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ARTICLE

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Neonatal testis growth recreated in vitro by two-dimensional organ spreading

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Abstract

Organ culture experiments can be hampered by central degeneration or necrosis due to the inadequate permeation of oxygen and nutrients, which deteriorates the function and growth of cultured tissues. In the current study, we aimed to overcome this limitation of organ culture through spreading the tissue two dimensionally on an agarose gel stand and molding into a disc shape by placing a ceiling of polydimethylsiloxane (PDMS) chip, which is highly oxygen permeable. By this, every part of the tissue can receive a sufficient supply of oxygen through PDMS as well as nutrients through the agarose gel below. This method not only prevented central necrosis of tissues, but also supported the tissue growth over time. In addition, such growth, as volume enlargement, could be easily measured. Under these conditions, we examined the effect of several factors on the growth of neonatal mouse testis, and found that follicle stimulating hormone (FSH) and insulin significantly promoted the growth. These results are in good agreement with previous in vivo reports. Notably, the growth achieved over 7 days in our in vitro system is almost comparable to, about 80% of, that observed in vivo. Thus, we successfully monitored the promotion of tissue growth beyond the limits of the conventional organ culture method. This extremely simple method could offer a unique platform to evaluate the growth as well as functional properties of organs, not only the testis but also others as well.

KEYWORDS

organ culture, PDMS, Sertoli cells, testis, tissue growth

1 | INTRODUCTION

Mammalian spermatogenesis takes place in the seminiferous tubules, which comprise a large part of the testes. The structure of a seminiferous tubule is built up by Sertoli cells and peritubular myoid cells, while spermatogenic germ cells reside in the tubule. The cell–cell contacts between germ and Sertoli cells are extremely intimate, reflecting the pivotal role of Sertoli cells in spermatogenesis. Between the tubules, several different types of cells, including Leydig cells, exist along with the vasculature. During fetal development, the testis first appears at around 10 days postcoitum (dpc) in the case of mice. Upon Sry expression within, the destined cells become Sertoli cells and testis formation actually starts, which is also recognized as the sexual differentiation of the gonads. The Sertoli cells secrete fibroblast growth factor 9 (FGF9), which induces the self-proliferation of Sertoli cells, the so-called first-wave proliferation, around 11 dpc (Brennan & Capel, 2004; Colvin, Green, Schmahl, Capel, & Ornitz, 2001; Schmahl, Kim, Colvin, Ornitz, & Capel, 2004; Ungewitter & Yao, 2012). Subsequently, other types of somatic cells emerge and differentiate to form the primitive architecture of the seminiferous tubules, which is completed by 14 dpc (Nel-Themaat et al., 2009; O'Shaughnessy & Fowler, 2010; Svingen & Koopman, 2013). From 15 dpc onward, activin-A secreted by Leydig cells triggers the second wave of Sertoli cell proliferation (Archambeault & Yao, 2010; Mendis, Meachem, Sarraj, & Loveland, 2011; Ungewitter & Yao, 2012). They continue to proliferate throughout the fetal period. After birth, on the other hand, their proliferation is supported by pituitary-derived FSH (Abel et al., 2008; Johnston et al., 2004), and continues until around 12 days postpartum (dpp; Kluin, Kramer, De, & Rooij, 1984; Vergouwen et al., 1991). Such growth of the fetal and postnatal testis is a unique phenomenon taking place in vivo and it is hardly observed under culture conditions in our experience (Kojima, Sato, Naruse, & Ogawa, 2016), nor in previous studies to our knowledge.

In our previous studies, we demonstrated that the mouse fetal, neonatal, and adult testis can be cultured to induce spermatogenesis on an agarose gel stand half-soaked in medium (Kojima et al., 2016; Sato et al., 2011, 2015). As for the fetal testis, spermatogenesis was induced with 14.5 dpc or older but not with younger mice, suggesting that the structural integrity of the seminiferous tubule is important for spermatogenic progression (Kojima et al., 2016). It has also been reported that the spermatogenic capacity of the testis depends on the number of Sertoli cells, because they are critical determinants of the adult testis sperm output (Orth, Gunsalus, & Lamperti, 1988; Sharpe, 1994). Therefore, promoting testis growth by the increased proliferation of Sertoli cells in vitro and identifying factors having such an effect would be useful for understanding and promoting male fertility. Although there have been several studies evaluating the cell kinetics of Sertoli cells in vitro for a relatively short period of time (Meehan et al., 2000; Schlatt et al., 1999), few studies have focused on actual testis growth, namely volume enlargement, or the elongation of seminiferous tubules.

We have cultured testis tissues in a microfluidics device made of polydimethylsiloxane (PDMS), which is oxygen permeable (Charati & Stern, 1998), to improve the culture microenvironment (Komeya et al., 2016). It actually ameliorated central necrosis, and maintained mouse spermatogenesis for over 6 months. Based on this experience, we considered that flattening neonatal testis tissue per se on agarose gel can prevent central necrosis. In the current study, we molded PDMS into a small board-shaped chip with a wide, shallow dent on one side, and placed it dent-side down over tissues on agarose gel. Beyond our expectations, tissues were not only free of central necrosis but also showed significant volume enlargement during the culture period, indicating that oxygen can be permeated through the PDMS chip, while nutrients diffuse from agarose gel almost evenly to every part of the tissue. Also, this PDMS ceiling allowed clearer observation of tissues owing to their flat shape, or reduced thickness. With this method, we evaluated several growth factors for their testes-growth-promoting effect, revealing that FSH and insulin were significantly effective.

2 | MATERIALS AND METHODS

2.1 | Mice and treatments

Histone H4-venus (H4V) transgenic mice (Makino et al., 2013; genetic background: C57BL/6 J-ICR mixed) and Sox9-EGFP knock-in mice (Nakamura et al., 2011; Nel-Themaat et al., 2009; genetic background: C57Bl/6J-129/SvEv-Swiss-ICR mixed) were used. The H4V signal is localized in the nucleus of germ cells from spermatogonia to spermatids, appearing as numerous green dots in the seminiferous tubules when observed with a stereomicroscope. The Sox9-EGFP, on the other hand, is expressed exclusively in the Sertoli cells in the testis. Mice were housed in air-conditioned rooms at 24 ± 1°C and 55 ± 5% humidity, with a 14-hr light and 10-hr dark cycle. Hard pellets of diet (Oriental Yeast Co. Ltd., Tokyo, Japan) were given ad libitum. Drinking water was acidified to pH 2.8–3.0 by HCl. Testes were obtained at 1.5 dpp, and decapsulated in PBS. Each testis as a whole was placed on an agarose gel stand for culturing. All of the animal experiments conformed to the Guide for Care and Use of Laboratory Animals and were approved by the Institutional Committee of Laboratory Animal Experimentation (Research Institute for Yokohama City University, Yokohama, Japan).

2.2 | PDMS-ceiling chip

The PDMS-ceiling (PC) chip was produced by mixing PDMS prepolymer and curing reagent (Silpot 184; DowCorning) at a 5:1 weight ratio. The mixture was poured over the mold master, placed in a vacuum chamber for degassing, and then moved to an oven to be heated at 72°C for 1.5 hr for curing. After cooling down, solidified PDMS was peeled off from the master without touching it with hands directly. Then, this disk of PDMS containing 32 chips was cut into individual chips using a cutter knife. The mold master was made by conventional photolithography and soft lithography techniques

(Duffy, McDonald, Schueller, & Whitesides, 1998; Fujii, 2002). Briefly, the material of the master mold, a negative-type photoresist (SU-8 2100 & 2075; MicroChem Co.) was poured on a 4-in. wafer and spin-coated over it to evenly achieve the target thicknesses, 60, 100, 140, and 180 μm, over the wafer. After prebaking, ultraviolet light was administered through a photomask to create a space for cultivation, followed by postbaking. The baked mold master was developed by incubation in propylene glycol monomethylether acetate (GODO, Tokyo, Japan) for 20–30 min, followed by rinsing in isopropanol (code 32435-70; Kanto Chemical, Kanto, Japan). This SU-8 mold master can be used repeatedly for the replica molding of PC chips. The photomask was designed with CAD software (AutoCAD; Autodesk Inc., San Rafael, CA) and fabricated with a laser lithography system.

2.3 | Culture method

To make an agarose gel stand for organ culturing, agarose powder (Dojindo Lab., Kumamoto, Japan) was dissolved in double-distilled water to 1.5% (wt/vol) and autoclaved. During the cooling, 33 ml of agarose solution was poured into 10-cm dishes to form an about 5-mm-thick gel. The gel was cut into about 1-cm^2 pieces and these were used as stands for testis tissue placement. Gels were submerged in the culture medium for more than 6 hr before use. Testes were transferred to the surface of agarose gel that was half-soaked in the medium. Each gel stand was loaded with a single testis. The protocol was reported previously in detail (Sato, Katagiri, Kubota, & Ogawa, 2013; Yokonishi, Sato, Katagiri, & Ogawa, 2013). Then, the PC chip was placed over the testis, dent-side down. Medium change was performed once a week. The culture incubator was supplied with 5% carbon dioxide in air and maintained at 34°C.

As for the culture medium, α-modified Eagle medium (αMEM; Invitrogen, Carlsbad, CA) supplemented with AlbuMAX (ThermoFisher Scientific, Waltham, MA) at 40 mg/ml (designated as AlbuMAX medium), was used unless otherwise stated. Germline stem cell medium (designated as GS medium) was originally formulated for culturing mouse spermatogonial stem cells (Kanatsu-Shinohara et al., 2003). It was slightly modified in this study and consisted of StemPro-34 SFM (Invitrogen) supplemented with StemPro supplement, 25 μg/ml insulin (Wako, Osaka, Japan), 100 μg/ml transferrin (Sigma Aldrich, St. Louis, MO), 60 μM putrescine (Sigma Aldrich), 30 nM sodium selenite (Sigma Aldrich), 6 mg/ml D-(+)-glucose (Wako), 38.5 μg/ml sodium pyruvate (Wako), 1 μl/ml DL-lactic acid (Sigma Aldrich), 5 mg/ml bovine serum albumin (BSA; Sigma Aldrich), 2 mM L-glutamine (Sigma Aldrich), 5×10^{-5} M 2-mercaptoethanol (Invitrogen), 1% (vol/vol) minimal essential medium (MEM) vitamin solution (Invitrogen), 1% (vol/vol) MEM nonessential amino acid solution (Invitrogen), 10^{-4} M ascorbic acid (Sigma Aldrich), 10 μg/ml D-biotin (Sigma Aldrich), 1% (vol/vol) antibiotic antimycotic (Invitrogen), 20 ng/ml recombinant human epidermal growth factor (Wako), 10 ng/ml human basic fibroblast growth factor (Wako), 10 ng/ml recombinant human glial cell line-derived neurotrophic factor (R&D Systems, Minneapolis, MN), 30 ng/ml β-estradiol (Sigma Aldrich), 60 ng/ml progesterone (Sigma Aldrich) and 1% fetal bovine serum (FBS), ES cell qualified (Invitrogen). For the 5-bromo-2′-deoxyuridine (BrdU) incorporation assay, testis tissues were cultured in the culture medium supplemented with 10 μM BrdU for 14 hr before tissue fixation.

2.4 | Observation

Testes being cultured were observed under a stereomicroscope equipped with an excitation light for ****** green fluorescent protein (GFP; Leica M 205 FA), and images were recorded as photographs once a week to evaluate the expansion of tissues and H4-Venus expression. For measuring the size of the cultured testis, the area of tissue in a picture was calculated using computer software (Leica Application Suite version 4.3.0).

2.5 | Histological and immunohistological examination

For histological examination, tissues were fixed in Bouin's fluid, embedded in paraffin, sectioned at 3 μm, and stained with hematoxylin and eosin. For immunohistological examination, sample tissues were fixed with 4% paraformaldehyde in PBS at 4°C overnight. Cryoprotection was then performed with solutions of 10%, 15%, and 20% (wt/vol) sucrose in PBS for 1 hr each in succession. Tissues were cryo-embedded in OCT compound (Sakura Finetechnical, Tokyo, Japan) and cut into 7-μm-thick sections. The cryosections were washed with 0.2% phosphate buffered saline and Tween 20 (PBT; 0.2% Triton X-100 in PBS) four times and then treated with Image-iT FX Signal Enhancer (Invitrogen) for 30 min. Incubation with primary antibodies in 0.2% PBT containing 5% BSA was performed overnight at 4°C, followed by rinsing four times with 0.2% PBT. Then secondary antibodies, Alexa Fluor 488, Alexa Fluor 555, or Alexa Fluor 647-conjugated goat anti-rat Ig G (1:200; Invitrogen) were applied for 1 hr at room temperature. Nuclei were counterstained with Hoechst 33342 dye. The following were used as primary antibodies: chicken anti-GFPantibody (1:1,000; Abcam, Cambridge, UK), rat anti-Tra98 antibody (1:1,000; Bioacademia, Osaka, Japan), goat anti-GFRα1 antibody (1:250; R&D Systems), rabbit anti-3βHSD (1:250; Trans Genic Inc., Fukuoka, Japan) and mouse anti-BrdU antibody (1:100; Santa Cruz Biotechnology, Dallas, TX). To count the number of germ cells in each section, Tra98-positive cells along with cells in total, by Hoechst stain, were counted manually in the representative pictures in three tissues each group. For BrdU detection, cryosections were treated with 2 N HCl at 37°C for 30 min and washed with PBS before primary antibody treatment. Specimens were observed with a confocal laser microscope (FV-1000D; Olympus, Tokyo, Japan). For terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL), apoptotic cells were detected with the In Situ Cell Death Detection Kit, TMR red (Sigma Aldrich).

2.6 | Flow cytometry

Testis tissues were digested with 2% collagenase in PBS for 20 min at 37°C, then followed by 0.25% trypsin for 10 min at 37°C. After stopping the reaction by adding Dulbecco's modified Eagle's medium + 10% FBS, they were pelleted by centrifugation after passing through a

cell strainer with a 40-μm pore size (Becton Dickinson, Franklin Lakes, NJ) and resuspended in PBS containing 3% (vol/vol) FBS. The samples were applied to flow-cytometer Guava easyCyte (Merck, Darmstadt, Germany). First, viable single cells were identified and selected by propidium iodide and profiling by forward and side scatter. Then, they were separated by GFP expression for counting and calculating Sox9-EGFP-positive cells. For intracellular protein detection, suspended cells were fixed with 2% paraformaldehyde in PBS for 20 min at room temperature. After a centrifugation, cell pellet was resuspended in methanol (90%)/PBS (10%) for 30 min on ice. The cells were washed with PBS containing 2% FBS two times and then incubate with chicken anti-GFP and rat anti-Tra98 antibodies in 0.2% PBT containing 5% BSA was performed overnight at 4°C, followed by rinsing two times with 2% FBS, and then secondary antibody, Alexa Fluor 488 of Alexa Fluor 647-conjugated goat anti-chicken and rat or rabbit Ig G (1:400; Invitrogen) were applied for 1 hr at room temperature. The cells were resuspented in PBS containing 3% (vol/vol) FBS and then applied to flow cytometer.

2.7 | Statistical analysis

The parametric multiple-comparison test (Tukey-Kramer) was performed to assess differences in the volume expansion level of testis tissues cultured by AlbuMAX medium supplemented with each supplement, covered by PC chips with depths of 60 μm during cultivation. A $p < 0.05$ was considered to indicate a significant difference.

3 | RESULTS

3.1 | Manufacturing of PC chip

Neonatal mouse testes, 1 dpp, were used for the experiment. When they were cultured using the conventional gas–liquid interphase method, on the half-soaked agarose gel stand, they formed a round dome-like shape with central regions occasionally showing degenerative or necrotic changes (Figure 1a). Namely, regions deep inside the tissue mass were prone to slow molecular exchanges, particularly involving

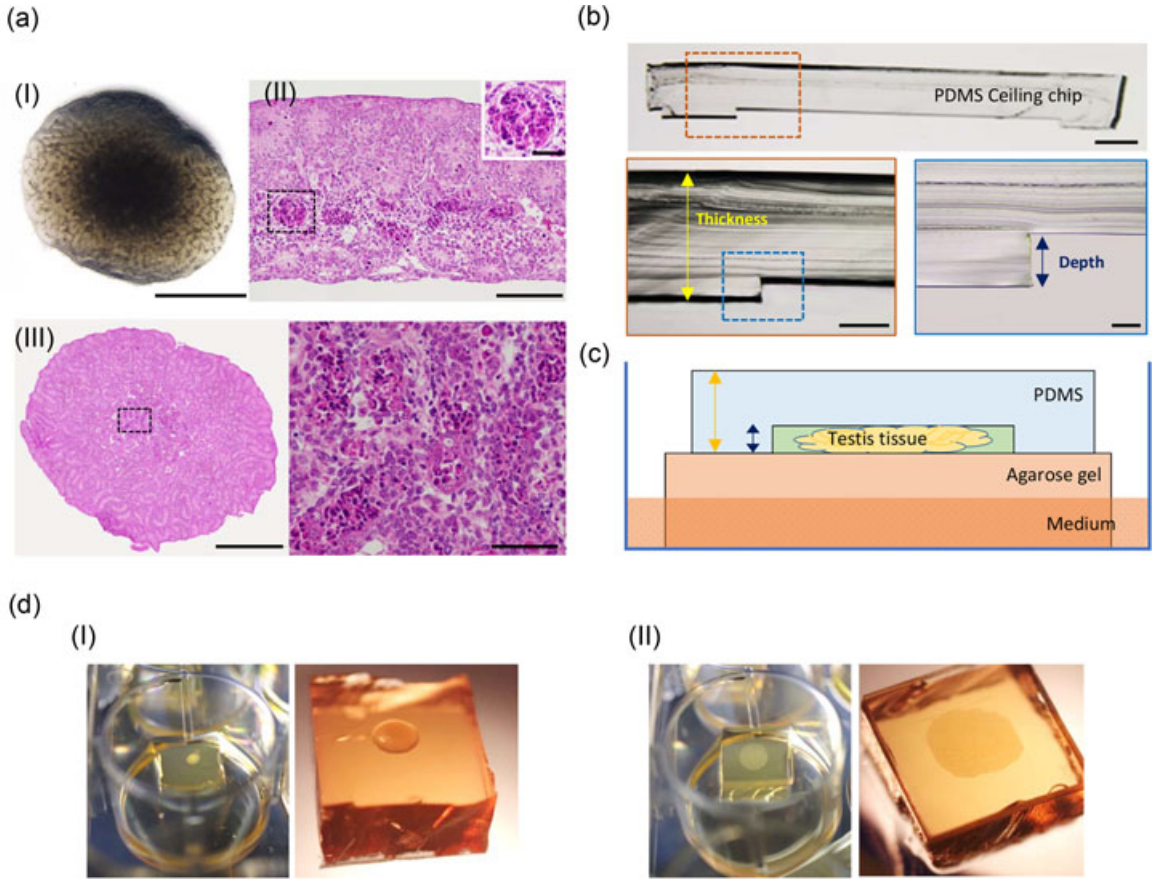

FIGURE 1 General image of testis tissues in organ culture. (a) The testis tissue on agarose gel, a conventional gas–liquid interphase method, adopted a dome-like shape and its center region showed necrotic changes. The tissue was taken from a 1-dpp mouse, and cultured for 7 days. (I) Stereomicroscopic bright-field image of cultured testis. (II) Histologic images showing vertical cross section. Inset is the enlarged image of the dashed-line square. (III) Histologic images showing transverse section. The dotted rectangular area is enlarged on the right. (b) A vertical cross-sectional view of the PDMS chip. The brown dotted rectangular area is enlarged in the middle left. The blue dotted rectangular area in the middle-left image is enlarged in the middle right. (c) A scheme of the PC method. Yellow line: Thickness. Blue line: Depth. (d) Comparison of testis organ culture between with and without PC covering. (I) The testis tissue on agarose gel without PC covering. (II) The testis tissue, covered with PC, showing two-dimensional spreading. Scale bars = (a) 1 mm (I), 100 μm (II), 25 μm (II, inset), 500 μm (III, left), and 50 μm (III, right), (b) 1 mm (upper), 500 μm (lower left), and 100 μm (lower right). dpp: days postpartum; PC: PDMS ceiling

oxygen. To solve this problem, one way would be to flatten the tissues by pressing to spread them widely on the agarose. Tissues spread on agarose gel, however, readily aggregate to return to a dome-like shape, possibly due to the surface tension of the covering medium along with occasional vibration that is unavoidable during handling. Thus, we manufactured a small chip made of PDMS for the ceiling, named the PDMS-ceiling (PC) chip (Figure 1b). The chip was 1–1.2 cm^2 and 1–1.5-mm thick, with a shallow dent, 4–8 mm^2, in the center on one side. The depth of the dent was designed as 60, 100, 140, or 180 μm (Figure 1b). They were actually 54–63, 100–115, 138–175, or 178–220 μm, respectively, when measured after being used. When tissues on agarose gel were covered with the PC chip, dent-side down, they spread in a given space of the dent, between the agarose gel and PDMS ceiling. They can obtain nutrients through the agarose below and oxygen through the PDMS above (Figure 1c). Actually, testis tissues placed on the agarose gel initially adopted a dome-like shape but then became flat when covered by the PC chip (Figure 1d). As the space volume of a dent with a size of 4–8 mm^2 and 60 μm deep is 0.96–3.84 mm^3, it is sufficient for the testis of a 1-dpp mouse (about 0.5 mm^3).

3.2 | Tissue growth under PDMS ceiling

A single whole neonatal mouse testis cultured with the conventional agarose method without the PC chip showed no marked changes in size during the 7 days of the experiment when observed under a stereomicroscope. On the other hand, when covered with the PC chip, they flattened into a disc shape. Notably, the apparent size of testis tissue increased day by day. This size increment became more prominent as the dent depth became shallower from 180 to 60 μm (Figure 2a). On Day 7, tissues were fixed and vertical sections were made, confirming that the tissue thickness corresponded well with the dent depth. In fact, the layers of the seminiferous tubule decreased as the dent depth became shallower; 3 or 4 at 180 μm, 3 at 140 μm, 2 at 100 μm, and 1 at 60 μm (Figure 2b). Those cultured neonatal testis tissues observed under the stereomicroscope were recorded as photographs. Using those images, the area of the tissue was calculated using image software. The changes in area values during 8 days were plotted on a graph. With a 60-μm dent depth, the area markedly increased, but this was not marked with 140 or 180 μm (Figure 2c). In addition, using the PC method, the height of the tissue mass was actually fixed by the dent depth; thus, the volume of tissue can be calculated by multiplying the height by the area, designated here as the tentative tissue volume (tV). Then, the area was converted to tV (Figure 2d). Notably, tV increased sharply in the dent at 60 and 100 μm, showing similar slopes. At 140 and 180 μm, on the other hand, tV increments were modest. We suggest that tissues with an increased thickness over 100 μm cannot grow sufficiently due to limited permeation, hampering the availability of oxygen and nutrients.

To obtain data on the degree of growth of the testis in vivo, mouse testes at 1, 4, and 7 dpp were decapsulated and covered with a PC chip, with a dent depth of 100 μm, to calculate their tV. Data showed that tV in vivo was about 0.5, 1.1, and 1.9 mm^3 at 1, 4, and 7 dpp, respectively (Figure 2e). As the growth achieved with the PC method on 7-day culture was around 0.9–1.1 mm^3 in tV, the in vitro growth using PC method reflected 50–60% of in vivo growth.

3.3 | Effect of culture media on tissue growth

With the PC method, it became possible to monitor the size of testis tissue during cultivation. In addition to the spatiophysical environment that the PC method conferred to the tissue, the chemical composition of culture medium naturally has a significant effect on growth. All through the above-mentioned experiment, α-MEM supplemented with AlbuMAX (AlbuMAX medium) was used as the medium (Sato et al., 2011). Then, instead of AlbuMAX medium, GS medium was tested with the initial aim of increasing the number of spermatogonia, because the GS medium was developed for that purpose and actually does so under cell culture conditions (Kanatsu-Shinohara et al., 2003). Unexpectedly, however, testis tissues cultured with GS medium enlarged markedly, being significantly larger than those cultured with AlbuMAX medium (Figure 3a). The volume of testis tissues on Day 6 was about 1.5 mm^3 in GS medium, while it was about 0.9 mm^3 in AlbuMAX medium, being 1.7-fold larger in the former (Figure 3b). The growth in GS medium was nearly comparable to that in vivo, reaching about 80% (1.5/1.9) of that in vivo (Figure 2e). These data suggest that a factor present in GS medium, but absent in AlbuMAX, promoted testis growth.

3.4 | Factors contributing to testis tissue growth

Several factors have been reported to promote testis growth, including FGF9, activin-A, and FSH. FGF9 and activin-A have been reported as Sertoli cell growth factors in the fetus (Archambeault & Yao, 2010; Brennan & Capel, 2004; Colvin et al., 2001; Schmahl et al., 2004; Ungewitter & Yao, 2012). FSH is also known to promote Sertoli cell growth after birth (Abel et al., 2008; Johnston et al., 2004). Although these factors were not included in the GS medium, we tested them by supplementing the AlbuMAX medium with them for the organ culture of 1-dpp mouse testis. Their tV was measured with the PC method, and the chip with a dent depth of 60 μm. FGF9 and activin-A showed no growth-promoting effect, while FSH increased the tV during the culture period of a week (Figure 4a). These results again support the validity of the PC method to faithfully reflect the growth of testis tissue in vivo. Next, five factors contained in GS medium: FGF2, epidermal growth factor (EGF), glial cell-line derived neurotrophic factor (GDNF), transferrin, and insulin, were examined one by one through adding each of them to the AlbuMAX medium. Through 22 experiments in total, insulin alone markedly promoted testis tissue growth (Figure 4b). The tV changes during the culture period were monitored to calculate the growth rate in AlbuMAX medium containing each factor, in comparison with the controls (AlbuMAX with no factor and GS medium). The growth rates, represented as the calculated slope and summarized in Figure 4c, showed that FSH and insulin had a testis growth-promoting effect. In particular, the effect of insulin was close to that of the GS medium, suggesting that insulin is the main factor in GS medium responsible for testis growth.

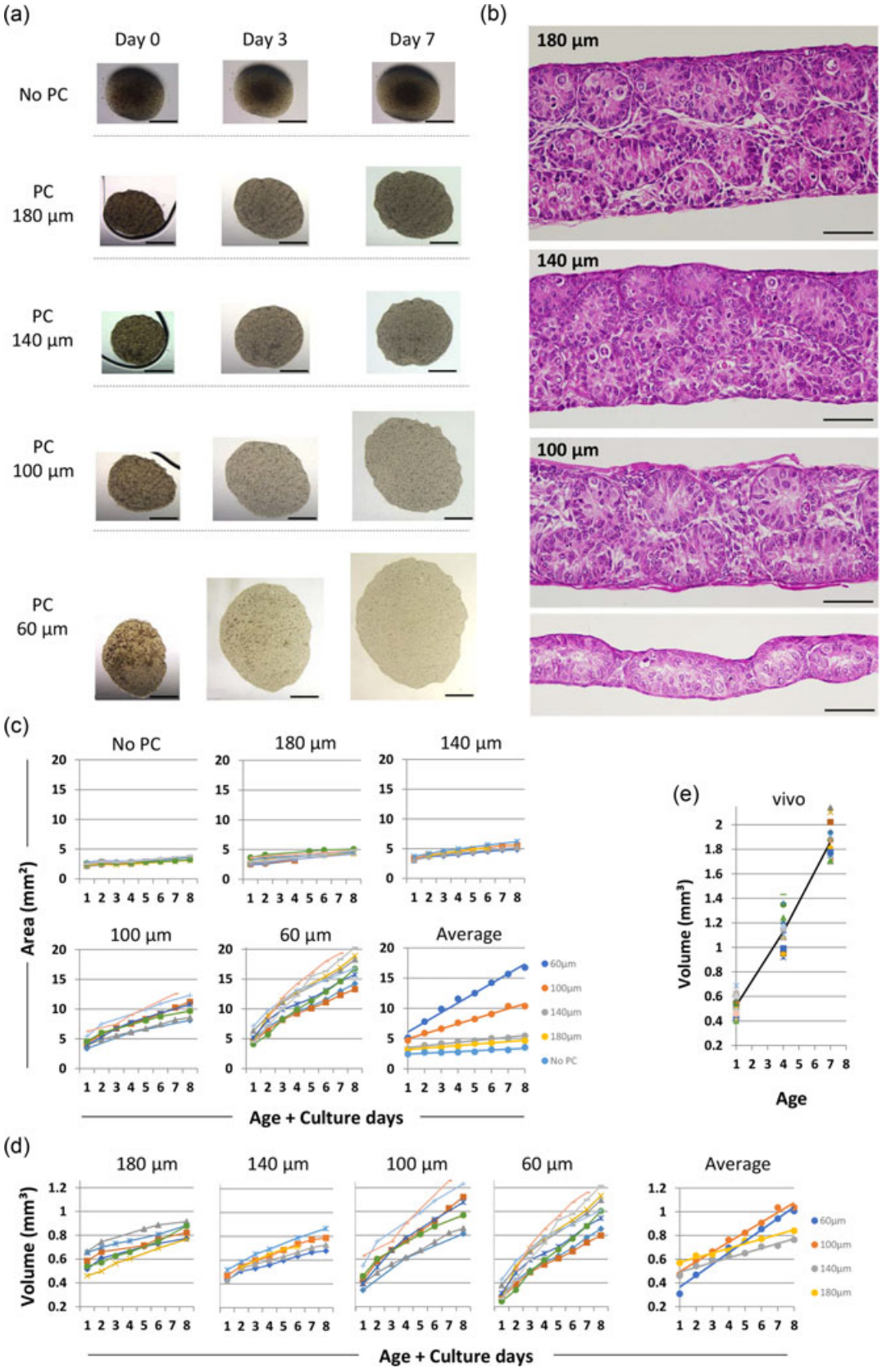

FIGURE 2 A 1-dpp mouse testis in organ culture with PC. (a) A stereomicroscopic view of testis tissues cultured with no PC and with PC chips with dent depth of 180, 140, 100, and 60 µm, on days 0, 3, and 7. (b) A histologic vertical cross-sectional view of testis cultured for 7 days with PC of four different dent-depths. (c) Area change of testis tissues cultured for 7 days with PC of 4 different dent-depths along with no PC. Each line corresponds to each sample. (d) Volume change of testis tissues cultured for 7 days with PC. (e) Volume change of testis in vivo. Scale bars = (a) 1 mm, (b) 50 µm. dpp: days postpartum; PC: PDMS ceiling

The initial aim using GS medium was to increase the number of germ cells, particularly spermatogonia. To evaluate this effect, mice carrying the histone H4-venus (H4V), thus expressing venus in their germ cells, were useful. The germ cells expressing H4V signals were easily detectable with our PC culture method as numerous green dots in the seminiferous tubules, when observed with a stereomicroscope under GFP excitation. In the H4V neonatal testis cultured in AlbuMAX medium, the venus-expressing germ cells were observed throughout the tissue for 8 days (Figure 4d). With GS medium, however, they decreased gradually and fewer germ cells remained, being mostly in the peripheral region, on Day 8. This was actually opposite to what we had expected. We considered whether that germ cell-diminishing effect might be related to the testis size enlargement. However, in testis tissues cultured in AlbuMAX medium supplemented with insulin, which promoted the testis growth, the H4V signal was maintained favorably compared with that in the control (Figure 4d). The histologic examination confirmed the presence of spermatogonia in control (AlbuMAX) and insulin-supplemented culture conditions and fewer in the testis cultured with GS medium (Figure 4e). To confirm this, immunohistochemistry with the antibody to TRA98, a germ-cell specific marker, was performed (Figure 4f). The TRA98-positive germ cells along with Hoechst-stained cells were counted to calculate the rate of germ cells in each group, confirming that germ cells decreased in GS medium but maintained in AlbuMAX and insulin (Figure 4f). The germ cell rate in the testis of 7.5 dpp mouse was counted to be 25.3 ± 5.5%. Taken together, the two effects of GS medium, one that enhances tissue growth and the other that reduces germ cells, can be separated. Nonetheless, the germ cell remaining in the tissue cultured with GS medium contained GFRα1-positive spermatogonia indicating the

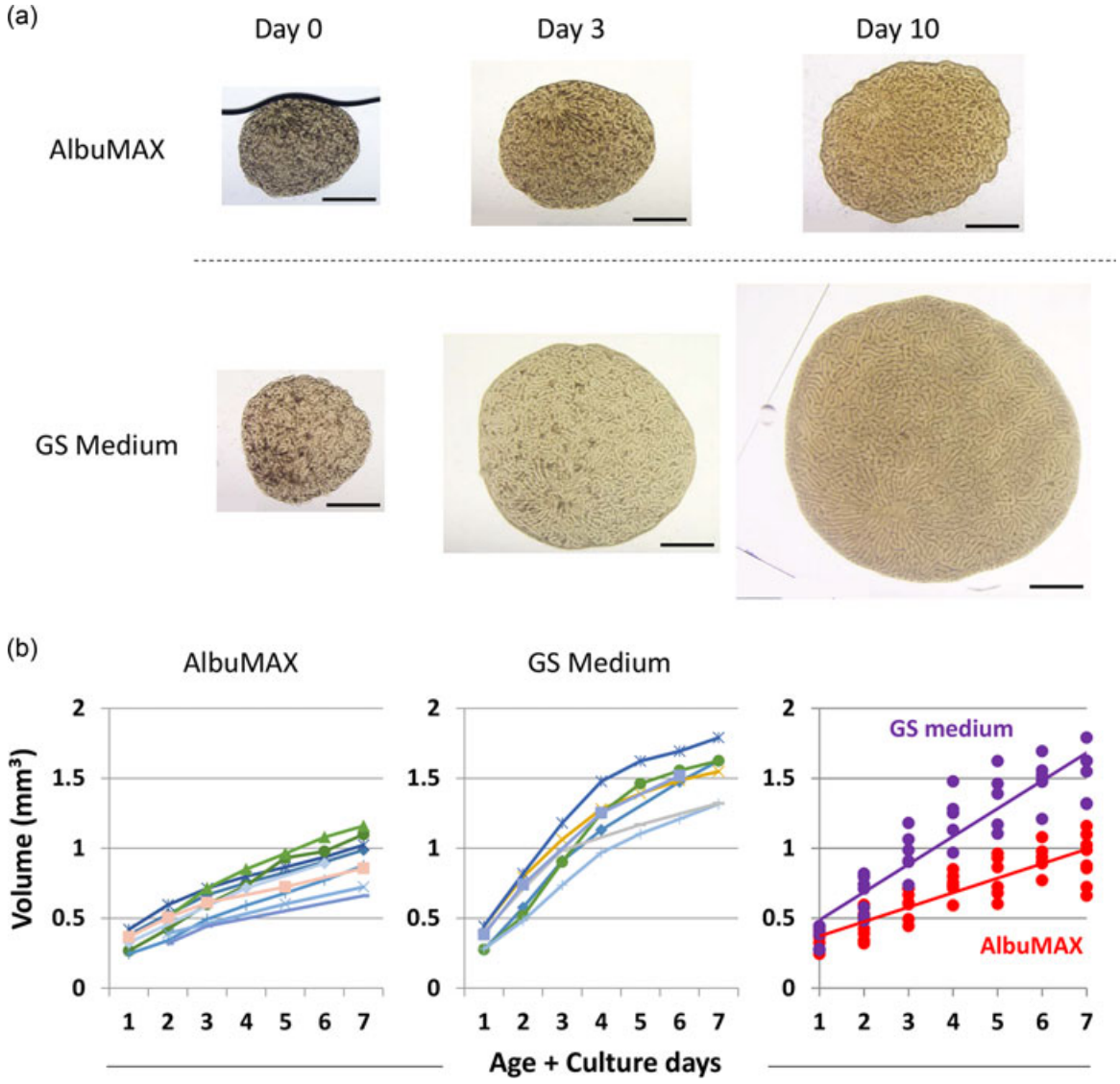

FIGURE 3 Volume expansion of 1-dpp mouse testis cultured in two media under PC. (a) A testis tissue cultured under the PC with a dent depth of 100 μm, with AlbuMAX or GS medium. Stereomicroscopic views of each testis on days 0, 3, and 10 are shown. (b) Volume changes of testis tissues cultured for 6 days under PC with a 60-μm depth and AlbuMAX or GS medium are shown in the left and center graphs, respectively. Each line corresponds to each sample. The approximate linearization of the volume change in each medium is indicated in the right graph. Scale bars = (a) 1 mm. GS: germline stem cell; PC: PDMS ceiling

presence of spermatogonial stem cell population (Supporting Information Figure 1).

3.5 | Cells responsible for the testis tissue enlargement

As the testis tissue enlargement with the GS medium was not aroused by germ cell proliferation, we next tried to identify cells responsible for that growth. For this, we used Sox9-EGFP mouse to faithfully identify Sertoli cells by their GFP expression. The neonatal testis tissues of this mouse responded similarly to different media as H4V mouse testis did (Figure 5a). The tissues cultured for 7 days were digested with enzymes for single cell isolation, which were applied to flow-cytometric analysis. GFP-positive Sertoli cells were counted among singly isolated viable cells in four groups (Figure 5b). Tissues cultured with GS medium contained increased number of Sertoli cells, while those cultured with insulin did not. This was an unexpected result considering the effect of insulin increasing the tissue volume (Figure 5a). We then fixed the single cells and applied antibodies to Tra98 and GFP for identifying germ and Sertoli cells, respectively, in the flow-cytometric analysis. The study demonstrated again that GS medium increased Sertoli cells significantly, while insulin did not (Figure 5c). Thus, the tissue-enlarging effect of insulin would be ascribed to proliferation of cells other than Sertoli cell and/or volume increment of each cell. When antibodies to 3β-HSD, for identifying Leydig cells, were used for

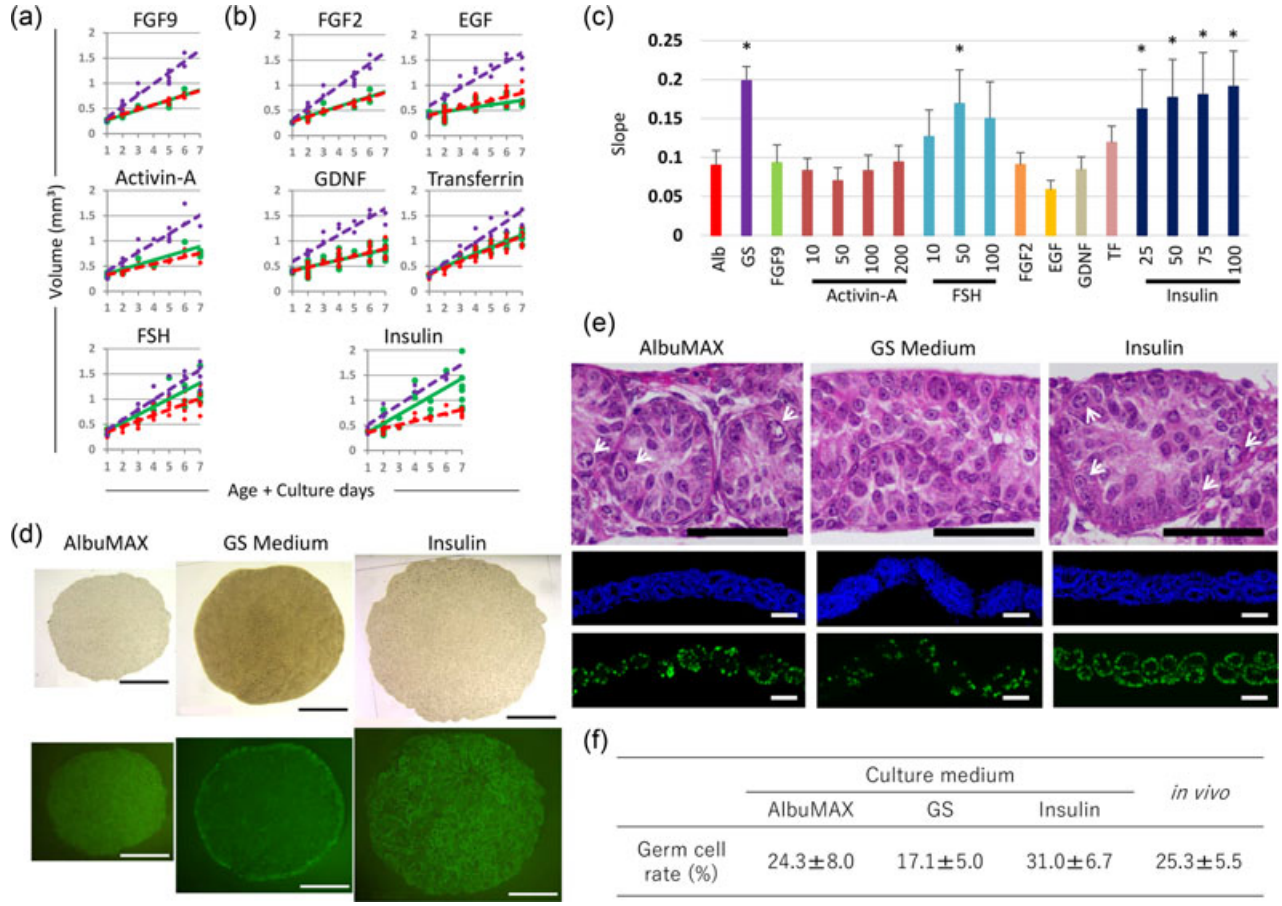

FIGURE 4 Testis growth was promoted by supplements in the culture medium. (a,b) Comparing the volume change of each testis cultured for 6 days with a 60-μm depth of PC in each medium, AlbuMAX medium (red dots and line), GS medium (purple dots and line), or AlbuMAX medium supplemented with factors designated at the top of each graph (green dots and line). Each dot corresponds to each sample. The line is the approximate linearization of the volume change. Concentration of each factor: FGF9 (10 ng/ml), activin-A (100 ng/ml), FSH (100 ng/ml), FGF2 (10 ng/ml), EGF (20 ng/ml), GDNF (10 ng/ml), transferrin (100 μg/ml), and insulin (25 μg/ml). Insulin showed significant difference to AlbuMAX medium. (c) The slope of each approximate line of volume change of testis tissues cultured in each medium. Concentration of each factor: FGF9 (10 ng/ml), Activin-A (10, 50, 100, 200 ng/ml), FSH (10, 50, 100 ng/ml), FGF2 (10 ng/ml), EGF (20 ng/ml), GDNF (10 ng/ml), transferrin (100 μg/ml), and insulin (25, 50, 75, 100 μg/ml). *Significant difference to AlbuMAX medium. (d) Stereomicroscopic views of bright-field and Venus-excited 1-dpp testes cultured for 8 days in medium, AlbuMAX medium, GS medium, and AlbuMAX medium supplemented with FSH (100 ng/ml) or insulin (50 μg/ml). Green indicates venus-positive cells. (e) Histological and immunohistological preparation of testis samples cultured with AlbuMAX medium, GS medium, and AlbuMAX medium supplemented with insulin. Nuclear stain with hoechst (blue) and immunostain with anti-TRA98 antibody (green) was performed. (f) Germ-cell rate among cells in total, mean ± *SD*, was calculated using three tissues in each culture group ($n = 3$). In vivo data was obtained from a mouse of 7.5 dpp. Scale bar = (D) 2 mm, (E) 50 μm. dpp: days postpartum; GS: germline stem cell; PC: PDMS ceiling

immunohistochemical staining and flow-cytometric analyses, Leydig cell frequency appeared ordinary and remained to be a minor component in the tissues in 3 culture media as in vivo control (Supporting Information Figure 2). As for germ cells, GS medium did not maintain them (Figure 5c), which was in agreement with the results obtained with H4V mouse (Figure 4d, e, f). AlubuMAX and insulin-supplemented AlbuMAX supported germ cell proliferation but not as much extent as in vivo control (Figure 5c). Then, we stained the H4V mouse testis tissues on culture Day 3 by BrdU to find cells actively proliferating. In both AlbuMAX and GS medium groups, Sertoli cells along with cells in the interstitium were labeled with BrdU in high frequency (Figure 5d). On the other hand, apoptotic cells identified by TUNEL stain were scattered but not many in both AlbuMAX and GS (Supporting Information Figure 3). Taken together, these data indicate that the enlargement of neonatal testis tissue cultured with PC method was a result of active proliferation of testicular somatic cells including Sertoli cells along with modest increment of germ cells. At the same time, it became obvious that such growth was influenced significantly by the composition of culture medium.

4 | DISCUSSION

It is rare for any single functional cell in a body to be located more than 30 μm away from a capillary (Hall, 2011, Chapter 16). In the testes, as the diameter of a seminiferous tubule reaches about 200 μm, cells at the center, mostly elongating spermatids, would exceed that distance from a capillary. Nonetheless, each seminiferous tubule is surrounded by a mesh work of capillaries, ensuring the supply of oxygen and nutrients. When explanted, and so losing that circulatory system, tissues are invariably exposed to uncontrolled oxygen tensions. Namely, while the outer region of the tissue may receive an adequate or even a surplus supply of oxygen, the central region almost inevitably suffers from a shortage of oxygen and undergoes degenerative or necrotic changes. As a rule in organ culture, tissues are generally placed at the interface between the liquid and gaseous phases, to facilitate gas exchange while retaining access to nutrients. Nonetheless, degeneration and necrosis in the central region frequently occur (Freshney, 2000, Chapter 24). Raising the oxygen tension in the incubator could be helpful in certain cases, such as whole embryo culture for a short period of time, but not so in

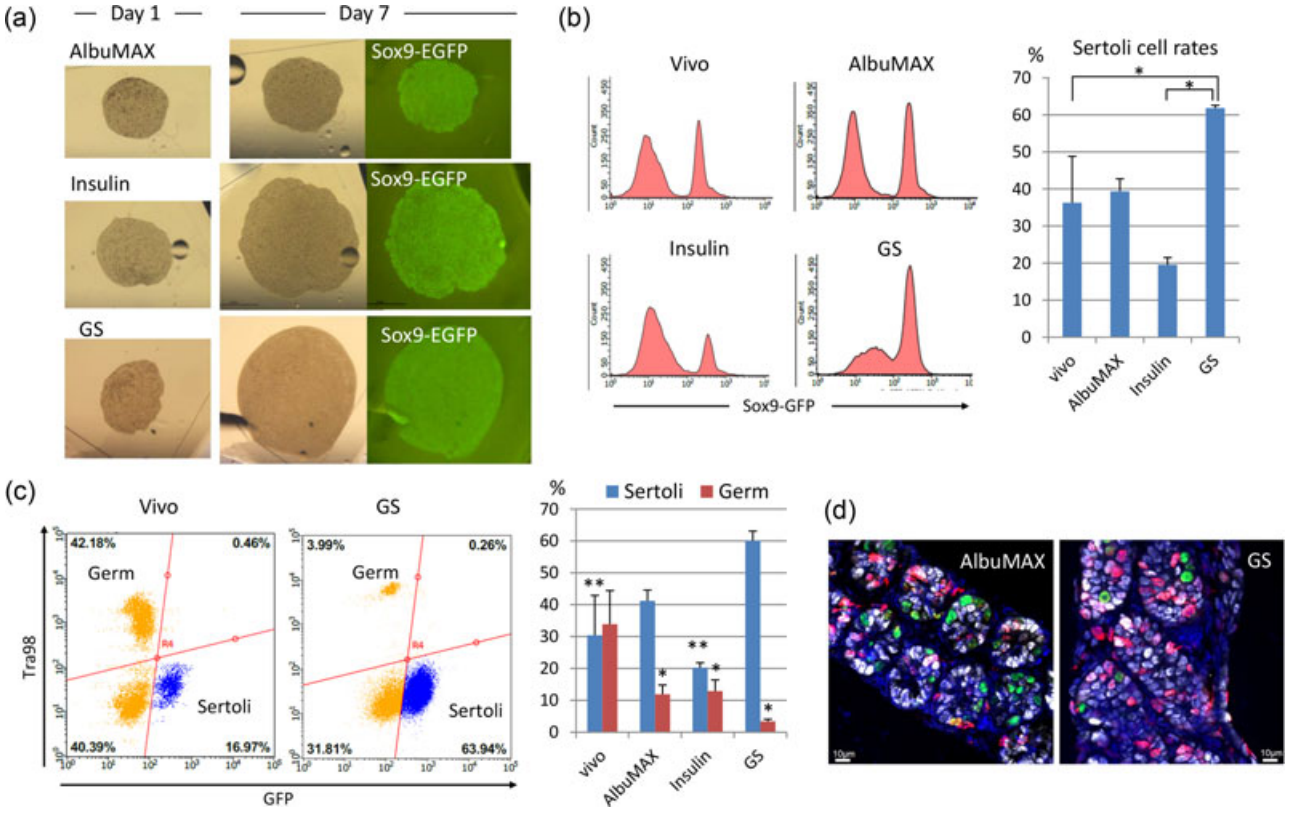

FIGURE 5 Flow-cytometric analysis of PC samples. (a) Neonatal testis of Sox9-EGFP mouse cultured under the PC with a dent depth of 100 μm, with AlbuMAX, AlbuMAX plus insulin, and GS medium (GS). Stereomicroscopic views of each testis on days 1 and 7 are shown. (b) Sox9-EGFP mouse testis cultured for 7 days were applied for flow cytometry. Rate of Sertoli cells among singly-isolated viable cells were summarized in the right graph. Tissue numbers examined were six for in vivo sample and three for each culture group. *Significant difference. (c) The singly-isolated cells were fixed and antibodies to Tra98 and GFP were applied, followed by flow-cytometric analysis. Germ (Tra98-positive) and Sertoli (Sox9-GFP-positive) cells were detected differentially. Data summarized were shown in the right graph. Tissue numbers examined were same as in (b). *Significant differences to vivo, **significant differences to GS. (d) Neonatal H4V mouse testis culture in media (AlbuMAX and GS) for 3 days were immunohistochemically examined with antibodies to venus (green, germ cells), BrdU (red, cells in S-phase), Sox9 (white, Sertoli cells), and counterstained with Hoechst (blue). GS: germline stem cell; PC: PDMS ceiling

most other cases, due to the toxic effect of oxygen becoming apparent during longer periods (Ellis-Hutchings & Carney, 2010; New, 1978; New, Coppola, & Terry, 1973). In recent years, trials to establish vascular systems in vitro have been keenly pursued, but none have been successful (Matsusaki, Case, & Akashi, 2014; Miller et al., 2012; Sekine et al., 2013; Seto et al., 2010; Takei, Sakai, Ono, Ijima, & Kawakami, 2006). In our previous attempt to introduce a microfluidic system in the organ culture experiments, we successfully prevented central degeneration and necrosis of testis tissues by decreasing their thickness to around 160 μm. In this microfluidic device, thin tissues obtained nutrients from the culture medium running above them and oxygen through PDMS below. In successful cases, mouse spermatogenesis was maintained for 6 months or beyond (Komeya et al., 2016). However, there were several drawbacks to that system: high cost, extensive labor, skills in high level, space occupation in an incubator, and a low number of tissues that could be examined at a time. Namely, the microfluidic device needs skills and experience to produce, taking a number of days to produce several devices. Mass production is not yet in practice. The number of tissues cultured in a device is small, possibly being only one. So, it becomes difficult to conduct an experiment involving multiple groups with several tissues in each group to obtain significance regarding particular experimental factors. On the other hand, the PC chip method we devised in this study is simpler both in terms of manufacturing and handling in experiments. Thus, it is cost-effective and less laborious for production. In addition, the PC chip is versatile in many aspects. For instance, a wide range of tissue sizes is acceptable, only requiring modification of the dent size. The number of samples cultured at one time can be increased as it simply depends on the number of chips and agarose gel blocks. Such a flexible method could not be expected in experiments using microfluidic devices. In principle, the PC method has the same effect of modifying the shape of tissues into a thin, flat form as in the microfluidic device. Every part of the tissue, even the central region, can equally receive oxygen through PDMS and nutrients through agarose gel. Thus, central degeneration and necrosis can be avoided. Not only that, to our surprise, the neonatal testis showed significant enlargement during a week of culture. There have been several reports addressing the issue of testis growth under culture conditions (Meehan et al., 2000; Schlatt et al., 1999). There are, however, few studies measuring the actual enlargement of the cultured tissue. In fact, organs and tissues have not been able to grow and enlarge under the culture conditions so far developed. The PC method, therefore, is brand new in this regard and has a significant potential in application to studies of organ culture and organoid experiments.

Making the tissue thin and flat yielded two other advantages. First, observation was more precise and clearer. In this study, a single whole testis of a 1-day-old mouse was spread in the PC chip with a dent depth of 60 μm to form a singly layered seminiferous tubule. Thus, every region of the testis was set in a single plane, allowing even observation of every part of the entire testis. Secondly, it became possible to measure the tissue volume easily and repeatedly while continuing the culture. In previous studies, the volume of the testis was calculated from its weight (Baines et al., 2008) or histological sections (Baker & O'Shaughnessy, 2001; Mendis et al., 2011). However, those methods cannot be applied to tissues in the middle of a culture experiment without disturbing them. To our knowledge, no studies have evaluated the growth of tissues as volume changes during cultivation. The present method makes this possible by modifying the form of tissue into a disc shape with a determined thickness, by which the area size of the disc directly corresponds to the volume. This meant that we were able to evaluate the effect of culture medium, and factors in it, on the growth of testis tissue in a precise manner.

The GS medium is used for culturing mouse spermatogonia for their proliferation (Kanatsu-Shinohara et al., 2003; Sato et al., 2013). Thus, the effect of GS medium to promote the enlargement of the neonatal mouse testis was not what we expected. To our surprise, the expansion was nearly comparable to that observed under in vivo conditions, while germ cells rather diminished. Flow-cytometric analyses indicated that the growth promotion by GS medium was mainly due to the proliferation of Sertoli cells. Then, we were interested in exploring the reason or factors responsible for that effect. Candidates should reside in the GS medium. We tested each of them, along with other factors reportedly responsible for the growth of Sertoli cells, namely FGF9, activin-A, and FSH, which were not contained in the GS medium. Interestingly, FGF9 and activin-A did not show the effect of testis growth promotion, while FSH did. This result is in agreement with the fact that the former two factors act in the fetal period while FSH does so postnatally, considering that the testis tissues cultured were neonatal. Among the five factors included in GS medium, insulin alone showed a growth-promoting effect which was nearly comparable to that shown with GS medium per se. Thus, insulin could be a main reason for the effect of the GS medium. It was reported that mice with knocked-out genes of both the insulin receptor and insulin-like growth factor-1 (IGF1) receptor specifically in Sertoli cells showed the significantly decreased growth of Sertoli cells, a reduced size of the testis, and decreased sperm production. This suggests that insulin–IGF signaling plays important roles in the regulation of the number of Sertoli cells. In addition, the testis weight of the wild-type mouse increased on subcutaneous FSH infusion, while it did not increase in the conditional KO mouse, indicating that the insulin/IGF signaling pathway could be responsible for the action of FSH on Sertoli cell proliferation (Griffeth, Bianda, & Nef, 2014; Pitetti et al., 2013). Contradictory to these reports and to the effect of GS medium that promoted Sertoli cell proliferation, insulin did not much increase the number of Sertoli cells even though it enlarged testis tissue as a whole. It is supposed that insulin, as a growth stimulating factor for a variety of cells, can stimulate various types of cells in the testis which made Sertoli cell's proliferation less significant than that of others. There are other factors that reportedly influence the number of Sertoli cells, including estradiol, thyroid hormone, transforming growth factor α, follistatin, forskolin, and somatostatin (Baines et al., 2008; Fumel et al., 2012; Krantic & Benahmed, 2000; Meehan et al., 2000; Oldknow et al., 2013; Petersen, Boitani, Fröysa, & Söder, 2001). The PC chip method

will be useful to test the effect of these and other factors on Sertoli cell proliferation and testis growth, which would give us a new point of view on the biology of testis growth during the developing stage.

The PC chip method provided unique culture conditions under which neonatal testis tissue exhibited natural growth almost proportional to what takes place in vivo. Furthermore, it even made it possible to monitor the growth accurately as their volume changed. Although only the testis was tested in this study, other tissues and organs could be applicable. Combining this method with other imaging technologies, like marking particular cell types, would provide a powerful tool to investigate the functional properties of those tissues. Due to its simplicity and versatility, the PC method could be applicable to various culture experiments.

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AUTHOR CONTRIBUTIONS

K. K. performed experiments and wrote the manuscript. T. F., H. K., and T. O. developed the basic idea of the chip device. H. N. and H. K. designed and fabricated a master mold for the of PC chip. M. K., H. Y., N. T. and T. S. performed culture experiments and discussed the results. Y. M. and Y. O. produced the Histone H4-venus (H4V) transgenic mice and gave advice regarding the experiments. H.A. provided Sox9-EGFP mouse. T.O. conceived the basic idea of the study and wrote the manuscript.

CONFLICTS OF INTEREST

The authors declare tha there are no conflicts of interests.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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