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In Vitro Reconstruction of Mouse Seminiferous Tubules Supporting Germ Cell Differentiation[1]

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ABSTRACT

Cells of testicular tissues during fetal or neonatal periods have the ability to reconstruct the testicular architecture even after dissociation into single cells. This ability, however, has not been demonstrated effectively in vitro. In the present study, we reconstructed seminiferous tubules in vitro that supported spermatogenesis to the meiotic phase. First, testicular cells of neonatal mice were dissociated enzymatically into single cells. Then, the cells formed aggregates in suspension culture and were transferred to the surface of agarose gel to continue the culture with a gas-liquid interphase method, and a tubular architecture gradually developed over the following 2 wk. Immunohistological examination confirmed Sertoli cells forming tubules and germ cells inside. With testicular tissues of *Acr*-GFP transgenic mice, the germ cells of which express GFP during meiosis, cell aggregates formed a tubular structure and showed GFP expression in their reconstructed tissues. Meiotic figures were also confirmed by regular histology and immunohistochemistry. In addition, we mixed cell lines of spermatogonial stem cells (GS cells) into the testicular cell suspension and found the incorporation of GS cells in the tubules of reconstructed tissues. When GS cells derived from *Acr*-GFP transgenic mice were used, GFP expression was observed, indicating that the spermatogenesis of GS cells was proceeding up to the meiotic phase. This in vitro reconstruction technique will be a useful method for the study of testicular organogenesis and spermatogenesis.

meiosis, reconstruction, seminiferous tubules, spermatogenesis, spermatogonial stem cells, testis

INTRODUCTION

Animal cells during developmental periods, such as embryonic, fetal, or neonatal periods, have a greater ability to undergo regeneration than cells in a developed body [1–3]. Testicular cells also show this ability by reconstructing the testicular architecture when they are dissociated into single cells. It was reported that testicular cells of neonatal pigs reconstructed a tubular structure when transplanted into the renal subcapsular space of SCID mice [4]. Grafting of rat and mouse testicular cells during the neonatal or pup period into the subcutaneous space of nude mice was also reported as having led to reconstructed tubular structures [5, 6]. Kita et al. [7] demonstrated reconstruction of the testicular architecture from dissociated neonatal mouse testicular cells in the subcutis of nude mice. In this case, cultured murine spermatogonial stem cells, germline stem (GS) cells, were intermingled with testicular cells. The GS cells colonized the reconstructed tubules and differentiated up to round spermatids, which were used for microinsemination to produce offspring. Later, it was also reported that the cells of fetal gonads (12.5 days postcoitum) reconstructed a testis or ovary in the renal subcapsular space and formed functional gametes [8]. These data proved the potential of immature testicular cells to reconstruct the original structure and regain functional properties. However, these results were all obtained in vivo and are believed to have depended on the microenvironment. In fact, one of the studies mentioned above was not able to reconstruct the tubular structure in vitro [5]. Several other trials to reconstruct the tubular structure under culture conditions have been performed, and neonatal or developing rat and mouse testicular cells showed a reconstructive ability in culture by forming cord-like structures [9–11]. These structures that formed in vitro, however, were not comparable to those that formed in vivo, let alone to the original seminiferous tubules.

Recently, we developed a new culture system supporting the full spermatogenesis of mice [12] by modifying a traditional organ culture method, gas-liquid interphase [13, 14]. GS cells also differentiated into sperm in vitro with this organ culture method and transplantation into testicular tissue before cultivation [15]. Based on this recent progress, the present study attempted to reconstruct the testicular architecture under culture conditions. We observed that the seminiferous tubular structure was reorganized with our culture method and supported spermatogenesis up to the meiotic phase. GS cells mixed with testicular cells also progressed to meiosis.

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MATERIALS AND METHODS

Animals

The ICR and C57BL/6 mice were purchased from Clea. Three lines of transgenic mice (mixture of C57BL/6 and ICR genetic backgrounds) carrying the *pCXN*-eGFP transgene [16] (*pCXN*-GFP transgenic mouse), *Acr*-GFP transgene [17, 18] (*Acr*-GFP transgenic mouse), and *Gsg2*-GFP transgene [19] (*Gsg2*- or *Haspin*-GFP transgenic mouse), respectively, were used as tissue sources and for the production of GS cell lines. All 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).

Reconstruction of Seminiferous Tubules

Testes of neonatal mice (age, 0.5–5.5 days postpartum [dpp]) were decapsulated and digested by 0.25% trypsin in PBS for 10 min at 37°C. The reaction was stopped by adding twice the volume of Dulbecco modified Eagle medium plus 10% fetal bovine serum. The cells were filtered through a membrane with a pore size of 40 μm (352340; Becton, Dickinson and Company). The singly dissociated cells were resuspended in α-Minimum Essential Medium (α-MEM; 12000-022; Invitrogen) supplemented with Knockout Serum Replacement (KSR; 10%, v/v; 10828-028; Invitrogen) and recombinant human glial cell line-derived neurotrophic factor (GDNF; 10 ng/ml; R&D Systems) or in the medium for GS cell culture as described below. Approximately 2×10^6 cells were introduced into each well of a 96-well, V-bottom plate (MS-9096V; Sumitomo Bakelite) and incubated at 34°C for 2 days for aggregation. The aggregates were gently aspirated with a pipette and transferred to the flat surface of agarose gel half-soaked in the medium of αMEM plus 10% KSR. When mixing GS cells with testicular cells, the cell ratio was approximately 1:2.

Culturing GS Cells

The GS cells were established as described previously [20]. Briefly, testicular cells were dissociated enzymatically and incubated overnight in dishes coated with 0.2% (w/v) gelatin to remove fibroblasts that had adhered to the base. The cells were collected by brisk pipetting and were plated into another well. After one to three passages, when fibroblast proliferation diminished, the cells were plated in wells with mouse embryonic fibroblast feeder cells. The culture medium consisted of StemPro-34 SFM (Invitrogen) supplemented with StemPro supplement (Invitrogen), recombinant human epidermal growth factor (20 ng/ml; Wako Pure Chemical Industries), human basic fibroblast growth factor (10 ng/ml; Becton Dickinson), recombinant human GDNF (10 ng/ml), and several other ingredients as described previously [15]. The cells were maintained at 37°C in an atmosphere of 5% carbon dioxide in air. Medium was changed every 2–4 days. Cell passage was arbitrary, depending on the proliferation state of GS cells in each well.

Gross and Histological Examination

For the evaluation of testicular tissue reconstruction, observation under a stereomicroscope and histological findings were used. The progression of spermatogenesis was evaluated by the area of GFP expression when transgenic mice were used. Under observation with a stereomicroscope equipped with an excitation light for GFP (SZX12; Olympus), the area showing GFP expression was roughly measured and classified into one of six degrees: ≤10%, 11%–30%, 31%–50%, 51%–70%, 71%–90%, and 91%–100% [14]. For histological examination, the specimens were fixed with Bouin 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) and periodic acid-Schiff (PAS).

Immunohistochemistry

For immunofluorescence staining, tissues fixed with 4% paraformaldehyde in PBS were cryoembedded in OCT compound (Sakura Finetechnical Co. Ltd.) and cut into sections (section thickness, 7 μm). Incubation with primary antibodies was performed overnight at 4°C, followed by rinsing twice with PBS, and then secondary antibodies were applied for 1 h at room temperature. Nuclei were counterstained with Hoechst 33342 dye. Specimens were observed with a confocal laser microscope (FV-1000D; Olympus). The following primary antibodies were used: rabbit anti-Sox9 antibody (1:200, Trans Genic, Inc.), rabbit anti-3β-HSD (1:00, Trans Genic, Inc.), rat anti-Tra98 antibody (1:500; Cosmo Bio), rat anti-GFP antibody (1:1000, Nakalai Tesque, Inc.), rabbit anti-SYCP1 antibody (1:600; Novus Biologicals), and rabbit anti-H2AX antibody (1:50; Novus Biologicals). The secondary antibodies used were goat anti-rabbit immunoglobulin (Ig) G and goat anti-rat IgG, conjugated with Alexa 488 or Alexa 555 (1:200; Molecular Probes).

RESULTS

Reconstruction of Seminiferous Tubules In Vitro

For a single experiment, testes obtained from a litter (n = 2–10 male mice; age, 0.5–5.5 dpp) were used. The enzymatically dissociated cells were cultured under suspension conditions that induced aggregation. During the first 24 h, the aggregates were still fragile. When left for 3 days or longer, they became round and very solid, which appeared to be unfavorable for subsequent tubulogenesis. In fact, when the aggregates were left for a longer time in the floating culture conditions, they became more compact, showing no sign of tubular formation (data not shown). Thus, on Day 2 of culture, the cell aggregates were transferred to the flat surface of agarose gel to continue cultivation according to our organ culture method [14] (Fig. 1A). The aggregates on the agarose gel showed gradual reorganization into a tubular structure that lasted approximately 2 wk (Fig. 1B).

Histologically, the initial change was observed in 2 days as sporadic cord formations. In 5 days, the cord formation further developed and became apparent. Tubular structures were confirmed at 14 days (Fig. 1B). However, the contour of the tubular structure was irregular and maze-like (Supplemental Fig. S1; all Supplemental Data are available online at www.biolreprod.org).

The cells constructing tubules were Sertoli cells, as shown by positive staining for Sox9 (Fig. 1C). In the tubules adjacent to Sertoli cells, germ cells were recognized as cells positive for Tra98 (Fig. 1C). Between tubules and at the periphery of the tissues, 3β-HSD-positive cells, presumably Leydig cells, were scattered (Fig. 1C). Germ cells were also observed in regular histological examination with H&E staining, which attached to the basement membrane of the tubules, suggesting that they were spermatogonia (Fig. 1D).

Spermatogenesis in Reconstructed Seminiferous Tubules

To monitor germ cell differentiation in the reconstructed tubules, we used *Acr*-GFP mice as a source of testicular cells, because they express GFP during the meiotic prophase. Among 40 reconstructed tissues in 13 experiments, expression of *Acr*-GFP was observed in 19 tissues (48%), indicating that spermatogenesis progressed at least up to the meiotic phase of stage IV pachytene [17] (Fig. 2A). The area of GFP expression in the tissues, however, was restricted to small portions: 10% or less in nine tissues, and 11%–30% in the 10 other tissues. The timing of GFP expression was from 30 to 51 days in the culture period (Table 1). With immunohistochemistry, germ cells positive for both SYCP1 and GFP were observed, supporting that meiosis was actually taking place (Fig. 2B). H2AX staining differentiated leptotene, zygotene, and late pachytene spermatocytes by its characteristic nuclear localization patterns (Supplemental Fig. S2) [21]. In addition, condensation of GFP was observed, which is a sign indicating that acrosome formation is in progress (Fig. 2C) [17, 18]. Regular histological sections with PAS staining demonstrated meiotic figures (Fig. 2D). Although very rare, acrosomal cap structures were also observed (Fig. 2, D and E). These data showed that meiosis and meiotic divisions in spermatogenesis were properly in progress in the reconstructed seminiferous tubules. However, it was very rare to observe the typical

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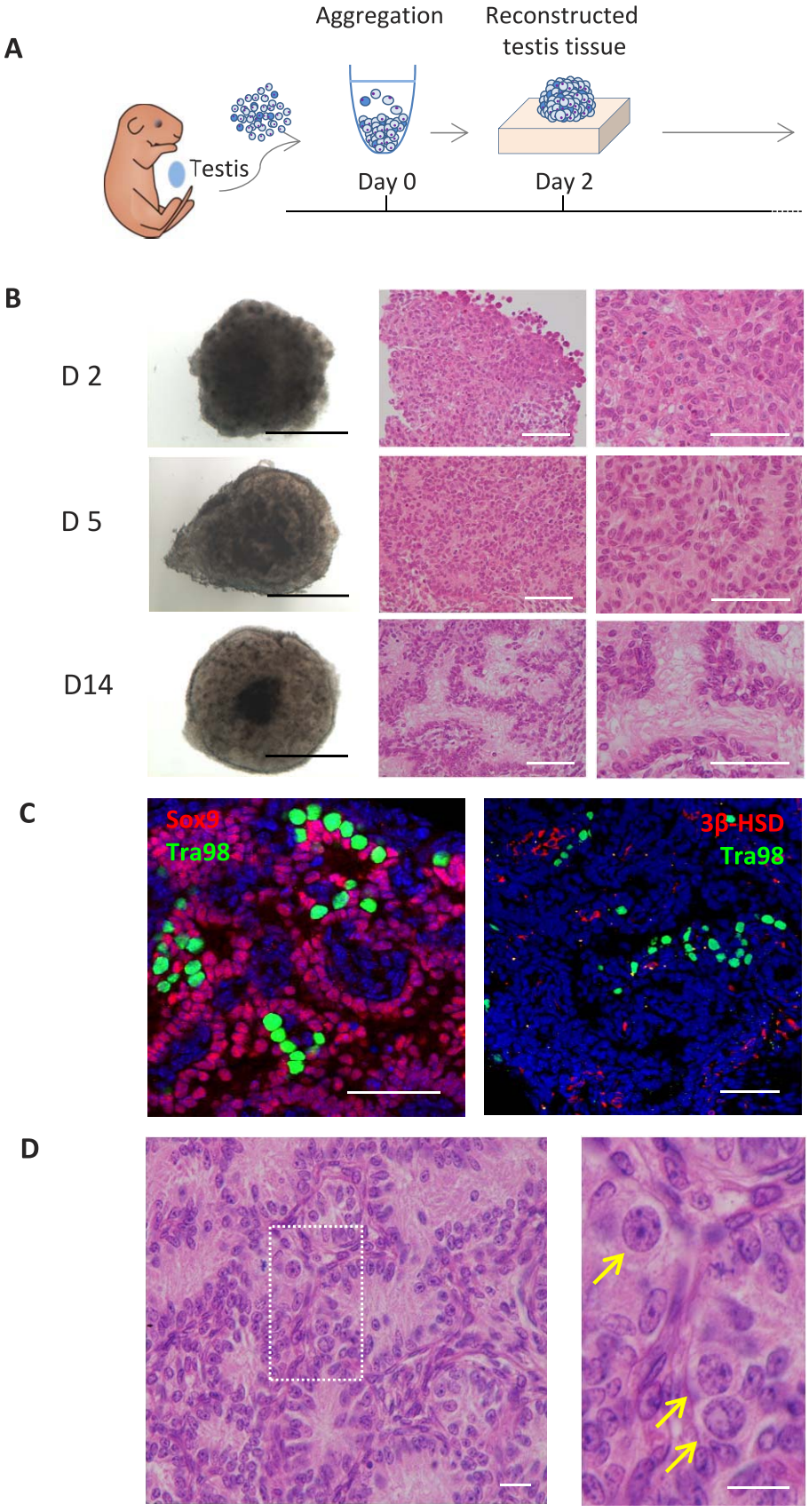

FIG. 1. In vitro reconstruction of tubular structure. **A**) Schematic presentation of experimental procedures. **B**) Stereomicroscopic and histological appearance of reconstructing tissues from mice (3.5 dpp). Bar = 1 mm (left) and 50 μm (center and right). **C**) Immunostaining of reconstructed tissues at 14 days in culture from mouse testicular cells (3.5 dpp). Sox9 (red; left), Tra98 (green), 3β-HSD (red; right), and Hoechst (blue) are shown. Bar = 50 μm. **D**) H&E staining of reconstructed tissues at 14 days in culture from mouse testicular cells (1.5 dpp). The area on the right corresponds to the boxed area on the left. Arrows indicate spermatogonia. Bar = 10 μm.

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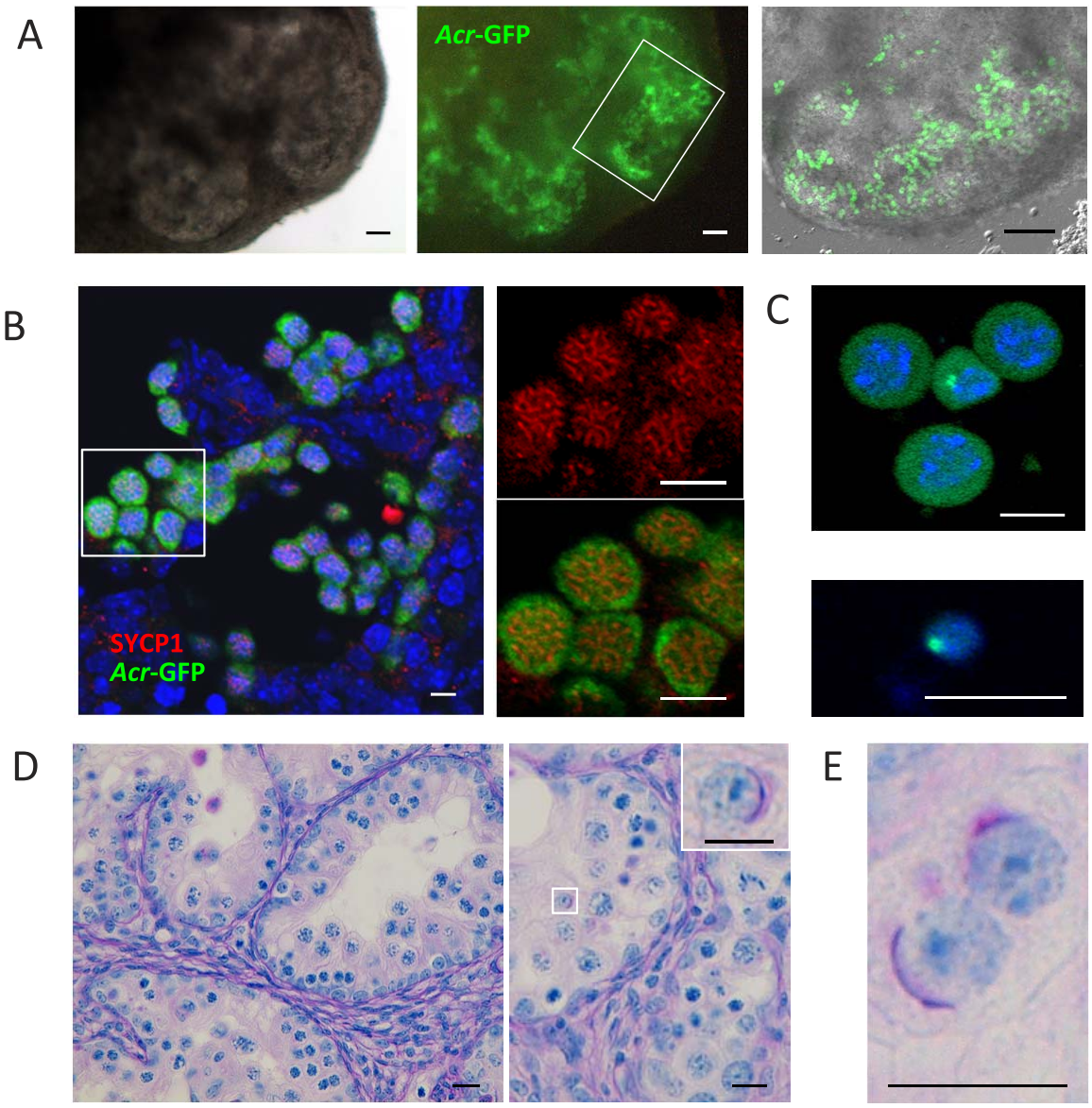

FIG. 2. Spermatogenesis in reconstructed tubules. **A**) A tissue reconstructed from *Acr*-GFP mouse testicular cells (1.5 dpp) at 61 days in culture. Bright view (left) and GFP-excitation view (center) are shown. On the right is a confocal image corresponding to the boxed area in the center. Bar = 100 μm. **B**) A reconstructed tubule from *Acr*-GFP mouse testicular cells (1.5 dpp) at 59 days in culture. Areas on the right correspond to the boxed area on the left. SYCP1 (red), GFP (green), and Hoechst (blue) are shown. Bar = 10 μm. **C**) *Acr*-GFP condensation during acrosome formation. Bar = 10 μm. **D**) Histology of reconstructed tissue, PAS stain, from *Gsg2*-GFP mouse testicular cells (3.5 dpp). Inset corresponds with the boxed area. Bar = 10 μm (inset, 5 μm). **E**) Round spermatids with a PAS-positive acrosomal cap at 59 days of culture. Bar = 5 μm.

association of germ cell types termed as stage of the seminiferous epithelium, which made us refrain from insisting the formation of haploid cell in the present study (Supplemental Fig. S3).

Induction of GS Cell Differentiation in the Reconstructed Seminiferous Tubules

We reasoned that cells from different sources can be combined for chimeric reconstruction of the testicular architecture. Then, GS cells derived from *pCXN*-GFP transgenic mice, which express GFP ubiquitously, were mixed with neonatal testicular cells of wild-type mice. Testicular cells were mixed with GS cells and led to aggregate formation. During the initial few days, the GFP-expressing GS cells were located diffusely in the cell aggregates. As the tubular formation progressed, the GFP signal decreased rapidly, probably because the GS cells outside the tubules were not able to survive and disappeared. In some cases, however, GS cells, having settled in the reconstructed tubules, started to proliferate and showed a gradual increase of GFP signals. In 4–8 wk, the GFP signals reached a maximum in those tissues (Fig. 3B). When the colonization of GS cells was judged based on the resurgence of GFP signals or localization at the periphery of tubules, it was observed in 12 of 21 reconstructed tissues (57%) in 15 experiments.

Immunohistochemical staining demonstrated that cells constructing tubules were positive for Sox9. Germ cells stained by GFP, being derived from GS cells, were in some tubules (Fig. 3C). The 3β-HSD-positive cells, presumably Leydig cells, were located between tubules (Fig. 3C).

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TABLE 1. Spermatogenesis in the reconstructed tissues.

Cell source	Reconstructed testicular tissues (n)	Tissues that expressed GFP (n [%])	Day of culture on which GFP expression began	GFP expression area (n [%])[a]		
				≤10%	11%–30%	>30%
Acr-GFP mice	40	19 (48)	30–51	9 (47)	10 (53)	0 (0)
Chimeric with *Acr*-GFP GS cells	19	7 (37)	17–31	4 (57)	3 (43)	0 (0)

[a] Values are presented as the number of tissues with GFP expression, with their percentage among all tissues showing GFP expression in parentheses.

To detect the differentiation of GS cells in the reconstructed tubules, we used GS cells derived from *Acr*-GFP transgenic mice. Among 19 reconstructed tissues chimeric with *Acr*-GFP GS cells, GFP expression was observed in seven (37%) (Fig. 3D and Table 1). The GFP expressions were observed during 17–37 days in culture. Taken together, the GS cells incorporated in the reconstructed tubules differentiated into the meiotic prophase, probably up to the pachytene stage of spermatocyte.

DISCUSSION

In the present study, we demonstrated that immature, dissociated testicular cells could reconstruct their original architecture under culture conditions. However, the architecture was not exactly comparable to the original one. Namely, the reconstructed seminiferous tubules had deformities like an uneven diameter and trifurcation leading to an irregular, maze-like configuration. Leydig cells were distributed irregularly in

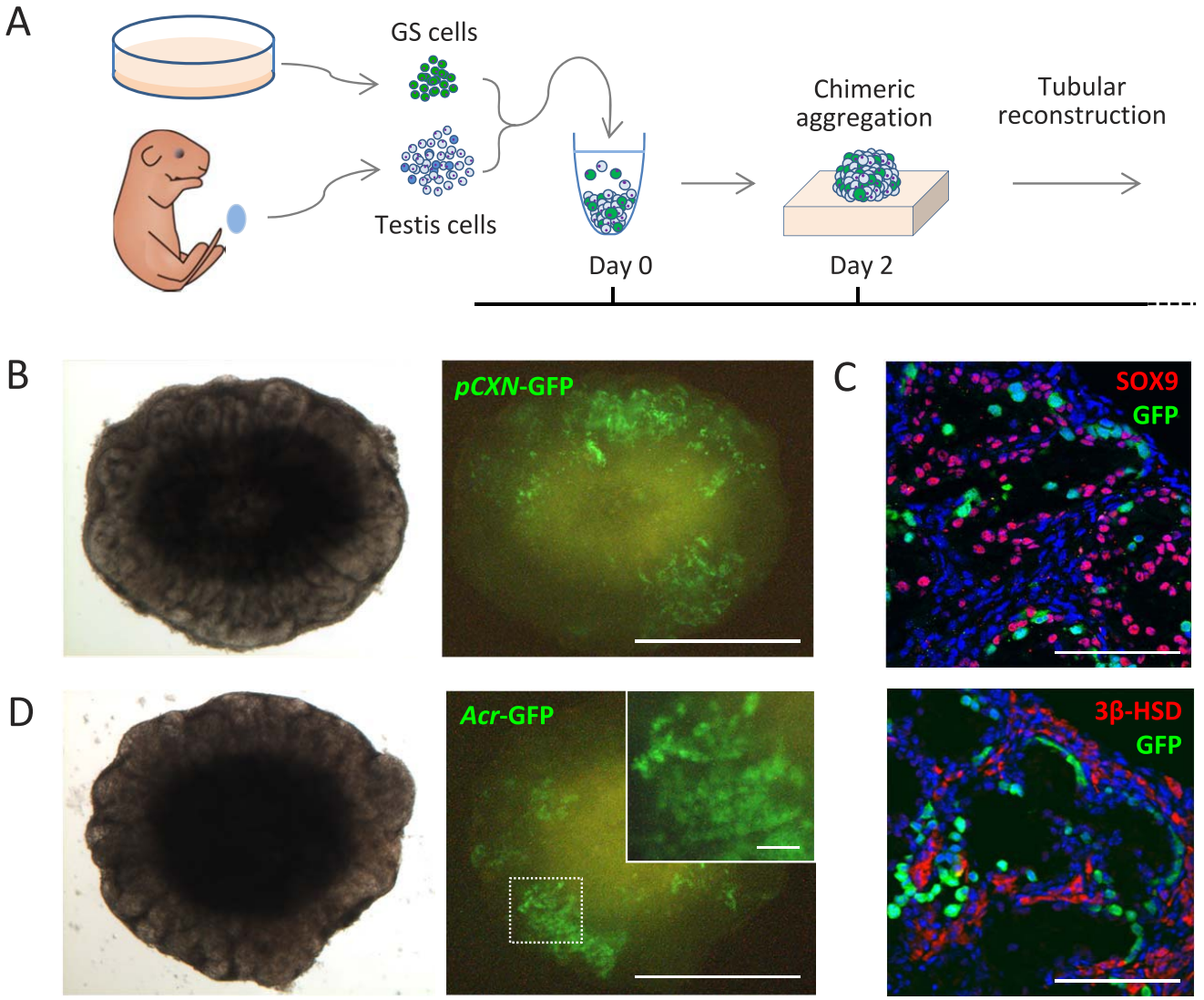

FIG. 3. Reconstruction of chimeric tubules with GS cells. **A**) Schematic presentation of experimental procedure for chimeric tissue reconstruction. **B**) GFP-GS cells mixed with testicular somatic cells from mice (2.5 dpp) at 7 wk of culture. Bright view (left) and GFP-excited view (right) are shown. Bar = 1 mm. C) Immunostaining of chimeric reconstructions. Germ cells derived from GS cells were marked with GFP (green). Sox9 (red; top), 3β-HSD (red; bottom), and Hoechst (blue) are also shown. **D**) GFP expression of *Acr*-GFP GS cells in a reconstructed tissue at 50 days of culture. Inset corresponds to the boxed area. Bars = 1 mm (inset, 100 μm).

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spaces between those tubular structures. Nonetheless, the in vitro reconstruction appeared comparable to that reconstructed in vivo, ectopically in subcutaneous spaces of mice, observed during the previous study [7]. This means that the microenvironment in a body is not necessary for the reconstruction of those testicular cells. One of the significant differences between in vivo and in vitro microenvironmental conditions would be the presence of microcirculatory systems. In fact, the presence of a microcirculatory system is mandatory for maintaining the homeostatic function of each organ and tissue. Based on the present results, however, we can argue that such microcirculatory systems, such as capillaries and lymph vessels, are not necessary for the reorganization of testicular cells. Nonetheless, it is also possible that further refinement of the reconstruction would need the microcirculatory systems.

On the other hand, we were not able to identify germ cell differentiation beyond the meiotic phase. This limitation may not be in vitro-specific, because it was also recognized in previous work involving ectopic reconstruction of the testicular structure in the subcutis [7]. The incomplete structure of the reconstructed tubules along with the unbalanced composition of each testicular somatic cell type, including Leydig and peritubular myoid cells, might be its cause. It was reported that the deformity of seminiferous tubules and lack of adult-type Leydig cells in desert hedgehog null mice are relevant to its spermatogenic failure [22]. The proper maturation of testicular somatic cells, especially Sertoli and Leydig cells, is mandatory for the normal progression of spermatogenesis. The cell dissociation and subsequent incomplete reorganization of the testicular structure could impair the maturation of those cells. Immature Sertoli cells in particular may not be able to build the blood-testis barrier, which plays an important role in the proper progression of spermatogenesis [23].

Another point that needs improvement is the efficiency of germ cell incorporation into the reconstructed tubules. To induce spermatogenesis over a larger area of the reconstructed tissues, germ or GS cells have to be incorporated effectively in the aggregates of Sertoli cells, because their aggregation is the initial step of tubular formation. During gonadogenesis in the embryo, primordial germ cells appear to have affinity with precursors of Sertoli cells. Such an affinity did not seem to be shared with the interaction between GS cells and neonatal Sertoli cells. For more efficient spermatogenesis in the reconstructed tubules, certain procedures may be needed to induce such an affinity without disturbing the tubular formation.

Our in vitro reconstruction method reliably established the initial phase of spermatogenesis and is easy to perform, so it will be a useful method for the study of testicular organogenesis and spermatogenesis. The combination of cells from different sources, not only germ cells but also different kinds of somatic cells, will be possible for testis reconstruction. When those cells become available from induced pluripotent stem (iPS) cells or other accessible cell sources [24, 25], testicular tissue fragments could be rebuilt in vitro from those cells without depending on the testis as a cell source in the future, which will make this method more useful and even practical for application to the study of human spermatogenesis.

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