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Establishment of an organ culture system to maintain the structure of mouse Müllerian ducts during development

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ABSTRACT. We previously showed that the anti-Müllerian hormone (AMH), infiltrating from the testis to the mesonephros reaches the cranial and middle regions of the Müllerian duct (MD) and induces their regression using an organ culture in mice. However, it is difficult to maintain structural integrity, such as the length and diameter and normal direction of elongation of the caudal region of the MD, in conventional organ culture systems. Therefore, the pathway of AMH to the caudal MD region remains uncharted. In this study, we established an organ culture method that can maintain the morphology of the caudal region of the MD. The gonad–mesonephros complex, metanephros, and urinary bladder of mouse fetuses at 12.5 dpc attached to the body trunk were cultured on agarose gels for 72 hr. The cultured caudal region of the mesonephros was elongated along the body trunk, and the course of the mesonephros was maintained in many individuals. In males, mesenchymal cells aggregated around the MD after culture. Moreover, the male MD diameter was significantly smaller than the female. Based on these results, it was concluded that the development of the MD was maintained in the present organ culture system. Using this culture system, AMH infiltration to the caudal region of the MD can be examined without the influence of AMH in the blood. This culture system is useful for clarifying the regression mechanism of the caudal region of the MD.

KEYWORDS: anti-Müllerian hormone (AMH), Müllerian duct regression, organ culture, secretion manner, sexual differentiation

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In mammals, the normal development of the gonads and reproductive tracts is important for reproduction. In males, the testis is crucial for spermatogenesis, and the male reproductive tract plays important roles in the maturation, storage, and transport of sperm. In females, the ovary is integral to oogenesis, and the female reproductive tract is central to fertilization, implantation, and embryogenesis. During gonadal development, the testis and ovary are derived from a common primordium. On the other hand, the male and female reproductive tracts are derived from different primordia, with the Wolffian duct (WD) and Müllerian duct (MD) serving as the primordia of the male and female reproductive tracts, respectively [19, 23]. In both sexes, the primordia form in the mesonephric region adjacent to the gonad during early development. After sex determination in the gonads, the MD regresses in males due to the binding of anti-Müllerian hormone (AMH), which is secreted by Sertoli cells in the testis, to the AMH receptor type 2 (AMHR2), which is expressed in the mesenchyme surrounding the MD [15, 16, 23]. The differentiation of the WD into the epididymis, vasa deferentia, and seminal vesicle glands is led by the binding of testosterone, which is secreted from the testis, to the androgen receptor expressed in the WD epithelium and the mesenchyme around the WD [2, 17, 23]. In females, which do not secrete these hormones, the MD differentiates into the oviduct, uterus, and upper part of the vagina, and the WD regresses [18, 23].

For MD regression, AMH secreted by the testis must migrate to the mesonephros and reach around the MD [15, 16, 23]. In the case of true hermaphrodite mice, which have an ovary on one side and a testis on the opposite side, MD regression occurs only on the testis side [10, 13, 21]. If AMH acts in an endocrine manner through the circulatory system, MD should regress on both sides. Hence, AMH, which reaches around the MD without relying on the circulatory system is thought to play important roles in MD regression [8, 22].

Organ culture systems have contributed significantly to studies on reproductive tracts [3, 4, 6, 20]. The filter culture method has been employed in numerous studies to investigate the mechanism underlying MD regression [3, 4, 6]. AMH was proven to cause

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MD regression when rat XX gonads were cultured with recombinant human AMH [20]. On the other hand, the filter culture method did not maintain the gonadal morphology well. After culture, mouse gonads exhibited a flattened appearance, and the morphology of AMH-producing Sertoli cells in the testes underwent degradation [7]. In our previous study, we employed the commonly used agarose gel method in gonadal studies to investigate the direct diffusion pathway of AMH from the testis to the mesonephros under conditions closely resembling those found *in vivo* [9]. We then showed that the infiltration of AMH to the mesonephros from the testis induces the regression of the cranial and middle regions of the MD (cranial MD or middle MD) in mice [9]. This finding was elucidated by the fact that the organ culture system is not affected by AMH in the circulatory system. However, using this culture method, the mesonephros of the caudal region not attached to the testis was strongly curved, and the morphology and diameter of the caudal region of the MD (caudal MD) were altered. Therefore, it has not been possible to analyze the mechanism underlying caudal MD regression. In this study, we established an organ culture method that can maintain the morphology of the caudal MD in mice to enable examination of the mechanisms underlying the caudal MD regression.

MATERIALS AND METHODS

Animals

Both male and female ICR mice were purchased from SLC Japan (Hamamatsu, Japan) and maintained as described elsewhere [9]. Fetuses at 12.5 days post coitum (dpc) were collected immediately after euthanasia, which was accomplished under deep anesthesia with 2% isoflurane and cervical dislocation. Noon of the day on which the mating plug was observed was designated as 0.5 dpc. This study was approved by the Institutional Animal Care and Use Committee of Kobe University (Permission #29-05-02) and carried out according to the Kobe University Animal Experimental Regulations.

Preparation of agarose gel blocks, cylindrical agar piece, and gel sheet

Agarose gel blocks of approximately 7 × 7 × 7 mm were prepared as described previously [7, 9]. A groove within each block was fashioned using 19-gauge needle sections (Terumo, Tokyo, Japan). Cylindrical agar pieces were prepared as described elsewhere [7]. They were cut to the same length as the culture pieces. Gel sheets of 1 × 7 mm were prepared in accordance with previous methods (Supplementary Fig. 1) [9]. In the conventional culture method, agarose gel blocks of about 6 × 6 × 2.5 mm were used. Each groove was made with 21-gauge needle sections (Terumo) and gel sheets of 0.8 × 5 mm were used as described previously [9].

Dissection and organ culture

The cranial portion above the liver was removed from a 12.5 dpc mouse fetus (Fig. 1A), in Dulbecco's Modified Eagle Medium (DMEM; with 4.5 g/L glucose, L-Gln, and HEPES, without sodium pyruvate; 08457-55; Nacalai Tesque, Kyoto, Japan). In addition, all intra-abdominal organs except the gonad–mesonephros complex, metanephros, and urinary bladder were removed (Fig. 1A). Next, the tail was resected at the 3 somites caudal to the base of the tail, and both hindlimbs were also resected (Fig. 1B). Then, the body trunk was split into right and left halves at the midline (Fig. 1C), and the cranial portion of the body was resected at a position 2 somites cranially from the most cranial part of the gonads (Fig. 1C). The body wall outside the mesonephros was excised (Fig. 1D), as was the region containing the neural tube (Fig. 1E). The reshaped culture piece was placed in a groove of agarose gel block with the dorsal side facing down (Fig. 1F and F'). A cylindrical agar piece was then placed on the medial side of the culture piece to prevent horizontal bending (Fig. 1F and F'). Furthermore, a gel sheet was placed over the culture piece to prevent deformation of the culture piece and to increase the permeability of the culture medium to the tissues (Fig. 1F and F'). The agarose gel block was placed in the center of a 24-well dish with a diameter of 15.4 mm as described previously [7, 9]. The well was filled with 520 µL of culture medium so that the gonad–mesonephros complex was positioned in the gas phase medium. It was then incubated at 37°C and 5% CO_2 for 72 hr. The culture medium was changed daily. After incubation, the gonad–mesonephros complex was detached from the body trunk and collected.

The conventional organ culture method, in which only the gonad–mesonephros complex was cultured, was performed according to the previous method [9]. The complex was placed in a groove on an agarose gel block, and a gel sheet was placed over the organ (Fig. 1G). The block was placed in the center of a 24-well dish with a diameter of 15.4 mm. The culture medium was 150 µL, ensuring that the complex was positioned in the gas phase medium, and then was incubated for 72 hr. The present organ culture method uses more culture medium than the conventional method because more nutrients are required for this culture due to the greater amount of tissue in the body trunk.

Histological analysis

The gonad–mesonephros complexes were fixed in modified Zamboni fixative containing 4% paraformaldehyde at 4°C for 24 hr, as described previously [9, 22]. The specimens were dehydrated with an ethanol series followed by xylene and then embedded in paraffin. Then, 5-µm-thick sections at 25-µm intervals were cut by a sliding microtome and placed on slide glasses that had been precoated with 2% 3-aminopropyltriethoxysilan (Shin-Etsu Chemical, Tokyo, Japan). The sections were then stored at −18°C until use. After deparaffinization and hydration, the sections were stained with hematoxylin and eosin (HE) for general histology and measurement of MD diameter. Immunohistochemical staining was carried out as described previously [9, 22]. For the primary antibody, an anti-AMH goat polyclonal antibody (1:12,000; sc-6886; Santa Cruz Biotechnology, Dallas, TX, USA) was used. For the secondary antibody, peroxidase-conjugated donkey anti-goat IgG (1:200, ab97112; Abcam, Cambridge, UK) was used.

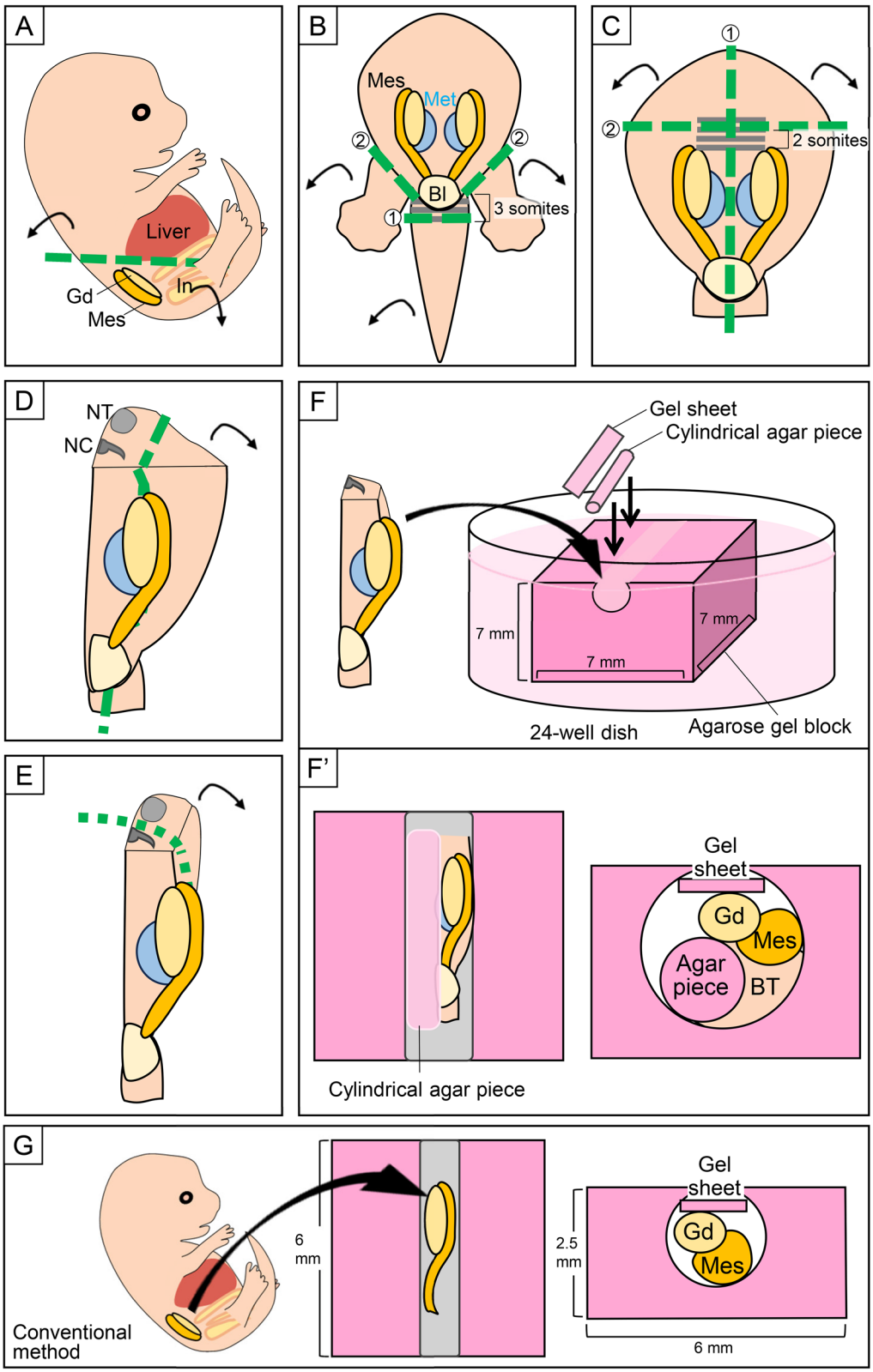

Fig. 1. Schematic diagram of the dissection and culture procedures. (**A**) The cranial side above the liver and the intestine (In) were removed from a mouse fetus at 12.5 dpc. (**B**) The tail was resected at the 3 somites caudally from the base of the tail, and both hindlimbs were also resected. (**C**) The body trunk (BT) was split into right and left halves at the midline, and the cranial side of the body was resected at a position 2 somites cranially from the most cranial part of the gonads (Gd). (**D**) The body wall outside the mesonephros (Mes) was resected. (**E**) The region containing the neural tube (NT) was resected. (**F** and **F'**) The reshaped culture piece was placed in a groove of agarose gel block with the dorsal side facing down. A cylindrical agar piece was placed on the medial side of the culture piece. A gel sheet was placed over the culture piece. (**G**) Conventional culture method. The gonad–mesonephros complex was extracted from the fetus and placed in a groove of agarose gel block. A gel sheet was placed over the gonad–mesonephros complex [9]. Bl, bladder; Met, metanephros; NC, notochord.

Measuring MD diameter

The MD diameter was measured as described previously [9]. In this study, the cranial MD was measured near the most cranial part of the gonad, the middle MD near the most caudal part of the gonad, and the caudal MD approximately 200 µm caudal to the most caudal part of the gonad (Fig. 2). The short diameters of the cranial, middle, and caudal MD were measured on four HE-stained sections for each individual, and the average of these measurements was calculated. The short diameter was used instead of the average of the long and short diameters in order to minimize measurement error when the MD was cut diagonally. Samples that had previously undergone measurements for cranial and middle MD diameters as detailed in [9] were also reevaluated. This time, the measurements encompassed diameters of the cranial, middle, and caudal MD, aligning with the definitions applied in this study. The Mann–Whitney *U* test was used to compare the degree of regression of MD in males and females after culture (BellCurve for Excel, version 3.23; SSRI, Tokyo, Japan). The results were considered significant when the *P*-value was less than 0.05.

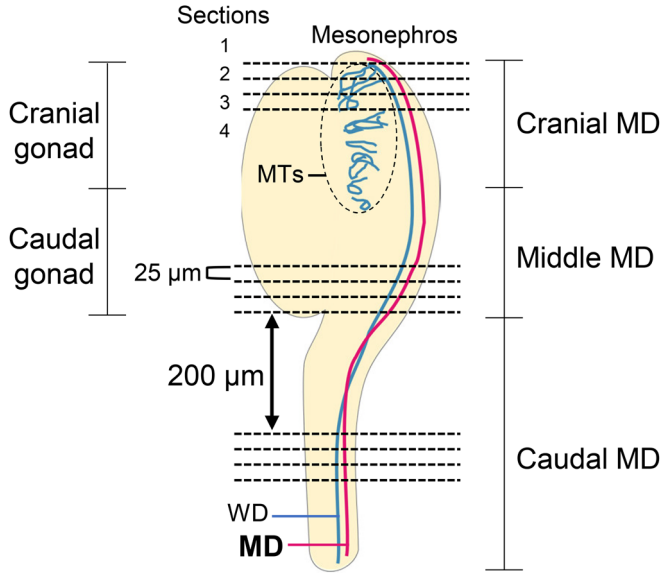

Fig. 2. Measurement position of the Müllerian duct (MD) diameter. The short diameters of the cranial, middle, and caudal MD were measured at intervals of 25 µm for four HE-stained sections. MTs, mesonephric tubules; WD, Wolffian duct.

RESULTS

The positional relationship between the metanephros and the gonad remained almost unchanged after culture (Fig. 3A and 3B). The mesonephros extended along the gonad, changed direction at the caudal end of the gonad, and continued its extension along the body trunk in mice (Fig. 3A and 3B). This differed from the elongation pattern when cultured solely with the mouse gonad–mesonephros complex (Fig. 3C). MD within the mesonephros elongated to the caudal end of the mesonephros after culture under the present culture method (Fig. 3A' and B'). The cultured testis (Fig. 3B) was thicker than that at 12.5 dpc but not thicker than that at 15.5 dpc (Fig. 3B and Supplementary Fig. 2A). The testis cord-like structures were observed in males (Fig. 3B).

Immunoreactivity for AMH was detected within the cultured mouse testis cord, and strong AMH immunoreactivities were also observed (Fig. 4A). On the other hand, the immunoreactivity of AMH was weaker in the cultured testis than in the testis at 13.5 dpc (Fig. 4B).

Histological analysis revealed the presence of testis cord-like structures in male mice (Fig. 5A and 5B), although they appeared less developed than those at 15.5 dpc (Supplementary Fig. 2B). Degenerating MD epithelial cells were observed in the cultured male MD, although the male MD formed a lumen (Fig. 5A'–C'). Furthermore, the mesenchymal cells around the male MD were more aggregated than those at 12.5 dpc but less aggregated than those at 15.5 dpc (Fig. 5A"–C" and Supplementary Fig. 2B). On the other hand, the female MD also showed lumen formation and the mesenchymal cells around the MD were not aggregated (Fig. 5D"–F"). In the WDs after culture, lumen formation and development progressed in males (Fig. 5A'–C'), whereas in females, numerous vacuoles were observed in the WD epithelium and regression advanced (Fig. 5D'–F').

The cranial, middle, and even caudal MD could be measured when cultured with the present culture method, unlike the case with MD cultured under our previous method, in which only the mouse gonad–mesonephros complex was cultured [9]. In males, the middle MD diameter was smaller than the cranial MD diameter, similar to the MD cultured with the gonad–mesonephros complex alone and that at 13.5 dpc (Fig. 6). In males, the caudal MD diameter was larger than the middle MD diameter, similar to the MD at 13.5 dpc (Fig. 6). In females, the middle MD diameter was smaller than the cranial MD, similar to the MD cultured with gonad–mesonephros complex alone and that at 13.5 dpc (Fig. 6). On the other hand, the caudal MD diameter was larger than the middle MD diameter, indicating a distinct tendency compared to the MD at 13.5 dpc (Fig. 6). Comparison of the MDs of males and females cultured by the present method revealed that the MD diameter was significantly smaller in males than in females for the cranial, middle, and caudal MD (Fig. 6).

DISCUSSION

When the mouse gonad–mesonephros complex was cultured using the organ culture system described in this study, the morphology of the mesonephros was maintained up to the caudal region, allowing for the measurement of MD diameter in the caudal region. As development progressed, the mesonephros near the caudal end of the testis became curved along the testis in mice (Supplementary Fig. 2A) [1, 14]. The curvature of the mesonephros near the caudal end of the testis was also observed during mesonephros culture using the present culture system. On the other hand, around 15.5 dpc, the mesonephros near the caudal end of the testis was strongly curved along the testis (Supplementary Fig. 2A) [1, 14], but the curvature was weaker in the mesonephros cultured with the present culture system (Fig. 3). The three-dimensional positioning of the metanephros (kidney), gonads, mesonephros, and the surrounding

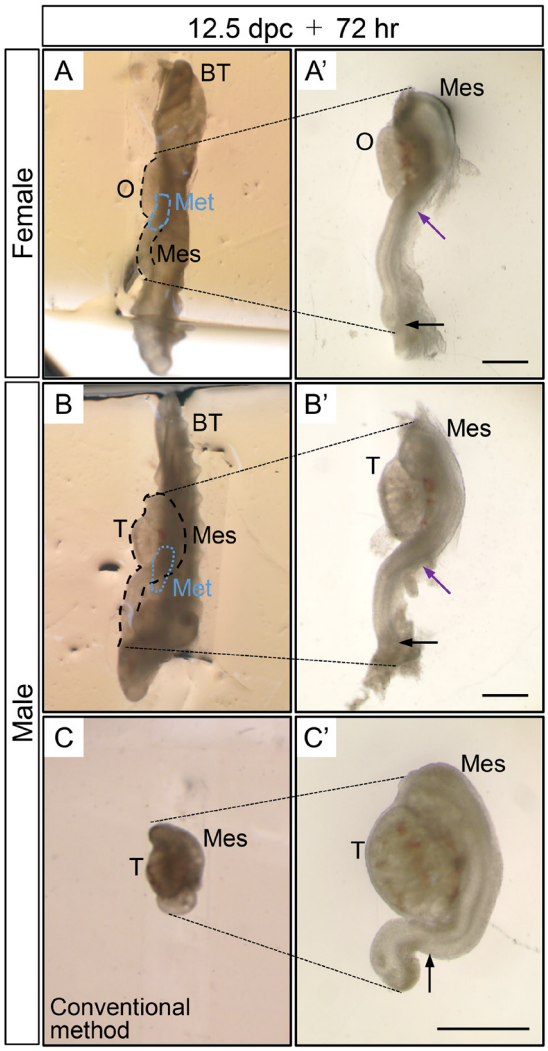

Fig. 3. Gonad–mesonephros (Mes) complex and body trunk (BT) after 72 hr of culture. (**A**) An ovary (O)–mesonephros complex and body trunk after culture. The body trunk extended straight within the groove of the agarose gel block. The mesonephros was elongated along the body trunk. (**A'**) The ovary–mesonephros complex was excised from the body trunk after culture. The mesonephros extended along the ovary, changed direction at the caudal end of the ovary (purple arrow), and continued its extension along the body trunk. (**B**) A testis (T)–mesonephros complex and body trunk after culture. The body trunk extended straight within the groove of the agarose gel block. The mesonephros was elongated along the body trunk. (**B'**) The testis–mesonephros complex excised from the body trunk after culture. The mesonephros extended along the testis, changed direction at the caudal end of the testis (purple arrow), and continued its extension along the body trunk. (**C** and **C'**) A testis–mesonephros complex cultured by the previous method, i.e., with the gonad–mesonephros complex alone [9]. The mesonephros of the caudal region not attached to the testis was strongly curved. Testis cord-like structures were clearly observed in the testis. Black arrows indicate the end of the Müllerian duct. Met, Metanephros.

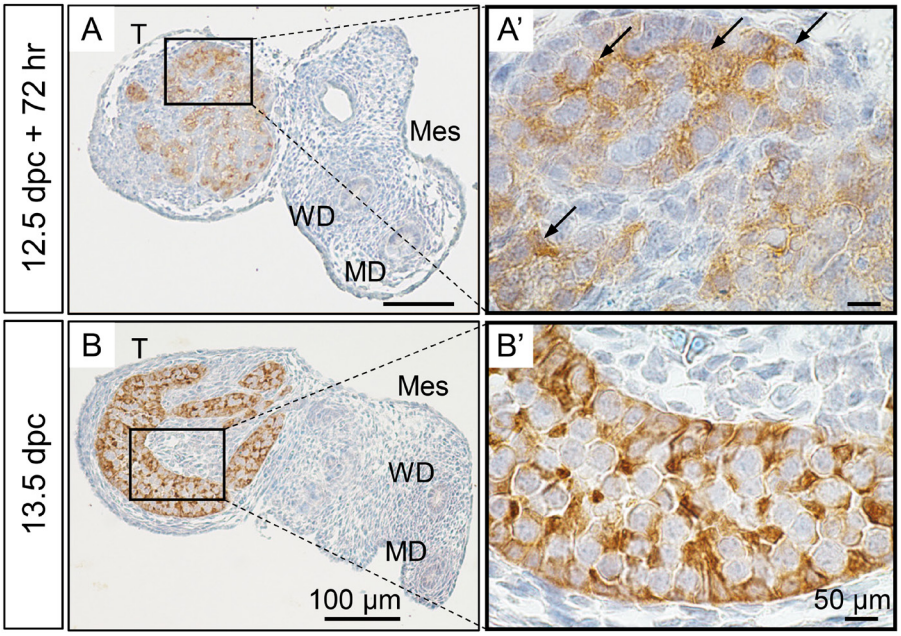

Fig. 4. Immunoreactivity of anti-Müllerian hormone (AMH) in sections of the testis (T)–mesonephros (Mes) complexes after 72 hr of culture and at 13.5 dpc. (**A**) Immunoreactivities for AMH were detected within the cultured testis cord, and strong AMH immunoreactivities were also observed (**A'**; arrows). (**B**) Immunoreactivities for AMH were detected within the testis cord at 13.5 dpc. Sertoli cells showed strong immunoreactivity for AMH (**B'**). MD, Müllerian duct; WD, Wolffian duct.

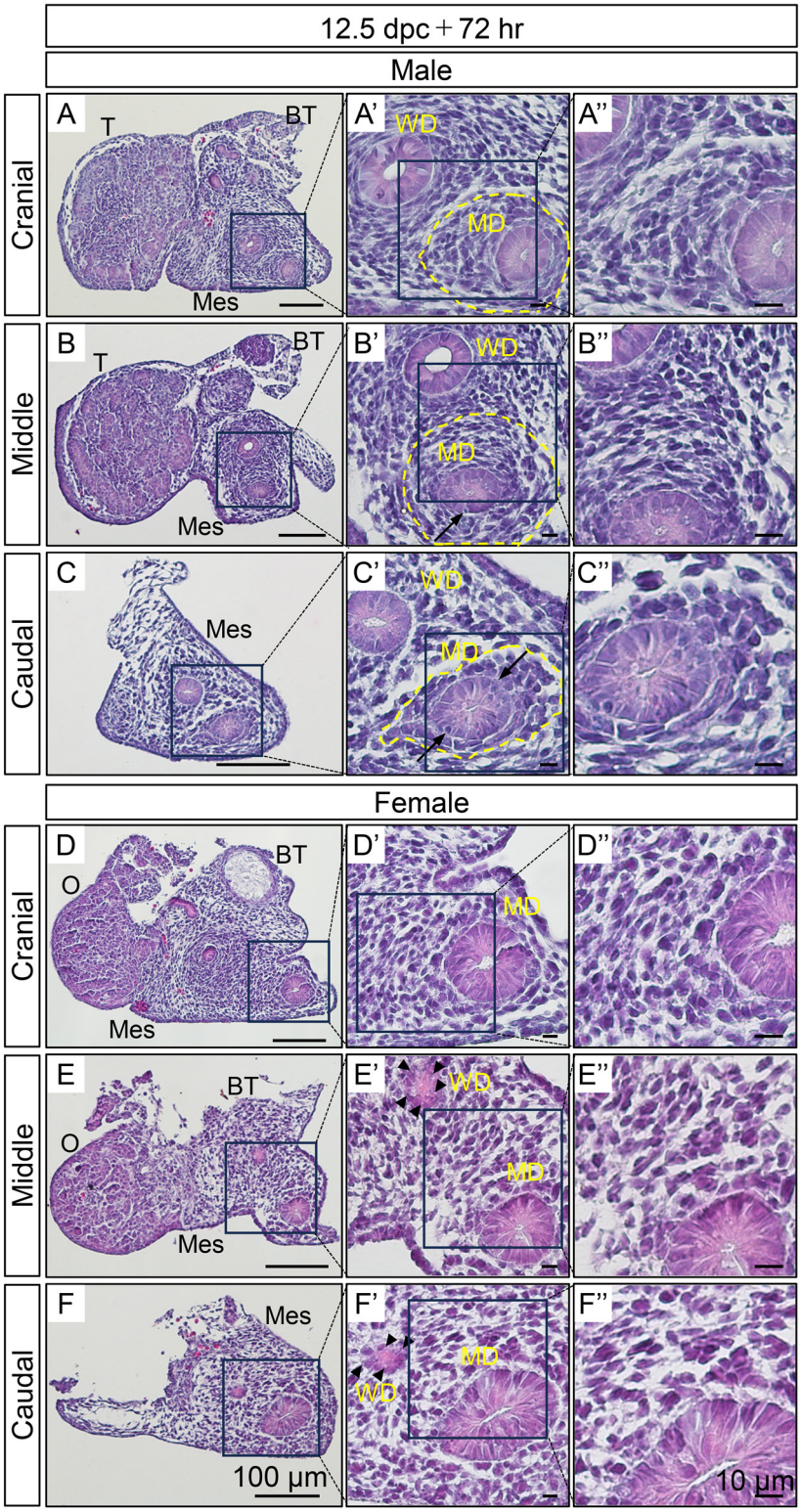

Fig. 5. HE-stained sections of male (**A–C**) and female (**D–F**) gonad–mesonephros (Mes) complexes after 72 hr of culture and enlarged images focusing on the reproductive tracts (**A'–F'**) and mesenchymal cells around the Müllerian duct (MD; **A"–F"**). In males (**A'–C'** and **A"–C"**), lumens were formed in the cranial, middle, and caudal MD. The mesenchymal cells around the cranial, middle, and caudal MD were aggregated (yellow dotted lines), and degenerating cells (arrows) were also observed. The lumen of the Wolffian duct (WD) was clearly formed. In females (**D'–F'** and **D"–F"**), lumens were formed in the cranial, middle, and caudal MD. Mesenchymal cells around the MD were not aggregated. Numerous vacuoles (arrowheads) were observed in the WD epithelium. BT, body trunk; O, ovary; T, testis.

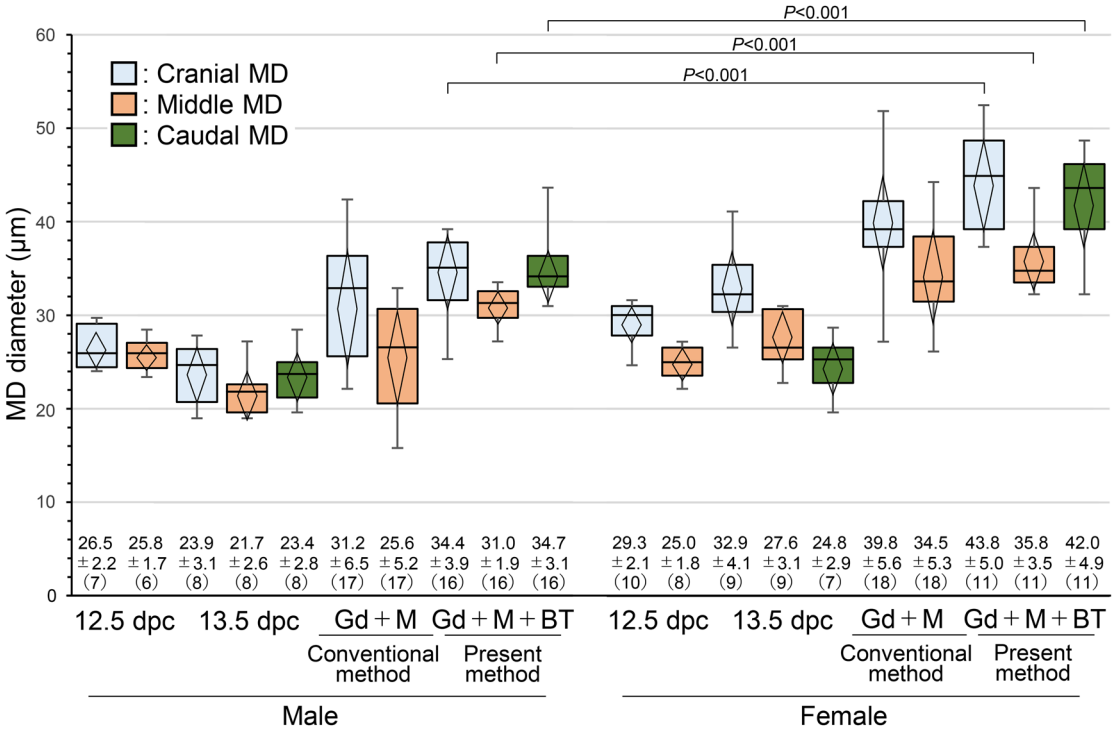

Fig. 6. The diameters of the cranial, middle, and caudal Müllerian ducts (MD) at 12.5 dpc, 13.5 dpc, and after 72 hr of culture with the gonad (Gd)–mesonephros (M) complex alone and with gonad–mesonephros complex attached to the body trunk (BT). The male and female caudal MD diameter at 12.5 dpc could not be measured because the MD had not extended to the caudal region. The male and female caudal MD cultured with the gonad–mesonephros complex alone could not be measured because the morphology of the caudal MD was not maintained. In males, the diameter of the middle MD was smaller than that of the cranial MD at 12.5 dpc, 13.5 dpc, and after 72 hr of culture by either culture method. At 13.5 dpc, the diameter of the caudal MD was larger than that of the middle MD. The caudal MD diameter was also larger than the middle MD diameter when the gonad–mesonephros complex was cultured attached to the body trunk. In females, the middle MD diameter was smaller than the cranial MD diameter at 12.5 dpc, 13.5 dpc, and after 72 hr of culture by either culture method. At 13.5 dpc, the diameter of the caudal MD was smaller than that of the middle MD. On the other hand, the caudal MD diameter was larger than the middle MD diameter when the complex was cultured attached to the body trunk. The diameters of the male cranial, middle, and caudal MD were significantly smaller than those of females ($P<0.001$). The rhombus in the boxplot indicates the mean ± SD. Data are presented as means ± SD. Numbers in parentheses are sample numbers.

fibers (ligament) plays a crucial role in the normal development of the MD [14]. In the present study, the positional relationship between the metanephros and gonad before and after culture remained nearly unchanged, and there was no descent of the testis. Additionally, although the body trunk curved *in vivo*, the cultured body trunk became straightened. These differences were thought to have affected the curvature of the caudal mesonephros cultured with the body trunk.

It has been reported that as development progresses in male mice, mesenchymal cells aggregate around the MD (Supplementary Fig. 2B) and MD epithelial cells degenerate, whereas in females mesenchymal cells do not aggregate around the MD [5]. The diameters of MD in male and female mice are similar at 12.5 dpc before the start of MD regression, whereas at 13.5 dpc, the diameters in males are smaller than in females [9]. In males, when the MD was cultured in the present study, the mesenchymal cells aggregated around the MD and the MD diameter was significantly smaller than in females. These results indicate that cranial, middle, and even caudal MD regression advanced in males when MD was cultured using the present culture system.

In the conventional organ culture system, it is difficult to maintain morphological integrity, such as the normal direction of elongation and the diameter of the caudal MD [9]. Therefore, it was demonstrated that AMH, which infiltrates the mesonephros from the testis, induces regression in both the cranial and middle MD in mice. However, the regression of the caudal MD could not be analyzed in the previous study [9]. The organ culture system used in the present study not only produced AMH in the testis cord and induced regression of the cranial and middle MD as shown in the previous study, but also maintained the morphology of the caudal mesonephros and advanced the regression of the caudal MD. This indicated that the organ culture method used in this study was a suitable system for evaluating the MD regression under conditions closely resembling those found *in vivo*, especially with regard to AMH influence. The cultured male WD developed and the cultured female WD regressed. On the other hand, the diameter of the caudal MD of females after culture differed from that of females at 13.5 dpc. Therefore, differences from *in vivo* conditions should be considered when using the present culture system to analyze female caudal MD.

MD regression is initiated from the cranial MD. This is attributed to the fact that the expression of AMHR2, the receptor for AMH, is initiated from the cranial MD in mice and rats [1, 20]. In male mice, AMHR2 expression starts at around 13.0 dpc and then progresses

in a sequential manner toward the caudal MD, reaching the most caudal part at 13.5 dpc [1]. On the other hand, MD regression does not advance continuously from the cranial region, but there are several regions where regression initiates earlier [11, 12]. MD remains intermittently at 15.5 dpc [11, 12] and then disappears at 18.5 dpc in male mice [5]. This phenomenon cannot be explained by the diffusion of AMH from the testis to the caudal mesonephros. It implies the presence of a mechanism that leads to local variations in AMH concentration, including AMH in the blood, as development progresses. However, the details of this mechanism have not yet been clarified. Analysis of caudal MD using the present culture system may help to elucidate the mechanism.

In this study, an organ culture system was established that could maintain the morphology of the caudal MD. Using this culture system, AMH was expressed in the cultured testis, and the regression of the cranial, middle, and caudal MD advanced. Therefore, the organ culture method in this study is useful for studying the regression mechanism of MD, including the caudal region.

CONFLICT OF INTEREST. The authors have no conflicts of interest.

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