Inc. 1997:95-121.

40. Carr AC, Frei B. Toward a new recommended dietary allowance for vitamin C based on antioxidant and health effects in humans. Am J Clin Nutr. 1999;69:1086-1107.

41. Bettger WJ, Boyce ST, Walthall BJ, Ham RG. Rapid clonal growth and serial passage of human diploid fibroblasts in a lipid-enriched synthetic medium supplemented with epidermal growth factor, insulin, and dexamethasone. Proc Natl Acad Sci U S A. 1981;78:5588-5592.

42. Chessebeuf M, Padieu P. Rat liver epithelial cell cultures in a serum-free medium: primary cultures and derived cell lines expressing differentiated functions. Vitro. 1984;20:780-795.

43. Wauthier E, Wauthier E, Turner R, et al. Hepatic stem cells and hepatoblasts: identification, isolation, and ex vivo maintenance. Methods Cell Biol. 2008;86:137-225.

44. Kanatsu-Shinohara M, Inoue K, Ogonuki N, Morimoto H, Ogura A, Shinozuka T. Serum- and feeder-free culture of mouse germline progeny. Biol Reprod. 2013;927:479-488.

45. Kubota H, Avarbock MR, Brinster RL. Culture conditions and sinusoid growth factors affect fate determination of mouse spermatogonia. Dev Biol. 2017;58:2229-2237.
47. Ye X, Skinner MK, Kennedy G, Chun J. Age-dependent loss of sperm production in mice via impaired lysophosphatidic acid signaling. *Biol Reprod*. 2008;79:328-336.

48. Kihara Y, Mizuno H, Chun J. Lysophospholipid receptors in drug discovery. *Exp Cell Res*. 2015;333:171-177.

49. Nguyen LN, Ma D, Shui G, et al. Mfsd2a is a transporter for the essential omega-3 fatty acid docosahexaenoic acid. *Nature*. 2014;509:503-506.

50. Warren L, Glick MC. Membranes of animal cells. II. The metabolism and turnover of the surface membrane. *J Cell Biol*. 1968;37:729-746.

51. Pasternak CA, Friedrichs B. Turnover of mammalian phospholipids. Rates of turnover and metabolic heterogeneity in cultured human lymphocytes and in tissues of healthy, starved and vitamin A-deficient rats. *Biochem J*. 1970;119:481-488.

52. Gallaher WR, Weinstein DB, Blough HA. Rapid turnover of principal phospholipids in BHK-21 cells. *Biochem Biophys Res Commun*. 1973;52:1252-1256.

53. Hishikawa D, Hashidate T, Shimizu T, Shindou H. Diversity and function of membrane glycoprophospholipids generated by the remodeling pathway in mammalian cells. *J Lipid Res*. 2014;55:799-807.

**SUPPORTING INFORMATION**

Additional Supporting Information may be found online in the Supporting Information section.

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