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Anatomy

Establishment of an organ culture system to induce Sertoli cell differentiation from undifferentiated mouse gonads

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ABSTRACT. Organ culture systems are useful for elucidating the process of testicular differentiation from mammalian undifferentiated genetically male gonads, as they permit various experiments, including experiments involving the control of gene expression. However, without addition of testicular differentiation-related factors, it is difficult to induce the formation of testis cord from immature gonads by a time point earlier 12 tail somites (ts) that corresponding to 11.0 days post coitum (dpc). In this study, we attempted to establish an organ culture system that induces testis formation from immature gonads (around 8 ts: 10.5 dpc) just before *Sry* (sex-determining region of the Y chromosome) expression. A paired gonad-mesonephros complex of around 8 ts was placed in the groove of an agarose gel block and put the semi-cylindrical piece of agarose gel to maintain the gonad morphology. The gonads were cultured in the gas phase for 96 hr. As a result, testis cord-like structures appeared in many genetically male gonads. Cells expressing the Sertoli cell markers Sox9 (SRY-box 9) and Amh (anti-Müllerian hormone) were observed, while granulosa cell marker Foxl2 (forkhead box L2) was not detected. In addition, Sox9- and Amh-expressing cells were observed throughout the entire gonad in many individuals. *Amh* mRNA expression was also upregulated. Surprisingly, formation of a partial testicular structure was observed from more immature gonads (6 ts). These results show that our gonadal organ culture system is useful for elucidating the regulation mechanism of *Sry* expression in undifferentiated bipotential gonads.

KEY WORDS: Amh (anti-Müllerian hormone), organ culture, Sertoli cell, testis differentiation, undifferentiated gonad

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In the early stage of testicular differentiation in mammals, Sertoli cell differentiation from undifferentiated supporting cells is essential [16, 33]. Expression of the sex determining gene *SRY/Sry* (sex-determining region of the Y chromosome) is important for Sertoli cell differentiation [18, 31]. *Sry* expression is stringently regulated spatio-temporally. Expression of *Sry* starts in the central region of the gonad at 11.0 days post coitum (dpc), and spreads throughout the entire gonad by 11.5 dpc [6, 16, 41]. Insufficient expression around 11.2 dpc in mice causes sex reversal [7, 16, 36]. The regulatory mechanism of *Sry* expression is complex, and many factors such as *Nr5a1* (nuclear receptor subfamily 5, group A, member 1), *Wt1* (Wilms tumor 1 homolog) and *Gadd45g* (growth arrest and DNA-damage-inducible 45 gamma) are known to be involved [32, 33, 37].

There are differences in the *Sry* sequence among mouse strains, and the capacity of testicular formation is different [1, 4, 30]. *Sry* sequences with weak activity harbored in many mouse strains with disorders of sex development may delay *Sry* expression

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and cause sex reversal, but the reason for such delayed *Sry* expression is unknown [2, 12, 36, 39, 40, 44]. Perturbation of histone demethylase *Kdm3a* (lysine (K)-specific demethylase 3A; Jmjd1a) expression also causes suppression of *Sry*, and causes sex reversal in the male gonads [19–21]. Although the involvement of *Gadd45g* and *Map3k4* (mitogen-activated protein kinase kinase kinase 4) has been speculated [38], the reason why *Sry* expression starts in the central region of the gonad has not yet been elucidated.

Undifferentiated gonads have been cultured on micropore filters since the 1970s, mainly for the purpose of studying germ cell differentiation [8, 26]. Organ cultures have been used to elucidate aspects of the regulatory mechanisms of testis differentiation, such as the migration of mesonephric cells to gonad [25], the importance of the central region of the gonad [15], the involvement of *Sox9* (SRY-box 9) in Sertoli cell differentiation [10] and the elucidation of critical windows of *Sry* expression [16]. Gonadal culture from 10.5 dpc (8 tail somites (ts); before *Sry* expression) does not result in Sertoli cell differentiation and testis cord formation in organ culture [26, 35]. Detailed studies in gonadal culture have shown that testis cannot be formed from the immature gonads before 12 ts, which correspond to about 11.0 dpc, without the addition of testicular differentiation-related factors such as Fgf9 (fibroblast growth factor 9), or forced expression of *Sry* [16]. Although partial testis differentiation has been reported in cultures of immature gonads [13, 23], testis cord formation throughout the gonads has not been reported. To elucidate the detailed mechanism of *Sry* expression, a gonadal culture system from the stage just before *Sry* expression started is essential. Here we attempted to establish the organ culture system which induces testis formation from immature gonads of around 8 ts (10.5 dpc).

MATERIALS AND METHODS

Animals

Both male and female ICR mice were purchased from SLC Japan (Hamamatsu, Japan) and maintained as described elsewhere [42]. Male fetuses at 10.5, 12.5, 13.5 and 14.5 dpc were collected immediately after euthanasia, which was accomplished under deep anesthesia with isoflurane. Noon of the day on which the mating plug was observed designated as 0.5 dpc. Tail somites (ts) were counted as described previously to ensure the accurate estimation of developmental stage [14]. Embryos at 10.5 dpc were sexed by multiplex PCR (polymerase chain reaction) as described previously [36]. This study was approved by the Institutional Animal Care and Use Committee (Permission #29-05-02) and carried out according to the Kobe University Animal Experimental Regulations.

Preparation of the agar blocks and semi-cylindrical agar piece

The needle sections of five 25-gauge needles (Terumo, Tokyo, Japan) were placed in parallel on the bottom of a 50 mm petri dish. Five m*l* of Dulbecco's Modified Eagle Medium (DMEM 4.5 g/*l* glucose with L-Gln; Nacalai Tesque, Kyoto, Japan) containing 1.5% agarose (Agarose S, NIPPON GENE, Toyama, Japan) was boiled for 2–3 min to dissolve completely and poured into a 50 mm petri dish. After taking out and inverting the solidified gel, the needles were removed to create a groove. The gel was cut into approximately rectangular blocks (6 × 9 × 2.5 mm) having a central groove (Fig. 1A). A semi-cylindrical gel piece of the same length as the gonad-mesonephros complex was prepared by cutting a cylindrical gel created in a glass capillary (Model G-1; Narishige, Tokyo, Japan; approximately 600 μm in diameter) with a scalpel blade (No.11; Feather Safety Razor, Osaka, Japan; Fig. 1B).

Dissection and organ culture

Paired gonad-mesonephros complexes around 10.5 dpc were excised from the embryos in DMEM with HEPES (4.5 g/*l* glucose with L-Gln and HEPES; Nacalai Tesque) and placed in the groove of the agarose gel block with the mesonephros side down. A semi-cylindrical gel piece was placed on each gonad-mesonephros complex to maintain the natural shape of the gonads by adding weight (Fig. 1C). To maintain that the gonads were in gas phase, 150 μ*l* of DMEM containing HEPES was added to a 24-well dish (well diameter 15.4 mm), and the dish was stored at 4°C until the start of culture. After harvesting all animals from one litter, the medium was replaced with an equivalent volume of culture medium and cultured at 37°C with 5% CO_2 for 96 hr. As the culture medium, DMEM (4.5 g/*l* glucose with L-Gln and sodium pyruvate) supplemented with 10% fetal bovine serum (FBS; BioWest, Nuaillé, France) or 10% KnockOut Serum Replacement (KSR; Gibco, Grand Island, NE, USA), 100 IU penicillin, 100 mg/m*l* streptomycin, 3.48 mM L-glutamine and 1.87 mM pyruvate was used, and exchanged every 24 hr.

Immunohistochemistry (IHC)

The gonad-mesonephros complexes before and after culture were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 2 hr at 4°C. The specimens were dehydrated with an ethanol series followed by xylene and then embedded in paraffin. Then, 5-μm-thick sections were cut by a sliding microtome and placed on slide glasses. Immunohistochemical staining was carried out as described previously [42]. For the primary antibody, we used an anti Amh (anti-Müllerian hormone) goat polyclonal antibody (1:24,000; sc-6886; Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-Sox9 rabbit polyclonal antibody (1:1,000; sc-20095; Santa Cruz Biotechnology) or anti-Foxl2 (forkhead box L2) goat polyclonal antibody (1:25,000; ab5096; Abcam, Cambridge, UK). For the secondary antibody, we used a Dako EnVision$^+$ system-HRP (horseradish peroxidase)-labeled polymer (Dako Japan, Tokyo, Japan) or peroxidase-conjugated donkey anti-goat IgG (ab97112; Abcam).

Quantitative PCR (qPCR)

The cultured gonad-mesonephros complexes were dissected to remove the non-gonadal region. One gonad was

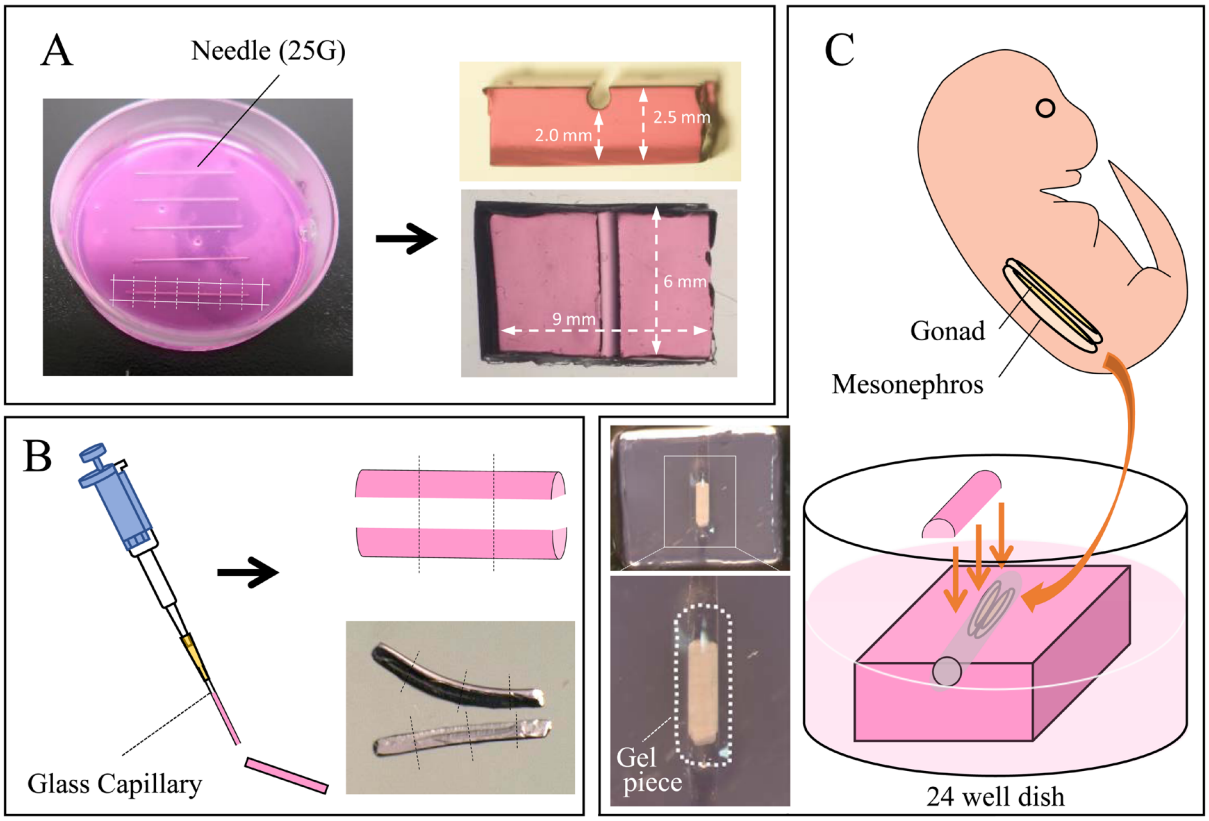

Fig. 1. Schematic diagram of the culture procedure. (A) The needle sections of five 25-gauge needles were placed parallel to the bottom of a 50 mm petri dish, and 5 m*l* of Dulbecco's Modified Eagle Medium (DMEM) containing 1.5% agarose was poured over them. Then, the portion of the gel containing the needle was cut out (white lines), and the needle was removed and the gel was cut into rectangular blocks (white dotted lines). (B) DMEM containing 1.5% agarose was sucked into a glass capillary attached to the tip of a micropipette. The gel was discharged after cooling, and then cut into small semi-cylindrical pieces with a scalpel blade. (C) A paired gonad-mesonephros complex was placed in the groove of the agarose gel block with the mesonephros side down. A semi-cylindrical gel piece was placed on the gonad-mesonephros complex to maintain the natural shape of the gonads by adding weight. To ensure that the gonads were in gas phase, 150 μl of culture medium was added to the 24-well dish.

used for qPCR and the other was used for IHC. Genetically male gonads at 10.5, 13.5 and 14.5 dpc were used as an experimental control. *Amh* mRNA expression levels were measured as described elsewhere [43]. The primers for *Amh* were TAACCCTTCAACCAAGCAGAGAA and GCGGGAATCAGAGCCAAA, and the annealing temperature was 57°C. *Gusb* (Glucuronidase, beta) and *Tbp* (TATA box binding protein) were used for normalizing gene [43]. The expression levels of *Amh* mRNA were compared using the Kruskal-Wallis test and Steel's multiple comparison test (Excel Statistics 2016, version 3.20; SSRI, Tokyo, Japan). The results were considered significant when the *P*-value was less than 0.05.

RESULTS

The thickness of undifferentiated male gonads (urogenital ridge) at 10.5 dpc (8 ts) before culture was very slight (Fig. 2A). After 4 days of culture with FBS-supplemented medium, the volume of the gonad was increased (Fig. 2B). Although the length of the gonad was extended only slightly, the thickness and breadth were greatly increased (Fig. 2B). The morphology of the cultured gonads was different from that of the gonads at 13.5 and 14.5 dpc, and resembled that at 12.5 dpc (Fig. 2C–E).

The Sertoli cell markers Sox9 and Amh were expressed throughout the entire gonads at 13.5 dpc, while the granulosa cell marker Foxl2 was not expressed (Fig. 3A–C). Testis cord-like structures containing cells expressing Sox9 (Fig. 3D) and Amh (Fig. 3E) appeared in the gonads cultured with FBS-supplemented medium, while the granulosa cell marker Foxl2 was not detected in the gonadal region (Fig. 3F). Sertoli cell marker-expressing cells appeared mainly on the mesonephros side and in the central region of the gonad. Approximately 40% of cultured gonads showed these results. In addition, about 20% of cultured gonads contained cells that strongly expressed Sox9 and Amh throughout the entire gonads, and expression of Foxl2 was not detected in any of the gonads (Fig. 3G–I). Sertoli cell marker-expressing cells also appeared throughout the entire gonad, when the gonads were cultured with KSR-supplemented medium (Fig. 3J and 3K). Approximately two-thirds of the gonads contained large numbers of Sertoli cells when the 56 gonads were classified by the expression of Sertoli cell markers (Table 1). Although there were differences between

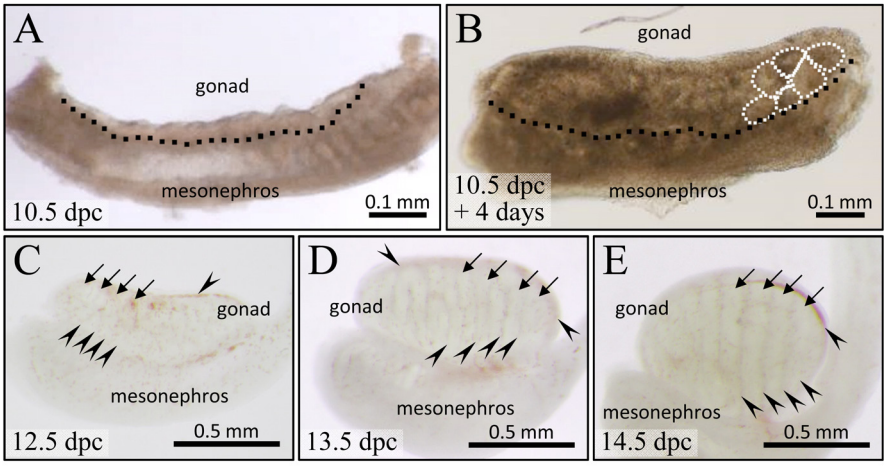

Fig. 2. Morphology of the undifferentiated male gonad in organ culture. (A) Before culture, the undifferentiated male gonad (urogenital ridge) at 10.5 dpc (12 ts) was very thin. (B) The volume of the gonad was increased after 4 days of culture. Although the length was extended only slightly, the thickness and breadth were greatly increased. Testis cord-like structures (white dotted line) appeared in the gonadal region, but no testis-specific vessels were observed. (C–E) The gonads increased in volume and became more rounded in shape, as the development progressed. The volume of the gonad at 13.5 and 14.5 dpc was larger than that of the cultured gonad. Testis cords (arrows) and testis-specific vessels (arrowheads) were observed in these gonads.

Table 1. Number of cultured gonads categorized by a Sertoli cell marker

Sertoli cell marker-expressing cells	Whole region	Mesonephros side	Central region	Not detected	Total
FBS-supplemented medium	4 (21%)	7 (37%)	4 (21%)	4 (21%)	19 (100%)
KSR-supplemented medium	15 (43%)	10 (29%)	6 (17%)	4 (15%)	35 (100%)
Total	19 (35%)	17 (31%)	10 (19%)	8 (15%)	54 (100%)

FBS, fetal bovine serum; KSR, KnockOut Serum Replacement.

the FBS and KSR groups, Sertoli cell markers were detected throughout the entire gonad in approximately one-third of cultured gonads. Moreover, in another one-third of the cultured gonads, large numbers of Sertoli cell marker-expressing cells appeared in more than half the total gonadal area. The gonads without Sertoli cell marker-expressing cells made up about one-sixth of the cultured gonads (Table 1).

In the male glands at 13.5 and 14.5 dpc, *Amh* mRNA expression was upregulated compared to that at 10.5 dpc. *Amh* mRNA expression was upregulated both in the gonads cultured in FBS- and those cultured in KSR-supplemented medium (Fig. 4). *Amh* mRNA expression in gonads that were Amh-negative by IHC was equivalent to that at 10.5 dpc (Fig. 4).

Surprisingly, formation of a partial testicular structure was also observed when more immature gonads (6 ts) were cultured (Fig. 5A). Although the proliferation of gonadal tissue was less than in the case of 8 ts, many cells expressing the Sertoli cell markers Sox9 and Amh appeared in the testis cord-like structure (Fig. 5B and 5C).

DISCUSSION

The organ culture system used in this study formed testis cord-like structures throughout the gonads, with expression of the Sertoli cell markers *Sox9* and *Amh*. The appearance of insufficiently differentiated Sertoli cells with *Sox9* expression without *Amh* expression has been reported in a mouse strain with disorders of sex development [42]. *Sry* induces expression of *Sox9* and differentiation of Sertoli cells from undifferentiated supporting cells. Differentiated Sertoli cells express their specific genes such as *Amh* [41]. The wide expression of *Amh* in addition to *Sox9* suggests that Sertoli cells in the cultured gonads were sufficiently differentiated. The gonads containing many Sertoli cells accounted for two-thirds of the cultured gonads, suggesting that this organ culture system was useful for elucidating the regulatory mechanism of *Sry*. Surprisingly, culture of more immature gonad (6 ts) than 8 ts (10.5 dpc), also induced Sertoli cell differentiation with *Amh* expression.

Although there are several methods for organ culture, filters [16, 26, 35] and agarose gels [5, 9, 13] are generally used in gonadal cultures. Culture on filters plays an important role in elucidating the masculinizing pathway [15, 16, 24]. In our experiments, the gonads became flat due to their own weight when cultured with the filter method. A small number of *Sox9*-expressing cells

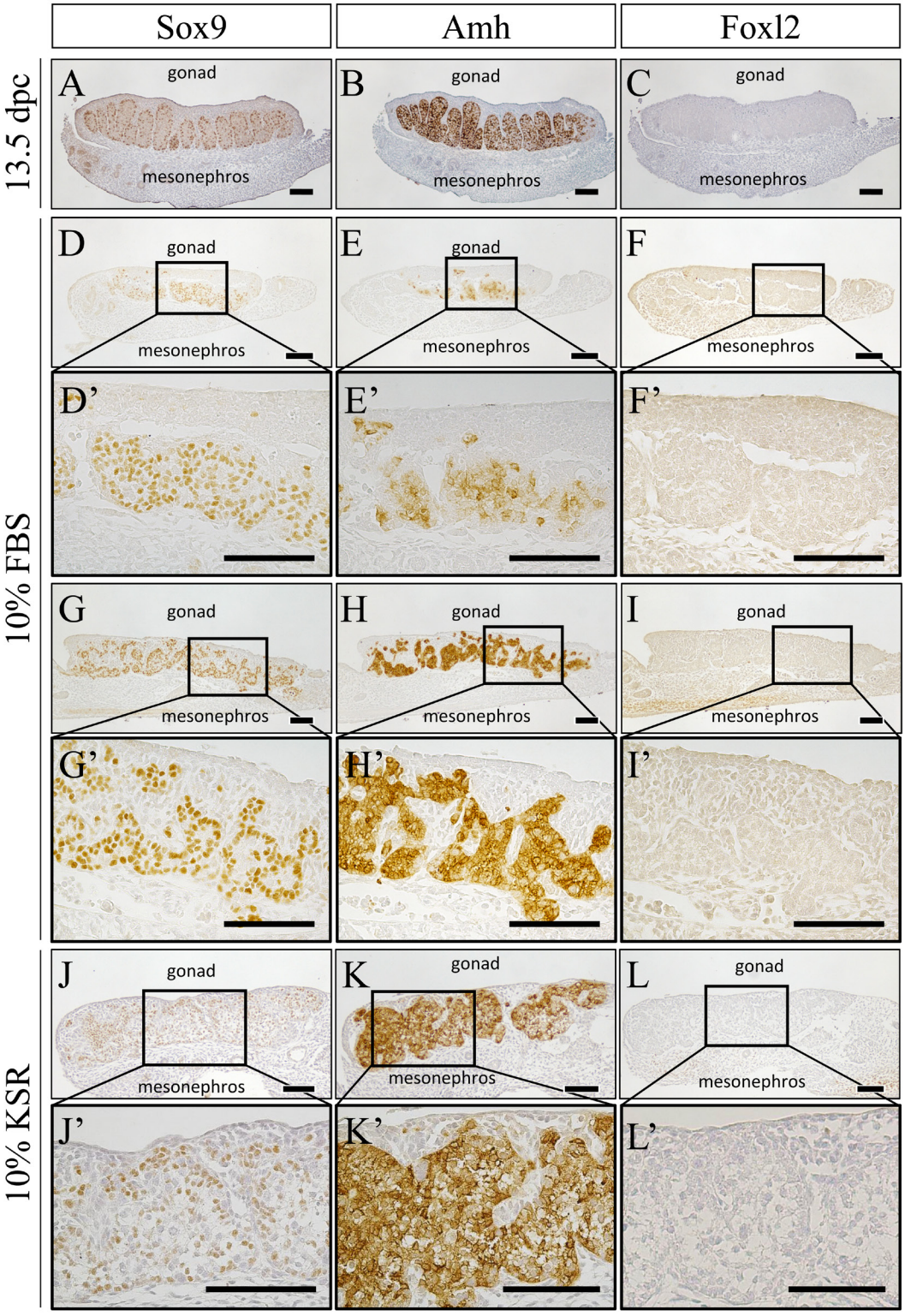

Fig. 3. Expression of markers of Sertoli and granulosa cells in the cultured gonads. (A–B) The Sertoli cell markers Sox9 (SRY-box 9) and Amh (anti-Müllerian hormone) were expressed in the testis cord of the male gonad at 13.5 dpc. (C) Granulosa cell marker Foxl2 (forkhead box L2) was not detected. (D) Sox9 was expressed in the testis cord-like structures cultured with FBS-supplemented medium. Sox9 was mainly expressed on the mesonephros side of the gonad and in the central region of the gonad. (E) Amh was expressed in almost the same region as Sox9, and some cells showed strong immunoreactivity. (F) Foxl2 was not detected in the gonadal region. (G) Some individuals exhibited Sox9 expression throughout the entire gonad. (H, I) Amh was also strongly expressed in the same region as Sox9, and Foxl2 was not expressed in the gonads. (J, K) Sertoli cell marker-expressing cells were also observed throughout the entire gonad, when the culture was performed using KSR-supplemented medium. (L) No Foxl2-expressing cells were detected in the gonadal region. Scale bars=100 μm. FBS, fetal bovine serum; KSR, KnockOut Serum Replacement.

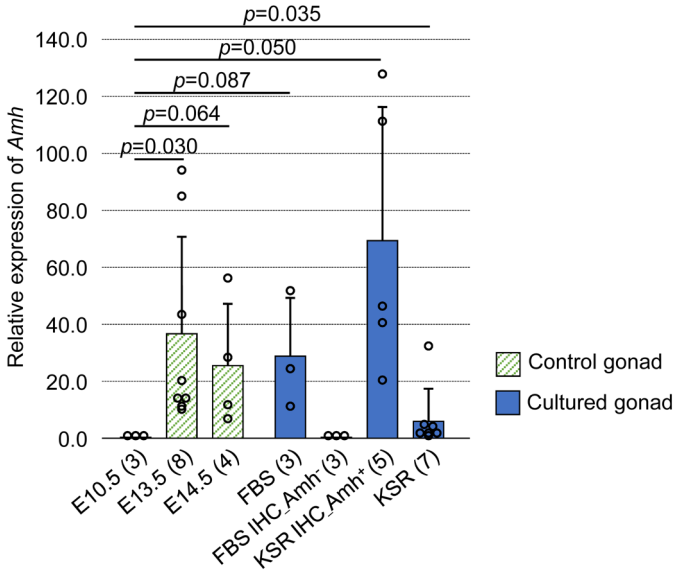

Fig. 4. Expression of *Amh* (anti-Müllerian hormone) mRNA in cultured genetically male gonads. *Amh* mRNA expression was upregulated in cultured male gonads (FBS, KSR IHC_Amh$^+$ and KSR). *Amh* expression in gonads that were immunohistologically Amh-negative was equivalent to that at 10.5 dpc (FBS IHC_Amh$^-$). Data are presented as the mean ± SD. FBS (fetal bovine serum), FBS-supplemented medium; IHC_Amh, immunohistologically Amh-positive or -negative gonad; KSR (KnockOut Serum Replacement), KSR-supplemented medium.

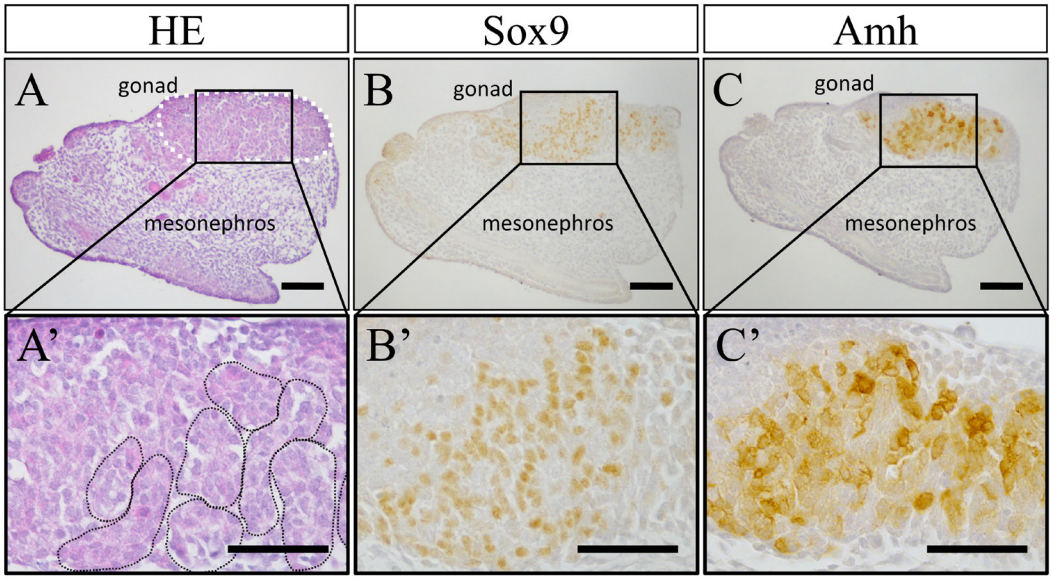

Fig. 5. Morphology of cultured genetically male gonads before 10.5 dpc (8 ts). The gonads were thickened and testis cord-like structures (black dotted line) were also observed when the culture was conducted using gonads more immature (6 ts) than 8 ts (A). The Sertoli cell markers Sox9 (SRY-box 9, B) and Amh (anti-Müllerian hormone, C) were both expressed in the testis cord-like structure. Scale bars=100 μm.

occasionally appeared in the cultured gonads (Supplementary Fig. 1). In addition, mesenchymal cells or the cells of supporting line occasionally migrated outside of the gonads and adhered to the culture substrate in the filter culture and the culture in plastic petri dishes (Supplementary Fig. 2). The importance of using a low adhesion culture material in the undifferentiated gonadal culture was presumed from the fact that the migrated cells did not adhere to the agarose gel block. An attempt was made to maintain the morphology by placing Matrigel or Mebiol Gel around the gonads. However, during the culture these gels gradually dissolved into culture medium and the gonads flattened (Supplementary Fig. 3). Hanging drop-culture has been widely used in germ cell culture in order to provides buoyancy to reduce the effects of vertical compression of the tissue due to its own weight [11, 17], has also used for gonadal culture [34]. In our experiments, the gonads were rounded under the hanging drop-culture, dead cells appeared centrally due to a lack of nutrition, and no testicular cord-like structure or *Sox9* expression was observed (Supplementary Fig. 4). Increased proliferation and cell viability, and lack of testicular cord formation have been reported under low gravity culture [27]. These results suggested that, in our own culture system, the proper biological pressure was provided through the maintaining the gonadal morphology by the addition of a semi-cylindrical piece of gel. A culture method using a small volume of culture medium suitable for RNA interference and inhibitory chemical additional experiments has also been reported [22, 29, 34]. The results of the

gonadal culture including the filter method may be improved if the morphology is maintained with a suitable holding material.

The glucose concentration is crucial in the testis development process, especially for Sertoli cell differentiation from undifferentiated gonads [24]. A widely used DMEM (high glucose) supplemented with FBS or KSR was used for the culture medium in our experiments [9, 29]. The success rates of the FBS- and KSR-supplemented groups were slightly different. The experiments of KSR-supplemented group were performed after those of the FBS-supplemented group. It was presumed that the differences between the two groups were not due to differences in the composition of FBS and KSR, but to differences in the culture technique. KSR does not contain steroid hormones such as estradiol and testosterone, and is useful for gonadal culture. KSR is also useful for experiments in which trophic factors must be avoided [42]. A commonly used DMEM contains phenol red, which has an estrogenic effect [3]. Gonadal culture experiments using DMEM without phenol red have been reported [28]. Our gonadal culture system showed no significant difference between the use of culture medium with phenol red and that without it (Supplementary Fig. 5). The phenol red containing DMEM, which can recognize the condition of the medium, was used because sufficient testis differentiation was induced. Sufficient oxygen is supplied and nutrients are also supplied through micropores in the filter method. Culture in the gas phase is considered important to ensure an adequate oxygen supply [29, 34]. The volume of the culture medium was adjusted to a position below the groove of the agarose gel block. Based on the high rate of Sertoli cell differentiation in our gonadal culture system, there appeared to be no problem in the culture medium or oxygen supply.

The following three steps has been standardized to improve the reproducibility of the culture system. First, the groove was created by using a commercially available needle. Second, a uniform gel piece was prepared to maintain the morphology of the gonad. And third, the gel block height was unified. The grooves in the agarose gel block of the gonadal culture system were created by using a commercially available injection needle. The advantages of using the injection needle are that the groove surface is smooth, uniform in diameter and reproducible, and the needle is easy to purchase. A commercially mounting mold also creates a smoother and more uniform groove than cutting the groove manually [9]. Although the size of the groove created by the commercially mounting mold was limited, the use of a thick injection needle allows culture from larger organs. The gonad-mesonephros complex culture from 13.5 and 14.5 dpc using the gel block with a thick groove made by a 21-gauge needle was effective in maintaining the morphology of the gonad and mesonephros [42]. The reproducibility of the semi-cylindrical gel piece to maintain the morphology of the gonad was improved by using microinjection glass capillaries. A cylindrical gel piece with the same volume as the semi-cylindrical gel piece was also tested to avoid cutting with a scalpel blade. The morphology of the gonads was not maintained because of the small diameter of the gel piece (Supplementary Fig. 6). It was presumed that the use of a thin rectangular plate would allow the gonads to maintain their morphology more stably. The reproducibility of the height of the agarose gel block was also improved by fixing the diameter of the petri dish and the amount of solution to be poured. Unifying the gel height and culture medium volume improved the reproducibility of the nutrient supply and oxygen supply. Although this standardization of the culture procedures improved the reproducibility of the culture system, the testis differentiation of the cultured gonads was still heterogeneous. The location of the gonad-mesonephros complex in the groove and the arrangement of the semi-cylindrical gel piece was presumed to affect the maintenance of morphology of the gonad, which affects testis differentiation.

These results indicated that our gonadal organ culture system induces Sertoli cell differentiation in many undifferentiated gonads. Partial *Amh* expression was also induced from more immature gonads (6 ts) than 10.5 dpc (8 ts), indicating that this gonadal organ culture system was useful for elucidating the regulatory mechanisms of *Sry* expression.

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